WHO FOOD ADDITIVES SERIES: 77

Prepared by the eighty-sixth meeting of the
Joint FAO/WHO Expert Committee
on Food Additives (JECFA)

Safety evaluation of certain food additives

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PREFACE

The monographs contained in this volume were prepared at the eighty-sixth meeting of the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), which met at WHO headquarters in Geneva, Switzerland, on 12–21 June 2018. These monographs summarize the data on selected food additives, including flavouring agents, reviewed by the Committee.

The eighty-sixth report of JECFA has been published by WHO as WHO Technical Report No. 1014. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1 of the current publication. The participants in the meeting are listed in Annex 3. A summary of the conclusions of the Committee with respect to the food additives discussed at the meeting is given in Annex 4.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives, the Codex Committee on Contaminants in Food and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and dietary exposure and toxicological monographs, such as those contained in this volume, on substances that they have considered.

The monographs contained in this volume are based on working papers that were prepared by WHO experts. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by J. Odrowaz, Toronto, Canada.

These monographs were prepared based on the evaluation of the original studies and the dossier provided by the sponsor(s) of the compound, of the relevant published scientific literature and of the data submitted by Codex members. When found consistent with the data of the original study, the monographs may contain parts of the text and tables of the dossier submitted by the sponsor(s), but not the sponsor(s)' conclusions. These monographs and their conclusions are based on an independent review of the available data and do not constitute an endorsement of the sponsor(s)' position.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the organizations participating in WHO concerning the legal status of any country, territory, city or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the organizations in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological or toxicological properties of or dietary exposure to the compounds evaluated in this publication should be addressed to: WHO Joint Secretary of the Joint FAO/WHO Expert Committee on Food Additives, Department

of Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

SPECIFIC FOOD ADDITIVES

Anionic methacrylate copolymer

First draft prepared by

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1. Explanation

Anionic methacrylate copolymer (AMC; E 1207; International Numbering System Number 1207; Chemical Abstracts Service [CAS] No. 26936-24-3; acrylates copolymers; methyl acrylate, methyl methacrylate, methacrylic acid polymer; methacrylic acid polymer with methyl acrylate and methyl methacrylate) is a copolymer manufactured from the monomers methacrylic acid (CAS No. 79-41-4), methyl methacrylate (CAS No. 80-62-6) and methyl acrylate (CAS No. 96-33-3) in the molar ratio of 7 : 3 : 1.

AMC has been evaluated by the European Food Safety Authority (EFSA) and is approved for use as a food additive in the European Union. Its use in the European Union is restricted to a maximum level of 100 000 mg/kg in solid food supplements, category 17.1 (EFSA, 2010).

The Committee considered three different copolymers: basic, anionic and neutral methacrylate copolymers. Each copolymer releases the active ingredients from within their coatings under different physiological conditions in different parts of the digestive tract. AMC is soluble above pH 7 and is used for its taste- and odour-masking properties; as protection from heat, light, moisture and oxidation; and to prevent the fast release of active ingredients once they leave the stomach.

AMC has not previously been evaluated by the Committee. The Committee evaluated the use of AMC as a coating or glazing agent for solid food supplements and foods for special medical purposes when provided in the form of solid food supplements such as capsules, pastilles, tablets, pills, pellets and powders, at levels not exceeding 10%, at the request of the Forty-ninth Session of the Codex Committee on Food Additives (FAO/WHO, 2017). AMC is also used in pharmaceuticals.

Toxicological data submitted for the evaluation included distribution studies, acute and short-term toxicity and genotoxicity studies as well as a developmental toxicity study. Limited data were also submitted on the residual monomers (methacrylic acid, methyl methacrylate, methyl acrylate). A comprehensive literature search retrieved data on the monomers but no additional

studies on AMC. The Committee considered the data on the residual monomers, as well as that for AMC itself, because the monomers are of low molecular weight and therefore likely to be absorbed from the gastrointestinal tract. Due to the low levels of residual monomers present in AMC, the Committee evaluated only absorption, distribution, metabolism and excretion (ADME) data and long-term toxicity and genotoxicity data on the monomers.

The Committee was also aware that AMC contains an oligomer fraction of up to 2%. As the lower end of the molecular weight range for all the constituent oligomers is greater than 1000 Da, with around 75% of the oligomer fraction having a molecular weight between 5000 and 10 000 Da, it is unlikely that the oligomers would be absorbed from the gastrointestinal tract. Therefore, the Committee did not consider the toxicological aspects of the oligomers.

Unless otherwise stated, the test substance used in the distribution and toxicity studies was prepared from an aqueous dispersion with 1.5% emulsifier and then freeze-dried to remove water. In all cases, doses have been expressed as dry weight of AMC.

The Committee evaluated toxicological and exposure data on sodium lauryl sulfate, polysorbate 80 and simethicone, residual components of AMC that can be present in the final product because they are used in the manufacture of the copolymer. The Committee concluded that these residual components did not pose a safety concern at the maximum estimated exposure levels.

1.1 Chemical and technical considerations

AMC is manufactured by emulsion polymerization of the monomers methacrylic acid, methyl methacrylate and methyl acrylate with water-soluble radical initiators. The product is purified by water vapour distillation and filtration to remove residual monomers, excess water, other volatile low molecular weight substances and coagulum.

AMC has a weight-average molecular weight of 280 000 Da and a number-average molecular weight of 77 000 Da.

Although organic solvents are not used in the manufacture of AMC, methanol may be present at a level not exceeding 1000 mg/kg as a result of hydrolysis of esterified carboxyl groups incorporated in the polymer. The copolymer is standardized as a 30% aqueous dispersion with sodium lauryl sulfate (0.3%) and polysorbate 80 (1.2%). Simethicone emulsion is used as an antifoaming agent during the manufacture and is present in the dispersion at not more than 20 mg/kg. The copolymer dispersion may contain residual monomers: methyl acrylate (not more than 1 mg/kg); methyl methacrylate (not more than 3 mg/kg); and methacrylic acid (not more than 1 mg/kg).

1.2 Literature search

Literature searches were carried out in PubMed and Google Scholar for genotoxicity studies (1990–2018) with the monomers. A literature search was also carried out for genetoxicity (2001–2018) and other toxicology studies (1997–2018) with methacrylic acid and methyl acrylate. Articles were incorporated if they included (genetic) toxicological information about the monomer on its own; articles were excluded if the information was not about the monomer on its own and did not include (genetic) toxicological information. Other toxicology searches were carried out for the monomer and specific species. Articles were included if they examined a toxicological end-point in one of the named species (or similar mammalian species); articles were rejected if there was no toxicological end-point or they did not include one of the named species (or a similar mammalian species). Review articles were manually searched to identify any articles that may not have been found via the database search.

The following keywords, search strings and limits were used in the PubMed and Google Scholar literature searches for genetic toxicology of methacrylic acid: "methacrylic acid AND gene* tox*" (from 1 January 2001); "methacrylic acid AND aberration" (from 1 January 2001); "methacrylic acid AND comet" (from 1 January 2001); "methacrylic acid AND micronucleus" (from 1 January 2001); "methacrylic acid AND Ames" (from 1 January 2001); "methacrylic acid AND chromatid exchange" (from 1 January 2001); "methacrylic acid AND muta*" (from 1 January 2001); "methacrylic acid AND adduct" (from 1 January 1990).

Due to the large number of articles retrieved, only the first 100 for any given search were considered.

The following keywords, search strings and limits were used in the PubMed and Google Scholar literature search for other toxicology studies of methacrylic acid: "methacrylic acid AND rat" (1997–2018); "methacrylic acid AND mouse" (1997–2018); "methacrylic acid AND rabbit" (1997–2018); methacrylic acid AND dog" (1997–2018); "methacrylic acid AND monkey" (1997–2018); "methacrylic acid AND non-human primate" (1997–2018); "methacrylic acid AND mice" (1997–2018); "methacrylic acid AND pig" (1997–2018).

Due to the large number of articles returned only the first 100 for any given search were considered.

The searches were repeated using the same search strings and limits but substituting "methyl acrylate" for "methacrylic acid".

A literature search was also conducted to identify any estimates of dietary exposure to AMC, using EBSCO Discovery Service. Medline, Food Science Source, Food Science and Technology Abstracts and ScienceDirect were searched as were a number of scientific, toxicological, food, nutrition and public

health–related journals. Search terms included "methacrylate copolymer" and "dietary exposure" or "dietary intake" or "consumption". These terms were also used in a general internet search to capture "grey" literature and other papers not included in the scientific literature. No exposure estimates additional to those submitted by the sponsor were retrieved.

2. Biological data

The Committee considered toxicological and exposure data on AMC, the residual monomers (methacrylic acid, methyl methacrylate, methyl acrylate) and residual components of AMC other than monomers that can be present in the final product because they are used in the manufacture of the copolymer (section 2.2.7).

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) AMC

Groups of rats (4/sex per group) received doses of 600 mg/kg body weight (bw) per day of AMC (30% dispersion in aqueous solution; dose expressed as dry weight of polymer) for 13 days. On day 14, a single radiolabelled dose was administered to each rat. Methacrylic acid monomers (radiolabelled on the carboxylic acid carbon moiety) were used to synthesize the radiolabelled test material, which was administered at approximately 370 000 Bq per animal. The groups were treated as follows: tissues were collected 24 hours (Group 1; animals nos 1–8) and 72 hours (Group 2; animals nos 9–16) after dosing with the radiolabelled test material; and faeces and urine were collected for 10 days post dosing with the radiolabelled test material after which Group 3 (animals nos 17–24) were necropsied. Terminal blood samples were collected and the following tissues and excreta examined: caecum, ileum, oesophagus and stomach, carcass (without skin, fur or feet), kidneys, rectum, colon, liver, duodenum and lymph nodes and urine (from bladder).

In Groups 1 and 2, the caecum wall had the highest concentration of radioactivity in the animals killed at 24 and 72 hours, accounting for 0.09% of the administered dose. Four animals killed at 24 or 72 hours had low levels of radioactivity in the colon wall, amounting to less than 0.01% of the administered dose. In Group 3 animals, tissue and tissue content amounted to less than 0.01% of the administered dose. Mean recovery in the faeces amounted to 92.38% of

the total dose after 72 hours and 94.07% (±3.42%) 10 days after the final dose. Radioactivity was detected at very low levels in the urine of four of the eight animals in this group; the study authors considered that this may have been due to contamination with faecal matter (Huntingdon Life Sciences, 1999).

(b) Residual monomers

Methacrylic acid

There are few ADME studies on methacrylic acid.

Methacrylic acid is well absorbed after oral or inhalational exposure. After inhalation exposure the majority of the dose was deposited in the upper respiratory tract (Morris & Frederick, 1995).

The European Chemicals Bureau Risk Assessment Report on methacrylic acid (European Commission, 2002) described the available ADME data after oral dosing as follows:

> After a single oral administration of the sodium salt of methacrylic acid to Wistar rats (540 mg/kg bw) methacrylic acid was detected in the blood serum by means of [high-performance liquid chromatography]. The maximum concentration was found after 10 min, whereas after 60 min no more methacrylic acid was detectable (Bereznowski, Mariewski & Somolenski, 1994).
>
> There are no studies which specifically address the metabolism of exogenously applied methacrylic acid. However, it is generally accepted that methacrylic acid-coenzyme-A is a naturally occurring intermediate of the valine pathway. Methacrylic acid-CoA is rapidly converted into (*S*)-3-hydroxyisobutyryl-CoA by the enzyme enoyl-CoA-hydratase. This pathway joins the citrate cycle, carbon dioxide, and water being the final products (Rawn, 1983; Shimomura et al., 1994; Boehringer, 1992).

Methacrylic acid does not undergo epoxidation (Albertini, 2017).

Methyl methacrylate

The ADME data on methyl methacrylate were summarized by the World Health Organization (WHO, 1998), as follows:

> Methyl methacrylate is rapidly absorbed and distributed following inhalation or oral administration to rats. On the basis of available data, methyl methacrylate appears to be rapidly metabolized to methacrylic acid [and methanol], which is subsequently converted to carbon dioxide via the tricarboxylic acid cycle in both experimental animals and humans….
>
> Methyl methacrylate is rapidly eliminated, primarily via the lungs in expired air. After oral or intravenous administration to rats, approximately 65% of the dose was exhaled

in the expired air as $^{14}CO_2$ within 2 hours (Bratt & Hathway, 1977). Lesser amounts are eliminated in the urine, and an even smaller fraction in the faeces. Owing to its rapid metabolism and excretion, there appears to be little potential for accumulation of methyl methacrylate within tissues (Government of Canada, 1993; ECETOC, 1995).

Methyl acrylate

In the rat, orally administered radiolabelled methyl acrylate was rapidly absorbed. The major route of excretion was in the urine (approximately 51% of the administered dose in 48 hours) followed by CO_2 exhalation (approximately 39% of the administered dose in 24 hours) (Sapota, 1993).

In an inhalation study in rats, between 1.5% and 8% of methyl acrylate was converted to thioester derivatives (Vodicka, Gut & Frantík, 1990).

2.2 Toxicological studies

2.2.1 Acute toxicity

In a study compliant with good laboratory practices (GLP), 2000 mg/kg bw of AMC (~100% polymer, freeze-dried; dose expressed as dry polymer) dissolved in 0.5% carboxymethylcellulose (CMC) was administered by gavage to rats (5/sex). Following a 14-day recovery period, the animals were killed and necropsied. During the recovery period, no abnormal clinical observations, no deaths, no statistically significant differences in body weights between test animals and historical controls were observed. No abnormalities were observed at necropsy (RTC, 1995).

2.2.2 Short-term studies of toxicity

(a) Rats

In a GLP-compliant 26-week oral toxicity study, four groups of rats (15/sex per group with an additional 10/sex of control and high-dose rats for the 4-week recovery study) were treated by gavage with AMC (30% dispersion in a 0.5% solution of CMC in distilled water; dose expressed as dry weight of polymer) at 0, 200, 500 or 1500 mg/kg bw per day. Feed consumption was monitored weekly and water consumption was measured over 3-day periods in weeks 5, 12 and 25. Blood and overnight urine samples were collected from all animals at 13 and 26 weeks and from recovery group animals in week 4 of the recovery period.

Two high-dose females and two low-dose males died during the study. One high-dose female died during blood collection, and one low-dose male showed poor health and was found to have a malignant histiocytoma with metastases in the thyroid, oesophagus and trachea. No treatment-related causes were evident for these deaths. No treatment-related effects were observed in

clinical observations of the animals, body weights, feed and water consumption, ophthalmoscopic examinations, terminal body and organ weights or microscopic observations. Haematocrit and haemoglobin levels were statistically significantly reduced in low-dose females in week 13 only, and white blood cell count was increased in high-dose females in week 26 only. Statistically significant clinical chemistry findings included: a reduction in sodium levels in low-dose males in week 13; an increase in sodium levels in low-dose females and high-dose males in week 13; a reduction in sodium levels in males in all dose groups in week 26; a reduction in calcium levels in mid- and high-dose males in week 26 with females at the same doses showing an increase in calcium levels; a decrease in urea and chloride levels in high-dose females after 26 weeks only; a decrease in potassium levels in high-dose males at 26 weeks; an increase in alanine aminotransferase (alanine transaminase; ALT) activity in low- and high-dose females and an increase in aspartate aminotransferase (aspartate transaminase; AST) in high-dose females only at 26 weeks; and an increase in cholesterol and total protein levels in high-dose females in week 13. None of these clinical chemistry parameters showed a dose–response relationship or continuity between time periods. Statistically significant urine analysis findings, which also showed no dose–response relationship or continuity between time periods, included decreased urine volume in low-dose females; increased specific gravity in mid- and high-dose females in week 13; and increased urine volume in treated recovery group animals. Decreases in thyroid and heart weights were also observed; these were considered to be spontaneous occurrences and not treatment related. Pale, firm contents were found in the stomachs of mid-dose (8/30) and high-dose animals (24/30; 5/20 high-dose recovery group animals). This was considered to be AMC agglomerating as a result of needing to administer the formulation in high doses. As no stomach lesions were found, the study authors considered that this agglomeration did not compromise the results of the study.

The no-observed-adverse-effect level (NOAEL) was 1500 mg/kg bw per day, the highest dose tested (RTC, 2000).

In a GLP-compliant study conducted according to Organisation for Economic Co-operation and Development Test Guideline (OECD TG) 407, rats (10/sex per group) received 0 (vehicle only), 200, 500 or 1500 mg/kg bw per day of AMC (~100% polymer powder, freeze-dried suspended in 0.5% CMC; dose expressed as the dry weight polymer) by gavage daily for 4 weeks. The control and high-dose groups included an additional five animals per sex that were killed after a 2-week recovery period. Body weights and mean feed consumption were recorded weekly. Ophthalmoscopic examinations were carried out before dosing and in week 4. Blood and overnight urine samples were collected just prior to necropsy in all groups.

No deaths occurred during the study. A slight reduction in body weight was observed during the recovery period in high-dose females. No differences were observed between test and control groups in feed consumption or ophthalmoscopic examinations. No treatment-related effects were noted on clinical chemistry, urine analysis or haematological parameters or organ weights. In low- and mid-dose females in week 4, increases in platelet counts and plasma urea levels were statistically significant but did not show a dose–response relationship. Statistically significant effects were observed on relative organ weights of brain (low- and mid-dose females), uterus (high-dose females) and spleen (low-dose females), pituitary (low- and mid-dose females). Kidney and adrenal weights increased in high-dose females after the 2-week recovery period. Overall, these findings were within historical control values or did not show a dose–response relationship, and were therefore considered not treatment related. Slight pigmentation was observed in the cortical tubular cells associated with the basophilia in the kidneys of three of the high-dose animals. This has been seen to form spontaneously in rats and the significance remains unclear.

The NOAEL was 1500 mg/kg bw per day, the highest dose tested (RTC, 1998).

(b) **Dogs**

In a GLP-compliant 4-week oral toxicity study, dogs (3/sex per group) received AMC at 0, 100, 200 or 400 mg/kg bw per day (doses expressed as dry weight of the polymer) in coated pellets containing 83% cellulose, 17% AMC and 1% glycerol monostearate. The animals were observed before and after dosing, and clinical signs and body weights were recorded weekly. Feed consumption was recorded daily. Water consumption was recorded before treatment and in weeks 1 and 4. Blood and urine samples were collected before treatment and in week 4.

Physical and ophthalmoscopic examinations revealed no treatment-related effects. No differences between groups were observed in mortality and clinical signs; body weight; feed and water consumption; haematological, clinical chemistry, urine and faecal analysis parameters; terminal body and organ weights; or macroscopic and microscopic observations. Reduced group mean plasma alanine aminotransferase (ALT) activity was observed in low- and high-dose males, but this was attributable to one animal in each group that showed low ALT activity at the start of the study. A statistically significant decrease in total cholesterol was observed in low-dose females and an increase in total protein was observed in mid-dose females. These changes were minor and did not show a dose–response relationship; they were therefore considered not treatment related. White granular material was found in the caecum, colon and rectum in some animals in all treatment groups. Analysis determined this to be primarily

cellulose and AMC. No macroscopic changes were observed in these tissues. Overall, no treatment-related effects were observed.

The NOAEL was 400 mg/kg bw per day, the highest dose tested (RTC, 2004).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) AMC

No long-term studies with AMC were available.

(b) Residual monomers

Methacrylic acid

No long-term chronic toxicity/carcinogenicity studies with methacrylic acid were available. The Committee noted that studies with methyl methacrylate, which is metabolized into methacrylic acid, showed no carcinogenic effects in mice, rats or hamsters (WHO, 1998).

Methyl methacrylate

A long-term toxicity study with methyl methacrylate given in drinking-water was summarized by WHO (1998) as follows:

> Data available on the effects of methyl methacrylate following ingestion are limited. In an early study (Borzelleca et al., 1964) in which organ to body weight ratios were determined and histopathological examination of a wide range of tissues as well as limited haematological and urine analyses were conducted, the relative kidney weight was increased in a small group of female rats (n = 25) exposed to 2000 ppm (mg/litre) methyl methacrylate in drinking-water for 2 years. This effect was not observed in the males, and histopathological examination revealed no damage. The authors also reported a decrease in fluid consumption in rats exposed to 2000 ppm. The NOAEL was therefore considered to be 2000 ppm (equivalent to a dose of about 146 mg/kg body weight per day for females and 121 mg/kg body weight per day for males, based on intake and body weight data presented by the authors). There were no treatment-related effects, based upon gross or histopathological examination, in extremely small groups of beagle dogs (n = 2) exposed to concentrations of up to 1500 ppm (mg/kg) methyl methacrylate (equivalent to a dose of about 38 mg/kg body weight per day) in their feed for 2 years (Borzelleca et al., 1964).

WHO used this study to derive a tolerable daily intake (TDI) of 1.2 mg/kg bw per day (WHO, 1998).

In chronic toxicity and carcinogenicity studies in mice, rats and hamsters with methyl methacrylate given by the inhalation route, the observed effects were, in general, similar to those reported in short-term studies, and included

inflammation and epithelial hyperplasia of the nasal cavity and degeneration of the olfactory sensory epithelium. There was no evidence of any carcinogenic effects (WHO, 1998).

In 1998, the International Agency for Research on Cancer (IARC) considered the same studies and concluded that evidence suggested a lack of carcinogenicity in animals (IARC, 1999).

Methyl acrylate

In a 2-year inhalation study, rats (86/sex per group) were exposed to methyl acrylate (purity >99.8%; main impurities methyl propionate and ethyl acrylate) by inhalation at 0, 15, 45 or 135 ppm (0, 53, 158 and 475 mg/m^3) in air for 6 hours per day on 5 days per week for 2 years. Interim kills took place after 12 and 18 months of exposure. No significant difference in mortality was observed between the groups. Incidence of soft-tissue sarcomas varied considerably between the groups but there was no dose-dependence. No increased frequency of any tumour type in any organ could be related to a carcinogenic effect of the test substance (Reininghaus, Koestner & Klimisch, 1991).

There are no epidemiological data in humans. The IARC (1999) concluded that there was inadequate evidence in experimental animals for carcinogenicity and that methyl acrylate was not classifiable as to its carcinogenicity in humans.

The Committee concluded that the long-term studies on methyl acrylate were inadequate to support the safety of this residual monomer when consumed orally.

2.2.4 Genotoxicity

The results of genotoxicity tests for AMC are summarized in Table 1 and for the residual monomers, in Tables 2, 3 and 4.

Of the several in vitro studies with methacrylic acid, the most useful data were from negative studies on reverse mutations in bacteria. Positive results were obtained in two comet assays and a γ-H2AX assay on human gingival fibroblasts (Szczepanska et al., 2012). There were no studies on chromosomal aberrations or gene mutation in mammalian cells and no in vivo studies on methacrylic acid. The Committee noted that there were insufficient data to reach a conclusion on the genotoxic potential of methacrylic acid.

Bacterial reverse mutation assays with methyl methacrylate gave negative results (Lijinsky & Andrews, 1980; Hachiya, Taketani & Takizawa, 1982; Waegemaekers & Bensink, 1984; NTP, 1986; Schweikl, Schmalz & Rackebrandt, 1998). Mixed results (i.e. positive, weakly positive or negative) were obtained in in vitro chromosomal aberration (NTP, 1986; Doerr, Harrington-Brock & Moore, 1989; Bigatti et al., 1994; Schweikl, Schmalz & Rackebrandt, 1998; Tuček

Table 1
Summary of genotoxicity studies with AMC

End-point	Test system/ species	Test compound	Concentration/dose	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537	Freeze-dried powder, ~100% purity, resuspended (±S9)	157, 313, 625, 1 250, 2 500, 5 000 mg/plate, plate incorporation and preincubation	Negative	RTC (1996a)
Reverse mutation	*Escherischia coli* WP2uvrA	Freeze-dried powder, ~100% purity, resuspended (±S9)	157, 313, 625, 1 250, 2 500, 5 000 mg/plate, plate incorporation and preincubation	Negative	RTC (1996a)
Gene mutation	L5178Y mouse lymphoma cell line	Freeze-dried suspension dose expressed as dry polymer (±S9)	7.2, 14.4, 28.8, 57.5, 115, 230 mg/mL	Negative	RCC (2001a)
Chromosomal aberration	Human lymphocytes	Freeze-dried powder, ~100% purity, resuspended (±S9)	10.8, 23.2, 50.0, 108, 232, 500, 1 080 mg/mL	Negative	RTC (1996b)
In vivo					
Micronucleus induction	NMRI mouse (bone marrow)	Freeze-dried suspension dose expressed as dry polymer	500, 1 000, 2 000 mg/kg bw per day, (top group assessed at 24 and 48 hours post dosing)	Negative	RCC (2001b)

AMC: anionic methacrylate copolymer; bw: body weight; S9: 9000 × *g* supernatant fraction from rat liver homogenate

Table 2
Genotoxicity studies with methacrylic acid

End-point	Test system/species	Concentration/dose	Result	Reference
In vitro				
Reverse gene mutation	*Salmonella typhimurium* TA1535, TA1537 and TA90, TA100	Up to 4 000 µg/plate	> 10 000	Weir (1965a)
Cytotoxic above 4 000 µg/plate (+S9)	Negative	Haworth et al. (1983)	> 10 000	Weir (1967)
DNA double strand breaks (alkaline comet assay)	Human gingival fibroblasts	0.5, 1, 5, 10 mmol/L (6 hours)	Positive	Szczepanska et al. (2012)
DNA double strand breaks (neutral comet assay)	Human gingival fibroblasts	0.5, 1, 5, 10 mmol/L (6 hours)	Positive	Szczepanska et al. (2012)
DNA double strand breaks (γ-H2AX assay)	Human gingival fibroblasts	10 mmol/L (6 hours)	Positive	Szczepanska et al. (2012)
Plasmid relaxation	pUC19 plasmid	0.5, 1, 5, 10 mmol/L (6 hours)	Negative	Szczepanska et al. (2012)
DNA double strand breaks (γ-H2AX assay)	Human gingival fibroblasts 6 hours	1.56, 5.21, 15.64 mmol/L	Positive [a]	Yang et al. (2017)

CAS: Chemical Abstracts Service; S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] Cells treated with methacrylic acid at 5.21 and 15.64 mmol/L. Methacrylic acid had a statistically significant increase in the number of DNA strand break–foci/cells compared with control cells. The study authors did not class this as positive as it was not being used as an assay for genotoxicity screening, per se.

Table 3
Genotoxicity studies with methyl methacrylate

End-point	Test system/species	Concentration/dose	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Up to 1 000 µg/plate for preincubation and plate incorporation (±S9)	Negative	Lijinsky & Andrews (1980)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 0.15, 0.29, 0.59, 1.18, 2.35, 4.70 mg/plate (±S9)	Negative	Hachiya, Taketani & Takizawa (1982)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	40–10 000 µg/plate	Negative	Waegemaekers & Bensink (1984)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA1535	0, 33, 100, 333, 1 000, 3 333, 6 666 µg/plate	Negative	NTP (1986)
Reverse mutation	*S. typhimurium* TA97a, TA98, TA100, TA102	0–12.5 mg/plate	Negative	Schweikl, Schmalz & Rackebrandt (1998)
Cytotoxicity	V79 cells	0.94, 4.7, 9.4 mg/mL	Positive	Pradeep & Sreekumar (2012)
Mutation frequency	L5178Y mouse lymphoma cells	1, 1.75, 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3 mg/mL	Negative at 1 and 1.75 mg/mL; positive at other doses	Doerr, Harrington-Brock & Moore (1989)
Chromosomal aberration	CHO cells	0, 750, 1 000, 1 600, 3 000 µg/mL	Positive (−S9) Positive at top dose only (+S9)	NTP (1986)
Chromosomal aberration	Human lymphocytes	Cells mixed with 0.14 g (mean weight) of methyl methacrylate (97.4%) and *N*,*N*-dimethyl-para-toluidine to form bone cement	Negative	Bigatti et al. (1994)
Chromosomal aberration	L5178Y mouse lymphoma cells	1, 1.75, 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3 mg/mL	Negative at 1 and 1.75 mg/mL; positive at other doses	Doerr, Harrington-Brock & Moore (1989)
Chromosomal aberration	V79B/HPRT cells	0, 10, 20 mmol/L	Weakly positive	Schweikl, Schmalz & Rackebrandt (1998)
Chromosomal aberration	Human peripheral lymphocytes	Occupationally exposed individuals	Weakly positive	Tuček et al. (2002)
Chromosomal aberration	CHO cells	9.33×10^{-1}; 9.33×10^{-2}; 9.33×10^{-3}, 9.33×10^{-4} mg/mL	Positive	Yang et al. (2003)
Micronucleus induction	L5178Y mouse lymphoma cells	1, 1.75, 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3 mg/mL	Negative at 1 and 1.75 mg/mL; weakly positive at other doses	Doerr, Harrington-Brock & Moore (1989)
Micronucleus induction	Chinese hamster V79 cells	10, 20, 30 mmol/L	Negative cytotoxic at top dose	Schweikl, Schmalz & Spruss (2001)

Table 3 (continued)

End-point	Test system/species	Concentration/dose	Result	Reference
SCE	CHO cells	0, 750, 1 000, 1 250, 1 500 µg/mL	Positive ±S9	NTP (1986)
SCE	Human peripheral lymphocytes	0.001–1 mg/100 mL	Equivocal	Cannas et al. (1987)
SCE	CHO cells	9.33×10^{-1}, 9.33×10^{-2}, 9.33×10^{-3}, 9.33×10^{-4} mg/mL	Positive	Yang et al. (2003)
Gene mutation	Mouse lymphoma L5178Y $tk^{+/-}$ cells	0, 0.125, 0.25, 0.5, 0.75, 1.0 µg/mL (±S9)	Positive	NTP (1986)
Gene mutation	Mouse lymphoma L5178Y $tk^{+/-}$ cells	0, 1 000, 1 750, 2 202, 2 400, 2 499, 2 601, 2 700, 2 799, 2 901, 3 000 µg/mL (−S9)	Positive	Moore et al. (1988)
Gene mutation	Mouse lymphoma L5178Y $tk^{+/-}$ cells	0–3 000 µg/mL (±S9)	Positive (+S9); negative (−S9)	Dearfield et al. (1991)
In vivo				
Chromosomal aberration	Human peripheral lymphocytes of 38 occupationally exposed males	0.9–71.9 ppm	Negative	Seiji et al. (1994)
SCE	Human peripheral lymphocytes from 31 occupationally exposed men	Mean atmospheric concentrations 0.7–21.6 ppm	Negative overall, positive when comparing peak exposures of staff who clean facilities manually with staff who do not	Marez et al. (1991)
SCE	Peripheral lymphocytes of 38 occupationally exposed males	0.9–71.9 ppm	Negative	Seiji et al. (1994)
Micronucleus induction	Mouse bone marrow; oral route	Single doses of 1.13, 2.26, 4.52 g/kg bw	Negative	Hachiya, Taketani & Takizawa (1982)
Micronucleus induction	Bone marrow cells of Wistar rats exposed by the inhalation route	150 ppm for 8 hours/day for 1 or 5 days	Positive after 1 day, negative after 5 days	Araújo et al. (2013)
Micronucleus induction	Human buccal mucosal cells	Dental technicians exposed to methyl methacrylate monomer from dental resins	Negative	Azhar et al. (2013)

bw: body weight; CHO: Chinese hamster ovary; ppm: parts per million; S9: 9000 × *g* supernatant fraction from liver homogenate; SCE: sister chromatid exchange; $tk^{+/-}$: thymidine kinase locus

et al., 2002; Yang et al., 2003) and sister chromatid exchange (SCE) assays (NTP, 1986; Cannas et al., 1987; Yang et al., 2003). One in vitro micronucleus assay was unequivocally negative (Schweikl, Schmalz & Spruss, 2001), whereas a second assay was negative at low concentrations but weakly positive at higher concentrations (Doerr, Harrington-Brock & Moore, 1989). Three mouse lymphoma assays for gene mutations were positive (NTP, 1986; Moore et al., 1988; Dearfield et al., 1991). A mouse bone marrow micronucleus assay was negative, but it is not clear if the target tissue was exposed to the test substance (Hachiya, Taketani & Takizawa, 1982). A rat micronucleus assay with exposure by

Table 4
Genotoxicity studies with methyl acrylate

End-point	Test system/species	Concentration/dose	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537	0, 3 µmol/plate	Negative (±S9)	Florin et al. (1980)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 0.15, 0.29, 0.59, 1.18, 2.35, 4.70 mg/plate (±S9)	Negative	Hachiya, Taketani & Takizawa (1982)
Reverse mutation	*S. typhimurium* TA100, TA1535, TA1537, TA98. TA97	0, 10, 33, 100, 167, 333, 667, 1 000, 1 667, 3 333, 10 000 µg/plate	Negative	Zeiger et al. (1987)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	1 250 µg/plate (±S9)	Negative	Waegemaekers & Bensink (1984)
Chromosomal aberration	Human peripheral lymphocytes	Occupationally exposed individuals	Weakly positive	Tuček et al. (2002)
Chromosomal aberration assay AS52 /XPRT	CHO cells	0, 10, 12.5, 15, 17.5, 20, 22.5, 25 µg/mL (±S9)	Negative	Oberly et al. (1993)
Chromosomal aberration	CHO cells suspension assay	0, 10, 12, 14, 15, 15.5, 16, 16.5, 17.5, 18, 18.5, 19, 19.5, 20, 20.5 µg/mL	Negative	Moore et al. (1991)
Chromosomal aberration	CHO cells monolayer assay	0, 5, 10, 20, 40, 45, 50, 55, 60, 65, 70, 75, 80 µg/mL	Negative	Moore et al. (1991)
Chromosomal aberration	CHO (*hgprt* [a]) cells	0, 14, 16, 18 µg/mL	Negative for gene mutations; positive for clastogenicity (small colonies) but only at cytotoxic doses	Moore et al. (1989)
Gene mutation; clastogenicity	Mouse lymphoma L5178Y $tk^{+/-}$ cells	0, 16, 22, 24 µg/mL (−S9)	Negative for gene mutations; positive for clastogenicity (small colonies) but only at cytotoxic doses	Moore et al. (1989)
Chromosomal aberration (assay)	Mouse lymphoma L5178Y $tk^{+/-}$ cells	0, 16, 22, 24 µg/mL (−S9)	Positive for gene mutations	Moore et al. (1988)
DNA deletion	*Saccharomyces cerevisiae* RS112	0, 200, 500, 1 000 µg/mL (±S9)	Positive −S9, equivocal +S9	Kirpnick et al. (2005)
Micronucleus induction	CHO cells	0, 890, 1 187, 1 582 µg/mL (−S9) 0, 1 187, 1 582, 2 109 µg/mL (+S9)	Negative −S9, equivocal +S9	Kirpnick et al. (2005)
In vivo				
Micronucleus induction	IP injection, Balb C male mice, bone marrow	37.5, 75, 150, 300 mg/kg bw	Clastogenic	Przybojewska, Dziubaltowska & Kowalski (1984)
Micronucleus induction	Mouse ddY male, oral route, bone marrow	Single doses unless stated: 1.13 (×4) 62.5, 125, 250 mg/kg bw	Negative	Hachiya, Taketani & Takizawa (1982)
Micronucleus induction	Mouse ddY NR bone marrow. Inhalation route for 3 hours	Up to 2 100 ppm	Negative	Sofuni et al. (1984)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; IP: intraperitoneal; ppm: parts per million; S9: 9000 × *g* supernatant fraction from liver homogenate; $tk^{+/-}$: thymidine kinase locus
[a] Also known as the hprt locus.

inhalation was positive following 1 day of exposure but negative following 5 days of exposure (Araújo et al., 2013). These results were judged by the Committee to be inconclusive.

The Committee concluded that there was some evidence of mutagenicity and clastogenicity in vitro, but there was a lack of adequate in vivo tests following up the equivocal findings.

In 1999, IARC considered all the studies listed in Table 4 apart from those of Kirpnick et al. (2005). Methyl acrylate did not induce mutations in bacteria. In mammalian cells treated in vitro, methyl acrylate induced mutations at the *tk* locus in mouse cells, in the absence of exogenous metabolic activation, but not at the *hgprt* locus in Chinese hamster ovary cells. It induced chromosomal aberrations in mouse and Chinese hamster cells in vitro. Of three micronucleus assays of methyl acrylate using mouse bone marrow in vivo, two (oral and inhalation routes) were negative. A third mouse micronucleus assay in bone marrow cells using intraperitoneal administration was positive. IARC concluded that the clastogenic activity of methyl acrylate seen in vitro is not readily detected in vivo (IARC, 1999).

The Committee considered the genotoxicity studies on methyl acrylate. Reverse mutation assays in bacteria were negative (Florin et al., 1980; Waegemaekers & Bensink, 1984). A well-performed genotoxicity study in L5178Y mouse lymphoma cells, without exogenous metabolism, produced positive outcomes for gene mutation and chromosomal aberrations, but only at cytotoxic concentrations (Moore et al., 1988). The in vivo study designs are limited, with methodological shortcomings. A mouse bone marrow micronucleus assay, using intraperitoneal dosing, showed an increase in micronucleus induction that was limited in magnitude and not dose related (Przybojewska, Dziubaltowska & Kowalski, 1984). A mouse bone marrow micronucleus assay using oral dosing was negative, but it is not clear if the target tissues were exposed to the test substance (Hachiya, Taketani & Takizawa, 1982).

The Committee concluded that the overall data were not sufficient to draw conclusions on the genotoxicity of methyl acrylate.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

No reproductive studies were available on AMC.

(b) Developmental toxicity

In a GLP-compliant study said to be conducted according to OECD TG 414, two groups of female rats were administered 0 (vehicle only) or 1000 mg/kg bw per day of AMC (freeze-dried powder, ~100% polymer; dissolved in 0.5% Na-CMC)

by gavage on gestation days 5–19. The Committee noted that only one test dose was used, which is not consistent with OECD TG 414. The females were observed daily and body weights and feed consumption measured regularly. Caesarean sections were carried out on day 20, and fetuses were examined in detail.

No abortions or preterminal deaths were noted in either group. No statistically significant differences were observed in weight gain or corrected weight gain, feed consumption, corpora lutea and implantations per dam, resorptions per litter, live birth index, postimplantation loss index, live fetuses per litter, sex ratio, fetal and placental weights, total malformations or litters affected. A statistically significant increase in gravid uterine weight in the test group ($P \leq 0.05$) was attributed to the slightly higher number of fetuses in this group. One treated animal showed hunched posture, pale skin, reduced activity and a rough coat on one day of the study, and a transient skin lesion was noted in another treated animal. Neither finding was considered to be treatment related. Five skeletal malformations were found in control group fetuses and three in the test group. One fetus with major visceral malformations was identified in the control group and none in the test group. Minor malformations were observed in fetuses from both groups but their distribution did not suggest a treatment-related effect.

The NOAEL was 1000 mg/kg bw for this study (Harlan, 2003), the only dose tested.

2.2.6 Special studies

(a) Cytotoxicity

In a GLP-compliant cytotoxicity study, AMC (residual monomers <1 mg/L) at concentrations of 5000, 3333, 2222, 1482, 988 or 658 mg/mL (concentrations expressed as dry polymer), the vehicle and a positive control were incubated with L929 mouse connective tissue cells for 72 hours.

Growth inhibition (88%) was observed in the positive control and at all concentrations of AMC (between 92% and 96%). The test compound was found to be cytotoxic at the tested concentrations (BSL, 1998a).

In a repeat of this study using preparation 4135F (obtained from the above formulation through "stress coagulation"; ~100% dry polymer; residual monomers methacrylic acid 9 mg/L; methyl acrylate 4 mg/L; methyl methacrylate 17 mg/L), the highest concentration of 200 mg/mL produced a growth inhibition of 32% and the lower concentrations induced cell growth inhibition of 7–23% (BSL, 1998b).

(b) **Dermal toxicity**

A single dose of 2000 mg/kg bw of AMC (mixed to a paste with sterile water; dose expressed as dried polymer) applied dermally to rats produced no effects on clinical signs, body weight or necropsy results (RTC, 1996c).

A single dose of 500 mg/kg bw of the AMC (mixed to a paste with sterile water; dose expressed as dried polymer) applied dermally to rabbits produced no effects on clinical signs, body weight or necropsy results (RTC, 1996d).

In a GLP-compliant study conducted according to OECD TG 406, female guinea-pigs (20/test group; 10 control animals). Three pairs of intradermal injections were administered to the clipped scapular region of each animal as follows: anterior, emulsified Freund's complete adjuvant; median, 2% AMC in Alembicol D; and posterior, 2% AMC in emulsified Freund's complete adjuvant. A volume of 0.1 mL was injected at each single site. Test animals then received the following injections: anterior, emulsified Freund's complete adjuvant; median, vehicle (Alembicol D); and posterior, vehicle mixed with emulsified Freund's complete adjuvant.

Minor reactions were observed at the injection sites following application of Freund's complete adjuvant (test and control groups), vehicle alone (control group) and AMC in vehicle (test group). No skin irritation was observed following 48 hours of exposure to AMC or the vehicle or during the 24-hour challenge phase of the experiment. Body weights were unaffected by treatment and no findings during necropsy suggested a treatment-related effect. Two animals in the control group died during the experiment, but these deaths were not considered treatment related (RTC, 1996e).

(c) **Inhalation toxicity**

Ten rats were exposed by nose-only inhalation to a 30% aqueous dispersion of AMC at an aerosol concentration of 3.999 mg/L air. No deaths, no clinical signs and no macroscopic pathology findings were observed. Marginal to marked losses in body weight were observed in two males and all the females, and slight growth retardation was observed in the other three males between days 1 and 4. These effects were transient and were followed by normal growth and body-weight gain (RCC, 2004).

(d) **Ocular toxicity**

In a GLP-compliant study conducted according to OECD TG 405, a 100 mg aliquot of dried AMC (~100% dried polymer) was introduced into one eye of each of three male rabbits. The eye was examined macroscopically after 1, 24, 48 and 72 hours and 7 days for signs of irritation and damage.

Evidence of corneal opacity (severity score 1), iris inflammation (severity score 1) and well-defined conjunctival irritation (severity score 2) was observed in the treated eye of all animals 1 hour after treatment but these had resolved after 24 hours. No indication of systemic toxicity or effects on body weight were identified (RTC, 1996f).

(e) Phototoxicity

In an in vitro study using the BALB/3T3 mouse fibroblast cell line, AMC (freeze-dried polymer) was dissolved in dimethyl sulfoxide (DMSO) and diluted to 100 mg/mL in Earle's balanced salt solution (EBSS). Cultured cells were incubated in the dark for 60 minutes with 1% DMSO in EBSS (negative control); chlorpromazine at 100, 31.6, 10, 3.16, 1, 0.316, 0.1 or 0.0316 mg/mL (positive control); test groups: test solution in vehicle at 100, 31.6, 10, 3.16, 1, 0.316, 0.1 and 0.0316 mg/mL (test groups); and EBSS buffer only. Following incubation, a plate was irradiated with ultraviolet A for 50 minutes while a duplicate plate was kept in the dark. After overnight incubation, cells were exposed to neutral red dye for 3 hours and washed. Dye uptake was measured spectrophotometrically.

Dye uptake did not differ significantly between groups.

AMC was considered to be non-phototoxic (BSL, 2002).

2.2.7 Residual components of AMC (other than monomers)

(a) Sodium lauryl sulfate

AMC contains 0.3% sodium lauryl sulfate, a surfactant permitted as a food additive in the United States of America at a maximum of 1000 parts per million (ppm). It has Generally Recognized as Safe (GRAS) status (USFDA, 2017). In a review of toxicity data (Committee for Human Medicinal Products, 2015), the NOAELs were as shown in Table 5.

Maximum exposure to AMC in children is 43 mg/kg bw per day (see section 3), from which it can be calculated that exposure to sodium lauryl sulfate is 0.12 mg/kg bw per day. Using the lowest NOAEL, a margin of exposure of greater than 400 was calculated, indicating that the presence of sodium lauryl sulfate at 0.3% in AMC is not of toxicological concern.

(b) Polysorbate 80

AMC contains 1.2% polysorbate 80 (polyoxyethylene(20)sorbitan monooleate). The Committee previously established an acceptable daily intake (ADI) of 0–25 mg/kg bw (Annex 1, reference *33*).

Maximum exposure to AMC in children is 43 mg/kg bw per day, from which it can be calculated that exposure to polysorbate 80 is 0.48 mg/kg bw per

Table 5
Summary of toxicity data on sodium lauryl sulfate

Species	Study length	NOAEL [a]	Reference
Rat	13 weeks	1 000 (50)	Walker et al. (1967)
Rat	2 years	10 000 (500)	Fukuzawa et al. (1978)
Rat	2 years	10 000 (500)	Fitzhugh & Nelson (1948)
Dog	1 year	20 000 (500)	Cosmetic Ingredient Review (1983)

bw: body weight; NOAEL: no-observed-adverse-effect level; ppm: parts per million
[a] NOAEL expressed as ppm in the diet and, in parentheses, the equivalent mg/kg bw per day.
Source: Committee for Human Medicinal Products (2015)

day. This is less than 2% of the ADI, indicating that the presence of polysorbate 80 at 1.2% in AMC is not of toxicological concern.

(c) Simethicone

AMC contains the antifoaming agent simethicone (dimethylpolysiloxane) at not more than 20 mg/kg (0.002%). The Committee has previously established an ADI of 0–1.5 mg/kg bw (Annex 1, reference *205*).

Maximum exposure to AMC in children is 43 mg/kg bw per day, from which it can be calculated that exposure to simethicone is 0.48 mg/kg bw per day. This is 0.06% of the ADI, indicating that the presence of simethicone at 0.002% in AMC is not of toxicological concern.

The Committee concluded that these residual components did not pose a safety concern at the maximum estimated exposure levels.

2.3 Observations in humans

No data on humans were available.

3. Dietary exposure

3.1 Introduction

AMC was evaluated by the Committee at the request of the Codex Committee on Food Additives at its Forty-ninth session (FAO/WHO, 2017). Codex asked the Committee to conduct a safety assessment of the use of AMC as a glazing or coating agent in food supplements.

Uses or proposed uses for AMC include in pharmaceuticals, food supplements and foods for special medical purposes. AMC has been used in pharmaceuticals since 1999. It is used as a glazing or enteric coating agent in pharmaceuticals and on solid food supplements, to protect the stomach against irritating ingredients or to protect sensitive nutrients against disintegration by stomach acid, bile acid and intestinal enzymes. It is also formulated as a pH-dependent delayed-release coating, for dissolution at above pH 7, to permit the release of active ingredients, for example, probiotics, in the colon (Eisele et al., 2016). AMC is used in products in single dosage unit forms – capsules, pastilles, tablets, pills and hard or soft gel capsules as well as multiparticulates such as powders, granules or pellets.

The copolymer is not used for liquid supplements, and this form was not included in this assessment.

3.2 Approach to the exposure assessment

JECFA reviewed exposure estimates calculated and submitted by the sponsor. The exposure assessment included information provided by the sponsor. This information included uses of the copolymer, the concentrations it is used in and estimates of exposure. The Committee also evaluated the EFSA exposure assessment (EFSA, 2010) and conducted a literature search (see section 1.2).

The Committee also undertook deterministic calculations to estimate exposure to the copolymer where national food consumption data were available. These data were primarily from the FAO/WHO Chronic Individual Food Consumption – Summary statistics (CIFOCOss) database (https://www.who.int/foodsafety/databases/en/) for Codex General Standard for Food Additives (GSFA) food category no. 13.6, "food supplements". Consumption data were also submitted for Australia and New Zealand. These are further discussed in section 3.4(c).

Exposures were reviewed or estimated for mean and high consumers as well as for adults and children.

In their assessment, the sponsor included children aged 6 years and older based on the assumption that younger children take supplements in liquid form. However, the Committee also included children aged between 1 and 6 years in its assessment based on evidence recorded in national dietary surveys (e.g. CIFOCOss) of consumption of solid supplements by children this age. In addition, some chewable tablets and soft gels are suitable for use by children from 2 years of age.

Estimates of exposure are included in the assessment for AMC, its monomers and the relevant metabolites based on the toxicological evaluation (section 3.5).

3.3 Estimates of exposure to AMC

3.3.1 Food supplements

(a) Estimates from the sponsor

The sponsor provided use levels of AMC in food supplements. Only small amounts of AMC are needed as its enteric properties are achieved at 5 mg/cm^2, which equates to approximately 30 mg for a 1 g tablet or dosage unit. For special applications, higher amounts are necessary in the coating, with the highest amount approximately 100 mg for a tablet weighing 1 g. This was the concentration used in the exposure assessments. Although larger unit weights are on the market, these do not represent the majority of the relevant products and they were not considered in this assessment.

It was assumed that if different dosage forms (tablets, powders, etc.) with different surface area to volume ratios were used, the same concentration applies.

Consumption amounts for food supplements were evaluated by the sponsor from previous evaluations conducted by EFSA (2004a, 2004b, 2005, 2006) and the Scientific Committee on Food (SCF) for other food additives used in this group (SCF, 1997, 2002). The number of dosage units consumed per day were between 3 and 7 for adults (12 for extreme users) and 2 for "children/young people" (ages between 6 and 18 years).

The sponsor also reviewed national consumption data from a range of dietary surveys; these mostly included data on frequency of consumption, but included some data for consumption amounts (for a summary, see Appendix 1). Consumption data from the Netherlands (Ocké, Buurma-Rethans & Fransen, 2005) indicated that 80% of users consumed one or two supplements twice a day (a total of 4 units/day), and 6% were high users who consumed four supplements 2 times per day (a total of 8 supplements/day). Having assumed that one of these supplements is in liquid form and that not all supplements contain AMC, the sponsor calculated consumption by adults to be 4 units/day, by heavy users to be 6 units/day and by children (not younger than 6 years) to be 2 units/day.

Based on these estimates and the assumption that AMC was present in the coatings of 1 g dosage units at 100 mg, the sponsor estimated exposures to AMC from expected consumption of food supplements to be 6.7 mg/kg bw per day in adults (10 in heavy users) and 13.3 mg/kg bw per day in children (Table 6).

The sponsor used a body weight of 15 kg for children 6 years and older. The WHO standard body weight for a 5 year old at the 50th percentile is 18.3 kg for boys and 18.2 kg for girls (WHO Multicentre Growth Reference Study Group, 2006). Therefore, the body weight used by the sponsor for this population group may be an underestimate, resulting in a worst case scenario estimate of exposure on a body weight basis.

Table 6
Estimated exposures to AMC by the sponsor from use in food supplements

Population	No. of units consumed/day [a]	Exposure	
		mg per day	mg/kg bw per day [b]
Adults – expected consumption	4	400	6.7
Adults – heavy user	6	600	10.0
Young people / children 6–18 years	2	200	13.3

AMC: anionic methacrylate copolymer; bw: body weight; no.: number
[a] One unit equates to 1 g of supplement containing 100 mg of AMC.
[b] Based on a mean body weight of 60 kg for adults and 15 kg for children.

Table 7
Estimated exposures to AMC from use in food supplements by high consumers

Population	No. of units consumed/day	Exposure			
		30 mg per 1 g dosage unit [a]		100 mg per 1 g dosage unit [b]	
		mg per day	mg/kg bw per day [c]	mg per day	mg/kg bw per day [c]
Adults 19–64 years	7	210	3.5	700	11.7
Children 4–18 years	2	60	2.4	200	8.0

AMC: anionic methacrylate copolymer; bw: body weight
[a] Concentration used for enteric properties.
[b] Highest coating level.
[c] Based on a mean body weight of 60 kg for adults and 25 kg for children.
Source: EFSA (2010)

(b) Estimates of exposure calculated by EFSA

EFSA estimated exposures to AMC from its use in food supplements (EFSA, 2010). They used two concentrations of AMC in supplements: 30 mg per 1 g dosage unit for enteric properties and 100 mg per 1 g dosage unit as the highest coating level. The assessment used consumption data from the British National Diet and Nutrition Surveys for high consumers (97.5th percentile) of food supplements for adults aged 19–64 years (Henderson, Gregory & Swan, 2002) and children aged 4–18 years (Gregory & Lowe, 2000). Seven and 2 dosage units per day were used for adults and children, respectively.

EFSA estimated exposures in high consumers using 30 mg of AMC per 1 g dosage unit at 3.5 mg/kg bw per day for adults and 2.4 mg/kg bw per day for children 4–18 years old. Exposures in high consumers using 100 mg AMC per 1 g dosage unit were estimated at 11.7 mg/kg bw per day for adults and 8.0 mg/kg bw per day for children (Table 7).

These estimates did not take into account that not all supplements contain AMC, and therefore may be overestimates. However, if a consumer was brand

loyal to a particular AMC-containing supplement that they consumed regularly, these estimates would be appropriate.

The Committee used the estimated exposures from the 100 mg per 1 g dosage unit scenario for its evaluation, this being a worst case scenario.

(c) National estimates of exposure calculated by the Committee

The Committee estimated exposure to AMC from food supplement use for a range of countries using consumption data in the CIFOCOss database (GSFA food category no. 13.6, "food supplements").

CIFOCOss includes data collected via national population dietary surveys of 2 or more days of individuals' food consumption (for further details, see http://www.who.int/foodsafety/databases/en/). Food consumption data are presented according to the CIFOCOss food classification system. For most food groups, this is based on the Codex raw commodity classification system; for some processed commodities, it is based on the Codex GSFA classification system. Food consumption data for a number of different age groups are represented in the CIFOCOss dataset based on the data collected in each of the national surveys. The Committee used consumption data for the general population and for children for the exposure estimates; the data were not split by sex.

Food supplement consumption data were available in CIFOCOss for nine countries (Brazil, Finland, Germany, Ireland, Italy, the Netherlands, Sweden, Thailand, the United Kingdom). However, as there were no quantitative consumption amounts for Thailand, that country was not included. Where there were fewer than 10 consumers per population group, these data were not used for exposure calculations as having too few consumers does not produce reliable estimates. Thus, data for Germany (children), Italy (infants, children and adolescents) and the Netherlands (toddlers and children) were excluded. The data from Brazil were also excluded as levels of consumption were at least 5 times higher than those of the other population groups included in the dataset; as no explanation was provided (e.g. what was included in the food group), these data were determined to be outliers and not retained for the exposure calculation. There were no consumption data for food supplements for infants in CIFOCOss.

Mean and 90th percentile consumption data per kg body weight for consumers only were used for the exposure calculations to take into account the specific body weights relevant to each country and population group. Concentrations of AMC used in the calculations were those provided by the sponsor: 100 mg per 1 g dosage unit.

Consumption data for use in the assessment for dietary supplements were also provided to the Committee for Australia and New Zealand (Food Standards Australia New Zealand [FSANZ], personal communication, 30 May 2018). The

Australian data were from the 2011/12 National Nutrition and Physical Activity Survey (ABS, 2015) based on 2 days of consumption data. The New Zealand data were from the 2002 Children's Nutrition survey (Parnell et al., 2003) and the 2008/09 NZ Adult Nutrition Survey (University of Otago and Ministry of Health, 2011) based on 1 day of consumption data. Liquid supplements were excluded from the derivation.

The actual consumption amounts as reported in the CIFOCOss database, shown in Table 8 in g/kg bw per day, were used for the exposure assessment calculations. The consumption amounts were not rounded to a number of dosage units for the exposure calculations, but were converted to 1 g units to compare with the number of dosage units used by the sponsor and EFSA. Based on mean consumption data, the number of dosage units consumed by adults ranged between 3 and 7 units per day and, at the 90th percentile, between 5 and 20 units per day. Similarly, based on mean consumption data, the number of dosage units consumed by children ranged between 1 and 4 per day and, based on 90th percentile consumption data, between 2 and 10 per day. For adults, the mean consumption range was the same as the range used by the sponsor and EFSA, but at the 90th percentile, the upper end of the consumption range was nearly 3 times as high. The lower end of the mean consumption range for children was similar to that used by the sponsor and EFSA, but the upper end of the range was about double and the upper end of the range for the 90th percentile was about 5 times higher. Apart from the values from Australia and New Zealand, the specific types of supplements – and whether this included liquid supplements – is unknown.

Exposures to AMC from use in food supplements estimated by the Committee from national food consumption data for adults at the mean ranged between 3.5 and 11.6 mg/kg bw per day and, at the 90th percentile, between 7.4 and 37.0 mg/kg bw per day (Table 8). For children, mean exposure ranged between 2.9 and 16.0 mg/kg bw per day and, at the 90th percentile, between 4.2 and 42.7 mg/kg bw per day. These estimates do not take into account that not all supplements contain AMC and therefore may be overestimates. However, if a consumer was brand loyal to a particular supplement that contained AMC and consumed it regularly, these estimates would be appropriate.

3.3.2 Pharmaceuticals

(a) Estimates from the sponsor

AMC has been used in pharmaceuticals in Europe since 1999, and exposure from this source was also considered in the estimates by the sponsor. The same concentration was used for pharmaceuticals as for food supplements (100 mg/1 g dosage unit). While the sponsor indicated that previous EFSA assessments were based on consumption of 10–12 units of pharmaceuticals per day, this was also

Table 8
Estimated national exposures to AMC from use as a food additive in food supplements [a]

Population / country	Age group (years)	No. of consumers	Percentage of consumers	Consumption (g/kg bw per day)		Exposure (mg/kg bw per day)	
				Mean	P90	Mean	P90
Adults							
Australia	≥19	2 284	38	0.03	0.08	3.5	7.7
Finland	18–64	663	42	0.04	0.08	4.2	7.6
	65–74	215	46	0.05	0.11	5.0	10.8
Ireland	18–64	246	26	0.04	0.09	4.3	8.6
Italy	18–64	98	4	0.08	0.20	8.2	20.0
	65–74	12	4	0.04	0.11	3.7	10.9
	≥75	17	7	0.12	0.37	11.6	37.0
New Zealand	≥15	576	12	0.05	0.09	4.7	9.1
United Kingdom	18–64	419	20	0.05	0.07	4.5	7.4
Children							
Australia	2–5	75	19	0.12	0.26	12.0	25.7
	6–18	236	18	0.04	0.06	3.6	6.3
Finland	3–9	56	22	0.16	0.43	16.0	42.7
New Zealand	5–14	144	4	0.05	0.09	4.8	8.8
Sweden	3–9	263	18	0.08	0.10	8.0	9.6
	10–17	178	17	0.03	0.04	2.9	4.2

AMC: anionic methacrylate copolymer; bw: body weight; no.: number; P90: 90th percentile
[a] Calculated by the Committee using national dietary survey data.

considered a conservative estimate given that not all pharmaceuticals contain AMC. As a result, 6 units/day was used in the exposure assessment for adults and 2/day for young children.

Exposures to AMC from use in pharmaceuticals were estimated to be 10 mg/kg bw per day for adults and 13.3 mg/kg bw per day for children (Table 9).

(b) Estimates of exposure calculated by EFSA

For its estimate of exposures to AMC from use in pharmaceuticals, EFSA (2010) also used two concentrations of AMC in supplements: 30 mg per 1 g dosage unit and 100 mg per 1 g dosage unit. The same number of dosage units used for food supplements were used in the assessment of pharmaceuticals: for adults, 7 units per day, and for children, 2 units per day.

EFSA estimated exposure to AMC when used in pharmaceuticals at 30 mg per 1 g dosage unit to be 3.5 mg/kg bw per day for adult high consumers and 2.4 mg/kg bw per day for children 4–18 years old. Exposure to AMC when used in pharmaceuticals at 100 mg per 1 g dosage unit was estimated for adults as 11.7

Table 9
Estimated exposures to AMC by the sponsor from use in pharmaceuticals

Population	Units consumed per day [a]	Exposure	
		mg per day	mg/kg bw per day [b]
Adults	6	600	10.0
Young people / children 6–18 years	2	200	13.3

AMC: anionic methacrylate copolymer; bw: body weight
[a] One unit is 1 g containing 100 mg of AMC.
[b] Based on a mean body weight of 60 kg for adults and 15 kg for children

Table 10
Estimated exposures to AMC from use in pharmaceuticals for high consumers

Population	No. of units consumed/ day	Exposure			
		30 mg per 1 g dosage unit [a]		100 mg per 1 g dosage unit [b]	
		mg per day	mg/kg bw per day [c]	mg per day	mg/kg bw per day [c]
Adults 19–64 years	7	210	3.5	700	11.7
Children 4–18 years	2	60	2.4	200	8.0

AMC: anionic methacrylate copolymer; bw: body weight
[a] Concentration used for enteric properties.
[b] Highest coating level.
[c] Based on a mean body weight of 60 kg for adults and 25 kg for children.
Source: EFSA (2010)

mg/kg bw per day for heavy users and 8.0 mg/kg bw per day for children 4–18 years old (Table 10).

These estimates did not take into account that not all pharmaceuticals contain AMC, and therefore are likely to be overestimates.

The Committee used the estimated exposures from the 100 mg per 1 g dosage unit scenario for its evaluation, this being a worst case scenario.

3.3.3 Foods for special medical purposes

AMC can be used in foods for special medical purposes as incomplete foods when these are presented in the same form of solid food supplements (e.g. tablets, capsules, etc.). The single dosage units are likely to contain the same concentration of AMC as food supplements, that is, 10% of the final dosage unit (100 mg/1 g unit).

Foods for special medical purposes are generally defined as those formulated for exclusive or partial dietary management of patients under medical supervision who have limited ability to digest or metabolize foods or nutrients or who have increased nutrient requirements. These products may contain higher concentrations of active ingredients compared with food supplements for the

general population. Foods for special medical purposes would not normally be consumed by the general population but by specific individuals who would generally be under medical supervision.

The duration of use of foods for special medical purposes depends on the medical indication and may vary from short-term to lifetime use. Consumption of foods for special medical purposes, and exposures to AMC from these, depends on the dosage instructions and/or the medical needs of the individual and are therefore highly variable.

No food consumption data for foods for special medical purposes were provided by the sponsor. While there are some consumption data for foods for special medical purposes in the CIFOCOss database, there is insufficient information to determine what foods specifically are included by each country in this category and therefore it was not possible to determine if it includes single dosage units. Therefore, the data extracted from CIFOCOss could not be used to estimate exposures from foods for special medical purposes.

A literature search found no consumption data for foods for special medical purposes. Because the Committee was unable to estimate dietary exposures from these foods, there is a degree of uncertainty in the Committee's exposure assessment and the overall safety evaluation. However, for individuals managing a condition or disease by consuming foods for special medical purposes, achieving a positive health status may be a priority greater than the need to consider exposure to AMC from these foods.

The sponsor noted that the AMC-containing foods used for special medical purposes are similar to food supplements. Given the conservative nature of the calculations, which are based on high consumers, foods for special medical purposes are not anticipated to increase exposures above that of food supplements and pharmaceuticals. However, there are no data or information to suggest this is the case.

The use of AMC in foods for special medical purposes was therefore not considered by the Committee.

3.4 Estimates of exposure to the monomers of AMC and relevant AMC metabolites

The Committee considered the data on the residual monomers, as well as that for AMC itself, because the monomers are of low molecular weight and therefore likely to be absorbed from the gastrointestinal tract. The AMC monomers are methacrylic acid, methyl methacrylate and methyl acrylate.

The Committee also considered total exposure to methacrylic acid; this took into account the summed exposure from the monomer and the hydrolysis

of methyl methacrylate to methacrylic acid (and alcohol). Estimated exposures to the metabolites and total exposures to methacrylic acid provided by the sponsor and the EFSA (2010) assessment were reviewed and the national estimates of exposure were calculated by the Committee.

The total monomer content is up to 0.01% of the copolymer, and this proportion was used to convert the estimates of exposure to the copolymer to an exposure to the total monomers.

The weight ratio of the monomers in the copolymer was used to convert the total exposure to all monomers to an exposure for each individual monomer.

The estimated total exposure to methacrylic acid was derived from the sum of the methacrylic acid monomer exposure and the methacrylic acid product of methyl methacrylate metabolism (based on a conversion using molecular weights of 100.1 for methyl methacrylate and 86.1 for methacrylic acid).

3.4.1 Estimates from the sponsor

Exposures to the monomers as estimated by the sponsor from uses in food supplements and pharmaceuticals are shown in Table 11. For adults, exposures from food supplements were 0.07–0.10 μg/kg bw per day for methacrylic acid, 0.17–0.25 μg/kg bw per day for methyl methacrylate and 0.43–0.64 μg/kg bw per day for methyl acrylate (range based on 4–6 dosage units/day). For children, the exposures from food supplements were 0.14 μg/kg bw per day for methacrylic acid, 0.34 μg/kg bw per day for methyl methacrylate and 0.86 μg/kg bw per day for methyl acrylate (based on 2 dosage units/day).

Exposures from pharmaceuticals for adults were 0.10 μg/kg bw per day for methacrylic acid, 0.25 μg/kg bw per day for methyl methacrylate and 0.64 μg/kg bw per day for methyl acrylate (based on 6 dosage units per day). Exposures from pharmaceuticals for children were exactly the same as for food supplements as it was assumed that the same number of dosage units were ingested.

The estimated total exposures to the metabolite methacrylic acid from food supplements were 0.21–0.32 μg/kg bw per day for adults (based on a range of 4–6 dosage units per day) and 0.43 μg/kg bw per day for children (from 2 dosage units/day). Adult exposure to total methacrylic acid from pharmaceuticals was 0.32 μg/kg bw per day (based on 6 dosage units/day) and for children was 0.43 μg/kg bw per day (based on 2 dosage units/day).

3.4.2 Estimates of exposure calculated by EFSA

EFSA (2010) estimated exposures to AMC monomers expressed as the metabolite methacrylic acid. The EFSA estimate was based on AMC in the coating at 10% of the final dosage unit (100 mg/1 g unit) (for both supplements and pharmaceuticals) and a total exposure to the copolymer from food supplements

Table 11
Sponsor-estimated exposures to AMC, AMC monomers and total methacrylic acid from uses in food supplements and pharmaceuticals

Source	Population	Exposure to AMC (mg/kg bw per day) [a]	Exposure to AMC monomers (µg/kg bw per day)				Total methacrylic acid exposure (µg/kg bw per day) [d]
			Total [b]	Methacrylic acid [c]	Methyl methacrylate	Methyl acrylate	
Food supplements	Adults	6.7–10.0	0.7–1.0	0.07–0.10	0.17–0.25	0.43–0.64	0.21–0.32
	Children	13.3	1.3	0.14	0.34	0.86	0.43
Pharmaceuticals	Adults	10.0	1.0	0.10	0.25	0.64	0.32
	Children	13.3	1.3	0.14	0.34	0.86	0.43

AMC: anionic methacrylate copolymer; bw: body weight
[a] Based on consumption of 4–6 dosage units per day of supplements and 6 dosage units per day of pharmaceuticals for adults; and 2 dosage units per day of supplements and 2 dosage units per day of pharmaceuticals for children. Body weights assumed to be 60 kg for adults and 15 kg for children.
[b] Based on a total monomer content of 0.01% of the copolymer.
[c] Not including from methyl methacrylate.
[d] Sum of the exposure to methacrylic acid monomers and methacrylic acid from methyl methacrylate, with a conversion using molecular weights.

and pharmaceuticals combined at 23.4 mg/kg bw per day for adults and 16.0 mg/kg bw per day for children. EFSA multiplied the value estimated for copolymer exposure by 0.01% to obtain the monomeric exposure, assuming equal residual amounts of monomer in relation to their molar ratio in the copolymer.

Exposure for adults to methacrylic acid from food supplements and pharmaceuticals combined was estimated by EFSA at 0.76 µg/kg bw per day for adults and 0.54 µg/kg bw per day for children.

The Committee also estimated exposure to the AMC monomers and relevant metabolites based on EFSA's estimated exposure to the copolymer. Exposures for adults from food supplements were 0.12 µg/kg bw per day for methacrylic acid, 0.30 µg/kg bw per day for methyl methacrylate and 0.75 µg/kg bw per day for methyl acrylate; and for children were 0.08 µg/kg bw per day for methacrylic acid, 0.20 µg/kg bw per day for methyl methacrylate and 0.52 µg/kg bw per day for methyl acrylate. Exposures from pharmaceuticals were estimated to be the same as for food supplements as the number of dosage units was assumed to be equal.

The Committee's estimates of exposures to total methacrylic acid, including weighting for the monomer weight ratio and converting based on molecular weights, were similar to those of EFSA when summed for food supplements and pharmaceuticals (Table 12). For adults, exposure to total methacrylic acid from food supplements alone was estimated at 0.37 µg/kg bw per day; for children, exposure was 0.26 µg/kg bw per day. Exposures from pharmaceuticals were exactly the same as for food supplements as the number of dosage units ingested was assumed to be the same.

Table 12

EFSA-estimated exposures to AMC, AMC monomers and total methacrylic acid for Europe from uses in food supplements and pharmaceuticals

Source	Population	Exposure to AMC (mg/kg bw per day) [a]	Exposure to AMC monomers (µg/kg bw per day)				Total methacrylic acid exposure (µg/kg bw per day) [e]
			Total [b,c]	Methacrylic acid [d]	Methyl methacrylate	Methyl acrylate	
Food supplements	Adults	11.7	1.2	0.12	0.30	0.75	0.37
	Children	8.0	0.8	0.08	0.20	0.52	0.26
Pharmaceuticals	Adults	11.7	1.2	0.12	0.30	0.75	0.37
	Children	8.0	0.8	0.08	0.20	0.52	0.26

AMC: anionic methacrylate copolymer; bw: body weight; EFSA: European Food Safety Authority

[a] EFSA estimates based on consumption of 7 dosage units per day of food supplements and of pharmaceuticals for adults and 2 dosage units per day of food supplements and of pharmaceuticals for children. Body weights assumed to be 60 kg for adults and 25 kg for children.

[b] Based on a total monomer content of 0.01% of the copolymer.

[c] Estimated by the Committee based on the EFSA (2000) estimate of copolymer exposure.

[d] Not including from methyl methacrylate.

[e] Sum of the exposure to methacrylic acid monomers and methacrylic acid from methyl methacrylate, with a conversion using molecular weights.

3.4.3 National estimates of exposure calculated by the Committee

The Committee estimated national exposures to the AMC monomers and to total methacrylic acid from uses of the copolymer in food supplements (Table 13).

Estimated exposures for adults to the monomer methacrylic acid at the mean ranged between 0.04 and 0.12 µg/kg bw per day and, at the 90th percentile, between 0.08 and 0.38 µg/kg bw per day; to methyl methacrylate at the mean ranged between 0.09 and 0.30 µg/kg bw per day and, at the 90th percentile, between 0.19 and 0.94 µg/kg bw per day; and to methyl acrylate at the mean ranged between 0.22 and 0.75 µg/kg bw per day and, at the 90th percentile, between 0.48 and 2.39 µg/kg bw per day.

Estimated exposures for children to the monomer methacrylic acid at the mean ranged between 0.03 and 1.60 µg/kg bw per day and, at the 90th percentile, between 0.04 and 0.43 µg/kg bw per day; to methyl methacrylate at the mean ranged between 0.07 and 0.41 µg/kg bw per day and, at the 90th percentile, between 0.11 and 1.09 µg/kg bw per day; and to methyl acrylate at the mean ranged between 0.19 and 1.03 µg/kg bw per day and, at the 90th percentile, between 0.27 and 2.75 µg/kg bw per day.

Estimated exposures for adults for the relevant metabolite, namely, total methacrylic acid at the mean ranged between 0.11 and 0.37 µg/kg bw per day and, at the 90th percentile, between 0.24 and 1.19 µg/kg bw per day; and for children at the mean ranged between 0.09 and 0.51 µg/kg bw per day and, at the 90th percentile, between 0.13 and 1.37 µg/kg bw per day.

These estimates do not take into account that not all supplements will contain AMC; as such, they are likely to be overestimates. However, if a consumer

Table 13

Committee-estimated exposures to AMC, AMC monomers and total methacrylic acid from AMC uses in food supplements based on national food consumption data [a]

		Exposure to monomers (µg/kg bw per day)								Total methacrylic acid exposure (µg/kg bw per day) [d]	
		Total [b]		Methacrylic acid [c]		Methyl methacrylate		Methyl acrylate			
Country	Population / years	Mean	P90	Mean	P90	Mean	P90	Mean	P90	Mean	P90
Australia	Adults ≥19	0.35	0.77	0.04	0.08	0.09	0.20	0.22	0.49	0.11	0.25
Finland	Adults 18–64	0.42	0.76	0.04	0.08	0.11	0.19	0.27	0.49	0.13	0.24
	Elderly adults 65–74	0.50	1.08	0.05	0.11	0.13	0.27	0.32	0.69	0.16	0.35
Ireland	Adults 18–64	0.43	0.86	0.04	0.09	0.11	0.22	0.28	0.56	0.14	0.28
Italy	Adults 18–64	0.82	2.00	0.08	0.20	0.21	0.51	0.53	1.29	0.26	0.64
	Elderly adults 65–74	0.37	1.09	0.04	0.11	0.09	0.28	0.24	0.70	0.12	0.35
	Very elderly adults ≥75	1.16	3.70	0.12	0.38	0.30	0.94	0.75	2.39	0.37	1.19
New Zealand	Adults ≥15	0.47	0.91	0.05	0.09	0.12	0.23	0.3	0.59	0.15	0.29
United Kingdom	Adults 18–64	0.45	0.74	0.05	0.08	0.12	0.19	0.29	0.48	0.14	0.24
Australia	Children 2–5	1.20	2.57	0.12	0.26	0.31	0.65	0.77	1.66	0.38	0.82
	Children 6–18	0.36	0.63	0.04	0.06	0.09	0.16	0.23	0.41	0.11	0.20
Finland	Other children 3–9	1.60	4.27	0.16	0.43	0.41	1.09	1.03	2.75	0.51	1.37
New Zealand	Children 5–14	0.48	0.88	0.05	0.09	0.12	0.22	0.31	0.56	0.15	0.28
Sweden	Other children 3–9	0.80	0.96	0.08	0.10	0.20	0.24	0.52	0.62	0.26	0.31
	Adolescents 10–17	0.29	0.42	0.03	0.04	0.07	0.11	0.19	0.27	0.09	0.13

AMC: anionic methacrylate copolymer; bw: body weight; P90: 90th percentile

[a] Based on consumption data for food supplements for consumers only.

[b] Total of methyl methacrylate, methacrylic acid and methyl acrylate based on total monomer content of 0.01% of the copolymer.

[c] Not including from methyl methacrylate.

[d] Sum of the exposure to methacrylic acid monomers and methacrylic acid from methyl methacrylate, with a conversion using molecular weights.

was brand loyal to a particular AMC-containing supplement that they consumed regularly, the exposures could be assumed to be appropriate estimates for those consumers.

3.5 Summary of the estimates of exposure for AMC for use in the safety evaluation

A summary of the estimates of exposure to AMC, its monomers and the relevant metabolite, methacrylic acid, was required for this evaluation. Although estimates of exposure to AMC from pharmaceuticals were reviewed, the Committee considered that this use should not be taken into account in the assessment of chronic dietary exposure for a healthy population, and only dietary exposures from food supplements were considered.

In order to capture the broad range of exposures from all sources of the estimates (the sponsor, EFSA and the Committee) for food supplements, the lower end of the range of mean exposures and the upper end of the range of high percentile exposures were used. The range of exposures used in the evaluation (Table 14) were those estimated by the Committee from the national exposures as the estimates from the sponsor and EFSA were within these ranges. This was the case for the exposures to the copolymer, monomers and metabolite.

The range of exposures were rounded for the evaluation as uncertainties are inherent in dietary exposure estimates and the results are a guide for risk characterization purposes.

4. Comments

4.1 Biochemical aspects

4.1.1 AMC

Radiolabelled AMC administered to rats was excreted in the faeces with mean recovery amounting to 92.38% of the total dose after 72 hours and 94.07% (±3.42%) of the total dose 10 days after dosing. Radioactivity was detected at very low levels in the urine of four of the eight treated animals in this group; this may have been due to contamination with faecal matter (Huntingdon Life Sciences, 1999).

4.1.2 Residual monomers

Methyl methacrylate is rapidly absorbed and distributed following inhalation or oral administration in rats. On the basis of available data, methyl methacrylate is metabolized to methacrylic acid and methanol, which is subsequently converted to carbon dioxide via the tricarboxylic acid cycle in both experimental animals and humans (Bratt & Hathway, 1977; Government of Canada; 1993; ECETOC, 1995).

Methyl acrylate is rapidly absorbed. About half the dose is exhaled as carbon dioxide, while the rest is excreted in the urine in the form of cysteine conjugates and other thioethers (Vodicka, Gut & Frantík, 1990; Sapota, 1993).

Methacrylic acid is rapidly absorbed after oral or inhalational exposure. After a single oral administration of the sodium salt of methacrylic acid to rats, the maximum concentration in blood was found after 10 minutes. After 60 minutes, no more methacrylic acid was detectable (Bereznowski, Mariewski & Somolenski, 1994).

4.2 Toxicological studies

4.2.1 AMC

In a GLP-compliant study, a single dose of 2000 mg/kg bw of AMC was administered by gavage to rats. Following a 14-day recovery period, there were no abnormal clinical observations, no deaths and no statistically significant differences in body weights between the test animals and historical controls. No abnormalities were observed at necropsy (RTC, 1995).

When gavage doses of 0, 200, 500 or 1500 mg/kg bw per day of AMC were administered to rats for 4 weeks, no treatment-related effects were observed. The NOAEL was 1500 mg/kg bw per day, the highest dose tested (RTC, 1998). In a GLP-compliant 26-week oral toxicity study in rats using gavage doses of 0, 200, 500 or 1500 mg/kg bw per day, there were also no treatment-related effects. Some statistically significant findings were observed, but these did not exhibit a dose–response relationship. The NOAEL was 1500 mg/kg bw per day, the highest dose tested (RTC, 2000). In a 4-week dog study, AMC was administered as copolymer-coated cellulose pellets at doses of the test substance at 0, 100, 200 or 400 mg/kg bw per day. There were no treatment-related effects. The NOAEL was 400 mg/kg bw per day, the highest dose tested (RTC, 2004).

Two reverse mutation assays in bacteria, a mouse lymphoma assay and a chromosomal aberration assay in vitro and an in vivo mouse micronucleus assay all gave negative results. The Committee concluded that AMC was not of concern for genotoxicity.

No long-term toxicity or carcinogenicity studies were available on AMC.

In a GLP-compliant developmental toxicity study, female rats administered AMC at 0 or 1000 mg/kg bw per day by gavage on gestation days 5–19 showed no treatment-related effects. The NOAEL was 1000 mg/kg bw per day, the only dose tested (Harlan, 2003).

Studies on cytotoxicity, dermal toxicity, inhalation toxicity, ocular toxicity and phototoxicity produced no effects.

4.2.2 Residual monomers

(a) Methyl methacrylate

In a long-term toxicity study in rats given methyl methacrylate in drinking-water at 0, 6, 60 or 2000 mg/L (equal to 0, 0.4, 4 and 121 mg/kg bw per day for males and 0, 0.5, 5 and 146 mg/kg bw per day for females, respectively) for 2 years, relative kidney weight increased in females at the highest dose but no treatment-related histopathological effects were observed in any organs or tissues (Borzelleca et al., 1964). Based on the results of this study, a TDI of 1.2 mg/kg bw per day was determined (WHO, 1998).

In long-term toxicity and carcinogenicity studies in mice, rats and hamsters given methyl methacrylate by inhalation, the observed effects were, in general, similar to those reported in the short-term studies but also included inflammation and epithelial hyperplasia of the nasal cavity and degeneration of the olfactory sensory epithelium. There was no evidence of any carcinogenic effects (Borzelleca et al., 1964; WHO, 1998).

Bacterial reverse mutation assays with methyl methacrylate gave mostly negative results. Mixed results (i.e. positive, weakly positive or negative) were obtained in in vitro chromosomal aberration and SCE assays. One in vitro micronucleus assay was unequivocally negative, whereas a second assay was negative at low concentrations but weakly positive at higher concentrations. Three mouse lymphoma assays for gene mutations were positive. A mouse bone marrow micronucleus assay was negative, but it is not clear if the target tissue was exposed to the test substance. A rat micronucleus assay with exposure by inhalation was positive following 1 day of exposure but negative following 5 days of exposure. These results were judged by the Committee to be inconclusive.

The Committee concluded that there was some evidence of mutagenicity and clastogenicity in vitro, but that there was a lack of adequate in vivo tests following up the equivocal in vitro findings.

(b) Methyl acrylate

In a 2-year inhalation study, groups of rats (n = 86/sex) were exposed to methyl acrylate by inhalation at 0, 15, 45 or 135 ppm (0, 53, 158 and 475 mg/m^3) in air for 6 hours per day on 5 days per week for 2 years. No significant difference in mortality was observed between the groups. The incidence of soft-tissue sarcomas varied considerably between the groups but there was no dose-dependence. No increased frequency of any tumour type in any organ could be related to a carcinogenic effect of the test substance (Reininghaus, Koestner & Klimisch, 1991).

There were no epidemiological data in humans. The IARC concluded that there was inadequate evidence in experimental animals for carcinogenicity

and that methyl acrylate was not classifiable as to its carcinogenicity in humans (IARC, 1999).

The Committee evaluated the genotoxicity studies on methyl acrylate. Reverse mutation studies in bacteria were negative. A well-performed genotoxicity study on L5178Y mouse lymphoma cells, without exogenous metabolism, produced positive outcomes for gene mutation and chromosomal aberrations but only at cytotoxic concentrations. The in vivo study designs have methodological shortcomings. A mouse bone marrow micronucleus assay using intraperitoneal dosing showed an increase in micronuclei induction, but this was limited in magnitude and not dose related. A mouse bone marrow micronucleus assay using oral dosing was negative, but it is not clear if the target tissues were exposed to the test substance. Overall, the Committee concluded that the data were not sufficient to draw conclusions on the genotoxicity of methyl acrylate.

(c) Methacrylic acid

There were no long-term chronic toxicity/carcinogenicity studies on methacrylic acid. The Committee noted that studies on methyl methacrylate, which is metabolized into methacrylic acid, found no carcinogenicity in mice, rats or hamsters (WHO, 1998).

There are several in vitro genotoxicity studies on methacrylic acid, but the most useful data were from negative studies on gene mutations in bacteria. Positive results were obtained in two comet assays and a γ-H2AX assay in human gingival fibroblasts (Szczepanska et al., 2012). There were no studies on chromosomal aberrations or gene mutation in mammalian cells and no in vivo studies on methacrylic acid. The Committee noted that there were insufficient data to reach a conclusion on the genotoxic potential of methacrylic acid.

4.3 Observations in humans

No human data were available on AMC.

4.4 Assessment of dietary exposure

The Committee evaluated exposure to AMC from its use as a glazing or coating agent in food supplements and foods for special medical purposes. As another major use of AMC is in pharmaceuticals, this use was also evaluated in the exposure assessment. The level of use of AMC is a maximum of 10%.

The Committee evaluated exposure to AMC and its residual monomers, methacrylic acid, methyl methacrylate and methyl acrylate. As the Committee evaluated the toxicology of methacrylic acid, a total exposure to this monomer

Table 14
Summary of range of estimated exposures to AMC, its monomers and total methacrylic acid, from uses in food supplements for mean and high exposures

	Range of estimated dietary exposures [a,b]				
Population group	AMC (mg/kg bw per day)	Methacrylic acid (µg/kg bw per day)	Methyl methacrylate (µg/kg bw per day)	Methyl acrylate (µg/kg bw per day)	Total methacrylic acid exposure (µg/kg bw per day) [c]
Adults	3.5–37	0.04–0.4	0.1–0.9	0.2–2.4	0.1–1.2
Children	2.9–43	0.03–0.4	0.1–1.1	0.2–2.8	0.1–1.4

AMC: anionic methacrylate copolymer; bw; body weight
[a] All estimates of exposure are presented as a range from the lowest of the mean exposures to the highest of the high exposures. The lower end of each range is the lowest of the estimated mean exposures, and the upper end of the range is the highest of the estimated high exposures.
[b] Includes exposures estimates submitted by the sponsor and EFSA (2010), and national estimates calculated by the Committee based on a concentration of 100 mg per 1 g dosage unit.
[c] The total methacrylic acid exposure is the sum of the exposure to methacrylic acid monomers and methacrylic acid from methyl methacrylate, with a conversion using molecular weights.

was estimated from the sum of the methacrylic acid monomer exposure and the methacrylic acid product of methyl methacrylate metabolism.

The exposure assessment included estimates provided by the sponsor and an evaluation by EFSA (2010) based on consumption of food supplements and pharmaceuticals. The Committee also estimated exposure based on national food consumption data for food supplements using the concentration proposed by the sponsor. The national consumption data were from CIFOCOss and data from Australia and New Zealand submitted to the Committee. A comprehensive literature search was also conducted; no additional studies were identified.

No quantitative estimates of exposure could be determined for foods for special medical purposes. The sponsor indicated that it is not anticipated that foods for special medical purposes would increase exposures above that of food supplements and pharmaceuticals, given the conservative nature of those calculations. In addition, the consumers of foods for special medical purposes will generally be under medical supervision, and exposures for these consumers are not relevant for the general healthy population. This use was not further evaluated by the Committee.

The total monomeric content of AMC is less than 0.01%. This level was used to calculate the exposure to individual monomers based on the ratio of each monomer in the copolymer. The total exposure to methacrylic acid is from the sum of the exposure to methacrylic acid monomers and methacrylic acid from methyl methacrylate with a conversion using molecular weights (Table 14).

The Committee noted that AMC is used in pharmaceuticals. Estimated exposures from this use from the sponsor and EFSA ranged between 8.0 and 13.3 mg/kg bw per day for adults and children. These estimates were within the range

of exposures from food supplements. However, the Committee considered that such use should not be taken into account in the assessment of long-term dietary exposure for a healthy population.

5. Evaluation

There were no concerns for the toxicity of AMC itself. However, the presence in AMC of the residual monomer methyl acrylate, for which it is not possible to conclude on genotoxic potential, and the insufficient carcinogenicity data for methyl acrylate preclude establishing an ADI for AMC.

The available toxicology data for AMC itself do not give rise to concerns for toxicity. The substance is poorly absorbed and is excreted in the faeces. In short-term and developmental toxicity studies, the NOAELs for AMC range from 400 to 1500 mg/kg bw per day, the highest doses tested. Estimated exposures to AMC range from 2.9 to 43 mg/kg bw per day.

Toxicological data on the residual monomers, apart from the genotoxicity data, do not give rise to concerns when taking into account the low exposures. Genotoxicity data for methyl methacrylate suggest a potential risk for mutagenicity and clastogenicity in vitro, and there is a lack of adequate data on genotoxicity in vivo. However, in carcinogenicity studies in mice, rats and hamsters with methyl methacrylate given by inhalation, there was no evidence of any carcinogenic effects. In a 2-year study in rats given methyl methacrylate in drinking-water, the NOAEL was 121 mg/kg bw per day; from this NOAEL, a TDI of 1.2 mg/kg bw was derived (WHO, 1998). Estimated exposures to methyl methacrylate range from 0.1 to 1.1 µg/kg bw per day, which are below the TDI.

Data on methyl acrylate are limited. ADME studies suggest that methyl acrylate is rapidly absorbed and excreted. The genotoxicity data are insufficiently adequate to draw conclusions on the genotoxic potential of methyl acrylate. Although a rat carcinogenicity study on methyl acrylate by the inhalation route was negative, no suitable long-term oral toxicity studies were available to support the safety of methyl acrylate. The Committee was unable to conclude on the safety of methyl acrylate as a residual monomer in AMC. Estimated exposures to methyl acrylate range from 0.2 to 2.8 µg/kg bw per day.

There were insufficient data to reach a conclusion on the genotoxic potential of methacrylic acid, and no long-term carcinogenicity studies were available. The Committee noted that there was evidence that methyl methacrylate is metabolized to methacrylic acid, and therefore the four negative long-term toxicity oral (via drinking-water) and inhalation carcinogenicity studies on methyl methacrylate could be used to support the safety of methacrylic acid. The Committee concluded that the exposure to methacrylic acid from the sum of the

levels present in AMC and as a metabolite of methyl methacrylate, ranging from 0.1 to 1.4 μg/kg bw per day, would be unlikely to be a health concern.

The Committee was unable to complete the evaluation of AMC. While the copolymer itself is not a health concern, genotoxicity concerns remain for the residual monomer methyl acrylate.

New specifications and a Chemical and Technical Assessment were prepared. The specifications were made tentative pending the completion of the safety evaluation of AMC.

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Basic methacrylate copolymer

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1. Explanation

Basic methacrylate copolymer (BMC; E 1205; International Numbering System 1205; Chemical Abstracts Service (CAS) No. 24938-16-7; basic butylated methacrylate copolymer; aminomethacrylate copolymer; aminoalkyl methacrylate copolymer E; butyl methacrylate (CAS No. 97-88-1), dimethylaminoethyl methacrylate (CAS No. 2867-47-2), methyl methacrylate (CAS No. 80-62-6) polymer; butyl methacrylate, methyl methacrylate, dimethylaminoethyl methacrylate copolymer) is a cationic copolymer manufactured using the monomers dimethylaminoethyl methacrylate [2-(dimethylamino) ethyl methacrylate], butyl methacrylate [*n*-butyl methacrylate] and methyl methacrylate in the molar ratio of 1:2:1.

BMC has been evaluated by European Food Safety Authority (EFSA) and is an approved food additive within the European Union where its use is restricted to a maximum level of 100 000 mg/kg in solid food supplements, category 17.1 (EFSA, 2010).

The Committee considered three different copolymers: basic, anionic and neutral methacrylate copolymers. Each copolymer releases the active ingredients from within their coatings under different physiological conditions in different parts of the digestive tract. BMC is soluble below pH 5 and is used for its taste- and odour-masking properties; as protection from heat, light, moisture and oxidation; and to prevent the fast release of active ingredients in the stomach.

BMC has not previously been evaluated by the Committee. The Committee evaluated the use of BMC as a coating or glazing agent for solid food supplements and foods for special medical purposes in the form of solid food supplements such as capsules, pastilles, tablets, pills, pellets and powders, at levels not exceeding 10%, at the request of the Forty-ninth Session of the Codex Committee on Contaminants in Food Additives (FAO/WHO, 2017). Another proposed use of BMC is for microencapsulation, which enhances the stability of micronutrients in food fortification, specifically for populations with nutrient deficiencies. BMC is also used in pharmaceuticals.

Toxicological data submitted for the evaluation included absorption, distribution, metabolism and excretion (ADME), acute and short-term toxicity

and genotoxicity studies as well as a developmental toxicity study. Limited data were also submitted on the residual monomers. A comprehensive literature search retrieved data on the monomers but no additional studies with BMC.

The Committee considered the data on the residual monomers because these are of low molecular weight and therefore likely to be absorbed from the gastrointestinal tract. As exposure to monomers from BMC is higher than to anionic methacrylate copolymer (AMC) and neutral methacrylate copolymer (NMC), the Committee evaluated ADME, short- and long-term toxicity studies, and genotoxicity and reproductive and developmental toxicity data on the monomers.

The Committee was also aware that BMC contains an oligomer fraction of up to 10%. As the lower end of the molecular weight range for all the constituent oligomers is greater than 1000 Da, and around two thirds of the oligomer fraction has a molecular weight of between 4000 and 5000 Da, it is unlikely that the oligomers would be absorbed from the gastrointestinal tract. Therefore, the Committee did not consider the toxicological aspects of the oligomers.

Unless otherwise stated, the test substance used in the distribution and toxicity studies was prepared from an aqueous dispersion and then freeze-dried to remove water. In all cases, doses have been expressed as dry weight of BMC.

The Committee evaluated toxicological and exposure data on 2-propanol, butanol and methanol, the residual components of BMC that can be present in the final products because they are used in the manufacture of the copolymer. The Committee concluded that these residual components do not pose a safety concern at the maximum estimated exposure levels.

1.1 Chemical and technical considerations

BMC is manufactured by a controlled polymerization process using a free radical donor initiator system. After completion of polymerization, the viscous copolymer solution is fed into an extruder to remove solvents and volatile substances, by continuous degassing through vacuum and heating. The solid granules of BMC formed in the extruder can be milled to a powder in which not more than 10% of the particles have a diameter less than 3 μm.

BMC has a weight-average molecular weight of 47 000 Da and number-average molecular weight of 22 000 Da.

During the manufacture of BMC, 2-propanol is added as a production aid. Most of the solvent evaporates during the polymerization and extrusion steps in the manufacture of BMC. The methyl methacrylate integrated in the polymer chain may hydrolyse to methacrylic acid and methanol. The methacrylic acid remains in the polymer chain but methanol is released. Similarly, butyl

methacrylate integrated in the polymer chain may hydrolyse to methacrylate and butanol. BMC may contain 2-propanol (not exceeding 0.5%); butanol (not exceeding 0.5%); and methanol (not exceeding 0.1%). The copolymer may also contain residual monomers: dimethylaminoethyl methacrylate (not exceeding 500 mg/kg); butyl methacrylate (not exceeding 200 mg/kg); and methyl methacrylate (not exceeding 50 mg/kg).

1.2 Literature search

A literature search carried out in PubMed and Google Scholar for basic methacrylate copolymer identified no additional relevant articles.

Literature searches were carried out in PubMed and Google Scholar for genetoxicity studies (1990–2018) with the monomers butyl methacrylate, methyl methacrylate and dimethylaminoethyl methacrylate. A literature search was also carried out for genetoxicity (2001–2018) and other toxicology studies (1997–2018) with methacrylic acid. Articles were selected if they included (genetic) toxicological information about the named monomer on its own; articles were excluded if the information was not about the monomer on its own and did not include (genetic) toxicological information. Other toxicology searches were carried out for the monomer and specific species. Articles were included if they examined a toxicological end-point in one of the named species (or similar mammalian species); articles were rejected if there was no toxicological end-point or they did not include one of the named species (or similar mammalian species). Review articles were manually searched to identify any articles that may not have been found via the database search.

The following keywords, search strings and limits were used in the PubMed and Google Scholar literature searches for butyl methacrylate: "butyl methacrylate AND gene* tox*" (from 1 January 1990); "butyl methacrylate AND mutation" (from 1 January 1990); "butyl methacrylate AND aberration" (from 1 January 1990); "butyl methacrylate AND comet" (from 1 January 1990); "butyl methacrylate AND micronucleus" (from 1 January 1990); "butyl methacrylate AND Ames" (from 1 January 1990); "butyl methacrylate AND chromatid exchange" (from 1 January 1990); "butyl methacrylate AND adduct" (from 1 January 1990); "butyl methacrylate AND muta*" (from 1 January 1990).

Due to the large number of articles retrieved, only the first 100 for any given search were considered.

The following keywords and limits were used in the PubMed and Google Scholar literature searches for dimethylaminoethyl methacrylate: "dimethylaminoethyl methacrylate AND gene* tox*" (from 1 January 1990); "dimethylaminoethyl methacrylate AND aberration" (from 1 January 1990);

"dimethylaminoethyl methacrylate AND comet" (from 1 January 1990); "dimethylaminoethyl methacrylate AND micronucleus" (from 1 January 1990); "dimethylaminoethyl methacrylate AND Ames" (from 1 January 1990); "dimethylaminoethyl methacrylate AND chromatid exchange" (from 1 January 1990); "dimethylaminoethyl methacrylate AND muta*" (from 1 January 1990); "dimethylaminoethyl methacrylate AND adduct" (from 1 January 1990).

Due to the large number of articles retrieved only the first 100 for any given search were considered.

The following keywords, search strings and limits were used in the PubMed and Google Scholar literature searches for genetic toxicology of methacrylic acid: "methacrylic acid AND gene* tox*" (from 1 January 2001); "methacrylic acid AND aberration" (from 1 January 2001); "methacrylic acid AND comet" (from 1 January 2001); "methacrylic acid AND micronucleus" (from 1 January 2001); "methacrylic acid AND Ames" (from 1 January 2001); "methacrylic acid AND chromatid exchange" (from 1 January 2001); "methacrylic acid AND muta*" (from 1 January 2001); "methacrylic acid AND adduct" (from 1 January 1990).

Due to the large number of articles retrieved, only the first 100 for any given search were considered.

The following keywords, search strings and limits were used in the PubMed and Google Scholar literature search for other toxicology (not genotoxicity) of methacrylic acid: "methacrylic acid AND rat" (1997–2018); "methacrylic acid AND mouse" (1997–2018); "methacrylic acid AND rabbit" (1997–2018); methacrylic acid AND dog" (1997–2018); "methacrylic acid AND monkey" (1997–2018); "methacrylic acid AND non-human primate" (1997–2018); "methacrylic acid AND mice" (1997–2018); "methacrylic acid AND pig" (1997–2018).

The same searches were carried out in Google Scholar with the same limits applied.

Due to the large number of articles returned only the first 100 for any given search were considered.

A literature search was also conducted to identify any estimates of dietary exposure to BMC, using EBSCO Discovery Service. Medline, Food Science Source, Food Science and Technology Abstracts and ScienceDirect were searched as were a number of scientific, toxicological, food, nutrition and public health–related journals. Search terms included "methacrylate copolymer" and "dietary exposure" or "dietary intake" or "consumption". These terms were also used in a general internet search to capture "grey" literature and other papers not included in the scientific literature. No exposure estimates additional to those already submitted by the sponsors were retrieved.

2. Biological data

The Committee reviewed the ADME, short- and long-term toxicity studies, genotoxicity data, and reproductive and developmental toxicity studies with BMC as well as with the residual monomers, *n*-butyl methacrylate, methyl methacrylate and 2-(dimethylamino)ethyl methacrylate). The Committee considered toxicological and exposure data on other residual components of BMC that are present in the final product but did not consider the toxicological aspects of the oligomers.

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) BMC

Absorption

Five adult male rats were housed in individual metabolism cages, and urine and faeces were collected at 24-hour intervals to establish background radioactivity levels. The animals were fasted overnight and intubated with (1 mL) of labelled BMC preparation (≥98% BMC) in hydrochloric acid and water (site of radiolabel not identified) with a specific radioactivity of 0.005 MBq/mg (calculated as approximately 0.5 MBq/kg body weight [bw]). Feed and water were available ad libitum throughout the experiment. Animals were killed 7 days after intubation and the following organs and tissues removed for radiochemical analysis: kidneys, liver, small and large intestine and contents, mesenteric lymph nodes, spleen and cardiac blood.

After 5 days, the mean radioactivity excreted in urine was 0.013% of the initial dose (after subtracting the activity in control urine, correcting for radiochemical efficiency and removing data from an outlier that clearly had faecal contamination). The greatest amount (0.006%) was excreted on day 1. Mean excretion of radioactivity in the faeces in the 5 days post dosing was 93.3% (range: 88.77–95.72%). The majority was excreted in the first 2 days. Based on the levels of radioactivity in urine, the study authors concluded that less than 0.02% of the radioactivity was absorbed (Life Science Research, 1979).

Distribution

Nine adult male rats were fasted overnight prior to the administration of a single oral dose of radiolabelled BMC preparation (≥98% BMC) in hydrochloric acid and water (site of radiolabel not identified). Groups of three animals were killed at 1, 3 and 14 days after intubation. The following organs and tissues were removed for radiochemical analysis: kidneys, liver, small and large intestine and

their contents, mesenteric lymph nodes, spleen and cardiac blood. Data for day 7 post intubation were from those animals used to investigate the absorption of the test substance (see section 2.1.1(a) Absorption).

Overall levels of radioactivity measured in blood and other tissues were similar to those obtained for the control animals. Increased levels of radioactivity were seen in the large intestine and the contents of the large and small intestines on days 1 and 3. The levels of radioactivity had returned to control levels by day 7. The increased levels in the large intestine on day 1 may reflect a possible contamination of the tissues by the contents.

The study authors concluded that no radioactive material was retained in the tissues (Life Science Research, 1979).

(b) **Residual monomers**

n-Butyl methacrylate

Data on the metabolism of *n*-butyl methacrylate have been described in the European Chemical Substances Information System (ESIS) IUCLID Dataset (EC, 2010) as follows:

> *n*-BMA [*n*-butyl methacrylate], like MMA [methyl methacrylate], is rapidly metabolized by body carboxylesterases... Hydrolysis of *n*-BMA yields methacrylic acid and *n*-butanol which are further metabolized by physiological pathways, methacroyl CoA being a physiological substrate of the valine pathway.

Methyl methacrylate

The ADME data on methyl methacrylate have been summarized by WHO (1998) as follows:

> Methyl methacrylate is rapidly absorbed and distributed following inhalation or oral administration to rats. On the basis of available data, methyl methacrylate appears to be rapidly metabolized to methacrylic acid [and methanol], which is subsequently converted to carbon dioxide via the tricarboxylic acid cycle in both experimental animals and humans…
>
> Methyl methacrylate is rapidly eliminated, primarily via the lungs in expired air. After oral or intravenous administration to rats, approximately 65% of the dose was exhaled in the expired air as $^{14}CO_2$ within 2 hours (Bratt & Hathway, 1977). Lesser amounts are eliminated in the urine, and an even smaller fraction in the faeces. Owing to its rapid metabolism and excretion, there appears to be little potential for accumulation of methyl methacrylate within tissues (Government of Canada, 1993; ECETOC, 1995).

2-(Dimethylamino)ethyl methacrylate

There were no in vivo studies on the toxicokinetics of 2-(dimethylamino)ethyl methacrylate. The available data on 2-(dimethylamino)ethyl methacrylate have been summarized in the European Chemicals Agency database (ECHA, 2015a) as follows:

> 2-(Dimethylamino)ethyl methacrylate was rapidly hydrolysed to methacrylic acid (CAS 79-41-4) and *N*,*N*,-dimethylaminoethanol (CAS 108-01-0) when incubated with simulated saliva or simulated intestinal fluid in vitro, but not with simulated gastric fluid. Up to 90% degradation was observed in simulated saliva after 4 hours at 37 °C and 86% degradation after incubation with simulated intestinal fluid for 4 hours at 37 °C. Degradation was below 8% after incubation with simulated gastric fluid for 4 hours at 37 °C.

2.2 Toxicological studies

2.2.1 Acute toxicity

BMC (≥98% BMC) suspended in water with 5% tragacanth gum was given by the oral route to mice (10/sex per dose) at 3, 5, 10, 15 and 20 g/kg bw. Additional groups (10/sex per dose) were dosed via the intraperitoneal route at 2, 3, 4 and 5 g/kg bw. Mortality and behavioural changes in the animals were observed for 24, 48 and 72 hours after dosing. Results are shown in Table 1.

In mice treated subcutaneously (doses and animal numbers not stated), a dose of 5 g/kg bw did not cause mortality or changes in behaviour in either sex for 72 hours after administration and the BMC preparation remained at the site of injection. No other details were provided.

In the same study, rats were exposed to 3.0 g/kg bw via the oral route, 2.0 g/kg bw via the intraperitoneal route and 1.0 g/kg bw via the subcutaneous route. No deaths or behavioural changes were observed in either sex for 72 hours after dosing (no other details were provided).

The study authors concluded that any oral, intraperitoneal and subcutaneous toxicity in rats would be at concentrations greater than 3.0, 2.0 and 1.0 g/kg bw, respectively (Nagasaki University, 1970).

Five groups of fasted rats (5/sex per group) were administered a single dose of BMC (≥98% BMC) dissolved in 1% aqueous hydroxyethyl methylcellulose mucilage by gavage. Doses were calculated to be 0, 7.5, 9.4, 11.9 and 15 g/kg bw for males and females. Behaviour, feed consumption and body weight were observed for 4 weeks after dosing, and the animals were dissected and examined.

Table 1
Mortality of mice per dose group in acute toxicity study with BMC

Dosing route / sex	Dose group (g/kg bw)	No. of deaths (hours after dosing)
Oral / males	5	1/10 (48 hrs)
	10	2/10 (72 hrs)
	15	3/10 (48 hrs); 2/10 (72 hrs) – 5/10 total
	20	3/10 (24 hrs); 3/10 (48 hrs); 1/10 (72 hrs) – 7/10 total
Oral / females	10	1/10 (48 hrs); 1/10 (72 hrs) – 2/10 total
	15	3/10 (48 hrs); 1/10 (72 hrs) – 4/10 total
	20	3/10 (24 hrs); 2/10 (48 hrs); 1/10 (72 hrs) – 6/10 total
Intraperitoneal / males	4	1/10 (24 hrs); 1/10 (48 hrs) – 2/10 total
	5	2/10 (48 hrs); 1/10 (72 hrs) – 3/10 total
Intraperitoneal / females	3	1/10 (24 hrs)
	4	2/10 (24 hrs); 1/10 (48 hrs) – 3/10 total
	5	1/10 (24 hrs); 3/10 (48 hrs) – 4/10 total

bw: body weight; no.: number
Source: Nagasaki University (1970)

No treatment-related effects were observed during the 4-week observation period or in macroscopic examination of the organs.

The oral median lethal dose (LD_{50}; 7 days) and oral toxicity threshold were both greater than 15 g/kg bw (LPT, 1970).

After a 12-hour fast, 13 rats were administered, via a stomach tube, BMC (≥98% BMC) pan-coated onto lactose granules with a final composition of 4.7% test substance and 95.3% lactose. The doses indicate the concentration of BMC preparation, but the animals would also have received the corresponding amount of lactose. One animal was dosed with water (50 g/kg bw), one with 1.2 g/kg bw test substance, two with 2.4 g/kg bw test substance, one or two with 4.7 g/kg bw test substance and one each with 5.9 and 7.05 g/kg bw test substance. Control animals were treated with corresponding amounts of lactose. Gavage administration of up to 100 g/kg bw lactose with (4.7%) and without lacquer substance did not show any effects. At (and above) 125 g/kg (equivalent to 5.9 g lacquer substance), the animals died 6–10 hrs after application.

The study authors concluded that the cause of death was the high amount of test substance administered rather than a response to the substance, per se. Gross examination showed abnormally full and bloated stomachs and intestines as well as kidneys with an increased blood supply.

As it was not possible to apply more than 5% of the BMC preparation to the lactose, a second experiment was carried out in which 5% and 10% BMC preparation (≥98% BMC) was sprayed on the animal feed. The animals fed for

24 hours. Feed with 5% BMC preparation was administered to five animals each at 2.5 and 4.0 g/kg bw. Feed containing 10% BMC preparation was administered to five animals each at 4.5 and 4.0 g/kg bw and 10 animals at 6.0 g/kg bw. After 14 days of observation no abnormal behaviour was noted and body weights increased at the normal rate. There was no evidence of changes in any organs.

In addition to the two studies described above, a range-finding study was performed for 3 days with 5% and 10% of lacquer solids applied to the feed. Two rats consumed 115 g/kg bw of feed containing 5% lacquer solids (equivalent to 5.8 g/kg bw of lacquer solids) and two rats consumed 50 g/kg bw of feed containing 10% lacquer solids (equivalent to 5 g/kg bw of lacquer solids). No abnormal behaviour, body-weight development or autopsy findings were noted (Battelle-Institute, 1966).

2.2.2 Short-term studies of toxicity

(a) BMC

Rats

In a 26-week dietary study, rats (20/sex per dosing group; 40 controls) were administered 500 and 2000 mg/kg bw per day of BMC (≥98% BMC) via feed. The test substance was sprayed onto the feed at a ratio of 1 : 9, and the treated feed was mixed in with the final feed to obtain the desired concentration. Control feed was sprayed with water. The treated feed was provided daily and adjusted weekly to body-weight gain and feed consumption. Animals were checked daily, and clinical signs and feed consumption were recorded. Body weights were recorded weekly. Blood samples were collected before the first dosing and after 6, 13, 18 and 26 weeks of treatment. All animals were killed at the end of the 26-week treatment period.

No changes in behaviour could be detected throughout the study. Faeces appeared normal, and no decreases in body weight, infections or deaths were recorded. No changes in blood parameters were detected. Female rats at 500 mg/kg bw per day showed a significant increase in total protein in week 13, but the study authors considered this not toxicologically relevant. No treatment-related findings were reported for urine. Occasional traces of protein and individual leucocytes in urine sediment were not considered effects of the test substance. No treatment-related histopathological changes could be detected in any of the 11 organs examined. Occasional findings of necrotic lesions in the lung and bronchiectasis, parasites in the intestine, cytomegaly in the salivary gland and changes in the prostate/uterus did not differ significantly between treated and non-treated animals.

The no-observed-adverse-effect level (NOAEL) was 2000 mg/kg bw per day, the highest dose tested (LPT, 1973).

Dogs

In a good laboratory practice (GLP)–compliant 28-day oral toxicity study, groups of dogs (3/sex per group) received 0, 100, 300 or 750 mg/kg bw per day of BMC (≥98% BMC) in gelatine capsules. Animals were treated for 28, 29 or 30 days, and necropsied 1 day later. Purity of the BMC preparation was not stated. Animals were checked daily for behaviour, coat condition, urine and faecal excretion, condition of body orifices and clinical signs. Heart rate, respiration rate and body temperature were checked prior to dosing and at the end of the study. All animals underwent ophthalmoscopic examination prior to dosing and at the end of the study. Viability was checked twice daily, in the morning and afternoon. Body weight was measured on the day after arrival at the laboratory, on the day of randomization, on the first day of treatment and weekly thereafter until the completion of the study and at necropsy. Feed consumption was measured daily. Blood (for haematological and clinical chemistry analyses) and urine samples were collected from fasting animals prior to treatment and after 28 days of treatment. A complete necropsy was performed. After external examination, the abdominal, thoracic and cranial cavities were macroscopically examined. The following organs were weighed: heart, brain, spleen, adrenals (×2), kidneys (×2), liver, lung, uterus, prostate, ovaries (×2), testes (with epididymis attached; ×2). Relative organ weights were calculated. Preserved organs and tissues from control and high-dose dogs and organs with macroscopic findings were histopathologically examined.

There were no unscheduled deaths. Vomiting of mucous material, parts of capsules or whole capsules occurred 25 minutes to 2 hours after dosing in mid- and high-dose animals for the first 4 days when feeding was 2 hours (days 1 and 2) or 30 minutes (days 3 and 4) after treatment. From day 5 until completion of the study, the animals were fed immediately after dosing and no further vomiting occurred. Although statistical significance was attained only in a few cases, there was a tendency to reduce body weight in high-dose animals. Feed consumption of mid- and high-dose males was significantly reduced compared with controls. There was a statistically significant decrease in the feed conversion ratio of high-dose males compared with controls. There were no statistically significant treatment-related daily clinical, heart rate, respiration rate, rectal body temperature, ophthalmoscopic or general findings, organ weight changes or macroscopic or microscopic observations. There were no biologically relevant haematological findings or toxicologically relevant clinical chemistry or urinary parameter findings.

The NOAEL was 750 mg/kg bw per day, the highest dose tested (Bien, 2003).

(b) Residual monomers

n-Butyl methacrylate

Data on short-term toxicity of *n*-butyl methacrylate have been described in the European Commission ESIS, IUCLID Dataset (European Commission, 2010) as follows:

> Whole body inhalation exposure of CDBR rats (sex not given) was conducted for 6 hr/day, 5 days/week at 310, 952, and 1891 [parts per million] ppm (OECD Guideline 412). All animals were necropsied at the end of the 4 week exposure period. Body weight, feed consumption, clinical signs, clinical chemistry, hematology, organ weight, and histopathologic evaluations were performed on all animals during this study. The only treatment related signs of toxicity observed were inactivity, lacrimation, eye squinting, and labored breathing. These signs were observed sporadically during exposure throughout the study in rats exposed to 952 or 1981 ppm *n*-BMA. Body weights and feed consumption were not affected by exposure to BMA. No deaths occurred at any concentration. At necropsy the only organ weight effect was a statistical increase in kidney weight to body weight ratio at 1981 ppm in males and females. However, the absolute kidney weights for this group were not statistically significantly increased. Therefore, in the absence of corresponding histologic effects, hematology, or clinical chemistry findings, this increase in relative kidney weight was judged as not being of toxicological significance. Macroscopic examination of the nasal cavities of the male and female rats exposed to 1981 ppm had slight and localized bilateral degeneration of the olfactory epithelium lining of the dorsal meati. One male and one female rat exposed to 952 ppm had similar changes in the olfactory epithelium. Rats exposed to 310 ppm had no exposure related nasal cavity macroscopic changes. On the basis of the most sensitive indicator of toxicity, the histopathologic changes seen in the nasal cavities, the [lowest-observed-effect level] LOEL was 952 ppm and the [no-observed-effect level] NOEL was 310 ppm.

Methyl methacrylate

There were numerous studies on the short-term toxicity of methyl methacrylate in mice, rats, hamsters and dogs, but the test substance was most commonly administered by inhalation. These were summarized by WHO (1998) as follows:

> In most sub-chronic studies conducted to date, rats and mice have been exposed to methyl methacrylate by inhalation. Effects observed most commonly in these investigations were decreases in body weight gain and irritation of the skin, nasal cavity, and eye at high concentrations (generally ≥500 ppm [2050 mg/m^3]) (Rohm & Haas Co., 1977; NTP, 1986). At higher concentrations, other effects, such as renal cortical necrosis and tubular

degeneration (rats and mice) and hepatic necrosis (mice), have also been reported (Tansy et al., 1980; NTP, 1986; Deichmann-Gruebler & Read, undated).

2-(Dimethylamino)ethyl methacrylate

A GLP-compliant 90-day repeated-dose oral toxicity study with 2-(dimethylamino) ethyl methacrylate conducted according to Organisation for Economic Co-operation and Development Test Guidelines (OECD TGs) 408 and 424 was summarized by ECHA (ECHA, 2015b) as follows:

> The oral toxicity of 2-2-(dimethylamino)ethyl methacrylate (CAS 2867-47-2, purity: 99.79%) when given by daily administration to rats, has been investigated over a period of 13 consecutive weeks and recovery from any treatment-related effects during a treatment-free period of 4 weeks. Three groups, each of 10 male and 10 female Sprague Dawley rats, received the test item by gavage at dosages of 100, 200 and 500 mg/kg/day for 13 consecutive weeks. A fourth similarly constituted group received the vehicle alone (corn oil) and acted as a control. Additional five male and five female animals were included in separate groups for neuropathology investigations. Control and high dose main and neuropathology groups included each 5 additional animals per sex to be sacrificed after 4 weeks of recovery.
>
> Minor signs of possible treatment-related effects of the test item, Dimethylaminoethyl methacrylate, were observed at in vivo and at post mortem in male and female rats only at the dose levels of 200 and 500 mg/kg/day, when administered by oral gavage for 13 consecutive weeks at the dosages of 100, 200 and 500 mg/kg/day. None of them were considered to be systemically adverse.
>
> No changes indicating a neuropathological effect of the treatment with the test item were observed at any of the dose levels tested.
>
> No significant changes were observed in the animals dosed at 100 mg/kg/day.
>
> Therefore, the high dose of 500 mg/kg/day, when administered daily for 13 consecutive weeks, was considered the No Observed Adverse Effect Level (NOAEL) for systemic toxicity.
>
> This study is acceptable and satisfies the guideline requirement for a 13 week oral toxicity study (OECD 408) in rats.

In an inhalation toxicity study, rats were exposed to 2-(dimethylamino) ethyl methacrylate at 0, 100 or 200 ppm using a constant flow pump for 6 hours/day, 5 days per week for 3 weeks. At 250 ppm (1608 mg/m^3), nose and eye irritation and laboured breathing were observed. Body-weight gain was slow. There were no changes in haematological parameters. No macroscopic or microscopic pathological effects on organs were observed. At 100 ppm (643 mg/m^3), no toxic effects were observed (Gage, 1970).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) BMC

No long-term studies with BMC were available.

(b) Residual monomers

No long-term studies with *n*-butyl methacrylate were available.

Methyl methacrylate

A long-term study in which methyl methacrylate was given in the drinking-water has been summarized by WHO (1998) as follows:

> Data available on the effects of methyl methacrylate following ingestion are limited. In an early study (Borzelleca et al., 1964) in which organ to body weight ratios were determined and histopathological examination of a wide range of tissues as well as limited haematological and urine analyses were conducted, the relative kidney weight was increased in a small group of female rats ($n = 25$) exposed to 2000 ppm (mg/litre) methyl methacrylate in drinking-water for 2 years. This effect was not observed in the males, and histopathological examination revealed no damage. The authors also reported a decrease in fluid consumption in rats exposed to 2000 ppm. The NOAEL was therefore considered to be 2000 ppm (equivalent to a dose of about 146 mg/kg body weight per day for females and 121 mg/kg body weight per day for males, based on intake and body weight data presented by the authors). There were no treatment-related effects, based upon gross or histopathological examination, in extremely small groups of beagle dogs ($n = 2$) exposed to concentrations of up to 1500 ppm (mg/kg) methyl methacrylate (equivalent to a dose of about 38 mg/kg body weight per day) in their feed for 2 years (Borzelleca et al., 1964).

Based on the results of this study, a tolerable daily intake (TDI) of 1.2 mg/kg bw per day was determined (WHO, 1998).

In the chronic toxicity and carcinogenicity studies in mice, rats and hamsters with methyl methacrylate given by inhalation, the observed effects were, in general, like those reported in short-term studies and included inflammation and epithelial hyperplasia of the nasal cavity and degeneration of the olfactory sensory epithelium. There was no evidence of any carcinogenic effects (WHO, 1998).

In 1999, the International Agency for Research on Cancer (IARC) considered the same studies and concluded that there is evidence suggesting lack of carcinogenicity in animals.

2-(Dimethylamino)ethyl methacrylate

No long-term studies are available on 2-(dimethylamino)ethyl methacrylate.

2.2.4 Genotoxicity

The results of genotoxicity tests with BMC are summarized in Table 2 and the results with for the residual monomers, in Tables 3, 4 and 5.

The Committee noted that *n*-butyl methacrylate was not genotoxic in a range of in vitro and in vivo studies covering all the relevant end-points.

Bacterial reverse mutation assays with methyl methacrylate gave mostly negative results (Lijinsky & Andrews, 1980; Hachiya, Taketani & Takizawa, 1982; Waegemaekers & Bensink, 1984; NTP, 1986; Schweikl, Schmalz & Rackebrandt, 1998). Mixed results (i.e. positive, weakly positive or negative) were obtained in in vitro chromosomal aberration (NTP, 1986; Bigatti et al., 1994; Doerr, Harrington-Brock & Moore, 1989; Schweikl, Schmalz & Rackebrandt, 1998; Tuček et al., 2002; Yang et al., 2003) and sister chromatid exchange (SCE) assays (NTP, 1986; Cannas et al., 1987; Yang et al., 2003). One in vitro micronucleus assay was unequivocally negative (Schweikl, Schmalz & Spruss, 2001), whereas a second assay was negative at low concentrations but weakly positive at higher concentrations (Doerr, Harrington-Brock & Moore, 1989). Three mouse lymphoma assays for gene mutations were positive (NTP, 1986; Moore et al., 1988; Dearfield et al., 1991). A mouse bone marrow micronucleus assay was negative, but it is not clear if the target tissue was exposed to the test substance (Hachiya, Taketani & Takizawa, 1982). A rat micronucleus assay with exposure by inhalation was positive following 1 day of exposure but negative following 5 days of exposure (Araújo et al., 2013). These results were judged by the Committee to be inconclusive.

The Committee concluded that there was some evidence of mutagenicity and clastogenicity in vitro, but there is a lack of adequate in vivo tests following up the equivocal findings.

2.2.5 Reproductive and developmental toxicity

(a) BMC

Multigeneration reproductive toxicity

There were no reproductive toxicity studies with BMC.

Developmental toxicity

Pregnant Wistar rats (20/group) were given BMC preparation (≥98% BMC) as 10% lacquer solids in their feed at 0 or 1000 mg/kg bw per day from gestation day 6 to 16, with adjustments made to compensate for the increase in body weight. Control animals received 1000 mg/kg bw per day of Tylose slime with their feed. During pregnancy, behaviour and outward appearance of the mothers was recorded. Body weight was determined on the first day of pregnancy, every day between gestational days 6 and 16, and before laparotomy. On gestation day 20,

Table 2
Summary of genotoxicity studies with BMC

End-point	Test system/ species	Test substance	Concentration/dose	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA1535, TA1537, TA1538, TA98, TA100	BMC (≥98% BMC) solubilized in DMSO	10, 100, 333, 1 000, 5 000 mg/plate (±S9) Plate incorporation and preincubation	Negative	Technical University of Darmstadt (1985)
Gene mutation assay	L5178Y mouse lymphoma cell line	BMC (≥98% BMC) solubilized in acetone and diluted in cell culture medium	4 hrs, −S9: 2.0, 3.9, 7.8, 15.6, 31.3 mg/mL 4 hrs, +S9: 2.0, 3.9, 7.8, 15.6 mg/mL 24 hrs, −S9: 1.9, 3.9, 7.8, 15.5, 31.0 mg/mL	Negative	RCC (2000)
In vivo					
Micronucleus assay	Swiss CD-1 mouse i.p. injection, bone marrow cells	BMC (≥98% BMC) solubilized in 15.6 mmol/L hydrochloric acid	500, 1 000, 2 000 mg/kg bw per day; 250, 500, 1 000 mg/kg bw per day (high-dose groups assessed at 24 and 48 hours post dosing)	Negative	RTC (2000)

BMC: basic methacrylate; bw: body weight; DMSO: dimethyl sulfoxide; i.p.: intraperitoneal; S9: 9000 × *g* supernatant fraction from rat liver homogenate

Table 3
Genotoxicity studies on *n*-butyl methacrylate

End-point	Test system/species	Concentration/dose	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA100, TA1535, TA98, TA1537 *Escherichia coli* WP2*uvr*A	−S9: 9.77, 19.5, 39.1, 78.1, 156, 313, 625 µg/plate +S9: 9.77, 19.5, 39.1, 78.1, 156, 313, 625, 1 250 µg/plate Preincubation method	Negative (±S9)	JECDB (2018a)
Reverse mutation	*S. typhimurium* TA100, TA1535, TA98, TA1537, TA1538	2.5, 8.2, 26, 84, 269 µg per 50 µL in plates (±S9) Preincubation method	Negative	ECHA (2018a)
Gene mutation assay (HPRT)	Chinese hamster lung fibroblasts (V79)	44.8, 88.8, 177.5, 355.0, 710.0; 1 420.0 µg/mL (±S9) 4 hrs exposure	Negative	ECHA (2018b)
Chromosomal aberration test	Chinese hamster lung fibroblasts	−S9 (short term and continuous for 6, 24 and 48 hrs): 0, 178, 355, 710, 1 420 µg/mL +S9, 6 hrs: 0, 355, 710, 1 420 µg/mL	Negative	ECHA (2018c); JECDB (2018a)
In vitro				
Erythrocyte micronucleus cytogenicity	Swiss mouse i.p. administration 24 and 48 hrs	0, 500, 1 000, 2 000 mg/kg bw	Negative	ECHA (2018d)

bw: body weight; HPRT: hypoxanthine-guanine phosphorybosyltransferase; i.p.: intraperitoneal; S9: 9000 × *g* supernatant fraction

Table 4
Genotoxicity studies on methyl methacrylate

End-point	Test system/species	Concentration/dose	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Up to 1 000 µg/plate for preincubation and plate incorporation (±S9)	Negative	Lijinsky & Andrews (1980)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 0.15, 0.29, 0.59, 1.18, 2.35, 4.70 mg/plate (±S9)	Negative	Hachiya, Taketani & Takizawa (1982)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	40–10 000 µg/plate	Negative	Waegemaekers & Bensink (1984)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA1535	0, 33, 100, 333, 1 000, 3 333, 6 666 µg/plate	Negative	NTP (1986)
Reverse mutation	*S. typhimurium* TA97a, TA98, TA100, TA102	0–12.5 mg/plate	Negative	Schweikl, Schmalz & Rackebrandt (1998)
Cytotoxicity	V79 cells	0.94, 4.7, 9.4 mg/mL	Positive	Pradeep & Sreekumar (2012)
Mutation frequency	L5178Y mouse lymphoma cells	1, 1.75, 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3 mg/mL	Negative at 1 and 1.75 mg/mL; positive at other doses	Doerr, Harrington-Brock & Moore (1989)
Chromosomal aberration	CHO cells	0, 750, 1 000, 1 600, 3 000 µg/mL	Positive (−S9) Positive at top dose only (+S9)	NTP (1986)
Chromosomal aberration	Human lymphocytes	Cells mixed with 0.14 g (mean weight) of methyl methacrylate (97.4%) and *N*,*N*-dimethyl-para-toluidine to form bone cement	Negative	Bigatti et al. (1994)
Chromosomal aberration	L5178Y mouse lymphoma cells	1, 1.75, 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3 mg/mL	Negative at 1 and 1.75 mg/mL; positive at other doses	Doerr, Harrington-Brock & Moore (1989)
Chromosomal aberration	V79B/HPRT cells	0, 10, 20 mmol/L	Weakly positive	Schweikl, Schmalz & Rackebrandt (1998)
Chromosomal aberration	Human peripheral lymphocytes	Occupationally exposed individuals	Weakly positive	Tuček et al. (2002)
Chromosomal aberration	CHO cells	9.33×10^{-1}; 9.33×10^{-2}; 9.33×10^{-3}, 9.33×10^{-4} mg/mL	Positive	Yang et al. (2003)
Micronucleus induction	L5178Y mouse lymphoma cells	1, 1.75, 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3 mg/mL	Negative at 1 and 1.75 mg/mL; weakly positive at other doses	Doerr, Harrington-Brock & Moore (1989)
Micronucleus induction	Chinese hamster V79 cells	10, 20, 30 mmol/L	Negative cytotoxic at top dose	Schweikl, Schmalz & Spruss (2001)
SCE	CHO cells	0, 750, 1 000, 1 250, 1 500 µg/mL	Positive ±S9	NTP (1986)
SCE	Human peripheral lymphocytes	0.001–1 mg/100 mL	Equivocal	Cannas et al. (1987)

Table 4 (continued)

End-point	Test system/species	Concentration/dose	Result	Reference
SCE	CHO cells	9.33×10^{-1}, 9.33×10^{-2}, 9.33×10^{-3}, 9.33×10^{-4} mg/mL	Positive	Yang et al. (2003)
Gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$ cells	0, 0.125, 0.25, 0.5, 0.75, 1.0 µg/mL (±S9)	Positive ±S9	NTP (1986)
Gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$ cells	0, 1 000, 1 750, 2 202, 2 400, 2 499, 2 601, 2 700, 2 799, 2 901, 3 000 µg/mL (−S9)	Positive	Moore et al. (1988)
Gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$cells	0–3 000 µg/mL (±S9)	Positive (+S9); negative (−S9)	Dearfield et al. (1991)
In vitro				
Chromosomal aberration	Human peripheral lymphocytes of 38 occupationally exposed males	0.9–71.9 ppm	Negative	Seiji et al. (1994)
SCE	Human peripheral lymphocytes from 31 occupationally exposed men	Mean atmospheric concentrations 0.7–21.6 ppm	Negative overall, positive when comparing peak exposures of staff who clean facilities manually with staff who do not	Marez et al. (1991)
SCE	Peripheral lymphocytes of 38 occupationally exposed males	0.9–71.9 ppm	Negative	Seiji et al. (1994)
Micronucleus induction	Mouse bone marrow; oral route	Single doses of 1.13, 2.26, 4.52 g/kg bw	Negative	Hachiya, Taketani & Takizawa (1982)
Micronucleus induction	Bone marrow cells of Wistar rats exposed by the inhalation route	150 ppm for 8 hours/day for 1 or 5 days	Positive after 1 day, negative after 5 days	Araújo et al. (2013)
Micronucleus induction	Human buccal mucosal cells	Dental technicians exposed to methyl methacrylate monomer from dental resins	Negative	Azhar et al. (2013)

bw: body weight; CHO: Chinese hamster ovary; ppm: parts per million; S9: 9000 × *g* supernatant fraction from liver homogenate; SCE: sister chromatid exchange; *tk*: thymidine kinase locus

Table 5
Genotoxicity studies on dimethylaminoethyl methacrylate

Assay	Test system/species	Concentration/dose	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA100, TA1535, TA98, TA1538, TA1537 Direct plate incorporation method	100, 500, 1 000, 2 500, 5 000 µg/plate (±S9)	Negative	ECHA (2018e)

Assay	Test system/species	Concentration/dose	Result	Reference
Reverse mutation	*S. typhimurium* TA100, TA1535, TA98, TA1537 *Escherichia coli* WP2*uvr*A Preincubation method (±S9) Confirmation test: TA98 and TA1537 (−S9)	156, 313, 625, 1 250, 2 500, 5 000 µg/plate (all species) 1 000, 1 500, 2 000, 2 500, 3 000, 3 500, 4 000, 4 500, 5 000 µg/plate	TA1537 −S9: positive TA1537 +S9: negative TA100, TA98, TA1535 ±S9: negative WP2*uvr*A ±S9: negative	Kusakabe et al. (2002); JECDB (2018b)
Bioluminescent bacterial genotoxicity test	Dark mutants of marine luminous bacteria (*Vibrio fischeri* M169)	0.01 to 20 mmol/L in a serial 1 in 2 dilution (12 concentrations)	Negative	Nomura et al. (2006)
Gene mutation (HPRT)	Chinese hamster lung fibroblasts (V79)	−S9: 31.25, 62.5, 125, 250, 500 µg/mL +S9: 62.5, 125, 250, 500, 1 000, 1 500, 2 000 µg/mL	Negative (±S9)	ECHA (2018f)
Chromosomal aberration	CHL/IU cells (Chinese hamster lung fibroblast cell line) ±S9 for 6 hours and −S9 for 24 and 48 hrs continuous treatment	−S9 (24 and 48 hr): 0, 20, 39, 78, 156, 313, 625 µg/mL ±S9 (6 hrs): 0, 200, 400, 600, 800, 1 400, 1 600 µg/mL	±S9 were positive for clastogenicity and negative for polyploidy, irrespective of length of dosing	Kusakabe et al. (2002); JECDB (2018b)
Chromosomal aberration	Human lymphocytes ±S9 3 hrs	−S9: 663.2, 884.3, 1 179 µg/mL +S9: 884.3, 1 179, 1 572 µg/mL	Positive	ECHA (2018g)
Cytotoxicity and genotoxicity (Comet assay)	MRC-5 cells (human cell line of fetal lung fibroblasts)	0.6, 3, 15, 75 µg/mL	Negative	Manojlovic et al. (2017)
In vivo				
Mammalian erythrocyte micronucleus	Swiss mouse 2 × i.p. administrations separated by 24 hours. Sampled 24 and 48 hrs after 2nd dose	1 000 mg/kg bw	Negative	ECHA (2018h)

bw: body weight; HPRT: hypoxanthine-guanine phosphorybosyltransferase ; i.p.; intraperitoneal; S9; 9000 × *g* supernatant fraction

the fetuses were removed by caesarean section and the following determined: fetus count, sex ratio and viability; number and size of resorption sites; number of corpora lutea and position of the fetuses in the uterus; fetal weights (fetuses weighing less than 70% of the mean weight of the litter were described as "runts"); external malformations; visceral (half of the fetuses, by body sections) and skeletal variations and malformations; and placental weight and number.

No treatment-related behavioural, body weight or feed consumption effects were seen in the dams. Variations in the number, size and morphological features of the placentae were within the range of natural fluctuations. No treatment-related effects were observed in the fetuses; all findings were within the range of normal variation. There were no runts, malformations or dead fetuses in the treated or control groups (LPT, 1968).

(b) **Residual monomers**

n-Butyl methacrylate

There were no reproductive toxicity studies with *n*-butyl methacrylate.

A developmental toxicity study in rats on four methacrylates, including *n*-butyl methacrylate, was conducted in rats after inhalational exposure for 6 hours/day on gestation days 6–20. The exposure concentrations for *n*-butyl methacrylate were 0, 100, 300, 600 or 1200 ppm. No significant increases in embryo/fetal lethality or fetal malformations were observed. Fetal toxicity evidenced by statistically significant decreases in fetal body weights was observed at 600 ppm and above. Statistically significant increases in the incidence of fetuses with skeletal variations and of fetuses with variations were noted at 1200 ppm. These developmental effects were observed in the presence of overt signs of maternal toxicity (Saillenfait et al., 1999).

Methyl methacrylate

No conventional reproductive toxicity studies with methyl methacrylate were available. A dominant lethal study in mice given 100, 1000 or 9000 ppm (410, 4100 and 36 900 mg/m^3, respectively) methyl methacrylate by inhalation for 6 hours/day for 5 days showed no effects on fertility (Anderson & Hodge, 1976).

