ISSN 1817-7077

FAO JECFA Monographs

27

COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives

92nd Meeting
Virtual meeting, 7 – 18 June 2021

Food and Agriculture Organization of the United Nations

World Health Organization

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Rome, 2022

Required citation:
FAO and WHO. 2022. *Compendium of Food Additive Specifications – Joint FAO/WHO Expert Committee on Food Additives (JECFA), 92nd Meeting Virtual meeting, 7–18 June 2021.*Joint FAO/WHO Expert Committee on Food Additives (JECFA) Monographs No. 27. Rome. https://doi.org/10.4060/cb8300en

ISSN 1817-7077 [Print]
ISSN 2664-7451 [Online]

ISBN 978-92-5-135627-2 [FAO]

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Virtual meeting, 7 – 18 June 2021

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INTRODUCTION

This volume of FAO JECFA Monographs contains specifications of identity and purity prepared at the 92nd meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), held virtually, 7 – 18 June 2021. 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 on-line searchable database of all JECFA specifications monographs from the FAO JECFA Monographs, which is available at: http://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: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-flav/en/. The databases have query pages and background information in English, French, Spanish, Arabic and Chinese. 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: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/technical-assessments/en/.

Contact and Feedback

More information on the work of the Committee is available from the FAO homepage of JECFA at: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/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

The Committee evaluated the safety of five (5) food additives (N) and revised the specifications (R) for two food additives.

Benzoic acid, its salts and derivatives (N)
Collagenase from Streptomyces violaceoruber expressed in *S. violaceoruber* (N)
β-Glucanase from Streptomyces violaceoruber expressed in *S. violaceoruber* (N)
Phospholipase A2 from Streptomyces violaceoruber expressed in *S. violaceoruber* (R)
Riboflavin from *Ashbya gossypii* (N)
Ribonuclease P from *Penicillium citrinum* (N)
Modified starches (R)

BENZOIC ACID

Prepared at the 92nd JECFA (2021) published in FAO JECFA MONOGRAPHS 27 (2021) superseding specifications prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996). Metals and arsenic specifications were revised at the 63rd JECFA (2004). A group ADI of 0- 5 mg/kg bw for benzoic acid and its calcium, potassium and sodium salts, expressed as benzoic acid, was established at the 27th JECFA (1996). Benzyl alcohol was evaluated at the twenty-third and forty-sixth meetings (Annex 1, references 50 and 122); benzyl acetate was evaluated at the eleventh, twenty-seventh, twenty-ninth, thirty-first, thirty-fifth, forty-first, and forty-sixth meetings (Annex 1, references 14, 62, 70, 77, 88, 107, and 122); benzyl benzoate was evaluated at the fifteenth and forty-sixth meetings (Annex 1, references 26 and 122); benzaldehyde was evaluated at the eleventh and forty-sixth meetings (Annex 1, references 14 and 122); and benzoic acid was evaluated at the sixth, ninth, seventeenth, twenty - seventh, and forty-sixth meetings (Annex 1, references 6, 11, 32, 62, and 122). At its forty-sixth meeting, the Committee evaluated the five benzyl derivatives as a group and maintained the group ADI of 0 - 5 mg/kg bw as benzoic acid equivalents (Annex 1, reference 122). At its nighty-second meeting the group ADI of 0 – 5 mg/kg bw was withdrawn and re- established to 0 - 20 mg/kg bw for benzoic acid, its salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate, expressed as benzoic acid equivalents (2021).

SYNONYMS Benzenecarboxylic acid, phenylcarboxylic acid, INS No. 210

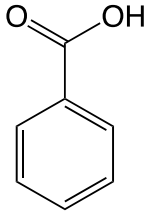
DEFINITION

Chemical names Benzoic acid

C.A.S. number 65-85-0

Chemical formula $C_7H_6O_2$

Structural formula



Formula weight 122.12

Assay Not less than 99.5% on the dried basis

DESCRIPTION White crystalline solid, usually in the form of scales or needles

FUNCTIONAL USES Antimicrobial preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Slightly soluble in water, freely soluble in ethanol
Melting range (Vol. 4)	121 - 123˚
Test for benzoate (Vol. 4)	Passes test Use 0.1 g of the sample with 0.1 g of calcium carbonate and 5 ml of water
pH (Vol. 4)	About 4.0 (solution in water)

PURITY

Loss on drying (Vol. 4)	Not more than 0.5% (over sulfuric acid, 3 h)
Sublimation test	Place a small amount of the sample in a dry test tube. Wrap the test tube about 4 cm from the bottom with moistened filter paper. Heat the test tube over a low flame. Benzoic acid sublimes and crystals deposit in the colder part of the test tube leaving no residue at the bottom.
Sulfated ash (Vol. 4)	Not more than 0.05%
Readily carbonizable substances (Vol. 4)	Passes test Dissolve 0.5 g of the sample, weighed to the nearest mg, in 5 ml of sulfuric acid TS. The colour produced should not be darker than a light pink ("Matching Fluid Q").
Chlorinated organic compounds (Vol. 4)	Not more than 0.07% (as Cl_2) Test 0.25 g of the sample dissolved in 10 ml of 0.1 N sodium hydroxide, using 0.5 ml of 0.01 N hydrochloric acid in the control.
Readily oxidizable substances	Passes test Add 1.5 ml of sulfuric acid to 100 ml of water, heat to boiling and add 0.1 N potassium permanganate in drops, until the pink colour persists for 30 sec. Dissolve 1 g of the sample, weighed to the nearest mg, in the heated solution, and titrate with 0.1 N potassium permanganate to a pink colour that persists for 15 sec. Not more than 0.5 ml should be required.
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, "Instrumental Methods."
METHOD OF ASSAY	Weigh, to the nearest mg, 2.5 g of the dried sample. Dissolve in 15 ml of warm ethanol previously neutralized using phenol red TS as indicator. Add 20 ml of water and titrate with 0.5 N sodium hydroxide, using phenolphthalein TS as indicator. Each ml of 0.5 N sodium hydroxide is equivalent to 61.06 mg of $C_7H_6O_2$.

CALCIUM BENZOATE

Prepared at the 92nd JECFA (2021) published in FAO JECFA MONOGRAPHS 27 (2021) superseding specifications prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996). Metals and arsenic specifications were revised at the 63rd JECFA (2004). A group ADI of 0 - 5 mg/kg bw for benzoic acid and its calcium, potassium and sodium salts, expressed as benzoic acid, was established at the 27th JECFA (1996). Benzyl alcohol was evaluated at the twenty-third and forty-sixth meetings (Annex 1, references 50 and 122); benzyl acetate was evaluated at the eleventh, twenty-seventh, twenty-ninth, thirty-first, thirty-fifth, forty-first, and forty-sixth meetings (Annex 1, references 14, 62, 70, 77, 88, 107, and 122); benzyl benzoate was evaluated at the fifteenth and forty-sixth meetings (Annex 1, references 26 and 122); benzaldehyde was evaluated at the eleventh and forty-sixth meetings (Annex 1, references 14 and 122); and benzoic acid was evaluated at the sixth, ninth, seventeenth, twenty-seventh, and forty-sixth meetings (Annex 1, references 6, 11, 32, 62, and 122). At its forty-sixth meeting, the Committee evaluated the five benzyl derivatives as a group and maintained the group ADI of 0 - 5 mg/kg bw as benzoic acid equivalents (Annex 1, reference 122). At its nighty-second meeting the group ADI of 0 - 5 mg/kg bw was withdrawn and re-established to 0 – 20 mg/kg bw for benzoic acid, its salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate, expressed as benzoic acid equivalents (2021).

SYNONYMS Calcium dibenzoate, calcium salt of benzenecarboxylic acid, calcium salt of phenolcarboxylic acid, INS No. 213

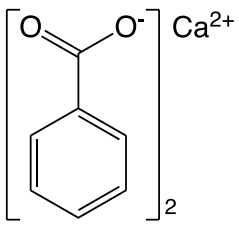
DEFINITION

Chemical names: Calcium benzoate

C.A.S. number: 532-25-3 (anhydrous)

Chemical formula: $C_{14}H_{10}CaO_4$ (anhydrous)
$C_{14}H_{10}CaO_4 \bullet H_2O$ (monohydrate)
$C_{14}H_{10}CaO_4 \bullet 3H_2O$ (trihydrate)

Structural formula:



Formula weight: 282.31 (anhydrous)
300.32 (monohydrate)
336.36 (trihydrate)

Assay: Not less than 99.0% on the dried basis

DESCRIPTION White or colourless crystals, or white powder

FUNCTIONAL USES Antimicrobial preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Sparingly soluble in ethanol
Test for benzoate (Vol. 4)	Passes test
Test for calcium (Vol. 4)	Passes test

PURITY

Loss on drying (Vol. 4)	Not more than 17.5% (105 ˚C, 4h)
Water insoluble matter	Not more than 0.3% Dissolve 10 g of the sample, weighed to the nearest mg, in 100 ml of hot water. Filter through a Gooch crucible, tared to an accuracy of ±0.2 mg, and wash any residue with hot water. Dry the crucible for 2 hours at 105 ˚C. Cool, weigh and calculate as percentage.
Acidity or alkalinity	Passes test Dissolve 2 g of the sample, weighed to the nearest mg, in 20 ml of freshly boiled water. Not more than 0.5 ml of either 0.1 N sodium hydroxide or 0.1 N hydrochloric acid should be required for neutralization, using phenolphthalein TS as indicator.
Fluoride (Vol. 4)	Not more than 10 mg/kg Weigh 5 g of the sample to the nearest mg and proceed as directed in the Limit Test (Method I or III).
Chlorinated organic compounds (Vol. 4)	Not more than 0.07% (as Cl_2) Test 0.25 g of the sample using 0.5 ml of 0.01 N hydrochloric acid in the control.
Readily oxidizable substances	Passes test Add 1.5 ml of sulfuric acid to 100 ml of water, heat to boiling and add 0.1 N potassium permanganate, dropwise, until the pink colour persists for 30 sec. Dissolve 1 g of the sample, weighed to the nearest mg, in the heated solution, and titrate with 0.1 N potassium permanganate to a pink colour that persists for 15 sec. Not more than 0.5 ml should be required.
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, “Instrumental Methods.”

METHOD OF ASSAY

Weigh accurately 0.6 g of the dried sample, dissolve in a mixture of 20 ml of water and 2 ml of dilute hydrochloric acid TS, and dilute to 100 ml with water. While stirring (preferably with a magnetic stirrer) add about 30 ml of 0.05 M disodium ethylenediaminetetraacetate from a 50-ml buret, then add 15 ml of sodium hydroxide TS, 40 mg of murexide indicator preparation (an alternative indicator is hydroxynaphthol blue, of which 0.25 g is used - in this case the naphthol green TS is omitted) and 3 ml of naphthol green TS, and continue the titration until the solution is deep blue in colour.

Each ml of 0.05 M disodium ethylenediamine tetraacetate is equivalent to 14.116 mg of $C_{14}H_{10}CaO_4$.

POTASSIUM BENZOATE

Prepared at the 92nd JECFA (2021) published in FAO JECFA MONOGRAPHS 27 (2021) superseding specifications prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996). Metals and arsenic specifications were revised at the 63rd JECFA (2004). A group ADI of 0 - 5 mg/kg bw for benzoic acid and its calcium, potassium and sodium salts, expressed as benzoic acid, was established at the 27th JECFA (1996). Benzyl alcohol was evaluated at the twenty-third and forty-sixth meetings (Annex 1, references 50 and 122); benzyl acetate was evaluated at the eleventh, twenty-seventh, twenty-ninth, thirty-first, thirty-fifth, forty-first, and forty-sixth meetings (Annex 1, references 14, 62, 70, 77, 88, 107, and 122); benzyl benzoate was evaluated at the fifteenth and forty-sixth meetings (Annex 1, references 26 and 122); benzaldehyde was evaluated at the eleventh and forty-sixth meetings (Annex 1, references 14 and 122); and benzoic acid was evaluated at the sixth, ninth, seventeenth, twenty-seventh, and forty-sixth meetings (Annex 1, references 6, 11, 32, 62, and 122). At its forty-sixth meeting, the Committee evaluated the five benzyl derivatives as a group and maintained the group ADI of 0 – 5 mg/kg bw as benzoic acid equivalents (Annex 1, reference 122). At its nighty-second meeting the group ADI of 0 - 5 mg/kg bw was withdrawn and re-established to 0 – 20 mg/kg bw for benzoic acid, its salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate, expressed as benzoic acid equivalents (2021).

SYNONYMS Potassium salt of benzenecarboxylic acid, potassium salt of phenylcarboxylic acid, INS No. 212

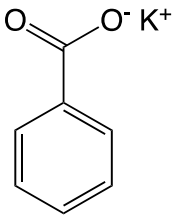
DEFINITION

Chemical names: Potassium benzoate

C.A.S. number: 532-25-2 (anhydrous)

Chemical formula: $C_7H_5KO_2$ (anhydrous)
$C_7H_5KO_2 \bullet 3H_2O$ (trihydrate)

Structural formula:



Formula weight: 160.22 (anhydrous)
214.27 (trihydrate)

Assay: Not less than 99.0% on the dried basis

DESCRIPTION White crystalline powder

FUNCTIONAL USES Antimicrobial preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Freely soluble in water, soluble in ethanol
Test for benzoate (Vol. 4)	Passes test Use a 10% solution of the sample
Test for potassium (Vol. 4)	Passes test Use a 10% solution of the sample

PURITY

Loss on drying (Vol. 4)	Not more than 26.5% (105˚, 4 hours)
Acidity or alkalinity	Passes test Dissolve 2 g of the sample, weighed to the nearest mg, in 20 ml of freshly boiled water. Not more than 0.5 ml of either 0.1 N sodium hydroxide or 0.1 N hydrochloric acid should be required for neutralization, using phenolphthalein TS as indicator.
Readily carbonizable substances (Vol. 4)	Passes test Dissolve 0.5 g of the sample, weighed to the nearest mg, in 5 ml of sulfuric acid TS. The colour produced should not be darker than a light pink ("Matching Fluid Q")
Chlorinated organic compounds (Vol. 4)	Not more than 0.07% (as Cl_2) Test 0.25 g of the sample using 0.5 ml of 0.01 N hydrochloric acid in the control
Readily oxidizable substances	Passes test Add 1.5 ml of sulfuric acid to 100 ml of water, heat to boiling and add 0.1 N potassium permanganate, dropwise, until the pink colour persists for 30 sec. Dissolve 1 g of the sample, weighed to the nearest mg, in the heated solution, and titrate with 0.1 N potassium permanganate to a pink colour that persists for 15 sec. Not more than 0.5 ml should be required.
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, “Instrumental Methods.”
METHOD OF ASSAY	Weigh to the nearest 0.1 mg, 2.5 to 3 g of the dried sample, and transfer to a 250-ml Erlenmeyer flask. Add 50 ml of water to dissolve the sample. Neutralize the solution, if necessary, with 0.1 N hydrochloric acid, using phenolphthalein TS as indicator. Add 50 ml of ether and a few drops of bromophenol blue TS and titrate with 0.5 N hydrochloric acid, shaking constantly the flask, until the colour of the indicator begins to change. Transfer the lower aqueous layer to another flask. Wash the ethereal layer with 10 ml of water, and add the washing and an additional 20 ml of ether to the separated aqueous layer. Complete the titration with the 0.5 N hydrochloric acid, shaking constantly the flask. Each ml of 0.5 N hydrochloric acid is equivalent to 80.11 mg of $C_7H_5KO_2$.

