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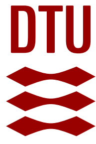

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WHO FOOD ADDITIVES SERIES: 75

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

Safety evaluation of certain food additives

Food and Agriculture Organization of the United Nations

World Health Organization

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World Health Organization, Geneva, 2019

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PREFACE

The monographs contained in this volume were prepared at the eighty-fourth 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 FAO headquarters in Rome, Italy, on 6–15 June 2017. These monographs summarize the data on selected food additives reviewed by the Committee.

The eighty-fourth report of JECFA has been published by WHO as WHO Technical Report No. 1007. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication. 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 and FAO 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.

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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:

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SAFETY EVALUATION OF SPECIFIC FOOD ADDITIVES

Brilliant Blue FCF (addendum)

First draft prepared by

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1. Explanation

Brilliant Blue FCF (Chemical Abstracts Service [CAS] No. 3844-45-9; International Numbering System for Food Additives [INS] No. 133) is a dye with a triphenylmethane base structure permitted as a food colour in the European Union, Japan, the United States of America (USA) and other regions. It is used for colouring breakfast cereals, cakes and cupcakes, candies, chewing gum, dairy products, decorations for baking, flavoured water and frozen treats.

The Committee previously evaluated the use of Brilliant Blue FCF as a food colour at the thirteenth meeting in 1969 (Annex 1, reference *19*). The specifications for Brilliant Blue FCF were prepared at the twenty-eighth Joint FAO/WHO Expert Committee on Food Additives (JECFA) meeting in 1984 and revised for metal specifications at the fifty-ninth meeting in 2002 (Annex 1, references *66* and *160*). An acceptable daily intake (ADI) of 0–12.5 mg/kg body weight (bw) was established by the Committee in 1969 (Annex 1, reference *19*). The ADI was based on a no-observed-adverse-effect level (NOAEL) of 5% (equivalent to 2500 mg/kg bw per day) derived from a chronic dietary toxicity study in rats (Hansen et al., 1966), with no explanation for the 200-fold uncertainty factor. More recent studies, including studies on absorption and excretion, biochemical effects, long- and short-term toxicity, carcinogenicity, genotoxicity, reproductive and developmental toxicity, and allergenicity, as well as studies on neurobehavioural effects and interaction with the membrane protein pannexin 1 (Panx1), have since become available.

Brilliant Blue FCF has been evaluated by the present Committee at the request of the Forty-eighth Session of the Codex Committee on Food Additives (FAO/WHO, 2016). Almost all of the new data were provided by the sponsor. Only eight additional publications were identified in a

literature search in Embase, PubMed and Scopus using the substance name, synonyms and the CAS number as search terms. The pre-1969 studies described below were considered by the Committee at the thirteenth meeting in 1969 (Annex 1, reference *19*).

1.1 Chemical and technical considerations

Brilliant Blue FCF consists mainly of disodium 3-[*N*-ethyl-*N*-[4-[[4-[*N*-ethyl-*N*(3-sulfobenzyl)amino]phenyl](2-sulfophenyl)methylene]-2,5-cyclohexadiene-1-ylidene]ammoniomethyl]benzenesulfonate and its isomers, together with subsidiary colouring matters, as well as sodium chloride and/or sodium sulfate as the principal uncoloured components. It is manufactured by condensing 2-formylbenzenesulfonic acid with a mixture of 3-[(*N*-ethyl-*N*-phenylamino)methyl]benzenesulfonic acid and its 2- and 4-isomers to form the leuco base precursor. Oxidation of the leuco base precursor with either chromium- or manganese-containing compounds produces the dye, which is isolated as the disodium salt. The dye contains not less than 85% total colouring matters. Impurities include unreacted starting material and reaction by-products (~2%), subsidiary colouring matters (≤6%), residual leuco base precursor (≤5%), unsulfonated primary aromatic amines (≤0.01% calculated as aniline), lead (≤2 mg/kg), chromium (≤50 mg/kg) and manganese (≤100 mg/kg).

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Brilliant Blue FCF was administered orally to rats as a 2% aqueous solution at 200 mg per rat. Almost the entire amount was excreted unchanged in the faeces within 40 hours. In a later investigation, the colour was found in bile in rats, rabbits and dogs after oral administration. In dogs, the amount in bile did not exceed 5% of the administered dose (Hess & Fitzhugh, 1953, 1954, 1955).

A dose of 0.27 mg/animal (~1.5 mg/kg bw) of ^{14}C-radiolabelled Brilliant Blue FCF (^{14}C on central methane; >99% pure) was given by gavage to intact (*n* = 5), bile duct–cannulated (*n* = 3) or bile duct–ligated (*n* = 5) female Sprague Dawley rats.

Only 0.27% of the dose was absorbed in intact animals and 2.05% in bile duct–ligated animals, with 91.1% and 97.3%, respectively, excreted in the faeces, and 91.7% and 99.4% of the total dose recovered. The total absorbed fraction included urinary ^{14}C excretion, expired $^{14}CO_2$ and residual radioactivity in internal organs and tissues over 96 hours. Biliary excretion was estimated to be 1.32% of the administered dose over 96 hours. The percentage of radioactivity expired as $^{14}CO_2$ was 0.04% of the dose in intact rats and 0.01% of the dose in bile duct–cannulated rats. Results obtained by thin-layer chromatography (TLC) of 24-hour urine and bile samples showed that about 95% of the excreted radioactivity was unaltered Brilliant Blue FCF and that about 5% was an unidentified metabolite or degradation product (Brown et al., 1980).

Almost complete excretion of Brilliant Blue FCF in the faeces was reported in another study in male and female rats. Following gavage administration of either 0.03 mg/kg bw or 3 mg/kg bw of ^{14}C-labelled Brilliant Blue FCF (^{14}C on central methane; radiochemical purity >95% by TLC), 99.9% and 95.4%, respectively, was recovered in faeces within 72 hours. Up to 0.5% was detected in the urine, which was speculated to represent cross-contamination of urine with faeces (Phillips et al., 1980).

In the same study, similar patterns of elimination were reported in pregnant rats following gavage administration of 3 mg/kg bw on day 8 of pregnancy, which was independent of prior 21-day dietary administration of unlabelled Brilliant Blue FCF at a dose of 30 mg/kg bw per day. Excretion was also almost completely via faeces in male mice and guinea-pigs, urinary excretion being less than 1%, regardless of dose level. TLC found no evidence of metabolites in rat or guinea-pig faeces. There was evidence of slower excretion in mice and guinea-pigs compared to rats.

Lack of gastrointestinal absorption and metabolism was further confirmed in situ using isolated loops of small intestine from guinea-pigs and mice. Negligible fractions of a 3 mg/kg bw dose were found in the bile of rats collected at 5 hours (<0.05%) and in day 11 fetuses (0.0004–0.0006%) of rats dosed orally on day 8 of gestation (Phillips et al., 1980).

In a more recently published study, the absorption of Brilliant Blue FCF on the lingual mucosa after licking lollipops was examined using an ex vivo porcine tongue system (Lucová et al., 2013). The tongue dorsum was exposed for 20 minutes to human saliva containing Brilliant Blue FCF (purity >85% of total colouring matters) at a reported concentration of 15 μg/cm^2. Fractions were analysed using an ultraviolet-visible spectrophotometer (limit of quantification: 6.6 ng/mL). Estimated distribution of the applied dose at 24 hours was 9.71 μg/cm^2 (~65%) remaining on the surface, 4.11 μg/cm^2 (27%) reaching the squamous epithelium and about 34 ng/cm^2 diffusing deeper than the surface layers (approximately 0.1% in the lamina propria and submucosa as well as 0.1% in the receptor fluid), from where it could be systemically bioavailable.

According to Peng et al. (2009), FD&C blue dye No. 1 (Brilliant Blue FCF) crosses the blood–brain barrier. However, the authors did not substantiate their statement with experimental data or any references. Based on a predictive absorption, distribution, metabolism and excretion (ADME) analysis (in silico), Park et al. (2009) stated that Brilliant Blue FCF may have higher brain permeability than colours such as Allura Red AC, amaranth, tartrazine and Sunset Yellow FCF.

In a pharmacokinetic study of biliary excretion in rats, spectrophotometry was used to analyse several food colours, including Brilliant Blue FCF, for excretion rate and protein binding (Iga, Awazu & Nogami, 1971). The average excretion ratios in 4 hours were 94.2%, 96.0% and 91.0% at doses of 3, 15 and 30 μmol/L, respectively. Equilibrium dialysis methods were used to analyse binding with plasma protein. Three millilitres of rat plasma in 30 mL isotonic pH 7.3 buffer solution containing Brilliant Blue FCF at a concentration of 1 μmol/mL was dialysed at 37 °C. The colour concentration of the buffer solution was determined after 160 hours of dialysis. The binding ratio of Brilliant Blue FCF with plasma protein was 65%.

In further studies, the authors investigated the relationship between the biliary excretion behaviour and the elimination from plasma of azo dyes and tryphenylmethane dyes in rats (Iga et al., 1971). Binding with bovine serum albumin and plasma protein was investigated using equilibrium dialysis methods. The analytical methods were the same as

described for the earlier study (Iga, Awazu & Nogami, 1971). The binding ratios of Brilliant Blue FCF with bovine serum albumin and plasma protein after 120 hours of dialysis at 4 °C were 16.2% and 39.7%, respectively.

2.1.2 Biotransformation

^{14}C-Radiolabelled Brilliant Blue FCF (^{14}C on central methane; >99% pure) was given by gavage to intact (*n* = 5), bile duct–cannulated (*n* = 3) or bile duct–ligated (*n* = 5) female Sprague Dawley rats. TLC of 24-hour urine and bile samples showed that about 95% of the excreted radioactivity was unaltered Brilliant Blue FCF and about 5% was an unidentified metabolite or degradation product (Brown et al., 1980).

Phillips et al. (1980) also used TLC but found no evidence for metabolites in rat and guinea-pig faeces. There are, however, no studies using more modern techniques to investigate biotransformation or identify metabolites.

2.1.3 Effects on enzymes and other biochemical parameters

The effect of Brilliant Blue FCF (among other food colour additives; purity not reported) on the activities of human phase I and II metabolic enzymes (CYP2A6, CYP3A4, UGT1A6 and UGT2B7) has been examined. Kuno & Mizutani (2005) and Mizutani (2009) reported that Brilliant Blue FCF is neither a substrate nor an inhibitor of any of the enzymes studied.

Eleven organic synthetic dyes used as food colours, including “Brilliant Blue” (purity 90.22%; chemical identity unclear), were tested to determine their effect on mitochondrial respiration in mitochondria isolated from rat liver and kidney. All food colours tested inhibited mitochondrial respiration (state III respiration, uncoupled), as measured with α-ketoglutarate or succinate as substrates. The extent of the inhibition varied widely, from 100% for erythrosine to 16% for tartrazine, at concentrations of 0.1 mg food colour per mg mitochondrial protein. Both rat liver and kidney mitochondria showed similar patterns of inhibition among the food colours tested. Brilliant Blue showed 78% inhibition of state III respiration rates of rat liver and kidney mitochondria with succinate as the substrate. The half maximal inhibitory concentration (IC_{50}) was 250 and 34 mg/g mitochondrial liver protein with α-ketoglutarate and succinate as substrates, respectively, and 28 mg/g mitochondrial kidney protein with succinate as substrate (Reyes, Valim & Vercesi, 1996).

In an in vitro study the *Xenopus* oocyte expression system was used to investigate the purinergic P2 receptors that interact with the membrane channel protein Panx1 in inflammasome signalling. Brilliant Blue FCF was shown to be a selective inhibitor of Panx1 channels, with an IC_{50} of 0.27 µmol/L, with no significant effect on the P2X7R receptor observed at concentrations as high as 100 µmol/L (Wang, Jackson & Dahl, 2013).

2.2 Toxicological studies

2.2.1 Acute toxicity

The oral median lethal dose (LD_{50}) in rats was reported to be higher than 2000 mg/kg bw (Lu & Lavallee, 1964).

In a dose-range finding experiment performed to identify the maximum tolerated dose for an in vivo comet assay, the LD_{50} in mice was higher than 2000 mg/kg bw (Sasaki et al., 2002).

2.2.2 Short-term studies of toxicity

(a) Mice

Mice were given 1200 mg Brilliant Blue FCF (called "Patentblau AE"; purity 37% colouring matter, the remainder sodium chloride and some sodium sulfate) in feed over 19 days. No adverse effects or other details were reported (Gross, 1961).

(b) Rats

Brilliant Blue FCF (purity not reported) tested for toxicity in weanling male Wistar rats (n = 5) for 21 days was reported to result in severe growth retardation when added to a low fibre diet at a level of 5% (equivalent to 5 g/kg bw, based on a conversion factor of 0.1). Addition of dietary fibre from edible burdock roots was reported to protect against this toxic effect (Tsujita et al., 1979).

Brilliant Blue FCF was tested for toxicity in male rats as part of two mixtures with tartrazine, Sunset Yellow and carmoisine (mixtures A and B) added to the diet for 30 or 60 days at a dose level for the combined mixture of 800 mg/kg bw per day (Aboel-Zahab et al., 1997). Neither the composition of the mixtures nor the concentration of each colour were specified. The Committee noted that this study cannot be considered

relevant for the evaluation of Brilliant Blue FCF because the investigation of mixtures of colours makes it impossible to attribute any effects directly to the test substance.

A substance described as "Brilliant blue (blue dye, N0.2) of the Imperial Chemicals Industries (ICI, England), from Cairo Food, Lona Company, Giza, Egypt" (purity not reported), which might be Brilliant Blue FCF, was tested in male Sprague Dawley albino rats (10/group) at a single dietary concentration of 124 mg/kg diet for 42 days, along with other food additives (tartrazine, carmoisine, *trans*-anethole propylene glycol and vanillin and their combinations).

Significant decreases in body weight, haemoglobin concentrations and red blood cell counts were reported for all treatment groups. Other changes reported include significant decreases in blood and liver levels of reduced glutathione and activities of glutathione-*S*-transferase and superoxide dismutase; and significant increases in activities of serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase and in blood levels of bilirubin, urea, creatinine, total protein and albumin. No effects on relative liver, kidney or heart weights were found (El-Wahab & Moram, 2013).

The Committee noted that the reliability of this study was limited because the identity and purity of the substance were unclear.

(c) Dogs

Twelve beagle dogs, 6–7 months of age, were fed Brilliant Blue FCF (purity not reported) at 0.0, 1.0% or 2.0% in the diet for up to 1 year (groups comprised one male and one female in the control group, two males and two females in the 1% group, and four males and two females in the 2% group). One dog at 2% died after 17 days and another at 1% after 46 weeks. Both deaths were attributed to viral infections. No clinical signs, gross lesions or microscopic pathology findings were attributed to the Brilliant Blue FCF exposure (Hansen et al., 1966).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

Groups of 57 male and 43 female mice were given Brilliant Blue FCF in the diet at 1 mg per day, and observed over 500–700 days. No evidence of carcinogenicity was found (Waterman & Lignac, 1958).

Brilliant Blue FCF (total colour content ≥85%; subsidiary colours ≤6%; sum of volatile matter chlorides and sulfates ≤15%) was given to ASH/CS1 mice (n = 48 males and 50 females) in the diet for up to 80 weeks at concentrations of 0, 0.015%, 0.15% or 1.5% (equivalent to 0, 20, 200 and 2000 mg/kg bw per day).

Significantly reduced body weight was reported, mainly in females at the highest dose. It was not clear if this was because of reduced feed consumption due to lower palatability of the test diet. No treatment-related changes in haematological parameters were observed in samples taken on weeks 13, 26 or 52 (10 mice/sex) and week 80 (all surviving mice). On week 13, significant reductions in reticulocyte counts were noted in males at 0.15% and both sexes at 1.5%. The low-dose group was not examined at this time point. At the highest dose, a significant decrease in the relative stomach weight of male mice and a significant increase in the relative brain weight of females, which appeared to be the result of the reduced female body weight, were reported. Complete histopathological examination of the control and high-dose animals was performed, but only the liver, kidney and selected tissues were examined in animals of the lower dietary levels, as was warranted by macroscopic observations. An increased incidence of glomerulonephritis was reported in all treated male but not female animals relative to the control group; the trend was not dose-dependent (14/44 in controls, 24/34 low dose, 17/30 mid dose, 24/44 high dose). An increased incidence of mild liver changes in male mice at the highest dose level was also observed (incidence of foam cells was 13/44 versus 2/44 in controls; incidence of fatty change was 11/44 versus 3/44 in controls). A statistically nonsignificant increase in reticulolymphatic tumours (lymphosarcoma and reticulum cell neoplasm) was noted at the highest dose level (8/44 versus 4/44 in controls) in male mice. A number of tumours observed randomly in single treated animals (but not in the controls) included squamous cell carcinoma of the stomach, thyroid adenoma, adrenal tumours, mesenteric lipoma and squamous

cell carcinoma of the skin. All of these tumours were considered to occur spontaneously and not be treatment related. The incidence of kidney tumours was increased in the 0.15% dietary group (six adenomas and one adenocarcinoma) compared with the control group and the high dietary group (only a single animal in each). No kidney tumours were identified in the low-dose group of males or any treated females. The authors noted that although kidney tumours are unusual in most strains of mice, they are common in the ASH/CS1 strain.

It was concluded that Brilliant Blue FCF was not carcinogenic in mice. The NOAEL was 0.15% (equivalent to 200 mg/kg bw per day) based on the slight reduction in weight gain and changes in organ weights of doubtful significance and some liver effects at the highest dose (Rowland et al., 1975).

The Committee noted that the study was performed before Organisation for Economic Co-operation and Development (OECD) guidelines and good laboratory practice (GLP) requirements were established and that its reliability was limited (e.g. only summary tables were available with no data on individual animals; the males were weighed individually while the females were weighed in caged groups of five).

Brilliant Blue FCF was given to Charles Rivers CD-1 mice (60/sex per group; 4 weeks old) in the diet for 24 months (104 weeks) at 0, 0.5%, 1.5% or 5% (equal to 0, 661, 2064 and 7354 mg/kg bw per day for males and 0, 819, 2562 and 8966 mg/kg bw per day for females, respectively). Separate male and female control groups ($n = 60$/sex) were used (Borzelleca, Depukat & Hallagan, 1990). The test material was reported to be certified by the United States Food and Drug Administration (USFDA) as Brilliant Blue FCF at 90% purity with the remaining 10% consisting of subsidiary colours, volatile chlorides and sulfates, and uncombined intermediates. Animals were monitored for clinical signs of toxicity twice daily, at least every 5 hours. Body weights and feed consumption were measured weekly for the first 14 weeks, biweekly on weeks 16–26 and monthly thereafter. Detailed physical examinations and palpation for masses were conducted weekly. Haematological parameters were evaluated at 3, 6, 12, 18 and 24 months (10/group, randomly selected). All animals that died spontaneously, were euthanized in a moribund condition or were killed as scheduled were necropsied, and organs were weighed. Histological

examinations were conducted on all animals from the two control groups and the highest dose group.

At the end of the study, 25 and 23 males survived in the control groups and 25, 28 and 33 in the low-, middle- and high-dose groups, respectively; and 24 and 31 females survived in the control groups and 31, 28 and 31 in the treatment groups, respectively. Blue staining of the hair, exposed skin and faeces was reported in all treatment groups, but there were no overt signs of treatment-related toxicity. There were no changes in feed consumption. The lower body weights ($P < 0.01$) recorded in different periods in both sexes at 1.5% or 5% were attributed to the replacement of caloric dietary content with the non-nutrient Brilliant Blue FCF. Except for occasional statistically significant decreases in mean haemoglobin, haematocrit and leukocyte counts, considered of no toxicological relevance, there were no treatment-related changes in haematological parameters.

Overall, there were no toxicologically relevant adverse effects from Brilliant Blue FCF exposure in the diet. Lifetime incidence of neoplasms in mice treated with Brilliant Blue FCF in the diet did not differ from the incidence in control groups. Except for an increased incidence of haemangiomas in females at 5%, the incidence rates of various lesions in mice in the 0.5% and 1.5% treatment groups were similar to the incidence rates in the controls. However, these changes were not statistically significant in the adjusted Fisher trend test.

It was concluded that there were no treatment-related neoplasms. The NOAEL was 5%, the highest dose tested (equal to 7354 mg/kg bw per day for males and 8966 mg/kg bw per day for females) (IRDC, 1981a; Borzelleca, Depukat & Hallagan, 1990). The Committee noted that survival at the end of the study was about 50% in both control and treatment groups. The Committee further noted that the study was performed before OECD guidelines were established but that the study was in compliance with USFDA GLP Regulations of 20 June 1979.

(b) **Rats**

Brilliant Blue FCF was administered at a dietary level of 4% to five male and five female rats for 600 days. Gross staining of the glandular stomach and some granular deposits in the stomach were observed, but no tumours (Willheim & Ivy, 1953).

Eighty-five rats were fed a diet containing 0.1% of Brilliant Blue FCF for their lifespan. The daily exposure was 10–15 mg. No tumours were found (Klinke, 1955).

Groups of 15 male and 15 female Wistar rats were given diets containing 0, 0.3% and 3% of Brilliant Blue FCF for 75 weeks (Mannell, Grice & Allmark, 1962). There were no adverse effects on growth, and the haematological findings were within normal ranges.

In its previous evaluation at the thirteenth meeting (Annex 1, reference *19*), the Committee established an ADI of 0–12.5 mg/kg bw based on a 2-year toxicity study in which 24 male and female weanling Osborne–Mendel rats, evenly divided by sex, were fed a diet containing 0, 0.5%, 1.0%, 2.0% or 5.0% Brilliant Blue FCF (purity not reported) (Hansen et al., 1966). The NOAEL was 5.0% (equivalent to 2500 mg/kg bw per day), the highest dose tested.

In a chronic toxicity study with a reproductive phase and in utero exposure phase, Charles Rivers CD rats (*n* = 60/sex per group; ~4 weeks old) received Brilliant Blue FCF in the diet for 17 weeks at 0, 0.1%, 1% or 2% (calculated to provide 0, 50, 514 and 1073 mg/kg bw per day in males and 0, 62, 631 and 1318 mg/kg bw per day in females). Separate male and female control groups (*n* = 60/sex) were used (IRDC, 1981b; Borzelleca, Depukat & Hallagan, 1990). These animals were designated the F_0 generation. One male and one female from the same treatment group for 15 consecutive days after 62 days of exposure to Brilliant Blue FCF. The chronic exposure phase was conducted on randomly selected F_1 animals (two F_1 rats/sex per litter) assigned to the control and treatment groups (70/sex per group) at the same dietary levels as the F_0 parental animals. The reproductive and developmental outcomes of this study are described below (section 2.2.5(a)). A minimum of 30 months (120 weeks) of exposure was required for the chronic exposure phase or until reduction in survival reached 12 rats/sex in any group. The maximum exposure was 116 weeks for F_1 males and 111 weeks for F_1 females.

The test material was the same as that used in the mouse study, namely USFDA-certified Brilliant Blue FCF (purity 90% with the remaining 10% consisting of subsidiary colours, volatile chlorides and sulfates, and uncombined intermediates). F_0 and F_1 animals were monitored for clinical signs of toxicity twice daily, at intervals at least 5

hours apart. Body weights and feed consumption were measured weekly for the first 14 weeks, biweekly on weeks 16–26, and monthly thereafter. Body weights were also recorded on gestation days 0, 6, 15 and 21 for F_0 females and on lactation days 0, 4, 14 and 21 for F_0 females and F_1 offspring. Physical examination and palpation for masses were conducted weekly. Haematological parameters were evaluated at 3, 6, 12, 18 and 24 months and at the end of the study (116 and 111 weeks for males and females, respectively) (10/group, randomly selected). Clinical chemistry and urine analysis were performed on samples collected after 25 months for males and 26 months for females. All animals that died spontaneously, were euthanized in a moribund condition or were killed as scheduled were necropsied, and their organs were weighed. All animals from the two control groups and the highest dose group were histologically examined as were randomly selected animals from the other treatment groups (10/sex per group) at the interim kill at 12 months.

At the end of the study, 18 and 13 males survived in the control groups and 12, 12 and 18 in the low-, middle- and high-dose groups, respectively; and 28 and 24 females survived in the control groups and 23, 28 and 10 in the treatment groups, respectively (414 were killed in extremis or died spontaneously or accidentally prior to the end of the study). Blue staining of the hair, exposed skin and faeces was reported in all treatment groups, but no signs of overt toxicity related to treatment were reported. Except for reduced body weights in females in the 2% dose group from week 90 to the end of the study (15% reduced terminal body weight, $P < 0.01$, from week 102), there were no significant differences in body weight between treated and control animals. Despite the relatively high mortality observed, there was no dose-related trend in either males or females, although survival of high-dose females was significantly decreased ($P < 0.01$) compared to the control groups (10/60 in the high-dose group versus 28/60 in control group 1 and 24/60 in control group 2). None of the differences between control and treated animals in haematological, clinical chemistry or urine analysis parameters were statistically significant or considered treatment related. A slight increase in feed consumption in males and females at the 2% dose level was significant ($P < 0.05$) on random weeks. No treatment-related gross or histological changes were found at necropsy or follow-up histological evaluation either at the interim kill or at the end of the study. Lifetime incidence of neoplasms in treated rats did not differ

from the incidence in control groups. Microscopic examination of the macroscopically observed masses in animals at 0.1% and 1% Brilliant Blue FCF revealed only random lesions that were considered spontaneous.

It was concluded that there were no treatment-related neoplasms. The NOAEL was the highest dose tested in males (equivalent to 1073 mg/kg bw per day) and the middle dose in females (equivalent to 631 mg/kg bw per day) based on decreased mean body weight and survival at the highest dose level. The Committee noted that the study was performed before OECD guidelines were established but that it was in compliance with USFDA GLP Regulations of 20 June 1979.

2.2.4 Genotoxicity

No mutagenic activity has been observed with Brilliant Blue FCF in several in vitro mutagenicity studies conducted in *Salmonella typhimurium*, *Bacillus subtilis* and *Escherichia coli* (Auletta, Kuzava & Parmar, 1977; Brown, Roehm & Brown, 1978; Bonin & Baker, 1980; Haveland-Smith & Combes, 1980; Kawachi et al., 1980; Bonin, Farquharson & Baker, 1981; Ishidate et al., 1984). Positive findings were reported in two in vitro chromosomal aberration assays (Kawachi et al., 1980; Ishidate et al., 1984), one in vitro micronucleus assay (Kus & Eroglu, 2015) and one in vitro comet assay (Pandir, 2016), but these studies had some shortcomings.

Negative results were obtained in vivo in a micronucleus assay in bone marrow (Hayashi et al., 1988) and a comet assay in the stomach, colon, liver, kidney, bladder, lung, brain and bone marrow of mice (Sasaki et al., 2002). The Committee noted that while these two in vivo studies are not fully compliant with the current versions of OECD guidelines 474 and 489, respectively, they were sufficiently reliable to be considered in the assessment.

The results of these studies are summarized in Table 1.

Based on the available data, the Committee concluded that there is no concern with respect to genotoxicity of Brilliant Blue.

2.2.5 Reproductive and developmental toxicity

(a) Reproductive toxicity

In a 3-generation reproductive toxicity study, Brilliant Blue FCF (purity 92%) was given in the diet to male and female Long–Evans rats at concentrations calculated to provide dose levels of 0, 10, 100, 300 or 1000

Table 1
Genotoxicity of Brilliant Blue FCF in vitro and in vivo

Test system	Test object	Concentration	Result	Reference
In vitro				
Reverse mutation	*Salmonella. typhimurium* TA98, TA100	100–10 000 µg/plate[a,b]	Negative	Bonin & Baker (1980)
	S. typhimurium TA98, TA100	320, 1 000, 3 200, 10 000 µg/plate[c]	Negative	
	S. typhimurium TA98, TA100, TA1535, TA1537, TA1538	320, 1 000, 3 200, 10 000 µg/plate[c,d]	Negative	
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	10, 100, 1 000, 10 000 µg/plate[b,c,d]	Negative	Bonin, Farquharson & Baker (1981)
		320, 1 000, 3 200, 10 000 µg/plate[c,d,e]	Negative	
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	50, 250, 1 000 µg/plate[c,d]	Negative	Brown, Roehm & Brown (1978)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	1, 10, 100, 1 000, 10 000 µg/plate[c,d]	Negative	Auletta, Kuzava & Parmar (1977)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA92, TA94	Up to 5 000 µg/plate[d,f]	Negative	Ishidate et al. (1984)
Reverse mutation	*S. typhimurium* TA98	Not reported	Negative	Kawachi et al. (1980)
Reverse mutation	*S. typhimurium* TA1538	10 mg/mL[d,g]	Negative	Haveland-Smith & Combes (1980)
Reverse mutation	*Bacillus subtilis* (rec assay)	Not reported	Negative	Kawachi et al. (1980)
Reverse mutation	*Escherichia coli* WP2 trp uvrA	10 mg/mL[d,h]	Negative	Haveland-Smith & Combes (1980)
Chromosomal aberration test	Chinese hamster fibroblast cell line (CHL)	Up to 5 000 µg/mL[h]	Positive[i]	Ishidate et al. (1984)
Chromosomal aberration test	Hamster lung fibroblasts	Not reported	Positive[j]	Kawachi et al. (1980)
Micronucleus assay	Human lymphocytes	10, 20, 30, 40 mg/mL[k]	Positive[l]	Kus & Eroglu (2015)
Comet assay	Human sperm cells	50, 100, 200, 500 µg/mL[l]	Positive[m]	Pandir (2016)
In vivo				
Bone marrow micronucleus assay	ddY Mice (M)	500, 1 000, 2 000 mg/kg bw[n]	Negative	Hayashi et al. (1988[o])
		1 010 mg/kg bw per day[p]	Negative	
Comet assay	ddY Mice (M)	2 000 mg/kg bw[q,r]	Negative[s]	Sasaki et al. (2002)

bw: body weight; M: male; OECD: Organisation for Economic Co-operation and Development; S9: 9000 × *g* supernatant fraction from liver homogenate; USFDA: United States Food and Drug Administration
[a] Range finder.
[b] Plate incorporation method.
[c] With and without S9.
[d] Material supplied by USFDA.
[e] Four samples from different suppliers tested, detailed results for one sample reported.
[f] The preincubation method was used in the presence of S9.
[g] Fluctuation test with 72–96 hour incubation at 37 °C.
[h] Treated for 24 hours and 48 hours; D_{20} (dose at which aberrations were found in 20% of cells): 4.45 mg/mL; frequency of cells with aberrations per unit dose: 3.
[i] Gaps not excluded from the evaluation; cytotoxicity not reported; purity not reported.
[j] No details were reported.
[k] Without S9.
[l] Incubated continuously for 72 hours at 37 °C; does not comply with OECD study design (cells were exposed for 72 hours; only 500 cells/concentration analysed; purity not reported; no positive control; identity of negative control not reported). Increase of micronucleus frequency was accompanied by cytotoxicity.
[m] High % tail DNA in all control groups (>50%); purity not reported.
[n] Single intraperitoneal administration with sampling 26 hours later.
[o] Appears by the synonym CI Acid Blue 9 (the CAS No. 3844-45-9 complies with the JECFA specification); 1000 polychromatic erythrocytes (PCEs) scored per animal.

Table 1 (continued)

[p] Four daily intraperitoneal administrations with sampling 24 hours after the final administration.
[q] Administered via oral gavage.
[r] Groups of animals were killed 3 or 24 hours after single dose administration.
[s] Tissues examined included glandular stomach, colon, liver, kidney, bladder, lung, brain and bone marrow; four animals per dose group; 50 nuclei per organ per animal measured.

mg/kg bw per day. In each generation, 10 males and 20 females were treated with the same dose range of Brilliant Blue FCF or vehicle control. Dose formulations were adjusted for body weights. The first generation of parents (F_0) was mated twice; one of the second generation of parents (F_{1b}) was mated three times, and one of the third generation of parents (F_{2b}) was mated once (offspring: F_{3a}). Dams were allowed to raise their F_{2a} and F_{2b} offspring to weaning. The F_{1b} dams were killed on gestation day 19 after the third mating and their uterine contents were examined. Offspring that were not selected for subsequent mating were killed at weaning and necropsied. Parental body weights, feed consumption, offspring survival and growth were recorded. F_{1b} and F_{3a} animals (5/sex per dietary dose level) were necropsied, and certain tissues were preserved. Histopathological examination of selected tissues of control and high-dose F_{1b} and F_{3a} animals was undertaken.

The mean body weights of the high-dose groups was lower than that of the control group in nursing offspring and in F_1 and F_2 males and females. There were no differences between treated and control animals in adult mortality, mating success, pregnancy and fertility rates, length of gestation period, offspring survival or sex, litter survival or necropsy findings in the animals killed on gestation day 19. Macroscopic and microscopic examinations of F_{1b} and F_{3a} animal tissues showed no treatment-related findings. Survival rates were compared using the chi-square test, but no other statistical analyses were shown (BioDynamics Inc., 1971). The Committee noted that the study was performed before OECD guidelines or GLP requirements were established, but the study design was based on a USFDA-approved protocol.

A single-generation reproductive toxicity study was conducted as part of a chronic toxicity study in rats (IRDC, 1981b; Borzelleca, Depukat & Hallagan, 1990). The test material was USFDA-certified Brilliant Blue FCF of 90% purity with the remaining 10% consisting of subsidiary colours,

volatile chlorides and sulfates, and uncombined intermediates. Brilliant Blue FCF was given to 60 animals/sex per group (~100 days of age) for 62 days at dietary levels of 0, 0.1%, 1% and 2% (equivalent to 0, 50, 514 and 1073 mg/kg bw per day for males and 0, 62, 631 and 1318 mg/kg bw per day for females, respectively). One male and one female from the same treatment group were mated for 15 consecutive days. Body weights of the dams were recorded on gestation days 0, 4, 14 and 21.

There were no differences between controls and treated animals in fertility, gestation, parturition, lactation, pup survival through weaning or number of live and stillborn pups. Dam mortality was not treatment related. One female in the 0.1% group, one male and one female in the 1.0% group, and one male in the 2.0% group died.

The NOAELs for reproductive end-points were 1073 mg/kg bw per day for males and 1318 mg/kg bw per day for females, the highest dose levels tested. The Committee noted that the study was performed before OECD guidelines were established but that it was in compliance with USFDA GLP Regulations of 20 June 1979.

Brilliant Blue FCF (purity >85%) was tested for reproductive toxicity and the potential induction of neurobehavioural effects in Crlj:CD1 mice (10/sex per group; 5 weeks of age at treatment start) at dietary levels of 0, 0.08%, 0.24% and 0.72% (equivalent to 111–407, 347–1287 and 1032–3856 mg/kg bw per day). Administration commenced 4 weeks prior to mating (F_0 mice mated at 9 weeks of age for 5 days) and continued through gestation (14 days), lactation (from birth to weaning, ~4 weeks) and through to 4–11 weeks of age in the F_1 generation. Selected reproductive and neurobehavioural parameters were measured.

There were no significant differences between control and treated F_0 mice in body weights or reproductive success, except for significantly increased average body weight of dams in the high-dose group in the second week of lactation. No treatment-related differences were observed in offspring survival, body weights or neurobehavioural parameters (Tanaka et al., 2012).

The authors concluded that the high dose of Brilliant Blue FCF resulted in a few significant effects on behavioural development and no significant effects on reproduction. The Committee noted that the study was not performed according to GLP and did not meet current

OECD guidelines (e.g. only some selected reproductive parameters were measured).

(b) **Developmental toxicity**

Brilliant Blue FCF (purity 92%) was given to pregnant Long–Evans rats by oral gavage (in 0.5% methylcellulose at 1 mL/100 g bw) from gestation day 6 to 15 at 0, 200, 600 or 2000 mg/kg bw per day (*n* = 22–24/group). Three vehicle control groups (*n* = 22 each) were included and a positive control group of animals (*n* = 22) treated with 30 mg/kg bw per day of Trypan Blue (injected subcutaneously from day 7 through day 9). Dams that survived to the end of the treatment period were killed on day 20. The number of corpora lutea recorded and uterine contents examined. All fetuses were examined for malformations and about two thirds were examined for skeletal ossification, variations and anomalies. Statistical comparisons between test and vehicle control groups were made with chi-square test, *t*-test or *F*-test, as appropriate.

There was no evidence of fetal toxicity or any developmental abnormalities that could be attributed to the administration of Brilliant Blue FCF (BioDynamics Inc., 1972b).

In a parallel developmental toxicity study, Brilliant Blue FCF (purity 92%, the same batch as in the rat study described above) was given orally (in 0.5% methylcellulose at 1 mL/kg bw) to pregnant New Zealand White rabbits on gestation days 6–18 at 0, 20, 60 or 200 mg/kg bw per day (*n* = 18, 19 and 15, respectively). Three vehicle control groups (*n* = 15–17/group) were included and a positive control group of animals (*n* = 15) treated with 150 mg/kg bw per day of thalidomide. Rabbits that survived to the end of the study were killed on day 20. The number of corpora lutea were recorded and uterine contents examined. All fetuses were weighed, examined macroscopically for externally visible defects and visceral anomalies and examined for skeletal ossification, variations and anomalies. Statistical comparisons between test and vehicle control groups were made with chi-square test, *t*-test or *F*-test, as appropriate.

There was no evidence of fetal toxicity or anomalies that could be attributed to the administration of Brilliant Blue FCF (BioDynamics Inc. 1972a).

The Committee noted that these two studies were performed before OECD guidelines and GLP requirements were established. The protocols

were, however, based upon segment II (Teratology Study) of the USFDA "Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use" from 1966.

2.2.6 Special studies

(a) Allergenicity

Brilliant Blue FCF (purity not reported) did not cause significant degranulation or histamine release from rat mast cells as demonstrated in vitro and ex vivo using mast cells harvested from the peritoneal cavity of male Sprague Dawley rats (Kreindler, Slutsky & Haddad, 1980). The rats were given the colour in drinking-water for 3 months at concentrations found in coloured soft drinks. The degranulation was investigated microscopically and the histamine release was assayed spectrophotofluorometrically. The authors concluded that Brilliant Blue FCF does not release histamine by an immunological mechanism.

No evidence of sensitization potential was found when Brilliant Blue FCF was tested in a local lymph node assay (SCCNFP, 2004).

(b) Neurotoxicity

Brilliant Blue FCF (purity not reported) was among other food colours evaluated in neuroblastoma cells in vitro for synergistic effects on developmental neurotoxicity parameters (Lau et al., 2006). Inhibition of neurite outgrowth over 24 hours was used as a marker of developmental neurotoxicity. Neurite outgrowth length was reduced in cells treated with Brilliant Blue FCF (0.05–500 nmol/L) in combination with L-glutamic acid (0.5–100 µmol/L), although each of them alone resulted in relatively weak inhibition of neurite outgrowth. The authors concluded that the combined treatment produced a synergistic inhibitory effect.

Park et al. (2009) examined the potential developmental toxicity in the central nervous system of Brilliant Blue FCF (purity not reported) as a single substance or in combination with other food colours by assessing neural progenitor cell survival and proliferation in vitro and the numbers of new neuronal progenitor cells in young ICR mice in vivo. Cells in culture were treated with Brilliant Blue FCF at concentrations of 100 nmol/L to 1 mmol/L at 10-fold increments for 12, 24 or 48 hours. Cell toxicity and proliferation were measured with the MTT assay. Only the highest

concentration resulted in reduced cell proliferation and survival after 48 hours of exposure. Cell death was also noted at the highest concentration after 12 and 24 hours and at the second highest concentration after 24 hours.

In the second part of the study, male mice (5 weeks old) were treated with Brilliant Blue FCF by oral gavage for 2 weeks, at 0.426, 4.26 or 42.6 mg/kg bw per day (equal to 10-, 100- and 1000-fold the estimated daily dietary exposure to Brilliant Blue FCF of consumers in the Republic of Korea, that is, ~4% of ADI of 12.5 mg/kg bw per day). New neuronal growth was evaluated in brain sections following staining for bromodeoxyuridine (BrdU; 100 mg/kg bw per day) administered by intraperitoneal injections twice daily for 3 days; the mice were killed 1 day after the last injection. There were no effects on body weight. No differences in BrdU incorporation were noted in animals treated with Brilliant Blue FCF alone. Decreased staining, implying reduced neuronal growth, was reported in animals treated with binary combinations of 1 mmol/L of each colour, including Brilliant Blue FCF with tartrazine, for 48 hours. The combination of Brilliant Blue FCF and tartrazine resulted in significantly decreased numbers of newly generated cells in adult mouse hippocampus at the highest dose (42.6 mg/kg bw per day Brilliant Blue FCF and 147.6 mg/kg bw per day tartrazine). According to the authors, this indicates adverse actions on hippocampal neurogenesis. However, it is not clear to what extent this effect was due to Brilliant Blue FCF (Park et al., 2009).

In a reproductive toxicity study in Crlj:CD1 mice (10/sex per group; 5 weeks of age at the start of treatment) (described in section 2.2.5), Tanaka et al., (2012) tested Brilliant Blue FCF (purity >85%) for potential induction of neurobehavioural effects at dietary levels of 0, 0.08%, 0.24% or 0.72% (equivalent to 111–407, 347–1287 and 1032–3856 mg/kg bw per day). Administration commenced 4 weeks prior to mating (F_0 mice mated at 9 weeks of age for 5 days) and continued through gestation (14 days), lactation (from birth to weaning, ~4 weeks) and through to 4–11 weeks of age in the F_1 generation.

Statistically significant trends of increased movement time ($P = 0.019$) in the exploratory behaviour test was noted in F_0 females after 3 weeks of treatment. However, there was a trend to decreased average time

of rearing (P = 0.027). A significant trend (P < 0.01) of delayed surface righting in F_1 males and females was observed on postnatal day 4, and the difference was also significant between both the high-dose male and female offspring and control groups. However, when the same test was run on postnatal day 7, there were no significant differences for either sex. There were also no effects in the inclined plane test (postnatal days 4 and 7), in cliff avoidance (postnatal day 7), in the swimming test (postnatal day 7) or olfactory orientation (postnatal day 14). The authors also described some statistically significant trends in exploratory behaviour in F_1 males and females, although they noted that the effects in males appeared inconsistent (Tanaka et al., 2012).

The Committee noted that the study was not performed according to GLP and did not meet the 2007 OECD Test Guideline 426 (e.g. concerning the number of animals per group).

In a study examining the association of artificial colour exposure and behavioural and learning outcomes, Brilliant Blue FCF (purity not reported) was included in a mixture with eight other colouring agents (erythrosine, Ponceau 4R, Sunset Yellow, Allura Red, amaranth, tartrazine, azorubine and Indigo Carmine) given by oral gavage to female Wistar Han rats (n = 15/group), each at a dose equal to their respective ADI. The animals received a dose of 12.5 mg/kg bw per day of Brilliant Blue FCF. The dams were treated for 1 week prior to mating, 5 days during mating and throughout pregnancy (with males untreated except for during the mating period). F_1 offspring were weaned 1 month after birth. At 12 weeks of age, the brains of the F_1 generation were isolated and tested for protein expression of the subunits NR2A and NR2B of the *N*-methyl-*D*-aspartate receptors and subunits a4, b2 and a7 of the nicotinic acetylcholine receptors (nAChRs) in brain tissue homogenates (hippocampi) by Western blotting and image density analysis.

In males, protein expression levels of NR2B and nAChR b2 was significantly increased (17% and 6.7%, respectively), whereas expression of nAChR a4 was significantly decreased (5.67%) compared with controls (P < 0.05). In contrast, a 14% decrease in NR2B protein levels was reported in females (P < 0.05). However, it is not clear to what extent the observed effects were due to Brilliant Blue FCF (Ceyhan et al., 2013).

In a developmental neurobehavioural study, Brilliant Blue FCF (purity not reported) was given to female Wistar albino rats (*n* = 15/group) before and during gestation in a mixture of eight other colouring agents (erythrosine, Ponceau 4R, Sunset Yellow, tartrazine, amaranth, Allura Red, azorubine and Indigo Carmine). The mixture was given in water by oral gavage at concentrations calculated to deliver a dose of each colouring agent equivalent to their NOAEL; for Brilliant Blue FCF, the dose was 600 mg/kg bw per day. The effects of the mixture on spatial working memory in offspring was assessed using the Morris water maze test, and behavioural and locomotor effects were assessed using the open-field and forced-swim tests.

No adverse effects were reported on spatial working memory and no depressive behaviour was reported in offspring. A few parameters of locomotor activity were significantly increased including "latency to locate the visible platform" (a measure of motivation) and forced-swim test parameters; anxiolytic-like effect in the open-field test; and mobility time in the forced-swim test. The authors suggested that the findings implied a sex-related increase in motility and a decrease in motivation and anxiety. It is, however, not clear to which extent the effects observed were due to Brilliant Blue FCF (Doguc et al., 2015).

(c) Skin cancer

Brilliant Blue FCF was tested for skin carcinogenicity in Swiss Webster mice (50/sex per group), among other colouring substances (Carson, 1984). Brilliant Blue FCF (0.1 mL of a 1% aqueous suspension) was applied to 6 cm^2 of clipped skin twice per week for 18 months. Untreated and vehicle negative controls were included. The treatment with Brilliant Blue FCF did not increase the incidence of skin neoplasms compared to control animals, unlike treatment with the positive control, 3,4-benzopyrene in acetone, which did. It was concluded that Brilliant Blue FCF is unlikely to cause skin cancer as a result of repeated dermal exposure.

2.3 Observations in humans

Two out of 24 urticaria patients reported sensitivity to "Brilliant Blue" (purity not reported, chemical identity unclear) when tested using a patch

test for allergic response to drug excipients. The condition of these patients was exacerbated by treatment with antihistamines (Shah, 2010).

No evidence of skin irritation or sensitization was reported in 207 human volunteers who underwent daily skin applications of a 5% aqueous solution of Brilliant Blue FCF or its aluminium lake. Testing included an induction phase of three applications per week for 3 weeks, followed by a challenge application 12 days later (BIBRA, 1990).

Three cases of chronic, unexplained pruritic skin disorders responded to medication changes related to the presence of Brilliant Blue FCF (Swerlick & Campbell, 2013). Because these medications also contained other dyes and excipients, it is not clear to what extent the response observed was due to Brilliant Blue FCF.

There are several reports in which the use of Brilliant Blue FCF in enteral feeding solutions has been associated with toxicity, including 12 deaths (WHO, 2003; Maloney & Brand, 2016). Its use in enteral feeding solutions was to facilitate detection and/or monitoring of pulmonary aspiration in patients fed via an enteral feeding tube. Some of the reported cases were associated with serious complications such as refractory hypotension, metabolic acidosis and death. A drip-chamber dye pellet tubing system that delivers 10 mg of dye per hour was used in several cases (Maloney & Brand, 2016). In a case with sepsis who developed green urine, serum and skin while receiving enteral feedings tinted with Brilliant Blue FCF, the concentrations of Brilliant Blue FCF in serum and urine samples were 10 and 100 µg/mL, respectively (Maloney & Brand, 2016). A causal relationship between systemic absorption of Brilliant Blue FCF and the reported outcomes has not been definitively established (WHO, 2003).

3. Dietary exposure

The Committee prepared a review of dietary exposure of Brilliant Blue FCF based on information from the European Food Safety Authority (EFSA, 2010); the Council of Scientific and Industrial Research (CSIR), Food Standards Australia New Zealand (FSANZ, 2012); India (Dixit et al., 2011; Dixit, Khanna & Das, 2013); the Kuwait Institute for Scientific Research (Husain et al., 2006); Konkuk University, Republic of Korea (Ha et al., 2013); and USFDA (Doell et al., 2016) (Table 2).

Table 2
Summary information on studies of dietary exposure to Brilliant Blue FCF

Organization (reference)	Concentration data	Sampling strategy	Consumer groups	Food consumption data	Estimates of dietary exposure
USFDA (Doell et al., 2016)	Analytical levels in foods	Targeted selection using label and/or colour	USA population 2 years and older; children 2–5 years; boys 13–18 years)	Individual dietary records food diaries. 2 days; 10–14 days	Mean; P90 scenarios[a,b,c]
EFSA (EFSA, 2010)	Budget method; MPLs; use levels in foods		Adults; Children 1–5 years (United Kingdom: 1.5–4.5 years)	Individual dietary records or 24-hour recall (depending on survey), 2–7 days	Mean; P95 or P97.5 scenarios[a,b,c]
FSANZ (FSANZ, 2012)	Analytical levels in foods in combination with use levels (for confectionery)	Targeted selection using label and/or colour	2 years and above; 2–5 years; 6–12 years; 13–18 years; 19–24 years; 25 years and above	24-hour recall, 2 days	Mean; P90 scenarios[b,c]
Konkuk University, Republic of Korea (Ha et al., 2013)	Analytical levels in foods	Market share	1–2 years; 3–6 years; 7–12 years; 13–19 years; 20–29 years; 30–49 years; 50–64 years; 65 years and above; male; female; upper 95th consumers	24-hour recall	Mean; P95 scenarios[b]
CSIR, India (Dixit et al., 2011; Dixit, Khanna & Das, 2013)	Analytical levels in foods	Targeted selection using colour in combination with market share	4–6 years; 7–9 years; 10–12 years; 13–15 years; 16–18 years	Limited household survey; food frequency recall	Mean; P95 scenarios[b]
Kuwait Institute for Scientific Research (Husain et al., 2006)	Analytical levels in foods	Targeted selection using label and/or colour	5–14 years (one year per group, male or female)	24-hour recall; 2 days	Mean; Scenarios[b]

CSIR: Council of Scientific and Industrial Research; FSANZ: Food standards Australia New Zealand; EFSA: European Food Safety Authority; MPL: maximum permitted level; USFDA: United States Food and Drug Administration; P90: 90th percentile; P95: 95th percentile; P97.5: 97.5th percentile

[a] The lowest analytical value for the colour additive was assigned to each food code.

[b] The analytical results were averaged for a given food code.

[c] The highest analytical value for the colour additive was assigned to each food code.

Since these studies were based on individual dietary records, analytical levels in foods or reported use levels, the Committee did not consider it necessary to include dietary exposure estimates based on the budget method, poundage data or diet models.

