Safety evaluation of certain food additives

Food and Agriculture
Organization of the
United Nations

World Health
Organization

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[1] For use in formulas for special medical purposes intended for infants.

PREFACE

The monographs contained in this volume were prepared at the eighty-seventh 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 4–13 June 2019. These monographs summarize the data on selected food additives reviewed by the Committee.

The eighty-seventh report of JECFA has been published by WHO as WHO Technical Report No. 1020. 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 Residues of Veterinary Drugs in Foods and the Codex Committee on Contaminants in Food regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and dietary exposure and toxicological monographs, such as those contained in this volume, on substances that they have considered.

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

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

Any comments or new information on the biological or toxicological properties of or dietary exposure to the compounds evaluated in this publication should be addressed to: WHO Joint Secretary of the Joint FAO/WHO Expert Committee on Food Additives, Department of Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

SAFETY EVALUATIONS OF SPECIFIC FOOD ADDITIVES

Black carrot extract

First draft prepared by

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1. Explanation

Black carrot extract (International Numbering System for Food Additives [INS] 163(vi)) is an anthocyanin-containing food colour obtained by acidic aqueous extraction from the root of black, purple or red carrot. The main colouring components are five cyanidin-based anthocyanins.

Black carrot extract has not been evaluated previously by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee previously evaluated anthocyanins, including the anthocyanin-containing food colour grape skin extract (INS 163(ii)), at its twenty-sixth meeting (Annex 1, reference *59*). At that meeting, the Committee established an acceptable daily intake (ADI) for anthocyanins in grape skin extract of 0–2.5 mg/kg body weight (bw), based on a no-observed-adverse-effect level (NOAEL) of 225 mg/kg bw per day expressed as anthocyanins from a two-generation reproductive toxicity study in rats (Cox & Babish, 1978a).

Black carrot extract was placed on the agenda of the present meeting for assessment of its safety, dietary exposure and specifications, at the request of the Fiftieth Session of the Codex Committee on Food Additives (FAO/WHO, 2018). In response to the call for data, a submission was received, which included studies identified from the publicly available literature and information on specifications and dietary exposure. A comprehensive literature search was also carried out in PubMed using the following search terms: anthocyanins, acute; cyanidin, genotox*; cyanidin, carcino*; cyanidin, in vivo; cyanidin, blood, absor*; cyanidin, toxicity; pelargonidin, in vivo; pelargonidin, toxicity; peonidin, toxicity; peonidin, in vivo; malvidin, toxicity; malvidin, in vivo; delphinidin, toxicity; delphinidin, in vivo; petunidin, toxicity; petunidin, in vivo. The Cochrane database of studies was also searched using the following search terms: black carrot; cyanidin; anthocyanin; peonidin; pelargonidin; malvidin; delphinidin; petunidin. These literature searches retrieved a number of additional studies, primarily on human pharmacokinetics and absorption, distribution, metabolism and excretion (ADME), and one additional genotoxicity study.

Given the similar aglycone structures of anthocyanins, the large number of studies on anthocyanins from various sources published since the previous assessment of grape skin extract and the lack of toxicity data on black carrot extract itself (only one genotoxicity study was submitted), the Committee

Fig. 1
General anthocyanin aglycone structure indicating substitution positions
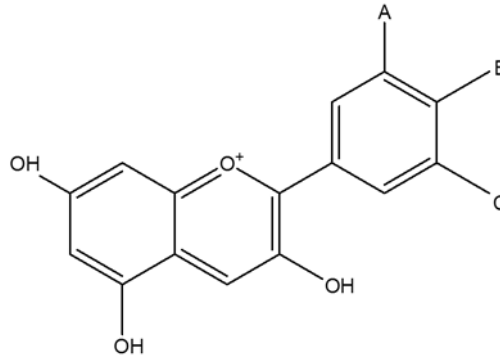
Aglycone	A	B	C
Cyanidin[a,b,c]	-OH	-OH	-H
Pelargonidin[a,b]	-H	-OH	-H
Delphinidin[b]	-OH	-OH	-OH
Peonidin[a,b]	-O-CH$_3$	-OH	-H
Petunidin[b]	-OH	-OH	-O-CH$_3$
Malvidin[a,b]	-O-CH$_3$	-OH	-O-CH$_3$

[a] Found in black carrot extract.
[b] Found in grape skin extract.
[c] The five main anthocyanins in black carrot extract are formed from this aglycone.
Source: Modified from Annex 1, reference *59*

decided to review the available data on anthocyanins as a whole. The studies described below therefore include previously evaluated studies on grape skin extract (published prior to 1982) as well as new studies on materials containing anthocyanins from a range of sources.

1.1 **Chemical and technical considerations**

Anthocyanins are a large group of related compounds consisting of aglycones such as cyanidin or pelargonidin (Fig. 1) combined with sugars such as galactose or glucose and acylating agents such as caffeic acid or *p*-coumaric acid (Overall et al., 2017).

Black carrot extract contains five main anthocyanins formed from the aglycone cyanidin substituted at the central hydroxyl position with a sugar moiety consisting of galactose, glucose and/or xylose. Three of the five anthocyanins are acylated with *p*-coumaric, ferulic or sinapinic acid (Smeriglio et al., 2018). One of the five main anthocyanins in black carrot extract is shown in Fig. 2. Anthocyanins in black carrot extract are also formed from other aglycones

Fig. 2

Cyanidin 3-*p*-coumaroylxylosylglucosylgalactoside, one of the five main anthocyanins in black carrot extract
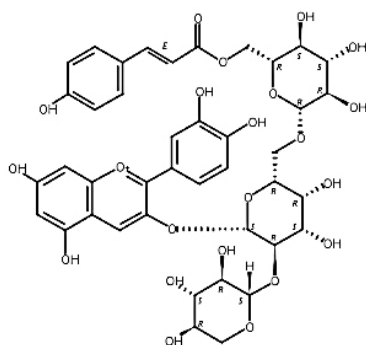
Source: PubChem

(malvidin, pelargonidin and peonidin; Fig. 1), which are present in minor amounts along with other polyphenols. Other components include proteins, carbohydrates, lipids, fibres, minerals and water. In contrast to black carrot extract, the predominant aglycone found in anthocyanins in grape skin extract is malvidin (Benmeziane et al., 2016).

Black carrot extract is produced by aqueous acidic extraction of the crushed, ground or milled roots of black, purple or red carrot (*Daucus carota* L., ssp. *sativus*) followed by fermentation to decrease sugars. Methanol or ethanol may be produced during the fermentation step. The anthocyanins may be concentrated by ultrafiltration, reverse osmosis or adsorption onto a polymeric resin followed by desorption with ethanol, isopropyl alcohol and/or water. The commercial product may be a liquid or spray-dried powder.

Black carrot extract is intended for use in colouring dairy-based desserts, processed fruit products, processed vegetable products, confectionery, chewing gum, cereals, pastas and noodles, cereal/starch-based desserts, processed rice and soy products, cakes, cookies, pies, preserved egg products, condiments (vinegar, mustard), sauces and gravies, dietetic foods and dietary supplements, non-alcoholic beverages and alcoholic beverages.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution, metabolism and excretion

(a) Studies in experimental animals

Groups of eight male Wistar rats received either an anthocyanin-rich diet (containing 20 g of blackberry extract per kilogram; ~35% anthocyanins as cyanidin-3-*O*-glucoside [C3G]; type of extract not specified) or a control diet for 12 days, after which animals were euthanized and blood and urine were collected. Blackberries contain only cyanidin-based anthocyanins. The heart, prostate, testes, epididymal white adipose tissue and bladder were removed and processed for analysis. Urine samples from the rats fed the anthocyanin-rich diet contained cyanidin-based anthocyanins as well as methylated and glucuronide-conjugated derivatives. Also present was a methylated derivative of cyanidin (peonidin) in the form of peonidin-3-*O*-glucoside. C3G was found at the highest concentrations in tissue, in bladder (1.37 ± 0.55 nmol/g tissue; 664 ± 227 ng/g tissue) followed by prostate (0.257 ± 0.081 nmol/g tissue; 125 ± 39 ng/g tissue). Lower concentrations were also detected in plasma, testes, adipose and heart tissues (Felgines et al., 2009).

Groups of eight male rats were provided with either a control diet or a diet containing monomeric anthocyanin extracts from chokeberry, bilberry or grape (type of extract not described) at a concentration of 3.85 g/kg for 13 weeks. After 13 weeks, animals were euthanized, and blood and urine samples were taken. No effects were observed on body weights or feed consumption. The anthocyanin profile of the different berries varied significantly, with the primary anthocyanins (in order of concentration, but figures not provided) in chokeberry being cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabidoside and cyanidin-3-*O*-xyloside; in bilberry, malvidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-galactoside, delphinidin-3-*O*-glucoside, delphinidin-3-*O*-galactoside, petunidin-3-*O*-galactoside and cyanidin-3-*O*-arabinoside; and grape, petunidin-3-*O*-glucoside, malvidin-3,5-diglucoside, peonidin-3,5-diglucoside, petunidin-3-*O*-(coumaroyl-glucosyl) and malvidin-3-*O*-arabinoside. Primary anthocyanins identified following chokeberry ingestion were cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, peonidin-3-*O*-galactoside and peonidin-3-*O*-arabinoside; following bilberry ingestion: peonidin-3-*O*-glucoside, malvidin-3-*O*-galactoside and malvidin-3-*O*-glucoside; and following grape ingestion: malvidin-3,5-diglucoside, peonidin-3,5-diglucoside and malvidin-3-*O*-glucoside. Total excretion of anthocyanins was found to vary depending on the type of berry ingested, with rats receiving

chokeberry excreting higher total anthocyanins than the other two groups. A full analysis of metabolites was not carried out in this study (He et al., 2006).

(b) **Studies in humans**

In humans, anthocyanins can be absorbed intact or hydrolysed to the aglycone and then absorbed. They may also be degraded to phenolic compounds by the gut microbiome before absorption. The primary route of metabolism by the microbiome appears to be cleavage of the heterocyclic flavylium ring followed by dihydroxylation or decarboxylation (Fang, 2014; Zhang et al., 2018). Lactase phlorizin hydrolase, which has been isolated from mammalian small intestine, has been found to deglycosylate flavonoids such as anthocyanins (Day et al., 2000).

In previous evaluations of anthocyanins or anthocyanin-rich extracts by JECFA (anthocyanins and grape skin extract; Annex 1, reference *59*) and the European Food Safety Authority (EFSA, 2013), ADME studies have indicated that only small amounts of anthocyanins were absorbed by humans – between 1% and 2%. Previous assessments have therefore concluded that anthocyanins primarily pass through the body unchanged. In more recent studies using stable ^{13}C-labelled C3G in human volunteers, bioavailability of around 12% has been reported, and a range of different metabolites have been identified, including carbon dioxide in breath and anthocyanin conjugates along with vanillic acid, ferulic acid, hippuric acid and 4-hydroxybenzaldehyde in urine (Czank et al., 2013; de Ferrars et al., 2014). The increasing sensitivity of methods of analysis and a greater awareness of the metabolites that need to be investigated are likely to be the primary reasons for this difference between estimated absorption levels. Two recent reviews concluded that anthocyanins are absorbed to a greater degree than previously thought (Lila et al., 2016; Kay et al., 2017).

Literature searches for this evaluation identified a number of ADME studies that predate and generally support the study by Czank et al. (2013), and these have not been described in this review (Miyazawa et al., 1999; Tsuda, Horio & Osawa, 1999; Bub et al., 2001; Matsumoto et al., 2001; Milbury et al., 2002; Mülleder, Murkovic & Pfannhauser, 2002; Wu, Cao & Prior, 2002; Frank et al., 2003; Passamonti et al., 2003; Talavéra et al., 2003, 2004; Harada et al., 2004; Ichiyanagi et al., 2005, 2006; McDougall et al., 2005; Riso et al., 2005; Stoner et al., 2005; Wu et al., 2005; Wu, Pittman & Prior, 2006; Yi et al., 2006; Galvano et al., 2007; Carkeet, Clevidence & Novotny, 2008; Hassimotto, Genovese & Lajolo, 2008).

In a review, it is stated that anthocyanins can be absorbed intact, but the rate and extent of absorption are dependent on the size of the molecule, the type of sugar moiety, the degree of acylation and the matrix in which the anthocyanin mixture is consumed. Anthocyanins with a greater degree of hydroxylation

on the single ring, such as peonidin, malvidin and delphinidin, tend to be less stable. Anthocyanins have been found in the bloodstream within minutes of consumption, leading to the conclusion that absorption can occur through the stomach wall. Absorption has also been reported primarily in the jejunum, with lower levels of absorption in the ileum, in rats and mice. First-pass metabolism appears to be important in anthocyanin metabolism (Fang, 2014).

An earlier review concentrated primarily on the influence of the gut microbiome on the metabolism of anthocyanins. The authors concluded that the gut microbiome is an important site of metabolism of anthocyanins and other related molecules and that changes in the microbiome may have a significant effect on the metabolic products produced following the consumption of anthocyanins (Williamson & Clifford, 2010).

Cereal bars and crackers containing purple wheat, rich in anthocyanins, were prepared and provided to eight male and eight female volunteers who were asked to consume both on separate days with at least a 5-day interval between them while consuming an otherwise low-anthocyanin diet. Following consumption of either wheat product, blood samples were taken at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 hours, and urine samples were taken during the following time periods: baseline, 0–2, 2–4, 4–6, 6–8 and 24 hours after consumption. Although serving sizes were different (bars were 40 g, crackers were 30 g), both products contained 6.7 mg of total anthocyanins (anthocyanin composition varied slightly). The cereal bars contained 2.9 mg C3G, 0.2 mg cyanidin-3-O-rutinoside and 1.4 mg peonidin-3-O-glucoside, with 2.2 mg non-identified anthocyanins, whereas the crackers contained 2.6 mg C3G, 0.2 mg cyanidin-3-O-rutinoside and 1.2 mg peonidin-3-O-glucoside, with 2.7 mg non-identified anthocyanins. No intact anthocyanins or their primary metabolites were identified in plasma. Ferulic acid was identified in plasma, which may be as a result of anthocyanin metabolism, but may also be due to the high levels of ferulic acid in the purple wheat. A higher plasma ferulic acid concentration was observed following consumption of the crackers compared with the cereal bars (31 vs 9 ng/mL; $P = 0.009$), which may be due to the composition of the products and therefore the bioavailability of the phenolic compounds. Primary urinary metabolites were hippuric acid and ferulic acid, and male volunteers were found to excrete higher amounts of anthocyanin metabolites in their urine compared with females (17.1 vs 11.6 μg; $P = 0.017$). Higher concentrations of phenolic urinary metabolites were found following consumption of the crackers (totals in pooled urine after 24 hours were 6750 ± 4400 μg phenolic acids and 400 ± 293 μg ferulic acid following cereal bar consumption and 10 000 ± 6660 μg phenolic acids and 620 ± 570 μg ferulic acid following cracker consumption; $P = 0.034$), which again is probably due to differences in the food matrices. Postprandial plasma interleukin 6 (IL-6)

and tumour necrosis factor alpha (TNF-α) were unaffected by the consumption of purple wheat–containing products (Gamel et al., 2018).

Three volunteers on a low-antioxidant diet were asked to consume 100 g saskatoon berry slurry (homogenized 1:1 mixture of saskatoon berries and water). Blood samples were taken 0.5, 1.5 and 4 hours after consumption of the slurry, and urine samples were collected 0–0.5, 0.5–1.5 and 1.5–4 hours after consumption. The anthocyanin composition of saskatoon berries was as follows: cyanidin-3-*O*-galactoside, 123.5 ± 10.2 mg/100 g; C3G, 29.7 ± 2.5 mg/100 g; cyanidin-3-*O*-arabinoside, 13.0 ± 1.7 mg/100 g; cyanidin-3-*O*-xyloside, 10.1 ± 1.7 mg/100 g; and quercetin-3-*O*-galactoside, 47.3 ± 7.0 mg/100 g. Cyanidin-3-*O*-galactoside was the primary intact anthocyanin measured in both urine and plasma, but all others were also detected at lower levels. Peonidin-3-*O*-glucoside was also detected in urine and plasma. Metabolites were not analysed in this study (Liu et al., 2018).

Following consumption of 24 g of freeze-dried strawberries (divided into two equal doses taken prior to lunch and dinner each day) per day by 19 healthy older adults (aged 60–75 years) for 90 days, pelargonidin glucuronide was found to be the primary metabolite and was found even after an overnight fast, suggesting that strawberry anthocyanins can persist in the circulation. In a control group (*n* = 19), no anthocyanins were identified in plasma samples. The primary metabolites identified following strawberry consumption were urolithins (although identified in only 13 of the 19 strawberry consumers and no consumers of the control beverage) and phenolic acids, in particular hippuric acid, with mean levels in the plasma of 46.0 ± 9.0 μg/L in the group receiving the strawberry beverage and 15.2 ± 1.5 μg/L in the control group (Sandhu et al., 2018).

Two healthy volunteers were asked to consume 125 g red raspberry puree daily for 4 weeks. On days 1 and 28, blood samples were collected at 0, 0.5, 1, 2, 3, 4, 5, 5.5, 6, 7, 8 and 24 hours and urine samples were collected at 0, 0–4, 4–8, 8–24, 24–36 and 36–48 hours following red raspberry consumption. In addition, a breastfeeding mother was asked to consume 125 g fresh red raspberries per day for 1 week, with breast milk samples being taken before and 2 hours after raspberry consumption on days 1 and 7. Analysis of four raspberry samples (including fresh raspberries, raspberry puree, frozen raspberries and raspberry powder) gave a total polyphenol content of between 71.6 and 281.0 mg/100 g fresh weight red raspberries. Glycosides of cyanidin, pelargonidin and peonidin, which constituted 31–66% of the total polyphenols, were identified, and these predominantly consisted of cyanidin-3-*O*-sophoroside (43–81%) and C3G (14–22%). The concentration of anthocyanins was highest in the frozen red raspberries (143.0 mg/100 g) and lowest in the fresh red raspberries (22.3 mg/100 g). In total, 62 metabolites were attributed to the consumption of red raspberries in the three

biological matrices analysed. These included parent anthocyanins (cyanidin-3-O-sophoroside, cyanidin 3-O-glucosyl-rutinoside, C3G, pelargonidin-3-O-sophoroside and cyanidin-3-O-rutinoside) and anthocyanin conjugates (cyanidin-3-O-glucuronide, methyl cyanidin-3-O-sophoroside and peonidin-3-O-glucoside). Urolithin A glucuronide and urolithin B glucuronide were identified in plasma, urine and breast milk, whereas urolithin A was identified only in urine samples. The free and conjugated phenolic acids differed in plasma, urine and breast milk, including benzoic acids, benzoates, benzaldehydes, phenylacetic acids, phenylpropionic acids, phenylcinnamic acids, phenylvalerolactones and their glucuronidated and sulfated derivatives (Zhang et al., 2018).

In a small study, a 10 g serving of an ethanolic bilberry extract containing 15 anthocyanins (total 2.2 g[1]), based on the aglycones delphinidin, cyanidin, petunidin, malvidin and peonidin and the three sugar residues glucose, galactose and arabinose in the 3-O position, was provided to five healthy females and five (otherwise healthy) ileostomy patients after they had consumed a low-polyphenol diet for 48 hours. Ileostomy fluid was collected at 0, 1, 2, 4, 6 and 8 hours, urine was collected at 0–2, 2–4, 4–8 and 8–24 hours and blood samples were taken at 0, 1, 2, 4 and 8 hours after bilberry consumption. In ileostomy patients, 29.6% of the initial anthocyanin dose was eliminated in the ileostomy fluid as intact anthocyanins within the first 2 hours, and 20% was eliminated as degradation products. Maximal plasma anthocyanin levels were reached after 1 hour and decreased steadily thereafter. In healthy volunteers, maximal plasma anthocyanin levels were reached at 2 hours (and were higher than in ileostomy patients), and a slower reduction in plasma concentration was observed. In ileostomy patients, the majority of the anthocyanins and degradants had been excreted in the urine within 4 hours. Metabolites in the plasma of ileostomy patients were generally found to be glucuronide conjugates. The amounts of anthocyanins in the plasma and urine of healthy volunteers were 79% and 44% higher than the amounts found in the plasma and urine of ileostomy patients, showing that the large intestine is an important site of absorption of anthocyanins from bilberry extract. Some absorption did occur in ileostomy patients, and the authors proposed that this takes place primarily in the jejunum, which was present in both ileostomy patients and healthy volunteers. Degradants in plasma, such as 4-hydroxybenzoic acid, gallic acid (3,4,5-trihydroxybenzoic acid), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), phloroglucinol aldehyde (2,4,6-trihydroxybenzaldehyde) and 3-O-methylgallic acid (3,4-dihydroxy-5-methoxybenzoic acid), were found to be up to 20 times higher in plasma and 38

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[1] Calculated using the molecular weight of C3G (449.388 g/mol), the predominant anthocyanin in this bilberry extract.

times higher in urine than the parent anthocyanins in patients and volunteers (Mueller et al., 2017).

In a small crossover study, nine healthy participants aged 18–78 years were asked to consume 12 g of strawberry powder in a milk- or water-based beverage both with and without food. The concentrations of pelargonidin-3-*O*-glucoside, pelargonidin glucuronide and pelargonidin-3-*O*-rutinoside were analysed, and it was determined that the oral bioavailability of anthocyanins in a milk-based beverage consumed under fasting conditions was significantly lower (~50%; $P < 0.05$) than with the water-based preparation. When the beverages were consumed after a meal, the bioavailability of anthocyanins was not significantly different between the water- and milk-based beverages (Xiao et al., 2017).

In a single-blind crossover trial, volunteers aged 20–45 years with a body mass index (BMI) range of 25–33 were provided with a beverage containing 25 g of freeze-dried wild blueberry powder or placebo with a meal, and plasma was collected over 24 hours. Parent anthocyanins in plasma peaked at 2 hours after ingestion, whereas phase II metabolites, including glucuronide conjugates of peonidin, delphinidin, cyanidin and petunidin, peaked at 2.6, 6.3, 7 and 8.8 hours, respectively. The authors concluded that this biphasic response suggests a significant role of enterohepatic recirculation in anthocyanin metabolism (Zhong et al., 2017).

Ten healthy men aged 18–35 years were provided with a single serving of 450 mL cranberry juice containing 16.2 mg anthocyanins (cyanidin-3-*O*-arabinoside, 6.8 mg; cyanidin-3-*O*-galactoside, 1.7 mg; peonidin-3-*O*-arabinoside, 2.1 mg; peonidin-3-*O*-galactoside, 2.0 mg; plus other minor anthocyanins), with a total polyphenol content of 787.5 mg per serving (primarily comprising proanthocyanins, although composition not specified). Blood samples were taken at 0, 1, 2, 4, 6, 8 and 24 hours after consumption. Sixty polyphenol metabolites were identified after cranberry juice consumption. Primary metabolites identified in plasma were hippuric acid, 2,3-dihydroxybenzoic acid, phenylacetic acid and catechol-*O*-sulfate. Primary metabolites identified in urine were hippuric acid, α-hydroxyhippuric acid, 4-hydroxyhippuric acid and phenylacetic acid (Feliciano et al., 2016).

In a placebo-controlled crossover trial, 14 overweight adults aged 18–45 years with a BMI range of 25–29.9 were provided with a strawberry-based beverage and two placebo drinks at each of three visits. Beverages were provided 2 hours before a meal, with a meal or 2 hours after a meal. Blood samples were taken hourly for 10 hours after consumption of the first beverage. Maximal blood concentrations of pelargonidin-based anthocyanins and areas under the concentration–time curve (AUCs) were observed when the strawberry drink was consumed 2 hours before a meal (Sandhu et al., 2016).

Table 1
The pharmacokinetic parameters of anthocyanins from fresh and fermented red cabbage

Parameter	Anthocyanin source	
	Fresh red cabbage	Fermented red cabbage
Elimination constant (h^{-1})	0.25 ± 0.03	0.23 ± 0.02
$t_{1/2}$ (h)	2.73 ± 0.11	3.02 ± 0.14
MRT$_{0-t}$ (h)	3.75 ± 0.19	4.32 ± 0.12
T_{max} (h)	2.0	2.0
C_{max} (nmol/L)	86.39 ± 3.51	64.02 ± 4.87
AUC$_{0-t}$ (nmol·h/L)	471.43 ± 14.11	350.93 ± 9.56

AUC: area under the concentration–time curve; C_{max}: maximum concentration; MRT: mean residence time; t: time; $t_{1/2}$: half-life; T_{max}: time to reach maximum concentration

Source: Wiczkowski, Szawara-Nowak & Romaszko (2016)

In a randomized crossover study, seven female and six male volunteers were provided with a meal containing 240 g of either fresh red cabbage or fermented red cabbage following consumption of a low-polyphenol diet over 72 hours. The red cabbage meals were tailored to provide a total anthocyanin dose of 6 mg/kg bw from the fresh cabbage and 5 mg/kg bw from the fermented cabbage. Blood samples were taken at 0, 0.5, 1, 2, 4, 6, 12 and 24 hours after cabbage consumption. Urine samples were taken at 0, 0–1, 1–2, 2–4, 4–6, 6–8, 8–12 and 12–24 hours after intake. Twenty different cyanidin glucosides were identified in both fresh and fermented red cabbage samples, primarily triglucosides. In plasma and urine, methylated, glucuronidated and sulfated forms of cyanidin were found. The primary metabolites identified in plasma and urine were cyanidin triglucosides substituted with coumaroyl, caffeoyl, feruloyl or sinapoyl groups. The increase in plasma concentration in the first 30 minutes was significantly more rapid from fermented cabbage, and the proportion of anthocyanins present in plasma was higher compared with fresh cabbage in this period. The plasma anthocyanin concentration and excretion rate of anthocyanins between 1 and 6 hours post-consumption were significantly higher following consumption of the fresh cabbage compared with fermented cabbage. The pharmacokinetic parameters for anthocyanins from fresh and fermented red cabbage can be found in Table 1. The authors concluded that the anthocyanins in fresh red cabbage were significantly more bioavailable that those in fermented red cabbage, probably due to the food matrix or saturation of absorption mechanisms (Wiczkowski, Szawara-Nowak & Romaszko, 2016).

Three male and three female volunteers aged 18–65 years on an anthocyanin-free diet were provided with two capsules containing a total of 500 mg of aronia extract (type of extract not specified). Blood samples were taken at 0, 0.5, 1, 2, 4, 6, 9, 12 and 24 hours after dosing, and urine samples were collected

at 0, 2, 4, 6, 9, 12 and 24 hours after dosing. Anthocyanins present in aronia berries were cyanidin-3-O-galactoside, C3G, cyanidin-3-O-arabinoside and cyanidin-3-O-xyloside. The total dose of aronia berry extract contained 306 mg total phenols as gallic acid equivalents, including 45.1 mg anthocyanins, 41.9 mg proanthocyanidins as catechin equivalents, 9.9 mg flavonols and 36.9 mg hydroxycinnamic acids. Protocatechuic acid, hippuric acid, 3-(4-hydroxyphenyl)-propionic acid, C3G and peonidin-3-O-galactoside were recovered in plasma after aronia consumption. C3G reached a peak concentration in plasma of 0.059 ± 0.024 µg/mL 1.6 hours after dosing, whereas polyphenol metabolites peaked at 1 hour for protocatechuic acid, 2.67 hours for peonidin-3-O-galactoside, 5.33 hours for hippuric acid and 6.33 hours for 3-(4-hydroxyphenyl)propionic acid. The plasma AUC values ranged from 0.054 ± 0.013 µg·h/mL for protocatechuic acid to 22.4 ± 4.4 µg·h/mL for hippuric acid. Among the plasma metabolites, hippuric acid accounted for 85.2% of the total polyphenols measured by their AUCs. Protocatechuic acid, 3,4-dihydroxyphenylacetic acid, hippuric acid, ferulic acid, 3-(4-hydroxyphenyl)propionic acid, cyanidin-3-O-galactoside, C3G, cyanidin-3-O-arabinoside and peonidin-3-O-galactoside were recovered in urine samples. Cyanidin-3-O-galactoside reached a peak concentration of 0.004 ± 0.001 mg/mg creatinine at 4.67 hours after dosing. The time to reach the maximum concentration (T_{max}) and the maximum concentration (C_{max}) for C3G and cyanidin-3-O-arabinoside were 6 hours and 0.010 ± 0.006 mg/mg creatinine and 4 hours and 0.020 ± 0.006 mg/mg creatinine, respectively. Phenolic acids peaked at 4 hours for protocatechuic acid, 4.67 hours for peonidin-3-O-galactoside, 4.83 hours for 3-(4-hydroxyphenyl)propionic acid, 5.33 hours for ferulic acid and 6.33 hours for both 3,4-dihydroxyphenylacetic acid and hippuric acid. The urine AUC ranged from 0.016 ± 0.005 mg·h/mg creatinine for cyanidin-3-O-galactoside to 2430 ± 360 µg·h/mg creatinine for hippuric acid. Among the urinary metabolites, hippuric acid accounted for 98.5% of the total increase in polyphenols after dosing with aronia extract. Differences were observed between individuals, with some individuals showing plasma anthocyanin levels below the limit of detection and some showing relatively high levels. Significant differences were also observed in T_{max} values, especially in plasma (Xie et al., 2016).

Five male and five female healthy volunteers aged 23–27 years consumed either a grape and blueberry juice mixture or a smoothie made with grape juice and blueberry puree in a double-blind crossover trial. Both the juice and the smoothie contained anthocyanins based on malvidin (juice 47%, smoothie 38%), peonidin (juice 23%, smoothie 19%), delphinidin (juice 11%, smoothie 19%), petunidin (juice 11%, smoothie 12%) and cyanidin (juice 8%, smoothie 12%). The aglycones were glycated with glucose, galactose and arabinose, and some were acylated with p-coumaric acid or acetic acid. Blood samples were taken 0, 10, 20, 30, 60, 90 and 120 minutes after consumption, and urine samples

Table 2

Plasma pharmacokinetic parameters of anthocyanins from raspberries

Compound	*n*	C_{max} (ng/L) (mean ± SD)	T_{max} (h)
C3G	7	89.9 ± 44.9	1
Cyanidin-3-*O*-glucuronide	3	47.8 ± 47.8	4
3′,4′-Dihydroxyphenylacetic acid	7	30 266 ± 14 965	6
Ferulic acid-4′-sulfate	9	12 889 ± 3 839	1
Ferulic acid-4′-*O*-glucuronide	9	6 666 ± 741	1.5
Isoferulic acid-3′-*O*-glucuronide	9	5 184 ± 741	1.5
4′-Hydroxyhippuric acid	9	17 565 ± 2 702	1

C_{max}: maximum concentration; C3G: cyanidin-3-*O*-glucoside; *n*: number of participants in whom metabolite was detected; SD: standard deviation; T_{max}: time to reach maximum concentration
Source: Ludwig et al. (2015)

were taken in the following time frames: 0–3, 3–6, 6–9, 9–12, 12–15 and 15–24 hours. All participants received both the smoothie and the juice twice. Urinary metabolite recovery and pharmacokinetics did not vary between smoothie and juice. Blueberry and grape juices/purees contained a lower percentage of cyanidin derivatives compared with black carrot extract (Kuntz et al., 2015).

Five female and four male volunteers on a low-polyphenol diet consumed 300 g of homogenized raspberries containing a total of 181.1 mg anthocyanins: cyanidin-3-*O*-sophoroside (107 ± 4 mg), cyanidin-3-*O*-(2″-*O*-glucosyl)-rutinoside (42.4 ± 1.5 mg), C3G (16.6 ± 0.4 mg), cyanidin-3-*O*-rutinoside (11.9 ± 0.6 mg), cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside (2.0 ± 0.07 mg), pelargonidin-3-*O*-sophoroside (0.7 ± 0.0 mg), pelargonidin-3-*O*-glucoside (0.5 ± 0.0 mg) and cyanidin-3,5-*O*-diglucoside (traces). Total urine was collected for 24 hours prior to raspberry consumption and in the following periods after consumption: 0–4, 4–8, 8–24, 24–32 and 32–48 hours. Blood samples were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 24 hours after raspberry consumption. Table 2 gives the metabolites in plasma and their pharmacokinetic parameters. Except for low levels of C3G, none of the raspberry anthocyanins was detected in urine. Low levels of peonidin-3-*O*-glucoside were detected in the first 8 hours after raspberry consumption; this anthocyanin is not present in raspberries, which suggests that methylation may be a minor route of metabolism of anthocyanins. The following metabolites of anthocyanins were identified in urine (amounts are total in all urine): 4-hydroxybenzoic acid (884.0 ± 662.0 µg), 3,4-dihydroxybenzoic acid (traces), 3-methoxy-4-hydroxybenzoic acid (traces), 4-hydroxybenzoic acid-3-sulfate (65.5 ± 21.8 µg), 3-hydroxybenzoic acid-4-sulfate (43.6 ± 21.8 µg), 3′,4′-dihydroxyphenylacetic acid (487.6 ± 218.6 µg), 3′-methoxy-4′-hydroxyphenylacetic acid (36.4 ± 36.4 µg), 4′-hydroxyhippuric acid (3142 ± 370.8 µg), hippuric acid (42.8 ± 9.9 mg), caffeic acid-3′-sulfate (260.2 ± 26.0 µg),

Table 3
Pharmacokinetic parameters for the five primary metabolites observed in each of plasma and urine[a]

Metabolite	C_{max1} (ng/mL)	T_{max1} (h)	C_{max2} (ng/mL)	T_{max2} (h)	AUC_{0-24h} (ng·h/mL)
Serum					
Protocatechuic acid	1 600 ± 1 600	8.4 ± 1.4	–	–	14 100 ± 18 500
Quercetin	340.2 ± 121.8	1.4 ± 1.3	463.0 ± 183.8	7.8 ± 1.9	2 984.9 ± 2 368.6
Vanillic acid	1 997.9 ± 360.9	0.7 ± 0.3	1 854.3 ± 378.2	6.1 ± 0.5	2 617.7 ± 1 721.6
Salicylic acid	53.5 ± 91.8	1.0 ± 0.8	36.6 ± 55.6	4.6 ± 2.3	635.7 ± 1 257.6
4-Hydroxybenzoic acid	197.7 ± 88.1	0.8 ± 0.7	198.6 ± 76.4	7.2 ± 2.3	492.4 ± 723.8
Urine					
Protocatechuic acid	21.1 ± 28.4	9.3 ± 7.2	–	–	95.9 ± 207.1
4-Hydroxyphenylacetic acid	9.6 ± 7.4	6.6 ± 1.7	6.7 ± 6.8	10.0 ± 0.0	78.1 ± 102.1
p-Coumaric acid	1.8 ± 2.0	5.4 ± 1.0	–	–	15.9 ± 22.7
3,4-Hydroxyphenylacetic acid	2.9 ± 4.1	7.0 ± 2.4	–	–	15.6 ± 21.0
4-Hydroxy-3-methoxyphenylacetic acid	1.8 ± 1.3	6.2 ± 2.2	0.74 ± 0.58	10.0 ± 0.0	9.8 ± 7.9

AUC: area under the concentration–time curve; C_{max}: maximum concentration; T_{max}: time to reach maximum concentration
[a] Values given as mean ± standard deviation.
Source: McKay et al. (2015)

dihydrocaffeic acid-3′-sulfate (288.5 ± 78.7 μg), ferulic acid (1.1 ± 0.4 mg) and sulfated and glucuronidated metabolites of ferulic acid and isoferulic acid. Of the initial dose of anthocyanins in the raspberries, 15% was identified in the urine, although this did not include hippuric acid, as the background level of hippuric acid was high and it was difficult to identify which came from sources other than the raspberries (Ludwig et al., 2015).

Five male and five female healthy volunteers aged 50–70 years were asked to consume a diet low in phenol and polyphenols for 48 hours prior to consumption of a cranberry juice cocktail. The anthocyanins present in the serving were peonidin-3-O-galactoside (68.0 mg), peonidin-3-O-arabinoside (41.0 mg), cyanidin-3-O-arabinoside (23.7 mg), cyanidin-3-O-galactoside (16.5 mg), peonidin-3-O-glucoside (5.2 mg) and C3G (1.1 mg). Blood was collected at 0, 0.25, 0.5, 1–6, 10 and 24 hours. Urine was collected every 2 hours for 10 hours, then at 24 hours. The pharmacokinetic parameters for the five primary metabolites (based on AUC) found in each of serum and urine can be found in Table 3. A bimodal distribution was found for some of the metabolites, which can be seen in the C_{max2} and T_{max2} columns of Table 3. Lower concentrations of other metabolites, unchanged anthocyanins and glucuronide conjugates of anthocyanin aglycones were detected (McKay et al., 2015).

Four male and 13 female volunteers aged 24–60 years consumed a single serving of 250 mL of blueberry juice containing 216 mg anthocyanins expressed as C3G equivalents. The anthocyanin content of the blueberry juice

was not investigated by the authors, but they do reference other sources, which state that blueberry juice contains at least 15 anthocyanin monoglycosides, some diglucosides and some anthocyanins with acylating groups; blueberries do not contain pelargonidin and contain only 6% peonidin. Urine was collected for 24 hours after blueberry juice consumption. The mean baseline urinary anthocyanin level was 20.26 ± 6.536 ng/mL. The first urine samples contained a mean urinary anthocyanin level of 416.7 ± 92.38 ng/mL. Predominant identified metabolites were aglycones, especially aglycone glucuronides. Anthocyanin metabolites based on pelargonidin were identified, which suggests ongoing dehydroxylation and demethylation of other anthocyanins via the colonic microbiome (Kalt et al., 2014).

Eight male volunteers aged 18–45 years (mean age 27.8 ± 8.1 years) received a single bolus dose of 500 mg ^{13}C-labelled C3G in the form of gelatine capsules after overnight fasting. After dosing, the following samples were taken at the stated time periods: blood (0.5, 1, 2, 4, 6, 24 and 48 hours), urine (individual voids between 0 and 6 hours; total voids between 6 and 24 hours and between 24 and 48 hours), breath (0.5, 1, 2, 4, 6, 24 and 48 hours) and faeces (all voids between 0 and 6, 6 and 24, and 24 and 48 hours). Urine was the primary route of elimination over the first 6 hours, with 66.33% ± 13.22% of total ^{13}C recovery, followed by breath, with 28.94% ± 6.89% of total ^{13}C recovery. Over the period from 6 to 48 hours, faeces were the main route of elimination. Of the total bolus dose of ^{13}C, 43.9% ± 25.9% was recovered in urine, faeces and breath, with high interindividual variation (range 15.1–99.3%). The relative bioavailability calculated from the recovery in urine and breath was 12.3% ± 1.38% (mean recovery of ^{13}C in urine was 5.4%, and in breath, 6.9%). Metabolites identified included carbon dioxide from breath and phase II conjugates of C3G and cyanidin (cyanidin glucuronide, methyl cyanidin glucuronide and methyl C3G glucuronide), degradants (protocatechuic acid and phloroglucinaldehyde), phase II conjugates of protocatechuic acid (including protocatechuic acid-3-glucuronide, protocatechuic acid-4-glucuronide, protocatechuic acid-3-sulfate, protocatechuic acid-4-sulfate, vanillic acid, isovanillic acid, vanillic acid glucuronide, isovanillic acid glucuronide, vanillic acid sulfate, isovanillic acid sulfate, methyl 3,4-dihydroxybenzoate, 2-hydroxy-4-methoxybenzoic acid and methyl vanillate), phenylacetic acids (3,4-dihydroxyphenyl acetic acid and 4-hydroxyphenylacetic acid), phenylpropenoic acids (caffeic acid and ferulic acid) and hippuric acid in urine. In serum, C3G reached a peak concentration of 0.14 ± 0.05 µmol/L (equal to 67.9 ± 24.2 µg/L) at 1.81 ± 0.16 hours post-consumption, whereas its degradation products peaked at 0.72 ± 0.23 µmol/L. The major metabolites of C3G in serum were phase II conjugates of protocatechuic acid, ferulic acid and hippuric acid, which peaked between the 6- and 24-hour collection time points at concentrations between 0.94 ± 0.37 and 2.35 ±

0.15 µmol/L. In urine, C3G and its degradants reached a peak at 2 hours post-consumption at 1.78 ± 0.18 µmol/L (863.0 ± 87.3 µg/L) and 0.48 ± 0.10 µmol/L, respectively. Also, phase II conjugates of C3G and cyanidin were identified in the urine at a maximum concentration of 1.17 ± 0.52 µmol/L at 1 hour and consisted of cyanidin glucuronide, methylated cyanidin glucuronide and methylated C3G glucuronide. Phase II conjugates of protocatechuic acid peaked in the urine at 24 hours (5.54 ± 0.49 µmol/L), phenylacetic and phenylpropenoic acids at 48 hours (1.51 ± 0.75 mmol/L) and hippuric acid at 24 hours (5.21 ± 3.43 µmol/L). Out of the total ^{13}C recovered in the urine, 36.72% ± 14.39% could be accounted for as ^{13}C-labelled metabolites, with the specific urinary recovery of metabolites ranging from 36.47 ± 20.27 ng for C3G to 1124.89 ± 830.70 ng for phase II conjugates of protocatechuic acid. Total metabolites present in faeces reached a maximal concentration at 24 hours post-consumption (43.16 ± 18.32 µmol/L), with phase II conjugates of protocatechuic acid reaching a peak concentration of 6.94 ± 3.59 µmol/L at 48 hours, whereas the majority of faecal metabolites were composed of phenylpropenoic and phenylacetic acids (41.69 ± 11.55 µmol/L), which peaked at 6–24 hours. The hippuric acid content of faeces reached a peak of 0.42 ± 0.17 µmol/L (33.3 ± 13.5 µg/L) at 24 hours. Out of the total ^{13}C recovered in faeces, 2.01% ± 0.01% could be accounted for as ^{13}C-labelled phenolic metabolites, with the specific faecal recovery of metabolites ranging from 0.44 ng for C3G to 56.00 ± 34.64 ng for phenylacetic and propenoic acids (Czank et al., 2013).

In a follow-up study, further analysis of the metabolites from C3G suggested that the parent compound is primarily converted to protocatechuic acid and then ultimately metabolized to vanillic acid, ferulic acid, hippuric acid and 4-hydroxybenzaldehyde, all metabolites that are excreted easily from the body (de Ferrars et al., 2014).

Six male and six female volunteers aged 20–45 years were provided with five capsules containing 200 mg black bean seed coat extract (ethanol extract rich in C3G, but exact dose of anthocyanins not determined) per day for 14 days. Blood samples were taken at 0, 0.25, 0.5, 1, 1.5, 2, 4 and 6 hours after dosing on days 1 and 14. Plasma concentrations of C3G were analysed, and the pharmacokinetic parameters can be found in Table 4. Results were not significantly different between days 1 and 14, and therefore the authors concluded that C3G did not accumulate in tissues (Jeon et al., 2012).

Six male and six female volunteers participated in a three-treatment crossover study separated by 3-week washout periods. Treatment consisted of 250 g purple carrot sticks served raw, 250 g purple carrot sticks served microwave-cooked and 500 g purple carrot sticks served microwave-cooked. Blood samples were taken at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7 and 8 hours. Urine samples were collected at baseline and every 2 hours until 16 hours. From 16 to 24 hours, urine was pooled. A diet free of anthocyanins was consumed

Table 4
Pharmacokinetic parameters of C3G from black bean seed coat extract

Parameter	Day 1	Day 14	*P*-value
AUC$_{last}$ (ng·h/mL)	4.26 ± 1.40	4.85 ± 1.65	0.073 7
C_{max} (ng/mL)	2.10 ± 0.60	2.17 ± 0.68	0.665 9
T_{max} (h)	0.73 ± 0.29	0.75 ± 0.34	0.883 7
$t_{½}$ (h)	1.58 ± 0.33	1.47 ± 0.29	0.305 6

AUC: area under the concentration–time curve; C_{max}: maximum concentration; $t_{½}$: half-life; T_{max}: time to reach maximum concentration
Source: Jeon et al. (2012)

by the participants throughout the study. The samples of purple carrot were analysed, and five anthocyanins were identified: cyanidin-3-*O*-(xylose-glucose-galactoside), cyanidin-3-*O*-(xylose-galactoside), cyanidin-3-*O*-(xylose-sinapoyl-glucose-galactoside), cyanidin-3-*O*-(xylose-feruloyl-glucose-galactoside) and cyanidin-3-*O*-(xylose-coumaroyl-glucose-galactoside). Anthocyanin content varied, with the raw carrots containing 1.852 μmol/g (1758 μg/g) and cooked carrots containing 1.428 μmol/g (1356 μg/g).[2] Total anthocyanin contents of the raw, cooked (250 g) and cooked (500 g) treatments were 463, 357 and 714 μmol, respectively (440, 339 and 678 mg, respectively). Cooking did not appear to have an effect on the bioavailability of the anthocyanins. Absorption efficiency was greater in the non-acylated anthocyanins than in the acylated anthocyanins. Compared with similar studies using liquid substrates, the authors noted that the transit time from consumption to appearance in plasma was long, but that the carrot matrix needed to be broken down by the digestive tract to a greater extent than in other studies. Acylated anthocyanins were found to have shorter half-lives for gastrointestinal absorption. Non-acylated anthocyanins were eliminated more slowly than their acylated counterparts. Glucuronidated or sulfated metabolites were not identified in blood or urine following consumption of purple carrots, suggesting that these anthocyanins are absorbed and excreted as intact molecules (Novotny, Clevidence & Kurilich, 2012).

In a controlled randomized crossover study, 10 male and eight female volunteers consumed 1 L of anthocyanin-containing blood orange juice (containing 53.09 mg total anthocyanins per litre, including delphinidin-3-*O*-glucoside, 3.96 mg/L; C3G, 25.79 mg/L; cyanidin-3-*O*-(6-malonylglucoside), 17.88 mg/L) per day divided into three portions to be taken at breakfast, lunch and dinner and blood orange juice containing no anthocyanins for a 4-week period, separated by a 4-week washout period. Blood and urine samples were

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[2] All calculated assuming a molecular weight of 949.841 g, the molar weight of cyanidin-3-*O*-(xylose-sinapoyl-glucose-galactoside) (Chemical Abstracts Service No. 142630-71-5), the predominant anthocyanin detected by the authors in all carrot treatments.

taken weekly during the study. Plasma anthocyanin levels remained undetectable throughout the study. Urinary excretion of delphinidin-3-*O*-glucoside, C3G and cyanidin-3-*O*-(6-malonylglucoside) showed a statistically significant increase (*P* < 0.05) between baseline and all weekly urine samples in the group receiving blood orange juice. Indicators of platelet function and leukocyte activation were not significantly affected by supplementation with either type of orange juice (Giordano et al., 2012).

Five male and four female volunteers on a low-phenolic diet consumed 300 g of raspberries containing a total anthocyanin content of 125 mg[3] comprising cyanidin-3-*O*-sophoroside and cyanidin-3-*O*-(2-*O*-glucosyl)rutinoside, which represented 50% and 30% of the total anthocyanins, respectively. Other minor anthocyanins identified included C3G, pelargonidin-3-*O*-sophoroside, cyanidin-3-*O*-rutinoside and pelargonidin-3-*O*-(2-*O*-glucosyl)rutinoside. Urine was collected during the following periods: 0–4, 4–7, 7–24, 24–32 and 32–48 hours. Hippuric acid was the primary metabolite excreted in urine and made up 86.5% of the total phenolic acids in urine; a mean increase of 236% was observed after raspberry consumption when compared with baseline values. Other minor phenolic acids were elevated statistically significantly from baseline values, including 4-hydroxymandelic acid, 3,4-dihydroxyphenylacetic acid and 3-(4-hydroxyphenyl)lactic acid. As part of the same study, faecal samples from the subjects were incubated with anthocyanins. After 48 hours, approximately 20% of the anthocyanins remained intact. Phenolic acids elevated from controls after incubation with faecal samples included catechol, resorcinol, pyrogallol, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, tyrosol and 3-(3-hydroxyphenyl)propionic acid (González-Barrio, Edwards & Crozier, 2011).

Carrot smoothies made from black, orange or white carrots (devoid of carotenoids) were consumed by five female volunteers aged 21–26 years in an unblinded crossover study with a 2-week wash-out period between smoothies. β-Carotene levels were equalized across the black and orange carrot smoothies at 10.3 mg per serving. Five anthocyanins were identified in the black carrot smoothies, but these were not reported in the paper. Blood samples were taken at 0, 1, 2, 4, 6, 8, 12, 16, 24, 32, 72 and 144 hours after each smoothie. Blood samples were analysed for β-carotene levels only. Anthocyanins in the black carrot smoothies were found to have no effect on absorption of β-carotene from the carrot smoothies (Arscott, Somin & Tanumihardjo, 2010).

A single dose of 480 mL cranberry juice given to 15 volunteers (two females, 13 males; aged 62 ± 8 years) with coronary artery disease contained 835 mg total polyphenols and 94.5 mg anthocyanins. Blood and urine samples were obtained at 0, 1, 2, 3 and 4 hours after consumption of the juice. Anthocyanins were

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[3] Calculated assuming a molecular weight equal to that of cyanidin-3-*O*-sophoroside (611.529 g/mol), the primary anthocyanin found in raspberries.

Table 5

Pharmacokinetic parameters of anthocyanins in plasma following consumption of acai berry juice or pulp

Parameter	Pulp		Juice	
	Mean	SD	Mean	SD
T_{max} (h)	2.17	0.11	2.00	0.22
C_{max} (ng/L)	2 321.35	230.30	1 138.51	142.19
$t_{½}$ (h)	6.56	3.05	3.00	0.56
AUC_{last} (ng·h/L)	8 568.30	972.08	3 314.04	604.71

AUC: area under the concentration–time curve; C_{max}: maximum concentration; SD: standard deviation; $t_{½}$: half-life; T_{max}: time to reach maximum concentration
Source: Mertens-Talcott et al. (2008)

quantified in the plasma and urine. The main anthocyanins found in cranberry juice were cyanidin-3-*O*-galactoside (18.7 mg), C3G (1.58 mg), cyanidin-3-*O*-arabinoside (16.5 mg), peonidin-3-*O*-galactoside (30.8 mg), peonidin-3-*O*-glucoside (5.85 mg) and peonidin-3-*O*-arabinoside (21.0 mg). The same anthocyanins were found in plasma and urine after dosing, along with malvidin-3-*O*-glucoside. All anthocyanins reached maximal plasma concentrations at 1.5 hours after consumption (Milbury, Vita & Blumberg, 2010).

Twelve volunteers aged 21–31 years participated in a double-blind crossover study, with each receiving 7 mL of acai berry pulp, clarified acai juice, an antioxidant-free control beverage containing fructose and purple food colouring or apple sauce per kilogram body weight. Acai berry pulp was found to contain 972 ± 27 mg total anthocyanins (as C3G and cyanidin-3-*O*-rutinoside) per kilogram; the juice was found to contain 531 ± 0.2 mg total anthocyanins (as C3G and cyanidin-3-*O*-rutinoside) per kilogram (proportions not given in either matrix); and the apple sauce did not contain anthocyanins. Blood samples were taken prior to consumption and 0.5, 1, 2, 4, 6 and 12 hours after consumption. Urine samples were collected for the periods 0–3, 3–6, 6–9, 9–12 and 12–24 hours after consumption. The pharmacokinetic parameters of the anthocyanins in plasma can be found in Table 5. No anthocyanins were detected following consumption of the antioxidant-free control beverage or the apple sauce. Pharmacokinetic parameters for anthocyanins in urine were not measured (Mertens-Talcott et al., 2008).

Six healthy volunteers (three males and three females aged 20–24 years) were asked to drink 1 L of blood orange juice (containing C3G at 43 mg/L and cyanidin-3-*O*-(6-malonylglucoside) at 28 mg/L), after which blood, urine and faecal samples were collected for 24 hours. At baseline, none of the serum samples contained anthocyanins (native or conjugated) or any of the monitored phenolic acids, including protocatechuic acid. Only C3G and protocatechuic acid were observed in serum within 6 hours of ingestion. The pharmacokinetic

parameters for C3G were as follows: C_{max} of 1.9 ± 0.6 nmol/L (921.2 ± 290.9 ng/L) and T_{max} of 0.5 hour. The concentration decreased to 1.1 ± 0.2 nmol/L (533.3 ± 97.0 ng/L) 2 hours after ingestion and reached a plateau during the next 4 hours. The AUC value was 7.0 ± 1.2 nmol·h/L (3393.9 ± 581.8 ng/L). For protocatechuic acid, the C_{max} was 492 ± 62 nmol/L (75.9 ± 9.6 mg/L) at T_{max} 2 hours; 6 hours after blood orange juice consumption, the protocatechuic acid concentration returned close to baseline. The blood protocatechuic acid AUC value was 11.0 ± 2.3 μmol·h/L (1695 ± 354 μg·h/L). At baseline, urine samples did not contain protocatechuic acid or any form of anthocyanin. Urine samples up to 24 hours after juice consumption contained both parent anthocyanin and glucuronidated and methylated compounds, whereas protocatechuic acid was never found. The maximum concentration of cyanidin glucosides in the native form in urine was reached at 2 hours, and the concentration returned to baseline 24 hours after juice consumption. Protocatechuic acid was the main metabolite of cyanidin glucosides, accounting for 44.4% of the ingested dose within 6 hours of consumption (Vitaglione et al., 2007).

Three male and three female healthy volunteers aged 43 ± 5 years consumed 200 g strawberries (containing 179 μmol [77.6 mg] pelargonidin-3-*O*-glucoside) after an overnight fast. Urine samples were taken before and 0–2, 2–4, 4–6, 6–8, 8–12 and 12–24 hours after strawberry consumption. Urine samples were split, with half being analysed fresh by high-performance liquid chromatography and half frozen and analysed after defrosting. Anthocyanins were not detected in the urine collected before consumption of the strawberries. In fresh urine samples collected after strawberry consumption, the following anthocyanins and metabolites were identified: pelargonidin monoglucuronides, pelargonidin-3-*O*-glucoside, pelargonidin sulfate and pelargonidin. In frozen samples, peaks for the pelargonidin monoglucuronides, pelargonidin sulfate and pelargonidin were much reduced compared with fresh samples. Pelargonidin monoglucuronides formed approximately 83% of the total urinary metabolites (Felgines et al., 2003).

Four females aged 67 ± 4 years fasted overnight, then consumed 720 mg anthocyanins in the form of 12 g elderberry extract (route of preparation not specified, but dried extract administered as an aqueous solution) containing mainly cyanidin-3-sambubioside, C3G and maltodextrin (proportions not given). Blood samples were taken before dosing and at 10, 20, 30 and 40 minutes and 1, 2, 4, 6 and 24 hours after dosing. Urine samples were collected before dosing and 0–2, 2–4, 4–6, 6–8, 8–12 and 12–24 hours after dosing. In plasma, the main anthocyanins identified were ions of cyanidin-3-sambubioside and C3G, as well as the aglycone in minor amounts. No glucuronides or sulfates of the anthocyanins were identified in plasma. Table 6 gives the pharmacokinetic parameters for total anthocyanins and the main individual components in

Table 6

Pharmacokinetic parameters for total anthocyanins and the main individual components in plasma of four healthy volunteers

Anthocyanins	Medium	C_{max} (mg/L)[a]	T_{max} (min)	$t_½$ (min)
Total	Plasma	32.1–97.9 (mean 56.6)	71.3	132.6
Cyanidin-3-sambubioside	Plasma	22.6	71.3	168.9
C3G	Plasma	24.7	65.0	97.3

C3G: cyanidin-3-*O*-glucoside; C_{max}: maximum concentration; $t_½$: half-life; T_{max}: time to reach maximum concentration
[a] Calculated using the molecular weight for cyanidin-3-sambubioside (581.503 g/mol), the primary anthocyanin in this elderberry extract, according to the authors.
Source: Cao et al. (2001)

plasma. The total amount of the anthocyanins excreted during the 24 hours after consumption of elderberry anthocyanins was calculated to be 397.0 ± 45.1 μg (C3G equivalents). The excretion rate of the total anthocyanins was 77.2 ± 10.9 μg/h during the first 4 hours and 13.4 ± 1.6 μg/h during the second 4 hours (Cao et al., 2001).

(c) **In vitro studies**

On incubation with human faecal microbiota, the glycosidic links of C3G and cyanidin-3-*O*-rutinoside were cleaved, and some cyanidin was converted to protocatechuic acid and other lower molecular weight compounds. The four primary metabolites identified were cyanidin (which was transiently present), protocatechuic acid, one other unidentified metabolite and one unidentified conjugate (Aura et al., 2005).

In an isolated rat small intestine, C3G was absorbed unchanged through the gut wall. Although not stated in the text, it is assumed that the rat microbiome had been removed in this study (Andlauer et al., 2003). Anthocyanins in the diet have also been shown to modulate faecal concentrations of some enterobacteria, including *Bifidobacterium* and *Enterococcus* (Boto-Ordóñez et al., 2014).

2.2 Toxicological studies

If information is available on the good laboratory practice (GLP) status of the studies described below, it is noted in the study description.

2.2.1 Acute toxicity

Dried red cabbage, extracted with ethanol and then dried to form a powder (anthocyanins not identified or quantified), was administered to groups of eight Swiss mice in a single dose of 1000, 2000, 3000, 4000 or 5000 mg extract per kilogram body weight in an Organisation for Economic Co-operation and

Development (OECD)–compliant study. Animals were observed for 72 hours. No adverse effects were identified (Thounaojam et al., 2011).

Mice and rats were administered anthocyanins (cyanidin, petunidin and delphinidin mixture extracted from currants, blueberries and elderberries) at doses ranging from 0 to 25 000 mg/kg bw for mice and from 0 to 20 000 mg/kg bw for rats. Following intravenous or intraperitoneal administration, toxic doses of anthocyanins produced sedation, convulsions and, finally, death. Oral doses were not acutely toxic (Pourrat et al., 1967).

Acute toxicity was assessed in Wistar rats (five of each sex per group), with test animals receiving *Cleistocalyx nervosum* var. *paniala* fruit extract (aqueous extract rich in C3G) at a dose of 5000 mg/kg bw and control animals receiving vehicle. After 15 days of observation, animals were euthanized, and a gross necropsy was carried out. No adverse effects were observed during the study, and no treatment-related effects were observed at necropsy (Charoensin et al., 2012).

In a GLP-compliant study conducted in accordance with OECD Test Guideline 425, a blend of anthocyanins derived from wild blueberry extract, bilberry extract, cranberry powder, elderberry extract, raspberry seed powder and strawberry powder (types of extracts not specified, anthocyanins not quantified or characterized) was used. Acute toxicity was assessed in three female rats using a 25% weight per volume extract suspension in distilled water administered by gavage. The dose was 5000 mg/kg bw. Fourteen days after dosing, animals were euthanized and necropsied. No adverse effects were noted in any of the test animals (Bagchi et al., 2006).

Grape seed extract (ethanol and water extraction; anthocyanins not characterized) was administered via gavage to groups of five male and five female F344/DuCrj rats at a dose of 0, 2000 or 4000 mg/kg bw. Slight decreases in body weight were observed on day 1 in the high-dose group compared with controls, but not on subsequent days. No adverse effects were noted (Yamakoshi et al., 2002).

The above acute toxicity studies are summarized in Table 7.

2.2.2 Short-term studies of toxicity

Short-term studies of the toxicity of anthocyanin-containing test materials are summarized in Table 8 and described below.

(a) Mice

Dried red cabbage, extracted with ethanol and then dried to form a powder (anthocyanins not identified or quantified), was administered to groups of eight Swiss mice for 28 days at a dose of extract of 0, 1000, 2000 or 3000 mg/kg bw per day. After 28 days, blood samples were collected, mice were sacrificed, and brain,

Table 7

Acute toxicity studies in experimental animals

Species	Test material	Route	LD$_{50}$ (mg/kg bw)	Reference
Mice	Dried red cabbage powder[a]	Oral	No effects observed up to 5 000 mg/kg bw	Thounaojam et al. (2011)
Mice	Cyanidin, petunidin and delphinidin mixture extracted from currants, blueberries and elderberries	Intraperitoneal	4 110	Pourrat et al. (1967)
		Intravenous	840	Pourrat et al. (1967)
		Oral	25 000	Pourrat et al. (1967)
Rats	*Cleistocalyx nervosum* var. *paniala* fruit extract (aqueous extract rich in C3G)	Oral	No effects observed up to 5 000 mg/kg bw	Charoensin et al. (2012)
Rats	A blend of anthocyanins derived from wild blueberry extract, bilberry extract, cranberry powder, elderberry extract, raspberry seed powder and strawberry powder (types of extracts not specified)[a]	Oral	No effects observed up to 5 000 mg/kg bw	Bagchi et al. (2006)
Rats	Grape seed extract (ethanol and water extraction)[a]	Oral	No effects observed up to 4 000 mg/kg bw	Yamakoshi et al. (2002)
Rats	Cyanidin, petunidin and delphinidin mixture extracted from currants, blueberries and elderberries	Intraperitoneal	2 850	Pourrat et al. (1967)
		Intravenous	240	Pourrat et al. (1967)
		Oral	20 000	Pourrat et al. (1967)

bw: body weight; C3G: cyanidin-3-*O*-glucoside; LD$_{50}$: median lethal dose
[a] Anthocyanins not quantified or characterized.

Table 8

Short-term studies of toxicity of anthocyanin-containing test materials

Species	Test material	Doses	Findings	Reference
Fifteen-day study				
Guinea-pigs	Anthocyanins in the diet (details of the anthocyanins not available)	3 000 mg/kg diet	No adverse effects reported	Pourrat et al. (1967)
Twenty-eight-day study				
Swiss mice	Dried red cabbage powder, ethanol extraction (anthocyanins not identified or quantified)	0, 1 000, 2 000 and 3 000 mg/kg bw per day	Reduced feed consumption and body weight in 2 000 and 3 000 mg/kg bw per day groups not considered to be treatment related owing to palatability of the test substance	Thounaojam et al. (2011)
Ninety-day studies				
F344/DuCrj rats	Grape skin extract (~2% anthocyanins and >15% proanthocyanins)	Anthocyanin doses: 0, 2, 12 and 66 mg/kg bw per day for males and 0, 2, 14 and 72 mg/kg bw per day for females	Hypertrophy and basophilia in the parotid glands, but shown to be an adaptive response in follow-up study; some calcification in the kidneys observed in all dose	Inoue et al. (2013, 2014)

Table 8 (continued)

Species	Test material	Doses	Findings	Reference
			groups, including controls, but top-dose group showed a statistically significant increase in severity	
F344/DuCrj rats	Purple corn colour (containing 26.4% C3G)	C3G 0, 84, 249 and 935 mg/kg bw per day for males and 0, 89, 272 and 1 016 mg/kg bw per day for females	A number of statistically significant differences in the top-dose group compared with controls	Nabae et al. (2008)
Rats	Grape seed extract (anthocyanins not quantified)	0, 434, 860 and 1 788 mg/kg bw per day for males and 0, 540, 1 052 and 2 167 mg/kg bw per day for females	Some statistically significant findings, but not thought to be treatment related	Bentivegna & Whitney (2002)
Rats	Grape skin extract (2.6% anthocyanins)	Anthocyanins 0 and 46.2 mg/kg bw per day for males and 0 and 54.9 mg/kg bw per day for females	Some statistically significant findings, but not thought to be treatment related	Bentivegna & Whitney (2002)
F344/DuCrj rats	Grape seed extract (ethanol and water extraction; anthocyanins not characterized or quantified)	0, 13.3 ± 0.4, 129.1 ± 3.5 and 1 409.8 ± 49.8 mg/kg bw per day for males and 0, 14.8 ± 0.5, 154.0 ± 4.7 and 1 501.1 ± 55.1 mg/kg bw per day for females	No treatment-related effects observed	Yamakoshi et al. (2002)
Weanling Wistar rats	Anthocyanin extract (details of the anthocyanins not available)	3 000 or 6 000 mg/day	No treatment-related effects observed	Pourrat et al. (1967)
Beagle dogs	Grape colour powder (containing 40% grape pigments, further details not provided)	0, 1 875 and 3 750 mg/kg bw per day	No treatment-related effects observed	Becci et al. (1983b)
Beagle dogs	Grape skin extract (2.4% anthocyanins)	0, 45 and 90 mg/kg bw per day expressed as anthocyanins	No treatment-related effects observed	Cox & Babish (1978b)

bw: body weight; C3G: cyanidin-3-*O*-glucoside

heart, lungs, liver, spleen, kidney and adrenals were removed, examined and weighed. Animals also underwent a gross necropsy. Blood samples were analysed for white blood cell count, haemoglobin, red blood cell count, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red cell distribution width coefficient of variance, red cell distribution width standard deviation, platelets, mean platelet volume, red cell distribution width and plateletcrit (total mass of platelets).

No statistically significant findings were recorded in organ weights or blood parameters. Feed consumption was found to be reduced in the 2000 mg/kg bw per day group ($P < 0.05$) and the 3000 mg/kg bw per day group ($P < 0.01$), and a related decrease in body weight gain was observed in these groups, but this is likely to be due to the palatability of the test substance rather than a toxicological finding (Thounaojam et al., 2011).

(b) **Rats**

In an OECD-compliant 90-day toxicity study, four groups of 10 male and 10 female F344/DuCrj rats were provided with a pelleted diet supplemented with 0, 2, 10 or 50 g grape skin extract per kilogram (equal to 0, 0.1, 0.6 and 3.3 g/kg bw per day for males and 0, 0.1, 0.7 and 3.6 g/kg bw per day for females, respectively). The type of extract was not specified or fully characterized, although the authors stated that it contained about 2% anthocyanins and more than 15% proanthocyanins. Doses of anthocyanins were therefore 0, 2, 12 and 66 mg/kg bw per day for males and 0, 2, 14 and 72 mg/kg bw per day for females, respectively. Animals were checked daily, and body weights were measured weekly. Blood samples were taken at the end of the study. Blood was analysed for red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count, white blood cell count, leukocyte count and reticulocyte count. Serum biochemistry parameters measured were total protein, albumin, albumin/globulin ratio, conjugated and free bilirubin, triglycerides, total cholesterol, blood urea nitrogen, creatinine, sodium, chloride, potassium, calcium, inorganic phosphate, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase. At necropsy, the following organs were weighed and then examined histopathologically: brain, heart, lungs, thymus, spleen, liver, kidneys, adrenal glands, ovaries and testes. The following organs were also examined histopathologically: pituitary gland, nasal cavity, eyes, Harderian gland, spinal cord, salivary glands (parotid, submandibular and sublingual), stomach, small intestine, large intestine, pancreas, urinary bladder, skin, mammary glands, mesenteric lymph nodes, trachea, oesophagus, thyroid glands, tongue, aorta, thigh muscle, sternum, femur, sciatic and trigeminal nerves, epididymides, seminal vesicles, prostate, uterus and vagina.

The hair around the mouth of all animals receiving grape skin extract was stained purple or black. All animals in the 50 g/kg feed group produced black-coloured faeces. No statistically significant changes in body weight were observed in any group during the study. In the top-dose males, statistically significant increases in feed consumption were observed compared with controls in weeks 2, 3, 4 and 13, and no effects on feed consumption were observed in the other groups. No statistically significant differences in body weight or absolute organ

weights were found between test and control groups. Relative brain weights were increased in both sexes at the top dose, and relative weights of heart and testes were increased in the top-dose males.

Mean corpuscular volume was increased slightly, but statistically significantly, compared with controls in males at 50 g/kg feed only, but no other haematological effects were observed. In the top-dose group, total bilirubin was increased in males, but decreased in females. Statistically significant decreases in triglycerides were observed in the top-dose males and females. Chloride was increased in males at 2 and 50 g/kg feed only.

The parotid glands were enlarged in all animals in the 50 g/kg feed group, but only one male in the 10 g/kg feed group showed enlargement, with no other effects in the parotid gland observed in other animals. Microscopically, this gland was found to have severe hypertrophy, and basophilia was diffusely observed in the epithelial cells in the animals of the 50 g/kg feed group. In the other dose groups, including controls, no hypertrophy was observed, but some epithelial cells were found to be basophilic, and diffuse vacuolation was observed. The kidneys of females were found to exhibit minimal calcification of the proximal tubules in all dose groups, including the controls, but this incidence was statistically significantly increased to mild calcification in half of the top-dose females.

Owing to the effects observed in the parotid gland and in the kidneys of female rats, the NOAEL was 10 g/kg feed (equal to anthocyanin doses of 12 and 14 mg/kg bw per day for males and females, respectively) (Inoue et al., 2013).

In a follow-up study, using a similar protocol, male rats received 5% grape skin extract either in the diet or via gavage for 4 weeks to ascertain whether effects on the parotid gland were attributable to an adaptive effect caused by direct exposure of the oral cavity to the astringent grape skin extract. Doses were not provided; however, assuming that body weights and specifications were similar to those in the previous study, anthocyanin doses would be 66 and 72 mg/kg bw per day for males and females, respectively. Control animals received a basal diet or distilled water administered via gavage. Half of the animals were sacrificed after the 4-week dosing period, and half after a 2-week recovery period. Parotid glands were isolated after sacrifice and examined.

Macroscopically, enlargement of the parotid glands was observed in the group receiving grape skin extract in the feed. Microscopically, diffuse, severe hypertrophy and basophils were observed in the glandular epithelial cells of the parotid glands of the group receiving the grape skin extract in the feed. These changes were still apparent, but less severe, after the 2-week recovery period. No hypertrophic findings were observed in the parotid glands of rats in the grape skin extract gavage or water control groups after the 4-week treatment. Slight basophilic change without hypertrophy was observed in the parotid gland epithelium in the

control and gavage treated groups, but not the grape skin extract feed group, after the 4-week treatment. The size and number of secretory granules increased, the endoplasmic reticulum within these appeared increased (although it is not clear whether this relates to size or number), and the electron density in the secretory granules decreased in the glandular epithelial cells of the parotid glands in the grape skin extract feed group compared with the controls. No adverse effects on organelles were observed, and no proliferating cell nuclear antigen-positive glandular epithelial cells were observed in the hypertrophic parotid glands in the grape skin extract feed group. The kidneys were not investigated in this study (Inoue et al., 2014).

The Committee agreed that these findings demonstrated that the effects observed in the parotid glands in Inoue et al. (2013) were an adaptive response to the test article and were not a toxicological finding.

Purple corn colour (containing 26.4% C3G and 57.7% other polyphenols) was included in the diets of groups of 10 male and 10 female F344/DuCrj rats at 0, 5, 15 or 50 g/kg for 90 days (equal to 0, 318, 942 and 3542 mg/kg bw per day for males and 0, 337, 1031 and 3849 mg/kg bw per day for females, expressed as the purple corn colour material). The doses of C3G were therefore 0, 84, 249 and 935 mg/kg bw per day for males and 0, 89, 272 and 1016 mg/kg bw per day for females. Animals were observed daily and weighed weekly, and feed and water consumption was measured over a 2-day period before each weighing. Urine samples were collected in week 13 and analysed for protein, glucose, ketones, bilirubin, occult blood and urobilinogen, specific gravity, electrolytes (sodium, potassium and chloride), volume, appearance, sediments and pH. Blood samples were collected at sacrifice in week 13 and analysed for erythrocyte counts, total leukocyte counts, haemoglobin concentration, haematocrit value, platelet count, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration. Prothrombin time and activated partial thromboplastin time were also measured. Clinical chemistry parameters included aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, blood urea nitrogen, creatinine, glucose, total cholesterol, phospholipid, triglyceride, total protein, albumin, albumin/globulin ratio, inorganic phosphate, calcium, magnesium, sodium, potassium and chloride. At necropsy, the following organs were weighed from all animals: heart, spleen, thymus, adrenals, pituitary, thyroids (including parathyroid), lungs, salivary glands, liver, kidneys, testes, seminal vesicles, prostate, ovaries (including oviducts), uterus and brain. Samples of these organs and of the aorta, lymph nodes, nasal cavity (turbinates), trachea, tongue, oesophagus, stomach, small intestine, large intestine, pancreas, urinary bladder, epididymides, vagina, spinal cord, sciatic nerve, eye, Harderian gland, skin, mammary gland, skeletal muscle, bone and bone marrow, Zymbal's gland,

and any gross lesions from rats in the control and top-dose groups were subject to histopathology.

Stained fur and black faeces were observed in the 15 and 50 g/kg feed groups. Body weights in females in the 50 g/kg feed group were significantly elevated in week 1 when compared with controls, but this was not sustained over the course of the study. Brown urine was noted in the 50 mg/kg feed group, and specific gravity was elevated in females in the 15 and 50 g/kg feed groups and in all treated males. A statistically significant decrease in pH was found in males of the 5 and 50 g/kg feed groups, an increase in potassium was observed in both males and females in the 15 and 50 g/kg feed groups, and chloride was elevated in both sexes in the 50 g/kg feed group. Platelet count was statistically significantly decreased in males in the 50 g/kg feed group and in females in all dose groups. Statistically significant increases in mean corpuscular haemoglobin were observed in males in the 50 g/kg feed group, and statistically significant attenuation of prothrombin time was observed in females at 50 g/kg feed. No ophthalmological abnormalities were observed throughout the study. Alanine aminotransferase and blood urea nitrogen were statistically significantly decreased in males in the 50 g/kg feed group. Total cholesterol, phospholipid and triglyceride values were statistically significantly decreased for both sexes of rats fed purple corn colour in the diet at 50 g/kg. Total protein and albumin were statistically significantly decreased in males in the 15 and 50 g/kg feed groups, and the albumin/globulin ratio was statistically significantly increased in females in the 5 and 50 g/kg feed groups. A statistically significant decrease in calcium and a statistically significant increase in chloride were noted in males at 50 g/kg feed. Statistically significant alterations of aspartate aminotransferase, sodium and potassium were noted in male or female rats, but without apparent dose dependence.

A slight, but statistically significant, decrease in relative liver and thymus weights and a statistically significant increase in relative pituitary weights were found in males in the 50 g/kg feed group. Statistically significant decreases in relative thymus weights in males at 5 g/kg feed and in relative pituitary weights in females at 15 g/kg feed did not show dose dependence. Relative salivary gland weights were statistically significantly increased in both sexes of rats at 50 g/kg feed. Statistically significant changes were also found in relative heart, kidney and thyroid weights, but no dose dependence was observed. Mineralization was found in the medulla of the kidneys in all the females in both the control and 50 g/kg feed groups, but only in two control males and no males at 50 g/kg feed. According to the authors, statistically significant effects did not show a dose–response relationship or were not backed up by other, related observations and were therefore not considered to be related to the test material.

The authors concluded that the NOAEL for this study was the top dose tested, 50 g/kg feed (Nabae et al., 2008). However, the Committee concluded that

as a number of statistically significant findings were observed in the top-dose group, including effects on haematological and clinical chemistry parameters, decreased relative liver and thymus weights and increased pituitary weights in males and increased salivary gland weights in both sexes, the NOAEL was 15 g/ kg feed (equal to 942 mg/kg bw per day for males and 1031 mg/kg bw per day for females, expressed as the purple corn colour material; equivalent to 249 mg/ kg bw per day for males and 272 mg/kg bw per day for females, expressed as C3G). The Committee agreed that the effects in the kidney of the female rats were commonly found spontaneously in female rats and therefore were not toxicologically relevant.

In a 90-day GLP-compliant study, groups of 20 male and 20 female rats were provided with diets supplemented with either grape seed extract at a concentration of 0, 6.3, 12.5 or 25 g/kg feed or grape skin extract at 25 g/kg feed (types of extract were not specified). Mean grape seed extract intake was calculated by the authors to be equal to 0, 434, 860 and 1788 mg/kg bw per day for males and 0, 540, 1052 and 2167 mg/kg bw per day for females. Mean grape skin extract intake was calculated to be 0 and 1778 mg/kg bw per day for males and 0 and 2111 mg/kg bw per day for females. Grape skin extract was found to contain 2.6% by weight anthocyanins; grape seed extract was not analysed for anthocyanin content. Anthocyanin intake from grape skin extract was therefore calculated to be 0 and 46.2 mg/kg bw per day for males and 0 and 54.9 mg/kg bw per day for females. Animals were observed regularly for adverse effects, and blood samples were taken twice during the treatment period (at 4 and 13 weeks). Necropsy was carried out on all animals at the end of the 13-week treatment period.