SODIUM BENZOATE

Prepared at the 92nd JECFA (2021) published in FAO JECFA MONOGRAPHS 27 (2021) superseding specifications prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996). Metals and arsenic specifications were revised at the 63rd JECFA (2004). A group ADI of 0 - 5 mg/kg bw for benzoic acid and its calcium, potassium and sodium salts, expressed as benzoic acid, was established at the 27th JECFA (1996). Benzyl alcohol was evaluated at the twenty-third and forty-sixth meetings (Annex 1, references 50 and 122); benzyl acetate was evaluated at the eleventh, twenty-seventh, twenty-ninth, thirty-first, thirty-fifth, forty-first, and forty-sixth meetings (Annex 1, references 14, 62, 70, 77, 88, 107, and 122); benzyl benzoate was evaluated at the fifteenth and forty-sixth meetings (Annex 1, references 26 and 122); benzaldehyde was evaluated at the eleventh and forty-sixth meetings (Annex 1, references 14 and 122); and benzoic acid was evaluated at the sixth, ninth, seventeenth, twenty-seventh, and forty-sixth meetings (Annex 1, references 6, 11, 32, 62, and 122). At its forty-sixth meeting, the Committee evaluated the five benzyl derivatives as a group and maintained the group ADI of 0 – 5 mg/kg bw as benzoic acid equivalents (Annex 1, reference 122). At its nighty-second meeting the group ADI of 0 - 5 mg/kg bw was withdrawn and re-established to 0 – 20 mg/kg bw for benzoic acid, its salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate, expressed as benzoic acid equivalents (2021).

SYNONYMS Sodium salt of benzenecarboxylic acid, sodium salt of phenylcarboxylic acid, INS No. 211

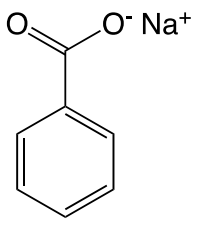
DEFINITION

Chemical names: Sodium benzoate

C.A.S. number: 532-32-1

Chemical formula: $C_7H_5NaO_2$

Structural formula:



Formula weight: 144.11

Assay: Not less than 99.0% on the dried basis

DESCRIPTION White, crystalline powder, flakes or granules

FUNCTIONAL USES Antimicrobial preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Freely soluble in water, sparingly soluble in ethanol
Test for benzoate (Vol. 4)	Passes test Use a 10% solution of the sample
Test for sodium (Vol. 4)	Passes test

PURITY

Loss on drying (Vol. 4)	Not more than 1.5% (105˚, 4h)
Acidity or alkalinity	Passes test Dissolve 2 g of the sample, weighed to the nearest mg, in 20 ml of freshly boiled water. Not more than 0.5 ml of either 0.1 N sodium hydroxide or 0.1 N hydrochloric acid should be required for neutralization, using phenolphthalein TS as indicator.
Readily carbonizable substances (Vol. 4)	Passes test Dissolve 0.5 g of the sample, weighed to the nearest mg, in 5 ml of sulfuric acid TS. The colour produced should not be darker than a light pink ("Matching Fluid Q")
Chlorinated organic compounds (Vol. 4)	Not more than 0.07% (as Cl_2) Test 0.25 g of the sample using 0.5 ml of 0.01 N hydrochloric acid in the control
Readily oxidizable substances	Passes test Add 1.5 ml of sulfuric acid to 100 ml of water, heat to boiling and add 0.1 N potassium permanganate, dropwise, until the pink colour persists for 30 sec. Dissolve 1 g of the sample, weighed to the nearest mg, in the heated solution, and titrate with 0.1 N potassium permanganate to a pink colour that persists for 15 sec. Not more than 0.5 ml should be required.
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, “Instrumental Methods.”
METHOD OF ASSAY	Weigh, to the nearest mg, 3 g of the sample previously dried for 4 h at 105˚C and transfer to a 250-ml Erlenmeyer flask. Add 50 ml of water to dissolve the sample. Neutralize the solution, if necessary, with 0.1 N hydrochloric acid, using phenolphthalein TS as indicator. Add 50 ml of ether and a few drops of bromophenol blue TS, and titrate with 0.5 N hydrochloric acid, shaking the flask constantly, until the colour of the indicator begins to change. Transfer the lower aqueous layer to another flask. Wash the ethereal layer with 10 ml of water, and add the washing and an additional 20 ml of ether to the

separated aqueous layer. Complete the titration with the 0.5 N hydrochloric acid, shaking constantly the flask.

Each ml of 0.5 N hydrochloric acid is equivalent to 72.05 mg of $C_7H_5NaO_2$.

COLLAGENASE FROM STREPTOMYCES VIOLACEORUBER

New specifications were prepared at the 92nd JECFA (2021), published in FAO JECFA Monographs 27 (2021). An ADI of "not specified" was established at the 92nd JECFA (2021).

SYNONYMS Microbial collagenase, collagenase; collagen peptidase; collagen protease; collagenase A; collagenase I; interstitial collagenase; matrix metalloproteinase; metallocollagenase

SOURCES Produced by controlled fed-batch fermentation of non-pathogenic, non-toxigenic strain of *Streptomyces violaceoruber*. The secreted collagenase is separated from the biomass solids and concentrated using sedimentation followed by a series of filtration steps. The collagenase liquid concentrate may be formulated into either a liquid or a powder enzyme preparation using food-grade stabilizing and preserving agents.

Active principles Collagenase

Systematic names and numbers Microbial collagenase; EC 3.4.24.3;
CAS No. 9001-12-1

Reaction catalysed Digestion of native collagen in the triple helical region at Gly bonds.

With synthetic peptides, a preference is shown for Gly at P3 and P1', Pro and Ala at P2 and P2', and hydroxyproline, Ala or Arg at P3'

Secondary enzyme activities No significant levels of secondary activities

DESCRIPTION Brown powder or liquid

FUNCTIONAL USES Enzyme preparation
Used for the degradation of collagen to tenderise meat and sausage casings. The collagenase preparation is also used in the manufacture of collagen hydrolysates used in food supplements.

GENERAL SPECIFICATIONS Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Collagenase activity The sample shows collagenase activity. See description under TESTS.

TESTS

METHOD OF ASSAY

Collagenase activity

Principle

Collagenase activity is determined spectrophotometrically by measuring the hydrolysis of a defined peptide substrate by Collagenase at 570 nm.

One unit of activity is defined as the quantity of collagenase required to liberate 1 µmol of glycine per minute under the conditions of the assay.

Apparatus

Water bath with circulation
Vortex mixer
UV-Vis spectrophotometer
pH meter

Reagents and solutions

- 1 M Tris· HCl Buffer, pH8.0: Dissolve 121.14 g of tris (hydroxymethyl) aminomethane (CAS No. 77-86-1) in about 700 ml of deionised water. Adjust to pH 8.0 with 1 N Hydrochloric acid. Dilute to 1000 ml with deionised water.
- Dilution solution 1: Dilute 50 ml of 1 M Tris· HCl Buffer (pH 8.0) with deionised water to 1000 ml.
- Enzyme diluent solution: Dilute 50 ml 1 M Tris·HCl Buffer (pH 8.0) with about 500 ml of deionised water. Dissolve 14.7 g of calcium chloride dihydrate (CAS No. 10035-04-8) in this solution. Dilute with deionised water to 1000 ml.
- 4 M Acetic acid solution: Dilute 115.1 ml of acetic acid (CAS No. 64-19-7) with deionised water to 500 ml.
- 4 M Sodium acetate solution: Dissolve 164 g of anhydrous sodium acetate (CAS No. 127-09-3) in about 300 ml of deionised water. Dilute to 500 ml with deionised water.
- 4 M Acetate buffer, pH 5.0: Add drops of 4 M acetic solution to 500 ml of 4 M sodium acetate solution up to a pH of 5.0 ± 1%.
- Trichloroacetic solution: Dissolve 4.0 g of trichloroacetic acid (CAS No. 76-03-9) in about 300 ml deionised water. Dilute to 400 ml with deionised water.
- 5% Sodium hydroxide solution: Dissolve 10.0 g of sodium hydroxide (CAS No. 1310-73-2) in 100 ml deionised water. Dilute to 200 ml with deionised water.
- 30% Ninhydrin solution: Dissolve 15 g of ninhydrin (CAS No. 485-47-2) in 30 ml of 2-methoxyethanol (CAS No. 109-86-4). Dilute to 50 ml with 2-methoxyethanol.
- Ninhydrin solution: Dilute 50 ml of 30% Ninhydrin Solution with 4 M Acetate Buffer, pH 5.0 to 500 ml.
- 50% Ethanol: Dilute 500 ml of ethanol with deionised water to 1000 ml.
- Glycine stock solution: Dissolve 150.14 mg of glycine (CAS No. 56-40-6; use of certified reference material or reference material is preferred) reference material in 100 ml of Dilution Solution 1. Dilute with Dilution Solution 1 to 200 ml.

Substrate solution

Dissolve 0.136g of Z-Gly-Pro-Leu-Gly-Pro (Peptide Institute, Inc.) in 1 ml DMSO (CAS No. 67-68-5). Dilute this solution with Dilution solution 1 to 50 ml.

Sample preparation

Dilute the enzyme to 0.033 - 0.1 U/ml using the Enzyme diluent solution.

Standard Curve Preparation

Prepare standard curve as follows.

Set up seven 100 ml volumetric flasks and label them Standards 1-6 and one as Blank (No. 7). Dilute the Glycine stock solution with Dilution solution 1, as shown in the table below.

No.	Glycine stock solution, ml	[Glycine] in the flask, µmol/ml	[Glycine] in the test tube, µmol/ml
1	6.00	0.6	0.04
2	5.00	0.5	0.033
3	4.00	0.4	0.027
4	3.00	0.3	0.02
5	2.00	0.2	0.133
6	1.00	0.1	0.007
7	0.00	(0) blank	0

1. Set up seven test tubes; label them as Standards 1-6 and Blank.
2. Add 0.4 mL of Standards 1-6 and Blank to the correspondingly labelled test tubes.
3. Add 0.5 ml of 1% Trichloroacetic solution, 0.1 ml of 5% Sodium hydroxide solution and 1.0 ml of Ninhydrin solution to each test tube. Mix well.
4. Cover the test tubes loosely and heat them in a boiling water bath for 30 min.
5. Cool the tubes in ice water for 30 min.
6. Add 4.0 ml of 50% Ethanol solution to each of the seven test tubes and mix the tubes well.
7. Measure the absorbance for the resulting solutions 570 nm against the blank.
8. Plot the absorbance of the solutions against the concentration of glycine (µmol/ml) in the test tube. Determine the slope and y-intercept of the standard curve.

Procedure

For each Sample preparation to be analysed, test in duplicate as follows.

1. Prepare 2 test tubes. Label one as Sample and one as Blank.
2. Add 0.2 ml of Substrate solution to the Sample test tube and Incubate for 5 min in a 30°C water bath.

3. Add 0.2 ml of the Sample preparation to the Sample test tube; mix and allow to react for 10 min in a 30°C water bath.
4. After exactly 10 min, add 0.1 ml of Trichloroacetic solution to the tube to stop the reaction.
5. Add 0.5 ml of 5 % Sodium hydroxide solution to the Sample test tube and mix well.
6. Add 1.0 ml of Ninhydrin Solution to the Sample test tube and mix using a vortex mixer.
7. Add 0.2 ml of the Sample preparation, 0.5 ml of Trichloroacetic solution, 0.2 ml of Substrate solution, 0.5 ml of 5% Sodium hydroxide solution and 1.0 ml of Ninhydrin solution to the Blank test tube.
8. Cover both test tubes with a glass bead and heat the tubes in boiling water for 30 min.
9. Cool the test tubes in ice water for 30 min.
10. Add 4.0 ml of 50% Ethanol solution to both Sample and Blank tubes and shake well.
11. Measure the absorbance of the Sample test tube (A_T) and Blank test tube (A_B) at 570 nm.

Calculation

Calculate the activity of each sample in U/g as follows:

$$Collagenase\ Activity, U/g = \frac{(A_T - A_B) - b}{m} \times \frac{6}{C \times 0.2 \times 10}$$

Where:

A_T is the absorbance of the Reaction solution;
A_B is the absorbance of the Blank solution;
b is the y-intercept of the standard curve;
m is the slope of the standard curve;
6 is the total volume of the Reaction solution (ml);
0.2 is the volume of the Sample preparation in the Reaction solution (ml);
10 is the reaction time (min);
C is the concentration of enzyme in the Sample preparation (g/ml).

β - GLUCANASE FROM *STREPTOMYCES VIOLACEORUBER*

New specifications were prepared at the 92nd JECFA (2021), published in FAO JECFA Monographs 27 (2021). An ADI of "not specified" was established at the 92nd JECFA (2021).

SYNONYMS endo-1,3-β-glucanase; laminarinase; laminaranase; oligo-1,3-glucosidase; endo-1,3-β-glucanase; callase; β-1,3-glucanase; kitalase; 1,3-β-D-glucan 3-glucanohydrolase; endo-(1,3)-β-D-glucanase; (1→3)-β-glucan 3-glucanohydrolase; endo-1,3-β-D-glucanase; endo-1,3-β-glucosidase; 1,3-β-D-glucan glucanohydrolase

SOURCES Produced by controlled fed-batch fermentation of a non-pathogenic, non-toxigenic strain of *Streptomyces violaceoruber*. The secreted β-glucanase is separated from the biomass and concentrated using sedimentation followed by a series of filtration steps. The β-glucanase concentrate may be formulated into either a liquid or a powder enzyme preparation using food-grade stabilizing and preserving agents.

Active principles — Glucan endo-1,3-β-D-glucosidase

Systematic names and numbers — 3-β-D-Glucan glucanohydrolase; EC 3.2.1.39;
CAS No. 9025-37-0

Reaction catalysed — Hydrolysis of the (1->3)- β-D-glucosidic linkages in (1->3)- β-D-glucans to produce corresponding β-glucans and glucose

Secondary enzyme activities — No significant levels of secondary activities

DESCRIPTION Brown, liquid or powder

FUNCTIONAL USES Enzyme preparation
Used in the manufacture of beer, yeast and mushroom extracts.

GENERAL SPECIFICATIONS Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

β-Glucanase activity — The sample shows beta-glucanase activity.
See description under TESTS.

TESTS

METHOD OF ASSAY

β-Glucanase activity — Principle

Assay measures the amount of glucose produced by spectrophotometry using the phenol-sulfuric acid method when curdlan is treated with β-glucanase.

One unit of activity is defined as the quantity of beta-glucanase required to produce 1µmol of D-glucose per minute under the conditions of the assay.

Apparatus

Water bath with circulation
UV-Vis spectrophotometer
pH meter
Vortex mixer

Reagents and Solutions

- 0.5 M Hydrochloric acid: Prepare by mixing 9 ml of 6.0 M hydrochloric acid with 99 ml of deionised water.
- Phenol solution (5%; w/v): Dilute 25 g of phenol (anhydrous), adjusted for purity, to 500 ml with deionised water.
- 1 M Acetic acid: Prepare by adding deionised water to 60 g of acetic acid and fill to 1 litre using a measuring flask.
- 1 M Sodium Acetate solution: Prepare by adding distilled water to 82 g of anhydrous sodium acetate and fill to 1 litre in a measuring flask.
- 1 M Acetate buffer (pH 5.0): Prepare by mixing 1 M acetic acid and 1 M sodium acetate and adjust the pH to 5.0.
- Enzyme diluent solution: Dilute 1 M Acetate buffer (pH 5.0) ten-fold in deionised water.
- D-Glucose stock solution: Transfer 0.901 g D-glucose to a 500 ml volumetric flask; dissolve in and dilute to volume with deionised water.