3.1 Assessments based on individual dietary records

3.1.1 Exposure estimates reported by USFDA

Brilliant Blue FCF is approved in the USA for general use in food at levels consistent with good manufacturing practice (GMP). In a study completed by the USFDA (Doell et al., 2016), approximately 600 samples from 52 broad food categories were analysed for Brilliant Blue FCF (Harp, Miranda-Bermudez & Barrows, 2013). Samples were selected based on a previous survey on food labels. Brilliant Blue FCF was found in products in 36 of the 52 food categories at concentrations ranging from the limit of detection (LOD; 1 mg/kg) to over 10 000 mg/kg (in the category “Frostings and Icings”; the second highest value (233 mg/kg) was found in the category “Ice Cream Cones”). If Brilliant Blue FCF was not included in the ingredient list, it was presumed it was not present in the food. However, if it was declared on the label, but the result for the colour was below the LOD, Brilliant Blue FCF was presumed present in the food at the LOD.

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 exposure survey for the United States population. Three population groups were used for the exposure estimate: the overall population 2 years and older; children aged 2–5 years; and teenage boys aged 13–18 years. Exposures were estimated based on “eaters-only”, that is, 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 Brilliant Blue FCF 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 (Table 3).

For all three groups “Juice Drinks” were the main contributor (20–25% of total exposure). Other major contributing food groups were “Ice Cream Cones”, “Soft Drinks”, “Breakfast Cereal” and “Decoration/Chips for Baking”.

Table 3
Eaters-only exposure to Brilliant Blue FCF for the United States population based on NHANES 2-day and NET-NID 10- to 14-day food consumption data

Study / Population age in years	% Eaters	Dietary exposure (mg/kg bw per day)					
		Low-exposure scenario		Average-exposure scenario		High-exposure scenario	
		Mean	P90	Mean	P90	Mean	P90
NHANES 2-day food consumption data							
≥2	92	0.01	0.03	0.02	0.05	0.04	0.09
2–5	96	0.04	0.07	0.07	0.1	0.1	0.2
Boys 13–18	93	0.02	0.03	0.02	0.05	0.04	0.1
NET-NID 10- to 14-day food consumption data							
≥2	99	0.01	0.02	0.02	0.04	0.04	0.06
2–5	100	0.02	0.04	0.04	0.07	0.1	0.1
Boys 13–18	100	0.01	0.03	0.02	0.04	0.04	0.06

bw: body weight; NET-NID: National Eating Trends – Nutrient Intake Database; NHANES: National Health and Nutrition Examination Survey; P90: 90th percentile
Source: Doell et al. (2016)

3.1.2 Exposure estimates reported by EFSA

The EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) has re-evaluated the safety of Brilliant Blue FCF when used as a food additive (EFSA, 2010). A tiered approach was used.

Dietary exposure estimates using a budget method or maximum permitted levels (MPLs) of use are not included in the review since a more refined estimate using maximum use levels or maximum level determined by analysis was available (Table 4).

Refined exposure estimates were performed using national consumption data from a United Kingdom consumption survey (Tennant, 2008). For children (1–10 years old), estimates have been calculated for the United Kingdom (Tennant, 2008) and for 11 European countries as part of the EXPOCHI consortium (Belgium, Cyprus, the Czech Republic, Finland, France, Germany, Greece, Italy, the Netherlands, Spain and Sweden) (Huybrechts et al., 2011). The United Kingdom population was selected as representative of adult European Union consumers for Brilliant Blue FCF exposure estimates because this survey likely represented the highest exposed population in the European Union.

In the refinement using the maximum reported use levels (Table 4) made available by the United Kingdom Food Standards Agency (FSA), the

Table 4
Maximum permitted levels of use and maximum reported use levels of Brilliant Blue FCF in beverages and foodstuffs used for the refined exposure assessment

Food group	Maximum permitted level (mg/L)[a]	Maximum reported use level (mg/L)
Beverages		
Fruit wines, cider and perry	200	5[b]
Nonalcoholic flavoured drinks	100	65[b]
Liquid food supplements/dietary integrators	100	100[c]
Spirituous beverages	200	200[b]
Aromatized wines, aromatized wine-based drinks and aromatized wine-product cocktails	200	200[c]
Foodstuffs		
Complete formulas and nutritional supplements for use under medical supervision	50	20[b]
Complete formulas for weight control intended to replace total daily food intake or an individual meal	50	50[c]
Soups		
Flavoured processed cheese	100	100[c]
Fish paste and crustaceans paste		
Smoked fish		
Savoury snack products and savoury coated nuts		
Meat and fish analogues based on vegetable proteins		
Desserts including flavoured milk products	150	0.1[b]
Edible ices	150	145[b]
Fine bakery wares	200	200[b]
Candied fruit and vegetables, Mostarda di frutta	200	200[c]
Preserves of red fruits		
Extruded or expanded savoury snack products		
Pre-cooked crustaceans	250	250[c]
Confectionery	300	300[b]
Mustard	300	300[c]
Fish roe		
Solid food supplements/dietary integrators		
Sauces, seasonings, pickles, relishes, chutney and piccalilli	500	500[c]
Salmon substitutes		
Surimi		
Decorations and coatings	500	500[b]
Edible cheese rind and edible casings	*Quantum satis*	100[d]
Edible casings	*Quantum satis*	500[d]

[a] According to the European Parliament and Council Directive 94/36/EC.
[b] Maximum use level or maximum level determined by analysis.
[c] Maximum permitted level.
[d] *Quantum satis* data.
Source: EFSA (2010)

Food Safety Authority of Ireland (FSAI), the Union of European Beverage Associations (UNESDA) and the Confederation of the Food and Drink Industries of the European Union (CIAA), the dietary exposure estimate for Brilliant Blue FCF showed a mean dietary exposure for the adult population of the United Kingdom of 0.6 mg/kg bw per day and of 3.0 mg/kg bw per day for high-level consumers (97.5th percentile exposure from beverages plus per capita average from the rest of the diet).

The mean dietary exposure of European children (aged 1–10 years and weighing 16–29 kg) ranged from 0.2 to 2.1 mg/kg bw per day, and from 0.6 to 4.8 mg/kg bw per day at the 95th percentile. The main contributors to the total anticipated mean exposure to Brilliant Blue FCF (>10% in all countries) were nonalcoholic beverages (13–53%), fine bakery wares (12–64%) and sauces, seasonings, pickles, relishes, chutney and piccalilli (14–60%). Confectionery accounted for 19–24% of exposure in two countries, while extruded or expanded savoury snack products accounted for 17% of exposure in one country.

3.1.3 Exposure estimates based on individual dietary records (FSANZ)

FSANZ has published an analytical survey to quantify actual levels of permitted synthetic colours in foods and beverages in Australia. Brilliant Blue is permitted at 70 mg/L in beverages and 290 mg/kg in other foods. In 2006, 396 samples were collected and analysed for added colours. These results were combined with results of a South Australian survey of synthetic colours in foods, sampled in 2004, to give information for a total of 651 samples (FSANZ, 2008). The dietary exposure estimates for children have been updated (FSANZ, 2012) using food consumption data from the 2007 Australian Children's Nutrition and Physical Activity Survey and updated information on typical use level data from the confectionery industry. A comparison of the FSANZ 2008 analytical data with the concentrations provided by industry showed only showed negligible differences.

The FSANZ 2008 survey did not specifically analyse for synthetic colours in the "lake" form. Lake colours are formed when a synthetic dye is combined with a metallic salt substrate such as aluminium hydroxide. Due to their insolubility in water and stability in light, lake colours are generally used to colour the coating of panned sugar confectionery (Downham & Collins, 2000). After the publication of the added colours survey (FSANZ, 2008), industry provided FSANZ with concentration data on the usage of

lake colours in confectionery. These concentration data were used in the revised dietary exposure estimates for children aged 2–16 years (Table 5).

The main contributors in the four age groups were (in varying order within each age group) flavoured milk (14–25%); ice cream and edible ices (11–25%); soft drinks (12–30%); and cakes, muffins and pastries (11–15%).

3.1.4 Exposure estimates based on individual dietary records (Others)

The dietary exposure to Brilliant Blue FCF in the Republic of Korea was estimated based on food consumption data for consumers and concentrations in processed foods (Ha et al., 2013). Brilliant Blue FCF was found in samples of biscuits, candies, chocolate, chewing gum, ice cream, beverages, pickled vegetables and liquor. For the average consumer in the Republic of Korea (using average concentrations of all samples in a food group), the estimated mean and 95th percentile for dietary exposure were 0.01 and 0.03 mg/kg bw per day, respectively. For a high-exposure consumer (using the average of positive samples), the estimated mean and 95th percentile for dietary exposure were 0.05 and 0.12 mg/kg bw per day, respectively.

Brilliant Blue FCF is permitted in specific foods in levels up to 100 mg/kg in India. Dietary exposures to Brilliant Blue FCF for children in 16 states of India from selected food groups (bakery products, beverages, candyfloss, chewing gums, coated candies, hard-boiled sugar confectioneries, jam and jellies, ice candy/ice creams, mouth fresheners and sugar toys) have been estimated (Dixit et al., 2011). A limited household survey on the food consumption patterns of 518 subjects was conducted and the exposure to colours was assessed through the food frequency recall method. Estimated dietary exposure for Brilliant Blue FCF (average consumption, average concentration values) for children aged 4–6, 10–12, 13–15 or 16–18 years were 0.1 mg/kg bw per day while for children aged 7–9 years the estimated dietary exposure was 0.2 mg/kg bw per day. Estimated dietary exposure (high consumption, high concentration values) for children aged 4–6, 7–9, 10–12, 13–15 or 16–18 years were 0.4–1.0 mg/kg bw per day.

Dietary exposure to Brilliant Blue FCF in school children in Kuwait from selected food groups (biscuits, cakes and ice cream, candy, chips

Table 5
Estimated dietary exposures (consumers only) to Brilliant Blue FCF for Australian consumers

Age group	Consumers		Mean colours scenario (mg/kg bw per day)		Maximum colours scenario (mg/kg bw per day)	
(years)	*n*	%[a]	Mean	P90	Mean	P90
≥ 2[b]	7 761	56	0.01	0.03	0.05	0.12
2–5[c]	1 086	92	0.02	0.05	0.1	0.24
6–12[c]	1 956	94	0.02	0.04	0.07	0.17
13–16[c]	1 090	89	0.01	0.03	0.05	0.11

bw: body weight; P90: 90th percentile
[a] Consumers as a % of total respondents.
[b] FSANZ (2008).
[c] FSANZ (2012).

and puffed snacks, chocolates, drinks and juices, chewing gum, jelly and lollypops) have been estimated (Husain et al., 2006). Consumption was estimated from a 24-hour dietary recall involving 3142 male and female, Kuwaiti and non-Kuwaiti, children between the ages of 5 and 14 years enrolled in private and public, primary and intermediate schools. A total of 344 foodstuffs were analysed for their contents of artificial food colour additives, including Brilliant Blue FCF. The estimated dietary exposure (average values) of Brilliant Blue FCF for children aged 5–14 years (in age groups of one year, male or female) was 0.1–2.4 mg/kg bw per day. Children aged 6 years had the highest estimated exposure (1.7–2.4 mg/kg bw per day) while children aged 13 and 14 years had the lowest (0.1–0.23 mg/kg bw per day). The Committee noted that this study had been used in estimating dietary exposures to tartrazine at a previous meeting (82nd meeting) where the Committee dismissed the relevance of the study as not being representative. The current Committee considered that, although the survey was not nationally representative, it was of positive value in the assessment of Brilliant Blue FCF and therefore its results are included herein.

3.2 Evaluation of estimates of dietary exposure

Estimates of dietary exposure to Brilliant Blue FCF published by EFSA, FSANZ, India, Kuwait, the Republic of Korea and USFDA are summarized in Table 6.

Table 6
Estimated dietary exposures to Brilliant Blue FCF based on individual dietary records (mg/kg bw per day)

		Mean	High percentile	Mean	High percentile
USFDA (Doell et al., 2016)		Consumers only, population ≥2 years		Consumers only, children 2–5 years	
2-day food consumption data	Average-exposure scenario	0.02	0.05[a]	0.07	0.10[a]
	High-exposure scenario	0.04	0.09[a]	0.10	0.20[a]
10–14-day food consumption data	Average-exposure scenario	0.02	0.04[a]	0.02	0.04[a]
	High-exposure scenario	0.04	0.06[a]	0.04	0.06[a]
FSANZ (FSANZ, 2012)		Consumers only, population ≥2 years		Consumers only, children 2–5 years	
	Mean colours scenario	0.01	0.03[a]	0.02	0.05[a]
	Maximum colours scenario	0.05	0.12[a]	0.10	0.24[a]
EFSA (EFSA, 2010)		Population ≥18 years		Children 1–10 years	
	Maximum reported use levels	0.06	3.0[b]	0.2–2.1	0.6–4.8[c]
Konkuk University, Republic of Korea (Ha et al., 2013)		Consumers			
		0.01	0.03[c]	–	–
CSIR, India (Dixit et al., 2011)				Children 4–18 years	
	Average-exposure scenario	–	–	0.1–0.2	–
	High-exposure scenario	–	–	–	0.4–1.0[c]
Kuwait Institute for Scientific Research (Husain et al., 2006)				Children 5–14 years	
	Average-exposure scenario	–	–	0.1–2.4	–

CSIR: Council of Scientific and Industrial Research; EFSA: European Food Safety Authority; FSANZ: Food Safety Australia New Zealand; USFDA: United States Food and Drug Administration
[a] 90th percentile.
[b] 97.5th percentile exposure from beverages plus per capita average from the rest of diet.
[c] 95th percentile.

The estimates of dietary exposure to Brilliant Blue FCF calculated by EFSA (4.8 mg/kg bw per day, 95th percentile) were much higher than those of USFDA and FSANZ (both 0.2 mg/kg bw per day for children at the 90th percentile) and the Republic of Korea (0.03 mg/kg bw per day at the 95th percentile for whole population). Estimates for India and Kuwait were also lower than the EFSA estimates, but higher than the estimates from USFDA and FSANZ. The Committee concluded that the higher values in the EFSA estimates were due to the use of maximum reported

use levels, as opposed to the use of the analysed levels for foods in the other studies.

4. Comments

4.1 Biochemical aspects

When Brilliant Blue FCF was administered orally to rats, almost the entire dose was excreted unchanged in the faeces within 40 hours. The colour was also found in the bile of rats, rabbits and dogs after oral administration. Only 5% of the dose administered was excreted in the bile of dogs (Hess & Fitzhugh, 1953, 1954, 1955). In other studies, absorption of Brilliant Blue FCF was about 0.5% in rats (Brown et al., 1980; Phillips et al., 1980), with more than 99% of total intake excreted in the faeces and less than 1% recovered in the urine. Results of TLC of urine and bile samples 24 hours after ingestion showed that about 95% of excreted radioactivity was unaltered ^{14}C-radiolabelled Brilliant Blue FCF and that about 5% was unidentified metabolite(s) or degradation product(s). Mass spectrometric analysis was, however, not used.

An ex vivo porcine tongue system showed that about 0.2% of Brilliant Blue FCF diffused through the surface oral mucosa layers (Lucová et al., 2013).

Equilibrium dialysis methods have demonstrated that Brilliant Blue FCF binds to rat plasma protein (Iga, Awazu & Nogami, 1971; Iga et al., 1971). The extent of binding of Brilliant Blue FCF with plasma protein was 65% after 160 hours of dialysis at 37 °C.

In an in vitro study in which the *Xenopus* oocyte expression system was used for pharmacological investigations on purinergic P2 receptors that interact with the membrane channel protein Panx1 in inflammasome signalling, Brilliant Blue FCF was shown to be a selective inhibitor of Panx1 channels, with an IC_{50} of 0.27 µmol/L; no significant effect on the P2X7R receptor was observed at concentrations as high as 100 µmol/L (Wang, Jackson & Dahl, 2013). The Committee was aware that Panx1 activation/inhibition is one of several signalling pathways involved in various physiological processes at the cellular level (e.g. immune function) and that there are many exogenous and endogenous modulators of these

pathways. Interactions of substances with P2 receptors and Panx1 are an active area of research, particularly in development of drug treatments for diverse chronic diseases. The Committee noted that a similar pattern of channel inhibition was observed with Fast Green FCF, and further research may clarify if the inhibition of Panx1 observed in an in vitro system has any relevance for the safety assessment for substances in food.

4.2 Toxicological studies

The acute toxicity of Brilliant Blue FCF is low. The LD_{50} in mice (Sasaki et al., 2002) and rats (Lu & Lavallee, 1964) was higher than 2000 mg/kg bw.

In a 1-year dietary study, 12 dogs were fed Brilliant Blue FCF (purity not reported) at 0%, 1% or 2%. No clinical signs, gross lesions or microscopic pathological findings were attributed to exposure to Brilliant Blue FCF (Hansen et al., 1966).

The long-term toxicity of Brilliant Blue FCF was investigated in three studies in mice and five in rats.

No evidence of treatment-related carcinogenicity was found when male and female mice were fed Brilliant Blue FCF at a dose of 1 mg/kg bw per day over 500–700 days (Waterman & Lignac, 1958).

The administration of Brilliant Blue FCF to male and female mice for up to 80 weeks in the diet at concentrations of 0%, 0.015%, 0.15% or 1.5% (equivalent to 0, 20, 200 and 2000 mg/kg bw per day, respectively) resulted in slight reduction in weight gain and increased incidence of foam cells in the liver at the highest dose (Rowland et al., 1975). The NOAEL was 0.15% (equivalent to 200 mg/kg bw per day).

In a long-term toxicity study in which Brilliant Blue FCF was fed to male and female mice for 24 months (104 weeks) at dietary concentrations of 0%, 0.5%, 1.5% or 5% (equal to 0, 661, 2064 and 7354 mg/kg bw per day for males and 0, 819, 2562 and 8966 mg/kg bw per day for females, respectively), the NOAEL was 5% (equal to 7354 mg/kg bw per day), the highest concentration tested (IRDC, 1981a; Borzelleca, Depukat & Hallagan, 1990). The Committee noted that the survival at the end of the study was about 50% in both control and treated groups.

When Brilliant Blue FCF was fed to male and female rats at a dietary level of 4% for 600 days, there were no treatment-related tumours (Willheim & Ivy, 1953). In another long-term toxicity study in which rats

were fed a diet containing 0.1% Brilliant Blue FCF over their lifetime (daily intake 10–15 mg), no treatment-related tumours were found (Klinke, 1955). Similarly, when male and female rats were fed diets containing 0%, 0.3% or 3% Brilliant Blue FCF for 75 weeks, no treatment-related adverse effects were observed on tumour incidence, growth or haematological findings (Mannell, Grice & Allmark, 1962).

In its previous evaluation at the thirteenth meeting (Annex 1, reference *19*), the Committee established an ADI of 0–12.5 mg/kg bw based on a 2-year toxicity study in which male and female rats were fed a diet containing 0%, 0.5%, 1.0%, 2.0% or 5.0% Brilliant Blue FCF. The NOAEL was 5.0% (equivalent to 2500 mg/kg bw per day), the highest concentration tested (Hansen et al., 1966).

In a long-term toxicity study that included an in utero exposure phase, Brilliant Blue FCF was fed to the F_0 rats for up to 17 weeks at levels of 0%, 0.1%, 1% or 2% (calculated to provide doses of 0, 50, 514 and 1073 mg/kg bw per day for males and 0, 62, 631 and 1318 mg/kg bw per day for females, respectively). The F_1 animals were administered Brilliant Blue FCF at the same dose levels for up to 116 weeks for males and 111 weeks for females. The NOAEL was 1% (equal to 631 mg/kg bw per day), based on 15% decreased mean terminal body weight and decreased survival of female rats at the highest dose level (IRDC, 1981b; Borzelleca, Depukat & Hallagan, 1990).

The Committee concluded from these studies in mice and rats that there is no concern with respect to carcinogenicity of Brilliant Blue FCF.

No mutagenic activity has been observed with Brilliant Blue FCF in several in vitro mutagenicity studies conducted in *S. typhimurium*, *B. subtilis* and *E. coli*. Positive findings were reported in two in vitro chromosomal aberration assays, one in vitro micronucleus assay and one in vitro comet assay in mammalian cells, but these studies had a number of shortcomings (Kawachi et al., 1980; Ishidate et al., 1984; Kus & Eroglu, 2015; Pandir, 2016). In contrast, negative results were obtained in an in vivo micronucleus assay in bone marrow (Hayashi et al., 1988) and a comet assay in the stomach, colon, liver, kidney, bladder, lung, brain and bone marrow of mice (Sasaki et al., 2002). Based on the available data, the Committee concluded that there is no concern with respect to genotoxicity of Brilliant Blue FCF.

No treatment-related adverse reproductive effects were found in a single-generation study in male and female rats fed Brilliant Blue FCF at doses up to 1318 or 1073 mg/kg bw per day, respectively (IRDC, 1981b; Borzelleca, Depukat & Hallagan, 1990). Similarly, no treatment-related adverse effects were seen in a 3-generation study in rats treated with Brilliant Blue FCF at doses up to 1000 mg/kg bw per day (BioDynamics Inc., 1971). In developmental toxicity studies, no adverse effects were reported in rats treated with Brilliant Blue FCF at doses up to 2000 mg/kg bw per day (BioDynamics Inc., 1972a) or in rabbits at doses up to 200 mg/kg bw per day (BioDynamics Inc., 1972b).

Other studies have reported no evidence for allergenicity (Kreindler, Slutsky & Haddad, 1980), skin irritation (BIBRA, 1990), dermal sensitization (BIBRA, 1990) or skin cancer (Carson, 1984) as a result of treatment with Brilliant Blue FCF.

In a one-generation study on neurobehavioural development in mice (Tanaka et al., 2012), Brilliant Blue FCF was given in the diet at concentrations of 0%, 0.08%, 0.24% or 0.72% (equal to 0, 111–407, 347–1287 and 1032–3856 mg/kg bw per day, respectively, exposure depending on gestational age). The high dose of Brilliant Blue FCF resulted in a few statistically significant effects on neurobehavioural development (exploratory behaviour and surface righting response). However, the Committee noted that the effects on exploratory behaviour were inconsistent and that there were no effects from exposure to Brilliant Blue at any dose in several other neurobehavioural tests in this study. The Committee concluded that the findings were not robust enough to be used in the safety assessment.

4.3 Observations in humans

Case reports describe the use of Brilliant Blue FCF in enteral feeding solutions associated with discoloration of skin, urine and serum and toxicity, including 12 deaths (WHO, 2003; Maloney & Brand, 2016). The Committee noted that these case reports relate to seriously ill patients, particularly those with increased gut permeability (e.g. patients with sepsis), and that a causal relationship with Brilliant Blue FCF has not been established.

4.4 Assessment of dietary exposure

Estimates of dietary exposure to Brilliant Blue FCF published by EFSA (EFSA, 2010), FSANZ (FSANZ, 2012), the USFDA (Doell et al., 2016), India (Dixit et al., 2011), Kuwait (Husain et al., 2006) and the Republic of Korea (Ha et al., 2013) were available to the Committee. The estimate of dietary exposure to Brilliant Blue FCF calculated by EFSA (4.8 mg/kg bw per day for children at the 95th percentile) was much higher than those of the USFDA and FSANZ (both 0.2 mg/kg bw per day for children at the 90th percentile) and the Republic of Korea (0.03 mg/kg bw per day for the whole population at the 95th percentile). Estimates from India and Kuwait were also lower than the EFSA estimates, but higher than the estimates from the USFDA and FSANZ. The Committee considered that the higher values in the EFSA estimates were due to the use of maximum reported use levels, whereas the other studies used mean analysed levels. The Committee concluded that the use of the more conservative EFSA estimate of 5 mg/kg bw per day should be considered in the safety assessment for Brilliant Blue FCF.

5. Evaluation

The Committee concluded that the available data support the revision of the ADI for Brilliant Blue FCF and that the study on long-term toxicity in rats should be considered as the pivotal study (IRDC, 1981b; Borzelleca, Depukat & Hallagan, 1990). In this study, a NOAEL of 631 mg/kg bw per day was identified, based on a 15% decrease in mean terminal body weight and decreased survival of females at 1318 mg/kg bw per day. The Committee established an ADI of 0–6 mg/kg bw based on this NOAEL by applying an uncertainty factor of 100 for interspecies and intraspecies differences.

The Committee noted that the conservative dietary exposure estimate of 5 mg/kg bw per day (95th percentile for children) is less than the upper limit of the ADI of 0–6 mg/kg bw established for Brilliant Blue FCF and concluded that dietary exposure to Brilliant Blue FCF for children and all other age groups does not present a health concern.

The previous ADI of 0–12.5 mg/kg bw was withdrawn.

At the present meeting, the existing specifications for Brilliant Blue FCF were revised, and a maximum limit for manganese was added. High-performance liquid chromatography (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 or aqueous ammonium acetate.

The specifications were revised, and a Chemical and Technical Assessment was prepared.

6. References

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β-Carotene-rich extract from *Dunaliella salina*

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1. Explanation

β-Carotene-rich extract from *Dunaliella salina* is a natural orange food colour. It is used in a wide range of food and beverages as a colour, including cider, malt beverages, water-based flavoured drinks, margarines, cheeses, cake fillings, custards, yogurts, processed nuts, precooked pastas and noodles and other

products. Intended use levels of the product range from 20 to 1200 mg/kg, depending on the food item or category.

Carotenes from natural sources (including carotenes from *D. salina*) were reviewed at the thirty-first, thirty-fifth and forty-first meetings of the Committee (Annex 1, references *77*, *88* and *107*). At the thirty-first meeting, the Committee concluded that the group acceptable daily intake (ADI) of 0–5 mg/kg body weight (bw) established by the eighteenth Committee for the sum of the synthetic carotenoids β-carotene, β-apo-8′-carotenal and β-apo-8′-carotenoic acid methyl and ethyl esters was not applicable to natural carotenes as they did not comply with the specifications for β-carotene. At the thirty-fifth and forty-first meetings, the Committee considered the available data inadequate to establish an ADI for the dehydrated algal carotene preparations or for the vegetable oil extract of *D. salina*. At the fifty-seventh meeting, the group ADI for synthetic β-carotene was extended to include β-carotene from *Blakeslea trispora* (Annex 1, reference *154*).

The Committee was asked by the Forty-eighth Session of the Codex Committee on Food Additives (FAO/WHO, 2016) to evaluate carotenes from *D. salina*. New short-term animal studies as well as studies on genotoxicity and developmental toxicity were submitted. A comprehensive literature search was conducted on carotenes from *D. salina* using the search terms "beta-carotene", "*Dunaliella salina*" and "*D. salina*" in PubMed. The Committee also considered a limited number of publications on β-carotene from other sources that became available since the previous evaluation. In light of the information submitted, the Committee limited the assessment to a vegetable oil preparation of a β-carotene-rich d-limonene extract of *D. salina*, hereafter referred to as *D. salina* d-limonene extract.

1.1 Chemical and technical considerations

β-Carotene-rich d-limonene extract of *D. salina* is produced from *D. salina*, an extreme halotolerant alga that inhabits natural and human-made salt lakes and ponds. The carotene-rich alga is harvested and concentrated, and the carotenoids are extracted using an essential oil rich in d-limonene. The resulting extract is saponified, purified, centrifuged, evaporated and finally mixed with a vegetable oil to obtain a commercial product with a carotene content of about 30% by weight. β-Carotene accounts for more than 95% of the carotene content of the extracted material as a mixture of *trans* and *cis* isomers in a ratio of approximately 2:1 by weight. The remainder of the carotene content includes α-carotene, lutein, zeaxanthin and cryptoxanthin. In addition to the colour pigments and vegetable oil used for standardization, d-limonene extracts of *D. salina* contain lipids and

other fat-soluble components naturally occurring in the source material, such as fatty acids, long-chain alcohols, alkenes and waxes. The composition of these fat-soluble components is primarily a mixture of fatty acids common to vegetable oils used in foods.

Carotenoids are naturally occurring pigments that are responsible for the bright colours of various fruits and vegetables, including citrus fruits, carrots and tomatoes. β-Carotene, a provitamin A, is the most common of these carotenoids, consisting of an unsaturated chain containing identical substituted ring structures at each end.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and metabolism

Several studies have shown that rodents absorb very little intact β-carotene compared with humans. The main site of carotenoid metabolism by β-carotene-15,15′-dioxygenase in rodents is the intestinal mucosa. Activity of 15,15′-dioxygenase enzyme in rat intestine homogenates was reported to be 750 nmol/min per mg homogenate protein (During, Albaugh & Smith, 1998; During et al., 1999). β-carotene is also metabolized in peripheral tissues such as lung, kidney, liver and fat (Wang et al., 1992; Redlich et al., 1996). Serum β-carotene levels in rats fed a diet containing 2.4 or 12.7 mg of β-carotene for 14 days were very low (0.5 to 0.6 μg/dL), and no β-carotene was found in the liver or adipose tissue This level is reportedly about 1/1000th that of humans (Ribaya-Mercado et al., 1989).

(a) Ferret

White et al. (1993) investigated the use of the ferret as an animal model for the bioavailability of natural and synthetic β-carotenes in foods. Male, de-scented 8-week-old ferrets ($n = 26$) were maintained on a pelleted purified ferret diet with negligible carotenoid and vitamin A content. The diet was formulated to contain nutrient concentrations similar to those in published, commercial ferret diets, but to allow depletion of baseline tissue carotenoid and vitamin A stores. Feed and fluid intakes were measured daily. After consuming the purified diet for 16 days, four ferrets were randomly selected to determine baseline liver β-carotene and vitamin A stores. The remaining ferrets were randomly assigned to one of three groups ($n = 7$ or 8/group). For 10 days, the ad libitum tap water intake was

replaced with daily intake of 85 mL of one of three test beverages containing 18 μmol/L all-*trans*-β-carotene from natural or synthetic sources: Group 1 received 100% carrot juice with added L-ascorbic acid (0.078 mmol/L) filtered and diluted 50% (volume per volume) with water; Group 2 received a suspension of cold water-dispersible β-carotene beadlets in a mixed juice beverage; and Group 3 received a suspension of cold water-dispersible β-carotene beadlets in water. Ingestion of 85 mL of each beverage resulted in daily intake of 1.5 μmol of all-*trans*-β-carotene for all three groups.

There was no detectable α- or β-carotene in the sera or livers of any of the baseline group animals. All three groups accumulated β-carotene in liver after the 10-day treatment. However, total accumulation of β-carotene in sera, livers and adrenals of ferrets that consumed the carrot juice was significantly lower than that in the other two groups (White et al., 1993).

The mean vitamin A concentration in liver of the ferrets in the baseline group (killed after the initial 16-day consumption of the low vitamin A diet) was 0.50 ± 0.07 μmol/g. There were no significant differences between the three treatment groups in liver vitamin A concentrations or liver vitamin A stores at the end of the 10-day treatment period. The mean total liver vitamin A stores of animals that ingested the all-*trans*-β-carotene in the carrot juice, mixed juice and cold water vehicles (Groups 1, 2 and 3, respectively) were 19.2 ± 2.3, 21.3 ± 2.1 and 21.1 ± 2.3 μmol/g, respectively (Table 1).

(b) **Cow (preruminant calf)**

Poor et al. (1992) evaluated the preruminant calf as an animal model for the study of human carotenoid metabolism. Fifteen newborn male Holstein calves were fed a carotenoid-free milk replacer diet to maintain them in the preruminant state. After a 7-day adjustment period, three calves were euthanized. The remainder received a single oral dose of β-carotene (20 mg of water-soluble beadlets). Blood samples were collected periodically, and tissue samples were collected at termination after 1, 3, 6 and 11 days post dosing (n = 3 animals/group).

Serum β-carotene concentrations peaked between 12 and 30 hours post dosing and declined slowly afterwards. Serum data were fitted to a two-compartment model and yielded an elimination constant. Adrenal tissue showed significant concentrations of β-carotene at 24 hours post dosing, and levels remained elevated at 264 hours. Liver, spleen and lung β-carotene levels were significantly elevated 24 hours post dosing and rapidly declined thereafter. Adipose and kidney peak β-carotene concentrations were observed at 72 and 144 hours post dosing, respectively.

Table 1
Serum and tissue concentrations of all-*trans*-β-carotene in ferrets after 10-day treatment

Group	Serum (μmol/L)	Liver (nmol/g)	Adrenal (nmol/g)	Total liver vitamin A (μmol/g)
1	0.305 ± 0.135	2.00 ± 1.04	0.83 ± 0.98	19.2 ± 2.3
2	0.972 ± 0.127	10.27 ± 0.97	6.54 ± 0.92	21.3 ± 2.1
3	1.081 ± 0.135	9.38 ± 1.04	4.56 ± 0.98	21.1 ± 2.3

Source: White et al. (1993)

The authors concluded that the preruminant calf may be a suitable model for the study of absorption and metabolism of carotenoids in humans (Poor et al., 1992).

(c) Human

Human volunteers (12 male and 20 female) ate a low-carotene diet for 10 days. The volunteers were randomly assigned to five groups. Two groups received three capsules daily containing vegetable oil extracts of *Dunaliella salina* providing 8 or 24 mg β-carotene and 1.1 or 3.2 mg α-carotene, respectively; two groups received quantities of carrots that provided similar amounts of β-carotene and 6.3 and 18.9 mg α-carotene, respectively; and the remaining group received placebo (vegetable oil) capsules. The volunteers were treated for 7 days followed by a depletion phase of 7 days.

All volunteers in the treatment groups showed an increase in serum concentration of α- and β-carotenes, with half-lives averaging 7.8 and 12.4 days, respectively. Serum vitamin A levels remained unchanged throughout the study (Jensen et al., 1985).

Levels of β-carotene, retinol and α-tocopherol in lung tissues and bronchoalveolar cells from 21 patients undergoing open lung surgery were determined with reverse-phase high-performance liquid chromatography. The dietary exposure to β-carotene, retinol and α-tocopherol of each patient was assessed using a semiquantitative dietary questionnaire. Serum and bronchoalveolar tissue samples were obtained at the time of surgery.

Dietary and serum levels of carotenoids, β-carotene, retinol and α-tocopherol were consistent with previously reported values. Lung tissue levels of total carotenoids, β-carotene, retinol and α-tocopherol were 0.34 ± 0.36, 0.13 ± 0.27, 0.15 ± 0.06 and 9.60 ± 4.86 μg/g tissue, respectively. Lung tissue levels of total carotenoids, β-carotene and α-tocopherol (but not retinol) correlated well

with their serum levels, suggesting that serum and bronchoalveolar cell levels of these nutrients could be used to predict lung tissue levels (Redlich et al., 1996).

Stahl, Schwarz & Sies (1993) studied the uptake of all-*trans*-β-carotene, 9-*cis*-β-carotene and α-carotene from a natural carotene preparation from *Dunaliella salina* in humans.

All-*trans*-β-carotene and α-carotene were absorbed well. Serum peak concentrations occurred between 24 and 48 hours. The mean increase in serum concentration of α-carotene was 5.6% of the increase of the all-*trans*-β-carotene, consistent with the composition of these carotenoids in the test substance; 9-*cis*-β-carotene was not detected in human serum, even after repeated dosing. The authors suggested that this could be due to preferential absorption of all-*trans*-β-carotene, rapid distribution of 9-*cis*-β-carotene in tissue or the presence of isomerase activity that can convert 9-*cis* to all-*trans*-β-carotene (Stahl, Schwarz & Sies, 1993).

Gaziano et al. (1995) compared the absorption of all-*trans*-β-carotene and 9-*cis*-isomers from synthetic β-carotene and from oil-extracted β-carotene from *Dunaliella salina* after administration of these test substances over 30 days in 24 study participants. After an initial loading dose of 100 mg/person per day for 6 days, the participants received either 66 or 100 mg/day of β-carotene from *Dunaliella salina* or 50 mg/day of synthetic β-carotene for an additional 23 days. The levels of β-carotene in plasma were determined on days 8, 15, 22 and 29.

Irrespective of the source of β-carotene, serum levels of all-*trans*-β-carotene, 9-*cis*-β-carotene and total β-carotene increased significantly. In the group receiving β-carotene from *Dunaliella salina*, total β-carotene increased from 0.29 to 1.16 μmol/L, and the all-*trans* isomer accounted for 93% of the increase. In the group receiving synthetic β-carotene, total β-carotene increased from 0.27 to 1.89 μmol/L, with the all-*trans*-β-carotene accounting for 93.8% of this increase. Low-density lipoprotein was isolated from 16 of the study participants on day 29 of the study and analysed for β-carotene, lycopene, α-tocopherol and ubiquinol-10. There was a strong correlation between the change in total β-carotene concentration in plasma and the change in total β-carotene in isolated low-density lipoprotein (Gaziano et al., 1995).

Over a period of 5 months, von Laar et al. (1996) studied the absorption and steady state levels of synthetic β-carotene and natural β-carotene (extracted from *Dunaliella salina*) in serum of 14 patients with erythropoietic porphyria. Blood samples were drawn on days 0, 30 and 150, and β-carotene levels in sera were determined.

Levels of β-carotene on day 0 in patients who received β-carotene from *D. salina* varied between 0.6 and 1.4 nmol/mL (mean: 1.1 nmol/mL). On day 30, the mean value had risen to 4.6 nmol/mL and did not significantly increase by day 150. A similar pattern was observed in patients who received synthetic β-carotene. The authors reported no significant differences between the two sources of β-carotene on the therapeutic effect. No adverse reactions were reported (von Laar et al., 1996).

The activity of β-carotene-15,15′-dioxygenase, the primary enzyme responsible for oxidative cleavage of β-carotene to retinal, was measured in a study using two different human cell lines and human small intestine and liver preparations. β-Carotene-15,15′-dioxygenase activity in small intestinal mucosa preparations from five adults (44–89 years old) was 97.4 ± 39.8 pmol/hour per mg protein and from an infant (17 months old) was 20 pmol/hour per mg protein. No activity was detected in adult stomach tissue. The β-carotene-15,15′-dioxygenase activity in a subcellular preparation of human liver was reported to be 62 pmol/hour per mg protein in normal adult liver and 7 pmol/hour per mg protein for a liver exhibiting gross pathology. The maximum capacity of carotene cleavage by this 15,15′-dioxygenase was estimated to be 12 mg/day (one fifth by small intestine and four fifths by liver), assuming an optimal 15,15′-carotene/retinal cleavage ratio of 1:2 (During, Albaugh & Smith, 1998; During et al., 2001).

2.1.2 Effects on enzymes and other biochemical parameters

Wang et al. (1999) studied the effects of β-carotene alone, or in combination with cigarette smoke, on β-carotene and retinoid concentrations in serum and lung and expression of genes for retinoic acid receptors (RARs) and activator protein-1 (encoded by the *c-Jun* and *c-Fos* genes) in ferrets. Twenty-four male adult ferrets were randomly assigned to one of four groups (6 animals/group) as follows: (1) chronic exposure to cigarette smoke for 6 months; (2) β-carotene supplementation at 2.4 mg/kg bw per day for 6 months; (3) combination cigarette smoke and β-carotene supplementation (at 2.4 mg/kg bw per day) for 6 months; and (4) control (no smoke treatment and basal level of β-carotene, at 0.16 mg/kg per day).

The carotene concentration increased in plasma and lung tissue in the β-carotene supplemented group to 22-fold higher than the control group. The increase was significantly lower (8% over the control group) in the ferrets treated with both smoke and β-carotene. The same pattern of response was observed in lung tissue. Levels of β-carotene in lung after 6 months of treatment were 9 ± 1, 2618 ± 171 and 171 ± 22 pmol per 100 mg lung tissue in the control, β-carotene

supplemented and smoke-plus-β-carotene supplemented groups, respectively. The pathological evaluation of lung sections from the four groups showed that smoke exposure caused a mild aggregation and proliferation of macrophages in the lung tissue of ferrets. However, localized proliferation of alveolar type II cells and alveolar macrophages, and keratinized squamous epithelium were observed in all ferrets given the high-dose β-carotene supplement both with and without smoke, as compared with the control group and the group exposed to smoke alone. Keratinized squamous metaplasia was confirmed by immunohistochemistry with anti-keratin antibody in the lung sections of all ferrets given either high-dose β-carotene alone or high-dose β-carotene with smoke exposure. Analysis of RAR expression and activator protein-1 expression in the lung homogenate tissue showed that RARβ expression, but not RARα or RARγ, was decreased in all three treatment groups (smoke exposure, 18%; high-dose β-carotene, 62%; both, 73%) compared with the control group. Expression of *c-Jun* and *c-Fos* was up-regulated 3- to 4-fold in ferrets given a β-carotene supplement and exposed to smoke compared with the control animals.

The authors concluded that diminished retinoid signalling, resulting from the suppression of RARβ expression and overexpression of activator protein-1, could be a mechanism to enhance lung tumorigenesis after high-dose β-carotene supplementation and exposure to tobacco smoke (Wang et al., 1999).

2.2 Toxicological studies

At the forty-first meeting, JECFA evaluated acute toxicity studies in mice and rats treated with dried *Dunaliella* by gavage; short-term toxicity studies in mice and rats fed diets supplemented with dried *Dunaliella bardawil* and a maize oil extract of *Dunaliella*; a multigeneration reproductive toxicity study in rats raised on diets containing dehydrated *D. bardawil*; and a bacterial mutagenicity test in five strains (Annex 1, reference *107*). New data available for the present assessment are discussed in the following sections.

2.2.1 Acute toxicity

No new acute toxicity studies were available.

2.2.2 Short-term studies of toxicity

In a 90-day toxicity study that complied with Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 and good laboratory practices (GLP), Leuschner (2006a) treated Charles River rats (n = 10/sex per group) by gavage with a 0, 318, 954 or 3180 mg/kg bw per day vegetable oil preparation of *D. salina* d-limonene extract (containing 31% carotenes; calculated

from doses of 0, 100, 300 and 1000 mg/kg bw per day carotenes using a correction factor of 3.18). Study parameters included behaviour, external appearance, faeces, motility, functional observations, growth, feed and water intake, haematology, clinical biochemistry, urine analysis, ophthalmology, fertility and reproduction, organ weights and histopathology.

There were no test substance–related deaths. Treatment had no effect on functional observation parameters or on body weight, body weight gain, feed and drinking-water consumption or haematological or biochemical parameters. Ophthalmological examination of ocular structures found no lesions. No test substance–related effect was noted on the estrus cycle or spermatogenesis or on relative and absolute organ weights. No test substance–related histopathological findings were noted. Histomorphological examination of organs in high-dose rats showed superficial erosion of the mucosa with neutrophilic granulocytes and haemorrhages in the fundus region of the stomach in 1/10 males and 5/10 females. This was considered to be a local effect caused by the application of large amounts of the test substance.

The no-observed-adverse-effect level (NOAEL) was 3180 mg/kg bw per day of *D. salina* d-limonene extract, the highest dose tested (Leuschner, 2006a).

In a 90-day feeding study, Kuroiwa et al. (2006) fed Fischer rats (n = 10/sex per group) diets containing 0 (control), 0.63%, 1.25%, 2.5% or 5% *Dunaliella* carotene extracted from *Dunaliella* alga. The authors reported the average daily intakes of *Dunaliella* carotene to be 0, 352, 696, 1420 and 2750 mg/kg bw per day for males and 0, 370, 748, 1444 and 2879 mg/kg bw per day for females. The test substance was prepared from a stock solution of *Dunaliella* carotene extracted with soybean oil and containing 31.4% β-carotene that was mixed with basal powder and fed, ad libitum, for the duration of the study. The test substance and the diets were prepared weekly.

No mortality or treatment-related clinical signs were observed throughout the experimental period. Yellowish to reddish brown faeces were noted at 1.25% and higher, but no pigmentation was macroscopically observed at necropsy in the gastrointestinal tract, adipose tissue or other organs. Males at 2.5% and 5% showed a slight (6%) but significant ($P < 0.05$) reduction in body weight gain compared with the control group from week 5 to the end of the experiment. Feed intake in affected animals was not significantly different from controls. Organ weight measurements and histopathological evaluations found no treatment-related differences in toxicological end-points in any of the treatment groups and all were within standard values. A statistically significant increase in platelet counts was observed in males at 1.25% and 5% ($P < 0.01$) and in females at 2.5% ($P < 0.05$) and 5% ($P < 0.001$), but these changes were not considered toxicologically significant because they were within the typical

range for current toxicology studies. Slight but statistically significant increases in serum total cholesterol and calcium levels occurred in all treated males and females, with a dose-dependent trend in males, but all levels were within the stated control ranges for these end-points in this laboratory and were considered not toxicologically significant.

The study authors considered the NOAEL to be 1.25% (696 mg/kg bw per day) in males based on growth suppression observed at the 2.5% dose level, and 5% (2879 mg/kg bw per day) in females, the highest dose tested (Kuroiwa et al., 2006).

The Committee considered this decrease in body weight gain to not be biologically relevant because the magnitude of the change in body weight was slight (<10%). The Committee considered the NOAEL to be 5% in the diet (2750 mg/kg bw per day for males and 2879 mg/kg bw per day for females), the highest dose tested.

2.2.3 Long-term studies of toxicity and carcinogenicity

Long-term toxicity or carcinogenicity studies of *D. salina* d-limonene extract have not been performed.

2.2.4 Genotoxicity

In a GLP-compliant study conducted in accordance with OECD Guideline 471, Stien (2006) tested *D. salina* d-limonene extract (carotenoid content of 31.43%) with five *Salmonella typhimurium* strains (TA98, TA100, TA102, TA1535 and TA1537) in two separate experiments both with and without metabolic activation. Preliminary plate incorporation and preincubation tests, without metabolic activation, were performed in test strain TA100 using 10 concentrations of the test substance per test, ranging from 0.158 to 2500 μg/plate in the plate incorporation test and from 0.0316 to 500 μg/plate in the preincubation test.

Cytotoxicity (scarce background lawn and reduction of the number of revertants) was noted at concentrations of 158 μg/plate and higher in the plate incorporation test and 316 μg/plate and higher in the preincubation test. As a result, 316 μg/plate was chosen as the highest concentration for the main study.

No mutagenic effect was observed in the main study using five concentrations of *D. salina* d-limonene extract ranging from 3.16 to 316 μg/plate in any of the five test strains in plate incorporation and preincubation tests with and without metabolic activation (Stien, 2006).

Leuschner (2006b) tested *D. salina* d-limonene extract (carotenoid content of 31.43%) in a gene mutation assay in cultured mammalian cells (L5178Y $TK^{+/-}$) both with and without metabolic activation. In a preliminary experiment,

cytotoxicity (decreased cell survival) was noted at 208 μg/mL without metabolic activation but not with metabolic activation. As a result, 208 μg/mL was chosen as the highest concentration for the main experiments.

In two independent experiments, both carried out with and without metabolic activation, a concentration range of *D. salina* d-limonene extract at 13–208 μg/mL was found to be negative with respect to increasing the mutation frequency in the L5178Y $TK^{+/-}$ mammalian cell mutagenicity test, whereas the positive controls showed potent mutagenic effects.

These findings indicate that d-limonene extract from *D. salina* was not genotoxic under the conditions of this assay (Leuschner, 2006b).

In a GLP-compliant study conducted in accordance with OECD Guideline 474, Weimans (2002) determined the ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs) in an in vivo mouse bone marrow micronucleus test of *D. salina* d-limonene extract (carotenoid content 31.12%). The ratio of PCEs to NCEs in the bone marrow indicated cell toxicity. Mice in two groups of 10 animals (n = 5/sex) were each orally dosed with the test substance at 2000 mg/kg bw. They were killed 24 or 48 hours after administration. A limit test was considered sufficient since the test substance produced no adverse toxic effects in rat tests available at that time or in vitro genotoxicity tests. The test substance was formulated in sesame oil, which was also used as a negative control. Cyclophosphamide at a dose level of 30 mg/kg bw was used as positive control.

No systemic toxic symptoms were observed at any time during the study. All animals survived to scheduled death. No indication of bone marrow toxicity was noted in any group treated with the test substance. The highest ratio of PCE/NCE, 1.0, was observed in females after 24 hours and in males after 48 hours. Positive and negative controls gave expected results. Statistical analysis of the data showed no significant increase (Wilcoxon matched pairs test, $P > 0.05$) of the micronuclei ratio per 2000 PCEs. Under the conditions of the test, there was no evidence of either an aneugenic or clastogenic effect of the test substance leading to micronucleus formation in PCEs of treated mice 24 hours or 48 hours after oral administration of *D. salina* d-limonene extract at 2000 mg/kg bw (Weimans, 2002).

2.2.5 Reproductive and developmental toxicity

No reproductive toxicity studies were available for *D. salina* d-limonene extract.

The effect of orally administered *D. salina* d-limonene extract was evaluated during the critical phase of organogenesis in a study with a protocol

that followed OECD Test Guideline 414. Leuschner (2007) administered *D. salina* d-limonene extract in soybean oil by oral gavage to pregnant CD/Crl:CD (SD) dams (*n* = 25/group) at dose levels of the active ingredient β-carotene of 0, 318, 954 or 3180 mg/kg bw per day of the extract (calculated from doses of β-carotene of 0, 100, 300 or 1000 mg/kg bw per day using a correction factor of 3.18) from gestation day 6 to 19 (determined by presence of vaginal plug). Parameters investigated included mortality, functional observations, growth, feed and water consumption and, at necropsy, uterus and carcass weights and effects on fetuses including embryo/fetal survival or fetal body weights.

No maternal or developmental toxicity was observed at any dose level.

The NOAEL for maternal toxicity was 3180 mg/kg bw per day (1000 mg/kg bw per day, expressed as total carotenes), the highest dose tested. The NOAEL for developmental toxicity was 3180 mg/kg bw per day (1000 mg/kg bw per day, expressed as total carotenes), the highest dose tested (Leuschner, 2007).

2.3 Observations in humans

A human study investigating the potential effect of β-carotene supplementation in reducing cancer risk in high-risk individuals included 29 133 male smokers (1 pack/day for an average of 36 years) who received daily doses of 20 mg β-carotene for 5–8 years. After 2 years of treatment, median serum β-carotene levels had increased 17.5-fold in participants receiving the supplement compared with controls. Study participants receiving β-carotene had significantly higher lung cancer incidence (18%) and mortality (8%) compared with controls (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994).

In a similar study, 18 314 participants with a higher risk of cancer, including smokers, former smokers and asbestos-exposed male workers, received daily doses of 30 mg β-carotene and 25 000 International Units of vitamin A or controls. After 5 years of treatment, median serum β-carotene levels had increased 12-fold in the supplement group. After a 4-year follow-up, lung cancer incidence was significantly increased (28%) in smokers and asbestos workers receiving the supplement; overall, 17% more deaths were observed in this group (Omenn et al., 1996a,b).