Developmental toxicity studies on methyl methacrylate given by the inhalation route have been summarized by WHO (1998) as follows:

> In a well conducted study in Crl:CDBR rats, there was no embryotoxicity or fetotoxicity and no increase in the incidence of malformations or variations following exposure for 6 hours/day on days 6–15 of gestation to concentrations of methyl methacrylate that ranged from 99 to 2028 ppm (406-8315 mg/m^3; NOEL = 8315 mg/m^3). However, there were treatment-related effects on maternal body weight at all concentrations (Solomon et al., 1993). In an earlier study in which pregnant ICR mice were exposed to 1330 ppm (5450 mg/m^3) methyl methacrylate for 2 hours twice daily during days 6-15 of pregnancy, there were no developmental effects. Maternal toxicity was not addressed in the report (McLaughlin et al., 1978).
>
> … In early studies, developmental effects, including decreases in fetal weights, embryo-fetal deaths, and skeletal abnormalities, were observed in rats following inhalation of concentrations of methyl methacrylate that were toxic to the dams (Hodge & Palmer, 1977; Nicholas et al., 1979). Similar effects were reported in studies in mice in which maternal toxicity was not addressed (Tansy, 1975) and in studies in rats in which the protocol and results were not well documented (Luo et al., 1986).

Dimethylaminoethyl methacrylate

A GLP-compliant repeated-dose reproductive/developmental toxicity study with 2-(dimethylamino)ethyl methacrylate conducted according to OECD TG 422 was summarized in the ECHA database (ECHA, 2015c) as follows:

> SD (Crj: CD) rats received gavage doses of 0 (vehicle; corn oil), 40, 200 and 1000 mg/kg/day, for males starting from 14 days before mating for 43 days and in females from 14 days before mating to day 3 of lactation. The female animals were sacrificed on day 4 of lactation. There were no effects on the reproductive parameters such as the mating index, the fertility index, the number of corpora lutea or implantations, the implantation index, the delivery index, the gestation index and the gestation length or the parturition. Three females in the 1000 mg/kg/day group, however, lost all of their pups during the lactation period. Females in the 1000 mg/kg/day group showed the following adverse effects in the repeated oral dose test: death of 3 animals out of 12, late onset of twitching, chronic convulsion, the suppression of body weight gain, degeneration of nerve fibers in the brain and spinal cord, hyperplasia of the mucosa in die gastric tract, oedema and inflammatory cell infiltration in the forestomach, atrophy of the thymus, increase in the weight of the kidneys and the adrenals without histopathological changes. The pups from the females in the 1000 mg/kg/day group showed lower body weights, and the viability index of the pups was decreased due to maternal nursery activity. By external inspection, no abnormalities were found. Conclusion: Under the conditions of this study, the NOAEL for the reproductive/developmental toxicity is considered to be 200 mg/kg/day.

A GLP-compliant developmental toxicity study with 2-(dimethylamino) ethyl methacrylate was summarized in the ECHA database (ECHA, 2015d) as follows:

> In a developmental toxicity study according to OECD 414 Dimethylaminoethyl methacrylate (purity: 99.79%) was administered to female rats (Sprague-Dawley, (SD)) by oral gavage at dose levels of 0, 100, 300 and 600 mg/kg bw/day from days 6 through 19 of gestation. Neither clinical signs nor signs of reaction to treatment were noted in treated females that could be related to treatment. No significant differences were noted in body weight, food consumption, gravid uterus weight, litter data and macroscopic observation of treated females, when compared to controls. Due to the absence of dose-relation, the findings detected at the external, visceral and skeletal examination of foetuses from all groups were considered incidental. The maternal and developmental NOAEL was therefore 600 mg/kg bw/day.

2.2.6 Special studies

(a) Cytotoxicity

In a GLP-compliant cytotoxicity study, BMC preparation (≥98%; extracted for 24 hours with DMEM in 10% FCS [acronyms not defined in study report] at 37 °C, in compliance with ISO 10993-5 and 10993-12) at concentrations of 8.8%, 13.2%, 19.8%, 29.6%, 44.4 or 66.7% volume per volume (v/v) as well as the vehicle (DMEM), a negative control and a positive control were incubated with L929 mouse connective tissue cells for 72 hours.

Growth inhibition (100%) was observed in the positive control. The negative control showed 19% growth inhibition, and the test extract showed between 3% and 10% growth inhibition (BSL, 1997a).

(b) Dermal toxicity

In a GLP-compliant study conducted according to OECD TG 402, BMC preparation (≥98%) moistened sufficiently with cotton seed oil, at a dose of 2000 mg/kg bw, was applied uniformly over approximately 10% of the total body surface area of rats (5/sex per group) for 24 hours.

During the 14-day observation period, there were no compound-related deaths or changes in feed intake or body-weight gain. There was no evidence of gross pathology of organs at necropsy (BSL, 2001a).

In a GLP-compliant study, 0.5 g of BMC preparation (≥98% BMC), moistened sufficiently with cotton seed oil, was applied to one side of each of three rabbits. The area was semioccluded, and the test substance was left in place for 4 hours. The other (untreated) side served as control. After 4 hours, bandages were removed and the site was examined after 1, 24, 48 and 72 hours.

No signs of irritation or other clinical signs were observed (BSL, 1997b).

In a GLP-compliant study conducted according to OECD TG 406, BMC preparation (≥98% BMC) was applied to a gauze patch moistened with isotonic saline and applied to one flank of female guinea-pigs (20/group, with 10 controls) on days 0, 6 and 13. On day 27, the test substance was applied to the untreated flank of control and treated animals. Skin reactions were observed after 24, 48 and 72 hours.

None of the treated animals showed any allergic reactions or sensitization compared with control animals (BSL, 1997c).

(c) Ocular toxicity

In a GLP-compliant study conducted according to OECD TG 405, 0.1 g of BMC preparation (≥98% BMC) was applied to the lower conjunctival sac of one eye in

three female rabbits. The other (untreated) eye served as control. Observations were made at 1, 24, 48 and 72 hours.

No signs of irritation were observed in any of the animals at 48 and 72 hours. A slight redness was visible 1 hour after application in all animals and after 24 hours in one animal. No signs of corrosion or irreversible effects were observed in any of the animals (BSL, 2001b).

(d) Phototoxicity

In a GLP-compliant in vitro study, using the BALB/3T3 mouse fibroblast cell line, BMC (≥98% BMC) was dissolved in dimethyl sulfoxide (DMSO) and diluted to 100 mg/mL in Earle's balanced salt solution (EBSS). Cultured cells were incubated in the dark for 60 minutes with 1% DMSO in EBSS (negative control); chlorpromazine at 100, 31.6, 10, 3.16, 1, 0.316, 0.1 or 0.0316 mg/mL (positive control); the test solution in the vehicle at 100, 31.6, 10, 3.16, 1, 0.316, 0.1 or 0.0316 mg/mL (test groups); and EBSS buffer only. Following incubation, a plate was irradiated with ultraviolet A (UVA) for 50 minutes while a duplicate plate was kept in the dark. After overnight incubation, cells were exposed to neutral red dye for 3 hours and washed. Dye uptake was measured spectrophotometrically.

Dye uptake did not differ significantly between groups.

BMC was considered to be non-phototoxic (BSL, 2001c).

2.2.7 Residual components of BMC (other than monomers)

The Committee considered toxicological and exposure data on other residual components of BMC that are present in the final product.

(a) 2-Propanol

2-Propanol has been evaluated by Joint FAO/ WHO Expert Committee on Food Additives (JECFA) as a flavouring agent. JECFA concluded 2-propanol was of no safety concern when used as a flavouring agent based on short-term, genotoxicity and reproductive and developmental studies. In a 13-week drinking-water study in rats, the NOAEL was 870 mg/kg bw per day. Three reverse mutation assays and two forward mutation assays in Chinese hamster ovary (CHO) cells (*hprt* locus) were negative. A rat developmental toxicity study showed no treatment-related effects in dams or offspring at the NOAEL of 400 mg/kg bw per day. In a rabbit developmental toxicity study, the NOAEL for maternal effects was 240 mg/kg bw per day and for developmental effects was 480 mg/kg bw per day. In a 3-generation study, the NOAEL was 1380 mg/kg bw per day (Annex 1, reference *138*).

Maximum exposure to BMC in children is 135 mg/kg bw per day. Based on BMC containing 2-propanol at levels not exceeding 0.5%, exposure

to 2-propanol would be 0.65 mg/kg bw per day. This gives margins of exposure (MOEs) of 370 or greater compared with the NOAELs, indicating that the presence of 2-propanol at 0.5% in copolymer BMC is not of toxicological concern.

(b) Butanol

Butanol is metabolized to butyric acid via the aldehyde, and then goes through the fatty acid and tricarboxylic acid pathways to be metabolized to innocuous products.

JECFA initially evaluated butanol as an extraction solvent and later as a flavouring agent. The NOAEL in a 28-day rat dietary administration study was 940 mg/kg bw per day. The Committee concluded that butanol was of no safety concern when used as a flavouring agent (Annex 1, reference *132*).

In a rat maternal and developmental toxicity study, the NOAEL for *n*-butanol (administered in drinking-water) was 1454 mg/kg bw per day. Fetal body weights were reduced at 5654 mg/kg bw per day but not at lower doses (Ema et al., 2005). In another drinking-water study looking at developmental effects, the lowest-observed-adverse-effect level (LOAEL) was 300 mg/kg bw per day (Sitarek, Berlińska & Barański, 1994). In a draft report, the USEPA derived a benchmark dose for a 10% response ($BMDL_{10}$) of 26 mg/kg bw per day from the Sitarek, Berlińska & Barański (1994) study based on dilatation of the lateral and/or third ventricle of the fetal brain. An uncertainty factor of 300 (10 for interspecies differences × 10 for intraspecies differences × 3 for database gaps) was applied to derive an oral reference dose of 0.09 mg/kg bw per day. The USEPA noted that this was the only effect and that fetal body weight was unaffected up to the highest dose of 5000 mg/kg bw per day (USEPA, 2011).

While genotoxicity data are limited, several reverse mutation assays were negative, and butanol did not increase the frequency of micronuclei or SCEs in CHO cells. A chromosomal aberration assay in V79 cells was negative. Studies on deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) replication have shown that *n*-butanol can inhibit these processes. There is also a positive forward mutation assay in CHO cells.

There were no long-term toxicity or carcinogenicity studies with butanol.

Maximum exposure to BMC in children is 135 mg/kg bw per day. Based on BMC containing butanol at levels not exceeding 0.5%, exposure to butanol would be 0.65 mg/kg bw per day. This gives MOEs of 40 compared with the $BMDL_{10}$, 2200 compared with the other developmental toxicity study, and 1445 compared with the 28-day oral NOAEL.

It can be concluded that the presence of butanol at 0.5% in BMC is not of toxicological concern.

(c) Methanol

JECFA previously evaluated methanol as an extraction solvent and concluded that its use "should be restricted to that determined by good manufacturing practice, which is expected to result in minimal residues … unlikely to have any significant toxicological effects" (Annex 1, reference *23*).

A USEPA Integrated Risk Information System (IRIS) review of methanol derived an oral reference dose of 2 mg/kg bw per day from a mouse developmental toxicity study of inhalation (Rogers et al., 1993) using route-to-route extrapolation of dose.

In a recent review, Moon (2017) calculated that an acute oral intake of approximately 3.16–11.85 g/person of pure methanol could cause blindness.

Maximum exposure to BMC in children is 135 mg/kg bw per day. Based on BMC containing methanol at levels not exceeding 0.1%, exposure to methanol would be 0.13 mg/kg bw per day, which is 6.5% of the reference dose.

It can be concluded that the presence of methanol at 0.1% in copolymer BMC is not of toxicological concern.

2.3 Observations in humans

No data on humans were available.

3. Dietary exposure

3.1 Introduction

BMC was evaluated by the Committee at the request of the Codex Committee on Food Additives from its Forty-ninth session (FAO/WHO, 2017). Codex asked the Committee to conduct a safety assessment on the use of BMC as a glazing or coating agent in food supplements.

Uses or proposed uses for BMC include in pharmaceuticals, food supplements, foods for special medical purposes and nutrient/micronutrient encapsulation for food fortification. BMC has been used for decades as an excipient in the pharmaceutical industry. It is used as a coating or glazing agent on food supplements. It has taste- and odour-masking and moisture-protection properties (Eisele, Haynes & Rosamilia, 2011), and it also protects against heat, light and oxidation.

BMC is useful for single dosage units such as tablets, hard and soft gel capsules and multiparticulates such as powders, granules or pellets. BMC dissolves

in acidic pH and thus allows fast release of active ingredients in the stomach. These properties mean it is useful for coating vitamin and mineral supplements, herbal extracts or supplements with unpleasant odours (e.g. branched-chain amino acids).

There were two sponsors for BMC, one proposing uses of BMC in food supplements and foods for special medical purposes, and the other proposing uses of BMC as a coating to enhance the stability of micronutrients used for food fortification. BMC can effectively encapsulate nutrients with a wide range of chemical and physical characteristics. It protects nutrients during transportation, storage, cooking and other conditions of heat, humidity or light, prevents interactions of the nutrients with other nutrients or substances in the food, and delivers them to the stomach for dissolution and absorption. It is used to assist in addressing micronutrient deficiencies by fortifying staple foods. Target populations are those living in low resource settings with limited access to a varied and nutritious diet, particularly women and children in developing countries where nutritional deficiencies are prevalent.

3.2 Approach to the exposure assessment

JECFA reviewed exposure estimates that were calculated and submitted by the sponsors. The exposure assessment included information provided by the sponsors. This information included uses of the copolymer, concentrations it is used in and estimates of exposure. A review of the EFSA (2010) exposure assessment for the copolymer was also included. The Committee also evaluated the EFSA exposure assessment independently and conducted a literature search (see section 1.2).

The Committee also undertook deterministic calculations to estimate exposure to the copolymer where national food consumption data were available. These data were primarily from the FAO/WHO Chronic Individual Food Consumption – Summary Statistics (CIFOCOss) database (https://www.who.int/foodsafety/databases/en/) for Codex General Standard for Food Additives (GSFA) food category no 13.6, "food supplements". Consumption data were also submitted for Australia and New Zealand. The details for these calculations are discussed further in section 3.4.1.

Exposures were reviewed or estimated for mean and high consumers as well as for adults and children.

The sponsor requesting uses in food supplements only included children aged 6 years and older based on the assumption that younger children take supplements in liquid form. However, the Committee also included children aged between 1 and 6 years in its assessment based on evidence recorded in national dietary surveys (e.g. CIFOCOss) of consumption of solid supplements

by children this age. In addition, some chewable tablets and soft gels are suitable for use by children from 2 years of age.

Estimates of exposure are included in the assessment for BMC and its monomers (section 3.5).

3.3 Estimates of exposure to BMC

3.3.1 Food supplements

(a) Estimates from the sponsor

The sponsor provided use levels of BMC in food supplements. Only small amounts of BMC are needed as effective taste masking and moisture protection can be achieved with as little as 1–5 mg/cm^2. This equates to approximately 6–10 mg for a 1 g tablet or dosage unit. For special applications, higher amounts are necessary in the coating, with the highest amount approximately 100 mg for a 1 g tablet or dosage unit. This was the concentration used in the exposure assessments. Although larger unit weights are on the market, these do not represent the majority of the relevant products and they were not considered in this assessment.

It was assumed that if different dosage forms (tablets, powders, etc.) with different surface area to volume ratios were used, the same concentration applies.

Consumption amounts for food supplements were evaluated by the sponsor from previous evaluations conducted by EFSA (2004a,b, 2005, 2006) and the Scientific Committee on Food (SCF) for other food additives used in this group (SCF, 1997, 2002). The number of dosage units consumed per day were calculated as between 3 and 7 for adults (12 for extreme users) and 2 for "children/young people" (ages between 6 and 18 years).

The sponsor also reviewed national consumption data from a range of dietary surveys, which mostly included data on frequency of consumption but also some data for consumption amounts (for a summary, see Appendix 1). Consumption data from the Netherlands (Ocké, Buurma-Rethans & Fransen, 2005) indicated that 80% of users consumed one or two supplements twice a day (a total of 4 supplements/day), and 6% were high users who consumed four supplements twice a day (a total of 8 supplements/day). Having assumed that one of these supplements is in liquid form and that not all supplement types contain BMC, the sponsor calculated consumption by adults to be 4 units per day, by heavy users to be 6 units per day and by children (not younger than 6 years) to be 2 units per day.

Based on these estimates and the assumption that BMC was present in the coatings of 1 g dosage units at 100 mg, the sponsor estimated exposures to BMC from expected consumption of food supplements to be 6.7 mg/kg bw per day in adults (10 in heavy users) and 13.3 mg/kg bw per day in children (Table 6).

Table 6
Exposures to BMC estimated by the sponsor from use in food supplements

Population	No. of units consumed/ day[a]	Exposure	
		mg per day	mg/kg bw per day[b]
Adults – expected consumption	4	400	6.7
Adults – heavy user	6	600	10.0
Young people / children 6–18 years	2	200	13.3

BMC: basic methacrylate copolymer; bw: body weight; no.: number
[a] One unit equates to 1 g containing 100 mg of BMC.
[b] Based on a mean body weight of 60 kg for adults and 15 kg for children.

The sponsor used a body weight of 15 kg for children 6 years and older. WHO standard body weight for a 5 year old at the 50th percentile is 18.3 kg for boys and 18.2 kg for girls (WHO Multicentre Growth Reference Study Group, 2006). Therefore, the body weight used by the sponsor for this population group may be an underestimate, resulting in a worst case scenario estimate of exposure on a body-weight basis.

(b) Estimates of exposure calculated by EFSA

EFSA estimated exposures to BMC from its use in food supplements (EFSA, 2010) using two concentrations: 30 mg per 1 g dosage unit for taste masking and moisture protection and 100 mg per 1 g dosage unit as the highest coating level. The assessment used consumption data from the British National Diet and Nutrition Surveys for high consumers (97.5th percentile) of food supplements for adults aged 19–64 years (Henderson, Gregory & Swan, 2002) and children aged 4–18 years (Gregory & Lowe, 2000). Seven and 2 dosage units per day were used for adults and children, respectively.

EFSA estimated exposures in high consumers using 30 mg of BMC per 1 g dosage unit at 3.5 mg/kg bw per day for adults and 2.4 mg/kg bw per day for children 4–18 years. Exposures in high consumers using 100 mg BMC per 1 g dosage unit were estimated at 11.7 mg/kg bw per day for adults and 8.0 mg/kg bw per day for children 4–18 years (Table 7). These estimates did not take into account that not all supplements contain BMC, and therefore they may be overestimates. However, if a consumer was brand loyal to a particular BMC-containing supplement they consumed regularly, these estimates would be appropriate.

The Committee used the estimated exposures from the 100 mg per 1 g dosage unit scenario for its evaluation, this being a worst case scenario.

Table 7
Estimated exposures to BMC from use in food supplements by high consumers

Population	No. of units consumed/day	Exposure			
		30 mg per 1 g dosage unit [a]		100 mg per 1 g dosage unit [b]	
		mg per day	mg/kg bw per day [c]	mg per day	mg/kg bw per day [c]
Adults 19–64 years	7	210	3.5	700	11.7
Children 4–18 years	2	60	2.4	200	8.0

BMC: basic methacrylate copolymer; bw: body weight; no.: number
[a] Concentration used for taste masking and moisture protection.
[b] Highest coating level.
[c] Based on a mean body weight of 60 kg for adults and 25 kg for children.
Source: EFSA (2010)

(c) National estimates of exposure calculated by the Committee

The Committee estimated exposure to BMC from food supplement use for a number of countries using consumption data in the CIFOCOss database (GSFA food category no. 13.6, "food supplements").

CIFOCOss includes data collected via national population dietary surveys of 2 or more days of individuals' food consumption (for further details, see http://www.who.int/foodsafety/databases/en/). Food consumption data are presented according to the CIFOCOss food classification system. For most food groups, this is based on the Codex raw commodity classification system; for some processed commodities, it is based on the Codex GSFA classification system. Food consumption data for a number of different age groups are represented in the CIFOCOss dataset based on the data collected in the countries' national surveys. The Committee used consumption data for the general population and for children for the exposure estimates; the data were not split by sex.

Food supplement consumption data were available in CIFOCOss for nine countries (Brazil, Finland, Germany, Ireland, Italy, the Netherlands, Sweden, Thailand, the United Kingdom). However, as there were no quantitative consumption amounts for Thailand, that country was not included. The data were not used for exposure calculations where there were fewer than 10 consumers per population group as this number was too low for an estimate to be reliable. Thus data for Germany (children), Italy (infants, children and adolescents) and the Netherlands (toddlers and children) were excluded. The data from Brazil were also excluded as the amounts consumed were at least 5 times higher than those of the other population groups included in the dataset; as no explanation was provided (e.g. what was included in the food group), these data were determined to be outliers and not retained for the exposure calculation. There were no consumption data for food supplements for infants in CIFOCOss.

Mean and 90th percentile consumption data per kg of body weight for consumers only were used for the exposure calculations to take into account the specific body weights relevant to each country and population group. Concentrations of BMC used in the calculations were those provided by the sponsor: 100 mg per 1 g dosage unit.

Consumption data for dietary supplements for Australia and New Zealand were also provided to the Committee for use in the assessment (Food Standards Australia New Zealand, personal communication, 30 May 2018). The Australian data were from the 2011/12 National Nutrition and Physical Activity Survey (ABS, 2015) based on 2 days of consumption data. The New Zealand data were from the 2008/09 NZ Adult Nutrition Survey (University of Otago and Ministry of Health, 2011) and the 2002 Children's Nutrition survey (Parnell et al., 2003) based on 1 day of consumption data. Liquid supplements were excluded from the derivation.

The actual consumption amounts as reported in the CIFOCOss database, shown in Table 8 in g/kg bw per day, were used for the exposure assessment calculations. The consumption amounts were not rounded to a number of dosage units for the exposure calculations but were converted to 1 g units to compare with the number of dosage units used by the sponsor and EFSA. Based on mean consumption data, the number of dosage units consumed by adults ranged between 3 and 7 units per day and, at the 90th percentile, between 5 and 20 units per day. Similarly, based on mean consumption data, the number of dosage units consumed by children ranged between 1 and 4 per day and, based on 90th percentile consumption data, between 2 and 10 per day. For adults, the mean consumption range was the same as the range used by the sponsor and EFSA, but at the 90th percentile, the upper end of the consumption range was up to nearly 3 times as high. The lower end of the mean consumption range for children was similar to that used by the sponsor and EFSA, but the upper end of the range was about double and the upper end of the range for the 90th percentile was about 5 times higher. Apart from the values from Australia and New Zealand, the specific types of supplements – and whether this included liquid supplements – is unknown.

Exposures to BMC from use in food supplements estimated by the Committee from national food consumption data for adults at the mean ranged between 3.5 and 11.6 mg/kg bw per day and, at the 90th percentile, between 7.4 and 37.0 mg/kg bw per day (Table 8). For children, mean exposure ranged between 2.9 and 16.0 mg/kg bw per day and, at the 90th percentile, between 4.2 and 42.7 mg/kg bw per day. These estimates do not take into account that not all supplements will contain BMC and therefore may be overestimates. However, if a consumer was brand loyal to a particular supplement that contained BMC and consumed it regularly, these estimates would be appropriate.

Table 8
Estimated national exposures to BMC from use as a food additive in food supplements [a]

Population / country	Age group (years)	No. of consumers	Percentage of consumers	Consumption (g/kg bw per day)		Exposure (mg/kg bw per day)	
				Mean	P90	Mean	P90
Adults							
Australia	≥19	2 284	38	0.03	0.08	3.5	7.7
Finland	18–64	663	42	0.04	0.08	4.2	7.6
	65–74	215	46	0.05	0.11	5.0	10.8
Ireland	18–64	246	26	0.04	0.09	4.3	8.6
Italy	18–64	98	4	0.08	0.20	8.2	20.0
	65–74	12	4	0.04	0.11	3.7	10.9
	≥75	17	7	0.12	0.37	11.6	37.0
New Zealand	≥15	576	12	0.05	0.09	4.7	9.1
United Kingdom	18–64	419	20	0.05	0.07	4.5	7.4
Children							
Australia	2–5	75	19	0.12	0.26	12.0	25.7
	6–18	236	18	0.04	0.06	3.6	6.3
Finland	3–9	56	22	0.16	0.43	16.0	42.7
New Zealand	5–14	144	4	0.05	0.09	4.8	8.8
Sweden	3–9	263	18	0.08	0.10	8.0	9.6
	10–17	178	17	0.03	0.04	2.9	4.2

BMC: basic methacrylate copolymer; bw: body weight; no.: number; P90: 90th percentile
[a] Calculated by the Committee using national dietary survey data

3.3.2 Pharmaceuticals

(a) Estimates from the sponsor

BMC has been used in pharmaceuticals in Europe since 1999, and exposure from this source was also considered in the estimates by the sponsor. The same concentration was used for pharmaceuticals as for food supplements (100 mg/1 g dosage unit). While the sponsor indicated that previous EFSA assessments were based on consumption of 10–12 units of pharmaceuticals per day, this was considered a conservative estimate given that not all pharmaceuticals contain BMC. As a result, 6 units/day was used in the exposure assessment for adults and 2/day for young children.

Exposures to BMC from use in pharmaceuticals were estimated by the sponsor as 10 mg/kg bw per day for adults and 13.3 mg/kg bw per day for children (Table 9).

Table 9
Estimated exposures to BMC by the sponsor from use in pharmaceuticals

Population	No. of units consumed/ day [a]	Exposure	
		mg per day	mg/kg bw per day [b]
Adults	6	600	10.0
Young people / children 6–18 years	2	200	13.3

BMC: basic methacrylate copolymer; bw: body weight; no.: number
[a] One unit is 1 g containing 100 mg of BMC.
[b] Based on a mean body weight of 60 kg for adults and 15 kg for children

(b) Estimates of exposure calculated by EFSA

For its estimate of exposures to BMC from its use in pharmaceuticals, EFSA (2010) used the same two concentrations as used for exposure via supplements, that is, 30 mg per 1 g dosage unit and 100 mg per 1 g dosage unit. The same number of dosage units used for food supplements were used for pharmaceuticals: 7 units per day for adults and 2 units per day for children.

EFSA estimated exposure to BMC when used in pharmaceuticals at 30 mg per 1 g dosage unit to be 3.5 mg/kg bw per day for adult high consumers and 2.4 mg/kg bw per day for children 4–18 years old. Exposure to BMC when used in pharmaceuticals at 100 mg per 1 g dosage unit was estimated for adults as 11.7 mg/kg bw per day for heavy users and 8.0 mg/kg bw per day for children 4–18 years old (Table 10).

These estimates did not take into account that not all pharmaceuticals contain BMC, and therefore are likely to be overestimates.

The Committee used the estimated exposures from the 100 mg per 1 g dosage unit scenario for its evaluation, this being a worst case scenario.

3.3.3 Foods for special medical purposes

BMC can be used in foods for special medical purposes as incomplete foods when these are presented in the same form of solid food supplements (e.g. tablets, capsules, etc.). The single dosage units are likely to contain the same concentration of BMC as food supplements, that is, 10% of the final dosage unit (100 mg/1 g unit).

Foods for special medical purposes are generally defined as those formulated for exclusive or partial dietary management of patients under medical supervision who have limited ability to digest or metabolize foods or nutrients or who have increased nutrient requirements. These products may contain higher concentrations of active ingredients than food supplements for the general population. Foods for special medical purposes would not normally

Table 10
Estimated exposures to BMC from use in pharmaceuticals for high consumers

Population	No. of units consumed/day	Exposure			
		30 mg per 1 g dosage unit [a]		100 mg per 1 g dosage unit [b]	
		mg per day	mg/kg bw per day[a]	mg per day	mg/kg bw per day[a]
Adults 19–64 years	7	210	3.5	700	11.7
Children 4–18 years	2	60	2.4	200	8.0

BMC: basic methacrylate copolymer; bw: body weight; no.: number
[a] Concentration used for taste masking and moisture protection.
[b] Highest coating level.
[c] Based on a mean body weight of 60 kg for adults and 25 kg for children.
Source: EFSA (2010)

be consumed by the general population but by specific individuals who would generally be under medical supervision.

The duration of use of foods for special medical purposes depends on the medical indication and may vary from short-term to lifetime use. Consumption of foods for special medical purposes, and exposures to BMC from these, depends on the dosage instructions and/or the medical needs of the individual and are therefore highly variable.

No food consumption data for foods for special medical purposes were provided by the sponsor. While the CIFOCOss database has some consumption data for foods for special medical purposes, there is insufficient information to determine what specific foods are included by each country in this category. It was therefore not possible to determine if these include single dosage units. As such, the data from the CIFOCOss database could not be used to estimate exposures from foods for special medical purposes.

A literature search found no consumption data for foods for special medical purposes.

As a result, the Committee was not able to estimate dietary exposures from foods for special medical purposes. Therefore, there is a degree of uncertainty in the Committee's exposure assessment and the overall safety evaluation. However, for individuals managing a condition or disease by consuming foods for special medical purposes, achieving a positive health status may be a priority greater than the need to consider exposure to BMC from these foods.

The sponsor noted that the BMC-containing foods used for special medical purposes are similar to food supplements. Given the conservative nature of those calculations, which are based on high consumers, foods for special medical purposes are not anticipated to increase exposures above that of food supplements and pharmaceuticals. However, there are no data or information to suggest this is the case.

The use of BMC in foods for special medical purposes was therefore not considered by the Committee.

3.3.4 Fortified foods

A second sponsor proposed the use of BMC for micronutrient encapsulation to fortify food and protect the nutrients from deterioration due to adverse conditions such as heat, humidity and light exposure during transportation, storage and cooking. Microencapsulating nutrients is suggested as being beneficial when fortifying staple foods to address micronutrient deficiencies in particular countries or regions (e.g. sub-Saharan Africa, southeast Asia) and population subgroups (e.g. pregnant women, young children) where deficiencies exist. Uses in food fortification aside from this use were not mentioned, although they may be a possibility.

The dietary exposure to BMC from food fortification will depend on the nutrient, and the proportion of the recommended daily intake to be met by the fortification. The nutrients considered included both vitamins and minerals. They were vitamin A (retinyl palmitate), folic acid, vitamin B12 (cobalamin), vitamin D3, thiamin, riboflavin, pantothenic acid, pyridoxine, biotin, iron, zinc and iodine.

The dietary exposure estimates by the sponsor for a range of micronutrients were based on providing all (100%) or a proportion (75% and 50%) of the United States/Canadian recommended dietary allowance (RDA) or adequate intake (AI). For adults, the highest RDA or AI from any group was selected as a worst case scenario. The highest RDA or AI was for lactating women for all nutrients except for folate and iron, where the values for pregnant women were used; and for vitamin D, where the values for adults older than 70 years were used. For children, the values for 1–3 year olds were used. RDAs were used for all nutrients except for pantothenic acid and biotin where AIs were used.

The concentration of BMC used for encapsulation differs for each micronutrient and ranges from approximately 50% to 99%. The copolymer concentration (i.e. the percent weight of copolymer relative to the total weight of the composition) depends on the specific micronutrient and the presence of other excipients. Some micronutrients are more readily encapsulated in the copolymer and therefore require a relatively lower copolymer percentage (e.g. approximately 96% for vitamin B2); others have a higher copolymer percentage (e.g. approximately 99% for zinc). In the cases where there are additional excipients (e.g. hyaluronic acid for iron), the copolymer percent is significantly reduced (e.g. approximately 71%) because the excipient makes up a large portion of the composition.

Each micronutrient-specific concentration was used for the dietary exposure calculations.

In order to calculate the dietary exposure to BMC from each micronutrient, the sponsor estimated the proportion of the nutrient in the whole microencapsulation particle (i.e. nutrient + copolymer + excipients) and then estimated the amount of particles needed to meet the RDA/AI. Based on the proportion of copolymer in the particle, the exposure to BMC was subsequently estimated.

The estimates of "total" dietary exposure to BMC as estimated by the sponsor was the sum of BMC exposures from each of the individual micronutrients.

Conversion factors were used to convert the form of the nutrient used for fortification to the equivalent amount of the form specified for the RDA/AI. This was done by the sponsor for vitamin A and folate. For Vitamin A, the fortificant as retinyl palmitate was adjusted by a factor of 1.8 to equal the RDA for retinol. For folate, the fortificant used was folic acid which was adjusted by a factor of 0.6 to equal the RDA for dietary folate equivalents.

If micronutrients were co-encapsulated, a copolymer concentration different than the individual encapsulated micronutrients would result because the presence of each material can impact the encapsulation process of the other. However, this difference in copolymer concentration is not expected to be large. While co-encapsulation is possible, multiple individually encapsulated nutrients can simply be combined as a physical mixture. In this case the copolymer concentration will be the weight-average amount of the starting copolymer concentrations. For example, if equal parts of the Vitamin B2 (96% copolymer) and zinc (99% copolymer) compositions were mixed, the resulting copolymer concentration would be 97.5%. The dietary exposures estimated by the sponsor were based on individual nutrient encapsulation as this provided a conservative estimate of exposure to BMC, rather than estimating exposure based on co-encapsulation.

When assessing single micronutrients, estimated dietary exposures to BMC for adults when providing 100% of the RDA ranged between 0.03 and 20.4 mg/kg bw per day; when providing 75% of the RDA exposures were between 0.02 and 15.3 mg/kg bw per day; and when providing 50% of the RDA were between 0.01 and 10.2 mg/kg bw per day. The lowest exposures were from encapsulating vitamin D; the highest from when encapsulating zinc (Table 11). For children 1–3 years, when providing 100% of the RDA, exposures to BMC ranged between 0.05 and 26.1 mg/kg bw per day; when providing 75% of the RDA exposures were between 0.04 and 19.5 mg/kg bw per day; and when providing 50% of the RDA were between 0.03 and 13.0 mg/kg bw per day. The lowest exposures were from encapsulating vitamin B12, and the highest from when encapsulating vitamin B5 (pantothenic acid).

For adults, total dietary exposures to BMC (as a sum of exposures from all nutrients assessed) when providing 100% of the RDA was 44.5 mg/kg bw per day; when providing 75% of the RDA exposures was 33.4 mg/kg bw per day; and when providing 50% of the RDA was 22.3 mg/kg bw per day. For children 1–3 years, total exposures to BMC when providing 100% of the RDA was 91.8 mg/kg bw per day; when providing 75% of the RDA was 68.8 mg/kg bw per day; and when providing 50% of the RDA was 45.9 mg/kg bw per day (Table 11).

It is extremely difficult to estimate accurately the dietary exposure to BMC via nutrient microencapsulation for food fortification. The dietary exposure would vary considerably in different populations and population subgroups for a number or reasons. The estimated dietary exposures will differ in populations where nutrient reference values (e.g. RDAs/AIs) differ from those in the United States of America (USA) and Canada, and it is the level of nutrient requirement that is the target to be met from the fortification. Actual exposures will also depend on the population of interest (as different populations and population subgroups have different nutrient requirements); the nutrient being used; the food being fortified; the level of fortification required; and the amount of the food being consumed by each population being assessed. It may be that only a single level of fortification can be incorporated into a staple food that is consumed in differing amounts by different population subgroups, and therefore the population may not meet the RDA or equivalent nutrient reference value.

The 100% scenario assumes that there are no other dietary sources of the nutrient; this may be the case depending on the fortification program. If, for example, other foods that contain naturally occurring sources of the nutrient are consumed a level of fortification that meets the requirement may cause some population groups to approach or exceed the upper safe level of the nutrient. It could be assumed that the 75% and 50% of the RDA exposure scenarios take into account that other foods provide these nutrients. Specific population intakes from naturally occurring sources would need to be evaluated before a level of fortification could be determined, which would then affect the level of exposure to BMC.

An added degree of uncertainty in the dietary exposure estimates would be population specific. The number of nutrients used for fortification at any time would need to be based on the needs of the specific population or population subgroup.

RDAs are relevant to individuals not populations. The estimated average requirements (EARs) for a population are usually established at the mean nutrient intake plus 2 standard deviations, meaning if population nutrient targets are the aim, rather than RDAs for individuals, the fortification level would be higher and therefore the copolymer exposure would also be higher.

Table 11

Sponsor-estimated dietary exposure to BMC from proposed uses in food fortification for adults ≥19 years and children 1–3 years

	Intake of BMC from use at various percentages of micronutrient RDA/AI (mg/kg bw per day)[a]					
	Adults [a]			Children [b]		
Micronutrient	**100%**	**75%**	**50%**	**100%**	**75%**	**50%**
Vitamin A (Retinyl palmitate)	0.5	0.4	0.3	0.6	0.5	0.3
Vitamin B1 (Thiamin)	1.3	1.0	0.7	6.6	4.9	3.3
Vitamin B2 (Riboflavin)	0.7	0.5	0.4	3.5	2.6	1.7
Vitamin B5 (Pantothenic Acid)	5.2	3.9	2.6	26.1	19.5	13.0
Vitamin B6 (Pyridoxine)	1.7	1.3	0.9	8.7	6.5	4.4
Vitamin B7 (Biotin)	0.2	0.1	0.1	0.8	0.6	0.4
Vitamin B9 (Folic acid)	4.0	3.0	2.0	5.0	3.7	2.5
Vitamin B12 (Cobalamin)	0.03	0.03	0.02	0.05	0.04	0.03
Vitamin D3	0.03	0.02	0.01	0.1	0.1	0.1
$FeSO_4$ (Iron)	10.0	7.5	5.0	13.0	9.7	6.5
$ZnSO_4$ (Zinc)	20.4	15.3	10.2	25.5	19.1	12.8
KIO_3 (Iodine)	0.4	0.3	0.2	1.9	1.4	0.9
Total from all listed micronutrients	44.5	33.4	22.3	91.8	68.8	45.9
Total, excluding zinc	24.1	18.1	12.0	66.2	49.7	33.1

BMC: basic methacrylate copolymer; bw: body weight; RDA/AI: recommended dietary allowance/adequate Intake

[a] Daily intake values (in mg/kg bw) were calculated by assuming a 60 kg adult body weight.

[b] Daily intake values (in mg/kg bw) were calculated by assuming a 12 kg child body weight.

The Committee noted that the estimated dietary exposures provided by the sponsor were to provide a presumed worst case scenario of the level of dietary exposure to BMC from micronutrient encapsulation for food fortification for the purpose of the safety assessment. The 100% scenario was used by the Committee for its evaluation as the worst case. This scenario may lead to overestimates of dietary exposure for some populations or population subgroups because they may have a lower RDA than that used in the calculations; they may not require 100% of the RDA to be provided via food fortification; and they may require different or fewer nutrients to be provided via food fortification.

Estimates of dietary exposures at a national level would be needed for safety assessments if use of BMC for this purpose is permitted nationally. The risk/benefit of dietary exposure to BMC from fortified foods would also need to be assessed at a national level based on the nutritional needs of each specific malnourished or target population or population group.

3.4 Estimates of exposure to the monomers of BMC

The BMC monomers are *n*-butyl methacrylate, methyl methacrylate and 2-(dimethylamino)ethyl methacrylate. The Committee reviewed estimated exposures to each monomer provided by the sponsor and from the EFSA (2010) assessment and calculated national estimates of exposure.

The total monomer content is up to 0.3% of the copolymer. Therefore, this proportion was used to convert the estimates of exposure to the copolymer to an exposure to the total monomers.

The weight ratio of the monomers was used to convert the total exposure to all monomers to an exposure for each individual monomer.

3.4.1 Estimates from the sponsor

Exposures to the monomers as estimated by the sponsor from uses in food supplements and pharmaceuticals are shown in Table 12. For adults, exposures from food supplements were 5.0–7.5 µg/kg bw per day for *n*-butyl methacrylate, 10.1–15.0 µg/kg bw per day for 2-(dimethylamino)ethyl methacrylate and 5.0–7.5 µg/kg bw per day for methyl methacrylate (range based on 4–6 dosage units per day). For children, the exposures from food supplements were 10.0 µg/kg bw per day for *n*-butyl methacrylate, 20.0 µg/kg bw per day for 2-(dimethylamino) ethyl methacrylate and 10.0 µg/kg bw per day for methyl methacrylate (based on 2 dosage units per day).

Exposures from pharmaceuticals for adults were 7.5 µg/kg bw per day for *n*-butyl methacrylate, 5.0 µg/kg bw per day for 2-(dimethylamino)ethyl methacrylate and 7.5 µg/kg bw per day for methyl methacrylate (based on 6 dosage units per day). Exposures from pharmaceuticals for children were exactly the same as for food supplements as it was assumed that the same number of dosage units were ingested.

It has been noted that 2-(dimethylamino)ethyl methacrylate is not detected when analysing the copolymer, and it was assumed that it is not particularly labile from the polymer matrix. It may also be assumed that 2-(dimethylamino)ethyl methacrylate does not contribute to the exposure and therefore the monomeric exposure may be lower than estimated.

The Committee calculated exposures to the monomers based on the dietary exposures to BMC from use in micronutrient encapsulation for food fortification estimated by the sponsor (Table 12). For adults, the estimated exposures to the BMC monomers from uses in micronutrient encapsulation for food fortification at 100% of the RDA/AI (Table 13) ranged from 0.02 to 15.3 µg/kg bw per day for *n*-butyl methacrylate; from 0.04 to 30.6 µg/kg bw per day for 2-(dimethylamino)ethyl methacrylate; and from 0.02 to 15.3 µg/kg bw per day for methyl methacrylate across the individual nutrients assessed. For children, the

Table 12
Sponsor-estimated exposures to BMC and BMC monomers from uses in food supplements and pharmaceuticals

Source	Population	Exposure to BMC (mg/kg bw per day) [a]	Exposure to BMC monomers (µg/kg bw per day)			
			Total [b]	*n*-butyl methacrylate	2-(dimethylamino) ethyl methacrylate	Methyl methacrylate
Food supplements	Adults	6.7–10.0	20.1–30.0	5.0–7.5	10.1–15.0	5.0–7.5
	Children	13.3	39.9	10.0	20.0	10.0
Pharmaceuticals	Adults	10.0	30.0	7.5	15.0	7.5
	Children	13.3	39.9	10.0	20.0	10.0

BMC: basic methacrylate copolymer; bw: body weight
[a] Based on consuming 4–6 dosage units per day of supplements and 6 dosage units per day of pharmaceuticals for adults; and 2 dosage units for each of supplements and pharmaceuticals per day for children. Body weight 60 kg for adults and 15 kg for children.
[b] Based on total monomer content of 0.3% of the copolymer.

exposures ranged from 0.04 to 19.5 µg/kg bw per day for *n*-butyl methacrylate; from 0.1 to 39.1 µg/kg bw per day for 2-(dimethylamino)ethyl methacrylate; and from 0.04 to 19.5 µg/kg bw per day for methyl methacrylate across the individual nutrients assessed.

As a total exposure from all nutrients assessed, dietary exposures to the monomers for adults were 33 µg/kg bw per day for *n*-butyl methacrylate; 67 µg/kg bw per day for 2-(dimethylamino)ethyl methacrylate; and 33 µg/kg bw per day for methyl methacrylate. For children, total exposure from all nutrients assessed was 69 µg/kg bw per day for *n*-butyl methacrylate; up to around 140 µg/kg bw per day for 2-(dimethylamino)ethyl methacrylate; and 69 µg/kg bw per day for methyl methacrylate (Table 13).

3.4.2 Estimates of exposure calculated by EFSA

EFSA (2010) estimated exposures to BMC monomers expressed as the metabolite methacrylic acid; EFSA did not present exposures to each of the individual monomers. The Committee estimated exposure to the monomers (total monomers and individual monomers) (Table 14) based on the EFSA estimated exposure to the copolymer using the 10% coating scenario, 0.3% of monomers in the copolymer and weighting for the weight of each monomer in the copolymer. While EFSA did not include exposure to 2-(dimethylamino)ethyl methacrylate as it was not detected in the copolymer, the Committee included it as a worst case scenario and to be consistent with the other completed calculations.

For adults, exposures from supplements were 8.8 µg/kg bw per day for *n*-butyl methacrylate, 17.6 µg/kg bw per day for 2-(dimethylamino)ethyl methacrylate and 8.8 µg/kg bw per day for methyl methacrylate. For children, exposures from supplements were 6.0 µg/kg bw per day to *n*-butyl methacrylate,

Table 13
Estimated exposures to BMC monomers from uses in micronutrient encapsulation for food fortification [a]

	Exposure to monomers (µg/kg bw per day)			
Population group / micronutrient	**Total [b]**	***n*-Butyl methacrylate**	**2-(Dimethylamino) ethyl methacrylate**	**Methyl methacrylate**
Adults				
Vitamin A (Retinyl palmitate)	1.6	0.4	0.8	0.4
Vitamin B1 (Thiamin)	4.0	1.0	2.0	1.0
Vitamin B2 (Riboflavin)	2.1	0.5	1.0	0.5
Vitamin B5 (Pantothenic Acid)	15.6	3.9	7.8	3.9
Vitamin B6 (Pyridoxine)	5.2	1.3	2.6	1.3
Vitamin B7 (Biotin)	0.5	0.1	0.3	0.1
Vitamin B9 (Folic acid)	11.9	3.0	6.0	3.0
Vitamin B12 (Cobalamin)	0.1	0.02	0.05	0.02
Vitamin D3	0.1	0.02	0.04	0.02
$FeSO_4$ (Iron)	30.0	7.5	15.0	7.5
$ZnSO_4$ (Zinc)	61.3	15.3	30.6	15.3
KIO_3 (Iodine)	1.1	0.3	0.6	0.3
Total from all listed micronutrients	133.5	33.4	66.8	33.4
Total, excluding zinc	72.3	18.1	36.1	18.1
Children				
Vitamin A (Retinyl palmitate)	1.9	0.5	0.9	0.5
Vitamin B1 (Thiamin)	19.8	4.9	9.9	4.9
Vitamin B2 (Riboflavin)	10.4	2.6	5.2	2.6
Vitamin B5 (Pantothenic Acid)	78.2	19.5	39.1	19.5
Vitamin B6 (Pyridoxine)	26.1	6.5	13.1	6.5
Vitamin B7 (Biotin)	2.5	0.6	1.2	0.6
Vitamin B9 (Folic acid)	14.9	3.7	7.4	3.7
Vitamin B12 (Cobalamin)	0.2	0.04	0.1	0.04
Vitamin D3	0.3	0.1	0.2	0.1
FeSO4 (Iron)	38.9	9.7	19.5	9.7
ZnSO4 (Zinc)	76.6	19.1	38.3	19.1
KIO3 (Iodine)	5.6	1.4	2.8	1.4
Total from all listed micronutrients	275.3	68.8	137.6	68.8
Total, excluding zinc	198.7	49.7	99.4	49.7

BMC: basic methacrylate copolymer; bw: body weight
[a] Highest exposures from any population subgroup from uses in micronutrient encapsulation for food fortification to meet 100% of the USA/Canadian recommended dietary allowance (RDA) or adequate intake (AI).
[b] Based on total monomer content of 0.3% of the copolymer.

12.0 µg/kg bw per day to 2-(dimethylamino)ethyl methacrylate and 6.0 µg/kg bw per day to methyl methacrylate.

Table 14
Estimated exposures to BMC and BMC monomers based on EFSA data

Source	Population	Exposure to BMC (mg/kg bw per day) [a]	Exposure to monomers (µg/kg bw per day) [b]			
			Total [c]	*n*-Butyl methacrylate	2-(dimethylamino) ethyl methacrylate	Methyl methacrylate
Food supplements	Adults	11.7	35.1	8.8	17.6	8.8
	Children	8.0	24.0	6.0	12.0	6.0
Pharmaceuticals	Adults	11.7	35.1	8.8	17.6	8.8
	Children	8.0	24.0	6.0	12.0	6.0

BMC: basic methacrylate copolymer; bw: body weight; EFSA: European Food Safety Authority
[a] Calculated by EFSA (2010). Based on consuming 7 dosage units per day for each of supplements and pharmaceuticals for adults and 2 dosage units per day for each of supplements and pharmaceuticals for children. BMC concentration of 100 mg coating per supplement. Body weight 60 kg for adults and 25 kg for children.
[b] Calculated by the Committee from EFSA (2010) copolymer exposure estimates from uses in food supplements and pharmaceuticals.
[c] Based on total monomer content of 0.3% of the copolymer.

Exposures from pharmaceuticals were exactly the same as for food supplements as it was assumed that the same number of dosage units were ingested.

3.4.3 National estimates of exposure calculated by the Committee

The Committee estimated national exposures to BMC monomers from uses of the copolymer in food supplements (Table 15).

Estimated exposures for adults to *n*-butyl methacrylate at the mean ranged between 2.6 and 8.7 µg/kg bw per day and, at the 90th percentile, between 5.6 and 27.8 µg/kg bw per day; to 2-(dimethylamino)ethyl methacrylate at the mean ranged between 5.2 and 17.4 µg/kg bw per day and, at the 90th percentile, between 11.2 and 55.6 µg/kg bw per day; and for methyl methacrylate at the mean ranged between 2.6 and 8.7 µg/kg bw per day and, at the 90th percentile, between 5.6 and 27.8 µg/kg bw per day.

Estimated exposures for children to *n*-butyl methacrylate at the mean ranged between 2.7 and 12.0 µg/kg bw per day and, at the 90th percentile, between 3.1 and 32.1 µg/kg bw per day; to 2-(dimethylamino)ethyl methacrylate at the mean ranged between 4.4 and 24.1 µg/kg bw per day and, at the 90th percentile, between 6.3 and 64.1 µg/kg bw per day; and to methyl methacrylate at the mean ranged between 2.2 and 12.0 µg/kg bw per day and, at the 90th percentile, between 3.1 and 32.1 µg/kg bw per day.

These estimates do not take into account that not all supplements will contain BMC. Therefore, they are likely to be overestimates. However, if a consumer was brand loyal to a particular BMC-containing supplement that they consumed regularly, the exposures could be assumed to be appropriate estimates for those consumers.

Table 15
Committee-estimated exposures to BMC and BMC monomers based on national food consumption data [a]

		Exposure to monomers (µg/kg bw per day)							
		Total [b]		*n*-Butyl methacrylate		2-(Dimethylamino) ethyl methacrylate		Methyl methacrylate	
Country	Population / years	Mean	P90	Mean	P90	Mean	P90	Mean	P90
Australia	Adults ≥19	10	23	2.6	5.8	5.2	11.5	2.6	5.8
Finland	Adults 18–64	13	23	3.2	5.7	6.3	11.3	3.2	5.7
	Elderly adults 65–74	15	32	3.8	8.1	7.5	16.2	3.8	8.1
Ireland	Adults 18–64	13	26	3.2	6.5	6.4	13.0	3.2	6.5
Italy	Adults 18–64	25	60	6.2	15.0	12.3	30.0	6.2	15.0
	Elderly adults 65–74	11	33	2.8	8.2	5.6	16.3	2.8	8.2
	Very elderly adults ≥75	35	111	8.7	27.8	17.4	55.6	8.7	27.8
New Zealand	Adults ≥15	14	27	3.5	6.8	7.0	13.6	3.5	6.8
United Kingdom	Adults 18–64	14	22	3.4	5.6	6.8	11.2	3.4	5.6
Australia	Children 2–5	36	77	9.0	19.3	18.0	38.6	9.0	19.3
	Children 6–18	11	19	2.7	4.7	5.4	9.5	2.7	4.7
Finland	Other children 3–9	48	128	12.0	32.1	24.1	64.1	12.0	32.1
New Zealand	Children 5–14	14	26	3.6	6.6	7.1	13.1	3.5	6.6
Sweden	Other children 3–9	24	29	6.0	7.2	12	14.4	6.0	7.2
	Adolescents 10–17	9	13	2.2	3.1	4.4	6.3	2.2	3.1

AMC: anionic methacrylate copolymer; bw: body weight; P90: 90th percentile
[a] Based on consumption data for food supplements for consumers only.
[b] Total of methyl methacrylate, 2-(dimethylamino)ethyl methacrylate and *n*-butyl methacrylate based on total monomer content of 0.3% of the copolymer.

3.5 Summary of the estimates of exposure for BMC for use in the safety evaluation

A summary of the estimates of exposure to BMC and its monomers was required for this evaluation. Although estimates of exposure to BMC from pharmaceuticals were reviewed, the Committee considered that this use should not be taken into account in the assessment of chronic dietary exposure for a healthy population, and only dietary exposures from food supplements and food fortification were considered.

In order to capture the broad range of exposures from all sources of the estimates (the sponsors, EFSA and the Committee) for food supplements, the lower end of the range of mean exposures and the upper end of the range of high percentile exposures were used. The range of exposures used in the evaluation (Table 16) were those estimated by the Committee from national exposures as the estimates from the sponsors and EFSA were within these estimates. This was the case for the exposures to the copolymer and the monomers.

Table 16
Summary of the range of estimated exposures to BMC and its monomers for average and high exposures

		Range of estimated dietary exposures [a,b]			
			Monomer exposure (µg/kg bw per day)		
Population group	Source of exposure	Copolymer exposure (mg/kg bw per day)	*n*-Butyl methacrylate	2-(Dimethylamino) ethyl methacrylate	Methyl methacrylate
Adults	Food supplements	3.5–37	2.6–28	5.2–56	2.6–28
	Micronutrient encapsulation for food fortification c	0.03–45	0.02–33	0.04–67	0.02–33
	All sources	3.5–82	2.6–61	5.2–120	2.6–61
Children	Food supplements	2.9–43	2.2–32	4.4–64	2.2–32
	Micronutrient encapsulation for food fortification [c]	0.05–92	0.04–69	0.1–140	0.04–69
	All sources	3.0–135	2.2–100	4.4–200	2.2–100

bw: body weight; RDA/AI: recommended dietary allowance/adequate intake
[a] All estimates of exposure are presented as a range from the lowest of the average exposures to the highest of the high exposures. The lower end of the range is the lowest of the estimated mean exposures, and the upper end of each range is the highest of the estimated high exposures. The lower end of each range is the lowest of the estimated mean exposures, and the upper end of each range is the highest of the estimated high exposures.
[b] Includes exposure estimates submitted by the sponsor and EFSA (2010) and national estimates calculated by the Committee. Based on a concentration of 100 mg per 1 g dosage unit.
[c] Based on meeting 100% of the United States/Canadian RDA/AI. The lower end of each range is lowest exposure for a single nutrient and the upper end of each range is the sum of exposure from 12 nutrients.

For estimates of exposure from micronutrient encapsulation for food fortification, the only estimates were provided by the sponsor. To summarize these for the evaluation, a range was used based on the lowest exposure from a single nutrient, to the sum of exposures from all nutrients.

The range of exposures were rounded for the evaluation as uncertainties are inherent in dietary exposure estimates and the results are a guide for risk characterization purposes.

4. Comments

4.1 Biochemical aspects

4.1.1 BMC

When radiolabelled BMC (purity >98%) was administered to adult rats in two separate studies, the majority was excreted in the faeces within 2–3 days of administration. Levels of radioactivity in the gut returned to normal within 7

days. From the levels of radioactivity in the urine, it can be concluded that less than 0.02% of the radioactivity was absorbed (Life Science Research, 1979).

4.1.2 Residual monomers

There were no toxicokinetic studies on 2-(dimethylamino)ethyl methacrylate. Studies using simulated saliva or intestinal fluids show that this residual monomer is rapidly hydrolysed with 86–90% degradation (ECHA, 2015a).

Methyl methacrylate is rapidly absorbed and distributed following inhalation or oral administration to rats. Methyl methacrylate is metabolized to methacrylic acid and methanol, which is subsequently converted to carbon dioxide via the tricarboxylic acid cycle in both experimental animals and humans (Bratt & Hathway, 1977; Government of Canada, 1993; ECETOC, 1995).

Like methyl methacrylate, *n*-butyl methacrylate, is rapidly metabolized by carboxylesterases. Hydrolysis of *n*-butyl methacrylate yields methacrylic acid and *n*-butanol, which are further metabolized, with methacryl CoA, a physiological substrate of the valine pathway (European Commission, 2010).

4.2 Toxicological studies

4.2.1 BMC

In acute toxicity studies in mice, no effects were observed after oral, intraperitoneal and subcutaneous administration of BMC (purity >98%) at 3.0, 2.0 and 1.0 g/kg bw, respectively, although deaths occurred at higher doses via the intraperitoneal and oral routes (Nagasaki University, 1970). In rats, the oral LD_{50} was greater than 15 g/kg bw (LPT, 1970). Orally administered lactose granules (125 g) coated in test material (BMC at 5.9 g/kg bw) resulted in deaths in rats, but this mortality was attributed to the amount of the administered dose. When doses of BMC of up to 6 g/kg bw were administered to rats in feed, no adverse effects were observed (Battelle-Institute, 1966).

Short-term toxicity studies were available in rats and dogs. A 6-month study in rats administered BMC (purity >98%) in feed at 500 or 2000 mg/kg bw per day showed no treatment-related effects (LPT, 1973). A 28-day study in dogs administered BMC (>98% BMC) in gelatine capsules showed no treatment-related effects at doses of 100–750 mg/kg bw per day (Bien, 2003).

No long-term toxicity or carcinogenicity studies were available on the copolymer itself.

A reverse mutation assay in bacteria, a gene mutation assay in mammalian cells and an in vivo mouse micronucleus assay all produced negative results. The Committee concluded that BMC was not of concern for genotoxicity.

A developmental study in rats showed no treatment-related effects at the only dose tested, 1000 mg/kg bw per day, when administered in feed between gestation days 6 and 16 (LPT, 1968).

Studies of cytotoxicity, dermal toxicity, ocular toxicity and phototoxicity with BMC showed no effects.

4.2.2 Residual monomers

(a) 2-(Dimethylamino)ethyl methacrylate

A short-term toxicity study with 2-(dimethylamino)ethyl methacrylate in which rats were given gavage doses of 0, 100, 200 or 500 mg/kg bw per day for 13 weeks showed no treatment-related adverse effects. The NOAEL was 500 mg/kg bw per day, the highest dose tested (ECHA, 2015b).

No long-term toxicity studies were available on 2-(dimethylamino)ethyl methacrylate. In genotoxicity studies, 2-(dimethylamino)ethyl methacrylate was negative in two reverse mutation assays in bacteria, apart from one isolated positive response in *Salmonella typhimurium* TA1537 in the absence of metabolic activation (negative in the presence of metabolic activation), and negative in a gene mutation assay in mammalian cells in vitro. The monomer was positive for clastogenicity in two chromosomal aberration tests in mammalian cells in vitro but negative in a GLP-compliant in vivo mouse erythrocyte micronucleus test conducted according to OECD TG 474. The Committee concluded that 2-(dimethylamino)ethyl methacrylate does not raise concerns for genotoxicity in vivo.

In a reproductive toxicity study with 2-(dimethylamino)ethyl methacrylate, rats received gavage doses of 0, 40, 200 and 1000 mg/kg per day from 14 days before mating, for 43 days in males and until lactation day 3 in females. There were no effects on reproductive parameters. At the highest dose, maternal toxicity was observed and there were some deaths among dams, with total loss of litters in some dams during the lactation period, reduced pup body weight and reduced pup viability. The NOAEL was 200 mg/kg bw per day (ECHA, 2015c).

In a developmental toxicity study, 2-(dimethylamino)ethyl methacrylate was administered to female rats by gavage at dose levels of 0, 100, 300 or 600 mg/kg bw per day from gestation days 6 to 19. There were no treatment-related effects on maternal or embryo/fetal parameters. The NOAEL was 600 mg/kg bw per day, the highest dose tested (ECHA, 2015d).

(b) Methyl methacrylate

In most of the numerous studies on the short-term toxicity of methyl methacrylate in mice, rats, hamsters and dogs, the monomer was administered by inhalation.

Most commonly observed effects were decreases in body-weight gain and irritation of the skin, nasal cavity and eye at high concentrations (generally 500 ppm [2050 mg/m^3]). At even higher concentrations, renal cortical necrosis and tubular degeneration (in rats and mice) and hepatic necrosis (in mice) were also reported (WHO, 1998).

In a long-term toxicity study in rats given methyl methacrylate in drinking-water at 0, 6, 60 or 2000 mg/L (equal to 0, 0.4, 4 and 121 mg/kg bw per day for males and 0, 0.5, 5 and 146 mg/kg bw per day for females, respectively) for 2 years, relative kidney weight in females increased at the highest dose, but no treatment-related histopathological effects were observed in any organ or tissue (Borzelleca et al., 1964). Based on the results of this study, a TDI of 1.2 mg/kg bw per day was determined (WHO, 1998).

In long-term toxicity and carcinogenicity studies in mice, rats and hamsters given methyl methacrylate by inhalation, the observed effects were, in general, similar to those reported in the short-term studies but also included inflammation and epithelial hyperplasia of the nasal cavity and degeneration of the olfactory sensory epithelium. There was no evidence of any carcinogenic effects (Borzelleca et al., 1964).

Bacterial reverse mutation assays with methyl methacrylate gave mostly negative results (Lijinsky & Andrews, 1980; Hachiya, Taketani & Takizawa, 1982; Waegemaekers & Bensink, 1984; NTP, 1986; Schweikl, Schmalz & Rackebrandt, 1998). Mixed results (i.e. positive, weakly positive or negative) were obtained in in vitro chromosomal aberration (NTP, 1986; Bigatti et al., 1994; Doerr, Harrington-Brock & Moore, 1989; Schweikl, Schmalz & Rackebrandt, 1998; Tuček et al., 2002; Yang et al., 2003) and SCE assays (NTP, 1986; Cannas et al., 1987; Yang et al., 2003). One in vitro micronucleus assay was unequivocally negative (Schweikl, Schmalz & Spruss, 2001), whereas a second assay was negative at low concentrations but weakly positive at higher concentrations (Doerr, Harrington-Brock & Moore, 1989). Three mouse lymphoma assays for gene mutations were positive (NTP, 1986; Moore et al., 1988; Dearfield et al., 1991). A mouse bone marrow micronucleus assay was negative, but it is not clear if the target tissue was exposed to the test substance (Hachiya, Taketani & Takizawa, 1982). A rat micronucleus assay with exposure by inhalation was positive following 1 day of exposure but negative following 5 days of exposure (Araújo et al., 2013). These results were judged by the Committee to be inconclusive.

The Committee concluded that there was some evidence of mutagenicity and clastogenicity in vitro. There was a lack of adequate in vivo tests following up the equivocal findings.

There were no conventional reproductive toxicity studies on methyl methacrylate. A dominant lethal study in mice given 100, 1000 or 9000 ppm (410, 4100 and 36 900 mg/m3, respectively) of methyl methacrylate by inhalation for

6 hours/day for 5 days showed no effects on fertility (Anderson & Hodge, 1976).

In older studies on mice and rats, developmental effects including decreases in fetal weight and increases in embryo/fetal death and skeletal abnormalities were observed following inhalation of methyl methacrylate at concentrations that were toxic to the dams (Hodge & Palmer, 1977; Nicholas, Lawrence & Autian, 1979).

In another older developmental toxicity study, pregnant mice were exposed to 1330 ppm (5450 mg/m^3) of methyl methacrylate for 2 hours, twice daily, during gestation days 6–15. There were no adverse developmental effects (McLaughlin et al., 1978).

In a developmental toxicity study, rats were given methyl methacrylate by inhalation at concentrations from 9 to 2028 ppm (406–8315 mg/m^3) for 2 hours daily from gestation days 6 to 15. There were no treatment-related adverse effects. The NOAEL was 8315 mg/m^3 (Solomon et al., 1993).

(c) *n*-Butyl methacrylate

In an inhalation study, rats were exposed to *n*-butyl methacrylate at concentrations of 310, 952 or 1891 ppm for 6 hours/day, 5 days/week for 4 weeks. Microscopic examination of the nasal cavities of the male and female rats exposed to 1891 ppm showed slight and localized bilateral degeneration of the olfactory epithelium lining of the dorsal meati. One male and one female rat exposed to 952 ppm had similar changes in the olfactory epithelium. Rats exposed to 310 ppm had no exposure-related microscopic changes in the nasal cavity (European Commission, 2010).

No long-term studies were available on *n*-butyl methacrylate.

n-Butyl methacrylate tested negative in a range of in vitro and in vivo mutagenicity studies covering all the relevant end-points.

In a developmental toxicity study in rats on four methacrylates, including *n*-butyl methacrylate, animals were exposed by inhalation for 6 hours/day on gestation days 6–20. The exposure concentrations for *n*-butyl methacrylate were 0, 100, 300, 600 or 1200 ppm. Fetal toxicity was evident as decreases in fetal body weight at 600 ppm or greater. These exposure levels of *n*-butyl methacrylate were also maternally toxic. No significant increases in embryo/fetal deaths or fetal malformations were observed (Saillenfait et al., 1999).

4.3 Observations in humans

No human data were available on BMC.

4.4 Assessment of dietary exposure

The Committee evaluated exposure to BMC from its use as a glazing or coating agent in food supplements and foods for special medical purposes as well as for micronutrient encapsulation for food fortification. Because another major use of BMC is in pharmaceuticals, this use was also evaluated in the exposure assessment. The level of use of BMC in food supplements, pharmaceuticals and foods for special medical purposes is a maximum of 10%. The level of use of BMC for food fortification differs depending on the nutrient or group of nutrients being microencapsulated.

The Committee evaluated exposure to BMC for the copolymer and its monomers (*n*-butyl methacrylate, 2-(dimethylamino)ethyl methacrylate and methyl methacrylate).

The exposure assessment included submitted estimates and an evaluation by EFSA based on consumption of food supplements and pharmaceuticals. The Committee also estimated exposure based on national food consumption data for food supplements using the concentration proposed by the sponsor. National consumption data were from the CIFOCOss. Data from Australia and New Zealand were submitted to the Committee. A second sponsor provided estimates of dietary exposure from use in micronutrient encapsulation for food fortification. These estimates included exposures for 12 nutrients at a level that met 100% of their respective recommended dietary allowance (RDA). The exposure was estimated for each nutrient individually and also for the sum of all 12 nutrients.

A comprehensive literature search was also conducted; no additional studies were identified.

No quantitative estimates of exposure could be determined for foods for special medical purposes. The sponsor indicated that it is not anticipated that foods for special medical purposes would increase exposures above that of food supplements and pharmaceuticals, given the conservative nature of those calculations. In addition, the consumers of foods for special medical purposes will generally be under medical supervision, and exposures for these consumers are therefore not relevant for the general healthy population. This use was therefore not further considered by the Committee.

The total monomeric content of BMC is less than 0.3%. This level was therefore used to calculate the exposure to total monomers from the copolymer exposure. Estimates of exposure to the individual monomers were based on exposure to total monomers, taking into account the ratio of each individual monomer in the copolymer.

The estimated exposures to BMC and its monomers for adults and children from uses in food supplements and in micronutrient encapsulation for food fortification are shown in Table 16. All estimates of exposure from uses

in food supplements are presented as a range from the lowest of the average exposures to the highest of the high exposures. All estimates of dietary exposure from micronutrient encapsulation for food fortification are presented as a range from the lowest exposure for a single nutrient to the exposure for the sum of the 12 nutrients assessed.

The Committee noted that the exposures estimated by the sponsor were to provide a presumed worst case for the purpose of the safety evaluation. The Committee also noted that national estimates of exposure would be required for the evaluation of safety based on their own nutrient reference values, fortification needs and food consumption patterns.

Estimates of exposure to BMC and its monomers from all sources combined (food supplements and micronutrient encapsulation for food fortification) are also shown in Table 16. The Committee noted that the upper end of the range representing high exposures from all sources is a worst case estimate, and unlikely in terms of actual long-term exposure to BMC.

The Committee noted that BMC is used in pharmaceuticals. Estimated exposures from this use from the sponsor and EFSA ranged between 8.0 and 13.3 mg/kg bw per day for adults and children. These estimates were within the range of exposures from food supplements. However, the Committee considered that such use should not be taken into account in the assessment of long-term dietary exposure for a healthy population.

5. Evaluation

The Committee concluded that the use of BMC that complies with the specifications established at the current meeting is not a safety concern when the food additive is used as a coating or glazing agent for solid food supplements and for foods for special medical purposes and micronutrient encapsulation for food fortification.

An acceptable daily intake (ADI) "not specified" was established for BMC.

The available toxicology data for BMC do not give rise to concerns for toxicity. The substance is poorly absorbed and is excreted in the faeces. In short-term and developmental toxicity studies, the NOAELs for BMC range from 750 to 2000 mg/kg bw per day, the highest doses tested.

Toxicological data on the residual monomers do not give rise to concerns when taking into account the low exposures. 2-(Dimethylamino) ethyl methacrylate and *n*-butyl methacrylate do not give rise to concerns for genotoxicity. Long-term, reproductive and developmental toxicity studies do

not suggest a risk for health at the estimated exposure levels. Genotoxicity data for methyl methacrylate suggest a potential for mutagenicity and clastogenicity in vitro, but there is a lack of adequate in vivo genotoxicity data. However, in carcinogenicity studies in mice, rats and hamsters given methyl methacrylate by inhalation, there was no evidence of any carcinogenic effects. In a 2-year study in rats given methyl methacrylate in drinking-water, the NOAEL was 121 mg/kg bw per day; from this NOAEL, a TDI of 1.2 mg/kg bw per day was derived (WHO, 1998). Estimated exposures to methyl methacrylate range from 2.2 to 100 μg/kg bw per day, which are below the TDI.

New specifications and a Chemical and Technical Assessment were prepared.

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Erythrosine (consolidated monograph)

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1. Explanation

Erythrosine (International Numbering System for Food Additives [INS] No. 127; Chemical Abstracts Service [CAS] No. 16423-68-0) is a xanthene dye permitted as a food colour in China, the European Union, the USA and other regions. It is used for colouring foods including baked goods, breakfast cereals, confectionery products, dairy products, decorations for baking, dressings and sauces, dried fruit, frostings and icings, frozen breakfast foods, frozen treats, hot beverages, juice drinks and processed foods (fish, meat and egg products).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) previously evaluated the safety of erythrosine at its eighth, thirteenth, eighteenth, twenty-eighth, thirtieth, thirty-third and thirty-seventh meetings (Annex 1, references *8*, *19*, *35*, *66*, *73*, *83* and *94*) and for dietary exposure at its fifty-third meeting (Annex 1, reference *143*). Toxicological monographs or monograph addenda were published after the thirteenth, eighteenth, twenty-eighth, thirtieth, thirty-third and thirty-seventh meetings (Annex 1, references *20*, *36*, *67*, *74*, *84* and *95*). At its eighteenth meeting, the Committee allocated an acceptable daily intake (ADI) of 0–2.5 mg/kg body weight (bw). This ADI was reduced at the twenty-eighth meeting to 0–1.25 mg/kg bw and made temporary following observations that erythrosine produced effects on thyroid function in short-term toxicity studies in rats and that, in long-term toxicity studies, male rats receiving 4% erythrosine in the diet developed benign thyroid tumours.

At the thirtieth meeting, the Committee reduced the temporary ADI to 0–0.6 mg/kg bw, based on biochemical effects of erythrosine on thyroid hormone metabolism and regulation in rats. The Committee at the thirtieth meeting requested further data from pharmacokinetic studies relating the amount of erythrosine absorbed to the amount ingested to enable the establishment of a correlation between blood/tissue erythrosine levels and effects on the thyroid. At the thirty-third meeting, the Committee further reduced the temporary ADI to 0–0.05 mg/kg bw. This ADI was based on a no-observed-adverse-effect level (NOAEL) from a study showing slightly increased thyroid-stimulating hormone (TSH) responsiveness in humans ingesting erythrosine at 60 mg per person per day (equivalent to 1 mg/kg bw per day) for 14 days, and applying an uncertainty factor[1] of 20. The Committee again requested the pharmacokinetic studies required by the previous Committee. At its thirty-seventh meeting, the Committee re-evaluated previously reviewed studies that had since been published and newer studies on thyroid physiology in rats. The Committee concluded that the thyroid tumours in male rats previously reported in long-term toxicity studies were secondary to thyroid hormone changes and species-specific sensitivity.

[1] The previous Committee used the term "safety factor".

In view of the differences in thyroid physiology between humans and rats, the Committee based its evaluation on the human data and allocated an ADI of 0–0.1 mg/kg bw on the NOAEL of 60 mg per person per day from the 14-day study in human subjects (equivalent to 1 mg/kg bw per day), with application of an uncertainty factor of 10.

At the present meeting, the Committee re-evaluated erythrosine at the request of the Forty-ninth Session of the Codex Committee on Food Additives (FAO/WHO, 2017).

A toxicological dossier that included new studies on genotoxicity, reproductive and developmental toxicity, neurological effects and hypersensitivity was submitted. A comprehensive literature search was conducted in March 2018 using the PubMed database of the United States National Library of Medicine. The linked search terms "erythrosine" and "toxicity" yielded 77 references, three of which were relevant to the present assessment as most of the remainder had been previously evaluated or had been identified by the sponsor. This Committee also considered studies evaluated at previous meetings.

A comprehensive literature search for dietary exposure estimate references using the search terms "erythrosine" and "exposure" or "intake" or "consumption" was also conducted in the PubMed and Scopus databases. These terms were also used in a general internet search. In addition to the references submitted by the sponsor, five references relevant to the exposure assessment were retrieved.

The previous monographs have been expanded and are reproduced in this consolidated monograph.

1.1 Chemical and technical considerations

Erythrosine consists of the disodium salt of 2-(2,4,5,7-tetraiodo-6-oxido-3-oxoxanthen-9-yl)benzoate monohydrate and subsidiary colouring matters. Sodium chloride and/or sodium sulfate are the principal uncoloured components. Erythrosine is manufactured by iodination of fluorescein, the condensation product of resorcinol and phthalic anhydride. Impurities include unreacted starting materials (≤0.4%), subsidiary colouring matters except fluorescein (≤4%), fluorescein (≤20 mg/kg), inorganic iodides (≤0.1%), lead (≤2 mg/kg) and zinc (≤50 mg/kg).

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

No new information on absorption, distribution and excretion of erythrosine has been published since the previous evaluations.

The Committee previously noted that when erythrosine was given by stomach tube to adult male rats at levels as high as 150 mg/rat, recovery in the excreta after 5 days was 102%. After intravenous administration of erythrosine at 3 mg/kg bw, an average of 55% of the administered quantity was recovered in bile and 1.3% in urine. No glucuronic acid conjugation was found (Annex 1, reference *36*).

Rats excreted erythrosine mainly in faeces (55–72%). No colour was identified in urine and only a small amount (0.4–1.7%) in bile (Daniel, 1962).

Webb, Fonda & Brouwer (1962) studied the possibility that iodide may be liberated from erythrosine and disturb thyroid function. Erythrosine was found to be metabolically stable, with 100% of the amount ingested excreted, with iodine content intact, after administration of 500 mg/kg bw in rats.

When erythrosine was administered twice weekly by stomach tube to rats (200–250 g) at doses of 5, 10, 15 or 50 mg/rat for 6 months, excretion of the dye was mainly in unchanged form in faeces (Bowie, Wallace & Lindstrom, 1966).

The urinary and faecal excretion of ^{14}C- and of ^{125}I-labelled erythrosine were studied in rats of both sexes either without pretreatment or following dosing with unlabelled erythrosine at dietary levels of 0.5% or 4.0% for 7 days. The distribution of the compound in tissues and body fluids was also studied.

The radioactivity from both radiolabels was excreted predominantly in faeces, mainly within 48 hours; less than 1% of the dose was excreted in urine. Blood and plasma radioactivity reached maximum levels by 1 hour, while levels in liver and kidneys peaked after 4–12 hours. The activity in blood and tissues was very low, suggesting that erythrosine is not extensively absorbed from the gastrointestinal tract. Of the tissues examined (liver, kidneys, thyroid, brain and pituitary), the highest levels of radioactivity were found in liver (maximally 0.145% of the dose of ^{14}C; 0.188% of the dose of ^{125}I). Thyroid residues of ^{14}C were at trace or nondetectable levels, while levels of ^{125}I were detectable but low (maximally approximately 0.01% of the dose), indicating that neither erythrosine nor its ring-containing metabolites accumulated in the thyroid. The magnitude of the ^{125}I levels in the thyroid was so low that it was not possible to conclude whether the activity resulted from ^{125}I-iodide in the dose or from ^{125}I-iodide

formed by a small degree of metabolic deiodination of erythrosine. No ^{14}C or ^{125}I was detectable in the brain or pituitary (Obrist et al., 1986).

Human studies also indicate that only a small proportion of ingested erythrosine is absorbed from the gastrointestinal tract (Ingbar, Garber & Gluckin, 1983; Ingbar, Garber & Burrows, 1984).

2.1.2 Biotransformation

No new information has been published since the previous evaluations.

Small amounts of metabolites in the Obrist et al. (1986) rat study, believed to be isomeric diiodo- and triiodofluoresceins, were detected in urine, faeces, plasma and tissue extracts from liver and kidneys.

2.1.3 Effects on enzymes and other biochemical parameters

Diemair & Hauser (1951) found that erythrosine at 200–400 mg/L inhibited the activity of pepsin but had no effect on lipase activity.

The effect of erythrosine on the activities of human phase I and phase II metabolic enzymes (CYP2A6, CYP3A4, UGT1A6 and UGT2B7) and P-glycoprotein activity indicates that erythrosine is not a substrate for any of these enzymes nor an inhibitor of CYP2A6. However, erythrosine was reported to be a noncompetitive inhibitor of UGT1A6 and UGT2B7 with half maximal inhibitory concentration (IC_{50}) values of 0.09 mmol/L (0.05 mmol/L; Mizutani, 2009) and 0.18 mmol/L, respectively (Kuno & Mizutani, 2005). Erythrosine also inhibited CYP3A4 and P-glycoprotein activity with IC_{50} values of 0.0079 and 0.0156 mmol/L, respectively (Mizutani, 2009). Erythrosine inhibition of UGT1A6 activity was prevented by the antioxidant enzyme superoxide dismutase, but not by catalase, in a dose-dependent manner. This effect indicating that the mechanism of inhibition is mediated by generation of reactive oxygen species (superoxide anion), presumably from oxidation of erythrosine by light under these experimental conditions.

Daily oral doses of ^{131}I-labelled erythrosine at over 1 mg inhibited uptake of ^{131}I by the thyroid of treated rats (Marignan, Boucard & Gelis, 1965).

Erythrosine could be an adventitious source of iodide (Vought, Brown & Wolfe, 1972). In humans, oral administration of 16 mg of erythrosine daily for 10 days resulted in an increase in protein-bound iodine (PBI) from 6 to 11 µg per 100 mL serum after 15–20 days, followed by a sharp decline in iodine levels in the following 10 days, with a gradual return to the initial value in 3 months (Andersen, Keiding & Nielsen, 1964).

Protein-bound and total blood iodine levels were elevated in rats given erythrosine by stomach tube twice weekly at doses of 5, 10, 15 or 50 mg/rat for 6 months. However, in experiments with rats and gerbils, the elevated PBI was

Table 1
Acute toxicity of erythrosine

Species	Sex	Route	LD_{50} (mg/kg bw)	Reference
Mouse	Male and female	Oral	6 800	Butterworth et al. (1976a)
	Male	Oral	2 558	Yankell & Loux (1977) [a]
	Male and female	Intraperitoneal	360	Butterworth et al. (1976a)
	Unknown	Intravenous	370	Waliszewski (1952)
Rat	Male	Oral	1 895	Lu & Lavallee (1964)
	Male and female	Oral	1 840	Hansen et al. (1973a)
	Male and female	Oral	7 100	Butterworth et al. (1976a)
	Female	Oral	2 891	Yankell & Loux (1977) [a]
	Unknown	Intraperitoneal	300	Emerson & Anderson (1934)
	Male and female	Intraperitoneal	350	Butterworth et al. (1976a)
Gerbil	Unknown	Oral	1 930	USFDA (1969)
Rabbit	Unknown	Intravenous	200	Emerson & Anderson (1934)

bw: body weight; JECFA: Joint FAO/WHO Expert Committee on Food Additives; LD_{50}: median lethal dose; USFDA: United States Food and Drug Administration
[a] Not previously evaluated by JECFA.

due to interference by erythrosine in PBI determinations (Bowie, Wallace & Lindstrom, 1966; USFDA, 1969).

For more effects of erythrosine on other biochemical parameters, see section 2.2.6(e).

2.2 Toxicological studies

2.2.1 Acute toxicity

Studies of acute toxicity in mice, rats, gerbils and rabbits are summarized in Table 1. The results show that erythrosine has low oral acute toxicity.

2.2.2 Short-term studies of toxicity

No new short-term studies with erythrosine have been published since the previous evaluations.

(a) Rats

Carworth Farm E strain SPF rats (15/sex per dose group) received 0, 0.25%, 0.5%, 1.0% or 2.0% erythrosine in the diet (equal to 0, 160, 330, 690 and 1750 mg/kg bw per day for males and 0, 170, 370, 730 and 1470 mg/kg bw per day for females, respectively) for 90 days.

No adverse test substance–related effects were noted in body weight, feed intake or haematology or blood and urine analysis parameters. Organ

weights were normal except for dose-related increases in absolute and relative caecal weights. However, the histology was normal. Absolute and relative thyroid weights were increased at 2.0%. Histopathological examination showed pigment deposition in renal tubules in females at 2.0% and in males in all but the lowest levels, in a dose-related manner; no other abnormalities were seen. In addition, total PBI was raised at all levels in a dose-related manner with protein-bound erythrosine in serum, and non-PBI also increased with increasing administered concentrations of erythrosine. Thyroxine iodine, however, remained unchanged. Thyroid activity was not impaired at any dietary level as indicated by the normal histopathology of the organ, the lack of effect on serum thyroxine (T_4) levels and the normal rates of oxygen consumption in the treated animals.

The NOAEL was 0.25% in the diet (equal to 160 mg/kg bw per day) based on pigment deposition in renal tubules at 0.5% and higher dietary levels in males (Butterworth et al., 1976a).

Female Sprague Dawley rats (12–20/group) were exposed to erythrosine in the diet at concentrations of 0 or 0.2% (equivalent to 0 and 100 mg/kg bw per day) for either 6 or 12 months. During the final 12 weeks of the experimental period, a slight decrease in body-weight gain was observed in rats exposed for 12 months. Other parameters such as feed consumption, haematology, clinical chemistry, urine analysis parameters and organ weights were comparable in treated and control rats in both the 6- and 12-month groups. Sporadic pathological changes were observed in treated and control rats (Sekigawa et al., 1978).

(b) **Gerbils**

Gerbils (15/sex per group; 30/sex in the control group) were fed erythrosine in the diet twice a week at concentrations of 200, 750 or 900 mg/kg bw for 19 months (animals at 900 mg/kg bw received 1200 mg/kg bw twice a week for the first 3 months). Body-weight decreases were seen in male gerbils at all feeding levels but in females only at 900 mg/kg bw. PBIs were elevated due to interference of erythrosine with PBI determinations. No other haematological differences or adverse gross pathological findings were noted. Histopathology was not performed (USFDA, 1969).

(c) **Dogs**

Two-year feeding studies were conducted with beagle dogs (3/sex per group) at dietary levels of erythrosine of 0, 0.5%, 1.0% or 2.0% (equivalent to 0, 125, 250 or 500 mg/kg bw per day, respectively).

There were no deaths. No gross or microscopic pathological changes related to colour administration were observed (Hansen et al., 1973a).

(d) Pigs

Large White pigs (3/sex per group; ~20 kg each) were fed erythrosine in the diet at dose levels of 0, 167, 500 or 1500 mg/kg bw per day for 14 weeks.

Levels of serum T_4 were decreased in treated pigs compared with controls. There were dose-related increases in the serum levels of PBI, non-PBI and protein-bound erythrosine in all treated animals. A dose-related increase in thyroid weight was noted, although the differences were statistically significant only in female pigs at the higher dose levels (500 and 1500 mg/kg bw per day). There were no pathological changes in the thyroid of treated pigs (Butterworth et al., 1976b).

The Committee noted that all these short-term studies were performed before the initiation of good laboratory practice (GLP) and the establishment of Organisation for Economic Co-operation and Development (OECD) guidelines.

2.2.3 Long-term studies of toxicity and carcinogenicity

One carcinogenicity study in rats (Hiasa et al., 1988) had not previously been evaluated.

(a) Mice

A total of 122 male and female mice (mixed breed from five different strains; aged 50–100 days) were given a diet containing erythrosine at 1 mg/animal per day. A negative control group consisted of 168 mice; two positive control groups, which were given α-aminotoluene and dimethylaminoazobenzene, were also included. A number of the animals were killed after an observation period of 500 days and the remainder after 700 days. The formation of liver tumours was noted in animals in the positive control groups after approximately 200 days. The incidence of tumours in mice fed the colour was not significantly greater than the incidence in the negative controls (Waterman & Lignac, 1958).

In a chronic study in 70 mice fed erythrosine at 1% or 2% (equivalent to 1300 and 2600 mg/kg bw per day), because only small number of animals survived the experiment and only a small number of tumours were found no effect on tumour formation could be attributed to the colour (USFDA, 1969).

Charles River CD-1 mice (60/sex per group) were exposed to erythrosine in the diet at concentrations of 0 (2 control groups were used), 0.3%, 1.0% or 3.0% (equal to 0, 424, 1474 and 4759 mg/kg bw per day for males and 0, 507, 1834 and 5779 mg/kg bw per day for females, respectively) for 24 months. With the exception of significantly decreased body weights (throughout the entire study) of males and females at 3.0%, erythrosine did not adversely affect other

investigated parameters (mortality, feed intake and haematology, gross pathology and histopathology) at any concentration.

The NOAEL was 1.0% of erythrosine in the diet (equal to 1474 mg/kg bw per day) based on the significantly decreased body weights at the 3.0% concentration (equal to 4759 mg/kg bw per day) in male mice (Richter et al., 1981, later published as Borzelleca & Hallagan, 1987).

ICR mice (50/sex per group, 45/sex in the control group; 27–38 g; 7 weeks old) were fed diets containing erythrosine at concentrations of 1.25% or 2.5% (equivalent to 1625 and 3250 mg/kg bw per day, respectively) for 18 months. The mice received erythrosine in cube diet for the first 20 weeks; thereafter, erythrosine was mixed with the basic powder diet. All animals in the experimental groups were fed the basic diet free of erythrosine for an additional 6 months, after which they were killed and autopsied. Mortality was greater in treated animals than in controls (approximately 61% of the animals at 2.5% died, 59% at 1.25% and 36% controls). Erythrosine did not adversely affect body-weight gains. Incidence of lymphocytic leukaemia was high in treated animals in both experimental groups, and sporadic cases of pulmonary adenomas were observed. However, the frequency of both lesions was in the range expected to occur spontaneously in this strain of mice.

Under the conditions of the study, erythrosine was not carcinogenic to ICR mice (Yoshii & Isaka, 1984).

(b) **Rats**

Rats (5/sex) were fed erythrosine at 4% in the diet (equivalent to 2000 mg/kg bw per day) for up to 18 months. Gross staining was observed in the glandular stomach and small intestine, and granular deposits were observed in the stomach, small intestine and colon. Hepatic cirrhosis was noted in 1/4 rats that survived up to 12 months. Fifty control animals observed for 20 months or longer failed to develop tumours or hepatic cirrhosis (Willheim & Ivy, 1953).

Weanling rats (12/sex per group) were fed erythrosine at 0, 0.5%, 1.0%, 2.0% or 5.0% (equivalent to 0, 250, 500, 1000 and 2500 mg/kg bw per day, respectively) in the diet for 2 years.

Slight growth depression was observed in the animals at 5% erythrosine. All treated animals had distended caeca, but microscopic examination of these distended caeca revealed only normal histology. Statistical evaluations found no significant changes in organ weights at the highest dietary level. There was some diarrhoea at the 5% level. There were no differences in survival between the test groups.

The NOAEL was 0.5% in the diet (equivalent to 250 mg/kg bw per day) based on the distended caeca observed at 1% of erythrosine in the diet (equivalent to 500 mg/kg bw per day; USFDA, 1969).

Osborne–Mendel rats (12/sex per group) were fed 0, 0.5%, 1.0%, 2.0% or 5.0% erythrosine in the diet (equivalent to 0, 250, 500, 1000 and 2500 mg/kg bw per day, respectively) for 2 years.

Growth depression was observed in rats at 5% erythrosine. Relative spleen weight was depressed in all treated males and in females at the 5% level. Slight caecal enlargement, which increased with dose, was noted at 1% but the histology of the enlarged caeca was normal. No other gross or histopathological findings related to colour administration were noted.

The NOAEL was 0.5% in the diet (equivalent to 250 mg/kg bw per day) based on the caecal enlargement observed at the concentration of 1% (equivalent to 500 mg/kg bw per day) (Hansen et al., 1973a).

Rats (25/sex per group; 50/sex in the control group; 100 days old) were fed erythrosine at 0, 0.5%, 1.0%, 2.0% or 4.0% in the diets (equivalent to 0, 250, 500, 1000 and 2000 mg/kg bw per day, respectively) for 86 weeks. Other groups (25/sex per group) were intubated twice a week for 85 weeks with erythrosine at 0, 100, 235, 750 or 1500 mg/kg bw. After this treatment, the animals were kept on normal diets for the remainder of the 2 years of the study. Body-weight decreases were seen at the 2% and 4% levels. The elevated PBI was due to interference by erythrosine with PBI determinations rather than due to thyroid dysfunction. T_4 iodine levels were not affected. There were no other haematological differences and no anaemia was seen. No adverse gross pathology was noted; histopathology examinations did not show any colour-related abnormalities.

The NOAEL was 1% in the diet (equivalent to 500 mg/kg bw per day) based on the body-weight decreases observed at the concentration of 2% (equivalent to 1000 mg/kg bw per day) (Hansen et al., 1973b).

A long-term toxicity and carcinogenicity study in Charles River CD rats consisted of an in utero phase and an F_1 phase. In the in utero phase, rats (60/sex per group) were fed either the basal diet (controls) or erythrosine in the diet at concentrations of 0.1%, 0.5% or 1.0% (equal to 49, 251 and 507 mg/kg bw per day for males and 61, 307 and 641 mg/kg bw per day for females, respectively) for approximately 2 months before mating. Female rats were weighed on gestation days 0, 6, 15 and 21 and on lactation days 0, 4, 14 and 21. In the F_1 phase, weanling rats (70/sex per group) were fed erythrosine in the diet at the same concentrations as in the in utero phase, for 30 months. Two concurrent control groups (70/sex per group) received the basal diet with no colour.

There were no consistent significant compound-related effects on fertility, gestation, parturition, lactation, pup survival through weaning or numbers of liveborn and stillborn pups. In the main study (F_1 phase), there were no consistent significant compound-related effects on physical parameters; behaviour; mortality; feed consumption; or haematological, clinical chemistry, urine analysis or ophthalmological parameters. Mean body-weights of control and treated rats did not differ significantly during the exposure period. The gross pathological changes noted could not be attributed to treatment with erythrosine. The incidence of nonneoplastic lesions was comparable between treated and control groups. There was a statistically significant increase in the incidence of benign thyroid tumours (follicular adenomas): 6/68 females at 1.0% versus 0/140 controls. The incidence of malignant tumours in treated rats was comparable with that of the controls (Brewer et al., 1981).

Charles River CD weanling rats (70/sex per group) were given erythrosine in the diet at concentrations of 0 or 4.0% (equal to 0 and 2464 mg/kg bw per day for males and 0 and 3029 mg/kg bw per day for females, respectively) for approximately 29 months.

There were no consistent significant compound-related effects on physical parameters, behaviour, mortality, feed consumption or haematological, clinical chemistry, urine analysis or ophthalmological parameters. Mean body-weights of treated rats (both sexes) were slightly lower than those of the control rats throughout the study. These differences were statistically significant except at weeks 3–5 and 122 (males) and at weeks 0–4, 6 and 114 (females). The mean absolute and relative thyroid weights of treated males were more than twice those of the controls. Histopathological examination revealed that the incidence of thyroid hyperplasia (follicular and C-cell) was significantly increased in treated males. There was a statistically significant increase in the incidence of follicular adenoma of the thyroid in treated male rats (16/68) compared with controls (0/69). The incidence of malignant tumours, including thyroid C-cell and follicular carcinomas, was comparable in treated and control rats (Brewer et al., 1982).

The results of the two long-term feeding studies in rats (Brewer et al., 1981, 1982) were later published as Borzelleca, Capen & Hallagen (1987). In the statistical analyses, thyroid follicular cell adenomas and carcinomas were treated as separate tumour classes. The authors concluded that erythrosine at a level of 4% in the diet for 128 weeks induces an increased incidence of thyroid follicular cell adenomas in male rats (15/69 compared to 1/69 in controls). The incidence of thyroid follicular cell carcinomas (3/69) did not differ statistically significantly from the control value (2/69). In females at 4%, the incidence of thyroid follicular

cell adenomas (5/68) or carcinomas (0/68) did not differ from controls (5/66 and 0/66, respectively). In female rats fed 0.1%, 0.5% or 1% erythrosine in the diet, an increase in the number of adenomas was observed: 1/68, 3/67 and 5/68, respectively, versus 1/138 control females. However, this increase was not statistically significant. The incidence of carcinomas in females were 0/68, 0/67 and 1/68, respectively, versus 0/138 controls. In males at 0.1%, 0.5% and 1.0%, incidences of adenomas (0/67, 2/68 and 1/69 versus 0/139) and carcinomas (3/67, 1/68 and 3/69 versus 0/139) were not considered significantly different.

The NOAEL was 0.5% in the diet (equal to 251 mg/kg bw per day) based on the increased incidence of thyroid follicular cell hyperplasia in males at the dietary concentration of 1% (equal to 507 mg/kg bw per day; Borzelleca, Capen & Hallagen, 1987).

The microscopic findings in the thyroids in the Brewer et al. (1981, 1982) and Hansen et al. (1973b) studies and the statistical analyses used were reviewed (FD&C Red No. 3 Review Panel, 1987; Federal Register, 1990) and slight discrepancies in the diagnoses of adenomas/carcinomas were reported. When the incidence of adenomas and carcinomas was combined for the statistical evaluation, the incidence was increased in males fed 4% erythrosine in the diet (18/68 versus 2/68 in control males). A statistically significant increase in combined adenomas and carcinomas was also found in male rats fed 0.1%, 0.5% and 1.0% erythrosine for 122 weeks (3 + 3/64, 7 + 1/66, 1 + 3/57, respectively, versus 0 + 1/128 in controls). In the female rats, a significant increase in tumour yield was only found at the 1.0% level (5 + 1/68 versus 1 + 0/138 in controls).

At its thirty-seventh meeting, JECFA considered it appropriate to combine thyroid follicular cell adenomas and carcinomas in the statistical analyses in view of evidence that adenomas are an earlier stage of carcinomas in the thyroid. The present Committee concurred.

Couch et al. (1983) undertook a study to investigate whether the thyroid tumours found after chronic feeding of erythrosine to male rats at a dietary concentration of 4.0% (equivalent to 2000 mg/kg bw per day) was a result of excess iodine (either as a contaminant of the colour or as iodine metabolized from the colour) or from another, non-iodine-related property of erythrosine. Charles River CD rats (35/sex per dose group) were continuously exposed to one of the following diets for 27 weeks: unadulterated diet; NaI (sodium iodide) at 80 µg/g diet; purified erythrosine at 4% in the diet; purified erythrosine at 4% in the diet plus NaI at 80 µg/g diet; purified erythrosine at 4% in the diet plus NaI at 160 µg/g diet; or commercial erythrosine at 4.0% in the diet.

Exposure to commercial erythrosine at 4% in the diet resulted in hyperthyroidism, that is, TSH and T_4 levels were elevated while triiodothyronine

(T_3) concentrations were depressed. Changes in clinical chemistry parameters, body weight and feed consumption were also indicative of hyperthyroidism. Additional purification of the commercial preparation of erythrosine to remove free iodide did not modify these effects. Similarly, these responses were not observed after feeding a diet spiked with NaI only.

This study demonstrated that the thyroid changes observed in this and other studies with erythrosine are associated with increased TSH concentrations. However, the mechanism for these effects of erythrosine could not be determined from the results of this study (Couch et al., 1983).

The thyroid glands were subsequently examined by electron microscopy (see section 2.2.6(e)).

Pathogen-free F344 rats (50/sex per group; 6 weeks old) were fed diets containing erythrosine at levels of 1.25% or 2.5% (equivalent to 625 and 1250 mg/kg bw per day, respectively) for 18 months. For the first 20 weeks of treatment, erythrosine was given in pelleted diet; for the remaining weeks, it was given in powder diet. The control group (30/sex) received an erythrosine-free diet. Rats exposed to erythrosine were killed at 18 months and the control rats at 24 months.

Histopathological examinations revealed sporadic cases of spontaneous neoplasms (tumours of the genital system, the endocrine system, the haematopoietic system and digestive system), but at a frequency similar across the treated and control groups. No pathological changes were observed in the thyroid glands. Only histopathological parameters were reported.

The NOAEL was 2.5% of erythrosine in the diet (equivalent to 1250 mg/kg bw per day), the highest dietary concentration tested (Fukunishi et al., 1984).

The promoting effect of erythrosine on thyroid tumours after a single intraperitoneal injection of *N*-bis(2-hydroxypropyl)nitrosamine (DHPN) was evaluated in young male Wistar rats with either intact thyroid glands or partial thyroidectomies. Erythrosine was provided at 4% in the diet (equivalent to 4000 mg/kg bw per day) for 19 weeks following DHPN injection at the beginning of the treatment period at a dose of 2800 mg/kg bw and with thyroidectomy performed on week 4.

In partially thyroidectomized rats, the incidence of DHPN-induced thyroid follicular adenomas was nonstatistically significant higher (91%) and the incidence of DHPN-induced thyroid follicular carcinomas was statistically significantly higher (41%) in erythrosine-fed rats than DHPN-only treated rats (63% and 0%, respectively). Serum TSH levels (5.5 ± 3.1 ng/mL) and T_4 levels (4.4 ± 1.5 µg/dL) were statistically significantly higher in erythrosine-fed rats than in the DHPN-only group (1.5 ± 0.5 ng/mL and 3.4 ± 0.5 µg/dL, respectively). In non-thyroidectomized rats, the incidence of DHPN-induced thyroid follicular

adenomas (52% versus 35%) or carcinomas (5% versus 0%) was not significantly increased in the erythrosine-fed group compared with the DHPN-only group. The incidence of papillary carcinoma (21%) was significantly higher in erythrosine-fed rats compared with DHPN-only rats (0%). Serum T_4 levels (6.7 ± 0.9 µg/dL), but not TSH levels (1.4 ± 0.9 µg/dL), were significantly increased in erythrosine-fed rats compared with DHPN-only rats (3.5 ± 0.6 ng/mL and 1.6 ± 0.2 µg/dL, respectively).

The authors concluded that erythrosine promoted the development of thyroid tumours in partially thyroidectomized rats but not in non-thyroidectomized rats (Hiasa et al., 1988).

Considering no effect was reported in rats with intact thyroid glands, the Committee concluded that this study does not provide reliable evidence for the carcinogenicity of erythrosine.

Twenty rats were injected subcutaneously with 1 mL of a 5% aqueous solution of erythrosine weekly for 596 days (85 weeks). The total quantity of colour administered was 2.65 g/animal. Seven rats survived 300 days or longer. No tumours were observed (Umeda, 1956).

Eighteen rats were injected subcutaneously with aqueous solutions of erythrosine at 12 mg/animal once per week for 2 years. No tumours were observed at the injection sites or elsewhere in the body (Hansen et al., 1973a).

(c) Gerbils

Mongolian gerbils (15–16/sex per group; ~6 months old) were fed diets containing erythrosine at levels of 1.0%, 2.0% or 4.0% for 105 weeks. Control groups (31/sex) were fed erythrosine-free diets.

All treated animals exhibited a statistically significant dose-related decrease in body-weight gain compared with the controls. In general, there were slight and in some isolated cases, significant, depressions of haematocrit and haemoglobin values and leukocyte and reticulocyte counts in treated animals. Relative heart, liver and spleen weights were significantly decreased in both sexes at 2.0% and 4.0%. Dose-related changes in thyroids, such as enlargement of follicles and, in some cases, focal hyperplasia, were observed in treated animals. Histopathological examinations did not reveal any treatment-related effects (Collins & Long, 1976).

Effects of intubated with erythrosine were also examined by the same research group. Mongolian gerbils (20–26/sex; ~6 months old) received erythrosine dissolved in water by stomach intubation at dose levels of 200, 750 or 900 mg/kg bw twice weekly for 97 weeks. A control group (32/sex) was intubated

with distilled water only. The dosages were administered in a volume of 10 mL/kg bw.

No treatment-related adverse effects were seen in clinical toxicity, mortality, body-weight gain, haematological parameters, organ weights or gross pathological and histopathological parameters (Collins & Long, 1976).

2.2.4 Genotoxicity

The results of in vitro and in vivo genotoxicity tests of erythrosine are summarized in Table 2.

A large number of in vitro and in vivo genotoxicity tests have been conducted on erythrosine. No genotoxic or mutagenic activity has been observed in the majority of in vitro assays (Kada, Tutikawa & Sadaie, 1972; Auletta, Kuzava & Parmar, 1977; Brown, Roehm & Brown, 1978; Muzzall & Cook, 1979; Bonin & Baker, 1980; Haveland-Smith, Combes & Bridges, 1981; Tarján & Kurti, 1982; Ishidate et al., 1984; Cifone & Myhr, 1984; Jaganath & Myhr, 1984a; Kombrust & Barfknecht, 1985; Cameron et al., 1987; Lakdawalla & Netrawali, 1988a; Miyachi & Tsutsui, 2005; Mpountoukas et al., 2010). Positive findings were reported in several in vitro assays conducted in *Bacillus subtilis*, yeast strain D7 and XV185–14C, mouse lymphoma L5178Y $TK^{+/-}$ cells, V79 Chinese hamster lung cells and HepG2 cells (Ishidate et al., 1984; Matula & Downie, 1984; Cameron et al., 1987; Lakdawalla & Netrawali, 1988b; Rogers et al., 1988; Hagiwara et al., 2006; Chequer et al., 2012). However, the Committee noted that these studies had some shortcomings including increased genotoxicity observed only at concentrations in the range of cytotoxicity, no application of metabolic activation, analysis of only 100 metaphases or inclusion of gaps in the evaluation.

Chromosomal aberrations were found in two in vivo studies (Mekkawy, Massoud & El-Zawahry, 2000; Devi et al., 2004), but the Committee noted that these two studies were not performed according to the current OECD guideline, as only 50 or 100 metaphases were analysed for each animal and no positive control group was included. There were also some other significant shortcomings including poor reporting of data and scoring of the aberrations in one of the studies (Mekkawy, Massoud & El-Zawahry, 2000). Negative results were obtained in the other in vivo assays (Tarján & Kurti, 1982; Ivett & Myhr, 1984; Kombrust & Barfknecht, 1985; Zijno et al., 1994).

Given that all of the studies with positive test outcomes had limitations in experimental design and reporting, the Committee concluded that there is no safety concern with respect to genotoxicity of erythrosine.

Table 2
Genotoxicity of erythrosine in vitro and in vivo

End-point	Test system	Concentration or dose	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	1, 10, 100, 1 000, 10 000 µg/plate	Negative [a]	Auletta, Kuzava & Parmar (1977)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	50, 250, 1 000 µg/plate	Negative [a]	Brown, Roehm & Brown (1978)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	0.2, 2, 20, 400 µg/plate	Negative [a]	Muzzall & Cook (1979)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	320, 1 000, 3 200, 10 000 µg/plate	Negative [a]	Bonin & Baker (1980)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1537	Unknown	Negative [a]	Tarján & Kurti (1982)
Reverse mutation	*S. typhimurium* TA92, TA94, TA98, TA100, TA1535, TA1537	1, 2, 5, 10 mg/plate	Negative [a]	Ishidate et al. (1984)
Reverse mutation	*S. typhimurium*	Unknown	Negative [a]	Jaganath & Myhr, (1984a)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	1, 3, 10, 33, 100 µg/plate	Negative [a]	Cameron et al. (1987)
Reverse mutation	*S. typhimurium* TA97a, TA98, TA100, TA102, TA104	20, 200, 2 000 µg/plate	Negative [b]	Lakdawalla & Netrawali (1988a)
Reverse mutation	*Escherichia coli*	0.5 g/100mL	Positive [c]	Lück, Wallnofer & Bach (1963); Lück & Rickerl (1960)
Reverse mutation	*E. coli* WP2*uvr*A; *S. typhimurium* TA1538	0.5 mg/mL	Negative [d]	Haveland-Smith & Combes (1980)
Reverse mutation	V79 Chinese hamster lung cells	200 µg/mL	Positive [e]	Rogers et al. (1988)
DNA repair/damage	*Bacillus subtilis* 17A, 45AT	1 mg/mL	Negative [f]	Kada, Tutikawa & Sadaie (1972)
DNA repair/damage	*E. coli* WP2	1 000 µg/plate	Negative [f]	Haveland-Smith, Combes & Bridges (1981)
DNA repair/damage	Rat hepatocytes	1×10^{-3} – 1 mmol/L	Negative [g]	Kombrust & Barfknecht (1985)
DNA repair/damage	HepG2 cells	0.1, 0.2, 2.0, 10, 25, 50, 70 µg/mL	Positive [h]	Chequer et al. (2012)
Forward mutation	Mouse lymphoma L5178Y $TK^{+/-}$	Unknown	Negative [i]	Cifone & Myhr (1984)
Forward mutation	Mouse lymphoma L5178Y $TK^{+/-}$	60–160 µg/mL	Positive [j]	Cameron et al. (1987)
Forward mutation	*B. subtilis* 168, her-9	0.01, 0.1, 1 mg/mL	Positive [k]	Lakdawalla & Netrawali (1988b)
Gene conversion	*Saccharomyces cerevisiae* BZ34	5 mg/mL	Negative [l]	Sankaranarayanan & Murthy (1979)
Gene conversion	*S. cerevisiae* D5	Unknown	Negative [l]	Jaganath & Myhr (1984b)
Gene conversion	*S. cerevisiae* D5, D7, XV185–14C	0.1–10 mg/mL	Negative (D5), Positive (D7, XV185–14C) [m]	Matula & Downie (1984)
Micronucleus induction	V79 Chinese hamster lung cells	300 µg/mL	Positive [n]	Rogers et al. (1988)
Micronucleus induction	HepG2 cells	0.1, 0.2, 2.0, 10, 25, 50, 70 µg/mL	Positive [o]	Chequer et al. (2012)
Chromosomal aberration	Chinese hamster fibroblast cell line	3 doses, up to 5 mg/mL	Positive only at 5 mg/mL [p]	Ishidate et al. (1984)

End-point	Test system	Concentration or dose	Result	Reference
Chromosomal aberration	Syrian hamster embryo cells	33, 110, 330 µmol/L	Positive only at 330 µmol/L [q]	Hagiwara et al. (2006)
SCE	V79 Chinese hamster lung cells	200, 300, 400 µg/mL	Negative [r]	Rogers et al. (1988)
SCE	Syrian hamster embryo cells	11, 33, 110 µmol/L	Negative [s]	Miyachi & Tsutsui (2005)
SCE	Human peripheral blood cells	0.02, 0.2, 0.5, 1 mmol/L	Negative [t]	Mpountoukas et al. (2010)
In vivo				
Micronucleus induction	Mice	Unknown	Negative [u]	Tarján & Kurti (1982)
Micronucleus induction	Mice	Unknown	Negative [u]	Ivett & Myhr (1984)
Micronucleus induction	Male CD1 mice	24, 80, 240 mg/kg bw	Positive only at 24 mg/kg bw [v]	Lin & Brusick (1986); Brusick (1984, 1989)
Micronucleus induction	Male B6C3F1 mice	50, 100, 200 mg/kg bw	Negative [w]	Zijno et al. (1994)
DNA repair/damage	Male Sprague Dawley rats	200 mg/kg bw	Negative [x]	Kombrust & Barfknecht (1985)
DNA repair/damage	Male ddY Mice	50, 100, 2 000 mg/kg bw	Positive [y]	Kawaguchi, Sasaki & Tsuda (2001); Sasaki et al. (2002)
Chromosomal aberration	Male Swiss albino mice	80, 400 mg/kg diet	Positive [z]	Mekkawy, Massoud & El-Zawahry (2000)
Chromosomal aberration	Male rats	64, 128, 256 mg/kg bw	Positive [aa]	Devi et al. (2004)
SCE	Male B6C3F1 mice	50, 100, 200 mg/kg bw	Negative [w]	Zijno et al. (1994)
Somatic mutation and recombination	*Drosophila melanogaster*	1, 3, 6 mg/mL	Inconclusive [bb]	Sankaya, Selvi & Erko (2012)

bw: body weight; S9: 9000 × *g* supernatant fraction from liver homogenate from induced rats; SCE: sister chromatid exchange

[a] Plate incorporation test with and without S9 mix (metabolic activation).

[b] In a preincubation test, activation was by rat liver S9 mix or rat caecal cell-free extract.

[c] A very slight but statistically significant mutagenic effect on *E. coli* was observed at a concentration of 0.5 g/100 mL. The xanthene molecule itself was the causative factor and the substituent groups only modified the effect.

[d] Tests with and without metabolic activation.

[e] In a V79 hepatocyte-mediated mutation assay, colony size was reduced at 200 µg/mL and lethality was >90% at 400 µg/mL. Thus, increased genotoxicity was observed only at concentrations well in the range of cytotoxicity.

[f] Rec assay.

[g] Rat hepatocyte primary culture/DNA repair (HPC/DR) assay.

[h] DNA damage occurred only at the two highest doses in the in vitro comet assay.

[i] Forward mutation assay in mouse lymphoma L5178Y $TK^{+/-}$ cells.

[j] Forward mutation assay in mouse lymphoma L5178Y $TK^{+/-}$ cells yielded positive results both with and without S9 mix. These results were in contrast to the results obtained by Lin & Brusick (1986).

[k] In a *B. subtilis* multigene sporulation assay, positive results were reported in *B. subtilis* excision repair-proficient strain 168 but not in the excision repair-deficient strain her-9 (exc).

[l] The gene conversion assay in diploid yeast was conducted with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as a positive control.

[m] The gene conversion assay in diploid yeast was conducted with ethyl methanesulfonate (EMS; 1000 µg/mL) as the positive control. Positive results were reported in yeast strains D7 and XV185–14C but not in D5.

[n] In a V79 hepatocyte-mediated mutation assay with and without metabolic activation, an increase in micronucleus frequency in the absence of increased hepatocytes was seen at 300 µg/mL.

[o] The cytokinesis-block micronucleus cytome assay was performed with doxorubicin as the positive control. Increased micronuclei frequencies were found. This test used a nonstandard cell type (HepG2 cells) and was not performed according to the current OECD guideline.

[p] In a chromosomal aberration assay in hamster cells with no metabolic activation, only a weak positive result was seen at 5 mg/mL.

[q] In a chromosomal aberration assay in hamster cells, a positive result was seen at 330 µmol/L in the presence of S9. This assay was not performed according to the current OECD guideline as only 100 metaphases were analysed and gaps were not excluded from the evaluation.

[r] A V79 hepatocyte-mediated mutation assay was conducted with and without metabolic activation.

[s] Cells were incubated for 24 hours in the presence of 5-bromodeoxyuridine in the dark. Colcemid was added 3 hours before the end of treatment.

[t] Cells were incubated for 72 hours in the presence of 5-bromodeoxyuridine. Colchicine was added at 70 hours.

[u] Detailed information not provided.

[v] Triethylenemelamine (TEM, 3 mg/kg bw) was used as the positive control. A positive response was observed only at the low dose.

Table 2 (continued)

[w] Mitomycin C (MMC, 1 mg/kg bw) was used as the positive control. Two doses were administered 24 hours apart. Target tissue exposure was demonstrated.
[x] *O*-Aminoazotoluene was the positive control in this rat hepatocyte primary culture/DNA repair (HPC/DR) assay.
[y] The comet assay was performed in isolated nuclei from the glandular stomach, colon, liver, kidneys, urinary bladder, lungs, brain and bone marrow of mice. Positive results were found 3 hours after exposure in glandular stomach, colon and urinary bladder at 100 and 2000 mg/kg bw and in lung only at 2000 mg/kg bw. No DNA damage was evident in these tissues at 24 hours.
[z] Erythrosine induced chromosomal aberrations after male rats were treated at 0.08 and 0.4 g/kg diet for 30 days. No positive control was used. This study was not performed according to the current OECD guideline.
[aa] Oral administration of erythrosine to male mice for 30 days resulted in significant increase of the percentage of chromosomal aberrations in the intermediate and the high-dose groups. No positive control was used. This study was not performed according to the current OECD guideline.
[bb] The somatic mutation and recombination test (SMART) (wing spot test) performed is currently obsolete. Standard cross design was used.

2.2.5 Reproductive and developmental toxicity

Three reproductive toxicity studies in mice and two developmental toxicity studies in rats have been published since the previous evaluation.

(a) Reproductive toxicity

Mice

To test the effects of erythrosine on spermatogenesis, adult male albino mice (10/group) were given erythrosine by gavage at dose levels of 68 or 136 mg/kg bw per day for 21 days. Lactate dehydrogenase isozyme activity in spermatocytes (a marker of progression of meiotic cycle and of testicular toxicity), spermatocyte counts and sperm motility were measured 1 day after the final dose.

Testicular lactate dehydrogenase isozyme activity was significantly decreased by 71.8% and 68.6% of the control value, at the low and high dose, respectively. The average epididymal sperm count was decreased to 50% and 33.9% of control values, respectively, and sperm motility was decreased to 57% and 80.5% of control values, respectively. Sperm morphology was also evaluated in mice treated at daily gavage doses of 340, 680 or 1360 mg/kg bw for 5 consecutive days and killed 35 days after the final dose. A significant increase in the incidence of abnormal sperm head morphology, by 57% and 74.7% at 680 and 1360 mg/kg bw per day, respectively, was reported. The authors suggested erythrosine affects testicular function and reproductive performance.

The lowest-observed-adverse-effect level (LOAEL) was 68 mg/kg bw per day based on the decreased epididymal sperm count and sperm motility at all dose levels (Abdel-Aziz et al., 1997).

In a more recent study, erythrosine was given to Swiss male albino mice (10/group) by gavage at 64, 128 or 256 mg/kg bw per day for 30 days. Sperm count was decreased at the mid-and high-dose levels, whereas sperm motility was significantly decreased at all dose levels. A significant increase in sperm abnormalities was also reported at the mid-and high-dose levels.

The LOAEL was 64 mg/kg bw per day based on decreased sperm motility at all dose levels (Vivekanandhi et al., 2006).

Erythrosine was administered to Crj:CD-1 mice (10/sex per group) at dietary concentrations of 0, 0.005%, 0.015% or 0.045% (equivalent to 0, 7.5, 22.5 and 67.5 mg/kg bw per day, respectively). The animals were treated from 5 weeks of age in the (parental generation (F_0) to 9 weeks of age in the first filial generation (F_1) and were monitored for changes in selected reproductive and neurobehavioural parameters.

There were no effects on litter size, litter weight or sex ratio at birth. A significant increase in mean body-weight of male and female offspring at the mid dose was reported during the lactation period. The author attributed these changes to body size at birth and did not consider them related to treatment. There were no significant changes in behavioural development parameters in either males or females during the lactation period. Significant changes in exploratory behaviour and movement were reported in high-dose animals; this was dose related in adult F_0 and F_1 females and in male F_1 offspring.

The author concluded that the administration of erythrosine produced few adverse effects in reproductive and neurobehavioural parameters in mice (Tanaka, 2001).

In view of the lack of consistency in the data, the poor absorption of erythrosine, the evidence that erythrosine does not penetrate the blood–brain barrier and the lack of evidence of behavioural toxicity in the Vorhees et al. (1983) rat study with dose levels equivalent to up to 1000 mg/kg bw per day, the Committee concluded that the findings did not provide robust evidence of behavioural effects.

Rats

Charles River CD rats (23–25/sex per group) received erythrosine in the diet at concentrations of 0, 0.25%, 1.0% or 4.0% (equal to 0, 149, 613 and 2957 mg/kg bw per day for males and 0, 255, 990 and 4760 mg/kg bw per day for females, respectively) for 3 consecutive generations. The F_0 parental rats were fed diets for 69 days prior to mating. Slight to moderate reductions in mean maternal body-weight gain were noted in females of all generations at the 1.0% and 4.0% concentrations. Slight to moderate reductions in mean pup body-weights were recorded at the 4.0% level on lactation days 0, 4, 14 and 21 in all generations. These reductions were statistically significant only on lactation day 21. There were no consistent compound-related effects on the reproductive performance of males and females or on pup survival at any concentration in any generation.

The NOAEL was 0.25% (equal to 255 mg/kg bw per day) based on the reduced maternal body-weight gain noted in females at 1.0% and 4.0% concentrations of erythrosine in the diet (Aldridge et al., 1981; later published as Borzelleca & Hallagan, 1990).

Adult Sprague Dawley rats (18–22 pairs of males and females/group; weighing 200–220 g) were fed diets containing erythrosine at levels of 0, 0.25%, 0.5% or 1.0% (equivalent to 0, 250, 500 and 1000 mg/kg bw per day) for 2 weeks before mating and during the mating period. The diets were continued for the females throughout gestation and lactation and were provided continuously to their offspring until they were 90–100 days old. Animals in the positive control group did not receive erythrosine in the diet, but offspring were injected daily with 50 mg/kg bw of hydroxyurea on postnatal days 2–10. Two years later, a second experiment replicated the first using the same dose groups and numbers of animals per group. In both experiments, body weights and feed consumption were measured in parental animals and reproductive success in females. Offspring were assessed for behavioural signs of toxicity plus body weight, feed consumption, physical development and brain weight.

There were no reductions in parental or offspring weight or feed consumption in erythrosine-treated animals. Pre-weaning offspring mortality was significantly increased in the 0.5% and 1.0% concentration groups in the first experiment but not the second. Erythrosine did not adversely affect mean litter size in either experiment. No dose-dependent behavioural effects in erythrosine-treated groups were replicated across the two experiments.

The authors concluded that these studies provided no evidence that erythrosine, via dietary exposure at levels as high as 1.0%, is psychotoxic to developing rats.

The NOAEL was 1.0% in the diet (equivalent to 1000 mg/kg bw per day), the highest dietary concentration tested (Vorhees et al., 1983).

(b) Developmental toxicity

To assess developmental toxicity and teratogenicity, erythrosine was administered to pregnant Osborne–Mendel rats by gavage or in drinking-water in two separate studies. Erythrosine was administered by gavage at doses of 0, 15, 30, 100, 200, 400 or 800 mg/kg bw per day or in drinking-water at levels of 0.05%, 0.1%, 0.2% or 0.4% erythrosine (equal to 64, 121, 248 and 472 mg/kg bw per day) on gestation days 0–20 and were killed on gestation day 20.

In the gavage study, statistically significant increases were reported in feed consumption at 400 mg/kg bw per day, while increases at 30 and 800 mg/kg bw per day were borderline significant. A significant but isolated increase in

maternal weight gain in dams at 30 mg/kg bw per day on days 0–7 was considered unrelated to treatment. There were no dose-related changes in maternal clinical observations, implantation efficiency, fetal viability, fetal size (weight and length) or skeletal or visceral development. There was no dose-related evidence of teratogenic effects. The authors concluded that erythrosine did not result in fetal or maternal toxicity or teratogenicity at the gavage levels tested.

The NOAEL was 800 mg/kg bw per day, the highest dietary concentration tested (Collins et al., 1993a).

In the drinking-water study, reduced water consumption in erythrosine-treated animals compared with controls reached statistical significance only sporadically and did not show dose dependence. Increased feed consumption was noted at 0.2%, but maternal body-weight gain in any group did not differ significantly from controls. There were no dose-related changes in maternal clinical observations, implantation efficiency, fetal viability, fetal size (weight and length) or visceral development. There was no dose-related evidence of teratogenic effects. Random statistically significant skeletal variations were not dose related.

Based on these data, erythrosine did not result in fetal or maternal toxicity or teratogenicity at the concentrations tested. The NOAEL was 0.4% (equal to 472 mg/kg bw per day), the highest dose tested (Collins, Black & Ruggles, 1993).

2.2.6 Special studies

(a) Sensitization

Erythrosine increased the release of serotonin (5-hydroxytryptamine; 5-HT) in rat basophilic leukaemia cells (RBL-2H3) in vitro. The authors indicated that erythrosine might modify the immediate-type allergic reactions (Tanaka et al., 1995).

Considering the limited systemic bioavailability of erythrosine via the oral route, the Committee concluded that this finding was of no biological significance.

Sugihara et al. (2004) reported that erythrosine induced iodine allergy in guinea-pigs. However, Bär & Griepentrog (1960) reported no sensitization activity of erythrosine in another experiment with guinea-pigs.

(b) Cancer chemopreventive activity

Cancer chemopreventive effects of erythrosine were investigated in vitro and in vivo. Erythrosine decreased the activation of Epstein–Barr virus (EBV) antigen by tumour promoter 12-*O*-tetradecanoylphorbol-13-actate (TPA) in cultured Epstein–Barr virus (EBV) genome-carrying lymphoblastoid Raji cells. In addition, erythrosine significantly decreased the incidence of skin tumours in

female ICR mice in a skin carcinogenesis assay, following topical application of 7,12-dimethylbenz[a]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-actate (TPA) as inducer–promoter system (Kapadia et al., 1998).

(c) Effects on hepatic function

The influence of a mixture of six artificial food colours (erythrosine, Allura Red, New Coccine, Brilliant Blue, tartrazine and Fast Green FCF) on the toxicity of the carcinogenic heterocyclic amine 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-b]indole (Trp-P-1) was investigated using primary cultured rat hepatocytes. Hepatocytes were isolated and cultured from rats fed a diet containing the mixture of food colours at half the amount of their respective ADI for 4 weeks. Trp-P-1 was subsequently administered to the hepatocytes at various concentrations for 12 hours. The mixture of food colours did not affect the decrease in cell viability or protein and DNA synthesis induced by Trp-P-1 but slightly augmented the decrease in gluconeogenesis and ureagenesis that Trp-P-1 induced. The authors suggested that the daily intake of artificial food colours may impair hepatic functions such as gluconeogenesis and ureagenesis when dietary carcinogens such as Trp-P-1 are exposed to liver cells (Ashida et al., 2000).

The Committee noted that the use of mixtures in these studies does not permit any observed effects to be ascribed to individual components, including erythrosine.

(d) Effects on macromolecular binding

In order to evaluate the modulating ability of erythrosine on the aggregation of amyloid-beta (Aβ) peptide fibrils, erythrosine (0.5, 5, 25, 50, 250 or 500 µmol/L) was added to a 50 µmol/L Aβ monomer solution incubated for up to 3 days at 37 °C. Erythrosine reduced the polymerization of Aβ monomers in a concentration-dependent manner and was shown to bind to the amino terminal of Aβ peptides.

The authors suggested that considering its safety profile, erythrosine may be used as a nontoxic modulator of Aβ-fibril aggregation in the prevention of Alzheimer disease progression (Wong & Kwon, 2011).

(e) Effects on thyroid function and morphology

In vivo studies were performed to investigate the effects of erythrosine on the metabolism of ^{125}I-labelled T_4 and T_3 by liver homogenates from rats given daily intraperitoneal injections of erythrosine at 2.5–250 mg/kg bw. Erythrosine caused a dose-dependent inhibition of the deiodination of T_4 and of the generation of T_3. At dose levels greater than 10 mg/kg bw, the proportionate reduction in the deiodination of ^{125}I-T_4 exceeded the reduction in the generation of ^{125}I-

T_3, indicating that pathways of T_4 metabolism other than those leading to T_3 formation were also inhibited.

The authors concluded that erythrosine may inhibit the 5-monodeiodination of T_4 to reverse T_3 (rT_3) because erythrosine inhibited the 5-monodeiodination of T_3 to 3,3′-diiodothyronine. Fluorescein did not exhibit similar effects. It was considered unlikely that erythrosine would produce similar effects in humans at the usual doses ingested. This conclusion was supported by studies in human volunteers given oral doses of erythrosine of up to 25 mg daily for 7 days, when no changes in serum T_4, T_3 or rT_3 concentrations were detected (Ruiz & Ingbar, 1982; Garber et al., 1981).

Sprague Dawley rats (15 males/group) received 0, 0.25%, 0.50%, 1.0%, 2.0% or 4.0% erythrosine in the diet (equivalent to 0, 125, 250, 500, 1000 and 2000 mg/kg bw per day, respectively) for 7 months. After 6 months, each group was divided into three subgroups of 5 rats each. During the next month, one subgroup received T_3 at 15 µg/kg bw per day by subcutaneous injection, the second subgroup received the saline vehicle and the third subgroup continued the treatment with no injections. Blood samples (orbital puncture) were obtained just before study start and at monthly intervals thereafter, and serum thyroid hormone levels were determined. At scheduled kill, pituitary glands and liver segments were excised and used to perform in vitro studies of T_4 metabolism.

Serum thyrotropin (TSH) levels varied and, although mean serum TSH values were higher at 4% than in the control group or at 0.5% over the first 6 months of the study, the differences were not statistically significant. Serum TSH concentrations in the subgroups that received injections of T_3 during the final month were below detection limits (15 µU/mL). Serum T_4 concentrations in animals at 4% erythrosine were elevated relative to baseline and control values during the 6 months of the study, whereas the values for animals in the control group and at the 0.5% erythrosine did not differ significantly from baseline values or from each other. Serum T_4 concentrations were below the level of detection in all animals that were injected with T_3 during the seventh month of the study. Serum T_3 concentrations in all three groups decreased significantly with time; at 4% erythrosine in the diet, T_3 concentrations were significantly lower than controls at 1, 2, 4 and 5 months. The effects of 0.5% erythrosine on T_3 levels were unclear as significant depressions relative to controls were only observed at 1 and 2 months. In rats at 4% erythrosine, serum rT_3 concentrations increased approximately 7-fold and remained elevated throughout the 6 months of the study. In both control and 0.5% groups, serum rT_3 concentrations did not differ significantly from baseline values or from each other. Serum rT_3 was undetectable in animals injected with T_3 during the final month of the study.

In vitro metabolism of ^{125}I-labelled T_4 was greatly altered in liver homogenates from rats fed 4.0% erythrosine in the diet, with degradation of T_4

decreasing to approximately 40% of control values, with an associated decrease of about 75% in the generation of ^{125}I-iodide and of 80% in the generation of ^{125}I-T_3 from ^{125}I-T_4. Percentage degradation of T_4 and generation of iodide and T_3 in liver homogenates from rats fed 0.5% erythrosine were similar to controls.

In vitro metabolism of T_4 was examined in pituitary glands from control and 1%, 2% and 4% dose group animals. Overall, ^{125}I-T_4 degradation and generation of ^{125}I-iodide appeared to be higher in the 2% and 4% groups than in controls, but none of the differences were statistically significant.

The results were interpreted as indicating that the primary effect of high doses of erythrosine on thyroid hormones is inhibition of type I 5′-monodeiodination of T_4 to T_3. As a consequence, TSH secretory mechanisms were activated in the pituitary. The increases in serum rT_3 levels were considered to arise from both increased availability of the T_4 precursor and inhibition of metabolism of rT_3 by 5′-monodeiodination (Ingbar, Ballman & Braverman, 1984).

In studies on female rats identical to the Ingbar, Ballman & Braverman (1984) study on male rats outlined above, similar results were obtained in that erythrosine at dietary concentrations of 4% caused an increase in serum T_4, rT_3 and TSH concentrations, a decrease in hepatic deiodination of T_4 to T_3, and an increase in deiodination in the pituitary. Hepatic generation of T_3 from T_4 was also diminished following dietary administration of 0.5% erythrosine, but to a lesser degree, and no changes in serum thyroid–related hormones could be detected. No changes in the metabolism of T_4 were observed in liver homogenates from rats fed 0.25% dietary erythrosine (Ingbar, 1985).

In both the male (Ingbar, Ballman & Braverman, 1984) and female (Ingbar, 1985) rat studies described above, ultrastructural examination of thyroids of the rats fed 4% erythrosine in the diet for 6 months revealed enhanced synthetic and secretory activity consistent with prolonged hyperstimulation by TSH. Less marked changes were seen at the 0.5% concentration, and the changes were generally less in females than males similarly treated.

Adult male Sprague Dawley rats (13/group) were fed 0, 0.5%, 1.0% or 4.0% erythrosine in the diet (equivalent to 0, 500, 1000 and 4000 mg/kg bw per day, respectively), NaI at 100 mg/kg bw per day or fluorescein at 1000 mg/kg bw per day for 3 weeks. The rats then underwent an in vivo thyrotropin-releasing hormone (TRH; TSH-releasing hormone) provocative test (100 ng/100 g bw).

Of all the treatments, only 4% dietary erythrosine produced an exaggerated response to TRH: 10 minutes after TRH injection, the serum TSH levels were 80% greater than controls ($P < 0.01$). Erythrosine also produced a dose-dependent increase in total serum T_4 levels, significant ($P < 0.01$) at the 1% and 4% concentrations. At the 4% level, there was also a significant increase in serum T_3 levels and a significant decrease in T_3 resin uptakes. The free T_4 indices

were significantly elevated after treatment with 1% or 4% dietary erythrosine, but the free T_3 indices were not.

The data indicate that feeding with 4% erythrosine disrupts the normal negative feedback regulatory mechanism of the pituitary–thyroid axis; the TRH hyper-responsiveness in the presence of elevated serum T_4 and T_3 levels suggests that the defect did not arise from a conventional antithyroid mechanism. The data also suggest that the effect of erythrosine on TSH release was due to intact dye or an iodinated metabolite rather than the fluorescein nucleus (Witorsch, Jennings & Schwartz, 1984).

Male Sprague Dawley rats (160/group) were administered erythrosine at concentrations of 0, 0.25% or 4.0% in the diet (equal to 0, 147.1 and 2514.3 mg/kg bw per day) for 60 days. Physical observations of all animals were performed and body weights and feed consumption measured pretest and at weekly intervals during treatment. Up to 20 animals/group were necropsied at days 0, 3, 7, 10, 14, 21, 30 and 60. Serum was prepared from blood samples taken from the abdominal aorta at each kill time and analysed by radioimmunoassays for thyrotropin (TSH), T_4, T_3 and rT_3. Thyroid and pituitary were weighed at each interval and organ to body-weight ratios calculated. Gross postmortem examinations were conducted on the thyroid and pituitary only.

Three rats fed 4.0% erythrosine in the diet died spontaneously during the second week of the study. The animals fed 4% erythrosine in the diet lost weight during the first week of the study, and the mean body-weights were significantly lower than control values throughout the study (13% at week 1 and 17% at week 8). Feed consumption of the animals at the 4.0% dietary level was significantly lower than the control value at week 1, but after week 2 it was comparable. This probably reflected a palatability problem during the first 2 weeks. The absolute pituitary weights of males at 4% erythrosine were statistically significantly lower than control values at days 7, 10, 14, 21 and 60. The differences were considered to reflect the body weight differences between the high-dose animals and the controls. The absolute thyroid/parathyroid weights of the rats at the 4% level were generally lower than the control values, but the differences were slight and may have been due to the body-weight differences between these groups. The relative weights of these organs were significantly greater at day 21; otherwise, relative weights were only slightly greater and not significant. Thyroid/parathyroid absolute and relative weights of the rats fed 0.25% erythrosine were significantly lower at day 60; otherwise, they were comparable to controls. Gross postmortem examinations of thyroid and pituitaries did not show any treatment-related changes (Kelly & Daly, 1988).

The analysis of serum hormone levels in these rats revealed a slight increase in serum TSH levels in the control rats during the 60-day experimental

period. The baseline (day 0) TSH level was significantly lower than the levels on days 21, 30 and 60. In the 0.25% group, serum TSH concentrations were significantly increased above baseline (day 0) levels at days 14, 21, 30 and 60. When compared with the TSH levels in control animals, a significant increase was observed at days 21, 30 and 60. In the 4.0% group, the TSH levels were significantly increased over the baseline (day 0) level and the corresponding control levels at all time points. When compared with the 0.25% group the serum TSH levels in the high-dose group were significantly greater at days 3, 7, 10 and 14. Serum T_4 concentrations were increased over baseline and control values at days 10 and 14 in the 0.25% group, while in the 4.0% group the T_4 concentrations were increased at all time points. Furthermore, the high-dose animals had significantly greater T_4 concentrations than the low-dose animals at days 7, 10, 21, 30 and 60. Serum T_3 concentrations in the low-dose rats were comparable to the control values except for a decrease at day 30. In the high-dose rats serum T_3 concentrations were significantly lower than baseline (day 0) and control values at all time points. In addition, serum T_3 concentrations were decreased compared with those of the low-dose animals on days 3, 10, 14, 21, 30 and 60. Serum rT_3 concentrations were increased above baseline (day 0) in the low-dose group at days 7, 10, 14, 21, 30 and 60 and increased above control values at days 10, 14 and 21. A marked increase in serum rT_3 over controls and low-dose animals was seen in the high-dose group at all time points. The results indicate that the ingestion of a dietary concentration of 4% erythrosine induces a rapid and sustained increase in serum TSH, T_4 and rT_3 and a comparable decrease in serum T_3 concentrations, and that these changes are also induced, but are less pronounced, at 0.25% erythrosine in the diet. These findings are consistent with erythrosine inhibiting the deiodination of T_4 and rT_3 in the 5′-position, resulting in a decreased production of T_3 from T_4 and a decreased deiodination of rT_3, respectively (Braverman & DeVito, 1988).