No treatment-related effects on clinical observations, body weights or body weight gains were observed. A small, but statistically significant, increase in feed consumption was observed in males fed grape seed extract or grape skin extract at 25 g/kg feed. This was likely due to the high level of non-nutritive extracts. Some statistically significant differences between control and test animals were observed in clinical chemistry (albumin/globulin ratio in males and females fed grape seed extract at 12.5 or 25 g/kg feed, chloride in males fed grape seed extract or grape skin extract at 25 g/kg feed, inorganic phosphorus in males fed grape skin extract at 25 g/kg feed, alkaline phosphatase in females fed grape seed extract at 25 g/kg feed), but values were within historical control ranges and were unlikely to be test substance related. Statistically significant ($P < 0.05$) decreases in absolute and relative heart weights were noted in female rats treated with grape skin extract at 25 g/kg in the diet; females fed grape seed extract at 12.5 g/kg feed exhibited a decrease in relative heart weight only. These observations are not considered treatment related, as they were not observed in males, they

did not exhibit a dose–response relationship among grape seed extract–treated groups and there was no correlated microscopic pathology. In male rats receiving grape skin extract at 25 g/kg feed, a statistically significant ($P < 0.05$) increase in the occurrence of a common renal cortical inflammation was observed in 11 of 20 animals, compared with an occurrence in four of 20 control animals. The severity in most cases was minimal. The increased frequency was not observed in female rats of the same treatment group. This finding is commonly seen in male rats, increasing in frequency and severity with age, and was not considered to be treatment related. The thymus weight as a percentage of brain weight was statistically significantly lower in males receiving grape seed extract at 12.5 g/kg feed, but no effects were found in other groups.

In summary, the NOAELs in this study were 1788 mg/kg bw per day for grape seed extract (the highest dose tested) and 1778 mg/kg bw per day for grape skin extract (with anthocyanin doses of 46.2 mg/kg bw per day for males and 54.9 mg/kg bw per day for females), the only dose tested (Bentivegna & Whitney, 2002).

Groups of 10 male and 10 female F344/DuCrj rats were given a diet containing grape seed extract (ethanol and water extraction; anthocyanins not characterized or quantified) at 0, 0.2, 2 or 20 g/kg feed (equal to 0, 13.3 ± 0.4, 129.1 ± 3.5 and 1409.8 ± 49.8 mg/kg bw per day for males and 0, 14.8 ± 0.5, 154.0 ± 4.7 and 1501.1 ± 55.1 mg/kg bw per day for females, respectively) for 90 days. Routine observations were made throughout the study, and haematology, blood chemistry and urine analysis were carried out at the end of the study. The following organs were weighed: brain, pituitary, thyroid with parathyroid, thymus, lung, heart, liver, spleen, kidneys, adrenals, stomach, testes, ovaries, epididymides, seminal vesicles, prostate, uterus, urinary bladder and submandibular glands. In the high-dose and control animals, all of the above organs plus the thoracic aorta, trachea, tongue, oesophagus, duodenum, ileum, jejunum, caecum, colon, rectum, pancreas, lymph nodes (mandibular and mesenteric), mammary glands, spinal cord, sciatic nerves, skin, eyes, optic nerves, Harderian glands, sternum, femur and skeletal muscles were examined histopathologically.

No treatment-related differences in haematology, clinical chemistry or urine analysis parameters were observed between groups. Statistically significant differences in the 2 g/kg feed group included higher epididymides weights in males (920.7 ± 37.0 mg vs 863.4 ± 44.2 mg in the controls), lower absolute thymus weight in females (127.8 ± 16.9 mg vs 161.7 ± 37.7 mg in the controls) and lower relative thymus weight in females (75.8 ± 9.9 mg/100 g bw vs 92.9 ± 15.4 mg/100 g bw in the controls). Statistically significant changes at 0.2 g/kg feed included a lower relative stomach weight in females (474.6 ± 15.6 mg/100 g bw vs 502.2 ± 44.3 mg/100 g bw in the controls). No treatment-related effects on body weights

were observed, and gross examination at necropsy found no treatment-related changes. Some microscopic changes were observed in some organs of both sexes in the control group and in the high-dose group; however, as no relationship between incidence and severity was observed, the authors concluded that these were not treatment related.

The NOAEL for this study was 1409.8 mg/kg bw per day expressed as grape seed extract, the highest dose tested (Yamakoshi et al., 2002).

Weanling male and female Wistar rats (20 of each sex per group) were fed a diet containing anthocyanin extract (details of the anthocyanins not available) at levels equivalent to 3000 or 6000 mg/day for a period of 90 days. A group of concurrent controls was also used in the study. The doses of anthocyanin administered were estimated to be 5 and 10 times, respectively, the level that a human would ingest. No differences in survival, growth or histopathology of the principal tissues were observed between the test animals and controls at the termination of the study.

The NOAEL for this study was 6000 mg anthocyanin extract per day, the highest dose tested (Pourrat et al., 1967).

(c) Guinea-pigs

Guinea-pigs received anthocyanin (details of the anthocyanins not available) in the diet at a concentration of 3000 mg/kg feed for 15 days. No adverse effects were reported (Pourrat et al., 1967).

(d) Dogs

Groups of four male and four female beagle dogs were fed diets containing grape colour powder (containing 40% grape pigments, further details not provided) at 0, 75 or 150 mg/kg feed (equivalent to 0, 1875 and 3750 mg/kg bw per day, respectively) for 90 consecutive days. A further control group received a diet containing 9% maltodextrin. Animals were observed daily, and body weight and feed consumption were measured weekly. Blood samples were taken in weeks 7 and 13 and analysed for haematocrit, total and differential leukocyte count, sedimentation rate, platelet count, reticulocyte count, haemoglobin, serum proteins, glucose, blood urea nitrogen, bilirubin, sodium, potassium, chloride and activities of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. Urine samples (it is not clear when urine samples were taken) were analysed for pH, specific gravity, colour, appearance, ketones, protein, bilirubin and occult blood and examined microscopically for sediment. An ophthalmological examination took place in all animals before and at the end of treatment. After 90 days, all animals were sacrificed, and a gross necropsy was performed, with the following organs weighed: brain, pituitary gland, heart,

thyroid gland, liver, kidneys, spleen, adrenal glands and testes or ovaries. These organs were also examined histopathologically, along with the pancreas, lung, epididymides, prostate or uterus, skeletal muscle, sciatic nerves, gall bladder, stomach, small intestine, large intestine, urinary bladder, lymph node, salivary gland, mammary gland, spinal cord, sternum and marrow, eyes, skin and all gross lesions.

The faeces of all animals fed grape colour powder were purple or blue after day 2 of dosing. Body weight gain in the high-dose group was statistically significantly reduced compared with controls (*P* < 0.05). No treatment-related effects on feed consumption, haematological parameters, urinary parameters or organ weights were observed. Gross necropsy and histopathological examination also showed no treatment-related effects (Becci et al., 1983b).

Male and female beagle dogs (four of each sex per dose) received grape skin extract (approximately 2.4% anthocyanins by weight) at 0, 75 or 150 g/kg feed (equivalent to 0, 1875 and 3750 mg/kg bw per day, respectively; anthocyanin intake was 0, 45 and 90 mg/kg bw per day, respectively) for 90 days. No differences in body weight, growth, survival, haematology, biochemistry, urine analysis, organ weights or pathological lesions (gross or microscopic) were noted between control and treated animals (Cox & Babish, 1978b).

2.2.3 Long-term studies of toxicity and carcinogenicity

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

2.2.4 Genotoxicity

Genotoxicity studies conducted using anthocyanins are summarized in Table 9.

Only one study in Table 9, a comet assay, was carried out using black carrot extract. An aqueous extract of black carrot juice was prepared by mixing black carrot juice with cell culture medium, ultrasonic treatment for 4 minutes and two centrifugation steps to produce a coloured but transparent liquid. This aqueous extract was incubated with HT29 clone 19A human colon cells for short-term tests (15–60 minutes) or longer-term tests (24–48 hours). Each experiment was performed in triplicate. Test and control group results were shown in graphical figures, but no description of the methods used to prepare the control experiments or the substances used were given by the authors. Statistically significant differences were found between the tail fluorescence of test and control groups from C3G concentrations equal to and greater than 88 μmol/L. Cell viability was also found to differ statistically significantly from controls at 88 μmol/L or higher. Therefore, genotoxicity was observed only at cytotoxic doses (Glei et al., 2003).

Table 9

Genotoxicity studies carried out on anthocyanins or anthocyanin-rich substances

End-point	Test system/ species	Test substance	Concentrations/ doses	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100	*Cleistocalyx nervosum* var. *paniala* fruit pulp	0.8, 4, 20, 100, 200 mg/mL	Negative	Charoensin et al. (2012)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	Grape seed extract[a]	TA98/TA100: 0, 19, 39, 78, 156, 313, 625, 1 250 µg/plate TA1535/TA1537: 0, 156, 313, 625, 1 250, 2 500, 5 000 µg/plate	Negative ±S9	Yamakoshi et al. (2002)
Reverse mutation	*S. typhimurium* TA1538, *Escherichia coli* Wf2, *Saccharomyces cerevisiae*	Anthocyanin (further details not provided)	0.5 mg/mL	Negative ±S9	Haveland-Smith (1981)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Cyanidin and delphinidin	<0.01 revertant per nanomole test agent for both test substances	Negative ±S9	Brown & Dietrich (1979)
Reverse mutation	*S. typhimurium* TA98	Cyanidin chloride	47.7, 477 mg/plate	Negative ±S9	MacGregor & Jurd (1978)
Chromosomal aberration assay	Chinese hamster lung cells	Grape seed extract[a]	9.4, 18.8, 37.5 mg/mL for 48 h and 18.8, 37.5, 75.0, 150.0, 300.0 mg/mL for 6 h	Negative ±S9	Yamakoshi et al. (2002)
Comet assay	HT29 clone 19A cells (human colon tumour cells)	Black carrot aqueous extract	88–350 µmol C3G equivalents per litre; 24–48 h incubation	Positive at cytotoxic concentrations	Glei et al. (2003)
Micronucleus assay	Human T lymphocytes	Cyanidin 3-*O*-β-glucopyranoside	6.25, 12.5, 25, 50, 100 µg/mL	Negative	Fimognari et al. (2004)
In vivo					
Comet assay: effects on liver and kidney tissues and peripheral blood	Male Swiss albino mice	Acai berry pulp	3.33, 10.0, 16.67 g/ kg bw	Negative	Ribeiro et al. (2010)
Liver micronucleus induction	Wistar rats	*Cleistocalyx nervosum* var. *paniala* fruit aqueous extract	1 000 mg/kg bw	Negative	Charoensin et al. (2012)
Bone marrow micronucleus assay	Male Swiss albino mice	Acai berry pulp (acute, single dose)	3.33, 10.0, 16.67 g/ kg bw	Negative	Ribeiro et al. (2010)
Bone marrow micronucleus assay	Male Swiss albino mice	Acai berry pulp (daily dose for 14 days)	3.33, 10.0, 16.67 g/ kg bw	Negative	Ribeiro et al. (2010)
Bone marrow micronucleus assay	Male Crl:CD-1 (ICR) BR mice	Grape skin extract (2.6% anthocyanins)[a]	500, 1 000, 2 000 mg/ kg bw via gavage	Negative	Erexson (2003)
Bone marrow micronucleus assay	Male Crl:CD-1 (ICR) BR mice	Grape seed extract[a]	500, 1 000, 2 000 mg/ kg bw via gavage	Negative; decrease in PCE:NCE ratio in 2 000 mg/kg bw group,	Erexson (2003)

Table 9 (continued)

End-point	Test system/ species	Test substance	Concentrations/ doses	Result	Reference
				showing cytotoxicity	
Peripheral blood micronucleus assay	ddY mice	Grape seed extract[a]	0, 0.5, 1, 2 g/kg bw	Negative	Yamakoshi et al. (2002)

bw: body weight; NCE: normochromatic erythrocytes; PCE: polychromatic erythrocytes; S9: 9000 × g supernatant fraction from rat liver homogenate
[a] Type of extract not specified. Anthocyanins not characterized or quantified.

The Committee noted that one mouse bone marrow micronucleus assay with a grape seed extract (type of extract not specified, and the anthocyanins were not characterized or quantified) gave negative results for micronuclei in the bone marrow, but a decrease in the ratio of polychromatic erythrocytes to normochromatic erythrocytes in the 2000 mg/kg bw group (the top-dose group) was observed, which suggests cytotoxicity to the bone marrow (Erexson, 2003).

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Groups of 25 male and 25 female Sprague Dawley rats were fed diets containing grape colour powder (containing anthocyanins, primarily peonidin, malvidin, delphinidin and petunidin, and other components, including tartaric acid, tannins, sugars, minerals and amino acids; amounts not specified) at 0, 75 or 150 g/kg feed (equivalent to 0, 7500 and 15 000 mg/kg bw per day, respectively). A further control group received a diet containing 9% maltodextrin. After 3 weeks of feeding, all rats in the F_0 generation were paired for mating. Female rats were fed their respective diets throughout mating, gestation and lactation. The number of pregnant females, number of pups born alive or dead, and survival were recorded. The F_1 generations were culled to five animals of each sex per litter on day 4 postpartum, and the pups were weighed on days 0, 4 and 21. Two males and two females were selected from each litter and used for a 90-day feeding study in which they received the same doses of grape colour powder as their parents. At this point, the F_0 generation animals were sacrificed, and a gross necropsy was performed. After 13 weeks of feeding, the F_1 generation animals were paired and mated. The females continued on their respective diets during mating, gestation and lactation. Reproductive parameters were measured as previously, and at day 21, the F_2 pups and the F_1 adults were sacrificed, and a gross necropsy was carried out. Throughout the experiment, animals were observed daily, and body weight and feed consumption were measured weekly except during the mating period. Ophthalmic examinations of the F_1 generation were carried out in weeks 0 and 13, and blood samples were taken in weeks 6 and 13 and analysed for

haematocrit, total and differential leukocyte count, platelet count, haemoglobin, total protein, glucose, blood urea nitrogen, bilirubin, sodium, potassium, chloride and activities of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. In the F_1 generation, a gross necropsy was performed, with the following organs weighed: epididymides, heart, thyroid gland, liver, kidneys, spleen, adrenal glands, uterus and testes or ovaries. These organs were also examined histopathologically, along with the brain, pituitary gland, spinal cord, eyes, lungs, stomach, pancreas, large and small intestines, urinary bladder, seminal vesicles, lymph nodes, skin, sternum and all gross lesions.

Body weight gain in females in the top-dose group was statistically significantly reduced compared with controls ($P < 0.05$). This was likely a result of the reduced caloric intake due to the high concentration of test substance in the feed. Several statistically significant differences in haematology and clinical chemistry were found in the F_1 generation, but these did not show a dose–response relationship or were not considered to be treatment related: a decrease in erythrocyte count was observed in the 75 g/kg feed group at 13 weeks only; chloride level was elevated in males in the 75 g/kg feed group at 6 weeks only; and decreases were observed in alkaline phosphatase activity in females in the 75 g/kg feed group and in potassium levels in females in the 75 and 150 g/kg feed groups at 6 weeks only. No ophthalmic effects were observed. Absolute and relative liver weights were statistically significantly lower in males fed grape colour powder at 75 or 150 g/kg feed compared with controls. Absolute adrenal gland weights were decreased in males in the groups receiving grape colour powder at 75 or 150 g/kg feed and in females receiving 150 g/kg feed ($P < 0.05$). Relative thyroid weights were decreased in males in all dose groups compared with controls; absolute thyroid weight was lower in the top-dose group only. These effects on organ weights were not considered treatment related as they did not show a dose–response relationship. One animal died in the 9% maltodextrin control group, but no other unscheduled mortalities occurred. No treatment-related effects on feed consumption were observed. Gross necropsy and histopathological examination also showed no treatment-related effects (Becci et al., 1983a).

As the anthocyanin level of the grape colour powder was not provided, a NOAEL could not be derived from this study.

In a two-generation reproduction study, Sprague Dawley rats ingested a grape skin extract preparation that was prepared by spray drying the liquid form of the extract after addition of a carrier material (maltodextrin). The preparation contained approximately 3% anthocyanins (composition not specified). The test groups received grape skin extract at a concentration of 75 or 150 g/kg feed throughout the study (equivalent to 7500 and 15 000 mg/kg bw per day expressed as grape skin extract; equivalent to 225 and 450 mg/kg bw per day expressed as

anthocyanins). There were two concurrent control groups, one receiving the basal diet, the other receiving a diet containing 9% of the maltodextrin used as a carrier to the grape skin extract preparation. At week 6 and at termination of the study, haematology, blood serum chemistry and urine analyses were carried out in the F_1 group. At week 18 of the study, rats in the F_1 group were sacrificed, absolute and relative organ weights were determined, and a complete histological study was carried out on the principal organs and tissues. The F_2 generation (10 per litter culled at 4 days) was maintained for 21 days postpartum, then necropsied.

No differences in reproductive performance or indices, including pup viability, were apparent between control and dosed groups. At the high dose, both the F_1 and F_2 rats exhibited lower body weights compared with the concurrent controls. Body weights of the F_2 pups in the 75 g/kg feed group were marginally depressed. However, it should be noted that the decrease in body weights was accompanied by a concomitant decrease in feed intake. There were no compound-related effects on haematology, blood serum chemistry or urine analysis parameters in the F_1 group. Decreases in weights of the liver, adrenal and thyroid occurred in the 150 g/kg feed group in the F_1 generation. There were no compound-related histological effects (Cox & Babish, 1978a).

The 1982 JECFA ADI of 0–2.5 mg/kg bw anthocyanins from grape skin extract was based on this study, with the intake of 225 mg/kg bw per day divided by an uncertainty factor of 100 and rounded up to 2.5 mg/kg bw per day.

(b) Developmental toxicity

The anthocyanin glycosides (an extract from currants, blueberries and elderberries) were reported not to be teratogenic in rats, mice or rabbits when given at doses of 1500, 3000 or 9000 mg/kg bw per day over three successive generations (Pourrat et al., 1967).

2.2.6 Special studies

Twenty-five polyphenols were identified in a study using crude black carrot extract prepared using a solution of methanol, water and acetic acid (70:29.5:0.5 by volume) with dried extract dissolved in a solution of 4% formic acid and acetonitrile (90:10 by volume). Anthocyanins formed 78.06% of these by weight. Using a number of chemical and in vitro tests, crude black carrot extract was found to have strong antioxidant and free-radical scavenging properties and promoted cell viability in human lymphocytes (Smeriglio et al., 2018).

Lymphocytes exposed to the oxidizing agent *tert*-butyl hydroperoxide showed decreased cell mortality when incubated with the crude black carrot extract compared with cells exposed to *tert*-butyl hydroperoxide alone (Smeriglio et al., 2018).

The chick chorioallantoic membrane assay was used to ascertain the degree of anti-angiogenicity caused by the crude black carrot extract. A significant decrease in blood vessel formation was found in eggs exposed to the crude black carrot extract compared with control eggs or eggs exposed to retinoic acid, a positive control (Smeriglio et al., 2018).

2.3 Observations in humans

In a 12-week randomized controlled trial in healthy male and female former smokers aged between 18 and 65 years, 500 mg aronia extract (n = 25) or placebo (n = 24) was consumed daily. Aronia extract was an ethanolic extract containing 45.1 mg anthocyanins (anthocyanins not characterized or quantified). Fasting blood samples were collected at baseline and in weeks 6 and 12, and urine samples were collected at baseline and in week 12. Following consumption of aronia extract, fasting plasma total cholesterol was reduced by 8% (P = 0.0140), low-density lipoprotein cholesterol was reduced by 11% (P = 0.0285) and low-density lipoprotein receptor protein was reduced in peripheral blood mononuclear cells (P = 0.0036) at 12 weeks compared with the placebo group. Increases in urinary peonidin-3-*O*-galactoside, 3-(4-hydroxyphenyl)propionic acid and unmetabolized cyanidin-3-*O*-galactoside were associated with lower plasma total cholesterol and low-density lipoprotein cholesterol in the aronia group. No changes in blood pressure or biomarkers of inflammation and oxidative stress were observed. Aronia polyphenols reduced total and low-density lipoprotein cholesterol, but did not improve biomarkers of oxidative stress or chronic inflammation, in former smokers (Xie et al., 2017).

In a 12-week randomized controlled trial, 160 participants aged 40–75 years with prediabetes or early untreated diabetes were assigned to receive either purified anthocyanins at 320 mg/day or placebo. Anthocyanins present were delphinidin-3-*O*-glucoside, delphinidin-3-*O*-galactoside and delphinidin-3-*O*-arabinoside, making up 58% of the dose; C3G, cyanidin-3-*O*-galactoside and cyanidin-3-*O*-arabinoside, making up 33% of the dose; malvidin-3-*O*-glucoside, malvidin-3-*O*-galactoside and malvidin-3-*O*-arabinoside, making up 3% of the dose; petunidin-3-*O*-glucoside, petunidin-3-*O*-galactoside and petunidin-3-*O*-arabinoside, making up 2.5% of the dose; peonidin-3-*O*-glucoside, peonidin-3-*O*-galactoside and peonidin-3-*O*-arabinoside, making up 2.5% of the dose; and delphinidin-3-*O*-rutinoside and cyanidin-3-*O*-rutinoside, making up 1% of the dose. The anthocyanin group was composed of 25 males and 55 females, and the control group, 29 males and 51 females. The baseline parameters for glycaemic control, insulin resistance and lipids were comparable between the two groups. Statistically significant differences were observed in the net changes of glycated haemoglobin A1c (a marker for glycaemic control; −0.14%, 95% confidence interval [CI]: −0.23% to −0.04%; P = 0.005), low-density lipid cholesterol (−0.2

mmol/L, 95% CI: −0.38 to −0.01; P = 0.04), apolipoprotein A1 (0.09 g/L, 95% CI: 0.02–0.17; P = 0.02) and apolipoprotein B (−0.07 g/L, 95% CI: −0.13 to −0.01; P = 0.01), but not in other biomarkers. There was a statistically significant reduction in insulin resistance in the anthocyanin group that was not observed in the placebo group. No differences were found between men and women. Ten adverse events were reported in this study, with three in the control group and seven in the anthocyanin group; these included dark stools (n = 5), insomnia (n = 1), abdominal pain (n = 1), diarrhoea (n = 1), dizziness (n = 1) and skin rash (n = 1) (Yang et al., 2017).

In a randomized, controlled, four-arm crossover trial, 21 adults (aged >18 years with a waist circumference of >110 cm) with insulin resistance were provided with a high-fat and high-carbohydrate meal along with a beverage containing 0, 10, 20 or 40 g of freeze-dried strawberry powder. Blood samples were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 5 and 6 hours. Up to 6 hours after the meal, serum insulin concentrations were statistically significantly reduced in the top-dose group (P < 0.05). Serum glucose was not statistically significantly different between groups, but the mean insulin:glucose ratio was statistically significantly different between the top-dose group and the other groups (P < 0.05). Blood pelargonidin glucuronide concentration was inversely associated with mean insulin concentration in the 20 g and 40 g strawberry groups (Park et al., 2016).

Six young adults (aged 18–35 years) and seven older adults (aged 55 years or older) with self-reported, uncontrolled hypertension participated in a crossover trial in which they consumed either 3 × 100 mL anthocyanin-rich cherry juice (69 mg red pigment per 100 g juice, anthocyanins not characterized or quantified) served at 0, 1 and 2 hours or 300 mL served at 0 hour with a 1-week washout period in between. Resting systolic and diastolic blood pressures and heart rate were measured in triplicate at baseline and 2 and 6 hours after juice consumption. Blood samples were also taken at baseline and 2 and 6 hours post-consumption, and phenolic acids (ferulic acid, isoferulic acid, caffeic acid, *p*-coumaric acid, syringic acid, vanillic acid, 4-hydroxybenzoic acid, *tert*-cinnamic acid) were measured to confirm absorption of the anthocyanins. After consumption of 300 mL of cherry juice, significant reductions in systolic blood pressure (P = 0.002), diastolic blood pressure (P = 0.008) and heart rate (P = 0.033) were observed at 2 hours, but these had returned to normal by 6 hours post-dosing. A similar trend was observed following ingestion of 3 × 100 mL juice over 2 hours, but this did not reach statistical significance (Kent et al., 2015).

In 24 overweight adults (BMI 25–33.5; 14 women, 10 men), a single strawberry beverage (containing 39.04 mg total anthocyanins per serving: 28.11 mg pelargonidin-3-*O*-glucoside, 0.55 mg pelargonidin-3-*O*-diglucoside, 2.48 mg pelargonidin-3-*O*-malonylglucoside, 5.43 mg pelargonidin-3-*O*-rutinoside, 0.32 mg pelargonidin-3-*O*-malonylrhamnoside and 1.61 mg C3G) was found

to statistically significantly reduce the postprandial inflammatory response as measured by high-sensitivity C-reactive protein ($P = 0.02$) and IL-6 ($P < 0.05$) induced by a high-carbohydrate, moderate-fat meal compared with a control strawberry-flavoured beverage. It was also associated with a reduction in insulin response ($P < 0.05$) (Edirisinghe et al., 2011).

In a randomized, placebo-controlled, acute crossover study, 11 female and nine male volunteers on a low-flavonoid diet for the previous 72 hours consumed 250 mL of a 20% blackcurrant juice drink (containing 30.5 mg delphinidin and 20 mg cyanidin per serving, total anthocyanins not given) or placebo. Urine and blood samples were taken regularly for 8 hours, and vascular reactivity was assessed using laser Doppler imaging. No effects on vascular reactivity were observed throughout the study, and no effects on biomarkers of endothelial function or lipid risk factors were observed. Delphinidin-3-*O*-rutinoside and cyanidin-3-*O*-rutinoside were the main anthocyanins excreted in urine, with delphinidin-3-*O*-glucoside also detected. Anthocyanins detected in urine were 0.021% ± 0.003% of the dietary intake of delphinidin glycosides and 0.009% ± 0.002% of the dietary intake of cyanidin glycosides (Jin et al., 2011).

In a double-blind placebo-controlled parallel study, 56 healthy postmenopausal women were divided into test and placebo groups. For 12 weeks, the test group ingested four capsules, each containing 125 mg C3G from an elderberry extract (type of extract not specified), per day. Blood samples were collected to test compliance and to identify a number of biomarkers of cardiovascular risk: C-reactive protein, IL-6, TNFα, TNF receptors 1 and 2, endothelin-1, chemokine ligand 5, fasting glucose, cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and triglycerides. Biomarkers for liver and kidney function were also assessed (plasma concentrations of total bilirubin, albumin, urea and creatinine and activities of alkaline phosphatase, alanine aminotransferase and gamma-glutamyl transferase). No treatment-related effects were observed throughout the study (Curtis et al., 2009).

Twenty healthy females aged 18–40 years were divided into two groups, with 11 receiving 3×250 mL cranberry juice per day and nine receiving the same volume of a placebo drink for 2 weeks. Blood and urine samples were taken at weeks −1, 0, 1 and 2. Total cholesterol, triglycerides, high-density lipoproteins and low-density lipoproteins were unaffected by cranberry juice. Plasma total homocysteine remained unchanged. Similarly, the antioxidant potential of the plasma (measured as reduced glutathione, ferric-reducing antioxidant potential and electron spin resonance) and the activity of the phase II metabolizing enzymes glutathione peroxidase, catalase and superoxide dismutase did not differ statistically significantly from baseline values in both groups. Urinary malondialdehyde was unchanged by cranberry juice consumption. Endogenous

DNA damage in lymphocytes remained unchanged in both treatment groups over the study period. Similarly, despite a statistically significant decrease ($P < 0.05$) in both treatment groups with time, there was no difference in the background excretion rate of the oxidized purine nucleoside 8-oxo-deoxyguanosine (measured in urine) in volunteers fed cranberry juice or placebo. Supplementation with cranberry juice did not affect either endogenous DNA strand breakage or oxidized pyrimidines in isolated lymphocytes. Moreover, DNA strand breakage induced in response to oxidative stress (hydrogen peroxide treatment) was similar in lymphocytes isolated from both treatment groups, indicating that the cellular antioxidant capacity of the volunteers was unchanged by supplementation (Duthie et al., 2006).

3. Dietary exposure

Black carrot extract (INS 163(vi)) has not been evaluated previously by the Committee. This food colour is not authorized in the Codex General Standard for Food Additives (GSFA), meaning that there is no current provision of uses. The Committee received submissions from the International Association of Colour Manufacturers (IACM, 2018) and the Natural Food Colours Association (NATCOL, 2018). In their submissions to JECFA, the sponsors had proposed the use of this food colour in 77 categories and subcategories of foods using the Codex GSFA at typical and maximum use levels. The sponsors provided typical and maximum use levels because the majority of food products in most food categories would not be intensely coloured and so a minority of food products would contain the maximum reported use level. The anthocyanin content in black carrot extracts reported by the sponsors ranges from 0.8% to 14.5%, with a proposed standardized content of 9%. The sponsors reported that their proposed use levels (typical and maximum) of black carrot extract are expressed as total anthocyanins (in milligrams per kilogram), the compounds of toxicological relevance to be used in the safety assessment of the food colour. Table 10 summarizes the higher typical and the higher maximum use levels from both sponsors' submissions.

3.1 International exposure estimates

The Committee evaluated the submission of the sponsors and reported international estimates of dietary exposure to black carrot extract according to the typical and maximum proposed use levels listed in Table 10. In their submission, the sponsors provided different assessments of the dietary exposure

Table 10

Typical and maximum proposed use levels of black carrot extract expressed as total anthocyanins from IACM and NATCOL according to the GSFA food categories and subcategories

Food category	Name of food category	Proposed use level of total anthocyanins (mg/kg)	
		Typical (higher)	Maximum (higher)
01.0	**Dairy products and analogues, excluding products of category 02.0**		
01.1.4	Flavoured fluid milk drinks	50	100
01.4.4	Cream analogues	50	100
01.5.2	Milk and cream powder analogues	50	100
01.6.1	Unripened cheese	50	100
01.6.2.2	Rind of ripened cheese	50	100
01.6.4.2	Flavoured processed cheese, including containing fruit, vegetables, meat, etc.	50	100
01.6.5	Cheese analogues	50	100
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	50	115
02.0	**Fats and oils, and fat emulsions**		
02.4	Fat-based desserts excluding dairy-based dessert products of food category 01.7	50	100
03.0	**Edible ice, including sherbet and sorbet**	50	180
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	Processed fruits		
04.1.2.1	Frozen fruit	22	92
04.1.2.2	Dried fruit	25	92
04.1.2.3	Fruit in vinegar, oil, or brine	50	100
04.1.2.4	Canned or bottled (pasteurized) fruit	50	100
04.1.2.5	Jams, jellies, marmalades	50	100
04.1.2.6	Fruit-based spreads (e.g. chutney) excluding products of food category 04.1.2.5	50	100
04.1.2.7	Candied fruit	50	100
04.1.2.8	Fruit preparations, including pulp, purees, fruit toppings and coconut milk	22	92
04.1.2.9	Fruit-based desserts, including fruit-flavoured water-based desserts	50	100
04.1.2.10	Fermented fruit products	22	92
04.1.2.11	Fruit fillings for pastries	22	100
04.1.2.12	Cooked fruit	22	92
04.2	Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds		
04.2.2	Processed vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds	15	53
04.2.2.3	Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), and seaweeds in vinegar, oil, brine, or soybean sauce	50	100
04.2.2.4	Canned or bottled (pasteurized) or retort pouch vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), and seaweeds	15	53
04.2.2.5	Vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed purees and spreads (e.g. peanut butter)	15	53

Table 10 (continued)

Food category	Name of food category	Proposed use level of total anthocyanins (mg/kg)	
		Typical (higher)	Maximum (higher)
04.2.2.6	Vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed pulps and preparations (e.g. vegetable desserts and sauces, candied vegetables) other than food category 04.2.2.5	15	53
04.2.2.7	Fermented vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera) and seaweed products, excluding fermented soybean products of food categories 06.8.6, 06.8.7, 12.9.1, 12.9.2.1 and 12.9.2.3	50	100
05.0	**Confectionery**		
05.1.4	Cocoa and chocolate products	120	180
05.1.5	Imitation chocolate, chocolate substitute products	120	180
05.2.1	Hard candy	18	290
05.2.2	Soft candy	50	290
05.2.3	Nougats and marzipans	74	180
05.3	Chewing gum	50	290
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	200	300
06.0	**Cereals and cereal products, derived from cereal grains, from roots and tubers, pulses, legumes and pith or soft core of palm tree, excluding bakery wares of food category 07.0**		
06.3	Breakfast cereals, including rolled oats	100	200
06.4	Pastas and noodles and like products (e.g. rice paper, rice vermicelli, soybean pastas and noodles)	120	180
06.4.2	Dried pastas and noodles and like products	100	200
06.4.3	Pre-cooked pastas and noodles and like products	120	180
06.5	Cereal and starch based desserts (e.g. rice pudding, tapioca pudding)	120	180
06.6	Batters (e.g. for breading or batters for fish or poultry)	50	100
06.7	Pre-cooked or processed rice products, including rice cakes (Oriental type only)	120	180
06.8.1	Soybean-based beverages	120	180
07.0	**Bakery wares**		
07.1.2	Crackers, excluding sweet crackers	40	70
07.2.1	Cakes, cookies and pies (e.g. fruit-filled or custard types)	30	60
10.0	**Eggs and egg products**		
10.3	Preserved eggs, including alkaline, salted, and canned eggs	23	115
10.4	Egg-based desserts (e.g. custard)	50	100
12.0	**Salts, spices, soups, sauces, salads and protein products**		
12.1	Protein products other than from soybeans	50	100
12.2.2	Seasonings and condiments	50	90
12.3	Vinegars	12	92
12.4	Mustards	50	115
12.6.1	Emulsified sauces and dips (e.g. mayonnaise, salad dressing, onion dips)	50	92
12.6.2	Non-emulsified sauces (e.g. ketchup, cheese sauce, cream sauce, brown gravy)	50	100
12.6.3	Mixes for sauces and gravies	50	100
12.7	Salads (e.g. macaroni salad, potato salad) and sandwich spreads excluding cocoa- and nut-based spreads of food categories 04.2.2.5 and 05.1.3	50	100

Food category	Name of food category	Proposed use level of total anthocyanins (mg/kg)	
		Typical (higher)	Maximum (higher)
13.0	**Foodstuffs intended for particular nutritional uses**		
13.3	Dietetic foods intended for special medical purposes (excluding products of food category 13.1)	50	100
13.4	Dietetic formulae for slimming purposes and weight reduction	4	82
13.5	Dietetic foods (e.g. supplementary foods for dietary use) excluding products of food categories 13.1–13.4 and 13.6	4	82
13.6	Food supplements	4	82
14.0	**Beverages, excluding dairy products**		
14.1	Non-alcoholic ("soft") beverages	11	69
14.1.2.4	Concentrates for vegetable juice	2	69
14.1.3.2	Vegetable nectar	2	69
14.1.3.4	Concentrates for vegetable nectar	12	173
14.1.4	Water-based flavoured drinks, including "sport," "energy" or "electrolyte" drinks and particulated drinks	50	100
14.1.4.1	Carbonated water-based flavoured drinks	10	69
14.1.4.2	Non-carbonated water-based flavoured drinks, including punches and ades	10	69
14.1.4.3	Concentrates (liquid or solid) for water-based flavoured drinks	25	173
14.2	Alcoholic beverages, including alcohol-free and low-alcoholic counterparts	10	45
14.2.1	Beer and malt beverages	10	45
14.2.2	Cider and perry	30	100
14.2.4	Wines (other than grape)	30	80
14.2.5	Mead	15	45
14.2.6	Distilled spirituous beverages containing more than 15% alcohol	30	80
14.2.7	Aromatized alcoholic beverages (e.g. beer, wine and spirituous cooler-type beverages, low alcoholic refreshers)	30	80
15.0	**Ready-to-eat savouries**		
15.1	Snacks – potato, cereal, flour or starch based (from roots and tubers, pulses and legumes)	30	80
15.2	Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)	150	300
15.3	Snacks – fish based	150	300

GSFA: Codex General Standard for Food Additives; IACM: International Association of Colour Manufacturers; NATCOL: Natural Food Colours Association

to black carrot extract based on the budget method and individual consumption data from Europe.

As black carrot extract would be present only in processed foods, the Committee did not perform an assessment with the commodity-based food consumption information from the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) cluster diets database. The Committee did not consider the estimates of dietary exposure to black carrot extract based on the budget method that were submitted by the sponsors, because the estimates were judged to be too conservative owing

to the high levels of black carrot extract in solid and liquid foods that were used in the calculations. Therefore, the Committee decided to report its own estimates based on the budget method, which was considered to be more representative than the sponsors' estimates.

3.1.1 Screening by the budget method

Dietary exposure to black carrot extract using the maximum use levels proposed by the sponsors was estimated using the budget method, with the assumptions described in Environmental Health Criteria 240 (WHO, 2009).

In the case of black carrot extract with a total anthocyanin content standardized to 9%, the maximum use levels proposed by the sponsor (Table 10) that were considered to be most representative of levels to which consumers might be exposed were 100 mg/L in beverages and 180 mg/kg in solid foods. More specifically, the levels of consumption of foods and beverages considered are maximum physiological levels of consumption – i.e. the daily consumption of 0.1 L of non-milk beverages per kilogram of body weight and the daily consumption of 100 kcal/kg bw from foods (equivalent to 0.05 kg/kg bw based on an estimated energy density of 2 kcal/g). The default proportions of solid foods and beverages containing the food additive that were considered to be adequate were assumed to be 12.5% and 25%, respectively, for adults and 25% and 100%, respectively, for children. In fact, even though black carrot extract may be used in a variety of solid and liquid foods, which could represent more than 25% of processed foods, it is unlikely that a person would systematically choose all processed solid and liquid foods with the same colour added.

For adults, it is assumed that a typical adult weighs 60 kg and consumes 1.5 L of beverages and 375 g of solid foods containing black carrot extract daily. The theoretical maximum daily dietary exposure for adults would therefore be $(100 \times 0.1 \times 0.25) + (180 \times 0.05 \times 0.125) = 2.5 + 1.125 = 3.625$ mg/kg bw per day.

For children, it is assumed that a typical 3-year-old child weighs 15 kg and consumes 1.5 L of beverages and 94 g of solid foods containing black carrot extract daily. The theoretical maximum daily dietary exposure for children would therefore be $(100 \times 0.1 \times 1) + (180 \times 0.05 \times 0.25) = 10 + 2.25 = 12.25$ mg/kg bw per day.

3.1.2 Assessment based on individual food consumption data

Dietary exposures to anthocyanins based on their use as food colour additives are available only for European populations. The sponsors noted that a comparison of dietary patterns in the European Union (EU) and the USA by the United States Department of Agriculture's Economic Research Service (Mitchell, 2004) and from data available in the Global Dietary Database (https://www.globaldietarydatabase.org/) shows that patterns of food intake in the USA and the

EU are comparable. Therefore, dietary exposure estimates obtained for European populations are representative of exposures of populations in the USA as well.

EFSA estimated dietary exposure to anthocyanins (E 163) for the EU population using maximum permitted levels (MPLs) according to Council Directive 94/36/EC and reported use levels in 2013 (EFSA, 2013). MPLs of anthocyanins (E 163) are approved in Europe for use in quantum satis[4] in 52 categories and subcategories of foods up to 200 mg/kg in breakfast cereals (only fruit-flavoured breakfast cereals) under Commission Regulation (EU) No. 1129/2011 of November 2011 amending Annex II to Regulation (EC) No. 1333/2008.

It is noted that specifications for anthocyanins defined in Commission Regulation (EU) No. 231/2012 do not include information on the types and percentage content of individual anthocyanins in the source material. Therefore, it is assumed that the dietary exposure calculated by EFSA refers to total anthocyanins.

Dietary exposure to anthocyanins (E 163) from their use as food additives has been calculated using data on reported use levels from NATCOL combined with national consumption data from the Comprehensive European Food Consumption Database for EU population groups (toddlers, children, adolescents, adults, the elderly). For calculation of chronic exposure, intake statistics have been calculated based on individual average consumption over the total survey period, excluding surveys with only 1 day per subject. High-level consumption was calculated only for those foods and population groups where the sample size was sufficiently large to allow calculation of the 95th percentile. Calculations were performed using individual body weights. Thus, for the EFSA assessment, food consumption data were available from 26 different dietary surveys carried out in 17 different European countries.

High-level exposure (typically 95th percentile exposure of consumers only) was calculated by adding the 95th percentile of exposure from one food group (i.e. the one having the highest value) to the mean exposure resulting from the consumption of all other food groups. This is based on the assumption that an individual might be a high-level consumer of one food category and would be an average consumer of the others. This approach has been tested several times by EFSA in its evaluations of food colours and has shown reasonable correlation with high-level total dietary exposures when using raw food individual consumption data. Therefore, this approach was preferred for the calculations based on maximum reported use levels in order to avoid excessively conservative estimates. However, EFSA noted that its estimates should be considered as being conservative, as it is assumed that all processed foods contain the colour added at the maximum reported use levels. Exposure was estimated using the Food

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[4] The amount that is enough.

Table 11

Summary of estimated dietary exposures to anthocyanins (E 163) from their use as food additives using maximum reported use levels for five population groups

	Dietary exposure (mg/kg bw per day)				
	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	Elderly (>65 years)
Mean	1.5–4.0	1.5–4.7	1.0–2.5	0.7–1.9	0.5–1.1
High level[a]	3.4–6.9	2.7–7.8	1.6–3.9	1.1–3.4	0.9–2.3

bw: body weight
[a] Typically 95th percentile of consumers only.

Additives Intake Model (FAIM), available on the EFSA website (https://www.efsa.europa.eu/en/applications/foodingredients/tools).

Table 11 summarizes the estimated dietary exposures to anthocyanins (E 163) from their use as food additives for all five population groups.

Main food categories contributing to dietary exposure to anthocyanins (E 163) using maximum reported use levels (>5% contribution to the total mean exposure in several surveys and population groups) were "Flavoured fermented milk products including heat treated products", "Processed meat", "Processed fruits and vegetables", "Flavoured drinks" and "Fine bakery wares".

With regard to use levels evaluated at this meeting, the Committee noted differences between proposed subcategories and current reported typical and maximum use levels by the sponsors and those use subcategories and levels considered by EFSA in its re-evaluation of the safety of anthocyanins (E 163) as a food additive in 2013. Main differences were noted for confectionery, cereals and cereal products, and beverage food categories, with more subcategories currently proposed for uses by the sponsors compared with what was considered at that time in the EFSA opinion. In contrast, some food categories were considered in the EFSA opinion that were not currently proposed for uses by the sponsor, in particular processed meat, which was a main contributor to overall exposure in the EFSA assessment, up to 30–50% in population groups, corresponding to average dietary exposures across groups of 0.5–2.4 mg/kg bw per day.

Since the publication of the EFSA opinion on anthocyanins, the exposure methodology approach followed by EFSA has been updated with more refined assessment, incorporating both typical and maximum use levels into the assessment of dietary exposure for both brand-loyal and non-brand-loyal consumer scenarios. More food consumption surveys conducted since the 2013 EFSA opinion were also considered, according to the updated Comprehensive European Food Consumption Database for all countries in Europe.

Overall, the Committee considered the updated EU assessment of dietary exposure to anthocyanins that was provided by the sponsors (IACM and

Table 12

Summary of refined estimates of dietary exposures to anthocyanins from their proposed uses as a food colour as listed by the sponsors in Table 10 in EU population groups

	Dietary exposure (mg/kg bw per day)				
	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	Elderly (>65 years)
Mean	0.3–1.3	0.2–1.1	0.1–0.5	0.1–0.3	<0.1–0.3
95th percentile[a] (brand-loyal scenario)	0.3–6.9	0.5–4.8	0.2–3.1	0.4–2.0	0.1–1.6
95th percentile[a] (non-brand-loyal scenario)	<0.1–2.4	<0.1–2.3	0.2–1.0	0.2–1.7	0.1–0.8

bw: body weight; EU: European Union
[a] Typically 95th percentile of consumers only, estimated by adding the 95th percentile of exposure from the food group having the highest value to the mean exposure resulting from the consumption of all other food groups considered (EFSA, 2017).

NATCOL) as being the most accurate. In this update, a refined dietary exposure assessment for EU populations was performed by Tennant & Klingenberg (2016) using the typical and maximum use levels proposed by the sponsors (see Table 10) in their submission with the two additional exposure scenario approaches that are now currently used in the EFSA opinion:

- the brand-loyal consumer scenario, in which it is assumed that an individual is a long-term brand-loyal consumer of one food category containing the food additive at the highest reported level and consumes the remaining food categories at the typical mean reported use levels;
- the non-brand-loyal consumer scenario, representing the general population, which is based on the assumption that an individual is not loyal to any specific brand available on the market and is exposed to the food additive at the typical mean reported use levels for all food categories.

In these scenarios, the high-level exposure (typically the 95th percentile of consumers only) is estimated by adding the 95th percentile of exposure from the food group having the highest value to the mean exposure resulting from the consumption of all other food groups considered (EFSA, 2017).

Table 12 summarizes the estimated dietary exposures to anthocyanins from their proposed uses as a food colour as listed by the sponsors in Table 10 for EU population groups.

The Committee noted that the mean estimated dietary exposures to anthocyanins from their use as a food colour ranged from less than 0.1 mg/kg

bw per day in the elderly population up to 1.3 mg/kg bw per day for toddlers. The 95th percentile exposure for consumers only ranged from 0.1 mg/kg bw per day in the elderly population up to 6.9 mg/kg bw per day for toddlers in the brand-loyal scenario, whereas it ranged from less than 0.1 mg/kg bw per day for toddlers and children up to 2.4 mg/kg bw per day for toddlers in the non-brand-loyal scenario.

In these estimates, the main foods contributing to the overall exposure to anthocyanins were non-alcoholic beverages, flavoured fermented desserts and cider.

3.2 Dietary exposure from natural sources

The Committee also considered the typical exposure to anthocyanins from natural sources entering into the regular diet as relevant for the assessment of exposure to anthocyanins from all sources, including its proposed uses as a food colour. Anthocyanins are naturally present in foods such as fruits (apple, blackberry, blueberry, chokeberry, cranberry, black and red currant, elderberry, gooseberry, grapes, nectarines, peaches, plums, raspberry and strawberry), vegetables (black beans, small red beans, cabbages, eggplant, lettuces, onions and red radishes), nuts (pistachios), chocolate, tea and wine. As published by Wu et al. (2006), concentrations of total anthocyanins vary considerably between products. Using the values reported by Wu et al. (2006), the mean exposures to anthocyanins in 2016 in the USA using the National Health and Nutrition Examination Survey (NHANES) 2001–2002 data (USDA, 2016) and in 2013 in the EU using data from the Irish population (EFSA, 2013) were estimated to be, respectively, 12.5 mg/person per day and 8.6 mg/person per day, which correspond to approximate mean exposures of 0.1–0.2 mg/kg bw per day for a 60 kg adult. Using the Comprehensive European Food Consumption Database, updated since the last EFSA opinion, Tennant & Klingenberg (2016) reported potential mean dietary exposures to anthocyanins for EU population groups ranging from 0.05 mg/kg bw per day for adolescents up to 1.6 mg/kg bw per day for adults and up to 4 mg/kg bw per day for toddlers.

4. Comments

4.1 Biochemical aspects

The previous Committee, in its evaluation of grape skin extract, concluded that anthocyanins are not absorbed by humans to any great extent (<2%) and pass through the body unchanged (Annex 1, reference 59). More recent studies have

shown anthocyanins to be absorbed up to about 12% (e.g. Czank et al., 2013; Lila et al., 2016; Kay et al., 2017); therefore, previously evaluated studies on the ADME of anthocyanins have not been included below.

A number of studies have been carried out in humans to investigate the ADME of anthocyanins. Anthocyanins can be absorbed intact or hydrolysed to the aglycone and then absorbed. They may also be degraded to phenolic compounds by the gut microbiota before absorption. The primary route of metabolism by the microbiota appears to be cleavage of the heterocyclic flavylium ring followed by dihydroxylation or decarboxylation (Fang, 2014; Zhang et al., 2018). The rate and extent of absorption are dependent on the size of the molecule, the type of sugar moiety, the degree of acylation and the matrix in which the anthocyanin mixture is consumed (Fang, 2014). The gut microbiome is likely to be an important site of metabolism of anthocyanins, and changes in the microbiome may have a significant effect on the metabolic products produced following the consumption of anthocyanins (Williamson & Clifford, 2010).

In recent studies in human volunteers using stable ^{13}C-labelled C3G, an anthocyanin found in grape skin extract and purple corn colour, bioavailability of about 12% (5% in urine and 7% in breath) was reported. Several metabolites were identified, including carbon dioxide in breath and anthocyanin conjugates along with vanillic acid, ferulic acid, hippuric acid and 4-hydroxybenzaldehyde in urine (Czank et al., 2013; de Ferrars et al., 2014).

4.2 Toxicological studies

A number of acute and short-term toxicity studies were identified using anthocyanins from a range of sources, including dried fruits and vegetables and extracts of these. In many cases, the anthocyanins in the test material were not identified or quantified (Pourrat et al., 1967; Yamakoshi et al., 2002; Bagchi et al., 2006; Thounaojam et al., 2011; Charoensin et al., 2012).

In the acute toxicity studies, no effects were observed at oral test substance doses up to 25 000 mg/kg bw (Pourrat et al., 1967; Yamakoshi et al., 2002; Bagchi et al., 2006; Thounaojam et al., 2011; Charoensin et al., 2012).

No short-term studies were carried out using black carrot extract. A number of short-term studies in a range of species using test substances containing anthocyanins were identified. No treatment-related effects were observed in a 28-day mouse study using dried red cabbage powder (Thounaojam et al., 2011), two 90-day studies in rats given grape seed extract (Bentivegna & Whitney, 2002; Yamakoshi et al., 2002), one 90-day study in rats given grape skin extract (Bentivegna & Whitney, 2002), one 90-day study in rats given an anthocyanin extract (Pourrat et al., 1967) and two 90-day studies in dogs, one using grape colour powder and one grape skin extract (Cox & Babish, 1978b; Becci et al.,

1983a). In addition, no effects were observed in a 15-day study in guinea-pigs given anthocyanins in the diet (Pourrat et al., 1967).

In a study in rats fed a diet supplemented with grape skin extract at 0, 2000, 10 000 or 50 000 mg/kg feed (equal to 0, 100, 600 and 3300 mg/kg bw per day for males and 0, 100, 700 and 3600 mg/kg bw per day for females, respectively) for 90 days, the anthocyanin content was not characterized, but the test material was said to contain approximately 2% anthocyanins (anthocyanin doses were therefore 0, 2, 12 and 66 mg/kg bw per day for males and 0, 2, 14 and 72 mg/kg bw per day for females, respectively). In this study, calcification of the proximal tubules of the kidney was identified in females in all dose groups, including controls, but the severity was significantly higher in the group receiving 50 000 mg/kg in the diet. A NOAEL of 14 mg/kg bw per day expressed as anthocyanins (10 000 mg/kg feed expressed as grape skin extract, equal to 700 mg/kg bw per day) was identified (Inoue et al., 2013).

In a study by Nabae et al. (2008), in which rats were administered purple corn colour (containing 26.4% C3G) in the diet at 0, 5000, 15 000 or 50 000 mg/kg (equal to C3G doses of 0, 84, 249 and 935 mg/kg bw per day for males and 0, 89, 272 and 1016 mg/kg bw per day for females, respectively) for 90 days, a number of statistically significant findings were observed at the top dose, including effects on haematological and clinical chemistry parameters and relative organ weights. Although the authors concluded that the NOAEL was the highest dose tested, the Committee was of the opinion that the effects observed at 50 000 mg/kg feed were toxicologically relevant and identified a NOAEL of 15 000 mg/kg feed (equal to 249 mg/kg bw per day).

No long-term toxicity or carcinogenicity studies are available.

Eight in vitro and seven in vivo genotoxicity studies are available, but only one assay (an in vitro comet assay in human colon cancer cells) used black carrot extract as the test material (Glei et al., 2003). This study showed positive results only at cytotoxic concentrations. No findings were observed for any of the anthocyanin-containing test materials that would raise concerns for genotoxicity (MacGregor & Jurd, 1978; Brown & Dietrich, 1979; Haveland-Smith, 1981; Yamakoshi et al., 2002; Erexson, 2003; Glei et al., 2003; Fimognari et al., 2004; Ribeiro et al., 2010; Charoensin et al., 2012).

Two multigeneration reproductive toxicity studies are available. One of these used grape colour powder administered to rats in the diet at 0, 7500 or 15 000 mg/kg bw per day, but the anthocyanins in the test material were not quantified. There were no treatment-related findings (Becci et al., 1983a). In a second study, using a grape skin extract preparation (containing 3% anthocyanins; composition of anthocyanins not given) administered to rats in the diet at a concentration of 0, 75 000 or 150 000 mg/kg feed (equivalent to 0, 7500 and 15 000 mg/kg bw per day, respectively, estimated to be 0, 225 and 450 mg/kg

bw per day expressed as anthocyanins), decreases in liver, adrenal and thyroid weights were observed in the top-dose group of the F_1 generation. The NOAEL identified by the previous Committee for this grape skin extract preparation was 75 000 mg/kg feed (equivalent to 7500 mg/kg bw per day and estimated to be 225 mg/kg bw per day expressed as anthocyanins) (Cox & Babish, 1978a). The ADI for anthocyanins from grape skin extract established by the previous Committee was based on this study, with application of an uncertainty factor of 100 to the NOAEL and rounding.

The anthocyanin glycosides (an extract from currants, blueberries and elderberries) were reported not to be teratogenic in rats, mice or rabbits when given at doses of 1500, 3000 or 9000 mg/kg bw per day over three successive generations (Pourrat et al., 1967).

4.3 Observations in humans

A number of studies have been carried out in humans to identify biological effects of anthocyanins. Although no toxicity issues have been identified from these studies, the study designs limit their suitability for deriving safe levels of anthocyanins.

4.4 Assessment of dietary exposure

In the submission to the Committee, the sponsors proposed the use of black carrot extract as a food colour at typical and maximum use levels (expressed as total anthocyanins in milligrams per kilogram) in 77 food categories and subcategories as specified in the Codex GSFA. The anthocyanin content in black carrot extract reported by the sponsors ranges from 0.8% to 14.5%, with a standardized content of 9%.

The Committee considered the European estimates of dietary exposure to anthocyanins, provided by the sponsors, as being the most representative of actual exposure. The Committee noted that the mean estimated dietary exposures to total anthocyanins ranged from less than 0.1 mg/kg bw per day for the elderly population up to 1.3 mg/kg bw per day for toddlers. The 95th percentile exposure for consumers only ranged from 0.1 mg/kg bw per day for the elderly population up to 6.9 mg/kg bw per day for toddlers in the brand-loyal scenario, whereas the 95th percentile exposure ranged from less than 0.1 mg/kg bw per day for toddlers and children up to 2.4 mg/kg bw per day for toddlers in the non-brand-loyal scenario. The main foods contributing to the overall exposure to anthocyanins were non-alcoholic beverages, flavoured fermented desserts and cider.

The Committee also considered typical exposure to anthocyanins from natural sources. Anthocyanins are naturally present in foods such as fruits, vegetables, nuts, chocolate, tea and wine. The mean dietary exposure to anthocyanins in the USA using NHANES 2001–2002 data (USDA, 2016) was 0.2 mg/kg bw per day for a 60 kg adult. In Europe, the mean dietary exposure to anthocyanins using the Comprehensive European Food Consumption Database (Tennant & Klingenberg, 2016) ranged from 0.05 mg/kg bw per day for adolescents to 1.6 mg/kg bw per day for adults and up to 4 mg/kg bw per day for toddlers.

The Committee noted that the European dietary exposures to anthocyanins from natural sources as described in the current evaluation are higher than the mean dietary exposure of 0.3 mg/kg bw per day that was reported for Europe by EFSA (2013), which at that time was based on one national dietary survey from Europe.

With regard to use levels evaluated at this meeting, the Committee noted differences between the sponsors' reported current and proposed use levels of black carrot extract expressed as total anthocyanins and those that were considered in the EFSA (2013) evaluation. The main difference was for the food category processed meat, which is not proposed as a food to which anthocyanins could be added. In the EFSA (2013) evaluation, processed meat was the main food contributing to overall exposure to total anthocyanins, contributing up to 30–50% of the average dietary exposures across Europe (0.5–2.4 mg/kg bw per day).

5. Evaluation

There are no data on the toxicity of black carrot extract, with the exception of one genotoxicity test. Nevertheless, the Committee noted the large number of studies on other sources of anthocyanins published since anthocyanins were last evaluated by JECFA in 1982, including toxicity studies in animals and ADME studies in humans.

The Committee concluded that the effects observed with one anthocyanin-containing test material cannot be extrapolated to another anthocyanin-containing test material based on the available information. This is because the test articles in the metabolism and toxicity studies evaluated at this meeting were very heterogeneous and often not fully described and/or the anthocyanin content of the test material was too low and variable. This agrees with the conclusion of the previous Committee (Annex 1, reference 59).

Owing to the lack of toxicological data on black carrot extract, the Committee was not able to draw conclusions on its safety. To proceed with the

assessment of black carrot extract, at least a 90-day toxicological study on a well-characterized extract representative of the material of commerce would be required.

The Committee concluded that the total mean dietary exposure to anthocyanins from naturally occurring sources and added black carrot extract using the non-brand-loyal scenario ranges from 0.1 to 1.9 mg/kg bw per day for the adult population (18+ years old) and from 0.1 to 5.3 mg/kg bw per day for children (<18 years old).

In these estimates, the Committee noted that the use of black carrot extract itself as proposed by the sponsors contributes as much as 25% to the total mean dietary exposure to anthocyanins, including from naturally occurring sources. The Committee noted that the ADI for grape skin extract established by the previous Committee in 1982 was not reconsidered as part of this assessment and remains unchanged.

At the present meeting, new specifications for the spray-dried powder form of black carrot extract were prepared. The specifications were made tentative pending the submission of further information on the material of commerce (see section 5.1 below).

A Chemical and Technical Assessment was prepared.

5.1 **Recommendations**

To proceed with the assessment of black carrot extract, at least a 90-day toxicological study on a well-characterized extract representative of the material of commerce would be required.

The specifications were made tentative pending the submission of further information on the material of commerce, including a full characterization of the proteins, carbohydrates, lipids, fibre, minerals and non-anthocyanin polyphenol components in five lots each of the liquid and powder forms of black carrot extract.

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Brilliant Black PN

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1. Explanation

Brilliant Black PN (International Numbering System for Food Additives [INS] 151; Chemical Abstracts Service No. 2519-30-4) is a synthetic disazo dye used as a food colouring agent. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) first evaluated Brilliant Black PN at its eighteenth meeting (Annex 1, reference *35*) and established a temporary acceptable daily intake (ADI) of 0–2.5 mg/kg body weight (bw), based on a no-observed-adverse-effect level (NOAEL) of 500 mg/kg bw per day obtained from a chronic rat study. An additional uncertainty factor of 2 was applied because the ADI was temporary, pending the submission of metabolic, reproductive and embryotoxicity studies.

At the twenty-second meeting of JECFA (Annex 1, reference *47*), the requested metabolic, reproductive and embryotoxicity studies were not submitted. In addition, the Committee indicated that the etiology and pathology of ileal cysts observed in a 90-day toxicity study in pigs submitted at that meeting should be determined. The Committee maintained the temporary ADI.

At the twenty-fifth meeting of JECFA (Annex 1, reference *56*), multigeneration reproductive toxicity and teratogenicity studies were submitted, both showing no toxicologically relevant effects. A metabolic study was also submitted. No further information on the ileal cysts in pigs was available. Therefore, the Committee established a new ADI of 0–1 mg/kg bw on the basis of the no-effect level of 100 mg/kg bw per day in the pig study.

Brilliant Black PN was placed on the agenda of the present meeting for re-evaluation of its safety, evaluation of its dietary exposure and revision of its specifications, at the request of the Forty-ninth Session of the Codex Committee on Food Additives (FAO/WHO, 2017).

Studies on the effects of Brilliant Black PN on enzymes and other biochemical parameters, genotoxicity studies, studies on the toxicity of metabolites and a study on non-allergic hypersensitivity in children were submitted. Additional literature searches in Medline, Toxline, Scopus and SciFinder using the keywords Brilliant Black, clinical, toxicology, genotoxicity, metabolism, absorption, excretion and ADME did not identify any additional relevant publications. The sponsor submitted use levels of Brilliant Black PN in three main food categories as well as dietary exposure estimates reported in the literature. The text below consolidates the newly submitted information with information provided in previous JECFA evaluations.

1.1 Chemical and technical considerations

Brilliant Black PN is intended for use in colouring confectionery, decorations and coatings, desserts including flavoured milk products, edible cheese rind, edible ices, fine bakery wares, fish and fish products, non-alcoholic flavoured drinks, non-dairy beverages, sauces and seasonings, and savoury snacks.

Brilliant Black PN consists mainly of tetrasodium 4-(acetylamino)-5-hydroxy-6-[2-[7-sulfo-4-[2-(4-sulfophenyl)diazenyl]-1-naphthalenyl]diazenyl]-1,7-naphthalenedisulfonate and subsidiary colouring matters. Sodium chloride and/or sodium sulfate are the principal uncoloured components. Brilliant Black PN is manufactured by diazotizing 4-aminobenzenesulfonic acid (sulfanilic acid [SA]), coupling with 8-aminonaphthalene-2-sulfonic acid (1,7-Cleve's acid), diazotizing the product and coupling with 4-(acetylamino)-5-hydroxy-1,7-naphthalenedisulfonic acid (*N*-acetyl K acid). The dye is isolated as the tetrasodium salt. Impurities include unreacted starting materials and reaction by-products (≤0.8%), subsidiary colouring matters (≤4%), unsulfonated primary aromatic amines (≤0.01% calculated as aniline) and lead (≤2 mg/kg).

2. Biological data

In addition to new toxicity studies with Brilliant Black PN and its metabolites that had not been previously assessed, the present Committee considered relevant data that had been available at the twenty-fifth meeting of JECFA (Annex 1, reference *57*) and for which the original studies were available to the current Committee.

2.1 Biochemical aspects

No new data were available on the absorption, distribution, metabolism and elimination of Brilliant Black PN. Previously available data were extensively described in the monograph of the twenty-fifth meeting of JECFA (Annex 1, reference *57*) and are summarized below.

2.1.1 Absorption, distribution and excretion

In a rat study (no route specified), the faeces and urine of animals treated with Brilliant Black PN were collected, and the presence of the unchanged substance was noted in the faeces but not in the urine. Quantitative determination of the dye in the faeces indicated the presence of 0.6% of the total amount administered to the rats. No details of the sampling were given (Piekarski, 1960).

The fate of orally administered Brilliant Black PN, labelled on the Cleve's acid moiety with ^{14}C, was investigated in three male and two female Wistar rats. One male rat and one female rat were habituated to a diet containing 3% unlabelled dye prior to dosing. The dose administered varied from 1 to 6 mg/kg bw for the non-habituated animals and from 8 to 10 mg/kg bw for animals pretreated with the colour in the diet. Radioactivity was excreted mainly in the faeces (94–98%), with less than 5% in urine, within 40 hours. After 40 hours, the gastrointestinal tract contained 0–0.6% of the administered radioactivity; trace amounts were detected in kidneys and residual carcasses, and no measurable radioactivity was found in liver or blood. Habituation to Brilliant Black PN did not alter its excretion patterns (Anon., 1980).

2.1.2 Biotransformation

In the study described in section 2.1.1 above in which rats were orally administered Brilliant Black PN (Anon., 1980), one major and several minor metabolites were detected in the faeces, whereas two major and at least four minor metabolites were identified in urine. It was concluded that Brilliant Black PN was virtually completely degraded by the gut flora of the rat and that metabolites containing the Cleve's acid moiety were poorly absorbed from the gastrointestinal tract. Habituation to Brilliant Black PN did not alter its metabolic patterns (Anon., 1980).

The metabolism of Brilliant Black PN was investigated qualitatively and quantitatively in both rats and humans. Oral administration of 20–100 mg of Brilliant Black PN to rats resulted in the detection of SA and the two isomers 4-acetamido-1-naphthylamine-6-sulfonic acid and 4-acetamido-1-naphthylamine-7-sulfonic acid (ANSA) in the urine, whereas these metabolites, together with 1,4-diaminonaphthalene-6-sulfonic acid (DSA), 8-acetamido-1-hydroxy-2-naphthylamine-3,5-disulfonic acid (AHNDA) and traces of unchanged dye, were detected in the faeces. When Brilliant Black PN was administered intraperitoneally to rats at 10–100 mg, SA, ANSA, DSA and AHNDA were identified in the faeces, whereas these metabolites, 1-(4′-sulfophenylazo)-4-naphthylamine-6-sulfonic acid (SNSA) and traces of unchanged dye were excreted in the urine. Following an intravenous administration of Brilliant Black PN to rats, SNSA, ANSA and traces of unchanged dye were excreted in the bile.

Differences in metabolism in rats following oral and intraperitoneal administration associated with the finding of Brilliant Black PN, SNSA and SA in the gastrointestinal tract of rats treated orally indicated that the gut microflora cleaved both azo links in the dye, whereas liver azoreductases preferentially cleaved the azo group linking the two naphthalene rings. Quantitative estimations were limited to SA, ANSA, SNSA and unchanged dye. The amount of SA excreted

in urine and faeces (about 90% of the administered dose) indicated an almost complete reduction of the azo link between the benzene and naphthalene rings. Significant amounts of SNSA (6–8% of the administered dose) appeared in the urine at all doses. The combined total of SA and SNSA excreted shows that all the Brilliant Black PN administered is metabolized by reduction of at least one azo link.

In humans, SA was the only metabolite identified in urine following the administration of an oral dose of 240 mg of Brilliant Black PN, and the amount (37.1%) was similar to that observed in rats (44.5%). Analyses of faeces were not carried out (Ryan & Welling, 1970).

2.1.3 Effects on enzymes and other biochemical parameters

The ability of Brilliant Black PN to inhibit the hippurate and liver-like anion transporter systems was assessed using transverse slices of the kidneys of female rats. Inhibition of uptake of radiolabelled o-iodohippurate and iodipamide by the hippurate and liver-like anion transporter systems, respectively, was measured in tissues that were incubated with Brilliant Black PN concentrations of 0.01, 0.1 and 1.0 mmol/L at 37 °C for 30 minutes with shaking. Negative controls included tissues incubated with the dye at 0 °C and untreated tissue slices incubated in the presence of the designated radiolabelled compound. Per cent uptake of radiolabelled o-iodohippurate or iodipamide was calculated as a percentage of controls, corrected for non-specific uptake at 0 °C. Brilliant Black PN was shown to have a dose-dependent decrease of radiolabelled o-iodohippurate and iodipamide uptake in the renal cortex slices, which was interpreted as inhibition of the hippurate and liver-like anion transport systems, respectively (Carlson, 1977).

In Chinese hamster ovary cells (CHO-K1) stably transfected with either A_1 or A_3 human receptors, Brilliant Black PN was assessed for its potential interference with the purinergic G protein–coupled receptors with adenosine as endogenous ligand in high-throughput screening drug discovery assays, as it is commonly used as a quencher of extracellular fluorescence. Receptor activity was measured using the calcium mobilization assay, a whole-cell competitive binding assay with deuterated 8-cyclopentyl-1,3-dipropylxanthine and live cell imaging. Brilliant Black PN (5–500 μmol/L) had no significant effect on receptor stimulation by the non-selective agonist 5′-(N-ethylcarboxamido)adenosine or on A_1 receptor antagonism by 8-cyclopentyl-1,3-dipropylxanthine (10 pmol/L – 10 μmol/L). However, Brilliant Black PN at 500 μmol/L significantly inhibited the other A_1 and A_3 receptor antagonists, N-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]-quinazolin-5-yl]benzene acetamide (10 pmol/L – 10 μmol/L) in CHO-A_3 cells and xanthine amine congener (10 pmol/L – 30 μmol/L) and a xanthine amine

congener derivative fluorescently tagged with dipyrrometheneboron difluoride (10 nmol/L – 10 μmol/L) in both CHO-A_1 and CHO-A_3 cells. Xanthine amine congener–mediated antagonism was reversed within 1 minute by Brilliant Black PN at 500 μmol/L. Results of kinetic analysis showed that Brilliant Black PN is an allosteric modulator of ligand affinity of these antagonists, rather than decreasing the solubility or aggregation of the antagonists in solution. The authors suggested that, based on the activity of Brilliant Black PN on human A_1 and A_3 receptors, further studies are needed to understand its effect as a potential allosteric modulator of other G protein–coupled receptors (May, Briddon & Hill, 2010). The Committee noted that the effects on adenosine receptors were observed only at high (500 μmol/L) concentrations of Brilliant Black PN. In view of the poor absorption of Brilliant Black PN, the Committee did not consider this study relevant to the evaluation.

2.2 Toxicological studies

No new data were available on the acute toxicity, short-term toxicity, long-term toxicity or carcinogenicity of Brilliant Black PN. Studies previously evaluated at the twenty-fifth meeting of JECFA (Annex 1, reference *57*) are summarized below.

2.2.1 Acute toxicity

The acute toxicity of Brilliant Black PN was evaluated in mice and rats using oral, intraperitoneal and intravenous routes of administration. The results of these studies are summarized in Table 1.

2.2.2 Short-term studies of toxicity

(a) Rats

Five rats (strain and sex not specified) were treated orally with Brilliant Black PN at 1500 mg/kg bw for 22 days, and no Heinz bodies were found (DFG, 1957).

Groups of 16 male and 16 female weanling rats were fed daily diets containing Brilliant Black PN (purity 83.6%) at a concentration of 0, 3000, 10 000 or 30 000 mg/kg feed (equivalent to 0, 300, 1000 and 3000 mg/kg bw per day, respectively) for 90 days. Growth retardation associated with diminished feed intake was evident only in males at the 30 000 mg/kg feed level. No abnormalities in haematology, liver or kidney function were observed. Weights of testes and kidneys increased in males at the 30 000 mg/kg feed level only. No adverse histopathological findings were seen (Gaunt et al., 1967).

Table 1
Summary of acute toxicity studies with Brilliant Black PN

Species	Route of administration	LD$_{50}$ (mg/kg bw)	Reference
Mouse	Oral	>5 000	DFG (1957)
	Oral	>2 000	Gaunt et al. (1967)
	Intraperitoneal	550–1 000	Gaunt et al. (1967)
Rat	Oral	>5 000	Gaunt et al. (1967)
	Intraperitoneal	900–1 200	Gaunt et al. (1967)
	Intraperitoneal	>2 000	DFG (1957)
	Intravenous	~2 500	DFG (1957)

bw: body weight; LD$_{50}$: median lethal dose

(b) Pigs

Groups of three male and three female 10-week-old pigs were dosed orally with Brilliant Black PN (minimum purity 82%) at 0, 100, 300 or 900 mg/kg bw per day for 90 days. The colourant was administered mixed with a quantity of diet and syrup prior to the main feed. No adverse effects were observed on growth, haematology, urine analyses, serum transaminase activities or organ weights. Cysts containing mucus and fibrin were found in the mucosa of the ileum of four pigs given 900 mg/kg bw per day and one pig given 300 mg/kg bw per day. It was suggested by the authors that this might have been due to an irritant effect of the dye caused by local high concentrations due to bolus dosing and therefore unlikely to occur in human diets. They concluded that the NOAEL in pigs was 100 mg/kg bw per day (Gaunt et al., 1969). The Committee at the twenty-fifth meeting (Annex 1, reference 56) established an ADI of 0–1 mg/kg bw on the basis of this NOAEL. In the current submission, the sponsor reiterated the authors' argument that the cysts might have been due to an irritant effect of local high concentrations of Brilliant Black PN related to the way in which the substance was administered as a bolus in a small amount of feed. The present Committee considered that this explanation lacked plausibility, as the upper parts of the gastrointestinal tract were not affected, as would be anticipated for an irritant effect.

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

Groups of 30 male and 30 female mice (CFW strain) were fed diets containing Brilliant Black PN (minimum purity 82%) at 1, 2500, 5000 or 10 000 mg/kg feed for 80 weeks. Groups of 60 male and 60 female mice with similar ranges of body weight served as controls. No dose-related effects in mortality, body weight

gain, haematology, relative organ weights or histopathology were observed. The incidence of tumours in the treated mice did not exceed that of the control animals at dietary concentrations up to 10 000 mg/kg feed (providing an intake of 1300 mg/kg bw per day, as reported by the authors). The authors concluded that the NOAEL in this study was approximately 1300 mg/kg bw per day, the highest dose tested (Drake et al., 1977).

(b) **Rats**

A group of 10 rats received 0.5 mL of a 1% solution (= 5 mg) of Brilliant Black PN twice weekly for 365 days and was observed for 653 days. The total amount administered per animal was 0.5 g. Two animals died prematurely, but no tumours were noted (DFG, 1957).

A group of 10 rats was given Brilliant Black PN in their drinking-water at 5 g/L (average dose 500 mg/kg bw per day) for 384 days and observed for 545 days. Total intake per animal was 20 g. No tumours were seen (DFG, 1957).

In another experiment, a group of 10 rats was given Brilliant Black PN in their drinking-water at 5 g/L (average dose 460 mg/kg bw per day) for 502 days and observed for 923 days. Total intake per animal was 50 g. No tumours were noted (DFG, 1957).

Groups of 16 rats were fed Brilliant Black PN in the diet at 1000 mg/kg feed (average dose 60 mg/kg bw per day) for 410 days and were observed for 761 days. The total dose per animal was 5.6 g. One rat died prematurely. No tumours were observed (Hecht & Wingler, 1952; DFG, 1957).

Groups of 24 male and 24 female weanling rats were fed for 2 years on diets containing Brilliant Black PN at a concentration of 0, 1000, 5000 or 10 000 mg/kg feed (equal to 0, 40, 180 and 360 mg/kg bw per day for males and 0, 45, 240 and 470 mg/kg bw per day for females, respectively). No treatment-related effects on mortality, feed intake, body weight gain, haematology, serum chemistry, renal concentration tests, organ weights or incidence of pathological findings, including tumours, were found. In this study, the NOAEL was 10 000 mg/kg feed (equal to 360 mg/kg bw per day), the highest dose tested (Gaunt et al., 1972).

2.2.4 **Genotoxicity**

The genotoxic potential of Brilliant Black PN was evaluated in a bacterial reverse mutation assay using the standard method and the Prival modification method, which is adequate to reduce the azo compounds to free amines (Seifried et al., 2006). Mutations in mammalian cells have been investigated in an L5178Y Tk$^{+/-}$ mouse lymphoma assay, which covers both gene mutation and chromosomal aberrations (Seifried et al., 2006). The gene mutation assay in mammalian cells (Seifried et al., 2006) was equivocal in the presence of metabolic activation, which

Table 2
Summary of in vitro and in vivo genotoxicity studies with Brilliant Black PN

End-point	Test system	Concentrations/doses	Result	Reference
In vitro				
Reverse mutation	1st experiment: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	1st experiment: 10, 33, 100, 333, 1 000, 3 333 and 10 000 µg/plate ±S9, plate incorporation method	Negative[a]	Seifried et al. (2006)
	2nd experiment: *S. typhimurium* TA98, TA100	2nd experiment: 10, 33, 100, 333, 1 000, 3 333 and 10 000 µg/plate ±S9, preincubation method under reductive conditions (Prival modification)		
Gene mutation (*Tk* locus)	L5178Y Tk$^{+/-}$ mouse lymphoma cells	470–3 922 µg/mL ±S9, 4 h treatment	Negative[b] −S9 Equivocal +S9	Seifried et al. (2006)
Unscheduled DNA synthesis	Isolated rat hepatocytes	0.01–1 mmol/L, 4 h treatment	Negative	Kornbrust & Barfknecht (1985)
Micronucleus induction	Human peripheral blood lymphocytes	8.67, 86.7 and 867 µg/mL, 24 h treatment −S9	Positive[c]	Macioszek & Kononowicz (2004)
DNA breakage (comet assay)	Human peripheral blood lymphocytes	8.67, 86.7 and 867 µg/mL, 1 h treatment −S9	Positive[d]	Macioszek & Kononowicz (2004)
In vivo				
Unscheduled DNA synthesis	Sprague Dawley rats	500 mg/kg bw by gavage	Negative[e]	Kornbrust & Barfknecht (1985)

bw: body weight; DNA: deoxyribonucleic acid; OECD TG: Organisation for Economic Co-operation and Development Test Guideline; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *Tk*: thymidine kinase

[a] Incomplete set of bacterial strains: TA102 and *Escherichia coli* WP2 strains missing.

[b] Dose range selected based on cytotoxicity. The study essentially complies with current OECD TG 490, but long-treatment −S9 is missing.

[c] Insufficient reliability due to an inadequate treatment schedule and shortcomings in technical procedures. Treatment with the test compound was performed 20 hours after lymphocyte stimulation for 24 hours in the absence of cytochalasin B, which was added 44 hours after stimulation, after the test compound was washed out. The relevant OECD TG 487 indicates that treatment with the test compound should be performed 44–48 hours after lymphocyte stimulation for 24 hours in the presence of cytochalasin B. In addition, the cytological preparations showed in the reported images indicate an inadequate quality for the analysis of micronuclei in binucleated cells (e.g. absence of cytoplasm, size of micronuclei and condensation of chromatin).