A systematic review and meta-analysis of nine randomized controlled trials investigating β-carotene supplementation and cancer risk was subsequently conducted. Thirty-one articles met the initial inclusion criteria for the literature search, and 13 of these were retained for the meta-analysis. The data included in the meta-analysis covered a total of 180 702 study participants and 1852

lung cancer cases. The analysis showed no increase in incidence of lung cancer in heavy smokers receiving supplemental doses of β-carotene from 6 to 15 mg/day for about 5–7 years. There was a low but significant increase in relative risk for individuals receiving supplemental β-carotene at 20–30 mg day (or in combination with other antioxidants) compared with those receiving the placebo. This effect was not observed in lower dose groups (Druesne-Pecollo et al., 2010).

3. Dietary exposure

The Committee considered dietary exposure to β-carotene from *D. salina* d-limonene extract assuming its uses as a food additive in the same food categories and at the same maximum use levels (β-carotene basis) as previously evaluated β-carotene additives.

JECFA evaluated carotenoids at several previous meetings, but dietary exposure (specifically to lutein, a naturally occurring carotenoid) was discussed only at its sixty-third meeting in 2004 (Annex 1, reference *173*). Based on dietary exposure data from a number of studies in North America and the United Kingdom, the Committee reported that the exposure to lutein from natural sources was in the range of 1–2 mg per day. In 2012, European Food Safety Authority (EFSA) evaluated the use of β-carotenes (E 160a (i) and E 160a (ii)) as a food additive (EFSA, 2012). Based on European food consumption data and usage data of β-carotene reported by the Natural Food Colours Association (NATCOL), mean and high (97.5th percentile) exposures of 0.06 and 0.11 mg/kg bw per day, respectively, were estimated for United Kingdom adults. Assuming a mean adult body weight of 60 kg, these exposures equalled 3.6 and 6.6 mg/day, respectively. For children, the corresponding mean and high exposure estimates ranged from 0.03 to 0.22 and 0.09 to 0.43 mg/kg bw per day, respectively, across European countries. Using a mean body weight of 15 kg, mean and high exposures of 0.5-3.3 and 1.4-6.5 mg/day, respectively, were calculated.

EFSA also reported mean exposure estimates to β-carotene from the diet derived from the European Nutrition and Health Report 2009 (Elmadfa, 2009; referenced in EFSA, 2012). This report provided mean exposure estimates to β-carotene from nine European countries (Czech Republic, Denmark, Finland, Germany, Ireland, Italy, Norway, Poland and Sweden) for children aged 4–6 years and from 13 European countries (Austria, Czech Republic, Denmark, Finland, Germany, Hungary, Ireland, Italy, Lithuania, Poland, Spain, Sweden and the United Kingdom) for adults. These estimates were based on different methods of assessing food consumption. The mean β-carotene exposure via the diet ranged from 1.1 to 3.9 mg/day for children and 1.4 to 5.6 mg/day for adults.

No data were provided for high-level consumers. EFSA estimated that in view of the large standard deviations reported for the mean exposures, the dietary β-carotene exposure from the diet would very likely be considerably higher at high percentiles reaching 10 mg/day. Based on these results, EFSA concluded that the amount of β-carotene likely to be ingested from the diet would be in the range of 5–10 mg/day (EFSA, 2012).

The Committee did not calculate international estimates of dietary exposure to β-carotene from *D. salina* d-limonene extract using either the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) cluster diets or the FAO/WHO Chronic Individual Food Consumption Database – Summary statistics (CIFOCOss) database. The reason for this was that the proposed use of β-carotene from *D. salina* d-limonene extract by the sponsor was not expected to change the current exposure to β-carotene through the diet. In addition, only maximum use levels were available which, if used in an assessment, would potentially have resulted in very high, unrealistic exposure estimates based on the assumption that all foods consumed within the 79 food categories would contain β-carotene from *D. salina* d-limonene extract at the maximum use level.

Instead, the Committee conducted a literature review of the exposure to β-carotene from the diet, covering the last 10 years. As chemical analyses of β-carotene in food cannot distinguish β-carotene added to food from that which occurs naturally, these exposure estimates reflect total dietary exposure to β-carotene. The results are listed in Table 2. Due to differences in the way the exposure to β-carotene has been estimated (e.g. 24-hour recall, food frequency questionnaire, dietary record), the exposures cannot be compared across countries. Overall, the mean or median exposure to β-carotene ranged from 1.4 to 11 mg/day in adults. For children, data from Europe showed a maximum mean exposure of 7.3 mg/day; globally, few data were available for children's dietary exposures (Table 2). For high-percentile consumers of foods containing β-carotene, exposures were as high as 13.7 mg/day (adults in Europe). Based on this review, the Committee concluded that a high daily exposure to β-carotene of 15 mg (0.25 mg/kg bw for a 60 kg individual) was most appropriate for use in a safety assessment.

D. salina d-limonene extract also contains other non-carotene components, including vegetable oil, algal lipids and d-limonene. Vegetable oil is a natural component of food and therefore of no toxicological concern. Algal lipids are estimated to be present in the extract in the range of 20–35%. Assuming a high exposure to β-carotene of 15 mg/day, that this exposure comes completely from *D. salina* d-limonene extract and that the material contains 30% β-carotene, the exposure to algal lipids would be maximally 18 mg/kg (0.3 mg/kg bw per

Table 2
Dietary exposures to β-carotene as reported in the literature

Country / region	Population group	Mean age or age range (years)	Exposure (mg/day)		Reference
			Mean	High	
Australia[a]	Adults	54.8[b]	5.1–5.3[c,d]	–	Hodge et al. (2009)
China	Adults	60.6[b]	4.6–5.0[c]	8.9–9.2[c,e]	Chen et al. (2015)
China	Adults (females)	48.2	6.5	–	Wang et al. (2014)
Czech Republic	Adults	58.2[b]	4.5–5.6[c,d]	–	Stepaniac et al. (2016)
Europe[f]	Children	4–6	1.1–3.9	2.9–10.3[e]	Elmadfa (2009)
	Adults	–	1.4–5.6	2.4–13.7[e]	
France	Adults	50.4[b]	3.6– 4.2[c]	–	Lassale et al. (2016)
Italy	Infants and toddlers	0–3	1.2	3.9[g]	Sette et al. (2010)
	Children	3–10	2.1	5.5[g]	
	Adolescents	10–18	2.4–2.6[c]	6.0–6.3[c,g]	
	Adults	18–65	3.0–3.1[c]	7.2–7.3[c,g]	
	Elderly adults	≥65	3.2–3.4[c]	7.4–7.8[c,g]	
Japan	Adults	–	1.6–3.0	4.2–6.4[e]	Yabuta et al. (2016)
Poland	Children	10–12	7.3d	–	Kopeć et al. (2013)
Poland	Adults	57.6[b]	6.9–8.0[c,d]	–	Stepaniac et al. (2016)
Republic of Korea	Adults (female)	60.6	5.0	12.5[e]	Kim et al. (2016)
Russian Federation	Adults	58.2[b]	8.7–10.8[c,d]	–	Stepaniac et al. (2016)
Spain	Adults	18–64	1.5	–	Beltrán-de-Miguel, Estévez-Santiago & Olmedilla-Alonso (2015)

[a] Includes people living in Australia, but born in Australia, Greece, Italy and the United Kingdom.
[b] Average age of males and females, respectively.
[c] Range refers to exposure estimates for males and females.
[d] Reported exposure is median (50th percentile, P50).
[e] Estimated by the Committee as mean exposure + 2 × standard deviation.
[f] For children, Czech Republic, Denmark, Finland, Germany, Ireland, Italy, Norway, Poland and Sweden were included. For adults, Austria, Czech Republic, Denmark, Finland, Germany, Hungary, Ireland, Italy, Lithuania, Poland, Spain, Sweden and the United Kingdom were included.
[g] The 95th percentile of exposure.

day for a 60 kg individual). d-Limonene is estimated to be present at lower levels in the extract, not more than 0.3%. Based on the same assumptions as for algal lipids, the exposure to limonene would be maximally 0.2 mg/day (0.003 mg/kg bw per day for a 60 kg individual).

4. Comments

4.1 Biochemical aspects

β-Carotene is absorbed and detected in human serum and liver when consumed as an extract from *D. salina* or as synthetic β-carotene (Redlich et al., 1996).

Peak levels of β-carotene in human serum occur between 24 and 48 hours after ingestion (Stahl, Schwarz & Sies, 1993). Absorption appears to be linear when doses up to 30 mg are ingested, but the degree of absorption decreases at higher concentrations (Woutersen et al., 1999). Absorption of β-carotene varies between 10% and 90% in humans and is dependent on various conditions, such as the food matrix and nutritional status of the individual (Wang et al., 1993; von Laar et al., 1996; Woutersen et al., 1999). In humans, the major storage sites for carotenoids are the liver and adipose tissue, and hepatic and adipose tissue levels tend to correlate with serum levels (Gaziano et al., 1995; Redlich et al., 1996). In human serum, most of the β-carotene is present as the all-*trans* isomer, in spite of significant intake of the 9-cis isomer (Stahl, Schwarz & Sies, 1993; Rock, 1997; Woutersen et al., 1999).

In contrast to humans, mice, rats, hamsters and rabbits have very low levels of serum and tissue β-carotene due to the very high activity of intestinal β-carotene-15,15′-dioxygenase that efficiently converts β-carotene to retinal (During, Albaugh & Smith, 1998; Woutersen et al., 1999; During et al., 2001). On this basis, the Committee concluded that these species are not suitable models for the evaluation of β-carotene in humans.

The toxicokinetics of β-carotene in ferrets and preruminant calves have been shown to be similar to the absorption of β-carotene in humans. Ferrets that consumed 18 μmol/L of β-carotene as a suspension in water for 16 days after a β-carotene elimination period of 10 days were shown to accumulate β-carotene in serum, liver and adrenal tissue (White et al., 1993). β-Carotene was also significantly increased in liver, spleen, lung and serum of preruminant calves fed a single oral dose of 20 mg. Serum levels were still elevated 264 hours post dosing (Poor et al., 1992).

4.2 Toxicological studies

At the present meeting, the Committee evaluated two new 90-day studies in rats, in vitro and in vivo genetic toxicity assays and a developmental toxicity study in rats conducted using a *D. salina* d-limonene extract. The Committee deemed these studies useful for evaluating the toxicity of the non-β-carotene portion of the extract.

In a 90-day study submitted to the Committee for this evaluation, rats were treated by gavage with *D. salina* d-limonene extract (containing 31% carotenes) at doses of 0, 318, 954 or 3180 mg/kg bw per day (calculated from doses of 0, 100, 300 and 1000 mg/kg bw per day carotenes using a correction factor of 3.18). Superficial erosion of the mucosa with infiltration of neutrophilic granulocytes and haemorrhages in the fundus region of the stomach were observed in one

female and five males in the high-dose group (Leuschner, 2006a). The Committee concluded that the findings in the fundus were most likely due to a local effect of the high concentration of the test material given as a bolus, and identified the NOAEL to be 3180 mg/kg bw per day of *D. salina* d-limonene extract, the highest dose tested.

In another 90-day study, rats were fed diets containing 0, 0.63%, 1.25%, 2.5% or 5% of an oil extract of carotenes from *Dunaliella* alga (species not specified). The Committee noted that although the test substance was not specified, based upon the reported percentage of β-carotene (31.4%) and the description of the material, this is likely a *D. salina* d-limonene extract. The average doses of the *Dunaliella* carotene extract were reported as 0, 352, 696, 1420 and 2750 mg/kg bw per day for males and 0, 370, 748, 1444 and 2879 mg/kg bw per day for females (Kuroiwa et al., 2006). Although the authors identified a NOAEL of 1.25% based on a 6% reduction in body weight gain in males at 2.5% and 5%, the Committee considered this not to be a toxicologically relevant effect. The Committee concluded that the NOAEL was 5% (2750 mg/kg bw per day) of *Dunaliella* extract, the highest concentration tested.

No long-term toxicity and carcinogenicity studies were available for *D. salina* d-limonene extract.

The *D. salina* d-limonene extract tested negative in genotoxicity assays, including the bacterial reverse mutation assay in five strains of *S. typhimurium*, the forward gene mutation assay in cultured mammalian cells ($TK^{+/-}$ L5178Y) with and without metabolic activation and an in vivo mouse bone marrow micronucleus test. No concerns for genotoxicity were identified (Leuschner, 2006b,c; Stien, 2006).

No reproductive toxicity studies were available for the *D. salina* d-limonene extract.

D. salina d-limonene extract (carotene content 31%) was administered to pregnant rats from gestation day 6 to 19 by oral gavage at doses of 0, 318, 954 or 3180 mg/kg bw per day of the extract (calculated from doses of 0, 100, 300 and 1000 mg/kg bw per day carotenes using a correction factor of 3.18). No maternal or developmental toxicity was observed (Leuschner, 2007).

4.3 Observations in humans

No studies were available on the *D. salina* d-limonene extract.

The Committee noted two independent trials of heavy smokers (at least 1 pack/day for 36 years on average) who received β-carotene supplements. In the first study, participants received β-carotene (20 mg/day) supplementation, with or without α-tocopherol supplementation (Alpha-Tocopherol, Beta Carotene

Cancer Prevention Study Group, 1994). In the second study, participants received β-carotene (30 mg/day) + retinol (25 000 International Units of vitamin A) (Omenn et al., 1996a,b). Both studies showed increased, rather than the hypothesized decreased, incidence of lung cancer. A subsequent systematic review of nine randomized clinical trials showed no increase in the incidence of lung cancer in heavy smokers at supplemental doses of β-carotene from 6 to 15 mg/day for about 5–7 years (Druesne-Pecollo et al., 2010).

4.4 Assessment of dietary exposure

The Committee considered dietary exposure to β-carotene from *D. salina* d-limonene extract assuming its uses as a food additive in the same food categories and at the same maximum use levels (β-carotene basis) as previously evaluated β-carotene additives. The Committee concluded that dietary exposure to β-carotene would not change, as the extract will provide β-carotene at a level equivalent to that from other β-carotene food additives.

The Committee therefore reviewed dietary exposures to β-carotene reported in the literature. Estimates of dietary exposure from the following regions/countries were included in this review: Australia (Hodge et al., 2009), China (Wang et al., 2014; Chen et al., 2015), the Czech Republic (Stepaniak et al., 2016), "Europe" (Elmadfa, 2009; referenced in EFSA, 2012), France (Lassale et al., 2016), Italy (Sette et al., 2010), Japan (Yabuta et al., 2016), Republic of Korea (Kim et al., 2016), Poland (Kopeć et al., 2013; Stepaniak et al., 2016), Russian Federation (Stepaniak et al., 2016) and Spain (Beltrán-de-Miquel, Estévez-Santiago & Olmedilla-Alonso, 2015). As chemical analyses of β-carotene in food cannot distinguish β-carotene added to food from that occurring naturally, these dietary exposure estimates reflect total dietary exposure to β-carotene.

Overall, mean or median dietary exposures to β-carotene ranged from 1.4 to 11 mg/day in adults. For children, data from Europe showed a maximum mean exposure of 7.3 mg/day; globally, few data were available for children's dietary exposures. For high-percentile consumers of foods containing β-carotene, dietary exposures were as high as 13.7 mg/day (adults in Europe).

The Committee concluded that a high daily dietary exposure to β-carotene of 15 mg (0.25 mg/kg bw for a 60 kg individual) is appropriate for use in safety assessment. Using this dietary exposure estimate and the assumptions that all the β-carotene in the diet comes from this extract and that the extract contains 30% β-carotene, 35% algal lipids (upper level of a range of 20–35%) and 0.3% d-limonene (maximum amount), dietary exposure to the other toxicologically relevant constituents of this extract would be 18 mg/day (0.3 mg/kg bw per day

for a 60 kg individual) for algal lipids and 0.2 mg/day (0.003 mg/kg bw per day for a 60 kg individual) for d-limonene.

5. Evaluation

The Committee noted that the total dietary exposure to β-carotene is not expected to increase when *D. salina* d-limonene extract is used as a food colour.

The Committee has also considered the basis for the ADI established for the group of carotenoids by the Committee at the eighteenth meeting. The group ADI (0–5 mg/kg bw) was derived using a four-generation study in rats with a NOAEL for β-carotene of 50 mg/kg bw per day with application of a safety factor of 10 because of the natural occurrence of carotenoids in the human diet and the low toxicity observed in animal studies. This ADI applies to the use of β-carotene as a colouring agent and not to its use as a food supplement.

Data that have become available since the previous evaluation show large differences in absorption of β-carotene between rodent species and humans. Specific β-carotene-15,15′-dioxygenase activity with β-carotene as substrate in the intestine of rodents is nearly 1 million-fold higher than that of humans. The Committee considered that rodents are inappropriate animal models for establishing an ADI for β-carotene because of the virtual absence of systemic absorption in rodents.

The Committee noted that the toxicity of the other components of the *D. salina* d-limonene extract can be evaluated using the results of rodent studies. The *D. salina* d-limonene extract used in the toxicological studies contained β-carotene at approximately 30%, algal lipids at 20–35% and diluent vegetable oil at 35–50%. The *D. salina* d-limonene extract did not show genotoxicity in the evaluated studies. Short-term toxicity studies in rats give a NOAEL equal to 3180 mg/kg bw per day, the highest dose tested. No effects were observed in a developmental toxicity study in rats. No long-term toxicity or reproductive studies have been conducted with the *D. salina* d-limonene extract. Correction of the dose used to derive the NOAEL for *D. salina* d-limonene extract of 3180 mg/kg bw per day for the percentage of the algal component (20–35%) gives an adjusted NOAEL of 636–1113 mg/kg bw per day for the algal lipid component of the test substance. The margin of exposure for the algal lipid component in the *D. salina* d-limonene extract is 2120–3710 using a dietary exposure of 18 mg/day (0.3 mg/kg bw per day). The Committee concluded that dietary exposure to the algal component of the extract does not pose a health concern.

The Committee concluded that there was no health concern for the use of β-carotene-rich extract from *D. salina* when used as a food colour and in

accordance with the specifications established at this meeting. This conclusion was reached because total dietary exposure to β-carotene will not increase and there are no toxicity concerns for the non-carotene components of the extract. The Committee emphasized that this conclusion applies to the use of this extract as a food colour, not as a food supplement.

A specifications monograph and a Chemical and Technical Assessment were prepared.

5.1 Recommendations

The Committee recommends that the group ADI for the sum of carotenoids, including β-carotene, β-apo-8′-carotenal and β-apo-8′-carotenoic acid methyl and ethyl esters, be re-evaluated in light of evidence that shows very low absorption of β-carotene in rodents and rabbits in contrast to humans.

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Fast Green FCF

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1. Explanation

Fast Green FCF (Chemical Abstracts Service No. 2353-45-9; INS No. 143) is a dye with a synthetic triphenylmethane base structure permitted as a food colour in Japan, the USA and other regions. It is used for colouring breakfast cereals, cakes and cupcakes, drink mixers and frozen treats.

The Committee previously evaluated Fast Green FCF at its thirteenth, twenty-fifth, twenty-ninth and thirtieth meetings (Annex 1, references *20*, *56*, *70* and *73*). At its thirteenth meeting, the Committee established an acceptable daily intake (ADI) of 0–12.5 mg/kg body weight (bw) based on a long-term feeding study in rats. At its twenty-fifth meeting, the ADI of 0–12.5 mg/kg bw was made temporary pending the results of adequate long-term feeding studies and a multigeneration reproduction/teratogenicity study. At the twenty-ninth meeting, two long-term toxicity and carcinogenicity studies and a three-generation reproductive study became available. It was noted that a mouse oral carcinogenicity study was negative but that in the rat study an increased incidence of urothelial hyperplasia and/or neoplasia of the bladder was observed at the highest dose level. The biological significance of observed differences in benign and malignant tumours at other sites was considered questionable since, apart from the bladder, complete histopathological examination was not performed on the low- and intermediate-dose groups. The temporary ADI was extended to permit complete histopathological examination of all groups of rats and biometric examination of the data. At its thirtieth meeting, a review of the histopathological data from the rat oral carcinogenicity study was performed from which it was concluded that inappropriate statistical tests had been performed on some of the data by the testing laboratory. The Committee concluded that Fast Green FCF was noncarcinogenic in rats and established an ADI of 0–25 mg/kg bw, based on a long-term study of toxicity in rats.

At the present meeting, the Committee re-evaluated this colour at the request of the Forty-eighth Session of the Codex Committee on Food Additives (FAO/WHO, 2016). In response to the Committee's request for further data, new studies on genotoxicity and neurological effects were submitted. The Committee

also considered other related information which has become available in the literature since its last evaluation.

The search of the scientific literature was conducted in March 2017 using the PubMed database of the United States National Library of Medicine. Use of the linked search terms "Fast Green" and "toxicity" yielded 32 references, some of which had been evaluated by previous Committees or identified by the sponsor. Several other studies used Fast Green FCF as a marker in their methodology (e.g. staining); only one of these references, Ashida et al. (2000), was considered relevant for the present assessment.

The previous monograph has been expanded and is reproduced in this consolidated monograph. References from 1986 onward were not considered by the previous Committees. References from before 1986 that had not been evaluated by the previous Committees are indicated in the text.

1.1 Chemical and technical considerations

Fast Green FCF consists mainly of disodium 3-[*N*-ethyl-*N*-[4-[[4-[*N*-ethyl-*N*-(3-sulfobenzyl)amino]-phenyl](4-hydroxy-2-sulfophenyl)methylene]-2,5-cyclohexadien-1-ylidene]ammoniomethyl]-benzenesulfonate and its isomers, together with subsidiary colouring matters, as well as sodium chloride and/or sodium sulfate as the principal uncoloured components. It is manufactured by condensing 2-formylhydroxybenzenesulfonic acid with a mixture of 3-[(*N*-ethyl-*N*-phenylamino)methyl]benzenesulfonic acid and its 2- and 4-isomers to form the leuco base precursor. Oxidation of the leuco base precursor with either chromium- or manganese-containing compounds produces the dye, which is purified to contain not less than 85% total colouring matters and isolated as the disodium salt. Impurities include unreacted starting material and reaction by-products (approximately 2%), subsidiary colouring matters (≤6%), residual leuco base precursor (≤5%), unsulfonated primary aromatic amines (≤0.01% calculated as aniline), lead (≤2 mg/kg), chromium (≤50 mg/kg) and manganese (≤100 mg/kg).

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

No new information has been published since the previous evaluations.

Rats and dogs were given 200 mg Fast Green FCF orally. In the rat, almost all the administered colour was excreted unchanged in faeces collected over 36 hours, and no colour was found in the urine. In the dog, the amount of Fast Green FCF in bile never exceeded 5% of the administered dose. It was concluded that the quantity found in the bile provides a reasonable estimate of the amount absorbed from the gastrointestinal tract (Hess & Fitzhugh, 1953; 1954; 1955).

Following intravenous injection of Fast Green FCF in rats, over 90% of the colour was excreted in the bile within 4 hours (Iga, Awazu & Nogami, 1971). Fast Green FCF was found to have a high binding affinity for plasma protein, and it was suggested that the binding ratio with plasma protein was also one of the factors in the biliary excretion (Gangolli, Grasso & Golberg, 1967; Iga, Awazu & Nogami, 1971; Gangolli et al., 1972).

2.1.2 Biotransformation

No data were available.

2.1.3 Effects on enzymes and other biochemical parameters

In an in vitro study, the *Xenopus* oocyte expression system was used to investigate the purinergic P2 receptors that interact with the membrane channel protein pannexin 1 (Panx1) in inflammasome signalling. Fast Green FCF was shown to be a selective inhibitor of Panx1 channels, with a half maximal inhibitory concentration (IC_{50}) of 0.27 μmol/L. Fast Green FCF did not significantly inhibit the P2X7R receptor (Wang, Jackson & Dahl, 2013). The Committee was aware that Panx1 activation/inhibition is one of several signalling pathways involved in various physiological processes at the cellular level (e.g. immune function) and that there are many exogenous and endogenous modulators of these pathways. Interactions of substances with P2 receptors and Panx1 are an active area of research, particularly in development of drug treatments for diverse chronic diseases. The Committee noted that a similar pattern of channel inhibition was observed with Brilliant Blue FCF. Further research may clarify if the inhibition of Panx1 observed in in vitro is relevant for the safety assessment of substances in food.

2.2 Toxicological studies

2.2.1 Acute toxicity

Studies of acute toxicity of Fast Green FCF administered to rats and dogs by the oral route are summarized in Table 1.

Table 1
Acute toxicity of Fast Green FCF

Species	Sex	Route	LD_{60} (mg/kg bw)	Reference
Rat	Male	Oral	>2 000	Lu & Lavallee (1964)
Dog	Unknown	Oral	>200	Radomski & Deichmann (1956)

bw: body weight; LD_{50}: median lethal dose

2.2.2 Short-term studies of toxicity

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

(a) Dogs

Four beagle dogs per group, equally divided by sex, were fed Fast Green FCF at 0, 1.0% or 2.0% in the diet (equal to 0, 269 and 695 mg/kg bw per day) for 2 years. Histopathology findings attributable to Fast Green FCF were limited to green blobs of pigment in the renal cortical tubular epithelial cytoplasm of one male dog at the high-dose level. One female dog at the high-dose level showed slight interstitial nephritis and slight bone marrow hyperplasia (Hansen et al., 1966).

2.2.3 Long-term studies of toxicity and carcinogenicity

No new long-term toxicity studies have become available since the previous evaluations.

(a) Mice

Groups of 50 male and 50 female C_3HeB/FeJ mice were fed diets containing 1.0% or 2.0% Fast Green FCF (equivalent to 1500 or 3000 mg/kg bw per day) for 2 years; 100 mice of each sex served as controls. After 78 weeks, 56 control animals, 27 animals in the 1.0% treatment group and 17 animals in the 2.0% treatment group were still alive. Microscopic examination found no treatment-related lesions.

The no-observed-adverse-effect level (NOAEL) was 2.0% in the diet (equivalent to 3000 mg/kg bw per day), the highest dietary concentration tested (Hansen et al., 1966).

Groups of 60 (120 controls) male and female Charles River CD1 mice were fed diets containing Fast Green FCF at 0, 0.5%, 1.5% or 5.0% (equal to 0, 842, 2465 and 8806 mg/kg bw per day for males and 0, 1141, 3392 and 11 805 mg/kg bw per day for females) from 43 days of age for approximately 24 months. Haematological examinations of 10 animals/sex per group were undertaken at

3, 6, 12 and 18 months. All animals that died or were euthanized in a moribund condition and all the animals that survived to study end underwent detailed necropsy. The following tissues from all the animals from the control and 5% dose group were examined: adrenals, aorta, bone and marrow (femur), brain (three sections), eyes (with optic nerve), gall bladder, gastrointestinal tract (oesophagus, stomach, duodenum, ileum, caecum, colon), heart, kidneys, liver, lung, lymph nodes (mesenteric and mediastinal), mammary gland, nerve (sciatic), ovaries, pancreas, pituitary, prostate, salivary gland, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, testes with epididymides, thymus, thyroid/parathyroid, trachea, urinary bladder, uterus and gross lesions/tissue masses. In addition, gross changes/tissue masses of all animals in the lower-dose groups were examined histopathologically.

No treatment-related effects on mortality were observed. The mean body weights of females in the 5% dose group were consistently lower than that of controls (10% at termination of the study). The mean body weights of males in the 5% dose group were lower than controls at weeks 52 and 78 (5% and 6%, respectively) and in the 1.5% dose group at week 78 (6%). Slight reductions in haemoglobin, haematocrit and erythrocyte counts were noted in the high-dose males at 18 months but no other consistent or dose-related haematological changes were observed. Histopathological examination did not find any treatment-related lesions, and the incidence, origins and histology of benign and malignant neoplasms did not differ significantly between controls and treated animals.

The previous Committee concluded that the NOAEL was 5% Fast Green FCF in the diet, the highest dose tested. The present Committee noted that the mean body weights of females in the 5% dose group were consistently lower than that of controls after the study start, indicating an adverse effect at this dose.

The present Committee concluded that the NOAEL was 1.5% (equal to 3392 mg/kg bw per day) based on the lower body weights observed at 5% (equal to 11 805 mg/kg bw per day) in females (Hogan & Knezevich, 1981).

The Committee noted that the study was performed before the establishment of Organisation for Economic Co-operation and Development (OECD) guidelines and the requirement for good laboratory practices (GLP). The study was, however, inspected by a quality assurance unit and met the requirements established by the United States Food and Drug Administration (USFDA).

(b) Rats

Groups of 50 weanling Osborne-Mendel rats, evenly divided by sex, were fed diets containing 0, 0.5%, 1.0%, 2.0% or 5.0% of Fast Green FCF (equivalent to 0, 250, 500, 1000 and 2500 mg/kg bw per day) for 2 years. No effects on growth or

mortality were observed. Microscopic examination found no treatment-related lesions.

The NOAEL was 5.0% in the diet (equivalent to 2500 mg/kg bw per day), the highest dietary concentration tested (Hansen et al., 1966).

The Committee noted that the study was performed before OECD guidelines and GLP requirements were established.

Fast Green FCF was fed at a dietary level of 4.0% (equivalent to 2000 mg/kg bw per day) to five male and five female rats for 18–20 months. This procedure resulted in gross staining of the forestomach, glandular stomach, small intestine and colon. Granular deposits were noted in the stomach. No tumours were observed (Willheim & Ivy, 1953).

A carcinogenicity study with an in utero phase was carried out in Charles River albino rats. Groups of 60 (120 controls) male and female rats of the F_0 generation were fed diets containing 0, 1.25%, 2.5% or 5.0% Fast Green FCF (equal to 0, 722, 1486 and 3184 mg/kg bw per day for males and 0, 926, 1863 and 4021 mg/kg bw per day for females) for 2 months prior to mating and throughout gestation and lactation. Following the reproductive phase, a maximum of four animals of each sex per litter were randomly selected from the F_1 generation for the long-term carcinogenicity study. Groups of 70 animals/sex per group were given Fast Green FCF in the diet at the same concentrations as the parent generation. An interim kill of 10 animals/sex per group was carried out after 12 months; the remaining animals continued to receive the test diets for 29 months (males) or 31 months (females). Haematological and clinical chemistry tests and urine analysis were performed on 10 rats/sex per group at 3, 6, 12, 18 and 24 months. All the animals that died or were euthanized in a moribund condition during the study and all F_1 animals killed at interim and terminal kills were necropsied. The following tissues from these animals as well as all survivors from the control and 5% dose groups were examined: adrenals, aorta, bone and marrow (femur), brain (3 sections), eyes (with optic nerve), gastrointestinal tract (oesophagus, stomach, duodenum, ileum, caecum, colon), heart, kidneys, liver, lung, lymph nodes (mesenteric, mediastinal), mammary gland, nerve (sciatic), ovaries, pancreas, pituitary, prostate, salivary gland, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, testes with epididymides, thymus, thyroid/parathyroid, trachea, urinary bladder, uterus and gross lesions/tissue masses. In addition, gross changes/tissue masses in all animals in the lower-dose groups and, subsequently, bladder of males at 1.25% and 2.5% also underwent histopathological examination.

During the premating period, no treatment-related effects were seen on mortality or body weight gain, but there was a dose-related increase in

feed consumption. There were no treatment-related effects on the number of successful pregnancies or pup viability at birth, but pup mortality was increased in the 5% dose group during lactation days 4–14. The Committee noted that this was due to high mortality in one litter (i.e. all 13 pups of one dam died). Mean pup weight was reduced in all treatment groups at the end of the lactation period, most markedly in the high-dose group.

In the F_1 generation, mortality was slightly higher in all treatment groups than in controls but not in a dose-related manner. Mean body weights of males at 2.5% and 5% were lower than controls at week 1 (about 5%), consistent with the lower mean pup weight noted for these groups prior to random selection of offspring for the F_1 generation. Mean body weights of males at 5% remained consistently lower than controls throughout the study (10% at study termination) despite their elevated feed intake. Mean body weights for all groups of treated females were comparable to controls in the first year of the study. Mean body weights of the females at 5% were lower (9%) than controls after 78 weeks; mean body weights of the females at 2.5% were lower (7%) than controls after 2 years. As with males, feed consumption in the high-dose females was higher than in controls. As there was no dose-dependency in the decrease in body weights in either males or females, the Committee considered these effects on body weight as not adverse. Fasting blood glucose levels were elevated in females in all treatment groups at 3 and 12 months, females at 1.25% and 2.5% at 18 months and males in all treatment groups at 12 and 18 months.

At the interim (12-month) kill, the mean absolute and relative thyroid weights were elevated in the high-dose males while the relative kidney weights were elevated in the high-dose females. At study end, the relative thyroid weights were elevated in males at 2.5% and 5% and females at 5%; relative kidney weights were elevated in both sexes at 5% and females at 2.5%. No treatment-related effects were seen in urine analyses or haematological, physical or ophthalmological examinations.

Histopathological examination found an increased incidence of urothelial hyperplasia in treated males and of urinary bladder transitional cell/ urothelial neoplasms in males at 5%. Nonstatistically significant increases in testicular Leydig cell tumours and neoplastic nodules in the liver (now called hepatocellular adenomas) were also observed. Time-to-tumour analyses of pathology incidence data confirmed the increased incidence of bladder tumours, and the incidence of several other tumour types showed statistically significant treatment-related differences, including neoplastic nodules in liver (males and females), female mammary adenomas and pituitary adenomas, male parathyroid adenomas, male thyroid medullary carcinomas, female uterine leiomyosarcomas and male testicular interstitial/Leydig cell tumours.

In nonneoplastic pathology, chronic nephropathy was a common finding in all groups but the severity was greater in females at 5%. Other lesions did not appear to be related to treatment (Knezevich & Hogan, 1981).

At the twenty-ninth meeting, the Committee noted an increased incidence of urothelial hyperplasia and/or neoplasia of the bladder in the Knezevich & Hogan (1981) study. However, the biological significance of the differences observed in benign and malignant tumours at other sites was considered questionable since, apart from the bladder, complete histopathological examination was not performed on the low- and intermediate-dose groups.

A review of the histopathological data from the Knezevich & Hogan (1981) study concluded that the testing laboratory had performed inappropriate statistical tests to analyse some of the data. After conducting appropriate statistical procedures, the reviewers concluded that there were no significant differences in tumour incidence between combined control groups and the high-dose group with respect to tumours of the liver, testes or thyroid; data on the tumours in the tissues from the low- and intermediate-dose groups were not examined. In a blind review of the slides of the bladders from all dose groups, only three proliferative lesions – two benign (papilloma) and one malignant (carcinoma) lesion – were observed in bladders of male rats in the high-dose group and a treatment-related increase in the incidence or severity of transitional cell hyperplasia was not detected. The presence of only three neoplasms in the high-dose male group, two of which were questionable, and the absence of evidence of a preneoplastic process in the hyperplasia indicated lack of a neoplastic effect of Fast Green FCF on the bladder (Dua, Chowdury & Moch, 1982; O'Donnell, 1982; USFDA, 1982a,b).

Based on this information, the Committee concluded at its thirtieth meeting that Fast Green FCF is noncarcinogenic in this species. Therefore, the NOAEL was identified as 5.0% in the diet (equal to 3184 mg/kg bw per day for males and 4021 mg/kg bw per day for females), the highest dietary concentration tested (Knezevich & Hogan, 1981). The present Committee concurred with this conclusion.

The Committee noted that although the study was performed before OECD guidelines and GLP requirements were established, it met USFDA quality assurance requirements.

Eighteen weanling Osborne-Mendel rats of both sexes received weekly subcutaneous injections of approximately 30 mg (1 mL of a 3% aqueous solution) of Fast Green FCF for 94–99 weeks. Subcutaneous fibrosarcomas were noted at the site of injection in 15 animals (Nelson & Hagan, 1953; Hansen et al., 1966).

Two groups of 16 female rats (control groups of 10 rats) were given subcutaneous injections of 0.5 mL of a 3% or 6% solution of Fast Green FCF in distilled water (15 or 30 mg, respectively, per injection). The colour used in the experiment was certified as 92% pure and was supplied as the disodium sulfonate salt. The 10 control rats were given distilled water injections. At first, injections of 6% were given 3 times a week; after 17 weeks it became necessary to reduce the dose to 3%, and both groups were given injections of 3% twice weekly for 9 more weeks. For the remaining 22 weeks, both groups were usually injected once a week, although occasionally two injections a week were tolerated. Growth inhibition was observed. Of the 16 animals at 6% Fast Green FCF solution, 13 had fibrosarcomas; 10 of the 12 animals at 3% Fast Green FCF also developed fibrosarcomas. None of the controls had neoplastic tissue at the site of injection (Hesselbach & O'Gara, 1960).

Subcutaneous injection of 1 mL of a 0.8% solution of Fast Green FCF in distilled water twice weekly (no further details given) resulted in histopathological changes suggestive of subsequent sarcoma formation that was not associated with chemical carcinogenic potential (Grasso & Golberg, 1966).

The Committee previously concluded, at its thirteenth, twenty-fifth and twenty-ninth meetings, that the production of local sarcomata at the site of subcutaneous injection in rats is not considered to constitute evidence of carcinogenicity by the oral route.

(c) Hamsters

No tumours were produced in 12 hamsters injected subcutaneously with 1 mg of Fast Green FCF dissolved in 0.1 mL water for 330 days (Price et al., 1978).

2.2.4 Genotoxicity

The results of in vitro and in vivo genotoxicity tests of Fast Green FCF are summarized in Table 2 and described in detail below. Although 10 of the 18 available genotoxicity tests were negative, four in vitro and four in vivo studies yielded positive results.

An Ames test and an in vitro chromosomal aberration assay were positive only in a crude sample when Fast Green FCF samples of different purities were assessed. However, no information on cytotoxicity was given, only 100 metaphases were scored and gaps were not excluded from the evaluation. The authors also stated that a sample of Fast Green FCF, purity 90.7%, was negative in the in vitro chromosomal aberration assay (Ishidate et al., 1984).

The Committee concluded that this study did not indicate a concern for genotoxicity.

Table 2

Genotoxicity of Fast Green FCF 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	50, 250, 1 000 µg/plate	Negative[a]	Brown, Roehm & Brown (1978)
	S. typhimurium TA98, TA100, TA1535, TA1537, TA1538	50, 250, 1 000 µg/plate	Negative[b]	Bonin, Farquharson & Baker (1981)[c]
	S. typhimurium TA92, TA94, TA98, TA100, TA1535, TA1537	1, 2, 5, 10 mg/plate	Positive in one crude sample[d]	Ishidate et al. (1984)
	S. typhimurium TA98, TA100	Unknown	Negative[e]	Kawachi et al. (1980)[c]
	Escherichia coli	Unknown	Negative[f]	Rosenkranz & Leifer (1980)
DNA damage	*Bacillus subtilis* 17A, 45AT	1 mg/mL	Negative[g]	Kada, Tutikawa & Sadaie (1972)
	B. subtilis	Unknown	Negative[h]	Kawachi et al. (1980)
Gene conversion	*Saccharomyces cerevisiae* BZ34	5 mg/mL	Negative[i]	Sankaranarayanan & Murthy (1979)
Chromosomal aberrations	Chinese hamster fibroblast cell line	3 doses, up to 4 mg/mL	Positive in one crude sample[j]	Ishidate et al. (1984)
	Chinese hamster ovary cell line	20 µmol/L	Positive[k]	Au & Hsu (1979)
	Hamster lung fibroblasts	Unknown	Negative[l]	Kawachi et al. (1980)
Cell transformation	Fischer rat embryo cells	1, 10, 100 µg/mL	Positive at 1 µg/mL in the second experiment only[m]	Price et al. (1978)
In vivo				
Bone marrow micronucleus induction	Male Swiss albino mice	100, 200, 400, 800 mg/kg bw	Positive[n]	Misra & Misra (1986)
	Male ddY mice	250, 500, 1 000, 2 000 mg/kg bw	Negative[o]	Hayashi et al. (1988)
Chromosomal aberrations	Male Swiss albino mice	1 (plus 1 mg/kg bw nitrite), 2 mg/kg bw	Positive[p]	Giri et al. (1986)
	Male Swiss albino mice	250 (plus nitrite at 250 mg/kg bw), 500, 500 (plus nitrite at 500 mg/kg bw), 1 000 mg/kg bw	Positive[q]	Das & Giri (1988)
Sister chromatid exchange	Male Swiss albino mice	5, 5 (plus nitrite at 5 mg/kg bw),10, 12.5 (plus nitrite at 12.5 mg/kg bw), 25, 25 (plus nitrite at 25 mg/kg bw), 50, 50 (plus nitrite at 50 mg/kg bw), 100 mg/kg bw	Positive[r]	Giri & Mukherjee (1990)
DNA damage	Male ddY Mice	2 000 mg/kg bw	Negative[s]	Sasaki et al. (2002)

bw: body weight. S9: 9000 × *g* supernatant fraction from liver homogenate

[a] Plate tests were performed with and without S9 mix.

[b] Plate tests were conducted with and without S9 mix.

[c] Not evaluated by previous Committees.

[d] Plate tests were conducted with and without S9 mix. Fast Green FCF samples were tested and only a crude sample was positive by inducing 347 revertants in TA100 at 10 mg/plate with S9 mix.

[e] With and without metabolic activation.

[f] DNA-polymerase-deficient *E. coli* was used to determine the DNA-modifying activity.

[g] The bacterial DNA repair tests were conducted by rec-assay.

[h] The bacterial DNA repair tests were conducted by rec-assay with and without metabolic activation.

Table 2 (continued)

[i] The gene conversion assay in diploid yeast was conducted without metabolic activation.
[j] The cells were exposed to three Fast Green FCF samples for 24 and 48 hours with no metabolic activation systems were applied. Only a crude sample was positive at 2 and 4 mg/mL at 24 and 48 hours.
[k] The mean number of breaks per metaphase induced by Fast Green FCF was 0.36 at 20 µmol/L for 5 hours. No positive control was used.
[l] Tests were conducted with and without metabolic activation.
[m] Two experiments were conducted, and only one had positive results.
[n] No positive control substance was used and the purity of Fast Green FCF was unknown.
[o] The purity of Fast Green FCF was 89.2%. Mitomycin C (2 mg/kg bw) was used as the positive control. Evidence of genotoxicity was assessed based on increased frequency of micronucleated polychromatic erythrocytes (MNPCEs) for each animal (1000 polychromatic erythrocytes [PCEs] per animal were examined).
[p] Bone marrow chromosomes were examined following 30-day oral treatment of mice with Fast Green FCF with and without nitrite. A significant increase in the frequency of chromosomal aberrations was found with the dye-treated and dye-plus-nitrite-treated groups when compared with the controls. No positive control was used.
[q] Bone marrow chromosomes were examined following 90-day oral treatment of mice with Fast Green FCF with and without nitrite. A significant increase in the frequency of chromosomal aberrations was found with the dye-treated and dye-plus-nitrite-treated groups when compared with the controls. No positive control was used.
[r] Sister chromatid exchange (SCE) in mouse bone marrow cells was studied after intraperitoneal injection of Fast Green FCF with and without nitrite. Mitomycin C (2.5 mg/kg bw) was used as the positive control. A significant increase in SCE frequency was found with the dye-treated and dye-plus-nitrite-treated groups when compared with the controls.
[s] The comet assay was performed in isolated nuclei from the glandular stomach, colon, liver, kidney, urinary bladder, lung, brain and bone marrow of mice.

Another in vitro chromosomal aberration assay found chromosome damage, but only summary data were given, there was no information on purity and it was unclear whether gaps were excluded from the evaluation. The authors indicated that this damage was probably due to the impurities in the sample, but did not identify the impurities (Au & Hsu, 1979).

Fast Green FCF gave positive results in an in vitro cell transformation study at the lowest concentration tested (1 µg/mL) but not at 10 or 100 µg/mL, and only in the second experiment (Price et al., 1978).

For three nonstandard in vivo assays performed in the same laboratory, chromosomal aberrations were reported with Fast Green FCF alone or in combination with nitrite (Giri et al., 1986; Das & Giri, 1988; Giri & Mukherjee, 1990). The Committee noted that the purity of Fast Green FCF was not reported in these publications and that in two of these studies no positive control was used.

An in vivo bone marrow micronucleus assay also showed positive results after intraperitoneal administration in male Swiss albino mice of Fast Green FCF at up to 400 mg/kg bw. In this study, no positive control was used, the purity of Fast Green FCF was not stated and intraperitoneal administration at 800 mg/kg bw resulted in all the animals dying (Misra & Misra, 1986).

In an attempt to duplicate these results, Hayashi et al. (1988) found consistently negative results in an in vivo bone marrow micronucleus assay after single intraperitoneal injections of Fast Green FCF (89.2% purity) of up to 2000 mg/kg bw into male ddY mice and after 4 injections at 24-hour intervals of the test substance at 250 mg/kg bw. A more recent and well conducted in vivo comet assay on murine tissues (glandular stomach, colon, liver, kidneys, urinary

bladder, lungs, brain and bone marrow) found clearly negative results 3 and 24 hours after treatment (Sasaki et al., 2002).

The Committee noted that the Hayashi et al. (1988) and Sasaki et al. (2002) in vivo studies were not fully compliant with the current versions of the OECD guidelines 474 and 489, respectively. However, they were considered sufficiently reliable to be used in this assessment.

Given that all of the genotoxicity studies with positive test outcomes had limitations in experimental design and reporting, and an in vivo mouse bone marrow micronucleus assay and an in vivo mouse tissue comet assay were clearly negative, the Committee concluded that there is no safety concern with respect to genotoxicity of Fast Green FCF.

2.2.5 Reproductive and developmental toxicity

No new reproductive and developmental toxicity studies have become available since the previous evaluations.

(a) Multigeneration reproductive toxicity

A 3-generation reproduction study was carried out on Fast Green FCF in Long–Evans rats at dose levels of 0, 10, 100, 300 or 1000 mg/kg bw per day. The F_0 parents (10 males, 20 females) were given Fast Green FCF in the diet 2 weeks before the first mating and continued to be treated daily throughout the gestation, lactation and post-weaning phases for three successive generations. The F_0 rats were mated twice. F_{1a} litters were necropsied at weaning, and selected F_{1b} animals (10 males, 20 females) were used for subsequent breeding. Following an 80-day growth period, F_{1b} animals were mated 3 times and their offspring treated identically to the F_{1a} and F_{1b} generations. Following the third mating, half of the pregnant F_{1b} dams were killed on gestation day 19, the uterine contents were examined for total embryos/resorption sites and the number of corpora lutea per ovary were recorded. The remaining F_{1b} dams were allowed to deliver normally (F_{2c}) and were killed at weaning. The F_{2b} animals were mated once and allowed to raise their offspring (F_{3a}) to weaning when both parents and offspring were killed.

Gross necropsies were performed on all parent animals and on F_{1a}, F_{2a}, F_{2c} and F_{3a} offspring at weaning. Selected tissues from five animals of each sex/dose from the F_{1b} parents and the F_{3a} generation at weaning were fixed at necropsy, and the following tissues examined histopathologically from the control and high-dose group: stomach, ileum, jejunum, colon, liver, spleen, heart, lungs, adrenals, kidneys, urinary bladder, thyroid, ovaries and uterus or testes.

No treatment-attributable effects were observed with respect to feed consumption, body weight, adult mortality, mating performance, pregnancy and

fertility rates, gestation length, litter survival, resorption rates, necropsy findings or offspring survival, weights and sex. There were no treatment-attributable macroscopic or microscopic tissue abnormalities of either F_{1b} or F_{3a} animals.

The NOAEL for reproductive toxicity was 1000 mg/kg bw per day, the highest dose tested (Smith, 1973).

The Committee noted that no information on the developmental toxicity of Fast Green FCF was available. However, information on the developmental toxicity of a structurally closely related substance (Brilliant Blue FCF, which differs only by one OH group) was available. No developmental toxicity of Brilliant Blue FCF was reported in rats at doses up to 2000 mg/kg bw per day or in rabbits at doses up to 200 mg/kg bw per day (BioDynamics Inc., 1972a,b). The Committee concluded there is no concern with respect to developmental toxicity of Fast Green FCF.

2.2.6 Special studies

(a) Effects on hepatic function

The influence of a mixture of six commonly used artificial food colours, erythrosine, Allura Red, new coccine (Ponceau 4R), 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 from rats fed a diet containing the mixture of the food colours at concentrations of half their respective ADIs for 4 weeks. Trp-P-1 was applied to the cultured hepatocytes at various concentrations for 12 hours.

The mixture of food colours did not affect the Trp-P-1-induced decrease in cell viability or protein and DNA synthesis, but slightly augmented the Trp-P-1-induced decrease in gluconeogenesis and ureogenesis. Ashida et al. (2000) suggested that the daily intake of artificial food colours may impair hepatic functions such as gluconeogenesis and ureogenesis when liver cells are exposed to dietary carcinogens such as Trp-P-1.

The Committee noted that the use of mixtures in these studies does not permit ascribing any observed effects to individual components.

(b) Neurological effects

Fast Green FCF was tested for its effect on synaptic events in whole-cell voltage clamped hippocampal interneurons using the patch clamp technique. The assay was performed on freshly isolated tissue from male Wistar rats and maintained in artificial cerebrospinal fluid (NaCl at 120 mmol/L, KCl at 3.5 mmol/L, $CaCl_2$ at 2.5 mmol/L, $MgSO_4$ at 1.3 mmol/L, NaH_2PO_4 at 1.25 mmol/L, $NaHCO_3$ at

25 mmol/L and glucose at 25 mmol/L). Miniature synaptic events were recorded in whole-cell voltage clamped hippocampal interneurons of the stratum radiatum of the CA1 hippocampus area.

Fast Green FCF added at concentrations of 0.1, 0.3 or 1 mg/mL (0.125, 0.375 and 1.2 mmol/L, respectively) reduced the frequency of synaptic activity in a dose-dependent manner. Frequency was reduced to 21% of the control frequency at 1 mg/mL (1.2 mmol/L). The effect was reversible upon removal of the test substance. The half maximal effective concentration (EC_{50}) was estimated to be 200 mg/mL (250 mmol/L). No effect was detected on the magnitude of current or the decay time constant. Van Hooft (2002) suggested that Fast Green FCF may inhibit the release of neurotransmitters at a presynaptic level.