Male Sprague Dawley rats (80/group) were administered erythrosine at concentrations of 0, 0.03%, 0.06% and 4.0% in the diet (equal to 0, 17.5, 35.8 and 2671.7 mg/kg bw per day, respectively) for a maximum of 60 days. Control animals (100 males) received standard laboratory diets. Physical observations, body weight and feed consumption measurements were performed on all animals pretest and at weekly intervals during the study period. To determine baseline data, 20 control animals were bled for radioimmunoassays of TSH, T_4, T_3 and rT_3 and killed on test day 0, prior to the initiation of dosing. Additional necropsy intervals were staggered so that an additional 20 animals/group were bled at days 7, 21, 30 and 60. Brain, pituitary and thyroid were weighed and organ to body weight and organ to brain weight ratios were calculated for all animals. Gross postmortem examinations of thyroids, pituitary and brains of all animals were performed.

In the animals at the 4% dietary level, a substantial loss of body weight and decreased feed consumption during week 1 of the study, probably due to poor palatability of the diet, resulted in statistically significantly lower body weights of the animals throughout the study period. The absolute and relative thyroid/parathyroid weights of the animals fed 4.0% erythrosine were increased at days 21 and 30 and relative organ to body weights were increased at day 60. The absolute pituitary weights and relative (to brain weight) pituitary weights of animals at the 4.0% dietary level were lower than control values at day 7. In the 0.03% group, absolute and relative thyroid/parathyroid weights were greater than corresponding control values at day 21 and 30 but comparable to control values at days 7 and 60. In conclusion, there were no consistent or dose-related changes in organ weight, absolute or relative at the lower doses. Gross postmortem examination of the thyroid, pituitaries and brain did not reveal any treatment-related effects (Kelly & Daly, 1989).

Analysis of serum hormone levels found no significant changes in serum TSH, T_4, T_3 or rT_3 concentrations in the 0.03% and 0.06% groups during the 60-day treatment period. In the 4.0% group, TSH concentrations were significantly greater than the corresponding control values at days 21, 30 and 60; a 41% increase at 7 days was not statistically significant compared with the control value. Serum TSH concentrations were also significantly greater than those of the 0.03% group at days 21, 30 and 60 and those of the 0.06% group at day 30. In the 4.0% group, serum T_4 concentrations were slightly elevated above those of controls during the treatment period; however, the increase was only statistically significant on day 30. In the high-dose animals, serum T_3 concentrations were significantly lower than controls at all time points. Serum rT_3 concentrations were markedly increased in the high-dose animals compared with controls or animals fed 0.03% and 0.06% erythrosine at all time points (Braverman & DeVito, 1989).

Ultrastructural examination by electron microscopy of the thyroid glands from the 27-week toxicity study (Couch et al., 1983; see section 2.2.3(b)) showed that rats fed commercial erythrosine had hypertrophy of follicular cells with increased development of synthetic and secretory organelles (rough endoplasmic reticulum, Golgi apparatuses and long microvilli). These changes were interpreted as representing mild to moderate stimulation of follicular cells consistent with elevated serum T_4 levels. Lysosomal bodies in treated rats receiving erythrosine were described as larger and more irregular and electron dense than controls; they appeared to be closely associated or fused with the limiting membrane of colloid droplets, a process involved in secretion of thyroid hormones. The degree of thyroid stimulation and the accumulation of colloid droplets and lysosomes in follicular cells were described as greater in males than in females. Ultrastructural indications of long-term thyroid stimulation appeared

greater in rats fed commercial erythrosine than in rats fed purified colour with supplemental iodide (Capen, n.d., a).

Examination by electron microscopy of the thyroids from the 7-month Ingbar, Ballman & Braverman (1984) study (see above) found the follicular cells from rats fed erythrosine to have ultrastructural features of a dose-dependent stimulation of synthetic and secretory activity, that is, hypertrophy with increased development of secretory organelles, most marked in rats fed 4% dietary erythrosine. These features were considered consistent with a response to long-standing TSH stimulation. The ultrastructural changes were reversible upon administration of T_3 during the final month of the study. A dose-dependent accumulation of numerous lysosome-like bodies in the follicular cells of the treated rats was considered not to be a response to TSH stimulation alone. Similar, but less marked changes, in follicular cell stimulation and accumulation of lysosome-like bodies were described in rats fed erythrosine at 0.25% and 0.5% (Capen, n.d., b).

(f) Neurological effects

The effects of erythrosine on neurobehavioural and related parameters were assessed in four separate groups of young male adult Charles Foster rats treated with a single administration of 1, 10, 100 or 200 mg/kg bw of erythrosine by gavage. Motor activity (frequency of vertical rearing motor activity) was assessed in group 1 at various times between 0 and 9 hours after dosing; steady-state levels of serotonin, its metabolite 5-hydroxyindoleacetic acid (5-HIAA) and of monoamine oxidase-A (MAO-A) in brain tissue were assessed in group 2 at 2 and 7 hours after dosing; the effect of erythrosine pretreatment on serotonin accumulation and 5-HIAA reduction rates induced by the MAO inhibitor pargyline was assessed in group 3 at 2 hours after erythrosine administration; and the effect of MAO-A and MAO-B inhibitors on motor activity was assessed in group 4 every 30 minutes between 0 and 9 hours after dosing. Motor activity was measured as the number of rearing events in each 5-minute observation period.

A transient, dose-related and statistically significant decrease in motor activity was observed at doses of erythrosine of 10 mg/kg bw and higher; these peaked at 2 hours post dose but were no longer statistically significant by 3–4 hours at the low dose and by 7 hours at the mid and high dose (group 1). A parallel transient decrease in steady-state levels of serotonin in medullary pons, hypothalamus and hippocampus, a decrease in steady-state levels of 5-HIAA in the hippocampus and an increase in MAO-A activity in the hippocampus were also reported at 2 hours and recovered by 7 hours (group 2). Similar decreases were observed in the pargyline-induced serotonin accumulation and 5-HIAA

reduction rates (group 3). The motor activity decrease associated with the 100 mg/kg bw dose of erythrosine was moderated by administration of MAO-A and MAO-B inhibitors when given individually and was prevented when these were given together (group 4). All effects were transient and had returned to normal levels within 7 hours (Dalal & Poddar, 2009).

In a follow-up repeated-dose study from the same research group, erythrosine was administered to young male adult Charles Foster rats at dose levels of 1, 10 and 100 mg/kg bw per day by gavage for 15 or 30 days to investigate the role of serotonin and MAO-A activity in the effect of erythrosine on motor activity (Dalal & Poddar, 2010). A similar study design and the same methodologies were used as in the previous study (Dalal & Poddar, 2009). Unlike the decrease in motor activity following single-dose administration of erythrosine (as reported in the earlier study), repeated-dose administration for either 15 or 30 days resulted in transient, dose-related and statistically significant induction of hyperactivity (increased rearing motor activity) at 10 and 100 mg/kg bw per day; this peaked at 2 hours after the final administration. As in the earlier Dalal & Poddar (2009) study, this effect was no longer statistically significant by 3–4 hours in the 10 mg/kg bw dose groups either after 15 or 30 days of treatment; by 7 hours in the 100 mg/kg bw dose group after 15 days of treatment; and by 9 hours in the 100 mg/kg bw dose groups after 30 days of treatment. A parallel increase in brain serotonin levels was noted at 10 and 100 mg/kg bw per day following either 15 or 30 days of treatment. A nonstatistically significant decrease in 5-HIAA levels and a statistically significant decrease in MAO-A activity were also reported at 100 mg/kg bw per day after 30 days of treatment only. Erythrosine pretreatment for 15 or 30 days resulted in higher pargyline-induced serotonin accumulation in brain regions but did not affect the 5-HIAA levels. The ratio of 5-HIAA to serotonin was significantly decreased in the medullary pons, hypothalamus, hippocampus and corpus striatum of animals at 100 mg/kg bw per day after 30 days of treatment and in medullary pons and hypothalamus after 15 days of treatment. The ratio of 5-HIAA to serotonin was decreased at 10 mg/kg bw per day only after 30 days of treatment and only in medullary pons and hippocampus. Erythrosine treatment at 10 and 100 mg/kg bw per day for 15 and 30 days resulted in significantly increased plasma corticosterone levels that were further increased after pargyline treatment. The authors interpreted the discrepancies in the effects of erythrosine on motor activity and brain serotonin levels after single-dose and repeated-dose administration to be the result of corticosterone levels (Dalal & Poddar, 2010) and therefore a secondary stress-mediated effect.

In view of the lack of consistency in the data, the poor oral absorption of erythrosine, the evidence that erythrosine does not penetrate the blood–brain barrier and the lack of signs of behavioural toxicity in the Vorhees et al. (1983) rat study with dose levels equivalent to up to 1000 mg/kg bw per day, the Committee

concluded that the findings in the Dalal & Poddar (2009, 2010) studies did not provide robust evidence of behavioural effects and could not be used in the risk assessment.

(g) Other endocrine effects

The ability of erythrosine to inhibit the activity of aromatase, an enzyme that catalyses the conversion of androgens to estrogens, was reported in an in vitro model system using rat ovarian microsomes. The IC_{50} was 0.32 μmol/L (Satoh et al., 2008).

Although aromatase has been a target for endocrine-disrupting chemicals, the Committee concluded that this finding was of no biological significance given the limited systemic bioavailability of erythrosine via the oral route and the absence of reproductive and developmental toxicity in in vivo studies.

No estrogenic activity (based on normal uterine weights) was detected in a group of five young rats given subcutaneous injections of erythrosine at 250 mg/kg bw in aqueous solution twice daily for 3 days (Graham & Allmark, 1959).

2.3 Observations in humans

(a) Effects on thyroid function

No biologically significant increases in plasma inorganic iodine or in urinary iodine were noted in six patients (sex not reported; aged 25–68 years) after oral exposure to 1.9 μmol/day (1.68 mg/day) of erythrosine for 10 days. In other assays of thyroid function, thyroid radioiodine uptake and levels of T_4 and PBI in plasma remained unchanged (Bernstein, Haugen & Frey, 1975).

Five volunteers (4 men and 1 woman, aged 21–35 years) received erythrosine in the diet at 5, 10 and 25 mg/day, increasing in weekly increments for a period of 3 weeks. Total serum iodine and PBI increased slowly and slightly in association with the weekly increasing doses of erythrosine. Serum T_4, T_3, TSH and erythrosine, urinary iodine, erythrosine excretion and T_3-resin uptake remained unchanged throughout the 3 weeks. Increases in serum PBI and total serum iodine during the exposure period indicate that a portion of the iodine ingested as erythrosine was absorbed from the gastrointestinal tract. No changes in serum concentrations of TSH, T_4 and T_3 indicate that both thyroid function and thyroid regulatory mechanisms were unaffected (Ingbar, Garber & Gluckin, 1983).

Human volunteers were given single doses of 75–80 mg of erythrosine labelled with ^{131}I in a milk shake or lemonade. The volunteers also received a daily dose of 5 drops of saturated potassium iodide solution to block thyroid uptake of ^{131}I. Whole-body radiolabel counts were carried out; complete stool and urine samples were examined for activity, and blood samples were daily examined for total serum ^{131}I and for T_4 and T_3.

Faecal excretion of ^{131}I was about 100% in four participants; in two participants, lower recoveries (80% and 90%) were achieved, but this was probably due to incomplete collection as the unrecovered activity was not detected in whole-body counts, urinary ^{131}I or serum. Urinary excretion of the radiolabel over 48 hours did not exceed 0.38% of the dose in any participant, and the urinary counts after 48 hours were similar to background counts. Whole-body counts indicated that ^{131}I-erythrosine was eliminated rapidly and nearly completely, less than 1% of the administered dose remaining after 7 days. The small amount of remaining activity declined exponentially in the 7–14 days after administration, with the half-life averaging 8.4 ± 2.1 days. Extrapolation of this slow phase to zero time indicated a potential initial body retention of 1.2 ± 0.4% of the administered dose. Negligible quantities of ^{131}I appeared in the serum, never exceeding 0.013% of the dose per litre of serum. Serum T_4 and T_3 concentrations were not significantly altered.

These results indicate that erythrosine did not affect thyroid hormone levels at the single oral dose levels tested (Ingbar, Garber & Burrows, 1984).

Healthy men (10/group) were given erythrosine orally (in capsules) for 14 days at doses of 20, 60 or 200 mg/day. Assays for serum T_4, T_3, rT_3, T_3-charcoal uptake, TSH, PBI, total iodide and total urinary iodide excretion were carried out on days 1, 8 and 15; TRH test were performed on days 1 and 15. This study was conducted in accordance with relevant requirements for conduct of human research.

There were no significant changes in serum T_3, T_4, rT_3 or T_3-charcoal uptake in any group. At 200 mg/day, the mean basal serum TSH concentration increased from 1.7 ± 0.1 on day 1 to 2.2 ± 0.1 μU/mL on day 15 ($P < 0.05$), and the mean peak TSH increment after TRH increased from 6.3 ± 0.5 to 10.5 ± 1.0 μU/mL ($P < 0.05$). There were no significant changes in basal or peak TSH responses at the two lower dose levels. Significant dose-related increases in serum total iodide and PBI concentrations occurred in all three dose groups, and significant dose-related increases in urinary iodide excretion occurred at 60 and 200 mg/day.

These data indicate that the increase in TSH secretion was related to the effect of increased serum iodide rather than a direct effect of erythrosine on thyroid hormone secretion or peripheral metabolism (Gardner et al., 1987).

The statistical design and interpretation of the Gardner et al. (1987) study was re-evaluated independently in relation to the effects on basal TSH concentration and maximum TSH increment after TRH provocation. With respect to basal TSH, there was no evidence for variation due to treatment when appropriate statistical methods were used to control for apparent initial differences between treatment groups. The maximum TSH increment following TRH provocation was slightly but significantly increased at 200 mg/day only (Crump & Farrar, 1987).

At its thirty-third meeting, JECFA concluded that the NOAEL with respect to thyroid function in humans was 60 mg/person per day (equivalent to 1 mg/kg bw per day) based on a small change in TSH responsiveness to TRH detected at the highest dose level of 200 mg/person per day. The present Committee concurred.

In a study designed to determine whether relatively small supplementary amounts of iodine in the diet would affect thyroid function, healthy, euthyroid human participants were given iodine at 250, 500 or 1500 µg/day for 14 days. The doses were selected to correspond to the amounts of iodine that might be bioavailable from the doses of erythrosine used in the Gardner et al. (1987) study.

Following administration of 1500 µg/day, there were small but significant decreases in serum T_4 and T_3 concentrations and a small compensatory increase in serum TSH concentrations and in the TSH response to TRH. However, all values remained within the normal range. No changes occurred following daily administration of 250 or 500 µg I_2 (Paul et al., 1988).

(b) Sensitivity

In a case study report, a patient presented hypersensitivity to denture materials. However, it was unclear whether this hypersensitivity was attributable to the use of erythrosine in these materials because further data on the constituents of the nylon denture base resin were not available (Barclay et al., 1999).

Erythrosine has been reported to induce hyperactivity in children, but documentation is lacking (Hallas-Møller et al., 2002). Inhibition of brain tissue ATPases and active re-uptake of neurotransmitters reported in vitro at high concentrations of erythrosine has been suggested as a possible mechanism for hyperactivity (Mailman et al., 1980; Mailman & Lewis, 1981). However, given the minimal bioavailability of erythrosine and the lack of evidence that it can cross the blood–brain barrier, the Committee concluded that these findings have little biological relevance.

3. Dietary exposure

The Committee previously evaluated dietary exposure to erythrosine at its fifty-third meeting, but significant changes in methodology of exposure assessments makes it difficult to compare the assessments.

3.1 Food uses

The Codex Alimentarius Commission has finalized authorization of erythrosine use in foods and beverages with maximum permitted levels ranging between 30 and 200 mg/kg, as noted in the General Standard of Food Additives (GSFA) (CODEX STAN 192-1995, 2017w) (Table 3).

The European Food Safety Authority (EFSA) has also established restricted maximum levels for use of erythrosine (E 127) in Europe; the use is restricted to cocktail and candied cherries and Bigarreaux cherries (94/36/EC, Annex IV) at maximum levels of 200 mg/kg and 150 mg/kg, respectively.

The United States Food and Drug Administration (USFDA) has not established maximum levels for this or other food colouring agents, but allows use according to good manufacturing practices (GMP) in a number of foods as listed in the Code of Federal Regulations (21 CFR 70 and 21 CFR 74).

Because erythrosine has been authorized for use in foods and beverages for many years, several countries have conducted exposure assessments based on household economic surveys or individual dietary records. Such high-tier intake assessments are more accurate than the comparatively uncertain estimates based on the budget method or "poundage" (disappearance) data. Consequently, the Committee assessed intake of erythrosine primarily using data from the most recent national studies.

As highlighted in the previous JECFA evaluation of erythrosine (Annex 1, reference *143*), this colour is also widely used in pharmaceutical products. Therefore, nondietary intake was also considered.

3.2 Assessment of dietary exposure

The Committee reviewed dietary exposure estimate references submitted by the sponsor. Five additional relevant references were retrieved as a result of a comprehensive literature search conducted in the PubMed and Scopus databases.

As highlighted by EFSA (2011), because erythrosine is only authorized in very few specified foodstuffs, estimating exposure based on the budget method is inappropriate. Moreover, several countries and regions have conducted exposure assessments based on household economic surveys, for example, India (Dixit

Table 3
GSFA maximum permissible levels of erythrosine (INS 127)

Food category no.	Food category	Max. level (mg/kg)	Notes
4.1.2.7	Candied fruit	200	For use in cocktail cherries and candied cherries only.
4.2.2.7	Fermented vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera) and seaweed products, excluding fermented soybean products of food categories 06.8.6, 06.8.7, 12.9.1, 12.9.2.1 and 12.9.2.3	30	–
5.3	Chewing gum	50	–
5.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	100	–
8.2	Processed meat, poultry and game products in whole pieces or cuts	30	Excluding products conforming to the Standard for Cooked Cured Ham (CODEX STAN 96-1981) and the Standard for Cooked Cured Pork Shoulder (CODEX STAN 97-1981). For use in decoration, stamping, marking or branding the product only; in glaze, coatings or decorations for fruit, vegetables, meat or fish only.
8.3	Processed comminuted meat, poultry and game products	30	Excluding products conforming to the Standard for Corned Beef (CODEX STAN 88-1981) For use in decoration, stamping, marking or branding the product only; in products conforming to the Standard for Luncheon Meat (CODEX STAN 89-1981) and Cooked Cured Chopped Meat (CODEX STAN 98-1981) at 15 mg/kg to replace loss of colour in product with binders only.

GSFA: General Standard of Food Additives; INS: International Numbering System for Food Additives; max.: maximum; no.: number

et al., 2011), or individual dietary records, for example, the USA (Doell et al., 2016; Bastaki et al., 2017), the Republic of Korea (Ha et al., 2013), Ireland and Northern Ireland (Gilsenan & Gibney, 2004) and Australia and New Zealand (FSANZ, 2010); these constitute a high-tier approach of exposure assessment. Consequently, the Committee chose to use these refined data to assess erythrosine exposure rather than less certain estimates that are based on budget method and "poundage" (disappearance) data. The national studies used for the dietary exposure assessment of erythrosine are described in Table 4.

An exposure estimate based on the food consumption data in Europe (EFSA Comprehensive European Food Consumption Database) with the Food Additives Intake Model (FAIM) 2.0 was conducted. In order to provide estimates at an international level, an exposure estimate based on the FAO/WHO Chronic individual food consumption database – Summary statistics (CIFOCOss) was also conducted.

Table 4
Summary of dietary exposure from published national studies of dietary intake

Country/region Organization Reference	Use or concentration data	Consumption data	Populations; ages (years)	Exposure assessment (mg/kg bw per day)		
				Mean	P90 or P95 [a]	Max.
Australia + New Zealand FSANZ FSANZ (2010)	Use data from the sponsor + current ML + proposed ML [b]	Different national consumption surveys + commercial and domestic recipes, food composition and food label data	Australian population >2	<0.010	0.010	0.010
			Australian children 2–6	<0.010	0.020	0.020 0.010
			New Zealand population >2	<0.010	0.010	
Ireland and Northern Ireland Institute of European Food Studies Gilsenan & Gibney (2004)	Irish National Food Ingredient Database (INFID)	North/South Ireland Food Consumption Survey (NSIFCS) (2001)	Adults	0.003 1	0.009 4	–
			Acceptable reporters	–	0.010	–
			Under reporters	–	0.000 5	_
India Indian Institute of Toxicology Research Dixit et al. (2011)	Indian survey specific to colours [c]	Household survey of food consumption patterns of 518 respondents	Children 4–6	–	–	–
			Children 7–9			
			Children 10–12			
			Adolescents 13–15			
			Adolescents 16–18			
Republic of Korea Universities of the Republic of Korea Ha et al. (2013)	Survey of the Republic of Korea specific to colours	Korea National Health and Nutrition Survey (Ministry of Health and Welfare, 2009)	The entire population of consumers	0.000 7	0.001 9	0.028 7
USA IACM Bastaki et al. (2017)	Industry survey + food colour manufacturers + Mintel International Group Ltd [d]	NHANES	USA population	0.000 3	0.001 1	0.003 8
			Children 2–5	0.001	0.002 6	0.011 4
			Children 6–12	0.006	0.001 9	0.006 5
			Adolescents 13–18	0.003	0.001	0.002 7
			Adults ≥19	0.000 2	0.000 6	0.002 4
USA USFDA Doell et al. (2016)	Survey specific to synthetic colours	NHANES NPD NET-NID 10–14 day food consumption pattern	USA population > 2	0.030	0.050	0.050
			Children 2–5	0.090	0.090	0.100
			Adolescent boys 13–18	0.020	0.040	0.050
Rastogi et al. (2015)	Measured concentration in paediatric syrups [e]	Scenarios from the recommendations on the syrup bottles	Not a population	–	–	–

bw: body weight; IACM: International Association of Color Manufacturers; FSANZ: Food Standards Australia New Zealand; max.: maximum; P90: 90th percentile; P95: 95th percentile; ML: maximum levels; NHANES: National Health and Nutrition Examination Survey; NPD NET-NID: National Eating Trends – Nutrient Intake Database by NPD Group, Inc.; USA: United States of America; USFDA: United States Food and Drug Administration

[a] Mean 95th percentile values are provided when available; otherwise, mean 90th percentile values are provided.

[b] Exposure assessment conducted in order to investigate the possibility of extending the use of erythrosine.

[c] Of the samples that used permitted colours, 53% had levels in excess of the maximum level. Not included in the final exposure assessment.

[d] The use levels reported by industry are consistent with the concentrations measured analytically by the USFDA (Doell et al., 2016).

[e] Nondietary intakes. Not taken into account in the final exposure assessment.

3.2.1 Assessments based on "poundage" (disappearance) data

The sponsor estimated an intake of 68 μg/kg bw per day for erythrosine based on the following assumptions:

- In the USA, approximately 91 815 kg of FD&C Red No. 3 dye were certified in 2016 for use in food, drugs and cosmetics combined.
- Daily intake from consumption of all the certified annual volume was estimated to be 8114 μg/person or 135 μg/kg bw per day.
- Of this, an average of 50% of the dye was estimated to be used in food and beverages in the USA (domestic use only).
- Based on a population of "consumers only", estimated to be 10% of the total population, the average daily intake was approximately 4057 μg/person from consumption of foods and beverages, corresponding to 68 μg/kg bw per day assuming a mean adult body weight of 60 kg.

3.2.2 Assessments based on data from household economic surveys

In a study conducted by the Food Toxicology Division of the Indian Institute of Toxicology Research (Dixit et al., 2011), 2867 samples from 10 broad food categories were analysed for erythrosine (and other synthetic colours) in 16 Indian states. The study focused on children. Sampled food commodities were identified as preferentially consumed by children through a food frequency questionnaire that included bakery products, beverages, candyfloss, chewing gums, coated candies, hard-boiled sugar confectioneries, jam and jellies, ice candy/ice creams, mouth fresheners and sugar toys. Samples were randomly chosen from street-vended retail outlets with a sampling design that enabled a homogeneous collection per market location, city and state. Erythrosine was found in 1.2% of the samples, at 11–556 mg/kg.

A limited household survey on the food consumption patterns of 518 survey respondents was used to assess dietary intakes focusing on the following age groups, in years: 4–6, 7–9, 10–12, 13–15. The intake of colours was assessed using the food frequency recall method and a food frequency questionnaire that asked for information on the respondent's name, age, sex and frequency of intake of specified coloured food commodities.

Three different scenarios of exposure were estimated: a scenario with mean levels of detected colours and mean consumption of foods; a scenario with 95th percentile levels of detected colours and mean consumption of foods; and a scenario with 95th percentile levels of detected colours and 95th percentile consumption of foods.

Because 53% of the samples contained erythrosine above maximum levels and because food consumption data were from an economic survey, the

resulting exposure assessment is an unrealistic overestimate. As a result, the Committee decided not to include this study in the final exposure assessment.

3.2.3 Assessments based on individual dietary records

(a) National studies

Australia and New Zealand

Following a request to extend the use of erythrosine from a single food that is consumed in low amounts (i.e. preserved cherries) to a food additive preparation that would be added to products such as icing and frostings used in other foods that are more widely consumed (e.g. cakes, biscuits, fancy breads), the Food Standards Australia New Zealand (FSANZ) estimated dietary exposure to erythrosine according to existing and proposed permissions for both Australia and New Zealand.

Dietary exposure was estimated using the FSANZ DIetary Modelling Of Nutritional Data (DIAMOND) computer program by combining food consumption with proposed levels of use of erythrosine in foods. As these proposed permissions were adopted, it reflects the current dietary exposure at maximum levels.

Ireland and Northern Ireland

The estimated exposure to erythrosine for the combined population of Ireland and Northern Ireland was based on the 2001 North/South Ireland Food Consumption Survey (NSIFCS) and usage in specific foods in the food supply established using the Irish National Food Ingredient Database (INFID) (Gilsenan & Gibney, 2002).

Dietary data were derived from the NSIFCS database which provides 7-day dietary records for a nationally representative sample of 1379 adults (aged 18–64 years) from the Republic of Ireland and Northern Ireland in the United Kingdom.

Two scenarios of exposure are proposed using both the maximum permitted level for each food product: one for consumers only and one for the total population. For both scenarios, exposure for acceptable reporter, under-reporters and total population are provided.

Republic of Korea

The dietary intakes of erythrosine in the Republic of Korea were estimated based on food consumption data for consumers and concentrations in processed foods (Ha et al., 2013).

Food consumption data were obtained from the Korea National Health and Nutrition Survey (Korea Ministry of Health and Welfare, 2009), which used a 24-hour recall method.

Food samples were selected based on stratified random sampling by taking into account their market share and a total of 704 food samples collected to determine the content of colours. Erythrosine was found in eight of 309 samples of candy, three of 40 samples of chocolate and one of 56 samples of chewing gum.

Two scenarios of exposure were estimated: one for average consumers using mean concentrations of all samples in a food group and one for high intake consumers using average of positive samples. The estimated mean and 95th percentile for dietary exposure based on these two scenarios are provided in Table 4.

USA

In a study by the USFDA (Doell et al., 2016), approximately 600 samples from 52 broad food categories were analysed for erythrosine (and other synthetic colours) using a USFDA-

validated high-performance liquid chromatography method with photodiode array detection (Petigara Harp et al., 2013). Samples were selected based on a previous survey of food labels.

Erythrosine was found in 16 of the 52 food categories and quantified in six food categories.

Two different sets of food consumption data from 2007–2010 were used for the dietary exposure estimates: a 2-day and a 10- to 14-day dietary intake survey for the population in the USA. Three population groups were used for the exposure estimate: 2 years and older, children 2–5 years old and teenage boys 13–18 years old.

Exposures were estimated based on "eaters-only" (individuals in the population who consumed one or more of the included foods over the survey period). Three different exposure scenarios were performed: a low-exposure scenario, where the lowest analytical value for erythrosine was assigned to the corresponding food code in the consumption survey; an average-exposure scenario, where the analytical results were averaged for a given food code; and a high-exposure scenario, where the highest analytical value was assigned to each food code. Dietary exposures were estimated at the mean and at the 90th percentile for each population for each food category.

Estimated dietary intakes from the average- and high-exposure scenarios based on the 10- to 14-day dietary intake survey are provided in Table 4. Only results from this consumption survey are provided since per cent eaters are greater for the exposures estimated using the 10- to 14-day food consumption data than for those estimated using the 2-day data for all three populations.

Decoration/chips for baking was the food category that contributed the most to exposure for the United States population aged 2 years and older.

While this food category was a major contributor to exposure for the other two populations, the food category contributing the most to exposure for children aged 2–5 years and teenage boys aged 13–18 years was ice cream cones. Frostings and icings, frozen dairy dessert/sherbet, soft candy/gummies, meal replacement drinks/bars, cookies, toaster pastries, ice pops and frozen fruit bars were also major contributing categories for all three populations.

The International Association of Color Manufacturers (IACM) recently published a new estimate of daily intake for erythrosine (and other colours) based on reported use levels and on information on the proportion of foods currently available in the marketplace that contain food colour (Bastaki et al., 2017). Typical and maximum use levels for erythrosine within 15 selected food/beverage general categories were provided by the IACM. The percentage of food products in the marketplace that contain erythrosine was calculated by the Mintel Group Ltd based on finished product labels between January 2011 and February 2015.

Food consumption data were obtained from the National Health and Nutrition Examination Survey (NHANES).

Estimates of the individual intakes were generated with the proprietary Foods and Residues Evaluation Program (FARE). Four different scenarios of exposure were calculated, all of them for consumers only: typical use levels with mean consumption and 95th percentile of consumption and maximum use levels with mean consumption and 95th percentile of consumption. Estimated dietary intakes from three of these four scenarios are provided in Table 4.

The use levels reported by the industry (Bastaki et al., 2017) are consistent with the concentrations measured analytically by the USFDA (Doell et al., 2016). However, exposure estimates from the IACM exposure assessment (Bastaki et al., 2017) are much lower than those from USFDA (Doell et al., 2016). The difference may come from the estimation of the intake; this was weighed by the frequency of colour presence in each category by Bastaki et al. (2017), whereas Doell et al. (2016) assumed 100% colour presence.

(b) **Europe**

In 2011, EFSA re-evaluated erythrosine as food additive, basing the exposure assessment on the data from the Gilsenan & Gibney (2004) study. EFSA concluded that at current levels of use, mean intake estimates for adults were 0.0031 mg/kg bw per day and 95th percentile intake estimates were 0.01 mg/kg bw per day.

(c) **International**

Food consumption data

The CIFOCOss provides summary statistics of national food consumption surveys (2 days or longer) from different countries.

Consumption data were available for 35 countries: Argentina, Australia, Bangladesh, Belgium, Brazil, Bulgaria, Burkina Faso, China, Cyprus, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Indonesia, the Islamic Republic of Iran, Ireland, Italy, Japan, Latvia, Mexico, the Netherlands, Philippines, Poland, Republic of Korea, Spain, Sweden, Thailand, Turkey, Uganda, United Kingdom, Uruguay and the USA.

For each country, several surveys are sometimes available and the overall database contains 57 surveys.

Consumption data for each study is described by population or age group according to 13 categories: general population, adults, adult women, women of childbearing age, adolescents, elderly, very elderly, infants, children, children (2–16 years), children (2–6 years), children (<6 years) and toddlers.

Link with maximum permissible levels

In order to link the food consumption data with the maximum permissible levels, matches were made between the Codex food code data provided in the GSFA (CODEX STAN 192-1995, 2017w) and CIFOCOss food codes (Table 5).

As no perfect match was possible, in order to have a maximum scenario, the following choices were made:

- As no data on 04.1.2.7 "Candied fruit" specifically were in the CIFOCOss, this category was matched against the larger food category, "Other processed fruit" (code 04.1.2), when no other precise descriptions were available; this means that other foods from 04.1.2 category (e.g. 04.1.2.4, 04.1.2.5, etc.) were excluded. This choice is extreme as not all processed fruits consumed are candied fruit.
- As no specific data on 04.2.2.7 "Fermented vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera) and seaweed products" were in the CIFOCOss, this category was matched against the larger food category "Other vegetables, nes, other processing" (code 04.2.2) when no other precise descriptions were available, including when the following precisions were provided: "Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)"; "Bulb vegetables, processed"; "Pulses processed, nes"; "Leafy vegetables, processed"; "Fruiting vegetables, other than cucurbits, processed"; "Legume vegetables, processed"; "Roots and tubers other processed, nes"; "Root vegetables, processed"; "Sweet corn preserved or frozen"; and "Roots and tubers processed". This choice is extreme as not all foods from the category "Other vegetables, nes, other processing" are fermented vegetables (including mushrooms

Table 5

Food groups and maximum levels of erythrosine used for the estimation of exposure based on the CIFOCOss

Codex code	Codex name	CIFOCOss code	CIFOCOss name	Maximum levels (mg/kg)
04.1.2.7	Candied fruit	04.1.2	Other processed fruits (excluding dried and juice), nes	200
04.2.2.7	Fermented vegetable and seaweed products	04.2.2	Other vegetables, nes, other processing	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2a	Other vegetables, nes, other processing	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2b	Other vegetables, nes, other processing	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2c	Other vegetables, nes, other processing	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2d	Other vegetables, nes, other processing	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2e	Other vegetables, nes, other processing	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2f	Other vegetables, nes, other processing	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2g	Other vegetables, nes, other processing	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2h	Processed nuts, including coated nuts and nut mixtures	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2i	Bulb vegetables, processed	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2k	Pulses processed, nes	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2l	Pulses processed, nes	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2m	Pulses processed, nes	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2n	Pulses processed, nes	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2o	Pulses processed, nes	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2p	Pulses processed, nes	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2q	Leafy vegetables, processed	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2r	Fruiting vegetables, other than cucurbits, processed	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2s	Legume vegetables, processed	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2t	Roots and tubers other processed, nes	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2u	Root vegetables, processed	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2v	Sweet corn preserved or frozen	30
04.2.2.7	Fermented vegetable and seaweed products	04.2.2	Roots and tubers processed	30
08.2	Processed meat, poultry and game products in whole pieces or cuts	08.0	Processed meat and meat products, nes	30
08.3	Processed comminuted meat, poultry and game products	08.0	Processed meat and meat products, nes	30
05.3	Chewing gum	no data	no data	50
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	no data	no data	100

CIFOCOss: Chronic Individual Food Consumption database – Summary statistics; ML: maximum levels; nes: not elsewhere specified

and fungi, roots and tubers, pulses and legumes, and aloe vera) and seaweed products.

- "Processed meat, poultry and game products in whole pieces or cuts" (08.2 in the Codex) and "Processed comminuted meat, poultry and game products" (08.3 in the Codex) were both matched with "Processed meat and meat products, nes" (08.0 in the CIFOCOss) as no other foods from the 08 family were found.
- As no data on "Chewing gum" and "Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces" were in the CIFOCOss, the Committee decided not to consider these categories. Although this may have led to an underestimated exposure, it was assumed that these items were only consumed at low levels because they were not included in any food consumption survey.

Dietary exposure

Because there are a number of foods with permissions for erythrosine (e.g. candied fruit, chewing gum, decorations, fermented vegetables and processed meats), the mean consumption amounts from all survey respondents were used to calculate exposure deterministically. Concentration levels were set up at the maximum levels. This is highly unrealistic as it means that all foods from the food categories described in Table 6 consumed by all individuals contain erythrosine at the maximum level.

3.3 Nondietary intake

As highlighted in the previous JECFA evaluation of erythrosine, this colour is also widely used in pharmaceutical products (>3000 products in Europe contain erythrosine, according to a report of the European Commission in 1998). In this previous evaluation, the maximum quantity of erythrosine that could be ingested with a single capsule, sugar-coated pill or 1 mL of a liquid preparation were estimated to be 0.013 mg/kg bw per day based on known use levels and assuming a 70 kg body weight (Annex 1, reference *143*). Assuming consumption of 6 pills per day by an adult, an exposure of approximately 0.1 mg/kg bw per day was estimated.

Rastogi et al. (2015) measured the quantity of erythrosine in some paediatric syrups and estimated the related exposure to erythrosine due to the consumption of syrup by children. The intake of colours was assessed based on the doses recommended on the syrup bottles. A single dose (10–15 mg/kg) of the syrup formulation taken orally 3 or 4 times per day was used to build the exposure scenario. Daily intake was estimated as ranging from 331 to 1102 µg/day for the minimum scenario and from 441 to 1469 µg/day for the maximum

Table 6

Dietary exposure per population and per cluster of countries estimated from CIFOCOss from the mean and the 95th percentile of total consumption

		Total consumption (mg/kg bw per day)			
		Mean		p95	
Population	**Cluster diet**	**Min.**	**Max.**	**Min.**	**Max.**
Adolescents	All diets	0	0.07	0	0.13
	7	0	0.03	0	0.07
	8	0	0.03	0	0.07
	10	0	0.07	0	0.12
	11	0	0.05	0	0.09
	15	0	0.07	0	0.13
Adult women	All diets	0	0	0	0.01
	9	0	0	0	0.02
	13	0	0	0	0.01
	16	0	0	0	0
Adults	All diets	0	0.08	0	0.18
	7	0	0.03	0	0.04
	8	0	0.02	0	0.04
	10	0	0.05	0	0.12
	11	0	0.04	0	0.09
	15	0	0.08	0	0.17
Women of childbearing age	All diets	0	0	0	0.03
	10	0	0	0	0
Children	All diets	0	0.04	0	0.08
	9	0	0.01	0	0.03
	10	0	0.04	0	0.08
Children <6 years old	All diets	0	0	0	0.01
	10	0	0	0	0.01
Elderly	All diets	0	0.06	0	0.14
	7	0	0.04	0	0.09
	8	0	0.03	0	0.05
	10	0	0.02	0	0.06
	11	0	0.05	0	0.12
	15	0	0.06	0	0.13
General population	All diets	0	0.06	0	0.11
	5	0	0	0	0
	9	0	0.01	0	0.03
	10	0	0.06	0	0.13
Infants	All diets	0	0.02	0	0.05
	10	0	0.02	0	0.04
Toddlers	All diets	0	0.21	0	0.42
	7	0	0.21	0	0.43

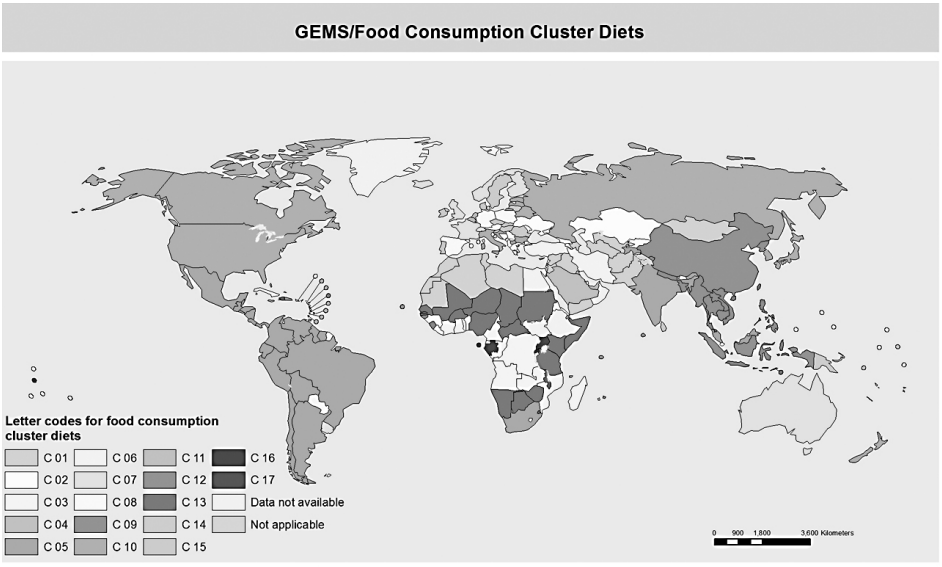
Table 6 (continued)

		Total consumption (mg/kg bw per day)			
		Mean		p95	
Population	Cluster diet	Min.	Max.	Min.	Max.
	8	0	0.08	0	0.17
	10	0	0.05	0	0.16
	11	0	0.15	0	0.33
Very elderly	All diets	0	0.07	0	0.14
	7	0	0.04	0	0.09
	8	0	0.03	0	0.06
	10	0	0.02	0	0.04
	11	0	0.06	0	0.13
	15	0	0.07	0	0.14

bw: body weight; CIFOCOss: Chronic Individual Food Consumption database – Summary statistics; max.: maximum; min.: minimum; p95: 95th percentile

Fig. 1

Description of the food consumption cluster diets used in Table 6.



scenario. This corresponds to 61.2% and 81.6%, respectively, of the ADI (0.1 mg/kg bw per day), when corrected for individual body weight.

Because exposure through pharmaceuticals is only occasional in a healthy population and because the focus of JECFA is chronic exposure in a healthy population, the nondietary exposure presented here was not taken into account in the final exposure assessment.

4. Comments

4.1 Biochemical aspects

Erythrosine is poorly absorbed and mainly excreted unchanged in rat faeces (Daniel, 1962; Webb, Fonda & Brouwer, 1962; Bowie, Wallace & Lindstrom, 1966).

Following oral administration of radiolabelled erythrosine, less than 1% of the dose was excreted in the urine. Blood and plasma radioactivity reached maximum levels by 1 hour, while liver and kidney levels peaked after 4–12 hours; no radioactivity was detectable in the brain or pituitary. No erythrosine was accumulated in the thyroid or other tissues (Obrist et al., 1986).

No additional metabolic or kinetic studies have become available since the previous evaluation by the Committee.

4.2 Toxicological studies

Erythrosine has low oral acute toxicity in mice, rats, gerbils and rabbits.

A short-term toxicity study with erythrosine showed pigment deposition in renal tubules in female rats at the 2% dietary level and in males at all but the lowest dietary levels, in a dose-related manner. The NOAEL was 0.25% in the diet (equal to 160 mg/kg bw per day) (Butterworth et al., 1976a). No compound-related effects were observed in other short-term toxicity studies in rats, gerbils, dogs and pigs. No additional short-term toxicity studies have become available since the previous evaluation by the Committee.

Several long-term oral toxicity studies showed no compound-related increases in tumour incidences in mice, rats and gerbils. In one long-term study of oral toxicity (Richter et al., 1981, later published as Borzelleca & Hallagan, 1987), mice showed decreased body weights at 3.0% erythrosine in the diet. The NOAEL was 1.0% erythrosine in the diet (equal to 1474 mg/kg bw per day). Body

weight decreases were also observed in a rat study (Hansen et al., 1973b). The NOAEL was 1% erythrosine in the diet (equivalent to 500 mg/kg bw per day).

Two long-term feeding studies with erythrosine found an increase in the incidence of thyroid follicular cell adenomas in male rats (Brewer et al., 1981, 1982, later published as Borzelleca et al., 1987). The previous Committee considered the occurrence of thyroid follicular tumours in rats secondary to hormonal effects based on results from studies on thyroid function and morphology. Another study indicated that erythrosine promoted the development of thyroid follicular tumours in partially thyroidectomized rats but not in non-thyroidectomized rats (Hiasa et al., 1988). The present Committee noted that the rat is not considered a suitable model for potential effects on the thyroid in humans (IPCS, 2007).

A large number of in vitro and in vivo genotoxicity tests have been conducted on erythrosine. The Committee confirmed that the overall weight of evidence indicates that erythrosine is not genotoxic.

Two short-term oral toxicity studies indicated that erythrosine may affect testicular function in mice. Abdel-Aziz et al. (1997) observed decreased epididymal sperm counts and sperm motility at dose levels of 68 and 136 mg/kg bw per day and a significant increase in the incidence of abnormal sperm head morphology at doses of 680 and 1360 mg/kg bw per day. Vivekanandhi et al. (2006) showed decreases in sperm motility and sperm counts and increases in sperm abnormalities, with a LOAEL of 64 mg/kg bw per day. No effects on sperm or on fertility were observed in rats in long-term toxicity studies and a multigeneration study conducted with higher doses administered via the diet (Aldridge et al., 1981, later published as Borzelleca & Hallagen, 1987; Brewer et al., 1981, 1982, later published as Borzelleca, Capen & Hallagen, 1987).

No developmental toxicity was reported in rats.

In a newly available reproductive toxicity study in mice, administration of erythrosine at dietary concentrations of 0, 0.005%, 0.015% or 0.045% (equivalent to 0, 7.5, 22.5 and 67.5 mg/kg bw per day, respectively) resulted in no adverse effects on reproductive parameters or functional developmental parameters in offspring. Significant changes in some measures of exploratory behaviour and motor activity were reported in high-dose dams and their offspring (Tanaka, 2001). Motor activity and brain serotonin levels in rat single-dose (Dalal & Poddar, 2009) and repeated-dose (Dalal & Poddar, 2010) studies were inconsistent. In view of the lack of consistency in the data, the poor oral absorption of erythrosine, the evidence that erythrosine does not penetrate the blood–brain barrier and the lack of evidence of behavioural toxicity in a rat study with dose levels equivalent to up to 1000 mg/kg bw per day (Vorhees et al., 1983), the Committee concluded that the findings in the Tanaka (2001) and Dalal & Poddar (2009, 2010) studies did not provide robust evidence of behavioural effects and could not be used in the risk assessment.

4.3 Observations in humans

Studies in human volunteers showed increased blood iodine levels but no changes in thyroid hormone levels in repeated-dose studies of up to 25 mg/day for 3 weeks or single-dose studies of up to 80 mg (Ingbar, Garber & Gluckin, 1983; Ingbar, Garber & Burrows, 1984). A study with 30 healthy male volunteers found that erythrosine slightly increased TSH responsiveness to TRH, but there were no effects on other thyroid hormone parameters at the highest dose level of 200 mg per person per day for 14 days (Gardner et al., 1987). No effect was observed at 60 mg per person per day. At its thirty-third meeting, the Committee concluded that the NOAEL was 60 mg per person per day (equivalent to 1 mg/kg bw per day), and the present Committee concurred.

4.4 Assessment of dietary exposure

The Committee previously evaluated dietary exposure to erythrosine at its fifty-third meeting, but significant changes in methodology for the exposure assessments makes comparing the previous and the current assessment difficult.

A comprehensive literature search was conducted. In addition to those provided by the sponsor, five references were found relevant for the assessment of the dietary exposure. The Committee reviewed published estimates of dietary exposure to erythrosine conducted in several countries. The Committee also conducted a conservative assessment using consumption data from 36 countries from the CIFOCOss database and Codex maximum use levels. A summary of the dietary exposure estimates is provided in Table 7.

Dietary exposure estimates based on individual consumption data and maximum use levels range from 0 to 0.4 mg/kg bw. Because erythrosine has been in use for many years, national estimates based on analytical data were also available to the Committee. National exposure estimates based on analytical data range from 0.00 to 0.05 mg/kg bw per day for adults and from 0.00 to 0.09 mg/kg bw per day for children, considering consumers only.

The Committee also noted that exposure through pharmaceuticals was previously estimated to occur at up to approximately 0.1 mg/kg bw per day in specific populations, generally over a short period of time. However, the Committee considered that such exposure should not be taken into account in the assessment of exposure to erythrosine as a food additive when looking at long-term exposure in a healthy population.

The Committee considered the approach based on analytical data to be more realistic for preparing long-term dietary exposure estimates than the approach based on maximum use levels. Because the scenarios available from the

Table 7
Summary of the range of estimates of dietary exposure for erythrosine

Source of estimates	Population	Range of estimated dietary exposures (mg/kg bw per day)	
		Means	High percentile (P90 and P95) [a]
Analytical levels [b]	Children	0.00–0.01	0.00–0.09
	Adolescents	0.00–0.02	0.00–0.04
	Adults	0.00–0.03	0.00–0.05
Maximum use levels [c]	Toddlers	0–0.2	0–0.4
	Children	0–0.1	0–0.1
	Adolescents	0–0.1	0–0.1
	Adults	0–0.1	0–0.2
	Elderly adults	0–0.1	0–0.1

bw: body weight; P90: 90th percentile; P95: 95th percentile
[a] The upper bound of the range is the maximum of the 90th and 95th percentiles.
[b] Studies from the Republic of Korea (Ha et al., 2013) and the USA (Doell et al., 2016; Bastaki et al., 2017) for consumers only.
[c] From published national studies (Gilsenan & Gibney, 2004; FSANZ, 2010) and the Committee's assessment based on consumption data from CIFOCOss for 36 countries.

national studies examined consumers only, the Committee concluded that the highest estimate of 0.09 mg/kg bw per day for children should be considered in the safety assessment of erythrosine.

5. Evaluation

The evidence newly available at this meeting indicates that there are no concerns with respect to genotoxicity and reproductive and developmental toxicity of erythrosine. The previously established ADI of 0–0.1 mg/kg bw is based on a NOAEL of 60 mg per person per day (equivalent to 1 mg/kg bw per day for a 60 kg person) identified in a human study, with a default uncertainty factor of 10. In this study (Gardner et al., 1987), minimal effects on thyroid function were observed at 200 mg per person per day (equivalent to 3.3 mg/kg bw per day). Effects in experimental animals were observed at doses at least 60-fold higher than the NOAEL in this human study; these effects supported the use of the human data as the basis for the ADI.

The Committee concluded that the new data that have become available since the previous evaluation do not give reason to revise the ADI and confirmed the previous ADI of 0–0.1 mg/kg bw.

The Committee noted that the dietary exposure estimate for erythrosine of 0.09 mg/kg bw per day (95th percentile for children) was close to the upper bound of the ADI. Given that this estimate of exposure is for children and it is

a high percentile for consumers only, such a level is unlikely to occur every day over a lifetime. Therefore, the Committee concluded that dietary exposures to erythrosine for all age groups do not present a safety concern.

At the present meeting, the existing specifications for erythrosine were revised. High-performance liquid chromatographic (HPLC) methods were added for determining subsidiary colouring matters and organic compounds other than colouring matters. The method of assay was changed to visible spectrophotometry, and spectrophotometric data were provided for the colour dissolved in water.

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Indigotine (consolidated monograph)

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1. Explanation

Indigotine (International Numbering System for Food Additives [INS] No. 132) consists of a mixture of disodium 3,3′-dioxo-[delta2,2′-biindoline]-5,5′-disulfonate (the principal component; Chemical Abstracts Service [CAS] No. 860-22-0), disodium 3,3′-dioxo-[delta2,2′-biindoline]-5,7′-disulfonate (an isomer) and subsidiary colouring matters. Indigotine is used in China, the European Union, Japan, the USA and other regions. It is used for colouring foods including blueberry bagels, breakfast cereals, cakes and cupcakes, candies including chocolate, chewing gum, dairy products, decorations for baking, frozen treats, sauces and seasonings.

The Committee evaluated the safety of indigotine at its thirteenth and eighteenth meeting (Annex 1, references *19* and *35*). At its eighteenth meeting, the Committee established an acceptable daily intake (ADI) of 0–5 mg/kg body weight (bw) based on a no-observed-adverse-effect level (NOAEL) of 500 mg/kg bw per day (1% in the diet) in a chronic dietary toxicity study in rats (Hansen et al., 1966), and prepared specifications for indigotine (Annex 1, reference *35*). At its seventy-third meeting, the Committee revised the specifications (Annex 1, references *202*).

Indigotine was on the agenda for re-evaluation of safety and revision of specifications at the request of the Forty-ninth Session of the Codex Committee on Contaminants in Food Additives (FAO/WHO, 2017).

A toxicological dossier was submitted summarizing the available toxicity data, together with relevant study reports and publications. Dietary exposure estimates references were also submitted.

A literature search was conducted following the Joint FAO/WHO Expert Committee on Food Additives (JECFA) guidance (WHO JECFA Secretariat, 2017). The keywords used in the searches included ("indigotine" OR "Indigo Carmine") AND ("toxicology" OR "toxicity") or ("indigotine" OR "Indigo Carmine"). The databases searched included PubMed (1975–2018 March 06; 1261 records); Web of Science (1950–2017 April 06; 11 records); MEDLINE (1975–2018 February 28; 1251 records); Scopus (Elsevier) (1950–2017 April 06; 6 records), AGRIS (1975–2018 February 28; 114 records); Cochrane Library (1975–2018 February 28; 1 record); Embase (1975–2018 February 28; 487 records); Directory of Open Access Journals(1975–2018 February 28; 88 records)/ GIM(1975–2018 February 28; 46 records); and CINAHL (1975–2018 February 28; 657 records). Eight of the records retrieved added to the toxicological data submitted to the Committee for this meeting.

A comprehensive literature search for dietary exposure estimate references was also conducted in the PubMed and Scopus databases using the search terms "indigotine" or "indigo carmine" and "exposure" or "intake"

or "consumption". These terms were also used in a general Internet search. In addition to the references submitted by the sponsor, five references relevant to the exposure assessment were retrieved.

1.1 Chemical and technical considerations

Indigotine is an indigoid dye. Indigotine consists of a mixture of disodium 3,3′-dioxo-[delta2,2′-biindoline]-5,5′-disulfonate, disodium 3,3′-dioxo-[delta2,2′-biindoline]-5,7′-disulfonate (an isomer) and subsidiary colouring matters. Sodium chloride and/or sodium sulfate are the principal uncoloured components. Indigotine is manufactured by heating indigo in the presence of sulfuric acid. The indigo (or indigo paste) is manufactured by the fusion of *N*-phenylglycine (prepared from aniline and formaldehyde) in a molten mixture of sodamide and sodium and potassium hydroxides under ammonia pressure. It is isolated and subjected to purification procedures prior to sulfonation. Impurities include unreacted starting materials (≤0.5%), subsidiary colouring matters (≤1%), unsulfonated primary aromatic amines (≤0.01% calculated as aniline) and lead (≤2 mg/kg).

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

The sodium salt of ^{35}S-labelled indigo 5,5-disulfonic acid, which was prepared by sulfonating indigo with ^{35}S-labelled sulfuric acid, was administered by stomach tube to male Osborne–Mendel rats (4/dose group) at doses of 0, 5, 25 or 50 mg/rat (approximately equivalent to 10.4–14.5, 52–72 and 104–145 mg/kg bw, respectively). Only 1.6–2.1% of the radioactivity appeared in urine while 61–80% was recovered in faeces within 3 days.

In order to determine whether the faecal elimination was due to the lack of absorption from the gastrointestinal tract or due to excretion into bile, indigotine was administered to a single anaesthetized rat by stomach tube. Of the 20 mg dose of indigotine administered, 0.004% was excreted via bile.

After intravenous injection of 1.4 mg/kg bw of ^{35}S-labelled indigotine to bile duct–cannulated rats with ligated urethras, 63% of the radioactivity appeared in urine and about 10% in bile within 6 hours. Almost all the biliary excretion occurred in the first 30 minutes.

When 5-sulphoanthranilic acid was orally administered to rats at doses of 1, 10 or 100 mg/rat, 19.8–27.7% of the dose was excreted in urine after 48 hours, demonstrating that this metabolite of indigotine is absorbed from the gastrointestinal tract to a greater extent than the parent compound (Lethco &Webb, 1966).

The Committee concluded that indigotine is poorly absorbed from the gastrointestinal tract.

Microchemical methods have shown that filterable indigotine in the blood plasma of rabbits is excreted through the glomeruli. The filterable fraction amounted to about 15%, while 75% of the colour was excreted via kidney tubules. The lumina of the tubules and Bowman's capsules were injected with solutions of indigotine. The lowest concentration to leave detectable traces of the colour in kidneys was 10 mg%. When an increased dose of colour was injected and the interval between the injection and the excision of the kidney shortened, the colour was detected in the intracapsular.spaces. The tubular excretion of indigotine resembled, quantitatively, excretion of phenol red (Kempton, Bott & Richards, 1937).

2.1.2 Biotransformation

(a) In vitro

In vitro incubation of ^{35}S-labelled indigotine with intestinal contents of rats for 48 hours resulted in loss of colour and formation of isatin-5-sulphonic acid and 5-sulphoanthranilic acid. The study authors proposed that these two metabolites are formed by the action of intestinal bacteria on indigotine and that they are absorbed and eliminated in urine to a small extent (Lethco &Webb, 1966).

In an in vitro study, indigotine was metabolized to four unidentified metabolites in the presence of caecal microflora of rats. The time period required to degrade 50% of the original colour concentration was 54 minutes (Singh, Das & Khanna, 1993).

(b) In vivo

^{35}S-labelled indigotine was administered by stomach tube to male Osborne–Mendel rats (4/dose group) at doses of 0, 5, 25 or 50 mg/rat (approximately equivalent to 10.4–14.5, 52–72 and 104–145 mg/kg bw, respectively). The radioactivity in urine after a single oral dose of 50 mg/rat was identified as unchanged indigotine, isatin-5-sulphonic acid and 5-sulphoanthranilic acid (0.53%, 0.63% and 0.28% of the dose, respectively). The metabolites isatin-5-sulphonic acid and 5-sulphoanthranilic acid were identified in bile from 2 hours after dosing and in urine from 1–2 hours after dosing. Following intravenous administration of 1.4

mg/kg bw, both metabolites appeared in urine after 2 hours and in bile after 4 hours. After 6 hours, 71% of the radioactivity in urine was the parent compound, 19% was isatin-5-sulphonic acid and 10% was 5-sulphoanthranilic acid, which amounted to 45%, 12% and 6% of the administered dose, respectively (Lethco & Webb, 1966).

2.1.3 Effects on enzymes and other biochemical parameters

(a) In vitro

In an in vitro study, bovine liver microsomes were used as source of cytochrome P450 (CYP) 2A6, UGT1A6 and UGT2B7. Indigotine significantly inhibited CYP2A6 monooxygenase activity in a noncompetitive manner, with a median inhibitory concentration (IC_{50}) of 0.05 mmol/L. UGT1A6 and UGT2B7 activity was not affected by indigotine (Kuno & Mizutani, 2005; Mizutani, 2009).

In a reporter gene assay using recombinant yeast, the aryl hydrocarbon receptor–mediated induction of microsomal drug-metabolizing enzyme activities of indirubin, indigo and indigotine were compared. Indigotine, a sulfate derivative of indigo, did not have an inducing effect on drug-metabolizing enzymes and did not induce CYP1A1/2 (Sugihara et al., 2004).

2.2 Toxicological studies

2.2.1 Acute toxicity

In acute oral toxicity studies, the oral median lethal dose (LD_{50}) values were 2000 mg/kg bw or higher (Table 1). In addition, in order to set the appropriate dose for an in vivo comet assay, indigotine was administered orally at up to the limit dose of 2000 mg/kg in up to five male ddY mice. The approximate LD_{50} was reported to be 2000 mg/kg bw(Sasaki et al., 2002).

2.2.2 Short-term studies of toxicity

(a) Mice

A 42-day toxicity study with adult male Swiss albino (B-6) mice (5/dose group; 4–5 weeks old) investigated the effect of indigotine on the reproductive organs. Indigotine was administered in the standard diet at doses of 0, 17 or 39 mg/kg bw per day. The test material (referred to as "E number 132; FD&C Blue No. 2; CI 73015"), which was bought at a local market, was manufactured and packaged by a company in Jodhpur (Rajasthan, India). At the end of the experiment, body weight, organ weights, results of histopathological examinations of testes, sperm density and sperm motility were recorded.

Table 1
Acute toxicity of indigotine

Animal	Route	LD_{50} (mg/kg bw)	Reference
Mice	Oral	2 500	USFDA (1969)
Mice	Subcutaneous	405	USFDA (1969)
Mice	Oral	2 000	Sasaki et al. (2002)
Rats	Oral	2 000	Lu & Lavalleé (1964)
Rats	Intravenous	93	USFDA (1969)

bw: body weight; LD_{50}: median lethal dose; USFDA: United States Food and Drug Administration

A statistically significant increase in body weight and a statistically significant decrease in the average weight of testes were reported at both dose levels. Compared with the control, testicular sperm density was statistically significantly reduced at the high dose and sperm motility was statistically significantly decreased at both doses. Histopathological findings in testes included a statistically significant reduction in the average diameter of the seminiferous tubules at both dose levels; severe histopathological changes in the testis architecture, described as thickening of the tubular basement membrane; arrest of spermatogenesis at the spermatid stage; and necrotic sperm debris within the tubular lumen. In the high-dose group, dissolution of the tubular basement membrane and exfoliation of cells in the lumen were also observed (Dixit & Goyal, 2013).

Because the composition and the purity of the test substance was unknown and it may have been a non-food grade material, the Committee considered this study not suitable for risk assessment purposes.

(b) Rats

Male albino rats (strain unknown; 10/group) were fed a diet containing a mixture of Brown HT (C.I.20285) and indigotine (C.I.73015) at a dose of 800 mg/kg bw per day (relative content of the two colours not stated) for a total of 30 or 60 days or for 60 days followed by a 30-day withdrawal period. A number of parameters were evaluated: body weight, haematology, liver and kidney functions, blood glucose, serum and liver lipids, liver nucleic acids (DNA and RNA), thyroid hormones (triiodothyronine [T_3] and thyroxine [T_4]), growth hormone) and histopathology of liver, kidneys and stomach. Changes observed included a significant decrease in body weights, reversible increases in liver enzymes and total protein, a reversible decrease in total serum cholesterol and high density lipoprotein (HDL)-cholesterol and a reversible decrease in neutrophil count. Blood vessel congestion and haemorrhage in both liver and renal sections were

also reported. No histopathological findings in the stomach were seen (Aboel-Zahab et al., 1997).

The Committee noted that the exact concentration of the individual components of mixture was not specified. Owing to the unclear dose of indigotine and its administration as a mixture, the Committee considered this study not suitable for risk assessment purposes.

Isatin-5-sulfonic acid, one of the metabolites of indigotine, was fed to groups of 3-week-old rats (10/sex per dose group) at dietary concentrations of 0, 0.25%, 0.5%, 1% or 2% for 13 weeks. Gross and histopathological examinations were conducted after termination of treatment.

The NOAEL was 2% (equivalent to 2000 mg/kg bw), the highest dose tested (Annex 1, reference *39*; USFDA,1969).

(c) Pigs

Large White pigs (3/sex per dose group; 10 weeks old) were given indigotine (purity >85%; conforming to the specification of the British Standards Institution, 1967, for indigo carmine for use in foodstuffs) at dose levels of 0, 150, 450 or 1350 mg/kg bw per day for 90 days.

Haemoglobin levels and red blood cell counts were slightly reduced after 45 and 90 days in males at the highest dose tested. There were no treatment-related effects on growth, urinalysis and serum analysis parameters or organ weights. Histological examination revealed liver abscesses in one low-dose male, but this was considered not treatment related. No other macroscopic or microscopic findings were seen (Gaunt et al., 1969).

As the reduction in haemoglobin levels and red blood cell counts were small and seen only in males, the Committee identified the NOAEL as 1350 mg/kg bw per day at the eighteenth meeting (Annex 1, reference *35*).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

A new long-term toxicity study in mice has become available since the previous evaluation of indigotine.

In a long-term combined toxicity and carcinogenicity study, mice (Charles Rivers CD-1, COBS (ICR-derived); 60/sex per dose group, two control groups; 42 days old) were fed indigotine (FD&C Blue No. 2, certified by USFDA; 93% purity, 7% volatile matter) at dietary concentrations of 0, 0.5%, 1.5% or 5.0% for 22 months for males and 23 months for females (equal to 0, 825, 2477 and 8259 mg/kg bw per day for males and 0, 945, 2836 and 9456 mg/kg bw per day for females, respectively). Animals were observed for mortality, morbidity and gross signs

of toxicity twice daily. Detailed physical examinations for signs of toxicity and palpation for masses were conducted weekly. Body weight and feed consumption were determined weekly through the first 14 weeks, biweekly at weeks 16–26 and monthly thereafter. Subgroups (10/sex per dose group) were randomly selected for haematological tests, including haemoglobin concentration, erythrocyte and total and differential leukocyte counts and erythrocyte morphology, at 3, 6, 12 and 18 months. All animals from the two control groups and the highest dose group (5%) as well as any other animals that were found to have gross lesions or masses underwent complete histopathological examinations. Brain, gonad, kidney, liver, spleen and thyroid weights and relative weights were recorded.

There was a moderate incidence of blue-green discolouration of the gastrointestinal tract in all treated animals, with occasional concomitant discolouration of liver, gall-bladder and urine. This discolouration was attributed to the test substance. The few gross abnormalities noted were random, with no relation to treatment or dose. The most frequent observations were cystic and/or polypoid uterine enlargement and ovarian cysts. These changes were incidental and are associated with ageing. Focal discolouration (red, tan, grey, brown) occurred at a moderate incidence in heart, lungs, kidneys, liver, uterus and spleen. Irregular kidney and liver shapes and enlargement of spleen and mesenteric nodes were also unrelated to treatment. Several nonneoplastic changes, including amyloidosis of kidneys, ileum, duodenum, adrenal glands, ovaries, spleen and liver were of the type and incidence common in ageing animals. Neoplastic lesions were found in lungs, liver, lymph nodes and uteri of mice from all treatment groups. Neither the incidence of neoplasms nor their primary locations and histopathological characteristics differed significantly between control and treated animals. There were no statistically significant differences for time-to-tumour analyses between the control and treated animals (Borzelleca & Hogan, 1985; EFSA, 2014).

The Committee concluded that indigotine is not carcinogenic. The NOAEL was 5% in the diet (equal to 8259 mg/kg bw per day), the highest dose tested.

Mice (Charles River CD1; 30/sex per dose group; 60/sex in the control group) were fed diets containing indigotine at 0.2%, 0.4%, 0.8% or 1.6% (conforming to the specifications of the British Standards Institution, purity ≥85%) for 80 weeks.

Treatment had no effect on the death rate, body-weight gain, organ weights or histopathology including the incidence of tumours. The only effect seen was slight anaemia in mice at 0.8% or 1.6% indigotine showed slight anaemia (Hooson et al., 1975).

The eighteenth Committee concluded that indigotine at dietary levels of up to 1.6% does not exert any carcinogenic effect on mice and that the "no-

untoward-effect level" was 0.4% (equivalent to an intake of approximately 550 mg/kg bw per day) (Annex 1, references *35* and *36*).

Groups of young adult mice (25/sex per group) received weekly subcutaneous injections of 2.5 mg of indigotine (obtained commercially and certified as FD&C Blue No. 2) as a 1% aqueous solution for 104 weeks. The control group (25/sex) were injected with 0.25 mL of physiological saline.

Many mice died from acute convulsions immediately after injection of the test dye, but otherwise no deleterious effects attributable to the subcutaneous injections were noted. Tumours were randomly distributed among test and control groups (Hansen et al., 1966).

(b) **Rats**

A new long-term combined toxicity and carcinogenicity study including an in utero treatment phase has become available since the previous evaluation of indigotine.

Charles River CD rats (60/sex per group, two concurrent control groups) were fed indigotine (FD&C Blue No. 2; certified by USFDA; purity 93%, 7% volatile matter) at 0, 0.5%, 1.0% and 2.0% for approximately 2 months prior to mating. After random selection of animals from the first filial generation (F_1), the long-term phase of the study was initialized, with indigotine administered to groups of rats (70/sex per group) at the same dietary levels of 0.5%, 1.0% or 2.0% (equal to 304, 632 and 1282 mg/kg bw per day for males and 363, 775 and 1592 mg/kg bw per day for females, respectively). Two control groups received a basal diet. The exposure duration was 29 months in males and 30 months in females.

No consistent compound-related adverse effects were seen. Staining of the hair, hair loss and alterations such as focal and diffuse retinopathy, conjunctivitis, uveitis and cataracts occurred in some males and females in all groups. There were random statistically significant differences between treated and control rats in body weight, feed consumption and clinical chemistry test findings. Feed consumption appeared to increase in a dose-related manner, probably due to the influence of the nonnutritive character of the high concentrations of the colour. Age-related chronic nephropathy occurred in all groups as did testicular atrophy of the seminiferous tubules, accompanied by oligospermia or aspermia, in male rats. The incidences of pituitary gland neoplasms (female and male), transitional-cell neoplasms of the urinary bladder (male) and malignant mammary gland neoplasm (carcinoma and adenocarcinoma, female) were increased in the high-dose group but not to an extent that differed significantly between the treated and control groups. A statistically significant ($P < 0.05$) increase in the incidence of malignant mammary gland tumours was noted in high-dose males. The incidence

of carcinoma in the highest dose group was 5.9% (3/51 animals in total), while none were found in the combined control groups (0/114 animals; the incidence of benign tumours was 3.5% [4/114 animals]). The incidence in gliomas in high-dose males (9.9%,7/71) was also significantly increased compared with controls (2.9%) or low-dose (2.9%) or mid-dose (2.9%) animals. There was no increased incidence of gliomas in female rats. Based on the lack of a clear dose–response relationship, no concurrent decrease in survival time and that historical control data suggesting a total incidence of gliomas of 4.4% in male rats, the increased incidence of gliomas in males was considered not biologically relevant.

Under the conditions of the study, indigotine showed no evidence of any toxicity including carcinogenicity (Borzelleca, Hogan & Koestner, 1985; EFSA, 2014).

The Committee concluded that the NOAEL was 1.0% in the diet (equal to 632 mg/kg bw per day) based on the statistically significant increase in incidence of gliomas and mammary gland tumours in male rats at the highest dose level of 2.0% in the diet (equal to 1282 mg/kg bw per day).

In a two-generation feeding study with continuous indigotine exposure, parental Wistar rats (15 treated males, 26 treated females, 20/sex in the control group; 50 g bw at the start of the experiment) were fed a diet containing 1% of indigotine (equivalent to 500 mg/kg bw per day) for 2 years. To investigate spontaneous tumour development in this strain of rats, an additional control group (100/sex) was fed the control diet. After 6 months, some animals were mated (number of animals mated per group was not specified). During lactation, dams were fed a diet without the test substance. F_1 rats (20/sex per treated group; 5/sex for control group) were weaned 2–3 weeks after birth at a body weight of 25–35 g, and fed a diet containing 0 or 1% indigotine thereafter for 2 years. All animals were observed over their lifespan or until euthanized if moribund. After macroscopic inspection, neoplasms and conspicuous alterations of tissues were examined microscopically. As no differences in behaviour, growth or survival time were observed, the results for the treated parental (P) and F_1 animals and for the control animals of the two generations were combined.

No adverse effects on fertility, body weight, organ changes or average survival time were observed. The incidence of malignant and benign tumours was no higher in treated animals than in control animals; in addition, the treated animals survived longer (20.6 versus 18.1 months). Rats fed indigotine at a concentration of 1% in the diet did not show any treatment-related pathological alterations or tumour development (Oettel et al., 1965).

Indigotine (obtained commercially and certified as FD&C Blue No. 2) was fed to groups of Osborne–Mendel weanling rats (12/sex per group) at

dose levels of 0, 0.5%, 1.0%, 2.0% or 5.0% for 2 years. The growth of males was significantly inhibited at 2.0% and 5.0%. Slight but statistically nonsignificant growth depression was observed in the females at the same dosages. There were no treatment-related effects on mortality, organ weights or haematological, gross or microscopic pathological parameters.

The NOAEL was 1% in the diet (equivalent to 500 mg/kg bw per day) based on inhibition of growth in males at 2.0% (Hansen et al., 1966).

Sprague Dawley rats (10/sex per dose group) were given first 1 mL of a 2% solution subcutaneously (10 injections) and later only 0.5 mL of a 0.5% solution of indigotine. The reduction in the concentration was due to local irritations at the injection site. The animals were injected twice weekly and a total 55 injections were administered over a period of 7 months. Control groups received injections of 50% glucose or 0.9% sodium chloride. All animals were observed for the duration of their lifespan.

No changes in organs were detectable clinically or histopathologically. No local tumours and only one auxiliary tumour were found in the test animals (Oettel et al., 1965).

Osborne–Mendel rats (40/sex; 4 weeks old) received weekly injections (in the axillary region) of 20 mg of indigotine (obtained commercially and certified as FD&C Blue No. 2) at a 2% aqueous solution for 2 years. Controls (50/sex) received an equivalent volume of saline solution.

Survival of test animals did not differ from that of controls. Of the 80 injected rats, 14 developed a fibrosarcoma at the site of injection and one saline-injected rat developed a fibroma at the injection site. No other effects were observed (Hansen et al., 1966).

(c) **Dogs**

Beagle dogs (2/sex per treated group; 1/sex in the control group; ~6 months old) were given 1% or 2% of indigotine (obtained commercially and certified as FD&C Blue No. 2) in the diet for 2 years. Two dogs on the high concentration diet died at 19 weeks; they were replaced and another dog was added to the control group. Two more dogs on the high concentration diet died, at 21 and 40 weeks; one dog on the low concentration diet died at 36 weeks and one control dog died at 34 weeks. Deaths were due to virus infections.

No clinical signs, gross lesions or microscopic pathological findings were attributable to the test substance (Hansen et al., 1966).

2.2.4 Genotoxicity

New in vitro and in vivo genotoxicity studies with indigotine have become available since the previous evaluation. The results of these and earlier genotoxicity tests with indigotine are summarized in Table 2.

In a series of bacterial mutagenicity assays with *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 with and without metabolic activation, no mutagenicity was reported (Auletta, Kuzava & Parmar, 1977; Brown, Roehm & Brown, 1978; Bonin & Baker, 1980; Haveland-Smith & Combes, 1980; Ishidate et al., 1984; Cameron et al., 1987). Indigotine was tested for mutagenicity at a concentration of 5 g/L in cultures of *Escherichia coli*. No mutagenic effect was found (Lück & Rickerl, 1960). No genotoxicity was reported with respect of DNA damage in a rec and pol assay of *E. coli* strains with indigotine (Haveland-Smith & Combes, 1980), in a rat hepatocyte primary cultures for DNA repair assay (Kornbrust & Barfknecht, 1985) and in two comet assays in human colon adenocarcinoma cell line and MCF-7 breast cancer cells respectively (Davies et al., 2006; Masannat et al., 2009). Positive findings were reported in two in vitro assays in *S. typhimurium* strains and Chinese hamster fibroblast cell line (Hesbert et al., 1984; Ishidate et al., 1984) and undetermined (equivocal) results in one gene mutation assay in mouse lymphoma L5178Y $TK^{+/-}$ cells, respectively (Cameron et al., 1987). However, the Committee noted that these studies had some shortcomings (effects only reported at an excessive concentrations without information on cytotoxicity and only for one sampling time; absence of a dose–response relationship in mutant frequency; gaps in the evaluation; results requiring repeated testing at lower concentrations).

No genotoxic or mutagenic activity of indigotine was observed in the majority of in vivo assays (Hesbert et al., 1984; Durnev et al., 1995; Sasaki et al., 2002; Sarikaya, Selvi & Erkoç, 2012; Whitwell, 2013). Chromosomal aberrations were reported in an in vivo chromosomal aberration study (Giri et al., 1986) and a sister chromatid exchange assay (Giri & Mukherjee, 1990). However, the Committee noted technical shortcomings that call into question the reliability of the in vivo chromosomal aberration study (Giri et al., 1986), including that it was not performed according to the current OECD guideline; only 60 metaphases were analysed for each animal; the presence of abnormal vertical control cells with more than 10 aberrations per cell, which may have been due to inappropriate preparation of slides; the limited absorption of the test compound in vivo; and the low administered dose. The Committee also noted that the increased frequency of sister chromatid exchanges (Giri & Mukherjee, 1990) was within the normal range for negative control animals in this assay, and the outcome was considered not relevant for the safety assessment.

Table 2
Summary of results of genotoxicity tests with indigotine

End-point	Test system	Route	Concentration	Results	Reference
In vitro					
Reverse mutation	*Escherichia coli* laboratory strain and ATCC 12408	–	0.5 g/100 mL (−S9)	Negative [a]	Lück & Rickerl (1960)
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	–	1–10 000 µg/plate (±S9)	Negative [b]	Auletta, Kuzava & Parmar (1977)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	–	50–1 000 µg/plate (±S9)	Negative [c]	Brown, Roehm & Brown (1978)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	–	320–10 000 µg/plate (±S9)	Negative [d]	Bonin & Baker (1980)
Reverse mutation	*S. typhimurium* TA1538 *E. coli* WP2*trpuvr*A	–	1 mg/mL (±S9)	Negative [e]	Haveland-Smith & Combes (1980)
Reverse mutation	*S. typhimurium* TA92, TA94, TA98, TA100, TA1535, TA1537	–	5 mg/plate (+S9)	Negative	Ishidate et al. (1984)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1538	–	10, 100, 250, 500, 1 000, 2 500 µg/plate (±S9)	Mutagenicity towards TA98 and TA1538, when concentration ≥250 µg/plate, +S9 [f]	Hesbert et al. (1984)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	–	333–10 000 µg/plate (±S9)	Negative [g]	Cameron et al. (1987)
Reverse mutation	*S. typhimurium* TA98, TA100	–	33–3 333 µg/plate (+S9)	Negative [h]	Cameron et al. (1987)
Gene mutation	Mouse lymphoma L5178Y $TK^{+/-}$ cells	–	971–2 000 µg/mL (−S9) 92–556 µg/mL (+S9)	Indeterminate [i]	Cameron et al. (1987)
Chromosomal aberration	Chinese hamster fibroblast cell line	–	12 mg/mL, 24-hour treatment	Positive [j]	Ishidate et al. (1984)
DNA repair	*E. coli* WP2*trpuvr*A, WP67*trpuvr*A*pol*A, WP100*trpuvr*ArecA	–	1 mg/mL (±S9)	Negative [k]	Haveland-Smith & Combes (1980)
DNA repair	Rat hepatocyte primary cultures / DNA repair (HPC/DR)	–	1×10^{-6} to 1×10^{-3} mol/L	Negative [l]	Kornbrust & Barfknecht (1985)
DNA induction (Comet assay)	Human colon adenocarcinoma cell line (Caco-2 cell line)	–	0.1%	Negative	Davies et al. (2006)
DNA induction (Comet assay)	MCF-7 breast cancer cells; HB-2 cell line	–	0.001–0.4%	Negative [m]	Masannat et al. (2009)
In vivo					
Chromosomal aberration	Swiss albino mice; M; bone marrow	Oral (once a day by gavage for 30 days)	2 mg/kg bw	Positive [n]	Giri et al. (1986)

Table 2 (continued)

End-point	Test system	Route	Concentration	Results	Reference
Chromosomal aberration	C57BL/6 mice bone marrow; M	Oral (once a day for 5 days)	1.4 or 14 mg/kg bw (5/ group)	Negative	Durnev et al. (1995)
Micronucleus induction	Swiss CD1 mice; M	Oral	0.1, 0.5, 1, 2 g/kg bw	Negative [o]	Hesbert et al. (1984)
Micronucleus induction	Outbred Han Wistar Crl:WI(Han) rats; M; bone marrow	Oral	Range-finding study: 300, 2 000, 3 000 mg/kg bw	No obvious adverse effects were seen	Whitwell (2013)
			Formal study: 300, 2 000, 3 000 mg/kg bw	Negative [p]	
SCE	Swiss albino mice; M; bone marrow cells	Intraperitoneal	5, 10, 25, 50 100 mg/kg bw	Increased frequency of SCEs at 25, 50 and 100 mg/kg bw	Giri & Mukherjee (1990)
DNA induction (Comet assay)	ddY mice; M	Oral	2 000 mg/kg bw	Negative [r]	Sasaki et al. (2002)
Somatic mutation and recombination	*Drosophila melanogaster*	Feed	0.25, 0.5, 1 mg/mL	Negative [s]	Sarıkaya, Selvi & Erkoç (2012)

bw: body weight; M: male; S9: 9000 × *g* supernatant fraction from rat liver homogenate; SCE: sister chromatid exchange

[a] Negative control: The same medium as the test group.

[b] Positive control (−S9): nitroquinoline-*N*-oxide (4-NQO) and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG); positive control (+S9): benzo[a]pyrene (BaP).

[c] Negative control: sodium dithionite; positive controls (−S9): ethylmethane sulfonate, methylmethane sulfonate, 9-aminoacridine, nitroquinoline-*N*-oxide (4-NQO), aminoacridine; positive control (+S9): 2-aminoacridine.

[d] Negative control: medium; positive controls: methylmethanesulfonate, 4-nitroquinoline-*N*-oxide (4-NQO), 9-aminoacridine, 2-acetylaminofluorene and aflatoxin B1.

[e] Positive control (−S9): furacin (5-nitro-2-furfurylidene semicarbazone); RMS (Rimmel Mahogany Silk); positive control (+S9): tris-(2,3-dibromopropyl) phosphate; 2-acetylaminofluorene.

[f] Negative control: dimethylsulfoxide; positive control (−S9): 4-nitro-1,2-paraphenylene diamine; (+S9): 2-aminoanthracene.

[g] Plate incorporation assay. Purity of the test substance >85%. Negative control: dimethylsulfoxide, acetone; positive control (−S9): sodium azide, 9-aminoacridine, 2-nitrofluorene; positive control (+S9):2-aminoanthracene.

[h] The flavin-mononucleotide (FMN) preincubation modification assay. Purity of the test substance >85%; negative control: solvent; positive control (+S9):trypan blue.

[i] The result of L5178Y TK$^{+/-}$ mouse lymphoma assay was judged as undetermined as a significantly elevated, but non-dose-related increase in mutant frequency was found when the substance was tested in the presence of S9 and no response in the absence of S9. In this study, negative control: solvent; positive control (−S9): ethylmethanesulfonate; positive control(+S9): 3-methylcholanthrene.

[j] Result judgement: negative: incidence <4.9%; equivocal: incidence 5.0–9.9%; positive: incidence >10.0%. In this study, 10% polyploidy and 3% of structural aberration was observed at 24 hours.

[k] Positive control(−S9): ethidiumbromide, furacin (5-nitro-2-furaldehyde semicarbazone), mitomycin C.

[l] Positive control: *o*-aminoazotoluene; negative control: carmoisine.

[m] Negative control: PBS; Positive control: 100 µmol/L of H2O2.

[n] Abnormal judgement: chromatid and chromosomal breaks, chromatid and chromosomal exchanges and cells > 10 aberrations with groups of 10 rats.

[o] The positive control was cyclophosphamide, via intraperitoneal administration (0.025 g/kg bw).

[p] Test substance (purity >91%) was dissolved in 1% (weight/volume) methylcellulose. The positive control was cyclophosphamide (CPA).

[q] The positive control was mitomycin C (2.5 mg/kg bw); the negative control vehicle was distilled water.

[r] Groups of 4 mice were treated once orally. Slides were prepared 3 or 24 h after treatment.

[s] Wings were scored for the presence of spots of mutant cells that can result from either somatic mutation or somatic recombination. The frequencies of spots per wing in the treated series were compared with those of the distilled water control groups.

As all the studies with positive test outcomes had several limitations in terms of experimental design or reporting, the Committee concluded that there are no safety concerns with respect to genotoxicity of indigotine.

2.2.5 Reproductive and developmental toxicity

New reproductive toxicity studies have become available since the last evaluation.

(a) Rats

In a three-generation reproduction study, Charles River CD rats (10 males and 20 females/dose group) were fed diets containing indigotine (purity ~93%) at dose levels of 0, 2.5, 25, 75 or 250 mg/kg bw per day.

Blue fur and faeces were reported in the two highest dose groups. No differences in gestation, viability and lactation indices were reported between exposed animals and controls. Statistically significant but not dose-dependent decreases in fertility were reported for F_2 females at 2.5 and 25 mg/kg bw per day. Fertility indices were also reduced in F_2 males. No statistically significant changes in the fertility index were observed in F_3 animals. As the effects on fertility indices in F_2 animals were not dose related and not identified in F_1 and F_3 animals, they were considered not compound related. Examination of the ovaries and uteri of F_1 dams killed on gestation day 19 revealed no gross anatomical abnormalities. No unusual changes were observed in stillborn pups or in pups that died during the study. No compound-related gross or microscopic pathological lesions were noted in any of the F_1 or F_3 rats that were killed and necropsied. No compound-related changes in organ weights were observed in the F_1 rats (Borzelleca, Goldenthal & Wazeter, 1986).

Ina 2-generation teratogenicity study at the same laboratory, pregnant Charles River CD rats (20/test group) were administered indigotine (purity ~91%) on gestation days 6–15 at dose levels of 0, 25,75 and 250 mg/kg bw per day. All animals were observed twice daily for signs of toxicity. Dams and offspring were examined for numbers of live and dead term fetuses, implantation sites and early and late resorptions.

No significant compound-related adverse effects were noted in maternal appearance, behaviour, body weight or mortality or fetal body weight, viability or abnormalities (cited as "Anonymous 1972a" in Annex 1, reference *36*, subsequently published as Borzelleca et al., 1987).

A 2-generation toxicity study with continuous exposure (except during lactation) to indigotine at 0 or 1% in the diet was conducted in Wistar rats. Both P animals (15 males and 26 females) and F_1 animals (20/sex per group; 5/sex in the control group) were given diets containing 0 or 1% indigotine (equivalent to 0 or 500 mg/kg bw per day) for 2 years and observed over their lifespan (or until euthanized if moribund).

No adverse effects were observed on fertility, growth or survival. Reproductive and developmental parameters were poorly reported (Oettel et al., 1965; EFSA, 2014).

(b) Rabbits

The potential teratogenicity of indigotine was evaluated in a two-generation study on rabbits. Male and female Dutch Belted rabbits (10 pregnant rabbits/group) were administered indigotine (purity ~91%) on gestation days 6–18 by gavage at doses of 0, 25, 75 or 250 mg/kg bw per day. Rabbits were observed twice daily during gestation for signs of toxicity and killed 1 day before term; appropriate maternal and fetal parameters were evaluated.

There were no consistent, significant compound-related adverse effects on any of the maternal and fetal parameters evaluated.

Under the conditions of this study, indigotine did not exert any teratogenicity or other developmental toxicity in rabbits (cited as "Anonymous 1972b" in Annex 1, reference *36*, subsequently published as Borzelleca et al., 1987).

2.2.6 Special studies

Five rats were given subcutaneous injections of an aqueous solution 250 mg/kg bw of indigotine) twice daily for 3 days and killed on day 4. No estrogenic activity was detected (uterine weight remained normal), but the animals lost body weight (Graham & Allmark, 1959).

In experiments with guinea-pigs it was found that indigotine had no sensitization activity (Bär & Griepentrog, 1960).

2.3 Observations in humans

Children (21 boys, 22 girls, 3–14 years old) who presented with angiooedema and/or urticaria and who responded to an additive-free diet were challenged with different food additives in a double-blind study.

Of the 43 children tested, 24 reacted to one or more additives. Out of 19 children orally challenged with 0.1 mg of indigotine, three reacted; two out of the three children reacted also to other additives. The authors noted that atopy was less common in these patients than in the general population and that food additive intolerance resulting in urticaria was not associated with atopy, which suggests that it is not an immunoglobulin E (Ig E)–mediated phenomenon (Supramaniam & Warner, 1986).

3. Dietary exposure

Indigotine (E 132) is a dye with an indigoid base structure that was previously assessed for its safety as a food colour by JECFA in 1969 and 1974.

This is the first time the Committee has evaluated dietary exposure to indigotine.

3.1 Food uses

The Codex Alimentarius Commission has finalized authorization of indigotine use in foods and beverages with maximum permitted levels ranging between 50 and 450 mg/kg, as noted in the General Standard of Food Additives (GSFA; CODEX STAN 192-1995, 20174; Table 3).

The European Food Safety Authority (EFSA) has also established maximum levels for use of indigotine in foods and beverages in Europe that range between 50 and 500 mg/kg.

The USFDA has not established maximum levels for this or other food colouring agents but allows use according to good manufacturing practices in a number of foods as listed in the Code of Federal Regulations (21 CFR 70 and 21 CFR 74).

Because indigotine has been authorized for use in foods for many years, several countries have conducted exposure assessments based on household economic surveys or individual dietary records. Such intake assessments are more accurate than the comparatively uncertain estimates based on the budget method or "poundage" (disappearance) data. Consequently, the Committee assessed the intake of indigotine primarily using data from the most recent national studies.

Indigotine is authorized in 51 different food categories but with specific restrictions of use and subject to national legislation of the importing country. For example, indigotine is authorized in fresh eggs at a maximum level of 300 mg/kg but for use in decorating, stamping, marking or branding of the product only (CODEX STAN 192-1995, 20174). Consequently, an exposure scenario based on maximum levels for each food category where indigotine is authorized would require too many unrealistic assumptions regarding the concentration in foods, which is why such an exposure assessment was not conducted.

3.2 Assessment of dietary exposure

The Committee reviewed dietary exposure estimate references submitted by the sponsor were reviewed. Five relevant references were retrieved as a result of the

Table 3
GSFA maximum permissible levels of indigotine (INS132)

Food category no.	Food category	Max. level (mg/kg)	Notes
01.1.2	Dairy-based drinks, flavoured and/or fermented (e.g. chocolate milk, cocoa, eggnog, drinking yoghurt, whey-based drinks)	300	Excluding chocolate milk.
01.6.1	Unripened cheese	200	For use in surface treatment only.
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	150	–
01.6.2.2	Rind of ripened cheese	100	–
01.6.5	Cheese analogues	200	For use in surface treatment only; subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
01.6.4.2	Flavoured processed cheese, including containing fruit, vegetables, meat, etc.	100	–
02.3	Fat emulsions mainly of type oil-in-water, including mixed and/or flavoured products based on fat emulsions	300	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
02.1.3	Lard, tallow, fish oil and other animal fats	300	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
02.4	Fat-based desserts excluding dairy-based dessert products of food category 01.7	150	–
03.0	Edible ices, including sherbet and sorbet	150	–
04.1.2.11	Fruit fillings for pastries	150	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
04.2.2.3	Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), and seaweeds in vinegar, oil, brine or soybean sauce	150	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
04.1.2.5	Jams, jellies, marmalades	300	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
04.1.2.6	Fruit-based spreads (e.g. chutney) excluding products of food category 04.1.2.5	300	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
04.1.2.7	Candied fruit	200	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
04.2.2.6	Vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed pulps and preparations (e.g. vegetable desserts and sauces, candied vegetables) other than food category 04.2.2.5	200	Excluding tomato-based sauces; Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
04.1.2.8	Fruit preparations, including pulp, purees, fruit toppings and coconut milk	150	Excluding coconut milk; Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.

Food category no.	Food category	Max. level (mg/kg)	Notes
04.2.2.7	Fermented vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera) and seaweed products, excluding fermented soybean products of food categories 06.8.6, 06.8.7, 12.9.1, 12.9.2.1 and 12.9.2.3	300	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
04.1.2.9	Fruit-based desserts, including fruit-flavoured water-based desserts	150	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
05.2	Confectionery including hard and soft candy, nougats, etc. other than food categories 05.1, 05.3 and 05.4	300	–
05.3	Chewing gum	300	–
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	300	–
05.1.4	Cocoa and chocolate products	450	Products conforming to the Standard for Chocolate and Chocolate Products (CODEX STAN 87-1981) may only use colours for surface decoration.
05.1.5	Imitation chocolate, chocolate substitute products	300	–
06.5	Cereal and starch based desserts (e.g. rice pudding, tapioca pudding)	150	–
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	200	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
09.1.1	Fresh fish	300	For use in decoration, stamping, marking or branding the product only; For use in fish roe only; For use in glaze, coatings or decorations for fruit, vegetables, meat or fish only.
09.2.1	Frozen fish, fish fillets, and fish products, including molluscs, crustaceans and echinoderms	300	For use in surimi and fish roe products only.
09.4	Fully preserved, including canned or fermented fish and fish products, including molluscs, crustaceans and echinoderms	300	–
09.3.3	Salmon substitutes, caviar and other fish roe products	300	–
09.2.4.1	Cooked fish and fish products	300	For use in surimi and fish roe products only.
09.2.5	Smoked, dried, fermented, and/or salted fish and fish products, including molluscs, crustaceans and echinoderms	300	For use in smoked fish products only; Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
09.3.4	Semi-preserved fish and fish products, including molluscs, crustaceans and echinoderms (e.g. fish paste), excluding products of food categories 09.3.1 – 09.3.3	300	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
09.2.4.2	Cooked molluscs, crustaceans and echinoderms	250	For use in glaze, coatings or decorations for fruit, vegetables, meat or fish only.
10.1	Fresh eggs	300	For use in decoration, stamping, marking or branding the product only; Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.

Table 3 (continued)

Food category no.	Food category	Max. level (mg/kg)	Notes
10.4	Egg-based desserts (e.g. custard)	300	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
11.4	Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings)	300	Subject to national legislation of the importing country aimed, in particular, at consistency with Section 3.2 of the Preamble.
12.2.2	Seasonings and condiments	300	–
12.4	Mustards	300	–
12.5	Soups and broths	50	–
12.6	Sauces and like products	300	–
13.3	Dietetic foods intended for special medical purposes (excluding products of food category 13.1)	50	–
13.4	Dietetic formulas for slimming purposes and weight reduction	50	–
13.5	Dietetic foods (e.g. supplementary foods for dietary use) excluding products of food categories 13.1–13.4 and 13.6	300	–
13.6	Food supplements	300	–
14.2.2	Cider and perry	200	–
14.1.4	Water-based flavoured drinks, including "sport", "energy" or "electrolyte" drinks and particulated drinks	100	–
14.2.4	Wines (other than grape)	200	–
14.2.6	Distilled spirituous beverages containing more than 15% alcohol	300	–
14.2.7	Aromatized alcoholic beverages (e.g. beer, wine and spirituous cooler-type beverages, low alcoholic refreshers)	200	–
15.1	Snacks – potato, cereal, flour or starch based (from roots and tubers, pulses and legumes)	200	–
15.2	Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)	100	–

GSFA: General Standard on Food Additives; INS: International Numbering System for Food Additives; max.: maximum; no.: number

comprehensive literature search (see section 1): Husain et al., 2006; FSANZ, 2008; Dixit et al., 2011; Ha et al., 2013; and Kist-van Holthe et al. 2015.

Several countries have conducted exposure assessments based on household economic surveys (Dixit et al., 2011) or individual dietary records (Gilsenan & Gibney, 2004; FSANZ, 2008; Ha et al., 2013; EFSA, 2014; Doell et al., 2016; Bastaki et al., 2017); these constitute a high-tier approach of exposure assessment. The Committee decided to use these refined data for the assessment

of indigotine intake (Table 4) rather than the unrealistic estimates based on budget method or "poundage" (disappearance) data.

3.2.1 Assessments based on "poundage" (disappearance) data

The sponsor estimated an intake of 140 µg/kg bw per day for the dye and 36 µg/kg bw for the lake based on the following assumptions:

- In the USA, approximately 164 397 kg of FD&C Blue No. 2 dye and 107 673 kg of FD&C Blue No. 2 lake were certified for use in food, drugs and cosmetics combined in 2016;
- Daily intake from consumption of all the certified annual volume was estimated to be 18 161 µg/person for the dye and 11 895 µg/person for the colour lake, or 302 µg/kg bw per day for the dye and 198 µg/kg bw per day for the colour lake;
- Of this, averages of 58% of the dye and 23% of the colour lake were estimated to be used in food and beverage in the USA (domestic use only); and
- Based on a population of "consumers only" estimated to be 10% of the total population, the average daily intake is approximately 8427 µg/person of the dye and 2189 µg/person of the lake, from consumption of foods and beverages, corresponding to 140 and 36 µg/kg bw per day, respectively, assuming a mean adult body weight of 60 kg.

3.2.2 Assessments based on data from household economic surveys

(a) India

In a study conducted by the Food Toxicology Division of the Indian Institute of Toxicology Research (Dixit et al., 2011), 2867 samples from 10 broad food categories were analysed for indigotine (and other synthetic colours) in 16 Indian states. The study focused on children and sampled food commodities were identified as preferentially consumed by children through a food frequency questionnaire (bakery products, beverages, candyfloss, chewing gums, coated candies, hard-boiled sugar confectioneries, jam and jellies, ice candy/ice creams, mouth fresheners and sugar toys). Samples were randomly chosen from street vendors using a sampling design that enabled homogeneous collection per market location, city and state.

Indigotine was not detected in any samples so no further dietary intake estimate was assessed in this study.

Table 4
Summary of the national studies used for the present assessment of dietary intake

Country/region Organization / Reference	Use or concentration data	Consumption data	Exposure assessment (mg/kg bw per day)			
			Populations, ages (years)	Mean	P90 or P95 [a]	Max.
Australia FSANZ FSANZ (2008)	Australian survey specific to colours (as part of the surveillance programme in 2006)	National Nutrition Survey (NNS)	Australian population >2	0.00	0.00	0.00
			Children 2–5	0.00	0.00	0.00
			Children 6–12	0.00	0.00	0.00
			Adolescents 13–18	0.00	0.00	0.00
			Adults 19–24	0.00	0.00	0.00
			Adults >25	0.00	0.00	0.00
India Indian Institute of Toxicology Research Dixit et al. (2011)	Indian survey specific to colours	Household survey of the food consumption patterns of 518 respondents	Children 4–6	–[b]	–[b]	–[b]
			Children 7–9			
			Children 10–12			
			Adolescents 13–15			
			Adolescents 16–18			
Kuwait Kuwait Institute for Scientific Research Husain et al. (2006)	Determination of colour additives in 344 foods items	A 24-h dietary recall survey conducted twice on 3 141 boys and girls from 58 schools	Children 5–14	0.003	0.300	–
Netherlands EMGO Institute for Health and Care Research Kist-van Holthe et al. (2015)	Maximum levels	Specific food diary study with children in Amsterdam	Children 5–12	–	–	0.140
Republic of Korea Universities of the Republic of Korea Ha et al. (2013)	Survey of the Republic of Korea specific to colours	Korea National Health and Nutrition Survey (Ministry of Health and Welfare, 2009)	The entire population of consumers	0.00	0.00	0.005 2
USA IACM Bastaki et al. (2017)	Industry survey + food colour manufacturers + Mintel International Group Ltd	NHANES	USA population	0.000 7	0.002 3	0.011 3
			Children 2–5	0.002 8	0.010 4	0.036 9
			Children 6–12	0.001 5	0.004 9	0.023 6
			Adolescents 13–18	0.000 7	0.003 1	0.012 2
			Adults ≥19	0.000 5	0.001 3	0.008 7
USA USFDA Doell et al. (2016)	Measured concentration	NHANES+ 10–14 day food consumption	USA population >2	0.010	0.020	0.040
			Children 2–5	0.030	0.060	0.100
			Adolescent boys 13–18	0.010	0.020	0.060

bw: body weight; EFSA: European Food Safety Authority; EU: European Union; FSANZ: Food Standards Australia New Zealand; IACM: International Association of Color Manufacturers; NHANES: National Health and Nutrition Examination Survey; P90: 90th percentile; P95: 95th percentile; USFDA: United States Food and Drug Administration; USA: United States of America

[a] P95 exposure assessment data are provided when available; otherwise, P90 exposure assessment data are provided.

[b] Indigotine was not detected in any samples. No further dietary intake estimates were conducted.

3.2.3 Assessments based on individual dietary records

(a) National studies

Australia

An analytical survey to quantify actual levels of all permitted synthetic colours and two natural colours in foods and beverages in Australia was conducted as part of the surveillance programme in 2006 (FSANZ, 2008).

A total of 396 individual samples of processed foods and beverages were sampled over a 3-month period between June and August 2006. The foods and beverages included were confectionery, ice cream, cheese, yoghurt, margarine, flavoured milk, flavoured soy beverages, soft drink, cordial, fruit drink, alcoholic drinks, biscuits, cakes, pastries, savoury snacks, breakfast cereal, pre-prepared meals, processed meats, sauces, toppings, jams/conserves and jelly. A small number of products that claimed to contain "no added colours" or "no artificial colour", was also sampled. The concentrations of all colours tested in foods and beverages were below the respective maximum level for all but six samples.

Consumption data came from the 1995 Australian National Nutrition Survey (NNS)that surveyed 13 858 people aged 2 years and above using a 24-hour food recall methodology.

Dietary exposure was assessed for the Australian population aged 2 years and above; children aged 2–5 years; children aged 6–12 years; adolescents aged 13–18 years; adults aged 19–24 years; and adults aged 25 years and above.

Two different scenarios of exposure were examined: the mean and the maximum colours scenarios. For the dietary modelling of the mean colours scenario, mean analytical concentrations of survey foods were assigned to their respective food subgroups. The maximum colours scenario investigates the situation where products with the highest levels of colours detected may be consumed, and dietary modelling was conducted based on assigning the maximum analytical concentrations of the survey foods to their respective food subgroups (Table 4).

Kuwait

Husain et al.(2006) assessed intakes of indigotine (and other food colour additives) for 5- to 14-year-old children in Kuwait. Consumption data were based on a 24-hour dietary recall conducted twice on 3141 male and female Kuwaiti and non-Kuwaiti children from 58 schools representative of the country thanks to a proportional stratified sampling procedure.

A sample of 344 foods items were grouped into nine categories: biscuits, cakes and ice cream, candy, chips and puffed snacks, chocolates, drinks and juices, chewing gum, jelly and lollipops was analysed for indigotine by high-performance liquid chromatography with diode array detector (HPLC–DAD).

Indigotine was detected and quantified in candies at 2.9–151 mg/kg and in chewing gums at 1.4–36 mg/kg.

The average daily intakes of indigotine calculated based on a mean scenario (average amounts of coloured foods consumed and average levels of the colour additives in those foods) are provided in Table 4.

Netherlands

A survey was conducted in 2012–2013 in Amsterdam to identify the presence of indigotine (and other colours) in a variety of products available in supermarkets and grocery shops (Kist-van Holthe et al., 2015). Of the 550 different products from 40 supermarkets, grocery shops and Turkish and Moroccan shops checked for the presence of artificial food colourings, indigotine was detected in 12.

Food consumption data from 121 children were collected; only six were identified as consuming products likely to contain indigotine. The dietary exposure of these six children was assessed using a maximum level scenario, and their dietary intakes were found to range from 0.06 to 0.14 mg/kg bw per day.

The assessed exposures are described in this section and reported in Table 5 for information. However, the Committee considered the methodology to be not well described and the results to be uncertain.

Republic of Korea

The dietary intakes of indigotine in the Republic of Korea were estimated based on food consumption data for consumers and concentrations in processed foods (Ha et al., 2013).

Food consumption data were obtained from the Korea National Health and Nutrition Survey (Korea Ministry of Health and Welfare, 2009) using a 24-hour recall method.

Food samples were selected based on stratified random sampling (KFDA 2004) considering their market share and a total of 704 food samples were collected to determine the content of colours. Indigotine was found in two samples of 309 samples of candy.

Two scenarios of exposure were estimated: one for an average consumers using average concentration of all samples in a food group and one for a high intake consumer using average of positive samples. The estimated mean and 95th percentile for dietary exposure based on these two scenarios are provided in Table 4.

USA

In a study by the USFDA, approximately 600 samples from 52 broad food categories were analysed for indigotine (and other synthetic colours) using a

USFDA-validated HPLC–DAD (Petigara Harp, Miranda-Bermudez E & Barrows, 2013). Samples were selected based on a preceding survey on food labels (Doell et al., 2016). Indigotine was found in 12 of the 52 food categories and quantified in five food categories.

Two different sets of food consumption data from 2007–2010 were used for the dietary exposure estimates: a 2-day and a 10–14-day dietary intake survey for the USA population. Three population groups were used for the exposure estimate: the population 2 years and older, children 2–5 years and teenage boys aged 13–18 years.

Exposures were estimated based on "eaters-only" (individuals in the population who consumed one or more of the included foods over the survey period). Three different exposure scenarios were performed (1) a low-exposure scenario, where the lowest analytical value for indigotine was assigned to the corresponding food code in the consumption survey; (2) an average-exposure scenario, where the analytical results were averaged for a given food code; and (3) a high-exposure scenario, where the highest analytical value was assigned to each food code. Dietary exposures were estimated at the mean and at the 90th percentile for each population for each food category.

Estimated dietary intakes from the average and high-exposure scenarios based on the 10–14-day dietary intake survey are provided in Table 4. Only results from this consumption survey are provided in Table 4 since per cent eaters are greater for the exposures estimated using the 10–14 day food consumption data than for those estimated using the 2-day data for all three populations.

Breakfast cereal was the food category that contributed the most to exposure for all three populations. The "Decoration/Chips for Baking", "Frozen Dairy Dessert/Sherbet", "Toaster Pastries, Cakes and Cupcakes, Chocolate, and Frostings and Icings" categories were also major contributors for all three populations, while "Pudding" was also a major contributor for the USA population 2 years and older and children aged 2–5 years.

The International Association of Color Manufacturers (IACM) recently published a new estimate of daily intake for indigotine (and other colours) based on reported use levels and on information on the proportion of foods currently available in the marketplace that contain food colour (Bastaki et al., 2017). Typical and maximum use levels for indigotine within selected food categories were provided by the IACM for 15 food/beverage general categories. The per cent of food products in the marketplace that contain indigotine were provided by the Mintel International Group Ltd, based on finished product labels between January 2011 and February 2015.

Food consumption data were obtained from the National Health and Nutrition Examination Survey (NHANES).

Estimates of the individual intakes were generated with the proprietary Foods and Residues Evaluation Program (FARE®). Four different scenarios of exposure were calculated, all of them for consumers only: typical use levels with mean consumption and 95th percentile of consumption and maximum use levels with mean consumption and 95th percentile of consumption. Estimated dietary intakes from these 4 scenarios are provided in Table 4.

The use levels reported by the industry by Bastaki et al. (2017) are consistent with the concentrations measured analytically by the USFDA in Doell et al. (2016).

Exposure estimates from the IACM exposure assessment (Bastaki et al., 2017) are much lower than those from USFDA (Doell et al., 2016). The difference may thus come from the estimation of the intake which was weighed by the frequency of colour presence in each category in Bastaki et al. (2017), whereas Doell et al. (2016) assumed 100% colour presence.

(b) Europe

EFSA carried out an exposure assessment for indigotine in 2014 in the context of the re-evaluation of indigotine as a food additive (EFSA, 2014).

Consumption data came from the EFSA Comprehensive European Food Consumption Database (Comprehensive Database), which has been populated with detailed national food consumption data since 2010. To calculate chronic exposure, intake statistics were calculated based on individual average consumption over the total survey period, excluding surveys with only one day per subject as these were considered inadequate for assessing chronic exposure. High percentile exposure was only calculated for those foods and population groups where the sample size was sufficiently large to allow calculation of the 95th percentile of exposure (EFSA, 2011). Thus, the assessment for indigotine was based on 26 different dietary surveys carried out in 17 European countries.

Chronic exposure was estimated for the following population groups: toddlers, children, adolescents, adults and elderly adults.

The food categories in which the use of indigotine is authorized were selected from the nomenclature of the EFSA Comprehensive Database (FoodEx classification system food codes), at a detailed level (up to FoodEx Level 4). The estimate did not take into account eight food categories not referenced in the EFSA Comprehensive Database, which might have resulted in an underestimation of the exposure. For seven other food categories, the restrictions that apply to the use of indigotine were not taken into account, and therefore the whole food category was considered for the exposure estimate, which resulted in an overestimation of the exposure.

Table 5
Exposure estimates for the three EFSA scenarios

	Exposure per age group (mg/kg bw per day)				
	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	Elderly adults (>65 years)
Maximum level scenario					
Mean	0.9–3.8	0.8–3.2	0.3–1.5	0.3–1.0	0.1–0.6
P95	2.8–7.1	1.8–6.7	1.0–3.2	0.7–2.3	0.5–1.4
Brand-loyal scenario					
Mean	0.1–0.4	0.1–0.3	0.04–0.2	0.02–0.1	0.02–0.1
P95	0.3–0.8	0.2–0.8	0.1–0.5	0.1–0.3	0.1–0.2
Non-brand-loyal scenario					
Mean	0.02–0.2	0.02–0.1	0.01–0.1	0.01–0.4	0.003–0.03
P95	0.1–0.3	0.1–0.3	0.03–0.1	0.02–0.1	0.01–0.1

bw: body weight; EFSA: European Food Safety Authority; P95: 95th percentile of consumers

Three different scenarios of exposure were estimated: a regulatory maximum level exposure assessment scenario based on the maximum levels as set in Annex II to Regulation No 1333/2008; a brand-loyal consumer scenario that assumes that a consumer is exposed long-term to the food additive present at the maximum reported use/analytical levels for one food category; and a non-brand-loyal consumer scenario that assumes the population is exposed long term to the food additive present at the mean reported use/analytical levels in food (Table 5).

The actual use levels of indigotine in foods were provided by industry and Member States following a public call for concentration data (usage and/or analytical data).

The EFSA Panel noted that the three main contributing food categories for most exposed age groups showed high number of analytical data far below maximum levels or had no use level reported combined with no detection in limited analytical data. The EFSA Panel thus considered the maximum level scenario to not be realistic.

4. Comments

4.1 Biochemical aspects

Although indigotine was poorly absorbed from the gastrointestinal tract in rats, one of its metabolites, 5-sulfoanthranilic acid, formed through microbial

fermentation, was absorbed to a greater extent. After administration of a single oral dose of 50 mg/rat, unchanged indigotine, isatin-5-sulfonic acid and 5-sulfoanthranilic acid were identified in urine (0.53%, 0.63% and 0.28% of the dose, respectively). The metabolites isatin-5-sulfonic acid and 5-sulfoanthranilic acid were also identified in the bile (Lethco & Webb, 1966). After in vitro incubation of indigotine with caecal microflora of rats, four unidentified metabolites were found (Singh, Das & Khanna, 1993).

In vitro, indigotine significantly inhibited the CYP2A6 monooxygenase activity in a noncompetitive manner, with an IC_{50} of 0.05 mmol/L (Kuno & Mizutani, 2005; Mizutani, 2009).

4.2 Toxicological studies

Indigotine has a low acute toxicity. No adverse effects were seen in pigs fed indigotine (purity >85%) at dose levels of 0, 150, 450 or 1350 mg/kg bw per day for 90 days (Gaunt et al., 1969).

When mice were fed diets containing 0.2%, 0.4%, 0.8% or 1.6% indigotine (purity ≥85%) for 80 weeks, the only effect seen was a slight anaemia in animals at 0.8% or 1.6% (Hooson et al., 1975). The previous Committee concluded that indigotine was not carcinogenic and that the "no-untoward-effect level was 0.4% of the diet, equivalent to an intake of approximately 550 mg/kg/day" (Annex 1, references *35* and *36*).

In a 2-year combined toxicity and carcinogenicity study, mice were fed indigotine (purity 93%; 7% volatile matter) at dietary concentrations of 0, 0.5%, 1.5% or 5.0% (equal to 0, 825, 2477 and 8259 mg/kg bw per day, respectively). A moderate incidence of blue-green discolouration of the gastrointestinal tract, with occasional discolouration of the liver, gall-bladder and urine, was observed at all doses. There was no evidence of carcinogenicity. The NOAEL was 5% in the diet (equal to 8259 mg/kg bw per day), the highest dose tested (Borzelleca & Hogan, 1985).

In a long-term combined toxicity and carcinogenicity study, which included an in utero phase, indigotine (purity 93%, 7% volatile matter) was fed to rats at dietary levels of 0, 0.5%, 1.0% or 2.0% (equal to 0, 304, 632 and 1282 mg/kg bw per day, respectively). Treatment began approximately 2 months prior to mating, and the long-term phase was initiated after random selection of the F_1 animals. No consistent substance-related adverse effects were noted, with the exception of statistically significant increases in incidences of malignant mammary gland tumours and gliomas in males, but not females, at the highest dose. The incidence of mammary gland tumours (carcinomas/adenocarcinomas) in high-dose males was 5.9% (3/51 compared with 0/114 control animals). The

incidence of gliomas in high-dose males was 9.9% (7/71) compared with 2.9% in controls (4/140 animals). No increase was seen in the low-and mid-dose groups (incidence 2.9%, 2/70 animals) or in female rats (Borzelleca, Hogan & Koestner, 1985).

The previous Committee noted the statistically significant increase in incidence of malignant mammary gland tumours and gliomas in male rats at the highest dose level of 1282 mg/kg bw per day. In the absence of any indications for genotoxicity, the Committee concluded that the NOAEL in this study was 632 mg/kg bw per day.

Rats fed a diet containing 1% indigotine (equivalent to 500 mg/kg bw per day) for 2 years showed no treatment-related pathological signs, and fewer malignant and benign tumours than the controls; they also survived for longer than the controls (Oettel et al., 1965). When indigotine was fed to groups of rats at dietary levels of 0, 0.5%, 1.0%, 2.0% or 5.0% for 2 years, the only effect seen was a reduced growth in males at 2.0% and 5.0% (Hansen et al., 1966). The NOAEL was 1% in the diet, equivalent to 500 mg/kg bw per day.

Indigotine tested negative in a series of bacterial mutagenicity assays as well as in in vitro mammalian cell chromosomal aberration assays and a number of comet assays. Indigotine was also not genotoxic in in vivo micronucleus tests in mice and rats and a comet assay in mice. The Committee concluded that indigotine does not raise any concerns with respect to genotoxicity.

No reproductive or developmental toxicity was observed in one 3-generation rat study (doses up to 250 mg/kg bw); two 2-generation rat studies (doses up to 250 or 500 mg/kg bw); one rat teratogenicity study (doses up to 250 mg/kg bw); and two rabbit teratogenicity studies (doses up to 250 mg/kg bw).

4.3 Assessment of dietary exposure

Dietary exposure to indigotine has not been previously reviewed by the Committee.

A comprehensive literature search was conducted. Five references in addition to those submitted by the sponsor were considered relevant for the assessment of the dietary exposure. The Committee reviewed published estimates of dietary exposure to indigotine conducted in several countries and regions. A summary of these estimates, showing the results from mean and high percentile calculations, is provided in Table 6.

As indigotine has provisions in 51 GSFA food categories, an exposure assessment based on maximum use levels was considered by the Committee to be unrealistic. Moreover, analytical and reported use levels for the main contributing food categories were far below the maximum use levels, reinforcing

Table 6
Summary of the range of estimates of dietary exposure for indigotine

		Range of estimated dietary exposures (mg/kg bw per day)	
Source of estimate	**Population**	**Mean**	**High percentile (P90 and P95) [a]**
National [b]	Children	0.00–0.03	0.0–0.3
	Adolescents	0.00–0.01	0.00–0.03
	Adults	0.00–0.01	0.00–0.02
Europe [c]	Toddlers	0.0–0.2	0.3–0.8
	Children	0.0–0.1	0.2–0.8
	Adolescents	0.0–0.1	0.1–0.5
	Adults	0.0–0.4	0.1–0.3
	Elderly adults	0.0–0.03	0.1–0.2

bw: body weight; P90: 90th percentile; P95: 95th percentile
[a] The upper bound of the range is the maximum of the 90th and 95th percentiles.
[b] Data from Australia (FSANZ, 2008),Kuwait (Husain et al., 2006); the Netherlands (Kist-van Holthe et al., 2015); the Republic of Korea (Ha et al., 2013); and the USA (Doell et al., 2016; Bastaki et al., 2017).
[c] Based on consumption data from 17 European countries (EFSA, 2014).

the Committee's decision not to consider estimates based on maximum use levels for the exposure assessment.

Because indigotine has been authorized for use for many years, estimates based on analytical data were available and were considered by the Committee to be more appropriate for determining long-term dietary exposure estimates. Exposure estimates based on analytical data range from 0.0 to 0.8 mg/kg bw per day for adults, adolescents and children at the 95th percentile. Because of the conservative assumptions in high percentile exposure estimates, the Committee concluded that an estimate of 0.8 mg/kg bw per day for children and toddlers should be considered in the safety assessment for indigotine.

5. Evaluation

The Committee noted that indigotine is poorly absorbed from the gastrointestinal tract, has a low acute toxicity, is not genotoxic and does not show any potential for reproductive or developmental toxicity. The previous Committee identified a NOAEL of 500 mg/kg bw per day from a 2-year rat study of 1% indigotine in the diet and established an ADI of 0–5 mg/kg bw. The current Committee considered the new data that had become available since the previous evaluation as well as previously evaluated studies. In one long-term toxicity study, slight anaemia was observed in mice fed diets with 0.8% or 1.6% indigotine. In another long-term toxicity study, body-weight gain was reduced in male rats at 2.0% and 5.0%

indigotine in the diet. In a third long-term toxicity study, increased incidences of malignant mammary gland tumours and gliomas were observed in male rats at 1282 mg/kg bw per day but not at 304 and 632 mg/kg bw per day.

The Committee concluded that there are no reasons to revise the ADI and confirmed the ADI of 0–5 mg/kg bw.

The Committee noted that the conservative dietary exposure estimate of 0.8 mg/kg bw per day (95th percentile for children and toddlers) is less than the upper limit of the ADI of 0–5 mg/kg bw established for indigotine. The Committee concluded that dietary exposure to indigotine for all age groups does not present a health concern.

The existing specifications for indigotine were revised. HPLC methods were added for determining subsidiary colouring matters and organic compounds other than colouring matters. The method of assay was changed to visible spectrophotometry, and spectrophotometric data were provided for the colour dissolved in water.

The specifications monograph was revised, and a Chemical and Technical Assessment was prepared.

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Lutein and lutein esters from *Tagetes erecta* and zeaxanthin (synthetic) (addendum)

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1. Explanation

Lutein esters from *Tagetes erecta* (International Numbering System for Food Additives [INS] No. 161b(iii)) and lutein from *Tagetes erecta* (INS No. 161b(i)) are used as food colouring agents and nutrient supplements in a wide range of baked goods and baking mixes, beverages and beverage bases, breakfast cereals, chewing gum, dairy product analogues, egg products, fats and oils, frozen dairy desserts and mixes, gravies and sauces, soft and hard candy, infant and toddler foods, milk products, processed fruits and fruit juices, soups and soup mixes at levels ranging from 2 to 330 mg/kg.

Zeaxanthin (INS No. 161h(i)) is used as a nutritional supplement and colour in a wide range of foods such as baked goods, beverages, chewing gum, egg products, fats and oils, gravies and sauces, hard and soft candy, infant and toddler foods (other than infant formula), milk products, processed fruits and fruit juices, soups and soup mixes at levels ranging from 0.5 to 70 mg/kg.

Lutein esters contain lutein (all-*E*,3*R*,3*R*,6*R*)-β, ε-carotene-3,3-diol, which is a naturally occurring oxygenated xanthophyll pigment and a macular carotenoid. Lutein occurs with its isomeric xanthophyll, zeaxanthin, in many foods but particularly in vegetables and fruit. Lutein occurs either esterified to fatty acids or in a non-esterified "free" form. Studies show that *meso*-zeaxanthin, a structurally related xanthophyll of lutein, and zeaxanthin may originate from foodstuffs that are sources of these xanthophylls, rather than being derived from the bioconversion of retinal lutein (Nolan et al., 2013). Extracts containing xanthophylls (free and/or esterified) are used as colours and as nutritional supplements in a wide range of applications.

At the thirty-first meeting, the Committee prepared tentative specifications for xanthophylls obtained from *Tagetes erecta* petals, but no toxicological evaluation was performed (Annex 1, reference *77*). Xanthophyll preparations (*Tagetes* extract) containing lutein esters at low concentrations were evaluated at the fifty-fifth and fifty-seventh meetings (Annex 1, references *149* and *154*). The tentative specifications (Annex 1, reference *151*) were subsequently superseded by full specifications (Annex 1, reference *156*). At the sixty-third meeting, the Committee evaluated biochemical data and the results of toxicological and human studies on *Tagetes* preparations with a high content of non-esterified lutein (>80%) and established a group acceptable daily intake (ADI) of 0–2 mg/kg body weight (bw) per day for lutein from *Tagetes erecta* and synthetic zeaxanthin (Annex 1, reference *173*). At the seventy-ninth meeting, the Committee evaluated the results of new toxicity studies on preparations with a higher content of xanthophyll esters (>60%), identified as "lutein esters from *Tagetes erecta*" (Annex 1, reference *220*). A temporary ADI "not specified" for

lutein esters from *Tagetes erecta* was established; the ADI was made temporary because the specifications for lutein esters from *Tagetes erecta* were tentative.

At the eighty-second meeting, the Committee evaluated additional information on manufacturing and composition of lutein esters from *Tagetes erecta* that permitted the adoption of final specifications and the removal of the temporary designation (Annex 1, reference *230*). An ADI "not specified" for lutein esters from *Tagetes erecta* was established. The Committee was unable to consider establishing a group ADI "not specified" for lutein esters from *Tagetes erecta*, lutein from *Tagetes erecta* and synthetic zeaxanthin and related xanthophylls, and recommended that this be taken up at a future meeting.

At the present meeting, newly available data for lutein, zeaxanthin and *meso*-zeaxanthin were submitted by the sponsor. Additional data that became available since the previous evaluations were obtained as a result of literature searches in Medline, Toxline, Scopus and SciFinder using the following keywords: lutein, zeaxanthin, meso_zeaxanthin, clinical, toxicology, genotoxicity, metabolism, absorption, excretion, ADME and *Tagetes erecta*. Because of the large number of hits for publications that refer to effects that were related to efficacy or health claims but not to safety, an appropriate selection policy was applied to retrieve only those studies designed to investigate end-points relevant to safety. As a result, two other studies were identified.

The Committee assessed available toxicity studies for re-evaluation of safety, dietary exposure and specifications to consider establishing a group ADI "not specified" for lutein and lutein esters from *Tagetes erecta* and zeaxanthin (synthetic).

1.1 Chemical and technical considerations

Lutein, lutein esters (including lutein dipalmitate) and zeaxanthin are members of a group of pigments known as xanthophylls; they have no provitamin A activity.

Tagetes extract (INS No. 161b(ii)) is obtained by hexane extraction of dried petals of marigold (*Tagetes erecta* L.), with subsequent removal of the solvent. The major colouring principles are lutein (3*R*,3′*R*,6′*R*-β,ε-carotene-3,3′-diol) and lutein dipalmitate (helenien; β,ε-carotene-3,3′-diol dipalmitate). Hydroxy derivatives of carotenes together with other oxy derivatives, such as epoxides, may also be present in *Tagetes* extract. The product may contain fats, oils and waxes that occur naturally in the plant material. The articles of commerce are usually further formulated to standardize the colour content or to obtain water-soluble or dispersible products.

Lutein esters from *Tagetes erecta* (INS No. 161b(iii)) is a purified extract obtained from marigold (*Tagetes erecta* L.) oleoresin, which is extracted

using organic solvents. The preparation contains lutein esters, of which lutein dipalmitate accounts for the major part; a smaller proportion of zeaxanthin esters is also present. The balance of the extract is made up of naturally occurring waxes.

Lutein from *Tagetes erecta* (INS No. 161b(i)) is a purified extract obtained from marigold (*Tagetes erecta* L.) oleoresin, which is extracted using organic solvents. The final product, after saponification and crystallization, contains lutein as the major component and a smaller proportion of zeaxanthin.

Zeaxanthin (synthetic) (INS No. 161h(i)) is the synthetic all-*trans* isomer of zeaxanthin (3*R*,3′*R*-β,β-carotene-3,3′-diol) produced by the Wittig reaction from raw materials that are commonly used in the production of other carotenoids with application in foods. Minor quantities of *cis*-zeaxanthins and by-products 12-apozeaxanthinal, parasiloxanthin, diatoxanthin and triphenyl phosphine oxide may be present in the final product.

2. Biological data

In addition to new toxicity studies with lutein, zeaxanthin and *meso*-zeaxanthin that had not been previously assessed, the Committee reconsidered the biological and clinical data for lutein and lutein esters that had been available at the seventy-ninth meeting (Annex 1, reference *220*).

2.1 Biochemical aspects

2.1.1 Absorption, distribution and elimination

The absorption, distribution, metabolism, excretion (ADME) of lutein and lutein esters from *Tagetes erecta* were extensively described in the monographs of the sixty-third and seventy-ninth Joint FAO/WHO Expert Committee on Food Additives (JECFA) meetings (Annex 1, references *173* and *220*). The absorption of lutein requires transfer of carotenoids from digested food, emulsification by bile and lipolysis by pancreatic lipases into the micellar fraction for absorption by intestinal cells. These processes can be influenced by a number of factors, such as dietary fat, which can promote absorption, and dietary fibre, which can decrease absorption (Fernández-García et al., 2012). The process of absorption of carotenoids from esters is even more complex because it requires hydrolysis of the esters.

In the present evaluation, a number of studies conducted in experimental animals and humans indicate that lutein esters administered as dietary constituents or as nutritional supplements increase levels of lutein in blood and

lead to its accumulation in tissues (liver, adipose tissue and eyes). The overall bioavailability of lutein from lutein esters is likely to be low but may increase with high fat intake.

(a) Rats

Sheshappa et al. (2015) investigated food matrix effects in lutein-deficient rats. Plasma, liver and eye lutein levels were higher than the control when animals were administered lutein-mixed micelles containing either 3% fat, phosphatidyl choline or lyso-phosphatidyl choline. In contrast, when animals were administered lutein-mixed micelles containing pectin and mixed carotenoids, the plasma and liver levels of lutein were lower than the control. The observed changes in bioavailability were mainly a function of micellar size, but facilitated by lipids, confirming previous findings. Of note, treatment with pure lutein and lutein from a dried plant (*Basella alba*) at similar dietary level, resulted in the same response in the examined tissues.

(b) Monkeys

Jeon et al. (2017) found that increased intake of carotenoids from infant formula enhanced lutein deposition in sera and numerous tissues and selectively increased lutein concentrations in multiple regions of brain of infant rhesus macaques. Monkeys (2/group; 1–3 months old) were fed either a formula supplemented with lutein, zeaxanthin, β-carotene and lycopene or a control formula with low levels of these carotenoids, for 4 months. Plasma samples from all animals were analysed by high-performance liquid chromatography (HPLC).

Serum lutein in the supplemented group was 5 times higher than in the nonsupplemented group. All the brain regions examined showed a selective increase in lutein deposition in the supplemented monkeys. Lutein differentially accumulated across regions of the brain, with highest amounts in occipital cortex in both groups; β-carotene also accumulated, but zeaxanthin and lycopene were undetectable in any region of the brain. Supplemented infant monkeys had higher concentrations of lutein in peripheral retina but not in macular retina. Of adipose tissue sites, abdominal subcutaneous adipose tissue had the highest lutein levels, namely 3-fold higher in the supplemented infants. The supplemented formula enhanced carotenoid deposition in several other tissues.

Khachik et al. (2006a) supplemented the diet of female rhesus macaques with high doses of lutein, zeaxanthin or a combination of the two to investigate resulting plasma and ocular tissue levels of these carotenoids and their metabolites over time and to determine whether these high doses can cause ocular toxicity. Animals were divided into the following treatment groups: control (n = 3); lutein

(n = 5; lutein at 9.34 mg/kg bw per day and zeaxanthin at 0.66 mg/kg bw per day); zeaxanthin (n = 5; zeaxanthin at 10.00 mg/kg bw per day); and lutein/zeaxanthin combination (n = 5; lutein and zeaxanthin each at 0.5 mg/kg bw per day). After 12 months of daily supplementation, one control animal, two lutein-treated animals, two zeaxanthin-treated animals and all the combination-treated animals were killed. The remaining monkeys were killed after a 6-month supplementation-free recovery period.

Supplementing with lutein and/or zeaxanthin increased the mean plasma and ocular tissue concentrations of these carotenoids and their metabolites in the monkeys. The mean levels of both carotenoids in the retinas of the lutein- and zeaxanthin-treated animals after 1 year were significantly increased over baseline.

Vishwanathan et al. (2013) evaluated the relationship between retinal and brain levels of lutein and zeaxanthin in rhesus monkeys (*Macaca mulatta*). Rhesus monkeys were reared on diets devoid of xanthophylls and subsequently fed pure lutein or pure zeaxanthin, each at 3.9 μmol/kg bw per day (6/group). Another group of rhesus monkeys was fed a stock diet containing lutein at 0.26 μmol/kg bw per day and zeaxanthin at 0.24 μmol/kg bw per day (n = 5). Retina (4 mm macular punch, 4–8 mm annulus and periphery) and brain tissue (cerebellum, frontal cortex, occipital cortex and pons) from the same animals were analysed by reverse phase HPLC.

Lutein levels in the macula and annulus were significantly related to lutein levels in the cerebellum, occipital cortex and pons, both in bivariate analysis and after adjusting for age, sex and n–3 fatty acid status. In the frontal cortex, the relationship was marginally significant. Macular zeaxanthin levels were significantly related to zeaxanthin levels in the cerebellum and frontal cortex, while the relationship was marginally significant in the occipital cortex and pons in a bivariate model.

(c) Humans

Bohn (2008) reviewed what was then known about the bioavailability of non-provitamin A carotenoids and summarized the information on lutein and zeaxanthin as follows:

> Fatty acid esters of xanthophylls are generally assumed to be cleaved in vivo by carboxylic ester hydrolase originating from pancreas, prior to absorption. Lutein is then thought to undergo mainly acid catalyzed dehydration to form 3-hydroxy-3′,4′-dihydro-β,γ-carotene and 3-hydroxy-2′3′-didehydro-β,ε-carotene. Other possible degradation pathways of both lutein and zeaxanthin follow oxidation to monoketocarotenoids and

> diketocarotenoids such as ε,ε-carotene-3,3′-dione. In the human retina, a number of additional products have been detected.

However, after reviewing additional in vitro and in vivo (clinical and epidemiological) studies, Bohn (2008) concluded that:

> …numerous factors can impact carotenoid absorption, including the amount and possibly type of dietary lipids consumed, the polarity and isomer form of the carotenoid, the stability of the matrix to which the carotenoid was bound, and additional dietary factors such dietary fiber.

This would be no less relevant for lutein and zeaxanthin, two of the most investigated carotenoids.

Thürmann et al. (2005) characterized lutein plasma kinetics and investigated the effects of lutein intake on concentrations of other plasma carotenoids in a multiple dosing design study with 19 healthy volunteers. The study participants were asked to avoid consuming lutein- and zeaxanthin-rich vegetables and fruit during the study. Daily fat intake was restricted to 100 g. After a run-in period of 7 days, the participants were assigned to receive daily oral doses of lutein at 4.1 mg ($n = 8$) or 20.5 mg ($n = 8$) for 42 days or no lutein ($n = 3$). The supplement contained 8.3% zeaxanthin relative to 100% lutein. The time profiles of plasma xanthophyll concentrations were monitored over the dosing phase. Samples were collected frequently over the 42-day treatment period.

Steady state all-*E*-lutein concentrations exceeded baseline concentrations by approximately 3.5- and 10-fold for the low- and high-dose groups, respectively. Dose-normalized lutein bioavailability in the high-dose group was approximately 60% that of the low-dose group. Kinetic disposition half-life did not differ significantly between groups. On average, to reach a greater than 90% fraction of the steady state concentration, which is consistent with an effective half-life for accumulation of approximately 5.6 days, dosing was required for 18 days. Plasma kinetics of all-*E*-lutein were parallel to those of all-*E*-3′-dehydrolutein. Kinetic analysis indicated that all-*E*-3′-dehydrolutein was formulated from lutein. Lutein was well tolerated and did not affect the concentrations of other carotenoids.

Khachik et al. (2006b) investigated the effect of supplementing with commercially available lutein on the distribution of other carotenoids and their metabolites in the sera of study participants aged 60 years and older, with and without age-related macular degeneration. Interaction with the serum levels of other dietary carotenoids, retinol and α -tocopherol, was also investigated. In this randomized double-blind clinical trial, the 45 participants received daily

supplements of lutein (containing 5% zeaxanthin) at doses of 2.5, 5.0 or 10 mg/day for 6 months. At the end of the supplementation period, the participants were followed for a further 6 months. Blood samples were collected 1 week before initiation of supplementation and throughout the study at months 1, 3, 6, 9 and 12. Lutein, zeaxanthin and their metabolites and retinol and α-tocopherol were quantified in the sera by normal phase HPLC-UV/visible detection.

After 6 months of supplementation with 10 mg of lutein, mean serum levels of lutein had increased from 210 nmol/L at baseline to 1000 nmol/L. Mean serum levels of zeaxanthin had increased from 56 to 95 nmol/L. Similarly, the mean concentrations of carotenoid metabolites increased from 49 to 98 nmol/L for 3-hydroxy-β,ε-caroten-3′-one (3′-oxolutein); from 31 to 80 nmol/L for 3′-hydroxy-ε,ε-caroten-3-one; and from 19 to 25 nmol/L for ε,ε-carotene-3,3′-dione (all statistically significant at $P < 0.0001$). The serum levels of these carotenoids gradually declined over the 6 months after supplementation ceased. The increase in the serum levels of lutein/zeaxanthin correlated with increases in the serum levels of the metabolites that have previously been identified in the ocular tissues. Other major dietary carotenoids identified and quantified in the sera of the participants were α-carotene, β-carotene, phytofluene, phytoene, lycopene and its metabolites 2,6-cyclolycopene-1,5-diols I and II (cyclolycopenes), β-cryptoxanthin, α-cryptoxanthin and anhydrolutein. The analysis of the mean serum concentrations of these carotenoids in all participants found no interaction with the mean serum concentrations of the supplementary lutein at the three dose levels at various time points throughout the study.