[d] Insufficient reliability due to i) the absence of a cytotoxicity evaluation, ii) the use of an inadequate scoring system and iii) the positive control diepoxybutane showing a marked increase in tail moment. The latter is not plausible, as diepoxybutane is a DNA crosslinking agent, which reduces DNA migration instead of increasing it.

[e] The overall low sensitivity of the unscheduled DNA synthesis assay in detecting rodent carcinogens and/or in vivo genotoxicants, highlighted by the analysis of the European Union Reference Laboratory for alternatives to animal testing database, confirms previous conclusions on the lower predictive value of the unscheduled DNA synthesis assay compared with the transgenic rodent assay and in vivo comet assays, supporting a more prominent role for the latter assays in regulatory testing strategies. For reassessment, test results may be considered as adequate to assess genotoxic potential only in cases of positive results. If the outcome of the unscheduled DNA synthesis assay is negative, however, other more reliable tests, such as the transgenic rodent assay or in vivo comet assay, would be needed to complete the assessment (EFSA, 2017).

normally would require follow-up, and aneugenicity was not tested for. Three additional in vitro tests in mammalian cells were also performed: an unscheduled DNA synthesis assay (Kornbrust & Barfknecht, 1985), an in vitro micronucleus test (Macioszek & Kononowicz, 2004) and a comet assay in human lymphocytes (Macioszek & Kononowicz, 2004). An in vivo unscheduled DNA synthesis assay in rats (Kornbrust & Barfknecht, 1985) was also available. The results obtained are presented in Table 2 and are generally negative. The positive findings obtained

in the in vitro micronucleus test and comet assay are considered to be unreliable due to major shortcomings, as listed in the relevant table notes.

The Committee noted that the equivocal results observed in the mouse lymphoma assay would have needed confirmatory in vitro follow-up studies and that aneugenicity was not covered by any of the available valid studies. However, the Committee further noted that, as also pointed out by Jung, Steinle & Anliker (1992), sulfonated aromatic amines (such as those generated by the azoreduction of Brilliant Black PN), in contrast with their unsulfonated analogues, have no or very low genotoxic potential; in addition, the structurally related azo dye Allura Red AC, bearing sulfonation of the aromatic rings, showed reliable negative findings in an in vivo comet assay (liver and glandular stomach), in a transgenic gene mutation assay in rats (liver and glandular stomach) and in a bone marrow micronucleus test (Honma, 2015).

The Committee concluded that, overall, the data did not indicate concern with respect to the genotoxicity of Brilliant Black PN.

2.2.5 Reproductive and developmental toxicity

No new data on reproductive or developmental toxicity were available. Studies previously evaluated at the twenty-fifth meeting of JECFA (Annex 1, reference *57*) are summarized below.

In a preliminary study, Brilliant Black PN was administered to four groups of 15 pregnant SPF-derived Wistar rats by gavage at a dose of 0, 250, 500 or 2500 mg/kg bw per day from day 0 to day 19 of pregnancy. In a second study involving four groups of 30 pregnant rats, a similar protocol was used. On day 21, the animals were killed, and ovaries and uterus were removed. The number of corpora lutea in each ovary was recorded, and the fetuses were examined. Live fetuses, embryonic and fetal resorptions and dead fetuses were counted, and the number and position of implantation sites were recorded. In the second study, half the fetuses in the control and top-dose groups were examined for skeletal malformations, and half for visceral defects. No abnormalities in condition or behaviour of the dams were observed in either study. At necropsy, no signs of embryotoxicity or teratogenicity were observed. One fetus in the top-dose group showed a complexity of malformations, but this was considered to be a fortuitous finding. It was concluded that ingestion of Brilliant Black PN at doses up to 2500 mg/kg bw per day was without teratogenic effect in the rat (Koeter, 1979).

A multigeneration study was performed on Wistar rats fed Brilliant Black PN at a dietary concentration of 0, 1000, 10 000 or 30 000 mg/kg feed for three successive generations. One litter was reared from each of the F_0, F_1 and F_2 parents. Each generation started with 60 animals of each sex in the control group and 40 of each sex in the test groups. After a 9-week test period, 24 males

and 24 females from the control group and 14 males and 14 females from each test group were used for a teratogenicity study; the remainder were used for the reproduction study. No adverse effects were observed with respect to fertility, litter size and weight, general condition, male/female ratio, growth during lactation, survival or maturation. Necropsy of parent rats and pups at weaning did not reveal any treatment-related changes in organ weights other than caecal enlargement in the 30 000 mg/kg feed group. Gross and microscopic examinations of the F_3 generation at weaning did not reveal any abnormality due to treatment, and no adverse effects were seen in the teratogenicity study. It was concluded that Brilliant Black PN did not exert any teratogenic effects or adverse effects on reproductive function of Wistar rats when fed at dietary concentrations up to 30 000 mg/kg feed (equivalent to 1500 mg/kg bw per day) for three successive generations (Koeter & Dreef-Van der Meulen, 1980).

2.2.6 Special studies

(a) Toxicity of sulfanilic acid

Brilliant Black PN is reduced by intestinal microflora mainly to four metabolites, and complete azoreduction results in SA, a sulfonated aromatic amine. The toxicity of SA has been assessed in several studies.

SA was administered to Wistar rats (five of each sex per group) by oral gavage at a dose of 0, 63, 250 or 1000 mg/kg bw per day for 28 days. The study was conducted in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 407. No clinical signs of toxicity, body weight changes or adverse effects on haematology, biochemistry, gross necropsy, organ weights or histopathology were noted at any dose. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (ECHA, 2010a).

The genotoxic potential of SA was evaluated in several bacterial reverse mutation assays and three in vitro mammalian cell assays (sister chromatid exchange, unscheduled DNA synthesis and gene mutation assays). The results of these assays are presented in Table 3 and indicate that SA does not show any genotoxic potential in vitro.

In a reproductive and developmental toxicity study compliant with OECD Test Guideline 421, SA was administered to Wistar rats (12 of each sex per group) daily by oral gavage at a dose of 0, 62.5, 250 or 1000 mg/kg bw per day over 41–79 days. Male rats were dosed for at least 6 weeks (an average of 8 weeks), including a period of 2 weeks prior to mating, through the mating period and up to the day before termination. Female rats were dosed for at least 6 weeks (an average of 8–9 weeks), including a period of 2 weeks prior to mating, at least two complete estrous cycles, time to conception, the duration of the pregnancy, at least 4 days after delivery, and up to and including the day before termination.

Table 3
Summary of in vitro genotoxicity studies with SA

End-point	Test system	Concentrations	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA97, TA98, TA100, TA1535	Five concentrations up to 1 500 μg/plate ±S9, preincubation method	Negative[a]	Zeiger et al. (1988)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	*Experiment 1:* Doses up to 1 000 μg/plate ±S9, plate incorporation method *Experiment 2:* Doses up to 500 μg/plate ±S9, preincubation method	Negative[a]	Chung, Fulk & Andrews (1981)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Eight concentrations up to 10 000 μg/plate ±S9, plate incorporation method	Negative[a]	Jagannath (1985)
Sister chromatid exchange	Chinese hamster ovary cells	167, 500, 1 670 or 5 000 μg/mL ±S9	Negative	Ivett (1985)
Unscheduled DNA synthesis	Human peripheral blood lymphocytes	Eight concentrations from 25 to 2 500 μg/mL, 18–19 h treatment	Negative	Cifone (1985b)
Gene mutation (*Tk* locus)	L5178Y Tk$^{+/-}$ mouse lymphoma cells	Six concentrations from 1 500 to 5 000 μg/mL ±S9, 4 h treatment	Negative[b]	Cifone (1985a)

DNA: deoxyribonucleic acid; OECD TG: Organisation for Economic Co-operation and Development Test Guideline; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *Tk*: thymidine kinase
[a] Incomplete set of bacterial strains: TA102 and *Escherichia coli* WP2 strains missing.
[b] Dose range selected based on cytotoxicity. The study essentially complies with current OECD TG 490, but long-treatment −S9 is missing.

Females that showed no evidence of copulation were placed into cohabitation with males for a second mating period.

Mild systemic signs of toxicity at the highest dose were not corroborated by histopathological examinations. No notable clinical signs of toxicity, body weight changes or other treatment-related adverse effects on haematology, biochemistry, gross necropsy, organ weights or histopathology were noted at any dose. No reproductive or developmental parameters were adversely affected by treatment with SA at any dose. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (ECHA, 2010b).

2.3 Observations in humans

The ability of Brilliant Black PN and five additional azo dyes (i.e. Tartrazine, Sunset Yellow, Azorubine, Amaranth, Ponceau) to induce an immunoglobulin E (IgE)–independent response such as chronic urticaria through an idiopathic as opposed to an autoimmune (IgE antibody–mediated) etiology was evaluated in a double-blind, placebo-controlled food challenge (Ehlers et al., 1998). This

test is commonly used to diagnose food allergies (Bégin & Nadeau, 2013). Six children with moderate to severe chronic urticaria lasting at least 3 months over a 2-year period were placed on an elimination diet that restricted exposure to preservatives, dyes or antioxidants for 3 weeks. They were selected for double-blind, placebo-controlled food challenge testing after examination of their patient history to ensure that symptoms were non-IgE mediated. Six subjects were orally administered 5 mg of Brilliant Black PN in gelatine capsules once daily after the restricted diet was completed and until allergic symptoms appeared. When positive reactions were observed, testing was stopped until symptoms of non-allergic hypersensitivity reactions ceased. In cases of severe reactions, symptomatic treatment was provided to patients. Out of the six patients, one child exhibited a positive response to all tested azo dyes, including Brilliant Black PN (Ehlers et al., 1998).

3. Dietary exposure

The sponsor proposes the use of Brilliant Black PN for 16 food subcategories belonging to three main food categories at typical and maximum use levels as listed in Table 4. Currently, Brilliant Black PN is authorized for use in food category "01.1.4 Flavoured fluid milk drinks", excluding chocolate milk, at a maximum permitted level (MPL) of 150 mg/L, as specified in the Codex General Standard for Food Additives (GSFA) (FAO/WHO, 2018). Brilliant Black PN (INS 455) was last evaluated by the Committee at the twenty-fifth meeting in 1981 (Annex 1, reference 56); no dietary exposure assessment was performed at that meeting.

The Committee evaluated the estimates of dietary exposure to Brilliant Black PN provided by the sponsor. Because Brilliant Black PN would be added to processed foods only, the Committee did not perform an international exposure assessment using the commodity-based food consumption data of the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) cluster diets based on the provided use levels. Also, the Committee did not perform an exposure assessment using the FAO/WHO Chronic Individual Food Consumption data – summary statistics (CIFOCOss) database. The description of the foods recorded in this database was often not specific enough for a relevant link between these foods and the provided use levels. Additionally, many of the recorded foods related to raw food commodities.

Table 4

Typical and maximum proposed use levels of Brilliant Black PN by food category of the Codex GSFA

Food category	Name of food category	Use level (mg/kg) Typical	Maximum
05.0	**Confectionery**		
05.2	Confectionery including hard and soft candy, nougats, etc. other than food categories 05.1, 05.3 and 05.4		
05.2.1	Hard candy	50	300
05.2.2	Soft candy	50	300
05.3	Chewing gum	10	10
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	50	500
09.0	**Fish and fish products, including mollusks, crustaceans, and echinoderms**		
09.3	Semi-preserved fish and fish products, including mollusks, crustaceans, and echinoderms		
09.3.3	Salmon substitutes, caviar, and other fish roe products	300	300
09.4	Fully preserved, including canned or fermented fish and fish products, including mollusks, crustaceans, and echinoderms	300	300
14.0	**Beverages, excluding dairy products**		
14.1	Non-alcoholic ("soft") beverages		
14.1.2	Fruit and vegetable juices		
14.1.2.1	Fruit juice	10	10
14.1.2.2	Vegetable juice	10	10
14.1.4	Water-based flavoured drinks, including "sport," "energy," or "electrolyte" drinks and particulated drinks	10	100
14.1.4.1	Carbonated water-based flavoured drinks	10	100
14.1.4.2	Non-carbonated water-based flavoured drinks, including punches and ades	10	100
14.1.4.3	Concentrates (liquid or solid) for water-based flavoured drinks	10	100
14.2	Alcoholic beverages, including alcohol-free and low-alcoholic counterparts		
14.2.2	Cider and perry	10	200
14.2.4	Wines (other than grape)	10	200
14.2.6	Distilled spirituous beverages containing more than 15% alcohol	10	200
14.2.7	Aromatized alcoholic beverages (e.g. beer, wine and spirituous cooler-type beverages, low alcoholic refreshers)	10	200

GSFA: General Standard for Food Additives

3.1 Exposure estimates provided by sponsor

The sponsor provided estimates of dietary exposure to Brilliant Black PN based on the budget method and data for the Australian population (FSANZ, 2008, 2012), European populations (EFSA, 2015) and schoolchildren living in Kuwait (Sawaya et al., 2007). The Committee did not consider the estimates based on the budget method, because more refined estimates were available.

In the studies considered by the Committee, Brilliant Black PN was referred to as Brilliant Black (FSANZ, 2008, 2012; Sawaya et al., 2007) or Brilliant

Black BN (EFSA, 2015). These terms refer to the same chemical as Brilliant Black PN.

3.1.1 Australia

In Australia, Food Standards Australia New Zealand (FSANZ) conducted a study on the concentrations of food colours in foods and beverages available in Australia (FSANZ, 2008). In total, FSANZ (2008) collected 396 foods and beverages that had been identified to contain food colours and analysed them for the presence of 16 food colours, including Brilliant Black PN. The concentrations were combined with those of 255 food and beverage samples collected and analysed in a similar study conducted by the South Australia Department of Health in 2004. The dietary exposure was calculated by combining the concentration data with individual food consumption data for the general Australian population aged 2 years and above and for five different subgroups, including:

1) children aged 2–5 years;
2) children aged 6–12 years;
3) adolescents aged 13–18 years;
4) adults aged 19–24 years; and
5) adults aged 25 years and above.

The food consumption data used for the exposure assessment were derived from the 1995 Australian National Nutrition Survey. FSANZ (2008) addressed children in more detail, because they consume more food per kilogram body weight, resulting in a higher exposure than for adults, and because food colours are often added to foods that may appeal more to children. The exposure was calculated according to two scenarios:

1) a mean scenario, based on the mean analytical concentration per food group; and
2) a maximum scenario, based on the maximum analytical concentration per food group.

In 2012, FSANZ updated the exposure assessment for all 16 food colours for children aged 2–5 years, 6–12 years and 13–16 years using food consumption data from the 2007 Australian National Children's Nutrition and Physical Activity Survey. The resulting exposure estimates replaced those for children aged 2–18 years reported in 2008. Also, the estimates for the general population aged 2 years and above were no longer relevant because of this update.

Brilliant Black PN was detected in cakes, muffins and pastries, confectionery, and jelly at mean concentrations ranging from 0.06 to 0.24 mg/

kg. The highest concentration, 2.5 mg/kg, was detected in cakes, muffins and pastries. Compared with the other food colours, Brilliant Black PN was present in only a very limited number of food groups at low concentrations (FSANZ, 2008).

The estimated mean dietary exposure to Brilliant Black PN in the mean scenario ranged from 0.00 mg/kg bw per day for adults 19 years of age and above to 0.001 mg/kg bw per day for children 2–5 years of age (FSANZ, 2008, 2012). The estimated high (90th percentile) dietary exposure in the mean scenario ranged from 0.00 mg/kg bw per day for adults 19 years of age and above to 0.002 mg/kg bw per day for children 2–5 years of age (FSANZ, 2008, 2012).

In the maximum scenario, the estimated mean dietary exposure to Brilliant Black PN ranged from 0.00 mg/kg bw per day for adults 19 years of age and above to 0.004 mg/kg bw per day for children 2–5 years of age (FSANZ, 2008, 2012). The estimated high (90th percentile) dietary exposure ranged from 0.003 mg/kg bw per day for children 13–16 years of age to 0.01 mg/kg bw per day for children 2–5 years of age (FSANZ, 2012) and for adults 19 years of age and above (FSANZ, 2008).

All these dietary exposure estimates refer to the exposure in persons who consumed foods that may contain Brilliant Black PN (consumers only).

3.1.2 Europe

The European Food Safety Authority (EFSA) estimated the dietary exposure to Brilliant Black PN in Europe in 2010 (EFSA, 2010). This assessment was, however, updated in 2015 (EFSA, 2015). The Committee has reported here only on the dietary exposure results of the more recent 2015 assessment.

EFSA (2015) calculated the dietary exposure to Brilliant Black PN based on MPLs according to Annex II of Regulation (EC) No. 1333/2008, use levels provided by the industry and analytical concentrations provided by five European countries. These data were combined with individual food consumption data from the Comprehensive European Food Consumption Database. At that time, the Comprehensive European Food Consumption Database contained consumption data from 26 dietary surveys from 17 European countries covering toddlers (1–2 years), children (3–9 years), adolescents (10–17 years) and adults (18 years and above).

According to Annex II of Regulation (EC) No. 1333/2008, the use of Brilliant Black PN is permitted in 36 food categories with MPLs ranging from 50 to 500 mg/kg and in two food categories at quantum satis.[1] Typical (mean) and maximum use levels ($n = 20$) were provided for 11 food categories, and analytical concentrations were provided for 24 food categories. EFSA (2015) calculated the dietary exposure to Brilliant Black PN using three exposure scenarios, one

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[1] The amount that is enough.

using MPLs and the other two using the provided use levels and analytical concentrations according to two refined exposure scenarios:

1) the regulatory maximum level exposure assessment scenario, using MPLs;

2) the brand-loyal consumer scenario, in which it was assumed that persons are exposed to Brilliant Black PN at the maximum reported use level or analytical concentration, whichever is higher, for one food category and at the typical (mean) reported use level or the mean of the analytical concentrations, whichever is higher, for the remaining food categories; and

3) the non-brand-loyal consumer scenario, in which it was assumed that persons are exposed to Brilliant Black PN at the typical (mean) reported use level or the mean of the analytical concentrations, whichever is higher, for all food categories.

Analytical concentrations below the limit of detection or limit of quantification were assumed to contain the additive at a level of half the limit value.

EFSA (2015) calculated the exposure for five age groups in the Comprehensive European Food Consumption Database. For this, adults were divided into the age groups 18–64 years and 65 years and above. Not all 36 food categories were included in the exposure assessment because of missing use levels or analytical concentrations (refined exposure scenarios) or because the food categories could not meaningfully be mapped to the food records in the Comprehensive European Food Consumption Database (all three exposure scenarios). In total, 26 food categories were included in the regulatory maximum level exposure assessment scenario, and 21 food categories were included in the two refined exposure scenarios.

The exposure estimates are reported in Table 5. Important sources of exposure in all scenarios across age groups were fine bakery wares and flavoured fermented milk products.

EFSA (2015) considered the results of the refined exposure assessment to be most representative of the exposure to Brilliant Black PN. This refined assessment included all food categories for which the sponsor has proposed use levels (Table 4), except for food categories "05.4 Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces", "14.1.2.1 Fruit juice" and "14.1.2.2 Vegetable juice". Food category 05.4 could not be meaningfully mapped to the consumption data in the Comprehensive European Food Consumption Database. Food categories 14.1.2.1 and 14.1.2.2 were not considered, because Brilliant Black PN is not authorized for use in these two food categories according to Annex II of Regulation (EC) No. 1333/2008. Food categories that were included in the EFSA

Table 5
Dietary exposure to Brilliant Black PN in children and adults in Europe

Scenario[a] and exposure level[b]	Estimated dietary exposure (mg/kg bw per day)[c] per age group				
	Toddlers (12–35 months)	**Children (3–9 years)**	**Adolescents (10–17 years)**	**Adults (18–64 years)**	**Elderly (65+ years)**
Regulatory maximum level exposure assessment scenario					
Mean	0.89–3.88	0.78–2.92	0.23–1.63	0.28–1.17	0.14–0.57
High	2.75–6.35	1.79–6.11	0.65–3.33	0.78–2.52	0.54–1.39
Brand-loyal scenario					
Mean	0.08–0.84	0.16–0.74	0.13–0.42	0.05–0.30	0.03–0.20
High	0.25–1.71	0.42–1.83	0.41–1.04	0.23–0.69	0.11–0.60
Non-brand-loyal scenario					
Mean	0.03–0.17	0.03–0.13	0.01–0.05	0.01–0.05	0.01–0.04
High	0.11–0.30	0.07–0.28	0.03–0.12	0.03–0.10	0.02–0.09

bw: body weight
[a] For a description of the scenarios, see text.
[b] High exposure: 95th percentile.
[c] Range represents the lowest to highest estimate in each age group across dietary surveys.
Source: EFSA (2015)

assessment, but for which no use levels were proposed by the sponsor, were "03.0 Edible ices", "07.2 Fine bakery wares", "12.2.2 Seasonings and condiments", "12.5 Soup and broths", "12.4 Mustard" and "15.1 Potato-, cereal-, flour- and starch-based snacks", as well as food category "01.4 Flavoured fermented milk products".

3.1.3 Kuwait

In Kuwait, dietary exposure to Brilliant Black PN was estimated based on analysed concentrations in 344 coloured foods commonly consumed by children and food consumption data from a 2-day 24-hour recall dietary survey administered to 3141 male and female children aged 5–14 years from 58 schools (Sawaya et al., 2007). The dietary survey was conducted by the Kuwait Institute for Scientific Research and the Ministry of Health's Nutrition and Food Administration. Foods analysed were identified in the 24-hour recall and belonged to the food categories biscuits, cakes, ice cream, candy, chips and puffed snacks, chocolates, drinks and juices, chewing gum, jelly and lollipops. The foods were analysed for six food colours, including Brilliant Black PN. The exposure was calculated per age and sex by multiplying the average consumption per food category with the average analysed Brilliant Black PN concentration and dividing the result by the average body weight per age and sex.

Brilliant Black PN was detected only in candy at concentrations of 0.2–0.4 mg/kg. The mean estimated dietary exposure to Brilliant Black PN ranged

Table 6
Overview of dietary exposures to Brilliant Black PN

	Exposure (mg/kg bw per day)	
Source	**Mean**	**High**
Australia[a]		
Mean analytical concentrations	0.00–0.001	0.00–0.002[b]
Maximum analytical concentrations	0.00–0.004	0.003–0.01[b]
Europe		
MPLs	0.14–3.88	0.54–6.35[c]
Use levels and analytical concentrations		
Brand-loyal scenario	0.03–0.84	0.11–1.83[c]
Non-brand-loyal scenario	0.01–0.17	0.02–0.30[c]
Kuwait[d]		
Analytical concentrations	0.000 2–0.000 3	–

bw: body weight; MPL: maximum permitted level
[a] Exposure only for consumers of foods containing Brilliant Black PN.
[b] Ninetieth percentile of exposure.
[c] Ninety-fifth percentile of exposure.
[d] Children.
Sources: Australia: FSANZ (2008, 2012); Europe: EFSA (2015); Kuwait: Sawaya et al. (2017)

from 0.0002 mg/kg bw per day in most age groups to maximally 0.0003 mg/kg bw per day in girls aged 6 and boys aged 7 and 10 (Sawaya et al., 2007).

3.2 Overview of the dietary exposure to Brilliant Black PN

Table 6 summarizes the estimated dietary exposures to Brilliant Black PN. The lowest exposure estimates were those for Australia (FSANZ, 2008, 2012) and children from Kuwait (Sawaya et al., 2017). These estimates were based on analytical concentrations of Brilliant Black PN in foods that may contain the food additive. EFSA based its refined assessment on use levels from the food industry and analytical concentrations from five European countries. The dietary exposure in Europe based on these data could be as high as 1.8 mg/kg bw per day in children 3–9 years of age (Table 6). The assessment of EFSA included almost all food categories for which the sponsor submitted use levels, as well as other food categories that contributed significantly to exposure, including fine bakery wares and flavoured fermented milk drinks.

The analytical concentrations of Brilliant Black PN in relevant foods available in Australia (FSANZ, 2008, 2012) and Kuwait (Sawaya et al., 2017) showed that Brilliant Black PN is used in a limited number of foods at low levels. In the refined European assessment, it was assumed that Brilliant Black PN is used in all food categories for which use levels and/or analytical concentrations

were provided and that all foods within these categories contain the colour at the provided use level or analytical concentration, whichever is higher. The exposure estimates for the European population are therefore very likely to be conservative.

4. Comments

4.1 Biochemical aspects

In rats, Brilliant Black PN is poorly absorbed, with 94–98% of administered doses up to 10 mg/kg bw excreted in the faeces within 40 hours and less than 5% detected in the urine within 40 hours (Anon., 1980). Differences in metabolism following oral and intraperitoneal administration indicate that metabolism by intestinal flora leads to complete azoreduction (cleavage of both azo sites), whereas azoreductases in liver preferentially cleave the azo site between the two naphthalene rings, resulting in sulfonated aromatic amines (Ryan & Welling, 1970).

In humans, SA was the only metabolite identified in urine following oral administration of a 240 mg dose of Brilliant Black PN, and the amount of metabolite was similar to that observed in rats (Ryan & Welling, 1970).

In vitro, Brilliant Black PN was shown to induce a dose-dependent decrease in the uptake of radiolabelled *o*-iodohippurate and iodipamide in rat renal cortex slices, which was interpreted as inhibition of the hippurate and liver-like anion transport systems (Carlson, 1977).

Brilliant Black PN was identified as a novel allosteric modulator of adenosine receptors using Chinese hamster ovary cells stably transfected with either A_1 or A_3 human receptors (May, Briddon & Hill, 2010). The Committee noted that the effects on adenosine receptors were observed only at high (500 µmol/L) concentrations of Brilliant Black PN. In view of the poor absorption of Brilliant Black PN, the Committee did not consider this study relevant to the evaluation.

4.2 Toxicological studies

In previously evaluated studies, Brilliant Black PN was not acutely toxic by the oral route in mice or rats (LD_{50} > 5000 mg/kg bw) (DFG, 1957; Gaunt et al., 1967) and showed no signs of toxicity in mice in a long-term study at doses up to 1300 mg/kg bw per day (Drake et al., 1977) or in rats in short-term studies with dietary concentrations up to 30 000 mg/kg feed (equivalent to 3000 mg/kg bw

per day) and long-term studies with dietary concentrations up to 10 000 mg/kg feed (equal to 360 mg/kg bw per day) (Gaunt et al., 1967, 1972) and no evidence of carcinogenicity in mice or rats (Gaunt et al., 1972; Drake et al., 1977). In rats, there was no reproductive toxicity or teratogenicity at dietary concentrations up to 30 000 mg/kg feed (equivalent to 1500 mg/kg bw per day) (Koeter & Dreef-Van der Meulen, 1980) and no teratogenicity at doses up to 2500 mg/kg bw per day (Koeter, 1979).

The only adverse findings reported previously were cysts containing mucus and fibrin in the ileal mucosa of pigs administered Brilliant Black PN at 300 mg/kg bw per day (one of six pigs) or 900 mg/kg bw per day (four of six pigs) for 90 days. The NOAEL in this study was 100 mg/kg bw per day (Gaunt et al., 1969). The Committee at the twenty-fifth meeting (Annex 1, reference 56) established an ADI based on this NOAEL. In the current submission, the sponsor reiterated the authors' argument that the cysts might have been due to an irritant effect of local high concentrations of Brilliant Black PN related to the way in which the substance was administered as a bolus in a small amount of feed. The present Committee considered that this explanation lacked plausibility, as the upper parts of the gastrointestinal tract were not affected, as would be anticipated for an irritant effect.

Several new in vitro genotoxicity studies (Kornbrust & Barfknecht, 1985; Macioszek & Kononowicz, 2004; Seifried et al., 2006) and one new in vivo genotoxicity study (Kornbrust & Barfknecht, 1985) were available to the Committee and were generally negative. The gene mutation assay in mammalian cells (Seifried et al., 2006) was equivocal in the presence of metabolic activation, which normally would require follow-up, and aneugenicity was not tested for. The positive findings obtained in the in vitro micronucleus test and comet assay are considered to be unreliable due to major shortcomings in study design. Read-across from a structurally related food colour (Allura Red AC) (Honma, 2015) and the lack of genotoxicity of other sulfonated aromatic amines such as those generated by the azoreduction of sulfonated azo dyes (Jung, Steinle & Anliker, 1992) were taken into consideration. The Committee concluded that, overall, the data did not indicate concern with respect to the genotoxicity of Brilliant Black PN.

The metabolite SA (the only metabolite found in human urine) did not show genotoxic activity or adverse effects in a 4-week study or in an OECD-compliant reproductive and developmental toxicity study in rats administered doses up to 1000 mg/kg bw per day (ECHA, 2010a,b).

4.3 Observations in humans

A study in six young patients with moderate to severe chronic urticaria found that one child exhibited IgE-independent responses to all tested azo dyes, including Brilliant Black PN (Ehlers et al., 1998). The Committee noted that this study is not informative for the present evaluation.

4.4 Assessment of dietary exposure

Brilliant Black PN is proposed by the sponsor for use in 16 food subcategories belonging to three main food categories of the Codex GSFA: "5. Confectionery", "9. Fish and fish products, including mollusks, crustaceans, and echinoderms" and "14. Beverages, excluding dairy products". The typical use levels range from 10 to 300 mg/kg, and the maximum use levels from 10 to 500 mg/kg. Currently, Brilliant Black PN is authorized for use only in food category "01.1.4 Flavoured fluid milk drinks", excluding chocolate milk, at an MPL of 150 mg/L, as specified in the GSFA (FAO/WHO, 2018).

The Committee used only those dietary exposure estimates that were considered to be most representative of actual exposure. These estimates were based on use levels and/or analytical concentrations combined with food consumption data from Australia (FSANZ, 2008, 2012), Europe (EFSA, 2015) and Kuwait (Sawaya et al., 2017) and are summarized in Table 7.

The dietary exposures to Brilliant Black PN in Australia and Kuwait were estimated using analytical concentrations measured in relevant foods, which resulted in low exposure estimates, as Brilliant Black PN was present in only a limited number of food groups at low levels (FSANZ, 2008, 2012; Sawaya et al., 2017). The high exposure (90th percentile) to Brilliant Black PN was maximally 0.01 mg/kg bw per day for children up to 16 years of age in Australia, based on the highest levels analysed per food group (FSANZ, 2012). This dietary exposure estimate refers to the exposure in persons who had consumed at least one of the foods that contained Brilliant Black PN (consumers only).

For Europe, the dietary exposure was estimated for different age groups using food consumption data from several European countries combined with MPLs, use levels and/or analytical concentrations, according to three exposure scenarios (EFSA, 2015). Given that the dietary exposure estimates for Australia and Kuwait were so low, the Committee considered the non-brand-loyal scenario to best reflect the dietary exposure to Brilliant Black PN. In this scenario, it is assumed that persons are exposed to a food additive at the typical (mean) reported use level or mean of the analytical concentrations for all relevant food categories and that all foods belonging to an authorized food category contain the food additive at that level. The mean dietary exposure to Brilliant Black PN

Table 7
Dietary exposure to Brilliant Black PN

Country/region	Dietary exposure (mg/kg bw per day)	
	Mean	**High**
Australia[a]		
Mean	0.00–0.001	0.00–0.002[b]
Maximum	0.00–0.004	0.003–0.01[b]
Europe[c]	0.01–0.17	0.02–0.30[d]
Kuwait[e]	0.000 2–0.000 3	–

bw: body weight
[a] Exposure only for consumers of foods containing Brilliant Black PN based on mean and maximum analytical concentrations.
[b] Ninetieth percentile of exposure.
[c] Non-brand-loyal scenario, based on mean use levels and analytical concentrations.
[d] Ninety-fifth percentile of exposure.
[e] Children; based on analytical concentrations.
Sources: Australia: FSANZ (2008, 2012); Europe: EFSA (2015); Kuwait: Sawaya et al. (2017)

ranged from 0.01 mg/kg bw per day for adolescents, adults 18–64 years of age and adults 65+ years of age to 0.17 mg/kg bw per day for toddlers in this scenario. The high dietary exposure (95th percentile) ranged from 0.02 mg/kg bw per day for adults 65+ years of age to 0.30 mg/kg bw per day for toddlers. The food categories included in this scenario overlapped largely with those for which use levels are proposed by the sponsor, in addition to other food categories for which no use levels were proposed, such as "Edible ices", "Fine bakery wares", "Seasonings and condiments", "Soup and broths", "Mustard" and "Potato-, cereal-, flour- and starch-based snacks". The Committee noted that the food category "Fine bakery wares" was the most important contributor to the dietary exposure to Brilliant Black PN across all age groups in Europe.

The Committee concluded that the high dietary exposure to Brilliant Black PN of 0.3 mg/kg bw per day, based on European data, is appropriate for use in a risk assessment.

5. Evaluation

The Committee concluded that the newly available information does not give reason to revise the previously established ADI of 0–1 mg/kg bw based on the short-term toxicity study in pigs. The Committee therefore retained the ADI for Brilliant Black PN.

The Committee noted that the range of estimated dietary exposures for Brilliant Black PN was below the upper end of the ADI and concluded that dietary exposure to Brilliant Black PN does not present a safety concern.

At the present meeting, the specifications for Brilliant Black PN were revised. Analytical methods for determining subsidiary colouring matters and organic compounds other than colouring matters were replaced with more specific and sensitive high-performance liquid chromatography methods. The existing titrimetric method for the assay of Brilliant Black PN was replaced with a visible spectrophotometric method.

A Chemical and Technical Assessment was prepared.

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Carotenoids (provitamin A)

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1. Explanation

β-Carotene (Chemical Abstracts Service [CAS] No. 7235-40-7) and β-apo-8′-carotenal (CAS No. 1107-26-2) are provitamin A carotenoids that are used as colours in a wide range of foods and beverages. Currently, both food additives are authorized for use in 79 food categories at maximum permitted levels (MPLs) ranging from 50 mg/kg up to 1200 mg/kg as specified in the Codex General Standard for Food Additives (GSFA) (FAO/WHO, 2018a).

A group acceptable daily intake (ADI) of 0–5 mg/kg body weight (bw) for β-carotene, β-apo-8′-carotenal and β-apo-8′-carotenoic acid methyl and ethyl esters was first established at the tenth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (Annex 1, reference *13*). At its eighteenth meeting, the Committee considered additional data and reaffirmed the decision of the tenth meeting (Annex 1, reference *35*). The group ADI was derived using a four-generation study in rats with a no-observed-adverse-effect level (NOAEL) for β-carotene of 50 mg/kg bw per day and application of an uncertainty factor of 10 because of the natural occurrence of provitamin A carotenoids in the human diet and the low toxicity observed in animal studies.

β-Carotenes from natural sources 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 ADI of 0–5 mg/kg bw established for the sum of the synthetic carotenoids β-carotene, β-apo-8′-carotenal and β-apo-8′-carotenoic acid methyl and ethyl esters by the eighteenth Committee 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 *Dunaliella salina*.

At the fifty-seventh meeting (Annex 1, reference *154*), the Committee undertook a re-evaluation of β-carotene for use as a food colour, but focused its assessment on the production and analytical characteristics of β-carotene produced from *Blakeslea trispora*. The Committee considered that the source organisms, the production process and the composition of β-carotene from *B. trispora* do not raise specific concerns and that the material should be considered toxicologically equivalent to chemically synthesized β-carotene, for which an ADI of 0–5 mg/kg bw was established by the Committee at its tenth meeting. Therefore, the Committee established a group ADI of 0–5 mg/kg bw for synthetic β-carotene and β-carotene derived from *B. trispora*.[1]

β-Carotene-rich extract from *D. salina* was evaluated at the eighty-fourth meeting (Annex 1, reference *234*). The Committee observed that data that had become available since the previous evaluation showed differences in absorption of β-carotene between rodent species and humans. The Committee considered that rodents were inappropriate animal models for establishing an ADI for β-carotene because of the virtual absence of systemic absorption of β-carotene in rodents, but that the non-β-carotene components of *D. salina* d-limonene extract could be evaluated using the results of rodent studies. The Committee recommended 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.

β-Carotene, β-apo-8′-carotenal, β-carotene from *B. trispora* and β-apo-8′-carotenoic acid methyl and ethyl esters were placed on the agenda of the present meeting for an assessment of their safety, dietary exposure and specifications in response to the recommendation of the eighty-fourth meeting of the Committee. The present Committee considered a submission that comprised

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[1] The present Committee was aware that two group ADIs for carotenoids had been established at previous meetings and that synthetic β-carotene had been included in both group ADIs. The Committee speculated that the Committee at the fifty-seventh meeting did not recognize that synthetic β-carotene was already part of a group ADI and included it in a new group ADI.

a review of information on synthetic β-carotene and β-apo-8′-carotenal that had become available since the eighteenth meeting. A targeted literature search was additionally carried out by searching the PubMed database using the following keywords: β-carotene, β-apo-8′-carotenal, absorption, distribution, metabolism, excretion, toxicity and clinical.

The Committee noted that no data were submitted on β-apo-8′-carotenoic acid methyl and ethyl esters. These food colours were therefore removed from the agenda.

1.1 Chemical and technical considerations

Provitamin A and xanthophyll carotenoids are natural pigments that are synthesized by plants and are responsible for the bright colours of various fruits and vegetables. Many different carotenoids are present in foods, and most have antioxidant activity. The most abundant carotenoid, β-carotene, consists of a highly branched, unsaturated chain with identical substituted ring structures at each end. β-Carotene and β-apo-8′-carotenal are provitamin A carotenoids.

1.1.1 β-Carotene, synthetic

Commercially available β-carotene, synthetic (International Numbering System for Food Additives [INS] 160a(i)) may be synthesized via a double Wittig condensation process or Grignard synthesis with enol ether condensations using a range of vitamin A precursors, including their phosphonium salts. The products of commerce may exist in multiple formulations, including water-dispersible forms, those that are water soluble and microcrystals prepared by spray drying and bound to food-grade carriers and antioxidants. Solvents used in manufacture may include dichloromethane, hexane, methanol, methylcyclohexane, toluene, acetone, ethanol, ethyl acetate, heptane, isobutyl alcohol and isopropyl alcohol (EFSA, 2012a). The colouring principle of β-carotene, synthetic consists predominantly of all-*trans*-β-carotene (*E*-isomer) together with minor amounts of other carotenoids. The total colouring matters content is not less than 96% (expressed as β-carotene).

1.1.2 β-Carotene from *Blakeslea trispora*

β-Carotene from *Blakeslea trispora* (INS 160a(iii)) is obtained by co-fermentation using a mixed culture of the two sexual mating types (+) and (−) of natural strains of the fungus that are non-pathogenic and non-toxigenic. The compound is isolated from the fungal biomass by solvent extraction and crystallized. The main articles of commerce are suspensions in food-grade vegetable or plant oil and water-dispersible powders. These formulations are made for ease of use and in order to improve stability, as carotenes easily oxidize. β-Carotene from *B. trispora* may also contain other

carotenoids, of which λ-carotene accounts for the major part, at concentrations up to 3%. As in synthetically produced β-carotene, the colouring principle of β-carotene from *B. trispora* consists predominantly of all-*trans*-β-carotene. The total colouring matters content is not less than 96% (expressed as β-carotene).

1.1.3 β-Apo-8′-carotenal

β-Apo-8′-carotenal (INS 160a(vi)) occurs naturally in various plant materials as an aldehydic carotenoid. The product of commerce is synthetically produced using multiple mechanisms that may include the use of vitamin A precursor molecules and Wittig-type condensation reactions. Sequential chemical reactions are carried out to produce the final material, which exists predominantly as the all-*trans* (*E*) isomer. The articles of commerce may be diluted and stabilized as suspensions in edible fats or oils, emulsions and water-dispersible powders. The total colouring matters content is not less than 96%.

1.1.4 β-Carotene-rich extract from *Dunaliella salina*

β-Carotene-rich extract from *Dunaliella salina* is produced from *D. salina*, an extremely 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.

2. Biological data

2.1 Biochemical aspects

2.1.1 β-Carotene

(a) Rats

β-Carotene is absorbed into enterocytes and centrally cleaved to give two retinal molecules. Retinal is reduced to retinol by the enzyme retinaldehyde reductase

and then esterified to form retinyl esters by lecithin:retinol acyltransferase and packaged with chylomicrons. Chylomicrons containing retinyl esters are released into the lymph and then the bloodstream and rapidly taken up into the liver (reviewed in Blomhoff et al., 1991; Blomhoff, Green & Norum, 1992).

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 (the primary enzyme responsible for oxidative cleavage of β-carotene to retinal) in rodents is the intestinal mucosa. The activity of β-carotene-15,15′-dioxygenase in rat intestine homogenates was reported to be 750 nmol/min per milligram homogenate protein (During, Albaugh & Smith, 1998; During et al., 1999).

The absorption and tissue distribution of β-carotene were investigated in groups of six female Sprague Dawley rats (170–190 g) administered β-carotene (Hoffmann-La Roche; 10% weight per weight [w/w] β-carotene beadlets) in the diet at 0, 20, 200 or 2000 mg/kg feed (equivalent to 0, 2, 20 and 200 mg/kg bw per day, respectively) for up to 21 weeks. A recovery group was maintained until day 182. Plasma β-carotene concentrations increased with dose to a maximum of approximately 0.15–0.2 µg/mL by 2 weeks and then remained relatively stable over the remainder of the treatment period. Concentrations returned rapidly to pretreatment levels during the recovery period. In investigated tissues, the highest concentrations of β-carotene were found in the liver (50 µg/g), adrenal gland (40 µg/g) and ovary (16 µg/g). Concentrations of β-carotene in the heart, kidney, lung, skin, brain and muscle were generally much lower (up to approximately 0.3–0.4 µg/g) (Shapiro, Mott & Machlin, 1984).

Three groups of 15 female Osborne Mendel rats (200–300 g) were given β-carotene (Hoffman-La Roche; 10% w/w beadlets) in the diet at 10 000 mg/kg feed (equivalent to 1000 mg/kg bw per day) for 17 weeks. A control group received the test diet without the addition of beadlets. Three rats from one β-carotene-treated group were killed at each of 3, 4, 5, 6 and 8 weeks, and rats in the remaining β-carotene-treated groups were killed at 14 or 17 weeks. Serum and skin samples were collected for analysis of β-carotene. Mean serum concentrations of β-carotene were 25.7 ± 11.8 ng/mL, 70.0 ± 23.0 ng/mL, 65.7 ± 12.8 ng/mL and 34.0 ± 7.3 ng/mL at 3, 8, 14 and 17 weeks, respectively. β-Carotene levels in skin generally increased in a time-dependent manner from 46.2 ng/g at 3 weeks to 134.8 ng/g at 17 weeks (Wamer, Giles & Kornhauser, 1985).

Male Sprague Dawley rats (n = 2–4; 10 months old) were administered β-carotene (Sigma Chemicals, St Louis, Missouri, USA; purity not stated) in the diet for 2 weeks to achieve a dose of 0, 4 or 20 mg/kg bw per day. At the end of the study period, rats were killed, and serum, liver and adipose tissue samples were analysed for β-carotene, retinol and retinyl esters. β-Carotene was not detected in the serum of control rats. Mean serum β-carotene concentrations were 0.006

Table 1

Plasma concentrations of β-carotene in male and female rats administered β-carotene in a 13-week oral toxicity study

Dose (mg/kg bw per day)	Sex	Mean plasma β-carotene concentration ± SD (µg/mL)				
		Week 2	Week 7	Week 12	Week 17	Week 22
0 (placebo control)	Males	ND	ND	ND	ND	ND
	Females	ND	ND	ND	ND	ND
250	Males	0.47 ± 0.28	0.39 ± 0.22	0.45 ± 0.21	0.01 ± 0.01	ND
	Females	0.53 ± 0.39	0.86 ± 0.61	0.83 ± 0.71	0.02 ± 0.02	ND
500	Males	0.54 ± 0.35	0.37 ± 0.27	0.52 ± 0.35	0.02 ± 0.02	ND
	Females	0.35 ± 0.15	0.68 ± 0.34	0.68 ± 0.47	0.02 ± 0.02	ND
1 000	Males	0.43 ± 0.20	0.32 ± 0.16	0.51 ± 0.22	0.03 ± 0.02	ND
	Females	0.54 ± 0.15	0.78 ± 0.21	0.73 ± 0.30	0.03 ± 0.02	ND

bw: body weight; ND: not detected; SD: standard deviation
Source: Buser & Arceo (1995)

µg/mL and 0.005 µg/mL in the 4 and 20 mg/kg bw per day groups, respectively. β-Carotene was not detected in the liver or adipose tissue of control or treated animals (Ribaya-Mercado et al., 1989).

In a 13-week toxicity study, Wistar rats (20 of each sex per group) were administered β-carotene (Hoffman-La Roche; present in a water-soluble formulation at approximately 10%) in the diet at a target dose of 0, 250, 500 or 1000 mg/kg bw per day, followed by a recovery period. One control group was given a placebo admixture, whereas a second, standard control group received feed alone. Plasma concentrations of β-carotene were assessed in six rats of each sex per group during weeks 2, 7 and 12 of the treatment period and weeks 3 and 8 of the recovery period (study weeks 17 and 22; Table 1). In general, plasma β-carotene concentrations in males and females were similar at 2 weeks. Plasma levels in males remained generally stable at weeks 7 and 12, whereas levels in females appeared to increase until 7 weeks and then plateau. Substantial elimination of plasma β-carotene was observed after a 3-week recovery period; by week 22, β-carotene concentrations were below the limit of detection in all animals. Tissue levels of β-carotene were not investigated. An orange-red discolouration of the faeces was observed in animals in the 500 and 1000 mg/kg bw per day groups. A reversible orange-yellow discolouration in the adipose tissue and liver was reported in females in each of the β-carotene treatment groups (Buser & Arceo, 1995).

Sprague Dawley rats (three of each sex per group; 4 months of age) were administered β-carotene (Hoffmann-La Roche; 10% w/w beadlets) in the diet at 0 (placebo beadlets), 1000 or 10 000 mg/kg feed for 8 weeks. After this period, rats were given a single dose of 0.74 MBq (0.5 mg) [^{14}C]β-carotene in olive oil by

oral gavage. Blood was taken at 0, 4, 8, 24, 48 and 72 hours, and radioactivity was measured in the plasma. Radioactivity in the plasma of placebo beadlet controls peaked at 4 hours and then rapidly declined. Over 95% of the radioactivity in the plasma was detected in the retinol fraction following saponification, whereas none was associated with β-carotene. Radioactivity in the liver was mainly detected as retinol (88–94%) following saponification and inversely related to the dose administered in the feed. Small amounts of radioactivity were recovered in the liver as β-carotene (Krinsky et al., 1990).

Male Sprague Dawley weanling rats were fed a retinol-deficient diet for 60 days. At the end of the period, groups of six depleted rats were fed for 7 days with a retinol-deficient diet that was supplemented with retinol at 7.5 mg/kg feed (equivalent to 3750 μg/kg bw per day), synthetic all-*trans*-β-carotene at 12, 29 or 48 mg/kg feed (equivalent to 600, 1450 and 2400 μg/kg bw per day, respectively), dry algae providing β-carotene at 29, 58 or 112 mg/kg feed (equivalent to 1450, 2900 and 5600 μg/kg bw per day, respectively) or an algal oil extract providing β-carotene at 16 mg/kg feed (equivalent to 800 μg/kg bw per day). An additional control group received no supplementation. At the end of the treatment period, the rats were killed and their livers removed for analysis of retinol (and retinyl esters), retinol isomers and β-carotene. In the control group, concentrations of β-carotene, retinol and retinyl esters in liver were below the limit of detection. A dose-related increase in the liver concentrations of retinol (and retinyl esters) was observed in retinol- and all β-carotene-treated groups. β-Carotene was detected in the liver at concentrations of 1.0, 22.1, 27.5 and 12.8 μg/g wet weight in the retinol (7.5 mg/kg feed), synthetic all-*trans*-β-carotene (29 mg/kg feed), dry algal extract (β-carotene at 29 mg/kg feed) and algal oil extract groups (β-carotene at 16 mg/kg feed), respectively. Blood was not analysed for retinol or β-carotene (Ben-Amotz, Mokady & Avron, 1988).

(b) Ferrets

Female 6- to 12-month-old ferrets (*n* = 2–3 per group) were administered β-carotene (Sigma Chemicals, St Louis, Missouri, USA; purity not stated) in the diet for 2 weeks to achieve a dose of 0, 4 or 20 mg/kg bw per day. At the end of the study period, the animals were fasted for 7–15 hours and killed. Mean serum β-carotene concentrations were 0.006 μg/mL, 0.153 μg/mL and 0.415 μg/mL in the control, 4 and 20 mg/kg bw per day groups, respectively. Increased concentrations of β-carotene were also observed in the liver of treated groups compared with control animals. Liver retinyl ester concentrations appeared higher in the β-carotene-treated groups but were not statistically significantly different, probably as a result of the small group sizes (Ribaya-Mercado et al., 1989).

Male ferrets (n = 4–6 per group, 1234 ± 98 g) were given β-carotene (Hoffmann-La Roche; β-carotene beadlets) in the diet at 80 μg/g feed (equivalent to 12.6 mg/kg bw per day) for 3 weeks. A control group received the test diet only, without the addition of beadlets. Serum β-carotene concentrations increased from a mean of 0.006 μg/mL before feeding to 3.1 μg/mL after 3 weeks on the test diet. In tissues, levels of β-carotene were reported in the following organs/tissues, in descending order from highest to lowest: liver >> adrenal, small intestine, stomach, colon > kidney > muscle, bladder, adipose tissue, lung, skin > brain and eye. Retinol levels in the liver of treated animals were approximately 5-fold higher than in the liver of controls, although the differences were not statistically significant (Ribaya-Mercado et al., 1992).

In serum of ferrets treated with β-carotene in the diet (80 μg/g wet feed) for 3 weeks, most β-carotene was associated with high-density lipoprotein, with smaller amounts associated with low-density lipoprotein, very low density lipoprotein and lipoprotein-deficient serum (Ribaya-Mercado et al., 1993).

Twelve fasted male ferrets were fed a single dose of β-carotene (Hoffmann-La Roche; 10% β-carotene beadlets) at 10 mg/kg bw and killed at 8 hours, 16 hours, 3 days or 11 days. A control group that did not receive β-carotene was killed at 3 days. Blood and tissue samples were taken for analysis of β-carotene. A peak mean β-carotene concentration of 0.97 μg/mL was observed in serum between 7 and 8 hours. Liver showed the highest levels of β-carotene, followed by kidney and spleen. Lower levels of β-carotene were found in lung, muscle and adipose tissue. No statistically significant effects on tissue vitamin A levels were observed (Gugger et al., 1992).

(c) Rhesus monkeys

Five adult female rhesus monkeys were given 1.85 MBq (1.26 mg) of [^{14}C]-β-carotene in olive oil by oral gavage. Radioactivity was measured in the plasma at 0, 1, 2, 4, 8, 24, 48 and 72 hours. Radioactivity was detected in the plasma at 4 hours, with peak levels observed between 8 and 24 hours after dosing. Upon fractionation, radioactivity was recovered associated with retinol and also with β-carotene. In the analysed organs, liver contained most of the radioactivity, mainly as retinol (85–95%), with smaller amounts recovered as β-carotene or other unidentified fractions. Some radioactivity was detected in other organs; in general, however, insufficient counts were obtained to allow fractionation by high-performance liquid chromatography (HPLC) (Krinsky et al., 1990).

(d) Humans

Although the mechanism of intestinal β-carotene absorption and metabolism appears to be comparable in animal models and humans, marked differences in

cleavage rates and consequently bioavailability between species have been shown (Ribaya-Mercado et al., 1992; Van Vliet, Schreurs & Van den Berg, 1995; During, Albaugh & Smith, 1998; During et al., 1999, 2001; Woutersen et al., 1999).

The activity of β-carotene-15,15′-dioxygenase was measured in a study using two different human cell lines and human small intestine and liver preparations. β-Carotene-15,15′-dioxygenase activities in small intestinal mucosal preparations were 97.4 ± 39.8 pmol/h per milligram protein in preparations from five adults (44–89 years old) and 20 pmol/h per milligram protein in a preparation from an infant (17 months old). No activity was detected in adult stomach tissue. The β-carotene-15,15′-dioxygenase activities in a subcellular preparation of human liver were reported to be 62 pmol/h per milligram protein in normal adult liver and 7 pmol/h per milligram protein for a liver exhibiting gross pathology. The maximum capacity of β-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′-β-carotene:retinal cleavage ratio of 1:2 (During, Albaugh & Smith, 1998; During et al., 2001).

In an early study, [³H]β-carotene dissolved in 2 mL olive oil and emulsified in 50 mL skim milk was fed to two patients who had polyethylene cannulae inserted into the thoracic duct of the neck to collect lymph. Absorption of radioactivity occurred mainly between 3 and 10 hours and accounted for approximately 9–17% of the administered radioactivity in the two patients. The radiolabel was mainly associated with chylomicrons and present as labelled retinyl esters (60–70%). Labelled β-carotene was reported to comprise 20–30% of the absorbed radioactivity after ingestion of [³H]β-carotene (Goodman et al., 1966).

In a similarly designed study, [³H]β-carotene ((Hoffman-La Roche; specific activity 39.6 MBq/mg) or [³H-¹⁴C]β-carotene (specific activity 0.56 MBq/mg) in vegetable oil was fed to four patients who had polyethylene cannulae inserted into the thoracic duct in the neck to collect lymph. About 9–17% of the administered radioactivity was recovered via the lymphatic pathway in three patients, with the main peak observed between 4 and 8 hours after feeding. Recovered radioactivity was mainly present as retinyl esters (68–88%), with smaller amounts of radioactivity recovered as β-carotene (2–28%). However, a different profile was observed in the fourth patient, in whom approximately 90% of recovered radioactivity in the lymph was associated with β-carotene. The authors speculated that this patient may have had a block in the enzymatic processes involved in the conversion, but no follow-up observations were conducted (Blomstrand & Werner, 1967).

A 41-year-old healthy male was placed on a low-carotenoid diet for 48 hours and then orally administered 1.0 mg [¹³C]β-carotene as an emulsion with a light breakfast. Plasma was analysed for [¹³C]β-carotene, [¹³C]retinol and [¹³C]-

retinyl esters for 22 hours after dosing. The three analytes labelled with the stable isotope ^{13}C were detected by 3 hours; at 5 hours, the molar ratio of the labelled species of retinyl ester:retinol:β-carotene was 4.5:1.5:1, respectively. After 9 hours, administered ^{13}C was recovered in the plasma mainly associated with retinol, with small amounts of labelled β-carotene and retinyl esters also reported. The authors concluded that most of the absorbed dose of β-carotene is converted to vitamin A, with retinyl esters predominating (Parker et al., 1993).

[^{14}C]β-Carotene (306 µg; 7.4 kBq; radioactivity and chemical purity >98%) was orally administered as a single dose to a 35-year-old, non-smoking male in good health. Concentrations of [^{14}C]β-carotene, [^{14}C]retinyl esters, [^{14}C]-retinol and [^{14}C]retinoic acids were measured in the plasma using accelerator mass spectrometry. Mass balance of the stool samples over 2 days indicated that approximately 40% of the radioactivity was absorbed. Radioactivity was detected in the plasma 5.5 hours after dosing. Similar kinetic profiles involving a number of local maxima were observed for radiolabelled β-carotene and retinyl esters over the first 24 hours. The peak concentration (C_{max}) for both analytes was observed 21.3 hours after treatment. For [^{14}C]β-carotene, the terminal decay slope indicated an elimination half-life of 40 days and a turnover time of 58 days. [^{14}C]-Retinol concentrations began to rise at 5.5 hours and, after an initial absorption peak between 8.5 and 10 hours, rose linearly to peak at 28 hours. Disappearance of retinol from plasma was biexponential after day 7. The terminal slope phase for [^{14}C]retinol was not apparent until approximately 77 days post-dosing, and a half-life of 209 days was estimated. The radiolabel was mainly excreted in the faeces, with smaller amounts of radioactivity recovered in the urine (Dueker et al., 2000).

A healthy male volunteer, 30 years of age, was given a single oral dose of [^{14}C]β-carotene (543 ng; 3.7 kBq) with a milkshake. Blood samples were drawn prior to dosing, at regular intervals until 36 hours and at various times until day 166 for analysis of ^{14}C by accelerator mass spectrometry. It was estimated that approximately 65% of the radiolabel was absorbed, on the basis that about 35% of the radioactivity was recovered in the first collection of faeces. The radioactivity profile in the plasma occurred as four peaks from 0.15 to 3 days after dosing. Radioactivity recovered as retinyl esters reached a peak at 0.15 day then declined, probably representing uptake by the liver. A peak associated with retinol began to rise after about 0.2 day, possibly due to retinol secretion from the liver associated with retinol binding protein. Radioactivity associated with β-carotene occurred as two broad peaks. A peak tentatively identified as β-apo-8′-carotenal was found in the plasma on the third day after dosing. Approximately 16% of the radioactivity was eliminated in the urine (Ho et al., 2007).

Eight healthy adults, aged 36 ± 8 years, were administered a single oral dose of [^{14}C]β-carotene (Hoffmann-La Roche; 1.01 nmol; 3.7 kBq) with a

milkshake. Blood samples were drawn prior to dosing, at regular intervals until 36 hours and at various times until day 166 for analysis of ^{14}C by accelerator mass spectrometry. Elimination of ^{14}C via faeces and urine was measured for mass balance calculations. The apparent absorption was estimated as 47% ± 13%, on the basis that 53% ± 13% of the radiolabel was eliminated in the faeces over the first 7.5 days. Radioactivity was detected in plasma at 0.04 day, with a C_{max} reached at 0.3 day (T_{max}). The ^{14}C plasma profiles for β-carotene were determined for three subjects. Plasma [^{14}C]β-carotene appeared as two peaks: the first at 0.1–0.25 day, and a second broad peak between days 1 and 3. The radioactivity associated with retinyl esters in the plasma occurred as two peaks, one at 0.3 day and the second at about 0.6 day, which was 3.5 times greater than that for β-carotene. A single peak of radioactivity was observed for retinol. Mean elimination of radioactivity in the urine was approximately 6.5% (Ho et al., 2009).

The metabolism and absorption of 20 mg [^{13}C]β-carotene (Euriso-top Saint Aubin, France; chemical purity >97%; stable isotope purity 99%) were studied in healthy men who were intragastrically and intraduodenally intubated. The men were randomly assigned to consume a lipid-rich control meal ($n = 3$) or a lipid-rich test meal ($n = 7$). Digesta and blood were collected, and extracts were analysed for ^{13}C-labelled β-carotene, retinol, retinyl palmitate, β-apo-carotenals, β-apo-carotenols or β-apo-carotenoic acids. Digesta contained low levels of β-apo-carotenals. Although anticipated retinoids were found in the blood of subjects consuming [^{13}C]β-carotene, no asymmetric β-apo-carotenals, β-apo-carotenols or β-apo-carotenoic acids were observed in the plasma or triglyceride-rich lipoprotein samples of treated subjects (Kopec et al., 2018).

Thirty-two adult subjects (12 males; 20 females) between the ages of 18 and 60 were placed on a low-carotene diet for 10 days, followed by a 7-day treatment phase with β-carotene and then another low-carotene diet period. Subjects were divided into five groups, consuming 1) 24 mg β-carotene (three capsules per day; algal extract from *D. salina*), 2) 24 mg β-carotene (207.3 g carrots per day), 3) 8 mg β-carotene (one capsule per day; algal extract from *D. salina*), 4) 8 mg β-carotene (69.1 g carrots per day) or 5) 0 mg β-carotene (three placebo capsules). Following the depletion period, mean serum β-carotene concentrations ranged from 0.11 to 0.13 μg/mL in the five treatment groups. Mean serum concentrations of β-carotene at the end of the treatment phase were 0.38, 0.28, 0.26, 0.22 and 0.10 μg/mL, respectively. Serum β-carotene concentrations declined during the final depletion phase in all treated groups. No significant differences in serum retinyl ester concentrations were seen during the study period (Jensen et al., 1985).

Sixteen healthy adults (six males; 10 females), aged 18–60 years, were placed on a 10-day low-carotene diet. Subjects were then randomly divided into three groups consuming a single dose of 1) three β-carotene capsules (algal extract

from *D. salina* containing 24 mg β-carotene in corn oil), 2) 207.3 g carrots (24 mg β-carotene) or 3) three β-carotene-free placebo capsules for 7 days. Serum all-*trans*-β-carotene concentrations increased from a mean of approximately 0.08 to 0.44 μg/mL in β-carotene capsule–treated subjects and from 0.11 to 0.35 μg/mL in subjects who consumed carrots. Serum *cis*-β-carotene concentrations were also reported to be slightly increased; however, the *trans*:*cis* ratio was approximately 9:1. No significant increase in serum β-carotene concentration was observed in placebo-treated subjects (Jensen et al., 1987).

β-Carotene (Hoffman-La Roche) was given to 34 healthy male and female subjects, aged 20–65 years, in capsules at a dose of 0, 15, 30, 45, 90 or 150 mg as part of a phase 1 trial to evaluate plasma kinetics and toxicity. Three dosing schedules were used: a single dose, a 3-day loading dose and a daily dose for 30 days. No signs of toxicity or yellowing of the skin were observed in any of the participating subjects. Marked interindividual variation in plasma β-carotene concentrations was observed in subjects administered the single doses. Peak plasma concentrations in subjects were generally observed between 6 and 24 hours, and the time to reach baseline was 4–12 days. Subjects who received β-carotene at doses of 30–150 mg for 3 consecutive days generally showed dose-related increases in plasma β-carotene compared with baseline levels. However, there remained some significant variation between individuals at the 90 and 150 mg doses. Plasma levels of three subjects who received 45 mg β-carotene for 30 days showed peak β-carotene concentrations of approximately 1.5–3.0 μg/mL, which then declined gradually at the end of dosing. Retinol concentrations in plasma were not altered in subjects who received single, loading or daily doses of β-carotene (Dimitrov et al., 1986).

Sixty-one healthy male and female volunteers, aged 21–63 years, were given β-carotene (Solatene®; Hoffmann-La Roche) in capsules according to a number of different dosing and dietary regimens lasting up to 8 weeks: 15 or 45 mg/day for 8 weeks, 45 mg/day for 3 weeks (high-fat), 45 mg/day for 3 weeks (low-fat), 45 mg every fifth day for 4 weeks, 90 mg every sixth day for 4 weeks, 45 mg loading for 2 weeks followed by weekly maintenance for 5 weeks, or placebo. Yellowing of the skin was observed in two participants after ingestion of 45 mg of β-carotene per day for 3 weeks; however, no adverse signs of toxicity clearly attributable to treatment were observed. Marked individual differences in plasma β-carotene concentrations were observed in subjects who received 15 or 45 mg of β-carotene daily for 8 weeks. A return to baseline was reached between 10 and 30 days after discontinuation of dosing. Individuals administered 45 mg β-carotene per day and maintained on a high-fat diet had higher plasma β-carotene concentrations than those on low-fat diets from day 2 through days 34–37. Plasma retinol levels did not change throughout the studies, independent of the dose and dosing schedule (Dimitrov et al., 1988).

β-Carotene concentrations were measured in the serum of 300 male participants, aged 50–75 years, enrolled in a pilot study to assess the feasibility of conducting a lung cancer prevention trial. The men were orally administered β-carotene (Hoffman-La Roche) in capsule form at a dose of 15 mg/day or a placebo daily for 10 months. Average serum β-carotene concentrations were 0.16 μg/mL at baseline and had increased approximately 10-fold to 1.7 μg/mL at 10 months. Serum total carotenoid concentrations approximately doubled from baseline levels over the course of the study. Retinol concentrations in serum were not affected by treatment. Yellowing of the skin was not observed in any subject, and there were no reported adverse effects (Constantino et al., 1988).

The relationship between plasma β-carotene concentration and variables including age, sex and baseline plasma β-carotene concentration was investigated in 582 subjects (403 men, 179 women; aged 29–84 years) who received 50 mg of β-carotene (BASF; 10% dry powder) in a capsule per day for 1 year as part of a randomized controlled trial of supplementation with β-carotene to prevent skin cancer recurrence. Median plasma β-carotene concentrations increased from 0.18 μg/mL at baseline to 1.7 μg/mL at 1 year; however, significant intersubject variability was observed in response to the fixed dose. A multivariate analysis showed that plasma β-carotene concentration prior to dosing was the best predictor of the increase in plasma levels after dosing. Other variables, including smoking status, sex and subject leanness, accounted for relatively little of the variability in subjects' plasma β-carotene response. No adverse effects were reported (Nierenberg et al., 1991).

2.1.2 β-Apo-8′-carotenal

(a) Rats

Five male Wistar rats, aged 9–10 weeks, were given a single oral dose of [6,7-^{14}C]-β-apo-8′-carotenal (3.0 MBq/mg) formulated as beadlets by oral gavage at a dose of 1.3 mg/kg bw. One animal was killed 3 hours after dosing for collection of a terminal blood sample. The remaining animals were placed individually into metabolism cages, and blood samples (about 0.3 mL) were taken at 1, 2, 4, 6, 8, 10 and 24 hours following dosing. Urine and faeces were collected from 0 to 24 hours and from 24 to 48 hours post-dosing. Animals were killed at 48 hours, and a terminal blood sample was collected.

Radiolabelled β-apo-8′-carotenal and its metabolites were at least 25% absorbed from the gastrointestinal tract of rats. Total radioactivity in plasma reached a C_{max} of 342 ng β-apo-8′-carotenal equivalents per gram after 10 hours (T_{max}). Radioactivity was eliminated from the plasma with a half-life of 21 hours. In the tissues, radioactivity was mainly recovered in the liver and kidney (1598 and 1139 ng β-apo-8′-carotenal equivalents per gram, respectively), with

Fig. 1
Metabolic pathway of β-apo-8′-carotenal in rats

lower concentrations recovered in the small intestine, colon, fat and stomach. Radioactivity was mainly eliminated via faeces (49.4%), with smaller amounts excreted in the urine (14.6%). In the plasma of the animal terminated at 3 hours after dosing, at least six metabolite fractions were separated by HPLC. β-Apo-8′-carotenal, β-apo-8′-carotenol, β-apo-8′-carotenoic acid and fatty acid conjugates represented 5.8%, 11.0%, 17.6% and 13.9% of the plasma radioactivity, respectively. In the liver, retinol and fatty acid conjugates of retinol represented 16.2% and 43.2% of the radioactivity recovered in the apolar fraction. The major radioactive residues eliminated via faeces were identified as β-apo-8′-carotenal, representing 18% of the dose applied, β-apo-8′-carotenoic acid (8%) and β-apo-8′-carotenol (1%). In urine, at least 13 radioactive polar metabolite fractions were characterized, each representing up to 2% of the dose applied (Rümbeli, Ringenbach & Elste, 2007).

A metabolic pathway was proposed (Fig. 1).

Groups of 10 male and 10 female Sprague Dawley rats (6 weeks of age) were administered β-apo-8′-carotenal (Apocarotenal 10% WS/N; containing

Table 2

Mean concentrations of β-apo-8′-carotenal and three metabolites in the plasma at weeks 13/14 in a 13-week dietary toxicity study of β-apo-8′-carotenal in rats

Analyte	Mean concentration ± standard deviation (µg/mL)				
	0 mg/kg bw per day (standard control)	0 mg/kg bw per day (placebo control)	10 mg/kg bw per day	30 mg/kg bw per day	100 mg/kg bw per day
Males					
β-Apo-8′-carotenal	ND	ND	0.21 ± 0.09	0.25 ± 0.10	0.58 ± 0.27
β-Apo-8′-carotenol	ND	ND	0.06 ± 0.01	0.13 ± 0.03	0.30 ± 0.09
β-Apo-8′-carotenoic acid	ND	ND	0.49 ± 0.10	1.02 ± 0.13	1.85 ± 0.53
Polar metabolite	ND	ND	1.56 ± 0.26	3.03 ± 0.51	5.09 ± 1.29
Females					
β-Apo-8′-carotenal	ND	ND	0.13 ± 0.08	0.26 ± 0.11	0.66 ± 0.25
β-Apo-8′-carotenol	ND	ND	0.09 ± 0.04	0.23 ± 0.06	0.61 ± 0.13
β-Apo-8′-carotenoic acid	ND	ND	0.63 ± 0.16	1.75 ± 0.58	3.54 ± 0.77
Polar metabolite	ND	ND	2.21 ± 0.49	5.39 ± 1.43	9.89 ± 2.29

bw: body weight; ND: not detected
Source: Edwards et al. (2007)

β-apo-8′-carotenal at 116 g/kg, β-carotene at 3.2 g/kg, crocentindialdehyde at 0.42 g/kg) in the diet at a dose of 0 (standard control), 0 (placebo control), 10, 30 or 100 mg/kg bw per day for 90 days. Additional groups of five male and five female rats were treated at 0 (standard control), 0 (placebo control) or 100 mg/kg bw per day for 13 weeks, followed by a 4-week recovery period. Mean plasma concentrations of β-apo-8′-carotenal were similar for males and females at each respective dose at day 2 and weeks 8 and 13/14 (values for weeks 13/14 are shown in Table 2). However, a clear sex difference was identified, with the concentrations of β-apo-8′-carotenol, β-apo-8′-carotenoic acid and an unidentified polar metabolite generally 2- to 3-fold higher in females. β-Apo-8′-carotenal and its metabolites were virtually cleared from the plasma during the recovery period in both sexes (data not shown). In liver samples, mean concentrations for β-apo-8′-carotenal were 3- to 8-fold higher in females than in males at weeks 13/14 (Table 3). Liver levels of β-apo-8′-carotenal and its metabolites remained markedly elevated in females following the recovery period. Total liver retinol levels were increased approximately 2- to 3-fold in all treated groups; however, there was no clear relationship with dose (Edwards et al., 2007).

In a developmental toxicity study, groups of 25 mated female Sprague Dawley rats were administered β-apo-8′-carotenal (Apocarotenal 10% WS/N; β-apo-8′-carotenal at 116 g/kg, β-carotene at 3.2 g/kg, crocentindialdehyde at 0.42 g/kg) by incorporation of beadlets in the diet from gestation days (GDs) 6

Table 3

Mean concentrations of β-apo-8′-carotenal and three metabolites in the liver in a 13-week dietary toxicity study of apo-8′-carotenal in rats

Analyte	Mean concentration ± standard deviation (µg/mL)				
	0 mg/kg bw per day (standard control)	0 mg/kg bw per day (placebo control)	10 mg/kg bw per day	30 mg/kg bw per day	100 mg/kg bw per day
Males					
β-Apo-8′-carotenal	ND	ND	1.32 ± 0.39	4.33 ± 1.66	10.77 ± 4.87
β-Apo-8′-carotenol	ND	ND	1.37 ± 0.24	2.85 ± 0.94	5.12 ± 1.97
β-Apo-8′-carotenoic acid	ND	ND	5.10 ± 1.19	13.75 ± 4.69	24.44 ± 10.70
Polar metabolite	ND	ND	3.41 ± 0.54	6.74 ± 1.28	8.84 ± 2.39
Females					
β-Apo-8′-carotenal	ND	ND	3.78 ± 1.47	14.08 ± 5.33	84.35 ± 30.32
β-Apo-8′-carotenol	ND	ND	3.02 ± 1.18	7.39 ± 1.92	18.78 ± 4.99
β-Apo-8′-carotenoic acid	ND	ND	8.62 ± 3.00	30.02 ± 9.11	148.99 ± 87.05
Polar metabolite	ND	ND	5.47 ± 0.96	11.02 ± 1.58	20.20 ± 4.31

bw: body weight; ND: not detected
Source: Edwards et al. (2007)

to 20 at a concentration of 2064, 10 344 or 51 720 mg/kg feed (equal to 20, 100 and 495 mg/kg bw per day, respectively). One control group was administered placebo beadlets, and a second was given standard diet. Animals were killed on GD 20, at which time plasma and liver samples were collected from five animals per group for measurement of β-apo-8′-carotenal, β-apo-8′-carotenol, β-apo-8′-carotenoic acid and a polar metabolite. No β-apo-8′-carotenal or any of the three metabolites were detected in plasma or liver samples in either of the control groups (Tables 4 and 5). β-Apo-8′-carotenal and the three metabolites were detected in plasma and liver in all three treated groups, with the concentrations increasing with dose (Loget et al., 2006).

(b) **Humans**

Six healthy adult male subjects were given a single oral dose of 41 mg β-apo-8′-carotenal in peanut oil as part of a light breakfast. Blood samples were taken at 0, 2, 5, 8, 11.5, 15.5, 24 and 32 hours and then at 2, 4, 6, 8 and 17 days after dosing. Serum was analysed by HPLC for serum carotenoids and retinol. It was reported that β-apo-8′-carotenal did not appear in plasma in significant amounts (data not shown). β-Apo-8′-carotenol and β-apo-8′-carotenyl palmitate were identified as the two major metabolites in the plasma and reached their maximum concentrations of 0.29 and 0.23 µmol/L at 11 and 6 hours, respectively. 3-Apo-8′-carotenoic acid was also detected in serum, but not quantified. It was

Table 4

Mean plasma concentrations of β-apo-8′-carotenal and three metabolites in pregnant female Sprague Dawley rats administered β-apo-8′-carotenal

Analyte	Mean concentration ± standard deviation (μg/mL)				
	0 mg/kg bw per day (standard control)	0 mg/kg bw per day (placebo control)	20 mg/kg bw per day	100 mg/kg bw per day	495 mg/kg bw per day
β-Apo-8′-carotenal	ND	ND	0.32 ± 0.05	1.68 ± 0.36	2.58 ± 0.43
β-Apo-8′-carotenol	ND	ND	0.19 ± 0.05	0.98 ± 0.12	1.62 ± 0.26
β-Apo-8′-carotenoic acid	ND	ND	1.10 ± 0.22	4.27 ± 0.42	5.69 ± 1.22
Polar metabolite	ND	ND	3.69 ± 1.06	11.12 ± 1.89	13.17 ± 2.73

bw: body weight; ND: not detected
Source: Loget et al. (2006)

Table 5

Mean liver concentrations of β-apo-8′-carotenal and three metabolites in pregnant female Sprague Dawley rats administered β-apo-8′-carotenal

Analyte	Mean concentration ± standard deviation (μg/mL)				
	0 mg/kg bw per day (standard control)	0 mg/kg bw per day (placebo control)	20 mg/kg bw per day	100 mg/kg bw per day	495 mg/kg bw per day
β-Apo-8′-carotenal	ND	ND	10.16 ± 3.31	66.88 ± 31.52	126.37 ± 28.18
β-Apo-8′-carotenol	ND	ND	5.40 ± 2.28	19.93 ± 5.58	31.01 ± 7.12
β-Apo-8′-carotenoic acid	ND	ND	22.98 ± 7.49	82.34 ± 26.12	114.39 ± 20.72
Polar metabolite	ND	ND	9.29 ± 1.60	21.41 ± 3.84	24.72 ± 4.53

bw: body weight; ND: not detected
Source: Loget et al. (2006)

stated that retinyl palmitate was slightly, but statistically significantly, elevated in all subjects at 12–32 hours (data not shown). Concentrations of β-carotene, lycopene and other endogenous carotenoids did not change detectably (Zeng, Furr & Olson, 1992).

2.2 **Toxicological studies**

2.2.1 **Acute toxicity**

(a) **β-Carotene**

The oral median lethal dose (LD_{50}) of β-carotene was greater than 2000 mg/kg bw in rats and greater than 8000 mg/kg bw in dogs (Table 6).

Table 6
Acute toxicity of β-carotene

Species	Sex	Test substance	Route	LD$_{50}$ (mg/kg bw)	Reference
Rat	Male and female	β-Carotene (purity 99.3%)	Oral	>2 000	Buser (1992)
Rat	Male and female	β-Carotene (purity 98.2%)	Oral	>2 000	Strobel (1994)
Rat	Male and female	β-Carotene biomass from *B. trispora*	Oral	>2 000	Kluifthooft (2001)[a]
Dog	Not specified	β-Carotene	Oral	>8 000	Nieman & Klein Obbink (1954)[a]

bw: body weight; LD$_{50}$: median lethal dose
[a] Previously considered by JECFA.

Table 7
Acute toxicity of β-apo-8′-carotenal

Species	Sex	Test substance	Route	LD$_{50}$ (mg/kg bw)	Reference
Mouse	Not stated	β-Apo-8′-carotenal (purity not stated)	Oral	>10 000	Anonymous (1966)[a]
Rat	Female	10% β-apo-8′-carotenal	Oral	>232[b]	Loget & Arcelin (2006a)
Rat	Female	10% β-apo-8′-carotenal	Oral	>2 000	Loget & Arcelin (2006b)

bw: body weight; LD$_{50}$: median lethal dose
[a] Previously considered by JECFA.
[b] Test article was administered at 2000 mg/kg bw, corresponding to a β-apo-8′-carotenal dose of 232 mg/kg bw.