Substrate solution

Dilute 3 g of curdlan (Fujifilm Wako Pure Chemical Industries Ltd.; product code 034-0991, or equivalent) to 30 g in 0.1 M acetate buffer (pH 5.0) in a beaker, with stirring. Prepare immediately before use. Cool the solution in a water bath during and after preparation.

Sample preparation

Dilute the sample to 0.045 - 0.230 U/ml in a glass container using Enzyme diluent solution.

Standard curve preparation

Prepare a standard curve as follows.
Set up five 100 ml volumetric flasks and label them Standards 1- 5. Dilute aliquots of the D-Glucose stock solution with deionised water as shown in the table below.

Standard No.	D-Glucose stock solution, ml	[D-Glucose] in the volumetric flask, mmol/ml	[D-Glucose] in the test tube, µmol/ml
1	1	0.1	14.3

2	2	0.2	28.6
3	4	0.4	57.1
4	6	0.6	85.7
5	8	0.8	114.3

1. Accurately measure 0.4 ml of each Standard solution into a test tube; add 0.4 ml of Phenol solution (5%; w/v) to each tube and shake thoroughly.
2. Incubate the test tubes in a water bath held at 37 °C for approximately 10 min.
3. Add 2 ml of concentrated sulfuric acid vertically to each tube using an automatic dispenser; vigorously mix the solutions using a vortex mixer.
4. Allow the solutions to stand at room temperature for approximately 30 min.
5. Record the absorbance of each solution at 490 nm against a deionised water blank.
6. Plot the absorbance of the solutions against the concentration of D-glucose (µmol/ml) in the test tube. Determine the slope and y-intercept of the standard curve.

Procedure

For each Sample preparation to be analysed, test in duplicate as follows.

1. Accurately measure 1.2 ml of the Substrate Solution into each of nine test tubes and incubate for 5 min in a 37 °C water bath.
2. Add 0.2 ml of the Sample preparation to each tube; mix and allow to react for 30 min in the 37 °C water bath.
3. After exactly 30 min, add 0.2 ml of 0.5 M hydrochloric acid to each tube to stop the reactions.
4. Transfer the solutions to individual Eppendorf tubes and centrifuge all tubes at 4 °C and 15000 rpm for 10 min.
5. Recover the supernatant from each tube using a 1 ml syringe and remove the insoluble material with Millex-LH (4 mm; 0.45 µm).
6. Accurately measure 0.4 ml of the supernatant liquid from the each of the Eppendorf tubes into a corresponding test tube; add 0.4 ml of Phenol solution (5%; w/v) to each of the nine tubes.
7. Mix the test tubes using a vortex mixer, then incubate for approximately 10 min in a 37 °C water bath.
8. Add 2 ml of sulfuric acid vertically to each tube using an automatic dispenser; vigorously mix the solutions using a vortex mixer.
9. Allow the solutions to stand at room temperature for approximately 30 min. Record the absorbance at a wavelength of 490 nm.
10. Prepare Enzyme blank solutions as follows: for each enzyme sample being tested, combine 0.2 ml of the Sample preparation and 0.2 ml of 0.5 M Hydrochloric acid in a test tube. Add 1.2 ml of the Substrate solution to the tube and follow the process above, beginning at Step 4.

Calculation

Calculate the activity of each sample in U/g as follows:

$$\beta - glucanase\ Activity, U/g = \frac{(A_1 - A_2) - b}{m} \times \frac{1.6}{C \times 0.2 \times 30}$$

Where

A_1 is the absorbance of the Reaction solution

A_2 is the absorbance of the Enzyme blank solution

b is the y-intercept of the standard curve

m is the slope of the standard curve

1.6 is the total volume of the Reaction solution (ml)

0.2 is the volume of Sample preparation in the Reaction solution (ml)

30 is the reaction time (min)

C is the concentration of enzyme in the Sample preparation (g/ml)

PHOSPHOLIPASE A2 FROM STREPTOMYCES VIOLACEORUBER

New specifications were prepared at the 92nd JECFA (2021), published in FAO JECFA Monographs 27 (2021). An ADI of "not specified" was established at the 92nd JECFA (2021).

SYNONYMS Lecithinase A; phosphatidase; phosphatidolipase; phospholipase A

SOURCES Produced by controlled fed-batch fermentation of a non-pathogenic, non-toxigenic strain of *Streptomyces violaceoruber*. The secreted phospholipase A2 is separated from the biomass and concentrated using sedimentation followed by a series of filtration steps. The phospholipase A2 concentrate may be formulated into either a liquid or a powder enzyme preparation using food-grade stabilizing and preserving agents.

Active principles: Phospholipase A2

Systematic names and numbers: Phosphatidylcholine 2-acylhydrolase; EC 3.1.1.4;
CAS No. 9001-84-7

Reaction catalysed: Hydrolysis of the sn-2 ester bonds of diacylphospholipids to form 1-acyl-2-lysophospholipids and free fatty acids

Also acts on phosphatidylethanolamine, choline plasmalogen and phosphatides, removing the fatty acid attached to the sn-2 position. Requires Ca^{2+}.

Secondary enzyme activities: No significant levels of secondary activities

DESCRIPTION Brown, liquid or powder

FUNCTIONAL USES Enzyme preparation
Used in the manufacture of enzyme-modified egg yolk, lecithin, cereal flour, dairy products, and degumming of vegetable oil.

GENERAL SPECIFICATIONS Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Phospholipase A2 activity: The sample shows Phospholipase A2 activity.
See description under TESTS.

TESTS

METHOD OF ASSAY

Phospholipase A2 activity

Principle

Phospholipase A2 activity is determined spectrophotometrically by measuring the hydrolysis of a phosphatidylcholine substrate and measuring the amount of fatty acid released at 550 nm.

One unit of activity is defined as the quantity of enzyme required to liberate 1 µmol of fatty acid from L-α-phosphatidylcholine per minute under the conditions of the assay.

Apparatus

Water bath with circulation
Ice bath
Sonicator
UV-Vis spectrophotometer
pH meter
Vortex mixer

Reagents and Solutions

- 1.0 M Tris-HCl Buffer (pH 8.0): Dissolve 60.5 g of tris(hydroxymethyl)aminomethane in approximately 250 ml of deionised water. Adjust pH to 8.0 ± 0.02 with 2 M HCl. Make up to 500 ml with deionised water.
- Triton·X-100: 10% (v/v)
- 1 M Calcium chloride solution (freshly made)
- NEFA-C Test Kit (Product No. 279·75401, Fujifilm Wako Pure Chemical Industries, Ltd. Equivalent test kits may be used; follow instructions provided by the manufacturer. The procedure included below is based on the Fujifilm Wako Pure Chemical Industries kit); includes Oleic Acid, 1 mEq/l, Solvent A, and Solvent B.
- Reagent A Solution: From the NEFA-C test kit, add 10 ml of Solvent A into the Colour Reagent A bottle and mix well gently. This solution can be stored for 1 week at 4°C.
- Reagent B Solution: From the NEFA-C test kit, add 20 ml of Solvent B into the Colour Reagent B bottle and mix well gently. This solution can be stored for 1 week at 4°C.
- Enzyme diluent solution: Combine 10 ml of 10% Triton X-100 solution, 5 ml of 1 M Calcium Chloride solution, and 100ml of 1.0 M Tris-HCl buffer (pH 8.0) and dilute to 1 litre with deionized water. The pH of this solution should be 8.0.

Substrate solution

Weigh 1 g L-α-phosphatidylcholine from soybean (Sigma P-3644 or equivalent) into a small beaker. Add 0.16 ml 10% Triton X-100, 0.2 ml of 1 M calcium chloride and 19.64 ml of deionised water. Sonicate in an ice bath until completely suspended. Transfer 15 ml of the prepared solution to a volumetric flask. Dilute to 30 ml with 0.1 M Tris-HCl buffer (pH 8.0) prior to use. This solution should be prepared fresh.

Sample preparation

Dilute the enzyme to 0.5 - 1.0 U/ml using the Enzyme diluent solution.

Standard curve preparation

Prepare a standard curve as follows.

Dilute the oleic acid standard (from the NEFA-C kit) with deionised water, as shown in the table below.

Standard No.	Oleic acid, ml	Deionised water, ml	[Oleic acid], µmol/ml
1	1.00	0.00	1.0
2	0.80	0.20	0.8
3	0.60	0.40	0.6
4	0.40	0.60	0.4
5	0.20	0.80	0.2
6	0.00	1.00	0 (Blank)

1. Transfer 0.5 ml Reagent A to each of six test tubes (label as Reagent A 1-6, respectively). Incubate the tubes in a water bath held at 37°C for about 3 min.
2. Add 50 µl oleic acid Standard solution into the corresponding test tube (1-6) containing Reagent A. Incubate for an additional 10 min.
3. Add 1 ml Reagent B to each of the six test tubes from Step 2. Allow to react for not less than 10 min.
4. Remove the test tubes from the water bath and place in cold water immediately.
5. Measure the absorbance value at 550 nm and calculate the CF values and Average CF value (for standards numbered 1-5) according to the table below.

Standard No.	[Oleic Acid], µmol/ml	OD_{550}	($OD_{550\ Sample}$ - $OD_{550\ Blank}$)	CF= ($OD_{550\ Sample}$ − $OD_{550\ Blank}$) / [Oleic acid]	Average CF (ACF), Stds. 1-5
1	1.0				
2	0.8				
3	0.6				
4	0.4				
5	0.2				
6	0 (Blank)				

Procedure

1. For each enzyme sample tested, set up four test tubes; label two tubes as Enzyme Reagent A and label two tubes as Enzyme Blank Reagent A. Add 0.5 ml Reagent A to each tube, and incubate for not less than 7 min in a 37°C water bath.
2. Prepare four additional test tubes and label two tubes as Enzyme Sample and label two as Enzyme Blank Sample. Add 0.5 ml of Substrate solution into each of these four tests tubes; incubate for 5 min in a 37°C water bath.
3. Add 50 µl of Sample preparation to the two Enzyme Sample tubes and allow to react for 10 min in the water bath. After the 10 min reaction time, separately transfer 50 µl from each of the Enzyme Sample tubes

into the two prewarmed Enzyme Reagent A tubes from Step 1. Allow to react for 10 min in the water bath.

4. Separately transfer 50 µl from each of the Blank Sample tubes into the two prewarmed Blank Reagent A tubes in Step 1.
5. Add 1 ml of Reagent B to each of the Enzyme Reagent A and Blank Reagent A tubes and allow to react for 10 min in the water bath.
6. Remove the Enzyme Reagent A and Blank Reagent A test tubes from the water bath and place in cold water.
7. Measure the absorbance value of the solutions from Step 6 at 550 nm within 20 min.

Calculation

Calculate the activity of each sample, using the average absorbance values for Enzyme Reagent A (test sample) and Blank Reagent A (blank sample).

$$Phospholipase\ A2\ activity\ (U/g) = \frac{(OD550_{Test\ Sample} - OD550_{Blank\ Sample}) \times 0.55}{C \times 0.05 \times 10 \times ACF}$$

Where:

0.55 is the volume of the Substrate solution combined with the Sample preparation (ml)

C is the concentration of the Sample preparation (g/ml)

0.05 is the volume of the Sample preparation added to the tubes (ml)

10 is the reaction time (min)

ACF is the average CF for standard tubes 1-5 from the Standard preparation

RIBOFLAVIN FROM *ASHBYA GOSSYPII*

New specifications prepared at the 92nd JECFA (2021) and published in FAO JECFA Monographs 27 (2021). A group ADI of not specified for riboflavin from Bacillus subtilis, riboflavin from Ashbya gossypii, synthetic riboflavin and riboflavin-5-phosphate was established at the 92nd JECFA (2021).

SYNONYMS Riboflavin from *Eremothecium gossypii*, vitamin B2; lactoflavin.

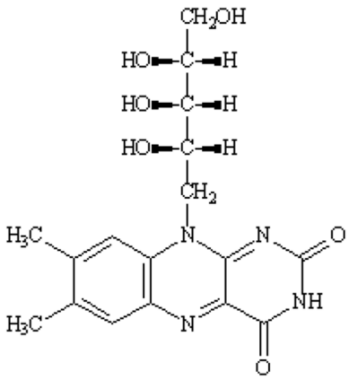
DEFINITION Prepared by fermentation with a non-pathogenic and non-toxicogenic self-cloned strain *of Ashbya gossypii* genetically modified for riboflavin overproduction. The fermentation is stopped by autolysis. Several filtration and precipitation/ crystallisation steps result in a dry product, containing not less than 98% of riboflavin, free of fermentation media components including the production organism.

Chemical names Riboflavin; 3,10-dihydro-7,8-dimethyl-10-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]benzo-[g]pteridine-2,4-dione; 7,8-dimethyl-10-(1'- D-ribityl)isoalloxazine.

C.A.S. number 83-88-5

Chemical formula $C_{17}H_{20}N_4O_6$

Structural formula



Formula weight 376.37

Assay Not less than 98.0% and not more than 101.0%, calculated on the dried basis.

DESCRIPTION Yellow to orange-yellow crystalline powder.

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol.4) Very slightly soluble in water; practically insoluble or insoluble in ethanol and acetone.

Spectrophotometry Using the aqueous solution from the Assay, determine the absorbance (A)

(Vol. 4)	at 267 nm, 375 nm and 444 nm. The ratio A_{375}/A_{267} is between 0.31 and 0.33. The ratio A_{444}/A_{267} is between 0.36 and 0.39.
Specific rotation	[alpha] 20, D: Between -115°C and -135°C Dry the sample at 100°C for 4 h. Dissolve 50.0 mg in 0.05 N sodium hydroxide (free from carbonate) and dilute to 10ml with the same solvent. Measure the optical rotation within 30 min of dissolution.
Colour reaction	Dissolve about 1 mg of sample in 100 ml of water. The pale greenish-yellow colour disappears on the addition of mineral acids and alkalis.

PURITY

Loss on drying (Vol.4)	Not more than 1.5% (105°C, 4 h).
Sulfated ash (Vol.4)	Not more than 0.1% Test 2 g of the sample.
Impurities	Lumiflavin: not more than 0.025% See description under TESTS.
Lead (Vol.4)	Not more than 1mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on principles of methods described in Volume 4 (under "General Methods, Metallic Impurities").

TESTS

PURITY TESTS

Impurities

Impurities are determined by liquid chromatography (Vol. 4)

[Note: The following procedure may be used to determine: lumiflavin (impurity A), lumichrome (impurity B), 6,7-dimethyl-8-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]pteridine-2,4(3H,8H)-dione (impurity C), 8-hydroxymethyl riboflavin (impurity D), unspecified impurities and total organic impurities. However, the only requirement for this specification is the determination of lumiflavin.]

Standards and Reagents:

- Riboflavin for peak identification certified reference standard (CRS) (containing impurities C and D), Ph.Eur. (Cat. Code: Y0000757).
- Sodium acetate trihydrate (CAS # 6131-90-4)
- Orthophosphoric acid
- Sodium hydroxide solution (0.5 M and 0.1 M)
- Acetic acid, 30%w/v
- Acetonitrile, HPLC grade with transmittance more than 95% at 210 nm
- Deionised water, HPLC grade

Preparation of solutions**:** (Note: Riboflavin is light sensitive. Prepare the solutions immediately before use and protect from light.)

Solution A: Weigh 13.6 g of sodium acetate trihydrate, dissolve in deionized water and make up to volume in a 1 litre volumetric flask.

Test solution: Accurately weigh 0.12 g of sample into a 100 ml volumetric flask, add 10 ml of 0.1 M sodium hydroxide solution, ultrasonicate to dissolve the sample and dilute to 100 ml with Solution A.

