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1056

Evaluation of certain food additives

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

Food and Agriculture Organization of the United Nations

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

Geneva, 11–20 June 2024

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List of acronyms and abbreviations

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and elimination
AGDU	α-glucosidase units
AMP	adenosine-5′-monophosphate
AMPDU	adenosine-5′-monophosphate deaminase unit
aPTT	activated partial thromboplastin time
BDXU	birchwood D(+)-xylanase unit
bw	body weight
CAS	Chemical Abstracts Service
CCFA	Codex Committee on Food Additives
CIFOCOss	FAO/WHO chronic individual food consumption database
EC	Enzyme Commission
EFSA	European Food Safety Authority
EHC 240	Environmental Health Criteria 240
FAO	Food and Agriculture Organization of the United Nations
FSANZ	Food Standards Australia New Zealand
GMP	Good Manufacturing Practices
GSFA	General Standard for Food Additives
HPLC	high-performance liquid chromatography
IARC	International Agency for Research on Cancer
IMO	isomalto-oligosaccharide
IMP	inosine-5′-monophosphate
INS	International Numbering System for Food Additives
JECFA	Joint FAO/WHO Expert Committee on Food Additives
ML	maximum level
MOE	margin of exposure
MS	mass spectrometry
MSDI	maximized survey-derived intake
MSG	monosodium glutamate
NOAEL	no-observed-adverse-effect level
NSR	nisin resistance protein
NTXU	new thermostable endoxylanase unit
PCR	polymerase chain reaction
RTD	ready-to-drink
TGU	transglucosidase activity unit
TMDI	theoretical maximum daily intake
TOS	total organic solids
uHPLC	ultra-high-performance liquid chromatography
USA	United States of America

WFBI	Westerdijk Fungal Biodiversity Institute
WHO	World Health Organization

1. Introduction

The Ninety-ninth meeting of the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) met in Geneva from 11 to 20 June 2024. The meeting was opened on behalf of WHO and FAO by Dr Moez Sanaa, Head of Standards and Scientific Advice on Food and Nutrition, Department of Nutrition and Food Safety, and Ms Angeliki Vlachou, FAO Joint Secretary, Food Safety Officer, Agrifood Systems and Food Safety Division, respectively.

Dr Sanaa extended a warm welcome to the experts attending the Ninety-ninth meeting and expressed gratitude for their valuable time and expertise dedicated to JECFA. He emphasized that the scientific advice provided by JECFA plays a pivotal role in ensuring the safety, quality and integrity of the global food supply. The contribution of JECFA to the field of food safety is of utmost significance, as its evaluations and recommendations not only protect public health but also establish international food standards that facilitate global trade. The impactful work of JECFA reaches millions of consumers and fosters trust in the food supply chain. Dr Sanaa sincerely thanked each expert for their exceptional commitment, voluntary contributions and unbiased approach driven by a passion for science and public service, which form the bedrock of the success of JECFA. The dedication and expertise demonstrated by JECFA experts exemplify the highest standards of professional excellence and integrity. The Committee was reminded of the critical importance of upholding rigorous scientific standards, and ensuring that debates and discussions are grounded in solid evidence and reflect the latest scientific advancements and innovations.

Ms Vlachou welcomed the experts to the meeting, expressing her deep gratitude for their acceptance of the invitation to participate. She acknowledged their valuable time and expertise dedicated to JECFA and commended their efforts in preparing for this meeting. Ms Vlachou encouraged the experts to engage in open and constructive dialogue based on the core United Nations values. She underscored that the scientific advice provided by JECFA ultimately ensures that food safety, quality measures and standards are based on sound scientific principles. Ms Vlachou also highlighted that the work of JECFA, along with the provision of scientific advice in other areas of food safety, remains a top priority for the FAO.

1.1 Declarations of interests

The Joint Secretariat informed the Committee that all experts participating in the Ninety-ninth meeting had completed declaration of interest forms. No conflicts of interest were identified.

1.2 Adoption of the agenda

After discussion among the experts, the agenda was amended to (i) change the names of three of the enzymes to: adenosine-5′-monophosphate deaminase from *Aspergillus* sp.; endo-1,4-β-xylanase from *Rasamsonia emersonii* expressed in *Aspergillus niger*; and glucosidase from *Aspergillus niger* expressed in *Trichoderma reesei* exhibiting α-glucosidase and transglucosidase activity; (ii) change the name of nisin to nisin A; (iii) reorder the agenda items alphabetically by amended name; and (iv) add JECFA enzyme numbers as decided at the Ninety-fifth JECFA meeting *(1)*. These changes were reflected in both the report and relevant monographs. The meeting agenda was adopted with no further modifications (Annex 1).

1.3 Meeting summary

See Annex 2 for a summary of food additives and processing aids discussed, as well as specifications revised.

Reference

1. Evaluation of certain food additives and contaminants: ninety-fifth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2023 (WHO Technical Report Series, No. 1042, https://iris.who.int/handle/10665/370106, accessed 1 July 2024).

2. General considerations

2.1 Lack of data for food additives prioritized by the Codex Committee on Food Additives (CCFA) for re-evaluation by JECFA

During the meeting, the Committee noted that CCFA prioritized certain food additives for JECFA re-evaluation. The Committee was extremely disappointed to find that no new data on the microbiological effects were submitted for natamycin and nisin of relevance to the request from CCFA. In addition, no new toxicological data were submitted for nisin. For polyglycerol esters of fatty acids, no new toxicological data were submitted or found in a literature search.

The Committee would like to remind CCFA of the limited resources of JECFA, and recommends that CCFA place greater emphasis on ensuring the availability of new data before a food additive is prioritized for JECFA re-evaluation.

2.2 Mapping food categories of the General Standard for Food Additives (GSFA) to the FoodEx2 classifications

At its Eighty-ninth meeting, the Committee concluded that an appropriately refined dietary exposure assessment for Sucrose esters of fatty acids (INS [International Numbering System for Food Additives] No. 473) and Sucrose oligoesters, type I and type II (INS No. 473a) could not be undertaken using the FAO/WHO chronic individual food consumption database (CIFOCOss) because of the inability to map it to the large number of food categories with use levels provided. It was concluded that food category mapping between the FoodEx2 categories *(1)* used for the food consumption data and GSFA food categories was needed. This issue with calculations of exposure also arose at the current meeting for the dietary exposure assessment of Polyglycerol esters of fatty acids (INS No. 475).

The Committee is aware of the work currently being undertaken by a group of CCFA members to map the GSFA food categories to the FoodEx2 food classification system, and requests that the mapping be finalized as soon as practicable.

The mapping, together with submissions of food industry data on uses and use levels for food additives under evaluation by the Committee, will enable more refined estimates of dietary exposure to be undertaken for a greater number of countries. This will inevitably better support the CCFA by providing clear conclusions on the safety assessments of food additives and will assist in the establishment of its priority list of food additives for re-evaluation by JECFA.

2.3 Enzyme submissions

The Committee reiterated the conclusions from the Ninety-fifth meeting *(2)* that, when considering enzymes as processing aids, the submissions from the sponsor did not always conform to the requirements set out in the appendix of section 9.1.4.2 of the second edition of *Principles related to specific groups of substances*, chapter 9 of Environmental Health Criteria 240 (EHC 240) *(3)*. The Committee recommends that sponsors use the checklist (provided in Annex 3) and supply the requested information, at a minimum as a link to the required information, among their submission documents. The Committee asked the JECFA Secretariat to include a reference to the checklist in future calls for data for enzymes.

Sponsors are reminded of the requirement to provide a statement detailing the enzyme activity as per the checklist. To clarify, this statement should take the following format: "One unit of XX enzyme activity is defined as the amount of enzyme required to convert one (1) μmole of substrate to product per minute under the conditions of the test". The method that is submitted should be sufficiently detailed to be easy to apply in any laboratory; it should not require unique or expensive equipment (such as an autoanalyser), a calibrant with unique assigned activity or other restricted substances.

References

1. The food classification and description system FoodEx2 (revision 2). Parma: European Food Safety Authority; 2015.
2. Evaluation of certain food additives and contaminants: ninety-fifth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2023 (WHO Technical Report Series, No. 1042, https://iris.who.int/handle/10665/370106, accessed 1 July 2024).
3. Section 9.1.4.2. Enzymes. Chapter 9. Principles related to specific groups of substances, second edition. In: Environmental health criteria 240. Rome: Food and Agriculture Organization of the United Nations; Geneva: World Health Organization; International Programme on Chemical Safety (IPCS); 2020 (https://www.who.int/docs/default-source/food-safety/publications/section9-1-4-2-enzymes.pdf?sfvrsn=e238e86e_2, accessed 3 July 2024).

3. Specific food additives (other than flavouring agents)

3.1 Safety evaluations

3.1.1 Adenosine-5′-monophosphate deaminase (JECFA99-1) from *Aspergillus sp.*

Explanation

At its Fifty-third Session the CCFA *(1)* requested that JECFA evaluate the safety of adenosine-5′-monophosphate (AMP) deaminase (Enzyme Commission [EC] No. 3.5.4.6; CAS [Chemical Abstracts Service] No. 9025-10-9) from a non-genetically modified filamentous fungus *Aspergillus oryzae* DEA 262 for use as a processing aid. The present Committee allocated the unique JECFA enzyme identifier *(2)*[1] JECFA99-1 to this enzyme preparation.

However, the Committee did not have adequate information to confirm the identity of the production organism, as discussed below. For the current evaluation, the Committee therefore referred to the enzyme as AMP deaminase (JECFA99-1) from *Aspergillus* sp. Consequently, the Committee could not determine which class this enzyme belongs to, in accordance with the criteria described in EHC 240 *(3)*.

The previous Committee evaluated an AMP deaminase from *Streptomyces murinus* at its Eighty-ninth meeting *(4)*[2] for which an ADI "not specified" *(5)*[3] was established.

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

The enzyme catalyses the hydrolytic deamination of AMP to produce inosine-5′-monophosphate (IMP) and free ammonia. The enzyme preparation is intended for use as a processing aid in the production of nucleotide-rich foods and food ingredients, specifically fish hydrolysates from fish roe/tissues, vegetable or fruit pastes/purees (including tomato purees), yeast extracts, fruit juices and juice concentrates, and yeast extracts that can be added to a wide range of foods (e.g. breads, cakes, cookies, yogurts, fresh cream, ganache, Asian-style sauces, soups, ice cream and custard products).

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[1] At its Ninety-fifth meeting the Committee decided that an identification system would be used for all enzyme preparations, consisting of the JECFA meeting number followed by the number reflecting the order of the enzyme in the report.

[2] A full list of JECFA publications is provided as Annex 4.

[3] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting for clarification of the term ADI "not specified".

The Committee evaluated the submitted data, but a literature search could not be conducted because of the lack of information about the production organism.

Genetic background

The sponsor identified the production strain as *Aspergillus oryzae* DEA 262 and deposited it in August 2005 at the Fungal Biodiversity Centre (renamed in 2017 as the Westerdijk Fungal Biodiversity Institute or WFBI) of the Royal Netherlands Academy of Arts and Sciences, Utrecht. In response to the Committee's request for more information about the identity of the production organism, the sponsor provided an identification report for *Aspergillus* strain DEA 56-111 by WFBI (dated August 2022). The strain DEA 56-111 was provided to WFBI by the sponsor. WFBI identified strain DEA 56-111 as *A. sojae* or *A. parasiticus* based on results from polymerase chain reaction (PCR) analyses of the calmodulin and the β-tubulin genes. WFBI also reported results from metabolite tests at the Petri-dish level indicating that strain DEA 56-111 did not produce 26 mycotoxins, including four aflatoxins, a characteristic that differentiates *A. sojae* from *A. parasiticus*. Combining the results from the PCR and metabolite analyses, WFBI concluded that the *Aspergillus* strain DEA 56-111 is *A. sojae*. However, the Committee noted that the test to identify metabolites at the Petri-dish level may not represent metabolite production under manufacturing conditions.

The Committee noted that the sponsor deposited the *Aspergillus* strain DEA 56-111 at the National Institute of Technology and Evaluation Biological Resource Center, Japan as *A. sojae* DEA 56-111 in November 2022.

The Committee was not provided with any data to demonstrate that the *Aspergillus* strain DEA 56-111 is the same as DEA 262, the production strain used to manufacture JECFA99-1. Additionally, the Committee could not conclude whether the identity of the *Aspergillus* strain DEA 56-111 is *A. parasiticus or A. sojae*, based on: (i) the equivocal results from the phylogenetic analysis based on sequenced PCR fragments reported by WFBI; and (ii) a lack of data to support the absence of toxic metabolites under manufacturing conditions using the *Aspergillus* strain DEA 56-111.

The Committee therefore did not have adequate information to confirm the identity of the production organism.

Chemical and technical considerations

The AMP deaminase is produced by pure culture fermentation under controlled conditions. Manufacture of the AMP deaminase enzyme preparation includes fermentation, recovery/purification and formulation. After fermentation, the enzyme is recovered, purified and concentrated. The resulting enzyme concentrate may be spray- or freeze-dried, formulated and standardized into a solid enzyme

preparation. The entire process is performed in accordance with current Good Manufacturing Practices (GMP) with food-grade raw materials.

The primary sequence of the enzyme consists of 560 amino acids with a calculated molecular weight of 63.2 kDa. The enzyme is not expected to have any significant secondary catalytic activity.

AMP deaminase catalyses the hydrolytic deamination of AMP to produce IMP and free ammonia expressed as AMP deaminase unit (AMPDU). One AMPDU is defined as the amount of enzyme required to hydrolyse AMP substrate to produce 1 μmol of IMP per minute under specified conditions (pH 5.5, T = 30 ± 0.5 °C, 10 min). The mean activity from three batches of the solid enzyme concentrate was 2613 U/g, and 2540 U/mL for one batch of liquid enzyme concentrate.

The enzyme preparation is intended for use as a processing aid in the production of nucleotide-rich foods and food ingredients. The recommended maximum level of AMP deaminase is 85.6 mg total organic solids (TOS)/kg food substrate.

The enzyme is heat-inactivated during food processing and is therefore not expected to have any technological function in the final food.

Biological data

Assessment of potential allergenicity

The Committee evaluated the potential for allergenicity of the enzyme preparation based on a homology search of the amino acid sequence of this enzyme with those of known allergens using the AllergenOnline *(6)* and Allergen *(7)* databases, according to bioinformatics criteria recommended by EHC 240 *(3)*. A search for matches with 35% or more 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[4] of 0.1 or less did not identify a homology to any known allergens. The Committee noted that the enzyme would be heat-denatured under conditions of food processing. No data relevant to the digestibility of the enzyme preparation were submitted.

The Committee concluded that the dietary exposure to the enzyme preparation is not anticipated to pose a risk for allergenicity.

Toxicological studies

The Committee reviewed data from a 13-week oral toxicity study in rats and three genetic toxicity studies. The reports of the 13-week oral toxicity study *(8)*, the bacterial reverse mutation test *(9)* and the in vitro chromosome aberration

[4] The E-value selected for a search tends to be larger for searches for short sequences ($E < 0.1$) than for long sequences ($E < 1 \times 10^{-7}$), as the likelihood of random matches is greater in the search for shorter sequences.

assay *(10)* stated that the enzyme concentrate was derived from *A. oryzae*. The report of the in vitro micronucleus test *(11)* stated that the enzyme concentrate was derived from *A. sojae*.

The Committee evaluated the submitted toxicity studies. However, the Committee could not confirm the identity of the production organism that the enzyme is derived from or whether the test material used in the studies is representative of the current article of commerce.

In the 13-week study in rats *(8)*, the enzyme concentrate was administered by gavage at dose levels of up to 1984 mg TOS/kg body weight (bw) per day. Based on the absence of any treatment-related adverse effects, the Committee identified a no-observed-adverse-effect level (NOAEL) of 1984 mg TOS/kg bw per day, the highest dose tested.

The enzyme concentrate was negative in the bacterial reverse mutation test *(9)*, the in vitro chromosome aberration assay *(10)* and the in vitro micronucleus assay *(11)*. The Committee did not identify a concern for genotoxicity based on the results for the materials tested.

A comparison of the amino acid sequence of the enzyme with those of known protein toxins *(12)* revealed no biologically relevant homology. The Committee therefore concluded that the enzyme was unlikely to be a toxin.

Assessment of dietary exposure

The Committee evaluated the dietary exposure to this enzyme preparation, proposed for use in the production of nucleotide-rich foods and food ingredients.

The Committee reviewed a conservative estimate of dietary exposure derived using the budget method based on default assumptions and use levels of 85.6 mg TOS/kg of solid food and non-milk beverages. The theoretical maximum daily intake (TMDI) was estimated to be 2.68 mg TOS/kg bw per day.

The Committee also reviewed a refined estimate of dietary exposure from the European Food Safety Authority (EFSA) based on the same use levels and consumption data for individuals from the Comprehensive European Food Consumption Database *(13)*. The highest estimated dietary exposure across all of the countries and population groups assessed was 0.005 mg/kg bw per day for children (age 3–9 years) at the 95th percentile.

For the dietary exposure assessment, it was assumed that 100% of the enzyme remains in the final food. The Committee noted that the enzyme is heat-inactivated during the processing of food and food ingredients.

The Committee concluded that the dietary exposure estimate of 0.005 mg TOS/kg bw per day was appropriate for use in the evaluation.

Evaluation

The Committee concluded that dietary exposure to this AMP deaminase enzyme preparation is not anticipated to pose a risk for allergenicity.

The Committee identified a NOAEL of 1984 mg TOS/kg bw per day, the highest dose tested, in a 13-week study in rats.

The Committee did not identify a concern for oral toxicity or genotoxicity based on the submitted studies.

Because of a lack of information to confirm the identity of the production organism or whether the test material used in the toxicity studies is representative of the current article of commerce, the Committee could not complete the safety evaluation of this enzyme preparation.

A toxicological monograph with a dietary exposure assessment was prepared.

A specifications monograph and a chemical and technical assessment based on the data submitted by the sponsor were drafted, but could not be finalized for publication because of the lack of data regarding the identity of the production organism.

