

WHO FOOD ADDITIVES SERIES: 83

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

Safety evaluation of certain food additives



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World Health Organization, Geneva, 2022



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PREFACE

The monographs contained in this volume were prepared at the ninety-second 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 virtually on 7–18 June 2021. These monographs summarize the data on specific food additives reviewed by the Committee.

The ninetieth report of JECFA has been published by WHO as WHO Technical Report No. 1032. Reports and other documents resulting from previous meetings of JECFA are listed in [Annex 1](#), and the participants in the meeting are listed in [Annex 3](#). A summary of the conclusions of the Committee with respect to the food additives discussed at the meeting is given in [Annex 4](#).

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

The monographs contained in this volume are based on working papers that were prepared by WHO and FAO experts. An acknowledgement is given at the beginning of each monograph to those who prepared the working papers. The monographs were edited by E. Heseltine, Saint Léon-sur-Vézère, France.

The monographs are based on evaluations of original studies and the dossiers provided by the sponsor(s) of the compound, of the relevant published scientific literature and of data submitted by Codex members. When consistent with the data from the original study, the monographs may contain parts of the text and tables of the dossier submitted by the sponsor(s), but not the sponsor(s)' conclusions. The monographs and their conclusions are based on independent reviews of the available data and do not constitute endorsement of the sponsor(s)' position.

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 (jecfa@who.int).



**SAFETY EVALUATION OF SPECIFIC FOOD ADDITIVES
(OTHER THAN FLAVOURING AGENTS)**



Benzoic acid, its salts and derivatives (addendum)

First draft prepared by

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1. Explanation

The Committee first evaluated benzoic acid and its salt, sodium benzoate, at its sixth meeting ([Annex 1](#), reference 6). A group acceptable daily intake (ADI) of 0–5 mg/kg body weight (bw) for benzoic acid and sodium benzoate (expressed as benzoic acid) was established at that meeting. The group ADI was based on the absence of any observed adverse effects over four successive generations, two of which involved lifetime dietary exposure to benzoic acid at a maximal concentration of 1% (equivalent to 500 mg/kg bw per day). The potassium and calcium salts were subsequently included in the group ADI for benzoic acid at the ninth, seventeenth, twenty-seventh and forty-sixth meetings ([Annex 1](#), references 11, 32, 62 and 122).

The current request to re-evaluate benzoic acid and its salts was made by the Codex Committee on Food Additives at its Forty-ninth Session (1). The sponsor provided an extended one-generation study of reproductive toxicity (Organization for Economic Co-operation and Development [OECD] 443) and findings relative to the chemical-specific adjustment factor, default uncertainty factors and intake assessment assumptions for benzoate.

Dietary exposure to benzoic acids and its salts was evaluated by the Committee at its fifty-first and eightieth meetings ([Annex 1](#), references 137, 138, 223 and 224).

Benzoic acid and its salts are used as food preservatives, whereas derivatives such as benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate are used as flavouring agents. Benzyl alcohol and benzyl benzoate are used as carrier solvents in foods. The Committee has evaluated benzyl derivatives when used as flavouring agents, most recently at its fifty-seventh meeting ([Annex 1](#), references 154, 155). Benzyl acetate was evaluated at the eleventh, twenty-seventh, twenty-ninth, thirty-first, thirty-fifth, forty-first and forty-sixth meetings ([Annex 1](#), references 14, 62, 70, 77, 88, 107 and 122). Benzaldehyde and benzyl alcohol were evaluated at the eleventh, twenty-third and forty-sixth meetings ([Annex 1](#), references 14, 50 and 122).

As all these structurally related compounds are metabolized along common pathways to benzoate in both rodents and humans ([Fig. 1](#), below), the Committee at its forty-sixth meeting evaluated benzyl acetate, benzyl alcohol, benzaldehyde, benzoic acid and the benzoate salts (calcium, potassium and sodium) together and included them in a group ADI of 0–5 mg/kg bw when expressed as benzoic acid equivalents ([Annex 1](#), reference 122).

A comprehensive literature search from January 2002 up to March 2021 was performed in PubMed and TOXLINE with the following search strings: benzoic acid OR 65-85-0 AND short-term toxicity; benzoic acid OR 65-85-0 AND (reproductive OR developmental toxicity); benzoic acid OR 65-

85-0 AND genotoxicity; benzoic acid OR 65-85-0 AND (long-term toxicity OR carcinogenicity); benzoic acid OR 65-85-0 AND allergenicity; sodium benzoate OR 532-32-1 OR potassium benzoate OR 582-25-2 AND short-term toxicity; sodium benzoate OR 532-32-1 OR potassium benzoate OR 582-25-2 AND (reproductive OR developmental toxicity); sodium benzoate OR 532-32-1 OR potassium benzoate OR 582-25-2 AND genotoxicity; sodium benzoate OR 532-32-1 OR potassium benzoate OR 582-25-2 AND (long-term toxicity OR carcinogenicity); sodium benzoate OR 532-32-1 OR potassium benzoate OR 582-25-2 AND allergenicity. Forty-nine publications were considered relevant and further evaluated. The Committee decided to use relevant toxicity publications from the literature reviewed by the European Food Safety Authority (EFSA) in 2016 and those 20 studies published after 2016 that were considered relevant for the evaluation.

In this addendum, the term “benzoic acid, its salts and derivatives” refers to benzoic acid, the benzoate salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate.

1.1 Chemical and technical considerations

Benzoic acid ($C_7H_6O_2$; Chemical Abstracts Services [CAS] No. 65-85-1; International Numbering System [INS] 210) occurs naturally in organic tissues and can be generated in fermented products. As benzoic acid has antibacterial and antifungal activities, it has applications in food manufacture.

Benzoic acid is synthesized by liquid-phase oxidation of toluene with oxygen in the presence of a cobalt-containing catalyst (2). During oxidation, several by-products are formed, such as benzaldehyde, benzyl alcohol and benzyl benzoate; small amounts of benzyl formate, benzyl acetate, biphenyl and methyl biphenyls and phthalic acid may also be formed.

For food and pharmaceutical uses, benzoic acid is purified by further processing, including sublimation, recrystallization and neutralization. Treatment with amines and rinsing are required to remove phthalic acid. Benzoic acid has been used as a preservative or flavouring agent in food, cosmetic, hygiene and pharmaceutical products. To extend its application in foods, the following water-soluble salts have been produced by neutralization: sodium benzoate ($C_7H_5NaO_2$; CAS No. 532-32-1; INS 211), potassium benzoate ($C_7H_5KO_2$; CAS No. 582-25-2; INS 212) and calcium benzoate ($C_{14}H_{10}CaO_4$; CAS No. 2090-05-3; INS 213).

2. Biological data

2.1 Biochemical aspects

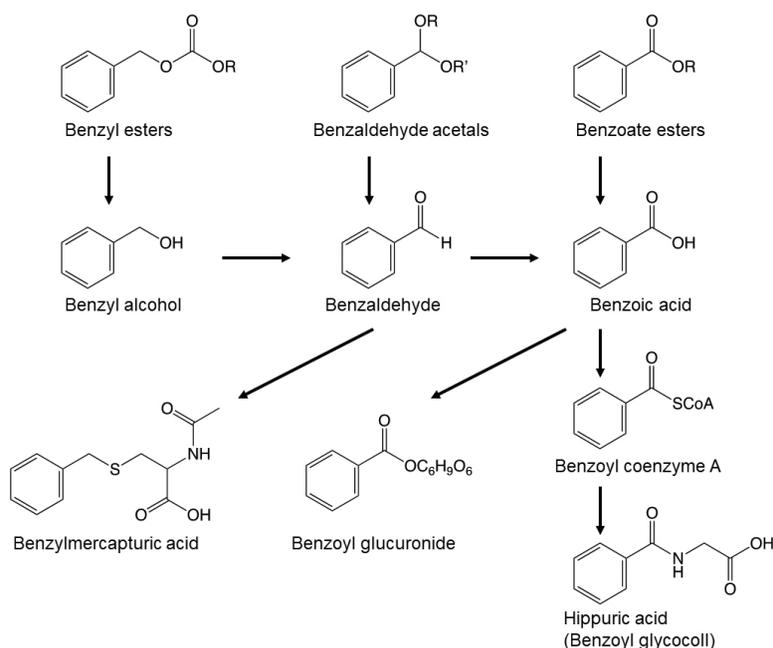
2.1.1 Absorption, distribution and excretion

The Committee noted previously that benzoic acid is rapidly absorbed, metabolized primarily in the liver and completely excreted in the urine (> 90%) as hippuric acid (major metabolite) and benzoyl glucuronide ([Annex 1](#), reference 32).

Conjugation with glycine and glucuronic acid occurs mainly in the liver and much more rapidly than oxidation of benzoic acid. In humans, rabbits and rats, benzoic acid is excreted almost entirely as hippuric acid, whereas dogs excrete more conjugated glucuronic acid than hippuric acid ([Annex 1](#), reference 32). Normal urinary excretion of hippuric acid in humans was estimated to be 1.0–1.25 g per day, equivalent to 0.7–1.7 g of benzoic acid ([Annex 1](#), reference 32). [Fig. 1](#) summarizes the metabolism of benzoic acid and benzyl derivatives ([Annex 1](#), reference 149, 150).

Fig. 1

Metabolism of benzyl derivatives



Toxicokinetics (TK) models to predict biologically relevant internal exposures of laboratory animal species and humans have recently been used to determine the distribution of benzoic acid (3,4). This approach is not commonly used for food additives because very few have any relevant human therapeutic applications, so the appropriate kinetic data are not available. Sodium benzoate, however, is used clinically to treat urea cycle disorders, as it increases waste nitrogen excretion (via glycine conjugation) and thereby improves clinical outcomes (5,6).

The purpose of TK models is either to reduce the uncertainty factor commonly used in extrapolating findings from laboratory animals to humans or simply to compare the margin of exposure (MOE) of the plasma concentrations. Two separate TK approaches for benzoic acid have been published. The first is a classical data-based compartmental model, described by Zu et al. (4), and the second involves development of a physiologically based pharmacokinetics (PBPK) or toxicokinetics model (3).

Zu et al. (4) used a classical model, in which the whole body is considered to be one compartment with first-order absorption and Michaelis–Menten (i.e., saturable) elimination via the urine. The characteristics of any compartment are hypothetical, in that they are chosen for the purpose of describing the data rather than based a priori on any physiological characteristics of the organism. As a result, classical models such as that used by Zu et al. are most useful for interpolation, i.e., within the range of doses, dose routes and species in which the data were generated. Zu et al. qualitatively concluded from published data (7–11) that the following parameters are essentially the same in humans and rats: time to maximum plasma concentration (T_{\max}), saturable conjugation with glycine to give hippuric acid, limited distribution to tissues after exposure to its salts or precursors and elimination in urine.

Zu et al. (4), following the IPCS guidance document for use of data in dose/concentration–response assessment (12), considered that the available data on the area under the plasma concentration–time curves (AUCs), clearance and maximum biotransformation rate (V_{\max}) were sufficient and suitable. They selected dose-normalized AUC values as the main dose metric because they argued that they provided a specific comparison of chronic and systemic exposure to benzoic acid in human and animal data in vivo. As there were no suitable TK data on oral exposure in rats, Zu et al. compared the AUC values after intravenous administration in humans and rats. For this calculation, they converted the intravenous doses (in mg/m²) into oral doses by dividing by a conversion factor of 37, which is based on a default body surface area for an adult weighing 60 kg (13). For consistency, they converted the administered dose of sodium benzoate into an internal benzoic acid equivalent dose from the ratio of their respective molecular weights. As shown in Table 1, the dose-normalized

Table 1

Pharmacokinetics of sodium benzoate in humans after intravenous administration

No. of people or animals	Benzoic acid equivalent dose (mg/kg bw)	Mean AUC (µg.h/mL)	Reference
Human			
6	22.9	20.3 (3.6)	0.9 (0.2)
6	45.8	114.9 (31.3)	2.5 (0.7)
6	91.6	562.8 (142.3)	6.1 (1.6)
17	85.9	564.6 (103.9)	6.6 (1.2)
3	126.0	1599.1 (463.1)	12.6 (3.6)
Rat			
5–8	24.4	10.4 (0.8)	0.4 (0.03)
5–8	61.1	58.2 (6.3)	1.0 (0.1)
5–8	122.3	239.5 (22.5)	2.0 (0.2)
5–8	244.2	1100.2 (89.2)	4.5 (0.4)

AUC increases with dose, suggesting that the clearance of circulating benzoic acid is non-linear. In contrast, the AUC for the main metabolite, hippuric acid, increased proportionately with dose. Because of saturation of this phase-II conjugation pathway with glycine, it was considered to be the most likely cause of any toxicity of benzoic acid at high levels of exposure.

To confirm the difference in the rate of catalysis, the authors compared published values for the hepatic V_{\max} of benzoic acid–glycine conjugation in rat and human liver. A comparison of the V_{\max} ratio shows a relatively consistent ratio of about 0.4, indicating that humans have approximately a 2.5-times greater metabolic capacity at doses that span metabolic saturation (6–200 µM).

Zu et al. (4) compared dose-normalized AUCs at the most comparable doses of benzoic acid in the studies in humans and in rats (8) to calculate an interspecies TK or PK factor. These ranged from 2.1 at the lowest dose (23 mg/kg bw and 24 mg/kg bw, respectively) to 6.3 at the highest dose (126 mg/kg bw and 122 mg/kg bw, respectively). Owing to the large inter-individual variation in the AUC for people at the highest dose, Zu et al. considered that the high-dose comparison was not reliable. Hence, they compared AUCs at dose levels that are most closely comparable to the ADI with the levels experienced by the general population (here, the lowest dose groups) and selected a chemical-specific adjustment factor of 2 for interspecies differences in TK. On this basis, a total uncertainty factor of 50 is proposed, which is based on the usual 10-fold for intraspecies variation with a reduction of twofold for interspecies variation.

The PBPK model described by Hoffman and Hanneman (3) differs from the classical compartmental model in that it is composed of compartments with

realistic tissue volumes that are linked by blood flow. Other parameters used in the model account for chemical-specific characteristics that are measured independently in both humans and laboratory animals. The chemical-specific parameters may include tissue solubility (i.e., partition coefficients), binding and metabolism. This model has a potential advantage over the classical model in that it can be used for extrapolation (i.e., across dose ranges, among animal species and between routes of exposure).

For their PBPK analysis of benzoic acid, Hoffman and Hanneman (3) selected a seven-compartment model (i.e., blood, liver, brain, adipose, testes/ovaries, rapidly and poorly perfused tissues) that had previously been applied to the herbicide atrazine (14) and the nerve agent, soman (15). For the precursors of benzoic acid, namely benzyl acetate, benzyl alcohol and benzaldehyde, a three-compartment model (i.e., blood, liver and remaining body) was also found to be optimal for determining plasma concentrations of benzoic acid. “Lumping” or grouping tissues that show similar kinetics to form fewer compartments is a commonly used approach to reduce the dimensionality and complexity of whole-body PBPK models (16). Hoffman and Hanneman (3) did not discuss whether lumping would yield an equally good fit to the data for benzoic acid as for its precursors, although this would appear to be important for increasing confidence in the reliability of the PBPK output, especially as Kubota and Ishizaki (7) were able confidently to assign the kinetics of benzoic acid in humans after oral administration to a simple one-compartment model with first-order rate absorption and Michaelis–Menten elimination, suggesting that there is very little distribution to tissues.

From a comparison of benzoic acid concentrations in rat and human plasma (Table 2), the authors concluded that the rat–human PK (or TK) factor for benzoic acid that reflects the AUC at steady state can be calculated as 0.3–0.4. This ratio could be used instead of the conventional interspecies PK factor of 4 to derive a health-based guidance value.

Under the steady-state condition considered in this PBPK modelling study, the interspecies TK factor is essentially determined by the values of hepatic clearance in rats and humans. The results of the PBPK modelling indicate high clearance in humans (extraction ratio, 0.86) but poor clearance in rats (extraction ratio, 0.37). The low hepatic clearance in rats (i.e., Michaelian constant for conversion of benzoic acid to hippuric acid) estimated in this modelling study may be an underestimate, as it is based only on a single intravenous dose of 122 mg/kg bw in rats (17). In contrast, the metabolism of benzoic acid in humans is determined mainly by the hepatic blood flow. The clearance of rapidly metabolized chemicals in both rats and humans is approximately equal to liver blood flow, and the interspecies TK factor is close to 4, the default value. In order to replace this default value, the Committee considers that confidence in the

Table 2

Steady-state plasma concentrations of benzoic acid and hippuric acid after simulated exposure to benzoic acid

Repeated dietary exposure to benzoic acid (mg/kg bw per day)	Benzoic acid concentration in rat plasma (ng/mL)	Benzoic acid concentration in human plasma (ng/mL)	Plasma concentration ratio (human/rat)
1	25.7	8.4	0.3
5	128.8	42.6	0.3
10	259.1	86.6	0.3
50	1338	486.2	0.4
100	2800.2	1220.9	0.4

estimated metabolic clearance (V_{\max} , K_m) in rats and humans is fundamental and should be confirmed at several other doses.

2.1.2 Effects on enzymes and other biochemical parameters

No additional information has become available since the previous evaluation by the Committee.

As described by JECFA 1973 ([Annex 1](#), reference 32), benzoic acid inhibits pepsin digestion, and sodium benzoate inhibits trypsin digestion of fibrin, but they have no effect on amylase or lipase activity. Benzoic acid is a potent specific inhibitor of D-amino acid oxidase.

2.2 Toxicological studies

2.2.1 Acute toxicity

No additional information has become available since the previous evaluation by the Committee.

The acutely toxic dose (LD_{50}) of benzoic acid in mice ranged from 200 to 1200 mg/kg bw ([Annex 1](#), reference 117). The LD_{50} of sodium benzoate was reported to be 2700 mg/kg bw in rats and 2000 mg/kg bw in rabbits and dogs ([Annex 1](#), reference 32).

The effects of sodium benzoate in zebrafish larvae were studied in the fish embryo acute toxicity test (18). The Committee considered that this test does not have predictive relevance for humans.

2.2.2 Short-term studies of toxicity

Male albino Wistar rats (n = 4 per group) were fed increasing amounts of sodium benzoate either alone or in combination with ascorbic acid to study effects on haematological parameters (19). Treatment with sodium benzoate

alone consisted of 1.0 or 10 mg/kg bw per day for 21 non-consecutive days (not stated how many days required to achieve 21 treatment days). No statistically significant changes were reported in animals at the lower dose, while the higher dose induced an anaemic condition. The authors also reported high white blood cell counts among animals fed the basal diet alone, suggesting biased statistical significance for this parameter. It was not clear whether the high white blood cell counts indicated an infection in control animals, which would have affected other parameters measured in this study.

Short-term studies on sodium benzoate evaluated previously by the Committee showed increased mortality in mice given 3 g/day and in rats at a dose of 5% in the diet ([Annex 1](#), reference 32). Other reported effects in rats were body-weight loss, reduction of phospholipids in the liver and of the potassium concentration in skeletal muscle, loss of coordination, tremor and convulsions. Addition of glycine to the diet reduced the toxic effects. In guinea-pigs, doses of benzoate plus benzoic acid of 150 mg/kg bw (not clear whether this was the only or the highest dose tested) given for 65 days had no adverse effects.

2.2.3 Long-term studies of toxicity and carcinogenicity

A mixture of 13 chemicals including sodium benzoate was tested in 40 Sprague Dawley (CD-SD) rats (20 males and 20 females) divided into four groups of 10, which were exposed for 18 months. The route of exposure (diet or drinking-water) was not reported (20). The mixture comprised carbaryl, dimethoate, glyphosate, methomyl, methyl parathion, triadimefon, calcium disodium ethylene diamine tetraacetate, ethylparaben, butylparaben, bisphenol A, aspartame, acacia gum and sodium benzoate. Doses were set to decreasing amounts of the no-observed adverse effect levels (NOAELs) identified by the authors from published risk assessments of the individual substances. There was no control group of animals not treated with the mixture. The Committee considers that the results of this study are not applicable to the evaluation of sodium benzoate as a food additive.

At its seventieth meeting ([Annex 1](#), reference 193), the Committee reported a study on 20 male and 30 female rats fed a diet containing 1.5% benzoic acid for 18 months; 13 male and 12 female rats served as controls (21). Fifteen animals in the test group and three in the control group died. The test animals showed reduced body weight and food intake. Further experiments on groups of 20 test animals and 10 controls of another strain provided similar findings.

At its fifty-seventh meeting ([Annex 1](#), reference 154), the Committee reviewed the studies evaluated in the previous monographs and an additional study, in which benzaldehyde was administered in corn oil by gavage to rats at 200 or 400 mg/kg bw per day for 103 weeks and to mice at 200 or 400 mg/kg bw per day (males) or 300 or 600 mg/kg bw per day (females) for 103 weeks. On the

basis of these studies, the Committee concluded that neither benzyl acetate nor benzyl alcohol is carcinogenic. As in the studies in mice and rats given benzyl acetate in corn oil by gavage, increased incidences of pancreatic acinar cell adenomas in rats and of papillomas of the forestomach in mice were noted after administration of benzaldehyde. The Committee concluded, however, that the results of studies in which the compound was administered in the diet were more relevant to its safety assessment as a food additive than those in which it was given in corn oil by gavage. In its present evaluation, the Committee concluded that previously reviewed long-term studies in mice and rats on benzyl derivatives, benzyl alcohol, benzaldehyde, benzyl acetate, benzoic acid and sodium benzoate did not indicate carcinogenic potential.

2.2.4 Genotoxicity

Sodium benzoate in combination with sunset yellow (CAS No. 2783-94-0) was tested *in vivo* in female albino rats to assess effects on chromosomal aberration in bone marrow cells and in the comet assay in liver cells (22). An unidentified test on DNA fragmentation of apoptotic liver cells was also performed, in which concentrations of the sunset yellow and sodium benzoate combination were administered in drinking-water to six animals per group for 12 weeks. The choice to test only female animals, the doses chosen and the treatment schedule are not explained in the publication. Only two animals were tested for chromosomal aberrations; it is not clear how many animals were tested in the comet assay. Cytotoxicity was not assessed for either test, and the analyses of chromosomal damage did not follow any internationally recognized procedure. A combination of chromosomal aberrations and chromatid aberrations was recorded. The authors reported positive findings for both targets; however, this is not compatible with a genotoxic chemical agent that should induce only chromatid aberrations. The result could be due to the limited quality of the cytogenetic preparations, as indicated by the illustrations in the publication. The Committee considered that this study was not suitable for evaluation of the genotoxicity of sodium benzoate. In addition to the limitations of the experimental design and procedure, sodium benzoate was administered always in combination with sunset yellow in drinking-water.

In 2016, EFSA (23) concluded that positive or equivocal results for the clastogenicity *in vitro* of benzoic acid and its sodium and potassium salts (24–26) had not been reproduced in well-performed, relevant *in vivo* studies in the alkaline comet assay (27), the rodent bone marrow chromosomal aberration assay or the dominant lethal assay in rats (unpublished reports).

The results of studies of genotoxicity *in vitro* and *in vivo* on benzyl derivatives, including benzoic acid, were reviewed by the Committee at its fifty-

seventh meeting ([Annex 1](#), reference 122). During that meeting, the Committee concluded that, in view of the mainly negative results in assays *in vitro* and the uniformly negative results in well-recognized assays *in vivo*, the group of benzyl derivatives, including benzoic acid, is not genotoxic *in vivo*.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

An extended one-generation study of reproductive toxicity with benzoic acid (CAS No. 65-85-0, purity > 99.9%) in CrI:CD (Sprague–Dawley) rats, conducted according to OECD 443 EOGRT guideline and according to good laboratory practice (GLP), was available to the Committee for this evaluation (28,29). The study included additional assessments of reproductive toxicity in the F1 generation, and the F2 offspring were followed up until postnatal day (PND) 91.

Four F0 groups of rats ($n = 30/\text{sex}$ per group) were given control basal diet supplemented with 0, 7500, 11 500 or 15 000 ppm benzoic acid for 14 consecutive days before mating, continuing through to the day of euthanasia or until initiation of fasting before scheduled necropsy. The target dose levels of benzoic acid were 0, 500, 750 and 1000 mg/kg bw per day. The mean calculated consumption of benzoic acid from the recorded body weights and food consumption for each animal were reported at relevant life stages for the F0, F1 and F2 generations ([Table 3](#)). Male rats were given benzoic acid at a constant concentration throughout the study, and female rats were given benzoic acid at a constant concentration in the diet during pre-mating and mating periods, while dosing was adjusted during gestation and lactation according to past body weight and food consumption to compensate for the higher caloric demand during these periods.

The F1 and F2 generations were exposed to benzoic acid *in utero* during gestation, in maternal milk during the pre-weaning period and by direct exposure in the diet from weaning until euthanasia. The dietary concentrations of benzoic acid for the F1 and F2 generations were adjusted from PND 21 through PND 70 and for F1 females in cohort 1B during gestation and lactation according to past control food consumption and body weight data for age-matched animals to maintain constant target doses of 0, 500, 750 and 1000 mg/kg bw per day.

F1 animals were divided into three cohorts after weaning to evaluate reproductive and developmental toxicity (cohort 1, A and B), developmental neurotoxicity (cohort 2, A and B) and immunotoxicity (cohort 3 and 3A). Cohort 1A animals were evaluated at PND 91 (including oestrous cycles and sperm evaluations). Cohort 1B animals were maintained for assessment of reproductive performance and to generate F2 offspring. Cohort 2B was evaluated for brain morphology on PND 22; cohort 2A was maintained until PND 78 to test neurobehavioural effects and adult neuropathology. Cohort 3A served

Table 3
Mean calculated benzoic acid consumption (mg/kg bw per day) at relevant life stages (F0, F1 and F2 generations)

Dietary concentration (ppm)	Target dose (mg/kg bw per day)	Males		Females		
		Before mating	After mating	Before mating	During gestation	During lactation
F0 generation						
7500	500	526	389	526	484	512
11 500	750	821	601	821	721	767
15 000	1000	1069	807	1069	965	1020
F1 generation						
		PND 21–98 (post-weaning, before mating)	After mating	PND 21–98 (post-weaning, before mating)	During gestation	During lactation
7500	500	499	349	535	499	512
11 500	750	747	542	790	712	771
15 000	1000	987	689	1024	957	1020
F2 generation						
		PND 21–91 (post-weaning)		PND 21–91 (post-weaning)		
750	500	491		504		
11 500	750	729		737		
15 000	1000	969		988		

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as a positive control group for the sheep red blood cell (sRBC) immunization challenge beginning on PND 54 and for comparison with cohort 3 (Table 4).

The parameters evaluated in this study were clinical signs, body weight, body weight gain, food consumption, oestrous cycles, reproductive performance, parturition, litter viability and survival, pre- and post-weaning developmental landmarks (e.g., anogenital distance, areolae/nipple anlage and retention vaginal, potency and balanopreputial separation), neurobehaviour, thyroid hormones, clinical pathology, gross necropsy, sperm parameters, immunophenotyping and T-cell-dependent antibody response assay, organ weights, histopathology, brain measurements, neuropathological and morphometric examinations.

No treatment-related effects were reported on F0 survival rates at any dose tested; however, one female in the control group was found dead on lactation day 0, which the authors concluded was related to parturition. Clinical observations, mean body weights, body weight gain, food consumption and food efficiency were not affected. There were no treatment-related effects on reproductive performance (males or females), mean days of gestation, processes of parturition, mean number of implantation sites or on ovarian follicle counts, oestrous cycles

Table 4
Offspring allocation by cohort

Cohort	No. selected	Evaluation
1A	1 pup/sex per litter per group (up to 20/sex per group)	Primary assessment of reproductive and developmental toxicity
1B	1 pup/sex per litter per group (up to 25/sex per group)	Follow-up reproductive assessment
2A	1 pup/litter per group (up to 12/sex per group from as many litters as possible)	Neurobehavioural testing (startle response, motor activity, functional observational battery and learning and memory) and neuropathology on PND 78
2B	1 pup/litter per group (up to 12/sex per group, from as many litters as possible)	Neuropathology on PND 22
3	1 pup/litter per group (up to 10/sex per group, from as many litters as possible)	Primary antibody response in sRBC challenge beginning on PND 54
3A	1 pup/litter per group (up to 10/sex in the control group, from as many litters as possible)	Positive control group for the sRBC challenge beginning on PND 54

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or spermatogenic parameters at any dose tested. Clinical pathology, levels of thyroxine and thyroid-stimulating hormone, organ weight changes or gross macroscopic or microscopic findings were not related to treatment.