Reference solution A: Dilute 1.0 ml of the test solution to 10 ml with solution A. Dilute 1.0 ml of this solution to 100 ml with Solution A.

Reference solution B: Dissolve the contents of a vial of riboflavin peak identification certified reference standard in 1 ml of a mixture of 1 volume of Mobile phase B and 9 volumes of Mobile phase A.

Reference solution C (For impurities A and B): Dissolve 10 mg of sample in 1 ml of 0.5 M sodium hydroxide. Expose to daylight for 1.5 h. Add 0.5 ml of 30 % w/v acetic acid and dilute to 100 ml with deionised water.

Procedure:

Use a HPLC consisting of a high precision binary pump and an autosampler

Column: Octadecylsilyl, end-capped (25-cm x 4.6-mm x 5-µm).

Flow rate: 1.0 ml/min

Detector: UV/Diode array, 267 nm

Injection volume: 10 µl

Gradient conditions:

Mobile phase:

- A: Orthophosphoric acid in deionised water (1:1000 v/v)
- B: Acetonitrile

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0-5	90	10
5-20	90 → 80	10 → 20
20-25	80	20
25-35	80 → 50	20 → 50
35-45	50	50

Identification of impurities: Use the chromatogram supplied with riboflavin for peak identification CRS and the chromatogram obtained with Reference solution B to identify the retention times of impurities C and D.

Inject the Test solution, Reference solution A, Reference solution B, and Reference solution C and calculate the relative retention times (RRT) with respect to riboflavin (retention time = about 16 min), approximately: Impurity C = 0.2; impurity D = 0.5; impurity A = 1.4; impurity B = 1.9.

System suitability:

Resolution: Minimum 5 between the peaks due to impurities A and B in the chromatogram obtained with Reference solution C.

The chromatogram obtained with Reference solution B is similar to the chromatogram supplied with riboflavin for peak identification CRS.

Correction factors for calculation of impurities:

Multiply the peak areas in the chromatogram of the Test solution corresponding to the following impurities by the appropriate correction factor: lumiflavin, impurity A = 0.7.

[Note: For information only - if calculation of other impurities is desired, the following correction factors may be used: impurity B = 1.4; impurity C = 2.3; impurity D = 1.4.]

Use corrected peak area to determine the content of lumiflavin in the sample (max 0.025%): The corrected peak area for lumiflavin in the chromatogram of the Test solution is less than 0.25 times the area of the principal peak in the chromatogram obtained with Reference solution A.

METHOD OF ASSAY

Carry out the assay in subdued light. Accurately weigh and suspend 65.0 mg of the sample in 5 ml of water, ensuring that it is completely wet, in an amber coloured 500 ml volumetric flask. Add 5 ml of 2 N sodium hydroxide solution and dissolve. As soon as dissolution is complete, add 100 ml of water and 2.5 ml of glacial acetic acid and dilute to 500ml with water. Place 20 ml of this solution in an amber coloured 200 ml volumetric flask, add 3.5 ml of a 1.4% w/v solution of sodium acetate and dilute to 200 ml with water. Measure the absorbance (A) of the solution at 444 nm.

$$\%Riboflavin = \frac{A \times 5000}{328 \times W}$$

A: absorbance of the sample solution at 444 nm
W: weight of sample, g
328: Specific absorbance of riboflavin at 444 nm

RIBONUCLEASE P FROM *PENICILLIUM CITRINUM*

New specifications prepared at the 92nd JECFA (2021) and published in FAO JECFA Monographs 27 (2021). An ADI of "not specified" was established at the 92nd JECFA (2021).

SYNONYM RNase P

SOURCES Produced by controlled fermentation of a pure culture of non-pathogenic, non-toxigenic strains of *Penicillium citrinum*, either by solid– or liquid–state fermentation techniques. The enzyme is secreted by the microbial cells and is subsequently separated from the resulting cell biomass using a series of filtration steps. The liquid enzyme concentrate is spray dried with food grade dextrin to produce the commercial enzyme preparation.

Active principles: Endonuclease

Systematic names and numbers: Ribonuclease P; EC 3.1.26.5;
CAS No. 71427-00-4

Reaction catalysed: Endonucleolytic cleavage of RNA, removing 5'-nucleotides

Secondary enzyme activities: No significant levels of secondary activities

DESCRIPTION White to dark brown powder

FUNCTIONAL USES Enzyme preparation
Used in the production of processed yeast products and flavouring substances and preparations with naturally occurring RNA.

GENERAL SPECIFICATIONS Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Ribonuclease P activity: The sample shows ribonuclease P activity.
See description under TESTS.

TESTS

METHOD OF ASSAY

Ribonuclease P activity

Principle

Ribonuclease P activity is determined by measuring the production of phosphate that results from the hydrolysis of adenosine 3'-monophosphate, used as a substrate. After a 15-minute reaction, the absorbance of the reaction mixture is measured spectrophotometrically at 750 nm, enabling

the determination of the ribonuclease P activity based on comparison to a disodium hydrogen phosphate standard curve.

One unit of ribonuclease P activity is defined as the quantity of enzyme required to liberate 1 µmol of phosphate (as phosphoric acid) in 1 min at the specified conditions.

Apparatus
Water bath with circulation
UV-Vis spectrophotometer
pH meter
Vortex mixer

Reagents and solutions

- Amidol solution: Dissolve 0.50 g of 2,4-diaminophenol dihydrochloride (Amidol; CAS No. 137-09-47) and 10.0 g of sodium hydrogen sulfite in about 25 ml of deionised water; dilute to 50 ml with deionised water. Filter through medium-porosity filter paper (Toyo Roshi Kaisya, Ltd. grade No.2).
- 1 N Hydrochloric acid: Dilute 98.5 ml of hydrochloric acid (37%) to 1 litre with deionised water.
- 8.5% Sodium chloride solution: Dilute 8.5 g of sodium chloride to 100 ml with deionised water.
- 6% Perchloric acid solution: Dilute 20 ml of perchloric acid (60%) to 200 ml with deionised water.
- Sodium barbital - sodium acetate solution: Dissolve 5.88 g of sodium barbital (sodium 5,5-diethylbarbiturate;
  CAS No. 144-02-5) and 2.34 g of sodium acetate in 100 ml of deionised water; dilute to 200 ml with deionised water.
- Sodium barbital buffer (pH 5.0): Combine 100 ml of Sodium barbital-sodium acetate solution with 40 ml of 8.5% Sodium chloride solution and 200 ml of deionised water. Adjust to pH 5.0 with 1 N Hydrochloric acid. Dilute to 500 ml with deionised water.
- Ammonium heptamolybdate solution:
  Dissolve 8.30 g of ammonium heptamolybdate tetrahydrate (CAS No. 12054-85-2) in about 70 ml of deionised water; dilute to 100 ml with deionised water.
- 0.01 M Disodium hydrogen phosphate standard solution: Dissolve 0.142 g of disodium hydrogen phosphate (CAS No. 7558-79-4) reference material in deionised water; dilute to 100 ml with deionised water (Standard stock solution).

Substrate solution
Dissolve 20 mg of adenosine 3'-monophosphate (CAS No. 84-21-9) in 10 ml of Sodium barbital buffer (pH 5.0). Filter through a PVDF membrane filter (Whatman GD/X PVDF, 0.45 µm).

Sample preparation
Prepare the sample enzyme solution at a dilution expected to catalyse the release of phosphoric acid within the range of the standard curve under the conditions prescribed. Dissolve an accurately weighed amount of ribonuclease P in deionised water. Prepare serial dilutions as necessary.

Standard curve preparation

Prepare a standard curve as follows.

Set up five 100-ml volumetric flasks and label them Standards 1-5. Dilute aliquots of the Standard stock solution with deionised water as shown in the table below.

Standard No.	Standard stock solution (ml)	[Phosphate] in the volumetric flask, µmol/ml	[Phosphate] in the test tube, µmol/ml
1	1.0	0.1	0.0098
2	5.0	0.5	0.049
3	10.0	1.0	0.098
4	15.0	1.5	0.147
5	20.0	2.0	0.196

1. Transfer 0.5 ml of each of the Standard solutions to individual test tubes.
2. Add 4 ml of 6% Perchloric acid solution to each tube and mix.
3. Add 0.4 ml of the Amidol solution to each tube and mix.
4. Add 0.2 ml of Ammonium heptamolybdate solution to each tube and mix.
5. Measure the absorbance of each solution at 750 nm.
6. Create a standard blank in a sixth test tube in the same manner, using 0.5 ml of water instead of a Standard solution. Subtract the absorbance of the standard blank from the absorbance of each of the Standard solutions. Plot the absorbance of the solutions against the concentration of phosphate (µmol/ml) in the test tube. Determine the slope and y-intercept of the standard curve.

Procedure

For each Sample preparation to be analysed, test in duplicate as follows.

1. Transfer 0.4 ml of the Substrate solution to each of two test tubes and place tubes in a water bath maintained at 70 °C ± 0.5 °C for 5 min.
2. Add 0.1 ml of the Sample preparation to each tube and mix. Incubate the tubes in the water bath at 70°±0.5° for exactly 15 min.
3. At the end of the 15 min reaction period, add 4 ml of 6% Perchloric acid solution to the tubes and mix.
4. Add 0.4 ml of Amidol solution and mix.
5. Add 0.2 ml of Ammonium heptamolybdate solution and mix.
6. Cool the tubes to room temperature and immediately measure the absorbance at 750 nm (Reaction solution).
7. For each Sample preparation to be analysed, prepare duplicate Enzyme blank solutions as follows. Transfer 0.4 ml of the Substrate solution to each of two test tubes, add 4 ml of 6% Perchloric acid solution to the tubes and mix. Add 0.1 ml of the Sample preparation and mix. Add 0.4 ml of Amidol solution and mix. Add 0.2 ml of Ammonium heptamolybdate solution and mix. Measure the absorbance at 750 nm.

Calculation

Calculate the activity of each sample in U/g, using the average absorbance values for the Reaction solution and the Enzyme blank solution:

$$\text{Ribonuclease P (U/g)} = \frac{[(A_1 - A_2) - b]}{m} x \left[\frac{5.1}{(C\ x\ 0.1\ x\ 15)}\right]$$

Where

A_1 is the absorbance of the Reaction solution.
A_2 is the absorbance of the Enzyme blank solution.
b is the y-intercept of the standard curve.
m is the slope of the standard curve.
5.1 is the total volume of the Reaction solution (ml).
0.1 is the volume of Sample preparation in the Reaction solution (ml).
15 is the reaction time (min).
C is the concentration of enzyme in the Sample preparation (g/ml).

MODIFIED STARCHES

Prepared at the 92nd JECFA (2021) and published in FAO JECFA Monograph 27 (2021), superseding specifications prepared at the 86th JECFA (2018) and published in FAO JECFA Monographs 22 (2018).

MODULAR MONOGRAPH consisting of "GENERAL SPECIFICATIONS"[(a)] that contains common specifications to all modified starches (INS 1400, 1401, 1402, 1403, 1404, 1405, 1410, 1412, 1413, 1414, 1420, 1422, 1440, 1442, 1450, 1451), and 8 ANNEXES that contain specifications related to the chemical treatments of native starches:

ANNEX 1[(a)]–Fragmentation
ANNEX 2[(a)]–Bleaching
ANNEX 3[(a)]–Esterification and/or crosslinking with phosphorus containing compounds
ANNEX 4[(a)]–Acetylation
ANNEX 5[(a)]–Oxidation
ANNEX 6[(a)]–Esterification with octenylsuccinic anhydride
ANNEX 7[(a)]–Etherification with propylene epoxide
ANNEX 8[(a)]–Esterification and crosslinking with adipic anhydride

The General specifications are applicable to the following modified starches, each of which should additionally fulfill the specifications of the ANNEXES as follows:

Modified Starch	INS	Annex	ADI	STATUS
Dextrin roasted starch	1400	1	N.S.[(1)]	F[(3)]
Acid treated starch	1401	1	N.S.[(1)]	F[(3)]
Alkaline treated starch	1402	1	N.S.[(1)]	F[(3)]
Bleached starch	1403	2	N.S.[(1)]	F[(3)]
Oxidized starch	1404	5	N.S.[(1)]	F[(3)]
Enzyme-treated starch	1405	1	N.S.[(1)]	F[(3)]
Monostarch phosphate	1410	3	N.S.[(1)]	F[(3)]
Distarch phosphate	1412	3	N.S.[(1)]	F[(3)]
Phosphateddistarch phosphate	1413	3	N.S.[(1)]	F[(3)]
Acetylated distarch phosphate	1414	3, 4	N.S.[(1)]	F[(3)]
Starch acetate	1420	4	N.S.[(1)]	F[(3)]
Acetylated distarchadipate	1422	4, 8	N.S.[(1)]	F[(3)]
Hydroxypropyl starch	1440	7	N.S.[(1)]	F[(3)]
Hydroxypropyldistarch phosphate	1442	3, 7	N.S.[(1)]	F[(3)]
Starch sodium octenylsuccinate	1450	6	N.S.[(1)]	F[(3)]
Acetylated oxidized starch	1451	4, 5	N.S.[(2)]	F[(3)]

Should any of the modified starches be subjected to additional chemical treatment, the appropriate specifications outlined in the respective ANNEX should be met. Consequently, for all starches subject to additional fragmentation or bleaching the specifications of ANNEXES 1 and/or 2 respectively should be met.

[(a)]Prepared at the 92nd JECFA (2021) and published in FAO JECFA Monograph 27 (2021), superseding specifications prepared at the 86th JECFA (2018), published in FAO JECFA Monographs 22 (2018).

[(1)]An ADI "not specified" was established at the 26th JECFA (1982).

[(2)]An ADI "not specified" was established at the 57th JECFA (2001).