Thurnham, Trémel & Howard (2008) evaluated the absorption of macular pigments from a food supplement by determining plasma levels of carotenoids following daily exposure of human volunteers (10 men and 9 women) to a capsule containing 10.8 mg of lutein, 1.2 mg of (3*R*,3′*R*)-zeaxanthin and 8.0 mg of *meso*-zeaxanthin for 22 days.

(3*R*,3′*R*)-Zeaxanthin was about 50% more actively retained by the body than lutein (although the difference was not significant in women) and 2.5–3.0 times more actively retained than *meso*-zeaxanthin. Compared with literature values for the pure substances, uptake of lutein and zeaxanthin into plasma appeared to be slightly depressed by the presence of *meso*-zeaxanthin. The lower plasma response to *meso*-zeaxanthin probably indicates that it is less well absorbed than zeaxanthin. However, studies with pure *meso*-zeaxanthin would be needed to confirm that the lower plasma response to *meso*-zeaxanthin was not due to the large amount of lutein in the supplement.

The interindividual variability in lutein bioavailability in humans and the relation between this variability and the fasting blood lutein concentration were

associated with single nucleotide polymorphisms involved in this phenomenon. Borel et al. (2014) identified 29 single nucleotide polymorphisms in 15 genes, which explains most of the variance in the postprandial chylomicron lutein response.

In a randomized double-blind placebo-controlled trial, Ma et al. (2009) investigated the plasma response of healthy participants (n = 37) who received a daily dose of 6 or 12 mg lutein or a placebo for 12 weeks, followed by 6 weeks without supplementation. The volunteers avoided excessive consumption of food items known to contain large amounts of lutein during the study. Dietary intake was estimated by food frequency questionnaires at baseline and at study termination. Serum levels of lutein and β-carotene were measured by HPLC at weeks 0, 1, 3, 6, 9, 12 and 18.

No significant sex differences were found in serum concentrations of lutein. Serum lutein levels positively correlated with dietary lutein, retinol equivalents, vitamin C, vitamin E, β-carotene and fat intake, after adjustment for caloric intake, but not with body mass index. After 12 weeks of supplementation, lutein levels had increased approximately 1.8-fold in the 6 mg dose group and 2.3-fold in the 12 mg dose group, having reached a plateau at week 9. Following cessation of supplementation, serum concentrations of lutein decreased to baseline values at week 18. No adverse events or reductions in serum β-carotene were observed throughout the study.

Evans et al. (2013) investigated the bioavailability of lutein and zeaxanthin from two different beadlet formulations. Study participants (n = 48) received a single dose of 20 mg of lutein in a starch matrix or cross-linked to an alginate matrix. Plasma concentrations of lutein and zeaxanthin were measured at 0, 1, 3, 6, 9, 12, 14, 24, 26, 28, 32, 36, 48, 72, 168 and 672 hours.

The mean plasma area under the concentration–time curve (AUC) for 0–72 hours and 0–672 hours, $AUC_{0-72\ h}$ and $AUC_{0-672\ h}$, and maximum plasma concentration (C_{max}) for total lutein and zeaxanthin and their all-*E*-isomers were significantly increased ($P < 0.001$) from pre-dose concentrations in response to both formulations. There was no difference between the two test items in the time for lutein to reach the maximum plasma concentration (T_{max}). However, by 14 hours after dosing, total plasma lutein increased by 7% with cross-linked alginate matrix and by 126% with starch matrix. Total lutein $AUC_{0-72\ h}$ and $AUC_{0-672\ h}$ were 1.8-fold and 1.3-fold higher, respectively, for starch matrix than for cross-linked alginate matrix. Both preparations were well tolerated by study participants.

The authors concluded that bioavailability of lutein and zeaxanthin is related to the matrix in which the carotenoids are embedded.

A more recent review focused on intra- and interindividual differences in carotenoid bioavailability and tissue concentrations in humans and therefore possibly their association with disease risk. In addition to the food matrix, factors affecting carotenoid ADME include existing disease states (e.g. colitis), lifestyle habits (e.g. smoking), sex and age. Genetic variations, including single nucleotide polymorphisms that govern carotenoid metabolism, also explain interindividual differences. For instance, digestive enzymes that foster micellization, uptake/efflux transporter expression, cleavage enzymes, intracellular transporters, secretion into chylomicrons, carotenoid metabolism in the blood and liver and distribution to target tissues such as adipose tissue or macula depend on the activity of these proteins. Human microbiota may also play a role in carotenoid bioavailability (Bohn et al., 2017).

2.1.2 Biotransformation

In a dietary study with rhesus monkeys (*M. mulatta*) fed a xanthophyll-free diet supplemented with pure lutein or pure zeaxanthin for 12–92 weeks, Albert et al. (2008) found 3′-dehydrolutein to be a common metabolite of both lutein and zeaxanthin. Two diastereomers, (3*R*,6′*R*)-3′-dehydrolutein and (3*R*,6′*S*)-3′-dehydrolutein, were identified and shown to be present in nearly equimolar amounts. These findings were considered comparable to the results obtained by Thürmann et al. (2005) with human plasma.

In mammals, β,β-carotene-9′,10′-oxygenase (BCO2) is responsible for mediating the cleavage of xanthophylls at specific sites on the polyene chain. This enzyme, localized in the mitochondria, has been proposed to prevent potentially toxic accumulation of these pigments in tissues (von Lintig, 2012).

Li et al. (2014) reported that the binding affinities between human BCO2 and lutein, zeaxanthin and *meso*-zeaxanthin are 10- to 40-fold weaker than those for mouse BCO2. This suggests that ineffective capture of carotenoids by human BCO2 prevents cleavage of xanthophyll carotenoids. These results explain how primates uniquely concentrate xanthophyll carotenoids at high levels in retinal tissue.

However, later studies found that primate BCO2s are active enzymes that are able to cleave all of the three major macular pigment xanthophylls and that BCO2 expression is a regulated process controlled in part by induction of oxidative stress within mitochondria (Babino et al., 2015).

Together, these findings indicate that, although carotenoids play physiologically beneficial roles in human health, their possible excessive accumulation, which could be related to harmful cellular pathologies, is restrained by the actions of BCO2. The low carotenoid status of patients with

chronic diseases, generally associated with inflammation and oxidative stress, supports this finding.

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Lutein

Mice

Nidhi & Baskaran (2013) investigated the acute toxicity of highly purified lutein (purity >97%; extracted from *Tagetes* petals using solvents and applying a saponification step) in mice. Male lutein-deficient mice (10/group) were administered single doses of 0 (refined peanut oil), 0.57, 100, 1000 or 10 000 mg/kg bw by gavage. The animals were monitored for 14 days for signs of morbidity and mortality, and then killed. Blood and tissue samples were processed to examine haematological, blood chemistry and histopathological changes.

There were no unscheduled deaths or treatment-related clinical signs of toxicity. Ophthalmological and gastrointestinal examinations did not reveal any differences between treated and control groups. No internal gross abnormalities were observed in any of the treated groups at necropsy. Plasma biochemical indices and haematological and histopathological examinations did not show any significant variations between the control and lutein-fed mice (data not shown).

The oral median lethal dose (LD_{50}) was greater than 10 000 mg/kg bw (Nidhi & Baskaran, 2013).

Rats

Ravikrishnan et al. (2011) investigated the acute oral toxicity of a lutein preparation in female Wistar rats. The preparation, extracted from *Tagetes* petals using hexane, followed by saponification and forced isomerization, contained at least 80% carotenoids, of which 67% was lutein and 13.5% were zeaxanthin and *meso*-zeaxanthin in equal proportions. Animals received a single dose of the preparation in refined sunflower oil, by gavage, at a dosage of 2000 mg/kg bw, and were monitored for 14 days.

There were no deaths or treatment-related toxicological signs. No treatment-related gross pathological abnormalities were seen at necropsy.

The oral LD_{50} was greater than 2000 mg/kg bw (Ravikrishnan et al., 2011).

Harikumar et al. (2008) evaluated the acute toxicity of lutein (purity 85%; extracted from marigold flowers using hexane, followed by saponification and crystallization) in female Wistar rats.

There were no deaths and, with the exception of reduced feed intake and diarrhoea in all groups, which was attributed to the sunflower oil vehicle, no test material–related effects were observed at concentrations of up to 4 g/kg bw.

The oral LD_{50} was greater than 4 g/kg bw (Harikumar et al., 2008).

(b) Zeaxanthin

Ravi et al. (2014) investigated the acute toxicity of a zeaxanthin-rich paprika extract containing approximately 65% zeaxanthin (as *R*,*R*′-isomer) and other carotenoids (lutein at 0–1%, β-carotene at 5–15% and β-cryptoxanthin at 4–6%) in female Wistar rats (n = 4). The test material was administered by gavage as a single dose of 0 (refined safflower oil) or 2000 mg/kg bw. The animals were monitored for 14 days for any signs of morbidity or mortality and then killed and necropsied.

There were no unscheduled deaths, treatment-related toxicological signs or treatment-related gross pathological abnormalities.

Based on these results, the authors considered that zeaxanthin-rich extract was neither toxic nor lethal in rats at the acute dose of 2000 mg/kg bw.

The oral LD_{50} was greater than 2000 mg/kg bw (Ravi et al., 2014).

(c) *Meso*-zeaxanthin

Xu et al. (2013) assessed the acute toxicity of a preparation containing *meso*-zeaxanthin at 85% of total xanthophylls after oral administration at up to 10 g/kg bw in mice (10/sex) and rats (10/sex). The animals were observed for 2 weeks.

No toxic effects or morbidity were seen.

The oral LD_{50} was greater than 10 g/kg bw (Xu et al., 2013).

2.2.2 Short-term toxicity

(a) Lutein

Mice

Nidhi & Baskaran (2013) investigated the short-term toxicity of highly purified lutein (>97%; extracted from *Tagetes* petals using solvents, followed by saponification) in male lutein-deficient mice. Animals (10/dose group) were treated by gavage with a daily dose of lutein dispersed in peanut oil (200 µL) at 0 (peanut oil control), 100 or 1000 mg/kg bw per day for 4 weeks. At the end of the treatment period, the animals were killed and blood and tissue samples were collected to assess changes in haematological, blood chemistry and histopathological parameters.

There were no unscheduled deaths or treatment-related abnormal clinical symptoms in any of the treated groups. Ophthalmological examination did not reveal any abnormalities in eyes, the main site of lutein accumulation.

Red blood cell count was lower in high-dose animals, but the values fell within normal laboratory reference data limits and were not associated with changes in related red cell mass parameters (packed cell volume) or red cell indices (mean cell volume, mean corpuscular haemoglobin concentration [MCHC] or mean corpuscular haemoglobin [MCH]) or with microscopic changes in haemopoietic tissues. The decrease was considered incidental or a biological variation and not a treatment-related adverse effect. The statistically significant ($P < 0.05$) dose-related increases in aspartate aminotransferase and alanine aminotransferase activities did not correlate with histopathological changes in liver or other hepatic parameters in plasma (e.g. bilirubin concentration or alkaline phosphatase activity). No significant effects or changes were observed in other assessed parameters. At necropsy, no gross lesions or treatment-related changes in absolute or relative organ weights were noted in lutein-fed mice compared with controls. Yellow coloration of the mucosal surface of the duodenum and caecum, likely due to the adhesion of lutein to the gastrointestinal tract, did not correspond with histopathological changes in intestine.

The no-observed-adverse-effect level (NOAEL) was 1000 mg/kg bw per day, the highest dose tested (Nidhi & Baskaran, 2013).

Rats

Harikumar et al. (2008) investigated the 4-week and 13-week toxicities of free lutein (purity 85%; extracted from marigold flowers using hexane, followed by saponification and crystallization) in rats. In both studies, Wistar rats (5/sex per group) were administered the test preparation once daily by gavage at 0 (vehicle control), 4, 40 or 400 mg/kg bw per day. The animals were monitored for mortality, clinical and behavioural symptoms and adverse reactions to lutein. Feed consumption and body weight were recorded every fifth day. At the end of each treatment period, the animals were killed and necropsied. In addition, in the 4-week study, blood was collected by heart puncture for haematological and serum chemistry assessment, and selected organs (liver, lungs, thymus, spleen, kidneys, brain and eyes) were weighed and histopathologically examined.

In the 4-week study, no signs of toxicity were observed in Wistar rats up to concentrations of 400 mg/kg bw per day, with the exception of slight, though statistically significant, increases in high-density lipoprotein cholesterol levels in some treated groups. In the 13-week study, no adverse clinical signs of toxicity were noted. None of the investigated study parameters were affected by lutein (Harikumar et al., 2008).

It should be noted that both studies included small sample sizes (5 animals/sex per dose group) and only a small number of tissues were examined.

Ravikrishnan et al. (2011) investigated the toxicity of a lutein preparation (≥80% carotenoids, of which 67% was lutein and 13.5% were zeaxanthin and *meso*-zeaxanthin in equal proportions; extracted from *Tagetes* petals using hexane, followed by saponification and forced isomerization) in Wistar rats. Rats (10/sex per group) were treated with the test item by gavage once daily at dose levels of 0 (vehicle control), 4, 40 or 400 mg/kg bw per day for 90 consecutive days. Two additional groups of control and high-dose animals were observed for a subsequent treatment-free 28-day recovery period to detect persistence of or recovery from toxic effects. Clinical signs, feed consumption and body weight were recorded. Urine and blood were collected from all animals during the final week of treatment or the recovery period for clinical evaluation (urine analysis, haematological and serum chemistry parameters). At scheduled necropsy (13 weeks for main groups; 17 weeks for recovery groups), relevant organs from all animals were weighed and over 40 tissues and organs were microscopically and histopathologically examined.

Mortality, clinical observations, feed consumption and growth were not affected by treatment. Urine analysis found no treatment-related effects, with all observations within historical control data ranges. In male rats in all treated groups, a statistically significant decrease in clotting time was recorded, and in low- and high-dose female rats, erythrocyte count and haemoglobin levels were significantly decreased compared with control values. However, these changes were not observed in both sexes, did not correlate with other standard red cell parameters, were small and were not associated with a dose–response relationship; therefore, these changes were not considered treatment related. No treatment-related significant effects on serum chemistry parameters were observed. Absolute organ weights were not affected by treatment. A statistically significant decrease in brain weight (in low- and mid-dose males) and liver weight (in high-dose recovery males) compared with the relevant control groups was observed. However, these statistically significant variations were not considered treatment related as they were within normal biological limits. Macroscopic and microscopic investigations did not reveal any treatment-related effects. Minor lesions were consistent with those typically found in rats of this strain and age; the lesions were considered spontaneous and/or incidental in nature.

The NOAEL was 400 mg/kg bw per day, the highest dose tested (Ravikrishnan et al., 2011).

(b) Lutein diacetate

Santhosh Kumar et al. (2009) determined the toxicity of lutein diacetate (containing 69.0% lutein diacetate, 2.1% zeaxanthin diacetate, 0–5% free lutein, 0–2% free zeaxanthin and 15–30% waxes; extracted from *Tagetes erecta*,

purified and refined in crystalline form) in Sprague Dawley rats. Animals (10/sex per group) were treated by gavage once daily at dose levels of 0 (vehicle control, corn oil), 2.1, 22.5 or 210 mg/kg bw per day for 90 consecutive days. Two additional control and high-dose groups were observed for a subsequent 28-day treatment-free recovery period to detect persistence of or recovery from any toxic effects. The study was conducted in accordance with Organisation for Economic Co-operation and Development Test Guideline (OECD TG) 408. All rats were observed for clinical signs, mortality, ophthalmological abnormalities, neurological changes (functional observational battery) and body weight and feed consumption changes. At the end of the treatment and recovery periods, haematological and clinical chemistry parameters were investigated. At scheduled kill, rats underwent a detailed necropsy, and liver, adrenal glands, kidneys, ovaries, testes, epididymides, uterus, thymus, spleen, brain and heart were weighed. Over 40 full sets of tissues and organs from the high-dose and control groups were histopathologically examined. All gross lesions from rats in all dose groups were also examined for histological changes.

No treatment-related clinical signs were observed in low- and mid-dose animals. Stained (light brown) faeces, likely due to the presence of the pigment in the gastrointestinal tract, were noted in the high-dose group; in the high-dose recovery group, the stained faeces were only noted during the first 4 days of recovery. There were no ophthalmological or neurological effects. Compared with the control group, no changes in mean body-weight, net body-weight gains or feed intake were noted. There were no effects on absolute organ weights or relative (to body weight) organ weights ratios. Lutein diacetate treatment did not affect the haematological or clinical chemistry parameters assessed. Histological examinations did not reveal any treatment-related microscopic changes. Minor changes noted in some of the parameters in the study were not considered treatment related.

The results of this study suggest that lutein diacetate administered orally at doses of up to 210 mg/kg bw per day does not cause toxicological or histopathological abnormalities.

The NOAEL was 210 mg/kg bw per day, the highest dose tested (Santhosh Kumar et al., 2009).

(c) **Zeaxanthin**

Ravi et al. (2014) studied the toxicity of a zeaxanthin-rich paprika extract in Wistar rats. Animals (10/sex per group) were treated once daily with the extract suspended in safflower oil at dose levels of 0, 4, 40 or 400 mg/kg bw per day for 90 consecutive days. The test item was a solvent extract that contained approximately 65% zeaxanthin (as *R*,*R*′-isomer) with other carotenoids (lutein, 0–1%; β-carotene,

5–15%; β-cryptoxanthin, 4–6%). Two satellite groups of control and high-dose animals were observed for a further treatment-free 28-day recovery period. The study was compliant with OECD TG 408. Feed consumption, body weight and a wide battery of clinical end-points were recorded. Urine and blood were collected on day 91 from the 90-day study animals and on day 119 from the recovery group animals for clinical evaluations (urine analysis, haematological and serum chemistry parameters). After necropsy on day 91, relevant organs of all animals were weighed, and over 40 tissues and organs were examined microscopically and histopathologically.

No treatment-related clinical signs or mortalities were observed. Statistically significant effects on feed consumption occurred occasionally, but these were considered incidental as they were not dose related but sporadic. There were no treatment-related adverse effects on haematological parameters in either male or female rats. Small statistically significant changes in mean corpuscular haemoglobin concentration, mean corpuscular haemoglobin and platelet counts were not dose related but almost comparable between groups; they were considered incidental.

There were no treatment-related variations in clinical chemistry parameters at any of the doses tested in any of the groups. Slight but statistically significant ($P < 0.05$) increases in sodium were observed in all treated females and males as well as increases in total protein, urea and globulin in females. These changes were considered toxicologically and biologically nonsignificant as no microscopic changes were observed in any of the dependent organ/tissue. Observed variations in a few other parameters were considered incidental as there was no dose-dependent trend or corresponding microscopic changes in any of the associated organs/tissues. In the recovery study, statistically significant ($P < 0.05$) decreases in alanine aminotransferase (high-dose recovery group males) and potassium (high-dose recovery group females) were observed; these were considered not treatment related as there were no such findings at 91 days. There were no treatment-related variations in urine analysis parameters at any of the doses tested in either sex. Organ weights in control and treated animals were comparable; in some groups in the main study, differences in weights of thymus and spleen were reported but were not dose dependent and corresponding microscopic changes were absent. Macroscopic and microscopic examinations did not reveal any abnormal findings. A few observations were consistent with lesions that occur typically in rats of the age used in the study.

The NOAEL was 400 mg/kg bw per day (equivalent to a dose of zeaxanthin of 260 mg/kg bw per day), the highest dose tested (Ravi et al., 2014).

Vijayabalaji et al. (2014) investigated the potential toxicity of a zeaxanthin suspension in safflower oil (14%) in Sprague Dawley rats. Animals (10/sex per

group) were treated by gavage once daily for 90 consecutive days at dose levels of 0 (vehicle control), 110, 220 or 550 mg/kg bw per day (corresponding to zeaxanthin at 0, 16.5. 33 and 87.5 mg/kg bw per day). The study was compliant with OECD TG 408. All animals were observed for clinical signs, mortality, ophthalmological abnormalities and neurological (functional observational battery), body-weight and feed consumption changes. At the end of treatment period, coagulation parameters, clinical chemistry and urine evaluations were investigated. After necropsy on day 91, relevant organs of all animals were weighed and specified organs were microscopically and histopathologically examined. No treatment-related abnormal clinical signs were observed at any of the doses tested in either sex, other than the brownish-red discoloration of the faeces in animals treated with test item. There were no unscheduled deaths and no treatment-related neurological abnormalities at any of the doses tested in either sex, except for a statistically significant decrease in total and ambulatory motor activity counts in mid-dose group males. This variation was considered incidental and not treatment related as no such variations were observed in the high-dose group. Mean body weights, body-weight gains and feed consumption were also not affected.

No treatment-related changes in any of the haematological parameters were identified. No statistically significant variations in any of the urine analysis parameters studied were observed. Terminal body weights and organ weights (both absolute and relative) of treated groups were comparable to concurrent vehicle controls. Statistically significant increases in absolute and relative brain weights relative to vehicle control groups were observed in low- and mid-dose females, respectively. However, these increases were inconsistent and not dose dependent, and there were no corresponding histopathological findings; these variations were not considered treatment related. No test item–related gross and histopathological findings were observed in any organs or tissues of treated males or females at any dose. Overall, daily oral administration was well tolerated.

The NOAEL was 550 mg/kg bw per day (corresponding to zeaxanthin at 87.5 mg/kg bw per day), the highest dose tested (Vijayabalaji et al., 2014).

(d) *Meso*-zeaxanthin

Thurnham & Howard (2013) investigated the potential toxicity of a commercial *Tagetes* preparation that contained, in the main batch tested, 344 g/kg of carotenoids, of which 210 g/kg was *meso*-zeaxanthin, 53 g/kg was zeaxanthin and 76 g/kg was lutein. According to the authors, the lutein esters in the raw extract were converted by alkaline hydrolysis to a large extent into *meso*-zeaxanthin. Han Wistar rats (15/sex in the control and high-dose groups; 10/sex in the low- and mid-dose groups) were administered the test preparation by gavage at dose

levels of 0, 2, 20 or 200 mg/kg bw per day of *meso*-zeaxanthin (carotenoids at approximately 0, 3, 30 and 300 mg/kg bw per day). After 13 weeks, 10 animals/group were necropsied, and the remaining control and highest-dose animals were observed over a 28-day recovery period prior to necropsy. Animals underwent routine clinical investigations. Blood was collected for routine haematological assessments, and macroscopic and histopathological examinations took place at necropsy.

No compound-related clinical, biochemical or pathological signs or symptoms were noted at any of the doses tested in either sex.

The NOAEL was 200 mg/kg bw per day, the highest dose tested (Thurnham & Howard, 2013; originally reported by Chang, 2006).

Xu et al. (2013) assessed the toxicity of a preparation containing *meso*-zeaxanthin at 85% of total xanthophylls in Sprague Dawley rats. More details on the product were not given, but all toxicity dosing was standardized to pure *meso*-zeaxanthin based on its purity. Animals (10/sex per group) were treated by gavage once daily for 90 consecutive days at dose levels of 0 (vehicle control), 300, 600 or 1200 mg/kg bw per day. All animals were observed for general behaviour and toxicological signs. Body weight and feed consumption were measured and physical examinations performed. Blood samples were collected in the middle of the study (day 42) and 1 day after the final dose (day 90) for haematological and blood chemistry assessments. After necropsy, liver, kidneys, spleen and testes/ovaries were weighed and organ-to-body weight ratios were calculated. Histopathological examinations of these tissues and intestine were conducted.

No statistically significant effects of the test material on clinical observations, body weight, feed consumption, organ weights or haematological and blood chemistry measures were reported. Histopathological examination found significantly increased liver vacuolar degeneration at 600 and 1200 mg/kg bw per day as well as significantly increased inflammatory cell infiltration within liver lobules and spotted liver cell necrosis with inflammatory cell infiltration at 1200 mg/kg bw per day compared with the negative control group.

The authors proposed the NOAEL of *meso*-zeaxanthin in rats to be 300 mg/kg bw per day under the conditions of this study (Xu et al., 2013).

The Committee noted that the reported histopathological findings in liver at 600 and 1200 mg/kg bw per day were observed only in very few animals of both sexes and therefore considered these findings of no biological relevance.

2.2.3 Long-term toxicity/carcinogenicity

In the Khachik et al. (2006a) study evaluating plasma levels and deposition in ocular tissue of lutein and/or zeaxanthin and their metabolites over time (section

2.1.1), biomarkers associated with nephrotoxicity analysis were measured and fundus photography and retinal histopathological assessments performed on the same macaque monkeys.

Supplementation with lutein and zeaxanthin at high doses (9.34 and 10.00 mg/kg bw per day, respectively) or their combination (0.5 mg/kg bw per day of each) did not cause ocular toxicity and had no effect on biomarkers (excreted urinary creatinine and proteins) associated with kidney toxicity.

2.2.4 Genotoxicity

The genotoxic potentials of lutein, zeaxanthin and *meso*-zeaxanthin were evaluated in several bacterial reverse mutation assays, two in vitro chromosome aberration assays in human lymphocytes and Chinese hamster ovary cells and in an in vivo micronucleus test in mouse bone marrow erythrocytes. The results of these assays are presented in Table 1. This combination of tests fulfils the basic requirements to cover the three genetic end-points (i.e. gene mutations and structural and numerical chromosome aberrations).

Based on the results of these studies the Committee concluded that there was no concern with respect to genotoxicity for these macular pigments.

2.2.5 Reproductive and developmental toxicity studies

Xu et al. (2013) assessed a preparation containing *meso*-zeaxanthin at 85% of total xanthophylls for effects on mice sperm. Male ICR mice (7/group) were treated with *meso*-zeaxanthin in distilled water at dose levels of 1.25, 2.5 or 5.0 g/kg bw per day by gavage at 20 mL/kg bw, daily for 5 days. Mitomycin C (MMC) at 2.0 mg/kg bw per day was used as positive control, while distilled water was administered to animals of the vehicle control group. A subgroup (5/group) was selected at random from each group and killed 35 days after the first dose administration. The epididymides was isolated and assessed for sperm abnormality. Morphology was examined on a total of 1000 sperm.

No statistically significant difference in sperm abnormality ratios was observed in any treated group compared with the vehicle control group, while a statistically significant increase ($P < 0.01$) was seen in the positive control group.

(a) Multigeneration reproductive toxicity

Clode (2018) investigated the effects of synthetic zeaxanthin on gonadal function, estrous cycle, mating behaviour, conception, pregnancy, parturition, lactation, weaning and the growth and development of offspring in a 2-generation study. The study was compliant with OECD TG 416 and good laboratory practices. The test item (zeaxanthin content 10%) was administered in the diet to parental generation (P) rats (Crl:WI(Glx/BRL/Han)BR; 24/sex per dose group) at dose

Table 1

Summary of in vitro and in vivo genotoxicity studies with lutein, zeaxanthin and *meso*-zeaxanthin

End-point	Test system	Test substance	Concentration	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA97, TA98, TA100 and TA102	Free lutein (80% purity)	334–1 335 µg/plate, ±S9	Negative [a]	Wang et al. (2006)
Reverse mutation	*S. typhimurium* TA1535, TA1537, TA98, TA100 and TA102	Lutemax 2020™ (from *Tagetes erecta*) containing 67% lutein, 13.5% zeaxanthin and 13.5% meso-zeaxanthin	31, 63, 125, 250 and 500 µg/plate, ±S9	Negative [b]	Ravikrishnan et al. (2011)
Reverse mutation	*S. typhimurium* TA1535, TA1537, TA98 and TA100; *Escherichia coli* WP2uvrA	Commercial *Tagetes* preparation containing 30.4% lutein, 17.05% zeaxanthin and 51.15% *meso*-zeaxanthin	10.0, 33.3, 100, 333, 1 000 and 5 000 µg/plate, ±S9	Negative [c]	Thurnham & Howard (2013)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100 and TA102	Preparation containing *meso*-zeaxanthin at 85% of total xanthophylls	40, 200, 1 000 and 5 000 µg/plate, ±S9	Negative [d]	Xu et al. (2013)
Reverse mutation	*S. typhimurium* TA1535, TA1537, TA98, TA100 and TA102	Paprika extracts (OmniXan™) containing 65% zeaxanthin and lutein 0–1%	62.5, 125, 250, 500 and 1 000 µg/plate, ±S9	Negative [e]	Ravi et al. (2014)
Reverse mutation	*S. typhimurium* TA1535, TA1537, TA98, TA100 and TA102	Commercial preparation (ZeaONE™) containing 14% zeaxanthin in safflower oil	1st experiment: 312.5, 625, 1 250, 2 500 and 5 000 µg/plate, ±S9 (5%) 2nd experiment: 128, 320, 800, 2 000 and 5 000 µg/plate, ±S9 (10%)	Negative [e]	Ghosh et al. (2014)
Chromosome aberration	Chinese hamster ovary cells	Free lutein (80% purity)	1st experiment: 66.8, 133.5 and 267.0 µg/mL, ±S9, 6-h treatment 2nd experiment: 66.8, 133.5 and 267.0 µg/mL,–S9, 24- and 48-h treatment	Negative [f]	Wang et al. (2006)
Chromosome aberration	Human peripheral blood lymphocytes	Commercial preparation (ZeaONE™) containing 14% zeaxanthin in safflower oil	156, 312, 625, 1 250 and 2 500 µg/mL, ±S9	Negative [g]	Ghosh (2014)
In vivo					
Micronucleus induction	Mouse, males and females	Preparation containing 85% *meso*-zeaxanthin of total xanthophylls	1 250, 2 500 and 5 000 mg/kg bw	Negative [h]	Xu et al. (2013)

bw: body weight; OECD TG: Organisation for Economic Co-operation and Development Guideline; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Limited validity: A single experiment was performed (plate incorporation method) using an incomplete set of bacterial strains (TA1535 is missing).

[b] Dose range selected based on solubility and precipitation of test item. The study essentially complies with current OECD TG 471.

[c] A single experiment was performed. Precipitation of test material was observed at 333 µg/plate and higher concentrations.

[d] Limited validity: An incomplete set of bacterial strains was used (TA1535 is missing). Two independent experiments were performed, but no modification was introduced in the second experiment. Only four concentrations were tested. No precipitation or cytotoxicity was reported.

[e] Dose range selected based on solubility, precipitation and cytotoxicity of test item. The study essentially complies with current OECD TG 471.

[f] Limited validity: Inadequate setting of harvesting times; limited number of metaphases scored; and no cytotoxicity evaluation performed.

[g] The study complies with the current OECD TG 473.

[h] Two doses were administered by gavage at 0 and 24 hours. Examinations were 6 hours after the final dose (i.e. at 30 hours). There were no increases in group mean micronuclei in treated groups compared with vehicle control, but there was no evidence of target tissue exposure because all treated groups exhibited proportions of immature erythrocytes (polychromatic erythrocytes) that were similar to vehicle controls.

levels of 455, 1364 or 4545 mg/kg bw per day (corresponding to 50, 150 and 500 mg/kg bw per day of active zeaxanthin ingredient). Two additional groups received either the control (basal) diet alone or placebo beadlets incorporated in the diet at the same concentrations as the zeaxanthin beadlets in the high-dose group. The animals received the test diets for 10 weeks before pairing and until necropsy. The P females were allowed to litter and rear their offspring (F_{1a}) to weaning. Animals (24/sex per dose group) were randomly selected to form the main study generation (F_1). Direct treatment of the F_1 animals started at weaning and continued for 10 weeks post weaning (maturation phase), pairing and until necropsy. All F_1 females were allowed to litter and rear their offspring (F_{2a}) to weaning. F_{1a} weanlings (10/sex per group) were randomly selected for assessment of functional and behavioural development. Samples of plasma and liver from the parental adults and F_{1a} weanlings pups were analysed for zeaxanthin.

There were no adverse effects on mating and fertility, pregnancy outcome, pup survival, pup growth or on seminology or organ weight data in parental animals. There were no treatment-related macroscopic findings at necropsy or microscopic findings at histopathological examination. There were also no adverse effects on F_1 animal learning or memory in the swimming maze, physical development, functional and behavioural development or motor activity. Although there were intergroup variations during the pairing phase of the study, there were no effects on mating or fertility. The mean numbers of implantations and pup survival were similar across all groups.

In the F_1 high-dose group, mean pup body-weight gain between postpartum days 4 and 7 was slightly lower than controls. No effect on maternal feed consumption was recorded between postnatal days 4 and 7. Mean pup body weight at weaning was similar to the controls and the lower pup body weight was considered nonadverse. There were no adverse effects on seminology or organ weight data. There were no macroscopic findings at necropsy or microscopic findings at histopathological examination, and the number of ovarian follicles were unaffected by treatment.

There were no adverse effects of treatment on the spleen, brain or thymus weights of the selected F_{2a} weanlings.

There was no evidence of toxicity in either generation, but the coats of the treated animals, particularly those at high dose, became stained with the test article. Routine histopathological examination of P and F_1 adult reproductive tissues (right testis, right epididymis, seminal vesicles, prostate, coagulating gland, ovaries, uterus/cervix and vagina) found no treatment-attributable changes.

The NOAEL was 4545 mg/kg bw per day (corresponding to the nominal dosage of 500 mg/kg bw per day of zeaxanthin), the highest dose tested (Clode, 2018).

2.3 Observations in humans

2.3.1 Clinical studies

In its review of lutein (free form) from *Tagetes erecta* at its sixty-third meeting, the Committee indicated that dietary lutein is well tolerated in humans (Annex 1, reference *173*). At its seventy-ninth meeting, the Committee evaluated a number of studies in humans that investigated the effects of the lutein esters, administered primarily as nutritional supplements and as therapeutic agents for age-related macular degeneration, on plasma levels of lutein and the level of the xanthophyll-containing macular pigment (Annex 1, reference *220*). Although these human studies were not designed as part of the safety assessment process, it was noted that these published studies did not report any adverse effects with doses of lutein esters from *Tagetes erecta* of up to 30 mg/day for up to 140 days.

The new available data are described below.

(a) Infants

Breast milk contains appreciable levels of lutein derived from the mother's diet (Capeding et al., 2010). Several studies compared the intake of this carotenoid from lutein-fortified infant formula against control diet and/or breast milk diet.

Capeding et al. (2010) evaluated an experimental infant formula containing lutein in a 16-week prospective, randomized controlled double-blind study with parallel groups of healthy term infants fed either control formula or experimental formula (fortified with lutein at 200 µg/L). Of the 232 infants aged 14 days or less who were initially randomized, 220 (94.8%) completed the study (110 with lutein, 110 as control). The primary end-point was weight gain (g/day) from baseline to week 16. Safety was assessed by monitoring events throughout the study and evaluating selected blood chemistry test results at week 16.

No differences between treatment groups were found as determined by net weight gain and serum chemistry by study end.

Bettler et al. (2010) compared the bioavailability of lutein in control infant formula (lutein at 20 µg/L) and fortified infant formulas (lutein at 45, 120 and 225 µg/L) with the bioavailability in breast milk (~20 µg/L). The lutein formula–fed groups included 6 or 7 infants per group and the breastfed group included 14 infants. Lutein concentrations in serum were determined at the beginning and end of the 12-week study. At week 12, the human breast milk group had a 6-fold

higher geometric mean serum lutein (69.3 µg/L) than the unfortified formula group (11.3 µg/L). Mean serum lutein increased from baseline in each formula group except the unfortified group. At the end of the study, infants fed formula fortified with lutein at 45, 120 and 225 µg/L had mean serum lutein levels of 35.9, 197.5 and 192.1 µg/L. These data suggest that approximately 4 times more lutein than is found in human milk is needed in infant formula to achieve similar serum lutein concentrations in breastfed and formula-fed infants. The safety evaluation concluded that infant growth was within normal limits as assessed by mean weight gain in g per day as well as *Z*-scores in weight, length and head circumference. No adverse formula-related events were observed.

Kon et al. (2014) evaluated an experimental infant formula fortified with, among other ingredients, lutein at 11.4 µg/100 mL, for safety, gastrointestinal tolerance and effects on growth. Of the 23 infants aged 10 days to 2 months who received the formula, 17 completed the 28-day study. The lutein was derived from marigold (*Calendula*), but no further details were given. No control group was included.

The formula was well tolerated and supported growth of the evaluated infants.

In a study designed to test the hypothesis that neonatal supplementation with lutein in the first hours of life reduces neonatal oxidative stress in the immediate postpartum period, Perrone et al. (2014) conducted a randomized double-blind control trial with 150 newborns (control, n = 47; treated, n = 103). Before first breastfeeding, infants received either 0.28 mg lutein or control vehicle. Total hydroperoxide, advanced oxidation protein products and biological antioxidant potential were investigated in blood samples at birth from the umbilical cord and from the infant 48 hours after birth.

Supplementation increased biological antioxidant potential and reduced total hydroperoxide. No treatment-related adverse effect was documented in the lutein-supplemented infants.

Costa et al. (2013) investigated the effects of lutein supplementation on biological antioxidant status in preterm infants in a randomized clinical trial. Infants (n = 77; gestational age <34 weeks) were randomly assigned to receive a daily dose of lutein and zeaxanthin (0.5 + 0.02 mg/kg bw per day; n = 38) or placebo (n = 39) from day 7 of life until the fortieth week of postmenstrual age or until discharge (6 weeks of administration). The free lutein was prepared from *Tagetes oleoresin* and included a saponification and crystallization stage, which meets current JECFA specifications. Statistically significant linear correlations between plasma lutein and zeaxanthin concentrations and total antioxidant

status was evident. However, the total antioxidant status did not differ statistically significantly during the study period. Plasma concentrations of total cholesterol, triglycerides and high-density lipoprotein cholesterol at the weekly determinations did not differ between the groups during the study. Health status was monitored in depth; there were no significant differences between groups with respect to baseline characteristics and the main morbidities (respiratory distress syndrome, necrotizing enterocolitis, bronchopulmonary dysplasia, intraventricular haemorrhage and bacterial sepsis) and therapies. Lutein at 0.5 mg/kg bw per day for 6 weeks was well tolerated by preterm infants.

(b) **Adults**

In the Ma et al. (2009) study designed to evaluate serum levels of lutein and β-carotene in human volunteers who received a daily dose of 6 or 12 mg lutein for 12 weeks (section 2.1.1), no significant adverse events or changes in biochemical or haematological indices were observed throughout the study.

Molldrem et al. (2004) used a randomized blinded 3 × 3 cross-over intervention to evaluate and compare lutein uptake and clearance after ingestion of genetically selected lutein-containing yellow carrots (1.7 mg/day of lutein), white carrots (0 mg/day of lutein; negative control) or a lutein supplement (1.7 mg/day of commercial pure crystalline lutein dissolved in oil; positive control). The study participants (5 men and 4 women; aged 23–28 years) remained in the study for 50 days, including a 1-week washout period before the study and between each 7-day long treatment.

Mean peak changes in serum lutein concentration from baseline were 0.31, 0.19 and 0.04 μmol/L for the supplement, yellow carrot and white carrot treatments, respectively. $AUC_{0-14\ d}$ differed significantly ($P < 0.0001$) between treatments. Lutein from the yellow carrot treatment was 65% as bioavailable as that from the supplement. The $AUC_{0-14\,d}$ for β-carotene also showed that the yellow carrot treatment maintained peak serum β-carotene concentrations, whereas the lutein supplement treatment was associated with a decrease.

In the Khachik et al. (2006b) study designed to evaluate the effect of commercially available lutein supplementation on the distribution of lutein, zeaxanthin and their metabolites in the sera of study participants aged 60 years and older (section 2.1.1), no toxicity or side-effects were associated with supplementation with lutein up to a dose of 10 mg per day, based on results of liver function and visual function tests.

Sherry et al. (2014) determined the impact of lutein supplementation on breast milk and plasma of lactating women and on the plasma of their 2–3 month old breastfed infants. Eighty-nine lactating women who were 4–6 weeks postpartum were randomly assigned to receive 0 (placebo), 6 or 12 mg per day of lutein. The nutritional supplements were consumed for 6 weeks while the mothers followed their usual diets. Breast milk carotenoids were measured weekly by HPLC, and maternal plasma carotenoid concentrations were measured at the beginning and end of the study. Infant plasma carotenoid concentrations were assessed at the end of the study.

Lactating women were found to be highly responsive to lutein supplementation. Maternal supplementation also affected plasma lutein concentrations in the infant. No significant differences were found between dietary lutein plus zeaxanthin intake and carotenoid concentrations in breast milk and plasma or body mass index at baseline. Total lutein plus zeaxanthin concentrations in breast milk were greater in the low- and high-dose groups than in the placebo group (140% and 250%, respectively; $P < 0.0001$) as well as in maternal plasma (170% and 250%, respectively; $P < 0.0001$) and infant plasma (180% and 330%, respectively; $P < 0.05$). Lutein supplementation did not affect levels of other carotenoids in lactating women or their infants.

3. Dietary exposure

The Forty-ninth Session of the Codex Committee on Contaminants in Food Additives requested that the Committee re-evaluate information on safety, dietary intake and specifications to extend the group ADI "not specified" for lutein esters to include lutein from *Tagetes erecta*, synthetic zeaxanthin and related xanthophylls (FAO/WHO, 2017a).

The sixty-third JECFA meeting estimated dietary exposure to lutein from *Tagetes erecta* (Annex 1, reference *173*). The sixty-third Committee reported estimates for the intake of lutein from natural sources in the range of 1–2 mg/day (approximately 0.01–0.03 mg/kg bw per day) based on a number of studies in North America and the United Kingdom. Simulations considering proposed levels of use as a food ingredient resulted in an estimated mean intake and 90th percentile intake of lutein plus zeaxanthin of approximately 7 and 13 mg/day, respectively (equivalent to 0.12 and 0.22 mg/kg bw per day).

At the seventy-ninth meeting, the use of lutein esters from *Tagetes erecta* as well as synthetic zeaxanthin was considered to be substitutional for the use of lutein from *Tagetes erecta* (Annex 1, reference *220*).

Lutein esters from *Tagetes erecta* were used in the same food categories and at the same use levels as those evaluated for lutein from *Tagetes erecta* at the sixty-third meeting. Based on their estimated mean and 90th percentile dietary exposures for lutein plus zeaxanthin of approximately 7 and 13 mg/day, respectively (equivalent to 0.12 and 0.22 mg/kg bw per day, respectively), and assuming a conversion factor of 1.8 to reflect the increased molecular weight due to the fatty acid moieties, the seventy-ninth Committee estimated that exposure to lutein esters from *Tagetes erecta* would be up to 24 mg/day (equivalent to 0.4 mg/kg bw per day, assuming a 60 kg body weight).

The European Food Safety Authority (EFSA, 2010) estimated the mean exposure to lutein naturally occurring in the diet to be up to 2.5 mg/day for both adults and children, equivalent to 0.04 and 0.1 mg/kg bw per day, respectively. A refined assessment of exposure to lutein from *Tagetes erecta* was carried out by EFSA (2012) based on updated use levels for lutein from *Tagetes erecta*, Estimated exposures to lutein were 0.1 mg/kg bw per day at the mean and of 0.3 mg/kg bw per day at the 97.5th percentile in the case of adults from the United Kingdom and ranging from 0.1 to 0.4 mg/kg bw per day at the mean and from 0.1 to 1.0 mg/kg bw per day at the 95th/97.5th percentile in the case of children from 11 European countries (section 3.2.1(a)).

A comprehensive literature search for exposure from the xanthophylls, intake of supplements and natural occurrence of lutein esters was conducted following the JECFA Secretariat guidance (FAO/WHO, 2017b). The keywords used in the searches included ((lutein OR zeaxanthin OR xanthophyll) AND (intake OR exposure)) published after 2009, ((lutein OR zeaxanthin OR xanthophyll) AND supplement) and (Lutein esters). In addition, searches using literature links, Google Scholar and Technical University of Denmark (DTU) Library search facilities were performed. The literature search retrieved 20 additional references relevant to the dietary exposure assessment.

3.1 Food uses

Lutein and zeaxanthin are present in vegetables (especially leafy green vegetables such as spinach, chard, parsley and kale) and some animal products (e.g. eggs). Amounts and ratios of xanthophylls vary between species and cultivars and depend on growing conditions, preparation methods, etc. (Holden et al., 1999). The xanthophylls can be also be found in esterified forms, especially in fruits and cereals (Breithaupt & Bamedi, 2001; Mercadante et al., 2017; Paznocht et al., 2018); esterification may influence the bioavailability (Norkus et al., 2010; Mercadante et al., 2017).

The Committee did not explicitly assess exposure to *meso*-zeaxanthin although it is present in some commercial dietary supplements.

In dietary supplements based on *Tagetes erecta*, the ratio between lutein and zeaxanthin usually is around 5 : 1.

In the sponsor's submission for the present assessment, lutein, lutein esters and synthetic zeaxanthin were intended for use as food colours. When used as a food colour, these substances are substitutional on a molar basis, and exposures are expressed as lutein throughout the monograph.

Lutein occurs naturally in breast milk and can be added to infant formula as a nutritive substance (Australian Federal Register of Legislation, 2015). However, infants were not included in the present JECFA assessment as the ADI is not applicable to this population group.

The sponsor provided proposed use levels of lutein, lutein esters and synthetic zeaxanthin in a number of foods. The proposed use levels used to estimate dietary exposure at the sixty-third and seventy-ninth JECFA meetings (Annex 1, references *173* and *220*) were adjusted in line with information from the sponsor on the expected use levels based on the stoichiometric equivalence of the three components, lutein, zeaxanthin and lutein esters from *Tagetes erecta* and zeaxanthin (synthetic) (Table 2).

3.2 Assessment of dietary exposure

3.2.1 Estimated dietary exposure from use as food colour using food consumption data

EFSA (2012) estimated exposure to lutein using the maximum reported use levels provided by the food industry (Table 3), combined with consumption data from national surveys of individuals aged 1 year and older conducted in 11 countries in Europe. Exposure for European adults to lutein from *Tagetes erecta* was estimated to be 0.1 mg/kg bw per day at the mean and 0.3 mg/kg bw per day at the 97.5th percentile. For European children, the estimated exposure ranged from 0.1 to 0.4 mg/kg bw per day at the mean and from 0.1 to 1.0 mg/kg bw per day at the 95th/97.5th percentile.

The Committee agreed that a high exposure to lutein and zeaxanthin and their esters (in combination) from use as food colour of 0.4 mg/kg bw per day for adults and 1 mg/kg bw per day for children would be used for the exposure assessment. These estimates were higher than those estimated by the Committee at the sixty-third meeting (Annex 1, references *173*).

Table 2

Proposed use levels for lutein and lutein esters from *Tagetes erecta* and zeaxanthin (synthetic)

GSFA category		Use levels [a] (mg/kg)		
No.	Name	Lutein + zeaxanthin from *Tagetes erecta*	Zeaxanthin (synthetic)	Lutein esters from *Tagetes erecta* [b]
01.1.2	Flavoured milk and milk drinks	16	16	23
01.2.1	Fermented milk beverages	3.1	3.1	4.7
01.3.3	Imitation milks	10	10	15
1.5	Dry milk	16	16	23
01.5.2	Soy milks	7.5	7.5	11
1.7	Frozen yoghurt	10	10	15
	Yoghurt	16	16	23
02.2.1.2	Margarine-like spreads	120	120	180
5.2	Hard candy	80	80	121
	Chewy and nougat candy	30	30	45
	Fruit Snacks	30	30	45
5.3	Chewing gum	397	397	594
6.3	Ready-to-eat cereals	43–157	43–157	78–282
6.5	Instant and regular hot cereals	10	10	15
07.1.2	Crackers and crispbreads	80	80	120.6
10.2	Liquid, frozen or dried egg substitutes	48	48	72
12.5.1	Canned Soups	3.1	3.1	4.7
12.6.1	Salad dressings	60–120	60–120	108–216
12.6.2	Tomato-based sauces	3.1	3.1	4.7
13.2	Junior, strained and toddler type baby foods [c]	7.1–169	7.1–169	13–303
13.4	Meal replacements	10	10	15
	Milk-based meal replacements	16	16	23
14.1.1.1	Bottled water	2.5	2.5	3.8
14.1.2.1	Fruit juice	10	10	15
14.1.2.2	Vegetable juice	10	10	15
14.1.3	Nectars	10	10	15
14.1.4	"Energy", "sport" and isotonic drinks	10	10	15
14.1.4.1	Carbonated beverages	10	10	15
	Fruit-flavoured drinks	10	10	15
14.1.5	Tea, ready-to-drink	3.1	3.1	4.7
15.1	Cereal and energy bars	60	60	90

GSFA: General Standard for Food Additives; no.: number

[a] When a range of use levels is reported for a proposed food use, particular foods may differ with respect to their serving size.

[b] Intended use levels of lutein esters have been estimated by assuming a conversion factor of 1.8 to reflect the increased molecular weight due to the fatty acid moieties.

[c] Does not include infant formula.

Table 3
Use levels for lutein used in the exposure estimate

Beverages and foodstuffs	Maximum reported use level
Drinks (mg/L)	
Nonalcoholic flavoured drinks	15
Foods (mg/kg)	
Confectionery	45
Decorations and coatings	60
Fine bakery wares (egg, Viennoiserie, biscuits, cakes, wafers)	10
Edible ices	20
Desserts including flavoured milk products	15
Sauces, seasonings (e.g. curry powder, tandoori), pickles, relished, chutney, piccalilli	20
Snacks: dry, savoury potato, cereal or starch-based snack products, extruded or expanded savoury snack products	60
Savoury snack products and savoury coated nuts	60
Edible cheese rind and edible chasings	120
Soups	10
Meat and fish analogues based on vegetable protein	2.9

Source: EFSA (2012)

3.2.2 Dietary exposure from use in dietary supplements

There are currently no internationally recommended dietary intake levels for lutein and zeaxanthin. The Republic of Korea Health Functional Code recommends 10–20 mg/day as lutein (Ministry of Food and Drug Safety, 2008). An intake of 6 mg of lutein and zeaxanthin per day for adults has been suggested as reducing the risk of age-related macular degeneration (Rasmussen & Johnson, 2013). Eisenhauer et al. (2017) indicated that lower intake levels may reduce the risk of age-related macular degeneration, while other studies fail to find significant effects of dietary supplements with lutein/zeaxanthin – at least for healthy individuals eating a healthy diet (Trumbo & Ellwood, 2006; Zampatti et al., 2014).

Dietary supplements in the form of tablets or capsules with 10–40 mg of lutein and/or zeaxanthin are commercially available. Suppliers of these products recommend daily intakes in the range of 10–40 mg lutein and zeaxanthin; a daily dose of 20 mg is often recommended. Breithaupt & Schlatterer (2005) found lutein concentrations from 0.25 to 20 mg per serving size in 14 products.

The Committee assumed a high exposure of 40 mg lutein and zeaxanthin (in combination) per day for adults (equivalent to 0.67 mg/kg bw per day) to assess dietary exposure to lutein and zeaxanthin from their use as dietary supplements. A dietary exposure from the use of lutein and zeaxanthin as dietary supplements of 0.67 mg/kg bw per day was also assumed for children.

3.2.3 Dietary exposure from natural occurrence in food

Lutein and zeaxanthin and their esters occur naturally in foods. Analytical procedures sometimes include hydrolysis that could include the esters in the measured concentrations of lutein and zeaxanthin. However, such hydrolysis may also have a negative effect on the content of lutein and zeaxanthin (Khachik, Beecher & Whittaker, 1986; Riso & Porrini, 1997). It is possible to analyse the free and esterified forms directly (Young et al., 2007; Abdel-Aal & Rabalski, 2015; Ziegler et al., 2015), but esters of xanthophylls are not always addressed in papers listing analytical results for the xanthophylls (Burkhard & Böhm, 2007; Perry, Rasmussen & Johnson, 2009).

At the sixty-third meeting, the Committee reported estimates for the dietary exposure to lutein from natural sources to be in the range of 1–2 mg/day (approximately 0.01–0.03 mg/kg bw per day) and that exposure to zeaxanthin was in the same range. Estimates were based on a number of studies in North America and the United Kingdom.

EFSA (2012) estimated the exposure to lutein naturally occurring in the diet at a mean of 2.5 mg/day for both children and adults, equivalent to 0.1 mg/kg bw per day for children and 0.04 mg/kg bw per day for adults. The exposure to lutein from the diet at the 95th percentile was estimated to be 7 mg/day for both children (equal to 0.28 mg/kg bw per day) and adults (equal to 0.12 mg/kg bw per day). Estimates were based on studies from the United States, Canada and European countries.

In studies from China, the Republic of Korea and European countries (Table 4), exposure estimates ranged from 1.2 mg/day (Spain) to 8 mg/day (China), equal to 0.02 and 0.13 mg/kg bw per day, respectively, for adults at 60 kg.

For the exposure assessment, the Committee agreed to use 0.13 mg/kg bw per day of lutein and zeaxanthin (in combination) as the value for high exposure from natural occurrence in food for adults and 0.69 mg/kg bw per day in children. These estimates were higher than those estimated by the Committee at the sixty-third meeting.

3.2.4 Aggregated exposure

The aggregated dietary exposure from use of lutein, zeaxanthin and their esters from *Tagetes erecta* and/or zeaxanthin (synthetic) as food colour, food supplements and in combination with the natural occurrence of these xanthophylls was estimated to be 1.2 mg/kg bw per day for adults and 2.4 mg/kg bw per day for children (Table 5).

Table 4
Estimated daily exposure of lutein and zeaxanthin from naturally occurring sources in food

Country	Compound(s)	Population, sex, age	Estimated daily exposure (mg/d)	Dietary intake method	Source of concentration data	Reference
China	Lutein + zeaxanthin	Females (25–70 y)	3.3–7.0 (25th–75th percentile, group 1) 3.5–8.0 (25th–75th percentile, group 2)	Food frequency questionnaire ($n = 561$) Food frequency questionnaire ($n = 561$)	US Department of Agriculture nutrient database	Wang et al. (2014)
Republic of Korea	Lutein + zeaxanthin	Children (2–18 y) [a]	3.1 (mean)	24-h dietary recall ($n = 8\,502$)	Food composition table from the Republic of Korea	Lee et al. (2013)
		Adults (>18 y) [b]	5.2 (mean)			
		Children (2–18 y) [c]	1.5 (mean)			
		Adults (>18 y) [d]	2.6 (mean)			
Netherlands	Lutein + zeaxanthin	Adults	2.4 (median in lowest quartile) 5.3 (median in highest quartile)	Food frequency questionnaire ($n = 37\,846$)	Dutch food composition table	Sluijs et al. (2015)
Spain	Lutein + zeaxanthin	Adults	1.2	24-h dietary recall (n = 3 000)	Spanish database of carotenoid content in foods	Estévez-Santiago, Beltrán-de-Miguel & Olmedilla-Alonso (2016)
Spain	Lutein + zeaxanthin	Pregnant women	3.1 (mean)	Semiquantitative food frequency questionnaire ($n = 740$)	Spanish and US food composition tables	Vioque et al. (2013)
United Kingdom	Lutein	Females (18–40 y)	0.15–0.51 (25th–75th percentile) 0.5–1.8 (25th–75th percentile)	Food frequency questionnaire 24h dietary recall ($n = 64$)	UK dietary composition tables	Coathup, Wheeler & Smith (2016)

[a] Children of the Republic of Korea meeting national dietary recommendations for fruit/vegetables.
[b] Adults of the Republic of Korea meeting national dietary recommendations for fruit/vegetables.
[c] Children of the Republic of Korea not meeting national dietary recommendations for fruit/vegetables.
[d] Adults of the Republic of Korea not meeting national dietary recommendations for fruit/vegetables.

4. Comments

4.1 Biochemical aspects

The ADME of lutein and lutein esters from *Tagetes erecta* was extensively described in the monographs at the sixty-third and seventy-ninth meetings (Annex 1,

Table 5
Estimates of high dietary exposure to lutein, zeaxanthin and their esters from *Tagetes erecta* and zeaxanthin (synthetic)

	Estimated dietary exposure, mg/kg bw per day (%) [a]	
Source of exposure	**Adults**	**Children**
Food colour [b]	0.4 (33)	1 (42)
Dietary supplements [c]	0.67 (56)	0.67 (28)
Natural occurrence [d]	0.13 (11)	0.69 (29)
Aggregated exposure	1.2	2.4

bw: body weight
[a] Estimated dietary exposure in mg/kg bw per day and, in parentheses, the estimated exposure as a percentage of the total (aggregated) exposure.
[b] Based on assessments from previous meetings of the Committee (JECFA 2005) and by EFSA (2012).
[c] Estimated from commercial recommendations and intervention studies.

references *174* and *221*). The absorption of lutein involves emulsification by bile, followed by lipolysis by pancreatic lipases into the micellar fraction for absorption by intestinal cells. Absorption of xanthophylls from esters requires hydrolysis. Hydrolysis is an efficient process as esterified lutein is not normally found in human serum. These processes have been shown to be influenced by food matrices, for example, dietary fibres reduce absorption whereas dietary fats promote absorption due to the hydrophobic nature of these xanthophylls. The bioavailability of lutein following administration of lutein esters has been shown to be equivalent to the administration of free lutein.

Sheshappa et al. (2015) investigated the influence of food matrices in lutein-deficient rats. Lutein levels in plasma, liver and eyes were higher than in the controls when animals were administered lutein-mixed micelles containing either 3% fat, phosphatidyl choline or lyso-phosphatidyl choline. In contrast, the administration of lutein-mixed micelles containing pectin and mixed xanthophylls resulted in lutein levels that were lower than in the controls. Evans et al. (2013) found that in humans given a single dose of 20 mg of lutein in a starch-based matrix or cross-linked to an alginate matrix, lutein from the starch-based product was better absorbed than lutein from the alginate one.

In humans, dietary supplements of lutein (containing 5% zeaxanthin) at 10 mg/day for 6 months increased the mean serum level of lutein from a baseline value of 210 to 1000 nmol/L and of zeaxanthin from 56 to 95 nmol/L (Khachik et al., 2006b).

Albert et al. (2008) observed that monkeys fed a xanthophyll-free diet supplemented with either pure lutein or pure zeaxanthin for 12–92 weeks formed 3′-dehydrolutein in plasma. In addition, two 3′-dehydrolutein diastereomers, (3*R*,6′*S*)- and (3*R*,6′*R*)-3′-dehydrolutein, were present in nearly equimolar concentrations. The authors considered these findings to be comparable to those

in human plasma after dietary supplementation with either lutein or zeaxanthin at doses from 1 to 20.5 mg/day for 42 days (Thürmann et al., 2005).

4.2 Toxicological studies

The Committee previously concluded that the NOAEL for lutein was 200 mg/kg bw per day, the highest dose tested in a 13-week rat toxicity study, and 1000 mg/kg bw per day, the highest dose tested in a rat developmental toxicity study. For lutein esters from *Tagetes erecta*, the NOAEL was 1000 mg/kg/bw per day (equivalent to 540 mg/kg bw per day of lutein), the highest dose tested in both a 13-week and a developmental toxicity study in rats.

The studies reviewed by the Committee confirm that lutein, zeaxanthin and *meso*-zeaxanthin, tested in their free form, are of very low toxicity. The only substance-related finding was discoloration of faeces and fur or skin in animals at higher doses. The NOAELs in short-term and long-term toxicity studies were approximately 210–400 mg/kg bw per day for lutein, 87.5–260 mg/kg bw per day for zeaxanthin and 200–300 mg/kg bw per day for *meso*-zeaxanthin in rats and 10 mg/kg bw per day for lutein and zeaxanthin in monkeys. These NOAELs were generally the highest dose levels tested (Ravikrishnan et al., 2011; Thurnham & Howard, 2013; Ravi et al., 2014).

A 2-generation reproductive toxicity study of zeaxanthin in rats indicated that administration of up to 500 mg/kg bw per day, the highest dose tested, did not cause any adverse effects.

Based on previously and newly available data, the Committee considered that lutein, zeaxanthin and *meso*-zeaxanthin did not raise concerns with respect to genotoxicity.

4.3 Observations in humans

The Committee previously reviewed the results of clinical studies in which lutein and lutein esters from *Tagetes erecta* were given as nutritional supplements or therapeutic agents for age-related macular degeneration (Annex 1, references *173* and *220*). Although these studies were not designed as safety assessments, lutein and lutein esters were found to be well tolerated. This was confirmed based on evaluations of newly available clinical studies. These clinical studies included healthy and preterm infants given lutein in infant formula at concentrations equal to 0.5 mg/kg bw per day for 6 weeks. Doses for adults were up to 10 mg/person per day (Khachik et al., 2006b; Costa et al., 2013).

4.4 Assessment of dietary exposure

At the sixty-third meeting, the Committee estimated mean and 90th percentile dietary exposure to lutein from *Tagetes erecta* as approximately 7 and 13 mg/day, respectively (equivalent to 0.12 and 0.22 mg/kg bw per day, assuming a 60 kg body weight) (Annex 1, reference *173*).

At the seventy-ninth meeting, the use of lutein esters from *Tagetes erecta* was considered to be substitutional for the use of lutein from *Tagetes erecta*.

The present Committee estimated exposure to lutein, zeaxanthin and their esters from *Tagetes erecta* and zeaxanthin (synthetic). When used as a food colour, these substances are substitutional on a molar basis, and exposures are expressed as lutein throughout the report. The estimates included exposure from the use in food supplements. Exposure to *meso*-zeaxanthin was not explicitly assessed by the Committee although it is present in some commercial food supplements.

A comprehensive literature search retrieved 20 additional references relevant to the dietary exposure assessment.

Dietary exposure from the use of lutein, zeaxanthin and their esters and zeaxanthin (synthetic) as a food colour was estimated as 0.3 mg/kg bw per day for adults and 1 mg/kg bw per day for children by EFSA (2012). These estimates, based on maximum reported use levels and national consumption data from 11 European countries, were higher than those estimated by the Committee at the sixty-third meeting.

Dietary exposure from the use of lutein, zeaxanthin and their esters and zeaxanthin (synthetic) in food supplements was estimated by the Committee from dosage information on product labels and from intervention studies to be 0.67 mg/kg bw per day for both adults and children.

Dietary exposure from natural occurrence in food was estimated as 0.13 mg/kg bw per day for adults and 0.69 mg/kg bw per day for children. These estimates were based on national food consumption data from China for adults (Wang et al., 2014) and from the Republic of Korea for children (Lee et al., 2013). These estimates were also higher than the previous Committee's estimates.

The present Committee estimated a conservative aggregated high dietary exposure from the use of lutein, zeaxanthin and their esters from *Tagetes erecta* and zeaxanthin (synthetic) as food colour and from food supplements in combination with the natural occurrence of these xanthophylls to be 1.2 mg/kg bw per day for adults and 2.4 mg/kg bw per day for children (Table 6).

Table 6
Estimates of high dietary exposure to lutein, zeaxanthin and their esters from *Tagetes erecta* and zeaxanthin (synthetic)

	Estimated dietary exposure, mg/kg bw per day (%) [a]	
Source of exposure	**Adults**	**Children**
Food colour	0.4 (33)	1 (42)
Food supplements	0.67 (56)	0.67 (28)
Natural occurrence	0.13 (11)	0.69 (29)
Aggregated exposure	1.2	2.4

bw: body weight
[a] Estimated dietary exposure in mg/kg bw per day and, in parentheses, the estimated exposure as a percentage of the total (aggregated) exposure.

5. Evaluation

Free lutein, lutein esters and free zeaxanthin including *meso*-zeaxanthin are biochemically and toxicologically equivalent. The esters of lutein and zeaxanthin are hydrolysed in the gastrointestinal tract, and systemic exposure is to free lutein and zeaxanthin, compounds that differ only in the position of a single double bond. Lutein and zeaxanthin are naturally present in food. In addition, both xanthophylls are essential constituents of the primate retina; biologically controlled mechanisms and pathways exist for absorption of both xanthophylls from the diet and their distribution within the body and deposition in the retina.

The Committee concluded that there were sufficient toxicological data to complete a safety assessment of lutein and lutein esters from *Tagetes erecta*, synthetic zeaxanthin and *meso*-zeaxanthin. The Committee considered the available toxicological data together with the dietary exposure of the general population.

No adverse effects were observed in a broad range of toxicological studies of free lutein, lutein esters and free zeaxanthin and *meso*-zeaxanthin in laboratory animals and in clinical studies in humans. Results from a new 2-generation reproductive toxicity study of zeaxanthin in rats indicated no adverse effects at up to 500 mg/kg bw per day, the highest dose tested.

The estimated dietary exposure from the use of free lutein, lutein esters and free zeaxanthin as colours or food supplements are in the same order of magnitude as the intakes from foods where these xanthophylls are naturally present.

Based on the absence of toxicity in a wide range of studies with NOAELs of approximately 210–400 mg/kg bw per day for lutein, 87.5–500 mg/kg bw per day for zeaxanthin and 200–300 mg/kg bw per day for *meso*-zeaxanthin and estimated dietary exposure of up to 2.4 mg/kg bw per day, the Committee

established a group ADI "not specified" for lutein from *Tagetes erecta*, lutein esters from *Tagetes erecta* and zeaxanthin (synthetic).

Meso-zeaxanthin was not included in this group ADI as specifications are not currently available.

The group ADI of 0–2 mg/kg bw for lutein from *Tagetes erecta* and zeaxanthin (synthetic) was withdrawn.

The specifications for lutein from *Tagetes erecta* were revised, and the Chemical and Technical Assessment was updated. The specifications for lutein esters from *Tagetes erecta* and zeaxanthin (synthetic) were maintained.

6. References

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Neutral methacrylate copolymer

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1. Explanation

Neutral methacrylate copolymer (NMC; E 1206; International Numbering System for Food Additives [INS] No. 1206; Chemical Abstracts Service [CAS] No. 9010-88-2; ethyl acrylate methyl methacrylate polymer; ethyl acrylate methyl methacrylate polymer; ethyl acrylate polymer with methyl methacrylate; methyl methacrylate ethyl acrylate polymer; methyl methacrylate polymer with ethyl acrylate) is a copolymer manufactured from the monomers ethyl acrylate (CAS No. 140-88-5) and methyl methacrylate (CAS No. 80-62-6) in the molar ratio of 2 : 1. NMC is described in the European Pharmacopoeia (2017) and the United States Pharmacopeia and National Formulary (USP, 2017).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) considered three different copolymers: basic, anionic and neutral methacrylate copolymers. Each copolymer releases the active ingredients from within their coatings under different physiological conditions in different parts of the digestive tract. NMC is insoluble in aqueous media and its release is not pH dependent. NMC is used in sustained-release formulations that enable continuous dissolution of an active ingredient over a defined time. Release periods can be controlled by changing the amount of copolymer used.

NMC has not previously been considered by the Committee. The Committee evaluated the use of NMC as a coating or glazing agent for solid food supplements such as capsules, pastilles, tablets, pills, pellets and powders, at levels not exceeding 20%, at the request of the Codex Committee on Food Additives at its Forty-ninth session (FAO/WHO, 2017). NMC is also used in pharmaceuticals.

Toxicological data submitted for evaluation included absorption, distribution and excretion studies, acute and short-term toxicity and genotoxicity studies as well as a developmental toxicity study. Limited data were also submitted on the residual monomers. A comprehensive literature search retrieved data on the residual monomers but no other studies on NMC. The Committee also considered the data on the residual monomers because the monomers are of low molecular weight and therefore likely to be absorbed from the gastrointestinal tract. Due to the low levels of residual monomers present in NMC, the Committee

considered only the absorption, distribution, metabolism and excretion (ADME), long-term toxicity and genotoxicity data on the monomers.

The Committee was also aware that NMC contains an oligomer fraction of between 0.06% and 0.13%. As the lower end of the molecular weight range for all the constituent oligomers is greater than 5000 Da, it is unlikely that they would be absorbed from the gastrointestinal tract. Therefore, the Committee did not consider the toxicological aspects of the oligomers.

Unless otherwise stated, the test substance used in the absorption, distribution and excretion and toxicity studies was prepared from an aqueous dispersion with 0.7% emulsifier and then freeze-dried to remove water. In all cases, doses have been expressed as dry weight of NMC.

The Committee evaluated toxicological and exposure data on polyethylene glycol monostearyl ether, methanol and ethanol, residual components of NMC that can be present in the final product because they are used in the manufacture of the copolymer. The Committee concluded that these residual components do not pose a safety concern at the maximum estimated exposure levels.

1.1 Chemical and technical considerations

NMC is manufactured by emulsion polymerization of the monomers ethyl acrylate and methyl methacrylate with water-soluble radical initiators. The product is purified by water vapour distillation and filtration to remove residual monomers, excess water, other volatile low molecular weight substances and coagulum.

NMC has a weight-average molecular weight of 600 000 Da and a number-average molecular weight of 220 000 Da.

Although organic solvents are not used in the manufacture of NMC, methanol may be present at a level not exceeding 100 mg/kg and ethanol may be present at a level not exceeding 1000 mg/kg. The copolymer is standardized as a 30% aqueous dispersion with polyethylene glycol monostearyl ether (0.7%). The copolymer dispersion may contain the residual monomers methyl methacrylate (not more than 50 mg/kg) and ethyl acrylate (not more than 20 mg/kg).

1.2 Literature search

A literature search for NMC in PubMed and Google Scholar identified no additional relevant articles. Literature searches were carried out in PubMed and Google Scholar for genetoxicity studies (1990–2018) with the monomerethyl acrylate and methyl methacrylate. Articles were included if they included (genetic) toxicological information about the named monomer on its own; articles

were excluded if the information was not about the monomer on its own and did not include (genetic) toxicological information. Other toxicology searches were carried out for the monomer and specific species. Articles were included if they examined a toxicological end-point in one of the named species (or similar mammalian species); articles were rejected if there was no toxicological end-point or they did not include one of the named species (or similar mammalian species). Review articles were manually searched to identify any articles that may not have been found via the database searches.

A literature search was also conducted to identify any estimates of exposure to NMC, using EBSCO Discovery Service. Medline, Food Science Source, Food Science and Technology Abstracts and ScienceDirect were also searched as were a number of scientific, toxicological, food, nutrition and public health–related journals. Search terms included "methacrylate copolymer" and "dietary exposure" or "dietary intake" or "consumption". These terms were also used in a general internet search to capture "grey" literature and other papers not included in the scientific literature. No exposure estimates additional to those submitted by the sponsor were retrieved.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) NMC

In a study not conducted according to good laboratory practice (GLP), a preparation of 30% NMC in an aqueous dispersion containing 1.5% nonylphenol ethoxylate labelled with ^{14}C (site of radiolabel not specified) was administered to rats at 600 mg/kg body weight (bw) per day (dry weight of polymer) by gavage. Faeces and urine were collected daily for 7 days and analysed for radioactivity. After 7 days, animals were killed and the organs analysed for radioactivity.

An average of 0.0092% of the radioactive dose was excreted in the urine, the majority within 48–72 hours. An average of 97.6% of the radioactive dose was excreted in the faeces within 48 hours. The radioactivity in tissues of treated animals did not differ significantly from that of control animals. In conclusion, the test substance was poorly absorbed and quickly eliminated from the body of rats (HSL, 1986).

(b) **Residual monomers**

Ethyl acrylate

As cited in Health Canada (2011):

> Toxicokinetic studies in experimental animals show that ethyl acrylate is absorbed and metabolized rapidly following oral and inhalation exposure (Stott & McKenna, 1984; Ghanayem, Burka & Matthews, 1987). The two major routes of metabolism are hydrolysis of the ester linkage and conjugation with glutathione (GSH). Hydrolysis of ethyl acrylate is catalyzed by the carboxylesterases, resulting in the production of ethanol and acrylic acid (Miller et al., 1981; Frederick, Udinsky & Finch 1994). Ethanol is further metabolized under catabolic process and acrylic acid goes through the propionate degradative pathway of cellular metabolism, resulting in the formation of carbon dioxide in both cases. Conjugation with GSH can occur either spontaneously by a Michael addition or can be mediated by GSH transferase (Ghanayem, Burka & Matthews, 1987; Potter & Tran, 1992). Inhalation of ethyl acrylate in rats resulted in non-protein sulfhydryl (NPSH) depletion most pronounced in liver followed by blood, brain and lungs (Vodicka, Gut & Frantík 1990). In oral gavage toxicity studies in rodents, significant reduction of NPSH in the forestomach and the glandular stomach was observed suggesting that conjugation at the site of contact might be an important detoxification process (deBethizy et al., 1987). Following GSH conjugation, ethyl acrylate is rapidly eliminated by urinary excretion (deBethizy et al., 1987). Although theoretically possible, no evidence was available for the generation of epoxidation products in ethyl acrylate metabolism (Delbressin, van Balen & Seutter-Berlage, 1982; deBethizy et al., 1987).

Methyl methacrylate

The absorption, distribution, metabolism and excretion (ADME) data on methyl methacrylate have been summarized by the World Health Organization (WHO, 1998), as follows:

> Methyl methacrylate is rapidly absorbed and distributed following inhalation or oral administration to rats. On the basis of available data, methyl methacrylate appears to be rapidly metabolized to methacrylic acid [and methanol], which is subsequently converted to carbon dioxide via the tricarboxylic acid cycle in both experimental animals and humans….
>
> Methyl methacrylate is rapidly eliminated, primarily via the lungs in expired air. After oral or intravenous administration to rats, approximately 65% of the dose was exhaled in the expired air as $^{14}CO_2$ within 2 hours (Bratt & Hathway, 1977). Lesser amounts are eliminated in the urine, and an even smaller fraction in the faeces. Owing to its rapid

metabolism and excretion, there appears to be little potential for accumulation of methyl methacrylate within tissues (Government of Canada, 1993; ECETOC, 1995).

2.2 Toxicological studies

2.2.1 Acute toxicity

Groups of rats (5/sex per group) were administered a single dose of one of two preparations of the test substance in feed. Doses of NMC were calculated to be 27.2 and 28.2 g/kg bw for males and females, respectively, for the first preparation (further details not available) and 25.2 and 27.2 g/kg bw for males and females, respectively, for the second preparation (20% NMC in a dry preparation of coated standard rat diet containing 1.0% nonylphenol ethoxylate). The animals were observed for 4 weeks, killed and necropsied.

No treatment-related effects were observed during the observation period or at macroscopic examination of the organs (LPT, 1971).

Groups of dogs (2/sex per group) were administered a single dose of one of two preparations of the test substance in feed. No control animals were included in this study. Doses of NMC were calculated to be 9100 mg/kg bw for the first preparation (further details not available) and 7950 mg/kg bw for the second preparation (20% NMC in a dry preparation of coated standard dog diet containing 1.0% nonylphenol ethoxylate). The animals were observed for 14 days, killed and necropsied.

No treatment-related effects were observed during the observation period or at the necropsy. Urine analysis also did not show any treatment-related effects (LPT, 1972a).

2.2.2 Short-term studies of toxicity

(a) Rats

Groups of rats (10/sex per control, low- and mid-dose group; 15/sex in the highest dose group) were administered NMC (freeze-dried then suspended in distilled water) by gavage at 0, 500, 1000 or 2000 mg/kg bw per day for 35 days. It is not clear if the doses were expressed in dry weight of polymer or of the preparation. One control group received distilled water and another group Aerosil (a synthetic amorphous silica [INS 551]) at 70 mg/kg bw (equivalent to the Aerosil content of the test substance). Clinical signs were assessed daily, and body weight and feed and water consumption measured 3 times in the first week and twice weekly thereafter. Blood and urine samples were collected after dosing and analysed. Ten animals per group were killed directly after dosing and the remaining five from the high-dose group were killed following a 2-week recovery period.

Treated and control animals did not differ significantly in body-weight gain or in urine and haematological analysis parameters. Fluctuations in feed and water consumption were not dose dependent and were considered not treatment related. Significant reductions in erythrocyte counts were seen in mid- and high-dose males and in erythrocyte count, haemoglobin and haematocrit in low-dose females. No changes were observed in haematocrit or haemoglobin values in males. Significant changes in serum biochemistry and organ weights were observed, but these did not show a dose–response relationship and were not considered treatment related. Histopathological examination found some changes, but these were considered to be spontaneous and not treatment related.

The no-observed-adverse-effect level (NOAEL) was 2000 mg/kg bw per day, the highest dose tested (BoZo Research Center, 1981).

In a pre-GLP study, rats (20/sex per group) were administered 0, 500 or 2000 mg/kg bw per day of NMC (10% NMC in a dry preparation of coated rat standard diet containing 0.5% nonylphenol ethoxylate) in feed for 6 months. Body weights were measured weekly, and blood and urine samples collected before dosing and after 6, 13, 18 and 26 weeks of treatment. After 6 months, the animals were killed and necropsied.

Feed and water consumption, behaviour, body and organ weights and haematological, clinical chemistry and urinary analysis findings did not differ significantly between groups. Histological examination did not find any treatment-related changes.

The NOAEL was 2000 mg/kg bw per day, the highest dose tested (LPT, 1972b).

(b) **Dogs**

In a GLP-compliant study, groups of dogs (3/sex per group) were administered NMC (40% NMC in an aqueous dispersion containing 2.0% nonylphenol ethoxylate in the form of coated cellulose pellets contained in transparent gelatine capsules) at 0, 50, 125 or 250 mg/kg bw per day for 26 weeks. Control and high-dose groups included additional animals (3/sex per group) for a 3-week recovery period. Animals were examined daily before and after dosing, and feed and water consumption was recorded. Clinical signs and body weights were recorded weekly. A physical examination by a veterinarian was carried out monthly, and ophthalmological tests and electrocardiographs were performed 3 times during the treatment period. Blood, urine and faeces samples were collected before dosing and in treatment weeks 13 and 26.

During treatment, one control animal had reduced activity and liquid or soft faeces. Body-weight gain was reduced in high-dose males and females

(statistically significant in males only). Compared with controls, body weights were also reduced in low- and mid-dose males but not statistically significantly. No effects were seen in low- and mid-dose females. Compared with controls, feed consumption was slightly reduced in high-dose males and in all treated females. No treatment-related findings were observed during ophthalmological tests or electrocardiographs. A slight reduction in erythrocyte count in high-dose males was not considered severe enough to suggest toxicological significance; the reduction was still apparent after the 3-week recovery period. Minor differences between test and control groups in haematology, clinical chemistry and urine and faecal parameters had no dose–response relationships and were not considered treatment related. Changes in absolute and/or relative organ weights, observed in high-dose males only, included statistically significant decreases in epididymis and prostate gland, decreases in spleen and increases in lungs and testes. Because histopathological examination did not find any treatment-related effects in these tissues, these changes were not considered toxicologically significant. A white granular substance was found in the gut of some animals, but its occurrence did not seem to be dose related. Histopathological examination did not reveal any treatment-related effects.

The study author considered the effects on body weight and feed consumption to be related to the physical characteristics of the test material.

The Committee agreed with the study author that the NOAEL was 250 mg/kg bw per day, the highest dose tested (Research Toxicology Centre, 2007a).

(c) Pigs

Groups of minipigs (3/sex per group) were administered NMC (coated onto cellulose pellets) at a dose of 0, 500, 1000 or 2000 mg/kg bw per day (equivalent to 0, 113, 227 and 454 mg/kg bw per day dry NMC) by gavage daily for 28 days. One male in the top-dose group was euthanized on day 25 because of tissue damage caused during the gavage procedure.

There were no treatment-related clinical signs or changes in body or organ weights; feed and water consumption; or physical, ophthalmoscopic, haematological, clinical chemistry, urine or faecal analysis parameters. Necropsy of the animal that was euthanized found dark patches in the testes, kidneys, thyroid, lungs and lymph nodes as well as inflammation, most likely due to the gavage accident. White granular material was found in the aorta and oesophagus of this animal and in the gastrointestinal tract of other test animals. This was considered to be due to the physical characteristics of NMC and not biologically relevant. Slight mucosal/submucosal oedema was identified in the caecum and colon of two high-dose and one mid-dose male. Centrilobular yellow/brown pigmentation and mild focal fibrosis were found in the liver of one high-dose

female. Other macroscopic/microscopic findings, including a mass in the epididymis of a treated male and small testes, were within the historical control ranges and considered spontaneous occurrences.

Because of the unexplained pigmentation and fibrosis in the liver of one high-dose animal, the NOAEL was considered to be 227 mg/kg bw per day (Research Toxicology Centre, 2006).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) NMC

No long-term studies with NMC were available.

(b) Residual monomers

Ethyl acrylate

Groups of B6C3F1 mice (50/sex per dose group) were given ethyl acrylate (in corn oil) by intragastric instillation at a dose of 0, 100 or 200 mg/kg bw per day, 5 days/week for 103 weeks. Dose-related increases in the incidences of hyperkeratosis, hyperplasia and inflammation of the forestomach were reported in both sexes. Statistically significant positive trends in the incidence of squamous cell neoplasms were reported in the forestomachs of treated males and females (NTP, 1986a).

Groups of F344/N rats (50/sex per dose group) were given ethyl acrylate (in corn oil) by gavage, at a dose of 0, 100 or 200 mg/kg bw per day, 5 days/week for 103 weeks. Dose-related increases in the incidence of non-neoplastic lesions were observed in the forestomach of males and females. Statistically significant positive trends in the incidence of squamous cell papillomas were reported in males and females. Statistically significant positive trends in the incidence of squamous cell carcinomas were reported in males (NTP, 1986a).

In contrast to the gavage studies, no neoplastic effects were observed in experimental animals administered ethyl acrylate by other routes. In a drinking-water study, Wistar rats were administered ethyl acrylate at 0, 6–7, 60–70 or 2000 parts per million (ppm; approximately 0, 0.84–0.98, 9.4–9.8 and 280 mg/kg bw per day, respectively) for 2 years. Histopathological examination found no treatment-related lesions (Borzelleca et al., 1964).

In inhalation carcinogenicity studies, no treatment-related neoplastic lesions were observed in Fischer 344 rats and B6C3F1 mice exposed to ethyl acrylate at 0, 25 or 75 ppm (approximately 0, 100 and 310 mg/m^3, respectively), 6 hours/day, 5 days/week for 27 months (Miller et al. 1985). In another study conducted by Miller et al. (1985), Fischer 344 rats and B6C3F1 mice were exposed to ethyl acrylate at 0 or 5 ppm (approximately 20 mg/m^3) for 24 months. No neoplastic effects were observed.

In a dermal carcinogenicity study, male C3H/HeJ mice were administered 800 mg/kg bw per day of ethyl acrylate, 3 times/week for life. No epidermal tumours were observed (DePass et al., 1984).

The relevance of forestomach tumours in rodents to potential carcinogenic targets in humans has been the subject of much investigation. An International Agency for Research on Cancer (IARC) working group concluded that in evaluating the relevance of the induction of forestomach tumours in rodents to human cancer, the exposure conditions in the experiments have to be taken into consideration (IARC, 2003). Exposure conditions during oral administration are unusual (particularly if dosing is by gavage) in that physical effects may result in high local concentrations of test substances in the forestomach and prolonged exposure of the epithelial tissue. Agents that only produce tumours in the forestomach in rodents after prolonged treatment through non-DNA reactive mechanisms may be less relevant to humans since human exposure to such agents would need to surpass time-integrated dose thresholds in order to elicit the carcinogenic response.

JECFA concluded that the appearance of forestomach lesions in the 2-year bioassays in rodents in which ethyl acrylate was administered at high concentrations by gavage has no relevance to humans. The results are attributable to the irritating effect of high bolus doses of ethyl acrylate delivered to the contact site (forestomach) by gavage, and not the effects of systemic concentrations in the whole animal (Annex 1, reference *174*).

Health Canada (2011) reached a similar conclusion more recently.

Methyl methacrylate

A long-term toxicity study with methyl methacrylate in drinking-water was summarized by WHO (1998) as follows:

> Data available on the effects of methyl methacrylate following ingestion are limited. In an early study (Borzelleca et al., 1964) in which organ to body weight ratios were determined and histopathological examination of a wide range of tissues as well as limited haematological and urine analyses were conducted, the relative kidney weight was increased in a small group of female rats (n = 25) exposed to 2000 ppm (mg/litre) methyl methacrylate in drinking-water for 2 years. This effect was not observed in the males, and histopathological examination revealed no damage. The authors also reported a decrease in fluid consumption in rats exposed to 2000 ppm. The NOAEL was therefore considered to be 2000 ppm (equivalent to a dose of about 146 mg/kg body weight per day for females and 121 mg/kg body weight per day for males, based on intake and body weight data presented by the authors). There were no treatment-related effects, based upon gross or histopathological examination, in extremely small groups of beagle dogs (n = 2) exposed

to concentrations of up to 1500 ppm (mg/kg) methyl methacrylate (equivalent to a dose of about 38 mg/kg body weight per day) in their feed for 2 years (Borzelleca et al., 1964).

WHO used this study to derive a tolerable daily intake (TDI) of 1.2 mg/kg bw per day (WHO, 1998).

In chronic toxicity and carcinogenicity studies in mice, rats and hamsters with methyl methacrylate given by the inhalation route, the observed effects were, in general, similar to those reported in short-term toxicity studies and included inflammation and epithelial hyperplasia of the nasal cavity and degeneration of the olfactory sensory epithelium. There was no evidence of any carcinogenic effects (WHO, 1998).

In 1994, IARC considered the same studies and concluded that there is evidence suggesting lack of carcinogenicity in animals (IARC, 1994).

2.2.4 Genotoxicity

The results of genotoxicity tests with NMC are summarized in Table 1. The results of genotoxicity tests with the residual monomers are summarized in Tables 2, 3, 4 and 5.

Negative results were reported in gene mutation studies in bacteria with ethyl acrylate. In the in vitro forward mutation assay in mouse lymphoma cells, ethyl acrylate gave uniformly positive results in the absence of metabolic activation when incubated with L5178Y $tk^{+/-}$ cells at cytotoxic concentrations (Moore et al., 1989). In a mouse lymphoma assay in which the relationship between cytotoxicity and mutation frequency was investigated, the ethyl acrylate–induced mutagenic response was found to be directly related to the time- and concentration-dependent decrease in NPSH levels and subsequent mitochondrial membrane impairment (Ciaccio et al., 1998). There were both positive and negative results in in vitro sister chromatid exchange (SCE) assays and in chromosomal aberration assays with metabolic activation, but there was no evidence of clastogenicity in the absence of metabolic activation.

In in vivo studies, one early mouse micronucleus study using intraperitoneal dosing, gave positive results (Przybojewska, Dziubałtowska & Kowalski, 1984). However, three other micronucleus studies in mice—one dermal exposure (Tice, Nylander-French & French, 1997), two by intraperitoneal injection (Ashby, Richardson & Tinwell, 1989; Kligerman et al., 1991) all gave negative results, including in one study that replicated the conditions of the earlier positive study (for details, see Annex 1, reference *174*). An in vivo point mutation assay, a chromosomal aberration assay and an SCE assay were all negative (Kligerman et al., 1991; Ellis-Hutchings et al., 2018).

The Committee concluded that the genotoxic potential observed in some in vitro studies was not expressed in vivo.

Table 1
Genotoxicity studies with NMC

End-point	Test system/ species	Test substance	Concentration/dose	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA 100, TA1537	NMC preparation (no further details available) (±S9)	313, 625, 1 250, 2 500, 5 000 mg/plate	Negative	Life Science Research (1977)
Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	40% NMC in an aqueous dispersion containing 2.0% nonylphenol ethoxylate (±S9)	Equal to 1.2, 4, 13.2, 40, 133.2, 400, 1 000, 2 000 µg/ plate; plate incorporation and preincubation methods	Negative	RCC (2005)
Gene mutation	L5178Y mouse lymphoma cell line	40% NMC in an aqueous dispersion containing 2.0% nonylphenol ethoxylate (±S9)	Equal to 156, 312, 625, 1 250, 2 500 µg/mL	Negative	RCC (2001)
In vivo					
Micronucleus	Hsd:ICR (CD-1) mouse route of administration	40% NMC in an aqueous dispersion containing 2.0% nonylphenol ethoxylate	500, 1 000, 2 000 mg/kg bw per day	Negative	Research Toxicology Centre (2007b)

bw: body weight; NMC: neutral methacrylate copolymer

Table 2
Genotoxicity studies with ethyl acrylate

End-point	Test system/species	Concentration/dose	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535	Up to 3 333 µg/plate	Equivocal (±S9)	Haworth et al. (1983)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	30–2 000 µg/plate	Negative	Waegemaekers & Bensink (1984)
Reverse mutation	*S. typhimurium* YG7018pin3E	2 000 µg/plate	Negative	Emmert et al. (2006)
Reverse mutation	*S. typhimurium* TA102	15–5 000 µg/plate	Negative	Kirkland et al. (2016)
Sex-linked recessive lethal mutations	*Drosophila melanogaster*	40 000 mg/kg feed	Negative	Valencia et al. (1985)
Chromosomal loss	*Saccharomyces cerevisiae*	2.5–25 mg/mL	Negative (ethyl acrylate alone); positive (ethyl acrylate in combination with propionitrile)	Zimmermann & Mohr (1992)
Chromosomal aberration, gene mutation	CHO cells	0, 21, 22, 23, 24 µg/ mL (−S9 only)	Positive for chromosomal aberration [a]; negative for gene mutation	Moore et al. (1989)
Chromosomal aberration	CHO cells	0–300 µg/mL [b] (poor copy received, figures not legible)	Positive (+S9), Negative (−S9)	Loveday et al. (1990)
Chromosomal aberration	CHO cells suspension	0, 14, 15, 16, 18, 20, 21, 22, 22.5, 23, 24, 25 µg/mL	Negative	Moore et al. (1991)
Chromosomal aberration	CHO cells monolayer	0, 5, 10, 20, 30, 40, 50, 60, 70, 75, 80 µg/mL	Negative	Moore et al. (1991)

End-point	Test system/species	Concentration/dose	Result	Reference
Chromosomal aberration	Rat i.p. injection; splenocytes	125, 250, 500, 1 000 mg/kg	Positive, but only after mitogenic stimulation and after 23-h exposure	Kligerman et al. (1991)
Chromosomal aberration	*S. cerevisiae*	0, 230, 368, 551, 642, 733, 823, 824, 914, 1 095 µg/mL	Positive	Zimmerman & Mohr (1992)
Chromosomal aberration	Human peripheral lymphocytes	Occupationally exposed individuals	Weakly positive	Tuček et al. (2002)
SCE	CHO cells	0, 0.5, 1.5, 5, 15, 50, 51.5, 103, 150, 155 µg/mL (±S9)	Weakly positive (+S9); negative (−S9)	Loveday et al. (1990)
SCE	I.p. injection; splenocytes	125, 250, 500, 1 000 mg/kg	Negative	Kligerman et al. (1991)
Micronucleus induction	V79 Chinese hamster lung fibroblasts, 3-h treatment, 21 h recovery	16, 20 µg/mL (−S9 only)	Positive [a]	Fowler et al. (2012)
Micronucleus induction	V79 CHO cells, 3-h treatment, 21-h recovery	20, 32 µg/mL (−S9 only)	Positive [a]	Fowler et al. (2012)
Micronucleus induction	Chinese hamster lung cells, 3-h treatment, 21-h recovery	39, 40 µg/mL (−S9 only)	Positive [a]	Fowler et al. (2012)
Micronucleus induction	V79 Chinese hamster lung fibroblasts, 24-h treatment	1, 4 µg/mL (−S9 only)	Positive	Fowler et al. (2012)
Micronucleus induction	CHO cells, 24-h treatment	10, 12 µg/mL (−S9 only)	Negative	Fowler et al. (2012)
Micronucleus induction	Chinese hamster lung cells, 24-h treatment	7, 14 µg/mL (−S9 only)	Positive [a]	Fowler et al. (2012)
Micronucleus induction	Human lymphocytes, 3-h treatment, 21-h recovery	38, 50 µg/mL (−S9 only)	Positive [a]	Fowler et al. (2012)
Micronucleus induction	Human lymphoblast TK6, 3-h treatment, 21-h recovery	20, 25 µg/mL (−S9 only)	Positive [a]	Fowler et al. (2012)
Micronucleus induction	Human HepG2 hepatocarcinoma cells, 3-h treatment, 21-h recovery	20, 25 µg/mL(−S9 only)	Positive in 1 of 2 repeats at same dose	Fowler et al. (2012)
Micronucleus induction	Human lymphocytes, 24-h treatment	10 µg/mL (−S9 only)	Negative	Fowler et al. (2012)
Micronucleus induction	Human Lymphoblast TK6, 24-h treatment	10 µg/mL (−S9 only)	Positive in 1 of 2 repeats at same dose	Fowler et al. (2012)
Micronucleus induction	Human HepG2 hepatocarcinoma cells, 24-h treatment	77 µg/mL (−S9 only)	Positive in 1 of 2 repeats at same dose	Fowler et al. (2012)
Micronucleus induction	Human lymphoblast TK6 cells	6 µg/mL	Negative	Whitwell et al. (2015)
Micronucleus induction	Human lymphoblast WIL2-NS cells	9 µg/mL	Negative	Whitwell et al. (2015)
Micronucleus induction	Human lymphoblast TK6 cells	6 µg/mL in the presence of cytochalasin B	Equivocal	Whitwell et al. (2015)
Micronucleus induction	Human lymphoblast WIL2-NS cells	6 µg/mL in the presence of cytochalasin B	Equivocal	Whitwell et al. (2015)

Table 2 (continued)

End-point	Test system/species	Concentration/dose	Result	Reference
Micronucleus test/ induction	Mouse lymphoma L5178Y cells	12, 18 µg/mL (−S9)	Equivocal	Whitwell et al. (2015)
Gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$	Up to 20 µg/mL (+S9)	Positive	McGregor et al. (1988)
Gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$	0, 20, 25, 30, 37.5 µg/mL (−S9 only)	Positive for gene mutation and chromosomal aberration [a]	Moore et al. (1988)
Chromosomal aberration; gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$	0, 20, 25, 30, 37.5 µg/mL (−S9 only)	Positive for gene mutation and chromosomal aberration [a]	Moore et al. (1989)
Gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$	10, 20, 50, 100, 150, 200 µg/mL (±S9)	Positive (±S9) [a]	Dearfield et al. (1991)
Gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$	0, 10, 15, 20, 25, 27.5, 30, 32.5, 35, 40 µg/mL (±S9)	Positive [a]	Ciaccio et al. (1998)
Decrease in NPSH levels	Mouse lymphoma L5178Y *tk*$^{+/-}$	0, 10, 20, 30, 40, 50 µg/mL (±S9)	Significant reduction in NPSH	Ciaccio et al. (1998)
Mitochondrial rhodamine 123 uptake	L5178Y *tk*$^{+/-}$	0, 10, 20, 30, 40, 50 µg/mL (±S9)	Negative at 2 h; positive at +4 h	Ciaccio et al. (1998)
DNA damage	L5178Y *tk*$^{+/-}$ (alkaline elution)	0, 10, 15, 20, 25, 27.5, 30, 32.5, 35, 40, 50 µg/mL (±S9)	Negative	Ciaccio et al. (1998)
In vivo				
Point mutation	Rats and mice; gavage administration	0, 8, 20, 50 mg/kg bw	Negative	Ellis-Hutchings et al. (2018)
DNA strand break	Male Fischer 344 rats forestomach epithelial cells (alkaline elution)	40 mg in solution per rat via gavage	Negative	Morimoto et al. (1990)
DNA strand break	Peripheral blood leucocytes after dermal exposure in Tg.AC (v-Ha-ras) mice	0, 60, 300, 600 µmol/L	Negative	Tice, Nylander-French & French (1997)
Chromosomal aberration	I.p. injection; splenocytes	125, 250, 500, 1 000 mg/kg bw	Negative	Kligerman et al. (1991)
SCE	I.p. injection, splenocytes	125, 250, 500, 1 000 mg/kg bw	Negative	Kligerman et al. (1991)
Micronucleus induction	Peripheral blood leucocytes after dermal exposure in Tg.AC (v-Ha-ras) mice	0, 60, 300, 600 µmol/L	Negative	Tice, Nylander-French & French (1997)
Micronucleus induction	I.p. injection; splenocytes	125, 250, 500, 1 000 mg/kg bw	Negative	Kligerman et al. (1991)
Micronucleus induction	I.p. injection, male BALB/c mice, after 30 h, bone marrow	0, 65, 461, 738, 812 mg/kg bw	Negative, positive in repeat at same dose	Ashby, Richardson & Tinwell (1989)
Micronucleus induction	I.p. injection, male and female C57BL/6J mice after 24, 48 and 72 h, bone marrow	Up to 738 mg/kg bw	Negative	Ashby, Richardson & Tinwell (1989)

End-point	Test system/species	Concentration/dose	Result	Reference
Micronucleus induction	I.p. injection, male C57BL/6J mice after 30 h, bone marrow	Up to 738 mg/kg bw	Negative	Ashby, Richardson & Tinwell (1989)
Micronucleus induction	I.p. injection, male mice, bone marrow	112.5, 225, 450, 900, 1 800 mg/kg bw	Positive	Przybojewska, Dziubałtowska & Kowalski (1984)

bw: body weight; CHO: Chinese hamster ovary; i.p.: intraperitoneal; NPSH: non-protein sulfhydryl; SCE: sister chromatid exchange; *tk*: thymidine kinase
[a] Positive results found at cytotoxic concentrations only.
[b] Poor copy of text resulted in figures and data being illegible.

Table 3
Genotoxicity studies with methyl methacrylate

End-point	Test system/species	Concentration/dose	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Up to 1 000 µg/plate for preincubation and plate incorporation (±S9)	Negative	Lijinsky & Andrews (1980)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 0.15, 0.29, 0.59, 1.18, 2.35, 4.70 mg/plate (±S9)	Negative	Hachiya, Taketani & Takizawa (1982)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	40–10 000 µg/plate	Negative	Waegemaekers & Bensink (1984)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA1535	0, 33, 100, 333, 1 000, 3 333, 6 666 µg/plate	Negative	NTP (1986b)
Reverse mutation	*S. typhimurium* TA97a, TA98, TA100, TA102	0–12.5 mg/plate	Negative	Schweikl, Schmalz & Rackebrandt (1998)
Cytotoxicity	V79 cells	0.94, 4.7, 9.4 mg/mL	Positive	Pradeep & Sreekumar (2012)
Mutation frequency	L5178Y mouse lymphoma cells	1, 1.75, 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3 mg/mL	Negative at 1 and 1.75 mg/mL; positive at other doses	Doerr, Harrington-Brock & Moore (1989)
Chromosomal aberration	CHO cells	0, 750, 1 000, 1 600, 3 000 µg/mL	Positive (−S9) Positive at top dose only (+S9)	NTP (1986b)
Chromosomal aberration	Human lymphocytes	Cells mixed with 0.14 g (mean weight) of methyl methacrylate (97.4%) and *N*,*N*-dimethyl-para-toluidine to form bone cement	Negative	Bigatti et al. (1994)
Chromosomal aberration	L5178Y mouse lymphoma cells	1, 1.75, 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3 mg/mL	Negative at 1 and 1.75 mg/mL; positive at other doses	Doerr, Harrington-Brock & Moore (1989)
Chromosomal aberration	V79B/HPRT cells	0, 10, 20 mmol/L	Weakly positive	Schweikl, Schmalz & Rackebrandt (1998)
Chromosomal aberration	Human peripheral lymphocytes	Occupationally exposed individuals	Weakly positive	Tuček et al. (2002)
Chromosomal aberration	CHO cells	9.33×10^{-1}, 9.33×10^{-2}, 9.33×10^{-3}, 9.33×10^{-4} mg/mL	Positive	Yang et al. (2003)

Table 3 (continued)

End-point	Test system/species	Concentration/dose	Result	Reference
Micronucleus induction	L5178Y mouse lymphoma cells	1, 1.75, 2.2, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3 mg/mL	Negative at 1 and 1.75 mg/mL; weakly positive at other doses	Doerr, Harrington-Brock & Moore (1989)
Micronucleus induction	Chinese hamster V79 cells	10, 20, 30 mmol/L	Negative; cytotoxic at top dose	Schweikl, Schmalz & Spruss (2001)
SCE	CHO Cells	0, 750, 1 000, 1 250, 1 500 µg/mL	Positive (±S9)	NTP (1986b)
SCE	Human peripheral lymphocytes	0.001–1 mg/100 mL	Equivocal	Cannas et al. (1987)
SCE	CHO cells	9.33×10^{-1}, 9.33×10^{-2}, 9.33×10^{-3}, 9.33×10^{-4} mg/mL	Positive	Yang et al. (2003)
Gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$ cells	0, 0.125, 0.25, 0.5, 0.75, 1.0 µg/mL (±S9)	Positive (±S9)	NTP (1986b)
Gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$ cells	0, 1 000, 1 750, 2 202, 2 400, 2 499, 2 601, 2 700, 2 799, 2 901, 3 000 µg/mL(−S9)	Positive	Moore et al. (1988)
Gene mutation	Mouse lymphoma L5178Y *tk*$^{+/-}$ cells	0–3 000 µg/mL (±S9)	Positive (+S9); negative (−S9)	Dearfield et al. (1991)
In vivo				
Chromosomal aberration	Human peripheral lymphocytes (38 occupationally exposed men)	0.9–71.9 ppm	Negative	Seiji et al. (1994)
SCE	Human peripheral lymphocytes (31 occupationally exposed men)	Mean atmospheric concentrations 0.7–1.6 ppm	Negative overall, positive when comparing peak exposures of staff who clean facilities manually with staff who do not	Marez et al. (1991)
SCE	Peripheral lymphocytes of 38 occupationally exposed males	0.9–71.9 ppm	Negative	Seiji et al. (1994)
Micronucleus induction	Mouse bone marrow, oral route	Single doses of 1.13, 2.26, 4.52 g/kg bw	Negative	Hachiya, Taketani & Takizawa (1982)
Micronucleus induction	Bone marrow cells of Wistar rats exposed by the inhalation route	150 ppm for 8 hours/day for 1 or 5 days	Positive after 1 day, negative after 5 days	Araújo et al. (2013)
Micronucleus induction	Human buccal mucosal cells	Dental technicians exposed to methyl methacrylate monomer from dental resins	Negative	Azhar et al. (2013)

bw: body weight; CHO: Chinese hamster ovary; ppm: parts per million; SCE: sister chromatid exchange; *tk*: thymidine kinase

Bacterial reverse mutation assays with methyl methacrylate gave negative results (Lijinsky & Andrews, 1980; Hachiya, Taketani & Takizawa, 1982; Waegemaekers & Bensink, 1984; NTP, 1986b; Schweikl, Schmalz & Rackebrandt, 1998). Mixed results (i.e. positive, weakly positive or negative) were obtained

Table 4
Exposures to NMC estimated by the sponsor from use in food supplements

		Exposure	
Population	**No. of units consumed/day [a]**	**mg per day**	**mg/kg bw per day [b]**
Adults – expected consumption	2	400	6.7
Adults – heavy user	3	600	10.0
Young people / children 6–18 years	1	200	13.3

bw: body weight; NMC: neutral methacrylate copolymer; no.: number
[a] One unit is 1 g containing 200 mg of NMC.
[b] Adults = 60 kg body weight; children = 15 kg body weight.

Table 5
Estimated exposures to NMC from use in food supplements by high consumers

		Exposure	
Population	**No. of units consumed/day [a]**	**mg per day**	**mg/kg bw per day [b]**
Adults (19–64 years)	7	1 400	23.3
Children (4–18 years)	2	400	16.0

bw: body weight; NMC: neutral methacrylate copolymer; no.: number
[a] One unit is 1 g tablet or dosage unit containing 200 mg of NMC.
[b] Based on a mean body weight of 60 kg for adults and 25 kg for children.
Source: EFSA (2010)

in in vitro chromosomal aberration (NTP, 1986b; Doerr, Harrington-Brock & Moore, 1989; Bigatti et al., 1994; Schweikl, Schmalz & Rackebrandt, 1998; Tuček et al., 2002; Yang et al., 2003) and SCE assays (NTP, 1986b; Cannas et al., 1987; Yang et al., 2003). One in vitro micronucleus assay was unequivocally negative (Schweikl, Schmalz & Spruss, 2001), whereas another was negative at low concentrations but weakly positive at higher concentrations (Doerr, Harrington-Brock & Moore, 1989). Three mouse lymphoma assays for gene mutations were positive (NTP, 1986b; Moore et al., 1988; Dearfield et al., 1991). A mouse bone marrow micronucleus assay was negative, but it is not clear if the target tissue was exposed to the test substance (Hachiya, Taketani & Takizawa, 1982). A rat micronucleus assay with exposure by inhalation was positive following 1 day of exposure but negative following 5 days of exposure (Araújo et al., 2013). These results were judged by the Committee to be inconclusive.

The Committee concluded that there was some evidence of mutagenicity and clastogenicity in vitro, but there was a lack of adequate in vivo tests following up the equivocal findings.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

No reproductive studies with NMC were available.

(b) Developmental toxicity

Pregnant rats (20/group) were given NMC (10% in a dry preparation of coated standard rat diet containing 0.5% nonylphenol ethoxylate) at 0, 500 or 2000 mg/kg bw per day in feed on gestation days 6–15. On pregnancy day 19, the dams were killed and internal organs, including uterus, and fetuses were examined.

No treatment-related effects were observed. Behaviour, external appearance, faeces, body weight and feed intake were similar in all groups. There were no differences between groups in numbers of corpora lutea, implantation loss or numbers of fetuses (live or dead) per dam. No treatment-related effects were observed in the fetuses including the number and weight, resorption and variation rates and skeletal malformations.

The NOAEL was 2000 mg/kg bw per day, the highest dose tested (LPT, 1974a).

Pregnant rabbits (10/group) were given NMC (10% in dry polymer in standard rabbit feed containing 0.5% nonylphenol ethoxylate) in feed at 0, 500 or 2000 mg/kg bw per day on gestation days 6–19. Laparotomy was performed on the pregnancy day 29 and the contents of the uterus were examined.

No treatment-related effects were observed. Behaviour, external appearance, faeces, body weight and feed and water intake were similar in all groups. There were no significant differences between groups in numbers of corpora lutea, implantation loss or numbers of fetuses (live or dead) per dam. No treatment-related effects were observed in the fetuses including the number and weight, resorption and variation rates and skeletal malformations.

The NOAEL was 2000 mg/kg bw per day, the highest dose tested (LPT, 1974b).

2.2.6 Special studies

(a) Cytotoxicity

In a GLP-compliant study, an NMC preparation (30% NMC in an aqueous dispersion containing 1.5% nonylphenol ethoxylate; residual monomers found to be <1 mg/L) at concentrations equal to 1500, 1000, 667, 445, 296 and 197 µg/mL, the vehicle (DMEM) and a positive control were incubated with L929 mouse connective tissue cells for 72 hours. Growth inhibition was observed in the positive control (88%) and the highest concentration tested (35%).

NMC was found to be cytotoxic at the highest tested concentration (BSL, 1998a).

(b) Dermal toxicity

In a GLP-compliant study, 0.5 mL of NMC (30% in an aqueous dispersion containing 1.5% nonylphenol ethoxylate) was applied to one side of three rabbits, occluded and kept in place for 4 hours. The other side of each animal acted as control. After 4 hours, the bandages were removed and the sites examined 1, 24, 48 and 72 hours later. No signs of irritation or other clinical signs of toxicity were observed (BSL, 1998b).

In a GLP-compliant study in line with Organisation for Economic Co-operation and Development (OECD) test guideline (TG) 406, 20 female guinea-pigs were exposed to the test substance. A group of 10 guinea-pigs acted as controls. The test area was clipped and a patch loaded with NMC (30% in an aqueous dispersion containing 1.5% nonylphenol ethoxylate) applied. The area was occluded for 6 hours. Control animals were given saline solution. This process was repeated on days 6 and 13 following the first application. On day 27, test animals were challenged with the test substance in a repeat of the day 0 protocol. Skin reactions were recorded 30, 54 and 72 hours after the challenge.

Sensitization was not observed in any of the test animals (BSL, 1998c).

(c) Inhalation toxicity

In a GLP-compliant study, albino rats (5/sex) were exposed by nose-only inhalation to NMC (30% in an aqueous dispersion containing 1.5% nonylphenol ethoxylate) for a single 4-hour period at an aerosol concentration of 3.960 mg/L air.

No deaths, no clinical signs or macroscopic pathological findings were observed. Slight transient body-weight loss was seen in 2/5 females from days 1 to 4, and slight growth retardation was observed in one female between days 8 and 15. It was not clear if this was treatment related. No effects were observed in the other females or any of the males (RCC, 2004).

(d) Ocular toxicity

In a GLP-compliant study carried out in line with OECD TG 405, 0.1 mL of NMC (30% in an aqueous dispersion containing 1.5% nonylphenol ethoxylate) was instilled into the lower conjunctival sac of one eye of each of three rabbits. The other eye acted as control. Observations were made at 1, 24, 48 and 72 hours.

No signs of irritation, corrosion or irreversible effects were observed in any of the animals (BSL, 2002).

(e) **Residual components of NMC (other than monomers)**

Polyethyleneglycol monostearyl ether

There are few toxicity data on polyethyleneglycol monostearyl ether (CAS No. 9005-00-9) other than results of acute toxicity studies (rat oral LD_{50} = 2.9 g/kg bw) and evidence that it is an eye irritant. The European Chemicals Agency (ECHA) lists it in the European Union Classification, Labelling and Packaging of substances and mixtures (CLP regulation) only for acute toxicity (harmful if swallowed) and eye damage (causes serious eye damage), stating that data are lacking for all other categories of toxicity data (ECHA, 2018).

Polyethyleneglycol monostearyl ether is not a food additive. It is used as an emulsifier/surfactant/wetting agent/detergent/solubilizer in cosmetics, household and industrial cleaning products, pharmaceuticals and pesticide formulations.

In 1995, the United States Environmental Protection Agency (USEPA) listed polyethyleneglycol monostearyl ether as an "inert ingredient of minimal concern", that is, the lowest of four USEPA categories of concern for pesticide formulation ingredients that are not active (1: toxicity concern; 2: potentially toxic; 3: unknown toxicity; 4: minimal concern) (GPO, 1995).

Maximum exposure to NMC in children is 86 mg/kg bw per day. Based on NMC containing 0.7% polyethyleneglycol monostearyl ether, exposure to polyethyleneglycol monostearyl ether is calculated to be 0.6 mg/kg bw per day.

Based on the USEPA listing NMC as an inert ingredient and the estimated exposure, the presence of polyethyleneglycol monostearyl ether at 0.7% in copolymer NMC is not of toxicological concern.

2.3 Observations in humans

No data on humans were available.

3. Dietary exposure

3.1 Introduction

NMC was evaluated by the Committee at the request of the Codex Committee on Food Additives at its Forty-ninth session (FAO/WHO, 2017). Codex asked the Committee to conduct a safety assessment on the use of NMC as a glazing or coating agent in food supplements.

The exposure assessment included information provided by the sponsor. This information included uses of the copolymer, concentrations it is used in and estimates of exposure.

Uses or proposed uses for NMC include in pharmaceuticals, food supplements and foods for special medical purposes. NMC has been used in sustained-release formulations in the pharmaceutical industry for many years. NMC is also used as a glazing or coating agent in food supplements that require sustained-release formulations (e.g. nutrients). Such formulations allow the continuous dissolution of the active ingredient over a defined time. NMC is used in products in single dosage unit forms – hard or soft gel capsules, pastilles, tablets, pills as well as multiparticulates such as pellets, granules and powders.

The release from a dosage unit coated with NMC is time dependent, rather than triggered by pH. The copolymer does not dissolve but forms a porous gel that allows the active substances to be released slowly. NMC is particularly useful where high peaks of the active substance in the blood should be avoided and an exposure over a broader time period is of benefit. A sustained-release dosage form allows intake only once per day instead of multiple times. In combination with certain other ingredients in the coating formulation, the gel-forming properties can enable an enteric release in the small intestine for substances that would need protection from gastric acid or that may irritate the mucosa, such as fish oil or garlic oil.

As noted by the sponsor, the copolymer is not used for liquid supplements, and this form was not included in this assessment.

3.2 Approach to the exposure assessment

JECFA reviewed exposure estimates that were calculated and submitted by the sponsor. The sponsor included a review of the exposure assessment of the copolymer by European Food Safety Authority (EFSA, 2010). The Committee also evaluated the EFSA (2010) exposure assessment and conducted a literature search (see section 1.2).

The Committee also undertook deterministic calculations to estimate exposure to the copolymer where national food consumption data were available. These data were primarily from the FAO/WHO Chronic Individual Food Consumption – Summary statistics (CIFOCOss; https://www.who.int/foodsafety/databases/en/) database for General Standard on Food Additives (GSFA) food category code 13.6, "food supplements". Consumption data were also submitted for Australia and New Zealand. These are further discussed in section 3.4.1.

Exposures were reviewed or estimated for mean and high consumers as well as for adults and children.

In their assessment, the sponsor included children aged 6 years of age and older based on the assumption that younger children take supplements in liquid form. However, the Committee also included children between 1 and 6 years in its assessment based on evidence recorded in national dietary surveys (e.g. CIFOCOss) of consumption of solid supplements by children this age. In addition, some chewable tablets and soft gels are suitable for use by children from 2 years of age.

Estimates of exposure are included in the assessment for NMC and its monomers (see section 3.5).

3.3 Estimates of exposure to NMC

3.3.1 Food supplements

(a) Estimates from the sponsor

The sponsor provided use levels of NMC in food supplements. The conventional amount of NMC for a sustained-release profile is in the range of 10% to 20% (16.7–33.4 mg/cm^2). This equates to up to 200 mg per 1 g tablet or dosage unit. This was the concentration used in the exposure assessments. Although larger unit weights are on the market, these do not represent the majority of the relevant products and were not considered in this assessment.

It was assumed that if different dosage forms (tablets, powders, etc.) with different surface area to volume ratios were used, the same concentration applies.

Consumption amounts for food supplements were evaluated by the sponsor from previous evaluations conducted by EFSA (2004a,b, 2005, 2006) and the Scientific Committee on Food (SCF) for other food additives used in this food group (SCF, 1997, 2002). The number of dosage units consumed per day were calculated as between 3 and 7 units for adults (12 for extreme users) and 2 for "children/young people" (ages between 6 and 18 years).

The sponsor also reviewed national consumption data from a range of dietary surveys, which mostly included data on frequency of consumption but also some data on consumption amounts (summary provided in Appendix 1). Consumption data from the Netherlands (Ocké, Buurma-Rethans & Fransen, 2005) indicate that 80% of users consume one or two supplements twice a day (total of 4 supplements per day), and 6% were high users who consumed 4 supplements twice a day (a total of 8 supplements per day). The sponsor assumed that one of these supplements was liquid and, because of the limited range of uses for supplements containing NMC (i.e. the polymer is used only for specific nutrients where a delayed release is required, or where peaks in blood

levels need to be avoided, or where lower doses of irritating active ingredients are needed to reduce mucosal irritation), a smaller proportion of supplements would contain NMC. In addition, given that NMC is used in sustained-release formulations, a smaller number of dosage units are needed as they are less likely to be consumed multiple times a day. Based on these assumptions, the sponsor calculated consumption by adults to be 2 units per day, by adult heavy users to be 3 units per day, and by children (not younger than 6 years) to be 1 unit per day.

The sponsor estimated exposures to NMC from expected consumption of food supplements to be 6.7 mg/kg bw per day based in adults (10 in heavy users) and 13.3 mg/kg bw per day in children (Table 4).

The sponsor used a body weight of 15 kg for children 6 years and older. The WHO standard body weight for a 5 year old at the 50th percentile is 18.3 kg for boys and 18.2 kg for girls (WHO Multicentre Growth Reference Study Group, 2006). Therefore, the body weight used by the sponsor for this population group may be an underestimate, resulting in a worst case scenario estimate of exposure on a body weight basis.

(b) Estimates of exposure calculated by EFSA

EFSA estimated exposures to NMC from its use in food supplements (EFSA, 2010) using a concentration of 200 mg per 1 g dosage unit. The assessment used consumption data from the British National Diet and Nutrition Surveys for high consumers (97.5th percentile) of food supplements for adults aged 19–64 years (Henderson, Gregory & Swan, 2002) and children aged 4–18 years (Gregory & Lowe, 2000). Seven and 2 dosage units per day were used for adults and children, respectively.

EFSA estimated exposures in high consumers using 200 mg of NMC per 1 g dosage unit at 23.3 mg/kg bw per day for adults and 16.0 mg/kg bw per day for children 4–18 years (Table 5). These estimates did not take into account that not all supplements contain NMC, as noted by the sponsor, and therefore they may be overestimates. However, if a consumer was brand loyal to a particular NMC-containing supplement they consumed regularly, the exposures would be appropriate.

(c) National estimates of exposure calculated by the Committee

The Committee estimated exposure to NMC from food supplement use for a number of countries using consumption data in the CIFOCOss database (GSFA food category no. 13.6, "food supplements").

CIFOCOss includes data collected via national population dietary surveys of 2 or more days of individuals' food consumption (for further details, see http://www.who.int/foodsafety/databases/en/). Food consumption data are

presented according to the CIFOCOss food classification system. For most food groups, this is based on the Codex raw commodity classification system; for some processed commodities, it is based on the Codex GSFA classification system. Food consumption data for a number of different age groups are represented in the CIFOCOss dataset based on the data collected in the countries' national surveys. The Committee used consumption data for the general population and for children for the exposure estimates; the data were not split by sex.

Food supplement consumption data were available in CIFOCOss for nine countries (Brazil, Finland, Germany, Ireland, Italy, the Netherlands, Sweden, Thailand, the United Kingdom). However, as there were no quantitative consumption amounts for Thailand, that country was not included. The data were not used for exposure calculations where there were fewer than 10 consumers per population group as this number was too low for an estimate to be reliable. Thus data for Germany (children), Italy (for infants, children and adolescents) and the Netherlands (toddlers and children) were excluded. The data from Brazil were also excluded as the amounts consumed were at least 5 times higher than those of all other population groups included in the dataset, as no explanation was provided (e.g. what was included in the food group), these data were determined to be outliers and not retained for the exposure calculation. There were no consumption data for food supplements for infants in CIFOCOss.

Mean and 90th percentile consumption data per kg of body weight for consumers only were used for the exposure calculations to take into account the specific body weights relevant to each country and population group. Concentrations of NMC used in the calculations were those provided by the sponsor: 200 mg per 1 g dosage unit.

Consumption data for dietary supplements for Australia and New Zealand were also provided to the Committee for use in the assessment (Food Standards Australia New Zealand, personal communication, 30 May 2018). The Australian data were from the 2011/12 National Nutrition and Physical Activity Survey (ABS, 2015) based on 2 days of consumption data. The New Zealand data were from the 2008/09 NZ Adult Nutrition survey (University of Otago and Ministry of Health, 2011) and the 2002 Children's Nutrition survey (Parnell et al., 2003) based on 1 day of consumption data. Liquid supplements were excluded when deriving the consumption amounts.

The actual consumption amounts as reported in the CIFOCOss database, shown in Table 6 in g/kg bw per day, were used for the exposure assessment calculations. The consumption amounts were not rounded to a number of dosage units for the exposure calculations, but were converted to 1 g units to compare with the number of dosage units used by the sponsor and EFSA. Based on mean consumption data, the number of dosage units consumed by adults ranged between 3 and 7 units per day and, at the 90th percentile, between 5 and 20 units

per day. Similarly, based on mean consumption data, the number of dosage units consumed by children ranged between 1 and 4 per day and, at the 90th percentile, between 2 and 10 per day. For adults, the mean consumption range was the same as the range used by the sponsor and EFSA, but at the 90th percentile, the upper end of the consumption range was up to nearly 3 times as high. The lower end of the mean consumption range for children was similar to that used by the sponsor and EFSA, but the upper end of the range was about double and the upper end of the range for the 90th percentile was around 5 times higher. Apart from the values from Australia and New Zealand, the specific types of supplements – and whether this included liquid supplements – in unknown.

Exposures to NMC from use in food supplements estimated by the Committee from national food consumption data for adults at the mean ranged between 6.9 and 23.2 mg/kg bw per day and, at the 90th percentile, between 14.9 and 74.1 mg/kg bw per day (Table 6). For children, mean exposure ranged between 5.8 and 32.1 mg/kg bw per day and at the 90th percentile between 8.3 and 85.5 mg/kg bw per day. These estimates do not take into account that not all supplements will contain NMC and therefore may be overestimates. However, if a consumer was brand loyal to a particular supplement that contained NMC and consumed it regularly, these estimates would be appropriate.

3.3.2 Pharmaceuticals

(a) Estimates from the sponsor

NMC has been used in pharmaceuticals in Europe since 1972, and exposure from this source was also considered in the estimates by the sponsor. The same concentration was used for pharmaceuticals as for food supplements (200 mg/1 g dosage unit). While the sponsor indicated that previous EFSA assessments were based on consumption of 10–12 units of pharmaceuticals per day, this was considered a conservative estimate given that not all pharmaceuticals contain NMC. NMC use in pharmaceuticals represents only a small proportion of all oral drugs (25%) (Eisele et al., 2013). As a result, 3 units/day was used in the exposure assessment for adults and 1/day for young children.

Exposures to NMC from use in pharmaceuticals were estimated by the sponsor as 10 mg/kg bw per day for adults and 13.3 mg/kg bw per day for children (Table 7).

(b) Estimates of exposure calculated by EFSA

For its estimate of exposures to NMC from its use in pharmaceuticals, EFSA (2010) used the same concentration of NMC as used for exposure via supplements, that is, 200 mg per 1 g dosage unit. The same number of dosage units used for food

Table 6
Estimated national exposures to NMC from use as a food additive in food supplements [a]

Population / country	Age group (years)	No. of consumers	Percentage of consumers	Consumption (g/kg bw per day)		Exposure (mg/kg bw per day)	
				Mean	P90	Mean	P90
Adults							
Australia	≥19	2 284	38	0.03	0.08	6.9	15.3
Finland	18–64	663	42	0.04	0.08	8.4	15.1
	65-74	215	46	0.05	0.11	10.1	21.6
Ireland	18-64	246	26	0.04	0.09	8.6	17.3
Italy	18-64	98	4	0.08	0.20	16.5	40.0
	65-74	12	4	0.04	0.11	7.4	21.7
	≥75	17	7	0.12	0.37	23.2	74.1
New Zealand	≥15	576	12	0.05	0.09	9.4	18.2
United Kingdom	18–64	419	20	0.05	0.07	9.0	14.9
Children							
Australia	2–5	75	19	0.12	0.26	24.0	51.4
	6–18	236	18	0.04	0.06	7.2	12.7
Finland	3–9	56	22	0.16	0.43	32.1	85.5
New Zealand	5–14	144	4	0.05	0.09	9.5	17.5
Sweden	3–9	263	18	0.08	0.10	16.0	19.2
	10–17	178	17	0.03	0.04	5.8	8.3

bw: body weight; NMC: neutral methacrylate copolymer; no.: number; P90: 90th percentile
[a] Calculated by the Committee using national dietary survey data.

Table 7
Estimated exposures to NMC by the sponsor from use in pharmaceuticals

Population	No. of units consumed/day [a]	Exposure	
		mg per day	mg/kg bw per day [b]
Adults	3	600	10.0
Young people / children 6–18 years	1	200	13.3

bw: body weight; NMC: neutral methacrylate copolymer; no.: number
[a] One unit is 1 g tablet or dosage unit containing 200 mg of NMC.
[b] Based on a mean body weight of 60 kg for adults and 25 kg for children.

supplements were used for pharmaceuticals: 3 units per day for adults and 1 unit per day for young children.

EFSA estimated exposures to NMC when used in pharmaceuticals at 200 mg per 1 g dosage unit to be 23.3 mg/kg bw per day for adult high consumers and 16.0 mg/kg bw per day for children 4–18 years old (Table 8).

Table 8
Estimated exposures to NMC from use in pharmaceuticals for high consumers

Population	No. of units consumed/day [a]	Exposure	
		mg per day	mg/kg bw per day [b]
Adults 19–64 years	7	1 400	23.3
Young people/ children 4-17 years	2	400	16.0

bw: body weight; NMC: neutral methacrylate copolymer; no.: number
[a] One unit is 1 g tablet or dosage unit containing 200 mg of NMC.
[b] Based on a mean body weight of 60 kg for adults and 25 kg for children.
Source: EFSA (2010)

3.3.3 Foods for special medical purposes

NMC can be used in foods for special medical purposes as incomplete foods when these are presented in the same form of solid food supplements (e.g. tablets, capsules, etc.). The single dosage units are likely to contain the same concentration of NMC as food supplements, that is,20% of the final dosage unit (200 mg/1 g unit).

Foods for special medical purposes are generally defined as those formulated for exclusive or partial dietary management of patients under medical supervision who have limited ability to digest or metabolize foods or nutrients or who have increased nutrient requirements. These products may contain higher concentrations of active ingredients than food supplements for the general population. Foods for special medical purposes products would not normally be consumed by the general healthy population but by specific individuals who would generally be under medical supervision.

The duration of use of foods for special medical purposes depends on the medical indication and may vary from short-term to lifetime use. Consumption of foods for special medical purposes, and exposures to NMC from these, depends on the dosage instructions and/or the medical needs of the individual and are therefore highly variable.

No food consumption data for foods for special medical purposes were provided by the sponsor. While the CIFOCOss database has some consumption data for foods for special medical purposes, there is insufficient information to determine what specific foods are included by each country in this category. It was therefore not possible to determine if these include single dosage units. As such, the data from the CIFOCOss database could not be used to estimate exposures from foods for special medical purposes.

A literature search found no consumption data for foods for special medical purposes.

As a result, the Committee was not able to estimate dietary exposures from foods for special medical purposes. Therefore, there is a degree of uncertainty in the Committee's exposure assessment and the overall safety evaluation. However, for individuals managing a condition or disease utilizing foods for special medical purposes may be a priority above the need to consider exposure to NMC from these foods.

The sponsor noted that the NMC-containing foods for special medical purposes are similar to food supplements. Given the conservative nature of those calculations, which are based on high consumers, foods for special medical purposes are not anticipated to increase exposures above that of food supplements and pharmaceuticals. However, there are no data or information available to suggest this is the case.

The use of NMC in foods for special medical purposes was therefore not considered further by the Committee.

3.4 Estimates of exposure to the monomers of NMC

The NMC monomers are methyl methacrylate and ethyl acrylate. The Committee reviewed estimated exposures to each monomer provided by the sponsor and from the EFSA (2010) assessment and calculated national estimates of exposure.

The total monomer content is up to 0.01% of the copolymer. Therefore, this proportion was used to convert the estimates of exposure to the copolymer to an exposure to the total monomers.

The weight ratio of the monomers was used to convert the total exposure to all monomers to an exposure for each individual monomer.

3.4.1 Estimates from the sponsor

Exposures to the monomers as estimated by the sponsor from uses as food supplements and in pharmaceuticals are shown in Table 9. For adults, exposures from food supplements were 0.19–0.30 µg/kg bw per day for methyl methacrylate and 0.47–0.70 µg/kg bw per day for ethyl acrylate (range based on 2–3 dosage units per day). For children, the exposures from food supplements were 0.30 µg/kg bw per day for methyl methacrylate and 0.94 µg/kg bw per day for ethyl acrylate (based on 1 dosage unit per day).

Exposures from pharmaceuticals for adults were 0.30 µg/kg bw per day for methyl methacrylate and 0.70 µg/kg bw per day for ethyl acrylate (based on 3 dosage units per day). Exposures from pharmaceuticals for children were exactly the same as for food supplements as it was assumed that the same number of dosage units was ingested.

Table 9
Sponsor-estimated exposures to NMC monomers from uses in food supplements and pharmaceuticals

Source	Population	Exposure to NMC (mg/kg bw per day) [a]	Exposure to NMC monomers (µg/kg bw per day) [b]		
			Total [b]	Methyl methacrylate	Ethyl acrylate
Food supplements	Adults	6.7–10.0	0.7–1.0	0.19–0.30	0.47–0.70
	Children	13.3	1.3	0.39	0.94
Pharmaceuticals	Adults	10.0	1.0	0.30	0.70
	Children	13.3	1.3	0.39	0.94

bw: body weight; NMC: neutral methacrylate copolymer
[a] Based on consuming 2–3 supplement dosage units per day and 3 dosage units for pharmaceuticals for adults; and 1 dosage unit for each of supplements and pharmaceuticals per day for children. Body weight 60 kg for adults and 15 kg for children.
[b] Based on a total monomer content of 0.01% of the copolymer.

3.4.2 Estimates of exposure calculated by EFSA

EFSA (2010) estimated exposures to NMC monomers expressed as the metabolites; EFSA did not present exposures to each of the individual monomers. The Committee estimated exposure to the monomers from food supplements and pharmaceuticals (Table 10) based on the EFSA estimated exposure to the copolymer using the 20% coating scenario, 0.01% of monomers in the copolymer and weighting for the weight of each monomer in the copolymer.

For adults, exposures from supplements were 1.38 µg/kg bw per day for methyl methacrylate and 3.28 µg/kg bw per day for ethyl acrylate (based on 7 dosage units per day). For children, exposures from supplements were 0.95 µg/kg bw per day for methyl methacrylate and 2.25 µg/kg bw per day for ethyl acrylate (based on 2 dosage units per day).

Exposures from pharmaceuticals were exactly the same as for food supplements as the same number of dosage units was assumed.

3.4.3 National estimates of exposure calculated by the Committee

The Committee estimated national exposures to NMC monomers (methyl methacrylate and ethyl acrylate) from uses of the copolymer in food supplements (Table 11).

Estimated exposures for adults to ethyl acrylate at the mean ranged between 0.49 and 1.63 µg/kg bw per day and, at the 90th percentile, between 1.05 and 5.21 µg/kg bw per day. Estimated exposures for adults to methyl methacrylate at the mean ranged between 0.20 and 0.69 µg/kg bw per day and, at the 90th percentile, between 0.44 and 2.19 µg/kg bw per day.

Estimated exposures for children to ethyl acrylate at the mean ranged between 0.41 and 2.26 µg/kg bw per day and, at the 90th percentile, between

Table 10

Estimated exposures to NMC and NMC monomers from uses in food supplements and pharmaceuticals based on EFSA data

Source	Population	Exposure to NMC (mg/kg bw per day) [a]	Monomeric exposure (µg/kg bw per day) [b]		
			Total [c]	Methyl methacrylate	Ethyl acrylate
Food supplements	Adults	23.3	4.7	1.38	3.28
	Children	16.0	3.2	0.95	2.25
Pharmaceuticals	Adults	23.3	4.7	1.38	3.28
	Children	16.0	3.2	0.95	2.25

bw: body weight; EFSA: European Food Safety Authority NMC: neutral methacrylate copolymer

[a] Based on consuming 7 dosage units per day for adults and 2 dosage units per day for children for each of food supplements and pharmaceuticals. Body weight 60 kg for adults and 25 kg for children.

[b] Calculated by the Committee from EFSA (2010) copolymer exposure estimates from uses in food supplements and pharmaceuticals.

[c] Based on a total monomer content of 0.01% of the copolymer.

Table 11

Committee-estimated exposures to NMC and NMC monomers based on national food consumption data [a]

Population / country	Years	Monomeric exposure (µg/kg bw per day)					
		Total [b]		Methyl methacrylate		Ethyl acrylate	
		Mean	P90	Mean	P90	Mean	P90
Adults							
Australia	≥19	0.7	1.5	0.20	0.45	0.49	1.08
Finland	18–64	0.8	1.5	0.25	0.45	0.59	1.06
	65–74	1.0	2.2	0.30	0.64	0.71	1.52
Ireland	18–64	0.9	1.7	0.25	0.51	0.60	1.22
Italy	18–64	1.7	4.0	0.49	1.19	1.16	2.81
	65–74	0.7	2.2	0.22	0.64	0.52	1.53
	≥75	2.3	7.4	0.69	2.19	1.63	5.21
New Zealand	≥15	0.9	1.8	0.28	0.54	0.66	1.28
United Kingdom	18–64	0.9	1.5	0.27	0.44	0.64	1.05
Children							
Australia	2–5	2.4	5.1	0.71	1.52	1.69	3.62
	6–18	0.7	1.3	0.21	0.38	0.50	0.89
Finland	3–9	3.2	8.6	0.95	2.53	2.26	6.01
New Zealand	5–14	1.0	1.8	0.28	0.52	0.67	1.23
Sweden	3–9	1.6	1.9	0.47	0.57	1.13	1.35
	10–17	0.6	0.8	0.17	0.25	0.41	0.59

bw: body weight; NMC: neutral methacrylate copolymer; P90: 90th percentile

[a] Based on consumption data for food supplements for consumers only.