F1-generation animals showed no treatment-related effects on the number of pups born, live litter size, percentage of males at birth, postnatal survival, clinical observations, preweaning body weights, necropsy findings, anogenital distance or developmental landmarks. Macroscopic findings in pups found dead or examined at scheduled necropsy (PND 21) were reported not to be related to benzoic acid treatment. Overall body weights and body weight gains were not affected by treatment in cohorts 2A and 2B. Lower final body weights were reported in males at 15 000 ppm in cohort 2B group (48 g) at PND 22, but the weights were within the historical laboratory control range for Sprague-Dawley rats at PND21 (37.6–59.8 g, mean 48.7 g). Reproductive performance (to generate F2 pups), oestrous cyclicity and spermatogenic parameters were not affected at any dose. The other evaluations of each of the cohorts (males or females), as listed in Table 4, were not affected by benzoic acid treatment. Cohorts 3 and 3A, designed to determine immunotoxicity, showed no significant overall changes in anti-sRBC-immunoglobulin M levels as compared with controls. Statistically significant changes were observed in female rats at 15 000 ppm benzoic acid, but the authors considered that they were not related to treatment, as they were observed in only two female animals that were very high responders and not in males. They concluded that dietary benzoic acid exposure had no adverse effect on the humoral immune system.

F2-generation animals showed no benzoic acid-related effects on reproductive parameters. The mean number of pups born, live litter size, percentage of males per litter at birth and postnatal survival between birth and PND 0 and up to PND 21 were not affected by treatment. Any differences observed were not statistically significant, nor were they dose-related. Offspring body weights were unaffected overall. Most animals survived to scheduled necropsy. Animals that died or were euthanized in extremis showed no treatment-related internal findings at necropsy. No statistically significant clinical chemistry changes, macroscopic findings or weights of pup organs were observed at any dose.

At its seventeenth meeting ([Annex 1](#), reference 32), the Committee reviewed a four-generation reproductive toxicity study in rats. Three groups of 20 male and 20 female rats were pair-fed for 8 weeks on diets containing 0%, 0.5% or 1% benzoic acid and thereafter fed ad libitum over four generations. Two generations were fed for their entire lifespan, and the third and fourth generations were autopsied after 16 weeks. No adverse effects were observed on growth, fertility, lactation or lifespan. Post-mortem examination showed no abnormalities. In another experiment, 20 male and 30 female rats were fed a diet containing 1.5% benzoic acid for 18 months, with 13 male and 12 female rats as controls. Fifteen animals in the test group and three in the control group died. The test animals showed reduced body weight and food intake. Repeated experiments on groups of 20 test animals and 10 controls of another strain gave similar findings. At its seventeenth meeting, the Committee concluded that the NOAEL in rats was 10 000 ppm (1%) in the diet, equivalent to 500 mg/kg bw per day.

(b) Developmental toxicity

The effects of sodium benzoate on neural tube development were studied in chicken embryos (30). Groups of fertile, specific pathogen-free eggs with 12 embryos were incubated until stage 9 of development according to the Hamburger–Hamilton series (31) in the presence of increasing concentrations of sodium benzoate. The Committee considered that this study has no predictive relevance for humans in the context of evaluating sodium benzoate as a food additive.

2.2.6 Special studies

The effects of sodium benzoate on HCT-116 colon cancer cell viability due to NFκB modulation were studied (32). HCT-116 cells were incubated with 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100 or 200 mM sodium benzoate, and cell viability was evaluated in the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Sodium benzoate significantly inhibited cell viability at concentrations ≥ 6.25 mM. The viability of the non-tumorigenic L929 fibroblast

cell line was not affected at 6.25 mM sodium benzoate, but higher concentrations (12.5–50 mM) were cytotoxic. Loss of cell viability in HCT-116 cells was mediated by apoptosis, as shown by annexin V-PE staining and flow cytometry. Sodium benzoate increased nuclear NFkB-p65 and Bim protein levels and NFkB activation of HCT-116 colon cancer cells at cytotoxic concentrations (6.25–200 mM).

A screening method based on gene expression (CARCINOscreen®) was used to predict hepatic carcinogenicity of sodium benzoate and 2,6-diaminotoluene in a 28-day repeated dose study in which Crl:CD(SD) and Crl:WI(HAN) male rats (n = 3/group) were fed 100 mg/kg bw sodium benzoate per day by gavage (33). Effects on liver tissue were assessed by microarray analysis of total extracted RNA. Predictive scores obtained in Sprague-Dawley and Wistar rats indicated that sodium benzoate is not carcinogenic in either strain. The predictive results were confirmed by negative histopathology in the livers of both strains of rats.

2.2.7 Special studies in humans

Sodium benzoate has been used to treat patients with inborn errors of urea cycle enzymes that result in hyperammonaemia. Long-term therapeutic doses of 250–500 mg/kg bw per day have been used to facilitate an alternative pathway for nitrogen excretion in the form of glycine (7,9,34). At these doses, clinical signs are rare and, in most cases, limited to anorexia and vomiting, especially after intravenous bolus infusions.

A randomized double-blind, placebo-controlled, 6-week trial was conducted to test the effects of sodium benzoate on patients with behavioural and psychological symptoms of dementia (35). Forty-nine patients (30 women and 19 men) were randomized to 6 weeks of treatment with 250–1500 mg sodium benzoate per day, and 48 (32 women and 16 men) were randomized to placebo. The primary outcomes were scored on the Alzheimer disease assessment scale–cognitive subscale and behavioural pathology on the Alzheimer disease rating scale. Laboratory measurements were also performed, on estradiol and follicle-stimulating hormone activity in plasma and serum, respectively. No statistically significant changes in follicle-stimulating hormone or estradiol levels or on estradiol to hormone ratios were reported between treated and placebo groups, either among women and men or among men grouped separately. No adverse effects were reported.

(a) Allergenicity

In patch-testing of 41 patients with a diagnosis of allergic contact cheilitis, benzoic acid gave 12% positive results and allergic-relevant reactions (36). The aim of the study was to determine the prevalence of allergic contact cheilitis in patients with

non-actinic cheilitis and to identify the most relevant allergens. Patients without actinic cheilitis ($n = 91$) identified from an institutional database underwent patch testing of a series of antigens determined by a dermatologist. Benzoic acid was classified as one of the most relevant preservative-derived allergens in the study.

The American Contact Dermatitis Society has added benzoic acid to its core allergen series (37), which gives dermatologists who are using TRUE test standard allergens as their baseline series a logical, graded tool to increase the number of allergens tested.

In 2016, EFSA (23) reviewed the available data on allergenicity, hypersensitivity and intolerance for sodium benzoate (Table 5).

In its previous evaluations, the Committee noted reports of allergic responses to sodium benzoate. The Committee concluded that the human data available indicate that benzoic acid and sodium benzoate can trigger allergic reactions in some individuals when consumed in foodstuffs.

(b) Immune responses

The immunotoxicity of sodium benzoate was studied *in vitro* by monitoring changes in the expression of cytokines, cell surface receptors and cell cycle distribution on cultured T-cells and B-cells (43). Cultured splenocytes isolated from inbred strains of female Balb/c or Swiss mice (8–10 weeks old) were exposed *in vitro* to 0, 100, 500, 1000 or 2500 $\mu\text{g/mL}$ sodium benzoate for 72 h. Cytotoxicity was determined by the MTT assay. Lymphocyte proliferation was determined by tritiated thymidine uptake after treatment with concanavalin A or lipopolysaccharides. The cell cycle phase distribution of sodium benzoate-treated cells was monitored by staining with propidium iodide solution and analysis by flow cytometry. Mixed lymphocyte response was monitored by fold proliferation of cells determined by the relative increase in uptake of tritiated thymidine in responder cells. Immunophenotyping was conducted by flow cytometry on labelled cells with specific B or T cell surface antibodies. Samples were collected from concanavalin A- or lipopolysaccharides-stimulated splenocytes for estimation of TH1/TH2/TH17 cytokines. Significant ($P < 0.05$) cytotoxicity was observed in cultured splenocytes only at 2500 $\mu\text{g/mL}$, the highest concentration tested. Lymphocyte proliferation assays showed lower proliferative responses with concanavalin A or lipopolysaccharide stimulation in the presence of 1000 $\mu\text{g/mL}$ than in controls and at 100 $\mu\text{g/mL}$ sodium benzoate without concanavalin A or lipopolysaccharide stimulation. The mixed lymphocyte response of Balb/c splenocytes against allogenic antigens was decreased (47%) with treatment at 100 $\mu\text{g/mL}$. The authors stated that sodium benzoate treatment (not clear which doses were tested) did not induce significant cell cycle arrest at G1 phase

Table 5
Human data on allergenicity to sodium benzoate

Available information	Reported response	Diagnosis	Follow-up	Reference
One female patient aged 19 years Ingestion of sodium benzoate in foodstuffs	Anaphylactic reaction	Challenge with 20 mg sodium benzoate induced itching on arms and generalized itching	Adherence to benzoate-free diet prevented recurrence of symptoms	38
Four of nine patients Oral provocation tests	Atopic dermatitis Increased leukotriene production	Benzoate induced 10 times more mean leukotriene production in basophils than in patients negative to oral provocation test		39
One of 47 subjects	Repeated episodes of acute urticarial angio-oedema	Ingestion of 75 mg sodium benzoate induced positive reaction to IgE test for food allergens		40
20 of 226 patients aged 12–60 years (mean, 40.2 years) Double-blind placebo-controlled study	Sneezing, rhinorrhoea, nasal blockage, nasal itching (symptoms of rhinitis)	Sodium benzoate can be considered a trigger or aggravating factor		41
One female patient aged 75 years 6-year history of diffuse pruritus	Results confirmed by a second series of two double-blind placebo-controlled challenges with sodium benzoate	Introduction of 100 mg sodium benzoate induced pruritus within 24 h	Adherence to a benzoate-free diet prevented recurrence of symptoms	42

Source: reference 23

($P > 0.05$) as compared with controls. The relative percentages of S phase (24% less) and G2/M phase (22% less) cells were significantly lower than in the untreated control. Exposure to sodium benzoate (not clear which doses were tested) did not affect the relative expression of CD3 and CD4 on T cells or the relative expression of CD86 receptors on B cells, although expression of other surface markers was reduced. Cytokine analysis showed reduced production of IL-4, IL-16, IFN- γ and IL-17 upon treatment with concanavalin A (T-cell stimulator) in the presence of sodium benzoate, whereas treatment with lipopolysaccharides (B-cell stimulator) decreased IL-6, IF- γ and TNF- α in the presence of sodium benzoate. It is not clear which doses of sodium benzoate were tested for either of these results. The authors suggested that sodium benzoate suppresses the functional responses of T-cell and B-cell lymphocytes.

The Committee noted that a one-generation study of toxicity in Sprague-Dawley rats exposed to benzoic acid at up to 1000 mg/kg bw per day did not show significant changes in immunophenotyping or on the T-cell-dependent antibody response assay (see Multigeneration reproductive toxicity).

3. Dietary exposure

The Committee considered studies of dietary exposure to benzoic acid (INS 210) and its sodium (INS 211), potassium (INS 212) and calcium (INS 213) salts. Benzoic acid and its salts are endorsed for use in 59 food categories at maximum permitted levels (MPLs) ranging from 200 mg/kg up to 5000 mg/kg, as specified in the Codex General Standard for Food Additives (GSFA). The MPLs are all expressed as benzoic acid.

Dietary exposure to benzoic acids and its salts was evaluated by the Committee at its fifty-first and eightieth meetings ([Annex 1](#), references 137 and 138). At its fifty-first meeting, in 1998, the Committee considered national dietary exposure estimates of benzoic acid salts (benzoates) from all food categories based on maximum limits specified in national food standards and by the European Union; only Japan's dietary exposure estimates were based on analytical concentrations of benzoates in foods. Estimates of national mean dietary exposure ranged from 0.18 mg/kg bw per day in Japan to 2.3 mg/kg bw per day in the USA for the total population. High exposure estimates were 7.3 mg/kg bw per day in the USA and 14 mg/kg bw per day in China. Important contributors to dietary exposure to benzoates in the majority of the national estimates were carbonated water-based flavoured drinks. Soya sauce was the main contributor in China and the second major contributor in Japan.

At its eightieth meeting, in 2015, the Committee estimated dietary exposure to benzoates from consumption of non-alcoholic beverages ([Annex 1](#), references 223 and 224). The Committee combined consumption data from the FAO/WHO Chronic Individual Food Consumption Data – Summary statistics (CIFOCos) database from 25 countries with average typical reported use levels of benzoates in water-based flavoured drinks from various countries, which ranged from 83 to 209 mg/L. The mean dietary exposure of consumers of non-alcoholic beverages ranged from 0.1 mg/kg bw per day in the very elderly in France (based on 83 mg/L) to 4.1 mg/kg bw per day in children aged 1–5 years in South Africa (based on 209 mg/L). High exposure estimates ranged from 0.2 (95th percentile) mg/kg bw per day in the very elderly in Denmark (based on 83 mg/L) to 10.9 (97.5th percentile) mg/kg bw per day in the same children in South Africa (based on 209 mg/L). The Committee also conducted a review of the literature published since 2000 on dietary exposure to benzoates from all foods and beverages. Mean and high (95th percentile) dietary exposure to benzoates in the general population ranged from 0.01 (mean) mg/kg bw per day in the Republic of Korea to 3.1 (high) mg/kg bw per day in China. From this literature review, the Committee concluded that, in most countries, water-based flavoured drinks contributed most to dietary exposure to benzoates.

At the eightieth meeting, the sponsor provided estimates of dietary exposure to benzoates from non-alcoholic beverages for four countries (Brazil, Mexico, South Africa and the USA). These estimates were not considered at that meeting because they were based on maximum reported use levels or national maximum permitted levels. For the current meeting, the sponsor provided new estimates of dietary exposure to benzoates from water-based flavoured drinks for Brazil, Canada, Mexico and the USA (44,45) based on maximum use levels and market-volume weighted average use levels.

The Committee did not use the CIFOcOss database and the use levels for water-based flavoured drinks provided by the sponsor (45) to estimate dietary exposure to benzoates at its current meeting. The sponsor provided maximum use levels and market volume-weighted average use levels determined by weighting brand-specific reported use levels for a given beverage type according to the brand's corresponding market volume share. Maximum reported use levels were not used, as they are considered to produce highly conservative estimates of dietary exposure to benzoates for the general population based on mean and high (e.g., 95th percentile) consumption levels per age group and country from the CIFOcOss database. The market volume-weighted average use levels were not considered appropriate as they do not account for the fact that people may be loyal to a certain food, such as a water-based flavoured drink. Use of these levels would thus underestimate the dietary exposure of such consumers, for whom it is important to determine the level of risk. The Committee also noted that the market volume-weighted average use levels – i.e., 39 to 197 mg/kg – overlapped with the average typical levels used by the Committee at its eightieth meeting (i.e., 83–209 mg/kg) to estimate dietary exposure based on the CIFOcOss database.

3.1 Estimated dietary exposure

At its current meeting, the Committee evaluated estimates of dietary exposure to benzoates from water-based flavoured drinks for Brazil, Canada, Mexico and the USA provided by the sponsor (44,45). At its eightieth meeting, the Committee reviewed publications on dietary exposure to benzoates from all foods from 2000–2015. Therefore, the Committee performed a literature search from January 2015 to March 2021 in PubMed, which resulted in four publications that were considered relevant for evaluation. These publications comprised dietary exposure estimates for Europe (23), India (46) and the Islamic Republic of Iran (47,48). Dietary exposure estimates reported for a salt of benzoic acid were converted to exposure estimates for benzoic acid on the basis of molecular weight.

3.1.1 Estimates of dietary exposure provided by the sponsor

The use levels provided by the sponsor are suitable for calculating dietary exposure to benzoic acid according to a “brand-loyal” exposure scenario based on individual food consumption data. In such a scenario, a maximum reported use level is mapped to the amount of the food consumed that contributes most to dietary exposure to an additive (i.e., the “brand-loyal” food), and a typical use level is used for other foods that may contain the food additive. An average use level weighted according to the brand’s market volume can be used when “brand loyalty” is already addressed.

The sponsor provided estimates of dietary exposure to benzoates (as benzoic acid) from water-based flavoured drinks in a “brand-loyal” scenario for Brazil and Mexico (45) and for Canada and the USA (44). Dietary exposure was estimated by combining individual food consumption data from those countries with maximum use and market volume-weighted average use levels for different types of water-based flavoured drinks per country (Table 6). Dietary exposure was estimated for the total population, irrespective of whether individuals reported consumption of water-based flavoured drinks that could contain benzoic acid on at least one the total number of recording days in the dietary survey period, which was 2 days for Brazil, Canada and the USA and 7 days for Mexico. Dietary exposure was also calculated for the group of individuals who did report consumption of these drinks, the so-called “consumers-only” group. To estimate the “brand-loyal” dietary exposure of these two population groups, a maximum reported use level of up to 438 mg/kg was mapped to consumption amounts of regular carbonated soft drinks with a pH > 3.5 and the Codex interim maximum permitted level of 250 mg/kg to those with a pH ≤ 3.5. Drinks with a lower pH (more acidic) require less preservatives to achieve the desired technological function. A market volume-weighted average use level was used for the other water-based flavoured drinks (Table 6). Regular carbonated soft drinks were selected as the “brand-loyal” food because this beverage type contributed most to the total mean daily exposure.

Dietary exposure to benzoates was calculated for children from age 1 year up to adults by multiplying the mean consumption amount of the different types of water-based flavoured drinks per individual across the survey days by either the maximum reported use level or the Codex interim maximum use level for regular carbonated soft drinks and the market volume-weighted average use level for the other beverage types. The resulting dietary exposure estimates for each beverage type were summed and adjusted for body weight to obtain the individual daily dietary exposure expressed per kilogram body weight. The mean and 95th percentile were calculated from the resulting distribution of individual daily exposures. Dietary exposure estimates for the total population and consumers

Table 6

Average and maximum use levels of benzoates (as benzoic acid) in water-based flavoured drinks in four countries

Type of drink	Use levels (mg/kg)							
	Brazil		Canada		Mexico		USA	
	Average ^a	Maximum	Average ^a	Maximum	Average ^a	Maximum	Average ^a	Maximum
Low- and no-calorie carbonated soft drinks	186	297	174	690 ^c	169	299	197	400 ^c
Regular carbonated soft drinks	104	297	79	438 ^c	37	299	115	428 ^c
Flavoured water drinks	0	0	0	0	172	205	4	197
Fruit juice-based drinks ^b	76	339	0	0	89	247	0	0
Energy drinks	144	366	109	429	102	300	111	431
Sports drinks	39	263	0	0	0	0	0	0
Ready-to-drink tea	66	271	56	91	74	197	67	196
Ready-to-drink coffee	0	0	0	0	0	0	0	0

Source: reference 45

^a Market volume-weighted average use level^b Including concentrates.^c Use level reported for product with a pH > 3.5.

only are listed in Table 7 for the total population and for consumers only. The mean dietary exposure of the total population ranged from 0.15 mg/kg bw per day for children aged 2–7 years in Canada to 1.6 mg/kg bw per day for children aged 1–7 years in Mexico. The corresponding high (95th percentile) dietary exposure estimates were 1.6 mg/kg bw per day for adults in Brazil and Canada to 5.1 mg/kg bw per day in children aged 1–7 years in Mexico. For consumers only, the high (95th percentile) dietary exposure could increase to 6.1 mg/kg bw per day for 2–7-year olds in Canada. This high exposure was due to reporting by 4 of 77 children of a mean daily consumption of up to 673 mL of regular carbonated soft drinks on the two survey days.

3.1.2 Estimates for the European population

In 2016, EFSA (23) estimated dietary exposure to benzoic acid (E 210) and benzoates (sodium (E 211), potassium (E 212) and calcium benzoate (E 213)) from use levels provided by industry and analytical concentrations provided by European Union Member States after a public call for data. In this section, benzoic acid and its salts are referred to as benzoates.

Table 7

Dietary exposure to benzoates (as benzoic acid) from water-based flavoured drinks in four countries for two population groups according to a “brand-loyal” scenario^{a,b}

Country and age group (years)	Dietary exposure (mg/kg bw per day) ^c			
	Total population		Consumers only ^d	
	Mean	High	Mean	High
Brazil				
10–17	0.63	2.7	1.1	3.2
≥ 18	0.37	1.6	0.70	2.1
Total	0.41	1.8	0.78	2.4
Canada				
2–7	0.15	0.89	1.5	6.1
8–17	0.48	2.4	1.2	2.9
≥ 18	0.31	1.6	0.9	2.6
Total	0.33	1.7	1.0	2.7
Mexico				
1–7	1.6	5.1	1.8	5.5
8–17	1.5	4.8	1.7	4.9
≥ 18	1.1	3.8	1.3	4.1
Total	1.2	4.3	1.5	4.6
USA				
1–7	0.44	2.7	1.1	3.7
8–17	0.83	3.2	1.2	3.8
≥ 18	0.77	3.0	1.2	3.5
Total	0.74	2.9	1.2	3.6

Sources: references 44 and 45

^a In the “brand-loyal” scenario, consumption amounts of regular carbonated soft drinks, averaged across the survey days, were multiplied by maximum use levels of up to 438 mg/kg (pH > 3.5) or the Codex interim maximum permitted level of 250 mg/kg (pH ≤ 3.5). Consumption amounts of the other water-based flavoured drinks, averaged across the survey days, were multiplied by the market volume-weighted average use levels.

^b For the water-based flavoured drinks and use levels, see Table 6.

^c High exposure: 95th percentile.

^d Consumers-only are consumers who reported consumption of a water-based flavoured drink that could contain benzoates on at least one of the total number of recording days in the dietary survey: 2 days for Brazil, Canada and the USA and 7 days for Mexico.

Use levels for benzoates were provided for nine of the 32 food categories in which benzoates are authorized in the European Union according to Annex II to Regulation (EC) No. 1333/2008. The food category for which the majority of use levels were provided was flavoured drinks (excluding dairy-based drinks), with a maximum reported use level of 150 mg/kg. The analytical concentrations referred to benzoic acid and also covered foods in which benzoates are not authorized in the European Union. The presence of benzoic acid in these food categories could be due to natural occurrence (e.g., in fruit) or due to the use of benzoates as preservatives in food additives, food enzymes and flavouring preparations according to Parts 2, 3 and 4 of Annex III to Regulation (EC) No.

133/2008, respectively. EFSA calculated dietary exposure to benzoates from the use levels and analytical concentrations of 26 food categories for which use of benzoates as food additive is authorized (i.e., dataset 1).

EFSA calculated dietary exposure to benzoates in a “brand-loyal” scenario, in which it was assumed that individuals are exposed to benzoates at the maximum reported use level or analytical concentration, whichever is highest, for all foods in the main contributing food category at the individual level and at the typical (mean) reported use level or analytical concentration, whichever is highest, for all foods in the remaining food categories. Analytical concentrations were included in the assessment according to a medium-bound scenario, in which samples with a reported analytical concentration below the limit of detection (LOD) or quantification (LOQ) were assumed to contain benzoic acid at a concentration equal to LOD/2 or LOQ/2, respectively. Analytical concentrations that exceeded the MPL set in Annex II to Regulation (EC) No. 1333/2008 were not considered in the assessment.

The use levels and analytical concentrations were combined with individual food consumption data from the Comprehensive European Food Consumption Database, which, at the time, contained data from 33 dietary surveys in 19 European countries, covering infants (< 1 year), toddlers (1–2 years), children (3–9 years), adolescents (10–17 years) and adults aged ≥ 18 –64 years and ≥ 65 years. Dietary exposure to benzoates (as benzoic acid) was calculated for all individuals in these age groups by multiplying the use level or analytical concentration per food category by the summed consumption amounts of all foods within that category for each individual. Total daily dietary exposure for each individual was determined by first adding exposure estimates derived for each relevant food category across survey days for that individual, dividing it by the individual’s body weight and finally averaging over the number of survey days. This resulted in a distribution of individual mean dietary exposure estimates per day. The mean and 95th percentile exposure estimates were calculated per survey and for each population group (Table 8).

The food categories that contributed most to dietary exposure to benzoates used as food additives were diverse. They included, in no particular order:

- processed fruit and vegetables: infants, adults and the elderly;
- other confectionery: children and adolescents;
- processed fish and fishery products: all age groups except infants;
- sauces: adults and the elderly;
- salads and savoury-based sandwich spreads: children, adolescents, adults and the elderly;
- flavoured drinks: all age groups except the elderly;

Table 8

Dietary exposure to benzoates (as benzoic acid) from their use as food additives in the total European population for a “brand-loyal” scenario^{a,b}

Exposure level ^c	Dietary exposure (mg/kg bw per day) per population group ^d					
	Infants (12 weeks–11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	Adults (≥ 65 years)
Mean	0.07–0.8	0.6–3.2	0.7–2.6	0.4–1.9	0.4–2.0	0.2–1.6
High	0.4–3.2	1.9–7.0	2.7–7.1	1.5–5.3	1.2–5.1	0.6–4.3

Source: reference 23

^a For a description of the “brand-loyal” scenario, see section 3.1.2.^b The concentrations used in this assessment were from dataset 1 (23).^c High exposure: 95th percentile.^d Range represents the lowest and highest estimates for each age group in the dietary surveys included in the assessment.

- flavoured fermented milk products: toddlers only.

EFSA (23) noted that information from Mintel’s Global New Products Database, an online database of newly introduced packaged foods onto the worldwide market, indicated that the main food categories containing foods labelled with benzoates in the European Union were carbonated soft drinks, table sauces and fish products.

EFSA (23) also calculated the dietary exposure to benzoates according to the “brand-loyal” scenario using use levels and analytical concentrations for food categories for which use of benzoates as food additives is authorized in the European Union with analytical concentrations of food categories that may contain benzoates due to natural occurrence or their use as preservatives in food additives, food enzymes and flavouring preparations. This assessment included 77 food categories. An increase in dietary exposure by a factor of two to three was observed over the estimates listed in Table 8. The 95th percentile estimated dietary exposure to benzoates could increase to 20 mg/kg bw per day for toddlers. In this assessment, unprocessed fruits and vegetables contributed most to exposure. The Committee noted that, of the 233 reported analytical concentrations for unprocessed fruits and vegetables, 213 (88%) were below the LOD or LOQ and that the LOD could be as high as 37.8 mg/kg and the LOQ as high as 113 mg/kg. Inclusion of these concentrations at a mean level of 57 mg/kg and a 95th percentile concentration of 470 mg/kg in a “brand-loyal” exposure assessment could have resulted in overestimation of dietary exposure and of the contribution of unprocessed fruits and vegetables to dietary exposure to benzoates.

EFSA considered the “brand-loyal” scenario relevant for assessing the safety of benzoates, because of possible loyalty to brands of certain types of flavoured drinks in which use of benzoates is authorized. Furthermore, EFSA

considered that real exposure to benzoates as food additives in European countries is overestimated because of the assumption that all foods in a food category contain benzoates at the mean or high use level or analytical concentration.

3.1.3 Estimates for India

Dietary exposure of schoolchildren in Tirupati, India, to sodium benzoate was estimated in a total diet study (46), in which 65 foods were analysed for sodium benzoate. The foods were sauces, pickles, soft drinks, fruit juices, jellies and jams. Food consumption data for 960 schoolchildren aged 2–7, 8–14 and 15–19 years were obtained with a 24-h dietary record method. The concentrations of benzoic acid and reported consumption amounts of foods per child were combined to calculate the mean dietary exposure for the three age groups.

The sodium benzoate concentrations ranged from 135 to 196 mg/kg in pickles, 355 to 482 mg/kg in sauces, 57 to 104 mg/kg in soft drinks, 93 to 163 mg/kg in fruit juices, 261 to 356 mg/kg in jellies and 336 to 418 mg/kg in jams. Mean dietary exposure to sodium benzoate was reported in mg/day. At average body weights per age group of 20, 35 and 50 kg, mean exposures of 1.5, 1.2 and 1.1 mg/kg bw per day as sodium benzoate and 1.3, 1.0 and 0.9 mg/kg bw per day as benzoic acid were calculated by the Committee, respectively. The main contributors to dietary exposure were soft drinks and fruit juice. The authors noted that some children aged 2–7 years had an exposure > 5 mg/kg bw per day because of high consumption relative to their body weights. The Committee noted that the exposure estimates are based on a 1-day 24-h dietary recall, which may result in higher estimates than those over a longer period.