(b) **β-Apo-8′-carotenal**

The oral LD$_{50}$ of β-apo-8′-carotenal was greater than 10 000 mg/kg bw in mice and greater than 2000 mg/kg bw in rats (Table 7).

2.2.2 **Short-term studies of toxicity**
(a) **β-Carotene**

Groups of five male and five female Wistar rats were administered β-carotene derived from *B. trispora* (Gist-Brocades B.V.; purity 86.8%) at a dietary concentration of 0, 2, 10 or 50 g/kg feed for 28 days (equal to 0, 150, 765 and 3809 mg/kg bw per day for males and 0, 162, 817 and 4175 mg/kg bw per day for females, respectively). Additional groups were fed a diet containing β-carotene at 50 g/kg feed (equal to 4038 mg/kg bw per day for males and 4155 mg/kg bw per day for females) from a different batch (purity 98.3%) or a diet containing β-carotene at 50 g/kg feed (equal to 3797 mg/kg bw per day for males and 4083 mg/kg bw per day for females) from a commercial supplier (purity 97.4%). No deaths occurred during the study, and clinical signs were limited to orange discolouration of the fur and furless parts of the skin in animals given diets containing β-carotene at 10 or 50 g/kg feed. Orange discolouration of the gastrointestinal contents of nearly all animals fed β-carotene was also observed. No treatment-related adverse

effects on body weight, feed consumption, haematology, clinical chemistry or organ weights were observed. Females given β-carotene from a commercial supplier showed a slight, but statistically significant, increase in the plasma level of phospholipids compared with controls. No treatment-related lesions were found on gross macroscopic or histopathological examination. The NOAEL in this study for β-carotene derived from *B. trispora* was 50 g/kg feed (equal to 3809 mg/kg bw per day), the highest dietary concentration tested (Van Beek, Catsburg & Wijnans, 1997).

In a good laboratory practice (GLP)– and guideline-compliant study, groups of 10 male and 10 female F344/DuCrj rats were administered β-carotene (San-Ei Gen F.F.I., Inc.; purity 97.4% as β-carotene from *B. trispora*) in the diet at 0, 2000, 10 000 or 50 000 mg/kg feed (equal to 0, 118, 581 and 3127 mg/kg bw per day for males and 0, 126, 663 and 3362 mg/kg bw per day for females, respectively) for 90 days. No deaths occurred during the study period. Red discolouration of the faeces was observed in all treated groups, and red staining of the fur occurred in the 10 000 and 50 000 mg/kg feed treatment groups. There were no treatment-related adverse effects on body weight, feed and water consumption, urine analysis, haematology, serum biochemistry, ophthalmology or organ weights. Gross macroscopic and histopathological examinations did not reveal any treatment-related lesions. The NOAEL was 50 000 mg/kg feed (equal to 3127 mg/kg bw per day), the highest dietary concentration tested (Nabae et al., 2005).

Groups of 20 male and 20 female Wistar rats (age not stated) were administered β-carotene (Hoffman-La Roche; present in a water-soluble formulation at approximately 10%) in the diet at a target dose of 0, 250, 500 or 1000 mg/kg bw per day (mean achieved intakes were 0, 253, 507 and 1016 mg/ kg bw per day for males and 0, 253, 507 and 1013 mg/kg bw per day for females, respectively) for 13 weeks. The GLP status of this study was not reported. One control group was given a placebo admixture, whereas a second, standard control group received feed alone. At the end of the treatment period, 14 rats of each sex per group were killed and underwent gross and histopathological examinations. The remaining six rats of each sex per group served as recovery animals and were fed a diet containing placebo formulation for a further 8 weeks prior to necropsy. Plasma levels of β-carotene were assessed during weeks 2, 7 and 12 of the treatment period and weeks 3 and 8 of the recovery period. The plasma measurements are reported in section 2.1. There were no treatment-related clinical signs except for an orange-red discolouration of the faeces from animals in the mid- and high-dose groups. No treatment-related mortalities or adverse effects on body weight gain, feed consumption, ophthalmoscopy, haematology, clinical chemistry, urine analysis or organ weights were reported. At necropsy, a slight to moderate orange-yellow discolouration of the adipose tissue and/or liver

was observed in a number of females from all treated groups. At the end of the recovery period, this discolouration was no longer observed, with the exception of one female in each of the low- and mid-dose groups. No treatment-related histopathological changes were observed in any tissue. The authors considered that the absence of histopathological findings in the liver correlating with the orange discolouration indicated that the pigmented material was diffuse in the tissue and not localized in any specific cells. The NOAEL in this study was 1013 mg/kg bw per day, the highest dose tested (Buser & Arceo, 1995).

(b) β-Apo-8′-carotenal

(i) Rats

Groups of five male and five female Sprague Dawley rats (6 weeks of age) were administered β-apo-8′-carotenal (Apocarotenal 10% WS/N; β-apo-8′-carotenal at 116 g/kg, β-carotene at 3.2 g/kg, crocentindialdehyde at 0.42 g/kg; as beadlets) in the diet for 28 days at a target dose of 0, 20, 100 or 500 mg/kg bw per day (mean achieved intakes were 0, 20, 100 and 501 mg/kg bw per day for males and 0, 20, 102 and 507 mg/kg bw per day for females, respectively). An additional group of five male and five female rats received placebo-treated diets containing the beadlets but not the test article over a similar treatment period. The study was not conducted according to GLP or specific test guidelines.

No mortality was observed during the study period. Orange-coloured skin and orange/red faeces were observed in all treated groups. A statistically significant reduction in group mean body weight was observed in females at 500 mg/kg bw per day on day 28 (Table 8). Body weight gain was statistically significantly lower in males and females at 500 mg/kg bw per day and females at 100 mg/kg bw per day over the 28-day period. Feed consumption at the high dose appeared lower than the control value, but the decrease did not achieve statistical significance.

Statistically significant increases in absolute and relative liver weights were observed in females at 100 and 500 mg/kg bw per day. Alanine aminotransferase (ALT) levels were statistically significantly elevated in all male groups and in females at 100 or 500 mg/kg bw per day. Aspartate aminotransferase (AST) levels were also statistically significantly increased in males at 20 and 500 mg/kg bw per day and in females at 100 or 500 mg/kg bw per day. Statistically significant increases in creatinine and total bilirubin levels were seen in all treated female groups. A statistically significant increase in creatinine levels was also noted in males dosed at 500 mg/kg bw per day (Table 9). There were no treatment-related effects on haematological parameters or urinary specific gravity or volume.

At necropsy, most animals showed yellow or orange discolouration of skin and tissues. Upon histopathological examination, one male and all females

Table 8

Terminal body weight and body weight gain in a 4-week dietary toxicity study of apo-8′-carotenal in rats

Target dose (mg/kg bw per day)	Mean body weight, day 1 (g)	Mean body weight, day 28 (g)	Body weight change, days 1–28 (g)
Males			
0	202 ± 19	377 ± 24	175 ± 18
0 (placebo)	208 ± 12	386 ± 8	178 ± 4
20	207 ± 16	381 ± 26	174 ± 12
100	206 ± 8	373 ± 25	167 ± 18
500	199 ± 18	341 ± 30	142 ± 21**
Females			
0	143 ± 8	218 ± 15	74 ± 8
0 (placebo)	144 ± 15	214 ± 21	69 ± 6
20	152 ± 9	219 ± 7	66 ± 9
100	151 ± 5	211 ± 14	59 ± 12*
500	141 ± 7	191 ± 5**	50 ± 5***

bw: body weight; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Loget & Morgan (2006)

Table 9

Absolute liver weight and clinical chemistry parameters in a 4-week dietary toxicity study of apo-8′-carotenal in rats

Target dose (mg/kg bw per day)	AST (IU/L)	ALT (IU/L)	Creatinine (µmol/L)	Total bilirubin (µmol/L)	Absolute liver weight (g)
Males					
0	87 ± 10	77 ± 11	52 ± 2	1.0 ± 0.5	17.09 ± 0.95
0 (placebo)	87 ± 3	77 ± 11	48 ± 3*	1.3 ± 0.6	18.73 ± 0.74
20	102 ± 4*	107 ± 14**	52 ± 2	1.0 ± 0.2	19.54 ± 3.38
100	96 ± 5	125 ± 15***	52 ± 1	1.2 ± 0.5	20.03 ± 1.34
500	110 ± 14***	118 ± 12***	56 ± 4*	1.5 ± 0.2	18.21 ± 2.04
Females					
0	74 ± 3	54 ± 2	46 ± 2	0.7 ± 0.2	10.25 ± 1.03
0 (placebo)	80 ± 8	54 ± 16	46 ± 2	0.7 ± 0.4	10.03 ± 1.33
20	77 ± 7	62 ± 13	49 ± 2*	1.3 ± 0.3*	11.24 ± 0.54
100	96 ± 27*	82 ± 21*	54 ± 3***	1.6 ± 0.5**	12.23 ± 1.25**
500	92 ± 5**	77 ± 18*	56 ± 2***	2.2 ± 0.4***	12.48 ± 0.07**

ALT: alanine aminotransferase; AST: aspartate aminotransferase; bw: body weight; IU: international units; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Loget & Morgan (2006)

at 500 mg/kg bw per day exhibited minimal eosinophilic droplet formation in the tubular epithelial cells of the outer renal cortices. The droplets consisted of both intracellular microvesicular and macrovesicular droplets, with some also being extruded into the tubular lumen. There were no other treatment-related histological findings. The authors considered the histological changes in the kidney to be of equivocal clinical significance, however, under the conditions of the study and identified a NOAEL of 100 mg/kg bw per day (Loget & Morgan, 2006).

The Committee disagreed with the study authors' conclusion and identified a NOAEL of 100 mg/kg bw per day on the basis of reduced body weight and body weight gain in rats at 500 mg/kg bw per day. The Committee noted the presence of eosinophilic droplets mainly in the kidneys of female rats, but did not consider the finding to be adverse on the basis that they were not linked to other lesions or any other signs of nephropathy.

Groups of 10 male and 10 female Sprague Dawley rats (6 weeks of age) were administered β-apo-8′-carotenal (Apocarotenal 10% WS/N; β-apo-8′-carotenal at 116 g/kg, β-carotene at 3.2 g/kg, crocentindialdehyde at 0.42 g/kg; as beadlets) in the feed for 90 days at a dose of 0 (control), 0 (placebo beadlets), 10, 30 or 100 mg/kg bw per day. Additional groups of five male and five female rats were treated at 0 (control), 0 (placebo beadlets) or 100 mg/kg bw per day for 13 weeks, followed by a 4-week recovery period. The study was designed in compliance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 and conducted in accordance with GLP.

One female rat in the placebo group died during the study period; however, this was not considered related to treatment. Orange or red discolouration of the faeces was observed in all treated groups from day 7 of treatment, and orange discolouration of the skin was seen at 30 and 100 mg/kg bw per day after 28 days. Skin discolouration persisted in recovery animals for 2 weeks. There were no signs of neurotoxicity in a functional observational battery, and no treatment-related effects on body weight or feed consumption or upon ophthalmoscopic examination were observed.

Liver weights (absolute and relative) were increased in high-dose females, but the increase was not evident in recovery animals. Serum ALT (66%), AST (63%) and creatinine (18%) were increased in females at the high dose relative to controls, but were not elevated after the recovery phase. Urine analysis did not reveal statistically significant effects related to treatment.

At gross necropsy, discolouration of body tissues was observed in high-dose animals and persisted throughout the recovery period. Histological evaluation was performed on all animals receiving the test article at 0 or 100 mg/kg bw per day. Livers of females and kidneys of males and females were also examined in

Table 10

Incidence and severity of the main microscopic findings in the liver and kidney in a 13-week dietary toxicity study of apo-8′-carotenal in rats

Target dose (mg/ kg bw per day)	Liver		Kidney		
			Droplets, eosinophilic		
	Multinucleate hepatocyes	Inflammatory cell foci	Minimal	Mild	Total incidence (recovery)
0 (control)					
Male	0	4	0	0	0 (0)
Female	0	0	0	0	0 (0)
0 (placebo)					
Male	0	0	0	0	0 (0)
Female	0	1	0	0	0 (0)
10					
Male	0	0	2	0	2
Female	0	5*	10***	0	10***
30					
Male	0	0	1	0	1
Female	3	0	10***	0	10***
100					
Male	0	5	3	0	3 (1)
Female	2	9***	5*	5*	10*** (2)

bw: body weight; *: $P < 0.05$; ***: $P < 0.001$
Source: Edwards et al. (2007)

the 0 (placebo), 10 or 30 mg/kg bw per day groups. Minimal eosinophilic droplets were observed in the kidneys of both sexes at all doses, increasing in severity in high-dose females. An increased incidence of multinucleate hepatocytes was seen in females from the 30 mg/kg bw per day group, and a statistically significant increase in the incidence of inflammatory cell foci was observed in the livers of high-dose females. Table 10 shows the main microscopic findings.

The study authors noted that eosinophilic droplet accumulation in the rat kidney is a finding associated with accumulation of α2u-globulins within phagolysosomes of the tubular epithelium. This protein droplet accumulation is commonly seen in α2u-globulin nephropathy in male rats in which there is a sequence of functional changes such as tubular degeneration followed by cellular proliferation, formation of granular casts in tubules with dilatation, necrosis, chronic progressive nephropathy and renal tubule hyperplasia and neoplasia. In this study, the effects were observed mainly in female rats and were not linked to other lesions or any other signs of nephropathy. On that basis, the study authors considered that eosinophilic droplet accumulation in the kidney was not adverse.

The study authors considered that the increased incidence of multinucleate hepatocytes in females was indicative of a regenerative response and was correlated with a higher than usual incidence of inflammatory cell foci, increased liver weight and elevated serum ALT and AST levels at the high dose. The study authors identified a NOAEL of 10 mg/kg bw per day on the basis of hepatic effects at 30 mg/kg bw per day (Edwards et al., 2007).

A report amendment was issued in 2008 in which the study pathologist and peer reviewer agreed that hepatocellular multinucleation is an adaptive change and not adverse. Accordingly, the NOAEL was considered to be 30 mg/kg bw per day, on the basis of an increased incidence of hepatic inflammatory cell foci in females at 100 mg/kg bw per day (Perry & Shearer, 2008).

The renal histological slides were re-examined in 2012. The pathologist noted that the main change was the presence of ill-defined clumps or droplets of eosinophilic material in the cytoplasm of proximal tubules, mainly in female rats. The material appeared as dull, pale brown areas within the tubule cytoplasm and was not autofluorescent under ultraviolet illumination. The material was also observed in male rats, but at lower density. In males, there was also an intensification of the normal pattern of autofluorescent cytoplasmic hyaline droplets in the proximal convoluted tubules of all treated groups.

Examination found no evidence of necrosis or single-cell death at any dose. At 100 mg/kg bw per day, the occasional tubule cell containing eosinophilic material appeared to be detached into the tubule lumen, and an occasional mitotic figure was present in the cortex.

Following the recovery period, the density of eosinophilic material had decreased in males and females at 100 mg/kg bw per day, but remained more prominent in females, involving up to approximately one half of the cortical tubules. In the opinion of the study pathologist, the eosinophilic material was likely to represent the accumulation of test material or a derivative during normal renal processing (Hard, 2012).

The Committee considered the reports of Edwards et al. (2007), Perry & Shearer (2008) and Hard (2012) and identified a NOAEL of 30 mg/kg bw per day on the basis of hepatic effects in high-dose female rats and renal changes (cell detachments and mitotic figures) in high-dose animals. The Committee noted that higher plasma and liver concentrations of β-apo-8′-carotenal or its metabolites were achieved in female rats (section 2.1.2) compared with males and considered this to be consistent with the toxicological findings in this study.

Groups of 16 male rats received carotenal intragastrically at 0, 100 or 500 mg/kg bw per day, 5 days per week, for 34 weeks. No adverse effects on body weight gain, general health, survival, liver and kidney function or organ weights were seen. The testicular weight of the high-dose test group was significantly

lower than that of the controls. Microscopic findings were normal except for granular pigment deposition in the liver and kidneys of test animals. Fertility, as shown by monthly mating of four females, was not affected (Anonymous, 1962, 1966).

(ii) Dogs

Groups of male (*n* = 3–4) and female (*n* = 2–3) dogs received 0, 0.1 or 1.0 g of carotenal daily per animal for 14 weeks. All remained healthy, and no statistically significant effects were noted. No pathological lesions related to the test substance were seen at necropsy. Peripheral blood parameters, liver function tests, serum enzymes and blood urea were normal. Vitamin A and carotenal levels of serum, liver, kidney, adrenal and mesenteric fat were measured. The kidney level of vitamin A was 3–5 times that of controls. The serum level of carotenal was elevated in the group receiving 1.0 g, and there was an occasional trace in the group administered 0.1 g. Tissue amounts were variable. The only microscopic finding was pigmentation of the adipose tissue, kidney and adrenal cortex. Organ weights were normal (Bagdon, Impellizzeri & Osadca, 1962).

2.2.3 **Long-term studies of toxicity and carcinogenicity**

(a) **β-Carotene**

(i) Mice

Groups of 100 male and 100 female CD1 mice, aged 34 ± 1 days, were administered β-carotene in the feed at a target dose of 0, 100, 250, 500 or 1000 mg/kg bw per day (mean achieved intakes were 0, 100, 252, 501 and 1010 mg/kg bw per day for males and 0, 101, 249, 496 and 1008 mg/kg bw per day for females, respectively) for 2 years. The test material was formulated in water-soluble beadlets with a β-carotene (Hoffman-La Roche) concentration of approximately 11.5%. Two control groups were included, one given feed containing placebo beadlets and one given standard feed alone. The study began prior to the issue of GLP regulations; however, these were implemented during the course of the study. Mice were monitored daily for clinical signs, and animals were checked weekly from week 26 to record the appearance and location of palpable masses. Ophthalmoscopy was performed on all surviving mice during week 102. Males and females were killed after 104 and 105 weeks of treatment, respectively, and macroscopic and histopathological assessments of selected organs and tissues were performed. No clinical chemistry, haematology or urine analyses were conducted.

Red discolouration of faeces was observed in all animals that received the red pigment, as well as a red or yellow staining of the ventral or dorsal fur due to contact with the test material. No other clinical signs were observed. Treatment with β-carotene did not adversely affect survival, and there were no adverse

Table 11

Incidence of lung tumours (adenoma and/or adenocarcinoma) in male mice administered β-carotene in a 2-year study

Neoplasm	Incidence of lung tumours					
	0 mg/kg bw per day (untreated control)	0 mg/kg bw per day (placebo control)	100 mg/kg bw per day	250 mg/kg bw per day	500 mg/kg bw per day	1 000 mg/kg bw per day
Lung adenoma	3/100	5/100	7/100	3/100	7/100	6/100
Lung adenocarcinoma	10/100	15/100	18/100	17/100	23/100	17/100

bw: body weight
Source: Buser & Hummler (1983a)

effects on body weight development or feed consumption. No treatment-related effects were found on ophthalmoscopy. There was some evidence for a higher incidence of lung tumours in β-carotene-treated and placebo-treated male mice (Table 11). Trend analysis found a statistically significant trend using nominal doses in comparison with the untreated controls ($P = 0.045$), but analyses using the actual doses or in comparison with the placebo controls were not statistically significant. The incidence of lung tumours in the male control mice (13%) was low in comparison with control values from three concurrent long-term studies with the same strain (20%, 28% and 36%) and did not show a clear dose–response relationship. It was concluded that the apparent increased incidences of lung tumours in treated mice were due to the unusually low incidence in the control mice. There was no evidence of a treatment-related effect on the incidence of lung tumours in females or on the incidence of neoplastic changes in any other organ in either sex. Non-neoplastic changes were limited to the presence of vacuolated cells lining the liver sinusoids in some treated animals. These cells were considered to potentially be stellate cells (referred to as perisinusoidal fat storing cells or Ito cells in the study report). As stellate cells store vitamin A, these changes were considered to be an expected effect following long-term administration of a vitamin A precursor and not of toxicological relevance.

The NOAEL in this study was the target dose of 1000 mg/kg bw per day (equal to a mean achieved intake of 1008 mg/kg bw per day), the highest dose tested (Buser & Hummler, 1983a).

(ii) Rats

Sprague Dawley CD rats (85 of each sex per group) were fed diets containing water-soluble beadlets containing β-carotene (Hoffman-La Roche; concentration approximately 11.5%) at a target dose of 0, 100, 250, 500 or 1000 mg/kg bw per day (mean achieved intakes were 0, 75.2, 194, 399 and 821 mg/kg bw per day for

males and 0, 93.6, 237, 489 and 988 mg/kg bw per day for females, respectively) for a duration of 114 (females) and 116 (males) weeks. One control group was given placebo beadlets, whereas another control group received standard diet. Rats were selected from the F_{1A} generation of a concurrent multigeneration study (Buser & Hummler, 1982) when sufficient litters reached 21 days of age. After 52 weeks of treatment, 10 animals of each sex per dose were killed and subjected to macroscopic and microscopic examinations as well as organ weight analysis. After 78 weeks of treatment, a further five animals of each sex per dose were killed and subjected to macroscopic examination and organ weight analysis. Blood and urine samples were collected from the interim kill animals at 6-month intervals and from the main study animals at week 104 and at termination. Ophthalmoscopy was performed periodically.

The only clinical signs considered to be related to treatment with β-carotene were a red discolouration of faecal pellets in all treated animals and a yellow/orange discolouration of fur, predominantly among rats administered 1000 mg/kg bw per day. Mortalities among treated male rats were higher than expected within the first 28 weeks and were frequently associated with haemorrhagic episodes. Haematological analysis found prolonged blood clotting times in placebo- and β-carotene-treated males, which was attributed to high doses of vitamin E (used as an antioxidant in the beadlets) interfering with vitamin K activity. Supplementing the diet of all beadlet-treated animals, from week 29, with a vitamin K analogue resulted in blood clotting tests within normal limits, and mortalities in males in the following weeks were within the normal range. During the later stages of the study, mortality was reduced in all groups receiving beadlets compared with standard diet controls, and this was attributed to lower feed intake and body weights in these animals. Feed intake of β-carotene-treated animals was reduced compared with that of standard controls, but similar to that of placebo controls. Rats in the standard control group gained statistically significantly more weight than animals of the beadlet-treated groups. During the first 26 weeks, body weight gains for rats treated with 100, 250 or 500 mg/kg bw per day were similar to those of the placebo controls, whereas body weight gains for rats given 1000 mg/kg bw per day were lower than in these groups. Between weeks 27 and 105, there was a dose-related reduction in body weight gain in β-carotene-treated groups compared with the placebo control group (Table 12). At week 52, the mean body weights of males given 1000 mg/kg bw per day were greater than 10% lower than those of the placebo controls; at week 78, mean body weights of males and females in the high-dose group were greater than 10% lower than those of the placebo controls; and at week 105, mean body weights of males given 500 and 1000 mg/kg bw per day and females given 1000 mg/kg bw per day were greater than 10% lower than those of placebo controls (Table 13).

Table 12

Group mean body weight changes in rats administered β-carotene in a long-term toxicity and carcinogenicity study

Week range	Mean body weight change (g) ± standard deviation					
	0 mg/kg bw per day (untreated control)	0 mg/kg bw per day (placebo control)	100 mg/kg bw per day	250 mg/kg bw per day	500 mg/kg bw per day	1 000 mg/kg bw per day
Males						
1–26	446 ± 74.7***	378 ± 65.9	369 ± 65.8	362 ± 61.9	358 ± 73.5	328 ± 54.0***
27–52	144 ± 54.9***	95 ± 33.6	89 ± 44.9	89 ± 37.0	78 ± 38.7*	65 ± 36.1***
53–78	91 ± 63.3	75 ± 41.9	54 ± 36.1***	47 ± 32.0***	41 ± 58.9***	38 ± 33.3***
79–105	42 ± 113.4	36 ± 80.7	20 ± 53.4	26 ± 47.7	34 ± 36.8	15 ± 46.6
106–118	−35.6 ± 103.0	−37.4 ± 36.6	−34.0 ± 22.4	−13.6 ± 27.9	6.2 ± 27.9	−8.6 ± 30.6
Females						
1–26	206 ± 48.2***	163 ± 32.2	144 ± 29.1***	151 ± 32.1*	153 ± 33.8	137 ± 29.7***
27–52	107 ± 42.9***	38 ± 23.0	30 ± 20.8	28 ± 17.8*	26 ± 21.1**	26 ± 16.5**
53–78	98 ± 50.5***	52 ± 34.1	29 ± 25.8***	34 ± 27.8***	30 ± 25.6***	20 ± 18.1***
79–105	76 ± 70.9**	44 ± 38.5	36 ± 26.1	31 ± 38.5	43 ± 29.1	36 ± 30.2
106–114	3.7 ± 30.9	10.1 ± 29.6	6.6 ± 22.9	12.6 ± 23.7	11.0 ± 13.7	9.0 ± 17.3

bw: body weight; *: $P < 0.05$; **: $P < 0.01$; *** $P < 0.001$ (analysis of variance and Student's t-test)
Source: Hummler & Buser (1983)

Table 13

Group mean body weights in rats administered β-carotene in a long-term toxicity and carcinogenicity study

Week	Mean body weight[a] (g)					
	0 mg/kg bw per day (untreated control)	0 mg/kg bw per day (placebo control)	100 mg/kg bw per day	250 mg/kg bw per day	500 mg/kg bw per day	1 000 mg/kg bw per day
Males						
52	794	664	661	646	629	592 (~11% ↓)
78	879	732	715	689	678	627 (~14% ↓)
104	951	756	749	706	677* (11% ↓)	647 (~14% ↓)
Females						
52	473	355	333	338	330	323
78	561	403	360	374	362 (~10% ↓)	342 (~15% ↓)
104	654	433	400	405	392	371 (~14% ↓)

bw: body weight; *: $P < 0.05$ (analysis of variance and Student's t-test)
[a] Body weight changes of 10% or more compared with placebo control are shown in parentheses.
Source: Hummler & Buser (1983)

No treatment-related adverse effects on ophthalmoscopy, haematology, blood chemistry, urine analysis, organ weights, or macroscopic or histopathological parameters were found. There was no treatment-related effect on the incidence of any tumour type. The study authors considered the NOAEL to be the target dose of 1000 mg/kg bw per day (equal to a mean achieved intake of 821 mg/kg bw per day), the highest dose tested (Hummler & Buser, 1983). The Committee disagreed with the study authors' conclusion and identified the NOAEL as the target dose of 250 mg/kg bw per day (equal to a mean achieved intake of 194 mg/kg bw per day), based on consistently decreased body weight and body weight gain in male and female rats compared with placebo controls at 500 and 1000 mg/kg bw per day.

(iii) Dogs

In a GLP-compliant study, groups of eight male and eight female beagle dogs were treated with β-carotene (Hoffman-La Roche) at a target dose of 0, 50, 100 or 250 mg/kg bw per day via water-soluble beadlets that were mixed with the feed. Two control groups were included, one treated with placebo beadlets and one given standard diet. Two animals of each sex per group were killed after 52 weeks, and a further three animals of each sex in groups given β-carotene were fed placebo for the final 16 weeks from week 88. The remaining three β-carotene-treated animals of each sex were treated for 104 weeks.

One female administered 250 mg/kg bw per day and one male given 50 mg/kg bw per day died during the course of the study, but these deaths were not considered to be related to treatment. Treatment-related clinical signs were restricted to red discolouration in the faeces and orange staining of extremities and fur, attributable to the colour of the test material. No adverse effects on feed and water consumption, ophthalmoscopy, haematology, clinical chemistry, urine analysis or organ weights were observed. Body weight gain was slightly reduced in animals given placebo or β-carotene beadlets, but no dose–response relationship was observed. Recovery animals had a significant body weight loss after they were withdrawn from dosing with β-carotene. The underlying reason for the body weight loss was unclear, but there was no relationship to the doses at which the animals had been previously treated. Irregular pale orange foci were observed on the liver surface in a number of treated animals at the 52-week necropsy and in all but one treated or recovery animal at 104 weeks. Histopathological changes were restricted to vacuolated cells in periportal areas of the liver, considered to be perisinusoidal fat storage cells, in all dogs given β-carotene, including the recovery animals. As these cells are able to store vitamin A, the finding was considered an expected effect following long-term administration of a vitamin A precursor. The study authors suggested that the persistence of the finding

in recovery animals likely indicates a large excess of vitamin A precursors or vitamin A itself over the animals' requirements. In the absence of any indications of related degenerative findings or clinical chemistry markers of toxicity, these changes were not considered adverse.

The NOAEL in this study was 250 mg/kg bw per day, the highest dose tested (Buser & Hummler, 1983b).

(b) β-Apo-8′-carotenal

In an early study, Wistar rats were administered β-apo-8′-carotenal (source and purity not specified) in the diet at 1000 mg/kg feed to 1) a first generation of rats for 2 years (20 of each sex per group), 2) their offspring for a period of 2 years (14–15 of each sex per group) and 3) a third generation of rats for 1 year (5–12 of each sex per group). The average dose over the course of the study was reported to be 40 mg/kg bw per day. There were no treatment-related effects on mortality. Body weight was slightly lower in treated females of the first generation and males and females of the second and third generations (up to 13%). No effects on absolute organ weights or haematological parameters were reported. Orange-yellow discolouration of the body fat and yellow to ochre discolouration of the liver were observed in treated groups. Histopathological examination of an unspecified number of animals showed golden yellow to yellow-brown, iron-free pigments in the liver and kidneys of treated animals. No microscopic changes were observed in the lungs, heart, spleen, bone marrow, stomach, duodenum, jejunum, pancreas, ovaries, skeletal musculature, thyroid or skin. Degenerative changes reported in the epithelium of the seminiferous tubules were slightly, but not statistically significantly, more frequent ($P = 0.17$) in the animals treated with β-apo-8′-carotenal than in the controls. The study authors considered that the result was due to an unusually low rate of atrophies in the control group and not related to treatment. Tumour rates and reproductive parameters were not affected (Schärer & Studer, 1961).

2.2.4 Genotoxicity

(a) β-Carotene

β-Carotene derived from *B. trispora* was tested for mutagenic activity in *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, with and without an exogenous metabolic activation system from rat liver, in two independent studies with five concentrations of the test substance. Negative controls were tested with dimethyl sulfoxide, and positive controls with sodium azide, 9-aminoacridine, benzo[*a*]pyrene, 2-nitrofluorene and 2-aminoanthracene. No mutagenic activity of β-carotene was observed (Kluifthooft, 2001).

β-Carotene derived from *B. trispora* was tested for its ability to induce chromosomal aberrations in cultured Chinese hamster ovary cells in two independent assays with and without exogenous metabolic activation. The material was dissolved in dimethyl sulfoxide and tested in serial dilutions from 25 to 0.1 mg/mL. A vehicle control and a positive control with cyclophosphamide were included. No clastogenic effects were recorded (Kluifthooft, 2001).

Algal carotene with 30% β-carotene content was tested for mutagenic activity in *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537, with and without an exogenous metabolic activation system from rat liver. The test material was dissolved in tetrahydrofuran and tested at five concentrations ranging from 3.16 to 316 µg/mL. The vehicle served as a negative control, and positive controls were sodium azide, 9-aminoacridine, 2-nitrofluorene, methyl methane sulfonate, cyclophosphamide and 2-aminoanthracene. No increase in revertant colonies was observed under the conditions of the study (Cognis Deutschland GmbH & Co. KG, 2006a).

Algal carotene with 30% β-carotene content was tested for mutagenicity in mouse lymphoma cells in the L5178Y thymidine kinase (TK) mammalian mutagenicity test, in the presence and absence of exogenous metabolic activation. The test material was dissolved in tetrahydrofuran and tested at concentrations of 13–208 µg/mL. The negative control was the vehicle, and positive controls were methyl methane sulfonate and 3-methylcholanthrene. No mutagenic activity was observed (Cognis Deutschland GmbH & Co. KG, 2006b).

A 30% solution of β-carotene from *D. salina* in sesame oil (C020072; Cognis Deutschland GmbH & Co.) was tested for the ability to induce micronuclei in bone marrow cells in vivo in a GLP-compliant study following OECD Test Guideline 474. The test substance was administered by oral gavage to 10 male and 10 female NMRI mice at a C020072 dose of 2000 mg/kg bw (equivalent to a β-carotene dose of 600 mg/kg bw), and 10 control animals of each sex were administered sesame oil. Cyclophosphamide was used as a positive control and was administered to five males and five females at a dose of 30 mg/kg bw. Bone marrow cells were collected from five males and five females in the negative control and test groups at 24 hours and 48 hours after dosing, and from all animals given cyclophosphamide 24 hours after application. No increases in the frequency of micronuclei compared with negative controls were found at either time point following application of the test substance. Cyclophosphamide produced the expected statistically significant increase in the incidence of micronuclei compared with negative controls, confirming the validity of the test system (Weimans, 2003).

(b) β-Apo-8′-carotenal

β-Apo-8′-carotenal (Apocarotenal 10% WS/N beadlets; β-apo-8′-carotenal at 116 g/kg, β-carotene at 3.2 g/kg, crocentindialdehyde at 0.42 g/kg) was tested for mutagenic activity in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 at concentrations of 8.7–277.9 μg/plate, with and without an exogenous metabolic activation system from rat liver. The highest concentration was limited by the maximum achievable solubility of the test article in the vehicle, dimethyl sulfoxide. Negative and positive solvent controls were included for all experiments. β-Apo-8′-carotenal was not mutagenic under the conditions of the study (Loget & Johnson, 2006).

β-Apo-8′-carotenal (C30-Aldehyd; purity 69.2%) was tested for mutagenic activity in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2 uvrA at concentrations of 20–5000 μg/plate (all tester strains) or 20–6000 μg/plate (TA100), in the presence and absence of a metabolic activation system from rat liver. The vehicle was 1-methyl-2-pyrrolidone. Precipitation of the test substance was observed at concentrations of 100 μg/plate and above. Cytotoxicity was occasionally observed, depending on the strain and test conditions, from 2500 μg/plate. No increase in the number of revertants was observed for *Salmonella* strains TA98, TA1535 and TA1537 or *E. coli* WP2 uvrA. A concentration-related increase in the number of revertants was observed in the TA100 strain in the presence and absence of metabolic activation. Negative and positive controls showed expected revertant rates. β-Apo-8′-carotenal was mutagenic in the bacterial reverse mutation assay under the conditions of the study. Given the low test material purity, it is possible that impurities may have contributed to the result (Engelhardt & Hoffmann, 1998).

The ability of a suspension of β-apo-8′-carotenal (sum of isomers: 95.50% + crocetindialdehyde 0.47%) in methyl cellulose (0.5%) to induce chromosomal aberrations was tested in Chinese hamster ovary cells in two independent experiments in an in vitro mammalian chromosomal aberration test. An increased frequency of chromosomal aberrations was observed in the absence and presence of metabolic activation at concentrations associated with approximately 50% cytotoxicity or greater, but not at lower concentrations, at which moderate to low cytotoxicity was observed. These increases were of questionable biological relevance given that they were observed only at doses associated with significant cytotoxicity. The proportion of cells in negative and positive controls in which structural aberrations were observed fell within historical control ranges. There were no consistent increases in numerical aberrations (Loget & Whitwell, 2006).

β-Apo-8′-carotenal (Apocarotenal 10% WS/N beadlets; β-apo-8′-carotenal at 116 g/kg) was administered by oral gavage to groups of six male rats at a dose of 0 (vehicle control), 200, 400 or 800 mg/kg bw per day on 2 consecutive

days in a mammalian erythrocyte micronucleus test. An additional placebo group received beadlets without the test substance at a level equivalent to the 800 mg/kg bw per day dose group. Analysis of plasma demonstrated systemic exposure to β-apo-8′-carotenal and three metabolites. Treatment with β-apo-8′-carotenal did not induce statistically significant increases in the frequency of micronucleated erythrocytes compared with the concurrent controls at doses up to 800 mg/kg bw per day (Loget & Beevers, 2006).

2.2.5 Reproductive and developmental toxicity

(a) β-Carotene

(i) Multigeneration reproductive toxicity

In a three-generation reproductive toxicity study conducted in accordance with GLP regulations, Sprague Dawley CD rats (age not specified) were administered β-carotene (Hoffman-La Roche; beadlets containing approximately 11.5% β-carotene) in the feed at concentrations targeting doses of 0, 100, 250, 500 and 1000 mg/kg bw per day (mean achieved doses were 0, 93, 243, 482 and 956 mg/kg bw per day for males and 0, 111, 281, 554 and 1108 mg/kg bw per day for females, respectively, in the F_0 generation; 0, 94, 246, 472 and 966 mg/kg bw per day for males and 0, 108, 286, 603 and 1174 mg/kg bw per day for females, respectively, in the F_{1B} generation; and 0, 89, 225, 475 and 895 mg/kg bw per day for males and 0, 102, 269, 542 and 1029 mg/kg bw per day for females, respectively, in the F_{2B} generation). One group of control rats received a standard diet, whereas a second control group received a diet containing placebo beadlets. Animals in the F_0 generation (35 males and 70 females per group) were maintained on their respective diets for 63 days prior to mating. For each generation, two litters were obtained. The first litters were killed and examined macroscopically, whereas young from the second litters were selected as the basis of the second and third generations. Additional organ weight analysis and histopathological examinations were performed on selected offspring (F_{3B}) of the final mating.

No clinical signs of toxicity were observed in treated animals. A tan or orange/red faecal discolouration and yellow/orange/red tinge to the fur and extremities were seen in animals given diets containing β-carotene, attributable to the colour of the β-carotene. Thirteen males and one female in the F_0 generation died or were killed during the study. All were from groups treated with β-carotene. In the F_{1B} and F_{2B} generations, only three males and one female died. Although clinical signs were not conclusive and observations of the gastrointestinal tract at postmortem were hampered by the intensity of colouration of the contents due to β-carotene, there was evidence of internal haemorrhage in several animals. The authors hypothesized that the male mortalities were most probably due to internal haemorrhage as a result of the high concentration of beadlets in the diet, leading

to increased vitamin E intake (vitamin E was used as an antioxidant in the beadlet formulation) and a vitamin K deficiency. The higher concentration of beadlets in the diet given to the F_0 generation for the first 5 weeks (20%, subsequently reduced to 14%) may have been a critical factor underlying the different mortality rates in the F_0 and subsequent generations. Supporting evidence for this hypothesis came from a long-term study with the same test material (Hummler & Buser, 1983), in which prolonged prothrombin times, haemorrhages and mortality could be corrected by dietary supplementation with a vitamin K analogue. Another possible contributing factor was an episode of infection with sialodacryoadenitis virus, which occurred during the period of 20% beadlet inclusion in the diet. No treatment-related effects on feed consumption, body weight, organ weights or histopathological investigations were observed. Mating performance, pregnancy rate and the mean duration of gestation were not affected by treatment, and no effects on litter size, pup mortality or pup weight were observed. Macroscopic evaluation of the F_0 generation found some evidence of smaller seminal vesicles in males given diets with beadlets. However, histopathological examination did not identify any abnormalities, mating performance had not been impaired and the observation was not found in F_{1B} or F_{2B} adult males. The NOAELs for reproductive toxicity, parental toxicity and offspring toxicity were all 895 mg/kg bw per day, the highest dose tested (Buser & Hummler, 1982).

(ii) Developmental toxicity

In a guideline- and GLP-compliant developmental toxicity study, groups of 40 mated female albino rats were administered β-carotene via beadlets (Hoffman-La Roche; 10% β-carotene) mixed into the diet at concentrations targeting doses of 0, 250, 500 and 1000 mg/kg bw per day (mean achieved doses were 0, 303, 602 and 1220 mg/kg bw per day, respectively) from GDs 7 to 16. Controls were given feed containing placebo beadlets. On GD 21, a subgroup of dams from each treatment group (17–18 per group) was killed, and the uteri and fetuses were examined. Dams in the second subgroup (14–17 per group) gave birth, and on lactation day 23, dams and young were killed and examined.

There were no maternal deaths or clinical signs of maternal toxicity. In the high-dose group, body weight gain of the dams was significantly reduced compared with controls during the period of treatment (weight gain of 11.2 ± 11.3 g vs 20.0 ± 8.1 g, respectively). These animals gained more weight than controls from GDs 17 to 21, so that over the entire gestation period, there were no statistically significant differences in body weight gain between groups (Table 14).

No indications of embryotoxicity or teratogenicity were observed in the study. Among the pups that were reared until lactation day 23, there were no

Table 14

Mean body weight and body weight gain in a developmental toxicity study in which pregnant rats were administered β-carotene

Parameter	0 mg/kg bw per day	250 mg/kg bw per day	500 mg/kg bw per day	1 000 mg/kg bw per day
Mean starting body weight of females (g)	220.1 ± 9.4	222.4 ± 12.1	220.5 ± 9.7	218.7 ± 13.0
Body weight gain (g), GDs 1–7 (before treatment)	21.9 ± 6.1	20.9 ± 7.7	21.6 ± 5.7	23.5 ± 5.6
Body weight gain (g), GDs 7–17 (during treatment)	20.0 ± 8.1	16.9 ± 8.6	16.3 ± 11.2	11.2 ± 11.3***
Body weight gain (g), GDs 17–21	46.5 ± 14.2	64.1 ± 12.6***	51.8 ± 15.3	54.5 ± 13.4*
Body weight gain (g), GDs 1–21	88.4 ± 18.8	102.0 ± 17.9**	89.8 ± 19.8	89.2 ± 20.1

bw: body weight; GD: gestation day; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test)
Source: Kistler (1981)

indications of any functional abnormalities, and no adverse effects on survival, body weight or weights of the liver, kidney and heart were observed.

The NOAEL for embryo and fetal toxicity and for maternal toxicity was the target dose of 1000 mg/kg bw per day (equal to a mean achieved dose of 1220 mg/kg bw per day), the highest dose tested (Kistler, 1981).

In a GLP-compliant developmental toxicity study conducted in accordance with relevant national guidelines, groups of 20 mated female albino rabbits were given β-carotene in seed oil by oral gavage at a dose of 0, 100, 200 or 400 mg/kg bw per day on GDs 7–19. All rabbits were killed on GD 30, and the uteri and fetuses were removed and examined. There were no treatment-related maternal deaths or clinical signs of toxicity. Body weight development was similar in all groups. There was no indication of any embryotoxic or teratogenic effects of β-carotene. The NOAEL for embryo and fetal toxicity and for maternal toxicity was 400 mg/kg bw per day, the highest dose tested (Kistler, 1982).

(b) β-Apo-8′-carotenal

(i) Multigeneration reproductive toxicity

No studies were identified.

(ii) Developmental toxicity

In a dose range–finding study, groups of six mated female Sprague Dawley rats were administered β-apo-8′-carotenal (Apocarotenal 10% WS/N; β-apo-8′-carotenal at 116 g/kg, β-carotene at 3.2 g/kg, crocentindialdehyde at 0.42 g/kg) by incorporation of beadlets in the feed from GDs 6 to 20 at concentrations targeting

doses of 0, 20, 100 and 500 mg/kg bw per day (mean achieved doses were 0, 18, 95 and 471 mg/kg bw per day, respectively). Two control groups were included, one given placebo beadlets and one given standard diet. The study was not GLP compliant but was conducted following the test facility's standard operating procedures, which were in accordance with GLP regulations. All animals were killed on GD 20. None of the females died over the course of the study. The only clinical signs associated with treatment were orange-coloured faeces in animals given 100 or 500 mg/kg bw per day and orange-coloured integuments in all treated animals. No adverse effects on body weight gain, feed consumption or embryo-fetal survival were observed. No external malformations of fetuses were seen in any group (Loget & Marsden, 2006).

In a GLP- and guideline-compliant study, groups of 25 mated female Sprague Dawley rats were administered β-apo-8′-carotenal (Apocarotenal 10% WS/N; β-apo-8′-carotenal at 116 g/kg, β-carotene at 3.2 g/kg, crocentindialdehyde at 0.42 g/kg) by incorporation of beadlets in the feed from GDs 6 to 20 at concentrations targeting doses of 0, 20, 100 and 500 mg/kg bw per day (mean achieved doses were 0, 20, 100 and 495 mg/kg bw per day, respectively). One control group was administered placebo beadlets, and a second was given standard diet. Animals were killed on GD 20, at which time plasma and liver samples were collected from five animals per group for measurement of β-apo-8′-carotenal, β-apo-8′-carotenol, β-apo-8′-carotenoic acid and a polar metabolite (results are shown in). Two females were found dead during the course of the study, but these deaths were not considered to be attributable to treatment. Treatment-related clinical signs were restricted to orange-coloured integuments in almost all treated animals, orange-coloured faeces in all animals given 100 or 500 mg/kg bw per day and orange-stained fur in some animals in the high-dose group. Slight reductions in mean body weight gain and feed consumption were observed over the first 9 days of treatment in the 100 and 500 mg/kg bw per day groups compared with animals given the standard control diet. After this period, feed consumption was similar to that in the standard controls, whereas net body weight gain over the entire treatment period was slightly, but not statistically significantly, lower (~6–7%) in the 100 and 500 mg/kg bw per day groups. No treatment-related macroscopic abnormalities were observed in dams, apart from orange-coloured organs and tissues, consistent with the colour of the test item. There were no treatment-related adverse effects on embryo-fetal survival, fetal weight or the incidence of external, visceral or skeletal malformations. The Committee considered the NOAEL for maternal toxicity and for embryo and fetal toxicity to be the target dose of 500 mg/kg bw per day (equal to a mean achieved dose of 495 mg/kg bw per day), the highest dose tested (Loget et al., 2006).

2.2.6 **Special studies**

(a) **β-Carotene**

In a series of experiments, groups of 10–24 male A/J mice, 5–6 weeks old, were exposed to tobacco smoke at a concentration of 140 mg/m^3 of total suspended particulate matter, 6 hours per day, 5 days per week, for either 4 or 5 months, followed by a recovery period in air for 4 or 5 months, or for 9 months without a recovery period. β-Carotene was administered to mice as beadlets in the diet at a concentration of 0, 50, 500 or 5000 mg/kg feed (equivalent to 0, 6.5, 65 and 650 mg/kg bw per day, respectively) either during or following the tobacco smoke exposure. The control diet contained beadlets without β-carotene. At 9 months, increases in lung tumour multiplicities and incidences were observed in smoke-treated animals. β-Carotene did not significantly affect tumour development under the conditions of the study (Obermueller-Jevic et al., 2002).

Thirty-six adult male ferrets (1.0–1.2 kg) were assigned to six groups and administered β-carotene (Sigma Chemicals, St Louis, Missouri, USA) dissolved in corn oil orally at a dose of 0, 0.43 or 2.4 mg/kg bw per day for 6 months, either with or without concomitant smoke exposure. No clinical signs were reported, and there were no effects on body weight. No pathological changes in the lung tissue of ferrets in the control group or in the low-dose β-carotene group were observed at 6 months. Localized proliferation of alveolar macrophages and keratinized squamous epithelium were reported in the lung tissue of the high-dose β-carotene group. Severe focal proliferation of alveolar cells, squamous metaplasia and destruction of alveolar walls were reported in the high-dose β-carotene group that was also smoke exposed (Liu et al., 2000).

Seven-week-old female ferrets were administered β-carotene (DSM Nutritional Products Ltd, Basel, Switzerland) as a water-soluble formulation (beadlets) containing 10% β-carotene orally every morning for 6 months dissolved in 200 μL of water at a dose of 0.8 or 3.2 mg/kg bw per day. Benzo[*a*]pyrene was also administered to the ferrets, such that the dose groups were 1) control, 2) β-carotene at 0.8 mg/kg bw per day, 3) β-carotene at 3.2 mg/kg bw per day, 4) benzo[*a*]pyrene at 8 mg/kg bw per day and 5) benzo[*a*]pyrene at 8 mg/kg bw per day plus β-carotene at 0.8 mg/kg bw per day. The authors reported that no significant lesions were observed in the lungs of any animal of the different groups studied. No signs of squamous metaplasia were found in the lungs of treated ferrets (Fuster et al., 2008).

2.3 **Observations in humans**

2.3.1 **β-Carotene**

The association between β-carotene intake and cancer risk has been evaluated in a number of observational studies and extensively reviewed (Block, Patterson

& Subar, 1992; Steinmetz & Potter, 1996; Ziegler, Mayne & Swanson, 1996; Woutersen et al., 1999). It was concluded that intake of β-carotene and fruits and vegetables appears to confer protection against cancers at different sites, with the most consistent effect being a protective effect against lung cancer. Consequently, a number of large, high-quality randomized controlled trials have investigated whether β-carotene supplementation at doses of 20–50 mg/day for durations of up to 12 years reduces cancer risk in human populations.

A double-blinded, randomized, placebo-controlled trial (known as the Alpha-Tocopherol, Beta Carotene Cancer Prevention, or ATBC, Study) enrolled 29 133 male smokers aged 50–69 years, 7282 of whom received β-carotene supplementation at 20 mg/day for 5–8 years, with a median of 6.1 years. Median plasma concentrations of β-carotene increased approximately 18-fold from baseline (from 0.17 to 3.0 μg/mL). A higher incidence of lung cancer was observed among the men who received β-carotene than among those who did not (relative risk [RR] 1.18; 95% confidence interval [CI]: 1.03–1.36). β-Carotene intake had no effect on the incidence of other cancers. Total mortality was higher (RR 1.08; 95% CI: 1.01–1.16) among those men who received β-carotene than among those who did not, primarily due to more deaths from lung cancer and ischaemic heart disease. The elevated risk was related to those who smoked at least one pack of cigarettes per day and was not seen in subjects who smoked less (ATBC Study Group, 1994; Woutersen et al., 1999).

Post-intervention follow-up assessment for cancer incidence, cause-specific deaths and all-cause mortality lasted for 6 and 8 years, respectively. No statistically significant overall difference in lung cancer incidence was observed between β-carotene recipients and non-recipients during the post-intervention follow-up period (RR 1.06; 95% CI: 0.91–1.20). Relative risk of death for β-carotene recipients compared with non-recipients was 1.07 (95% CI: 1.02–1.12). The higher mortality rate of β-carotene recipients observed compared with non-recipients at the end of the intervention period returned towards null within 4–6 years of follow-up after stopping the supplementation (ATBC Study Group, 2003).

The post-intervention effects of α-tocopherol (50 mg/day) and β-carotene (20 mg/day) were further investigated in 25 563 men followed for 18 years for cancer incidence and all causes of mortality through national registries. Supplementation with either α-tocopherol or β-carotene had no significant effects on post-trial cancer incidence. Mortality from prostate cancer was statistically significantly higher among subjects who received β-carotene; however, the relevance of this finding is not clear, because β-carotene had no impact on post-trial prostate cancer incidence or overall mortality (Virtamo et al., 2014).

The effects of 50 mg/day tocopherol or 20 mg/day β-carotene supplementation, or both, on liver cancer incidence and chronic liver disease

mortality in the ATBC Study were studied. No overall effects on the incidence of liver cancer or on mortality from chronic liver disease were observed over the course of 24 years of follow-up either during the intervention period or in the post-intervention follow-up period (Lai et al., 2014).

A 2018 study analysed whether β-carotene supplementation in the ATBC Study influenced pneumonia risk in 14 564 Finnish male smokers. β-Carotene had no effect on pneumonia risk for the non-smoker participants or the average incidence of pneumonia (RR 1.07; 95% CI: 0.89–1.29), but heterogeneity in the effect was observed with the level of cigarette smoking exposure. In a subgroup of 990 participants who initiated smoking at or above 21 years of age and smoked at least 21 cigarettes per day at the study baseline, an increased incidence (RR 4; 95% CI: 1.63–10) of pneumonia was observed. β-Carotene supplementation had no effect on the number of cases of pneumonia among a subgroup of 4290 participants who had quit smoking (Hemila, 2018).

The Physicians' Health Study, conducted in the USA, was a large, randomized, double-blinded, placebo-controlled trial with 2×2 factorial design conducted on 22 071 healthy male physicians with no history of cancer, myocardial infarction, stroke or transient cerebral ischaemia. The study tested the effect of aspirin with or without β-carotene supplementation on the prevention of cardiovascular disease and cancer. Mean plasma β-carotene concentration increased 4-fold from baseline, from 0.3 to 1.2 μg/mL. Owing to a clear preventive effect of aspirin on myocardial infarction, this arm was stopped early, and the study continued with β-carotene only. The β-carotene intervention group ($n = 11$ 036) received 50 mg β-carotene every second day for a period of 12 years, while the remaining 11 035 received a placebo. The study found that β-carotene had no effect on the risk of any malignant neoplasm (RR 0.98; 95% CI: 0.91–1.06). No significant effect for β-carotene, compared with placebo, was found for cases of lung cancer, death from cancer, all-cause death, cardiovascular disease, myocardial infarction or stroke. Overall, it was concluded that 12 years of β-carotene supplementation (5 years combined with aspirin followed by 7 years without concomitant intervention) at 50 mg on alternate days produced neither benefit nor harm in terms of the incidence of malignant neoplasms, cardiovascular disease or death from all causes (Hennekens et al., 1996).

A study analysed the development of age-related cataracts in the 22 071 participants in the Physicians' Health Study. There was no difference between the β-carotene and placebo groups in the overall incidence of cataract (RR 1.00; 95% CI: 0.91–1.09) or cataract extraction. A subgroup analysis of current smokers appeared to show an attenuation of excess risk of cataract (RR 0.74; 95% CI: 0.57–0.95). There was no significant difference in the number of cases between current non-smokers and placebo (Christen et al., 2003).

A prospective study nested within the Physicians' Health Study investigated whether supplemental β-carotene intake during radiotherapy for prostate cancer is associated with an increased risk of prostate cancer death or metastases. The study population consisted of a cohort of 383 men treated with radiation therapy for prostate cancer while randomized to receive 50 mg β-carotene on alternate days or placebo. With a median follow-up of 10.5 years, the intake of β-carotene was not associated with an increased risk of prostate cancer death or metastases compared with that of placebo (Margalit et al., 2012).

The Beta-Carotene and Retinol Efficacy Trial (CARET), conducted in the USA, involved 18 314 participants who were smokers or ex-smokers or exposed to asbestos. Participants were given daily doses of 30 mg β-carotene and 25 000 international units (IU) of vitamin A, as retinyl palmitate, or a placebo. After 5 years of treatment, median serum β-carotene levels had increased from 0.15 to 2.1 µg/mL in the β-carotene treatment groups. Lung cancer incidence was increased by 28% in the supplemented group (RR 1.28; 95% CI: 1.04–1.57), and total mortality increased 17% in smokers and asbestos workers treated with β-carotene and retinyl palmitate (RR 1.17; 95% CI: 1.03–1.33), compared with controls. The incidence of other tumours was not affected (Omenn et al., 1996a,b). The results obtained after termination of the study (2 years earlier than planned) showed a higher incidence of lung cancer and total mortality in the supplemented group. However, because the two compounds were administered in combination, it is not possible to ascribe the effects observed in this study to β-carotene alone. A post-intervention analysis of CARET carried out 6 years after the trial was stopped reported that the post-intervention relative risks for lung cancer (RR 1.12; 95% CI: 0.97–1.31) and all-cause mortality (RR 1.08; 95% CI: 0.99–1.17) persisted, but were no longer statistically significant (Goodman et al., 2004).

The Women's Antioxidant Cardiovascular Study was a randomized trial that occurred between 1995 and 2005, involving 8171 female health professionals at least 40 years of age with either a history of cardiovascular disease or three or more cardiovascular disease risk factors. Subjects were randomly assigned to receive vitamin C (ascorbic acid, 500 mg every day), vitamin E (α-tocopherol acetate, 600 IU every other day), β-carotene (50 mg every other day) or their respective placebos for an average of 9.4 years. Compared with placebo, β-carotene supplementation did not affect cancer incidence (RR 1.00; 95% CI: 0.85–1.17) or cancer mortality (RR 0.84; 95% CI: 0.62–1.13). No statistically significant effect of β-carotene treatment on the risk of developing type 2 diabetes (RR 0.97; 95% CI: 0.85–1.11) was observed in women at high risk of cardiovascular disease. No statistically significant differences in other adverse effects were reported (Lin et al., 2009; Song et al., 2009).

A randomized controlled trial conducted in the USA investigated whether β-carotene reduced the risk of new cancers in 1805 subjects (70:30 males to females) diagnosed with a non-melanoma skin cancer. Participants (n = 913) assigned to the treatment group received capsules containing 50 mg β-carotene (BASF, Wyandotte, Michigan, USA) per day for 5 years and were followed up for another 5 years. After 1 year of treatment, the plasma β-carotene concentrations in the placebo and treated groups were 0.19 μg/mL and 1.62 μg/mL, respectively. No difference in the incidence of the first new non-melanoma skin cancer was observed between the intervention and the placebo groups. Skin yellowing was observed in some participants in the β-carotene treatment group; however, no adverse signs of toxicity were reported (Greenberg et al., 1990).

A community-based, randomized, placebo-controlled trial that lasted for 4.5 years and enrolled 1621 residents of south-east Queensland, Australia, aged between 20 and 69 years, investigated the effectiveness of β-carotene in preventing skin cancers, compared with sunscreen. Daily supplementation of 30 mg β-carotene did not result in either beneficial or harmful effects on the rates of basal cell or squamous cell types of skin cancer. No adverse effects that were clearly attributable to β-carotene supplementation were reported (Green et al., 1999). A randomized, placebo-controlled trial on 903 subjects less than 55 years of age enrolled in the Green et al. (1999) study investigated whether regular use of sunscreen compared with discretionary use of β-carotene supplements compared with placebo can delay skin ageing. No overall effect on skin ageing was observed in the studied population after 4.5 years of β-carotene supplementation at 30 mg/day (Hughes et al., 2013).

A randomized controlled trial was conducted to determine whether β-carotene was effective in the prevention of oral/pharyngeal/laryngeal (head and neck) cancer in humans. The subject population included 264 patients (20–79 years of age) who had been curatively treated for a recent early-stage squamous cell carcinoma of the oral cavity, pharynx or larynx. Patients were assigned randomly to receive 50 mg β-carotene per day or placebo and were followed for up to 90 months for the development of second primary tumours and local recurrences. β-Carotene supplementation produced a persistent 9- to 10-fold increase in median plasma β-carotene concentrations. Skin yellowing was reported more frequently in patients randomized to β-carotene than in those receiving placebo; however, no other clinical signs attributable to treatment were observed. There were no statistically significant differences between β-carotene and placebo groups for development of second primary tumours or local recurrences (Mayne et al., 2001). An analysis of urine samples (n = 55) from both smokers and non-smokers enrolled in this study did not show significant differences in the excretion of F2-isoprostanes between β-carotene and placebo groups (Mayne et al., 2004).

Several systematic reviews and meta-analyses of randomized controlled trials have evaluated the effects of β-carotene supplementation on a range of health end-points. A meta-analysis of eight randomized controlled trials assessed the effect of vitamin E and β-carotene provided as supplements on all-cause mortality and cardiovascular death. The β-carotene trials involved 138 113 participants with daily intake of β-carotene ranging from 20 to 50 mg and intervention periods ranging from 1.4 to 12 years. The meta-analysis concluded that β-carotene supplementation led to a slightly, but statistically significantly, increased odds ratio (OR) of all-cause mortality (OR 1.07; 95% CI: 1.02–1.11) and cardiovascular death (OR 1.10; 95% CI: 1.03–1.17). However, the pooled estimates were not exclusively drawn from randomized controlled trials in which β-carotene was the only intervention, and therefore the findings cannot be directly attributed to β-carotene alone (Vivekananthan et al., 2003).

In 2013, Bjelakovic, Nikolova & Gluud published a meta-analysis of the association between antioxidants and all-cause mortality based on a previous systematic review and meta-analysis by the same researchers. It concluded that β-carotene, considered to be administered singly in seven of the included randomized controlled trials with 43 019 subjects, at doses ranging between 25 and 50 mg/day, slightly, but statistically significantly, increased all-cause mortality (RR 1.06; 95% CI: 1.02–1.10) when compared with placebo. β-Carotene used singly or in combination with other antioxidants versus placebo in 26 trials, including 173 006 participants at doses of 1.2–50 mg/day, statistically significantly increased mortality (RR 1.05; 95% CI: 1.01–1.09). However, it was not possible to separate any effect of β-carotene from that of the other antioxidants used in the interventions.

In a meta-analysis of nine randomized controlled trials, there was no overall effect of β-carotene supplementation on the incidence of all cancers combined (RR 1.01; 95% CI: 0.98–1.04). The incidences of lung and stomach cancers were statistically significantly increased in individuals given β-carotene at 20–30 mg/day (RR 1.16; 95% CI: 1.06–1.27 for lung cancer; RR 1.34; 95% CI: 1.06–1.70 for stomach cancer) and in populations with only smokers and asbestos workers (RR 1.20; 95% CI: 1.07–1.34 for lung cancer; RR 1.54; 95% CI: 1.08–2.19 for stomach cancer) compared with the placebo. β-Carotene supplementation did not affect the incidence of other cancer types analysed. A meta-analysis of studies that used lower doses of β-carotene (6 or 15 mg/kg bw per day) did not show an increase in all-site cancer incidence or lung cancer. However, β-carotene was co-administered with other substances in these studies, and the populations were not the same as in the ATBC and CARET studies with regard to their smoking status and asbestos exposures. In addition, the authors noted a number of limitations with their analyses, suggesting that the results should be interpreted with some caution, including possible confounding, because subjects

were administered β-carotene in combination with other antioxidants, the small number of randomized controlled trials and problems associated with assessing the statistical significance of subgroup analyses (Druesne-Pecollo et al., 2010).

In a meta-analysis of six randomized controlled trials with pure β-carotene arms, including 40 544 total participants, 20 290 in β-carotene supplement groups and 20 254 in placebo groups, β-carotene supplements at doses of 30–75 mg/day had no preventive effect on either cancer incidence (RR 1.08; 95% CI: 0.99–1.18) or cancer mortality (RR 1.00; 95% CI: 0.87–1.15). Analysed by type of cancer, there was no statistically significant effect on the incidence of lung cancer, colorectal cancer, head and neck cancer, skin cancer or prostate cancer; however, β-carotene statistically significantly increased the incidence of urothelial cancer (RR 1.35; 95% CI: 1.01–1.81). No statistically significant associations were found for smoking status, dose or duration of treatment in the subgroup analyses (Jeon et al., 2011).

2.3.2 β-Apo-8′-carotenal

No studies were identified.

3. Dietary exposure

The Committee considered dietary exposure to β-carotene and β-apo-8′-carotenal (INS 160e). β-Carotene includes synthetic β-carotene (160a(i)), β-carotene from *Blakeslea trispora* (160a(iii)) and β-carotenes, vegetable (160a(ii)). β-Carotene and β-apo-8′-carotenal are authorized for use in 79 food categories at MPLs ranging from 50 mg/kg up to 1200 mg/kg as specified in the Codex General Standard for Food Additives (GSFA) (FAO/WHO, 2018a).

During several meetings of the Committee (1993, 2001 and 2017), carotenoids were evaluated. During its eighty-fourth meeting in 2017, the Committee discussed β-carotene as part of a request from a sponsor for the use of β-carotene from *D. salina* extract at the same maximum use levels as for the already approved β-carotene additives (Annex 1, reference *234*). As β-carotene from *D. salina* extract would substitute for the other β-carotene additives in food and would not be used additionally, the expected exposure to β-carotene was not likely to be changed due to its use. Therefore, the Committee did not perform a dietary exposure assessment. Instead, the Committee conducted a literature review into the exposure to β-carotene from food, covering the last 10 years. Based on this review, 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 assessments. This dietary exposure estimate includes exposure to β-carotene from natural sources and from its use as an additive.

As part of the current evaluation, the sponsor provided typical (mean) and maximum use levels of β-carotene and β-apo-8′-carotenal in 33 and 12 food categories, respectively. These levels are listed in Table 15 together with the corresponding MPLs. Because β-carotene and β-apo-8′-carotenal would be added to processed foods only, the Committee did not perform an international exposure assessment based on the provided use levels using the commodity-based food consumption data of the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) database cluster diets. Also, the Committee did not perform an international exposure assessment using the FAO/WHO Chronic Individual Food Consumption data – summary statistics (CIFOCOss) database. The description of the foods recorded in this database was often not specific enough for a relevant link between these foods and the provided use levels. Additionally, many of the recorded foods related to raw food commodities.

The Committee evaluated the estimates of dietary exposure to β-carotene and β-apo-8′-carotenal provided by the sponsor. As these estimates included refined estimations of the dietary exposure to both compounds, the Committee did not use the budget method to calculate a theoretical high level of exposure to β-carotene and β-apo-8′-carotenal based on the use levels provided by the sponsor.

3.1 Exposure estimations

The sponsor provided information on dietary exposure to β-carotene and β-apo-8′-carotenal from two evaluations of the European Food Safety Authority (EFSA, 2012a, 2014) and a study on dietary exposure to β-carotene in France, Germany and the United Kingdom (Tennant et al., 2004). Furthermore, the sponsor also calculated dietary exposure to β-carotene using the EFSA Food Additives Intake Model (FAIM).[2] The Committee noted that EFSA published an evaluation of β-apo-8′-carotenal in 2012 (EFSA, 2012b). As this evaluation was superseded by the 2014 EFSA evaluation, the Committee has reported on the exposure results of this more recent evaluation only.

3.1.1 β-Carotene

In 2012, β-carotene was evaluated by EFSA using MPLs as specified in Council Directive 94/36/EC and maximum use levels provided by the Natural Food

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[2] https://www.efsa.europa.eu/en/applications/foodingredients/tools

Table 15

Maximum permitted levels and typical (mean) and maximum use levels of β-carotene and β-apo-8′-carotenal in food categories of the Codex GSFA for which use levels were provided by the sponsor

Food category code	Food category name	MPL (mg/kg)	β-Carotene		β-Apo-8′-carotenal	
			Typical	Maximum	Typical	Maximum
01.1.4	Flavoured fluid milk drinks	150	2	5	–	–
01.3.2	Beverage whiteners	100	2	2	–	–
01.4.4	Cream analogues	20	2	2	–	–
01.6.1	Unripened cheese	100	–	–	0.4	0.4
01.6.2.1	Ripened cheese, includes rind	100	3	3	–	–
01.6.4	Processed cheese	100	1	3	–	–
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	100	5	20	–	–
02.2.1	Butter	25	2	3.6	–	–
02.2.2	Fat spreads, dairy fat spreads and blended spreads	35	3	10	–	–
02.3	Fat emulsions mainly of type oil-in-water, including mixed and/or flavoured products based on fat emulsions	200	3	10	–	–
03.0	Edible ices, including sherbet and sorbet	200	1	70	–	–
04.1.2.4	Canned or bottled (pasteurized) fruit	200	6	12	–	–
04.1.2.5	Jams, jellies, marmalades	200	5	5	–	–
04.1.2.9	Fruit-based desserts, including fruit-flavoured water-based desserts	150	4	4	–	–
04.2.2.4	Canned or bottled (pasteurized) or retort pouch vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), and seaweeds	50	6	12	–	–
05.2	Confectionery including hard and soft candy, nougats, etc. other than food categories 05.1, 05.3 and 05.4	100	6	31	20	104
05.3	Chewing gum	100	8	20	14	25
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	100	5	50	3	11
06.3	Breakfast cereals, including rolled oats	200	1	50	–	–
06.4.3	Pre-cooked pastas and noodles and like products	1 200	20	40	–	–
06.5	Cereal and starch based desserts (e.g. rice pudding, tapioca pudding)	150	4	4	6.9	10.8

| Food category code | Food category name | MPL (mg/kg) | Use levels (mg/kg) | | | |
| | | | β-Carotene | | β-Apo-8′-carotenal | |
			Typical	Maximum	Typical	Maximum
07.1.2	Crackers, excluding sweet crackers	1 000	7	40	–	–
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	100	7	40	1.8	3.5
09.2	Processed fish and fish products, including mollusks, crustaceans, and echinoderms	100	–	–	12.5	15
10.4	Egg-based desserts (e.g. custard)	150	4	4	–	–
12.2.2	Seasonings and condiments	500	3	10	50	50
12.4	Mustards	300	5	5	–	–
12.5	Soups and broths	300	7	7	8.5	14
12.6	Sauces and like products	500	3	10	50	50
13.6	Food supplements	300	–	–	8.5	260
14.1.4	Water-based flavoured drinks, including "sport,""energy," or "electrolyte" drinks and particulated drinks	100	2	15	3.4	9.6
14.2.2	Cider and perry	200	1	2	–	–
14.2.4	Wines (other than grape)	200	3	2.5	–	–
14.2.6	Distilled spirituous beverages containing more than 15% alcohol	200	2	2.5	–	–
15.1	Snacks – potato, cereal, flour or starch based (from roots and tubers, pulses and legumes)	100	4	4	–	–
15.2	Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)	100	4	4.1	–	–

GSFA: General Standard for Food Additives; MPL: maximum permitted level

Colours Association (NATCOL) (EFSA, 2012a). According to this directive, β-carotene was authorized for use in various foods at quantum satis,[3] except for sausages, pâtés and terrines, which have an MPL of 20 mg/kg. In 2009, EFSA had also received maximum use levels from the Confederation of the Food and Drink Industry of the European Union (CIAA; currently known as FoodDrinkEurope), which were also used in the assessment.

In this assessment, EFSA used food consumption data from the EXPOCHI ("Individual food consumption data and exposure assessment studies for children") consortium to calculate the dietary exposure to β-carotene in combination with the maximum use levels provided. This consortium included food consumption data for children aged 1–10 years from 10 European countries (Huybrechts et al., 2010). Additionally, EFSA calculated the dietary exposure to β-carotene based on food consumption data for adults (19 years and above) and children (1.5–4.5 years) from the United Kingdom. For the assessment, summary statistics for consumption (mean for all population and 95th/97.5th percentile for consumers only) were used. No dietary exposure was assessed using MPLs, as almost all authorizations were at quantum satis. The dietary exposure results are listed in Table 16.

EFSA also reported on mean estimates of dietary exposure to β-carotene from food of nine European countries derived from the European Nutrition and Health Report 2009 (Elmadfa et al., 2009). Based on the results of that study, EFSA concluded that the exposure to β-carotene from food would be in the range of 5–10 mg/day. The dietary exposure estimates based on the CIAA data exceeded this dietary exposure estimate (Table 16). EFSA considered the data reported by NATCOL to better reflect the actual situation of use.

The sponsor also provided the Committee with estimates of dietary exposure to β-carotene obtained with the web-based EFSA FAIM. This model contains food consumption data from the Comprehensive European Food Consumption Database. With FAIM, the sponsor calculated the dietary exposure to β-carotene with the use levels listed in Table 15 for three exposure scenarios:

1) maximum usage scenario, based on maximum use levels per food category;
2) brand-loyal scenario, based on maximum use level for one food category and typical (mean) use levels for the other food categories;
3) non-brand-loyal scenario, based on typical (mean) use levels for all food categories.

The exposure was calculated for six age groups, as listed in Table 17. In the maximum usage scenario, the food category "Fine bakery wares" contributed

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[3] The amount that is enough.

Table 16

Dietary exposure to β-carotene from its use as a food additive in the European population

Supplier maximum reported use levels and exposure parameter	Dietary exposure (mg/kg bw per day)	
	Adults UK (19+ years)	**Children UK[a] and EXPOCHI[b]** (1–10 years)
CIAA		
Mean	1.5	2.2–11.4
P95 or P97.5[c,d]	3.2	4.5–21.3
NATCOL		
Mean	0.06	0.03–0.22
P95 or P97.5[c,d]	0.11	0.09–0.43

bw: body weight; CIAA: Confederation of the Food and Drink Industry of the EU (currently FoodDrinkEurope); EXPOCHI: individual food consumption data and exposure assessment studies for children; NATCOL: Natural Food Colours Association; P95: 95th percentile; P97.5: 97.5th percentile; UK: United Kingdom

[a] Refers to children aged 1.5–4.5 years.

[b] Refers to children aged 1–10 years from Belgium (2–6 years), Czech Republic (4–10 years), Finland (1, 3, 7–9 years), France (3–10 years), Germany (1–10 years), Greece (4–6 years), Italy (1–10 years), the Netherlands (2–6 years), Spain (1–10 years) and Sweden (3–10 years).

[c] For the United Kingdom populations, 97.5th percentile exposure estimates were based on the 97.5th percentile consumption of beverages plus average consumption for the rest of the diet.

[d] For children (1–10 years), 95th percentile exposure estimates were based on the 95th percentile consumption of one food category and average consumption for the rest of the diet.

Source: EFSA (2012a)

Table 17

Dietary exposure to β-carotene from its use as a food additive in the European population (sponsor submission)

Scenario[a] and exposure level[b]	Dietary exposure (mg/kg bw per day)[c]					
	Infants (<1 year)	**Toddlers** (1–2 years)	**Other children** (3–9 years)	**Adolescents** (10–17 years)	**Adults** (18–64 years)	**Elderly** (≥65 years)
Maximum usage scenario						
Mean	0.08–0.23	0.26–0.54	0.23–0.50	0.09–0.28	0.05–0.15	0.04–0.17
High	0.28–0.63	0.54–1.00	0.45–0.90	0.18–0.49	0.11–0.34	0.10–0.38
Brand-loyal scenario						
Mean	0.04–0.09	0.08–0.28	0.07–0.24	0.04–0.14	0.03–0.09	0.02–0.08
High	0.09–0.27	0.16–0.45	0.13–0.45	0.06–0.27	0.05–0.20	0.06–0.19
Non-brand-loyal scenario						
Mean	0.03–0.07	0.07–0.19	0.06–0.15	0.03–0.08	0.02–0.06	0.02–0.05
High	0.09–0.20	0.12–0.28	0.10–0.28	0.05–0.15	0.04–0.12	0.03–0.09

bw: body weight

[a] For a description of the scenarios, see text.

[b] High exposure: 95th percentile.

[c] Range represents the lowest to highest country estimate in each age group across dietary surveys.

most to the exposure. Therefore, this food category was mapped to the maximum use level in the brand-loyal scenario. In the non-brand-loyal scenario, the food

categories contributing most to the mean exposure were "Pasta", "Processed fruit and vegetables" and "Flavoured fermented milk products".

In 2004, the exposure to β-carotene was calculated based on food consumption data for adults from France, Germany and the United Kingdom and use levels in processed foods, fortified foods and food supplements (Tennant et al., 2004). The use levels were confirmed by chemical analysis. The dietary exposure ranged from 0.53 mg/day at the mean up to 1.9 mg/day at the 97.5th percentile. The main sources of exposure to β-carotene were carbonated soft drinks and dilutable soft drinks. In this study, food manufacturers indicated that β-carotene is used only in yellow/orange drinks and that these drinks cover 15% of the European Union market for non-alcoholic flavoured drinks.

3.1.2 **β-Apo-8′-carotenal**

In 2014, EFSA performed a dietary exposure assessment for β-apo-8′-carotenal using MPLs as specified in Annex II of Regulation (EC) No. 1333/2008, reported use levels, analytical concentrations and individual food consumption data from the Comprehensive European Food Consumption Database (EFSA, 2014). At that time, the Comprehensive European Food Consumption Database contained consumption data from 26 dietary surveys from 17 European countries covering toddlers (1–2 years), children (3–9 years), adolescents (10–17 years) and adults (18 years and above).

According to Annex II of Regulation (EC) No. 1333/2008, the use of β-apo-8′-carotenal is permitted in 37 food categories with MPLs ranging from 50 to 500 mg/kg and in two food categories at quantum satis. Typical (mean) and maximum use levels were provided for 13 food categories by the food industry. Cyprus provided 47 analytical concentrations in soft drinks. EFSA estimated the dietary exposure to β-apo-8′-carotenal using only MPLs and the provided use levels according to two refined exposure scenarios:

1) the maximum use level per food category (UL1);
2) the maximum use level per food category, supplemented with MPLs for those food categories with no use levels (UL2).

The analytical data were not used.

EFSA calculated the exposure for five age groups in the Comprehensive European Food Consumption Database. For this, adults were divided into the age groups 18–64 years and 65 years and above. The exposure estimates are reported in Table 18. Important sources of exposure in all scenarios were the food categories "Fine bakery wares" and "Flavoured fermented milk products".