[b] Total of methyl methacrylate and ethyl acrylate based on total monomer content of 0.01% of the copolymer.

0.59 and 6.01 μg/kg bw per day. Estimated exposures for children to methyl methacrylate at the mean ranged between 0.17 and 0.95 μg/kg bw per day and, at the 90th percentile, between 0.25 and 2.53 μg/kg bw per day.

These estimates do not take into account that not all supplements will contain NMC and therefore are likely to be overestimates. However, if a consumer was brand loyal to a particular supplement that contained NMC which they consumed on a regular basis, the exposures could be assumed to be appropriate estimates for those consumers.

3.5 Summary of the estimates of exposure for NMC for use in the safety evaluation

A summary of the estimates of exposure to NMC and its monomers was required for this evaluation. Although estimates of exposure to NMC from pharmaceuticals were reviewed, the Committee considered that this use should not be taken into account in the assessment of chronic dietary exposure for a healthy population, and only dietary exposures from food supplements were considered.

In order to capture the broad range of exposures from all sources of the estimates (the sponsor, EFSA and the Committee) for food supplements, the lower end of the range of mean exposures was used and the upper end of the high percentile exposures. The range of exposures used in the evaluation (Table 12) were those estimated by the Committee from national exposures as the estimates from the sponsor and EFSA were within these estimates. This was the case for the copolymer and monomer exposures.

The range of exposures were rounded for the evaluation given as uncertainties are inherent in dietary exposure estimates and the results are a guide for risk characterization purposes.

4. Comments

4.1 Biochemical aspects

4.1.1 NMC

In a pre-GLP study, NMC was found to be poorly absorbed and quickly eliminated from the body when single doses of ^{14}C-labelled NMC at 600 mg/kg bw per day were administered to rats by gavage. An average of 97.6% of the radioactivity was excreted in the faeces within 48 hours. Seven days after dosing, levels of

radioactivity in tissues of treated animals did not differ significantly from that of control animals (HSL, 1986).

4.1.2 Residual monomers

Methyl methacrylate is rapidly absorbed and distributed following inhalation or oral administration to rats. Methyl methacrylate is metabolized to methacrylic acid (and methanol), which is subsequently converted to carbon dioxide via the tricarboxylic acid cycle in both experimental animals and humans (Bratt & Hathway, 1977; Government of Canada, 1993; ECETOC, 1995).

Ethyl acrylate is rapidly absorbed and metabolized following inhalation or oral administration in rats. Metabolism occurs via hydrolysis of the ester linkage by carboxylesterases, forming ethanol and acrylic acid, both of which are ultimately metabolized to carbon dioxide, or via conjugation of ethyl acrylate with glutathione. Following conjugation with glutathione, ethyl acrylate is rapidly eliminated via urinary excretion (Health Canada, 2011).

4.2 Toxicological studies

4.2.1 NMC

Two acute toxicity studies were available, in rats and in dogs. Rats received doses of 25.2–28.2 g/kg bw in their feed; dogs received doses of 7950 or 9100 mg/kg bw in their feed. No treatment-related effects were seen during the observation period or at macroscopic examination of the organs. Urine analysis also found no treatment-related effects (LPT, 1971, 1972a).

Two short-term toxicity studies were available in rats. In the first study, doses of 0, 500, 1000 or 2000 mg/kg bw per day were given by gavage for 35 days. It is not clear if the doses were expressed in dry weight of polymer or of the preparation. No treatment-related effects were observed (BoZo Research Center, 1981). In the second study, doses of 0, 500 or 2000 mg/kg bw per day were given in the feed for 6 months. No treatment-related effects were observed (LPT, 1972b). In both studies, the NOAELs were 2000 mg/kg bw per day, the highest doses tested. In a 26-week study, dogs were given NMC at doses of 0, 50, 125 or 250 mg/kg bw per day administered as NMC-coated cellulose pellets. Apart from decreases in body-weight gain and feed consumption at the highest dose, and the presence of white granular material in the gut, attributed to the physical characteristics of the test material, no treatment-related effects were observed. The NOAEL was 250 mg/kg bw per day, the highest doses tested (Research Toxicology Centre, 2007a). A 28-day study in mini pigs, in which NMC was given by gavage as NMC-coated cellulose pellets, the calculated doses were 0, 113, 227

and 454 mg/kg bw per day. Other than the presence of white granular material in the intestines, attributed to the physical characteristics of the test substance, no treatment-related effects were found. The NOAEL was 454 mg/kg bw per day, the highest dose tested (Research Toxicology Centre, 2006).

Two reverse mutation studies in bacteria (Life Science Research, 1977; RCC, 2005), one in vitro mouse lymphoma assay (RCC, 2001) and an in vivo mouse micronucleus assay (Research Toxicology Centre, 2007b) gave negative results. The Committee concluded that the NMC does not give rise to concern for genotoxicity.

No long-term toxicity or carcinogenicity studies with NMC were available.

Developmental toxicity studies were available in rats and rabbits, both using doses of 0, 500 or 2000 mg/kg bw per day in the feed during the period of organogenesis. No treatment-related effects were observed. The NOAEL for both studies was 2000 mg/kg bw per day, the highest doses tested (LPT, 1974a,b).

Studies on dermal, inhalation and ocular toxicity found no effects (BSL, 1998b,c, 2002; RCC, 2004). NMC was found to be cytotoxic at the highest dose tested of 1500 μg/mL (BSL, 1998a).

4.2.2 Residual monomers

(a) Methyl methacrylate

In a long-term toxicity study in rats given methyl methacrylate in drinking-water at 0, 6, 60 or 2000 mg/L (equal to 0, 0.4, 4 and 121 mg/kg bw per day for males and 0, 0.5, 5 and 146 mg/kg bw per day for females, respectively) for 2 years, relative kidney weight increased in females at the highest dose but no treatment-related histopathological effects were observed in any organs or tissues (Borzelleca et al., 1964). Based on the results of this study, a TDI of 1.2 mg/kg bw per day was determined (WHO, 1998).

In long-term toxicity and carcinogenicity studies in mice, rats and hamsters given methyl methacrylate by inhalation, the observed effects were, in general, similar to those reported in the short-term toxicity studies but also included inflammation and epithelial hyperplasia of the nasal cavity and degeneration of the olfactory sensory epithelium. There was no evidence of any carcinogenic effects (Borzelleca et al., 1964).

Bacterial reverse mutation assays with methyl methacrylate gave mostly negative results (Lijinsky & Andrews, 1980; Hachiya, Taketani & Takizawa, 1982; Waegemaekers & Bensink, 1984; NTP, 1986b; Schweikl, Schmalz & Rackebrandt, 1998). Mixed results (i.e. positive, weakly positive or negative) were obtained in in vitro chromosomal aberration (NTP, 1986b; Doerr, Harrington-Brock & Moore, 1989; Bigatti et al., 1994; Schweikl, Schmalz & Rackebrandt, 1998; Tuček

et al., 2002; Yang et al., 2003) and SCE assays(NTP, 1986b; Cannas et al., 1987; Yang et al., 2003). One in vitro micronucleus assay was unequivocally negative (Schweikl, Schmalz & Spruss, 2001), whereas a second assay was negative at low concentrations but weakly positive at higher concentrations (Doerr, Harrington-Brock & Moore, 1989). Three mouse lymphoma assays for gene mutations were positive (NTP, 1986b; Moore et al., 1988; Dearfield et al., 1991). A mouse bone marrow micronucleus assay was negative, but it is not clear if the target tissue was exposed to the test substance (Hachiya, Taketani & Takizawa, 1982). A rat micronucleus assay with exposure by inhalation was positive following 1 day of exposure but negative following 5 days of exposure (Araújo et al., 2013). These results were judged by the Committee to be inconclusive.

The Committee concluded that there was some evidence of mutagenicity and clastogenicity in vitro. There was a lack of adequate in vivo tests following up the equivocal findings.

(b) Ethyl acrylate

Studies in mice and rats administered ethyl acrylate at 0, 100 or 200 mg/kg bw per day for 103 weeks by gavage showed an increased incidence of squamous cell hyperplasia and papillomas in the forestomach of both species, and, in male rats, squamous cell carcinomas in the forestomach. In contrast, studies using other routes of administration including via inhalation (with concentrations up to 310 mg/m^3) and in the drinking-water (with doses up to 280 mg/kg bw per day) showed no such effects. An IARC working group concluded that the mechanism of formation of the forestomach tumours in rodents is not relevant to humans; rather it can be attributed to the irritating effect of high bolus doses of ethyl acrylate delivered to the contact site (forestomach) by gavage (IARC, 2003). This conclusion was recently confirmed by Health Canada (2011). The Committee concluded that ethyl acrylate is not a carcinogenic risk to humans.

Genotoxicity results for ethyl acrylate are mixed, with some positive results in vitro, some negative results in reverse mutation assays in bacteria and positive results in a mouse lymphoma assay. There were both positive and negative results in in vitro SCE assays and in chromosomal aberration assays with metabolic activation, but no evidence of clastogenicity in the absence of metabolic activation.

In in vivo studies on ethyl acrylate, one early mouse micronucleus study using intraperitoneal dosing gave positive results (Przybojewska, Dziubałtowska & Kowalski, 1984). However, three other micronucleus studies in mice (one dermal exposure, two by intraperitoneal injection) all gave negative results (Annex 1, reference *174*). A chromosomal aberration assay and an in vivo SCE assay and a recent point mutation assay in mice were also negative. The Committee

concluded that the genotoxic potential observed in some in vitro studies was not expressed in vivo. The Committee noted that Health Canada (2011) reached a similar conclusion.

4.3 Observations in humans

No human data were available on NMC.

4.4 Assessment of dietary exposure

The Committee evaluated exposure to NMC from its use as a glazing or coating agent in food supplements and foods for special medical purposes. As another major use of NMC is in pharmaceuticals, this use was also considered in the exposure assessment. The level of use of NMC is a maximum of 20%.

The Committee evaluated exposure to NMC for the copolymer and its monomers, methyl methacrylate and ethyl acrylate. The exposure assessment included estimates submitted by the sponsor and an evaluation by EFSA (2010) based on consumption of food supplements and pharmaceuticals. The Committee also estimated exposure based on national food consumption data for food supplements using the concentration proposed by the sponsor. The national consumption data were from CIFOCOss and data submitted to the Committee from Australia and New Zealand. A comprehensive literature search was also conducted; no additional studies relevant to the exposure assessment were found.

No quantitative estimates of exposure could be determined for foods for special medical purposes. The sponsor indicated that it is not anticipated that foods for special medical purposes would increase exposures above that of food supplements and pharmaceuticals given the conservative nature of those calculations. In addition, the consumers of foods for special medical purposes will generally be under medical supervision, and exposures for these consumers are therefore not relevant for the general healthy population. This use was therefore not further considered by the Committee.

The total monomeric content of NMC is less than 0.01%. This level was used to calculate the exposure to total monomers from the copolymer exposure. Estimates of exposure to the individual monomers were based on the exposure to total monomers, taking into account the ratio of each individual monomer in the copolymer.

All estimates of exposure are presented as a range from the lowest of the average exposures to the highest of the high exposures.

The estimated exposures to NMC and its monomers from uses in food supplements are shown in Table 12.

Table 12
Summary of the range of estimated exposures to NMC and its monomers from uses in food supplements for average and high exposures

	Range of estimated dietary exposures [a,b]		
	Copolymer exposure (mg/kg	Monomer exposure (µg/kg bw per day)	
Population group	bw per day)	Methyl methacrylate	Ethyl acrylate
Adults	6.9–74	0.2–2.2	0.5–5.2
Children	5.8–86	0.2–2.5	0.4–6.0

bw: body weight; NMC: neutral methacrylate copolymer
[a] All estimates of exposure are presented as a range from the lowest of the average exposures to the highest of the high exposures. The lower end of the range is the lowest of the estimated mean exposures, and the upper end of each range is the highest of the estimated high exposures.
[b] Includes exposure estimates submitted by the sponsor and EFSA (2010) and national estimates calculated by the Committee, based on a concentration of 200 mg per 1 g dosage unit.

The Committee noted that NMC is used in pharmaceuticals. Estimated exposures from this use from the sponsor and EFSA (2010) ranged between 10.0 and 23.3 mg/kg bw per day for adults and children. These estimates were within the range of exposures from food supplements. However, the Committee considered that such use should not be taken into account in the assessment of long-term dietary exposure for a healthy population.

5. Evaluation

New specifications for NMC were prepared and made tentative, requiring a suitable validated method for its assay. A Chemical and Technical Assessment was prepared.

The Committee concluded that the use of NMC that complies with the specifications established at the current meeting is not of safety concern when the food additive is used as a coating or glazing agent for solid food supplements and for foods for special medical purposes.

The Committee therefore established an acceptable daily intake (ADI) "not specified" for NMC. The ADI "not specified" was made temporary because the specifications are tentative.

The available toxicology data for NMC do not give rise to concerns for toxicity. The substance is poorly absorbed and is excreted in the faeces. In short-term and developmental toxicity studies, the NOAELs for NMC range from 454 to 2000 mg/kg bw per day, and these were the highest doses tested. Estimated exposures to NMC range from 5.8 to 86 mg/kg bw per day.

Toxicological data on the residual monomers do not give rise to concerns when taking into account the low exposures. Genotoxicity data for methyl

methacrylate suggest a potential risk for mutagenicity and clastogenicity in vitro, and there is a lack of adequate data on genotoxicity in vivo. However, in carcinogenicity studies in mice, rats and hamsters given methyl methacrylate by inhalation, there was no evidence of any carcinogenic effects. In a 2-year drinking-water study on methyl methacrylate in rats, the NOAEL was 121 mg/kg bw per day, from which a TDI of 1.2 mg/kg bw per day was derived (WHO, 1998). Estimated exposures to methyl methacrylate range from 0.2 to 2.5 μg/kg bw per day, which are below the TDI.

Although there were some positive genotoxicity findings for ethyl acrylate, the Committee concluded that the genotoxic potential observed in some in vitro studies was not expressed in vivo. Long-term toxicity studies on ethyl acrylate in mice and rats produced forestomach tumours, but the Committee concurred with the conclusions of IARC (2003) and, more recently, Health Canada (2011), that the mechanism of forestomach tumour formation in rats and mice is not relevant to humans. The Committee was also reassured by the long-term carcinogenicity studies on ethyl acrylate that did not use gavage as the route of administration; these suggest that ethyl acrylate is not carcinogenic at the doses tested. In the 2-year drinking-water study on ethyl acrylate, the NOAEL was 280 mg/kg bw per day. Estimated dietary exposures to ethyl acrylate range from 0.4 to 6 μg/kg bw per day. The margin of exposure based on the highest estimated exposure was calculated to be 46 000.

Assessments of dietary exposure to methyl methacrylate and ethyl acrylate due to their residual occurrence in NMC suggest that exposure to these monomers from the uses of NMC is not a safety concern.

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Spirulina extract

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1. Explanation

Spirulina extract (International Numbering System for Food Additives [INS] 134) is used as a food additive, as a blue colouring agent. Spirulina extract is used in food in China, the European Union, Japan, Mexico and the United States of America (USA), among others. It is used as a colour in a wide range of foods and beverages including flavoured dairy products, cheese, dairy-based desserts, processed fruits and vegetables, baked goods and baking mixes, alcoholic and non-alcoholic beverages and beverage bases, breakfast cereals, cocoa products, confectionery products (including soft and hard candy and chewing gum), egg products, gravies and sauces, herbs and spices, condiments and soup and soup mixes. It is also used as a colouring agent in nutritional supplements and in pharmaceuticals. Intended use levels range from 400 to 40 000 mg/kg, depending on the food item or category and the colour strength of the formulated spirulina extract used.

Spirulina extract has not been evaluated previously by the Committee. It was placed on the agenda for its use as a food colour at the request of the Forty-ninth Session of the Codex Committee on Food Additives (FAO/WHO, 2017).

A toxicological dossier submitted by the sponsor summarized the available toxicity data on spirulina, together with relevant study reports and publications. A few of the studies were conducted using spirulina extract, but most toxicity studies used dried spirulina as the test material. A comprehensive literature search retrieved a further 17 relevant toxicological study reports.

The literature search for texts relevant to the toxicological assessment was conducted following the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Secretariat guidance (WHO JECFA Secretariat, 2017) using the keywords spirulin* OR arthrospir*. The databases searched included EMBASE (1974–2017 December 26; 222 records for human studies, 835 records for other studies); Ovid MEDLINE (1946–2017 December 27; 119 records for human studies, 692 records for other studies); CAB Abstracts (1973–2017 Week 50), Food Science and Technology Abstracts (1969–2017 December Week 5); Global Health (1973–2017 Week 50); International Pharmaceutical Abstracts (1970–2017 December 28); 7 records for human studies, 625 records for other studies). Seventeen of the records retrieved added to the toxicological data submitted to the Committee for this meeting.

The literature search for references relevant to the dietary exposure assessment, also conducted following the JECFA Secretariat guidance (2017), used the following keywords: ((exposure OR intake) AND (Spirulina OR Arthrospira OR phycobili* OR *phycocyanin*)). In addition, searches were performed using literature links, Google Scholar and DTU Library search facilities. Nine references relevant to dietary exposure assessment were retrieved. Information on dosing in

intervention studies were used in assessment of exposure from food supplements, but no information on occurrence or exposure from to use of Spirulina products in food were identified.

1.1 Chemical and technical considerations

Spirulina extract is produced from *Arthrospira platensis* (commonly called *Spirulina platensis*), an edible cyanobacterium cultivated in open or covered ponds or in bioreactors. Commercial cultivation occurs in alkaline aqueous medium containing sodium bicarbonate, nitrates, phosphates, sulfates and other nutrients including trace minerals. No herbicides or additional solvents are used during the cultivation of *A. platensis* or in the manufacture of spirulina extract. Cultivation conditions are optimized to control for contaminating organisms.

Following cultivation, the culture is harvested, concentrated, washed and prepared as a paste or dried to a powder. The dried or fresh biomass undergoes aqueous extraction and pH adjustment, as needed. Following extraction, the mixture undergoes centrifugation and filtration to remove cell debris and water-insoluble components. The resulting aqueous phase contains proteins, carbohydrates, minerals and two phycobiliproteins (also referred to as phycocyanins) that impart the blue colour. The mixture is concentrated to the desired pigment concentration and then pasteurized and/or sterilized before packaging. Spirulina extract products may undergo additional standardization and/or drying to achieve the desired formulation and pigment concentration. Commercially available products occur in either liquid (aqueous) or powder form with a wide range of pigment concentrations.

The primary colouring principles in spirulina extract are *C*-phycocyanin (Chemical Abstracts Service [CAS] No. 11016-15-2; European Inventory of Existing Commercial Chemical Substances [EINECS] No. 234-248-8; ~30 kDa) and allophycocyanin (no CAS number assigned; ~105 kDa), in various ratios, with *C*-phycocyanin occurring in higher proportions. Phycocyanins are complexes of proteins with the pigment molecule phycocyanobilin (Glazer & Fang, 1973). The total content of *C*-phycocyanin and allophycocyanin in spirulina extract varies depending on the desired colour effect and degree of dilution of the extract. Phycocyanin concentrations in spirulina extract range from 1.5–15% in liquid products to 1.5–65% in powder products, as the sum of *C*-phycocyanin and allophycocyanin.

Commercial spirulina extracts typically contain peptides and proteins (10–90%, dry weight, including the proteins complexed with phycocyanobilin), carbohydrates and polysaccharides (≤65%, dry weight), fat (<1%, dry weight), fibre (<6%, dry weight), minerals/ash (<6%, dry weight) and water (<6% for

powder products and ≤95% for liquid formulations). Spirulina extract may contain trace amounts (<1%) of carotenoids and chlorophylls, which are largely removed during production.

2. Biological data

2.1 Biochemical aspects

Absorption, distribution, metabolism and excretion data on spirulina extract were not available. Spirulina extract was described as consisting of various proportions of protein and carbohydrates as well as much smaller amounts of fibre and fats, all of which are digestible like other common dietary constituents.

An in vitro simulated gastric fluid digestion assay demonstrated that the protein portion of the *C*-phycocyanin is quickly digested by pepsin into small chromopeptides (peptides bound to a chromophore) consisting of 2–13 amino acid residues (Minic et al., 2016). The chemical structure of the chromophore phycocyanobilin is similar to biliverdin, a non-reduced form of bilirubin (Schram & Kroes, 1971). Once absorbed, the phycocyanobilin is expected to be metabolized and excreted similarly to bilirubin, that is, through the bile and into the faeces (McCarty, 2007; Eriksen, 2008).

No evidence of bioaccumulation of colour matter was observed in repeated-dose studies with laboratory animals fed spirulina extract or dried spirulina.

2.2 Toxicological studies

The toxicity studies evaluated included acute, short-term and long-term oral toxicity studies; in vivo and in vitro genotoxicity assays; and reproductive and developmental oral toxicity studies. Submitted special studies tested the utility of dried spirulina as a dietary source of protein and other nutrients and its ability to permit normal growth and maintain animal health. Observations in humans consisted mostly of nutritional studies, clinical studies and trials and case reports.

As previously noted, most of the tests were conducted with dried spirulina and not spirulina extract. Based on the similarity of the constituents and the high concentrations of the dried spirulina test materials used in the toxicity studies, the Committee considered the studies to be acceptable in the evaluation of spirulina extract. In some studies with spirulina extract, the phycocyanin content was reported; no study with dried spirulina reported the phycocyanin content.

The Committee also noted that the source of the test material in some studies was *Spirulina maxima*, as opposed to *S. platensis*. Given the chemical, genetic and nutritional similarity of these two species of edible cyanobacteria (Ciferri, 1983; Scheldeman et al., 1999; Kwei et al., 2011), this was considered acceptable in the evaluation of spirulina extract. Most of the reports did not state if the studies were compliant with good laboratory practice (GLP) or Organisation for Economic Co-operation and Development (OECD) or other guidelines. Nevertheless, the Committee concluded that the studies were of acceptable quality and the findings valid.

2.2.1 Acute toxicity

Acute oral toxicity studies were conducted with fresh or dried *S. platensis* biomass as the test material. In other studies, a cold-water extract or a freeze-dried acid precipitate of dried *S. platensis* biomass was used as the test material. In general, a single oral dose was administered to the test animals. After dosing, the animals were observed for between 7 and 21 days and killed.

No deaths, signs of clinical toxicity or gross pathologies were noted in any study. When reported, no treatment-related body or organ weight changes or histological differences between treated and control groups were noted. Regardless of the test material used, the oral median lethal dose (LD_{50}) was always determined to be greater than the highest dose tested. The results are summarized in Table 1.

2.2.2 Short-term studies of toxicity

(a) Mice

CF1 mice (10/sex per dose group; 6 weeks old) received diets containing dried *S. maxima* at concentrations of 0, 10%, 20% or 30% (equivalent to dried spirulina doses of 0, 15 000, 30 000 and 45 000 mg/kg body weight (bw) per day, respectively) for 13 weeks. The diets were mixed to be nutritionally balanced. Animals were observed daily for mortality and signs of clinical toxicity. Individual body weights were recorded at treatment initiation and then twice weekly until scheduled kill at week 13. Feed and water consumption over a 24-hour period were measured immediately before each weighing. At study termination, blood samples were collected for haematology and blood chemistry assessments: haemoglobin concentration, total red blood cell (RBC) counts, packed cell volume and total and differential leukocyte counts; aspartate aminotransferase (AST), alanine aminotransferase (also known as alanine transaminase, ALT) and alkaline phosphatase (ALP) activities; and glucose, urea nitrogen, cholesterol and protein. All animals were necropsied and macroscopic anomalies were recorded. Brain, heart, liver, spleen, kidneys, gonads and seminal vesicles were weighed and

Table 1
Acute toxicity of *Spirulina platensis* or *Spirulina maxima*

Species	Sex	Route	Test material	LD_{50} (mg/kg bw)	Reference
Mouse	Male	Oral	Extract of *Spirulina maxima*	> 3 000	Romay, Ledón & González (1998) [a]
Mouse	Male	Oral	Fresh *S. platensis*	> 30 000	Hutadilok-Towatana et al. (2008) [b]
Mouse	Male	Oral	Dried *S. platensis*	> 10 000	Hutadilok-Towatana et al. (2008) [b]
Mouse	Female	Oral	Dried *S. platensis*	> 5 000	Andrica et al. (2015) [c]
Rat	Female	Oral	Dried *S. platensis*	> 800	Krishnakumari, Ramesh & Venkataraman (1981) [d]
Rat	Male	Oral	Extract of *S. maxima*	> 3 000	Romay, Ledón & González (1998) [e]
Rat	Male and female	Oral	Extract of *S. platensis*	> 5 000	Naidu et al. (1999) [f]
Rat	Male and female	Oral	Extract of *S. platensis*	> 5 000	Chen et al. (2016) [g]

bw: body weight; LD_{50}: median lethal dose; OECD: Organisation for Economic Co-operation and Development

[a] Male OF_1 mice (number of animals per dose group not stated; 22–25 g bw) were administered spirulina extract from *S. maxima* (extraction method and *C*-phycocyanin content not reported) in order to determine the LD_{50} using the Litchfield and Wilcoxon method. Animals were observed for 14 days and then necropsied.

[b] Male Swiss mice (30–36 g bw) were administered either fresh or dried *S. platensis* in order to determine the LD_{50} using the up and down method. Animals were observed for 7 days and then necropsied.

[c] In an escalating-dose study compliant with GLP and OECD Guideline 425, a single female SKH1 mouse (25–30 g bw) was administrated powdered *S. platensis* at a dose of 2000 mg/kg bw. After surviving 24 hours, this mouse received a second dose of 5000 mg/kg bw and four other mice received doses of 2000 mg/kg bw. After surviving 24 hours, three of these four mice received a second dose of 5000 mg/kg bw. All mice were observed for 14 days and then necropsied.

[d] Female adult CFT-Wistar rats (6 animals/group: 200–250 g bw) received a single dose of dried *S. platensis* at 0, 200, 400 or 800 mg/kg bw. The test material was mixed in water (control) and administered as slurry. Animals were observed for 14 days and then necropsied.

[e] Male Sprague Dawley rats (no. of animals per dose group not stated; 170–250 g bw) were administered spirulina extract from *S. maxima* (extraction method and *C*-phycocyanin content not reported) in order to determine the LD_{50} using the Litchfield and Wilcoxon method. Animals were observed for 14 days and then killed.

[f] CFT-Wistar rats (8/sex per dose group; 200–250 g bw) received a water extract of fresh acid-precipitated and then freeze-dried *S. platensis* (phycocyanin content 26%), mixed in basal diet, at a dose of 0, 250, 500, 1000, 2000 or 5000 mg/kg bw. Animals were observed for 21 days and then necropsied.

[g] In a study compliant with OECD Guideline 420 study protocol (personal communication), Sprague Dawley rats (10/sex per dose group; initial mean bw ~245 g for males and ~190 g for females) received a cold-water extract of dried *S. platensis* (24% phycocyanin content) at a dose of 0, 1000, 3000 or 5000 mg/kg bw. Animals were observed for 15 days and then necropsied.

relative organ to body weight ratios calculated. Samples of these organs and of stomach and duodenum were processed for microscopic examination. Statistical evaluation of means was performed using Scheffe test for multiple comparison, and differences were considered significant at $P < 0.05$.

There were no deaths or clinical signs of toxicity. All treated animals excreted dark faeces (no explanation was provided). Males and females at 30% diet were described as having feed and water consumption slightly lower than their respective controls (no data were provided), but no differences were noted between the other groups and the controls. The study authors considered this difference to be of no toxicological relevance because body weights and body weight gains did not differ significantly between the groups. No toxicologically relevant haematological or blood chemistry differences between treated and control groups were observed. However, it was noted that male and female mice fed the 20% and 30% diets had a dose-dependent and statistically significant decrease in cholesterol concentrations compared to their respective controls, an effect not considered to be adverse. The authors speculated that the decrease in cholesterol concentrations may be due to increased faecal excretion of cholesterol

Table 2
Seminal vesicle weight relative to body weight of male mice treated with dried *Spirulina maxima* for 13 weeks

Concentration of *Spirulina maxima* in the diet (%)	Mean relative weight ± SD [a]
0	0.17 ± 0.02
10	0.28 ± 0.02*
20	0.38 ± 0.04**
30	0.41 ± 0.04**

SD: standard deviation; *: $P < 0.05$, **: $P < 0.01$
[a] $N = 10$.
Source: Salazar et al. (1998)

(no data were provided). Organ weights (no data were provided) and relative organ weights did not differ significantly between treated and control groups with the exception of a dose-dependent increase in relative weight of the seminal vesicles (Table 2). Although this finding was acknowledged by the study authors, they did not provide an explanation.

At necropsy, no macroscopic differences between treated and control groups were noted. Microscopic examination of the tissues – including the seminal vesicles – of animals fed the 30% diets found no significant histological differences compared to controls.

In the absence of any observed toxicity, including histopathological changes in the seminal vesicles, the no-observed-adverse-effect level (NOAEL) was 30% dried *S. maxima* in the diet (equivalent to a dose of dried spirulina of 45 000 mg/kg bw per day), the highest concentration tested (Salazar et al., 1998).

C57BL/6 mice (8/sex per dose group; 4 weeks old) received diets containing dried *S. platensis* at concentrations of 0, 2.5% or 5% (equivalent to doses of dried spirulina of 0, 3750 and 7500 mg/kg bw per day, respectively) for 6 months. The authors stated that they based their study on the International Conference on Harmonisation (ICH) guideline S4 ("Duration of Chronic Toxicity Testing in Animals"), endorsed by the United States Food and Drug Administration (USFDA). The diets were mixed to be nutritionally balanced. Animal body weight was recorded every other week. Feed intake was not measured. Blood samples were collected at 2, 4 and 6 months, after a 4-hour fast. No haematological assessment was conducted; blood chemistry parameter assessments were limited to ALT and AST activities. At scheduled kill at 6 months, no necropsies were conducted and only the liver was weighed and processed for histological examination.

No deaths occurred during treatment and there were no clinical signs of toxicity. Body weights of treated and control animals did not differ significantly. At 6 months, male mice fed 2.5% and 5% diets showed a dose-dependent decrease in AST activity compared to controls. The study authors considered this decrease treatment related but not toxicologically relevant because only increases in AST activity are associated with tissue damage; a possible explanation for the decrease was not given. Histopathological examination of the liver found no treatment-related effects. Mild to moderate lipidosis was observed in all groups with no association with treatment.

In the absence of any observed toxicity, the NOAEL was 5% of *S. platensis* in the diet (equivalent to a dose of dried spirulina of 7500 mg/kg bw per day), the highest concentration tested (Yang et al., 2011).

In a study to assess the effect of dietary *S. platensis* on lipid metabolism, male C57BL/6 mice (7–8/group; 4 weeks old) received diets containing dried *S. platensis* at concentrations of 0, 2.5% or 5% (equivalent to doses of dried spirulina of 0, 3750 and 7500 mg/kg bw per day, respectively) for 6 months. The diets were mixed to be nutritionally balanced. The standard suite of observations were not made (i.e. no general clinical observations, no measurements of body weight, no determination of feed consumption, no assessment of haematological parameters, no necropsy or histological examination). However, blood samples were collected every month, after a 4-hour fast, and total cholesterol and total triglycerides were measured. At scheduled kill at 6 months, a liver sample was processed to assess cholesterol-related gene expression and for western blot analysis.

No deaths or clinical signs of toxicities were reported. At 6 months, the 5% group had a statistically significant decrease in plasma total cholesterol and triglyceride levels compared to the control group. The authors did not consider these observations toxicologically relevant as they were not associated with adverse events. No changes were observed in the expression of hepatic cholesterol or lipid metabolism genes in the spirulina-fed mice compared to the control group.

No toxicity was observed under the conditions of the study (Yang et al., 2014).

(b) Rats

Wistar rats (10/sex per dose group; 57 g mean bw) received diets containing dried *S. maxima* at concentrations of 0, 10%, 20% or 30% (equivalent to doses of dried spirulina of 0, 10 000, 20 000 and 30 000 mg/kg bw per day, respectively) for 13 weeks. The diets were mixed to be nutritionally balanced, using soy oil meal. A second control group was fed a stock diet (as opposed to a diet containing

soy oil meal). Animals were observed daily for mortality and signs of clinical toxicity. Individual body weights were recorded weekly. Feed consumption was measured per cage (5 rats/cage) for the first 4 weeks and then on weeks 11 and 12, and expressed as g of feed/rat per day. Blood samples from individual animals were collected on week 12 to measure the following haematological parameters: haemoglobin concentration, haematocrit, RBC count, total and differential white blood cell (WBC) counts. Urine samples were collected on week 13, after a 16-hour period without water. The samples were examined for appearance, pH measurement and the semiquantitation of glucose, protein, blood, ketones and microscopic constituents. At scheduled kill (week 13), blood samples were collected and clinical blood chemistry parameters including glutamic-oxaloacetic transaminase (now known as aspartate transaminase or aspartate aminotransaminase, AST), glutamic-pyruvic transaminase (now known as alanine transaminase or ALT) and ALP activities and total protein assessed. The animals were necropsied, macroscopic abnormalities were noted and heart, brain, lungs, kidneys, liver, spleen, testes/ovaries, seminal vesicles, thymus, thyroid, adrenals and pituitary gland were weighed. Samples of these organs, as well as of pancreas, trachea, salivary glands, mammary glands, uterus, prostate, epididymides, gastrointestinal tract, urinary bladder, skeletal muscle, spinal cord, femoral nerve sternum, aorta and skin were prepared for microscopic examination. Statistical differences between the groups were assessed using the Student *t*-test or χ^2 test, with a value of $P \leq 0.05$ considered statistically significant.

There were no deaths in any of the treated groups. There was one death in the stock diet group (no explanation was provided and the animal was replaced). No signs of clinical toxicity were noted in any group. All treated animals had dark faeces (no explanation was provided). Feed consumption and body weights did not differ statistically significantly although the mean body weights of treated groups tended to be about 3–4% lower than that of controls. No toxicologically relevant differences in haematological or urine or blood clinical chemistry parameters were observed between the groups. Organ weights in treated and control animals did not differ significantly, with the exception of relative weights of seminal vesicles. In this case, males at 20% and 30% of the diet showed a dose-dependent statistically significant increase in relative organ weight (Table 3). No explanation was provided for this difference, but macroscopic or microscopic examination of the organ did not suggest any toxicity. No macroscopic or microscopic differences between treated and control group organs or tissues were noted.

In the absence of any observed toxicity, including histopathological changes in the seminal vesicles, the NOAEL was 30% dried *S. maxima* in the diet (equivalent to a dose of dried spirulina of 30 000 mg/kg bw per day), the highest concentration tested (Chamorro et al., 1988).

Table 3
Seminal vesicle weight relative to body weight of male rats treated with *Spirulina maxima* for 13 weeks

Concentration of *Spirulina maxima* in the diet (%)	Mean relative weight ± SD [b]
0 (stock)	0.31 ± 0.014
0 (soya oil meal)	0.32 ± 0.012
10	0.36 ± 0.011
20	0.39 ± 0.025*
30	0.42 ± 0.023*

SD: standard deviation; *: $P < 0.05$
[a] Relative to body weight
[b] $N = 10$.
Source: Chamorro et al. (1988).

Sprague Dawley rats (6/sex per dose group; 5 weeks old; 150–300 g bw) were administered *S. platensis* by gavage either fresh at doses of 0, 300, 600 or 1200 mg/kg bw per day or dried at 0, 30, 60 or 120 mg/kg bw per day, respectively, for 12 weeks. The test material was administered as a suspension in water at volume of 5 mL/kg bw. Animals were observed daily for mortality and signs of clinical toxicity. Feed and water intake were recorded daily. Individual body weights were recorded twice a week. Blood samples for haematology and clinical chemistry assessments were collected every 4 weeks, after a 12-hour fast, and the following parameters measured: packed cell volume and total and differential WBC counts; AST, ALT and ALP activities; total and direct bilirubin; glucose, creatinine, urea nitrogen, uric acid, albumin and total protein; and total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides; and sodium and potassium levels. At scheduled kill at week 12, necropsies were conducted and macroscopic anomalies recorded. Heart, liver, spleen and kidneys were weighed and samples of these organs processed for microscopic examinations. Statistical analyses used one-way analysis of variance (ANOVA) at the 95% confidence level and a least significance difference (LSD) test for comparison of differences between control and treated groups, with statistical significance set at $P < 0.05$.

No deaths or clinical signs of toxicity were observed during the treatment period. Treated animals excreted faeces that was darker than that of controls. Daily feed and water intake as well as body weights of treated and control groups did not differ. At scheduled kill, males given fresh spirulina at 1200 mg/kg bw per day had a statistically significant decrease in total cholesterol compared to controls, whereas females given dried spirulina at 120 mg/kg bw per day had a statistically significant decrease in HDL cholesterol, but not total cholesterol, compared to controls; otherwise, there were no significant differences between the groups in

clinical chemistry measurements. The authors considered these findings to be normal variations. With the exception of non-dose-related increases in monocyte concentrations in some groups at scheduled kill, there were no statistically significant changes in haematological parameters. The authors considered this to be due to the trauma induced by daily gavage. This conclusion is consistent with the absence of any macroscopic or microscopic anomalies in the organs examined and an absence of treatment-related differences in organ weights.

No toxicity was observed under the conditions of the study (Hutadilok-Towatana et al., 2008).

CFT-Wistar rats (8 sex/group; 21 days old; 200–250 g bw) received diets containing a water extract of *S. platensis* (freeze-dried precipitate of homogenized, acidified and centrifuged fresh *S. platensis*; 26% phycocyanin content) at concentrations of 0, 500, 1000, 2000 or 4000 mg/kg feed (equivalent to doses of spirulina extract of 0, 50, 100, 200 and 400 mg/kg bw per day, respectively) for 14 weeks. The diets were mixed to be nutritionally balanced. Animals were observed daily for mortality and signs of clinical toxicity. Feed intake was recorded daily and body weights weekly. All animals were necropsied at scheduled kill at week 14 and macroscopically examined. Liver, lungs, heart, kidneys, spleen, brain, adrenals and gonads were weighed and tissue samples processed for microscopic examination. Immediately prior to scheduled kill, blood samples were collected for haematological and clinical chemistry assessments including RBC count; haemoglobin content; packed cell volume; total and differential WBC counts; and lactate dehydrogenase, ALP, AST and ALT activities. Statistical analyses were conducted using Student *t*-test.

There were no treatment-related deaths and no clinical signs of toxicity. Feed intake and body weights did not differ significantly between treated and control groups (data provided only for the highest dose group). Absolute and relative organ weights did not differ between treated and control groups. No statistically significant differences in haematological or blood clinical chemistry parameters were observed, and none were seen in macroscopic or microscopic examinations.

In the absence of any observed toxicity, the NOAEL for the water extract of *S. platensis* was 4000 mg/kg feed (equivalent to a dose of spirulina extract of 400 mg/kg bw per day), the highest concentration tested (Naidu et al., 1999).

Sprague Dawley rats (10/sex per dose group; 6–9 weeks old) were administered phycocyanin (isolated from food grade spirulina powder purity 1.0 (A260/A280); phycocyanin content not reported) by gavage at doses of spirulina extract of 0, 120, 400 or 4000 mg/kg bw per day for 12 weeks. Six animals per dose group were killed at 12 weeks; the remainder were observed over a 4-week

recovery period (16 weeks total). Observations were made daily on mortality, clinical signs of toxicity and faecal excretion, and weekly on body weight and feed and water intake. At weeks 12 and 16, samples were processed for haematological assessment including haemoglobin concentration and RBC, platelet and total and differential WBC counts (neutrophil and lymphocytes only); ALT, AST and ALP activities; albumen, total bilirubin, blood urea nitrogen, creatinine, glucose and creatine kinase; and triglyceride and total cholesterol. At scheduled kill, animals were examined externally and internally for anomalies. Adrenals, thymus, testes/ovaries, prostate, uterus, heart, liver, spleen, lungs, kidneys and brain were weighed and relative organ to body weights calculated. Samples of these organs as well as thyroid, stomach, duodenum, ileum, colon, pancreas, eyes, optic nerves, sternum and bone marrow were processed for histopathological assessments. Statistical analyses were conducted with pairwise comparisons using least significance difference (LSD) *t*-test; $P > 0.05$ was considered statistically significant.

No deaths or signs of clinical toxicity were observed. Body weights and feed and water intake did not differ significantly between the groups at 12 or 16 weeks (no data provided). Haematological and clinical chemistry measurements and relative organ to body weight ratios were not affected by treatment at 12 or 16 weeks. No gross anomalies or histopathological changes were observed with treatment.

No toxicity was observed under the conditions of the study (Song et al., 2012).

Sprague Dawley rats (6/sex per dose group; initial bw ~100 g) were administered a suspension of freeze-dried silicon-enriched *S. platensis* by gavage at doses of dried spirulina of 28.5, 57 or 285 mg/kg bw per day for 90 days. The test material was grown in a culture in the presence of sodium metasilicate, which resulted in the *S. platensis* containing 1% silicon. Another group received an aqueous suspension of crude *S. platensis* (not silicon enriched) by gavage at a dried spirulina dose of 285 mg/kg bw per day for 90 days. Control animals received tap water. All animals were observed twice daily for mortality and signs of clinical toxicity. Individual body weights and feed intake were measured daily. All animals were fasted overnight prior to scheduled kill on day 90 and blood samples were collected to measure haematological and clinical chemistry parameters including RBC count, haemoglobin concentration, mean cell volume, mean cell haemoglobin, total and differential WBC counts and platelet count; urea, creatinine, cholesterol, triglycerides, chloride, phosphate and bilirubin; and ALT, AST and γ-glutamyltransferase (GGT) activities. The liver was perfused and weighed and then frozen for assessment of oxidative status (superoxide dismutase [SOD], glutathione peroxidase [GPx] and catalase [CAT] activities) and inflammatory status (interleukin-6 [IL-6] and tumour necrosis factor-α

[TNF-α]). Other organs, including heart, spleen and kidneys, were weighed. Histopathological assessments were not conducted. Statistical assessment of the data was conducted using one-way ANOVA followed by Student *t*-test.

No animals died during the study and no signs of clinical toxicity were observed. There were no statistically significant differences between control and treated groups in feed (data provided) or water (data not provided) intake. Body weight gains and body weights did not differ significantly between the groups. Haematological parameters did not differ significantly between the groups with the minor exception of a nearly 50% decrease in basophils in females at 57 mg/kg bw per day of silicon-enriched spirulina compared to the water control group (0.32 ± 0.06% vs 0.17 ± 0.03%; $P < 0.05$). According to the authors, this value was within the historical control range and not toxicologically relevant. Clinical chemistry measurements did not differ significantly between the control and treated groups, with a few minor exceptions. Triglyceride concentrations were decreased in females (only) treated with the non-silicon-enriched spirulina (0.80 ± 0.13 mmol/L) and with silicon-enriched spirulina at dried spirulina doses of 28.5 and 57 mg/kg bw per day (0.90 ± 0.17 and 0.64 ± 0.06, respectively, vs 2.21 ± 0.32 mmol/L for the water control; $P < 0.05$). According to the authors, these values were also within the historical control range and not adverse. In addition, ALT activity was decreased in females treated with non-silicon-enriched spirulina (26.2 ± 2.92 U/L) and with silicon-enriched spirulina at dried spirulina doses of 57 and 285 mg/kg bw per day (29.50 ± 1.65 and 23.00 ± 1.63, respectively, vs 37.2 ± 2.35 U/L for the water control). These decreases were not considered toxicologically relevant. Liver oxidative status and inflammatory status were not affected by treatment. Relative organ to body weights in control and treated groups were not significantly different, with one exception: females treated with silicon-enriched spirulina at the dried spirulina dose of 57 mg/kg bw per day had an increase in relative heart weight of about 10% (0.38 ± 0.01 vs 0.34 ± 0.01 for the water control; $P < 0.05$). The authors did not consider this effect relevant.

No toxicity was observed under the conditions of the study (Vidé et al., 2015).

Sprague Dawley rats (10/sex per dose group; mean initial bw for males ~260 g and for females ~200 g) were administered a cold-water extract of *S. platensis* (24% phycocyanin content) by gavage at spirulina extract doses of 0, 750, 1500 or 3000 mg/kg bw per day for 14 days. Body weights were measured on days 1, 8 and 14. Other measurements included ophthalmological examinations (time of assessment not provided); haematological assessments including RBC and WBC counts, haemoglobin concentration, mean cell volume, mean cell haematocrit, mean corpuscular haemoglobin concentration and platelet concentration; urine analysis (no details provided); blood coagulation assays (no details provided);

and serum chemistry assays, including AST, ALP, ALT and GGT activities and total protein, uric acid, glucose and creatinine concentrations. Necropsies were performed at scheduled kill.

There were no treatment-related differences between the groups in any of the parameters measured.

In the absence of any observed toxicity, the NOAEL for the cold-water extract of *S. platensis* was 3000 mg/kg bw per day, the highest dose tested (Chen et al., 2016).

Male Sprague Dawley rats (8/treated group; 4/control group; 6–8 weeks old) received diets containing freeze-dried *S. platensis* at concentrations of 0 or 20% (equivalent to a dried spirulina dose of 0 and 12 000 mg/kg bw per day; feed consumption data not provided) for 1 month. All diets were nutritionally balanced. Animals were observed daily for mortality and signs of clinical toxicity. Detailed physical examinations were conducted at treatment initiation, weekly during the study and at scheduled kill at week 4; these included assessments of fur, motor activity, eyes and orifices. Body weights were also recorded at these times, and mean body weight and mean body-weight gain calculated. During study week 3, animals were placed in metabolic cages for 24 hours, and water and feed intake as well as urine and faeces production were measured. At scheduled kill, blood samples were collected for clinical chemistry assessments of plasma urea and creatinine; ALP, ALT and AST activities; and total, LDL and HDL cholesterol. All animals were necropsied and the following examined: external surfaces; all orifices; cranial, thoracic, abdominal and pelvic cavities; and all viscera. Brain, heart, kidneys, liver, bladder, caecum, colon and spleen were weighed, and tissues samples processed for microscopic examination. Other, nonstandard parameters investigated included assessments of oedema in the tibio-tarsal joint, blood pressure, oxidative stress and antioxidant status of kidney and liver, expression of lipid metabolism-related genes in the liver and faecal lipid excretion. Statistical analyses used an unpaired *t*-test with Mann–Whitney correction; $P < 0.05$ was considered statistically significant.

All animals survived treatment and no signs of clinical toxicity were observed. Production of faeces was increased in treated animals compared to controls. There were no significant differences between treated and control groups with respect to body weights, body-weight gain or feed intake. Treated animals had greater daily water intake (27.18 ± 1.24 vs 21.53 ± 1.68 mL for control animals; $P < 0.05$) and greater urine production (12.63 ± 0.99 vs 7.00 ± 1.29 mL for control animals; $P < 0.01$). Blood clinical chemistry parameters did not differ significantly between the groups, with the exception of triglyceride values, which were significantly reduced (53.5 ± 10.9 vs 187 ± 62 mg/dL for control animals; $P < 0.05$), and HDL cholesterol values, which were significantly

increased (72.7 ± 2.4 vs 55.0 ± 5.0 mg/dL for control animals; $P < 0.05$). No significant changes in absolute weight or relative to body weight of brain, heart, spleen, caecum, kidneys or bladder were observed. A significant reduction in relative liver weight was observed in the treated group compared to the control group (4.70 ± 0.48 vs 3.32 ± 0.01). Microscopic examination of selected organs showed no anomalies, with the exception of one treated animal that had areas of crystalloid appearance within an amorphous substance in its bladder. None of the findings were considered toxicologically relevant as they were singular events or not adverse.

The authors suggested that the increase in water intake and faeces and urine production may be related to the increase in sodium intake in treated animals (30 mg/day vs 15 mg/day in control animals).

No toxicity was observed under the conditions of the study (Bigagli et al., 2017).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Rats

Sprague Dawley rats (14–18/sex per dose group; 4 weeks old) were fed diets containing a water extract of spirulina (8–9% phycocyanin content; species of *Spirulina* not stated) at a concentration of 0 or 1% (equivalent to a dose of spirulina extract of 0 and 500 mg/kg bw per day) for up to 12 months. Groups of animals were killed at 3 months (3/sex per dose group), 6 months (5/sex per dose group) and 12 months (6–10/sex per dose group). Feed intake was not measured. Individual animal body weights were measured weekly. Immediately prior to interim and final kills at 3, 6 and 12 months, blood samples were collected for haematology and clinical chemistry assessments including haematocrit, urea nitrogen, serum protein, uric acid, creatinine, total cholesterol, total bilirubin, calcium inorganic phosphorous and ALP, lactate dehydrogenase, AST and ALT activities. In addition, the following organs were weighed: brain, heart, liver, spleen, kidney (right), kidney (left), pituitary, adrenals, thyroid, testes and ovaries. Organ to body weight ratios were not calculated. The statistical analyses tests used were not described.

There were no signs of clinical toxicity in treated animals (the study report made no mention of mortality). Body weights in control and treated groups did not differ significantly at any time during the study. At scheduled kill at 12 months, the haematological and clinical chemistry findings in the control and treated groups did not differ significantly, except for a statistically significant decrease in ALP activity (190.9 ± 100.2 vs 302.7 ± 38.2 mU/mL, $P < 0.05$) and inorganic phosphorus concentration (4.5 ± 0.6 vs 6.7 ± 1.8 mg/dL, $P < 0.05$) in treated females. However, these values were considered within normal ranges of

Table 4
Selected absolute organ weights in rats treated with *Spirulina* extract in the diet for 12 months

	Mean absolute weight ± SE per sex per treatment group			
	Males		Females	
Organ	Control group (*N* = 8)	Treated group (*N* = 9) [a]	Control group (*N* = 8)	Treated group (*N* = 9) [a]
Pituitary (mg)	13.69 ± 2.22	16.50 ± 3.04*	–	–
Adrenal (mg)	37.63 ± 8.05	45.75 ± 5.93*	–	–
Thyroid (mg)	21.88 ± 41.20	26.83 ± 4.58*	–	–
Liver (g)	–	–	10.41 ± 1.94	8.67 ± 1.14*
Kidney (right) (g)	–	–	0.93 ± 0.11	0.83 ± 0.07*

SE: standard error; *: $P < 0.05$ (statistical method used not stated)
[a] Equivalent to a dose of spirulina extract of 0 and 500 mg/kg bw per day.
Source: Takemoto (1980)

variation and not toxicologically relevant. Absolute organ weights in the control and treated groups did not differ statistically significantly except for increases in weights of pituitary gland, adrenal glands and thyroid gland in treated males and decreases in weights of liver, spleen and right kidney in treated females compared with their respective controls (Table 4). However, the author stated that these differences were within normal range of variation for these organs and that there were no corresponding macroscopic or microscopic differences in treated animals. It was implied that the differences in absolute organ weight were not biologically relevant.

No toxicity was observed under the conditions of the study (Takemoto, 1980).

Wistar rats (20/sex per dose group; age and bw not stated) received diets containing dried *Spirulina* (species not stated) at concentrations of 10%, 20% or 30% (equivalent to a dried spirulina dose of 5000, 10 000 and 15 000 mg/kg bw per day, respectively) for 84 weeks. Two control groups (20/sex per dose group; age and body weight not stated) were established, one receiving a commercial diet (not described) and the second a soy-based diet (not described), both nutritionally balanced. Feed intake was not measured. All animals were weighed at 48, 64 and 80 weeks, at which time blood samples were collected for haematological assessments: haemoglobin concentration, haematocrit, RBC count and total and differential WBC counts. Functional renal tests measuring phenol red elimination were also conducted at these times. Urine density and urine ALT activity were also measured. Immediately prior to scheduled kill (week 84), blood samples were collected for clinical chemistry assessments: glucose; urea nitrogen; AST, ALT and ALP activities; and total serum protein. Heart, brain, lungs, kidneys,

liver, spleen, testes, ovaries, seminal vesicles, thyroid, adrenals and pituitary were weighed and macroscopically examined. Samples from the following organs and tissues were processed for microscopic examination: pancreas, trachea, salivary glands, mammary glands, uterus, prostate, epididymides, gastrointestinal tract, bladder, skeletal muscle, spinal cord, femoral nerves, aorta and skin. Student *t*-test or Wilcoxon text were used for statistical analyses.

Survival was similar in all groups (11–13 males and 9–12 females/group). According to the author, body weights did not differ significantly between the groups until the fourth month, when the control group on the commercial diet started to gain more weight than the other groups (no data were provided). After 21 months, body weights did not differ significantly between the soy-based control group and the spirulina-fed groups. The few statistically significant haematological differences between the groups at 48, 64 and 84 weeks were neither time nor dose dependent, and were not considered treatment related or adverse. Similarly, the renal function tests showed one or two statistically significant differences at 48, 64 and 84 weeks that were not considered biologically relevant. The clinical chemistry findings at 84 weeks showed no statistically significant difference between the soy-based control group and the spirulina-fed groups. Relative organ to body weight ratios did not differ statistically significantly between the groups, with the exception of females fed the commercial diet, which had an increased relative adrenal gland weight compared with that of the soy control group. However, this increase was similar to that seen in the spirulina-fed animals and was not considered biologically relevant. Macroscopic and microscopic examinations, including of tumours, found no statistically significant differences between the groups.

Based on the absence of toxicity, the NOAEL for dried spirulina was 30% in the diet (equivalent to a dried spirulina dose of 15 000 mg/kg bw per day), the highest concentration tested (Chamorro-Cevallos, 1980).

2.2.4 Genotoxicity

The results of several genotoxicity assays with various preparations of *S. platensis*, *S. maxima* and one unknown species of *Spirulina* are summarized in Table 5. With the exception of Chen et al. (2016), none of the studies stated whether a guideline was followed or GLP applied. Results were negative in all the assays.

2.2.5 Reproductive and developmental toxicity

(a) Mice

Pregnant CD-1 mice (15–22/group; 9–10 weeks old; initial mean bw 27 ± 1 g) received diets containing dried *S. platensis* at concentrations of 0, 10%, 20% or 30% (equivalent to dried spirulina doses of 0, 15 000, 30 000 and 45 000 mg/kg

Table 5
Results of assays for genotoxicity with *Spirulina* species

Test system	Test object	Test material	Concentration/dose	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium*	Cold-water extract of *Spirulina platensis*	0, 50, 150, 500, 1 500 or 5 000 µg/mL	Negative	Chen et al. (2016) [a]
Chromosomal aberration	CHO cell line	Cold-water extract of *S. platensis*	0, 50, 150, 500, 1 500 or 5 000 µg/mL	Negative	Chen et al. (2016) [b]
In vivo					
Chromosomal aberration	Mouse bone marrow cell	Water extract of unspecified *Spirulina* species	0, 17, 33 or 67 mg/kg bw	Negative	Ghoshal, Mukhopadhyay & Mukherjee (2001) [c]
Dominant lethal mutation	Male mouse	Dried *S. maxima*	0, 10%, 20% or 30% in the diet for 5 days	Negative	Chamorro & Salazar (1989) [d]
Dominant lethal mutation	Male mouse	Dried *S. maxima*	0, 10%, 20% or 30% in the diet for 5 days/week for 10 weeks	Negative	Chamorro & Salazar (1989) [e]
Dominant lethal mutation	Male rat	Dried *S. maxima*	0, 10%, 20% or 30% concentration in the diet for 5 days	Negative	Chamorro, Salazar & Pages (1996) [f]
Dominant lethal mutation	Female rat	Dried *S. maxima*	0, 10%, 20% or 30% concentration in the diet for 5 days	Negative	Chamorro, Salazar & Pages (1996) [g]

bw: body weight; CHO: Chinese hamster ovary

[a] No details were provided other than that the test material was a cold-water extract of dried *S. platensis* (26% phycocyanin content) and that the assay was conducted in the absence or presence of S9 metabolic activation using *S. typhimurium* tester strains. The author stated that the study was compliant with OECD Guideline 471 (personal communication).

[b] No details were provided other than that the test material was a cold-water extract of dried *S. platensis* (26% phycocyanin content) and that the assay was conducted in the absence or presence of S9 metabolic activation in CHO cells. The author stated that the study was compliant with OECD Guideline 473 (personal communication).

[c] The test material was described as a tablet containing 500 mg of *Spirulina* species as well as the colours brilliant blue lake and yellow oxide of iron. An aqueous extract was prepared (details not provided), and single doses were administered by gastric intubation to Swiss mice (3/group; sex not stated; 8–10 weeks old; 25–30 g bw). A positive control group received cyclophosphamide (20 mg/kg bw) by intraperitoneal injection. After 16.5 hours, all animals were injected with colchicine (0.04% per kg bw) and killed 90 minutes later. Bone marrow cells were processed according to the Preston et al. (1987) method. Chromosomal aberrations (including chromatid gaps, chromosome gaps, chromatid breaks, chromosome breaks and rearrangements) were examined in a total of 150 cells (50 cells/animal). The vehicle and positive control yielded expected results. Data were analysed for statistical significance by Cochran–Armitage test for trend. Results were considered significant when $P < 0.05$.

[d] The test material, described as *S. maxima* powdered algae, was mixed into the diet. A mixture of vegetable and animal protein was used to maintain the protein in the algae-containing diets at the same level as in the control diet. Male CD-1 mice (10/group) were fed the diets for 5 days. After treatment, each male mated with two nulliparous females for 1 week. Mating was confirmed by the presence of a vaginal plug (designated gestation day 0). Female mice were replaced every week for 6 weeks. Females were killed on gestation day 12–14, and uterus and ovaries were examined. The numbers of implantation sites, corpora lutea, live embryos and early or late deaths were recorded. Data were analysed for statistical significance against respective controls using the *t*-test. Results were considered significant when Z value = 0.05.

[e] The test material, described as powdered *S. maxima* algae, was mixed into the diet. Male CD-1 mice (10/group) were fed the diets for 5 days/week for 10 weeks. After treatment, each male mated with two nulliparous females for 1 week and then with other females for a second week. Kill time, examinations and assessment of results of pregnant females were identical to those described in footnote d.

[f] The test material, described as a spray-dried *Spirulina* species, was mixed into the diet as described in footnote d. Sexually mature male Wistar rats (10/group; 210–230 g bw) were fed the diets for 5 days. After treatment, each male mated with two nulliparous females for 1 week. Females were replaced with new females every week for 8 weeks. Females were killed on gestation day 14. The uterus and ovaries were examined, and the numbers of pregnant females, implantation sites, corpora lutea, live embryos and resorptions were recorded. Frequency of pre- and postimplantation losses was analysed for statistical significance by χ^2 test. Results were considered significant when $P < 0.05$.

[g] The test material, described as a spray-dried *Spirulina* species, was mixed into the diet as described in footnote d. Sexually mature female Wistar rats (10/group; 210–230 g bw) were fed the diets for 5 days. After treatment, each female was caged with a single male overnight. The next morning, a vaginal wash was used to verify the presence of sperm and that mating had occurred. This process was repeated for 19 days (4–5 estrus cycles) or until copulation was detected. If copulation had not occurred after I7 days, the male was replaced. All pregnant dams were killed on gestation day 14. The uterus and ovaries were examined, and the numbers of implantation sites, corpora lutea, live embryos and resorptions were recorded.

bw per day, respectively) on gestation days 7–13, 1–13 or 1–19. All diets were nutritionally balanced. Maternal body weights were recorded on gestation days 6, 13 and 19. On gestation day 19, the dams were killed, and uterus and fetuses of each were examined. Fetuses were examined for external, visceral and skeletal abnormalities. Maternal observations included numbers of animals mated, pregnant, with normal litter, with affected litter, with resorptions, with fetal anomalies and with resorptions plus fetal anomalies. Fetal parameters measured included numbers of implantations/dam and live fetuses/dam; total number of fetuses; numbers of normal fetuses, affected fetuses, resorptions and fetuses with anomalies; and mean fetal body weight. Statistical analyses were conducted using Student *t*-test or χ^2 test.

Maternal body weights were not affected by treatment, with the exception of dams fed the diet with spirulina on gestation days 1–19. These groups of dams had similar body-weight gain (range: 73.3–76.2 g), but all were statistically significantly heavier ($P < 0.05$) than the control group (67.6 g). As there were no corresponding toxicological changes, the increase in body-weight gain was not considered toxicologically relevant. Other maternal parameters were also not affected by treatment. The incidences of external, visceral or skeletal anomalies were not affected by treatment.

Based on the absence of toxicity, the NOAEL for dried *S. platensis* was 30% in the diet (equivalent to a dried spirulina dose of 45 000 mg/kg bw per day), the highest concentration tested (Chamorro & Salazar, 1990).

In a reproductive toxicity study, CD-1 mice (10 males and 20 females/test group; mean bw 25 g) received diets containing dried *S. maxima* at concentrations of 0, 10%, 20% or 30% (equivalent to dried spirulina doses of 0, 15 000, 30 000 and 45 000 mg/kg bw per day, respectively). Males were fed the diets for 9 weeks and females for 2 weeks prior to mating; females continued their diets during gestation. Mating was confirmed by the presence of a vaginal plug (designated gestation day 0). During the study the following parameters were recorded: mortality, clinical signs of toxicity, body weight, mating performance and pregnancy rate. Females were killed on gestation day 18, and ovaries and uteri were removed and examined to determine the numbers of corpora lutea and implantations. Gravid uterine weight and body weight of live fetuses, and the numbers of dead fetuses and resorptions (total, early and late) were also determined. Preimplantation and postimplantation losses were calculated. Live fetuses were examined for external, internal and skeletal anomalies. Statistical analyses were conducted using Student *t*-test and χ^2 test. The authors stated that the study was based on ICH guideline S5(R2), "Detection of toxicity to reproduction for medicinal products & toxicity to male fertility".

In males, treatment had no effect on mating success, body-weight gain, feed intake or mortality. Similarly, treatment had no effect in females on the frequency of estrus cycles, feed intake during preconception, days required for successful mating, copulation rate, pregnancy rate, weight gain during pregnancy, feed intake during gestation, net maternal weight gained during pregnancy, gravid uterus weight or mortality. Also not affected by treatment were the following: the total number of corpora lutea, the number of corpora lutea/dam, the number of implantation sites, preimplantation loss, total number of live fetuses, number of live fetuses per litter, number of dead fetuses, number of litters totally resorbed, postimplantation loss and combined fetal weight. The incidences of external, visceral and skeletal anomalies were not affected by treatment.

No treatment-related effects were seen in surviving F_0 females in terms of gestation length, parturition status, feed intake during lactation or incidence of death during treatment or parturition. No treatment-related effects were observed on the number of implantation sites, the total number of live pups at birth or the number of live pups per dam; pup birth weights; postimplantation loss; external abnormalities; survival rate (lactation days 0–4); and weaning rate (lactation days 4–21). An exception was a statistically significant decrease, compared with controls, in pup birth weights in the 30% spirulina group (male pups: 1.50 ± 0.08 vs 1.75 ± 0.04 g; female pups: 1.42 ± 0.06 vs 1.69 ± 0.04 g; $P < 0.05$). Pup survival rates on lactation days 0–4 were also decreased in the group fed 30% spirulina (70.1% vs 91.1% for controls).

The NOAEL for dried *S. maxima* was 30% in the diet (equivalent to a dried spirulina dose of 45 000 mg/kg bw per day), the highest concentration tested (Chamorro et al., 1997).

In the same study, peri- and postnatal toxicities were also assessed. Pregnant CD-1 mice (F_0; 20/group; age and body weights not reported) received diets as described above from gestation day 15 to lactation day 21. Animals were observed daily for mortality, signs of clinical toxicity and feed consumption during lactation. Gestation length and parturition status were also recorded. At birth, the numbers of live and dead pups were counted, and the pups examined for external anomalies. Postimplantation loss was calculated. Pup body weight and viability were recorded.

At weaning, F_1 pups were separated by sex and reared to maturity. At about 10 weeks of age, 10 males were mated with 10 females from the same treatment group. The ensuing F_2 pups were examined for survival, body weight and external, visceral and skeletal anomalies. Pre- and postimplantation losses were also calculated.

In males, treatment had no effect on mating success, body-weight gain, feed intake or mortality. Similarly, treatment had no effect in females on the frequency of estrus cycles, feed intake during preconception, days required for

successful mating, copulation rate, pregnancy rate, weight gain during pregnancy, feed intake during gestation, net maternal weight gained during pregnancy, gravid uterus weight or mortality. Also not affected by treatment were the following: the total number of corpora lutea, the number of corpora lutea/dam, the number of implantation sites, preimplantation loss, total number of live fetuses, number of live fetuses per litter, number of dead fetuses, number of litters totally resorbed, postimplantation loss and combined fetal weight. The incidences of external, visceral and skeletal anomalies were not affected by treatment.

No treatment-related effects were seen in surviving F_0 females in terms of gestation length, parturition status, feed intake during lactation or incidence of death during treatment or parturition. No treatment-related effects were observed on the number of implantation sites, the total number of live pups at birth or the number of live pups per dam; pup birth weights; postimplantation loss; external abnormalities; survival rate (lactation days 0–4); and weaning rate (lactation days 4–21). An exception was a statistically significant decrease, compared with controls, in pup birth weights in the 30% spirulina group (male pups: 1.50 ± 0.08 vs 1.75 ± 0.04 g; female pups: 1.42 ± 0.06 vs 1.69 ± 0.04 g; $P < 0.05$). Pup survival rates on lactation days 0–4 were also decreased in the group fed 30% spirulina (70.1% vs 91.1% for controls).

No treatment-related effects were observed on F_1 reproductive performance or litter values of F_2 pups. In addition, external, internal and skeletal examinations revealed no treatment-related anomalies in the F_2 pups.

Based on the absence of reproductive toxicity, the NOAEL for dried *S. maxima* was 30% in the diet (equivalent to a dried spirulina dose of 45 000 mg/kg bw per day), the highest concentration tested.

Based on the lower body weight and survival rate in F_1 pups, the NOAEL for developmental toxicity of dried *S. maxima* was 20% in the diet (equivalent to a dried spirulina dose of 30 000 mg/kg bw per day) (Chamorro et al., 1997).

(b) **Rats**

Mated female Wistar rats (18–22/group; mean bw: 210 ± 20 g) received diets containing dried *Spirulina* (species not stated) at concentrations of 0, 10%, 20% or 30% (equivalent to dried spirulina doses of 0, 10 000, 20 000 and 30 000 mg/kg bw per day, respectively) on gestation days 7–14, 1–14 or 1–21. (Note: Each treatment period had its own group of control animals.) All diets were nutritionally balanced. Maternal body weights were recorded on gestation days 1, 7, 14 and 21. On gestation day 21, dams were killed, and uterus and fetuses of each dam examined. Fetuses were examined for external, visceral and skeletal abnormalities. Maternal observations included numbers of animals mated, pregnant, with normal litter, with affected litter, with resorptions, with fetal

anomalies and with resorptions plus fetal anomalies. Fetal parameters measured included number of implantations/dam; total number of fetuses; numbers of live fetuses/dam, of normal fetuses, of affected fetuses, of fetuses resorbed and of fetuses with anomalies; and mean fetal body weight. Statistical analyses was conducted using Student *t*-test or χ^2 test.

Dam total body-weight gains did not differ statistically significantly between the groups. Although the dams on the 20% spirulina diet on gestation days 1–14 showed a statistically significant increase in body-weight gain relative to controls on days 7 and 14, total body-weight gain did not differ at scheduled kill on day 21. No effect was seen on body-weight gain in the higher dose group. The Committee considered this finding incidental as it was not dose related and therefore not toxicologically relevant. There were no differences with respect to other maternal observations or fetal parameters, with the exception of mean fetal body weight; this was statistically significantly increased in fetuses of dams fed diets containing spirulina at a concentration of 30% on gestation days 1–4 and 1–21 compared with their respective controls (days 1–4: 3.3 ± 0.3 vs 3.1 ± 0.4 g for control; days 1–21: 3.2 ± 0.3 vs 3.0 ± 0.4 g for control; $P < 0.05$, Student *t*-test). According to the authors, this difference did not correlate with an increase in dam body weight or a decrease in litter size; they considered this difference treatment related but not adverse. The incidences of fetal external, visceral or skeletal anomalies were not affected by treatment.

Based on the absence of toxicity, the NOAEL for dried spirulina was 30% in the diet (equivalent to a dried spirulina dose of 30 000 mg/kg bw per day), the highest concentration tested (Chamorro & Salazar, 1989).

In a reproductive toxicity study, Wistar rats (10 males and 20 females/group; age and body weights not reported) received diets containing dried *S. maxima* at concentrations of 0, 10%, 20% or 30% (equivalent to 0, 6000, 12 000 and 18 000 mg/kg bw per day, respectively). All diets were nutritionally balanced. Males were fed the diets for 9 weeks and females for 2 weeks prior to mating. Females remained on the diet until they were killed, either at the end of gestation or at pup weaning. Mating was confirmed by the presence of a vaginal plug (designated gestation day 0). During the study, the following parameters were recorded: mortality, clinical signs of toxicity, body weight, mating performance, pregnancy rate and gestation length.

Ten females were killed on gestation day 20; ovaries and uterus were removed and examined to determine the numbers of corpora lutea and implantations. The body weight of live fetuses and the numbers of live fetuses, dead fetuses and resorptions (total, early and late) were also determined. Live fetuses were examined for external, internal and skeletal anomalies.

The remaining 10 females were observed twice daily for the initiation of parturition (the day of birth was considered lactation day 1). Pups were observed at birth and pup sex was determined on the day of birth. Viability was assessed from birth to lactation day 21, and pup body weights were recorded on lactation days 1, 4, 14 and 21. Pups were also assessed for the occurrence of developmental markers, such as pinnae unfolding, appearance of primary coat of down hair, upper incisor eruption, eye opening, surface righting reflex, free-fall righting and startle reflex. When possible, pups that died were necropsied. After weaning (day 21), pups were killed and examined externally for abnormalities.

Statistical analyses were conducted using Student *t*-test and χ^2 test. The authors stated that the study was based on ICH guideline S5(R2), "Detection of toxicity to reproduction for medicinal products & toxicity to male fertility".

All males and females survived the premating period. Treatment did not affect mating success or mean body-weight gain in males. In females, treatment did not affect the number that became pregnant, fertility rate, number pregnant at gestation day 20, number surviving to postpartum day 21 postpartum, gestation length, mean body-weight gain during pregnancy or mean body-weight gain postpartum. One control female died, but necropsy found no macroscopic changes to explain the death. One mid-dose female had total litter resorption; this was not considered toxicologically relevant because it was a singular event and was not observed at the higher concentration. There were no significant differences between the control and treated groups with respect to numbers of corpora lutea, total implantations or pre- and postimplantation loss. There were no significant differences with respect to the numbers of live and dead fetuses or the mean fetal body weights. One fetus in the 10% group had hydrocephaly and two fetuses from different 30% group litters had hydrocephaly and bilateral hydronephrosis. There was no increase in skeletal malformations with treatment.

In females that were allowed to deliver, there were no differences in the numbers of live or dead pups at birth, survival rate or weaning rate. One control died during parturition and one dam in the 10% group was killed for humane reasons (respiratory problems). One dam at 30% had total litter loss at birth; otherwise there was no difference in mortality at birth. Pre-weaning development markers did not differ between the groups. There were no differences between the groups with respect to external anomalies in the pups, although one pup in the 30% group had unilateral anophthalmy (missing an eye). Overall these effects were not considered toxicologically relevant because they were singular events and did not show a dose-related effect.

In the same study, peri- and postnatal toxicity was also assessed. Pregnant Wistar rats (10/group) received diets as described above from gestation day 17 to lactation day 21. All diets were nutritionally balanced. Animals were observed daily for mortality and signs of clinical toxicity. Pregnancy rate, live pregnant

rats at delivery, gestation length and parturition status were also recorded. At birth, the F_1 pups were examined for external anomalies, sex determined and numbers of live pups counted. Survival rate was determined from birth to day 4 and viability from birth to day 21. Body weights were recorded on lactation days 1, 4, 14 and 21. Pups were also assessed for the occurrence of developmental markers, such as pinnae unfolding, appearance of primary coat of down hair, upper incisor eruption, eye opening, surface righting reflex, free-fall righting and startle reflex.

At weaning, pups were separated by sex and raised to maturity. At 10 weeks of age, 10 males were mated with 10 females from the same treatment group. The ensuing F_2 pups were examined for survival, body weight and external, visceral and skeletal anomalies. The F_1 uteri were examined for pre- and postimplantation losses.

There were no treatment-related effects on mortality, clinical signs of toxicity or body weight. No treatment-related effects were observed on F_1 developmental markers, reproductive performance or litter values of F_2 pups. External, internal and skeletal examinations of F_2 pups found no treatment-related anomalies compared to control animals.

Based on the absence of reproductive toxicity, the NOAEL for dried *S. maxima* was 30% in the diet (equivalent to a dose of dried spirulina of 18 000 mg/kg bw per day), the highest concentration tested.

Based on the absence of developmental toxicity, the NOAEL for dried *S. maxima* was 30% in the diet (equivalent to a dose of dried spirulina of 18 000 mg/kg bw per day), the highest concentration tested (Salazar et al., 1996).

S. platensis was also tested as a dietary supplement in a reproductive study in rats. Pregnant Albino rats (10/dose group; 150 g bw) received a casein-based control diet or a diet containing *S. platensis* at a concentration of 48% (providing 22% dietary protein) at gestation day 0 (based on presence of sperm in vaginal smear of the mated female). Feed intake was slightly reduced in the spirulina-fed rats compared with the control animals (16.83 ± 1.3 vs 18.8 ± 1.42 g), but body-weight gain was comparable (96.2 ± 10.41 vs 95.2 ± 9.1 g). The number of pups per litter was greater in the spirulina-fed rats than the control animals (12.6 ± 0.8 vs 9.6 ±1.0) as was the total litter weight (56.6 ± 5.71 vs 49.6 ± 4.7 g). However, the mean pup birth weight was less in the spirulina-fed rats than the control animals (4.57 ± 0.39 vs 5.16 ± 0.26 g).

No toxicity was observed under the conditions of the study (Kapoor & Mehta, 1993).

(c) Hamsters

Mated female Golden hamsters (about 20/group; bw range: 100–120 g) received diets containing dried *S. platensis* at concentrations of 0, 10%, 20% or 30% (equivalent to a dose of dried spirulina of 9000, 18 000 and 27 000 mg/kg bw per day) from gestation day 7 to 11, 1 to 11 or 1 to 14. All diets were nutritionally balanced. Maternal body weights were recorded on gestation days 6, 10 and 14. On gestation day 14, the dams were killed, and uterus and fetuses of each dam were examined. Fetuses were examined for external, visceral and skeletal abnormalities. Maternal observations included numbers of animals mated, pregnant, with normal litter, with affected litter, with resorptions, with fetal anomalies and with resorptions plus fetal anomalies. Fetal parameters measured included numbers of implantations/dam, of fetuses, of live fetuses/dam, of normal fetuses, of affected fetuses, of resorbed fetuses, and of fetuses with anomalies and mean fetal body weight. Statistical analyses were conducted using Student *t*-test.

Maternal and fetal weights were not affected by treatment. Other maternal observations were also not affected by treatment. The incidences of external, visceral or skeletal anomalies were not affected by treatment.

Based on the absence of toxicity, the NOAEL for dried *S. platensis* was 30% in the diet (equivalent to a dose of dried spirulina of 27 000 mg/kg bw per day), the highest concentration tested (Chamorro et al., 1987).

2.2.6 Special studies

(a) Nutritional studies in animals

Dried spirulina consists of about 45–70% protein and contains vitamins and minerals (Ciferri, 1983; Becker & Venkataraman, 1984). Because of the relatively high protein content, spirulina has been assessed in a number of nutritional studies in laboratory animals and in livestock.

In one study, rats were given diets containing dried spirulina at a concentration of 25% (equivalent to a dried spirulina dose of 12 500 mg/kg bw per day) for 75 weeks (Boudène, Collas & Jenkins, 1975). In a second study, rats received diets containing dried spirulina at a concentration of 40% (equivalent to a dried spirulina dose of 40 000 mg/kg bw per day) for 16 weeks (Tranquille et al., 1994). In both studies, no toxicologically relevant differences to control animals were observed with respect to mortality, general health or body weights. The long-term study also found no difference between treated and control animals in tumour incidence (Boudène, Collas & Jenkins, 1975), while the short-term study found no differences between treated and controls animals with respect to absolute organ weights or histopathological measures (Tranquille et al., 1994).

In other short-term toxicity studies (6–24 weeks), dried spirulina fed at relatively high dietary concentrations (16–27%; equivalent to dried spirulina doses of 10 000–26 700 mg/kg bw per day) showed no toxicologically relevant differences between treated and control animals with respect to mortality, general health, body weight, organ weights or histopathological findings (Yoshino et al., 1980; Becker & Venkatraman, 1984; Mitchell et al., 1990). These studies were limited in terms of observations from a toxicological perspective; nevertheless no toxicities were observed with the relatively high doses tested.

Dried spirulina has also been used as a dietary supplement in livestock feed for dairy cows (1.18% in the diet for 90 days, equivalent to 180 mg/kg bw per day, based on 7500 g of feed per day and 500 kg bw; Kulpys et al., 2009); piglets (14% in the diet for 13 days, equivalent to 56 000 mg/kg bw per day; Yap et al., 1982); pigs (0.2% in the diet for about 12 weeks, equivalent to 56 mg/kg bw per day, based on 2250 g of feed/day and 60 kg bw; Saeid et al., 2013; Simkus et al., 2013); chickens (up to 21% in the diet for 21 days, equivalent to 26 250 mg /kg bw per day based on 50 g of feed/day and 0.4 kg bw; Evans, Smith & Moritz, 2015); and rabbits (up to 15% in the diet for 24 days, equivalent to 7300 mg/kg bw per day, based on 186 g of feed/day and 3.8 kg bw; Peiretti & Melineri, 2008).

The treated animals were in general good health, and there were no adverse effects on body-weight gain.

2.3 Observations in humans

2.3.1 Case reports

A 52-year-old man ingested spirulina supplements (species not stated; doses not stated) daily for 5 weeks. He developed hepatotoxicity as indicated by elevated levels of AST, ALT, bilirubin and GGT. A lymphocyte stimulation test for spirulina was positive. The man had a history of hypertension, hyperlipidaemia and type 2 diabetes but no history of liver disease. The symptoms resolved within 5 weeks of discontinuation of spirulina supplements and all other medications (Iwasa et al., 2002).

Although the report authors concluded that the hepatotoxicity was due to spirulina consumption, the adverse effect may have been a result of the patient's use of simvastatin, a drug with well-documented hepatotoxicity (Marles et al., 2011).

A 57-year-old man with pemphigus vulgaris, an autoimmune disease, consumed dietary supplements containing *Spirulina* (species and dose not stated) and *Gingko biloba* twice a day for about 7 days. He experienced a flare-up of his

pemphigus vulgaris. The symptoms resolved within 2 weeks of discontinuation of spirulina and *Gingko biloba* and all multivitamin supplements. The authors suggested that immune stimulation by spirulina may have exacerbated the autoimmune disease (Lee & Werth, 2004).

A 45-year-old woman consumed a dietary supplement containing *S. platensis* (dose not stated), cayenne pepper, methylsulfonylmethane and *Aphanizomenon flos-aquae* for 2 days and developed rosacea. She had a history of hypertension, chronic migraines and reportedly spontaneously resolved fibromyalgia. She stopped using the dietary supplement, but the rosacea continued for weeks and did not respond to standard treatment. She began using the dietary supplement again and the condition worsened. She stopped using the dietary supplement again and her condition slowly improved. The patient was found to be heterozygous for a genetic polymorphism that results in increased production of the inflammatory cytokine tumour necrosis factor-α (TNF-α). The authors suggested that the woman's genetics predisposed her to autoimmune diseases and that immune stimulation by spirulina may have exacerbated her condition (Lee & Werth, 2004).

An 82-year-old woman consumed a dietary supplement containing *S. platensis* (dose not stated) for 1 year before developing bullae on her trunk and extremities. She stopped taking the dietary supplement and, with treatment, her condition resolved within 3 months. The dietary supplement tested positive in an intro interferon-γ release test. The authors suggested that the condition may have been caused by the immunostimulatory property of the algae (Kraigher et al., 2008).

A 28-year-old man consumed a dietary supplement containing *S. platensis* (100% dried spirulina) at a dose of 3 g/day for 1 month. He subsequently developed acute rhabdomyolysis. He had no genetic predisposition for the condition and he was not taking medications or illicit drugs. He stopped using the supplement and, with treatment, the condition resolved within 4 days. The authors concluded that the spirulina supplement was the cause of the acute rhabdomyolysis; they speculated that the presence of the algae neurotoxin β-*N*-methylamino-L-alanine (BMAA) in the supplement was the reason for the rhabdomyolysis but provided no evidence (Mazokopakis et al., 2008).

The authors of the United States Pharmacopoeia safety evaluation on dietary supplement use of spirulina noted that β-*N*-methylamino-L-alanine (BMAA) has never been reported in spirulina (Marles et al., 2011).

A 14-year-old man ingested five tablets containing *S. platensis* and, 6 hours later, developed urticarial, labial oedema and asthma. He tested positive in a skin prick test and an oral challenge test with spirulina. The condition resolved with treatment. The investigators isolated the protein fraction from the tablets and tested it against immunoglobulin E (IgE) from the patient. They identified two main IgE-binding fractions. These fractions were isolated, digested with trypsin and then subject to mass mapping by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis. The IgE-binding fractions were identified as the α- and β-chains of *C*-phycocyanin. The authors concluded that *C*-phycocyanin was the allergen responsible for the reaction (Petrus et al., 2010).

A 49-year-old woman developed a rash over large parts of her body after taking dietary supplements containing *Spirulina* (species, dose and duration of use were not stated). She discontinued use of the supplements but the rash continued for 2 months. After 5 months, she developed muscle weakness and was admitted to hospital. Tests showed that she had elevated creatine kinase levels. Histological examination of muscle tissue revealed the presence of necrotizing muscle fibres and mononuclear cells.

The authors suggested that the use of spirulina could result in inflammatory myopathy (Konno et al., 2011; abstract only).

A newborn male hospitalized on the first day of his life due to the development of seizures was found to have increased levels of serum calcium, ionized calcium, serum phosphate and calcitriol. He also had low levels of parathyroid hormone and demonstrated calciuria. The infant was diagnosed with hypercalcaemia, was treated accordingly and recovered. The mother had begun taking a daily dietary supplement of vitamin D at the start of her pregnancy and a dietary supplement of *S. platensis* (dose not stated) from her fourth month of pregnancy until term. She also had elevated calcitriol levels.

The authors considered the infant's condition likely due to the mother's intake of the spirulina supplement (Moulis et al., 2012).

A 17-year-old male ingested a single supplement containing *S. platensis* (300 mg tablet consisting of 295.5 mg dried spirulina, 3 mg silicon dioxide, 1.2 mg inulin and 0.3 mg magnesium stearate) and developed symptoms of anaphylaxis within 10 minutes. The symptoms included tingling of the lips, angioedema of the face, an itching exanthema and urticaria of arms and trunk, nausea, abdominal pain, wheezing, dyspnoea and inspiratory stridor. The patient had a history of atopic dermatitis, asthma and allergic rhinitis. He was treated and recovered. This was the first time the patient had ingested the dietary supplement. A skin

prick test of each of the constituents of the dietary supplement determined that spirulina was the allergic component (Le, Knulst & Röckmann, 2014).

A critical evaluation of the safety of spirulina as a dietary supplement was conducted by the United States Pharmacopeia. *S. maxima*, *S. platensis* and *S. fusiformis* were all considered, these being the species most commonly used in dietary supplements. In addition to case reports, adverse events reported by the USFDA's MedWatch, the Canada Vigilance Program, Therapeutic Goods Administration in Australia, the Medicines and Healthcare Products Regulatory Agency in the United Kingdom and the Uppsala Monitoring Centre in Sweden were assessed. The number of adverse events reports from each monitoring group was relatively small, that is, fewer than 16 from any one group over a period of time that ranged from 3 to 13 years. Showing causation between the use of spirulina and the adverse event was often confounded by the use of additional supplements or medicines, complicated medical histories and lack of details on the use of the product. The authors concluded, based on the evidence, that spirulina as a dietary supplement, when consumed in the range of 1–10 g/day (although 40 g/day was not uncommon) did not pose a serious risk to health (Marles et al., 2011).

2.3.2 Clinical studies

A number of clinical studies investigated the antioxidation and anti-inflammation properties of spirulina and phycocyanin. Safety-related end-points were largely limited to reporting tolerability of the test material and measurement of a small number of clinical chemistry parameters, often related to blood cholesterol.

A group of 36 volunteers (18–65 years old; 16 men and 20 women) received daily oral supplements containing 4.5 g of *S. maxima* for 6 weeks. Individual blood samples were drawn after a 12-hour fast, prior to study start and at study termination (week 6). Blood chemistry parameters measured included plasma glucose, total triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol and AST activity. Also measured were systolic and diastolic blood pressure, height, weight and body mass index (BMI).

No clinical signs of toxicity were observed and the treatment was well tolerated (Torres-Duran, Ferriera-Hermosillo & Juarez-Oropeza, 2007).

A randomized double-blind, placebo-controlled study was conducted with 78 volunteers (60–87 years old; 43 men and 35 women) received a daily oral supplement containing 8 g of *Spirulina* (species not stated) or a placebo for 16 weeks. Individual blood samples were drawn after a 12-hour fast prior to study start and at study termination (week 16). Blood chemistry measurements included

total triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol and fasting blood sugar. Also measured were systolic and diastolic blood pressure, height, weight, BMI, body fat and lifestyle habits (i.e, alcohol consumption, cigarette smoking).

No clinical signs of toxicity were observed and the treatment was well tolerated (Park et al., 2008).

A group of 52 volunteers (37–61 years old; 32 men and 20 women) received a daily oral supplement containing 1 g of *Spirulina* (species not stated) for 12 weeks. Individual blood samples were drawn after a 12-hour fast prior to study start and at study termination at week 12. Blood chemistry measurements included total triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, non-HDL cholesterol and fasting blood sugar. Also measured were systolic and diastolic blood pressure, height, weight, BMI and lifestyle habits (i.e. cigarette smoking).

Compared to initial observations, the final observations noted statistically significant changes in total triglycerides (↓16.3%), total cholesterol (↓8.9%), HDL cholesterol (↑3.5%), LDL cholesterol (↓10.1%) and non-HDL (↓10.8%). No clinical signs of toxicity were observed and the treatment was well tolerated (Mazokopakis et al., 2014a).

A group of 15 volunteers (29–62 years old; 13 men and 2 women) received a daily oral supplement containing 6 g of *Spirulina* (species not stated) for 6 months. Individual blood samples were drawn after a 12-hour fast prior to study start and at study termination (month 6). Blood chemistry measurements included haemoglobin; total triglycerides, total cholesterol, HDL cholesterol and LDL cholesterol; fasting blood sugar and insulin; and AST, ALT and GGT activities. Other measurements included systolic and diastolic blood pressure, height, weight, BMI, waist circumference body fat and lifestyle habits (i.e. cigarette smoking). Sonograms were taken to determine the extent of carotid steatosis. The final observations noted statistically significant changes, compared with initial observations, in total triglycerides (↓24.8%), total cholesterol (↓9.1%), HDL cholesterol (↑4.2%) and LDL cholesterol (↓9.6%). Also observed at the end of the study were changes in haemoglobin (↑4.21%), AST activity (↓38.5%), ALT activity (↓37.5%) and GGT activity (↓26.7%). Body weight also decreased with treatment (↓8.1%). No clinical signs of toxicity were observed and the treatment was well tolerated (Mazokopakis et al., 2014b).

Two pilot studies were conducted with 12 volunteers with chronic joint pain (age range: 40–72 years old; BMI range: 18–36 kg/m^2). In the first study, volunteers ingested a daily dietary supplement containing 1 g of a water extract

of *S. platensis* (40% phycocyanin) for 4 weeks. The second study was a placebo-controlled, single-blind, crossover study with the volunteers ingesting a daily dietary supplement containing 0, 250 or 500 mg of a water extract of *S. platensis* (40% phycocyanin) for 1 week, separated by a 1-week washout period. Pain was assessed using a visual analogue scale (VAS 1–100) at study start and termination.

The results showed a non-dose-related reduction in pain with the consumption of the dietary supplement. No adverse effects were noted (Jensen et al., 2016a).

A randomized double-blind placebo-control study was conducted in 24 volunteers with chronic joint pain (test group: 2 men and 10 women; control group: 3 men and 9 women; age range: 25–66 years old; BMI range: 21–35 kg/m^2). The test material was a water extract of *S. platensis* (40% phycocyanin). The test group ingested a daily dietary supplement containing 2.3 g of *S. platensis* (about 1 g phycocyanin) and the control group a colour-matched rice flour powder placebo for 2 weeks. Pain was assessed using a VAS (1–100) at study start and termination. Blood samples were taken at study start and termination to measure platelet activation, serum P-selectin levels, activated partial thromboplastin time, thrombin clotting time, fibrinogen activity and AST and ALT activities.

Pain was reduced in the treated group compared with controls (e.g. primary pain at rest was reduced by about 20 points on the VAS). The treated group also had a mild reduction in AST activity compared with the control value (~18 vs 23 IU/L). Other parameters were not affected by treatment. No adverse effects were noted (Jensen et al., 2016b).

2.3.3 Nutritional studies

Spirulina has historically been used as a food by the Aztecs of Mexico and the Kanembu of the Republic of Chad (Ciferri, 1983; Habib et al., 2008; Marles et al., 2011). More recently, several studies described the use of spirulina as a food and dietary supplement for malnourished individuals (reviewed by Siva Kiran, Madhu & Satyanarayana, 2015). No signs of clinical toxicity were reported in any of these historical comments or in the more recent studies with spirulina as a dietary supplement.

Children with or without HIV infection (~40/group; ~15 months old) were given 10 g of dried *S. platensis* with their meals (10 g/day) for 8 weeks. Two other similar groups did not receive spirulina with their meals.

Weight gain was better in the children receiving spirulina; they gained 15 g/day compared with 10 g/day in the control group. Children without HIV infection gained 25 g/day compared with controls at 20 g/day. The addition

of spirulina to the diets was well tolerated; no clinical toxicities were reported (Simpore et al., 2005).

Groups of malnourished children (~15 months old; 170/group) were given diets containing misola (a mixture of millet, soy and peanut) alone, misola plus *Spirulina* (species not stated; 10 g/day) or traditional meals plus *Spirulina* (species not stated; 10 g/day) for 8 weeks. A fourth group of 40 children (<5 years old) was given traditional meals for 8 weeks. All diets were calorically balanced. Misola plus spirulina resulted in better body-weight gain than misola alone (34 vs 20 g/day). Traditional meals plus spirulina also resulted in greater body-weight gain than traditional meals alone (25 vs 15 g/day). The addition of spirulina to the diets was well tolerated; no clinical toxicities were reported (Simpore et al., 2006).

Groups of persons with HIV (~80/group) ingested 10 g/day of *Spirulina* (species not stated) or a placebo for 6 months. No relevant nutritional differences between the two groups were observed at the end of the study (Yamani et al., 2009).

Groups of poorly nourished adolescent girls (13–15 years old; 100/group) received 1 g/day of *Spirulina* (species not stated) or placebo for 6 days per week for 2 months. The spirulina group showed fewer signs of fatigue, paleness of skin and dental caries than the control (Dewan, 2014).

Women in the 28th week of pregnancy (460/group) received a daily dietary supplement containing 1.5 g of *Spirulina* (species not stated) or one containing iron and folic acid, up to day 42 after delivery. Both groups had similar pregnancy outcomes (e.g. number of births, mode of delivery, incidence of haemorrhage at delivery, incidence of stillbirth, incidence of malformation). The spirulina-supplemented mothers and their infants had higher haemoglobin levels than the mothers and their infants in the other supplement group. The authors considered the spirulina supplement to be well tolerated (Niang et al., 2017).

3. Dietary exposure

Spirulina extract has not been evaluated previously by the Committee. At the present meeting, the Committee was requested to evaluate the use of Spirulina extract as a food colour by the Forth-ninth Session of the Codex Committee on Food Additives (FAO/WHO, 2017).

The Committee received a submission from colour manufacturing industry that provided an estimate of dietary exposure to spirulina extract and phycocyanins. In this submission exposure from use as a food colour was estimated using the budget method (FAO/WHO, 2009); exposure from use in coatings of food supplements was estimated from expected use levels and consumption; and the exposure from use as an ingredient in food was estimated using information from four Generally Recognized as Safe (GRAS) notices from the USA.

The present exposure assessment is based on phycocyanin content. Consumption of spirulina or components of spirulina occurs from uses other than food colour, that is, dried spirulina in dietary supplements, dried spirulina and spirulina extract as food ingredients, and spirulina extract in coatings for dietary supplements and pharmaceuticals. In order to assess the aggregated dietary exposure from these uses, exposures have been normalized based on phycocyanin content. This was considered appropriate because the colour component of spirulina extract is due to its content of phycocyanins (*C*-phycocyanin and allophycocyanin). This approach allowed for a comparison of the two test substances used in the toxicological assessments, dried spirulina and spirulina extract. Use and exposure levels from the literature are in some cases based on spirulina (in dry form) or on spirulina extracts (in dry form or in solution) without an explicit description of phycocyanin content. This diversity in the substances assessed adds to the uncertainty of the assessments.

Spirulina products are also used in cosmetics (Pandey, Pandey & Sharma, 2013), but there are no reliable estimates of exposure from this use.

3.1 Food uses

Spirulina is used as a nutritional supplement, as a food colour in countries in Central America and Asia and as a colouring food in the USA and the European Union.

The sponsor proposed typical and maximum use levels of Spirulina extract in a number of foods and in dietary supplements. Data were obtained from a targeted industry survey. Levels of phycocyanins in the foods were calculated by the sponsor from the phycocyanin content across a range of spirulina extracts (2.8–25.2% in foods and beverages and up to 28% in dietary supplements) (Table 6).

3.2 Assessment of dietary exposure

3.2.1 Dietary exposure from use as food colour – budget method

The sponsor included an assessment of dietary exposure to phycocyanins based on the use of spirulina as a food additive (colour). The budget method can be

Table 6

Proposed typical and maximum use levels for spirulina extract as provided by the sponsor

GSFA category		Spirulina extract (INS 134) use levels (ppm)		Phycocyanin		
				Content	Use levels (ppm)	
No.	Name	Highest typical	Highest max.	Highest %	Highest typical	Highest max.
01.0	Dairy products and analogues, excluding products of category 02.0					
01.1.4	Flavoured fluid milk drinks	2 000	10 000	25	100	2 500
01.6.1	Unripened cheese	2 000	8 000	25	250	1 250
01.6.2.1	Ripened cheese, includes rind	2 000	5 000	25	500	1 250
01.6.2.2	Rind of ripened cheese	4 000	8 000	25	1 000	2 000
01.6.4.2	Flavoured processed cheese, including containing fruit, vegetables, meat, etc.	1 000	5 000	25	250	1 250
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	2 000	10 000	25	250	1 250
02.0	Fats and oils, and fat emulsions					
02.4	Fat-based desserts excluding dairy-based dessert products of food category 01.7	3 000	20 000	25	250	1 250
03.0	Edible ices, including sherbet and sorbet	2 000	10 000	25	375	2 000
04.0	Fruits and vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds					
04.1.2.1	Frozen fruit	1 500	8 000	25	375	2 000
04.1.2.2	Dried fruit	1 500	8 000	25	375	2 000
04.1.2.3	Fruit in vinegar, oil or brine	1 500	8 000	25	375	2 000
04.1.2.4	Canned or bottled (pasteurized) fruit	1 500	8 000	25	375	2 000
04.1.2.5	Jams, jellies, marmalades	1 500	8 000	25	375	2 000
04.1.2.6	Fruit-based spreads (e.g. chutney) excluding products of food category 04.1.2.5	1 500	8 000	25	375	2 000
04.1.2.7	Candied fruit	3 000	10 000	25	375	2 000
04.1.2.8	Fruit preparations, including pulp, purees, fruit toppings and coconut milk	3 000	10 000	25	375	2 000
04.1.2.9	Fruit-based desserts, including fruit-flavoured water-based desserts	3 000	8 000	25	375	2 000
04.1.2.10	Fermented fruit products	1 500	8 000	25	375	2 000
04.1.2.11	Fruit fillings for pastries	3 000	10 000	25	375	2 000
04.1.2.12	Cooked fruit	1 500	8 000	25	375	2 000
04.2	Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds	1 500	8 000	25	375	2 000
04.2.2.2	Dried vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds	1 500	8 000	25	375	2 000
04.2.2.3	Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera) and seaweeds in vinegar, oil, brine or soybean sauce	1 500	8 000	25	375	2 000
04.2.2.4	Canned or bottled (pasteurized) or retort pouch vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera) and seaweeds	1 500	8 000	25	375	2 000

GSFA category		Spirulina extract (INS 134) use levels (ppm)		Phycocyanin		
				Content	Use levels (ppm)	
No.	Name	Highest typical	Highest max.	Highest %	Highest typical	Highest max.
04.2.2.5	Vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed purees and spreads (e.g. peanut butter)	1 500	8 000	25	375	2 000
04.2.2.6	Vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed pulps and preparations (e.g. vegetable desserts and sauces, candied vegetables) other than food category 04.2.2.5	1 500	8 000	25	375	2 000
04.2.2.8	Cooked or fried vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera) and seaweeds	1 500	8 000	25	375	2 000
05.0	Confectionery					
05.1.1	Cocoa mixes (powders) and cocoa mass/cake	1 500	8 000	25	375	2 000
05.1.2	Cocoa mixes (syrups)	2 000	8 000	25	375	2 000
05.1.3	Cocoa-based spreads, including fillings	2 000	8 000	25	375	2 000
05.1.4	Cocoa and chocolate products	2 500	8 000	25.2	630	2 000
05.1.5	Imitation chocolate, chocolate substitute products	2 000	8 000	25	375	2 000
05.2	Confectionery including hard and soft candy, nougats, etc. other than food categories 05.1, 05.3 and 05.4	3 600	12 000	25	774	2 580
05.2.1	Hard candy	7 500	15 000	25.2	1 890	3 780
05.2.2	Soft candy	15 000	30 000	25.2	3 780	7 560
05.2.3	Nougats and marzipans	2 000	8 000	25	375	2 000
05.3	Chewing gum	25 000	40 000	25.2	6 300	10 080
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	15 000	30 000	25.2	3 780	7 560
06.0	Cereals and cereal products, derived from cereal grains, from roots and tubers, pulses, legumes and pith or soft core of palm tree, excluding bakery wares of food category 07.0					
06.3	Breakfast cereals, including rolled oats	2 000	10 000	25	375	2 000
06.4.3	Pre-cooked pastas and noodles and like products	1 500	8 000	25	375	2 000
06.5	Cereal and starch based desserts (e.g. rice pudding, tapioca pudding)	2 000	10 000	25	100	2 500
06.6	Batters (e.g. for breading or batters for fish or poultry)	400	10 000	25	100	2 500
06.7	Pre-cooked or processed rice products, including rice cakes (Oriental type only)	400	10 000	25	100	2 500
06.8.1	Soybean-based beverages	3 000	10 000	25	100	2 500
06.8.2	Soybean-based beverage film	400	10 000	25	100	2 500
06.8.3	Soybean curd (tofu)	400	10 000	25	100	2 500
06.8.4.1	Thick gravy-stewed semi-dehydrated soybean curd	400	10 000	25	100	2 500
06.8.4.2	Deep fried semi-dehydrated soybean curd	400	10 000	25	100	2 500
06.8.4.3	Semi-dehydrated soybean curd, other than food categories 06.8.4.1 and 06.8.4.2	400	10 000	25	100	2 500
06.8.5	Dehydrated soybean curd (kori tofu)	400	10 000	25	100	2 500
06.8.6	Fermented soybeans (e.g. natto, tempe)	400	10 000	25	100	2 500
06.8.7	Fermented soybean curd	400	10 000	25	100	2 500

Table 6 (continued)

GSFA category		Spirulina extract (INS 134) use levels (ppm)		Phycocyanin		
				Content	Use levels (ppm)	
No.	Name	Highest typical	Highest max.	Highest %	Highest typical	Highest max.
06.8.8	Other soybean protein products	400	10 000	25	100	2 500
07.0	Bakery wares					
07.1.1.1	Yeast-leavened breads and specialty breads	1 500	8 000	25	375	2 000
07.1.1.2	Soda breads	1 500	8 000	25	375	2 000
07.1.2	Crackers, excluding sweet crackers	5 000	10 000	25	375	2 000
07.1.3	Other ordinary bakery products (e.g. bagels, pita, English muffins)	1 500	8 000	25	375	2 000
07.1.4	Bread-type products, including bread stuffing and bread crumbs	1 500	8 000	25	375	2 000
07.1.5	Steamed breads and buns	1 500	8 000	25	375	2 000
07.1.6	Mixes for bread and ordinary bakery wares	1 500	8 000	25	375	2 000
07.2.1	Cakes, cookies and pies (e.g. fruit-filled or custard types)	1 500	8 000	25	375	2 000
07.2.2	Other fine bakery products (e.g. doughnuts, sweet rolls, scones and muffins)	5 000	10 000	25	375	2 000
07.2.3	Mixes for fine bakery wares (e.g. cakes, pancakes)	1 500	8 000	25	375	2 000
08.0	Meat and meat products, including poultry and game					
08.2.1.1	Cured (including salted) non-heat-treated processed meat, poultry and game products in whole pieces or cuts	400	10 000	25	100	2 500
08.2.1.2	Cured (including salted) and dried non-heat-treated processed meat, poultry and game products in whole pieces or cuts	400	10 000	25	100	2 500
08.2.1.3	Fermented non-heat-treated processed meat, poultry and game products in whole pieces or cuts	400	10 000	25	100	2 500
08.2.2	Heat-treated processed meat, poultry and game products in whole pieces or cuts	400	10 000	25	100	2 500
08.2.3	Frozen processed meat, poultry and game products in whole pieces or cuts	400	10 000	25	100	2 500
08.3.1.1	Cured (including salted) non-heat-treated processed comminuted meat, poultry and game products	400	10 000	25	100	2 500
08.3.1.2	Cured (including salted) and dried non-heat-treated processed comminuted meat, poultry and game products	400	10 000	25	100	2 500
08.3.1.3	Fermented non-heat-treated processed comminuted meat, poultry and game products	400	10 000	25	100	2 500
08.3.2	Heat-treated processed comminuted meat, poultry and game products	400	10 000	25	100	2 500
08.3.3	Frozen processed comminuted meat, poultry and game products	400	10 000	25	100	2 500
08.4	Edible casings (e.g. sausage casings)	1 500	8 000	25	375	2 000
09.0	Fish and fish products, including mollusks, crustaceans and echinoderms					
09.3.1	Fish and fish products, including mollusks, crustaceans and echinoderms, marinated and/or in jelly	400	10 000	25	100	2 500
09.3.2	Fish and fish products, including mollusks, crustaceans and echinoderms, pickled and/or in brine	400	10 000	25	100	2 500

GSFA category		Spirulina extract (INS 134) use levels (ppm)		Phycocyanin		
				Content	Use levels (ppm)	
No.	Name	Highest typical	Highest max.	Highest %	Highest typical	Highest max.
09.3.3	Salmon substitutes, caviar and other fish roe products	400	10 000	25	100	2 500
09.3.4	Semi-preserved fish and fish products, including mollusks, crustaceans and echinoderms (e.g. fish paste), excluding products of food categories 09.3.1–09.3.3	400	10 000	25	100	2 500
09.4	Fully preserved, including canned or fermented fish and fish products, including mollusks, crustaceans and echinoderms	400	10 000	25	100	2 500
10.0	Eggs and egg products					
10.2.3	Dried and/or heat coagulated egg products	400	10 000	25	100	2 500
10.3	Preserved eggs, including alkaline, salted and canned eggs	400	10 000	25	100	2 500
10.4	Egg-based desserts (e.g. custard)	2 000	10 000	25	100	2 500
11.0	Sweeteners, including honey	0	0	0	0	0
12.0	Salts, spices, soups, sauces, salads, protein products					
12.2	Herbs, spices, seasonings and condiments (e.g. seasoning for instant noodles)	1 500	8 000	25	375	2 000
12.2.2	Seasonings and condiments	1 500	8 000	25	375	2 000
12.3	Vinegars	300	8 000	25	75	2 000
12.4	Mustards	400	8 000	25	100	2 000
12.5.1	Ready-to-eat soups and broths, including canned, bottled and frozen	400	10 000	25	100	2 500
12.5.2	Mixes for soups and broths	400	10 000	25	100	2 500
12.6.1	Emulsified sauces and dips (e.g. mayonnaise, salad dressing, onion dip)	3 000	10 000	25	100	2 500
12.6.2	Non-emulsified sauces (e.g. ketchup, cheese sauce, cream sauce, brown gravy)	3 000	10 000	25	100	2 500
12.6.3	Mixes for sauces and gravies	3 000	10 000	25	100	2 500
12.6.4	Clear sauces (e.g. fish sauce)	3 000	10 000	25	100	2 500
12.7	Salads (e.g. macaroni salad, potato salad) and sandwich spreads excluding cocoa- and nut-based spreads of food categories 04.2.2.5 and 05.1.3	400	10 000	25	100	2 500
12.8	Yeast and like products	400	10 000	25	100	2 500
12.9.1	Fermented soybean paste (e.g. miso)	1 500	8 000	25	375	2 000
12.9.2	Soybean sauce	1 500	8 000	25	375	2 000
12.1	Protein products other than from soybeans	1 500	8 000	25	375	2 000
13.0	Foodstuffs intended for particular nutritional uses					
13.3	Dietetic foods intended for special medical purposes (excluding products of food category 13.1)	3 000	10 000	25	100	2 500
13.4	Dietetic formulae for slimming purposes and weight reduction	3 000	10 000	25	100	2 500
13.5	Dietetic foods (e.g. supplementary foods for dietary use) excluding products of food categories 13.1–13.4 and 13.6	3 000	10 000	25	100	2 500
13.6	Food supplements	3 600	12 000	28	1 008	3 360
14.0	Beverages, excluding dairy products					
14.1.4.1	Carbonated water-based flavoured drinks	3 000	10 000	25	100	2 500

Table 6 (continued)

GSFA category		Spirulina extract (INS 134) use levels (ppm)		Phycocyanin		
				Content	Use levels (ppm)	
No.	Name	Highest typical	Highest max.	Highest %	Highest typical	Highest max.
14.1.4.2	Non-carbonated water-based flavoured drinks, including punches and ades	3 000	10 000	25	100	2 500
14.1.4.3	Concentrates (liquid or solid) for water-based flavoured drinks	2 000	10 000	25	100	2 500
14.2	Alcoholic beverages, including alcohol-free and low-alcoholic counterparts	400	10 000	25	100	2 500
14.2.1	Beer and malt beverages	400	10 000	25	100	2 500
14.2.2	Cider and perry	400	10 000	25	100	2 500
14.2.4	Wines (other than grape)	400	10 000	25	100	2 500
14.2.5	Mead	400	10 000	25	100	2 500
14.2.6	Distilled spirituous beverages containing more than 15% alcohol	400	10 000	25	100	2 500
14.2.7	Aromatized alcoholic beverages (e.g. beer, wine and spirituous cooler-type beverages, low alcoholic refreshers)	400	10 000	25	100	2 500
15.0	Ready-to-eat savouries					
15.1	Snacks – potato, cereal, flour or starch based (from roots and tubers, pulses and legumes)	3 000	8 000	25	375	2 000
15.2	Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)	1 500	8 000	25	375	2 000
15.3	Snacks – fish based	1 500	8 000	25	375	2 000
16.0	Prepared foods	400	10 000	25	100	2 500

GSFA: General Standard on Food Additives; INS: International Numbering System for Food Additives; max.: maximum; no.: number; ppm: parts per million

used to estimate the theoretical maximum level of phycocyanins in those foods and beverages that are likely to contain the food colour (Hansen, 1979; FAO/WHO, 2009). It can also be used to estimate the theoretical maximum daily intake of a food additive based on maximum use levels.

The highest maximum proposed level of spirulina extract in any foodstuff is 30 000 mg/kg for solid food excluding chewing gum (40 000 mg/kg including chewing gum) and 10 000 mg/L for beverages excluding dairy products. The content of phycocyanins may vary in spirulina extracts. Using the highest content of phycocyanins in the spirulina extracts proposed for use by the sponsor (28%) as a worst case scenario for all foods, these uses transform to 8400 mg/kg for solid food (excluding chewing gum) and 2800 mg/L for beverages (excluding dairy products). If chewing gum is included in the calculation, the estimated exposure from solid foods would be 11 200 mg/kg. For the assessment, the proportion of solid foods and beverages containing phycocyanins is assumed to be the default proportions 12.5% and 25%, respectively, for adults, and 25% and

100%, respectively, for children. If 8400 mg/kg is used as the level in solid foods and 2800 mg/L is used for beverages, excluding dairy products, the theoretical maximum daily intake of phycocyanins is calculated to be 123 and 385 mg/kg bw per day for adults and children, respectively.

3.2.2 Dietary exposure from use in dietary supplements

Commercial recommendations for the use of dried spirulina as a food supplement range from 2 to 10 g per day. Intervention studies using spirulina dietary supplements have reported exposure to spirulina in the same range: 2–7.5 g/day (Braakhuis & Hopkins, 2015); 1 g/day (Zeinalian et al., 2017); 3 g/day (Johnson et al., 2016); 10 g/day (Ngo-Matip et al., 2014); 10 g/day (adults and children; Grobler et al., 2013); and 4–5 g/day (Yu et al., 2012). The maximum dose at 10 g/day is equivalent to 2000 mg/day of phycocyanins based on 20% phycocyanin content in products from the high end of the range reported in GRAS notices for commercial dried spirulina. The maximum dose is equivalent to 33 and 133 mg/kg bw per day for adults and children, respectively.

3.2.3 Dietary exposure from use as a food ingredient

Spirulina and spirulina extract are used as food ingredients in the USA. Exposure to spirulina from use as a food ingredient has been estimated in four GRAS notices (Table 7). A substantial overlap exists between these uses of spirulina and spirulina extract as a food ingredient. Therefore, the Committee agreed to a conservative estimate of 2000 g/person per day, expressed as phycocyanin, for the total exposure from the use of spirulina and spirulina extract as food ingredients. This equates to an exposure to phycocyanin of 33 and 133 mg/kg bw per day for adults and children, respectively.

3.2.4 Dietary exposure from use in coatings of dietary supplements

Estimates of dietary exposure to phycocyanins from use of spirulina extract in coatings for food supplements were provided by the sponsor. According to sponsor, spirulina extract used in coatings for dietary supplements would typically be 12% (weight per weight), and the coating weight gain would typically be 3–5% of the tablet. Therefore, for a 1000 mg tablet or capsule, the amount of spirulina extract is typically 3.6–6.0 mg/tablet. Based on a phycocyanin content of 28%, this equates to 1.0–1.7 mg/tablet. Studies in the Netherlands show average daily use of dietary supplements by adults (users only) to be 0.5–2 tablets per day, with about 5–10% of adult users consuming 4 or more tablets per day (Ocké, Buurma-Rethans & Fransen, 2005). Based on a consumption of 4 tablets/day for adults and an assumed consumption of 2 tablets/day for children, phycocyanin exposure for adult high consumers (4 tablets/day) is estimated to be up to 6.7

Table 7
Estimates of dietary exposure to phycocyanins from the use of spirulina products as food ingredients

GRN	Product	Intended use	*C*-phycocyanin	Phycocyanin (crude)	Basis for exposure estimate	Exposure estimate		Reference
						Spirulina (g/day)	Phycocyanin (mg/day)	
127	Dried biomass of *Arthrospira platensis*	Specialty food bars (e.g. granola, breakfast or energy bars); powdered nutritional drink mixes such as "smoothies"; "healthy" snacks such as popcorn; and as a condiment in salads and pasta, at levels ranging from 0.5 to 3 g/serving	55 mg/g	120 mg/g	High-end consumer	6	720	Dore (2003)
394	Dried biomass of *A. platensis*, also known as *Spirulina platensis* (Spirulina)	Granola bars, cereal bars, protein bars and power bars, meal replacements and mixes, sports beverages, energy drinks, energy soft drinks, fruit juices, low calorie fruit and vegetable juice drinks, low fat soy milk and medical foods	–	12–19%	P90 for population aged ≥1 year at 10% market share	1.35	257	Cho (2011)
417	Dried biomass of *A. platensis*, also known as *S. platensis* (spirulina)	Beverages and beverage bases (nonalcoholic); breakfast cereals; fresh fruits and fruit juices; frozen dairy desserts and mixes (including ice cream); grain products and pastas; milk products; plant protein products; processed fruits and fruit juices; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes	–	15–19%	High-end consumer	6	1 140	Endres (2012)
424	*C*-phycocyanin-enriched water extract of the cyanobacterium *A. maxima* or *A. platensis* (also known as *S. maxima* or *S. platensis*)	The addition of spirulina extract to food categories is intended to replace consumption of other forms of spirulina added to foods in the same respective food categories. Hence, for consumers who already purchase food products containing spirulina, spirulina extract is not expected to add additional exposure to spirulina or its components from the same categories of foods	>30%	–	–	–	–	Newman (2012)

GRN: GRAS [Generally Recognized as Safe] notice number; P90: 90th percentile

Table 8
Estimates of dietary exposure to phycocyanin from the use of spirulina products

	Exposure to phycocyanin, mg/kg bw per day (%) [a]	
Use (source of phycocyanins)	**Adults**	**Children**
Food colour (spirulina extract) [b]	123 (65)	385 (59)
Dietary supplement (dried spirulina) [c]	33 (17)	133 (20)
Food ingredient (dried spirulina and spirulina extract) [d]	33 (17)	133 (20)
Dietary supplement coating (spirulina extract) [e]	0.1 (0.06)	0.2 (0.03)
Aggregated exposure	189	651

bw: body weight; GRAS: Generally Recognized as Safe
[a] Estimated dietary exposure in mg/kg bw per day and, in parentheses, the estimated exposure as a percentage of the total (aggregated) exposure.
[b] Estimated using the budget method (Hansen, 1979; FAO/WHO, 2009).
[c] Estimated from commercial recommendations and intervention studies.
[d] Estimated based on GRAS notices.
[e] Estimates provided by the sponsor.

mg/day and for children high consumers is estimated to be 3.4 mg/day. This is equivalent to 0.1 and 0.2 mg/kg bw per day for adults and children, respectively.

3.2.5 Dietary exposure from use in coatings of pharmaceuticals

No information from the use of phycocyanins in coatings of pharmaceuticals was available. Exposure from this use is estimated to be at the same levels as for the use in coatings for dietary supplements, that is, up to 0.1 and 0.2 mg/kg per day for adults and children, respectively. However, the Committee considered that such use should not be taken into account in the assessment of long-term dietary exposure in a healthy population.

3.2.6 Aggregated exposure

The Committee estimated the aggregated exposure to spirulina and spirulina extract from their uses as food colours, dietary supplements, food ingredients and coatings of dietary supplements to be 190 and 650 mg/kg bw per day, expressed as phycocyanins, for adults and children, respectively (Table 8).

4. Comments

4.1 Biochemical aspects

ADME data on spirulina extract are not available.

Spirulina extract is described as consisting of various proportions of proteins and carbohydrates as well as much smaller amounts of fibre and fats. These extract components are digested through normal biochemical pathways like other common dietary constituents.

An in vitro simulated gastric fluid digestion assay demonstrated that the protein portion of the *C*-phycocyanin is rapidly digested by pepsin into small chromopeptides, consisting of two to 13 amino acids (Minic et al., 2016). Because the chemical structure of the chromophore phycocyanobilin is very similar to biliverdin, a non-reduced form of bilirubin (Schram & Kroes, 1971), phycocyanobilin is expected to be metabolized and excreted like bilirubin, through the bile and into the faeces (McCarty, 2007; Eriksen, 2008).

No evidence of bioaccumulation of coloured matter was observed in repeated-dose toxicity studies in laboratory animals fed spirulina extract or dried spirulina.

4.2 Toxicological studies

As noted, most of the tests conducted used dried spirulina and not spirulina extract. However, based on the similarity of the constituents and the high concentrations of the dried spirulina test materials used in the toxicity studies, the Committee considered this acceptable in the evaluation of spirulina extract. In some studies conducted with spirulina extract, the phycocyanin content was reported; no study conducted with dried spirulina reported its phycocyanin content. The Committee also noted that the source of the test material in some studies was *S. maxima*, as opposed to *S. platensis*. Given the chemical, genetic and nutritional similarity of these two species of edible cyanobacteria (Ciferri, 1983; Scheldeman et al., 1999; Kwei et al., 2011), this was considered acceptable in the evaluation of spirulina extract. Most reports did not state if the studies were GLP or guideline compliant. However, the Committee concluded that the studies were of acceptable quality and the findings valid.

In acute gavage toxicity studies, no clinical signs of toxicity were observed in mice administered spirulina extract (phycocyanin content not reported) at a dose of up to 3000 mg/kg bw (Romay, Ledón & González, 1998); in rats administered spirulina extract (24–26% phycocyanin content) at a dose of up to 5000 mg/kg bw (Naidu et al., 1999; Chen et al., 2016); or in rats administered dried spirulina at a dose of up to 10 000 mg/kg bw (Hutadilok-Towatana et al., 2008).

No toxicity was seen in rats administered spirulina extract (24% phycocyanin content) by gavage at a dose of 3000 mg/kg bw per day for 14 days (Chen et al., 2016); in rats administered spirulina extract (phycocyanin content

not reported) by gavage at doses of up to 4000 mg/kg bw per day for 12 weeks (Song et al., 2012); in rats fed diets with spirulina extract (26% phycocyanin content) at a concentration of 0.4% (equivalent to 400 mg/kg bw per day) for 14 weeks (Naidu et al., 1999); or in rats in a long-term toxicity study where the dietary concentration of spirulina extract (8–9% phycocyanin content) was 1.0% (equivalent to 500 mg/kg bw per day) for 12 months (Takemoto, 1980).

In a short-term mouse toxicity study conducted with dietary concentrations of dried spirulina of up to 5% (equivalent to 7500 mg/kg bw per day) for 6 months, the NOAEL was the highest concentration tested (Yang et al., 2011). In a set of well-conducted toxicity studies conducted with dietary concentrations of dried spirulina of up to 30% for 13 weeks, the NOAELs were the highest concentrations tested (equivalent to 45 000 and 30 000 mg/kg bw per day in mice and rats, respectively) (Chamorro et al., 1988; Salazar et al., 1998). Several additional short-term feeding studies conducted with dried spirulina in mice (Yang et al., 2014) and rats (Hutadilok-Towatana et al., 2008; Vidé et al., 2015; Bigagli et al., 2017) showed no toxicity under the conditions of the studies. Similarly, no evidence of systemic toxicity or carcinogenicity was observed in long-term feeding studies in rats where the dietary concentration of dried spirulina was 30% (equivalent to 15 000 mg/kg bw per day) for 84 weeks (Chamorro-Cevallos, 1980).

With dried spirulina, a dose-related increase in relative seminal vesicle weight was the only treatment-related effect observed in 13-week-long toxicity studies in mice (Salazar et al., 1998) and rats (Chamorro et al., 1988). This finding was not reproduced in other short-term or long-term toxicity studies where seminal vesicle weight was investigated. As there were no histopathological changes in the seminal vesicles and no effects on reproduction in the test animals, the Committee considered this finding of no toxicological relevance.

A bacterial reverse mutation assay and in vitro and in vivo chromosomal aberration assays with spirulina extract (26% phycocyanin content) showed no evidence of genotoxicity (Ghoshal, Mukhopadhyay & Mukherjee, 2001; Chen et al., 2016). Dominant lethal assays conducted in mice and rats with dried spirulina also showed no evidence of genotoxicity under the conditions of the assays (Chamorro & Salazar, 1989; Chamorro, Salazar & Pages, 1996).

There was no evidence of reproductive toxicity when mice and rats were fed dietary concentrations of dried spirulina of up to 30% (equivalent to 45 000 and 18 000 mg/kg bw per day, respectively) prior to mating (male mice and rats for 9 weeks, female mice for 8 weeks, female rats for 2 weeks) and during mating and gestation. No toxicity was observed in dams or pups when mice, rats and hamsters were fed dietary concentrations of dried spirulina up to 30% (equivalent to 45 000, 30 000 and 27 000 mg/kg bw per day, respectively) over the entire gestation period (Chamorro et al., 1987, 1997; Chamorro & Salazar, 1990).

In a 2-generation toxicity study in which dosing was limited to F_0 parental females from gestation day 15 through to lactation day 21, mice were fed diets containing dried spirulina at 0, 10%, 20% or 30% (equivalent to 0, 15 000, 30 000 and 45 000 mg/kg bw per day, respectively). F_1 and F_2 offspring were not directly exposed to the test material. At 30% dried spirulina in the diet, reduced F_1 pup weight at birth and reduced F_1 survival rate on postnatal days 0–4 were reported; no effects were observed in the F_2 animals (Chamorro et al., 1997). The Committee noted that there was no effect on pup survival rate in a 2-generation toxicity study in which F_0 female rats were exposed from gestation day 17 to lactation day 21, in a dosing schedule similar to that used in the Chamorro et al. (1997) mouse study (Salazar et al., 1996). There were also no effects on fetal weight or pup weight at birth in several other reproductive and developmental toxicity studies in the mouse, rat and hamster.

Nutritional studies of dried spirulina fed to rats, rabbits, pigs, sheep and cows showed that the animals maintained good health (Madeira et al., 2017) even when the dietary concentrations were very high (e.g. up to 40% of the diets in rats, equivalent to 40 000 mg/kg bw per day). From a toxicological perspective, these nutritional studies were limited in terms of their observations.

4.3 Observations in humans

Observations in humans included case reports, clinical studies and nutritional studies.

The case reports of adverse effects were relatively few considering the long history and widespread use of dried spirulina as a food ingredient and dietary supplement (Marles et al., 2011). A few reports cited adverse immunological reactions, such as allergy, associated with the ingestion of dried spirulina dietary supplements. The Committee noted that dried spirulina was well tolerated in clinical and nutritional studies conducted with gram quantities consumed daily for months.

4.4 Assessment of dietary exposure

Dietary exposure to spirulina extract from its use as a food colour was assessed by the present Committee.

A comprehensive literature search retrieved nine references relevant for the assessment of dietary exposure. Consumption of spirulina or components of spirulina occurs from uses other than food colour, that is, dried spirulina in dietary supplements, dried spirulina and spirulina extract as food ingredients, and spirulina extract in coatings for dietary supplements and pharmaceuticals.

In order to assess the aggregated dietary exposure from these uses, exposures have been normalized based on phycocyanin content. This was considered appropriate since the colour component of spirulina extract is due to its content of phycocyanins (*C*-phycocyanin and allophycocyanin). This approach allowed for a comparison of the two test substances used in the toxicological assessments, dried spirulina and spirulina extract.

Dietary exposure expressed as phycocyanins from the use of spirulina extract as a food colour was estimated using the budget method (Hansen, 1979; FAO/WHO, 2009). The theoretical maximum daily exposure was estimated to be 123 and 385 mg/kg bw per day for adults and children, respectively. The conversion to phycocyanin content was based on the content of phycocyanins (28%) in the spirulina extracts proposed for use by the sponsor.

Dietary exposure expressed as phycocyanins from the use of dried spirulina in dietary supplements was estimated to be 33 and 133 mg/kg bw per day for adults and children, respectively. This estimate was based on dosage information on product labels and from intervention studies. The conversion to phycocyanin content was based on phycocyanin content (20%) in products from the high end of the range reported in GRAS notices for commercial dried spirulina.

Dietary exposure expressed as phycocyanins from the use of dried spirulina or spirulina extract as food ingredients was estimated for high consumers to be 33 and 133 mg/kg bw per day for adults and children, respectively. This estimate was based on proposed uses in four GRAS notices, with the conversion to phycocyanin content based on information in the GRAS documentation.

Dietary exposure expressed as phycocyanins from use of spirulina extract in coatings of dietary supplements was estimated for high consumers to be 0.1 and 0.2 mg/kg per day for adults and children, respectively. The conversion to phycocyanin content was based on the assumption that the concentration was the same as for food colour, that is, 28% in spirulina extract.

The Committee noted that spirulina extract could be used in coatings of pharmaceuticals comparable to the use in coatings for dietary supplements, resulting in a similar dietary exposure (0.1 mg/kg bw per day for adults and 0.2 mg/kg bw per day for children). However, the Committee considered that such use should not be taken into account in the assessment of long-term dietary exposure in a healthy population.

The Committee estimated a conservative aggregated exposure to dried spirulina and spirulina extract from all the assessed uses to be 190 and 650 mg/kg bw per day for adults and children, expressed as phycocyanins (Table 9). Based on this assessment, the estimated exposure to phycocyanins from the use of dried spirulina and spirulina extract as a food colour contributes approximately 60% to this total exposure and as dietary supplements and food ingredients contributes

Table 9
Estimates of dietary exposure to phycocyanins from the use of spirulina products

	Exposure to phycocyanins, mg/kg bw per day (%) [a]	
Use (source of phycocyanins)	**Adults**	**Children**
Food colour (spirulina extract)	123 (65)	385 (59)
Dietary supplements (dried spirulina)	33 (17)	133 (20)
Food ingredients (dried spirulina and spirulina extract)	33 (17)	133 (20)
Dietary supplement coating (spirulina extract)	0.1 (0.06)	0.2 (0.03)
Aggregated exposure	190	650

bw: body weight
[a] Estimated dietary exposure in mg/kg bw per day and, in parentheses, the estimated exposure as a percentage of the total (aggregated) exposure.

approximately 20% each, while the contribution from the use in coatings of dietary supplements is negligible.

5. Evaluation

The Committee established a temporary acceptable daily intake (ADI) "not specified" for spirulina extract. The ADI was based on the absence of toxicity in repeated-dose animal studies conducted with spirulina extract and dried spirulina. These included well-conducted short-term toxicity studies in mice and rats fed dried spirulina at doses of up to 45 000 and 30 000 mg dried spirulina/kg bw per day, respectively. Assuming a phycocyanin content of 10% based on commercial dried spirulina, the doses of phycocyanin were estimated to be 4500 and 3000 mg/kg bw per day, respectively. No evidence of carcinogenicity or systemic toxicity was observed in long-term toxicity studies in rats fed spirulina extract or dried spirulina. There were no concerns regarding genotoxicity. Reproductive and developmental toxicity were not of concern based on the absence of toxicity in feeding studies conducted with dried spirulina in mice, rats and hamsters.

Expressed as phycocyanins, estimated dietary exposure from the use of spirulina extract as a food colour, based on the budget method, and exposure to spirulina extract and dried spirulina from other dietary sources including food ingredients, dietary supplements, and coatings of dietary supplements was 190 mg/kg bw for a 60 kg adult and 650 mg/kg bw for a 15 kg child. The Committee concluded that this dietary exposure does not present a health concern.

The ADI "not specified" was made temporary due to the tentative nature of the specifications.

5.1 Recommendations

A new tentative specifications monograph and a Chemical and Technical Assessment were prepared.

The Committee received limited analytical data on spirulina extract. In order to remove the tentative designation from the specifications, the following information on the products of commerce is requested by December 2019:

- Full compositional characterization of commercial products in both liquid and powder forms;
- Full compositional characterization of the aqueous extract before formulation/standardization;
- Validated analytical methods for identification of the substance with a suitable specificity (including validation data and representative batch data); and
- Validated analytical methods for the determination of the purity of the substance with a suitable specificity (including validation data and representative batch data).

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SPECIFIC FLAVOURING AGENTS

Alicyclic primary alcohols, aldehydes, acids and related esters (Group 7) (addendum)

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1. Evaluation

1.1 Introduction

The Committee evaluated an additional three flavouring agents belonging to the group of alicyclic primary alcohols, aldehydes, acids and related esters. These three flavouring agents have not previously been evaluated by the Committee.

The Committee also re-evaluated six previously evaluated flavouring agents in this group.

The Committee previously evaluated 26 members of this group of flavouring agents at its fifty-ninth meeting (Annex 1, reference *160*), and 11 members of this group at its seventy-third meeting (Annex 1, reference *202*). The Committee concluded that all 37 flavouring agents were of no safety concern at estimated dietary exposures.

The additional flavouring agents are the mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde (No. 2253), (1-methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl)cyclopropyl)methanol (No. 2254), and (±)-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, ethyl ester (No. 2255). These three flavouring agents have not been reported to occur as natural components of foods.

The six previously evaluated flavouring agents in this group that were re-evaluated at the present meeting are *p*-mentha-1,8-dien-7-al (perillaldehyde; No. 973), *p*-mentha-1,8-dien-7-ol (No. 974), *p*-mentha-1,8-dien-7-yl acetate (No. 975), formyl-6,6-dimethylbicyclo[3.1.1]hept-2-ene (No. 980), myrtenol (No. 981) and myrtenyl acetate (No. 982). These six flavouring agents are all reported to occur as natural components of foods, including bergamot oil, blackberry, black tea, cumin, ginger, grapefruit oil, kabosu oil, kumquat oil, lamb's lettuce, lemon peel oil, lime oil, mandarin oil, orange juice and oil, pepper, peppermint, pistachio, spearmint, thyme, yuzu oil and other foods (Nijssen, van Ingen-Visscher & Donders, 2017).

Two of the six previously evaluated flavouring agents, *p*-mentha-1,8-dien-7-al (No. 973) and formyl-6,6-dimethylbicyclo[3.1.1]hept-2-ene (No. 980), contain an α,β-unsaturated aldehyde group that is considered to be a structural alert for genotoxicity (Eder et al., 1993). The remaining four re-evaluated flavouring agents in this group are not α,β-unsaturated aldehydes, but are structurally related to Nos 973 and 980. Additional in vitro and in vivo genotoxicity data were available for all six of these previously evaluated flavouring agents. Also, one of the two major components of an additional flavouring agent evaluated by the Committee at the present meeting (No. 2253, which is a mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde) contains an α,β-unsaturated aldehyde group. In vitro and in vivo genotoxicity data on No. 2253 were also available for evaluation.

The evaluation of the three additional members of this group and the re-evaluation of six previously considered flavouring agents in this group was conducted using the revised Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *230*).

1.2 Assessment of dietary exposure

The total annual volumes of production of the nine flavouring agents belonging to the group of alicyclic primary alcohols, aldehydes, acids and related esters are 36 kg in Europe, 34 kg in the USA, 3030 kg in Japan and 28 kg in Latin America [3, 4]. More than 97% of the annual production volume in Japan is accounted for by *p*-mentha-1,8-dien-7-al (No. 973). More than 97% of the annual production volume in Europe and the USA are accounted for by three flavouring agents: *p*-mentha-1,8-dien-7-al (No. 973); *p*-mentha-1,8-dien-7-yl acetate (No. 975); and formyl-6,6-dimethylbicyclo[3.1.1]hept-2-ene (No. 980). More than 96% of the annual production volume in Latin America is accounted for by three flavouring agents, *p*-mentha-1,8-dien-7-al (No. 973), formyl-6,6-dimethylbicyclo[3.1.1] hept-2-ene (No. 980) and myrtenol (No. 981).

Annual volumes of production in the USA for the three additional flavouring agents belonging to the group of alicyclic primary alcohols, aldehydes, acids and related esters are 0.4 kg each for Nos 2253 and 2254 and 0.1 kg for No. 2255. Annual production volume for these three flavouring agents in Europe, Japan or Latin America is reported as 0 kg (IOFI, 2017a,b).

Dietary exposures were estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, and the higher of the two values for each flavouring agent is reported in Table 1. The SPET and MSDI method values are in the range of 30–1500 and 0.01–780 μg/day, respectively, with the SPET yielding the highest estimate for each flavouring agent. The estimated daily dietary exposure was highest for the mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde (No. 2253) (1500 μg/day, the SPET value obtained for nonalcoholic beverages).

1.3 Absorption, distribution, metabolism and excretion

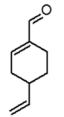
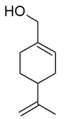
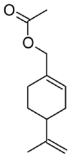
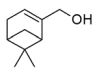
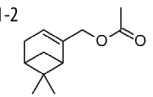
Information on the absorption, distribution, metabolism and excretion (ADME) of this group of flavouring agents was described in the reports of the fifty-ninth and seventy-third meetings (Annex 1, references *160* and *202*). No additional information was available for this meeting.