The Committee noted that Fast Green FCF is poorly absorbed and that the colour was only active at high concentrations in vitro. The Committee concluded that these results are not relevant to the present evaluation.

2.3 Observations in humans

No data were available.

3. Dietary exposure

The Committee reviewed dietary exposures for Fast Green FCF based on individual dietary records from the USFDA (Doell et al., 2016) and Konkuk University, Republic of Korea (Ha et al., 2013). In these studies, Fast Green FCF was detected in only a few food groups – mainly in the ice cream group – and the exposure estimates reflect use in only these foods (Table 3). Fast Green FCF was included in the analytical programs of studies in Australia (FSANZ, 2008) and Kuwait (Husain et al., 2006), but it was not detected and no dietary exposure estimates were reported. The USFDA analysed 44 food samples for Fast Green FCF; the colour was detected in two food groups (cereal and sherbet) at levels of 1.9–2.9 mg/kg (Harp, Miranda-Bermudez & Barrows, 2013).

Since the exposure estimates from the USA and the Republic of Korea were based on only a few findings in a limited number of food groups, the Committee performed a conservative dietary exposure assessment using the FAO/WHO Chronic Individual Food Consumption Database – Summary statistics (CIFOCOss) database and Codex maximum levels.

Table 3
Studies reviewed for the present assessment of dietary exposure to Fast Green FCF

Organization	Concentration data	Sampling strategy	Consumer groups	Food consumption data	Estimates of dietary exposure	Scenarios
USFDA (Doell et al., 2016)	Analytical levels in foods	Targeted using label and/or colour	USA population ≥2 years; children 2–5 years; boys 13–18 years	Individual dietary records and food diaries 2 days; 10–14 days	Mean P90[a] P95[b]	Low[c] Medium[d] High[e]
Konkuk University, Republic of Korea (Ha et al., 2013)	Analytical levels in foods	Market share	1–2 years; 3–6 years; 7–12 years; 13–19 years; 20–29 years; 30–49 years; 50–64 years; ≥65 years; male; female; upper 95th consumers	24-hour recall	Mean P95[b]	Medium[d]

USFDA: United States Food and Drug Administration; P90: 90th percentile; P95: 95th percentile
[a] 90th percentile.
[b] 95th percentile.
[c] The lowest analytical value for the colour additive was assigned to each food code.
[d] The analytical results were averaged for a given food code.
[e] The highest analytical value for the colour additive was assigned to each food code.

3.1 Assessments based on model diets

3.1.1 Exposure estimates based on CIFOCOss database

The CIFOCOss database[1] currently contains summary statistics from 55 surveys from 36 countries, each with survey durations of 2 days or more. The countries, grouped in 17 Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) clusters diets (WHO, 2012), are culturally and economically comparable. The database provides summary statistics of food categorization, grouped in three tiers, with more than 600 items at the most detailed tier. The food categories from the Codex General Standard for Food Additives (GSFA) (FAO/WHO, 2015) were matched with the CIFOCOss detailed food groups to assign the Codex maximum levels for the calculations. These assignments are listed in Table 4.

The mean and the 95th percentile of the dietary exposure were calculated within each survey, age class and CIFOCOss food group. For each survey a mean and a high exposure were calculated. The high exposure was calculated as the sum of the 95th percentile exposure for the food group with the highest 95th percentile exposure and the mean exposure for each of the remaining food groups. For each age group, Table 5 lists the number of clusters and surveys included in the ranges given for the mean and the high exposure estimates. The high value of 12 mg/kg

[1] Detailed data (January 2017) were made available to the Committee by the World Health Organization.

Table 4
Food groups and Codex maximum levels of Fast Green FCF used in the CIFOCOss calculations

CIFOCOss food group	GSFA code	Codex maximum levels (mg/kg or mg/L)
Dairy-based desserts (e.g. pudding, fruit or flavoured yogurt)	01.7	100
Fat-based desserts excluding dairy-based dessert products	02.4	
Edible ices, including sherbet and sorbet	03	
Bullets or lollipop	05.2	
Cakes, cookies and pies (e.g. fruit-filled or custard types)	07	
Meat from mammals other than marine mammals, nes	08.1	100
Game (mammalian) meat		
Beef and other bovines meat		
Goat and other caprines		
Horse and other equines		
Kangaroo meat		
Pork and other porcines		
Rabbit meat		
Sheep and other ovines		
Poultry meat, nes		
Game (poultry) meat		
Chicken meat		
Duck meat		
Goose meat		
Quail meat		
Turkey meat		
Unprocessed meat and offals, nes		
Processed meat and meat products, nes	08.2	100
Fish-based composite food	10.4	
Dietary supplements, food supplements	13.6	600
Flavoured milk	01.1.4	100
Canned or bottled (pasteurized) fruits	04.1.2.4	200
Jams, jellies, marmalades	04.1.2.5	400
Other processed fruits (excluding dried and juice), nes	04.1.2.8	100
Papaya, dried	Average of 04.2.2.3, 04.2.2.4 and 04.2.2.7	200[a]
Raspberries, red, black, dried		
Pulses processed, nes		
Roots and tubers processed		
Roots and tubers other processed, nes		
Brassica vegetables, head cabbages, flowerhead cabbages, processed		
Bulb vegetables, processed		
Fruiting vegetables, other than cucurbits, processed		
Leafy vegetables, processed		
Legume vegetables, processed		
Root vegetables, processed		

Table 4 (continued)

CIFOCOss food group	GSFA code	Codex maximum levels (mg/kg or mg/L)
Other vegetables, nes, other processing		
Other vegetables, unprocessed, nes		
Strawberry, dried	04.2.2.7	100
Processed fish and fish products (including molluscs, crustaceans and echinoderms)	09.2.4.1	100
Tuna, preserved		
Salmon, preserved		
Smoked, dried, fermented, and/or salted fish and fish products, including molluscs, crustaceans and echinoderms	09.2.5	
Mayonnaise	12.6.1	100
Mustard sauce		
Sauces and like products, nes		
Energy drinks	14.1.4	100
Isotonic drink		
Nonalcoholic ("soft") beverages, nes		
Distilled spirituous beverages containing more than 15% alcohol	14.2.6	100
Beer and malt beverages, nes	14.2.7	
Beer of barley		
Beer of maize		
Alcoholic beverages, nes		

CIFOCOss: Chronic Individual Food Consumption Database – Summary statistics; GSFA: General Standard for Food Additives; nes: not elsewhere specified
[a] Average of 04.2.2.3 (300 mg/kg), 04.2.2.4 (200 mg/kg) and 04.2.2.7 (100 mg/kg).

Table 5
Exposure estimates for Fast Green FCF per age group based on CIFOCOss database and Codex maximum levels

Population[a]	No. of clusters	No. of surveys	Range for mean exposure across dietary surveys (mg/kg bw per day)	Range for high-level exposure across dietary surveys (mg/kg bw per day)
Adults	5	19	0.49–1.5	1.0–4.8
Adolescents	5	17	0.50–2.9	1.2–12
Children	7	30	0.25–2.7	1.0–7.5
Toddlers	4	9	0.93–2.8	2.1–8.7
Infants	1	2	0.19–0.37	0.57–6.9

bw: body weight; CIFOCOss: Chronic Individual Food Consumption Database – Summary statistics; No.: number
[a] Ages not defined in the version of CIFOCOss database available.

bw per day for adolescents is strongly influenced by the contribution of United Kingdom adolescent consumers of nonalcoholic soft drinks.

Table 6
Food groups contributing most to total mean exposure to Fast Green FCF

Population[a]	Food group contributing most to the mean exposure	No. of surveys	Range of contributions (%)
Adults	Beer and malt beverages, nes	10	9–44
	Nonalcoholic ("soft") beverages, nes	7	12–57
	Diet beverages	2	22–24
Adolescents	Nonalcoholic ("soft") beverages, nes	13	17–100
	Roots and tubers other processed, nes	1	39
Children	Nonalcoholic ("soft") beverages, nes	20	7–100
	Roots and tubers other processed, nes	3	31–43
	Canned or bottled (pasteurized) fruits	2	1–13
	Other vegetables, unprocessed, nes	1	30
Toddlers	Nonalcoholic ("soft") beverages, nes	6	2–30
	Roots and tubers other processed, nes	2	37–38
	Canned or bottled (pasteurized) fruits	1	13
Infants	Roots and tubers other processed, nes	1	62

nes: not elsewhere specified; No.: number
[a] Ages not defined in the version of CIFOCOss database available.

For each age group, Table 6 lists the CIFOCOss food group contributing most to the total mean exposure within each survey, the range of these contributions and the number of surveys included in this range. Only food groups that contributed at least 10% to the total mean exposure are included in the table.

3.2 Assessments based on individual dietary records

3.2.1 Exposure estimates reported by USFDA

In a study completed by the USFDA (Doell et al., 2016), approximately 600 commercially available food samples from 52 broad categories were analysed for Fast Green FCF (and other synthetic colours) using a validated liquid chromatography method with photodiode array detection (Harp, Miranda-Bermudez & Barrows, 2013). Samples were selected based on a previous survey of food labels. Fast Green FCF was found in products in two of the 52 food categories at concentrations just over the limit of detection (LOD; 1 mg/kg) to 1.2 mg/kg (in the categories "Cakes and cupcakes" and "Ice Cream, frozen yogurt, sherbet"). 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 exposure survey for the United States population. Three population groups were used for the exposure estimate: United States 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 but showed no difference in exposure to Fast Green FCF. Dietary exposures were estimated at the mean and at the 90th percentile for each population for each food category (Tables 7 and 8).

The "Frozen dairy dessert/Sherbet" food group was the dominant contributor (95–97% of total exposure) to exposure to Fast Green FCF in the USA population. Table 8 lists the major contributing food groups for the three age groups.

3.2.2 Exposure estimates reported by Konkuk University, Republic of Korea

The dietary exposure to Fast Green FCF in the Republic of Korea was estimated based on food consumption data for consumers and concentrations in processed foods (Ha et al., 2013). Fast Green FCF was found in 2/40 samples of ice cream. For the average consumer in the Republic of Korea (using the average concentration of all samples in a food group), the estimated mean and 95th percentile for dietary exposure were both below 0.001 mg/kg bw per day. For a high exposure consumer (using the average concentration of positive samples), the estimated mean and 95th percentile for dietary exposure were 0.001 and 0.003 mg/kg bw per day, respectively.

The dietary exposure estimates based on the CIFOCOss model diets and Codex maximum levels uses a very conservative approach and shows mean dietary exposures 0.5–1.5 mg/kg bw per day for adults and 0.3–3 mg/kg bw per day for toddlers, children and adolescents. Ranges for high exposures (in mg/kg bw per day) were 1–5 for adults, 1–9 for toddlers and children, and 0.5–12 for adolescents.

3.3 Evaluation of estimates of dietary exposure

Using the CIFOCOss data for adolescents, the dietary exposure is estimated to be 12 mg/kg bw per day (95th percentile).

The USFDA assessments based on USA food consumption data and analytical levels estimate mean dietary exposure in the range 0.01–0.04 mg/kg bw per day for children aged 2–5 years, teenage boys aged 13–18 years and the population aged 2 years and older. The 90th percentile exposures were in the range of 0.02–0.09 mg/kg bw per day. The estimates were derived from a generally lower-bound approach and were based on foods found to contain Fast Green FCF, which can explain the differences with the conservative approach of the CIFOCOss model.

The Committee concluded that the higher dietary exposure estimates prepared using the CIFOCOss model were due to the use of Codex maximum

Table 7
Estimated daily exposure to Fast Green FCF for the United States population based on NHANES food consumption data, 2007–2010

		Estimated exposure (mg/kg bw per day)			
		2 days food consumption		10–14 days food consumption	
Age–sex group (years)	**% eaters[a]**	**Mean**	**90th percentile**	**Mean**	**90th percentile**
Children (2–5)	15	0.04	0.09	0.02	0.03
Boys (13–18)	14	0.03	0.06	0.01	0.02
Population (≥2)	15	0.02	0.04	0.01	0.02

bw: body weight; NHANES: National Health and Nutrition Examination Survey
[a] Individuals who consumed one or more of the included foods over the survey period.
Source: Doell et al. (2016)

Table 8
Major contributors to Fast Green FCF exposure in the USA population based on the 2-day mean average-exposure scenario

	% of total exposure per population group		
Food group	**Population ≥2 years**	**Children 2–5 years**	**Boys 13–18 years**
Frozen dairy dessert/Sherbet	95	97	97
Drink mixers	2	–	–
Breakfast cereal	1	1	1
Cake and cupcakes	1	1	–

Source: Doell et al. (2016)

levels as opposed to mean analysed levels for all foods used by the USFDA and the Republic of Korea.

The Committee concluded that the conservative estimate of 12 mg/kg bw per day, prepared using the CIFOCOss model, should be considered in the safety assessment for Fast Green FCF.

4. Comments

4.1 Biochemical aspects

Absorption of orally administered Fast Green FCF was shown to be less than 5%; almost all the administered colour was excreted unchanged in the faeces of the rats (Hess & Fitzhugh, 1953, 1954, 1955).

In an in vitro study, in which the *Xenopus* oocyte expression system was used for pharmacological investigations on purinergic P2 receptors that interact with the membrane channel protein Panx1 in inflammasome signalling, Fast Green FCF was shown to be a selective inhibitor of Panx1 channels, with an IC_{50} of 0.27 µmol/L, and did not significantly inhibit the P2X7R receptor (Wang, Jackson & Dahl, 2013). The Committee was aware that Panx1 activation/inhibition is one of several signalling pathways involved in various physiological processes at the cellular level (e.g. immune function) and that there are many exogenous and endogenous modulators of these pathways. Interactions of substances with P2 receptors and Panx1 are an active area of research, particularly in development of drug treatments for diverse chronic diseases. The Committee noted that a similar pattern of channel inhibition was observed with Brilliant Blue FCF, and further research may clarify if the inhibition of Panx1 observed in an in vitro system has any relevance for the safety assessment for substances in food.

4.2 Toxicological studies

Fast Green FCF has low oral acute toxicity in rats (Lu & Lavallee, 1964) and dogs (Radomski & Deichmann, 1956).

A short-term study of toxicity revealed no compound-related effects in dogs fed Fast Green FCF at 0%, 1.0% or 2.0% of the diet (equal to 0, 269 and 695 mg/kg bw per day, respectively) for 2 years (Hansen et al., 1966).

Two previously reviewed long-term studies of oral toxicity showed no compound-related effects in mice and rats. The NOAEL was 2% Fast Green FCF in the diet (equivalent to 3000 mg/kg bw per day) in mice and 5.0% (equivalent to 2500 mg/kg bw per day) in rats (Hansen et al., 1966).

No treatment-related increase in tumour incidence was found in a mouse carcinogenicity study (Hogan & Knezevich, 1981). At the twenty-ninth meeting, the Committee concluded that the NOAEL was 5% Fast Green FCF in the diet, the highest dose tested. The present Committee noted that the mean body weights of females in the 5% dose group were consistently lower than those of controls after the commencement of the study (−10% compared with relevant controls at termination of the study). The Committee considered this decrease in body weights to be a treatment-related adverse effect and concluded that the NOAEL was 1.5% Fast Green FCF (equal to 3392 mg/kg bw per day), based on the lower body weights observed at 5% (equal to 11 805 mg/kg bw per day) in females.

A carcinogenicity study in rats reported an increased incidence of urothelial hyperplasia and/or neoplasia of the bladder (Knezevich & Hogan, 1981). However, a peer review of the histopathological data showed that Fast

Green FCF is noncarcinogenic in this species (Dua, Chowdury & Moch, 1982; O'Donnell, 1982; USFDA, 1982a,b). The previous Committee agreed with this conclusion at its thirtieth meeting and concluded that the NOAEL in this dietary study was 5% Fast Green FCF (equal to 3184 mg/kg bw per day), the highest dose tested (Annex 1, reference *73*). The present Committee concurred with this conclusion.

Whereas 10 of the 18 available genotoxicity tests were negative, four in vitro and four in vivo studies yielded positive results. Given that all of the studies with positive test outcomes had several limitations in experimental design and reporting, whereas an in vivo mouse bone marrow micronucleus assay (Hayashi et al., 1988) and an in vivo mouse tissue comet assay (Sasaki et al., 2002) were clearly negative, the Committee concluded that there is no concern with respect to genotoxicity of Fast Green FCF.

No reproductive toxicity was reported at doses up to 1000 mg/kg bw per day over three generations of rats (Smith, 1973).

No developmental toxicity studies were available. However, information on the developmental toxicity of the structurally related substance Brilliant Blue FCF, which differs from Fast Green FCF by a single hydroxyl group, was available. No developmental toxicity was reported in rats treated with Brilliant Blue FCF at doses up to 2000 mg/kg bw per day or in rabbits at up to 200 mg/kg bw per day (BioDynamics Inc. 1972a,b). Based on these findings, the Committee concluded that there is no concern for developmental toxicity for Fast Green FCF.

4.3 Observations in humans

No data were available.

4.4 Assessment of dietary exposure

Estimates of dietary exposure to Fast Green FCF published by the Republic of Korea (Ha et al., 2013) and the USFDA (Doell et al., 2016) were available. Because the estimates were based on only a few findings in a limited number of food groups, the Committee conducted a conservative assessment using the CIFOCOss database and Codex maximum levels.

Dietary exposure to Fast Green FCF was estimated to be 12 mg/kg bw per day for adolescents, the age group with the highest exposure, at the 95th percentile. This estimate was much higher than those of both the USFDA (0.09 mg/kg bw per day for children at the 90th percentile) and the Republic of Korea (0.003 mg/kg bw per day for the whole population at the 95th percentile). The Committee concluded that these differences were due to the use of Codex

maximum levels, in contrast to the estimates from the USFDA and the Republic of Korea, which used mean analysed levels for all foods.

The Committee concluded that the conservative estimate of 12 mg/kg bw per day, prepared using CIFOCOss data, should be considered in the safety assessment for Fast Green FCF.

5. Evaluation

The Committee at previous meetings concluded that Fast Green FCF is not carcinogenic. The evidence newly available at this meeting indicates that there is no concern with respect to genotoxicity of Fast Green FCF. The ADI of 0–25 mg/kg bw established previously by the Committee was based on a long-term rat dietary study in which a NOAEL of 5% Fast Green FCF (equivalent to 2500 mg/kg bw per day), the highest concentration tested, was identified (Hansen et al., 1966).

The Committee concluded that the new data that had become available since the previous evaluation gave no reason to revise the ADI and confirmed the ADI of 0–25 mg/kg bw. The Committee noted that the conservative dietary exposure estimate for Fast Green FCF of 12 mg/kg bw per day (95th percentile for adolescents) was below the upper bound of the ADI. The Committee concluded that dietary exposures to Fast Green FCF for adolescents and all other age groups do not present a health concern.

At the present meeting, the existing specifications for Fast Green FCF were revised, and a maximum limit for manganese was added. High-performance liquid chromatography (HPLC) methods were added to determine subsidiary colouring matters and organic compounds other than colouring matters. The assay method was changed to visible spectrophotometry, and spectrophotometric data were provided for the colour dissolved in water or aqueous ammonium acetate.

The specifications monograph was revised, and a Chemical and Technical Assessment was prepared.

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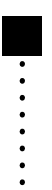

Gum ghatti

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1. Explanation

Gum ghatti (Chemical Abstracts Service [CAS] No. 9000-26-6), also known as Indian gum, ghatti gum or gum ghati, is the dried gummy exudate from wounds in the bark of *Anogeissus latifolia* Wallich (family Combretaceae), a large tree native to India and Sri Lanka (Al-Assaf, Phillips & Amar, 2009). Gum ghatti is used as a thickening agent and stabilizer. It is permitted as a food additive in the USA.

Gum ghatti was previously evaluated at the twenty-sixth and twenty-ninth Joint FAO/WHO Expert Committee on Food Additives (JECFA) meetings (Annex 1, references *59* and *70*). Heavy metal specifications were revised at the fifty-seventh JECFA meeting (Annex 1, reference *154*). No acceptable daily intake (ADI) could be established at the twenty-sixth or twenty-ninth meetings because of insufficient data, but the Committee did not make specific recommendations for further studies; no monographs were prepared.

At the present meeting, the Committee evaluated gum ghatti at the request of the Forty-eighth Session of the Codex Committee on Food Additives (FAO/WHO, 2016). A toxicological dossier was submitted. Two new 90-day rat studies as well as genotoxicity studies have become available since the previous evaluations. To address any data gaps for gum ghatti, the safety data on other polysaccharide-based gums were considered based on their similar general structure, chemical and functional properties, technical uses, lack of absorption as intact substances and their metabolism in the lower gastrointestinal tract.

A comprehensive literature search up to April 2017 was performed in PubMed and TOXLINE using the following search terms: "gum ghatti"/"ghati", "ghatti"/"ghati gum", "gatifolia", "CAS no 9000-28-6", "toxicity", "mutagenicity", "teratologic", "evaluation". Although the search resulted in five additional papers, these did not add further relevant data to those submitted to the Committee for this meeting.

1.1 Chemical and technical considerations

Unprocessed gum ghatti occurs as both amorphous "tears" of various sizes and as broken irregular pieces. It is light to dark brown in colour, has little or no odour and is available commercially in the form of brown tears or grey to reddish-grey powder. The product in commerce is manufactured by collecting the dried translucent exudate as tears, partially dissolving these in water and filtering. The final product is sterilized and dried to a gummy, lump form, or spray-dried to a powder form.

Gum ghatti consists mainly of a calcium salt (or occasionally magnesium) salts of high molecular weight and water-soluble complex polysaccharides. The hydrolysis of the polysaccharide yields L-arabinose, D-galactose, D-glucuronic acid and D-mannose, and small amounts of D-xylose and L-rhamnose. The reported average molar ratio of the various units is L-arabinose:D-galactose:D-glucuronic acid:D-mannose:D-xylose:L-rhamnose = 40:25:20:7:1:1 (Sakai et al., 2013). Gum ghatti also contains protein-bound arabinogalactan units, tannins and moisture. The average molecular weight of gum ghatti is in the order of several hundred kDa (Kang et al., 2015).

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Data on the absorption, distribution and excretion of gum ghatti were not available at the last evaluation by the Committee, and no additional information has become available since. However, based on its chemical composition, it is reasonable to assume that gum ghatti, like similar gums, will not be significantly degraded or absorbed in the stomach or small intestine. Instead, it is probable that gum ghatti, like gum arabic, passes into the caecum where it is enzymatically degraded and fermented by the microflora in the large intestine to hydrogen gas, carbon dioxide and short chain fatty acids, which can be absorbed and metabolized (Ali, Ziada & Blunden, 2009).

Gum arabic has been found to be fermented mainly to acetate, propionate and butyrate, which can be absorbed and metabolized by normal metabolic pathways. Short chain fatty acids have been found to significantly affect intestinal and liver metabolism as sources of energy or metabolic effectors (Ali, Ziada & Blunden, 2009).

2.1.2 Biotransformation

No information was available.

2.1.3 Effects on enzymes and other biochemical parameters

No information was available.

2.2 Toxicological studies

2.2.1 Acute toxicity

No new information was available on acute toxicity. One study that included a test of acute toxicity had not been previously described (Newell & Maxwell, 1972). Male Sprague Dawley rats (n = 10; 200–250 g) were fasted overnight prior to administration of gum ghatti suspended in corn oil at a dose of 10 000 mg/kg body weight (bw). There were no deaths and no symptoms were observed except for a "transient depression of the rats" for a few hours after dosing.

The Committee noted that this study was performed prior to the introduction of good laboratory practice (GLP) regulations and the report was very limited.

2.2.2 Short-term toxicity

Male Sprague Dawley rats (200–250 g; non-fasted) were administered gum ghatti suspended in corn oil at a daily dose of 5000 mg/kg bw for 5 days. No unusual or adverse effects were observed (Newell & Maxwell, 1972).

The Committee noted that this study was performed prior to GLP and the report was very limited.

Two 90-day rat studies were performed in compliance with GLP and quality assurance (QA) and in accordance with Organization for Economic Co-operation and Development (OECD) Test Guideline 408. The test substance, Gatti Gum SD (previously called Gatifolia SD), a grey to reddish-grey spray-dried powder, was mixed with basal diet AIN-93M. Because the test substance was found to be stable in the prepared feed for only 58 days, two batches of feed containing the test substance were prepared for both studies. Five lots of the test substance were prepared, analysed and certified in accordance with specifications and standards for food additives in Japan. Six sugar components in each lot were characterized; the total sugar composition was 84.2–86.5%.

In the first study, Davis & Lea (2011; also described in Maronpot et al., 2013) administered gum ghatti in a purified basal diet, AIN-93M, to Sprague Dawley rats (n = 10/sex per group) at concentrations of 0 (control), 0.5%, 1.5% and 5% (equal to 0, 337, 1018 and 3044 for males and 0, 396, 1149 and 3308 mg/kg bw per day for females, respectively). The dose levels were selected from a previous 14-day dose range–finding study (Davis & Lea, 2014).

No clinical signs of toxicity were observed in any of the animals and all animals survived to study end. After 90 days of treatment, the animals were killed and all major tissues were fixed for analysis.

There were no differences in mean feed consumption and body weight gains between the treatment and control groups. No significant differences were

found in ophthalmologic and neurological observations, urine analyses or blood clotting times.

In haematological analyses, there was a statistically significant increase in monocyte concentration in each of the treated groups of female rats compared with controls. The increases were above historical control values but were not dose-related, being of similar magnitude in each treated group. In contrast, a statistically significant, dose-dependent decrease in monocyte concentration was observed in male rats. A decrease in albumin level was observed in male rats in the 0.5% and 5% dietary dose groups. Although statistically significant, this decrease was slight and not dose-dependent.

An increase in mean ovary weight was observed at the 0.5% dose level (relative mean weight) and at 5% (absolute and relative mean weights). A decrease in absolute brain weight in male rats was observed at the 5% dose level. The Committee considered these effects not treatment related because they were not dose related, not observed in both sexes and/or not accompanied by any histopathological correlates.

In both males and females, exposure to 5% gum ghatti in the diet was significantly associated with increased empty and full caecal weights. This increase was apparent both in absolute and relative tissues weights. In addition, a significant association with increased empty caecal weights was observed in females at 0.5%. Minimal to moderate histopathological changes were observed in the caecum of high-dose males; 6/10 displayed minimal to mild caecal mucosal hyperplasia and/or minimal to mild crypt elongation. No lesions were found in female rat caeca. In 2/10 female rats exposed to 5% gum ghatti in the diet, moderate inflammation of the colon with loss of mucosa, haemorrhage and necrosis was diagnosed as ulcerative colitis. No significant lesions were observed in the colon of the male rats.

Because of the lesions observed in the colon of female rats, fixed colon wet tissues from all male and female rats were thoroughly re-examined in a peer review to see if any additional lesions could be identified. Moderate lymphoid hyperplasia was observed in one male control and in one male at 5% gum ghatti in the diet. In addition, one female control rat displayed minimal congestion of the mucosa. In the three remaining high-dose rats, no significant lesions were found in the colon. No lesions other than those identified in the initial histopathology evaluation were found. Microscopic evaluation of tissues in all other investigated organs found no significant lesions in the high-dose (5%) animals.

The effects on caecal weights seen in both sexes at 5% gum ghatti in the diet have been reported in other toxicity studies of poorly digestible polysaccharides and gum products (Tulung, Rémésy & Demigné, 1987; Wyatt et al., 1988; Levrat et al., 1991; Doi et al., 2006; Ali, Ziada & Blunden, 2009; Hagiwara et al., 2010). The increase in caecal weights is considered to be the result of absent or incomplete

absorption of gum in the small intestine leading to microbial fermentation of the gum in the caecum and colon (Newberne, Conner & Estes, 1988). The ulcerative colitis observed in 2/10 female rats at 5% was not seen in any other dose group or in males, and no reason could be identified for the occurrence of ulcers in females only. Other effects on monocytes, albumin, brain and ovarian weight were either sporadic and/or did not show a dose-response relationship.

Based on the results of this study a no-observed-adverse-effect level (NOAEL) of 3044 mg/kg bw per day, the highest dose tested, was identified for male rats, and of 1149 mg/kg bw per day for female rats, based on the incidence of ulcerative colitis observed at the highest dose (3308 mg/kg bw per day).

In order to evaluate the relevance of the focal colonic ulcerations, the possible role of the AIN-93M diet and the possibility that intrinsically susceptible littermates had been randomly assigned to the same group, Davis (2012; also described in Maronpot et al., 2013) conducted a study in female Sprague Dawley rats (*n* = 20/group) to compare two different basal diets (AIN-93M and NIH-07), with a focus on histopathology of colon. The female rats were fed diets containing 0 (control) or 5% gum ghatti, equal to 0 or 3671 mg/kg bw per day for rats given the AIN-93M diet and 3825 mg/kg bw per day for rats given the NIH-07 diet.

The study parameters were essentially the same as in the Davis & Lea (2011) study (described above) except that neurological assessments and urine analyses were not carried out and histopathological assessment was restricted to caecum and colon. To circumvent the possibility that intrinsically susceptible littermates could have been randomly assigned to the same group, rats ordered for the second study were specifically requested to be non-littermates.

No clinical signs of toxicity were observed in any of the animals and all animals survived the 90-day treatment. There were no associations between exposure to gum ghatti and feed consumption, body weight gain, tissue weights or ophthalmological parameters.

Haematological analysis found that prothrombin time was significantly decreased in rats administered 5% gum ghatti in the AIN-93M diet, while activated partial thromboplastin time and red blood cell distribution width were significantly increased compared to control rats fed the AIN-93M diet. In contrast, no differences in haematological end-points were observed in the female rats fed the NIH-07 diet. In the initial 90-day study, the only change noted in females exposed to 5% gum ghatti in the AIN-93M diet was an increase in monocytes.

In clinical chemistry analyses, chloride and alkaline phosphatase levels increased significantly and potassium, albumin and phosphorus levels decreased significantly in treated females fed the AIN-93M diet compared with the controls.

In treated females fed the NIH-07 diet, globulin levels were significantly decreased and calcium levels increased. The clinical chemistry changes were not consistent across the groups in the two studies, however (AIN-93M diet versus NIH-07 diet in this, the Davis, 2012, study versus AIN-93M in the initial, Davis & Lea, 2011, study). This indicates that the changes may not be related to gum ghatti exposure.

Pituitary gland weight was significantly increased in treated animals fed the AIN-93M diet compared with controls; however, no change in pituitary gland weight was observed in treated rats fed the NIH-07 diet or in the initial Davis & Lea, 2011, study.

As in the first study, empty caecal weights (absolute and relative) were increased in treated animals in both AIN-93M and NIH-07 diets compared with their respective controls. Full caecal weights (absolute and relative) were also increased in treated rats fed the AIN-93M diet compared with rats fed the carrier diet alone, but not in treated rats fed the NIH-07 diet. Histopathological changes were not apparent in the caecum of animals from any dose group.

A focal colonic ulcer associated with an acute inflammation was found in one of the control rats. The histology of this ulcer was similar to those observed in the first study. A common observation in all groups in the second study was focal lymphoid hyperplasia of the colon; it was found in 5/20 rats fed the AIN-93M diet alone, 5/20 rats fed the AIN-93M diet with 5% gum ghatti, 2/20 rats fed the NIH-07 diet alone and in 3/20 rats fed the NIH-07 diet with 5% gum ghatti. The severity of this lesion ranged from minimal to marked, with no apparent association with gum ghatti exposure.

A panel of experts in a post-study pathology working group examined the colon lesions and 50% of the colon and caecum sections from both 90-day studies. The expert group reached the conclusion that the ulceration of the colon was a sporadic event that was not associated with exposure to gum ghatti and that the caecal changes diagnosed in 6/10 male rats in the first study were "normal variations". The caecal changes could not be confirmed as being caecal crypt hyperplasia and/or crypt elongation; rather, they may have been affected by variable handling of the tissues while fixing, trimming, embedding and preparing of tissue sections. Moreover, the pathology expert group did not find the increase in caecal weight toxicologically relevant.

The Committee considered the increase in caecal weight at the 5% dose of gum ghatti in both 90-day studies to be an adaptive effect in the rat that has no relevance to humans. Based on the results of these two studies, a NOAEL of 3044 mg/kg bw per day, the highest dose tested in male rats, was identified (equal to 2590 mg/kg bw per day after correcting for purity).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.2.4 Genotoxicity

New information has become available since the last evaluations by the Committee at the twenty-sixth and twenty-ninth meeting. The results of these newly available genotoxicity studies as well as the results of older studies are summarized in Table 1.

No evidence for genotoxic potential of gum ghatti was found in several in vitro or in vivo studies at concentrations or dose levels that met or exceeded the levels suggested in OECD guidelines.

It should be noted that the older genotoxicity studies of gum ghatti (Newell & Maxwell, 1972; Mortelmans, 1981; Prival, Simmon & Mortelmans, 1991) were conducted prior to OECD guidelines and were generally not certified for compliance with GLP and QA, whereas the newer genotoxicity studies (Swarts, 2010a,b; Davis & Hobbs, 2011; also reported in Hobbs et al., 2012) were all performed according to OECD guidelines and were certified for compliance with GLP and QA.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

No information was available.

(b) Developmental toxicity

No new information has become available since the last evaluation. The results of the previously submitted studies are summarized below as no toxicological monographs were prepared following the Committee's previous evaluations of gum ghatti.

Food and Drug Research Laboratories Inc. (1972a) investigated the developmental toxicity effects of gum ghatti on four animal species: mice, rats, hamsters and rabbits. In each study, gum ghatti (described as "fine tan powdered material"; purity not specified) was administered daily to the female animals by oral intubation gavage using anhydrous corn oil as vehicle, at a volume of 1.0 mL/kg bw. The day of mating was considered to be gestation day 0. Gum ghatti was administered daily during major organogenesis, defined as the period between implantation and closure of the hard palate. In each study, the controls were described as "sham treated"; the report does not specify whether this means the controls received the vehicle.

Groups of pregnant albino CD-1 mice (*n* = 20–21/group) were administered 0, 17, 80, 370 or 1700 mg/kg bw per day of gum ghatti (purity not

Table 1
Genotoxicity of gum ghatti in vitro and in vivo

End-point	Test system	Route of administration	Concentration	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538, *Escherichia coli* WP2uvrA	–	0.3–100 µg/plate, ±S9	Negative[a]	Mortelmans (1981); Prival, Simmon & Mortelmans (1991)
Forward and reverse mutations	Host-mediated assay, related in vitro tests (mouse) *S. typhimurium* G-46, TA1530	–	5% w/v	Negative	Newell & Maxwell (1972)b
Recombination frequency	Host-mediated assay, related in vitro tests (mouse) *Saccharomyces cerevisiae* D-3	–	1% w/v	Negative	Newell & Maxwell (1972)[b]
Adverse effects on metaphase chromosomes	Cytogenetic assay WI-38 cells (human embryonic lung)	–	0.16–16 µg/mL	Negative	Newell & Maxwell (1972)[b]
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA97a, E. coli WP2uvrA, pKM101	–	125–6 000 µg/plate, ±S9	Negative[c]	Swarts (2010a); Hobbs et al. (2012)
Chromosomal aberration	Chinese hamster ovary cells (CHO)	–	1 000–6 000 µg/mL, ±S9	Negative[c,d]	Swarts (2010b); Hobbs et al. (2012)
In vivo					
Chromosomal aberration in germ cells	Dominant lethal test Rat	Oral	30, 2 500, 5 000 mg/kg bw	Negative	Newell & Maxwell (1972)[b]
Adverse effects to metaphase chromosomes	Cytogenetic assay (bone marrow) Rat	Oral	30, 2 500, 5 000 mg/kg bw	Negative	Newell & Maxwell (1972)[b]
DNA damage Micronucleus induction	Comet/micronucleus combination assay B6C3f1 Mouse; male	Oral	1 000, 1 500, 2 000 mg/kg bw	Negative	Davis & Hobbs (2011); Hobbs et al. (2012)

bw: body weight; S9: 9000 × *g* supernatant fraction from rat liver homogenate; w/v: weight per volume
[a] Plate incorporation method.
[b] Methodologies used to conduct the tests in Newell & Maxwell (1972) are described in the reference (IACM, 1972).
[c] Preincubation method used.
[d] In the in vitro chromosomal aberration study in Chinese hamster ovary cells (CHO), a statistically significant increase in the polyploidy index after 4 hours of exposure was reported, but only at a single dose (1000 mg/mL) with metabolic activation. In addition, a weak but significant increasing trend in polyploidy index was found after continuous exposure for 19 hours, but this was not accompanied by any significant increases in any single treatment culture, relative to vehicle controls. These results were considered by the study authors to be incidental findings and not an indication of a true positive response. The results did not demonstrate any decrease in viable cell counts or mitotic index, or any increase in chromosome aberrations (as measured by the percentage of damaged cells in each culture) for any of the treatment cultures compared to vehicle controls (Swarts, 2010b; also reported in Hobbs et al., 2012).

stated) on gestation days 6–15. Aspirin at 150 mg/kg bw per day was given to the positive controls. Body weights were recorded on gestation days 0, 6, 11, 15 and 17. All animals were observed daily for appearance and behaviour with particular attention paid to feed consumption in order to better recognize any abnormalities resulting from anorexic effects in the pregnant animal. On gestation day 17, all mice underwent caesarean section under anaesthesia. The numbers of

implantation sites, resorption sites and live and dead fetuses and body weights of the live pups were recorded. The urogenital tract of each dam was examined in detail for anatomical normality. All fetuses underwent gross examination for external congenital abnormalities; one third of the fetuses from each litter were examined for visceral abnormalities, and the remaining two thirds were examined for skeletal defects.

There were no treatment-related adverse effects in the dams; on the numbers of implantations, resorptions or live or dead fetuses; or on the frequency of external, soft tissue or skeletal tissue abnormalities in mice treated with gum ghatti at up to the highest dose (1700 mg/kg bw per day) relative to sham-treated controls (Food and Drug Research Laboratories Inc., 1972a).

The Committee noted that no soft tissue abnormalities were recorded for the positive controls, although the dose of aspirin may have been too low to induce abnormalities.

In the same study, groups of pregnant albino Wistar-derived rats (n = 20–24/group) were administered 0, 17, 80, 370 or 1700 mg/kg bw per day of gum ghatti (purity not stated) on gestation days 6–15. Aspirin at 250 mg/kg bw per day was given to the positive controls. Body weights were recorded on gestation days 0, 6, 11, 15 and 17. All animals were observed daily for appearance and behaviour with particular attention paid to feed consumption in order to better recognize any abnormalities resulting from anorexic effects in the pregnant animal. On gestation day 20, all the rats underwent caesarean section under anaesthesia. The numbers of implantation sites, resorption sites and live and dead fetuses and the body weights of the live pups were recorded. The urogenital tract of each dam was examined in detail for anatomical abnormalities. All fetuses underwent gross examination for external congenital abnormalities. One third of the fetuses from each litter were examined for visceral abnormalities, and the remaining two thirds were examined for skeletal defects.

Between gestation days 9 and 19, there were four, zero, one, one and five maternal deaths at 0, 17, 80, 370 and 1700 mg/kg bw per day, respectively. In the 48–72 hours prior to death (between gestation days 9 and 19), severe diarrhoea and urinary incontinence with anorexia were observed in the dams that died. Pathological investigations of the viscera revealed a marked petechial haemorrhage in the mucosa of the small intestine, but no other findings. According to the authors, there were no adverse effects on the dams at the lower doses of 17, 80 and 370 mg/kg bw per day.

The number and type of abnormalities in fetal soft and skeletal tissues in the high-dose group did not differ from the number found to occur spontaneously in the sham-treated controls. There were no treatment-related adverse effects on numbers of implantations, resorptions or live or dead fetuses or seen in gross,

soft tissue or skeletal tissue examinations relative to sham-treated controls at any dose, including in surviving rats at the highest dose (Food and Drug Research Laboratories Inc., 1972a).

The Committee noted that the positive control group showed an increased number of resorptions, a reduced number of live fetuses, reduced fetal weights and increases in soft tissue and skeletal abnormalities.

Groups of golden hamsters (*n* = 20/group) were administered 0, 17, 80, 370 or 1700 mg/kg bw per day of gum ghatti (purity not stated) on gestation days 6–10. Aspirin at 250 mg/kg bw per day was given to the positive controls. Body weights were recorded on gestation days 0, 8, 10 and 14. All animals were observed daily for appearance and behaviour with particular attention paid to feed consumption in order to better recognize any abnormalities resulting from anorexic effects in the pregnant animal. On gestation day 14, all the hamsters underwent caesarean section under anaesthesia. The numbers of corpora lutea, implantation sites, resorption sites and live and dead fetuses and body weights of the live pups were recorded. The urogenital tract of each dam was examined in detail for anatomical abnormalites. All fetuses underwent gross examination for external congenital abnormalities. One third of the fetuses from each litter were examined for visceral abnormalities, and the remaining two thirds were examined for skeletal defects.

There were, according to the authors, no treatment-related adverse effects on the dams, on the number of implantations, resorptions or live or dead fetuses or on gross, soft tissue or skeletal abnormalities in hamsters treated with gum ghatti up to 1700 mg/kg bw per day relative to sham-treated controls (Food and Drug Research Laboratories Inc., 1972a). The Committee noted that soft tissue abnormalities were reported in one hamster treated at 370 and one at 1700 mg/kg bw per day gum ghatti and in one positive control.

Groups of pregnant Dutch Belted rabbits (*n* = 15/group) were administered 0, 7, 33, 150 or 700 mg/kg bw per day of gum ghatti (purity not stated) on gestation days 6–18. In addition, 6-amino nicotinamide at 2.5 mg/kg bw (administered once, on gestation day 9) was given to the positive controls. Body weights were recorded on gestation days 0, 6, 12, 18 and 29. The appearance and behaviour of all the animals were observed daily with particular attention to feed consumption in order to better recognize any abnormalities resulting from anorexic effects in the pregnant animal. On gestation day 29, all the pregnant rabbits (*n* = 12) underwent caesarean section under anaesthesia and the numbers of corpora lutea, implantation sites, resorption sites and live and dead fetuses and body weights of the live pups were recorded. The urogenital tract of each doe was examined in detail for anatomical abnormalities and all the fetuses were examined

for external congenital abnormalities. The live fetuses in each litter were placed in an incubator for 24 hours to assess neonatal survival, after which the surviving pups were killed and examined for visceral abnormalities (by dissection). The fetuses were then cleared of cellular matter in potassium hydroxide, stained with Alizarin Red S dye and examined for skeletal defects.

At the highest dose (700 mg/kg bw per day), following severe diarrhoea and urinary incontinence with anorexia, 10/12 pregnant rabbits died between gestation day 10 and 18, and 10 aborted. There were no gross pathology findings in the few surviving does except for haemorrhagic spots in the small intestine. At the second highest dose (150 mg/kg bw per day), 5/14 pregnant does died between gestation day 12 and 20, and five aborted. At mid dose (33 mg/kg bw per day), 3/14 pregnant does died between gestation day 16 and 26, and four aborted. At the lowest dose (7 mg/kg bw per day), no deaths or abortions were observed. It should be noted that 3/13 sham-treated does died between gestation day 13 and 14, and three aborted. All the dams that died aborted prior to death.

At 33, 150 and 700 mg/kg bw per day, 21%, 36% and 83%, respectively, of the pregnant rabbits died. In addition, 23% of the sham-treated does died.

According to the authors, up to 33 mg/kg bw per day of the test material had no discernible effect on nidation or on maternal and fetal survival. However, at 150 and 700 mg/kg bw per day, significant maternal toxicity ensued and a majority of the animals died. It was concluded that the test substance was not a teratogen in the rabbit (Food and Drug Research Laboratories Inc., 1972a).

The Committee noted that only two high-dose pregnant does survived to term and that three sham-treated pregnant does died. An increase in both soft tissue and skeletal abnormalities was observed in the positive control group.

2.3 Observations in humans

No information was available.

3. Dietary exposure

3.1 Exposure assessment

The Committee evaluated dietary exposure information from a submission provided by a sponsor and prepared estimates of dietary exposure from potential use scenarios.

3.1.1 Budget method

The Committee concluded that no screening by the budget method would be appropriate for this assessment.

3.1.2 Production volume data

The sponsor's submission to the Committee stated that the global production of gum ghatti was estimated to be 1 000 000–1 500 000 kg. As gum ghatti is used in several industry applications other than as a food additive, only a small fraction of the gum ghatti currently produced is expected be used in food (Ido et al., 2008). A personal communication included with the submission stated that internal industry estimates suggest that current global production volumes for food use is 50 000–60 000 kg.

With a conservative estimate of global production volume for food use of 100 000 kg (taking into account incomplete reporting), and the consumer "eaters-only" population is assumed to be 10% of the total population in countries where gum ghatti is known to be currently approved (as noted above), then the dietary exposure can be estimated as follows:

[100 000 kg*1 × 10^9 µg/kg]/[(395 × 10^6 persons)*365 days] = 694 µg/person per day,

or, for a 60 kg individual:

[694 µg/person per day]/[60 kg bw per person] = 11.6 µg/kg bw per day.

The Committee noted that this very low estimate of dietary exposure reflects the current low usage of gum ghatti in food. It would be unrealistic as a measure of long-term consumption by consumers of food products containing gum ghatti.

3.2 International estimates of dietary exposure

As with other gums with similar technical effects, gum ghatti is used only in processed food products. Therefore, the Committee concluded that the use of commodity-based food consumption information from the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) would not be appropriate for this evaluation.

3.3 National estimates of dietary exposure

No national estimates of exposure were submitted to the Committee and no reports were found in the literature.

The submission reviewed by the Committee noted that gum ghatti is used in a number of countries. The only reported use levels were from the USA, however, and the Committee was unable to find any information on usual use in the other countries. In the USA, gum ghatti is generally recognized as safe for use in foods at the maximum usage levels (see Table 2).

Gum Ghatti is also used as a flavouring ingredient in the USA, although at significantly lower use levels (Burdock, 2009).

3.4 Exposure scenario based on submitted use levels

The submission to the Committee contained two reports outlining use levels for gum ghatti in a number of General Standard for Food Additives (GSFA) food categories (Tables 3 and 4). The Committee prepared an estimate of exposure based on these levels.

Table 3 contains use levels for foods in GSFA categories 1.1.4 "Flavoured fluid milk drinks" and categories 14.1 and 14.2 (various beverage categories). The maximum use level for milk beverages was 150 mg/L; for nonalcoholic beverages, 100 mg/L; and for alcoholic beverages, 300 mg/L. Using food consumption data from the USA, the Committee completed a scenario assessment of dietary exposure by assuming 250 g/day consumption of milk beverages (95th percentile); 900 g/day of nonalcoholic beverages (95th percentile); and 750 g/day of alcoholic beverages (95th percentile). The Committee estimated dietary exposure to gum ghatti would be 350 mg/day or 6 mg/kg bw per day for a 60 kg individual[1].

Table 4 contains a more extensive list of foods potentially containing gum ghatti. Additional food categories include dairy- and fruit-based desserts, candies, pastas and prepared noodles, bakery wares, seasonings and spices, comminuted meat products and food for special dietary uses and supplements. The Committee concluded that only consumption of pre-cooked pastas and noodles and similar products containing gum ghatti at the proposed use level of 6000 mg/kg would result in a dietary exposure different from that in the scenario discussed above. The exposure to gum ghatti from consumption of 60 g of prepared noodles[1] containing the maximum level would be approximately 360 mg/day (6 mg/kg bw per day for a 60 kg individual). Addition of this exposure

[1] Food consumption data taken from the reported 95th percentile levels in the USA from the NPD Group's National Eating Trends survey (proprietary data from the United States Food and Drug Administration provided to the Committee).

Table 2
Maximum usage levels generally recognized as safe for gum ghatti in food in the USA

Food (as served)	% in final food product	Function
Beverages and beverage bases, nonalcoholic	0.2	Emulsifier and emulsifier salt
All other food categories	0.1	Emulsifier and emulsifier salt

Table 3
Use levels of gum ghatti in foods in GSFA categories 1.1.4 "Flavoured fluid milk drinks" and categories 14.1 and 14.2 (various beverage categories)

GSFA Food category		Concentration in final food product (mg/L)	
		Mean	Max.
1.1.4	Flavoured fluid milk drinks	5.0	150.0
14.1	Non-alcoholic ("soft") beverages	5.0	100.0
14.1.2	Fruit and vegetable juices	5.0	100.0
14.1.4	Water-based flavoured drinks, including "sport," "energy" or "electrolyte" drinks and particulated drinks	5.0	100.0
14.1.4.1	Carbonated water-based flavoured drinks	5.0	100.0
14.1.4.3	Concentrates (liquid or solid) for water-based flavoured drinks	5.0	100.0
14.2	Alcoholic beverages, including alcohol-free and low-alcoholic counterparts	10.0	300.0
14.2.1	Beer and malt beverages	10.0	300.0
14.2.6	Distilled spirituous beverages containing more than 15% alcohol	10.0	300.0

GSFA: General Standard for Food Additives; max.: maximum

Table 4
Use levels of gum ghatti in foods potentially containing gum ghatti per GSFA Food category

GSFA Food category no.	GSFA Food category description	Max. use level (mg/kg final food product)
01.0	Dairy products and analogues, excluding products of food category 02.0	
01.1.2	Dairy-based drinks, flavoured and/or fermented (e.g. chocolate milk, cocoa, eggnog, drinking yoghurt, whey-based drinks)	150
1.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	36
04.0	Fruits and vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds	
04.1.2.9	Fruit-based desserts, including fruit-flavoured water-based desserts	80
05.0	Confectionery	
05.2.1	Hard candy	800
05.2.2	Soft candy	80
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	

Table 4 (continued)

GSFA Food category no.	GSFA Food category description	Max. use level (mg/kg final food product)
06.4.3	Pre-cooked pastas and noodles and like products	6 000
07.0	Bakery wares	
7.2	Fine bakery wares (sweet, salty, savoury) and mixes	40
08.0	Meat and meat products, including poultry and game	
08.3.2	Heat-treated processed comminuted meat, poultry and game products	42
12 .0	Salts, spices, soups, sauces, salads and protein products	
12.2.1	Herbs and spices	600
12.2.2	Seasonings and condiments	350
13 .0	Foodstuffs intended for particular nutritional uses	
13.3	Dietetic foods intended for special medical purposes (excluding products of food category 13.1)	10 000
13.5	Dietetic foods (e.g. supplementary foods for dietary use) excluding products of food categories 13.1–13.4 and 13.6	120
13.6	Food supplements	77 660
14 .0	Beverages, excluding dairy products	
14.1	Non-alcoholic ("soft") beverages	100
14.1.2	Fruit and vegetable juices	100
14.1.3	Fruit and vegetable nectars	
14.1.4	Water-based flavoured drinks, including "sport", "energy" or "electrolyte" drinks and particulated drinks	100
14.1.4.1	Carbonated water-based flavoured drinks	48
14.1.4.2	Non-carbonated water-based flavoured drinks, including punches and ades	136
14.1.4.3	Concentrates (liquid or solid) for water-based flavoured drinks	240
14.1.5	Coffee, coffee substitutes, tea, herbal infusions and other hot cereal and grain beverages, excluding cocoa	20
14.2	Alcoholic beverages, including alcohol-free and low-alcoholic counterparts	300
14.2.1	Beer and malt beverages	300
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)	32

GSFA: General Standard for Food Additives; max.: maximum; no.: number

to that from the uses modelled above results in a conservative dietary exposure estimate of 12 mg/kg bw per day.