Recommendations

The Committee requires the following information to be submitted before the enzyme preparation can be considered for review at a future meeting:

- results from whole genome sequencing, using appropriate technologies, to confirm the identity of the current production organism (genus, species and strain);
- data demonstrating that the current large-scale production conditions do not lead to the synthesis of toxic secondary metabolites;
- data demonstrating multigenerational stability of the current production organism;
- results from five batches of the article of commerce produced by the current production organism showing the absence of mycotoxins;
- a robust method of enzyme activity assay using commercially available standards that does not use a proprietary enzyme as a calibrant; and
- data to determine whether the batches of test materials used in the already submitted toxicological studies are representative of the current article of commerce.

3.1.2 Butterfly pea flower extract

Explanation

Butterfly pea flower extract is an anthocyanin-containing, blue food colouring agent prepared by the ultra-filtered aqueous extraction of dried *Clitoria ternatea* flowers.

Butterfly pea flower extract was placed on the agenda of the present meeting at the request of the Fifty-third Session of the Codex Committee on Food Additives *(1)*. A submission was received in response to the call for data, which included data on specifications, toxicity and dietary exposure.

The previous Committee had not evaluated butterfly pea flower extract. However, the previous Committee had evaluated several food colours derived from natural sources *(4,14–18)*, including anthocyanin-containing food colours *(5,19)*.

There is information in the literature for several anthocyanin-containing plant extracts *(20)*. The anthocyanin content of plant extracts will vary based on many factors, including growing conditions, different parts of the plant and extraction methods. Anthocyanins were considered at the Eighty-seventh JECFA meeting *(5)* where another anthocyanin-containing food colour was evaluated (black carrot extract), and it was concluded that "the effects observed with one anthocyanin-containing test material cannot be extrapolated to another anthocyanin-containing test material based on the available information".

The focus of the present evaluation was *C. ternatea* flower extracts (principally aqueous extracts) as well as information on the principal anthocyanins (delphinidin derivatives) and flavonols (quercetin and kaempferol derivatives) identified in butterfly pea flower extract.

In response to the call for data, the results of a literature search conducted on 9 October 2023 were provided to the Committee *(20)*. In addition, a systematic literature search up to January 2024 was conducted by the Committee using PubMed, Embase, Medline, Global Health, CAB Abstracts, Food Science and Technology Abstracts, and the Cochrane Central Register of Controlled Trials, with search terms related to various toxicological end-points (e.g. "toxic*", "adverse*", "cancer*", "gene*", "pharmacokinetic*", "adverse events" and "allergens") pertaining to "butterfly pea flower extract" and "*Clitoria ternatea*".

Chemical and technical considerations

C. ternatea (commonly known as butterfly pea) is a perennial leguminous plant belonging to the Fabaceae family *(21,22)*. Although its native origin is not clear, butterfly pea grows optimally in tropical and subtropical climates. The plant produces pentamerous, conch- or pea-shaped flowers used to colour foods and drinks *(23,24)*.

Butterfly pea flower extract is the aqueous extract of dried flowers. The residual plant material is removed by filtration from the aqueous extract, which is further processed by ultrafiltration to remove residues of high molecular weight (> 2500 Da), for example, plant proteins. The extract is then concentrated to a liquid product that is standardized on colour intensity with an anthocyanin content of approximately 2%, and subsequently pasteurized.

Butterfly pea flowers contain high levels of anthocyanins, which are water-soluble polyphenols *(25)*. They are composed of anthocyanidins, which are based on the 2-phenyl-1-benzopyrylium chromophore (flavylium cation; CAS No. 14051-53-7) *(5)*. The primary chromophore in butterfly pea flower extract is delphinidin, which is responsible for the blue colour. The delphinidin derivatives include polyacylated glucose chains of various lengths or glucosyl groups at the 3′ and 5′ positions (ternatins) *(26)* that alter the shade of the blue colour *(25,27)*. Thirty-four delphinidin derivatives, separated and quantitated by ultra-high-performance liquid chromatography (uHPLC)–photodiode array–mass spectrometry (MS)/MS, were identified with various degrees of acylation with coumaric and/or malonic acid *(25,28–31)*. The most prominent are Dp-tri-glu+(cou-glu)-di-cou+malonic (Ternatin D2) and Dp-tri-glu+tri-(cou-glu)-cou+malonic (Ternatin B1).

The delphinidin derivatives were also characterized by alkaline saponification to remove acyl groups and by acid hydrolysis to remove acyl and sugar groups, followed by uHPLC-MS/MS analysis. Analysis of an alkali-saponified sample of butterfly pea flower extract resulted in peaks representing delphinidin-tri- and delphinidin-di-glycoside. Analysis of an acid-hydrolysed sample of butterfly pea flower extract resulted in peaks representing delphinidin-3-glucoside and delphinidin *(25,28,29)*. The Committee determined that an assay based on the quantification of the alkali-saponified and acid-hydrolysed products was appropriate.

The product of commerce consists of 42–62% water, 22–43% carbohydrates, 8–12% proteins, 4–7% ash, 4–6% total polyphenols (1.5–1.9% anthocyanins and 2.0–3.6% flavonols) and 0.1–0.2% lipids.

Biochemical aspects

Butterfly pea flower extract is a complex and variable mixture of substances. In particular, butterfly pea flower extract contains 34 anthocyanins and eight flavonols; however, the proportions of the anthocyanins differ between batches of the commercial product, and some of the anthocyanins and flavonols detected in the commercial product were not detected in the batches used for biochemical and toxicological testing. It is not possible to determine whether the test materials were representative of the article of commerce.

No information concerning the absorption, distribution, metabolism or elimination of butterfly pea flower extract was available. The Committee noted that analysis of a limited number of urine samples from the submitted short-term toxicity test in male and female rats, exposed to butterfly pea flower extract in the diet for 4 days at 3201 or 3549 mg/kg bw per day, respectively (test material not completely characterized), resulted in "greenish"-coloured urine. Anthocyanin was detected in the urine of treated animals at concentrations ranging from 2.2 to 8.4 mg/L *(32)*.

Anthocyanins are absorbed in the stomach or small intestine, potentially via active transporters, and absorption appears dependent on the size of the molecule, the type of sugar moiety, the degree of acylation and the dietary matrix *(33)*. Absorption of the flavonol components of butterfly pea flower extract (i.e. quercetin and kaempferol derivatives) is anticipated to be low *(34,35)* but will likely depend on the food matrix *(36)*. In silico estimates of bioavailability for components of *C. ternatea* flower suggest that, although the glycosidic forms of delphinidin, quercetin and kaempferol may show low potential for oral absorption, the aglycones may show higher absorption, based partially on the smaller molecular weights and lower water solubility of the aglycones *(37,38)*.

Toxicological studies

No acute oral toxicity studies were available for butterfly pea flower extract. Quercetin is well tolerated in rats at dietary doses of approximately 1900 mg/kg bw per day *(39)*. Low acute oral toxicity has been reported for kaempferol in mice (lethal dose [LD_{50}] > 10 000 mg/kg bw) *(40)*.

In a 90-day study, rats were fed diets containing butterfly pea flower extract (test material not completely characterized) at doses of 0, 99, 1735 or 3201 mg/kg bw per day in males and 0, 102, 1740 or 3549 mg/kg bw per day in females *(41)*. The only potentially toxicologically relevant findings noted by the Committee were statistically significant ($P < 0.05$) increases in activated partial thromboplastin time (aPTT) in the high-dose males (10% increase) and females (13% increase), as well as in the mid-dose females (12% increase). However, since the prolonged clotting times in the treated animals were relatively small compared with the range of concurrent and reported historical control aPTT values, the Committee concluded that these effects were not adverse. Overall, the Committee identified a NOAEL of 3201 mg/kg bw per day, the highest dose tested in male rats.

The Committee noted mechanistic studies at high intravenous doses/concentrations showing that delphinidin, quercetin and kaempferol can have anticoagulant effects *(42–44)*.

No information concerning the chronic oral toxicity of butterfly pea flower extract was available. Quercetin has been shown to induce cancer in

two rat studies *(39,45)*; however, the carcinogenic responses appear to be strain dependent. For example, in the earlier study, quercetin induced intestinal and urinary bladder tumours at dietary concentrations of 1000 mg/kg feed (equal to approximately 50 mg/kg bw per day) in male and female albino rats *(45)*. In the later study, quercetin induced renal tubular neoplasms (primarily adenomas) in male F344/N rats at dietary concentrations of 40 000 mg/kg feed (equal to approximately 1900 mg/kg bw per day) *(39)*. These observations were possibly related to exacerbated chronic progressive nephropathy, an effect with no relevance to humans *(46)*. Notably, Takanashi et al. *(47)* reported that quercetin showed no evidence of carcinogenicity in male and female ACI rats exposed to 1000 mg/kg feed (equivalent to 50 mg/kg bw per day) in the diet for 540 days. Takanashi et al. *(47)* also report that no treatment-related tumours were observed following chronic dietary exposure of male and female ACI rats to kaempferol at concentrations of 400 mg/kg feed (equivalent to 20 mg/kg bw per day) for 540 days.

No toxicologically relevant increases in mutations were observed in the bacterial reverse mutation assay, and no toxicologically relevant structural and numerical chromosomal aberrations were observed in the in vitro micronucleus assay with butterfly pea flower extract (test materials not completely characterized) *(48,49)*. Although delphinidin inhibits topoisomerase at concentrations of as low as 2.5 μM and has produced positive results in the comet assay at relatively high concentrations (> 50 μM) *(50)*, the Committee noted that the comet assay is only an indicator assay for genotoxicity and that there are no definitive genotoxicity results for delphinidin. Although positive genotoxicity results have been reported for quercetin and kaempferol in vitro, neither flavonol is genotoxic in vivo *(51,52)*. Overall, the Committee concluded that the available studies on butterfly pea flower extract do not raise a concern for genotoxicity in vivo.

No information concerning the reproductive and developmental toxicity of butterfly pea flower extract was available. At a relatively high dose of quercetin (2000 mg/kg bw per day), reduced fetal weight was observed in rats following exposure on gestation days 6–15 *(39,51)*.

It has been suggested that polyphenols can generally exert both estrogenic and antiestrogenic effects via differential binding to estrogen receptors α and/or β *(53)*. Nikolaichuk et al. *(54)* reported that a methanol-water (4:1) *C. ternatea* flower extract (test material not completely characterized) demonstrated an estrogenic response in the endocrine yeast antagonist-verified estrogen/androgen (pYAVES/pYAVAS) screening bioassay. Andres et al. *(55)* summarized that, although quercetin has induced estrogenic effects in female rodents (primarily in immature animals), the outcome of the rodent studies is "inconsistent". Oh et al. *(56)* and Wang et al. *(57)* provided in vitro data showing that kaempferol has both estrogenic and antiestrogenic activity, depending on concentration.

Because of the presence of allergenic proteins in plants from the pea family *(58)*, the potential presence of allergenic proteins in butterfly pea flower extract was investigated using a weight-of-evidence approach. Most allergenic proteins or glycoproteins have molecular weights in the range of 5–100 kDa *(59)*. The filtration method used to prepare the butterfly pea flower extract removes all molecules with molecular weights in excess of 2.5 kDa. There are no reports of allergic reactions in the published literature following butterfly pea flower extract exposure. An amino acid sequence comparison with all sequenced proteins derived from *C. ternatea* against the amino acid sequences from known allergens was predominantly negative *(20)*. Of the few *C. ternatea* proteins that showed some sequence similarities with known allergenic proteins, the molecular weights of the identified proteins were all in excess of 5 kDa. Based on these considerations, the Committee concluded that it is unlikely that butterfly pea flower extract is allergenic.

Observations in humans

Chusak et al. *(60)* and Thilavech et al. *(61)* described two clinical trials with *C. ternatea* flower extract. Although these trials were not designed to assess safety, no adverse events were reported by study participants following single doses of up to 2 g *C. ternatea* flower extract (test materials not completely characterized; equivalent to approximately 33 mg/kg bw per day assuming an average body weight of 60 kg).

Adverse events that have been reported by clinical trial participants following short-term quercetin supplementation include gastrointestinal effects, headache and mild tingling of the extremities *(55)*.

Assessment of dietary exposure

Butterfly pea flower extract is proposed for use as a blue colour in 18 GSFA food categories including: flavoured fluid milk drinks; beverage whiteners; dairy-based desserts; fat emulsions; edible ices; fruit preparations; confectionery; chewing-gum; decorations, topping and sweet sauces; breakfast cereals; crackers; carbonated water-based flavoured drinks; non-carbonated water-based flavoured drinks; cider and perry; wines; distilled spirituous beverages; aromatized alcoholic beverages; and snacks (potato, cereal, flour or starch-based). The maximum use level of butterfly pea flower extract proposed in the final food ranged from 4 to 50 000 mg/kg.

The Committee reviewed estimates of dietary exposure to butterfly pea flower extract prepared by the sponsor from data on worldwide national individual food consumption data from 40 surveys across Brazil, the United Kingdom and the USA as well as Africa and Asia, based on at least 2 complete

days of food consumption data, and proposed maximum use levels matching the 18 GSFA food categories.

In these estimates, it was assumed that percentages of foods within a food category likely to contain butterfly pea flower extract provide a reasonable representation for the proportion of blue-coloured foods consumed in each food use. Adjusted use levels were derived by applying factors for the assumptions that only a portion of a food category or food would be expected to include colouring (e.g. coated sweets) at the proposed maximum use levels.

The ranges of mean and high (95th percentile) estimates of dietary exposure to butterfly pea flower extract based on adjusted use levels were < 0.1 to 16 and < 0.1 to 21.2 mg/kg bw per day for infants (age 11 months) and toddlers (age 1–3 years), 0.2–10.8 and 1.6–31.1 mg/kg bw per day for children (age 2–12 years) and adolescents (age 10–17 years), and 0.1–6.7 and 0.8–31.0 mg/kg bw per day for adults (age ≥ 18 years), respectively.

Depending on the country dataset, the main food categories contributing more than 10% to the total mean dietary exposure to butterfly pea flower extract in children and adult populations were ready-to-eat cereals, fruit drinks, carbonated soft drinks, ready-to-drink (RTD) tea, RTD dairy and non-dairy drinks, ice cream and frozen dairy desserts. Sports and energy drinks, soft and hard candy, crackers and other snacks, and plain corn, tortilla and multigrain chips were contributors for children, and alcoholic beverages and RTD nutritional beverages were contributors for adults.

The Committee noted that it was unclear to what extent the adjusted levels will reflect actual use levels in commercially available foods, as butterfly pea flower extract has not yet been commercially available on a global scale. However, it was considered that these estimates of dietary exposure were conducted according to internationally accepted methodologies, reflecting what might be the relevant exposure scenario if the food additive was marketed globally.

The Committee concluded that the highest high-level exposure of 31.1 mg/kg bw per day for adolescents should be considered in the safety assessment of butterfly pea flower extract.

Evaluation

Limited toxicological information is available for butterfly pea flower extract compared with that available for other food colours derived from natural sources for which previous Committees have derived ADIs, for example Jagua blue *(4)*, curcumin *(14)*, paprika extract *(16)* and spirulina extract *(18)*. Additionally, the Committee expressed concerns that the composition of the batches of butterfly pea flower extract used in the submitted studies (i.e. 90-day study and genotoxicity studies) differ from the commercial material and that the composition (i.e. concentration of the individual substances) was not fully defined in the submitted studies.

With these reservations regarding the representativeness of the materials tested, the Committee (i) noted that the 90-day dietary study on butterfly pea flower extract in rats indicated a NOAEL of 3201 mg/kg bw per day; (ii) did not identify a concern for in vivo genotoxicity of the test material; and (iii) noted that butterfly pea flower extract is unlikely to be allergenic. The available information from the literature on the aglycone flavonols (i.e. quercetin and kaempferol) in butterfly pea flower extract, as well as information on other extracts of *C. ternatea* flower, indicated potential estrogenic activity. The Committee noted that the highest high-level dietary exposure was 31.1 mg/kg bw per day in adolescents.

Because of the limited nature of the toxicological data, and the uncertainties concerning the specifications for the commercial product and the characterization of the test materials in the submitted toxicity studies, the Committee was unable to complete the safety assessment of butterfly pea flower extract.

A toxicological monograph with a dietary exposure assessment was prepared.

A specifications monograph and a chemical and technical assessment were drafted but could not be finalized for publication because of a lack of critical information.

Recommendations

The following information is required to complete the specifications for butterfly pea flower extract:

- quantitative composition of non-colouring components (e.g. carbohydrates, proteins and plant lipids) of butterfly pea flower extract from at least five batches of the article of commerce;
- detailed methods for determination of water content, Brix and colour strength; and
- analysis of the article of commerce using both alkali saponification and acid hydrolysis.

In addition, the following information is required to complete the safety assessment for butterfly pea flower extract:

- studies on reproductive and developmental toxicity with a test material that is representative of the article of commerce, given the indications of systemic exposure and possible estrogenic activity of the polyphenol constituents (i.e. delphinidin, quercetin and kaempferol);
- quantitative characterization of the test articles used in the already submitted toxicity studies to assess whether they are representative of the article of commerce; and

- if the article of commerce differs substantially from the test material used in the already submitted toxicity studies (90-day and genotoxicity studies), new studies on the same end-points.

3.1.3 Endo-1,4-β-xylanase (JECFA99-2) from *Bacillus subtilis* expressed in *Bacillus subtilis*

Explanation

At the request of the CCFA at its Fifty-third Session *(1)*, the Committee evaluated the safety of endo-1,4-β-xylanase (EC No. 3.2.1.8; CAS No. 9025-57-4) from *Bacillus subtilis* expressed in *Bacillus subtilis* strain. The previous Committee had not evaluated this enzyme preparation. The present Committee allocated the unique JECFA enzyme identifier JECFA99-2[5] to this enzyme preparation.

The term "endo-1,4-β-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 that is used in the toxicity studies; and the term "enzyme preparation" refers to the product formulated for commercial use.