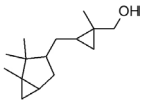
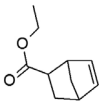
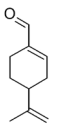
1.4 Consideration of genotoxicity data

The Committee considered new genotoxicity data on six members of this group. The six members were evaluated at the fifty-ninth meeting (Annex 1, reference *160*) and re-evaluated at the present meeting: *p*-mentha-1,8-dien-7-al (No. 973), *p*-mentha-1,8-dien-7-ol (No. 974), *p*-mentha-1,8-dien-7-yl acetate (No. 975),

Table 1

Summary of the results of the safety evaluations of alicyclic primary alcohols, aldehydes, acids and related esters used as flavouring agents [a,b,c,d]

Flavouring agent	No.	CAS no. and structure		*Step 4* [e] Does the highest dietary exposure estimate exceed the threshold of toxicological concern?	Condusion based on current estimated dietary exposure
Structural class I					
Mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde	2253	1049017-63-1 	1049017-68-6 	No, SPET: 1 500	No safety concern
p-Mentha-1,8-dien-7-ol	974	536-59-4 		No, SPET: 1 000	No safety concern
p-Mentha-1,8-dien-7-yl acetate	975	15111-96-3 		No, SPET: 1 000	No safety concern
Formyl-6,6-dimethylbicyclo[3.1.1]hept-2-ene	980	564-94-3 		No, SPET: 1 000	No safety concern
Myrtenol	981	515-00-4 		No, SPET: 1 000	No safety concern
Myrtenyl acetate	982	1079-01-2 		No, SPET: 1 000	No safety concern

Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the highest dietary exposure estimate exceed the threshold of toxicological concern?	Conclusion based on current estimated dietary exposure
Structural class II				
(1-Methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl) cyclopropyl)methanol	2254	198404-98-7 	No, SPET: 30	No safety concern
Structural class III				
(±)-Bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, ethyl ester	2255	10138-32-6 	No, SPET: 60	No safety concern
Flavouring agent excluded at Step 1 of the Procedure				
p-Mentha-1,8-dien-7-al (perillaldehyde)	973	2111-75-3 	NA	Genotoxicity data for *p*-mentha-1,8-dien-7-al raise concerns for potential genotoxicity

bw: body weight; CAS: Chemical Abstracts Service; MSDI: maximized survey-derived intake; NA: not applicable; No.: number; SPET: single-portion exposure technique

[a] Thirty-seven flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *160* and *202*). Six of those flavouring agents (Nos 973–975 and 980–982) and three additional flavouring agents in this group (Nos 2253–2255) were also evaluated at the present meeting.

[b] *Step 1*: Genotoxicity data for one of the nine flavouring agents in this group, *p*-mentha-1,8-dien-7-al (No. 973), raise concerns for potential genotoxicity. Genotoxicity data for the remaining eight flavouring agents in this group do not indicate potential genotoxicity.

[c] *Step 2*: The additional flavouring agents in this group are in structural class I (No. 2253), class II (No. 2254) and class III (No. 2255). The five re-evaluated flavouring agents in this group are in structural class I.

[d] *Step 3*: Dietary exposures were estimated using both the SPET and the MSDI method, and the higher of the two values for each flavouring agent is reported in Table 1. The SPET gave the highest estimate for each flavouring agent. Dietary exposure values are expressed in µg/day.

[e] *Step 4*: The thresholds for human dietary exposure for structural classes I, II and III are 1800, 540 and 90 µg/day, respectively.

formyl-6,6-dimethylbicyclo[3.1.1]hept-2-ene (No. 980), myrtenol (No. 981) and myrtenyl acetate (No. 982). Two of these six flavouring agents, *p*-mentha-1,8-dien-7-al (No. 973) and formyl-6,6-dimethylbicyclo[3.1.1]hept-2-ene (No. 980), contain an α,β-unsaturated aldehyde group considered to be a structural alert for genotoxicity (Eder et al., 1993).

The Committee considered the new genotoxicity data for No. 973 and noted that there were reproducible positive results in a reverse mutation assay in the TA98 strain of *Salmonella typhimurium* (Bowen, 2011). In addition, a study in rats investigated micronucleus induction in bone marrow and DNA damage in liver and duodenum (comet assay) (Beevers, 2014a,b). In this good laboratory practice (GLP)–compliant study, the micronucleus assay was conducted according to Organisation for Economic Co-operation and Development (OECD) guideline 474 while the comet assay was conducted in accordance with published guidelines developed by an expert working group (Burlinson et al., 2007). The results of the micronucleus assay indicated that *p*-mentha-1,8-dien-7-al (No. 973) did not induce an increase in micronucleated polychromatic erythrocytes. Although the comet assay did not indicate DNA damage in duodenum compared to the negative control, a 3-fold increase in DNA strand breaks (statistically significant, $P < 0.001$) was observed in liver at the highest dose tested (700 mg/kg bw per day), and there was a dose-dependent trend in the response ($P < 0.001$). Based on these new data, the Committee concluded that there are concerns for potential genotoxicity for *p*-mentha-1,8-dien-7-al (No. 973).

For No. 980, new in vitro reverse mutation (McGarry, 2016a; Dakoulas, 2017) and micronucleus induction (McGarry, 2016b; Lloyd, 2017) studies were negative. Also available were new in vivo assays on micronucleus induction in bone marrow and DNA damage in liver and duodenum (Pant, 2018) conducted in the same laboratory and using the same protocols as used for the study on No. 973. The results of the micronucleus assay indicated no increase in micronucleated polychromatic erythrocytes and the results of the comet assay indicated no DNA damage in duodenum or liver. The Committee concluded that there are no concerns for potential genotoxicity for formyl-6,6-dimethylbicyclo[3.1.1]hept-2-ene (No. 980).

The remaining four re-evaluated flavouring agents in this group are not α,β-unsaturated aldehydes but are structurally related to Nos 973 and 980. Additional in vitro and in vivo genotoxicity data were available on these flavouring agents for evaluation at the present meeting. None of these studies indicated potential for genotoxicity.

One of the two major components of an additional flavouring agent evaluated by the Committee at the present meeting (No. 2253, which is a mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde) also contains an α,β-unsaturated aldehyde group, and in vitro and in vivo

genotoxicity data on No. 2253 were available for evaluation. In a bacterial reverse mutation assay, No. 2253 was negative in all five strains tested in both the presence and absence of metabolic activation (Sokolowski, 2011). In an in vitro mammalian chromosomal aberration assay, No. 2253 induced an increase in the percentage of aberrant cells (Hall, 2011a). However, a GLP-compliant in vivo micronucleus induction assay in mice, conducted in accordance with OECD guideline 474, was negative (Hall, 2011b). The Committee concluded that there are no concerns for potential genotoxicity for the mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde (No. 2253).

1.5 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. Genotoxicity data on the three additional flavouring agents in this group (Nos 2253–2255) and five of the six re-evaluated flavouring agents in this group do not indicate that these flavouring agents have the potential for genotoxicity. For one of the re-evaluated flavouring agents, *p*-mentha-1,8-dien-7-al (No. 973) the Committee concluded that there were concerns for genotoxicity. Therefore, No. 973 was not further considered using the revised Procedure for the Safety Evaluation of Flavouring Agents.

Step 2. In applying the revised Procedure, the Committee assigned six flavouring agents (Nos 974, 975, 980–982 and 2253) to structural class I, one flavouring agent (No. 2254) to structural class II and one flavouring agent (No. 2255) to structural class III (Cramer, Ford & Hall, 1978).

Step 3. Dietary exposures were estimated using both the MSDI method and the SPET.

Step 4. The highest estimated dietary exposures for all eight flavouring agents were below the threshold of toxicological concern applicable to each flavouring agent. The Committee therefore concluded that these eight flavouring agents would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the eight flavouring agents belonging to this group of alicyclic primary alcohols, aldehydes, acids and related esters that were considered at the present meeting (Nos 974, 975, 980–982 and 2253–2255).

1.6 Consideration of combined intakes from use as flavouring agents

The Committee previously considered the potential combined intakes for this group of alicyclic primary alcohols, aldehydes, acids and related esters and did not identify any safety concerns. The three additional flavouring agents in this

group (Nos 2253–2255) have low MSDI values (0.01–0.04 μg/day) and therefore would make a negligible contribution to the combined intake of this group.

1.7 Consideration of additional data on previously evaluated flavouring agents

In addition to new genotoxicity data on the six flavouring agents that were re-evaluated at the present meeting, the Committee considered additional data on several other previously evaluated flavouring agents in this group. Acute toxicity data were evaluated for Nos 961, 967, 977 and 981, and studies of genotoxicity were evaluated for Nos 967, 977–979 and 984. These new toxicological data support the conclusions of previous Committee evaluations that these flavouring agents are not safety concerns.

1.8 Conclusions

In previous evaluations of flavouring agents in this group of alicyclic primary alcohols, aldehydes, acids and related esters, studies of hydrolysis; ADME; acute, short-term and long-term toxicity; and genotoxicity were available. None of the 37 previously evaluated flavouring agents raised safety concerns.

At the present meeting, the Committee concluded that the three flavouring agents (Nos 2253–2255) that are additions to the group of alicyclic primary alcohols, aldehydes, acids and related esters, would not give rise to safety concerns at current estimated dietary exposures.

The Committee also concluded that five of the six previously evaluated flavouring agents in this group (Nos 974, 975 and 980–982) that were re-evaluated at the present meeting do not give rise to safety concerns. For one of the re-evaluated flavouring agents, *p*-mentha-1,8-dien-7-al (perillaldehyde; No. 973), the Committee concluded that there were concerns for potential genotoxicity. Therefore No. 973 was not further considered using the revised Procedure.

2. Relevant background information

2.1 Explanation

This monograph addendum summarizes data relevant to the safety evaluation of three additional flavouring agents (Nos 2253–2255) and six previously evaluated

flavouring agents (Nos 973–975 and 980–982) in this group of alicyclic primary alcohols, aldehydes, acids and related esters.

2.2 Additional considerations on dietary exposure

Annual volumes of production and daily dietary exposures estimated using both the MSDI method and the SPET for each flavouring agent are reported in Table 2. None of the three additional flavouring agents considered at the current meeting have been reported to occur as natural components of foods.

2.3 Biological data

2.3.1 Biochemical aspects: ADME

Information on the ADME for this group of flavouring agents was described in the reports of the fifty-ninth and seventy-third meetings (Annex 1, references *160* and *202*). No additional information was available for this meeting.

2.3.2 Toxicological studies

(a) Acute toxicity

Oral median lethal dose (LD_{50}) values have been reported for one of the three additional flavouring substances (No. 2253) and four previously evaluated substances (Nos 961, 967, 977 and 981) in this group. These are summarized in Table 3. The low acute toxicity of these flavouring agents is consistent with findings in previous evaluations.

(b) Short-term studies of toxicity

Results of short-term studies of toxicity with the mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde (No. 2253) and for (1-methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl)cyclopropyl) methanol (No. 2254) are summarized in Table 4 and described below.

(i) Mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde (No. 2253)

In a 7-day range-finding study, groups of Wistar rats (3/sex per dose group) were administered a mixture of 1-vinyl-3-cyclohexenecarbaldehyde (~65%) and 4-vinyl-1-cyclohexenecarbaldehyde (~30%) by gavage at 0, 100, 300 or 1000 mg/kg bw per day. Clinical observations, feed consumption and body weights were recorded periodically. After the dosing period, the animals were killed and necropsied.

Table 2

Annual volumes of production and daily dietary exposures of alicyclic primary alcohols, aldehydes, acids and related esters used as flavouring agents in Europe, the USA, Japan and Latin America

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Natural occurrence in foods
		MSDI [b]		SPET [c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
Mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde (2253)				1 500	25	–
Europe	ND	ND	ND			
USA	0.4	0.04	0.001			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
(1-Methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl)cyclopropyl)methanol (2254)				30	1	–
Europe	ND	ND	ND			
USA	0.4	0.04	0.001			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
(±)-Bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, ethyl ester (2255)				60	1	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
***p*-Mentha-1,8-dien-7-al (973)**				1 000	17	+
Europe	10	0.8	0.01			
USA	9	0.9	0.02			
Japan	2 950	777	13			
LATAM	17	0.9	0.02			
***p*-Mentha-1,8-dien-7-ol (974)**				1 000	17	+
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	72	19	0.3			
LATAM	0.4	0.02	0.000 4			
***p*-Mentha-1,8-dien-7-yl acetate (975)**				1 000	17	+
Europe	12	1	0.02			
USA	5	0.5	0.01			
Japan	5	1	0.02			
LATAM	0.3	0.02	0.000 3			
Formyl-6,6-dimethylbicyclo[3.1.1]hept-2-ene (980)				1 000	17	+
Europe	13	1	0.02			
USA	19	2	0.03			

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Natural occurrence in foods
		MSDI [b]		SPET [c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
Japan	2	0.5	0.01			
LATAM	4	0.2	0.004			
Myrtenol (981)				1 000	17	+
Europe	0.5	0.04	0.001			
USA	0.1	0.01	0.000 2			
Japan	1	0.3	0.004			
LATAM	6	0.3	0.006			
Myrtenyl acetate (982)				1 000	17	+
Europe	1	0.08	0.001			
USA	ND	ND	ND			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
Total						
Europe	36					
USA	34					
Japan	3 030					
LATAM	28					

bw: body weight; LATAM: Latin America; MSDI: maximized survey-derived intake; ND: no data reported; +: reported to occur naturally in foods (Nijssen, van Ingen-Visscher & Donders, 2017); –: not reported to occur naturally in foods; SPET: single-portion exposure technique; USA: United States of America

[a] From International Organization for the Flavor Industry (2017a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × $(1 \times 10^9$ µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 45×10^6 for Europe, 33×10^6 for the USA, 13×10^6 for Japan, and 62×10^6 for LATAM; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (IOFI, 2017a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (IOFI, 2017b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

Table 3

Results of oral acute toxicity studies with alicyclic primary alcohols, aldehydes, acids and related esters used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
2253	Mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde	Rat; F	>2 000	Sieber (2011)
961	Cyclohexanecarboxylic acid	Rat; M/F	3 300	Moran, Easterday & Oser (1980)
967	(2,2,3-Trimethylcyclopent-3-en-1-yl)acetaldehyde	Rat; M/F	>2 000	ECHA (2017a)
967	(2,2,3-Trimethylcyclopent-3-en-1-yl)acetaldehyde	Rat: M/F	4 100	ECHA (2017b)

Table 3 (continued)

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
977	2,6,6-Trimethylcyclohexa-1,3-dienyl methanal	Mouse; M/F	>5 000	Hosseinzadeh et al. (2013)
977	2,6,6-Trimethylcyclohexa-1,3-dienyl methanal	Rat; M	>5 000	Hosseinzadeh et al. (2013)
977	2,6,6-Trimethylcyclohexa-1,3-dienyl methanal	Rat; F	300–2 000	Tarcai (2015)
981	Myrtenol	Rat; M/F	2 500 (M); 630 (F)	Leuschner (2001)

bw: body weight; ECHA: European Chemicals Agency; F: female; LD_{50}: median lethal dose; M: male

Table 4

Results of short-term studies of toxicity with alicyclic primary alcohols, aldehydes, acids and related esters used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups [a] / no. of animals per group [b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
2253	Mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde	Rats; M/F	3/6	Gavage	7	NA [c]	Braun (2011)
2253	Mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde	Rats; M/F	3/10	Gavage	28	150	Braun (2012)
2254	(1-Methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl) cyclopropyl)methanol	Rats; M/F	3/10	Gavage	28	20	Braun (2000)

bw: body weight; F: female; M: male; NA: not applicable; no.: number; NOAEL: no-observed-adverse-effect level
[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] Range-finding study.

On the day after dosing, test article–related changes were noted in all high-dose males and females. All rats in this treatment group had swaying gait, decreased activity, sedation, prostration and ruffled fur. These rats were considered moribund and were removed from the study. All animals at 100 and 300 mg/kg bw per day survived until the end of the study. No clinical signs of toxicity or changes in feed consumption or body weights were noted in rats treated with 100 or 300 mg/kg bw per day. Terminal body weights of both sexes were lower at 300 mg/kg bw per day (by 4% in males and 7% in females) and in females at100 mg/kg bw per day (by 7%) compared with controls. Absolute organ weights of treated males and females were similar to those of respective controls with the exception of slightly increased absolute liver weights for males

at 300 mg/kg bw per day. No treatment-related macroscopic changes were noted at necropsy in either sex at any dose level (including the animals at 1000 mg/kg bw per day group that were removed from the study).

Based on the results of this 7-day range-finding toxicity study, dose levels of 50, 150 and 450 mg/kg bw per day were proposed for a subsequent 28-day study with No. 2553 (Braun, 2011).

In an OECD guideline–compliant 28-day study, groups of Wistar rats (5/sex per dose group) were administered a mixture of 1-vinyl-3-cyclohexenecarbaldehyde (~65% w/w) and 4-vinyl-1-cyclohexenecarbaldehyde (~30% w/w) at 0, 50, 150 or 450 mg/kg bw per day by gavage. All animals were monitored daily for clinical signs of toxicity. Body weights and feed consumption were recorded weekly. In the fourth week of treatment, all animals were assessed for sensory reactivity, grip strength and motor activity. Haematological, clinical chemistry and urine analyses were performed at the end of the dosing period, and all animals were killed and necropsied. Organs and tissues from control and high-dose animals and any gross lesions in rats from other groups underwent histopathological examinations.

There were no unscheduled deaths, abnormal clinical observations or differences in grip strength in rats of either sex at any dose level. High-dose females showed consistently reduced locomotor activity compared with control females. Locomotor activity values in treated males remained unchanged. No significant changes in feed consumption or mean body weights were reported. There were no treatment-related effects on haematological, clinical chemistry, urine analysis, gross pathological or histopathological parameters or on organ weights.

The no-observed-adverse-effect level (NOAEL) for the mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde was 150 mg/kg bw per day based on reduced locomotor activity in females at 450 mg/kg bw per day (Braun, 2012).

(ii) (1-Methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl)cyclopropyl)methanol (No. 2254)

In an OECD guideline–compliant 28-day gavage study, groups of Wister rats (5/sex per dose group) were administered (1-methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl)cyclopropyl)methanol (purity 89%) by gavage at 0, 20, 100 or 500 mg/kg bw per day for a period of 28 days. Clinical observations were recorded daily. Body weights and feed consumption were recorded weekly. A functional observational battery and locomotor activity and grip strength tests were performed during the fourth week. At scheduled kill, blood was taken for clinical chemistry and haematological analyses. At necropsy, organs were weighed and

histopathological examinations performed of control and high-dose animals and any gross lesions identified in rats from other groups. Kidneys of low- and mid-dose animals were examined to establish a no-effect level.

There were no unscheduled deaths during the study. Mean body weights and body-weight gains in treated animals were similar to controls. A transient reduction in feed consumption was noted only in high-dose females. Locomotor activity and grip strength in treated animals was similar to controls. No clinical signs of toxicity or toxicologically relevant haematological changes were observed.

Test article–related reduction in plasma glucose was noted in males at 100 mg/kg bw per day and in both sexes at 500 mg/kg bw per day. Increased levels of total cholesterol, triglycerides and phospholipids were recorded in females at 500 mg/kg bw per day. Increased γ-glutamyltransferase activity and calcium levels were noted in both sexes at 500 mg/kg bw per day. Lower potassium levels were noted in both sexes at 500 mg/kg bw per day. All other differences in clinical chemistry parameters were considered incidental or of no toxicological significance. A statistically significant increase in absolute liver weights was reported

(c) Genotoxicity

Studies of genotoxicity on alicyclic primary alcohols, aldehydes, acids and related esters evaluated for the present meeting are summarized in Table 5.

Positive results in an in vitro chromosomal aberration assay were observed for the mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde (No. 2253); however, an in vitro reverse mutation assay and an in vivo assay of micronucleus induction in mice were negative.

Positive results in an in vitro chromosomal aberration assay were observed for (±)-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, ethyl ester (No. 2255), however, in vitro reverse and forward mutation assays, an in vitro micronucleus induction assay, and an in vivo assay of micronucleus induction in mice were negative.

For *p*-mentha-1,8-dien-7-al (No. 973), reproducible positive results were observed in a reverse mutation assay in the TA98 strain of *S. typhimurium* (Bowen, 2011). In addition, a study in rats investigated micronucleus induction in bone marrow and DNA damage in liver and duodenum (comet assay) (Beevers, 2014a,b). In this GLP-compliant study, the micronucleus assay was conducted according to OECD guideline 474, while the comet assay was conducted in accordance with published guidelines developed by an expert working group (Burlinson et al., 2007). The results of the micronucleus assay indicated that *p*-mentha-1,8-dien-7-al (No. 973) did not induce an increase in micronucleated polychromatic erythrocytes. Although the comet assay did not indicate DNA

Table 5

Studies of genotoxicity of alicyclic primary alcohols, aldehydes, acids and related esters used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2253	Mixture of 1-vinyl-3-cyclohexene carbaldehyde and 4-vinyl-1-cyclohexene carbaldehyde	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2uvrA	3–5 000 µg/plate (±S9) [a,b]	Negative	Sokolowski (2011)
2253	Mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexenecarbaldehyde	Chromosomal aberration	Chinese hamster V79 cells	0.08, 0.16 and 0.3 µL/mL (+S9) 16–93 µL/mL (−S9)	Positive	Hall (2011a)
2254	(1-Methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl)cyclopropyl)methanol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	1c–5 000 µg/plate (±S9) [a,d]	Negative	Sokolowski (2012)
2254	(1-Methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl)cyclopropyl)metha nol	Chromosomal aberration	Chinese hamster V79 cells	5, 10 and 20 µg/mL (−S9, 4-h exposure) 2.5, 5 and 10 µg/mL (−S9,18-h exposure) 2.5, 7.5 and 15 µg/mL (−S9, 28-h exposure) 15, 30 and 45 µg/mL (+S9, 4-h exposure)	Negative	Chetelat (2000)
2255	(±)-Bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, ethyl ester	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	5–5 000 µg/plate (±S9)	Negative	May (2010)
2255	(±)-Bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, ethyl ester	Micronucleus induction	Human peripheral lymphocyte cells	46.5, 215, 598 µg/mL (−S9, 3-h exposure and 21-h recovery) 300 and 580 µg/mL (+S9, 3-h exposure and 21-h recovery) 5, 20 and 40 µg/mL (−S9, 20-h exposure)	Negative	May (2012)
2255	(±)-Bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, ethyl ester	Chromosomal aberration	Human peripheral lymphocyte cells	129, 215 and 359 µg/mL (−S9, 3-h exposure with 18-h recovery) 480, 495 and 525 µg/mL (+S9, 3-h exposure with 18-h recovery) 80, 160 and 240 µg/mL (−S9, 21-h exposure) 500, 550 and 575 µg/mL (+S9, 3-h exposure with 18-h recovery)	Negative (3-h treatment, ±S9; and 21-h treatment, +S9); positive (21-h treatment, −S9)	Pritchard (2010)
2255	(±)-Bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, ethyl ester	Forward mutation	Mouse lymphoma L5178Y cells	5–450 µg/mL (−S9, 3-h exposure) 5–850 µg/mL (+S9, 3-h exposure) 5–400 µg/mL (−S9, 24-h exposure)	Negative	Pritchard (2011)

Table 5 (continued)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
967	(2,2,3-Trimethylcyclopent-3-en-1-yl)acetaldehyde	Reverse mutation	*S. typhimurium* TA100	15–5 000 µg/plate(±S9) [e]	Negative	ECHA (2017c)
973	*p*-Mentha-1,8-dien-7-al	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.3–5 000 µg/plate (±S9) [f,g] 8–5 000 µg/plate (±S9) [h,i]	Negative for all strains except TA98 (−S9)	Bowen (2011)
973	*p*-Mentha-1,8-dien-7-al	Micronucleus induction	Human peripheral blood lymphocytes	80, 100, 110 and 120 µg/mL (−S9, 3-h exposure and 21-h recovery) 100, 120 and 140 µg/mL (+S9, 3-h exposure and 21-h recovery) 20, 25 and 35 µg/mL (−S9, 24-h exposure)	Negative	Lloyd (2009)
973	*p*-Mentha-1,8-dien-7-al	Forward mutation	Mouse lymphoma L5178Y cells	10–150 µg/mL (−S9, 3-h exposure) 20–200 µg/mL (+S9, 3-h exposure) 10–120 µg/mL (−S9, 3-h exposure) 25–200 µg/mL (+S9, 3-h exposure) 4–40 µg/mL (−S9, 24-h exposure)	Negative	Lloyd (2012)
973	*p*-Mentha-1,8-dien-7-al	Forward mutation	Mouse lymphoma L5178Y cells	1–1 520 µg/mL (±S9, 4- and 24-h exposure) [j] 25, 50 and 100 µg/mL (−S9, 24-h exposure) 100, 250 and 325 µg/mL (−S9, 4-h exposure) [k] 100, 225 and 275 µg/mL (+S9,,4-h exposure) [l]	Equivocal	Roy (2016)
974	*p*-Mentha-1,8-dien-7-ol	Micronucleus induction	Human peripheral blood lymphocytes	1–1 520 µg/mL (±S9, 4- and 24-h exposure) [j] 25, 50 and 100 µg/mL (−S9, 24-h exposure) 100, 250 and 325 µg/mL (−S9, 4-h exposure) [k] 100, 225 and 275 µg/mL (+S9, 4-h exposure) [l]	Negative	Roy (2016)
974	*p*-Mentha-1,8-dien-7-ol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	1–3 300 µg/plate (±S9) [a,m,n]	Negative	Wagner (2016)
975	*p*-Mentha-1,8-dien-7-yl acetate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	5–5 000 µg/plate (±S9) [o] 1.6–1 600 µg/plate (±S9) [p,q]	Negative	Lloyd (2016a)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
975	*p*-Mentha-1,8-dien-7-yl acetate	Micronucleus induction	Human peripheral blood lymphocytes	100, 120 and 130 µg/mL (−S9, 3-h exposure plus 21-h recovery) 150, 240 and 280 µg/mL (+S9, 3-h exposure plus 21-h recovery) 30, 60 and 80 µg/mL (−S9, 24-h exposure)	Negative	Lloyd (2016b)
975	*p*-Mentha-1,8-dien-7-yl acetate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	1.6–5 000 µg/plate (±S9) 16–500 µg/plate (−S9, only TA98, TA1535 and TA1537) [r] 78–2 500 µg/plate (−S9, only TA100 and TA102) [r] 156–5 000 µg/plate (+S9, only TA98 and TA100) [r,s] 78–2 500 µg/plate (+S9, only TA1535, TA1537 and TA102) [r,s] 25–400 µg/plate (−S9, only TA100) 50–800 µg/plate (+S9, only TA100) [s] 25–400 µg/plate (+S9, only TA100, TA102, TA1535 and TA1537) [s]	Negative	Beevers (2010a)
975	*p*-Mentha-1,8-dien-7-yl acetate	Micronucleus induction	Human peripheral blood lymphocytes	80, 90 and 110 µg/mL (−S9, with 3-h exposure with 21-h recovery) 200, 300 and 400 µg/mL (+S9, with 3-h exposure plus 21-h recovery) 20–100 µg/mL (−S9, 24-h exposure)	Negative	Whitwell (2010a)
977	2,6,6-Trimethylcyclohexa-1,3-dienyl methanal	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	1.6–5 000 µg/plate (±S9) [t] 125–5 000 µg/plate (−S9) 63–5 000 µg/plate (+S9, only TA98, TA100 and TA1535) [s] 63–2 000 µg/plate (+S9, only TA1537 and TA102) [s] 16–500 µg/plate (21-h exposure) [s]	Negative	Beevers (2010b)
977	2,6,6-Trimethylcyclohexa-1,3-dienyl methanal	Micronucleus induction	Human peripheral blood lymphocytes	40, 60 and 90 µg/mL (−S9, 3-h exposure plus 21-h recovery) 80–140 µg/mL (+S9, 3-h exposure plus 21-h recovery) 4, 8 and 12 µg/mL (−S9, 24-h exposure)	Negative	Whitwell (2010a)
978	2,6,6-Trimethyl-1-cyclohexen-1-acetaldehyde	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	5–5 000 µg/plate (±S9) [u] 5–1 600 µg/plate (±S9) [v,w] 5– 5 000 µg/plate (+S9, only in *E. coli* WP2*uvr*A) [x]	Negative	Bhalli (2015a)

Table 5 (continued)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
978	2,6,6-Trimethyl-1-cyclohexen-1-acetaldehyde	Micronucleus induction	Human peripheral blood lymphocytes	12, 17 and 30 µg/mL (−S9, 24-h exposure) 23, 51, 57 and 63 µg/mL (−S9, 3-h exposure) 96, 131 and 180 µg/mL (+S9, 3-h exposure)	Negative	Bhalli (2015b)
979	2,6,6-Trimethyl-1 & 2-cyclohexen-1-carboxaldehyde	Reverse mutation	S. typhimurium TA98, TA100, TA1535 and TA1537; *E. coli* WP2uvrA	5–5 000 µg/ plate (±S9) [w] 5–1 600 µg/plate (−S9, only *S. typhimurium* strains) [v] 16–5 000 µg/plate (+S9, *S. typhimurium* strains; ±S9, WP2*uvr*A)[y] 5–5 000 µg/plate (+S9, only in *E. coli* WP2uvrA)[z,aa] 5–1 600 µg/plate (−S9, only in TA1537) [z,aa]	Negative	Bhalli (2015c)
979	2,6,6-Trimethyl-1 & 2-cyclohexen-1-carboxaldehyde	Micronucleus induction	Human peripheral blood lymphocytes	18–59 µg/mL (−S9, 24-h exposure) 204, 227 and 239 µg/mL (−S9, 3-h exposure) 251, 279 and 309 µg/mL (+S9, 3-h exposure)	Negative	Bhalli (2015d)
980	Formyl-6,6-dimethylbicyclo [3.1.1]hept-2-ene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	1.5–5 000 µg/plate (±S9) [aa]	Negative	Dakoulas (2017)
980	Formyl-6,6-dimethylbicyclo [3.1.1]hept-2-ene	Micronucleus induction	Human peripheral blood lymphocytes	50, 130 and 180 µg/mL (−S9) and 25, 200 and 350 µg/mL (+S9, 3-h exposure plus 21-h recovery) 15, 25 and 34 µg/mL (−S9, 24-h exposure)	Negative	McGarry (2016b)
980	Formyl-6,6-dimethylbicyclo [3.1.1]hept-2-ene	Micronucleus induction	Human peripheral blood lymphocytes	100, 160 and 200 µg/mL (−S9, 3-h exposure plus 21-h recovery) and 100, 200, 300 and 350 µg/mL (+S9, 3-h exposure plus 21-h recovery) 10, 20, 30 and 32 µg/mL (−S9, 24-h exposure) 30, 45, 65 and 75 µg/mL (−S9, 24-h treatment with 24-h recovery)	Negative	Lloyd (2017)
980	Formyl-6,6-dimethylbicyclo [3.1.1]hept-2-ene	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	5–5 000 µg/plate (±S9) [bb] 80–5 000 µg/plate (±S9) [p,cc]	Negative	McGarry (2016a)
981	Myrtenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	5–5 000 µg/plate (±S9) [dd,ee] 16–5 000 µg/plate (±S9) [ff]	Negative	Bhalli (2015e)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
981	Myrtenol	Micronucleus induction	Human peripheral blood lymphocytes	368, 387, 451 and 475 µg/mL (−S9) 407, 451 and 475 µg/mL (+S9, 3-h exposure) 31, 47 and 53 µg/mL (−S9, 24-h exposure)	Negative	Bhalli (2015f)
982	Myrtenyl acetate	Micronucleus induction	Human peripheral blood lymphocytes	20, 60, 80 and 90 µg/mL (3-h exposure plus 21-h recovery) 150, 250 and 325 µg/mL (−S9, 3-h exposure plus 21-h recovery) 10, 20 and 40 µg/mL (−S9, 24-h exposure)	Negative	McGarry (2016c)
982	Myrtenyl acetate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	5–5 000 µg/plate (±S9) [dd] 3.3–2 000 µg/plate (±S9) [p,gg]	Negative	McGarry (2016d)
984	Santalol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	5–5 000 µg/plate (±S9) [hh,ii] 1.6–1 600 µg/plate (−S9) [jj]	Negative	Bhalli (2014a)
984	Santalol	Micronucleus induction	Human peripheral blood lymphocytes	63–260 µg/mL (±S9, 3-h exposure) 84, 88 and 103 µg/mL (−S9, 3-h exposure) [ll] 103, 128 and 142 µg/mL (+S9, 3-h exposure) [ll] 8.6–21 µg/mL (−S9, 24-h exposure)	Negative	Bhalli (2014b)
In vivo						
2253	Mixture of 1-vinyl-3-cyclohexenecarbaldehyde and 4-vinyl-1-cyclohexene carbaldehyde	Micronucleus induction	Mice; M	500, 1 000 and 2 000 mg/kg bw	Negative	Hall (2011b)
2255	(±)-Bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, ethyl ester	Micronucleus induction	Mice; M	312, 625 and 1 250 mg/kg bw, intraperitoneal	Negative	Roy (2013)
973	*p*-Mentha-1,8-dien-7-al	Micronucleus induction	Rats; M	175, 350 and 700 mg/kg bw per day for 3 days	Negative	Beevers (2014a)
973	*p*-Mentha-1,8-dien-7-al	Comet assay	Rats; M	175, 350 and 700 mg/kg bw	Negative for duodenum. Positive for liver. Compared to the negative control, a 3-fold increase in DNA strand breaks (statistically	Beevers (2014a,b)

Table 5 (continued)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
					significant, $P < 0.001$) was observed in liver at 700 mg/kg bw per day, and there was a dose-dependent trend in the response ($P < 0.001$).	
977	2,6,6-Trimethylcyclohexa-1,3-dienyl methanal	Comet assay	Mice; M	73 [mm] and 364 mg/kg bw (3-h exposure)	Negative	Hosseinzadeh & Sadeghnia (2007)
980	Formyl-6,6-dimethylbicyclo [3.1.1]hept-2-ene	Micronucleus induction	Rats, M	125, 250 and 500 mg/kg bw per day for 3 days	Negative	Pant (2018)
980	Formyl-6,6-dimethylbicyclo [3.1.1]hept-2-ene	Comet assay	Rats, M	125, 250 and 500 mg/kg bw	Negative	Pant (2018)

bw: body weight; S9: metabolic activation using 9000 × *g* supernatant fraction from rat liver homogenate

[a] Both preincubation and plate incorporation used.

[b] Cytotoxicity observed at higher concentrations with and without metabolic activation in all strains used in both experiments.

[c] Dose used only without S9; preincubation method.

[d] Cytotoxicity observed at higher concentration in strains TA98, TA100, TA1535 and TA1537, with and without metabolic activation.

[e] Slightly cytotoxic at 1500 µg/plate; background lawn was mostly absent at 5000 µg/plate.

[f] Cytotoxicity observed at 1000 and/or 5000 µg/plate in all strains in the presence or absence of S9.

[g] Extensive cytotoxicity at all concentrations observed in the presence of S9.

[h] Treatments in the presence of S9 were modified with the inclusion of a preincubation step.

[i] Cytotoxicity observed at 320, 800 and 2000 µg/plate and above in all strains in the presence and absence of S9.

[j] Assay was repeated because adequate cytotoxicity was not reached during the 4-hour exposure.

[k] In the repeat micronucleus assay, cytotoxicity was observed at ≥325 µg/mL in the 4-hour exposure group in the absence of S9.

[l] In the repeat micronucleus assay, cytotoxicity was observed at ≥275 µg/mL in the 4-hour exposure group in the presence of S9.

[m] Cytotoxicity observed at 3300 µg/plate when using the plate incorporation method.

[n] Cytotoxicity observed at ≥100 or ≥333 µg/plate when using the preincubation method.

[o] Cytotoxicity observed at ≥500 µg/plate for all strains in the absence of S9 and in TA1537 in the presence of S9; and at ≥1600 µg/plate for the remaining strains in the presence of S9.

[p] Treatments in the presence of S9 were modified to include a preincubation step.

[q] Cytotoxicity observed at ≥500 µg/plate for all strains in the absence of S9 and in TA1535 and TA102 in the presence of S9; and at ≥160 µg/plate in TA98, TA100 and TA1537 in the presence of S9.

[r] Excessive cytotoxicity observed in all strains in the presence of S9 and in TA100 in the absence of S9.

[s] Preincubation step used.

[t] Cytotoxicity observed at 5000 µg/plate in all strains in the presence or absence of S9 and at 1000 µg/plate in TA102 and TA1537 in the presence of S9.

[u] Cytotoxicity observed at ≥1600 µg/plate in all strains both in the presence and absence of S9, and at ≥500 µg/plate in TA98, TA100 and TA1537 in the absence of S9.
[v] Independent confirmatory assay.
[w] Cytotoxicity observed at ≥1600 µg/plate in all strains in the presence and absence of S9, and at ≥5000 µg/plate in *E. coli* WP2*uvr*A in the presence of S9.
[x] Cytotoxicity observed at ≥1600 µg/plate in the presence of S9.
[y] Cytotoxicity observed at ≥1600 µg/plate in all strains in the presence or absence of S9, and at 5000 µg/plate in WP2*uvr*A in the presence or absence of S9.
[z] Repeat confirmatory assay.
[aa] Cytotoxicity observed at the highest tested dose.
[bb] Cytotoxicity observed at ≥1600 µg/plate in all strains in the presence and absence of S9.
[cc] Cytotoxicity observed at ≥1250 µg/plate in all strains in the absence of S9 and at ≥300 µg/plate in all strains in the presence of S9.
[dd] Cytotoxicity observed at ≥500 µg/plate in all tested strains in the presence or absence of S9.
[ee] Cytotoxicity observed in all *S. typhimurium* strains at 5000 µg/plate in the presence or absence of S9 and at ≥1600 µg/plate in TA98 and WP2*uvr*A in the presence and absence of S9.
[ff] Cytotoxicity observed in all *S. typhimurium* strains at 5000 µg/plate in the presence and absence of S9 and at ≥1600 µg/plate in *E. coli* WP2*uvr*A in the absence of S9.
[gg] Cytotoxicity observed at ≥320 µg/plate in TA98, TA100, TA1535 and TA1537 in the absence and presence of S9 and at 2000 µg/plate in TA102 in the presence and absence of S9.
[hh] Cytotoxicity observed in all strains at ≥1600 µg/plate in the presence of S9 and at 5000 µg/plate in TA98 and WP2*uvr*A in the presence of S9; and at ≥160 µg/plate in all strains in the absence of S9 and at ≥1600 µg/plate in WP2*uvr*A in the absence of S9.
[ii] Cytotoxicity observed in all strains at ≥1600 µg/plate in the presence of S9 and at ≥500 µg/plate in TA98 and 5000 µg/plate in WP2*uvr*A in the presence of S9.
[jj] Cytotoxicity observed at ≥160 µg/plate in all *S. typhimurium* strains and at ≥50 µg/plate in TA1537. No cytotoxicity observed in WP2*uvr*A.
[ll] Repeat micronucleus assay.
[mm] Given 45 minutes prior to intraperitoneal administration of methyl methanesulfonate.

damage in duodenum compared to the negative control, a 3-fold increase in DNA strand breaks (statistically significant, $P < 0.001$) was observed in liver at the highest dose tested (700 mg/kg bw per day), and there was a dose-dependent trend in the response ($P < 0.001$). Based on these new data, the Committee concluded that there are concerns for potential genotoxicity for *p*-mentha-1,8-dien-7-al (No. 973).

In vitro genotoxicity assays on other flavouring agents in this group for which data were available (Nos 2254, 967, 974, 975, 977–982 and 984) were all negative. In vivo genotoxicity data were also available for Nos 977 and 980; these studies gave negative results.

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Carvone and related substances (addendum)

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1. Evaluation

1.1 Introduction

The Committee evaluated five flavouring agents belonging to the previously evaluated group of carvone and structurally related substances. The Committee re-evaluated two flavouring agents, (+)-carvone (No. 380.1; *d*-carvone) and

(–)-carvone (No. 380.2; *l*-carvone), and evaluated three additional flavouring agents. These three additional flavouring agents included two esters, pinocarvyl isobutyrate (No. 2242) and carvyl palmitate (No. 2243), and one alicyclic secondary alcohol, 6-hydroxycarvone (No. 2244). The evaluations were conducted using the revised Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *230*). (+)-Carvone (No. 380.1) and (–)-carvone (No. 380.2) were re-evaluated because of new data had become available.

The Committee previously evaluated nine members of this group of flavouring agents at its fifty-first meeting (Annex 1, reference *137*). The Committee concluded that all nine flavouring agents were of no safety concern at estimated dietary exposures.

Carvone (Nos 380.1 and 380.2) was evaluated at the eleventh meeting (Annex 1, reference *14*) at which a conditional[1] acceptable daily intake (ADI) of 0–1.25 mg/kg body weight (bw) for the (+)- and (–)-enantiomers was established. At the twenty-third meeting (Annex 1, reference *50*), a temporary ADI of 0–1 mg/kg bw was established for (+)- and (–)-carvone. This temporary ADI was extended at the twenty-fifth, twenty-seventh, thirtieth and thirty-third meetings (Annex 1, references *56*, *62*, *73* and *83*). At its thirty-seventh meeting, the Committee determined that the (+)- and (–)-enantiomers of carvone should be evaluated separately (Annex 1, reference *94*). The Committee established an ADI for (+)-carvone of 0–1 mg/kg bw per day based on a no-observed-effect level (NOEL) of 93 mg/kg bw per day from a 3-month toxicity study in rats. The temporary ADI for (–)-carvone was not extended because insufficient data were available for the toxicological evaluation of this enantiomer. The Committee at its fifty-first meeting maintained the ADI of 0–1 mg/kg bw for (+)-carvone (No. 380.1) (Annex 1, reference *137*).

Two of the five flavouring agents, (+)-carvone (No. 380.1) and (–)-carvone (No. 380.2), have been reported to occur naturally in foods, mainly botanicals. (+)-Carvone (No. 380.1) has been reported in *Carum* (caraway) and *Anethum* (dill). (–)-Carvone (No. 380.2) has been reported in the oils of *Mentha* (spearmint) (Nijssen, Van Ingen-Visscher & Donders, 2017).

A comprehensive literature search for toxicological data was performed in Scopus using the names and Chemical Abstracts Service (CAS) numbers of the flavouring agents under evaluation in this group of flavouring agents; no additional relevant references were identified.

[1] "Conditional ADI is a term no longer used by Joint FAO/WHO Expert Committee on Food Additives to signify a range above the 'unconditional ADI', which may signify an acceptable intake when special problems, different patterns of dietary intake, and special groups of the population that may require consideration are taken into account." (FAO/WHO, 2009)

1.2 Assessment of dietary exposure

The total annual volume of production of the three new flavouring agents belonging to the group of carvone and structurally related substances is 2 kg in Japan (IOFI, 2017a,b). The total production volume for the flavouring agents presented for re-evaluation, (+)-carvone (No. 380.1) and (−)-carvone (No. 380.2), is 48 300 kg in Europe, 53 700 kg in the USA, 1780 kg in Japan and 6340 kg in Latin America (IOFI, 2017a,b). Separate production volumes for the two enantiomers are not available.

Dietary exposure was estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, with the highest values reported in Table 1. The estimated daily dietary exposure is highest for carvone (Nos 380.1 and 380.2) at 37 800 μg/day, the SPET value obtained from beer and malt beverages. The MSDI values for carvone range from 350 to 5573 μg/day for the four regions. For the other flavouring agents, the estimated daily dietary exposures range from 0.05 to 0.3 μg/day (MSDI values) and from 3 to 450 μg/day (SPET values).

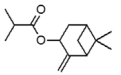
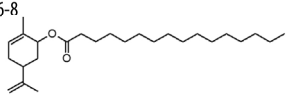
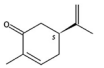
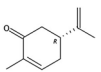
1.3 Absorption, distribution, metabolism and excretion (ADME)

Relevant information apart from that described in the monographs of the eleventh, twenty-third, thirty-seventh and fifty-first meetings (Annex 1, references *15*, *51*, *95* and *138*) was available on the ADME of the flavouring agents belonging to the group of carvone and structurally related substances.

In general, carboxylesterases or esterases catalyse the hydrolysis of esters to their corresponding alcohol and carboxylic acid. The carvyl esters in this group (Nos 2242 and 2243) can be expected to readily hydrolyse to pinocarveol (No. 1403) and carveol (No. 974), respectively, and their respective carboxylic acids. Carvone (Nos 380.1 and 380.2) is metabolized to carveol, dihydrocarveol, carvonic acid, dihydrocarvonic acid and uroterpenolone in humans (Engel, 2001). Oxidation of (+)- or (−)-carvone by human or rat liver microsomes is stereospecific; (+)-carvone is oxidized exclusively to (+)-carveol and (−)-carvone to (−)-carveol, with (−)-carvone having a significantly higher affinity for microsomal enzymes (lower apparent Michaelis constant [K_m]). Only (−)-carveol is converted to a glucuronide conjugate by human or rat liver microsomes (Jäger et al., 2000). This is expected to impact the toxicity profiles of the enantiomers and their metabolites. 6-Hydroxycarvone is expected to undergo conjugation with glucuronic acid or glutathione followed by excretion.

Table 1

Summary of the results of the safety evaluations of carvone and structurally related substances used as flavouring agents [a,b]

Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the highest dietary exposure estimate exceed the threshold of toxicological concern?	Condusion based on current estimated dietary exposure
Structural class I				
Pinocarvyl isobutyrate	2242	929116-08-5 	No, SPET: 450	No safety concern
Carvyl palmitate	2243	929222-96-8 	No, SPET: 3	No safety concern
Structural class II				
6-Hydroxycarvone	2244	51200-86-3 	No, SPET: 4	No safety concern
Flavouring agents not evaluated according to the revised Procedure				
(+)-Carvone	380.1	2244-16-8 	A review of the ADI is recommended based on an evaluation of all biochemical and toxicological data. Also, data are needed for an assessment of oral exposure to (+)-carvone from all sources to complete the evaluation for (+)-carvone.	
(−)-Carvone	380.2	6485-40-1 	Additional toxicological data on (−)-carvone are necessary. Also, data are needed for an assessment of oral exposure to (−)-carvone from all sources to complete the evaluation for (−)-carvone.	

ADI: acceptable daily intake; CAS: Chemical Abstracts Service; no.: number; NR: not relevant; SPET: single-portion exposure technique

[a] Nine flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *137*).

[b] Step 2: Two additional flavouring agents in this group are in structural class I and one additional flavouring agent in this group is in structural class III.

[c] The threshold of toxicological concern for human dietary exposure for structural class I is 1800 µg/day and for structural class III is 90 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest estimated daily dietary exposures calculated using the SPET.

1.4 Flavouring agents not evaluated according to the revised Procedure for the Safety Evaluation of Flavouring Agents

Exposure to (+)-carvone (No. 380.1) and (−)-carvone (No. 380.2) occurs from different food and non-food sources as well as from their use as flavouring agents. Both enantiomers of carvone occur naturally in food and are used in one or more of the following applications: pesticides, feed additives, veterinary products, personal care products, natural insect repellents, food supplements and herbal medicinal products (EFSA, 2014; Nijssen, Van Ingen-Visscher & Donders, 2017).

For the current re-evaluation of carvone (Nos 380.1 and 380.2), additional biochemical data and studies of acute toxicity and genotoxicity were submitted by the flavour industry. The Scientific Committee of the European Food Safety Authority (EFSA) published an evaluation of carvone based on data from the flavour and pesticide industries. An ADI of 0.6 mg/kg bw per day for (+)-carvone was established, but an ADI for (−)-carvone could not be established due to lack of data. The highest level of aggregated exposure to (+)-carvone was at the level of the ADI for (+)-carvone. The highest level of aggregated exposure to (−)-carvone was 3-fold higher than that of (+)-carvone (EFSA, 2014).

The Committee previously established an ADI for (+)-carvone of 0–1 mg/kg bw per day based on a NOEL of 93 mg/kg bw per day from a 3-month toxicity study in rats (National Toxicology Program, 1989). At the current meeting, the Committee noted that when identifying the NOEL, no correction was made for the 5-day (rather than 7-day) dosing scheme. Shortly before the meeting, data from the pesticide industry (studies of acute toxicity, short-term toxicity and a two-generation study) were made available to the Committee by the sponsor. The Committee considered these data and concluded that a review of the ADI for (+)-carvone is recommended based on the evaluation of all biochemical and toxicological data. Also, additional data are needed for an exposure assessment for the oral exposure to (+)-carvone from all sources to complete the re-evaluation of (+)-carvone (No. 380.1).

The Committee previously concluded that the ADI for (+)-carvone could not be extended to (−)-carvone because insufficient data were available for the toxicological evaluation of this enantiomer (Annex 1, reference *94*). Data are also needed in an oral exposure assessment to (−)-carvone from all sources to complete the re-evaluation of (−)-carvone (No. 380.2).

The Committee therefore did not re-evaluate (+)-carvone (No. 380.1) and (−)-carvone (No. 380.2) according to the revised Procedure for the Safety Evaluation of Flavouring Agents at the current meeting.

1.5 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. There are no structural alerts for genotoxicity for the additional flavouring agents (Nos 2242–2244) in this group. Chemical-specific genotoxicity data on flavouring agents previously evaluated within this group do not indicate that the flavouring agents in this group have the potential to be genotoxic.

Step 2. In applying the revised Procedure for the Safety Evaluation of Flavouring Agents to Nos 2242–2244, the Committee assigned Nos 2242 and 2243 to structural class I and No. 2244 to structural class III (Cramer, Ford & Hall, 1978).

Steps 3 and 4. The highest estimated dietary exposures of Nos 2242–2244 are below their respective thresholds of toxicological concern (i.e. 1800 µg/day for structural class I and 90 µg/day for structural class III). The Committee therefore concluded that these three flavouring agents would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the three flavouring agents (Nos 2422–2244) in the group of carvone and structurally related substances.

1.6 Consideration of combined intakes from use as flavouring agents

The three additional flavouring agents in the group of carvone and structurally related substances have low MSDIs (0.05–0.3 µg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

1.7 Consideration of additional data on previously evaluated flavouring agents

In the previous evaluations of substances in this group of carvone and structurally related substances, studies of biochemistry, acute toxicity, short-term and long-term toxicity and genotoxicity were available (Annex 1, references *15*, *51*, *95* and *138*). None of the nine flavouring agents of this group raised safety concerns.

Besides data on carvone (see the section "Flavouring agents not evaluated according to the revised Procedure for the Safety Evaluation of Flavouring Agents"), additional biochemical data on carveol (No. 381), a study of acute toxicity with *p*-menthan-2-one (No. 375) and studies of genotoxicity with dihydrocarveol (No. 378) and carveol (No. 381) were available. These data support the conclusions of the previous evaluation.

1.8 Conclusions

The Committee concluded that the three flavouring agents pinocarvyl isobutyrate (No. 2242), carvyl palmitate (No. 2243) and 6-hydroxycarvone (No. 2244), which are additions to the group of carvone and structurally related substances evaluated previously, do not give rise to safety concerns at current estimated dietary exposures.

The Committee did not re-evaluate (+)-carvone (No. 380.1) and (−)-carvone (No. 380.2) according to the revised Procedure given the lack of information on the oral exposure from all sources, the need to review the ADI of (+)-carvone and the lack of toxicological data on (−)-carvone.

For (+)-carvone (No. 380.1), the Committee concluded that a review of the ADI is recommended based on the evaluation of all biochemical and toxicological data. In addition, data are needed for an assessment of oral exposure to (+)-carvone from all sources to complete the re-evaluation. This could not be completed during the current meeting.

For (−)-carvone (No. 380.2), the Committee concluded that additional toxicological data are necessary. Also, data are needed for an assessment of oral exposure to (−)-carvone from all sources to complete the re-evaluation.

The ADI for (+)-carvone is maintained pending review of the ADI at a future meeting. The Committee recommends that the re-evaluation is completed within 3 years.

2. Relevant background information

2.1 Explanation

This monograph addendum summarizes key aspects relevant to the safety evaluation of three flavouring agents (Nos 2242–2244) that are additions to the group of carvone and related substances evaluated previously by the Committee at its eleventh, twenty-third, thirty-seventh and fifty-first meetings (Annex 1, references *15*, *51*, *95* and *138*). Additional data relevant for the re-evaluation of carvone (Nos 380.1 and 380.2) were submitted by the flavour industry. In addition, EFSA published an evaluation of carvone from all sources (EFSA, 2014). This evaluation was based on data available from the flavouring industry as well as from the pesticide industry. The data from the pesticide industry were made available to the Committee by the data owner shortly before the meeting and were not included in the monograph (see section 1.4). Several summaries of additional studies available on the website of the European Chemicals Agency

(ECHA) were submitted, but the study reports of these studies were not available for the current evaluation and therefore were not included in this monograph.

2.2 Additional considerations on dietary exposure

Carvone (Nos 380.1 and 380.2) is the only substance in this group of flavouring agents reported to occur naturally in foods, mainly botanicals (Stofberg & Grundschober, 1987; Nijssen, van Ingen-Visscher & Donders, 2017). Quantitative natural occurrence data and a consumption ratio reported for carvone (Nos 380.1 and 380.2) indicate that exposure occurs predominantly from natural occurrence in food (i.e. consumption ratio > 1) (Stofberg & Kirschman, 1985; Stofberg & Grundschober, 1987).

Annual volumes of production of this group of flavouring agents as well as the daily dietary exposures calculated using both the MSDI method and the SPET are summarized in Table 2.

In addition to their use as flavouring agents, exposure to carvone (Nos 380.1 and 380.2) can occur from different food and non-food sources.

2.3 Biological data

2.3.1 Biochemical data: ADME

Relevant information additional to that described in the monograph of the eleventh, twenty-third, thirty-seventh and fifty-first meetings (Annex 1, references *15*, *51*, *95* and *138*) was available on the ADME of the flavouring agents belonging to the group of carvone and structurally related substances.

(a) Carvone (Nos 380.1 and 380.2)

The in vivo metabolism of (+)- and (−)-carvone (Nos 380.1 and 380.2) was investigated in human volunteers (3/sex per group). The study was reported to comply with ethical guidelines and be approved by the 'Freie Ethikkommission München'. The volunteers were given 1 mg/kg bw of (+)- or (−)-carvone in full-fat milk and 24-hour urine samples were collected prior to ingestion (controls) and post ingestion. The participants were given a controlled diet throughout the study, from 24 hours before the collection of the control urine sample.

Five metabolites were detected for both enantiomers after treatment of the samples with sulfatase and glucuronidase: dihydrocarvonic acid (α-4-dimethyl-5-oxo-3-cyclohexene-1-acetic acid), carvonic acid (α-methylene-4-methyl-5-oxo-3-cyclohexene-1-acetic acid), uroterpenolone (5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one), carveol and dihydrocarveol.

Table 2

Annual volumes of production and daily dietary exposures for carvone and three additional related substances used as flavouring agents in Europe, the USA, Japan and LATAM

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Annual volume of consumption via natural occurrence in foods (kg) [d]	Consumption ratio [e]
		MSDI [b]		SPET [c]			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
Pinocarvyl isobutyrate (2242)				450	8	–	NA
Europe	ND	ND	ND				
USA	ND	ND	ND				
Japan	1	0.3	0.004				
LATAM	ND	ND	ND				
Carvyl palmitate (2243)				3	0.1	–	NA
Europe	ND	ND	ND				
USA	ND	ND	ND				
Japan	0.2	0.05	0.000 9				
LATAM	ND	ND	ND				
6-Hydroxycarvone (2244)				4	0.1	–	NA
Europe	ND	ND	ND				
USA	ND	ND	ND				
Japan	0.8	0.2	0.004				
LATAM	ND	ND	ND				
Total for additional flavouring agents							
Europe	ND						
USA	ND						
Japan	2						
LATAM	ND						
Carvone (380.1 and 380.2)				37 800	630	156 477	3
Europe	48 300	4 034	67				
USA	53 700	5 573	93				
Japan	1 780	469	8				
LATAM	6 340	350	6				
Total							
Europe	48 300						
USA	53 700						
Japan	1 782						
LATAM	6 340						

bw: body weight; LATAM: Latin America; MSDI: maximized survey-derived intake; ND: no data reported; SPET: single-portion exposure technique; USA: United States of America; –: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (IOFI, 2017a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:

(annual volume, kg) × (1 × 10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 45 × 10^6 for Europe, 33 × 10^6 for the USA, 13 × 10^6 for Japan and 62 × 10^6 for LATAM; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (IOFI, 2017a,b).

MSDI (µg/kg bw per day) calculated as follows:

(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

Table 2 (continued)

[c] SPET (μg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (IOFI, 2017b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (μg/kg bw per day) calculated as follows:
(μg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.
[d] Quantitative data for the USA reported by Stofberg & Grundschober (1987).
[e] The consumption ratio is calculated as follows:
(annual volume of consumption via natural occurrence in food, kg)/(most recent annual volume as a flavouring agent, kg).

10-Hydroxycarvone, an in vivo metabolite previously identified in rabbits, could not be detected (Engel, 2001).

In a follow-up study, the formation of dihydrocarvonic acid, carvonic acid and uroterpenolone was investigated by analysing urine collected from a human volunteer given 15 mg (200 μg/kg bw) of either 9,9-[^{2}H]-carvone or 9-[^{13}C]-carvone. Analysis of the metabolites after treatment of the samples with sulfatase and glucuronidase indicated that carvonic acid is formed by oxidation at the methyl carbon of the isopropenyl group of carvone and dihydrocarvonic acid was formed by oxidation at the methylene position, most likely via an epoxide intermediate. Uroterpenolone was found to be exclusively formed by oxidation at the methylene carbon of the isopropenyl group of carvone, and thus most probably by hydrolysis of the carvone epoxide (Engel, 2002).

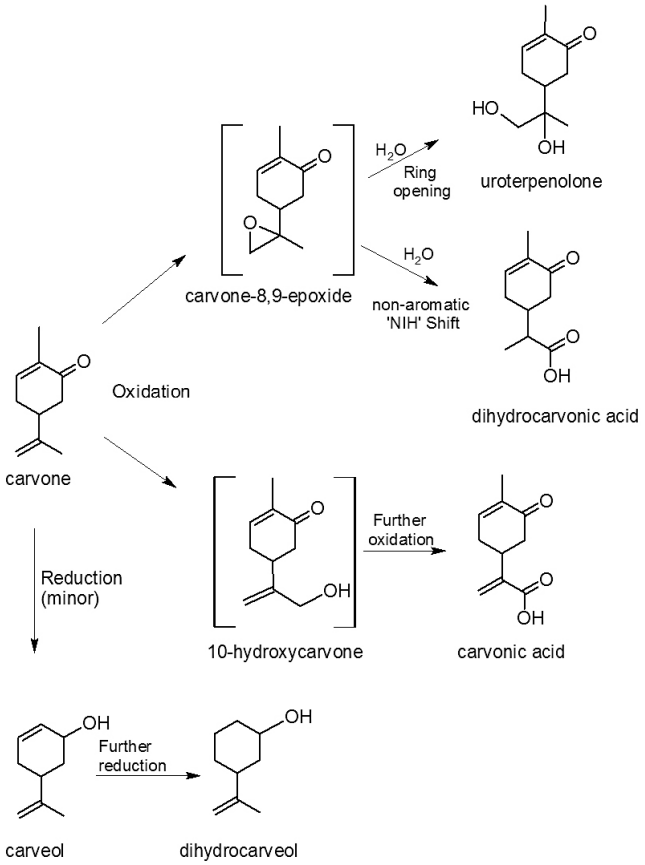
The proposed metabolic pathways for these three metabolites are shown in Fig. 1.

In an in vitro experiment using human and rat liver microsomes, (+)-carvone was exclusively metabolized to (+)-carveol and (−)-carvone to (−)-carveol, indicating stereospecific metabolism of carvone. The K_m values determined for the formation of (−)-carveol were significantly lower than for (+)-carveol in both rat and human liver microsomes, indicating that (−)-carvone has a significantly higher affinity for microsomal enzymes. The maximum velocity (V_{max}) was higher in human liver microsomes than in rat microsomes. Incubation of (+)- and (−)-carveol enantiomers with rat and human liver microsomes in the presence of uridine *S′*-diphosphoglucuronic acid (UDPGA) resulted in only (−)-carveol forming conjugates with glucuronic acid. The Committee noted that this would impact the toxicity profiles of the enantiomers and their metabolites. The V_{max} for glucuronide formation was more than 4-fold higher for the rat liver preparations than the human liver preparations, while K_m values were similar (Jäger et al., 2000).

In a toxicokinetic study, 16 healthy male volunteers ingested five capsules of an immediate release formulation containing 36 mg of peppermint oil and 20 mg of caraway oil, with 250 mL water after a 10-hour fast. The study protocol

Fig. 1

Proposed metabolic pathways of carvone resulting in the formation of dihydrocarvonic acid, carvonic acid, uroterpenolone, carveol and dihydrocarveol



Source: Engel (2001, 2002)

was reported to comply with Declaration of Helsinki ethical guidelines and was approved by an independent ethical committee. The carvone (and menthol) content was not determined, but the study authors stated that in general caraway oil consists of 50–65% of (+)-carvone. Blood samples were collected at 0, 20, 40, 60, 90, 120 and 150 minutes and 3, 4, 5, 6, 8, 10 and 15 hours after administration.

Following cleavage of conjugates by treatment with glucuronidase/arylsulfatase, carvone concentrations in plasma were determined by gas chromatography–mass spectrometry (GC-MS) analysis. On the first day of the study, one participant had slight fever and nausea; his plasma levels had very low levels of menthol. These results were excluded from further evaluation. The

following toxicokinetic parameters were determined for carvone: maximum plasma concentration (C_{max}) was 14.8 ± 10.4 ng/mL; the total bioavailability ($AUC_{0-\infty}$) was 28.9 ± 20.0 ng·h/mL; the time to reach the maximum plasma concentration (T_{max}) was 1.3 ± 0.6 hours, and the half-life ($t_{½}$) was 2.4 ± 1.2 hours (Mascher, Kikuta & Schiel, 2001).

(b) Carvone (Nos 380.1 and 380.2) and carveol (No. 381)

Species and sex differences in the metabolism of carveol and carvone were investigated with liver microsomes and/or recombinant cytochrome P450 (CYP) of mice, rats, guinea-pigs, rabbits, dogs, monkeys and humans.

Male rat liver microsomes had the highest rate of conversion of (+)-carvone to (+)-carveol of the species tested (about 50-fold higher than female rat liver microsomes and about 20-fold higher than human liver microsomes). In humans, CYP2C9 and CYP2C19 showed the highest activity for this conversion, followed by (in order of decreasing activity) CYP3A4, CYP 2E1. CYP2C18, CYP2C8, CYP 2B6 and CYP2A6. In rats, CYP2C11 and CYP2B1 showed the highest activity, followed by (in order of decreasing activity) CYP3A2, CYP3A1 and CYP2C12. Conversion of (+)-carveol to (+)-carvone was observed in incubations with dog, rabbit and guinea-pig liver microsomes, but not with human, rat, mouse or monkey liver microsomes. Incubations with rabbit CYP1A2 and CYP2B4 showed conversion of (+)-carvone and (−)-carvone into, respectively, (+)-carveol and (−)-carveol, and conversion of (+)-carveol and (−)-carveol into respectively (+)-carvone and (+)-carveol (Shimada, Shindo & Miyazawa, 2002).

2.3.2 Toxicological studies

Relevant information additional to that already available and described in the monographs of the eleventh, twenty-third, thirty-seventh and fifty-first meetings (Annex 1, references *15*, *51*, *95* and *138*) was available on the acute toxicity and genotoxicity of the flavouring agents belonging to the group of carvone and structurally substances.

(a) Acute toxicity

In acute toxicity studies in rats conducted according to Organisation for Economic Cooperation and Development (OECD) guidelines and certified for Good Laboratory Practice (GLP) and Quality Assurance (QA), oral median lethal dose (LD_{50}) values for (+)-carvone (No. 380.1) were 4900 and 5900 mg/kg bw in males and females, respectively (Gardner, 1986) and for *p*-menthan-2-one (No. 375) were >2000 and 4735 mg/kg bw (Leuschner, 2001) (Table 3).

Table 3

Results of oral acute toxicity studies with carvone and structurally related substances used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
375	*p*-Menthan-2-one	Rat; M, F	M: >2 000 F: 4 735	Leuschner (2001)
380.1	(+)-Carvone	Rat; M, F	M: 5 900 F: 4 900	Gardner (1986)

bw: body weight; F, female; LD_{50}: median lethal dose; M, male; no.: number

(b) Genotoxicity studies

Studies of genotoxicity of carvone and structurally related substances used as flavouring agents are summarized in Table 4 and described below.

(i) In vitro

Negative results were obtained when *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and/or *Escherichia coli* WP2*uvrA* were incubated with (+)-carvone (No. 380.1), (−)-carvone (No. 380.2) or dihydrocarveol (No. 378) at concentrations up to 5000 µg/plate, in the presence and absence of S9 metabolic activation (Glover, 1987; Stammati et al., 1999; Thompson, 2016). The study by Thompson (2016) was certified for compliance with QA and GLP and was performed according to OECD Test Guideline 471 ("Bacterial Reverse Mutation Assay", 1997).

In an SOS chromotest, (+)-carvone (No. 380.1) gave negative results when incubated in concentrations up to 0.25 µmol with *E. coli* strain PQ37 (Stammati et al., 1999).

In a DNA repair assay, (+)-carvone (No. 380.1) was incubated with *E. coli* WP2*trp*E65 and its isogenic DNA-repair deficient derivative CM871*trp*E65, *uvrA*155, *recA*56, *lexA*. In this filter disc assay, 25–80 µmol of (+)-carvone was applied to agar plates infused with each *E. coli* strain. A dose-dependent increase in the visible inhibition zone was observed for (+)-carvone in the repair-deficient strain, indicating the capacity of the test compound to induce DNA damage (Stammati et al., 1999).

In a DNA repair assay (umu test), an increase in β-galactosidase activity was detected when 1234.6 µg/mL carvone (No. 380; enantiomer not specified) was incubated with *S. typhimurium* strain TA1535 containing plasmid pSK1002 carrying fused gene *umuC'-'lacZ* in the presence but not in the absence of S9 (Ono, Somiya & Kawamura, 1991).

No increase in the frequency of binucleated cells with micronuclei was observed when carveol (No. 381) and dihydrocarveol (No. 378) were tested in a

Table 4
Studies of genotoxicity in vitro with carvone and structurally related substances used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
378	Dihydrocarveol	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2*uvr*A	1.5–5 000 µg/plate, ±S9	Negative [a]	Thompson (2016)
378	Dihydrocarveol	Micronucleus induction	Human peripheral blood lymphocytes	First experiment: 96, 192, 288, 384 and 480 (−S9 only), µg/mL, ±S9 Second experiment: 96, 192, 240 and 288 µg/mL, −S9	Negative [b]	Morris (2016)
380	Carvone (isomer not specified)	DNA repair	*S. typhimurium* TA1535 (with plasmid pSK1002/*umuC'-'lacZ*)	1 234.6 µg/mL, ±S9	Positive (+S9) [c]	Ono, Somiya & Kawamura (1991)
380.1	(+)-Carvone	Reverse mutation	*S. typhimurium* TA98 and TA100	6.25, 12.5 and 25 µmol/plate, ±S9	Negative [d]	Stammati et al. (1999)
380.1	(+)-Carvone	SOS chromotest	*E. coli* PQ37	0.025–0.25 µmol	Negative [e]	Stammati et al. (1999)
380.1	(+)-Carvone	DNA repair	*E. coli* WP2 *trpE65* and *E. coli* CM871 *trpE65*, *uvrA155*, *recA56*, *lexA*	25–80 µmol/plate	Positive [f]	Stammati et al. (1999)
380.2	(−)-Carvone	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0.5–5 000 µg/plate, ±S9	Negative [g]	Glover (1987)
381	Carveol	Micronucleus induction	Human peripheral blood lymphocytes	First experiment: 90, 180 and 360 µg/mL, ±S9 Second experiment: 45, 90, 180 and 360 µg/mL, −S9	Negative [h]	Morris (2014)

No.: number; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Two independent experiments using the plate incorporation method and preincubation method, respectively. In all strains, slight toxicity was observed at 5000 µg/plate in the plate incorporation assay and from 500–1500 µg/plate onwards in the preincubation assay (except in strain TA98 in the absence of S9).

[b] In the first experiment, cells were exposed for 4 h, followed by 24-h recovery. In the second experiment, cells were exposed for 24 h, followed by 24-h recovery. Haemolysis was observed from 192 µg/mL onwards in both experiments.

[c] UMU test. In this test, the umu operon is induced by DNA-damaging agents and the intensity of DNA repair is measured by β-galactosidase activity produced from the fused gene.

[d] Plate incorporation method.

[e] This test measures the increased expression of specific stress genes ("SOS genes", specifically *sfiA*) as a consequence of DNA damage or an inhibition of DNA synthesis. *E. coli* strain PQ37 contains the *sfiA* gene, a deletion in the *lac* region with a *uvrA* mutation and an *rfa* mutation that increases the permeability of the cells to chemical agents.

[f] Filter disc assay.

[g] Two independent experiments using the preincubation method were conducted. In the preliminary toxicity assay, a dose range of 0.5–5000 µg/plate was used in all strains. Toxicity was observed at 5000 µg/plate in all strains and from 500 µg/plate onwards in TA1537. In the main assays, concentrations of up to 1000 µg/plate (strains TA135 and TA1537) and 1500 µg/plate (strains TA98 and TA100; toxicity was observed at 1500 µg/plate) were tested in the presence of S9 and concentrations up to 150 µg/plate (strains TA98, TA1535 and TA1537) and 500 µg/plate (TA100) were tested in the absence of S9.

[h] In the first experiment, cells were exposed for 4 hours, followed by 28-h recovery. In the absence of S9, haemolysis was observed at the highest concentrations, and in the presence of S9 at the two highest concentrations. In the second experiment, cells were exposed for 24 h, followed by 28-h recovery. Haemolysis was observed at the highest concentration.

micronucleus assay using cultured human peripheral blood lymphocytes in the absence and presence of S9 metabolic activation. Both assays were performed according to OECD Test Guideline 487 ("In Vitro Mammalian Cell Micronucleus Test", 2010; 2014) and were certified for compliance with QA and GLP (Morris, 2014, 2016).

(ii) Conclusion for genotoxicity

In the previous evaluation of this group of flavourings agents (Annex 1, reference *138*), negative or equivocal results were obtained with carvone (Nos 380.1 and 380.2), carveol (No. 381) and carvyl acetate (No. 382) in in vitro genotoxicity tests including bacterial reverse mutation assays, a rec assay, a sister chromatic exchanges assay and a chromosomal aberration assay. Also, in long-term studies of toxicity and carcinogenicity with (+)-carvone in mice, no evidence for carcinogenicity was observed (Annex 1, reference *94*).

For the present evaluation, additional in vitro genotoxicity data were available on (+)-carvone (No. 380.1), (−)-carvone (No. 380.2), dihydrocarveol (No. 378) and carveol (No. 381). Negative results were obtained in bacterial reverse mutation assays, micronucleus assays and an SOS gene mutation assay. Results from two non-standard in vitro DNA repair assays (one with (+)-carvone and one with carvone [enantiomer not specified]) were positive, but these results were not in line with those of other assays. Altogether, the Committee concluded that there is no concern for genotoxicity of carvone and related substances under the conditions of oral human intake.

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Furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers (Group 8) (addendum)

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1. Evaluation

1.1 Introduction

The Committee re-evaluated 39 flavouring agents belonging to the group of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids

and related esters, sulfides, disulfides and ethers (Nos 1491–1526 and 2103–2105). At three previous meetings, the Committee noted that there were positive in vitro genotoxicity data for several members of this group and there was a paucity of in vivo genotoxicity data to allay concern (Annex 1, references *178*, *190* and *211*). Based on these considerations, the Committee previously concluded that the Procedure for the Safety Evaluation of Flavouring Agents could not be applied to this group.

The concerns with this group arose primarily from the carcinogenicity of furan itself, which is believed to involve a reactive genotoxic metabolite formed by epoxidation and opening of the furan ring. Furan is not a member of this group of flavouring agents, but all the members of the group contain a furan ring with substituents of varying complexity. Four members of this group, namely 2-methylfuran (No. 1487), 2,5-dimethylfuran (No. 1488), 2-ethylfuran (No. 1489) and 2-butylfuran (No. 1490), have short-chain alkyl substituents on the furan ring; although considered at previous meetings, these four members are no longer supported by industry and were not considered in this re-evaluation.

At the present meeting, the Committee considered additional studies of in vitro genotoxicity (for seven flavouring agents: Nos 1495, 1497, 1503, 1504, 1511, 1514 and 1520) and in vivo genotoxicity (for four flavouring agents: Nos 1491, 1497, 1503 and 1511). Additional short-term studies of toxicity for two flavouring agents in this group (Nos 1491 and 1500) were also available.

For the present meeting, newly submitted studies of in vitro genotoxicity (for seven flavouring agents: Nos 1495, 1497, 1503, 1504, 1511, 1514 and 1520) and in vivo genotoxicity (for four flavouring agents: Nos 1491, 1497, 1503 and 1511) were considered by the Committee. New short-term studies of toxicity with two flavouring agents in this group (Nos 1491 and 1500) were also evaluated.

Twenty of these 39 flavouring agents (Nos 1491–1494, 1497, 1499, 1503–1505, 1508–1513, 1520–1522, 2104 and 2105) have been reported to occur as natural components in foods including cheese, chicken, cocoa, coffee, honey, rye bread, spirituous beverages, tomatoes, wheaten bread, wine and other foods (Nijssen, van Ingen-Visscher & Donders, 2017).

The re-evaluations of the 39 flavouring agents in this group were conducted using the revised Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *230*).

1.2 Assessment of dietary exposure

The total annual volumes of production of the 39 flavouring agents belonging to the group of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers is 585

kg in Europe, 798 kg in the USA, 246 kg in Japan and 490 kg in Latin America (LATAM; IOFI, 2017a,b). More than 83%, 70%, 93% and 85% of the annual production volume in Europe, the USA, Japan and LATAM, respectively, is accounted for by 2-furyl methyl ketone (No. 1503).

Dietary exposures were estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, and the higher of the two values for each flavouring agent is reported in Table 1. Estimated daily dietary exposures range from 0.3 to 1200 μg/person (SPET values) and from 0.01 to 61 μg/person (MSDI method values). The estimated daily dietary exposure is highest for 2,5-dimethyl-3-oxo-(2*H*)-fur-4-yl butyrate (No. 1519) at 1200 μg/person, the SPET value for nonalcoholic beverages. The SPET yielded the highest estimated daily dietary exposure in each case with the exception of phenethyl 2-furoate (No. 1517; MSDI = 2 μg/person).

Annual volumes of production of this group of flavouring agents and the daily dietary exposures calculated using both the SPET and the MSDI method are summarized in Table 2.

1.3 Absorption, distribution, metabolism and excretion

Detailed information on the absorption, distribution, metabolism and excretion (ADME) of this group of flavouring agents was described in the monograph of the sixty-ninth meeting of the Committee (Annex 1, reference *191*). At that meeting, the Committee noted that the biotransformation processes applicable to members of this group of furan-substituted flavouring agents are, in large part, dependent on the presence or absence of specific functional groups attached to the furan ring. The Committee also noted that at higher dose levels, low relative molecular mass alkyl furans (e.g. 2-methylfuran) can undergo ring oxidation to yield reactive 2-ene-1,4-dicarbonyl intermediates that can react with protein and DNA. For example, furan has limited metabolic options and is biotransformed via ring oxidation to an enedialdehyde species that is a potent hepatotoxin. The Committee further noted that the presence of an extended side-chain attached to the furan ring would reduce the potential for epoxidation of the double bond and provide a site for detoxication via metabolism and elimination.

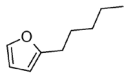
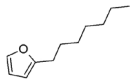
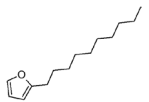
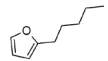
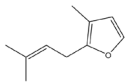
No new data on the ADME of specific members of this group of flavouring agents were available for the present meeting.

1.4 Consideration of genotoxicity data

Additional studies of genotoxicity on furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides,

Table 1

Summary of the results of the safety evaluations of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers used as flavouring agents [a,b,c,d]

Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the higher of the two predicted dietary exposure estimates exceed the threshold of toxicological concern value for the structural class? [e]	*Step 5* DDoes a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Structural relative name (No.) and Structure	Conclusion based on current estimated dietary exposure
Structural class III						
2-Pentylfuran	1491	3777-69-3 	Yes, SPET: 180	Yes. The NOAEL of 30 mg/kg bw per day in a 90-day study in rats (Bauter, 2017) is 10 000 times greater than the estimated dietary exposure to No. 1491 when used as a flavouring agent.	NR	No safety concern
2-Heptylfuran	1492	3777-71-7 	No, SPET: 6	NR	NR	No safety concern
2-Decylfuran	1493	83469-85-6 	Yes, SPET: 300	Yes. The NOAEL of 30 mg/kg bw per day in a 90-day study in rats (Bauter, 2017) for the related substance 2-pentylfuran (No.1491) is 6 000 times greater than the estimated dietary exposure to No. 1493 when used as a flavouring agent.	2-Pentylfuran (No. 1491) 	No safety concern
3-Methyl-2-(3-methylbut-2-enyl)-furan	1494	15186-51-3 	Yes, SPET: 300	Yes. The NOAEL of 45 mg/kg bw per day for the related substance 3-(2-furyl) acrolein (No. 1497) (Lough, 1985) is 9 000 times greater than the dietary exposure to No. 1494 when used as a flavouring agent.	3-(2-Furyl)acrolein (No. 1497) 	No safety concern

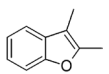
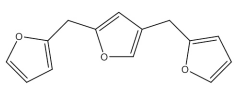
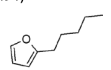
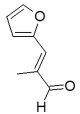
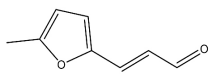
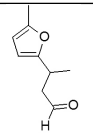
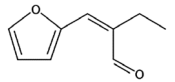
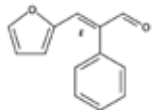
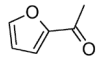
Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the higher of the two predicted dietary exposure estimates exceed the threshold of toxicological concern value for the structural class? [e]	*Step 5* DDoes a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Structural relative name (No.) and Structure	Conclusion based on current estimated dietary exposure
2,3-Dimethylbenzofuran	1495	3782-00-1 	Yes, SPET: 100	Yes. The NOAEL of 0.6 mg/kg bw per day (Long, 1977) is 360 times greater than the estimated dietary exposure to No. 1495 when used as a flavouring agent.	NR	No safety concern
2,4-Difurfurylfuran	1496	64280-32-6 	Yes, SPET: 400	Yes. The NOAEL of 30 mg/kg bw per day (Bauter, 2017) for the related substance 2-pentylfuran (No.1491) is 4 500 times greater than the estimated dietary exposure to No. 1496 when used as a flavouring agent.	2-Pentylfuran (No. 1491) 	No safety concern
3-(2-Furyl)acrolein	1497	623-30-3	Yes, SPET: 500	Yes. The NOAEL of 45 mg/kg bw per day (Lough, 1985) is 5 400 times greater than the dietary exposure to No. 1497 when used as a flavouring agent.	NR	No safety concern
2-Methyl-3(2-furyl)acrolein	1498	874-66-8 	Yes, SPET: 250	Yes. The NOAEL of 45 mg/kg bw per day (Lough, 1985) for the related substance 3-(2-furyl) acrolein (No. 1497) is 10 800 times greater than the dietary exposure to No. 1498 when used as a flavouring agent.	3-(2-Furyl)acrolein (No. 1497) 	No safety concern
3-(5-Methyl-2-furyl)prop-2-enal	1499	5555-90-8 	Yes, SPET: 1 000	Yes. The NOAEL of 45 mg/kg bw per day (Lough, 1985) for the related substance 3-(2-furyl) acrolein (No. 1497) is 2 700 times greater than the estimated dietary exposure to No. 1499 when used as a flavouring agent.	3-(2-Furyl)acrolein (No. 1497) 	No safety concern

Table 1 (continued)

Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the higher of the two predicted dietary exposure estimates exceed the threshold of toxicological concern value for the structural class? [e]	*Step 5* DDoes a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Structural relative name (No.) and Structure	Conclusion based on current estimated dietary exposure
3-(5-Methyl-2-furyl)butanal	1500	31704-80-0 	No, SPET: 50	NR	NR	No safety concern
2-Furfurylidene-butyraldehyde	1501	770-27-4 	Yes, SPET: 250	Yes. The NOAEL of 45 mg/kg bw per day (Lough, 1985) for the related substance 3-(2-furyl) acrolein (No. 1497) is 10 800 times greater than the estimated dietary exposure to No. 1501 when used as a flavouring agent.	3-(2-Furyl)acrolein (No. 1497) 	No safety concern
2-Phenyl-3-(2-furyl)prop-2-enal	1502	65545-81-5 	No, SPET: 40	NR	NR	No safety concern
2-Furyl methyl ketone	1503	1192-62-7 	Yes, SPET: 350	Yes. The NOAEL of 25 mg/kg bw per day (Lough, 1985) is 4 300 times greater than the estimated dietary exposure to No. 1503 when used as a flavouring agent.	NR	No safety concern

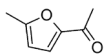
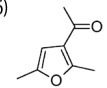
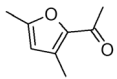
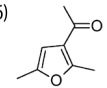
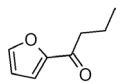
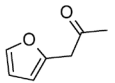
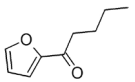
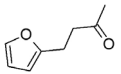
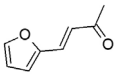
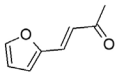
Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the higher of the two predicted dietary exposure estimates exceed the threshold of toxicological concern value for the structural class? [e]	*Step 5* DDoes a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Structural relative name (No.) and Structure	Conclusion based on current estimated dietary exposure
2-Acetyl-5-methylfuran	1504	1193-79-9 	Yes, SPET: 100	Yes. The NOAEL of 10 mg/kg bw per day (Van Miller & Weaver, 1987) for the related substance 3-acetyl-2,5-dimethylfuran (No. 1506) is 6 000 times greater than the estimated dietary exposure to No. 1504 when used as a flavouring agent.	3-Acetyl-2,5-dimethylfuran (No. 1506) 	No safety concern
2-Acetyl-3,5-dimethylfuran	1505	22940-86-9 	Yes, SPET: 1 000	Yes. The NOAEL of 10 mg/kg bw per day (Van Miller & Weaver, 1987) for the related substance 3-acetyl-2,5-dimethylfuran (No. 1506) is 600 times greater than the estimated dietary exposure to No. 1505 when used as a flavouring agent.	3-Acetyl-2,5-dimethylfuran (No. 1506) 	No safety concern
3-Acetyl-2,5-dimethylfuran	1506	10599-70-9 	Yes, SPET: 200	Yes. The NOAEL of 10 mg/kg bw per day (Van Miller & Weaver, 1987) is 3 000 times greater than the estimated dietary exposure to No. 1506 when used as a flavouring agent.	NR	No safety concern
2-Butyrylfuran	1507	100113-53-9 	Yes, SPET: 625	Yes. The NOAEL of 25 mg/kg bw per day (Lough, 1985) for the related substance 2-furyl methyl ketone (No. 1503) is 2 400 times greater than the estimated dietary exposure to No. 1507 when used as a flavouring agent.	2-Furyl methyl ketone (No. 1503) 	No safety concern

Table 1 (continued)

Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the higher of the two predicted dietary exposure estimates exceed the threshold of toxicological concern value for the structural class? [e]	*Step 5* DDoes a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Structural relative name (No.) and Structure	Conclusion based on current estimated dietary exposure
(2-Furyl)-2-propanone	1508	6975-60-6 	Yes, SPET: 150	Yes. The NOAEL of 25 mg/kg bw per day (Lough, 1985) for the related substance 2-furyl methyl ketone (No. 1503) is 10 000 times greater than the estimated dietary exposure to No. 1508 when used as a flavouring agent.	2-Furyl methyl ketone (No. 1503) 	No safety concern
2-Pentanoylfuran	1509	3194-17-0 	Yes, SPET: 1 000	Yes. The NOAEL of 25 mg/kg bw per day (Lough, 1985) for the related substance 2-furyl methyl ketone (No. 1503) is 1 500 times greater than the estimated dietary exposure to No. 1509 when used as a flavouring agent.	2-Furyl methyl ketone (No. 1503) 	No safety concern
1-(2-Furyl)butan-3-one	1510	699-17-2 	Yes, SPET: 400	Yes. The NOAEL of 30 mg/kg bw per day (Gill & Van Miller, 1987) for the related substance 4-(2-furyl)-3-buten-2-one (No. 1511) is 4 500 times greater than the estimated dietary exposure to No. 1510 when used as a flavouring agent.	4-(2-Furyl)-3-buten-2-one (No. 1511) 	No safety concern
4-(2-Furyl)-3-buten-2-one	1511	623-15-4 	Yes, SPET: 699	Yes. The NOAEL of 30 mg/kg bw per day (Gill & Van Miller, 1987) is 2 600 times greater than the estimated dietary exposure to No. 1511 when used as a flavouring agent.	NR	No safety concern

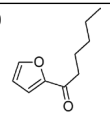
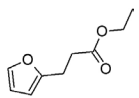
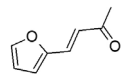
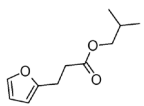
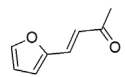
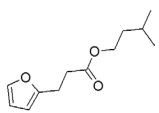
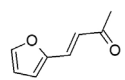
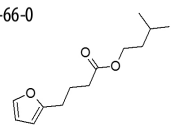
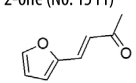
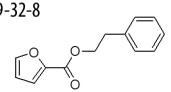
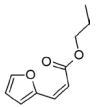
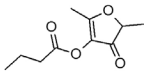
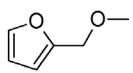
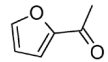
Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the higher of the two predicted dietary exposure estimates exceed the threshold of toxicological concern value for the structural class? [e]	*Step 5* DDoes a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Structural relative name (No.) and Structure	Conclusion based on current estimated dietary exposure
Pentyl 2-furyl ketone	1512	14360-50-0 	No, SPET: 15	NR	NR	No safety concern
Ethyl 3-(2-furyl)propanoate	1513	10031-90-0 	Yes, SPET: 463	Yes. The NOAEL of 30 mg/kg bw per day (Gill & Van Miller, 1987) for the related substance 4-(2-furyl)-3-buten-2-one (No. 1511) is 3 900 times greater than the estimated dietary exposure to No. 1513 when used as a flavouring agent.	4-(2-Furyl)-3-buten-2-one (No. 1511) 	No safety concern
Isobutyl 3-(2-furan)propionate	1514	105-01-1 	Yes, SPET: 400	Yes. The NOAEL of 30 mg/kg bw per day (Gill & Van Miller, 1987) for the related substance 4-(2-furyl)-3-buten-2-one (No. 1511) is 4 500 times greater than the estimated dietary exposure to No. 1514 when used as a flavouring agent.	4-(2-Furyl)-3-buten-2-one (No. 1511) 	No safety concern
Isoamyl 3-(2-furan)propionate	1515	7779-67-1 	Yes, SPET: 400	Yes. The NOAEL of 30 mg/kg bw per day (Gill & Van Miller, 1987) for the related substance 4-(2-furyl)-3-buten-2-one (No. 1511) is 4 500 times greater than the estimated dietary exposure to No. 1515 when used as a flavouring agent.	4-(2-Furyl)-3-buten-2-one (No. 1511) 	No safety concern
Isoamyl 3-(2-furan)butyrate	1516	7779-66-0 	Yes, SPET: 150	Yes. The NOAEL of 30 mg/kg bw per day (Gill & Van Miller, 1987) for the related substance 4-(2-furyl)-3-buten-2-one (No. 1511) is 12 000 times greater than the estimated dietary exposure to No. 1516 when used as a flavouring agent.	4-(2-Furyl)-3-buten-2-one (No. 1511) 	No safety concern

Table 1 (continued)

Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the higher of the two predicted dietary exposure estimates exceed the threshold of toxicological concern value for the structural class? [e]	*Step 5* DDoes a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Structural relative name (No.) and Structure	Conclusion based on current estimated dietary exposure
Phenethyl 2-furoate	1517	7149-32-8 	No, MSDI: 2	NR	NR	No safety concern
Propyl 2-furanacrylate	1518	623-22-3 	No, SPET: 18	NR	NR	No safety concern
2,5-Dimethyl-3-oxo-(2*H*)-fur-4-yl butyrate	1519	114099-96-6 	Yes, SPET: 1 200	Yes. The NOAEL of 200 mg/kg bw per day (Kelly & Bolte, 2003) for the related substance 2,5-dimethyl-4-hydroxyl-3(2*H*)-furanone (No. 1446) is 10 000 times greater than the estimated dietary exposure to No. 1519 when used as a flavouring agent.	2,5-dimethyl-4-hydroxyl-3(2*H*)-furanone (No. 1446) 	No safety concern
Furfuryl methyl ether	1520	13679-46-4 	Yes, SPET: 200	Yes. The NOAEL of 25 mg/kg bw per day (Lough, 1985) for the related substance 2-furyl methyl ketone (No. 1503) is 7 500 times greater than the estimated dietary exposure to No. 1520 when used as a flavouring agent.	2-Furyl methyl ketone (No. 1503) 	No safety concern

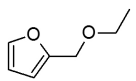
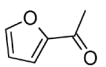
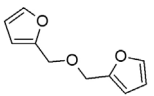
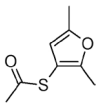
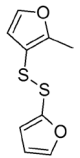
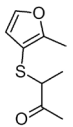
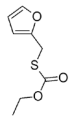
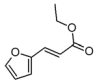
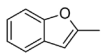
Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the higher of the two predicted dietary exposure estimates exceed the threshold of toxicological concern value for the structural class? [e]	*Step 5* DDoes a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Structural relative name (No.) and Structure	Conclusion based on current estimated dietary exposure
Ethyl furfuryl ether	1521	6270-56-0 	Yes, SPET: 125	Yes. The NOAEL of 25 mg/kg bw per day (Lough, 1985) for the related substance 2-furyl methyl ketone (No. 1503) is 12 000 times greater than the estimated dietary exposure to No. 1521 when used as a flavouring agent.	2-Furyl methyl ketone (No. 1503) 	No safety concern
Difurfuryl ether	1522	4437-22-3 	No, SPET: 6	NR	NR	No safety concern
2,5-Dimethyl-3-furanthiol acetate	1523	55764-22-2 	No, SPET: 2	NR	NR	No safety concern
Furfuryl 2-methyl-3-furyl disulfide	1524	109537-55-5 	No, SPET: 10	NR	NR	No safety concern
3-[(2-Methyl-3-furyl)thio]-2-butanone	1525	61295-44-1 	No, SPET: 0.3	NR	NR	No safety concern

Table 1 (continued)

Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the higher of the two predicted dietary exposure estimates exceed the threshold of toxicological concern value for the structural class? [e]	*Step 5* DDoes a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Structural relative name (No.) and Structure	Conclusion based on current estimated dietary exposure
O-Ethyl *S*-(2-furylmethyl)thiocarbonate	1526	376595-42-5 	No, SPET: 9	NR	NR	No safety concern
(*E*)-Ethyl 3-(2-furyl)acrylate	2103	53282-12-5 	No, SPET: 20	NR	NR	No safety concern
Di-2-furylmethane	2104	1197-40-6 	No, SPET: 40	NR	NR	No safety concern
2-Methylbenzofuran	2105	4265-25-2 	No, SPET: 3	NR	NR	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; MSDI: maximized survey-derived intake; No.: number; NOAEL: no-observed-adverse-effect level; NR: not reported; SPET: single-portion exposure technique

[a] The Committee re-evaluated 39 flavouring agents belonging to the group of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers. All 39 flavouring agents have been considered previously by the Committee.

[b] *Step 1*: Genotoxicity data on the flavouring agents in this group do not raise concerns for genotoxicity.

[c] *Step 2*: All 39 flavouring agents belong to structural class III.

[d] *Step 3*: Dietary exposures were estimated using both the SPET and the MSDI method, and the higher of the two values for each flavouring agent is reported. Dietary exposure values are expressed in µg/day.

[e] *Step 4*: The threshold for human dietary exposure for structural class III is 90 µg/day.

disulfides and ethers were evaluated for the present meeting. A total of 16 in vitro genotoxicity studies were available for seven flavouring agents (Nos 1495, 1497, 1503, 1504, 1511, 1514 and 1520), and a total of eight in vivo genotoxicity studies were available for four flavouring agents (Nos 1491, 1497, 1503 and 1511).

A positive result was observed for 2-furyl methyl ketone (No. 1503) in an in vitro sister chromatid exchange assay. However, an in vitro chromosomal aberration assay was negative and in vivo studies of DNA damage (comet assays) and micronucleus induction were also negative. All other in vitro and in vivo genotoxicity assays considered at the present meeting were negative.

The Committee concluded that the newly available in vitro and in vivo genotoxicity data evaluated at the present meeting allay the previous concerns of the Committee. Those concerns arose primarily from the carcinogenicity of furan itself and from some positive genotoxicity findings for four flavouring agents with short-chain alkyl substituents on the furan ring. Those four flavouring agents, namely 2-methylfuran (No. 1487), 2,5-dimethylfuran (No. 1488), 2-ethylfuran (No. 1489) and 2-butylfuran (No. 1490), are no longer supported by industry, and were not considered in this re-evaluation.

1.5 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. Genotoxicity data on members of this group of 39 flavouring agents do not raise concerns for genotoxicity.

Step 2. In applying the revised Procedure for the Safety Evaluation of Flavouring Agents, the Committee assigned all 39 flavouring agents to structural class III (Cramer, Ford & Hall, 1978).

Step 3. Dietary exposures were estimated using both the MSDI method and the SPET (Table 1).

Step 4. The highest estimated dietary exposures for 14 flavouring agents (Nos 1492, 1500, 1502, 1512, 1517, 1518, 1522–1526, 2103–2105) are below the class III threshold of toxicological concern (i.e. 90 μg/day). The Committee therefore concluded that these 14 flavouring agents would not pose a safety concern at current estimated dietary exposures.

The highest estimated dietary exposures for 25 flavouring agents (Nos 1491, 1493–1499, 1501, 1503–1511, 1513–1516, 1519–1521) are above the class III threshold of toxicological concern. Therefore, these 25 flavouring agents proceeded to Step 5 of the Procedure.

Step 5. For 2-pentylfuran (No. 1491) the no-observed-adverse-effect level (NOAEL) of 30 mg/kg body weight (bw) per day from a 90-day study in rats (Bauter, 2017) provides an adequate margin of exposure (MOE) of 10 000 in

relation to the estimated dietary exposure to No. 1491 when used as a flavouring agent. This NOAEL is also appropriate for assessment of the structurally related flavouring agents 2-decylfuran (No. 1493) and 2,4-difurfurylfuran (No. 1496). This NOAEL provides adequate MOEs of 6000 and 4500 in relation to the estimated dietary exposure to No. 1493 and No. 1496, respectively, when used as flavouring agents.

For 3-(2-furyl)acrolein (No. 1497), the NOAEL of 45 mg/kg bw per day from a 90-day study in rats (Lough, 1985) provides an adequate MOE of 5400 in relation to the estimated dietary exposure to No. 1497 when used as a flavouring agent. This NOAEL is also appropriate for assessment of the structurally related flavouring agents 3-methyl-2-(3-methylbut-2-enyl)-furan (No. 1494), 2-methyl-3(2-furyl)acrolein (No. 1498), 3-(5-methyl-2-furyl)prop-2-enal (No. 1499) and 2-furfurylidene-butyraldehyde (No. 1501) and provides adequate MOEs of 9000, 10 800, 2700 and 10 800 in relation to the estimated dietary exposures to Nos 1494, 1498, 1499 and 1501, respectively, when used as flavouring agents.

For 2,3-dimethylbenzofuran (No. 1495), the NOAEL of 0.6 mg/kg bw per day from a 90-day study in rats (Long, 1977) provides an adequate MOE of 360 in relation to the estimated dietary exposure to No. 1495 when used as a flavouring agent.

For 3-acetyl-2,5-dimethylfuran (No. 1506), the NOAEL of 10 mg/kg bw per day from a 14-day study in rats (Van Miller & Weaver, 1987) provides an adequate MOE of 3000 in relation to the estimated dietary exposure to No. 1506 when used as a flavouring agent. The NOAEL of 10 mg/kg bw per day for No. 1506 is also appropriate for assessment of the structurally related flavouring agents 2-acetyl-5-methylfuran (No. 1504) and 2-acetyl-3,5-dimethylfuran (No. 1505) and provides adequate MOEs of 6000 and 600 in relation to the estimated dietary exposures to 1504 and 1505, respectively, when used as flavouring agents.

For 2-furyl methyl ketone (No. 1503), the NOAEL of 25 mg/kg bw per day obtained from a 90-day study in rats (Lough, 1985) provides an adequate MOE of 4300 in relation to the estimated dietary exposure to No. 1503 when used as a flavouring agent. The NOAEL of 25 mg/kg bw per day for No. 1503 is also appropriate for assessment of the structurally related flavouring agents 2-butyrylfuran (No. 1507), (2-furyl)-2-propanone (No. 1508), 2-pentanoylfuran (No. 1509), furfuryl methyl ether (No. 1520) and ethyl furfuryl ether (No. 1521) and provides adequate MOEs of 2400, 10 000, 1500, 7500 and 12 000, respectively, in relation to the estimated dietary exposures to Nos 1507, 1508, 1509, 1520 and 1521, respectively, when used as flavouring agents.

For 4-(2-furyl)-3-buten-2-one (No. 1511), the NOAEL of 30 mg/kg bw per day from a 14-day study in rats (Gill & Van Miller, 1987) provides an adequate MOE of 2600 in relation to the estimated dietary exposure to No. 1511 when used as a flavouring agent. The NOAEL of 30 mg/kg bw per day for No. 1511

is also appropriate for assessment of the structurally related flavouring agents 1-(2-furyl)butan-3-one (No. 1510), ethyl 3-(2-furyl)propanoate (No. 1513), isobutyl 3-(2-furan)propionate (No. 1514), isoamyl 3-(2-furan)propionate (No. 1515), isoamyl 3-(2-furan)butyrate (No. 1516) provides adequate MOEs of 4500, 3900, 4500, 4500 and 12 000 in relation to the estimated dietary exposures to Nos 1510, 1513, 1514, 1515 and 1516, respectively, when used as flavouring agents.

For 2,5-dimethyl-3-oxo-(2*H*)-fur-4-yl butyrate (No. 1519), the NOAEL of 200 mg/kg bw per day for the structurally related substance 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (No. 1446) obtained from a 2-year study in rats (Kelly & Bolte, 2003) provides an adequate MOE of 10 000 in relation to the estimated dietary exposure to No. 1519 when used as a flavouring agent.

Based on the adequate margins of exposure for each of the 25 flavouring agents considered at Step 5 of the Procedure, the Committee concluded that these 25 flavouring agents (Nos 1491, 1493–1499, 1501, 1503–1511, 1513–1516, 1519–1521) would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the 39 flavouring agents belonging to this group of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers that were considered at the present meeting (Nos 1491–1526 and 2103–2105).

1.6 Consideration of combined intakes from use as flavouring agents

Twenty-five of the flavouring agents in this group have MSDI values of less than 0.1 μg/day, and the four highest MSDI values are 3, 6, 13 and 61 μg/day. The Committee considered that combined intakes of members of this group of flavouring agents do not raise safety concerns.

1.7 Consideration of secondary components

Two flavouring agents in this group (Nos 1519 and 1524) have a minimum assay value of less than 95% (see Annex 3). For No. 1519, the major secondary components are 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (present at 1–3%) and butyric acid (present at 1–3%). The SPET value for No. 1519 is 1200 μg/day and 3% of this value is 36 μg/day, which is below the class III threshold of toxicological concern (90 μg/day). Butyric acid (Joint FAO/WHO Expert Committee on Food Additives [JECFA] flavour No. 87) was evaluated at the forty-ninth meeting, and it was concluded that there were no safety concerns from its use as a flavouring agent (Annex 1, reference *131*). The major secondary components of No. 1519 are therefore not considered to present a safety concern at estimated dietary exposures from the use of No. 1519 as a flavouring agent.

For No. 1524, the major secondary component is di-(2-methyl-3-furyl) disulfide (present at 6–7%). The SPET value for No. 1524 is 10 μg/day and 7% of this value is 0.7 μg/day, which is below the class III threshold of toxicological concern (90 μg/day). The major secondary component of No. 1524 is therefore not considered to present a safety concern at estimated dietary exposures from the use of No. 1524 as a flavouring agent.

1.8 Conclusions

The Committee concluded that the 39 previously evaluated flavouring agents in this group of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers that were re-evaluated at the present meeting (Nos 1491–1529 and 2103–2105) do not give rise to safety concerns. The Committee concluded that the additional genotoxicity data on members of this group allay the concerns that were raised at previous meetings.

2. Relevant background information

2.1 Explanation

This monograph summarizes data relevant to the re-evaluation of 39 flavouring agents (Nos 1491–1526 and 2103–2105) in this group of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers.

2.2 Additional considerations on dietary exposure

Annual volumes of production and daily dietary exposures estimated using both the MSDI method and the SPET for each flavouring agent are reported in Table 2.

2.3 Biological data

2.3.1 Biochemical aspects: ADME

Detailed information on the ADME for this group of flavouring agents was described in the monograph of the sixty-ninth meeting of the Committee (Annex

Table 2

Annual volumes of production and daily dietary exposures of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers used as flavouring agents in Europe, the USA, Japan and LATAM

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Natural occurrence in foods
		MSDI [b]		SPET [c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
2-Pentylfuran (1491)				180	3	+
Europe	30	3	0.04			
USA	15	2	0.03			
Japan	1	0.3	0.004			
LATAM	5	0.3	0.005			
2-Heptylfuran (1492)				6	0.1	+
Europe	0.1	0.01	0.000 1			
USA	ND	ND	ND			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
2-Decylfuran (1493)				300	5	+
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
3-Methyl-2-(3-methylbut-2-enyl)-furan (1494)			300	5		+
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
2,3-Dimethylbenzofuran (1495)			100	2		+
Europe	0.6	0.05	0.000 8			
USA	0.1	0.01	0.000 2			
Japan	0.1	0.03	0.000 4			
LATAM	1	0.06	0.000 9			
2,4-Difurfurylfuran (1496)			400	7		+
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
3-(2-Furyl)acrolein (1497)				500	8	+
Europe	1	0.08	0.001			
USA	0.2	0.02	0.000 3			
Japan	0.1	0.03	0.000 4			
LATAM	0.1	0.01	0.000 1			

Table 2 (continued)

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Natural occurrence in foods
		MSDI [b]		SPET [c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
2-Methyl-3(2-furyl)acrolein (1498)				250	4	–
Europe	0.1	0.01	0.000 1			
USA	ND	ND	ND			
Japan	0.1	0.03	0.000 4			
LATAM	6	0.3	0.006			
3-(5-Methyl-2-furyl)prop-2-enal (1499)			1 000	17		
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
3-(5-Methyl-2-furyl)butanal (1500)			50	1		
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
2-Furfurylidene-butyraldehyde (1501)			250	4		
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
2-Phenyl-3-(2-furyl)prop-2-enal (1502)			40	1		
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
2-Furyl methyl ketone (1503)			350	6	+	
Europe	490	41	0.7			
USA	560	58	1			
Japan	230	61	1			
LATAM	420	23	0.4			
2-Acetyl-5-methylfuran (1504)			100	2		
Europe	5	0.4	0.007			
USA	1	0.1	0.002			
Japan	0.1	0.03	0.000 4			
LATAM	1	0.06	0.000 9			
2-Acetyl-3,5-dimethylfuran (1505)			1 000	17		
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Natural occurrence in foods
		MSDI [b]		SPET [c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
3-Acetyl-2,5-dimethylfuran (1506)			200	3		
Europe	ND	ND	ND			
USA	6	0.6	0.01			
Japan	0.1	0.03	0.000 4			
LATAM	0.2	0.01	0.000 2			
2-Butyrylfuran (1507)				625	10	–
Europe	ND	ND	ND			
USA	0.2	0.02	0.000 3			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
(2-Furyl)-2-propanone (1508)			150	3	+	
Europe	0.6	0.05	0.000 8			
USA	ND	ND	ND			
Japan	1	0.3	0.004			
LATAM	0.1	0.01	0.000 1			
2-Pentanoylfuran (1509)				1 000	17	+
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
1-(2-Furyl)butan-3-one (1510)			400	7		
Europe	ND	ND	ND			
USA	3	0.3	0.005			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
4-(2-Furyl)-3-buten-2-one (1511)			699	12		
Europe	29	2	0.04			
USA	2	0.2	0.003			
Japan	1	0.3	0.004			
LATAM	7	0.4	0.006			
Pentyl 2-furyl ketone (1512)			15	0.3	+	
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	ND	ND	ND			
LATAM	0.1	0.01	0.000 1			
Ethyl 3-(2-furyl)propanoate (1513)			463	8		
Europe	1	0.08	0.001			
USA	ND	ND	ND			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			

Table 2 (continued)

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Natural occurrence in foods
		MSDI [b]		SPET [c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
Isobutyl 3-(2-furan)propionate (1514)			400	7		
Europe	12	1	0.02			
USA	54	6	0.09			
Japan	5	1	0.02			
LATAM	49	3	0.05			
Isoamyl 3-(2-furan)propionate (1515)			400	7		
Europe	ND	ND	ND			
USA	130	13	0.2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
Isoamyl 4-(2-furan)butyrate (1516)			150	3		
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
Phenethyl 2-furoate (1517)			1	0.02	–	
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	6	2	0.03			
LATAM	0.1	0.01	0.000 1			
Propyl 2-furanacrylate (1518)			18	0.3		
Europe	ND	ND	ND			
USA	1	0.1	0.002			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
2,5-Dimethyl-3-oxo-(2*H*)-fur-4-yl butyrate (1519)			1 200	20		
Europe	15	1	0.02			
USA	27	3	0.05			
Japan	ND	ND	ND			
LATAM	0.1	0.01	0.000 1			
Furfuryl methyl ether (1520)			200	3	+	
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.9	0.2	0.004			
LATAM	ND	ND	ND			
Ethyl furfuryl ether (1521)			125	2	+	
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Natural occurrence in foods
		MSDI [b]		SPET [c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
Difurfuryl ether (1522)				6	0.1	+
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.2	0.05	0.000 9			
LATAM	ND	ND	ND			
2,5-Dimethyl-3-furanthiol acetate (1523)			2	0.03		
Europe	0.1	0.01	0.000 1			
USA	ND	ND	ND			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
Furfuryl 2-methyl-3-furyl disulfide (1524)			10	0.2		
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
3-[(2-Methyl-3-furyl)thio]-2-butanone (1525)			0.3	0.01		
Europe	0.1	0.01	0.000 1			
USA	ND	ND	ND			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
***O*-Ethyl *S*-(2-furylmethyl)thiocarbonate (1526)**			9	0.2		
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.1	0.03	0.000 4			
LATAM	ND	ND	ND			
(*E*)-Ethyl 3-(2-furyl)acrylate (2103)			20	0.3		
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
LATAM	ND	ND	ND			
Di-2-furylmethane (2104)				40	0.7	+
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.1	0.03	0.000 4			
LATAM	ND	ND	ND			
2-Methylbenzofuran (2105)			3	0.1	+	
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.1	0.03	0.000 4			
LATAM	ND	ND	ND			

Table 2 (continued)

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Natural occurrence in foods
		MSDI [b]		SPET [c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
Total						
Europe	585					
USA	798					
Japan	246					
LATAM	490					

bw: body weight; LATAM: Latin America; MSDI: maximized survey-derived intake; ND: no data reported; +: reported to occur naturally in foods, but quantitative estimate of intake not available (Nijssen, van Ingen-Visscher & Donders, 2017); No.: number; –: not reported to occur naturally in foods; SPET: single-portion exposure technique; USA: United States of America

[a] From International Organization for the Flavor Industry (2017a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 45×10^6 for Europe, 33×10^6 for the USA, 13×10^6 for Japan, and 62×10^6 for LATAM; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (IOFI, 2017a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (IOFI, 2017b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

1, reference *191*). No new data for members of this group of flavouring agents were available for the present meeting.

2.3.2 Toxicological studies

(a) Short-term studies of toxicity

Results of short-term studies of toxicity with 2-pentylfuran (No. 1491) and 3-(5-methyl-2-furyl) butanal (No. 1500) are summarized in Table 3 and described below.

(i) 2-Pentylfuran (No. 1491)

In a 14-day range-finding study, groups of Sprague Dawley rats (5/sex per group) were provided diets containing 2-pentylfuran (No. 1491, purity 99.8%) at concentrations of 0, 1200, 3000 or 6000 parts per million (ppm). These dietary concentrations correspond to calculated mean intakes of 0, 106, 258 and 477 mg/kg bw per day for males and 0, 112, 263 and 491 mg/kg bw per day for females, respectively). However, analysis of feed gave low levels of recovery of the test substance with up to only 59% measurable on the day of preparation and 29% of the target concentration measurable 10 days after preparation.

Table 3

Results of short-term studies of toxicity with furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups [a] / no. of animals per group [b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
1491	2-Pentylfuran	Rat; M/F	3/10	Diet	14	– [c]	Bauter (2016)
1491	2-Pentylfuran	Rat; M/F	3/20	Gavage	90	30	Bauter (2017)
1500	3-(5-Methyl-2-furyl)butanal	Rat; M/F	3/6	Diet	14	52	Kappeler (2013a)
1500	3-(5-Methyl-2-furyl)butanal	Rat; M/F	3/20	Diet	90	51	Kappeler (2013b)

bw: body weight; F: female; M: male; NA: not applicable; no.: number; NOAEL: no-observed-adverse-effect level

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] Analysis of feed after 10 days indicated that the test article concentrations were as low as 30% of the target concentrations. It was therefore not possible to estimate the achieved doses in this study.

No deaths or clinical signs of toxicity were observed during the study. Reduced feed consumption and body-weight gain were observed for two high-dose males, one mid-dose female and four high-dose females. There were no abnormal findings at necropsy. Because analysis of feed after 10 days indicated that the test article concentrations were as low as 30% of the target concentrations, it was not possible to estimate the achieved doses in this study (Bauter, 2016).

In a 90-day oral toxicity study performed according to Organisation for Economic Co-operation and Development test guideline 408 and United States Food and Drug Administration good laboratory principles, groups of Sprague Dawley rats (10/sex per group) were gavaged with 2-pentylfuran (No. 1491; purity 95.7%) at doses of 0, 30, 100 or 150 mg/kg bw per day. No deaths were attributable to 2-pentylfuran administration. The mid- and high-dose groups exhibited increased incidences of hypersalivation and nasal discharge and decreased body-weight gain. Feed consumption in test and control groups remained comparable. No test article–related effects were evident upon ophthalmological examination. There were no test article–related gross pathology findings.

The following clinical chemistry and haematological parameters were affected in mid- and high-dose males and females: decreased serum glucose and triglycerides; increased total protein, albumin and globulin; decreased haematocrit,

haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration; and increased platelet counts. Mid- and high-dose males showed increased prothrombin times with no effects on other coagulation parameters. At the mid and high dose, increased sorbitol dehydrogenase and/or bilirubin levels correlated with increased liver weights and hepatocellular hypertrophy. A slight increase in bilirubin was observed in low-dose males; however, as no adverse pathology correlate was evident, this finding was considered not adverse. Hepatocellular hypertrophy was observed in mid- and high-dose females and in all treated groups of males; at the low dose, no effects on liver weight and no adverse clinical chemistry or haematology findings were observed.

High-dose males showed increased phosphorous levels, and high-dose females showed increased calcium. Decreased chloride levels were noted in mid- and high-dose females. Urine analysis revealed increased urobilinogen with decreased specific gravity and/or protein concentration for the mid- and high-dose males and in all female treatment groups. The altered urinary parameters in low-dose females were not considered toxicologically relevant because they were within the historical control range for the test laboratory and there were no clinical chemistry or histopathology correlates.

In all treated females and mid- and high-dose males, pigment-laden macrophages were increased in the spleen, which is likely to be associated with alterations in several erythrocyte parameters that were observed at the mid and high dose (described above). This finding was not considered to be adverse because macrophages appeared to be otherwise normal.

Increased relative (to body weight) brain and testes weights were observed in high-dose males. Mid- and high-dose males and females showed increased relative kidney weights, while mid- and high-dose males showed increased relative spleen weights. These relative organ weight differences can be attributed to the lower terminal body weights in these groups.

The NOAEL for 2-pentylfuran was 30 mg/kg bw per day based on treatment-related adverse effects at 100 mg/kg bw per day and higher doses in both sexes (Bauter, 2017).

(ii) 3-(5-Methyl-2-furyl)butanal (No. 1500)

In a 14-day range-finding study, groups of Sprague Dawley rats (3/sex per group) were fed diets designed to deliver 0, 50, 300 or 750 mg/kg bw per day of 3-(5-methyl-2-furyl)butanal (No 1500, purity >99%). The mean achieved doses were calculated to be 0, 52, 303 and 740 mg/kg bw per day for males and 0, 52, 298 and 772 mg/kg bw per day for females, respectively.

There were no deaths or clinical signs of toxicity. Body weight gains were lower in both sexes at 300 and 750 mg/kg bw per day (6.5% and 15% lower for males, respectively, and 8.3% and 6.1% lower for females, respectively), compared with the control groups. Feed consumption in high-dose males and females was lower over days 0–7 than over days 7–14, suggesting an initial lack of palatability for the diet at the highest dietary concentration tested. Feed consumption at the low and mid dose was comparable to controls.

Based on reduced body-weight gain at the mid dose (calculated to be 298 mg/kg bw per day), which was not accompanied by reduced feed consumption, the NOAEL was 52 mg/kg bw per day, the low dose tested (Kappeler, 2013a).

The toxicity of 3-(5-methyl-2-furyl)butanal (No. 1500; purity >99%) was assessed in groups of Sprague Dawley rats (10/sex per group) in a 90-day study at dietary concentrations resulting in achieved doses of 0, 10, 51 or 252 mg/kg bw per day for males and 0, 10, 51 or 251 mg/kg bw per day for females, respectively.

All animals survived to the scheduled necropsy. There were no test item–related effects on body weight or feed consumption. No adverse effects on haematological measures, coagulation or serum chemistry parameters were noted for any group. There were no test item–related clinical observations, ophthalmological or macroscopic findings or effects on urine analysis parameters at any dietary concentration. Higher relative (to final body weight) liver weight was noted for high-dose males than for controls. Minimal to moderate centrilobular hepatocellular vacuolation was also observed in high-dose males.

The NOAEL was 51 mg/kg bw per day based on increased relative liver weight and centrilobular hepatocellular vacuolation in male rats at 252 mg/kg bw per day (Kappeler, 2013b).

(b) Genotoxicity

Studies of genotoxicity on furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers evaluated for the present meeting are summarized in Table 4. A total of 16 studies of in vitro genotoxicity were available for seven flavouring agents (Nos 1495, 1497, 1503, 1504, 1511, 1514 and 1520), and a total of eight studies of in vivo genotoxicity were available for four flavouring agents (Nos 1491, 1497, 1503 and 1511).

A positive result was observed for 2-furyl methyl ketone (No. 1503) in an in vitro assay of sister chromatid exchange (Sujatha, 2008); however, an in vitro chromosomal aberration assay was negative (Sujatha, 2008) and in vivo studies of DNA damage (comet assay) and micronucleus induction were also negative (Beevers, 2016).

All other in vitro and in vivo genotoxicity assays were uniformly negative.

Table 4

Studies of genotoxicity of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration/ dose	Results	Reference
In vitro						
1495	2,3-Dimethylbenzofuran	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2*uvr*A	1.5–5 000 µg/plate [a,b] 0.5–5 000 c µg/plate [a,d]	Negative	Thompson (2016)
1495	2,3-Dimethylbenzofuran	Micronucleus induction	Human peripheral blood lymphocytes	46–184 µg/mL [d] 46–184 µg/mL [e] 23–115 µg/mL [f]	Negative	Morris (2016)
1497	3-(2-Furyl)acrolein	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.32–5 000 µg/plate [a,b] 8–5 000 µg/plate [a,d]	Negative	Lillford (2010)
1497	3-(2-Furyl)acrolein	Micronucleus induction	Human peripheral blood lymphocytes	50, 75, 120, 150 µg/mL [b] 50, 65 and 90 µg/mL [d] 25, 30 and 40 µg/mL [d]	Negative	Whitwell (2010)
1497	3-(2-Furyl)acrolein	Micronucleus induction	Human peripheral blood lymphocytes	55, 70, 95 and 110 µg/mL [d]	Negative	Whitwell (2012)
1503	2-Furyl methyl ketone	Sister chromatid exchange	Human peripheral blood lymphocytes	240, 480 and 720 ppm	Positive	Sujatha (2008)
1503	2-Furyl methyl ketone	Chromosomal aberration	Human peripheral blood lymphocytes	240, 480 and 720 ppm	Negative	Sujatha (2008)
1504	2-Acetyl-5-methylfuran	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	5–5 000 µg/plate [a]	Negative	Bhalli (2014a)
1504	2-Acetyl-5-methylfuran	Micronucleus induction	Human peripheral blood lymphocytes	613, 875 and 1 250 µg/mL [f] 613, 875 and 1 250 µg/mL [e] 613, 875 and 1 250 µg/mL [d]	Negative	Bhalli (2014b)
1511	4-(2-Furyl)-3-buten-2-one	Reverse mutation	*S. typhimurium* TA102	1.6–5 000 µg/plate [a,b] 78–5 000 µg/plate [a,d]	Negative	Kilford (2010)
1511	4-(2-Furyl)-3-buten-2-one	Micronucleus induction	Human peripheral blood lymphocytes	100, 250 and 300 µg/mL [e] 65, 80 and 100 µg/mL [d] 40, 55 and 65 µg/mL [f]	Negative	Lloyd (2009)
1511	4-(2-Furyl)-3-buten-2-one	Micronucleus induction	Human peripheral blood lymphocytes	100– 325 µg/mL [e]	Negative	Lloyd (2011)
1514	Isobutyl 3-(2-furan)propionate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	5–5000 µg/plate [a] 16–5 000 µg/plate [a] 16–5 000 µg/plate [a]	Negative	Bhalli (2015)

No.	Flavouring agent	End-point	Test object	Concentration/ dose	Results	Reference
1514	Isobutyl 3-(2-furan)propionate	Micronucleus induction	Human peripheral blood lymphocytes	175–374 µg/mL [f] 305–438 µg/mL [e] 824–1 017 µg/mL [d]	Negative	Bhalli (2017)
1520	Furfuryl methyl ether	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	5–5 000 µg/plate [a,b] 160–5 000 µg/plate [a,d]	Negative	Higton (2015)
1520	Furfuryl methyl ether	Micronucleus induction	Human peripheral blood lymphocytes	600, 900 and 1 121 µg/mL [e] 50, 125 and 175 µg/mL [d] 400, 700 and 900 µg/mL [f]	Negative	Whitwell (2015)
In vivo						
1491	2-Pentylfuran	Micronucleus induction in bone marrow	Han Wistar rats; M	43, 85 and 170 mg/kg bw per day for 3 days (gavage)	Negative	Keig-Shevlin (2014)
1491	2-Pentylfuran	Comet assay in liver	Han Wistar rats; M	43, 85 and 170 mg/kg bw per day for 3 days (gavage)	Negative	Keig-Shevlin (2014)
1497	3-(2-Furyl)acrolein	Micronucleus induction in bone marrow	Han Wistar rats; M	125, 250 and 500 mg/kg bw per day for 3 days (gavage)	Negative	Beevers (2013a)
1497	3-(2-Furyl)acrolein	Comet assay in liver	Han Wistar rats; M	125, 250 and 500 mg/kg bw per day for 3 days (gavage)	Negative	Beevers (2013a)
1503	2-Furyl methyl ketone	Micronucleus induction in peripheral blood reticulocytes	Muta™ mice; M	15, 30 and 60 mg/kg bw per day for 28 days	Negative	Beevers (2016)
1503	2-Furyl methyl ketone	Mutation frequency at *lacZ* transgene (liver and duodenum)	Muta™ mice; M	15, 30 and 60 mg/kg bw per day for 28 days	Negative	Beevers (2016)
1511	4-(2-Furyl)-3-buten-2-one	Micronucleus induction in bone marrow	Han Wistar rats; M	25, 50 and 100 mg/kg bw per day for 3 days	Negative	Beevers (2013b)
1511	4-(2-Furyl)-3-buten-2-one	Comet assay (liver and duodenum)	Han Wistar rats; M	25, 50 and 100 mg/kg bw per day for 3 days	Negative	Beevers (2013b)

bw: body weight; M: males; ppm: parts per million; S9: 9000 × *g* supernatant fraction from rat liver homogenate (metabolic activation)
[a] All strains/dose levels tested with and without S9.
[b] Plate incorporation method.
[c] Not tested in strains TA1535, TA1537, TA98 or TA100.
[d] Preincubation method.
[e] Three hours of treatment in the presence of S9.
[f] Three hours of treatment in the absence of S9 activation.
[g] Twenty-four hours of treatment in the absence of S9.

(c) Conclusions for genotoxicity

The previous concerns raised by the Committee for this group of furan-substituted flavouring agents arose primarily from the carcinogenicity of furan itself, which is believed to involve a reactive genotoxic metabolite formed by epoxidation and opening of the furan ring. Furan is not a member of this group of flavouring agents, but all the members of the group contain a furan ring with substituents of varying complexity. The Committee previously noted that the presence of an extended side-chain attached to the furan ring would reduce the potential for epoxidation of the double bond and provide a site for detoxication via metabolism and elimination. Four members of this group that were considered at previous meetings, namely 2-methylfuran (No. 1487), 2,5-dimethylfuran (No. 1488), 2-ethylfuran (No. 1489) and 2-butylfuran (No. 1490), have short-chain alkyl substituents on the furan ring, are no longer supported by industry, and were not considered in this re-evaluation.

The negative findings in the in vitro and in vivo genotoxicity assays considered at the present meeting collectively provide a strong weight of evidence for an absence of genotoxic potential for members of this group of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers.

3. References

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Linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters (addendum)

First draft prepared by
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1. Evaluation

1.1 Introduction

The Committee evaluated two additional flavouring agents belonging to the group of linear and branched-chain aliphatic, unsaturated, unconjugated alcohols,

aldehydes, acids and related esters. One of the flavouring agents was a linear unsaturated aldehyde, *trans*-6-octenal (No. 2240) and the other was a branched unsaturated alcohol, 2,6-dimethyl-5-heptenol (No. 2241). The evaluations were conducted using the revised Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *230*).

The Committee evaluated 42 other members of this group of flavouring agents at its fifty-first meeting (Annex 1, reference *137*). For 41 of the 42 substances in this group, the Committee concluded that there were no safety concerns at estimated dietary exposures. The evaluation of the remaining substance, ethyl 2-methyl-3,4-pentadienoate (No. 353), was completed at the sixty-eighth meeting (Annex 1, reference *187*). The Committee evaluated 20 other members of this group of flavouring agents at its sixty-first meeting (Annex 1, reference *166*). The Committee concluded that there were no safety concerns at estimated dietary exposures with respect to all of these 20 flavouring agents. The Committee evaluated nine other members of this group of flavouring agents at its seventy-sixth meeting (Annex 1, reference *211*). The Committee concluded that all nine flavouring agents were of no safety concern at estimated dietary exposures.

Both of the additional flavouring agents (Nos 2240 and 2241) in this group have been reported to occur naturally and can be found in ginger (Nijssen, van Ingen-Visscher & Donders, 2017).

A comprehensive literature search for the additional flavouring agents was conducted in PubMed; no relevant studies were identified.

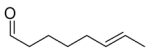
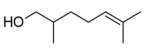
1.2 Assessment of dietary exposure

The total annual volume of production of the two flavouring agents is 0.2 kg (IOFI, 2017a,b). The volume of the annual production in the USA is completely accounted for by *trans*-6-octenal (No. 2240). In Japan, the production volume is completely accounted for by 2,6-dimethyl-5-heptenol (No. 2241).

Dietary exposures were estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, with the highest values reported in Table 1. The higher estimated daily dietary exposure is for 2,6-dimethyl-5-heptenol (No. 2241), at 300 µg/day, the SPET value obtained from non-alcoholic soft beverages. For *trans*-6-octenal (No. 2240), the estimates of daily dietary exposures via the MSDI method and SPET are 0.03 and 40 µg/day, respectively.

Table 1

Summary of the results of the safety evaluations of two additional flavouring agents in the group of linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters [a,b]

Flavouring agent	No.	CAS no. and structure	*Step 4* [c] Does the highest dietary exposure estimate exceed the threshold of toxicological concern?	*Step 5* Does a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Conclusion based on current estimated dietary exposure
Structural class I					
trans-6-Octenal	2240	63196-63-4 	No, SPET: 40	NR	No safety concern
2,6-Dimethyl-5-heptenol	2241	4234-93-9 	No, SPET: 300	NR	No safety concern

CAS: Chemical Abstracts Service; no.: number; NOAEL: no-observed-adverse-effect level; NR: not relevant; SPET: single-portion exposure technique

[a] Seventy-one flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *137*, *166*, *187* and *211*).

[b] *Step 2*: Two flavouring agents (Nos 2240–2241) are in structural class I.

[c] The threshold for human dietary exposure for structural class I is 1800 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure, which was calculated using SPET.

1.3 Absorption, distribution, metabolism and excretion

Information on the absorption, distribution, metabolism and excretion (ADME) of the flavouring agents belonging to the group of linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters has been described in the monographs of the fifty-first, sixty-first, sixty-eighth and seventy-sixth meetings (Annex 1, references *138*, *167*, *188* and *212*).

The aliphatic esters are expected to hydrolyse to their analogous unsaturated aliphatic alcohol and carboxylic acids during passage through the gastrointestinal tract (Gangolli & Shilling, 1968; Junge & Heymann, 1979; Heymann, 1980). The resulting linear and branched-chain unsaturated primary alcohols are expected to be absorbed and further oxidized to their analogous aldehydes and acids, and rapidly absorbed (Dawson, Holdsworth & Webb, 1964; Gaillard & Derache, 1965). The absorbed aldehydes are oxidized to their analogous unsaturated carboxylic acids. These unsaturated carboxylic acids undergo further enzymatic conversion prior to entry into the β-oxidation pathway, where they are later fully metabolized to carbon dioxide and water via the citric acid cycle.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. There are no adequate genotoxicity data on the additional flavouring agents. However, there are no structural alerts for these compounds. Data from related flavouring agents indicate that these do not show genotoxic potential based on uniformly negative genotoxicity data.

Step 2. In applying the Procedure for the Safety Evaluation of Flavouring Agents to *trans*-6-octenal (No. 2240) and 2,6-dimethyl-5-heptenol (No. 2241), the Committee assigned both flavouring agents to structural class I (Cramer, Ford & Hall, 1978).

Steps 3 and 4. Dietary exposures using both the MSDI method and SPET have been determined. The highest estimated dietary exposures of both flavouring agents in structural class I were below the threshold of concern (i.e. 1800 µg/person per day for class I). The Committee therefore concluded that both flavouring agents (Nos 2240–2241) would not pose a safety concern at current estimated dietary exposures.

Annual volumes of production of the two flavouring agents belonging to this group of

flavouring agents (Nos 2240 and 2241) and the daily dietary exposures calculated using both MSDI and SPET methods are summarized in Table 2.

1.5 Consideration of combined intakes from use as flavouring agents

The two additional flavouring agents in this group of linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters have low MSDIs (0.01–0.03 µg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

1.6 Consideration of secondary components

One flavouring agent in this group, 2,6-dimethyl-5-heptenol (No. 2241), has a minimum assay of less than 95% (see Annex 3). The major secondary component, 2,6-dimethyl-5-heptenal (No. 349), present at 1–6%, does not to present a safety concern at estimated dietary exposures from the use of No. 2241 as a flavouring agent. The sum of 2,6-dimethyl-5-heptenol and 2,6-dimethyl-5-heptenal is not less than 95%.

Table 2

Annual volumes of production of linear and branched-chain unsaturated, unconjugated alcohols, aldehydes, acids and related esters used as flavouring agents in Europe, the USA, Japan and Latin America

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Annual volume in naturally occurring foods (kg)[d]	Consumption ratio [e]
		MSDI [b]		SPET [c]			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
***trans*-6-Octenal (2240)**				40	0.7	+	NA
Europe	ND	ND	ND				
USA	ND	ND	ND				
Japan	0.1	0.03	0.000 4				
LATAM	ND	ND	ND				
2,6-Dimethyl-5-heptenol (2241)				300	5	+	NA
Europe	ND	ND	ND				
USA	0.1	0.01	0.000 2				
Japan	ND	ND	ND				
LATAM	ND	ND	ND				
Total							
Europe	ND						
USA	0.1						
Japan	0.1						
LATAM	ND						

bw: body weight; LATAM: Latin America; NA, not available; ND, no intake data reported; No.: number; +, reported to occur naturally in foods (Nijssen , van Ingen-Visscher & Donders, 2017), but no quantitative data.

[a] From the IOFA (2017a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1×10^9 µg/kg)/population × survey correction factor × 365 days), where population (10%, "eaters only") = 45×10^6 for Europe, 33×10^6 for the USA, 13×10^6 for Japan and 62×10^6 for LATAM; and where correction factor = 0.8 for International Organization of the Flavor Industry's Global Poundage Survey and the Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume, respectively, was reported in the poundage surveys (IOFI, 2017a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/ day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/ day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (IOFI, 2017b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/ day)/body weight), where body weight = 60 kg. Slight variations may occur from rounding.

[d] Quantitative data for the United States reported by Stofberg & Grundschober (1987).

[e] The consumption ratio is calculated as follows:
(annual consumption via food, kg)/(most recent reported volume as a flavouring substance, kg)

1.7 Consideration of additional data on previously evaluated flavouring agents

In the previous evaluation of substances in this group of linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters, studies of biochemistry, acute toxicity, short-term and long-term toxicity and genotoxicity were available (Annex 1, references *137*, *166*, *187* and *211*). None of the 71 flavouring agents in this group raised safety concerns.

No adequate studies on the additional flavouring agents were available. For previously evaluated flavouring agents in this group, studies of acute toxicity (Nos 336, 1269, 1286 and 1640), studies of short-term toxicity (Nos 330, 332 and 333) and studies of genotoxicity (Nos 315, 329, 330, 333, 334, 346, 349, 1272, 1286 and 1637) were available. The studies available for the present evaluation support the conclusions drawn by previous safety evaluations.

1.8 Conclusions

The Committee concluded that these two flavouring agents *trans*-6-octenal (No. 2240) and 2,6-dimethyl-5-heptenol (No. 2241), which are additions to the group of linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of two flavouring agents *trans*-6-octenal (No. 2240) and 2,6-dimethyl-5-heptenol (No. 2241), which are additions to the group of linear and branch-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters evaluated previously.

2.2 Additional considerations on exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using SPET for each flavouring agent are reported in Table 2.

2.3 Biological data

2.3.1 Biochemical aspects: ADME

No additional information related to the ADME of these flavouring agents has been reported since the submission of the most recent monograph (Annex 1, references *138*, *167*, *188* and *212*).

2.3.2 Toxicological studies

Information related to the short-term and long-term toxicity and genotoxicity of these flavouring agents has been reported since the submission of the most recent monograph (Annex 1, references *138*, *167*, *188* and *212*).

(a) Acute toxicity

Oral median lethal dose (LD_{50}) values have been reported for four previously evaluated flavouring agents in this group. These are summarized in Table 3. In acute oral toxicity studies in rats, LD_{50} ranges were determined to be >2000–5000 mg/kg body weight (bw). In addition, one acute oral toxicity study in mice gave an LD_{50} of 9500 mg/kg bw for 9-decenal (No. 1286) (Johnson, 1979; Sauer, 1979; Collier & Wilson, 1983; Arcelin, 2000; Sanders, 2002).

(b) Short-term studies of toxicity

Results of a short-term study of toxicity with 10-undecenal (No. 330) are summarized in Table 4. The study details are summarized below.

