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1037

Evaluation of certain food additives

Ninety-second report of the Joint FAO/WHO Expert Committee on Food Additives

Food and Agriculture Organization of the United Nations

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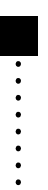

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Ninety-second meeting of the Joint FAO/WHO Expert Committee on Food Additives

Virtual meeting, 7–18 June 2021

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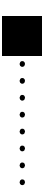
List of abbreviations and acronyms

ADI acceptable daily intake
bw body weight
CCFA Codex Committee on Food Additives
CSAF chemical-specific adjustment factor
EFSA European Food Safety Authority
FAD flavin-adenine dinucleotide
FAO Food and Agricultural Organization of the United Nations
GSFA General Standard for Food Additives
INS International Numbering System
JECFA Joint Expert Committee on Food Additives
LD_{50} median lethal dose
MOE margin of error
MPL maximum permitted level
MRUL maximum reported use level
NOAEL no-observed-adverse-effect level
TMDI theoretical maximum daily intake
TOS total organic solids
WHO World Health Organization



1. Introduction

The ninety-second meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was convened by video conference from 7 to 18 June 2021. The meeting was opened on behalf of the Director-General of the Food and Agriculture Organization of the United Nations (FAO) by Dr Markus Lipp (Food Systems and Food Safety Division, FAO) and on behalf of the Director-General of the World Health Organization (WHO) by Mr Kim Petersen (Programme Manager, Department of Nutrition and Food Safety, WHO). Dr Lipp welcomed all meeting participants and stressed that, despite the challenges of the ongoing COVID-19 pandemic, the work of JECFA had progressed, and the Committee continued to provide sound scientific advice to the Codex Alimentarius Commission and Member States. He reminded the participants of their responsibility to impart the least biased, best scientific advice possible and that they had been invited to serve solely in their capacity as experts to provide sound scientific advice and not as representatives of their employer or country. He closed by reiterating his sincere gratitude to all participants for providing their time and expertise to the present JECFA meeting.

Mr Petersen welcomed all meeting participants on behalf of WHO and thanked all experts for their commitment and dedication to the work of JECFA. He underlined the importance of their work in relation to the work of the Codex Alimentarius Commission in developing international food safety standards.

1.1 Procedural matters

Owing to travel restrictions and lockdowns due to the response to the COVID-19 pandemic in many countries, it was not possible to convene a physical meeting and it was instead decided to hold it online by video-conferencing. In view of the time differences in the countries of the invited experts, the only possible time for a video-conference was restricted to a 4-h time slot (12:00–16:00 CET) each day. This allowed only 40% of the usual daily length (8–10 h) of a typical JECFA meeting. Although the experts participated fully, they noted that online meetings do not permit the necessary in-depth, robust scientific discussions that are characteristic of JECFA meetings and are therefore not a suitable substitute for face-to-face meetings. In particular, the experts felt that the online format did not foster the atmosphere of trust, inclusiveness and openness that has marked all physical JECFA meetings. The experts considered that the success of the ninety-second meeting was due mainly to the cohesion among them stemming from the trust built on the relationships they had formed during previous face-to-face meetings. The experts also decried the significant difficulty of holding any

informal meetings outside the scheduled meeting times because of the widely differing time zones. Perhaps the greatest loss due to the virtual meeting format rather than in-person meetings is in efficiency in solving issues that arise shortly before or during the meeting that require immediate input from individuals or small groups of both FAO and WHO representatives. Indeed, this deficiency means that fewer food additives can be evaluated within a two-week meeting.

The experts emphasized further that an invitation to a physical JECFA meeting at FAO or WHO headquarters gives rise to more significant recognition by the expert's employer of the weight, reach, responsibility and workload required for full participation in a JECFA meeting. The same degree of acknowledgement is not granted by employers for online meetings, as the experts remain available locally. This lack of recognition of the workload and significance of participation in a JECFA meeting led to an increase in other demands on the experts, resulting in more distractions and more frequent scheduling conflicts. The experts concluded that, cumulatively, such factors would be counterproductive for participation in future JECFA meetings if FAO and WHO maintained the online-only format.

In recognition of the difficulties and the tremendous efforts made, the Joint FAO/WHO Secretariat expressed its deep gratitude to all the experts for their commitment and flexibility, not least as the scheduled meeting times were exceedingly inconvenient for many.

The meeting report was adopted on 18 June 2021.