The previous Committee evaluated several other endo-1,4-β-xylanases, including an endo-1,4-β-xylanase from *Thermomyces lanuginosus* expressed in *Fusarium venenatum* at its Sixty-first meeting *(14)*; an endo-1,4-β-xylanase from *Bacillus subtilis* expressed in *B. subtilis* at its Sixty-third meeting *(62)*; and a mixed β-glucanase, cellulase and endo-1,4-β-xylanase from *R. emersonii*, and a mixed β-glucanase and endo-1,4-β-xylanase from *Disporotrichum dimorphosporum* at its Eightieth meeting *(63)*, for which an ADI "not specified"[6] was established. At its Ninety-fifth meeting, the Committee evaluated an endo-1,4-β-xylanase from *B. licheniformis* expressed in *B. licheniformis* and established a temporary ADI "not specified" *(2)*. The ADI "not specified" was made temporary because of the tentative nature of the specifications.

The previous Committee evaluated several food enzymes from *B. subtilis*, such as an α-amylase *(64)*, an asparaginase *(2)*, and a mixed carbohydrase and protease *(65)*, and established an ADI "not specified" or ADI "not limited"[7] for these enzyme preparations. On this basis, the present Committee concluded that endo-1,4-β-xylanase from *Bacillus subtilis* strain expressed in *B. subtilis* met the criteria of a Class I, Type iii enzyme, as described in EHC 240 *(3)*. A Class I, Type iii

[5] At its Ninety-fifth meeting the Committee decided that an identification system would be used for all enzyme preparations, consisting of the JECFA meeting number followed by the number reflecting the order of the enzyme in the report.

[6] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting for clarification of the term ADI "not specified".

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

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 I, Type iii enzymes, the Committee evaluated the submitted information.

The enzyme catalyses the hydrolysis of the (1→4)-β-D-xylosidic linkages in (arabino)xylans resulting in the formation of (1→4)-β-D-xylans of different chain lengths. The enzyme preparation is intended for use as a processing aid for baking applications.

The Committee evaluated the submitted data and conducted a literature search in PubMed (all fields) with the linked search terms "endo-1,4-β-xylanase" AND "*Bacillus subtilis*", identifying 149 references. One of the identified references was the safety evaluation of the enzyme preparation conducted by EFSA and was considered relevant to the current evaluation *(66)*.

Genetic background

The production organism *B. subtilis* is a non-pathogenic and non-toxigenic ubiquitous bacterium commonly recovered from soil, water sources, food, air, animals and plants *(67–69)*. *B. subtilis* has a history in the production of enzymes intended for use in food processing. Endo-1,4-β-xylanase produced by *B. subtilis* (including recombinant *B. subtilis* strains) has been approved in many countries including Argentina, Australia, Brazil, Canada, China, France, India, Japan, Mexico and New Zealand.

The production strain was obtained from *B. subtilis* TD046 by a series of mutations including those to modify the native xylanase gene, suppress the ability to sporulate and increase the ability to produce extracellular enzymes. The stability of the production strain was confirmed by its performance over at least 10 successive subcultures, corresponding to more than 100 generations. The production strain is deposited in the Belgian Co-ordinated Collection of Microorganisms at the University of Gent.

Chemical and technical considerations

Endo-1,4-β-xylanase is produced by pure culture fermentation of the *B. subtilis* production strain. Manufacture of the enzyme preparation includes fermentation, recovery and formulation. After fermentation, the broth containing the endo-1,4-β-xylanase enzyme is separated from the biomass via a series of filtration steps, purification by ion exchange chromatography and concentration steps. The resulting enzyme concentrate is formulated and standardized into a liquid enzyme preparation. The entire process is performed in accordance with current GMP and using food-grade raw materials. The enzyme concentrate is tested to ensure that it is free from the production organism and any antibiotic activity.

Specifications for potential chemical and microbiological contaminants of the endo-1,4-β-xylanase preparation meet the requirements of JECFA *(70)*.

The primary sequence of the mature protein of endo-1,4-β-xylanase produced by *B. subtilis* consists of 185 amino acids; its calculated molecular weight is 20 kDa as confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The endo-1,4-β-xylanase produced by *B. subtilis* is not expected to have any significant secondary catalytic activities.

The activity of endo-1,4-β-xylanase is determined spectrophotometrically by measuring the release of xylose from birchwood xylan substrate in the presence of 3,5-dinitrosalicylic acid at 570 nm (pH 6.0, T = 50 °C, 15 min) and is measured in birchwood D(+)-xylanase units (BDXU). One BDXU is defined as the amount of enzyme that liberates 1 μmole of reducing sugars (xylose equivalents) from birchwood xylan per minute at pH 6.0 and 50 °C. The mean activity from seven batches of the liquid enzyme concentrate is 14 288 BDXU/mL.

Endo-1,4-β-xylanase catalyses the endohydrolysis of (1→4)-β-D-xylosidic linkages. The enzyme preparation is intended for use as a processing aid in baking applications. The endo-1,4-β-xylanase enzyme preparation is intended to be used at a maximum level of 0.333 mg TOS/kg raw material. 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 endo-1,4-β-xylanase enzyme is heat-inactivated during food processing and is therefore not expected to have any technological function in the final food.

Biological data

Assessment of potential allergenicity

The Committee evaluated the potential for allergenicity of the enzyme preparation based on a homology search of the amino acid sequence of the enzyme with those of known allergens in the AllergenOnline *(71)* and Allergen *(72)* databases, according to bioinformatics criteria recommended by EHC 240 *(3)*. 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 with an E-value[8] of 0.1 or less did not identify a homology to any known allergen. The Committee noted that the enzyme would be heat-denatured under conditions of food processing. No data relevant to the digestibility of the enzyme preparation were submitted.

[8] The E-value selected for a search tends to be larger for searches for short sequences ($E < 0.1$) than for long sequences ($E < 1 \times 10^{-7}$), as the likelihood of random matches is greater in the search for shorter sequences.

The Committee concluded that the dietary exposure to the enzyme preparation is not anticipated to pose a risk for allergenicity.

Toxicological studies

In a 13-week study in rats *(73)*, the enzyme concentrate was administered by gavage at doses of up to 147.3 mg TOS/kg bw per day. Based on the absence of any treatment-related adverse effects, the Committee identified a NOAEL of 147.3 mg TOS/kg bw per day, the highest dose tested.

The enzyme concentrate was negative in a bacterial reverse mutation test *(74)* and an in vitro chromosome aberration assay *(75)*. The Committee therefore had no concerns about the potential for genotoxicity of the enzyme concentrate.

A comparison of the amino acid sequence of the enzyme with those of known protein toxins *(76)* revealed no biologically relevant homology. The Committee therefore concluded that the enzyme was unlikely to be a toxin.

Assessment of dietary exposure

The Committee evaluated the dietary exposure to this endo-1,4-β-xylanase preparation, proposed for use as a processing aid for baking applications.

The Committee estimated the TMDI of the TOS derived from this xylanase preparation, specifically for solid foods based on a use level of 0.334 mg TOS/kg flour, to be 0.003 mg TOS/kg bw per day using the budget method. Additionally, an estimate of dietary exposure was provided by the sponsor, based on per capita bread consumption by high consumers in Europe, of 0.0011 mg TOS/kg bw per day. EFSA conducted a more refined dietary exposure assessment based on baking applications with a recommended use level of up to 0.752 mg TOS/kg flour, using food consumption data for individuals from the EFSA Comprehensive European Food Consumption Database *(13)*. The estimated mean dietary exposures were up to 0.005 mg TOS/kg bw per day for toddlers (age 12–35 months) and the estimated 95th dietary exposures were up to 0.008 mg TOS/kg bw per day for toddlers and children (age 1–9 years).

For the dietary exposure assessment, it was assumed that 100% of the enzyme remains in the final food. The Committee noted that the enzyme is heat-inactivated during the processing of foods and food ingredients.

The Committee concluded that the dietary exposure estimate of 0.008 mg TOS/kg bw per day was appropriate for use in the evaluation.

Evaluation

The Committee concluded that dietary exposure to this endo-1,4-β-xylanase enzyme preparation is not anticipated to pose a risk for allergenicity.

The Committee identified a NOAEL of 147.3 mg TOS/kg bw per day, the highest dose tested, in a 13-week study in rats.

Comparison of this NOAEL with the estimated dietary exposure of 0.008 mg TOS/kg bw per day gives a margin of exposure (MOE) of more than 18 000. Based on this MOE and the lack of concern for genotoxicity, the Committee established an ADI "not specified" for endo-1,4-β-xylanase (JECFA99-2) from *Bacillus subtilis* expressed in *Bacillus subtilis* when used in the applications specified, at the levels of use specified and in accordance with current GMP.

A toxicological monograph with a dietary exposure assessment was prepared.

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

3.1.4 Endo-1,4-β-xylanase (JECFA99-3) from *Rasamsonia emersonii* expressed in *Aspergillus niger*

Explanation

At the request of the CCFA at its Forty-sixth Session *(77)*, the Committee evaluated the safety of an endo-1,4-β-xylanase (EC No. 3.2.1.8; CAS No. 9025-57-4) from *Rasamsonia emersonii* (previously named *Talaromyces emersonii*) expressed in *Aspergillus niger*. The previous Committee had not evaluated this enzyme preparation. The present Committee allocated the unique JECFA enzyme identifier[9] JECFA99-3 to this enzyme preparation to distinguish it from similarly named enzyme preparations.

The term "endo-1,4-β-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 that is used in the toxicity studies; and the term "enzyme preparation" refers to the formulated product for commercial use.

The previous Committee evaluated several other endo-1,4-β-xylanases, including an endo-1,4-β-xylanase from *Thermomyces lanuginosus* expressed in *Fusarium venenatum* at its Sixty-first meeting *(14)*; an endo-1,4-β-xylanase from *Bacillus subtilis* expressed in *B. subtilis* at its Sixty-third meeting *(62)*; and a mixed β-glucanase, cellulase and endo-1,4-β-xylanase from *R. emersonii*, and a mixed β-glucanase and endo-1,4-β-xylanase from *Disporotrichum dimorphosporum*, at its Eightieth meeting *(63)*, for which an ADI "not specified"[10] was established. At

[9] At its Ninety-fifth meeting the Committee decided that an identification system would be used for all enzyme preparations, consisting of the JECFA meeting number followed by the number reflecting the order of the enzyme in the report.

[10] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting for clarification of the term ADI "not specified".

its Ninety-fifth meeting, the Committee evaluated an endo-1,4-β-xylanase from *B. licheniformis* expressed in *B. licheniformis* and established a temporary ADI "not specified" *(2)*. The ADI "not specified" was made temporary because of the tentative nature of the specifications.

The previous Committee evaluated several food enzymes from *A. niger*, such as asparaginase *(15)* and 3-phytase *(78)*, and established an ADI "not specified" for these enzyme preparations. On this basis, the present Committee concluded that endo-1,4-β-xylanase (JECFA99-3) from *R. emersonii* expressed in *A. niger* met the criteria of a Class I, Type iii enzyme, as described in EHC 240 *(3)*. A Class I, Type iii enzyme is produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Although toxicity studies and a dietary exposure assessment are not required for Class I, Type iii enzymes, the Committee evaluated the submitted data.

The enzyme catalyses the hydrolysis of 1,4-β-D-xylosidic linkages within xylan chains to form oligomers of 1,4-β-xylan and 1,4-β-arabinoxylan. The enzyme preparation is intended to be used as a processing aid in: (i) brewing processes to hydrolyse arabinoxylans in cereal cell walls, reduce wort viscosity and improve filtration; (ii) baking and other cereal-based applications to improve dough characteristics and handling; (iii) fruit and vegetable processes to facilitate the liquefaction and/or softening of fruit and vegetable materials, and juice clarification; and (iv) plant-based analogues of milk and milk products to hydrolyse arabinoxylans to improve conversion yield and increase dry matter recovery.

The Committee evaluated the submitted data and conducted a literature search in Google Scholar with the linked search terms "xylanase" OR "9025-57-4" AND "*Aspergillus niger*" AND "*Rasamsonia emersonii*" OR "*Talaromyces emersonii*" AND "safety" OR "toxi*" OR "allerg*". This search identified 186 references. None of the identified publications was considered relevant to the present toxicological evaluation. The Committee identified three additional references considered relevant to the allergenicity assessment.

Genetic background

The production organism *Aspergillus niger* is a non-pathogenic filamentous fungus found in cereals, cereal grains and spoiled foods *(79)*. It has long been recognized as a source organism for production of enzymes intended for use in food processing *(80–82)*. In their natural habitats, *A. niger* strains have the potential to produce mycotoxins and other secondary metabolites; however, the *A. niger* production strain expressing the endo-1,4-β-xylanase does not produce mycotoxins under the production conditions used *(83)*.

The non-toxigenic and non-pathogenic *A. niger* production strain was obtained from parental strain *A. niger* NRRL 3122. The gene encoding

endo-1,4-β-xylanase was synthesized in vitro from a cDNA coding sequence obtained from *R. emersonii* and included in an endo-1,4-β-xylanase expression cassette. The recipient strain is transformed with seven copies of the expression cassette. The expression of the endo-1,4-β-xylanase gene is controlled by the native promoter and terminator of the recipient strain. The strain lineage used to construct the production strain has been stable for more than 30 years. The production strain does not contain any antibiotic resistance markers and the expression cassette is not transferrable to other microbes. The *A. niger* endo-1,4-β-xylanase production strain is deposited in the culture collection of the WFBI.

Chemical and technical considerations

Endo-1,4-β-xylanase is produced by pure culture fermentation of the *A. niger* production strain. Manufacture of the enzyme preparation includes fermentation, recovery and formulation. After fermentation, the broth containing the endo-1,4-β-xylanase enzyme is separated from the biomass via a series of filtration steps, followed by concentration steps. The resulting enzyme concentrate is formulated into either a liquid or microgranulate preparation. The entire process is performed in accordance with current GMP and with food-grade raw materials. The commercial enzyme preparation is tested to ensure that it is free from the production organism and any antimicrobial activity. Specifications for potential chemical and microbiological contaminants of the endo-1,4-β-xylanase preparation meet the requirements of JECFA *(70)*.

One new thermostable endoxylanase unit (NTXU) is defined as the amount of enzyme required to liberate 0.06 µmole of *p*-nitrophenol per minute at pH 4.5 and 37 °C. The activity of endo-1,4-β-xylanase is determined spectrophotometrically by measuring the yellow colour of *p*-nitrophenol released by the hydrolysis of the *p*-nitrophenyl-β-D-xylopyranoside substrate under the assay conditions. The mean activity from five batches of the enzyme concentrate is 19 827 NTXU/g.

Endo-1,4-β-xylanase catalyses the hydrolysis of 1,4-β-D-xylosidic linkages within xylan chains to form oligomers of 1,4-β-xylan and 1,4-β-arabinoxylan. The liquid enzyme preparation is intended for use as a processing aid in brewing processes, fruit and vegetable processing, and in the production of plant-based analogues of milk and milk products, at a maximum level of 27.6 mg TOS/kg raw material. The microgranulate enzyme preparation is intended for use as a processing aid in baking and other cereal-based applications at a maximum level of 27.6 mg 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 endo-1,4-β-xylanase is heat-inactivated during food processing. It is not expected to have any technological function in the final food.

Biological data

Assessment of potential allergenicity

The Committee used a weight-of-evidence approach, as described in EHC 240 *(3)*, to assess this endo-1,4-β-xylanase for allergenicity. This was achieved by conducting a search of the available literature for evidence of oral sensitization to xylanase, assessing this endo-1,4-β-xylanase for resistance to proteolysis in an in vitro digestion assay, and comparing the amino acid sequence of this endo-1,4-β-xylanase with that of known protein allergens.

Cases of respiratory and skin contact allergies following occupational inhalation of aerosols containing xylanase and/or dermal exposure to xylanase have been reported *(84–87)*. However, the Committee noted that some individuals who had been sensitized to enzymes via the respiratory route could also ingest the same allergen without showing any clinical symptoms *(88–90)*. This is supported by the absence of evidence of oral sensitization to xylanase in the literature. Xylanases are also commonly found in food (e.g. cereal crops) *(91,92)*, and there are no indications for allergic reactions as a result of their ingestion.

The results of a simulated gastric and intestinal fluid digestion assay indicated that this endo-1,4-β-xylanase is likely to be resistant to proteolysis following human dietary exposure *(93)*. However, this endo-1,4-β-xylanase is denatured by heat during the various manufacturing processes. Resistance to proteolysis does not necessarily trigger a concern for allergenicity. In cases where proteins are denatured because of food processing conditions (i.e. baking applications), which is the case for this endo-1,4-β-xylanase, the tertiary conformation of the enzyme molecule is disrupted. In general, these alterations in conformation are associated with a decrease in antigenic reactivity in humans; in the majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins *(94–97)*.

The amino acid sequence of this endo-1,4-β-xylanase was also compared with those of known allergens using the AllergenOnline *(98)* and Allermatch *(99)* online databases, based on bioinformatics criteria recommended by EHC 240 *(3)*. A search for matches having more than 35% identity over a sliding window of 80 amino acids, a search for sequence identity for eight contiguous amino acids and a full-length FASTA sequence search with an E-value[11] cut-off value of less than 1×10^{-7} did not identify a homology to any known allergens.

Based on the results of the bioinformatics analysis, a simulated gastric and intestinal fluid digestion assay, the heat-denaturation of the enzyme during

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

food processing and the absence of any evidence of sensitization following oral exposure to xylanase, the Committee concluded that the risk of allergenicity upon dietary exposure to this endo-1,4-β-xylanase is low.

Toxicological studies

In a 13-week study in rats *(100)*, the enzyme concentrate was administered by gavage at doses up to 1850 mg TOS/kg bw per day. No treatment-related adverse effects were observed in any of the evaluated parameters. The Committee identified a NOAEL of 1850 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 *(101)* or an in vitro mammalian chromosomal aberration assay *(102)*. The Committee had no concerns about potential genotoxicity of the enzyme concentrate.

A comparison of the amino acid sequence of this endo-1,4-β-xylanase with those of known protein toxins and virulence factors *(103–105)* revealed no biologically relevant homology *(106)*. The Committee therefore concluded that this enzyme is unlikely to be a toxin.