The food categories included in the UL1 assessment were the same as those for which the sponsor proposed use levels (Table 15), except for food

Table 18

Dietary exposure to β-apo-8′-carotenal from its use as a food additive in the European population

Scenario[a] and exposure level[b]	Dietary exposure (mg/kg bw per day)[c]				
	Toddlers (1–2 years)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	Adults 65+ (≥65 years)
Maximum permitted levels					
Mean	1.0–3.9	1.0–3.3	0.5–1.7	0.3–1.1	0.2–0.6
High	2.8–7.4	2.4–6.7	1.0–3.4	0.8–2.4	0.6–1.5
UL1					
Mean	0.02–0.25	0.02–0.24	0.02–0.14	0.01–0.09	0.01–0.06
High	0.06–0.49	0.07–0.46	0.07–0.32	0.04–0.20	0.04–0.14
UL2					
Mean	0.9–2.5	0.7–2.2	0.2–0.8	0.2–0.5	0.1–0.4
High	2.4–6.1	1.4–5.1	0.5–1.8	0.4–1.2	0.4–0.9

bw: body weight; UL: use level
[a] For a description of the UL1 and UL2 scenarios, see text.
[b] High exposure: 95th percentile.
[c] Range represents the lowest to highest country estimate in each age group across dietary surveys.
Source: EFSA (2014)

category "05.4 Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces". This food category was not included in the EFSA assessment, because the maximum use level of this food category could not meaningfully be mapped to any of the foods recorded in the Comprehensive European Food Consumption Database (EFSA, 2014). However, EFSA noted that the foods covered by this food category were included in the assessment via other food categories. The maximum use levels used in the UL1 (and UL2) scenario were identical to those proposed by the sponsor (Table 15).

3.2 Overview of estimates of dietary exposure to β-carotene and β-apo-8′-carotenal

Table 19 summarizes the dietary exposures to β-carotene and β-apo-8′-carotenal from their use as food additives in Europe. The exposure estimates for β-carotene by EFSA obtained with the use levels of the CIAA were not included, because the Committee considered the estimates obtained with the data reported by NATCOL to better reflect actual exposure (section 3.1.1). The Committee also considered the exposure estimates of the UL1 scenario for β-apo-8′-carotenal to better reflect actual exposure compared with the UL2 scenario (section 3.1.2).

The dietary exposures to β-carotene and β-apo-8′-carotenal estimated by EFSA and with FAIM very likely overestimate the actual dietary exposures to

Table 19

Overview of dietary exposure estimates for β-carotene and β-apo-8′-carotenal from their use as food additives in the European population

Source and concentration data	Dietary exposure (mg/kg bw per day)[a]			
	β-Carotene		β-Apo-8′-carotenal	
	Mean	High	Mean	High
EFSA				
MPLs	–	–	0.2–3.9	0.6–7.4[b]
Use levels[c,d]	0.03–0.22	0.09–0.43[e]	0.01–0.25	0.04–0.49[b]
EFSA FAIM				
MPLs	0.04–0.54	0.10–1.00[b]	–	–
Use levels				
Brand loyal	0.02–0.28	0.05–0.45[b]	–	–
Non–brand loyal	0.02–0.19	0.03–0.28[b]	–	–
France, Germany, United Kingdom[f]				
Use levels	0.01	0.03[g]	–	–

bw: body weight; EFSA: European Food Safety Authority; FAIM: Food Additives Intake Model; MPL: maximum permitted level; NATCOL: Natural Food Colours Association
[a] Range represents the lowest to highest country estimate.
[b] High exposure: 95th percentile.
[c] Maximum use levels for β-carotene from NATCOL (Table 16).
[d] Maximum use levels for β-apo-8′-carotenal according to UL1 scenario (Table 18).
[e] High exposure: 95th or 97.5th percentile.
[f] Exposure estimated by the present Committee using a 60 kg adult body weight.
[g] High exposure: 97.5th percentile.
Sources: EFSA: EFSA (2012a, 2014); EFSA FAIM: sponsor submission; France, Germany, United Kingdom: Tennant et al. (2004)

these food additives. In all exposure scenarios, all foods in a given food category were assumed to contain the food additive at the typical or maximum use level. In reality, not all foods will be yellow coloured, and, for those that are, colours other than β-carotene are also available. Additionally, EFSA pointed out that β-apo-8′-carotenal is a niche colour for very specific applications (e.g. adjusting the shade of orange colour resulting from the use of other colours, mainly β-carotene) and that the information of the food industry provided "some evidence that β-apo-8′-carotenal is only used in 0.8 % of all products in the category 'flavoured drinks with sugar' or 'flavoured drinks with sweeteners'" (EFSA, 2014). Tennant et al. (2004) noted that food manufacturers indicated that β-carotene is used only in yellow/orange drinks and that these drinks cover 15% of the European Union market for non-alcoholic flavoured drinks.

4. Comments

4.1 Biochemical aspects

4.1.1 β-Carotene

β-Carotene is absorbed into enterocytes and centrally cleaved to give two retinal molecules. Retinal is reduced to retinol by the enzyme retinaldehyde reductase and then esterified to form retinyl esters by lecithin:retinol acyltransferase and packaged with chylomicrons. Chylomicrons containing retinyl esters are released into the lymph and then the bloodstream and rapidly taken up into the liver (reviewed in Blomhoff et al., 1991; Blomhoff, Green & Norum, 1992). Although the mechanism of intestinal β-carotene absorption and metabolism appears to be comparable in animal models and humans, marked differences in cleavage rates and consequently bioavailability between species have been shown (Ribaya-Mercado et al., 1992; Van Vliet, Schreurs & Van den Berg, 1995; During, Albaugh & Smith, 1998; During et al., 1999, 2001; Woutersen et al., 1999).

In a short-term toxicity study in rats administered β-carotene at a dose of 0, 250, 500 or 1000 mg/kg bw per day for 13 weeks, plasma β-carotene concentrations ranged from 0.3 to 0.9 μg/mL (Buser & Arceo, 1995). In other studies in rats administered β-carotene at doses up to 1000 mg/kg bw per day for up to 21 weeks, plasma β-carotene concentrations ranged from below the limit of detection to about 0.2 μg/mL (Shapiro, Mott & Machlin, 1984; Wamer, Giles & Kornhauser, 1985; Ribaya-Mercado et al., 1989). More than 95% of radioactivity in plasma and approximately 88–94% of radioactivity in liver were identified as retinol in rats administered 0.5 mg (0.74 MBq) radiolabelled β-carotene. β-Carotene was not detected in plasma (Krinsky et al., 1990).

In human subjects, the absorption of β-carotene has been estimated to be in the range of 40–65% (Dueker et al., 2000; Ho et al., 2007, 2009). In human subjects administered radiolabelled β-carotene, radioactivity in lymph was mainly associated with chylomicrons as retinyl esters, with approximately 20–30% of the absorbed radioactivity recovered as β-carotene (Goodman et al., 1966; Blomstrand & Werner, 1967). Following the administration of ^{13}C-labelled β-carotene to humans, most of the absorbed dose was converted to vitamin A (Parker et al., 1993). Excretion of radioactivity occurred mainly via the faeces, with smaller amounts in the urine (Dueker et al., 2000; Ho et al., 2007, 2009).

A number of studies in human subjects also investigated plasma levels of β-carotene following dosing for up to 12 years with pharmacological amounts of β-carotene. The most informative of these were a number of randomized controlled trials. Mean or median plasma β-carotene levels increased from 0.3 to 1.2 μg/mL in subjects administered 50 mg β-carotene every second day

(Hennekens et al., 1996); from 0.17 to 3.0 µg/mL in subjects administered 20 mg β-carotene per day (ATBC Study Group, 1994); and from 0.15 to 2.1 µg/mL in subjects administered 30 mg β-carotene per day with 25 000 IU vitamin A (Omenn et al., 1996a,b).

Based on the observed differences in cleavage rates and bioavailability of β-carotene between rats and humans, the Committee reaffirmed the conclusion of the eighty-fourth meeting that this species is not suitable for the evaluation of β-carotene in humans. Absorption and tissue disposition studies with β-carotene in mice or dogs were not available to the Committee.

4.1.2 β-Apo-8′-Carotenal

Radiolabelled β-apo-8′-carotenal and its metabolites were at least 25% absorbed from the gastrointestinal tract of rats. Total radioactivity in plasma reached a peak concentration after 10 hours and was eliminated with a half-life of 21 hours. β-Apo-8′-carotenal and its metabolites β-apo-8′-carotenol, β-apo-8′-carotenoic acid and fatty acid conjugates were identified in the plasma. Radioactivity was recovered in the liver as retinol and fatty acid conjugates of retinol, demonstrating conversion of β-apo-8′-carotenal to vitamin A. Elimination of radioactivity occurred mainly via faeces, with smaller amounts excreted in the urine (Rümbeli, Ringenbach & Elste, 2007).

A clear sex-related difference was seen in a 13-week toxicity study in which female rats showed higher concentrations of β-apo-8′-carotenal and/or its metabolites in the plasma and liver compared with males (Edwards et al., 2007).

β-Apo-8′-carotenal did not appear in plasma in significant amounts in human male volunteers given a single oral dose of 41 mg β-apo-8′-carotenal. β-Apo-8′-carotenol and β-apo-8′-carotenyl palmitate were identified as the two major metabolites in the plasma and reached their maximum concentrations of 0.29 and 0.23 µmol/L at 11 and 6 hours, respectively. 3-Apo-8′-carotenoic acid was also detected in serum, but the concentrations were not determined (Zeng, Furr & Olson, 1992).

4.2 Toxicological studies

4.2.1 β-Carotene

β-Carotene has low acute oral toxicity in rats and dogs (Nieman & Klein Obbink, 1954; Buser, 1992; Strobel, 1994; Kluifthooft, 2001).

No target organ toxicity was observed in short- or long-term studies in rats or dogs administered β-carotene (Buser & Hummler, 1983a,b; Hummler & Buser, 1983; Buser & Arceo, 1995; Van Beek, Catsburg & Wijnans, 1997; Nabae et al., 2005).

β-Carotene was not carcinogenic in mice or rats (Buser & Hummler, 1983a; Hummler & Buser, 1983).

β-Carotene was not genotoxic in vitro or in vivo (Kluifthooft, 2001; Weimans, 2003; Cognis Deutschland GmbH & Co. KG, 2006a,b).

There was no evidence of reproductive or developmental toxicity in studies in rats or rabbits (Kistler, 1981, 1982; Buser & Hummler, 1982).

4.2.2 β-Apo-8′-carotenal

β-Apo-8′-carotenal has low acute oral toxicity in mice (Anonymous, 1966) and rats (Loget & Arcelin, 2006a,b).

Two new short-term studies in rats were available to the Committee. In a 28-day study, rats were given β-apo-8′-carotenal in the feed at a target dose of 0, 20, 100 or 500 mg/kg bw per day. A NOAEL of 100 mg/kg bw per day was established on the basis of reduced body weight and body weight gain in rats at 500 mg/kg bw per day (Loget & Morgan, 2006). The Committee noted the presence of eosinophilic droplets mainly in the kidneys of female rats, but did not consider the finding to be adverse on the basis that the droplets were not linked to other lesions or any other signs of nephropathy.

In a follow-up 90-day study, male and female rats were administered β-apo-8′-carotenal in the feed at a target dose of 0, 10, 30 or 100 mg/kg bw per day. Liver weight and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly increased in female rats at the high dose relative to controls. Upon histopathological examination, a significant increase in the incidence of inflammatory cell foci was seen in the liver of female rats at 100 mg/kg bw per day. In the kidney, an increase in the incidence of minimal eosinophilic droplets was observed in females at all doses, increasing in severity in high-dose females, but there was no evidence of necrosis or single-cell death at any dose. Findings of tubular injury were generally limited to the occasional tubular epithelial cell containing eosinophilic material appearing to be detached from the tubule and the presence of mitotic figures in the cortex in males and females at 100 mg/kg bw per day (Edwards et al., 2007). The Committee identified a NOAEL of 30 mg/kg bw per day, on the basis of increased liver weight, serum ALT and AST activities and incidence of inflammatory cell foci in the liver of high-dose female rats and evidence of tubular injury in the kidney of high-dose males and females. The Committee noted that higher plasma and liver concentrations of β-apo-8′-carotenal or its metabolites were achieved in female rats compared with males and considered this to be consistent with the toxicological findings of this study.

No new long-term toxicity or carcinogenicity studies were available to the Committee. In an early study, rats were administered β-apo-8′-carotenal in the

diet at 1000 mg/kg feed to 1) a first generation of rats for 2 years, 2) their offspring for 2 years and 3) a third generation of rats for 1 year. The average dose over the course of the study was reported to be 40 mg/kg bw per day. Histopathological examination of the liver and kidneys of treated animals did not identify any adverse effects (Schärer & Studer, 1961).

β-Apo-8′-carotenal was not mutagenic in *Salmonella typhimurium* strain TA98, TA100, TA102, TA1535 or TA1537 at concentrations of 8.7–277.9 μg/plate, with or without metabolic activation (Loget & Johnson, 2006). In an earlier study, conducted with β-apo-8′-carotenal of low purity (69.2%), a concentration-related increase in the number of revertants was observed only in strain TA100, in the absence and presence of metabolic activation. The study authors noted that this result could be associated with impurities in the test material (Engelhardt & Hoffmann, 1998). In an in vitro mammalian chromosomal aberration test, an increased frequency of chromosomal aberrations was observed in the absence and presence of metabolic activation at concentrations associated with approximately 50% cytotoxicity or greater, but not at lower concentrations, where moderate to low cytotoxicity was observed. The study authors noted that these increases were of questionable biological relevance given that they were observed only at doses associated with significant cytotoxicity (Loget & Whitwell, 2006). In an in vivo mammalian erythrocyte micronucleus test, treatment with β-apo-8′-carotenal did not induce statistically significant increases in the frequency of micronucleated erythrocytes compared with the concurrent controls at doses up to 800 mg/kg bw per day (Loget & Beevers, 2006). The Committee concluded that the weight of evidence suggests that there is no concern for genotoxicity of β-apo-8′-carotenal.

No reproductive toxicity studies were available to the Committee. In a GLP- and guideline-compliant developmental toxicity study, rats were administered β-apo-8′-carotenal in the feed from GDs 6 to 20 at a dose of 0, 20, 100 or 495 mg/kg bw per day. No maternal or developmental toxicity was observed. The NOAEL for maternal toxicity and for embryo and fetal toxicity was 495 mg/kg bw per day, the highest dose tested (Loget et al., 2006).

4.3 **Observations in humans**

The association between β-carotene intake and cancer risk has been evaluated in a number of observational studies and extensively reviewed (Block, Patterson & Subar, 1992; Steinmetz & Potter, 1996; Ziegler, Mayne & Swanson, 1996; Woutersen et al., 1999). It was concluded that intake of β-carotene and fruits and vegetables appears to confer protection against cancers at different sites, with the most consistent effect being a protective effect against lung cancer. Consequently,

a number of large, high-quality randomized controlled trials have investigated whether β-carotene supplementation at doses of 20–50 mg/day for durations of up to 12 years reduces cancer risk in human populations.

In the ATBC Study, a higher incidence of lung cancer (RR 1.18; 95% CI: 1.03–1.36) and total mortality (RR 1.08; 95% CI: 1.01–1.16) was observed among the men who received β-carotene at doses of 20 mg/day for between 5 and 8 years. The elevated risk was related to those who smoked at least one pack of cigarettes per day and was not seen in subjects who smoked less (ATBC Study Group, 1994; Woutersen et al., 1999). In CARET, participants who were smokers or ex-smokers, or were exposed to asbestos, were given daily doses of 30 mg β-carotene and 25 000 IU vitamin A as retinyl palmitate for 5 years. Lung cancer incidence and total mortality were increased by 28% (RR 1.28; 95% CI: 1.04–1.57) and 17% (RR 1.17; 95% CI: 1.03–1.33), respectively, in the supplemented group (Omenn et al., 1996a,b).

The Committee noted that the effects observed in heavy smokers and asbestos workers in the ATBC and CARET studies were not seen in population subgroups that were not at increased risk of lung cancer. In the Physicians' Health Study, β-carotene administered to subjects at 50 mg every second day for a period of 12 years did not affect the number of cases of lung cancer, mortality from cancer, all-cause mortality, cardiovascular disease, myocardial infarction or stroke (Hennekens et al., 1996). No effects on cancer incidence or total mortality were seen in a number of other smaller randomized controlled trials in which β-carotene was administered at doses of up to 50 mg/day for durations of up to approximately 9 years (Greenberg et al., 1990; Green et al., 1999; Mayne et al., 2001; Lin et al., 2009; Song et al., 2009; Hughes et al., 2013).

4.4 Assessment of dietary exposure

β-Carotene and β-apo-8′-carotenal are proposed by the sponsor for use at typical and maximum use levels in 33 and 12 food categories of the Codex GSFA, respectively. For β-carotene, the typical (mean) and maximum use levels ranged from 1 to 20 mg/kg and from 2 to 70 mg/kg, respectively. Corresponding ranges for β-apo-8′-carotenal were 0.4–50 mg/kg and 0.4–260 mg/kg. Currently, both food additives are authorized for use in 79 food categories at MPLs ranging from 50 mg/kg up to 1200 mg/kg, as specified in the GSFA (FAO/WHO, 2018a).

The Committee used the exposure estimates submitted by the sponsor, which more closely represent actual exposure. These estimates were based on use levels combined with food consumption data from Europe (EFSA, 2012a, 2014) and on a study on dietary exposure to β-carotene based on food consumption data from France, Germany and the United Kingdom (Tennant et al., 2004).

Table 20

Overview of dietary exposure estimates for β-carotene and β-apo-8′-carotenal from their use as food additives in the European population

Source	Dietary exposure (mg/kg bw per day)			
	β-Carotene		β-Apo-8′-carotenal	
	Mean	**High**	**Mean**	**High**
EFSA[a]	0.03–0.22	0.09–0.43[b]	0.01–0.25	0.04–0.49[c]
EFSA FAIM[d]	0.02–0.19	0.03–0.28[c]	–	–
France, Germany, United Kingdom[e,f]	0.009	0.03[g]	–	–

bw: body weight; EFSA: European Food Safety Authority; FAIM: Food Additives Intake Model
[a] All age groups; maximum use levels.
[b] High exposure: 95th or 97.5th percentile.
[c] High exposure: 95th percentile.
[d] All age groups; typical (mean) use levels.
[e] Adults; typical (mean) use levels.
[f] Exposure estimated by the present Committee using a 60 kg adult body weight.
[g] High exposure: 97.5th percentile.
Sources: EFSA: EFSA (2012a, 2014); EFSA FAIM: sponsor submission; France, Germany, United Kingdom: Tennant et al. (2004)

Furthermore, the sponsor also reported on the exposure to β-carotene calculated with the EFSA FAIM. The dietary exposure estimates are listed in Table 20.

The Committee concluded that the exposure to β-carotene from its use as a food additive at typical (mean) use levels estimated with EFSA FAIM is appropriate for use in risk assessment. The upper level of 0.28 mg/kg bw per day refers to the exposure in children aged 1–9 years. For adults aged 18 and above, the upper level of exposure to β-carotene equals about 0.1 mg/kg bw per day. The Committee acknowledged that these dietary exposure estimates were overestimations due to the assumption that β-carotene is used in all foods belonging to the relevant food categories.

The Committee considered that the high daily exposure estimate for β-apo-8′-carotenal of 0.49 mg/kg bw per day overestimates the exposure to this additive, owing to the assumption that all foods contained the additive at the maximum use level. The Committee therefore concluded that the high daily dietary exposure to β-carotene of 0.28 mg/kg bw per day may also be used for risk assessment of β-apo-8′-carotenal.

5. Evaluation

The Committee reaffirmed the conclusion from the eighty-fourth meeting that rats are not an appropriate model for deriving an ADI for β-carotene due to the relatively low bioavailability of β-carotene in rats compared with humans.

Therefore, the Committee withdrew the two group ADIs of 0–5 mg/kg bw for 1) the sum of the synthetic carotenoids β-carotene, β-apo-8′-carotenal and β-apo-8′-carotenoic acid methyl and ethyl esters and 2) synthetic β-carotene and β-carotene derived from *B. trispora*, which were based on a NOAEL from a rat study.

The Committee considered that no adverse health effects were observed in the general population in large, well-conducted human intervention studies in which healthy participants were administered between 20 and 50 mg β-carotene per day for up to 12 years, in addition to the background exposure from the diet.

An additional elevated risk of lung cancer and total mortality was seen in heavy smokers (at least one pack per day) and asbestos workers in intervention studies in which participants were administered 20 mg β-carotene per day for 5–8 years or 30 mg β-carotene per day and 25 000 IU vitamin A for 5 years. The Committee noted that a generally accepted explanation for the cause of these effects has not been identified. The Committee was unable to reach any conclusion about risk from β-carotene exposure in heavy smokers.

For the remainder of the general population, the Committee concluded that the estimated high exposure to β-carotene at 9 mg/day for a 30 kg child and 6 mg/day for a 60 kg adult from its current uses as a food additive, in addition to background exposure from the diet, would not be expected to be a safety concern. This conclusion includes synthetic β-carotene, β-carotene derived from *B. trispora* and β-carotene-rich extract from *D. salina*.

The Committee was unable to establish a group ADI for synthetic β-carotene, β-carotene derived from *B. trispora*, β-carotene-rich extract from *D. salina* and β-apo-8′-carotenoic acid methyl and ethyl esters because a group ADI is applicable to the general population, which includes heavy smokers. The Committee noted that it is very unlikely that it will ever be possible to establish a group ADI because further data from the heavy smoker population cannot be gathered ethically.

Because β-apo-8′-carotenoic acid methyl and ethyl esters were previously evaluated on the basis of β-carotene and because no new data were submitted, the Committee was unable to complete an evaluation on β-apo-8′-carotenoic acid methyl and ethyl esters.

The present Committee established an ADI of 0–0.3 mg/kg bw for β-apo-8′-carotenal on the basis of a NOAEL of 30 mg/kg bw per day in a 13-week study in rats and application of an uncertainty factor of 100. An additional uncertainty factor to take into account the short duration of the study was not considered necessary because renal injury and hepatic lesions observed in the 13-week study at 100 mg/kg bw per day were not observed in the 2-year study at 40 mg/kg bw per day, the single dose tested.

Estimated dietary exposure to β-apo-8′-carotenal of 0.28 mg/kg bw per day was at the upper end of the ADI established by the Committee (i.e. 0–0.3 mg/kg bw). The Committee noted that the estimated dietary exposure is overestimated and concluded that the current use of β-apo-8′-carotenal as a food additive will not pose a safety concern.

The specifications for β-carotene, synthetic, β-carotene from *B. trispora* and β-apo-8′-carotenal were revised to replace an identification test for carotenoids with additional spectrophotometric requirements.

β-Carotene-rich extract from *D. salina* was on the agenda of the current meeting at the request of the Fiftieth Session of CCFA (FAO/WHO, 2018b) to revise the maximum limit on arsenic. The Committee received sufficient analytical data. Based on the arsenic levels from several batches of the product of commerce, the existing specifications were revised from 1 mg/kg to 3 mg/kg. The Chemical and Technical Assessment was revised.

5.1 Recommendations

The Committee noted that the use levels of β-carotene and β-apo-8′-carotenal provided by the sponsor were much lower than the corresponding MPLs as specified in the GSFA, and that the sponsor indicated that the majority of the MPLs are not justifiable from a technological point of view. Also, use levels were not provided for all authorized food categories. The Committee recommended that the Codex Alimentarius Commission should review current uses of β-carotene (synthetic β-carotene, β-carotene from *B. trispora* and β-carotene-rich extract from *D. salina*) and β-apo-8′-carotenal in the GSFA, including the MPLs and the food categories in which these food additives may be used.

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Gellan gum

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1. Explanation

Gellan gum (International Numbering System for Food Additives [INS] 418; Chemical Abstracts Service No. 71010-52-1) is used as a gelling agent, stabilizer and thickener in a wide range of foods and beverages listed in the Codex General Standard for Food Additives (GSFA), under the conditions of good manufacturing practice. It is commercially available in three different forms – namely, high-acyl, low-acyl and low-acyl clarified.

Gellan gum was previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives at its thirty-seventh meeting, at which an acceptable daily intake (ADI) "not specified" was established (Annex 1, reference *95*). This ADI "not specified" was based on the absence of adverse effects in toxicological studies in mice, rats, dogs and prepubertal rhesus monkeys and in a limited study on tolerance of gellan gum in humans. The Committee pointed out that the potential laxative effect of gellan gum at high dietary exposures should be taken into account when gellan gum is used as a food additive (Annex 1, reference *95*).

Gellan gum was evaluated by the Committee at its forty-ninth and seventy-ninth meetings for revision of specifications only (Annex 1, references *124* and *222*). At the seventy-ninth meeting, the Committee evaluated a request to include ethanol as an additional extraction solvent during the processing of gellan gum. The Committee at that meeting included ethanol in the specifications monograph and established a numerical limit of 50 mg/kg for residual ethanol (Annex 1, reference *222*).

At the present meeting, the Committee evaluated gellan gum for use in formulas for special medical purposes for infants (GSFA food category 13.1.3; referred to as "FSMPs" below) and re-evaluated the limit for residual ethanol in the specifications of gellan gum, at the request of the Fiftieth Session of the Codex Committee on Food Additives (CCFA) (FAO/WHO, 2018). Although the request from CCFA included the use of gellan gum in infant formula (GSFA food category 13.1.1) and follow-up formula (GSFA food category 13.1.2), only data supporting the use of gellan gum in FSMPs were received. Therefore, the Committee did not evaluate the use of gellan gum in infant formula or follow-up formula.

The low-acyl clarified form of gellan gum would be added directly to ready-to-feed FSMPs or would be used as a component of concentrated liquid fortification products[1] formulated with hydrolysed protein and/or amino acids (for addition to human milk or infant formula). According to the sponsor, these liquid fortification products also belong to food category 13.1.3. Gellan gum would be used to increase thickness and maintain homogeneity for better delivery of nutrients to the infant. It would also be used as a component of a stabilizer system, which contains octenyl succinic anhydride–modified corn starch (starch sodium octenyl succinate) (INS 1450). The target gellan gum concentration in the fed products (FSMPs, fortified human milk or fortified infant formula) is approximately 40 mg/L. Owing to manufacturing variability, the maximum gellan gum concentration requested is 50 mg/L.

At the present meeting, the Committee considered the submitted data, including new unpublished and published studies. A comprehensive literature search on gellan gum in PubMed did not identify any additional relevant published studies on biochemical or toxicological aspects. The literature search was conducted in February 2019 using the PubMed database of the United States National Library of Medicine. Use of the linked search terms "gellan" and "food" yielded 156 references, of which five were potentially relevant. Use of the linked search terms "gellan" and "gastrointestinal" yielded 27 references, of which four were potentially relevant. Three of these were also found using "gellan" and "food" as search terms, resulting in a total of six potentially relevant references. These six references were also submitted by the sponsors. Studies from the previously published toxicological monograph, new studies that had become available since the thirty-seventh meeting and older studies not previously reviewed by the Committee are described below in a consolidated monograph.

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[1] Nutritional supplements designed to increase the total energy, protein and micronutrient delivery to preterm infants (Kim et al., 2015).

Fig. 1
Chemical structure of gellan gum backbone
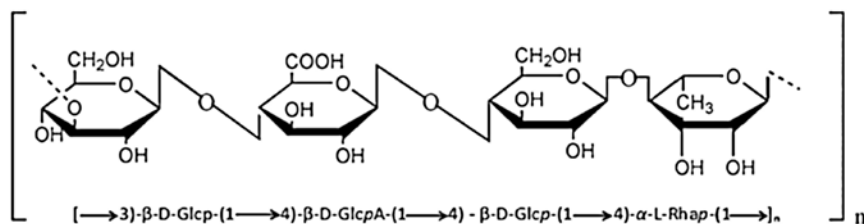
1.1 **Chemical and technical considerations**

Gellan gum is a high-molecular-weight (>500 000 Da) anionic polysaccharide that is produced by a controlled pure culture fermentation of the non-pathogenic Gram-negative bacterium *Pseudomonas elodea* (reclassified as *Sphingomonas elodea*) in the presence of a carbon source, a nitrogen source and inorganic salts. The fermentation broth is pasteurized to kill viable cells, and the gellan gum is recovered via precipitation with food-grade isopropanol or ethanol to obtain the high-acyl form (native gellan gum). Controlled treatment with hot alkali prior to alcohol precipitation results in deacylation and yields gellan gum with varying degrees of acylation, including the low-acyl form. Low-acyl gellan gum can be further filtered to obtain low-acyl clarified gellan gum. The gelling properties of the articles of commerce are controlled by the addition of metal ions such as sodium, potassium and calcium to neutralize the glucuronic acid. By-products of fermentation include polyhydroxybutyrate, enzymes and viable cells of the production organism, which are removed and/or inactivated during processing. The resulting gellan gum is separated, dried and milled.

In its native form, gellan gum is linear; it is composed of β-D-glucopyranosyl, β-D-glucuronopyranosyl and L-rhamnopyranosyl units in molar ratios of 2:1:1 (Fig. 1). Native gellan gum also contains an acetyl and a glyceryl group bound to the glucose adjacent to the glucuronic acid residues.

Different types of gellan gum were used in the toxicological studies evaluated at the thirty-seventh meeting (Annex 1, reference *95*) and in the new studies (on experimental animals and humans) available for the current meeting. The gellan gum used in the acute toxicity studies (Coate et al., 1980; Wolfe & Bristol, 1980), the 13-week oral rat study (Batham et al., 1983) and the genotoxicity studies by Robertson, Nichols & Bokelman (1985a,b,c) and Robertson et al. (1985) was the low-acyl form, with greater than 95% polysaccharide content.

The gellan gum samples used in the 52-week study in dogs (Batham, Kalichman & Osborne, 1986), the long-term studies in mice and rats (Batham, Engel & Osborne, 1987a,b), the genotoxicity study by Ivett (1989), the special studies by Gordon (1990a,b) and the studies in human adults (Eastwood, Brydon & Anderson, 1987; Anderson et al., 1990) were a blend of five products containing 58.5% polysaccharide (no further information available) with varying degrees of acylation. Low-acyl clarified gellan gum was used in the study in neonatal pigs (Lanphear, 2016), the clinical trials in infants (Barrett-Reis, 2013; Thoene et al., 2016; Kumar et al., 2017; Schanler et al., 2018) and the commercial products on which the post-marketing surveillance data were available (Mako, 2015, 2016, 2017). The specific purity of the batches used was not provided. Based on the certificates of analyses of three representative batches submitted, these products are expected to contain greater than 94% polysaccharide. Characterization information on the gellan gum used in the other studies described below was not available.

2. Biological data

2.1 Biochemical aspects

2.1.1 In vivo

At the thirty-seventh meeting (Annex 1, reference *95*), the Committee evaluated the results of three separate experiments in which rats were orally administered single doses of radiolabelled gellan gum to assess the kinetics of gellan gum. The absorption, distribution and excretion of gellan gum were studied using a dually radiolabelled (^{3}H and ^{14}C) preparation. The use of dual labelling allowed simultaneous quantification of both polysaccharide and "protein" fractions of gellan gum. The gellan gum was prepared in separate fermentations using ^{3}H-labelled glucose or ^{14}C-labelled glucose as the carbon source. The ^{3}H product was subjected to a multistage purification process to give a relatively pure ^{3}H-labelled polysaccharide. This was added to the medium of the ^{14}C fermentation and was then precipitated in isopropanol to yield a product with the polysaccharide fraction labelled with both isotopes and the non-polysaccharide (or "protein") fraction labelled only with ^{14}C.

In the first experiment, one male and one female Sprague Dawley rat were gavaged with single doses of ^{3}H/^{14}C-labelled gellan gum (approximately 960 mg/kg body weight [bw]; approximately 148 kBq). Expired air was collected

for 24 hours after dosing. Less than 0.55% of the administered radioactivity was detected as ^{14}C.

In the second experiment, four male and three female Sprague Dawley rats were dosed with a single gavage dose of ^{3}H/^{14}C-labelled gellan gum (approximately 870 mg/kg bw; 107–152 kBq ^{14}C; 25.9–33.3 kBq ^{3}H). Urine and faeces were collected for 7 days, at which time the animals were sacrificed and their tissues analysed for residual radioactivity. Females excreted 86.8% and 1.9% of the administered ^{14}C in their faeces and urine, respectively. Males excreted 86.0% of the dosed ^{14}C in the faeces and 3.3% in the urine. Females excreted 4.1% of the dosed ^{3}H in their urine and 100.1% in their faeces, whereas males excreted 3.6% of the total ^{3}H in their urine and 99.6% in their faeces. In all animals, the activities of ^{3}H in tissues (blood, brain, liver, kidney, lung, muscle, skin, heart and carcass) were too low to be quantified accurately. Tissue and carcass ^{14}C radioactivity averaged 3.8% of the dose for male rats and 3.0% of the dose for female rats.

In the third experiment, one male and four female Sprague Dawley rats were gavaged with radiolabelled gellan gum at a dose of about 1000 mg/kg bw, and blood samples were collected from the tail vein at different time intervals over a 7-day period. Data were reported as ^{14}C disintegrations per minute per millilitre blood (^{3}H disintegrations per minute per millilitre blood were not reported). The peak level of radioactivity, which amounted to about 0.4% of the administered radioactivity, occurred about 5 hours after dosing (Selim, 1984).

These three experiments indicate that gellan gum is poorly absorbed in rats following oral exposure and is primarily excreted in the faeces. Based on the results of the short-term toxicity studies in rats described in section 2.2.2(a) below (Edwards & Eastwood, 1995; Shimizu et al., 1999), gellan gum produces lower levels of short-chain fatty acids (SCFAs) in the colon compared with other dietary fibres (e.g. cellulose) known to be degraded by the intestinal microflora. Therefore, gellan gum is not as readily degraded by intestinal bacteria. Additionally, gellan gum has been shown to significantly increase stool output and decrease gastrointestinal transit time in rats (Edwards & Eastwood, 1995; Tetsuguchi et al., 1997; Shimizu et al., 1999; Innami, Shimizu & Kudoh, 2000).

In addition, the study in human volunteers described in section 2.3.1 below provided no evidence of gellan gum absorption. No changes in blood glucose or insulin levels that would suggest gellan gum digestion and uptake of the monosaccharides were reported. No statistically significant changes in hydrogen or methane levels in breath and no consistent changes in SCFA levels were observed, indicating that gellan gum also was not subject to microbial degradation. Based on the increases observed in faecal weights and faecal water content, gellan gum acted as a faecal bulking agent (Eastwood, Brydon & Anderson, 1987; Anderson, Brydon & Eastwood, 1988). Variable effects on

gastrointestinal transit times were observed (Anderson, Brydon & Eastwood, 1988).

2.1.2 **In vitro**

Adiotomre et al. (1990) investigated the effect of gellan gum on nutrient absorption, sterol metabolism, caecal fermentation and faecal bulking in a series of in vitro studies. In the test material (molecular weight [MW] 500–1000 kDa; purity and level of acylation not specified), one of the glucose residues in the tetrasaccharide repeat unit carried acetic ester groups at C-6 and a glyceric ester group at C-2. Glucose and bile (taurocholic acid) dialysis studies were used to mimic events occurring in the jejunum and the ileum, respectively, whereas fermentation studies with fresh human microbiota were used to mimic events occurring in the large intestine (via SCFA production and water-holding capacity). Compared with the control used in this study (wheat bran), gellan gum reduced glucose and taurocholic acid movement in the dialysis studies. Gellan gum increased the amount of total SCFAs (37.2 vs 15.5 mmol/L) and the water-holding capacity after fermentation (3.08 vs 0.91 g/g). The major SCFAs produced included acetic acid and propionic acid, with smaller amounts of butyric, isobutyric, valeric and isovaleric acids (Adiotomre et al., 1990). The increase in the amount of SCFAs was not confirmed in the in vivo studies described in section 2.1.1 above.

2.2 **Toxicological studies**

2.2.1 **Acute toxicity**

The median lethal dose (LD_{50}) and the median lethal concentration (LC_{50}) for gellan gum (low-acyl form containing >95% polysaccharide) from studies evaluated at the thirty-seventh meeting (Annex 1, reference *95*) are given in Table 1.

Gellan gum is practically non-toxic to rats when administered as a single large oral dose (5000 mg/kg bw).

2.2.2 **Short-term studies of toxicity**

In addition to the short-term studies of toxicity by Batham et al. (1983), Selim (1984) and Batham, Kalichman & Osborne (1986) that were evaluated by the Committee at its thirty-seventh meeting (Annex 1, reference *95*), several other studies on gellan gum are available.

(a) **Rats**

Seven Wistar rats (body weight at study initiation was approximately 150 g) were given a basal diet (45 g non-starch polysaccharides per kilogram feed) for 4

Table 1

Acute toxicity of gellan gum

Species	Sex	Route	LD$_{50}$ / LC$_{50}$	Reference
Rat	Male + female	Oral	>5 000 mg/kg bw	Wolfe & Bristol (1980)
	Male + female	Inhalation	>5.09 mg/L	Coate et al. (1980)

bw: body weight; LC$_{50}$: median lethal concentration; LD$_{50}$: median lethal dose

weeks followed by basal diet supplemented with 50 g gellan gum (MW 500–1000 kDa; purity and level of deacylation not specified) per kilogram feed (equivalent to 5000 mg/kg bw per day) for an additional 4 weeks. A control group of 23 rats was maintained on basal diet for 8 weeks. Feed and water were provided ad libitum. Body weight gain was monitored weekly. During the final 3 days of the study, rats were housed individually in metabolism cages to measure daily feed intake and to collect faeces. At study termination, rats were euthanized, and the caecum and large intestine and their contents were removed, separated and weighed. SCFAs (a byproduct of colonic bacterial fermentation) were analysed in the faecal samples collected on the last 2 days of the study.

Feed consumption (calculated without the contribution of the added non-starch polysaccharides) was statistically significantly increased (+18%) in the treatment group compared with the control group, whereas final mean body weight was lower (−11%; not statistically significant). Gellan gum statistically significantly increased dry (blotted) caecal tissue weight (+26%) and decreased dry caecal content weight (−40%). Caecal pH was slightly, but statistically significantly, increased (+8%). Gellan gum also statistically significantly increased wet and dry faecal output (+87% and +70%, respectively) and absolute faecal water weight (+102%). Dietary administration of gellan gum had no effects on the total amount of SCFAs in the caecum or on daily SCFA faecal output, although gellan gum statistically significantly increased the concentration of total SCFAs in dry caecal tissue (+27%) and decreased the concentration of SCFAs in wet caecal tissue (−29%). Based on levels of individual SCFAs, gellan gum statistically significantly increased caecal levels of acetic acid (+8%) and *n*-butyric acid (+46%) and decreased levels of propionic acid (−34%). In the faeces, only levels of acetic acid were statistically significantly changed (−11%). Gellan gum had a significant effect on stool output (increasing both wet and dry weights), which the authors attributed to "some remaining physical structure" (undigested polysaccharide) (Edwards & Eastwood, 1995).

To study the effects of gellan gum on gastrointestinal function and on surface structure of the intestinal mucosa, two groups of 13 male Sprague Dawley

rats (3 weeks of age) were administered cellulose (control) or gellan gum (purity, molecular weight and level of deacylation not specified) at a dietary concentration of 50 g/kg (equivalent to 5000 mg/kg bw per day) for 4 weeks. Feed and water were provided ad libitum. Body weight and feed consumption were measured every 2 days. Gastrointestinal transit time was measured 5 days prior to completion of the feeding period. At termination, tissue samples from the ileum, caecum and colon were obtained for analysis by electron microscopy, and the weight and pH of the contents of the caecum and colon were measured.

Body weight gains and final body weights were slightly, but not statistically significantly, higher for the treatment group, and feed intakes did not differ between the two groups. Faecal lumps from treated rats were smaller and rounder and had a blacker colour in comparison with the controls. The number of faecal lumps was greater, but total wet weight was not statistically significantly different, in comparison with the controls. The wet weight of caecal contents was statistically significantly lower in the gellan gum group compared with controls (approximately 2.5 g and 3.2 g, respectively), whereas the wet weight of the caecum, pH values and water percentage of caecal contents were not different. Wet colon weights were comparable between the two groups, but the wet weight of the colon contents was statistically significantly increased for gellan gum–fed rats compared with controls (approximately 1.0 g and 0.6 g, respectively). Gastrointestinal transit time was statistically significantly shorter for gellan gum–treated rats compared with controls (approximately 28 and 35 hours, respectively). No morphological changes were reported in the colons of rats treated with gellan gum compared with the control group. In the ileum and caecum, the tops of the microvilli adhered to one another and were covered by intestinal content in rats treated with gellan gum.

The authors considered it probable that gellan gum was not fermented to a significant degree, and they indicated that the higher amount of undigested gellan gum could have formed a highly viscous gel in the gastrointestinal tract, which caused the morphological changes in the intestinal mucosal fine structure (Tetsuguchi et al., 1997).

Two experiments were performed in which groups of seven (experiment 1) or eight (experiment 2) Sprague Dawley rats (4 weeks of age) were given cellulose (control) or gellan gum (MW 600–700 kDa; purity and level of deacylation not specified) at a dietary concentration of 50 g/kg feed (equivalent to 5000 mg/kg bw per day) for 4 weeks. All rats were fed a basal diet for 7 days prior to the study periods. Feed and water were provided ad libitum. Body weight and feed intake were measured weekly. In both experiments, blood samples were taken from the heart at termination to measure total cholesterol and high-density lipoprotein cholesterol serum concentrations. In addition, in experiment 1, liver,

small intestine, caecum, colon and rectum were weighed, and the lengths of the small intestine and colon plus rectum were determined. Caecal content weight and pH were measured. Faecal output, gastrointestinal transit time and faecal bile acid excretion were recorded. Also, liver lipid concentrations were measured. In experiment 2, blood samples were taken from the portal vein and abdominal aorta. The caecum was removed and weighed, and caecal content weight and pH were determined. SCFA and lactic acid levels in caecal contents were analysed. Ammonia concentrations were analysed in portal venous blood and caecal contents.

No effects on body weight were observed. Feed intake was slightly, but statistically significantly, lower (–5%) in rats treated with gellan gum in experiment 1 only. Caecum weight, weight of caecal contents and caecal pH also did not differ between control and test animals in either experiment. Colon plus rectum weights of gellan gum–treated animals were statistically significantly increased in both experiments (+26%); however, no difference was reported in the length of the colon plus rectum. The weight and length of the small intestines of test and control animals were comparable. Gellan gum statistically significantly increased the number of faecal lumps per day (+100%) and moisture content of the faeces (+118%) and statistically significantly decreased the dry weight of the faeces (–14%) and gastrointestinal transit time (–25%). Faecal pH was slightly, but statistically significantly, decreased (–5%). No diarrhoea was reported in any of the animals. No statistically significant differences were reported in blood ammonia concentrations; however, ammonia concentration in the caecal contents was statistically significantly decreased in the treatment group (–52%). No changes were observed in serum cholesterol concentrations, liver weights or liver cholesterol, triacylglycerol and phospholipid concentrations. Faecal bile acid analysis revealed only a statistically significant increase in levels of ω-muricholic acid (+150%) in the treatment group, but total faecal bile acid content was comparable with that of the control group. When SCFA concentrations in total caecal contents were determined, no statistically significant differences were reported; statistically significant reductions in levels of propionic acid and total SCFAs were reported when expressed per gram of caecal contents (–43% and –25%, respectively).

The authors concluded that gellan gum is little degraded or fermented by intestinal bacteria, and the decrease in gastrointestinal transit time suggests that gellan gum promotes evacuation (Shimizu et al., 1999; Innami, Shimizu & Kudoh, 2000).

In a further study by the same group, groups of seven male Sprague Dawley rats (4 weeks of age) were provided cellulose (control) or gellan gum (MW 600–700 kDa; purity and level of deacylation not reported) at a dietary

concentration of 50 g/kg feed (equivalent to 5000 mg/kg bw per day) for 4 weeks. Rats were fed a basal diet for 7 days prior to the study period. Feed and water were provided ad libitum. At termination, blood samples were taken from the heart. The caecum was removed, and its contents were weighed. The pH and SCFA (acetate, propionate and butyrate) and lactate concentrations of the caecal contents were measured. Additionally, caecal microflora count (bifidobacteria, lactobacilli, streptococci, clostridia and Enterobacteriaceae) was determined. Caecal content homogenates were examined for bifidobacteria-proliferating activity in vitro.

No statistically significant differences were reported between the treated rats and the controls. The results of this study suggest that gellan gum does not have any effects on caecal tissue or content weight, pH, SCFA concentrations or microflora growth (Shimizu et al., 2001).

Male and female Sprague Dawley rats (20 of each sex per group) were fed dietary concentrations of gellan gum (low-acyl form containing >95% polysaccharide) ranging up to 60 g/kg feed (equivalent to 6000 mg/kg bw per day) for 13 weeks. Although the animals experienced symptoms of a sialodacryoadenitis viral infection, all survived treatment, and there were no adverse effects associated with the feeding of gellan gum (Batham et al., 1983).

(b) Dogs

Diets containing gellan gum (containing 58.5% polysaccharide) at a concentration of 0, 30, 45 or 60 g/kg feed (equivalent to 0, 750, 1125 and 1500 mg/kg bw per day, respectively, assuming dry laboratory chow diet) were fed to groups of five beagle dogs of each sex for a period of 52 weeks. The dogs were observed daily for clinical signs of toxicity, and body weights and feed consumption were measured. Ophthalmoscopic examinations were performed pretreatment and after 12, 24, 39 and 51 weeks. Haematology and clinical chemistry were measured pretreatment and after 6, 13, 25, 39 and 50 weeks. After 52 weeks, all animals were killed and grossly examined. The following organs and tissues were removed, processed and examined for histopathological lesions: adrenals, aorta, bone and marrow, brain, caecum, colon, duodenum, epididymis, eyes, gall bladder, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes, mammary gland, oesophagus, optic nerves, ovaries and oviducts, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid and parathyroid, tongue, trachea, urinary bladder and uterus.

All animals survived treatment. Feed intake was higher in the treated groups than in the controls. There were no adverse effects associated with the

feeding of gellan gum to beagle dogs for a period of 1 year (Batham, Kalichman & Osborne, 1986).

(c) Rhesus monkeys

Prepubertal rhesus monkeys (two of each sex per group) were dosed by oral gavage with gellan gum at a concentration of 0, 1000, 2000 or 3000 mg/kg bw per day for 28 days. No overt signs of toxicity were reported (Selim, Fuller & Burnett, 1984).

2.2.3 Long-term studies of toxicity and carcinogenicity

Other than the studies evaluated by the Committee at its thirty-seventh meeting (Annex 1, reference 95), no additional long-term studies of toxicity and carcinogenicity with gellan gum are available.

(a) Mice

Groups of 50 male and 50 female Swiss Crl mice were fed gellan gum (containing 58.5% polysaccharide) admixed in the diet at 0, 10, 20 or 30 g/kg feed (equivalent to 0, 1500, 3000 and 4500 mg/kg bw per day, respectively) for 96 weeks for males and 98 weeks for females. All animals were examined twice daily for mortality and morbidity. Physical examination for the presence of palpable masses was initiated on a weekly basis starting in week 26. Body weights and feed consumption were measured for 7-day periods on a weekly basis for the first 26 weeks of treatment and every 2 weeks thereafter. At necropsy, a complete gross pathological examination was performed on the following organs and tissues of the animals from the control and 30 g/kg feed groups: adrenals, aorta (thoracic), bone (sternum), brain (forebrain, midbrain and hindbrain), caecum, colon, duodenum, epididymis, eyes, Harderian gland, heart, ileum, jejunum, kidneys, lacrimal gland, liver (sample of two lobes), lung (sample of two lobes), lymph nodes (mandibular and mesenteric), mammary gland (inguinal), nasal turbinates, oesophagus, optic nerves, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid lobes (and parathyroids), tongue, trachea, urinary bladder, uterus, vagina, Zymbal's gland and all gross lesions. Only the liver, kidneys, ovaries, testes, adrenals, pituitary, lungs and heart were examined for animals of the 10 and 20 g/kg feed groups.

There were no effects on either body weight gain or feed consumption that were attributable to the feeding of gellan gum. No neoplastic or non-neoplastic changes were associated with the feeding of gellan gum (Batham, Engel & Osborne, 1987a).

(b) **Rats**

Groups of 50 F_1 generation Sprague Dawley rats of each sex were exposed to gellan gum (containing 58.5% polysaccharide) in utero and continued on gellan gum diets for approximately 104 weeks. The dietary concentrations of gellan gum were 0, 25, 38 and 50 g/kg feed (equivalent to 0, 1250, 1900 and 2500 mg/kg bw per day, respectively). The rats were observed for clinical signs of toxicity daily for the first 4 weeks of treatment and weekly thereafter. Individual body weights and feed consumption were measured on a weekly basis for the first 26 weeks of treatment and every 2 weeks thereafter. Funduscopic and biomicroscopic examinations were conducted on the control and 50 g/kg feed groups during weeks 1, 13, 26, 52, 78 and 103. Clinical chemistry and haematological samples were collected in weeks 13, 25, 39 and 51. The same organs and tissues as those listed in the description of the mouse study by Batham, Engel & Osborne (1987a) were examined for histopathological changes at study termination.

After 104 weeks, ophthalmoscopic examinations, haematology, clinical chemistry and organ weight data revealed no changes that could be attributed to the feeding of gellan gum. Survival of treated male rats was poor when compared with that of controls, whereas treated female rats exhibited better survival than their concurrent controls. Male rats fed gellan gum at 38 and 50 g/kg feed exhibited lower body weights after 76 weeks. The initial body weights were 5.2% and 3.4% lower than the control values at 38 and 50 g/kg feed, respectively. The authors concluded that in spite of the initial body weight deficit, the growth pattern for these treated groups was identical to that of the controls. In addition, this effect was not seen in either the females or any other species tested. There is no basis to suggest that the lower body weights observed in the male rats are indicative of toxicity.

No neoplastic or non-neoplastic changes that could be associated with the feeding of gellan gum were observed. The authors concluded that under the conditions of this bioassay, gellan gum was non-carcinogenic to Sprague Dawley rats (Batham, Engel & Osborne, 1987b).

2.2.4 Genotoxicity

The Committee previously evaluated three in vitro genotoxicity studies on gellan gum (low-acyl form containing >95% polysaccharide), including a bacterial reverse mutation assay, a DNA repair assay (unscheduled DNA synthesis assay) and a gene mutation assay (V79/hypoxanthine–guanine phosphoribosyltransferase assay) (Robertson, Nichols & Bokelman, 1985a,b; Robertson et al., 1985). All three studies showed no evidence of genotoxicity.

Two additional studies were available for the present evaluation: an alkaline elution assay (Robertson, Nichols & Bokelman, 1985c – summary

Table 2
Results of genotoxicity studies with gellan gum

End-point	Test system	Concentrations/doses	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	10, 30, 100, 300, 1 000 µg/plate	Negative[a]	Robertson et al. (1985)
DNA repair	Rat hepatocytes	3, 5, 10, 20 mg/mL	Negative	Robertson, Nichols & Bokelman (1985b)
Gene mutation	Chinese hamster V79 lung fibroblasts	3, 5, 10, 20 mg/mL	Negative	Robertson, Nichols & Bokelman (1985a)
DNA damage	Rat hepatocyte alkaline elution assay	0.1, 0.3, 1.0 mg/mL	Not valid[b]	Robertson, Nichols & Bokelman (1985c)[c]
In vivo				
Micronucleus induction	Mice, male and female	0, 45, 225, 450 mg/kg bw for 2 days	Negative[d]	Ivett (1989)

bw: body weight; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] Both with and without rat liver S9 fraction.
[b] The assay was not valid because gellan gum was found to react with diaminobenzoic acid to form a fluorescent product that interfered with measurements of DNA.
[c] Only study summary was available.
[d] One dose administered by oral gavage on each of 2 consecutive days. Termination and bone marrow collection at 24 or 48 hours after treatment. No bone marrow toxicity was reported.

only; low-acyl form of gellan gum containing >95% polysaccharide) and a good laboratory practice (GLP)–compliant in vivo micronucleus assay (Ivett, 1989; gellan gum containing 58.5% polysaccharide). In the alkaline elution assay, gellan gum was found to react with diaminobenzoic acid to form a fluorescent product that interfered with measurements of DNA. The authors therefore concluded that the assay was not valid (Robertson, Nichols & Bokelman, 1985c), and the Committee agreed with this conclusion. Gellan gum gave negative results in the in vivo micronucleus assay (Ivett, 1989). However, the Committee noted that this result is not unexpected, given the poor absorption of gellan gum.

Considering the results of all available genotoxicity studies (Table 2) as well as the chemical structure of gellan gum, the Committee concluded that there is no concern for genotoxicity of gellan gum.

2.2.5 **Reproductive and developmental toxicity**

(a) **Multigeneration reproductive toxicity**

Groups of 26 male and 26 female CD (Sprague Dawley) rats were administered gellan gum in their diets at a concentration of 0, 25, 38 or 50 g/kg feed. Males were treated for 70 days prior to mating and for 3 weeks after mating. Females were treated for 14 days prior to mating and throughout mating, gestation and lactation. Selection was made for the pups (F_1) of this mating; they were allowed to mature and were mated to form the F_2 generation.

There were no treatment-related effects on mating or fertility index, conception rate, length of gestation, length of parturition, number of live pups, number of dead pups, post-implantation loss index, survival index on day 4, 7, 14 or 21 or lactation index for any of the generations (Robinson, Thibault & Procter, 1985a).

The long-term toxicity and carcinogenicity study in rats (Batham, Engel & Osborne, 1987b; see section 2.2.3 above) also included an in utero exposure phase. The results from the in utero phase (Batham, Pinsonneault & Procter, 1985) were not evaluated by the Committee at its thirty-seventh meeting (Annex 1, reference 95) and are described below. The study was certified for compliance with GLP.

Groups of 75 male and 75 female Sprague Dawley rats were administered gellan gum (EX4967, lot no. 86001A, 58.5% polysaccharide) in their diet at a concentration of 0, 25, 38 or 50 g/kg feed 63 days prior to mating and throughout the mating period. Females were also treated throughout gestation and lactation. In males, dietary concentrations of gellan gum were equal to 0, 1670–2880, 2570–4440 and 3520–5660 mg/kg bw per day, respectively, in the premating period. In females, dietary concentrations of gellan gum were equal to 0, 2060–2940, 3180–4510 and 4270–5790 mg/kg bw per day, respectively, in the premating period and during the first 2 weeks of gestation. The F_0 generation was examined clinically twice daily. Body weights and feed consumption were measured at least weekly. Times of onset and completion of parturition were recorded where possible, and any sign of dystocia was recorded. Behaviour of the dams immediately postpartum was observed. Numbers of live and dead pups were recorded, and pups were examined for malformations and weighed. Pups with malformations were killed and placed in Bouin's fluid for internal examination. This was also done for pups born dead or found dead before or at postnatal day (PND) 7. On PND 0, the litters were randomly culled to 10 pups (five of each sex) where possible. The general condition of the pups was evaluated daily during the lactation period. Pups dying after PND 7 were given a full macroscopic examination. Pups were weighed on PNDs 4, 7, 14 and 21. On PND 21, the pups were weaned, and at least one male and one female pup from each litter were selected for the long-term toxicity and carcinogenicity study (see section 2.2.3). Those weanlings not selected were killed without further examination. All animals found dead during the study were subjected to external and internal gross examinations. F_0 generation males were killed after the mating period and were given a gross necropsy. Females that failed to mate were killed approximately 26 days after the end of the mating period; females that mated but did not litter by day 26 post-coitum were killed, and these animals were given a detailed macroscopic examination. The F_0 generation dams were killed on PNDs

21–23 and given a macroscopic examination. The uteri were removed, and the number of implantation site scars was recorded. Testes, ovaries and uteri were preserved, as well as any other tissues deemed to be abnormal.

No treatment-related deaths or clinical signs occurred in the parental generation. In F_0 males, body weights of the highest-dose group were statistically significantly lower than control values between week 7 and the end of the study. However, final body weight was only slightly lower (–3%) in the highest-dose group compared with the control group. Feed consumption by males and females in the treatment groups was frequently higher than in the control group. This is considered to be related to the high levels of non-nutritive gellan gum in the diet. These effects are not considered to be adverse. The fertility indices and conception rates in all treated groups were comparable with control values. A statistically significant decrease (–7%) in mating index was observed in the mid-dose group only. Maternal performance, assessed by gestation index, length of gestation, duration of parturition, numbers of live and dead pups at birth, sex ratio, number of implantation scars and post-implantation losses, was unaffected by gellan gum treatment.

For the pups (F_1 generation), the viability and lactation indices in the gellan gum–treated groups were comparable with control values. Male pup weights in the mid-dose group were slightly (up to 6%), but statistically significantly, lower than those of the control group males on PNDs 0, 4 and 14, but not on PNDs 15 and 21. Male pup weights in the lowest- and highest-dose groups and in the females were not statistically significantly different. These incidental findings were therefore not considered to be treatment related.

Treatment of male and female rats of the F_0 generation with gellan gum at dietary concentrations up to 50 g/kg feed did not produce any clear evidence of parental, reproductive or offspring toxicity. The no-observed-adverse-effect level (NOAEL) for parental, reproductive and offspring toxicity was 50 g/kg feed (equal to 3520 and 4270 mg/kg bw per day for males and females, respectively), the highest dose tested (Batham, Pinsonneault & Procter, 1985).

(b) Developmental toxicity

Gellan gum was fed to groups of 25 pregnant female Sprague Dawley rats at a dietary concentration of 0, 25, 38 or 50 g/kg feed during days 6–15 of gestation. Gellan gum had no fetotoxic or teratogenic effects on rats when ingested in the diet at concentrations up to 50 g/kg feed (Robinson, Thibault & Procter, 1985b).

2.2.6 Special study in neonatal pigs

To assess the safety of gellan gum specifically as a component of infant formula, a 21-day study was conducted in neonatal pigs to investigate potential effects of

gellan gum on growth and development, with emphasis on the gastrointestinal tract and immune system (Lanphear, 2016). The Committee previously considered studies in neonatal pigs as appropriate toxicological models for human infants for whom infant formula may be provided as the sole source of nutrition (Annex 1, reference *221*). The study was certified for compliance with GLP. Groups of six male and six female domestic Yorkshire crossbred swine (body weight approximately 2.0–2.5 kg for males and 1.8–2.9 kg for females), born on lactation day 0, were transferred to the test facility on lactation day 2 and housed individually. They were administered gellan gum (low-acyl clarified gellan gum; lot no. 5E2154A) at a concentration of 0, 41 or 205 mg/L in milk replacer (equal to 0, 19 and 100 mg/kg bw per day for males and 0, 20 and 100 mg/kg bw per day for females, respectively[2]), beginning on lactation day 2 (designated study day 1). The animals were given the milk replacer at a dosing volume of 500 mL/kg bw per day via a feeding device in six doses per day (approximately 83.33 mL/kg bw per dose, 3 ± 0.25 hours between doses) for 21 days. All animals were observed at least twice daily for morbidity and mortality and the availability of feed. Detailed clinical observations were conducted twice weekly. A complete physical examination was conducted on all animals on days 1 and 5. Individual body weights were recorded daily during the first week of the study and every other day thereafter. Individual feed consumption was recorded daily. Blood samples for haematology and clinical chemistry were collected from the vena cava on days 7 and 21. Additional blood samples were collected at termination for peripheral blood leukocyte analysis, leukocyte phenotype determinations and immunogenicity analysis. At terminal necropsy, urine was collected by cystocentesis, macroscopic examinations were conducted on all animals, body weights were recorded and gross lesions were examined microscopically. In addition, the adrenal glands, bone with marrow, brain, eyes, gall bladder, gut-associated lymphoid system, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, heart, kidneys, liver, lymph nodes, pancreas, spleen and thymus were weighed and examined microscopically.

All animals survived to the scheduled termination on study day 22. Clinical signs reported were comparable among all groups and were not considered to be treatment related, as they were low in incidence or comparable with those of controls. Physical examination findings were within limits of variation commonly encountered in the age and species and therefore considered acceptable. Findings included soft faeces in two females of the low-dose group and one male of the high-dose group on day 1 and in three males of the low-dose group and two males of the high-dose group on day 5. These findings were transient and not considered to be toxicologically relevant. Mean body weights in treated animals

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[2] In the study report (Lanphear, 2016), different doses were erroneously reported. Revised tables on compound consumption values were submitted by the sponsor (Anonymous, 2016).

were not statistically significantly different from control values throughout the study period. Statistically significantly lower mean feed consumption (−19%) was reported on day 2 in females of the high-dose group. However, as a similar decrease in feed consumption was not reported in male animals and as feed consumption of test animals at all other intervals was comparable with control values, this transient effect was considered to be unrelated to treatment. No effects were observed on feed efficacy, which was considered indicative of good growth.

Several statistically significant changes in haematological parameters were observed. In males, an increase in neutrophils (+68%) was observed in the low-dose group on day 21. Observations in females included a decrease in mean corpuscular volume (−7%) in the low-dose group on day 7, a decrease in platelets (−30%) in the high-dose group on day 7, decreases in lymphocytes (−19% and −21%, respectively) in the low- and high-dose groups on day 21 and a decrease (−38%) in monocyte count in the high-dose group on day 7. These changes were minor, not consistent among sexes and/or dose groups and/or were resolved by day 21. Therefore, these were not considered to be treatment related. Activated partial thromboplastin time was statistically significantly decreased (−13%) in males of the high-dose group on day 21, and fibrinogen levels were increased (+25%) in males of the high-dose group on day 7. Clinical chemistry analysis revealed a statistically significant increase in alanine aminotransferase levels in males and females of the high-dose group (+22% and +20%, respectively) on day 7, which was resolved by day 21. Also, a statistically significant decrease (−10%) in phosphorus levels was reported in high-dose females on day 21. The differences in coagulation and clinical chemistry parameters were small, transient and/or not reported in both sexes and were therefore not considered to be treatment related. Peripheral blood leukocyte analyses did not reveal treatment-related effects on the relative proportions or per cent gated values for lymphocytes, mature T cells, helper T cells, cytotoxic T cells, B cells or natural killer cells. All values were considered to be within expected ranges for biological variation.

No treatment-related alterations were observed among urine analysis parameters (volume, specific gravity, pH) in either sex at any dose. Gastrointestinal content pH values of males and females of the treatment groups were comparable with control values. In the kidneys, pelvic dilatation (hydronephrosis) was noted in one male and two females of the low-dose group and two males and two females of the high-dose group. Red foci of the kidney were observed in one male and two females of the low-dose group only. The severity of these observations was classified as "mild", which means that, in general, the lesion is easily identified but of limited severity and the lesion probably does not produce any functional impairment. According to the author, pelvic dilatation can be occasionally noted as a background finding in pigs of this age, based on historical control data. The Committee noted that the prevalence of pelvic dilatation in

this study was higher than the background prevalence from the historical control data (prevalence 2/40 in male and female pigs aged 1.5–19 months). However, as the severity was mild and there were no microscopic correlates, the Committee considered these findings in the kidney not to be of toxicological relevance. In the skin, subcutaneous cysts were noted in one male in the control group and three females in the high-dose group. The skin cysts were correlated with microscopic cavitations; however, the cause of the cysts was not apparent microscopically and likely consistent with focal trauma of unknown origin. The lesions were therefore considered incidental and not related to test article administration based on concurrent presence in a control animal and lack of a dose–response relationship. No statistically significant changes were observed in absolute organ weights. Incidental findings in relative organ weights were considered to be unrelated to treatment.

No test article–related microscopic findings were observed. Histological examination of non-glandular stomach revealed variable acute inflammation, hyperkeratosis and/or erosion in all groups of animals, including concurrent controls (Table 3). The non-glandular stomach lesions were considered to be most likely incidental, as the highest incidence of erosion and acute inflammation was observed in males of the low-dose group, and the highest incidence of hyperkeratosis was observed in males of the high-dose group and females of the control group. Furthermore, the stomach lesions reported in this study (erosion and hyperkeratosis) are recognized as common observations in swine (Embaye, Thomlinson & Lawrence, 1990, data from pigs aged 10–22 weeks; Krakowka & Ellis, 2006, data from gnotobiotic swine infected with *Helicobacter pylori*–like bacteria). Therefore, these non-glandular stomach lesions were not considered to be related to the test article administration. Other microscopic findings were reported in several organs, including the spleen, kidneys and liver. Considering the severity of the findings (mostly minimal), lack of a dose–response relationship, single or low incidence rates and concurrent occurrence in the controls, these additional microscopic observations were also considered to be unrelated to test article administration and/or consistent with observations occasionally noted in pigs of this age.

The NOAEL of gellan gum in this study was 205 mg/L (equal to 100 mg/kg bw per day), the highest dose tested (Lanphear, 2016).

2.2.7 Other special studies

(a) Immunogenicity

BALB/c mice were given cellulose (control) or gellan gum (purity, molecular weight and level of deacylation not specified) at a dietary concentration of 50 g/kg feed (equivalent to 7500 mg/kg bw per day), and the effects on antigen production

Table 3

Histological changes reported at terminal necropsy in the stomachs of neonatal pigs fed gellan gum in milk replacer

Microscopic findings	Severity[b]	Incidence of finding[a]					
		Males			Females		
		0 mg/L	41 mg/L	205 mg/L	0 mg/L	41 mg/L	205 mg/L
Stomach, cardia							
Infiltration, mononuclear cell	Minimal	0	0	0	1	0	0
Stomach, fundus							
Infiltration, mononuclear cell	Minimal	2	5	4	2	3	2
Polyarteritis	Minimal	0	0	0	0	1	0
Stomach, non-glandular							
Erosion	Total	0	4	0	0	2	3
	Minimal	0	1	0	0	2	3
	Mild	0	3	0	0	0	0
Hyperkeratosis	Total	2	2	4	4	3	1
	Minimal	1	0	1	0	1	1
	Mild	1	0	2	1	1	0
	Moderate	0	2	1	3	1	0
Infiltration, mononuclear cell	Minimal	0	0	0	1	1	0
Inflammation, acute	Total	2	5	2	0	2	4
	Minimal	2	1	2	0	2	1
	Mild	0	2	0	0	0	2
	Moderate	0	2	0	0	0	1
Stomach, pylorus							
Dilatation	Minimal	0	1	0	0	0	0
Infiltration, mononuclear cell	Minimal	5	5	4	5	4	2
Polyarteritis	Minimal	0	0	0	0	1	0

bw: body weight

[a] Total number of animals is six males and six females at each dose.

[b] *Minimal*: the amount of change present barely exceeds that which is considered to be within normal limits; *Mild*: in general, the lesion is easily identified but of limited severity. The lesion probably does not produce any functional impairment; *Moderate*: the lesion is prominent, but there is significant potential for increased severity. Limited tissue or organ dysfunction is possible; *Severe*: the degree of change is either as complete as considered possible or great enough in intensity or extent to expect significant tissue or organ dysfunction.

Source: Lanphear (2016)

were investigated with and without immunization with 2,4,6-trinitrophenol–keyhole limpet haemocyanin. Statistically significant increases in serum antibody titre (with immunization) and immunoglobulin G concentrations (with and without immunization) were reported in the gellan gum group compared with the controls. The low-molecular-weight fraction of caecal contents of mice treated with gellan gum showed a higher in vitro splenocyte proliferation activity compared with the cellulose-fed controls (Funada et al., 2000). No further study details are available (paper in Japanese with English abstract and tables only).

(b) **Effects on gastrointestinal tract mucosa**

Male Sprague Dawley rats (13–14 per group) were administered gellan gum (EX4967; lot no. 86001A; 72.8% total dietary fibre) at a dietary concentration of 0, 12.5, 25 or 50 g/kg feed (equivalent to 0, 625, 1250 and 5000 mg/kg bw per day, respectively) to study the effects on intestinal morphology and cytokinetics in comparison with cellulose, as part of a larger study on several food polymers. The study was certified for compliance with GLP. Rat pairs were fed their respective diets for 25 days before being injected with the labelling reagent 5-bromo-2′-deoxyuridine. The first rat of each pair was killed 70 minutes after the scheduled injection, and the second rat 24 hours after the scheduled injection. The intestine was removed, and the lengths and weights of the small intestine, caecum and large intestine and the width of the caecum were measured. Four intestinal sections were collected for cytokinetic measurements, and five for morphometric measurements.

Treatment with gellan gum did not cause any statistically significant effects on body weight, feed consumption or intestinal length, size and mass compared with cellulose. According to the author, the effects of gellan gum on intestinal morphology and cytokinetics can best be described as representing normal changes. The author concluded that the results of this study do not indicate that gellan gum has any adverse effect on intestinal morphology or cytokinetics (Gordon, 1990a).

(c) **Mineral retention**

To assess potential effects on mineral retention, groups of eight male Sprague Dawley rats were administered gellan gum (EX4967; lot no. 86001A; 72.8% total dietary fibre) at a concentration of 0, 12.5, 25 or 50 g/kg feed (equivalent to 0, 1250, 2500 and 5000 mg/kg bw per day, respectively) for 8 weeks. To ensure that the experiment was sensitive enough to identify an effect on mineral retention in rats, threshold concentrations of minerals (concentrations that ensure adequate growth without providing excess) were used in the basal diet. Controls were administered a diet containing cellulose at 50 g/kg feed (equivalent to 5000 mg/kg bw per day). Additional control groups received a diet without food polymer or a diet containing cellulose at 50 g/kg feed and standard (higher) mineral concentrations instead of threshold concentrations, but these latter two groups were not included in the statistical analyses. Body weights were recorded daily. Feed intakes were initially measured every other day and then every day near the end of the study. The following parameters were measured at termination: blood haemoglobin and haematocrit, liver (wet weight) iron and non-haem iron concentrations, liver (dry weight) iron, copper and magnesium concentrations, serum zinc, copper and magnesium concentrations, femur (dry weight) calcium, phosphorus, magnesium and zinc

concentrations, and carcass (dry weight) calcium, phosphorus, magnesium, iron, zinc, copper and manganese concentrations.

No effects on body weight gain, feed consumption or liver, femur and carcass weights were observed. No statistically significant differences in mineral levels were observed between rats of the high-dose group and the controls (cellulose group), except for a statistically significantly lower carcass copper concentration in the rats treated with gellan gum. However, this was considered to be an incidental finding and was considered not to be related to treatment, as the decreases were not dose related and as rats fed control diet with cellulose and standard (higher) mineral copper concentrations had lower carcass copper concentrations than rats fed the control diet with cellulose and threshold copper concentrations.

In conclusion, gellan gum did not have adverse effects on growth and mineral retention at dietary concentrations up to 50 g/kg feed (equivalent to 5000 mg/kg bw per day), the highest dose tested (Gordon, 1990b).

2.3 **Observations in humans**

2.3.1 **Studies in adults**

At its thirty-seventh meeting (Annex 1, reference *95*), the Committee evaluated one confidential study in human volunteers (Eastwood, Brydon & Anderson, 1987). This study (published as Anderson, Brydon & Eastwood, 1988) and a follow-up study (Anderson et al., 1990) have now been published. The summary from the previous monograph is combined with the information from the two publications.

Five female and five male volunteers, all normal in health and free from gastrointestinal disease, were given gellan gum (containing 58.3% polysaccharide) at a dose of 175 mg/kg bw per day for 7 days, after which the dose was increased to 200 mg/kg bw per day for a further 16 days (Eastwood, Brydon & Anderson, 1987; Anderson, Brydon & Eastwood, 1988). The treatment period was preceded by a 7-day control period. Plasma biochemistry parameters, haematological indices, urine analysis parameters, blood glucose and plasma insulin concentrations and breath hydrogen and methane concentrations were monitored on the first day of the control period and repeated on the last day of the treatment period. On the last day of the control and treatment periods, 24-hour urine was collected, and aliquots were tested for reducing sugars, protein and blood. Five-day stool samples were collected during days 2–6 and 16–20 of the control and treatment periods, respectively, after consumption of 40 radiopaque markers to assess gastrointestinal transit time and completeness of collection.

Treatment with gellan gum did not have any effects on plasma biochemistry parameters, urine analysis parameters or blood glucose, plasma

insulin or breath hydrogen and methane concentrations. No changes were observed in haematological parameters, except that an increase in percentage of eosinophils (18% and 11%, respectively, vs 3% during the control period) was observed in two male subjects after treatment with gellan gum. The values of 18% and 11% correspond to 1.1×10^9/L and 0.6×10^9/L, respectively, and the former concentration was reported to fall outside the normal range. None of the subjects reported any allergic responses. One female subject reported abdominal distension for the first few days of dietary supplementation, and two female participants reported an increase in flatulence. Based on the increases observed in faecal weights and faecal water content, gellan gum appeared to act as a faecal bulking agent. In two males and two females, the colonic transit time increased, but it decreased in the six other volunteers. Gellan gum ingestion was associated with variable effects on faecal fat, bile acid content, neutral sterols and SCFAs. Serum cholesterol measurements showed a statistically significant decrease in female subjects following gellan gum ingestion (−13%), but no changes in serum cholesterol levels in males or in any of the other serum lipid indices in either males or females were observed.

The authors concluded that the ingestion of gellan gum caused no adverse effects in any of the volunteers (Eastwood, Brydon & Anderson, 1987; Anderson, Brydon & Eastwood, 1988).

To exclude possible sensitizing effects of gellan gum, a follow-up study was performed in which 10 male and 10 female volunteers consumed gellan gum (containing 58.3% polysaccharide) at a dose of 175 mg/kg bw per day for 7 days, after which the dose was increased to 200 mg/kg bw per day for the next 14 days (Anderson et al., 1990). The gellan gum intervention period (days 8–28) was preceded (days 1–7) and followed (days 29–36) by 7-day control periods. The two males who had shown increased eosinophil percentages in the previous study (Eastwood, Brydon & Anderson, 1987; Anderson, Brydon & Eastwood, 1988) were included in this study, along with two females from the same study. A skin-prick test with gellan gum was performed on the subjects on day 1. Blood samples for haematology and clinical chemistry were collected on days 1, 8 and 36 (control samples) and on days 15, 22 and 29 (test samples). Additionally, 24-hour urine and faecal samples were collected 3 times per week. Blood samples from the males who showed increased eosinophil percentages in the previous study (as well as six other blood samples, but details on these samples are not provided in the study report) were also analysed by paper radioimmunosorbent test and radioallergosorbent test for total immunoglobulin E (IgE) and allergen-specific IgE, respectively (Hamburger, 1989).

The skin-prick test revealed weal and/or erythema values of 2–5 (on a 10-point scale) for three of the 10 male subjects, whereas no female subjects

had any reaction. The two male subjects with indications of eosinophilia in the previous study (Eastwood, Brydon & Anderson, 1987; Anderson, Brydon & Eastwood, 1988) tested negative in the skin-prick test. Although evidence of weal and erythema in response to gellan gum may suggest an allergy, the reaction scores were low. There were no changes in urine analysis, haematological or clinical chemistry parameters, no positive reactions in haemoccult tests, and no allergic reactions among the subjects during or following gellan gum dietary supplementation. One female subject reported slight abdominal distension during the initial days of dietary supplementation, as she did in the previous study. The two male subjects who showed elevated eosinophil concentrations in the previous study demonstrated normal eosinophil concentrations in the follow-up study. Also, no changes in IgE levels were observed, and their radioallergosorbent tests were negative, so there was no evidence of sensitivity to gellan gum in these two subjects. Overall, IgE levels were occasionally elevated in some samples, but no IgE specific for gellan gum was detected in the serum of participants.

Based on the results, the authors concluded that gellan gum in high doses is non-sensitizing (Hamburger, 1989; Anderson et al., 1990).

2.3.2 Studies in infants

Four paediatric clinical studies were conducted in preterm infants (gestational age <33 weeks, birth weight <2000 g) with human milk fortification (HMF) products containing gellan gum (low-acyl clarified form) for consumption by preterm and/or very low birth weight infants (Barrett-Reis, 2013 [published as Kim et al., 2015]; Thoene et al., 2016; Kumar et al., 2017; Schanler et al., 2018). The primary aim of the studies was to assess the effects of the HMF products on growth and to assess the tolerability of the HMF products. The HMF products evaluated in these studies included both powdered and liquid products. The liquid products included both acidified (sterilized by acidification) and non-acidified (sterilized by heat treatment) products. Gellan gum was an ingredient of the non-acidified liquid HMF (NAL-HMF) product. For the four studies taken together, 214 infants were given human milk with an NAL-HMF product containing gellan gum, and 226 infants received human milk with an HMF product without gellan gum (117 infants were given an acidified liquid HMF [AL-HMF] product, and 109 received a powdered HMF product). The HMF products were administered enterally for between 29 and 40 days. The tested products resulted in a gellan gum concentration in milk of 40 mg/L, which is 20% lower than the maximum concentration of 50 mg/L proposed in the request to Codex. The dietary gellan gum exposure ranged from approximately 3.2 to 6 mg/kg bw per day.

