WHO FOOD ADDITIVES SERIES: 86

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

Safety evaluation of certain food additives

Food and Agriculture Organization of the United Nations

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World Health Organization, Geneva, 2023

Food and Agriculture Organization of the United Nations

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PREFACE

The monographs contained in this volume were prepared at the Ninety-fifth meeting of the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), which met virtually on 6–17 and 22 June 2022. These monographs summarize the data on specific food additives reviewed by the Committee.

The Ninetieth and Ninety-fourth reports of JECFA have been published by WHO as WHO Technical Report No. 1032 and No. 1041, respectively. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1, and the participants of the meeting are listed in Annex 3. A summary of the conclusions of the Committee with respect to the food additives discussed at the meeting is given in Annex 4.

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

The monographs contained in this volume are based on working papers that were prepared by WHO and FAO experts. An acknowledgement is given at the beginning of each monograph to those who prepared the working papers. The monographs were edited by E. Rowan, Contin, United Kingdom of Great Britain and Northern Ireland.

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

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

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

α-Amylase (JECFA95-1) from *Geobacillus stearothermophilus* expressed in *Bacillus licheniformis*

First draft prepared by
Joel Rotstein,[1] Tracy Hambridge,[2] Utz Mueller,[3] Feng-Qin Li[4] and Eugenia Dessipri[5]

[1] Ottawa, Ontario, Canada
[2] Food Standards Australia New Zealand, Majura Park, Australian Capital Territory, Australia
[3] Perth, Western Australia, Australia
[4] China National Center for Food Safety Risk Assessment, Beijing, China
[5] General Chemical State Laboratory, Athens, Greece

1. Explanation

At the request of the Codex Committee on Food Additives (CCFA) at its Forty-eighth Session *(1)*, the Committee evaluated the safety of α-amylase (Enzyme Commission No. 3.2.1.1; Chemical Abstracts Service [CAS] No. 9000-90-2)

from *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) expressed in *Bacillus licheniformis*. The Committee had not previously considered this enzyme preparation. The present Committee allocated the unique JECFA enzyme identifier JECFA95-1 to distinguish it from similarly named enzyme preparations. The Committee had previously evaluated several other α-amylases, including from *G. stearothermophilus*, and α-amylase from *G. stearothermophilus* expressed in *Bacillus subtilis* (Annex 1, reference 94), for which an acceptable daily intake (ADI) "not specified"[1] was established.

The term "α-amylase" refers to the enzyme and its amino acid sequence; the term "enzyme concentrate" refers to the fermentation product containing the enzyme of interest, which is used in the toxicity studies; and the term "enzyme preparation" refers to the product formulated for commercial use.

The Committee has previously assessed the safety of enzyme preparations derived from *B. licheniformis* (e.g. pullulanase, Annex 1, reference 205; serine protease, Annex 1, reference 205), and established an ADI "not specified" for these enzyme preparations. On this basis, the present Committee considered that α-amylase (JECFA95-1) from *G. stearothermophilus* expressed in *B. licheniformis* met the criteria of a Class 1, Type iii enzyme, as described in Environmental Health Criteria (EHC) 240 *(2)*. A Class 1, Type iii enzyme preparation is produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Although toxicity data and a dietary exposure assessment are not required for Class 1, Type iii enzymes, the Committee evaluated the submitted data.

The enzyme catalyses the endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units. The enzyme preparation is intended to be used as a processing aid in starch/carbohydrate processing, for example, potable alcohol production.

The Committee conducted a literature search in Google Scholar with the linked search terms "alpha-amylase" AND "*Geobacillus stearothermophilus*" and "alpha-amylase" AND "*Bacillus licheniformis*". This search identified 298 references. None of the identified publications provided additional toxicity data relevant to this evaluation.

1.1 Genetic background

The production organism *B. licheniformis* is a non-pathogenic and non-toxigenic bacterium. It is ubiquitous in nature, having been isolated from environments as

[1] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

diverse as freshwater, saltwater, soil, plants, animals and air *(3)*. *B. licheniformis* has a history in the production of enzymes intended for use in food processing.

The production strain JSF07-170-3 was obtained from *B. licheniformis* Bra 7 strain by inactivation of the genes encoding α-amylase, chloramphenicol resistance and a sporulation factor. The α-amylase gene from *G. stearothermophilus* ASP-154 strain was modified and transferred into *B. licheniformis* Bra 7. The donor strain was deposited in the American Type Culture Collection (ATCC) as *B. stearothermophilus* ATCC 39709. The stability of the introduced gene sequence was confirmed by the production strain performance over at least 60 generations.

1.2 Chemical and technical considerations

This α-amylase is produced by pure culture fermentation of the *B. licheniformis* production strain. Manufacture of the enzyme preparation includes fermentation, recovery and formulation. After fermentation, the broth containing the α-amylase enzyme is separated from the biomass via a series of filtration steps, then concentrated. The resulting enzyme concentrate is formulated and standardized into a liquid preparation. The entire process is performed in accordance with current Good Manufacturing Practices (GMP) and with food-grade raw materials. The enzyme concentrate is tested to ensure that it is free from the production organism and any antibiotic activity.

The primary sequence of α-amylase produced by *B. licheniformis* consists of 486 amino acids; its molecular weight calculated from the determined amino acid sequence is 55 kDa. The α-amylase produced by *B. licheniformis* is not expected to have any significant subsidiary or secondary activity.

One α-amylase unit (AAU) is defined as the amount of enzyme required to hydrolyse 10 mg of starch per minute under specified conditions (pH 6.0, T = 60 °C). The activity of α-amylase is determined spectrophotometrically by measuring the release of *p*-nitrophenol from *p*-nitrophenyl maltoheptoside at 410 nm (pH 5.6, T = 25 °C, 5 min), compared with a proprietary enzyme standard with activity expressed in AAU. The mean activity from three batches of the liquid enzyme concentrate was 32 377 AAU/g.

α-Amylase catalyses the endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units. The enzyme preparation is intended for use as a processing aid in starch/carbohydrate processing, brewing/cereal beverage processing and potable alcohol production at maximum levels of 31.6 mg total organic solids (TOS)/kg raw material. The TOS includes the enzyme of interest and residues of organic materials (e.g. proteins, peptides and carbohydrates) from the production organism during manufacture.

The α-amylase enzyme is inactivated by heat or removed during processing. It is not expected to have any technological function in the final food.

2. Biological data

2.1 Assessment of potential allergenicity

Since no single assay or property can distinguish allergens from non-allergens, α-amylase was assessed for allergenicity using a weight-of-evidence approach. This was achieved by comparing its amino acid sequence with that of known protein allergens, as recommended by the Codex Alimentarius Commission *(4)*, and assessing its resistance to proteolysis in vitro in simulated gastric fluid (SGF) and intestinal fluid (SIF) assays *(5)*.

Using the FASTA search algorithm *(6)*, the full-length amino acid sequence of α-amylase (486 amino acids) was compared with 2171 peer-reviewed allergen sequences (using an expectation value [E-value] of < 0.1) in the Food Allergy Research and Resource Program (FARRP) AllergenOnline database *(7)*. Comparisons between highly homologous proteins yielded E-values approaching zero, indicating a very low probability that such matches would occur by chance (larger E-values indicate a lower degree of similarity).

The analyses also included a search for amino acid matches between α-amylase and other proteins with > 35% identity over a sliding window of 80 amino acids and full identity over eight contiguous amino acids. The results indicated that, in a search over a sliding window of 80 amino acids, the amino acid sequence of α-amylase matched two α-amylases from *Aspergillus oryzae*: α-amylase A type-1/2 and Taka-amylase A. Neither of these proteins are food allergens. The α-amylase did not align with any known food allergen. There were no matches over eight contiguous amino acids.

In the SGF assay, α-amylase (5 mg/mL) was added to SGF (pH ~ 1.2, with 10 units pepsin/mg protein) and incubated at 37 °C *(5)*. In addition to α-amylase (~ 54 kDa), bovine serum albumin (~ 66 kDa) and β-lactoglobulin (~ 18 kDa) were also included as the positive and negative controls, respectively. Pepsin is a broad-spectrum protease that preferentially hydrolyses peptide bonds between the aromatic amino acids Phe, Trp and Tyr *(8)*. At multiple timepoints (0, 0.5, 1, 2, 5, 10, 20, 30 and 60 min), a sample of each pepsin digest was removed and applied to a gradient 4–12% polyacrylamide gel (molecular weight range: 2–200 kDa) for electrophoresis. During electrophoresis, negatively charged denatured proteins migrate through the gel according to their molecular weight and, once stained, establish a band pattern characteristic of the proteins within

the sample. Coomassie blue protein staining revealed that α-amylase had been completely hydrolysed to a series of low-molecular-weight peptides (≤ 3 kDa) in < 0.5 min. In contrast, bovine serum albumin took slightly longer (~ 1 min) for the 66 kDa band to become indistinguishably faint, and β-lactoglobulin as an 18-kDa band persisted throughout the 60-min digestion period.

A second set of samples was prepared for a Western blot analysis. Protein bands in the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) that had been transferred to a suitable membrane by electroblotting were visualized using alkaline phosphatase immunodetection. The Western blot analyses showed that no immunodetectable α-amylase was present on the membranes following < 0.5 min of exposure to SGF.

In a SIF assay, α-amylase (5 mg/mL) was added to SIF (pH ~ 7.5; with 1% pancreatin weight by volume [w/v]) and incubated at 37 °C. At multiple timepoints (0, 1, 2, 5, 10, 20, 30, 60 and 360 min), a sample was removed from the mixture and subjected to SDS-PAGE analysis and Western blotting as described for the SGF assay. In addition to the α-amylase samples, the gels were loaded with two positive controls: β-lactoglobulin (~ 18 kDa) and bovine serum albumen (~ 66 kDa). The SIF assay showed that the positive controls gave the expected result (i.e. complete digestion within 1 min), but that α-amylase was not digested after 6 hours.

Based on the results of an in silico assessment of potential allergenicity and digestive stability in simulated protein hydrolysis studies, the Committee concluded that dietary exposure to this α-amylase is not anticipated to pose a risk for allergenicity.

2.2 Toxicological studies

All toxicity studies with the α-amylase concentrate were conducted with the same test material, that is, the enzyme concentrate GC358 (batch no. 1661077022; TOS 8.5% w/v).

2.2.1 Acute toxicity

An acute oral toxicity study *(9)* using a fixed dose protocol was conducted in Wistar Hannover SPF (HanTac:WH) female rats in compliance with Good Laboratory Practice (GLP) and according to Organisation for Economic Co-operation (OECD) guideline no. 420 *(10)*. In a sighting study, one animal received a single dose of the test material of 1776 mg TOS/kg body weight (bw) by gavage. This concentration was the highest possible dose in a volume of 20 mL/kg bw, which was the volume limit of the test. Clinical signs of toxicity were monitored 0.25, 1, 3 and 6 hours after treatment, and then daily for 14 days. Body weight was

recorded at arrival, prior to treatment on day 1 and on days 2, 3, 8 and 15, and body weight gain was calculated. The animal was killed on day 15 after treatment and a necropsy was conducted. Over the course of the study, the animal appeared in good health and gained body weight; necropsy showed no abnormalities. Based on these results, this dose was chosen for the main study.

In the main study, four female rats each received a single dose of the test material of 1776 mg TOS/kg bw by gavage. The protocol and parameters used in the sighting study were repeated in the main study. The results showed that during the study all animals remained in good health and gained body weight, and necropsy showed no abnormalities.

2.2.2 Short-term studies of toxicity

A 13-week oral toxicity study *(11)* in Sprague-Dawley SPF (Ntac:SD) rats was conducted according to GLP and OECD guideline no. 408 *(12)*. Groups of rats (10 per sex per group) received a single dose of the enzyme concentrate of 0, 8.9, 22.27 or 66.81 mg TOS/kg bw per day (dose volume was 5 mL/kg bw) by gavage for 13 weeks. The vehicle used was 0.9% saline.

The Committee noted that the highest dose tested in this study was unexpectedly low compared with the dose tested in the acute oral toxicity study, which showed no toxicity. The sponsor was unable to provide a rationale for the selection of doses.

All animals were observed daily for mortality and clinical signs of toxicity, and weekly for more detailed observation. Body weight and feed consumption for all animals were recorded weekly. Water consumption was recorded twice per week for each cage (two animals per cage). Ophthalmoscopic examination was performed on all animals prior to the start of the study and on animals in the control and high-dose groups at study termination. A functional observation battery, which included an open field test (grip strength and motor activity) and stimuli-induced tests (auditory, visual and tactile), was conducted during weeks 12 and 13 on animals in the control and high-dose groups. Blood and urine samples were collected from all animals prior to study termination for clinical chemistry and haematology measurements. At study termination, a macroscopic examination was conducted and selected organs were removed, weighed and processed for histopathology. A microscopic examination was conducted on selected organs from animals in the control and high-dose groups.

No deaths occurred during the study and no treatment-related signs of clinical toxicity were observed. There were no treatment-related effects observed in open field or stimuli-induced tests. There were no treatment-related differences between treated and control animals with respect to the ophthalmoscopic examinations. There were no treatment-related significant differences between

groups regarding measurements of body weight, feed intake and absolute or relative organ weights.

A statistically significant higher water consumption was observed in females given 22.27 mg TOS/kg bw per day compared with control females (91.8 ± 8.9 and 71.4 ± 15.0 g per animal, respectively; $P < 0.05$) from day 84 to day 87. The finding was considered incidental since it was limited in duration, not dose related and appeared only in females in the middle-dose but not the high-dose group.

Haematology results showed that the percentages of reticulocytes were statistically lower in males receiving 66.81 mg TOS/kg bw per day compared with control males (1.72 ± 0.18 and 2.05 ± 0.34, respectively; $P < 0.05$, Student's t-test; historical control mean: 2.32, range: 2.00–2.87). Associated parameters, such as the number of reticulocytes and mean cell volume, were not significantly different from control males; the differences in reticulocyte percentage were therefore considered incidental. The results also showed a statistically lower percentage of neutrophils in females receiving doses of 8.9 and 66.81 mg TOS/kg bw per day compared with the female controls (10.5 ± 4.5, 5.0 ± 2.4 [$P < 0.01$], 7.1 ± 4.1 and 6.6 ± 2.5 [$P < 0.05$] for control, low-, middle- and high-dose groups, respectively; historical control mean: 7.82, range: 4.80–13.10). Because all values were within the historical range and a dose–response effect was not demonstrated, the effect was considered not toxicologically relevant. Finally, a statistically higher percentage of lymphocytes was observed in females receiving 8.9, 22.27 or 66.81 mg TOS/kg bw per day compared with control females (88.3 ± 4.6, 93.5 ± 2.8 [$P < 0.01$], 92.5 ± 4.0 [$P < 0.05$] and 92.4 ± 3.0 [$P < 0.05$] for control, low-, middle- and high-dose groups, respectively; historical control mean: 90.48, range: 84.60–94.10). Because this effect was relatively small, a dose–response effect was not demonstrated and the values were within the range of the historical controls, the effect was considered not toxicologically relevant.

Microscopic examination of the urine showed statistically lower amounts of epithelial cells and crystals in middle- and high-dose females compared with control females. Urinalysis also revealed statistically lower amounts of urates in high-dose females compared with control females. Increases in the amounts of epithelial cells and urates in urine can indicate urinary tract infection or renal damage; however, decreases are not an adverse effect. The findings were considered not toxicologically relevant.

Macroscopic findings revealed a diminished size of the prostate gland in one high-dose male, and a diminished size of the seminal vesicles in one male in each of the middle- and high-dose groups. These observations were not associated with any microscopic findings, and were considered not toxicologically relevant.

Microscopic findings revealed minimal focal acinar atrophy in the pancreas of 3 out of 10 high-dose females. None was observed in the control

females. A historical control finding from a single study showed that the lesion occurred in 2 out of 10 females. The finding was not observed in any of the male groups, and the incidence of the finding was considered consistent with normal variation. The observation was considered not treatment related and not toxicologically relevant.

In the absence of any adverse effects, a no-observed-adverse-effects limit (NOAEL) of 67 mg TOS/kg bw per day was identified (rounded by the Committee from 66.81 mg TOS/kg bw per day), the highest dose tested.

2.2.3 Genotoxicity

Genotoxicity was assessed in a bacterial reverse mutation test *(13)* and an in vitro mammalian chromosome aberration test *(14)*. The studies were conducted in compliance with GLP and in accordance with OECD guideline nos 471 *(15)* and 473 *(16)*, respectively.

The results of the bacterial reverse mutation and in vitro mammalian chromosome aberration tests were negative (Table 1). These results provide evidence that this α-amylase concentrate is not mutagenic or clastogenic in vitro.

The Committee had no concerns about potential genotoxicity of this α-amylase enzyme concentrate.

2.2.4 Other studies

This α-amylase was evaluated for potential toxicity by searching for homology in the amino acid sequence of the enzyme compared with proteins in the UNIPROT database *(17)*, as well as a subset of this database that has 6247 sequences that are manually annotated as animal toxins and 6736 as venom proteins *(18)*. The basic local alignment search tool (BLAST) search using a threshold E-value of 0.1 revealed sequence matches with other amylases, but no matches for toxin or venom sequences. The BLAST findings indicated that α-amylase does not have any amino acid sequence similarities to any known protein toxin.

2.3 Observations in humans

No information was available.

Table 1
Genotoxicity of α-amylase concentrate

End-point	Test system	Concentration	Results	Reference
In vitro reverse mutation[a]	*Salmonella typhimurium* TA1535, TA102, TA100, TA1537 and TA98	8.95–895 µg TOS/plate ± S9[b]	Negative	Edwards *(13)*
In vitro mammalian chromosome aberration[c]	Primary cultured human peripheral lymphocytes	*First main assay* 3 h exposure ± S9: 72, 143, 286 µg TOS/mL	Negative	Edwards *(14)*
		Second main assay 18 h exposure (3 h + S9 followed by 15 h – S9): 72, 143, 286 µg TOS/mL	Negative	
		18 h exposure (18 h – S9): 36, 72, 143 µg TOS/mL	Negative	

[a] A treat-and-plate assay was conducted (not described in OECD guidance). In this assay, tester strains were incubated with test material for 3 hours at 37 °C. After this period, the tester strains were centrifuged, and the pellets were resuspended in buffer. The bacteria were again centrifuged, and the bacteria were suspended again in buffer. An aliquot of this final suspension was added to top agar, which was then plated onto minimal glucose agar plates. These plates were incubated at 37 °C for 72 hours, after which the colonies were counted. A preliminary toxicity test was conducted with strain TA98 with concentrations ranging from 50 to 5000 µg/plate in the absence and presence of S9. The main tests were conducted twice (in some cases repeat tests were conducted). All concentrations of the test substance were assayed three times. All positive control substances yielded expected results.

[b] S9 is a liver homogenate from Aroclor 1254-treated rats combined with co-factors that were required for mixed function oxidase activity.

[c] A dose-range-finding study was conducted with five concentrations of the α-amylase concentrate. Three concentrations were selected for the two main assays, with the highest concentration inducing a 50% reduction in mitotic index. In the first main assay, cultures were treated for 3 hours in the absence or presence of S9. In the second main assay, cultures were treated for 18 hours in the absence of S9, or for 3 hours in the presence of S9 followed by 15 hours in the absence of S9. All cultures were harvested 18 hours after the start of treatment. All cultures were treated with Demecolcine, an inhibitor of mitosis, 2 hours prior to harvest. At harvest cultures were centrifuged and the supernatant discarded. Cell pellets were resuspended in a KCl solution, which causes the cells to swell, and then incubated for 10 minutes, centrifuged and supernatants removed. Cells were fixed onto slides, stained and scored for chromosomal aberrations (i.e. metaphase analysis) and for cytotoxicity. Both main assays were repeated at least twice.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to the TOS from the α-amylase from *G. stearothermophilus* expressed in *B. licheniformis* enzyme preparation. The enzyme is intended for use in starch/carbohydrate processing, brewing and cereal beverage production, and potable alcohol production; these uses were therefore considered for the dietary exposure assessment. The submission included an estimate of dietary exposure based on the budget method, a screening method used to determine the theoretical maximum daily intake (TMDI) of food additives *(19,20)*. The method takes into account the maximum physiological levels of consumption of solid food and non-milk beverages, the energy density of foods, the concentration of the food additive in solid foods and non-milk beverages, and the proportion of solid foods and non-milk beverages that may contain it. The method provides a conservative estimate of dietary exposure. Further details of the budget method can be found in EHC 240 *(21)*.

3.2 Dietary exposure assessment

The estimated TMDI provided by the sponsor was based on a number of inputs to the budget method. The amount of food consumed was assumed to be 0.025 kg/kg bw per day for solid foods and 0.1 L/kg bw per day for non-milk beverages. With regards to the proportion of solid food and non-milk beverages containing the enzyme preparation, EHC 240 *(5)* refers to commonly used default proportions of 12.5% for solid foods and 25% for non-milk beverages. Foods processed with this α-amylase enzyme preparation are proposed to be added to a variety of foods intended to be consumed by the general population. The sponsor therefore assumed that 50% of solid food and 25% of non-milk beverages are processed and would contain this enzyme preparation.

The maximum use level of the enzyme present in the final solid foods of 0.44 mg TOS/kg food was used in the budget method calculation by the sponsor. This level was based on uses in starch/carbohydrate processing at a maximum use level of 8.8 mg TOS/kg raw material and a maximum amount of the ingredient in the final food of 5%. For non-milk beverages, the concentration in the final beverage was dependent upon the proposed use. The level used in the budget method calculation by the sponsor was 5.37 mg TOS/L for use in brewing/cereal beverage processing, based on a maximum use level of 31.6 mg TOS/kg raw material and 17% use in the final beverage. The sponsor did not use the highest maximum concentration from all beverage uses of 83.1 mg TOS/L, noting that this was for potable alcohol production in which it can correctly be assumed that the enzyme protein and other organic solids are removed during distillation. The sponsor provided additional information, noting that: "… in potable alcohol production, solids are separated from the fermentation slurry at the end of fermentation and any enzyme protein precipitate will be removed with the solids. The liquids are then distilled. The distilled alcohol is subsequently filtered through a molecular sieve at temperatures well over boiling to adsorb further traces of water and water-soluble protein." The Committee accepted this assumption based on this technical information and because it would be unlikely for all non-milk beverage consumption to be from potable alcohol or distilled spirits. However, the use of a concentration of 5.37 mg TOS/L for all non-milk beverages is still a conservative assumption for the dietary exposure calculation. It assumes that the potable alcohol that makes up a part of the total non-milk beverage consumption would still contain a small amount of TOS from the enzyme preparation.

The resulting TMDIs of TOS from this α-amylase enzyme preparation were estimated by the sponsor to be 0.0055 mg TOS/kg bw per day for solid foods and 0.134 mg TOS/kg bw per day for non-milk beverages for a total of 0.14 mg TOS/kg bw per day.

The Committee independently calculated dietary exposure to the TOS from the enzyme preparation using the budget method. All inputs and assumptions were the same as for the estimate by the sponsor, with the exception of the consumption amount for solid foods. For the consumption amount the Committee assumed that the maximum physiological requirement for solid food (including milk) is 0.05 kg/kg bw per day. This is the level used in a budget method calculation where there is potential for the enzyme to be present in baby foods or general-purpose foods that may be consumed by infants and young children, and represents a worst-case scenario. The TMDI of TOS from this α-amylase enzyme preparation as estimated by the Committee for solid food was 0.011 mg TOS/kg bw per day and 0.134 mg TOS/kg bw per day for non-milk beverages, for a total of 0.2 mg TOS/kg bw per day (rounded from 0.145 mg TOS/kg bw per day).

The Committee concluded that the dietary exposure estimate of 0.2 mg TOS/kg bw per day was appropriate for use in the evaluation. For the purposes of the dietary exposure assessments conducted by both the sponsor and the Committee, it was assumed that the enzyme is not removed and/or inactivated during final processing of ingredients or foods, and that 100% of the TOS from the enzyme preparation remains in the ingredient or final food. In reality, the enzyme is either removed or inactivated during the processing of food ingredients, and will have no function in the final food.

4. Comments

4.1 Assessment of potential allergenicity

Potential immunological cross-reactivity with known allergens was assessed by comparing the amino acid sequence of α-amylase with those of known allergens *(7,18)*. The analyses included a search for amino acid matches between α-amylase and other proteins with more than 35% identity over the full length of the α-amylase and over a sliding window of 80 amino acids, as well as full identity over eight contiguous amino acids.

The results indicated that the amino acid sequence of the α-amylase aligned with Asp o 21, an α-amylase originating from the fungus *A. oryzae*. This is a respiratory allergen associated with occupational rhinitis and asthma in bakers. When compared with the α-amylase amino acid sequence, the enzymes showed a 37.5% identity over a sliding window of 80 amino acids, with 25.1% identity over the full length of the protein. No identity over eight contiguous amino acids was observed. The α-amylase did not align with any known food allergen.

The α-amylase was assessed for its resistance to proteolysis using SGF and SIF assays in vitro *(5)*. In the SGF assay, SDS-PAGE and Western blot analyses showed that the α-amylase was readily converted to short-chain peptides within 30 seconds of exposure to SGF.

The Committee concluded that dietary exposure to this α-amylase is not anticipated to pose a risk for allergenicity.

4.2 Toxicological studies

An acute oral toxicity study was conducted in female rats with the enzyme concentrate as the test material *(9)*, and no toxicity was observed at 1776 mg TOS/kg body weight (bw), the only dose tested.

A 13-week oral toxicity study was conducted in rats with the enzyme concentrate as the test material *(11)*. The enzyme concentrate was administered via gavage at doses up to 66.81 mg TOS/kg bw per day. In the absence of any adverse effects, a NOAEL of 67 mg TOS/kg bw per day was identified (rounded by the Committee from 66.81 mg TOS/kg bw per day), the highest dose tested.

The enzyme concentrate was not genotoxic in a bacterial reverse mutation assay *(13)* and in an in vitro mammalian chromosome aberration assay *(14)*. The Committee had no concerns about potential genotoxicity of this α-amylase enzyme concentrate.

A comparison of the amino acid sequence of the enzyme with those of known protein toxins *(17,18)* revealed no biologically relevant homology. The Committee concluded that the enzyme was unlikely to be a toxin.

4.3 Assessment of dietary exposure

The Committee evaluated two estimates of dietary exposure to TOS from this α-amylase enzyme preparation, one submitted by the sponsor and the other estimated by the Committee. Both estimates were derived using the budget method, a screening method based on maximum physiological levels of consumption of solid foods and non-milk beverages, and on maximum use levels of the enzyme preparation. Maximum use levels of 0.44 mg TOS/kg for solid foods and 5.37 mg TOS/L for non-milk beverages were used. The highest concentration from all proposed uses for non-milk beverages was not used in the budget method calculation in this assessment. It was assumed that the highest TOS of 83.1 mg/kg for the production of potable alcohol would not be present in the final distilled product as a result of the production process. In addition, the Committee noted that the entire consumption of non-milk beverages would not be distilled alcoholic beverages, which supports this assumption. In both dietary

exposure estimates it was assumed that 50% of solid foods and 25% of non-milk beverages would contain the enzyme preparation. Different assumptions were made about the amount of solid foods consumed. The resulting TMDIs from solid food and non-milk beverages were 0.14 mg TOS/kg bw per day by the sponsor and 0.2 mg TOS/kg bw per day by the Committee. The Committee concluded that the dietary exposure estimate of 0.2 mg TOS/kg bw per day was appropriate for use in the evaluation. For the dietary exposure assessment, it was assumed that 100% of the TOS from the enzyme preparation remains in the final food. The Committee noted that the enzyme is either removed or inactivated during the processing of food ingredients, and will have no function in the final food.

5. Evaluation

The Committee concluded that dietary exposure to this α-amylase enzyme preparation is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 67 mg TOS/kg bw per day, the highest dose tested, in a 13-week oral toxicity study in rats. When this NOAEL is compared with the dietary exposure estimate of 0.2 mg TOS/kg bw per day, a margin of exposure (MOE) of more than 330 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary ADI "not specified"[2] for α-amylase (JECFA95-1) from *G. stearothermophilus* expressed in *B. licheniformis*, when used in the applications specified, at the levels of use specified and in accordance with current GMP. This ADI "not specified" was made temporary because of the tentative nature of the specifications.

5.1 Recommendations

The Committee requested the following information, by the end of 2023, to complete the safety assessment:

- validated method of analysis to determine α-amylase activity, including the validation report;
- unit definition for α-amylase activity based on the method of assay; and
- analytical data using the validated method for at least five different batches of commercially available products.

[2] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

6. References

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α-Amylase (JECFA95-2) from *Geobacillus stearothermophilus* expressed in *Bacillus licheniformis*

First draft prepared by
Rosalind Dalefield,[1] Tracy Hambridge,[2] Josef Schlatter,[3] Ivan Stankovic[4] and Daniel Folmer[5]

[1] Food Standards Australia New Zealand, Wellington, New Zealand
[2] Food Standards Australia New Zealand, Majura Park, Australian Capital Territory, Australia
[3] Zürich, Switzerland
[4] Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia
[5] Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), United States of America (USA)

1. Explanation

At the request of the CCFA at its Forty-seventh session *(1)*, the Committee evaluated the safety of α-amylase (Enzyme Commission No. 3.2.1.1; CAS

No. 9000-90-2) from *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) expressed in *Bacillus licheniformis*. The Committee has not previously evaluated this enzyme preparation. The present Committee allocated the unique JECFA enzyme identifier JECFA95-2 to distinguish it from similarly named enzyme preparations. The Committee had previously evaluated several other α-amylases, including from *G. stearothermophilus*, and α-amylase from *G. stearothermophilus* expressed in *Bacillus subtilis* (Annex 1, reference 94), for which an ADI "not specified"[1] was established.

The term "α-amylase" refers to the α-amylase enzyme and its amino acid sequence; the term "enzyme concentrate" refers to the fermentation product containing the enzyme of interest, which is used in the toxicity studies; and the term "enzyme preparation" refers to the product formulated for commercial use.

The Committee has previously assessed the safety of enzyme preparations derived from *B. licheniformis* (e.g. pullulanase, Annex 1, reference 205; serine protease, Annex 1, reference 211) and established an ADI "not specified" for these enzyme preparations. On this basis, the present Committee considered that α-amylase (JECFA95-2) from *G. stearothermophilus* expressed in *B. licheniformis* met the criteria of a Class 1, Type iii enzyme, as described in EHC 240 *(2)*. A Class 1, Type iii enzyme preparation is produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Although toxicity data and a dietary exposure assessment are not required for Class 1, Type iii enzymes, the Committee evaluated the submitted data.

The enzyme catalyses the endohydrolysis of (1→4)-α-D-glucosidic linkages in amylose and amylopectin. The α-amylase enzyme preparation is intended for use as a processing aid in starch/carbohydrate processing, brewing/cereal beverage processing, potable alcohol production and the removal of starch in sugar processing.

The Committee conducted literature searches in PubMed and EBSCO. Searches were conducted with the linked search terms "α-amylase" AND "stearothermophilus" AND ("safety" OR "toxic" OR "allergy"). Searches were repeated using "alpha-amylase". Similar searches were conducted in both databases replacing "stearothermophilus" with "licheniformis". Finally, searches were conducted using the names of both organisms, together with "safety" OR "toxic" OR "allergy". One publication of peripheral relevance, which was considered at the Thirty-seventh JECFA meeting (Annex 1, reference 95), was identified as a result of all literature searches.

[1] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

1.1 Genetic background

The parental strain *B. licheniformis* Ca63 (also named DSM 9552) is a natural isolate deposited in Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The α-amylase production strain NZYM-BC is non-pathogenic and non-toxigenic, and was developed from the *B. licheniformis* Si3 cell lineage derived from the *B. licheniformis* parental strain Ca63. The Si3 cell lineage has a long history of safe use at Novozymes in the production of food enzymes.

The source of the α-amylase gene is a *G. stearothermophilus* strain that has a history of safe use in the production of food enzymes. The inserted DNA was integrated into the chromosome, and the genetic stability of the production stain was confirmed by Southern blot analysis.

The α-amylase production strain is marker free, and it does not produce secondary metabolites of toxicological concern to humans. It is monitored by Novozymes' Master Cell Bank and Working Cell Bank. The seed material for the fermentation is derived from the Working Cell Bank.

1.2 Chemical and technical considerations

The α-amylase enzyme preparation is produced by controlled submerged fed-batch fermentation of a pure culture of *B. licheniformis* NZYM-BC production strain. The manufacture of α-amylase enzyme preparation consists of three steps: fermentation (preparation of inoculum, seed and main fermentation), recovery and formulation. After fermentation, the microorganism is separated from the liquid broth containing the excreted enzyme, the broth is purified and concentrated to increase the enzyme content, and the concentrate is formulated into either a liquid or granulate enzyme preparation. The preparation is formulated using food-grade ingredients for the purpose of stabilizing, standardizing and preserving the food enzyme preparation. The α-amylase enzyme preparation method complies with FAO/WHO specifications (Annex 1, reference 185).

The α-amylase enzyme is not expected to be active in the final food as, depending upon the application, it will have been denatured via processing through high temperatures or low pH, or physically removed from the final food by clarification or filtration.

2. Biological data

2.1 Assessment of potential allergenicity

α-Amylase was assessed as a potential allergen in 2012, with a search strategy based on FAO/WHO 2001 recommendations *(3)*. The amino acid sequence of the enzyme was compared with the amino acid sequences of known allergens in the FARRP AllergenOnline *(4)* and the WHO/International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-committee *(5)* databases. Three homology analyses were performed: (i) more than 35% identity over the full length of the alignment; (ii) more than 35% identity using a sliding window of 80 amino acids and a suitable gap penalty; and (iii) as for (ii) but with scaling enabled, in order to identify windows shorter than 80 amino acids but with high identity.

Using the 80 amino acid window search, 10 matches with at least 35% identity were found; all of these were the allergen Asp o 21, an α-amylase originating from the fungus *Aspergillus oryzae*. The 80 amino acid window search with scaling enabled also yielded matches to this allergen, but no homology to any other allergens. The allergen is described in the WHO/IUIS Allergen Nomenclature Sub-committee database as an occupational respiratory allergen, associated with symptoms such as asthma and rhinitis in bakers. There are only four individual case reports of possible food allergy to this allergen, of which three were associated with occupational exposure *(6–9)*. Furthermore, when aligned and compared over the full length, the Asp o 21 α-amylase and this α-amylase are only 21.2% identical. A full-length comparison has been reported to be more reliable than the sliding window approach *(10)*.

The Committee repeated the AllergenOnline search using an updated version (Version 21, updated 14 February 2021), with no other matches identified.

No information on digestibility in SGF or SIF was submitted.

The Committee considered that dietary exposure to this α-amylase is not anticipated to pose a risk of allergenicity.

2.2 Toxicological studies

The toxicological testing of the enzyme was conducted on two test batches of α-amylase concentrates, designated PPY 31071 and PPY 23880, both of which were α-amylase enzyme concentrates without any addition of additives or other standardization or stabilization ingredients. Test batch no. PPY 31071, which was used for the genotoxicity assays, was produced by the current production strain; test batch no. PPY 23880, used for the 90-day repeat-dose study in

rats, was produced by a previous production strain in the same strain lineage. Both batches were characterized. Compared with batch no. PPY 31071, batch no. PPY 23880 had a slightly lower α-amylase activity (89% of the activity of batch no. PPY 31071), a slightly higher water content (89.2 w/w% compared with 87 w/w%), a slightly higher ash content (4.6 w/w% compared with 3.1 w/w%) and a moderately lower TOS (6.2 w/w% compared with 9.9 w/w%). No other differences between the two batches were detected. Both batches comply with JECFA specifications for chemical and microbiological purity of food enzymes (Annex 1, reference 185). The Committee concluded that batch no. PPY 23880 is sufficiently similar to batch no. PPY 31071 to be used in the present safety assessment.

2.2.1 Acute toxicity

No information was available.

2.2.2 Short-term studies of toxicity

A 13-week oral toxicity study was performed in rats using batch no. PPY 23880 *(11)*. The study was conducted in 2005 under GLP conditions and in accordance with OECD guideline no. 408 as revised in 1997 *(12)*. The test article was provided to the testing laboratory as a liquid that contained 6.2% w/w TOS. SPF Sprague-Dawley rats (10 per sex per dose group) of age 33–37 days, acclimatized to standard laboratory environmental conditions for 13 days prior to the start of the study, were used. Tap water was used as the vehicle and negative control article. The rats received a single dose of α-amylase by gavage of 0, 1.0, 3.3 or 10 mL/kg bw per day, equivalent to 0, 70, 220 or 660 mg TOS/kg bw per day, respectively, at a volume of 10 mL/kg bw. The test article was completely soluble in water, and the expected enzyme activity was verified in samples taken during weeks 1, 6 and 13 of the in-life phase.