Assessment of dietary exposure

The Committee evaluated two estimates of dietary exposure to the TOS from this endo-1,4-β-xylanase enzyme preparation submitted by the sponsor. Using the budget method, a TMDI of 0.33 mg TOS/kg bw per day (0.245 mg TOS/kg bw per day for solid foods and 0.085 mg TOS/kg bw per day for non-milk beverages) was estimated by the sponsor for the enzyme preparation. The sponsor also provided an estimate of dietary exposure based on the recommended use levels in the various applications (i.e. baking, brewing, production of fruit and vegetable products, and production of plant-based analogues of milk and milk products) and the Food Enzyme Intake Model of EFSA. The 95th percentile dietary exposure estimate for baking was combined with the mean dietary exposure estimates for the other applications, resulting in dietary exposure estimates of 0.024–0.380 mg TOS/kg bw per day in toddlers (age 12–35 months) and 0.053–0.151 mg TOS/kg bw per day in adults (age 18–64 years).

The Committee concluded that the highest estimate of dietary exposure to the endo-1,4-β-xylanase enzyme preparation of 0.380 mg TOS/kg bw per day in toddlers 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 denatured by heat during the processing of foods and food ingredients.

Evaluation

The Committee concluded that the risk of allergenicity upon dietary exposure to this endo-1,4-β-xylanase is low. The Committee identified a NOAEL of 1850 mg TOS/kg bw per day, the highest dose tested in the 13-week study in rats. Comparison of this NOAEL with the highest estimated dietary exposure of 0.380 mg TOS/kg bw per day in toddlers gave an MOE of more than 4800. On the basis of this MOE and lack of concern about genotoxicity, the Committee established an ADI "not specified"[12] for this endo-1,4-β-xylanase (JECFA99-3) from *Rasamsonia emersonii* expressed in *Aspergillus niger* when used in the applications specified, at the levels of use specified and in accordance with GMP.

A toxicological monograph with a dietary exposure assessment was prepared.

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

3.1.5 Glucosidase from *Aspergillus niger* expressed in *Trichoderma reesei* exhibiting α-glucosidase (JECFA99-4a) and transglucosidase (JECFA99-4b) activity

Explanation

At the request of the CCFA at its Forty-eighth Session *(107)*, the Committee evaluated the safety of glucosidase from *Aspergillus niger* expressed in *Trichoderma reesei* exhibiting α-glucosidase (EC No. 3.2.1.20) and transglucosidase (EC No. 2.4.1.24) activity. The previous Committee had not evaluated any α-glucosidase or transglucosidase enzyme preparations. The present Committee allocated the unique JECFA enzyme identifiers[13] JECFA99-4a and JECFA99-4b for the enzyme preparations used for their α-glucosidase and transglucosidase activities, respectively.

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

The Committee has previously evaluated several food enzymes from *T. reesei*, such as cellulase *(108)* and glucoamylase *(109)*, and established an ADI "not specified" for these enzyme preparations. On this basis, the present

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

[13] At its Ninety-fifth meeting the Committee decided that an identification system would be used for all enzyme preparations, consisting of the JECFA meeting number followed by the number reflecting the order of the enzyme in the report.

Committee concluded that glucosidase from *A. niger* expressed in *T. reesei* exhibiting α-glucosidase (JECFA99-4a) and transglucosidase (JECFA99-4b) activity met the criteria of a Class I, Type iii enzyme, as described in EHC 240 *(3)*. A Class I, Type iii enzyme is produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Although toxicity studies and a dietary exposure assessment are not required for Class I, Type iii enzymes, the Committee has evaluated the submitted data.

This enzyme catalyses both hydrolytic and transfer reactions on incubation with α-D-glucooligosaccharides. The enzyme preparation is intended to be used for its α-glucosidase activity in the manufacture of potable alcohol, organic acids (e.g. lactic acid, citric acid and succinic acid) and monosodium glutamate (MSG), and for its transglucosidase activity in the manufacture of isomalto-oligosaccharide (IMO) syrups from a variety of sources (e.g. corn, wheat and rice).

The Committee conducted a literature search in Google Scholar with the linked search terms "transglucosidase" OR "trans-glucosidase" OR "trans glucosidase" OR "alpha-glucosidase" OR "alpha glucosidase" OR "9001-42-7" OR "9030-12-0" AND "Aspergillus niger" AND "Trichoderma reesei" AND "safety" OR "toxi*" OR "allerg*". This search identified 114 references. None of the identified publications was considered relevant to the present evaluation.

Genetic background

The production organism *T. reesei* is a non-pathogenic and non-toxigenic wood rot fungus *(110–112)* with a history in the production of enzymes intended for use in food processing.

The production strain was developed from *T. reesei* recipient strain RL-P37 to overexpress the glucosidase gene obtained from *A. niger*. The *A. niger* glucosidase gene expression cassette (promoter, signal peptide, enzyme coding sequence, terminator and selectable marker) was randomly integrated into the genome of the host using a spore electroporation method. Whole genome sequencing data show that one copy of the gene expression cassette was inserted. The stability of the introduced genes was confirmed by Southern blot analysis. No antibiotic resistance genes were introduced. The production strain is deposited in the Genencor International Culture Collection in the USA.

Chemical and technical considerations

Glucosidase is produced by controlled fermentation of a pure culture of the *T. reesei* production strain. Manufacture of the enzyme preparation includes fermentation at controlled temperature, pressure and pH (inoculum, seed and main fermentation), recovery and formulation. After fermentation, the broth containing the glucosidase is separated from the biomass via multiple filtration

steps; this is followed by concentration, stabilization and polish filtration. The concentrated enzyme is formulated, stabilized and standardized as a liquid enzyme preparation. The entire process is performed in accordance with current GMP and with food-grade raw materials. The enzyme preparation conforms to the General Specifications for Enzyme Preparations used in Food Processing *(70,113)*.

The glucosidase enzyme produced by the *T. reesei* strain consists of 960 amino acids and its calculated molecular weight is 106.2 kDa. Under different conditions, the glucosidase exhibits either α-glucosidase activity or transglucosidase activity. It is not expected to show other significant secondary catalytic activities.

Transglucosidase activity is determined by incubating a diluted enzyme solution with a 10% maltose solution in 0.02 M acetate buffer, pH 4.0 at 50 °C for 60 min and measuring the amount of trisaccharide produced using high-performance liquid chromatography (HPLC). One transglucosidase activity unit (TGU) is defined as the amount of enzyme that will produce 1 μmole of trisaccharide per minute under the assay conditions. The mean activity from three batches of the liquid enzyme concentrate is 6964 TGU/g.

α-Glucosidase activity, expressed in α-glucosidase units (AGDU), can be determined spectrophotometrically by measuring the yellow colour of *p*-nitrophenol released by the hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside substrate at 420 nm, pH 10. One AGDU is defined as the amount of enzyme required to generate 1.0 μmole of *p*-nitrophenol per minute under the assay conditions (pH 4.8, 30 °C).

The α-glucosidase activity in the article of commerce is standardized based on the TGU. An enzyme preparation with an activity of 2000 TGU/mL will have an activity corresponding to approximately 30 AGDU/mL.

The enzyme preparation is used for its α-glucosidase activity in the production of potable alcohol, organic acids (e.g. lactic, citric and succinic) and MSG. The enzyme preparation is also used for its transglucosidase activity in the manufacture of IMO syrups from a variety of sources (e.g. corn, wheat and rice).

Glucosidase enzyme preparations are formulated to 1700–2400 TGU/g based on the intended uses. The maximum use level of the enzyme preparation for its α-glucosidase activity is 235 mg TOS/kg substrate. The maximum use level of the enzyme preparation for its transglucosidase activity is 88 mg TOS/kg substrate.

The enzyme is heat-inactivated or removed during the processing of foods and food ingredients. It is not expected to have any technological function in the final food. Any remaining enzyme activity in the final food would be negligible.

Biological data

Assessment of potential allergenicity

The Committee evaluated the potential for allergenicity of this glucosidase by comparing the amino acid sequence of the enzyme with known allergens using the AllergenOnline *(114)* and Allermatch *(115)* online databases, based on bioinformatics criteria recommended by EHC 240 *(3)*. A search for matches having more than 35% identity over a sliding window of 80 amino acids, a search for sequence identity for eight contiguous amino acids and a full-length FASTA sequence search with an E-value[14] cut-off of less than 1 produced no matches with known allergens, indicating that this enzyme is unlikely to share any immunological cross-reactivity with known allergens. A simulated gastric fluid digestion assay with the glucosidase enzyme preparation demonstrated complete digestion to short peptides and amino acids after only 30 seconds *(116)*. The Committee also noted that the enzyme is heat-denatured or removed during the processing of foods and food ingredients.

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

Toxicological studies

In the acute oral toxicity study in rats *(117)*, no toxicity was observed at a dose of 2352 TOS/kg bw for the enzyme concentrate, the only dose tested.

In an 18-week study in rats *(118)*, the enzyme concentrate was administered by gavage at doses up to 74.8 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 74.8 mg TOS/kg bw per day, the highest dose tested, for the enzyme concentrate.

The enzyme concentrate was not genotoxic in a bacterial reverse mutation assay *(119)* or an in vitro mammalian chromosomal aberration assay *(120)*. The Committee had no concerns about potential genotoxicity of the enzyme concentrate.

A comparison of the amino acid sequence of this glucosidase with those of known protein toxins *(121)* revealed no biologically relevant homology *(122)*. The Committee therefore concluded that this glucosidase is unlikely to be a toxin.

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

Assessment of dietary exposure

This enzyme preparation is used for both its transglucosidase and α-glucosidase activities. The transglucosidase activity is used to produce IMO syrups from various sources. The α-glucosidase activity is utilized in the production of potable alcohol, organic acids (e.g. lactic, citric and succinic acids) and MSG. The Committee evaluated the estimate of the dietary exposure to the TOS from the combined use of both enzymatic applications using the budget method. Considering the highest use levels of 22.03 TOS/kg in cereals and confectionary products for solid foods, and 6.71 mg TOS/kg in liquid soup for non-milk beverages, the TMDI was estimated to be 0.443 mg TOS/kg bw per day.

For the dietary exposure assessment, it was assumed that 100% of the enzyme remains in the final food. The Committee noted that the enzyme is heat-denatured or removed during the processing of foods and food ingredients. Any remaining enzyme in the final food would be considered negligible.

The Committee concluded that the dietary exposure estimate of 0.443 mg TOS/kg bw per day was appropriate for use in the evaluation.

Evaluation

The Committee concluded that dietary exposure to this glucosidase is not anticipated to pose a risk for allergenicity. The Committee also had no concerns about potential genotoxicity of the enzyme concentrate. The Committee identified a NOAEL of 74.8 mg TOS/kg bw per day, the highest dose tested, for the enzyme concentrate in the 18-week study in rats. Comparison of this NOAEL with the estimated dietary exposure of 0.443 mg TOS/kg bw per day gave an MOE of 169.

The Committee noted that the selection of such a low dose as the highest dose tested in the 18-week oral toxicity study, as well as the use of the budget method for the dietary exposure assessment, greatly influenced the resulting MOE. Although this MOE is small, this glucosidase from *A. niger* expressed in *T. reesei* exhibiting α-glucosidase (JECFA99-4a) and transglucosidase (JECFA99-4b) activity meets the criteria of a Class I, Type iii enzyme for which toxicity studies and a dietary exposure assessment are not required. The Committee therefore had no concerns with the MOE calculated in the present evaluation.

The Committee therefore established an ADI "not specified" for glucosidase from *A. niger* expressed in *T. reesei* exhibiting α-glucosidase (JECFA99-4a) and transglucosidase (JECFA99-4b) activity when used in the applications specified, at the levels of use specified and in accordance with GMP.

A toxicological monograph with a dietary exposure assessment was prepared.

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

3.1.6 Natamycin

Explanation

Natamycin (synonym pimaricin) is an antimycotic agent of the polyene macrolide class of antimicrobials used as a surface treatment of cheeses and dried sausages. Natamycin exerts its fungicidal activity by preventing the growth of fungi and germination of fungal spores through binding to ergosterol located in fungal cellular membranes.

At its Fifty-first Session, the CCFA requested a re-evaluation of the safety of natamycin including any data on "(i) promoting antimicrobial resistance, as well as speeding up virulence and pathogenic potential of food-borne human pathogens; and (ii) unbalancing the immunity and other bodily functions due to effects on gastrointestinal microflora; dietary intake and specifications" *(123)*.

Natamycin was evaluated by the previous Committee at its Twelfth, Twentieth, Fifty-seventh and Sixty-seventh meetings *(70,124–126)*. At the Twelfth meeting, the Committee established a conditional ADI[15] of 0–0.25 mg/kg bw. The conditional ADI was converted to an ADI of 0–0.3 mg/kg bw at the Twentieth meeting based on observations of gastrointestinal effects in humans *(125)*. At the Fifty-seventh meeting, the Committee confirmed the previously established ADI of 0–0.3 mg/kg bw for natamycin *(126)*.

The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) evaluated natamycin in 2017 and concluded that the available evidence was inadequate to draw a conclusion on the genotoxicity and carcinogenic potential of natamycin *(127)*. JMPR did not establish an ADI for natamycin because of the inadequate database available to the 2017 meeting.

The sponsor submitted studies investigating the toxicity and microbiological effects of natamycin. Comprehensive literature searches were also undertaken on the toxicological, antimicrobial resistance and dietary exposure aspects of this assessment in databases such as PubMed and Web of Science from 2001 to 2024, using keywords including "natamycin", "pimaricin", "metabolite", "toxicity", "polyene", "microbiome", "intestinal microbiota", "microbiota", "microbiome", "gastrointestinal microbiota", "gastrointestinal microbiome", "antimicrobial resistance", "dietary intake", "dietary exposure", "occurrence" and "concentration".

[15] The meeting report describes how a "conditional ADI" was allocated either for specified uses or when the data were insufficient for an unconditional ADI. The term "conditional ADI" is no longer used by the Committee.

Chemical and technical considerations

Natamycin, chemical name 22-(3-amino-3,6-dideoxy-b-D-manno-pyranosol) oxy-1,3,26- trihydroxy-12-methyl-10-oxo-6,11,28-trioxiatri[22.3.1.05.7]ocatosa-8,14,16,18,20-pantanene-25-carboxylic acid, is a polyene macrolide antimycotic agent produced by submerged fermentation of strains of Actinomycetes such as *Streptomyces natalensis*, *S. lydicus* and *S. chattanoogensis (128)*. Production processes have been optimized for natamycin yield *(129)*. Natamycin is produced at a commercial scale by a batch-fed cultivation of either *S. natalensis* or *S. gilvosporeus* in a medium containing a carbon source such as starch or molasses, and a fermentable nitrogen source such as corn steep liquor, soy bean meal or casein at pH 6–8, at a temperature of 26–30 °C for 96 hours. After fermentation, the substance is isolated by extraction from broth or by extraction of the mycelium. Since natamycin has poor aqueous solubility, it accumulates as crystals (0.5–20 µm) and is separated from the biomass *(130)*. The isolated material after downstream processing contains at least 95.0% natamycin ($C_{33}H_{47}NO_{13}$), calculated on the anhydrous basis. It is a white to creamy-white crystalline powder with little taste or odour. The commercial products may contain 6–8% water. Under influence of light or acidic conditions, natamycin decomposes into inactive substances, such as mycosamine, aponatamycin and di-natamycinolidediol *(131–133)*.

Biochemical aspects

The Committee has previously evaluated studies performed in rats and dogs investigating the distribution and elimination of natamycin after oral administration of ^{14}C-natamycin. The Committee concluded that natamycin is poorly absorbed and eliminated primarily in the faeces *(126)*.

At the current meeting, the Committee evaluated an in vitro model investigating the fate of natamycin in the large intestine and its possible degradation by healthy human adult microbiota. Natamycin concentrations were reduced to below the level of quantification (0.1 mg/kg) within a 5–6-hour period in the test system. When the microbiota were inactivated by a 10-minute heat treatment at 80 °C, natamycin concentrations were reduced to 27% of the initial concentration at the end of an 8-hour time period *(134)*. The Committee considered this study to be of limited utility given the substantial decreases of natamycin that were also observed in the presence of heat-inactivated microbiota.

Cytochrome P450 activity was significantly decreased in rat hepatic microsomes following oral gavage administration of natamycin at doses of 1, 3 or 10 mg/kg bw per day for 6 days. No change in cytochrome P450 activity was seen at a dose of 0.3 mg/kg bw per day *(135)*.

Toxicological studies

In a 13-week feeding study in rats given natamycin at up to 2000 mg/kg in the feed (equal to 204 mg/kg bw per day), the NOAEL was 42 mg/kg bw per day based on reduced body weights compared with controls *(136)*. In a 1-year study in rats given natamycin at up to 1500 mg/kg in the feed (equal to 78 mg/kg bw per day), the NOAEL was 26 mg/kg bw per day based on decreased body weight compared with controls *(137)*.

No new long-term toxicity or carcinogenicity studies were available to the Committee for evaluation. In a study previously evaluated by the Committee, groups of male and female rats received natamycin in the feed for 2 years providing doses equivalent to 0, 6.25, 12.5, 25 or 50 mg/kg bw per day. The numbers and types of tumours found in natamycin-treated rats were not significantly different from those in untreated animals *(126)*. The Committee also noted that there was no evidence of the presence of preneoplastic lesions as an indicator of a proliferative response, or an increase in tumour incidence, in the 1-year rat study at doses of natamycin of up to 78 mg/kg bw per day *(137)*.

The presence of an epoxide ring on the natamycin molecule is a structural alert for genotoxicity. However, natamycin was not mutagenic in gene mutation tests in bacterial or mammalian cells and did not induce chromosome aberrations or an increase in unscheduled DNA synthesis in mammalian cells in vitro *(138–142)*. No increase in the frequency of micronucleated polychromatic erythrocytes was observed in an in vivo study in mice *(143,144)*. This study is considered to be of limited relevance because it is not clear that there was exposure to the bone marrow; however, the Committee noted that in vivo genotoxicity tests are not required when negative results are obtained in in vitro tests.