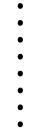
1.2 Declarations of interests

The Secretariat informed the Committee that all experts participating in the ninety-second JECFA meeting had completed a declaration of interests form. The declarations were assessed to determine the extent to which any interest could be reasonably expected to influence the experts' judgement. The declared interests were considered unlikely to impair any individual's objectivity or have any significant influence on the impartiality, neutrality and integrity of their work. Neither FAO nor WHO received any public comments in response to the online posting of the names and brief biographies of the individuals considered for participation in the expert meeting. The interests of all participants were disclosed at the beginning of the meeting to all attendees.

1.3 Adoption of the agenda

After discussion between the experts and the sponsor, the name of the enzyme listed on the agenda as "phosphodiesterase" was changed to "ribonuclease P", and

this change was reflected on the agenda and in the subsequent monograph and specifications. Spirulina was removed from the agenda as the sponsor could not provide the information necessary for establishing full specifications before the meeting. The meeting agenda was adopted with no further modification.



2. Toxicological evaluations and exposure assessments

2.1 Benzoic acid, its salts and derivatives

2.1.1 Explanation

The Committee first evaluated benzoic acid and its salt, sodium benzoate, at its sixth meeting (Annex 1, reference 6) in 1962. 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. This group ADI was based on the absence of any observed adverse effects in rats 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). Dietary exposure to benzoic acid and its salts (benzoates) was evaluated by the Committee at its fifty-first and eightieth meetings (Annex 1, references 137, 138, 223 and 224). At its eightieth meeting, in 2015, the Committee estimated dietary exposure to benzoates through consumption of water-based flavoured drinks on the basis of reported average typical use levels up to 209 mg/L. The highest high dietary exposure estimates were for consumers-only of these drinks, which was up to 10.9 mg/kg bw per day for toddlers and young children. After a literature review, the Committee concluded that, in most countries, water-based flavoured drinks contribute most to dietary exposure to benzoates.

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 also 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 and 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, 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 re-affirmed the group ADI of 0–5 mg/kg bw expressed as benzoic acid equivalents (Annex 1, reference 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 a report of an extended one-generation reproductive toxicity study according to OECD 443 and two published reports on the use of default uncertainty factors for benzoic acid. In addition, the sponsor provided dietary exposure estimates for benzoates from water-based flavoured drinks for Brazil, Canada, Mexico and the USA based on maximum use levels and market volume-weighted average use levels of benzoates (expressed as benzoic acid).

A comprehensive literature search for toxicological and biochemical data from January 2002 to March 2021 was conducted in PubMed/TOXLINE; 49 publications were considered relevant and further evaluated. As benzoic acid and its salts were evaluated by the European Food Safety Authority (EFSA) from the literature available up to 2016, the Committee decided to use only 20 studies published after 2016 and those published since 2002 that the Committee relevant for this evaluation.

At its eightieth meeting, the Committee reviewed publications on dietary exposure to benzoates from all foods between 2000 and 2015. Therefore, the Committee performed a literature search from January 2015 to March 2021 in PubMed and found four publications that were considered relevant for the evaluation.

2.1.2 Chemical and technical considerations

Benzoic acid ($C_7H_6O_2$; CAS No. 65-85-1; 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).

2.1.3 Biochemical aspects

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

Two pharmacokinetics approaches have been proposed in order to reduce the default uncertainty (safety) factor used to set the current ADI for benzoic acid, its salts and derivatives (e.g., benzyl acetate, benzyl alcohol and benzaldehyde). Zu et al. *(3)* applied the procedures described in the IPCS Guidance document for use of data in dose/concentration–response assessment *(4)* to derive a chemical-specific adjustment factor (CSAF) from data on rat and human pharmacokinetics. A reduction of four to two times was proposed for the pharmacokinetics subfactor usually applied to account for inter-species differences. This reduction resulted in an overall uncertainty factor of 50 (i.e., 2 × 2.5 × 10), rather than the default 100 (i.e. 4 × 2.5 × 10). The Committee noted that a CSAF can be set for a food additive only when suitable pharmacokinetics data are available for both the relevant experimental animal species and humans. For deriving a CSAF for benzoic acid, data are available from the use of sodium benzoate at doses as high as 500 mg/kg bw per day in the long-term treatment of patients with inborn errors of urea cycle enzymes that result in hyperammonaemia.