Two female rats, one in the control group and one in the middle-dose group, died prior to scheduled termination. These deaths were attributed to oesophageal perforation during dosing and epistaxis during blood collection, respectively. There were no treatment-related effects on clinical observations, motor/behavioural test performance, body weight changes, feed consumption, ophthalmological findings, group mean haematology values, group mean coagulation values, or absolute or relative organ weights. The middle and high doses (220 and 660 mg TOS/kg bw per day, respectively) of α-amylase concentrate were associated with increased consumption of water. The group mean values for plasma calcium were significantly higher in middle- and high-dose male rats compared with that for control males, and the group mean value for plasma phosphorus was also elevated in high-dose males relative to that for control males.

Distension of the caecum was found on necropsy in four males and two females in the high-dose groups, and these changes corresponded microscopically to luminal dilatation of the caecum. All these differences between treatment groups and sex-matched controls were attributed to the high salt content of the enzyme concentrate, and not considered to be adverse. Based on the absence of adverse effects, the Committee concluded that the NOAEL was the highest dose given, equivalent to 660 mg TOS/kg bw per day.

2.2.3 Genotoxicity

Two genotoxicity assays were available: a bacterial reverse mutation assay *(13)* and an in vitro micronucleus assay *(14)* (Table 1). Both assays were conducted under GLP conditions using test batch no. PPY 31071, which was produced by the current production strain. The bacterial reverse mutation assay was conducted in accordance with OECD guideline no. 471 as revised in July 1997 *(15)*, although the exposure of the test strains of bacteria in liquid culture (treat-and-plate) method used is not specified in any guidelines. This method was used because the enzyme preparation is likely to include free histidine and tryptophan, which could cause false positive responses if the conventional exposure method was used. The micronucleus assay was also conducted in 2010, in compliance with the OECD draft proposal for new guideline no. 487 in 2009 *(16)*. The Committee concluded that the α-amylase concentrate at ≤ 5000 µg dry matter (DM)/mL is not mutagenic under the conditions of these assays.

2.2.4 Other studies

No information concerning homology with known protein toxins was provided. The Committee conducted a search for homology between the amino acid sequence of this α-amylase and toxins in the UNIPROT database *(17)*. The BLAST search using a threshold E-value of 0.1 showed sequence matches with other amylases but no matches to toxin or venom sequences, indicating that the amino acid sequence of this α-amylase is not similar to any known protein toxin.

2.3 Observations in humans

No information was available.

Table 1
Genotoxicity of α-amylase concentrate

End-point	Test system	Concentration	Results	Reference
In vitro reverse mutation[a]	*Salmonella typhimurium* TA1535, TA100, TA1537 and TA98, and *Escherichia coli* WP2uvrApKM101	156–5000 µg DM/plate ± S9[b]	Negative	Pedersen *(13)*
In vitro mammalian chromosome aberration[c]	Primary cultured human peripheral lymphocytes	*First main assay* 3 h exposure ± S9: 3000–5000 µg DM/mL	Negative	Whitwell *(14)*
		Second main assay 18 h exposure (3 h + S9 followed by 15 h – S9): 3000–5000 µg DM/mL	Negative	
		18 h exposure (18 h – S9): 3000–5000 µg DM/mL	Negative	

DM: dry matter.

[a] In this assay, tester strains were incubated with test material for 3 hours at 37 °C. After this period, the tester strains were centrifuged and the pellets were resuspended in buffer. The bacteria were again centrifuged, and the bacteria were again suspended in buffer. An aliquot of this final suspension was added to top agar, which was then plated onto minimal glucose agar plates. These plates were incubated at 37 °C for 72 hours, after which the colonies were counted. All concentrations of the test substance were assayed three times. All positive control substances yielded expected results.

[b] S9 was a liver homogenate from Aroclor 1254-treated rats combined with co-factors that were required for mixed function oxidase activity.

[c] On the basis of a preliminary dose-range-finding study, three concentrations were selected for the two main assays. In the first main assay, cultures were treated for 3 hours in the absence or presence of S9. In the second main assay, cultures were treated for 18 hours in the absence of S9, or for 3 hours in the presence of S9 followed by 15 hours in the absence of S9. All cultures were harvested 18 hours after the start of treatment. All cultures were treated with Cytochalasin B, an inhibitor of mitosis, added to the post wash-off medium. At harvest cultures were centrifuged and the supernatant discarded. Cell pellets were resuspended in a KCl solution, which causes the cells to swell, and then incubated for 10 minutes, centrifuged and supernatants removed. Cells were fixed onto slides, stained and scored for chromosomal aberrations (i.e. metaphase analysis) and for cytotoxicity. Both main assays were repeated at least twice.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to the TOS from this α-amylase enzyme preparation from *G. stearothermophilus* expressed in *B. licheniformis*. The enzyme is intended for use in starch processing, alcohol distilling processes, brewing and other cereal beverage processes, cereal processing, and fruit and vegetable processing; these uses were therefore considered for the dietary exposure assessment. The submission included an estimate of dietary exposure based on the budget method, a screening method used to determine the TMDI of food additives *(18,19)*. The method takes into account maximum physiological levels of consumption of solid food and non-milk beverages, the energy density of foods, the concentration of the food additive in solid foods and non-milk beverages, and the proportion of solid foods and non-milk beverages that may contain it. The method provides a conservative estimate of dietary exposure. Further details of the budget method can be found in EHC 240 *(20)*.

3.2 Dietary exposure assessment

The estimated TMDI provided by the sponsor was based on a number of inputs to the budget method. The first was the amount of food consumed, for which 0.025 kg/kg bw per day of solid foods and 0.1 L/kg bw per day of non-milk beverages were used. The proportion of solid food and non-milk beverages containing the enzyme preparation was also included. EHC 240 *(20)* refers to commonly used default proportions of 12.5% for solid foods and 25% for non-milk beverages. Foods processed with this α-amylase enzyme preparation are proposed to be added to a variety of foods intended to be consumed by the general population. The sponsor therefore assumed that 50% of solid foods and 25% of non-milk beverages are processed and would contain the enzyme preparation. The additional assumption that processed foods contain 25% starch (or starch-derived) dry matter and processed beverages contain 10% starch hydrolysates was also made by the sponsor. The highest dosage in both solid foods and non-milk beverages was 9.48 mg TOS/kg starch-based raw material.

The standard budget method calculation was used to estimate dietary exposure to the TOS from solid foods and non-milk beverages. The resulting TMDIs of TOS from this α-amylase enzyme preparation were estimated by the sponsor to be 0.0296 mg TOS/kg bw per day for solid foods and 0.0237 mg TOS/kg bw per day for non-milk beverages for a total of 0.0533 mg TOS/kg bw per day.

The sponsor additionally considered special applications in which the enzyme is used to remove a small amount of starch in sugar processing and fruit and vegetable processing. For sugar processing, in which a maximum dosage of 0.079 mg TOS/kg sugar dry matter is used and it is assumed that 50% of solid food consumed is processed, the resulting TMDI is 0.001 mg TOS/kg bw per day. For fruit and vegetable processing, in which a maximum dosage of 0.158 mg TOS/L fruit juice is used and it is assumed that 25% of non-milk beverages is fruit juice, the resulting TMDI is 0.004 mg TOS/kg bw per day. The TMDIs for these special applications are lower than the TMDIs for the separate solid food and non-milk beverage components of the budget method calculation undertaken by the sponsor; the budget method calculation was therefore deemed by the Committee to cover these special applications.

The Committee independently calculated dietary exposure to the TOS from this α-amylase enzyme preparation using the budget method. All inputs and assumptions remained the same as for the estimate by the sponsor, with the exception of the consumption amount for solid foods. The Committee assumed the maximum physiological requirement for solid food (including milk) is 0.05 kg/kg bw per day. This is the level used in a budget method calculation where there is potential for the enzyme to be present in baby foods or general-purpose foods that may be consumed by infants and young children, and represents a worst-

case scenario. The TMDI of the TOS from this α-amylase enzyme preparation as estimated by the Committee was 0.059 mg TOS/kg bw per day for solid food and the same as the sponsor (0.024 mg TOS/kg bw) for non-milk beverages, for a total of 0.08 mg TOS/kg bw per day (rounded from 0.083 mg TOS/kg bw per day).

The Committee concluded that the dietary exposure estimate of 0.08 mg TOS/kg bw per day was appropriate for use in the evaluation.

For the purposes of the dietary exposure assessment conducted by both the sponsor and the Committee, it was assumed that the enzyme is not removed or inactivated during the final processing of ingredients or foods, and that 100% of the TOS from the enzyme preparation remains in the ingredient and final food. However, the enzyme is in reality either removed or inactivated by high temperatures during processing of food ingredients, such that it will have no function in the final food.

4. Comments

4.1 Assessment of potential allergenicity

Potential immunological cross-reactivity with known allergens was assessed by the sponsor in 2012 according to recommended criteria *(3)*. The amino acid sequence of α-amylase was compared with those of known allergens in two databases *(4,5)*. Three homology analyses were performed: (i) more than 35% identity over the full length of the alignment; (ii) more than 35% identity using a sliding window of 80 amino acids and a suitable gap penalty; and (iii) as for (ii) but with scaling enabled, in order to identify windows shorter than 80 amino acids but with high identity. Using the 80 amino acid window search, with and without scaling, several matches with more than 35% identity were found; all of these were the allergen Asp o 21, an α-amylase originating from the fungus *A. oryzae*. This is a respiratory allergen associated with occupational rhinitis and asthma in bakers. However, when compared over the full length of the sequence, the Asp o 21 α-amylase and this α-amylase from *B. stearothermophilus* expressed in *B. licheniformis* are only 21.2% identical.

The Committee repeated the comparison of the amino acid sequence of the enzyme with known allergens based on the recommended bioinformatics criteria *(3,21)* and as outlined in EHC 240 *(2)*. The amino acid sequence of the enzyme was compared with those of known allergens in the current version of the FARRP AllergenOnline database *(4)*. A search for matches with more than 35% identity in a sliding window of 80 amino acids, a search for exact matches

over contiguous stretches of eight amino acids and a full-length FASTA sequence search did not identify homology to any allergens not previously identified by the sponsor in 2012.

No information concerning the digestibility of this α-amylase in SGF or SIF was available. The Committee noted that a similar α-amylase (JECFA95-1) from *G. stearothermophilus* expressed in *B. licheniformis* was readily hydrolysed in SGF.

The Committee concluded that dietary exposure to this α-amylase is not anticipated to pose a risk for allergenicity.

4.2 Toxicological studies

A 13-week study of oral toxicity in rats of an α-amylase enzyme concentrate from a closely related production strain of *B. licheniformis (11)* was available and was considered relevant based on safe strain lineage. The enzyme concentrate was administered by gavage at dosages up to 660 mg TOS/kg bw per day. No treatment-related adverse effects were observed. The Committee identified a NOAEL of 660 mg TOS/kg bw per day, the highest dose tested.

The enzyme concentrate was not genotoxic in either a bacterial reverse mutation assay *(13)* or an in vitro micronucleus induction assay in human peripheral blood lymphocytes *(14)*. The Committee had no concerns about potential genotoxicity of the enzyme concentrate.

The Committee compared the amino acid sequence of the enzyme with those of known protein toxins *(17)*. There was no biologically relevant homology (~ 16%). The Committee therefore concluded that the enzyme is unlikely to be a toxin.

4.3 Assessment of dietary exposure

The Committee evaluated two estimates of dietary exposure to the TOS from this α-amylase enzyme preparation, one submitted by the sponsor and the other estimated by the Committee. Both estimates were derived with the budget method, a screening method based on maximum physiological levels of consumption of solid foods and non-milk beverages, and on maximum use levels of the enzyme preparation. A maximum use level of 9.48 mg TOS/kg starch-based raw material for both solid foods and non-milk beverages was used. It was assumed that 50% of solid foods and 25% of non-milk beverages in the food supply are processed and would contain the enzyme preparation, and that processed solid foods contain 25% starch (or starch-derived) dry matter and processed beverages contain 10% starch hydrolysates. Different assumptions were made about the amount of solid

foods consumed. The resulting TMDI from solid food and non-milk beverages was 0.0533 mg TOS/kg bw per day by the sponsor and 0.08 mg TOS/kg bw per day by the Committee. The Committee concluded that the dietary exposure estimate of 0.08 mg TOS/kg bw per day was appropriate for use in the evaluation. For the dietary exposure assessment, it was assumed that 100% of the TOS from the enzyme preparation remains in the final food. The Committee noted that the enzyme is either removed or inactivated during the processing of food ingredients and will have no function in the final food.

5. Evaluation

The Committee concluded that dietary exposure to this α-amylase enzyme preparation is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 660 mg TOS/kg bw per day, the highest dose tested in a 13-week oral toxicity study in rats. When this NOAEL is compared with the dietary exposure estimate of 0.08 mg TOS/kg bw per day, a MOE of more than 8000 can be calculated. Based on this MOE and the lack of concern about genotoxicity, the Committee established a temporary ADI "not specified"[2] for α-amylase (JECFA95-2) from *G. stearothermophilus* expressed in *B. licheniformis*, when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications.

5.1 Recommendations

The Committee requested the following information, by the end of 2023, to complete the safety assessment:

- validated method of analysis to determine α-amylase activity, including the validation report;
- unit definition for α-amylase activity based on the method of assay; and
- analytical data using the validated method for at least five different batches of commercially available products.

[2] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

6. References

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α-Amylase (JECFA95-3) from *Rhizomucor pusillus* expressed in *Aspergillus niger*

First draft prepared by
Rosalind Dalefield,[1] Polly E. Boon,[2] Josef Schlatter,[3] Ivan Stankovic[4] and Jannavi Srinivasan[5]

[1] Food Standards Australia New Zealand, Wellington, New Zealand
[2] Department for Food Safety, Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment, Bilthoven, Netherlands (Kingdom of the)
[3] Zürich, Switzerland
[4] Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia
[5] Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), USA

1. Explanation

At the request of the CCFA at its Forty-eighth session *(1)*, the Committee evaluated the safety of α-amylase (Enzyme Commission No. 3.2.1.1; CAS No. 9000-90-2) from *Rhizomucor pusillus* expressed in *Aspergillus niger*. The Committee has not

previously evaluated this enzyme preparation. The present Committee allocated the unique JECFA enzyme identifier JECFA95-3 to distinguish it from similarly named enzyme preparations. The Committee has previously evaluated several α-amylases from other sources, for which an ADI "not specified"[1] was established (e.g. Annex 1, reference 94).

The term "α-amylase" refers to the α-amylase enzyme and its amino acid sequence; the term "enzyme concentrate" refers to the fermentation product containing the enzyme of interest, which is used in the toxicity studies; and the term "enzyme preparation" refers to the product formulated for commercial use.

The Committee has previously assessed the safety of enzyme preparations derived from *A. niger* (e.g. asparaginase, Annex 1, reference 190; 3-phytase, Annex 1, reference 211), and established an ADI "not specified" for these enzyme preparations. On this basis, the present Committee considered that α-amylase (JECFA95-3) from *R. pusillus* expressed in *A. niger* met the criteria of a Class 1, Type iii enzyme, as described in EHC 240 *(2)*. A Class 1, Type iii enzyme preparation is produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Although toxicity data and a dietary exposure assessment are not required for Class 1, Type iii enzymes, the Committee evaluated the submitted data.

The enzyme catalyses the endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units, which results in the degradation of starch and related polysaccharides. The enzyme preparation is intended for use as a processing aid in starch processing, beverage alcohol (distilling) processes, and baking and cereal-based processes.

The Committee conducted literature searches in PubMed and EBSCO. Searches were conducted with the linked search terms "α-amylase" AND "*Rhizomucor pusillus*" AND ("safety" OR "toxic" OR "allergy"). No studies relevant to the safety evaluation were found.

1.1 Genetic background

The production organism, *A. niger*, is a non-pathogenic, filamentous fungus that is ubiquitous in the environment. It has a history of use in the production of enzymes intended for use in food processing and citric acid production *(3)*.

The non-toxigenic, non-pathogenic *A. niger* production strain was constructed through a combination of chemical mutagenesis and genetic modifications of the parental strain. The production strain was obtained by inactivation of several genes encoding for major secreted proteins, deletion of a

[1] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

gene encoding a protease, and disruption of genes responsible for the production of oxalic acid and fumonisin. The expression plasmid containing the α-amylase gene from *R. pusillus*, an optimized *A. niger* promoter, a transcriptional terminator and a selection marker were integrated by targeted homologous recombination. The stability of the integration and the absence of any genes of concern were confirmed by Southern blot hybridization. The production strain is deposited in DSMZ, Germany.

1.2 Chemical and technical considerations

α-Amylase enzyme is produced by controlled fermentation of a pure culture of the *A. niger* production strain. The manufacture of the α-amylase enzyme preparation includes fermentation, recovery and formulation. After fermentation, the broth containing the α-amylase enzyme is recovered from the biomass by multiple filtration and concentration steps. The resulting concentrate is formulated into a powder preparation. The entire process is performed in accordance with current GMP using food-grade raw materials. The enzyme concentrate is tested to be free of the production organism and any antibiotic activity.

The primary sequence of α-amylase enzyme produced by *A. niger* consists of 583 amino acids; its molecular weight calculated from the determined amino acid sequence is 63.5 kDa. The α-amylase produced by *A. niger* is not expected to have any secondary or subsidiary activities.

A unit definition of activity was not provided. The activity of α-amylase is determined spectrophotometrically (405 nm) by measuring the hydrolysis of a 4,6-ethylidene-(G7)-*p*-nitrophenyl-(G1)-α-D-maltoheptaoside (ethylidene-G7PNP) substrate by the enzyme, compared with a proprietary enzyme standard. The activity was expressed in units relative to an enzyme of a declared strength. The mean activity of α-amylase from four batches of enzyme concentrate was 57.2 units/g.

α-Amylase catalyses the endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units. The enzyme preparation is intended for use as a processing aid in starch processing, beverage alcohol (distilling) processes, and baking and cereal-based processes at a maximum level of 407 mg TOS/kg of starch-based raw material. The TOS includes the enzyme of interest and residues of organic materials (e.g. proteins, peptides and carbohydrates) from the production organism during manufacture.

The α-amylase enzyme is inactivated by heat or removed during processing, and is not expected to have any technological function in the final food.

2. Biological data

2.1 Assessment of potential allergenicity

This α-amylase was assessed as a potential allergen in 2013, with a search strategy based on the 2001 recommendations of FAO/WHO *(4)*. The amino acid sequence of the enzyme was compared with the amino acid sequences of known allergens in the FARRP AllergenOnline *(5)* and the WHO/IUIS Allergen Nomenclature Sub-committee *(6)* databases. Searches included (i) more than 35% identity over the full length of the alignment; (ii) more than 35% identity using a sliding window of 80 amino acids and a suitable gap penalty; and (iii) as for (ii) but with scaling enabled, in order to identify windows shorter than 80 amino acids but with high identity.

The 80 amino acid searches, with and without scaling, identified three matches: Asp o 21 and Asp o 21.0101, both of which are names for α-amylase originating from the fungus *A. oryzae*; and Sch c 1, an amyloglucosidase synthesized by the fungus *Schizophyllum commune*. These allergens are identified by the WHO/IUIS Allergen Nomenclature Sub-committee database as respiratory allergens. The identity over the full-length search was 32.4% for the *A. oryzae* α-amylase, and 25.2% for the *S. commune* amyloglucosidase. No hits were found for 100% identity over eight amino acids. A full-length comparison has been reported to be more reliable than the sliding window approach *(7)*. There are only four individual case reports of possible food allergy to Asp o 21, of which three were associated with occupational exposure *(8–11)*. No cases of possible food allergy from Sch c 1 were located by literature search. The Committee repeated the AllergenOnline search using the current version (Version 21, updated 14 February 2021) with no other matches identified.

No information on digestibility in SGF or SIF was submitted.

The Committee considered that dietary exposure to this α-amylase is not anticipated to pose a risk of allergenicity.

2.2 Toxicological studies

The available toxicological studies were conducted on the enzyme concentrate designated batch no. PPY 33598. This batch was produced according to the same method as the commercial enzyme preparation, but differs from the enzyme preparation in that no additives or other standardization or stabilization ingredients were included.

2.2.1 Acute toxicity

No information was available.

2.2.2 Short-term studies of toxicity

A 13-week oral toxicity study was conducted in Wistar rats using batch no. PPY 33598 (TOS 13.3 w/w%) *(12)*. The study was conducted under GLP conditions and in compliance with OECD guideline no. 408 as revised in 1997 *(13)*. Wistar rats of age 5 weeks (10 per sex per dose group), acclimatized to standard laboratory conditions for 7 days prior to the start of the study, were used. Tap water was used as the vehicle and negative control article. Rats received a single dose of α-amylase concentrate of 0, 140, 462 or 1400 mg TOS/kg bw per day by gavage, at a dose volume of 10 mL/kg bw. The enzyme activity of dose formulations was measured in samples collected during weeks 1, 7 and 13 of the in-life phase, and found to exceed the anticipated level at the highest dose administered.

One male rat in the middle-dose group (462 mg TOS/kg bw per day) was found dead on day 76 of the study. On necropsy the death was found to be the result of pleuritis, secondary to gavage accident. There were no test-article-related mortalities. There were no treatment-related effects on clinical signs, group mean body weights, group mean feed consumption, neurobehavioural assessments, ophthalmology, clinical pathology, organ weights, organ weight ratios, gross necropsy findings or histopathological findings. Compared with the control group, males in the high-dose group (1400 mg TOS/kg bw per day) had a significantly decreased group mean serum creatinine value and a significantly higher group mean serum calcium value, but the values remained within the normal historical control range. A small number of statistically significant differences in group mean values between treatment groups and their sex-matched controls was considered to be incidental because no dose–response relationship was evident, and group mean values remained within historical control ranges.

In females, transient differences in group mean water consumption values were observed between the low- and high-dose groups. Compared with the control group: relatively high group mean values for mean corpuscular volume (MCV), and relatively high absolute and relative thymus weight, were observed in the low-dose group; relatively high group mean alkaline phosphatase and relatively low group mean total cholesterol were observed in the middle-dose group; relatively high group mean serum chloride was observed in the high-dose group; and relatively high group mean serum glucose values were observed in all dose groups.

In males, statistically significant differences from the control group included relatively high group mean serum protein and low group mean brain

weight relative to body weight in the low-dose group, and high absolute and relative group mean prostate weight in the middle-dose group. The Committee concluded that the NOAEL for the enzyme concentrate in rats was 1400 mg TOS/kg bw per day, the highest dose tested.

2.2.3 Genotoxicity

Two genotoxicity assays were available: a bacterial reverse mutation assay *(14)* and an in vitro micronucleus assay *(15)*. Both assays were conducted under GLP conditions, using enzyme concentrate batch no. PPY 33598.

The bacterial reverse mutation assay was conducted in general accordance with OECD guideline no. 471 as revised in 1997 *(16)*, although the exposure in liquid culture (the treat-and-plate method) is not described in any guideline. This method was used because an enzyme concentrate is likely to contain free histidine and tryptophan, which could cause a so-called feeder effect on bacterial colony growth and result in false positive results. The in vitro micronucleus assay was conducted in compliance with OECD guideline no. 487 as revised in 2010 *(17)*.

The results of both genotoxicity assays were negative (Table 1). These results provide evidence that this α-amylase concentrate is not mutagenic or clastogenic. The Committee had no concerns regarding potential genotoxicity of this α-amylase enzyme concentrate.

2.2.4 Other studies

Protein sequences containing the word "toxin" were extracted from UniProt *(18)*. Each of the sequences was placed in its own FASTA file, and the sequence of α-amylase from *R. pusillus* expressed in *A. niger* was placed in a separate file. Alignment of each sequence with the α-amylase was carried out using the sequence alignment software Clustal, version 2.0.10. The identity percentage of this α-amylase sequence or of the compared toxin sequence was calculated, whichever was longest. This method was chosen because the toxin sequences have different lengths. By always using the longest sequence, artificial high scores from very short or very long toxins are avoided. The largest homology encountered was 16.3%, indicating that the homology to any toxin sequence in this database was random and very low.

2.3 Observations in humans

No information was available.

Table 1
Genotoxicity of α-amylase concentrate

End-point	Test system	Concentration	Results	Reference
Bacterial reverse mutation assay[a]	*Salmonella typhimurium* TA1535, TA100, TA1537 and TA98, and *Escherichia coli* WP2uvrApKM101	156–5000 µg DM/plate ± S9[b]	Negative	Pedersen *(14)*
Mammalian chromosomal aberration assay[c]	Primary cultured human peripheral lymphocytes	*First main assay* 3 h exposure + S9: 500–5000 µg/mL	Negative	Whitwell *(15)*
		Second main assay 18 h exposure (3 h + S9 followed by 15 h – S9): 500–5000 µg/mL	Negative	
		18 h exposure (18 h – S9): 500–5000 µg/mL	Negative	

DM: dry matter.
[a] In this assay, tester strains were incubated with test material for 3 hours at 37 °C. After this period, the tester strains were centrifuged and the pellets were resuspended in buffer. The bacteria were again centrifuged, and the bacteria were again suspended in buffer. An aliquot of this final suspension was added to top agar, which was then plated onto minimal glucose agar plates. These plates were incubated at 37 °C for 72 hours, after which the colonies were counted. All concentrations of the test substance were assayed three times. All positive control substances yielded expected results.
[b] S9 was a liver homogenate from Aroclor 1254-treated rats combined with co-factors that were required for mixed function oxidase activity.
[c] On the basis of a preliminary dose-range-finding study, three concentrations were selected for the two main assays. In the first main assay, cultures were treated for 3 hours in the absence or presence of S9. In the second main assay, cultures were treated for 18 hours in the absence of S9, or for 3 hours in the presence of S9 followed by 15 hours in the absence of S9. All cultures were harvested 18 hours after the start of treatment. All cultures were treated with Cytochalasin B, an inhibitor of mitosis, added to the post wash-off medium. At harvest cultures were centrifuged and the supernatant discarded. Cell pellets were resuspended in a KCl solution, which causes the cells to swell, and then incubated for 10 minutes, centrifuged and supernatants removed. Cells were fixed onto slides, stained and scored for chromosomal aberrations (i.e. metaphase analysis) and for cytotoxicity. Both main assays were repeated at least twice.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to the TOS from the enzyme preparation. The enzyme is intended for use in starch processing, alcohol distilling processes, and baking and other cereal-based processes; these uses were therefore considered for the dietary exposure assessment. The submission included an estimate of dietary exposure based on the budget method, a screening method used to determine the TMDI of food additives *(19,20)*. The method takes into account maximum physiological levels of consumption of solid foods and non-milk beverages, the energy density of foods, the concentration of the food additive in solid foods and non-milk beverages, and the proportion of solid foods and non-milk beverages that may contain it. The method provides a conservative estimate of dietary exposure. Further details of the budget method can be found in EHC 240 *(21)*.

3.2 Dietary exposure assessment

The estimated TMDI provided by the sponsor was calculated using a high level of consumption of solid foods and of non-milk beverages; the maximum use level of the enzyme preparation in solid foods and in non-milk beverages; and the proportion of solid foods and of non-milk beverages that may contain the enzyme preparation. EHC 240 *(21)* refers to a daily consumed amount of 0.05 kg/kg bw of solid foods (based on 100 kcal/kg bw as the maximum energy intake over the course of a lifetime from solid foods) and 0.1 L/kg bw of non-milk beverages, and default proportions of 12.5% for solid foods and 25% for non-milk beverages. Food ingredients processed with the enzyme preparation are proposed to be added to a variety of foods intended to be consumed by the general population. The sponsor therefore assumed that 50% of solid foods and 25% of non-milk beverages are processed, and that processed solid foods contain 25% starch (or starch-derived) dry matter and processed beverages contain 10% starch hydrolysates. These assumptions resulted in an overall proportion of 12.5% for solid foods and 2.5% for non-milk beverages that contain the enzyme preparation. The maximum use level in both solid foods and non-milk beverages was 407 mg TOS/kg starch-based raw material.

The budget method calculation was used to estimate dietary exposure to the TOS from the enzyme preparation. The sponsor used the standard inputs, with the exception of the consumption of solid foods and the proportion of non-milk beverages that contain the enzyme preparation as explained above. For the consumption of solid foods, the sponsor assumed a maximum energy intake over the course of a lifetime from solid foods of 50 kcal/kg bw per day instead of 100 kcal/kg bw per day, resulting in a daily consumption of solid foods of 0.025 kg/kg bw. The resulting TMDIs were 1.27 mg TOS/kg bw per day for solid foods and 1.02 mg TOS/kg bw per day for non-milk beverages, for a total of 2.29 mg TOS/kg bw per day.

The Committee also calculated dietary exposure to the TOS from the enzyme preparation using the budget method. All inputs and assumptions were the same as used by the sponsor, with the exception of the consumed amount for solid foods. The Committee assumed the maximum physiological requirement for solid foods (including milk) to be 0.050 kg/kg bw per day from EHC 240 *(21)*. This is the amount used in a budget method calculation where there is potential for the enzyme to be used in baby foods or general-purpose foods that may be consumed by infants and young children, and represents a worst-case scenario. The TMDIs were 2.54 mg TOS/kg bw per day for solid foods and 1.02 mg TOS/kg bw per day for non-milk beverages, for a total of 3.56 mg TOS/kg bw per day (rounded to 4.0 mg TOS/kg bw per day by the Committee).

For the purposes of the dietary exposure assessment conducted by both the sponsor and the Committee, it was assumed that the enzyme is not removed or inactivated during the processing of food ingredients, and that 100% of the TOS from the enzyme preparation remains in the final food. In reality, the enzyme is either removed or inactivated by high temperatures during the processing of food ingredients, and will have no function in the final food.

4. Comments

4.1 Assessment of potential allergenicity

Potential immunological cross-reactivity with known allergens was assessed by the sponsor in 2012 according to recommended criteria *(4)*. The amino acid sequence of α-amylase was compared with those of known allergens in two databases *(5,6)*. Three homology analyses were performed: (i) more than 35% identity over the full length of the alignment; (ii) more than 35% identity using a sliding window of 80 amino acids and a suitable gap penalty; and (iii) as for (ii) but with scaling enabled, in order to identify windows shorter than 80 amino acids but with high identity. No biologically relevant matches were found in either database.

The Committee repeated the comparison of the amino acid sequence of the enzyme with known allergens based on the recommended bioinformatics criteria *(4,22)* and as outlined in EHC 240 *(2)*. The amino acid sequence of the enzyme was compared with those of known allergens in the current version of the FARRP AllergenOnline database *(5)*. A search for matches with more than 35% identity in a sliding window of 80 amino acids, a search for exact matches over contiguous stretches of eight amino acids and a full-length FASTA sequence search did not identify homology to any allergens.

No information concerning the digestibility of this α-amylase in SGF or SIF was available.

The Committee concluded that dietary exposure to this α-amylase is not anticipated to pose a risk for allergenicity.

4.2 Toxicological studies

A 13-week oral toxicity study of this α-amylase concentrate was conducted in rats *(12)*. The enzyme concentrate was administered by gavage at doses up to 1400 mg TOS/kg bw per day. No treatment-related adverse effects were observed.

The Committee identified a NOAEL of 1400 mg TOS/kg bw per day, the highest dose tested.

An enzyme concentrate of this α-amylase was not genotoxic in either a bacterial reverse mutation assay *(14)* or an in vitro micronucleus induction assay in human peripheral blood lymphocytes *(15)*. The Committee had no concerns about potential genotoxicity of this α-amylase enzyme concentrate.

A comparison of the amino acid sequence of the enzyme with those of known protein toxins *(18)* revealed no biologically relevant homology. The Committee therefore concluded that the enzyme was unlikely to be a toxin.

4.3 Assessment of dietary exposure

The Committee evaluated two estimates of dietary exposure to TOS from this α-amylase enzyme preparation, one submitted by the sponsor and the other estimated by the Committee. Both estimates were derived with the budget method, a screening method based on maximum physiological levels of consumption of solid foods and non-milk beverages, and on maximum use levels of the enzyme preparation. A maximum use level of 407 mg TOS/kg starch-based raw material for both solid foods and non-milk beverages was used. It was assumed that 50% of solid foods and 25% of non-milk beverages are processed, and that processed solid foods contain 25% starch (or starch-derived) dry matter and processed beverages contain 10% starch hydrolysates. Different assumptions were made about the amount of solid foods consumed. The resulting TMDIs from solid foods and non-milk beverages were 2.29 mg TOS/kg bw per day by the sponsor and 4 mg TOS/kg bw per day by the Committee. The Committee concluded that the dietary exposure estimate of 4 mg TOS/kg bw per day was appropriate for use in the evaluation. For the dietary exposure assessment, it was assumed that 100% of the TOS of the enzyme preparation remains in the final food. The Committee noted that the enzyme is either removed or inactivated during the processing of food ingredients, and will have no function in the final food.

5. Evaluation

The Committee concluded that dietary exposure to this α-amylase enzyme preparation is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 1400 mg TOS/kg bw per day, the highest dose tested in a 13-week oral toxicity study in rats. When this NOAEL is compared with the dietary exposure estimate of 4 mg TOS/kg bw per day, a MOE of more than 350 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary

ADI "not specified"[2] for α-amylase (JECFA95-3) from *R. pusillus* expressed in *A. niger*, when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications.

5.1 Recommendations

The Committee requested the following information, by the end of 2023, to complete the safety assessment:

- validated method of analysis to determine α-amylase activity, including the validation report;
- unit definition for α-amylase activity based on the method of assay; and
- analytical data using the validated method for at least five different batches of commercially available products.

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Amyloglucosidase (JECFA95-4) from *Rasamsonia emersonii* expressed in *Aspergillus niger*

First draft prepared by
Joel Rotstein,[1] Tracy Hambridge,[2] Utz Mueller,[3] Maria Beatriz de Abreu Gloria[4] and Stephan G. Walch[5]

[1] Ottawa, Ontario, Canada
[2] Food Standards Australia New Zealand, Majura Park, Australian Capital Territory, Australia
[3] Perth, Western Australia, Australia
[4] Faculty of Pharmacy, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[5] Chemisches und Veterinäruntersuchungsamt Karlsruhe, Karlsruhe, Baden-Württemberg, Germany

1. Explanation

At the request of the CCFA at its Forty-eighth Session *(1)*, the Committee evaluated the safety of amyloglucosidase (Enzyme Commission No. 3.2.1.3; CAS No. 9032-08-0) from *Rasamsonia emersonii* (formerly *Talaromyces emersonii*) expressed in *Aspergillus niger*. The Committee had not previously considered

this enzyme preparation. The present Committee allocated the unique JECFA enzyme identifier JECFA95-4 to this enzyme preparation. The Committee had previously evaluated amyloglucosidase from *A. niger* (Annex 1, reference 77), for which an ADI "not specified"[1] was established.

The term "amyloglucosidase" refers to the enzyme and its amino acid sequence; the term "enzyme concentrate" refers to the fermentation product containing the enzyme of interest, which is used in the toxicity studies; and the term "enzyme preparation" refers to the product formulated for commercial use.

The Committee has previously assessed the safety of enzyme preparations derived from *A. niger* (e.g. asparaginase, Annex 1, reference 190; phytase, Annex 1, reference 211) and established an ADI "not specified" for these enzyme preparations. On this basis, the present Committee considered that this amyloglucosidase (JECFA95-4) from *R. emersonii* expressed in *A. niger* met the criteria of a Class 1, Type iii enzyme, as described in EHC 240 *(2)*. A Class 1, Type iii enzyme preparation is produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Although toxicity data and a dietary exposure assessment are not required for Class 1, Type iii enzymes, the Committee evaluated the submitted data.

Amyloglucosidase hydrolyses (1→4)-α as well as (1→6)-α linkages in starch. The enzyme preparation is intended for use as a processing aid in starch processing.

The Committee conducted a literature search in Google Scholar with the linked search terms "amyloglucosidase", "*Rasamsonia emersonii*" and "*Aspergillus niger*". A total of 112 hits were found, but none was considered relevant to this safety evaluation. A second literature search was conducted in Google Scholar with the linked search terms "amyloglucosidase", "*Talaromyces emersonii*" and "*Aspergillus niger*". This search identified 108 references. None of the identified publications provided additional toxicity data relevant to this evaluation.

1.1 Genetic background

The production organism, *A. niger*, is a non-pathogenic filamentous fungus that is ubiquitous in the environment. It has a history of use in the production of enzymes intended for use in food processing and citric acid production *(3,4)*. The genetic modification includes the introduction of the amgT gene encoding for glucoamylase from *T. emersonii*, which has been reclassified as *R. emersonii (5,6)*.

The non-toxigenic and non-pathogenic production strain was constructed through a combination of chemical mutagenesis and genetic modifications of the

[1] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

parental strain. The expression plasmid contained a promoter sequence obtained from *A. niger* BO-1, the amyloglucosidase gene obtained from *R. emersonii*, a terminator sequence obtained from *A. niger* BO-1 and an antibiotic marker. The stability of the introduced amyloglucosidase gene was confirmed by Southern blot hybridization. The production strain was deposited in DSMZ, Germany.

1.2 Chemical and technical considerations

Amyloglucosidase is produced by controlled fermentation of a pure culture of the *A. niger* production strain. The manufacture of the amyloglucosidase enzyme preparation includes fermentation, recovery and formulation. After fermentation, the broth containing the amyloglucosidase enzyme is separated from the biomass by sedimentation followed by several filtration steps. The resulting concentrate is formulated into a liquid preparation. The entire process is performed in accordance with current GMP and using food-grade raw materials. The enzyme concentrate is tested to be free from the production organism and any antibiotic activity, as well as free from mycotoxins (ochratoxin A [OTA] and fumonisin B2 [FUM]). It has residual α-amylase activity.