The Committee also noted that the European Agency for the Evaluation of Medicinal Products *(145)* reported that negative results were obtained in a bacterial reverse mutation test, a mouse lymphoma mutation assay and a chromosomal aberration assay. Overall, based on the available evidence, the Committee concluded that there is no concern for the genotoxicity of natamycin.

No new studies on reproductive or developmental toxicity were available to the Committee for evaluation. In reproductive and developmental toxicity studies evaluated by the previous Committee no effects were observed at natamycin doses of up to 50 mg/kg bw per day *(126)*.

Observations in humans

No new relevant human studies were available to the Committee for evaluation at the current meeting. In a study evaluated by the previous Committee, natamycin tablets were administered to 10 adults with various mycoses at doses of up to 1000 mg/day. The duration of dosing ranged from 13 to 334 days *(146)*. The previous Committee identified that the dose level causing no toxicological effects was 200 mg/day, equivalent to approximately 3 mg/kg bw per day *(125)*.

Microbiological effects

The previous Committee has evaluated studies on the microbiological effects of natamycin, including the mechanisms of action and fungal resistance, as well as the effect on the fungal microbiota; the previous Committee concluded that no evidence of resistance had been recorded in clinical use of natamycin, and no natural resistance against natamycin was known in fungi. Furthermore, the previous Committee found that the risk of trace exposure of natamycin on fungal microbiota would have a minimal effect *(126)*.

Natamycin inhibits fungi by binding to ergosterol, the major membrane sterol found in fungi, which is lacking in mammals and bacteria. No evidence of resistance mechanisms has been recorded. Attempts to adapt fungal strains to tolerate higher concentrations of natamycin have not been successful, showing only marginal increases in tolerance upon prolonged exposure to natamycin *(147)*. Mechanisms of protection against natamycin identified in bacteria are only relevant for natamycin-producing *Streptomyces* spp., as bacteria are not inhibited by natamycin *(148)*.

Considering the current available data, along with the previous evaluation of JMPR *(127)*, the Committee concluded that there is no concern for the induction of antimicrobial resistance. There were no available data to suggest that natamycin induces the virulence and pathogenic potential of bacteria.

The Committee concluded that the overall risk of natamycin having a disrupting effect on the microbiome of the human gastrointestinal tract is low.

Assessment of dietary exposure

The previous Committee assessed the dietary exposure to natamycin at its Fifty-seventh and Sixty-seventh meetings *(70,126)*. At those meetings the estimates of dietary exposure ranged between 0.006 and 0.1 mg/kg bw per day for mean and high exposures.

Estimates of dietary exposure at the national level considered at the current meeting included those submitted by the sponsor, from the literature and calculated by the Committee. There were estimates available for more than 40 countries across all WHO regions. The dietary exposure estimates were based on different types of foods and concentrations. These included GSFA permissions for cheeses and processed meats, local permissions, or proposed use levels in other food groups such as yoghurts and yoghurt products, and beverages.

For the national estimates of dietary exposure considered at the current meeting (Table 3.1), mean estimates ranged up to 0.12 mg/kg bw per day for children and up to 0.09 mg/kg bw per day for adults. Estimates of high dietary exposure ranged up to 0.25 mg/kg bw per day for children and up to 0.18 mg/kg bw per day for adults. Cheeses were most commonly the highest contributors to dietary exposures.

Table 3.1
Range of national estimates of dietary exposure to natamycin

Population group	Age range	Mean (mg/kg bw per day)	High[a] (mg/kg bw per day)
Children	Included up to 14, 17, 18 and 19 years	< 0.001 to 0.12	< 0.001 to 0.25
Adults	Included from 10, 15, 18 and 19 years	< 0.001 to 0.09	< 0.001 to 0.18

[a] A range of high percentiles was reported, including 90th to 97.5th percentile.

The Committee noted that the upper end of the range of dietary exposure estimates considered at the current meeting is higher than the estimates reported by previous Committees. This is because of the greater number of national estimates available for the current meeting, capturing a greater degree of variability in food consumption patterns and resulting dietary exposures.

The Committee concluded that the highest estimate of dietary exposure of 0.25 mg/kg bw per day in children was appropriate to use in the evaluation.

Evaluation

Based on the available data, the Committee concluded that there is no concern for the induction of antimicrobial resistance and that the risk of natamycin having a disrupting effect on the microbiome of the human gastrointestinal tract is low.

The Committee re-affirmed the ADI of 0–0.3 mg/kg bw for natamycin established by the previous Committee at its Twentieth meeting. The Committee further noted that the NOAELs in the new 13-week and 1-year studies in rats (42 and 26 mg/kg bw per day, respectively), with the application of a 100-fold uncertainty factor, support the current ADI of 0–0.3 mg/kg bw.

The Committee noted that dietary exposure estimates for natamycin of up to 0.25 mg/kg bw per day for all population subgroups assessed were below the ADI, and concluded that dietary exposure to natamycin was not of toxicological concern.

A toxicological monograph addendum, including a section on microbiological effects, with a dietary exposure assessment was prepared.

The existing specifications monograph was revised.

3.1.7 Nisin A

Explanation

Nisin is a group of antimicrobial polypeptides produced mainly by *Lactococcus lactis* subsp. *lactis*. Several natural nisin variant molecules produced by *L. lactis* have been identified in the literature, including nisin A, nisin Q and nisin Z *(149,150)*. Although nisin evaluations by the previous Committee were indicated to be for nisin, which is consistent with the current request received from

CCFA, the first and the only commercially available product until approximately 2005 is specifically nisin A. In previous evaluations data were only received to support specifications for nisin A. For the current meeting, no new data were received for specifications; the Evaluation therefore focused only on nisin A, and the name of the substance is amended.

At its Fifty-first Session, the Codex Committee on Food Additives requested the re-evaluation of the safety of nisin including any data on the "appropriateness of retaining nisin in the GSFA due to emerging data on nisin role in: (i) promoting antimicrobial resistance, as well as speeding up virulence and pathogenic potential of food-borne human pathogens; and (ii) unbalancing the immunity and other bodily functions due to effects on gastrointestinal microflora" *(123)*.

There are GSFA provisions for the use of nisin as an antimicrobial preservative in 19 food categories, including in processed cheeses, pasteurized dairy products and processed meats. At its Twelfth meeting the Committee established an ADI of 0–33 000 units of nisin per kilogram body weight *(124)*. At its Seventy-seventh meeting, the Committee withdrew the previous ADI and established an ADI of 0–2 mg/kg bw for nisin *(109)*. The ADI was established on the basis of a NOAEL of 225 mg/kg bw per day from a 90-day study in rats and applying a 100-fold uncertainty factor *(109)*. The Committee considered that the use of an additional uncertainty factor to account for the short duration of the pivotal study was not necessary since no substance-related effects were observed.

No new toxicological data were submitted for the present evaluation; however, the Committee performed a literature search on nisin up to March 2024. The search terms "nisin" AND "toxicity", "nisin" AND "toxicity" AND "414-45-5", "nisin" AND "immunological effects", and "nisin" AND "metabolism" were applied to search ScienceDirect, PubMed, Scopus and Google Scholar. From this literature search, four studies were identified.

Literature searches on the microbiological effects of nisin and on dietary exposure to nisin were undertaken to identify any additional information not already submitted to the Committee. Databases including Medline, Food Science Source, PubMed, Web of Science and ScienceDirect up to April 2024 were searched. Keywords in the searches included "nisin", "lantibiotic", "microbiome", "intestinal microbiota", "gut microbiota", "gut microbiome", "gastrointestinal microbiota", "gastrointestinal microbiome", "antimicrobial resistance", "resistance mechanism", "susceptibility testing", "bioavailability", "dietary intake", "intake", "dietary exposure" or "consumption", "occurrence" and "use level" as well as the genus/species of specific intestinal bacteria with the Boolean operators (AND, OR, NOT). Additionally, sponsor evaluations of the risks of induction of antimicrobial resistance and biological activity were submitted.

Chemical and technical considerations

Nisin A is obtained by fermentation using strains of *Lactococcus lactis* subsp. *lactis*, containing *nis*A gene. Nisin A is a small antimicrobial peptide (3354 Da) belonging to the lantibiotic class of bacteriocins (Class I, molecular weight < 5 kDa). It is a cationic, hydrophobic, 34-amino acid peptide that contains one lanthionine and four b-methyl-lanthionine rings, and includes the unusual amino acids dehydroalanine, dehydrobutyrine, lanthionine and β-methyllanthionine.

The antimicrobial activity, stability and water solubility of nisin A are pH dependent *(151)*. Antimicrobial activity is inversely dependent on pH value. Nisin A is stable at low temperatures; however, its stability at high temperatures is strongly dependent on pH *(152)*. The water solubility ranges from 12% (soluble) at pH 2.5, to 4% (sparingly soluble) at pH 5 and insoluble at pH > 7.

Under appropriate and well controlled fermentation conditions, strains of *L. lactis* subsp. *lactis* containing *nisA* gene produce the polypeptide nisin A that, after purification and concentration steps, is then stabilized and standardized to 2.2–2.5% (w/w) of the active ingredient and formulated with more than 50% sodium chloride with a typical batch containing 75%. In the past, nisin A processing was based on fermentation in a sterilized medium using non-fat milk. Current processes are tending towards the use of non-milk sources such as yeast extract and a carbohydrate source. Time, temperature and pH are strictly controlled to achieve optimum nisin A production *(153)*.

One IU is defined as the amount of nisin A required to inhibit the growth of one bacterial cell (*L. lactis* subsp. *cremoris*) in 1 mL of broth. This amount is 0.025 μg of nisin A (= 1 IU) *(154,155)*; 1 μg of nisin A is therefore equivalent to 40 IU.

Biochemical aspects

The previous Committee concluded that nisin administered by gavage to rats was hydrolysed and inactivated in the intestinal tract, with no biologically active nisin being detected in the colon or caecum *(109)*. A study in dogs in which nisin was found in the serum of only one out of 18 animals dosed with nisin was inconclusive.

Toxicological studies

The previous Committee considered nisin to be a substance of low oral toxicity. Nisin was found not to be carcinogenic or mutagenic, and not associated with any reproductive or developmental toxicity *(109)*.

In a study used by the previous Committee in establishing the ADI, rats were exposed for 90 days to doses of nisin A in the diet of 225 and 239 mg/kg bw per day for males and females, respectively *(156,157)*. A control group

was treated with sodium chloride. Any observed changes were considered by the previous Committee to be related to the high intake of sodium chloride (2196 and 2423 mg/kg bw per day for males and females, respectively). The Committee identified a NOAEL of 225 mg/kg bw per day, the highest dose tested, in this study.

In a 90-day study not previously considered by the Committee, nisin (1000 IU/mg) was administered to female mice as a single concentration of 200 mg/kg diet (equal to 27 mg/kg bw per day). Histopathological examination showed mild to moderate vacuolar degeneration in hepatocytes in half the animals tested (5/10) without affecting alanine transaminase and aspartate transaminase blood levels *(158)*. No additional details about the nisin preparation used in this study were provided. The Committee noted that commercial nisin preparations typically contain more than 50% sodium chloride. The Committee concluded that the mild histopathological observations in the liver were likely related to the high intake of sodium chloride and not nisin.

In a study on the immune system not evaluated by the previous Committee mice were fed a commercial preparation of nisin A at 0, 50 or 100 mg/kg diet (equivalent to 0, 7.5 or 15 mg/kg bw per day, respectively) for 30, 75 or 100 days in each dietary group *(159)*. Statistically significant changes in some of the immune parameters were not dose or time dependent, and the Committee considered that they were not toxicologically relevant.

Cytotoxicity of nisin has been evaluated by the previous Committee. The Committee concluded that purified nisin was cytotoxic to a number of eukaryotic cell types in vitro. Two new in vitro studies confirmed the conclusion by the previous Committee *(160,161)*.

Observations in humans

There was no information on human studies *(109)* in the previous evaluation. No new information was found for this evaluation.

Microbiological effects

Nisin evaluations by the previous Committee have included limited information on the microbiological effects of nisin, including the mechanisms of action and of resistance *(109)*. To consider whether nisin presents a risk for development of antimicrobial resistance, the Committee has evaluated available in vitro studies on resistance mechanisms in nisin-producing and pathogenic bacteria, and in the possible transmission of resistance between bacteria. Multiple natural variants of nisin exist, and most microbiological studies are carried out using nisin A or Z, which differ by a single amino acid. The Committee noted that – given the similarity of the nisin variants A and Z, with similar susceptibility to proteolytic activity, and excellent and comparable antimicrobial activity, solubility and pH

stability – studies with both substances (or in some cases not specified nisin variants) have been included in the evaluation.

Mode of action

The primary antimicrobial action of nisin involves binding to lipid II molecules in the cytoplasmic membrane of susceptible Gram-positive cells. Lipid II are precursor molecules involved in the synthesis of the bacterial cell wall. The insertion of nisin into the membrane causes disruption of membrane function. Nisin shows little or no activity against Gram-negative bacteria because of their different cell structure involving an outer membrane, which masks the primary target lipid II and prevents interference by nisin.

The nisin peptide consists of two structural domains, an N-terminal lipid II binding domain linked to the pore-forming C-terminal rings by a short three-residue hinge region. The binding of nisin to lipid II will inhibit cell wall synthesis, which initially has a bacteriostatic (growth inhibiting) effect but ultimately a bactericidal (killing) effect. The consequences of the formation of pores result in lethal loss of membrane integrity, depletion of the transmembrane potential and the leakage of cellular constituents. Nisin also impairs microbial membranes independently of lipid II and prevents outgrowth of bacterial spores. Nisin therefore has multiple antimicrobial activities based on both high-affinity targets and low-affinity membrane interactions, which make it particularly difficult for bacteria to become nisin resistant *(162)*.

Antimicrobial resistance

Nisin resistance can arise from mutations that induce changes in the membrane and cell wall, reducing the acidity of the extracellular medium to stimulate the binding of nisin to the cell wall and induce its degradation, prevent the insertion of nisin into the membrane, and transport or extrude nisin out across the membrane. Despite the widespread use of nisin as a food additive, reports of acquired nisin resistance have been limited to in vitro studies with adaptation to nisin under laboratory conditions.

Through studies on *Streptococcus bovis*, Mantovani and Russell *(163)* showed that the bacterial cells could survive in liquid medium containing nisin (1 μM) if the initial number of cells and adaptation time was high enough (> 8 hours) to obtain sporadic mutations. Nisin caused an initial decrease in the viability of nisin-sensitive cultures, but the viable cell number eventually increased; it was suggested that changes in teichoic acids, which are anionic glycopolymers in the bacterial cell wall, and cell wall charge caused the tolerance. The nisin-resistant cells showed unchanged or higher sensitivity towards a range of antibiotics, with the exception of ampicillin (for which higher resistance was observed).

Gravesen et al. *(164)* obtained spontaneous nisin-resistant *Listeria monocytogenes* mutants, typically having a two- to fourfold increase in the minimally inhibitory concentration of nisin, after sequential passaging in media containing 10–20 μg/mL nisin. The mutants demonstrated various changes in gene expression linked to cell wall biosynthesis, which was suggested to possibly affect the cell wall composition and thereby alter the sensitivity to compounds targeting the cell wall. The mutants also had an isolate-specific increase in sensitivity to different antibiotics, including the beta-lactams targeting the cell wall.

Naturally occurring mechanisms of resistance include enzymatic degradation of nisin. In early studies of nisin resistance, nisinase, a dehydropeptide reductase that can inactivate nisin through an enzymatic reaction, was detected from several bacterial species (*Lactobacillus plantarum*, *Streptococcus thermophilus*, *Clostridium botulinum*, *L. lactis* subsp. *cremoris*, *E. faecalis* and *S. aureus*) *(165)*. However, this nisinase has not been thoroughly purified, cloned or sequenced, and there has never been a conclusive study indicating the presence of this nisinase in the nisin producer *L. lactis (166)*.

The genetic background is known for a nisin-degrading protease named nisin resistance protein (NSR) and several other resistance mechanisms. The encoding gene *nsr* was originally observed as a specific nisin resistance gene located on a 60-kb plasmid in the nisin non-producer *L. lactis* subsp. *lactis* biovar diacetylactis. NSR is capable of proteolytically cleaving the C-terminal tail of nisin, thereby inactivating and reducing the antimicrobial activity of nisin by 100-fold *(167)*. Since then, several groups have isolated nisin-resistant lactococcal strains containing *nsr* on a plasmid, although NSR is only produced by *L. lactis* in a strain-specific manner.

van Gijtenbeek et al. *(168)* screened a collection of 710 *L. lactis* strains by whole genome sequencing and examined the effect of nisin on the *L. lactis* strains during milk fermentations. In contact with nisin, 279 (39%) *L. lactis* strains had a loss of acidification (nisin sensitive), 101 (14%) *L. lactis* strains had unaltered acidification (nisin resistant) and, in the remaining strains, the effect of nisin resulted in a range of delays in the onset of acidification. van Gijtenbeek et al. *(168)* analysed the collection for genes encoding key proteins involved in nisin biosynthesis (a nisin structural gene: *nisA*; the nisin biosynthetic machinery: *nisB*, *nisT*, *nisC* and *nisP*; *nis* gene regulation: *nisR* and *nisK*), nisin immunity (*nisI* and *nisFEG*) and nisin degradation (*nsr*). Nisin degradation by NSR was found to be a common feature present in 38% of the *L. lactis* strains examined and, of all strains, only 6.3% contained a full nisin biosynthesis cassette. Several nisin producers that also carried the *nsr* gene were identified.

Since nisin targets any Gram-positive bacterial cytoplasmic membrane, including that of *L. lactis* itself, nisin-producing *L. lactis* strains co-express proteins that confer nisin self-resistance. These are NisI, a membrane-associated

lipoprotein, and NisFEG, an ABC transporter *(168)*. ABC transporters are part of two-component systems, which are major signal transduction pathways that allow bacteria to detect and respond to environmental and intracellular changes. A group of two-component systems has been shown to be involved in the response against antimicrobial peptides, including nisin.