The Committee used the European Food Safety Authority (EFSA) Food Additive Intake Model (Version 1.0) with the use levels from Table 3 to estimate dietary exposure to gum ghatti. The results are shown in Table 5. The estimated exposure for adults was 6 mg/kg bw per day.

Finally, the Committee considered, in light of the scant information available on the current uses of gum ghatti, a scenario in which gum ghatti would

Table 5
Dietary exposure estimates using the EFSA Food Additive Intake Model (Version 1.0) and use levels from Table 3

	Range for mean across dietary surveys (mg/kg bw per day)		Range for high level across dietary surveys (mg/kg bw per day)	
Population[a]	**Min.**	**Max.**	**Min.**	**Max.**
Toddlers	0.216	3.033	1.263	25.805
Children	0.193	3.410	0.490	15.240
Adolescents	0.168	0.648	0.339	9.518
Adults	0.175	0.849	0.567	6.011
Elderly adults	0.145	0.476	0.287	6.187

bw: body weight; EFSA: European Food Safety Authority; max.: maximum; min.: minimum
[a] European country population group descriptors have the following definitions: infants, <12 months; toddlers, 12–35 months; other children, 3–9 years; adolescents, 10–17 years; adults, 18–64 years; elderly adults, 65–74 years; very elderly adults, ≥75 years.

substitute directly for other food gums in processed foods. At the seventy-first JECFA meeting, the dietary exposure to OSA-modified gum arabic was estimated at "less than 20 mg/kg bw per day"; this was reaffirmed at the eighty-second meeting.

3.5 Conclusion

The Committee considered that a dietary exposure of 12 mg/kg bw per day was suitable for use in a safety assessment of gum ghatti.

4. Comments

4.1 Biochemical aspects

Absorption, distribution, metabolism and excretion data on gum ghatti were not available. However, similar to other gums and dietary fibres, gum ghatti is unlikely to be significantly digested or absorbed in the stomach or small intestine. Based on its chemical composition, gum ghatti is expected to be enzymatically degraded and fermented by the microflora in the large intestine to hydrogen gas, carbon dioxide and short-chain fatty acids, which can be absorbed and metabolized (Ali, Ziada & Blunden, 2009).

4.2 Toxicological studies

In an acute toxicity study in male rats (Newell & Maxwell, 1972), no deaths were reported at 10 000 mg/kg bw, the highest dose tested.

Two new 90-day studies of the toxicity of gum ghatti (purity 85%) have been performed in rats.

In the first study (Davis & Lea, 2011), rats were fed a basal diet (AIN-93M) containing 0%, 0.5%, 1.5% or 5% gum ghatti (equal to 0, 337, 1018 and 3044 mg/kg bw for males and 0, 396, 1149 and 3308 mg/kg bw per day for females, respectively). Although haematological and clinical chemistry effects were observed, they were not dose related, not found in both sexes and/or not correlated with any histopathological findings.

Increased caecal weights were observed in the male and female rats at 5% gum ghatti. In addition, in 6 out of 10 high-dose males, minimal to mild mucosal hyperplasia and/or minimal to mild crypt elongation were observed in the caecum, whereas no lesions were found in the caecum of female rats. In 2/10 high-dose females, ulcerative colitis was observed in the colon; no significant lesions were observed in the colon of male rats.

In order to evaluate the relevance of the ulcerative colitis, the possible role of the AIN-93M diet and the possibility that intrinsically susceptible littermates had been randomly assigned to the same group, a second study tested two different basal diets (AIN-93M and NIH-07) containing 0 or 5% gum ghatti (equal to 0 and 3671 mg/kg bw per day for rats fed the AIN-93M diet and 0 and 3825 mg/kg bw per day for rats fed the NIH-07 diet) (Davis, 2012). The study deviated from OECD Test Guideline 408, as only one dose was tested in only one sex (female) and the histopathological examination did not include the full range of recommended organs, because the aim of this second study was to follow up on the observations reported in female rats at the highest dose in the first study.

Increased empty caecal weights were observed in animals exposed to gum ghatti in both diets. Full caecal weights (absolute and relative) were also increased in rats exposed to gum ghatti in the AIN-93M diet but not the NIH-07 diet. Focal lymphoid hyperplasia of the colon was observed in all study groups, but there was no association with the dietary exposure to gum ghatti, and the authors concluded that these findings were incidental and not treatment related. A pathology working group subsequently concluded that the ulcerative colitis observed in the colon of the female rats was a sporadic event not associated with the dietary exposure to gum ghatti and that the caecal changes in male rats could not be confirmed as caecal crypt hyperplasia/crypt elongation (Maronpot et al., 2013).

The Committee noted that the effects on caecal weights observed in both sexes at 5% gum ghatti have also been reported in other toxicity studies of poorly

digestible polysaccharides and gum products (Tulung, Rémésy & Demigné, 1987; Wyatt et al., 1988; Levrat et al., 1991; Doi et al., 2006; Ali, Ziada & Blunden, 2009; Hagiwara et al., 2010). The increase in caecal weight is considered to be the result of microbial fermentation of undigested and unabsorbed gum in the lower large intestine (Newberne, Conner & Estes, 1988). The Committee considered this increase in caecal weight at 5% dietary gum ghatti to be an adaptive, rather than adverse, response. Based on the results of the new 90-day studies (Davis & Lea, 2011; Davis, 2012), the Committee identified a NOAEL of 3044 mg/kg bw per day (equal to 2590 mg/kg bw per day, corrected for purity), the highest dose tested.

No long-term studies of the toxicity or carcinogenicity of gum ghatti were available.

In vitro and in vivo genotoxicity studies have recently been conducted. These, together with earlier in vitro and in vivo studies, showed no evidence for a genotoxic potential of gum ghatti. The Committee concluded that there were no genotoxicity concerns for gum ghatti.

No reproductive toxicity studies were available for gum ghatti.

Studies on developmental toxicity of gum ghatti administered by oral gavage were performed in mice, rats, hamsters and rabbits (Food and Drug Research Laboratories, Inc., 1972a). In mice and hamsters dosed at 0, 17, 80, 370 or 1700 mg/kg bw per day, there were no treatment-related adverse effects on the dams. There were also no treatment-related adverse effects on the numbers of implantations, resorptions or live and dead fetuses or on the frequency of external, soft tissue or skeletal abnormalities.

In the rat, there were four, zero, one, one and five maternal deaths at 0, 17, 80, 370 and 1700 mg/kg bw per day, respectively. Severe diarrhoea and urinary incontinence with anorexia were observed in the 2–3 days prior to death. Petechial haemorrhage was observed in the mucosa of the small intestine of the dams that died. There were no treatment-related adverse embryo-fetal effects at any dose, including in those rats that survived at the highest dose tested.

In the rabbit study, there were 15 animals per dose group. There were 3, 0, 3, 5 and 10 maternal deaths in the 0, 7, 33, 150 and 700 mg/kg bw per day dose groups, respectively. As with rats, severe diarrhoea and urinary incontinence with anorexia were observed in the 2–3 days prior to death. In addition, all animals aborted prior to death. Petechial haemorrhage was observed in the mucosa of the small intestine of the does that died. There were no treatment-related adverse embryo-fetal effects at any dose, including in the two pregnant rabbits that survived at the highest dose tested.

These developmental toxicity studies were performed prior to OECD guidelines or GLP standards and do not comply with several modern standards/guidelines: the purity of the substance was not stated; the treatment period covered the major phase of organogenesis but did not extend to the end of gestation; and

the rationale for dose selection in all four studies was not presented. In addition, none of the study reports presented the clinical observations, feed consumption, gravid uteri weights or statistical analyses of the results. The Committee also noted that there were maternal deaths at high doses in mice, rats and, in particular, rabbits in developmental toxicity studies on other gums conducted by the same laboratory at about the same time, in which the test substance was also administered by oral gavage. The Committee considered that this may have been due to the difficulty of administering high concentrations of viscous substances by gavage. They further noted that no treatment-related adverse maternal or developmental effects were reported in surviving high-dose animals in studies on gum ghatti and other gums. Despite the deficiencies in the study methods and reporting and the occurrence of maternal deaths, there were no effects on embryo-fetal growth or development at doses up to 1700 mg/kg bw per day.

In view of the gaps in the database for gum ghatti (i.e. the absence of any long-term toxicity or carcinogenicity studies, the limitations of the developmental toxicity studies and the lack of any reproductive studies), the Committee considered data on structurally related gums. The gum most closely related to gum ghatti is gum arabic (also known as gum acacia); the two gums have similar monosaccharide profiles with respect to L-arabinose, L-rhamnose, D-galactose and D-glucuronic acid (Pitthard & Finch, 2001; Akiyama, Yamazaki & Tanamoto, 2011).

Developmental toxicity studies on other gums in mice, rats, hamsters and rabbits (Food and Drug Research Laboratories Inc., 1972b) were conducted by the same laboratory that conducted the developmental toxicity studies on gum ghatti. As such, they may have had similar limitations. A more recent combined fertility and developmental toxicity study of gum arabic in rats (Collins et al., 1987) was previously evaluated by the Committee, which considered that this study did not give cause for concern about the safety of gum arabic (Annex 1, reference *89*). EFSA (2017) also described more recent fertility studies in rats (Morseth & Ihara, 1989; Huynh et al., 2000) and considered that these studies did not give cause for concern about the safety of gum arabic. Based on the combined fertility and developmental toxicity study in rats (Collins et al., 1987), an overall NOAEL of 10 647 mg/kg bw per day (the highest dose tested) was identified for reproductive, developmental and parental effects. The Committee noted that reproductive and developmental toxicity studies on other gums (carob bean gum [FAS 16], cassia gum [FAS 62], gellan gum [FAS 28], guar gum [FAS 8], karaya gum [FAS 24], tara gum [FAS 21], tragacanth [FAS 20], xanthan gum [FAS 21]) also previously evaluated by the Committee (Annex 1, references *39*, *57*, *72*, *74*, *84*, *95* and *197*) did not raise any health concerns for reproductive or developmental effects.

Overall, the Committee concluded that there were no health concerns for gum ghatti regarding reproductive or developmental effects.

Previously evaluated carcinogenicity studies of gum arabic in mice and rats conducted by the United States National Toxicology Program (National Toxicology Program, 1982) found no indications of any treatment-related increases in tumour incidence at dietary gum arabic concentrations of 2.5% and 5.0% (equivalent to 1250 and 2500 mg/kg bw per day for rats and 3750 and 7500 mg/kg bw per day for mice). The Committee noted that other previously evaluated chronic toxicity/carcinogenicity studies in mice and rats (carob bean gum, gellan gum, guar gum, tara gum, xanthan gum) also raised no health concerns regarding carcinogenic potential.

4.3 Observations in humans

No observations of gum ghatti in humans were available. However, three human studies on gum arabic found that daily ingestion by adults of up to 30 g (equivalent to 500 mg/kg bw per day for a 60 kg individual) over 18–21 days was well tolerated (Ross et al., 1983; Sharma, 1985; Cherbut et al., 2003). Furthermore, Ross et al. (1983) found that gum arabic could not be detected in the stool, indicating complete fermentation in the colon.

4.4 Assessment of dietary exposure

The Committee received one assessment of dietary exposure to gum ghatti from the sponsor and prepared estimates of dietary exposure based on model diets and potential use scenarios using food consumption data from the European Union and the USA.

The sponsor's submission noted that gum ghatti is used in a number of countries. The only use levels reported were from the USA. The Committee was unable to find information on the typical use levels in other countries. The submission to the Committee contained two reports outlining use levels for gum ghatti in a number of GSFA food categories. The Committee prepared estimates of dietary exposure based on these levels.

One report contains use levels for foods in GSFA categories 1.1.4 "Flavoured fluid milk drinks" and categories 14.1 and 14.2 (various beverage categories). The maximum use level for the milk beverages was 150 mg/L; for nonalcoholic beverages, 100 mg/L; and for alcoholic beverages, 300 mg/L. Using food consumption data from the USA, the Committee completed a scenario assessment of dietary exposure by assuming 250 g/day consumption of milk beverages (95th percentile), 900 g/day nonalcoholic beverages (95th percentile);

and 750 g/day of alcoholic beverages (95th percentile). The estimated dietary exposure to gum ghatti would be 350 mg/day or 6 mg/kg bw per day for a 60 kg individual. The use of these maximizing assumptions in the preparation of the estimate from these three broad food groups results in a highly conservative estimate of chronic dietary exposure to gum ghatti.

The second report contains a more extensive list of foods potentially containing gum ghatti. The Committee concluded that only consumption of noodles containing gum ghatti at a use level of 6000 mg/kg would result in a dietary exposure different from that in the scenario discussed above. The exposure to gum ghatti from consumption of 60 g of prepared noodles containing the maximum level would be approximately 360 mg/day (6 mg/kg bw per day for a 60 kg individual), doubling the previous scenario estimate (12 mg/kg bw per day).

The Committee also used the EFSA Food Additive Intake Model (Version 1.0) with the use levels from the sponsor's report to estimate dietary exposure to gum ghatti. The estimated exposure for adults was 6 mg/kg bw per day.

The Committee considered that a dietary exposure of 12 mg/kg bw per day was suitable for use in a safety assessment of gum ghatti.

5. Evaluation

Because limited toxicological data on gum ghatti were available, ADIs were not established at previous meetings (Annex 1, references *59* and *70*). The present Committee evaluated two new 90-day studies in rats that did not show adverse effects at doses up to 3044 mg/kg bw per day, the highest dose tested (equal to 2590 mg/kg bw per day when corrected for purity). The Committee took into account the lack of systemic exposure to gum ghatti because of its high molecular weight and polysaccharide structure, its lack of toxicity in short-term studies, the lack of concern for genotoxicity and the absence of treatment-related adverse effects in studies of gum arabic and other polysaccharide gums with a similar profile.

The Committee concluded that gum ghatti is unlikely to be a health concern and established an ADI "not specified" for gum ghatti that complies with the specifications.

Therefore, the Committee concluded that the estimated dietary exposure to gum ghatti of 12 mg/kg bw per day does not represent a health concern.

The specifications were revised based on submitted information and available literature. An high-performance liquid chromatography (HPLC) method for the identification of the gum constituents was added to replace the thin-layer

chromatography method. One identity method, using a mercury-containing reagent, was removed. L-Rhamnose was added as one of the constituents of gum ghatti, based on current literature reports.

The specifications were revised, and a Chemical and Technical Assessment was prepared.

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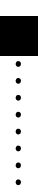

Jagua (Genipin–Glycine) Blue

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1. Explanation

Jagua (Genipin–Glycine) Blue (Chemical Abstracts Service [CAS] No. 1314879-21-4) is the product of the reaction between stoichiometric equivalents of genipin extracted from the unripe *Genipa americana* Linne (Rubiaceae) fruit and glycine, resulting in a blue-coloured genipin–glycine polymer and dimers. This report refers to the blue-coloured genipin–glycine polymer and dimer content of Jagua (Genipin–Glycine) Blue as the "blue polymer" content. *Genipa americana* fruit has traditionally been used for the preparation of juices, jellies, marmalades and liquors (Ramos-de-la-Peña et al., 2015).

Jagua (Genipin–Glycine) Blue is permitted for use as a food colour in Colombia.

Jagua (Genipin–Glycine) Blue has not been evaluated previously by the Committee. It was on the agenda at the request of the Forty-eighth Session of the Codex Committee on Food Additives (FAO/WHO, 2016).

The sponsor provided a dossier containing chemical, technical, dietary exposure and toxicological data, including unpublished in vitro studies, genotoxicity studies and in vivo toxicological studies in rats and dogs.

At the present meeting, the Committee reviewed a series of unpublished studies relevant to the human health risk assessment of Jagua (Genipin–Glycine) Blue. A comprehensive literature search of peer-reviewed literature on *Genipa americana* or the structurally related colour Gardenia Blue was undertaken in PubMed; 54 records were retrieved, but only two suitable publications on Gardenia Blue were identified. One suitable reference, a long-term toxicity study on Gardenia Blue, was identified and added to the toxicological data submitted to the Committee for this meeting. A literature search of articles published since 1900 on the occurrence of and dietary exposure to Jagua (Genipin–Glycine) Blue was conducted in Web of Science using the search terms "Jagua", "Genipa", "Jenipapo" and ""Huito" in the areas "Food science technology", "Nutrition dietetics", "Plant sciences" and "Chemistry". A total of 164 records were retrieved, but none were relevant in relation to occurrence in food or dietary exposure to Jagua (Genipin–Glycine) Blue.

The name was changed from "Jagua extract" to "Jagua (Genipin–Glycine) Blue" because the name "Jagua extract" was not adequately descriptive.

1.1 Chemical and technical considerations

Genipa americana L. is a small to medium-sized tree (UNCTAD, 2005) that belongs to the Rubiaceae family and is native to central and tropical South America (Djerassi, Gray & Kincl, 1960; Ueda, Iwahashi & Tokuda, 1991). The

plant yields edible berries referred to as jagua fruit, chipara, guayatil, maluco, caruto or huito (Ramos-de-la-Peña et al., 2015) in Spanish and as genipap in English.

The unripe jagua fruit contains high levels of a cyclopentan-[C]-pyran skeleton class of compound, called iridoids (Dinda, Debnath & Harigaya, 2007a,b). Genipin is a unique iridoid in its ability to crosslink with primary amines present in amino acids and proteins, in the presence of oxygen, to produce high molecular weight water-soluble blue pigments (Touyama et al., 1994a,b; Fujikawa et al., 1987; Paik et al., 2001; Park et al., 2002; Cho et al., 2006; Lee, Lee & Jeong, 2009).

Jagua (Genipin–Glycine) Blue is a deep blue/black colour obtained by treating peeled and ground pulp of unripe fruits of *G. americana* L. with water. The resulting juice is filtered and treated with a stoichiometric amount of glycine based on the concentration of genipin in the water extract; it is heated at 70 °C for 2 hours, until the blue colour is completely formed. The product is centrifuged, concentrated and/or dried. Unreacted genipin is considered an impurity of Jagua (Genipin–Glycine) Blue. The liquid product is obtained by concentrating the Jagua (Genipin–Glycine) Blue up to 20–50°Bx and formulating with food-grade glycerine or other permitted food additives. Alternatively, a powder is obtained, after concentrating the Jagua (Genipin–Glycine) Blue to 20°Bx, mixing with a food-grade carrier, then spray-drying and sieving.

The Jagua (Genipin–Glycine) Blue product in commerce contains a blue polymer (20–40%) and three blue dimers (approximately 1.5%) as colouring matters. The remaining components of the product are carbohydrates (>55%), protein (approximately 7%) and water (approximately 5%). The blue polymer composed of repeating dimers has the molecular formula $(C_{27}H_{25}O_8N_2)_n$ and an average molecular weight of 6000 Da. The molecular formulae of the three identified dimers are $C_{28}H_{28}O_8N_2$ (CAS No. 1313734-13-2), $C_{27}H_{25}O_8N_2$ (CAS No. 104359-67-3) and $C_{27}H_{24}O_8N_2$ (CAS No. 1313734-14-3). The blue polymer and the three dimers have been identified by nuclear magnetic resonance spectroscopy (NMR (^{1}H, ^{13}C)), infrared spectroscopy (IR), mass spectroscopy (MS) and high-performance liquid chromatography (HPLC). The Jagua (Genipin–Glycine) Blue is stable and has no decomposition products under normal storage conditions.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

In a study that was not compliant with good laboratory practices (GLP), the permeability of Jagua Blue (batch 5314034; 200 mg/mL, equivalent to 67.5 mg/mL, expressed as "blue polymer") was assessed across Caco-2 epithelial monolayers. Ranitidine (10 μmol/L) was used as a low permeability control; talinolol (10 μmol/L) as a P-gp efflux control; and warfarin (10 μmol/L) as a high permeability control for apical to basolateral transport. Lucifer Yellow, a nonpermeable dye, was used in control wells to verify that the monolayer was properly formed. Receiver side buffer was sampled and evaluated by the sponsor using ultraviolet–visible spectroscopy. Control media were analysed using liquid chromatography–tandem mass spectrometry. The apparent permeability rate coefficient for Jagua (Genipin–Glycine) Blue was lower than the rate coefficient for the low permeability control, ranitidine, suggesting poor passive absorption. However, some efflux at a single concentration was seen, suggesting that some of the blue-coloured material in the test article may be a substrate for an efflux transporter (Gilbert, 2015).

As part of a 90-day repeated-dose study in dogs (section 2.2.2(b)), Jagua (Genipin–Glycine) Blue (33.79%) chromophores were monitored in plasma using a low sensitivity spectrophotometric (590 nm) assay (analytical range 1–2.5 mg/mL). There was no detectable activity in any of the plasma samples at any time on day 1 or day 91 after daily dosing with extracts containing up to 338 mg/kg body weight (bw) of "blue polymer" (Mancari, 2016).

2.1.2 Biotransformation

No information was available.

2.2 Toxicological studies

2.2.1 Acute toxicity

A group of female Wistar Crl:WI(Han) rats ($n = 5$; 9–10 weeks) were treated with Jagua (Genipin–Glycine) Blue (33.05% active component; batch no. 5313014) by gavage at a dose of 2000 mg/kg bw (equal to 660 mg/kg bw on a "blue polymer" basis). The test item, provided to the laboratory as a dark blue solid, was suspended in sterile water at a concentration of 0.2 g/mL and administered at a dose volume of 10 mL/kg bw. The study was certified as compliant with GLP and

quality assurance (QA). It was performed in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 420.

No abnormal clinical signs were observed during the study. All five animals survived until the end of the study, and no signs of toxicity were observed. No treatment-related macroscopic findings were noted at necropsy.

The oral median lethal dose (LD_{50}) was greater than 2000 mg/kg bw (equal to 660 mg/kg bw on a "blue polymer" basis; Allingham, 2014a).

2.2.2 Short-term studies of toxicity

(a) Rats

In a dose range–finding study, Allingham (2014b) administered Jagua (Genipin–Glycine) Blue (batch no. 5313014) to groups of Wistar Crl:WI(Han) rats (*n* = 3/sex per group; 9–10 weeks at study start) by gavage at 0, 10, 50, 100, 500 or 1000 mg/kg bw per day (equal to 0, 3, 17, 33, 165 and 330 mg/kg bw per day on a "blue polymer" basis) for 28 days. High-performance liquid chromatography with ultraviolet detection (HPLC-UV) determined the blue polymer content to be 33.05% of the extract. The study was described as conforming with GLP, but it was not audited by a QA unit. The study conformed to the OECD Test Guideline 408. Observations included clinical signs, body weights, feed consumption, functional observational battery, haematology, clinical chemistry, urine analysis, organ weights and macroscopic pathology.

There were no deaths during the study. There were no effects on body weight gain or feed consumption. Effects on piloerection are listed in escalating dose order: slight (1, 2, 1, 3, 3, 3 males and 0, 0, 0, 1, 2, 3 females) or moderate (0, 0, 0, 3, 3, 1 male animals only). Some animals were also noted to move their bedding immediately after test substance administration (0, 0, 0, 2, 1, 3 males and 0, 0, 1, 3, 3, 1 females). Other clinical findings were sporadic and were assumed to not be treatment related. Dark discoloration of the kidneys and testes at 1000 mg/kg bw per day were attributed to the intense colour of the test item.

This study (Allingham, 2014b) was used to determine the high dose (1000 mg/kg bw per day of Jagua (Genipin–Glycine) Blue) for the 90-day repeated-dose rat study described below.

Allingham et al. (2014) administered Jagua (Genipin–Glycine) Blue (batch no. 5313014) to groups of Wistar Crl:WI(Han) rats (*n* = 10/sex per group; 7–8 weeks at study start) by gavage at 0, 100, 300 or 1000 mg/kg bw per day (equal to 0, 33, 99 and 330 mg/kg bw per day on a "blue polymer" basis) for 90 days. An additional five male and five female rats were observed for a further 28 days following the last dose. These recovery group animals were also treated at the highest dose. The doses were measured and verified in study weeks 1,

5, 9 and 13 using HPLC-UV (590 nm) to detect the "blue polymer" content, which represented 33.05% of the extract. The study was certified as GLP and QA compliant and conformed with OECD Test Guideline 408. Observations included clinical signs, body weight, feed consumption, functional observational battery, motor activity, ophthalmoscopy, haematology, clinical chemistry, urine analysis, organ weights and macroscopic and microscopic pathology (Allingham et al., 2014).

There were no deaths during the study. Mid-dose females were reported to have higher feed consumption on days 15–21 (36.4 g/day vs 21.2 g/day for controls), though the study did not report the statistical significance of this finding. High-dose males had a slight (8%) yet significant lower body-weight gain at the end of the recovery period. Discoloured faeces were noted in all treatment groups from day 32 (for the high-dose animals) and day 56 (for the low-dose animals) until the end of the treatment period; the discoloration was presumed to be due to the colour of the test article. Additional cage-side observations included transient crust (one control male, one recovery group male, two high-dose males, five high-dose females), alopecia (two recovery group males, five recovery group females, one low-dose female, four high-dose females) and a transient necrosis of the left shoulder in a single control male. The study authors concluded that these effects were not treatment related. No significant findings were seen in the functional observational battery. No statistically significant differences in haematological values were reported. The only statistically significant differences in clinical chemistry values were lower alkaline phospatase activity in recovery group males and higher urea levels in high-dose females compared with controls. The report authors noted that the values were within the range for historical controls. Results from urine analysis tests were similar in control and treated animals except for one mid-dose male with elevated bilirubin (2 mg/dL), erythrocytes (250 cells/μL) and leukocytes (500 cells/μL) and low pH (5). The report authors noted that the changes were not associated with any clinical biochemistry, haematological or macro/histopathological changes and concluded that these changes were not treatment related.

Pathological findings included dilated pelvis in control (7/20), low-dose (4/20), mid-dose (11/20) and high-dose (8/20) animals. One control animal had an enlarged kidney; one mid-dose male, four high-dose males and four high-dose females had dark discoloration on a kidney; one control had a red discoloration on the thymus and red discoloration on axillary lymph nodes; one low-dose male and one high-dose male had dark discoloration on the liver; one high-dose male had an enlarged liver; and one mid-dose male had dark foci on the lungs. Five control, three low-dose, two mid-dose and two high-dose females had fluid distension of the uterus/cervix, with one at the low dose and one at the high dose having it in both horns, and one mid-dose female having two horns 5

mm in diameter. One control, one low-dose and one mid-dose female had a small uterus/cervix. One mid-dose female had a black focus on mucosa of the fundus of the stomach. The findings were not dose dependent and control animals were similarly affected. The findings were of low incidence and related to the estrus state of the animals (small or fluid filled findings in the uterus). The study authors did not consider these findings to be treatment related.

Changes in organ weights were as follows: statistically significant decreases in absolute kidney weight in low-dose males (89% of control weight) and mid-dose males (90% of control weight), but not in the high-dose groups nor relative to body weight; statistically significant increases in brain weights relative to body weight in low-dose males (11% above controls), but not mid-dose or high-dose animals. Adrenal gland weights in high-dose females were slightly yet statistically significantly lower than that of controls (83% of controls) compared with brain weights of the respective animals. There were no histopathological correlates to the change in adrenal gland weights and male animals were unaffected. Slightly lower absolute thyroid/parathyroid gland weights (~67% of controls; not statistically significantly) were observed in male but not female high-dose animals. No treatment-related alterations were observed in the thyroid/parathyroid glands (macroscopically or microscopically) at the end of the treatment period. All recorded histopathological findings were within the range of spontaneous background alterations in Wistar rats of these ages. The changes were considered not toxicologically relevant because they were not dose responsive, not seen in both sexes or not related to histopathological correlates.

The NOAEL was 330 mg/kg bw per day of Jagua (Genipin–Glycine) Blue on a “blue polymer” basis, the highest dose tested (Allingham et al., 2014).

(b) Dogs

Mancari (2016) administered Jagua (Genipin–Glycine) Blue (batch no. 5314034; 33.79% “blue polymer” as determined by HPLC-UV [590 nm]) to groups of beagle dogs (n = 3/sex per dose) by gavage at 0, 250, 500 or 1000 mg/kg bw per day in water (0, 85, 169 and 338 on a “blue polymer” basis) for 90 days. The measured doses on two occasions were found to be 102.3–104.1% and 92.9–94.2% of the expected value. The study was certified as following OECD GLP, but it deviated from OECD Guideline No. 409, which recommends using a minimum of four animals per sex per dose. Furthermore, clinical chemistry and urine analysis were performed at the beginning and end of the study rather than monthly or additionally at the midway point, as recommended; the dogs were around 1 year old at study initiation, when the guideline recommends starting by 9 months of age; and the epididymis, thymus and uterus were collected, preserved and

examined at necropsy, but not weighed. Nevertheless, the Committee considered these deviations to be minor and not affecting the validity of the study.

Study observations included clinical signs, body weights, feed consumption (qualitative), ophthalmoscopy, electrocardiography, haematology, clinical chemistry, urine analysis, organ weights and macroscopic and microscopic pathology.

There were no deaths during the study. Clinical signs noted were blood in urine and faeces in one mid-dose male on day 2 and one low-dose male on day 42. Blue faeces were observed throughout the study in all treated animals, which was assumed to be due to the colour of the test article.

One high-dose male lost approximately 15% of its body weight, but other variations in body weight were small and there was no dose relationship. No treatment-related ophthalmoscopic or electrocardiographic changes were noted. Compared with pretest values, a 26%, 8%, 9% and 18% decrease in white blood cells was observed in control, low-, mid- and high-dose males, respectively, and in high-dose females (20%), with inter-animal variability. This decrease in white blood cells was due to a decrease in both lymphocytes and neutrophils. Lymphocytes were also decreased by 11% in low-dose females and by 12% in mid-dose females. An approximate 10% decrease in red blood cells, haemoglobin and haematocrit was observed in both sexes at the high dose. Compared with pretest values, red blood cells, haemoglobin and haematocrit decreased by 5–11%, 4–10% and 5–14%, respectively, in high-dose animals, and all post-test values were above the 5th percentile for the historical controls. Prothrombin time and prothrombin time ratio were significantly increased in high-dose females ($P < 0.01$; Dunnett test), though values remained within the normal range for dogs (Sodikoff, 1995). Prothrombin time also increased in mid-dose males ($P < 0.05$; Dunnett test). No other changes in coagulation parameters were observed. A 1.7-fold increase in total cholesterol was recorded in one high-dose female. A slight to marked dose-related increase in total serum bilirubin (from 0–0.09 at pretest to 0.1–0.28 mg/dL on day 88) was seen in both sexes at all doses; changes for mid- and high-dose females were significant ($P < 0.05$ and $P < 0.01$, respectively, Cochran–Cox test) and changes for high-dose males were significant ($P < 0.05$; Dunnett test). The authors noted that the test results may have been affected by the colour of the test article: most of the treated animals at all doses had green urine and the intensity of the colour correlated with treatment dose.

The Committee noted that the change in colour of urine and serum bilirubin that the study author attributed to the colour of the test article suggests that a coloured component of the test substance was absorbed. All microscopic findings recorded were reported to be within the range of spontaneous background alterations that may be recorded in beagle dogs of this age.

The NOAEL was the highest dose tested, 338 mg/kg per day on a "blue polymer" basis (Mancari, 2016).

2.2.3 Long-term studies of toxicity and carcinogenicity

There were no long-term studies of Jagua (Genipin–Glycine) Blue.

Gardenia blue is also a genipin–amino acid/peptide polymer, and is expected to have a structure similar to Jagua (Genipin–Glycine) Blue. One 104-week toxicity study in F344 rats of a blue polymer derived from geniposide extracted from *Gardenia jasminoides* was reported in the literature (Imazawa et al., 2000), and it has been included in this monograph for read-across of the findings.

Geniposide and eight other iridoid forms were extracted from *G. jasminoides* with methanol. The glucosides were mixed with defatted soybean protein and treated with β-glycosidase and protease in a buffer solution, resulting in a blue polymer with a melting-point of 118–120 °C and molecular weight of 15 600 ± 400. The test article was not further described in terms of its composition, polymer structure, blue polymer content or impurities.

Fischer 344/DuCrj (F344) rats (n = 50/sex per dose; 6 weeks old) were exposed to the blue polymer at concentrations of 0, 2.5% and 5% in the diet (equal to 0, 1077 and 2173 mg/kg bw in males and 0, 1267 and 2533 mg/kg bw in females). The study was not reported as compliant with OECD guidelines or GLP. Feed consumption was measured once every 5 weeks and body weight was measured once a week for the first 5 weeks and every 5 weeks thereafter. The surviving rats were killed at 104 weeks after an overnight fast. Haematology measurements included white blood cells, red blood cells, haemoglobin, haematocrit and platelets. At necropsy, brain, submaxillary gland, lungs, heart, liver, spleen, adrenal glands, kidneys and testes were weighed. Tumours, major organs and tissues fixed in formalin and paraffin and stained for histopathological examination. There was no reported change in mean body weight or survival. Survival rates for the 0, 2.5% and 5% dose groups were 68%, 68% and 52% in males and 88%, 82% and 74% in females, respectively. No changes were found in organ weights except for a significant increase in left lung weight in males in the 5% group. The report noted some unspecified and occasional variation from controls, but these changes were neither consistent nor dose related. Faeces, but not urine, in treated animals were reported as blue. No treated group showed significant increases in tumour incidence over the control.

The NOAEL was 5% in the diet (2173 mg/kg bw per day in males and 2533 mg/kg bw per day in females), the highest dose tested (Imazawa et al., 2000).

2.2.4 Genotoxicity

The genotoxicity of Jagua (Genipin–Glycine) Blue has been investigated in three genotoxicity studies. A bacterial reverse mutation assay was conducted in accordance with OECD Test Guideline 471, an in vitro mouse lymphoma assay in accordance with OECD Test Guideline 476 and an in vivo mammalian micronucleus induction assay in accordance with OECD Test Guideline 474. All studies were certified for compliance with GLP and QA.

No genotoxic potential of Jagua (Genipin–Glycine) Blue was identified. The results from three genotoxicity studies with Jagua (Genipin–Glycine) Blue are summarized in Table 1.

2.2.5 Reproductive and developmental toxicity

No information was available.

2.2.6 Special studies

(a) Allergenicity

Jagua (Genipin–Glycine) Blue was tested for the presence of 26 known and characterized contact allergens by gas chromatography–mass spectrometry. None were detected (Arnoult, 2013).

(b) In vitro studies

Jagua (Genipin–Glycine) Blue was found not to penetrate the skin in an in vitro percutaneous penetration assay (Lati, 2013); to be a nonirritant in a hen egg chorioallantoic membrane assay; and to be nonphototoxic in an in vitro 3T3 NRU phototoxicity test (Parmantier, 2013a,b).

The Committee did not find the tests pertinent for evaluating the risk of use of Jagua (Genipin–Glycine) Blue as a food colourant.

2.3 Observations in humans

A QA-compliant repeated insult patch test was conducted to determine the irritation and/or sensitization potential of Jagua (Genipin–Glycine) Blue after repeated application, under occlusive patch test conditions, to the skin of 52 volunteers with self-perceived sensitive skin. Approximately 0.2 mL of a 1.5% solution of Jagua (Genipin–Glycine) Blue in distilled water was applied to the skin for 24 hours, with 24–48 hour intervals between applications, for a total of nine applications. No dermatitis or allergic contact dermatitis was seen in any of the volunteers during the induction or challenge phases of the study (Hollenback, Miller & Erianne, 2013).

Table 1
Genotoxicity of Jagua (Genipin–Glycine) Blue in vitro and in vivo

End-point	Test system	Route of administration	Concentration/dose	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	–	3.16–5 000 µg/plate, ±S9	Negative[a]	Kraft (2013)
Gene mutation	Mouse lymphoma L5178Y $TK^{+/-}$ cells	–	First experiment: 500–4 250 µg/mL, −S9 250–5 000 µg/mL, +S9 Second experiment: 100–3 250 µg/mL, −S9 600–5 000 µg/mL, +S9	Negative[b]	Trenz (2013)
In vivo					
Micronucleus induction	Mouse; male and female	Single Intraperitoneal	400, 1 000 and 2 000 mg/kg bw	Negative[c]	Wessels (2013)

bw: body weight; TK: thymidine kinase; S9: 9000 × *g* supernatant fraction from liver homogenate

[a] Two separate experiments were performed. The first experiment used the plate incorporation method and the second experiment the preincubation method. Cytotoxicity was noted in the plate incorporation method assays using strains TA1537 at 2500 µg/plate without metabolic activation, strains TA98 and TA1535 at ≥2500 µg/plate without metabolic activation. Cytotoxicity was also noted in the preincubation method assays using strain TA100 at ≥316 µg/plate without metabolic activation, strain TA1537 at ≥100 µg/plate without metabolic activation and strain TA1537 at ≥2500 µg/plate with metabolic activation.

[b] Two separate experiments were performed. Relative total growth was reduced at the highest concentrations in both experiments. The global evaluation factor was not exceeded and no dose–response relationship was observed. Colony sizing showed no clastogenic effects with or without metabolic activation. Positive responses were obtained for appropriate positive controls (methyl methanesulfonate [MMS] and ethyl methanesulfonate [EMS] without activation and benzo[a]pyrene with activation).

[c] The dose of 2000 mg/kg bw was determined to be the maximum tolerated dose in a preliminary toxicity test. Moderate transient toxicity was observed in the highest test group and mild to moderate in the mid-dose test group. Micronuclei were examined in peripheral blood drawn at 44 hours and also for the control and high-dose group at 68 hours.

The Committee did not find the tests pertinent for evaluating the risk of use of Jagua (Genipin–Glycine) Blue as a food colourant.

3. Dietary exposure

3.1 Introduction

Estimates of dietary exposure to Jagua (Genipin–Glycine) Blue prepared by the sponsor, based on dietary data for the USA population, and estimated use levels and use frequencies were made available to the Committee. In addition, the Committee completed a conservative assessment using the WHO/FAO Chronic Individual Food Consumption Database – Summary statistics (CIFOCOss) and

proposed maximum use levels provided by the sponsor. No actual use data were presented or found in the literature.

3.2 Dietary exposure assessment

3.2.1 Assessments based on model diets

(a) Exposure estimates based on CIFOCOss database

The CIFOCOss database[1] currently contains summary statistics from 55 surveys from 36 countries, each with survey durations of 2 days or more. The countries are grouped into 17 Global Environment Monitoring System (GEMS) clusters (WHO, 2012) that are culturally and economically comparable. The database provides summary statistics of food categorization, grouped in three tiers, with more than 600 items at the most detailed tier. The food categories provided as examples for proposed uses by the sponsor were matched with the CIFOCOss detailed food groups to assign the maximum proposed use levels for the calculations. These assignments are listed in Table 2.

The mean and the 95th percentile of the dietary exposure were calculated within each survey, age class and CIFOCOss food group. For each survey, a mean and a high exposure were calculated. The high exposure was calculated as the sum of the 95th percentile exposure, for the food group with the highest 95th percentile exposure, and the mean exposure for each of the remaining food groups. For each age class, Table 3 lists the number of clusters and surveys included in the ranges given for the mean and the high exposure estimates.

For each age group, Table 4 lists the CIFOCOss food group contributing most to the total mean exposure within each survey, the range of these contributions and the number of surveys included in this range. Only food groups that contributed at least 10% to the total mean exposure are included in the table.

3.2.2 Assessments based on individual dietary records

(a) Exposure estimates based on Brazilian consumption data

The sponsor submitted estimates on dietary exposure from Jagua (Genipin–Glycine) Blue (“Jenipapo Concentrate”) based on average consumption data from Brazil (whole population), submitted by the Instituto Brasileiro de Geografia e Estatística (IBGE) and proposed use levels. The estimated average dietary exposure at 100% use in proposed food items was 32.7 mg/day as Jagua (Genipin–Glycine) Blue (corresponding to 13 mg/day calculated as “blue polymer” from Jagua (Genipin–Glycine) Blue, using a conversion factor of 0.4). The proposed

[1] Detailed data (January 2017) were made available to the Committee by the World Health Organization.

Table 2
Food groups and maximum use levels of "blue polymer" from Jagua (Genipin–Glycine) Blue used in the CIFOCOss calculations

CIFOCOss food group	Sponsor-proposed food group	Proposed maximum use level (mg/kg or mg/L)[a]
Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	Average of yoghurt, pudding	180
Yoghurt, cheese and milk-based dessert for infants and young children	Baby food	120
Food for infants and small children, nes		
Ready-to-eat meal for infants and young children		
Cereal-based food for infants and young children		
Fruit juice and herbal tea for infants and young children		
Bullets or lollipop	Candy	120
Cream	Cream	240
Edible ices, including sherbet and sorbet	Ice cream	
Dairy products, nes	Dairy-based drinks, flavoured and/or fermented (e.g. chocolate milk, cocoa, eggnog, drinking yoghurt, whey-based drinks) (GMP)	
Fermented milks (plain)	Flavoured milk	120
Chantilly		
Flavoured milk		
Other cocoa products (including chocolate), nes	Cocoa and chocolate products (GMP)	240
Cocoa butter		
Cocoa mass		
Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)	Nougats and marzipans (GMP)	240
Tree nuts, processed, nes		
Fruit juice, nes	Fruit drinks	40
Vegetable juice, nes		
Cakes, cookies and pies (e.g. fruit-filled or custard types)	Jams Gelatins	120
Gum		
Sugar products and confectionaries, nes		
Other processed fruits (excluding dried and juice), nes		
Canned or bottled (pasteurized) fruits		
Jams, jellies, marmalades		
Other processed fruits (excluding dried and juice), nes		
Diet beverages	Soft drink	40
Nonalcoholic ("soft") beverages, nes	Energy drink	
Energy drinks	Sport drink	
Isotonic drink		
Soy drink	Soy milk	120
Soy milk powder		

CIFOCOss: Chronic Individual Food Consumption Database – Summary statistics; GMP: good manufacturing practices; nes: not elsewhere specified
[a] Recalculated from total Jagua (Genipin–Glycine) Blue to "blue polymer" from Jagua (Genipin–Glycine) Blue using 0.4 as a conversion factor.

Table 3
Exposure estimates for "blue polymer" from Jagua (Genipin–Glycine) Blue per age group based on CIFOCOss database and maximum use levels proposed by sponsor

Population[a]	Number of clusters	Number of surveys	Range for mean exposure across dietary surveys (mg/kg bw per day)	Range for high-level exposure across dietary surveys (mg/kg bw per day)
Adults	5	19	0.18–0.7	0.46–1.5
Adolescents	5	17	0.25–1.6	0.69–5.1
Children	7	30	0.07–3.8	0.57–9.8
Toddlers	4	9	1.68–3.6	3.79–10.9
Infants	10	2	2.42–3.7	7.69–12.4

bw: body weight; CIFOCOss: Chronic Individual Food Consumption Database – Summary statistics
[a] Ages in years were not defined in the available version of CIFOCOss database.

Table 4
Food groups contributing most to total mean exposure to "blue polymer" from Jagua (Genipin–Glycine) Blue

Population[a]	Food group contributing most to the mean exposure	Number of surveys	Range of contributions (%)
Adults	Fermented milks (plain)	6	22–47
	Nonalcoholic ("soft") beverages, nes	3	25–63
	Diet beverages	2	27–30
Adolescents	Nonalcoholic ("soft") beverages, nes	8	28–74
	Edible ices, including sherbet and sorbet	2	6–22
	Fermented milks (plain)	1	31
Children	Fermented milks (plain)	5	21–43
	Nonalcoholic ("soft") beverages, nes	5	52–64
	Edible ices, including sherbet and sorbet	2	10–34
	Fermented milks (plain)	1	83
	Fruit juice, nes	1	70
Toddlers	Ready-to-eat meal for infants and young children	3	41–49
	Soy drink	2	3–14
	Dairy products, nes	1	18
	Fermented milks (plain)	1	60
	Food for infants and small children, nes	1	61
	Fruit juice and herbal tea for infants and young children	1	10
Infants	Fermented milks (plain)	1	41
	Ready-to-eat meal for infants and young children	1	47

nes: not elsewhere specified
[a] Ages in years were not defined in the available version of CIFOCOss database.

use frequency of 30% reduces the exposure to 3.9 mg/day (calculated as “blue polymer” from Jagua (Genipin–Glycine) Blue).

(b) Exposure estimates based on USA consumption data

The sponsor submitted estimated daily exposures to Jagua (Genipin–Glycine) Blue in selected foods based on foods reported to be consumed in the What We Eat in America (WWEIA) dietary component of the National Health and Nutrition Examination Surveys (NHANES) 2009–2012.

The amounts of coloured food consumed were reduced using expectations of use frequencies based on flavour and food types. The list of all food codes reported consumed in NHANES 2009–2012 that would fall into the target food types was reviewed. When available, the flavours specified for foods under each food type were used to identify which foods can be found to be blue coloured or potentially blue coloured. When flavours were not specified for foods under a given food type, all foods for that food type were conservatively assumed to be coloured blue. Baby products were excluded from selection.

Furthermore, the amounts of coloured food consumed were reduced based on expectations of the portion of food items that would be coloured (e.g. for candy-coated candies with chocolate or non-chocolate interiors, it was assumed that 50% of the weight of the candy was the coating that could be coloured).

Finally, the expected use level was reduced from the maximum proposed use level to a lesser level, based on the intensity of the colour of the representative products.

Estimated dietary exposures based on these assumptions are shown in Table 5.

3.3 Evaluation of estimates of dietary exposure

The dietary exposure estimate for “blue polymer” from Jagua (Genipin–Glycine) Blue based on the CIFOCOss model diets and proposed maximum use levels uses a very conservative approach and shows mean exposures (in mg/kg bw per day) in the range of 0.2–0.7 for adults, 0.3–2 for adolescents, 0.07–4 for children and 2–4 for toddlers. Ranges (in mg/kg bw per day) for high exposures were 0.5–2 for adults, 0.7–5 for adolescents, 0.6–10 for children and 4–11 for toddlers.

The assessment based on Brazilian consumption data and proposed use levels estimated the dietary exposure at 4 mg/day for the whole population.

The dietary exposure estimates based on USA food consumption data and proposed use levels, refined with assumptions of expected use levels and use frequencies, were significantly lower. The estimated daily mean exposure was 0.2 mg/kg bw per day for children, and 0.06 mg/kg bw per day for adolescents and

Table 5
Estimated daily exposure to "blue polymer" from Jagua (Genipin–Glycine) Blue from proposed uses based on 2-day food consumption data from NHANES 2009–12

		Estimated exposure (mg/kg bw per day)[a]	
Age–sex group (years)	**% eaters**	**Mean**	**90th percentile**
Children (2–5)	90	0.17	0.37
Teenage boys (13–18)	85	0.06	0.14
Population (≥2)	76	0.06	0.14

bw: body weight; NHANES: National Health and Nutrition Examination Surveys
[a] Submitted data were recalculated from total Jagua (Genipin–Glycine) Blue to "blue polymer" from Jagua (Genipin–Glycine) Blue using 0.4 as conversion factor.

for the general population 2 years and older. The 90th percentile estimates were 0.4 mg/kg bw per day for children, and 0.14 mg/kg bw per day for adolescents and for the general population 2 years and older.

Using the CIFOCOss data from USA for children less than 6 years old, the dietary exposure is estimated at 11 mg/kg bw per day (95th percentile).

The estimates of the dietary exposure for "blue polymer" from Jagua (Genipin–Glycine) Blue calculated by this method were much higher than those of the sponsor (0.4 mg/kg bw per day for children at the 90th percentile). The Committee concluded that this was due to the use of reduction factors for use levels and use frequencies by the sponsor.

4. Comments

4.1 Biochemical aspects

The molecular weight and chemical properties of the "blue polymer" of Jagua (Genipin–Glycine) Blue suggest that the polymer is unlikely to be absorbed intact from the gastrointestinal tract. A size distribution analysis showed that less than 1.5% of the mixture contained dimers with molecular weights of around 500 Da, which could be absorbed. An in vitro study using a Caco-2 cell intestinal barrier model showed that "blue polymer" has poor passive penetration, but there is some evidence to suggest that a small proportion of Jagua (Genipin–Glycine) Blue, possibly the smallest coloured molecular species (such as genipin–glycine dimers (molecular weight approximately 500 Da) or other coloured low molecular weight components), was actively transported (Gilbert, 2015). No "blue polymer" was detectable in the plasma of dogs in an oral gavage repeated-dose study (tested up to 338 mg/kg bw per day, limit of quantification 1 mg/mL) on

day 1 or 91 following dosing with Jagua (Genipin–Glycine) Blue (Mancari, 2016). No investigations into biotransformation of the "blue polymer" were undertaken.

4.2 Toxicological studies

Results from an oral gavage acute toxicity test in the rat showed no adverse effects at the highest tested dose of 660 mg/kg bw (Allingham, 2014b).

Results from oral gavage 90-day repeated-dose toxicity studies in rats and dogs showed no adverse effects at 330 mg/kg bw per day or 338 mg/kg bw per day of "blue polymer", respectively, the highest doses tested (Allingham et al., 2014; Mancari, 2016). The dog study deviated from the relevant OECD guideline, but the Committee considered these deviations to be minor and to not affect the validity of the study. The Committee noted that in dogs the urine was coloured green with an intensity that appeared to be in proportion to the administered dose, and there was an increase in measured serum bilirubin values attributed to the interference of the test article with the analytical method, suggesting that some of the "blue polymer" had been absorbed from the gastrointestinal tract. Green-coloured urine was not observed in rats. In 90-day animal studies, all treated animals had faeces that were coloured blue, which is consistent with poor absorption of the high molecular weight component of the "blue polymer" from the gastrointestinal tract.