The primary sequence of amyloglucosidase produced by *A. niger* consists of 591 amino acids; its molecular weight by calculation from the determined amino acid sequence is 62.8 kDa. The amyloglucosidase produced by *A. niger* is not expected to have any secondary or subsidiary activity.

The activity of amyloglucosidase is determined spectrophotometrically (340 nm) by measuring the hydrolysis of maltose compared with a proprietary enzyme standard, with activity expressed in units per gram. A unit definition of activity was not provided. The mean activity of amyloglucosidase from four batches of the liquid enzyme concentrate was 400 units/g.

Amyloglucosidase catalyses the hydrolysis of (1→4)-α and (1→6)-α glycosidic linkages in starch. The amyloglucosidase enzyme preparation is intended for use as a processing aid in starch/carbohydrate processing, baking, brewing/cereal beverage processing and potable alcohol production. The amyloglucosidase enzyme preparation is used at a maximum level of 931 mg TOS/kg starch-based raw material for solid foods and 1090 mg TOS/kg starch-based raw material for non-milk beverages. The TOS includes the enzyme of interest and residues of organic materials (e.g. proteins, peptides and carbohydrates) derived from the production organism during the manufacturing process. The amyloglucosidase enzyme is inactivated by heat or removed during processing, and is not expected to have any technological function in the final food.

2. Biological data

2.1 Assessment of potential allergenicity

Amyloglucosidase from *R. emersonii* expressed in *A. niger* was assessed as a potential allergen with the use of bioinformatics consistent with recommended criteria *(7–9)*. The amino acid sequence of the amyloglucosidase was compared with the sequences of known allergens in two databases *(10,11)*. The analyses included a search for matches between the amyloglucosidase and other proteins with: (i) more than 35% identity over the full length of the protein and over a sliding window of 80 amino acids (using a threshold of $E < 1 \times 10^{-7}$); (ii) with more than 35% identity over a sliding window of 80 amino acids with scaling enabled (allowing for the identification of matches with high identity, but over windows shorter than 80 amino acids); and (iii) a search for 100% identity over eight contiguous amino acids. Comparisons between highly homologous proteins yielded E-values approaching zero, indicating a very low probability that such matches would occur by chance (larger E-values indicate a lower degree of similarity).

Both allergen databases revealed a match to a reported respiratory allergen, the amyloglucosidase Sch c 1 found in split-gill mushrooms (*Schizophyllum commune*) *(12,13)*. This allergen showed 60–84% identity with amyloglucosidase over a sliding window of 80 amino acids (with and without scaling), 47.9% identity over the full length of the protein and 10 matches based on a 100% identity over eight contiguous amino acids. The Committee acknowledged that amyloglucosidase could pose a risk as a respiratory allergen, which when ingested may also cause a food allergy *(14,15)*. Although *S. commune* is consumed as a traditional food throughout Asia and Central America, there are currently no published reports of it being linked to a food allergen. The Committee concluded that there was insufficient information on digestibility to conduct its weight-of-evidence approach, and was therefore unable to complete the assessment of the potential for allergenicity from dietary exposure to this amyloglucosidase.

2.2 Toxicological studies

A. niger is a common organism in food; many strains are well established as a source of this enzyme, and numerous toxicity studies of various strains have not demonstrated any hazard to human health at the specified levels of use *(16)* (Annex 1, reference 211). *A. niger* has no potential to synthesize aflatoxins *(4)*, but can potentially synthesize the mycotoxins FUM and OTA *(17)*. However, through several genetic modifications, the *A. niger* production strain used for

this preparation no longer has the ability to synthesize either FUM or OTA. This conclusion is supported by the absence of any FUM or OTA in the preparation at a detection limit of 0.001 mg/kg.

The amyloglucosidase enzyme concentrate used as the test material in the 13-week oral toxicity study was not derived from the production strain that is the subject of this evaluation, but from another production strain that had undergone the same genetic modifications. The Committee considered it to be an acceptable substitution because of the similarity of the lineage of the two organisms, as well as the absence of any significant differences between the two enzyme concentrates with respect to chemical purity (e.g. the presence of heavy metals, OTA, FUM and antimicrobial activity).

The Committee noted that the TOS of the test material used in the 13-week oral toxicity study (batch no. PPY 24900) was greater than the TOS of the test material used in the genotoxicity studies (batch no. PPY 32789) (13.9% and 9.3% TOS, respectively). The sponsor explained that this difference was partly because the test material (batch no. PPY 32789) used to make the commercial product was less concentrated than the test material (batch no. PPY 24900) used in the 13-week oral toxicity study. The enzyme concentrates were manufactured identically.

2.2.1 Acute toxicity

No information was available.

2.2.2 Short-term studies of toxicity

A 13-week oral toxicity study *(18)* in Sprague-Dawley (Crl:CD (SD) BR) rats was conducted according to GLP and OECD guideline no. 408 *(19)*. The test material was prepared as a series of graded concentrations in purified water to provide doses at the constant-volume dosage of 10 mL/kg bw. Every animal in every dose group (10 per sex) received a single dose of the enzyme concentrate of 0, 150, 480 or 1470 mg TOS/kg bw per day by gavage for 13 weeks.

No animals died as a result of the treatment. No treatment-related clinical signs of toxicity were observed in any group. One animal, a male rat receiving 480 mg TOS/kg bw per day, was killed for humane reasons during week 3 of treatment: the animal was observed to have clinical signs of toxicity, including underactive behaviour, body tremors and abnormal gait. The cause of morbidity was identified as meningitis in the brain and spinal cord. This finding was considered incidental and not related to treatment.

Overall, body weight and body weight gains were not significantly different between groups. During the first 2 weeks of treatment, a few groups showed slightly lower body weight gains compared with the control body

weight gains, but these were not dose related and were transient in nature. The observation was considered not toxicologically relevant.

Feed consumption was consistently slightly lower for males receiving the high dose (1470 mg TOS/kg bw per day) than for control animals. The overall difference was slight (6%) and had no effect on the general health of the animals. The difference was considered not toxicologically relevant. Feed efficiency and water consumption were not affected by treatment.

Ophthalmic examination did not reveal any changes that were considered related to treatment.

Sensory reactivity and motor activity were not affected by treatment. Forelimb grip strength values in males in all treated groups were statistically lower than that for the control group (1.43, 1.18, 1.26 and 1.21 kg for control and low-, middle- and high-dose groups, respectively; $P < 0.01$, Student's t-test); however, there was no dose–response relationship and the lower values were all within the historical control range (0.62–1.33 kg). It was noted that the forelimb grip strength of the control group exceeded historical control values. The effect on forelimb grip strength was considered not toxicologically relevant.

Haematology observations showed that the haematocrit of high-dose males (0.450 L/L ± 0.0183) was statistically significantly lower than that for the control group (0.472 L/L ± 0.0127; $P < 0.01$). The difference was considered slight and all values in this high-dose group were within the historical control range (0.416–0.492 L/L). No related parameters were affected. No females were affected; the difference was considered to reflect normal biological variation. Haematology was considered not affected by treatment.

Blood chemistry results found that plasma glucose concentrations in all treated male groups were statistically significantly higher than for the control group (6.48 ± 0.917, 7.32 ± 1.04, 7.52 ± 0.508 and 7.38 ± 0.793 mmol/L for the control, low-, middle- and high-dose groups, respectively; $P < 0.05$). The results were within the historical control range (5.29–9.89 mmol/L). The differences were considered slight, not dose related, within the historical control range of values and not observed in female animals. On this basis, the differences were considered not toxicologically relevant. The creatinine concentrations in all female treated groups were statistically significantly higher than for the control group (39 ± 3.3, 45 ± 3.0, 47 ± 3.6 and 48 ± 6.2 mmol/L for control, low-, middle- and high-dose groups; $P < 0.01$). The results for the female treated groups were very similar to or within the historical control range (47–62 mmol/L). It was noted that the creatinine concentration in the control group was relatively low compared with the treated animals, and low compared with the historical control range. The effect was considered slight, consistent with the historical control range values and not observed in male animals. The effect was considered not toxicologically relevant.

Table 1
Histopathological findings in the adrenal cortex of male rats

Dose (mg TOS/kg bw per day)		0	150	480	1470
No. animals		10	10	9	10
Total incidence of cortical vacuolation		2	2	4	5
Incidence of severity of cortical vacuolation:					
	Minimal	2	2	3	4
	Slight	0	0	1	1
	Moderate	0	0	0	0

TOS: total organic solids.

Table 2
Historical histopathological findings in the adrenal cortex of control male rats from nine studies and a recovery phase from a tenth study[a]

Study no.	1	2	3	4	5	6	7	8	9	10	Historical control totals
No. animals	12	12	10	10	10	10	10	10	9	5	98
Total incidence of cortical vacuolation	2	0	6	4	3	0	0	0	3	2	20
Incidence of severity of cortical vacuolation:											
Minimal	0	0	6	4	3	0	0	0	3	2	18
Slight	2	0	0	0	0	0	0	0	0	0	2
Moderate	0	0	0	0	0	0	0	0	0	0	0

[a] All studies were 13 weeks in duration and conducted at two facilities during 2005–2006. The historical range of the incidence of cortical vacuolation in male rats in the control studies was 0–60%. The range reflects the results of nine 13-week toxicity studies and a recovery phase from a tenth study (five animals).

Organ weights and organ weight relative to body weight values were not significantly different between treated and control groups. Macroscopic pathological findings were common, and none appeared to be related to treatment.

Histopathology examination revealed a dose-related increase in the incidence and severity of cortical vacuolation in the adrenal cortex of middle- and high-dose males when compared with the control group (2/10, 2/10, 4/9 and 5/10 animals for control, low-, middle- and high-dose groups, respectively; Table 1). The incidence of slight severity (the highest level of severity observed) tended to increase with dose (0/10, 0/10, 1/9 and 1/10 for control, low-, middle- and high-dose groups, respectively; Table 1). The incidence was within the historical control incidence range (0–60%; Table 2) and the severity of the lesion was consistent with the historical control (Table 2). The lesion was not associated with

Table 3
Genotoxicity of amyloglucosidase enzyme concentrate

End-point	Test system	Concentration	Results	Reference
In vitro reverse mutation[a]	*Salmonella typhimurium* TA1535, TA100, TA1537 and TA98, and *Escherichia coli* WP2 uvrApKM101	143–4560 mg TOS/mL ± S9[b]	Negative	Pedersen *(20)*
In vitro micronucleus induction	Cultured human peripheral lymphocytes	3-h exposure:[c] 46.5–465 mg TOS/mL ± S9	Negative	Whitwell *(21)*
		24-h exposure:[d] 46.5–465 mg TOS/mL ± S9	Negative	

TOS: total organic solids.
[a] A treat-and-plate assay was conducted (not described in OECD guidance). In this assay, tester strains were incubated with test material for 3 hours at 37 °C. After this period, the tester strains were washed twice by centrifugation, and the pellets were resuspended after each wash in phosphate buffer. After washing, the bacteria pellets were suspended again in buffer. An aliquot of this final suspension was added to top agar, which was then plated onto minimal glucose agar plates. These plates were incubated at 37 °C for 72 hours, after which the colonies were counted. All concentrations of the test substance and the positive control chemicals were assayed three times, and the negative control substance was assayed five times.
[b] S9 was a liver homogenate from Aroclor 1254-treated male rats combined with co-factors that were required for mixed function oxidase activity.
[c] "3-h exposure" refers to 3 hours of exposure of the lymphocytes to the test material, followed by 21 hours to express the potential effect (total treatment time 24 hours). All concentrations of the test substance and the positive control chemicals were assayed twice, and the negative control substance was assayed four times.
[d] "24-h exposure" refers to 24 hours of exposure of the lymphocytes to the test material, followed by 24 hours to express the potential effect (total treatment time 48 hours). All concentrations of the test substance and the positive control chemicals were assayed twice, and the negative control substance was assayed four times.

other histopathological changes such as inflammation or necrosis. The finding was not observed in females. The Committee concluded that the incidence and severity of the vacuoles were consistent with historical control findings and not toxicologically relevant.

In the absence of any adverse effect, a NOAEL of 1500 mg TOS/kg bw per day was identified (rounded by the Committee from 1470 mg TOS/kg bw per day), the highest dose tested.

2.2.3 Genotoxicity

A liquid form of amyloglucosidase enzyme concentrate (batch no. PPY 32789; TOS: 9.3%) was mixed in water and sterilized by filtration to form a standardized test material, and was then tested for genotoxicity in a bacterial reverse mutation assay *(20)* and an in vitro micronucleus induction assay *(21)*. The studies were conducted in compliance with GLP and in accordance with OECD guideline nos 471 *(22)* and 487 *(23)*.

The results of the bacterial reverse mutation and in vitro micronucleus induction assays were negative (Table 3). These results provide evidence that the amyloglucosidase enzyme concentrate is not mutagenic or clastogenic in vitro.

The Committee had no concerns about potential genotoxicity of this amyloglucosidase enzyme concentrate.

2.2.4 Other studies

Friis *(9)* evaluated the amyloglucosidase from *R. emersonii* expressed in *A. niger* for potential toxicity using bioinformatics and searching for a homology in the amino acid sequence of the enzyme compared with sequences of known toxins in the UniProt database *(24)*. The identity percentage to the amyloglucosidase or the toxin sequence was calculated, depending on which was longest. The FASTA search showed the largest homology was about 16%, matching to the CdiA toxin fragment from *Escherichia coli*.[2] The results showed very little homology between the amyloglucosidase and any of the toxic proteins in the database. From these results, the Committee concluded that amyloglucosidase is unlikely to be homologous to any known protein toxin.

2.3 Observations in humans

No information was available.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to the TOS from the amyloglucosidase from *R. emersonii* expressed in *A. niger* enzyme preparation. The enzyme is intended for use in starch processing, alcohol distilling processes, brewing processes, baking processes, and fruit and vegetable processing; these uses were therefore considered for the dietary exposure assessment. The submission included an estimate of dietary exposure based on the budget method, a screening method used to determine the TMDI of food additives *(25,26)*.

The method takes into account maximum physiological levels of consumption of solid food and non-milk beverages, the energy density of foods, the concentration of the food additive in solid foods and non-milk beverages, and the proportion of solid foods and non-milk beverages that may contain it. The method provides a conservative estimate of dietary exposure. Further details of the budget method can be found in EHC 240 *(27)*.

[2] Contact-dependent growth inhibition (Cdi) is a process where a secretion system consisting of a CdiA toxin and a CdiB transporter inhibits the growth of other *E. coli* strains. CdiB is an outer membrane transporter that releases its CdiA toxin to the cell surface.

3.2 Dietary exposure assessment

The estimated TMDI provided by the sponsor was based on a number of inputs to the budget method. The amount of food consumed was assumed to be 0.025 kg/kg bw per day of solid foods and 0.1 L/kg bw per day of non-milk beverages. The proportion of solid food and non-milk beverages containing the amyloglucosidase enzyme preparation was also included; EHC 240 *(27)* cites default proportions of 12.5% for solid foods and 25% for non-milk beverages. Food ingredients processed with this enzyme preparation are proposed to be added to a variety of foods intended to be consumed by the general population. The sponsor therefore assumed that 50% of solid foods and 25% of non-milk beverages are processed and would contain the enzyme preparation. The sponsor also assumed that processed foods contain 25% starch (or starch-derived) dry matter, and processed beverages contain 10% starch hydrolysates. The highest dosage in solid foods was 931 mg TOS/kg starch-based raw material, and for non-milk beverages was 1090 mg TOS/kg starch-based raw material. For the purposes of the dietary exposure assessment, the sponsor used concentrations of TOS derived from the test material for batch no. PPY 24900. As noted in Section 2.2 (toxicological studies), the TOS of 13.9% for batch no. PPY 24900 was greater than that of the concentrate used in the commercial product (9.3% TOS for batch no. PPY 32789). The concentrations used in the dietary exposure assessment therefore represent a worst-case scenario, and the TMDI would be lower than what was calculated.

The sponsor used the standard budget method calculation to estimate the dietary exposure to the TOS from solid foods and non-milk beverages. The resulting TMDIs of the TOS from the amyloglucosidase enzyme preparation were estimated by the sponsor to be 2.90 mg TOS/kg bw per day for solid foods and 2.73 mg TOS/kg bw per day for non-milk beverages, for a total of 5.63 mg TOS/kg bw per day.

The sponsor also considered a special application where the enzyme is used to remove a small amount of starch in fruit and vegetable processing. Using a maximum dosage of 39.9 mg TOS/L fruit juice and an assumption that 25% of non-milk beverages is fruit and vegetable juice, the resulting TMDI is 1.0 mg TOS/kg bw per day. The TMDI for this special application is lower than the TMDI for the non-milk beverage component of the budget method calculation undertaken by the sponsor; the budget method calculation was therefore deemed by the Committee to cover this special application.

The Committee independently calculated dietary exposure to the TOS from the enzyme preparation using the budget method. All inputs and assumptions were the same as for the estimate by the sponsor, with the exception of the consumption amount for solid foods. The Committee assumed a

maximum physiological requirement for solid food (including milk) of 0.05 kg/kg bw per day. This is the level used in a budget method calculation where there is potential for the enzyme to be in baby foods, or in general-purpose foods that may be consumed by infants and young children, and represents a worst-case scenario. The TMDI of the TOS from the amyloglucosidase enzyme preparation was estimated by the Committee as 5.8 mg TOS/kg bw per day for solid foods and 2.7 mg TOS/kg bw (the same as the sponsor) for non-milk beverages. This resulted in a total of 9 mg TOS/kg bw per day (rounded from 8.5 mg/kg bw per day).

The Committee concluded that the dietary exposure estimate of 9 mg TOS/kg bw per day was appropriate for use in the evaluation.

For the purposes of the dietary exposure assessment conducted by both the sponsor and the Committee, it was assumed that the enzyme is not removed and/or inactivated during final processing of ingredients or foods, and that 100% of the TOS from the enzyme preparation remains in the ingredient and final food. In reality, the enzyme is inactivated by high temperatures or removed during the processing of food ingredients, and will have no function in the final food.

4. Comments

4.1 Assessment of potential allergenicity

The amino acid sequence of the amyloglucosidase was compared with the sequences of allergens *(9–11)*, consistent with the recommended criteria *(7)*. The databases each identified a single match to a respiratory allergen, the amyloglucosidase Sch c 1 found in *S. commune* (split-gill mushroom) *(12,13)*. The allergen showed 60–84% identity with amyloglucosidase over a sliding window of 80 amino acids, 47.9% identity over the full length of the protein and 10 matches based on a 100% identity over eight contiguous amino acids. *S. commune* is consumed as food in India, Mexico and other countries, and there are no reports of it being associated with food allergy. There are no reports of food allergy associated with similar amyloglucosidases.

The Committee noted the significant amino acid sequence homology of this amyloglucosidase with the known respiratory allergen Sch c 1, and that the respiratory allergen is associated with occupational asthma in bakers *(28)*. In the absence of digestibility data, the Committee was unable to complete the assessment of the potential for allergenicity from dietary exposure to this amyloglucosidase.

4.2 Toxicological studies

A 13-week oral toxicity study in rats was conducted *(18)*. The test material was an amyloglucosidase concentrate not derived from the production strain that is the subject of this evaluation, but from a previous production strain. The Committee considered it to be relevant to the current evaluation because of the similarity of the lineage of the two organisms, and the absence of any significant differences between the two enzyme concentrates with respect to chemical purity. The enzyme concentrate was administered by gavage at doses up to 1470 mg TOS/kg bw per day. A dose-related increase in the incidence and severity of cortical vacuolation in the adrenal cortex of males was observed compared with control animals. The incidence and severity of the lesions were consistent with the historical control values. Further, it was not associated with other histopathological changes such as inflammation, necrosis or other signs of histopathology, and was confined to one sex. The Committee concluded that this finding was not toxicologically relevant. In the absence of any relevant adverse effect, a NOAEL of 1500 mg TOS/kg bw per day was identified (rounded by the Committee from 1470 mg TOS/kg bw per day), the highest dose tested.

The enzyme concentrate yielded negative results when tested in an in vitro bacterial reverse mutation assay *(20)* and an in vitro micronucleus induction assay *(21)*. The Committee had no concerns about potential genotoxicity of this amyloglucosidase concentrate.

A comparison of the amino acid sequence of the enzyme with those of known protein toxins was conducted *(9,24)*, which revealed no biologically relevant homology. The Committee concluded that the enzyme is unlikely to be a toxin.

4.3 Assessment of dietary exposure

The Committee evaluated two estimates of dietary exposure to the TOS from this amyloglucosidase enzyme preparation, one submitted by the sponsor and the other estimated by the Committee. Both estimates were derived with the budget method, a screening method based on maximum physiological levels of consumption of solid foods and non-milk beverages, and on maximum use levels of the enzyme preparation. Maximum use levels of 931 mg TOS/kg starch-based raw material for solid foods and 1090 mg TOS/kg starch-based raw material for non-milk beverages were used. These TOS values were derived from a batch of the test material with a higher proportion of TOS compared with that of the commercial product; the TMDI would therefore be lower than what has been calculated. It was assumed that 50% of solid foods and 25% of non-milk beverages are processed and would contain the enzyme preparation, and that processed

solid foods contain 25% starch (or starch-derived) dry matter and processed beverages contain 10% starch hydrolysates. Different assumptions were made about the amount of solid foods consumed. The resulting TMDIs from solid food and non-milk beverages were 5.63 mg TOS/kg bw per day by the sponsor and 9 mg TOS/kg bw per day by the Committee. The Committee concluded that the dietary exposure estimate of 9 mg TOS/kg bw per day was appropriate for use in the evaluation. For the dietary exposure assessment, it was assumed that 100% of the TOS from the enzyme preparation remains in the final food. The Committee noted that the enzyme is either removed or inactivated during the processing of food ingredients and will have no function in the final food.

5. Evaluation

The Committee noted that amyloglucosidase may pose a risk as a respiratory allergen. In the absence of any information regarding its stability within the gastrointestinal tract, the Committee could not complete the assessment of the risk for allergenicity from dietary exposure to this amyloglucosidase enzyme preparation.

The Committee identified a NOAEL of 1500 mg TOS/kg bw per day in a 13-week study of oral toxicity in rats. When this NOAEL, the highest dose tested, is compared with the conservative dietary exposure estimate of 9 mg TOS/kg bw per day, a MOE of more than 160 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary ADI "not specified"[3] for amyloglucosidase (JECFA95-4) from *R. emersonii* expressed in *A. niger* when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications and the inability to complete the allergenicity assessment.

5.1 Recommendations

The Committee requested the following information, by the end of 2023, to complete the safety assessment:

- digestibility data in order to complete the allergenicity assessment;

[3] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

- validated method of analysis to determine amyloglucosidase activity, including the validation report;
- unit definition for amyloglucosidase activity based on the method of assay; and
- analytical data using the validated method for at least five different batches of commercially available products.

6. References

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Asparaginase (JECFA95-5) from *Pyrococcus furiosus* expressed in *Bacillus subtilis*

First draft prepared by
Shruti V. Kabadi,[1] Michael DiNovi,[2] Suzanne M.F. Jeurissen,[3] Kristie Laurvick[4] and Imad Toufeili[5]

[1] Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), USA
[2] Baltimore (MD), USA
[3] Department for Food Safety, Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment, Bilthoven, Netherlands (Kingdom of the)
[4] Food Standards, United States Pharmacopeia, Rockville (MD), USA
[5] Department of Nutrition and Food Sciences, American University of Beirut, Beirut, Lebanon

1. Explanation

At the request of the CCFA at its Forty-seventh Session *(1)*, the Committee evaluated the safety of asparaginase (Enzyme Commission No. 3.5.1.1; CAS

No. 9015-68-3) from *Pyrococcus furiosus* expressed in *Bacillus subtilis*. The Committee has not previously evaluated this enzyme preparation. The present Committee allocated the unique JECFA enzyme identifier JECFA95-5 to this enzyme preparation. The Committee had previously evaluated several other asparaginases – for example, an asparaginase from *Aspergillus oryzae* expressed in *A. oryzae* (Annex 1, reference 187) and an asparaginase from *A. niger* expressed in *A. niger* (Annex 1, reference 190) at its Sixty-eighth and Sixty-ninth meetings, respectively – for which an ADI "not specified"[1] was established.

The term "asparaginase" refers to the asparaginase enzyme and its amino acid sequence; the term "enzyme concentrate" refers to the fermentation product containing the enzyme of interest, which is used in the toxicity studies; and the term "enzyme preparation" refers to the product formulated for commercial use.

The Committee previously evaluated several food enzymes from *B. subtilis*, such as an α-amylase (Annex 1, reference 94) and a mixed carbohydrase and protease (Annex 1, reference 26), and established an ADI "not specified" and ADI "not limited"[2], respectively, for these enzyme preparations. On this basis, the present Committee considered that asparaginase (JECFA95-5) from *P. furiosus* expressed in *B. subtilis* met the criteria of a Class 1, Type iii enzyme, as described in EHC 240 *(2)*. A Class 1, Type iii enzyme is produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Although toxicity data and a dietary exposure assessment are not required for Class 1, Type iii enzymes, the Committee evaluated the submitted data.

The enzyme catalyses the hydrolysis of free asparagine to aspartic acid and ammonia. The enzyme preparation is intended for use as a processing aid in baking and the production of breakfast cereal dry materials, in potato processing, and in coffee and cocoa processing.

The Committee conducted a literature search in PubMed (all fields) with the linked search terms "Asparaginase" AND "*Pyrococcus furiosus*", and "Asparaginase" AND "*Bacillus subtilis*", which identified 10 and 35 references, respectively. None of the identified references was relevant to the toxicological evaluation of this enzyme preparation.

1.1 Genetic background

The production organism, *B. subtilis*, belongs to the genus *Bacillus* and family *Bacillaceae*. *B. subtilis* is a soil- and plant-living saprophyte that is recognized as a non-pathogenic and non-toxigenic species for humans, animals and plants *(3,4)*.

[1] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

[2] The expression ADI "not limited" is no longer used by JECFA and has been replaced by ADI "not specified".

B. subtilis species have been used for years as recombinant organisms for the manufacture of a variety of bio-industrial products such as food-grade enzymes, vitamins, antibiotics and additives, and in the production of natto by solid-state fermentation of soybeans *(5)*. Unlike the toxicogenic strain *B. cereus*, the parent strain of *B. subtilis* is not expected to elicit a toxic response upon oral exposure *(6)*.

The recipient strain *B. subtilis* PP2982 was constructed from the parental strain A164 (deposited as *B. subtilis* ATCC 6051a) through a series of gene-modification steps. The modification steps introduce deletion genes encoding several proteases. Genes essential to sporulation and formation of surfactin were also deleted to improve product safety and stability *(7)*, and to facilitate the isolation of asparaginase. The modification also included the insertion of a hybrid *Bacillus* promoter, comprising promoter regions from *B. licheniformis, B. amyloliquefaciens* and *B. thuringiensis*, and an integration fragment that allows site-specific integration on the genome of the recipient strain. The resulting recipient strain is therefore non-sporulating, protease-deficient and surfactin-negative *(7,8)*.

The production strain was obtained by transforming the recipient *B. subtilis* PP2982 strain with the plasmid containing the asparaginase encoding gene from *P. furiosus* ATCC43587. The pMOL2930 plasmid included the structural gene of asparaginase from *P. furiosus* ATCC43587, a terminator sequence T_{amyL} from *B. licheniformis*, and an integration fragment that enables site-specific integration on the genome of the recipient strain. Only the elements between the promoter fragment and the integration region of the pMOL2930 plasmid were inserted at three specific loci in the recipient strain. The marker was removed during the integration of the expression cassette into the recipient strain; the production strain does not maintain any genetic elements conferring resistance to antibiotics. The absence of the genes encoding antibiotic resistance was verified by Southern blot analysis using relevant gene probes. The presence of the expected genes encoding for asparaginase at the three loci in the production strain was confirmed by polymerase chain reaction. The stability of the introduced genes was confirmed by comparing the band pattern of the production strain with that of strains from replicate production batches by Southern blot analysis *(7,8)*.

1.2 Chemical and technical considerations

Asparaginase is produced by controlled fermentation of a pure culture of a genetically modified strain of *B. subtilis*. The manufacture of the asparaginase enzyme preparation includes fermentation (seed, pre- and main culture), recovery and formulation. After fermentation, the broth containing the asparaginase

enzyme is separated from the biomass; this is followed by several filtration steps and concentration. The resulting concentrate is stabilized by adding sodium chloride and adjusting the pH for optimal storage. The liquid concentrate is formulated by adding sorbitol, water, sodium chloride, sodium benzoate and potassium sorbate to the final liquid asparaginase enzyme preparation. A powdered enzyme preparation is produced by spray-drying the liquid concentrate, followed by standardization with corn flour, sodium chloride and dextrin. The entire process is performed according to GMP and using food-grade raw materials.

The asparaginase enzyme produced by the *B. subtilis* production strain is a dimer, and each monomer consists of 326 amino acids; by calculation from the determined amino acid sequence, the molecular weight of the asparaginase monomer is 35.8 kDa *(8)*. The enzyme concentrate is tested to be free from the production organism and any antibiotic activity, and does not exhibit α-amylase, glucoamylase, lipase or protease side activities. Asparaginase has an optimum temperature of more than 90 °C and an optimum pH of 9 (at 70 °C); the enzyme is active at temperatures up to at least 90 °C and within a pH range of 4–9.

The activity of asparaginase is determined spectrophotometrically by condensing the ammonia produced by the enzymatic hydrolysis of asparagine with α-ketoglutarate and measuring the amount of nicotinamide adenine dinucleotide (NADH, H^+) utilized spectrophotometrically at 340 nm. The asparaginase activity is expressed in thermostable asparaginase units (TASU) per gram relative to an asparaginase standard. The mean activity of asparaginase from three batches of the enzyme concentrate was 54 267 TASU/g.

Asparaginase catalyses the hydrolysis of free asparagine to aspartic acid and ammonia by hydrolysing the amide in free asparagine. Asparaginase enzyme preparations are intended for use as processing aids in baking and the production of breakfast cereal dry materials, in potato processing, and in coffee and cocoa processing. The enzyme preparation is added to reduce the levels of asparagine that lead to the generation of acrylamide in foods that undergo Maillard browning.

The asparaginase enzyme preparations are standardized to an activity of 6000 TASU/g, and are recommended for use at a maximum of 15 000 TASU/kg of final food. The commercial enzyme preparations are supplied as a light-brown liquid or light-yellow powder at approximately 0.8% TOS. The TOS includes the enzyme of interest and residues of organic materials (e.g. proteins, peptides and carbohydrates) derived from the production organism during the manufacturing process.

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

2. Biological data

2.1 Assessment of potential allergenicity

The Committee evaluated the potential for allergenicity of the enzyme preparation based on the recommended bioinformatics criteria *(9,10)* and as outlined in EHC 240 *(2)*. A homology search was conducted in which the amino acid sequence of asparaginase from *P. furiosus* expressed in *B. subtilis* was compared with the amino acid sequences of known allergens using the AllergenOnline *(11)* and Allergen *(12)* databases. A search for matches with > 35% identity in a sliding window of 80 amino acids, a search for exact matches over contiguous stretches of eight amino acids, and a full-length FASTA sequence with an E-value of < 0.1 did not identify a homology to any known allergens. Comparisons between highly homologous proteins yield E-values approaching zero, indicating very low probability that such matches would occur by chance (larger E-values indicate a lower degree of similarity). No data on the digestibility of the enzyme preparation in the gastrointestinal tract were available. Based on the results of the bioinformatic analysis, the Committee concluded that the dietary exposure to the enzyme preparation is not anticipated to pose a risk of allergenicity.

2.2 Toxicological studies

The test material in the evaluated studies was a brown liquid enzyme concentrate (batch no. PPV33595) with a declared dry matter content of 13.1% w/w, a TOS content of 11.4% w/w and an activity of 55 200 TASU/g.

2.2.1 Acute toxicity

No data on the acute toxicity of the enzyme preparation were available.

2.2.2 Short-term studies of toxicity

The Committee evaluated a 13-week oral toxicity study *(13)* in which 40 male and 40 female Sprague-Dawley SPF Ntac:SD rats (10 rats per treatment group per sex) from Taconic Europe A/S (Denmark) were given the liquid enzyme concentrate by gavage at dose levels of 0, 0.121, 0.398 or 1.207 g TOS/kg bw per day, corresponding to 0, 58 457, 192 907 and 584 568 TASU/kg bw per day, respectively. The study was certified for compliance with GLP regulations and the study report included a Quality Assurance (QA) statement. The study was conducted in accordance with OECD guideline no. 408 *(14)*. The vehicle used for preparation of test samples was tap water and the dose volume was 10 mL/kg.

No mortality was reported. No treatment-related effects in terms of clinical signs, body weights, feed and water consumption, or ophthalmoscopic observations were reported. No treatment-related effects in terms of the parameters assessed to evaluate motor activity in the open field test and reactivity to different stimuli and grip strength were reported.

The mean haemotocrit level (± SD) was statistically significantly lower ($P < 0.05$) in male rats of the middle-dose group (40.2 ± 3.8 mL/100 mL) than for the control group (43.8 ± 2.1 mL/100 mL). However, this difference was because of a very low level for one animal that was concluded to be an incidental finding and not treatment related. The mean prothrombin time (± SD) of male rats of the high-dose group (16.8 ± 0.8 s) was statistically significantly higher ($P < 0.01$) than for the control group (15.6 ± 0.6 s). Because no statistically significant changes in the mean prothrombin time were observed in females, this was concluded to be an incidental finding that was not treatment related. The mean percentage basophils (± SD) in female rats of the high-dose group (0.0 ± 0.0) was statistically significantly lower ($P < 0.01$) than for the control group (0.6 ± 0.5). However, the value was within the historical control range and no such changes in other leucocyte counts were observed. This observation was therefore considered not to be toxicologically relevant.

An assessment of clinical chemistry indicated that male rats of the middle- and high-dose groups had statistically significantly higher mean albumin/globulin ratios (1.88 ± 0.11 and 1.89 ± 0.09, respectively; $P < 0.05$) than the control group (1.74 ± 0.14). However, no statistically significant changes in albumin or globulin levels were reported in any of the treated groups in males and females compared with the control groups; this observation was therefore considered not to be toxicologically relevant. The mean cholesterol level in female rats of the high-dose group (2.51 ± 0.25 mmol/L) was statistically significantly lower ($P < 0.05$) than for the control group (2.87 ± 0.34 mmol/L). A similar, albeit not statistically significant, decrease in cholesterol levels was observed in female rats of the low- and middle-dose groups, as well as in male rats of the middle- and high-dose groups, compared with the control groups. Even though the decrease in cholesterol levels in both sexes was dose dependent, and possibly related to treatment, the observed changes were within the historical control range; this finding was therefore considered not to be toxicologically relevant.

An assessment of absolute and relative organ weights indicated that the mean relative liver weight (percentage of the body weight ± SD) of male rats of the middle-dose group (885.3 ± 69.4) was statistically significantly higher ($P < 0.05$) than for the control group (840.4 ± 148.0). However, this effect was not reported in male rats of the high-dose group or observed in any of the treated groups of female rats; this observation was therefore considered to be an incidental finding that was not treatment related. The absolute and relative weights (percentage of

the body weight and of the brain weight) of the prostrate were reported to be lower in male rats of the low-dose group than in the control group. However, these changes were not dose dependent as they were not observed in high-dose male rats, and were therefore considered not to be toxicologically relevant.

Some incidental findings were observed upon a macroscopic and microscopic examination of selected organs and tissues. However, these findings were not consistently reported across all rats within any particular group. These findings were also within the background incidence of such observations associated with the age and strain of the rats, and were therefore considered not to be toxicologically relevant.

Based on the absence of any adverse effects, the Committee identified a NOAEL of 1.207 g TOS/kg bw per day, the highest dose tested.

2.2.3 Genotoxicity

The Committee evaluated two in vitro genotoxicity studies with the liquid enzyme concentrate (batch no. PPV33595): an Ames assay *(15)* and a mammalian cell micronucleus assay *(16)*. Both studies were certified for compliance with GLP, and the study reports included QA statements. The Ames assay was conducted in accordance with OECD guideline no. 471 *(17)*, except for the use of the treat-and-plate method. The in vitro micronucleus assay was conducted in accordance with OECD guideline no. 487 *(18)*. A standard solution of 5% w/v dry matter was prepared using deionized water and sterilized by filtration for the Ames assay. The test substance preparations were added to the cultures at a concentration of 10% v/v for the in vitro micronucleus assay. The results of both studies were negative, providing evidence that the enzyme concentrate is not mutagenic or clastogenic in vitro. The Committee therefore had no concerns about potential genotoxicity of the enzyme concentrate.

The results of the two genotoxicity studies performed on the enzyme concentrate are summarized in Table 1 below.