Similar species-specific, nisin-associated immunity and regulatory genes have been found, including SpiFEG and NsuFEG on rare occasions in *Streptococcus infantarius (169)*, BceRS/BceAB in *Bacillus subtilis* and BceABRS in *Streptococcus mutans (170)*. These genes encoding resistance mechanisms are not nisin specific as they are part of complex stress-response systems involving gene operons and promoter regions, and are not readily transferred to other bacteria.

Overall, the identified nisin resistance mechanisms are either unstable or complex, typically involving changes in the lipid membrane composition (by mutations) or signal pathways that are not readily transmitted by genomic elements. In the literature review, the Committee did not find any evidence of the natural transfer of acquired nisin resistance and heterologous expression of nisin self-resistance and, currently, there is no definitive evidence that in vivo exposure to nisin significantly contributes to induction or transmission of antimicrobial resistance.

Effect of nisin on the gut microbiome

There are few in vivo studies that have investigated the possible antimicrobial activity of nisin in the lower gastrointestinal tract. In addition, there is a paucity of reports providing quantitative data on the amount of intact nisin that reaches the intestines and impacts the intestinal microbiota. The previous Committee has concluded that the risk of active nisin reaching the intestines was low because of the enzymatic degradation of nisin. Newly available literature was evaluated.

In a study by Gough et al. *(171)* conducted using in vitro test systems designed to simulate human oral, gastric and small intestinal digestion, no intact nisin A applied to the system could be detected, with the authors concluding that nisin was primarily digested by pancreatin. In a follow-up in vivo study intended to evaluate the protective effect of starch matrices on nisin through the digestive tract in an animal model, Gough et al. *(172)* analysed faecal pellets of mice consuming starch dough and starch gel containing a commercial preparation of nisin A (54–161 mg/kg bw). They observed a nisin-protecting effect of the starch matrix and detected nisin and nisin digestion products in the faecal samples, but found them to have low antibacterial activity against *Lactococcus lactis* subsp. *cremoris* in agar diffusion assays. Using 16S rRNA high-throughput sequencing, they showed that diets including starch matrices containing nisin altered the mouse microbiota, but that similar alterations were also seen from starch matrices alone.

O'Reilley et al. *(173)* demonstrated intact nisin Z in piglet faeces after gut transit that retained antimicrobial activity, albeit using a diet that provided a relatively high dose (150 mg/kg bw) of high-potency nisin.

These two studies, which determined the presence of biologically active nisin in animal model faeces, were based on a high intake of nisin (54–161 mg/kg bw).

The Committee considered a number of in vivo and in vitro studies investigating the ability of nisin to modulate the gut microbiota. These studies used a variety of concentrations of nisin, often including therapeutic concentrations, or the use of capsules to ensure delivery of intact nisin to the large intestine.

In vivo studies on the effect of nisin on the microbiota in animal models were evaluated, including in mice *(174)*, chicken *(175,176)* and piglets *(173)*. However, the differences in nisin concentrations, animal models, microbiological methods and investigated (groups of) bacteria do not add sufficient and consistent information on the effects of nisin on specific genera or species of bacteria commonly found in the microbiome, or the more generic groups of Gram-positive and -negative bacteria, to draw conclusions on the overall effect of nisin.

Studies using metagenomic (community) DNA sequencing of intestinal microbiota allow for a better evaluation of the changes in composition of bacteria compared with traditional microbiology enumeration assays, as this methodology allows for a more complete overview of the presence and relative abundance of present microorganisms. O'Reilly et al. *(173)* used a porcine model to study the impact of orally ingested nisin on the gut microbiome. Nisin Z (≥ 38 000 IU/mg) was fed to piglets (age 34 days) either encapsulated in an ethyl cellulose preparation (850 mg/kg bw) or non-encapsulated (150 mg/kg bw) in the diet for 3 consecutive days. Metagenomic analyses of faecal samples showed a modulation of the gut microbiota in both nisin treatment groups compared with controls, illustrated by a change in the relative abundances of certain Gram-positive bacterial groups, including Firmicutes and *Bifidobacterium pseudocatenulatum*, but also decreases in some Gram-negative bacteria. This effect of nisin Z on the microbiota was transient as the microbiome microbial composition returned to levels similar to untreated groups by 3 days post-treatment.

Le Blay et al. *(177)* evaluated the antimicrobial activity of nisin A and nisin Z against 21 species of intestinal bacteria in vitro. The sensitivity of nisin A and nisin Z at 8.2 µg per well to intestinal microbiota using the agar-well diffusion test indicated that all Gram-positive intestinal bacteria (including *Bifidobacterium* spp., *Lactobacillus* spp. and *Clostridium* spp.), with the exception of *Streptococcus salivarius*, were inhibited to some extent depending upon the species tested. Nisin Z exhibited higher inhibitory effects than nisin A for many of the tested Gram-positive bacteria. Gram-negative bacteria *Bacteroides*

thetaiotaomicron, *Bacteroides vulgatus* and *Escherichia coli* were resistant to both nisin A and nisin Z.

The Committee concluded that, under certain experimental conditions, nisin can still retain some biological activity when entering the large intestine and can have a transient, modulating role on the microbiota. However, there are no reports of nisin showing any deleterious or dysbiotic effect on the microbiota.

Assessment of dietary exposure

The previous Committee assessed the dietary exposure to nisin at its Seventy-seventh meeting *(109)*. Estimates of dietary exposure reviewed ranged between 0.008 and 0.07 mg/kg bw per day across all mean and high exposures.

Estimates of dietary exposure at the national level considered at the current meeting included those submitted to the Committee, from the literature and calculated by the Committee. There were estimates available for more than 40 countries across all WHO regions. The dietary exposure estimates were based on different types of foods and concentrations. These included GSFA permissions, local permissions or other uses (e.g. proposed use levels; as a processing aid). GSFA permissions include cheeses, processed meats, other dairy products, fine bakery wares, desserts, liquid egg products, salads, soups, sauces and dips.

For all national estimates of dietary exposure considered at the current meeting (Table 3.2), mean estimates ranged up to 0.23 mg/kg bw per day for children and up to 0.07 mg/kg bw per day for adults. Estimates of high dietary exposure ranged up to 0.78 mg/kg bw per day for children and up to 0.23 mg/kg bw per day for adults. Major contributors to dietary exposure were commonly processed meats, cheeses and fermented milk products.

Table 3.2
Range of national estimates of dietary exposure to nisin

Population group	Age range	Mean (mg/kg bw per day)	High[a] (mg/kg bw per day)
Children	Up to 14, 17 or 18 years	0.001–0.23	< 0.001 to 0.78
Adults	From 15, 18 or 19 years and older	< 0.001 to 0.07	< 0.001 to 0.23

[a] A range of high percentiles was reported, including 90th to 97.5th percentile.

The Committee noted that the upper end of the range of dietary exposure estimates considered at the current meeting is higher than the estimates reported by the Committee at its Seventy-seventh meeting *(109)*. This is because of the greater number of national estimates available for the current meeting, capturing a greater degree of variability in food consumption patterns and resulting dietary exposures.

The Committee concluded that the highest estimate of dietary exposure of 0.78 mg/kg bw per day in children was appropriate to use in the evaluation.

Evaluation

Based on the available data, the Committee concluded that there is no concern for the induction of antimicrobial resistance, and that the risk of nisin having a disrupting effect on the microbiome of the human gastrointestinal tract is low.

The new toxicological information available for this evaluation did not provide any reason to revise the ADI for nisin *(109)*. The Committee re-affirmed the ADI of 0–2 mg/kg bw for nisin established by the previous Committee at the Seventy-seventh meeting *(109)*, but noted that the critical toxicological studies were conducted with nisin A; the Committee therefore concluded that the ADI applies only to nisin A.

The Committee noted that the dietary exposure estimates for nisin of up to 0.78 mg/kg bw per day for all population subgroups assessed were below the ADI for nisin A, and concluded that dietary exposure to nisin was not of toxicological concern.

A toxicological monograph addendum, including a section on microbiological effects, with a dietary exposure assessment was prepared.

The existing specifications monograph was revised.

3.1.8 Polyglycerol esters of fatty acids

Explanation

Polyglycerol esters of fatty acids (INS No. 475) is a food additive with the functional use as an emulsifier. A conditional ADI[16] of 0–25 mg/kg bw was established at the Ninth JECFA meeting *(178)* based on a NOAEL of 2500 mg/kg bw identified in a long-term study with rats. This was converted to an ADI of 0–25 mg/kg bw at the Seventeenth JECFA meeting *(179)* and was reaffirmed at the Thirty-fifth JECFA meeting *(180)*. The Committee was asked by the Fifty-third session of CCFA *(1)* to re-evaluate Polyglycerol esters of fatty acids, since the physical working group on the GSFA of the Fifty-first session of CCFA *(123)* had noted that there may be new information available that could raise the ADI.

Literature searches were conducted in Web of Science and PubMed. Searches were conducted with the search term "Polyglycerol fatty acid esters" or linked search terms "Polyglycerol" AND "fatty acid esters" ("safety" OR "toxicity" OR "genotoxicity" OR "toxic"). Searches did not identify any new relevant studies, and none was provided by the sponsor. The Committee noted that the

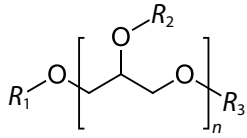
[16] A term no longer used by JECFA to signify a range above the "unconditional ADI", which may signify an acceptable intake when special problems, different patterns of dietary intake and special groups of the population that may require consideration are taken into account.

new information referred to in the Fifty-first session of CCFA *(123)* appears to be limited to the Scientific Opinion by EFSA in 2017 *(181)*, and that no new toxicological data were available to EFSA that had not been previously considered by JECFA.

Chemical and technical considerations

Polyglycerol esters of fatty acids are mixed esters formed by partially esterifying polymerized glycerols (polyglycerols) with edible food-grade fats, oils or fatty acids. The additive may contain minor amounts of mono-, di- and triglycerides, free glycerol and polyglycerols, free fatty acids and sodium salts of fatty acids. The degree of polymerization of the polyglycerol portion of the additive varies and is specified by a number (such as tri-) that is related to the average number of glycerol residues per polyglycerol molecule. JECFA specifications consider that the polyglycerol moiety is composed of not less than 70% of di-, tri- and tetraglycerols and not more than 10% of polyglycerols equal to or higher than heptaglycerol. Polyglycerol esters of fatty acids comprise a large group of closely related compounds that have varying properties depending on the carbon chain length of the fatty acid moieties and the number of glycerol monomers in the polyglycerol group. The physical and chemical properties of the Polyglycerol esters of fatty acids depend upon the degree of polymerization of the polyglycerol, the degree of esterification and the nature of the fatty acids. As a result, no single structure, molecular formula or molecular weight can be assigned. The general structural formula for Polyglycerol esters of fatty acids is shown in Fig. 3.1, where the average value of n is approximately 3, and R_1, R_2 and R_3 may each be a fatty acid moiety or hydrogen.

Fig. 3.1
General structural formula for Polyglycerol esters of fatty acids



Biochemical aspects

No new data on biochemical aspects were identified. Polyglycerol esters of fatty acids include a large group of closely related compounds. Many of the individual components are normal constituents of the human diet, namely glycerol, mono-, di- and tri-fatty acid esters, and individual fatty acids, with the exception of the artificially produced polymers of glycerol *(182)*. It has been shown that Polyglycerol esters of fatty acids are rapidly hydrolysed to fatty acids and polyglycerols after ingestion. The fatty acid moiety of polyglycerol esters

has been shown to be rapidly absorbed and utilized, whereas the polyglycerols were absorbed and eliminated, and limited retention was seen in the carcass of rats *(183)*.

Toxicological studies

No new toxicological data on Polyglycerol esters of fatty acids (INS No. 475) have been identified.

In previously available data, no adverse effects were observed in any of the short-term dietary studies in rats with inclusion of up to 10% Polyglycerol esters of fatty acids (equivalent to 9000 mg/kg bw per day), the highest dose tested *(183)*. There were no adverse effects in the 80-week or 62-week dietary studies in mice using doses up to 7500 mg/kg bw per day, or in a 2-year study in rats using doses up to 2500 mg/kg bw per day *(183)*. There were no effects on fertility or reproduction in a three-generation study in rats *(183)*.

Observations in humans

No new data were available.

Assessment of dietary exposure

Dietary exposure to Polyglycerol esters of fatty acids (INS No. 475) has not been evaluated previously. For the current meeting, in response to the JECFA call for data, one submission including dietary exposure estimates for Europe was received from the European Food Emulsifiers Manufacturers Association.

Polyglycerol esters of fatty acids are authorized for use in the GSFA in 56 food categories at maximum levels (MLs) ranging from 20 mg/kg up to 20 000 mg/kg.

Because there can be large differences in permitted local uses and use levels compared with the 56 GSFA provisions (e.g. 16 in Europe), the Committee conducted its own international estimates of dietary exposure to Polyglycerol esters of fatty acids. These were conducted using the current 56 GSFA food category provisions and national individual food consumption data for populations in Australia, New Zealand and Europe, which could be applicable to other populations.

The upper end of the estimates of the mean and high-level dietary exposures to Polyglycerol esters of fatty acids were 268 and 537 mg/kg bw per day for infants (age ≤ 11 months) and toddlers (age 1 to < 3 years), 222 and 377 mg/kg bw per day for children (age 3–14 years) and adolescents (age 10–17 years), and 108 and 196 mg/kg bw per day for adults (age ≥ 18 years), respectively.

Depending on the country dataset, the main food categories contributing more than 10% to the total mean dietary exposure to Polyglycerol esters of fatty

acids in children and adult populations were breakfast cereals; breads and rolls; fine bakery wares; water-based flavoured drinks; and coffee, tea, herbal infusions and other hot beverages.

The Committee also reviewed more specific estimates of dietary exposure that were performed by EFSA and Food Standards Australia New Zealand (FSANZ) based on their local permissions.

For the EFSA assessment, seven use levels were provided by the industry for three of the 16 categories with authorized uses. It was noted by EFSA that, of the seven use levels provided by industry in Europe, one referred to chewing gum and six referred to niche products for fillings of various sponge cakes, fine bakery wares and chocolate eggs.

The FSANZ assessment was based on current authorized uses in seven food categories (whipped, thickened light cream; shortening; margarine and similar products; oil emulsions; cakes; dairy and fat-based desserts, dips and snacks; and sauces and toppings) at maximum permitted levels.

In the EFSA assessment, the highest mean and 95th percentile estimates of dietary exposure were 2.6 and 6.4 mg/kg bw per day. In the Australia and New Zealand assessment, the highest mean and 90th percentile estimates of exposure were 14 and 36 mg/kg bw per day.

Overall, the Committee noted that potential estimates of dietary exposure based on the MLs for the 56 GSFA food categories were highly conservative in comparison to dietary exposure estimates that are based on local permissions (Australia, New Zealand and Europe). The main reason is that for all exposure scenarios it was assumed that all foods in a category contain the food additive at the maximum levels, but also because of the fewer uses and levels of use (seven food categories for Australia and New Zealand, and three out of 16 food categories for Europe, compared with 56 food categories in the GSFA).

Evaluation

At its Seventeenth meeting *(179)*, the Committee established an ADI of 0–25 mg/kg bw for Polyglycerol esters of fatty acids, based on a long-term study in rats in which there were no effects at 2500 mg/kg bw, the highest dose tested. In the absence of any new toxicological information, the present Committee re-affirmed the ADI of 0–25 mg/kg bw.

The Committee understands that the new information referred to in the CCFA request to re-evaluate Polyglycerol esters of fatty acids was only the EFSA Scientific Opinion *(181)*. In its re-evaluation of the food additive Polyglycerol esters of fatty acids, EFSA concluded that it does not raise any safety concerns at the use and use levels reported by industry, and there was no need for a numerical ADI. This conclusion was based on several considerations: no adverse effects observed in short- or long-term toxicity studies, no genotoxic potential identified and low dietary exposure to this additive (up to 6.4 mg/kg bw per day) *(181)*.

The present Committee's estimated dietary exposures to Polyglycerol esters of fatty acids based on the MLs for 56 GSFA food categories were up to 537 mg/kg bw per day. These estimates exceed the ADI by up to about 20-fold at the highest 95th percentile, which indicates a potential health concern. However, the Committee recognized that the dietary exposure estimates are highly conservative but could not refine them in the absence of more data on uses and use levels.

At the present meeting, the Committee reviewed the specifications for Polyglycerol esters of fatty acids. The Committee lowered the existing limit for lead; added limits for arsenic, cadmium and mercury; replaced the "test for fatty acids" with a "test for free fatty acids"; and removed the test for "acids". The Committee also clarified that the test for "polyglycerols" is the "polyglycerol determination in polyglycerol esters" test found in the FAO JECFA Monographs *(184)*. To mitigate the occurrence of contaminants coming from the raw materials (fats, oils and fatty acids) used in the manufacture of Polyglycerol esters of fatty acids, only edible food-grade precursors are used, particularly those prepared in a manner consistent with the Codex Alimentarius *Code of practice for the reduction of 3-monochloropropane-1,2-diol esters (3-MCPDEs) and glycidyl esters (GEs) in refined oils and food products made with refined oils (185)*.

A toxicological monograph addendum with a dietary exposure assessment was prepared.

The existing specifications monograph was revised.

Recommendations

The Committee makes the following recommendations.

- Considering the potential high exceedance of the ADI based on the estimated dietary exposures, the CCFA should review and revise current uses of Polyglycerol esters of fatty acids in the GSFA, including the maximum permitted levels and the food categories in which this food additive is permitted to be used.
- The food industry should provide use levels of Polyglycerol esters of fatty acids by the end of 2026 to enable more refined estimates of dietary exposure to be calculated by the Committee. When these data are provided, the Committee will reconsider the safe use of Polyglycerol esters of fatty acids.
- Dietary exposure estimates are required from a larger number of countries before the Committee can draw robust conclusions about the safety of use of Polyglycerol esters of fatty acids. These should be based on industry use levels where possible. The Committee encourages Member States to provide dietary exposure estimates by the end of 2026.