3.1.4 Estimates for the Islamic Republic of Iran

Dietary exposure to sodium benzoate from orange juice was estimated in the Islamic Republic of Iran (47) from analysis of sodium benzoate in 30 samples of orange juice purchased at local markets in Tehran and per capita food consumption based on the annual orange juice production in the country. The concentrations of sodium benzoate in orange juice ranged from 12.23 to 56.80 mg/kg. From these concentrations, the daily dietary exposure to sodium benzoate was estimated to be 1.11 mg/kg bw per capita (or 0.94 mg/kg bw per capita as benzoic acid). The Committee noted that no information was provided on the amount of orange juice consumed, the body weight or the actual sodium benzoate concentration used to obtain this dietary exposure estimate.

In a second study in the Islamic Republic of Iran (48), dietary exposure to sodium benzoate was estimated from cake, toast, tomato paste, mayonnaise, carbonated soft drinks and *olovieh* salad. A total of 103 samples of these foods were purchased at local supermarkets in Kashan and analysed. Sodium benzoate

was found in all 15 mayonnaise samples at 161.68–296.2 mg/kg and in all 19 samples of carbonated soft drinks at 2.15–131 mg/kg. No sodium benzoate was found in the other food samples above the LOQ of 4 mg/kg. The Committee noted that the authors did not explain why the lowest reported concentration of sodium benzoate was below the LOQ. To estimate dietary exposure to sodium benzoate from the analysed concentrations, mean sodium benzoate concentrations of 243.42 mg/kg in mayonnaise and 61.75 mg/kg in carbonated soft drinks were combined with a mean consumption of 3.4 g/day of mayonnaise and 144 mL/day of carbonated soft drinks. Assuming an average body weight of 60 kg, total dietary exposure to sodium benzoate was estimated at 0.16 mg/kg bw per day (or 0.14 mg/kg bw per day for benzoic acid). Consumption of carbonated soft drinks accounted for 94% of dietary exposure to sodium benzoate. The Committee noted that the dietary exposure estimate reported in the publication was expressed in mg/kg bw per day, while it should have been expressed as µg/kg bw per day.

3.2 Overview of estimated dietary exposure

Table 9 summarizes the estimates of dietary exposure to benzoates (as benzoic acid) considered at the present meeting for the total population. The summary also includes the estimates of dietary exposure to benzoates derived from the literature and summarized by the Committee at its eightieth meeting (**Annex 1**, references 223 and 224). The dietary exposure estimates from the literature were from scientific papers published in 2000–2015 and refer to dietary exposure to benzoates from all foods and beverages for the total population. The dietary exposure estimates summarized in **Table 9** should be interpreted with care as they include a number of different foods, were calculated by different methods and different food consumption and concentration data and are relevant for different age groups.

From its literature review, the Committee concluded at its eightieth meeting that the largest contributor to the estimated dietary exposure to benzoates in most countries was water-based flavoured drinks, which contributed up to 80% for the total population of Brazil in a study in 2002 in which benzoates were analysed in a limited number of foods: soft drinks, fruit juices, margarine, yoghurt and cheese (49). In the study by EFSA (23), the contribution of water-based flavoured drinks to dietary exposure to benzoates, including only the food categories for which use of benzoates as a food additive is authorized was 6.9–40.3% for adults aged ≥ 65 years up to 14.8–92.5% for adolescents.

Benzoic acid may also occur in food due to natural occurrence, such as in berries, or may be present due to the use of benzoates in food additives, food enzymes and flavouring preparations. The concentrations in food due to

Table 9
Overview of estimated dietary exposure of the total population to benzoates (as benzoic acid)^a from their use as food additives

Country or source	Foods included	Source concentrations	Consumption	Dietary exposure (mg/kg bw per day) ^{b,c}		
				Children ^d	Mean	High
Brazil	Water-based flavoured drinks	Maximum and market weighted use levels	Individual food consumption data	0.63	2.7	0.41
Canada				0.15–0.48	0.89–2.4	0.33
Mexico				1.5–1.6	4.8–5.1	1.2
USA				0.44–0.83	2.7–3.2	0.74
Europe ^e	Whole diet	Maximum and average use / analytical concentrations	Individual food consumption data	0.07–3.2	0.4–7.1	0.07–3.2
India	Pickles, sauces, soft/fruit drinks, jellies, jams	Analytical concentrations	Individual food consumption data	0.9–1.3	–	–
Islamic Republic of Iran	Orange juice Cake, toast bread, tomato paste, mayonnaise, carbonated soft drinks, <i>olovieh</i> salad	Analytical concentrations	Per capita Mean consumption	–	0.94 ^g 0.14	–
Eightieth meeting	Whole diet	Use / analytical concentrations	Individual food consumption data ^h	0.1–1.5	0.4–3.9	0.01–1.5
Literature ^h						0.2–3.1

Sources: references 23, 44–48; Annex 1, reference 224.

^a Exposure estimates for India and the Islamic Republic of Iran refer to dietary exposure to sodium benzoate converted to benzoic acid according to molecular weight. As no information was provided for the estimates from the literature summarized by the Committee at its eightieth meeting, they were assumed to refer to benzoic acid.

^b High exposure: 95th percentile

^c Dietary exposure for Brazil, Canada, Europe, Mexico and the USA were calculated for a "brand-loyal" scenario. For more details, see section 3.1.1.

^d Children aged 10–17 years in Brazil, 2–17 years in Canada, 1–17 years in Europe, Mexico, the USA and the eightieth meeting, and 2–19 years in India.

^e The general population of Europe comprises people aged from 12 weeks to ≥ 65 years; for Mexico, the USA and estimates from the literature, people aged ≥ 1 year, for Brazil ≥ 10 years and for Canada ≥ 2 years. The ages for the general population in the Islamic Republic of Iran assessments were not specified.

^f The European countries were Austria, Belgium, Bulgaria, Czechia, Cyprus, Denmark, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Romania, Spain, Sweden and United Kingdom.

^g The Committee noted that no information was provided on the consumption level of orange juice, the body weight and the actual sodium benzoate concentration used to obtain this dietary exposure estimate.

^h Refers to literature-derived estimates for benzoates from all foods for the total population as published between 2000 and 2015 and summarized by the Committee at its eightieth meeting. These estimates are for Australia, Austria, Belgium, Brazil, China, Denmark, France, Ireland, Italy, Lebanon, New Zealand, Republic of Korea, Saudi Arabia, Serbia and United Kingdom.

ⁱ Exposure estimates for Brazil were based on per capita estimates of consumption.

^j High exposure in children is the 97.5th percentile.

these two sources are not usually high. For example, concentrations up to 29 mg/kg have been reported in strawberries (50), and up to 5 mg/kg is allowed in cheese due to use of benzoic acid at a maximum level of 12 000 mg/kg in rennet (23). In view of the wide range of foods in which benzoic acid may occur due to natural occurrence and the use of benzoates in food additives, food enzymes and flavouring preparations, however, the contribution of these two sources to dietary exposure to benzoic acid may not be negligible. Dietary exposure of the European population could increase to 20 mg/kg in a “brand-loyal” scenario (see section 3.1.2); however, these estimates were highly conservative because of the assumption that all unprocessed fruits and vegetables contain benzoic acid.

To protect individuals who eat foods that may contain a food additive, exposure of “consumers-only” can be estimated by including only individuals who report consumption of these foods. The sponsor provided dietary exposure estimates for consumers only of water-based flavoured drinks that could contain benzoates in four countries (Table 7). The 95th percentile dietary exposure estimates were up to 6.1 mg/kg bw per day for 2–7-year-olds in Canada. At its eightieth meeting, the Committee also estimated dietary exposure of consumers-only of water-based flavoured drinks from CIFOcOs data and an average reported typical use level. The highest mean exposure calculated was 4.1 mg/kg bw per day, and the highest high (95th percentile) estimate was 10.9 mg/kg bw per day, both for children aged 1–5 years in South Africa. The Committee at its current meeting noted an even higher estimated 95th percentile of exposure for toddlers in Germany of 15.9 mg/kg bw per day; however, this estimate was based on only a few children and was considered not statistically robust. Furthermore, the high estimates were based on the highest average typical use level in water-based flavoured drinks reported by various countries: 209 mg/kg. This average use level is at the upper range of average use levels in these drinks as reported by the sponsor (i.e., 39–197 mg/kg; Table 6) and higher than the reported maximum use level of 150 mg/kg in flavoured drinks in Europe (23).

At its eightieth meeting, the Committee also summarized estimates of dietary exposure to benzoates from all foods and beverages as reported in the literature. The dietary exposure of consumers only was reported to be up to 6.8 mg/kg bw per day for preschool children in Austria for the mean and up to 9 mg/kg bw per day for young children in Denmark for the 95th percentile estimate. The Committee at its current meeting noted that the Danish estimate was based on a total population approach but could be considered to refer to consumers only because of the broad range of foods included in the assessment. The Committee also noted that the dietary exposure estimates for Austria and Denmark were made in 2012 and 2010, respectively, and have been superseded by the more recent assessment by EFSA (23) for Europe, which included food consumption data and analytical concentrations for both countries. Therefore,

the estimates for these two countries reviewed at the eightieth meeting were not considered further by the Committee at its current meeting.

The most complete assessment of dietary exposure to benzoates from their use as food additives was performed for the European population, covering 26 of 32 food categories that could contain benzoates as food additives according to Annex II to Regulation (EC) No. 1333/2008 (23). Dietary exposure according to a “brand-loyal” scenario could be as high as 7.1 mg/kg bw per day for children aged 3–9 years (Table 8). This estimate, and the dietary exposure estimates of benzoates for Brazil, Canada, Mexico and the USA are overestimates of the actual mean or high dietary exposure to benzoates in these countries, as it is assumed in these assessments that all foods in a food category that could contain benzoates did in fact contain benzoates at the maximum or (market volume-weighted) average use level or analytical level. Furthermore, not all foods contain preservatives, and, for those that do, other food additives with the same function in foods are available.

4. Comments

4.1 Biochemical aspects

The Committee noted previously that benzoic acid is rapidly absorbed, primarily metabolized in the liver and completely excreted in the urine as hippuric acid (major metabolite) and benzoyl-glucuronide.

4.2 Toxicological studies

In studies previously evaluated by the Committee, the oral acute toxicity (LD_{50}) of benzoic acid ranged from 200 to 1200 mg/kg bw in mice to 2700 mg/kg bw for sodium benzoate in rats and 2000 mg/kg bw in rabbits and dogs.

A large number of short-term studies on benzoic acid, sodium benzoate and its benzyl derivatives were evaluated previously by the Committee, none of which showed effects at doses up to 1000 mg/kg bw per day.

The Committee had previously reviewed long-term studies in mice and rats on benzyl derivatives, benzyl alcohol, benzaldehyde, benzyl acetate, benzoic acid and sodium benzoate and concluded that the data did not indicate carcinogenic potential.

The Committee had previously reviewed studies on genotoxicity, and, although positive results were seen in some in vitro studies, the results of in vivo

studies were consistently negative. The present Committee concluded that there was no concern about the genotoxicity of benzoic acid, its salts or derivatives.

The Committee previously evaluated a four-generation reproductive toxicity study in rats (51), in which the highest dose tested, 10 000 ppm (1%) in the diet, equivalent to 500 mg/kg bw day, was not associated with any toxicological effect. On the basis of these results, the Committee at its previous meetings established a group ADI of 0–5 mg/kg bw for benzoic acid, the benzoate salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate, expressed as benzoic acid equivalents, applying a default uncertainty factor of 100.

In a reproductive toxicity study conducted according to OECD 443 extended one-generation reproductive toxicity test guideline, doses of 0, 500, 750 or 1000 mg benzoic acid/kg bw per day were given in the diet to rats through F₀, F₁ and F₂ generations (28,29). The study included offspring cohorts that were assessed for potential developmental immunotoxicity and developmental neurotoxicity. No treatment-related adverse effects were observed on reproductive performance, estrous cycles, parturition, litter viability or survival, pre- or post-weaning developmental landmarks, neurobehaviour, thyroid hormones, clinical pathology, gross necropsy, organ weights, histopathology or sperm parameters. Immunophenotyping and T-cell-dependent antibody responses, organ weights, histopathological examination, neuropathology and brain morphometry in the offspring were not affected by the treatment. The Committee identified a NOAEL of 1000 mg/kg bw per day, the highest dose tested, for reproductive and developmental toxicity.

4.3 Allergenicity

The available human data indicate that benzoic acid and its sodium salt can trigger intolerance and allergic reactions in some individuals when ingested in food.

4.4 Assessment of dietary exposure

Benzoic acid and its salts are endorsed for use in 59 food categories at MPLs ranging from 200 mg/kg up to 5000 mg/kg, as specified in the Codex GSFA, all expressed as benzoic acid. At its current meeting, the Committee evaluated estimates of dietary exposure to benzoates from water-based flavoured drinks submitted by the sponsor for Brazil, Canada, Mexico and the USA (44,45), based on maximum use levels of benzoates (expressed as benzoic acid) of up to 438 mg/kg in regular carbonated soft drinks and market volume-weighted average

use levels ranging from 39 to 197 mg/kg. In addition, dietary exposure estimates from the literature were assessed for Europe (23), India (46), the Islamic Republic of Iran (47,48) and other countries, as reviewed by the Committee at its eightieth meeting (Annex 1, references 223 and 224). Table 9 gives an overview of the dietary exposure estimates, all expressed as benzoic acid. The estimates of dietary exposure for Brazil, Canada, Mexico and the USA and for Europe are “brand-loyal estimates”, which account for brand loyalty by mapping the consumption of such foods at a maximum reported use level and that of other foods that may contain benzoates at a typical use level or at a market volume-weighted average use level. The estimates of dietary exposure to benzoates for Europe covered 26 of the 32 food categories for which the use of benzoates is authorized in the European Union according to Annex II to Regulation (EC) No. 1333/2008.

Benzoic acid may also occur naturally in foods, such as in berries, but the concentrations are usually not high. In Europe, benzoates may also be present in food due to their use as preservatives in food additives, food enzymes and flavouring preparations according to Annex III to Regulation (EC) No 1333/2008. The concentrations of benzoic acid in food due to this use are also not expected to be high; however, in view of the wide range of foods in which benzoic acid may occur naturally and from use of benzoates in food additives, food enzymes and flavouring preparations, dietary exposure to benzoic acid from these two sources may not be negligible.

The most complete assessment of dietary exposure to benzoates from their use as food additives was performed for the European population. Dietary exposure in a brand-loyal scenario could be as high as 7.1 mg/kg bw per day for children aged 3–9 years (Table 9). The Committee considered that this high estimate of dietary exposure was the most suitable estimate currently available for evaluating dietary exposure to benzoates expressed as benzoic acid, as it accounts for people who are loyal to brands of foods, such as water-based flavoured drinks, over a long period. In addition, this estimate applies to the majority of foods to which benzoates may be added as additives in the European Union. The estimate was also considered conservative enough to include dietary exposure to benzoic acid from natural sources and from authorized use of benzoates as preservatives in food additives, food enzymes and flavouring preparations in the European Union. The Committee further noted that this high exposure estimate exceeds the high dietary exposure estimates for consumers only reported by the sponsor.

4.5 Benzene as a reaction product in benzoic acid-containing beverages

Benzene is a known human carcinogen after chronic inhalation (52). During the early 2000s, it was found in trace quantities in some soft drinks and other beverages, where it might have been formed during storage by radical-initiated decarboxylation of benzoic acid (53). Studies have indicated higher concentrations of benzene in beverages that contain benzoate and ascorbic acid (54,55).

Following investigations by a number of national regulatory agencies and the development of mitigation strategies, analyses of reformulated products demonstrated that benzene could not be detected in some samples and that benzene levels were commonly < 5 ng/mL in all others (54–57). The Committee at its present meeting considered these findings in its assessment of the safety of foods containing benzoic acid and related compounds.

Estimated dietary exposure to benzene from beverages and foods is low. An assessment made as part of an examination of the use of the concept of MOE presented estimates of dietary exposure to benzene from beverages of 8 ng/kg bw per day and from food of 3–50 ng/kg bw per day. With a lower confidence limit of the benchmark dose representing a 10% extra risk of 17.6 mg/kg bw per day derived from dose–response modelling of data on carcinogenicity in experimental animals, the MOEs were calculated to be 2×10^6 for beverages and 6×10^6 for food (58).

The Committee noted that food and beverages contribute much less benzene to human exposure than other sources, such as inhalation, vehicle fuel fumes and cigarette smoking (57–59). WHO (60) has set a guideline for benzene in drinking-water at 10 µg/L.

5. Evaluation

The Committee at previous meetings established a group ADI of 0–5 mg/kg bw for benzoic acid, its salts and derivatives expressed as benzoic acid on the basis of a four-generation reproductive toxicity study in rats that showed no toxicological effects at the highest dose tested, 10 000 ppm (1%) in the diet, equivalent to 500 mg/kg bw per day. A default uncertainty factor of 100 was applied to the dose of 500 mg/kg bw per day.

At its present meeting, the Committee evaluated a new extended one-generation reproductive toxicity study on benzoic acid. This study showed no treatment-related adverse effects, indicating a NOAEL of 1000 mg/kg bw per day, the highest dose tested. As no adverse effects were noted in any of the repeated

dose studies in the toxicology database, the Committee decided to use this NOAEL as the basis for a revised group ADI.

The Committee evaluated two approaches to refining the uncertainty factor to be used in establishing an ADI and concluded that the chemical-specific adjustment factor approach of Zu et al. (4) was the more appropriate. The Committee therefore applied a factor of 2 for interspecies variation in pharmacokinetics instead of the default factor of 4. An overall uncertainty factor of 50 (2 for interspecies pharmacokinetics variation \times 2.5 for interspecies pharmacodynamics variation \times 10 for interindividual variation) was applied to the NOAEL of 1000 mg/kg bw per day identified in the new one-generation reproductive toxicity study in rats. The Committee established a group ADI of 0–20 mg/kg bw. This group ADI applies to benzoic acid, the benzoate salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate, expressed as benzoic acid equivalents. The Committee withdrew the previous group ADI of 0–5 mg/kg bw.

The Committee noted that the high dietary exposure estimate, expressed as benzoic acid, of 7.1 mg/kg bw per day for children aged 3–9 years does not exceed the group ADI of 0–20 mg/kg bw. The Committee considered this dietary exposure to be of no concern. In addition, the Committee noted that the highest estimates of dietary exposure, expressed as benzoic acid, from water-based flavoured drinks evaluated by the Committee at its eightieth meeting also did not exceed the group ADI of 0–20 mg/kg bw ([Annex 1](#), references 223 and 224). These dietary exposure estimates do not include contributions from benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate due to their use as flavouring agents; however, dietary exposure to these derivatives is expected to be low and would be covered by the dietary exposure estimates to benzoic acid and its salts as food additives.

At its previous meeting, the Committee identified reports of human idiosyncratic intolerance and of allergy to benzoate. The Committee noted that intolerance and allergenicity to benzoate may pose a health concern to sensitive individuals.

The Committee noted that benzene can be formed as a reaction product in benzoic acid-containing beverages. On the basis of the available information, the Committee concluded that exposure to benzene from soft drinks and other foods formulated with benzoic acid or its salts is of little concern from a public health perspective.

An addendum to the toxicology and dietary exposure monograph was prepared.

The specifications were revised, and a chemical and technical assessment was prepared.

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Collagenase from *Streptomyces violaceoruber* expressed in *S. violaceoruber*

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1. Explanation

At the request of the Codex Committee on Food Additives (CCFA) at its Fifty-first Session (1), the Committee evaluated the safety of collagenase (microbial



collagenase; Enzyme Commission No. 3.4.24.3; Chemical Abstracts Services Registry Number: 9001-12-1) from *Streptomyces violaceoruber* pCol, which it had not previously considered.

The enzyme catalyses the hydrolysis of peptide bonds in collagen. The collagenase enzyme preparation is intended for use as a processing aid in the production of meat and sausage casings and in the production of collagen hydrolysates used as ingredients in food supplements and special purpose foods.

In this monograph, the expression “collagenase” refers to the collagenase enzyme and its amino acid sequence, the expression “enzyme concentrate” refers to the test material used in the toxicity studies, and the expression “enzyme preparation” refers to the product formulated for commercial use.

At its present meeting, the Committee considered the submitted data. It also conducted a literature search in Google Scholar with the linked search terms “collagenase” AND “*Streptomyces violaceoruber*”, which identified 27 references. One reference (2) was relevant to this toxicological evaluation; however, it was based entirely on the studies in the submitted dossier.

1.1 Genetic background

The production organism, *S. violaceureuber*, also referred to as *S. lividans* or *S. coelicolor*, belongs to the genus *Streptomyces*. *S. violaceureuber* is non-pathogenic, non-toxicogenic, occurs in nature as a component of soil (3) and has a history in the production of enzymes intended for use in food processing (4).

The *S. violaceureuber* pCol production strain was obtained by transforming a plasmid containing the following: a promoter sequence obtained from *S. avermitilis* ATCC 31267, the collagenase gene obtained from *S. violaceoruber* NBRC 15146, a terminator sequence obtained from *S. cinnamoneus* NBRC 12852 and a selectable marker. The resulting plasmid was incorporated into the host organism, *S. violaceoruber* 1326, by electroporation. The stability of the introduced sequences was confirmed by cultivating the production strain over three generations, measuring collagenase activity each time. The final enzyme preparations were tested for the absence of antibiotic resistance gene by PCR. The production strain has been deposited at the National Institute of Technological Evaluation in Japan.

1.2 Chemical and technical considerations

Collagenase (designated EC No. 3.4.24.3 by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology) is produced by controlled fermentation of a pure culture of the *S. violaceoruber* production

strain. The manufacture of the collagenase enzyme preparation includes fermentation (pre-, seed and main fermentation), recovery and formulation. After fermentation, the broth containing the collagenase enzyme is separated from the biomass by sedimentation; this is followed by three filtration steps. The resulting liquid preparation is formulated with water and glycerol. Two powdered enzyme preparations are produced by further filtering and freeze-drying of the liquid filtrate, followed by formulation with dextrin. The entire process is performed in accordance with current good manufacturing practices and with food-grade raw materials. The primary sequence of collagenase produced by *S. violaceoruber* consists of 865 amino acids; its molecular weight by calculation from the determined amino acid sequence is 92.4 kDa. The enzyme concentrate is tested to be free from the production organism and any antibiotic activity.

The activity of collagenase activity is determined spectrophotometrically by measuring the hydrolysis of a defined peptide substrate by collagenase at 570 nm; one unit of activity is defined as the quantity of enzyme required to liberate one $\mu\text{mol}/\text{min}$ of glycine under the conditions of the assay. The mean activities of collagenase from three batches each of the liquid and the two powder enzyme preparations were 477 U/g, 122 U/g and 2690 U/g, respectively.

The collagenase enzyme preparations are intended for use as processing aids in the production of meat and sausage casings and in the production of collagen hydrolysates used as ingredients in foods, such as those for specific dietary uses (for example, foods for special nutritional purposes, sports foods, health foods) and in dietary supplements.

The collagenase enzyme preparations are used at maximum levels of 1188 mg TOS/kg raw material (as a liquid) and 1566 mg TOS/kg raw material (as a powder) for hydrolysates, and 36 mg TOS/kg raw material (as a powder) for meat processing. The TOS includes the enzyme of interest and residues of organic materials, such as proteins, peptides and carbohydrates, derived from the production organism during the manufacturing process.

The collagenase enzyme is inactivated by heat treatment prior to use of the final foods. If present, it is expected that collagenase will be digested, as would any other protein occurring in food, but no data are available on its digestibility.

2. Biological data

2.1 Biotransformation

No information was available.

2.2 Assessment of potential allergenicity

The collagenase from *S. violaceoruber* pCol. was assessed as a potential allergen by bioinformatics, consistent with the criteria recommend by FAO/WHO and others (5–7). The amino acid sequence of the enzyme (865 amino acids; 92.4 kDa) was compared with the sequence of known allergens present in the AllergenOnline (<http://www.allergenonline.org/databasefasta.shtml>; accessed 10 February 2020) and Allermatch (<http://allermatch.org/>; accessed 27 February 2020) databases. A search for matches with > 35% identity over a sliding window of 80 amino acids and a search for sequence identity of eight contiguous amino acids were conducted in both databases. No matches were found. A full-length FASTA sequence search was conducted with an E-value¹ cut-off of < 0.1, and no matches were found. From this information, it was concluded that the enzyme was not similar to any known allergen and was not anticipated to pose an allergenic risk.

2.3 Toxicological studies

2.3.1 Acute toxicity

A GLP-compliant acute oral toxicity study in Sprague-Dawley SPF (CrI:CD (SD)) rats was conducted according to OECD test guideline 423 (8). The test material was a powdered concentrate of collagenase from *S. violaceoruber* pCol (TOS: 93.96%) mixed in water (200 mg/mL powder concentrate). A group of three female rats, 8 weeks old at the start of treatment, received a dose of 2000 mg/kg bw, equal to 1879.2 mg TOS/kg bw, by oral gavage with a dose volume of 10 mL/kg bw. The experiment was repeated with a second group of three female rats (8 weeks old at start of treatment). In both experiments, animals were observed for up to 14 days, and then necropsied. No deaths, clinical signs of toxicity or anomalies in body weight gain or body weight were observed in either experiment. Gross pathological examination found no abnormalities. The LD₅₀ of the enzyme concentrate was concluded to be > 2000 mg/kg bw or 1879.2 mg TOS/kg bw.

2.3.2 Short-term studies of toxicity

A GLP-compliant 13-week oral toxicity study in Sprague-Dawley SPF (CrI:CD(SD)) rats was conducted according to OECD test guideline 408 (9). The test material was a powdered collagenase concentrate (TOS: 93.96 %), which was

¹ Comparisons between highly homologous proteins yield expectation values (E-values) approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. The E-value threshold selected for a search tends to be larger with searches of short sequences ($E < 0.1$) than with the long sequences ($E < 1 \times 10^{-7}$), as the likelihood of random matches is greater in the search of shorter sequence matches.

mixed in water. Groups of animals ($n = 10/\text{sex}$ per dose group) received an oral gavage dose of 0, 62.5, 250 or 1000 mg/kg bw per day of the enzyme concentrate, equal to 0, 58.7, 234.9 and 939.6 mg TOS/kg bw per day, respectively, for 90 days. Animals consumed feed and water ad libitum throughout the study, except for an overnight fast before scheduled blood sampling. Standard guideline parameters were measured in all animals and included general health (observed three times per day); clinical signs of toxicity (detailed observations made once before the start of treatment and then weekly); neurobehavioural testing (observations made once before the start of treatment and then at week 12); ophthalmoscopic examination (observations made once before the start of treatment and then at week 12); body weights (measured three times in the first week and then twice weekly); food intake (measured twice in the first week and then weekly); water consumption (measured on the day before urinalysis); haematology, blood chemistry and urinalysis (all measured at the end of treatment). At termination, a necropsy was conducted on all animals, absolute organ weights were measured, organ weights relative to body weights were calculated, and samples of tissues and organs were collected for microscopic examination.

All animals survived the treatment period, and no treatment-related clinical signs of toxicity were observed in any group. No statistically significant treatment-related or toxicologically relevant changes in body weights, food consumption, ophthalmological examination, urinalysis, haematology, gross pathology or histopathology were reported.

The results of urinalysis showed a trend towards lower urine pH with increasing dose in both sexes. This trend was considered to be due to the ingestion of acidic test suspensions (pH ranged from 5.12 at 62.5 mg/kg bw per day to 5.06 at 1000 mg/kg bw per day test material). A statistically significant reduction in the excretion of sodium (26%) was observed in males at the high dose when compared with controls (mean control \pm SD: 1.9 ± 0.7 mmol/24 h; high dose \pm SD: 1.4 ± 4.5 ; $P \leq 0.05$; Dunnett's two-sided test). This observation was considered not to be toxicologically relevant, as there was no change in blood sodium levels and the levels were within the historical range (mean sodium excretion \pm SD, $n=316$: 1.85 ± 0.59 mmol/24 h). Statistically significant reductions in mean corpuscular volume and mean corpuscular haemoglobin were observed in males at the high dose (3% and 4%, respectively) when compared with controls ($P \leq 0.05$; Dunnett's two-sided test). These differences were slight and shown to be within the historical range (mean corpuscular volume, mean \pm SD, $n=252$: 49.9 ± 1.7 fL; control \pm SD: 52.9 ± 1.5 fL; high dose \pm SD: 51.4 ± 0.8 fL; mean corpuscular haemoglobin, mean \pm SD, $n=252$: 17.6 ± 0.7 pg; control: 17.8 ± 0.7 pg; high dose: 17.1 ± 0.4 pg). On this basis, they were considered not toxicologically relevant.