2.2.4 Other studies

The enzyme preparation was evaluated for potential toxicity by a homology search in which the amino acid sequence of the enzyme was compared with known toxins in the UniProt database *(19)*. No biologically relevant homology (~ 17%) of the enzyme with the known toxins in the database was identified. The Committee concluded that the enzyme is unlikely to be a toxin.

2.3 Observations in humans

No information was available.

Table 1
In vitro genotoxicity studies of the asparaginase liquid enzyme concentrate

End-point	Test system	Concentration	Results	Reference
Ames (bacterial reverse mutation) assay	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2uvrApKM101	133, 272, 544, 1088, 2175 and 4350 µg TOS/mL ± S9[a]	Negative; no toxicity of the test substance was observed; growth stimulation in terms of increases in viable counts was observed in some test samples with the *E. coli* strain compared with the solvent control, but there was no correlation with the revertant colony counts	Pendersen *(15)*
In vitro mammalian cell micronucleus assay	Primary cultured human peripheral lymphocytes	193, 374, 760, 1509 and 3018 µg TOS/mL ± S9 for a 3-hour treatment[b] and − S9 for a 20-hour treatment[c]	Negative; no toxicity of the test preparation was observed	May *(16)*

[a] Treat-and-plate method, selective incubation, followed by viable counting.
[b] Test preparations added to cultures at 10% v/v and cultures incubated at 37 °C for 3 hours followed by centrifugation of cells, replacement of medium with fresh medium, and addition of cytochalasin B at a final concentration of 6 µg/mL; cultures incubated for additional 17 hours until scheduled harvest time.
[c] Test preparations added to cultures at 10% v/v in the presence of cytochalasin B at a final concentration of 6 µg/mL, and cultures subjected to 20 hours of continuous treatment in the absence of S9 mix.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to asparaginase from *P. furiosus* expressed in *B. subtilis*. The enzyme is intended for use in various food manufacturing processes including, but not limited to, baking and other cereal-based processes, potato processing, and coffee and cocoa processing. The sponsor prepared an estimate of dietary exposure based on the budget method, a screening method used to determine the TMDI of food additives. The method takes into account a theoretical maximum physiological level of consumption for solid food and non-milk beverages based on the energy density of the food, the concentration of the food additive in foods and non-milk beverages, and the proportion of foods and non-milk beverages that may contain it. The method provides a screening estimate of dietary exposure (Tier 1). Further details of the budget method can be found in EHC 240 *(20)*.

3.2 Dietary exposure assessment

The estimated TMDI derived using the budget method is based on the inputs outlined above. The first input is the amount of food consumed, which is assumed to be 25 g/kg bw per day for solid foods and 100 mL/kg bw per day for non-milk beverages. The second input is the proportion of food and non-milk beverages

containing the enzyme preparation. Foods processed with this asparaginase enzyme preparation are proposed to be added to a variety of foods intended to be consumed by the general population; for the budget method calculation, the sponsor therefore made the assumptions that 50% of solid food and 25% of non-milk beverages are processed.

The maximum level of the enzyme present in the final solid foods was reported to be 15 000 TASU/kg food, which equates to 27.8 mg TOS/kg food. For non-milk beverages, the maximum use level was taken to be 12 500 TASU/kg coffee or cocoa beans. To account for production of the final beverage from the beans, it was also assumed that 1000 mL coffee or cocoa is made from 60 g of roasted (and milled) beans, and that 60 g of roasted beans is dosed with 12 500 × 60/1000 = 750 TASU. This corresponds to a maximum TOS of 1.39 mg/L in the coffee beverages.

The standard budget method calculation was used to estimate dietary exposure to the TOS from solid foods and non-milk beverages. The resulting TMDIs of asparaginase from *P. furiosus* expressed in *B. subtilis* were estimated by the sponsor to be 0.348 mg TOS/kg bw per day for solid foods and 0.035 mg TOS/kg bw per day for non-milk beverages, for a total of 0.383 mg TOS/kg bw per day or 0.4 mg TOS/kg bw per day after rounding.

The sponsor noted that young children could consume biscuits including ingredients that were prepared using the asparaginase enzyme preparation. A budget estimate for children was therefore conducted making the following assumptions. The energy requirement of young children between 12 and 36 months of age was estimated to be 400 kJ/kg bw per day, equivalent to 96 kcal/kg bw per day. The available energy content in biscuits was estimated to be between 440 and 540 kcal/100 g, depending upon type of biscuit considered. A maximum of 25% of the energy requirements of young children would be covered by the intake of biscuits, and the energy content of a biscuit is 500 kcal/100 g. The maximum intake of biscuits would be 24 kcal/kg bw per day, equivalent to 4.8 g/kg bw per day of biscuit. The TMDI of the food enzyme by young children via biscuits would therefore be 27.8 mg TOS per kg/1000 g per kg × 4.8 g = 0.133 mg TOS/kg bw per day. Noting that this estimate is lower than that calculated above, the sponsor concluded that the larger estimate covers children in the safety assessment.

The Committee concluded that the estimate of dietary exposure from the overall budget method, 0.4 mg TOS/kg bw per day, is appropriate for use in the safety assessment.

4. Comments

4.1 Assessment of potential allergenicity

The Committee evaluated the potential for allergenicity of the enzyme preparation based on the recommended bioinformatics criteria *(9,10)* and as outlined in EHC 240 *(2)*. The amino acid sequence of the enzyme was compared with those of known allergens in two online databases *(11,12)*. A search for matches with more than 35% identity in a sliding window of 80 amino acids, a search for exact matches over contiguous stretches of eight amino acids and a full-length FASTA sequence search did not identify homology to any known allergens. No data on the digestibility of the enzyme preparation were available. Based on the results of the bioinformatics analysis, the Committee concluded that dietary exposure to the enzyme is not anticipated to pose a risk for allergenicity.

4.2 Toxicological studies

In a 13-week study of oral toxicity in rats *(13)*, the enzyme concentrate was mixed in water and administered by gavage at dose levels of up to 1207 mg TOS/kg bw per day. The only effect that was dose and possibly treatment related was a decrease in mean cholesterol levels in both sexes, but the observed changes were within the historical control range. The Committee therefore concluded that this finding was not toxicologically relevant. Based on the absence of any adverse effects, the Committee identified a NOAEL of 1207 mg TOS/kg bw per day, the highest dose tested.

The enzyme concentrate was negative in a bacterial reverse mutation test *(15)* and in an in vitro mammalian cell micronucleus assay *(16)*. The Committee therefore had no concerns about potential genotoxicity of the enzyme concentrate.

A comparison of the amino acid sequence of the enzyme with those of known protein toxins *(19)* revealed no biologically relevant homology (~ 17%). The Committee therefore concluded that the enzyme was unlikely to be a toxin.

4.3 Assessment of dietary exposure

The Committee considered one estimate of dietary exposure to TOS from this asparaginase enzyme preparation, which was submitted by the sponsor. The standard budget method calculation, a screening method based on maximum physiological levels of consumption of solid foods and non-milk beverages, and on

maximum use levels of the enzyme preparation, was used. The resulting TMDIs of TOS from the enzyme preparation were estimated by the sponsor to be 0.348 mg TOS/kg bw per day for solid foods and 0.035 mg TOS/kg bw per day for non-milk beverages, for a total of 0.383 mg TOS/kg bw per day (or 0.4 mg TOS/kg bw per day after rounding). The sponsor noted that young children could consume biscuits including ingredients that were prepared using the asparaginase enzyme preparation, and conducted an estimate for children using the budget method. The TMDI of the TOS from the enzyme preparation for biscuits consumed by young children was estimated to be 0.133 mg TOS/kg bw per day. The Committee concluded that the higher estimate of dietary exposure from the overall budget method, 0.4 mg TOS/kg bw per day, was appropriate for use in the evaluation. For the dietary exposure assessment, it was assumed that 100% of the TOS from the enzyme preparation remains in the final food. The Committee noted that the enzyme is inactivated or removed during the processing of food ingredients, and will have no function in the final food.

5. Evaluation

The Committee concluded that dietary exposure to this asparaginase enzyme preparation is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 1207 mg TOS/kg bw per day, the highest dose tested, in a 13-week study of oral toxicity in rats. When this NOAEL is compared with the dietary exposure estimate of 0.4 mg TOS/kg bw per day, a MOE of more than 3000 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary ADI "not specified"[3] for asparaginase (JECFA95-5) from *P. furiosus* expressed in *B. subtilis* when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications.

5.1 Recommendations

The Committee requested the following information, by the end of 2023, to complete the safety assessment:

- validated method of analysis to determine asparaginase activity, including the validation report;

[3] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

- unit definition for asparaginase activity based on the method of assay; and
- analytical data using the validated method for at least five different batches of commercially available products.

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β-Amylase (JECFA95-6) from *Bacillus flexus* expressed in *Bacillus licheniformis*

First draft prepared by
Shruti V. Kabadi,[1] Polly E. Boon,[2] Suzanne M.F. Jeurissen,[2] Atsuko Tada[3] and Stefan G. Walch[4]

[1] Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), USA
[2] Department for Food Safety, Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment, Bilthoven, Netherlands (Kingdom of the)
[3] Division of Food Additives, National Institute of Health Sciences, Kanagawa, Japan
[4] Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, Karlsruhe, Baden-Württemberg, Germany

1. Explanation

At the request of the CCFA at its Forty-eighth Session *(1)*, the Committee evaluated the safety of β-amylase (Enzyme Commission No. 3.2.1.2; CAS No. 9000-91-3) from *Bacillus flexus* expressed in *B. licheniformis*. The Committee

has not previously evaluated any β-amylase enzyme preparation. The present Committee allocated the unique JECFA enzyme identifier JECFA95-6 to this enzyme preparation.

The term "β-amylase" refers to the β-amylase enzyme and its amino acid sequence; the term "enzyme concentrate" refers to the fermentation product containing the enzyme of interest, which is used in the toxicity studies; and the term "enzyme preparation" refers to the product formulated for commercial use.

The Committee previously evaluated several food enzymes from *B. licheniformis* – for example, a carbohydrase (α-amylase) (Annex 1, reference 70) and an α-amylase containing a genetically engineered α-amylase gene from *B. licheniformis* (Annex 1, reference 166) at its Twenty-ninth and Sixty-first meetings, respectively – and established an ADI "not specified"[1] for these enzyme preparations. On this basis, the present Committee considered that β-amylase (JECFA95-6) from *B. flexus* expressed in *B. licheniformis* met the criteria of a Class 1, Type iii enzyme, as described in EHC 240 *(2)*. A Class 1, Type iii enzyme is produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Although toxicity data and a dietary exposure assessment are not required for Class 1, Type iii enzymes, the Committee evaluated the submitted information.

The enzyme catalyses the hydrolysis of the (1→4)-α-D-glucosidic linkages in polysaccharides. The enzyme preparation is intended for use as a processing aid, typically for starch processing in the manufacture of food products, such as syrups, containing maltose.

The Committee conducted a literature search in PubMed (all fields) with the linked search terms "beta-amylase" AND "Bacillus flexus", and "beta-amylase" AND "*Bacillus licheniformis*", which identified 3 and 52 references, respectively. No additional toxicity data were identified.

1.1 Genetic background

The production organism *B. licheniformis* belongs to the genus *Bacillus* and family *Bacillaceae*. *B. licheniformis* is a soil- and plant-living saprophyte that is recognized as a non-pathogenic species for humans, animals and plants *(3)*. *B. licheniformis* species have been used for years as recombinant organisms for the production of a variety of bio-industrial products such as food-grade enzymes, vitamins, antibiotics and additives *(4)*. Unlike the toxicogenic strain *B. cereus*, the parent strain of *B. licheniformis* is not expected to elicit a toxic response upon oral exposure *(5)*.

[1] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

The β-amylase production strain NZYM-JA *(6,7)* is developed from the *B. licheniformis* recipient strain. During development, the recipient strain is modified at several chromosomal loci to inactivate genes encoding a number of proteases and a major α-amylase. A gene essential for sporulation is deleted, eliminating the ability to sporulate. Additional genes encoding unwanted proteins that can be present in the culture supernatant are also deleted, to improve product purity, safety and stability. The *bmyFzyn2* expression cassette, encoding the β-amylase gene from a *B. flexus* donor, is then inserted at selected loci using a plasmid *(6)*. Southern blot analysis is performed to confirm the number and position of *bmyFzyn2* copies inserted in the production strain. Plasmids containing genes of concern are subsequently removed from the strain, demonstrated by genome sequence analysis and a loss of antibiotic resistance. The production strain is deposited in DSMZ, Germany.

1.2 Chemical and technical considerations

β-Amylase is produced by pure culture fermentation of the *B. licheniformis* production strain (NZYM-JA) *(6,7)*. Manufacture of the β-amylase enzyme preparation includes fermentation processes (inoculum, seed and main fermentation), recovery and formulation. After fermentation, the broth containing the β-amylase enzyme is separated from the biomass, then concentrated by multiple filtration steps. The resulting concentrate is then formulated as a liquid preparation. For example, a liquid food enzyme preparation is formulated with sorbitol, sodium benzoate, potassium sorbate, etc. The entire process is performed in accordance with current GMP and with food-grade raw materials. The enzyme concentrate is tested to ensure that it is free from the production organism and any antibiotic activity. The primary sequence of β-amylase produced by the *B. licheniformis* strain consists of 515 amino acids; its molecular weight by calculation from the determined amino acid sequence is 57.6 kDa *(7)*. The β-amylase has no secondary activity.

β-Amylase activity is determined spectrophotometrically by measuring the hydrolysis of (1→4)-α-D-glucan substrate (maltohexaose) at 540 nm, compared with a proprietary enzyme standard with activity expressed in β-amylase units (BAMU); 1 BAMU is defined as the amount of enzyme required to hydrolyse 1 μmol maltohexaose per minute under conditions of the assay (pH 5.5, T = 37 °C). In test samples, β-amylase acts on the non-reducing end of maltohexaose (G6) to form maltose (G2) and maltotetraose (G4). The G4 produced has a stronger reaction than G6 in the presence of lactose-oxidase and O_2 to form H_2O_2. In the presence of peroxidase, the H_2O_2 activates the oxidative condensation of 4-aminoantipyrine and *N*-ethyl-*N*-sulfopropyl-*m*-toluidin to

form a purple product, which can be quantified by its absorbance at 540 nm. The β-amylase enzyme is active at temperatures of up to approximately 70 °C (with an optimum of 30–65 °C at pH 5) and within a pH range of 3–10 (with an optimum of pH 4–9.5 at 60 °C).

β-Amylase catalyses the hydrolysis of the (1→4)-α-D-glucosidic linkages in polysaccharides (e.g. amylose and amylopectin) to produce maltose and the remaining polymer from the non-reducing ends of the chain. The enzyme preparation is intended for use as a processing aid in a variety of food applications, typically for starch processing in the production of syrups containing maltose. The first step in starch conversion is liquefaction, when the starch is gelatinized and hydrolysed by α-amylases into maltodextrins. The β-amylase enzyme preparation is then added to hydrolyse maltodextrins in the saccharification step. The mean TOS of three representative food enzyme concentrate batches was 10.9% (11 000 BAMU/g, 100.9 BAMU/mg TOS), and TOS of the toxicology test batch (batch no. PPY36295) was 11.5% (9544 BAMU/g, 83.0 BAMU/mg TOS). The recommended dosage for the food enzyme in starch processing is up to 10 000 BAMU/kg of starch-based raw material (99.1 mg TOS/kg). The TOS includes the enzyme of interest and residues of organic materials (e.g. proteins, peptides and carbohydrates) from the production organism during manufacture.

The β-amylase enzyme is inactivated and removed during processing, when the syrup is purified by filtration, carbon treatment and ion exchange. It is not expected to have any technological function in the final food.

2. Biological data

2.1 Assessment of potential allergenicity

The Committee evaluated the potential for allergenicity of β-amylase from *B. flexus* expressed in *B. licheniformis* using a weight-of-evidence approach including the recommended bioinformatics criteria *(8,9)* and as outlined in EHC 240 *(2)*.

A homology search was conducted in which the amino acid sequence of β-amylase from *B. flexus* expressed in *B. licheniformis* was compared with the amino acid sequences of known allergens using the AllergenOnline *(10)* and Allergen *(11)* databases. A search for matches with > 35% identity in a sliding window of 80 amino acids with a full-length FASTA sequence search with an E-value of < 0.1 identified homology to one known allergen, *Triticum aestivum* (Tri a 17.0101). Comparisons between highly homologous proteins yield E-values approaching zero, indicating very low probability that such matches would occur by chance (larger E-values indicate a lower degree of similarity). Tri a 17 and

β-amylase showed a highest identity of 42.47%, with an identity of 31.4% across the full alignment. In contrast, the sponsor stated that Tri a 17 and β-amylase from *B. flexus* expressed in *B. licheniformis* showed a highest identity of 44.7%, with an identity of 25.7% across the full alignment. The differences in the predictions of the bioinformatic analysis conducted by the Committee and the sponsor are the result of the sponsor using an older version (version 19) of the AllergenOnline database. A search for exact matches over contiguous stretches of eight amino acids generated two hits, which also indicated homology to Tri a 17.

T. aestivum (Tri a 17) has been classified as a food allergen by WHO and the IUIS (Allergen database), based on the results of a study described in a recently published article *(12)*. This article reported that wheat β-amylase with Tri a 17 bound to immunoglobulin E (IgE) of patients with a recognized wheat allergy. In this study, IgE reactivity of three forms of Tri a 17 (Tri a 17_clone, Tri a 17_inactive and Tri a 17_active) was assessed using non-denaturing radioallergosorbent test (RAST)-based IgE dot blot analysis with sera from 17 patients with a wheat food allergy. Tri a 17_clone and Tri a 17_inactive were recognized by 24% (4 out of 17) of the wheat-food-allergic patients with varying intensities, whereas Tri a 17_active was recognized by 41% (7 out of 17) of wheat-food-allergic patients, indicating the presence of conformational IgE epitopes and linear epitopes. The reported IgE reactivity was specific to patients with a known wheat food allergy and was not observed in non-allergic individuals, grass-pollen-allergic patients or baker's asthma patients. A logistic regression analysis to assess the relative risk of developing wheat-induced anaphylaxis indicated a 24-fold higher probability for β-amylase-reactive patients. Further, FcεRI-expressing rat basophilic leukaemia (RBL) cells were sensitized with sera from two wheat-food-allergic patients that showed IgE reactivity to Tri a 17. The cells were incubated with increasing concentrations of Tri a 17_active that showed that Tri a 17 induced degranulation of humanized RBL cells and released hexosaminidase for both wheat-food-allergic patients tested.

In the same study *(12)*, the relative enzymatic activity of β-amylase was evaluated with respect to pH and temperature. The enzyme was highly reactive in the acidic pH range of 4–7 with maximum activity reported at pH 5. However, as the pH increased further, the relative enzymatic activity decreased such that 50% of the enzymatic activity was lost at pH 8. An assessment of the melting temperature of Tri a 17 at different pH values showed that the protein was most stable in slightly acidic conditions, with the highest melting temperature of 59 °C observed at pH 5.5, with a reduction to lower values as the pH increased and a sharp drop in stability at pH 4. The study concluded that wheat β-amylase with Tri a 17 is associated with severe allergic reactions upon wheat ingestion by sensitized people with a known wheat allergy.

The sponsor submitted a report of an in vitro digestibility study performed with β-amylase from *B. flexus* expressed in *B. licheniformis* (bmyFzyn2; product formulation Secura; batch LZN200005) *(13)*. In this study, the enzyme sample was added to SGF preheated to 37 °C and containing pepsin from porcine gastric mucosa (such that the resulting pH was ~ 1.15), and then incubated at the same temperature. After 2, 30 and 180 minutes, samples were qualitatively analysed using SDS-PAGE with Coomassie brilliant blue (CBB) staining. The study showed that all proteins of the enzyme preparation were digested by the earliest time point of 2 minutes, indicating that the enzyme has the potential to be readily digested in the acidic conditions of the stomach. The Committee also noted that the enzyme preparation would be removed or inactivated during the manufacturing process.

Although the Committee acknowledged that the enzyme is homologous to the known food allergen Tri a 17, when taking into account the results of the digestibility study, the Committee concluded that dietary exposure to the enzyme is not anticipated to pose a risk for allergenicity.

2.2 Toxicological studies

The test material in the evaluated studies was a brownish liquid enzyme concentration (batch no. PPY36295) with a declared dry matter content of 12.7% w/w, a TOS content of 11.5% w/w and an activity of 9544 BAMU/g.

2.2.1 Acute toxicity

No data on acute toxicity were available.

2.2.2 Short-term studies of toxicity

An 13-week oral toxicity study *(14)* was conducted in which groups of 40 male and 40 female Sprague-Dawley Crl:CD(SD) rats (10 rats per treatment group per sex) received the enzyme concentrate by gavage at doses of 0, 0.120, 0.396 or 1.199 g TOS/kg bw per day, corresponding to 0, 9954, 32 849 and 99 544 BAMU/kg bw per day, respectively. The study was certified for compliance with GLP and QA, and was conducted in accordance with OECD guideline no. 408 *(15)*. The vehicle used for preparation of test samples was reverse-osmosis water, and the dose volume of administration was 10 mL/kg bw.

No mortality or effects on the general appearance or behaviour of tested animals were reported. Some effects on body weights and on consumption of feed and water were observed; however, these effects were not dose dependent or consistently reported across both sexes, and were therefore not considered to be treatment related or toxicologically relevant. No treatment-related effects on

sensory reactivity, grip strength or motor activity were reported. The group mean forelimb grip strength (± standard deviation) for female rats in the middle-dose (1 ± 0.11 kg) and high-dose (0.91 ± 0.11 kg) groups was statistically significantly higher ($P < 0.05$) than for controls (0.84 ± 0.1 kg); however, all group mean scores were within the historical control data range, and no such observations were reported in males. The effects on grip strength in females were therefore not considered to be toxicologically relevant. No treatment-related effects on motor scores and ophthalmoscopic observations were reported.

A haematological examination of the collected blood samples indicated changes in some parameters, but none of these observations was concluded to be treatment related or toxicologically relevant. There was a small decrease in mean haemoglobin and haematocrit levels at all doses in males; however, these changes were not dose related or observed in females. Decreases in mean erythrocyte and mean reticulocyte counts were also observed in males and in females, respectively, in the high-dose groups; however, these observed changes were within the historical control range, and therefore not considered to be toxicologically relevant. A decrease in mean platelet counts was observed for all doses in females. However, since no changes in clotting times (prothrombin and activated partial thromboplastin times) were reported, this was not considered to be toxicologically relevant. A reduction in mean eosinophil counts was observed for all doses in females. Since these changes were not associated with a dose–response relationship and all individual values were within the historical control range, these effects were not considered to be toxicologically relevant.

An assessment of blood chemistry revealed some statistically significant differences between treatment and control groups. However, these changes were not dose dependent, were confined to one sex and/or individual values were within the historical control range. These changes were therefore not considered to be toxicologically relevant.

An assessment of organ weights indicated that all differences between the treatment and control groups were not dose dependent and were confined to one sex. Any changes in organ weights were therefore not considered to be treatment related or toxicologically relevant.

Any reported findings of a macroscopic as well as microscopic examination of selected organs were observed to be minor and consistent with the common spontaneous background changes observed within the particular species and strain of rats. These findings were therefore not considered to be treatment related or toxicologically relevant.

Based on the absence of adverse effects, the Committee identified a NOAEL of 1.199 g TOS/kg bw per day, the highest dose tested.

Table 1
In vitro genotoxicity studies on β-amylase liquid enzyme concentrate

End-point	Test system	Concentration	Results	Reference
Ames (bacterial reverse mutation) assay	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2uvrApKM101	156, 313, 625, 1250, 2500, 5000 µg TOS/mL ± metabolic activation (±S9)[a]	Negative; slight toxicity at higher dose levels in some test strains – particularly TA100 (−S9) in both experiments, and TA1537 (+S9) in experiment 1 and TA1537 (+S9) in experiment 2 – was observed, but not consistently across all tested dose levels, strains and conditions; growth stimulation in terms of increases in viable counts was observed in some test strains compared with the solvent control, but there was no correlation with the revertant colony counts	Lund *(16)*
In vitro human lymphocyte micronucleus assay	Human lymphocytes in culture of whole blood drawn from two healthy non-smoking female donors	0.1814–50 mg TOS/mL with final concentrations of 18.14–5000 µg TOS/mL for 3+21-hour (±S9) and 24+24-hour (−S9) treatments in the cytotoxicity range-finding experiments, and 5–50 mg TOS/mL with final concentrations of 500–5000 µg TOS/mL for 3+21-hour (±S9) and 24+24-hour (−S9) treatments in the micronucleus experiments[b]	Negative; no marked changes in osmolality or pH were observed at the highest concentration of 5000 µg TOS/mL compared with the solvent controls in the cytotoxicity range-finding test; no dose-dependent cytotoxicity of the test preparation was observed	Whitwell *(17)*

TOS: total organic solids.
[a] Treat-and-plate method and selective incubation, followed by viable counting.
[b] Separate cytotoxicity range-finding and micronucleus experiments. Test preparations were added to cultures such that the final culture volume was 10 mL, incubated at 37 °C for the designated period of time (3 hours of treatment and 21 hours of recovery, or 24 hours of treatment and 24 hours of recovery) followed by centrifugation of cells, replacement of medium with fresh medium and resuspension of cells an appropriate number of times (until cell pellets were clean).

2.2.3 Genotoxicity

The Committee evaluated two in vitro genotoxicity studies conducted with the enzyme concentrate – a bacterial reverse mutation assay (Ames assay) *(16)* and a human lymphocyte micronucleus assay *(17)* – summarized in Table 1 above. Both studies were certified for compliance with appropriate GLP regulations, and the study reports included QA statements. The Ames assay was conducted in accordance with OECD guideline no. 471 *(18)*, except for the use of the treat-and-plate method. The in vitro micronucleus assay was conducted in accordance with OECD guideline no. 487 *(19)*. The results of both studies were negative, indicating that the enzyme concentrate is not mutagenic or clastogenic in vitro. The Committee therefore had no concerns about potential genotoxicity of the enzyme concentrate.

2.2.4 Other studies

The enzyme was evaluated for potential toxicity by a homology search in which the amino acid sequence of the enzyme was compared with known toxins in the UniProt database *(20)*. The UniProt database was used to extract protein sequences that contain the word "toxin" in the description field, which resulted in 182 686 entries. ClustalW software *(21)* was used to align the identified sequences to the sequence of β-amylase from *B. flexus* expressed in *B. licheniformis*. The highest homology encountered when running the sequence homology search was ~ 16%, indicating that the enzyme is unlikely to be a toxin.

2.3 Observations in humans

No information was available.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to TOS from the enzyme preparation. Because the enzyme is intended for use in starch processing, this use was considered for the dietary exposure assessment. The submission included an estimate of dietary exposure based on the budget method *(22)*, a screening method used to determine the TMDI of food additives *(23,24)*. The method, which provides a conservative estimate of dietary exposure, takes into account the maximum physiological levels of consumption of solid foods and non-milk beverages, the energy density of foods, the concentration of the food additive in solid foods and non-milk beverages, and the proportion of solid foods and non-milk beverages that may contain it.

3.2 Dietary exposure assessment

The estimated TMDI provided by the sponsor was based on: (1) a high level of consumption of solid foods and of non-milk beverages; (2) the maximum use level of the enzyme preparation in solid foods and in non-milk beverages; and (3) the proportion of solid foods and of non-milk beverages that may contain the enzyme preparation. EHC 240 *(22)* refers to a daily consumed amount of 0.05 kg/kg bw of solid foods, based on 100 kcal/kg bw as the maximum energy intake over the course of a lifetime from solid foods, a daily consumed amount of 0.1 L/kg bw

of non-milk beverages, and default proportions of 12.5% for solid foods and 25% for non-milk beverages. Food ingredients processed with the enzyme preparation are proposed to be added to a variety of foods intended to be consumed by the general population. The sponsor therefore assumed that 50% of solid foods and 25% of non-milk beverages are processed, and that processed solid foods contain 25% starch (or starch-derived) dry matter and processed beverages contain 13% starch. These assumptions resulted in an overall proportion of 12.5% for solid foods and 3.25% for non-milk beverages that contain the enzyme preparation. The maximum use level in both solid foods and non-milk beverages was 99.1 mg TOS/kg starch-based raw material.

The budget method calculation was used to estimate the dietary exposure to TOS from the enzyme preparation. The sponsor used the standard inputs, except for the consumption of solid foods and the proportion of non-milk beverages that contain the enzyme preparation as described in the previous paragraph. For the consumption of solid foods, the sponsor assumed 50 kcal/kg bw per day as the maximum energy intake over the course of a lifetime from solid foods, resulting in a daily consumption of 0.025 kg/kg bw. The resulting TMDIs were 0.31 mg TOS/kg bw per day for solid foods and 0.33 mg TOS/kg bw per day for non-milk beverages, for a total of 0.64 mg TOS/kg bw per day. In its review of the sponsor's calculation, the Committee noted that the TMDI for non-milk beverages would be 0.32 mg TOS/kg bw per day, resulting in a total TMDI of 0.63 mg TOS/kg bw per day.

The Committee independently calculated dietary exposure to TOS from the enzyme preparation using the budget method. All inputs and assumptions were the same as those used by the sponsor except for the consumed amount for solid foods. The Committee used a maximum physiological requirement for solid foods (including milk) of 0.050 kg/kg bw per day, as reported in EHC 240 *(22)*. This is the amount used in a budget method calculation where there is potential for the enzyme to be used in baby foods, or in general-purpose foods that may be consumed by infants and young children, and represents a worst-case scenario. The TMDIs were 0.62 mg TOS/kg bw per day for solid foods and 0.32 mg TOS/kg bw per day for non-milk beverages, for a total of 0.94 mg TOS/kg bw per day.

For the purposes of the dietary exposure assessments conducted by the sponsor and the Committee, it was assumed that the enzyme is not removed or inactivated during the processing of food ingredients, and that 100% of the TOS from the enzyme preparation remains in the final food. In reality, the enzyme is either removed or inactivated by high temperatures during the processing of food ingredients, and will have no function in the final food.

4. Comments

4.1 Assessment of potential allergenicity

The Committee evaluated the potential for allergenicity of the enzyme preparation using a weight-of-evidence approach including the recommended bioinformatics criteria *(8,9)* and as outlined in EHC 240 *(2)*. The amino acid sequence of the enzyme was compared with those of known allergens in two online databases *(10,11)*. A search for matches with more than 35% identity in a sliding window of 80 amino acids and a full-length FASTA sequence search identified homology to one known food allergen, *Triticum aestivum* (Tri a 17.0101). The Committee determined that Tri a 17 and β-amylase showed a highest identity of 42.47%, with an identity of 31.4% across the full alignment. In contrast, the sponsor stated that Tri a 17 and β-amylase showed a highest identity of 44.7%, with an identity of 25.7% across the full alignment. The difference is the result of the sponsor using an older version (version 19) of the AllergenOnline database. A search for exact matches over contiguous stretches of eight amino acids generated two hits, which also indicated homology to Tri a 17. A recently published article *(12)* reported that wheat β-amylase with Tri a 17 is associated with allergic reactions upon wheat ingestion by sensitized people with a known wheat allergy.

An in vitro digestibility study *(13)* of the enzyme preparation with SGF showed that all proteins of the enzyme preparation were digested at the earliest time point of 2 minutes, indicating that the enzyme has the potential to be digested readily in the acidic conditions of the stomach. The Committee also noted that the enzyme preparation would be removed or inactivated during the manufacturing process.

Although the Committee acknowledged that the enzyme is homologous to a known food allergen, when taking into account the results of the digestibility study, the Committee concluded that dietary exposure to the enzyme was not anticipated to pose a risk for allergenicity.

4.2 Toxicological studies

In a 13-week study of oral toxicity in rats *(14)*, the enzyme concentrate was mixed in water and administered by gavage at doses of up to 1199 mg TOS/kg bw per day. The Committee did not identify any treatment-related or toxicologically relevant effects in any of the assessed parameters. Based on the absence of any adverse effects, the Committee identified a NOAEL of 1199 mg TOS/kg bw per day, the highest dose tested.

The enzyme concentrate was negative in a bacterial reverse mutation test *(16)* and in an in vitro mammalian cell micronucleus assay *(17)*. The Committee therefore had no concerns about potential genotoxicity of the enzyme concentrate.

A comparison of the amino acid sequence of the enzyme with those of known protein toxins *(20)* revealed no biologically relevant homology (~ 16%). The Committee therefore concluded that the enzyme was unlikely to be a toxin.

4.3 Assessment of dietary exposure

The Committee evaluated two estimates of dietary exposure to TOS from this β-amylase enzyme preparation, one submitted by the sponsor and the other estimated by the Committee. Both estimates were derived using the budget method, a screening method based on maximum physiological levels of consumption of solid foods and non-milk beverages, and on maximum use levels of the enzyme preparation. A maximum use level of 99.1 mg TOS/kg starch-based raw material for both solid foods and non-milk beverages was used. It was assumed that 50% of solid foods and 25% of non-milk beverages are processed, and that processed solid foods contain 25% starch (or starch-derived) dry matter and processed beverages contain 13% starch. Different assumptions were made about the amount of solid foods consumed. The resulting TMDIs from solid foods and non-milk beverages were 0.63 mg TOS/kg bw per day by the sponsor and 1 mg TOS/kg bw per day by the Committee. The Committee concluded that the dietary exposure estimate of 1 mg TOS/kg bw per day was appropriate for use in the evaluation. For the dietary exposure assessment, it was assumed that 100% of the TOS from the enzyme preparation remains in the final food. The Committee noted that the enzyme is either inactivated or removed during the processing of food ingredients, and will have no function in the final food.

5. Evaluation

The Committee concluded that dietary exposure to this β-amylase enzyme preparation is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 1199 mg TOS/kg bw per day, the highest dose tested, in a 13-week study of oral toxicity in rats. When this NOAEL is compared with the dietary exposure estimate of 1 mg TOS/kg bw per day, a MOE of around 1200 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary ADI "not specified"[2] for

[2] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

β-amylase (JECFA95-6) from *B. flexus* expressed in *B. licheniformis* when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications.

5.1 Recommendations

The Committee requested the following information, by the end of 2023, to complete the safety assessment:

- validated method of analysis to determine β-amylase activity, including the validation report; and
- analytical data using the validated method for at least five different batches of commercially available products.

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Lipase (JECFA95-7) from *Thermomyces lanuginosus* and *Fusarium oxysporum* expressed in *Aspergillus oryzae*

First draft prepared by
Catherine A. Smith,[1] Diane Benford,[2] Michael DiNovi,[3] Shayla West-Barnette[4] and Jannavi R. Srinivasan[4]

[1] Food Directorate, Health Canada, Ottawa, Canada
[2] Cheddington, United Kingdom
[3] Baltimore (MD), USA
[4] Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), USA

1. Explanation

At the request of the CCFA at its Forty-eighth Session *(1)*, the Committee evaluated the safety of a lipase (triacylglycerol lipase; Enzyme Commission No. 3.1.1.3; CAS No. 9001-62-1) from *Thermomyces lanuginosus* and *Fusarium oxysporum* expressed in *Aspergillus oryzae*. The Committee had not previously evaluated this enzyme preparation. The present Committee allocated the unique

JECFA enzyme identifier JECFA95-7 to this enzyme preparation. The Committee had previously evaluated several other lipases, including lipase from *A. oryzae* at its Eighteenth meeting for which an ADI "not specified"[1] was established (Annex 1, reference 35). At its Eighty-ninth meeting, the Committee noted the specifications for lipase from *A. oryzae* var. had been withdrawn at its Fifty-fifth meeting because the requested data had not been submitted (Annex 1, reference 145). The consequences of the withdrawal of specifications on the ADI were never addressed and, as a result, the Committee decided to withdraw the ADI of "not specified" for lipase from *A. oryzae* var. at its Eighty-ninth meeting and recommended the reconsideration of the ADI at a future meeting (Annex 1, reference 246).

The term "lipase" refers to the triacylglycerol lipase enzyme and its amino acid sequence; the term "enzyme concentrate" refers to the fermentation product containing the enzyme of interest, which is used in the toxicity studies; and the term "enzyme preparation" refers to the formulated product for commercial use.

The Committee previously assessed the safety of enzyme preparations derived from *A. oryzae* (e.g. asparaginase and phospholipase A1; Annex 1, reference 187) and established an ADI "not specified" for these enzyme preparations. On this basis, the present Committee considered that lipase (JECFA95-7) from *T. lanuginosus* and *F. oxysporum* expressed in *A. oryzae* met the criteria of a Class 1, Type iii enzyme as described in EHC 240 *(2)*. A Class 1, Type iii enzyme is produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Although toxicity data and a dietary exposure assessment are not required for Class 1, Type iii enzymes, the Committee evaluated the submitted data.