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4. Flavouring agents

4.1 Specifications of identity and purity

4.1.1 Revised specifications

The Committee received information in support of the revision of the full specifications for 10 flavouring agents that were on the agenda of the present meeting (JECFA Nos 482, 487, 498, 499, 511, 528, 533, 554, 555 and 560).

The Committee revised specifications for *S*-methyl thioacetate (No. 482) based on data from 15 lots of the commercial product. The assay minimum was revised from 98% to 96%, and the specific gravity was revised to 1.021–1.025. The Flavis No. (12.149), the solubility in ethanol (soluble) and the odour (pungent, garlic, sharp cheese) were added. In addition, the chemical name was revised to *S*-methyl ethanethioate, and the synonyms and the solubility were also revised.

For *S*-methyl 3-methylbutanethioate (No. 487), the Committee revised the specifications based on data from 13 lots of the commercial product. The specific gravity was revised to 0.935–0.947. The Flavis No. (12.157), the solubility in ethanol (soluble) and the odour (pungent, fruity and onion, garlic) were added. In addition, the synonyms and the solubility were revised.

For 4,5-dihydro-3(2*H*) thiophenone (No. 498), the Committee revised the specifications based on data from 19 lots of the commercial product. The specific gravity was revised to 1.194–1.207. The Flavis No. (15.012), the solubility (minimally soluble in water and organic solvents) and the solubility in ethanol (soluble) were added. In addition, the synonyms were revised.

For 2-methyltetrahydrothiophen-3-one (No. 499), the Committee revised the specifications based on data from 18 lots of the commercial product. The assay minimum was revised from 99% to 98%, the refractive index was revised to 1.505–1.520 and the specific gravity was revised to 1.115–1.126. The Flavis No. (15.023), the solubility in ethanol (soluble), physical form (colourless to yellow liquid) and the odour (earthy, garlic, fruity) were added. In addition, the synonyms and the solubility were revised.

For 1-butanethiol (No. 511), the Committee revised the specifications based on data from 14 lots of the commercial product. The refractive index was revised to 1.440–1.452 and the specific gravity was revised to 0.830–0.848. The Flavis No. (12.010) was added. In addition, the solubility and the solubility in ethanol were revised.

For *o*-toluenethiol (No. 528), the Committee revised the specifications based on data from 21 lots of the commercial product. The refractive index was revised to 1.570–1.582 and the specific gravity was revised to 1.050–1.059. The Flavis No. (12.027), the solubility (insoluble in water; soluble in fats) and the

solubility in ethanol (moderately soluble) were added. In addition, the synonyms were revised.

For bis(methylthio)methane (No. 533), the Committee revised the specifications based on data from 18 lots of the commercial product. The specific gravity was revised to 1.047–1.067. The Flavis No. (12.118) and the solubility in ethanol (soluble) were added. In addition, the synonyms and the solubility were revised.

For 3-mercaptohexyl acetate (No. 554), the Committee revised the specifications based on data from 15 lots of the commercial product. The assay minimum was revised from 81.7% to 95%, and the other requirements listed as "also contains min. 8.2% 3-mercaptohexanol and 9.7% 3-acetylmercaptohexyl acetate" were deleted. The refractive index was revised to 1.455–1.472 and the specific gravity was revised to 0.987–0.997. The Flavis No. (12.234), the solubility in ethanol (soluble) and the odour (fruity, with grapefruit/citrus notes, and sulfur undertone) were added. In addition, the chemical name was revised to 3-sulfanylhexyl acetate, and the synonyms and the solubility were revised.

For 3-mercaptohexyl butyrate (No. 555), the Committee revised the specifications based on data from 17 lots of the commercial product. The assay minimum was revised from 90% to 98%, the refractive index was revised to 1.457–1.469 and the specific gravity was revised to 0.960–0.968. The Flavis No. (12.235), the solubility in ethanol (soluble) and the odour (fruity, with grapefruit/citrus notes and sulfur undertone) were added. In addition, the chemical name was revised to 3-sulfanylhexyl butanoate, and the synonyms and the solubility were revised.

For 3-mercapto-2-pentanone (No. 560), the Committee revised the specifications based on data from 19 lots of the commercial product. The refractive index was revised to 1.465–1.471. The Flavis No. (12.031) and the odour (raw-meat, garlic, sulfur) were added. In addition, the chemical name was revised to 3-sulfanylpentan-2-one and a synonym (3-mercapto-2-pentanone) was added. The solubility and the solubility in ethanol were also revised.

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Annex 1

Meeting agenda

Food and Agriculture Organization of the United Nations

World Health Organization

99th JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES (JECFA)
Geneva, 11–20 June 2024

1. Opening

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

3. Election of Chairperson and Vice-chairperson, appointment of rapporteurs

4. Adoption of agenda

5. Matters of interest arising from previous sessions of the Codex Committee on Food Additives (CCFA)

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

7. Evaluations

   Toxicological evaluation, exposure assessment and establishment of specifications

   7.1 Food additives

   - Butterfly pea flower extract
   - Natamycin (INS No. 235)
   - Nisin (INS No. 234)
   - Polyglycerol esters of fatty acids (INS No. 475)

   7.2 Processing aids

   - Adenosine-5′- monophosphate deaminase from *Aspergillus oryzae*
   - Endo-1,4-β-xylanase from *Bacillus subtilis* produced by *B. subtilis* LMG S-28356

- Transglucosidase/alphaglucosidase from *Trichoderma reesei* expressing an alphaglucosidase gene from *Aspergillus niger*
- Xylanase from *Talaromyces emersonii* expressed in *Aspergillus niger*

Revision of specifications

7.3 Flavouring agents

- 3-Mercaptohexyl acetate (554)
- 3-Mercaptohexyl butyrate (555)
- 1-Butanethiol (511)
- 3-Mercapto-2-pentanone (560)
- bis(Methylthio)methane (533)
- *o*-Toluenethiol (528)
- *S*-Methyl thioacetate (482)
- *S*-Methyl 3-methylbutanethioate (487)
- 4,5-Dihydro-3(2*H*) thiophenone (498)
- 2-Methyltetrahydrothiophen-3-one (499)

8. Errata

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

10. Adoption of the report

Annex 2

Toxicological information and information on specifications

Table A2.1
Food additives evaluated toxicologically, assessed for dietary exposure and specifications

Food additive	JECFA enzyme identifier	Specifications	ADIs and other conclusions on toxicology and dietary exposure
Adenosine-5′-monophosphate deaminase from *Aspergillus* sp.	JECFA99-1	No[a]	Because of a lack of information to confirm the identity of the production organism and whether the test material used in the toxicity studies is representative of the current article of commerce, the Committee could not complete the safety evaluation of this enzyme preparation.
Butterfly pea flower extract	–	No[a]	Because of the limited nature of the toxicological data and the uncertainties concerning the specifications for the commercial product and the characterization of the test materials in the submitted toxicity studies, the Committee was unable to complete the safety assessment of butterfly pea flower extract.
Endo-1,4-β-xylanase from *Bacillus subtilis* expressed in *Bacillus subtilis*	JECFA99-2	N	The Committee concluded that dietary exposure to this endo-1,4-β-xylanase enzyme preparation is not anticipated to pose a risk for allergenicity. The Committee identified a NOAEL of 147.3 mg TOS/kg bw per day, the highest dose tested, in a 13-week study in rats. Comparison of this NOAEL with the estimated dietary exposure of 0.008 mg TOS/kg bw per day gives an MOE of more than 18 000. Based on this MOE and the lack of concern for genotoxicity, the Committee established an ADI "not specified"[b] for endo-1,4-β-xylanase (JECFA99-2) from *Bacillus subtilis* expressed in *Bacillus subtilis* when used in the applications specified, at the levels of use specified and in accordance with current GMP.
Endo-1,4-β-xylanase from *Rasamsonia emersonii* expressed in *Aspergillus niger*	JECFA99-3	N	The Committee concluded that the risk of allergenicity upon dietary exposure to this endo-1,4-β-xylanase is low. The Committee identified a NOAEL of 1850 mg TOS/kg bw per day, the highest dose tested in the 13-week study in rats. Comparison of this NOAEL with the highest estimated dietary exposure of 0.380 mg TOS/kg bw per day in toddlers gave an MOE of more than 4800. On the basis of this MOE and lack of concern about genotoxicity, the Committee established an ADI "not specified" for this endo-1,4-β-xylanase (JECFA99-3) from *R. emersonii* expressed in *A. niger* when used in the applications specified, at the levels of use specified and in accordance with GMP.

Food additive	JECFA enzyme identifier	Specifications	ADIs and other conclusions on toxicology and dietary exposure
Glucosidase from *Aspergillus niger* expressed in *Trichoderma reesei* exhibiting α-glucosidase and transglucosidase activity	JECFA99-4a, JECFA99-4b	N	The Committee concluded that dietary exposure to this glucosidase is not anticipated to pose a risk for allergenicity. The Committee also had no concerns about potential genotoxicity of the enzyme concentrate. The Committee identified a NOAEL of 74.8 mg TOS/kg bw per day, the highest dose tested, for the enzyme concentrate in the 18-week study in rats. Comparison of this NOAEL with the estimated dietary exposure of 0.443 mg TOS/kg bw per day gave an MOE of 169. The Committee therefore established an ADI "not specified" for glucosidase from *A. niger* expressed in *T. reesei* exhibiting α-glucosidase (JECFA99-4a) and transglucosidase (JECFA99-4b) activity when used in the applications specified, at the levels of use specified and in accordance with GMP.
Natamycin	–	R	Based on the available data, the Committee concluded that there is no concern for the induction of antimicrobial resistance and that the risk of natamycin having a disrupting effect on the microbiome of the human gastrointestinal tract is low. The Committee re-affirmed the ADI of 0–0.3 mg/kg bw for natamycin established by the previous Committee at its Twentieth meeting. The Committee further noted that the NOAELs in the new 13-week and 1-year studies in rats (42 and 26 mg/kg bw per day, respectively), with the application of a 100-fold uncertainty factor, support the current ADI of 0–0.3 mg/kg bw.
Nisin A	–	R	Based on the available data, the Committee concluded that there is no concern for the induction of antimicrobial resistance, and that the risk of nisin having a disrupting effect on the microbiome of the human gastrointestinal tract is low. The new toxicological information available for this evaluation did not provide any reason to revise the ADI for nisin. The Committee re-affirmed the ADI of 0–2 mg/kg bw for nisin established by the previous Committee at the Seventy-seventh meeting, but noted that the critical toxicological studies were conducted with nisin A; the Committee therefore concluded that the ADI applies only to nisin A.
Polyglycerol esters of fatty acids	–	R	At its Seventeenth meeting, the Committee established an ADI of 0–25 mg/kg bw for Polyglycerol esters of fatty acids, based on a long-term study in rats in which there were no effects at 2500 mg/kg bw, the highest dose tested. In the absence of any new toxicological information, the present Committee re-affirmed the ADI of 0–25 mg/kg bw.

ADI: acceptable daily intake; GMP: Good Manufacturing Practices; MOE: margin of exposure; N: new specification; NOAEL: no-observed-adverse-effect limit; R: revised specifications; TOS: total organic solids.

[a] Specifications were drafted but could not be finalized for publication because of a lack of critical information. Information is required to complete the specifications.

[b] The reader is referred to the Technical Report of the Eighty-seventh JECFA meeting for clarification of the term ADI "not specified".

Table A2.2
Flavouring agents considered for specifications only

Food additive	No.	Specification
S-methyl thioacetate	482	R
S-methyl 3-methylbutanethioate	487	R
4,5-dihydro-3(2*H*) thiophenone	498	R
2-methyltetrahydrothiophen-3-one	499	R
1-Butanethiol	511	R
o-Toluenethiol	528	R
bis(Methylthio)methane	533	R
3-Mercaptohexyl acetate	554	R
3-Mercaptohexyl butyrate	555	R
3-Mercapto-2-pentanone	560	R

R: revised specification.

Annex 3

JECFA enzyme submission checklist

Table A3.1

Information to be provided by the sponsor for the safety assessment of enzyme preparations for use in foods

No.	Class(es)[a]	Information required	Details/rationale	Information to be provided by sponsor (document title, section, page number)
Enzyme classification and description of active components of enzyme preparation				
1.	All	Name of enzyme(s)	e.g. Triacylglycerol phosphodiesterase	
2.	All	Systematic name(s) and number(s)	EC/IUBMB no.; CAS no. (where appropriate)	
3.	All	Molecular weight(s)	As determined by SDS PAGE, gel filtration chromatography etc.	
4.	All	Amino acid sequence(s)	Predicted and determined primary amino acid sequence	
5.	All	Catalytic activity	All reactions catalysed including any secondary activities, conditions under which catalysis occurs (e.g. pH, temperature)	
6.	All	Historical use(s) in food-based applications	Evidence of commercial food use, including from the parent strain or the lineage (e.g. as a processing aid in the manufacture of bakery products, pasta and noodles, in egg yolk and in oil degumming)	
7.	All	Use levels in food(s)	Express each use as total organic solids (TOS) in mg/kg food	
8.	All	Fate in final food(s)	Is the enzyme active, inactive or removed? How is the enzyme inactivated/removed?	
9.	All	Existing safety evaluations	Include any existing health-based guidance values (e.g. ADI)	
Details about the production organism				
10.	All	Identity of the production organism	Identify genus, species, strain	
11.	I (iii), II	Host/recipient organism	Identify genus, species	
12.	I (iii), II	Donor/source of genetic material	e.g. Identify source of genetic material by genus, species (native, modified or synthetic)	

No.	Class(es)[a]	Information required	Details/rationale	Information to be provided by sponsor (document title, section, page number)
13.	I (iii), ii	Details of genetic modification: (i) to host genome;	History of development of host strain (e.g. deletion of gene clusters that encode for aflatoxins, modifications that make host extracellular protease deficient or make it non-sporulating, etc.), identification of genes removed/added	
		(ii) addition of rDNA (gene of interest from another microorganism) to host microorganism through mobile genetic elements	Donor/source of genetic material, details on how the genetic element was designed and the identity of genes on the element, stability information, copy numbers, whether it integrates or does not integrate into host genome, etc. Evidence that genetic material does not contain genes coding for virulence factors, protein toxins, or any enzymes that may be involved in the synthesis of mycotoxins.	
14.	I (iii), II	Genetic modification techniques	Site-directed mutagenesis, chemical mutagenesis, recombinant DNA technology, etc.	
15.	I (iii), II	Description of intended and non-specific effects resulting from genetic modification and any changes carried out to prevent unwanted side reactions/products	e.g. An intended effect may be increased yield; a non-specific effect may be activation of toxin production. Rectification measures may include genetic modifications, specific fermentation conditions etc.	
16.	All	Deposit information (if applicable)	e.g. ATCC no.	
Production of enzyme concentrate and preparation				
17.	All	Detailed manufacturing process	For enzymes in Class I(i) and Class I(ii), and Class II enzymes derived from plants and animals, manufacturing details are required. For enzymes in Class I(iii) and Class II produced by microorganisms, include details describing controlled fermentation inputs and conditions; the steps taken to retain genetic modifications; and further processing, purification and concentration steps. Indicate how production strains are maintained under conditions that ensure the absence of genetic drift and, when used in the production of enzyme preparations, indicate the methods and conditions that are applied to ensure consistency and reproducibility from batch to batch. Such conditions must ensure the absence of toxin production by the source organism and prevent the introduction of microorganisms that could be the source of toxic or other undesirable substances.	

No.	Class(es)[a]	Information required	Details/rationale	Information to be provided by sponsor (document title, section, page number)
18.	All	Formulation ingredients	Identify the carriers, diluents, excipients, supports and other additives and ingredients (including processing aids) used in the production, stabilization and application of enzyme preparations, which must be acceptable for food use. In order to distinguish the proportion of the enzyme preparation derived from the source material as opposed to that contributed by diluents and other additives and ingredients, individual specifications require a statement of percentage TOS defined: % TOS = 100 – (A + W + D) where A = % ash, W = % water and D = % diluents and/or other additives and ingredients. TOS content is usually expressed in milligrams or micrograms TOS per kilogram body weight per day.	
Specifications and data required for enzyme concentrates and preparations				
19.	All	Description	Physical form of the enzyme preparation (liquid, semiliquid or dried product)	
20.	All	Purity	Impurities including elemental and microbiological impurities. Analytical test methods, validation data, representative batch data (minimum of 5 batches) are required.	
21.	All	Enzyme characterization	Enzyme activity (including method of assay, activity unit definition), molecular weight determination for the enzyme and other specific identification techniques. A universally usable test method to define enzyme activity present in the preparation should be submitted. Analytical test methods, validation data, representative batch data (minimum of 5 batches) are required.	
22.	All	Analysis of at least five non-consecutive batches of the enzyme concentrate (for enzymes in Class II, at least one of which should have been used for toxicological testing)	e.g. TOS, enzyme activity, protein concentration, impurities, absence of antibiotic inactivating proteins, etc.	
23.	All	Composition of at least five non-consecutive batches of the product(s) of commerce (enzyme preparation)	e.g. Stabilizers, pH adjustment agent, carriers, diluents, preservatives, etc.	
24.	I (iii), II	Information on carryover of allergens from the fermentation media to the enzyme concentrate	Identification of major food allergens in media components	

No.	Class(es)[a]	Information required	Details/rationale	Information to be provided by sponsor (document title, section, page number)
25.	I (iii), II	Evidence for absence of recombinant DNA and production organisms in the enzyme concentrate		
Assessment of potential allergenicity of the enzyme				
26.	I (iii), II	Comparison of the amino acid sequence of the enzyme to known allergens	In silico comparison of primary amino acid structure with allergen databases to confirm the absence of sequence homology with known allergenic proteins. (i) Sequence homology (35% of a sliding window of 80 amino acids) (ii) Sequence identity in contiguous stretches of 8 amino acids within the enzyme sequence. All the information resulting from the sequence homology comparison between an expressed enzyme and known allergens should be reported. If any of the identity scores equal or exceed 35%, this is considered to indicate significant homology and needs to be scientifically considered in the context of a safety assessment for enzymes in food.	
27.	I (iii), II	Proteolysis resistance/digestibility of the enzyme	e.g. Simulated gastric fluid studies, etc.	
Toxicology				
28.	II	Results of toxicological testing of the enzyme concentrate	It is necessary to conduct toxicological studies in order to establish an ADI: (i) 90-day oral toxicity test in a rodent species; and (ii) two short-term genotoxicity tests (mutagenicity and clastogenicity): (a) for gene-mutation in bacteria and (b) for chromosomal aberrations (preferably in vitro)	
29.	I (iii), II	Bioinformatic analysis of the amino acid sequence for potential matches with known toxins	Explanation of the analysis and interpretation should be provided	

No.	Class(es)[a]	Information required	Details/rationale	Information to be provided by sponsor (document title, section, page number)
Dietary exposure assessment				
30.	II	Estimate of dietary exposure to the enzyme preparation calculated on the basis of TOS. Separate dietary exposure situations may need to be considered with respect to the enzymes described in Classes I (iii) and II, depending on whether they are (i) enzyme preparations added directly to food and not removed; (ii) enzyme preparations added to food but removed from the final product according to GMP; or (iii) immobilized enzyme preparations that are in contact with food only during processing.	Express the dietary exposure as mg TOS/kg bw per day; provide an explanation of the methodology used to derive the estimated dietary exposure	
31.		Additional information and comments	Additional items considered helpful in the safety assessment	

ADI: acceptable daily intake; ATCC: American Type Culture Collection; CAS: Chemical Abstracts Service; EC: Enzyme Commission; GMP: Good Manufacturing Practices; IUBMB: International Union of Biochemistry and Molecular Biology; TOS: total organic solids.