There were no long-term toxicity or carcinogenicity studies available on Jagua (Genipin–Glycine) Blue. To address the data gap, one non-GLP carcinogenicity study in rats on a structurally related genipin-based blue polymer from *Gardenia jasminoides* (Gardenia Blue) was considered. The Gardenia Blue used in the study was formed from a mixture of genipin and a protease digest of soy proteins, resulting in different amino acids attached to genipin. The Committee noted that the purity of blue polymer in the Gardenia Blue was not described. At concentrations up to 5% in the diet (equal to 2173 mg/kg bw per day in the males and 2533 mg/kg bw per day in the females), there were no treatment-related adverse effects or changes in tumour incidence (Imazawa et al., 2000).

There was no evidence of genotoxicity of Jagua (Genipin–Glycine) Blue in vitro, with bacterial reverse mutation assays and a mouse lymphoma assay, or in vivo, with a mouse micronucleus assay. The Committee concluded that there was no concern with regard to genotoxicity.

No reproductive or developmental toxicity studies on Jagua (Genipin–Glycine) Blue were available; there were also no available reproductive or developmental toxicity studies on Gardenia Blue.

4.3 Observations in humans

No relevant human studies were available.

4.4 Assessment of dietary exposure

Estimates of dietary exposure to Jagua (Genipin–Glycine) Blue prepared by the sponsor based on dietary data for the United States population, estimated use levels and use frequencies were available to the Committee. Additionally, a conservative assessment using the CIFOCOss database and maximum use levels provided by the sponsor was performed by the Committee. The 95th percentile estimates of dietary exposure for Jagua (Genipin–Glycine) Blue on a "blue polymer" basis calculated by the Committee were 11 mg/kg bw per day for children and 5 mg/kg bw per day for adolescents. These estimates were much higher than those calculated by the sponsor. The difference between the sponsor's estimates and the Committee's estimates was due to the use by the sponsor of lower use levels and use frequencies.

The Committee concluded that the conservative estimate of 11 mg/kg bw per day for children and 5 mg/kg bw per day (for adolescents), prepared using the CIFOCOss model, should be considered in the safety assessment for Jagua (Genipin–Glycine) Blue on a "blue polymer" basis.

5. Evaluation

The Committee noted that in 90-day toxicity studies with Jagua (Genipin–Glycine) Blue in the dog and rat, no treatment-related adverse effects were found at the highest doses tested; in addition, genotoxicity tests were negative and no treatment-related adverse effects were observed in a carcinogenicity study with the structurally related food colour, Gardenia Blue. Based on the coloration of the urine in the dogs and the increase in serum bilirubin test values, which was attributed to interference of the test article with the analytical method, the Committee concluded that some component of the Jagua (Genipin–Glycine) Blue is absorbed and excreted, most likely the dimers or other coloured low molecular weight component; the dimers make up less than 1.5% of Jagua (Genipin–Glycine) Blue. However, the Committee noted that the highest doses tested in both 90-day studies were only 330 and 338 mg/kg bw per day (expressed on a "blue polymer" basis) in rats and dogs, respectively. The Committee was concerned that the possible effects of the low molecular weight species that could be absorbed would not have been adequately investigated.

A comparison of the dietary exposure estimate (11 mg/kg bw per day) with the NOAEL from the 90-day studies of oral toxicity in rats and dogs (approximately 330 mg/kg bw per day) gives a margin of exposure of approximately 30.

Because of the limited biochemical and toxicological database and the low margin of exposure, the Committee was unable to complete the evaluation for Jagua (Genipin–Glycine) Blue.

A new tentative specifications monograph and a Chemical and Technical Assessment were prepared.

The Committee raised concern regarding potential toxicity of low molecular weight fraction of the total colouring matter in Jagua (Genipin–Glycine) Blue. The Committee recommends additional biochemical and toxicological information (e.g. absorption, distribution, metabolism and excretion studies, long-term toxicity, carcinogenicity, reproductive and developmental toxicity studies), including the use of higher doses of the "blue polymer", including the dimers, in order to complete an evaluation of the safety of Jagua (Genipin–Glycine) Blue.

To support the above, additional information is required on:

- Characterization of the low molecular weight components of the "blue polymer".
- A validated method for the determination of dimers.
- Data on concentrations of dimers from five batches of the commercial product.

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Metatartaric acid

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1. Explanation

Metatartaric acid (Chemical Abstracts Service No. 56959-20-7/39469-81-3; INS No. 353), a polymer of L(+)-tartaric acid, is used as a food additive in winemaking in the following countries and regions: Argentina, Australia, Brazil, Canada, Chile, the European Union, New Zealand, Norway, Paraguay, the Russian Federation, South Africa, Turkey and Uruguay.

Metatartaric acid, which was not previously evaluated by the Committee, was evaluated at the request of the Codex Committee on Food Additives (CCFA) at its Forty-eighth Session (FAO/WHO, 2016). It is proposed for use in winemaking at a level of good manufacturing practice. The data that were submitted in response to the call for data related to its use as a food additive in winemaking only.

The safety of L(+)-tartaric acid and DL-tartaric acid and their sodium and potassium salts was evaluated at the seventeenth and twenty-first meetings of the Committee (Annex 1, references *32* and *44*). At its seventeenth meeting, the Committee established a group acceptable daily intake (ADI) of 0–30 mg/kg body weight (bw) for L(+)-tartaric acid and its sodium, potassium, potassium–sodium salts, expressed as L(+)-tartaric acid. Specifications were available to the Committee for L(+)-tartaric acid (only interim additional data were available; no further action was taken) at its nineteenth meeting (Annex 1, reference *38*). At its twenty-first meeting, the Committee reaffirmed the ADI for the L(+)-tartrate monosodium salt and the existing specifications for L(+)-tartaric acid, but did not establish an ADI for monosodium DL-tartrate.

L(+)-Tartaric acid, the naturally occurring form of tartaric acid, occurs in many fruits and wines. Tartrate crystals (potassium bitartrate and calcium tartrate) develop naturally in wine and are the major cause of sediment in bottled wines. In order to prevent sedimentation, metatartaric acid has been used in wine since 1955 (OIV, 2012, 2017; Guise et al., 2014).

At the present meeting, the Committee reviewed a short-term toxicity study and a genotoxicity study of metatartaric acid. A literature search was conducted in a number of databases for articles from 1950 to 6 April 2017. The keywords used in the searches included "metatartaric acid" OR "tartaric acid" OR "tartrate" AND "toxicology" OR "toxicity". References were retrieved for "metatartaric acid" from PubMed (5 records), Web of Science (11 records), MEDLINE (6 records), Scopus (Elsevier; 6 records) and AGRIS (10 records); none were retrieved from Embase/Cochrane Library/Directory of Open Access Journals/GIM/CINAHL. However, none of these retrieved studies were considered relevant for the safety assessment of metatartaric acid. References retrieved for "tartaric acid" and "tartrate" from PubMed (63 records), BIOSIS (163 records), MEDLINE (195 records), CINAHL (94 records), AGRIS (94 records), Embase (47 records), Cochrane Library (16 records), Directory of Open Access Journals (32 records) and GIM (4 records) identified three other toxicity studies.

The previous monograph on L(+)- and DL-tartaric acid has been expanded and is reproduced in this consolidated monograph that includes metatartaric acid and L(+)-tartaric acid/tartrate. Studies on L(+)- and DL-tartaric acid from 1977 onward had not been previously reviewed by the Committee.

1.1 Chemical and technical considerations

Metatartaric acid is typically manufactured using L(+)-tartaric acid from natural sources. It is formed by the intermolecular esterification between the carboxylic group of one L-tartaric acid unit and the secondary alcohol group of another molecule of L-tartaric acid, which may be followed by further intermolecular and intramolecular esterification reactions (Sprenger et al., 2015). The primary components of metatartaric acid are the L-tartaric acid monomer, ditartrate monoester and diester, and polyester chains of varying degrees of polymerization. The average molecular weight range has been determined in commercial products to be 2.2–8.9 kDa, with a polydispersity index up to 50. Metatartaric acid is used as a stabilizer and sequestrant in wine to prevent growth and precipitation of potassium bitartrate and calcium tartrate crystals (Marchal & Jeandet, 2009). Stability studies in wine indicated that it undergoes hydrolysis to tartaric acid over time, but the rate of hydrolysis is dependent on pH and storage temperature (Ribéreau-Gayon et al., 2006; Morello, 2012).

Metatartaric acid is produced by heating L-tartaric acid from grapes at 150–170 °C under atmospheric or reduced pressure for less than 1 hour (Ribéreau-Gayon et al., 2006). This process produces a colourless liquid, which is cooled, dried and ground into an off-white powder. Variations in production

temperature, pressure and time allow manufacturers to alter the degree of esterification in the final product.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Metatartaric acid

Metatartaric acid, a dispersed polymer of tartaric acid units linked together by ester bonds, is anticipated to undergo rapid enzyme-mediated hydrolysis to L(+)-tartaric acid once exposed to carboxylesterases in the gastrointestinal tract (Heymann, 1980; Anders, 1989).

(b) L(+)-Tartaric acid

Rats

The excretion of L(+)-tartaric acid in the urine after oral administration was investigated in rats, guinea-pigs, rabbits and dogs.

In the rat, 68–99% of a 400 mg/kg bw dose of tartaric acid was recovered unchanged.

In the guinea-pig, 13–27% of the dose was recovered from doses ranging from 100 to 800 mg/kg bw.

In the rabbit, 90–99% of a 50 mg/kg bw dose was recovered. When the dose was raised to 100, 200 or 300 mg/kg bw, 21–23%, 15–26% and 2–3%, respectively, was found in urine.

In the dog, doses lower than 600 mg/kg bw were fully excreted in the urine (83–100%); with higher doses (600–1500 mg/kg bw), the recovery diminished to 50–60% and was associated with slight renal changes (Underhill et al., 1931a).

Following oral gavage administration of L(+)-tartaric acid at 1000 mg/kg bw, Gry & Larsen (1978) determined that the average percentage of tartrate recovered in urine was 72.9% in Wistar rats (n = 5/sex), 26% in female Danish Landrace pigs (n = 3) and 3.6% in female guinea-pigs (n = 11).

In a study investigating the disposition of monosodium tartrate, two groups of male CFY rats (n = 10/group) received either a dose of monosodium [^{14}C]L(+)-tartrate or of monosodium [^{14}C]DL-tartrate at 2730 mg/kg bw per day by oral intubation for 7 days. Three hours after the last dose, one animal per

group was killed and radioactivity was measured in whole blood, plasma, bone and kidneys. Thereafter, a rat from each group was killed at intervals over the next 12 days. One rat at each kill time underwent whole-body autoradiography.

In a second study, two groups of male rats (*n* = 8) received either monosodium [^{14}C]L(+)-tartrate or [^{14}C]DL-tartrate at 2570 mg/kg bw per day by oral intubation for 7 days. All the animals were killed 6 hours after administration of the final dose, and the livers and kidneys were removed.

Radioactivity concentrations in plasma following dosing with monosodium [^{14}C]L(+)-tartrate at 2730 mg/kg bw declined biphasically, with calculated half-lives of 3 and 53 hours, respectively. Whole-body autoradiography at 3 hours after monosodium [^{14}C]L(+)-tartrate administration showed most of the radioactivity to be in the gastrointestinal tract, liver, kidneys and bone. From 24 to 192 hours after the last dose, radioactivity was only detected in bone. Dosing with monosodium [^{14}C]DL-tartrate at 2730 mg/kg bw resulted in peak plasma levels 3 hours after the last dose with the half-lives for the biphasic decline in plasma at 15 and 58 hours. Autoradiography at 3 hours after monosodium [^{14}C]DL-tartrate administration showed radioactivity to be mainly in the gastrointestinal tract, liver, kidneys and bone. Radioactivity was still detected in bone at 24 hours after the last dose and in granular deposits in the kidneys at 192 hours after the last dose. At 96 hours after dosing, radioactivity in the kidneys and bones of rats treated with [^{14}C]DL-tartrate was twice that of rats treated with [^{14}C]L(+)-tartrate; this also corresponded with an increased relative kidney weight. Renal retention of tartrate was attributed to precipitation of calcium DL-tartrate in the tubules, and histopathological examinations showed crystalluria, which was not observed in rats treated with [^{14}C]L(+)-tartrate (Down et al., 1977).

Following administration to rats of a single oral dose of monosodium [^{14}C]L(+)-tartrate at 400 mg/kg bw, 70.1% of the radioactivity was excreted in urine, 15.6% in expired air and 13.6% in the faeces within 48 hours. Excretion of the labelled material in the urine was almost complete within 12 hours and in the expired air within 24 hours. After the same dose was administered by intravenous injection, 81.8%, 7.5% and less than 1% of the labelled material was excreted in urine, expired air and faeces, respectively (Chasseaud, Down & Kirkpatrick, 1977).

Following administration to rats of a single oral dose of ^{14}C-labelled sodium tartrate at 18.8 mg/kg bw, 51.5% of the radioactivity was excreted unchanged in urine within 24 hours, 21.8% was expired as CO_2 within 6 hours. After intraperitoneal injection, 63.1% was excreted in urine within 24 hours and 9.4% as CO_2 within 6 hours. The intestinal absorption in rats was 81% of the tartrate dose and the urinary excretion 70% of the dose.

As a larger portion of the ^{14}C was expired as respiratory CO_2 after oral or intracaecal administration than after parenteral administration, this indicates that some of the tartrate was metabolized in the intestines, probably by bacteria in the caecum (Chadwick et al., 1978).

Humans

In contrast to rats, only 12% of an oral dose of sodium tartrate with radioactive tracer (2, 5 or 10 g/person; *n* = 5/group) was recovered unchanged in human urine, while 46% of the dose was recovered as expired CO_2. Following intravenous administration, 63.8% of the dose was recovered in the urine of a single human participant, while a further 18% was expired as $^{14}CO_2$.

The large difference in urinary excretion after oral and intravenous administration indicated that only a small proportion of an oral dose is absorbed. Since only small amounts of radiolabelled tartrate were recovered in human urine following oral administration, tartrate is likely fermented by microorganisms in the large intestine (Chadwick et al., 1978).

A study was undertaken to demonstrate the concentration range of tartrate in human urine following dietary exposure to 280 mL of grape juice containing tartaric acid at 2.0 g/L (i.e. 560 mg, but reported to be 590 mg). Urine collected over 24 hours from 23 human volunteers (*n* = 23; sex not reported) was tested for tartrate after 1 day consuming a diet free of tartaric acid followed by 1 day of the restricted diet plus the grape juice to a total of 560 mg tartaric acid. Concentrations of urinary tartrate ranged from 7.4 to 282 μg/mg of creatinine.

The authors concluded that urinary tartrate levels in humans depended heavily on the diet and gastrointestinal microflora, and that there was no clinical benefit in routinely measuring tartrate levels in urine (Lord, Burdette & Bralley, 2005).

In a randomized cross-over feeding trial, 21 healthy men consumed a single dose of 100, 200 or 300 mL of wine at dinner, followed by a 7-day washout period during which the participants avoided consuming wine or grape-based products. A strongly significant correlation was found between wine consumption and urinary tartaric acid (Regueiro et al., 2014).

In a study using ion-chromatographic determination of L-tartrate in urine samples, urinary excretion of tartrate was measured in healthy people eating a normal diet (*n* = 19), in practising vegetarians (*n* = 26) and in idiopathic calcium stone formers (*n* = 33). The results confirmed the critical dependence of tartrate excretion on the composition of the diet, with the excreted tartrate

Table 1
Acute toxicity of tartaric acid

Species	Sex	Route	Test substance	LD_{50} (mg/kg bw)	Reference
Mice	Not reported	Oral	Sodium tartrate	4 360	Locke et al. (1942)
Rabbits	Male	Oral	Disodium tartrate	>3 680	Locke et al. (1942)

bw: body weight; LD_{50}: oral median lethal dose

mainly from dietary sources and entirely exogenous in origin (Petrarulo et al.,1991).

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Metatartaric acid

No information was available.

(b) L(+)-Tartaric acid

Mice

The oral median lethal dose (LD_{50}) of sodium tartrate in mice was reported to be 4360 mg/kg bw (Locke et al., 1942).

Rabbits

Three out of seven male rabbits died following oral administration of disodium tartrate at an average dose of 5290 mg/kg bw, while six male rabbits survived an average oral dose of 3680 mg/kg bw (Locke et al., 1942). Renal damage was observed only after the intravenous administration of tartaric acid at doses of 0.2–0.3 g in rabbits and rats (Bodansky, Gold & Zahm, 1942; Gold & Zahm, 1943).

Dogs

Tartaric acid at a dose of 5000 mg/kg bw was reported to be fatal in a dog when administered by stomach tube (Sourkes & Koppanyi, 1950).

2.2.2 Short-term studies of toxicity

(a) Metatartaric acid

In an 18-week oral toxicity study, metatartaric acid (with 27–35% esterified tartaric acid) was given to Wistar rats (*n* = 15/sex per group) in their drinking-

water at concentrations of 0, 0.1%, 0.5% or 3.0% (0, 80, 330 and 1810 mg/kg bw per day for males and 0, 130, 520 and 2520 mg/kg bw per day for females, respectively, based on the animals' water consumption). Another three groups of rats (n = 5/sex per group) were treated similarly for either 2 or 6 weeks.

A urine concentration and dilution test was undertaken at the end of weeks 2, 6 and 18. Rats deprived of water for 6 hours were given water at 25 mL/kg bw. The specific gravity of their urine was measured twice in a 2-hour period following this administration of water. At weeks 6 and 18, the specific gravity of urine collected during a 4-hour period after 16 hours of water deprivation was measured again.

Relative to controls, feed consumption was reduced significantly ($P < 0.01$) in males, by 7% in the middle-dose group and 20% in the high-dose group, and in females ($P < 0.05$), by 6% in the middle-dose group and 9% in the high-dose group. Nevertheless, body weights were only reduced (4%; $P < 0.001$) in high-dose male rats. Treated rats also consumed less water; in males, water consumption was 19.5%, 35.5% and 45.7% less than controls at the low, mid and high dose, respectively, while in females water consumption was 17%, 36% and 50% less than controls at the low, mid and high dose, respectively.

Relative to controls, all groups of rats treated for 2, 6 or 18 weeks excreted less urine, and urine of a higher specific gravity, when deprived of water for 6 hours or during the 2-hour period following a water load. In contrast, at week 18 only male rats in the middle- and high-dose groups excreted urine of lower specific gravity and in larger volumes than control animals in the 16–20 hour period after the water load. Urinary constituents were normal in the treated animals. Some significant differences in relative organ weights (brain, heart, spleen, kidneys, stomach, caecum and gonads) were observed in high-dose male and female rats at 6 or 18 weeks; these were related to the low terminal body weights of the animals. Both high-dose sexes showed an increase in relative kidney weight but without any accompanying histopathological changes. Haematology and blood chemistry examinations found no treatment-related adverse effects (Ingram et al.,1982).

The Committee noted that most of the observed effects – reduced feed and water consumption; reduced body weight; urine with increased specific gravity – were directly attributable to the unpalatability of metatartaric acid in drinking-water. As a result, the Committee considered that this study was not suitable for use in this safety assessment.

(b) L(+)-Tartaric acid

Rabbit

Three rabbits survived 17 consecutive daily feedings of disodium tartrate at an average dosage of 1150 mg/kg bw; three out of six rabbits died after being administered average dosages of 3680 mg/kg bw over 6–19 consecutive daily feedings (Locke et al., 1942).

Male New Zealand White rabbits (*n* = 15) fed 7.7% sodium tartrate in the diet (equivalent to 2310 mg/kg bw per day) for 22 weeks showed no evidence of toxicity either in terms of body and organ weight (testes and thyroid) changes or pathology (Packman, Abbott & Harrisson, 1963).

Dog

The test substance, "tartaric acid", was given in daily oral doses of 990 mg/kg bw to each of four dogs for 90–114 days. Casts appeared in the urine of three dogs; the blood chemistry remained normal except in one dog that developed azotaemia and died within 90 days. Weight changes varied from a weight gain of 30% to a loss of 32% (Krop & Gold, 1945).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Metatartaric acid

No information was available.

(b) L(+)-Tartaric acid

No new long-term toxicity studies were available since the previous evaluation of L(+)-tartaric acid.

A previously unpublished 2-year feeding study in rats that supports the ADI for tartaric acid had since been published (Hunter et al., 1977). Groups of Sprague Dawley rats (*n* = 35/sex) were fed diets described as containing monosodium L(+)-tartrate at concentrations of 0, 25 600, 42 240, 60 160 or 76 800 mg/kg bw per day (reported to be equal to a dose of L(+)-tartaric acid of 0, 710, 1220, 1840 and 2460 mg/kg bw per day for males and 0, 930, 1600, 2360 and 3200 mg/kg bw per day for females, respectively) for 104 weeks.

The survival of rats at 42 240, 60 160 or 76 800 mg/kg bw per day was better than that of the controls; this correlated with the lower feed consumption of these groups and a reduced body-weight gain. There were no adverse clinical signs or ophthalmoscopic changes. Haematology and urology tests did not show any treatment-related effects, nor were there any changes in macroscopic

pathology or organ weights. Histopathological examination of tissues did not show any evidence of toxicity or tumour induction attributable to treatment with the test substance. No treatment-related adverse effects were seen, even at the highest dose.

However, the Committee noted that the dose conversion reported used the molecular weight for disodium tartrate rather than monosodium tartrate to calculate the doses of L(+)-tartaric acid. Using monosodium tartrate, the Committee calculated the doses to be 0, 770, 1400, 1900 and 2680 mg/kg bw per day for males and 0, 1030, 1780, 2630 and 3550 mg/kg bw per day for females, respectively. As a result, the Committee concluded that the no-observed-adverse-effect level (NOAEL) for L(+)-tartaric acid in the study was 2680 mg/kg bw per day, the highest tested dose.

2.2.4 Genotoxicity

(a) Metatartaric acid

In a newly submitted in vitro reverse mutation assay, two independent experiments were performed. The test system consisted of *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535. Negative-control substances included demineralized H_2O, dimethyl sulfoxide and ethanol. Positive-control substances in tests without metabolic activation were 4-nitro-1,2-phenylenediamine for TA97a, TA98 and TA102; and sodium azide for TA100 and TA1535. Positive-control substances in tests with metabolic activation were 2-aminoanthracene for TA97, TA100, TA102 and TA1535, and benzo[a]pyrene for TA98. The supernatant fraction used for metabolic activation was from the livers of male Sprague Dawley rats that had been treated intraperitoneally with Aroclor 1254 at 500 mg/kg bw.

A test substance is considered to have mutagenic potential in the reverse mutation assay if there is a reproducible increase (by at least a factor of 2) in the number of revertant colonies per plate. A concentration-related increase over the range tested is also taken as a sign of mutagenic activity.

The first experiment was performed using the plate incorporation method, with concentrations of 50–5000 μg/plate (actual concentrations ranged from 47 to 4709 μg/plate, with or without metabolic activation). The second experiment was performed using the preincubation method with concentrations of 156–5000 μg/plate (actual concentrations ranged from 157 to 5037 μg/plate, with or without metabolic activation). The positive controls produced the expected increase in the number of revertants. Results of both experiments were negative; metatartaric acid showed no genotoxic potential (Andres, 2016).

(b) L(+)-Tartaric acid

In two in vitro assays, reverse mutation and chromosomal aberration, L(+)-tartaric acid showed no genotoxic potential. In contrast, sodium L(+)-tartrate was negative in the reverse mutation assay but yielded a positive result in the chromosomal aberration test. The Committee noted that potential cytotoxicity was not tested and that gaps were counted in the chromosomal aberration test. The Committee concluded that these factors call into question the reliability of this study. In addition, 1 mg/mL of the related compound, L(+)-tartaric acid, was shown to be negative in the same assay.

In an in vivo micronucleus test in mice, sodium L(+)-tartrate was also negative using single intraperitoneal doses up to 3600 mg/kg bw (Hayashi et al., 1988).

The results of the studies are summarized in Table 2.

2.2.5 Reproductive and developmental toxicity

(a) Metatartaric acid

No information was available.

(b) L(+)-Tartaric acid

Teratology studies have been conducted in mice, rats, hamsters and rabbits. The highest doses tested were 274 mg/kg bw per day (for 10 days) in mice; 181 mg/kg bw per day (for 10 days) in rats; 225 mg/kg bw per day (for 5 days) in hamsters; and 215 mg/kg bw per day (for 13 days) in rabbits, administered during the period of organogenesis.

Tartaric acid did not produce teratogenic effects in either soft or skeletal tissues at the highest doses tested. Likewise, there were no effects on nidation, or maternal or fetal survival rates (FDRL, 1973).

2.2.6 Immunotoxicity

The potential immunotoxicity of L-tartaric acid was evaluated in a rapid screening protocol in which groups of 30 female CD1 mice were given L-tartaric acid orally at doses of 750, 1500 or 3000 mg/kg bw per day for 5 days. A group of control animals was also evaluated. The animals received an infectious challenge on day 3 of dosing and immunization on day 5. The antibody plaque-forming cell response was measured 4 days later. Deaths and survival were monitored for 10 days after infection.

There were no statistically significant differences in spleen weight, thymus weight, spleen cellularity, anti-sheep red blood cell or plaque-forming cell response, or death due to *Listeria* infection between test and control animals (Gaworski et al., 1994).

Table 2
Genotoxicity of L(+)-tartaric acid and sodium L(+)-tartrate

End-point	Test substance	Test system	Concentration	Result	Reference
In vitro					
Reverse mutation[a]	L(+)-tartaric acid[b,c]	*Salmonella typhimurium* strains TA92, TA94, TA98, TA100, TA102, TA1535, TA1537	Up to 10.0 mg/plate ± S9[d]	Negative[e]	Ishidate et al. (1984)
	Sodium L(+)-tartrate[f,g]	*Salmonella typhimurium* strains TA92, TA94, TA100, TA1535, TA1537 and TA9	Up to 5.0 mg/plate ± S9[d]	Negative[e]	Ishidate et al. (1984)
Chromosomal aberration	L(+)-tartaric acid[b,c]	Chinese hamster fibroblast cell line	Up to 1.0 mg/mL[h]	Negative	Ishidate et al. (1984)
	Sodium L(+)-tartrate[f,i]	Chinese hamster fibroblast cell line	Up to 15.0 mg/mL[h]	Positive	Ishidate et al. (1984)
In vivo					
Micronucleus	Sodium L(+)-tartrate[f,g]	Male ddY mice bone marrow cells	0, 900, 1 800, 2 700, 3 600 mg/kg bw[i]	Negative	Hayashi et al. (1988)

bw: body weight; S9: 9000 × *g* supernatant fraction from liver homogenate
[a] Using the Ames method.
[b] Purity of the test substance: 99.9%.
[c] Solvent: phosphate buffer.
[d] The S9 was prepared from the liver of Fischer rats pretreated 5 days before with polychlorinated biphenyls (500 mg/kg bw of Kanechlor KC-400 in olive oil, by intraperitoneal injection).
[e] Negative result indicates that no significant increases in the numbers of revertant colonies were detected in any *S. typhimurium* strains at the maximum dose.
[f] Purity of the substance: 99.5%.
[g] Solvent: physiological saline.
[h] The cells were exposed to each sample at three different doses for 24 and 48 hours. No metabolic activation systems were applied. The maximum dose of each sample was selected by a preliminary test, in which the dose needed for 50% cell-growth inhibition was estimated using a cell densitomer.
[i] The test substance was administered in a single intraperitoneal injection, and the sampling time was 26 hours.

2.3 Observations in humans

(a) Metatartaric acid

No information was available.

(b) L(+)-Tartaric acid

A fatal case of tubular nephropathy following accidental ingestion of 30 g tartaric acid has been reported (Robertson & Lönnell, 1968).

Sodium tartrate at daily doses of up to 10 or even 20 g has been used in medical practice as a laxative. Sodium tartrate was tested as a laxative in a clinical study involving daily doses of 10 g given to 26 study participants for an average of 11.8 doses; laxative responses occurred in 66% of the participants. The only observed side-effects were nausea or vomiting and abdominal cramps (Gold & Zahm, 1943).

In a human study involving ingestion of L-tartrate at 225 mg/kg bw per day, one volunteer was treated for 2 consecutive days while another was treated for 4 consecutive days). There was no evidence of renal toxicity: creatinine clearance was normal and there was no proteinuria (Chadwick et al., 1978).

3. Dietary exposure

The sponsor submitted dietary exposure estimates for metatartaric acid in wine at a maximum use level of 100 mg/L using the European Food Safety Authority Food Additives Intake Model (FAIM, version 1.0). However, the Committee considered that this did not reflect long-term dietary exposure to metatartaric acid in wine.

Since metatartaric acid is proposed as a winemaking additive, it is expected to be consumed by those of drinking age. The legal minimum age for drinking alcoholic beverages in most countries is 18 years, but the range is from 16 to 21 years (IARD, 2016).

The Committee conducted international dietary exposure assessments for metatartaric acid in wine using the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) cluster diets database. Per capita wine consumption ranged from 0.24 to 88 g/day across the 17 cluster diets (WHO, 2012). The per capita dietary exposure estimates for metatartaric acid ranged from 0.0004 (G14) to 0.2 mg/kg bw per day (G07), assuming a 60 kg average body weight and 100 mg/L of metatartaric acid as the maximum use level.

The Committee also prepared international estimates of dietary exposure to metatartaric acid using wine (14.2.3.1 "Still grape wine" and 14.2.3.3 "Fortified grape wine, grape liquor wine and sweet grape wine") consumption levels from the FAO/WHO Chronic Individual Food Consumption Data – Summary statistics (CIFOCOss) database and 100 mg/L of metatartaric acid as the maximum use level. Estimates were based on 51 consumption datasets from 19 countries across eight GEMS/Food cluster diets (G05, G06, G07, G08, G09, G10, G11 and G15) were used.

Table 3 summarizes the exposure estimates to metatartaric acid for adults. The mean exposure estimates to metatartaric acid in wine for the adult population ranged from 0.000 07 to 0.2 mg/kg bw per day. This range was within the range calculated using data from the GEMS/Food cluster diets database. The maximum mean dietary exposure estimate to metatartaric acid for adult consumers of wine was 0.3 mg/kg bw per day, and the maximum 95th percentile dietary exposure estimate for adult consumers of wine was 0.8 mg/kg bw per day.

At the national level, the mean and 95th percentile dietary exposures for the United States adult consumers of wine to metatartaric acid were 0.1 and 0.3 mg/kg bw per day, respectively. The Committee also estimated dietary exposures to metatartaric acid using the consumption data of wine for Australian and New Zealand adults and the maximum use level of 100 mg/L metatartaric acid. The estimated dietary exposures to metatartaric acid for 95th percentile adult

Table 3
Estimates of dietary exposure to metatartaric acid in wine in adults (CIFOCOss data plus Committee-prepared estimates)

Group	Mean total population (mg/kg bw per day)	Mean consumers of wine (mg/kg bw per day)	95th percentile consumers of wine (mg/kg bw per day)
CIFOCOss	0.000 07–0.2	0.004–0.3	0.02–0.8
Australian[a]	0.08[b]	–	1.3[b]
New Zealand[c]	0.05[b]	–	1.3[b]
USA adults[d]	–	0.1	0.3

bw: body weight; CIFOCOss: FAO/WHO Chronic Individual Food Consumption Database – Summary statistics
[a] 0.05 L/day for mean respondents irrespective of whether they consumed wine, and 0.8 L/day for 95th percentile consumers of wine.
[b] Using an average body weight of 60 kg/person.
[c] 0.03 L/day for mean respondents irrespective of whether they consumed wine, and 0.75 L/day for the 95th percentile consumers of wine. Consumption data from the 1995 Australian National Nutrition Survey.
[d] 66 g/day (1.1 g/kg bw per day) for mean USA adult consumers of wine and 200 g/day (3.4 g/kg bw per day) for 95th percentile adult consumers of wine from the 1997 New Zealand National Nutrition Survey food consumption data.

consumers of wine were 1.3 mg/kg bw per day for both Australians and New Zealanders.

The Committee assumed that metatartaric acid hydrolyses to an approximately equivalent concentration of tartaric acid. The Committee noted that the dietary exposure to metatartaric acid for the highest 95th percentile adult consumers of wine (1.3 mg/kg bw per day, expressed as L(+)-tartaric acid) is appropriate for use in this safety assessment.

4. Comments

4.1 Biochemical aspects

(a) Metatartaric acid

Metatartaric acid, a dispersed polymer of tartaric acid units linked together by ester bonds, is anticipated to undergo rapid enzyme-mediated hydrolysis to L(+)-tartaric acid once exposed to carboxylesterases in the gastrointestinal tract.

(b) L(+)-Tartaric acid

The disposition of L(+)-tartaric acid following ingestion appears to differ markedly between most of the animal species investigated (rats, rabbits, dogs and pigs) and humans. In rats, rabbits, dogs and pigs, most of the ingested tartrate is absorbed and excreted unchanged (50–100%) in the urine (Underhill et al., 1931a; Gry

& Larsen, 1978). The extent of absorption and urinary excretion of unchanged tartrate in guinea-pigs (13–27%) is similar to that observed in humans (12%) (Underhill et al., 1931b; Chadwick et al., 1978).

In rats, 15–22% of ingested tartrate was exhaled as carbon dioxide. Microbial fermentation was confirmed following intracaecal administration, when 66% of the administered dose (18.8 mg/kg bw) was exhaled as radiolabelled carbon dioxide, while less than 2% of the administered dose was absorbed and excreted in urine (Chasseaud, Down & Kirkpatrick, 1977; Chadwick et al., 1978). In rats, the concentration–time curve for radiolabelled L(+)-tartrate suggested a short half-life in plasma of around 3 hours (Down et al., 1977).

Apart from its excretion in urine, there is evidence of extensive microbial fermentation of L(+)-tartaric acid to carbon dioxide in humans: very little unchanged tartrate (<5%) has been detected in faeces. Although the concentration of radiolabelled carbon dioxide exhaled by humans 1 hour after intravenous dosing was small (18%), suggesting metabolism by tissue enzymes, up to 46% of the label was exhaled 4 hours after oral dosing (Chadwick et al., 1978).

4.2 Toxicological studies

(a) Metatartaric acid

No acute toxicity studies were available.

Rats exposed to metatartaric acid in their drinking-water at concentrations up to 3.0% for 18 weeks had markedly reduced body weight due to a dose-related reduction in feed and water intake, owing to the poor palatability of metatartaric acid in water at all concentrations tested (Ingram et al., 1982). As a result, the Committee considered this study to be unsuitable for a risk assessment of metatartaric acid.

Metatartaric acid was not genotoxic in a reverse mutation assay.

No long-term toxicity and carcinogenicity, reproductive toxicity or developmental toxicity studies were available.

(b) L(+)-Tartaric acid

The LD_{50} of sodium tartrate in mice was reported to be 4360 mg/kg bw; for disodium tartrate in male rabbits, it was greater than 3680 mg/kg bw (Locke et al., 1942).

The Committee noted that no new long-term toxicity studies had become available since the previous evaluation of L(+)-tartaric acid. However, the previously unpublished toxicity study that supports the ADI for tartaric acid had since been published. In that study, no treatment-related adverse effects were observed in rats with diets containing monosodium L(+)-tartrate at

concentrations of 0, 25 600, 42 240, 60 160 or 76 800 mg/kg bw (reported to be equal to L(+)-tartaric acid doses of 0, 710, 1220, 1840 and 2460 mg/kg bw per day for males and 0, 930, 1600, 2360 and 3200 mg/kg bw per day for females, respectively) (Hunter et al., 1977). The Committee noted that the conversion reported in the publication used the molecular weight for disodium tartrate rather than monosodium tartrate to calculate the doses of L(+)-tartaric acid. Using monosodium tartrate, the Committee calculated the doses to be 0, 770, 1400, 1900 and 2680 mg/kg bw per day for males and 0, 1030, 1780, 2630 and 3550 mg/kg bw per day for females, respectively. The Committee concluded that the NOAEL for L(+)-tartaric acid in the study was 2680 mg/kg bw per day, the highest tested dose.

In two in vitro assays including reverse mutation (*S. typhimurium* strains TA92, TA1535, TA100, TA1537, TA94 and TA98) and chromosomal aberration (Chinese hamster fibroblast cell line), L(+)-tartaric acid showed no genotoxic potential at concentrations up to 1 mg/mL. However, although sodium L(+)-tartrate was negative in the reverse mutation assay, it was positive in a chromosomal aberration test at high concentrations of up to 15 mg/mL (Ishidate et al., 1984). The Committee noted that no testing of potential cytotoxicity was performed and that gaps had been counted in the chromosomal aberration test. The Committee concluded that these factors call into question the reliability of this study. In addition, the related compound, L(+)-tartaric acid, at 1 mg/mL was shown to be negative in the same assay. Sodium L(+)-tartrate was also negative using single intraperitoneal doses up to 3600 mg/kg bw in an in vivo micronucleus test in mice (Hayashi et al., 1988).

4.3 Assessment of dietary exposure

The sponsor requested the use of metatartaric acid as a food additive in wine at a maximum use level of 100 mg/L. The Committee conducted international dietary exposure assessments for metatartaric acid in wine using the GEMS/Food cluster diets database. The dietary exposure estimates for metatartaric acid ranged from 0.0004 (G14) to 0.2 mg/kg bw per day (G7) (per capita), assuming a 60 kg body weight and 100 mg/L of metatartaric acid as the maximum use level. The Committee also prepared international estimates of dietary exposure to metatartaric acid using wine (food category no. 14.2.3.1 “Still grape wine” and food category no. 14.2.3.3 “Fortified grape wine, grape liquor wine and sweet grape wine”) consumption levels from the CIFOCOss database and 100 mg/L of metatartaric acid as the maximum use level. The estimates of mean dietary exposure to metatartaric acid for adult consumers of wine ranged up to 0.3 mg/kg bw per day, and the highest 95th percentile dietary exposures in adult consumers

of wine reached 0.8 mg/kg bw per day. The Committee prepared dietary estimates to metatartaric acid in wine using consumption data from the 1995 Australian National Nutrition Survey, the 1997 New Zealand National Nutrition Survey and the USA National Health and Nutrition Examination Surveys, with the maximum use level of 100 mg/L. These estimates were 1.3, 1.3 and 0.3 mg/kg bw per day for the 95th percentile exposures for adult consumers of wine, respectively.

The Committee assumed that metatartaric acid hydrolyses to an approximately equivalent concentration of tartaric acid. The Committee noted that the dietary exposure to metatartaric acid for the highest 95th percentile adult consumers of wine (1.3 mg/kg bw per day, expressed as L(+)-tartaric acid) is appropriate for use in this safety assessment.

5. Evaluation

As metatartaric acid undergoes enzymatic hydrolysis to tartaric acid prior to systemic absorption, the biochemical and toxicological data on tartaric acid considered at previous meetings are relevant to the safety assessment of the metatartaric acid. Additional information to support the safety assessment of metatartaric acid includes the absence of any effects in a bacterial reverse mutation test. The Committee evaluated a series of studies that had become available since L(+)-tartaric acid was last evaluated. The body of evidence suggests no change to the group ADI previously established for L(+)-tartaric acid and its sodium, potassium and potassium–sodium salts, expressed as L(+)-tartaric acid.

The Committee concluded that metatartaric acid (when used in winemaking) should be included in the group ADI of 0–30 mg/kg bw for L(+)-tartaric acid and its sodium, potassium and potassium–sodium salts, expressed as L(+)-tartaric acid.

The Committee noted that the dietary exposure estimate for metatartaric acid for adult consumers of wine was 4% of the upper bound of the ADI, and concluded that dietary exposure to metatartaric acid in wine at the maximum use level of 100 mg/L does not present a health concern.

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Tamarind seed polysaccharide

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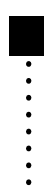

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1. Explanation

Tamarind seed polysaccharide (Chemical Abstract Service [CAS] Number 39386-78-2) is produced from the hulled seeds of *Tamarindus indica* Linne. Tamarind seed polysaccharide is a xyloglucan. Xyloglucans are a type of dietary fibre naturally present in the cell wall of plants and are abundant in rice, vegetables and fruits (Shibuya & Iwasaki, 1978; Kato & Matsukura 1994; Kato, 1995; Kato, Ito & Watanabe, 2001). Tamarind seed polysaccharide is permitted for use as a thickener, stabilizer, emulsifier and gelling agent in a variety of food products in China, Japan, the Republic of Korea and the USA.

Tamarind seed polysaccharide (Fig. 1) has not been previously evaluated by Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee evaluated tamarind seed polysaccharide at the request of the Forty-eighth Session of the Codex Committee on Food Additives (FAO/WHO, 2016).

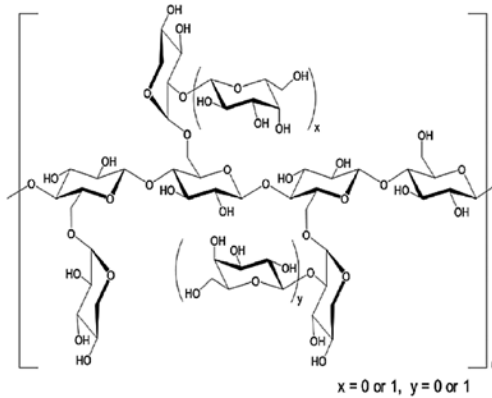
A toxicological dossier for tamarind seed polysaccharide was submitted. A comprehensive literature search was also conducted. The keywords used in the searches included tamarind OR glyloid OR glyate OR Imlees OR Imli or Imlis OR 39386-78-2 (CAS Registry Number). The databases searched included EMBASE (1974–2017 February 6; 480 records); Ovid MEDLINE (1946–2017 February 07; 210 records); CAB Abstracts (1973–2017 Week 4), Food Science and Technology Abstracts (1969–2017 Week 5) and Global Health and International Pharmaceutical Abstracts (1970–2017 February 07), which retrieved 123 records; SCOPUS (to 7 February 2017; 33 records); and Reactions Weekly (Ovid journals; 1 record). None of the records retrieved added to the toxicological data submitted to the Committee for this meeting.

To address any data gaps, the Committee also considered safety data on other polysaccharide-based gums on the basis of their similar general structure, chemical and functional properties, technical uses, lack of absorption as intact substances and metabolism to normal dietary constituents (e.g. short-chain fatty acids) as a result of microbial fermentation in the large intestine.

1.1 Chemical and technical considerations

The tamarind tree is a large evergreen widely distributed in subtropical and tropical zones (Williams, 2006). *T. indica* L. is a monotypic genus and belongs to

Fig. 1
Structural formula of tamarind seed polysaccharide



the subfamily Caesalpinioideae of the family Leguminosae (Fabaceae). The seeds of the tamarind fruit are smooth, glossy, flattened and oblong-shaped (Duke, 1981). Tamarind seed polysaccharide is also known as tamarind seed gum, tamarind gum, tamarind xyloglucan, tamarind seed xyloglucan and tamarind galactoxyloglucan.

Every part of the *T. indica* L. tree is used as food or in traditional medicine in most tropical countries (De Caluwé, Halamová & Van Damme, 2010). Traditional uses in food rely on the aroma and flavouring properties of the tamarind fruit, in its fresh or dried form. It is also used in herbal medicinal therapies (Williams, 2006).

Tamarind seed polysaccharide is produced from tamarind seeds that are sieved and toasted to remove the black testa (seed coat). The light brown tamarind kernel obtained is then pulverized and sieved to obtain tamarind kernel powder. The kernels contain 65–72% carbohydrate (polysaccharide and free sugars), 15–23% protein, 4–7% fat, 2–3% ash and 0.7–8% crude fibre, reported on a dry matter basis (Duke, 1981). The tamarind kernel powder is treated with methanol, and the pH is adjusted during treatment; this is followed by centrifugation to physically separate the insoluble tamarind seed polysaccharide from the supernatant, which contains the protein, fat and minerals. The polysaccharide is dried, pulverized, sieved and mixed with bulking agents to standardize the product. Depending on the pH treatment, downstream filtration, and acid or alkali treatment, products differing by viscosity can be manufactured.

Tamarind seed polysaccharide is composed of a linear chain of D-glucose units linked by β(1–4) glycosidic bonds. Single D-xylose units are attached to about 75% of these D-glucose units via α(1–6) bonds. Single D-galactose units are attached to some of the D-xylose units through β(1–2) bonds. The molar ratio

of glucose:xylose:galactose is about 4:3:1 (Gidley et al., 1991). The tendency of xyloglucans to self-associate gives rise to a wide range of reported molecular weights (400–6000 kDa) (Nishinari et al., 2009).

2. Biological data

2.1 Biochemical aspects

Absorption, distribution, metabolism and excretion data for tamarind seed polysaccharide were not available. Based on the size and chemical composition, it was considered that tamarind seed polysaccharide, like other dietary fibres, would not be absorbed intact and would not be digested by enzymes in the gastrointestinal tract (Cummings & Englyst, 1987). Based on its chemical composition, tamarind seed polysaccharide is likely to be degraded and fermented by intestinal bacteria in the lower gastrointestinal tract. It has been estimated that more than 75% of tamarind seed polysaccharide is fermented (Ministry of Health, Labour and Welfare, 2003).

This fermentation process, similar to that of other nondigestive polysaccharides such as locust (carob) bean gum, cassia gum and tara gum, would yield hydrogen gas, carbon dioxide and short-chain fatty acids. All of these products would be expected to be absorbed, metabolized or excreted.

Evidence supporting such a fermentation process includes the results of a 14-day dietary study in rats, which showed that oligosaccharides of tamarind seed polysaccharide generate short-chain fatty acids (specifically, lactic acid, propionic acid and butyric acid) in greater amounts in the caeca of test animals than is generated by control rats fed a non-fibre diet (Ebihara & Nakamoto, 1998). In vitro studies demonstrated that human microflora can also degrade and ferment tamarind seed polysaccharide (Hartemink et al., 1996). Specific bacteria in the large intestine in humans are capable of enzymatic hydrolysis of the glucan backbone of xyloglucans, which lead to degradation and subsequent fermentation (Hartemink et al., 1996; Larsbrink et al., 2014).

2.2 Toxicological studies

The toxicological tests summarized below used commercially available products. The manufacturing process for tamarind seed polysaccharide product has changed with time, such that the earlier products contained about 80% dietary fibre and the later products about 85% dietary fibre. The remaining 15–20% of the

tamarind seed polysaccharide product consisted of water, carbohydrates, protein and fat—normal dietary constituents that are not expected to pose a health concern.

2.2.1 Acute toxicity

Acute oral toxicity studies were conducted with tamarind seed polysaccharide (purity: 80.8% tamarind seed polysaccharide) in mice and rats of both sexes. The results are summarized in Table 1. The highest dose tested in these studies was 5000 mg/kg body weight (bw), the limit of the test.

In all the studies, the animals received a single oral dose of tamarind seed polysaccharide after fasting overnight and were observed for 2 weeks (with the exception of the 1-week-long Isozaki & Yoshida, 1962–1964, study). No deaths, clinical signs of toxicity or gross pathologies were observed in any of the studies. Tamarind seed polysaccharide was considered to have very low acute oral toxicity in mice and rats. The oral median lethal dose (LD_{50}) in mice and rats was greater than 5000 mg/kg bw or 4000 mg/kg bw when corrected for purity.

With one exception (Hachiya et al., 1985), the authors of the studies did not provide a statement on good laboratory practice (GLP) or of guidelines followed.

2.2.2 Short-term studies of toxicity

(a) Mice

In a 13-week range-finding study for a 78-week carcinogenicity study, Sano et al. (1996) fed groups of male and female B6C3F1 mice (10/sex per group; 5 weeks old) diets containing tamarind seed polysaccharide (purity: 80.8%) at concentrations of 0, 0.625%, 1.25%, 2.5% or 5% for 13 weeks (equal to 0, 1000, 1900, 3900 and 8200 mg/kg bw per day for males and 0, 1300, 2700, 5400 and 10 600 mg/kg bw per day for females, respectively). Although the study was performed prior to the establishment of Organisation for Economic Co-operation and Development (OECD) GLP or study guidelines, it was considered to be well-conducted.

Animals were observed daily for mortality and clinical signs of toxicity, and were weighed weekly. Feed and water consumption were monitored over a 2-day period prior to each weighing. Prior to termination, animals were fasted overnight and blood samples were collected for haematological and blood chemistry measurements. Haematological measures included erythrocyte counts, leukocyte counts, haemoglobin concentrations and haematocrit values. Blood chemistry parameters assessed included total protein, albumin, albumin/globulin ratio, aspartate transaminase, alanine transaminase, alkaline phosphatase, total bilirubin and urea nitrogen. At termination, necropsies were conducted, organs weighed (brain, heart, liver, spleen, kidneys, adrenals, testes and ovaries) and

Table 1
Acute toxicity of tamarind seed polysaccharide

Species	Sex	Route	LD_{50} (mg/kg bw)	Reference
Mouse	Male	Oral	>1 500	Isozaki & Yoshida (1962–1964)[a]
Mouse	Male and female	Oral	>5 000	Hachiya et al. (1985)[b]
Mouse	Male and female	Oral	>5 000	Noda et al. (1988)[c]
Mouse	Male and female	Oral	>2 000	Takizawa, Hachiya & Birukawa (1993)[d]
Rat	Male and female	Oral	>5 000	Hachiya et al. (1985)[e]
Rat	Male	Oral	>5 000	Noda et al. (1988)[f]
Rat	Male and female	Oral	>5 000	Noda et al. (1988)[g]

bw: body weight; LD_{50}: median lethal dose; OECD: Organisation for Economic Co-operation and Development
[a] Groups of ICR-JCL male mice (6/group; 30 g bw) received a single oral dose of tamarind seed polysaccharide at 1000 or 1500 mg/kg bw. Animals were observed for 7 days.
[b] According to the authors, the test was conducted in compliance with United States Environmental Protection Agency or OECD guidelines. Groups of ddY mice (≥5–10/sex per group; 5–7 weeks old) received a single gavage dose of tamarind seed polysaccharide of up to 5000 mg/kg bw.
[c] Groups of ddY mice (10/sex per group; 4 weeks old) received a single gavage dose of tamarind seed polysaccharide at 0 or 5000 mg/kg bw (vehicle not described).
[d] Groups of ddY mice (5/sex per group; 9 weeks old) received a single gavage dose of tamarind seed polysaccharide at 0 (vehicle: distilled water) or 2000 mg/kg bw.
[e] According to the authors, the experiment was conducted in compliance with United States Environmental Protection Agency or OECD guidelines. Groups of Sprague Dawley rats (≥5–10/sex per group; 5–6 weeks old) received a single gavage dose of tamarind seed polysaccharide.
[f] Groups of male Wistar rats (5/group; 4 weeks old) received a single gavage dose of tamarind seed polysaccharide at 5000 mg/kg bw.
[g] Groups of Sprague Dawley rats (5/sex per group; 4 weeks old) received a single gavage dose of tamarind seed polysaccharide at a dose of 5000 mg/kg bw.

histopathology performed (on the organs listed above as well as on lymph nodes, bone marrow, thymus, tongue, salivary glands, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, pancreas, gall bladder, urinary bladder, prostate, seminal vesicle, mammary gland, uterus, vagina, femur, sternum, skin, subcutis, eyes, Harderian glands, spinal cord and all grossly visible lesions). A full histopathological examination was conducted only in control and 5% groups.