The enzyme hydrolyses ester linkages of triacylglycerides and phospholipids. The enzyme preparation is intended to be used as a processing aid in baking and in the processing of cereal-based foods. The Committee conducted a literature search in Google Scholar with the linked search terms "lipase" AND "*Aspergillus oryzae*" AND "*Thermomyces lanuginosus*" OR "*Humicola lanuginose*" AND "safety" OR "tox". A total of 480 hits were found. One reference *(3)* was identified that summarized a series of toxicity studies conducted with lipase from *Humicola lanuginosa* (now known as *T. lanuginosus*) expressed in *A. oryzae*; however, not enough information was available to adequately compare the lipase used in these studies to the lipase described in the present monograph. This reference was therefore not considered relevant for the present toxicological evaluation. Two additional references *(4,5)* were identified in the literature search that described the same studies summarized by Greenough et al. *(3)*; for the reason described above, neither reference was considered relevant to the present toxicological evaluation.

[1] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

1.1 Genetic background

The production organism, *A. oryzae*, is a non-pathogenic, filamentous fungus found in cereals, cereal grains and spoiled foods *(6)*. It has a history of use as a source organism in the production of food enzymes *(6–8)*.

The non-toxigenic, non-pathogenic *A. oryzae* production strain was obtained by a combination of chemical mutagenesis and genetic modifications of the parental strain. The parental strain was obtained from the Institute for Fermentation, Osaka, Japan. The production strain was obtained by inactivation of genes encoding for a major secreted protein, deletion of genes encoding for three proteases, and disruption of genes responsible for the production of kojic acid and mycotoxins. The expression plasmid used in the transformation contained a lipase gene created from portions of lipase genes from *T. lanuginosus* and *F. oxysporum*, an optimized *A. niger* promoter, an *A. niger* terminator and a selectable marker. The stability of the integration and the absence of any genes of concern in the production strain were confirmed by Southern blot hybridization.

1.2 Chemical and technical considerations

Lipase is produced by controlled submerged fermentation of a pure culture of the *A. oryzae* production strain. Manufacture of the enzyme preparation includes fermentation, recovery and formulation. After fermentation, the broth containing the lipase enzyme is separated from the biomass via a series of filtration steps, and concentrated. The resulting concentrate is formulated into a powder preparation. The entire process is performed in accordance with current GMP using food-grade raw materials. The final enzyme preparation contains no major food allergens from the fermentation medium and is free from the production organism and any antibiotic activity.

The primary sequence of lipase enzyme produced by *A. oryzae* consists of 317 amino acids; its molecular weight calculated from the determined amino acid sequence is 35 kDa. The lipase produced by *A. oryzae* is not known to have any significant subsidiary or secondary activity.

The activity of lipase expressed in lipase units (LU) is determined by measuring the rate of butyric acid released from tributyrin; 1 LU is defined as the amount of enzyme required to liberate 1 μmole of titratable butyric acid per minute under the conditions of the assay. The mean activity of lipase from four batches of the enzyme concentrate was 10 450 LU/g.

Lipase catalyses the hydrolysis of ester linkages in triacylglycerol and phospholipids. The enzyme preparation is intended for use as a processing aid in baking and in the processing of cereal-based foods up to a maximum level of 20 mg TOS/kg flour. The TOS includes the enzyme of interest and residues of

organic materials (e.g. proteins, peptides and carbohydrates) derived from the production organism during the manufacturing process.

The lipase enzyme is inactivated by heat during processing. It is not expected to have any technological function in the final food.

2. Biological data

2.1 Assessment of potential allergenicity

Two bioinformatics analyses were submitted by the sponsor *(9,10)* comparing the amino acid sequence of this lipase with the sequences of known allergens in two online databases *(11,12)*. No matches were found with > 35% identity over the full length of the protein, > 35% identity over a sliding window of 80 amino acids (with or without scaling) or 100% identity over eight contiguous amino acids. The Committee repeated the comparison of the amino acid sequence of this enzyme with known allergens in two online databases *(13,14)* based on bioinformatics criteria recommended by FAO/WHO *(15)*, Codex Alimentarius *(16)* and JECFA (Annex 1, reference 223). Searches for matches with > 35% identity over a sliding window of 80 amino acids and for sequence identity of eight contiguous amino acids were conducted in both databases. No matches were found. A full-length FASTA sequence search was also conducted with an E-value cut-off of < 0.1 and no matches were found. Comparisons between highly homologous proteins yield E-values approaching zero, indicating very low probability that such matches would occur by chance (larger E-values indicate a lower degree of similarity). No data on the digestibility of this enzyme in the gastrointestinal tract were submitted. The Committee considered that dietary exposure to this lipase is not anticipated to pose a risk for allergenicity.

2.2 Toxicological studies

The test material used in these studies was described as a liquid enzyme concentrate (Lipopan Xtra; batch no. PPW 26090) that was produced according to the same method as the commercial enzyme preparation, except that the additives and other standardization or stabilization ingredients normally added to the commercial enzyme preparation were not added to the test material. The lipase enzyme concentrate had a declared dry matter content of 11.9% (w/w), a TOS content of 10.3% (w/w) and an enzyme activity of 11 300 LU/g.

2.2.1 Acute toxicity

No information was available.

2.2.2 Short-term studies of toxicity

The cumulative effects of subchronic oral exposure to the lipase enzyme concentrate was evaluated in a study of male and female Sprague-Dawley SPF rats aged 5 weeks at the start of the treatment *(17)*. The 13-week oral toxicity study complied with GLP and was conducted in accordance with OECD guideline no. 408 *(18)* with the exception of the ophthalmoscopic examinations, which were not performed at the end of the treatment period. The liquid lipase enzyme concentration was administered at doses of 0, 1.0, 3.3 or 10.0 mL/kg bw per day (equivalent to 0, 108, 356.6 or 1080.2 mg TOS/kg bw per day or 0, 11 800, 39 100 or 118 500 LU/kg bw per day) by gavage for 93 days (10 rats per sex per group). For the low and intermediate doses, the liquid enzyme concentrate was diluted with the vehicle (tap water) prior to dosing. Rats in the high-dose group were given the undiluted liquid enzyme concentrate, and controls rats were given tap water. One male rat in the middle-dose group had difficulty breathing after gavage on the second day of treatment and died approximately 10 minutes later. The death was likely linked to complications associated with the administration of the test material by gavage, and the Committee did not consider it to be a treatment-related death. The rat that died was substituted by an additional rat on the second day of the study. The replacement rat was necropsied 1 day earlier than planned and was therefore only dosed for 92 days.

All rats survived until scheduled necropsy, except for the single male rat in the middle-dose group that died on the second day of treatment. As mentioned in the previous paragraph, this was not considered to be a treatment-related death. No clinical signs of toxicological relevance were observed in any group during the daily or weekly observations. There were also no treatment-related differences in body weight or in the consumption of feed or water. A statistically significant ($P < 0.05$) decrease in time spent moving and moves/count (not defined by the study authors in the study report, and no other information was available) were reported in female rats from the high-dose group in the open field test. However, the magnitude of these effects was small (~ 3% decrease in both parameters compared with the control group) and these findings were therefore considered incidental by the Committee.

In males, a statistically significant ($P < 0.01$ for the low-dose group; $P < 0.05$ for the middle- and high-dose groups) increase in the percentage of lymphocytes and a statistically significant ($P < 0.05$ for low- and middle-dose groups; $P < 0.01$ for high-dose group) decrease in the percentage of neutrophils were observed in all three treatment groups; however, because similar increases

in lymphocytes and decreases in neutrophils were observed in all dose groups, neither effect appeared to be dose dependent. A statistically significant ($P < 0.05$) decrease in neutrophils was also observed in male rats in the low- and middle-dose groups. In females, a statistically significant ($P < 0.05$) decrease was observed in neutrophils in the low-dose group as well as a statistically significant ($P < 0.05$) increase in the percentage of eosinophils in the middle-dose group. None of these observations was considered toxicologically relevant because there was no apparent dose–response relationship, they were not observed in both sexes, they were not associated with changes in histology and/or they were within the range of historical controls (95% confidence interval) reported by the study authors.

A statistically significant ($P < 0.05$) decrease in aspartate aminotransferase was observed in males in the high-dose group, and there was a trend towards a dose-dependent decrease in the low- and middle-dose groups. This finding was considered incidental since it was observed in only one sex and was not associated with changes in liver histology. A statistically significant ($P < 0.05$) decrease in potassium levels was observed in female rats in the middle-dose group. This finding was considered independent of treatment since it was not observed in both sexes and there was no apparent dose–response relationship.

A statistically significant ($P < 0.05$) decrease in absolute brain weight was observed in male rats in the low-dose group. This effect was only observed in one sex with no apparent dose–response relationship; it was therefore not considered to be toxicologically relevant. There was no evidence of any treatment-related macroscopic or microscopic findings in any dose group. The study authors reported that the opthalmoscopic examination of the eyes was not performed at the end of treatment prior to necropsy, which was a deviation from OECD guideline no. 408 *(18)*. However, a later histopathological examination did not yield any evidence of pathological findings in the eyes of any rat from any dose group.

In the absence of any toxicologically relevant treatment-related findings, the Committee identified a NOAEL of 1080.2 mg TOS/kg bw per day for the lipase enzyme concentrate, which was the highest dose tested.

2.2.3 Genotoxicity

The lipase enzyme concentrate, dissolved in water, was tested for genotoxicity in an in vitro bacterial mutagenicity assay with five bacterial strains – *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2 uvrA *(19)* – and an in vitro micronucleus assay in cultured human peripheral blood lymphocytes *(20)*. Both studies complied with GLP and were conducted in accordance with OECD guideline nos 471 *(21)* and 487 *(22)*, respectively.

Table 1
Genotoxicity of lipase enzyme concentrate

Assay	Test system	Concentration	Result	Reference
In vitro bacterial reverse mutation assay	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	3-h exposure: 16–515 µg TOS/mL (±S9)[a]	Negative[b]	*(19)*
	Escherichia coli WP2 uvrA	3-h exposure: 16–515 µg TOS/plate (±S9)[a]	Negative[b]	
In vitro micronucleus assay	Cultured human peripheral lymphocytes	3-h exposure: 309–515 µg TOS/mL (+S9)	Negative	*(20)*
		20-h exposure: 185–515 µg TOS/mL (−S9)	Negative	

TOS: total organic solids.
[a] Treat-and-plate assay.
[b] Significant toxicity was observed in the experiments with *S. typhimurium* TA1537 in the presence of S9 but was found to be negative in a third experiment using a heat-inactivated sample of the enzyme concentrate, indicating that lipase activity was not related to the observation of toxicity. The bacterial reverse mutation assay was therefore considered negative in this strain under test conditions.

The results of the bacterial reverse mutation assay and in vitro micronucleus assay were negative (Table 1), indicating that the lipase enzyme concentrate was not mutagenic or clastogenic in vitro under test conditions. The Committee had no concerns about potential genotoxicity of the lipase enzyme concentrate.

2.2.4 Other studies

A sequence homology search was submitted by the sponsor *(10)* comparing the amino acid sequence of this lipase with proteins in the UniProt database *(23)* that contained the word "toxin" but not "fragment", using the sequence alignment software ClustalW 2.0.10 *(24)*. The identity percentage to the lipase protein sequence or the compared toxin (whichever was longest) was calculated based on the length of each sequence as well as the number of identical residues. The highest homology was reported to be ~ 18%, indicating that the lipase amino acid sequence had no biologically relevant homology with toxin protein sequences in this database. The Committee therefore concluded that this lipase is unlikely to be a toxin.

2.3 Observations in humans

No information was available.

3. Dietary exposure

3.1 Introduction

The Committee evaluated one submission from the sponsor on dietary exposure to this lipase from a genetically modified strain of *A. oryzae*. The lipase is used as a processing aid in baking and in the manufacture of cereal-based products. The sponsor prepared an estimate of dietary exposure based on the reported consumption of baked goods in Europe. The method uses data (publicly available in 2012) from the European Food Safety Authority (EFSA) Comprehensive European Food Consumption Database.

3.2 Dietary exposure assessment

The estimated TMDI was derived by combining the maximum average intake for "grains and grain-based products" as reported by 17 European countries for all age groups, corrected for the final amount of flour needed to produce 100 g of baked goods, with the highest proposed use level of the enzyme. These inputs were 7.64 g flour/kg bw per day for food consumption (10.7 g baked goods/kg bw per day, corrected for processing, which equates to approximately 750 g baked goods per day for an individual of weight 70 kg) and 2200 LU/kg for the highest proposed use level of the enzyme preparation (which equates to 20 mg TOS/kg flour). The combination of these inputs results in an estimated dietary exposure of 0.153 mg TOS/kg bw per day, or 0.2 mg TOS/kg bw per day after rounding.

Because of the assumption of a large daily consumption of baked goods (one that is unlikely to vary significantly in regions other than Europe), and the postulation that all baked goods would contain the enzyme at the maximum use level, the Committee concluded that the estimated dietary exposure is appropriate for use in the safety assessment.

4. Comments

4.1 Assessment of potential allergenicity

Two bioinformatics analyses were submitted by the sponsor *(9,10)* comparing the amino acid sequence of this lipase with the sequences of known allergens in two online databases *(11,12)*. A search for amino acid sequence matches with more than 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 were conducted in both databases and did not identify any matches. The Committee repeated the comparison of the amino acid sequence of this enzyme with known allergens in two online databases *(13,14)* based on bioinformatics criteria recommended by FAO/WHO *(15)*, Codex Alimentarius *(16)* and JECFA (Annex 1, reference 223), and did not identify any matches. No data on the digestibility of this enzyme in the gastrointestinal tract were submitted. The Committee considered that dietary exposure to this lipase is not anticipated to pose a risk for allergenicity.

4.2 Toxicological studies

In a 13-week study of oral toxicity in rats *(17)*, the enzyme concentrate was administered by gavage at doses up to 1080.2 mg TOS/kg bw per day. No toxicologically relevant treatment-related effects were observed in any of the evaluated parameters. The Committee identified a NOAEL of 1080 mg TOS/kg bw per day (rounded from 1080.2 mg TOS/kg bw per day) for the enzyme concentrate, which was the highest dose tested.

The enzyme concentrate was not genotoxic in a bacterial reverse mutation assay *(19)* or in vitro micronucleus assay under test conditions *(20)*. The Committee had no concerns about potential genotoxicity of the enzyme concentrate.

A comparison of the amino acid sequence of this lipase with those of known protein toxins (UniProt database) revealed no biologically relevant homology (< 18%) *(10)*. The Committee therefore concluded that this lipase is unlikely to be a toxin.

4.3 Assessment of dietary exposure

The Committee evaluated one estimate of dietary exposure to TOS from this lipase enzyme preparation, submitted by the sponsor. It combined a high estimate of daily consumption of baked goods (flour at 7.64 g/kg bw per day) with the maximum use level to treat flour (20 mg TOS/kg flour). The TMDI was calculated as 0.153 mg TOS/kg bw per day (0.2 mg TOS/kg bw per day after rounding). For the dietary exposure assessment, it was assumed that 100% of the TOS from the enzyme preparation remains in the final food. The Committee noted that the enzyme is inactivated during the processing of food ingredients and will have no function in the final food.

5. Evaluation

The Committee concluded that dietary exposure to this lipase enzyme preparation is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 1080 mg TOS/kg bw per day, the highest dose tested, in the 13-week study of oral toxicity in rats. When this NOAEL is compared with the dietary exposure estimate of 0.2 mg TOS/kg bw per day, a MOE of more than 5000 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established an ADI "not specified"[2] for lipase (JECFA95-7) from *T. lanuginosus* and *F. oxysporum* expressed in *A. oryzae* when used in the applications specified, at the levels of use specified and in accordance with current GMP.

A new specifications monograph and a chemical and technical assessment were prepared.

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Xylanase (JECFA95-9) from *Bacillus licheniformis* expressed in *Bacillus licheniformis*

First draft prepared by
Szabina Stice,[1] Sue Barlow,[2] Orish Ebere Orisakwe,[3] Hae Jung Yoon,[4] Atsuko Tada[5] and Kristie Laurvick[6]

[1] Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), USA
[2] Brighton, East Sussex, United Kingdom
[3] Department of Experimental Pharmacology and Toxicology, University of Port Harcourt, Rivers State, Nigeria
[4] Korea Food and Drug Administration, Seoul, Republic of Korea
[5] Division of Food Additives, National Institute of Health Sciences, Kanagawa, Japan
[6] Food Standards, United States Pharmacopeia, Rockville (MD), USA

1. Explanation

At the request of the CCFA at its Fifty-first Session *(1)*, the Committee evaluated the safety of xylanase (endo-1,4-β-xylanase, Enzyme Commission No. 3.2.1.8; CAS No. 9025-57-4) from *Bacillus licheniformis* expressed in *B. licheniformis*. The Committee had not previously evaluated this enzyme preparation. The

present Committee allocated the unique JECFA enzyme identifier JECFA95-9 to this enzyme preparation. The Committee had previously evaluated several other xylanases, including xylanase from *B. subtilis* expressed in *B. subtilis*, and xylanase from *Thermomyces lanuginosus* expressed in *Fusarium venenatum* (Annex 1, references 167, 174), for which an ADI "not specified"[1] was established.

The term "xylanase" refers to the endo-1,4-β-xylanase enzyme and its amino acid sequence; the term "enzyme concentrate" refers to the fermentation product containing the enzyme of interest, which is used in the toxicity studies; and the term "enzyme preparation" refers to the product formulated for commercial use.

The Committee previously assessed the safety of enzyme preparations derived from *B. licheniformis* (e.g. pullulanase, Annex 1, reference 205; serine protease, Annex 1, reference 211) and established an ADI "not specified" for these enzyme preparations. On this basis, the present Committee considered that xylanase (JECFA95-9) from *B. licheniformis* expressed in *B. licheniformis* met the criteria of a Class 1, Type iii enzyme as described in EHC 240 *(2)*. A Class 1, Type iii enzyme preparation is produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Although toxicity data and a dietary exposure assessment are not required for Class 1, Type iii enzymes, the Committee evaluated the submitted data.

Xylanase catalyses the endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans including arabinoxylans into oligosaccharides of variable lengths. The enzyme is intended for use as a processing aid in the manufacture of baked goods and cereal-based products.

The Committee conducted a literature search in Google Scholar with the linked search terms "xylanase" AND "*Bacillus licheniformis*", which resulted in 8270 references. None of the identified references was relevant to the toxicological evaluation of this enzyme preparation.

1.1 Genetic background

The production organism *B. licheniformis* belongs to the genus Bacillus. *B. licheniformis* is a common Gram-positive, saprophytic bacterium that is considered to be non-pathogenic and non-toxigenic. It is common in foods including agricultural products such as cereals, and has a long history in the production of enzymes intended for use in food processing *(3–8)* (Annex 1, references 72, 167).

[1] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

The *B. licheniformis* xylanase production strain was obtained from recipient strain *B. licheniformis* BW302 using phage integration technology plasmid pBW120 *(9)*. During this step, two copies of the xylanase gene expression cassette, encoding the genetically engineered variant xylanase gene (formerly named *xyl264*), were inserted at the selected loci using plasmid pBW120 *(9)*. Southern blot analyses confirmed that the expected two copies of the xylanase gene were correctly inserted at the expected loci in the recipient strain to create the production strain *B. licheniformis* NZYM-CE *(10)*. The stability of the inserts was confirmed by Southern blot analysis. Southern blot and genome sequence analyses were performed on the production strain to verify the absence of relevant genes of concern, including antibiotic resistance markers. The production strain is deposited at DSMZ, Germany.

1.2 Chemical and technical considerations

Xylanase is produced by controlled fermentation of a pure culture of the *B. licheniformis* production strain. The manufacture of the xylanase enzyme preparation includes fermentation processes (inoculum, seed and main fermentation), recovery, and formulation at controlled temperature, pressure and pH. After fermentation, the broth containing the xylanase is separated from the biomass via multiple filtration steps; this is followed by concentration, stabilization and polish filtration. The concentrated enzyme is formulated into either a liquid or a powder enzyme preparation using food-grade ingredients for stabilization, standardization and preservation. The entire process is performed in accordance with current GMP and with food-grade raw materials. The enzyme concentrate is tested to ensure that it is free from the production organism and antibiotic activity. The final enzyme preparation is not expected to contain any major food allergens from the fermentation medium. The enzyme preparation conforms to the general specifications for enzyme preparations used in food processing (Annex 1, references 184, 185).

The xylanase enzyme produced by the *B. licheniformis* strain consists of 407 amino acids; its molecular weight by calculation from the determined amino acid sequence is 45.4 kDa *(10)*. The xylanase produced by *B. licheniformis* is not expected to have any secondary or subsidiary activities.

Xylanase activity is determined spectrophotometrically (405 nm) by measuring the formation of complexes of 4-hydroxybenzoic acid hydrazide and reducing carbohydrates released by the hydrolysis of wheat arabinoxylan substrate. The xylanase activity is calculated from a standard curve of an enzyme standard and the result is given in units/g. The unit is defined relative to the enzyme standard based on the amount of wheat arabinoxylan substrate degraded

per minute under standard conditions (pH 6.0; 50 °C). The mean activity of xylanase from three batches of enzyme concentrate was 4377 units/g.

Xylanase catalyses the endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans including arabinoxylans (also called pentosans). The enzymatic hydrolysis of arabinoxylans results in the generation of (1→4)-β-D-xylan oligosaccharides of variable lengths. These oligosaccharides are natural constituents of cereal-containing foodstuffs. The enzyme preparations are intended for use as a processing aid in the manufacture of baked goods and cereal-based products to improve dough structure, facilitate the handling of dough, and produce a uniform and predictable appearance in the dry product. The xylanase enzyme is active at temperatures up to approximately 70 °C (with an optimum of 45–60 °C at pH 6) and within a pH range of 4.5–9 (with an optimum of pH 6–8 at 37 °C).

The mean TOS of the toxicology test material (batch no. PPQ33502) was 9.7%. There is no normal or maximum use level, and the food enzyme is used according to the *quantum satis* principle. The sponsor recommended a dosage for the food enzyme in baked goods and cereal-based products of up to 60 units/kg flour (1 mg TOS/kg flour).

The xylanase enzyme exerts its function during dough handling and is either removed or inactivated by heat during the drying, boiling or steaming step. It is not expected to have any technological function in the final foods. Any residual enzyme present in the final food will be subjected to digestion in the gastrointestinal system, as for most other proteins; however, no data were available on its digestibility.

2. Biological data

2.1 Assessment of potential allergenicity

Xylanase from *B. licheniformis* expressed in *B. licheniformis* was evaluated by the sponsor in 2022 for potential allergenicity according to the bioinformatics criteria recommended by EFSA *(11)* and by FAO/WHO *(12)*. Four types of sequence homology assessments with known food allergens found in online databases AllergenOnline *(13)* and Allergen *(14)* were conducted: (i) search for > 35% identity in the amino acid sequence of xylanase using a window of 80 amino acids and a suitable gap penalty; (ii) as for (i) but with scaling enabled; (iii) alignment of xylanase with each of the allergens and identifying hits with > 35% identity over the full length of the alignment; and (iv) 100% identity over eight contiguous amino acids (the threshold E-value was not specified). No significant homology was found between the xylanase and any of the allergens

in the databases mentioned above. No data were available on the digestibility of xylanase in the gastrointestinal tract.

The Committee concluded that dietary exposure to xylanase from *B. licheniformis* expressed in *B. licheniformis* is not anticipated to pose a risk for allergenicity.

2.2 Toxicological studies

The test material in the evaluated studies was a brown liquid enzyme concentrate (batch no. PPQ33502) with a declared dry matter content of 11.7% w/w, a TOS content of 9/7% and an activity of 3670 units/g.

2.2.1 Acute toxicity

No information was available.

2.2.2 Short-term studies of toxicity

A 13-week gavage toxicity study *(15)* was conducted in Sprague-Dawley SPF Ntac:SD rats in compliance with GLP and in accordance with OECD guideline no. 408 *(16)*.

The test item, xylanase (batch no. PPQ33502), was mixed in water at concentrations to deliver doses in a volume of 10 mL/kg bw. Groups of 10 animals of each sex per dose group, of age approximately 5 weeks at the start of treatment, received a single dose by gavage of 0, 102, 336.7 or 1020 mg TOS/kg bw per day (equal to 0, 3861, 12 741 or 38 608 units/kg bw per day, respectively) for 13 weeks. Animals consumed feed and water *ad libitum* throughout the study, except for approximately 17 hours on Day 88 when none of the animals in the study had access to water during urine sampling.

No treatment-related signs were recorded at the clinical examination (clinical observations, open field and stimuli tests, and ophthalmoscopy), on body weight gain or on food consumption. The test item had no treatment-related effects on the clinical chemistry, haematology and coagulation parameters, or on the urinalysis and urine microscopy. At necropsy, no microscopic or macroscopic treatment-related findings were observed. For high-dose males, a higher water intake was seen on occasions during the study. Furthermore, a larger volume of urine was collected from these animals, with the urine having a lighter colour and a higher specific gravity. A tendency towards higher specific gravity in females in the middle- and high-dose groups was also seen, together with an overall lower water intake. Because the overall water intake was affected differently in the two sexes (decreased in females but increased in males), and no other observations in

the study indicated changes in the renal function, these findings were considered not toxicologically relevant.

In conclusion, gavage treatment of rats with xylanase for 13 weeks at dose levels of up to 1020 mg TOS/kg bw per day in a dose volume of 10 mL/kg bw per day did not cause any test-item-related changes. The Committee therefore concluded that the NOAEL for both females and males for xylanase was 1020 mg TOS/kg bw per day.

2.2.3 Genotoxicity

Genotoxicity of the enzyme concentrate (batch no. PPQ33502) was assessed in a bacterial reverse mutation assay *(17)* and an in vitro micronucleus assay *(18)*. The studies were conducted in compliance with GLP and in accordance with OECD guideline nos 471 *(19)* and 487 *(20)*.

The test results show that xylanase did not induce gene mutations in bacteria and did not induce micronuclei in cultured human peripheral blood lymphocytes (HPBL) under the experimental conditions of the above studies (Table 1). The Committee had no concerns about potential genotoxicity of xylanase.

2.3 Observations in humans

No information was available.

3. Dietary exposure

3.1 Introduction

Xylanases are naturally present in many raw materials (including wheat, barley and malt) and have been used extensively in various food applications such as in starch processing, beverage alcohol processes, and in brewing and baking processes *(22)*. Xylanase from *B. licheniformis* expressed in *B. licheniformis* has been permitted as a processing aid at the maximum dosage necessary to achieve the desired enzymatic reaction in Australia, Brazil, Canada, China, Denmark and many other countries. The sponsor submitted recommended use levels of up to 60 units/kg flour (1 mg TOS/kg flour) in baking processes.

Table 1
Genotoxicity of xylanase

Assay	Test system	Concentration	Result	Reference
In vitro reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2uvrApKM101	15–485 µg TOS/mL[a,b] ± S9	Negative	Pedersen *(17)*
In vitro micronucleus assay	Cultured human peripheral lymphocytes[c]	3 + 21 h exposure: 291–485 µg TOS/mL ± S9	Negative	Whitwell *(18)*
		24 + 24 h exposure: 48.5–485 µg TOS/mL – S9	Negative	

TOS: total organic solids.
[a] 1 mL of test substance solution was used in each test.
[b] A treat-and-plate assay was conducted (not described in OECD guidance, but recognized as a suitable approach for testing enzymes) *(21)*. Bacteria were exposed to the test substance in a phosphate-buffered nutrient broth for 3 hours with 5 mg dry matter per mL as the highest concentration. After incubation, the test substance was removed by centrifugation prior to plating. The study was conducted with and without Aroclor 1254-induced rat liver S9 metabolic activation system. All results were confirmed by conducting two complete and independent experiments. These conditions had no obvious influence on the revertant colony count. Toxicity of the test substance to the bacteria, or any increases in revertant numbers that met the criteria for a positive or equivocal response, were not observed.
[c] HPBL were exposed to xylanase for 3 hours in the absence and presence of Aroclor 1254-induced S9. These cultures were sampled 24 hours after the beginning of treatment. In addition, an extended 24-hour treatment with 24-hour recovery in the absence of S9 was included. These cultures were sampled 48 hours after the beginning of the treatment. The final concentration range of these three experiments was 500–5000 µg/mL. As the positive and negative controls produced the appropriate responses, the study was considered valid. Treatment of HPBL with xylanase in the absence and presence of S9 resulted in frequencies of micronucleated binucleate (MNBN) cells that were similar to and not significantly higher than those observed in concurrent vehicle controls for all concentrations analysed. The MNBN cell frequency of all xylanase-treated cultures fell within historical vehicle control (normal) ranges.

3.2 Dietary exposure assessment

As no use for xylanase in non-milk beverages was proposed, the Committee estimated the TMDI of xylanase in solid foods only. This TMDI estimate was based on the common assumptions used in the budget method, a screening method applied to food additives *(23)*. In this budget method calculation, the Committee used a maximum use level of 1 mg TOS/kg solid foods (assuming that all solid foods contain the maximum use level of flour) and a consumption value for solid foods of 0.025 kg/kg bw per day, and assumed that 50% of solid foods may contain the enzyme. The resulting TMDI of xylanase was estimated to be 0.01 mg TOS/kg bw per day for solid foods.

The sponsor provided a dietary exposure estimate for xylanase based on the summarized intake of grains and grain-based products given in the EFSA Comprehensive European Food Consumption Database. The sponsor noted a consumption of 10.7 g baked goods/kg bw per day (the highest average from 17 countries), corresponding to a consumption of flour of 7.64 g/kg bw per day and a resulting TMDI of 0.0076 mg TOS/kg bw per day. However, the Committee noted that this estimate was included in the EFSA 2019 estimate *(10)*. From this EFSA 2019 assessment, based on the maximum use levels recommended for the respective food processes (baking processes and cereal-based processes), and individual data from the EFSA Comprehensive European Food Consumption Database and technical conversion factors, mean dietary exposure was estimated to be 0.001–0.002 mg TOS/kg bw per day in adults (≥ 18 years) and elderly

population groups, and the 95th percentile dietary exposure was estimated to be 0.002–0.010 mg TOS/kg bw per day in infants.

For the dietary exposure assessment, it was assumed that 100% of the TOS from the enzyme preparation remains in the final food. The Committee noted that the enzyme is inactivated during processing of food ingredients, and will have no function in the final food.

4. Comments

4.1 Assessment of potential allergenicity

Xylanase from *B. licheniformis* expressed in *B. licheniformis* was evaluated by the sponsor in 2022 for potential allergenicity according to the bioinformatics criteria recommended by EFSA *(11)* and FAO/WHO *(12)*. The amino acid sequence of the enzyme was compared with those of known allergens in two online databases *(13,14)*. A search for matches with more than 35% identity in a sliding window of 80 amino acids, a search for exact matches over contiguous stretches of eight amino acids and a full-length FASTA sequence search did not identify a homology to any known allergens. No biologically relevant homology was found between the xylanase and any of the allergens in the databases mentioned above. No data were available on the digestibility of xylanase in the gastrointestinal tract. The Committee concluded that dietary exposure to xylanase from *B. licheniformis* expressed in *B. licheniformis* is not anticipated to pose a risk for allergenicity.

4.2 Toxicological studies

In a 13-week study of general toxicity in rats, no toxicologically relevant treatment-related effects were observed when the enzyme concentrate was administered by gavage at doses up to 1020 mg TOS/kg bw per day *(15)*. A NOAEL was identified as the highest dose tested (i.e. 1020 mg TOS/kg bw per day).

The enzyme concentrate was negative in a bacterial reverse mutation assay *(17)* and an in vitro mammalian micronucleus assay *(18)*. The Committee therefore had no concerns about potential genotoxicity of the xylanase enzyme concentrate.

4.3 Assessment of dietary exposure

The Committee evaluated estimates of dietary exposure to TOS in this xylanase enzyme preparation. The Committee used the budget method to estimate the TMDI of xylanase in solid foods, based on a maximum use level of 1 mg TOS/kg food (assuming that all solid foods contain the maximum use level for flour), a consumption value of 0.025 kg/kg bw per day, and the assumption that 50% of solid foods may contain the enzyme. The Committee noted that the method provided a conservative estimate of dietary exposure, and the resulting TMDI of xylanase was estimated to be 0.01 mg TOS/kg bw per day for solid foods. The sponsor submitted a more refined dietary exposure estimate for xylanase based on the summarized intake of grains and grain-based products given in the EFSA Comprehensive European Food Consumption Database. However, the Committee noted that this estimate has been included in those that EFSA estimated in 2019 *(10)*; the Committee also noted and concluded that the highest estimate of dietary exposure to xylanase of 0.01 mg TOS/kg bw per day for infants at the 95th percentile, calculated with maximum use levels recommended for the respective food processes and individual data from the EFSA Comprehensive European Food Consumption Database, should be considered in the evaluation. For the dietary exposure assessment, it was assumed that 100% of the TOS from the enzyme preparation remains in the final food. The Committee noted that the enzyme is inactivated during the processing of food ingredients, and will have no function in the final food.

5. Evaluation

The Committee concluded that dietary exposure to this xylanase enzyme preparation is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 1020 mg TOS/kg bw per day, the highest dose tested, in the 13-week study of oral toxicity in rats. When this NOAEL is compared with the dietary exposure estimate of 0.01 mg TOS/kg bw per day, a MOE of more than 100 000 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee allocated a temporary ADI "not specified"[2] for xylanase (JECFA95-9) from *B. licheniformis* expressed in *B. licheniformis* when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications.

[2] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting (Annex 1, reference 243) for clarification of the term ADI "not specified".

5.1 Recommendations

The Committee requested the following information, by the end of 2023, to complete the safety assessment:

- validated method of analysis to determine xylanase activity, including the full validation report;
- unit definition for xylanase activity based on the method of assay; and
- analytical data using the validated method for at least five different batches of commercially available products.

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SAFETY EVALUATION OF GROUPS OF RELATED FLAVOURING AGENTS

Alicyclic ketones, secondary alcohols and related esters (addendum)

First draft prepared by
Szabina Stice,[1] Maria Beatriz de Abreu Gloria,[2] Michael DiNovi,[3] Daniel Folmer[1] and Suzanne M.F. Jeurissen[4]

[1] Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), USA
[2] Faculty of Pharmacy, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[3] Baltimore (MD), USA
[4] Department for Food Safety, Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment, Bilthoven, Netherlands (Kingdom of the)

1. Evaluation

1.1 Introduction

At the request of the CCFA at its Fifty-first session *(1)*, the Committee evaluated an additional two flavouring agents in the group of alicyclic ketones, secondary alcohols and related esters for the first time. In addition, the Committee considered new data for 10 previously evaluated flavouring agents in this group

and data on three structurally related substances: the formyl and acetate esters of 4-*tert*-butylcyclohexanol and 2-*tert*-butylcyclohexanone.

The Committee evaluated 25 members of this group at its Fifty-ninth meeting (Annex 1, reference 160) and 12 members of this group at its Seventy-third meeting (Annex 1, reference 202). The Committee concluded that none of the 37 flavouring agents was a safety concern at the estimated dietary exposures.

The additional flavouring agents in this group are *trans*-4-*tert*-butylcyclohexanol (No. 2263) and caryophylla-3(4),8-dien-5-ol (No. 2264) (mixture of CAS No. 38284-26-3 and CAS No. 34298-31-2). Both flavouring agents have been reported to occur naturally; No. 2263 occurs in white wine and No. 2264 occurs in clary sage, clove bud, pepper and Scotch spearmint oil *(2–4)*.

The two additional members of this group were evaluated according to the revised Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference 230).

A literature search for toxicological data was performed in Google Scholar, PubMed, Embase and Web of Science using the names and CAS numbers of the flavouring agents under evaluation in this group; no additional relevant references were identified.

1.2 Assessment of dietary exposure

The total annual volume of production of the two flavouring agents in the group of alicyclic ketones, secondary alcohols and related esters is 0.2 kg in the USA *(5,6)*. No production volumes were reported for Japan, Europe or Latin America.

Dietary exposures were estimated with both the single portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method; the higher of the two values for each flavouring agent is reported in Table 1. The estimated daily dietary exposure is higher for *trans*-4-*tert*-butylcyclohexanol (No. 2263) (6000 µg/day, SPET value). For the other flavouring agent, caryophylla-3(4),8-dien-5-ol (mixture) (No. 2264), the estimated daily dietary exposure ranged from 0.01 to 300 µg/day, with the SPET yielding the higher estimate.

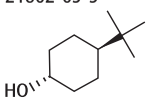
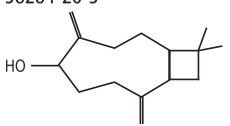
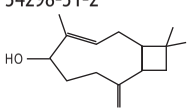
Annual volumes of production of this group of flavouring agents and the daily dietary exposures calculated with both the MSDI method and the SPET are summarized in Table 2 *(2–7)*.