[a] Class I: enzymes derived from sources that are considered safe for consumption and for which toxicological evaluations are not normally required. Type i: enzymes obtained from edible tissues of plants or animals commonly used as foods. Type ii: enzymes derived from microorganisms that are traditionally accepted as constituents of foods or are normally used in the preparation of foods. Type iii: enzymes derived from a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain. Class II: enzymes derived from sources that are NOT considered safe for consumption and are not in any of the sub-categories listed above.

Annex 4

Reports and other documents resulting from previous JECFA meetings

Table A4.1
Publications resulting from previous JECFA meetings

JECFA meeting no. (year)	Topic	Publications resulting from meeting (year of publication)
1 (1956)	Food additives	TRS 129 (1957), NMRS 15 (1957)
2 (1957)	Food additives	TRS 144 (1958), NMRS 17 (1958)
3 (1958)	Food additives	Specifications for identity and purity of food additives, Vol. I (1962)
4 (1959)	Food additives	Specifications for identity and purity of food additives, Vol. II (1963)
5 (1960)	Food additives	TRS 220 (1961), NMRS 29 (1961)
6 (1961)	Antimicrobials and antioxidants	TRS 228 (1962), NMRS 31 (1962)
7 (1963)	Food additives	TRS 281 (1964), NMRS 35 (1964)
8 (1964)	Food additives	TRS 309 (1965), NMRS 38 (1965), NMRS 38B (1966)
9 (1965)	Food additives	TRS 339 (1966), NMRS 40 (1966), NMRS 40A, B, C (1966)
10 (1966)	Food additives	TRS 373 (1967), NMRS 43 (1967)
11 (1967)	Food additives	TRS 383 (1968), NMRS 44 (1968), NMRS 44A (1968), NMRS 44B (1969)
12 (1968)	Food additives	TRS 430 (1969), NMRS 45 (1969), NMRS 45A (1969)
13 (1969)	Food additives	TRS 445 (1970), NMRS 46 (1970), NMRS 46A, 46B (1970)
14 (1970)	Food additives	TRS 462 (1971), NMRS 48 (1971), NMRS 48A, B, C (1971), FAS 1, 2, 3 (1972)
15 (1971)	Food additives	TRS 488 (1972), NMRS 50 (1972), NMRS 50A, B, C (1972)
16 (1972)	Food additives and contaminants	TRS 505 (1972), NMRS 51 (1972), NMRS 51A (1972), FAS 4 (1972)
17 (1973)	Food additives	TRS 539 (1974), NMRS 53 (1974), NMRS 53A (1974), FAS 5 (1974)
18 (1973)	Food additives	TRS 557 (1974), NMRS 54 (1974), NMRS 54A, B (1975), FAS 6, 7 (1975)
19 (1974)	Food additives	TRS 576 (1975), NMRS 55 (1975), NMRS 55A (1975), NMRS 55B (1976), FAS 8 (1975), FAS 9 (1976)
20 (1975)	Food additives	TRS 599 (1976), FNM 1 (1976), FNM 1B (1977), FAS 10 (1976), FAS 11 (1977)
21 (1977)	Food additives	TRS 617 (1977), NMRS 57 (1977), FAS 12 (1977)
22 (1978)	Food additives	TRS 631 (1978), FAS 13 (1978), FNP 4 (1978), FNR 7 (1978),
23 (1979)	Food additives	TRS 648 (1980), FAS 14 (1980), FNP 12 (1979)

JECFA meeting no. (year)	Topic	Publications resulting from meeting (year of publication)
24 (1980)	Food additives	TRS 653 (1981), FAS 15 (1980), FNP 17 (1980)
25 (1981)	Food additives	TRS 669 (1981), FAS 16 (1981), FNP 19 (1981)
26 (1982)	Food additives and contaminants	TRS 683 (1982), FAS 17 (1982), FNP 25 (1982)
27 (1983)	Food additives and contaminants	TRS 696 (1984), FAS 18 (1983), FNP 28 (1983), FNP 5 (Revision 1) (1983)
28 (1984)	Food additives and contaminants	TRS 710 (1984), FAS 19 (1984), FNP 31/1 (1984), FNP 31/2 (1984)
29 (1985)	Food additives and contaminants	TRS 733 (1986), FAS 20 (1987), FNP 34 (1986)
30 (1986)	Food additives and contaminants	TRS 751 (1987), FAS 21 (1987), FNP 37 (1986)
–	–	Principles for the safety assessment of food additives and contaminants in food. WHO Environmental Health Criteria, No. 70, 1987 (https://apps.who.int/iris/handle/10665/37578)
31 (1987)	Food additives and contaminants	TRS 759 (1987), FAS 22 (1988), FNP 38 (1988)
32 (1987)	Veterinary drug residues in food	TRS 763 (1988), FAS 23 (1988), FNP 41 (1988)
33 (1988)	Food additives and contaminants	TRS 776 (1989), FAS 24 (1989)
34 (1989)	Veterinary drug residues in food	TRS 788 (1989), FAS 25 (1990), FNP 41/2 (1990)
35 (1989)	Food additives and contaminants	TRS 789 (1990), FAS 26 (1990), FNP 49 (1990)
36 (1990)	Veterinary drug residues in food	TRS 799 (1990), FAS 27 (1991), FNP 41/3 (1991)
37 (1990)	Food additives and contaminants	TRS 806 (1991), FAS 28 (1991)
–	–	Compendium of food additive specifications, from 1992 (https://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/)
38 (1991)	Veterinary drug residues in food	TRS 815 (1991), FAS 29 (1991), FNP 41/1 (1991), FNP 5 (Revision 2) (1991)
39 (1992)	Food additives and naturally occurring toxicants	TRS 828 (1992), FAS 30 (1993)
40 (1992)	Veterinary drug residues in food	TRS 832 (1993), FAS 31 (1993), FNP 41/5
41 (1993)	Food additives and contaminants	TRS 837 (1993), FAS 32 (1993)
42 (1994)	Veterinary drug residues in food	TRS 851 (1995), FAS 33 (1994), FNP 41/6 (1994)
43 (1994)	Veterinary drug residues in food	TRS 855 (1995), FAS 34 (1995), FNP 41/7 (1995)
44 (1995)	Food additives and contaminants	TRS 859 (1995), FAS 35 (1996)
45 (1995)	Veterinary drug residues in food	TRS 864 (1996), FAS 36 (1996), FNP 41/8 (1996)
46 (1996)	Food additives and contaminants	TRS 868 (1997), FAS 37 (1997)
47 (1996)	Veterinary drug residues in food	TRS 876 (1998), FAS 38 (1998), FNP 41/9 (1997)
48 (1997)	Veterinary drug residues in food	TRS 879 (1998), FAS 39 (1997), FNP 41/10 (1998)
49 (1997)	Food additives and contaminants	TRS 901 (1999), FAS 40 (1998)

JECFA meeting no. (year)	Topic	Publications resulting from meeting (year of publication)
50 (1998)	Veterinary drug residues in food	TRS 888 (1999), FAS 41 (1998), FNP 41/11 (1999)
51 (1998)	Food additives	TRS 891 (2000), FAS 42 (1999)
52 (1999)	Veterinary drug residues in food	TRS 893 (2000), FAS 43 (2000), FNP 41/12 (2000)
53 (1999)	Food additives and contaminants	TRS 896 (2000), FAS 44 (2000)
54 (2000)	Veterinary drug residues in food	TRS 900 (2001), FAS 45 (2000), FNP 41/13 (2000)
55 (2000)	Food additives and contaminants	TRS 901 (2001), FAS 46 (2001)
56 (2001)	Mycotoxins in food	TRS 906 (2002), FAS 47 (2001), FNP 74 (2001)
57 (2001)	Food additives and contaminants	TRS 909 (2002), FAS 48 (2002)
58 (2002)	Veterinary drug residues in food	TRS 911 (2002), FAS 49 (2002), FNP 41/14 (2002)
59 (2002)	Food additives	TRS 913 (2002), FAS 50 (2003)
60 (2002)	Veterinary drug residues in food	TRS 918 (2003), FAS 51 (2003), FNP 41/15 (2003)
61 (2003)	Food additives and contaminants	TRS 922 (2004), FAS 52 (2004)
62 (2004)	Veterinary drug residues in food	TRS 925 (2004), FAS 53 (2005), FNP 41/16 (2004)
63 (2004)	Food additives	TRS 928 (2005), FAS 54 (2005)
64 (2005)	Food additives	TRS 930 (2005), FAS 55 (2006), FNP 82 (2006)
65 (2006)	Food additives	TRS 934 (2006), FAS 56 (2006), Mono 1(1–4) (2006)
66 (2006)	Veterinary drug residues in food	TRS 939 (2006), FAS 57 (2006), Mono 2 (2006)
67 (2007)	Food additives and contaminants	TRS 940 (2007), FAS 58 (2007), Mono 3 (2007)
68 (2007)	Food additives and contaminants	TRS 947 (2008), FAS 59 (2008), Mono 4 (2007)
69 (2008)	Food additives	TRS 952 (2009), FAS 60 (2009), Mono 5 (2009)
70 (2008)	Veterinary drug residues in food	TRS 954 (2009), FAS 61 (2009), Mono 6 (2009)
71 (2009)	Food additives	TRS 956 (2010), FAS 62 (2010), Mono 7 (2009)
72 (2010)	Contaminants in food	TRS 959 (2011), FAS 63 (2011), Mono 8 (2011), Mono 9 (2010)
73 (2010)	Food additives and contaminants	TRS 960 (2011), FAS 64 (2011), Mono 10 (2010)
74 (2011)	Veterinary drug residues in food	TRS 966 (2012), FAS 65 (2011), Mono 11 (2011)
75 (2011)	Veterinary drug residues in food	TRS 969 (2012), FAS 66 (2012), Mono 12 (2012)
76 (2012)	Food additives	TRS 974 (2012), FAS 67 (2012), Mono 13 (2012)
77 (2013)	Food additives and contaminants	TRS 983 (2014), FAS 68 (2013), Mono 14 (2013)
78 (2013)	Veterinary drug residues in food	TRS 988 (2014), FAS 69 (2014), Mono 15 (2014)
79 (2014)	Food additives	TRS 990 (2015), FAS 70 (2015), Mono 16 (2014)

JECFA meeting no. (year)	Topic	Publications resulting from meeting (year of publication)
80 (2015)	Food additives and contaminants	TRS 995 (2016), FAS 71 (2015), FAS 71/1 (2016), Mono 17 (2015)
81 (2015)	Veterinary drug residues in food	TRS 997 (2016), FAS 72 (2016), Mono 18 (2016)
82 (2016)	Food additives	TRS 1000 (2016), FAS 73 (2017), Mono 19 (2016)
83 (2016)	Contaminants in food	TRS 1002 (2017), FAS 74 (2018)
84 (2017)	Food additives	TRS 1007 (2017), FAS 75 (2019), Mono 20 (2017)
85 (2017)	Veterinary drug residues in food	TRS 1008 (2018), FAS 76 (2019), Mono 21 (2018)
86 (2018)	Food additives	TRS 1014 (2019), FAS 77 (2019), Mono 22 (2018)
87 (2019)	Food additives	TRS 1020 (2019), FAS 78 (2020), Mono 23 (2019)
88 (2019)	Veterinary drug residues in food	TRS 1023 (2020), FAS 79 (2020), Mono 24 (2020)
89 (2020)	Food additives	TRS 1027 (2021), FAS 80 (2021), Mono 25 (2021)
90 (2020)	Contaminants in food	TRS 1032 (2022), FAS 81 (2022)
91 (2021)	Food additives and contaminants	TRS 1036 (2022), FAS 82 (2023), Mono 26 (2021)
92 (2021)	Food additives	TRS 1037 (2022), FAS 83 (2023), Mono 27 (2022)
93 (2022)	Contaminants in food	TRS 1040 (2023), FAS 84, Mono 28 (2023)
94 (2022)	Veterinary drug residues in food	TRS 1041 (2022), FAS 85 (2023), Mono 29 (2023)
95 (2022)	Food additives	TRS 1042 (2023), FAS 86 (2023), Mono 30 (2023)
96 (2023)	Food additives	TRS 1050 (2023), FAS 87 (2024), Mono 31 (2023)
97 (2023)	Food additives	TRS 1051 (2024), FAS 88, Mono 32 (2024)
98 (2024)	Veterinary drug residues in food	TRS 1055 (2024), FAS 89, Mono 33

FAO: Food and Agriculture Organization of the United Nations; FAS: WHO Food Additives Series; FNP: FAO Food and Nutrition Paper; JECFA: Joint FAO/WHO Executive Committee on Food Additives; Mono: FAO JECFA Monograph; NMRS: FAO Nutrition Meetings Report Series; TRS: WHO Technical Report Series; WHO: World Health Organization.

Notes: (i) The JECFA report that is accepted on the last day of the meeting is published as part of the WHO Technical Report Series. (ii) The JECFA monograph that includes the background information on which the report is based is published as part of the WHO Food Additives Series. (iii) Revised and new specifications are published as part of the FAO Food and Nutrition Papers and FAO JECFA Monographs.

SELECTED WHO PUBLICATIONS OF RELATED INTEREST

Evaluation of certain veterinary drug residues in food
Ninety-eighth report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1055, 2024 (114 pages)

Evaluation of certain food additives
Ninety-seventh report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1051, 2024 (88 pages)

Evaluation of certain food additives
Ninety-sixth report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1050, 2024 (88 pages)

Safety evaluation of certain food additives
Ninety-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives
WHO Food Additives Series, No. 86, 2023 (192 pages)

Safety evaluation of certain contaminants in food
Ninetieth meeting of the Joint FAO/WHO Expert Committee on Food Additives
WHO Food Additives Series, No. 81, 2023 (404 pages)

Evaluation of certain food additives
Ninety-fifth report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1042, 2023 (98 pages)

Toxicological evaluation of certain veterinary drug residues in food
Ninety-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives
WHO Food Additives Series, No. 85, 2023 (134 pages)

Safety evaluation of certain contaminants in food
Ninety-first meeting of the Joint FAO/WHO Expert Committee on Food Additives
WHO Food Additives Series, No. 82, 2023 (362 pages)

Evaluation of certain contaminants in food
Ninety-third report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1040, 2023 (68 pages)

Evaluation of certain food additives
Ninety-second report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 1037, 2022 (76 pages)

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Evaluation of certain food additives

This report represents the conclusions of a Joint FAO/WHO Expert Committee convened to evaluate the safety of certain food additives and enzymes, to review and prepare specifications for the identity and purity of these food additives and enzymes, and to review specifications for the identity and purity of certain flavouring agents.

The report provides a summary of the Committee's evaluations of technical, toxicological and dietary exposure data for eight specific food additives: adenosine-5′-monophosphate deaminase (JECFA99-1) from *Aspergillus sp.*, butterfly pea flower extract, endo-1,4-β-xylanase (JECFA99-2) from *Bacillus subtilis* expressed in *Bacillus subtilis*, endo-1,4-β-xylanase (JECFA99-3) from *Rasamsonia emersonii* expressed in *Aspergillus niger*, glucosidase from *Aspergillus niger* expressed in *Trichoderma reesei* exhibiting α-glucosidase (JECFA99-4a) and transglucosidase (JECFA99-4b) activity, natamycin, nisin A and Polyglycerol esters of fatty acids. The Committee was unable to complete the safety evaluations of adenosine-5′-monophosphate deaminase (JECFA99-1) from *Aspergillus sp.* and of butterfly pea flower extract because of a lack of critical information.

The specifications for natamycin, nisin A and Polyglycerol esters of fatty acids were revised. New specifications were prepared for endo-1,4-β-xylanase (JECFA99-2) from *Bacillus subtilis* expressed in *Bacillus subtilis*, endo-1,4-β-xylanase (JECFA99-3) from *Rasamsonia emersonii* expressed in *Aspergillus niger*, and glucosidase from *Aspergillus niger* expressed in *Trichoderma reesei* exhibiting α-glucosidase (JECFA99-4a) and transglucosidase (JECFA99-4b) activity. The specifications for 10 flavouring agents (*S*-methyl thioacetate, *S*-methyl 3-methylbutanethioate, 4,5-dihydro-3(2*H*) thiophenone, 2-methyltetrahydrothiophen-3-one, 1-butanethiol, *o*-toluenethiol, bis(methylthio)methane, 3-mercaptohexyl acetate, 3-mercaptohexyl butyrate and 3-mercapto-2-pentanone) were revised.

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