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COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives

Ninety-ninth Meeting
Geneva, 11–20 June 2024

Food and Agriculture Organization of the United Nations

World Health Organization

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
WORLD HEALTH ORGANIZATION
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Joint FAO/WHO Expert Committee on Food Additives
Geneva, 11–20 June 2024

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Introduction

This volume of FAO JECFA Monographs contains specifications prepared at the Ninety-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), held in Geneva, from 11 to 20 June 2024. The specifications monographs are one of the outputs of JECFA's risk assessment of food additives, and should be read in conjunction with the safety evaluation, reference to which is made in the section at the head of each specifications monograph. Further information on the meeting discussions can be found in the summary report of the meeting (see Annex 1), and in the full report which will be published in the WHO Technical Report series. Toxicological monographs of the substances considered at the meeting will be published in the WHO Food Additive Series.

Specifications monographs prepared by JECFA up to the 65th meeting, other than specifications for flavouring agents, have been published in consolidated form in the Combined Compendium of Food Additive Specifications which is the first publication in the series FAO JECFA Monographs. This publication consists of four volumes, the first three of which contain the specifications monographs on the identity and purity of the food additives and the fourth volume contains the analytical methods, test procedures and laboratory solutions required and referenced in the specifications monographs. FAO maintains an online searchable database of all JECFA specifications monographs from the FAO JECFA Monographs, which is available at: https://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/. The specifications for flavourings evaluated by JECFA, and previously published in FAO Food and Nutrition Paper 52 and subsequent Addenda, are included in a database for flavourings specifications. All specifications for flavourings that have been evaluated by JECFA since its 44th meeting, including the 79th meeting, are available in the online searchable database at the JECFA website at FAO: https://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-flav/en/. The databases have query pages and background information in Arabic, Chinese, English, French, and Spanish. Information about analytical methods referred to in the specifications is available in the Combined Compendium of Food Additive Specifications (Volume 4), which can be accessed from the query pages.

An account of the purpose and function of specifications of identity and purity, the role of JECFA specifications in the Codex system, the link between specifications and methods of analysis, and the format of specifications, are set out in the Introduction to the Combined Compendium, which is available in shortened format online on the query page, which could be consulted for further information on the role of specifications in the risk assessment of additives.

Chemical and Technical Assessments (CTAs) for some of the food additives have been prepared as background documentation for the meeting. These documents are available online at: https://www.fao.org/food-safety/resources/publications/en/.

Contact and feedback

More information on the work of the Committee is available from the FAO homepage of JECFA at: https://www.fao.org/food-safety/scientific-advice/en/. Readers are invited to address comments and questions on this publication and other topics related to the work of JECFA to jecfa@fao.org

Specifications for certain food additives

New and revised specifications

At its ninety-ninth meeting, the Committee evaluated the safety of four (4) food additives, four (4) processing aids and revised the specifications for ten (10) flavouring agents.

The Committee drafted specifications but could not finalize them for publication because of lack of critical information for:

- Adenosine-5c-monophosphate deaminase from *Aspergillus* sp. (JECFA99-1) (No[a])
- Butterfly pea flower extract (No[a])

The Committee prepared new specifications for:

- Endo-1,4-β-xylanase from *Bacillus subtilis* expressed in *Bacillus subtilis* (JECFA99-2) (N)
- Endo-1,4-β-xylanase from *Rasamsonia emersonii* expressed in *Aspergillus niger*
- (JECFA99-3) (N)
- Glucosidase from *Aspergillus niger* expressed in *Trichoderma reesei* exhibiting α-glucosidase and transglucosidase activity (JECFA99-4a, JECFA99-4b) (N)

The Committee revised the specifications for:

- Natamycin (R)
- Nisin A (R)
- Polyglycerol esters of fatty acids (R)

The Committee revised the specifications only for the following favouring agents:

- S-methyl thioacetate (482) (R)
- S-methyl 3-methylbutanethioate (487) (R)
- 4,5-dihydro-3(2H) 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)

N: New specifications
R: Revised specifications
No[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.

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

New specifications prepared at the 99th JECFA (2024), published in FAO JECFA Monographs 34 (2025). An ADI "not specified" was established at the 99th JECFA (2024)

SYNONYMS	Endo-(1→4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-1,4-xylanase; endo β 1,4 xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; endo 1,4-β-xylanase; β-D-xylanase
SOURCES	Produced by a genetically modified non-pathogenic and non toxigenic strain of *Bacillus subtilis* expressing the endo 1,4 β xylanase gene from *B. subtilis* under controlled submerged fed-batch pure culture fermentation. The endo 1,4 β xylanase is recovered from the fermentation broth by the separation of cellular biomass, concentration by ultrafiltration, purification by ion exchange chromatography and microfiltration. The final liquid product is formulated and standardized to the desired activity.
Active principles	Endo-1,4-β-xylanase
Molecular weight	With an apparent molecular weight of 20 kDa equivalent to the calculated molecular weight of the enzyme
Systematic names and numbers	4-β-D-xylan xylanohydrolase; IUBMB number: 3.2.1.8; CAS number: 9025-57-4.
Reaction catalysed	Endohydrolysis of 1,4-b-D-xylosidic linkages in xylans (including arabinoxylans) resulting in the generation of (1→4)-b-D-xylan oligosaccharides of different lengths.
Secondary enzyme activities	No significant secondary activities
DESCRIPTION	Brown liquid
FUNCTIONAL USES	Enzyme preparation Used as a processing aid in baking applications
GENERAL SPECIFICATIONS	Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Endo-1,4-β-xylanase activity	The sample shows endo-1,4-β-xylanase activity See description under TESTS

TESTS

METHOD OF ASSAY

Endo-1,4-β-xylanase activity

PRINCIPLE

The activity of endo-1,4-β-xylanase is determined using birchwood xylan as substrate. The analytical principle is based on hydrolysis of xylan to reducing sugars (xylose equivalents) at pH 6.0, 50 °C and 15 min. The released reducing sugars is measured spectrophotometrically at 570 nm. The endo 1,4 xylanase activity is quantified relative to a reference enzyme standard and expressed in Birchwood D(+) Xylanase Units/ml (BDXU/ml).

One BDXU is defined as the amount of enzyme that liberates 1 µmol of reducing sugars (xylose equivalents) from birchwood xylan per minute per ml at pH 6.0 and 50 °C.

MATERIALS AND EQUIPMENT

- Analytical balance (precision: 0.001 g)
- 96-well Microplate
- Spectrophotometer
- pH Meter
- Vortex mixer
- Thermostated water bath
- Positive displacement pipets with tips

REAGENTS AND SOLUTIONS

- Deionized water
- Citric acid monohydrated ($C_6H_{10}O_8$)
  Di-sodium hydrogen phosphate dehydrated ($Na_2HPO_4 \cdot 2H_2O$)
- Birchwood xylan
- Sodium hydroxide (NaOH)
- 37% Hydrochloric acid (HCl)
- D(+) xylose
- 2-hydroxy-3,5-dinitrobenzoic acid (DNS, $C_7H_4N_2O_7$)
- 29% Sodium hydroxide (NaOH)
  Potassium sodium tartrate tetrahydrate ($NaKC_4H_4O6 \cdot 4H_2O$)
- Reference endo-1,4-β-xylanase of known activity in BDXU/g

SOLUTIONS

50 mM Citrate Phosphate Buffer (pH 6.0)

- Weigh 10.5 g citric acid monohydrate and 8.9 g $Na_2HPO_4 \cdot 2H_2O$, respectively and dissolve in 600 ml deionized water in a 1 000-ml volumetric flask by mixing on shaker at 500 rpm.
- Adjust pH to 6.0 with 29% NaOH
- Make up the volume to 1 000 ml with deionized water.
- Store at 4 °C in a closed container. Stable for 1 week.

5% NaOH Solution (w/v)

- Weigh 3.0 g NaOH and dissolve in 60 ml deionized water in a beaker.
- Mix on shaker at 500 rpm to dissolution.

3% Birchwood Xylan Solution

- Weigh 6.0 g birchwood xylan and dissolve in 60 ml of 5% NaOH solution in a 250-ml beaker by mixing on shaker 300 rpm for 20 minutes.
- Add 100 mL of 50 mM citrate phosphate buffer and continue to mix.
- Adjust pH to 6.0 with 37% HCI.
- Quantitatively transfer to a 200-ml volumetric flask and make up to volume with deionized water.
- Prepare the solution on the day of assay.

40 mM Xylose

- Weigh 0.300 g D(+) xylose in a 50-ml volumetric flask and dissolve in a small amount of deionized water on shaker at 500 rpm.
- Make up the volume to 50 ml with deionized water and mix.
- Aliquot the solution in 1-ml tubes and freeze at -20 °C.

1 M NaOH Solution

Weigh 16.0 g NaOH and dissolve in 400 ml deionized water in a 600-ml beaker by mixing on shaker at 500 rpm to dissolution.

DNS Solution

Weigh 10.0 g DNS in a 1 000-ml beaker and add 300 ml deionized water.

Mix on shaker at 500 rpm and heat to 100 °C to dissolution.

Add 400 ml of 1M NaOH and continue to mix on shaker at 500 rpm.

Add 300 g potassium sodium tartrate tetrahydrate and continue to mix on shaker at 500 rpm to dissolution.

Transfer the solution to a 1 000-ml volumetric flask and make up the volume to 1 000 ml with deionized water.