1.3 Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination (ADME) of the flavouring agents in the group of alicyclic ketones, secondary alcohols and related esters was provided in the monographs from the Fifty-

Table 1

Summary of the results of safety evaluations of alicyclic ketones, secondary alcohols and related esters used as flavouring agents[a,b,c,d]

Flavouring agent	No.	CAS No. and structure	*Step 4* Does intake exceed the threshold of toxicological concern?[e]	*Step 5* Does a NOAEL exist for the flavouring agent or a structural relative that provides an adequate MOE?[f]	Comments on predicted metabolism	Conclusion based on current estimated dietary exposure
Structural Class I						
trans-4-*tert*-Butylcyclohexanol	2263	21862-63-5 	Yes; SPET: 6000 µg/day	Yes. The NOAEL of 240 mg/kg bw per day for *trans*-4-*tert*-butylcyclohexanol in a 13-week study in rats *(9)* is 2400 times the estimated dietary exposure of No. 2263 when used as a flavouring agent.	–[g]	No safety concern
Caryophylla-3(4),8-dien-5-ol (mixture)	2264	38284-26-3  34298-31-2 	No; SPET: 300 µg/day	NA	–[g]	No safety concern

CAS: Chemical Abstracts Service; MOE: margin of exposure; NA: not applicable; NOAEL: no-observed-adverse-effect level; SPET: single portion exposure technique.

[a] Thirty-seven flavouring agents in this group were previously evaluated by the Committee (Annex 1, references 160 and 202).

[b] Step 1: There are no structural alerts for genotoxicity for the additional two flavouring agents (Nos 2263 and 2264), and data on genotoxicity of No. 2263 do not indicate potential for genotoxicity.

[c] Step 2: Both flavouring agents are in structural class I.

[d] Step 3: Dietary exposures were estimated by both the SPET and MSDI method, and the higher of the two values for each flavouring agent is reported. SPET gave the higher estimate for each flavouring agent. All dietary exposure values are expressed in µg/day.

[e] Step 4: The threshold for human intake for structural class I is 1800 µg/day.

[f] The margin of exposure was calculated based on the highest daily per capita intake calculated either by the SPET or MSDI method for a body weight of 60 kg.

[g] The substances are detoxicated by glucuronic acid conjugation and excretion in the urine.

Table 2
Annual volumes of production of alicyclic ketones, secondary alcohols and related esters used as flavouring agents in Japan and the USA, and in Europe and Latin America

Flavouring agent (No.; CAS No.)	Most recent annual volume (kg)[a]	Dietary exposure				Annual volume in naturally occurring foods (kg)[d]	Consumption ratio[e]
		MSDI[b]		SPET[c]			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
***trans*-4-*tert*-Butylcyclohexanol (2263; 21862-63-5)**				6000	100		0.5
Japan	ND	ND	ND				
USA	0.1	0.01	0.000 2				
Europe	ND	ND	ND				
Latin America	ND	ND	ND				
Caryophylla-3(4),8-dien-5-ol (2264; mixture of 38284-26-3 and 34298-31-2)				300	5	+	0.5
Japan	ND	ND	ND				
USA	0.1	0.01	0.0002				
Europe	ND	ND	ND				
Latin America	ND	ND	ND				
Total							
Japan	ND						
USA	0.2						
Europe	ND						
Latin America	ND						

CAS: Chemical Abstracts Service; IOFI: International Organization of the Flavor Industry; MSDI: maximized survey-derived intake; ND: no data; SPET: single portion exposure technique; US FDA: United States Food and Drug Administration; +: reported to occur naturally in foods *(2)*, but no quantitative data.

[a] From IOFI *(5,6)*. Values positive, but any values < 0.1 kg were reported as 0.1 kg.

[b] Intake (µg/person per day) calculated as: (annual volume, kg) x (1 x 10^9 µg/kg)/(population x survey correction factor x 365 days), where population (10%, eaters only) = 13 x 10^6 for Japan, 33 x 10^6 for the USA, 45 x 10^6 for Europe and 62 x 10^6 for Latin America; correction factor = 0.8 for IOFI Global Poundage Survey; and the IOFI Interim Poundage and Use Levels Survey only 80% of the annual flavour volume, respectively, was reported in the poundage surveys *(5,6)*. Intake (µg/kg bw per day) calculated as: (µg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/person per day) calculated as: (US FDA standard food portion in g/day) x (highest usual use level) *(6)*. (µg/person per day)/body weight, where body weight = 60 kg. Slight variations are due to rounding.

[d] Quantitative data for the USA reported by Stofberg & Grundschober *(7)*.

[e] Consumption ratio calculated as: (annual consumption via food in kg)/(most recent reported volume as a flavouring substance in kg). The consumption ratio for No. 2263 was calculated based on the natural occurrence of No. 2263 in white wine: 0.0001–0.0003 ppm in Lukić et al. *(3)* and 0.05 ppm in Oliveira et al. *(4)*.

ninth and Seventy-third meetings (Annex 1, references 160 and 202). Specific information on one of the additional flavouring agents (No. 2263) evaluated at this meeting has become available.

Metabolic studies on alicyclic ketones indicate that they are converted to the corresponding secondary alcohols, which can be further oxidized to ketones or form conjugates with glucuronic acid or sulfate prior to urinary elimination.

1.4 Application of the revised Procedure for the Safety Evaluation of Flavouring Agents

Step 1. There are no structural alerts for genotoxicity for the additional two flavouring agents (Nos 2263 and 2264) in this group. Chemical-specific genotoxicity data on flavouring agents previously evaluated in this group and on the additional flavouring agents do not indicate any genotoxic potential.

Step 2. In applying the revised Procedure for the Safety Evaluation of Flavouring Agents to the additional two flavouring agents, the Committee assigned both flavouring agents (Nos 2263 and 2264) to structural class I *(8)*.

Step 3. Dietary exposures were estimated with both the MSDI method and the SPET and are presented in Table 1.

Step 4. The highest estimated dietary exposure to one flavouring agent (No. 2264) in structural class I is below the threshold of toxicological concern for the class (i.e. 1800 µg/person per day). The Committee therefore concluded that this flavouring agent (No. 2264) is not a safety concern at the current estimated dietary exposure. The highest estimated dietary exposure of the other flavouring agent (No. 2263) in structural class I is above the threshold of toxicological concern for that class (i.e. 1800 µg/person per day). Evaluation of this flavouring agent therefore proceeded to Step 5.

Step 5. For *trans*-4-*tert*-butylcyclohexanol (No. 2263), the NOAEL of 240 mg/kg bw per day in a 13-week gavage study in male and female rats *(9)* provides an adequate MOE (2400) relative to the SPET estimate of 6000 µg/day (or 100 µg/kg bw per day for a 60-kg person).

Table 1 summarizes the evaluations of the two flavouring agents in the group of alicyclic ketones, secondary alcohols and related esters that were considered at the present meeting (Nos 2263 and 2264).

1.5 Consideration of combined intakes from use as flavouring agents

The Committee previously considered the potential combined intake for this group of alicyclic ketones, secondary alcohols and related esters, and identified no safety concerns. The two additional flavouring agents in this group (Nos 2263 and 2264) have a low MSDI value (0.01 µg/day). According to the screening assessment for combined intake recommended by the Committee at its Seventy-third meeting (Annex 1, reference 202), the Committee concluded that consideration of combined intakes is not necessary because the additional flavouring agents would not contribute significantly to the combined intake of this group.

1.6 Consideration of additional data on previously evaluated flavouring agents

The Committee considered additional data on 10 of the 37 previously evaluated flavouring agents in this group. Studies of short-term toxicity (Nos 1109, 1114 and 2053), reproductive and developmental toxicity (Nos 1109, 1114 and 2053) and genotoxicity (Nos 1093, 1099, 1109, 1111, 1114, 1115, 2051–2053 and 2057) were available. These new toxicological data support the conclusions of the previous evaluations that these flavouring agents would not give rise to safety concerns.[1]

1.7 Conclusion

In the previous evaluations of 37 substances in this group of alicyclic ketones, secondary alcohols and related esters, studies of ADME, acute toxicity, short-term and long-term toxicity, and genotoxicity were evaluated (Annex 1, references 160 and 202). None raised safety concerns.

Studies of ADME, acute toxicity, short-term toxicity, developmental toxicity and genotoxicity were available for one of the two additional flavouring agents (No. 2263). For the structurally related substance 4-*tert*-butylcyclohexyl acetate, studies of short-term toxicity, reproductive and developmental toxicity, and genotoxicity were available. In addition, studies of genotoxicity were available for the structurally related substances 2-*tert*-butylcyclohexanone and the formate ester of 4-*tert*-butylcyclohexanol.

The Committee concluded that the two additional flavouring agents (Nos 2263 and 2264) would not give rise to safety concerns at the current estimated dietary exposures.

The Committee also concluded that the additional data presented do not give rise to safety concerns and further support the safety of the 37 previously evaluated flavours in this group.

2. Relevant background information

2.1 Explanation

This addendum summarizes the key data relevant to the safety evaluation of two flavouring agents (Nos 2263 and 2264) that are additions to the group of alicyclic ketones, secondary alcohols and related esters (Table 1). Thirty-seven

[1] In line with the screening assessment for combined intake recommended by the Committee at its Seventh-third meeting (Annex 1, reference 202).

other flavouring agents in this group were evaluated previously at the Fifty-ninth and Seventy-third meetings (Annex 1, references 160 and 202).

2.2 Additional considerations on dietary exposure

Both additional flavouring agents have been reported to occur naturally in food, *trans*-4-*tert*-butylcyclohexanol (No. 2263) in white wine and caryophylla-3(4),8-dien-5-ol (mixture) (No. 2264) in clary sage, clove bud, pepper and Scotch spearmint oil *(2)*. Quantitative natural occurrence data and a consumption ratio reported for *trans*-4-*tert*-butylcyclohexanol (No. 2263) indicate that exposure occurs predominantly from its use as a flavouring agent (i.e. consumption ratio < 1) *(7,10)*.

Annual volumes of production and dietary exposures estimated both as the MSDI and with the use of SPET for each flavouring agent are reported in Table 2.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

Several studies on the ADME of alicyclic ketones, secondary alcohols and related esters were described in a previous monograph and addendum (Annex 1, references 160 and 202). Studies on one of the new flavouring agents (No. 2263) evaluated at this meeting and its precursor ketone are described in the following sections.

trans-4-*tert*-Butylcyclohexanol (No. 2263)

The metabolic fate of the precursor ketone, 4-*tert*-butylcyclohexanone, was investigated (along with the 2- and 3- isomers) in female albino rabbits. The animals (n = 6) received a single dose of 3.7 mmol/kg bw, or 578 mg/kg bw (calculated with formula weight equal to 156.26 g/mol) of 4-*tert*-butylcyclohexanone by gavage. Urine samples were collected over 24 hours following treatment. Analysis of urinary metabolites indicated that 4-*tert*-butylcyclohexanone was reduced in vivo mainly to *trans*-4-*tert*-butylcyclohexanol (74% *trans* and 26% *cis*), which was excreted in urine as β-D-glucuronide conjugates at an average of 80% of the administered dose (range: 74–87%) *(11)*.

Male Fischer 344 rats received *tert*-butylcyclohexane (n = 8) at a dose of 800 mg/kg bw or vehicle only (n = 6 control animals) every other day for 14 days *(12)*. Urine samples were collected over 48 hours following the first treatment with

the test substance, and were treated with glucuronidase or sulfatase to hydrolyse conjugates. Both *cis*- and *trans*-4-*tert*-butylcyclohexanol were recovered from urine, with the *trans*-isomer being the major metabolite accounting for 53.8% of all urinary metabolites. The *cis*-isomer was a minor metabolite (1% of total metabolites). The relative abundance of the *trans*-isomer may reflect its easier conjugation with glucuronic acid relative to *cis*-4-*tert*-butylcyclohexanol. The difficulty in glucuronidation of the *cis*-isomer could then, conceivably, result in further hydroxylation to facilitate urinary excretion by creating more polar metabolites (diols) *(12)*. It is not clear what fraction of the administered dose was recovered in the urine.

2.3.2 Toxicological studies

New information related to the short-term toxicity (Nos 1109, 1114 and 2053), reproductive and developmental toxicity (Nos 1109, 1114 and 2053), and genotoxicity (Nos 1093, 1099, 1109, 1111, 1114, 1115, 2051–2053 and 2057) for previously evaluated flavouring agents in this group has been reported since the submission of the most recent monographs (Annex 1, references 160 and 202). For one of the two additional flavouring agents in this group (No. 2263), studies of short-term toxicity, developmental toxicity and genotoxicity were available. In addition, studies of short-term toxicity, reproductive and developmental toxicity, and genotoxicity were available for 4-*tert*-butylcyclohexyl acetate, a structurally related substance. Moreover, studies of genotoxicity were available for the structurally related substances 2-*tert*-butylcyclohexanone and the formate ester of 4-*tert*-butylcyclohexanol.

(a) Acute toxicity

In a study of oral acute toxicity with *trans*-4-*tert*-butylcyclohexanol (No. 2263; batch no. Oc 072 358 HV5, purity unknown) in female rats that was compliant with OECD guideline no. 420 *(13)* a median lethal dose (LD_{50} value) of more than 2000 mg/kg bw was reported *(14)*.

The result of the above study is consistent with the finding in the previous evaluations that the acute oral toxicity of alicyclic ketones, secondary alcohols and related esters is low.

(b) Short-term studies of toxicity

Results of short-term studies of toxicity were available for one of the additional flavouring agents in this group, *trans*-4-*tert*-butylcyclohexanol (No. 2263), and for the previously evaluated flavouring agents 2-*sec*-butylcyclohexanone (No. 1109), 3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one (No. 1114) and 3,3,5-trimethylcyclohexyl acetate (No. 2053). In addition, results of short-term

Table 3

Results of studies of oral short-term and long-term toxicity with alicyclic ketones, secondary alcohols and related esters used as flavouring agents

CAS No.	Flavouring agent	Species; sex	No. of test groups[a]; no. per group[b]	Route	Duration (days)	NOAEL/LOAEL (mg/kg bw per day)	Reference
Short-term toxicity							
2263	*trans*-4-*tert*-Butylcyclohexanol	Rat; M/F	3; 10	Gavage	14	NR[c]	Sieber *(15)*
2263	*trans*-4-*tert*-Butylcyclohexanol	Rat; M/F	5; 20–30[d]	Gavage	90	240[e]	Sieber *(9)*
1109	2-sec-Butylcyclohexanone	Rat; M/F	3; 20	Diet	28 (M) 51–56 (F)[f] 42 (F)[g]	151 (M)[e] 226 (F)[e]	van Otterdijk *(16)*
1114	3-Methyl-2-(2-pentenyl)-2-cyclopenten-1-one	Rat; M/F	3; 6	Diet	14	NR[c]	Leroy *(17)*
1114	3-Methyl-2-(2-pentenyl)-2-cyclopenten-1-one	Rat; M/F	3; 20	Diet	28	100[e,h]	Leroy *(17)*
2053	3,3,5-Trimethylcyclohexyl acetate	Rat; M/F	3; 10	Gavage	14	NR[c]	Nam *(18)*
2053	3,3,5-Trimethylcyclohexyl acetate	Rat; M/F	3; 24	Gavage	42 (M) ~56 (F)	150[e]	Nam *(19)*
NA[i]	4-tert-Butylcyclohexyl acetate	Rat; M/F	3; 10[d]	Gavage	28/21/18[j]	300[k]	Britton *(20)*
NA[i]	4-tert-Butylcyclohexyl acetate	Rat; M/F	3; 6	Diet	14	NR[c]	Rashid *(21)*
NA[i]	4-tert-Butylcyclohexyl acetate	Rat; M/F	3; 10	Diet	28	1000[e]	Rashid *(22)*

F: female; LOAEL: lowest-observed-adverse-effect level; M: male; NA: not applicable; NOAEL: no-observed-adverse-effect level; NR: not reported.
[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] Not reported because of the short duration of the study.
[d] High-dose group had an additional 10 animals as recovery group.
[e] NOAEL.
[f] Females with offspring.
[g] Females with no offspring.
[h] The NOAEL was 1500 mg/kg diet, equivalent to a target dose of 100 mg/kg bw per day. The actual exposure was not measured.
[i] Structurally related substance.
[j] Control, low-, middle-, and high-dose animals were treated for 28, 28, 21 and 18 days, respectively.
[k] LOAEL.

studies of toxicity were available for 4-*tert*-butylcyclohexyl acetate, a structurally related substance. These studies *(9,15–22)* are summarized in Table 3 and described in the following sections.

(i) *trans*-4-*tert*-Butylcyclohexanol (No. 2263)

In a 14-day dose-range-finding study, groups of male and female SPF-Wistar rats (five per sex per group) were given 0 (vehicle control), 100, 300 or 1000 mg *trans*-4-*tert*-butylcyclohexanol/kg bw per day (No. 2263; batch no. 5, purity 97.0%) formulated in a vehicle of 10% ethanol in corn oil by gavage *(15)*. The author of the study noted that the selected vehicle was the only vehicle in

which these concentrations could be achieved, and that this level of ethanol intake is well tolerated based on published data *(23)*. There were no *trans*-4-*tert*-butylcyclohexanol-related mortalities during the study. Slightly reduced activity and slight salivation in one male animal in the high-dose group were reported. No differences in feed consumption or mean absolute body weight were reported. However, weekly body weight gain was statistically significantly reduced in males of the high-dose group. In addition, statistically significant increases in mean absolute and relative-to-body weights of liver (20% and 22%, respectively) and kidneys (15% and 18%, respectively) were observed in high-dose males. In females, statistically significant increases in absolute and relative liver weight were observed in the middle-dose (16% and 14%, respectively) and the high-dose (34% and 31%, respectively) groups, and increases in absolute and relative adrenal weight were observed (24% and 20%, respectively) in the high-dose group. There were no treatment-related macroscopic findings at necropsy. Based on the results of this dose-range-finding study, 800 mg/kg bw per day was selected as the highest dose for a subsequent 90-day toxicity study *(15)*.

In the subsequent 90-day oral toxicity study, performed in compliance with OECD guideline no. 408 *(24)* and Swiss GLP Ordinance, groups of SPF-Wistar rats (10 per sex per group) were given 0, 80, 240 or 800 mg/kg bw per day of *trans*-4-*tert*-butylcyclohexanol (No. 2263; batch no. 5, purity 97.0%) formulated in a vehicle of 10% ethanol in corn oil by gavage *(9)*. Two control groups were included in order to assess the effects caused by the ethanol in the vehicle; one received corn oil only (referred to as the control group) and the other received 10% ethanol in corn oil (referred to as the vehicle group). A 28-day recovery period followed 91 days of treatment of an additional five rats per sex in the control, vehicle and high-dose groups. Animals in the vehicle and high-dose groups received the vehicle (i.e. 10% ethanol in corn oil) to avoid ethanol withdrawal symptoms.

No treatment-related mortality or changes in ophthalmology, feed consumption, behaviour, functional observational battery, grip strength or locomotion activity were observed at any dose level. Temporary clinical signs of dyspnoea and salivation were noted in the high-dose groups, although never affecting more than 30% of the group at the same time. At the high dose, statistically significant reductions in body weight (by up to 9.5%) and body weight gain were observed in males after the second and first week, respectively, and in females (by up to 5% for body weight) until week 8, compared with the vehicle group (10% ethanol in corn oil). These reductions were reversed during the recovery period. Reduced body weight gain was also noted in females of the middle-dose group during the first half of the treatment period compared with the vehicle group, but these were reversed during the remainder of the treatment period. Compared with the vehicle group, increased prothrombin time and

decreased partial thromboplastin time, and changes in reticulocyte fluorescence, were noted in the high-dose group; however, these remained within historical control ranges and returned to control levels during recovery.

Clinical chemistry parameter changes were observed in the low-dose (increased calcium and phosphorus levels in females), middle-dose (increased urea, potassium and phosphorus levels in females) and high-dose (decreased glucose and albumin/globulin ratio) groups; increased urea, creatinine, cholesterol, triglyceride, phospholipid, calcium, phosphorus levels and globulin concentration in males and females; increased protein and lactate dehydrogenase levels in males; and increased potassium and alkaline phosphatase levels in females. None of these changes was dose related, and all parameters returned to control levels during recovery. Of these, only triglyceride levels in females and phospholipid and globulin levels in males reached values outside the historical control ranges at the high dose, with no dose–response reported.

Urinalysis revealed increased urinary volume in high-dose males and urinary protein concentration in high-dose males and females. These remained within historical control values and were reversed during recovery. All reversible changes, except for urinalysis, were considered by the Committee to be adaptive. Urinalysis changes at the high dose were correlated with kidney histopathological findings. Kidney lesions – such as hyaline droplets, tubular basophilia, granular casts and mononuclear foci – were observed in male animals at all dose levels. They were noted with lower incidence and severity in females and low- and middle-dose male groups. Hyaline casts (3/10 and 7/10 animals) and tubular dilation (7/10 and 10/10 animals) were observed in the middle- and high-dose male groups, respectively, and tubular cysts (2/10) in the high-dose males. None of these was reported in animals treated with corn oil or vehicle groups, except for hyaline casts in 1/10 male animals in the vehicle group. In females, increased incidence and severity of tubular basophilia (3/10), minimal granular casts (1/10), tubular dilation (8/10) and tubular cysts (1/10) were noted in the high-dose group only. None of these changes was noted in female control and vehicle groups, except for 1/10 tubular basophilia in both control and vehicle groups. The incidence and severity of tubular basophilia, granular casts, mononuclear cell foci, tubular dilation and hyaline casts persisted in a few high-dose males through the recovery period, as well as mononuclear foci in one female.

Macroscopic observations in the liver included accentuated lobular pattern in two and three males in the middle- and high-dose groups, respectively, correlated with microscopic findings of low incidence of centrilobular hepatocellular hypertrophy of minimal severity in the middle-dose (1/10 males) and high-dose (2/10 males and 4/10 females) groups. Minimal hepatocellular hypertrophy observed in 4/10 females at the high dose, 1/10 male at the middle dose and 2/10 males at the high dose were considered adaptive in the absence of

other findings or lesions. Hepatocellular hypertrophy was not reported in any other groups. Significantly increased relative (to body weight) liver weight in males in the high-dose group (31%) and significantly increased relative kidney weights in males in the middle-dose (22%) and high-dose (51%) groups were observed. A slight increase in relative kidney weights was also observed in the low-dose males (18%), along with a slight increase in relative liver weight in high-dose females (11%) and middle-dose males (12%). Organ weight increases correlated with the microscopic findings in livers and kidneys and were reversible during recovery, except the relative kidney weight increase in high-dose males.

Considering that the test item is chemically very similar to other compounds known to bind to $\alpha_{2\mu}$-globulin, the cause of the hyaline droplets, tubular basophilia and casts observed in males, the kidney effects in males were most likely caused by $\alpha_{2\mu}$-globulin nephropathy, and therefore not relevant to humans *(25)*. Forestomach ulceration (1/10 females in the middle-dose group and 1/10 males in the high-dose group) and minor incidence and severity of epithelial hyperplasia (4/10 high-dose males, 1/10 middle-dose females and 3/10 high-dose females), hyperkeratosis (1/10 middle-dose males and females, 9/10 high-dose males and 5/10 high-dose females) and focal erosion (1/10 low-dose and 2/10 high-dose males; 1/10 middle-dose females) in the glandular stomach were also observed. Hyperkeratosis in the glandular stomach in males was sometimes seen along minimal parakeratosis at the high dose. Forestomach and glandular stomach alterations were attributed to a local irritation effect of administration of the substance by gavage that resolved during the recovery period. Similar local irritation findings were also noted in the nasal cavity, nasopharyngeal duct and pharynx and were attributed to regurgitation following gavage. These local effects were treatment related but were not considered to be toxicologically relevant by the Committee.

Other microscopic findings included minimal focal or multifocal glandular inflammation in the prostate, characterized by degenerating granulocytic cell infiltrates and inspissated colloid in three animals in the high-dose group, and non-inflammatory concretions in a single animal in the middle-dose group. These findings resolved completely in all animals during the recovery period. The only difference reported between animals treated with corn oil and the vehicle group was reduced potassium levels in vehicle-treated males compared with corn-oil-treated males during the 4-week recovery period. As no changes in potassium levels were observed after 13 weeks of treatment, this finding was considered incidental *(9)*.

Based on the histopathological findings in kidneys of females in the high-dose group, and in the absence of any other adverse effects in middle-dose animals apart from the signs of $\alpha_{2\mu}$-globulin renal pathology in males that is

not relevant to humans, a NOAEL of 240 mg/kg bw per day for *trans*-4-*tert*-butylcyclohexanol to rats by gavage was identified by the Committee.

(ii) 2-*sec*-Butylcyclohexanone (No. 1109)

A combined 28-day oral toxicity study *(16)* with the reproductive/developmental toxicity screening test with 2-*sec*-butylcyclohexanone (No. 1109; batch no. VE00478017, purity > 97.0%) was performed according to OECD guideline no. 422 *(26)* and United States Environmental Protection Agency (US EPA) Office of Prevention, Pesticides and Toxic Substances (OPPTS) guideline no. 870.3650 *(27)*. Groups of Wistar Han rats (10 per sex per group) were given 0, 650, 2000 or 6000 mg/kg of 2-*sec*-butylcyclohexanone in feed daily, equal to mean daily exposures of: 0, 50, 157 and 354 mg/kg bw per day, respectively, during pre-mating, and 0, 46, 143 and 403 mg/kg bw per day, respectively, during mating for males; and 0, 47, 134 and 332 mg/kg bw per day, respectively, during pre-mating, 0, 72, 174 and 414 mg/kg per day, respectively, during post-coitum, and 0, 153, 400 and 832 mg/kg bw per day, respectively, during lactation for females. The mean exposure to the test compound over the entire study period was 0, 48, 151 and 377 mg/kg bw per day for males and 0, 88, 226 and 508 mg/kg bw per day for females. Male animals were exposed for a minimum of 28 days in total, including 14 days before mating. Females were exposed for a total of either 42 days (if no offspring) or 51–56 days (those with offspring), including 14 days before mating, during mating and gestation, and a minimum of 13 days after delivery. The part of the study on reproductive and developmental toxicity screening is described in the section titled Reproductive and developmental toxicity.

No mortalities were attributable to 2-*sec*-butylcyclohexanone treatment. Piloerection was reported in all animals of the high-dose group and hunched posture in most females and one male of the high-dose group, and piloerection was also noted in females of the middle-dose group. Decreased body weights (up to 14% after 4 weeks of treatment in males, and up to 10% in week 1 and up to 5% during lactation in females) and body weight gain was reported in high-dose groups only. The final body weights were about 15% lower in males and 20% lower in females in the high-dose groups compared with controls. These decreases were related to a significant reduction in feed consumption, which was up to 70% in males and females in the first 2 days (indicative of poor palatability of the substance), and on average 35% and 25% in the pre-mating and mating periods in males, and 30, 40 and 50% in females during pre-mating, post-coitum and lactation, respectively.

Functional tests showed statistically significant lower fore- and hindlimb grip strength values in females of the high-dose group. There were no corroborative changes in other measures in the neuromuscular domain

(including gait, air righting reflex and motor activity). Grip strength values in treated females remained in the normal range for females of this strain and age. The lower grip strength was therefore not considered to reflect an adverse effect on neuromuscular function. The author of the study indicated that it could not be excluded that this effect may reflect the general toxicity observed at the high dose. In males, statistically significant lower mean forelimb grip strength was observed in the middle-dose group only, and was therefore considered unrelated to treatment.

Decreases in reticulocyte numbers (up to 31%) and red blood cell distribution width (up to 10%) were statistically significant in high-dose females. A statistically significant increase in mean corpuscular volume was observed in females of the middle-dose group only, and was therefore considered unrelated to treatment. There were no other changes in haematology or in coagulation parameters. No changes were found in thyroxine (T4) levels in males; based on this, T4 was not measured in females, and thyroid-stimulating hormone (TSH) was not measured in either males or females.

Clinical chemistry changes were reported only for the high-dose groups, except for increased bilirubin in both sexes that was observed both in the middle- and high-dose groups (40% and 2.4-fold, respectively). The changes in the high-dose groups included increased total protein (6%), albumin (8%) and cholesterol (27%) in males; and increased plasma alanine aminotransferase (ALAT) activity (38%), bile acids (5.5-fold), potassium (15%) and calcium (7%) in females. The higher mean ALAT plasma activity was the result of a single female with a 3.3-fold increase and was not considered related to treatment. Levels of protein, albumin and bile acids remained within historical control ranges. The haematological and clinical chemistry changes were not considered adverse because of an absence of correlated changes in haemoglobin or numbers of red blood cells, or the presence of other pathology findings.

No treatment-related abnormalities or lesions were observed in gross pathology. Increased relative liver weights were reported in both males and females of the high-dose groups (26% and 18%, respectively). In the absence of histopathological findings, the increases were not considered adverse. Absolute and relative thymus weights were statistically significantly decreased in males (29% and 14%, respectively) and in females (39% and 24%, respectively) of the high-dose groups. Other changes in (relative) organ weights were not considered related to treatment (but rather the result of decreased body weight or pregnancy status), were not dose related or the change direction was not toxicologically relevant.

In histopathology, changes in the kidneys included a dose-related increased incidence and severity of hyaline droplet accumulation, granular casts and/or tubular basophilia in males, primarily in the middle- and high-dose

groups. These changes were associated with a small, but statistically significant, increase in relative kidney weight in the high-dose group only. These findings, consistent with $\alpha_{2\mu}$-globulin renal pathology (not considered relevant to human health risk assessment) *(25)* in male rats, were not observed in females. In high-dose females, observations included slight yellow-brown pigmentation of the spleen consistent with haemosiderin release; minimal epithelial hypertrophy of the urinary bladder characterized by uniform, diffuse thickening without apparent increased cellularity; minimal to slight increase in bone marrow adipocytes; and minimally decreased bone marrow cellularity. None of these findings was considered adverse.

Lastly, in the thymus minimal to slight lymphoid atrophy was noted in high-dose males and females, and correlated with decreased thymus absolute and relative weights. However, these morphological changes were of minimal severity and not considered adverse.

Based on clinical signs of toxicity (piloerection and/or hunched posture) and reduced body weight and feed intake at the highest dose, the Committee identified a NOAEL of 2000 mg/kg feed for parental toxicity, equal to 151 and 226 mg/kg bw per day in males and females, respectively, in agreement with the study author. This NOAEL excludes the histopathological findings in the kidneys of male rats based on the lack of relevance to humans *(16)*.

(iii) 3-Methyl-2-(2-pentenyl)-2-cyclopenten-1-one (No. 1114)

In a 14-day dose-range-finding study, groups of male and female Wistar Han rats (three per sex per group) were fed diets containing 0, 1500, 5000 or 15 000 mg/kg of 3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one (referred to as Jasmone Cis.; No. 1114; batch no. SC00011133, purity 99.6%, or 87.7% for *cis*- and 11.9% for *trans*-isomers) *(17)*. These dietary levels were calculated to achieve target doses of 0, 100, 300 and 1000 mg/kg bw per day, which were selected based on acute toxicity data (LD_{50} ~ 4000 mg/kg bw). There were no treatment-related mortalities or clinical signs of toxicity during the study. Body weight loss in the first 4 days and reduced mean feed consumption and reduced overall body weights (6% and 7% in males and females, respectively) were observed in the high-dose groups. Body weight gain was not affected after day 4 and feed consumption recovered in week 2. All animals were subject to full necropsy, and liver and kidney weights were recorded. Dose-dependent increases in mean absolute liver weights in males (up to 24%) and females (up to 33%), and non-dose-related increases in mean absolute kidney weights in males (up to 11%) and in females (up to 8%), were observed. Although no histopathological evaluation was performed, the author considered it likely that these increases were related to the test item because the effects were seen in the subsequent main study (described in the

following paragraph). Based on the absence of severe toxicity in this study, the author concluded that these dietary levels should be tolerable for a study of longer duration *(17)*.

A combined 28-day dietary toxicity study *(17)* with the reproductive/developmental toxicity screening test was performed for 3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one (No. 1114; batch no. SC00011133, purity 99.6%, or 87.7% for *cis* and 11.9% for *trans*-isomers) following OECD guideline no. 422 *(28)* and US EPA OPPTS guideline nos 870.3650 *(27)*, 870.3550 *(29)* and 870.3050 *(30)*. Groups of Wistar Han rats (10 per sex per group) were fed diets containing 0, 1500, 5000 or 15 000 mg/kg of 3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one. These dietary concentrations correspond to target mean daily intakes of 0, 100, 300 and 1000 mg/kg bw per day, respectively, but the actual exposure was not provided in the study report. Male animals were exposed for 28 days in total, including 14 days before mating. Females were exposed for 14 days before mating, during mating and gestation, and for 4 days after delivery. (The study was performed prior to 2016 and according to 1996 OECD guideline no. 422 *(28)*, which called for dosing until day 4 postnatally. The Committee noted that, according to the current version of OECD guideline no. 422 *(26)*, exposure should be continued for at least 13 days of lactation.) The part of the study on reproductive and developmental toxicity screening is described in the section titled Reproductive and developmental toxicity.

No mortalities and no clinical signs of toxicity were attributable to 3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one administration. Weight loss in high-dose males (up to 11.8%) and females (up to 9.14%) was reported in the first week of study. Body weights of middle-dose males were lower until termination, and lower for high-dose males and females (up to 18% less in males by termination, and up to 8% less at the end of pre-mating and up to 17% less at termination in females), than for control animals. During gestation, lower body weight gain (16, 17 and 37% for low, middle- and high-dose groups, respectively) and lower body weights (8, 8 and 20% for low, middle- and high-dose groups, respectively) were reported. Body weight gain recovered during lactation, but body weights remained lower in all treated females compared with controls, and statistically significantly different at the high dose (17% less). Lower feed consumption was reported in middle- and high-dose males, and in females at all dietary levels, primarily during the first week and continued through termination (up to 26% during gestation and 35% during lactation), indicative of poor palatability.

There were no adverse effects of treatment on functional outcomes either in males or females. No toxicologically relevant differences in haematology, coagulation or clinical chemistry were reported at any dietary level. Slight but statistically significant higher mean cholesterol and triglyceride concentrations

were noted for both sexes in the high-dose groups, which remained within historical control ranges and were not considered toxicologically relevant.

Statistically significant increased relative testes and epididymides weights, slightly lower (but not statistically significant) absolute and relative weights of the seminal vesicles and coagulating glands, and lower absolute ovary weights were reported in high-dose groups. These were not considered adverse as there were no treatment-related histological findings in the reproductive organs in males or females.

Minimal or slight hepatocyte hypertrophy that was considered non-adverse but adaptive was observed in 7/10 high-dose males and 4/10 middle-dose females compared with none in the controls. Relative liver weights of all treated males were higher than in controls (9, 26 and 21% for low-, middle- and high-dose males, respectively) and higher in middle-dose (16%) and high-dose (32%) females during the main phase of the study. Renal cortical tubular degeneration was observed in 3/10 (middle-dose) and 5/10 (high-dose) males, an observation that was not made in the controls or in the females. This was associated with minimal or slight cortical tubular hypertrophy and moderately increased cortical tubular hyaline droplets accumulation. These findings are consistent with male-rat-specific $\alpha_{2\mu}$-globulin nephropathy that is not relevant to human risk assessment *(25)*. Increased relative kidney weight was also reported in high-dose males (23%).

At the highest dose, changes observed in the thymus, adrenal glands and spleen of males and/or females were considered secondary to overall toxicity and reduced body weights also observed at that dietary level. Changes included minimal to moderate thymus atrophy (one male and three females) and reduced relative thymus weights (by 13% in males and 46% in females); minimal or slight zona fasciculata vacuolation of adrenal glands (4/5 males); an absence of expected increase of extramedullary haematopoiesis in the spleen of pregnant females (seen in dams of all other groups); and lower spleen weights in females (22% less). However, overall haematopoiesis was not reduced. Paraovarian cysts were reported in one high-dose female, but were not considered treatment related and were unlikely to have caused the litter loss that was also seen in that animal (described further in the section on reproductive and developmental toxicity).

The author of the study identified a NOAEL for dietary exposure of 3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one for rats to be 1500 mg/kg in the diet for males, equivalent to 100 mg/kg bw per day. This was based on lower body weight and feed consumption, and pathology findings reported for 5000 and 15 000 mg/kg feed. The author did not identify a NOAEL for females in this study because of a statistically significant reduction in body weight, and in feed consumption and pathology findings, at all dietary levels. The severity of the findings in the 15 000 mg/kg group, which led to lower terminal mean

body weights (−18% and −17% for the males and females, respectively), was in excess of a maximum tolerated dose *(17)*. The Committee noted that the kidney pathology findings in middle- and high-dose males are not relevant to humans. It is not clear which pathology findings in females the study author is referring to. Based on the adverse effects on body weight and feed intake that were observed, the Committee identified a NOAEL for parental toxicity of 1500 mg/kg in the diet, equivalent to a target dose of 100 mg/kg bw per day.

(iv) 3,3,5-Trimethylcyclohexyl acetate (No. 2053)

In a 14-day dose-range-finding study, groups of male and female Sprague-Dawley rats (five per sex per group) received 0, 100, 300 or 1000 mg 3,3,5-trimethylcyclohexyl acetate/kg bw per day (No. 2053; batch no. 10300020, purity 99.7%) by gavage, formulated in corn oil *(18)*. The test doses were based on information on acute toxicity of this substance (LD_{50} > 2000 mg/kg bw) in rats. There were no test-substance-related mortalities during the study. Clinical signs of toxicity included salivation and/or perineum soiling in high-dose males and females, and in middle-dose females. There were no differences in body weights, body weight gains or feed consumption between treated and control males or females. No differences were observed in clinical chemistry parameters at any dose level in either sex. Statistically significant increases in absolute and relative liver weight in females and in relative kidney weight in males were reported for the high-dose groups. Macroscopic findings at necropsy included bilateral enlarged kidneys and thickening of stomach in high-dose males. Based on the organ weight and macroscopic findings for the high-dose groups, the author suggested that doses of up to 500 mg/kg bw per day should be tolerable for a study of longer duration.