The results of the studies are summarized in Table 4. The Committee noted that the HMF products tested differed in several ways (protein content,

Table 4

Summary of paediatric studies involving preterm/very low birth weight infants provided HMF products with gellan gum

Reference	Study design, study population, dosing regimen and duration	Gellan gum concentration and exposure	Study parameters	Study outcomes
Barrett-Reis (2013); Kim et al. (2015)	Prospective, randomized, controlled, unblinded, multicentre trial Preterm infants (M, F; gestational age ≤33 weeks, birth weight 700–1 500 g) Human milk supplemented with NAL-HMP product containing gellan gum (n = 66) or with P-HMP[b] product (n = 63) (enterally fed) Treatment started within 72 hours after enteral feeding intake of 100 mL/kg bw per day was reached Duration: 29 days or until hospital discharge, whichever came first	40 mg/L 5.7 mg/kg bw per day[c]	Anthropometric indices (body weight, body length, head circumference) Tolerance Serum biochemistry (study days 1, 15 and 29) Intake Morbidity	Comparable weight gain, body length and head circumference gain Both fortification products were well tolerated No statistically significant differences in morbidity outcomes; low incidence of necrotizing enterocolitis (1.5% in NAL-HMF group, 3.2% in P-HMF group) and sepsis (4.5% in NAL-HMF group, 3.2% in P-HMF group) in both groups Statistically significantly higher protein intake in NAL-HMF group due to higher protein content of NAL-HMF product Statistically significantly increased blood urea nitrogen and prealbumin levels in NAL-HMF group on study days 15 and 29; both groups were well within reference ranges
Thoene et al. (2016)	Retrospective chart review Preterm infants (M, F; estimated gestational age ~30 weeks; birth weight <2 000 g) Human milk supplemented with P-HMP[b] (n = 46), AL-HMP[a] (n = 23) or NAL-HMP[a] product (n = 51; containing gellan gum) (enterally fed) Treatment started after enteral feeding intake of 80–100 mL/kg bw per day was reached Duration: up to estimated gestational age of 36 weeks if still hospitalized	40 mg/L 3.2–4 mg/kg bw per day	Anthropometric indices (body weight, body length, head circumference) Enteral feeding data Laboratory data (lowest CO_2 values and maximum blood urea nitrogen) Clinical outcome data (bronchopulmonary dysplasia, retinopathy of prematurity [stage 2 or greater], intraventricular haemorrhage [stage 3 or greater], necrotizing enterocolitis, death)	Growth was slower in AL-HMF group compared with P-HMF and NAL-HMF groups Median lowest CO_2 values were statistically significantly lower in AL-HMF group Three infants in AL-HMF group developed necrotizing enterocolitis (3/23) compared with no infants in the other two groups Incidence of retinopathy of prematurity was statistically significantly higher in P-HMF group compared with NAL-HMF group
Kumar et al. (2017)	Prospective pilot trial Preterm infants (M, F; gestational age ≤31 weeks, birth weight <1 500 g)	40 mg/L 5.7 mg/kg bw per day[c]	Anthropometric indices (body weight, body length, head circumference) Enteral feeding data Clinical chemistry	Body weight gain and growth velocity were higher in NAL-HMF group Incidence of metabolic acidosis was lower in NAL-HMF group (6.6%) compared with AL-HMF group (56.2%)

Table 4 (continued)

Reference	Study design, study population, dosing regimen and duration	Gellan gum concentration and exposure	Study parameters	Study outcomes
	Human milk supplemented with AL-HMF[d] (n = 16) or NAL-HMF[a] (n = 15; containing gellan gum) product (enterally fed) Treatment started after enteral feeding intake of 100 mL/kg bw per day was reached Duration: until discharge			Reticulocyte counts were higher in NAL-HMF group compared with AL-HMF group No major gastrointestinal complications, including necrotizing enterocolitis, reported in either group; abdominal distension resulted in stopping of feeding for ≥24 hours in 25% of the AL-HMF group and none of the NAL-HMF group
Schanler et al. (2018)	Prospective, randomized, controlled, unblinded, parallel, multicentre study Preterm infants (M, F; gestational age ≤32 weeks, birth weight 700–1 500 g) Human milk supplemented with AL-HMF[d] (n = 78) or NAL-HMF[a] (n = 82; containing gellan gum) product (enterally fed) Treatment started within 72 hours after enteral feeding intake of 80 mL/kg bw per day was reached Duration: 29 days or until hospital discharge, whichever came first	40 mg/L ≥3.4 mg/kg bw per day (6 mg/kg bw per day at target volume of 150 mL/kg bw per day)	Anthropometric indices (body weight, body length, head circumference) Feeding tolerance Morbidity Biochemical evaluations Duration of parenteral nutrition Medication and dietary supplement use Adverse events	Body weight gain in NAL-HMF group was higher during study days 1–15 Tolerance of NAL-HMF was higher; infants in AL-HMF group had more feedings withheld due to abdominal distension, gastric residuals and vomiting, and more diaper dermatitis AL-HMF product contained 10% more protein; however, blood urea nitrogen values were lower in AL-HMF group Infants in the AL-HMF group had more metabolic acidosis than infants fed NAL-HMF product (27% vs 5%) There were no differences in morbidity outcomes More adverse events occurred in the AL-HMF group than in the NAL-HMF group

AL-HMF: acidified liquid human milk fortification; bw: body weight; F: female; HMF: human milk fortification; M: male; NAL-HMF: non-acidified liquid human milk fortification; P-HMF: powdered human milk fortification

[a] NAL-HMF product containing hydrolysed protein and gellan gum at 220 mg/L. Fortified milk was prepared by adding 5 mL NAL-HMF product to 25 mL of human milk. Therefore, gellan gum concentration in fortified milk is approximately 40 mg/L (information provided by sponsor, not included in study report).

[b] P-HMF product contains intact protein and no gellan gum.

[c] Infants in the NAL-HMF group consumed 142.5 mL of fortified milk per kilogram body weight, equal to a gellan gum dose of approximately 5.7 mg/kg bw per day.

[d] AL-HMF product contains no gellan gum.

[e] Infants in the NAL-HMF group consumed 143.6 mL of fortified milk per kilogram body weight, equal to a gellan gum dose of approximately 5.7 mg/kg bw per day.

protein type [hydrolysed vs intact], powder vs liquid, acidified vs non-acidified, different food additives), so these studies do not provide information specifically about gellan gum. However, these studies did show that the tested NAL-HMF products containing gellan gum were generally well tolerated. No adverse effects on growth, haematological or biochemical parameters, or clinical outcomes were reported with NAL-HMF products containing gellan gum in comparison with the other HMF products tested, except for an increase in reticulocyte count in the pilot study by Kumar et al. (2017). The authors indicated that this could possibly be explained by the ferrous sulfate that was given to the NAL-HMF product group because of the lower iron content of NAL-HMF products compared with AL-HMF products, but noted that this finding needs to be further investigated in larger studies.

Two abstracts containing limited information reported two additional studies in infants (Perkey & Zimmerman, 2015; Rice et al., 2015). In the first study, anthropometric data (weight, head circumference and body length) were collected on infants (n = 38) in a neonatal intensive care unit who were given HMF products with or without additional protein for 1 month. Thereafter, infants (n = 60) were given a new extensively hydrolysed protein concentrated liquid HMF product (no details provided in the abstract, but the sponsor indicated that this was an NAL-HMF product containing gellan gum), and the same data were collected for 3 months. No adverse events were reported throughout the trial. Average daily weight gains were reported to be higher for infants provided the new NAL-HMF product compared with infants given the older HMF product (24 vs 21 g/day). Lower average head circumference gains and length gains were reported in the group of infants provided NAL-HMF product, but the authors indicated that inconsistencies in these two measurements were a limitation of the study (Perkey & Zimmerman, 2015).

In the second study, growth parameters of very low birth weight infants (n = 56; mean gestational age 28 weeks; mean birth weight 1088 g) fed powdered HMF product with protein, acidified HMF product, concentrated HMF product with protein or hydrolysed protein HMF product were reported. No further information about the HMF products was provided in the abstract, but the sponsor indicated that the hydrolysed protein HMF product was an NAL-HMF product containing gellan gum. The study authors reported a marked effect of type of HMF product on the evaluated growth outcome parameters, with poorest outcomes reported in infants fed acidified HMF product (Rice et al., 2015).

Given the lack of information on, among other study details, the test material and study setup, the Committee considered these abstracts to be of limited value for the evaluation of gellan gum.

2.3.3 **Post-marketing surveillance**

Gellan gum is used as a food additive in the USA, the European Union and approximately 40 other countries. In the USA, gellan gum is currently approved as an ingredient of a concentrated liquid fortification product. This fortification product is used as a nutritional supplement to add to human milk intended for premature and low birth weight infants. Three post-marketing surveillance reports were submitted for a hydrolysed protein concentrated liquid HMF product (Mako, 2015, 2016, 2017). The reports covered the periods 1 May 2014 – 30 September 2015, 1 October 2015 – 30 June 2016 and 1 July 2016 – 31 December 2016. The concentrated liquid was sold in 5 mL packets containing gellan gum (low-acyl clarified form) at 220 mg/L, corresponding to approximately 1.1 g gellan gum per packet. Based on the number of packets distributed and assuming that the average total daily dose for a preterm infant would be eight packets per day, the total number of patient treatment days was calculated to be 2 832 569. The estimated number of exposed infants was 70 815, based on an assumed treatment period of 40 days. The daily exposure was estimated to be 8.8 mg gellan gum per day or 5.9 mg/kg bw per day, assuming a body weight of 1.5 kg.

Table 5 provides a summary of the adverse events reported. The total number of adverse event reports received included 15 patient deaths, 73 non-fatal serious adverse events and 87 non-serious adverse events. None of the serious adverse events was confirmed to be related to the intake of the HMF product. Of the 15 patients who died, eight were reported to have developed fatal necrotizing enterocolitis. Additionally, among the other non-fatal serious adverse events, 45 involved necrotizing enterocolitis. Necrotizing enterocolitis is an important serious gastrointestinal disorder causing morbidity and mortality in premature infants, and incidences ranging from 2.6% to 11% have been reported (Guthrie et al., 2003; Lee & Polin, 2003; Stoll et al., 2010; Yee et al., 2012). However, the incidence of necrotizing enterocolitis coincident with the use of the HMF product was only 0.075% (53 case reports/70 815 patients exposed). One patient death was reported to be due to *Cronobacter sakazakii* infection. Other deaths were related to intestinal perforation, pulmonary disorder and pyrexia. Other serious effects included blood in stool, renal failure/insufficiency, hyperkalaemia and hypersensitivity. The total incidence of hyperkalaemia was 0.025% (18/70 815). Based on questionnaires of 132 hospitals caring for premature infants (<30 gestational weeks), reported incidences of hyperkalaemia for premature infants ranged from 0% to 40% (Mildenberger & Versmold, 2002; Kwak et al., 2013). Hypersensitivity was reported in only two infants (0.0028%; 2/70 815). The most commonly reported non-serious adverse events included abdominal distension, blood in stool (lower degree of severity compared with when recorded as serious adverse event), bloating, diarrhoea, flatulence, hypoglycaemia, intolerance, loose

Table 5

Adverse events reported for NAL-HMF product for period 1 May 2014 – 31 December 2016

Type of adverse event	Number	Rate[a]
Death	15	0.052
Serious non-fatal adverse event		
Blood in stool	3	0.011
Hyperkalaemia	18	0.064
Hypersensitivity	2	0.007 1
Necrotizing enterocolitis	45	0.16
Renal failure/insufficiency	7	0.025
Other (not specified)	2	0.007 1
Total	77[b]	0.27
Non-serious adverse events		
Abdominal distension	3	0.011
Blood in stool[c]	29	0.10
Bloating	16	0.056
Diarrhoea	3	0.011
Flatulence	2	0.007 1
Hypoglycaemia	7	0.025
Loose stool	8	0.028
Tolerance	11	0.039
Vomiting	12	0.042
Other (not specified)	5	0.017
Total	96[d]	0.34
Total number of adverse events	188	0.66

NAL-HMF: non-acidified liquid human milk fortification
[a] Expressed as number of adverse events per 10 000 patient treatment days (total number of patient treatment days = 2 832 569).
[b] When calculated as one incidence per patient, the total number is 73, because some patients had multiple adverse events.
[c] Lower degree of severity compared with when recorded as serious adverse event.
[d] When calculated as one incidence per patient, the total number is 87, because some patients had multiple adverse events.
Sources: Mako (2015, 2016, 2017)

stools and vomiting. The total incidence of these non-serious adverse events was low (<1:10 000).

Overall, the post-marketing surveillance data for the period May 2014 – December 2016 show that the use of gellan gum was well tolerated when administered to infants through its use in a hydrolysed protein concentrated liquid HMF product (Mako, 2015, 2016, 2017).

3. Dietary exposure

Gellan gum (INS 148) is proposed for use as a thickener at a maximum concentration of 50 mg/L (0.005%) in food category 13.1.3, "Formulae for special

medical purposes for infants" (FSMPs), of the Codex GSFA. Furthermore, gellan gum will also be used as a component of concentrated liquid fortification products formulated with hydrolysed protein and/or amino acids for addition to human milk or infant formula. The level of gellan gum in the fed products (fortified human milk and fortified infant formula) should also be maximally 50 mg/L. Currently, gellan gum is authorized for use as a food additive in numerous food categories within the GSFA at levels needed for good manufacturing practice (GMP). As these authorized uses relate only to food categories that are not relevant for infants, these uses are not further discussed.

The Committee evaluated dietary exposure estimates submitted by the sponsor and additionally estimated the dietary exposure to gellan gum based on a high level of consumption of infant formula as proposed by the Scientific Committee of the European Food Safety Authority (EFSA, 2017). The Committee did not estimate dietary exposure to gellan gum using the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) database cluster diets or the FAO/WHO Chronic Individual Food Consumption data – summary statistics (CIFOCOss) database. These databases did not contain meaningful information about the consumption of infant formula.

No consumption data were available for FSMPs, fortified human milk or fortified infant formula. In the dietary exposure estimations submitted by the sponsor and performed by the Committee, it was assumed that the consumption levels of these products were similar to those of "regular" infant formula.

3.1 **Exposure estimates submitted by sponsor**

The sponsor submitted estimates of dietary exposure to gellan gum from its use in FSMPs, fortified human milk and fortified infant formula at a maximum gellan gum concentration of 50 mg/L based on different estimations of the consumption of these products:

1) enteral feeding volumes for preterm infants;
2) WHO-recommended consumption of infant formula;
3) estimated energy requirements (EERs) for fully formula fed infants; and
4) reported consumed amounts of infant formula.

These consumption estimations were the same as those used by the Committee at its eighty-second meeting (Annex 1, reference *232*) in the evaluation of the dietary exposure to carob bean gum, pectin and xanthan gum from their use in

Table 6

Estimated exposure to gellan gum from its use in FSMPs, fortified human milk and fortified infant formula at a maximum concentration of 50 mg/L based on WHO-recommended consumption of infant formula

Age of infant (months)	Recommended consumption of infant formula (mL/kg bw per day)[a]	Estimated exposure to gellan gum (mg/kg bw per day)
0[b]	60	3.0
1	150	7.5
3	150	7.5
6	150	7.5

bw: body weight; FSMP: formula for special medical purposes for infants; WHO: World Health Organization
[a] Based on WHO recommendations for infant formula consumption (WHO, 2009).
[b] Newborn.

infant formula, except for the enteral feeding volumes. The different assessments submitted by the sponsor are summarized below.

3.1.1 Exposure using enteral feeding volumes for preterm infants

Enteral feeding volumes for preterm infants are cited to be in the range of 150–180 mL/kg bw per day (Kim, 2016; Kumar et al., 2017). At the requested maximum gellan gum concentration of 50 mg/L, these volumes would result in an exposure to gellan gum of 7.5–9 mg/kg bw per day for preterm infants.

3.1.2 Exposure using WHO-recommended consumption of infant formula

Exposure to gellan gum was estimated using WHO-recommended average daily consumption of infant formula (WHO, 2009; Table 6). Based on the proposed maximum concentration of gellan gum of 50 mg/L, the estimated mean exposure to gellan gum ranged from 3.0 mg/kg bw per day in the newborn infant to 7.5 mg/kg bw per day in infants 1–6 months of age (Table 6).

3.1.3 Exposure using estimated energy requirements for fully formula fed infants

Infant formula consumption was also estimated assuming that infant formula is consumed in amounts that meet the EERs for fully formula fed infants. Standard body weights and the median EERs for infants up to 6 months of age were obtained from the Joint FAO/WHO/United Nations University report on human energy requirements (FAO/WHO/UNU, 2004). A common formula energy density of 67 kcal/100 mL (280 kJ/100 mL) was used to convert the EERs to the amount of formula consumed.

Using this approach, the estimated exposure to gellan gum ranged from 6.0 mg/kg bw per day for boys aged 5–6 months to 8.4 mg/kg bw per day for boys

Table 7

Estimated exposure to gellan gum from its use in FSMPs, fortified human milk and fortified infant formula at a maximum concentration of 50 mg/L based on standard median EERs for fully formula fed infants

Age of infants (months)	Body weight (kg)[a]	Average EERs (kcal/day)[a]	Volume of formula consumed (mL/day)[b]	Estimated exposure to gellan gum (mg/kg bw per day)
Boys				
0–1	4.58	518	773	8.4
1–2	5.50	570	851	7.7
2–3	6.28	596	890	7.1
3–4	6.94	569	849	6.1
4–5	7.48	608	907	6.1
5–6	7.93	639	954	6.0
Girls				
0–1	4.35	464	693	8.0
1–2	5.14	517	772	7.5
2–3	5.82	550	821	7.1
3–4	6.41	537	801	6.3
4–5	6.92	571	852	6.2
5–6	7.35	599	894	6.1

bw: body weight; EER: estimated energy requirement; FAO: Food and Agriculture Organization of the United Nations; FSMP: formula for special medical purposes for infants; UNU: United Nations University; WHO: World Health Organization

[a] Median body weights and average EERs reported by the Joint FAO/WHO/UNU expert report on human energy requirements (FAO/WHO/UNU, 2004).

[b] Volume of ingested formula based on standard energy density of 67 kcal/100 mL (280 kJ/100 mL).

aged 0–1 month (Table 7). It should be noted that the energy requirements of formula-fed infants are higher than those of breastfed infants (FAO/WHO/UNU, 2004) and that the exposure to gellan gum in children fed (fortified) human milk may therefore be lower than reported below.

The sponsor also considered 14- to 27-day-old formula-fed boys and girls with high (95th percentile) EERs of 149 and 146 kcal/kg bw per day, respectively (Fomon, 1993). Using the same assumptions as for the calculations based on the median EERs, the high exposure to gellan gum was estimated to be 11 mg/kg bw per day in both populations.

3.1.4 Exposure using reported consumed amounts of infant formula

The sponsor also estimated the exposure to gellan gum based on the German Dortmund Nutritional and Anthropometrical Longitudinally Designed (DONALD) study (Kersting et al., 1998). In this study, mean and high (95th percentile) consumptions of infant powder (dry) at 3 and 6 months of age were reported. Using typical preparation instructions for infant formula (13 g of

Table 8

Estimated exposure to gellan gum from its use in FSMPs, fortified human milk and fortified infant formula at a maximum concentration of 50 mg/L based on actual levels of consumption of infant formula by infants at 3 and 6 months of age (German data)

Age of infants (months)	Consumption of infant formula (dry)[a] (g/kg bw per day)		Estimated exposure to gellan gum[b] (mg/kg bw per day)	
	Mean	High[c]	Mean	High[c]
3	17.1	24.4	6.6	9.4
6	8.6	15.8	3.3	6.1

bw: body weight; FSMP: formula for special medical purposes for infants
[a] Mean and high consumption levels of dry infant formula and follow-on formula as reported by Kersting et al. (1998).
[b] Assuming a reconstitution factor of 13 g of powdered infant formula per 100 mL of ready-to-feed formula.
[c] High: 95th percentile.

powdered infant formula to yield 100 mL of ready-to-feed formula), the mean exposure to gellan gum at 3 and 6 months was estimated to be 6.6 and 3.3 mg/kg bw per day, respectively. Corresponding high exposures were 9.4 and 6.1 mg/kg bw per day, respectively (Table 8).

3.2 Exposure using consumption of infant formula proposed by Scientific Committee of EFSA

In 2017, the Scientific Committee of EFSA published guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age (EFSA, 2017). In this guidance, the Scientific Committee proposed the use of a high daily consumption value of 260 mL infant formula per kilogram body weight when performing an exposure assessment. This high value was based on all the available data at that time on the reported consumption of infant formula and also covers the potential high consumption of preterm infants on formula feeding (EFSA, 2017). Based on this high daily consumption value of 260 mL of infant formula per kilogram body weight for infants up to 4 months of age and the maximum gellan gum concentration of 50 mg/L, the exposure to gellan gum was estimated to be 13 mg/kg bw per day.

3.3 Overview of the estimates of exposure to gellan gum from its use in infant formula for special medical purposes

Table 9 summarizes the dietary exposure to gellan gum from its use in FSMPs, fortified human milk and fortified infant formula at a maximum concentration of 50 mg/L in the fed product as submitted by the sponsor and as estimated by the

Table 9

Overview of the exposure estimates for gellan gum from its use in FSMPs, fortified human milk and fortified infant formula at a maximum concentration of 50 mg/L

Source	Age (months)[a]	Exposure (mg/kg bw per day)	
		Mean	**High**
Enteral feeding	0	9[b]	
WHO-recommended consumption	0	3.0	–
	1–6	7.5	–
EERs of fully formula fed infants	0–1	8.2	11
	2–3	7.1	–
	5–6	5.8	–
Reported consumption	3	6.6	9.4
	6	3.3	6.1
Scientific Committee of EFSA	0–4	–	13

bw: body weight; EER: estimated energy requirement; EFSA: European Food Safety Authority; FSMP: formula for special medical purposes for infants
[a] Age of 0 month refers to newborns.
[b] Based on an enteral feeding volume of 180 mg/kg bw per day (see section 3.1.1).

Committee. The mean exposure was highest in infants aged 0–1 month based on mean EERs for fully formula fed infants. The highest high exposure was based on the high daily consumption value of 260 mL infant formula per kilogram body weight, as proposed by the Scientific Committee of EFSA.

4. Comments

4.1 Biochemical aspects

At its thirty-seventh meeting (Annex 1, reference *95*), the Committee concluded, on the basis of rat studies with radiolabelled gellan gum, that gellan gum is poorly absorbed and primarily excreted in the faeces following oral administration (Selim, 1984).

For the present meeting, data from short-term studies in rats and human volunteer studies were submitted. A study in human volunteers provided no evidence of gellan gum absorption (Anderson, Brydon & Eastwood, 1988). No statistically significant increases in SCFA production were reported in animals or humans following gellan gum exposure, suggesting limited microbial degradation of gellan gum in the gastrointestinal tract. Increases were observed in faecal weights and water content, indicating that gellan gum may be a faecal bulking agent (Eastwood, Brydon & Anderson, 1987; Anderson, Brydon & Eastwood,

1988; Edwards & Eastwood, 1995; Tetsuguchi et al., 1997; Shimizu et al., 1999). In rats, reduced gastrointestinal transit times were reported after exposure to gellan gum (Tetsuguchi et al., 1997; Shimizu et al., 1999; Innami, Shimizu & Kudoh, 2000), whereas in a study in human volunteers, variable effects on gastrointestinal transit times were observed (Anderson, Brydon & Eastwood, 1988).

4.2 Toxicological studies

At the thirty-seventh meeting (Annex 1, reference *95*), the Committee noted that gellan gum exhibited low acute oral toxicity, with an LD_{50} value greater than 5000 mg/kg bw in rats (Wolfe & Bristol, 1980). Gellan gum did not cause adverse effects in a 90-day study in rats at doses up to 60 000 mg/kg feed (equivalent to 6000 mg/kg bw per day), a 52-week study in dogs at doses up to 60 000 mg/kg feed (equivalent to 1500 mg/kg bw per day, assuming dry laboratory chow diet) and a 28-day study in prepubertal rhesus monkeys at doses up to 3000 mg/kg bw per day via gavage (Batham et al., 1983; Selim, Fuller & Burnett, 1984; Batham, Kalichman & Osborne, 1986).

For the current meeting, an additional series of short-term studies in rats was available, focusing mainly on the gastrointestinal system (Edwards & Eastwood, 1995; Tetsuguchi et al., 1997; Shimizu et al., 1999, 2001; Innami, Shimizu & Kudoh, 2000). In all these studies, the animals were given gellan gum at a dietary concentration equivalent to 5000 mg/kg bw per day for 4 weeks. In the study by Tetsuguchi et al. (1997), slight morphological changes in the intestinal mucosa were observed microscopically. The variations in the gastrointestinal mucosa were considered by the authors to be related to the gellan gum–induced increase in the viscosity of the intestinal contents, rather than a direct effect of gellan gum, and were not considered to be adverse, and the Committee agreed with this conclusion.

In summary, no adverse effects were reported in any of the short-term studies.

Two additional studies were conducted to specifically assess the potential effects of gellan gum on the gut epithelium and on mineral retention, respectively, using dietary concentrations equivalent to up to 5000 mg/kg bw per day (Gordon, 1990a,b). No adverse effects on intestinal morphology were reported after exposure of rats for 25 days (Gordon, 1990a). Gellan gum did not affect growth or mineral retention after exposure of rats for 8 weeks (Gordon, 1990b).

Both available long-term toxicity and carcinogenicity studies were previously reviewed by the Committee at its thirty-seventh meeting (Annex 1, reference *95*). No treatment-related adverse effects or histopathological changes were reported following administration of gellan gum at dietary concentrations

up to 30 000 mg/kg feed (equivalent to 4500 mg/kg bw per day) in mice for up to 98 weeks or 50 000 mg/kg feed (equivalent to 2500 mg/kg bw per day) in rats for 104 weeks (Batham, Engel & Osborne, 1987a,b).

The Committee previously evaluated three in vitro genotoxicity studies on gellan gum, including a bacterial reverse mutation assay, a DNA repair assay (unscheduled DNA synthesis assay) and a gene mutation assay (Robertson, Nichols & Bokelman, 1985a,b; Robertson et al., 1985). These studies all showed no evidence of genotoxicity.

Two additional genotoxicity studies were available for the present evaluation. In an alkaline elution assay, gellan gum was found to react with diaminobenzoic acid, forming a fluorescent product that interfered with DNA measurements. The authors therefore concluded that the assay was not valid (Robertson, Nichols & Bokelman, 1985c), and the Committee agreed with this conclusion. Gellan gum gave negative results in an in vivo micronucleus assay (Ivett, 1989). However, the Committee noted that this result is not unexpected, given the poor absorption of gellan gum.

Considering the results of all available genotoxicity studies as well as the chemical structure of gellan gum, the Committee concluded that there is no concern for genotoxicity.

No adverse effects were reported in the reproductive and developmental toxicity studies in rats that were evaluated at the previous meeting (Robinson, Thibault & Procter, 1985a,b).

For the current meeting, the results of the in utero phase (Batham, Pinsonneault & Procter, 1985) of the chronic toxicity and carcinogenicity study in rats (Batham, Engel & Osborne, 1987b) were available. Treatment of the animals with gellan gum started 63 days prior to mating and was continued throughout mating, gestation and lactation. The NOAEL for parental, reproductive and offspring toxicity was 50 000 mg/kg feed (equal to 3520 mg/kg bw per day), the highest dose tested.

To assess the safety of gellan gum specifically as a component of infant formula, a study in neonatal pigs was submitted (Lanphear, 2016). These pigs were fed milk replacer with gellan gum (low-acyl clarified product) at a concentration of 0, 41 or 205 mg/L (equal to 0, 19 and 100 mg/kg bw per day for males and 0, 20 and 100 mg/kg bw per day for females, respectively). The neonatal pigs were fed the gellan gum–containing milk replacer during the first 3 weeks of life (starting 2 days after birth) as the sole source of nutrition to model the 0- to 12-week period of development in human infants in which infant formula or (fortified) human milk may be provided as the sole source of nutrition. The aim of this study was to investigate potential effects of gellan gum on growth and development, with emphasis on the gastrointestinal tract and immune system. No gross or microscopic changes were reported in the small or large intestine of the

neonatal pigs. The incidence of pelvic dilatation in the kidneys (hydronephrosis) was higher than the background incidence in historical controls. However, as the severity was mild and there were no microscopic correlates, the Committee considered these findings in the kidney to be of no toxicological relevance. Histopathological examination of the non-glandular stomach revealed variable acute inflammation, hyperkeratosis and/or erosion in all groups of animals, including concurrent controls. The author considered the non-glandular stomach lesions likely to be incidental, as no dose–response relationship was observed and as the stomach lesions reported were recognized as common observations in pigs. The Committee agreed with this conclusion. The NOAEL for gellan gum was 205 mg/L (equal to 100 mg/kg bw per day), the highest dose tested (Lanphear, 2016).

4.3 Observations in humans

Results from a previously evaluated, limited study on tolerance of gellan gum in adults indicated that daily oral doses of up to 200 mg/kg bw administered over a 23-day period did not elicit any adverse reaction, although faecal bulking effects were observed in most subjects. In two males, an increase in the percentage of eosinophils was observed, and the number of eosinophils in one of the subjects was reported to fall outside the normal range (Eastwood, Brydon & Anderson, 1987; Anderson, Brydon & Eastwood, 1988). Therefore, a follow-up study was performed to exclude possible sensitizing effects of gellan gum in 20 human volunteers, among whom were the two male volunteers who had elevated eosinophils in the previous study (Hamburger, 1989; Anderson et al., 1990). No allergic reactions were observed among the subjects during or following gellan gum dietary supplementation, and no changes were observed in haematological parameters. Based on the results, the Committee concluded that there are no indications that gellan gum is sensitizing (Hamburger, 1989; Anderson et al., 1990).

Four paediatric clinical studies were conducted in preterm infants (gestational age <33 weeks, birth weight <2000 g) with HMF products containing gellan gum (low-acyl clarified form) for consumption by preterm and/or very low birth weight infants (Barrett-Reis, 2013; Thoene et al., 2016; Kumar et al., 2017; Schanler et al., 2018). The products evaluated in these studies included powdered HMF products, AL-HMF products (sterilized by acidification) and NAL-HMF products (sterilized by heat treatment). Gellan gum was an ingredient of the NAL-HMF products. For the four studies taken together, 214 infants received human milk with NAL-HMF products containing gellan gum, and 226 infants received human milk with HMF products without gellan gum. The infants were enterally fed with human milk fortified with HMF products for 29–40 days. The

gellan gum concentration in the fortified human milk was approximately 40 mg/L, and the dietary exposure ranged from approximately 3 to 6 mg/kg bw per day. No adverse effects on growth, haematological or biochemical parameters, or clinical outcomes were reported with NAL-HMF products containing gellan gum when compared with the other HMF products tested, except for an increase in reticulocyte count in a pilot study by Kumar et al. (2017). The authors indicated that this could possibly be explained by the ferrous sulfate that was given to the NAL-HMF group because of the lower iron content of NAL-HMF products compared with AL-HMF products, but noted that this finding would need confirmation in larger studies. The Committee noted that the HMF products tested differed in several ways (protein content, protein type [hydrolysed vs intact], powder vs liquid, acidified vs non-acidified, different food additives), so these studies do not provide information specifically about gellan gum. However, these studies did show that the tested NAL-HMF products containing gellan gum were generally well tolerated.

Post-marketing surveillance data over a 2.5-year period showed that the use of gellan gum (low-acyl clarified form) was well tolerated when administered to preterm infants through its use in an HMF product resulting in concentrations in human milk of approximately 40 mg/L (Mako, 2015, 2016, 2017).

4.4 Assessment of dietary exposure

At the current meeting, the Committee estimated the dietary exposure to gellan gum from its use in FSMPs and in concentrated liquid fortification products for addition to human milk or infant formula, as proposed by the sponsor. The requested maximum concentration of gellan gum in the fed products (FSMPs, fortified human milk or fortified infant formula) is 50 mg/L. Dietary exposure to gellan gum was assessed using consumption data for infant formula based on enteral feeding volumes for preterm infants, WHO-recommended consumption levels, consumption levels based on EERs and actual reported consumption levels.

Based on the different consumption levels, the dietary exposure to gellan gum at the requested maximum concentration of 50 mg/L in fed products was estimated to range from 3.0 to 13 mg/kg bw per day. The dietary exposure of 13 mg/kg bw per day was based on a high level of consumption of infant formula of 260 mL/kg bw per day as derived by the Scientific Committee of EFSA (EFSA, 2017). This high consumption level also covers the potential high consumption of preterm infants on formula feeding (EFSA, 2017).

The Committee noted that no dietary exposure assessment was performed for gellan gum for any food uses at the previous meeting (Annex 1, reference 95).

5. Evaluation

The Committee previously established an ADI "not specified" for gellan gum (Annex 1, reference *95*). The ADI "not specified" was based on the absence of toxicity in animal studies, including long-term studies in mice and rats and a 52-week study in dogs in which animals were fed gellan gum at doses up to, respectively, 4500 mg/kg bw per day, 2500 mg/kg bw per day and 1500 mg/kg bw per day.

Several additional in vitro studies, animal studies and human data related to the safety of gellan gum have become available since the Committee's last evaluation. Results confirm the absence of any adverse effects arising from exposure to gellan gum. Therefore, the Committee retained the previously established ADI "not specified" for gellan gum.

ADIs established on the basis of the usually provided toxicology data are not applicable to infants up to the age of 12 weeks. The previously evaluated toxicity studies did not include direct oral administration to neonatal animals and thus did not address safety for the young infant age group. At the present meeting, a 21-day neonatal pig study using low-acyl clarified gellan gum, which modelled the 0- to 12-week period of development in human infants, was evaluated. The NOAEL was 100 mg/kg bw per day, the highest dose tested. Based on this NOAEL and the high estimate of dietary exposure of infants to gellan gum of 13 mg/kg bw per day (based on the requested maximum concentration of gellan gum of 50 mg/L and the high level of consumption of infant formula of 260 mL/kg bw per day), a margin of exposure (MOE) of 7.7 was calculated.

To interpret an MOE related to exposure in infants, the Committee has previously established several considerations that need to be addressed (Annex 1, reference *220*). If these considerations are met, MOEs in the region of 1–10 may indicate low risk for the health of 0- to 12-week-old infants exposed to the food additive through infant formula (Annex 1, reference *220*). The considerations relevant for the current evaluation of gellan gum for use in FSMPs and liquid fortification products for addition to human milk or infant formula are as follows:

- No adverse effects were observed in any of the studies available, indicating that the toxicity of gellan gum is low.
- The NOAEL was the highest dose tested in a study in neonatal pigs, which are considered a relevant animal model for human infants.
- Clinical studies in preterm infants support the tolerability of HMF products containing gellan gum resulting in concentrations of gellan gum in human milk up to approximately 40 mg/L.
- Post-marketing surveillance data over a 2.5-year period showed that the use of gellan gum was well tolerated when administered

to preterm infants through its use in an HMF product resulting in concentrations in human milk of approximately 40 mg/L.

- The dietary exposure estimate was based on the requested maximum concentration of gellan gum of 50 mg/L.

- A high level of consumption of infant formula (260 mL/kg bw per day) was used to assess the dietary exposure.

Based on these considerations, the Committee concluded that the MOE of 7.7 calculated for the use of gellan gum in FSMPs and liquid fortification products for addition to human milk or infant formula at a maximum level of 50 mg/L in the fed product indicates low risk for the health of infants, including preterm infants, and that its proposed use is therefore of no safety concern. This conclusion applies only to the use of low-acyl clarified gellan gum. The Committee recognizes that there is variability in medical conditions among infants requiring these products and that these infants would normally be under medical supervision.

The Committee discussed the request to revise the limits on residual ethanol. Based on the data submitted, the Committee concluded that the use of ethanol in the manufacturing of gellan gum is not a safety concern when used in accordance with GMP. The specification for ethanol was removed, and the existing specifications for gellan gum were revised. The specifications were made tentative, pending submission of new methods for characterizing the three forms of gellan gum in commerce by 2021.

A Chemical and Technical Assessment was prepared.

5.1 **Recommendations**

The specifications were made tentative, pending submission of new methods for characterizing the three forms of gellan gum in commerce by 2021. Specific information required is as follows:

- A method to differentiate the three commercial forms of gellan gum – i.e. high-acyl, low-acyl and low-acyl clarified.

- A method to determine the degree of acylation.

- Validation data for the above methods, including detailed description of the sample preparation.

- Data from five non-consecutive commercial batches of material using the proposed validated methods for all three forms of gellan gum.

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Potassium polyaspartate

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1. Explanation

Potassium polyaspartate (International Numbering System for Food Additives [INS] 456) is a food additive intended to be used as a stabilizer to prevent tartrate crystal precipitation in wine at a proposed maximum use level of 300 mg/L. Potassium polyaspartate is produced from L-aspartic acid and potassium hydroxide.

Potassium polyaspartate has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). L-Aspartic acid is a

component of the sweetener aspartame, which was evaluated by the Committee at its nineteenth, twentieth, twenty-first, twenty-third, twenty-fourth, twenty-fifth and eighty-second (specifications only) meetings (Annex 1, references *38*, *41*, *44*, *50*, *53*, *56* and *230*), and the use of L-aspartic acid as a flavouring agent was evaluated by the Committee at its sixty-third meeting (Annex 1, reference *173*). Potassium hydroxide is a food additive (INS 525; Chemical Abstracts Service [CAS] No. 1310-58-3) that was evaluated by the Committee at its ninth meeting (Annex 1, reference *11*).

Potassium polyaspartate was placed on the agenda of the present meeting at the request of the Fiftieth Session of the Codex Committee on Food Additives (CCFA) (FAO/WHO, 2018a) for an assessment of its safety, dietary exposure and specifications. The sponsor submitted unpublished toxicological studies and published papers. Two additional relevant publications were identified in a literature search. The Committee also considered the components of potassium polyaspartate using previous JECFA evaluations and other reviews. The sponsor provided details of typical and maximum use levels in wine and a dietary exposure assessment for Europe. Published estimates of dietary exposure noted by the sponsor were also reviewed. A literature search did not identify any additional estimates of dietary exposure.

1.1 Chemical and technical considerations

Potassium polyaspartate is produced from L-aspartic acid in a two-step process. During the first step, heating of solid L-aspartic acid leads to solid-phase polycondensation and production of polysuccinimide. Racemization occurs during this step (Kokufuta, Suzuki & Harada, 1978), leading to the occurrence of both D- and L-aspartic acid in the final product. The water-insoluble polysuccinimide obtained is subsequently treated with aqueous potassium hydroxide under controlled conditions, which leads to hydrolysis, opening of the succinimide rings and production of the water-soluble potassium salt. The product contains approximately 70% β-peptide bonds and 30% α-peptide bonds. The final spray-dried potassium polyaspartate is a low-molecular-weight, polydisperse polymer with a weight-average molecular weight of approximately 5000 Da and a number-average molecular weight of about 1000 Da. Up to 20% has a molecular weight of less than 1000 Da.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Absorption

There were no in vivo data on the absorption of potassium polyaspartate.

In an in vitro study, the absorption of potassium polyaspartate (purity 90.6%, Nanochem (USA), batch no. KHK-S040412-1) in water through a cell layer was investigated using differentiated human colon carcinoma (Caco-2) cells as a model for the intestinal barrier (as described by Sambuy et al., 2005) in order to simulate intestinal absorption. Cell viability was assessed using the MTT assay (a colorimetric assay that is based on the cleavage of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] to a purple formazan derivative when incubated with live cells; Mosmann, 1983) at concentrations up to 2 mg/mL, and the barrier integrity of the monolayer was assessed by measuring the transepithelial electrical resistance at potassium polyaspartate concentrations of 0.5 and 1 mg/mL. A solution of 20% ethanol was used as the positive control in the transepithelial electrical resistance assay. Potassium polyaspartate was not cytotoxic up to 2 mg/mL and did not have an effect on barrier integrity, whereas the positive control affected barrier integrity. The microbiuret assay was used to quantify polypeptides, and the ninhydrin method was used to quantify free amino acids. The calibrations were made with potassium polyaspartate concentrations ranging from 0 to 0.250 mg/mL and L-aspartic acid concentrations ranging from 0 to 8119 ng/mL. The absorption of potassium polyaspartate through the Caco-2 monolayer was measured in triplicate after incubation for 24 hours at 37 °C. According to the study report, the concentrations of both potassium polyaspartate and L-aspartic acid were below the limits of detection of the microbiuret and ninhydrin methods in the basolateral portion of the model. The detection limits were not reported for these methods; however, apart from the blanks (0 mg/mL), the lowest potassium polyaspartate and L-aspartic acid concentrations used for calibration were 0.017 mg/mL and 507 ng/mL, respectively, and the linearity was satisfactory (R^2 close to 1) (Restani, 2015).

Samples from the in vitro absorption assay were also analysed by high-performance liquid chromatography (HPLC) with fluorescence detection analysis, which had a limit of detection of 0.7 mg/L and a limit of quantification of 2.1 mg/L. According to this analysis, a transfer of about 5% potassium polyaspartate from the apical to the basolateral site of the Caco-2 model was observed (Vassanelli, 2015).

(b) **Distribution**

There were no data on distribution.

(c) **Excretion**

There were no data on excretion.

2.1.2 **Biotransformation**

There were no in vivo data on biotransformation.

In an in vitro study, the proteolysis of potassium polyaspartate (dry weight 92.4%, purity 90.6%, Nanochem (USA), batch no. KHK-S040412-1) was investigated using pepsin (from porcine gastric mucosa) and pancreatin (from porcine pancreas) in order to simulate gastrointestinal proteolysis. Potassium polyaspartate at a concentration of 3 mg/mL was incubated with pepsin at an enzyme/protein ratio of 1:60 (weight per weight [w/w]) and pH 1.27–2.80 for 5, 10 or 120 minutes at 37 °C. An aliquot of samples was analysed by measuring the undigested proteins with the microbiuret method (Itzhaki & Gill, 1964) and the release of the amino acids with the ninhydrin method (Moore & Stein, 1954; Moore, 1968). At the end of the peptic incubation, a solution of borate buffer containing pancreatin at 0.5 mg/mL was added to the remaining aliquots of samples in the ratio 1:3.5 (volume per volume), and the pH was adjusted to 6.8. The final pancreatin/potassium polyaspartate ratio was 1:21 (w/w). The solution was further incubated for 4 or 24 hours. Samples at different times of incubation (5, 10 or 120 minutes in the presence of pepsin, or 120 minutes in the presence of pepsin and a further 4 or 24 hours in the presence of pancreatin) were heated at 100 °C for 10 minutes to stop the enzymatic activity. Proteolysis was quantified by measuring the undigested polyaspartate using the microbiuret method. In addition, the ninhydrin method, which detects primary amines, was used to assess proteolysis to amino acids (free amino acids or terminal amino groups in polypeptides) released during in vitro digestion at different pH levels: for peptic digestion, for up to 2 hours in 0.06 N hydrochloric acid at pH 1–2; and for pancreatic digestion, for up to 24 hours in a hydrochloric acid/borate buffer at pH 6.8. The in vitro digestion of potassium polyaspartate was performed in triplicate. No statistically significant differences between data on undigested polyaspartate and on amino acids from the beginning to the end of the enzymatic attack were observed (Restani, 2015).

The Committee noted that the HPLC method that was applied in the in vitro absorption assay (where it was shown to be more sensitive than the microbiuret and ninhydrin methods; see section 2.1.1 above) was not used in this in vitro digestion assay.

2.1.3 **Effects on enzymes and other biochemical parameters**

There were no studies on effects on enzymes and other biochemical parameters.

2.2 **Toxicological studies**

2.2.1 **Acute toxicity**

There were no data on acute toxicity.

2.2.2 **Short-term studies of toxicity**

In a dose range–finding study performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 407 (2008) and in compliance with OECD principles of good laboratory practice (GLP), potassium polyaspartate (CAS No. 64723-18-8,[1] purity 98.0% w/w on dry matter, batch no. KHKS-040412) was administered to groups of five Wistar rats of each sex via oral gavage at a dose of 0, 60, 125, 250, 500 or 1000 mg/kg body weight (bw) per day for 14 days. Analytical-grade water was used as the vehicle control.

Feed consumption and body weight gain were unaffected in all dose groups. There was no mortality, and no treatment-related incidences of clinical abnormalities were noted in the course of the study. There were no treatment-related effects on haematological and clinical chemistry parameters or on absolute and relative organ weights. As there was no mortality and there were no signs of gross pathological changes in adrenals, brain, heart, kidney, liver, spleen and testes, no histological microscopic examinations were carried out (Gumaste, 2014a; Galbusera et al., 2017).

A 90-day oral toxicity study was performed with potassium polyaspartate (purity 98.0% w/w on dry matter, batch no. KHKS-040412) in Wistar rats according to OECD Test Guideline 408 (1998) and in compliance with OECD principles of GLP. Additional parameters, such as estrous cycle, thyroid hormones and histopathology of tissues of the endocrine system, as described in OECD Test Guideline 407 (2008), were included in order to investigate potential endocrine effects and to obtain information on neurotoxicity and immunotoxicity. Based on the 14-day dose range–finding study, the doses applied via oral gavage to groups of 10 Wistar rats of each sex were 0, 250, 500 and 1000 mg/kg bw per day. Analytical-grade water was used as the vehicle control. Additionally, groups of five rats of each sex received water or potassium polyaspartate at a dose of 1000

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[1]   This CAS number was assigned to "L-aspartic acid, homopolymer, potassium salt" by CAS. However, it has become clear that the food additive is actually based on D- and L-aspartic acid (see section 1.1). Therefore, this CAS number does not correspond to the food additive. As yet, no CAS number has been assigned to the racemate.

mg/kg bw per day and were further observed for a period of 28 days following the 90-day treatment to evaluate the reversibility of potential effects. The rats were examined daily for signs of toxicity, morbidity and mortality. They were subjected to detailed clinical examination before initiation of the study and weekly thereafter during the treatment period, during the recovery period and at termination. Ophthalmoscopic examination was conducted on control and high-dose animals before initiation of the study and at termination of treatment. In the thirteenth week of treatment, animals were additionally examined to assess sensory reactivity, grip strength and motor activity. Body weight and feed consumption were recorded weekly. Laboratory investigations were performed on blood and urine at termination of the treatment and at the end of the recovery period. All animals sacrificed terminally were subjected to a detailed necropsy, and weights of kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries and heart were recorded. Histopathological evaluation was performed on all tissues (brain, spinal cord, eye, pituitary, thyroid, parathyroid, spleen, thymus, adrenals, pancreas, trachea, lungs, heart, aorta, oesophagus, stomach, duodenum, jejunum, terminal ileum, colon, rectum, liver, kidneys, urinary bladder, prostate, seminal vesicle, epididymides, testes, ovaries, uterus, skin, sciatic nerve, bone marrow [smear], mammary gland, mesenteric lymph node, axillary lymph node and salivary glands) in all rats from the control and high-dose groups.

No treatment-related effects were observed. The no-observed-adverse-effect level (NOAEL) of potassium polyaspartate was 1000 mg/kg bw per day, the highest dose tested (Gumaste, 2014g; Galbusera et al., 2017).

2.2.3 Long-term studies of toxicity and carcinogenicity

There were no studies on long-term toxicity or carcinogenicity.

2.2.4 Genotoxicity

Potassium polyaspartate (dry matter 93.0%, NanoChem Solutions Inc. (USA), batch no. KHKS-040412) was investigated in a bacterial gene mutation assay according to OECD Test Guideline 471 (1997) and in compliance with OECD principles of GLP. It was tested in five strains of *Salmonella typhimurium* (TA97a, TA98, TA100, TA102 and TA1535) in a preincubation assay in the presence and absence of metabolic activation (S9 mix). No precipitates or bacteriotoxic effects were observed. Potassium polyaspartate did not induce an increase in the frequency of revertant colonies under any conditions of this study. The results of this assay were negative (Mane, 2014a; Galbusera et al., 2017).

Potassium polyaspartate (dry matter 93.0%, NanoChem Solutions Inc. (USA), batch no. KHKS-040412) was investigated in an in vitro mammalian

Table 1

Genotoxicity of potassium polyaspartate in vitro

End-point	Test system	Concentrations	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA97a, TA98, TA100, TA102 and TA1535	0, 50, 150, 500, 1 500, 5 000 μg/plate, ±S9	Negative[a]	Mane (2014a); Galbusera et al. (2017)
Micronucleus induction	Human peripheral blood lymphocytes	0, 500, 1 500, 5 000 μg/ mL, ±S9	Negative[b]	Mane (2014b); Galbusera et al. (2017)

OECD: Organisation for Economic Co-operation and Development; S9: 9000 × *g* supernatant fraction from rat liver homogenate. The S9 fraction was prepared from phenobarbitone and β-naphthoflavone-induced livers of Wistar rats.

[a] Two separate experiments were performed, both using the preincubation method.

[b] Limited reliability because the time schedule for the use of cytochalasin B was not consistent with OECD Test Guideline 487. After 48 hours of culture initiation, the cells were exposed to potassium polyaspartate for 3 and 20 hours, respectively, in the absence of metabolic activation and for 3 hours in the presence of metabolic activation. Cytochalasin B was added at 68 hours, and cells were harvested at 96 hours after culture initiation. According to OECD Test Guideline 487, cytochalasin B should have been added directly after the 3-hour treatment and should have been present during the 20-hour treatment.

cell micronucleus test according to OECD Test Guideline 487 (2010) and in compliance with OECD principles of GLP. It was tested in human peripheral blood lymphocytes in the presence and absence of metabolic activation (S9 mix). No precipitates were observed. Very low cytotoxicity (only slight cytostasis as determined with the cytokinesis-block proliferation index) was observed at 5000 μg/mL, the highest concentration tested. Potassium polyaspartate did not induce an increase in the frequency of micronuclei under any conditions of this study. Thus, it was not clastogenic or aneugenic in human lymphocytes, and the results of this assay were negative (Mane, 2014b; Galbusera et al., 2017).

The Committee noted that the time schedule for the use of cytochalasin B was not consistent with OECD Test Guideline 487, which limits the reliability of this study. However, the Committee considered that, taking the chemical structure of potassium polyaspartate into account, there is no concern with respect to its genotoxicity.

These two studies of the genotoxicity of potassium polyaspartate are summarized in Table 1.

2.2.5 **Reproductive and developmental toxicity**

There were no specific studies on reproductive and developmental toxicity; however, the 90-day oral toxicity study performed with potassium polyaspartate (see section 2.2.2 above) did not reveal any effects on the estrous cycle or on the weights and histopathology of testes, epididymides, seminal vesicles, uterus or ovaries (Gumaste, 2014b; Galbusera et al., 2017).

2.2.6 Special studies

(a) Immune responses

The results of the 90-day oral toxicity study performed with potassium polyaspartate (see section 2.2.2 above) did not indicate any immunotoxic or immunomodulatory potential (Gumaste, 2014b; Galbusera et al., 2017).

In an in vitro study, the human promyelocytic cell line THP-1 was used as a surrogate for monocytes. CD86 cell surface marker content and interleukin 8 (IL-8) release were quantified by flow cytometry and the enzyme-linked immunosorbent assay, respectively, as markers of immune cell activation (as suggested by Corsini et al., 2014). Potassium polyaspartate was tested at a concentration of 2 mg/mL. Although statistically significant increases in both CD86 expression and IL-8 release were induced by lipopolysaccharide that was used as a positive control, potassium polyaspartate did not activate THP-1 cells (Restani, 2015).

(b) Neurotoxicity

No specific study on neurotoxicity was available; however, the 90-day oral toxicity study performed with potassium polyaspartate (see section 2.2.2 above) in which additional parameters were investigated to provide information on neurotoxicity using a functional observational battery (including examinations of posture, respiration, palpebral closure, lacrimation, salivation, skin and hair coat, urination, defecation, locomotor activity, rearing, gait, touch response, tail pinch, pupil constriction in response to light, righting reflex, auditory response, head shaking in response to air blow in ear, landing foot splay, grip strengths of forelimbs and hindlimbs) did not reveal any signs of neurological dysfunction (Gumaste, 2014b; Galbusera et al., 2017).

(c) Thyroid function

No specific study on thyroid function was available; however, in the 90-day oral toxicity study performed with potassium polyaspartate (see section 2.2.2 above), histopathological investigations performed on thyroid and parathyroid did not reveal any treatment-related effects. In addition, blood concentrations of triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) were measured at termination of the treatment and at the end of the recovery period. In three male and three female rats, T_4 values were below the limit of detection. As standard deviations of mean T_3, T_4 and TSH levels in blood were broad and similar standard deviations were observed in vehicle controls, the observed effects were not considered to be treatment related (Gumaste, 2014b; Galbusera et al., 2017).

(d) **Potential effects on the bioavailability of minerals naturally present in food**

In order to investigate the potential interference of potassium polyaspartate with the bioavailability of metals, the binding properties of potassium polyaspartate were tested with calcium, iron and magnesium as model substances. A solution of potassium polyaspartate was incubated with a known amount of each of these minerals and loaded onto a column for size-exclusion chromatography. Twenty-five aliquots of the eluted solution were then quantitatively analysed for potassium polyaspartate by the microbiuret method, and those aliquots that showed higher potassium polyaspartate concentrations than others were also analysed for the amount of bound mineral by inductively coupled plasma optical emission spectrometry. The analysis showed that the percentage of bound minerals in these aliquots was lower than in the incubation solution. Based on this observation and on comparison of calculated binding capacities of potassium polyaspartate for calcium, magnesium and iron ions (8.2, 4.9 and 7.9 mg/L, respectively) with the natural presence of these minerals in wine (50–150, 50–150 and 1–5 mg/L, respectively), the authors of the study considered that the binding capacity of potassium polyaspartate is saturated by the minerals present in wine and that the effect of potassium polyaspartate on the bioavailability of metals is negligible (Colombo et al., 2019).

The Committee noted that the study had several shortcomings in design and reporting (e.g. the incubation was done only at one concentration and pH and no proof of any equilibrium was provided, the mineral concentrations were not measured in all aliquots, the reaction mixture was not investigated in the presence of wine and food matrix, the meaning of "bound mineral" was unclear) and was not published in a peer-reviewed journal.

2.3 **Observations in humans**

No information was available.

2.4 **Studies on L- and D-aspartic acid and potassium**

Because the aspartic acid incorporated in the polyaspartate backbone is in an L- and D- configuration, the Committee considered L- and D-aspartic acid resulting from possible breakdown of potassium polyaspartate, as well as potassium.

2.4.1 **L-Aspartic acid**

L-Aspartic acid is a non-essential amino acid that occurs in food. It is also a component of the intense sweetener aspartame. Because L-aspartic acid results

from the hydrolysis of aspartame, the toxicity of and dietary exposure to L-aspartic acid were considered by the Committee in the course of its evaluations of the use of aspartame. The Committee concluded that L-aspartic acid generated from aspartame was not a safety concern at current dietary exposure to aspartame (Annex 1, reference *54*).

When the use of L-aspartic acid as a flavouring agent was evaluated by the Committee at its sixty-third meeting, the Committee concluded that there was no safety concern at current dietary exposures when L-aspartic acid was used as a flavouring agent (Annex 1, reference *174*).

The European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources Added to Food noted in its scientific opinion on the re-evaluation of aspartame as a food additive that aspartic acid is itself a neurotransmitter and can be converted to the more potent excitatory neurotransmitter glutamate. The Panel concluded that aspartic acid generated from aspartame was not of safety concern at the acceptable daily intake (ADI) of 40 mg/kg bw per day that was derived for aspartame (EFSA, 2013).

Oral administration of monopotassium L-aspartate by gavage to 35-day-old male Wistar rats at a single dose of 3.8 g/kg bw (expressed as aspartic acid) resulted in an increased plasma aspartate level (98 μmol/100 mL vs 2.8 μmol/100 mL in control animals) and induced hypothalamic lesions in 50% (4/8) of the treated rats (Itoh et al., 1979).

In a 90-day feeding study in Fischer 344 rats that received a diet containing L-aspartic acid at 0, 0.5, 12.5, 25 or 50 g/kg feed (equal to 0, 26.9, 697, 1417 and 2770 mg/kg bw per day for males and 0, 28.7, 715, 1470 and 2966 mg/kg bw per day for females, respectively), tubular dilatation was observed in kidneys of males (at 25 and 50 g/kg feed), and effects on submandibular and parotid glands (at 25 and 50 g/kg feed) were observed in both sexes. The NOAEL was 12.5 g/kg feed (equal to 697 mg/kg bw per day for males and 715 mg/kg bw per day for females) (Tada et al., 2008).

According to a review by Garlick (2004), administration of a 10 g bolus dose of aspartic acid to humans did not result in a change in secretion of pituitary hormones, and administration of aspartic acid at doses of 75–130 mg/kg bw per day as a food supplement during short or prolonged exercise regimes did not result in adverse effects.

In an evaluation of different aspartates as mineral sources added for nutritional purposes to food supplements, EFSA (2008) noted that reported estimates of dietary exposure to L-aspartic acid are about 3 times lower than the NOAEL of approximately 700 mg/kg bw per day identified in the 90-day study performed by Tada et al. (2008) in which tubular dilatation was observed in kidneys of males and effects on salivary glands were observed in both sexes at higher doses. EFSA further noted that uses of different aspartates such as

potassium aspartate as food supplements, resulting, at the proposed use levels, in exposure to L-aspartate of about 0.3–1.1 g and 1–4 g in young people and adults, respectively, could be of safety concern because the margins of safety as calculated based on this NOAEL were considered too low (EFSA, 2008).

2.4.2 D-Aspartic acid

D-Aspartic acid is an endogenous amino acid that is involved in the development of the nervous system, plays a role in the neuroendocrine system, including hormone synthesis, has neuronal activities and is implicated in male fertility (A. D'Aniello et al., 1996; G. D'Aniello et al., 2005; Furuchi & Homma, 2005; A. D'Aniello, 2007; Errico et al., 2008, 2009, 2012; Topo et al., 2009; S. D'Aniello et al., 2011; Katane & Homma, 2011; Ota, Shi & Sweedler, 2012; Di Fiore et al., 2016, 2018; Genchi, 2017).

D-Aspartic acid is present in the human brain (Man et al., 1987) and accumulates with age in the central nervous system white matter, but not in grey matter (Man et al., 1983). This is reflected by an increase in the D-aspartic acid/L-aspartic acid ratio with age (Man et al., 1987). Free D-aspartic acid can be metabolized by D-amino acid oxidase (EC 1.4.3.3), which is expressed in brain, spinal cord, liver, renal proximal tubule cells and the proximal and middle small intestine of mice and humans (Pollegioni, Sacchi & Murtas, 2018).

D-Aspartic acid has been suggested for therapeutic uses in the treatment of diseases related to myelin dysfunction and *N*-methyl-D-aspartate receptor hypofunction, including schizophrenia and cognitive deficits (Errico et al., 2018; de Rosa et al., 2019) and multiple sclerosis (Afraei et al., 2017; Nicoletti et al., 2019).

(a) Animal data

A daily oral administration of D-aspartic acid in drinking-water at a concentration of 20 mmol/L (equivalent to about 480 mg/kg bw per day, based on a conversion factor of 0.18 for subacute drinking-water studies performed with young mice; EFSA, 2012; WHO, 2016) to a group of six male C57BL/6 mice for 1 month resulted in about a 5-fold increase in the level of D-aspartate in the hippocampus in comparison with the control group and produced an enhanced long-term potentiation and a loss of depotentiation in the CA1 area of the hippocampus (Errico et al., 2008).

In a 28-day toxicity study, groups of seven female Sprague Dawley rats received deionized water (control group) or D-aspartic acid in drinking-water at a daily dose of 50 mg/kg bw. Administration of D-aspartic acid resulted in a 4.8-fold increase in the D-aspartate level in liver and blood serum, respectively, and an 8.2-fold increase in the D-aspartate level in kidney homogenates, compared

with the control group, whereas no increase was observed in the D-aspartate level in brain homogenates. No signs of general toxicity or effects on feed and water consumption, development of body weight or organ weight or serum parameters and no histopathological changes in liver or kidneys were observed (Schieber et al., 1997).

There is experimental evidence for an L-isomer-selective transport of aspartic acid at the blood–brain barrier, according to which L-aspartic acid, but not D-aspartic acid, undergoes efflux transport from the brain to the blood across the rat blood–brain barrier; in contrast, the uptake of aspartic acid in brain parenchymal cells is not stereospecific (Hosoya et al., 1999; Tetsuka et al., 2003).

Oral administration of sodium D-aspartate in drinking-water daily at a concentration of 20 mmol/L (equivalent to about 130 mg/kg bw per day, expressed as aspartic acid, based on a conversion factor of 0.05 for subacute drinking-water studies performed with adult rats; EFSA, 2012; WHO, 2016) to a group of 10 male adult Wistar rats for 12 days resulted in a 1.5-fold increase in the serum level of luteinizing hormone and a 2-fold increase in the level of testosterone, compared with the control group (Topo et al., 2009).

Oral administration of sodium D-aspartate in drinking-water daily at a concentration of 40 mmol/L (equivalent to about 260 mg/kg bw per day, expressed as aspartic acid, based on a conversion factor of 0.05 for subacute drinking-water studies performed with adult rats; EFSA, 2012; WHO, 2016) to a group of 12 adult male Wistar rats for 12–16 days resulted in a 2.7-fold increased level of D-aspartate in the hippocampus and improved the cognitive capability of the animals to find a hidden platform in the Morris water maze test, compared with the control group. The authors of the study concluded that D-aspartic acid is involved in learning and memory processes (Topo et al., 2010).

Oral administration of sodium D-aspartate in drinking-water daily at a concentration of 20 mmol/L (equivalent to about 130 mg/kg bw per day, expressed as aspartic acid, based on a conversion factor of 0.05 for subacute drinking-water studies performed with adult rats; EFSA, 2012; WHO, 2016) to adult male Wistar rats (number of rats per group not reported) for 12 days resulted in statistically significantly increased levels of testosterone in testes (about 2-fold) and serum (about 2-fold), androstenedione in testes (about 1.8-fold) and serum (about 1.5-fold) and luteinizing hormone in serum (about 2.5-fold), compared with the control group, whereas no statistically significant effects were observed with L-aspartate (Raucci, D'Aniello & Di Fiore, 2014).

Oral administration of D-aspartate (cation not reported) in drinking-water daily at a concentration of 20 mmol/L (equivalent to about 130 mg/kg bw per day, expressed as aspartic acid, based on a conversion factor of 0.05 for subacute drinking-water studies performed with adult rats; EFSA, 2012; WHO, 2016) to a group of five adult male Wistar rats for 15 days resulted in statistically

significantly increased levels of testosterone in testes (about 1.7-fold) and serum (about 2-fold), compared with the control group (Santillo et al., 2014).

Oral administration of D-aspartic acid in drinking-water daily at a concentration of 20 mmol/L (equivalent to about 130 mg/kg bw per day, based on a conversion factor of 0.05 for subacute drinking-water studies performed with adult rats; EFSA, 2012; WHO, 2016) to a group of five male Wistar rats for 30 days resulted in increased levels of sex hormones in the brain (with about a 2-fold increased level of testosterone and about 1.3-fold increased levels of progesterone and 17β-estradiol compared with control animals) (Di Fiore et al., 2018).

There are no longer-term (>1 month) oral toxicity studies on D-aspartic acid and no toxicity studies on racemic mixtures of D- and L-aspartic acid.

(b) **Human data**

Consumption of a solution of dietary supplements containing 3.12 g sodium D-aspartate in 10 mL (equivalent to 52 mg/kg bw per day at a body weight of 60 kg, which is equivalent to a dose of D-aspartic acid of 45 mg/kg bw per day) and three vitamins for 12 consecutive days by 23 healthy male human volunteers resulted in statistically significant increases in the levels of luteinizing hormone (1.3-fold) and testosterone (1.4-fold) in blood serum, whereas no statistically significant changes were observed in the placebo group (Topo et al., 2009).

In a randomized, double-blinded, placebo-controlled human study, groups of 10 volunteers participated in 28 days of heavy resistance training while ingesting either placebo or D-aspartic acid at 3 g/day (equal to 40 mg/kg bw per day, based on the mean reported body weight of about 75 kg). Serum levels of testosterone, luteinizing hormone, gonadotropin-releasing hormone and estradiol were unchanged with resistance training and D-aspartic acid supplementation. Adverse effects (i.e. feelings of irritability, nervousness, rapid heart rate and headache) were reported by 2/10 participants who consumed D-aspartic acid and by 1/10 participants of the placebo group (Willoughby & Leutholtz, 2013).

In a clinical trial in which groups of five overweight or obese men consumed a D-aspartic acid/sodium nitrate/vitamin D_3 dietary supplement (either one or two servings per day) that contained 3120 mg D-aspartic acid (equal to 35 mg/kg bw per day in the one-serving group, based on the mean reported body weight of 88 kg, and 61 mg/kg bw per day in the two-servings group, based on the mean reported body weight of 102 kg) for 28 days, no statistically significant changes in blood testosterone or estradiol levels between day 0 and day 28 were observed (Bloomer et al., 2015).

In a randomized, double-blinded, placebo-controlled clinical trial, three groups of eight resistance-trained men participated in 14 days of resistance

training while ingesting a placebo or 3 or 6 g of D-aspartic acid per day (equal to 38 mg/kg bw per day in the 3 g group, based on the mean reported body weight of about 80 kg, and 71 mg/kg bw per day in the 6 g group, based on the mean reported body weight of about 85 kg). The daily dose of 6 g D-aspartic acid resulted in slightly, but statistically significantly, decreased levels of total testosterone (12% decrease) and free testosterone (15% decrease) in blood without any concurrent changes in estradiol, sex hormone binding globulin or albumin levels. The lower dose had no significant effect on either testosterone marker (Melville, Siegler & Marshall, 2015).

Oral administration of D-aspartate (cation not reported) at a daily dose of 2660 mg (equivalent to 44 mg/kg bw per day at a body weight of 60 kg) for 4 weeks to 16 patients who were affected by a progressive form of multiple sclerosis resulted in enhanced synaptic plasticity reserve compared with the control group of 15 patients who received a placebo. The authors of the study therefore hypothesized that D-aspartate may represent a therapeutic opportunity to counteract disability progression in multiple sclerosis (Nicoletti et al., 2019).

(c) Systematic review of animal and human data

In a systematic review of 23 animal studies, three of which involved oral exposure of rats (Topo et al., 2009; Raucci et al., 2014; Santillo et al., 2014), and four human studies (Topo et al., 2009; Willoughby & Leutholtz, 2013; Bloomer et al., 2015; Melville, Siegler & Marshall, 2015), the authors concluded that exogenous D-aspartic acid enhances testosterone levels in male animals at oral doses of around 130 mg/kg bw per day, whereas studies in humans, in which daily doses ranging from 36 to 70 mg/kg bw per day were consumed as dietary supplements, yielded inconsistent results. The authors noted that the inconsistent results obtained in these human trials could be due to limitations in the study design, such as short-term supplement duration (12–28 days) and small sample sizes (N = 10–23 in the supplemented groups) (Roshanzamir & Safavi, 2017).

2.4.3 Potassium

Potassium was evaluated by the Committee in the course of the evaluation of potassium hydroxide as a food additive (INS 525) at its ninth meeting (Annex 1, reference *11*). The result of the evaluation was an ADI "not limited"[2] for potassium hydroxide.

Serum levels of potassium usually rise only moderately in response to potassium intake, even in the case of a short-term (2–24 weeks) high potassium intake of 1755 mg/day, which resulted in an increase in potassium serum levels by only 0.17 mmol/L (6.6 mg/L) (Cappuccio et al., 2016).

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[2]   Now called ADI "not specified".

The United States National Academies of Sciences, Engineering, and Medicine (Stallings, Harrison & Oria, 2019) and the EFSA Panel on Dietetic Products, Nutrition and Allergies (EFSA, 2016a) noted that potassium intakes of about 2500–3000 mg/day with food supplements have been shown not to have adverse effects in healthy adults.

3. Dietary exposure

3.1 Introduction

The CCFA, at its Fiftieth Session (FAO/WHO, 2018a), asked the Committee to conduct a safety assessment on the use of potassium polyaspartate as a stabilizer to prevent tartrate crystal precipitation in wine; therefore, a dietary exposure assessment was undertaken. This is the first time the Committee has evaluated dietary exposure to potassium polyaspartate.

The sponsor noted that potassium polyaspartate will be used in wines at typical use levels of 100–200 mg/L and a maximum use level of 300 mg/L, depending on the instability of the wine to be treated. These three levels of use were evaluated in the dietary exposure assessment.

The Codex General Standard for Food Additives (GSFA) food category for wine is 14.2.3 Grape wines, which includes still grape wine, sparkling and semi-sparkling grape wines, and fortified grape wine, grape liquor wine and sweet grape wine. Therefore, all of these types of wine were considered as part of the dietary exposure assessment. Cider and perry were not included.

3.2 Approach to the dietary exposure assessment

The dietary exposure assessment included a review of the information provided by the sponsor. This information included the proposed use of potassium polyaspartate, use levels, alcohol consumption figures and an estimate of dietary exposure. The sponsor also provided summaries of the evaluations conducted by EFSA (2016b) and Food Standards Australia New Zealand (FSANZ, 2018a,b). FSANZ also submitted its dietary exposure assessments for Australia and New Zealand directly to the Committee. As the EFSA and FSANZ dietary exposure assessments were available to the Committee in full, each was reviewed in detail by the Committee.

The scientific literature was reviewed to identify any additional estimates of dietary exposure to potassium polyaspartate. EBSCO Discovery Service was

used, and databases including Medline, Food Science Source, Food Science and Technology Abstracts and Science Direct were searched, as well as a number of journals related to science, toxicology, food, nutrition and public health. Search terms included "potassium polyaspartate" and "dietary exposure" or "dietary intake" or "consumption". These terms were also used in a general Internet search to capture other papers or reports not included in the scientific literature. These searches did not result in any estimates of dietary exposure to potassium polyaspartate not already submitted to the Committee or noted by the sponsor.

A screening assessment of dietary exposure using the budget method is usually conducted or reviewed by the Committee for food additive evaluations. The budget method produces a conservative estimate of dietary exposure, as it is based on large consumption amounts of 6 L of non-milk beverages and 3 kg of food per day. As potassium polyaspartate is proposed for use in only one specific food group (wine) and not a broad range of foods and beverages, the Committee did not undertake a dietary exposure assessment using the budget method, as it was determined that the method was not appropriate in this context. In addition, other more refined national estimates of dietary exposure were available to the Committee for review that are more appropriate for evaluating chronic dietary exposures.

The Committee also undertook deterministic calculations to estimate dietary exposure to potassium polyaspartate for countries that had national consumption data for wine and where dietary exposure assessments were not currently available. The consumption data used for these calculations were taken from the FAO/WHO Chronic Individual Food Consumption database – summary statistics (CIFOCOss) (FAO/WHO, 2017, 2018b). Food consumption data incorporated into CIFOCOss are from national dietary surveys with 2 or more days of data. The details regarding the specific data extracted and how they were used for these calculations are discussed further in section 3.3.3.

The FAO/WHO Global Individual Food consumption data Tool (GIFT) database (FAO/WHO, 2019) was searched to determine whether there were any consumption data for wines that were appropriate to use for estimating dietary exposures. Of the eight countries with data in the database (Bangladesh, Bolivia, Plurinational State of, Burkina Faso, Italy, Lao People's Democratic Republic, Philippines, Uganda, Zambia), only one (Italy) had data on consumption of wine. As Italy was included in the dietary exposure estimates undertaken by EFSA, the data were not extracted from GIFT to undertake a separate assessment.

The Committee also estimated international dietary exposures to potassium polyaspartate using the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) cluster diets (FAO/WHO, 2018c). This database includes diets for 17 clusters, which are based on consumption data derived from food balance sheets. The

data included in the diets are mostly for foods expressed on a raw commodity basis and therefore are not usually used to estimate dietary exposures to food additives. However, the diets also include some semi-processed and processed commodities, including wine (food code 564) and vermouths and similar products (food code 565); therefore, it was possible to estimate dietary exposures using this data source. The details regarding how the data were used and how the calculations were undertaken are discussed further in section 3.3.3.

Dietary exposures were reviewed or estimated for average and high consumers, as well as for both adults and children. Food consumption data for wine were available for children. Wine can be consumed by this population subgroup as a result of its inclusion as an ingredient in mixed dishes, such as casseroles or sauces. The sponsor indicated that the food additive is heat stable, so it is assumed not to be lost upon heating/cooking. As it would be in the final food, and as children would be exposed to it through the diet, children were included in the dietary exposure assessment.

The sponsor indicated that potassium polyaspartate is stable in wine; therefore, it is assumed that the wine still contains the food additive at the end of its storage life when it is consumed.

The proposed use levels are expressed in milligrams per litre. Some national food consumption data for wines are expressed in grams. The specific gravity of wine is generally between 0.99 and 1 for still and sparkling wines and up to 1.03–1.04 for fortified and dessert wines (FSANZ, 2019). Therefore, for the purpose of this assessment, it is assumed that the volume of wine is equivalent to the number of grams (i.e. a specific gravity of 1), such that a use level of potassium polyaspartate of 100 mg/L is equivalent to 100 mg/kg. Any slight variations in specific gravity are not likely to influence the estimates of dietary exposure or the conclusions based on these.

The L-aspartic acid monomer is a primary component in the manufacture of potassium polyaspartate. Aspartic acid is, however, incorporated into the polyaspartate backbone in both the L- and D- configurations. Therefore, potential L- and D-aspartic acid exposures resulting from possible breakdown of potassium polyaspartate were considered by the Committee in the dietary exposure assessment and compared with L- and D-aspartic acid exposures from the background diet. Potassium exposures from possible breakdown of the food additive were also considered. Further details of how these assessments were undertaken are provided in section 3.4.

Table 2

Estimated dietary exposures to potassium polyaspartate using the EFSA FAIM model for various population groups as provided by the sponsor

| Population | Population subgroup | Estimated dietary exposures (mg/kg bw per day)[a] | | | |
| | | Use level 200 mg/L | | Use level 300 mg/L | |
		Mean	High	Mean	High
Children	Toddlers	0.0–0.0	0.04–0.3	0.0–0.01	0.05–0.4
	Children	0.0–0.04	0.0–1.0	0.0–0.06	0.0–1.5
	Adolescents	0.0–0.2	0.02–5.2	0.0–0.3	0.04–7.8
Adults	Adults	0.2–1.1	1.2–6.4	0.3–1.6	1.8–9.5
	Elderly	0.1–0.9	1.2–2.9	0.2–1.3	1.8–4.4

ADI: acceptable daily intake; bw: body weight; EFSA: European Food Safety Authority; FAIM: Food Additives Intake Model
[a] Calculated by the Committee based on the results provided by the sponsor on a %ADI basis and an ADI of 10 mg/kg bw per day.

3.3 Estimates of dietary exposure to potassium polyaspartate

3.3.1 Information provided by the sponsor

The sponsor provided use levels of potassium polyaspartate in wine: typical use levels of 100–200 mg/L and a maximum use level of 300 mg/L.

The sponsor provided data on the per capita consumption of wine in Australia and the proportion of different population groups who consumed different types of alcoholic beverages. As the FSANZ dietary exposure assessment for Australia was based on food consumption data for individuals from national dietary survey data, which provides a more accurate estimate of exposure for consumers of wine, the data provided by the sponsor were not considered further by the Committee.

The sponsor provided to the Committee the results of an exposure assessment it had undertaken using the EFSA Food Additives Intake Model (FAIM) template (version 2.0) from 2017 (https://www.efsa.europa.eu/en/applications/foodingredients/tools). The FAIM template is a screening tool used in estimating chronic dietary exposure. Dietary exposures to potassium polyaspartate were presented for toddlers, children, adolescents, adults and the elderly as a percentage of an ADI (an ADI of 10 mg/kg bw per day was used). The Committee extrapolated back from the %ADI results provided to determine the exposure estimates on a milligram per kilogram body weight per day basis (Table 2). For use levels of 200 mg potassium polyaspartate per litre, mean dietary exposures ranged between 0.0 and 0.2 mg/kg bw per day for children and between 0.1 and 1.1 mg/kg bw per day for adults, and high dietary exposures ranged between 0.0 and 5.2 mg/kg bw per day for children and between 1.2 and 6.4 mg/kg bw per day for adults. Based on a maximum use level of 300 mg/L, mean dietary exposures ranged between 0.0 and 0.3 mg/kg bw per day for children and

between 0.2 and 1.6 mg/kg bw per day for adults, and high dietary exposures ranged between 0.0 and 7.8 mg/kg bw per day for children and between 1.8 and 9.5 mg/kg bw per day for adults. The lowest exposures were for toddlers and children, and the highest exposures were for adults.

EFSA also considered these dietary exposures from FAIM in its evaluation (EFSA, 2016b) and concluded that they were overestimates; therefore, EFSA did not consider them further in its opinion. The version of FAIM available prior to 2017, from which the results in Table 2 were derived, used a high consumer model in which the highest exposure from one food category for consumers only was added to the mean exposure for the total population for all other food groups to get total dietary exposures. This methodology will produce an overestimate of dietary exposure compared with more refined estimates based on using dietary exposure data for individuals and deriving summary exposure statistics from a distribution of individual exposures. Given this and given that other, more refined estimates of dietary exposure were available for Europe from EFSA (2016b), the Committee did not include the results from the FAIM model in its evaluation.