Blood chemistry showed statistically significant increases in the values of aspartate and alanine aminotransferase, lactic acid dehydrogenase, total

cholesterol, triglyceride and phospholipids in one female at the high dose (Table 1). The serum and plasma of this animal were yellow, and total bilirubin was statistically significantly elevated (total bilirubin; control: 0.1 mg/dL; high-dose female: 0.5 mg/dL). The cause of these changes could not be established, as they could not be explained by any gross or microscopic pathology. No statistically significant changes in blood chemistry were observed in any other female or any of the males at any dose.

Statistically significant reductions in blood creatinine and chloride were observed in males at the high dose (14% and 2%, respectively) when compared with control values. The difference in blood creatinine was within the historical control range (mean control \pm SD: 0.29 ± 0.03 mg/dL; mean historical control \pm SD: 0.27 ± 0.04 mg/dL; mean high dose males \pm SD: 0.25 ± 0.03 mg/dL) and was considered not toxicologically relevant. The difference in blood chloride was also considered not to be toxicologically relevant, as there was no change in function, and the values were within the range of historical controls (mean historical control \pm SD (n = 242): 107 ± 2 mmol/L; mean control \pm SD: 106 ± 1 mmol/L; mean high dose \pm SD: 104 ± 2 mmol/L; $P \leq 0.01$, Dunnett's two-sided test).

Statistically significant increases in the absolute and relative weights of kidneys were observed in high-dose males when compared with control values (14% and 13 %, respectively; Table 2). No changes in renal function or histopathology were observed as compared with the control group. The effect appeared to be related to treatment, but no change in function suggested that the finding was toxicologically relevant.

The Committee considered that the outlying blood chemistry values of the one female at the high dose could be excluded, as the values appeared to be extreme (2–11 times greater than those for other animals in the group) and no other female was affected. The increases in absolute and relative kidney weights of the high-dose males were considered to be treatment-related, but, as no change in function was associated with these changes, they were considered not to be adverse. On this basis, the Committee identified a NOAEL of 940 mg TOS/kg bw per day (rounded from 939.6), the highest dose tested.

2.3.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.3.4 Genotoxicity

GLP-compliant genotoxicity studies were conducted according to OECD guidelines 471, 476, and 474. The powder form of the collagenase concentrate (TOS content, 93.96 %) was dissolved in water and tested in a bacterial reverse mutation assay (10,11), an in vitro mammalian cell gene mutation assay (mouse

Table 1

Summary of blood chemistry in female rats fed a powdered collagenase concentrate

Dose (mg kg bw per day)	0	62.5	250	1000	1000*	1000**
Number of animals	10	10	10	10	10	9
AST (IU/L)	55 ± 6	66 ± 31	53 ± 10	97 ± 137	487, 63, 50, 53, 48, 51, 46, 55, 48, 63	53 ± 6
ALT (IU/L)	24 ± 6	27 ± 11	23 ± 5	48 ± 77	267, 38, 24, 22, 20, 18, 20, 26, 23, 24,	24 ± 6
LDH (IU/L)	48 ± 17	65 ± 37	57 ± 16	93 ± 103	381, 81, 61, 94, 41, 33, 88, 68, 39, 45	61 ± 22
T-CHO (mg/dL)	79 ± 18	76 ± 15	89 ± 20	107 ± 50	238, 104, 109, 40, 89, 90, 118, 96, 106, 72	92 ± 22
TG (mg/dL)	24 ± 8	31 ± 20	42 ± 33	44 ± 34	125, 30, 34, 21, 53, 32, 23, 30, 77, 13	35 ± 18
PL (mg/dL)	148 ± 28	141 ± 23	168 ± 37	190 ± 79	400, 181, 189, 102, 176, 166, 181, 166, 198, 136	166 ± 28

AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactic acid dehydrogenase; T-CHO, total cholesterol; TG, triglyceride; PL, phospholipids. Values represent group means ± SD, except in the column 1000*, where individual animal values are shown. The values in bold are for one animal. Column 1000** shows mean values without those for one animal with extremely high values.

Table 2

Kidney weights of male rats fed a powdered collagenase concentrate

Dose (mg/kg bw per day)	0	62.5	250	1000
Number of animals	10	10	10	10
Body weight (g)	555 ± 65	533 ± 38	564 ± 52	558 ± 31
Kidney				
Absolute weight (g)	2.93 ± 0.23	2.92 ± 0.21	3.09 ± 0.21	3.34 ± 0.28**
Relative weight (g/100 g)	0.53 ± 0.03	0.55 ± 0.02	0.55 ± 0.03	0.60 ± 0.05**

* $P \leq 0.01$ significantly different from control group mean; Dunnett's two-sided test. Values represent group means ± SD.

lymphoma tk assay) (12) and an in vivo micronucleus induction assay in rats (13). The summary results of these studies are shown in Table 3.

The results of the bacterial reverse mutation assay were negative in all tester strains, except TA1535, in which weak but statistically significant increases in mutagenesis and growth inhibition were observed with and without metabolic activation at the highest concentration of 5000 µg/plate (equal to 4698 µg TOS/plate), with the preincubation method. In a confirmation assay with tester strains TA100 and TA1535, the results were negative with and without metabolic activation at the highest concentration of 5000 µg/plate, by the preincubation and treat-and-wash methods.

The results of the mammalian cell gene mutation assay were equivocal (one test result with continuous treatment without a 9000 × g supernatant fraction from liver homogenate (S9) was positive and one negative), and those of the in vivo micronucleus induction assay were negative.

Table 3
Genotoxicity of collagenase from *Streptomyces violaceoruber*

End-point	Test system	Concentration	Result	Reference
In vitro				
Reverse mutation	<i>Salmonella typhimurium</i> TA100, TA98, TA1535, TA1537 <i>Escherichia coli</i> WP2 uvrA	147-4698 µg TOS/plate ±S9	Negative in <i>Salmonella typhimurium</i> TA100, TA98, TA1537 <i>Escherichia coli</i> WP2 uvrA Positive in <i>Salmonella typhimurium</i> TA1535 at 4698 µg/plate ±S9	10
	<i>Salmonella typhimurium</i> TA100, TA1535	147-4698 µg TOS/plate ±S9	Negative	11
Mammalian cell gene mutation ^a	Mouse lymphoma cultured cell line L5178Y	147-4698 µg TOS/plate ±S9	Negative in short-term exposure -S9 Negative in short-term exposure +S9 up to 2349 µg TOS/mL Positive in continuous treatment -S9 at 4698 µg TOS/mL	12
		1889-4698 µg TOS/mL ±S9 (Confirmation test)	Negative in continuous treatment -S9 up to 4698 µg TOS/mL	
In vivo				
Micronucleus induction	Sprague-Dawley SPF rats	470-1879 mg TOS/kg bw	Negative	13

S9 is a 9000 × g supernatant fraction from liver homogenate. It contains microsomes and cytosol and is devoid of mitochondria. The livers used to obtain the S9 fraction were obtained from male Sprague-Dawley rats treated with intraperitoneal injections of phenobarbital (at 24-h intervals for 4 days; 30–60 mg/kg body weight) and a single injection of 5,6-benzoflavone (80 mg/kg) on the third day of phenobarbital injection. This treatment induced cytochrome P450 enzymes. The S9 fraction in combination with other factors (S9 mix) acts as an exogenous metabolic activation system.

^a For the study of mammalian cell gene mutation, a preliminary dose range-finding test was conducted, which consisted of a short (3-h exposure with or without S9 mix) and continuous treatment (25-h exposure without S9 mix) with the test material at a concentration of 75, 150, 300, 600, 1174, 2349 or 4698 µg TOS/mL. No precipitates were observed at any concentration. The relative survival was concentration-related and ranged from 100 to 29% under the different exposure conditions. The main test was conducted under the same conditions. Positive controls included cyclophosphamide (5 µg/mL) in the presence of S9 mix and methylmethane sulfonate (5 or 10 µg/mL) in the absence of S9 mix. The negative control was water. A result was considered positive if the test substance increased the mutation frequency by twice or more over the negative control value. In the main test, the negative and positive controls yielded the expected results. No statistically significant increases in mutant frequency were observed at any concentration after short-term treatment with or without S9, although severe cytotoxicity was observed at 4698 TOS µg/mL, the highest concentration with S9. A positive test result was obtained with continuous treatment without S9 at 4698 TOS µg/mL, the highest concentration tested. A confirmation test was conducted with continuous treatment and concentrations of 2010, 2410, 1928, 2312, 2772, 3329, 4000 and 4698 TOS µg/mL without S9. The results were negative at all tested concentrations; however, there was a concentration-dependent increase in mutation frequency.

Under the conditions of the studies, the collagenase concentrate was considered to be negative in the bacterial reverse mutation assay, equivocal in the mammalian cell gene mutation assay and negative in the in vivo micronucleus induction assay. The Committee had no concern about the genotoxicity of the collagenase enzyme concentrate.

2.3.5 Reproductive and developmental toxicity

No information was available.

2.4 Observations in humans

No information was available.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to collagenase from *S. violaceoruber*. The enzyme is intended for use in meat processing and for the production of collagen hydrolysates to be added to foods and dietary supplements; therefore, these uses were considered for the dietary exposure assessment. One of the two powdered forms of the enzyme preparation is to be used in meat processing and the other powdered form and the liquid form in the production of collagen hydrolysates. The sponsor noted that foods that could contain the enzyme are chicken and sausage casings, foods with special nutritional purposes, sports foods and other health foods. The submission included an estimate of dietary exposure based on the budget method, a screening method used to determine the theoretical maximum daily intake (TMDI) of food additives (14,15). The method takes into account maximum physiological levels of consumption of food and non-milk beverages, the energy density of foods, the concentration of the food additive in foods and non-milk beverages and the proportion of foods and non-milk beverages that may contain it. The method provides a conservative estimate of dietary exposure. Further details of the budget method can be found in chapter 6 of Environmental Health Criteria (EHC) 240 (16).

3.2 Dietary exposure assessment

The estimated TMDI provided by the sponsor was based on a number of inputs, the first being the proportion of food and non-milk beverages containing the enzyme preparation. EHC 240 (16) refers to commonly used default proportions of 12.5% for foods and 25% for non-milk beverages. Food ingredients processed with the specified collagenase preparation are proposed to be added to a variety of

foods intended to be consumed by the general population. Therefore, the sponsor used the default proportions in the budget method calculation.

The maximum level of the enzyme present in the final food from meat processing uses was 36.36 mg TOS/kg food. Use in solid foods was also from collagen hydrolysates, with a maximum level in the final food of 17.5 mg TOS/kg. This level was based on a maximum use level of ≤ 70 mg TOS/kg ingredient and a maximum amount of the ingredient in the final food of 25%. The amount of ingredient in the final food for this use was based on the maximum amount in ready-to-eat health food products on the market. The higher of the two solid food concentrations of 36.36 mg TOS/kg was used in the budget method calculation for the solid food part of the equation. For the collagen hydrolysates used in non-milk beverages, the concentration in the final beverage was 7 mg TOS/kg. This level was based on the maximum use level of ≤ 70 mg TOS/kg ingredient and the maximum amount of the ingredient in the final beverage of 10%. The amount of ingredient in the final food was based on the use of protein powder (as a surrogate for collagen hydrolysates) from the US Department of Agriculture Food and Nutrient Database for Dietary Studies 2015–16. For the collagen hydrolysates used in dietary supplements, the concentration in the final supplement was 70 mg TOS/kg (based on the maximum use level of ≤ 70 mg TOS/kg ingredient and the maximum amount of the ingredient in the final supplement of 100%). The standard budget method calculation was undertaken for estimating the dietary exposure to the TOS from solid foods and non-milk beverages. A separate calculation was undertaken to determine the exposure from dietary supplements based on a maximum daily dose of collagen from supplements on the US market of 24 g/day and a body weight of 60 kg.

The resulting TMDIs of collagenase were estimated to be 0.227 mg TOS/kg bw per day for solid foods, 0.175 mg TOS/kg bw per day for non-milk beverages and 0.028 mg TOS/kg bw per day for dietary supplements, for a total of 0.43 mg TOS/kg bw per day.

For the dietary exposure assessment, it was assumed that the enzyme is not removed and/or denatured during final processing of ingredients or foods and that 100% of the enzyme remains in the ingredient and final food. In reality, the enzyme is inactivated by high temperatures during processing of food ingredients, such that it will have no technological function in the final food.

4. Comments

4.1 Assessment of potential allergenicity

Collagenase from *S. violaceoruber* pCol. was assessed as a potential allergen with bioinformatics, consistent with the criteria recommend by FAO/WHO and others (5–7). The amino acid sequence of the enzyme was compared with the sequences of known allergens present in two online databases. No statistically significant matches were found in either database. The Committee concluded that the enzyme was not anticipated to pose an allergenic risk.

4.2 Toxicological studies

A study of acute oral toxicity in rats (8) was conducted with the enzyme concentrate (TOS: 93.6%), which was mixed in water and administered by gavage. The oral LD₅₀ of the enzyme concentrate was > 2000 mg/kg bw enzyme concentrate, equal to 1879.2 mg TOS/kg bw.

In a 13-week study of oral toxicity in rats (9), a powdered enzyme concentrate (TOS: 93.96%) was mixed in water and administered by gavage at 1000 mg /kg bw per day, equal to 939.6 mg TOS/kg bw per day, for 90 days. Adverse effects included several anomalies in blood chemistry, but these were observed only in one high-dose female. The cause of these changes could not be established; however, the Committee noted that the changes were extreme. The findings were considered not to be due to treatment, as only one animal was affected, and there was no indication of similar effects in any other animal. The results for the one animal were therefore excluded. The Committee identified a NOAEL of 940 mg TOS/kg bw per day (rounded by the Committee from 939.6), the highest dose tested.

A powdered enzyme concentrate (TOS content, 93.96%) was tested for genotoxicity in a bacterial reverse mutation test, an in vitro mammalian cell gene mutation assay (mouse lymphoma TK assay) and an in vivo micronucleus induction assay in rats (10–13). The results of the bacterial reverse mutation assay were negative, those of the mammalian cell gene mutation assay were equivocal, and those of the in vivo micronucleus induction assay were negative. The Committee had no concern about the genotoxicity of the collagenase enzyme concentrate.

4.3 Assessment of dietary exposure

The Committee evaluated an estimate of the TMDI of the collagenase enzyme preparation conducted with the budget method. The TMDI was based on the level of TOS in the collagenase enzyme preparation and its maximum proposed use levels (equivalent to ≤ 36.36 mg TOS/kg in solid foods and ≤ 7 mg TOS/kg in non-milk beverages) and an assumption that 12.5% of solid foods and 25% of the non-milk beverages contain the enzyme preparation. The TMDI also included exposure to dietary supplements based on maximum proposed use levels (70 mg TOS/kg) and a daily dose of 24 g/day. The resulting TMDI was 0.43 mg TOS/kg bw per day from solid food, non-milk beverages and dietary supplements. For the dietary exposure assessment, it was assumed that 100% of the enzyme remains in the final food. The Committee noted that the enzyme is inactivated during the processing of food ingredients and will have no function in the final food.

5. Evaluation

The Committee identified a NOAEL of 940 mg TOS/kg bw per day, the highest dose tested in a 13-week study of oral toxicity in rats. When this NOAEL was compared with the estimated dietary exposure of 0.43 mg TOS/kg bw per day, the MOE was > 2100 . In view of this MOE and the lack of concern about genotoxicity, the Committee established an ADI “not specified”² for collagenase from *S. violaceoruber*, when used in the applications specified and in accordance with good manufacturing practice.

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β -Glucanase from *Streptomyces violaceoruber* expressed in *S. violaceoruber*

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1. Explanation

At the request of the Codex Committee on Food Additives at its Fifty-first Session (1), the Committee evaluated the safety of β -glucanase (Enzyme Commission No.3.2.1.39; Chemical Abstract Services No. 9025-37-0) from *Streptomyces*

violaceoruber pGlu. The Committee had not evaluated this enzyme preparation previously.

The enzyme catalyses the hydrolysis of (1,3)- β -D-glucosidic linkages in (1,3)- β -D-glucans. The enzyme preparation is intended for use as a processing aid in the production of beer and in the manufacture of yeast and mushroom extracts for use as ingredients in seasonings.

In this monograph, the expression “ β -glucanase” refers to the β -glucanase enzyme and its amino acid sequence, the expression “enzyme concentrate” refers to the test material used in the toxicity studies, and the expression “enzyme preparation” refers to the product formulate for commercial use.

At the present meeting, the Committee considered the submitted data and conducted a literature search in Google Scholar with the linked search terms “ β -glucanase” AND “*Streptomyces violaceoruber*”, which identified 25 references; however, none were relevant for this toxicological evaluation.

1.1 Genetic background

The production organism, *S. violaceoreuber*, also referred to as *S. lividans* or *S. coelicolor*, belongs to the genus *Streptomyces*. *S. violaceoreuber* is non-pathogenic, non-toxicogenic, occurs in nature as a component of soil (2) and has a history in the production of enzymes intended for use in food processing (3).

The *S. violaceoreuber* pGlu production strain was obtained by transforming a plasmid containing the following: a promoter sequence obtained from *S. cinnamoneus* TH 2, the β -glucanase gene obtained from *S. violaceoruber* NBRC 15146 and a terminator sequence obtained from *S. cinnamoneus* NRBC 12852. The stability of the introduced sequences was confirmed by cultivating the production strain over multiple generations and measuring β -glucanase activity each time. The final enzyme preparations were tested for the absence of antibiotic resistance genes by PCR. The production strain has been deposited at the National Institute of Technology and Evaluation in Japan.

1.2 Chemical and technical considerations

β -Glucanase (designated EC No. 3.2.1.39 by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology) is produced by controlled fermentation of a pure culture of the *S. violaceoruber* production strain. The manufacture of the β -glucanase enzyme preparation includes fermentation (seed, pre- and main culture), recovery and formulation. After fermentation, the broth containing the β -glucanase enzyme is separated from the biomass; this is followed by multiple filtration steps and dispersion at controlled temperature,

pressure and pH. The resulting precipitate is formulated with glycerol to the final β -glucanase enzyme preparation. A powdered enzyme preparation is produced by further filtering and freeze-drying the liquid formulation, followed by standardization with sodium chloride. The entire process is performed in accordance with current good manufacturing practice and with food-grade raw materials. The primary sequence of β -glucanase produced by *S. violaceoruber* consists of 453 amino acids; its molecular weight by calculation from the determined amino acid sequence is 42.7 kDa. The enzyme concentrate is tested to be free from the production organism and any antibiotic activity.

The activity of β -glucanase is determined spectrophotometrically by measuring the hydrolysis of 1,3- β -D-glucan substrate by the enzyme at 490 nm; one unit of activity is defined as the quantity of enzyme required to catalyse the formation of 1 μ mol/min of glucose under the conditions of the assay. The mean activity of β -glucanase from three batches of the liquid and powder enzyme concentrates were 12 897 U/g and 23 041 U/g, respectively.

β -Glucanase catalyses the hydrolysis of the (1 \rightarrow 3)- β -D-glucosidic linkages in (1 \rightarrow 3)- β -D-glucans to produce D-glucose and β -glucans. The enzyme preparations are intended for use as processing aids in the manufacture of yeast and mushroom extracts for use as ingredients in seasonings and in the production of beer. The enzyme preparation is added to disrupt the cell walls of mushroom and yeast raw material to improve yield and residual filtration of the extract products; it is used as a clarifying and filtration aid in the production of beer.

The β -glucanase enzyme preparations are intended to be used at maximum levels of 151 mg TOS of powdered β -glucanase per kilogram raw material (TOS/kg) and 202 mg TOS of liquid β -glucanase/kg raw material. The TOS includes the enzyme of interest and residues of organic materials, such as proteins, peptides and carbohydrates, derived from the production organism during the manufacturing process.

The β -glucanase enzyme is inactivated by heat treatment during processing. It is not expected to have any technological function in the finished foods. If present in the finished food, it would probably be digested, like most other proteins, although no data were available on its digestibility.

2. Biological data

2.1 Biotransformation

No information was available.

2.2 Assessment of potential allergenicity

β -Glucanase from *S. violaceoruber* pGlu was assessed as a potential allergen by bioinformatics, consistent with the criteria recommend by FAO/WHO (4–6). The amino acid sequence of the enzyme (453 amino acids; 42.7 kDa) was compared with the sequence of known allergens present in the AllergenOnline (Version 21, updated 14 February 2021; <http://www.allergenonline.org/databasefasta.shtml>) and Allermatch (updated 31 March 2021; <http://allermatch.org/>) databases. A search for matches with > 35% identity over a sliding window of 80 amino acids and a search for sequence identity of eight contiguous amino acids were conducted in both databases.

In the AllergenOnline database, a full-length FASTA sequence search showed an identity score of 25% with an E-value of 0.86.³ A search with an 80-mer sliding window produced one match to collagen α -2(I) chain isoform X1 from *Salmo salar* (Atlantic salmon), which shared 36.29% identity. No matches were identified in the 8-mer search. The exceedance of the 35% identity threshold in the 80-mer sliding window search was considered slight, and the absence of significant homology in the full-length searches and the lack of matches in the eight amino acid exact searches suggested that β -glucanase would probably not cross-react with known allergens.

In the Allermatch database, a full-length sequence search with the Basic Local Alignment Search Tool (BLAST) produced matches of 38.4% with an E-value of 0.018 for collagen α -1(I) chain-like isoform X1 from *Lates calcarifer* (Asian sea bass) and 25.0% with an E-value of 0.87 collagen α -2(I) chain-like isoform X1 from *S. salar*. A search with an 80-mer sliding window produced matches that were 38.71% and 36.29% for collagen α -1(I) chain-like isoform X1 from *L. calcarifer* and *S. salar*, respectively. No exact matches were found in the eight amino acid exact search. Again, the exceedance of the 35% identity threshold in the 80-mer sliding window was considered slight, and the absence of significant homology matches in the full-length search and lack of matches in the eight amino acid exact searches suggested that β -glucanase would probably not cross-react with known allergens.

³ Comparisons between highly homologous proteins yield expectation values (E-values) approaching zero, indicating very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. The E-value selected for a search tends to be larger with searches of short sequences ($E < 0.1$) than with long sequences ($E < 1 \times 10^{-7}$), as the likelihood of random matches is greater in the search of shorter sequences.

2.3 Toxicological studies

2.3.1 Acute toxicity

A study of acute oral toxicity that was compliant with GLP was conducted in Sprague-Dawley SPF CrI:CD(SD) rats according to OECD test guideline 423 (7). The test material was a powdered concentrate of β -glucanase from *S. violaceoruber* pGlu (TOS: 95.33%) mixed in water. A group of three females, 8 weeks old at the start of treatment, received a dose of 2000 mg/kg bw, equal to 1906.6 mg TOS/kg bw, by oral gavage at a dose volume of 10 mL/kg bw. The experiment was repeated with a second group of three females. In both experiments, the animals were observed for up to 14 days and then necropsied. No deaths, clinical signs of toxicity or anomalies in body weight gain or body weight were observed in either experiment. Gross pathological examination showed no abnormalities. The LD₅₀ of the enzyme concentrate was > 2000 mg/kg bw, equal to 1906.6 mg TOS/kg bw.

2.3.2 Short-term studies of toxicity

A 2-week oral dose range-finding toxicity study was conducted in Sprague-Dawley strain SPF rats (Crj:CD(SD) IGS) according to the Guideline for proper conduct of animal experiments of the Science Council of Japan and other guidance (Act on Welfare and Management of Animals, 1973, 2006; Standards for raising and storing laboratory animals and reducing pain, 2006). The study was reviewed by the reliability assurance department in the research centre in which the study was conducted (8).

The test material was a powdered concentrate of β -glucanase from *S. violaceoruber* (TOS: 95.33%), which was mixed in water at concentrations to deliver doses in a volume of 10 mL/kg bw. Groups of five animals of each sex per dose group, 6 weeks old at the start of treatment, received a single oral gavage dose of 0, 100 or 1000 mg enzyme concentrate/kg bw per day (equal to 0, 95.33 and 953.3 mg TOS/kg bw per day, respectively) for 14 days. Animals consumed feed and water ad libitum throughout the study, except for an overnight fast before termination (day 14). Animals were monitored for mortality and clinical signs of toxicity three times each day, and body weights and food consumption were measured on days 1, 4, 7 and 14. Blood samples were collected for haematology and clinical chemistry immediately before termination. The haematological parameters studied were: red blood cell count, haemoglobin concentration, haematocrit, average red blood cell haemoglobin, platelet count and white blood cell count. Blood smears were prepared from all animals, but no microscopy was performed. The clinical chemistry parameters measured were alkaline phosphatase activity, total cholesterol, glucose, urea nitrogen, total protein and aspartate and alanine aminotransferase and lactate dehydrogenase activity. At termination, all animals were necropsied and examined for gross anomalies.

Absolute and relative organ weight-to-body weight values were recorded, and the adrenal gland, spleen, heart, lung, liver, kidney and testes/ovaries were assessed. Histopathological sections were prepared from the thyroid, parathyroid, adrenal gland, spleen, heart, lungs, tongue, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, liver, kidney and testes/ovary of all animals, but no histopathological findings were reported. Appropriate statistical analysis was conducted on the data collected on body weight, food consumption, haematology, clinical chemistry and organ weights.

The results showed no significant differences between control and treated groups with respect to any of the parameters measured. A single exception was a statistically significant increase in relative heart weight to body weight in the group of female rats given 1000 mg/kg bw per day enzyme concentrate as compared with female controls (dose: relative heart weight to body weight \pm SD; 0, 100, 1000 mg/kg bw per day enzyme concentrate: 0.38 ± 0.02 , 0.41 ± 0.02 , 0.41 ± 0.03 ; $P < 0.05$, Dunnett's test). The Committee considered that these findings were not toxicologically relevant, as they were slight and were not reproduced in a more robust 13-week study of oral toxicity (i.e., with longer duration, more parameters assessed and more animals per dose group). On the basis of these results, the doses for the 13-week oral toxicity were set at 0, 40, 200 and 1000 mg/kg bw per day (equal to 0, 38.13, 190.66, and 953.3 mg TOS/kg bw per day, respectively).

A 13-week oral toxicity study in Sprague-Dawley SPF CrI:CD(SD) rats was conducted according to GLP (9) and OECD test guideline 408 (10,11). The test material was a powdered β -glucanase concentrate (TOS: 95.33 %), which was mixed in water. Groups of 10 animals of each sex per dose group, 6 weeks old at the start of treatment, received a single oral gavage dose of 0, 40, 200 or 1000 mg/kg bw per day of enzyme concentrate (equal to 0, 38.13, 190.66 and 953.3 mg TOS/kg bw per day, respectively) for 90 days. The animals consumed feed and water ad libitum throughout the study, except for an overnight fast before scheduled blood sampling. The parameters measured in all animals, were general health (observed three times per day), clinical signs of toxicity (detailed observations made once before the start of treatment and then weekly), neurobehavioural testing (observations made once before the start of treatment and then at week 12), ophthalmoscopic examination (once before the start of treatment and then at week 12), body weights (measured three times in the first week and then twice weekly), food intake (measured twice in the first week and then weekly), water consumption (measured on the day before urinalysis), haematology, blood chemistry and urinalysis (all measured at the end of treatment). At termination, necropsy was conducted on all animals, absolute organ weights were measured (and organ weights relative to body weights were calculated), and samples were collected for microscopic examination.