A subsequent combined 28-day oral toxicity study with the reproductive/developmental toxicity screening test was performed for 3,3,5-trimethylcyclohexyl acetate following OECD guideline no. 422 *(28)* and in compliance with GLP. Groups of Sprague-Dawley CD IGS rats (12 per sex per group) received 0 (vehicle controls), 50, 150 or 500 mg/kg bw per day of 3,3,5-trimethylcyclohexyl acetate (No. 2053; batch no. 10300020, purity 99.7%) formulated in corn oil by gavage *(19)*. Male animals were exposed for 6 weeks in total (42 days), including 2 weeks before mating, during mating and 2 weeks after. Females were exposed for 2 weeks before mating, during mating and gestation, and for 5 days after delivery (approximately 8 weeks in total). (The study was performed prior to 2016 and according to 1996 OECD guideline no. 422 *(28)*, which called for dosing until day 4 postnatally. The Committee noted that, according to the current version of OECD guideline no. 422 *(26)*, exposure should be continued for at least 13 days of lactation.) Additional animals of control and high-dose groups (six

per sex per group) were treated for 6 weeks followed by a 2-week recovery period (referred to as recovery groups). The part of the study on reproductive and developmental toxicity screening is described in the section titled Reproductive and developmental toxicity.

No mortality was observed in male animals or in either sex of the recovery groups; two females of the main high-dose group were found dead on postpartum day (PPD) 3 and 5. Before the females were found dead, clinical signs such as soiled perineal region and staining around the mouth and/or haematuria were observed. Upon necropsy, small thymus (2/2) and spleen (1/2), enlargement of adrenals (1/2) and black focus in the forestomach (1/2) were observed in these dead females. Clinical signs of toxicity in surviving animals included salivation and/or perineum soiling in high-dose males and females, and in one middle-dose animal of each sex. These signs persisted through the recovery period for a few animals of the high-dose groups. There were no test substance effects on body weights except a slight, but statistically significant, increase in high-dose females on PPD 0 (+9.7% versus control).

Transient but statistically significant increases in feed consumption were recorded for high-dose males on day 42 for the main study group and on days 8, 21 and 36 of the recovery group. Feed consumption was higher in females of the middle- and high-dose main groups throughout the dosing period except for postnatal day (PND) 4, and reached statistical significance on gestational day (GD) 7 and for the high-dose group also on GD 14. A transient but statistically significant decrease in feed consumption was noted in females of the recovery group on day 29.

No treatment-related adverse effects were recorded in behavioural assessment, in the grip strength test, rotarod test and on spontaneous motor activity. A statistically significant increase in grip strength noted in high-dose males of the recovery group was not considered to be toxicologically significant because of its small magnitude. No treatment-related differences were observed in haematology, coagulation or clinical chemistry analysis. Any statistically significant differences recorded were incidental, small in magnitude and within historical control ranges.

Urinalysis revealed low incidence of proteinuria in two males and two females of the high-dose main group, and in one female of the high-dose recovery group. Occult blood and erythrocytes were found in the urine of one female of the middle-dose main group. In the absence of any relevant changes in histopathology, these findings were not considered toxicologically relevant. In histopathology, centrilobular hepatocyte hypertrophy was observed in 6/6 males and 4/6 females of the high-dose main group after 6 weeks compared with 0/6 control animals for both sexes, and was characterized by increased cytoplasmic volume. Since this finding was fully reversible by the end of the recovery period and there was

no associated inflammation or necrosis, it was considered an adaptive response to treatment at the high dose, as typically seen in the centrilobular region. In the kidneys, minimal incidence of hyaline droplets accumulation in the cortical tubular epithelium of mild to moderate severity was reported in all treated males but not in females. These lesions were not associated with degeneration, inflammation, necrosis or any other visible evidence of tubular injury, and were eliminated after the recovery period. This finding is consistent with $\alpha_{2\mu}$-globulin renal nephropathy specific to male rats and not relevant to humans *(25)*. There were no statistically significant differences in organ weights in males at any dose level that were considered treatment related. The only statistically significant difference in organ weights in females was increased absolute and relative thymus weight in the high-dose main group.

The Committee identified a NOAEL of 150 mg/kg bw per day for parental toxicity, based on the effects observed in high-dose females.

(v) Structurally related substances

4-*tert*-Butylcyclohexyl acetate (batch no. 1000976729, purity 99.4%) was tested in a 28-day oral toxicity study performed in accordance with OECD guideline no. 407 *(31)* and European Commission (EC) regulation no. 440/2008 *(32)*. Groups of five male and five female Crl:WI(Han) rats received 4-*tert*-butylcyclohexyl acetate at dose levels of 0 (vehicle control), 300, 500 or 1000/750 mg/kg bw per day by corn oil gavage for 28, 28, 21 or 7/11 days, respectively *(20)*. The test doses were selected based on the results of a preliminary study, where the highest dose (938 mg/kg bw per day) was tolerated after initial signs of toxicity during the first 4 days. In the main study, the highest dose level of 1000 mg/kg bw per day was reduced on day 8 to 750 mg/kg bw per day because of an increased incidence of convulsions and twitching/tremors, resulting in one death and early termination for five animals. An additional five animals per sex received the vehicle (corn oil) or the highest dose for 18 days, and were then maintained for a 14-day recovery period without treatment.

During the treatment period, one male of the high-dose group was found dead, and four females of the high-dose group and one female of the low-dose group were euthanized following severe, but transient, convulsions and other adverse clinical signs. Despite lowering the highest dose level from 1000 mg/kg bw per day to 750 mg/kg bw per day on day 8, twitching/tremors and convulsions persisted; the group was therefore terminated on day 19.

Clinical signs including excessive salivation, piloerection and changes in activity were observed in middle-dose males 1 hour after dosing, in addition to sporadic instances of twitching in females. The incidence and severity of these findings increased, and convulsions were observed in one middle-dose male and

one middle-dose female on day 21, leading to the termination of the dose group. Similar findings of twitching and excessive salivation were observed in both sexes of the low-dose group, and instances of convulsion were observed in 3/5 females of this group from day 22. Increased urination and water consumption were observed in animals of all treatment groups after 1, 2 and 3 weeks for the high-, middle- and low-dose groups, respectively.

During week 1, statistically significant lower mean body weight gains were observed for males of the middle-dose ($P < 0.01$) and high-dose ($P < 0.001$) groups. Reduced body weight gains were associated with decreased feed consumption in the same groups, indicative of poor palatability. However, mean group body weight gains were comparable between the high-dose and control groups over the entire administration period (days 0–19). High-dose males in the recovery group presented slightly higher body weight gains compared with the control recovery animals. In middle- and low-dose males, the overall group mean body weight gains were lower by 33% and 23%, respectively, compared with controls. Females in the main and recovery groups did not present statistically significant differences in body weights. Feed consumption in treated groups (main and recovery) was similar to that of the control group following the first week of treatment. The functional observational battery and motor activity assessment did not reveal any significant treatment-related changes in evaluated parameters.

During haematology and coagulation evaluations, increased mean white blood cell counts were reported in both sexes of the high-dose group: +12% in males (not statistically significant) and +29% (statistically significant) in females relative to the control group. This finding was not considered toxicologically relevant, and potentially represented a stress response in the affected animals. No other treatment-related changes to haematology parameters were noted. Non-significantly increased plasma creatinine levels were observed in both sexes of the middle-dose group only, but these increases were not associated with corresponding changes to urea levels, kidney weights (changes seen were not in the same animals), or macro- or microscopic findings.

Other clinical chemistry findings – such as higher aspartate aminotransferase levels in both sexes of the middle- and high-dose groups, decreased total protein levels in high-dose males and females of all groups, increased cholesterol concentrations in females of all groups, increased sodium and potassium concentration in middle- and high-dose females, and increased calcium concentration in middle-dose females – were not considered toxicologically relevant, since individual animal values were within the historical control range for the age and strain of animal, and the changes were small in magnitude. Slight differences in serum bile acid levels were attributed to the different times of day when blood samples were collected from control and

low-dose animals (mid-morning), and middle- and high-dose animals (mid-afternoon).

Relative liver weights were increased in high-dose males and all treated females, and relative kidney weights were increased in all treated males and females. The differences in absolute and relative liver and kidney weights were not observed in the recovery group for either sex. Macroscopic examination revealed abnormally pale or mottled kidneys in one low-dose, one middle-dose and three high-dose males. No treatment-related macroscopic findings were reported for any of the recovery animals.

Histopathological examinations revealed minimal follicular epithelial hypertrophy in thyroid glands for middle- and high-dose males (4/5 middle-dose males; 3/4 high-dose males) and minimal to slight feminization of mammary glands in middle- and high-dose males (2/5 middle-dose males; 2/4 high-dose males). For the high-dose group, only four males were examined histopathologically because of the degree of autolysis in the fifth male of the group. The changes in relative liver weights and increased incidences of follicular epithelial hypertrophy in the thyroid glands were considered secondary adaptive effects of hepatic enzyme induction, and a full recovery was observed during the recovery period. Male mammary gland feminization and oestrous cycle disturbance also recovered. Kidney findings in male rats also included minimal to moderate basophilic cortical tubules (1/5 control, 5/5 low-dose and 4/4 high-dose); slightly increased hyaline droplets in the proximal tubules (5/5 low-dose and 4/4 high-dose); minimal to slight focal tubular necrosis in the outer medulla (1/5 low-dose and 1/4 high-dose); and granular casts in the medulla (3/5 low-dose and 1/4 high-dose). These kidney findings were not reported in female rats of the same groups and are considered related to male-rat-specific $\alpha_{2\mu}$-globulin kidney pathology. Increased absolute and relative kidney weights were observed in low- and middle-dose females, and increased relative kidney weight in males of all dose levels. These changes were fully reversed during the recovery period. Evidence of minimal to moderate inflammation/fibroplasia was observed on the tails of four high-dose males, with epithelial hyperplasia and/or scab formation in 3/4 males. This microscopic finding was considered to be a result of the treatment for this dose group. Histopathological findings in the thyroid and mammary glands were not observed in recovery males.

Treatment-related changes in body weight gain, liver weights and histopathology in the middle- and high-dose groups were transient or adaptive, and were reversed during the recovery period. Kidney histopathological findings were species- and sex-specific, and consistent with documented pathology in male rats. Other changes were not toxicologically relevant. However, adverse effects in the central nervous system, including twitching and convulsions, were

gradually noted at all dose levels, including the low-dose groups of both males and females, after day 22 *(20)*.

In agreement with the study author, the Committee identified a lowest-observed-adverse-effect level (LOAEL) of 300 mg/kg bw per day based on the adverse effect on the central nervous system, including twitching and convulsions observed at all dose levels.

In a 14-day dose-range-finding study, groups of male and female Crl:WI(Han) rats (three per sex per group) were fed diets calculated to provide target doses of 0, 100, 300 and 1000 mg/kg bw per day of 4-*tert*-butylcyclohexyl acetate (batch no. 1001220504, purity 99%) *(21)*. The actual exposure levels were 0, 104, 325 and 968 mg/kg bw per day for males and 0, 96, 281 and 911 mg/kg bw per day for females.

There were no mortalities or treatment-related clinical observations during the study. Moderately lower body weight gains in males and females (by 33% and 22%, respectively) and mean terminal body weights (by 10% and 5%, respectively) were observed in the high-dose groups, considered attributable to decreased feed consumption. There were no effects on feed intake or body weight measurements in low- or middle-dose males. Lower body weight gains and mean terminal body weights were observed in females of the low- and middle-dose groups (by 24% and 25%, respectively) compared with the control group, which was associated with occasionally decreased feed intake values. The author noted the high inter-animal variation in the body weight parameters, and indicated that individual animal data were comparable between females of the low- and middle-dose groups and the control females. Findings observed at necropsy were considered spontaneous and normal for the species and age of rat. Based on the results of this study, the author concluded that the same dietary intake levels of 100, 300 and 1000 mg/kg bw per day should be tolerable for a longer duration study *(21)*.

A subsequent 28-day dietary toxicity study was conducted according to OECD guideline no. 407, during which 4-*tert*-butylcyclohexyl acetate (batch no. 1001220504, purity 99%) was administered to groups of male and female Crl:WI(Han) rats (five per sex per group) at dietary levels calculated to provide target doses of 0 (control), 100, 300 or 1000 mg/kg bw per day *(22)*. The actual dose levels were 0, 97, 299 and 1005 mg/kg bw per day for males and 0, 105, 296 and 980 mg/kg bw per day for females.

During the course of the 28-day dietary exposure period, no deaths were reported. The only clinical observations noted were pale faeces in all groups (including control animals) during week 4. Body weight gains and terminal body weights were moderately reduced in males (17%) and slightly reduced in females (7%) of the high-dose groups, and slightly reduced in males of the low- and middle-dose groups (up to 8%). In the high-dose group, decreased

feed consumption was noted compared with the control group (up to 23% in males and up to 14% in females). Lesser reductions (up to 15%) with no dose relationship were observed in low- and middle-dose males. The reduction of 17% in terminal body weight for high-dose males was considered to be a secondary response resulting from the unpalatability of the diet causing reduced feed intake, and therefore not adverse.

Haematological assessment revealed statistically significant, dose-dependent reductions in total leukocyte count, mainly the result of reduced absolute lymphocyte counts in middle- and high-dose males. Similar findings were reported in females of the same group, which only reached statistical significance ($P < 0.05$) for the high-dose group. Middle- and high-dose males also displayed statistically significant ($P < 0.05$) and dose-dependent reductions in reticulocyte counts that were not observed in females of the same dietary levels. Since individual animal total leukocyte counts and reticulocyte counts were largely within the historical control ranges, these reductions were not considered to be toxicologically relevant. Increased levels of plasma creatinine were observed in male rats of all intake levels that were statistically significant ($P < 0.001$). These changes were not considered to be toxicologically relevant because no associated changes in urea and electrolyte levels or histopathological findings were reported.

Statistically significant increased bile acid concentrations in the plasma were observed in both sexes of the high-dose group and males of the middle-dose group, but were attributable to the animals not being fasted prior to blood collection rather than from dietary exposure to 4-*tert*-butylcyclohexyl acetate. No exposure-related changes to functional observation battery parameters were noted in any animal receiving 4-*tert*-butylcyclohexyl acetate in the diet compared with the control group. Dose-dependent increases in relative liver weights were reported in both sexes that were statistically significant in the middle- and high-dose groups, with a greater change observed in males. Additionally, increased relative kidney weights were observed in males of all groups that were significantly higher in the middle- and high-dose groups, but were not noted for females.

Macroscopic examination did not reveal any treatment-related findings. Histopathology was only conducted on organs of control and high-dose animals, and findings included increased incidence of minimal centrilobular hypertrophy in the liver (0/5 control males and females, 2/5 high-dose males and 4/5 high-dose females); increased incidence of renal pathology in males only, such as the presence of hyaline droplets of minimal to moderate severity (0/5 control and 5/5 high-dose males); increased total incidence of minimal to slight basophilic cortical tubules (1/5 control and 3/5 high-dose); and increased incidence of follicular epithelial hypertrophy in the thyroid glands in males only (0/5 control and 2/5 high-dose). These histopathological findings were considered rodent-specific (renal pathology) or adaptive in nature (hepatic enzyme induction); the

low- and middle-dose groups were therefore not evaluated. The increased kidney weights and associated histopathological findings were only reported in male rats and were consistent with evidence of $\alpha_{2\mu}$-globulin renal pathology, with no relevance to humans *(25)*.

Based on these considerations, a NOAEL of 1000 mg/kg bw per day, the highest intake level tested, was identified for both sexes from the 28-day dietary toxicity study *(22)*.

(c) Genotoxicity studies

Studies of in vitro genotoxicity reported for alicyclic ketones, secondary alcohols and related esters are summarized in Table 4 *(33–58)* and briefly described in the following sections.

(i) Reverse mutation

No evidence of mutagenic potential was observed in bacterial reverse mutation assay with *trans*-4-*tert*-butylcyclohexanol (No. 2263), cyclohexyl acetate (No. 1093), 3,3,5-trimethylcyclohexanol (No. 1099), tetramethyl ethyl cyclohexenone isomers (No. 1111), isojasmone (No. 1115), 3,3,5-trimethylcyclohexyl acetate (No. 2053) and 4-(2-butenylidene)-3,3,5-trimethylcyclohex-2-en-1-one (No. 2057) in concentrations up to 5000 µg/plate in the presence and absence of metabolic activation *(33,35,37,41,46,50,53)*. All studies were performed according to OECD guideline no. 471 *(59)* and were certified for compliance with GLP and QA.

No evidence of mutagenic potential was observed in a bacterial reverse mutation assay with structurally related substances 2-*tert*-butylcyclohexanone (structurally related to 2-*sec*-butylcyclohexanone; No. 1109) and 4-*tert*-butylcyclohexyl acetate (which will yield the flavouring agent No. 2263 upon ester hydrolysis, and is therefore relevant to support the lack of its mutagenic potential) in concentrations up to 5000 µg/plate in the presence and absence of metabolic activation *(54,57)*. Both studies were performed according to OECD guideline no. 471 and were certified for compliance with GLP and QA.

In addition, no mutagenic potential was observed in bacterial reverse mutation assays performed with structurally related substances 4-*tert*-butylcyclohexanol and the formyl ester of 4-*tert*-butylcyclohexanol *(56,58)*. The study reports do not provide a statement on compliance with GLP or any OECD guidelines. Further, the study reports do not contain information on the substance used to induce enzyme production in the liver. Moreover, both studies tested concentrations that are lower than the recommended OECD concentrations because of cytotoxicity at higher doses.

Table 4

Studies of in vitro genotoxicity with alicyclic ketones, secondary alcohols and related esters used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
2263	*trans*-4-*tert*-Butylcyclohexanol	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA102	5, 15, 50, 150, 500, 1500, 5000 µg/plate[a,b]	Negative	Thompson & Shanks *(33)*
2263	*trans*-4-*tert*-Butylcyclohexanol	Chromosome aberration	Human peripheral blood lymphocytes	95.2, 166.7, 291.6 µg/mL[c]	Negative	Bohnenberger *(34)*
				54.4, 95.2, 166.7 µg/mL[d]	Negative	
				54.4, 95.2, 166.7 µg/mL[e]	Weakly positive	
				100, 150, 200 µg/mL[d]	Negative	
				180, 200, 220 µg/mL[e]	Negative	
1093	Cyclohexyl acetate	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2uvrA	1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate[a,b]	Negative	Dakoulas *(35)*
1093	Cyclohexyl acetate	Micronucleus induction	Human peripheral blood lymphocytes	400, 500, 800 µg/mL[c]	Negative	Dutta *(36)*
				300, 700, 1420 µg/mL[d]	Negative	
				100, 400, 800 µg/mL[e]	Negative	
1099	3,5,5-Trimethylcyclohexanol	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2uvrA	1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate[a,b]	Negative	Dakoulas *(37)*
1099	3,5,5-Trimethylcyclohexanol	Micronucleus induction	Human peripheral blood lymphocytes	100, 200, 450 µg/mL[c]	Negative	Roy *(38)*
				100, 200, 400 µg/mL[d]	Negative	
				25, 50, 115 µg/mL[e]	Negative	
1109	2-*sec*-Butylcyclohexanone	Forward mutation	Mouse lymphoma L5178Y/ $TK^{+/-}$ cells	12.5, 25, 50, 100, 150, 175, 200 µg/mL[c]	Negative	Verspeek-Rip *(39)*
				25, 50, 100, 150, 200, 225, 250, 275 µg/mL[d]	Negative	
				25, 50, 75, 100, 125, 150, 170, 180 µg/mL[e]	Negative	
1109	2-*sec*-Butylcyclohexanone	Micronucleus induction	Human peripheral blood lymphocytes	50, 200, 270 µg/mL[c]	Negative	Eurlings *(40)*
				50, 250, 300 µg/mL[d]	Negative	
				50, 70, 110 µg/mL[e]	Negative	
1111	Tetramethyl ethyl cyclohexenone (mixture of isomers)	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2uvrA	5, 16, 50, 160, 500, 1600, 5000 µg/mL[a,b]	Negative	Bhalli *(41)*
				16, 50, 160, 500, 1600, 5000 µg/plate[a,b]		
1111	Tetramethyl ethyl cyclohexenone (mixture of isomers)	Micronucleus induction	Human peripheral blood lymphocytes	103, 184, 226 µg/mL[c]	Negative	Bhalli *(42)*
				168, 244, 271 µg/mL[d]	Negative	
				37.2, 51.7, 57.5 µg/mL[e]	Negative	
1114	3-Methyl-2-(2-pentenyl)-2-cyclopenten-1-one	Forward mutation	Chinese hamster ovary cells (CHO K1)	37.5, 75, 150, 300, 350, 400, 450 µg/mL[e,f]	Negative	Kovacs *(43)*
1114	3-Methyl-2-(2-pentenyl)-2-cyclopenten-1-one	Micronucleus induction	Human peripheral blood lymphocytes	200, 300, 500 µg/mL[c,d]	Negative	Roy *(44)*
				25, 50, 85 µg/mL[e]	Negative	

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
1114	3-Methyl-2-(2-pentenyl)-2-cyclopenten-1-one	Micronucleus induction	Human peripheral blood lymphocytes	100, 400, 440 µg/mL[c]	Negative	Verbaan *(45)*
				100, 450, 495 µg/mL[d]	Negative	
				10, 50, 70 µg/mL[e]	Negative	
1115	Isojasmone	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2uvrA	5, 16, 50, 160, 500, 1600, 5000 µg/plate[a,b]	Negative	Bhalli *(46)*
				16, 50, 160, 500, 1600 µg/plate[a,b]		
				16, 50, 160, 500, 1600, 5000 µg/plate[a,b,g]		
1115	Isojasmone	Micronucleus induction	Human peripheral blood lymphocytes	138, 171, 180 µg/plate[c]	Negative	Bhalli *(47)*
				171, 199, 210, 221 µg/plate[d]	Equivocal	
				153, 170, 210 µg/plate[d]	Negative	
				31, 42.5, 47.2, 58.3 µg/plate[e]	Equivocal	
				16.2, 20.3, 39.6 µg/plate[e]	Negative	
2051	Cyclohexanone diethyl ketal	Micronucleus induction	Human peripheral blood lymphocytes	200, 400, 500 µg/mL[c]	Negative	Morris *(48)*
				200, 400, 500, 600 µg/mL[d]	Negative	
				200, 400, 500 µg/mL[e]	Negative	
2052	2-Cyclohexenone	Alkaline comet assay	Chinese hamster lung fibroblasts (V79 cells)	9.6, 28.8 µg/mL[h]	Positive	Janzowski et al. *(49)*
2053	3,3,5-Trimethylcyclohexyl acetate	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2uvrA	3, 10, 33, 100, 333, 1000, 2500, 5000 µg/plate[a,b,i]	Negative	Sokolowski *(50)*
2053	3,3,5-Trimethylcyclohexyl acetate	Forward mutation	Chinese hamster fibroblasts (V79 cells)	14.4, 28.8, 57.5, 230, 345 µg/mL[c]	Negative	Wollny *(51)*
				115, 230, 460, 690, 805, 1150 µg/mL[d]	Negative	
				7.2, 14.4, 28.8, 57.5 µg/mL[e]	Negative	
2053	3,3,5-Trimethylcyclohexyl acetate	Chromosome aberration	Human peripheral blood lymphocytes	36.8, 64.41, 112.71 µg/mL[c]	Negative	Sokolowski *(52)*
				64.41, 112.71, 197.25 µg/mL[d]	Negative	
				93.3, 163.3, 285.7 µg/mL[d]	Negative	
				53.3, 93.3, 163.3 µg/mL[e]	Negative	
2057	4-(2-Butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and TA102	15, 50, 150, 500, 1500, 5000 µg/plate[a,b]	Negative	Thompson & Bowles *(53)*
				5, 15, 50, 150, 500, 1500, 5000 µg/plate[a,i]	Negative	
NA[j]	2-*tert*-Butylcyclohexanone	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2uvrA	16.0, 50.0, 160, 500, 1600, 5000 µg/plate[a,b]	Negative	Bhalli *(54)*
NA[j]	2-*tert*-Butylcyclohexanone	Micronucleus induction	Human peripheral blood lymphocytes	13.6, 26.5, 40.3 µg/mL[e]	Negative	Bhalli *(55)*
				126, 155, 191 µg/mL[c]	Negative	
				150, 203, 249 µg/mL[d]	Negative	

Table 4 (continued)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
NA[j]	4-*tert*-Butylcyclohexyl acetate	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and TA1538	2, 4.7, 9 µg/plate[a,b,k]	Negative	Richold & Jones *(56)*
NA[j]	4-*tert*-Butylcyclohexyl acetate	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2uvrA	15, 50, 150, 500, 1500, 5000 µg/plate[a,i,l]	Negative	Thompson *(57)*
				1.5, 5, 15, 50, 150, 500, 1500 µg/plate[a,i,m]	Negative	
				0.5, 1.5, 5, 15, 50, 150, 500 µg/plate[a,i,n]	Negative	
NA[j]	4-*tert*-Butylcyclohexyl formate	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	0.0009, 0.009, 0.09, 0.9 µg/plate[a,b,k]	Negative	Richold & Jones *(58)*

[a] All strains/dose levels tested in the presence and absence of S9 activation.
[b] Plate incorporation method.
[c] 3- or 4-hour treatment in the absence of S9 activation.
[d] 3- or 4-hour treatment in the presence of S9 activation.
[e] 22- or 24-hour treatment in the absence of S9 activation.
[f] 5-hour treatment in the presence and absence of S9 activation.
[g] In *S. typhimurium* strains only.
[h] Incubation time with the test material was 1 hour. The assay was followed by formamidopyrimidine-DNA glycosylase treatment for 1, 2 and 3 hours, with no increase in oxidative DNA damage.
[i] Preincubation method.
[j] Structurally related substance.
[k] Concentrations lower than those recommended by the OECD were tested because of cytotoxicity.
[l] In *E. coli* only.
[m] In *S. typhimurium* strain TA1537 only.
[n] In *S. typhimurium* strains TA100, TA1535 and TA98 only.

(ii) Forward mutation

2-*sec*-Butylcyclohexanone (No. 1109) did not induce forward mutations in mouse lymphoma L5178Y/*TK*$^{+/-}$ cells in the presence or absence of metabolic activation *(39)*. The study was performed according to OECD guideline no. 490 *(60)* and in compliance with GLP.

There was no indication of mutagenic potential when 3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one (referred to as Cis-Jasmone; No. 1114) was tested in a forward mutation assay in Chinese hamster ovary K1 cells in the presence or absence of metabolic activation *(43)*. In addition, there was no indication of mutagenic potential when 3,3,5-trimethylcyclohexyl acetate (No. 2053) was tested in a forward mutation assay in Chinese hamster lung fibroblasts (V79) in the presence and absence of metabolic activation *(51)*. Both studies were performed according to OECD guideline no. 476 *(61)* and in compliance with GLP.

(iii) Chromosomal aberration

trans-4-*tert*-Butylcyclohexanol (No. 2263; batch no. 5, purity 99.5%) was tested for the potential to induce chromosome aberrations in HPBL in two separate

experiments *(34)* compliant with OECD guideline no. 473 *(62)* and GLP. In the first experiment, HPBL were incubated with *trans*-4-*tert*-butylcyclohexanol for 4-hour exposure periods with or without exogenous metabolic activation (phenobarbital/β-naphthoflavone-induced S9). Tested concentrations for both 4-hour treatments (with an 18-hour recovery period with and without exogenous metabolic activation) ranged over 10.2–1563 µg/mL (~ 10 mM). In the second experiment, human lymphocytes were continuously exposed to *trans*-4-*tert*-butylcyclohexanol (exposure period 22 hours) in the absence of S9 activation at concentrations of 10.2–1563 µg/mL, or were exposed for 4 hours in the presence of S9 activation at concentrations of 12.5–500 µg/mL. The continuous exposure (22 hours) treatment arm was repeated under similar conditions, but at a narrowed concentration range of 50–400 µg/mL.

Treatment with *trans*-4-*tert*-butylcyclohexanol for 4 hours in the first experiment did not result in any increases in the frequency of chromosome aberrations at the selected concentrations of 95.2, 166.7 and 291.6 µg/mL or 54.4, 95.2 and 166.7 µg/mL in the absence or presence of S9, respectively. Precipitation of the test item was observed at the end of treatment at 291.6 µg/mL in the absence of S9 and at 95.2 µg/mL and higher in the presence of S9. Dose selection for the second experiment was influenced by test item toxicity and the occurrence of precipitation in the first experiment. In the second experiment, there were no increases in the number of aberrations at the selected concentrations in the 4-hour treatment with S9 (100, 150 and 200 µg/mL). Precipitation of the test item was observed at the end of the treatment with 150 µg/mL and higher. Moreover, there was no increase in the number of aberrations at the selected concentrations in the 22-hour treatment without S9 (54.4, 95.2 and 166.7 µg/mL), except a slight but statistically significant increase at the highest concentration scored (166.7 µg/mL) in the latter (3.0%). In the repeated 22-hour experiment, higher concentrations of 180, 200 and 220 µg/mL were analysed but no increases were observed. The increase in the frequency of chromosome aberrations observed in the first continuous treatment was within the historical vehicle control range of the conducting laboratory (0.0–3.0%) and was not reproducible in the repeat experiment; it was therefore considered to be biologically irrelevant. Furthermore, no evidence of an increase in polyploid metaphases was noticed for any of the treatments compared with the control cultures. The study author therefore concluded that *trans*-4-*tert*-butylcyclohexanol did not induce chromosome aberrations under the experimental conditions in vitro *(34)*.

No induction of chromosome aberrations or polyploidy was observed with 3,3,5-trimethylcyclohexyl acetate (No. 2053) or *trans*-4-*tert*-butylcyclohexanol (No. 2263) in HPBL in the presence and absence of metabolic activation *(50)*. The studies were performed according to OECD guideline no. 473 *(62)* and in compliance with GLP.

(iv) Micronucleus induction

No statistically significant increases in micronuclei frequency were observed when HPBL were incubated with cyclohexyl acetate (No. 1093), 3,5,5-trimethylcyclohexanol (No. 1099), 2-*sec*-butylcyclohexanone (No. 1109), 5-ethyl-2,3,4,5-tetramethylcyclohexen-1-one (No. 1111), 3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one (referred to as *cis*-Jasmone, No. 1114), cyclohexanone diethyl ketal (No. 2051) or 2-*tert*-butylcyclohexanone (a structural relative of 2-*sec*-butylcyclohexanone; No. 1109) in the presence and absence of metabolic activation *(36,38,40,42,44,48,45,55)*. Nos 1093, 1099, 1109, 1111, 1114 and 2051 and 2-*tert*-butylcyclohexanone were therefore considered non-clastogenic and non-aneugenic under the conditions of the above studies. All studies were performed according to OECD guideline no. 487 *(63)* and in compliance with GLP.

Isojasmone (No. 1115; batch no. 0005665197, purity 96.2%) was assessed for its potential to induce micronuclei in HPBL in an in vitro study *(47)* conducted according to OECD guideline no. 487 *(63)*. Concentrations of isojasmone up to 300 µg/mL were evaluated for cytotoxicity in 3-hour treatments with S9 and up to 232 µg/mL without S9, while concentrations up to 71.9 µg/mL were evaluated in the 24-hour treatment without S9. Based on observed toxicity at the higher concentrations tested, the concentrations selected for micronuclei scoring for the 3-hour treatments were 138, 171 and 180 µg/mL in the absence of S9, and 171, 199, 210 and 221 µg/mL in the presence of S9. The concentrations scored for the 24-hour treatment were 31, 42.5, 47.2 and 58.3 µg/mL in the absence of S9. A statistically significant increase in micronuclei frequency was observed at 171 µg/mL only in the 3-hour treatment with S9 (the lowest concentration tested) and at 42.5 µg/mL only in the 24-hour treatment without S9 (second-lowest concentration tested). Neither increase was concentration dependent, and one of the two cultures for each of these concentrations had micronuclei frequencies that were within the historical vehicle control ranges of the laboratory for the respective exposure periods. The criteria for a positive response were not met for either of these observed increases, and were considered of questionable biological relevance by the study author. The 3-hour treatment with S9 and the 24-hour treatment without S9 were repeated at narrower concentration intervals of 137–300 µg/mL and 16.2–70 µg/mL, respectively. The selected concentrations for scoring of 153, 170 and 210 µg/mL (3-hour treatment, with S9) and 16.2, 20.3 and 39.6 µg/mL (24-hour treatment, without S9) did not present any statistically significant increases in micronucleus frequency. Since the increases observed in the initial experiment were not reproducible, they were considered biologically irrelevant; the study author concluded isojasmone to be non-clastogenic and non-aneugenic under all treatment conditions *(47)*.

(v) Alkaline comet

The potential of 2-cyclohexenone (No. 2052, purity ≥ 95%) to cause oxidative DNA damage was evaluated using a modified alkaline comet assay in V79 cells (Chinese hamster lung fibroblasts) *(49)* that provided no citation of GLP certification or QA. Following the 1-hour incubation in V79 cells, 2-cyclohexenone cultures displayed increased mean tail intensities, indicative of DNA damage, that were greater than 20% at 28.8 µg/mL (calculated with formula weight equal to 96.13 g/mol) (300 µmol/L) and were lower (5–7%) at 9.6 µg/mL (100 µmol/L). Cytotoxicity was less than 15% in all cultures. During the experiments, the cells were monitored for glutathione (GSH) depletion at 0, 1, 2 or 3 hours post-incubation, but only suspensions with viabilities greater than 80% were evaluated for GSH depletion. In V79 cells, 2-cyclohexenone at both concentrations (100 and 300 µmol/L) resulted in GSH depletion down to less than 20% of the control at the end of the 1-hour incubation (0-hour post-incubation) as previously reported *(64)* (Appendix 1, reference 203). At 3 hours post-incubation, increases in cellular GSH levels were reported for the other compounds tested, while GSH levels in 2-cyclohexenone cultures remained below 25% of the control. The contribution of an oxidative mechanism to DNA damage was assessed based on the sensitivity (increase) of the mean tail intensity to formamidopyrimidine-DNA glycosylase (FPG) activity. The presence of FPG-sensitive sites was evaluated at 0, 1, 2 and 3 hours post-incubation. The mean tail intensities following FPG incubation were comparable to control at 0 and 3 hours post-incubation with 2-cyclohexenone. A lack of increase in mean tail intensity following FPG treatment indicates the absence of either oxidation or alkylation mechanism on DNA by 2-cyclohexenone; 2-cyclohexenone therefore did not induce oxidative DNA damage. FPG has been reported to detect sites of both oxidative and alkylating modes of action *(65)*; a lack of sensitivity to FPG therefore also indicates the absence of both modes of DNA damage *(49)*.

Based on the absence of mutagenicity in the newly available Ames assay (above) and in assays evaluated at the previous meeting, and on the absence of clastogenicity in vivo reported in bone marrow micronucleus assays in mice and rats previously evaluated by the Committee (Annex 1, reference 202), there is no concern for genotoxicity of 2-cyclohexenone (No. 2052) when used as a flavouring agent.

(vi) Conclusions on genotoxicity

The additional data that are presented in the current addendum to the existing monographs for alicyclic ketones, secondary alcohols and related esters (Annex 1, references 161 and 202) show an absence of genotoxicity when members of this group are tested in assays following GLP that are consistent with

OECD guidelines using validated protocols. Representative flavouring agents of this group tested consistently negative in in vitro mutation assays conducted in *Salmonella typhimurium* and *Escherichia coli*, with and without metabolic activation, as well as in genotoxicity assays performed in mammalian cell lines. For 2-cyclohexenone (No. 2052), positive results were obtained in an in vitro Comet assay. However, based on the absence of mutagenicity in Ames assays, and on the absence of clastogenicity in vivo reported in bone marrow micronucleus assays in mice and rats previously evaluated by the Committee (Annex 1, reference 202), there is no concern for genotoxicity of 2-cyclohexenone (No. 2052) when used as a flavouring agent.

The newly presented data support the conclusion of the previous meetings that there is no concern for genotoxicity for alicyclic ketones, secondary alcohols and related esters when used as flavouring agents.

