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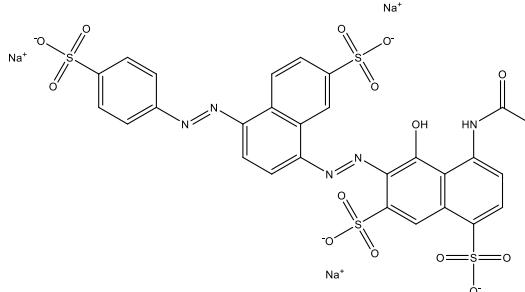
Specification Monograph prepared by the meeting of the Joint FAO/WHO
Expert Committee on Food Additives (JECFA), 87th Meeting 2019

BRILLIANT BLACK PN

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BRILLIANT BLACK PN

Prepared at the 87th JECFA and published in JECFA Monograph 23 (2019) superseding specifications prepared at the 28th JECFA (1984), published in FNP 31/1 (1984) and in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002). An ADI of 0·1 mg/kg bw was established at the 25th JECFA (1981) and confirmed at the 87th JECFA (2019).

SYNONYMS	INS No. 151, CI Food Black 1, CI (1975) No. 28440, Black PN, Brilliant Black BN
DEFINITION	<p>Brilliant Black PN consists of tetrasodium 4-(acetylamino)-5-hydroxy-6-[2-[7-sulfo-4-[2-(4-sulfophenyl)2pprox.2]-1-naphthalenyl]2pprox.2]-1,7-naphthalenedisulfonate and subsidiary colouring matters, as well as sodium chloride and/or sodium sulfate as the principal uncoloured components. Brilliant Black PN is manufactured by diazotizing 4-aminobenzenesulfonic acid (sulfanilic acid), coupling with 8-aminonaphthalene-2-sulfonic acid (1,7-Cleve's acid), diazotizing the product, and coupling with 4-(acetylamino)-5-hydroxy-1,7-naphthalenedisulfonic acid (N-acetyl K acid).</p> <p>Brilliant Black PN may be converted to the corresponding aluminium lake in which case only the requirements in the <i>General Specifications for Aluminium Lakes of Colouring Matters</i> apply.</p>
Chemical name	<p>Tetrasodium 4-acetamido-5-hydroxy-6-[7-sulfonato-4-(4-sulfonato-phenylazo)-1-naphthylazo]-1,7-naphthalenedisulfonate Tetrasodium salt of 4-(acetylamino)-5-hydroxy-6-[[(7-sulfo-4-[(4-sulfophenyl)azo]-1-naphthalenyl]azo]-1,7-naphthalenedisulfonic acid Tetrasodium;(6E)-4-acetamido-5-oxo-6-[[7-sulfonato-4-[(4-sulfonatophenyl)2pprox.2]naphthalen-1-yl]hydrazinylidene]naphthalene-1,7-disulfonate</p>
C.A.S. number	2519-30-4
Chemical formula	C ₂₈ H ₁₇ N ₅ Na ₄ O ₁₄ S ₄
Structural formula	

Formula weight	867.69
Assay	Not less than 80% total colouring matters
DESCRIPTION	Black powder or granules
FUNCTIONAL USES	Colour
CHARACTERISTICS	
IDENTIFICATION	
<u>Solubility</u> (Vol. 4)	Soluble in water, sparingly soluble in ethanol.
<u>Spectrophotometry</u> (Vol. 4)	Maximum wavelength approximately 572 nm Determine the UV-visible absorption spectrum of the sample dissolved in water.
PURITY	
<u>Loss on drying, chloride and sulfate as sodium salts</u> (Vol. 4)	Not more than 20% Determine chloride as sodium chloride, sulfate as sodium sulfate, and loss on drying (135°, 6 h) as described in Volume 4 (under "Specific Methods, Food Colours").
<u>Water insoluble matter</u> (Vol. 4)	Not more than 0.2%
<u>Lead</u> (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 (under "General Methods, Metallic Impurities").
<u>Subsidiary colouring matters</u>	Not more than 4% See description under TESTS
<u>Organic compounds other than colouring matters</u>	Not more than 0.8% sum of 4-(acetylamino)-5-hydroxy-1,7-naphthalenedisulfonic acid, 4-amino-5-hydroxynaphthalene-1,7-disulfonic acid, 8-amino-2-naphthalenesulfonic acid, sulfanilic acid, and 4,4'-(diamoamino)dibenzenesulfonic acid See description under TESTS
<u>Unsulfonated primary aromatic amines</u> (Vol. 4)	Not more than 0.01% calculated as aniline

Ether extractable matter (Vol. 4) Not more than 0.2%

TESTS

PURITY TESTS

Subsidiary colouring matters Determine subsidiary colouring matters content by reversed-phase HPLC (Vol. 4) using the following conditions:

- Column: Atlantis T3 RP18 (4.6 mm x 150 mm, 3 µm particle size) or equivalent
- Eluent A: 0.04 M ammonium acetate in water
- Eluent B: methanol
- Injection volume: 20 µl
- Column temperature: 35°
- Detector: UV-visible/diode array at 572 nm
- Flow rate: 0.8 ml/min

Gradient:

Elution time (min)	Eluent A (%)	Eluent B (%)
0	98	2
15	60	40
30	60	40
35	10	90
35.1	98	2
45	98	2

Reagents: HPLC grade

Standard:

- Brilliant Black PN (C.A.S. No. 2519-30-4) – USP
Brilliant Black PN RS or equivalent

Prepare standard solutions as required using 0.1 M ammonium acetate in water as the solvent.