Table 1
Results of urinalysis

Sex	Male			
Dose (mg/kg bw per day)	0	40	200	1000
Number of animals	10	10	10	10
Water intake (mL/24 h)	33 ± 5	35 ± 5	41 ± 11	42* ± 9
Osmolality (mOsm/kg)	2025 ± 274	2117 ± 292	2024 ± 348	2068 ± 280
Chloride (mmol/24 h)	2.7 ± 0.2	2.8 ± 0.7	3.4* ± 0.8	3.6** ± 0.8
Urine volume (mL/24 h)	13.8 ± 1.9	13.7 ± 4.2	16.8 ± 5.0	18.0 ± 3.7
Urine pH				
5.5	0	0	0	0
6.0	0	0	0	10
6.5	0	0	5	0
7.0	0	0	3	0
7.5	0	3	2	0
8.0	1	1	0	0
8.5	5	6	0	0
9.0	4	0	0	0
Sex	Female			
Dose (mg/kg bw per day)	0	40	200	1000
Number of animals	10	10	10	10
Water intake (mL/24 h)	28 ± 4	37 ± 11	33 ± 5	33 ± 10
Osmolality (mOsm/kg)	2157 ± 265	1752 ± 176	1742 ± 504	1941 ± 537
Chloride (mmol/24 h)	1.7 ± 0.4	1.8 ± 0.4	1.6 ± 0.4	2.0 ± 0.7
Urine volume (mL/24 h)	8.3 ± 2.1	10.6 ± 2.9	10.3 ± 4.5	11.8 ± 6.2
Urine pH				
5.5	0	0	0	1
6.0	0	0	4	7
6.5	1	0	1	0
7.0	3	1	4	2
7.5	0	1	1	0
8.0	1	2	0	0
8.5	4	4	0	0
9.0	1	2	0	0

The values are group mean values ± SD.

* $P \leq 0.05$; ** $P \leq 0.01$; statistically significantly different from control s; Dunnett's two-sided test.

The mean laboratory control value of water intake for males ($n = 226$) was 39 ± 9 mL/24 h (range, 23–75 mL/24 h).

The mean laboratory control value of chloride excretion for males ($n = 254$) was 2.57 ± 0.65 mmol/24 h (range, 0.58–4.30 mmol/24 h.)

All the animals survived, and no treatment-related clinical signs of toxicity were observed in any group. One remarkable observation in this study, however, was that the mean pH of excreted urine appeared to decrease (i.e., became more acidic) with increasing dose in animals of each sex (Table 1). At the highest dose of 1000 mg/kg bw per day, 10/10 males and 8/10 females excreted

urine with a pH ≤ 6 , while the pH of urine from most control animals was about 8.5. Although water intake also increased with increasing dose in both sexes, it was largely matched by a corresponding volume of excreted urine, of which the osmolality was not appreciably different from that of controls. The study authors noted that the pH of the powdered β -glucanase suspensions administered to the low-, middle- and high-dose groups was 4.68, 4.78, and 5.00, respectively. They concluded that the tendency towards a lower urinary pH of treated rats was a reactive change reflecting ingestion of acidic enzyme suspensions.⁴

A statistically significant reduction in the percentage of lymphocytes (10%) and a significant increase in the percentage of neutrophils (50%) in the differential white blood cell ratio were observed in the group of females treated with 1000 mg enzyme concentrate/kg bw per day as compared with the control group (Table 2). This observation was considered not to be toxicologically significant, as there were no significant differences in the numbers of lymphocytes or neutrophils between the two groups.

Statistically significant increases in relative liver and kidney weights were observed in males treated with 1000 mg/kg bw per day as compared with the control group (Table 3). These findings were considered not toxicologically significant, as the absolute organs weights in treated animals were not significantly different from organ weights in control animals, no significant change was seen in organ function (as indicated by clinical chemistry), and there were no histopathological anomalies.

An observation possibly related to treatment was an increased incidence of pancreatic focal acinar atrophy in males. The incidence of these lesions was 0/10, 3/10, 1/10 and 3/10 in the groups given 0, 40, 200 and 1000 mg/kg bw per day, respectively. The severity was graded as minimal, exception for one mild lesion in the highest dose group. The laboratory control incidence of the lesion in males was 0–30%; all lesions were graded as minimal. The lesion was considered not to be toxicologically significant because of the absence of a dose–response relation in incidence or severity, and the incidence was within normal variation.

A treatment-related observation was the presence of hyperplasia of squamous cells in the forestomach, which was noted only in males and females treated with 1000 mg/kg bw per day of the enzyme concentrate (Table 4). The incidence and severity were greater in males than in females, but the severity was graded as only mild in the worst cases.

The study authors concluded that the hyperplasia observed in the forestomach of the high-dose groups was the critical finding and determined a

⁴ Although the pH of the urine became more acidic with increasing dose, the test material was not more acidic with increasing dose. The sponsor explained that this phenomenon was the result of the physical properties of the test material. The low and mid-doses were suspensions and the high dose was a slurry. These conditions made pH measurement complicated and less precise.

Table 2
Results of haematological examinations

Sex	Female			
Dose (mg/kg bw per day)	0	40	200	1000
Number of animals	10	10	10	10±
Lymphocytes (%)	79.9 ± 5.1	78.1 ± 6.0	76.4 ± 5.5	71.8 ± 8.6*
Lymphocytes (102/μL)	40.7 ± 9.8	39.6 ± 12.9	32.1 ± 6.6	33.1 ± 14.8
Neutrophils (%)	15.9 ± 5.3	17.5 ± 5.8	18.9 ± 4.8	23.9 ± 8.1*
Neutrophils (102/μL)	8.1 ± 3.1	8.6 ± 3.2	8.0 ± 3.0	9.9 ± 2.7

The values are group mean values ± SD.

* $P \leq 0.05$; statistically different from the control group; Dunnett's two-sided test.

Table 3
Mean body, liver and kidney weights in males

Sex	Male			
Dose (mg/kg bw per day)	0	40	200	1000
Number of animals	10	10	10	10
Body weight (g)	529	542	520	536
Liver				
Absolute weight (g)	12.74	13.70	12.79	14.00
Relative weight (g/100 g)	2.41	2.52	2.46	2.61*
Kidney				
Absolute weight (g)	3.20	3.32	3.20	3.52
Relative weight (g/100 g)	0.60	0.61	0.62	0.65*

The values are group mean values ± SD.

* $P \leq 0.05$; statistically dsignificantly different from the control group; Dunnett's two-sided test

Table 4
Results of histopathological examination of the stomach

Sex	Male				Female			
Dose (mg/kg bw per day)	0	40	200	1000	0	40	200	1000
Number of animals	10	10	10	10	10	10	10	10
Stomach Minimal hyperplasia, squamous, forestomach	0	0	0	6	0	0	0	2
Mild	0	0	0	2	0	0	0	0

The severity of the lesions was graded as minimal, mild, moderate or severe.

NOAEL of 200 mg/kg bw per day. The Committee considered that this treatment-related effect was probably due to repeated dosing by gavage of the enzyme concentrate, which was an acidic substance and caused local irritation at a high concentration (see footnote 4). The finding was considered not toxicologically significant because humans who ingest these materials are unlikely to experience localized high concentrations. Further, because of differences in anatomy and function between rodents and humans, the lesion is considered not relevant to human health risk.

In the absence of any adverse treatment-related effects relevant to humans, a NOAEL of 950 mg TOS/kg bw per day was identified (rounded by the Committee from 953.3), the highest dose tested.

2.3.3 Long-term studies of toxicity

No information was available.

2.3.4 Genotoxicity

A powdered form of the β -glucanase enzyme concentrate, dissolved in water, was tested for genotoxicity in a bacterial reverse mutation assay (TOS: 95.33%) (12), a chromosome aberration assay in a Chinese hamster cell line (TOS: 95.33%) (13), an in vitro micronucleus assay in cultured human peripheral lymphocytes (TOS: 95.33%) (14) and an in vivo micronucleus assay in Sprague-Dawley rats (TOS: 97.05%) (15). All the studies complied with GLP and were conducted in accordance with the appropriate OECD test guideline (471, 473, 487, and 474, respectively).

The results of the bacterial reverse mutation, in vitro micronucleus and in vivo micronucleus assays were negative. Those of the chromosome aberration assay were positive after a 6-h exposure to the test material with metabolic activation, causing structural chromosome aberration in a concentration-dependent manner. No significant numerical aberrations were observed. Negative results were observed after 6 h of exposure in the absence of metabolic activation. According to the study authors, longer exposures (24 and 48 h) were not assessed because of the positive results after short exposure (6 h) with metabolic activation. The basis for the positive results was not explained.

The results provide evidence that the β -glucanase enzyme concentrate is not mutagenic in vitro and is not clastogenic in vivo. The enzyme concentrate was clastogenic in the in-vitro chromosomal aberration assay in a Chinese hamster lung cell line but not clastogenic in an in-vitro micronucleus assay in cultured human peripheral lymphocytes. Overall, the Committee had no concern about the genotoxicity of the β -glucanase enzyme concentrate (Table 5).

Table 5
Genotoxicity of β-glucanase enzyme concentrate

End-point	Test system	Concentration	Result	Reference
In vitro				
Reverse mutation	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537, <i>Escherichia coli</i> WP2 uvrA	298-4767 TOS µg/plate ± S9	Negative	12
Chromosomal aberrations ^a	Chinese hamster lung cell line (CHL/IU)	6 h exposure: 1907 TOS µg/mL +S9	Positive	13
		1268 TOS µg/mL +S9	Equivocal	
		565-847 TOS µg/mL +S9	Negative	
		1268-4290 TOS µg/ mL -S9	Negative	
		24 h exposure: 847-2860 TOS µg/mL -S9	Not assessed	
Micronucleus assay	Cultured human peripheral lymphocytes	4 h exposure: 238-1907 TOS µg/mL ±S9	Negative	14
		24 h exposure: 119-953 TOS µg/mL -S9	Negative	
In vivo				
Micronucleus assay	Sprague-Dawley SPF rat	485-1941 TOS mg/kg bw	Negative	15

S9, 9000 × g supernatant fraction from rat liver homogenate

^a For the chromosome aberration assays, the highest concentrations were determined from the results of preliminary cell growth inhibition tests. Cells in culture were exposed to the test material for 6 h with or without metabolic activation and were then rinsed and fresh media was added for another 18 h without metabolic activation. After a total of 22 h, cells were treated with colcemid, and, 2 h later, were harvested, and chromosome spreads were prepared. In a second test, cells in culture were exposed continuously to the test material for 24 h or 48 h without metabolic activation. Two hours before the end of the treatment period, the cells were treated with colcemid; then, 2 h later, the cells were harvested and chromosome spreads were prepared. The numbers of cells with structural aberrations and their types were recorded for 200 well-spread metaphases in each group. The number of polyploidy cells was recorded at the same time. Chromosomal aberrations were classified as numerical or structural (structural aberrations were further classified). The results of the 24-h and 48-h treatments without metabolic activation were not assessed as a positive result was observed in the 6-h test.

2.3.5 Reproductive and developmental toxicity

No information was available.

2.4 Observations in humans

No information was available.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to β -glucanase from *S. violaceoruber*. The enzyme is intended for use in beer manufacture and in the production of seasonings from yeast and mushroom extracts; therefore, these uses were considered for the dietary exposure assessment. The liquid form of the enzyme preparation is to be used in beer manufacture and both the liquid and powdered forms in the production of seasonings. The submission included an estimate of dietary exposure based on the budget method, a screening method used to determine the theoretical maximum daily intake (TMDI) of food additives (16,17). The method accounts for maximum physiological levels of consumption of food and non-milk beverages, the energy density of foods, the concentration of the food additive in foods and non-milk beverages and the proportion of foods and non-milk beverages that may contain it. The method provides a conservative estimate of dietary exposure. Further details of the budget method can be found in chapter 6 of EHC 240 (18).

3.2 Dietary exposure

The estimated TMDI provided by the sponsor was based on a number of inputs, the first being the proportion of food and non-milk beverages containing the enzyme preparation. EHC 240 (18) refers to commonly used default proportions of 12.5% for foods and 25% for non-milk beverages. Food ingredients processed with the specified β -glucanase preparation are proposed to be added to a variety of foods intended to be consumed by the general population. The proportion of both solid foods and non-milk beverages used in the budget method by the sponsor was 25%. This was higher than the commonly used default for solid foods stated in EHC 240 (18) because of the proposed use in seasonings, which the sponsor noted would result in a broader range of foods potentially containing the enzyme preparation.

The maximum level of the enzyme present in the final food was based on the maximum use level of the ingredient (the highest of the powdered and liquid uses for seasonings of 202 mg TOS/kg ingredient was considered a worst case for the solid food part of the budget method calculation) and the maximum amount of the ingredient in the final foods (4%). The amount of ingredient in the final food was derived from recipe databases from the United Kingdom Food Standards Agency and the US Department of Agriculture. This resulted in a maximum level of the enzyme in the final solid foods of 8.08 mg TOS/kg.

The maximum level of the enzyme present in final beverages was 1.9 mg TOS/kg. The resulting TMDIs of β -glucanase were estimated to be 0.101 mg TOS/kg bw per day for solid foods and 0.048 mg TOS/kg bw per day for non-milk beverages, for a total of 0.149 mg TOS/kg bw per day.

For the dietary exposure assessment, it was assumed that the enzyme is not removed and/or denatured during final processing of ingredients or foods and that 100% of the enzyme remains in the ingredient and final food. In reality, the enzyme is inactivated by high temperatures during processing of food ingredients such that it will have no technological function in the final food.

4. Comments

4.1 Assessment of potential allergenicity

The enzyme β -glucanase was assessed as a potential allergen with bioinformatics, consistent with the criteria recommend by FAO/WHO (4), Codex Alimentarius (5) and JECFA (6). The amino acid sequence of the enzyme was compared with the sequences of known allergens present in two publicly available databases. A search for matches with > 35% identity over a sliding window of 80 amino acids and a search for sequence identity with eight contiguous amino acids produced a small number of matches. Upon further examination, however, these matches were considered not significant. In view of the intended use and available information, the Committee anticipated that β -glucanase would not pose an allergenic risk.

4.2 Toxicological studies

A study of acute oral toxicity in rats (7) was conducted with the enzyme concentrate mixed in water and administered as a single gavage dose. The oral LD₅₀ was > 2000 mg/kg bw, equal to 1906.6 mg TOS/kg bw.

In a 2-week dose range-finding study in rats (8), no significant toxicity was observed when the enzyme concentrate was mixed in water and administered by gavage at doses up to 953.3 mg TOS/kg bw.

In a 13-week study of oral toxicity in rats (11), the enzyme concentrate was mixed in water and administered by gavage at doses up to 953.3 mg TOS/kg bw. The only treatment-related observation was hyperplasia of the forestomach in male and female rats at the high dose. This was considered not to be related to systemic toxicity but rather an artefact of gavage with increasing concentrations of

an acidic substance, which resulted in local irritation. The Committee identified a NOAEL of 950 mg TOS/kg bw per day (rounded by the Committee from 953.3), the highest dose tested.

The enzyme concentrate gave negative results in a bacterial reverse mutation assay, an in vitro micronucleus assay and an in vivo micronucleus assay (12–14). The enzyme concentrate gave negative results in an in vitro chromosomal aberration assay without metabolic activation and positive results with metabolic activation, after 6 h of exposure. Under the conditions of the in vitro chromosomal aberration assay with metabolic activation, the enzyme concentrate caused structural aberrations but did not induce polyploidy aberrations (13). The Committee had no concern about the genotoxicity of the enzyme concentrate.

4.3 Assessment of dietary exposure

The Committee evaluated an estimate of the TMDI of the β -glucanase enzyme preparation derived with the budget method. The TMDI was based on the level of TOS in the β -glucanase enzyme preparation and its maximum proposed use levels (equivalent to ≤ 8.08 mg TOS/kg in solid foods and ≤ 1.9 mg TOS/kg in non-milk beverages) and on the assumption that 25% of the food supply contains the enzyme preparation. The resulting TMDI was 0.15 mg TOS/kg bw per day (rounded by the Committee from 0.149) from both solid food and non-milk beverages. For the dietary exposure assessment, it was assumed that 100% of the enzyme remains in the final food. The Committee noted that the enzyme will be inactivated during the processing of food ingredients and will have no technical function in the final food.

5. Evaluation

The Committee identified an NOAEL of 950 mg TOS/kg bw per day, the highest dose tested in the 13-week study of oral toxicity in rats. Comparison of this NOAEL with the estimated dietary exposure of 0.15 mg TOS/kg bw per day gave an MOE > 6300 . On the basis of this MOE and lack of concern about genotoxicity, the Committee established an ADI “not specified”⁵ for β -glucanase from *S. violaceoruber* for the proposed uses and in accordance with good manufacturing practice.

⁵ The reader is referred to the Technical Report of the 87th JECFA meeting (Annex 1, reference 243) for clarification of the term “ADI not specified”.

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Phospholipase A2 from *Streptomyces violaceoruber* expressed in *S. violaceoruber*

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1. Explanation

At the request of the CCFA at its fifty-first Session (1), the Committee evaluated the safety of phospholipase A2 (Enzyme Commission No. 3.1.1.4) from *Streptomyces violaceoruber* for the first time.

In this report, the term “phospholipase A2” refers to the phospholipase A2 enzyme and its amino acid sequence, the term “enzyme concentrate” refers to the test material used in the toxicity studies, and the term “enzyme preparation” refers to the product formulated for commercial use.

The Committee at its present meeting considered the submitted data and conducted a literature search in the PubMed (all fields), Scopus (title, abstract, keywords) and Embase (title, abstract, keywords) with the linked search terms “phospholipase A2” and “streptomyces” or “violaceoruber”. The search yielded 118 unique references, none of which reported biochemical and/or toxicological studies on phospholipase A2 from *S. violaceoruber*.

1.1 Genetic background

The production organism, *S. violaceoruber*, also referred to as *S. lividans* or *S. coelicolor*, belongs to the genus *Streptomyces*. *S. violaceoruber* is non-pathogenic and non-toxicogenic and occurs in nature as a component of soil (2). It has a history of use in the production of enzymes intended for use in food processing (3).

The *S. violaceoruber* AS-10 production strain was obtained by transforming a plasmid containing an expression cassette with the phospholipase A2 encoding gene from *S. violaceoruber* NBRC 15146 donor, a suitable promoter and terminator encoding phospholipase D from *S. cinnamoneum* and a selectable marker, ligated with a plasmid obtained from *S. violaceoruber* ATCC 35287. The resulting plasmid was incorporated into the host organism, *S. violaceoruber* 1326, by electroporation. The stability of the introduced sequences was confirmed by cultivating the production strain over three generations and by measuring phospholipase A2 activity each time. The final enzyme preparations were tested for the absence of an antibiotic resistance gene by PCR. The production strain has been deposited at the National Institute of Technology and Evaluation in Japan.

1.2 Chemical and technical considerations

Phospholipase A2 is produced by controlled submerged fermentation of a pure culture of the *S. violaceoruber* production strain. Manufacture of the phospholipase A2 enzyme preparation includes fermentation (pre-, seed and main fermentation), recovery and formulation. After fermentation, the broth containing phospholipase A2 enzyme is separated from the biomass by sedimentation; this is followed by several filtration steps. The resulting liquid filtrate is formulated with water, sorbitol, potassium sorbate and sodium chloride to obtain the liquid phospholipase A2 enzyme preparation. A powdered enzyme preparation is produced by further filtering and freeze-drying the liquid filtrate,

followed by formulation with sodium chloride. The entire process is performed in accordance with current good manufacturing practices and with food-grade raw materials. The primary amino acid sequence of phospholipase A2 produced by *S. violaceoruber* consists of 151 amino acids; its molecular weight, calculated from the determined amino acid sequence, is 16.4 kDa. The enzyme preparation is tested for the absence of any of the major food allergens that are present in the fermentation medium. The enzyme concentrate is tested to ensure that it contains neither the production organism nor any antibiotic activity.

The activity of phospholipase A2 is determined spectrophotometrically by measuring the hydrolysis of a phosphatidylcholine substrate by the enzyme at 550 nm; one unit of activity is defined as the quantity of enzyme required to liberate 1 $\mu\text{mol}/\text{min}$ of fatty acid from L- α -phosphatidylcholine under the conditions of the assay. The mean activities of phospholipase A2 from three batches of the liquid and the powder enzyme concentrates are 10 400 U/g and 114 200 U/g, respectively.

Phospholipase A2 catalyses the hydrolysis of the SN-2 ester bonds of diacylphospholipids to form 1-acyl-2-lysophospholipids and free fatty acids; when added to food, this improves emulsification. The enzyme preparation is intended for use as a processing aid in the manufacture of enzyme-modified egg yolk, lecithin, cereal flour, dairy products and vegetable oil. The phospholipase A2 enzyme preparation is intended to be used as a processing aid at a maximum level of 105 mg total organic solids (TOS) of powdered phospholipase A2/kg raw material and 459 mg TOS of liquid phospholipase A2/kg raw material. The TOS includes the enzyme of interest and residues of organic materials, such as proteins, peptides and carbohydrates, derived from the production organism during the manufacturing process.

The phospholipase A2 enzyme is inactivated by heat treatment before use of the final foods. If present, it is expected that phospholipase A2 will be digested, as would most other protein occurring in food, but no data were available on its digestibility.

2. Biological data

2.1 Biotransformation

No data were available.

2.2 Assessment of potential allergenicity

Phospholipase A2 from *S. violaceoruber* was evaluated for potential allergenicity by the bioinformatics criteria recommended by FAO/WHO (4,5), as modified at the eightieth meeting of the Committee (Annex 1, reference 223). A homology search was conducted, in which the amino acid sequence of phospholipase A2 from *S. violaceoruber* was compared with the amino acid sequences of known allergens in the AllergenOnline database (<http://www.allergenonline.org/databasefasta.shtml>; version 19, February 2019) and in the Allermatch database (<http://allermatch.org/>; version July 2019). A search for matches with > 35% identity in a sliding window of 80 amino acids and a search for exact matches in an 8-amino acid window produced no matches. Additionally, a full-length FASTA sequence search was conducted with an E-value⁶ cut off of 0.1. No sequences were considered homologous with known allergens. Therefore, the Committee considered that dietary exposure to phospholipase A2 from *S. violaceoruber* is not anticipated to pose a risk of allergenicity. No data were available on the digestibility of phospholipase A2 in the gastrointestinal tract.

2.3 Toxicological studies

Toxicological studies of phospholipase A2 from *S. violaceoruber* AS-10 have been conducted with a powdered enzyme concentrate (Lot No. 04-3t-01, total organic solids [TOS], 95.6%; phospholipase A2 enzyme activity, 263,200 U/g).

2.3.1 Acute toxicity

In an oral acute toxicity study, two groups of three female Sprague-Dawley rats were given powdered enzyme concentrate as a suspension in water at a single dose of 2000 mg/kg bw (equal to 1912 mg TOS/kg bw) by gavage (7). The study was conducted according to the Organization for Economic Co-operation and Development (OECD) test guideline 423 (Acute Oral Toxicity – Acute Toxic Class Method, 2001) and was certified for compliance with good laboratory practice (GLP) and quality assurance (QA). Animals were observed for mortality and clinical signs frequently during the first 6 h after administration and then once daily during 14 days. Body weights were measured immediately before dosing and on days 1, 3, 7 and 14. After 14 days, the animals were killed by exsanguination under anaesthesia. External appearance and organs and tissues in the thoracic and abdominal cavities were visually examined. None of the animals died before study termination. No symptoms of toxicity or changes in

⁶ Comparisons between highly homologous proteins yield expectation values (E-values) approaching zero, indicating a very low probability that such matches would occur by chance. A larger E-value indicates a smaller degree of similarity.

body weight were reported. Upon necropsy, no gross pathological findings were observed. In the absence of deaths, the median lethal dose (LD₅₀) was estimated to be > 1912 mg TOS/kg.

2.3.2 Short-term studies of toxicity

(a) Rats

A dose range-finding study was performed in which groups of six male and six female Sprague-Dawley rats were given powdered enzyme concentrate as a suspension in water at a dose of 0, 100, 300 or 1000 mg/kg bw per day (equal to 0, 96, 287 or 956 mg TOS/kg bw per day) by oral gavage for 2 weeks (7). The study was certified for compliance with Article 18, 4-3 of the Japanese Enforcement Regulation of Pharmaceutical Affairs Law and with QA. Feed and water were available *ad libitum*. Animals were observed three times daily for clinical signs, and body weight and feed consumption were measured on days 1, 4, 7, 10 and 14. At study termination, blood samples were collected from the abdominal aorta for haematology and clinical chemistry analyses. Animals were killed by exsanguination, and a necropsy was performed. Macroscopic examination was performed on all organs and tissues, including those in the cephalic, thoracic and abdominal regions. The weights of adrenals, spleen, heart, lung (including bronchus), liver, kidney, testis and ovary were measured. These organs as well as the tongue, oesophagus, stomach, duodenum, jejunum, ileum (including Peyer's patches), caecum, colon, rectum and larynx were preserved for histopathological analyses.

All animals survived until study termination, and no overt signs of toxicity were observed. No significant differences in body weight, feed intake or haematology or clinical chemistry parameters were observed. On day 10, a statistically significant increase in feed consumption (+12%) was reported in males at the middle dose. At the end of the study, a statistically significant increase in relative spleen weight (+13%) was reported in females at the low dose. The Committee considered these findings as incidental and not treatment-related. No gross pathological findings were observed and therefore no histopathological examinations were done. On the basis of these results and in view of the length of the administration period for the subsequent 13-week oral toxicity study, the dose levels for that study were set at 38, 191 and 956 mg TOS/kg per day.

In the subsequent 13-week study, groups of 12 male and 12 female Sprague-Dawley rats were given powdered enzyme concentrate as a suspension in water at a dose of 0, 40, 200 or 1000 mg/kg bw per day (equal to 0, 38, 191 or 956 mg TOS/kg per day) by oral gavage for 13 weeks (8). The study was certified for compliance with GLP and QA and was conducted according to OECD test guideline 408 (Repeated Dose 90-day Oral Toxicity Study in Rodents, 1998). Feed

and water were available *ad libitum*. The animals were observed for clinical signs at least twice daily. Detailed home cage, in-hand and open field observations were made weekly, and sensory reactivity to stimuli and spontaneous movements were measured weekly. Body weight and feed consumption were recorded three times in week 1 and twice weekly thereafter. Ophthalmology (six animals/group) was conducted at the end of treatment. Manipulative tests and measurements of grip strength and spontaneous movement were performed on day 89 or 90 for all animals. Urine was analysed in week 13. For each animal, a 4-h urine sample was collected under deprivation of feed but with free access to water, and thereafter a 20-h urine sample was collected with free access to feed and water; 1-day water consumption was measured on the day before the urine samples were collected. At study termination, blood samples for haematology and blood chemistry analyses were collected from the abdominal aorta under anaesthesia, after fasting overnight, and the animals were killed by exsanguination. Macroscopic examination was performed and organs from all animals were weighed. Histopathological examination was conducted on approximately 50 tissues from the control and high-dose group animals only, with the exception of microscopic examination of the stomach and caecum of animals at the mid- and high-doses (see below).

No deaths and no overt symptoms of toxicity were observed. No effects were observed on body weight or feed consumption or on ophthalmological examination. Incidental effects were observed in rearing counts, including a statistically significant decrease in the rearing count in females at the mid dose in week 1 and a statistically significant increase in females at the low dose in week 6. A statistically significant decrease in grip strength of forelimb (–12%) and hindlimb (–21%) was observed in males at the high dose in week 13. Urinalysis showed a dose-related tendency towards a decreased urinary pH, which was considered to be related to administration of the test article. This was not considered to be toxicologically relevant, as no treatment-related histopathological effects were observed in the urinary tract. A statistically significant increase in mean corpuscular haemoglobin concentration (+1.5%) was observed in females at the mid dose only. In females at the mid- and high doses, a statistically significant increase in the percentage of lymphocytes (+10% in both groups) and a statistically significant decrease in the percentage of segmented neutrophils (–42% and –38%, respectively) were observed. As no changes were observed in total white blood cell count, this was considered not to be toxicologically relevant. Blood chemistry showed a small but statistically significant decrease in sodium concentration in males in all treated groups (–1%) and in chloride concentration in males at the high dose (–2%). An incidental but statistically significant decrease in relative kidney weight (–12%) was observed in low-dose females. A statistically significant increase in relative liver weight (+12%) was

observed in high-dose males, whereas absolute liver weights were not statistically significantly increased (+10%). Incidental gross pathological findings included enlargement of the trigeminal nerve in one high-dose male and a small thyroid in one low-dose female.

In the stomach, hyperplasia of the limiting ridge was observed in all animals at the high dose, one female at the mid dose, one male at the low dose and two males in the control group. Diffuse mucosal hyperplasia of the stomach was observed in four males and two females at the high dose, and globule leukocyte infiltration was observed in the stomachs of two males and five females at the high dose, one female at the mid dose and one male in the control group. The effects were classified as minimal or mild. The Committee considered that these effects in the stomach were probably local irritation due to administration of the enzyme concentrate by gavage and were not relevant to the human situation. In addition, minimal erosion of the glandular stomach was observed in one male at the high dose, and one female at the mid dose and one female at the high dose had a cyst in the stomach. White foci were observed in the stomachs of these females upon gross pathological examination.