[(3)]F: FULL

Summary Table							
GENERAL REQUIREMENTS							
IDENTIFICATION				**PURITY**			
Solubility	**Microscopy**	**Iodine Stain**	**Copper Reduction**	**Loss on Drying**	**Lead**	**Microbiological Criteria**	**Sulfur dioxide**
Insoluble in cold water	Granular structure typical of the starch source	Colour from dark blue to orange-red after addition of iodine TS	Red precipitate after addition of hot alkaline cupric tartrate to a test sample refluxed under acidic condition	Cereal starch ≤15.0%; Potato starch: ≤21.0%; Other starches: ≤18.0%	≤0.2mg/kg d.w. Pb (≤0.1 mg/kg) for starch sodium octenylsuccinate for infant formula	Aerobic Plate Count: ≤100,000 CFU/g; Yeasts and molds: ≤1,000 CFU/g; Total Coliforms: ≤100 CFU/g;	Modified cereal starches: ≤50 mg/kg d.w.; Other modified starches ≤10 mg/kg d.w.
SPECIFIC REQUIREMENTS							
Modified Starch	Annex	**IDENTIFICATION**		**PURITY**			
Dextrin roasted starch (INS 1400)	1	Dispersion test		No additional			
Acid treated starch (INS 1401)	1	Dispersion test		No additional			
Alkaline treated starch (INS 1402)	1	Dispersion test		No additional			
Bleached starch (INS 1403)	2	No additional		Carboxyl groups (≤0.1% d.w.); Residual oxidizing substances < 180 mg/kg calculated as H_2O_2			
Oxidized starch (INS 1404)	5	Hypochlorite oxidized starch		Carboxyl groups (≤1.3% d.w.); Residual oxidizing substances < 180 mg/kg calculated as H_2O_2			
Enzyme-treated starch (INS1405)	1	Dispersion index (Information Required); Reducing sugars (Information Required)		No additional			
Monostarch phosphate (INS 1410)	3	Phosphate groups		Phosphate (≤0.5% d.w. for potato or wheat starches; ≤0.4% d.w for other starches)			
Distarch phosphate (INS 1412)	3	Crosslinking		Phosphate (≤0.5% d.w. for potato or wheat starches; ≤0.4% d.w. for other starches)			
Phosphated distarch phosphate (INS 1413)	3	Crosslinking		Phosphate (≤0.5% d.w. for potato or wheat starches; ≤0.4% d.w. for other starches)			
Acetylated distarch phosphate (INS 1414)	3, 4	Acetyl group; Ester group; Crosslinking		Phosphate (≤0.14% d.w. for potato or wheat starches; ≤0.04% d.w. for other starches) Acetyl groups (≤2.5% d.w.); Ester groups (≤0.5% d.w.)			
Starch acetate (INS 1420)	4	Acetyl group; Ester group		Acetyl groups (≤2.5% d.w.); Ester groups (≤0.5% d.w.)			
Acetylated distarch adipate (INS 1422)	4, 8	Acetyl group; Ester group; Crosslinking		Acetyl groups (≤2.5% d.w.); Vinyl acetate (≤0.1 mg/kg); Ester groups (≤0.5%d.w.) Adipate groups (≤0.135% d.w.); Residual free adipic acid (≤0.025% d.w.)			
Hydroxypropyl starch (INS 1440)	7	Hydroxypropyl ether groups		Hydroxypropyl groups (≤7.0% d.w.); Propylene chlorohydrins (≤1 mg/kg d.w.)			
Hydroxypropyl distarch phosphate (INS 1442)	3, 7	Hydroxypropyl ether groups; Crosslinking		Phosphate (≤0.14% d.w. for potato or wheat starches; ≤0.04% d.w. for other starches) Hydroxypropyl groups (≤7.0% d.w.); Propylene chlorohydrins (≤1 mg/kg d.w.)			
Starch sodium octenylsuccinate (INS 1450)	6	No additional		Octenylsuccinyl groups (≤3% d.w.); Resiidual free octenylsuccinic acid (≤0.3% d.w.);			
Acetylated oxidized starch (INS 1451)	4, 5	Acetyl group		Acetyl groups (≤2.5% d.w.); Vinyl acetate (≤0.1 mg/kg); Ester groups (≤ 0.5% d.w.) Carboxyl groups (≤1.3% d.w.); Residual oxidizing substances < 180 mg/kg calculated as H_2O_2			

GENERAL SPECIFICATIONS FOR MODIFIED STARCHES

(VERSION 2021)

DEFINITION

Starch consists mainly of amylose and/or amylopectin which contain various amounts of naturally bound phosphate esters and counter ions. Amylose is a predominantly linear molecule of α-D-glucopyranosyl units linked by (1-4)-α-linkages. Amylopectin is a highly-branched polymer of α-D-glucopyranosyl units linked by (1-4)-α-linkagesand by (1-6)-α-linkages that constitute the branch points. Each glucose unit possesses a maximum of three hydroxyls that can undergo chemical substitution.

Native starches can be physically and/or chemically modified for improved functionality. The most common sources of native starch used in these modifications are various roots, tubers, cereals and legumes. Modified starches are used in applications requiring special properties not manifested by native starches.

Chemical modifications of food starches are often performed, in an aqueous suspension under controlled conditions of pH, time and temperature, unless otherwise indicated in the description of the respective Annex. After sufficient reaction time, the modified starch is recovered by filtration or centrifugation, washed with water, dried and packaged. The relevant modification reactions can be, separately or in combination, fragmentations (hydrolysis, oxidation, enzymatic), bleaching, oxidation, esterification, etherification or phosphorylation of one or more of the hydroxyl groups of the α-D-glucopyranosyl units or crosslinking using polyfunctional agents.

See the appropriate Annex or Annexes for the treatment that is applicable to individual modified starch products.

C.A.S numbers — See ANNEXES

DESCRIPTION — White or nearly white powder or granules or (if pre-gelatinized) flakes, or amorphous powder or coarse particles.

FUNCTIONAL USES — Thickener, stabilizer, binder, emulsifier

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) — Insoluble in cold water (if not pre-gelatinised); form typical colloidal solutions with viscous properties in hot water; insoluble in ethanol.

Microscopy — Passes test
See description under TESTS

Iodine stain — Passes test
See description under TESTS

Copper reduction — Passes test

See description under TESTS

PURITY

Loss on drying

Cereal starch: not more than 15.0%
Potato starch: not more than 21.0%
Other starches: not more than 18.0%
(120 °C, 4 h, vacuum not exceeding 100 mm Hg)

Lead (Vol. 4)

Not more than 0.2 mg/kg calculated on the dried basis

Not more than 0.1 mg/kg calculated on the dried basis for Starch sodium octenylsuccinate (INS 1450) for use in infant formula and formula for special medical purposes intended for infants (see Annex 6)

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

Microbiological criteria (Vol. 4)

Aerobic plate count: Not more than 100 000 CFU/g
Yeasts and molds: Not more than 1 000 CFU/g
Total coliforms: Not more than 100 CFU/g

Sulfur dioxide (Vol. 4)

Not more than 50 mg/kg calculated on the dried basis for modified cereal starches
Not more than 10 mg/kg calculated on the dried basis for other modified starches

TESTS

IDENTITY TESTS

Microscopy

Modified starches, unless pre-gelatinized, retain their granular structure and can be identified by microscopic observation. The typical Maltese cross is observed when the sample is examined in polarized light under crossed Nicol prisms.

Corn starch: Polygonal, rounded or spherical granules up to 35 µm diameter having a circular or several-rayed central cleft.

Potato starch: Irregular shaped, ovoid, and pear-shaped granules (approximately 30-100 µm diameter, occasionally >100 µm); both, the ovoid, the pear-shaped granules and the rounded granules have an eccentric hilum. All granules show clearly visible concentric striations.

Tapioca starch: Spherical granules with one truncated side (5–35 µm diameter) usually having a circular or several-rayed central cleft.

Wheat starch: large and small granules (10–60 µm diameter). The central hilum and striations are visible and barely visible.

Rice starch: polyhedral in shape and generally 3 to 15 µm in diameter.

Iodine stain	Add a few drops of Iodine TS to an aqueous suspension of the sample. The colour will range from dark blue to orange-red.
Copper reduction	Place about 2.5 g of a sample previously washed with water, in a boiling flask; add 10 ml of dilute hydrochloric acid (3%) and 70 ml of water; mix, reflux for about three hours and cool. Add 0.5 ml of the resulting solution to 5 ml of hot alkaline cupric tartrate TS. A copious red precipitate is produced.

ANNEX 1: ADDITIONAL SPECIFICATIONS FOR STARCHES MODIFIED BY FRAGMENTATION

(VERSION 2021)

APPLIES TO

Dextrin roasted starch (INS No. 1400)
Acid treated starch (INS No. 1401)
Alkaline treated starch (INS No. 1402)
Enzyme-treated starch (INS No. 1405)
All modified starches that are fragmented

SYNONYMS

Modified starch by fragmentation, converted starch, hydrolysed starch.

TREATMENT

The fragmentation of food starch results in products containing polymers with a lower average molecular weight and reduced viscosity.

a) Dextrin roasted starch (INS. 1400) is obtained by dry heating or roasting starch with hydrochloric acid or ortho-phosphoric acid.
b) Acid treated starch (INS. 1401) is obtained by treating a slurry or a suspension of starch with dilute hydrochloric acid, ortho-phosphoric acid, or sulfuric acid.
c) Alkaline treated starch (INS. 1402) is obtained by treating a suspension of starch with sodium hydroxide or potassium hydroxide.
d) Enzyme-treated starch (INS 1405) is obtained by treating a dispersion of starch with one or more food-grade amyolytic-enzymes.

The properties of the modified starches obtained by fragmentation vary depending on the source of the starch, reaction conditions (pH, time, temperature, reagents etc.) The alteration of the starch allows for applications that require reduced viscosity in hot solutions and/or typically utilise high levels of modified starches.

C.A.S number

9004-53-9 (Dextrins)
65996-63-6 (Starch, acid-hydrolysed)
68909-37-5 (Amylopectin, acid-hydrolysed)
9005-84-9 (Amylodextrin)
65996-64-7 (Starch, enzyme-hydrolysed)
1001439-91-3 (Amylopectin, enzyme-treated)

CHARACTERISTICS

IDENTIFICATION

Dispersion identity

Passes test.
See description under TESTS.

TESTS

IDENTITY TESTS

Dispersion test

Principle

The flow properties of an alkaline solution of the sample are evaluated visually. Fragmentation results in dramatic reduction in viscosity.

Reagents and Solutions

- 5 N potassium hydroxide: weigh 330 g of potassium hydroxide (purity 85%), dissolve by adding gradually to water and dilute to 1 litre.

Procedure

Suspend 12 g of sample in 50 ml of water and mix well. Add gradually 50 ml of 5 N potassium hydroxide with continued stirring. Observe the viscosity and texture of the dispersion using a handheld stir rod. The dispersion is flowable to water-thin (see Figure 1).

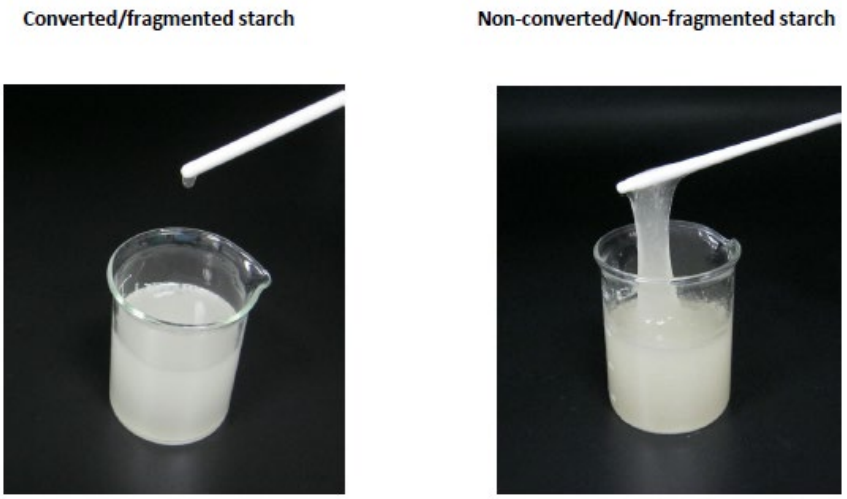


Figure 1: Characteristic pictures of dispersion test results for fragmented and non-fragmented starches.

ANNEX 2: ADDITIONAL SPECIFICATIONS FOR BLEACHED STARCHES

(VERSION 2021)

APPLIES TO

Bleached starch (INS No. 1403)
All modified starches that are bleached

TREATMENT

Peracetic acid and/or hydrogen peroxide, or sodium hypochlorite, sodium chlorite, sulphur dioxide, alternative permitted forms of sulphites, potassium permanganate, or ammonium persulfate

Bleaching is performed to improve appearance of the starch. Colour due to oxidation of traces of pigments such as phenolic compounds, carotenoids and xanthophylls, is removed. The process is intended to change only the colour. Residual reagents are either removed or limited to technically unavoidable levels.

CHARACTERISTICS

PURITY

Manganese (Vol. 4)

Not more than 50 mg/kg calculated on the dried basis

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

Residual oxidizing substances

Not more than 180 mg/kg, calculated as hydrogen peroxide.
See description under TESTS

Carboxyl groups (Vol. 4)

Not more than 0.1% calculated on the dried basis applying the correction for phosphate content as outlined in Note 6 of the method for starches containing phosphate such as potato starches, wheat starches and starches esterified with phosphorus containing compounds.

TESTS

PURITY TESTS

Residual oxidizing substances

Principle

Residual oxidizing substances are extracted in water and titrated with sodium thiosulfate in the presence of iodine and starch as indicator.

Reagents and solutions

- 0.002 N sodium thiosulfate: Prepare using Sodium thiosulfate TS as instructed for dilute solutions that are not stable (Vol. 4, Preparation and Methods of Standardization).
- Starch solution: Mix 1 g of soluble starch with 10 mg of red mercuric iodide and sufficient cold water to make a thin paste. Add 200 ml of boiling water, and boil for 1 minute with continuous stirring. Cool, and use only the clear solution. [NOTE: Commercially available, stabilized

starch indicator solutions may be used, including mercury-free solutions preserved with other compounds such as salicylic acid.]

Procedure

Weigh 4.0 g of sample to a glass-stoppered, 125 ml conical flask, and add 50.0 ml of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50 ml centrifuge tube, and centrifuge to clarify. Transfer 30.0 ml of the clear supernatant to a glass-stoppered, 125 ml conical flask. Add 1 ml of glacial acetic acid and 0.5 – 1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25 to 30 min in the dark. Add 1 ml of Starch solution and titrate with 0.002 N sodium thiosulfate to the disappearance of the starch-iodine colour. Prepare a blank using 30.0 ml water.

Calculate the residual oxidizing substances in mg/kg using the formula:

$$\text{Residual oxidizing substances} = \frac{V \times 34}{m} \times \frac{50}{30}$$

Where:
V is the volume of 0.002 N sodium thiosulfate necessary for the titration of the sample, corrected for the volume used in the blank determination if necessary (ml).
m is the mass of sample (g).

Each ml of 0.002 N sodium thiosulfate is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide. *Portions of this monograph are reproduced from USP-NF, with permission from The United States Pharmacopeial Convention.

ANNEX 3: ADDITIONAL SPECIFICATIONS FOR STARCHES ESTERIFIED AND/OR CROSSLINKED WITH PHOSPHORUS CONTAINING COMPOUNDS

(VERSION 2021)

APPLIES TO

Monostarch phosphate (INS No. 1410)
Distarch phosphate (INS No. 1412)
Phosphated distarch phosphate (INS No. 1413)
Acetylated distarch phosphate (INS No. 1414)
Hydroxypropyl distarch phosphate (INS No. 1442)

TREATMENT

The phosphorus containing compounds ortho-phosphoric acid, sodium or potassium ortho-phosphate and sodium tripolyphosphate, can be used for esterification and sodium trimetaphosphate or phosphorus oxychloride for crosslinking.

- Monostarch phosphate (INS 1410) is obtained by esterification of starch with ortho-phosphoric acid, or sodium or potassium ortho-phosphate, or sodium tripolyphosphate.
- Distarch phosphate (INS 1412) is obtained by crosslinking of starch with sodium trimetaphosphate or phosphorus oxychloride.
- Phosphated distarch phosphate (INS 1413) is obtained by crosslinking of starch with sodium trimetaphosphate or phosphorus oxychloride combined with esterification with ortho-phosphoric acid, or sodium or potassium ortho-phosphate, or sodium tripolyphosphate.
- Acetylated distarch phosphate (INS 1414) is obtained by crosslinking of starch with sodium trimetaphosphate or phosphorus oxychloride combined with esterification with acetic anhydride or vinyl acetate.
- Hydroxypropyl distarch phosphate (INS 1442) is obtained by crosslinking of starch with sodium trimetaphosphate or phosphorus oxychloride combined with etherification by propylene oxide.

Phosphorylation results in partial substitution of the 2, 3- or 6- position of the anhydro glucose unit unless the 6-position is occupied for branching. In the case of crosslinking, where a polyfunctional substituting agent, such as phosphorus oxychloride connects two chains, the structure can be represented by: Starch-O-R-O-Starch, where R = cross-linking group and starch refers to the linear and/or branched structure.