Store in a glass bottle and cap. This solution can be stored in the dark at room temperature for up to one week.

Reference Endo-1,4-β-xylanase Solution

Weigh 1.0 g of the reference endo-1,4-β-xylanase standard in a 100-ml volumetric flask and let it dissolve completely in 50 mM citrate phosphate buffer by placing it at 4 °C overnight or mixing at room temperature for approximately 30 min.

Filter the solution with filter paper on ice.

Make a dilution with 50 mM citrate phosphate buffer to approximately 1.7 BDXU/ml so that its net absorbance falls within the linear range of the assay after the subtraction of the blank.

Place the standard solution on ice (stable for 8 hours).

Prepare on the day of assay.

Sample

Dilute each sample with 50 mM citrate phosphate buffer to obtain a final net absorbance between 0.45 and 0.55.

Place the diluted samples on ice; samples are stable for 8 hours.

Assay Procedure

Prepare the following dilutions of xylose standard according to the table below with 40 mM xylose standard solution:

Dilutions of xylose standard with 40 mM xylose standard solution

TUBE #	1 (BLANK)	2	3	4	5	6
Deionized water (µl)	100	80	60	40	20	0
40 mM xylose standard solution (µl)	0	20	40	60	80	100
BDXU/ml equivalent	0	8	12	24	32	40

Arrange test tubes in triplicate per sample. Add 100 µl of sample to each test tube. A maximum of 10 samples can be determined simultaneously per assay.

Set up duplicate blanks per sample.

Set up two reference endo-1,4-β-xylanase samples, in triplicate.

Add 700 µl of 3% birchwood xylan substrate solution to each test tube of xylose standards, reference endo 1,4 β-xylanase standards, samples and blanks.

Mix well for 5 seconds and incubate at 50 °C for 15 minutes.

Add 1 ml DNS solution to each tube to stop the reaction.

Add 100 µll of sample solution to each test tube of the blank.

Place all the tubes in a water bath at 95 °C for 15 minutes. Cool all the tubes for 5 minutes in an ice bath.

Transfer 300 µl each of xylose standard, samples, reference endo-1,4-β-xylanase standards and blanks, respectively into a 96-well microplate and measure the absorbance at 570 nm.

CALCULATION

To determine the net ΔAbsorbance, subtract the average blank from the absorbance reading of all standards and samples.

Prepare the standard curve using linear regression where net absorbance is on the y-axis and concentration of xylose (BDXU/ml) on the x-axis.

The net absorbance should be in the range between 0.45 and 0.55

The correlation coefficient must be ³ 0.99.

Determine the concentration of each sample from linear regression using the following equation:

$$\frac{\text{BDXU}}{\text{ml}} = \frac{(\Delta\text{Abs} - \text{b})}{\text{a}} \times \frac{\text{Dilution}}{15} \times \frac{A_{ref1}}{A_{ref2}}$$

Where:

ΔAbs: average absorbance of sample
(or reference endo 1,4 β xylanase standard) – average absorbance of blank

b: Y intercept of the calibration curve

a: Slope of the calibration curve

15: Time in minutes

Aref1: Known endo-1,4-β-xylanase activity of the reference standard

Aref2: Measured Endo-1,4-β-xylanase activity of the reference standard

Note: The ratio of Aref1/Aref2 must be between 0.9 and 1.1

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

New specifications prepared at the 99th JECFA (2024), published in FAO JECFA Monographs 34 (2025). An ADI "not specified" was established at the 99th JECFA (2024).

SYNONYMS	Endo-(1→4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-1,4-xylanase; endo- β-1,4 xylanase; endo-1,4- β- D- xylanase; 1, 4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylano-hydrolase; endo-1,4-β-xylanase; β-D-xylanase
SOURCES	Produced by a genetically modified non-pathogenic and non-toxigenic strain of Aspergillus niger expressing the endo-1,4-β-xylanase gene from Rasamsonia emersonii under controlled, submerged, fed-batch pure culture fermentation. The secreted endo-1,4-βxylanase is recovered from the fermentation broth by the separation of the cellular biomass, concentrated by ultrafiltration, and purified by a series of filtration steps. The final liquid or microgranulated products are formulated and standardized to the desired activity.
Active principles	Endo-1,4-β-xylanase
Systematic names and numbers	4- β-D-xylan xylanohydrolase; IUBMB number: 3.2.1.8, CAS number: 9025-57-4.
Molecular weight	52 kDa
Reaction catalysed	Endohydrolysis of 1,4-β-D-xylosidic linkages in xylans (including arabinoxylans) resulting in the generation of (1→4)-β-D-xylan oligosaccharides of different lengths.
Secondary enzyme activities	No significant secondary enzyme activities.
DESCRIPTION	Off-white microgranulate or light brown to brown liquid.
FUNCTIONAL USES	Enzyme preparation Used in brewing and baking applications, fruit and vegetable processing, and in the production of plant-based analogues of milk and milk products.
GENERAL SPECIFICATIONS	Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Endo-1,4-β-xylanase activity	The sample shows endo-1,4-β-xylanase activity See description under TESTS

TESTS

METHOD OF ASSAY

Endo-1,4-β-xylanase activity

PRINCIPLE

Endo-1,4-β-xylanase activity is defined as the amount of free *p*-nitrophenol produced from the hydrolysis of the *p*-nitrophenyl- β-D-xylopyranoside substrate.

One unit of endo-1,4-β-xylanase activity is the amount of endo-1,4-β-xylanase required to liberate 0.06 µmole of *p*-nitrophenol per minute under the conditions of the assay (New Thermostable Xylanase Unit (NTXU)).

APPARATUS

- Spectrophotometer
- Thermostated water bath at 37 °C
- 10-ml test tubes
- Stopwatch

REAGENTS AND SOLUTIONS

- Deionized water
- Sodium acetate trihydrate (Merck 106267 or equivalent)
- Glacial acetic acid (Merck 100063 or equivalent)
- Triton X-100 (Merck 112298 or equivalent)
- *p*-nitrophenyl-beta-D-xylopyranoside substrate (Sigma, N2132)
- Endo-1,4-β-xylanase reference standard (Neogen® Megazyme E-XYAN4)
- *p*-nitrophenol standard solution, approx. 10 mmol/L (Sigma, N7660)
- Sodium carbonate, anhydrous (Merck, 106392 or equivalent)

SOLUTIONS

Acetate buffer, 30mM, pH 4.5

Weigh 4.4 g sodium acetate trihydrate in a 1 000-ml volumetric flask and dissolve in approximately 800 ml deionized water.

Adjust the pH to 4.5 with glacial acetic acid. Make up to volume with deionized water and mix.

May be stored in the refrigerator for up to one month.

Triton/acetate buffer, 30mM, pH 4.5

Dissolve 2.0 g Triton X-100 in 800 ml acetate buffer pH 4.5 in a

1 000-ml volumetric flask. Make up to volume and mix.

May be stored in the refrigerator for up to one month.

Sodium carbonate solution, 300 mM

Dissolve 1.59 g anhydrous sodium carbonate in 40 mL deionized water in a 50-mL volumetric flask. Make up to volume and mix.

May be stored in the refrigerator for 1 month.

***p*-nitrophenyl-β-D-xylopyranoside substrate solution, 7 mM**

Dissolve 0.050 ± 0.001 g *p*-nitrophenyl-beta-D-xylopyranoside in 20 ml acetate buffer in a 25-ml volumetric flask. Make up to volume and mix.

Always use a freshly prepared solution. Immediately before use, check that the pH is 4.5.

Note: At this substrate concentration, the maximum rate at which the enzyme can catalyse the reaction is not reached. The substrate concentration will affect enzyme activity; therefore, it is crucial to weigh the substrate within the range specified.

***p*-nitrophenol standards**

Prepare the following dilutions according to the table below with Triton/ acetate buffer:

TUBE	*P*-NITROPHENOL STANDARD SOLUTION [ml]	BUFFER TO BE ADDED [ml]	TOTAL DILUTION FACTOR	*P*-NP CONC [µM]
S1 (Blank)	0	2.000	-	-
S2	0.400	1.600	5	2 000
S3	0.300	1.700	6.67	1 500
S4	0.200	1.800	10	1 000
S5	0.100	1.900	20	500
S6	0.100	4.900	50	200

The actual concentration depends on the concentration of the p-nitrophenol batch used. This may differ slightly from 10 mM. This should be incorporated in the calculations.

Endo-1,4-β-xylanase reference standard

- Use an endo-1,4-β-xylanase reference standard of known activity. Thaw at room temperature.
- Accurately weigh (within 1 mg) in duplicate an amount corresponding to approximately 6 500 NTXU in a 50-ml volumetric flask.
- Dissolve each endo-1,4-β-xylanase reference standard in 40 ml acetate buffer, make up to volume and mix. This will be the endo-1,4-β-xylanase reference standard stock solution.
- Prepare a 15-fold dilution (concentrated standard) by pipetting 200 µl of endo-1,4-β-xylanase reference standard stock solution into 2.800 ml Triton/Acetate buffer.
- Prepare a 10-fold working dilution (diluted enzyme standard) by

pipetting 500 µl of endo-1,4-β-xylanase concentrated standard solution into 4.500 ml Triton/Acetate buffer.

Endo-1,4-β-xylanase sample preparation

- Working in duplicate, dilute the endo-1,4-β-xylanase sample to an activity of approximately 0.9 NTXU/ml using Triton/acetate buffer (based on its anticipated activity, make a series of dilutions as necessary, so that the activity of the final dilution is between 0.35 - 1.5 NTXU/ml).
- Store each diluted sample on ice.