In the caecum, minimal diffuse mucosal hyperplasia was observed in three males at the high dose, one female at the mid dose and one female at the high dose but not in the control groups. The Committee considered that the effects in the caecum were treatment-related adverse effects.

In the liver, microgranuloma were observed in 10 males and 10 females at the highest dose and in 8 males and 7 females in the control group. Incidental histopathological findings were observed in other organs and tissues. The incidence in the treated groups was at most 1 higher than in the control. These changes were considered not to be treatment related in view of their sporadic occurrence.

Two males at the high dose had extramedullary haematopoiesis in the spleen but no accompanying effects on haematological parameters. Minimal mineralization of the arterial wall of the lungs occurred in two males and two females at the high dose and in one male in the control group.

Although the effects at the high dose of 956 mg/kg bw per day were small or occurred at low incidence, they might have been related to treatment. On this basis, the Committee identified a no-observed-adverse-effect level (NOAEL) of 190 mg/kg per day (rounded by the Committee from 191 mg/kg bw per day).

2.3.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.3.4 Genotoxicity

The results of studies of genotoxicity *in vitro* with the powdered enzyme concentrate are summarized in Table 1. The bacterial reverse mutation study (9) was performed according to OECD test guideline 471 (Bacterial Reverse Mutation Test, 1997). The chromosomal aberration test was performed in accordance with OECD test guideline 473 (*In vitro* Mammalian Chromosome Aberration Test, 1997). Both studies were certified for compliance with GLP and QA. The results of both studies were negative, and the Committee concluded that there is no concern regarding the genotoxicity of the phospholipase A2 preparation.

2.3.5 Reproductive and developmental toxicity

No information was available.

2.3.6 Other studies

Phospholipase A2 from *S. violaceoruber* was evaluated for potential toxicity and virulence by a homology search in which the amino acid sequence of the enzyme was compared with known venom proteins, toxins and virulence factors in the Tox-Prot program (<https://www.uniprot.org/program/Toxins>; version released February 2021). The BLAST search did not result in a hit (E-value⁶ < 0.05). These findings indicate that phospholipase A2 is unlikely to be structurally related to any of the validated toxins or virulence factors currently in these databases.

2.4 Observations in humans

No information was available.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to phospholipase A2 from *S. violaceoruber* (PLA2). The enzyme is intended for use in the production of food ingredients, including egg yolks, lecithins, flour, vegetable oils, and milk; therefore, these uses were considered for the dietary exposure assessment. The sponsor noted that many foods that contain these ingredients could contain the enzyme. The submission included an estimate of dietary exposure based on the budget method, a screening method used to determine the theoretical maximum daily intake (TMDI) of food additives (11,

Table 1
Genotoxicity of the powdered phospholipase A2 concentrate in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation	<i>Salmonella typhimurium</i> TA98, TA100, TA1535 and TA1537 and <i>Escherichia coli</i> WP2uvrA	1.1–4780 µg TOS ^a /plate, ± S9	Negative ^b	9
Chromosomal aberration	Chinese hamster lung fibroblasts (CHL/IU cells)	Short exposure (6h): 149, 299, 598 and 1195 µg TOS ^a /mL, +S9; 598, 1195, 2390 and 4780 µg TOS ^a /mL, –S9 Continuous exposure (24h): 299, 598, 1195 and 2390 µg TOS ^a /mL, –S9 Continuous exposure (48h): 37.4, 74.7, 149 and 299 µg TOS ^a /mL, –S9	Negative ^c	10

S9: 9000 × g supernatant fraction from rat liver homogenate

^a Concentration in µg TOS/plate calculated from concentrations in µg/plate with the TOS content (95.6%) of the powdered enzyme concentrate.

^b A range-finding study (concentration range, 1.1–4780 µg TSO/plate; one plate/concentration) and a duplicate experiment (concentration range, 300–4780 µg TOS/plate; three plates/concentration) were performed with the preincubation method. No toxicity was reported.

^c In the short-exposure experiment, the cells were treated for 6 h with or without S9 and were harvested 18 h later. At the highest concentration, the relative cell growth (% of control) was approximately 66% with S9 and approximately 24% without S9. The highest concentration could not be evaluated for chromosomal aberrations. In the continuous exposure experiment, the cells were exposed continuously for 24 or 48 h without S9 and then harvested. After 24-h treatment, toxicity was observed at all concentrations (relative cell growth was, respectively, 44, 56, 44 and 44% at 299, 598, 1195 and 2390 µg TOS/mL). Only the lowest concentration could be evaluated for chromosomal aberrations. After 48-h treatment, the relative cell growth at the highest dose tested was 30%. No statistically significant increases in chromosomal aberrations were observed in the experiments. The Committee noted that only one analysable concentration was available after 24-h continuous exposure but concluded that this did not affect the validity of the study.

12). The method takes into account maximum physiological levels of consumption of food and non-milk beverages, the energy density of foods, the concentration of the food additive in foods and non-milk beverages and the proportion of foods and non-milk beverages that may contain it. The method provides a conservative estimate of dietary exposure. Further details of the budget method can be found in chapter 6 of Environmental Health Criteria 240 (EHC 240) (13).

3.2 Dietary exposure assessment

The sponsor provided information on PLA2 use levels (including different levels based on the liquid or solid form of the article of commerce) in some representative foods and ingredients that could be treated with the enzyme. However, as PLA2 can be used broadly in the diet, the assumptions used in the budget methods obviate the need for detailed information. The estimated TMDI provided by the sponsor was based on the proportion of food, milk and non-milk beverages containing the enzyme preparation, maximum TOS levels for each category and the percentage of ingredients formulated into final foods.

EHC 240 refers to commonly used default proportions of 12.5% for foods and 25% for non-milk beverages (13). As food ingredients processed with the

PLA2 preparation are proposed to be added to a variety of foods intended to be consumed by the general population, the sponsor used 25% as the default proportion for foods in the budget method calculation. Additionally, for the purpose of this assessment, the sponsor assumed that 25% of milk consumed would be treated with the enzyme. The sponsor conservatively assumed that exposure would be derived by summing the exposures from solid foods, non-milk beverages, and milk.

The maximum level of PLA2 present in the final food from solid food uses was 6.42 mg TOS/kg food. When the enzyme was used in non-milk beverages, the maximum concentration in the final beverage was 4.59 mg TOS/kg. For use in milk production, the maximum concentration in milk was 9.17 mg TOS/kg. The standard budget method calculation was undertaken for estimating the dietary exposure to the TOS from the three sources: solid foods, non-milk beverages and milk.⁷

The resulting TMDIs of PLA2 were estimated to be 0.08 mg TOS/kg bw per day for solid foods, 0.115 mg TOS/kg bw per day for non-milk beverages and 0.057 mg TOS/kg bw per day for milk, resulting in an overall total of 0.252 mg TOS/kg bw per day.

For the dietary exposure assessment, it was assumed that the enzyme is not removed during final processing of ingredients or foods and that 100% of the enzyme remains in the ingredient and final food. The enzyme is inactivated by high temperatures during processing of food ingredients and would have no technological function in the final food.

4. Comments

4.1 Biotransformation

No information was available.

4.2 Assessment of potential allergenicity

Phospholipase A2 from *S. violaceoruber* was evaluated for allergenicity according to the bioinformatics criteria recommended by FAO/WHO (4,5) and modified at

⁷ Consumption of milk in this dietary exposure calculation is considered to be additional to the usual inputs from the budget method. The consumption value for milk was taken from the FAO/WHO model diet for exposure assessments for veterinary drug residues of 1.5 L/day (equivalent to 0.025 kg/kg body weight for a 60-kg adult) (14).

the eightieth meeting of the Committee ([Annex 1](#), reference 223). The amino acid sequence of phospholipase A2 from *S. violaceoruber* was compared with those of known allergens in publicly available databases. A search for matches with > 35% identity in a sliding window of 80 amino acids, a search for sequence identity of 8 contiguous amino acids and a full-length FASTA sequence search produced no matches. Therefore, the Committee considered that dietary exposure to phospholipase A2 from *S. violaceoruber* is not anticipated to pose a risk of allergenicity.

4.3 Toxicological data

In a study of oral acute toxicity in rats with powdered phospholipase A2 concentrate, the LD₅₀ was estimated to be > 1912 mg TOS/kg (6).

No treatment-related effects were observed in a range-finding study in which rats were given powdered enzyme concentrate at doses up to 956 mg TOS/kg bw per day by oral gavage for 2 weeks (7).

In a 13-week study of oral toxicity in rats, treatment-related effects were observed on the caecum and the stomach when the powdered phosphodiesterase enzyme concentrate was administered by gavage (8). In the stomach, hyperplasia of the limiting ridge was observed in all animals at the high dose, one female at the mid dose, one male at the low dose and two males in the control group. Diffuse mucosal hyperplasia of the stomach was observed in four males and two females at the high dose, and globule leukocyte infiltration was observed in the stomachs of two males and five females at the high dose, one female at the mid dose and one male in the control group. The effects were classified as minimal or mild. The Committee considered that the effects in the stomach were probably local irritation due to administration of the enzyme concentrate by gavage and were not relevant to the human situation. In the caecum, minimal diffuse mucosal hyperplasia was observed in three males at the high dose, one female at the mid dose and one female at the high dose. The Committee considered that the effects in the caecum were treatment-related adverse effects. In addition, two males at the high dose had extramedullary haematopoiesis in the spleen, although no accompanying effects on haematological parameters were seen. Minimal mineralization of the arterial wall of the lungs occurred in two males and two females at the high dose and in one male in the control group. A statistically significant decrease in grip strength and a statistically significant increase in relative, but not absolute, liver weight were also observed in males at the high dose. Although the effects at the high dose of 956 mg/kg bw per day were small or occurred at a low incidence, they might have been related to treatment.

On this basis, the Committee identified a NOAEL of 190 mg/kg per day (rounded by the Committee from 191 mg/kg bw per day).

The powdered enzyme concentrate was not genotoxic in a bacterial reverse mutation assay (9) or in an in-vitro chromosomal aberration assay (10). The Committee had no concern with respect to the genotoxicity of the phospholipase A2 enzyme preparation.

5. Evaluation

The Committee identified a NOAEL of 190 mg TOS/kg bw per day in a 13-week study in rats. A comparison of the estimated dietary exposure of 0.25 mg TOS/kg bw per day with the NOAEL of 190 mg TOS/kg bw per day from the oral toxicity study gives a MOE of 760. On this basis and in the absence of concern about genotoxicity, the Committee established an ADI “not specified”⁸ for the phospholipase A2 enzyme preparation from *S. violaceoruber* when used in the applications specified and in accordance with good manufacturing practice.

A toxicology and dietary exposure monograph was prepared.

New specifications and a chemical and technical assessment were prepared.

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⁸ The reader is referred to the Technical Report of the 87th JECFA meeting (Annex 1, reference 243) for clarification of the term “ADI not specified”.

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Riboflavin from *Ashbya gossypii*

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1. Explanation

Riboflavin or 7,8-dimethyl-10-(1'-D-ribyl)isoalloxazine (7,8-dimethyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]benzo[g]pteridine-2,4-dione), commonly known as vitamin B2, was evaluated by JECFA previously (1) as a synthetic product or product of fermentation from a genetically modified strain of *Bacillus subtilis*. It is an essential nutrient and obligatory component of human and animal diets. It can be synthesized by all plants and many microorganisms but is not produced by higher animals. It serves as a precursor of flavin coenzymes such as flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) and is therefore involved in oxidative metabolism and other processes.

Riboflavin is widely used as a dietary supplement, in food fortification and as a food colour. Riboflavin and riboflavin-5'-phosphate sodium can be obtained by chemical synthesis or by the enzymatic processes of certain microbes in large-scale fermentation.

At its thirteenth meeting, the Committee established an ADI of 0–0.5 mg/kg bw for riboflavin in the absence of any adverse effects at the only dose tested of 50 mg/kg bw per day in a three-generation study of reproductive toxicity in rats (2). At its twenty-fifth meeting, the Committee included riboflavin and riboflavin-5'-phosphate, expressed as riboflavin, in a newly established group ADI of 0–0.5 mg/kg bw (3). At its fifty-first meeting, the Committee evaluated riboflavin produced by fermentation from *B. subtilis* and included it in the group ADI of 0–0.5 mg/kg bw for riboflavin and riboflavin-5'-phosphate, on the basis of its equivalence to riboflavin (4).

The CCFA at its Fifty-first session (5) requested the Committee to evaluate riboflavin from *Ashbya gossypii* as an alternative source of riboflavin for colouring purposes and as a nutrient source. At its present meeting, the Committee evaluated riboflavin from *A. gossypii* for use as a food colour for the first time. It did not review the nutrient properties of riboflavin but took into account dietary exposure from all sources of riboflavin, including as a nutrient.

A literature search on riboflavin from *A. gossypii* was conducted according to the JECFA guideline (6), with the keywords (“riboflavin” OR “vitamin B2”) AND (“*Ashbya gossypii*”). The databases searched were PubMed (1946–10 April 2021; 88 records), Web of Science (1946–10 April 2021; 141 records), MEDLINE (1946–10 April 2021; 80 records), Scopus (Elsevier) (1946–10 April 2021; 0 records), AGRIS (1946–10 April 2021; 11 records), Cochrane Library (1946–10 April 2021; 0 records), Embase (1946–16 January 2021; 0 records), Directory of Open Access Journals (1946–16 January 2021; 6 records), GIM (1946–16 January 2021; 0 records) and CINAHL (1946–16 January 2021; 0 records). Two of the records retrieved added to the toxicological data submitted to the Committee for this meeting. A literature search of studies of riboflavin from other sources was

also conducted. The keywords used were (“riboflavin” OR “vitamin B2”) AND (“toxicology” OR “toxicity”). The main database searched was PubMed (1998–10 April 2021; 4235 records); four of the records retrieved were added to the toxicological dossier.

1.1 Genetic background

The Committee at its present meeting evaluated the information provided by the sponsor on use of the filamentous fungus *A. gossypii* (*Eremothecium gossypii*) in the production of commercial riboflavin.

A. gossypii is a naturally occurring phenotypic riboflavin-overproducing organism (7), which possibly provides protection against ultraviolet radiation (8). Early development of *A. gossypii* strains for commercial production of riboflavin involved classical mutagenesis and strain selection to obtain a high riboflavin titre. The *A. gossypii* strain LU8907 was continuously developed into several commercial strains to further increase riboflavin production. The present production strain, *A. gossypii* LU11439, was constructed from the recipient strain *A. gossypii* LU8907. The recipient was modified by the addition of several genes from the wild-type *A. gossypii* strain under the control of translation elongation factor promoters and antibiotic resistance marker genes. The DNA sequences for transformation were prepared as linear, vector-free fragments and inserted by electroporation, followed by homologous recombination and targeted integration. The production strain was confirmed to be genetically stable and not to contain any transferable marker genes or sequences derived from vector DNA.

The complete sequence and annotation of the *A. gossypii* genome was published in 2004. It shows 95% homology and gene synteny to the genome of budding yeast, *Saccharomyces cerevisiae* (9,10). The complete genome sequences of the sponsor's first self-cloned production strain and of the published *A. gossypii* wild-type strain (ATCC 10895) were analysed for the presence of gene clusters encoding secondary metabolites. No gene clusters of polyketide synthases or non-ribosomal peptide synthases were identified. The genome of *A. gossypii* has no potential for production of secondary metabolites.

1.2 Chemical and technical considerations

Riboflavin from *A. gossypii* is obtained by fermentation under controlled conditions. Several filtration and precipitation or crystallization steps result in a dry powder containing $\geq 98\%$ riboflavin, free of fermentation medium components and the production organism. The entire process is carried out in accordance with current good manufacturing practice; all the raw materials used

in the manufacture are food-grade. Riboflavin is relatively stable during food processing and storage but is very sensitive to light.

Riboflavin was previously evaluated by JECFA (1) as a synthetic product (1987) and as a product of fermentation from *B. subtilis* (1999). Independently of the source, these additives contain > 98% and < 101% of riboflavin (on a dried basis). Residual moisture is present at < 1.5%.

Riboflavin is used as a food colour. It is used in products subjected to intense processing or storage (which results in partial loss of their natural colour), to standardize the colour of the food products or to impart a yellow hue to processed foods.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Riboflavin is absorbed actively and passively mainly in the proximal small intestine, partly in the large intestine and also in the colon (11–13). Riboflavin is absorbed by two mechanisms, a saturable active component that dominates at near-physiological vitamin concentrations and a passive component that is revealed under conditions of high levels of supplementation with riboflavin. The active transport mechanism is regulated by the Ca^{2+} /calmodulin pathway, which is highly riboflavin-specific and temperature-dependent. The protein kinase A system and cGMP-dependent protein kinase pathways are involved in absorption regulation (14). In plasma, some riboflavin is bound to albumin; however, a large portion is associated with immunoglobulins (IgA or IgG) for transport (15). When riboflavin is absorbed at high concentrations, little is stored in the body tissues, and the excess is excreted, primarily in the urine (16–18).

2.1.2 Biotransformation

Absorbed riboflavin is converted to FAD and FMN in the cellular cytoplasm of most tissues but mainly in the small intestine, liver, heart and kidney (19–21). The metabolism of riboflavin begins with ATP-dependent phosphorylation of riboflavin to FMN, catalysed by the enzyme flavokinase under hormonal control. FMN is then complexed with specific apoenzymes to form a variety of flavoproteins or is mainly converted to FAD by FAD synthetase (22). In a randomized controlled trial, baseline examination revealed median plasma concentrations of riboflavin, FMN and FAD in healthy elderly people of 10.5, 6.6

and 74 nmol/L, respectively, while only trace amounts of riboflavin were found in erythrocytes, with median concentrations of FMN and FAD of 44 and 469 nmol/L. After 12 weeks' supplementation with riboflavin (1.6 mg/day), these indicators, except for plasma FAD, had increased significantly, with the greatest changes in plasma riboflavin and erythrocyte FMN. Although FAD was the major form in plasma, plasma riboflavin and erythrocyte FMN were suggested to be useful indicators of riboflavin status in humans (23).

Riboflavin is metabolized in only small amounts (17,24). When riboflavin occurs in high concentrations in tissues, it is excreted mainly in the urine. It is catabolised to numerous metabolites. Lumichrome and lumiflavin were identified as metabolites of riboflavin in rats (25) and hydroxyethylflavin, formylmethylflavin and an unknown metabolite as metabolites in humans (26).

2.2 Toxicological studies

2.2.1 Acute toxicity

The oral LD₅₀ values for riboflavin from *A. gossypii* in rats were > 2500 mg/kg bw (Table 1).

2.2.2 Short-term studies of toxicity

(a) Rats

In a dose-range finding study, riboflavin derived from *A. gossypii* was administered to four groups of three rats of each sex at a dietary concentration of 0, 6000, 18 000 or 50 000 ppm for 2 weeks. There were no effects on feed consumption, body weight or clinical signs. Faeces were discoloured at all dietary concentrations, and urine was discoloured at the mid and high concentrations. No other effects were observed (27).

A 90-day oral toxicity study in rats treated with riboflavin derived from *A. gossypii*, which was conducted in 1998, was made available to the Committee for the current meeting. The study was performed under GLP and was compliant with OECD Guideline 408. Riboflavin from *A. gossypii* was administered to four groups of rats (10/sex per group, starting at 42 days of age) at a dietary concentration of 0, 500, 5000 or 50 000 ppm (equal to 0, 35, 362 or 3659 mg/kg bw per day in males and 0, 41, 410 or 4325 mg/kg bw per day in females). The rats were examined for signs of toxicity or mortality at least once a day. Feed consumption and body weight were determined weekly, and clinical examinations were conducted. Ophthalmological examinations were done before the start or after the end of dosing. Urine analysis, blood chemical and haematological and gross-pathological examinations were carried out at the end of the administration

Table 1

Acute toxicity of riboflavin from *Ashbya gossypii* after oral administration to Wistar rats

Purity of test substance	LD ₅₀ (mg/kg bw)	Reference
82.3% (feed grade)	> 2500	28
100.5% (food grade)	> 10 000	29

Source: reference 27

period. All animals were subjected to gross-pathological assessment, followed by histopathological examinations.

There were no deaths related to treatment, and treatment had no effects on body weight, body weight gain or feed consumption. It was reported that there were no “overt” changes in the volume of water consumption. Foci were detected in the kidneys of two females at the highest dose, which were due to tubular dilation in the cortex in one rat and a blood-filled cyst in the other. Both lesions were considered to be irrelevant to the treatment. Discoloration of faeces was seen in all three dose groups in both sexes. Discoloration of the skin, fur and urine was observed in both sexes at the highest dose (50 000 ppm), while discoloration of the stomach and the contents of the urinary bladder and caecum were seen only in males at this dose. The discoloration of both tissues and urine was clearly attributable to the intake and excretion of the coloured test substance; however, the Committee considers that the findings do not represent a toxicologically relevant effect.

At the end of the study, a positive nitrogen balance was observed in urine specimens from males at the high dose and females at the mid and high doses. The study authors attributed this to the nitrogen content of the test material, which caused discoloration of the urine. There were no treatment-related changes in other urine parameters, apart from slightly increased urinary volume with decreased specific gravity in females at the highest dose. This change was considered by the study authors possibly to indicate mild impairment of renal function. According to the authors, the NOAEL in this study was 5000 ppm (equal to 410 mg/kg bw per day) for females, on the basis of increased urinary volume with decreased specific gravity, and 50 000 ppm (equal to 3659 mg/kg bw per day), the highest dose tested, for males (27).

The Committee noted that the slightly increased urinary volume with decreased specific gravity in female rats did not exclude the possibility of increasing water consumption; however, data on water consumption were not provided in study report. As no other treatment-related changes were observed, the Committee concluded that the NOAEL for the test substance was 50 000 ppm, equal to 4325 mg/kg bw per day for females and 3659 mg/kg bw per day for

males, the highest dose tested. After adjustment for the purity of the test substance (82.3%), the NOAELs for riboflavin from *A. gossypii* were equal to 3559 mg/kg bw per day for females and 3011 mg/kg bw per day for males.

In a 90-day oral toxicity study in rats (30), which was reviewed previously by the Committee (4), two grades of riboflavin (purity, 98% or 96%) produced by fermentation from a genetically modified strain of *B. subtilis*, were fed in the diet at concentrations providing doses of 0, 20, 50 or 200 mg/kg bw per day. The experiment also included a group of rats fed with 98% pure synthetic riboflavin for comparison of toxicity. The Committee concluded that the NOEL for 98% or 96% pure riboflavin produced by fermentation from *B. subtilis* was 200 mg/kg bw per day, whereas that for chemically synthesized riboflavin was 50 mg/kg bw per day on the basis of decreases in haemoglobin and erythrocyte count (4).

Another 90-day oral toxicity study performed with riboflavin derived by fermentation from *B. subtilis* (containing 80.1% riboflavin, feed grade) had become available since the previous evaluation by the Committee (31). Riboflavin from *B. subtilis* was administered to groups of 10 rats of each sex in the diet to provide doses of 0, 50, 100 and 200 mg /kg bw per day. Additional groups of 5 rats of each sex were given the same treatment for 13 weeks and then observed during a 4-week recovery period. Blood and urine were collected at weeks 4 and 10 for biochemical analysis during the treatment period. Clinical signs, feed consumption and body weights were recorded periodically during acclimatization, at the end of treatment and at the end of the recovery period. Microscopic changes in the kidneys were seen at the end of treatment, which comprised eosinophilic granules in the renal tubules of males at the two highest doses (100 and 200 mg/kg bw per day); however, kidney morphology returned to normal after the 4-week recovery period. The authors considered that the NOAEL for the test material was 200 mg/kg bw per day, corresponding to 160 mg/kg bw per day of riboflavin.

2.2.3 Long-term studies of toxicity and carcinogenicity

No long-term toxicity or carcinogenicity studies were available.

2.2.4 Genotoxicity

(a) In vitro

Riboflavin from *A. gossypii* was evaluated in two bacterial reverse mutation assays in vitro with plate incorporation and pre-incubation methods, both with and without rat S9 metabolism (32,33), and in an in vitro micronucleus test with human peripheral blood lymphocytes (34). The results obtained are presented in Table 2; all were negative.

Table 2

Results of in vitro genotoxicity studies with riboflavin from *A. gossypii*

End-point	Test system	Concentrations	Purity of the test substance	Result	Reference
Reverse mutation	<i>S. typhimurium</i> TA1535, TA1537, TA98, TA100	20–5000 µg/plate, ± S9; plate incorporation and pre-incubation methods	99%	Negative ^a	32
Reverse mutation	<i>S. typhimurium</i> TA1535, TA1537, TA98, TA100 and <i>E. coli</i> WP2uvrA	33, 100, 333, 1000, 3050 and 6100 µg/plate, ± S9; plate incorporation and pre-incubation methods	80.8%	Negative ^{bc}	33
Micronuclei induction	Human peripheral blood lymphocytes	Range finding: 4.8–2475 µg/mL Experiments 4 h ± S9: 44.9, 25.7 and 14.7 µg/mL 20 h without S9: 114.0, 65.3 and 37.3 µg/mL	80.8%	Negative ^d	34

S9: 9000 × g supernatant fraction from rat liver homogenate

^a The study meets the requirements of the current OECD Guidelines for Testing of Chemicals No. 471 "Bacterial reverse mutation test", except that the tester strains *S. typhimurium* TA102 or *E. coli* WP2uvrA bearing an AT mutation were not used.

^b The highest dose (6100 µg/plate) corresponds to 5000 µg/plate of active ingredient.

^c The study meets the requirements of the current OECD Guidelines for testing of chemicals No. 471 "Bacterial reverse mutation test"

^d The study was not performed in compliance with the current OECD Guidelines for Testing of Chemicals No. 487 "In vitro mammalian cell micronucleus test" in respect of the treatment schedule and the addition of cytochalasin B at 4 h of treatment ± S9 and at 20 h of treatment without S9. Cytochalasin B was added to cultures 20 h after the beginning of treatment, possibly determining the loss of post-treatment micronucleated cells.

Despite some minor limitations of one of the bacterial reverse mutation assays (32) and the in vitro micronucleus test in human peripheral blood lymphocytes (34) (see footnotes to Table 2), the combination of these tests fulfils the basic requirement to cover the three genetic end-points (i.e., gene mutations, structural and numerical chromosome aberrations) for assessment of genotoxic potential. The results of these studies did not raise any concern about the genotoxicity of riboflavin from *A. gossypii*.

Another bacterial reverse mutation test with riboflavin from a fermentation source (strain unknown) has been conducted since the Committee's previous evaluation. The original study reports were not available to this Committee, and the description that follows is from EFSA (35). The reverse mutation assay was performed with riboflavin of 96% purity (feed grade, spiked with 2.31% 6,7-dimethyl-8-ribityllumazine (DMRL)) and riboflavin of 98% purity (tablet grade, spiked with 1.2% DMRL). DMRL is a known precursor of riboflavin in the biofermentation process and may occur as an impurity in the final product. In this study, riboflavin containing DMRL was evaluated in five *S. typhimurium* strains (TA97, TA98, TA100, TA102 and TA1535) in the standard plate incorporation assay and in the preincubation method in the presence and absence of S9, respectively. Upon addition of aliquots of the test material to the aqueous medium, precipitation was observed at 5 mg/plate. Weak toxicity was

observed at 1.25 mg/plate in strain TA102. The results obtained with these two riboflavin formulations were similar, and no mutagenic effects were observed (36,37).

(b) **In vivo**

No *in vivo* genotoxicity studies were available.

The Committee concluded that there is no concern with respect to genotoxicity for riboflavin from *A. gossypii*.

2.2.5 Reproductive and developmental toxicity

Rats

In a multigeneration study, groups of 10–12 rats of each sex were given 0 or 10 mg/day of riboflavin (source not stated), equivalent to approximately 50 mg/kg bw per day, assuming an average body weight of 200 g per rat, for 140 days from weaning for each of three consecutive generations. The authors stated that the riboflavin was fed to the animals (38); however, it is not clear that it was administered in the diet in this experiment, as the description of the test material, which precedes descriptions of the several experiments included in the paper, indicates that it was an aqueous suspension. Data on body weights were provided in tabular form, but no numerical information on other parameters was provided. The results were reported only as brief statements in the text. The body weights of treated animals were comparable to those of controls in all generations. The authors stated that there were no differences in development, growth, maturation or reproduction between treated and control groups and that the autopsies at the end of the test period showed no gross changes. Although this study is from 1942 and was previously described and evaluated by JECFA (2,3), this brief summary is included as it was this study that formed the basis of the ADI of 0–0.5 mg/kg bw for riboflavin established by the Committee in 1969.