3.3.2 **Regional and national estimates of dietary exposure**

Estimates of dietary exposure to potassium polyaspartate from its use in wine and wine products were available for Europe (derived from national estimates of dietary exposure based on national food consumption data) (EFSA, 2016b), Australia and New Zealand (FSANZ, 2018a) and were reviewed by the Committee. The Committee also estimated dietary exposures to potassium polyaspartate for a range of countries for which consumption data were available from CIFOCOss. Two versions of the database were examined to find relevant consumption data: the downloaded spreadsheet version from January 2017 (FAO/WHO, 2017) and the online version (FAO/WHO, 2018b). Consumption data for wine were available in CIFOCOss for some European countries; however, as EFSA had already conducted dietary exposure estimates for European countries using consumption data from the same dietary surveys from the Comprehensive European Food Consumption Database, they were not replicated by the Committee. The remaining countries with consumption data in CIFOCOss for which dietary exposures were calculated were Brazil and China from the 2017 version and the USA from the online version.

CIFOCOss includes national dietary surveys with 2 or more days of food consumption data for individuals collected from population surveys (for further details, see http://www.who.int/foodsafety/databases/en/). Food consumption data are presented in the spreadsheet version according to the CIFOCOss food classification system, which, for most food groups, is based on the Codex raw commodity classification system and the classification system in the Codex GSFA

for some processed commodities; food consumption data in the online version are based on FoodEx2. Food consumption data for a number of different age groups were represented in the CIFOCOss dataset based on the data collected in the countries' national surveys. Consumption data presented for the general population, adults and children were used for the dietary exposure estimates. The data were not split by sex.

Where there were no quantitative consumption amounts for wine in countries reporting consumers, the Committee was unable to include those countries in the assessment; this was the case for Thailand. Where there were fewer than 10 consumers for a population group, these data were not used for dietary exposure calculations, as this provided too few consumers to produce a reliable estimate. This excluded the data for China (children).

Mean and 90th percentile consumption figures per kilogram of body weight for consumers only were used for the dietary exposure calculations. This was to ensure that the body weights relevant to each country and population group specifically were taken into account.

Concentrations of potassium polyaspartate used in the dietary exposure calculations were those provided by the sponsor: typical use levels of between 100 and 200 mg/L and the maximum use level of 300 mg/L for wine.

Estimated national dietary exposures to potassium polyaspartate from use in wines are shown in Table 3. These include estimates calculated by the Committee from national food consumption data and those submitted to the Committee for review and exclude regional estimates for Europe. Details of how the estimated dietary exposures were calculated for each country are described in the subsections below.

From the national estimates available, dietary exposures to potassium polyaspartate for typical use levels of 100–200 mg/L for children ranged between 0.003 and 0.06 mg/kg bw per day at the mean and between 0.01 and 0.14 mg/kg bw per day at the 90th percentile; for adults, dietary exposures to potassium polyaspartate ranged between 0.07 and 0.68 mg/kg bw per day at the mean and between 0.23 and 1.52 mg/kg bw per day at the 90th percentile. Where dietary exposures for the general population were considered, mean exposures ranged between 0.09 and 0.70 mg/kg bw per day, and 90th percentile exposures ranged between 0.18 and 1.58 mg/kg bw per day.

For the maximum potassium polyaspartate use level of 300 mg/L, estimated dietary exposures for children ranged between 0.01 and 0.09 mg/kg bw per day at the mean and between 0.03 and 0.22 mg/kg bw per day at the 90th percentile; for adults, estimated dietary exposures ranged between 0.20 and 1.02 mg/kg bw per day at the mean and between 0.70 and 2.28 mg/kg bw per day at the 90th percentile. Where dietary exposures for the general population were

Table 3
National estimates of dietary exposure to potassium polyaspartate

Population group	Country	Age range	Number of consumers (proportion of respondents)	Dietary exposure for consumers only (mg/kg bw per day)					
				Additive use 100 mg/L		Additive use 200 mg/L		Additive use 300 mg/L	
				Mean	90th percentile	Mean	90th percentile	Mean	90th percentile
Children	New Zealand	5–14 years	99 (3)	0.03	0.07	0.06[a]	0.14[a]	0.09[a]	0.22[a]
	USA[b]	0–35 months	131 (5.1)	0.004	0.01	0.01	0.02	0.01	0.03
		3–5 years	170 (12.0)	0.004	0.01	0.01	0.03	0.01	0.04
		6–14 years	641 (14.8)	0.003	0.01	0.01	0.02	0.01	0.03
Adults	New Zealand	15+ years	870 (18)	0.34	0.76	0.68[a]	1.52[a]	1.02[a]	2.28[a]
	USA[b]	15–49 years	2 207 (23.4)	0.07	0.23	0.13	0.46	0.20	0.70
		50–74 years	1 227 (23.5)	0.08	0.27	0.17	0.53	0.25	0.80
		>75 years	351 (22.0)	0.09	0.28	0.19	0.56	0.28	0.83
General population	Australia	2+ years	1 541 (20)	0.35	0.79	0.70[a]	1.58[a]	1.05[a]	2.37[a]
	Brazil[b]	Not specified	275 (0.8)	0.26	0.54	0.52	1.07	0.78	1.61
	China[b]	Not specified	144 (0.2)	0.09	0.18	0.17	0.36	0.26	0.54

bw: body weight; CIFOCOss: Chronic Individual Food Consumption database – summary statistics
[a] Not included in the original assessment by FSANZ (2018a); calculated by the Committee.
[b] Calculated by the Committee based on consumption data from CIFOCOss.

considered, mean exposures ranged between 0.26 and 1.05 mg/kg bw per day, and 90th percentile exposures ranged between 0.54 and 2.37 mg/kg bw per day.

(a) Australia

Dietary exposure to potassium polyaspartate was estimated for Australia by FSANZ (2018a) following an application to permit the use of the food additive in wine at a maximum permitted level of 100 mg/L (FSANZ, 2018b).

The food consumption data for wine were from the 2011–2012 Australian National Nutrition and Physical Activity Survey, which surveyed 12 153 respondents aged 2 years and above. A 24-hour recall methodology was used, with 7735 (64%) of respondents completing a second 24-hour recall. Only respondents with 2 days of consumption data were included in the assessment, and the estimated exposures were averaged across the 2 days. Population weights for this proportion of the sample were applied to ensure representativeness for the Australian population. Foods included in the assessment were red and white wine, sparkling wine and fortified wine. Each individual respondent's consumption of wine was used to estimate each respondent's dietary exposure, including incorporation of exposure from wine from mixed dishes/recipes, and their own body weights were used to express exposures on a body weight basis. Consumption of wine for consumers only aged 2 years and above at the mean was 265 g/day and at the 90th percentile was 594 g/day.

The estimated dietary exposures for Australia (Table 3) based on a food additive use level of 100 mg/L for the population aged 2 years and above were 0.35 mg/kg bw per day at the mean and 0.79 mg/kg bw per day at the 90th percentile. The assessment undertaken by FSANZ evaluated dietary exposures based on the use level of 100 mg/L only. As the sponsor also indicated a typical use level of 200 mg/L and a maximum use level of 300 mg/L, the Committee calculated estimated dietary exposures at these use levels by multiplying the estimates based on 100 mg/L by 2 and 3, respectively. Therefore, estimated dietary exposures based on a food additive use level of 200 mg/L for the Australian population aged 2 years and above were 0.70 mg/kg bw per day at the mean and 1.58 mg/kg bw per day at the 90th percentile. Based on a food additive use level of 300 mg/L for the Australian population aged 2 years and above, estimated dietary exposures were 1.05 mg/kg bw per day at the mean and 2.37 mg/kg bw per day at the 90th percentile.

(b) Brazil

Dietary exposure to potassium polyaspartate was estimated for Brazil by the Committee using the typical and maximum use levels in wine. The food consumption data for the assessment were derived from CIFOCOss (version January 2017) for the food code 14.2.3.1 Still grape wine. Consumption data

at the mean (2.61 g/kg bw per day) and 90th percentile (5.36 g/kg bw per day) for consumers only (n = 275 consumers out of 34 003 respondents) across the general population were used.

The estimated dietary exposures for Brazil (Table 3) based on a food additive use level of 100 mg/L for the general population were 0.26 mg/kg bw per day at the mean and 0.54 mg/kg bw per day at the 90th percentile. Based on a food additive use level of 200 mg/L, the estimated dietary exposures were 0.52 mg/kg bw per day at the mean and 1.07 mg/kg bw per day at the 90th percentile. Based on a food additive use level of 300 mg/L, the estimated dietary exposures were 0.78 mg/kg bw per day at the mean and 1.61 mg/kg bw per day at the 90th percentile.

(c) China

Dietary exposure to potassium polyaspartate was estimated for China by the Committee using typical and maximum use levels in wine. The food consumption data for the assessment were derived from CIFOCOss (version January 2017) for the food code 14.2.3.1 Still grape wine. Consumption data at the mean (0.86 g/kg bw per day) and 90th percentile (1.82 g/kg bw per day) for consumers only (n = 144 consumers out of 65 359 respondents) across the general population were used. These data were from the 2002 China Nutrition and Health Survey.

The estimated dietary exposures for China (Table 3) based on a food additive use level of 100 mg/L for the general population were 0.09 mg/kg bw per day at the mean and 0.18 mg/kg bw per day at the 90th percentile. Based on a food additive use level of 200 mg/L, the estimated dietary exposures were 0.17 mg/kg bw per day at the mean and 0.36 mg/kg bw per day at the 90th percentile. Based on a food additive use level of 300 mg/L, the estimated dietary exposures were 0.26 mg/kg bw per day at the mean and 0.54 mg/kg bw per day at the 90th percentile.

(d) Europe

EFSA estimated dietary exposures to potassium polyaspartate for Europe (EFSA, 2016b) using individual consumption data from the Comprehensive European Food Consumption Database and two concentrations of potassium polyaspartate in wine: a proposed typical use level of 200 mg/L and a proposed maximum use level of 300 mg/L. EFSA included consumption of all wine (unrefined wine, white wine, red wine, sparkling wine) or fortified and liqueur wines (including fortified wine, vermouth and sherry) based on the FoodEx classification system.

National consumption data were available from 33 dietary surveys and 19 countries, and only those surveys with 2 days of data or more were included in the assessment. For children and adolescents, the countries included in

Table 4

Estimated dietary exposures to potassium polyaspartate for various population groups in Europe

Population	Age	Dietary exposure (mg/kg bw per day)[a]			
		Additive use 200 mg/L		Additive use 300 mg/L	
		Mean	95th percentile	Mean	95th percentile
Children	4–11 months	<0.001	<0.001	<0.001	<0.001
	12–35 months	0–0.005	0–0.002	0–0.007	0–0.004
	3–9 years	0–0.01	0–0.08	0–0.02	0–0.1
	10–17 years	0–0.01	0–0.08	0–0.02	0–0.1
Adults	18–64 years	0.01–0.2	0–1.0	0.02–0.4	0–1.4
	≥65 years	0.04–0.4	0.3–1.2	0.05–0.6	0.4–1.8

[a] It is not stated whether dietary exposures are for consumers only or for all respondents; however, they are assumed to be for all respondents, as zero exposures are not possible if expressed on a consumers only basis.

Source: EFSA (2016b)

the assessment were Austria, Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, the Netherlands, Spain, Sweden and the United Kingdom. For adults, the countries included in the assessment were Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, the Netherlands, Romania, Spain, Sweden and the United Kingdom. The age groups assessed by EFSA included infants (4–11 months), toddlers (12–35 months), children (3–9 years) and adolescents (10–17 years). Adults included two groups: 18–64 years and 65 years and older. For both adults and children, the countries with consumption data differed slightly, depending on the specific age group.

EFSA disregarded exposures for children (including infants to adolescents) based on the assumption that their dietary exposures would be from indirect consumption of wine from recipes, low and not relevant for the risk assessment. Despite this, the estimated dietary exposures for children were presented in the EFSA report. As the Committee had decided to include children in its evaluation, the EFSA dietary exposure results for children were reviewed.

A summary of the dietary exposures to potassium polyaspartate estimated for Europe is shown in Table 4. For a potassium polyaspartate use level of 200 mg/L, mean dietary exposures for children ranged from 0 to 0.01 mg/kg bw per day, and high exposures ranged from 0 to 0.08 mg/kg bw per day; for adults, mean dietary exposures ranged between 0.01 and 0.4 mg/kg bw per day, and high exposures ranged between 0 and 1.2 mg/kg bw per day. Based on a maximum use level of 300 mg/L, mean dietary exposures for children ranged from 0 to 0.02 mg/kg bw per day, and high exposures ranged from 0 to 0.1 mg/kg

bw per day; for adults, mean dietary exposures ranged between 0.02 and 0.6 mg/kg bw per day, and high exposures ranged between 0 and 1.8 mg/kg bw per day. The lowest exposures were for toddlers and children 3–17 years of age, and the highest were for adults overall, but especially for adults 65 years of age and older.

(e) New Zealand

Dietary exposure to potassium polyaspartate was estimated for New Zealand by FSANZ (2018a) following an application to permit the use of the food additive in wine at a maximum permitted level of 100 mg/L (FSANZ, 2018b).

The food consumption data for wine were from the 2002 New Zealand Children's Nutrition Survey, which surveyed 3275 respondents aged 5–14 years, and from the 2008–2009 New Zealand Adult Nutrition Survey, which surveyed 4721 respondents aged 15 years and above. A 24-hour recall methodology was used for both surveys, with 15% and 25% of respondents, respectively, completing a second 24-hour recall. Only data from day 1 were included in the assessment, and population weights were applied. Foods included in the assessment were red and white wine, sparkling wine and fortified wine. Each individual respondent's consumption of wine was used to estimate each respondent's dietary exposure, including incorporation of exposure from wine from mixed dishes/recipes, and their own body weights were used to express exposures on a body weight basis. Consumption of wine for consumers only for children aged 5–14 years was 13 g/day at the mean and 28 g/day at the 90th percentile; for adults 15 years and above, consumption of wine for consumers only was 249 g/day at the mean and 537 g/day at the 90th percentile.

The estimated dietary exposures for New Zealanders (Table 3) based on a food additive use level of 100 mg/L for children aged 5–14 years were 0.03 mg/kg bw per day at the mean and 0.07 mg/kg bw per day at the 90th percentile. For adults aged 15 years and above, the estimated dietary exposures were 0.34 mg/kg bw per day at the mean and 0.76 mg/kg bw per day at the 90th percentile.

The assessment undertaken by FSANZ evaluated dietary exposures based on the use level of 100 mg/L only. As the sponsor also indicated a typical use level of 200 mg/L and a maximum use level of 300 mg/L, the Committee calculated estimated dietary exposures at these levels by multiplying the estimates based on 100 mg/L by 2 and 3, respectively. Therefore, estimated dietary exposures based on a food additive use level of 200 mg/L for New Zealand children aged 5–14 years were 0.06 mg/kg bw per day at the mean and 0.14 mg/kg bw per day at the 90th percentile. For adults aged 15 years and above, estimated dietary exposures were 0.68 mg/kg bw per day at the mean and 1.52 mg/kg bw per day at the 90th percentile. Estimated dietary exposures based on a food additive use level of 300 mg/L for New Zealand children aged 5–14 years were 0.09 mg/kg bw per day at

Table 5
Consumption of wine for various population groups in the USA used in the dietary exposure calculations for potassium polyaspartate

Population group	Age	Number of consumers	Number of respondents	Consumers as proportion of respondents	Mean consumption, consumers only (g/day)	90th percentile consumption, consumers only (g/day)
Children	0–35 months	131	2 591	5.1	0.4	1.10
	3–5 years	170	1 418	12.0	0.7	2.5
	6–14 years	641	4 330	14.8	1.3	3.7
Adults	15–49 years	2 207	9 446	23.4	52.1	183.8
	50–74 years	1 227	5 215	23.5	70.1	223.0
	>75 years	351	1 595	22.0	69.3	205.8

the mean and 0.22 mg/kg bw per day at the 90th percentile. For adults aged 15 years and above, estimated dietary exposures were 1.02 mg/kg bw per day at the mean and 2.28 mg/kg bw per day at the 90th percentile.

(f) United States of America

Dietary exposure to potassium polyaspartate was estimated for the USA by the Committee using typical and maximum use levels in wine. The food consumption data for the assessment were derived from CIFOCOss (online version April 2019). The database was filtered for the foods wine, white wine, red wine, wine-like drinks, vermouth, and fortified and liqueur wines; however, the only food for which consumption data were reported was "wine and wine like drinks". Consumption data were available for a range of population groups, including 0–35 months, 3–5 years, 6–14 years, 15–49 years, 50–74 years and 75 years and above. Mean and 90th percentile consumption data for consumers only for both sexes combined were used.

The consumption data were not provided on a body weight basis. Mean body weights for each age group (sexes combined) were derived using the raw data from each respondent in the What We Eat in America – Food Commodity Intake Database, 2005–2010, which are the data for the CIFOCOss dataset. The mean body weights used were 10.5 kg for 0–35 months, 18.5 kg for 3–5 years, 43.2 kg for 6–14 years, 79.1 kg for 15–49 years, 83.4 kg for 50–74 years and 74 kg for 75 years and above. A summary of the consumption amounts used in the assessment is shown in Table 5.

The estimated dietary exposure to potassium polyaspartate for the USA (Table 3) based on a food additive use level of 100 mg/L for children ranged between 0.003 and 0.004 mg/kg bw per day at the mean and was 0.01 mg/kg bw per day at the 90th percentile; for adults, estimated dietary exposures ranged

between 0.07 and 0.09 mg/kg bw per day at the mean and between 0.23 and 0.28 mg/kg bw per day at the 90th percentile. Based on a food additive use level of 200 mg/L, estimated dietary exposure for children was 0.01 mg/kg bw per day at the mean and ranged between 0.02 and 0.03 mg/kg bw per day at the 90th percentile; for adults, estimated dietary exposures ranged between 0.13 and 0.19 mg/kg bw per day at the mean and between 0.46 and 0.56 mg/kg bw per day at the 90th percentile. Based on a food additive use level of 300 mg/L, estimated dietary exposure for children was 0.01 mg/kg bw per day at the mean and ranged between 0.03 and 0.04 mg/kg bw per day at the 90th percentile; for adults, estimated dietary exposures ranged between 0.20 and 0.28 mg/kg bw per day at the mean and between 0.70 and 0.83 mg/kg bw per day at the 90th percentile.

3.3.3 International estimates of dietary exposure

International estimates of dietary exposure to potassium polyaspartate were calculated by the Committee using the 17 GEMS/Food cluster diets. The cluster diets are supranational model diets based on food balance sheet data from individual countries and are for mean amounts of food per capita available for consumption. The diets include consumption amounts of raw or minimally processed foods, and wine is included in the dataset. They do not include consumption data for any population subgroups by either age or sex; therefore, they do not allow for separate assessments for groups such as children. As the consumption data are per capita, they will by default include the whole population, including children. The main use of this assessment in this context is for an international comparison to determine areas of the world that may have higher exposures to potassium polyaspartate compared with others. All clusters were included in the assessments based on the assumptions that there is a global food supply with foods traded between countries and that potassium polyaspartate would be in wines found in every cluster. Further information on the cluster diets can be found at https://www.who.int/foodsafety/databases/en/.

Mean estimates of dietary exposure were calculated using the cluster diet consumption amounts multiplied by the typical use levels of potassium polyaspartate of 100 and 200 mg/L and the maximum use level of 300 mg/L. In order for a high-percentile dietary exposure to be estimated, the mean exposure was multiplied by 2 in order to approximate a 90th percentile (FAO/WHO, 1985).

Consumption data for the clusters were not available on a body weight basis; therefore, estimates of dietary exposure were divided by a standard body weight of 60 kg to enable them to be expressed per kilogram body weight.

The estimates of dietary exposure are presented in Table 6. For the food additive use level of 100 mg/L, mean exposures ranged between 0.0004 and 0.15 mg/kg bw per day, and the 90th percentile exposures ranged between

Table 6

Per capita estimates of dietary exposure to potassium polyaspartate based on different levels of food additive use

| | Consumption (g/person per day) | | Dietary exposure (mg/kg bw per day)[a] | | | | | |
| | | | Additive use level 100 mg/L | | Additive use level 200 mg/L | | Additive use level 300 mg/L | |
Cluster	Wine (code 564)	Vermouths & similar (code 565)	Mean	90th percentile	Mean	90th percentile	Mean	90th percentile
G01	0.61	0.06	0.001	0.002	0.002	0.004	0.003	0.007
G02	11.97	0.56	0.02	0.04	0.04	0.08	0.06	0.13
G03	1.96	0.05	0.003	0.007	0.007	0.01	0.01	0.02
G04	1.16	0.05	0.002	0.004	0.004	0.008	0.006	0.01
G05	3.52	0.01	0.006	0.01	0.01	0.02	0.02	0.04
G06	3.97	0.04	0.007	0.01	0.01	0.03	0.02	0.04
G07	88.43	0.51	0.15	0.30	0.30	0.60	0.45	0.89
G08	61.70	0.71	0.10	0.21	0.21	0.42	0.32	0.62
G09	1.82	0.01	0.003	0.006	0.006	0.01	0.009	0.02
G10	24.90	0.17	0.04	0.08	0.08	0.17	0.13	0.25
G11	58.79	2.38	0.10	0.20	0.20	0.41	0.31	0.61
G12	5.26	0.58	0.01	0.02	0.02	0.04	0.03	0.06
G13	0.30	0.01	0.001	0.001	0.001	0.002	0.002	0.003
G14	0.22	0.02	0.000 4	0.001	0.001	0.002	0.001	0.002
G15	59.77	0.66	0.10	0.20	0.20	0.40	0.30	0.60
G16	0.52	0.01	0.001	0.002	0.002	0.003	0.003	0.005
G17	31.89	0.02	0.05	0.11	0.11	0.21	0.16	0.32

bw: body weight
[a] Assuming a body weight of 60 kg.

0.001 and 0.30 mg/kg bw per day. For the food additive use level of 200 mg/L, mean exposures ranged between 0.001 and 0.30 mg/kg bw per day, and the 90th percentile exposures ranged between 0.002 and 0.60 mg/kg bw per day. For the food additive use level of 300 mg/L, mean exposures ranged between 0.001 and 0.45 mg/kg bw per day, and the 90th percentile exposures ranged between 0.002 and 0.89 mg/kg bw per day.

The clusters with the highest estimates of dietary exposure to potassium polyaspartate (mean exposure over 0.4 mg/kg bw per day), in order from highest to lowest, are G07 (which consists of European countries, Australia, Bermuda and Uruguay), G08 (European countries), G11 (European countries) and G15 (European countries). A full list of countries in clusters G07, G08, G11 and G15 is shown in Appendix 1.

The Committee decided that the estimates of international dietary exposure would not be used for the purpose of the evaluation, as they are

based on per capita food consumption data, and more refined estimates of dietary exposure based on national food consumption data were available for that purpose. However, the Committee noted that the international estimates provided information on the countries of the world with higher dietary exposures compared with others. Countries with the highest exposures were from Europe and Australia. More refined estimates of dietary exposure were available for these countries; therefore, the evaluation by the Committee includes the relevant countries, and any determination of safety would therefore be based on countries at a higher level of risk.

3.4 Dietary exposure assessment for other relevant substances

3.4.1 Total aspartic acid

Aspartic acid is a non-essential amino acid that is a naturally occurring component of the diet. It can be found in animal foods, including meats, seafood and gelatine, and plant sources, such as grains, nuts, seeds, fruits, vegetables and legumes (FSANZ, 2019), dietary supplements and intense sweeteners, such as aspartame (at levels of around 40% of the sweetener; Institute of Medicine, 2005). The Committee also undertook a review of dietary exposure to total aspartic acid.

A literature search was undertaken to find estimates of dietary exposure to aspartic acid. EBSCO Discovery Service was used, and databases including Medline, Food Science Source, Food Science and Technology Abstracts and Science Direct were searched, as well as a number of journals related to science, toxicology, food, nutrition and public health. Search terms included "aspartic acid" and "intake" or "dietary intake" or "consumption". These terms were also used in a general Internet search to capture other papers not included in the scientific literature. The search would have picked up information in relation to total aspartic acid and/or its D- or L- forms. The only population-based estimates of dietary exposure to total aspartic acid found were from the USA (Institute of Medicine, 2005).

Estimated dietary exposures to total aspartic acid from the USA are from the 1988–1994 National Health and Nutrition Examination Survey (Institute of Medicine, 2005). The estimated dietary exposures to total aspartic acid from the background diet do not differentiate between the L- and D- forms and are simply reported as dietary exposures to "aspartic acid". It was therefore assumed that these exposures were for total aspartic acid. The mean dietary exposure to total aspartic acid for the population from food and supplements was 6.5 g/day (108 mg/kg bw per day based on a 60 kg body weight). The highest 90th percentile exposure across the age/sex groups reported was 12.0 g/day for males 19–30 years of age (200 mg/kg bw per day based on a 60 kg body weight). Although exposures

were described as being from food and supplements, it was not noted what supplements were included in the assessment or what contribution supplements made to the estimated exposures. The food composition dataset published by the USDA (2019) for "aspartic acid" has only one beverage supplement included in the dataset, and no dietary supplements in dosage form are included. It is not known whether this dataset formed the basis of the published dietary exposures to total aspartic acid.

Total aspartic acid exposures from use of potassium polyaspartate in wine resulting from a hypothetical 100% breakdown of potassium polyaspartate (as a worst-case assumption) were considered by the Committee to enable a comparison with dietary exposure to total aspartic acid from the background diet. The highest end of the range of estimated national high-percentile dietary exposures to potassium polyaspartate based on typical use levels in wine was 1.6 mg/kg bw per day (90th percentile exposure for Australia of 1.58, rounded). Based on the molecular weight (MW) of aspartic acid of 133.1 g/mol, it could be estimated that there are around eight monomers in the potassium polyaspartate polymer, which has a molecular weight of 1100 g/mol. Potassium makes up approximately 25% of the potassium aspartate monomer ($MW_p/MW_{monomer}$ = 39/154 = 0.25; this is supported by the specifications determined by the Committee at the present meeting for the assay, which specify that potassium should not be less than 23% on the dried basis). However, for the purpose of this dietary exposure assessment, in keeping with a tiered approach and assuming a worst-case scenario, the calculations do not make adjustments to specifically remove potassium, and it was assumed that the potassium polyaspartate is all broken down to aspartic acid and that there is no contribution from potassium. The Committee determined, therefore, that the exposures to total aspartic acid from potassium polyaspartate would be equivalent to dietary exposures to the food additive at a highest estimated amount of 1.6 mg/kg bw per day based on typical use levels. Assuming a 60 kg body weight, the dietary exposure to total aspartic acid from the food additive is 1.5% of the mean population dietary exposure to total aspartic acid from the background diet of 108 mg/kg bw per day (6.5 g/day) and 0.8% of the 90th percentile dietary exposure to total aspartic acid from the background diet of 200 mg/kg bw per day (12 g/day) (Table 7). Based on this conclusion, should a more refined estimate of dietary exposure have been conducted that specifically excluded the potassium within the monomer, the conclusion would remain the same – that exposure to total aspartic acid from the food additive is a small proportion of exposure to total aspartic acid from the background diet.

The same conclusion, that dietary exposures to aspartic acid from the food additive were a small proportion of exposures to aspartic acid from the background diet, was also reached by EFSA, based on its dietary exposure

Table 7

Estimated dietary exposures to total aspartic acid from typical use of potassium polyaspartate compared with exposures from the background diet

Source of dietary exposure	Measure	Total aspartic acid exposure
Total aspartic acid from use of potassium polyaspartate in wine	90th percentile	1.6 mg/kg bw per day
Total aspartic acid from background diet	Mean	108 mg/kg bw per day[a] (6.5 g/day[b])
	90th percentile	200 mg/kg bw per day[a] (12.0 g/day[b])
Contribution of additive to total aspartic acid exposure	Mean	1.5%
	90th percentile	0.8%

bw: body weight
[a] Based on a 60 kg body weight.
[b] Institute of Medicine (2005).

estimates. EFSA only described aspartic acid as such with no further descriptions or qualifiers as to "total" or otherwise. EFSA assumed a 4% breakdown of the food additive to aspartic acid based on simulated gastric digestion in vitro. Using the highest estimated level of exposure to the food additive (1.2 mg/kg bw per day, based on typical use levels, and 1.8 mg/kg bw per day, based on the maximum use level for the elderly), EFSA estimated an exposure to aspartic acid to be 0.04 mg/kg bw per day based on typical use levels and 0.07 mg/kg bw per day based on maximum use levels. EFSA compared this with exposures to aspartic acid from the diet (9.1 and 13 g/day or 130 and 186 mg/kg bw per day) to conclude that exposures to aspartic acid would increase by only 0.05% at the maximum level of use and considered this negligible. When assuming a hypothetical 100% breakdown of the food additive, EFSA estimated that aspartic acid exposures from the food additive would be less than 1.5% of aspartic acid exposures from the diet.

The proportions of the L- and D-aspartic acid forms in the background diet are not known for all foods. Therefore, it is difficult to estimate with certainty the amount of D- and L-aspartic acid consumed in total from the food additive and from the background diet.

3.4.2 **L-Aspartic acid**

L-Aspartic acid used in the manufacture of potassium polyaspartate also occurs naturally in food and can be consumed via dietary supplements and food additives such as aspartame.

According to EFSA (2013), aspartame is fully hydrolysed in the gastrointestinal tract to L-phenylalanine, L-aspartic acid and methanol. EFSA estimated the dietary exposures to L-aspartic acid from aspartame based on

Table 8

Estimated dietary exposures to L-aspartic acid from typical use of potassium polyaspartate in wine compared with exposures to total aspartic acid from the background diet

Source of dietary exposure	Measure	Dietary exposure
L-Aspartic acid from use of potassium polyaspartate in wine	90th percentile	0.8 mg/kg bw per day[a]
Total aspartic acid from background diet	Mean	108 mg/kg bw per day[b] (6.5 g/day[c])
	90th percentile	200 mg/kg bw per day[b] (12.0 g/day[c])
L-Aspartic acid exposure from use as additive as a % of total aspartic acid exposure from background diet	Mean	0.7%
	90th percentile	0.4%

bw: body weight
[a] Assumes that L-aspartic acid is 50% of total aspartic acid due to racemization.
[b] Based on a 60 kg body weight.
[c] Institute of Medicine (2005).

maximum permitted levels to be between 0.2 and 7.4 mg/kg bw per day at the mean and between 0.7 and 16.7 mg/kg bw per day at the 95th percentile for consumers in five age groups in Europe. When the estimate was based on reported use levels or analytical data, L-aspartic acid exposures from aspartame were between 0.2 and 7.4 mg/kg bw per day for mean exposures and between 0.6 and 16.3 mg/kg bw per day for high exposures.

The Committee estimated that dietary exposure to L-aspartic acid is up to 0.8 mg/kg bw per day from the typical use of potassium polyaspartate in wine. This is 50% of the total aspartic acid exposure of 1.6 mg/kg bw per day due to racemization and assumes that potassium polyaspartate is completely fermented in the colon and that the products of the fermentation are absorbed and bioavailable. The estimate of dietary exposure to L-aspartic acid is also based on the worst-case scenario noted above, assuming no contribution from the potassium in the potassium aspartate monomer. In order to put the L-aspartic acid exposures from wine into the context of the whole diet, they were compared with total aspartic acid exposures. Estimated dietary exposure to L-aspartic acid from food additive use was 0.7% of mean total aspartic acid exposure from the background diet and 0.4% of 90th percentile total aspartic acid exposure from the background diet (Table 8). The additional exposure to L-aspartic acid from the use of potassium polyaspartate in wine is negligible and would be within normal daily variation in dietary exposures.

3.4.3 D-**Aspartic acid**

D-Amino acids can occur in foods naturally, such as in cow, sheep and goat milk and fruits and vegetables such as apples, grapes, oranges, carrots, tomatoes

and cabbages (Genchi, 2017). They can also occur in foods as a result of food processing, such as from the application of high temperatures, strong acid and alkali treatments and fermentation (e.g. yoghurt, cheese, beer, wine, sourdough bread), or as a result of microbiological activity. D-Aspartic acid specifically occurs in milk (pasteurized and homogenized), yoghurt, cheese (Zagon, Dehne & Bogl, 1994), evaporated milk, foods that have undergone heat treatment such as toast and cooked meats (Csapó, Albert & Csapó-Kiss, 2009), savoury wheat-based crackers, wheat cake, Mexican pancakes and corn cakes (Finley, 1985), and also in dried milk powder and infant formula (as a result of milk pasteurization and homogenization and heating during the drying process) (Csapó, Albert & Csapó-Kiss, 2009).

D-Aspartic acid is present at low levels in raw and pasteurized milk (0.17–0.38 mg/L) and at somewhat higher levels in processed milk products such as kefir (3.5 mg/L), yoghurt (3.1 mg/L), curdled milk (2.5 mg/L), fresh goat cheese (3.8 mg/kg) and harzer cheese (3.7 mg/kg) (Brückner & Hausch, 1990).

Csapó et al. (2007) investigated the effects of the different manufacturing processes, starter culture and ripening on the D-aspartic acid content of cheese, with concentrations of 0.59–0.98 mg/100 g dry matter at the start of the process, depending on starter culture strain, and up to 1.5–2.4 mg/100 g dry matter after 63 days of ripening. The proportion of D-enantiomers was 28% at the start of manufacture, compared with 36% after 63 days of ripening. Another study of various cheeses (Csapó et al., 1997) showed a mean concentration of 58 μmol/100 g for D-aspartic acid. D-Aspartic acid has also been found in rice wine at levels ranging between not detected (<2.5 μmol/L) and 86 μmol/L (Miao et al., 2017).

Abe et al. (1999) measured mean levels of D-aspartic acid of 0.02–1.75 μmol/g in fish sauces across a number of countries and 0.02–0.99 μmol/g in fermented fish products (fish paste to which amino acid condiment was added).

D-Aspartic acid occurs in instant or roasted coffee, with almost none in green unroasted coffee (Zagon et al., 1994). One paper (Packard, 1982) noted a D-aspartic acid concentration in coffee of 200 mg/L. However, the Committee could not verify whether the concentration reported for instant coffee was based on the beverage in milligrams per millilitre or on dry coffee powder in milligrams per kilogram, as the reported concentration was taken from secondary literature, and the original publication was not available.

The presence of D-Asx (= D-aspartate + D-asparagine) in wines that were produced in Germany, France and Spain from 1967 to 2003 was reported by Ali, Pätzold & Brückner (2010). It was shown that concentrations of D-Asx in white wine (n = 16) were in the range of 0.5–10.0 mg/L, in red wine (n = 4) in the range of 2.3–12.0 mg/L, in ice wine (n = 2) in the range of 0.7–5.2 mg/L and in sparkling wine (n = 4) in the range of 1.2–4.0 mg/L. It is not clear whether this is due to a natural occurrence in grapes; however, considering the time period in which

Table 9
Estimated dietary exposure to D-aspartic acid for specific foods

Food	Concentration of D-aspartic acid (mg/kg or mg/L)	Approximate consumption amount (g/day for consumers)	Estimated dietary exposure (mg/kg bw per day)[a]
Milk	0.17–0.38[b]	250	0.001–0.002
Yoghurt	3[b]	150	0.01
Cheese	14.4[c]	40	0.01
Juice (beetroot)	10.2[d]	400	0.07
Beer	2.1[e]	800	0.03
White wine (no added potassium polyaspartate)	2.2[d]	400	0.02

bw: body weight
[a] Based on a 60 kg body weight.
[b] Brückner & Hausch (1990). Aspartate + asparagine calculated as aspartate.
[c] Csapó et al. (2007). Converted from concentration in dry matter to fresh weight based on a moisture content of 40%.
[d] Brückner & Hausch (1989a).
[e] Brückner & Hausch (1989b).

these wines were produced, it seems unlikely that these concentrations were due to the use of potassium polyaspartate in wine.

The Committee estimated that dietary exposure to D-aspartic acid from the typical use of potassium polyaspartate in wine is the same as for L-aspartic acid, up to 0.8 mg/kg bw per day. This is 50% of the total aspartic acid exposure of 1.6 mg/kg bw per day due to racemization, as noted above for L-aspartic acid. As was the case for total and L-aspartic acid, the estimate of dietary exposure to D-aspartic acid is also based on the worst-case scenario noted above, assuming no contribution from the potassium in the potassium aspartate monomer.

The Committee compared the dietary exposure to D-aspartic acid from the use of potassium polyaspartate in wine with that from other specific foods in the diet known to contain D-aspartic acid. Estimates were calculated for only a small number of foods (*n* = 6) for which concentration data were available and that are more common dietary components. These foods were milk, cheese, yoghurt, beer, wine and juice. The concentration of D-aspartic acid in foods from the literature was multiplied by an approximate daily consumption of those foods (Table 9). Dietary exposures to D-aspartic acid following consumption of the individual foods ranged between 0.001 and 0.07 mg/kg bw per day. In general, the dietary exposure to D-aspartic acid from each individual food in its own right was well below the estimated exposure of up to 0.8 mg/kg bw per day of D-aspartic acid that could potentially result from the use of potassium polyaspartate in wine.

However, this assessment of D-aspartic acid exposure from the background diet is from an incomplete list of foods that could contain D-aspartic acid. Food processing (e.g. heat treatment of protein, fermentation, drying of

foods) will result in partial conversion of L-aspartic acid to D-aspartic acid for a range of other foods. A more extensive investigation into the concentrations of D-aspartic acid across a broad range of foods would be required to enable the total dietary exposure to D-aspartic acid to be estimated. The Committee was unable to undertake this investigation during its present meeting. It is assumed, therefore, that total dietary exposure to D-aspartic acid summed from the total diet would be higher than estimated for the small number of individual foods considered in this assessment.

Aspartic acid is also found in dietary supplements, particularly sports-style supplements. Some do not specify the form of the aspartic acid, and many are specifically for D-aspartic acid. Products are offered in capsule or powder (made into a drink) forms. Dosage instructions result in daily intakes of aspartic acid or D-aspartic acid ranging from about 1.4 g/day to 3 g/day (for most products), with some up to 6 g/day. These D-aspartic acid intakes would be equivalent to 23, 50 and 100 mg/kg bw per day, respectively, assuming a 60 kg body weight.

3.4.4 **Potassium**

Dietary exposures to potassium were reviewed in order to determine whether exposure to potassium from the use of potassium polyaspartate in wine is of dietary significance.

The maximum use level of potassium polyaspartate in wine is 300 mg/L. This equates to a level of potassium of 75 mg/L, assuming that 25% of the potassium aspartate monomer is potassium based on molecular weights, as noted in section 3.4.1. This amount of potassium from the food additive is lower than the naturally occurring levels of potassium in wine, which generally range between 700 and 1200 mg/kg (FSANZ, 2019; USDA, 2019).

From the data on wine consumption available to the Committee for this assessment, wine consumption is around 600 mL/day for a high consumer (data from Australia of 594 g/day, rounded up). This equates to exposure to potassium from the food additive of 45 mg/day. Estimated usual mean dietary exposures to potassium generally range between 2000 and 3000 mg/day (University of Otago & Ministry of Health, 2011; Cogswell et al., 2012; Australian Bureau of Statistics, 2015; EFSA, 2016a). Therefore, the additional exposures to potassium from use of the food additive in wine would be within normal daily variation.

4. Comments

4.1 Biochemical aspects

There are no in vivo data on the absorption of potassium polyaspartate.

In vitro data on Caco-2 monolayers that were used to simulate gastrointestinal absorption (Restani, 2015; Vassanelli, 2015) suggest that the systemic bioavailability of potassium polyaspartate is low. Other in vitro data obtained with pepsin and pancreatin to simulate gastrointestinal digestion (Restani, 2015) suggest that potassium polyaspartate would not be cleaved in the stomach and the intestine. However, potassium polyaspartate could be digested by microbiota occurring in the human intestine. The Committee noted the absence of information on the extent of fermentation of polyaspartate.

4.2 Toxicological studies

No information on the acute toxicity of potassium polyaspartate was available.

A dose range–finding study in rats given potassium polyaspartate by oral gavage at a dose of 0, 60, 125, 250, 500 or 1000 mg/kg bw per day for 14 days showed no treatment-related adverse effects (Gumaste, 2014a; Galbusera et al., 2017).

In a 90-day toxicity study, rats were given potassium polyaspartate by oral gavage at a dose of 0, 250, 500 or 1000 mg/kg bw per day. No treatment-related adverse effects were observed. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Gumaste, 2014b; Galbusera et al., 2017).

No long-term toxicity or carcinogenicity studies were available.

A bacterial reverse mutation assay and an in vitro micronucleus assay in human lymphocytes gave negative results (Mane, 2014a,b; Galbusera et al., 2017). The Committee concluded that there is no concern with respect to the genotoxicity of potassium polyaspartate.

No specific studies on reproductive and developmental toxicity, neurotoxicity or immunotoxicity were available. However, the 90-day study described above included additional parameters that provide information on some of these end-points. No effects on the estrous cycle or on weights and histopathology of testes, epididymides, seminal vesicles, uterus or ovaries were observed. There were no signs of neurological dysfunction investigated using a functional observational battery approach. No treatment-related effects indicating an immunotoxic or immunomodulatory potential were observed. In addition, histopathological investigations performed on thyroid and parathyroid and blood concentrations of T_3, T_4 and TSH measured at termination of the treatment

found no treatment-related effects that indicated disturbance of thyroid function (Gumaste, 2014b; Galbusera et al., 2017).

Results from an in vitro study in which the human promyelocytic cell line THP-1 was used as a surrogate for monocytes did not provide any indication of an immune response as indicated by CD86 expression and IL-8 release (Restani, 2015).

4.3 **Observations in humans**

No information was available.

4.4 **Studies on L- and D-aspartic acid and potassium**

Because the aspartic acid incorporated in the polyaspartate backbone is in an L- and D- configuration, the Committee considered L- and D-aspartic acid resulting from possible breakdown of potassium polyaspartate, as well as potassium.

4.4.1 L-**Aspartic acid**

L-Aspartic acid is a non-essential amino acid that occurs in food. It is also a component of the intense sweetener aspartame. Because L-aspartic acid results from the hydrolysis of aspartame, the toxicity of and dietary exposure to L-aspartic acid were considered by the Committee in the course of its evaluations of the use of aspartame. The Committee concluded that L-aspartic acid generated from aspartame was not a safety concern at current dietary exposure to aspartame (Annex 1, reference *54*).

When the use of L-aspartic acid as a flavouring agent was evaluated by the Committee at its sixty-third meeting, the Committee concluded that there was no safety concern at current dietary exposures when used as a flavouring agent (Annex 1, reference *174*).

4.4.2 D-**Aspartic acid**

D-Aspartic acid is an endogenous amino acid that is involved in the development of the nervous system, plays a role in the neuroendocrine system, including hormone synthesis, has neuronal activities and is implicated in male fertility (A. D'Aniello et al., 1996; G. D'Aniello et al., 2005; Furuchi & Homma, 2005; A. D'Aniello, 2007; Errico et al., 2008, 2009, 2012; Topo et al., 2009; S. D'Aniello et al., 2011; Katane & Homma, 2011; Ota, Shi & Sweedler, 2012; Di Fiore et al., 2016, 2018; Genchi, 2017). D-Aspartic acid is present in the human brain and

accumulates with age in the central nervous system white matter, but not in grey matter (Man et al., 1983, 1987).

In a systematic review of 23 animal studies, three of which involved oral exposure of rats, and four human studies, the authors concluded that exogenous D-aspartic acid enhances testosterone levels in male animals at oral doses equivalent to around 130 mg/kg bw per day, whereas studies in humans, in which daily doses ranging from 36 to 70 mg/kg bw per day were consumed as dietary supplements, yielded inconsistent results. The authors noted that the inconsistent results obtained in these human trials could be due to limitations of the study designs, such as short-term supplement duration (12–28 days) and small sample sizes (N = 10–23 in the supplemented groups) (Roshanzamir & Safavi, 2017). The Committee agreed with this conclusion and noted that no NOAELs could be identified from the oral rat studies, as only single doses were tested.

There is experimental evidence for an L-isomer-selective transport of aspartic acid at the blood–brain barrier in the rat, whereby L-aspartic acid, but not D-aspartic acid, undergoes efflux transport from the brain to the blood; in contrast, the uptake of aspartic acid in brain parenchymal cells is not stereospecific (Hosoya et al., 1999; Tetsuka et al., 2003). However, the Committee noted that administration of D-aspartic acid to rats in drinking-water at a dose of 50 mg/kg bw per day for 28 days increased its levels in both liver and blood serum about 5-fold and in kidney homogenates 8-fold, but did not increase the D-aspartate level in brain homogenates (Schieber et al., 1997). The Committee also noted that while the study did not meet current standards applicable for repeated-dose 28-day oral toxicity studies in rats (OECD Test Guideline 407), no signs of general toxicity were detected, and histopathological evaluation of renal and hepatic tissues did not reveal any treatment-related pathological alterations.

The Committee further noted that free D-aspartic acid can be metabolized by D-amino acid oxidase, which is expressed in brain, spinal cord, liver, renal proximal tubule cells and the proximal and middle small intestine of mice and humans (Pollegioni, Sacchi & Murtas, 2018).

There are no longer-term (>1 month) oral toxicity studies on D-aspartic acid and no toxicity studies on racemic mixtures of D- and L-aspartic acid.

4.4.3 Potassium

Potassium was evaluated by the Committee in the course of the evaluation of potassium hydroxide as a food additive at its ninth meeting (Annex 1, reference *11*). The result of the evaluation was an ADI "not limited"[3] for potassium hydroxide.

Serum levels of potassium usually rise only moderately in response to potassium intake, even in the case of a short-term (2–24 weeks) high potassium

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[3] Now called ADI "not specified".

Table 10
Range of estimated dietary exposures[a] to potassium polyaspartate at typical and maximum use levels

Population group	Estimated dietary exposure (mg/kg bw per day)			
	Typical use level: 100–200 mg/L		Maximum use level: 300 mg/L	
	Mean	High[b]	Mean	High[b]
Children	0–0.06	0–0.14	0–0.09	0–0.22
Adults	0.01–0.68	0–1.52	0.02–1.02	0–2.28
General population	0.09–0.70	0.18–1.58	0.26–1.05	0.54–2.37

bw: body weight
[a] Includes estimates for Europe and national estimates submitted to and calculated by the Committee.
[b] High exposure is the 90th percentile for all estimates other than Europe, for which high exposure is the 95th percentile.

intake of 1755 mg/day, which resulted in an increase in potassium serum levels by only 0.17 mmol/L (6.6 mg/L) (Cappuccio et al., 2016).

4.5 Assessment of dietary exposure

A dietary exposure assessment for potassium polyaspartate was undertaken for the first time by the present Committee. The assessment was based on typical use levels in wine of 100–200 mg/L and a maximum proposed use level of 300 mg/L.

Estimated dietary exposures reviewed were those submitted by the sponsor based on EFSA's FAIM, an EFSA assessment based on the Comprehensive European Food Consumption Database (EFSA, 2016b) and national dietary survey data for Australia and New Zealand (FSANZ, 2018a). The Committee also calculated national estimates of dietary exposure based on food consumption data in CIFOCOss for Brazil, China and the USA.

A summary of the national dietary exposure estimates is shown in Table 10.

The estimates of dietary exposure to potassium polyaspartate based on the maximum use level are overestimates; instead, the dietary exposures based on typical use levels provide better estimates of chronic dietary exposures. Mean estimates of dietary exposure based on typical use levels are up to 0.7 mg/kg bw per day, and high exposures are up to 1.6 mg/kg bw per day.

L-Aspartic acid used in the manufacture of potassium polyaspartate also occurs naturally in food and can be consumed via dietary supplements and food additives such as aspartame. The Committee estimated that the dietary exposure for each of L- and D-aspartic acid is up to 0.8 mg/kg bw per day from the typical use of potassium polyaspartate in wine. This represents 50% of the total aspartic acid (both L- and D-aspartic acid) exposure of 1.6 mg/kg bw per

day due to racemization and assumes that potassium polyaspartate is completely fermented in the colon and that the products of the fermentation are absorbed and bioavailable.

Estimated dietary exposure to L-aspartic acid from the food additive use is around 1% of a mean population dietary exposure of 108 mg/kg bw per day (6.5 g/day) for total aspartic acid from the diet (natural and supplemental sources) (Institute of Medicine, 2005) and less than 1% of a high dietary exposure of 200 mg/kg bw per day (12.0 g/day) for total aspartic acid. The Committee concluded that the amount of additional L-aspartic acid in the diet from potassium polyaspartate is negligible and would be within normal daily variation in dietary exposures.

Dietary exposures to D-aspartic acid from six foods known to contain it (milk, cheese, yoghurt, beer, wine, juice) were estimated. Dietary exposures from the individual foods ranged between 0.001 and 0.07 mg/kg bw per day. The Committee was aware that this is an incomplete list of foods and also noted that food processing (e.g. heat treatment of protein, fermentation) will result in partial conversion of L-aspartic acid to D-aspartic acid for a range of other foods. Therefore, total dietary exposure to D-aspartic acid would be higher than estimated here.

The Committee considered the additional dietary exposure to potassium in the diet from use of the food additive and estimated a dietary exposure to potassium of about 45 mg/day for high consumers of wine. This is well below usual dietary exposures of between 2000 and 3000 mg/day, and the Committee concluded that the additional dietary exposure to potassium from use of the food additive in wine would be within normal daily variation.

5. Evaluation

In vitro data suggest that the systemic bioavailability of potassium polyaspartate is low and that it would not be cleaved in the stomach or the intestine. The NOAEL in a 90-day rat study on potassium polyaspartate was 1000 mg/kg bw per day, the highest dose tested. There was no concern for genotoxicity.

Potassium has been evaluated by the Committee in the course of its evaluation of potassium hydroxide (Annex 1, reference *11*), and the result of the evaluation was an ADI "not limited". Exposure to potassium that results from the use of potassium polyaspartate in wine would be within normal daily variation of background potassium exposure from the diet.

The Committee noted that no information on potential microbial fermentation in the human colon is available, but should that occur, there would

be potential exposure to L- and D-aspartic acid. L-Aspartic acid is a normal constituent of dietary protein, and systemic exposure to L-aspartic acid from the diet is much higher than potential exposure from the use of potassium polyaspartate in wine.

There are no relevant toxicological data on D-aspartic acid. In three studies, rats exposed to around 130 mg/kg bw per day showed effects on sex hormone levels. However, NOAELs have not been identified in these studies due to the use of single doses. The Committee noted that there is a margin of exposure of more than 100-fold between the potential human exposure to D-aspartic acid of up to 0.8 mg/kg bw per day and the effect level of 130 mg/kg bw per day.

The estimated dietary exposure to D-aspartic acid from typical use of potassium polyaspartate in wine (up to 0.8 mg/kg bw per day) would be expected to be lower than the exposure from non-added sources in the diet. The Committee noted that it had limited data on concentrations of D-aspartic acid in food, but that food processing (e.g. heat treatment of protein, fermentation) will result in partial conversion of L-aspartic acid to D-aspartic acid.

The Committee concluded that the use of potassium polyaspartate in wine at the maximum proposed use level of 300 mg/L is not of safety concern.

New specifications for potassium polyaspartate were prepared.

A Chemical and Technical Assessment was prepared.

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Appendix 1: Clusters with the highest estimates of dietary exposure to potassium polyaspartate

Cluster	Country
G07	Australia
	Bermuda
	Finland
	France
	Iceland
	Luxembourg
	Norway
	Switzerland
	United Kingdom
	Uruguay
G08	Austria
	Germany
	Poland
	Spain
G11	Belgium
	Netherlands
G15	Czech Republic
	Denmark
	Hungary
	Ireland
	Lithuania
	Portugal
	Romania
	Slovakia
	Slovenia
	Sweden

Rosemary extract (addendum)

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1. Explanation

Rosemary extract (International Numbering System for Food Additives [INS] 392) is an antioxidant food additive obtained from ground dried leaves of *Rosmarinus officinalis*. The antioxidant properties of rosemary extract are primarily attributed to its phenolic diterpene content – namely, carnosic acid and carnosol. Rosemary also contains several volatile components that contribute to its characteristic flavour. The rosemary extract for use as an antioxidant has a minimum ratio of total content of carnosic acid and carnosol to total volatile components of 15:1.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) previously evaluated rosemary extract at its eighty-second meeting (Annex 1, reference *230*). At that meeting, the Committee established a temporary acceptable daily intake (ADI) of 0–0.3 mg/kg body weight (bw) for rosemary extract, expressed as carnosic acid plus carnosol. This ADI was based on a no-observed-adverse-effect level (NOAEL) of 64 mg/kg bw per day, the highest dose tested in a short-term toxicity study in rats. An uncertainty factor of 200 was used, which includes an uncertainty factor of 100 and an additional uncertainty factor of 2 to account for the temporary designation of the ADI, pending the submission of studies to elucidate the potential developmental and reproductive toxicity of the rosemary extract under consideration. An additional uncertainty factor to account for the lack of a chronic toxicity study was not considered necessary, based on the absence of adverse effects in the short-term toxicity studies at doses up to and including the highest dose tested. The temporary ADI applies to the rosemary extract that met the specifications prepared at the eighty-second meeting.

Rosemary extract was placed on the agenda of the present meeting at the request of the Fiftieth Session of the Codex Committee on Food Additives (CCFA) (FAO/WHO, 2018) for an assessment of its safety, dietary exposure and specifications, including studies to elucidate its potential developmental and reproductive toxicity, information to validate the method of determination of residual solvents and data on typical use levels in food. A study on the reproductive and developmental toxicity of an acetone-based rosemary extract was submitted by the sponsors. In addition, a literature search identified five relevant studies

published after the eighty-second meeting of JECFA. The Committee reviewed the data on typical use levels in food that were provided for the present meeting. In addition, updated dietary exposure assessments based on maximum permitted levels were available for review, as were assessments based on typical use levels. A literature search was also undertaken; however, it did not identify any further information on typical use levels or estimates of dietary exposure to rosemary extract.

1.1 Chemical and technical considerations

No new manufacturing information was submitted. The Committee received validation data and information on the method for determination of ethanol and acetone used during the manufacturing of rosemary extract.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution, biotransformation and excretion

Wang et al. (2017) investigated the pharmacokinetics of three phenolic diterpene constituents (carnosic acid, carnosol and rosmanol) of an ethanol-based extract of dried leaves of rosemary (*Rosmarinus officinalis*) in male Sprague Dawley rats (six per dose group). Samples of venous blood (0.25 mL) were withdrawn at 0.08, 0.25, 0.50, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 hours after oral (gavage) administration of rosemary extract at a dose of 240, 820 or 2450 mg/kg bw. Concentrations of carnosic acid, carnosol and rosmanol in the plasma were determined by ultra-high-performance liquid chromatography–tandem mass spectrometry. The limit of quantification for carnosic acid in this study (10.75 ng/mL) was much lower than the limits of quantification of analytical methods used in two studies previously evaluated by JECFA – i.e. 265 ng/mL for Yan et al. (2009) and 5000 ng/mL for Doolaege et al. (2011). The pharmacokinetic parameters shown in Table 1 indicated that the three phenolic diterpenes of the rosemary extract reached a peak plasma concentration (C_{max}) after 0.2–0.7 hour (T_{max}) and were slowly eliminated thereafter, with elimination half-lives varying from 8 to 15 hours, depending on the dose and diterpene analysed. The C_{max} and area under the plasma concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$) values showed reasonably good agreement, with a proportional increase with dose (Table 1). The T_{max} values (mean ± standard deviation) for carnosic acid reported

Table 1

Pharmacokinetic parameters of major diterpenes in a rosemary extract administered by oral gavage to rats[a]

Diterpene constituent of the extract	Rosemary extract dose (mg/kg bw)	C_{max} (ng/mL)	T_{max} (h)	$t_{½}$ (h)	AUC_{0-t} (ng·h/mL)	$AUC_{0-∞}$ (ng·h/mL)
Carnosic acid	240	1 212 ± 344.23	0.30 ± 0.11	8.02 ± 2.08	9 142 ± 1 504	14 922 ± 10 211
	820	3 955 ± 515.85	0.30 ± 0.11	12.81 ± 5.14	28 490 ± 2 928	40 037 ± 32 222
	2 450	27 504 ± 1 881	0.50 ± 0.31	12.84 ± 1.23	145 707 ± 12 774	181 076 ± 19 700
Carnosol	240	216.56 ± 53.09	0.40 ± 0.13	12.29 ± 2.36	1 466 ± 190.18	1 819 ± 327.06
	820	413.34 ± 42.59	0.27 ± 0.15	13.86 ± 6.95	3 187 ± 316.05	7 358 ± 2 738
	2 450	1 480 ± 266.45	0.70 ± 0.45	12.83 ± 3.15	13 993 ± 3 895	17 428 ± 4 042
Rosmanol	240	77.20 ± 10.54	0.25 ± 0.00	8.88 ± 3.52	460.48 ± 54.92	552.10 ± 130.19
	820	187.90 ± 75.87	0.20 ± 0.18	9.75 ± 2.44	678.44 ± 56.94	825.96 ± 137.51
	2 450	633.41 ± 118.52	0.55 ± 0.27	15.36 ± 3.54	5 369 ± 927.05	7 985 ± 1 648

AUC: area under the plasma concentration–time curve; bw: body weight; C_{max}: maximum concentration; $t_{½}$: elimination half-life; T_{max}: time to reach maximum concentration
[a] Values expressed as mean ± standard deviation.
Source: Wang et al. (2017)

by Wang et al. (2017) – i.e. 18.0 ± 6.6 minutes for doses of 240 and 820 mg/kg bw and 30.0 ± 18.6 minutes for 2450 mg/kg bw – were markedly different from the T_{max} values described in the two studies (e.g. Yan et al., 2009: T_{max} = 125.6 ± 118.4 minutes; Doolaege et al., 2011: T_{max} = 136.6 ± 151.5 minutes) that had been previously reviewed by JECFA. Wang et al. (2017) suggested that their test article "was an *R. officinalis* extract not a monomeric compound (purity ≥ 98%)" as used by Yan et al. (2009). This explanation seems improbable, given the apparent assay specificity for carnosic acid in all three studies. An apparent double-peak phenomenon appearing in the plasma concentration versus time curves suggested a redistribution and enterohepatic recirculation of the administered compounds (Wang et al., 2017). However, in the studies by Doolaege et al. (2011) and Yan et al. (2009), there was no evidence of a second peak following intravenous dosing to support enterohepatic recirculation.

2.1.2 Effects on enzymes and other biochemical parameters

Pregnane X receptor (PXR) is known to regulate the expression of many genes, including those involved in drug metabolism. A study by Seow & Lau (2017) investigated whether carnosic acid and other major constituents of rosemary extracts (carnosol, ursolic acid and rosmarinic acid) were PXR activators in a dual luciferase reporter gene assay with human (hPXR), mouse (mPXR) and rat (rPXR) receptors. It was reported that carnosic acid, carnosol and ursolic acid, but not rosmarinic acid, activated both hPXR and mPXR, whereas carnosol and ursolic acid, but not carnosic acid or rosmarinic acid, activated rPXR. Carnosic acid (median effective concentration [EC_{50}] = 0.79 μmol/L), carnosol (EC_{50} = 2.22 μmol/L) and ursolic acid (EC_{50} = 10.77 μmol/L) were shown to activate hPXR, to transactivate the ligand-binding domain of hPXR and to recruit steroid receptor coactivators 1, 2 and 3. It was also shown that carnosic acid, carnosol and ursolic acid, but not rosmarinic acid, increased the hPXR target gene expressions in LS180 human colon adenocarcinoma cells, such as CYP3A4, UGT1A3 and ABCB1 mRNA expression. These findings suggest that carnosic acid (the most potent activator), carnosol and ursolic acid, but not rosmarinic acid, are hPXR agonists. The agonistic effects of carnosic acid on PXR were species specific, as carnosic acid activated hPXR and mPXR, but not rPXR. These new findings provide insight on the molecular basis for the PXR-mediated induction of expression of phase 1 and phase 2 enzymes of xenobiotic metabolism and membrane transport proteins by carnosic acid and other major constituents of rosemary extracts (Seow & Lau, 2017).

An aqueous extract of dried rosemary, with 18.72 ± 0.33 mg carnosic acid per gram of extract, was shown to be a potent in vitro inhibitor of pancreatic lipase (median inhibitory concentration [IC_{50}] = 4.31 ± 0.62 μg/mL) and a somewhat

weaker inhibitor of α-glucosidase (IC$_{50}$ = 76.80 ± 1.68 µg/mL) and α-amylase (IC$_{50}$ = 95.65 ± 2.73 µg/mL). The authors also estimated the bioaccessibility of carnosic acid in rosemary extract samples. It was found that, after incubating (in vitro) rosemary extract samples with simulated digestion fluids, the carnosic acid content of the rosemary extract decreased from 18.72 ± 0.33 mg/g to 7.17 ± 0.13 mg/g, resulting in an estimated bioaccessibility of 38.32% ± 21% (% bioaccessibility = [carnosic acid content of in vitro digested sample] / [carnosic acid content of the sample before in vitro digestion] × 100) (Ercan & El, 2018).

The potent in vitro inhibitory effects of rosemary extract on pancreatic lipase activity were consistent with findings of in vivo studies in mice (Harach et al., 2010; Ibarra et al., 2011) and rats (Romo Vaquero et al., 2012). Inhibition of lipase can impair fat absorption in the intestines, resulting in increased faecal fat elimination and body weight loss. Diarrhoea, bloating and flatulence are symptoms that may eventually occur if potent lipase inhibitors are associated with fatty diets.

2.2 Toxicological studies

2.2.1 Reproductive and developmental toxicity

(a) Reproductive toxicity

A good laboratory practice (GLP)–compliant study conducted according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 421 (Reproduction/Developmental Toxicity Screening Test) provided preliminary data on the potential reproductive toxicity of an acetone-based rosemary extract (Blunt, 2018). In this study, groups of 10 male and 10 female Crl:WI (Han) rats (weighing 303–403 g for males and 191–242 g for females) were fed a powdered diet containing a commercial acetone extract of rosemary (42.98% carnosic acid and 4.24% carnosol) at a concentration of 0, 2100, 3600 or 5000 mg/kg feed for at least 14 days prior to mating, during the time (variable) of conception and pregnancy, and through delivery up to the day before scheduled euthanasia (postpartum day 13). Concentrations of rosemary extract in the diet given to females were reduced to 0, 1050, 1800 and 2500 mg/kg feed from gestation day (GD) 20 until termination to account for enhanced feed intake during the suckling period. Estimated (average) rosemary extract doses administered over the study duration were as follows: 130, 219 and 316 mg/kg bw per day for males and 167, 276 and 401 mg/kg bw per day for females at the low, intermediate and high doses, respectively. Feed and water were available ad libitum throughout the study. Parental males and females (F$_0$) and their pups (F$_1$) were examined twice daily for mortality, morbidity and clinical signs of toxicity. Body weight of F$_0$ males was recorded weekly from the first day of treatment

until termination. F_0 males were euthanized and necropsied after all females had littered. Body weight of F_0 females was recorded weekly in the premating period, on days 0, 7, 14 and 20 of pregnancy and on days 1, 4, 7, 10 and 13 of lactation. Litter size and pup sex were determined at birth. On day 4 after birth, litters were randomly culled to yield four pups of each sex per litter, wherever possible, by removing extra pups. Anogenital distance was measured on postnatal day (PND) 1, whereas the number of nipples on male pups was counted on PND 12. Pup body weights were recorded on PNDs 1, 4, 7, 10 and 13. Pups were euthanized and necropsied on PND 13. Blood samples for thyroid hormone measurements were taken from parental males and females upon termination, from the extra pups after culling (on PND 4) (one pooled sample per litter) and from one male pup and one female pup per litter on PND 13. Thyroid glands from one male and one female pup per litter (from pups that had not been sampled for thyroid hormone measurements) were fixed, stained and examined under a microscope for histopathological abnormalities.

F_0 males of the high-dose group (5000 mg/kg feed) gained less weight than those of the control group over the pre-pairing, pairing and post-pairing periods (0–8 weeks; control, 96.6 ± 8.5 g; 5000 mg/kg feed, 76.0 ± 19.5 g; $P \leq$ 0.05, analysis of variance [ANOVA] and Dunnett's test, trend analysis). No treatment-related deaths or other clinical signs of toxicity, changes in absolute or relative organ weights (testes, epididymis, prostate and seminal vesicle), gross pathology and histopathology findings or thyroxine (T_4) blood levels were noted in parental males exposed to rosemary extract via the diet. As male reproductive performance was also unaffected by dietary exposure to rosemary extract, the reduced body weight gain in the high-dose group could be considered an isolated finding of minor toxicological relevance.

Control and rosemary extract–treated F_0 females showed comparable numbers and durations of estrous cycles as well as mating performance and fertility. Lengths of gestation of control and rosemary extract–treated females were similar as well. The body weight gain during pre-pairing and the first 2 weeks of pregnancy (GDs 0–7 and 7–14) did not differ between control and rosemary extract–treated females. During the third (last) week of pregnancy (GDs 14–20), females treated with the high dose of rosemary extract gained nearly 20% less weight than females of the control group (65.4 ± 8.1 g vs 52.4 ± 6.7 g; Kruskal-Wallis and Steel-Dwass post hoc test, $P \leq 0.01$). Nonetheless, maternal weight gain during lactation up to termination (PNDs 1–13) did not differ between females of the control group (37.3 ± 9.0 g) and those of the high-dose group (47.8 ± 12.8 g). On PND 1, mean pup body weight and litter sizes of control and rosemary extract–treated groups were comparable. These results indicated that the transient decrease in weight gain on GDs 14–20, noted in the high-dose

Table 2

Thyroid hormone and TSH concentrations in the serum of F_1 rats on PND 4 (total T_4, pooled samples) and PND 13 (total T_4, total T_3 and TSH)[a,b]

Concentration of rosemary extract in the diet[c] (mg/kg feed)	Total T_4 (µg/dL)			Total T_3 (nmol/L)		TSH (ng/mL)	
	PND 4	PND 13					
	Pool	Males	Females	Males	Females	Males	Females
0	2.566 ± 0.554 ($n = 10$)	4.821 ± 0.600 ($n = 10$)	4.786 ± 0.343 ($n = 10$)	0.48 ± 0.26 ($n = 9$)	0.44 ± 0.22 ($n = 9$)	0.891 ± 0.856 ($n = 10$)	0.822 ± 0.709 ($n = 9$)
2 100	2.455 ± 0.608 ($n = 6$)	4.563 ± 0.363 ($n = 9$)	4.480 ± 0.364* ($n = 9$)	0.72 ± 0.30 ($n = 9$)	0.68 ± 0.30 ($n = 8$)	1.070 ± 1.785 ($n = 9$)	0.849 ± 1.099 ($n = 9$)
3 600	2.437 ± 0.382 ($n = 9$)	3.971 ± 0.521** ($n = 10$)	4.046 ± 0.235*** ($n = 10$)	0.91 ± 0.36 ($n = 7$)	0.80 ± 0.40 ($n = 8$)	0.520 ± 0.485 ($n = 10$)	0.406 ± 0.303 ($n = 10$)
5 000	2.532 ± 0.501 ($n = 10$)	3.505 ± 0.474*** ($n = 10$)	3.579 ± 0.533*** ($n = 10$)	0.69 ± 0.28 ($n = 7$)	0.77 ± 0.33 ($n = 7$)	0.256 ± 0.267 ($n = 10$)	0.476 ± 0.281 ($n = 10$)
ANOVA	*P > 0.05*	*P < 0.000 1*	*P < 0.000 1*	*P > 0.05*	*P > 0.05*	*P > 0.05*	*P > 0.05*

ANOVA: analysis of variance; GD: gestation day; *n*: sample number; PND: postnatal day; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; *: $P \leq 0.05$; **: $P < 0.01$; ***: $P \leq 0.001$ (ANOVA and Dunnett's test)

[a] Parental (F_0) male and female rats and their offspring (F_1) were exposed to an acetone-based extract of rosemary added to the diet (0, 2100, 3600 and 5000 mg/kg feed) for 14 days before mating and during conception time, pregnancy, delivery and lactation until termination on PND 13.

[b] Data are shown as mean ± standard deviation. Statistical analyses were conducted by the Committee using the individual rat data provided in the final report of the study (Blunt, 2018).

[c] From GD 20 to termination (PND 13), concentrations of rosemary extract in the diet of female rats were reduced to 1050, 1800 and 2500 mg/kg feed to account for enhanced feed intake during late pregnancy and lactation.

Source: Blunt (2018)

group, was not associated with an adverse pregnancy outcome, and thus it could be considered a finding of minor toxicological relevance.

In the F_1 generation (terminated on PND 13), no rosemary extract treatment–related effects on survival, mean body weight or body weight gain, clinical signs of toxicity, anogenital distance, gross external morphological features or retention of nipples in male pups on PND 13 were observed. No treatment-related histopathological findings in the thyroid glands were recorded among F_1 rats fed diets containing rosemary extract at concentrations up to 5000 mg/kg feed. On PND 4, total T_4 levels were similar between rosemary extract–treated groups and controls (Table 2). On PND 13, a dose-related reduction in total-T_4 blood concentrations in male and female pups of rosemary extract–treated groups was observed, especially among mid- and high-dose pups compared with control pups ($P \leq 0.0001$) (Table 2). In F_1 rats, thyroid stimulating hormone (TSH) levels on PND 13 showed a rather high interindividual variability, with standard deviations around or larger than group means. TSH concentrations (non-GLP analysis) and total-triiodothyronine (total-T_3) concentrations (non-GLP analysis) for male and female pups and the statistical evaluations are shown in Table 2. Serum total-T_3 levels for rosemary extract–treated pups were generally

slightly higher than those recorded for males and females of the control group. The study investigators did not perform a statistical evaluation of these data, but an ANOVA analysis performed by the Committee revealed that this increase did not achieve statistical significance.

The NOAEL for reproductive toxicity and parental toxicity was the highest dose tested – i.e. 5000 mg/kg feed, equal to 316 mg/kg bw per day for males and 401 mg/kg bw per day for females, expressed as rosemary extract, equivalent to 149 mg/kg bw per day for males and 189 mg/kg bw per day for females, expressed as the sum of carnosic acid plus carnosol.

The toxicological relevance of the dose-related reduction of total-T_4 levels in male and female pups on PND 13 is unclear, and therefore a NOAEL for offspring toxicity could not be identified. Reductions of thyroid hormone levels, particularly during prenatal and/or postnatal development, are cause for concern. A longer follow-up of F_1 development, beyond mid-lactation (PND 13), is necessary to demonstrate that the reductions in total-T_4 serum concentrations were not adverse effects. The study also did not provide adequate evidence for the absence of developmental toxicity, given that no fetuses were examined.

(b) Developmental toxicity

The embryotoxicity of carnosic acid (purity not reported) was assessed in an in vitro screening assay based on the differentiation of murine embryonic stem cells into contracting cardiomyocytes (Liu et al., 2017). In the embryonic stem cell assay, the cytotoxicity of carnosic acid (IC_{50}) was determined in two murine permanent cell lines – namely, fibroblast (BALB/c-3T3) cells and embryonic stem cells. The concentration of carnosic acid that caused a 50% inhibition of murine embryonic stem cell differentiation into cardiomyocytes (ID_{50}) was also determined. 5-Fluorouracil, a known embryotoxic agent, was used as a positive control, and penicillin G was used as a negative control. Carnosic acid was toxic for fibroblasts (BALB/c-3T3) at a concentration of 26.28 ± 3.861 μg/mL (IC_{50}), whereas the IC_{50} was 5.771 ± 1.297 μg/mL for embryonic stem cells. Carnosic acid inhibited differentiation of murine embryonic stem cells into contracting cardiomyocytes (ID_{50} = 6.143 ± 0.575 μg/mL) at similar concentrations as the IC_{50} in murine embryonic stem cells. The effects (IC_{50} and ID_{50}) of control and test substances on these three end-points were applied to a biostatistical prediction model that leads to a classification of tested substances into one of three different classes according to potential in vivo embryotoxic potencies: strongly embryotoxic, weakly embryotoxic or not embryotoxic. Carnosic acid was classified as being weakly embryotoxic (Liu et al., 2017). According to Genschow et al. (2002), the embryonic stem cell assay exhibited a good overall test accuracy of 78% for classifying 20 chemicals with known in vivo embryotoxic potential. The embryonic stem cell test is an extensively used screening assay for

developmental toxicity that has been validated by the European Union Reference Laboratory for alternatives to animal testing (Paquette et al., 2008). Studies on the predictivity of the embryonic stem cell assay indicated a significant false-positive rate (approximately 40%), but a very low false-negative rate (approximately 7%) (Paquette et al., 2008).

2.3 Observations in humans

A small-scale clinical study (randomized, double-blinded and placebo-controlled) investigated the memory-enhancing effects of a combined ethanol extract of three plants (*Salvia officinalis*, *Melissa officinalis* and *Rosmarinus officinalis*) that are traditionally used in folk medicine in Europe. Forty-four participants (mean age 61 ± 9.26 years) were randomly assigned to a placebo or an active treatment (combined extract) group. During 14 consecutive days, placebo group participants received oral doses of an ethanol extract of *Myrrhis odorata* (thought to be therapeutically inactive) twice a day, while participants of the active treatment group received the combined three-plant ethanol extract twice a day. Immediate and delayed word recall tests were used to assess memory prior to (pre-dose baseline) and after the 14-day treatment.

Overall, there was no difference between placebo- and combined extract–treated groups in immediate and delayed recall test performances. Subgroup analysis, however, suggested that the combined extract promoted improvements in delayed word recall in the subgroup of participants under the age of 63 years. A chromatographic (liquid chromatography–mass spectrometry) analysis of the combined extract tentatively detected flavones and their glycosides, rosmarinic acid and diterpenoids. No adverse effects of the combined extract were reported (Perry et al., 2018).

The Committee noted that the lack of information on the administered dose and quantitative chemical composition of the combined extract and other methodological shortcomings of the trial, including the small number of participants and the lack of description of the treatment allocation concealment method and the test for the blinding status of the participants, limited the contribution of this clinical study to the evaluation of the safety of solvent-based rosemary extract for humans.

3. Dietary exposure

3.1 Introduction

The Committee evaluated chronic dietary exposure to rosemary extract for the first time at its eighty-second meeting (Annex 1, reference *230*) following a request to evaluate the food additive by CCFA. The dietary exposure to rosemary extract was re-evaluated at the current meeting.

The dietary exposure assessments reviewed at the eighty-second meeting included the following:

- Europe: based on food consumption data from the Comprehensive European Food Consumption Database for a range of European countries and European Union (EU) maximum permitted levels and proposed maximum permitted levels (in fat-based spreads) (EFSA, 2015);
- USA: based on consumption data from the 2011–2012 National Health and Nutrition Examination Survey (NHANES) and EU maximum permitted levels and proposed maximum permitted levels (assessment provided by the sponsors);
- United Kingdom: based on food consumption data from National Dietary and Nutrition Surveys (NDNSs) and proposed EU maximum permitted levels (EFSA, 2008); and
- United Kingdom: based on 2000–2001 NDNS data and EU maximum permitted levels and proposed maximum permitted levels (in fat-based spreads) (assessment provided by the sponsors).

None of these dietary exposure estimates included naturally occurring sources, flavouring uses or uses in colour or nutrient preparations. Estimates of dietary exposure were expressed on a carnosic acid plus carnosol basis.

For children, estimated dietary exposures to carnosic acid plus carnosol from the eighty-second meeting ranged between 0.05 and 0.44 mg/kg bw per day at the mean and between 0.11 and 0.81 mg/kg bw per day at the high (95th) percentile across all the assessments evaluated. For adults, estimated dietary exposures ranged between 0.03 and 0.17 mg/kg bw per day at the mean and between 0.09 and 0.37 mg/kg bw per day at the high percentile. The highest estimates of dietary exposure were for toddlers and children across the assessments evaluated. The main contributors to dietary exposure were noted as fine bakery wares and processed meat from the European Food Safety Authority (EFSA) assessments.

Dietary exposures for high consumers used for the evaluation at the eighty-second meeting were 0.09–0.81 mg/kg bw per day (as carnosic acid plus carnosol). It was noted by that Committee that these exposures may exceed the upper bound of the temporary ADI of 0.3 mg/kg bw per day up to 2.7-fold (for young children at the top end of the range of estimated dietary exposures). As the dietary exposures were based on the assumption that all foods for which use of the food additive was permitted contained the food additive at the maximum permitted level, the Committee concluded that the dietary exposures were conservative and that this exceedance does not necessarily represent a safety concern. The Committee at the eighty-second meeting requested that data on the typical use levels in foods be provided in order to refine the dietary exposure estimates.