C.A.S numbers

Monostarch phosphate
11120-02-8(Starch, phosphate)
63055-37-8 (Amylopectin, phosphate)

Distarch phosphate
55963-33-2(Distarch phosphate)
63055-37-8 (Amylopectin, phosphate)

Phosphated distarch phosphate
11120-02-8 (Starch, phosphate)
63055-37-8 (Amylopectin, phosphate)

Acetylated distarch phosphate
9067-33-8 (Starch, acetate, phosphate)

68130-14-3 (Acetylated distarch phosphate)
113894-91-0 (Amylopectin, acetate, phosphate)

Hydroxypropyl distarch phosphate
53124-00-8 (Hydroxypropyl distarch phosphate)
113894-92-1 (Hydroxypropyl amylopectin phosphate)

CHARACTERISTICS

IDENTIFICATION

Crosslinking — Passes test (does not apply to monostarch phosphate)
See description under TESTS.

Phosphate groups — Passes test (applies to monostarch phosphate)
See description under TESTS.

PURITY

Phosphate (calculated as phosphorus) (Vol. 4) — For monostarch phosphate (INS No. 1410), distarch phosphate (INS No. 1412), and phosphate distarch phosphate (INS No. 1413)

Not more than 0.5% calculated on the dried basis for potato or wheat starches
Not more than 0.4% calculated on the dried basis for other starches

For acetylated distarch phosphate (INS No. 1414) and hydroxypropyl distarch phosphate (INS No. 1442)

Not more than 0.14% calculated on the dried basis for potato and wheat starch
Not more than 0.04% calculated on the dried basis for other starches

TESTS

IDENTITY TESTS

Crosslinking

Principle
Granular starch is dispersed in a zinc chloride and ammonium chloride solution. While the salts aid in the dispersion of non-cross-linked granules, cross-linked granules resist full dispersion and show sedimentation at low concentration after 24 hours. Non-cross-linked starches should disperse and stay suspended. Monostarch phosphate is not cross-linked and therefore is not identified with this test.

Reagents and solutions
- Solution A: Combine 10 parts of zinc chloride ($ZnCl_2$), 26 parts of ammonium chloride (NH_4Cl) and 64 parts of deionised water by weight.

Procedure
Weigh 1.0 g of sample into a 150 ml Erlenmeyer flask and add 100 ml of Solution A. Heat the mixture in a boiling water bath for 10 min, stirring constantly. Cool to room temperature and transfer the entire mixture to a

100 ml graduated cylinder. After 24 hours distinct sedimentation is observed.

[Note: The sediment layer can range from dense and opaque to very slightly hazy. This test is not appropriate for high amylose starches, since most high amylose starches will give sediment when they are not cross-linked. Regular dent corn can also give false positive tests with a small clear layer on top, similar to very lightly cross-linked starches.]

Phosphate groups

Principle

Because of the phosphate group content, monostarch phosphate has anionic properties. It can be dyed with positively charged dyes such as methylene blue. The test is not specific to the phosphate groups.

Procedure

A 50 mg sample is kept in suspension for 5-10 min in 25 ml of a Methylene Blue TS, Diluted solution and stirred occasionally. After decantation of the excess solution, the starch is washed with distilled water. Microscopic inspection clearly shows colouring.

ANNEX 4: ADDITIONAL SPECIFICATIONS FOR ACETYLATED STARCHES

(VERSION 2021)

APPLIES TO

Acetylated distarch phosphate (INS No. 1414)
Starch acetate (INS No. 1420)
Acetylated distarch adipate (INS No. 1422)
Acetylated oxidized starch (INS No. 1451)

TREATMENT

This type of modified starch is obtained by esterification with acetic anhydride or vinyl acetate. Acetylation results in substitution of hydroxyl groups with acetyl esters.

Acetylated distarch phosphate (INS 1414) is obtained by reacting starch with sodium trimetaphosphate or phosphorus oxychloride and esterifying with acetic anhydride or vinyl acetate.
Starch acetate (INS 1420) is obtained by esterification of starches with acetic anhydride or vinyl acetate.
Acetylated distarch adipate (INS 1422) is obtained by esterification of starch with acetic anhydride and esterification/cross-linking with adipic anhydride.
Acetylated oxidized starch (INS 1451) is obtained by treatment of starch with sodium hypochlorite followed by esterification with acetic anhydride.

C.A.S numbers

Acetylated distarch phosphate
9067-33-8 (Starch, acetate phosphate)
68130-14-3 (Acetylated distarch phosphate)
113894-91-0 (Amylopectin, acetate phosphate)

Starch acetate
9045-28-7 (Starch acetate)
601464-73-0 (Amylopectin, acetate)

Acetylated distarch adipate
63798-35-6 (Acetylated distarch adipate)
63055-36-7 (Amylopectin, acetate hexanedioate)

Acetylated oxidized starch
68187-08-6 (Starch, acetate, oxidised)

CHARACTERISTICS

IDENTIFICATION

Specific reaction for Acetyl groups

Passes test
See description under TESTS.

Ester groups

Passes test
See description under TESTS.

PURITY

Acetyl groups

Not more than 2.5% calculated on the dried basis

See description under TESTS.

Vinyl acetate — Not more than 0.1 mg/kg
See description under TESTS.

TESTS

IDENTITY TESTS

Specific reaction for acetyl groups

Principle
Acetate is liberated upon saponification of acetylated starchand converted to acetone by heating with calcium hydroxide. The acetone thus produced stains blue with *o*-nitrobenzaldehyde.

Procedure
Suspend about 10 g of the sample in 25 ml water. Add 20 ml of 0.4 M NaOH. After shaking for 1 h filter the starch off and evaporate the filtrate in an oven at 110 °C. Dissolve the residue in a few drops of water and transfer to a test tube. Add calcium hydroxide and heat the tube. If the sample is acetylated starch, acetone vapours are produced. These produce a blue colour on a paper strip soaked in a fresh saturated solution of *o*-nitrobenzaldehyde in 2 M NaOH. The blue colour is more distinct when the original yellow colour of the reagents is removed with 1 drop of a 1 in 10 solution of hydrochloric acid.

Ester groups — The infrared spectrum of a thin film gives a typical absorption band at about 1720 cm^{-1} which is an indication for ester groups. The limit of detection is about 0.5% acetyl groups in the product.

PURITY TESTS

Acetyl groups — Accurately weigh about 5 g of the sample and transfer into a 250 ml conical flask. Suspend in 50 ml of water, add a few drops of phenolphthalein TS, and titrate with 0.1 M sodium hydroxide to a permanent pink end-point. Add 25.0 ml of 0.45 M sodium hydroxide, stopper the flask, and shake vigorously for 30 min, preferably with a mechanical shaker. [NOTE: The temperature should not exceed 30 °C as some starches may gelatinise]. Remove the stopper, wash the stopper and sides of the flask with a few ml of water, and titrate the excess alkali with 0.2 M hydrochloric acid to the disappearance of the pink colour. Record the volume, in ml of 0.2 M hydrochloric acid required as S.
Perform a blank titration on 25.0 ml of 0.45 M sodium hydroxide, and record the volume, in ml, of 0.2 M hydrochloric acid required as B.

$$\text{Acetyl groups (\%)} = \frac{(B-S)\times M\times 0.043\times 100}{W}$$

where
M is the molarity of hydrochloric acid solution.
W is the weight of sample, on the dried basis (g).
0.043 is the molar mass of the acetyl group (g/mmol).

Vinyl acetate

Principle
Determined by a headspace gas chromatographic method

Reagents and solutions

- Standard solution (0.1 mg/l)

Accurately weigh 0.1 g of vinyl acetate into a 100-ml volumetric flask containing an appropriate amount of water, dissolve and make up to volume with water. Dilute 1 ml of this solution to 100 ml with water. Dilute 1 ml of the resulting solution to 100 ml with water to make the working standard solution.

- Sample Preparation

Weigh an amount of sample equivalent to 5.0 g on the dried in a 20-ml vial (designed for headspace gas chromatography - add a stirring bar if automated stirring is not available). Add 5 ml of water to the vial, stopper tightly, and stir for 20 minutes.

- Standard addition solutions

Weigh an amount of sample equivalent to 5 g on the dried basis to each of two 20-ml vials (designed for headspace gas chromatography containing a stirring bar if automated stirring is not available). Add 2.5 ml of water and 2.5 ml of the working standard solution to the first vial (0.05 mg added vinyl acetate/kg starch); add 5 ml of the working standard to the second vial (0.1 mg added vinyl acetate/kg starch). Stopper the vials tightly. Stir for 20 minutes.

Procedure

HS-GC operating conditions

Headspace GC equipped with a flame ionisation detector (FID)
Column: fused silica tube (0.25 mm internal diameter and 10 m length) coated with a 3 m thick layer of styrene divinylbenzene polymer (CP-PoraBOND Q FUSED SILICA, Agilent Technologies or equivalent)
Carrier gas: nitrogen or helium
Column temperature constant at 90–110°
Flow rate: adjust so that the peak of vinyl acetate appears about 9–11 min after injection
Injector temperature: 200 °C
Detector temperature: 250 °C

Split ratio: 1:10
Headspace sampler conditions:
Equilibrium temperature in the vial: 70 °C
Equilibrium time in the vial: 30 min

Analyze the Sample preparation and the Standard addition solutions using the conditions given above. Plot the peak areas of vinyl acetate in all three solutions versus the amount of vinyl acetate added to the solutions in mg/kg starch. The plot should be linear. Extrapolate the plot to intercept the x axis. Report the concentration of vinyl acetate in the sample as equal to the absolute value at the x-intercept.

ANNEX 5: ADDITIONAL SPECIFICATIONS FOR STARCHES SUBJECTED TO OXIDATION

(VERSION 2021)

APPLIES TO	Oxidized starch (INS No. 1404) Acetylated oxidized starch (INS No. 1451)
TREATMENT	Sodium hypochlorite is used for oxidation. Oxidized starch (INS 1404) is obtained by treatment of starch with sodium hypochlorite. Acetylated oxidized starch (INS 1451) is obtained by treatment of starch with sodium hypochlorite followed by esterification with acetic anhydride. Oxidation involves the deliberate production of carboxyl groups.
C.A.S number	65996-62-5 (Starch, oxidized) 113894-86-3 (Amylopectin, oxidized 68187-08-6 (Starch, acetate, oxidized)

CHARACTERISTICS

IDENTIFICATION

Test for hypochlorite oxidized starch	Passes test See description under TESTS.

PURITY

Carboxyl groups (Vol. 4)	Not more than 1.3% calculated on the dried basis
Residual oxidizing substances	No more than 180 mg/kg, calculated as hydrogen peroxide. See description under TESTS in ANNEX 2.

TESTS

IDENTITY TESTS

Test for hypochlorite oxidized starch

Principle

Because of the carboxyl group content, hypochlorite-oxidized starch has anionic properties. It can be dyed with positively charged dyes such as methylene blue. The test may not distinguish between oxidized potato starch and non-oxidized potato starch or any starch with phosphate groups.

Procedure

A 50 mg sample is kept in suspension for 5-10 min in 25 ml of a Methylene Blue TS, Diluted solution and stirred occasionally. After decantation of the excess solution, the starch is washed with distilled water. Microscopic inspection clearly shows colouring, if the sample is hypochlorite-oxidized starch.

ANNEX 6: ADDITIONAL SPECIFICATIONS FOR STARCHES ESTERIFIED WITH OCTENYLSUCCINIC ANHYDRIDE

(VERSION 2021)

APPLIES TO Starch sodium octenylsuccinate (INS No. 1450)

TREATMENT Starch sodium octenylsuccinate is obtained by esterification of starch with octenylsuccinic anhydride. Either sodium hydroxide or sodium carbonate is used as a pH buffer for neutralisation.

C.A.S numbers 66829-29-6 (Sodium starch octenylsuccinate)
52906-93-1 (Starch octenylsuccinate)
125109-81-1 (Amylopectin, hydrogen 2-(1octen-1-yl)butanedioate)

CHARACTERISTICS

PURITY

Octenylsuccinate groups: Not more than 3% calculated on the dried basis
See description under TESTS.

Residual free octenylsuccinic acid: Not more than 0.3% calculated on the dried basis.
See description under TESTS.

TESTS

PURITY TESTS

Octenylsuccinate groups and residual free octenylsuccinic acid in Starch sodium octenyl succinate

Principle

Residual free octenylsuccinic acid in the sample is extracted and determined by HPLC/UV. Total octenylsuccinic content is determined using the same method after hydrolysis of the sample. Octenylsuccinate ester groups on the modified starch are calculated by subtraction of the residual free octenylsuccinic acid from the total.

Reagents and solutions

Octenylsuccinic anhydride:
2-Octen-1-ylsuccinic anhydride, mixture of cis and trans (>97%) (CAS: 42482-06-4)

0.1N potassium hydroxide:
Dissolve 1.65 g of potassium hydroxide (purity 85%), in water and dilute to 250 ml with water.

0.073mol/l phosphoric acid:
Dilute 1 ml of phosphoric acid (85%, density 1.686 g/cm3) to 200 ml with water.

Preparation of octenylsuccinic acid standard solutions

Accurately weigh 20 mg of octenylsuccinic anhydride, add 10 ml of 0.1 N potassium hydroxide, stopper and heat at 80 °C for 3 hours. After cooling, add 8 ml of 0.073 mol/l phosphoric acid and dilute with water to 20 ml.

Pipette 2 ml of this solution into a 20-ml volumetric flask and dilute with water. Calculate the concentration of this standard (C, mg/ml) as octenylsuccinic acid using the formula:

$$C = \frac{m \times 1.086}{20 \times 10}$$

where

m is the mass of octenylsuccinic anhydride used, mg.

1.086 is the molecular weight of octenylsuccinic acid divided by the molecular weight of octenylsuccinic anhydride .

Pipette 1 ml, 2 ml, 5 ml, and 10 ml of the resulting solution into four separate 20-ml volumetric flasks, and dilute each to volume with water.

Sample preparation A (for residual octenylsuccinic acid)

Accurately weigh about 100 mg of sample, add 20 ml of methanol, and shake for at least 18 h. Centrifuge the mixture at about 3000 rpm for 5 min pipette 10 ml of the supernatant, and evaporate to dryness under vacuum at 40 °C. Dissolve the residue and dilute with water in a 5 ml volumetric flask.

Sample preparation B (for total octenylsuccinic content)

Accurately weigh about 20 mg of sample, dissolve in 10 ml of 0.1 N potassium hydroxide, stopper and heat at 80 °C for 3 hours. After cooling, add 8 ml of 0.05 mol/l phosphoric acid, dilute with water to 20 ml.

Procedure

HPLC operating conditions:

Column: Octadecylsilane silica gel column (250 mm x 4.6 mm, 5µm) (L-Column ODS-V CERI or equivalent)

Column temperature: 40 °C

Detector: UV at 205 nm

Mobile phase: A 1:1 mixture of 0.1% (v/v) phosphoric acid solution/acetonitrile

Injection volume: 20 µl

Flow rate: Adjust the retention time of the main peak to about 9 min.

Inject Sample preparations A and B and the standard solutions into the chromatograph.

Sum the areas of the two main peaks of *cis*- and *trans*-2-octenylsuccinic acid for each standard solution and prepare a standard curve for octenylsuccinic acid (µg/ml) from the sums obtained and the concentrations of octenylsuccinic acid in the standard solutions.

Sum the areas of the two main peaks for the Sample preparations A and B. Determine the concentration of octenylsuccinic acid (C_{osA} and C_{osB}, µg/ml) in the Sample preparations A and B, respectively, from the standard curve.