Procedure

- All assay steps should be conducted in duplicate at temperature 37 °C.
- Add 800 µl sodium carbonate stop solution only to the tubes for the *p*-nitrophenol standards (S1-S6).
- Place all tubes for the *p*-nitrophenol blanks and standards (S1-S6), endo-1,4-β-xylanase reference standard, and endo-1,4-β-xylanase samples into the water bath and equilibrate for 5 min.
- Pipette 1 560 µl *p*-nitrophenyl-beta-D-xylopyranoside substrate solution into the blanks and standards (S1-S6) endo 1,4-β-xylanase reference standard and the endo 1,4 β xylanase sample, mix well, and place back in the water bath to equilibrate for 5 min.
- At t = 0, in order and with a fixed timing, pipette 130 µl of the diluted endo-1,4-β-xylanase reference standard solution into the tube prepared for the reference standard, pipette 130 µl of the endo-1,4-β-xylanase samples into the tubes prepared for samples, and pipette 130 µl of acetate buffer into the tubes prepared for enzyme blanks and standards (S1-S6).
- Mix well after addition and place back in the water bath.
- Starting at t = 1 000 s (16.7 min) and with the same time intervals as before, add 800 µl sodium carbonate stop solution to the tubes for the endo-1,4-β-xylanase reference standard and the endo-1,4-β-xylanase samples and mix immediately.
- Remove all tubes from the water bath and transfer an appropriate volume to 1 cm path length cuvettes.
- Measure the absorbance of all solutions at 405 nm. Zero the spectrophotometer with water.

Standard curve and calculations

One NTXU is defined as the amount of endo-1,4-β-xylanase required to liberate 0.06 µmole of *p*-nitrophenol per minute under the conditions of the assay

Standard Curve:

Plot the next absorbance as y-values against the concentration of the *p*-nitrophenol working solutions in µmol/ml as the x-values and prepare a standard curve using linear regression.

Calculations:

Calculate the slope of the calibration line of absorption at 405 nm as a function of the concentration of *p*-nitrophenol.

NTXU activity is calculated using the following equation:

$$\frac{\text{NTXU}}{\text{g}} = \frac{\Delta A \times D_f \times V}{\text{Slope} \times W \times 1\,000}$$

Or:

$$\frac{\text{NTXU}}{\text{ml}} = \frac{\Delta A \times D_f \times V}{\text{Slope} \times W \times 1\,000}$$

Where:

$$\Delta A = A_{sample} - A_{blank} \text{ [at 405 nm]}$$

D_f = dilution factor of sample

Slope = Slope of *p*-nitro-phenol calibration curve [l/µmol]

V = volume of volumetric flask used for initial sample dilution [ml]

W = initial weight of sample [g]

1 000 = conversion factor from l to ml

Report the average of the duplicate measurements.

Quality control check:

- Determine the activity of the endo-1,4-β-xylanase reference standard as the average of the duplicate measurements. A 5% deviation from the reference value is acceptable.
- Determine the molar extinction coefficient of the *p*-nitrophenol standards:

The molar extinction coefficient ε is calculated using the following equation:

$$\varepsilon = \text{Slope} \times \frac{V_{tot}}{V_s \times d}$$

Where:

Slope = Slope of *p*-nitro-phenol calibration curve [l/µmol]

V_{tot} = Total volume in culture tube after quenching [2490 µl]

V_s = Volume of sample [130 µl]

d = Length of path of light in the cuvette [1 cm]

The value of the molar extinction coefficient should be in the range of 0.0175 – 0.0195 $\mu M^{-1}cm^{-1}$

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

New specifications prepared at the 99th JECFA (2024), published in FAO JECFA Monographs 34 (2025). An ADI "not specified" was established at the 99th JECFA (2024).

SOURCES

Produced by a submerged fed-batch pure culture fermentation of a non-pathogenic and non-toxigenic strain of *Trichoderma reesei* for the expression of an enzyme-encoding gene from *Aspergillus niger*. The enzyme that is produced and secreted into the fermentation broth exhibits α-glucosidase or transglucosidase activity. It is separated from the biomass using multiple filtration steps to obtain the enzyme concentrate and is formulated and standardized as a liquid enzyme preparation.

The enzyme preparation is used for its α-glucosidase (JECFA99-4a) or transglucosidase (JECFA99-4b) activity. Transglucosidase activity is assayed and expressed in Transglucosidase Units (TGU). α-Glucosidase activity in the article of commerce is standardized based on the transglucosidase activity units (TGU).

The respective specifications are presented below in Annex 1 (JECFA99-4a) and Annex 2 (JECFA99-4b).

Annex 1: α-glucosidase (JECFA99-4a) from *Aspergillus niger* expressed in *Trichoderma reesei*

SYNONYMS	α-Glucosidase; Maltase; Glucoinvertase; Glucosidosucrase; Maltase-Glucoamylase; α-Glucopyranosidase;
Active principles	α-Glucosidase
Systematic names and numbers	α-D-Glucoside glucohydrolase; EC 3.2.1.20
Reaction catalysed	Hydrolysis of terminal, non-reducing (1→4)-linked α-D-glucose residues with release of α-D-glucose.
Secondary enzyme activities	Transglucosidase
DESCRIPTION	Brown liquid.
FUNCTIONAL USES	Enzyme preparation Used as a processing aid in the manufacture of potable alcohol, organic acids, and monosodium glutamate.
GENERAL SPECIFICATIONS	Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

α-Glucosidase activity	The sample shows α-glucosidase activity. See description under TESTS.
Transglucosidase activity	The sample shows transglucosidase activity. See description under TESTS in Annex 2.

TESTS

IDENTIFICATION TEST

α-Glucosidase activity

PRINCIPLE

The activity of α-glucosidase is determined spectrophotometrically by measuring the formation of yellow colour of *p*-nitrophenol released by the hydrolysis of *p*-nitrophenyl α-D-glucopyranoside (PNPG) substrate at 420 nm, at pH 10.0. This activity, under this assay condition, is expressed in α-Glucosidase Units (AGDU)/g or AGDU/ml.

One AGDU is defined as the amount of enzyme required to generate 1.0 µmole of *p*-nitrophenol per minute at pH 4.8 and 30 °C.

MATERIALS AND EQUIPMENT

- UV-Vis Spectrophotometer
- Magnetic stirrer
- Thermometer
- pH Meter
- Cuvettes, 10 mm path length

REAGENTS AND SOLUTIONS

Deionized water

Sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$)

Glacial acetic acid (CH_3COOH)

Sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$)

Sodium hydroxide (NaOH)

Substrate: *p*-nitrophenyl α-D-glucopyranoside (PNPG: $C_{12}H_{15}NO_8$; CAS No. 3767-28-0; N-4351 or N-4350 from Biosynth, or equivalent)

Standard: *p*-nitrophenol ($C_6H_5NO_3$; CAS No. 100-02-7; ≥ 99.0 %)

Assay buffer (Sodium acetate buffer (pH 4.8)): Weigh and dissolve 2.72 g of sodium acetate trihydrate in approximately 800 ml of deionized water with stirring. Adjust the pH to 4.8 with glacial acetic acid at room temperature with stirring. Make up to volume with deionized water in a 1 000-ml volumetric flask and mix well. The solution can be stored in a refrigerator in a closed container.

Stop Solution (0.2 M Borax solution): Weigh and dissolve 76.20 g of sodium borate decahydrate in approximately 800 ml of deionized water with stirring for about 30 minutes. Adjust the pH to 10.0 at room temperature with sodium hydroxide, then make up to volume with deionized water in a 1 000–ml volumetric flask. The solution can be stored at room temperature in a closed container.

Standard dilution buffer: Mix 500 ml of Assay buffer and 250 ml of Stop solution.

SUBSTRATE SOLUTION

Weigh 50.0 mg of PNPG in a 50-ml amber volumetric flask. Add approximately 40 ml of the Assay buffer to the flask and mix on a magnetic stirrer for 20-30 minutes until completely dissolved (Periodic heating in a warm water bath can also aid the dissolution). Make up to volume with the Assay buffer. The solution can be stored for up to 2 weeks in refrigerator.

STANDARD SOLUTIONS

- Standard stock solution (0.001 M *p*-nitrophenol solution): Dissolve 139.11 mg of *p*-nitrophenol previously dried (60 °C, maximum 4 h) in water in a 1-L volumetric flask and dilute to volume with water.
- Standard working solutions (0.005, 0.02 and 0.05 mM *p*-nitrophenol solutions):
  - Standard 0.005 mM: Add 1 ml of the Standard stock solution in a 200-ml volumetric flask, and dilute to volume with Standard dilution buffer
  - Standard 0.02 mM: Add 2 ml of the Standard stock solution in a 100-ml volumetric flask, and dilute to volume with Standard dilution buffer
  - Standard 0.05 mM: Add 5 ml of the Standard stock solution in a 100-ml volumetric flask and dilute to volume with Standard dilution buffer

SAMPLE SOLUTIONS

Work in duplicate. Accurately weigh a sample and dissolve with the Assay buffer in a volumetric flask to obtain a final net absorbance within the range of the Standard solutions prepared for the calibration curve (approximately 0.04 AGDU/ml). The sample solutions are typically stable for 6 hours at room temperature, but storage on ice is recommended.