3.2 Approach to the dietary exposure assessment

The dietary exposure assessment for the current meeting was based on information that has become available since the eighty-second meeting and included a review of maximum permitted levels and typical use levels of rosemary extract, as well as national and international estimates of chronic dietary exposure.

The dietary exposure assessment included a review of the information provided by the sponsors. This information included an estimate of dietary exposure for the USA based on typical use levels, with some levels up to maximum permitted levels for some foods. The sponsors also included a review of the most recent EFSA assessment (EFSA, 2018) and the Food Standards Australia New Zealand (FSANZ) assessment for both Australia and New Zealand (FSANZ, 2018a,b). The dietary exposure assessments for Australia and New Zealand were also submitted by FSANZ following consideration of an application to permit the use of rosemary extract in a range of foods (FSANZ, 2018a,b). Permissions for use were granted in January 2019. As the EFSA and FSANZ dietary exposure assessments were available to the Committee in full, each was reviewed independently by the Committee. The details for these assessments are discussed further in section 3.4.

The scientific literature was reviewed to identify any other information on typical use levels or estimates of dietary exposure to rosemary extract. EBSCO Discovery Service was used, and databases including Medline, Food Science Source, Food Science and Technology Abstracts and Science Direct were searched, as well as a number of journals related to science, toxicology, food, nutrition and public health. Search terms included "rosemary extract", "carnosic acid", "carnosol", "concentration", "use level" and "dietary exposure" or "dietary intake" or "consumption". These terms were also used in a general Internet search to capture other papers not included in the scientific literature. Although some

papers were identified that described studies to determine effective use levels of rosemary extract in foods for antioxidant properties, they did not include information on typical use levels in commercially available foods that would be relevant for the dietary exposure assessment. These searches did not result in any additional information on use levels or dietary exposure estimates not already noted by the sponsors or submitted to the Committee.

As the ADI is expressed as the sum of carnosic acid plus carnosol, the concentration data reviewed and the dietary exposure assessments were also expressed on this basis.

Dietary exposures were reviewed for mean consumers and high consumers and included both children and adults.

A screening assessment of dietary exposure for food additives using the budget method is usually conducted or reviewed by the Committee. The budget method produces a conservative estimate of dietary exposure, as it assumes consumption of 6 L of non-milk beverages and 3 kg of food per day, assuming a 60 kg body weight, and the highest maximum permitted levels from any of the foods containing the food additive. The Committee did not conduct a budget method calculation for rosemary extract at its eighty-second meeting. For this reason, and also because more refined estimates of dietary exposure based on typical use levels were available to the Committee for use in the current evaluation, the present Committee also did not conduct a budget method calculation.

Estimates of dietary exposure based on added sources of rosemary extract were reviewed. As carnosic acid and carnosol are naturally occurring substances, estimated dietary exposure from natural sources was also assessed. Concentrations of carnosic acid and carnosol in naturally occurring sources were also investigated. Rosemary extract is also used as a flavouring agent, as well as in colour preparations and nutrient preparations in the EU. However, these sources were not included in the dietary exposure assessment by the present Committee, as there were no available data on their use, and it was assumed that they would have minimal impact on the estimates of dietary exposure.

At the eighty-second meeting, the Committee did not calculate national estimates of dietary exposure using the FAO/WHO Chronic Individual Food Consumption database – summary statistics (CIFOCOss) (FAO/WHO, 2017, 2018b). This was also the case for the present Committee. There are few corresponding consumption data for Codex General Standard for Food Additives (GSFA) food categories where there are permissions to use rosemary extract. Any calculations using the data that were available would not have produced accurate or reliable estimates of dietary exposure, and they would have included a great degree of uncertainty. Many of the countries with data in CIFOCOss are from Europe; Australia and the USA also contribute to CIFOCOss. Estimates of dietary exposure based on individual dietary records from other sources were

already available to the Committee for these countries, so there was no need for the present Committee to replicate calculations for these countries.

At the eighty-second meeting, the Committee did not calculate international estimates of dietary exposure. That Committee noted that it is not appropriate to undertake international dietary exposure assessments using the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) cluster diets (FAO/WHO, 2018c) for rosemary extract. Food consumption data in the cluster diets are generally for raw commodities and only a small number of processed foods, not the extensive range of processed foods to which rosemary extract is permitted to be added.

3.3 Levels of use of rosemary extract

Concentrations in foods, whether naturally occurring or as maximum permitted or typical use levels, for the purpose of the dietary exposure assessment are expressed as carnosic acid plus carnosol, as this is the basis upon which the temporary ADI was established. As the assessment undertaken by the Committee at its eighty-second meeting was based on maximum permitted levels, the eighty-second Committee specifically requested information on typical use levels added to foods. These data were available to the current Committee and were reviewed at the present meeting.

3.3.1 Added sources

Rosemary extract is permitted for use in foods as an antioxidant in a number of countries. It is used in foods but also in preparations for colours and nutrients and as a flavouring.

The EU permits the use of rosemary extract as an antioxidant in a range of foods (n = 33 categories) at maximum permitted levels of between 15 and 400 mg/kg expressed on a whole weight or a fat basis (Annex II to Regulation (EC) No. 1333/2008 as amended). Rosemary extract is also approved for use in European Commission regulations for use in food colour preparations with a maximum level of 1000 mg/kg in the preparation and 5 mg/kg in the final product, in all food flavourings at a maximum level of 1000 mg/kg and in preparations of nutrients (e.g. β-carotene and lycopene) at a level of 1000 mg/kg in the preparation and 5 mg/kg in the final product.

Rosemary extract is permitted for use in Australia and New Zealand in 18 food categories. These are similar to the food categories in which rosemary extract is permitted to be used in the EU, but fewer than the categories permitted in the EU. The foods requested by the applicant to FSANZ were matched to the food classification system used in Standard 1.3.1 of the Australia New Zealand

Food Standards Code. The maximum permitted levels across the food groups ranged between 10 and 150 mg/kg (on an as consumed basis). Maximum permitted levels in Australia and New Zealand are generally lower than those permitted in the EU.

Typical use levels of rosemary extract (expressed as the sum of carnosic acid and carnosol) were available from two sources: the EFSA revised assessment, which included industry use data submitted as a result of a data call (EFSA, 2018); and FSANZ, the applicant to which provided both maximum permitted levels and typical use levels (noted in their report as usual use levels) for use in their assessment. The ranges of typical use levels provided to EFSA were 0.4–102 mg/kg for mean use levels and 1–200 mg/kg for maximum use levels. The applicant indicated that usual use levels for fats and oils are around 80% of the maximum permitted levels and around 50% for all other foods and ranged between 5 and 75 mg/kg.

Table 3 provides a summary of the maximum permitted levels and the typical use levels of rosemary extract in the EU and in Australia and New Zealand. Also included in Table 3 are the proposed maximum permitted levels for the GSFA as provided by the sponsors. There is also a summary of the typical use levels used by the sponsors for the dietary exposure assessment they conducted for the USA, which are based on a mixture of maximum permitted use levels from the EU and Australia and New Zealand and typical use levels used in the EFSA and FSANZ dietary exposure assessments.

3.3.2 Naturally occurring sources

Carnosic acid and carnosol occur naturally in foods. Key sources include rosemary (*R. officinalis* L.) and sage (*Salvia officinalis* and other *Salvia* species) (EFSA, 2018). As noted by the Committee at its eighty-second meeting, rosemary has been used for centuries as a culinary ingredient (as a seasoning) in its fresh and dried forms, as well as being used in oils, infusions and medicines.

At the present meeting, data on concentrations in naturally occurring sources presented in the EFSA and FSANZ assessments were reviewed. A literature search was also undertaken to find any additional concentration data. EBSCO Discovery Service was used with search terms including carnosic acid, carnosol, phenol*, antioxidant*, rosemary extract, concentration, source*, content, natural source*, food*, characterisation, dietary exposure, sage, rosemary, oregano, lamiaceae, spices, herbs, Rosmarinus, Salvia. Web searches were also undertaken using similar search terms and strings such as foods containing carnosic acid, foods containing carnosol, carnosic acid content, carnosic acid concentration, rosemary extract antioxidant, rosemary extract risk assessment and use level.

Table 3

Maximum permitted levels, proposed maximum permitted levels and typical use levels of rosemary extract (expressed as carnosic acid plus carnosol)

Food category	Food name	Concentrations of rosemary extract (expressed as carnosic acid plus carnosol) (mg/kg)					
		EU maximum permitted levels[a]	Mean (maximum) industry use levels provided to EFSA[a]	Australia and New Zealand maximum permitted levels[b]	Australia and New Zealand typical use levels	Proposed maximum permitted levels for GSFA from sponsors	Typical use levels from sponsors[c]
1.5	Dried milk for the manufacturing of ice cream	30	–	–	–	30	–
1.5	Milk powder for vending machines	200[d]	–	–	–	200[d]	–
2.1	Vegetable oils (excluding virgin oils and olive oils) and fat where content of polyunsaturated fatty acids is higher than 15% w/w of the total fatty acid, for the use in non-heat-treated food products	30[d]	–	–	–	30[d]	30[d]
2.1	Only fish oil and algal oil	50[d]	–	50	40	50[d]	50[d]
2.1	Lard, beef, poultry sheep and porcine fat	50[d]	–	–	–	50[d]	50[d]
2.1	Fat and oils for the professional manufacture of heat-treated foods	50[d]	–	–	–	50[d]	50[d]
2.1	Frying oils and frying fat, excluding olive oil and pomace oil	50[d]	–	–	–	50[d]	50[d]
2.2.2	Other fat and oil emulsions including spreads – only spreadable fats with a fat content less than 80%	100[d]	34 (43)	75	60	100[d]	75[d]
2.3	Vegetable oil pan spray – only fats and oils for the professional manufacture of heat-treated foods	50[d]	–	–	–	–	–
4.2.2.2 (4.2.4.1 EU)	Fruit and vegetable preparations excluding compote – only seaweed based fish roe analogues	200	–	–	–	200	–
4.2.5.4	Nut butters and nut spreads	200[d]	–	50	25	200[d]	50[d]
4.2.6	Dehydrated potato products	200	50 (108)	–	–	200	108
5.3	Chewing gum	200	–	–	–	200	–
5.4	Only sauces	100[d]	–	50	25	100[d]	50[d]
5.4	Icings and frostings	–	–	20	10	–	–
6.3	Processed cereal and meal products – only grain bars, breakfast bars, breakfast cereals	–	–	50	25	–	50

Concentrations of rosemary extract (expressed as carmosic acid plus carnosol) (mg/kg)

Food category	Food name	EU maximum permitted levels[a]	Mean (maximum) industry use levels provided to EFSA[a]	Australia and New Zealand maximum permitted levels[b]	Australia and New Zealand typical use levels	Proposed maximum permitted levels for GSFA from sponsors	Typical use levels from sponsors[c]
6.4	Flour products – Only for flour-based snacks e.g. pretzels, fritters, and crackers; not for noodles and pasta	–	–	10	5	–	–
6.4.5	Fillings stuffed dry pasta (ravioli and similar)	250[d]	–	–	–	250[d]	–
7.1.2	Crackers, excluding sweet crackers	–	–	–	–	200[d]	100[d]
7.2	Fine bakery wares (e.g. biscuits, cakes, pastries)	200[d]	102[d] (200[e])	40	20	200	–
8.1.2	Fresh meat, poultry and game comminuted, fat content <10%	–	–	–	–	15[d]	–
8.1.2	Fresh meat, poultry and game comminuted, fat content >10%	–	–	–	–	150[d]	–
8.2	Processed meat, poultry and game products in whole cuts or pieces, fat content <10%	–	–	15	7.5	–	–
8.2	Processed meat, poultry and game products in whole cuts or pieces, fat content >10%	–	–	37.5	18.75	–	–
8.2.1	Non-heat-treated processed meats, poultry and game products in whole cuts or pieces, fat content <10%	–	–	–	–	15	15
8.2.1	Non-heat-treated processed meats, poultry and game products in whole cuts or pieces, fat content >10%	–	–	–	–	150[d]	37.5[d]
8.2.1	Dehydrated meat	–	–	–	–	150	150
8.2.1	Non-heat-treated processed meat, fat content <10%	–	–	–	–	15	–
8.2.1	Non-heat-treated processed meat, fat content >10%	–	–	–	–	150[d]	30[d]
8.2.2	Heat-treated processed meats, poultry and game products in whole cuts or pieces, fat content <10%	–	–	–	–	15	15
8.2.2	Heat-treated processed meats, poultry and game products in whole cuts or pieces, fat content >10%	–	–	–	–	150[d]	37.5[d]
8.2.2	Dehydrated meat	–	–	–	–	150	150
8.2.3	Dried meat	–	–	150	75	–	–
8.3.1[e]	Non-heat-treated meat products – only dried sausages	100	–	–	–	100	100
8.3.1[e]	Non-heat-treated meat products – only meat with a fat content <10%, excluding dried sausages	15	–	–	–	15	–

Table 3 (continued)

Food category	Food name	Concentrations of rosemary extract (expressed as carnosic acid plus carnosol) (mg/kg)					
		EU maximum permitted levels[a]	Mean (maximum) industry use levels provided to EFSA[a]	Australia and New Zealand maximum permitted levels[b]	Australia and New Zealand typical use levels	Proposed maximum permitted levels for GSFA from sponsors	Typical use levels from sponsors[c]
8.3.1[e]	Non-heat-treated meat products – only meat with a fat content >10%, excluding dried sausages	150[d]	25 (30)	37.5	18.8	150[d]	30
8.3.1[e]	Non-heat-treated meat products – only dehydrated meat	150	–	150	75	150	150
8.3.2[e]	Heat-treated meat products – only meat with a fat content <10%, excluding dried sausages	15	–	–	–	15	–
8.3.2[e]	Heat-treated meat products – only meat with a fat content >10%, excluding dried sausages	150[d]	25 (30)	37.5	18.8	150[d]	30
8.3.2[e]	Heat-treated meat products – only dried sausages	100	–	100	50	100	100
8.3.2[e]	Heat-treated meat products – only dehydrated meat	150	–	150	75	150	150
9.2	Processed fish and fishery products including molluscs and crustaceans – only fish and fishery products including molluscs and crustaceans with a fat content <10%	15	51[d] (62[d])	–	–	15	62[d]
9.2	Processed fish and fishery products including molluscs and crustaceans – only fish and fishery products including molluscs and crustaceans with a fat content >10%	150[d]	51[d] (62[d])	–	–	150[d]	62[d]
9.3	Semi-preserved fish and fish products, including molluscs, crustaceans and echinoderms, fat content <10%	–	–	–	–	15	62[d]
9.3	Semi-preserved fish and fish products, including molluscs, crustaceans and echinoderms, fat content >10%	–	–	–	–	150[d]	62[d]
9.4	Fully preserved fish and fish products, including molluscs, crustaceans and echinoderms, fat content <10%	–	–	–	–	15	62[d]
9.4	Fully preserved fish and fish products, including molluscs, crustaceans and echinoderms, fat content >10%	–	–	–	–	150[d]	62[d]
10.2	Processed eggs and egg products	200	–	–	–	200	–
12.0	Salts and condiments (not for sauces)	–	–	40	20	–	–
12.2.2	Seasoning and condiments	200[d]	0.4 (1)	–	–	200[d]	50[d]

Food category	Food name	Concentrations of rosemary extract (expressed as carnosic acid plus carnosol) (mg/kg)					
		EU maximum permitted levels[a]	Mean (maximum) industry use levels provided to EFSA[a]	Australia and New Zealand maximum permitted levels[b]	Australia and New Zealand typical use levels	Proposed maximum permitted levels for GSFA from sponsors	Typical use levels from sponsors[c]
12.4	Mustard	100[d]	–	–	–	100[d]	–
12.5	Soups and broths	50	1.5 (3)	–	–	50	40
12.6	Sauces	100[d]	5 (5)	50[f]	25	100[d]	50[d]
15.1	Potato-, cereal-, flour- or starch-based snacks	50[d]	20 (20)	20	10	50[d]	40[d]
15.2	Processed nuts	200[d]	20 (20)	50	25	200[d]	50[d]
15.3	Fish-based snacks	–	–	–	–	50[d]	40[d]
17.1	Food supplements supplied in a solid form including capsules and tablets and similar forms, excluding chewable forms	400	138	–	–	400	138
17.2	Food supplements supplied in a liquid form	400	138	–	–	400	138
17.3	Food supplements supplied in a syrup-type or chewable form	400	138	–	–	400	138

EFSA: European Food Safety Authority; EU: European Union; GSFA: Codex General Standard for Food Additives; USA: United States of America; w/w: weight per weight

[a] EFSA (2018). These levels were also proposed by the sponsors for inclusion in the GSFA.
[b] Expressed on an as consumed basis. Usual use levels are 80% of the maximum permitted level for fats and oils and 50% of the maximum permitted level for all other foods (FSANZ, 2018a).
[c] Used by the sponsors in the dietary exposure assessment for the USA.
[d] Use levels expressed on a fat basis.
[e] Category 8.3 is comminuted meat in the GSFA.
[f] Classification 20.2.4 Sauces and toppings including mayonnaise, salad dressing, gravy, syrups.

A small number of papers were retrieved and reviewed, some containing quantitative data and others with no usable information or data.

EFSA (2018) retrieved data on concentrations in naturally occurring sources from databases and the literature. In its dietary exposure assessment, EFSA used concentrations of carnosic acid plus carnosol of 112 mg/kg in dried rosemary leaves, 12.18 mg/kg in fresh rosemary leaves and 5.3 mg/kg in dried sage. EFSA also noted that carnosic acid and carnosol have been detected but not quantified in other foods, such as other herbs, spices and teas. The applicant to FSANZ (2018a) noted that the concentrations of carnosol and carnosic acid in dried rosemary leaves are around 1–2 mg/g and 15–25 mg/g, respectively. This results in a range of concentrations of carnosic acid plus carnosol of between 16 000 and 27 000 mg/kg, with the midpoint of the range being 21 500 mg/kg. A conversion to a concentration in fresh leaves was undertaken based on energy content and resulted in a carnosic acid plus carnosol concentration of 7525 mg/kg.

Other papers have reported carnosic acid and carnosol concentrations in rosemary. Luis & Johnson (2005) (cited in EFSA, 2018, but concentration recalculated by the Committee) showed a mean concentration of 12 710 mg/kg (n = 6) in fresh rosemary. Concentrations in fresh rosemary have also been reported to be between 1270 and 22 790 mg/kg for carnosic acid and between 580 and 2380 mg/kg for carnosol (Ribeiro-Santos et al., 2015). Lugemwa (2012) used three different extractions to obtain carnosic acid concentrations in fresh rosemary of 32 500 mg/kg (ethyl acetate:ethanol:water), 26 300 mg/kg (acetone) and 20 500 mg/kg (methanol). For dried rosemary leaves, mean concentrations have been reported as 112 mg/kg (carnosic acid plus carnosol) (Loussouarn et al., 2017), 18 720 mg/kg (carnosic acid only) (Ercan & El, 2018) and 42 050 mg/kg (carnosic acid) and 3870 mg/kg (carnosol) (Okamura et al., 1994).

One paper reported lower concentrations of carnosic acid in dried sage (3760 mg/kg) than in dried rosemary (18 720 mg/kg) (Ercan & El, 2018). Hernandez-Hernandez et al. (2009) analysed extracts from rosemary and oregano leaves (*Origanum vulgare* L.). While not reporting concentrations in the plants themselves (only extracts), they showed lower concentrations of carnosic acid plus carnosol in fresh oregano (0.0005 mg/mg extract) and dried oregano (0.0007 mg/mg extract) compared with fresh rosemary (0.35 mg/mg extract). Carnosic acid has also been detected in marjoram (Vagi et al., 2005) (no quantitative values provided).

Ribeiro-Santos et al. (2015) noted that the levels of the bioactive compounds in rosemary, such as carnosic acid and carnosol, can vary greatly. They also noted that the levels depend on many factors, including variety, plant part, age of the plant, climatic conditions, time of harvest, drying and extraction, and analytical methods. The Committee also noted this variability in concentrations

of carnosic acid and carnosol in rosemary from its review of a small number of papers, in addition to the variability in concentrations of carnosic acid and carnosol in different herbs.

3.4 Estimates of dietary exposure to rosemary extract

3.4.1 Europe

At its eighty-second meeting, the Committee reviewed two estimates of dietary exposure to rosemary extract calculated by EFSA. The first, from 2008, was based on United Kingdom food consumption data and proposed maximum permitted levels in the EU (EFSA, 2008), and the second, from 2015, was based on the Comprehensive European Food Consumption Database, maximum permitted levels and an extension of use to fat-based spreads (EFSA, 2015).

For the present meeting, the Committee reviewed more recent estimates of dietary exposure for a number of scenarios published by EFSA (2018). The first was based on EU maximum permitted levels (as per Table 3) using consumption data that had been updated since the previous (2015) EFSA assessment. Twenty-six food categories were included in the maximum permitted levels scenario. Two refined assessments using industry use data (brand-loyal scenario and non-brand-loyal scenario) were used to provide a better estimate of dietary exposure. These were based on actual use levels provided by the food industry sourced from a call for data. Typical use levels were received for 12 of the 33 categories with permission to use rosemary extract. No analytical data were provided. For the brand-loyal assessment, it was assumed that the highest contributing food contained the maximum reported use level and all other foods contained the mean reported use level. For the non-brand-loyal scenario, it was assumed that all foods contained the mean reported use level. There was also a food supplement consumers only scenario, which included only individuals who reported consuming food supplements (excluding infants and toddlers), where the supplements contained the maximum use level and other foods contained the mean use level from industry. Food supplements were not included in the maximum permitted level or refined scenarios. Dietary exposures from naturally occurring sources estimated by EFSA are described further in section 3.5.

The food categories included in the assessment were those permitted in the EU with FoodEx (the EFSA Food classification and description system for exposure assessment) coding and matched to the consumption database also coded using FoodEx2 (version 2 of FoodEx). Concentrations expressed on a fat basis were converted to a whole weight basis for the dietary exposure calculations. Uses for colour preparations, nutrient preparations and flavourings were not included in the dietary exposure assessment.

For the assessment, the food consumption data used were from the Comprehensive European Food Consumption Database. Food consumption data are for individual respondents based on 2 days of data or more. National consumption data were available from 33 dietary surveys and 19 countries. Countries included in the refined assessment for children (including infants, toddlers and adolescents) were Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, the Netherlands, Spain, Sweden and the United Kingdom. For adults (including the elderly population), countries included Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, the Netherlands, Romania, Spain, Sweden and the United Kingdom. For both adults and children, the countries differed slightly, depending on the specific age group.

The age groups assessed by EFSA included infants (4–11 months), toddlers (12–35 months), children (3–9 years) and adolescents (10–17 years). Adults included two groups: adults aged 18–64 years and adults 65 years of age and older.

Data from the Mintel Global New Products Database were also provided to EFSA for its assessment. Over 4700 products in the database between January 2013 and February 2018 contained rosemary extract (INS 392). The proportion of categories permitted to contain rosemary extract that contained labels reporting its use ranged between 0.1% and 17.8%, with the mean being 1%. The food categories with the highest proportion containing rosemary extract were dry soup, followed by pizzas and stocks. These data show that the actual uptake of the permission to use rosemary extract within a category is low.

Estimated dietary exposures to carnosic acid plus carnosol for consumers only based on maximum permitted levels for children ranged between 0.03 and 0.44 mg/kg bw per day at the mean and between 0.08 and 0.85 mg/kg bw per day at the 95th percentile; for adults, dietary exposures ranged between 0.04 and 0.17 mg/kg bw per day at the mean and between 0.09 and 0.40 mg/kg bw per day at the 95th percentile. For the refined estimates based on industry use levels for the brand-loyal scenario, estimated dietary exposures for children ranged between <0.01 and 0.16 mg/kg bw per day at the mean and between <0.01 and 0.35 mg/kg bw per day at the 95th percentile; for adults, dietary exposures ranged between 0.01 and 0.05 mg/kg bw per day at the mean and between 0.02 and 0.15 mg/kg bw per day at the 95th percentile. For the refined non-brand-loyal scenario, estimated dietary exposures for children ranged between <0.01 and 0.09 mg/kg bw per day at the mean and between <0.01 and 0.20 mg/kg bw per day at the 95th percentile; for adults, dietary exposures ranged between <0.01 and 0.03 mg/kg bw per day at the mean and between 0.01 and 0.10 mg/kg bw per day at the 95th percentile. For the supplement users only scenario, estimated dietary exposures for respondents 3 years of age and older ranged between 0.01 and 0.09 mg/kg

bw per day at the mean and between 0.02 and 0.13 mg/kg bw per day at the 95th percentile (Table 4).

The dietary exposure assessment based on maximum permitted levels is similar to the EFSA results based on maximum permitted levels reviewed at the eighty-second meeting. The brand-loyal assessments provide lower estimates of dietary exposure compared with those based on maximum permitted levels by more than half at the top end of the range of estimates. The non-brand-loyal scenario produced lower estimates compared with the brand-loyal scenario. EFSA's conclusion based on consideration of all uncertainties was that the dietary exposures to rosemary extract would be overestimated based on its use as a food additive, even using the typical use levels.

The food categories contributing the most to the dietary exposure to rosemary extract for the maximum permitted level scenario for infants only were fats and oils essentially free from water (11–76%). For infants and all other population groups (toddlers, children, adolescents, adults and the elderly population), the main contributing food categories were meat products (7–77%), fine bakery wares (5–63%) and soups and broths (7–80%). The main food categories contributing to dietary exposure for the refined assessment (both brand-loyal and non-brand-loyal scenarios) were fine bakery wares (67–73%) and soups and broths (5–99%) for infants and fine bakery wares (30–94%) for the other population groups assessed.

3.4.2 Australia

Dietary exposure to rosemary extract was estimated for Australia by FSANZ (2018a) following an application seeking to permit the use of the food additive in a range of foods as per the levels shown in Table 3 (FSANZ, 2018b).

The food consumption data used for the dietary exposure assessment were from the 2011–2012 Australian National Nutrition and Physical Activity Survey, which surveyed 12 153 respondents aged 2 years and older. A 24-hour recall methodology was used, with 7735 (64%) respondents completing a second 24-hour recall. Only respondents with 2 days of consumption data were included in the assessment, and the estimated dietary exposures were averaged across the 2 days for each individual. Population weights for this proportion of the sample were applied to ensure representativeness for the Australian population. Each individual respondent's dietary exposure from all foods, including carry-over of the food additive use to mixed dishes, was summed, and their own body weights were used to express dietary exposures on a body weight basis.

FSANZ estimated dietary exposure for two food additive use only scenarios, the first based on maximum permitted levels and the second based on typical use levels (noted as "usual" use levels by FSANZ). Typical use levels

Table 4

Estimated national mean and high-level dietary exposures to carnosic acid plus carnosol for consumers only from use of rosemary extract as a food additive based on different types of concentration data

Population	Region/country	Population subgroup	Number of consumers	Percentage of consumers	Maximum permitted level scenario		Typical use level scenario	
					Mean	High[a]	Mean	High[a]
Children	New Zealand[b]	5–14 years	3 258	99.0	0.17	0.34	0.091	0.18
	USA	0–2 years	473	76.8	–	–	0.14	0.30
		3–11 years	1 273	99.8	–	–	0.09	0.20
		12–19 years, males	550	99.2	–	–	0.06	0.15
		12–19 years, females	564	99.3	–	–	0.05	0.16
	Europe	4 months – 17 years	NS	NS	0.03–0.44	0.08–0.85	–	–
		4 months – 17 years – brand loyal	NS	NS	–	–	<0.01–0.16	<0.01–0.35
		4 months – 17 years – non–brand loyal	NS	NS	–	–	<0.01–0.09	<0.01–0.20
Adults	New Zealand[b]	15+ years	4 620	98.0	0.078	0.15	0.041	0.083
	USA	20+ years, males	2 056	98.7	–	–	0.05	0.12
		20+ years, females	2 357	99.5	–	–	0.05	0.11
	Europe	18+ years	NS	NS	0.04–0.17	0.09–0.40	–	–
		18+ years – brand loyal	NS	NS	–	–	0.01–0.05	0.02–0.15
		18+ years – non–brand loyal	NS	NS	–	–	<0.01–0.03	0.01–0.10
General population	Australia	2+ years	7 701	99.6	0.084	0.18	0.043	0.093
	USA	All ages	7 273	98.3	–	–	0.06	0.14
Other groups	Europe	Supplement users only, 3+ years	NS	NS	–	–	0.01–0.09	0.02–0.13

bw: body weight; NS: not specified; USA: United States of America

[a] Europe = 95th percentile. Australia, New Zealand, USA = 90th percentile.

[b] Based on 1 day of dietary survey data. All other assessments based on 2 days of data.

were 80% of maximum permitted levels for fats and oils and 50% of maximum permitted levels for all other foods. Scenarios including naturally occurring sources of carnosic acid plus carnosol are described in section 3.5. Flavouring or colour preparations were not included in the assessment.

The estimated dietary exposures to carnosic acid plus carnosol for Australia for consumers only (Table 4) based on maximum permitted levels for the population aged 2 years and older were 0.084 mg/kg bw per day at the mean and 0.18 mg/kg bw per day at the 90th percentile. For the typical use scenario for the population aged 2 years and older, estimated dietary exposures for consumers only were lower than those based on maximum permitted levels and were 0.043 mg/kg bw per day at the mean and 0.093 mg/kg bw per day at the 90th percentile. Most (99.6%) respondents were consumers of carnosic acid plus carnosol for both scenarios. Estimated dietary exposures based on the typical use scenario are still likely to be overestimates, based on the assumption that 100% of foods within each category contained the food additive.

The main contributors to dietary exposure for both the maximum permitted level and typical use scenarios from use of the food additive were sauces and toppings (including mayonnaise and salad dressings) (42–45%), grain bars, breakfast bars and breakfast cereals (23–24%) and cookies, pancakes, waffles and sweet pastries (10–11%). For the usual use scenario, margarines with fat content <80% also contributed to dietary exposure (6–7%).

3.4.3 New Zealand

Dietary exposure to rosemary extract was estimated for New Zealand by FSANZ (2018a) following an application seeking to permit the use of the food additive in a range of foods as per the levels shown in Table 3 (FSANZ, 2018b).

The food consumption data used for the dietary exposure assessment were from the 2002 New Zealand National Children's Nutrition Survey, which surveyed 3275 respondents aged 5–14 years, and the 2008–2009 New Zealand Adult Nutrition Survey, which surveyed 4721 respondents aged 15 years and above. A 24-hour recall methodology was used for both surveys. While a second 24-hour recall was conducted on 25% of the respondents in each of the surveys, only consumption data from the first day for all respondents were included in the assessment. Population weights were applied to ensure representativeness for the New Zealand population for both surveys. Each individual respondent's dietary exposure from all foods, including carry-over of the food additive use to mixed dishes, was summed, and their own body weights were used to express dietary exposures on a body weight basis.

FSANZ estimated dietary exposure for two food additive use only scenarios, the first based on maximum permitted levels and the second based on

typical use levels (80% of maximum permitted levels for fats and oils and 50% of maximum permitted levels for all other foods). Scenarios including naturally occurring sources of carnosic acid plus carnosol are described in section 3.5. Flavouring or colour preparations were not included in the assessment.

The estimated dietary exposures to carnosic acid plus carnosol for New Zealanders for consumers only (Table 4) based on maximum permitted levels for children 5–14 years of age were 0.17 mg/kg bw per day at the mean and 0.34 mg/kg bw per day at the 90th percentile; for adults 15 years of age and older, dietary exposures were 0.078 mg/kg bw per day at the mean and 0.15 mg/kg bw per day at the 90th percentile. For the typical use scenario for children, estimated dietary exposures were lower than dietary exposures for the maximum permitted level scenario and were 0.091 mg/kg bw per day at the mean and 0.18 mg/kg bw per day at the 90th percentile; for adults, dietary exposures were 0.041 mg/kg bw per day at the mean and 0.083 mg/kg bw per day at the 90th percentile. For children, 99% of respondents were consumers of carnosic acid plus carnosol for both scenarios; for adults, the percentage was 98% for both scenarios. Estimated dietary exposures based on the typical use scenario are still likely to be overestimates based on the assumption that 100% of foods within each category contained the food additive.

The main contributors to dietary exposure for both the maximum permitted level and typical use scenarios from use of the food additive were sauces and toppings (including mayonnaise and salad dressings) (27–28%, children; 37–41%, adults), grain bars, breakfast bars and breakfast cereals (25–27%, children; 18–20%, adults), margarines with fat content <80% (9–14%, children; 11–17%, adults), cookies, pancakes, waffles and sweet pastries (18–19%, children; 9–11%, adults) and processed meat, poultry and game in whole cuts or pieces, fat content >10% (5%, adults).

3.4.4 United States of America

For the present meeting, the sponsors provided an updated assessment of dietary exposure to rosemary extract for the USA based on food and supplement consumption data from the 2013–2014 NHANES and typical use levels of rosemary extract, as available from the EFSA and FSANZ dietary exposure assessments. This differed from the assessment provided by the sponsors at the eighty-second meeting, which was based on 2011–2012 data from NHANES and EU maximum permitted levels of rosemary extract.

The NHANES data are derived from individuals using a 24-hour recall on 2 non-consecutive days and are collected across all seasons of the year. In total, 10 175 respondents were interviewed. Sample weights were used to express the data on a population basis.

The concentrations used for the dietary exposure assessment were established based on values as reported by the sponsors, which were within the range of the typical and maximum use levels in the EU and Australia and New Zealand. The concentrations are those listed as the typical use levels in the last column of Table 3. Concentrations expressed on a fat basis were converted to an as consumed basis based on the fat content of the food.

Food codes in the consumption survey were matched with food codes in the regulations for which rosemary extract permissions would apply. Product-specific adjustment factors were derived based on a standard survey recipe file.

No dietary exposure from the use of the food additive in colour preparations or flavourings was taken into account in this assessment.

Dietary exposures to carnosic acid plus carnosol from use of rosemary extract as a food additive for consumers only in the USA (Table 4) as estimated by the sponsors using typical use levels for children ranged between 0.05 and 0.14 mg/kg bw per day at the mean and between 0.15 and 0.30 mg/kg bw per day at the 90th percentile. For adults, mean dietary exposures were 0.05 mg/kg bw per day for both males and females, and dietary exposures ranged between 0.11 and 0.12 mg/kg bw per day at the 90th percentile. For the whole population of the USA, 98.3% were consumers of rosemary extract; when expressed on a total population basis, dietary exposures were 0.06 mg/kg bw per day at the mean and 0.14 mg/kg bw per day at the 90th percentile. These new estimates based on typical use levels were lower than the estimates reviewed by the Committee at the eighty-second meeting, which were based on maximum permitted levels.

The main contributors to total mean dietary exposure were soups and broths (33–51%), fine bakery wares (18–33%) and processed meat products (10–17%) across the population groups assessed.

3.5 Estimates of dietary exposure to other sources of carnosic acid and carnosol

At its eighty-second meeting, the Committee reviewed information provided by EFSA (2008) noting dietary exposure from dried rosemary of up to 150 mg carnosic acid plus carnosol per day (2.5 mg/kg bw per day based on a 60 kg body weight) based on dried rosemary containing 2% carnosic acid plus carnosol and 7.5 g per serving based on recipes for individuals. That Committee noted that there were no consumption data available for flavouring oils and that these would not contribute significantly to dietary exposure to carnosic acid and carnosol.

At the present meeting, estimates of dietary exposure to carnosic acid plus carnosol from naturally occurring sources were reviewed. Both the EFSA

(2018) and FSANZ (2018a) assessments contained estimates of dietary exposure to carnosic acid plus carnosol from naturally occurring sources.

The CIFOCOss database (FAO/WHO, 2017) was reviewed for consumption data for rosemary (fresh and dried), sage and marjoram to determine whether any estimates of dietary exposure could be calculated from these consumption data. Consumption data for Australia, New Zealand and European countries were excluded, as assessments of dietary exposure to carnosic acid and carnosol from naturally occurring sources had already been conducted for these countries. "Herbs not specified as to the type" were excluded, as it is unknown what specific type this includes and whether they would contain carnosic acid and carnosol. Although the data for the USA noted that there were consumers of marjoram (62% for the general population), no numerical consumption data were provided. No other countries had consumption data reported for the relevant herbs. Therefore, no estimates of dietary exposure were calculated by the Committee for naturally occurring sources of carnosic acid and carnosol using CIFOCOss data.

3.5.1 Dietary exposures from naturally occurring sources only

The present Committee reviewed the most recent EFSA estimates (EFSA, 2018) of dietary exposure to rosemary from the natural diet. EFSA included rosemary (dried at 112 mg carnosic acid plus carnosol per kilogram; fresh at 12.18 mg/kg), sage (dried at 5.3 mg/kg) and other undefined aromatic herbs (dried and fresh at the same concentration as sage, 5.3 mg/kg). Dietary exposures from the natural diet (Table 5) were estimated for children to be between 0.0 and 0.34 mg/kg bw per day at the mean and between 0.0 and 1.66 mg/kg bw per day at the 95th percentile. For adults, estimates were between 0.0 and 0.13 mg/kg bw per day at the mean and between 0.0 and 0.52 mg/kg bw per day at the 95th percentile. The highest estimate of 1.66 mg/kg bw per day at the 95th percentile was for toddlers. This represents 9 times the exposure from food additive use for this population group.

For Australia and New Zealand, dietary exposures to naturally occurring carnosic acid plus carnosol were estimated from consumption of rosemary, dried and fresh. The concentration of carnosic acid plus carnosol in dried rosemary used in the dietary exposure assessment was 21 500 mg/kg, which was the midpoint of the range provided by the applicant (as noted in section 3.3.2). The level in fresh rosemary used was 7525 mg/kg. For Australians 2 years of age and older, 5.8% of the population (n = 477) were consumers of carnosic acid plus carnosol from rosemary. The estimated dietary exposure was 0.048 mg/kg bw per day at the mean and 0.081 mg/kg bw per day at the 90th percentile. For New Zealand, 0.1% of children (n = 5) were consumers of carnosic acid plus carnosol

Table 5

Estimated dietary exposure to carnosic acid plus carnosol for consumers only from naturally occurring sources and from naturally occurring sources plus added sources

			Dietary exposure (mg/kg bw per day)					
			Naturally occurring only		Naturally occurring plus maximum permitted level scenario		Naturally occurring plus typical use level scenario	
Population	**Region/country**	**Age group**	**Mean**	**High**	**Mean**	**High**	**Mean**	**High**
Children	New Zealand[a]	5–14 years	0.023	na	0.17	0.34	0.091	0.18
	Europe[b]	4 months – 17 years	0.0–0.34	0.0–1.66	na	na	0.0–0.42[c]	na
Adults	New Zealand[d]	15+ years	0.18	0.30	0.08	0.16	0.043	0.085
	Europe[b]	18+ years	0.0–0.13	0.0–0.52	na	na	0.0–0.16[c]	na
General population	Australia[e]	2+ years	0.048	0.081	0.086	0.18	0.045	0.096

bw: body weight; na: not assessed, too few respondents to derive a reliable 90th percentile
[a] Proportion of consumers: naturally occurring only 0.1% ($n = 5$); natural plus added (both scenarios) 99% ($n = 3258$).
[b] The additive source is from the non-brand-loyal scenario.
[c] Mean exposure from naturally occurring sources added to mean exposure from added sources.
[d] Proportion of consumers: naturally occurring only 1.1% ($n = 52$); natural plus added (both scenarios) 98% ($n = 4620$).
[e] Proportion of consumers: naturally occurring only 5.8% ($n = 477$); natural plus added (both scenarios) 99.6% ($n = 7701$).

from rosemary, as were 1.1% of adults aged 15 years and over (*n* = 52). Estimated dietary exposures for New Zealand from naturally occurring sources were, for children, 0.023 mg/kg bw per day at the mean (there were not enough consumers to derive a robust 90th percentile) and, for adults, 0.18 mg/kg bw per day at the mean and 0.30 mg/kg bw per day at the 90th percentile.

The sponsors also provided some information regarding dietary exposure from naturally occurring sources. This was based on typical use of 1 tablespoon of rosemary in recipes, equivalent to approximately 3.75 g rosemary per serving (assuming four servings per recipe) (rosemary crusted lamb rack recipe: https://www.schwartz.co.uk/recipes/lamb/lamb-with-a-mint-and-rosemary-crust). If it is assumed that dried rosemary contains 2% carnosic acid and carnosol, this would equate to 75 mg of carnosic acid and carnosol per serving from a typical meal (equivalent to 1.25 mg/kg bw per day based on a 60 kg adult body weight). In comparison, estimated dietary exposures to carnosic acid plus carnosol from food additive use of rosemary extract in the USA range between 3.9 and 9.9 mg/day at the 90th percentile (corresponding to exposures of 0.30 and 0.16 mg/kg bw per day in infants and young children [0–2 years] and female teenagers [12–19 years], respectively, in Table 4). The sponsors concluded, therefore, that the estimated dietary exposure from rosemary extract based on the proposed typical use levels is well below that from levels of dried rosemary in the background diet.

Estimated dietary exposures from naturally occurring sources were generally higher in Europe, at the upper end of the range of estimated dietary exposures, than in Australia and New Zealand. This may be as a result of a higher consumption of herbs in Europe. It may also be a function of the consumption databases, with the use of recipes for Australia and New Zealand producing some smaller consumption amounts of herbs, which skews down the dietary exposure estimates.

3.5.2 Dietary exposures from naturally occurring and added sources combined

The Committee also considered the estimated dietary exposure when naturally occurring and added sources of carnosic acid plus carnosol were consumed together. Estimates were provided for Europe (EFSA, 2018) and for Australia and New Zealand (FSANZ, 2018a).

For Europe, summed mean exposures from the food additive use (non-brand-loyal scenario) and the natural diet (based on mean exposures only) (Table 5) resulted in estimated dietary exposures between 0.0 and 0.42 mg/kg bw per day for children and between 0.0 and 0.16 mg/kg bw per day for adults. The highest estimate of 0.42 mg/kg bw per day was for toddlers. Between 7% and 35% of dietary exposure in children from the combined sources comes from the

food additive (proportion increases as age group increases), and between 19% and 25% of dietary exposure in adults comes from the food additive.

FSANZ conducted an assessment that included the naturally occurring sources of carnosic acid plus carnosol and the added sources, for both the maximum permitted level scenario and the typical use level scenario. Estimated dietary exposures were calculated for each individual in the dietary surveys from both sources; population summary statistics were then derived.

For the Australian population aged 2 years and above, estimated dietary exposures from naturally occurring sources plus maximum permitted levels of the food additive were 0.086 mg/kg bw per day at the mean and 0.18 mg/kg bw per day at the 90th percentile. When typical use levels of rosemary extract were used, dietary exposures were 0.045 mg/kg bw per day at the mean and 0.096 mg/kg bw per day at the 90th percentile (Table 5). For Australia, 100% of respondents were consumers of carnosic acid plus carnosol for both scenarios.

For New Zealanders, when naturally occurring exposure is combined with that from maximum permitted levels of the food additive, dietary exposures for children were 0.17 mg/kg bw per day at the mean and 0.34 mg/kg bw per day at the 90th percentile; for adults, dietary exposures were 0.08 mg/kg bw per day at the mean and 0.16 mg/kg bw per day at the 90th percentile. For naturally occurring and typical use levels, estimated dietary exposures for children were 0.091 mg/kg bw per day at the mean and 0.18 mg/kg bw per day at the 90th percentile; for adults, dietary exposures were 0.043 mg/kg bw per day at the mean and 0.085 mg/kg bw per day at the 90th percentile (Table 5). For New Zealand children, 99% of respondents were consumers of carnosic acid plus carnosol for both scenarios, and for adults, the percentage was 98% for both scenarios.

Overall, for all Australian and New Zealand population groups and scenarios assessed, there was minimal impact when considering dietary exposures for consumers for the food additive use only and then adding naturally occurring sources, with an increase of between 0 and 0.01 mg/kg bw per day. For Australia, 97% of the dietary exposure was from food additive sources, and for New Zealand, 96–99% of dietary exposure was from food additive sources. Thus, the estimated values show that there was a minimal influence of naturally occurring sources on the distribution of individual dietary exposures, as population summary statistics remained similar when naturally occurring sources were included.

Differences in the proportion of dietary exposure coming from the food additive use in Europe compared with Australia and New Zealand are based on differences in the methodology used to derive the estimates of dietary exposure. For Europe, mean dietary exposure from the food additive was summed with mean dietary exposure from naturally occurring sources. For Australia and New Zealand, it was based on the distribution of dietary exposures from individuals.

3.6 Summary of estimates of dietary exposure

Only estimated dietary exposures based on typical use levels were used by the Committee for the evaluation, as they provide a more realistic estimate of chronic dietary exposure compared with those based on maximum permitted levels. The Committee determined that the most appropriate estimates of dietary exposure for Europe to use for the evaluation would be from the non-brand-loyal scenario, given that it most closely represents long-term chronic dietary exposures, being based on mean typical use levels.

Compared with estimated dietary exposures from the eighty-second meeting, where the estimates were based on maximum permitted levels, the estimates at the present meeting, based on typical use levels, were around half those at the top end of the range for both mean and high-percentile exposures.

The dietary exposure estimates based on typical use levels still incorporate a degree of uncertainty, as they assume that 100% of foods within a category contain the food additive at the specified level.

4. Comments

4.1 Biochemical aspects

In a pharmacokinetics study that investigated an ethanol-based extract of dried leaves of rosemary (Wang et al., 2017), rats were administered rosemary extract at a dose of 240, 820 or 2450 mg/kg bw by oral gavage. Plasma concentrations of carnosic acid and carnosol were determined up to 24 hours after administration. The T_{max} was approximately 0.5 hour. The C_{max} and $AUC_{0-\infty}$ values showed reasonably good agreement, with a proportional increase with dose. An apparent double-peak phenomenon in the plasma concentration versus time curves, suggesting redistribution and enterohepatic recirculation, was also observed (Wang et al., 2017). The Committee noted inconsistencies in C_{max} and T_{max} values between this study and two previously evaluated studies (Yan et al., 2009; Doolaege et al., 2011).

A study by Seow & Lau (2017) using a luciferase reporter gene assay with hPXR, mPXR and rPXR indicated that carnosol is an activator of all three receptors, whereas carnosic acid is a potent agonist of both hPXR and mPXR, but not rPXR. These new findings provide insight on the molecular basis for the PXR-mediated induction of expression of phase 1 and phase 2 enzymes of xenobiotic metabolism and membrane transport proteins.

An in vitro study by Ercan & El (2018) showed that a rosemary water extract with 18.7% carnosic acid was a potent inhibitor of pancreatic lipase.

4.2 Toxicological studies

A new OECD-compliant (Test Guideline 421) reproductive/developmental toxicity screening study in rats using an acetone extract of rosemary with a high content of carnosic acid was available (Blunt, 2018). Rats were administered rosemary extract in the diet at initial concentrations of 0, 2100, 3600 and 5000 mg/kg feed, which were later reduced in females from GD 20 to 0, 1050, 1800 and 2500 mg/kg feed (equal to 0, 130, 219 and 316 mg/kg bw per day for males and 0, 167, 276 and 401 mg/kg bw per day for females, respectively). No adverse effects were observed in parental males or females or in reproductive parameters. Gestation duration, litter size and pup body weight on PND 1 and pup survival and body weight gain until PND 13 (termination) were not affected by treatment. A clear dose-related reduction in total-T_4 serum levels in male and female pups was observed on PND 13. Histopathological examination of the thyroid gland (one male and one female pup per litter) showed no abnormality (Blunt, 2018). The Committee noted the high variability in the thyroid hormone measurements in the pups.

A NOAEL of 5000 mg/kg feed (equal to 316 mg/kg bw per day), the highest dose tested, was identified for reproductive and parental toxicity. The Committee noted that it was unclear whether the treatment-related effects on thyroid hormone levels in pups were adverse, and therefore a NOAEL for offspring toxicity could not be identified. The study also did not provide adequate evidence for the absence of developmental toxicity, given that no fetuses were examined.

One toxicological study on carnosic acid was identified in the literature search. Liu et al. (2017) tested carnosic acid in an in vitro screening assay for embryotoxic potential using mouse embryonic stem cells. The embryonic stem cell test is an extensively used screening assay for developmental toxicity that has been validated by the European Union Reference Laboratory for alternatives to animal testing (Paquette et al., 2008). Studies on the predictivity of the embryonic stem cell assay indicated a significant false-positive rate (approximately 40%), but a very low false-negative rate (approximately 7%) (Paquette et al., 2008). According to the results from this in vitro assay, carnosic acid is weakly embryotoxic (Liu et al., 2017).

4.3 **Observations in humans**

A small-scale clinical study (a randomized, double-blinded and placebo-controlled study) investigated the memory-enhancing effects of a combined ethanol extract of three plants, including *Rosmarinus officinalis*. No adverse effects of the combined ethanol extract following administration for 14 days were reported (Perry et al., 2018). The Committee noted that this study does not contribute to the evaluation.

4.4 **Assessment of dietary exposure**

The Committee first evaluated dietary exposure to rosemary extract (expressed as carnosic acid plus carnosol) at its eighty-second meeting (Annex 1, reference *230*). At that time, the estimates were based on maximum permitted and proposed levels. The Committee at that meeting noted that the dietary exposure estimates for high consumers of 0.09–0.81 mg/kg bw per day may exceed the upper bound of the temporary ADI by up to 2.7-fold. Based on the conservative nature of the dietary exposure assessments, the Committee requested that data on typical use levels in foods be provided in order to refine the dietary exposure estimates.

At the current meeting, typical use levels of rosemary extract (expressed as carnosic acid plus carnosol) from Europe (EFSA, 2018), Australia and New Zealand (FSANZ, 2018a) were available to the Committee for review. Dietary exposure assessments (expressed as carnosic acid plus carnosol) were also available based on typical use levels. These included estimates for Europe based on typical use levels in Europe (EFSA, 2018), estimates for Australia and New Zealand based on typical use levels for those countries (FSANZ, 2018a) and an assessment for the USA (from the sponsors) based on concentrations that were between the range of typical use and maximum permitted levels from the EU, Australia and New Zealand. Although estimates of dietary exposure were also provided based on maximum permitted levels, only estimates of dietary exposure based on typical use levels were used by the Committee in the evaluation. In addition, only non-brand-loyal results for Europe were used for the purpose of the evaluation.

For children, mean estimates of dietary exposure ranged between <0.01 and 0.14 mg/kg bw per day; high-percentile exposures ranged between <0.01 and 0.30 mg/kg bw per day. For adults, mean estimates of dietary exposure ranged between <0.01 and 0.05 mg/kg bw per day; high-percentile exposures ranged between 0.01 and 0.12 mg/kg bw per day. Estimated dietary exposures based on typical use levels were less than half those estimated by the Committee at the eighty-second meeting, which were based on maximum permitted levels, at the upper ends of the ranges of both mean and high-percentile exposures. Depending on the country, the main contributors to dietary exposure were fine

bakery wares, soups and broths, sauces and toppings (including mayonnaise and salad dressings) and processed meat products.

For the present meeting, estimates of dietary exposure from naturally occurring sources were available for Europe (rosemary and other herbs) (EFSA, 2018) and Australia and New Zealand (rosemary only) (FSANZ, 2018a). Estimates included dietary exposures from naturally occurring sources only and in combination with added sources.

For naturally occurring sources only, estimated dietary exposures for children ranged between 0.0 and 0.34 mg/kg bw per day for mean exposures and between 0.0 and 1.66 mg/kg bw per day for high-percentile exposures. Estimated dietary exposures for adults ranged between 0.0 and 0.18 mg/kg bw per day for mean exposures and between 0.0 and 0.52 mg/kg bw per day for high-percentile exposures. When dietary exposures from naturally occurring sources are combined with dietary exposures from added sources at typical use levels, the estimated mean and high-percentile dietary exposures were up to 0.42 mg/kg bw per day for children and up to 0.16 mg/kg bw per day for adults (both estimates from Europe; mean naturally occurring dietary exposure added to mean food additive dietary exposure, eliminating the high dietary exposure of up to 0.52 mg/kg bw per day). The contribution from naturally occurring sources was <1–4% for Australia and New Zealand, based on the distribution of dietary exposures for individuals, and 65–93% for Europe, based on summing mean dietary exposures from added and natural sources.

5. Evaluation

The Committee concluded that the new studies provided evidence for the absence of reproductive toxicity, but not for the absence of developmental toxicity. The Committee retained the temporary ADI of 0–0.3 mg/kg bw, pending the submission of studies on the developmental toxicity of rosemary extract and studies to elucidate whether the effects noted on rodent pup thyroid hormone levels can be replicated. The temporary ADI will be withdrawn if the requested studies are not submitted by the end of 2021.

Estimated mean and high-percentile dietary exposures to carnosic acid plus carnosol from use of rosemary extract as a food additive for all countries assessed based on typical use levels did not exceed the upper end of the temporary ADI of 0–0.3 mg/kg bw. The Committee noted that when dietary exposures from naturally occurring sources are combined with dietary exposures from added sources at typical use levels, the estimated dietary exposures for children were up to 0.42 mg/kg bw per day, which exceeds the ADI. The Committee noted that the

temporary ADI is based on the highest dose tested in a short-term toxicity study in rats and that in the newly submitted reproductive/developmental toxicity screening study, no effects on reproductive toxicity or on parental animals were observed at 316 mg/kg bw per day, the highest dose tested. Therefore, the Committee does not consider the slight exceedance of the ADI to be a safety concern.

The Committee removed the specification for ethanol. The specifications monograph for rosemary extract was revised, and the tentative status was removed.

5.1 Recommendations

Studies on the developmental toxicity of rosemary extract and studies to elucidate whether the effects noted on pup thyroid hormone levels can be replicated were identified as research needs to complete the evaluation. The Committee requests that this information be provided by the end of 2021.

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ANNEX 1

Reports and other documents resulting from previous meetings of the Joint FAO/WHO Expert Committee on Food Additives

1. General principles governing the use of food additives (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).

2. Procedures for the testing of intentional food additives to establish their safety for use (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).

3. Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants) (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. I. Antimicrobial preservatives and antioxidants, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).

4. Specifications for identity and purity of food additives (food colours) (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. II. Food colours, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).

5. Evaluation of the carcinogenic hazards of food additives (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).

6. Evaluation of the toxicity of a number of antimicrobials and antioxidants (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).

7. Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).

8. Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).

9. Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).

10. Specifications for identity and purity and toxicological evaluation of food colours. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.

11. Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).

12. Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases. FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.

13. Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.

14. Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non-nutritive sweetening agents (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.

15. Toxicological evaluation of some flavouring substances and non-nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.

16. Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.

17. Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.

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21. Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives. FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.

22. Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.

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28. Specifications for the identity and purity of some enzymes and certain other substances. FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.

29. A review of the technological efficacy of some antioxidants and synergists. FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.

30. Evaluation of certain food additives and the contaminants mercury, lead, and cadmium (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.

31. Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate. FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.

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34. Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers. FAO Food and Nutrition Paper, No. 4, 1978.

35. Evaluation of certain food additives (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.

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44. Evaluation of certain food additives (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.

45. Summary of toxicological data of certain food additives. WHO Food Additives Series, No. 12, 1977.

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59. Evaluation of certain food additives and contaminants (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.

60. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 17, 1982.

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62. Evaluation of certain food additives and contaminants (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983, and corrigenda.

63. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 18, 1983.

64. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 28, 1983.

65. Guide to specifications – General notices, general methods, identification tests, test solutions, and other reference materials. FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.

66. Evaluation of certain food additives and contaminants (Twenty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 710, 1984, and corrigendum.

67. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 19, 1984.

68. Specifications for the identity and purity of food colours. FAO Food and Nutrition Paper, No. 31/1, 1984.

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89. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 26, 1990.

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91. Evaluation of certain veterinary drug residues in food (Thirty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 799, 1990.

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93. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/3, 1991.

94. Evaluation of certain food additives and contaminants (Thirty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 806, 1991, and corrigenda.

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98. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 29, 1991.

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103. Compendium of food additive specifications: addendum 1. FAO Food and Nutrition Paper, No. 52, 1992.

104. Evaluation of certain veterinary drug residues in food (Fortieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 832, 1993.

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108. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 32, 1993.

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110. Evaluation of certain veterinary drug residues in food (Forty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 851, 1995.

111. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 33, 1994.

112. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/6, 1994.

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ANNEX 2

Abbreviations used in the monographs

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
AHNDA	8-acetamido-1-hydroxy-2-naphthylamine-3,5-disulfonic acid
AL-HMF	acidified liquid human milk fortification
ALT	alanine aminotransferase
ANOVA	analysis of variance
ANSA	4-acetamido-1-naphthylamine-6-sulfonic acid; 4-acetamido-1-naphthylamine-7-sulfonic acid
AST	aspartate aminotransferase
ATBC	Alpha-Tocopherol, Beta Carotene Cancer Prevention (Study)
AUC	area under the concentration–time curve
BMI	body mass index
bw	body weight
C3G	cyanidin-3-O-glucoside
CARET	Beta-Carotene and Retinol Efficacy Trial
CAS	Chemical Abstracts Service
CCFA	Codex Committee on Food Additives
CI	confidence interval
CIAA	Confederation of the Food and Drink Industry of the European Union (currently known as FoodDrinkEurope)
CIFOCOss	FAO/WHO Chronic Individual Food Consumption data – summary statistics
C_{max}	maximum concentration
DNA	deoxyribonucleic acid
DONALD	(German) Dortmund Nutritional and Anthropometrical Longitudinally Designed study
DSA	1,4-diaminonaphthalene-6-sulfonic acid
EC_{50}	median effective concentration
EER	estimated energy requirement
EFSA	European Food Safety Authority
EU	European Union
EXPOCHI	individual food consumption data and exposure assessment studies for children
F_0	parental generation
F_1	first filial generation

F_2	second filial generation
FAIM	Food Additives Intake Model
FAO	Food and Agriculture Organization of the United Nations
FoodEx	EFSA Food classification and description system for exposure assessment
FSANZ	Food Standards Australia New Zealand
FSMP	formula for special medical purposes for infants
GD	gestation day
GEMS/Food	Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme
GIFT	FAO/WHO Global Individual Food consumption data Tool
GLP	good laboratory practice
GMP	good manufacturing practice
GSFA	(Codex) General Standard for Food Additives
HMF	human milk fortification
HPLC	high-performance liquid chromatography
hPXR	human pregnane X receptor
IACM	International Association of Colour Manufacturers
IC_{50}	median inhibitory concentration for cell growth (cytotoxicity)
ID_{50}	median inhibitory concentration for cell differentiation
IgE	immunoglobulin E
IL-6	interleukin 6
IL-8	interleukin 8
INS	International Numbering System for Food Additives
IU	international units
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC_{50}	median lethal concentration
LD_{50}	median lethal dose
MOE	margin of exposure
MPL	maximum permitted level
mPXR	mouse pregnane X receptor
mRNA	messenger ribonucleic acid
MRT	mean residence time
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
n	number
NAL-HMF	non-acidified liquid human milk fortification
NATCOL	Natural Food Colours Association
NCE	normochromatic erythrocyte
ND	not detected
NDNS	National Dietary and Nutrition Survey (United Kingdom)

NHANES	National Health and Nutrition Examination Survey (USA)
No.	number
NOAEL	no-observed-adverse-effect level
NS	not specified
OECD	Organisation for Economic Co-operation and Development
OR	odds ratio
PCE	polychromatic erythrocyte
P-HMF	powdered human milk fortification
PND	postnatal day
PXR	pregnane X receptor
rPXR	rat pregnane X receptor
RR	relative risk
S9	$9000 \times g$ supernatant fraction from rat liver homogenate
SA	sulfanilic acid
SCFA	short-chain fatty acid
SD	standard deviation
SNSA	1-(4′-sulfophenylazo)-4-naphthylamine-6-sulfonic acid
t	time
$t_{1/2}$	half-life
T_3	triiodothyronine
T_4	thyroxine
TG	Test Guideline
Tk	thymidine kinase
T_{max}	time to reach maximum concentration
TNF-α	tumour necrosis factor alpha
TSH	thyroid stimulating hormone
UL	use level
vs	versus
WHO	World Health Organization
w/w	weight per weight

ANNEX 3

Joint FAO/WHO Expert Committee on Food Additives

Rome, 4–13 June 2019

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ANNEX 4

Toxicological information, dietary exposure information and information on specifications

Food additives evaluated toxicologically and assessed for dietary exposure

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
Black carrot extract	N[a], T[b]	The Committee concluded that the effects observed with one anthocyanin-containing test material cannot be extrapolated to another anthocyanin-containing test material. This is because the test articles used in metabolism and toxicity studies are very heterogeneous and often not fully described and/or the anthocyanin content of the test material is too low and variable.
		Only one genotoxicity study was available for black carrot extract. **Owing to the lack of toxicological data on black carrot extract, the Committee was not able to draw conclusions on its safety.** To proceed with its assessment, at least a 90-day toxicological study on a well-characterized extract representative of the material of commerce would be required.
		The Committee concluded that the total mean dietary exposure to anthocyanins from naturally occurring sources and added black carrot extract ranges from 0.1 to 1.9 mg/kg body weight (bw) per day for adults (18+ years) and from 0.1 to 5.3 mg/kg bw per day for children (<18 years). The Committee noted that the contribution of the use of the food colour itself to the total mean dietary exposure to anthocyanins including from naturally occurring sources is as high as 25%.
		The Committee noted that the ADI for grape skin extract established by the previous Committee in 1982 was not reconsidered as part of this assessment and remains unchanged.
Brilliant Black PN	R[c]	The Committee concluded that the newly available information does not give reason to revise the previously established ADI of 0–1 mg/kg bw based on a short-term toxicity study in pigs. **The Committee therefore retained the ADI for Brilliant Black PN.**
		The Committee noted that the range of estimated dietary exposures for Brilliant Black PN was below the upper end of the ADI and concluded that **dietary exposure to Brilliant Black PN does not present a safety concern.**
Carotenoids (provitamin A)	R[d]	The Committee reaffirmed the conclusion from the eighty-fourth meeting that rats are not an appropriate model for deriving an ADI for β-carotene due to the relatively low bioavailability of β-carotene in rats compared with humans. Therefore, **the Committee withdrew the two group ADIs of 0–5 mg/kg bw for (1) the sum of the synthetic carotenoids β-carotene, β-apo-8′-carotenal and β-apo-8′-carotenoic acid methyl and ethyl esters and (2) synthetic β-carotene and β-carotene derived from *Blakeslea trispora*[e]**, which were based on a no-observed-adverse-effect level (NOAEL) from a rat study.

(continued)

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
		The Committee considered that no adverse health effects were observed in the general population in large, well-conducted human intervention studies in which healthy participants were administered 20–50 mg β-carotene per day for up to 12 years, in addition to background exposure from the diet.
		An additional elevated risk of lung cancer and total mortality was seen in heavy smokers (at least one pack per day) and asbestos workers in intervention studies in which participants were administered 20 mg β-carotene per day for 5–8 years or 30 mg β-carotene per day and 25 000 IU vitamin A for 5 years. The Committee noted that a generally accepted explanation for the cause of these effects has not been identified. **The Committee was unable to reach any conclusion about risk from β-carotene exposure in heavy smokers.**
		For the remainder of the general population, the Committee concluded that the estimated high exposure to β-carotene of 9 mg/day for a 30 kg child and 6 mg/day for a 60 kg adult from its current uses as a food additive, in addition to background exposure from the diet, would not be expected to be a safety concern. This conclusion includes synthetic β-carotene, β-carotene derived from *B. trispora* and β-carotene-rich extract from *Dunaliella salina*.
		T**he Committee was unable to establish a group ADI for synthetic β-carotene, β-carotene derived from *B. trispora*, β-carotene-rich extract from *D. salina*, and β-apo-8'-carotenoic acid methyl and ethyl esters** because a group ADI is applicable to the general population, which includes heavy smokers. The Committee noted that it is very unlikely that it will ever be possible to establish a group ADI because further data from the population of heavy smokers cannot be gathered ethically.
		Because β-apo-8'-carotenoic acid methyl and ethyl esters were previously evaluated on the basis of β-carotene and because no new data were submitted, **the Committee was unable to complete an evaluation on β-apo-8'-carotenoic acid methyl and ethyl esters.**
		The present Committee established an ADI of 0–0.3 mg/kg bw for β-apo-8'-carotenal on the basis of a NOAEL of 30 mg/kg bw per day in a 13-week study in rats and application of an uncertainty factor of 100. An additional uncertainty factor to take into account the short duration of the study was not considered necessary because kidney and liver effects observed in the 13-week study at 100 mg/kg bw per day were not observed in a 2-year study at 40 mg/kg bw per day, the single dose tested.
		Estimated dietary exposure to β-apo-8'-carotenal of 0.3 mg/kg bw per day was at the upper end of the ADI established by the Committee (i.e. 0–0.3 mg/kg bw per day). The Committee noted that the estimated dietary exposure is overestimated and concluded that the **current use of β-apo-8'-carotenal as a food additive will not pose a safety concern.**

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
Gellan gum	R[f], T[g]	Available studies confirm the absence of any adverse effects arising from exposure to gellan gum. **The Committee retained the previously established ADI "not specified"[h] for gellan gum.** The Committee evaluated low-acyl clarified gellan gum for use in formulas for special medical purposes for infants. Based on a NOAEL of 100 mg/kg bw per day, the highest dose of low-acyl clarified gellan gum tested in a 21-day neonatal pig study, which modelled the 0- to 12-week period of development in human infants, and the high estimate of dietary exposure of infants to gellan gum of 13 mg/kg bw per day (based on the requested maximum concentration of gellan gum of 50 mg/L and the high level of consumption of infant formula of 260 mL/kg bw per day), a margin of exposure of 7.7 was calculated. The Committee concluded on the basis of several considerations (e.g. the low toxicity of gellan gum, the NOAEL being the highest dose tested, clinical studies in preterm infants and post-marketing surveillance data showing that gellan gum is well tolerated) that the margin of exposure of 7.7 calculated for the use of gellan gum in formulas for special medical purposes for infants and liquid fortification products for addition to human milk or infant formula at a maximum level of 50 mg/L in the fed product indicates low risk for the health of infants, including preterm infants, and that **its proposed use is therefore of no safety concern. This conclusion applies only to the use of low-acyl clarified gellan gum.** The Committee recognizes that there is variability in medical conditions among infants requiring these products and that these infants would normally be under medical supervision.
Potassium polyaspartate	N	In vitro data suggest that the systemic bioavailability of potassium polyaspartate is low and that potassium polyaspartate would not be cleaved in the stomach or the intestine. The NOAEL in a 90-day rat study on potassium polyaspartate was 1000 mg/kg bw per day, the highest dose tested. There was no concern for genotoxicity. Potassium has been evaluated by the Committee in the course of its previous evaluation of potassium hydroxide, and the result of the evaluation was an ADI "not limited". [i] Exposure to potassium that results from the use of potassium polyaspartate in wine would be within normal daily variation of background potassium exposure from the diet. Should microbial fermentation in the human colon occur, there would be potential exposure to L - and D-aspartic acid. L-Aspartic acid is a normal constituent of dietary protein, and systemic exposure to L-aspartic acid from the diet is much higher than potential exposure from the use of potassium polyaspartate in wine. There are no relevant toxicological data on D-aspartic acid. In three studies, rats exposed to around 130 mg/kg bw per day showed effects on sex hormone levels. However, NOAELs have not been identified in these studies due to the use of single doses. The Committee noted that there is a margin of exposure of more than 100-fold between the potential human dietary exposure to D-aspartic acid of up to 0.8 mg/kg bw per day and the effect level of 130 mg/kg bw per day.

(continued)

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
		The estimated dietary exposure to D-aspartic acid from typical use of potassium polyaspartate in wine (up to 0.8 mg/kg bw per day) would be expected to be lower than the exposure from non-added sources in the diet. The Committee noted that it had limited data on concentrations of D-aspartic acid in food, but that food processing (e.g. heat treatment of protein, fermentation) will result in partial conversion of L-aspartic acid to D-aspartic acid. **The Committee concluded that the use of potassium polyaspartate in wine at the maximum proposed use level of 300 mg/L is not of safety concern.**
Rosemary extract	R[i]	The Committee concluded that the new studies provided evidence for the absence of reproductive toxicity, but not for the absence of developmental toxicity. **The Committee retained the temporary ADI of 0–0.3 mg/kg bw, pending the submission of studies on the developmental toxicity of rosemary extract and studies to elucidate whether the effects noted on rodent pup thyroid hormone levels can be replicated.** The temporary ADI will be withdrawn if the requested studies are not submitted by the end of 2021. Estimated mean and high-percentile dietary exposures to carnosic acid plus carnosol from use of rosemary extract as an additive for all countries assessed based on typical use levels did not exceed the upper end of the temporary ADI (0–0.3 mg/kg bw per day). The Committee noted that when dietary exposures from naturally occurring sources are combined with dietary exposures from added sources at typical use levels, the estimated dietary exposures for children were up to 0.42 mg/kg bw per day, which exceeds the ADI. The Committee also noted that the temporary ADI is based on the highest dose tested in a short-term toxicity study in rats and that in the newly submitted reproductive/developmental toxicity screening study, no effects on reproductive toxicity or on parental animals were observed at 316 mg/kg bw per day, the highest dose tested. **Therefore, the Committee does not consider the slight exceedance of the ADI to be a safety concern.**

N: new specifications; R: existing specifications revised; T: tentative specifications

[a] For the spray-dried powder form of black carrot extract.

[b] The specifications were made tentative pending further information on the material of commerce, including a full characterization of the proteins, carbohydrates, lipids, fibre, minerals and non-anthocyanin polyphenol components in five lots each of the liquid and powder forms of black carrot extract.

[c] Analytical methods for determining subsidiary colouring matters and organic compounds other than colouring matters were replaced with more specific and sensitive high-performance liquid chromatography methods. The existing titrimetric method for the assay of Brilliant Black PN was replaced with a visible spectrophotometric method.

[d] The specifications for synthetic β-carotene, β-carotene from *B. trispora* and β-apo-8′-carotenal were revised to replace an identification test for carotenoids with additional spectrophotometric requirements. Based on the arsenic levels from several batches of the product of commerce for β-carotene-rich extract from *D. salina*, the existing specifications for arsenic were revised from 1 mg/kg to 3 mg/kg.

[e] The Committee was aware that two group ADIs for carotenoids had been established at previous meetings and that synthetic β-carotene had been included in both group ADIs. The Committee speculated that the Committee at the fifty-seventh meeting did not recognize that synthetic β-carotene was already part of a group ADI and included it in a new group ADI.

[f] The Committee concluded that the use of ethanol in the manufacturing of gellan gum is not a safety concern when used according to good manufacturing practice. The specification for ethanol was removed.

[g] The specifications were made tentative, pending submission of new methods for characterizing the three forms of gellan gum in commerce by 2021.

[h] 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.

[i] Now called an ADI "not specified" (see table note h).

[j] The Committee removed the specification for ethanol, and the tentative status of the specifications for rosemary extract was removed.

Food additives considered for specifications only

Food additive	Specifications
Cassia gum	T[a]
Citric and fatty acid esters of glycerol (CITREM)	R[b]
Metatartaric acid	R[c]
Mannoproteins from yeast cell walls	R[d]
Steviol glycosides	See note e

R: existing specifications revised; T: tentative specifications

[a] At the eighty-sixth meeting, the Committee updated the specifications for cassia gum by including the high-performance liquid chromatographic method received and removed their tentative status. Based on comments received about the method performance, the present Committee reviewed the method again and noted that additional investigations were required. Therefore, the Committee decided to make the specifications tentative until ongoing investigations are completed.

[b] The Committee received a suitable validated replacement method for an obsolete packed column gas chromatographic method for the determination of total citric acid content, along with performance characteristics of the method and data on the total citric acid content in products currently available in commerce, determined using that method. The Committee included the new method in the specifications and deleted the previous method. A new high-performance liquid chromatography method for the analysis of glycerol, supported by validation data, was provided and included in the revised specifications. The limit for glycerol was maintained. Data on the use of additional neutralizing salts in CITREM manufacture were received and added to the specifications. The lead limit for use of CITREM in infant formula was corrected to 0.5 mg/kg according to the previous evaluation. Data on the sulfated ash levels and the content of minerals in neutralized CITREM products were provided. The limit for sulfated ash was maintained for non-neutralized CITREM, and new limits were set for partially neutralized and for wholly neutralized CITREM. The tentative status of the specifications was removed.

[c] The Committee received information on optical rotation, infrared identification, free tartaric acid content, degree of esterification and molecular weight distribution, together with the analytical methods. The Committee revised the specifications for free tartaric acid, optical rotation, molecular weight and molecular weight distribution and included a specification for polydispersity index. The tentative status of the specifications for metatartaric acid was removed.

[d] The Committee revised the specifications monograph and noted that a change in the name of the food additive from "Yeast extracts containing mannoproteins" to "Mannoproteins from yeast cell walls" was appropriate. The Committee noted that all mannoproteins, regardless of the range of molecular weights, were included in the same specifications monograph and therefore specifying a range of average molecular weight and a method for measuring it was not essential. Data were also received for metallic impurities. The Committee reviewed the information received and decided that only a limit for lead was required. The tentative status of the specifications was removed.

[e] A framework was adopted for developing specifications for steviol glycosides by four different methods of production. Specifications for steviol glycosides produced by different production methods were included as annexes, as below:
- Annex 1: Steviol Glycosides from *Stevia rebaudiana* Bertoni (**revised** from the specifications monograph for Steviol glycosides from *Stevia rebaudiana* Bertoni prepared at the eighty-fourth meeting of JECFA (INS 960a)).
- Annex 2: Steviol Glycosides from Fermentation (specifications for Rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica* (INS 960b(i)) prepared at the eighty-second meeting of JECFA were **revised** to include other steviol glycosides from *Saccharomyces cerevisiae* and *Yarrowia lipolytica*).
- Annex 3: Enzyme Modified Steviol Glycosides (**new** specifications).
- Annex 4: Enzyme Modified Glucosylated Steviol Glycosides (**new** specifications, **tentative** pending further information concerning the analytical methods).

Flavouring agents considered for specifications only

Flavouring agent	No.	Specifications
Methyl propionate	141	R[a]
Ethyl oleate	345	R[b]
alpha-Methyl-beta-hydroxypropyl alpha-methyl-beta-mercaptopropyl sulfide	547	R[c]
Vanillin	889	R[d]
Ethyl vanillin	893	R[e]
2,2,3-Trimethylcyclopent-3-en-1-yl acetaldehyde	967	R[f]
alpha- and beta-Cyclocitral (50:50 mixture)	979	R[g]

(continued)

Flavouring agent	No.	Specifications
Sodium 2-(4-methoxyphenoxy)propanoate	1029	R[h]
2,2,6-Trimethyl-6-vinyltetrahydropyran	1236	R[i]

R: existing specifications revised

[a] The Committee revised the specific gravity to 0.912–0.918.

[b] The Committee revised the assay minimum to not less than 75% ethyl oleate. Specifications for the secondary components were also established: ethyl linoleate (3.4–11.5%), ethyl palmitate (0.4–5.1%), ethyl stearate (0.5–2.5%), ethyl laurate (1–2%) and other fatty acid ethyl esters.

[c] The Committee revised the refractive index to 1.512–1.522, the specific gravity to 1.040–1.050 and the assay minimum to 95%.

[d] The Committee revised the melting point to 81–84 °C.

[e] The Committee revised the melting point to 76–79 °C.

[f] The Committee revised the assay minimum to 93%, with a secondary component of up to 2% of gamma-campholenic aldehyde.

[g] The Committee revised the specifications to include the Chemical Abstracts Service (CAS) numbers for alpha-cyclocitral (CAS No. 432-24-6) and for the mixture of alpha- and beta-cyclocitral (CAS No. 52844-21-0). The Flavis and Council of Europe (COE) numbers for alpha- and beta-cyclocitral were also included. The refractive index range was revised to 1.4986–1.4991.

[h] The Committee revised the CAS number (150436-68-3) and Flavis number (08.127) to reflect the salt form. The melting point was revised to 184–190 °C. Identifiers and synonyms associated with the free acid were removed.

[i] The Committee changed the minimum assay to 95%, the refractive index to 1.442–1.452 and the specific gravity to 0.863–0.873.

This volume contains monographs prepared at the eighty-seventh meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Rome, Italy, from 4 to 13 June 2019.

The toxicological and dietary exposure monographs in this volume summarize the safety and/or dietary exposure data on specific food additives: black carrot extract, Brilliant Black PN, carotenoids (provitamin A), gellan gum, potassium polyaspartate and rosemary extract.

This volume and others in the WHO Food Additives series contain information that is useful to those who produce and use food additives and veterinary drugs and those involved with controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.