Using a physiologically based pharmacokinetics modelling approach to simulate benzoic acid concentrations in the plasma of rats and humans, Hoffman and Hanneman *(5)* incorporated a seven-compartment model (i.e., blood, liver, brain, adipose, testes/ovaries and rapidly and poorly perfused tissues) with input from three compartments (blood, liver and the remaining body) for some metabolic precursors (i.e., benzyl acetate, benzyl alcohol and benzaldehyde). After a comparison of simulated benzoic acid concentrations in rat and human plasma, the authors concluded that the pharmacokinetics subfactor used to account for inter-species differences was in the range of 0.3–0.4. Hoffman and Hanneman suggested that this fractional value could be used instead of the conventional interspecies pharmacokinetics factor of 4 to yield an overall uncertainty factor of 7.5–10 (i.e., 0.3–0.4 × 2.5 × 10). The Committee noted that, although the absorption and disposition of benzoic acid and its precursors is essentially the same, Hoffman and Hanneman provided no comparison of goodness-of-fit to experimental results with fewer compartments, which increases the model uncertainty. Furthermore, as the interspecies pharmacokinetics factor is essentially determined by hepatic clearance in both rats and humans, the low hepatic clearance in rats (i.e., the Michaelean constant for conversion of benzoic acid to hippuric acid) used in this model may be an underestimate, as it is based on the data after a single intravenous dose of 122 mg/kg bw.

2.1.4 Toxicological studies

In studies previously evaluated by the Committee, the oral acute toxicity (median lethal dose, 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 have been evaluated previously by the Committee, none of which showed effects at doses up to 1000 mg/kg bw per day.

No new long-term studies were found. The Committee 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 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 Committee concluded that there was no concern about the genotoxicity of benzoic acid, its salts and its derivatives.

The Committee previously evaluated a four-generation reproductive toxicity study in rats, 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 *(6)*. On the basis of these results, the Committee 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 an extended one-generation 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 F_0, F_1 and F_2 generations *(7, 8)*. 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 no-observed-adverse effect level (NOAEL) of 1000 mg/kg bw per day, the highest dose tested, for reproductive and developmental toxicity.

2.1.5 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.

2.1.6 Assessment of dietary exposure

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), all expressed as benzoic acid. At the 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 *(9, 10)*, 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 *(11)*, India *(12)*, the Islamic Republic of Iran *(13, 14)* and various other countries, as reviewed at the eightieth meeting of the Committee (Annex 1, references 223 and 224). Table 1 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 of Regulation (EC) No. 1333/2008.

Benzoic acid may also be present 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; however, the concentrations of benzoic acid in food are not expected to be high. 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, however, dietary exposure to benzoic acid 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 1). The Committee considered that this high estimate of dietary exposure was the most suitable estimate currently available for

Table 1

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

Country/ source	Foods included	Source concentrations	Consumption	Dietary exposure (mg/kg bw per day)[b,c] Children[d] Mean	Children[d] High	General population[e] Mean	General population[e] High
Brazil,	Water-based flavoured drinks	Maximum and market volume-weighted use levels	Individual food consumption data	0.63	2.7	0.41	1.8
Canada,				0.15–0.48	0.89-2.4	0.33	1.7
Mexico,				1.5–1.6	4.8-5.1	1.2	4.3
USA				0.44–0.83	2.7-3.2	0.74	2.9
Europe[f]	Whole diet	Maximum and average use; analytical concentrations	Individual food consumption data	0.07–3.2	0.4–7.1	0.07–3.2	0.4–7.1
India	Pickles, sauces, soft and fruit drinks, jellies, jams	Analytical concentrations	Individual food consumption data	0.9–1.3	–	–	4.3
Iran, Islamic Republic of	Orange juice	Analytical concentrations	Per capita	–	0.94[g]	–	2.9
	Cake, toast bread, tomato paste, mayonnaise, carbonated soft drinks, *olovieh* salad		Mean consumption		0.14		
Eightieth meeting literature[h]	Whole diet	Use / analytical concentrations	Individual food consumption data[i]	0.1–1.5	0.4–3.9[j]	0.01–1.5	0.2–3.1

Source: references *9–14*, Annex I, reference *244*.

[a] Exposure estimates for India and the Islamic Republic of Iran refer to dietary exposure to sodium benzoate but 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, Mexico and the USA and for Europe were calculated for a "brand-loyal" scenario. For more details see the text.

[d] Children aged 10–17 years in Brazil, 2–17 years in Canada, 1–17 years in Europe, Mexico, the USA and literature from the eightieth meeting, and 2–19 years for India.