There were no deaths or clinical signs of toxicity. No treatment-related adverse effects were observed with respect to body weights or feed or water consumption. The study authors stated that no haematological treatment-related effects were observed (no data were provided). Blood chemistry findings showed a very slight but statistically significant decrease in total protein levels in males at 0.625%, 2.5% and 5% groups (i.e., not in the 1.25% group) compared with controls (data not provided). The authors considered these findings not toxicologically significant because no other blood chemistry changes were observed. No treatment-related changes were observed at necropsy, including gross pathology, organ weight or histopathology.

In the absence of any toxicologically relevant findings, the no-observed-adverse-effect level (NOAEL) was a dietary concentration of 5%, equal to 8200 and 10 600 mg/kg bw per day for males and females, respectively, or 6600 and 8600 mg/kg bw per day when corrected for purity (Sano et al., 1996).

(b) **Rats**

In a 4-week study, Oka et al. (1962–1964) fed groups of male Donryu rats (10/group; mean bw 100 g) diets containing tamarind seed polysaccharide (purity: 80.8%) equivalent to doses of 0, 200 or 2000 mg/kg bw per day. The study was conducted prior to the establishment of OECD GLP or study guidance documents.

The animals were monitored daily for mortality. Feed consumption and body weights were measured daily. Immediately prior to termination, blood samples were drawn for haematology measures including red blood cell concentration, white blood cell concentration, Sahli value (an indication of haemoglobin concentration) and haematocrit. Three animals in the control group died on day 17 of the study (no explanation for the deaths was given).

The study authors did not report differences between treated and control groups in body weight, feed consumption or haematological measures; the graphical data they presented did not show a difference (no statistical analysis was provided). At necropsy, a mild pneumonia was seen in two animals dosed at 200 and three at 2000 mg/kg bw per day. According to the study authors, no treatment-related lesions were observed at necropsy (no data were provided).

In the absence of any observed toxicity, the NOAEL was 2000 mg/kg bw per day, equal to 1620 mg/kg bw per day when corrected for purity (Oka et al., 1962–1964).

In a 4-week study, groups of Crl:SD CD IGS rats (10/sex per group; 7–8 weeks old) were given ad libitum diets containing tamarind seed polysaccharide (purity: 85.5%; lot number: 12.02.28-1) at concentrations of 0, 40 000, 80 000 or 120 000 mg/kg feed (equal to 0, 3451, 6739 and 10 597 mg/kg bw per day for males and 0, 3602, 7190 and 10 691 mg/kg bw per day for females, respectively). The study was conducted according to OECD and United States Food and Drug Administration (USFDA) GLP requirements and in compliance with the appropriate guidelines.

Animals were observed at least twice daily for viability and were clinically examined prior to the first treatment and weekly thereafter until termination. Ophthalmological examinations were conducted according to the guidelines. Body weight was recorded on the first day of treatment and weekly thereafter and at termination. Body weight gain was calculated at weekly intervals and for the overall study. Feed consumption was measured weekly and feed efficiency, including the mean daily dietary intakes, were calculated. A functional observational battery was performed, and motor activity of all animals was assessed at week 4. Blood samples were collected to assess haematology and blood chemistry parameters and urine samples for urine analysis at weeks 2 and 4. Vaginal smears from all females were assessed microscopically to determine the animal's estrus stage. At

termination, all surviving animals were euthanized and necropsied. A standard set of organ wet weights was recorded. Samples of organs and other standard tissues as well as organs and tissues from the control and high-dose groups and any gross lesions from any group underwent histopathological examination.

All animals survived until study termination. All clinical observations seen in control and treated groups in both sexes were considered incidental and neither treatment- nor dose-related. All ophthalmological examinations were normal. Mean body weights of treated males and females did not differ significantly from those of control groups. However, there was a statistically significant decrease in mean body-weight gain in males at 80 000 (−14%; $P < 0.05$) and 120 000 mg/kg feed (−26%; $P < 0.001$) compared with controls during the first week of treatment only. The only statistically significant differences in feed consumption and feed efficiency occurred during the first week of treatment when mean daily feed consumption decreased in high-dose females (−10%; $P < 0.01$) and mean feed efficiency decreased in high-dose males (−15%; $P < 0.001$). These differences were transient and possibly due to the palatability of the feed; the differences were not considered toxicologically relevant. The findings from the functional observational battery and the motor activity assessments were comparable in treated and control groups.

Haematological, blood chemistry and urine analysis results were comparable in treated and control groups. Statistically significant differences between treated and control groups were slight and not dose-dependent. Gross necropsy findings occurred in single incidences and/or showed no dose relationship. Statistically significant decreases in absolute and relative organ weights occurred in adrenals and uterus with oviducts, but these findings were all within the range of historical control values found in the literature; were not dose-dependent; and were not associated with any clinical or histopathological changes. These differences were considered incidental and toxicologically insignificant. No microscopic findings were associated with treatment. The vaginal smears showed no cyclic changes that could be considered treatment-related.

In the absence of any toxicologically significant findings, the NOAEL for tamarind seed polysaccharide was 120 000 mg/kg feed (equal to 10 597 and 10 691 mg/kg bw per day for males and females, respectively, or 9113 and 9194 mg/kg bw per day, when corrected for purity), the highest concentration tested (Heimbach et al., 2013; Koetzner, 2013).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

In a 78-week carcinogenicity study, Sano et al. (1996) fed groups of male and female B6C3F1 mice (*n* = 50/sex per group; 6 weeks old) diets containing tamarind seed polysaccharide (purity: 80.8%) at concentrations of 0, 1.25% or 5% for 78 weeks (equal to 0, 1474 and 6658 mg/kg bw per day for males and 0, 2185 and 8575 mg/kg bw per day for females). The study was conducted prior to the establishment of OECD GLP or study guidelines documents, but was consistent with OECD Test Guideline 451 for carcinogenicity (2008).

Animals were observed daily for mortality and clinical signs of toxicity, and weights and feed and water consumption were measured once a week for the first 14 weeks and every 2 weeks thereafter. Immediately before termination, all animals were fasted overnight and blood samples were collected. Haematological parameters assessed included erythrocyte counts, leukocyte counts, haemoglobin concentrations and haematocrit values. Blood chemistry and urine analyses were not performed. At termination, each animal was necropsied and examined for gross changes. Brain, heart, liver, spleen, kidneys, adrenals, testes and ovaries were weighed and the organ to body weight ratio calculated for each. Bone marrow, caecum, colon, duodenum, eyes, femur, gall bladder, Harderian glands, ileum, jejunum, mammary gland, lungs, lymph nodes, oesophagus, pancreas, parathyroids, pituitary, prostate, rectum, thymus, thyroids, tongue, trachea, salivary glands, seminal vesicle, skin and subcutis, sternum, stomach, urinary bladder, uterus, vagina, spinal cord and all grossly visible lesions underwent histopathological examination. Missing from the standard suite of organs and tissues were the following: cervix, coagulating gland, epididymis, lacrimal gland, peripheral nerve and skeletal muscle. All males and females in the control and 5% groups underwent complete histological examination; histological examination of mice at 1.25% was restricted to spleen, lungs, liver, gall bladder, kidneys and all abnormal tissues.

The percentage survival of mice to 78 weeks did not significantly differ between treated and control groups. At 0, 1.25% and 5%, 46, 46 and 43 males and 50, 48 and 48 females survived. There were also no significant differences between treated and control groups with respect to clinical signs of toxicity. While mean body weights of males did not significantly differ between treated and control groups, the mean body weights of females at both doses were lower than that of the control group from week 34; by termination at week 78, mean female body weights were statistically significantly reduced, in a non-dose-related manner, by 11% for the low-dose group and 7% for high-dose group compared with the controls (Table 2). The study authors stated that there were no significant

Table 2
Mean (± SD) terminal body weights and relative organ weights in male and female B6CF1 mice per dietary dose of tamarind seed polysaccharide

Sex	Dose	No. of mice	Final body weight (g)[a]	Relative organ weight (%)[a]				
				Brain	Heart	Liver	Spleen	Kidney
M	0	46	38.8 ± 3.3	1.28 ± 0.12	0.56 ± 0.07	4.4 ± 1.3	0.27 ± 0.12	1.71 ± 0.14
	1.25%	46	39.6 ± 4.0	1.26 ± 0.14	0.54 ± 0.08	4.7 ± 1.8	0.29 ± 0.14	1.61 ± 0.19**
	5%	43	38.2 ± 3.8	1.32 ± 0.13	0.55 ± 0.07	4.7 ± 1.6	0.27 ± 0.12	1.66 ± 0.16
F	0	50	43.3 ± 7.0	1.25 ± 0.22	0.36 ± 0.06	3.3 ± 0.4	0.27 ± 0.08	0.97 ± 0.15
	1.25%	48	38.5 ± 6.5**	1.41 ± 0.24**	0.43 ± 0.11**	3.8 ± 0.9**	0.40 ± 0.48	1.14 ± 0.025**
	5%	48	40.2 ± 4.7*	1.33 ± 0.17*	0.40 ± 0.06**	3.6 ± 0.4**	0.31 ± 0.11*	1.04 ± 0.11*

M: male; F: female; No: number; SD: standard deviation; *: $P < 0.05$; **: $P < 0.01$ Student *t*-test
[a] Mean ± SD.
Source: Sano et al. (1996)

differences in feed or water consumption between treated and control groups (data not provided).

Haematological examinations showed a statistically significant decrease in the haemoglobin concentration in males at 5% (−5%; no statistics provided); the authors considered this not toxicologically significant as there were no significant changes in other haematological parameters. No other haematological differences were reported.

The absolute organ weight data were not presented. Relative organ weight data are shown in Table 2. The organ to body weight ratios in treated males did not differ statistically from the controls with the exception of kidney weights in the 1.25% dose group (−6%, $P < 0.01$). However, the difference was considered slight, and likely reflected the marginal increase in body weights in this group compared with controls. Several organ to body weight ratios were statistically significantly increased in females at 1.25% and 5% compared with controls, including the brain, heart, liver, spleen and kidneys. The differences were not dose-related. The study authors suggested that these differences were due to the reduced body-weight gain in the treated females, and the Committee noted that the effects on relative organ weights correlated with the non-dose-related effects on body weight. Necropsy findings showed no significant difference between treated and control groups in terms of gross pathology or histopathology.

All tumours were consistent with the type, severity and incidence expected in aged mice. The incidence of tumours was generally singular, with the exception of tumours in the lung, stomach, liver, mammary gland, uterus and lymphoma (Table 3). The study authors stated that nonneoplastic lesions in the

Table 3
Incidence of tumours in male and female B6CF1 mice per dietary dose of tamarind seed polysaccharide

	Incidence per dose and sex[a]					
	Male			Female		
Species	**Control**	**1.25%**	**5%**	**Control**	**1.25%**	**5%**
Liver						
Hepatocellular adenoma	5	3	6	0	0	0
Hepatocellular carcinoma	3	7	5	0	0	0
Haemangioma	0	2	2	0	0	0
Lung						
Adenoma	2	3	0	0	0	2
Adenocarcinoma	1	1	0	0	0	0
Mammary gland						
Fibroadenoma	NA	NA	NA	0	2	0
Haematopoietic system						
Malignant lymphoma / leukaemia	3	1	0	0	NA	0
Stomach						
Papilloma	1	1	2	0	1	0
Uterus						
Endometrial stromal polyp	NA	NA	NA	8	4	11

NA: not assessed
[a] Number of occurrences observed in all the mice ($n = 50$, except the females at 5%, where $n = 49$). Exceptions included sex-specific observations and findings that were not seen in the control or high-dose group.
Source: Sano et al. (1996)

treated groups were similar to those in both male and female controls (no data were provided).

Based on the absence of carcinogenicity and dose-related systemic toxicity, the NOAEL was 5%, equal to 6658 and 8575 mg/kg bw per day for males and females, respectively, or 5380 and 6929 mg/kg bw per day when corrected for purity (Sano et al., 1996).

(b) Rats

Groups of Sprague Dawley rats (20/sex per group; 8 weeks old) were given diets containing tamarind seed polysaccharide (purity: 80.8%) at concentrations of 0, 4%, 8% or 12% for 104 weeks (equal to 1.2–2.5, 2.3–5.3 and 3.8–8.3 g/kg bw per day for males and 1.4–3.1, 3.0–6.7 and 4.7–9.4 g/kg bw per day for females, respectively). The study was not described as having been conducted according to GLP or OECD guidelines.

The animals were observed daily for mortality and signs of clinical toxicity. Body weight and feed consumption were recorded every fourth week. Animals in extremis were euthanized. All animals were necropsied. Organ weights were recorded for all animals, and organ to body weight ratios were calculated for brain, heart, lung, liver, kidney, spleen, pituitary, thyroid, adrenal gland, testis, ovary and uterus. Organ and tissue samples of heart, aorta, lung, liver, kidney, urinary bladder, spleen, pancreas, tongue, submaxillary gland, stomach, mesentery, thyroid, adrenal, parathyroid, testis, prostate, bone, skeletal muscle, colon, ovary, uterus, bone femur and bone marrow were histopathologically examined. Blood samples taken by heart puncture at termination were chemically analysed for glucose, calcium, inorganic phosphorus, urea nitrogen, creatinine, cholesterol, total protein, albumin, albumin/globulin ratio, total bilirubin, lactate dehydrogenase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, sodium, potassium and chlorine. Additional blood samples were taken from amputated tail segments to assess haematological parameters, which included red blood cell concentration, haemoglobin concentration, haematocrit, clotting time, white blood cell concentration and differentiation of white blood cells. Urine samples were collected from animals housed in metabolic cages for 18 hours at weeks 6, 19, 31, 43, 57, 67, 85 and 104 for males and 6, 18, 30, 44, 58, 70, 85 and 103 for females. Urine analysis included assessment of urine volume as well as protein, urea nitrogen, creatinine, sodium, potassium and chlorine concentrations.

Survival, though poor, did not differ significantly in treated and control groups; in the control, 4%, 8% and 12% groups, 25%, 55%, 35% and 55% of males and 55%, 65%, 60% and 40% of females, respectively, survived to 104 weeks. No explanation was given for the higher than expected death rate in the male control group. Almost all animals survived up to week 70, after which survival decreased (Table 4). Control group males had the poorest survival; 65% survived at week 81 whereas all other groups had at least 80% survival. There was no dose-related effect on survival.

The authors stated that no differences in clinical signs of toxicity between the treated and control groups were observed (limited information provided).

There were no significant differences between the treated and control groups with respect to mean body weights and mean feed consumption (feed consumption data not provided).

Iida et al. (1978) reported that males consumed between 1.2 and 2.5, 2.3 and 5.3, and 4.3 and 8.3 g/kg bw per day and females between 1.5 and 3.1, 3.4 and 6.7 and 5.4 and 9.4 g/kg bw per day, respectively, at 4%, 8% and 12%. Based on data in the original laboratory report (Iida et al., 1977), the Committee calculated weighted averages for the different dose groups and sexes. Accordingly, at 4%, 8%

Table 4
Survival of rats fed tamarind seed polysaccharide in the diet for 2 years

	No. of surviving rats							
	Male				Female			
Weeks	**Control**	**4%**	**8%**	**12%**	**Control**	**4%**	**8%**	**12%**
0–4	20	20	20	20	20	20	20	20
69–72	18	17	19	17	19	18	19	16
73–76	16	17	16	17	19	18	19	16
77–80	13	16	16	17	19	18	19	16
81–84	13	16	16	17	18	17	19	16
85–88	12	14	16	16	18	16	19	15
89–92	11	14	14	16	16	16	17	12
93–96	9	13	9	15	15	14	17	10
97–100	7	11	8	13	13	13	13	10
101–104	5	11	7	11	11	13	12	8

no: number
Source: Iida et al. (1978)

and 12%, males consumed 1.62, 3.29 and 5.15 g/kg bw per day and females 1.93, 4.00 and 6.07 g/kg bw per day, respectively.

Individual and mean body weights and organ weights were presented separately for surviving animals and for animals that died during the study. In surviving animals, there was no dose-related effect observed with body or organ weight differences in either sex.

Histopathological findings were presented for all animals, as well as separately for surviving animals and animals that died during the study. All effects were age-related and none were treatment-related. Statistically significant differences between treated and control male groups were observed in the incidences of nephropathy and seminiferous tubule atrophy, but the incidence in the treated groups was lower than in the control groups and the effect was not dose-related. No other statistically significant differences were observed in males. A statistically significant difference between treated and control female groups was observed in the incidence of myocardial degeneration. An increased incidence was only observed in the 8% dose group and not the higher 12% dose group, and therefore the effect was considered not treatment-related. No other statistically significant differences between treated and control females were observed with respect to histopathological findings.

Tumour incidences were presented for all animals, as well as separately for surviving animals and for most animals that died or were killed in extremis during the study. The most frequently observed tumours were considered age-related and typical of this strain of rat (e.g. pituitary adenomas, mammary gland

tumours). No statistically significant increases in any tumour type were observed in treated males or females compared with controls except for the total incidence of mammary tumours in females at 4%, which nevertheless was not considered treatment-related.

Haematological assessment showed no statistically significant differences between treated and control groups with respect to any measured parameter in either sex. Blood chemistry assessment showed a statistically significant decrease in alkaline phosphatase activity in males at 12% compared with controls (−9%; $P < 0.05$). Statistically significant decreases in alanine transferase activity and blood urea nitrogen in females at 4% and 12% and a statistically significant reduction in potassium in females at 8% compared with the controls were observed. The study authors considered these changes slight and not toxicologically relevant as they were inconsistent with adverse reactions (i.e. decreases were observed). Urinary protein decreased statistically significantly in males at 12% compared with controls (−46%; $P < 0.05$), although this decrease was not considered toxicologically relevant. No urine analysis effects were observed in treated females.

Based on the absence of carcinogenicity and lack of dose-related systemic toxicity, the NOAEL was the highest concentration tested, equal to 5150 mg/kg bw per day for males and 6070 mg/kg bw per day for females, or 4161 and 4904 mg/kg bw per day, respectively, when corrected for purity (Iida et al., 1978).

2.2.4 Genotoxicity

The results of four in vitro genotoxicity assays with tamarind seed polysaccharide are summarized in Table 5. One bacterial reverse mutation study (Schreib, 2012; reported in Heimbach et al., 2013) was conducted in compliance with GLP and OECD Test Guideline 471 (1997), whereas another (Miyabe, 1993) was conducted in compliance with Japanese Ministry of Labour guidelines. Neither Kurita (1993) nor Ishidate, Sofuni & Kishi (1985), respectively, specified if the DNA repair assay and chromosomal aberration assay they conducted were GLP or guideline compliant. The test results were negative in all the assays.

Despite the limitations of some of these assays (e.g. solubility at higher concentrations), based on the negative results and the absence of chemical structural alerts the Committee concluded that there was no genotoxic concern with tamarind seed polysaccharide.

2.2.5 Reproductive and developmental toxicity

No data were available from specific studies on reproductive or developmental toxicity. Short- and long-term studies in mice and rats found no treatment-related adverse effects on reproductive tissues.

Table 5
Results of genotoxicity assays with tamarind seed polysaccharide

Test system	Test object	Concentration / dose (purity)	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2uvrA	0, 10.0, 31.6, 100, 316, 1 000, 2 500 and 5 000 µg/plate (purity: 85.5%; lot no. 12.02.28-1)	Negative	Schreib (2012)[a]
Reverse mutation	*S. typhimurium* TA98 and TA100	0, 0.2, 0.5, 1, 2, 5, 10 and 20 mg per plate (purity: 80.8%)	Negative	Miyabe (1993)[b]
DNA repair	*Bacillus subtilis* H17 and M45	15.6, 31.3, 62.5, 125, 250 and 500 µg per disc (purity: 80.8%)	Negative	Kurita (1993)[c]
Chromosomal aberration	Chinese hamster lung derived fibroblast cells	Maximum 2.0 mg/mL (purity: 80.8%)	Negative	Ishidate, Sofuni & Kishi (1985)[d]

DNA: deoxyribonucleic acid

[a] Plate incorporation and preincubation methods were used with and without metabolic activation (S9 liver microsomal fraction derived from male Sprague Dawley rats induced with phenobarbital/β-naphthoflavone). The solvent control was distilled water. A specific positive control was used for each tester strain and for each metabolic condition (i.e. with or without metabolic activation). Precipitation was observed in all tester strains when concentrations were 316 µg/plate or higher (with or without metabolic activation). No cytotoxicity was observed at any concentration tested.

[b] Preincubation method used with and without metabolic activation (S9 liver microsomal fraction derived from Sprague Dawley rats induced with phenobarbital/β-naphthoflavone). The solvent control was distilled water. No positive control was used. Two plates were tested at each concentration of the test material, and six plates were used for the solvent control. Summary data were provided.

[c] Assay was conducted with and without metabolic activation (S9 liver microsomal fraction derived from male Sprague Dawley rats induced with phenobarbital/β-naphthoflavone). The solvent control was sterile pure water. A positive control was used with or without metabolic activation (tryptophan pyrolysate 1 and mitomycin C, respectively). Detailed data were provided.

[d] The test material was incubated with the test object for 48 hours. The percentage of chromosomal structural aberrations was determined in 100 metaphases. The solvent control was physiological saline. No positive control was used. The authors noted that higher concentrations could not be tested due to problems with solubility. Summary data were provided.

2.2.6 Special studies – Allergenicity

The potential antigenicity of tamarind seed polysaccharide and tamarind kernel powder was assessed in sensitized guinea-pigs (Dainippon Pharmaceutical Co., Ltd, 1963) and in isolated guinea-pig intestinal tract using the Schultz-Dale method (on exposure to an antigen, contraction of smooth muscle, possibly due to histamine release, is considered an early step in a general anaphylactic reaction) (Geiger & Alpers, 1959).

Guinea-pigs (200–300 g bw; number and sex of animals per group and maintenance conditions not stated) were sensitized with tamarind seed polysaccharide (2% solution; purity 80%; no more than 3% protein), tamarind kernel powder (2% solution of commercial tamarind kernel powder; range of protein 16.9–22.7%) or egg albumen (0.2% solution) by three intraperitoneal injections of 0.5 mL/animal per day every other day for an unstated number of days (possibly 2 days of injections, based on the Schultz-Dale method). The animals were subjected to further experimentation 3 weeks after the last injection. A group of nonsensitized animals were also included.

Sensitized and nonsensitized guinea-pigs were challenged with a single intraperitoneal injection of a 2% tamarind seed polysaccharide or tamarind kernel

powder solution (0.5 mL/animal). Animals sensitized to tamarind kernel powder solution showed signs of mild anaphylactic shock, which included stimulated respiration, mild hiccups, incontinence and piloerection. Animals sensitized to tamarind seed polysaccharide and nonsensitized animals did not react to any challenge (Dainippon Pharmaceutical Co., Ltd, 1963).

Geiger & Alpers (1959) conducted an ex vivo experiment using a Magnus instrument and the Schultz-Dale method. Strips of intestinal tract from sensitized and nonsensitized guinea-pigs were assessed for their reactivity (ability to contract) to solutions of egg albumen, tamarind seed polysaccharide or tamarind kernel powder (all treatments used 0.2 mL of 2% solutions). Intestinal movements were recorded for 3 minutes after treatment with each of the solutions. Intestinal strips from animals sensitized with egg albumen or tamarind kernel powder reacted when treated with a corresponding solution. Intestinal strips from animals sensitized with tamarind seed polysaccharide did not react with a corresponding solution. Intestinal strips from nonsensitized animals did not react with any of the test solutions.

The authors of the study concluded that under the test conditions, tamarind kernel powder was antigenic and that tamarind seed polysaccharide was not.

The Committee considered the study of poor scientific quality and that it did not contribute to this safety assessment.

2.3 Observations in humans

Tamarind kernel powder is used as a sizing agent in textile and paper industries. Published case reports describe acute respiratory reactions in industry workers after inhalation of tamarind seed kernel powder (Murray, Dingwall-Fordyce & Lane, 1957; Tuffnell & Dingwall-Fordyce, 1957). The Committee considered these findings not relevant to this dietary exposure assessment.

No reports were found on food allergies or food intolerance following ingestion of tamarind seed polysaccharide. Given its long time use in several countries and the absence of reported intolerances or food allergies, either these populations have developed tolerance or allergy or intolerance to tamarind seed polysaccharide is uncommon.

In a randomized, open-label, parallel group, multicentre, controlled clinical study (Gnessi et al., 2015), individuals with acute diarrhoea (50/group) ingested two oral capsules containing xyloglucan (origin and description of xyloglucan not stated) and gelatine (Tasectan Plus®; 100 mg xyloglucan and 250 mg gelatine per capsule) every 6 hours for 3 days (to a total of 800 mg xyloglucan

per person per day) or another treatment (diosmectite or *Saccharomyces boulardii*). Xyloglucan was well tolerated and not associated with any adverse event.

Due to the insufficient description of the products used in this study, the Committee did not consider this study useful for safety assessment.

3. Dietary exposure

3.1 Dietary exposure estimates

The Committee received an assessment of dietary exposure to tamarind seed polysaccharides from one sponsor in response to the call for data.

3.1.1 Budget method

The Committee concluded that no screening by the budget method would be appropriate for this assessment.

3.1.2 Production volume data

The per capita dietary exposure to tamarind seed polysaccharide in Japan was calculated based on the quantity produced in 2011 (787 370 kg), the total population of Japan (128 million) and a food wastage rate, assumed to be 20%. The body weight used was 55.1 kg, based on the mean body weight of the adult Japanese population as specified by the Ministry of Health, Labour and Welfare (MHLW) and Food Safety Commission. Dietary exposure was estimated at 13.5 mg/person per day (0.24 mg/kg bw per day for a person weighing 55.1 kg).

3.2 International estimates of dietary exposure

The Committee concluded that because tamarind seed polysaccharide (like all gums used for the same technical effects) is used exclusively in processed foods, it would be inappropriate to estimate exposure using the commodity-level food consumption data used to derive the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) cluster diets. Therefore, there are no international estimates of exposure for tamarind seed polysaccharide.

3.3 National estimates of dietary exposure

Two national estimates of dietary exposure to tamarind seed polysaccharide were submitted for review by the Committee: Japan and the USA. For the safety assessment, the Committee adjusted the dietary exposure estimates to account for the content of tamarind seed polysaccharide in the test articles (85%) using a factor of 0.85.

3.3.1 Japan

Tamarind seed polysaccharide has long been available as a food additive in Japan and is used in many food products. The food categories and typical (0.1–0.5%) and maximum (0.2– 1.5%) use levels are based on use experience and study results for tamarind seed polysaccharide in Japan (Table 6). Tamarind seed polysaccharide usage is technologically self-limiting because food products become unacceptably viscous at higher usage levels.

Estimates of dietary exposure to tamarind seed polysaccharide were made by combining the use levels with 2014 food consumption data from the Japanese National Health and Nutrition Survey (Ministry of Health, Labour and Welfare, 2014). In the survey, 3648 households were randomly selected from 300 districts so that 8047 individuals aged 1 year and older participated. Both the typical and the maximum levels were combined with food consumption data for each category. The mean estimated dietary exposure per capita of tamarind seed polysaccharide was 39.5 mg/person per day (0.72 mg/kg bw per day for an individual weighing 55 kg) at the typical use level and 101.9 mg/person per day (1.85 mg/kg bw per day for an individual weighing 55 kg) at the maximum use level.

A market share factor of 5% was used to make these estimates, based on the assumption that tamarind seed polysaccharide production was only 4.4% of all thickeners produced in Japan. The Committee concluded that the use of this factor was inappropriate as it does not take product brand loyalty into account. Consequently, the Committee considered the dietary exposures to be 20-fold higher than those submitted to the Secretariat: the mean, typical use dietary exposure would be 790 mg/person per day (14 mg/kg bw per day) and the maximum use dietary exposures would be 2.0 g/person per day (37 mg/kg bw per day). After adjusting for the 85% polysaccharide in the test articles, the dietary exposures were estimated to be 670 mg per person per day (12 mg/kg bw per day) and 1.7 g per person per day (31 mg/kg bw per day).

3.3.2 United States of America

Estimates of exposure to tamarind seed polysaccharide in the USA were prepared in a manner parallel to that used for the Japanese assessment. Food consumption

Table 6
Food categories and use levels for tamarind seed polysaccharide in Japan

Food category			Codex GSFA food category number	Typical use level		Maximum use level	
				mg/kg	(%)	mg/kg	(%)
Cereals products	Wheat flour / flour	Bread (except sweet bread)	07.0	3 000	0.3	5 000	0.5
		Sweet bread	07.0	3 000	0.3	5 000	0.5
			01.7				
			02.4				
			04.1.2.8				
			05.1.3				
			10.4				
		Udon and fresh Chinese noodles	06.4	3 000	0.3	5 000	0.5
		Instant Chinese noodles	06.4	3 000	0.3	5 000	0.5
		Pasta	06.4	3 000	0.3	5 000	0.5
		Other flour products	06.6	3 000	0.3	5 000	0.5
Vegetables	Pickles		04.2.2.3	5 000	0.5	10 000	1.0
			04.2.2.7				
			04.2.2.8				
Fruit	Jam		04.1.2.5	5 000	0.5	10 000	1.0
			04.2.2.8				
	Fruit juice / fruit beverages		14.1.3	1 000	0.1	2 000	0.2
Fish and shellfish	Processed fish products	Tsukudani (boiled foods in sweetened soy sauce)	09.2.4.1	5 000	0.5	10 000	1.0
Meat	Meat	Ham and sausages	08.2.2	3 000	0.3	10 000	1.0
			08.3.2				
			08.3.3				
Milk	Milk and dairy products	Fermented milk and lactic acid bacteria beverages	01.2.1	1 000	0.1	5 000	0.5
		Other dairy products	01.4				
			01.7				
			03.0	1 000	0.1	5 000	0.5
Confectionery		Japanese traditional confectionery	04.2.2.6	5 000	0.5	15 000	1.5
			06.5				

Table 6 (continued)

Food category			Codex GSFA food category number	Typical use level		Maximum use level	
				mg/kg	(%)	mg/kg	(%)
		Cakes, buns and pastries	07.0	1 000	0.1	5 000	0.5
		Cookies	07.0	1 000	0.1	5 000	0.5
		Other confectionery	04.1.2.5	5 000	0.5	10 000	1.0
Beverages	Other beverages	Coffee and cocoa	01.1.2	1 000	0.1	2 000	0.2
Seasonings and spices	Seasonings	Sauces	12.6	5 000	0.5	10 000	1.0
		Mayonnaise	12.6 12.7	5 000	0.5	10 000	1.0
		Other seasonings	12.5 12.9.2.3	2 000	0.2	10 000	1.0
	Spices and others		12.4	1 000	0.1	10 000	1.0

GSFA: General Standard for Food Additives

data were taken from the 2003–2006 National Health and Nutrition Examination Surveys (NHANES) for all categories of food that might contain tamarind seed polysaccharide (CDC, 2006). A total of 16 783 individuals participated in the surveys during this period. These data were combined with the proposed maximum use levels for each category and summed to give a per capita estimate of the mean exposure. Market share was assumed to be 100%, and it was also assumed that all foods in each category would contain tamarind seed polysaccharide. As almost 100% of those surveyed consumed foods from at least one of the food categories in which tamarind seed polysaccharide is intended for use, the per capita exposures were identical to those for consumers.

Tamarind seed polysaccharide exposure is concentrated in three food categories: beverages, flour products, and sauces and condiments. The estimated mean and 90th percentile daily exposures to tamarind seed polysaccharide were reported to be 2.6 and 4.4 g/person per day, respectively. Expressed in terms of body weight (60 kg), the estimated mean and 90th percentile daily dietary exposures were 45 and 91 mg/kg bw per day, respectively. Adjustment of these estimates to account for the 85% content of tamarind seed polysaccharide in the test articles gives estimates of 2.2 and 3.7 g/person per day for the mean and 90th percentiles, respectively (38 and 77 mg/kg bw per day). The food categories and proposed maximum use levels for this analysis are shown in Table 7.

Table 7
USA food categories and maximum use levels for tamarind seed polysaccharide

Food category	Examples	Codex GSFA food category number	Maximum use level (%)
Ice cream	Ice cream, sorbet, gelato, frozen yogurt	01.7 02.4 03.0	0.3
Sauces and condiments	Barbecue, steak, demiglace, tomato, chilli, tabasco, curry, teriyaki, tare sauces; ketchup; gravy Tonkatsu, korokke, yakisoba, okonomiyaki sauces	12.6 12.4 12.9.2.3	1.5
Mayonnaise and dressings	Mayonnaise, reduced fat mayonnaise; Caesar, French, Italian, Ranch, Thousand Island, wafu dressings	12.6 12.7	1.0
Fruit preserves	Fruit spread, jam, jelly, apple sauce	04.1.2.5 0 4.1.2.8 0 4.1.2.9	1.0
Desserts	Pudding, Bavarian cream, mousse	01.7 10.4	0.2
Beverages	Fruit juice, reduced fat milk, cocoa drink	01.1.2 06.8.1 14.1.3 14.1.4.2	0.2
Pickles	Tsukemono (pickled foods), kimchi, pickled cucumber, pickled olives, pickled sauerkraut	04.2.2.3 04.2.2.7	1.0
Tsukudani (boiled foods in sweetened soy sauce)	Laver, mushroom and kelp tsukudani	04.2.2.8	1.0
Spreads and fillings	Custard cream, spreads	01.6.1 01.7 02.2.2 02.4 04.1.2.8 04.2.2.6 05.1.3 10.4	0.5
Flour products	Bread, pastry, cake, instant noodles, ramen, udon, dough, batter	06.4 06.6 07.0	0.5
Soups	Broth, consommé, creamy soups	12.5	0.2
All other food categories		01.4 01.7 01.6.1 01.6.4 01.6.5 04.1.2.9 05.1.4 05.1.5 05.2.2 05.3 05.4 11.4 12.6 13.4 13.5	0.5

GSFA: General Standard for Food Additives

4. Comments

4.1 Biochemical aspects

Absorption, distribution, metabolism or excretion data were not available on tamarind seed polysaccharide. Based on its size and chemical composition, tamarind seed polysaccharide, like other dietary fibres, is not expected to be absorbed intact or digested in the gastrointestinal tract (Cummings & Englyst, 1987). Based on its chemical composition, tamarind seed polysaccharide is expected to be enzymatically degraded and fermented by intestinal bacteria in the large intestine. The fermentation process would yield hydrogen gas, carbon dioxide and short-chain fatty acids, which could be absorbed and metabolized.

It has been estimated that more than 75% of tamarind seed polysaccharide is fermented (Ministry of Health, Labour and Welfare, 2003). This extensive fermentation process is similar to that for other nondigestive polysaccharides, such as carob bean gum, cassia gum and tara gum.

Evidence supporting such a fermentation process includes the results of a 14-day dietary study in rats, which showed that oligosaccharides of tamarind seed polysaccharide generate short-chain fatty acids (specifically, lactic acid, propionic acid and butyric acid) in the caeca of test animals in greater amounts than in control rats fed a non-fibre diet (Ebihara & Nakamoto, 1998). In vitro studies demonstrated that human microflora can also degrade and ferment tamarind seed polysaccharide (Hartemink et al., 1996). Specific bacteria that colonize the large intestine in humans are capable of enzymatic hydrolysis of the glucan backbone of xyloglucans, which would lead to fermentation (Hartemink et al., 1996; Larsbrink et al., 2014).

4.2 Toxicological studies

All toxicological tests were conducted using a commercial product in which the purity of the tamarind seed polysaccharide was between 80% and 85%. The remaining 15–20% included water, carbohydrates, protein and fat, which are normal dietary constituents that are not expected to pose a toxicological hazard. Tamarind seed polysaccharide is of low acute oral toxicity in mice and rats. The LD_{50} in each of these species was greater than 5000 mg/kg bw (4000 mg/kg bw when corrected for purity).

No toxicity was observed in a 13-week study in mice at concentrations of up to 50 000 mg/kg feed (equal to 8200 mg/kg bw per day, or 6642 mg/kg bw per day when corrected for purity) (Sano et al., 1996). There were no toxicologically relevant effects in a 4-week dietary study of tamarind seed polysaccharide in rats

at concentrations up to 120 000 mg/kg feed (equal to 10 597 mg/kg bw per day, or 9113 mg/kg bw per day when corrected for purity) (Heimbach et al., 2013; Koetzner, 2013).

Similarly, no toxicologically relevant effects, including treatment-related tumours, were observed in a 78-week study in mice at concentrations of up to 50 000 mg/kg feed (equal to 6658 mg/kg bw per day, or 5380 mg/kg bw per day when corrected for purity) (Sano et al., 1996). No treatment-related toxicity, including tumours, was observed in a 104-week study in rats at concentrations of up to 120 000 mg/kg feed (equal to 5150 mg/kg bw per day, or 4161 mg/kg bw per day when corrected for purity) (Iida et al., 1978). The highest doses tested in these toxicity studies routinely equalled or exceeded the recommended dose limit of 5% of the diet for rodent toxicity studies.

The Committee concluded that the pivotal study was the 104-week study in rats (Iida et al., 1978). This was a well-conducted study performed before the implementation of GLP. The NOAEL was 5150 mg/kg bw per day (corrected to 4161 mg/kg bw per day for purity), the highest dose tested.

Tamarind seed polysaccharide tested negative in bacterial reverse mutation assays and in an in vitro chromosomal aberration assay. Despite the limitations of some of these assays (due to the poor solubility of the test substance at higher concentrations), based on the absence of chemical structural alerts and negative results, the Committee concluded that for tamarind seed polysaccharide, there was no concern with respect to genotoxicity.

No reproductive or developmental toxicity studies were conducted with tamarind seed polysaccharide. The Committee noted that histopathological analysis of reproductive organs from long-term feeding studies in mice and rats did not identify any effects on reproductive tissues. The Committee also noted that reproductive and developmental toxicity studies on other polysaccharide gums previously evaluated by the Committee did not raise concerns for reproductive or developmental effects. For example, when cassia gum was assessed in a two-generation reproductive toxicity study in rats, it was shown not to cause reproductive toxicity at 50 000 mg/kg feed (equal to 5280 mg/kg bw per day), the highest concentration tested. In a developmental toxicity study in rats, cassia gum did not cause embryotoxicity or teratogenicity at 1000 mg/kg bw per day, the highest dose tested. In a developmental toxicity study in rabbits, cassia gum did not cause any adverse effects on dams or numbers of implantations, postimplantation losses or fetal defects at 1000 mg/kg bw per day, the highest dose tested.

Based on the absence of histopathological effects on reproductive tissues in long-term rodent studies, the lack of absorption of intact tamarind seed polysaccharide, the degradation and fermentation of tamarind seed polysaccharide into normal dietary constituents, and the absence of reproductive

or developmental toxicity observed with other polysaccharide gums, the Committee concluded that tamarind seed polysaccharide would be unlikely to pose a concern with respect to reproductive or developmental toxicity.

4.3 Observations in humans

No reports were found on food allergies or food intolerance to tamarind seed polysaccharide, despite its long-term use in several countries.

4.4 Assessment of dietary exposure

The Committee received an assessment of dietary exposure to tamarind seed polysaccharide from one sponsor in response to the call for data.

Two national estimates of dietary exposure to tamarind seed polysaccharide were included in the sponsor's submission and reviewed by the Committee: from Japan and the USA. These estimates of dietary exposure to tamarind seed polysaccharide were made by combining maximum use levels (assuming 85% polysaccharide in the commercial product) with 2014 food consumption data from the Japanese National Health and Nutrition Survey or with 2003–2006 food consumption data from USA National Health and Nutrition Examination Surveys. The estimated mean dietary exposure to tamarind seed polysaccharide ranged from 31 to 38 mg/kg bw per day, with the 90th percentile exposures up to 77 mg/kg bw per day. These estimates are conservative, in that it has been assumed that all products that might contain tamarind seed polysaccharide would contain the substance at the indicated maximum use levels. Tamarind seed polysaccharide would be likely to substitute for other gums.

The Committee concluded that the estimated dietary exposure of 75 mg/kg bw per day was suitable for use in this safety assessment.

5. Evaluation

5.1 Recommendations

The Committee established an acceptable daily intake (ADI) "not specified" for tamarind seed polysaccharide. This ADI was based on the absence of toxicity in repeated-dose animal studies of tamarind seed polysaccharide. These included long-term rodent studies in which mice were fed up to 6658 mg/kg bw per day (corrected to 5380 mg/kg bw per day, for purity) and rats were fed up to

5150 mg/kg bw per day in rats (corrected to 4161 mg/kg bw per day, for purity). In addition, there was no concern regarding genotoxicity. Reproductive toxicity and developmental toxicity were not considered a concern based on the lack of absorption of intact polysaccharide, the degradation and fermentation of tamarind seed polysaccharide into normal dietary constituents, and the absence of reproductive and developmental effects in other polysaccharide gums.

The estimated dietary exposure based on proposed uses and use levels was 75 mg/kg bw per day. The Committee concluded that this does not present a health concern.

A new specifications monograph and a Chemical and Technical Assessment were prepared.

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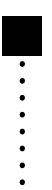

Yeast extracts containing mannoproteins

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1. Explanation

Yeast extracts containing mannoproteins are used as food additives in winemaking. Yeast mannoproteins are extracted from purified yeast (*Saccharomyces cerevisiae*) cell walls by enzymatic treatment with β-glucosidase or by physicochemical extraction with thermal treatment. Yeast mannoproteins are galactomannans consisting almost exclusively of mannose units bound to proteins or peptides.

The name was changed from "yeast mannoproteins" to "yeast extracts containing mannoproteins" because the name "yeast mannoproteins" was not adequately descriptive. The products in commerce are extracts containing yeast components and mannoproteins, and not pure mannoproteins. Yeast extracts containing mannoproteins have not been previously evaluated by the Committee. The compounds were evaluated at the present meeting at the request of the Forty-eighth Session of the Codex Committee on Food Additives (FAO/WHO, 2016). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) call asked for data on yeast mannoproteins in general; however, the only data that were submitted related to their use as a food additive in winemaking.

Wine contains significant concentrations of tartrates that can crystallize and precipitate during storage, resulting in unwanted sediment. Wine also contains small amounts of protein, which can produce a haze. Although yeast mannoproteins occur naturally in wine due to yeast fermentation, they are also added to inhibit the crystallization of tartrates and stabilize the proteins in the wine after bottling and during storage.

Yeast mannoproteins are approved for treatment of wine in Argentina, Australia, Canada, the European Union (Commission Regulation (EC) No. 2165/2005), New Zealand and the USA.

The sponsor submitted a dossier summarizing technological, toxicological and dietary exposure information relevant to the evaluation of yeast mannoproteins from *S. cerevisiae*. In addition, a literature search for toxicity data performed using multiple databases and search terms resulted in approximately 20 other potentially relevant papers. However, because few toxicological studies were available for yeast mannoproteins, relevant studies with *S. cerevisiae*, its constituents or substances derived from its fermentation were included in the assessment.

1.1 Chemical and technical considerations

Mannoproteins represent a large group of natural compounds from yeast (*S. cerevisiae*) in which polysaccharide chains are bound to proteins and peptides by covalent and noncovalent linkages (i.e. ionic interactions). The structures and molecular weights of mannoproteins vary, depending on the degree and type of glycosylation. The polysaccharide chains consist almost exclusively of mannose units linked by α-links forming a long α-1→6 linked backbone containing short α-1→2 and α-1→3 linked side-chains. Several of the side-chains may have phosphodiester linkages to other mannosyl residues. Yeast mannoproteins are extracted from purified yeast cell walls by enzymatic extraction using glucan 1,3-β-glucosidase (EC 3.2.1.58) or by thermal treatment. The enzyme hydrolyses the yeast cell wall, allowing the mannoproteins to be solubilized. The thermal treatment breaks the links with β-glucans in the cell wall to release the mannoproteins. The mannoproteins thus solubilized by either treatment are then separated from the insoluble cell wall material, concentrated and micro-filtered or ultra-filtered. The mannoproteins have molecular weights ranging from 20 kDa to more than 450 kDa.

There was limited information available to the Committee to fully characterize the yeast mannoprotein products in products of commerce. Information and data about the chemical composition of the range of commercial yeast mannoprotein products are required. There are also limited data available on the levels of yeast mannoproteins in wine. Wine contains yeast mannoproteins from the fermentation process as well as those added for the purpose of precipitating tartrates. This results in potential levels higher than 400 mg/L of yeast mannoproteins in the wine.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

In an in vivo study in rats (strain not indicated), Adrian & Frangne (1976) compared the digestibility of yeast cell walls in a control diet with one supplemented with 20% dried yeast cell walls. The digestibility of the supplemented diet was 92%, close to that of the control diet. The digestibility of the nitrogenous content was particularly high, indicating that cell wall proteins were available to the digestive tract proteases.

In an in vitro study, Moine-Ledoux (2003) compared the effect of reconstituted intestinal fluid (at pH 7.5 and containing pancreatic enzymes) on dry active yeasts, yeast cell walls and mannoproteins. Following incubation in the reconstituted intestinal fluid, mannoproteins were released from active dry yeasts and yeast cell walls. Further analysis of the mannoproteins in the reconstituted intestinal fluid showed that mannoproteins behave like proteins in terms of digestibility.

2.1.2 Biotransformation

Once mannoproteins have been hydrolysed by intestinal enzymes, further fermentation of the glucomannan moiety can occur through the action of bacterial glycoside hydrolases (glucomannanases and endoglucanases) in the large intestine (Liu et al. 2010; Bâgenholm 2017). These glycoside hydrolases hydrolyse the glycosidic chain into oligo- or monosaccharides, which may be subsequently processed in the regular glycosidic pathway into, among others, organic acids or alcohols (den Besten et al., 2013a,b).

2.1.3 Effects on enzymes and other biochemical parameters

In a study using immortalized human hepatocytes (Fa2N-4 cells), Schauss et al. (2012) found that a fermentation product of *S. cerevisiae* did not induce cytochrome P450 (CYP) CYP1A2 or CYP3A4 messenger ribonucleic acid (mRNA) or enzymatic activity and did not interfere with the induction of CYP1A2 by omeprazole or of CYP3A4 by rifampin (also known as rifampicin). The test article used in this study was described as the product of a proprietary fermentation process using *S. cerevisiae*, involving "both a unique substrate and a stress process". The test article, hereafter referred to as "yeast fermentate preparation", was also tested in several toxicology studies conducted by Schauss et al. (2012) (described in section 2.2). The whole liquid fermentate was dried, resulting in a product that contained the same nutrient/vitamin profile, cell wall

components including mannoproteins, and stress-induced defence metabolites. However, a more complete chemical characterization of the test article was not available.

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Acute oral toxicity

In a good laboratory practice (GLP)-compliant study that conformed with Organisation for Economic Co-operation and Development Test Guideline (OECD TG) 423, Sprague Dawley rats (*n* = 10/sex) received a single dose of 2000 mg/kg body weight (bw) of yeast fermentate preparation by gavage.

All treated rats survived to study end at day 15. No body-weight loss was noted and feed consumption was similar in treated and control rats. No clinical toxicological symptoms were observed and no gross pathological changes were found in the organs at necropsy (Schauss et al., 2012).

(b) Dermal irritation

In a GLP-compliant cutaneous irritation study conducted according to OECD TG 404, Richeux (2002ba) assessed the acute dermal irritant/corrosive effects of mannoproteins enzymatically extracted from *S. cerevisiae* yeast in albino New Zealand White rabbits. The test substance was applied (0.5 g) to the healthy skin of the right flank of each animal under a semi-occlusive dressing. On the left flank, and under the same conditions, 0.5 mL of distilled water was applied to an equivalent area of healthy skin. The cutaneous reactions were evaluated at 1, 24, 48 and 72 hours after the dressing was removed.

No macroscopic cutaneous reaction (erythema, oedema) was observed in any of the animals (Richeux, 2002a).

(c) Eye irritation

In a GLP-compliant ocular irritation study conducted according to OECD TG 405, Richeux (2002b) assessed the acute irritant/corrosive effect of mannoproteins enzymatically extracted from *S. cerevisiae* yeast by inserting the powder (0.1 g) directly into one eye of New Zealand White rabbits. The other eye served as control. Ocular reactions were evaluated at 1, 24, 48 and 72 hours after instillation.

Very mild ocular reactions (lachrymation and enanthema), limited to the conjunctiva, were observed 1 hour after application. These effects were totally reversed after 3 days (Richeux, 2002b).

2.2.2 Short-term studies of toxicity

González Pereyra et al. (2014) administered a daily dose of 10^8 viable cells or colony forming units (cfu) of *S. cerevisiae* RC016 suspended in phosphate-buffered saline (PBS) that was prepared daily to male Wistar rats (*n* = 6) by gavage for 60 days. There were no deaths or signs of illness. No changes were reported in the general health status of the animals, including behaviour, activity, posture, fur quality, feed and water intake. Histopathological examinations of liver, lungs, intestine and testes did not show any differences between treated and control animals (González Pereyra et al., 2014).