ASSAY TEST SOLUTIONS AND ASSAY BLANK

Prepare all Assay test solutions, and the Assay blank in duplicate.

- Pipette 500 µl of each Sample solution, or Assay buffer (as Assay blank) to the corresponding tube.
- Pre-heat the tubes in a 30 °C water bath for approximately 5 minutes and allow to equilibrate.
- Add 500 µl of Substrate solution to each tube at timed intervals, vortex, and replace the tube in the water bath.
- After exactly 5 minutes from the addition of Substrate solution, add 500 µl of Stop solution to each tube and mix well by vortex to terminate the reaction.

Measurements

Measure the absorbance of each of the Standard working solutions, Assay test solutions and Assay blanks at 420 nm using deionized water to zero the spectrophotometer in a 10-mm cell.

STANDARD CURVE AND CALCULATION

One AGDU is defined as the amount of α-glucosidase that will liberate 1.0 µmole of *p*-nitrophenol per minute from the Substrate solution under the conditions of the assay.

Standard Curve:

Plot the net absorbance as y-values against the concentration of the three Standard working solutions in µmole/ml as the x values and prepare a standard curve using linear regression. The correlation coefficient must be ≥0.999.

Calculation:

1. Calculate the net average absorbance of the Assay test solutions by subtracting the average absorbance of the two replicates of Assay blanks from the average absorbance of the two replicates of the Assay test solutions. Determine the average concentration of *p*-nitrophenol in the Assay test solutions in µmole of *p*-nitrophenol /ml using the standard curve.
2. Calculate the activity of the Sample solutions

$$\frac{\text{AGDU}}{\text{ml of sample solution}} = \frac{\text{M} \times 1.5}{0.5 \times 5}$$

Where:

M is the average concentration of *p*-nitrophenol in the Assay test solutions in µmole of *p*-nitrophenol/ml using Standard curve;

1.5 is the final volume of the test solutions, in ml;

5 is the reaction time, in min;

0.5 is the sample aliquot, in ml

Calculate the activity of the sample in AGDU per gram (g) using the following equation:

$$\frac{\text{AGDU}}{\text{g}} = \text{S} \times \frac{1}{\text{C}}$$

Where:

S = Activity of the Sample solution in AGDU/ml

C= Concentration of the Sample solution in g/ml

If needed, convert from AGDU/g to AGDU/ml based on the relative density of the sample.

An enzyme preparation with an activity of 2000 TGU/g (or TGU/ml) will have an activity corresponding to approximately 30 AGDU/g (or AGDU/ml).

Annex 2: Transglucosidase (JECFA99-4b) from *Aspergillus niger* expressed in *Trichoderma reesei*

SYNONYMS	Transglucosidase; 1,4-D-glucan 6-D-glucosyltransferase; oligoglucan-branching glycosyltransferase; D-glucosyltransferase
Active principles	Transglucosidase
Systematic names and numbers	1,4-D-glucan 6-D-glucosyltransferase; EC 2.4.1.24
Reaction catalysed	Transfers an α-D-glucosyl residue in a (1→4)- α-D-glucan to the primary hydroxy group of glucose, free or combined in a (1→4)- α-D-glucan.
Secondary enzyme activities	α-Glucosidase
DESCRIPTION	Brown liquid.
FUNCTIONAL USES	Enzyme preparation Used as a processing aid in the production of isomalto oligosaccharide syrups from a variety of sources.
GENERAL SPECIFICATIONS	Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Transglucosidase activity	The sample shows transglucosidase activity. See description under TESTS.

TESTS

IDENTIFICATION TEST

Transglucosidase activity

PRINCIPLE

The activity of transglucosidase is determined by incubating a diluted enzyme solution with a 10% (w/v) maltose solution in 0.02 M acetate buffer and measuring, using HPLC with RI detector, the amount of trisaccharide produced. This activity is expressed in Transglucosidase Units (TGU)/g or TGU/ml.

One TGU is defined as the amount of enzyme that produces one µmol trisaccharide per minute at pH 4.0 and 50 °C.

MATERIALS AND EQUIPMENT

- HPLC with RI detector
- Magnetic stirrer
- Thermometer
- pH Meter

REAGENTS AND SOLUTIONS

Acarbose ($C_{25}H_{43}NO_{18}$; CAS No. 56180-94-0;

BAY g 5421 from Bayer AG, or equivalent)

Concentrated sulfuric acid (≥ 96%)

Glacial acetic acid (CH_3COOH)

HPLC grade water

Sodium hydroxide (NaOH) AR or ACS Grade.

D-Glucose (anhydrous) ($C_6H_{12}O_6$; CAS No. 50-99-7) AR Grade

Substrate: D-Maltose monohydrate ($C12H22O11 \cdot H_2O$; CAS No. 6363-53-7) ≥ 99% (by HPLC) (Recommend purity test every six months)

D-Panose (anhydrous) (as qualitative standard) ($C_{18}H_{32}O_{16}$; CAS No. 33401-87-5)

Qualitative reference solution: Weigh 0.1 g of D-panose (anhydrous) in a 100-ml volumetric flask and dilute to volume with 0.01 N H_2SO_4.

2 N NaOH: Weigh 80.0 g NaOH and add it carefully with mixing to 500 ml of water in a 1-L beaker. Cool to room temperature, transfer to a 1-L volumetric flask and make up to volume with water.

Acetate buffer (0.02 M, pH 4.0): Add 2.30 ml glacial acetic acid to 1800 ml of water in a 2-L beaker. Adjust the pH to 4.00 ± 0.01 using 2 N NaOH, under stirring. Transfer to a 2 L volumetric flask and make up to volume with water. This solution can be stored in refrigerator.

Acarbose buffer: Weigh 258 mg of acarbose in a 100-ml volumetric flask. Make up to volume with Acetate buffer (0.02 M, pH 4.0) (Stock solution). This can be stored frozen in a suitable closed container. Pipette 10 ml of the Stock solution into a 1-L volumetric flask and make up to volume with Acetate buffer (0.02 M, pH 4.0) (Working solution). This solution can be stored in refrigerator.

0.01 N H_2SO_4 (for HPLC Mobile Phase): Weigh 2.05 g of conc. H_2SO_4, add it to 4.0 L of HPLC grade water and mix.

0.011 N H_2SO_4 (for sample diluent): Weigh 1.13 g of conc. H_2SO_4, add it to 2.0 L of HPLC grade water and mix.

SUBSTRATE SOLUTION (20 % (w/v) D-Maltose monohydrate in Acetate buffer)

Weigh 5.00 g of D-maltose monohydrate in a small beaker, add 20 ml of Acetate buffer (0.02 M, pH 4.0) and mix well. Quantitatively transfer to a 25-ml volumetric flask and make up to volume with acetate buffer (0.02 M, pH 4.0). Solution must be made fresh daily.

STANDARD SOLUTION (GLUCOSE SOLUTION (1 MG/ML))

Weigh 1.000 g of D-Glucose (anhydrous) in a 100 ml volumetric flask. Make up to volume with 0.01 N H_2SO_4. Pipette 10 ml of the solution in a 100-ml volumetric flask and make up to volume with 0.01 N H_2SO_4. This solution can be stored frozen in screw cap tubes.

SAMPLE SOLUTION

Accurately weigh at least 1.0 g (W1) of enzyme in a 100 ml volumetric flask (V1) and make up to volume with Acetate buffer (0.02 M, pH 4.0). Accurately dilute the solution 10 - 100 times with Acetate buffer (0.02 M, pH 4.0) to produce a dilution containing 0.2-1.0 TGU/ml (target 0.5 TGU/ml). (Vs/V2)

In the case that the product of the enzyme is considered insufficiently purified and may contain impurities such as glucoamylase, the following operations should be performed, instead of the above sample solution.

Weigh at least 1.0 g of enzyme in a 100-ml volumetric flask and make up to volume with Acarbose buffer working solution. Accurately dilute the solution 10-100 times with Acarbose buffer working solution to produce a dilution containing 0.2 1.0 TGU/ml (target 0.5 TGU/ml). Incubate the diluted enzyme at room temperature for at least 10 min before assay. Then use it as Sample solution. (Acarbose is added as an inhibitor to prevent interference by the other activity such as glucoamylase.

PROCEDURE

Perform the following procedure on all Sample solutions.

FOR ASSAY TEST SOLUTION

1. Prepare 3 tubes for each Sample solution. Pipette 500 µl of Substrate solution to the tubes. Pre-heat the tubes in a water bath at 50 °C for 10 minutes.
2. Add 500 µl of Sample solution to each tube at timed intervals, seal, vortex, and replace the tube in the water bath at 50 °C.
3. After exactly 60 minutes from the addition of Sample solution, terminate the reaction by removing the tube and immediately placing in boiling water bath for exactly 10 minutes. Cool to room temperature, add 9.0 ml of 0.011 N H_2SO_4 to each tube and mix by inversion.

FOR ASSAY BLANK

1. Prepare 2 tubes for assay blank of each Sample. Pipette 500 µl of Substrate solution to the tubes. Incubate in a water bath at 50 °C for exactly 60 minutes of incubation.
2. Remove the tubes from the water bath, add 500 µl of Sample solution to each test tube, seal, mix, and immediately place in boiling water bath for exactly 10 minutes. Cool to room temperature, add 9.0 ml of 0.011 N H_2SO_4 to each tube and mix by inversion.