Calculate residual and total octenylsuccinic acid using the formulae that follow.

$$\text{Residual free octenylsuccinic acid (\%)} = \frac{C_{osA}}{W_r}$$

$$\text{Total octenylsuccinic acid (\%)} = \frac{C_{osB}}{W_s} \times 2$$

Where

C_{osA} and C_{osB} are the octenylsuccinic acid concentrations (□g/ml) of Sample preparations A and B, respectively; W_r or W_s is the weight of the sample on the dried basis, in Sample preparations A and B (mg), respectively.

Octenylsuccinate groups (bound)(%)=

Total octenylsuccinic acid (%) - Residual free octenylsuccinic acid (%)

ANNEX 7: ADDITIONAL SPECIFICATIONS FOR STARCHES ETHERIFIED WITH PROPYLENE OXIDE

(VERSION 2021)

APPLIES TO	Hydroxypropyl starch (INS No. 1440) Hydroxypropyl distarch phosphate (INS No. 1442)
TREATMENT	Starch is etherified using propylene oxide. Hydroxypropyl starch (INS No. 1440) is obtained by etherification of starch with propylene oxide. Hydroxypropyl distarch phosphate (INS No. 1442) is obtained by cross-linking of starch with sodium trimetaphosphate or phosphorus oxychloride combined with etherification by propylene oxide. Hydroxypropylation results in substitution of hydroxyl groups with 2-hydroxypropyl ether.
C.A.S numbers	Hydroxypropyl starch 9049-76-7 (Hydroxypropyl starch) 74315-67-6 (Amylopectin, 2-hydroxypropyl ether) Hydroxypropyl distarch phosphate 53124-00-8 (Hydroxypropyl distarch phosphate) 113894-92-1 (Hydroxypropyl amylopectin phosphate)

CHARACTERISTICS

IDENTIFICATION

Hydroxypropyl ether groups	Passes test See description under TESTS.

PURITY

Hydroxypropyl groups	Not more than 7.0% calculated on the dried basis. See description under TESTS.
Propylene chlorohydrins	Not more than 1 mg/kg calculated on the dried basis. See description under TESTS.

TESTS

IDENTITY TESTS

Hydroxypropyl ether groups (USP29-NF34: U.S. Pharmacopeial Convention,	Reagents and solutions Ninhydrin reagent Prepare a solution containing 3% 1,2,3-triketohydrindene in a 5% aqueous solution of sodium bisulfite.

Hydroxylpropyl corn starch monograph, 2015. Reproduced with permission.)

Procedure

Weigh 100 mg of the sample into a 100-ml volumetric flask and add 12.5 ml of 2 N sulfuric acid. Prepare a sample of unmodified starch of the same source (i.e. corn or potato) in the same manner. Place the flasks in a boiling water bath and heat until the samples are in solution. Cool and dilute the contents to 100 ml with water. Transfer 1 ml of the solutions into two 25-ml graduated test tubes with glass stoppers and, with the tubes immersed in cold water, add dropwise 8 ml of concentrated sulfuric acid to each. Mix well and place the tubes in a boiling water bath for exactly 3 min. Immediately transfer the tubes to an ice bath until the solution is chilled. Add 0.6 ml of Ninhydrin reagent, carefully allowing the reagent to run down the walls of the test tubes. Immediately shake well and place the tubes in a 25 °C water bath for 100 min. Adjust the volume in each tube to 25 ml with concentrated sulfuric acid and mix by inverting the tubes several times (Do not shake).

A violet colour develops only in the modified sample within 5 min due to the presence of hydroxypropyl groups (starch ether). For all other non-hydroxypropyl treated starches a light pink colour is observed.

PURITY TESTS

Hydroxypropyl groups

Reagents and solutions

Ninhydrin reagent

Prepare a solution containing 3% 1,2,3-triketohydrindene in a 5% aqueous solution of sodium bisulfite.

Propylene glycol

Standard stock solution (1 mg/ml)

Accurately weigh 100 mg propylene glycol into a 100-ml volumetric flask, and dilute with deionised water to volume.

Working standard solutions

Transfer 1 ml, 2 ml, 3 ml, 4 ml, 5 ml of the Standard stock solution to five 100-ml volumetric flasks, dilute with deionised water to volume to prepare the working standard solutions of propylene glycol with concentrations 10 µg/m, 20 µg/m, 30 µg/m, 40 µg/m and 50 µg/ml of propylene glycol, respectively.

Procedure

Accurately weigh 50 -100 mg of the sample into a 100-ml volumetric flask and add 25 ml of 1 N sulfuric acid. Prepare a sample of unmodified starch of the same source (i.e. corn or potato) in the same manner. Place the flasks in a boiling water bath and heat until the samples are in solution. Cool and dilute the contents to 100 ml with deionised water. If necessary, dilute the sample further to assure the presence of no more than 4 mg of hydroxypropyl group per 100 ml, and then dilute the blank starch in the same proportion. Transfer 1 ml of the solutions into two 25-ml graduated test tubes with glass stoppers and, with the tubes immersed in cold water, add dropwise 8 ml of concentrated sulfuric acid to each. Mix well and place the tubes in a boiling water bath for exactly 3 min. Immediately transfer the tubes to an ice bath until the solution is chilled. Add 0.6 ml of Ninhydrin reagent, carefully allowing the reagent to run down the walls of the test tubes. Immediately shake well, and place the tubes in a 25 °C water bath for 100 min. Adjust the volume in each tube to 25 ml with concentrated sulfuric

acid and mix by inverting the tubes several times (Do not shake). Immediately transfer portions of the solutions to 1-cm cells and after exactly 5 min, measure the absorption (A) at 590 nm, using the starch blank as the reference.

Prepare a standard curve. Transfer 1-ml aliquots of the five Working standard solutions into a series of five 25-ml graduated test tubes with glass stoppers and follow the instructions above beginning from "…with the tubes immersed in cold water, add dropwise 8 ml of concentrated sulfuric acid to each..."

Calculation

$$\text{Hydroxypropyl groups (\%)} = \frac{C \times 0.776 \times F \times 10}{W}$$

where

C is the amount of propylene glycol in the sample solution determined from the standard curve (µg/ml);
0.776 is the ratio of the molecular weight of the hydroxypropyl group to the molecular weight of propylene glycol;
F is the dilution factor (if a further dilution has been necessary)
W is the weight of sample (mg), calculated on the dry weight basis.

Propylene chlorohydrins

Principle

The propylene chlorohydrins, 1-chloro-2 propanol (propylene chlorohydrin-1: PCH-1) and 2-chloro-1-propanol (propylene chlorohydrin-2: PCH-2), present in the (hydroxypropylated) starch sample are extracted with aqueous methanol with a water content of 50 g/l and determined by gas chromatography - mass spectrometry (GC-MS) with selected ion monitoring (SIM) using a standard curve and 3-chloro-1-propanol (propylene chlorohydrin-3: PCH-3) as an internal standard.

GC/MS operating conditions

GC equipped with an auto sampler system, a programmable temperature vaporization (PTV) injector or split/splitless injector, and a mass spectrometric detector (MS)
Column: Fused silica column coated with a polar phase (polyethylene glycol) (30 m × 0.25 mm i.d., 0.25 µm) (Restek Stabilwax-DA, Agilent Stabilwax or equivalent)
Carrier gas: He, purity > 99.995%
Flow rate: 1.0 ml/min
Colum temperature: 40 °C for 2 min, 16°/min to 220 °C, hold 220 °C for 10 min, cool down at 60 °C/min to 40 °C
Injector: Use PTV injector or split/splitless injector
PTV injector settings
PTV: splitless
Injector temperature: programme as follows
Initial 52 °C. Increase temperature to 250 °C at a rate of 14.5 °C/s Hold at 250 °C for 1 min 0.5 µl injected
Split/splitless injector settings
Injector temperature: 250 °C
Injection mode: splitless for 0.8 min
MS detector:

Ionisation: Electron ionization (EI) method
Source temperature: 230 °C
Quad temperature: 150 °C
Electron energy: 70 eV
Acquisition type: selected ion monitoring (SIM)
Solvent delay: 7.0 min
Scan time segments (adjust time as appropriate):
Time segment 1:
PCH-1; target ions = *m/z* of 79 and 81 start time 7.0 min
Time segment 2:
PCH-2; target ions = *m/z* of 62 and 65 start time 8.1 min
Time segment 3:
PCH-3; target ions = *m/z* of 76 and 78 start time 9.0 min
[NOTE: Set the instrument up to use the sum of the target ion signals for each analyte to construct the chromatograms.]

Reagents and solutions

1-Chloro-2 propanol (propylene chlorohydrin-1; PCH-1)
not less than 75% (with impurity not more than 25% PCH-2 - Aldrich Chemical Co., cat # 292087 or equivalent)

2-Chloro-1-propanol (propylene chlorohydrin-2; PCH-2)
≥ 97% (Aldrich Chemical Co., cat # 296708 or equivalent)

3-Chloro-1-propanol (propylene chlorohydrin-3; PCH-3)

≥ 98% (Aldrich Chemical Co., cat # C46403 or equivalent).

Preparation of standard solutions

PCH-1 Standard solutions: Using a stock standard solution of PCH-1 in methanol of 2 g/l, prepare two standard solutions in methanol containing 1 and 20 mg/l of PCH-1, respectively. Calculate the concentration of the standard solutions correcting for the purity of the standard used.

PCH-2 Standard solutions: Using a stock standard solution of PCH-2 in methanol of 2 g/l, prepare two standard solutions in methanol containing 1 and 20 mg/l of PCH-2, respectively. Calculate the concentration of the standard solutions correcting for the purity of the standard used.

Internal standard solution: Accurately weigh 120 mg of PCH-3, dissolve in methanol and dilute to 50 ml. Transfer 0.40 ml of the solution to a 250 ml volumetric flask and dilute with methanol.

PCH-1 Working standard solutions: Transfer to each of six 50-ml volumetric flasks 2 ml of Internal standard solution and 2.5 ml of water. Transfer to each flask the appropriate volume of the most suitable PCH-1 Standard solution to prepare a series of six solutions covering the dilution range of 0.06 to 1.0 mg/l PCH1. Dilute with methanol to volume.

PCH-2 Working standard solutions: Transfer to each of six 50-ml volumetric flasks 2 ml of internal standard solution and 2.5 ml of water. Transfer to each flask the appropriate volume of the most suitable PCH-2 Standard solution to prepare a series of six solutions covering the dilution range of 0.06 to 1.0 mg/l PCH2. Dilute with methanol to volume.

Sample preparation

Accurately weigh an amount of sample (m) corresponding to 8.0 g starch on the dried basis in a 50 ml stopped Erlenmeyer flask, add 36.8 ml of methanol and stir with a magnetic stirring bar. Add 1.60 ml of the Internal standard solution and a volume of water (v), such that:

$$v\ (ml) = 10\ - m$$

The total volume of the mixture is 40 ml; the water content is 50 g/l. Close the Erlenmeyer flask and stir for not less than 2 hours at room temperature. After 2 hours, allow the undissolved material to settle for approximately 20 min. Once the undissolved material has settled to the bottom of the flask, transfer an aliquot of the upper layer to a GC-vial.

Procedure

Inject 0.5 µl of each of the Working standard solutions of PCH-1 and PCH-2 into the chromatograph. From the resulting chromatograms, construct standard curves using the resulting peak area ratios of PCH-1 to the internal standard and PCH-2 to the internal standard against the concentration of PCH-1 and PCH-2, respectively, in mg/l.

Inject 0.5 µl of the Sample preparation. From the resulting chromatogram, obtain the peak area ratios of PCH-1 and PCH-2 to the internal standard.

Determine the concentration of PCH-1 and PCH-2 in the Sample preparation from the corresponding standard curves and calculate the contents of PCH-1, PCH-2 and total propylene chlorohydrins in the sample of starch using the following formula:

$$\text{Content}_{\text{PCH-1}} = C_{\text{PCH-1}} \times 40 \div 1000 \times 1 \div m_d \times 1000$$

$$\text{Content}_{\text{PCH-2}} = C_{\text{PCH-2}} \times 40 \div 1000 \times 1 \div m_d \times 1000$$

$$\text{Content}_{\text{PCH-total}} = \text{Content}_{\text{PCH-1}} + \text{Content}_{\text{PCH-2}}$$

where:

- $C_{\text{PCH-1}}$ and $C_{\text{PCH-2}}$: concentrations of PCH-1 and PCH-2 in the Sample preparation (mg/l), obtained from the standard curves;
- m_d the mass of the starch in the Sample preparation, on the dried basis (8.0 g);
- $\text{Content}_{\text{PCH-1}}$: concentration PCH-1 in the sample (mg/kg);
- $\text{Content}_{\text{PCH-2}}$: concentration PCH-2 in the sample (mg/kg);
- $\text{Content}_{\text{PCH-total}}$: concentration of total PCH in the sample (mg/kg).

ANNEX 8: ADDITIONAL SPECIFICATIONS FOR STARCHES CROSS-LINKED WITH ADIPIC ANHYDRIDE

(VERSION 2021)

APPLIES TO Acetylated distarch adipate (INS No. 1422)

TREATMENT Acetylated distarch adipate is obtained by esterification with acetic anhydride and crosslinking of starch with a mixed adipic/acetic anhydride reagent prepared by reacting adipic acid with acetic anhydride. After the reaction, the starch is both acetylated and cross-linked. The structure, where adipic anhydride connects two chains, can be represented by: Starch-O-R-O-Starch, where R = CO-$(CH_2)_4$-CO and starch refers to the linear and/or branched structure.

C.A.S numbers 63798-35-6 (Acetylated distarch adipate)
63055-36-7 (Amylopectin, acetate hexanedioate)

CHARACTERISTICS

IDENTIFICATION

Crosslinking Passes test
See description under TESTS in ANNEX 3.

PURITY

Adipate groups Not more than 0.135% calculated on the dried basis
See description under TESTS.

Free adipic acid Not more than 0.025% calculated on the dried basis.
See description under TESTS.

TESTS

PURITY TESTS

Adipate groups and free adipic acid Determine by gas chromatography after derivatisation

Principle

Free adipic acid (adipic acid not bonded to starch) in the sample is extracted and determined by capillary gas chromatography after trimethylsilyl-derivatisation. Total adipic acid is determined using the same method after hydrolysis of the sample and adipate groups are calculated by subtraction of the free adipic acid from the total.

Solutions and reagents

Adipic acid (>99%)

Glutaric acid (>99%)

Starch, unmodified (of the same botanical origin as the sample)

Sodium hydroxide solution (4N): weigh 40 g of NaOH, dissolve in water and dilute to 250 ml.
Concentrated HCl (36%)
Ethyl acetate
Sodium sulfate, anhydrous
N,*O*-Bis(trimethylsilyl)trifluoroacetamide
Pyridine

Internal standard solution (1 mg/ml): Accurately weigh 100 mg of glutaric acid, dissolve in water and dilute to 100 ml.

Standard stock solution (1 mg/ml): Accurately weigh 100 mg of adipic acid, dissolve in 90 ml of warm water, cool to room temperature, dilute to 100 ml and mix.

Working standard solutions (0.02 mg/ml, 0.1 mg/ml, 0.2 mg/ml and 0.4 mg/ml): Pipette 1 ml, 5 ml, 10 ml, and 20 ml of the Standard stock solution into four separate 50-ml volumetric flasks, and dilute to volume with deionised water.