(d) Reproductive and developmental toxicity

(i) *trans*-4-*tert*-Butylcyclohexanol (No. 2263)

An oral toxicity study *(66)* to assess the developmental toxicity of *trans*-4-*tert*-butylcyclohexanol (batch no. 6, purity 95.82% *trans*- and 3.59% *cis*-isomers) was performed following OECD guideline no. 414 *(67)* and GLP. Groups of pregnant female Hsd-Han: Wistar rats (24 per group) received 0 (vehicle control), 150, 300 or 600 mg/kg bw per day of *trans*-4-*tert*-butylcyclohexanol in corn oil by gavage in a volume of 10 mL/kg bw. Dams were exposed during GD 5–19 for a total of 15 days. The test doses were determined based on the results of a preliminary range-finding study of pregnant females (seven per group) given doses of 0, 100, 300 or 1000 mg/kg bw per day on GD 5–19. Clinical signs of toxicity were observed at 1000 mg/kg bw per day, including hypoactivity, dehydration, hunched posture, wet perineum and decreased body weights and feed consumption. Pre-terminal deaths of 2/7 rats on GD 8 and 11 occurred. Because of these deaths, the treatment of high-dose dams in the range-finding study was terminated on GD 10–12, and the dose of 1000 mg/kg bw per day was not considered as the maximum tolerable dose.

No mortality was reported for the dams and no gross lesions were observed at necropsy. Treatment-related clinical signs of hypoactivity and/or salivation were observed in 21/24 high-dose dams on different days of gestation. Maternal body weight was not affected by the treatment with the test substance at the low or middle doses. The group means of maternal body weights were statistically significantly reduced on GD 11, 14, 17 and 20 in the high-dose group compared with the vehicle control group. However, this reduction in body weights for the high-dose group was less than 10% relative to the vehicle control group. The maternal body weight gain was statistically significantly reduced during

the treatment period and throughout gestation for the middle-dose (13%) and high-dose (22%) groups compared with the vehicle control group. In addition, no differences between treated and control dams were observed for maternal and litter parameters.

In the fetuses, there was an incidence of slight kidney renal pelvis dilation in the middle-dose group. This variation is commonly observed in colonies of rats used in the experiment and was therefore not attributed to treatment. There was an incidence of situs inversus (mirror image transposition of thoracic and abdominal viscera) in a fetus in the vehicle control group. This solitary incidence was considered an incidental finding. In all tested groups, skeletal variations routinely observed in rat fetuses were reported that were not considered toxicologically relevant. Statistically significant higher rates of hypoplatic sternum were reported in the middle- and high-dose groups, but these values were within the historical control data range and therefore considered not treatment related. A statistically significantly increased incidence of rudimentary rib was reported in the high-dose group (61.11%, compared with 44.06% for the control group) that was attributed by the study author to maternal stress, as evidenced by significant reduction in body weight and feed intake during the treatment period.

The Committee identified the NOAEL for maternal toxicity to be 150 mg/kg bw per day because of the clinical signs of toxicity and reductions in body weight gain for the middle and high doses, and the NOAEL for developmental toxicity to be 600 mg/kg bw per day, the highest dose tested, because of the absence of any adverse effects attributable to the test item in the offspring *(66)*.

(ii) 2-*sec*-Butylcyclohexanone (No. 1109)

A combined 28-day oral toxicity study *(16)* with the reproductive/developmental screening test with 2-*sec*-butylcyclohexanone (No. 1109; batch no. VE00226417, purity > 97.0%) was performed according to OECD guideline no. 422 *(26)* and GLP. Groups of Wistar Han rats (10 per sex per group) were given 2-*sec*-butylcyclohexanone in feed at dietary concentrations of 0, 650, 2000 or 6000 mg/kg equal to mean daily exposures of: 0, 50, 157 or 354 mg/kg bw per day, respectively, for males; 0, 46, 143 or 403 mg/kg bw per day, respectively, for females during pre-mating; 0, 46, 143 or 403 mg/kg bw per day, respectively, for females during mating; and 0, 72, 174 or 414 mg/kg bw per day, respectively, for females post-coitum. The mean daily intake was 0, 153, 400 or 832 mg/kg bw per day for females during lactation. The mean daily intake was equal to 0, 48, 151 or 377 mg/kg bw per day for males and 0, 88, 226 or 508 mg/kg bw per day for females of the F0 generation over the entire testing period. Male animals were exposed for 28 days in total, including 14 days before mating. Females were exposed for a total of either 42 days (if no offspring) or 51–56 days (those with

offspring), including 13 days before mating, during mating and gestation, and a minimum of 13 days after delivery. The pups were not treated directly after delivery. The part of the study on repeated dose toxicity testing is described in a previous section.

One control, two low-dose and one high-dose F0 females were not pregnant despite verification of mating and the absence of abnormalities of the reproductive organs. No morphological abnormalities of the reproductive organs were observed in either males or females, and no abnormal spermatogenesis was observed at any dietary level. The oestrous cycle length and regularity was adversely affected in 7/10 females of the high-dose group (six were acyclic and one was irregular); despite that, 9/10 females in this group had normal litters. Only one acyclic female was reported in the middle-dose group and one with extended di-oestrous in the control group, both consistent with background incidence. There was no adverse effect on mating index, pre-coital index or fertility index (90, 80, 100 and 90% for control, low-, middle- and high-dose groups, respectively) at any intake level. A slightly, not statistically significant, lower mean number of implantation sites in the high-dose group (9.7) compared with the control group (10.9) was observed, likely because of high variability in control numbers, but remained within the historical control range (mean: 12; 5–95% range: 5–16; n = 387; 2015–2017). No adverse effects on gestation index and duration, parturition, maternal care, live birth index or post-implantation survival index (90, 90, 93 and 94% for control, low-, middle- and high-dose groups, respectively) were observed. Mean live litter size was slightly reduced in the high-dose group (9.7, 10.3, 11.5 and 9.0 for control, low-, middle- and high-dose groups, respectively) but was within the historical control range (mean: 11.5; 5–95% range: 5.0–15.0; n = 484; 2015–2017). The small reduction in implantation sites and the parallel reduced number of live pups and litter size were not dose related and were not considered adverse, but were likely the result of reduced maternal body weight and feed intake *(68)*. Pup mortality, viability index and lactation index were also unaffected by treatment. Sex ratio in the low-dose group differed statistically significantly from the control ratio, but this finding was considered unrelated to treatment as the sex ratios in the middle- and high-dose groups were similar to the control ratio. Anogenital distance and areola and nipple retention were not different between treated and control animals. Pup body weights in the high-dose group were 10% lower than control pups at birth and up to 30% lower on PND 13, and differences were statistically significant after PND 4 for male and PND 7 for female pups. Pup body weight reduction was considered an adverse developmental effect and was associated with clinical signs of toxicity in the dams, although no direct correlation was found with reduced maternal body weights and feed consumption in the high-dose group. T4 was measured in the plasma of pups at PND 13–15. T4 levels were

comparable between treated and control pups; T4 was therefore not measured in PND 1 samples and TSH was not measured in any samples collected. No clinical signs of toxicity and no abnormal macroscopic observations were reported at any intake level in the pups.

A NOAEL of 2000 mg/kg feed, equal to 151 and 226 mg/kg bw per day for males and females, respectively, was identified for reproductive toxicity because of an oestrous cycle adverse effect in the high-dose group. A NOAEL of 2000 mg/kg feed, equal to 151 and 226 mg/kg bw per day for males and females, respectively, was identified for developmental toxicity, because of reduced pup weight in the high-dose group *(16)*.

(iii) 3-Methyl-2-(2-pentenyl)-2-cyclopenten-1-one (No. 1114)

A combined 28-day oral toxicity study *(17)* with the reproductive/developmental screening test with 2-*sec*-butylcyclohexanone with 3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one (referred to as Jasmone Cis, No. 1114; batch no. SC00011133, purity 87.7% for *cis* and 11.9% for *trans*-isomers) was conducted according to OECD guideline no. 422 *(28)*, related US EPA OPPTS guideline nos 870.3650, 870.3550 and 870.3050 *(27,29,30)*, EC regulation no. 440/2008 *(32)* and GLP. Groups of Crl:WI(Han) rats (10 per sex per group) were given 0, 1500, 5000 or 15 000 mg/kg of 3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one in feed. These dietary concentrations correspond to target mean daily exposures of 0, 100, 300 or 1000 mg/kg bw per day, respectively, but the actual exposure was not provided in the study report. Male animals were exposed for 28 days in total, including 14 days before mating. Females were exposed for 14 days before mating, during mating and gestation, and 4 days after delivery. (The study was performed prior to 2016 and according to 1996 OECD guideline no. 422 *(28)*, which called for dosing until day 4 postnatally. The Committee noted that, according to the current version of OECD guideline no. 422 *(26)*, exposure should be continued for at least 13 days of lactation.) Males and females from the same group were paired one-to-one for a maximum of 13 days (mating period). GD 0 was recorded as the day of sperm presence in vaginal smears. The part of the study on repeated dose toxicity testing is described in a previous section.

There was no treatment-related effect on mating performance, fertility or any reproductive parameter in either males or females. No differences in gestation length or maternal behaviour were noted between treated and control animals. The control group showed a higher number of implantation sites (15.0) than the historical control range (9.5–13.5), and a higher number of delivered pups (13.6) relative to the historical control range (8.7–12.5) and the exposure group ranges (11.0–12.1). Ten pups from two litters of stillborn pups of the high-dose group resulted in a reduced live birth index (91.2%) compared with the control group

(97.1%). There were also seven stillborn pups from two litters of the low-dose group. The number of stillborn pups was not dose dependent (number of stillborn pups: 4, 7, 0 and 10 in control, low-, middle- and high-dose groups, respectively) and was considered to be incidental. There were three females with total litter death, all from the high-dose group. The only gross observation in these animals was several ovarian cysts in one animal. This was confirmed histologically as a paraovarian cyst but, because of the single occurrence of the finding, it was not considered test-item related. Paraovarian cysts are seen occasionally in untreated rats, and the finding was not considered likely to have caused any reproductive problem. Litter loss (dead or missing pups) in the high-dose group during the postnatal period resulted in a markedly reduced viability index (69.9%) and mean litter size on PND 4 (7.2) compared with control (100% and 13.2, respectively) and historical control data for viability index (94.1–100%). No difference in sex ratio and no clinical signs of toxicity in the surviving pups were recorded. Pup weights were statistically significantly lower in the high-dose group for males on PND 1 and for both sexes (−17%) by PND 4. There were no macroscopic findings in pups associated with maternal treatment.

In agreement with the study author, the Committee identified the NOAEL for reproductive toxicity to be the highest dose tested of 15 000 mg/kg feed, equivalent to a target dose of 1000 mg/kg bw per day, in the absence of any adverse findings in reproductive parameters. The NOAEL for developmental toxicity was identified to be 5000 mg/kg feed, equivalent to a target dose of 300 mg/kg bw per day maternal exposure, because of fetal loss, pup mortality and reduced pup weights at the high dose consistent with the severity of maternal toxicity *(17)*.

(iv) 3,3,5-Trimethylcyclohexyl acetate (No. 2053)

A combined 28-day oral toxicity study with the reproductive/developmental screening test with 3,3,5-trimethylcyclohexyl acetate (No. 2053; batch no. 10300020, purity 99.7%) was performed following OECD guideline no. 422 (1996) and in compliance with GLP *(19)*. Groups of Sprague-Dawley (Crl:CD) SPF rats (12 per sex per group) received 0 (vehicle controls), 50, 150 or 500 mg/kg bw per day formulated in corn oil by gavage. Male animals were exposed for 6 weeks in total (42 days) including 2 weeks before mating, during mating and 2 weeks after. Females were exposed for 2 weeks before mating, during mating and gestation, and 5 days after delivery (approximately 8 weeks in total). (The study was performed prior to 2016 and according to 1996 OECD guideline no. 422 *(28)*, which called for dosing until day 4 postnatally. The Committee noted that, according to the current version of OECD guideline no. 422 *(26)*, exposure should be continued for at least 13 days of lactation.) Females showing

no evidence of mating were dosed daily for 26 days after the last day of mating. Additional animals of control and high-dose groups (six per sex per group) were treated for 6 weeks followed by a 2-week recovery period. Males and females from the same group were paired one-to-one for a maximum of 2 weeks (mating period). GD 0 was recorded as the day of sperm presence in vaginal smears.

A statistically significant increase in dam body weight was noted in the high-dose main group on PPD 0 (+9.7% versus control), which was considered related to the test substance. Increased feed consumption was reported in dams in the middle and high-dose groups during gestation (on GD 7 and/or GD 14). There were no statistically significant differences in mating index, gestation length, fertility index of either males or females, delivery success or sex ratios between treated and control animals. Differences in pre-implantation loss rates (25.0, 18.8, 18.4 and 20.1%), post-implantation loss rates (12.4, 3.5, 6.2 and 12.4%), live birth indices (87.7, 96.5, 93.8 and 87.6%), mean litter sizes (14.0, 15.6, 14.4 and 14.1), and viability indices on PND 0 (97.6, 100.0, 99.4 and 98.5%) and on PND 4 (98.9, 95.1, 98.7 and 89.1%) in control, low-, middle- and high-dose groups, respectively, were not dose related and were not considered toxicologically relevant or adverse. Female pup weights were statistically significantly higher in the high-dose group (21.3%) compared with control pups, and this effect was not considered adverse. There were no adverse findings in external gross examinations.

The author of the study identified the NOAEL to be the highest dose tested of 500 mg/kg bw per day for male reproductive toxicity and the middle dose of 150 mg/kg bw per day for female reproductive toxicity. The NOAEL for developmental toxicity was identified to be 500 mg/kg bw per day, based on the absence of adverse effects up to the highest dose tested *(19)*. The Committee noted that the NOAEL that was identified by the study author for reproductive toxicity in females was based on the statistically significantly high value in feed consumption and its associated increase in body weights at 500 mg/kg bw per day. Since these effects are not considered as adverse reproductive end-points, the Committee identified a NOAEL for reproductive toxicity of 500 mg/kg bw per day, the highest dose tested, for both males and females.

(v) Structurally related substances

To determine appropriate dose ranges to evaluate developmental toxicity, 4-*tert*-butylcyclohexyl acetate (batch no. 1003915, purity 99.3%) was tested in a pilot study that complied with GLP *(69)*. Groups of eight pregnant female Sprague-Dawley Crl:CD rats received 4-*tert*-butylcyclohexyl acetate at levels of 0 (vehicle), 37.5, 50, 150 or 300 mg/kg bw per day during GD 7–20 by gavage in corn oil. Animals were monitored twice daily for viability, and post-dosing clinical observations were conducted hourly for 4 hours during the first 3 days

and within 1–2 hours thereafter. Body weights of all animals were measured during the acclimation period, on GD 0, and daily during the dosing and post-dosing periods. Feed consumption was also measured on GD 0, 7, 10, 12, 15, 18, 20 and 21. On GD 21, scheduled necropsies on thoracic, abdominal and pelvic viscera were performed on all dams. Pups were delivered by caesarean section and litters were evaluated for corpora lutea, implantations, litter sizes, live fetuses, early and late resorptions, fetal body weights, percentage of resorbed conceptuses and percentage of live male fetuses. External examinations were conducted on all fetuses for gross developmental abnormalities, including malformations and variations (developmental delays or accelerations).

All dams survived until necropsy. Treatment-related clinical observations included slight excessive salivation and the higher prevalence of urine-stained abdominal fur at 50, 150 and 300 mg/kg bw per day. Decreased body weight gains were observed at these three higher-dose levels after the first two doses, associated with reduced feed consumption during the same period. There were no differences in body weight gains or feed consumption between treated and control groups after this. At necropsy, no dams presented any gross lesions and pregnancy was confirmed for all animals. There were no effects related to treatment with 4-*tert*-butylcyclohexyl acetate on litter parameters. In addition, fetuses did not present any gross external alterations that were treatment related. Based on these findings, dose levels of 50, 150 and 300 mg 4-*tert*-butylcyclohexyl acetate/kg bw per day were recommended for the main developmental toxicity study *(69)*.

An oral developmental and reproductive toxicity study *(70,71)* was conducted on 4-*tert*-butylcyclohexyl acetate (batch no. 1003915, purity 99.3%) in accordance with GLP and US EPA OPPTS guideline no. S5A *(72)*. Groups of pregnant female Sprague-Dawley Crl:CD rats (25 per group) received 4-*tert*-butylcyclohexyl acetate by corn oil gavage at dose levels of 0 (vehicle), 40, 160 or 640 mg/kg bw per day in corn oil on GD 7–20 at a volume of 10 mL/kg bw. The test doses were selected based on the absence of significant toxicity in the preliminary study with the exception of the inclusion of a higher dose, so maternal toxicity could be expected. Rats were monitored daily for viability, mortality and clinical observations. Body weight measurements were recorded prior to study initiation, on the day of confirmed pregnancy (GD 0) and daily during treatment. Feed consumption was recorded on GD 0, 7, 10, 12, 15, 18, 20 and 21. All animals were subject to necropsy and gross examination at study termination. Maternal organs were removed and weighed, and selected organs and tissues were fixed for potential histopathological examination. Caesarean sections were performed, and the number and distribution of corpora lutea were noted. All fetuses were evaluated for external abnormalities, and approximately one half was evaluated for soft tissue alterations and one half for skeletal alterations.

One dam of the high-dose group was euthanized on GD 20 because of severe clinical observations that were attributable to the test substance, including decreased motor activity, ptosis, red perioral substance, extreme excessive salivation and apparent dehydration. Body weight loss and decreased feed consumption were noted for this female on GD 18–20. Necropsy revealed distention of the stomach that was filled with gas and yellow fluid. The litter of this dam included 17 dead fetuses, with no apparent gross external or skeletal malformations. Soft tissue examination of 8 of the 17 dead fetuses revealed dilation of the renal pelvis in one or both kidneys (5/8), considered indicative of developmental delays for the gestational ages of the fetuses at death.

Statistically significantly increased incidences ($P < 0.05$ or $P < 0.01$) of slightly to moderately excessive salivation in the middle- and high-dose groups, respectively, were noted within 1–2 hours of test substance administration. Other clinical findings in high-dose animals included the presence of red perioral substance, sparse hair coat, ungroomed coat and localized alopecia on the limbs and/or neck. Throughout the gestation and/or treatment period, statistically significant reductions in maternal feed consumption, body weight gain ($P < 0.01$) and body weight ($P < 0.05$) were observed in the high-dose group only. Body weight loss occurred during GD 7–9 (after two administrations of test substance), while body weight gain was statistically significantly reduced during gestation (GD 15–18 and GD 18–21). No effects on feed consumption or body weight were recorded at lower dose levels.

Necropsies of maternal animals that survived until scheduled termination did not reveal any dose-dependent, treatment-related macroscopic findings. Pregnancy was confirmed in 24–25 animals in all groups, including the control group. Fetal body weights from the high-dose group (combined sexes) were statistically significantly lower ($P < 0.01$) than the control group (−11%). There were no effects on the numbers of corpora lutea, implantations, live fetuses, early and late resorptions, litter sizes, percentages of resorbed conceptuses, or ratio of live male to female fetuses. Transient delays in fetal development were noted for fetuses of high-dose dams, and included an increased incidence of renal pelvis enlargement in one or both kidneys and reversible delays in ossification of the caudal vertebrae, fore- and hindlimb phalanges, and hindlimb metatarsals. These findings were associated with the significant reductions in fetal body weight, and with body weight and feed consumption decreases in the dams. No other dose-dependent or treatment-related gross external, soft tissue or skeletal fetal alterations were reported in any group.

On the basis of these findings, the maternal reproductive NOAEL and the developmental NOAEL were both identified to be the middle dose of 160 mg/kg bw per day *(70,71)*.

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ANNEX 1

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

1. General principles governing the use of food additives (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
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218. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 69, 2014.

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

220. Evaluation of certain food additives (Seventy-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 990, 2015.

221. Safety evaluation of certain food additives. WHO Food Additives Series, No. 70, 2015.

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223. Evaluation of certain food additives and contaminants (Eightieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 995, 2016.

224. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 71, 2015.

225. Compendium of food additive specifications. FAO JECFA Monographs 17, 2015.

226. Evaluation of certain veterinary drug residues in food (Eighty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 997, 2016.

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

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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.

231. Compendium of food additive specifications. FAO JECFA Monographs 19, 2016.

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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.

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235. Safety evaluation of certain contaminants in food. WHO Food Additives Series, No. 74, FAO JECFA Monographs 19 bis, 2018.

236. Compendium of food additive specifications. FAO JECFA Monographs 20, 2017.

237. Toxicological 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.

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247. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 79, 2021.

248. Evaluation of certain food additives (Ninety-second report of the Joint FAO/WHO Expert Committee on Food Additives) WHO Technical Report Series, No. 1037, 2020.

ANNEX 2

Abbreviations and acronyms used in the monographs

AAU	α-amylase unit
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and elimination
ALAT	alanine aminotransferase
ATCC	American Type Culture Collection
BAMU	β-amylase unit
BLAST	basic local alignment search tool
bw	body weight
CAS	Chemical Abstracts Service
CCFA	Codex Committee on Food Additives
COVID-19	coronavirus disease
DM	dry matter
DSMZ	Deutsche Sammlung von Mikrooorganismen und Zellkulturen
EC	European Commission
EFSA	European Food Safety Authority
EHC	Environmental Health Criteria
FAO	Food and Agriculture Organization of the United Nations
FARRP	Food Allergy Research and Resource Program
FPG	formamidopyrimidine-DNA glycosylase
FUM	fumonisin B2
GD	gestational day
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practices
GSH	glutathione
HPBL	human peripheral blood lymphocytes
IgE	immunoglobulin E
IUIS	International Union of Immunological Societies
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	median lethal dose
LOAEL	lowest-observed-adverse-effect level
LU	lipase unit
MNBN	micronucleated binucleate
MOE	margin of exposure
MSDI	maximized survey-derived intake
NOAEL	no-observed-adverse-effect level

OECD	Organisation for Economic Co-operation
OPPTS	Office of Prevention, Pesticides and Toxic Substances (of USEPA)
OTA	ochratoxin A
PND	postnatal day
PPD	postpartum day
QA	Quality Assurance
RBL	rat basophilic leukaemia
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SPET	single portion exposure technique
T4	thyroxine
TASU	thermostable asparaginase units
TMDI	theoretical maximum daily intake
TOS	total organic solids
TSH	thyroid-stimulating hormone
US EPA	United States Environmental Protection Agency
USA	United States of America
WHO	World Health Organization

ANNEX 3

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

Virtual meeting 6–17 and 22 June 2022

Members

Dr S. Barlow, Brighton, East Sussex, United Kingdom

Dr J.N. Barrows, Office of Cosmetics and Colors, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), United States of America

Dr D. Benford, Cheddington, United Kingdom (*Vice-chairperson*)

Dr R. Cantrill, Bedford, Nova Scotia, Canada (*Chairperson*)

Dr E. Dessipri, General Chemical State Laboratory, Athens, Greece

Dr M. DiNovi, Baltimore (MD), USA

Dr D.E. Folmer, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), United States of America (*Joint rapporteur*)

Dr S.M.F. Jeurissen, Department for Food Safety, Centre for Nutrition, Prevention and Health, National Institute for Public Health and the Environment, Bilthoven, Netherlands (Kingdom of the)

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

Dr U. Mueller, Perth, Western Australia, Australia (*Joint rapporteur*)

Professor O.E. Orisakwe, University of Port Harcourt, Port Harcourt, Rivers State, Nigeria

Dr J. Schlatter, Zürich, Switzerland

Dr J.R. Srinivasan, United States Food and Drug Administration, College Park (MD), United States of America

Dr S.G. Walch, Executive Director, Chemisches und Veterinäruntersuchungsamt, Karlsruhe, Germany

Experts

Dr P.E. Boon, Department for Food Safety, Centre for Nutrition, Prevention and Health, National Institute for Public Health and the Environment, Bilthoven, Netherlands (Kingdom of the) (*FAO dietary exposure expert*)

Dr R. Dalefield, Food Standards Australia New Zealand, Wellington, New Zealand (*WHO expert*)

Professor M.B. de Abreu Gloria, Faculty of Pharmacy, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil (*FAO expert*)

Ms T. Hambridge, Food Standards Australia New Zealand, Majura Park, Australian Capital Territory, Australia (*FAO dietary exposure expert*)

Dr S.V. Kabadi, Division of Food Contact Substances, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), USA (*WHO expert*)

Dr F.-Q. Li, China National Center for Food Safety Risk Assessment, Beijing, China (*FAO expert*)

Dr J. Rotstein, Ottawa, Ontario, Canada (*WHO expert*)

Dr C. Smith, Food Directorate, Health Canada, Ottawa, Canada (*WHO expert*)

Professor I. Stankovic, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia

Dr S. Stice, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), United States of America (*WHO expert*)

Dr A. Tada, Division of Food Additives, National Institute of Health Sciences, Kanagawa, Japan (*FAO expert*)

Dr I. Toufeili, Department of Nutrition and Food Sciences, American University of Beirut, Beirut, Lebanon (*FAO expert*)

Dr S. West-Barnette, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park (MD), United States of America (*FAO expert*)

Dr H.J. Yoon, Korea Food and Drug Administration, Seoul, Republic of Korea (*WHO expert*)

Secretariat

Ms N.Y. Ho, Department of Nutrition and Food Safety, World Health Organization, Geneva, Switzerland (*WHO joint secretary*)

Dr M. Lipp, Food Systems and Food Safety Division, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO joint secretary*)

Mr K. Petersen, Department of Nutrition and Food Safety, World Health Organization, Geneva, Switzerland (*WHO joint secretary*)

Dr E. Rowan, Contin, United Kingdom (*WHO technical editor*)

Ms A. Vlachou, Food Systems and Food Safety Division, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO joint secretary*)

ANNEX 4

Toxicological information and information on specifications

Food additives evaluated toxicologically and assessed for dietary exposure

Food additive	JECFA enzyme identifier	Specifications	ADIs and other conclusions on toxicology and dietary exposure
α-Amylase from *Geobacillus stearothermophilus* expressed in *Bacillus licheniformis*	JECFA95-1	N, T	The Committee concluded that dietary exposure to this α-amylase is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 67 mg TOS/kg bw per day, the highest dose tested in a 13-week oral toxicity study in rats. When this NOAEL is compared with the dietary exposure estimate of 0.2 mg TOS/kg bw per day, a MOE of more than 330 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary ADI "not specified"[a] for α-amylase (JECFA95-1) from *G. stearothermophilus* expressed in *B. licheniformis*, when used in the applications specified, at the levels of use specified and in accordance with current GMP. This ADI "not specified" was made temporary because of the tentative nature of the specifications.
α-Amylase from *Geobacillus stearothermophilus* expressed in *Bacillus licheniformis*	JECFA95-2	N, T	The Committee concluded that dietary exposure to this α-amylase is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 660 mg TOS/kg bw per day, the highest dose tested in a 13-week oral toxicity study in rats. When this NOAEL is compared with the dietary exposure estimate of 0.08 mg TOS/kg bw per day, a MOE of more than 8000 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary ADI "not specified" for α-amylase (JECFA95-2) from *G. stearothermophilus* expressed in *B. licheniformis*, when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications.
α-Amylase from *Rhizomucor pusillus* expressed in *Aspergillus niger*	JECFA95-3	N, T	The Committee concluded that dietary exposure to this α-amylase is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 1400 mg TOS/kg bw per day, the highest dose tested in a 13-week oral toxicity study in rats. When this NOAEL is compared with the dietary exposure estimate of 4 mg TOS/kg bw per day, a MOE of more than 350 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary ADI "not specified" for α-amylase (JECFA95-3) from *R. pusillus* expressed in *A. niger*, when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications.

Food additive	JECFA enzyme identifier	Specifications	ADIs and other conclusions on toxicology and dietary exposure
Amyloglucosidase from *Rasamsonia emersonii* expressed in *Aspergillus niger*	JECFA95-4	N, T	The Committee noted that amyloglucosidase may pose a risk as a respiratory allergen. In the absence of any information regarding its stability within the gastrointestinal tract, the Committee could not complete the assessment of the risk for allergenicity from dietary exposure to this enzyme. The Committee identified a NOAEL of 1500 mg TOS/kg bw per day in a 13-week study of oral toxicity in rats. When this NOAEL, the highest dose tested, is compared with the conservative dietary exposure estimate of 9 mg TOS/kg bw per day, a MOE of more than 160 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary ADI "not specified" for amyloglucosidase (JECFA95-4) from *R. emersonii* expressed in *A. niger* when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications and the inability to complete the allergenicity assessment.
Asparaginase from *Pyrococcus furiosus* expressed in *Bacillus subtilis*	JECFA95-5	N, T	The Committee concluded that dietary exposure to the enzyme preparation is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 1207 mg TOS/kg bw per day, the highest dose tested, in a 13-week study of oral toxicity in rats. When this NOAEL is compared with the dietary exposure estimate of 0.4 mg TOS/kg bw per day, a MOE of more than 3000 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary ADI "not specified" for asparaginase (JECFA95-5) from *P. furiosus* expressed in *B. subtilis* when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications.
β-Amylase from *Bacillus flexus* expressed in *Bacillus licheniformis*	JECFA95-6	N, T	The Committee concluded that dietary exposure to the enzyme preparation is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 1199 mg TOS/kg bw per day, the highest dose tested, in a 13-week study of oral toxicity in rats. When this NOAEL is compared with the dietary exposure estimate of 1 mg TOS/kg bw per day, a MOE of around 1200 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee established a temporary ADI "not specified" for β-amylase (JECFA95-6) from *B. flexus* expressed in *B. licheniformis* when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications.
Lipase from *Thermomyces lanuginosus* and *Fusarium oxysporum* expressed in *Aspergillus oryzae*	JECFA95-7	N	The Committee concluded that dietary exposure to this lipase is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 1080 mg TOS/kg bw per day, the highest dose tested in the 13-week study of oral toxicity in rats. When this NOAEL is compared with the dietary exposure estimate of 0.2 mg TOS/kg bw per day, a MOE of more than 5000 can be calculated.

Food additive	JECFA enzyme identifier	Specifications	ADIs and other conclusions on toxicology and dietary exposure
			Based on this MOE and the lack of concern for genotoxicity, the Committee established an ADI "not specified" for lipase (JECFA95-7) from *T. lanuginosus* and *F. oxysporum* expressed in *A. oryzae* when used in the applications specified, at the levels of use specified and in accordance with current GMP.
Phospholipase A2 (PLA2) from porcine pancreas expressed in *Aspergillus niger*	JECFA95-8	No[b]	Because of the late submission of highly relevant toxicological data, other missing information and time constraints, the Committee was unable to complete this evaluation. The Committee recommended that the evaluation of this enzyme preparation is completed at a future meeting.
Xylanase from *Bacillus licheniformis* expressed in *Bacillus licheniformis*	JECFA95-9	N, T	The Committee concluded that dietary exposure to this xylanase is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 1020 mg TOS/kg bw per day, the highest dose tested, in the 13-week study of oral toxicity in rats. When this NOAEL is compared with the dietary exposure estimate of 0.01 mg TOS/kg bw per day, a MOE of more than 100 000 can be calculated. Based on this MOE and the lack of concern for genotoxicity, the Committee allocated a temporary ADI "not specified" for xylanase (JECFA95-9) from *B. licheniformis* expressed in *B. licheniformis* when used in the applications specified, at the levels of use specified and in accordance with current GMP. The ADI "not specified" was made temporary because of the tentative nature of the specifications.

ADI: acceptable daily intake; GMP: Good Manufacturing Practices; MOE: margin of exposure; N: new specification; NOAEL: no-observed-adverse-effect limit; T: tentative specification; TOS: total organic solids.
[a] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting for clarification of the term ADI "not specified".
[b] No specifications were prepared. Information is required to prepare specifications.

Food additives considered for specifications only

Food additive	Specifications
Spirulina extract (INS 134)	R

R: revised specification.

Flavouring agents evaluated by the revised Procedure for the Safety of Evaluation of Flavouring Agents

Alicyclic ketones, secondary alcohols and related esters

Flavouring agent[a]	No.	Specifications	Conclusion based on current estimated dietary exposure
Trans-4-*tert*-butylcyclohexanol	2263	N	No safety concern
Caryophylla-3(4),8-dien-5-ol	2264	N	No safety concern

N: new specification.
[a] Both flavouring agents are in structural class I.

ANNEX 5

Corrigenda

The Committee discussed the tentative errata. One request was for the amendment of the name of the microorganism *Geobacillus stearothermophilus* used for the Glucosyl Steviol Glycosides (GSG) production (Steviol Glycosides Framework, annex 4) by replacing the existing name of the microorganism with *Anoxybacillus caldiproteoliticus* (or adding it as an alternative name). The Committee decided to refer this request to a later meeting in order to allow for a more careful evaluation of possible implications of this requested name change.

The other requests for corrections (below), reported to the JECFA Secretariat, were evaluated by the Committee and found to be necessary. However, these corrections will only be made in the electronic versions available in the online database.

Requests for revisions and additions reported to the JECFA Secretariat

Substance	Original text	Revised text	Additional information
Saccharin	An ADI of 0–5 mg/kg bw for saccharin and its Ca, K, Na salts was established at 41st JECFA.	A group ADI of 0–5 mg/kg bw for saccharin and its Ca, K, Na salts, expressed as Na saccharin, was established at 41st JECFA.	The reporting basis for saccharins should be revised as "For saccharin and its Ca, K, Na salts, expressed as Na saccharin"
Paprika oleoresin *Monograph*	Functional uses: colour, flavouring agent	Functional uses: flavouring agent	Correct functional class
Lysozyme *Monograph*	Functional uses: preservative (mainly to prevent the late blowing of cheese caused by *Clostridium tyrobutyricum*)	Functional uses: processing aid for cheese production	Correct functional class
β-carotene, synthetic INS 160a(i) *Monograph*	In the "purity test", "procedure" section, the impurity at relative retention time of 0.85 currently reads **all-*trans*-□-carotene (0.85)** In the "purity test", "calculation" section, the formula is wrong; the multiplication sign should be replaced by a subtraction sign	It should read "all-trans-γ-carotene". $\frac{A_{\text{"total"}} - A_{\text{"β-carotenes"}}}{A_{\text{"total"}}} \times 100$	Transcription errors
Jagua blue *Monograph*	Synonyms: Jagua blue	Add synonyms. Synonyms: Jagua blue, Genipapo, huito blue, huito, jagua.	Improves understanding
	Name: "Jagua (genipin-glycine) blue (Jagua blue)"	Name: jagua (genipin-glycine) blue	Transcription error

Substance	Original text	Revised text	Additional information
Steviol glycosides *Framework*	"Reagents" section (page 11) - Mobile phase A: Deionized water, HPLC or LC-MS grade, filtered using a 0.2-µm filter, with 0.1% formic acid or acetic acid. (Note: If only UV detection will be used, 20 mM sodium phosphate buffer at pH 2.6 or 0.01% trifluoroacetic acid may be used.)	"Reagents" section (page 11) to be amended to read: "0.01% formic acid or acetic acid."	Transcription error
Steviol glycosides *Framework*	The Molecular Weight RRF values in table 2: Rebaudioside B: 0.82 Steviolbioside: 0.83	The Molecular Weight RRF values located in table 2 (pages 13, 14) to be amended: Rebaudioside B: 0.83 Steviolbioside: 0.66	Transcription error
Steviol glycosides *Framework*	Calculate the concentration of minor steviol glycosides: Conc. (% w/w) = CX x MX x 100 / MA x Csample	Conc. (% w/w) = CX x MX x 100 / (MA x Csample)	Transcription error
Steviol glycosides *Framework*	"Equilibration" Powdered samples should be equilibrated in the lab not less than 12 hours before assaying.	"Equilibration" "Powdered samples and powdered standards should be equilibrated in the lab not less than 12 hours before assaying." Addition of Note: "An unopened reference standard with moisture listed on a Certificate of Analysis may be used without equilibrating."	Item for discussion: request for amendment to and addition of a note to the "Equilibration" section.
Steviol glycosides *Framework*	"Equilibration" The loss on drying of the equilibrated sample should be determined concurrently with performing the assay using the conditions in Annexes 1–4 (Vol. 4).	"Equilibration" "The loss on drying of the equilibrated sample should be determined concurrently with performing the assay using the conditions in Annexes 1–4 (Vol. 4). Karl Fischer titration may be used as an alternative to loss on drying for determining moisture of equilibrated samples and standards when performing the assay."	Item for discussion: request for addition of Karl Fischer titration as alternative

This volume contains monographs prepared at the ninety-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met virtually during 6–17 and 22 June 2022.

The toxicological and dietary exposure monographs in this volume summarize the safety and dietary exposure data on eight specific food additives: α-amlyase (JECFA95-1 and JECFA95-2) from *Geobacillus stearothermophilus* expressed in *Bacillus licheniformis*; α-amlyase (JECFA95-3) from *Rhizomucor pusillus* expressed in *Aspergillus niger*; amyloglucosidase (JECFA95-4) from *Rasamsonia emersonii* expressed in *Aspergillus niger*; asparaginase (JECFA95-5) from *Pyrococcus furiosus* expressed in *Bacillus subtilis*; β-amylase (JECFA95-6) from *Bacillus flexus* expressed in *Bacillus licheniformis*; lipase (JECFA95-7) from *Thermomyces lanuginosus* and *Fusarium oxysporum* expressed in *Aspergillus oryzae*; and xylanase (JECFA95-9) from *Bacillus licheniformis* expressed in *Bacillus licheniformis*. An addendum summarizes the safety and dietary exposure data on a group of related flavouring agents (alicyclic ketones, secondary alcohols and related esters).

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, those involved in the control of contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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