[e] The general population of Europe covers people aged from 12 weeks up 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 were not specified.

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

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

[h] Refers to literature-derived dietary exposure estimates for exposure to benzoates in 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 the 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.

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.

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

Benzene is a known human carcinogen after chronic inhalation *(15)*. 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 *(16)*. Studies have indicated higher concentrations of benzene in beverages that contain benzoate and ascorbic acid *(17, 18)*.

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 *(17–20)*. The present Committee 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 "margin of exposure" (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. Based on a benchmark dose level with a 10% extra risk of adverse effects ($BMDL_{10}$) of 17.6 mg/kg bw per day from dose–response modelling of data on experimental animals, the MOEs were calculated to be 2×10^6 for beverages and between 6×10^6 and 0.4×10^6 for food *(21)*.

The Committee noted that food and beverages make a much smaller contribution to human exposure than other sources, such as inhalation, vehicle fuel fumes and cigarette smoking *(20–22)*. WHO has set a guideline for benzene in drinking-water at 10 μg/L *(23)*.

2.1.8 Evaluation

The Committee previously 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. The previous Committee applied a default uncertainty factor of 100 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 acids. This study showed no treatment-related adverse effects, indicating a NOAEL of 1000 mg/kg bw per day, the highest dose tested.

The Committee evaluated two approaches to refining the uncertainty factor to be used in establishing an ADI and concluded that the CSAF approach of Zu et al. *(3)* was the most appropriate. The Committee therefore applied a CSAF of 2 for interspecies toxicokinetics variation instead of the default factor of 4.0. An overall uncertainty factor of 50 (2 for interspecies toxicokinetic variation × 2.5 for interspecies toxicodynamic variation × 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 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 conservative 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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2.2 Collagenase from *Streptomyces violaceoruber* expressed in *S. violaceoruber*

2.2.1 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; IUBMB EC No. 3.4.24.3) from *Streptomyces violaceoruber* pCol, which it had not previously considered.

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

At its present meeting, the Committee considered the submitted data and 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.

2.2.2 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-toxigenic, 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. violaceoreuber* pCol production strain was obtained by transforming a plasmid containing 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 and measuring collagenase activity each time. The final enzyme preparations were tested for the absence of an antibiotic resistance gene by polymerase chain reaction. The production strain has been deposited at National Institute of Technological Evaluation in Japan.

2.2.3 Chemical and technical considerations

Collagenase is produced by controlled fermentation of a pure culture of the *S. violaceoruber* production strain. 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 the liquid filtrate, followed by formulation with dextrin. The entire process is performed in accordance with current good manufacturing practices and with raw materials that are food-grade. The primary sequence of collagenase produced by *S. violaceoruber* consists of 865 amino acids; its molecular weight calculated from the determined amino acid sequence is 92.4 kDa. The enzyme concentrate is tested to ensure that it is free from the production organism and any antibiotic activity.

The activity of collagenase is determined spectrophotometrically by measuring the hydrolysis of a defined peptide substrate at 570 nm; one unit of activity is defined as the quantity of enzyme required to liberate one μmol/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 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 foods, such as those for special nutritional purposes, sports foods and health foods and in dietary supplements. The collagenase enzyme preparations are used at maximum levels of 1188 mg total organic solids (TOS)/kg raw material (as a liquid) or 36 mg TOS/kg raw material (as a powder) and 1566 mg TOS/kg raw material (as a powder). 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.

The collagenase enzyme is inactivated by heat treatment before use of the final foods. If it were present in the finished food it would probably be digested, like most other proteins, although no data were available on its digestibility.

2.2.4 Biological data

Biotransformation

No information was available.

2.2.5 Allergenicity

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

2.2.6 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 LD_{50} 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 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.

2.2.7 Observations in humans

No information was available.

2.2.8 Assessment of dietary exposure

The Committee evaluated an estimate of the theoretical maximum daily intake (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.

2.2.9 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"[1] for collagenase 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.

[1] 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".

References

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

2.3.1 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.

In this report, the term "β-glucanase" refers to the β-glucanase enzyme and its amino acid sequence, the term "enzyme concentrate" to the test material used in the toxicity studies and the term "enzyme preparation" to the formulated product for commercial use.

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

2.3.2 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, non-toxigenic, occurs in nature as a component of soil *(2)* and has a history of use in the production of enzymes intended for use in food processing *(3)*.