HPLC PROCEDURE

- Column: Cation exchange column with guard column (H type) (300 mm x 7.8 mm, 9 µm) with guard column (H type), for example Bio-Rad HPX-87H (cat. no. 125-0140) with Cation H guard cartridge (cat. no. 125-0129), or equivalent.
- Column temperature: 60 °C
- Mobile Phase 0.01N H_2SO_4
- Detector: Refractive Index
- Flow rate: 0.7 ml/min
- Injection volume: 20 µl
- Run Time: 15 min

The retention time of D-panose in the chromatogram of the Sample solution should be determined using the Qualitative reference solution.

(Approximate retention times are as follows: D-Panose 5.7 min, D-Glucose 7.7 min)

CALCULATION

Calculate the activity of the sample in TGU per gram (g) using the following equation:

$$\frac{\text{TGU}}{\text{g}} = \frac{(G_3^{spl} - G_3^{blk}) \times 1\,000 \times 2 \times 10 \times V_1 \times V_2}{G_1^{std} \times 504 \times 60 \times W_1 \times V_s \times 1.30}$$

Where:

G_3^{spl} = Area of trisaccharide peak (average) in Assay test solution

G_3^{blk} = Area of trisaccharide peak (average) in Assay blank

G_1^{std} = Area of glucose standard (1 mg/ml) peak (average of duplicate injection)

1 000 = Conversion of gram to mg

504 = Mol. wt. of trisaccharide (D-panose) (conversion to m moles)

2 = Conversion to 1 ml enzyme (0.5 ml used in assay)

10 = HPLC dilution factor

V_1 = Primary dilution total volume (ml)

V_2 = Secondary dilution total volume (ml)

W_1 = Primary dilution weight of sample (g)

V_s = Secondary dilution sample volume (ml)

1.30 = Calculation factor related to the use of glucose standard

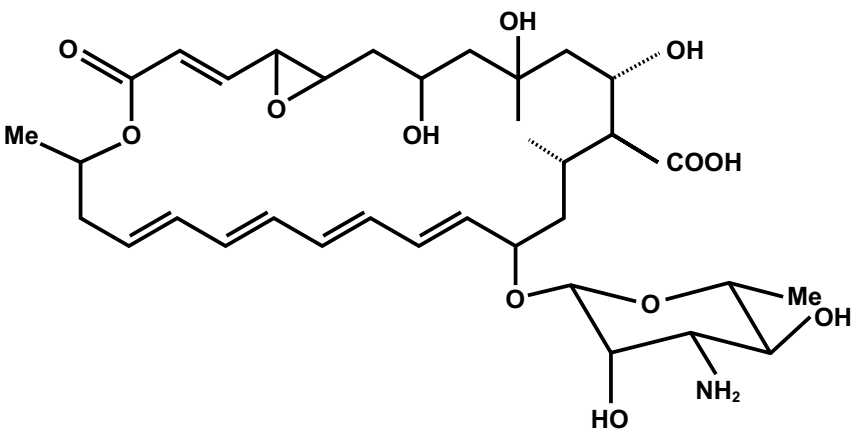
60 = Minutes of assay

One TGU is defined as the amount of enzyme that produces one μmol trisaccharide per minute under the assay condition.

If needed, convert from TGU/g to TGU/ml based on the relative density of the sample.

Natamycin

Revised specifications prepared at the 99th JECFA (2024) and published in FAO JECFA Monographs 34 (2025) superseding specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add 11 (2003). The Committee reaffirmed the ADI of 0- 0.3 mg/kg bw established at the 20th JECFA (1976).

SYNONYMS	Pimaricin; INS No. 235
DEFINITION	A fungicidal antimycotic of the polyene macrolide group. It is produced by several species of Streptomyces. The commercial product may contain up to three moles of water.
Chemical names	A stereoisomer of 22-(3-Amino-3,6-dideoxy-β-D-mannopyranosyloxy)-1,3,26-trihydroxy-12-methyl-10-oxo-6,11, 28-trioxatricyclo[22.3.1.$0^{5,7}$] octacosa-8,14,16,18,20-pentaene-25-carboxylic acid
C.A.S. number	7681-93-8
Chemical formula	$C_{33}H_{47}NO_{13}$
Structural formula	
Formula weight	665.74
Assay	Not less than 95.0% calculated on the dried basis
DESCRIPTION	White to creamy-white, almost odourless, crystalline powder
FUNCTIONAL USES	Fungicidal preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Practically insoluble in water, in lipid and in mineral oils; slightly soluble in methanol; soluble in glacial acetic acid and dimethylformamide.
Colour reaction	On adding a few crystals of the sample, on a spot plate, to a drop of - concentrated hydrochloric acid, a blue colour develops; - concentrated phosphoric acid, a green colour develops, which changes into pale-red after a few minutes
Infrared absorption	The infrared spectrum of a potassium bromide dispersion of the sample corresponds with the reference infrared spectrum in Appendix A (Main bands: 3 000-2 500 cm^{-1} Alcohol/Phenol O-H Stretch, 1716 cm^{-1} Carboxylic Acid C=O Stretch; 1570 cm^{-1} N-H bending of a primary amine).
Ultraviolet absorption (Vol. 4)	A solution of 5 mg/l of the sample in 0.1% glacial acetic acid in methanol has absorption maxima at about 290, 303 and 318 nm, a shoulder at about 280 nm and exhibits minima at about 250, 295.5 and 311 nm.

PURITY

Loss on drying (Vol. 4)	Not more than 8.0% (60 °C, over P_2O_5, pressure less than 5 mm Hg)
Specific rotation (Vol. 4)	α_D^{20} + 250° to + 295° (1% w/v solution in glacial acetic acid)
pH (Vol. 4)	5.0 - 7.5 (1.0% w/v suspension in deionized water)
Sulfated ash (Vol. 4)	Not more than 0.5% Test 2 g of the sample (Method I)
Arsenic (Vol. 4)	Not more than 1 mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the method described in Volume 4
Lead (Vol. 4)	Not more than 2 mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the method described in Volume 4.

TESTS

METHOD OF ASSAY

Transglucosidase activity

The purity of natamycin can be determined both spectrophotometrically and by HPLC. Data must be expressed as percentage on dry basis. If the process can take to the formation of natamycin methyl ester, the HPLC method must be used and the resolution of the two peaks of natamycin and natamycin methyl ester must be guaranteed.

(Note: Throughout this *Assay*, protect from direct light all solutions containing natamycin)

REAGENTS

Methanol (analytical or spectrophotometric grade, ≥99.9%); Aqueous methanol (mixing methanol and water (2+1 (v/v)); natamycin reference standard (minimum 95%).

NATAMYCIN STANDARD SOLUTIONS

Immediately before use, dissolve in a 100 ml volumetric flask, containing methanol, a known amount (50 mg) of standard natamycin, make up to the mark (solution A1); In a 50 ml volumetric flask, dilute 5 ml of solution A1 to 50 ml using the methanol-water solution (Solution B1); In a 50 ml volumetric flask, dilute 5 ml of solution B1 to 50 ml using the methanol-water solution to obtain the final standard natamycin (Solution C1): 5 mg/l of natamycin.

ASSAY PREPARATION

Immediately before use, dissolve in a 100 ml volumetric flask, containing methanol, a known amount (50 mg) of the unknown sample of natamycin (solution A2), proceed as described in Natamycin standard solution to obtain solutions B2 and C2 for the unknown sample.

Filter both solutions (standard and assay) through a syringe filter (0.45 μm) to obtain at least 3 ml of filtrate. Discharge the first drops.

SPECTROPHOTOMETRIC DETERMINATION

- **Calibration:** Record the spectrum of the Natamycin standard (Solution C1) in the range 300-340 nm, using the methanol-water solution as a blank. The spectrum should show a fingerprint with maxima at: 290, 303 and 318 nm, and minima at: 311 and 329 nm, respectively. Both the maximum and minimum values may vary slightly because of apparatus and solvents.
- **Sample solution:** Analogously to the standard solution, record the spectrum of the Natamycin unknown (Solution C2) in the range 300-340 nm, always using the methanol-water solution as a blank.
- Measure the absorbances, of both solutions, at 318 nm and at 311 and 329 nm, respectively.

- Read the net absorbances as:

$$\text{Net Abs} = \text{Abs318nm} - {}^{2}/_{3}\ \text{Abs311nm} - {}^{1}/_{3}\ \text{Abs329nm},$$

both for unknown sample and standard of natamycin.

- Calculate the concentration of natamycin in the unknown sample (mg/kg) as:

$$\text{Natamycin}\left(\frac{\text{mg}}{\text{kg}}\right) = \frac{\text{V(ml)}}{\text{Wsample(g)}} \times \frac{\text{NetAbs unknown sample}}{\text{NetAbs natamicin standard}} \times \text{natamicin standard}\left(\frac{\text{mg}}{\text{kg}}\right)$$

ALTERNATE METHOD

HPLC DETERMINATION:

Apparatus:

- HPLC equipped with an UV-Vis or Diode Array Detector (DAD); solvents HPLC purity grade.