2.2.6 Special studies

No special studies on riboflavin from *A. gossypii* were available.

2.3 Observations in humans

No studies or observations in humans treated with riboflavin from *A. gossypii* were available.

In a randomized, placebo-controlled, double-blind, cross-over trial, children aged 6–13 years with migraine were given 50 mg/day of riboflavin orally for 16 weeks. No adverse effect was reported (39). Similarly, in a study of 68

Japanese children aged 6–15 years with migraines who received 10 or 40 mg/day of riboflavin for 3 months, no adverse effects were identified (40). In further studies, in which 5–18-year-old children with migraines were given 200 or 400 mg/day of riboflavin for 12–24 weeks, several mild effects, including vomiting, increased appetite, tension headache and changes in urine colour, were observed during treatment. When adults with migraine were given riboflavin supplementation at 400 mg/day for 3–12 months, weight gain, dizziness, diarrhoea, upper abdominal pain and facial erythema were observed in a few patients (41–43). None of the effects were considered to be related to riboflavin treatment.

No adverse side-effects of riboflavin were mentioned in intervention studies with riboflavin in elderly people (44), elderly people with cardiovascular diseases (45), anaemic pregnant women (46) and colorectal polyp patients (47).

3. Dietary exposure

3.1 Use levels of riboflavin

At its forty-second session, the Codex Alimentarius Commission endorsed MPLs of riboflavin (synthetic, including riboflavin from *B. subtilis* and riboflavin phosphate sodium) of 30–1000 mg/kg when used as a food colour for 71 food categories in the GSFA (2019 online edition). In the European Union, riboflavin is permitted at *quantum satis* in all foodstuffs, with the exception of MPLs up to 100 mg/L in aromatized wine-based drinks (Annex II to Regulation (EC) No. 1333/2008). In many countries, including Australia, New Zealand, the Republic of Korea and the USA, riboflavin may be used in amounts consistent with good manufacturing practice.

The sponsor provided maximum reported use levels (MRULs) of 10–400 mg/kg riboflavin as a food colour for 29 food categories in Annex II to Regulation (EC) No. 1333/2008. The levels were originally reported by industry in response to a call for data for re-evaluation of riboflavin by EFSA (35). For the exposure assessment based on MPLs in the GSFA with the EFSA Food Additive Intake Model (FAIM) for exposure model, the 38 food categories in Annex II to Regulation (EC) No. 1333/2008 were matched to the 71 GSFA food categories (Table 3). Several GSFA food categories were matched to one food category in Annex II at the highest MPL for these GSFA food categories. The GSFA food categories edible cheese rind (01.6.2.2) and batters (06.6) were not matched, because no corresponding food categories were available in Annex II to Regulation (EC) No. 1333/2008.

Table 3
Maximum reported use levels (MRUL) and GSFA MPL of riboflavin for the food categories in Annex II to Regulation (EC) No. 1333/2008

Food category	MRUL (mg/kg)	GSFA MPL (mg/kg)
01.4 Flavoured fermented milk products, including heat-treated products	30	300
01.5 Dehydrated milk as defined in Directive 2001/114/EC	100	–
01.6 Cream and cream powder	100	–
01.7 Cheese and cheese products	100	–
01.7.1 Unripened cheese excluding products in category 16	100	300
01.7.2 Ripened cheese	100	300
01.7.4 Whey cheese	100	–
01.7.5 Processed cheese	100	300
01.8 Dairy analogues, including beverage whiteners	100	–
02.2 Fat and oil emulsions, mainly of water-in-oil type	–	300
03 Edible ices	50	500
04.1 Unprocessed fruit and vegetables	–	300
04.2 Processed fruit and vegetables	80	500
05.2.1 Other confectionary with added sugar	80	1000
05.2.2 Other confectionary without added sugar	80	1000
05.3.1 Chewing gum with added sugar	80	1000
05.3.2 Chewing gum without added sugar	80	1000
05.4 Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	80	1000
06.3 Breakfast cereals	–	300
06.5 Noodles	70	300
07.2 Fine bakery wares	38	300
08.2 Meat preparations as defined by Regulation (EC) No. 853/2004	–	1000
09.2 Processed fish and fishery products, including molluscs and crustaceans	25	300
09.3 Fish roe	100	1000
11.2 Other	–	300
12.2 Herbs, spices, seasonings	10	350
12.4 Mustard	150	300
12.5 Soups and broths	195	200
12.6 Sauces	10	350
12.7 Salads and savory-based sandwich spreads	–	300
12.9 Protein products, excluding products covered in category 1.8	–	50
13.2 Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding products in food category 13.1.5)	–	300
13.3 Dietary foods for weight control intended to replace total daily food intake or an individual meal (whole or part of the total daily diet)	–	300
14.1.4.1 Flavoured drinks with sugar	10	300
14.1.4.2 Flavoured drinks with sweetener	10	300
14.1.5 Coffee, tea, herbal and fruit infusions, chicory; tea herbal and fruit infusions and chicory extracts; tea, fruit and cereal preparations for infusions, as well as mixed and instant mixes of these products	10	–

Table 3 (continued)

Food category	MRUL (mg/kg)	GSFA MPL (mg/kg)
14.2.4 Fruit wine and made wine	–	300
14.2.7.1 Aromatized wines	–	100
14.2.8 Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with < 15% alcohol	–	100
15.1 Potato-, cereal-, flour- or starch-based snacks	400	1000
15.2 Processed nuts	–	1000
16 Desserts, excluding products covered in categories 1, 3, and 4	10	300
17 Food supplements as defined in Directive 2002/46/EC, excluding food supplements for infants and young children	–	300

3.2 Assessment of dietary exposure

The sponsor provided dietary exposure estimates for riboflavin based on GSFA MPLs and MRULs in combination with food consumption in FAIM 2.0. They estimated the dietary exposure to riboflavin for six age groups (infants, toddlers aged 12–35 months, children aged 3–9 years, adolescents aged 10–17 years, adults aged 18–64 years and elderly people > 65 years). In this model, food consumption data from 26 dietary surveys in 17 European countries were available. High-level dietary exposure was calculated by adding the 95th percentile of dietary exposure to one food category with the highest dietary exposure to the mean dietary exposure resulting from consumption of all other food categories. The results are listed in Table 4. Estimates of high dietary exposure to riboflavin based on MRULs were highest for children aged 3–9 years (1.1–3.6 mg/kg bw per day). EFSA reported that the food category “processed fruit and vegetables” contributed most to the exposure of all population groups to riboflavin and up to 70% in toddlers. High-level exposures based on GSFA MPLs were calculated in the same way. The estimates were highest for toddlers aged 12–35 months (15.4–25.5 mg/kg bw per day) and for children aged 3–9 years (1.1–3.6 mg/kg bw per day).

The Committee noted that EFSA had published an evaluation of riboflavin in 2013 (35), which was, however, based on the same MRULs and performed with an older version of FAIM than that used by the sponsor. As the evaluation was superseded by that of the sponsor, the Committee did not report its results.

3.3 Dietary exposure from other sources

Riboflavin is naturally present in a wide range of foods, particularly eggs, organ meat and milk; green vegetables also contain riboflavin. Riboflavin is also used

Table 4

Dietary exposure estimates of riboflavin (mg/kg bw per day) based on GSFA MPLs and MRULs in six population groups in the European Union

Age group	GSFA MPL × FAIM		MRUL × FAIM	
	Mean	High ^a	Mean	High ^a
Infants	3.5–7.9	12.3–16.3	0.4–0.9	1.2–2.2
Toddlers (12–35 months)	8.9–18.3	15.4–25.5	0.6–2.4	1.1–2.8
Children (3–9 years)	7.4–14.8	12.6–24.5	0.6–1.8	1.1–3.6
Adolescents (10–17 years)	3.6–8.6	7.0–15.5	0.3–1.1	0.7–2.4
Adults (18–64 years)	3.0–5.0	5.8–9.7	0.2–0.8	0.4–1.5
Elderly (> 65 years)	2.8–4.1	4.8–7.5	0.2–1.0	0.5–1.6

^a High refers to the 95th percentile of exposure from one food group with the highest exposure to the mean exposure resulting from consumption of all other food groups.

in food supplements and can be used as an additional nutrient source through food fortification.

EFSA (35) estimated dietary exposure to riboflavin naturally present in food in the European Union to be 0.05–0.09 mg/kg bw per day for children, 0.02–0.04 mg/kg bw per day for women and 0.02–0.03 mg/kg bw per day for men. The largest dietary contributor to exposure to riboflavin in western diets is milk and dairy products, which was estimated to be 25–30% (48). This food group is also the main contributor to dietary exposure to riboflavin in Spain, at 32.3% (48); Australia, at 27–28% (49) and New Zealand, at 23% (50,51).

Estimates of dietary exposure to riboflavin are also available from national dietary surveys in Australia, New Zealand, the Republic of Korea and the USA (Table 5). The food composition datasets used to estimate dietary exposures from national dietary surveys include naturally occurring levels and also any use of riboflavin as a food colour and as a fortificant. The estimated mean dietary exposures to riboflavin from food and beverages (excluding dietary supplements; day 1 data only) from the 2011–2012 Australian National Nutrition and Physical Activity Survey (49) was 1.9 mg/day (0.06 mg/kg bw per day) for children aged 2–17 years and 1.9 mg/day (0.03 mg/kg bw per day) for adults aged ≥ 18 years (52). In New Zealand, the estimated mean dietary exposure of children aged 5–14 years from food and beverages (excluding dietary supplements; day 1 data only) in the 2002 National Children’s Nutrition Survey (2) was 1.8 mg/day (0.05 mg/kg bw per day), and that of adults aged ≥ 15 years in the 2008 Adult Nutrition Survey (50) was 2.0 mg/day (0.03 mg/kg bw per day) (52). Dietary exposure to riboflavin in the Republic of Korea is similar to that from western diets (53), as determined in the 6th Korea National Health and Nutrition Examination survey, which includes foods, beverages and supplements. The dietary exposure of women was

Table 5
Estimates of dietary exposures to riboflavin from national dietary surveys

Country or region	Group	Average (mg/kg bw per day)
European Union	Children	0.05–0.09
	Women	0.02–0.04
	Men	0.02–0.03
Australia	Children aged 2–17 years	0.06
	Adults	0.03
New Zealand	Children aged 5–14 years	0.05
	> 14 years	0.03
Republic of Korea	Women	0.02
	Men	0.03
USA	Children aged 2–5 years	0.14
	Children aged 6–11 years	0.1
	Children aged 12–19 years	0.05
	Women	0.07
	Men	0.05

1.21 ± 0.01 mg/day (0.02 mg/kg bw per day), and that of men was 1.61 ± 0.03 mg/day (0.03 mg/kg bw per day). The main contributors to dietary exposure were cereals and grains (15–17%), meats (12–14%), vegetables (15–16%), eggs (13–17%) and food supplements (26–30%). Analysis of data from the 2003–2006 US National Health and Nutrition Examination Survey showed that the average daily exposure to riboflavin from foods and supplements children aged 2–5 years, children aged 6–11 years and adolescents aged 12–19 years was 2.1 mg (2.1/15; 0.14 mg/kg bw per day), 2.2 mg (2.2/23.1; 0.1 mg/kg bw per day) and 2.3 mg (2.3/45.8; 0.05 mg/kg bw per day), respectively, and that of adults was 4.5 mg in men (4.5/82; 0.05 mg/kg bw per day) and 4.7 mg (4.7/67.2; 0.07 mg/kg bw per day) in women (54).

4. Comments

4.1 Biochemical data

Riboflavin is absorbed actively and passively mainly in the proximal small intestine, partly in the large intestine and also in the colon (11–13). Riboflavin is absorbed by two mechanisms – a saturable active component that dominates at near-physiological vitamin concentrations and a passive component that is revealed under conditions of high levels of supplementation with riboflavin.

In plasma, some riboflavin is bound to albumin; however, a large portion of riboflavin is associated with immunoglobulins (A or G) for transport (15). When riboflavin is absorbed in high concentrations, little is stored in the body tissues, and the excess is excreted, primarily in the urine (16–18).

The metabolism of riboflavin begins with ATP-dependent phosphorylation to flavin mononucleotide, catalysed by the enzyme flavokinase under hormonal control. Flavin mononucleotide is then complexed with specific apoenzymes to form a variety of flavoproteins or is mainly converted to FAD by FAD synthetase (22). Although FAD was the major form in plasma, plasma riboflavin and erythrocytes flavin mononucleotide were suggested to represent riboflavin status in humans (23). Lumichrome and lumiflavin have been identified as metabolites of riboflavin in rats, while hydroxyriboflavin and formylmethylflavin have been identified as metabolites in human plasma (25,26).

4.2 Toxicological studies

The acute oral toxicity of riboflavin from *A. gossypii* is low, with an LD₅₀ of > 2500 mg/kg bw (28,29).

In a 90-day repeated oral toxicity study in rats (27), riboflavin from *A. gossypii* (purity, 82.3%, feed grade) was fed in the diet at a concentration of 0, 500, 5000 or 50 000 mg/kg diet, equal to 0, 35, 362 or 3659 mg/kg bw in males and 0, 41, 410 or 4325 mg/kg bw in females. Treatment had no effects on body weight, body weight gain or feed or drinking-water consumption. Foci were detected in the kidneys of two female rats at the highest dose, but these were considered not to be toxicologically relevant. After accounting for the purity of the preparation used, the Committee identified NOAELs of 3011 mg/kg bw per day for males and 3559 mg/kg bw per day for females, the highest doses tested.

In another 90-day oral toxicity study in rats (30), reviewed previously by the Committee, riboflavin from *B. subtilis* with a purity of 98% or 96% was fed in the diet at concentrations providing 0, 20, 50 or 200 mg/kg bw per day. The previous Committee (4) identified a NOAEL of 200 mg/kg bw per day.

EFSA (35) described a further 90-day oral toxicity study in rats (31) performed with riboflavin from *B. subtilis* (containing 80.1% riboflavin, feed grade). The test material was administered in the diet to provide doses of 0, 50, 100 and 200 mg/kg bw per day. Additional groups of rats were treated at the same doses for 13 weeks and then observed for a 4-week recovery period. Eosinophilic granules were observed in the renal tubules of male rats receiving 100 or 200 mg/kg bw per day at the end of the treatment period, but renal morphology returned to normal after the 4-week recovery period. The study authors pointed out that accumulation of hyaline droplets is associated with α -2 μ -globulin and

is considered to be a response specific to male rats and therefore not relevant to humans. EFSA (35) concurred with this consideration and concluded that the NOAEL for the test material in this study was 200 mg/kg bw per day, the highest dose tested, corresponding to 160 mg/kg bw per day expressed as riboflavin. The Committee at its present meeting agreed with this evaluation.

No studies of chronic toxicity or carcinogenicity with riboflavin from *A. gossypii* or riboflavin from any other source were available.

Riboflavin from *A. gossypii* (purity, 99% and 80.8%) was tested in two bacterial mutagenicity assays (32,33) and in an in vitro micronucleus induction assay in human lymphocytes (34). In spite of minor limitations, the combination of these tests fulfilled the basic requirements for an assessment of genotoxic potential, and the Committee concluded that there is no concern with respect to the genotoxicity of riboflavin from *A. gossypii*.

No reproductive or developmental toxicity was observed in a multigeneration study in which rats received riboflavin at a daily dose of 0 or 10 mg per rat (equivalent to 0 or approximately 50 mg/kg bw per day) from weaning for three generations (38). The dose of 50 mg/kg bw per day was used as the basis for the ADI of 0–0.5 mg/kg bw per day established by the Committee in 1969 (2). The Committee at its present meeting noted that the report of the study provided limited experimental data, and only one dose level was used.

In a series of intervention studies of oral administration of riboflavin, no adverse effects were reported in populations of children and adults, including healthy individuals and patients suffering from migraine, cardiovascular diseases, colorectal polyp or anaemia (39–47,56,57).

4.3 Dietary exposure

The Committee noted that riboflavin is endorsed for use in 71 food categories in the Codex GSA at MPLs of 30–1000 mg/kg, while riboflavin may be used in amounts consistent with national good manufacturing practice in Australia, New Zealand, the Republic of Korea and the USA and in the European Union. The sponsor provided MRULs for riboflavin of 10–400 mg/kg as a food colour for the 29 food categories in which it is authorized in the European Union according to Annex II to Regulation (EC) No. 1333/2008.

Estimates of dietary exposure to riboflavin from GSA MPLs and MRULs, in combination with food consumption data from the FAIM 2.0, by the sponsor were reviewed by the Committee. This model includes food consumption data from various European countries for six age groups. High-level dietary exposure estimates are calculated by adding the 95th percentile of dietary exposure to one food category at the highest dietary exposure to the mean dietary exposure

resulting from consumption of all other food categories. Estimated mean and high-level dietary exposure to riboflavin of the six age groups were 2.8–18.3 mg/kg bw per day and 4.8–25.5 mg/kg bw per day with GSFA MPLs and 0.2–2.4 mg/kg bw per day and 0.4–3.6 mg/kg bw per day with MRULs. The Committee noted that the MRULs for riboflavin as a food colour were well below the GSFA MPLs for most food categories.

Estimates of dietary exposure to riboflavin from all sources, including from its use as a food additive, are available from many national dietary surveys. EFSA (35) estimated dietary exposure to be in the range of 0.05–0.09 mg/kg bw per day for children and 0.02–0.04 mg/kg bw per day for adults. The Committee also noted estimates of dietary exposure to riboflavin from Australia (0.03 mg/kg bw per day for adults and 0.06 mg/kg bw per day for children; 52), New Zealand (0.03 mg/kg bw per day for adults and 0.05 mg/kg bw per day for children; 52), the Republic of Korea (0.02–0.03 mg/kg bw per day for adults; 53) and the USA (from 0.05 mg/kg bw per day for men to 0.14 mg/kg bw per day for children; 54). The group of milk and dairy products was the main contributor to dietary exposure to riboflavin in Spain, at 32.3% (48); Australia, at 27–28% (49); and New Zealand; at 23% (50,51).

The Committee concluded that the highest estimate of high-level dietary exposure to riboflavin of 3.6 mg/kg bw per day for children aged 3–9 years, calculated with the FAIM 2.0 with MRULs, should be considered in the safety assessment of riboflavin.

5. Evaluation

In its present evaluation of riboflavin from *A. gossypii*, the Committee noted that it has low acute toxicity and did not raise concern for genotoxicity. The NOAEL in a 90-day oral toxicity study in rats on riboflavin from *A. gossypii* was 3000 mg/kg bw per day (rounded by the Committee from 3011 mg/kg bw per day), the highest dose tested.

Comparison of the NOAEL of 3000 mg/kg bw per day with the estimate of dietary exposure of 3.6 mg/kg bw per day, based on maximum reported use levels, resulted in an MOE > 800. The Committee concluded that exposure to riboflavin from all sources does not represent a safety concern.

The NOAEL of 3000 mg/kg bw per day in the present evaluation of riboflavin from *A. gossypii* is considerably higher than the 50 mg/kg bw per day in the multigeneration study with a single dose level that was used by the previous Committee to establish an ADI of 0–0.5 mg/kg bw. The Committee at its present meeting noted that the toxicity database on riboflavin from various sources

reviewed previously by the Committee does not indicate any adverse effects. The Committee at its present meeting established a group ADI “not specified”⁹ for riboflavin, riboflavin-5'-phosphate, riboflavin from *B. subtilis* and riboflavin from *A. gossypii* and withdrew the previous group ADI of 0–0.5 mg/kg bw.

A toxicological and a dietary exposure monograph was prepared.

New specifications and a Chemical and Technical Assessment were prepared.

Future work

Regarding the previously established specifications for riboflavin and riboflavin from *B. subtilis*, the Committee proposes to:

- rename “riboflavin” as “riboflavin, synthetic”;
- replace the existing method for determination of lumiflavin in both specifications to avoid the use of chloroform; and
- delete the functional use of “nutrient supplement” from the specifications monograph on riboflavin from *B. subtilis*, as the Codex food additive definition does not include nutrients.

Recommendation

In view of information received at the current meeting that implies that riboflavin is no longer produced synthetically for use as a food additive, the Committee recommends that the CCFA reconsider the requirement for specifications for synthetically produced riboflavin.

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Ribonuclease P from *Penicillium citrinum*

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1. Explanation

At the request of the CCFA at its Fifty-first Session (1), the Committee evaluated the safety of ribonuclease P (IUBMB EC No. 3.1.26.5) from *Penicillium citrinum*, which it has not previously evaluated.

In this report, the term “ribonuclease P” refers to the ribonuclease P enzyme and its amino acid sequence, the term “powdered enzyme concentrate”

to the test material used in the toxicity studies submitted and the term “enzyme preparation” to the product formulated for commercial use.

The Committee at its present meeting considered the submitted data and searched the literature in the PubMed database (all fields), Scopus and Embase (title, abstract, keywords) with the linked search terms “ribonuclease” AND (“penicillium” OR “citrinum”). In total, 188 unique references were found, of which only two described biochemical and/or toxicological studies with ribonuclease P from *P. citrinum*. Most of the toxicological studies with ribonuclease P from *P. citrinum* described below were therefore submitted by the sponsor.

1.1 Genetic background

P. citrinum is a filamentous fungus that is ubiquitous in the environment. It occurs on various plants, including citrus fruits and wheat and other cereal grains (2). *Penicillium* species are recognized for use in food applications (3), including as a source organism in the production of ribonuclease P for use in food processing (4). The taxonomy of the source organism was confirmed from its macroscopic and microscopic characteristics. The *P. citrinum* production strain used in the manufacture of ribonuclease P was *P. citrinum* AE-RP. The strain was verified as from *P. citrinum* by phylogenetic analysis of the rDNA sequence from the results of a BLAST homology search in the APOLLON DB-FU ver.1.0 database, which includes all sequences in the International Nucleotide Sequence Database (GenBank/DBK/EMBL).

The *P. citrinum* production strain was obtained by conventional mutation with *N*-methyl-*N*'-nitrosoguanidine, ultraviolet light and monospore isolation of the parent strain, *P. citrinum* IAM 7003. The parent strain was originally housed at the Institute of Applied Microbiology Culture Collection; it is presently held at the Japan Collection of Microorganisms under *P. citrinum* JCM 22500.

P. citrinum is known to produce citrinin, a mycotoxin (5); however, citrinin is not produced in the manufacture of ribonuclease P by fermentation of *P. citrinum* AE-RP. *P. citrinum* is an occasional opportunistic human pathogen and has been identified rarely as a cause of pneumonia in immunocompromised individuals (6,7). No viable *P. citrinum* organisms are present in the enzyme preparation.

1.2 Chemical and technical considerations

Ribonuclease P is produced by controlled aerobic submerged batch fermentation of a pure culture of a selected strain of *P. citrinum* AE-RP. Ribonuclease P can also be produced by *P. citrinum* RP-4, but insufficient information was available

on the enzyme concentrate produced from this strain, and enzyme preparations manufactured with the RP-4 strain were not included in this evaluation. During fermentation, the enzyme is secreted into the fermentation broth by the microbial cells. Fermentation continues for a predetermined time or until the enzyme production rate decreases below a defined threshold. The enzyme is separated from the fermentation medium in a series of filtration steps. The biomass is pre-treated with flocculants and filtration aids to facilitate removal of cell material. Germ and polish filtration are performed as part of the recovery process to prevent microbial contamination. The liquid enzyme concentrate is spray-dried, and the activity is standardized with dextrin in production of the final powdered enzyme preparation. The entire process is performed in accordance with current good manufacturing practice with food-grade raw materials. The final ribonuclease P enzyme preparation does not contain the production strain. The enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing. The primary sequence of ribonuclease P produced by *P. citrinum* consists of 342 amino acids; its molecular weight by calculation from the determined amino acid sequence is 35 kDa.

Ribonuclease P catalyses the hydrolysis of RNA to monophosphate nucleotides. Ribonuclease P enzyme preparation is intended for use in processing yeast products and flavouring substances and preparations with naturally occurring RNA. The degradation of the RNA substrate in raw materials to produce free phosphonucleotides, specifically guanine and adenine, enhances the consistency and organoleptic (flavour) properties of the final food or food ingredient. Ribonuclease P activity is measured spectrophotometrically as the release of phosphate from adenosine 3'-phosphate. One unit is defined as the amount of enzyme that liberates one $\mu\text{mol}/\text{min}$ of phosphate under the assay conditions. The mean activity of ribonuclease P from three batches of the powdered enzyme concentrate was 112 600 U/g.

The mean TOS content of the enzyme concentrate is 444 mg/g. The TOS includes the enzyme of interest and residues of organic materials, such as proteins, peptides and carbohydrates, derived from the production organism during manufacture. Ribonuclease P enzyme preparation is used at concentrations up to 1000 mg TOS/kg raw material. Ribonuclease P is denatured and inactivated by high temperatures ($> 80\text{ }^{\circ}\text{C}$) during the production of processed yeast and has no technological effect in the final food. When used in the production of flavouring substances or flavouring preparations, the enzyme is either denatured or removed from the final product. Any carry-over of active ribonuclease P to food is negligible. If present, it is expected that ribonuclease P will be digested, as are most other proteins occurring in food, but no data were available on its digestibility.

2. Biological data

2.1 Biotransformation

No data were available.

2.2 Assessment of potential allergenicity

Ribonuclease P from *P. citrinum* was evaluated for allergenicity according to the bioinformatics criteria recommended by FAO/WHO (8,9), modified at the eightieth meeting of the Committee (Annex 1, reference 223). A homology search was conducted, in which the amino acid sequence of ribonuclease P from *P. citrinum* was compared with those of known allergens in the AllergenOnline database (<http://www.allergenonline.org/databasefasta.shtml>; version 19, February 2019) and in the Allermatch database (<http://allermatch.org/>; version July 2019). A search for matches with > 35% identity in a sliding window of 80 amino acids and a search for exact matches in an eight-amino acid window produced no matches. Additionally, a full-length FASTA sequence search was conducted with an E-value cut off of 0.1.¹⁰ No sequences were considered homologous with known allergens. Therefore, the Committee considered that dietary exposure to ribonuclease P from *P. citrinum* is not anticipated to pose a risk of allergenicity. No data were available on the digestibility of ribonuclease P in the gastrointestinal tract.

2.3 Toxicological studies

The range-finding study and 90-day study initially submitted by the sponsor were performed with a powdered concentrate of ribonuclease P from *P. citrinum* AE-RP (batch no. P7BA501; TOS content, 49.2%). The powdered enzyme concentrate had a ribonuclease activity of 72600 U/g, or 147.5 U/mg TOS (10,11).

The bacterial reverse mutation assay and chromosomal aberration assay initially submitted by the sponsor were performed with a powdered concentrate from *P. citrinum* AE-RP (batch no. RP06I20L12.SP1; TOS content, 42.5%). The powdered enzyme concentrate had a ribonuclease activity of 104 000 U/mg (12,13). The method used to determine the ribonuclease activity is no longer in use. A correction factor of 0.564 was provided by the sponsor to calculate the

¹⁰ Comparisons between highly homologous proteins yield expectation values (E-values) approaching zero, indicating a very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity.

ribonuclease activity (in U/g) that would be measured with the method currently in use. For this enzyme concentrate the enzyme activity would be 58 700 U/g or 138.1 U/mg TOS.

In addition to the studies on ribonuclease P from *P. citrinum* AE-RP initially submitted, the literature search resulted in toxicity studies with ribonuclease P from *P. citrinum* RP-4 (14,15), and, subsequently, the sponsor submitted the original study reports of most of the studies reported in these paper (16–19).

The manufacture of ribonuclease P from *P. citrinum* RP-4 includes a precipitation step with ethanol. Therefore, the composition of the enzyme concentrates obtained with *P. citrinum* AE-RP is different from those obtained with *P. citrinum* RP-4. The Committee concluded that the studies with ribonuclease from *P. citrinum* RP-4 were not relevant for the current evaluation of ribonuclease P from *P. citrinum* AE-RP and did not include them in their evaluation.

2.3.1 Acute toxicity

No data were available.