Schauss et al. (2012) conducted a GLP-compliant 90-day study in accordance with OECD TG 408 in Sprague Dawley rats (*n* = 80/sex; *n* = 20/group). The animals received by gavage 0, 30, 200 or 1500 mg/kg bw per day of a yeast fermentate preparation in water containing 1% methylcellulose. Direct ophthalmological examinations were performed on five males and five females of each dose group prior to treatment and on all high-dose and control animals once during week 11 of treatment. After the last treatment, blood samples were taken and examined for haematological and clinical chemistry parameters. Urine analysis was carried out once during week 12 on 10 males and 10 females. All animals underwent necropsy on day 91 after a 16-hour fast. Organ weights were recorded and histopathological examinations performed.

There were no deaths. No treatment-related changes in general state, external appearance or behaviour were observed in any of the animals. There were no significant differences in body weight or body weight gain compared with the controls. Amounts of food and water consumed was similar in all treated and control male and female rats. No ophthalmological changes were observed in control or high-dose groups. No treatment-related differences in the visual (finger approach) or auditory (startle) reactivity, pain perception (tall pinch), grip strength or motor activity were observed during examination of male and female rats at week 11. No treatment-related effects were observed in red blood cells, white blood cells, haemoglobin, haematocrit or platelet count values. There were no changes in blood coagulation rates as seen in prothrombin time measurements. Clinical chemistry data determined at the end of the 90-day study did not indicate any treatment-related toxicity. Individual values and group mean values were within physiological ranges. There were no differences in the volume, specific gravity or pH of urine between control and treated groups; nor were there any differences in glucose, blood or protein concentrations in the urine.

Gross necropsy found no treatment-related lesions. The few sporadic pathological changes observed were not treatment related. Internal examination showed that subcutaneous tissue, regional lymph nodes, fatty tissue, skeletal

muscles, joints and bone system were normal in all animals. No differences in the actual organ weights or relative organ weights were noted, and no treatment-related histopathological findings were observed. The test article was well tolerated at all doses for the duration of the 90-day study.

Based on these results, the no-observed-adverse-effect level (NOAEL) was 1500 mg/kg bw per day, the highest dose tested.

2.2.3 Long-term studies of toxicity and carcinogenicity

Schauss et al. (2012) conducted a GLP-compliant study, in accordance with the OECD TGs 408 and 452, in Sprague Dawley rats (n = 80/sex). The animals received 0, 20, 200 or 800 mg/kg bw per day of yeast fermentate preparation by gavage for 1 year. In addition to the examinations conducted as described in the 90-day study by the same authors (section 2.2.2), blood samples were collected at weeks 14 and 33 and before necropsy. Urine analysis was performed during weeks 13, 32 and 52.

Macroscopic, microscopic, serum chemistry, haematological, and histological examinations and urine analysis found no clinically significant results. Overall, the test article was well tolerated at all doses. A statistically significant decrease in water consumption over nonconsecutive weeks in the highest dose group was considered not clinically relevant. The nonneoplastic histopathological findings were spontaneous or incidental and typical of this strain of rat. The authors considered the low incidence of benign tumours in the female rats to be unrelated to treatment because of the lack of statistical significance and because the incidences fell within the historical control ranges (6.3–32% for mammary gland adenomas and 26.0–92.86% for pituitary adenomas).

Based on the lack of treatment-related results, the NOAEL was 800 mg/kg bw per day, the highest dose tested.

2.2.4 Genotoxicity

(a) In vitro

No evidence of genotoxicity was seen in a bacterial reverse mutation assay performed in accordance with OECD TG 471 on the yeast fermentate preparation (Ames test with *Salmonella typhimurium* strains TA97a, TA98, TA100, and TA1535, and *Escherichia coli* strain WP2 urvA [pKM101^{+}], with and without metabolic activation). Similarly, no evidence of genotoxicity was seen in a mouse lymphoma cell mutagenicity assay conducted in accordance with OECD TG 476 (mouse lymphoma L5178Y) (Schauss et al., 2012).

(b) In vivo

Madrigal-Santillán et al. (2010) examined the antigenotoxic effects of *S. cerevisiae* and its extracts using micronucleus assay, comet assay and sister chromatid exchange in mice. The researchers reported evidence for antimutagenic capacity of mannans (mannose with α-1,6 links and α-1,2 and α-1,3 branched side-chains) and glucans (glucose with links α-1,6 links and β-1,2 and β-1,3) branched side-chains) against the genotoxic effects of antineoplastic compounds such as cyclophosphamide and mitomycin C (Madrigal-Santillán et al., 2010).

González Pereyra et al. (2014) fed male Wistar rats a commercial basal diet that provided a daily dose of 10^8 viable cells or cfu of *S. cerevisiae* RC016 suspended in PBS (prepared daily) for 60 days. Genotoxicity and cytotoxicity were evaluated using the bone marrow micronucleus assay and the comet assay. Internal organs were macroscopically and microscopically examined.

Dietary administration of *S. cerevisiae* RC016 did not induce cytotoxicity or genotoxicity in rats (González Pereyra et al., 2014).

2.2.5 Reproductive and developmental toxicity studies

No data were available.

2.2.6 Special studies

(a) Allergenicity

A GLP-compliant sensitization study was conducted according to OECD TG 406 using an extract containing mannoproteins enzymatically extracted from *S. cerevisiae* yeast in albino guinea-pigs (Richeux, 2002c). Preliminary tests showed no necrosis after intradermal injection at the highest dose (40%). Topical application kept under an occlusive dressing for 24 hours at the maximum dose of 100% induced no cutaneous reaction. In contrast, a topical application kept under an occlusive dressing for 24 hours at the maximum dose of 100% after intradermal induction with physiological serum and topical application of distilled water (negative control) induced a slight erythema in two animals treated with the highest dose. After the first release phase, a macroscopic cutaneous reaction was noted (moderate erythema) in 5% of the animals in the treated group (1/20), 24 and 48 hours after removal of the occlusive dressing, at the 50% treated site. No reaction of cutaneous intolerance was observed in the negative controls or the 25% treated group. A second release phase was performed to confirm or invalidate these results after 11 days of rest. No macroscopic cutaneous reaction was noted following removal of the occlusive dressings.

Posadas et al. (2010) reported that 10% and 15% administration of mannoprotein E1, a mannoprotein-rich product from *S. cerevisiae*, in a liquid diet had a protective effect on intestinal tissue in Wistar rats (*n* = 58) infected with *S. typhimurium*. This protection was expressed as a lower pro-inflammatory response, with decreased production of the pro-inflammatory cytokines interleukin (IL)-6, tumour necrosis factor alpha (TNF-α) and IL-1β, and downregulation of toll-like receptor 5 (TLR5) in gut epithelium, as well as by inhibiting apoptosis.

(b) Animal nutrition studies

Póo & Millán (1990) and Aziz et al. (1997) showed that substitution of 50% of the protein ration in feed with *S. carlsbergensis* cells had no metabolic effects. Accordingly, in the European Union, *S. cerevisiae* yeasts or lysates are permitted in animal feed for any species, with no restriction on the amounts used (Directive 82/471/EEC and amendments; European Commission, 1982).

(c) Immune response

In a GLP-compliant study conducted in accordance with United States Food and Drug Administration (USFDA) guidelines, a yeast fermentate preparation was not mitogenic for human peripheral lymphocytes (Schauss et al., 2012).

Yuan et al. (2015) assessed the effects on immunity and uterine inflammation in transition cows of a yeast product derived from *S. cerevisiae*. Barn blocks of multiparous Holstein cows were randomly assigned to one of four treatments (*n* = 10/group) from 21 days before expected parturition to day 42 postpartum. Rations were supplemented with a product containing yeast culture plus enzymatically hydrolysed yeast (YC-EHY) at 0, 30, 60 or 90 g/day throughout the experiment. Cows were injected subcutaneously with ovalbumin on day −21, −7 and 14 to assess their humoral immune response.

Concentrations of colostrum immunoglobulin G were unaffected by treatments. Platelet count was increased by addition of YC-EHY. Increasing YC-EHY dose directly correlated with increases in plasma anti-ovalbumin immunoglobulin G levels following three ovalbumin challenges, suggesting that the treatments resulted in an enhanced humoral immunity. Increasing YC-EHY dose also correlated with quadratically increased faecal immunoglobulin A concentrations during early lactation. Uterine neutrophil counts were much greater in samples collected on day 7 than in those collected on day 42 (32.1 versus 7.6 ± 3.5% of cells), reflecting neutrophil infiltration immediately after calving, but no treatment-related effect was detected. Significant day effects were detected for mRNA of IL-6, IL-8, neutrophil myeloperoxidase and neutrophil elastase in

the uterine samples, reflecting greater abundance of these transcripts collected on day 7 than on day 42. The mRNA abundance of neutrophil myeloperoxidase and neutrophil elastase increased linearly with YC-EHY dose.

The authors concluded that supplementation with YC-EHY enhanced humoral and mucosal immunity and modulated uterine inflammatory signals and mammary gland health in transition dairy cows.

2.2.7 Special studies on receptors

Mannose is important in protein glycosylation. Its involvement as a component of diverse glycoepitopes in intra- and intercellular immunological processes is gaining recognition (Loke et al., 2016). Like other galactomannans, mannoproteins can interact with the mannose receptor, a G-coupled protein expressed on the cell surface and in the cytoplasm of various cells (Wild, Robinson & Winchester, 1983; Ezekowitz & Stahl, 1988; Tizard et al., 1989; Takahashi et al., 1998; Régnier-Vigouroux, 2003). Mannose receptors are also present in a soluble form in serum. Mannose receptors are a collectin of the family of C-type lectins, which require calcium for efficient binding (Hansen et al., 2016); as mannose receptors are capable of specifically recognizing mannose, they are also called mannose-binding lectin (Loh et al., 2017).

Mannose receptors bind mannose conjugated with structures such as polysaccharides and glycoproteins. They also bind free mannose, though less efficiently. Mannose receptors are expressed in the gastrointestinal tract and in a variety of cells (macrophages, dendritic cells and endothelial cells) in different organs (liver, spleen, lung, kidney, muscle, brain and the lymphatic system). Consequently, mannosylated proteins and mannose receptors may be involved in physiological mechanisms in the following ways:

- Innate immunity: by recognizing and facilitating (acting as an opsonin) the uptake by macrophages of microorganisms that express mannose on their outer membranes (Cui, Hsu & Mumper, 2003) and by activating the complement system (van Asbeck et al., 2008; Beltrame et al., 2015), which may in turn induce inflammatory reactions (Yamamoto et al., 1997). But mannose receptors also facilitate entry of microorganisms (Takahashi et al., 2012; de Pasquale et al., 2013; Borggren & Jansson, 2015) into macrophages or dendritic cells.
- Specific immunity: as mannose receptors expressed on dendritic cells contribute to the regulation of T-cell functionality (Burgdorf, Lukacs-Kornek & Kurts, 2006; Li et al., 2010; Ramberg et al., 2010; Schuette et al., 2016).

- Intracellular calcium regulation: binding of mannose glycoproteins to mannose receptors may result in increased intracellular calcium levels (Blackmore & Eisoldt, 1999).

In conclusion, increased intake of mannoproteins may be beneficial (impaired fixation of bacteria on gastrointestinal epithelial cells leads to a decreasing risk of infection) or detrimental (blockage of mannose receptors on macrophages resulting in a decrease in their capacity to eliminate infectious organisms that have an outer membrane rich in mannose) (Allavena et al., 2004).

The Committee acknowledged that mannoproteins might have some physiological effects following their binding to mannose receptors. However, the interpretation and relevance of this effect is still a matter of research and the results of studies in this area were not considered in this evaluation.

2.3 Observations in humans

Jensen et al. (2015) studied the anti-inflammatory properties of a dried fermentate derived from *S. cerevisiae* in vitro using cell-based bioassays, and in healthy humans using a topical application of the fermentate. Treatment of primary human polymorphonuclear cells in vitro resulted in reduced formation of reactive oxygen species and migratory activity towards inflammatory mediator leukotriene B4. In vivo, inflammatory responses to histamine-induced skin inflammation were significantly reduced at the inflamed sites treated with dried fermentate compared with the sites treated with placebo based on subjective scores of irritation ($P < 0.05$).

Bansal, Tadros & Bansal (2017) reported a case of allergy to beer, wine and cider resulting from immunoglobulin E reactivity to yeasts and moulds. The authors concluded that although cases of yeast allergy are extremely rare in the medical literature, they may in fact be under-recognized and should be considered in patients with reactions to alcoholic beverages and other yeast-containing products.

2.3.1 Special considerations – History of safe use of baker's yeast and related products

S. cerevisiae and its products have a long history of safe use in foods and beverages such as beer, wine and cider, and as a dietary or nutritional supplement (among others, Tucker & Woods, 1995; Pretorius, 2000; Zhang et al., 2005; Owens & McCracken, 2007; Ciamponi, Duckham & Tirelli, 2012; Salari et al., 2013; Marongiu et al., 2015).

The European Food Safety Agency (EFSA) concluded that baker's yeast is nontoxic and nonpathogenic (Pariza & Foster, 1983; Pariza & Johnson, 2001) and

Table 1
Concentrations of yeast mannoproteins naturally present in food

	Concentration (mg/kg or mg/L)	
Food item	**Mean**	**High**
Beer	192	507
Bread and pastries[a]	5 600	8 000
Yeast extracts	100 000	120 000
Wine[b]	350	500
Yeast-containing food supplements	16 000[c]	

[a] Based on the assumption that bread contains on average 3.5% yeast and up to 5% yeast (Cofalec, 2017), that 50% of yeast is cell wall and that 34% of the cell wall is released as mannoproteins. Note that the submission used a percentage of 16% of yeast mannoproteins in yeast (instead of 17%) to calculate the concentration of yeast mannoproteins in bread and pastries.
[b] Llauberes, Dubourdieu & Villettaz (1987); Dupin et al. (2000).
[c] The nature of this concentration, i.e. mean or high level, was not specified.

that it is not a major food allergen. EFSA included baker's yeast on the Qualified Presumption of Safety (QPS) list (EFSA, 2007).

The USFDA has approved dried yeast as an ingredient in food (21 C.F.R. 5172.896), and "generally recognized as safe" (GRAS) notices (GRN) have been accepted by USFDA with no questions regarding the use of genetically modified yeasts as starter cultures for wine (GRAS GRN No. 120 and GRAS GRN No. 175). Baker's yeast extract (21 C.F.R. s184.1933) has been affirmed by the USFDA as a GRAS flavouring agent and adjuvant at a level not to exceed 5% in food.

The Committee noted that most of the biological studies reported did not consider the variability in the composition of yeast mannoproteins (Lopez-Solis et al., 2017) and their possible interactions with other wine components (Wu, Guan & Zhong, 2015; Mekoue Nguela et al., 2016).

3. Dietary exposure

Yeast extracts containing mannoproteins are proposed for use at a recommended use level of 200 mg/L and at a maximum level of 400 mg/L in food category 14.2.3 "Grape wines" and its subcategories within the Codex General Standard for Food Additives (GSFA). Yeast mannoproteins also occur naturally in wine, foods including bread, pastries, beer and yeast extracts, and in yeast-containing food supplements. The concentration data for yeast mannoproteins naturally present in foods, as provided to the Committee, are shown in Table 1.

The Committee evaluated the sponsor's submission and prepared international estimates of dietary exposure to yeast mannoproteins using the FAO/WHO Chronic Individual Food Consumption Database – Summary

statistics (CIFOCOss) in combination with the recommended and maximum use levels in wine and the background occurrence of yeast mannoproteins in wine, bread, pastries and beer. No consumption data on yeast extracts and yeast-containing food supplements were available in CIFOCOss. Since yeast mannoproteins are present in processed foods, the Committee concluded that the use of the commodity-based food consumption information from the GEMS/Food Database cluster diets would not be appropriate for this evaluation.

3.1 Dietary exposure estimates based on food consumption data

3.1.1 Dietary exposure estimates submitted to the Committee by the sponsor

Two assessments of the dietary exposure to yeast mannoproteins, based on the following data, were made available to the Committee by the sponsor:

- French consumption data on bread, pastries, wine and beer in adults, assuming a population with a high consumption of bread and wine and therefore potentially a high exposure to yeast mannoproteins; and
- United States consumption data (90th percentile) on wine, beer, bread and pastries in adults, supplemented with estimated consumption of yeast extracts and yeast-containing food supplements.

Based on French food consumption data from the 1998–1999 Individual and National French Food Intake (INCA) study on bread, pastries, wine and beer, the mean exposure to yeast mannoproteins from food and from yeast extracts containing mannoproteins added to wine at a level of 300 mg/L was estimated to be 1.7 g/day (Volatier, 2000). In this assessment, a mean background level of yeast mannoproteins in wine of 125 mg/L was used based on a French study of the presence of yeast mannoproteins in commercial food products (Moine-Ledoux, 2003). Furthermore, it was assumed that bread and pastries contain 5% yeast and that 17% of the yeast consists of yeast mannoproteins. For beer, the mean concentration of 192 mg/L (Table 1) was used in the calculations.

Using food consumption data from the United States Department of Agriculture's 1994 and 1995 Continuing Survey of Food Intakes by Individuals and 1994 and 1995 Diet and Health Knowledge Survey (Wilson et al., 1997) in combination with concentrations (high) of yeast mannoproteins in foods and in yeast-containing food supplements (mean or high concentration not specified; Table 1), the background exposure to yeast mannoproteins was estimated to be 3.7 g/day.

Addition of yeast extracts containing mannoproteins to wine at the maximum level of 400 mg/L would result in a maximum additional exposure to

yeast mannoproteins of 0.02 g/day. This is about 0.5% of the estimated background exposure to yeast mannoproteins. The exposure to yeast mannoproteins via the intake of yeast-containing food supplements was estimated based on a recommended daily intake of 3.5 g according to the packaging label.

3.1.2 Dietary exposure estimates performed by Food Standards Australia New Zealand (FSANZ)

FSANZ performed a dietary exposure assessment of yeast mannoproteins for the Australian and New Zealand adult populations (≥18 years) based on food consumption data from the 1995 Australian National Nutrition Survey and the 1997 New Zealand National Nutrition Survey (FSANZ, 2008). Estimated mean dietary exposures to yeast mannoproteins from all added and background sources, including bread, pastries, wine, beer and yeast extracts of were estimated to be 0.42 and 0.35 g/day for adults from Australia and New Zealand, respectively. For consumers with a high consumption of wine (95th percentile), estimated dietary exposures to yeast mannoproteins from wine and other sources were 0.74 and 0.66 g/day, respectively. FSANZ used 125 mg/L as the mean background level of yeast mannoproteins in wine in their assessment and an added amount of 300 mg/L in wine in the high consumer scenario. The added amount used to assess the mean dietary exposure was not specified (FSANZ, 2008).

In the dietary exposure estimates provided by the sponsor and conducted by FSANZ, it was assumed that 100% of the yeast extracts containing mannoproteins added to wine was mannoproteins.

3.2 International assessment of the intake conducted by the Committee

3.2.1 Background concentration data

The Committee performed an international dietary exposure assessment of yeast mannoproteins using the background concentrations of yeast mannoproteins in food as listed in Table 1, excluding the concentrations in bread and pastries. Based on the assumption that bread contains on average 3.5% yeast (range: 2–5%; Cofalec, 2017) and that yeast contains 17% mannoproteins (assuming that 50% of yeast is the cell wall and 34% of the cell wall is released as mannoproteins), a mean concentration of yeast mannoproteins of 6000 mg/kg was used in the exposure assessment. The Committee noted that the background concentration of yeast mannoproteins in wine used in these exposure calculations is higher than the one used in the exposure calculations performed by the sponsor using the French consumption data (section 3.1.1) and by FSANZ (2008; section 3.1.2):

350 versus 125 mg/L. The Committee selected the highest reported level as part of a conservative approach.

3.2.2 Food consumption data and food mapping

The CIFOCOss[1] contains summary statistics of 55 surveys from 36 countries. These surveys cover at least 2 survey days. The database provides summary statistics of food consumption for about 750 items at a high level of classification. The statistics are available for different age groups per survey, ranging from infants to very elderly adults. Dietary exposure to yeast mannoproteins was calculated using CIFOCOss datasets on food consumption by adolescents (10–18 years), adults (18+ years) and the general population (ages not specified).

United States food consumption data were excluded from the exposure assessment. For the USA, the only relevant consumption data available in CIFOCOss were for the food category 14.2.3.3 "Fortified grape wine, grape liquor wine and sweet grape wine", resulting in very low exposure estimates for yeast mannoproteins compared with those of other countries included in CIFOCOss. Thai food consumption data for the general population were also not considered; no 95th percentile consumers of still grape wine were reported for this country.

Foods with concentration data (Table 1) were mapped to comparable food categories in CIFOCOss for the exposure assessment (Table 2). For yeast extracts and yeast-containing food supplements, no comparable food categories were present in the database. These sources of dietary exposure were therefore not considered in the exposure assessment using CIFOCOss.

3.2.3 Mean and high dietary exposure estimates

The Committee calculated a mean and high dietary exposure to yeast mannoproteins at a background level or with yeast extracts containing mannoproteins added to wine at the recommended or maximum use level. In these calculations, the Committee assumed that the yeast extracts (containing mannoproteins) were 100% mannoprotein.

(a) Mean dietary exposure

The mean background dietary exposure to yeast mannoproteins was calculated by combining a mean consumption of all relevant foods (Table 2) with the respective mean background concentration of yeast mannoproteins per food (Table 1), except for bread and pastries for which a mean concentration of 6000 mg/kg was used (section 3.2.1). The mean exposure to yeast mannoproteins, including yeast extracts containing mannoproteins added to wine, was estimated at the

[1] The January 2017 version was made available to the Committee by the World Health Organization.

Table 2
Mapping foods to assess the dietary exposure to yeast mannoproteins using CIFOCOss

	CIFOCOss	
Food[a]	**Food code**[b]	**Food description**
Beer	14.2.1	Beer and malt beverages
	14.2.1.1	Beer of sorghum
	14.2.1.2	Beer of millet
	14.2.1.3	Beer of barley
	14.2.1.6	Beer of maize
Bread and pastries	07.2.1	Cakes, cookies and pies (e.g., fruit-filled or custard types)
	CP 0179[a]	Wheat white bread
	CP 0179[b]	Wheat wholemeal bread
	CP 1250	Rye bread
	CP 5295	Corn bread
Wine	14.2.3.1	Still grape wine
	14.2.3.3	Fortified grape wine, grape liquor wine and sweet grape wine

CIFOCOss: FAO/WHO Chronic Individual Food Consumption Database – Summary statistics
[a] Foods for which concentrations of yeast mannoproteins were available (Table 1) and could be mapped to comparable food categories within CIFOCOss.
[b] Level 3 code of CIFOCOss.

recommended use level of 200 mg/L, resulting in overall concentration of yeast mannoproteins in wine of 550 mg/L (background + added).

The mean background exposure to yeast mannoproteins ranged from 0.1 to 21 mg/kg bw per day (Table 3). Addition of yeast extracts containing mannoproteins to wine at the recommended level resulted in an increase in the mean dietary exposure to yeast mannoproteins of less than 5% (<0.1–4.2%), except for adults and elderly adults in Finland. In these two population groups, the increase was 14% and 30%, respectively. However, despite this increase the overall (background + added) exposure to yeast mannoproteins remained low: 0.4 and 0.1 mg/kg bw per day, respectively. The exposure varied significantly within population groups (Table 3). This reflected differences in the consumption of bread and pastries, which contributed at least 90% of the exposure to yeast mannoproteins in almost all datasets.

(b) High dietary exposure

The high background dietary exposure was calculated by combining a high consumption level (95th percentile of consumers) of “Still grape wine” (food category 14.2.3.1), the wine that was consumed at the highest levels, with the high background level of yeast mannoproteins in wine of 500 mg/L (Table 1). To assess a high exposure, including the addition of yeast extracts containing mannoproteins to wine, the maximum use level of 400 mg/L was added to the

Table 3

Estimated exposures to yeast mannoproteins via the diet[a] at a background level or with yeast extracts containing mannoproteins added to wine (background + added) based on CIFOCOss data

Population group	Mean total population (mg/kg bw per day)		95th percentile consumer of wineb (mg/kg bw per day)	
	Background	**Background + added**	**Background**	**Background + added**
Adolescents (10–18 years)	6.1–21	6.1–21	8.0–21	8.5–21
Adults (18+ years)[c]	0.1–13	0.4–13	2.5–15	4.3–18
General population[d]	5.1–7.8	5.1–7.8	6.3–11	7.2–13

bw: body weight; CIFOCOss: FAO/WHO Chronic Individual Food Consumption Database – Summary statistics

[a] Exposure estimates do not include potential exposure via yeast extract and yeast-containing food supplements.

[b] Based on 95th percentile consumption of food category 14.2.3.1 "Still grape wine" (consumers only) and mean consumption levels of the other relevant foods (total population).

[c] Including the population groups adults, elderly adults and very elderly adults within CIFOCOss;

[d] Ages included in the general population are not specified within CIFOCOss.

high background level of yeast mannoproteins in wine resulting in an overall concentration of 900 mg/L. The other wine type coded in CIFOCOss (food category 14.2.3.3 "Fortified grape wine, grape liquor wine and sweet grape wine") (Table 2) and the other foods (bread, pastries and beer) were included in the high exposure assessment by combining the mean consumption with a mean concentration of yeast mannoproteins per food.

The background exposure to yeast mannoproteins of consumers with a high wine consumption ranged from 2.5 to 21 mg/kg bw per day (Table 3). Addition of yeast extracts containing mannoproteins to wine at the maximum level of 400 mg/L resulted in an increase in yeast mannoprotein exposure ranging from <1-30% per dataset (Table 3). The increase was significantly higher only among Finnish adults and elderly adults: 72% and 78%, respectively. This large increase was due to the relatively high consumption of wine in these two Finnish populations relative to the other sources of exposure to yeast mannoproteins. However, this increase did not result in an exposure to yeast mannoproteins outside the range of exposures estimated for the total group of adults present in CIFOCOss (Table 3): 4.3 and 5.0 mg/kg bw per day, respectively. In general, the highest increases in exposure were observed in the adult population (on average 20%) because this population group consumes wine at the highest levels.

(c) Overall

The estimated dietary exposures to yeast mannoproteins at the mean level increased less than 5% (relative to background estimates) after the addition of yeast extracts containing mannoproteins to wine at the recommended level of 200 mg/L. For adult consumers with a high wine consumption, the dietary exposure increased 20% on average after the addition of yeast extracts containing mannoproteins to wine at the maximum level of 400 mg/L. The highest estimated dietary exposure was 21 mg/kg bw per day in adolescents. Exposure to yeast mannoproteins was mainly determined by the consumption of bread and pastries, due to both the high consumption levels of these foods and a high concentration level (6000 mg/kg).

3.2.4 Exposure via yeast extracts and yeast-containing food supplements

The Committee did not consider the possible exposure to yeast mannoproteins via the consumption of yeast extracts and yeast-containing food supplements in the exposure assessments; no information on the consumption of these foods was available in CIFOCOss. To assess the possible exposure to yeast mannoproteins via yeast extracts, the Committee used a mean exposure to yeast mannoproteins of 0.15 g/day through this source as estimated by FSANZ for the adult populations of Australia and New Zealand (FSANZ, 2008). The highest exposure estimated to yeast mannoproteins was 21 mg/kg bw per day in adolescents (Table 3). For this population group, an additional exposure of about 3 mg/kg bw per day was calculated via the consumption of yeast extracts based on an average body weight of 52 kg in this population group (EFSA, 2012). Thus, the highest potential exposure in adolescents was estimated at 24 mg/kg bw per day.

For yeast-containing food supplements, the sponsor provided an intake level of 3.5 g/day (the recommended daily consumption on the packaging label) and a concentration level of 16 000 mg/kg (Table 1). However, the Committee considered this information to be unreliable, and did not quantify the exposure to yeast mannoproteins via this source.

3.3 Overall results of the dietary exposure estimates

Table 4 lists the estimated dietary exposures to yeast mannoproteins using food consumption data from France and the USA (as provided by the sponsor), those calculated by FSANZ (2008), and those calculated by the Committee. Due to the use of different background levels of yeast mannoproteins in wine, the exposure results are not completely comparable. In addition, the Committee also considered the exposure in adolescents whereas the other exposure estimates apply only to adults (≥18 years).

Table 4
Estimated dietary exposures to yeast mannoproteins, including via the addition of yeast extracts containing mannoproteinsa to wine

	Exposure (mg/kg bw per day)[a]	
Source[c]	**Mean**	**High**
France	28	–
USA	–	62
FSANZ	5.8–7.0	11–12.3
JECFA	3.4–24	7.3–24[d]

bw: body weight; CIFOCOss: FAO/WHO Chronic Individual Food Consumption Database – Summary statistics; FSANZ: Food Standards Australia New Zealand; USA: United States of America.
[a] In all calculations, it was assumed that the yeast extracts (containing mannoproteins) were comprised of 100% mannoproteins.
[b] Exposure estimates for France and the USA, and of FSANZ were converted to exposure estimates per kg body weight by division with a body weight of 60 kg.
[c] Exposure estimates for France and the USA (provided by the sponsor), and those of FSANZ relate to adult populations (≥18 years). Exposure estimates calculated by the Committee include adolescents (10–18 years), adults (≥18 years) and the general population (age not specified in CIFOCOss).
[d] Including an additional exposure to yeast mannoproteins of about 3 mg/kg bw per day via the consumption of yeast extracts (section 3.2.4).

The exposure based on the United States food consumption data was higher than the highest exposure calculated by the Committee. The reason for this is that high consumption levels of all foods were combined with high concentration levels of yeast mannoproteins (section 3.1). The exposure estimates of FSANZ and those based on French consumption data (section 3.1) were approximately within the range of the CIFOCOss estimates.

FSANZ (2008) estimated the exposure to yeast mannoproteins via bread and pastries by estimating first the consumption of yeast via these two foods based on recipes. Subsequently, the amount of yeast mannoproteins consumed was estimated by assuming that 17% of the yeast consists of mannoproteins. This difference in approach may also have contributed to the lower exposures reported by FSANZ (2008) compared with the Committee estimates. JECFA estimated the mean consumption of yeast via bread and pastries in adults (including the elderly and very elderly) at about 3 g/day within CIFOCOss (based on the assumption that bread and pastries contain 3.5% of yeast and an average adult weighs 60 kg). The amounts reported by FSANZ were about 1 g/day.

4. Comments

4.1 Biochemical aspects

No relevant absorption, distribution, metabolism or excretion studies were available for yeast mannoproteins. The Committee assumed that mannoproteins

extracted from *S. cerevisiae* in the test compound will behave similarly to those resulting from dietary exposure to the intact yeast or to other glucomannans consumed as part of a regular diet. Once mannoproteins have been hydrolysed by intestinal enzymes, the carbohydrate moiety can be fermented by intestinal microflora in the large intestine into, among others, organic acids or alcohols (den Besten et al., 2013a,b; Bâgenholm et al., 2017).

In a study using immortalized human hepatocytes (Fa2N-4 cells), a fermentation product of *S. cerevisiae* did not induce cytochrome P450 (CYP) CYP1A2 or CYP3A4 mRNA or enzymatic activity and did not interfere with the induction of CYP1A2 or CYP3A4 by omeprazole or rifampin (also known as rifampicin), respectively (Schauss et al., 2012). The test article used in this study was described as the product of a proprietary fermentation process using *S. cerevisiae*, involving "both a unique substrate and a stress process". The test article, hereafter referred to as "yeast fermentate preparation", was also tested in several toxicology studies. The yeast fermentate preparation is reported to contain cell wall components, including mannoproteins, components from the medium, fermentative by-products and stress-induced metabolites. However, a more complete chemical characterization of the test article was not available.

4.2 Toxicological studies

In male rats given a daily dose of 10^8 viable cells or cfu of *S. cerevisiae* RC016 by oral gavage for 60 days, no treatment-related effects were reported (González Pereyra et al., 2014).

In a 90-day study, groups of male and female rats were given 0, 30, 200 or 1500 mg/kg bw per day of a suspension of yeast fermentate preparation in water containing 1% methylcellulose. No deaths occurred, and no treatment-related changes in any of the parameters assessed at any dose were observed (Schauss et al., 2012).

Schauss et al. (2012) reported a chronic toxicity study in male and female rats administered 0, 20, 200 or 800 mg/kg bw per day of a suspension of yeast fermentate preparation in water containing 1% methylcellulose. No treatment-related or clinically relevant findings were reported in any of the parameters assessed at any dose.

A yeast fermentate preparation was negative in a bacterial reverse mutation assay and in a mouse lymphoma cell mutagenicity test (Schauss et al., 2012). Bone marrow micronucleus and comet assays were negative in male rats given 10^8 viable cells or cfu of *S. cerevisiae* RC016 daily for 60 days by oral gavage (González Pereyra et al., 2014).

No data were available regarding the carcinogenicity and reproductive or developmental toxicity of material relevant to yeast extracts containing mannoproteins.

The only study available with yeast extracts containing mannoproteins gave a negative result in a dermal sensitization study conducted on albino guinea-pigs (Richeux, 2002c).

Owing to the high content of mannose in yeast, the Committee assumed that yeast mannoproteins, like other galactomannans, can interact with mannose receptors (Tizard et al., 1989). Binding of mannosylated proteins to mannose receptors is involved in various physiological mechanisms, including innate and specific immunity. The consequence of increased binding of mannoproteins to mannose receptors and the relevance of such data are still a matter of research.

4.3 Observations in humans

Yeast fermentate preparation from *S. cerevisiae* was not mitogenic in human peripheral lymphocytes (Schauss et al., 2012).

Bansal, Tadros & Bansal (2017) reported one case of allergy to beer, wine and cider resulting from immunoglobulin E reactivity to yeasts and moulds.

4.4 Assessment of dietary exposure

Yeast extracts containing mannoproteins are proposed for use at a recommended use level of 200 mg/L and at a maximum level of 400 mg/L in food category 14.2.3 "Grape wines" and its subcategories within the Codex GSFA. Yeast mannoproteins also occur naturally in wine, as well as in other foods including bread, pastries, beer and yeast extracts, and in food supplements. The Committee evaluated the sponsor's submission and prepared international estimates of dietary exposure to yeast mannoproteins using the CIFOCOss database in combination with the recommended and maximum use levels in wine and the background occurrence of yeast mannoproteins in wine, bread, pastries and beer. No consumption data on yeast extracts and yeast-containing food supplements were available in the CIFOCOss. The dietary exposure was calculated using datasets in the CIFOCOss that were related to food consumption data for adolescents (10–18 years), adults (18+ years) and the general population (ages not specified), assuming that 100% of the yeast extract was mannoproteins.

The mean background exposure to yeast mannoproteins ranged from 0.1 to 21 mg/kg bw per day. In consumers with high consumption of wine, the background exposure ranged from 2.5 to 21 mg/kg bw per day. The highest background exposures were calculated for adolescents. Addition of yeast extracts

containing mannoproteins to wine at the recommended level resulted in an increase in the mean dietary exposure to yeast mannoproteins in the datasets of less than 5% (<0.1–4.2%), resulting in a range of exposure of 0.4–21 mg/kg bw per day. For consumers with high consumption of wine, the addition of yeast extracts containing mannoproteins to wine at the maximum level resulted in an increase of dietary exposure of, on average, 20%. The resulting high estimates of dietary exposure were 4.3–21 mg/kg bw per day. Dietary exposure to yeast mannoproteins was mainly (at least 90% in almost all datasets) determined by bread and pastries, due to both high consumption and a high concentration level. The additional dietary exposure to yeast mannoproteins via the consumption of yeast extract, based on FSANZ data (FSANZ, 2008), was estimated to be about 3 mg/kg bw per day.

5. Evaluation

The Committee noted that very few toxicity studies were available for the range of yeast extracts containing mannoproteins on the market. However, consumers are exposed to yeast mannoproteins from *S. cerevisiae* present in wine as well as in other fermented foods, including bread, pastries, beer and yeast extracts, and in food supplements. Therefore, the Committee considered that it was possible to use the available information relating to *S. cerevisiae* and its constituents for this evaluation. No indication of toxicity was identified from the available information, including the toxicological studies on one product that was poorly characterized (yeast fermentate preparation from *S. cerevisiae*). However, there were no data on reproductive and developmental toxicity or carcinogenicity for any relevant yeast preparation.

In addition to the natural presence of yeast mannoproteins in wine and the long history of consumption of yeast products in common foods, the Committee considered that the tentative product specifications for yeast extracts containing mannoproteins indicate that these do not contain chemical residues or microbiological contaminants of concern. In addition, the Committee estimated that the exposure to yeast mannoproteins due to the addition of yeast extracts containing mannoproteins to wine at the maximum level of 400 mg/L would result, on average, in a 20% increase in dietary exposure compared to the background exposure through the regular diet of 0.4–21 mg/kg bw per day, primarily driven by bread and pastries. These conservative dietary exposure estimates are based on the assumption that 100% of the yeast extracts containing mannoproteins is mannoproteins.

In considering the data and information regarding yeast and yeast-derived products, the Committee concluded that it is unlikely that there would be a health concern for the use of yeast extracts containing mannoproteins as a food additive for oenological uses at maximum use levels up to 400 mg/L for the stabilization of wine.

The Committee noted that any change in the uses and/or use levels of yeast extracts containing mannoproteins as a food additive will require a new evaluation.

A new tentative specifications monograph and a Chemical and Technical Assessment were prepared.

In order to remove the tentative designation of the specifications, the Committee requires chemical characterization of the product in commerce along with data to be able to complete specifications related to the use of yeast extracts containing mannoproteins in wine manufacture. The following information is required:

- Composition of yeast extracts containing mannoproteins as well as the processes used in their manufacture;
- Analytical data from five batches of each commercial product, including information related to impurities; and
- Data on concentrations of yeast mannoproteins in wine in which yeast extracts containing mannoproteins have been used.

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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).
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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.
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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.
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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.
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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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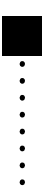

ANNEX 2

Abbreviations used in the monographs

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, and excretion
ANS	[EFSA Panel on]Food Additives and Nutrient Sources added to Food
bw	body weight
CAS	Chemical Abstracts Service
CCFA	Codex Committee on Food Additives
CIFOCOss	FAO/WHO Chronic Individual Food Consumption Database – Summary statistics
cfu	colony-forming unit
CSIR	Council of Scientific and Industrial Research
CYP	cytochrome P450
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
F	female
F	generation, e.g. F_0, F_{1a}, F_{1b}, F_{2a}, F_{2b}, etc.
FAO	Food and Agriculture Organization of the United Nations
FSANZ	Food Standards Australia New Zealand
GEMS/Food	Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme
GLP	good laboratory practice
GRAS	Generally Recognized as Safe
GRN	Generally Recognized as Safe (GRAS) notice
GSFA	General Standard for Food Additives
HPLC	high-performance liquid chromatography
IC_{50}	half maximal inhibitory concentration
INS	International Numbering System for Food Additives
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	median lethal dose
LOD	limit of detection
M	male
NA	not applicable
nAChR	nicotinic acetylcholine receptor
NCE	normochromatic erythrocytes
nes	not elsewhere specified
NET-NID	National Eating Trends – Nutrient Intake Database

NHANES	National Health and Nutrition Examination Survey
no./No.	number
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Cooperation and Development
OECD TG	Organisation for Economic Co-operation and Development Test Guideline
P90	90th percentile
P95	95th percentile
P97.5	97.5th percentile
Panx1	pannexin 1
PBS	phosphate-buffered saline
PCE	polychromatic erythrocytes
PND	postnatal day
QA	quality assurance
QPS	[EFSA] Qualified Presumption of Safety
RAR	retinoic acid receptor
S9	9000 × *g* supernatant fraction from liver homogenate
TLC	thin-layer chromatography
USA	United States of America
USFDA	United States Food and Drug Administration
v/v	volume per volume
WHO	World Health Organization

ANNEX 3

Joint FAO/WHO Expert Committee on Food Additives

Rome, 6–15 June 2017

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ANNEX 4

Toxicological information, dietary exposures 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
Brilliant Blue FCF	R[a]	The Committee concluded that the available data support the revision of the ADI for Brilliant Blue FCF. In a long-term toxicity study in rats, a no-observed-adverse-effect level (NOAEL) of 631 mg/kg body weight (bw) per day was identified, based on a 15% decrease in mean terminal body weight and decreased survival of females at 1318 mg/kg bw per day. **The Committee established an ADI of 0–6 mg/kg bw** based on this NOAEL by applying an uncertainty factor of 100 for interspecies and intraspecies differences. The Committee noted that the conservative dietary exposure estimate of 5 mg/kg bw per day (95th percentile for children) is less than the upper limit of the ADI of 0–6 mg/kg bw established for Brilliant Blue FCF and **concluded that dietary exposure to Brilliant Blue FCF for children and all other age groups does not present a health concern.** **The previous ADI of 0–12.5 mg/kg bw was withdrawn.**
β-Carotene-rich extract from *Dunaliella salina*	N	The Committee noted that data have become available since the previous evaluation that show large differences in absorption of β-carotene between rodents and humans. The Committee considered that rodents are inappropriate animal models for establishing an ADI for β-carotene. The Committee noted that the toxicity of the other components of the β-carotene-rich d-limonene extract of *D. salina* (hereafter referred to as *D. salina* d-limonene extract) can be evaluated using the results of rodent studies. A short-term toxicity study in rats gave a NOAEL of 3180 mg/kg bw per day, the highest dose tested. No long-term toxicity or reproductive studies have been conducted. The *D. salina* d-limonene extract did not show genotoxicity or developmental toxicity. Correction of the NOAEL of 3180 mg/kg bw per day for the percentage of the algal component (20–35%) gives an adjusted NOAEL of 636–1113 mg/kg bw per day for the algal lipid component of the *D. salina* d-limonene extract. The margin of exposure for this algal lipid component is 2120–3710 using a dietary exposure of 18 mg/day (0.3 mg/kg bw per day). The Committee concluded that exposure to the algal component of the extract does not pose a health concern. The Committee noted that the total dietary exposure to β-carotene is not expected to increase when *D. salina* d-limonene extract is used as a food colour. **The Committee concluded that there was no health concern for the use of β-carotene-rich extract from *D. salina* when used as a food colour in accordance with the specifications established at this meeting.** The Committee emphasized that this conclusion applies to the use of this extract as a food colour, not as a food supplement.

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
Fast Green FCF	R[a]	The ADI of 0–25 mg/kg bw established previously by the Committee was based on a long-term rat dietary that identified a NOAEL of 5% Fast Green FCF (equivalent to 2500 mg/kg bw per day), the highest concentration tested. The Committee concluded that the new data that had become available since the previous evaluation gave no reason to revise the ADI and **confirmed the ADI of 0–25 mg/kg bw.** The Committee noted that the conservative dietary exposure estimate for Fast Green FCF of 12 mg/kg bw per day (95th percentile for adolescents) was below the upper bound of the ADI. **The Committee concluded that dietary exposures to Fast Green FCF for adolescents and all other age groups do not present a health concern.**
Gum ghatti	R[b]	The Committee took into account the lack of systemic exposure to gum ghatti because of its high molecular weight and polysaccharide structure, its lack of toxicity in short-term studies, the lack of concern for genotoxicity and the absence of treatment-related adverse effects in studies of gum arabic and other polysaccharide gums with a similar profile. The Committee concluded that gum ghatti is unlikely to be of health concern and **established an ADI "not specified"c for gum ghatti that complies with the specifications.** **The Committee concluded that the estimated dietary exposure to gum ghatti of 12 mg/kg bw per day does not present a health concern.**
Jagua (Genipin–Glycine) Blue	N,T	The Committee noted that the highest doses tested in two 90-day toxicity studies in rats and dogs were only 330 and 338 mg/kg bw per day (expressed on a "blue polymer" basisd), respectively. The Committee was concerned that the possible effects of the low molecular weight component of the "blue polymer" that could be absorbed were not adequately investigated. A comparison of the dietary exposure estimate (11 mg/kg bw per day) with the NOAEL from the 90-day studies of oral toxicity in rats and dogs (approximately 330 mg/kg bw per day) gives a margin of exposure of about 30. Because of the limited biochemical and toxicological database and the low margin of exposure, **the Committee was unable to complete the evaluation for Jagua (Genipin–Glycine) Blue.**

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
Metatartaric acid	T	As metatartaric acid undergoes enzymatic hydrolysis to tartaric acid prior to systemic absorption, the biochemical and toxicological data on tartaric acid considered at previous meetings are relevant to the safety assessment of metatartaric acid. Previously evaluated and new studies suggest no change to the group ADI previously established for L(+)-tartaric acid and its sodium, potassium and potassium–sodium salts, expressed as L(+)-tartaric acid. **The Committee concluded that metatartaric acid (when used in winemaking) should be included in the group ADI of 0–30 mg/kg bw for L(+)-tartaric acid and its sodium, potassium, potassium–sodium salts, expressed as L(+)-tartaric acid.** The Committee noted that the dietary exposure estimate for metatartaric acid for adult consumers of wine was 4% of the upper bound of the ADI and **concluded that dietary exposure to metatartaric acid in wine at the maximum use level of 100 mg/L does not present a health concern.**
Tamarind seed polysaccharide	N	The Committee noted the absence of toxicity in long-term rodent studies and lack of concern regarding genotoxicity, reproductive toxicity and developmental toxicity, and **established an ADI "not specified"c for tamarind seed polysaccharide.** **The Committee concluded that the estimated dietary exposure of 75 mg/kg bw per day based on proposed uses and use levels does not present a health concern.**
Tannins (oenological tannins)	–	The Committee noted that the available data do not provide clear information on which tannin sources and individual tannin compounds are present in commercially used oenological tannins and, thus, how the oenological tannins would compare to the tannins used in the submitted studies. Therefore, it is not possible to establish which studies are relevant and, consequently, the extent of the data gaps. The information on biochemical aspects is incomplete, with the implications of repeated dosing on absorption, tissue distribution and interindividual variation needing consideration. In general, there are also few data available on reproductive and developmental toxicity and/or long-term toxicity for some or all of the tannins. **In the absence of specifications and identification of the products in commerce, the Committee concluded that it was not possible to evaluate tannins used in winemaking.**

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
Yeast extracts containing mannoproteins	N,T	In addition to the natural presence of yeast mannoproteins in wine and the long history of consumption of yeast products in common foods, the Committee considered that the tentative product specifications for yeast extracts containing mannoproteins indicate that these do not contain chemical residues or microbiological contaminants of concern. In addition, the Committee estimated that dietary exposure to yeast mannoproteins due to the addition of yeast extracts containing mannoproteins to wine at the maximum level of 400 mg/L would result, on average, in a 20% increase in dietary exposure compared to the background exposure through the regular diet of 0.4–21 mg/kg bw per day, primarily driven by bread and pastries. These conservative dietary exposure estimates are based on the assumption that 100% of the yeast extracts containing mannoproteins is mannoproteins. In considering the data and information regarding yeast and yeast-derived products, **the Committee concluded that it is unlikely that there would be a health concern for the use of yeast extracts containing mannoproteins as a food additive for oenological uses at maximum use levels up to 400 mg/L for the stabilization of wine.** The Committee noted that any change in the uses and/or use levels of yeast extracts containing mannoproteins as a food additive will require a new evaluation.

–: no specifications prepared; N: new specifications; R: existing specifications revised; T: tentative specifications

[a] A maximum limit for manganese was added. High-performance liquid chromatography (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 or aqueous ammonium acetate.

[b] An HPLC method for the identification of the gum constituents was added to replace the thin-layer chromatography (TLC) method. One identity method, using a mercury-containing reagent, was removed. L-Rhamnose was added as one of the constituents of gum ghatti, based on current literature reports.

[c] ADI "not specified" is used to refer to a food substance of very low toxicity that, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary exposure to the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice – i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect; it should not conceal food of inferior quality or adulterated food; and it should not create a nutritional imbalance.

[d] "Blue polymer" refers to the blue-coloured genipin–glycine polymer and dimer content of Jagua (Genipin–Glycine) Blue.

Food additives considered for specifications only

Food additive	Specifications
Microcrystalline cellulose	R[a]
Silicon dioxide, amorphous	R[b]
Sodium aluminium silicate	R[c]
Steviol glycosides	R[d]
Sucrose esters of fatty acids	R[e]

R: existing specifications revised

[a] The Committee assessed the information submitted on the solubility of microcrystalline cellulose and redesignated its solubility as "Insoluble in water and ethanol. Practically insoluble or insoluble in sodium hydroxide solution (50 g/L)".

[b] Silicon dioxide, amorphous was on the agenda at the present meeting for revisions related to pH, assay, loss on drying, loss on ignition and impurities. The Committee at its present meeting received the requested information. The tentative status was removed.

[c] At the current meeting, the Committee evaluated the data submitted for loss on ignition, impurities soluble in 0.5 mol/L hydrochloric acid and the suitability of the proposed assay method for the determination of aluminium, silicon and sodium. Information received on functional uses confirmed that the substance is used only as an anticaking agent. The tentative status was removed.

[d] The Committee received a validated HPLC–ultraviolet (UV) method for the assay of steviol glycosides, for which reference standards are commercially available. The presence of steviol glycosides that exist in small quantities is confirmed using an HPLC–mass spectrometric method and quantified using HPLC–UV data. The Committee also received assay data for three batches of a commercial product using the proposed methods. The Committee, at its present meeting, assessed the information received and replaced the existing assay. Two additional saccharides (galactose and arabinose) have been identified in the extracts of *Stevia rebaudiana* Bertoni since the last evaluation of steviol glycosides. The Committee included the two saccharides in the definition of the specifications for steviol glycosides from *S. rebaudiana* Bertoni. The tentative status was removed.

[e] The Committee assessed the information submitted on the solubility of sucrose esters of fatty acids and revised the solubility criterion. In addition, the Committee reviewed the information submitted on the chromatographic conditions for the separation of the compounds and revised the UV integration instructions.

This volume contains monographs prepared at the eighty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Rome, Italy, from 6 to 15 June 2017.

The toxicological and dietary exposure monographs in this volume summarize the safety and/or dietary exposure data on eight specific food additives: Brilliant Blue FC; β-carotene-rich extract from *Dunaliella salina*; Fast Green FCF; Gum ghatti; Jagua (Genipin–Glycine) Blue; metatartaric acid; tamarind seed polysaccharide; and Yeast extracts containing mannoproteins.

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 or are involved with controlling contaminants in food; government and food regulatory officers; industrial testing laboratories; toxicological laboratories; and universities.

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