Procedure

Preparation of standard curve solutions
Weigh 1.0 g of starch into each of four Erlenmeyer flasks, add 50 ml of water and 1 ml of Internal standard solution to each flask. Add 5 ml each of the four Working standard solutions, respectively. Stopper the flasks and shake them well to disperse the starch, add 50 ml of 4 N sodium hydroxide solution, and shake for 5 min. Place the flasks in a water bath, at room temperature, and cautiously add 20 ml of concentrated hydrochloric acid. Cool, and quantitatively separately transfer the contents of the flasks into four separation funnels with a small amount of deionised water. Extract each solution with three 100-ml portions of ethyl acetate. Collect the ethyl acetate layers separately in four dry Erlenmeyer flasks, add 20 g of anhydrous sodium sulfate, allow to stand for 10 min with occasional shaking, and filter into a rotary evaporator flask. Wash the Erlenmeyer flask and the residue on the filter paper twice with a small quantity of ethyl acetate and combine the washings with the filtrate. Evaporate the ethyl acetate under a reduced pressure of 6.7 kPa at a temperature below 40 °C. Remove the remaining ethyl acetate completely under a stream of nitrogen. The evaporation of ethyl acetate should be accomplished as quickly as possible. Successively add 2 ml of pyridine and 1 ml of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide to the residue in each flask and stopper the flasks. Allow the solutions to stand for 1 h, transfer 2 ml from each flask to four GC vials, and immediately stopper tightly. Use these solutions to construct a standard curve. (Internal standard 1 mg/g starch; standards 0.1 mg/g, 0.5 mg/g, 1 mg/g and 2 mg/g starch respectively.)

Preparation of test solution A (for residual free adipic acid)
Weigh accurately about 5 g of sample into an Erlenmeyer flask, add 100 ml of deionised water and 1 ml of the Internal standard solution. Shake well for 1 hour, and filter through a 0.45-µm membrane filter. To the filtrate, add exactly 1 ml of concentrated hydrochloric acid (in the case of pre-gelatinized starch or water-soluble starch, directly add 1 ml of concentrated hydrochloric acid to the resulting suspension without filtering), and transfer to a separation

funnel. Proceed as directed for the preparation of standard solutions, beginning with "…and wash the inside of the flask with a small amount of deionised water." Use this solution for the determination of residual free adipic acid (Internal standard 1 mg/5 g starch).

Preparation of test solution B (for total adipic acid)

Weigh accurately about 1 g of sample into an Erlenmeyer flask, add 50 ml of deionised water and exactly 1 ml of the Internal standard solution. Shake the mixture well to disperse the starch, add 50 ml of 4N sodium hydroxide solution and shake well for 5 min. Place the flask in a water bath at room temperature, and cautiously add 20 ml of concentrated hydrochloric acid. After cooling, transfer the contents of the flask to a separation funnel. Proceed as directed for the preparation of standard solutions, beginning with "…and wash the inside of the flask with a small amount of deionised water.". Use this solution for the determination of total adipic acid (Internal standard 1 mg/g starch).

GC operating conditions

GC equipped with a flame ionization detector (FID)
Column: Fused silica column coated with a mixture of 50% diphenyl and 50% dimethylpolysiloxane (15 m x 0.25 mm i.d., 0.25 µm)
Carrier gas: He
Column flow: 1.0 ml/min
Column temperature: hold at 120 °C for 5 min; increase temperature to 150 °C at a rate of 5 °C/min (glutaric and adipic acids elute at about 5 min and 8 min, respectively)
Injector temperature: 250°C
Detector temperature: 250°C
Injection volume: 1µl
Split ratio: 30:1

Inject the standard curve solutions into the chromatograph and construct a standard curve using the peak area ratios of adipic acid and glutaric acid versus the amounts of adipic acid in the standard solutions (in g). Inject Test solutions A and B and obtain the peak area ratio of adipic acid to glutaric acid for each Test solution. Determine the amount of adipic acid in each Test solution from the standard curve and calculate the percent of adipate groups using the following formulae:

$$\text{Free adipic acid, \%w/w} = [\text{CF} \div \text{MF}] \times 100$$

$$\text{Adipate groups, \%w/w} = [\text{CT} \div \text{MT} - \text{CF} \div \text{MF}] \times 100$$

where
CF: amount of the free adipic acid in Test solution A (g), determined from the standard curve;
MF: mass of the sample in Test solution A (g, on the dried basis);
CT: amount of the total adipic acid in Test solution B (g), determined from the standard curve;
MT: mass of sample in Test solution B (g, on the dried basis).

ANNEX 1: SUMMARY OF RECOMMENDATIONS FROM THE 92nd JECFA[1]

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES
Ninety-second meeting (Safety evaluation of certain food additives)
Virtual meeting, 7–18 June 2021

SUMMARY AND CONCLUSIONS

Issued on 9 July 2021

(Section on evaluation of benzoic acid, its salts and derivatives updated in August 2021)

A meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was held on a virtual online platform on 7–18 June 2021. The purpose of the meeting was to evaluate the safety of certain food additives and flavourings. The present meeting was the 92nd in a series of similar meetings. The tasks before the Committee were (a) to further elaborate principles governing the evaluation of food additives, (b) to undertake safety evaluations of certain food additives, (c) to review and prepare specifications for certain food additives and (d) to establish specifications for certain flavouring agents.

Owing to travel restrictions and lockdowns due to the response to the COVID-19 pandemic in many countries, it was not possible to convene a physical meeting and it was instead decided to hold it online by video-conferencing. In view of the time differences in the countries of the invited experts, the only possible time for a video-conference was restricted to a 4-h time slot (12:00–16:00 CET) each day. This allowed only 40% of the usual daily length (8–10 h) of a typical JECFA meeting.

Dr R. Cantrill served as Chairperson, and Dr D. Benford as Vice-Chairperson.

Mr Kim Petersen, World Health Organization (WHO), and Dr Markus Lipp, Food and Agriculture Organization of the United Nations (FAO), served as joint secretaries.

The Committee evaluated the safety of six food additives and revised the specifications for one group of food additives.

The report of the meeting will be published in the WHO Technical Report Series. The report will summarize the main conclusions of the Committee in terms of acceptable daily intakes and other toxicological, dietary exposure and safety recommendations. Information on deliberations and conclusions with regard to the specifications for the identity and purity of certain food additives examined by the Committee and on specifications for 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. Future work and recommendations arising from the summary report of the ninety-second meeting of JECFA are summarized in Annex 3. Annex 4 details the selection of compounds and observations by experts with regard to the feasibility of holding the expert meetings online rather than in-person.

Toxicological monographs summarizing the data that were considered by the Committee in establishing ADIs will be published in WHO Food Additives Series No. 83. New and revised specifications for the identity and purity of the compounds will be published in FAO JECFA Monographs 27.

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 92nd meeting and are not those in this volume of the FAO JECFA Monographs series.

Toxicological and dietary exposure information and information on specifications

Food additives evaluated toxicologically and assessed for dietary exposure

Food additive	Specifications	Acceptable daily intakes (ADIs) and other conclusions on toxicology and dietary exposure
Benzoic acid, its salts and derivatives	N	The Committee evaluated a new extended one-generation reproductive toxicity study on benzoic acid. This study showed no treatment-related adverse effects, indicating a NOAEL of 1000 mg/kg bw per day, the highest dose tested. **Applying a chemical specific adjustment factor of 2 for interspecies toxicokinetics variation instead of the default factor of 4.0, the Committee established a group ADI of 0-20 mg/kg bw, which applies to benzoic acid, the benzoate salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate, expressed as benzoic acid equivalents. The Committee withdrew the previous group ADI of 0–5 mg/kg bw. The Committee noted that the high dietary exposure estimate, expressed as benzoic acid, of 7.1 mg/kg bw per day for children aged 3–9 years does not exceed the group ADI of 0–20 mg/kg bw.**
Collagenase from *Streptomyces violaceoruber* expressed in *S. violaceoruber*	N	Negative results were observed in genotoxicity studies with a powdered enzyme concentrate. The Committee identified a NOAEL of 940 mg TOS/kg bw per day (rounded from 939.6), the highest dose tested in a 13-week study of oral toxicity in rats. The Committee identified a NOAEL of 940 mg TOS/kg bw per day, the highest dose tested in a 13-week study of oral toxicity in rats. Comparison of this NOAEL with the estimated dietary exposure of 0.43 mg TOS/kg bw per day gave a margin of exposure (MOE) of > 2100. **In view of this MOE and the lack of concern about genotoxicity, the Committee established an ADI "not specified"[2] for collagenase from *S. violaceoruber*, when used in the applications specified and in accordance with good manufacturing practice.**
β-Glucanase from *Streptomyces violaceoruber* expressed in *S. violaceoruber*	N	The Committee noted negative results in studies of genotoxicity and in studies of oral toxicity in rats. The Committee identified a NOAEL of 950 mg TOS/kg bw per day (rounded by the Committee from 953.3), the highest dose tested. Comparison of this NOAEL with the estimated dietary exposure of 0.15 mg TOS/kg bw per day gave an MOE > 6300. **On the basis of this MOE and the lack of concern about genotoxicity, the Committee established an ADI "not specified"[1] for β-glucanase from *S. violaceoruber*, for the proposed uses and in accordance with good manufacturing practice.**

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

Phospholipase A2 from *Streptomyces violaceoruber* expressed in *S. violaceoruber*	R	Negative results were obtained in genotoxicity tests. In a 13-week study of oral toxicity in rats, small effects were seen at low incidence at the high dose of 956 mg TOS/kg bw per day, which might have been related to treatment. The Committee therefore identified a NOAEL of 190 mg TOS/kg per day (rounded by the Committee from 191 mg TOS/kg bw per day). A comparison of the estimated dietary exposure of 0.25 mg TOS/kg bw per day with the NOAEL of 190 mg TOS/kg bw per day from the oral toxicity study gives a MOE of 760. **On this basis and in the absence of concern about genotoxicity, the Committee established an ADI "not specified"[1] for the phospholipase A2 enzyme preparation from *S. violaceoruber* when used in the applications specified and in accordance with good manufacturing practice.**
Riboflavin from *Ashbya gossypii*	N	The Committee noted that riboflavin from *A. gossypii* has low acute toxicity and does not raise concern for genotoxicity. The NOAEL from a 90-day oral toxicity study in rats was 3000 mg/kg bw per day, the highest dose tested. Comparison of this NOAEL with the estimated dietary exposure of 3.6 mg/kg bw per day, based on maximum reported use levels, resulted in an MOE > 800. **The Committee established a group ADI "not specified"[1] for riboflavin, riboflavin- 5´-phosphate, riboflavin from *B. subtilis* and riboflavin from *A. gossypii*, expressed as riboflavin. The Committee withdrew the previous group ADI of 0–0.5 mg/kg bw.**
Ribonuclease P from *Penicillium citrinum*	N	The Committee identified a NOAEL of 980 mg TOS/kg bw per day (the highest dose tested) in a 13-week study in which rats were treated with ribonuclease P concentrate from *P. citrinum* AE-RP by gavage. A comparison of the estimated dietary exposure of 1.3 mg TOS/kg bw per day with the NOAEL of 980 mg TOS/kg bw per day gives an MOE > 750. **On the basis of this MOE and the lack of concern for genotoxicity, the Committee established an ADI "not specified"[1] for the ribonuclease P enzyme preparation from *P. citrinum* AE-RP, used in the applications specified and in accordance with good manufacturing practice.**

N: new specifications, R: revised specifications

Food additives considered for specifications only

Food additive	Specifications
Modified starches	R

R: revised specifications

ANNEX 2: CORRIGENDA

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

Food additive	Original text	Revised text	Additional information
Riboflavin INS 101(i)	$\%\ Riboflavin = \frac{A \times 5000}{328 \times W} \times 1.367$	$\%\ Riboflavin = \frac{A \times 5000}{328 \times W}$	Correction to calculation in the method of assay; removal of a wrongly assigned factor
Riboflavin from *Bacillus subtilis* INS 101(iii)	$\%\ Riboflavin = \frac{A \times 5000}{328 \times W} \times 1.367$	$\%\ Riboflavin = \frac{A \times 5000}{328 \times W}$	Correction to calculation in the method of assay; removal of a wrongly assigned factor
Riboflavin 5´-phosphate sodium INS 101(ii)	CAS number 130-40-5	CAS number 130-40-5 (anhydrous) CAS number 6184-17-4 (dihydrate)	Current specifications provide the formula for the dihydrate but no applicable CAS number
Potassium polyaspartate	Missing "Method of assay"	Add "Method of assay" under "Purity tests" after the test entitled "Molecular weight and molecular weight distribution". Delete the bold text "Potassium polyaspartate", which appears in the test for "Molecular weight and molecular weight distribution", and replace with "Principle" (as the method of assay).	Correct errors in format of specifications monograph
Vol. 4 procedure			
Unsulfonated primary aromatic amines	See printed version of Vol. 4	See **revised text** below; modified text is in bold.	Correction to the range of the standard curve

Revised text:

Procedure

Preparation of standard aniline solution

Weigh **100 mg** of redistilled aniline into a small beaker, and transfer to a 100-mL volumetric flask, rinsing the beaker several times with water. Add 30 mL of 3 N hydrochloric acid, and dilute to the mark with water at room temperature. Dilute 10.0 m of this solution to 100 mL with water, and mix well. **Dilute 20.0 mL of this solution to 100 mL with water, and mix well (1 mL of this standard solution is equivalent to 20 µg of aniline).** Measure the following volumes of the standard aniline solution into a series of 100-mL volumetric flasks: 5 mL, 10 mL, 15 mL, 20 mL and 25 mL. Dilute to 100 mL with 1 N hydrochloric acid, and mix well **(100 mL of the resulting working standard solutions contains 100, 200, 300, 400 and 500 µg of aniline, respectively). Prepare all standard solutions freshly.**

Construction of standard curve

Pipette 10 mL of each **working standard** solution into clean, dry test tubes; cool them for 10 min by

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

Preparation and evaluation of a test solution

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

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

ANNEX 3: RECOMMENDATIONS AND FUTURE WORK

Riboflavin from *A. gossypii*

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

Future work

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

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

Ribonuclease P from *Penicillium citrinum*

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

ANNEX 4: PROCEDURAL MATTERS

Owing to travel restrictions and lockdowns due to the response to the COVID-19 pandemic in many countries, it was not possible to convene a physical meeting and it was instead decided to hold it online by video-conferencing. In view of the time differences in the countries of the invited experts, the only possible time for a video-conference was restricted to a 4-h time slot (12:00–16:00 CET) each day. This allowed only 40% of the usual daily length (8–10 h) of a typical JECFA meeting. Although the experts participated fully, they noted that online meetings do not permit the necessary in-depth, robust scientific discussions that are characteristic of JECFA meetings and are therefore not a suitable substitute for face-to-face meetings. In particular, the experts felt that the online format did not foster the atmosphere of trust, inclusiveness and openness that has marked all physical JECFA meetings. The experts considered that the success of the ninety-second meeting was due mainly to the cohesion among them stemming from the trust built on the relationships they had formed during previous face-to-face meetings. The experts also decried the significant difficulty of holding any informal meetings outside the scheduled meeting times because of the widely differing time zones. Perhaps the greatest loss due to the virtual meeting format rather than in-person meetings is in efficiency in solving issues that arise shortly before or during the meeting that require immediate input from individuals or small groups of both FAO and WHO representatives. Indeed, this deficiency means that fewer food additives can be evaluated within a two-week meeting.

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

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

The meeting report was adopted on 18 June 2021.

COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives

92nd Meeting, Virtual meeting, 7 – 18 June 2021

This document contains food additive specification monographs, analytical methods, and other information prepared at the ninety-second meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which was held virtually from 7 to 18 June 2021. The specification monographs provide information on the identity and purity of food additives used directly in foods or in food production. The main three 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.

ISBN 978-92-5-135627-2 ISSN 1817-7077

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CB8300EN/1/09.22