(i) 10-Undecenal (No. 330)

Rats

In a 90-day dietary toxicity study that was compliant with Organisation for Economic Co-operation and Development testing guideline (OECD TG) 408 and with good laboratory practice (GLP), groups of Sprague Dawley Crl:CD (SD) IGS BR rats (10/sex per group) were administered 10-undecenal (No. 330; lot no. VE00152883, purity 98.2%) at dietary concentrations of 200, 2000, 6000 or 20 000 mg/kg feed (equal to 14.3, 138.6, 382.3 and 1135.9 mg/kg bw per day, respectively). Body weight and clinical observations were made weekly throughout the exposure period. Feed consumption was recorded daily for each cage, and water consumption was assessed by visual inspection for large changes. Animals were observed daily for behavioural changes and signs of toxicity. Blood and urine samples were taken from all animals and analysed during week 7 and again after the end of the exposure period. In weeks 6 and 7, and again in weeks 12 and 13, an estrous cycle assessment was performed on all females. Ophthalmoscopic examinations were performed on the control

Table 3
Results of oral acute toxicity studies with linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
336	*cis*-3-Hexenyl *cis*-3-hexenoate	Rats; M, F	> 5 000	Sauer (1979)
336	*cis*-3-Hexenyl *cis*-3-hexenoate	Rats; M, F	> 2 000	Arcelin (2000)
1269	Isoprenyl acetate	Rats; M, F	> 2 000	Collier & Wilson (1983)
1286	9-Decenal	Mice; M, F	> 9 500[a]	Johnson (1979)
1640	(*Z*)-8-Tetradecenal	Rats; M, F	> 2 000	Sanders (2002)

bw: body weight; F: female; LD_{50}: median lethal dose; M: male; NR: not reported
[a] Calculated using a specific gravity of 0.95 g/mL.

Table 4
Results of short-term studies of toxicity with linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups/no. per group	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
330	10-Undecenal	Rats, M, F	3/5	Gavage	28	1 000	ECHA (2017a)[a,b]
330	10-Undecenal	Rats; M, F	4/10	Diet	90	14.3[c]	Liwska & Watson (2012)

bw: body weight; F: female; M: male; NA: not applicable, no.: number; NOAEL: no-observed-adverse-effect level; NOEL: no-observed -effect level
[a] Study summary only; the Committee could not confirm the NOAEL.
[b] Reported to be compliant with OECD TG 407; doses were 250, 500 and 1000 mg/kg bw per day
[c] No effect level as assessed by the study authors. The Committee did not determine the NOAEL.

and highest-exposure groups prior to day 1 of the exposure period and again during week 12. In week 12, all animals underwent functional performance tests, including forelimb/hindlimb grip strength and motor activity. Sensory response evaluation and detailed clinical observations were also performed on all animals during this week. At the end of the study, all animals were killed and necropsied; males underwent a sperm analysis. Organs were weighed and histopathological examinations of all animals performed.

Feed consumption was reduced in a concentration-related manner in both sexes in all but the low-exposure group. Males and females in the 6000 and 20 000 mg/kg feed groups and males in the 2000 mg/kg feed group had a concentration-related reduction in body weight throughout the study. Body-weight gain was reduced in weeks 1 and 2 for males in the 6000 mg/kg feed group; in weeks 1, 3 and 4 for females in the 20 000 mg/kg feed group; and during weeks 1 through 6, 8 and 13 for males in the 20 000 mg/kg feed group. Males treated with 2000, 6000 and 20 000 mg/kg feed had reduced absolute (21–30%) and

relative-to-body-weight (7–21%) adrenal weights. Males treated with 20 000 mg/kg feed had reduced absolute (39%) and relative-to-body-weight prostate weights (17%) as well as absolute (63%) and relative-to-body-weight (87%) seminal vesicle weights. Males treated with 6000 mg/kg feed had reduced absolute (13%) and relative-to-body-weight seminal vesicle weights (1%). Epididymides weights were increased on an absolute basis and decreased relative to body weight for males treated with 6000 and 20 000 mg/kg feed. Females in the 20 000 mg/kg feed group had reduced pituitary weights and uterine and cervix weights on an absolute and relative-to-body-weight basis (33% and 20%, 26% and 11%, respectively). Females in all dose groups had reduced heart weights (2–22%, absolute; 2–8%, relative to body weight). Epithelial acanthosis of the limiting ridge was observed in the stomachs of animals in the 2000 and 20 000 mg/kg feed groups and females in the 6000 mg/kg feed group. The study authors associated this effect with the route of administration and did not consider it a sign of systemic toxicity but due to the irritating nature of aldehydes on mucosal tissues. Minimal centrilobular hepatocellular hypertrophy was noted in males in all groups except the low-dose group. The study authors considered this to be an adaptive effect as it was not accompanied by degenerative or inflammatory changes. In both sexes in the 2000, 6000 and 20 000 mg/kg feed groups, blood chemistry analysis revealed a significant increase in alkaline phosphatase levels in weeks 7 and 13. Females in the 6000 and 20 000 mg/kg feed groups had lower total protein and albumin levels in weeks 7 and 13. Males in the 2000, 6000 and 20 000 mg/kg feed groups showed an increased albumin count and albumin/globulin ratio in week 7 and week 13, while males in the 200 mg/kg feed group only showed an increased albumin count in week 13. At week 13, cholesterol concentration was lower in females at 20 000 mg/kg feed and urea values were increased in both sexes at 20 000 mg/kg feed group.

Because treatment-related effects were noted in animals of both sexes at doses of 2000 mg/kg feed and above, a no-observed-effect level (NOEL) of 200 mg/kg feed (equivalent to 14.3 mg/kg bw per day) was identified (Liwska & Watson, 2012).

(c) Genotoxicity studies

Studies of genotoxicity in vitro and in vivo reported for linear and branch-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters are summarized in Table 5 and described below.

(i) In vitro

The results of one bacterial reverse mutation assay was reported for the additional flavouring agent *trans*-6-octenal (No. 2240). The study was neither GLP nor

Table 5

Studies of genotoxicity of linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters (addendum) used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration/ dose	Results	Reference
In vitro						
315	3-hexen-1-ol	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2*uvr*A	5–5 000 µg/plate [a,b]	Negative [c]	Bhalli (2014a)
315	3-hexen-1-ol	Micronucleus induction	Human peripheral blood lymphocytes	361–1 002 µg/mL [c,d] 641–1 002 µg/mL[e,f]	Negative	Bhalli (2014b)
329 & 330	Mixture of (9E)-9-undecenal, (9Z)-9-undecenal, & 10-undecenal[g]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2*uvr*A	1.7–5 000 µg/plate [h,i] 0.5–512 µg/plate [j] 9–164 µg/plate [k] 92–878 µg/plate [l] 492–5000 µg/plate [a]	Negative [a,c]	Verspeek-Rip (2014)
330	10-Undecanal	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	3–5 000 µg/plate 0.3–1 000 µg/plate [m]	Negative [a,c,n]	Sokolowski (2007)
330	10-Undecanal	Forward mutation	Chinese hamster ovary cells	84–840	Negative [o]	ECHA (2017b)
333	Oleic acid	Micronucleus induction	Human peripheral blood lymphocytes	20–240 µg/mL [c,p] 20–160 µg/mL [e,q]	Positive [r]	Morris (2014)
334	Methyl 1-hexenoate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2*uvr*A	1.5–5 000 µg/plate 5–5 000 µg/plate	Negative [a,c,s]	Dakoulas (2016a)
334	Methyl 1-hexenoate	Micronucleus induction	Human peripheral blood lymphocytes t	100–350 µg/mL[e,u] 100–700 µg/mL[t,u,v] 100–375 µg/mL[e,f]	Negative	Roy (2016a)
346	Methyl linoleate & methyl linolenate (mixture) w	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2*uvr*A	1.5–5 000 µg/plate 15–5 000 µg/plate	Negative [a,c]	Dakoulas (2016b)

No.	Flavouring agent	End-point	Test object	Concentration/ dose	Results	Reference
346	Methyl linoleate & methyl linolenate (mixture)	Micronucleus induction	Human peripheral blood lymphocytes	20–150 µg/mL [e,u] 50–100 µg/mL [u,v] 20–150 µg/mL [e,f]	Negative [x]	Roy (2016b)
349	2,6-Dimethyl-5-heptanal	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2*uvr*A	3–5 000 µg/plate [a,n]	Negative [c]	Sokolowski (2006)
1272	*cis*-3-Hexenyl formate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2*uvr*A	1.5–5 000 µg/plate [a,c,y] 15–5 000 µg/plate [n,c]	Negative	Thompson (2016)
1272	*cis*-3-Hexenyl formate	Micronucleus induction	Human peripheral blood lymphocytes	320–960 µg/mL [e,z] 640–1 280 µg/mL [v,z] 640–1 280 µg/mL [e,f]	Negative	Bowles (2016)
1286	9-Decenal	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0.000 001–0.001 µL/plate 0.000 000 5–0.000 002 µL/plate	–[a,aa]	Richold & Jones (1980)
1637	*cis*-9-octadecenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2*uvr*A	5–5 000 µg/plate 16–5 000 µg/plate	Negative [a,c]	Bhalli (2017a)
1637	*cis*-9-octadecenol	Micronucleus induction	Human peripheral blood lymphocytes	52.3–109 [e,bb] 142–197 [v,bb] 24.2–55 [e,f]	Equivocal [cc]	Bhalli (2017b)
2240	*trans*-6-octenal	Reverse mutation	*S. typhimurium* TA98, TA100	0.1–100 µg/plate	–[c,n,dd]	Kawaguchi (2012)
In vivo						
330	10-undecenal	Micronucleus induction	Mouse bone marrow PCE	500,1 000, 2 000 mg/kg bw [ee]	Negative [ff]	Honarvar (2007)

bw: body weight; F: female; M: male; NA: not applicable; PCE: polychromatic erythrocytes; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Plate incorporation method.

[b] Result was confirmed in a second assay.

[c] In the presence and absence of rat S9.

[d] 3-hour incubation with test article, 21-hour incubation without test article.

[e] In the absence of S9.

[f] 24-hour incubation with test article.

[g] Sum of 3 isomers was 91.9% purity.

[h] Dose range finding using all strains.

[i] *E. coli* WP2*uvr*A.

[j] Strains TA1535, TA1537 and TA98.

Table 5 (continued)

[k] Strains TA1535, TA1537, TA98 and TA100.
[l] Strains TA1535 and TA98.
[m] Strain TA98.
[n] Preincubation method.
[o] Study was only a summary. Independent evaluation was not possible.
[p] 4-hour incubation with test article followed by 28-hour incubation without test article.
[q] 24-hour incubation with test article followed by 28-hour incubation without test article.
[r] The study authors concluded the assay was negative. The 4-hour exposures with and without metabolic activation were negative. The 24-hour exposure was statistically significantly positive ($P < 0.01$) at the highest dose scored (100 µg/mL), and was above the historical control range for control samples at this laboratory.
[s] In the first assay, an increase of 1.9-fold was observed in the TA98 strain in the absence of S9. In the second assay, an increase of 1.7-fold was observed in the TA98 strain. The response was still judged to be negative.
[t] Only 1000 cells per dose were evaluated.
[u] 4-hour Incubation with test article followed by 20-hour incubation without test article.
[v] In the presence of rat S9.
[w] 48% methyl linoleate, 52% methyl linolenate.
[x] Statistically significant induction was seen in the 4-hour exposure group without S9 at the lowest exposure (20 µg/mL). There was no dose–response relationship and response was within the historical control. The conclusion was that the assay did not show micronucleus induction.
[y] Statistically significant change was seen in WP2*uvr*A strain with metabolic activation. The change was not considered to be an indication of genotoxicity because it was not dose responsive, the fold change was 1.5-times and it was not repeated in the preincubation experiment.
[z] 4-hour incubation with test article followed by 24-hour incubation without test article.
[aa] The authors reported the results as negative, but the Committee judged the study to be inconclusive because the report did not give an adequate description of the procedure used, the concentration spacing was 10-fold between doses, there was no measure of purity of the test article and the exposure was expressed as volume.
[bb] 3-hour incubation with test article followed by 24-hour incubation without test article.
[cc] Statistically significant increase in micronucleated binucleated cells was observed in the 37.8 µg/mL (0.75%) and 55 µg/mL (0.85%) exposures in the 24-hour treatment without S9 and in the 109 µg/mL (1.10%) exposure in the 3-hour treatment without S9. The authors concluded that these tests were negative because there were no concentration-related increases and the increases were within historical vehicle controls for female donors. The Committee judged the study to be equivocal.
[dd] The study authors reported the study to be negative. Only two strains were evaluated in the study, and the highest dose tested was too low. In addition, the positive control for with metabolic activation, 2-aminoanthracene, only induced a 1.5-fold increase in colonies in TA100 strain. The study was neither GLP nor OECD compliant. Historical control data were not provided.
[ee] Administered by oral gavage.
[ff] No evidence was provided that the test article reaches the bone marrow after ingestion.

OECD-guideline compliant and was only done using two of the recommended five strains for bacterial reverse mutation assays (*Salmonella typhimurium* TA98, TA100). In addition, the positive control, 2-aminoanthracene, only induced a 1.5-fold increase in colonies in the TA100 strain (Kawaguchi, 2012). The Committee evaluated the assay as inconclusive because it was inadequate for assessing genotoxicity.

Reports for bacterial reverse mutation assays were provided for previously evaluated flavouring agents in this group, Nos 315, 329, 330, 334, 346, 349, 1272, 1286 and 1637. The results of these assays were negative (Sokolowski, 2006, 2007; Bhalli, 2014a, 2017a; Verspeek-Rip, 2014; Dakoulas, 2016a,b; Thompson, 2016) except for the assay on 9-decenal (Richold & Jones, 1980). The assay on 9-decenal was judged to be inconclusive because the report inadequately described the procedure used, the between-dose concentration spacing was 10-fold, the report contained no measure of purity of the test article and the exposure was expressed as volume, which did not allow determination of the administered dose.

Reports with negative results for in vitro micronucleus induction assays were provided for previously evaluated flavouring agents in this group, Nos 315, 334, 346 and 1272 (Bhalli, 2014b; Bowles, 2016; Roy, 2016a,b). In addition to those reports, a report on an in vitro micronucleus assay on oleic acid (No. 333) was provided in which the authors concluded the results were negative (Morris, 2014). In this assay, no micronucleus induction was detected in the 4-hour exposures with or without metabolic activation. The number of micronuclei at 24-hour exposure was statistically significantly increased over the control ($P < 0.01$) at the highest dose scored (100 µg/mL) and was above the historical control range for control samples at this laboratory. The report authors discounted this result because it was within the historical control range noted in the OECD guidelines. However, the Committee assessed the results of this assay as positive. The Committee previously reviewed oleic acid at the fifty-first meeting and confirmed an acceptable daily intake (ADI) of not specified established at the twenty-ninth meeting (Annex 1, references *70* and *138*). No change was made to this ADI as a result of the new data.

Lastly, a report on an in vitro micronucleus induction assay on *cis*-9-octadecenol (No. 1637) was provided. Statistically significant increases in micronucleated binucleated cells were observed at the 37.8 µg/mL (0.75%) and 55 µg/mL (0.85%) exposures in the 24-hour treatment but not at the 47.2 µg/mL exposure (0.50%) without S9, or at the 109 µg/mL (1.10%) exposure in the 3-hour treatment without S9. The authors concluded that these tests were negative because there were no concentration-related increases and the increases were within historical vehicle controls for female donors (Bhalli, 2017b). The Committee judged the study to be equivocal.

One forward mutation assay on 10-undecanal (No. 330) was reported. While the results were reported as negative, the Committee was unable to evaluate the results because only a summary was provided (ECHA, 2017b, summary only).

(ii) In vivo

One in vivo micronucleus induction assay using mouse bone marrow polychromatic erythrocytes was reported on 10-undecenal (No. 330). The results were negative; however, the authors did not demonstrate that the test article had reached the bone marrow (Honarvar, 2007).

(d) **Conclusions for genotoxicity**

As no data was available on the new flavouring compounds, previously evaluated compounds with sufficiently similar structures and genotoxicity data were identified for read across. *cis*-3-Hexanol (No. 315) was identified as structurally similar to *trans*-6-octenal, and 2,6-dimethylheptanal (No. 349) was identified as structurally similar to 2,6-dimethyl-5-heptanol. The Committee's evaluation of the genotoxicity data on *cis*-3-hexanol (No. 315) and 2,6-dimethylheptanal (No. 349) at the fifty-first meeting identified no genotoxic potential (Annex 1, reference *137*).

The additional data presented in the current addendum to the existing monograph for linear and branch-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters in a weight-of-evidence approach is consistent with the evaluated flavourings in this group not presenting a risk of genotoxicity.

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Maltol and related substances (addendum)

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1. Evaluation

1.1 Introduction

The Committee evaluated two flavouring agents belonging to the group of maltol and related substances: one additional flavouring agent, ethyl maltol isobutyrate (No. 2252), and one flavouring agent, maltol (No. 1480), that was being re-evaluated. The evaluations were conducted according to the Procedure for the

Safety Evaluation of Flavouring Agents (Annex 1, reference *230*). Ethyl maltol isobutyrate (No. 2252) has not been previously evaluated by the Committee. Maltol (No. 1480) was re-evaluated because new in vitro and in vivo genotoxicity data had become available.

The Committee previously evaluated seven members of this group of flavouring agents at its sixty-fifth meeting (Annex 1, reference *178*). The Committee concluded that all seven flavouring agents were of no safety concern at estimated dietary exposures and maintained the previously established acceptable daily intakes (ADIs) of 0–1 mg/kg body weight (bw) for maltol (No. 1480) and 0–2 mg/kg bw for ethyl maltol.

Maltol (No. 1480) was evaluated at the eleventh meeting (Annex 1, reference *14*), when a temporary ADI of 0–1 mg/kg bw was established because no long-term studies of toxicity were available. At the eighteenth meeting (Annex 1, reference *35*), the Committee withdrew the temporary ADI because the results of long-term studies of toxicity requested at the previous meeting had not been made available. At the twenty-second meeting (Annex 1, reference *47*), the Committee evaluated new data on toxicity and established a temporary ADI of 0–0.5 mg/kg bw. At its twenty-fifth meeting, the Committee evaluated additional data and established an ADI of 0–1 mg/kg bw for maltol on the basis of a no-observed-effect level (NOEL) of 100 mg/kg bw in rats (Annex 1, reference *56*).

Ethyl maltol (No. 1481) was evaluated at the fourteenth meeting (Annex 1, reference *22*), when the Committee established an ADI of 0–2 mg/kg bw. At its eighteenth meeting (Annex 1, reference *35*), the Committee re-evaluated ethyl maltol and re-affirmed the ADI of 0–2 mg/kg bw.

Maltol (No. 1480) has been reported to occur naturally in a wide variety of foods such as wheat and rye breads, milk, butter, uncured pork, beer, cocoa, coffee, peanuts, soy proteins, beans and clams (Nijssen, van Ingen-Visscher & Donders, 2017). During baking (for example, of bread or beans) and roasting (for example, of cocoa, coffee, peanuts), simple sugars are partly converted to maltol (Belitz, Grosch & Schieberle, 2009; Nijssen, van Ingen-Visscher & Donders, 2017).

A literature search was performed in Scopus using the names and Chemical Abstracts Service (CAS) numbers of the flavouring agents under evaluation in this group of flavouring agents; two additional relevant studies were identified.

1.2 Assessment of dietary exposure

The total annual volume of production of the additional flavouring agent (ethyl maltol isobutyrate, No. 2252) is 29 kg in Japan (IOFI, 2017a,b). No production volume was reported for Europe, Latin America (LATAM) or the USA.

The total annual volume of production for the flavouring agent presented for re-evaluation, maltol (No. 1480), is approximately 83 200 kg in Europe, 87 600 kg in the USA, 12 500 kg in Japan and 47 600 kg in LATAM (IOFI, 2017a,b).

Dietary exposure was estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, with the highest values reported in Table 1. The estimated daily dietary exposure is highest for maltol (10 440 µg/day, SPET value for nonalcoholic "soft" beverages). The MSDI values for the four regions range from 2625 to 9091 µg/day. For ethyl maltol isobutyrate (No. 2252), the estimated daily dietary exposures were 8 µg/day (MSDI value) and 400 µg/day (SPET value).

1.3 Absorption, distribution, metabolism and excretion (ADME)

Information on the ADME of flavouring agents belonging to the group of maltol and related substances is described in the monograph of the sixty-fifth meeting (Annex 1, reference *179*).

Chemically, maltol is classified as a γ -pyrone. It is a hydroxyl-substituted 4*H*-pyran-4-one and is anticipated to be metabolized like phenol, which primarily undergoes phase II conjugation of the free hydroxyl substituent. Maltol and ethyl maltol are predominantly metabolized to sulfate and glucuronic acid conjugates, which are then eliminated in the urine (Rennhard, 1971). Ethyl maltol isobutyrate (No. 2252) is predicted to be hydrolysed to ethyl maltol and the corresponding simple aliphatic carboxylic acid (isobutyric acid) (Bennett, 1998).

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

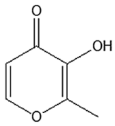
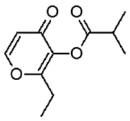
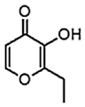
Step 1. There are no structural alerts for genotoxicity for maltol (No. 1480) or ethyl maltol isobutyrate (No. 2252). Chemical-specific genotoxicity data available for maltol (No. 1480) do not indicate that this flavouring agent has genotoxic potential.

Step 2. In applying the Procedure for the Safety Evaluation of Flavouring Agents, the Committee assigned one flavouring agent (No. 1480) to structural class II and one flavouring agent (No. 2252) to structural class III (Cramer, Ford & Hall, 1978).

Step 3. The highest dietary exposures were estimated using the SPET for both flavouring agents.

Step 4. The highest estimated dietary exposure for maltol (No. 1480) is above the threshold of toxicological concern (i.e. 540 µg/day for class II).

Table 1
Summary of the results of the safety evaluations of maltol and related substances used as flavouring agents [a,b]

Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the highest dietary exposure estimate exceed the threshold of toxicological concern?	*Step 5* [d] Does a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Related structure name (No.) and structure	Conclusion based on current estimated dietary exposure
Structural class II						
Maltol [e]	1480	118-71-8 	Yes, SPET: 10 440	Yes. The NOAEL of 125 mg/kg bw per day from a 90-day study in dogs (Gralla et al., 1969) is 720 times the estimated dietary exposure to No. 1480 when used as a flavouring agent.		No safety concern
Structural class II						
Ethyl maltol isobutyrate	2252	852997-28-5 	Yes, SPET: 400	Yes. The NOEL of 200 mg/kg bw per day in a 2-year dietary study in rats for the structurally related substance ethyl maltol (No. 1481) (Gralla et al., 1969) is 28 570 times the estimated dietary exposure to No. 2252 when used as a flavouring agent.	Ethyl maltol [f] (No. 1481) 	No safety concern

ADI: acceptable daily intake; bw: body weight; CAS: Chemical Abstracts Service; no.: number; NOAEL: no-observed-adverse-effect level; NOEL: no-observed-effect level; NR: not relevant; SPET: single-portion exposure technique

[a] Seven flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *178*).

[b] *Step 2*: One flavouring agent is in structural class II and one flavouring agent is in structural class III.

[c] The threshold of toxicological concern for human dietary exposure for structural class II is 540 µg/day and for structural class III is 90 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest estimated daily dietary exposures calculated using the SPET.

[d] The margin of exposure was calculated based on the highest estimated daily dietary exposure calculated using the SPET.

[e] The previously established ADI for maltol was withdrawn by the Committee.

[f] The previously established ADI for ethyl maltol of 0–2 mg/kg bw was maintained.

Accordingly, the evaluation of this flavouring agent proceeded to Step 5 of the Procedure.

The highest estimated dietary exposure for ethyl maltol isobutyrate (No. 2252) is above the threshold of toxicological concern (i.e. 90 µg/day for class III). Accordingly, the evaluation of this flavouring agent proceeded to Step 5 of the Procedure.

Step 5. For maltol, the no-observed-adverse-effect level (NOAEL) of 125 mg/kg bw per day from a 90-day study in dogs (Gralla et al., 1969) provides a margin of exposure of 720 in relation to the estimated daily dietary exposure to No. 1480 (SPET = 10 440 µg/day or 174 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that maltol (No. 1480) would not pose a safety concern at current estimated dietary exposures.

At its fourteenth meeting (Annex 1, reference *22*), the Committee established an ADI of 0–2 mg/kg bw for the structurally related substance ethyl maltol (No. 1481) on the basis of a NOEL of 200 mg/kg bw per day in a 2-year dietary study in rats (Gralla et al., 1969). This ADI was maintained at the sixty-fifth meeting (Annex 1, reference *178*). The NOEL of 200 mg/kg bw per day provides an adequate margin of exposure of 28 570 in relation to estimated daily dietary exposure to No. 2252 (SPET = 400 µg/day or 7 µg/kg bw per day) when used as a flavouring agent. The Committee concluded that ethyl maltol isobutyrate (No. 2252) would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the two flavouring agents (Nos 1480 and 2252) belonging to this group of maltol and related substances.

1.5 Consideration of combined intakes from use as flavouring agents

The additional flavouring agent, ethyl maltol isobutyrate (No. 2252), in the group of maltol and related substances has a low MSDI (8 µg/day). The Committee concluded that consideration of combined intakes is not necessary because the additional flavouring agent would not contribute significantly to the combined intake of this flavouring group.

1.6 Consideration of secondary components

Ethyl maltol isobutyrate (No. 2252) has a minimum assay of less than 95% (see Annex 1, reference *238*). The major secondary component in No. 2252, present at 2–3%, is ethyl maltol (No. 1481). This flavouring agent had been previously evaluated by the Committee (Annex 1, reference *178)* and did not pose a safety concern at the estimated dietary exposure.

1.7 Consideration of additional data on previously evaluated flavouring agents

For two previously evaluated flavouring agents in this group, maltol (No. 1480) and maltyl isobutyrate (No. 1482), additional studies of acute toxicity (No. 1482)

and genotoxicity (Nos 1480 and 1482) were available for the present evaluation. These additional data raised no safety concerns and support the previous safety evaluations.

However, the Committee could not verify the NOEL of 100 mg/kg bw in rats that was used to derive the ADI of 0–1 mg/kg bw for maltol at its twenty-fifth meeting because of uncertainties in the administered dose levels and the effects observed in several studies as described in the monograph of that meeting (Annex 1, reference *56*). The Committee identified a NOAEL of 125 mg/kg bw per day in a published 90-day study in dogs (Gralla et al., 1969). This NOAEL was used to complete the re-evaluation of maltol as a flavouring agent.

1.8 Conclusions

In the previous evaluation of flavouring agents in this group of maltol and related substances, biochemical data and studies of acute toxicity, short-term toxicity, long-term toxicity and carcinogenicity, genotoxicity and reproductive toxicity were available. None of the seven previously evaluated flavouring agents raised safety concerns based on the estimated dietary exposures and the biochemical and toxicological data available (Annex 1, reference *179*).

The Committee concluded that the two flavouring agents (Nos 2252 and 1480) under evaluation, one of which is an addition to the group of maltol and related substances evaluated previously, do not give rise to safety concerns at current estimated dietary exposures.

However, the Committee could not verify the NOEL of 100 mg/kg bw in rats that was used to derive the ADI of 0–1 mg/kg bw for maltol during its twenty-fifth meeting because of uncertainties in the administered dose levels and the effects observed in several studies described in the monograph of that meeting (Annex 1, reference *57*). The Committee concluded that access to either the original studies or submission of new data would be needed to reaffirm or amend the current ADI. The Committee therefore withdrew the ADI for maltol pending review of the appropriate data at a future meeting. The ADI for ethyl maltol was maintained.

2. Relevant background information

2.1 Explanation

This monograph addendum summarizes key aspects relevant to the safety evaluation of the flavouring agents ethyl maltol isobutyrate (No. 2252) and maltol (No. 1480) belonging to the group of maltol and related substances. This group was evaluated previously by the Committee at its sixty-fifth meeting (Annex 1, reference *178*). Whereas maltol was evaluated by the Committee at the eleventh, eighteenth, twenty-second, twenty-fifth and sixty-fifth meetings (Annex 1, references *15*, *35*, *47*, *56* and *178*), ethyl maltol isobutyrate was not.

2.2 Additional considerations on dietary exposure

Maltol (No. 1480) is the only substance in the group of maltol and related substances reported to occur in traditional foods (Stofberg & Grundschober, 1987; Nijssen, van Ingen-Visscher & Donders, 2017). Quantitative natural occurrence data and a consumption ratio reported for maltol (No. 1480) indicate that exposure occurs predominantly from consumption of the flavouring agent (i.e. consumption ratio < 1) (Stofberg & Kirschman, 1985; Stofberg & Grundschober, 1987).

Annual volumes of production of this group of flavouring agents as well as the daily dietary exposures calculated using both the MSDI method and the SPET are summarized in Table 2.

2.3 Biological data

2.3.1 Biochemical aspects: ADME

Information on the ADME of flavouring agents belonging to the group of maltol and related substances has previously been described in the monograph of the sixty-fifth meeting (Annex 1, reference *179*). In addition, information on ethyl maltol has also been described in the monographs of the fourteenth and eighteenth meeting (Annex 1, reference *23* and *36*). No additional information was available for the current meeting.

2.3.2 Toxicological studies

(a) Acute toxicity

In male and female rats, an oral median lethal dose (LD_{50}) of greater than 2000 mg/kg bw has been reported for the previously evaluated flavouring agent maltyl isobutyrate (No. 1482) (Arcelin, 2000). The study was in compliance with

Table 2
Annual volumes of production and daily dietary exposures for maltol and related substances used as flavouring agents in Europe, the USA, Japan and LATAM

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Annual volume of consumption via natural occurrence in foods (kg)[d]	Consumption ratio [e]
		MSDI [b]		SPET [c]			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
Maltol (1480)				10 440	174	38 694	0.44
Europe	83 200	6 950	116				
USA	87 600	9 091	152				
Japan	12 500	3 293	55				
LATAM	47 600	2 625	44				
Ethyl maltol isobutyrate (2252)				400	7	–	NA
Europe	ND	ND	ND				
USA	ND	ND	ND				
Japan	29	8	0.1				
LATAM	ND	ND	ND				
Total							
Europe	83 200						
USA	87 600						
Japan	12 529						
LATAM	47 600						

bw: body weight; LATAM: Latin America; MSDI: maximized survey-derived intake; ND: no data reported; SPET: single-portion exposure technique; –: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (IOFI, 2017a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1 × 10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 45 × 10^6 for Europe, 33 × 10^6 for the USA, 13 × 10^6 for Japan and 62 × 10^6 for LATAM; and where survey correction factor = 0.8 for the IOFI's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (IOFI, 2017a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (IOFI, 2017b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[d] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[e] The consumption ratio is calculated as follows:
(annual volume of consumption via natural occurrence in food, kg)/(most recent annual volume as a flavouring agent, kg)

Organisation for Economic Co-operation and Development Test Guideline (OECD TG) 423 ("Acute Oral Toxicity", 1996) and was certified for compliance with good laboratory practice (GLP) and quality assurance (QA).

This result is consistent with the finding in the previous evaluation that the acute oral toxicity of maltol and related substances is low (Annex 1, reference *179*).

(b) Genotoxicity studies

For the previously evaluated flavouring agents maltol (No. 1480) and maltyl isobutyrate (No. 1482) new studies of genotoxicity in vitro (Nos 1480 and 1482) and in vivo (No. 1480) are available. These are summarized in Table 3 and described below.

(i) In vitro

No evidence of mutagenicity was observed when maltol (No. 1480; up to 5 000 µg/plate) or maltyl isobutyrate (No. 1482; up to 10 000 µg/plate) were incubated with *Salmonella typhimurium* strains TA98, TA100, TA102, TA104, TA1535, TA1537 and/or TA1538 in the presence and absence of metabolic activation (Ballantyne, 2012; McConville, 1979; Watanabe-Akanuma, Inaba & Ohta, 2007). Only the study by Ballantyne (2012) was reported to be conducted according to OECD TG 471 ("Bacterial Reverse Mutation Test", 1997) and was certified for compliance with GLP and QA.

Watanabe-Akanuma, Inaba & Ohta (2007) also tested the mutagenicity of UV-irradiated maltol in a reverse mutation assay using the preincubation method without metabolic activation in *S. typhimurium* strains TA97, TA98, TA100 and TA104. Maltol treated with either UVA (230 µW/cm^2) for 5–30 minutes or UVC (610 µW/cm^2) for 3 minutes in sodium phosphate buffer (pH 7.4) prior to the exposure of bacterial cells gave positive results in strains TA97, TA100 and TA104. The mutagenicity was dependent on experimental conditions and was diminished in the presence of thiol compounds, at lower pH and when water and sodium chloride buffer were used instead of sodium phosphate buffer (Watanabe-Akanuma, Inaba & Ohta, 2007). The Committee considered this finding with UV-irradiated maltol not relevant for the assessment of the genotoxicity of maltol used as a flavouring agent.

Maltol (No. 1480) was evaluated for its ability to induce chromosomal damage or aneuploidy in an in vitro micronucleus assay using human peripheral blood lymphocytes (Whitwell, 2012). The study was conducted according to OECD TG 487 ("In Vitro Mammalian Cell Micronucleus Test", 2010) and was certified for compliance with GLP and QA. Maltol induced a statistically significant increase in the occurrence of micronucleated binucleated cells (MNBN) in human peripheral blood lymphocytes treated for 3 hours (with 21-hour recovery period) in the presence and absence of S9 metabolic activation system. In the presence of S9, frequencies of MNBN cells were significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for the highest two concentrations analysed (800 and 1262 µg/mL). The MNBN cell frequencies of both replicate cultures at each of these concentrations (and a single culture at a lower concentration analysed) exceeded historical control ranges. The highest

Table 3
Studies of genotoxicity with maltol and related substances used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
1480	Maltol	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.32–5 000 µg/plate, ±S9	Negative [a]	Ballantyne (2012)
				20.48–5 000 µg/plate, ±S9	Negative [b]	
1480	Maltol	Reverse mutation	*S. typhimurium* TA97, TA98, TA100 and TA104	500–1 500 µg/plate, –S9	Negative (maltol) Positive (UV-irradiated maltol) [c]	Watanabe-Akanuma, Inaba & Ohta (2007)
1480	Maltol	Micronucleus induction	Human peripheral blood lymphocytes	400, 800 and 1 262 µg/mL, ±S9	Positive [d]	Whitwell (2012)
				125, 200 and 300 µg/mL, –S9	Negative [e]	
1482	Maltyl isobutyrate [f]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	33.3–10 000 µg/plate, ±S9	Negative [g]	McConville (1979)
In vivo						
1480	Maltol	Micronucleus induction	Rat [h], M	70, 350 and 700 mg/kg bw per day	Negative [i]	Beevers (2013)
1480	Maltol	Comet assay	Rat [j], M	70, 350 and 700 mg/kg bw per day	Negative [i]	Beevers (2013)

bw, body weight; M: male; no.: number; S9: 9000 × *g* supernatant fraction from rat liver homogenate; UV: ultraviolet

[a] Plate incorporation assay. Toxicity was observed in strain TA102 only from 1000 µg/plate onwards (without S9) and from 200 µg/plate onwards (with S9).

[b] Plate incorporation assay (without S9) or preincubation method (with S9). Evidence of toxicity was observed at 5000 µg/plate in strain TA102 (with and without S9) and in strain TA100 (without S9 only).

[c] Preincubation assay. Maltol was not mutagenic, but maltol treated with either ultraviolet A (230 µW/cm^2) for 5–30 minutes or ultraviolet C (610 µW/cm^2) for 3 minutes in sodium phosphate buffer (pH 7.4) prior to the exposure of bacterial cells gave positive results in strains TA97, TA100 and TA104.

[d] Three-hour treatment followed by 21-hour recovery. In the presence of S9, frequencies of micronucleated binuclear cells were significantly ($P \leq 0.05$) higher than in concurrent vehicle controls for the highest two concentrations analysed. The highest concentration (with S9) induced 19% cytotoxicity. Without S9, frequencies of micronucleated binuclear cells were statistically significantly increased at all three concentrations. The highest concentration induced 24% cytotoxicity.

[e] Twenty-four-hour treatment without recovery. The maximum concentration tested was only 300 µg/mL as this concentration induced 57% cytotoxicity and higher concentrations resulted in even greater cytotoxicity.

[f] No certificate of analyses was included in the study report.

[g] Plate incorporation method. Evidence of toxicity was observed at the highest treatment concentration in all strains in the absence and presence of S9.

[h] Target organ was bone marrow.

[i] Three doses, administered by oral gavage at 0, 24 and 45 hours. Termination and sample collection at 48 hours after treatment.

[j] Target organ was liver.

concentration induced 19% cytotoxicity. In the absence of S9, frequencies of MNBN cells were significantly increased ($P \leq 0.05$) at all three concentrations (400, 800 and 1262 µg/mL). However, increases in MNBN cell frequencies that exceeded normal ranges were restricted to single cultures at each of the two highest concentrations analysed. The highest concentration induced 24% cytotoxicity. When lymphocytes were continuously treated with maltol for 24 hours (without a recovery period) in the absence of S9, no statistically significant increases in MNBN cells were observed. However, only concentrations up to 300 µg/mL could be tested, due to high cytotoxicity.

Subsequent mechanistic analysis of slides generated from the 3-hour treatments via the use of fluorescence in situ hybridization analysis with human pan-centromeric DNA probes demonstrated that micronuclei were generated via a predominantly clastogenic (chromosome breakage) mechanism (Whitwell, 2012).

(ii) In vivo

Maltol (No. 1480) was evaluated in a combined bone marrow micronucleus test and comet assay in male Han Wistar rats (Beevers, 2013). The micronucleus test was conducted in accordance with OECD TG 474 ("Mammalian Erythrocyte Micronucleus Test", 1997). The comet assay was performed in accordance with recommendations of the In Vivo Comet Assay Working Group of the International Workshop on Genotoxicity Testing (IWGT) workshops and current literature (Burlinson et al., 2007; Smith et al., 2008). The studies were certified for compliance with GLP and QA.

Rats were administered doses of 0, 70, 250 or 700 mg/kg bw at 0, 24 and 45 hours. The doses selected were based on the results of a range-finding study. In this range-finding study, 700 mg/kg bw was the maximum tolerable dose. Dose levels of 1000 mg/kg bw and above resulted in death. Dose levels of 500 and 700 mg/kg bw caused decreased activity and/or piloerection in all animals only after the first administration; no clinical signs of toxicity were observed on the second and third day of dosing. Reductions in individual body weights were noted following dosing at 500 mg/kg bw and above.

In the main study, no clinical signs of toxicity were observed following dosing at 70, 350 or 700 mg/kg bw and no test article-related effects on clinical chemistry were observed. A mean decrease in body weight of approximately 5 g (equivalent to ~2% reduction in body weight) was observed in animals dosed at 700 mg/kg/day. There was a minor dose-related reduction in the level of glycogen vacuolation in liver of treated rats compared with concurrent controls.

For detection of micronuclei, bone marrow was sampled 48 hours after the final treatment. No statistically significant increase in the incidence

of micronucleated polychromatic erythrocytes of the bone marrow of rats was observed. There was no evidence of bone marrow toxicity: the percentage of polychromatic erythrocytes in the treated groups did not differ from that in the vehicle control group and was comparable to the laboratory's historical control data (Beevers, 2013).

Plasma samples taken from six satellite animals dosed with 700 mg/kg bw (at 0, 24 and 45 hours) were analysed to confirm systemic exposure to maltol. In plasma samples of three rats collected 0.5, 2 and 8 hours after the final administration, maltol was detected in 2 of 3 animals after 0.5 and 2 hours but not at 8 hours. None was detected in the third animal. In plasma samples collected from the other three animals (collected 1, 4 and 24 hours after the final administration), maltol was detected after 1 hour but was no longer detectable after 4 or 24 hours (Mallinson & Hough, 2014). Because of these inconsistent results a second experiment was conducted. Six male rats were dosed with 700 mg/kg bw of maltol by oral gavage using the same dosing regimen as in the micronucleus test. Blood samples were collected 0.5, 1, 2 and 3 hours after the final dose. Maltol was detected in all plasma samples collected at 0.5, 1 and 2 hours after dosing and was also detected 3 hours after dosing in plasma samples of 3/6 rats. In the majority of the animals, peak plasma levels occurred at 0.5 hours after dosing. It was concluded that rats dosed with maltol at 700 mg/kg bw were systemically exposed, with peak plasma levels 0.5–1 hour after the final dose administration (Beevers, 2015).

The Committee noted that there was no evidence of bone marrow toxicity and systemic toxicity of maltol in the micronucleus assay. Based on the additional plasma analyses, which demonstrated the presence of maltol, the Committee assumed that the bone marrow was exposed.

In the concurrent in vivo comet assay, liver samples were examined for DNA damage. Maltol did not result in tail moments and tail intensities greater than the laboratory's historical control range. Two animals had tail moments and tail intensities notably higher than the vehicle control animals, but these values were not dose dependent and fell within the historical control data ranges. These differences were therefore not considered biologically relevant (Beevers, 2013).

Conclusions for genotoxicity

In the previous evaluation (Annex 1, reference *179*), equivocal or weakly positive results were obtained with maltol and ethyl maltol in some tests for genotoxicity in vitro, and positive or equivocal results were found in vivo with maltol but not with ethyl maltol. Based on the structural similarity of maltol to ascorbate, which is genotoxic in test systems similar to those in which maltol gave positive results, it was suggested that a similar mechanism might be responsible for its

mutagenicity, although this has not been tested experimentally. In animals, maltol is rapidly conjugated with glucuronic acid and sulfate in the liver and excreted as glucuronic acid and sulfate conjugates in the urine. Therefore, the Committee at its sixty-fifth meeting concluded that despite the structural similarity of maltol and ascorbate, it seems unlikely that the mutagenic activity of maltol would be expressed under the conditions of oral human intake (Annex 1, reference *179*). Also, in long-term studies of toxicity and carcinogenicity with maltol in rats and mice, no evidence for carcinogenicity was observed (Annex 1, reference *56*).

For the present evaluation, additional genotoxicity data on maltol (No. 1480) and maltyl isobutyrate (No. 1482) were available. Neither maltol (No. 1480) nor maltyl isobutyrate (No. 1482) showed mutagenic potential in bacterial reverse mutation assays in the absence or presence of metabolic activation. Maltol showed genotoxic potential in the absence and presence of metabolic activation in an in vitro micronucleus test in human peripheral blood lymphocytes but gave negative results in an in vivo combined bone marrow micronucleus test and liver comet assay in orally treated rats. The positive or equivocal in vivo results described in the previous evaluation were obtained from a micronucleus test in mice treated intraperitoneally with maltol and in a sex-linked recessive lethal mutation assay in *Drosophila melanogaster*. Both the intraperitoneal administration route and the assay in *D. melanogaster* are less relevant for the oral human exposure from the use of maltol as a flavouring agent.

The Committee concluded that there is no concern for genotoxicity of maltol when ingested by humans.

(c) Effects on enzymes

Maltol was shown to induce cytochrome P450 1A1 in murine hepatoma Hepa 1c1c7 cells. In addition, maltol induced aryl hydrocarbon receptor (AhR)-dependent luciferase reporter gene expression in stable transfected murine hepatoma H1L1.1c2 cells, suggesting an AhR-dependent mechanism (Anwar-Mohamed & El-Kadi, 2007).

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Menthol and structurally related substances (addendum)

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1. Evaluation

1.1 Introduction

The Committee evaluated seven flavouring agents belonging to the group of menthol and structurally related substances, which was previously evaluated. The Committee re-evaluated menthol (No. 427) and evaluated six additional flavouring agents. These included four menthyl esters (menthyl formate [No. 2246], menthyl propionate [No. 2247], *l*-menthyl butyrate [No. 2248] and

dimenthyl glutarate [No. 2250]), *dl*-isomenthol (No. 2249) and one polyether alcohol, (±)-2-[(2-*p*-menthoxy)ethoxy]ethanol (No. 2251). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *230*).

The Committee previously evaluated menthol and 13 other members of this group of flavouring agents at its fifty-first meeting (Annex 1, references *137*) and 10 additional members of the group at its sixty-ninth meeting (Annex 1, reference *190*). The Committee concluded that all 24 flavouring agents were of no safety concern at estimated dietary exposures.

Menthol (No. 427) was first evaluated by the Committee at the eleventh meeting when an unconditional acceptable daily intake (ADI) of 0–0.2 mg/kg body weight (bw) and a conditional[1] ADI of 0.2–2 mg/kg bw was established (Annex 1, reference *14*). At the eighteenth meeting, an ADI of 0–0.2 mg/kg bw was established for menthol (Annex 1, reference *35*). This ADI was maintained at the twentieth meeting. At the twentieth meeting, the Committee requested additional long-term studies of toxicity and carcinogenicity in rats, information on the average and likely maximum dietary exposure to menthol, clinical observations of humans with higher than average dietary exposure to menthol and studies of metabolism (Annex 1, reference *41*). At the fifty-first meeting, an ADI of 0–4 mg/kg bw was established on the basis of the no-observed-effect level (NOEL) of 380 mg/kg bw per day in a long-term study in rats, applying a safety factor of 100 and rounding to one significant figure (Annex 1, reference *137*).

Menthol (No. 427), menthyl formate (No. 2246) and *dl*-isomenthol (No. 2249) have been reported to occur naturally in foods and can be mainly found in peppermint oil and other *Mentha* species oils. Menthol (10–70%) and menthone (No. 429; 7–40%) are the principal constituents of peppermint oil (Nijssen, van Ingen-Visscher & Donders, 2017; Annex 1, reference *137*).

Menthol (No. 427) was re-evaluated because new data had become available since the previous evaluation. Menthol and the other flavourings were evaluated at the request of the Forty-eighth Session of the CCFA (FAO/WHO, 2016).

A comprehensive literature search was performed in Scopus; one additional reference was identified.

[1] "Conditional ADI" is a term no longer used by Joint FAO/WHO Expert Committee on Food Additives (JECFA) to signify a range above the "unconditional ADI", which may signify an acceptable intake when special problems, different patterns of dietary intake, and special groups of the population that may require consideration are taken into account" (IPCS, 2009).

1.2 Assessment of dietary exposure

The total annual volume of production for menthol (No. 427) is 296 000 kg in Europe, 496 000 kg in the USA, 146 000 kg in Japan and 127 000 kg in Latin America (LATAM; IOFI, 2017a,b).

The total annual volume of production of the six additional flavouring agents in the group of menthol and structurally related substances is 14 900 kg in Europe, 103 937 kg in the USA and 92 kg in Japan. No production volume was reported for LATAM (IOFI, 2017a,b).

Of the seven flavouring agents under evaluation by this current Committee, menthol (No. 427) accounts for more than 95% of the total annual production volume in Europe and 82% in the USA. Dimenthyl glutarate (No. 2250) accounts for almost all the remaining volume. Menthol (No. 427) accounts for more than 99% of the annual production volume in Japan and 100% in LATAM.

Dietary exposures were estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, with the highest values reported in Table 1. The highest estimated dietary exposure is for menthol (No. 427; 51 474 µg/day, MSDI value; the SPET value is 42 210 µg/day). For the other flavouring agents, the estimated daily dietary exposures range from 0.03 to 10 784 µg/day (MSDI values) and from 300 to 9000 µg/day (SPET values), with the SPET yielding the highest estimate in all but one case (dimenthyl glutarate [No. 2250]).

1.3 Absorption, distribution, metabolism and excretion (ADME)

Information on the ADME of the flavouring agents belonging to the group of menthol and structurally related substances has previously been described in the monographs of the eleventh, twentieth and fifty-first meetings (Annex 1, references *15*, *42* and *138*). Additional information was available for the current meeting.

The menthyl esters in this group (Nos 2246–2248 and 2250) can be expected to be readily hydrolysed to menthol and their respective carboxylic acids (Heymann, 1980; Anders, 1989). Similar to menthol, *dl*-isomenthol (No. 2249) is expected to be conjugated with glucuronic acid and be eliminated in the urine or faeces (Williams, 1940; Atzl et al., 1972; Yamaguchi, Caldwell & Farmer, 1994). The polyether alcohol conjugate of menthol, (±)-2-[(2-*p*-menthoxy)ethoxy] ethanol (No. 2251) is also expected to undergo conjugation with glucuronic acid and subsequent elimination in the urine and faeces.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. There are no structural alerts for genotoxicity for these flavouring agents. Chemical-specific genotoxicity data available for menthol (No. 427), *dl*-isomenthol (No. 2249) and (±)-2-[(2-*p*-menthoxy)ethoxy]ethanol (No. 2251) indicate that these flavouring agents are unlikely to be genotoxic based on the weight of evidence.

Step 2. In applying the Procedure for the Safety Evaluation of Flavouring Agents, the Committee assigned six flavouring agents (Nos 427, 2246–2250) to structural class I and one flavouring agent (No. 2251) to structural class III (Cramer, Ford & Hall, 1978).

Step 3. The highest dietary exposures were estimated using the SPET for five of the seven flavouring agents (Nos 2246–2249, 2251) and the MSDI method for menthol (No. 427) and dimenthyl glutarate (Nos 2250).

Step 4. The highest estimated dietary exposures for three of the six flavouring agents in structural class I (Nos 2246–2248) are below the threshold of toxicological concern (i.e. 1800 µg/day for class I). The Committee therefore concluded that these flavouring agents would not pose a safety concern at current estimated dietary exposures.

The highest estimated dietary exposures for the remaining three flavouring agents in structural class I (Nos 427, 2249 and 2250) are above the threshold of toxicological concern. Therefore, the evaluation of these flavouring agents proceeded to Step 5.

The highest estimated dietary exposure for the one flavouring agent in structural class III (No. 2251) is above the threshold of toxicological concern (i.e. 90 µg/day for class III). Therefore, the evaluation of this flavouring agent proceeded to Step 5.

Step 5. For menthol (No. 427), an ADI of 0–4 mg/kg bw was allocated on the basis of the NOEL of 380 mg/kg bw per day from a 2-year dietary study in rats, applying a safety factor of 100 and rounding to one significant figure (Annex 1, reference *137*). The NOEL of 380 mg/kg bw per day from a 2-year dietary study in rats (NCI, 1979) provides an adequate margin of exposure (MOE) of 440 in relation to the estimated dietary exposure to No. 427 (MSDI of 51 474 µg/day). The Committee therefore concluded that menthol (No. 427) would not pose a safety concern when used as a flavouring agent at current estimated dietary exposures.

For *dl*-isomenthol (No. 2249), the NOEL of 380 mg/kg bw per day from a 2-year dietary study in rats (NCI, 1979) of the structurally related substance menthol (No. 427) provides an adequate MOE of 7600 in relation to the estimated dietary exposure to No. 2249 (SPET value of 3000 µg/day). The Committee

therefore concluded that *dl*-isomenthol (No. 2249) would not pose a safety concern when used as a flavouring agent at current estimated dietary exposures.

For dimenthyl glutarate (No. 2250), the NOEL of 380 mg/kg bw per day from a 2-year dietary study in rats (NCI, 1979) for the structurally related substance menthol (No. 427) provides an adequate MOE of 2100 in relation to the estimated dietary exposure to No. 2250 (MSDI value of 10 784 μg/day). The Committee therefore concluded that dimenthyl glutarate (No. 2250) would not pose a safety concern when used as a flavouring agent at current estimated dietary exposures.

For (±)-2-[(2-*p*-menthoxy)ethoxy]ethanol (No. 2251), the NOEL of 30 mg/kg bw per day from a 90-day study in rats for the structurally related substance 3-*l*-menthoxypropane-1,2-diol (No. 1408; Wolfe, 1992) provides an adequate MOE of 1700 in relation to the estimated dietary exposure to No. 2251 (SPET value of 1000 μg/day). The Committee therefore concluded that (±)-2-[(2-*p*-menthoxy)ethoxy]ethanol (No. 2251) would not pose a safety concern when used as a flavouring agent at current estimated dietary exposures.

Table 1 summarizes the evaluations of the seven flavouring agents belonging to this group of menthol and structurally related substances (Nos 427 and 2246–2251).

1.5 Consideration of combined intakes from use as flavouring agents

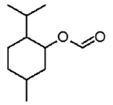
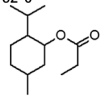
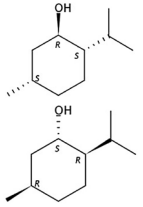
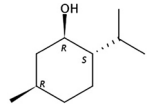
With the exception of dimenthyl glutarate (No. 2250), all additional flavouring agents to the group of menthol and structurally related substances have low MSDI values (range: 0.03–21 μg/day). The highest MSDI value for dimenthyl glutarate (No. 2250) is 10 784 μg/day. No production volume for No. 2250 was reported for Japan and LATAM. The Committee concluded that consideration of combined intakes is not necessary for Japan and LATAM because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

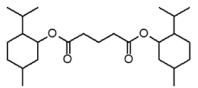
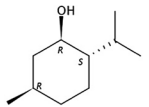
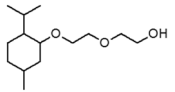
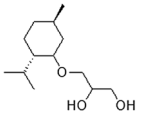
In the unlikely event that the flavouring agents with the common metabolite menthol (No. 427) in this group were to be consumed together with menthol (No. 427) on a daily basis, the estimated combined intakes[2] for the four flavouring agents (Nos 427, 429, 1414 and 2250) with the highest estimated dietary exposures (MSDI values) would be 24 622 μg/day in Europe and 65 627 μg/day in the USA. The estimated combined intake would therefore exceed the human threshold of toxicological concern (1800 μg/day for structural class I). However, the vast majority of the combined intake would be due to menthol alone,

[2] Combined intake was calculated on a molar basis relative to the formation of a common metabolite. In this case, the common metabolite is menthol, with a relative molecular mass of 157.

Table 1

Summary of the results of safety evaluations of menthol and six additional structurally related substances used as flavouring agents [a,b]

Flavouring agent	No.	CAS no. and structure	*Step 4* [e] Does the highest dietary exposure estimate exceed the threshold of toxicological concern?	*Step 5* [d] Does a NOAEL exist for the flavouring agent or a structurally related substance that provides an adequate MOE?	Related structure name (No.) and structure	Conclusion based on current estimated dietary exposure
Structural class I						
Menthyl formate	2246	2230-90-2 	No, SPET: 400	NR	NR	No safety concern
Menthyl propionate	2247	86014-82-6 	No, SPET: 600	NR	NR	No safety concern
l-Menthyl butyrate	2248	68366-64-3 	No, SPET: 300	NR	NR	No safety concern
dl-Isomenthol	2249	3623-52-7 	Yes, SPET: 3 000	Yes. The NOEL of 380 mg/kg bw per day from a 2-year dietary study in rats for the structurally related substance menthol (NCI, 1979) is 7 600 times the estimated dietary exposure to No. 2249 when used as a flavouring agent.	Menthol (No. 427) 	No safety concern

Flavouring agent	No.	CAS no. and structure	*Step 4* [c] Does the highest dietary exposure estimate exceed the threshold of toxicological concern?	*Step 5* [d] Does a NOAEL exist for the flavouring agent or a structurally related substance that provides an adequate MOE?	Related structure name (No.) and structure	Conclusion based on current estimated dietary exposure
Dimenthyl glutarate	2250	406179-71-3 	Yes, MSDI: 10 784	Yes. The NOEL of 380 mg/kg bw per day from a 2-year dietary study in rats for the structurally related substance menthol (NCI, 1979) is 2 100 times the estimated dietary exposure to No. 2250 when used as a flavouring agent.	Menthol (No. 427)	No safety concern
Menthol [e]	427	89-78-1 	Yes, MSDI: 51 474	Yes. The NOEL of 380 mg/kg bw per day from a 2-year dietary study in rats (NCI, 1979) is 440 times the estimated dietary exposure to No. 427 when used as a flavouring agent.	NR	No safety concern
Structural class III						
(±)-2-[(2-*p*-Menthoxy) ethoxy]ethanol	2251	28804-53-7 	Yes, SPET: 1 000	Yes. The NOEL of 30 mg/kg bw per day from a 90-day study in rats for the structurally related substance 3-*l*-menthoxypropane-1,2-diol (Wolfe, 1992) is 1 700 times the estimated dietary exposure to No. 2251 when used as a flavouring agent.	3-*l*-menthoxypropane-1,2-diol (No. 1408) 	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; MOE: margin of exposure; MSDI: maximized survey-derived intake; no.: number; NOAEL: no-observed-adverse-effect-level; NOEL: no-observed-effect level; no.: number; NR: not relevant; SPET: single-portion exposure technique

[a] Twenty-four flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *137* and *190*).

[b] *Step 2*: Six flavouring agents (Nos 427, 2246–2250) are in structural class I and one flavouring agent (No. 2251) is in structural class III.

[c] The thresholds for toxicological concern for structural class I and structural class III are 1800 µg/day and 90 µg/day, respectively. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest daily dietary exposure calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case except for Nos 427 and 2250 where the MSDI value was higher.

[d] The MOE was calculated based on the highest estimated daily dietary exposure using either the SPET or the MSDI method.

[e] The acceptable daily intake of menthol of 0–4 mg/kg bw established at the fifty-first meeting (Annex 1, reference *137*) was maintained.

for which an ADI of 0–4 mg/kg bw was previously established. The estimated combined intake does not exceed this ADI, which is equal to 240 mg/day for a 60 kg person. Also, as the flavouring agents are likely to be metabolized efficiently, they would not saturate metabolic pathways.

Therefore, the Committee concluded that combined intake would not raise safety concerns.

1.6 Consideration of additional data on previously evaluated flavouring agents

For eight previously evaluated flavouring agents in this group, additional studies of metabolism (No. 429); studies of acute toxicity (Nos 430, 1854 and 1856); and studies of genotoxicity (Nos 429, 430, 431, 445, 1856 and 1857) were available.

1.7 Conclusions

In the previous evaluations of substances in this group of menthol and structurally related substances, biochemical data; studies of acute toxicity, short-term toxicity, long-term toxicity and carcinogenicity, genotoxicity, developmental toxicity, immunotoxicity and sensitivity; and human data were available. None of the 24 previously evaluated flavouring agents raised safety concerns based on the estimated dietary exposures and the biochemical and toxicological data available (Annex 1, references *138* and *191*).

For the flavouring agent under re-evaluation, menthol (No. 427), additional biochemical data, studies of acute toxicity and genotoxicity and a case study were available. Studies of genotoxicity were available for the *dl*-isomenthol (No. 2249) and (±)-2-[(2-*p*-menthoxy)ethoxy]ethanol (No. 2251).

The studies available for the present evaluation raised no safety concerns and support the previous safety evaluations. The additional data on menthol did not indicate a need to revise the ADI of menthol (No. 427).

The Committee concluded that the seven flavouring agents under evaluation, six of which are additions to the group of menthol and structurally related substances evaluated previously, do not give rise to safety concerns at current estimated dietary exposures. The previously established ADI of 0–4 mg/kg bw for menthol was maintained.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of six flavouring agents (Nos. 2246–2251) that are additions to the group of menthol and structurally related substances evaluated previously by the Committee at its fifty-first and sixty-ninth meetings (Annex 1, references *138* and *191*). Additional data for menthol (No. 427) are also summarized. Several summaries of additional studies available on the website of the European Chemicals Agency (ECHA) were submitted. However, the study reports of these studies were not available for the current evaluation and therefore, these studies are not included in this monograph.

2.2 Additional considerations on exposure

Menthol and two of the six flavouring agents that are additions to the group of menthol and structurally related substances have been reported to occur naturally in foods (Stofberg & Grundschober, 1987; Nijssen, van Ingen-Visscher & Donders, 2017; Annex 1, reference *137*). Quantitative natural occurrence data and a consumption ratio reported for menthol (No. 427) indicate that exposure occurs predominantly from consumption of flavouring agents (i.e. consumption ratio < 1) (Stofberg & Kirschman, 1985; Stofberg & Grundschober, 1987).

Annual volumes of production of this group of flavouring agents as well as the daily dietary exposures calculated using both the MSDI method and the SPET are summarized in Table 2.

2.3 Biological data

2.3.1 Biochemical aspects: ADME

Information on the ADME of the flavouring agents belonging to the group of menthol and structurally related substances was described in the monographs of the eleventh, twentieth and fifty-first meeting (Annex 1, references *15*, *42* and *138*). Additional information was available for two compounds of this group, menthol (No. 427) and menthone (No. 429).

(a) Menthol (No. 427)

In a toxicokinetic study, 16 healthy male human volunteers ingested five capsules of an immediate release formulation containing 36 mg of peppermint oil and 20

Table 2

Annual volumes of production and daily dietary exposures for menthol and structurally related substances used as flavouring agents in Europe, the USA, Japan and LATAM

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Annual volume of consumption via natural occurrence in foods (kg)[d]	Consumption ratio [e]
		MSDI [b]		SPET [c]			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
Menthyl formate (2246)			400	7	+	NA	0.44
Europe	ND	ND	ND				
USA	ND	ND	ND				
Japan	0.1	0.03	0.000 4				
LATAM	ND	ND	ND				
Menthyl propionate (2247)			600	10	–	NA	NA
Europe	ND	ND	ND				
USA	ND	ND	ND				
Japan	11	3	0.05				
LATAM	ND	ND	ND				
***l*-Menthyl butyrate (2248)**			300	5	–	NA	
Europe	ND	ND	ND				
USA	ND	ND	ND				
Japan	81	21	0.4				
LATAM	ND	ND	ND				
***dl*-Isomenthol (2249)**			3 000	50	+	NA	
Europe	ND	ND	ND				
USA	10	1	0.02				
Japan	ND	ND	ND				
LATAM	ND	ND	ND				
Dimenthyl glutarate (2250)			9 000	150	–	NA	
Europe	14 900	1 131	19				
USA	103 917	10 784	180				
Japan	ND	ND	ND				
LATAM	ND	ND	ND				
(±)-2-[(2-*p*-Menthoxy)ethoxy] ethanol (2251)			1 000	17	–	NA	
Europe	ND	ND	ND				
USA	10	1	0.02				
Japan	ND	ND	ND				
LATAM	ND	ND	ND				
Total for additional flavouring agents							
Europe	14 900						
USA	103 937						
Japan	92						
LATAM	ND						

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Annual volume of consumption via natural occurrence in foods (kg)[d]	Consumption ratio [e]
		MSDI [b]		SPET [c]			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
Menthol (427)				42 210	704	211 140	0.43
Europe	296 000	22 477	375				
USA	496 000	51 474	858				
Japan	146 000	38 462	641				
LATAM	127 000	7 004	117				
Total							
Europe	310 900						
USA	599 937						
Japan	146 092						
LATAM	127 000						

bw: body weight; LATAM: Latin America; MSDI: maximized survey-derived intake; NA: not applicable; ND: no data reported; no.: number; +: reported to occur naturally in foods (Nijssen, van Ingen-Visscher & Donders, 2017), but no quantitative data; –: not reported to occur naturally in foods; SPET: single-portion exposure technique

[a] From International Organization of the Flavor Industry (IOFI, 2017a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × $(1 \times 10^9$ µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 45×10^6 for Europe, 33×10^6 for the USA, 13×10^6 for Japan and 62×10^6 for LATAM; and where survey correction factor = 0.8 for the IOFI's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (IOFI, 2017a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (IOFI, 2017b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[d] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[e] The consumption ratio is calculated as follows:
(annual volume of consumption via natural occurrence in food, kg)/(most recent annual volume as a flavouring agent, kg).

mg of caraway oil, with 250 mL water after a 10-hour fast. The menthol content of the peppermint oil was not determined. Blood samples were collected at 0, 20, 40, 60, 90, 120 and 150 minutes and 3, 4, 5, 6, 8, 10 and 15 hours after administration. The study protocol was reported to comply with Declaration of Helsinki ethical guidelines and was approved by an independent ethical committee.

Following cleavage of conjugates by treatment with glucuronidase/arylsulfatase, menthol concentrations in plasma were determined by gas chromatography–mass spectrometry (GC-MS) analysis. On the first day of the study, one participant had slight fever and nausea; his plasma levels had very low levels of menthol. These results were excluded from further evaluation. The following toxicokinetic parameters were determined for menthol: maximum plasma concentration (C_{max}) was 1492 ± 614 ng/mL; the total bioavailability ($AUC_{0-\infty}$) was 3226 ± 484 ng·h/mL; the time to reach the maximum plasma

concentration (T_{max}) was 1.7 ± 1.1 hours and the half-life ($t_{½}$) was 4.4 ± 2.2 hours (Mascher, Kikuta & Schiel, 2001).

In a phase I clinical study, healthy male human volunteers (6/dose group) received *l*-menthol at doses of 0, 80, 160 or 320 mg/person by spraying a 0.8% solution directly on to the gastric mucosa with a standardized spraying catheter. Venous blood samples were collected at 0, 5, 10, 30, 60, 120 and 240 minutes and 8, 12 and 24 hours after dosing and urine samples were collected before dosing (−12 to 0 h) and 0–4, 4–8, 8–12 and 12–24 hours post dosing. Plasma and urine samples were analysed for *l*-menthol and menthol glucuronide using GC-MS. Plasma and urine samples from the 320 mg dose group were also analysed for all metabolites using electrospray ionization mass spectrometry (ESI-MS).

Pharmacokinetic analysis of sampled plasma showed that the concentrations of *l*-menthol and menthol glucuronide reached peak concentrations within 1 hour of administration. In the three treatment groups, the C_{max} and $AUC_{0-\infty}$ values for *l*-menthol ranged from 32 to 48 ng/mL and from 40 to 108 ng·h/mL, respectively. The C_{max} and $AUC_{0-\infty}$ values for menthol glucuronide ranged from 5617 to 17 602 ng/mL and from 9437 to 31 855 ng·h/mL, respectively. The T_{max} and terminal half-life ($t_{½}$) of *l*-menthol ranged from 0.40 to 0.76 hours and from 1.03 to 1.77 hours, respectively. Over 24 hours, 65–68% of the administered dose was excreted in the urine as the glucuronide conjugate of menthol. Four metabolites were identified in plasma and 32 metabolites in urine samples from the 320 mg dose group. The principal metabolite identified in plasma and urine was menthol glucuronide. The other metabolites included mono-and di-hydroxylated menthol derivatives, most of which are excreted, in part, as glucuronic acid conjugates. Sulfate conjugates were also detected in urine and plasma (Hiki et al., 2011).

(b) Menthone (No. 429)

In an in vitro study, (−)-menthone was incubated with human liver microsomes. Two metabolites were identified: (+)-neomenthol (No. 428), the major metabolite, and 7-hydroxymenthone, a minor metabolite (Miyazawa & Nakanishi, 2006).

2.3.2 Toxicological studies

(a) Acute toxicity

For menthol (No. 427) and three other previously evaluated flavouring agents in the group (Nos 430, 1854 and 1856), additional acute toxicity data were available. These are summarized in Table 3 and described below.

In mice and rats, oral LD_{50} values for menthol (No. 427) ranged from 1470 to 2615 mg/kg bw (Steinhoff, 1974a,b; Leuschner, 1980).

Table 3
Results of oral acute toxicity studies with menthol and structurally related substances used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
427	Menthol [a]	Rat; F	2 615	Steinhoff (1974a)
427	Menthol [b]	Rat; F	2 602	Steinhoff (1974b)
427	Menthol [b]	Mouse; M	1 470 c	Leuschner (1980)
430	Isomenthone	Rat: F	2 119	Sanders (1999)
1854	*l*-Menthyl acetoacetate	Rat; F	>2 000	Sanders (2007)
1856	*l*-Piperitone	Rat; F	>2 000	Park (2016)

bw: body weight; F: female; LD_{50}: median lethal dose; M: male; no.: number
[a] Test compound was *l*-menthol.
[b] Test compound was racemic menthol.
[c] Only 2 animals/dose were tested.

In rats, the oral LD_{50} value for isomenthone (No. 430) in a test performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) No. 401 ("Acute Oral Toxicity") was 2119 mg/kg bw; for *l*-menthyl acetoacetate (No. 1854) in a test performed according to OECD TG No. 420 ("Acute Oral Toxicity – Fixed Dose Method") was greater than 2000 mg/kg bw; and for *l*-piperitone (No. 1856) in a test performed according to OECD TG No. 423 ("Acute Oral Toxicity – Acute Toxic Class Method") was greater than 2000 mg/kg bw (Sanders, 1999, 2007; Park, 2016). All three studies were certified for compliance with good laboratory practice (GLP) and quality assurance (QA).

These results were consistent with the findings in the previous evaluations that the oral acute toxicity of the flavourings agents in this group of menthol and structurally related substances is low.

(b) Short-term studies of toxicity

In a study of liver toxicity, male Wistar albino rats (5/group; 120 g) were administered 0 or 5 µg/kg bw of *dl*-menthol (No. 427; 0.6 µg of *dl*-menthol dissolved in 0.25 mL absolute alcohol) intraperitoneally 4 times at weekly intervals. An additional control group of five rats received no treatment.

Treated rats showed a significant decrease in mean body weight from week 4 onwards. All enzymic activities measured were significantly increased in liver homogenate and blood serum of treated rats compared with both the absolute alcohol and untreated control groups. Cholesterol, glycogen and triglyceride levels were also significantly increased in liver homogenates as were glucose levels in blood serum of the treated rats. No numerical data were included in the paper, but the included graphs showed that most increases were less than 2-fold. The exceptions were alanine aminotransferase activity in serum, which

was approximately 10 times higher in treated rats than in the control groups, and succinate dehydrogenase activities in liver homogenates and blood serum, which were approximately 2 times higher. No significant histopathological changes were observed in the livers of the treatment or control groups (Pereira et al., 2007).

The Committee noted that intraperitoneal administration is not a relevant route for human exposure to menthol used as flavouring agent.

(c) Genotoxicity studies

Studies of genotoxicity of menthol and structurally related substances used as flavouring agents are summarized in Table 4 and described below.

(i) In vitro

Bacterial reverse mutation assays

No evidence of mutagenicity was observed when *dl*-isomenthol (No. 2249), (±)-2-[(2-*p*-menthoxy)ethoxy]ethanol (No. 2251), menthol (No. 427), mixtures of menthone and (±)-isomenthone (Nos 429/430), menthyl acetate (No. 431), *l*-piperitone (No. 1856) or 2,6,6-trimethylcyclohex-2-ene-1,4-dione (No. 1857) were incubated with *Salmonella typhimurium* strains TA97a, TA98, TA100, TA102, TA1535, TA1537 and/or *Escherichia coli* WP2*uvr*A in the absence or presence of metabolic activation (Gomes-Carneiro, Felzenszwalb & Paumgartten, 1998; Bowles, 1999; Flügge, 2010; Yoshihiro, 2010; Bowen, 2011; Sokolowski, 2012a,b; Chang, 2016; Kirkland et al., 2016). With the exception of the studies by Gomes-Carneiro, Felzenszwalb & Paumgartten (1998), Yoshihiro (2010) and Kirkland et al. (2016), all the bacterial reverse mutation assays were conducted according to OECD TG No. 471 ("Bacterial Reverse Mutation Test") and were certified for compliance with GLP and QA.

Mammalian cell assays

There were no significant increases in mutant frequency at the *hprt* locus of V79 Chinese hamster cells exposed to a mixture of 76.1% menthone and 23.5% (±)-isomenthone (Nos 429/430), a mixture of 84% menthone and 15.7% (±)-isomenthone (Nos 429/430) or menthyl acetate (No. 431), with and without S9 metabolic activation (Morris, 2013a,b; Wollny, 2013). Similarly, L5178Y mouse lymphoma cells showed no significant increases in mutant frequency in the thymidine kinase locus (*tk*) when exposed to (±)-menthone 1,2-glycerol ketal (No. 445) in the absence or presence of an S9 metabolic activation system (Wollny, 2008). The gene mutation assays were conducted according to OECD TG No. 476 ("In vitro Mammalian Cell Gene Mutation", 1998) and were certified for compliance with GLP and QA.

Table 4

Studies of genotoxicity with menthol and structurally related substances used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2249	*dl*-Isomenthol	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	3.16–1 000 µg/plate, ±S9	Negative [a]	Flügge (2010)
2251	(+)-2-[(2-*p*-Menthoxy) ethoxy]ethanol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2*uvr*A	0.61–1 250 µg/plate, ±S9	Negative [b]	Yoshihiro (2010)
427	Menthol [c]	Reverse mutation	*S. typhimurium* TA97a, TA98, TA100 and TA102	5–800 µg/plate, ±S9	Negative [d]	Gomes-Carneiro, Felzenszwalb & Paumgartten (1998)
427	Menthol	Reverse mutation	*S. typhimurium* TA102	15–5 000 µg/plate, ±S9	Negative [e]	Kirkland et al. (2016)
427	Menthol [c]	Chromosomal aberration	CHL fibroblast cells	0.1, 0.2 and 0.3 mg/mL, ±S9	Negative	Matsuoka, Hayashi & Sofuni (1998)
427	Menthol	Comet assay	CHO cells	6.25, 12.5, 25, 50 and 100 µg/mL [f] 62.5, 125, 250, 500 and 1 000 µg/mL [g]	Negative	Kiffe, Christen & Arni (2003)
427	Menthol	Micronucleus induction	Mouse lymphocytes	50, 100, 125, 150, 175, 200 and 250 µg/mL, –S9 [h] 50, 100, 150, 200, 215, 225 and 230 µg/mL, +S9 [h] 40, 60, 80, 100, 150, 175 and 200 µg/mL, –S9 [i]	Negative [j]	Olivo (2016)
427	Menthol	Micronucleus induction	V79, CHL, CHO, HuLy, TK6 and HepG2 cells	Not reported, –S9	Negative	Fowler et al. (2012)
429/430	Menthone (76.1%)/ (±)-isomenthone (23.5%)	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvr*A	3–5 000 µg/plate, ±S9	Negative [k]	Sokolowski (2012a)
429/430	Menthone (84%) / (±)-isomenthone (15.7%)	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvr*A	1–5 000 µg/plate, ±S9	Negative [l]	Sokolowski (2012b)
429/430	Menthone (76.1%) / (±)-isomenthone (23.5%)	Gene mutation (*hprt*)	Chinese hamster V79 cells	22.5–360 µg/mL, ±S9	Negative [m]	Morris (2013a)
429/430	Menthone (84.5%)/ (±)-isomenthone (15.1%)	Gene mutation (*hprt*)	Chinese hamster V79 cells	12.5–300 µg/mL, ±S9	Negative [n]	Wollny (2013)
429/430	Menthone (76.1%) /(±)-isomenthone (23.5%)	Micronucleus Induction	Human peripheral blood lymphocytes	385.8, 771.5 and 1 543.0 µg/mL, –S9 [h] 96.4, 192.9 and 385.8 µg/mL, +S9 [h]	Negative [p]	Bohnenberger, (2013a)

Table 4 (continued)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
				96.4, 192.9 and 385.8 µg/mL, −S9 [c]		
				200.0, 300.0 and 400.0 µg/mL, −S9 [c]		
				300.0, 600.0 and 700.0 µg/mL, +S9 [h]		
431	Menthyl acetate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	5–5 000 µg/plate, ±S9	Negative [q]	Bowles (1999)
431	Menthyl acetate	Gene mutation (*hprt*)	Chinese hamster V79 cells	1.88–120 µg/mL, ±S9	Negative [r]	Morris (2013b)
431	Menthyl acetate	Micronucleus Induction	Human peripheral blood lymphocytes	120.8, 647.5, 1 133.1 and 1 983.0 µg/mL, ±S9 [h]	Negative [s]	Bohnenberger (2013b)
				39.5, 69.0 and 120.8 µg/mL, −S9 [c]		
				120.0, 140.0, 160.0 and 180.0 µg/mL, −S9 [c]		
445	(±)-Menthone 1,2-glycerol ketal	Gene mutation (tk+/−)	L5178Y mouse lymphoma cells	9.0–576.0, ±S9	Negative [t]	Wollny (2008)
1856	l-Piperitone	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvr*A	3–5 000 µg/plate, ±S9	Negative [u]	Chang (2016)
1857	2,6,6-Trimethylcyclohex-2-ene-1,4-dione	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.32–5 000 µg/plate, ±S9	Negative [v]	Bowen (2011)
1857	2,6,6-Trimethylcyclohex-2-ene-1,4-dione	Micronucleus Induction	Human peripheral blood lymphocytes	500, 1 000 and 1 522 µg/mL, −S9 [w]	Negative	Lloyd (2011)
				1 000, 1 250 and 1 522 µg/mL, +S9 [w]		
				300.0, 420.0 and 550.0 µg/mL, −S9 [i]		
In vivo						
427	Menthol	Comet assay	Sprague Dawley rats [Crl:CD(SD)]; M	500, 1 000 and 2 000 mg/kg bw per day	Negative [x]	Uno et al. (2015); Wada et al. (2015)
429	Menthone [y]	Micronucleus induction	NMRI mice; M, F	500, 1 000 and 2 000 mg/kg bw	Negative [z]	Honarvar (2009)

bw: body weight; CHL: Chinese hamster lung; CHO: Chinese hamster ovary; F: female; *hprt*: hypoxanthine–guanine phosphoribosyltransferase locus; M: male; S9: 9000 × *g* supernatant fraction from liver homogenate; *tk*: thymidine kinase locus

[a] Two independent experiments using the preincubation method and the plate incorporation method, respectively. Cytotoxicity was observed at the highest dose tested in all strains.

[b] Pre-incubation assay. The dose levels tested were 9.77–313 µg/plate (TA100 and TA1535), 0.61–19.5 µg/plate (TA98 and TA 1537) and 39.1–1250 µg/plate (WP2*uvr*A) in the absence of S9;, and 9.77–313 µg/plate (TA98, TA100, TA1535 and TA1537) and 39.1–1250 µg/plate for WP2*uvr*A in the presence of S9. Toxicity was observed from 9.77 µg/plate (TA98 and TA1537), 156 µg/plate (TA100 and TA1535) and 625 µg/plate (WP2*uvr*A) in the absence of S9. In the presence of S9, toxicity was observed at 625 µg/plate for WP2*uvr*A and from 156 µg/plate in all strains except WP2*uvr*A. Since a minimum of four non-toxic dose levels was not achieved for strains TA98 and TA1537, an additional experiment was conducted for these strains. A statistically significant increase in revertants was seen in TA1535 in the absence of S9 at 19.5 µg/plate. This was not confirmed in an additional study for this strain. Also, since this increase was observed at an intermediate dose level only, it was not considered to be toxicologically relevant.

[c] Test compound was (–)-menthol.
[d] Plate incorporation assay. In the absence of S9, (–)-menthol was tested at concentrations up to 700 µg/plate in TA97a, up to 600 µg/plate in TA98 and TA100 and up to 400 µg/plate in TA102. Cytotoxicity was observed in TA97a at the highest dose tested. In the presence of S9, (–)-menthol was tested at concentrations up to 700 µg/plate for TA100, 800 µg/plate for TA97a and TA98 and 500 µg/plate for TA102. Toxicity was observed in TA97a, TA98 and TA102 at the highest dose tested. The experiment was repeated at least once.
[e] Plate incorporation assay. Cytotoxicity was observed from concentrations of 1500 µg/plate onwards.
[f] Standard comet assay. Cytotoxicity was observed at 100 µg/mL.
[g] Modified "all cell" comet assay. The media containing the test compound and the media used for the washing steps were retained and cells were collected by centrifugation and combined with the cells by trypsination for electrophoresis. Cytotoxicity was observed at 500 and 1000 µg/mL.
[h] Incubation for 4 hours.
[i] Incubation for 24 hours.
[j] Two independent experiments were conducted. The study focused on statistical evaluation of the results; no detailed results were provided.
[k] Two independent experiments were conducted, a plate incorporation assay and a preincubation assay. At higher concentrations, the background growth was reduced and toxicity was observed in the presence and absence of S9 in all strains except WP2*uvr*A where no toxicity was observed in the absence of S9 using the plate incorporation method.
[l] Two independent experiments were conducted, a plate incorporation assay and a preincubation assay. At higher concentrations, the background growth was reduced and toxicity was observed in the presence and absence of S9 in all strains.
[m] Two independent experiments were conducted. In the first experiment, Chinese hamster V79 cells were exposed for 4 hours. In the second set of experiments, cells were exposed to the test substance at 45–360 µg/mL for 4 hours in the presence of S9 and for 24 hours in the absence of S9. The doses were based on a preliminary toxicity test.
[n] Two independent sets of experiments were conducted. In the first experiment, Chinese hamster V79 cells were exposed for 4 hours to the test substance at 12.5–200 µg/mL in the absence of S9 and at 25–400 µg/mL in the presence of S9. In the second set of experiments, cells were exposed for 4 hours in the presence of S9 and for 24 hours in the absence of S9, and the dose range tested was 25–400 µg/mL. The doses were based on a preliminary toxicity test.
[o] Exposure for 20 hours.
[p] Three independent experiments were conducted. In the first, human peripheral blood lymphocytes were exposed for 4 hours, in the presence and absence of S9, followed by a 16-hour recovery period. In the second experiment, the cells were exposed continuously for 20 hours in the absence of S9. In the third experiment, the cells were exposed continuously for 20 hours in the absence of S9 and 4 hours with 16-hour recovery period in the presence of S9. In the first experiment, in the absence of S9 mix, one single statistically significant increase in the number of micronucleated cells was observed at the highest evaluated concentration. This value was in the range of the historical control data and therefore not considered to be toxicologically relevant.
[q] In two independent experiments using the plate incorporation method, toxicity was observed in TA100 and TA1535 at concentrations of 1500 and/or 5000 µg/plate in the absence and presence of S9.
[r] Two independent experiments were conducted. In the first experiment, Chinese hamster V79 cells were exposed for 4 hours to menthyl acetate at 1.88–60 µg/mL in the absence of S9 and 7.5–120 µg/mL in the presence of S9. In the second experiments, the cells were exposed to 10–100 µg/mL for 4 hours in the presence of S9 and for 24 hours in the absence of S9. The doses were based on a preliminary toxicity test.
[s] Three independent experiments were conducted. In the first experiment, human peripheral blood lymphocytes were exposed for 4 hours, in the presence and absence of S9, followed by a 16-hour recovery period. In the second experiment, the cells were exposed continuously for 20 hour in the absence of S9 and for 4 h, followed by a 16-h recovery period, in the presence of S9. In the third experiment, the cells were exposed continuously for 20 h in the absence of S9.
[t] Two independent experiments were conducted. In the first set of experiments, L5178Y mouse lymphoma cells were exposed for 4 h to the test substance at 9.0–144.0 µg/mL in the absence of S9 and 18–288 µg/mL in the presence of S9. In the second set of experiments, cells were exposed for 4 h at 36–576 µg/mL in the presence of S9 and for 24 h at 9–144 µg/mL in the absence of S9. The doses were based on a preliminary toxicity test.
[u] Two independent assays were conducted, a plate incorporation and preincubation method. Concentrations tested ranged from 3–5000 µg/plate for all strains in both experiments, except for TA1535 and TA1537, which were tested at 10–5000 µg/plate in experiment 2. Toxicity was observed in all strains starting at concentrations ranging from 1000–5000 µg/plate. In TA1535, no toxicity was observed in the absence of S9.
[v] Two independent experiments were conducted using the plate incorporation method in the absence of S9, and using the plate incorporation method (experiment 1) and the preincubation method (experiment 2) in the presence of S9. Concentrations tested were 0.32–5000 µg/plate (experiment 1) and 156.3–5000 µg/plate (experiment 2). Toxicity was observed at 1000 and/or 5000 µg/plate in strains TA102 and TA1535 in the presence of S9 in experiment 1, and at 2500 and/or 5000 µg/plate in TA102 in the absence and presence of S9.
[w] Cells were treated for 3 hours followed by a 21-hour recovery period.
[x] The test compound was administered by oral gavage at 0, 24 and 45 hours. Animals were killed 3 hours after the final administration and liver and glandular stomach removed and examined.
[y] The test compound was l-menthone.
[z] One single dose was administered by oral gavage. Bone marrow was harvested 24 hours after administration for all three doses and 48 hours after administration for the highest dose only.

In micronucleus assays performed according to OECD TG 487 ("In Vitro Mammalian Cell Micronucleus Test", 2010), no increases in the frequency of micronucleated binucleated (MNBN) cells were observed in cultured human peripheral blood lymphocytes after exposure to a mixture of 76.1% menthone and 23.5% isomenthone (Nos 429/430), menthyl acetate (No. 431) or 2,6,6-trimethylcyclohex-2-ene-1,4-dione (No. 1857) in the absence of presence of S9 metabolic activation (Lloyd, 2011; Bohnenberger, 2013a,b). The studies were certified for compliance with GLP and QA.

No increase in the frequency of micronucleated binucleated (MNBN) cells was observed when menthol (No. 427) was incubated with mouse lymphocytes with or without S9 metabolic activation (Olivo, 2016).

In an in vitro micronucleus induction assay, menthol (No. 427) was incubated in six different rodent and human cell lines for 3 hours in the absence of S9. The concentrations tested were determined by range-finding experiments that assessed cytotoxicity, but no details on the concentrations used were provided. No increase in the frequency of micronucleated binucleated (MNBN) cells was observed in any of the cell lines (Fowler et al., 2012).

In a chromosomal aberration study, *l*-menthol (No. 427) was incubated with Chinese hamster lung fibroblast cells in the absence or presence of an S9 metabolic activation system. No increase in the frequency of aberrant cells was measured (Matsuoka, Hayashi & Sofuni, 1998).

In a study comparing the standard comet assay to a modified all cell comet assay, *dl*-menthol did not significantly affect DNA migration in either the standard or modified assays in Chinese hamster ovary cells (Kiffe, Christen & Arni, 2003).

(ii) In vivo

In a study performed using study protocol version 14.2 from the Japanese Center for the Validation of Alternative Methods (JaCVAM) with OECD TG No. 489 ("In vivo Mammalian Alkaline Comet Assay", 2014) for statistical analysis, Sprague Dawley [Crl:CD(SD)] rats (5 males/group) were administered menthol (No. 427) at 0, 500, 1000 or 2000 mg/kg bw per day by oral gavage at 0, 24 and 45 hours. Three hours after the final administration, the rats were killed and the liver and glandular stomach tissues excised.

Clinical signs observed included wet fur in one mid-dose rat and ananastasia (inability to stand up), ataxic gait and a decrease in spontaneous motor activity in one or more high-dose rats. Histopathological findings in liver and glandular stomach in rats at 2000 mg/kg bw per day dose group (only control and high-dose animals were tested) included diffuse hepatocellular hypertrophy and vacuolation and increased hepatocyte mitotic figures and ulcers and erosion

in the pyloric mucosa. The comet assay showed no significant differences between the treated and vehicle control groups in DNA damage in the liver and stomach tissues (Wada et al., 2015; data also reported in Uno et al., 2015).

In an in vivo mouse micronucleus induction assay performed according to OECD TG 474 ("Mammalian Erythrocyte Micronucleus Test") and certified as QA and GLP compliant, NMRI mice (5/sex per dose group) were administered a single dose of 0 (corn oil vehicle), 500, 1000 or 2000 mg/kg bw of *l*-menthone (No. 429) by oral gavage. Bone marrow was sampled 24 hours after administration of the test compound. In an additional group administered 2000 mg/kg bw, bone marrow was sampled 48 hours after administration.

One high-dose mouse died following administration of the test substance. Clinical signs included reduction of spontaneous activity in males and females at 1000 and 2000 mg/kg bw and ruffled fur in males and females at 500 mg/kg bw and higher doses. There was no evidence of bone marrow toxicity: the percentages of polychromatic erythrocytes in the treated groups did not differ from that in the vehicle control group and there were no significant increases in the incidences of micronucleated polychromatic erythrocytes in treated groups relative to the vehicle control group (Honarvar, 2009).

The Committee noted that the reported clinical signs indicated that bone marrow was exposed, but that there was no evidence of bone marrow toxicity in the micronucleus assay.

(iii) Conclusion

The additional genotoxicity assays for menthol (No. 427), including bacterial reverse mutation, chromosomal aberration, comet (in vitro and in vivo) and micronucleus assays, gave negative results.

Results of genotoxicity assays with the structurally related substances *dl*-isomenthol (No. 2249), (+)-2-[(2-*p*-menthoxy)ethoxy]ethanol (No. 2251), menthone (No. 429), isomenthone (No. 430), menthyl acetate (No. 431), *dl*-menthone 1,2-glycerol ketal (No. 445), *l*-piperitone (No. 1856) and 2,6,6-trimethylcyclohex-2-ene-1,4-dione (No. 1857) were also negative. These results are consistent with the findings in the previous evaluations that there is no concern for genotoxicity for menthol and structurally related flavouring agents.

(d) Human case study

An 86-year old man presented with neurological (coma, ataxia), cutaneous (skin lesions) and gastrointestinal (heartburn, intermittent diarrhoea) manifestations and was admitted to hospital. In the absence of an obvious cause for the coma and ataxia, a detailed history (including of dietary habits) was obtained. The patient

had ingested two bags of menthol-rich cough droplets per day for 20 years. The patient was advised to stop eating the menthol droplets and recovered fully 6 months after cessation (Baibars et al., 2012).

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Miscellaneous nitrogen-containing substances (addendum)

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1. Evaluation

1.1 Introduction

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated three additional flavouring agents belonging to the group of miscellaneous nitrogen-containing substances, which was evaluated previously. The additional flavouring agents included a triazole moiety with a thiopyridine side-chain, 2-(((3-(2,3-dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine (No.

2235); a benzothiadiazine moiety with a piperidinyl side-chain, (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one (No. 2236); and an *N*-pyrazole- and *N*-thiophene-substituted amide, 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide (No. 2237). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *230*).

The Committee considered whether (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one (No. 2236) belonged to the group of aliphatic and aromatic amines and amides. This group includes the structurally related 3[(4-amino-2,2-dioxido-1*H*-2,1,3-benzothiadiazin-5-yl)oxy]-2,2-dimethyl-*N*-propylpropanamide (No. 2082), which was evaluated at the seventy-sixth meeting (Annex 1, reference *211*). The Committee concluded that the additional flavouring agent should remain in this group. All three flavouring agents that were evaluated at this meeting are reported to be flavour modifiers.

The Committee previously evaluated 16 additional members of this group of miscellaneous nitrogen-containing substances at its sixty-fifth meeting (Annex 1, reference *178*); 14 additional members at its sixty-ninth meeting (Annex 1, reference *190*); two additional members at its seventy-sixth meeting (Annex 1, reference *211*); and two additional members at its seventy-ninth meeting (Annex 1, reference *220*). At each meeting, the Committee concluded that all of the evaluated flavouring agents were of no safety concern at estimated dietary exposures.

None of the additional flavouring agents (Nos 2235, 2236 and 2237) in this group have been reported to occur naturally.

A comprehensive literature search on data on the additional flavouring agents was conducted in the PubMed and Web of Science databases; no studies additional to those submitted by the sponsor were identified.

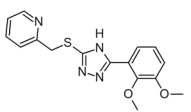
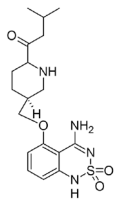
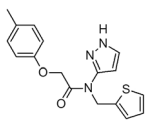
1.2 Assessment of dietary exposure

The total annual volume of production of the three flavouring agents belonging to the group of miscellaneous nitrogen-containing substances is 0.3 kg in the USA. No data on intake were reported for Europe, Japan or Latin America (IOFI, 2017a,b). Each of these flavouring agents contributes equally to the annual production volume in the USA.

Dietary exposures were estimated using both single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method. The highest values are reported in Table 1. The estimated daily dietary exposure

Table 1

Summary of the results of the safety evaluations of miscellaneous nitrogen-containing substances [a,b]

Flavouring agent	No.	CAS no. and structure	*Step 4* [c] Does the highest dietary exposure estimate exceed the threshold of toxicological concern?	*Step 5* [e] Does a NOAEL exist for the flavouring agent or a structurally related substance that provides an adequate margin of exposure?	Conclusion based on current estimated dietary exposure
Structural class I					
2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine	2235	902136-79-2 	Yes, SPET: 800	Yes, The NOAEL of 100 mg/kg bw per day (Karanewsky et al., 2016) is 7 500 times the estimated dietary exposure to No. 2235 when used as a flavouring agent.	No safety concern
(*S*)-1-(3-(((4-Amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl) piperidin-1-yl)-3-methylbutan-1-one	2236	1469426-64-9 	Yes, SPET: 750	Yes, The NOAEL of 100 mg/kg bw per day (Arthur et al., 2015) is 8 000 times the estimated dietary exposure to No. 2236 when used as a flavouring agent.	No safety concern
2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide	2237	1374760-95-8 	Yes, SPET: 600	Yes, The NOAEL of 100 mg/kg bw per day (Karanewsky et al., 2015) is 10 000 times the estimated dietary exposure to No. 2237 when used as a flavouring agent.	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; no.: number; NOAEL: no-observed-adverse-effect level; SPET: single-portion exposure technique

[a] Thirty-four flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *178*, *190*, *211* and *220*).

[b] *Step 2*: All three flavouring agents are in structural class III.

[c] The threshold for toxicological concern for structural class III is 90 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated using the SPET.

[d] The margin of exposure was calculated based on estimated dietary exposure calculated by the SPET.

is highest for 2-(((3-(2,3-dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl) pyridine (No. 2235), at 800 µg/person per day, the SPET value obtained from soups and broths. For each of the three flavouring agents the estimate from the MSDI method was 0.01 µg/day. For Nos 2236 and 2237 the highest SPET estimates were 750 and 600 µg/person per day, respectively.

1.3 Absorption, distribution, metabolism and excretion

Information on the absorption, distribution, metabolism and excretion (ADME) of the flavouring agents belonging to this group has previously been described in the monographs of the sixty-fifth, sixty-ninth, seventy-sixth and seventy-ninth meetings (Annex 1, references *179*, *191*, *212* and *221*). Additional information was made available for this meeting.

Metabolic studies show that Nos 2236 and 2237 have low bioavailability (Arthur et al., 2015; Karanewsky et al., 2016a). The data for No. 2235 showed that it was bioavailable with a short half-life ($t_{½}$) in rat plasma. No. 2235 was shown to be metabolized in toxicokinetic and in vivo metabolism studies in the rat (Karanewsky et al., 2016b). All these additional flavouring agents undergo limited hydrolysis, sulfoxidation or oxidation of the heterocyclic rings or are expected to be excreted unchanged in the faeces or urine (Arthur et al., 2015; Karanewsky et al., 2016a,b).

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. There are no structural alerts for genotoxicity for the three additional flavouring agents (Nos 2235–2237). Chemical-specific genotoxicity data available for each of these flavouring agents indicate that they lack the potential to be genotoxic.

Step 2. All three flavouring agents (Nos 2235–2237) were assigned to structural class III (Cramer, Ford & Hall, 1978).

Step 3. The highest dietary exposures were estimated using SPET (Table 1).

Step 4. The highest dietary exposure estimates of the three flavouring agents in structural class III were above the threshold of toxicological concern (i.e. 90 µg/person per day for class III). These flavouring agents proceeded to Step 5.

Step 5. For these flavouring agents, the no-observed-adverse-effect levels

(NOAELs) of 100 mg/kg body weight (bw) per day in rats, the highest dose tested in 90-day oral toxicity studies (Arthur et al., 2015; Karanewsky et al., 2016b), provide adequate margins of exposure (7500, 8000 and 10 000, respectively) relative to the highest estimated dietary exposure to No. 2235 (SPET = 800 μg/day), No. 2236 (SPET = 750 μg/day) and No. 2237 (SPET = 600 μg/day) when used as flavouring agents. The Committee therefore concluded that Nos 2235–2237 would not pose safety concerns at current estimated dietary exposures.

Table 1 summarizes the evaluations of the three flavouring agents belonging to this group of miscellaneous nitrogen-containing substances (Nos 2235–2237).

1.5 Consideration of combined intakes from use as flavouring agents

The three additional flavouring agents in this group of miscellaneous nitrogen-containing substances have low MSDIs (0.01 μg/day). The Committee concluded that consideration of combined intakes is not necessary because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

1.6 Consideration of additional data on previously evaluated flavouring agents

For previously evaluated flavouring agents in this group, studies of acute toxicity (Nos 1566 and 1889), genotoxicity (No. 1566) and reproductive/developmental toxicity (No. 2161) were available.

1.7 Conclusions

In previous evaluations of flavouring agents in this group of miscellaneous nitrogen-containing substances, biochemical data and studies of acute toxicity, short-term toxicity, long-term toxicity and carcinogenicity, genotoxicity, developmental and reproductive toxicity and thyroid toxicity were available. The estimated dietary exposures and the biochemical and toxicological data available did not raise any safety concerns for the 34 previously evaluated flavouring agents in this group (Annex 1, references *178*, *190*, *211* and *220*).

For the additional flavouring agents in this group, biochemical data (on Nos 2235–2237); studies of short-term toxicity (on Nos 2235–2237); studies on genotoxicity (on Nos 2235–2237 and a metabolite of 2237); and developmental studies (on Nos 2236 and 2237) were available.

The studies available on the previously evaluated and additional flavouring agents in this group available for the present evaluation raised no safety concerns and support the previous safety evaluations.

The Committee concluded that these three flavouring agents, which are additions to the group of miscellaneous nitrogen-containing substances evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of a group of three miscellaneous nitrogen-containing substances used as flavouring agents (Table 1), which are additions to the group of miscellaneous nitrogen-containing substances evaluated previously.

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using SPET for each flavouring agent are reported in Table 2.

2.3 Biological data

2.3.1 Biochemical aspects: ADME

Information related to the ADME of the flavouring agents belonging to the group of miscellaneous nitrogen-containing substances has been described in the reports of the sixty-fifth, sixty-ninth, seventy-sixth and seventy-ninth meetings (Annex 1, references *179*, *191*, *212* and *221*). Additional studies for all of the flavouring agents currently under evaluation are summarized below.

(a) 2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine (No. 2235)

Pharmacokinetic studies have shown 2-(((3-(2,3-dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine (No. 2235; lot no. BP110707; purity >99%) to be rapidly eliminated after intravenous administration in both sexes of rats. Elimination was slower following oral administration.

Table 2

Annual volumes of production of miscellaneous nitrogen-containing substances used as flavouring agents in Europe, the USA, Japan and Latin America

Flavouring agent (No.)	Most recent annual volume (kg) [a]	Dietary exposure				Annual volume in naturally occurring foods (kg)[d]	Consumption ratio [e]
		MSDI [b]		SPET [c]			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio) methyl)pyridine (2235)				800	13	–	NA
Europe	ND	ND	ND				
USA	0.10	0.01	0.000 2				
Japan	ND	ND	ND				
LATAM	ND	ND	ND				
(*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy) methyl)piperidin-1-yl)-3-methylbutan-1-one (2236)				750	13	–	NA
Europe	ND	ND	ND				
USA	0.10	0.01	0.000 2				
Japan	ND	ND	ND				
LATAM	ND	ND	ND				
2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide (2237)				600	10	–	NA
Europe	ND	ND	ND				
USA	0.10	0.01	0.000 2				
Japan	ND	ND	ND				
LATAM	ND	ND	ND				
Total							
Europe	ND						
USA	0.30						
Japan	ND						
LATAM	ND						

LATAM: Latin America; MSDI: maximized survey-derived intake; NA, not available; ND, no intake data reported; SPET: single-portion exposure technique; USFDA: United States Food and Drug Administration; +, reported to occur naturally in foods (Nijssen, van Ingen-Visscher & Donders, 2017), but no quantitative data; –, not reported to occur naturally in foods

[a] From the International Organization for the Flavor Industry (2017a,b). Values greater than zero but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 45×10^6 for Europe, 33×10^6 for the USA, 13×10^6 for Japan and 62×10^6 for LATAM; and where survey correction factor = 0.8 for IOFI Global Poundage Survey and the International Organization of the Flavor Industry's Global Interim Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (IOFI, 2017a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(USFDA standard food portion, g/day) × (highest usual use level) (IOFI, 2017b).
SPET (µg/person per day) calculated as follows:
(µg/day)/body weight), where body weight = 60 kg. Slight variations may occur from rounding.

Table 2 (continued)

[d] Quantitative data for the United States reported by Stofberg & Grundschober (1987).
[e] The consumption ratio is calculated as follows:
(annual consumption via food, kg)/(most recent reported volume as a flavouring substance, kg)

Sprague Dawley rats (4/sex) were administered a bolus intravenous dose of No. 2235 at 1 mg/kg bw in 10% ethanol/20 mmol/L of potassium phosphate buffer (pH 7.4). A single dose of No. 2235 at either 10, 30 or 100 mg/kg bw in 1% methylcellulose (MC) was also given by gavage to Sprague Dawley rats (4/sex per group).

Oral bioavailability (%F) was independent of dose; it was calculated to be 95.5%, 96.0% and 65.7% in males and 67.4%, 116.1% and 113.4% in females at doses of 10, 30 and 100 mg/kg bw, respectively. Plasma concentrations increased less than proportionally with dose following oral administration and peaked at 0.25–0.44 hours across all dose levels in both sexes. Systemically available No. 2235 was rapidly eliminated, with plasma half-lives for male and female rats of 0.19 and 0.16 hours, respectively, after intravenous administration and 0.80–1.7 hours for both sexes after gavage administration, with increasing half-lives at increased dose levels (Karanewsky et al., 2016b).

The toxicokinetic parameters of No. 2235 (lot no. 60287-12-001-R; purity >98.5%) were examined over the course of a 90-day dietary toxicity study in male and female CD[Crl:CD(SD)] rats (section 2.3.2 (b). For the toxicokinetic evaluation, rats (6/sex per dose group, 3 rats/sex in the control group) were given 0 (control), 10, 30 or 100 mg/kg bw per day of No. 2235 by dietary administration. Blood samples were collected on days 7 and 90, after the start of the dark cycle (18:00), from half of the treated animals per group at alternating time points, and at 1 hour from the controls.

The bioavailability of No. 2235 was found to be independent of sex based on mean and individual plasma concentration data from both sexes. Normalized area under the concentration–time curve (AUC) for female-to-male exposure ratios (accumulation ratios) ranged from 0.445 to 2.22. Therefore, toxicokinetic parameters were calculated from the combined mean plasma concentrations of both sexes. Peak plasma concentrations (C_{max}) increased greater than proportionally with dose (1 : 3 : 10) on day 7 (1 : 7.5 : 22.5), though proportionality was similar with dose on day 90 (1 : 3.7 : 12.3). C_{max} values were reached by 6 hours into the dark cycle for the low and mid doses on both days, and by 12 hours and 3 hours on day 7 and 90, respectively, for the high dose. The dose-normalized systemic concentration ($AUC_{0–24\,h/dose}$) was greater on day 90 than on day 7. Dose-

normalized accumulation ratios between days 7 and 90 ranged from 1.35 to 1.86 (Karanewsky et al., 2016b).

An in vitro metabolism study of No. 2235 (lot no. BP110707; purity >99%) was conducted using rat (Sprague Dawley) and human liver microsomes to determine the similarity in metabolic profile.

In 10 µmol/L of No. 2235 samples incubated at 37 °C for 60 minutes with rat or human microsomes in the presence of 1.3 mmol/L of nicotinamide adenine dinucleotide phosphate (reduced) (NADPH), about 18.4% and 54.9%, respectively, of the parent substance remained unchanged, based on the total peak area of the mass spectrum. Incubation with the rat microsomes resulted in two major metabolites, a sulfoxide via thioether oxidation metabolite, 2-(((3-(2,3-dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)sulfinyl)methyl)pyridine, and a 4′ -hydroxylated phenyl metabolite, 2,3-dimethoxy-4-(5-((pyridin-2-ylmethyl)thio)-1*H*-1,2,4-triazol-3-yl)phenol. These metabolites represented approximately 18.6% and 10.5%, respectively, of the initial parent peak area. Incubation with human microsomes resulted in an *O*-desmethyl metabolite, 2-methoxy-3-(5-((pyridin-2-ylmethyl)thio)-1*H*-1,2,4-triazol-3-yl)phenol, and a sulfoxide metabolite, 2-(((3-(2,3-dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl) sulfinyl)methyl)pyridine. These metabolites represented approximately 13.8% and 6.5%, respectively, of the initial parent peak area. Other metabolites were also detected at lower levels in both rat and human microsomal incubations. One of the major rat microsome metabolites, the 4′-hydroxylated phenyl metabolite, and one of the major human microsome metabolites, the *O*-desmethyl metabolite, were detected at relatively low levels in the microsomal incubations of the opposite species (~1.8% and 2.3%, respectively) (Karanewsky et al., 2016b).

In vivo metabolism of No. 2235 (lot no. BP110707; purity >99%) was also assessed in Sprague Dawley rats (4/sex). A single oral dose of 100 mg/kg bw in 1% methylcellulose was administered by oral gavage. Blood samples were collected 0, 0.25, 0.5, 1, 2, 4, 8 and 24 hours after dosing.

The Phase I metabolites detected in plasma included products of thioether oxidation and demethylation and hydroxylation of the phenyl moiety. The major metabolite in plasma after 1 hour was the sulfoxide of the parent compound (43.4%). The parent compound was detected at approximately 55.5%. All other metabolites, including the mono-*O*-desmethyl and 4′-hydroxyphenyl metabolites, were also present at lower concentrations (<0.45%) (Karanewsky et al., 2016b).

In summary, the data show that 2-(((3-(2,3-dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine (No. 2235) is orally bioavailable in

the rat (65–116%), with a short half-life following single dose intravenous administration (0.16–0.19 hours) and single dose gavage administration (<1.7 hours). In vivo metabolism in the rat was predominantly to the sulfoxide of the parent compound. Incubation with human microsomes resulted in formation of the *O*-desmethyl metabolite at approximately 2-fold higher concentrations than the sulfoxide metabolite.

(b) **(*S*)-1-(3-(((4-Amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl) piperidin-1-yl)-3-methylbutan-1-one (No. 2236)**

The pharmacokinetic profile of No. 2236 (batch no. 57926320; purity >99%) was evaluated in male and female Sprague Dawley rats after either a bolus intravenous injection at 1 mg/kg bw in buffered 10% dimethyl sulfoxide, with blood samples collected at 2, 5, 10 and 30 minutes and 1, 2, 4 and 8 hours after dosing, or after oral administration via gavage at concentrations of 10, 30 or 100 mg/kg bw in 1% methylcellulose, with blood samples collected at 0.25, 0.5, 1, 2, 4, 8 and 24 hours after dosing.

Oral bioavailability was minimal (0.67–0.2% in males and 1.73–0.44% in females) and decreased with dose. After intravenous administration, No. 2236 was rapidly eliminated, with a plasma half-life of approximately 0.3 hours for both sexes. The plasma half-lives of the systemically available substance after oral administration ranged between 1.45 and 4.25 hours, uncorrelated with dose. None of the microsomal metabolites (see below) were identified in the plasma of rats dosed orally at 100 mg/kg (Arthur et al., 2015).

The toxicokinetic parameters of No. 2236 (batch no. 105809368; purity 99.9%) were also assessed as part of a 90-day dietary toxicity study (section 2.3.2 (b)). CD[Crl:CD(SD)] rats (6/sex per treated group, 3/sex controls) were given the equivalent of 0 (control), 10, 30 or 100 mg/kg bw per day of the test substance in the diet. Blood samples were collected on days 7 and 90 from half of the treated animals per group at alternating time points, and at 1 hour from the controls.

The bioavailability of No. 2236 was variable and independent of sex and dose level, with %F ranging between 2.79% and 14.2% on day 7 and between 8.97% and 13.9% on day 90. C_{max} values were low and increased lower than proportionally with dose (1 : 3 : 10) on day 7 (1 : 1.5 : 2.4 in males) and on day 90 (1 : 4.1 : 5.5). The dose-normalized systemic concentration ($AUC_{0–24\ h/dose}$) was greater on day 90 than on day 7, with dose-normalized accumulation ratios between days 7 and 90 ranging from 0.98 to 2.69. $AUC_{0–24\ h/dose}$ and C_{max} were higher on day 90 than on day 7. The dose-normalized accumulation ratios on day 90 compared to day 7 (average of male and female values) for the low, mid and high dose levels were 0.98, 2.04 and 2.69, respectively (Arthur et al., 2015).

The in vitro biotransformation of No. 2236 (batch no. 57926320; purity >99%) was examined following incubation at 37 °C for 60 or 120 minutes with either rat (Sprague Dawley) or human liver microsomes in the presence of NADPH. More than 97% of the test substance remained unchanged and less than 3% of the parent compound was metabolized by microsomes from either species after a 60-minute incubation. Although not present in high concentrations, the metabolites identified included (but were not limited to) mono- and dihydroxylated metabolites, carbonyl reduction metabolites and amide hydrolysis metabolites (Arthur et al., 2015).

In summary, oral bioavailability of (*S*)-1-(3-(((4-Amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one (No. 2236) was low (up to 1.7%) in rat. Half-lives ranged from 1.5 to 4.25 hours. None of the microsomal metabolites were detected in the orally dosed rats.

(c) 2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide (No. 2237)

Pharmacokinetic studies with No. 2237 (batch no. 58705651; purity 99.1%) were undertaken in male and female Sprague Dawley [Crl:CD(SD)] rats and male beagle dogs following either single intravenous or single oral dose administration and in male CD-1 mice following single oral dose administration.

A single intravenous dose of 1 mg/kg bw of No. 2237 solution in polyethylene glycol 400 (PEG400) was given to rats (4/sex) and to male dogs (n = 2). Blood samples were collected at approximately 0, 2, 5, 10 and 30 minutes and 1, 2, 4 and 8 hours after administration.

A single oral dose of 10, 30 or 100 mg/kg bw of No. 2237 in 1% methylcellulose was administered to Sprague Dawley rats (4/sex per dose group) by gavage and to male dogs (3/dose group) in gelatine capsules. A single oral dose of 30 mg/kg bw of the parent compound in 1% methylcellulose was administered to CD-1 mice (28/dose group) by gavage. A separate group of CD-1 mice was given 20 mg/kg bw of the amine hydrolysis product, *N*-(2-thienylmethyl)-1*H*-pyrazol-3-amine (M179), as a hydrochloride salt. Blood samples were taken at 0, 15 and 30 minutes and 1, 2, 4, 8 and 24 hours after oral administration.

The oral bioavailability was independent of dose and ranged between 0.07% and 0.23% in male rats, 0.26% and 0.53% in female rats and 0.75% and 1.26% in male dogs. After intravenous administration, the test substance was rapidly eliminated from plasma, with a half-life of about 15–20 minutes in rats and 30 minutes in dogs. The plasma half-life after oral administration in male and female rats and male dogs was 0.6, 1.5 and 1.9 hours at 10 mg/kg bw, 0.9, 2.2 and 4.5 hours at 30 mg/kg bw and 3.0, 2.9 and 5.3 hours at 100 mg/kg bw, respectively. The systemically available concentration of No. 2237 in rats after oral

administration increased with dose in both sexes, was higher in female rats than in male rats, and reached a maximum within 1 hour. The plasma concentration in dogs was higher than in rats and reached a maximum within 1.7 hours. The bioavailability and plasma concentration of the parent compound in mice was comparable to that of rats.

The toxicokinetics of the amide hydrolysis products of the parent compound were also assessed. The parent compound was rapidly hydrolysed to the carboxylic acid, 2-(4-methylphenoxy)ethanoic acid (M166), and the amine, M179, in both species following intravenous administration. The maximum and the total plasma concentrations of the carboxylic acid after oral administration was higher than the respective concentrations of the parent compound in rats, dogs and mice. Rats differed from dogs and mice in the plasma levels of the amine, which were higher in the rat than the other species (Karanewsky et al., 2015).

An additional assessment of the toxicokinetic profile of No. 2237 was included in a 90-day oral toxicity study in male and female Sprague Dawley [Crl:CD(SD)] rats. Rats (6/sex per dose group, 3 rats/sex in the control group) were given 0 (control), 10, 30 or 100 mg/kg bw per day of No. 2237 by gavage. Single dose administrations demonstrated that hydrolysis of the amide bond in vivo resulted in the corresponding carboxylic acid and secondary amine, and that plasma levels of the parent compound were low in the 90-day study, but plasma levels of the carboxylic acid metabolite were higher. Therefore, toxicokinetic analysis was conducted for the carboxylic acid metabolite of No. 2237, 2-(4-methylphenoxy)ethanoic acid (M166). Blood was collected on days 1, 44 and 90 from all animals at multiple time points after gavage dosing. C_{max} was reached within 1 or 3 hours (T_{max}) after gavage administration regardless of dose or day of study. After M166 reached C_{max}, plasma concentration decreased exponentially with a half-life of between 1.43 and 4.41 hours.

Overall, the total systemically available concentration (AUC_{last}) increased with dose, but less than proportionally, and tended to be higher in females than males. Total plasma concentration increased minimally in female rats between study days 1 and 90, with ratios between 1.07 and 1.19, but was lower on day 90 than on day 1 in male rats, with ratios ranging from 0.413 to 0.701 (Karanewsky et al., 2015).

The in vitro biotransformation of No. 2237 was investigated following incubations of 10 µmol/L of the compound with liver microsomes from rats (Sprague Dawley), rabbits, pigs, dogs and humans for 10, 20 or 60 minutes.

The amide bond hydrolysis product, M179, was detected in microsomal incubations with or without NADPH. Rapid hydrolysis was observed with

rabbit microsomes (<15% parent compound remaining) before the addition of NADPH, indicative of a cytochrome P450 (CYP)-independent metabolic pathway in this species. The rate of CYP-dependent hydrolysis was highest in pig microsomes, followed by those of rat, human and dog, with 0.89%, 1.00%, 3.15% and 7.67%, respectively, of parent compound detected after 60 minutes. In total, 27 metabolites were identified across all species. The amine product of the amide bond hydrolysis, M179, was produced in smaller amounts by human microsomes compared to the microsomes of the other species tested. At time points when the remaining parent compound levels were comparable in the microsomal incubations of different species, the major human metabolites were products of the oxidative cleavage of the thienylmethyl group, hydroxylation of the 4-methylphenoxy moiety and oxidation of the thiophene ring. The metabolite profile in dogs was most similar to that of humans (Karanewsky et al., 2015).

In vivo metabolism of No. 2237 was also assessed in Sprague Dawley rats (4/sex) and in beagle dogs (3 males) after a single oral dose of 100 mg/kg bw in 1% methylcellulose administered by gavage (rats) or in gelatine capsules (dogs). Blood samples were collected at 0.25, 0.5, 1, 2, 4, 8 and 24 hours post dose.

Several Phase I metabolites, in addition to the amide hydrolysis products, were detected in plasma of rats and dogs. These included products of oxidative cleavage of the thienylmethyl group, hydroxylation of the pyrazole moiety, hydroxylation and oxidation of the 4-methylphenoxy moiety to the corresponding alcohol and carboxylic acid, and their secondary hydrolysis products to carboxylic acid/alcohol and dicarboxylic acid. Phase II metabolites identified were glucuronidation conjugates. The major metabolites in rat plasma after 1 hour were the amide hydrolysis products, the carboxylic acid of the 4-methylphenoxy moiety, a hydroxylation product of the pyrazole moiety and its glucuronide conjugate. The major metabolites in dog plasma after 1 hour were the same as for rat plasma, with one additional glucuronide conjugate and no hydroxylation product of the pyrazole moiety. The major metabolites were detected at levels higher than the parent compound after 1 hour. The amine hydrolysis product reached lower maximum concentrations and was more rapidly eliminated than the carboxylic acid or the parent compound in both rats and dogs. Other metabolites were also present at lower concentrations (Karanewsky et al., 2015).

In summary, oral bioavailability of 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide (No. 2237) was low and ranged from 0.07% to 0.53% in rats and from 0.75% to 1.26% in dogs. Plasma half-lives increased with increasing dose and were higher in dogs than rats. Plasma levels of the amine metabolite M179 were higher in rats than dogs. Comparative

in vitro CYP-dependent hydrolysis rates measured using microsomes were pig > rat > human > dog. Major metabolites in rats and dogs include the amide hydrolysis products, the carboxylic acid of the 4-methylphenoxy moiety, a hydroxylation product of the pyrazole moiety and its glucuronide conjugate, and one additional glucuronide conjugate. The major metabolites were detected at levels higher than the parent compound after 1 hour. The amine hydrolysis product reached lower maximum concentrations and had more rapid elimination than the carboxylic acid or the parent compound in both rats and dogs.

2.3.2 Toxicological studies

Information related to the acute toxicity, short-term toxicity, genotoxicity, and reproductive and developmental toxicity of these flavouring agents has been reported since the submission of the most recent monograph (Annex 1, references *179*, *191*, *212* and *221*).

(a) Acute toxicity

An oral median lethal dose (LD_{50}) of 260 ± 40 mg/kg bw in mice was reported for the previously evaluated 5,7-dihydro-2-methylthieno(3,4-d)pyrimidine (No. 1566) (DeProspo & Fegleman, 1974). The acute oral LD_{50} of 3-butenyl isothiocyanate (No. 1889) in rats (Sprague Dawley CD) was greater than 200 mg/kg bw but less than 2000 mg/kg bw (Sanders, 1997).

These new studies are summarized in Table 3.

(b) Short-term studies of toxicity

Short-term toxicity studies were completed for the three new flavouring agents (Nos 2235–2237) belonging to the group of miscellaneous nitrogen-containing substances. These studies are described in detail below and summarized in Table 4.

2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine (No. 2235)

In a 28-day dose range–finding study not conducted according to good laboratory practices (GLP), No. 2235 (lot no. 60287-12-001-R; purity >98.5%) was administered to CD[Crl:CD(SD)] rats (5/sex per dose level) at 0 (control), 10, 30 or 100 mg/kg bw per day in the diet. Body weight and feed consumption were evaluated throughout the 28-day testing period and used to adjust the concentration of the test substance in the feed. Ophthalmoscopic examinations were performed at the beginning and end of the testing period. Blood and urine samples were taken prior to necropsy. At necropsy, organs were weighed.

Table 3

Results of oral acute toxicity studies with miscellaneous nitrogen-containing substances used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1566	5,7-Dihydro-2-methylthieno(3,4-d)pyrimidine	Mice; M/F	260 ± 40	DeProspo & Fegleman (1974)
1889	3-Butenyl isothiocyanate	Rats; M/F	200–2000	Sanders (1997)

bw: body weight; LD_{50}: median lethal dose

Table 4

Results of short-term studies of toxicity with miscellaneous nitrogen-containing substances used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups [a] / no. of animals per group [b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
2235	2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine	Rats; M/F	3/20	Diet	28	100 [c]	Rose (2012); Karanewsky et al. (2016b)
2235	2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine	Rats; M/F	3/20	Diet	90	100 [c]	Rose (2013a); Karanewsky et al. (2016b)
2236	(*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy) methyl)piperidin-1-yl)-3-methylbutan-1-one	Rats; M/F	3/20	Diet	28	100 [c]	Rose (2013b); Arthur et al. (2015)
2236	(*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy) methyl)piperidin-1-yl)-3-methylbutan-1-one	Rats; M/F	3/20	Diet	90	100 [c]	Rose (2013c); Arthur et al. (2015)
2237	2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide	Rats; M/F	3/20	Gavage	28	100 [c]	Diehl (2013); Karanewsky et al. (2015)
2237	2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide	Rats; M/F	3/20	Gavage	90	100 [c]	Diehl (2014); Karanewsky et al. (2015)

bw: body weight; F, female; M, male; no.: number; NOAEL: no-observed-adverse-effect level

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] The NOAEL was identified as the highest dose level tested.

Overall, the test article was well tolerated by the test animals. There were no obvious clinical signs of toxicity. While there was a small decrease in weight gain relative to the control group in female rats at 30 and 100 mg/kg bw per day, the change was not statistically significant.

The NOAEL was 100 mg/kg bw per day, the highest dose tested (Rose, 2012; Karanewsky et al., 2016b).

In a GLP-compliant 90-day dietary toxicity study, No. 2235 (lot no. 60287-12-001-R; purity >98.5%) was provided in the diet to Sprague Dawley [Crl:CD(SD)] rats (20/sex per dose level) at 0, 10, 30 or 100 mg/kg bw per day, based on the results of the Rose (2012) 28-day dose range–finding study. Functional tests, including motor activity assessment, and opthalmoscopic examinations were conducted in all animals pre test and during the final week of test substance administration. On days 14 and 45 and at scheduled kill, samples for haematological and clinical chemistry evaluations were collected. Samples for coagulation evaluations and urine analysis were collected prior to scheduled kill (by asphyxiation before subsequent exsanguination). Major parameters assessed including survival, clinical signs, body-weight gain, feed consumption, organ weights, urine analysis and clinical chemistry values, haematology, organ weights and macroscopic observations.

The histopathology of the following organs and tissues of control and high-dose animals were examined: adrenal glands, aorta, bones with marrow (femur and sternum), bone marrow smear, brain (cerebrum, midbrain, cerebellum, medulla/pons), epididymides, oesophagus, eyes (with optic nerve), gut-associated lymphoid tissue, heart, tibiofemoral joints, kidneys, exorbital lachrymal glands, large intestine (caecum, colon, rectum), larynx, liver, lungs, lymph nodes (mandibular, mesenteric), mammary glands (females), sciatic nerves, ovaries with oviducts, pancreas, pituitary, prostate, salivary glands (mandibular, parotid, sublingual), seminal vesicles (males), biceps femoris, skin, small intestine (duodenum, ileum, jejunum), spinal cord (cervical, lumbar, thoracic), spleen, stomach (glandular, nonglandular), testes, thymus, thyroid gland (with parathyroid glands), tongue, trachea, ureters, urinary bladder, uterus with cervix, aina (females), gross lesions and tissues masses with regional lymph nodes. Histopathology of the target organs, gross lesions and tissue masses with regional lymph nodes of animals in all dose groups was also examined.

Mean body weight in females at 100 mg/kg bw per day was statistically significantly lower (7% lower; $P < 0.05$) than controls beginning at week 5. The animals also consumed 6–14% less feed during that time. The reduced body weight and feed consumption persisted throughout the study period. The same changes were not seen in male rats, and the study authors considered the differences in female body weight to be test substance–related but not adverse due to their small magnitude.

Statistically significant changes in haematological values were as follows: at day 14, low-dose males had a decrease in erythrocyte count and an increase in mean cell haemoglobin (MCH); high-dose males had a decrease in haematocrit;

low-dose females had a decrease in haemoglobin levels on day 14 and a decrease in monocyte count on day 45. Similar changes were not seen on the other sample collection days or in the opposite sex. There were no statistically significant effects on coagulation times. In clinical chemistry values, potassium was increased on day 45 in low- and high-dose male rats, urea nitrogen was reduced on day 14 in low-dose male rats, globulin was increased at the end of the study period in high-dose male rats, and cholesterol was increased on day 45 and at the end of the study period in high-dose male rats. In female rats, potassium was increased in low- and high-dose animals on day 14, phosphorus levels were increased in low-dose animals on day 14, and albumin and triglycerides were decreased in all three dose groups on day 45. Changes were not see in dose groups/sexes not mentioned above. There were no significant differences between groups in urine analysis values.

Macroscopic observations were mild or moderate in nature, and did not show a dose–response relationship. Absolute and relative epididymis weights were decreased in the low-dose males (12%). Relative liver weights were increased in low- and high-dose males. Relative seminal vesicle weights were decreased in low-dose males. Relative liver weights were increased in high-dose females and absolute thymus weights were decreased in high-dose females.

Minimal and mild cardiomyopathy was seen in both control and high-dose males and females in similar numbers. Chronic progressive nephropathy of minimal grade was also seen in the same number of control and high-dose males. One renal tubule carcinoma (amophophilic-vacuolar type) was seen in one low-dose female only. This tumour was determined to be incidental as it occurred in only one animal and one sex at the low dose; no other renal tubule tumours were observed in other animals. Mononuclear cell infiltration of the liver of minimal grade was seen in most of the animals in all groups. Hepatic centrilobular hypertrophy of minimal grade was seen in three high-dose animals but not in control, low- or mid-dose males. One high-dose female had a mild grade C-cell focal hyperplasia of the thyroid gland. One low-dose female had moderate transitional cell hyperplasia, one low-dose female had moderate papillary/nodular transitional hyperplasia of the urinary bladder.

The only test substance–related microscopic findings were minimal degree liver centrilobular hepatocyte hypertrophy in three males at 100 mg/kg bw per day. Centrilobular hepatocyte hypertrophy is usually representative of enzyme induction, which is considered an adaptive response. This effect correlated to increased liver weights in males at this specific dosage.

The NOAEL was 100 mg/kg bw per day, the highest dose level tested (Rose, 2013a; Karanewsky et al., 2016b).

(*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one (No. 2236)

In a non-GLP 28-day dose range–finding study, four groups of CD[Crl:CD(SD)] rats (8/sex per dose group) were administered No. 2236 (batch no. 57670265; purity 99%) in the feed at target dietary dose levels of 0, 10, 30 or 100 mg/kg bw per day. Actual consumption was 0, 8.9–9.4, 26.0–28.2 and 87.1–93.2 mg/kg bw per day, respectively. The study authors assessed body weight, feed consumption, clinical signs, ophthalmoscopic observations, organ weights, clinical chemistry values, survival and microscopic examination of the livers.

There were no statistically significant differences in the parameters evaluated.

The NOAEL was 100 mg/kg bw per day, the highest dose tested (Arthur et al., 2015; Rose, 2013b).

In a GLP-compliant 90-day combined dietary toxicity and toxicokinetic study, No. 2236 (lot no. CMLW-083/13-SS2; batch no. 105809368; purity 99.9%) was assessed in rats according to United States Food and Drug Administration (USFDA) subchronic toxicity testing guidelines (except for recommended dose levels). The test substance was administered in the diet to Sprague Dawley [Crl:CD(SD)] rats (20/sex per dose level) at 0, 10, 30 or 100 mg/kg bw per day, for 90 days, based on the results of the Rose (2013b) 28-day dose range–finding study. Average daily consumption was 0, 10.2–10.4, 30.7–30.8 and 102.5–103.9 mg/kg bw per day, respectively. Parameters evaluated included body-weight gain, survival and organ weights. Clinical signs, functional observational battery, ophthalmoscopic observations, feed consumption, urine analysis were evaluated and haematology, clinical chemistry and macroscopic observations were conducted at necropsy. Satellite groups (6/sex per dose) received the test substance at these dose levels for the toxicokinetic part of the studies (see section 2.3.1).

Histopathological examinations of the following organs and tissues of the control and high-dose animals were completed: adrenal glands, aorta, bone with marrow (femur and sternum), bone marrow smear, brain (cerebrum, midbrain, cerebellum, medulla/pons), epididymides, oesophagus, eyes (with optic nerve), gut-associated lymphoid tissue, heart, tibiofemoral joints, kidneys, exorbital lachrymal glands, large intestine (caecum, colon, rectum), larynx, liver, lungs, lymph nodes (mandibular, mesenteric), mammary glands (females), sciatic nerves, nose (4 sections), ovaries with oviducts, pancreas, pituitary, prostate, salivary glands (mandibular, parotid, sublingual), seminal vesicles (males), biceps femoris, skin, small intestine (duodenum, ileum, jejunum), spinal cord (cervical, lumbar, thoracic), spleen, stomach (glandular, nonglandular), testes, thymus,

thyroid gland (with parathyroid glands), tongue, trachea, ureters, urinary bladder, uterus with cervix, aina (females), gross lesions and tissue masses with regional lymph nodes. Histopathological examinations of target organs, gross lesions and tissue masses with regional lymph nodes were conducted in animals in all dose groups.

Two male rats died: the death of the control male was attributed to urogenital inflammation/obstruction and that of the low-dose male to accidental severe fracture and haemorrhage of the nose. Statistically significant changes in haematological parameters, coagulation times, body weight, urine analysis parameters, organ weights and macroscopic and microscopic organ observations were as follows: a decrease in triglyceride levels in high-dose males; a decrease in urine specific gravity in high-dose males and females; an increase in urine volume in high-dose females; a decrease in creatinine levels in high-dose females at the end of the study; and an increase in brain weights in low-dose females. All the statistically significant findings were considered not test article–related as they were within acceptable biological ranges or known test variability ranges. A mammary gland tumour in a female at 30 mg/kg bw per day and a brain tumour in a male at 100 mg/kg bw per day were considered unrelated to the test substance as no pre-neoplastic lesions were found in other animals and these types of neoplasms have been seen in control group SD rats in previous studies in this testing laboratory.

The NOAEL was 100 mg/kg bw per day, the highest dose level tested (Arthur et al., 2015; Rose, 2013c).

2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide (No. 2237)

In a non-GLP-compliant 28-day dose range–finding study, CD[Crl:CD(SD)] rats (8/sex) were administered No. 2237 (lot no. 58705651; purity 97%) at dose levels of 0, 10, 30 or 100 mg/kg bw per day. The study authors examined a variety of parameters, including survival, clinical signs, body weight, feed consumption, organ weights, clinical chemistry values, other macroscopic observations and histological and histopathological examinations of gross lesions, liver, stomach, duodenum, jejunum and ileum. Some statistically significant findings were noted, but were determined to be not test article–related because they were transitory, not dose responsive, small in magnitude or noted in both control and treated animals.

The NOAEL was 100 mg/kg bw per day, the highest dose tested (Diehl, 2013; Karanewsky et al., 2015).

In a GLP-compliant combined toxicity and toxicokinetic study, No. 2237 (batch no. 10640134; lot no. GXS-2013-052-1; purity 99.3%) was administered

by gavage to Sprague Dawley [Crl:CD(SD)] rats (10/sex per dose level) as a suspension in 1% methylcellulose at 0, 10, 30 or 100 mg/kg bw per day, based on the results of the Diehl (2013) 28-day dose range–finding study. Satellite groups (6/sex per dose group) for toxicokinetic studies (see section 2.3.1(c)) also received the test substance at the same dose levels.

Parameters evaluated included survival, clinical signs, body-weight gain, feed consumption, organ weights and haematological, clinical chemistry, urine analysis and macroscopic observations. Histopathological examinations of the following organs and tissues of control and high-dose animals were completed: adrenal glands, aorta, bone with marrow (femur and sternum), brain, cervix (females), epididymides (males), oesophagus, eye (with optic nerve), gut-associated lymphoid tissue, Harderian glands, heart, kidneys, lachrymal glands, large intestine (caecum, colon, rectum), liver, lungs, lymph nodes (mandibular, mesenteric), mammary glands, sciatic nerves, nasal cavity, ovaries with oviducts (females), pancreas, pituitary, prostate, salivary glands, seminal vesicles (males), skeletal muscle, skin, small intestine (duodenum, ileum, jejunum), spinal cord, spleen, stomach, testes, thymus, thyroid gland, parathyroid glands, tongue, trachea, ureters, urinary bladder, uterus and vagina (females). Histopathological examinations of target organs, gross lesions and tissue masses with regional lymph nodes were conducted in all animals. Functional observational battery and opthalmoscopic examinations of all main study animals were conducted pre test and during the final week of test substance administration. Samples for haematological and clinical chemistry evaluations were collected from all study animals during weeks 1 and 6 and prior to necropsy. Urine analysis and coagulation assessment samples were collected at scheduled kill only.

There were no test substance–related deaths during the study. The study authors reported that the only clinical sign of note was laboured breathing and abnormal breathing sounds observed once (in a single animal) during the study (day 63); these were most likely due to inadvertent aspiration during the gavage procedure, and this finding was considered incidental and not adverse. No test substance–related changes were observed in the functional observational battery or ophthalmoscopic observations. No differences relative to controls were observed in body-weight gain or body weight. Feed consumption was increased in females at 30 mg/kg bw per day from day 22 to 29 and day 43 to 90 (except for days 71–78). Mean corpuscular haemoglobin concentration (MCHC) in high-dose males was increased compared to controls, whereas reticulocyte and platelet levels were decreased during week 1 but not at other time points. Leukocyte levels were increased in mid-dose males before scheduled kill. Erythrocyte counts, haemoglobin levels and haematocrit were decreased in mid-dose animals during week 6. Eosinophil levels were increased in low- and mid-dose females during week 6. Alkaline phosphatase (ALP) activity was decreased in high-dose

males during week 1. Bilirubin was decreased in all dosed males during week 1. Triglyceride levels were increased in high-dose males during week 1. Total protein and albumin/globulin ratio was decreased in high-dose males before scheduled kill. Sodium levels were increased in mid- and high-dose males during week 1 and in low-dose males during week 6. Serum total protein levels were increased in mid-dose females before scheduled kill whereas albumin levels were increased during week 6. Glucose levels were increased in mid- and high-dose females during week 6. Calcium levels were increased in high-dose females during week 1. Phosphorus levels were increased in low-dose females during week 6. Chloride levels were decreased in mid-dose females during week 6. Thyroid gland weights were decreased in low-dose males. Lung weights were increased in high-dose females. No test substance–related effects were noted in other organ weights, haematological parameters, coagulation metrics, clinical chemistry results or urine analysis data. The changes seen were judged not to be test article–related for lack of dose–response relationship, because of their small magnitude, which were within historical control ranges, and because of a lack of consistency across time and between sexes. Macro- and microscopic changes were similar in control and high-dose animals.