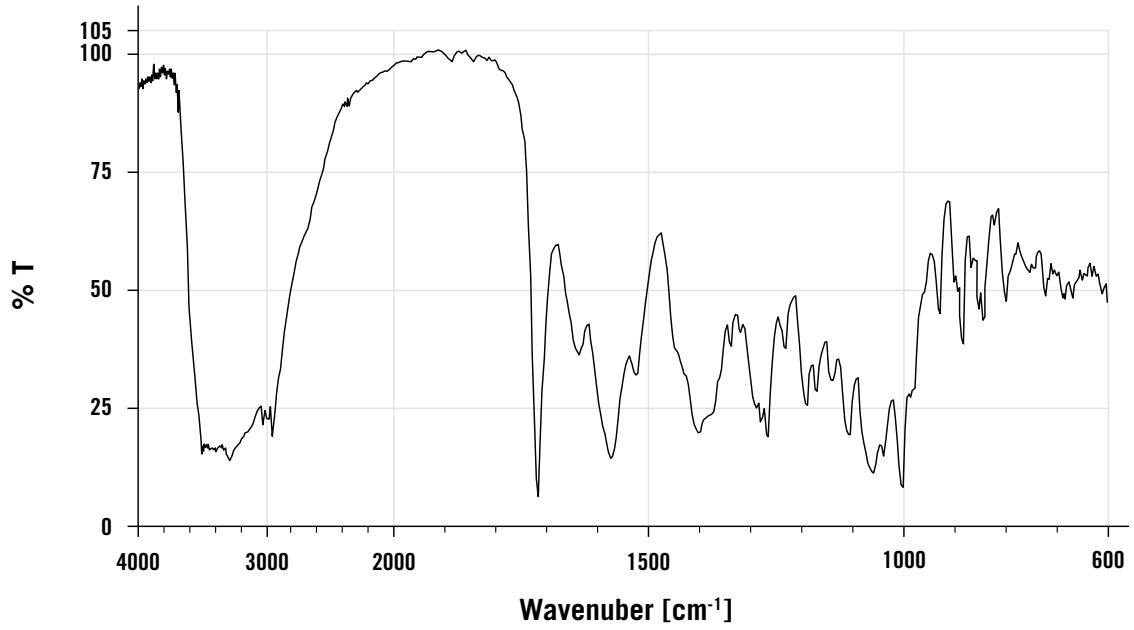
Procedure:

- Mobile phase: 30% acetonitrile-70% acidified water (0.1% glacial acetic) v/v
- Flow rate: isocratic at 0.8 mi/min
- Detector: DAD, ranges 250-360 nm, Quantitative at 305 nm.
- Column: RP-18, 250 mmx4.6 mm id, 5µm packed material, complete of guard column.
- Expected Retention Time: about 6 minutes.
- **External HPLC calibration curve and retention time of natamycin:** Pipet, into a series of 50 ml volumetric flasks, 1, 2, 4, 6 and 8 ml of standard natamycin (solution C1) and make up to the mark with the methanol-water solution to obtain standard solutions containing: 0.1, 0.2, 0.4, 0.6 and 0.8 mg/l of natamycin, respectively. Filter through a syringe filter (0.45µm) and inject into the HPLC. Establish the retention time of natamycin and build a calibration curve. Express natamycin in mg/kg.
- **Sample injection:** Inject the same aliquot of the sample (solution C2) into the HPLC, measure the area of the corresponding natamycin peak. Substitute it in the calibration curve to obtain the corresponding concentration of natamycin in the unknown sample.
- Adjust the injection volumes and dilutions of the samples to ensure that the response remains within the linear response range of the DAD detector.

Appendix 1

Reference Infrared Spectrum for natamycin

(kindly provided by Database of Japan's Specifications and Standards for Food Additives)



Nisin A

Revised specifications prepared at the 99th JECFA (2024) and published in FAO JECFA Monographs 34 (2025), superseding specifications for Nisin prepared at the 77th JECFA (2013). The Committee reaffirmed the ADI of 0-2 mg/kg bw established at the 77th JECFA.

SYNONYMS INS No. 234

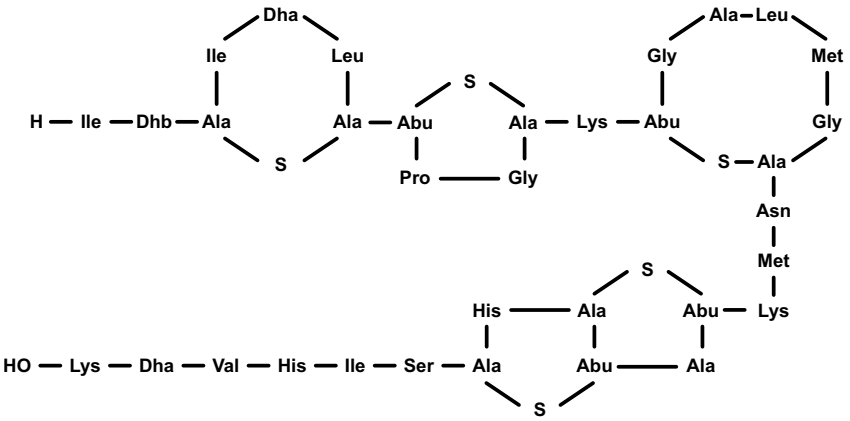
DEFINITION Nisin A, produced under appropriate fermentation conditions by *Lactococcus lactis* subsp. *lactis* strains, is an antimicrobial polypeptide. Nisin A is produced in a sterilized medium of non-fat milk solids or non-milk-based fermentation source, such as yeast extract and carbohydrate solids. The fermentation process is controlled for time and pH, until optimum nisin A production has been achieved. The nisin is then concentrated, recovered and purified from the fermentation medium by various methods, such as sterile injection, membrane filtration, acidification, salting out, ultrafiltration or spray-drying. The purified nisin A is then standardized with sodium chloride to achieve desired activity levels of nisin preparation. Nisin A is stable, at ambient temperatures and when heated, under acidic conditions (up to pH 3). Nisin A is commercially available as nisin preparation, which contains 2.5% w/w nisin A, >50% sodium chloride; the remaining components of the preparation are products of fermentation that include proteins and carbohydrates related to the starting material used for fermentation.

The activity of nisin A is measured in International Units (IU). One IU is defined as the amount in micrograms of nisin A required to inhibit the growth of 1 bacterial cell/1 ml of broth of Lactococcus lactis subsp. Cremoris. 1 IU of nisin A is equivalent to 0.025 µg.

C.A.S. number 1414-45-5

Chemical formula $C_{143}H_{230}O_{37}N_{42}S_7$ (Nisin A)

Structural formula



Abu =alpha-aminobutyric acid, Dha=dehydroalanine, Dhb=dehydrobutyrine (Nisin A)

Formula weight 3354.12 (Nisin A)

Assay Not less than 900 IU of nisin per mg (or 22.5 µg/mg)

DESCRIPTION White to light brown micronized powder

FUNCTIONAL USES Antimicrobial preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water at pH 2.5, sparingly soluble at pH 5, insoluble at pH>7 and in non-polar solvents

Differentiation from other antimicrobial substances Passes test

See description under TESTS

Nisin A Activity The sample shows nisin A activity

See description under METHOD OF ASSAY

PURITY

Loss on drying (Vol. 4) Not more than 3.0% (105 °C, 2 h)

Sodium Chloride (Vol. 4) Not less than 50%

LEAD (Vol. 4) Not more than 1 mg/kg

Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under "General methods, metallic impurities").

Microbiological criteria (Vol. 4) *Salmonella* species: Absent in 25 g of sample

Total coliforms: Less than 30 cfu per gram

Escherichia coli: Absent in 25 g of sample

TESTS

IDENTIFICATION TESTS

Differentiation from other antimicrobial substances

STABILITY IN ACIDIC CONDITIONS

Sample stock solution: Suspend 1 g of sample in 1 liter of 0.02 N hydrochloric acid to give a solution containing 1 000 IU/ml.

Sample Preparation: Make a dilution of the Sample stock solution with 0.02 N hydrochloric acid to arrive at a concentration of 50 IU/ml. Boil this solution for 5 min and measure the nisin A activity as directed under 'Determination of Nisin A Activity', in METHOD OF ASSAY.

The calculated nisin A concentration of the boiled sample should be 100% (+/- 5%) of the assay value indicating no significant loss of activity following this heat treatment.

STABILITY IN ALKALINE CONDITIONS

Adjust the pH of the unused portion of the boiled nisin A solution from 'Stability in acidic conditions' to 11.0 by adding 5 N sodium hydroxide. Heat the solution at 65 °C for 30 min, and then cool.

Adjust the pH to 2.0 by adding hydrochloric acid dropwise. Measure the nisin A activity as directed under 'Determination of Nisin A Activity' in METHOD OF ASSAY. Record loss of the antimicrobial activity of nisin A following this treatment. Total loss of the antimicrobial activity should be observed following the treatment described.

TOLERANCE OF *LACTOCOCCUS LACTIS* TO HIGH CONCENTRATIONS OF NISIN A

Prepare cultures of *L. lactis* (ATCC 11454, NCIMB 8586) in sterile skim (<1% fat) milk by incubating for 18 h at 30 °C. Prepare one or more flasks containing 100 ml of litmus milk and sterilize at 121 °C for 15 min. Suspend 0.1 g of sample in the sterilized litmus milk, and allow to stand at room temperature for 2 h. Add 0.1 ml of the *L. lactis* culture, and incubate at 30 °C for 24 h. Record *L. lactis* growth. *L. lactis* will grow at this concentration of sample (about 1 000 IU/ml); however, it will not grow in similar concentrations of other antimicrobial substances. (NOTE: This test will not differentiate nisin A from subtilin.)