2.3.2 Short-term studies of toxicity

(a) Rats

An oral dose range-finding study was performed in which groups of six male and six female Sprague-Dawley rats were given ribonuclease P powdered enzyme concentrate from *P. citrinum* AE-RP dissolved in water at a dose of 0, 500, 1000 or 2000 mg/day (equal to 246, 492 or 984 mg TOS/kg bw per day) by gavage for 14 days (10). The study was certified for compliance with the Japanese “Reliability standards of application data” and quality assurance. Rats were observed for deaths, clinical signs, body weight and feed consumption. Blood samples were collected for haematological and clinical chemistry at study termination. All rats were necropsied, and organ weights of adrenal gland, spleen, heart, lung, liver, kidney and testis/ovary were measured. Samples from 19 organs were preserved for histopathological examinations.

No treatment-related deaths or clinical signs were observed. Body weight and feed consumption were not affected. Incidental findings included a statistically significant increase (+13%) in serum glucose concentration in females at the low dose and a statistically significantly lower absolute ovary weight (–15%) in females at the mid dose. In males at the high dose, a statistically significant increase (+31%) in white blood cell count was observed, which was considered a minor change and was not observed in females. No abnormalities were observed at autopsy and therefore no histopathological examinations were

performed. In the absence of adverse effects, the same dose levels were selected for the subsequent 13-week study.

In the subsequent study, groups of 12 male and 12 female Sprague-Dawley rats were given ribonuclease P powdered enzyme concentrate from *P. citrinum* AE-RP dissolved in water at a dose of 0, 500, 1000 or 2000 mg/day (equal to 0, 246, 492 or 984 mg TOS/kg bw per day) by oral gavage for 13 weeks (11). The study was certified for compliance with GLP and quality assurance and was conducted according to Japanese guidelines for toxicity testing. The protocol was comparable to OECD test guideline 408 (repeated dose 90-day oral toxicity study in rodents, 1998), except that no functional observational tests were performed. Feed and water were available ad libitum. Animals were observed for clinical signs at least twice daily. Body weight and feed consumption were measured three times in the first week and twice a week thereafter. Ophthalmoscopic examinations were performed in week 13 (six animals per group). Urinalysis was also performed in week 13 for each animal, from which a 4-h urine sample was collected under deprivation of feed but free access to water; thereafter, a 20-h urine sample was collected with free access to feed and water. One-day water consumption was measured on the day before the urine samples were collected. At necropsy, blood samples were collected from the abdominal aorta for haematology and blood chemistry, and then all animals were killed by exsanguination. Macroscopic examination was performed on all organs and tissues, including those in the cephalic, thoracic and abdominal cavities. Organ weights were determined for brain, pituitary, thyroid including parathyroid, adrenal, thymus, spleen, heart, lung including bronchus, salivary gland, liver, kidney, testis, prostate, seminal vesicle, ovary and uterus. Histopathological examination was conducted on approximately 50 tissues from animals in the control and high-dose groups, on gross lesions and on tissues from an animal that died during the treatment period.

One female at the mid dose died during week 3 of treatment. The animal showed prone/lateral position, bradypnoea and hypothermia and was found dead immediately before dosing on day 17. Necropsy showed dark reddening of the cerebrum and cerebellum but no histopathological changes in these organs. Histopathological examination showed some changes that were classified as minimal or mild, including hypertrophy of cortical cells of the adrenals, necrosis of lymphocytes in the mesenteric lymph node, cell infiltration of lymphocytes in the pancreas, follicular atrophy in the spleen and necrosis of lymphocytes in the thymus. As the changes in the cerebrum and cerebellum were not observed in other treated animals, the death was considered not to be treatment-related.

No clinical signs were recorded in the other animals. No effects were observed on body weight, feed consumption, ophthalmoscopy, urinalysis or clinical chemistry. A statistically significant increase in reticulocyte percentage (+29%) was observed in males at the mid dose. The relative weight of the spleen

was statistically significantly increased (+7%) in males at the mid dose, and the absolute (+22%) and relative (+14%) weights of the adrenal glands in females at this dose were statistically significantly increased. As these changes were not dose-related, they were considered not to be treatment-related. Macroscopic examination revealed several changes in one or two animals per group, which included diverticulum in the ileum (one female at the high dose), a white focus in the kidney (one male at the high dose), a cyst in the kidney (one female at the high dose), a dark-red focus in the liver (one female at the high dose), a dark-red focus in the lung (one male at the mid dose and one male at the high dose), a cyst in the pituitary (one female at the low dose), a small spleen with a cyst (one female at the mid dose), a white focus in the spleen (one male at the mid dose) and a dark-red focus in the stomachs of two male control rats. These were considered to be incidental findings. No treatment-related histopathological findings were observed in the tissues and organs studied.

In view of the absence of treatment-related effects, the Committee identified a NOAEL of 980 mg TOS/kg bw per day (rounded by the Committee from 984 mg TOS/kg bw per day), the highest dose tested.

2.3.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.3.4 Genotoxicity

The results of studies of genotoxicity in vitro with the powdered ribonuclease P concentrate from *P. citrinum* AE-RP are summarized in [Table 1](#). The bacterial reverse mutation study (12) was performed according to OECD test guideline 471 (bacterial reverse mutation test, 1997). The chromosomal aberration test (13) was performed in accordance with OECD test guideline 473 (in vitro mammalian chromosome aberration test, 1997). Both studies were certified for compliance with GLP and quality assurance. The results of both studies were negative, and the Committee concluded that there is no concern regarding the genotoxicity of the ribonuclease P enzyme preparation.

2.3.5 Reproductive and developmental toxicity

No information was available.

2.4 Observations in humans

No information was available.

Table 1
Genotoxicity of the powdered ribonuclease P concentrate in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation	<i>Salmonella typhimurium</i> TA98, TA100, TA1535 and TA1537 and <i>Escherichia coli</i> WP2uvrA	21–2125 µg TOS ^a /plate, ± S9	Negative ^b	11
Chromosomal aberration	Human peripheral blood lymphocytes	Short exposure (3 h): 531, 1063 or 2125 µg TOS ^a /mL, ±S9;	Negative ^c	12
		Continuous exposure (24 h): 531, 1063 or 2125 µg TOS ^a /mL, –S9		

S9: 9000 × g supernatant fraction from rat liver homogenate

^a Concentration in µg TOS/plate is calculated from concentrations in µg/plate with the TOS content (42.5%) of the powdered enzyme concentrate.

^b A range-finding study (concentration range, 21–2125 µg TOS/plate; one plate/concentration, except for three plates/concentration for controls) and a duplicate experiment (concentration range, 133–2125 µg TOS/plate; three plates/concentration) were performed with the preincubation method. No toxicity was reported.

^c In the first experiment, the cells were treated for 3 h ± S9 and were harvested 21 h later. In the second experiment, the cells were exposed continuously for 24 h without S9 and then harvested. No statistically significant increase in the prevalence of chromosomal aberrations was observed. The test concentrations were based on the results of a growth inhibition test with the same durations of exposure. After short exposure, the relative cell growth (percentage of control) was approximately 40% with S9 and approximately 90% without S9 at the highest concentration of 2125 µg TOS/plate. After continuous exposure, the relative cell growth (percentage of control) was approximately 30% at the highest concentration tested.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to ribonuclease P from *P. citrinum*. The enzyme is intended for use in yeast processing and in the production of flavourings of animal, vegetable, or microbial origin. The yeast extracts are also used in dietary supplements. All these uses were considered in the dietary exposure assessment. The sponsor noted that many foods that contain these ingredients could contain the enzyme. The submission included an estimate of dietary exposure based on the budget method, a screening method used to determine the TMDI of food additives (20,21), which accounts for maximum physiological levels of consumption of food and non-milk beverages, the energy density of foods, the concentration of the food additive in foods and non-milk beverages and the proportion of foods and non-milk beverages that may contain it. The method provides a conservative estimate of dietary exposure. Further details of the budget method can be found in chapter 6 of EHC 240 (22).

3.2 Dietary exposure assessment

The sponsor provided information on use levels in some representative foods and ingredients that could be treated with the enzyme. As the enzyme can be used broadly in the diet, however, the assumptions used in the budget method do not require detailed information. The estimated TMDI provided by the sponsor

was based on the proportion of food and non-milk beverages containing the enzyme preparation, maximum TOS levels for each category and the percentage of ingredients formulated into final foods. Additionally, a maximum level of use and a residual level in dietary supplements were included.

EHC 240 refers to commonly used default proportions of 12.5% for foods and 25% for non-milk beverages (23). As food ingredients processed with the enzyme are proposed to be added to a variety of foods intended to be consumed by the general population, the sponsor used 25% as the default proportion for foods in the budget method calculation. The sponsor conservatively assumed that exposure would be derived by summing the exposures from solid foods, non-milk beverages and dietary supplements.

The maximum level of TOS from the enzyme present in the final food from solid food and non-milk beverage uses was 20 mg TOS/kg food and that of the enzyme as used in dietary supplements was 1000 mg TOS/kg. The standard budget method calculation was undertaken for estimating dietary exposure to the TOS from three sources: solid foods, non-milk beverages and dietary supplements. For the dietary supplements, it was assumed that a maximum of 30 g/day would be consumed.

The resulting TMDIs of ribonuclease P were estimated to be 0.25 mg TOS/kg bw per day from solid foods, 0.50 mg TOS/kg bw per day from non-milk beverages and 0.50 mg TOS/kg bw per day from dietary supplements, resulting in a total of 1.25 mg TOS/kg bw per day.

For the dietary exposure assessment, it was assumed that 100% of the enzyme remains in the ingredient and final food. The enzyme is removed or inactivated by high temperatures during processing of food ingredients and would have no technological function in the final food.

4. Comments

Biotransformation

No data were available.

Assessment of allergenicity

Ribonuclease P from *P. citrinum* was evaluated for potential allergenicity according to the bioinformatics criteria recommended by FAO/WHO (8,9) and modified at the eightieth meeting of the Committee ([Annex 1](#), reference 223).

The amino acid sequence of ribonuclease P from *P. citrinum* was compared with those of known allergens in publicly available databases. A search for matches with > 35% identity in a sliding window of 80 amino acids, a search for sequence identity of eight contiguous amino acids and a full-length FASTA sequence search produced no matches. Therefore, the Committee concluded that dietary exposure to ribonuclease P from *P. citrinum* would not be anticipated to pose a risk of allergenicity.

Toxicological studies

In addition to the studies submitted on ribonuclease P from *P. citrinum* AE-RP, the literature search resulted in toxicity studies with ribonuclease P from *P. citrinum* RP-4 (14,15). As manufacture of ribonuclease P from *P. citrinum* RP-4 includes a precipitation step with ethanol, the composition of the enzyme concentrates obtained with *P. citrinum* AE-RP is different from those obtained with *P. citrinum* RP-4. The Committee concluded that the studies with ribonuclease from *P. citrinum* RP-4 were not relevant for the current evaluation of ribonuclease P from *P. citrinum* AE-RP and did not include them in their evaluation.

In a 2-week dose range-finding study and a 13-week study of oral toxicity in rats with ribonuclease P from *P. citrinum* AE-RP, no treatment-related adverse effects were seen when the powdered enzyme concentrate was administered by gavage at doses up to 984 mg TOS/kg bw per day, the highest dose tested (10,11). The Committee identified a NOAEL of 980 mg TOS/kg bw per day (rounded by the Committee from 984 mg TOS/kg bw per day), the highest dose tested.

Powdered ribonuclease P concentrate from *P. citrinum* AE-RP was not genotoxic in a bacterial reverse mutation assay or in an in vitro chromosomal aberration assay (12,13). The Committee had no concern with respect to the genotoxicity of the preparation of ribonuclease P from *P. citrinum* AE-RP.

Observations in humans

No data were available.

Assessment of dietary exposure

The Committee evaluated an estimate of dietary exposure to ribonuclease P from *P. citrinum* submitted by the sponsor. The estimate was derived with the budget method and was based on maximum use levels of 20 mg TOS/kg for solid foods and for non-milk beverages and 1000 mg TOS/kg for dietary supplements and the

assumption that 25% of the food supply would contain the enzyme preparation. It was assumed that the maximum consumption of dietary supplements would be 30 g/day. The theoretical maximum daily intake was estimated to be 1.3 mg TOS/kg bw per day (rounded by the Committee from 1.25 mg TOS/kg bw per day). For the dietary exposure assessment, it was assumed that 100% of the enzyme remains in the ingredient and final food. The enzyme is removed or inactivated by high temperatures during processing of food ingredients and would have no technological function in the final food.

5. Evaluation

The Committee identified a NOAEL of 980 mg TOS/kg bw per day (the highest dose tested) in a 13-week study in which rats were treated with ribonuclease P concentrate from *P. citrinum* AE-RP by gavage. A comparison of the estimated dietary exposure of 1.3 mg TOS/kg bw per day with the NOAEL of 980 mg TOS/kg bw per day gives an MOE > 750. On the basis of this MOE and the absence of concern for genotoxicity, the Committee established an ADI “not specified”¹¹ for the ribonuclease P enzyme preparation from *P. citrinum* AE-RP, used in the applications specified and in accordance with good manufacturing practice.

A toxicology and dietary exposure monograph was prepared.

New specifications and a chemical and technical assessment were prepared.

5.1 Recommendations

Ribonuclease P can also be produced by *P. citrinum* RP-4, but insufficient information was available on the enzyme concentrate produced from this strain. To evaluate the safety of ribonuclease P from *P. citrinum* RP-4, toxicological studies with well-characterized enzyme concentrate are required.

6. References

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¹¹ The reader is referred to the technical report of the eighty-seventh meeting (Annex 1, reference X), for clarification of the term “ADI not specified”.

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ANNEX 1

Reports and other documents resulting from previous meetings of the Joint FAO/WHO Expert Committee on Food Additives

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244. Evaluation of veterinary drug residues in food (Eighty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives) WHO Technical Report Series, No. 1023, 2020.
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ANNEX 2

Abbreviations and acronyms used in the monographs

ADI	acceptable daily intake
AUC	area under the concentration–time curve
BLAST	basic local alignment search tool
bw	body weight
CAS	Chemical Abstracts Services
CCFA	Codex Committee on Food Additives
CIFOCoss	Chronic Individual Food Consumption database summary statistics
DMRL	6,7-dimethyl-8-ribityllumazine
EFSA	European Food Safety Authority
EHC	Environmental Health Criteria
FAD	flavin-adenine dinucleotide
FAIM	Food Additive Intake Model
FAO	Food and Agriculture Organization of the United Nations
FMN	flavin mononucleotide
GLP	good laboratory practice
GSFA	General Standard for Food Additives
INS	international numbering system
JECFA	Joint Expert Committee on Food Additives
LD ₅₀	acute toxic dose
LOD	limit of detection
LOQ	limit of quantification
MOE	margin of exposure
MPL	maximum permitted level
MRUL	maximum reported use level
NOAEL	no-observed-adverse-effect level
OECD	Organization for Economic Co-operation and Development
PBPK	physiologically based pharmacokinetics
PND	postnatal day
S9	9000 × <i>g</i> supernatant fraction from liver homogenate
SD	standard deviation
TK	toxicokinetics
TMDI	theoretical maximum daily intake
TOS	total organic solids
U	unit
USA	United States of America
V _{max}	maximum biotransformation rate



ANNEX 3

Participants in the ninety-second meeting of the Joint FAO/WHO Expert Committee on Food Additives

Members

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Ms K. Laurvick, Food Standards, United States Pharmacopeia, Rockville (MD), United States of America (*Co-rapporteur*)

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- Dr S.M.F. Jeurissen, Department for Food Safety, Centre for Nutrition, Prevention and Health, National Institute for Public Health and the Environment, Netherlands (*WHO temporary adviser*)
- Dr M. Lipp, Agriculture and Consumer Protection Department, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Joint Secretary*)
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- Mr K. Petersen, Department of Nutrition and Food Safety, World Health Organization, Geneva, Switzerland (*WHO Joint Secretary*)
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- Dr S.G. Walch, Executive Director, Chemisches und Veterinäruntersuchungsamt, Karlsruhe, Germany (*FAO expert*)
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- Dr H.J. Yoon, Korea Food and Drug Administration, Seoul, Republic of Korea (*WHO temporary adviser*)

ANNEX 4

Toxicological and 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
Benzoic acid, its salts and derivatives	N	<p>The Committee evaluated a new extended one-generation reproductive toxicity study on benzoic acid. This study showed no treatment-related adverse effects, indicating a NOAEL of 1000 mg/kg bw per day, the highest dose tested.</p> <p>Applying a chemical specific adjustment factor of 2 for interspecies toxicokinetics variation instead of the default factor of 4.0, the Committee established a group ADI of 0–20 mg/kg bw, which applies to benzoic acid, the benzoate salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate, expressed as benzoic acid equivalents. The Committee withdrew the previous group ADI of 0–5 mg/kg bw. The Committee noted that the high dietary exposure estimate, expressed as benzoic acid, of 7.1 mg/kg bw per day for children aged 3–9 years does not exceed the group ADI of 0–20 mg/kg bw.</p>
Collagenase from <i>Streptomyces violaceoruber</i> expressed in <i>S. violaceoruber</i>	N	<p>Negative results were observed in genotoxicity studies with a powdered enzyme concentrate. The Committee identified a NOAEL of 940 mg TOS/kg bw per day (rounded from 939.6), the highest dose tested in a 13-week study of oral toxicity in rats. The Committee identified a NOAEL of 940 mg TOS/kg bw per day, the highest dose tested in a 13-week study of oral toxicity in rats. Comparison of this NOAEL with the estimated dietary exposure of 0.43 mg TOS/kg bw per day gave a margin of exposure (MOE) of > 2100.</p> <p>In view of this MOE and the lack of concern about genotoxicity, the Committee established an ADI “not specified”¹² for collagenase from <i>S. violaceoruber</i>, when used in the applications specified and in accordance with good manufacturing practice.</p>
β -Glucanase from <i>Streptomyces violaceoruber</i> expressed in <i>S. violaceoruber</i>	No ³	<p>The Committee noted negative results in studies of genotoxicity and in studies of oral toxicity in rats. The Committee identified a NOAEL of 950 mg TOS/kg bw per day (rounded by the Committee from 953.3), the highest dose tested. Comparison of this NOAEL with the estimated dietary exposure of 0.15 mg TOS/kg bw per day gave an MOE > 6300.</p>

¹² The reader is referred to the Technical Report of the 87th JECFA meeting for clarification of the term “ADI not specified”.

(continued)

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
Phospholipase A2 from <i>Streptomyces violaceoruber</i> expressed in <i>S. violaceoruber</i>	R	<p>On the basis of this MOE and the lack of concern about genotoxicity, the Committee established an ADI “not specified” for β-glucanase from <i>S. violaceoruber</i>, for the proposed uses and in accordance with good manufacturing practice.</p> <p>Negative results were obtained in genotoxicity tests. In a 13-week study of oral toxicity in rats, small effects were seen at low incidence at the high dose of 956 mg TOS/kg bw per day, which might have been related to treatment. The Committee therefore identified a NOAEL of 190 mg TOS/kg per day (rounded by the Committee from 191 mg TOS/kg bw per day). A comparison of the estimated dietary exposure of 0.25 mg TOS/kg bw per day with the NOAEL of 190 mg TOS/kg bw per day from the oral toxicity study gives a MOE of 760.</p> <p>On this basis and in the absence of concern about genotoxicity, the Committee established an ADI “not specified” for the phospholipase A2 enzyme preparation from <i>S. violaceoruber</i> when used in the applications specified and in accordance with good manufacturing practice.</p>
Riboflavin from <i>Ashbya gossypii</i>	N	<p>The Committee noted that riboflavin from <i>A. gossypii</i> has low acute toxicity and does not raise concern for genotoxicity. The NOAEL from a 90-day oral toxicity study in rats was 3000 mg/kg bw per day, the highest dose tested. Comparison of this NOAEL with the estimated dietary exposure of 3.6 mg/kg bw per day, based on maximum reported use levels, resulted in an MOE > 800.</p> <p>The Committee established a group ADI “not specified” for riboflavin, riboflavin-5'-phosphate, riboflavin from <i>B. subtilis</i> and riboflavin from <i>A. gossypii</i>, expressed as riboflavin. The Committee withdrew the previous group ADI of 0–0.5 mg/kg bw.</p>
Ribonuclease P from <i>Penicillium citrinum</i>	N	<p>The Committee identified a NOAEL of 980 mg TOS/kg bw per day (the highest dose tested) in a 13-week study in which rats were treated with ribonuclease P concentrate from <i>P. citrinum</i> AE-RP by gavage. A comparison of the estimated dietary exposure of 1.3 mg TOS/kg bw per day with the NOAEL of 980 mg TOS/kg bw per day gives an MOE > 750.</p> <p>On the basis of this MOE and the lack of concern for genotoxicity, the Committee established an ADI “not specified” for the ribonuclease P enzyme preparation from <i>P. citrinum</i> AE-RP, used in the applications specified and in accordance with good manufacturing practice.</p>

N: new specifications, R: revised specifications

Food additives considered for specifications only

Food additive	Specifications
Modified starches	R

R: existing specifications revised

Revision of specifications and analytical methods**Modified starches****Explanation**

The Committee at its eighty-sixth meeting reviewed full specifications for three modified starches, International Numbering System (INS) 1404, 1420 and 1451, tentative specifications for the remaining 13 modified starches (INS 1400, 1401, 1402, 1403, 1405, 1410, 1412, 1413, 1414, 1422, 1440, 1442 and 1450) and data on the method of manufacture, identity and purity of all 16 modified starches. At the same meeting, the Committee drafted a modular specifications monograph entitled “Modified starches”, consisting of an explanatory introduction, “General specifications for modified starches”, applying to all 16 modified starches, and eight annexes with specifications applicable to individual modified starches according to their treatment(s) (see list below). The general specifications and annexes 1, 2, 3, 5, 7 and 8 were made tentative. Data and the information necessary to remove the tentative status and revise the modular specifications monograph were requested.

At its current meeting, the Committee reviewed the information and data received, revised the modular specifications monograph and removed the tentative status of the “General specifications” and annexes 1, 2, 3, 5, 7 and 8. Each modified starch should fulfil the specification requirements of the “General specifications” and in the applicable annexes.

Modified starches considered and applicable annexes

Modified starch	INS	Annex
Dextrin roasted starch	1400	1
Acid treated starch	1401	1
Alkaline treated starch	1402	1
Bleached starch	1403	2
Oxidized starch	1404	5
Enzyme-treated starch	1405	1
Monostarch phosphate	1410	3
Distarch phosphate	1412	3
Phosphated distarch phosphate	1413	3
Acetylated distarch phosphate	1414	3,4
Starch acetate	1420	4

(continued)

Modified starch	INS	Annex
Acetylated distarch adipate	1422	4, 8
Hydroxypropyl starch	1440	7
Hydroxypropyl distarch phosphate	1442	3, 7
Starch sodium octenylsuccinate	1450	6
Acetylated oxidized starch	1451	4, 5

ANNEX 5

Corrigenda

Food additive	Original text	Revised text	Additional information
Riboflavin INS 101(i)	$\% \text{ Riboflavin} = A \times 5000328 \times W \times 1.367\%$ $\text{riboflavin} = A \times 5000328 \times W \times 1.367$	$\% \text{ Riboflavin} = A \times 5000328 \times W\%$ $\text{riboflavin} = A \times 5000328 \times W$	Correction to calculation in the method of assay; removal of a wrongly assigned factor
Riboflavin from <i>Bacillus subtilis</i> INS 101(iii)	$\% \text{ Riboflavin} = A \times 5000328 \times W \times 1.367\%$ $\text{riboflavin} = A \times 5000328 \times W \times 1.367$	$\% \text{ Riboflavin} = A \times 5000328 \times W\%$ $\text{riboflavin} = A \times 5000328 \times W$	Correction to calculation in the method of assay; removal of a wrongly assigned factor
Riboflavin 5'-phosphate sodium INS 101(ii)	CAS number 130-40-5	CAS number 130-40-5 (anhydrous) CAS number 6184-17-4 (dihydrate)	Current specifications provide the formula for the dihydrate but no applicable CAS number
Potassium polyaspartate	Missing "Method of assay"	Add "Method of assay" under "Purity tests" after the test entitled "Molecular weight and molecular weight distribution". Delete the bold text "Potassium polyaspartate", which appears in the test for "Molecular weight and molecular weight distribution", and replace with "Principle" (as the method of assay).	Correct errors in format of specifications monograph
Vol. 4 procedure			
Unulfonated primary aromatic amines	See printed version of Vol. 4	See revised text below; modified text is in bold.	Correction to the range of the standard curve

Revised text:

Procedure

Preparation of standard aniline solution

Weigh **100 mg** of redistilled aniline into a small beaker, and transfer to a 100-mL volumetric flask, rinsing the beaker several times with water. Add 30 mL of 3 N hydrochloric acid, and dilute to the mark with water at room temperature. Dilute 10.0 mL of this solution to 100 mL with water, and mix well. **Dilute 20.0 mL of this solution to 100 mL with water, and mix well (1 mL of this standard solution is equivalent to 20 µg of aniline).** Measure the following volumes of the standard aniline solution into a series of 100-mL volumetric flasks: 5 mL, 10 mL, 15 mL, 20 mL and 25 mL. Dilute to 100 mL with 1 N hydrochloric acid, and mix well

(100 mL of the resulting working standard solutions contains 100, 200, 300, 400 and 500 µg of aniline, respectively). Prepare all standard solutions freshly.

Construction of standard curve

Pipette 10 mL of each **working standard** solution into clean, dry test tubes; cool them for 10 min by immersion in a beaker of ice water. To each tube, add 1 mL of the potassium bromide solution and 0.05 ml of the sodium nitrite solution. Mix, and allow the tubes to stand for 10 min in the ice-water bath while the aniline is diazotized. Into each of five 25-mL volumetric flasks, measure 1 mL of the R salt solution and 10 ml of the sodium carbonate solution. Pour each diazotized aniline solution into a separate flask containing R salt solution and sodium carbonate solution; rinse each test tube with a few drops of water. Dilute to the mark with water, stopper the flasks, mix the contents well, and allow them to stand for 15 min in the dark. Measure the absorbance of each coupled solution at 510 nm in 40-mm cells. As a reference solution, use a mixture of 10.0 mL of 1 N hydrochloric acid, 10.0 mL of the sodium carbonate solution and 2.0 mL of the R salt solution, diluted to 25.0 mL with water. **Construct a standard curve of the absorbance versus the weight (g) of aniline in each 100 mL of working standard solution.**

Preparation and evaluation of a test solution

Weigh, to the nearest 0.01 g, about 2.0 g of the colouring matter sample (W) into a separatory funnel containing 100 mL of water, rinse the sides of the funnel with a further 50 mL of water, swirling to dissolve the sample, and add 5 mL of 1 N sodium hydroxide. Extract with two 50-mL portions of toluene, and wash the combined toluene extracts with 10-mL portions of 0.1 N sodium hydroxide to remove traces of colour. Extract the washed toluene with three 10-mL portions of 3 N hydrochloric acid, and dilute the combined extract to 100 mL with water. Mix well. Call this "solution T". **Pipette** 10.0 mL of solution T into a clean, dry test tube, cool for 10 min by immersion in a beaker of iced water, add 1 mL of the potassium bromide solution, and proceed as described above for preparation of the **standard curve**, starting with addition of 0.05 mL of the sodium nitrite solution. Measure the absorbance of the coupled test solution at 510 nm in a 40-mm cell. Use a reference solution prepared from 10.0 mL of solution T, 10 mL of the sodium carbonate solution and 2.0 mL of the R salt solution diluted to 25.0 mL with water. From the **standard curve**, read the weight of aniline (WA) corresponding to the observed absorbance of the test solution.

Calculation: % unsulfonated primary aromatic amine (as aniline) = $100 \times WA/W$

This volume contains monographs prepared at the ninety-second meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met virtually from 7 to 18 June 2021.

The toxicological and dietary exposure monographs in this volume summarize data on the safety of and dietary exposure to specific food additives: benzoic acid, its salts and derivatives; collagenase from *Streptomyces violaceoruber* expressed in *S. violaceoruber*; β -glucanase from *Streptomyces violaceoruber* expressed in *S. violaceoruber*; phospholipase A2 from *Streptomyces violaceoruber* expressed in *S. violaceoruber*; riboflavin from *Ashbya gossypii*; and ribonuclease P from *Penicillium citrinum*.

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 in controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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