Based on these findings, the NOAEL was 100 mg/kg bw per day, the highest dose tested (Diehl, 2014; Karanewsky et al., 2015).

(c) Long-term studies of toxicity and carcinogenicity

No additional information was found.

(d) Genotoxicity

Studies of genotoxicity in vitro and in vivo with miscellaneous nitrogen-containing substances are summarized in Table 5.

In vitro

In vitro reverse mutation studies were reported for the three new flavouring agents (Nos 2235–2237) belonging to the group of miscellaneous nitrogen-containing substances. No evidence of mutagenicity was observed when Nos 2235–2237 were incubated with *Salmonella typhimurium* strains TA98, TA100, TA1535 or TA1537 or *Escherichia coli* WP2*uvr*A in the presence or absence of metabolic activation (Arthur et al., 2015; Karanewsky et al., 2015, 2016b). These studies were all reported to have been conducted in accordance with Organisation for Economic Co-operation and Development Test Guideline Test Guideline (OECD TG) No. 471 and were GLP compliant. An additional in vitro reverse mutagenicity assay was reported on flavouring No. 2237; however, the

Table 5
Studies of genotoxicity with miscellaneous nitrogen-containing substances used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2235	2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2*uvr*A	*S. typhimurium* strains: 16, 31, 63, 130, 250, 500, 1 000, 2 000 µg/plate [a,b] *S. typhimurium* strains: 130, 250, 500, 1 000, 2 000 µg/plate [b,c] *S. typhimurium* strains: 63 (TA1537 only), 130, 250, 500, 1 000, 2 000 µg/plate [a,d] *S. typhimurium* strains: 31, 63, 130, 250, 500, 1 000 µg/plate [c,d] *E. coli* strain WP2*uvr*A: 250, 500, 1 000, 2 000, 5 000 µg/plate [b,d,e]	Negative	Karanewsky et al. (2016b)
2235	2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine	Chromosomal aberration	HPBL	130, 250, 500 µg/mL [a,f] 63, 130, 250 µg/mL [c] 31, 63, 130, 250 µg/mL [a,h,j]	Negative [g]	Karanewsky et al. (2016b)
2235	2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine	Micronucleus induction	Chinese hamster ovary cells (CHO-WBL)	100, 200, 300 µg/mL [a,j] 50, 100, 200 µg/mL [c,j] 16, 31, 63 µg/mL [a,k]	Negative	Karanewsky et al. (2016b)
2236	(*S*)-1-(3-(((4-Amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvr*A	310, 630, 1 300, 2 500, 5 000 µg/plate [b,d,e]	Negative	Arthur et al. (2015)
2236	(*S*)-1-(3-(((4-Amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one	Chromosomal aberration	HPBL	630, 1 300, 2 500 µg/mL [a,f,g] 630, 1 300, 2 500 µg/mL [c,f,g] 130, 250, 500 µg/mL [a,h,g]	Negative [l]	Arthur et al. (2015)
2236	(*S*)-1-(3-(((4-Amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one	Micronucleus induction	Chinese hamster ovary cells (CHO-WBL)	625, 1 250, 2 500 µg/mL [e,f] 625, 1 250, 2 500 [a,m]	Negative	Arthur et al. (2015)
2237	2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvr*A	63, 130, 250, 500, 1 000 µg/plate [b,d,e,n]	Negative	Karanewsky et al. (2015)
2237	2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA97a and *E. coli* WP2*uvr*A	0.3, 1, 3, 10, 30, 100, 300, 1 000 µg/plate [b,d,e]	Negative [o]	Bruce (2017)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
2237	2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide	Chromosomal aberration	HPBL	58, 97, 160 µg/mL [a,f] 1.3, 2.5, 5.0 µg/mL [d,f] 23, 39, 65 µg/mL [a,g]	Negative	Karanewsky et al. (2015)
	N-(2-thienylmethyl)-1*H*-pyrazol-3-amine [p]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA97a and *E. coli* WP2*uvr*A	1.5, 5, 15, 50, 150, 500, 1 500, 5 000 µg/plate [b,e]	Negative	Karanewsky et al. (2015)
	N-(2-thienylmethyl)-1*H*-pyrazol-3-amine [p]	Chromosomal aberration	HPBL	1 130, 1 560, 2 160 µg/mL [a,q] 1 850, 2 000, 2 160 [a,p,r] 1 130, 1 560, 1 850 µg/mL [c,q] 25, 100, 225 µg/mL [a,h]	Positive [s]	Karanewsky et al. (2015)
1566	5,7-Dihydro-2-methylthieno(3,4-d)pyrimidine	Micronucleus induction	HPBL	500, 1 000, 1 520 µg/mL [a,t] 250, 500, 1 400 µg/mL [a,u] 500, 1 000, 1 520 µg/mL [c,t]	Negative	Roy (2016)
In vivo						
2237	2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide	Micronucleus induction	Mice; M,F [v]	500, 1 000, 2 000 mg/kg bw	Negative	Karanewsky et al. (2015)
M179	*N*-(2-thienylmethyl)-1*H*-pyrazol-3-amine [p]	Micronucleus induction	Mice [w]; M, F; bone marrow cells	M: 125, 250, 500 mg/kg bw F: 250, 500, 1 000 mg/kg bw	Negative	Karanewsky et al. (2015)
	N-(2-thienylmethyl)-1*H*-pyrazol-3-amine [p]	DNA damage	Mice [x]; M, F; liver cells	M: 125, 250, 500 mg/kg bw F: 250, 500, 1 000 mg/kg bw	Negative [y]	Karanewsky et al. (2015)

bw: body weight; F: females; HPBL: human peripheral blood lymphocytes; M: males; S9: 9000 × *g* supernatant fraction (metabolic activation)

[a] In the absence of metabolic activation.

[b] Plate incorporation method.

[c] In the presence of metabolic activation.

[d] Preincubation method.

[e] In the absence and presence of metabolic activation.

[f] 3-hour exposure.

[g] Only 200 metaphases were evaluated per concentration, which meets 1997 but not current OECD guidelines.

[h] 20-hour exposure.

[i] The 31 µg/mL concentration was evaluated for the numerical (not structural) chromosomal aberrations only, according to the supplemental information in Karanewsky et al. (2016b).

[j] 3-hour exposure with cells harvested after 19–21 hours.

[k] 19-hour exposure.

[l] Relative mitotic index did not meet 55 ± 5% for 3-hour incubation with and without metabolic activation, but old guidelines recommend targeting 50% with no suggested boundaries.

[m] 20.5-hour exposure.

Table 5 (continued)

[n] The guidelines suggest a maximum plate concentration of 5 mg/plate. Precipitation was noted at the highest dose in the plate incorporation method but no toxicity at the highest doses was noted.
[o] All the background levels of colonies were below historically seen levels from other laboratories. The authors noted that there were greater than 2-fold increases over controls in TA98 with and without metabolic activation, WP2*uvr*A with metabolic control, and TA100 and TA97a without metabolic activation. The Committee judged the study to be inadequate and therefore unreliable.
[p] Amine metabolite of flavouring agent No. 2237.
[q] 4-hour exposure.
[r] Repeated assay.
[s] The positive result was observed only at the highest concentration tested in the 4-hour exposure without metabolic activation. This result was not observed in the 4-h exposure with metabolic activation or in the 20-h continuous exposure without metabolic activation.
[t] 4-hour exposure with 20-hour recovery.
[u] 24-hour exposure with no recovery.
[v] Gavage administration.
[w] Same animals as used in the comet assay below.
[x] Same animals as used in the micronucleus assay above.
[y] 100 cells per animal were scored; 2014 and current OECD guidelines recommend using at least 150 cells per animal.

background number of colonies for TA98 and TA100 was below the historical averages and the Committee did not utilize the data (Bruce, 2017).

For all three of the new flavouring agents (Nos 2235–2237) belonging to the group miscellaneous nitrogen-containing substances and one amine metabolite of No. 2237 (M179), chromosomal aberration studies were reported using human peripheral blood lymphocytes (HPBLs) for short incubations in the presence or absence of an S9 metabolic activation system or longer incubations in the absence of S9 metabolic activation. For all three of the new flavouring agents (Nos 2235–2237), there was no evidence of structural or numerical chromosomal aberrations under the conditions of these studies (Arthur et al., 2015; Karanewsky et al., 2015, 2016b).

In the chromosomal aberration study with M179, the cells treated for 4 hours without an S9 metabolic activation system showed a statistically significant dose-dependent increase in structural aberrations at dose levels of 2000 and 2160 μg/mL (5.5–6%; $P \leq 0.01$) (Karanewsky et al., 2015).

These studies were all reported to have been conducted in accordance with OECD TG No. 473 and were GLP compliant.

After short incubations with Chinese hamster ovary cells (CHO-WBL) in the presence or absence of an S9 metabolic activation system and longer incubations in the absence of S9 metabolic activation, no evidence of micronucleus induction was seen for two of the new flavouring agents, Nos 2235 and 2236, and one previously evaluated flavouring agent, No. 1566, belonging to the group miscellaneous nitrogen-containing substances (Arthur et al., 2015; Karanewsky et al. 2016b; Roy, 2016)

These studies were all reported to have been conducted in general accordance with OECD TG No. 487 and were GLP compliant.

In vivo

2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide (No. 2237)

An in vivo micronucleus induction assay with No. 2237 was conducted according to OECD TG 474. Crl:CD-1(ICR) mice of both sexes were administered No. 2237 suspended in 1% methylcellulose in purified water by gavage with 0, 500, 1000 or 2000 mg/kg bw per day at a volume of 10 mL/kg for 3 consecutive days. The test animals were killed 18–24 hours after the last dose.

All animals appeared normal throughout the study. There were no test substance–related clinical signs or effects on body weight or feed consumption. The ratios of polychromatic erythrocytes to total erythrocytes (PCE/TE) were comparable in the treated groups and the control group. There was also no statistically significant increase in the incidence of micronucleated polychromatic erythrocytes (MNPCEs) in the treated groups. No. 2237 did not induce

micronuclei in CD-1 mice under the conditions of this study (Karanewsky et al., 2015).

N-(2-thienylmethyl)-1*H*-pyrazol-3-amine (M179; amine metabolite of No. 2237)

A combined in vivo micronucleus induction and comet assay with M179 was conducted according to OECD TG 474. Crl:CD-1(ICR) mice were administered M179 suspended in 1% methylcellulose in purified water, by gavage, at a volume of 10 mL/kg for 3 consecutive days. The doses levels of M179 were set at 0, 125, 250 or 500 mg/kg bw per day (males) and 0, 250, 500 or 1000 mg/kg bw per day (females) based on mortality and toxicity in a dose range–finding study. The mice were killed 3–4 hours after the final dose. Bone marrow was used in the micronucleus assay and liver in the comet assay.

Piloerection was seen in all treated animals and lethargy in animals treated at 500 and 1000 mg/kg bw per day. Prostration, irregular breathing and crusty eyes were observed in females at 1000 mg/kg bw per day. The PCE/TE ratios were comparable in the treated groups and the control group. There was no statistically significant increase in the incidence of MNPCEs in the treated groups.

M179 did not induce micronuclei in CD-1 mice under the conditions of this study. However, it is unclear if the test article reached the bone marrow.

In the comet assay, there was no change in the number of clouds or % tail DNA in the liver cells from treated animals (Karanewsky et al., 2015).

Conclusions on Genotoxicity

Bacterial reverse mutation assays were available for the three new members of this group (Nos 2235–2237), all of which had negative results. In vitro chromosomal aberration assays were also available for Nos 2235–2237 and for an amine metabolite of 2237. The assays on the flavouring agents had negative results; however, the results of the assay with the No. 2237 metabolite, M179, were positive. In vitro micronucleus induction assays of Nos 2235 and 2236 and the previously evaluated No. 1566 were negative.

In vivo studies with No. 2237 (micronucleus induction) and one of its metabolites (micronucleus induction and DNA damage using a comet assay) were negative. Based on the results of the in vivo assays and the negative results of the assay with No. 2237, the Committee determined that the weight of evidence supports no risk of genotoxicity for No. 2237.

(e) **Reproductive and developmental toxicity**

(*S*)-1-(3-(((4-Amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one (No. 2236)

The developmental toxicity of No. 2236 (batch no. 105809368; purity 99.9%) was evaluated in rats in dose range–finding and subsequent definitive studies according to USFDA Redbook and OECD TG to evaluate toxicity from impregnation to the day before giving birth. The test substance was administered to the animals to evaluate both maternal toxicity and any correlating effects on embryo/fetal development.

In the dose range–finding study, No. 2236 in 1% methylcellulose was administered via gavage to pregnant Crl:CD(SD) rats (8/dose level) at 0 (vehicle), 125, 250, 500 or 1000 mg/kg bw per day from gestation day 6 through 20. The dams were examined for mortality, morbidity, body weight, feed consumption and other clinical signs of toxicity. At scheduled kill, the uteri, placentae and ovaries were examined and the numbers of fetuses, corpora lutea, early and late resorptions and total implantations counted. The fetuses were examined for malformations and variations.

No toxicity in the dams or fetuses was reported (Arthur et al., 2015).

In the subsequent definitive maternal toxicity and embryo/fetal developmental study, No. 2236 in 1% methylcellulose was administered via gavage to presumed pregnant Crl:CD(SD) rats (25/dose level) at 0 (vehicle), 250, 500 or 1000 mg/kg bw per day from gestation day 6 through 20.

All animals were observed for changes in feed consumption, body weight, mortality and clinical signs. On gestation day 21, the uterus, ovaries and placentae were removed from each female and the number of fetuses was counted. The study authors recorded any early or late deaths of embryos and the numbers of corpora lutea and total implantations. Physical characteristics of maternal rats were examined, including uterine weight and body-weight changes. Fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

All the females survived the 21-day gestation period until the scheduled necropsy. No test substance–related clinical signs were noted at any dose level. All except one low-dose and one high-dose female were gravid. There were no significant differences in the numbers of fetuses, resorptions, implantation losses or corpora lutea (essential for maintaining a healthy pregnancy) or in fetal weight. While small differences in skeletal variations were observed, they occurred at about the same rate in the control group. One set of conjoined twins was noted in the high dose group.

The NOAEL for both maternal toxicity and embryo/fetal developmental toxicity was 1000 mg/kg bw per day, the highest dose tested (Arthur et al., 2015).

2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide (No. 2237)

The developmental toxicity of No. 2237 (batch no. 5870561 and purity 99.1% for the dose range–finding study; batch no. 0640134, lot no. GXS-2013-052-1 and purity 99.3% for the definitive developmental toxicity study) was assessed in a dose range–finding study, followed by a definitive developmental toxicity study.

In the dose range–finding part of the study, Karanewsky et al. (2015) administered No. 2237 (batch no. 5870561; purity 99.1%) in 1% methylcellulose by gavage to presumed pregnant Crl:CD(SD) rats (8/dosage level) at 0 (vehicle), 125, 250, 500 or 1000 mg/kg bw per day from gestation day 6 to 20. The animals were observed for mortality, clinical signs of toxicity, body weight and feed consumption. On gestation day 21, the animals were killed and the uterus, ovaries and placentae of each female were examined. The number of fetuses were counted.

Limited occurrences of clear and/or red material around the mouth were observed at all dose levels about 1 hour after dose administration during gestation days 17–20. Hair loss was also observed at 500 and 1000 mg/kg bw per day throughout the study. Feed consumption was reduced at 500 and 1000 mg/kg bw per day as were initial body-weight gain and final body weight. No remarkable internal findings were noted. All the females were gravid. The mean combined fetal weight in the highest dose group was 8.9% lower than the control group. No other effects on fetal growth, survival or morphology were noted.

Based on the results of this dose range–finding study, No. 2237 (batch no. 0640134; lot no. GXS-2013-052-1; purity 99.3%) in 1% methylcellulose was given via gavage once a day to presumed pregnant Crl:CD(SD) rats (25/dose level; approximately 11 weeks old at time of mating) at 0 (vehicle), 125, 300 or 1000 mg/kg bw per day from gestation day 6 through 20. The definitive study followed the relevant OECD test guidelines.

All rats were observed for survival, morbidity, clinical signs, feed consumption and body weight. On gestation day 21, uterus, ovaries and placentae of each female were removed and examined. The number of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Physical characteristics of dams were examined and overall body weight and uterine weight measured. Fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

All mated females were gravid except for one low-dose and one high-dose female. No females died before scheduled necropsy on gestation day 21. Some red material was observed near the mouth and nose about 1 hour after

administration of the 300 and 1000 mg/kg bw per day doses in 11 and 15 females, respectively. However, these signs were considered nonadverse because they were not seen the following day. No test substance–related clinical signs were observed in any treatment group.

All mated females were gravid except one at low dose and one at high dose. No females died before scheduled necropsy on 21. Some red material was observed near the mouth and nose about 1 hour after administration of the 300 and 1000 mg/kg bw per day doses in 11 and 15 females, respectively. However, these observations were considered nonadverse because they did not persist into the following day. No test substance–related clinical signs were observed in any treatment group.

Mean body-weight loss and lower feed consumption compared with controls were noted after dosing on treatment day 1 (gestation days 6–7) in the 1000 mg/kg bw per day group. This was followed by a significant mean body-weight gain on gestation days 7–8. The effects on body-weight change and feed consumption were transient and did not affect mean body weight for the overall treatment period; therefore, they were not considered to be adverse. There were no test substance–related changes in net body weight or gravid uterine weight.

No test substance–related clinical signs were observed at any of the three dose levels. Litter sizes and fetal body weight did not significantly differ between groups. Malformations observed in two low-dose and three mid-dose fetuses were not dose related and were within the performing laboratory's historical control ranges. Soft tissue developmental variation (renal papillae not developed or distended) exceeded the performing laboratory's control data for the low and high dose groups, but did not differ statistically significantly from concurrent controls. Other individual skeletal and soft tissue variations were noted in single fetuses. The study authors determined that the reported malformations were unrelated to test article administration.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested, for both maternal toxicity and embryo/fetal developmental toxicity (Karanewsky et al., 2015).

3-(1-((3,5-Dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)-imidazolidine-2,4-dione (No. 2161)

Dose range–finding and definitive developmental toxicity studies were conducted to determine if maternal exposure to No. 2161 from impregnation to one day before expected birth caused developmental toxicity (Karanewsky et al., 2016a). The definitive study followed the relevant OECD test guidelines for developmental toxicity testing.

In the dose range–finding part of the study, No. 2161 in 1% methylcellulose was administered by gavage to mated female CRl:CD(SD) rats (8/dose group;

~12 weeks old) on gestation days 6–20 at 0, 125, 250, 500 or 1000 mg/kg bw per day. The uterus, ovaries and placentae of the females were examined, and the number of fetuses counted. Two females at 1000 mg/kg bw per day delivered on day 21. One female at 500 mg/kg bw per day was nongravid. The study authors reported no test article–related clinical findings but provided no data. Cyclopia (proboscis-like nose, microstomia and bilateral anophthalmia) was noted in one fetus at 250 mg/kg bw per day. The study authors concluded that there were no test article–related effects on fetal or maternal toxicity up to the highest dose tested.

In the definitive part of the developmental toxicity study, No. 2161 (batch no. 110280840; lot no. 140491; purity 99.7%) in 1% methylcellulose was administered daily by gavage to mated Crl:CD(SD) (25/dose level; ~13 weeks old) at 0 (control), 125, 500 or 1000 mg/kg bw per day from gestation day 6 through 20. All animals were observed for mortality, moribundity, clinical signs of toxicity, body weight and feed consumption. On gestation day 21, uterus, ovaries and placentae of each female were examined. The numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. The fetuses were also examined for abnormalities, skeletal malformations and developmental variations. The statistics used for assessment were not described.

All female rats survived to scheduled necropsy on gestation day 21. Two control group females and three at 1000 mg/kg bw per day group were nongravid. The only significant change in mean feed consumption, an increase compared with that of control animals, was seen in high-dose dams on gestation days 8–9. No treatment-related macroscopic observations or clinical signs were noted at any of the dose levels. A nonsignificant non-dose-responsive increase in body weight and body-weight gain were observed in all treated rats compared with controls. A small, apparently dose-dependent but not statistically significant increase in early resorptions and postimplantation losses was noted; however, the values were within the performing laboratory's historical control data range. Female fetal weights for the 125 mg/kg bw per day group were significantly increased. The numbers of fetuses per group were about the same regardless of dose level (n = 320–364). Malformations were observed in 4, 3, 2 and 4 fetuses in the 0, 125, 500 and 1000 mg/kg bw per day dose groups, respectively. One fetus at 1000 mg/kg bw per day had a localized neck oedema; one fetus in the control group and one at 500 mg/kg bw per day had meningoencephalocele; and one fetus at 125 mg/kg bw per day had unilateral microphthalmia. One fetus at 1000 and another at 125 mg/kg bw per day had visceral malformations. The malformations in the pup at 1000 mg/kg bw per day were described as including "situs inversus (trachea, oesophagus, heart, great and major vessels, liver, stomach, pancreas, spleen, kidneys, adrenals, and/or intestine laterally transposed), lobular dysgenesis of the lungs (1 lobe present, bilateral), small atrio-ventricular valve (bilateral),

and interventricular septal defect for both fetuses, and a malpositioned vena cava (coursed between the right adrenal and kidney)." The malformation in the fetus in the 125 mg/kg bw per day group included a small left ventricle. In the control group, one fetus had lobular dysgenesis of the lung and two from the same litter had persistent truncus arteriosus. The study authors noted the rate of malformations were in single pups, at similar rates to the controls, and within the historical range. Visceral developmental variations were similar across treatment groups. Skeletal malformations at 1000 mg/kg bw per day included sternoschisis (1 fetus) and bilaterally bent femur and humerus (1 fetus); at 500 mg/kg bw per day included fused sternebrae (1 fetus); and at 125 mg/kg bw per day included severely malaligned sternebrae (2 fetuses). An increase in 27 presacral ribs at 100 mg/kg bw per day was noted because seven fetuses from one litter (eight in two litters versus one in one control litter) in that dose group had the variation. Skeletal variations were similar across treatment groups.

The NOAEL for both maternal toxicity and embryo/fetal development toxicity was 1000 mg/kg bw per day, the highest dose tested (Karanewsky et al., 2016a).

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Saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids (addendum)

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1. Evaluation

1.1 Introduction

The Committee evaluated two additional flavouring agents belonging to the group of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids that was evaluated previously. The additional flavouring agents were 8-methyldecanal (No. 2238) and 8-methylnonanal (No. 2239). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *230*). Neither of these substances had been previously evaluated.

The Committee evaluated 25 members of this group at its forty-ninth meeting (Annex 1, reference *131*). The Committee concluded that all 25 flavouring agents did not raise any safety concerns at estimated dietary exposures. At its seventy-sixth meeting, the Committee evaluated four additional members of this group of flavouring agents and concluded that all four were of no safety concern at estimated dietary exposures (Annex 1, reference *211*).

8-Methylnonanal (No. 2239) was reported to occur naturally in citrus fruits. 8-Methyldecanal (No. 2238) is not reported to occur naturally (Njissen, van Ingen-Visscher & Donders, 2017)

A comprehensive literature search was conducted in PubMed; no additional relevant studies were identified.

1.2 Assessment of dietary exposure

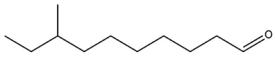
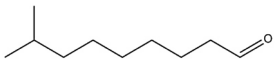
The total annual volume of production of the two flavouring agents belonging to the group of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids is 0.1 kg in the USA and 0.1 kg in Japan (IOFI, 2017a,b). The entirety of the annual production volume in the USA is accounted for by 8-methyldecanal (No. 2238). The entirety of the annual production volume in Japan is accounted for by 8-methylnonanal (No. 2239).

Dietary exposures were estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, with highest values reported in Table 1. The estimated daily dietary exposure is highest for 8-methyldecanal (No. 2238) (0.1 µg/day, SPET value). The highest estimated daily dietary exposure of 8-methylnonanal (No. 2239) is 0.03 µg/day (MSDI value).

Annual volumes of production of this group of flavouring agents and the daily dietary exposures calculated using both MSDI and SPET methods are summarized in Table 2.

Table 1

Summary of the results of the safety evaluations of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids used as flavouring agents [a,b]

Flavouring agent	No.	CAS no. and structure	*Step 4* [c] Does the highest dietary exposure estimate exceed the threshold of toxicological concern?	*Step 5* Does a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?	Conclusion based on current estimated dietary exposure
Structural class I					
8-Methyldecanal	2238	127793-88-8 	No, SPET: 0.1	NR	No safety concern
8-Methylnonanal	2239	3085-26-5 	No, MSDI: 0.03	NR	No safety concern

CAS: Chemical Abstracts Service; MSDI: maximized survey-derived intake; no.: number; NOAEL: no-observed-adverse-effect level; NR: not required; SPET: single-portion exposure technique

[a] Twenty-nine flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *131* and *211*).

[b] *Step 2*: Both flavouring agents are in structural class I.

[c] The threshold for toxicological concern for structural class I is 1800 µg/day, respectively. All dietary exposure values are expressed in µg per day. The dietary exposure value listed represents the highest daily per capita intake calculated either by SPET or MSDI. The SPET gave the highest estimated dietary exposure for No. 2238 and the MSDI gave the highest estimated dietary exposure for No. 2239.

Table 2

Annual volumes of production of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids used as flavouring agents in Europe, the USA, Japan and Latin America

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Annual volume in naturally occurring foods (kg) [d]	Consumption ratio [e]
		MSDI [b]		SPET [c]			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
8-Methyldecanal (2238)				0.1	0.002	–	NA
Europe	ND	ND	ND				
USA	0.1	0.01	0.000 2				
Japan	ND	ND	ND				
LATAM	ND	ND	ND				
8-Methylnonanal (2239)				0.003	0.000 1	+	NA
Europe	ND	ND	ND				
USA	ND	ND	ND				
Japan	0.1	0.03	0.000 4				
LATAM	ND	ND	ND				

Table 2 (continued)

Flavouring agent (No.)	Most recent annual volume of production (kg) [a]	Dietary exposure				Annual volume in naturally occurring foods (kg) [d]	Consumption ratio [e]
		MSDI [b]		SPET [c]			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
Total							
Europe	ND						
USA	0.1						
Japan	0.1						
LATAM	ND						

bw: body weight; LATAM: Latin America; MSDI: maximized survey-derived intake; NA, not available; ND, no intake data reported; no.: number; SPET: single-portion exposure technique; +, reported to occur naturally in foods (Nijssen van Ingen-Visscher & Donders, 2017), but no quantitative data; –, not reported to occur naturally in foods

[a] From the IOFI (2017a,b). Values greater than zero but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1 × 10^9 µg/kg)/population × survey correction factor × 365 days), where population (10%, "eaters only") = 45 × 10^6 for Europe, 33 × 10^6 for the USA, 13 × 10^6 for Japan and 62 × 10^6 for LATAM; and where survey correction factor = 0.8 for IOFI Global Poundage Survey and the Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (IOFI, 2017a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (IOFI, 2013b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/person per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[d] (USFDA standard food portion, g/day) × (highest usual use level) (IOFI, 2017b).
(µg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[e] Quantitative data for the United States reported by Stofberg & Grundschober (1987).

[e] The consumption ratio is calculated as follows:
(annual consumption via food, kg)/(most recent reported volume as a flavouring substance, kg).

1.3 Absorption, distribution, metabolism and excretion

Information on the absorption, distribution, metabolism and excretion (ADME) of the flavouring agents belonging to the group of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids has previously been described in the monograph of the forty-ninth meeting (Annex 1, references *132*). Additional information on the ADME of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids was available for this meeting. The previously described information as well as the additional information available for this meeting, on the ADME of the flavouring agents belonging to this group, are summarized as follows.

The substances in this group share common metabolic pathways, and are expected to be absorbed in the gastrointestinal tract (Dawson, Holdworth & Webb, 1964; Gaillard & Derache, 1965). Shorter branched-chain aliphatic alcohols, aldehydes and acids undergo β-oxidation cleavage, with intermediates metabolized to CO_2 via the tricarboxylic acid cycle. Longer alkyl chain length and branching increases susceptibility to both oxidation and glucuronidation in aliphatic

alcohols, as there is an increase in the affinity for UDP-glucuronosyltransferases (Iwersen & Schmoldt, 1995; Jurowich, Sticht & Käferstein, 2004). Longer and more substituted alcohols produce polar metabolites after undergoing ω-, ω-1 and β-oxidation and selective dehydrogenation and hydration, resulting in chain-shortening (Veenstra et al., 2009). The substances in this group are expected to be reduced to innocuous products via various metabolic pathways, or be excreted in the urine.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. There are neither structural alerts for genotoxicity nor chemical-specific genotoxicity data on the additional flavouring agents. New and previously evaluated data from other related flavouring agents indicate that these flavouring agents are not likely to be genotoxic. Therefore, the weight of evidence indicates that these additional saturated aliphatic acyclic branched-chain primary aldehyde flavouring agents are not likely to be genotoxic.

Step 2. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned both flavouring agents to structural class I (Cramer, Ford & Hall, 1978).

Steps 3 and 4. Dietary exposures using both MSDI method and SPET have been determined. The highest estimated dietary exposures of both flavouring agents in structural class I were below the threshold of concern (i.e. 1800 μg/person per day for Class I). The Committee therefore concluded that both flavouring agents (Nos 2238 and 2239) would not pose a safety concern at current estimated dietary exposures.

1.5 Consideration of combined intakes from use as flavouring agents

The two additional flavouring agents in this group of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids have low MSDIs (0.01–0.03 μg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

1.6 Consideration of additional data on previously evaluated flavouring agents

In the previous evaluation of substances in this group of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids, studies of biochemistry, acute toxicity, short-term and long-term toxicity, reproductive and developmental toxicity and genotoxicity were available (Annex 1, references *131* and *211*). None of the 29 flavouring agents of this group raised safety concerns.

For the present evaluation, no relevant background information was available for the two additional flavouring agents (Nos 2238 and 2239). For previously evaluated flavouring agents in this group, studies of acute toxicity (Nos 251, 252, 253, 254, 258, 260, 267, 268, 269 and 2176), studies of short-term toxicity (Nos 251, 252, 254, 258, 267, 269, 272 and 275), studies of long-term toxicity (No. 252), studies of genotoxicity (Nos 52, 251, 252, 253, 255, 259, 268, 270, 272 and 275), studies of reproductive and developmental toxicity (Nos 251, 252, 267, 268 and 272) and neurotoxicity (No. 251) were available.

There are positive genotoxicity data, not previously evaluated, for isobutyraldehyde (No. 252) in an in vitro and in vivo chromosomal aberration assay, an in vitro sister chromatid exchange (SCE) assay and an in vitro forward mutation assay; and for isobutyric acid (No. 253) in an in vitro forward mutation assay. The bacterial reverse mutation assays, and the in vivo micronucleus assays on isobutyraldehyde (No. 252), were negative. The in vivo chromosomal aberration assay using isobutyraldehyde (No. 252) was only positive at the highest dose, which produced notable signs of cytotoxicity. The negative in vivo results for isobutyraldehyde (No. 252) are consistent with the 2-year inhalational carcinogenicity mouse and rat assays that showed nasal toxicity but no carcinogenicity (Abdo, Haseman & Nyska, 1998; NTP, 1999). Therefore, the weight of evidence indicates that these saturated aldehyde flavouring agents are not likely to be genotoxic. The results of the remainder of the genotoxicity assays on flavouring agents in this group are negative.

The studies available for the present evaluation support the previous safety evaluations.

1.7 Conclusions

The Committee concluded that these two flavouring agents, which are additions to the group of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph addendum summarizes the key data relevant to the safety evaluation of a two saturated aliphatic alicyclic branched-chain primary aldehydes used as flavouring agents (Table 1), which are additions to the group of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids evaluated previously.

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using SPET for each flavouring agent are reported in Table 2.

2.3 Biological data

2.3.1 Biochemical aspects: absorption, distribution, metabolism and excretion

New information relating to the ADME of the flavouring agents belonging to the group of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids has been reported since the submission of the most recent monographs (Annex 1, references *132* and *212*). Additional data not previously reviewed by the Committee are summarized below.

(a) Isobutyl alcohol (No. 251)

In an in vitro metabolic study of aliphatic alcohols, the role of cytochrome P450 enzymes in the oxidation of longer chain or branched alcohols was investigated. Among other aliphatic alcohols, isobutyl alcohol (No. 251) at concentrations 0.5–300 mmol/L was incubated with liver microsomes from Sprague Dawley rats treated with 10% ethanol in drinking-water for 2 weeks. The reaction mixture was incubated at 37 °C for 10 minutes, including a 2-minute preincubation period before addition of nicotinamide adenine dinucleotide phosphate (reduced) (NADPH; 0.5 mmol/L final concentration). Following termination of the reaction and separation from microsomal proteins, the supernatant was mixed with 3-methyl-2-benzothiazolinone hydrazone (MBTH) for 1 hour at room temperature and then incubated with an oxidation mixture (200 mmol/L sulfamic acid and 44 mmol/L ferric chloride) for 20 minutes. The MBTH derivatives were analysed by high-performance liquid chromatography (HPLC).

The authors noted that the oxidation of long and branch-chain alcohols by rat liver microsomes was more efficient with increasing chain length due to higher enzyme affinity with increasing chain length, suggesting potential competitive inhibition of equimolar ethanol oxidation. Isobutyl alcohol (No. 251) was oxidized with an affinity (Michaelis constant; K_m) of 4.5 mmol/L and maximum velocity (V_{max}) of 12 nmol/min per mg microsomal protein. Glucuronidation of isobutyl alcohol was also assessed; K_m was 14.3 mmol/L and V_{max} was 33.3 nmol/min per mg microsomal protein. Ethanol was a competitive inhibitor of oxidation at low concentrations (5–10 mmol/L) and of glucuronidation at high concentrations (300–400 mmol/L) of other aliphatic alcohols (even though ethanol itself was not glucuronidated) (Iwersen & Schmoldt, 1995).

The glucuronidation of short-chained aliphatic alcohols including isobutyl alcohol (No. 251) at nine concentrations between 1 and 256 mmol/L was evaluated in vitro following incubation with human liver microsomes containing uridine diphosphoglucuronosyltransferase (UDPGT) in a reaction mixture for 1 hour. After reaction termination with ethyl acetate, 10 µL of *n*-butylglucuronide was added as an internal standard. The samples were centrifuged and extracted in organic solvent mix, and urea was added to the organic phase residue and analysed by gas chromatography–mass spectrometry (GC-MS).

Isobutyl alcohol was glucuronidated, resulting in 2.5% (volume per volume [v/v]) glucuronide after 3 hours with K_m of 43.7 mmol/L and V_{max} of 1486 nmol/min per mg microsomal protein, respectively. Isobutyl alcohol had a maximum turnover rate 2.5-times lower than isopentyl alcohol. Isobutyl alcohol also showed an affinity for UDPGT that was slightly higher than *n*-butanol and much lower than isopentanol. The study authors concluded that the rate of glucuronidation and affinity for UDPGT in aliphatic alcohols positively correlates with longer alkyl chains (Jurowich, Sticht & Käferstein, 2004).

In an in vivo study on the tissue distribution, metabolism and effects of four butyl alcohols on hepatic microsomal enzyme activity, isobutyl alcohol (No. 251) was given to rats at an oral dose of 500 mg/kg bw (vehicle not described).

Isobutyl alcohol reached peak blood concentration in 45–50 minutes with a peak serum concentration of 35 parts per million (ppm), the lowest of the tested butyl alcohols. Isobutyl alcohol was no longer detected in the blood 2–3 hours after dosage. Isobutyl alcohol in vitro metabolism produced detectable amounts of isobutyraldehyde (No. 252), isobutyric acid (No. 253) and trace amounts of acetone. When administered with pyrazole, an inhibitor of alcohol dehydrogenase, the peak blood concentration of isobutyl alcohol increased 10-fold.

The authors concluded that there was a correlation "between the mode of metabolism and biologic persistence of the various butanols and their differential ability to affect hepatic microsomal enzyme activity". However, no data were shown to support this conclusion and no data were shown on the effect of different butyl alcohols on enzyme activities (Bechtel & Cornish, 1975; abstract only).

The oxidation of small aliphatic alcohols that are typical by-products of ethanol consumption was assessed in enzyme assays with human liver alcohol dehydrogenase class I, II and III at concentrations in the range of the corresponding blood levels following consumption of alcoholic drinks (10–100 µmol/L). Isobutyl alcohol (No. 251) was primarily metabolized by alcohol dehydrogenase class I with K_m of 33 µmol/L and V_{max} of 0.19 µmol/min per mg protein. Isobutyl alcohol had the lowest affinity for alcohol dehydrogenase class I from among the other congeners (K_m of 0.14 mmol/L vs 0.17–0.32 mmol/L). Enzyme inhibition was evident at 1–10 mmol/L. Ethanol was found to be a competitive inhibitor of the oxidation of isobutyl alcohol and other alcohols. In reactions with 100 µmol/L of isobutyl alcohol and 10 mmol/L ethanol (within range of moderate alcohol consumption), the oxidation of isobutyl alcohol was inhibited by 92%, a degree higher than all other congeners evaluated (Ehrig et al., 1988).

(b) **Isobutyric acid (No. 253)**

The conjugation with glycine and carnitine of seven carboxylic acids, including isobutyric acid (No. 253), was evaluated following incubation of 1 mg/mL with rat hepatocytes or kidney tissue in the presence of 2 µmol/mL of ^{14}C-labelled glycine or carnitine.

The branched-chain carboxylic acids, isobutyric acid and pivalic acid (i.e. 2,2-dimethylpropanoic acid) were poor substrates for carnitine and glycine conjugation in both rat hepatocytes and kidney tissues. No carnitine conjugates of isobutyric acid were detectable in incubations of rat hepatocytes and only 0.24% of the ^{14}C-glycine was detected as an isobutyric acid conjugate. In rat kidney tissues, 2.9% of ^{14}C-carnitine and 0.2% of ^{14}C-glycine were conjugated with isobutyric acid (Kanazu & Yamaguchi, 1997).

The blood levels of endogenous free volatile fatty acids were measured in fasted human volunteers. Mean (± standard deviation [SD]; n = 6) levels of isobutyric acid (No. 253) were 30.7 ± 7.9 µg/100 mL serum, 143 ± 30 µg/100 mL plasma and 96 ± 21 µg/100 mL packed red cells. Isovaleric acid (No. 259) was found at 58.3 ± 35 µg/100 mL serum and at 194 ± 80 µg/100 mL plasma (n = 6 participants) but was not detected in packed red cells (Mahadevan & Zieve, 1969).

(c) **2-Ethyl-1-hexanol (No. 267)**

The binding of 2-ethyl-1-hexanol (No. 267), which is a di(2-ethylhexyl) phthalate (DEHP) metabolite, to proteins and nucleic acids was assessed in a pharmacokinetics study of DEHP. Male Fischer 344 rats were administered a single oral dose of 3.7×10^6 Bq of ^{14}C-radiolabelled 2-ethyl-1-hexanol. Radioactivity associated with liver homogenate or protein (190 mg/g liver), RNA (13.5 mg/g liver) and DNA (4 mg/g liver) was measured. Relative to the liver homogenate, 20% of the 2-ethyl-1-hexanol label was found associated with the protein and 2% with RNA fractions, but it was not found associated with DNA. However, DEHP and monoethylhexyl phthalic acid (MEHP) appeared to bind to rat liver DNA in vivo (Albro et al., 1982).

The metabolism of 2-heptanone, a metabolite of 2-ethyl-1-hexanol (No. 267), was evaluated following administration of a single dose of 3.7×10^5 Bq of [2-^{14}C]-2-heptanone (52 µg) to three female Fischer 344 rats via gavage.

Up to 95.5% of administered radioactivity was accounted for. Of that, 64% was exhaled as CO_2 by 24 hours, 15% was found in 24-hour urine samples and 1.5% in 24-hour faeces samples. The remaining radioactivity was detected within 48 hours in the gastrointestinal tract (4.7%), muscle (3.9%), skin (3.4%), liver (1.5%), blood (0.64%) and adipose tissue (0.32%), with lower amounts in bladder, kidneys, ovaries, thymus, pancreas, spleen, lungs, heart and brain. In addition, after administration of 170 mg/kg of unlabelled 2-ethyl-1-hexanol to three rats, the metabolites 2-heptanone (38.6 nmol/mL) and 2-ethylhexanoic acid (122 nmol/mL) were detected in 24-hour urine samples. After dosing three female rats with a single oral dose of 3.7×10^6 Bq of [2-^{14}C]-2-heptanone (520 µg) by gavage, 2-heptanone was found bound to macromolecules (protein and DNA) (Albro, Corbett & Schroeder, 1984).

In a study on the metabolism of aliphatic alcohols, three chinchilla rabbits were given 25 mmol/animal (3 kg bw) of 2-ethyl-1-hexanol (No. 267) in a single oral dose. On average, 86.9% of the dose was found in the urine, excreted as the glucuronide of the corresponding fatty acid (Kamil, Smith & Williams, 1953).

In summary, the substances in this group of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids are expected to be absorbed and reduced to innocuous products via various metabolic pathways or conjugated and excreted in the urine. Shorter branched-chain aliphatic alcohols, aldehydes and acids undergo β-oxidation cleavage, with intermediates metabolized to CO_2 via the tricarboxylic acid cycle. Longer alkyl chain length and branching increases susceptibility to both oxidation and glucuronidation in aliphatic alcohols as

there is an increase in the affinity for UDP-glucuronosyltransferases. Longer and more substituted alcohols produce polar metabolites after undergoing ω-, ω-1 and β-oxidation and selective dehydrogenation and hydration, resulting in chain shortening.

2.3.2 Toxicological studies

Information related to the short-term and long-term toxicity and genotoxicity of these flavouring agents has been reported since the publication of the most recent monographs (Annex 1, references *132* and *212*).

(a) Acute toxicity

Oral median lethal dose (LD_{50}) values have been reported for 10 previously evaluated flavouring agents in this group and are summarized in Table 3. Limited information was available on these data because only summaries are available in most cases. In a number of acute oral toxicity studies, LD_{50} values in male and female rats were in the range of 960 to >5000 mg/kg bw for isobutyl alcohol (No. 251), isobutyraldehyde (No. 252), isobutyric acid (No. 253), 2-methylbutyraldehyde (No. 254), 3-methylbutyraldehyde (No. 258), 2-methylpentanal (No. 260), 2-ethyl-1-hexanol (No. 267), 3,5,5-trimethyl-1-hexanol (No. 268), 3,5,5-trimethylhexanal (No. 269) and 3,7-dimethyloctanal (No. 2176) (Moreno, 1981; Serota, 1984; Nishimura et al., 1994; ECHA, 2017a,b,c,d,e,f,h,l,m,o).

These data demonstrate that the oral acute toxicity of this group of saturated aliphatic alicyclic branched-chain primary alcohols, aldehydes and acids is low.

(b) Short-term studies of toxicity

The results of several short-term toxicity studies with isobutyl alcohol (No. 251), isobutyraldehyde (No. 252), 2-methylbutanal (No. 254), isovaleraldehyde (No. 258), 2-ethyl-1-hexanol (No. 267), 3,5,5-trimethyl-1-hexanol (No. 268), 3,5,5-trimethylhexanal (No. 269), 3,7-dimethyl-1-octanol (No. 272) and 2-methylundecanal (No. 275) are summarized in Table 4. Studies with more weight and detail are described below.

(i) Isobutyl alcohol (No. 251)

In an inhalation study compliant with Organisation for Economic Co-operation and Development (OECD) C(81)30 test guideline, Sprague Dawley rats (10/sex; 20/sex at 0 and 2500 ppm) were exposed to No. 251 (purity 99%) via inhalation at atmospheres of 0, 250, 1000 or 2500 ppm (equivalent to 0, 98, 392 and 979

Table 3

Results of oral acute toxicity studies with saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
251	Isobutyl alcohol	Rats; M, F	>2 830 (M); 3 350 (F)	ECHA (2017b) [a]
251	Isobutyl alcohol	Rats; NR	2 460	Nishimura et al. (1994) [b]
252	Isobutyraldehyde	Rats; NR	960	Nishimura et al. (1994) [b]
253	Isobutyric acid	Rats; M, F	1 874	Serota (1984)
253	Isobutyric acid	Rats; M, F	2 230	ECHA (2017l) [a]
253	Isobutyric acid	Rats; M, F	2 510	ECHA (2017m) [a]
254	2-Methylbutyraldehyde	Rats; M	6 884	ECHA (2017a) [a]
258	3-Methylbutyraldehyde	Rats; M, F	5 740	ECHA (2017o) [a]
260	2-Methylpentanal	Rats; F	>5 000	ECHA (2017c) [a,c]
260	2-Methylpentanal	Rats; M, F	7 900	ECHA (2017d) [a]
267	2-Ethyl-1-hexanol	Rats; NR	2 049	Nishimura et al. (1994) [b]
268	3,5,5-Trimethyl-1-hexanol	Rats; M, F	>2 000	ECHA (2017e) [a]
268	3,5,5-Trimethyl-1-hexanol	Rats; M, F	3 250	ECHA (2017f) [a]
269	3,5,5-Trimethylhexanal	Rats; M, F	>2 000	ECHA (2017h) [a]
2176	3,7-Dimethyloctanal	Rats; NR	>5 000	Moreno (1981) [a]

bw: body weight; F: female; LD_{50}: median lethal dose; M: male; no.: number; NR: not reported

[a] Limited summary information was available; information in the table is as reported in the summary.

[b] Article in Japanese; only abstract was used.

[c] Study reports dosing up to 2000 mg/kg bw and an LD_{50} > 5000

Table 4

Results of oral short-term and long-term studies of toxicity and carcinogenicity with saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids

No.	Flavouring agent	Species; sex	No. of test groups [a] / no. of animals per group [b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
Short-term studies of toxicity							
251	Isobutyl alcohol	Rat; M, F	2/20–1/40	Inhalation	98	979	Li et al. (1999)
251	Isobutyl alcohol	Rats; M, F	3/20	Drinking-water	90	1 251 (F); 1 657 (M)	Schilling et al. (1997)
251	Isobutyl alcohol	Rats; M, F	3/60	Gavage	92	316 [c]	ECHA (2017n)[d]
252	Isobutyraldehyde	Mice; M, F	5/20	Inhalation	91	2.0	Abdo, Haseman & Nyska (1998); NTP (1999)
252	Isobutyraldehyde	Rats; M, F	5/20	Inhalation	91	1.6	Abdo, Haseman & Nyska (1998); NTP (1999)
252	Isobutyraldehyde	Rats; M, F	4/20	Gavage	90	600	Furukawa (2004)
254	2-Methylbutyraldehyde	Rats; M, F	4/20	Gavage	90	1 000	Hokibara (2004)
258	Isovaleraldehyde	Rats; M, F	4/20	Gavage	90	300	Hatano Research Institute (2005a)

No.	Flavouring agent	Species; sex	No. of test groups [a] / no. of animals per group [b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
267	2-Ethyl-1-hexanol	Rats; M, F	3/20	Inhalation	90	88	Klimisch et al. (1998)
269	3,5,5-Trimethylhexanal	Rats; M, F	3/10	Gavage	28	250 [e]	ECHA (2017i) [d]
272	3,7-Dimethyl-1-octanol	Rats; M, F	3/20	Gavage	90	150 (M); 30 (F)[f]	ECHA (2017j) [d]
275	2-Methylundecanal	Rats; M, F	1/24	Diet	84	19.1	Trubek Laboratories (1958) [d]
Long-term studies of toxicity							
252	Isobutyraldehyde	Mice; M, F	3/100	Inhalation	728	2	Abdo, Haseman & Nyska (1998); NTP (1999)
252	Isobutyraldehyde	Rats; M, F	3/100	Inhalation	735	1.6	Abdo, Haseman & Nyska (1998); NTP (1999)

bw: body weight; F: female; M: male; NOAEL: no-observed-adverse-effect level; NOEL: no-observed-effect level

mg/kg bw per day[1]). All experimental groups (excluding the control group) were exposed for 6 hours/day, 5 days/week, for approximately 14 weeks, excluding 1 day of neurobehavioural testing at weeks 4, 8 and 13. Behaviour was assessed using a functional observational battery and automated test of motor activity. Animals were killed after 14 weeks of exposure. For each dosage group, five rats/sex were prepared for neuropathological examination; another five rats/sex per dosage group underwent full histopathological and clinical pathological examinations. Tissues examined microscopically included adrenal glands, brain (5 coronal sections), eyes, heart, kidneys, liver, lungs, nose (3 sections), ovaries, testes, epididymides, skin, spleen, uterus and vagina. The remaining 10/sex control and high-dose groups rats were given a full gross necropsy.

One female rat at 2500 ppm was euthanized for humane reasons after 2 months. The animals at all exposure levels showed a slight decrease in response to external stimuli during exposure, specifically a light brush on the exposure chamber. In addition, a few rats at the highest dose showed a generalized depression of the nervous system in week 1, shown by their lack of response to a strong tap on the exposure chamber during the first 3 days of testing. No treatment-related abnormal clinical signs were observed after exposure at any level; nor were there statistically significant differences between the experimental and control groups in body weight, body-weight change, feed consumption,

[1] Converted as follows, assuming 100% absorption: From X ppm to Y mg/m^3, (X/24.45)*molecular weight (for isobutyraldehyde = 74.123). Adjusted for exposure duration = Y mg/m^3*(hours per day/24)*(days per week/7) = Z mg/m^3. Mg/kg bw per day = Z*60*alveolar ventilation rate (529 mL/min per kg for rat, Brown et al., 1997)*(1 m^3/1 000 000 mL).

absolute organ weight, organ-to-body-weight ratios or organ-to-brain-weight ratios. Similarly, no effects on motor activity or habituation were observed. The moribund female rat at the highest dose level suffered from paralysis of the hind limbs and an enlarged liver and spleen on day 70 of testing and was diagnosed postmortem with lymphoblastic leukaemia with two tumours on her vertebral column, which the study authors concluded were the cause of the paralysis and organ enlargement. Statistically significant increases in red blood cell count, haematocrit and haemoglobin parameters were observed in the highest-dose females. A significant increase in serum calcium levels was observed in the males of the middle exposure group. No other significant differences in the serum chemistry or haematological parameters were observed.

The no-observed-adverse-effect level (NOAEL) was 2500 ppm (equivalent to 979 mg/kg bw per day), the highest dose tested (Li et al., 1999).

In a 90-day oral toxicity study compliant with OECD Section 4 (part 408), Wistar rats (10/sex per dose group) were administered isobutyl alcohol (No. 251, purity 99.8%) via drinking-water at concentrations of 0 (vehicle), 1000, 4000 or 16 000 mg/L (equivalent to 0, 75, 300 and 1251 mg/kg per day for males and 0, 91, 385 and 1657 mg/kg bw per day for females, respectively, based on the authors' calculations). Feed consumption, water consumption and body weight were recorded prior to study commencement and weekly throughout the testing period. Clinical examinations were conducted daily, and ophthalmological, haematological and blood chemistry examinations were conducted at the end of the study. Necropsy, gross pathological assessments, body and organ weights and histopathology on some organs (not specified) were conducted at the end of the study.

No signs of gross toxicity were observed in any test group, although one control animal died on day 42. No changes were reported in organ weights or haematological or clinical chemistry assays. Histopathological examination showed sporadic changes in testes, spleen and kidneys of control and treated animals; these were not considered test article–related.

The NOAEL was 1251 mg/kg bw per day, the highest dose tested (Schilling et al., 1997).

(ii) Isobutyraldehyde (No. 252)

Mice

In a 13-week inhalation study compliant with good laboratory practices (GLP), 6-week-old B6C3F$_1$ mice (10/sex per dose group) were exposed to atmosphere exposures of 0, 500, 1000, 2000, 4000 or 8000 ppm of isobutyraldehyde (No. 252; lot nos. 5-202, E042283; purity 99%) for 6 hours/day, 5 days/week (equivalent

to 0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/kg bw per day [2]). Body weight and clinical observations were recorded weekly. All animals were necropsied, and brain, heart, right kidney, liver, lungs, right testis and thymus of each animal were weighed. All visible lesions and tissue masses were examined. A complete histopathologic examination was performed on all rats from the control and 2000 ppm groups. At 500 and 1000 ppm, the spleen, nose and thymus, which were identified as target organs, were examined in all animals. Sperm samples were collected from all male mice in the 0, 500, 1000, 2000 and 4000 ppm groups at the end of the study and evaluated for concentration, motility and per cent abnormality. Vaginal samples were collected for up to 12 consecutive days prior to the end of the study from all females in the 0, 500, 1000, 2000 and 4000 ppm groups and evaluated for relative frequency of estrous stages and estrous cycle length.

One male in the control group, another at 1000 ppm, nine at 4000 ppm and all at 8000 ppm died before the end of the study. All the females at 4000 and 8000 ppm died before the end of the study. Mean body-weight and body-weight gain of the treated male mice was similar to that of controls. Mean body-weight and body-weight gain of females at 1000 ppm were significantly lower than the controls. Kidney weights (both absolute and relative) in males at 1000 and 2000 ppm were significantly greater than the controls. The absolute liver weight of females at 1000 ppm and both the absolute and relative liver weights of females at 500 ppm were significantly lower than the controls. The absolute thymus weight of females at 1000 ppm and both the absolute and relative liver weights of females at 2000 ppm were significantly lower than the controls. Non-neoplastic lesions of the nasal cavity were found in male and female mice exposed to at least 1000 ppm. No exposure-related gross lesions were observed at necropsy. Because the effects did not appear to be dose related and no corresponding lesions were found, these effects were not considered exposure related.

The NOAEL was 2000 ppm (equivalent to 2.0 mg/kg bw per day) based on premature deaths at 4000 ppm (equivalent to 4.0 mg/kg bw per day) and higher doses (Abdo, Haseman & Nyska, 1998; NTP, 1999).

Rats

In a 13-week inhalation study, F344/N rats (10/sex per dose group) were exposed to 500, 1000, 2000, 4000 or 8000 ppm of isobutyraldehyde (No. 252; lot nos. 5-202, E042283, purity 99% each lot) for 6 hours/day, 5 days/week (equivalent to 0.4, 0.8, 1.6, 3.2 and 6.4 mg/kw bw per day[3] according to the study authors).

[2] Based on equivalent exposures provided for the 2-year carcinogenicity study also in the National Toxicology Program (NTP, 1999) report. The method for deriving this equivalent dose was not described.

[3] Based on calculations provided for the 2-year carcinogenicity study also in the NTP (1999) report. The method for deriving this equivalent dose was not described.

Body weight and clinical observations were recorded weekly. All animals were necropsied, and brain, heart, right kidney, liver, lungs, right testis and thymus of each animal were weighed. A complete histopathological examination was performed on all males from control and 4000 ppm groups and all females from control and 2000 ppm group. Epididymis, larynx, nose, spleen, testis, thymus and trachea were identified as target organs and were examined in all animals at 500, 1000, 2000 and 4000 ppm. Sperm samples were collected from all males at 0, 500, 1000, 2000 and 4000 ppm at the end of the study and evaluated for sperm concentration, motility and per cent abnormality. Vaginal samples were collected for up to 12 consecutive days prior to the end of the study from all females at 0, 500, 1000, 2000 and 4000 ppm and evaluated for relative frequency of estrous stages and estrous cycle length.

All rats at 8000 ppm died before the end of the study. Three male and six female rats at 4000 ppm as well as one female at 500 ppm died before the end of the study. The final mean body-weight of males at 4000 ppm and the mean body-weight gains of males and females at 4000 ppm were significantly lower than the controls. Rats at 4000 and 8000 ppm exhibited abnormal respiratory sounds, decreased activity, nasal discharge, prostration and slowed respiration. Significant changes were noted in relative kidney weight in males at 4000 ppm, liver and thymus weights in males at 4000 ppm and absolute brain weight in females at 4000 ppm; these were judged not to be biologically relevant. Exposure-related gross lesions were not observed. Rats at 8000 ppm exhibited congestion and severe necrosis of the epithelium, occasionally of the entire mucosa, of the nasal turbinates, accompanied by acute inflammation and accumulation of serous or fibropurulent exudate within the nasal passages. At 4000 ppm, males and females exhibited mild epithelial hyperplasia of the mucosa of the nasal cavity and nasopharynx, along with increased incidences of squamous metaplasia and mild acute inflammation. Males at 4000 and 8000 ppm and females at 4000 ppm exhibited mild osteodystrophy of the maxilloturbinates and nasoturbinates, along with inflammation of the overlying mucosa. In all male rats at 2000 and 4000 ppm as well as three female rats at 2000 ppm, minimal to mild degeneration of the olfactory epithelium was observed. Increased frequency of necrosis and degeneration of the larynx and trachea was observed in males at 8000 ppm. Spermatozoal motility at 500 and 100 ppm were reduced, though not in the higher dose groups. Females at 4000 ppm differed from controls in relative time spent in estrous stages. Exposure-related increase in alanine aminotransferase but not sorbitol dehydrogenase was reported.

Based on body-weight changes, survival and incidence and severity of chemical-related lesions observed at higher doses, the study authors determined that 2000 ppm was the maximally tolerated dose for use in the long-term carcinogenicity study. The NOAEL was determined to be 2000 ppm (equivalent

to 1.6 mg/kg bw per day) based on respiratory tract lesions seen at exposure concentrations of 1000 ppm (equivalent to 0.8 mg/kw bw per day) or greater (Abdo, Haseman & Nyska, 1998; NTP, 1999).

In a GLP-compliant 90-day oral toxicity study, CRJ:CD (SD) IGS rats (10/sex per group) were dosed, by gavage, with 0 (vehicle control), 20, 60, 200 or 600 mg/kg bw per day of isobutyraldehyde (No. 252; lot no. 8641192; purity 99.8%) dissolved in 0.5% weight per volume (w/v) sodium carboxymethylcellulose solution containing 0.3% v/v Tween 80. An additional control group received "purified water". The doses were based on the results of a preliminary 14-day study with five rats/sex per dose level of 60, 200 or 600 mg/kg bw per day, where thickening of the limiting ridge in the stomach was observed in females at 200 mg/kg bw and in both males and females at 600 mg/kg bw. The rats underwent ophthalmoscopic examination during acclimation and at the end of the study. The animals were observed twice daily for mortality, overall appearance and clinical signs of toxicity. Body weight and feed consumption were measured weekly. All the animals were fasted prior to scheduled kill, and samples were collected for urine analysis (3-hour samples for pH, protein and glucose and 21-hour samples for other parameters) and assessment of haematological, coagulation and clinical chemistry parameters. At necropsy, all animals underwent gross examination and selected organs were weighed. The following tissues were preserved for subsequent assessment: skin (right abdomen), mammary glands (female only), submaxillary lymph nodes, mesentery lymph nodes, sublingual gland, submaxillary gland, breast bone and right femur (including bone marrow), thymus, trachea, lungs (including bronchi), heart, thyroid gland, parathyroid glands, tongue, pharynx, oesophagus, stomach (proventriculus, glandular stomach), duodenum, jejunum, ileum (including Peyer's patch), appendix, colon, rectum, liver, pancreas, spleen, kidneys, adrenal glands, bladder, seminal vesicles (including coagulating gland), prostate, testes and epididymides, ovaries, uterine tube, uterus (horns and necks), vagina, brain (cerebrum, cerebellum), pituitary, sciatic nerve (right), skeletal muscles (right femur), spine (neck), nasal cavity (turbinate), eyeballs, Harderian glands and Zymbal gland. Preserved tissues from control and high dose groups and of unscheduled deaths were examined microscopically and the stomachs of all animals were examined due to macroscopic findings in high-dose animals. Specimens of liver and/or pancreas from two males in control group, one in the vehicle control and one in the high dose groups were examined microscopically.

There were no treatment-attributable deaths or clinical signs of toxicity. No test substance–related changes were noted in the ophthalmoscopic exam or on body weights, body-weight gain and feed consumption. Statistically significant haematological changes relative to vehicle control group included higher red blood cell count and increased activated partial thromboplastin time (APTT) in males at 20 mg/kg bw, lower white blood cell count in females at 60 mg/kg bw

and higher differential monocyte counts in males at 60 and 200 mg/kg bw; these were judged not to be test article related because of the lack of dose–response relationship. There were no differences in haematological parameter between control and high-dose males or females. No changes in treatment-related clinical chemistry parameters were reported. Significantly lower urine pH was noted in high-dose animals compared to control groups but with no related changes (e.g. in electrolyte levels). No differences were reported in absolute and relative organ weights in males or females at any dose level. At necropsy, thickening of the limiting ridges was observed in the stomach in 8/10 high-dose males and all high-dose females; this was considered treatment related. In the 200 mg/kg bw per day group, mild hyperplasia of the squamous epithelium was found in the forestomach of two males and four females and in the limiting ridges of the stomach of one male. Several other low incidence histopathological changes were reported at the high dose in males and females but with similar numbers in control animals.

The authors identified a NOAEL of 60 mg/kg bw per day based on the forestomach lesions at 200 mg/kg bw per day and higher dose levels. The Committee determined that the effects on the forestomach were likely a localized irritation effect and not relevant to the human safety assessment for use as a flavouring agent. As such, the Committee identified the NOAEL as 600 mg/kg bw per day, the highest dose tested (Furukawa, 2004).

(iii) 2-Methylbutanal (No. 254)

In a 90-day oral toxicity study, CRJ:CD(SD) IGS rats (10/sex per dose group) were dosed with 0 (corn oil; vehicle control), 30, 125, 300 or 1000 mg/kg bw per day of 2-methylbutanal (No. 254; lot no. 8631103; purity not stated) in corn oil by gavage. The dose range was selected based on the results of a preliminary 14-day study at dose levels of 400, 800, 1600 and 3200 mg/kg bw per day, where all animals died at the high dose and severe toxicity was observed at 1600 mg/kg bw per day. No adverse effects were observed at lower dose levels. Concentration in dosing solutions was verified to be within 93.5–109.9% of target. Ophthalmoscopic and fundus examination was performed on all rats before the start and at the end of the study. The animals were observed for clinical signs of toxicity twice daily, before and after dosing. Body weights were measured on day 1 and every 3–4 days throughout the study. Feed consumption was recorded for 3 days before the start of the study and then weekly until scheduled kill. Samples for urine analysis were collected from fasted animals on week 13 (6/sex per group) at 4 and 16 hours. Blood samples (10/sex per group) were collected from all animals fasted for at least 16 hours prior to necropsy, from the jugular vein for haematological and coagulation measurements and from the caudal vena cava for clinical chemistry

measurements. All animals underwent gross observations. Selected organs were weighed. Some organs from all animals were prepared for histopathological examinations, but only the tissues from control and high-dose animals and of unscheduled deaths were examined. The following were histopathologically examined: lungs/bronchi, trachea, heart, aorta, kidneys, urinary bladder, liver, pancreas, submaxillary gland, sublingual gland, parotid gland, tongue/laryngopharynx, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, thymus, spleen, mandibular lymph node, mesenteric lymph node, thyroid gland, parathyroid glands, pituitary, adrenal glands, epididymides, seminal vesicles, coagulation gland, prostate, ovaries, oviducts, uterus, vagina, Harderian glands, skin, mammary glands, cerebrum, cerebellum, medulla oblongata, spinal cord, sciatic nerves, bone and bone marrow (sternum and femur), skeletal muscle (thigh muscle), nasal cavity (nasoturbinate), testes, eyes, optic nerves and Zymbal gland. The stomachs of animals from all dose groups were examined microscopically as a result of the pathological findings in the stomachs of high-dose animals.

There were no treatment-related mortalities. "Flush of the auricles" (presumed to mean redness of the ear) was observed in all males and females at the high dose and in 5/10 males after 25 days at 300 mg/kg bw per day. No test substance–related ophthalmoscopic changes were noted. A transient decrease in body-weight gain in high-dose females was reported between days 36 and 78, with a parallel decrease in feed consumption on days 31–59; other changes were isolated and not dose dependent. Increased mean corpuscular volume was reported in high-dose males. In clinical chemistry, significantly increased triglycerides and albumin: globulin ratio and significantly decreased aspartate transaminase (AST) were reported in males at 1000 mg/kg bw per day. A significant increase of total bilirubin reported in females at 300 mg/kg bw per day was reported. No treatment-related differences were reported in absolute and relative organ weights or macroscopic findings at necropsy in males or females at any dose level, except for isolated instances that were not considered relevant to treatment. The only microscopic observations that were considered attributable to the test substance were limited to squamous cell hyperplasia in the forestomach that was very slight or slight at 125 mg/kg bw per day (6 males and 7 females) and 300 mg/kg bw per day (9 males and 9 females) and moderate in all animals at 1000 mg/kg bw; and accompanied by slight or moderate parakeratosis and hyperkeratosis (3 males at 125 mg/kg bw per day; 2 males at 300 mg/kg bw per day; all animals at 1000 mg/kg bw per day). Associated findings included very slight or slight submucosal oedema in the forestomach of two males at 125 mg/kg bw per day, one male at 300 mg/kg bw and one male and five females at 1000 mg/kg bw per day; and slight erosion in the forestomach in one female at 1000 mg/kg bw. Other findings included very slight squamous cell hyperplasia in one male and two females at 30 mg/kg bw (not

statistically significant); very slight dilation of the fundic gland in the glandular stomach in all treatment groups; and very slight submucosal oedema in the glandular stomach in one male at 125 mg/kg bw per day.

The study authors identified a NOAEL of 30 mg/kg bw per day based on the forestomach findings at higher dose levels. The Committee determined that the effects on the forestomach were likely a localized irritation effect and not relevant to the safety assessment for use of flavouring agents. Based on the lack of other identified effects, the Committee identified the NOAEL as 1000 mg/kg bw per day, the highest dose tested (Hokibara, 2004).

(iv) Isovaleraldehyde (No. 258)

In a 90-day oral toxicity study, CRJ:CD(SD)IGS rats (10/sex per dose group) were dosed with isovaleraldehyde (No. 258; lot no. 8627112; purity 99.4%) in corn oil by gavage at 0 (corn oil; vehicle control), 30, 100, 300 or 1000 mg/kg bw per day. The dose range was selected based on the results of a preliminary 14-day study at dose levels of 30, 100, 300 and 1000 mg/kg bw per day, where no evidence of adverse effects was observed. Concentration in dosing solutions was verified to be within 102–103% of target. The rats underwent ophthalmoscopic and fundus examination before the start and at the end of the study. The animals were observed daily for clinical signs of toxicity, before and after dosing. Body weights and feed consumption were measured on day 1 and then weekly until scheduled kill. Samples for urine analysis were collected from fasted animals on day 84 (5/sex per group) at 4 and 24 hours. Blood samples for haematological, coagulation and clinical chemistry measurements were collected from the abdominal inferior vena cava (10/sex per group) from all animals fasted for 18–24 hours prior to necropsy. At necropsy, all animals underwent gross examination. Selected organs were weighed, and tissues of select organs from control and high-dose animals and those that died prior to treatment end (unscheduled deaths) were prepared for histopathological examination. Histopathological examinations of the following were conducted: tissues with visible changes, brain, pituitary, "pith", "bulbs", optic nerves, Harderian glands, tongue, submandibular gland (including sublingual gland), cervical lymph node, lungs, trachea, bronchus, thyroid gland (including parathyroid glands), thymus, heart, pancreas, liver, kidneys, spleen, adrenal glands, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, mesenteric lymph nodes, bladder, testes, epididymides, seminal vesicle (including coagulation gland), prostate (ventral lobe), ovaries, uterus, oviduct, vagina, femur/bone marrow, breastbone/bone marrow muscle (crus), sciatic nerves, aorta, skin (abdominal) and mammary glands.

The only death, one high-dose male on day 27, was attributed to a dosing error. An increase in salivation was observed immediately after dosing

in males and females at 1000 mg/kg bw per day. No test substance–related changes were noted in the body weights, feed consumption, ophthalmoscopic or haematological examinations or urine analysis. In clinical chemistry, lower AST and alanine transaminase (ALT) levels in all treated males were attributed to one control animal with high values for these enzymes. Increased ALT and AST in females at 30 mg/kg bw per day were driven by values in one animal in this dose group. Increased absolute prostate weight was noted in males at 100 mg/kg bw group. No treatment-related macroscopic findings at necropsy were reported in any dose group. Histopathological observations included diffuse squamous cell hyperplasia of the forestomach in all males and females at 1000 mg/kg bw per day and in one male and one female at 300 mg/kg bw per day. In lungs and bronchi, there was an accumulation of foam cells in the alveoli, mineralization at the arterial wall and periarterial infiltration of eosinophil and mast cells in males and females at 1000 mg/kg bw per day. A focal osseous metaplasia at an alveolus was detected in males at 100 mg/kg bw per day. In the thyroid gland, slight ectopic thymic tissue was detected in one male receiving 1000 mg/kg bw per day. In the kidneys, focal fibrosis in the subcapsule was detected in one male at 1000 mg/kg bw per day and infiltration of lymphocyte and neutrophil in the pelvis and transitional epithelium was detected in one female at 1000 mg/kg bw per day. Histopathological findings in the submandibular gland, heart, liver, pancreas, spleen, prostate and Harderian glands and other findings in the lungs and kidneys were similar in control and treated animals.

The NOAEL was identified by the study author as 100 mg/kg bw per day based on the findings in the forestomach at 300 mg/kg bw per day and higher dose levels. The Committee determined that the effects on the forestomach were likely a localized irritation effect and not relevant to the human safety assessment for use as a flavouring agent. As such, the Committee identified the NOAEL as 300 mg/kg bw per day based on the effects in the lungs and bronchi at 1000 mg/kg bw per day (Hatano Research Institute, 2005).

(v) 2-Ethyl-1-hexanol (No. 267)

In a 90-day inhalation study compliant with GLP and OECD test guidelines, Wistar rats (10/sex per dose group) were exposed to 2-ethyl-1-hexanol (No. 267, purity 99.9%) at atmospheric exposures of 0, 15, 40 or 120 ppm for 6 hours/day, 5 days/week for a total number of 65 exposures over 90 days (equivalent to 0, 11, 29 and 88 mg/kg bw per day [4]). Vapour saturation at 20 °C occurs at 120 ppm.

[4] Converted as follows, assuming 100% absorption: From X ppm to Y mg/m^3, (X/24.45)*molecular weight (for 2-ethyl-1-hexanol = 130.231). Adjusted for exposure duration = Y mg/m^3*(hours per day/24)*(exposure days/total period = 65/90)) = Z mg/m^3. Mg/kg bw per day = Z*60*alveolar ventilation rate (529 mL/min per kg for rat, Brown et al., 1997)*(1 m^3/1 000 000 mL).

Body weights were measured prior to initial exposure and weekly throughout the study. Clinical signs and mortality were observed daily. Ophthalmological examinations were performed via ophthalmoscope before initial exposure and after the dosing period had ended. Haematological, clinical chemistry and clinical pathological examinations were conducted at the end of the study. Blood samples were collected via retro-orbital bleeding. All animals were necropsied, and gross pathology and organ weights were assessed. Histopathological examinations were conducted, but which organs these were was not specified.

Body-weight gain was decreased in females at 40 and 120 ppm on day 37. Males at 15 ppm had increased body weight on day 93. No clinical signs or ophthalmological effects were noted throughout the study. No animals died prematurely. In females, increased bilirubin concentration was noted at 120 ppm (4.07 μmol/L at 120 ppm; 2.99 μmol/L at 0 ppm) and decreased glucose levels at 15 ppm (6.98 mmol/L at 15 ppm; 7.81 mmol/L at 0 ppm). The authors concluded that these effects were not test article–related, as they only occurred in one sex and at one dose level. No increase in cyanide-insensitive-palmitoyl-CoA oxidation was observed. Exposure-related changes in organ weight was not observed in any group. No exposure-related macroscopic or histopathological changes were observed.

The NOAEL was 120 ppm (equivalent to 88 mg/kg bw per day), the highest dose tested (Klimisch et al., 1998).

(viii) 2-Methylundecanal (No. 275)

In a 12-week feeding study, rats (12/sex per dose group) were fed either an unsupplemented diet or one containing a mixture of aldehydes ad libitum. The dosages of the test mixture were calculated to be equal to 19.1 mg/kg bw per day of 2-methylundecanal (No. 275), undecanal (No. 107), 10-undecenal (No. 330) and lauric aldehyde (No. 110) along with 7 mg/kg bw per day of decanal (No. 104), 12.4 mg/kg bw per day of octanal (No. 98) and 28.6 mg/kg bw per day of nonanal (No. 101), totalling to 112 mg/kg bw per day of the aldehyde mixture. Growth and feed intake and physical appearance and behaviour were monitored weekly. After the 12-week exposure period, urine and blood samples (from 3 rats/sex) were analysed for urinary sugar and albumin levels and blood haemoglobin levels. All animals were autopsied upon study completion. All livers and kidneys were weighed.

Physical appearance and behaviour were similar in both the test and control groups. No significant difference was found in growth, feed intake or feed utilization. One animal died during the study, but autopsy revealed the cause to be an abdominal infection. Liver and kidney weights of rats in both groups were within normal limits. No other physiological effects were observed.

The author identified a NOAEL of 19.1 mg/kg bw per day. The Committee was unable to confirm this NOAEL based on the brevity of the information in the report (Trubek Laboratories, 1958).

(c) Long-term studies of toxicity and carcinogenicity

The results of long-term studies of toxicity and carcinogenicity with the representative aliphatic alicyclic hydrocarbon isobutyraldehyde (No. 252) are summarized in Table 4 and described below.

(i) Isobutyraldehyde (No. 252)

Mice

In a 2-year inhalation study, B6C3F$_1$ mice (50/sex per dose group) were exposed to 0, 500, 1000 or 2000 ppm of isobutyraldehyde (No. 252; lot no. E080289; purity 98%) for 6 hours/day, 5 days/week, for 104 weeks. These concentrations were calculated to be equivalent to doses of 0, 0.5, 1 or 2 mg/kg bw per day, as reported by the authors (NTP, 1999)[5]. All animals were observed twice daily. Body weight was recorded weekly for 13 weeks, monthly between weeks 14 and 92 and biweekly thereafter until the end of the study. Animals were observed for 3 to 7 days prior to necropsy. Necropsy was performed on all animals, and brain, heart, right kidney, liver, lungs, right testis and thymus of each animal were weighed. All visible lesions and tissue masses were examined, and a complete histopathologic examination was performed on all mice. Tissues examined microscopically included adrenal glands, bone marrow, brain, clitoral glands, oesophagus, gallbladder, heart, large intestine, small intestine, kidneys, larynx, liver, lungs, lymph nodes, mammary glands, nose, ovaries, pancreas, parathyroid glands, pituitary, preputial glands, prostate, salivary glands, skin, spleens, stomach, testes, thymus, thyroid gland, trachea, bladder and uterus. These tissues were preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin and stained with haematoxylin and eosin. A quality assessment pathologist reviewed slides from all tumours and potential target organs from all animals, including the heart, larynx, liver, lung, nose, skin, thyroid gland and trachea.

Increased exposure was found to lower survival rate among male mice, although the survival rate of males at 2000 ppm was only marginally lower than that of the control (control: 80%; 500 ppm: 74%; 1000 ppm: 70%; 2000 ppm: 60%). No significant difference in survival rate was found in the treated and untreated female groups (control: 57%; 500 ppm: 65%; 1000 ppm: 72%; 2000 ppm: 74%). No significant effect on mean body-weight was found in male mice. Female mice at 1000 and 2000 ppm lower mean body-weight than the controls in the second year of the study (weeks 53–104: control: 47.3 g; 500 ppm: 45.2 g; 1000 ppm:

[5] The method for deriving this equivalent dose was not described.

44.0 g; 2000 ppm: 42.2 g). No effect was found in tumour incidence. Animals at 1000 and 2000 ppm had an increased frequency of malignant lymphoma (males: control: 1/50; 500 ppm: 5/50; 1000 ppm: 1/50; 2000 ppm: 6/50; females: control: 12/50; 500 ppm: 13/50; 1000 ppm: 12/50; 2000 ppm: 19/50). Males at 2000 ppm showed a decreased frequency of hepatocellular adenoma or carcinoma (control: 27/49; 500 ppm: 25/50; 1000 ppm: 26/50; 2000 ppm: 18/50). Minimal to mild olfactory epithelial degeneration was found at a much higher rate at 1000 and 2000 ppm compared with controls. Two females at 1000 ppm, one female at 2000 ppm and one male at 2000 ppm had necrosis of the olfactory epithelium.

The authors concluded that although isobutyraldehyde was an upper respiratory tract toxicant in $B6C3F_1$ mice, it was not a carcinogen under the conditions of this bioassay. The Committee determined that the nasal and olfactory lesions were likely due to local irritation effects not relevant to human safety assessment for flavouring agents. The NOAEL was determined to be 2000 ppm (equivalent to 2 mg/kg bw per day), the highest dose tested (Abdo, Haseman & Nyska, 1998; NTP, 1999).

Rats

In a 2-year inhalation study, F344/N rats (50/sex per dose group) were exposed to 0, 500, 1000 or 2000 ppm of isobutyraldehyde (No. 252; lot no. E080289; purity 98%) for 6 hours/day, 5 days/week, for 105 weeks. These concentrations correspond to estimated doses of 0, 0.4, 0.8 and 1.6 mg/kg bw per day as reported by the authors[6] (NTP, 1999). All animals were observed twice daily. Body weight was recorded weekly for 12 weeks, monthly between weeks 14 and 91 and biweekly thereafter until the end of the study. Clinical findings were recorded every 4 weeks until week 91 and biweekly thereafter. Animals were observed for 3–7 days prior to necropsy. All animals were necropsied, and brain, heart, right kidney, liver, lungs, right testis and thymus of each animal were weighed. All visible lesions and tissue masses were examined, and a complete histopathologic examination was performed on all mice. Tissues examined microscopically included adrenal glands, bone marrow, brain, clitoral glands, oesophagus, heart, large intestine, small intestine, kidneys, larynx, liver, lungs, lymph nodes, mammary glands, nose, ovaries, pancreas, parathyroid glands, pituitary, preputial glands, prostate, salivary glands, skin, spleen, stomach, testes, thymus, thyroid gland, trachea, bladder and uterus. These tissues were preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin and stained with haematoxylin and eosin. A quality assessment pathologist reviewed slides from all tumours and potential target organs from all animals, including

[6] The method for deriving this equivalent dose was not described.

the adrenal glands, larynx, liver, lungs, mammary glands, nose, pituitary, skin, spleen, forestomach, trachea and testes.

No significant differences were found in the survival rates of the treated and control group rats (males: control: 12/50; 500 ppm: 15/50; 1000 ppm: 11/50; 2000 ppm: 10/50; females: control: 27/50; 500 ppm: 24/50; 1000 ppm: 24/50; 2000 ppm: 32/50). No significant effect was found on mean body-weight in any group. No clinical findings attributed to isobutyraldehyde exposure were identified by the study authors. A primary nasal neoplasm was observed in three animals: a polypoid adenoma in the anterior nasal section of a male at 1000 ppm, an adenoma of the vomeronasal organ in a male at 2000 ppm and an undifferentiated malignant neoplasm classified as a mesenchymal origin sarcoma in the posterior section of the nose in a female at 500 ppm. The study authors noted that spontaneous nasal neoplasms are rare in chamber control F344/N rats in the National Toxicology Program (NTP) historical database. Minimal to mild squamous metaplasia, mostly in the median septum, was found at a significantly higher rate in males at 1000 and 2000 ppm and in females at 500 ppm than in controls (males: control: 1/50; 500 ppm: 1/50; 1000 ppm: 10/50; 2000 ppm: 44/50; female: control: 1/50; 500 ppm: 11/50; 1000 ppm: 9/50; 2000 ppm: 44/50). Minimal to mild degeneration of the olfactory epithelium was common in males (44/50) and females (45/50) at 2000 ppm and not at all in controls. These groups also showed an increased incidence of suppurative inflammation (rhinitis), most often in the anterior nasal section. Mononuclear cell leukaemia was significantly more prevalent in females at 2000 ppm than controls (control: 12/50; 500 ppm: 19/50; 1000 ppm: 18/50, 2000 ppm: 25/50), although the study authors noted that the control group showed an atypically low rate and the rates in exposed groups were all within historical control ranges from 2-year NTP inhalation studies.

The study authors concluded that although isobutyraldehyde is an upper respiratory tract toxicant in F344 rats, it was not a carcinogen under the conditions of this bioassay. The Committee determined that the nasal olfactory lesions were likely due to local irritation effects not relevant to human safety assessment for flavouring agents. The NOAEL was determined to be 2000 ppm (equivalent to 1.6 mg/kg bw per day), the highest dose tested (Abdo, Haseman & Nyska, 1998; NTP, 1999).

(d) Studies of genotoxicity

No studies on the genotoxicity of the new flavouring agents (Nos 2238–2239) were reported. Studies of genotoxicity in vitro and in vivo reported for previously evaluated saturated aliphatic alicyclic branched-chain primary alcohols, aldehydes and acids are summarized in Table 5 and described below.

Table 5

Studies of genotoxicity of saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
251	Isobutyl alcohol	HPRT mutation	Chinese hamster V79 cells	7 931 µg/mL	Negative [a,b]	Kreja & Seidel (2002)
251	Isobutyl alcohol	Micronucleus induction	Chinese hamster V79 cells	815 or 3 928 µg/mL	Negative [b,c]	Kreja & Seidel (2002)
251	Isobutyl alcohol	DNA damage [d]	Chinese hamster V79 cells	3 928 or 20 012 µg/mL	Negative [b,c,e]	Kreja & Seidel (2002)
251	Isobutyl alcohol	DNA damage [d]	A549 cells	3 928 µg/mL	Negative [b,c,e]	Kreja & Seidel (2002)
251	Isobutyl alcohol	DNA damage [d]	Peripheral human blood sample	3 928 µg/mL	Negative [b,c,f]	Kreja & Seidel (2002)
252	Isobutyraldehyde	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537	100–10 000 µg/plate [g]	Negative [a,h,i]	Mortelmans et al. (1986); NTP (1999)
252	Isobutyraldehyde	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535, TA1537	10–10 000 µg/plate [j]	Negative [a,j] Equivocal [k]	NTP (1999)
252	Isobutyraldehyde	Reverse mutation	*S. typhimurium* TA100, TA102, TA104	50–5 000 µg/plate	Negative [l] Equivocal [m]	Dillon, Combes & Zeiger (1998)
252	Isobutyraldehyde	Micronucleus induction	Chinese hamster lung cells	Up to 721 µg/mL [n]	Negative	Fowler et al. (2012)
252	Isobutyraldehyde	Micronucleus induction	TK6 human lymphoblastoid cells	Up to 721 µg/mL [n]	Negative	Fowler et al. (2012.
252	Isobutyraldehyde	Forward mutation	Mouse L5178Y cells	62.5–1 000 µg/mL [c] 62.5–500 µg/mL [c]	Positive [c]	NTP (1999)
252	Isobutyraldehyde	Chromosomal aberration	Chinese hamster ovary cells	16–2 000 µg/mL	Positive [c]	NTP (1999)
252	Isobutyraldehyde	SCE	Chinese hamster ovary cells	5–500 µg/mL 16–1 250 µg/mL	Positive [a]	NTP (1999)
253	Isobutyric acid	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	1.6–70.8 µg/plate [a,o]	Negative	Jagannath (1982)
253	Isobutyric acid	Forward mutation	Mouse L5178Y cells	30–569 µg/mL [o,p]	Positive [q]	Cifone (1982)
253	Isobutyric acid	Chromosomal aberration	Chinese hamster ovary cells	95–3 132 µg/mL [o]	Negative	Galloway (1983)
253	Isobutyric acid	Micronucleus induction	Human blood lymphocytes	220, 440, 880 µg/mL [a,r] 220, 440, 880 µg/mL [c,s]	Negative	Morris (2014a)
255	2-methylbutyric acid	Reverse mutation	*S. typhimurium* TA97a, TA98, TA100, TA102, TA1535	50–5 000 µg/plate a,	Negative	Scheerbaum (2000)
259	Isovaleric acid	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	50–5 000 µg/plate [a,t]	Negative	Thompson &Bowles (1999)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
268	3,5,5-Trimethyl-1-hexanol	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	5–5 000 µg/plate [a,t]	Negative	Harnasch (1999b)
268	3,5,5-Trimethyl-1-hexanol	Chromosomal aberration	Chinese hamster lung cells	Unspecified [u,v]	Negative	Kusakabe et al. (2002)
270	2-Methyloctanal	Micronucleus induction	Human blood lymphocytes	60,90, 135 µg/mL [r,q] 22.5, 45, 90 µg/mL [c,r] 45, 90, 5112.5 µg/mL [c,s]	Inadequate/ Negative [w]	Morris (2014b)
272	3,7-Dimethyl-1-octanal	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	1.5–1 500 µg/plate [a,p]	Negative	King (2001)
275	2-Methylundecanal	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	5–5 000 µg/plate [a,p]	Negative	Harnasch (1999a)
In vivo						
52	Isoamyl alcohol	Micronucleus induction [x]	Male ICR(Crj:CD-1) mice	500–2 000 mg/kg bw	Negative	Hatano (2004a)
252	Isobutyraldehyde	Micronucleus induction [x]	Male B6C3F$_1$ mice	39–1 250 mg/kg bw	Negative	NTP (1999)
252	Isobutyraldehyde	Micronucleus induction x	Male B6C3F$_1$ mice	156–1 250 mg/kg bw	Negative	NTP (1999)
252	Isobutyraldehyde	Micronucleus induction [x]	Male F344/N rats	313–1 250 mg/kg bw	Negative	NTP (1999)
252	Isobutyraldehyde	Chromosomal aberration [x]	Male B6C3F$_1$ mice	500–2 000 mg/kg bw [y] 1 000–1 750 mg/kg bw	Positive	NTP (1999)

F: female; M: male; NA: not applicable; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from liver homogenate; SCE: sister chromatid exchange

[a] In the presence and absence of rat S9.
[b] Study report was not detailed enough to evaluate independently; described results are those of the study authors.
[c] In the absence of rat S9.
[d] Comet assay.
[e] 54 cells/experiment, 2 experiments.
[f] 54 cells/experiment, 1 experiment.
[g] 98% pure.
[h] Preincubation method.
[i] In the presence and absence of hamster S9.
[j] Maximum concentration ranged from 1000 to 10 000 µg/plate depending on strain and presence or absence of rat S9.
[k] Strain TA104 in the presence of rat S9.
[l] In strain TA100; in TA102 in the absence of S9 and in the presence of mouse S9; and in TA104 in the absence of S9.
[m] In strain TA102 in the presence of rat S9; in TA104 in the presence of mouse S9 or of rat S9.
[n] 3 hours treatment (±S9) with 21-hour recovery phase or 24-hour treatment in the absence of rat S9.
[o] Converted from 1.7–75 µL/plate using 0.949 density.
[p] Purity of test article not specified.
[q] With rat S9 incubation.
[r] 4-hour treatment.
[s] 24-hour treatment.
[t] Plate incorporation method.
[u] Publication specifies that the highest dose was 50% or less that 50% cytotoxic at 5 mg/mL or 10 mmol/L
[v] 6-hour incubation with and without S9 with further 18-hour incubation. Additional cultures were treated with 24- and 48-hour incubations without S9. The species the S9 was derived from was not specified.
[w] Highest dose concentration in 4 hours with S9 incubation was statistically significantly higher than control but within historical controls. Highest dose was not 55±5% cytotoxic for all conditions.
[x] In the bone marrow.
[y] 2000 mg/kg bw was lethal.

(i) In vitro

In vitro bacterial reverse mutation assays were reported for isobutyraldehyde (No. 252), isobutryric acid (No. 253), 2-methylbutyric acid (No. 255), isovaleric acid (No. 259), 3,5,5-trimethyl-1-hexanol (No. 268), 3,7-dimethyl-1-octanal (No. 272) and 2-methylundecanal (No. 275). The results all showed no evidence of mutagenicity (Jagannath, 1982; Mortelmans et al., 1986; Harnasch, 1999a,b; Thompson & Bowles, 1999; Scheerbaum, 2000; King, 2001) except for isobutyraldehyde (No. 252), for which two reports (Dillon, Combes & Zeiger, 1998; NTP, 1999) had equivocal results.

An hypoxanthine-guanine phosphoribosyltransferase (HPRT) mutation assay in Chinese hamster V79 cells of isobutyl alcohol (No. 251) was negative for HPRT mutation (Kreja & Seidel, 2002).

In vitro comet assays in Chinese hamster V79 cells, A549 cells and human peripheral blood lymphocytes treated with isobutyl alcohol were negative for signs of DNA (Kreja & Seidel, 2002).

Reports on in vitro micronucleus induction assays of isobutyl alcohol (No. 251), isobutyraldehyde (No. 252), isobutyric acid (No. 253) and 2-methyloctanal (No. 270) were all negative (Kreja & Seidel, 2002; Fowler et al., 2012, Morris, 2014a,b).

Forward mutation assays using mouse L5178Y cells of isobutyraldehyde (No. 252) or isobutyric acid (No. 253) were positive (NTP, 1999; Cifone, 1982).

An SCE assay of isobutyraldehyde (No. 252) was also positive (NTP, 1999).

Chromosomal aberration assays of isobutyric acid (No. 253) and of 2,5,5-trimethyl-1-hexanol (No. 268) were negative (Galloway, 1983; Kusakabe et al., 2002) but of isobutyraldehyde (No. 252) were positive (NTP, 1999).

(ii) In vivo

Micronucleus induction assays were performed with male ICR mice for isoamyl alcohol (No. 52) and B6C3F mice and F344 rats for isobutyraldehyde (No. 252). The results for all the in vivo micronucleus induction assays were negative.

The results of a chromosomal aberration assay reported on isobutyraldehyde (No. 252) was positive (NTP, 1999).

(iii) Conclusions on genotoxicity

An in vitro and in vivo chromosomal aberration assay, an in vitro SCE assay and an in vitro forward mutation assay (not previously evaluated) were positive for isobutyraldehyde (No. 252) and in an in vitro forward mutation assay was positive for isobutyric acid (No. 253). The reverse bacterial mutation assays as well as the in vivo micronucleus assays of isobutyraldehyde (No. 252) were negative. The

in vivo chromosomal aberration assay of isobutyraldehyde (No. 252) was only positive at the highest dose, which produced notable signs of cytotoxicity. The negative in vivo results on isobutyraldehyde (No. 252) are consistent with the 2-year carcinogenicity mouse and rat assays on isobutyraldehyde (No. 252) that showed local toxicity but no carcinogenicity (Abdo, Haseman & Nyska, 1998; NTP, 1999). The weight of evidence indicates that these saturated aldehyde flavouring agents are not likely to be genotoxic. The results of the remainder of the genotoxicity assays on flavouring agents in this group are negative or equivocal.

(e) Reproductive and developmental toxicity

3,5,5-Trimethyl-1-hexanol (No. 268)

In 90-day reproductive toxicity study compliant with OECD TG No. 422, 3,5,5-trimethyl-1-hexanol (No. 268) was administered via gavage to Crl:CD(SD) rats (12/sex per dose group) at 12, 60 or 300 mg/kg bw per day (ECHA, 2017g).

The numbers of implantations and births and the implantation index were all significantly reduced in mid- and high-dose animals. At birth, weight of mid-dose pups was slightly reduced, while weight of pups at 300 mg/kg bw per day was significantly reduced. The pups in both groups gained weight and were similar to the controls 4 days after birth. These groups also exhibited maternal toxicity, with 33% mortality at the highest dose. The survival index of pups at the high dose was significantly reduced in the first 4 days after birth. An unspecified number of pups from each group were killed and necropsied 4 days after birth; but no external aberrations were noted.

The study authors identified the NOAEL for maternal and embryo/fetal toxicity as 12 mg/kg bw per day and the NOAEL for teratogenicity as 300 mg/kg bw per day. Because only a summary report was available, the Committee was unable confirm this NOAEL (ECHA, 2017g).

Isobutyl alcohol (No. 251)

In a prenatal inhalation toxicity study, pregnant SPF-Wistar rats (25/dose group) were exposed to isobutyl alcohol (No. 251; purity 99.8%) at atmospheric concentrations of 0.5, 2.5 or 10 mg/L for 6 hours/day on days 6–15 postcoitum. These concentrations were equivalent to 95, 476 and 1904 mg/kg bw per day, respectively[7]. Body weights and clinical observations were recorded throughout the study. All rats were killed on day 20 postcoitum. All animals were necropsied, uteruses and ovaries were removed and weighed, and numbers of corpora lutea and

[7] Converted as follows, assuming 100% absorption: From X mg/L to Y mg/m^3, (X*1000 L/m^3). Adjusted for exposure duration = Y mg/m^3*(6 h per day/24) = Z mg/m^3. Mg/kg bw per day = Z*60*alveolar ventilation rate (529 mL/min per kg for rat, Brown et al., 1997)*(1 m3/1 000 000 mL).

implantation sites counted. Fetuses were removed and examined for soft tissue and skeletal abnormalities, as well as weight and sex. Two fetuses in the highest dose group were reported to have anasarca, one with cleft palate. The number of fetuses (litters) examined for soft tissue malformations and variations were 125 (21), 150 (23), 134 (23) and 122 (19) in the 0, 0.5, 2.5 and 10 mg/L dose groups, respectively. One fetus in the highest dose group had dilation of both ventricles. The number of soft tissue variations in treated animals were similar to or lower than the numbers in controls. The number of fetuses (litters) examined for skeletal malformations and variations were 134 (21), 164 (23), 149 (23) and 128 (19) in the 0, 0.5, 2.5 and 10 mg/L dose groups, respectively. Skeletal malformations and variations in fetuses from treated and control dams were similar in number and type.

The NOAEL was identified as 10 mg/L (equivalent to 1904 mg/kg bw per day), the highest concentration tested (Klimisch & Hellwig, 1995).

In a prenatal inhalation toxicity study, pregnant Himalayan Chbb:HM outbred rabbits (15/dose group) were exposed to isobutyl alcohol (No. 251; purity 99.8%) at 0.5, 2.5 or 10 mg/L air for 6 hours/day (equivalent to 30, 150 and 601 mg/kg bw per day, respectively[8]) during postinsemination days 7–19. Body weights and clinical observations were recorded throughout the study. All pregnant rabbits were killed on postinsemination day 29. All animals were necropsied, uteruses and ovaries were removed and weighted, and number of corpora lutea and implantation sites counted. Fetuses were removed and examined for soft tissue and skeletal abnormalities as well as weight and sex. One rabbit in each the 0.5 and 2.5 mg/L dose groups aborted. Exposure to the 10 mg/L dose resulted in slightly retarded in body-weight increase through the exposure period. A slight increase in placental weights at 2.5 mg/L group was attributed to the lower number of fetuses per dam in this group. No external or soft tissue malformations were noted. For soft tissue variations, an increase in the number of interventricular foramen/septum membranaceum was noted in the 10 mg/L group. Other findings were without concentration relationship or statistical significance between groups.

The NOAEL was as 10 mg/L (equivalent to 601 mg/kg bw per day), the highest concentration tested (Klimisch & Hellwig, 1995).

Isobutyraldehyde (No. 252)

In a prenatal developmental toxicity study compliant with OECD TG No. 414, pregnant SPF-Wistar rats (25/dose group) were exposed to isobutyraldehyde

[8] Converted as follows, assuming 100% absorption: From X mg/L to Y mg/m^3, (X*1000 L/m^3). Adjusted for exposure duration = Y mg/m^3*(6 h per day/24) = Z mg/m^3. Mg/kg bw per day = Z*60*alveolar ventilation rate (167 mL/min per kg for rabbit, Brown et al., 1997; https://www.rarc.wisc.edu/animal_health/normative_data.htmI for rabbit minute volume)*(1 m^3/1 000 000 mL).

(No. 252) vapours at concentrations of 3, 7.6 or 12 mg/L air (equivalent to 571, 1447 and 2285 mg/kg bw per day, respectively [9]). All animals were exposed for 6 hours/day, from gestation days 6 to 15, and killed on day 20 postcoitum.

A dose-related depression in body-weight development was noted in all treated dams, though this was not considered an adverse effect. Single mid- and high-dose fetuses each exhibited anasarca, but this was not considered to be treatment related. No signs of embryo/fetal toxicity were observed in any dose group. There were no changes in conception rate or numbers of viable fetuses and resorptions or mean numbers of corpora lutea or implantation sites.

The authors identified the NOAEL as 12 mg/L air (which corresponds to 2285 mg/kg bw per day), the highest concentration tested. Because only a summary report was available, the Committee was unable to confirm this NOAEL (ECHA, 2017k).

2-ethyl-1-hexanol (No. 267)

In a study on the relationship between maternal zinc metabolism and developmental toxicity, pregnant SD rats were orally administered 813, 1200 or 1625 mg/kg bw of 2-ethyl-1-hexanol (No. 267; lot no. and purity not stated) in corn oil. Pregnant rats were fed a diet containing Zn at 25 µg/g diet from gestation day 0 to 12.5. Eight hours after administration, all dams were intubated with 1.2×10^6 Bq of ^{65}Zn in a 0.75 mL 25% w/v slurry of Zn at 0.5 µg/g egg white diet in 0.9% NaCl. Dams were killed by asphyxiation 10 hours after ^{65}Zn administration. Blood samples were drawn, and the gravid uterus was excised, weighed and examined for numbers of implantation sites, embryos and resorptions sites. Embryos and amniotic sacs were pooled and weighed after removal. ^{65}Zn radioactivity was measured in various tissues and organs.

Maternal feed intake was significantly reduced in the two highest dose groups. These groups also exhibited increased Zn and metallothionine concentrations in the maternal liver. These dosages also resulted in a lower percentage of ^{65}Zn recovered in their embryos (Bui et al., 1998).

In a developmental toxicity study of di-(2-ethylhexyl)phthalate and its metabolites, monoethylhexyl phthalate and 2-ethyl-1-hexanol (No. 267), five male CD Sprague Dawley rats (3 days old) were administered 167 mg/kg bw of 2-ethyl-1-hexanol (No. 267; purity >95%) by gavage. The animals received subcutaneous injections of bromodeoxyuridine (BrdU) 21 hours after treatment and were killed 3 hours after the bromodeoxyuridine injection. The right testis of

[9] Converted as follows, assuming 100% absorption: From X mg/L to Y mg/m^3, (X*1000 L/m^3). Adjusted for exposure duration = Y mg/m^3*(6 h per day/24) = Z mg/m^3. Mg/kg bw per day = Z*60*alveolar ventilation rate (529 mL/min per kg for rat, Brown et al., 1997)*(1 m^3/1 000 000 mL).

each animal underwent morphological examination, while the left testis was used for bromodeoxyuridine immunostaining.

2-Ethyl-1-hexanol did not cause changes in Sertoli cells or gonocytes, while di-(2-ethylhexyl) phthalate caused abnormalities in both (Li et al., 2000).

3,7-dimethyl-1-octanol (No. 272)

In a reproductive toxicity study of five compounds, five male Wistar rats (13–14 weeks old) were administered 1000 mg/kg bw per day of 3,7-dimethyl-1-octanol (No. 272; batch no. Ch. 65872377L0; purity 98.3%) by gavage for 14 days. All animals were observed for clinical signs, body weight and feed consumption throughout the study and were killed one day after the final administration. Sperm analysis, gross pathological and histopathological examinations of the reproductive organs and haematological examinations were performed.

Clinical observations included slight to severe salivation. A decrease in feed consumption (−17%) during the first 3 days of exposure was noted. One animal had a stomach ulcer. Sperm analysis and histopathological, gross pathological and haematological examinations revealed no other abnormalities.

Based on these results, 3,7-dimethyl-1-octanol (No. 272) does not induce male reproductive toxicity at up to 1000 mg/kg bw per day (Flick, 2013).

In a reproductive toxicity study that was compliant with OECD TG No. 414, pregnant Wistar rats (25/dose group) were administered 3,7-dimethyl-1-octanol (No. 272; batch no. 00011277L0; purity 98.8%) in corn oil via gavage at 0, 50, 150 or 450 mg/kg bw per day, from gestation day 6 to 19. Feed consumption, body weight and clinical observations were recorded regularly throughout the study. All animals were killed on gestation day 20 and assessed for gross pathology. Numbers of corpora lutea and implantation sites were counted in each animal. All fetuses were removed and assessed for weight, sex and abnormalities. Half of the fetuses were examined for soft tissue abnormalities and the other half for skeletal abnormalities.

Transient salivation was noted in all high-dose animals, 24/25 mid-dose animals and 4 low-dose animals during the first post-dosing check (within 2 hours) but not the second (within 5 hours). Fetal weights at the high and low doses were lower than the control group; they were not considered test article related because there was no dose–response relationship and the weights were similar to means for historical controls. Soft tissue malformations were noted in fetuses in the control group (microphthalmia, hydronephrosis and hydroureter in one and malpositioned testis in the other) and one at mid dose (situs inversus). One skeletal malformation was reported for each dose group (shortened scapula and shortened humerus at 0 mg/kg bw per day; absent lumbar vertebra branched rib and intercostal rib at 50 mg/kg bw per day; multiple skeletal malformations

at 150 mg/kg bw per day; and thoracic hemivertebra and branched rib at 450 mg/kg bw per day. External and soft tissue variations were similar across dose groups. Increases in supernumerary thoracic vertebra (at 150 and 350 mg/kg bw per day) and supernumerary rib (14th) and cartilage present (at 450 mg/kg bw per day) were noted, although variations were noted in almost all fetuses in all dose groups. The authors considered this finding test article related, but because variations are expected to resolve, it was not considered adverse.

The NOAEL for maternal and fetal toxicity was 450 mg/kg bw per day, the highest dose tested (Schneider, Grauert & van Ravenzwaay, 2016).

(g) **Additional studies – neurotoxicity**

In a single-dose neurotoxicity study, rats (10/sex per group) were exposed to isobutyl alcohol vapours (No. 251; lot no. EH3639; purity >99.9%) at atmospheric concentrations of 0, 1500, 3000 or 6000 ppm (corresponding to oral doses of 0, 870, 1730 and 3460 mg/kg bw [10]). Neurobehavioural tests were performed before exposure and immediately after, 1 day after, 7 days after and 14 days after exposure. All the animals were killed 15 days after exposure. Five animals from each dosage group were perfused, and the remaining animals were given a complete gross necropsy. The study authors observed a "rapidly reversible general depression of the central nervous system" in high- and mid-dose group animals. A decrease in alertness was noted in high-dose females and a decrease in motor activity was noted in both sexes in the high dose group. One male exhibited a "slight, incoordinated gait", but these effects were thought to be as a result of the anaesthetic effect of the high concentration. Decrease in activity was observed at the low dose during exposure but not afterwards. Necropsies did not reveal any treatment-related effects in tissues or organs.

The lowest-observed-effect level was 1500 ppm (equivalent to 870 mg/kg bw) based on hypoactivity during testing in all dose groups (Li et al., 1994).

In a GLP-compliant, 3-month schedule-controlled operant behaviour (SCOB) study, Sprague Dawley rats (10 males/group) were trained to perform multiple fixed-ratio and fixed-interval schedules of food reinforcement. After training, the groups were exposed to isobutyl alcohol (No. 251, purity 99%) at atmospheric concentrations of 0, 250, 1000 or 2500 ppm for 6 hours/day, 5 days/week (65 exposures total; equivalent to 0, 98, 392 and 979 mg/kg bw per day [11]).

[10] Converted as follows, assuming 100% absorption: From X ppm to Y mg/m^3, (X/24.45)*molecular weight (for isobutyl alcohol = 74.123). Adjusted for exposure duration = Y mg/m^3*(6 h per day/24) = Z mg/m^3. Mg/kg bw per day = Z*60*alveolar ventilation rate (529 mL/min per kg for rat, Brown et al., 1997)*(1 m^3/1 000 000 mL).

[11] Converted as follows, assuming 100% absorption: From X ppm to Y mg/m^3, (X/24.45)*molecular weight (for isobutyl alcohol = 74.123). Adjusted for exposure duration = Y mg/m^3*(h per day/24)*(days per week/7) = Z mg/m^3. Mg/kg bw per day = Z*60*alveolar ventilation rate (529 mL/min per kg for rat, Brown et al., 1997)*(1 m^3/1 000 000 mL).

Clinical observations and body weight were recorded weekly. Ophthalmoscopic examinations were conducted on each animal prior to initial exposure and again during the final week of treatment. SCOB-dependent variables were analysed for 4 consecutive days before exposure and during exposure weeks 4, 8 and 13. All animals were killed 1 week after their final exposure.

All three concentrations of isobutyl alcohol caused a slight reduction in responsiveness to external stimuli during exposure, but no differences were noted immediately after exposure. It was concluded that these symptoms were likely temporary effects of acute exposure. No significant concentration–time dependent effects were observed in any SCOB parameters. No changes were observed in the weekly clinical observations, except singular incidences of skin abrasion and swollen genitalia that were not considered to be treatment related. No morphological or behavioural treatment-related effects were observed.

The NOAEL was 2500 ppm (equivalent to 979 mg/kg bw per day), the highest dose tested (Li et al., 1999).

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APPENDIX 1

Frequency and amount of food supplements consumed based on a range of surveys reviewed by the sponsor

Table A1.1.
Consumption of supplements from a range of studies including amount consumed and frequency of consumption

Country	Year	Survey	Respondents	Consumption amount	Consumption frequency	Reference
France	Not specified	7-day record and questionnaire	24–54 years ($n = 290$)	–	21% consume >1 supplement simultaneously 3.6% take supplements daily	Touvier et al. (2003)
Germany	1998	Nutrition survey	18–79 years ($n = 4\,030$)	–	25% women and 18% men took supplements at least once per week 43% consumed vitamin and/or mineral supplements at least once in the previous 12 months	Beitz et al. (2004)
Germany	2006	Health care study	Not stated	–	35% regularly, 17% temporarily, taking food supplements The most frequent users are elderly people	psychonomics (2006)
United Kingdom	2000/2001	National Diet and Nutrition Survey	Not stated	High consumers (P97.5): 2 tablets or capsules per day children 4–18 years	40% women, 29% men take supplements	Gregory & Lowe (2000)
United Kingdom	2000/2001	National Diet and Nutrition Survey	Not stated	7 tablets or capsules per day adults	Women 50–64 years represent the highest proportion of consumers (55%)	Henderson, Gregory & Swan (2002)
Ireland	Not specified	Food consumption survey	18–64 years	–	23% regularly take supplements	Kiely et al. (2001)
Netherlands	After 1998	Dutch National Food Consumption Survey and several monitoring and cohort surveys	Not stated	50% take 1 supplement 33% take 2 supplements 10% take 3 supplements 6% of users take 8 units (4 supplements twice daily)[a]	Mean frequency 4.9–15.4 per week 80% of users take 1 or 2 different supplements, at most twice per day	Ocké, Buurma-Rethans & Fransen (2005)
Netherlands	2003	Dutch National Food Consumption Survey		4.9% take 3 supplements 0.1% take 4 or more		

Abbreviation: P97.5: 97.5th percentile

[a] The sponsor used this for exposure estimates, assuming that some of these are liquid supplements and some do not use AMC in the coating, therefore assumed for adults 4/day for an average user, 6/day for a heavy user and 2 per day for children.

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ANNEX 1

Reports and other documents resulting from previous meetings of the Joint FAO/WHO Expert Committee on Food Additives

1. General principles governing the use of food additives (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. Procedures for the testing of intentional food additives to establish their safety for use (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants) (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. I. Antimicrobial preservatives and antioxidants, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. Specifications for identity and purity of food additives (food colours) (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. II. Food colours, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. Evaluation of the carcinogenic hazards of food additives (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. Evaluation of the toxicity of a number of antimicrobials and antioxidants (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. Specifications for identity and purity and toxicological evaluation of food colours. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.

11. Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).

12. Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases. FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.

13. Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.

14. Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non nutritive sweetening agents (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.

15. Toxicological evaluation of some flavouring substances and non nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.

16. Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.

17. Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.

18. Specifications for the identity and purity of some antibiotics. FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.

19. Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.

20. Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances. FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.

21. Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives. FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.

22. Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.

23. Toxicological evaluation of some extraction solvents and certain other substances. FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.

24. Specifications for the identity and purity of some extraction solvents and certain other substances. FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.

25. A review of the technological efficacy of some antimicrobial agents. FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.

26. Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants (Fifteenth report

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27. Toxicological evaluation of some enzymes, modified starches, and certain other substances. FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.

28. Specifications for the identity and purity of some enzymes and certain other substances. FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.

29. A review of the technological efficacy of some antioxidants and synergists. FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.

30. Evaluation of certain food additives and the contaminants mercury, lead, and cadmium (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.

31. Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate. FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.

32. Toxicological evaluation of certain food additives with a review of general principles and of specifications (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).

33. Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents. FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.

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ANNEX 2

Abbreviations used in the monographs

ACT	Australian Capital Territory
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, excretion
AhR	aryl hydrocarbon receptor
AI	adequate intake
ALP	alkaline phosphatase
ALT	alanine transaminase / alanine aminotransferase
AMC	anionic methacrylate copolymer
ANOVA	analysis of variance
ASAT	aspartate aminotransferase
AST	aspartate aminotransferase / aspartate transaminase
AUC	area under the concentration–time curve
$AUC_{0–14\ d}$	area under the concentration–time curve for days 0–14
$AUC_{0–\infty}$	area under the concentration–time curve from time 0 to infinity (total bioavailability)
Aβ	amyloid-beta peptide
BMA	n-butyl methacrylate
BMC	basic methacrylate copolymer
$BMDL_{10}$	benchmark dose for a 10% response
BMI	body mass index
bw	body weight
CAS	Chemical Abstracts Service
CCFA	Codex Committee on Food Additives
CCFA50	Fiftieth Session of the Codex Committee on Contaminants in Food Additives
CHO	Chinese hamster ovary
CIFOCOss	FAO/WHO Chronic Individual Food Consumption Database – Summary statistics
CITREM	citric and fatty acid esters of glycerol
CLP	Classification, Labelling and Packaging of substances and mixtures
C_{max}	maximum concentration
CMC	carboxymethylcellulose
CXG	Codex Guideline
CXS	Codex Standard
CYP	cytochrome P450

DEHP	di(2-ethylhexyl) phthalate
DHPN	*N*-bis(2-hydroxypropyl)nitrosamine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DR	dietary recall
EBSS	Earle's balanced salt solution
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EINECS	European Inventory of Existing Commercial Chemical Substances
EU	European Union
F	female
F	filial generation (F_0, F_1, F_2, etc.)
FAO	Food and Agriculture Organization of the United Nations
FD&C	US Federal Food, Drug and Cosmetic
FFQ	food frequency questionnaire
FSANZ	Food Standards Australia New Zealand
GC-MS	gas chromatography–mass spectrometry
GEMS	Global Environment Monitoring System
GEWR	glycerol ester of wood rosin
GGT	γ-glutamyltransferase or gamma-glutamyltransferase
GLP	good laboratory practice(s)
GMP	good manufacturing practices
GRAS	Generally Recognized as Safe
GRN	GRAS [Generally Recognized as Safe] notice number
GSFA	General Standard for Food Additives
GSH	glutathione
HDL	high density lipoprotein
5-HIAA	5-hydroxyindoleacetic acid
HPBL	human peripheral blood lymphocytes
HPC/DR	hepatocyte primary culture/DNA repair [assay]
HPLC	high-performance liquid chromatography
HPLC-UV	high-performance liquid chromatography with ultraviolet detection
HPRT	hypoxanthine-guanine phosphoribosyltransferase
i.p. or IP	intraperitoneal OR intraperitoneal injection
IACM	International Association of Color Manufacturers
IARC	International Agency for Research on Cancer
IC_{50}	half maximal inhibitory concentration
ICH	International Conference on Harmonisation
IgE	immunoglobulin E

INS	International Numbering System for Food Additives
IOFI	International Organization of the Flavor Industry
IP	intraperitoneal injection
IRIS	Integrated Risk Information System
IWGT	International Workshops on Genotoxicity Testing
JaCVAM	Japanese Center for the Validation of Alternative Methods
JECFA	Joint FAO/WHO Expert Committee on Food Additives
K_m	Michaelis constant (affinity)
LATAM	Latin America
LD_{50}	median lethal dose
LDL	low density lipoprotein
LOAEL	lowest-observed-adverse-effect level
LSD	least significance difference
M	male
MAO	monoamine oxidase-A
MBTH	3-methyl-2-benzothiazolinone hydrazone
MCH	mean corpuscular haemoglobin
MCHC	mean cell haemoglobin concentration
MNBN	micronucleated binucleated
MOE	margin of exposure
MSDI	maximized survey-derived intake
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
nes	not elsewhere specified
NHANES	National Health and Nutrition Examination Survey
NMC	neutral methacrylate copolymer
NNS	National Nutrition Survey
no. / No.	number
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NPSH	non-protein sulfhydryl
OECD TG	Organisation for Economic Co-operation and Development Test Guideline
OECD	Organisation for Economic Co-operation and Development
P	probability
P	parental animals
P90	90th percentile
P95	95th percentile
PBI	protein-bound iodine
ppm	parts per million
RBC	red blood cell

RDA	recommended dietary allowance
RNA	ribonucleic acid
rT_3	reverse T_3 (triiodothyronine)
S9	9000 × *g* supernatant fraction (metabolic activation)
SCE	sister chromatid exchange
SCF	Scientific Committee on Food
SCOB	schedule-controlled operant behaviour
SD	standard deviation
SE	standard error
SPET	single-portion exposure technique
$t_{½}$	half-life
T_3	triiodothyronine
TDI	tolerable daily intake
T_4	thyroxine
tk / TK	thymidine kinase
T_{max}	time to reach the maximum concentration (Cmax)
TNF-α	tumour necrosis factor-α
TRH	thyrotropin-releasing hormone (TSH-releasing hormone)
Trp-P-1	3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole
TSH	thyroid-stimulating hormone
UDPGT	uridine diphosphoglucuronosyltransferase
USA	United States of America
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
UV	ultraviolet
VAS	visual analogue scale
v/v	volume per volume
V_{max}	maximum velocity
w/v	weight per volume
WBC	white blood cell
WHO	World Health Organization

ANNEX 3

Joint FAO/WHO Expert Committee on Food Additives[1]

Geneva, 12–21 June 2018

Members

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Dr D. Benford, Cheddington, London, England, United Kingdom

Dr R. Cantrill, Halifax, Nova Scotia, Canada (*Vice-Chairperson*)

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Dr U. Mueller, Australian Pesticides and Veterinary Medicines Authority (APVMA), Symonston, Australian Capital Territory (ACT), Australia (*Joint Rapporteur*)

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Dr M. Veerabhadra Rao, Hyderabad, India

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ANNEX 4

Toxicological information and information on specifications

Food additives evaluated toxicologically and assessed for dietary exposure

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
Anionic methacrylate copolymer (AMC)	N, T[a]	**The Committee was unable to complete the evaluation of AMC.** While the copolymer itself is not of health concern, genotoxicity concerns remains for the residual monomer methacrylic acid. The specifications were made tentative pending the completion of the safety evaluation of AMC.
Basic methacrylate copolymer (BMC)	N	**The Committee established an ADI "not specified" for basic methacrylate copolymer.** The Committee concluded that the use of BMC that complies with the specifications established at the current meeting is not of safety concern when the food additive is used as a coating or glazing agent for solid food supplements and for foods for special medical purposes and micronutrient encapsulation for food fortification. The NOAELs for BMC ranged from 750-2000 mg/kg bw per day which were the highest doses tested. The Committee evaluated exposure to BMC for the copolymer and its monomers, (*n*-butyl methacrylate, 2-(dimethylamino)ethyl methacrylate and methyl methacrylate). Estimated exposures to BMC range from 3.0 to 135 mg/kg bw per day. The total monomeric content of BMC is less than 0.3%. The Committee concluded that the toxicological data on the residual monomers do not give rise to concerns when taking into account the low dietary exposures.
Erythrosine	R[b]	**The Committee concluded that the new data that have become available since the previous evaluation of erythrosine do not give reason to revise the ADI and confirmed the previous ADI of 0–0.1 mg/kg bw.** The Committee noted that the dietary exposure estimate for erythrosine of 0.09 mg/kg bw per day (95th percentile for children) was close to the upper bound of the ADI. Given that this estimate of exposure is for children and it is a high percentile for consumers only, such a level is unlikely to occur every day over a lifetime. Therefore, the Committee concluded that dietary exposures to erythrosine for all age groups do not present a health concern.
Indigotine	R[b]	The Committee considered the new data that had become available since the previous evaluation as well as previously evaluated studies and c**oncluded that there are no reasons to revise the ADI and confirmed the previous ADI of 0–5 mg/kg bw.** The Committee noted that the conservative dietary exposure estimate of 0.8 mg/kg bw per day (95th percentile for children and toddlers) is less than the upper bound of the ADI of 0–5 mg/kg bw. The Committee concluded that dietary exposure to indigotine for all age groups does not present a health concern.

(continued)

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
Lutein	R[c,d]	Free lutein, lutein esters and free zeaxanthin including *meso*-zeaxanthin are biochemically and toxicologically equivalent. At the present meeting the Committee concluded that there were sufficient toxicological data to complete a safety assessment of lutein and lutein esters from *Tagetes erecta*, synthetic zeaxanthin and *meso*- zeaxanthin. Free lutein, lutein esters and free zeaxanthin and *meso*-zeaxanthin are substances of low toxicity for which no adverse effects have been observed in a broad range of toxicological studies in laboratory animals and clinical studies in humans. Based on the absence of toxicity in a wide range of studies, **the Committee established a group ADI "not specified" for lutein from *Tagetes erecta*, lutein esters from *Tagetes erecta* and zeaxanthin (synthetic).** *Meso*-zeaxanthin was not included in this group ADI, as specifications are not currently available. **The group ADI of 0–2 mg/kg bw for lutein from Tagetes erecta and zeaxanthin (synthetic) was withdrawn.**
Neutral methacrylate copolymer (NMC)	N, T	**The Committee established an ADI "not specified" for NMC. The ADI "not specified" was made temporary because the specifications are tentative.** The Committee concluded that the use of NMC that complies with the specifications established at the current meeting is not of safety concern when the food additive is used as a coating or glazing agent for solid food supplements and for foods for special medical purposes. The NOAELs for NMC ranged from 454–2000 mg/kg bw per day, and these were the highest doses tested. The Committee evaluated exposure to NMC for the copolymer and its monomers (methyl methacrylate and ethyl acrylate). Estimated exposures to NMC range from 5.8 to 86 mg/kg bw per day. The total monomeric content of NMC is less than 0.01%. Toxicological data on the residual monomers do not give rise to concerns when taking into account the low dietary exposures.
Sorbitol syrup	–	Sorbitol syrup (INS 420(ii)) is currently included in the Codex General Standard for Food Additives (GSFA) although it has not been assigned an ADI or determined, on the basis of other criteria, to be safe. The Committee was therefore requested to consider the previous evaluations of sorbitol, hydrogenated glucose syrups and other relevant substances, and advise on the need for a separate evaluation of sorbitol syrup or if the ADI "not specified" for sorbitol is also applicable for sorbitol syrup. Based on the similarity of the chemical constituents of sorbitol syrup to the previously evaluated sorbitol, maltitol syrup and polyglycitol syrup, **the Committee concluded that there is no need for a separate evaluation of sorbitol syrup and established an ADI "not specified" for sorbitol syrup.**

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
Spirulina extract	N, T	**The Committee established a temporary ADI "not specified" for spirulina extract.** The ADI was based on the absence of toxicity in repeated-dose animal studies with spirulina extract and dried spirulina. **The ADI "not specified" was made temporary due to the tentative nature of the specifications.** Expressed as phycocyanins, estimated dietary exposure from the use of spirulina extract as a food colour based on the Budget method and exposure to spirulina extract and dried spirulina from other dietary sources, including food ingredients, dietary supplements, and coatings of food supplements was 190 mg/kg bw for adults (60 kg/person) and 650 mg/kg bw for a child (15 kg/person). The Committee concluded that this dietary exposure does not present a health concern.

–: no specifications prepared; N: new specifications; R: existing specifications revised; T: tentative specifications

[a] The specifications were made tentative pending the completion of the safety evaluation of AMC.

[b] At the current meeting, high-performance liquid chromatographic (HPLC) methods were added for determining subsidiary colouring matters and organic compounds other than colouring matters. The method of assay was changed to visible spectrophotometry, and spectrophotometric data were provided for the colour dissolved in water.

[c] The specifications for lutein esters from Tagetes erecta and zeaxanthin (synthetic) were maintained.

[d] At the current meeting, the identity test for melting range was deleted, the identity tests for carotenoids and spectrophotometry were updated, the test for propylene glycol was incorporated verbatim and the previous reference removed, and the method of assay was updated.

Food additives considered for specifications only

Food additive	Specifications
Cassia gum	R[a]
Citric and fatty acid esters of glycerol	R, T[b]
Glycerol ester of wood rosin	R[c]
Modified starches	R[d], T

R: existing specifications revised; T: tentative specifications

[a] The Committee, at its current meeting, received analytical methods and included the most suitable validated method in the specifications monograph. However, this method uses chloroform for the extraction of anthraquinones. Extraction with n-hexane and diethyl ether resulted in poor recovery of anthraquinones. The Committee recommends that the JECFA Secretariat be notified if an alternative extraction solvent is identified. The specifications were revised and the tentative status was removed.

[b] The Committee did not receive a replacement method for the obsolete packed column gas chromatographic method for the determination of total citric acid, in its specifications monograph. The Committee noted further that the method for total glycerol still uses chloroform. The Committee encouraged the submission of a method for total glycerol that eliminates the use of chloroform. Specifications were revised and made tentative pending the availability of data. Specifications will be withdrawn if suitable information is not provided by December 2019.

[c] The Committee received information on the manufacture of GEWR from the rosin obtained from the stumps of two additional species namely *Pinus halepensis* and *Pinus brutia* as source materials. Recognizing the natural variability of the composition of wood rosin, the Committee removed the restriction to certain pine species within the specifications. Since the specifications monograph for GEWR does not contain an assay, the Committee recommended that the JECFA Secretariat be notified upon the development and validation of an appropriate assay. The existing specifications were revised.

[d] The Committee reviewed data on the method of manufacture, identity, and purity of all 16 modified starches. Based on the information received, and available information the Committee noted that:

- All processes are performed under similar manufacturing conditions and result in minor chemical modifications. Given the chemical and physical similarities of modified starches, the Committee at previous meetings considered the application of a read-across approach to be appropriate for the toxicological evaluation of these substances.
- All 16 modified starches had been assigned an ADI of "not specified".
- All modified starches can be additionally bleached or fragmented; therefore revision in the specifications of bleached or fragmented starches would imply the revision of all 16 monographs;
- Microbiological specifications were not present in the existing specifications for all modified starches;
- Several specifications were common to all modified starches (such as for heavy metals impurities content and microbiological considerations). Revision of those common specifications would affect all 16 monographs;

(continued)

- As a result of the wide range of products manufactured, the identification tests required to unambiguously chemically characterize each modified starch in individual specifications may be cumbersome, potentially unavailable, and unlikely to reflect market requirements.
- It may not be possible to publish identification tests based on market requirements without unduly revealing proprietary information.
- Based on the points noted above, individual specifications for several modified starches may remain tentative for an indefinite period or may need to be withdrawn. The Committee therefore recommended that a new approach to the specifications monographs should be introduced to account for the chemical similarity between all modified starches, their functional diversity, the variety of chemicals used in their manufacture, and the corresponding diversity of impurities. The Committee recommended that all modified starches be included in a modular monograph titled 'Modified Starches' that contains common requirements [General specifications for modified starches] consisting of specifications that apply to all 16 modified starches (INS 1400, 1401, 1402, 1403, 1404, 1405, 1410, 1412, 1413, 1414, 1420, 1422, 1440, 1442, 1450, 1451), and annexes with specifications applicable to each individual modified starch based on the treatment(s) received. The Committee drafted a new modular specifications monograph titled "Modified starches" consisting of an explanatory introduction, "General specifications for modified starches," and eight annexes. The new modular specifications monograph for modified starches is printed in FAO Monograph 22, and will replace the 16 existing individual specifications for modified starches (INS 1400, 1401, 1402, 1403, 1404, 1405, 1410, 1412, 1413, 1414, 1420, 1422, 1440, 1442, 1450, 1451).

The specification for lead included in the General specifications be decreased from 2 mg/kg to 0.2 mg/kg. The limit of lead for starch sodium octenylsuccinate for use in infant formula and formula for special medical purposes intended for infants was set to 0.1 mg/kg in the General specifications.

The methods for the determination of free adipic acid and adipate groups, residual vinyl acetate, free octenyl succinic acid and octenyl succinate esters were revised and a method for the determination of propylene chlorohydrins was added.

Flavouring agents evaluated by the revised Procedure for the Safety Evaluation of Flavouring Agents

A. Alicyclic primary alcohols, aldehydes, acids and related esters

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
Mixture of 1-Vinyl-3-cyclohexenecarbaldehyde and 4-Vinyl-1-cyclohexenecarbaldehyde	2253	N	No safety concern
p-Mentha-1,8-dien-7-ol	974	N	No safety concern
p-Mentha-1,8-dien-7-yl acetate	975	N	No safety concern
Formyl-6,6-dimethylbicyclo[3.1.1]hept-2-ene	980	N	No safety concern
Myrtenol	981	N	No safety concern
Myrtenyl acetate	982	M	No safety concern
Structural class II			
(1-Methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl)cyclopropyl)methanol	2254	N	No safety concern
Structural class III			
(±)-Bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, ethyl ester	2255	N	No safety concern
Flavouring agent excluded at Step 1 of the Procedure			
p-Mentha-1,8-dien-7-al (Perillaldehyde)	973	M	Genotoxicity data for p-mentha-1,8-dien-7-al raise concerns for potential genotoxicity

N: new specifications
M: existing specifications maintained

B. Carvone and structurally related substances

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
Pinocarvyl isobutyrate	2242	N	No safety concern
Carvyl palmitate	2243	N	No safety concern
Structural class III			
6-Hydroxycarvone	2244	N	No safety concern
Flavouring agents not evaluated according to the revised Procedure			
(+)-Carvone	380.1	M	The Committee did not re- evaluate (+)-carvone (No. 380.1) according to the revised Procedure given the lack of information on the oral exposure from all sources and the need to review the ADI. A review of the ADI is recommended based on the evaluation of all biochemical and toxicological data. Also, data are needed for an exposure assessment for oral exposure to (+)-carvone from all sources to complete the evaluation for (+)-carvone.
(-)-Carvone	380.2	M	The Committee did not re- evaluate (-)-carvone (No. 380.2) according to the revised Procedure given the lack of information on the oral exposure from all sources and the lack of toxicological data.

M: existing specifications maintained; N: new specifications

C. Furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class III			
2-Pentylfuran	1491	M[a]	No safety concern
2-Heptylfuran	1492	M[a]	No safety concern
2-Decylfuran	1493	M[a]	No safety concern
3-Methyl-2-(3-methylbut-2-enyl)-furan	1494	M[a]	No safety concern
2,3-Dimethylbenzofuran	1495	M[a]	No safety concern
2,4-Difurfurylfuran	1496	M[a]	No safety concern
3-(2-Furyl)acrolein	1497	M[a]	No safety concern
2-Methyl-3(2-furyl)acrolein	1498	M[a]	No safety concern
3-(5-Methyl-2-furyl)prop-2-enal	1499	M[a]	No safety concern
3-(5-Methyl-2-furyl)butanal	1500	M[a]	No safety concern
2-Furfurylidene-butyraldehyde	1501	M[a]	No safety concern
2-Phenyl-3-(2-furyl)prop-2-enal	1502	M[a]	No safety concern
2-Furyl methyl ketone	1503	M[a]	No safety concern
2-Acetyl-5-methylfuran	1504	M[a]	No safety concern
2-Acetyl-3,5-dimethylfuran	1505	M[a]	No safety concern
3-Acetyl-2,5-dimethylfuran	1506	M[a]	No safety concern
2-Butyrylfuran	1507	M[a]	No safety concern
(2-Furyl)-2-propanone	1508	M[a]	No safety concern
2-Pentanoylfuran	1509	M[a]	No safety concern
1-(2-Furyl)butan-3-one	1510	M[a]	No safety concern

(continued)

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
4-(2-Furyl)-3-buten-2-one	1511	M[a]	No safety concern
Pentyl 2-furyl ketone	1512	M[a]	No safety concern
Ethyl 3-(2-furyl)propanoate	1513	M[a]	No safety concern
Isobutyl 3-(2-furan)propionate	1514	M[a]	No safety concern
Isoamyl 3-(2-furan)propionate	1515	M[a]	No safety concern
Isoamyl 3-(2-furan)butyrate	1516	M[a]	No safety concern
Phenethyl 2-furoate	1517	M[a]	No safety concern
Propyl 2-furanacrylate	1518	M[a]	No safety concern
2,5-Dimethyl-3-oxo-(2H)-fur-4-yl butyrate	1519	M[a]	No safety concern
Furfuryl methyl ether	1520	M[a]	No safety concern
Ethyl furfuryl ether	1521	M[a]	No safety concern
Difurfuryl ether	1522	M[a]	No safety concern
2,5-Dimethyl-3-furanthiol acetate	1523	M[a]	No safety concern
Furfuryl 2-methyl-3-furyl disulfide	1524	M[a]	No safety concern
3-[(2-Methyl-3-furyl)thio]-2-butanone	1525	M[a]	No safety concern
O-Ethyl S-(2-furylmethyl)thiocarbonate	1526	M[a]	No safety concern
(E)-Ethyl 3-(2-furyl)acrylate	2103	M[a]	No safety concern
di-2-Furylmethane	2104	M[a]	No safety concern
2-Methylbenzofuran	2105	M[a]	No safety concern

M: existing specifications maintained

[a] The text indicating that the safety evaluation for these flavouring agents had not been completed was removed from the specifications and the specifications were maintained as full

D. Linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
trans-6-Octenal	2240	N	No safety concern
2,6-Dimethyl-5-heptenol	2241	N	No safety concern

N: new specifications

E. Maltol and related substances

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class II			
Maltol	1480	M	No safety concern[a]
Structural class III			
Ethyl maltol isobutyrate	2252	N	No safety concern

M: existing specifications maintained N: new specifications

[a] The previously established ADI for maltol was withdrawn by the Committee.

F. Menthol and structurally related substances

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
Menthyl formate	2246	N	No safety concern
Menthyl propionate	2247	N	No safety concern
l-Menthyl butyrate	2248	N	No safety concern
dl-Isomenthol	2249	N	No safety concern
Dimenthyl glutarate	2250	N	No safety concern
Menthol	427	M	No safety concern[a]
Structural class I			
(±)-2-[(2-*p*-Menthoxy)ethoxy]ethanol	2251	N	No safety concern

M: existing specifications maintained N: new specifications
[a] The ADI of menthol of 0–4 mg/kg bw established at the fifty-first meeting was maintained

G. Miscellaneous nitrogen-containing substances

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class III			
2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine	2235	N	No safety concern
(*S*)-1-(3-(((4-Amino-2,2-dioxido-1*H*- benzo[c][1,2,6]thiadiazin-5- yl)oxy)methyl) piperidin-1-yl)-3- methylbutan-1-one	2236	N	No safety concern
2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide	2237	N	No safety concern

N: new specifications

H. Saturated aliphatic acyclic branched-chain primary alcohols, aldehydes, and acids

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
8-Methyldecanal	2238	N	No safety concern
8-Methylnonanal	2239	N	No safety concern

N: new specifications

Flavouring agents considered for specifications only

Flavouring agent	No.	Specifications
L-menthyl lactate	433	R[a]
L-malic acid	619	R[b]

(continued)

Flavouring agent	No.	Specifications
Glutamyl-valyl-glycine	2123	R[c]

[a] The CAS number was changed from 59259-38-0 to 61597-98-6 and the name to L-menthyl L-lactate.
[b] The specification for specific rotation were removed.
[c] The melting point range was revised.

This volume contains monographs prepared at the eighty-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Geneva, Switzerland, from 12 to 21 June 2018.

The toxicological and dietary exposure monographs in this volume summarize the safety and/or dietary exposure data on seven specific food additives (anionic methacrylate copolymer; basic methacrylate copolymer; erythrosine; indigotine; lutein and lutein esters from *Tagetes erecta* and zeaxanthin (synthetic); neutral methacrylate copolymer; and spirulina extract) and eight specific flavouring agents (alicyclic primary alcohols, aldehydes, acids and related esters; carvone and structurally related substances; furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters; linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters; maltol and related substances; menthol and structurally related substances; miscellaneous nitrogen-containing substances; and saturated aliphatic acyclic branched-chain primary alcohols, aldehydes and acids).

This volume and others in the WHO Food Additives series contain information that is useful to those who produce and use food additives and veterinary drugs and those involved with controlling contaminants in food: government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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