METHOD OF ASSAY

Transglucosidase activity

DETERMINATION OF NISIN A ACTIVITY

Principle

Nisin A activity, expressed in International Units (IU), refers to the amount of nisin A required to inhibit growth of 1 bacterial cell in 1 ml of broth. 1 IU of nisin A is equivalent to 0.025 µg. Commercial nisin A preparations consist typically of 2.5% w/w of nisin A, sodium chloride and other solids from the fermentation medium.

Solution A (*L. lactis* subsp. *cremoris* (ATCC 14365, NCDO 495)): Prepare a subculture daily by transferring one loopful of the test culture to a McCartney bottle of litmus milk and incubating at 30 °C for 24 h (subculture A).

Inoculate a suitable quantity of sterile skim milk with 2 percent of subculture A and place it in a water bath at 30 °C for 90 min. Use immediately.

Solution B (Standard solution of nisin A): Dissolve 1 g of Nisin A preparation in 1 L of 0.02 N hydrochloric acid and dilute to 1 000 IU/ml. Dilute a portion of the standard stock in 0.02 N hydrochloric acid to a final concentration of 50 IU/ml. Prepare a nisin A solution containing 5,000 IU/ml (125 micrograms/ml). Dissolve an accurately weighed quantity of standard nisin A in 0.02 N hydrochloric acid. Immediately before use, dilute the solution further with 0.02 N hydrochloric acid to give 50 units/ml (dilution factor 100).

Solution C (Unknown sample): Weigh an amount of sample sufficient to ensure that corresponding tubes of the sample and standard series match, i.e., within close limits, so that the nisin A content in the sample and standard are similar. Dilute the sample solution in 0.02 N hydrochloric acid to obtain an approximate concentration of 50 IU per ml (IU/ml).

Solution D (Resazurin): Prepare a 0.0125% w/v solution of resazurin in water immediately prior to use.

PROCEDURE

- Pipet volumes of the 50 IU/ml nùisin standard (Solution B) and unknown sample (Solution C) into two rows of ten 12-ml bacteriological test tubes as depicted in the table below.

Pipet volumes of nisin standard and unknown sample

Dilution number	1	2	3	4	5	6	7	8	9	10
Solution B (ml)	0.60	0.55	0.50	0.45	0.41	0.38	0.34	0.31	0.28	0.26
Solution C (ml)	0.60	0.55	0.50	0.45	0.41	0.38	0.34	0.31	0.28	0.26

- Add 4.6 ml of the inoculated milk (Solution A) to each by means of an automatic pipetting device. The addition of inoculated milk should be made in turn across each duplicate row of tubes containing the same nominal concentration, and not along each row of ten tubes (e.g., dilution 1 (C and B), dilution 2 (C and B), etc.).
- Place the tubes in a water-bath at 30 °C for 15 min, then cool in an ice-water bath while adding 1 ml resazurin solution to each.
- Add the resazurin solution in the same order as the addition of inoculated milk, using an automatic pipetting device. Thoroughly mix the contents of the tubes by shaking. Continue incubation at 30 °C in a water bath for a further 3 – 5 min.
- Examine the standard and sample tubes under fluorescent light in a black matte-finish cabinet. Compare the sample tube of the highest concentration that shows the first clear difference in colour (i.e., has changed from blue to mauve) with tubes of the standard to find the nearest match in colour. Make further matches at the next two lower concentrations of the sample with the standard. Interpolation of matches may be made at half dilution steps. Obtain three readings of the sample solution and average them. As the standard tubes contain known amounts of nisin A, calculate the concentration of nisin in the sample solution.
- If necessary, convert nisin A activity from IU to micrograms of nisin A, using the conversion factor 1 IU = 0.025 micrograms of nisin A.

Alternative method

Quantification of Nisin A using HPLC

- Apparatus and reagents: HPLC equipped with an UV-Vis or Diode Array Detector (DAD); solvents HPLC purity grade.
- **External HPLC calibration curve and retention time of nisin:** Use dilutions of solution B. Filtrate through a syringe filter (0.45µm) and inject into the HPLC. Establish the retention time of nisin and build a calibration curve. Express nisin in mg/kg.
- **Sample injection:** Inject the same aliquot of the unknown samp into the HPLC, measure the area of the corresponding nisin peak. Substitute it in the calibration curve to obtain the corresponding concentration of natamycin in the unknown sample.

Adjust the injection volumes and dilutions of the samples to ensure that the response remains within the linear response range of the detector.

Column: reversed-phase Jupiter, 5µm, C18, 300A,
250 mm × 4.6 mm or equivalent
Flow rate: 1.0 ml/min
Detector: 210-260 nm, quantitative at 214 nm
Solvents: A (H2O (0.1% Trifluoracetic acid); B (Acetonitrile/H2O / Trifluoracetic acid (90/10/0.1 V/V)
Gradient:

Time (min)	A (%)	B (%)
0	78	22
30	43	57
35	0	100
45	78	22

Expected Retention Time of nisin A: 25 minutes (35-37% of acetonitrile)

Polyglycerol esters of fatty acids

Revised specifications prepared at the 99th JECFA (2024) and published in FAO JECFA Monographs 34 (2025), superseding specifications prepared at the 35th JECFA (1989) and published in FNP 49 (1990). The Committee reaffirmed the ADI of 0 25 mg/kg bw established at the 35th JECFA (1989).

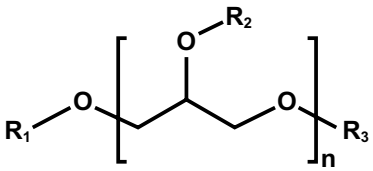
SYNONYMS Polyglycerol fatty acid esters, glycerin fatty acid esters;

INS No. 475

DEFINITION Polyglycerol esters of fatty acids are mixed esters formed by partially esterifying polymerized glycerols (polyglycerols) with fats, oils, or fatty acids. To mitigate the occurrence of contaminants coming from raw materials, only edible food grade precursors and those that have been 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" (CXC 79-2019) may be used. Polyglycerol esters of fatty acids 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 Polyglycerol esters of fatty acids varies, and is specified by a number (such as tri-) that is related to the average number of glycerol residues per polyglycerol molecule. A specified polyglycerol consists of a distribution of molecular species characteristic of its nominal degree of polymerization. By varying the proportions as well as the nature of the fats or fatty acids to be reacted with the polyglycerols, a large and diverse class of products may be obtained.

The article of commerce may be further specified as to saponification value, solidification point of the free fatty acids, iodine value, hydroxyl value and ash content.

Structural formula



where the average value of n is about 3 and R1, R2 and R3 each may be a fatty acid moiety or hydrogen

DESCRIPTION Light yellow to amber, oily to very viscous liquids; light tan to medium brown, plastic or soft solids; and light tan to brown, hard, waxy solids

FUNCTIONAL USES Emulsifier

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) From very hydrophilic to very lipophilic, but as a class tend to be dispersible in water and soluble in organic solvents

Free fatty acids (Vol. 4) Not more than 6% estimated as oleic acid (use equivalence factor (e) of 28.2)

PURITY

Polyglycerol determination in polyglycerol esters (Vol. 4) The polyglycerol moiety shall be composed of not less than 70% of di-, tri- and tetra- glycerols and shall contain not more than 10% of polyglycerols equal to or higher than heptaglycerol.

Arsenic (Vol. 4) Not more than 0.2 mg/kg

Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General methods, metallic impurities”).

Cadmium (Vol. 4) Not more than 0.2 mg/kg

Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General methods, metallic impurities”).

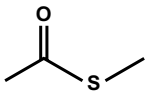
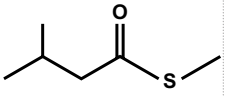
Lead (Vol. 4) Not more than 0.5 mg/kg

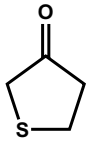
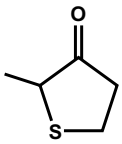
Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General methods, metallic impurities”).

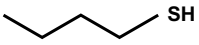
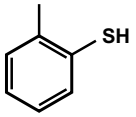
Mercury (Vol. 4) Note more than 0.5 mg/kg

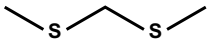
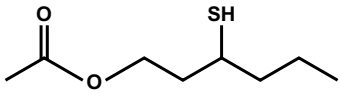
Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General methods, metallic impurities”).