The *S. violaceoruber* pGlu production strain was obtained by transforming a plasmid containing 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 for 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.

2.3.3 Chemical and technical considerations

β-Glucanase 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 of the liquid formulation, followed by standardization with sodium chloride. The entire process is performed in accordance with current good manufacturing practices and with food-grade raw materials. The primary sequence of β-glucanase produced by *S. violaceoruber* consists of 453 amino acids; its molecular weight calculated from the determined amino acid sequence is 42.7 kDa. The enzyme concentrate is tested to ensure that it is free of 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 μmol/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→3)-β-D-glucosidic linkages in (1→3)-β-D-glucans to produce D-glucose and β-glucans. The enzyme preparation is intended for use as a processing aid 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, with residual filtration of the extract products; it is used as a clarifying and filtration aid in the production of beer. The β-glucanase enzyme preparation is intended to be used at a maximum level of 151 mg TOS of powdered β-glucanase/kg raw material 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 manufacture.

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 it is present in finished foods, it would probably be digested, like most other proteins, although no data were available on its digestibility.

2.3.4 Biological data

Biotransformation

No information was available.

2.3.5 Assessment of potential allergenicity

β-Glucanase was assessed as a potential allergen according to 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 those of known allergens 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 of eight contiguous amino acids produced a small number of matches. Upon examination, however, these matches were considered not significant. In view of the intended use and available information, the Committee did not anticipate that β-glucanase would pose an allergenic risk.

2.3.6 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 LD_{50} was > 2000 mg/kg bw of the enzyme concentrate, 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 ≤ 1000 mg/kg bw, equal to 953.3 mg TOS/kg bw.

In a 13-week study of oral toxicity in rats *(9)*, the enzyme concentrate was mixed in water and administered at doses ≤ 1000 mg/kg bw, equal to 953.3 mg TOS/kg bw, to groups of rats by gavage. 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 *(10–12)*. 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 it did not induce polyploidy aberrations *(13)*. The Committee had no concern about the genotoxicity of the enzyme concentrate.

2.3.7 Observations in humans

No information was available.

2.3.8 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.

2.3.9 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 the lack of concern about genotoxicity, the Committee established an ADI "not specified"[1] for β-glucanase from *S. violaceoruber*, for the proposed uses 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.

References

1. Beta-glucanase from *Streptomyces violaceoruber* expressed in *S. violaceoruber*. In: List of substances scheduled for evaluation and request for data, addendum [to be presented at]: Joint FAO/WHO Expert Committee on Food Additives (JECFA), Eighty-ninth meeting, June 2–11, 2020. Rome: Food and Agriculture Organization of the United Nations; Geneva: World Health Organization; 2019:4 (http://www.fao.org/3/ca6809en/ca6809en.pdf).

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2.4 Phosphoripase A2 from *Streptomyces violaceoruber* expressed in *S. violaceoruber*

2.4.1 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*.

2.4.2 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-toxigenic 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.

2.4.3 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 μmol/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.

2.4.4 Biotransformation

No information was available.

2.4.5 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 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.

2.4.6 Toxicological data

In a study of oral acute toxicity in rats with powdered phospholipase A2 concentrate, the LD_{50} 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 no-observed-adverse-effect level (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 concerns with respect to the genotoxicity of the phospholipase A2 enzyme preparation.

2.4.7 Assessment of dietary exposure

The Committee evaluated one estimate of dietary exposure for phospholipase A2 from *S. violaceoruber* submitted by the sponsor. The enzyme is used in a broad spectrum of food and beverages, including milk. The estimate was derived with the budget method and was based on maximum use levels of 6.42 mg TOS/kg for solid foods, 4.59 mg TOS/kg for non-milk beverages and 9.17 mg TOS/kg for milk, and on the assumption that 25% of the food supply would contain the enzyme preparation. The theoretical maximum daily intake was estimated to be 0.25 mg TOS/kg bw per day (rounded by the Committee from 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 Committee noted that the enzyme will be inactivated during the processing of food ingredients and has no function in the final food.

2.4.8 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"[1] 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.

[1] 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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2.5 Riboflavin from *Ashbya gossypii*

2.5.1 Explanation

Riboflavin, or 7,8-dimethyl-10-(1′-D-ribityl)isoalloxazine, (7,8-dimethyl-10-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]benzo[*g*]pteridine-2,4-dione), is commonly

known as vitamin B2. It can be obtained by chemical synthesis or by fermentation. Riboflavin and riboflavin-5′-phosphate are used as food colours.

At its thirteenth meeting, the Committee established an ADI of 0–0.5 mg/kg bw for riboflavin on the basis of 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 *(1)*. 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 *(2)*. At its fifty-first meeting, the Committee evaluated riboflavin produced by fermentation from *Bacillus 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 *(3)*.

The CCFA at its Fifty-first Session requested the Committee to evaluate riboflavin from *Ashbya gossypii* as an alternative source of riboflavin for colouring purposes and as a nutrient source *(4)*. 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 toxicological dossier was received, with relevant study reports and publications. A comprehensive literature search on riboflavin from *A. gossypii* conducted on eight databases identified two additional studies for evaluation, and an additional search on riboflavin from other sources identified four additional studies for evaluation.

2.5.2 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 *(5)*, which possibly provides protection against ultraviolet radiation *(6)*. 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 a 95% homology and gene synteny to the genome of budding yeast, *Saccharomyces cerevisiae (7, 8)*. 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 in the genome. The genome of *A. gossypii* has no potential for production of secondary metabolites.

2.5.3 Chemical and technical considerations

Riboflavin is obtained from *A. gossypii* by fermentation under controlled conditions. Several filtration and precipitation or crystallization steps result in a highly purified food-grade dry powder containing not less than 98% riboflavin, free of fermentation medium components and the production organism. The entire process is carried out in accordance with current good manufacturing practices; all raw materials used in the manufacture are food-grade.

Riboflavin was evaluated previously by JECFA *(9)* as a synthetic product (1987) and as a product of fermentation from *B. subtilis* (1999). Independent of the source, these additives contain not less than 98% and not more than 101% of riboflavin (on a dried basis). Riboflavin is relatively stable during food processing and storage but is very sensitive to light.

2.5.4 Biochemical aspects

Riboflavin is absorbed actively and passively mainly in the proximal small intestine, partly in the large intestine and also in the colon *(10–12)*. 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 *(13)*. When riboflavin is absorbed in high concentrations, little is stored in the body tissues, and the excess is excreted, primarily in the urine *(14–16)*.

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 flavin-adenine dinucleotide (FAD) by FAD synthetase *(17)*. Although FAD was the major

form in plasma, plasma riboflavin and erythrocytes flavin mononucleotide were suggested to represent riboflavin status in humans *(18)*. Lumichrome and lumiflavin have been identified as metabolites of riboflavin in the rat, while hydroxyriboflavin and formylmethylflavin have been identified as metabolites in human plasma *(19, 20)*.

2.5.5 Toxicological studies

The acute oral toxicity of riboflavin from *A. gossypii* is low, with an LD_{50} of > 2500 mg/kg bw *(21, 22)*.

In a 90-day repeated-dose oral toxicity study in rats *(23)*, 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 bw, bw gain, feed or drinking-water consumption. Foci were detected in the kidneys of two female rats at the highest dose, but were considered not to be toxicologically relevant. When accounting for the purity of the preparation used, the Committee identified a NOAEL 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 *(24)*, reviewed previously by the Committee *(3)*, 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 identified a NOAEL of 200 mg/kg bw per day *(3)*.

The European Food Safety Authority (EFSA) *(25)* described a further 90-day oral toxicity study in rats (Bachmann et al., 2005) 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-2μ-globulin and is considered to be a response specific to male rats and therefore not relevant to humans. EFSA *(25)* 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 and in an in vitro micronucleus induction assay in human lymphocytes *(26–28)*. 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 *(29)*. 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 *(1)*. The present Committee noted that the report of the study provided limited experimental data, poor reporting and only one dose level was used.

In a series of intervention studies with 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 *(30–40)*.

2.5.6 Assessment of dietary exposure

The Committee noted that riboflavin is endorsed for use in 71 food categories in the Codex GSFA 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 maximum reported use levels (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 GSFA MPLs and MRULs, in combination with food consumption data from the Food Additive Intake Model 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 *(25)* 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; *41*), New Zealand (0.03 mg/kg bw per day for adults and 0.05 mg/kg bw per day for children; *41*), the Republic of Korea (0.02–0.03 mg/kg bw per day for adults; *42*) and the USA (from 0.05 mg/kg bw per day for men to 0.14 mg/kg bw per day for children; *43*). The group pf milk and dairy products group was the main contributor to dietary exposure to riboflavin in Spain at 32.3% *(44)*, Australia at 27–28% *(45)* and New Zealand at 23% *(46, 47)*.