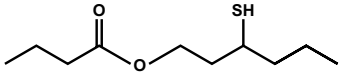
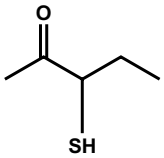
Revisions to existing flavour specifications

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20 °)	Other requirements
Status	Chemical Name	FLAVIS	M.W	Solubility in ethanol	Assay min %	S.G. (25 °)	
	Synonyms	COE	Physical form; Odour	B.P. °C	Acid value		Information required
Session		CAS					
482	**S-Methyl thioacetate**	3876	C_3H_6OS	Soluble in oil	MS, NMR	1.460-1.468	
Full	S-Methyl ethanethioate	12.149	90.14	Soluble	96%	1.021-1.025	
	S-Methyl acetothioate; Methanethiol acetate		Colourless to pale yellow liquid; pungent, garlic, sharp cheese	95-96 °C			
99t		1534-08-3					
487	**S-Methyl 3-methylbutanethioate**	3864	$C_6H_{12}OS$	Insoluble in water; Soluble in peanut oil	IR, MS, NMR, UV	1.455-1.461	
Full	S-Methyl 3-methylbutanethioate	12.157	132.22	Soluble	95%	0.935-0.947	
	Methylthiol isovalerate, Methanethiol isovalerate		Colourless liquid; pungent, fruity and onion, garlic	157 °C			
99th		23747-45-7					

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20 °)	Other requirements
Status	Chemical Name	FLAVIS	M.W	Solubility in ethanol	Assay min %	S.G. (25 °)	
	Synonyms	COE	Physical form; Odour	B.P. °C	Acid value		Information required
Session		CAS					
498	**4,5-Dihydro-3(2H) thiophenone**	3266	C_4H_6OS	minimally soluble in water and organic solvents	IR	1.527-1.531	
Full	4,5-Dihydro-3(2H)-thiophenone	15.012	102.15	Soluble	97%	1.194-1.207	
	3-Thiophanone; Tetrahydrothiophene-3-one, Tetrahydrothiophen-3-one	2337	Colourless to yellow liquid; Garlic, meaty, green vegetable, buttery	174-175 °C; 73 °C (15 mm Hg); 86-87 °C (25 mm Hg)			
99th		1003-04-9					
499	**2-Methyltetra-hydrothiophen-3-one**	3512	C_5H_8OS	Insoluble in water; soluble in fats	IR	1.505-1.520	
Full	4,5-Dihydro-2-methyl-3(2H)-thiophenone	15.023	116.18	Soluble	98%	1.115-1.126	
	2-Methyl-4,5-dihydro-3(2H)-thiophenone	11601	Colourless to yellow liquid; earthy, garlic, fruity	64-65 °C (11 mm Hg)			
99th		13679-85-1					

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20 °)	Other requirements
Status	Chemical Name	FLAVIS	M.W	Solubility in ethanol	Assay min %	S.G. (25 °)	
	Synonyms	COE	Physical form; Odour	B.P. °C	Acid value		Information required
Session		CAS					
511	**1-Butanethiol**	3478	$C_4H_{10}S$	Slightly soluble in water; slightly soluble in oils	IR	1.440-1.452	
Full	1-Butanethiol	12.010	90.19	Freely soluble	98%	0.830-0.848	
	Butyl mercaptan	526	Colourless to pale yellow liquid; Garlic or skunk-like	97-98.4 °C			
99th		109-79-5					
528	**o-Toluenethiol**	3240	C_7H_8S	Insoluble in water; Soluble in fats	IR	1.570-1.582	
Full	2-Methylbenzenethiol	12.027	124.2	Moderately soluble	95%	1.050-1.059	
	2-Methyl(thiophenol), 2-Methylthiophenol; o-Tolyl mercaptan	2272	Colourless to pale yellow liquid; Disagreeable odour	194-195 °C			
99th		137-06-4					

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20 °)	Other requirements
Status	Chemical Name	FLAVIS	M.W	Solubility in ethanol	Assay min %	S.G. (25 °)	
	Synonyms	COE	Physical form; Odour	B.P. °C	Acid value		Information required
Session		CAS					
533	**bis(Methylthio)methane**	3878	$C_3H_8S_2$	Very slightly soluble in water; soluble in fats	IR	1.530-1.538	
Full	bis(Methylthio)methane	12.118	108.22	Soluble	99%	1.047-1.067	
	Thioformaldehyde dimethyl acetal; bis(Methyl mercapto)methane; Methylenebis(methyl sulfide); Formaldehyde dimethyl mercaptal; 2,4-Dithiapentane; Formaldehyde dimethyl dithioacetal		Pale yellow, oily liquid; Fresh mustard	193 °C			
99th		1618-26-4					
554	**3-Mercaptohexyl acetate**	3851	$C_8H_{16}O_2S$	Insoluble in water; soluble in heptane	IR, MS, NMR	1.455-1.472	
Full	3-Sulfanylhexyl acetate	12.234	176.28	Soluble	95%	0.987-0.997	
	3-Mercaptohexyl acetate; 3-Thiohexyl ethanoate; 3-Thiohexyl acetate		Clear liquid; Fruity, with grapefruit/citrus notes, and sulfur undertone	186 °C			
99th		136954-20-6					

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20 °)	Other requirements
Status	Chemical Name	FLAVIS	M.W	Solubility in ethanol	Assay min %	S.G. (25 °)	
	Synonyms	COE	Physical form; Odour	B.P. °C	Acid value		Information required
Session		CAS					
555	**3-Mercaptohexyl butyrate**	3852	$C_{10}H_{20}O_2S$	insoluble in water; soluble in heptane	IR, MS, NMR	1.457-1.469	
Full	3-Sulfanylhexyl butanoate	12.235	204.33	Soluble	98%	0.960-0.968	
	3-Mercaptohexyl butyrate; 3-Thiohexyl butanoate; 3-Thiohexyl butyrate		Clear liquid; Fruity, with grapefruit/citrus notes, and sulfur undertone	196 °C			
99th		136954-21-7					
560	**3-Mercapto-2-pentanone**	3300	$C_5H_{10}OS$	Insoluble in water	MS	1.465-1.471	
Full	3-Sulfanylpentan-2-one	12.031	118.19	Miscible	98%	0.988-0.998	
	3-Mercapto-2-pentanone	2327	Colourless liquid; Raw-meat, garlic, sulfur	183 °C; 57 °C (15 mm Hg)			
99th		67633-97-0					

Annex 1: Summary and conclusions from JECFA99

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

Ninety-ninth meeting (Safety evaluation of certain food additives)

11–20 June 2024

SUMMARY AND CONCLUSIONS

Issued on 5 July 2024

The Ninety-ninth meeting of the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Executive Committee on Food Additives (JECFA) was held in Geneva from 11 to 20 June 2024. The purpose of the meeting was to evaluate the safety of certain food additives. The present meeting was the Ninety-ninth in a series of similar meetings. The tasks before the Committee were to (a) further elaborate principles governing the evaluation of food additives and enzymes; (b) undertake safety evaluations of certain food additives and enzymes; (c) review and prepare specifications for certain food additives and enzymes; and (d) review specifications for certain flavouring agents.

Dr D. Benford served as Chairperson and Dr R. Cantrill served as Vice-chairperson. Mr K. Petersen and Ms A. Vlachou served as joint secretaries.

The Committee evaluated the safety of four food additives and four processing aids, and revised the specifications for 10 flavouring agents.

The report of the meeting will be published in the WHO Technical Report Series (No. 1056). The report will summarize the main conclusions of the Committee in terms of acceptable daily intakes (ADIs) and other toxicological, dietary exposure and safety recommendations. Information on deliberations and conclusions with regards to the specifications for the identity and purity of certain food additives, enzymes examined by the Committee and the flavouring agents will also be included.

The participants are listed in Annex 1. Information of a general nature that the Committee wishes to disseminate quickly is provided in Annex 2. A related checklist to assist sponsors in the provision of information required for the safety assessment of enzyme preparations for use in foods is provided in Annex 3. Recommendations made by the Committee at the Ninety-ninth JECFA meeting are summarized in Annex 4.

Toxicological monographs summarizing the data that were considered by the Committee in establishing ADIs will be published in WHO Food Additives Series No. 90. New and revised specifications for the identity and purity of the compounds will be published in FAO JECFA Monographs No. 34.

More information on the work of JECFA is available at: http://www.fao.org/food-safety/scientific-advice/jecfa/en/ and https://www.who.int/foodsafety/en/.

[1] Please note that the annexes referred to in this document are to be found in the original summary of the ninety-ninth meeting and are not those in this volume of the FAO JECFA Monographs series.

Toxicological and dietary exposure information and conclusions

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 a margin of exposure (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.
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.

Food additive	JECFA enzyme identifier	Specifications	ADIs and other conclusions on toxicology and dietary exposure
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".

Favouring 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 2. General considerations

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.

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 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.

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 *(3)*. The Committee recommends that sponsors use the checklist (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).

Annex 3. JECFA enzyme submission checklist

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)	
13.	I (iii), ii	Details of genetic modification: (i) to host genome; (ii) addition of rDNA (gene of interest from another microorganism) to host microorganism through mobile genetic elements	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 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.	
16.	All	Deposit information (if applicable)	e.g. ATCC no.	

No.	Class(es)[a]	Information required	Details/rationale	Information to be provided by sponsor (document title, section, page number)
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.	
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 methto 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	
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 which are NOT considered safe for consumption and are not in any of the sub-categories listed above.

Annex 4. Recommendations and future work

Information required to be submitted for review

Food additive	JECFA enzyme identifier	Recommendations
Adenosine-5'-monophosphate deaminase from Aspergillus *sp.*	JECFA99-1	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.
Butterfly pea flower extract	-	The following information is required to complete the specifications for butterfly pea flower extract: quantitative composition of non-colouring components (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.
Polyglycerol esters of fatty acids	-	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.

ADI: acceptable daily intake; CCFA: Codex Committee on Food Additives; GSFA: General Standard for Food Additives.

COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives

Ninety-ninth Meeting, Geneva, 11–20 June 2024

This document contains food additive specification monographs, analytical methods, and other information prepared at the ninety-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which was held in Geneva from 11 to 20 June 2024. The specification monographs provide information on the identity and purity of food additives used directly in foods or in food production. The three main objectives of these specifications are to identify the food additive that has been subjected to testing for safety, to ensure that the additives are of the quality required for use in food or in processing and to reflect and encourage good manufacturing practice. This publication and other documents produced by JECFA contain information that is useful to all those who work with or are interested in food additives and their safe use in food.

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