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 Food Additive Intake Model 2.0 with MRULs, should be considered in the safety assessment of riboflavin.

2.5.7 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 from 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"[1] 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 toxicology and a dietary exposure monograph was prepared.

New specifications and a chemical and technical assessment were prepared.

2.5.8 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.

2.5.9 Recommendation

In view of information received at the current meeting which 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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2.6 Ribonuclease P from *Penicillium citrinum*

2.6.1 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 has not previously been 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.

2.6.2 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.

2.6.3 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 µmol/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 °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.

2.6.4 Biotransformation

No data were available.

2.6.5 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.

2.6.6 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 *(10, 11)*. 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.

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 *(12, 13)*. 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 *(14, 15)*. The Committee had no concern with respect to the genotoxicity of the preparation of ribonuclease P from *P. citrinum* AE-RP.

2.6.7 Observations in humans

No data were available.

2.6.8 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.

2.6.9 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 lack of concern for genotoxicity, the Committee established an ADI "not specified"[1] 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.

2.6.10 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.

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[1] 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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3. Revision of specifications and analytical methods

3.1 Modified starches

3.1.1 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) (Table 2). 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.

Table 2
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
Acetylated distarch adipate	1422	4, 8
Hydroxypropyl starch	1440	7
Hydroxypropyldistarch phosphate	1442	3, 7

Table 2 (continued)

Modified starch	INS	Annex
Starch sodium octenylsuccinate	1450	6
Acetylated oxidized starch	1451	4, 5

Corrigenda

The following requests for corrections, reported to the JECFA Secretariat, were evaluated by the 92nd JECFA meeting and found to be necessary. These corrections will be made, however, only in the electronic versions and in the online database of specifications.

Food additive	Original text	Revised text	Additional information
Riboflavin INS 101(i)	$\% Riboflavin = A \times 5000328 \times W \times 1.367$% riboflavin = A × 5000328 × W × 1.367	$\% Riboflavin = A \times 5000328 \times W$% riboflavin = A × 5000328 × W	Correction to calculation in the method of assay; removal of a wrongly assigned factor
Riboflavin from Bacillus subtilis INS 101(iii)	$\% Riboflavin = A \times 5000328 \times W \times 1.367$% riboflavin = A × 5000328 × W × 1.367	$\% Riboflavin = A \times 5000328 \times W$% riboflavin = A × 5000328 × 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			
Unsulfonated 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 m 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) =

$$100 \times WA/W$$

Annex 1

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

1. General principles governing the use of food additives (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
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208. Evaluation of certain veterinary drug residues in food (Seventy-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 969, 2012.

209. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 66, 2012.

210. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 12, 2012.

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213. Compendium of food additive specifications. FAO JECFA Monographs 13, 2012.

214. Evaluation of certain food additives and contaminants (Seventy-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 983, 2013.

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217. Evaluation of certain veterinary drug residues in food (Seventy-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 988, 2014.

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219. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 15, 2014.

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222. Compendium of food additive specifications. FAO JECFA Monographs 16, 2014.

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227. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 72, 2016.

228. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 18, 2016.

229. Safety evaluation of certain food additives and contaminants. Supplement 1: Non-dioxin-like polychlorinated biphenyls. WHO Food Additives Series, No. 71-1, 2016.

230. Evaluation of certain food additives (Eighty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1000, 2016.

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232. Safety evaluation of certain food additives. WHO Food Additives Series, No. 73, 2017.

233. Evaluation of certain contaminants in food (Eighty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1002, 2017.

234. Evaluation of certain food additives (Eighty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1007, 2017.

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237. Safety evaluation of certain food additives. WHO Food Additives Series, No. 75, 2019.

238. Evaluation of certain veterinary drug residues in food (Eighty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1008, 2018.

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240. Compendium of food additive specifications. FAO JECFA Monographs 22, 2018.

241. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 76, 2019.

242. Evaluation of certain food additives (Eighty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1014, 2019.

243. Evaluation of certain food additives (Eighty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives) WHO Technical Report Series, No. 1020, 2019.

244. Compendium of food additive specifications. FAO JECFA Monographs 23, 2019.

245. 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.

246. Evaluation of certain food additives (Eighty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1027, 2021.

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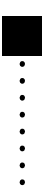

Annex 2

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 conclusions on toxicology and dietary exposure
Benzoic acid, its salts and derivatives	N	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. **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.**
Collagenase from *Streptomyces violaceoruber* expressed in *S. violaceoruber*	N	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. **In view of this MOE and the lack of concern about genotoxicity, the Committee established an ADI "not specified"[1] for collagenase from *S. violaceoruber*, when used in the applications specified and in accordance with good manufacturing practice.**
β-Glucanase from *Streptomyces violaceoruber* expressed in *S. violaceoruber*	N	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. **On the basis of this MOE and the lack of concern about genotoxicity, the Committee established an ADI "not specified"[1] for β-glucanase from *S. violaceoruber*, for the proposed uses and in accordance with good manufacturing practice.**

[1] The reader is referred to the Technical Report of the 87th JECFA meeting for clarification of the term "ADI not specified".

Food additive	Specifications	Acceptable daily intakes (ADIs) and other conclusions on toxicology and dietary exposure
Phospholipase A2 from *Streptomyces violaceoruber* expressed in *S. violaceoruber*	R	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 an MOE of 760. **On this basis and in the absence of concern about genotoxicity, the Committee established an ADI "not specified"[1] for the phospholipase A2 enzyme preparation from *S. violaceoruber* when used in the applications specified and in accordance with good manufacturing practice.**
Riboflavin from *Ashbya gossypii*	N	The Committee noted that riboflavin from *A. gossypii* 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. **The Committee established a group ADI "not specified"[1] for riboflavin, riboflavin- 5´-phosphate, riboflavin from *B. subtilis* and riboflavin from *A. gossypii*, expressed as riboflavin. The Committee withdrew the previous group ADI of 0–0.5 mg/kg bw.**
Ribonuclease P from *Penicillium citrinum*	N	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 lack of concern for genotoxicity, the Committee established an ADI "not specified"[1] for the ribonuclease P enzyme preparation from *P. citrinum* AE-RP, used in the applications specified and in accordance with good manufacturing practice.**

N: new specifications, R: revised specifications

Food additives considered for specifications only

Food additive	Specifications
Modified starches	R

R: revised specifications

[1] The reader is referred to the Technical Report of the 87th JECFA meeting for clarification of the term "ADI not specified".

Annex 3

Meeting agenda

Food and Agriculture Organization of the United Nations

World Health Organization

92nd JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES (JECFA)
7–18 June 2021

Virtual meeting: 12:00–16:00 (CET)

1. Opening

2. Declarations of Interests (information by the Secretariat on any declared interests and discussion, update by experts)

3. Election of Chairperson and Vice-Chairperson, appointment of Rapporteurs

4. Adoption of the agenda

5. Matters of interest arising from previous Sessions of the Codex Committee on Food Additives

6. Critical issues and questions from Working Papers (first brief round of discussion on all subjects to inform the full Committee)

7. Evaluations

   7.1. Food additives
   - Benzoic acid and its salts
   - Riboflavin from *Ashbya gossypii*

   7.2. Enzymes
   - β-Glucanase
   - Collagenase
   - Phosphodiesterase
   - Phospholipase A2

8. Other matters to be considered (general considerations).

9. Other matters as may be brought forth by the Committee during discussions at the meeting.

10. Adoption of the report.

SELECTED WHO PUBLICATIONS OF RELATED INTEREST

Evaluation of certain food additives
Eighty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1027, 2020 (106 pages)

Evaluation of certain 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 (116 pages)

Evaluation of certain food additives
Eighty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1020, 2019 (109 pages)

Evaluation of certain food additives
Eighty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1014, 2019 (156 pages)

Evaluation of certain veterinary drug residues in food
Eighty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1008, 2017 (150 pages)

Safety evaluation of certain food additives
Eighty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives
WHO Food Additives Series, No. 75, 2018 (244 pages)

Evaluation of certain food additives
Eighty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1007, 2017 (92 pages)

Safety evaluation of certain contaminants in food
Eighty-third meeting of the Joint FAO/WHO Expert Committee on Food Additives
WHO Food Additives Series, No. 74, 2018 (897 pages)

Evaluation of certain contaminants in food
Eighty-third report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1002, 2017 (166 pages)

Further information on these and other WHO publications can be obtained from
WHO Press, World Health Organization ▪ 1211 Geneva 27, Switzerland ▪ www.who.int/bookorders
tel.: +41 22 791 3264; **fax:** +41 22 791 4857; **email:** bookorders@who.int

Evaluation of certain food additives

This report presents the conclusions of a 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 *Stretomyces 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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