Sample solution (0.1 mg/ml):

Weigh accurately 100 ± 2 mg of sample into a 100 ml volumetric flask and dilute to volume with 0.1 M ammonium acetate in water. Dilute the solution, if required, to separate subsidiary colours from the primary colour component in order to improve their resolution.

Procedure:

Inject the standard and sample solutions. Integrate all peaks in the chromatogram of the sample solution. Identify the peak of Brilliant Black PN from the chromatogram of the standard solution. Determine the ratio of the sum of all peak areas not corresponding to Brilliant Black PN to the sum of all peak areas. Calculate the result for subsidiary colours as a percentage of the sample weight.

Organic compounds
other than colouring
matters

Determine organic compounds other than colouring matters content by reversed-phase HPLC (Vol. 4) using the following conditions:

- Column: Atlantis T3 RP18 (4.6 mm x 150 mm, 3 µm particle size) or equivalent
- Eluent A: 0.04 M ammonium acetate in water
- Eluent B: methanol
- Injection volume: 20 µl
- Column temperature: 35°
- Detector: UV-visible/diode array at 254 nm
- Flow rate: 0.8 ml/min

Gradient:

Elution time (min)	Eluent A (%)	Eluent B (%)
0	98	2
15	60	40
30	60	40
35	10	90
35.1	98	2
45	98	2

Reagents: HPLC grade

Standards:

- 4-(Acetylamino)-5-hydroxy-1,7-naphthalenedisulfonic acid (N-acetyl K acid) (C.A.S. 6409-21-8) – ChemTik, Cat. No. CTK2F3097 or equivalent
- 4-Amino-5-hydroxynaphthalene-1,7-disulfonic acid (K acid) (C.A.S. 130-23-4) – BOC Sciences, Cat. No. 130-23-4 or equivalent
- 8-Amino-2-naphthalenesulfonic acid (1,7-Cleve's acid) (C.A.S. 119-28-8) – TCI Cat. No. A0356 or equivalent
- Sulfanilic acid (4-aminobenzenesulfonic acid) (C.A.S. 121-57-3) – Sigma, Cat. No. 251917 or equivalent
- 4,4'-(Diazoamino)dibenzenesulfonic acid (DAADBSA) (C.A.S. 17596-06-4) – Wako Cat. No. 040 33231 or equivalent

Prepare standard solutions as required using the following solvents:

- Dissolve N-acetyl K acid, K acid, and sulfanilic acid in water
- Dissolve 1,7-Cleve's acid in methanol/water (1:1)
- Dissolve DAADBSA in water containing 1 drop of 50% sodium hydroxide in water

Sample preparation:

Weigh accurately 100 ± 2 mg of sample into a 100 ml volumetric flask and dilute to volume with 0.1 M ammonium acetate in water.

Procedure:

Inject the standard solutions. Integrate the chromatogram peaks obtained for N-acetyl K acid, K acid, 1,7-Cleve's acid, sulfanilic acid, and DAADBSA. Construct the relevant standard curves. Inject the sample solution and determine the concentration of each analyte from its respective standard curve. Calculate the percentage of each analyte in the sample and calculate their sum.

METHOD OF ASSAY

Determine total colouring matters content by spectrophotometry using Procedure 1 in Volume 4 (under "Specific Methods, Food Colours") and an appropriate solvent. Analyze immediately after preparation.
Using water as the solvent: absorptivity (a) = 53.0 $l/(g\cdot cm)$ and wavelength of maximum absorbance = 572 nm.

PULLULAN

New specifications prepared at the 65th JECFA and published in FNP 52 Add 13 (2005). An ADI 'not specified' was established was established at the 65th JECFA (2005).

SYNOMYS

INS No. 1204

DEFINITION

Linear, neutral glucan consisting mainly of maltotriose units connected by α-1,6 glycosidic bonds. It is produced by fermentation from a food grade hydrolysed starch using a non-toxin producing strain of *Aureobasidium pullulans*. After completion of the fermentation, the fungal cells are removed by microfiltration, the filtrate is heat-sterilized and pigments and other impurities are removed by adsorption and ion exchange chromatography.

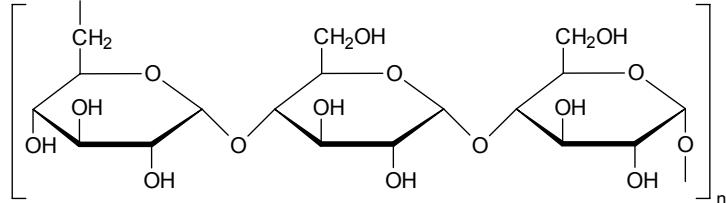
C.A.S. number

9057-02-7

Chemical formula

(C₆H₁₀O₅)_x

Structural formula



Assay

Not less than 90% of glucan on the dried basis

DESCRIPTION

White to off-white odourless powder

FUNCTIONAL USES

Glazing agent, film-forming agent, thickener

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Soluble in water, practically insoluble in ethanol

pH (Vol. 4)

5.0 - 7.0 (10% solution)

Precipitation with polyethylene glycol 600

Add 2 ml of polyethylene glycol 600 to 10 ml of a 2% aqueous solution of pullulan. A white precipitate is formed.

Depolymerization with pullulanase

Prepare two test tubes each with 10 ml of a 10% pullulan solution. Add 0.1 ml pullulanase solution having activity 10 units/g (refer to pullulanase activity, under Methods for enzyme preparations in Volume 4) to one test tube, and 0.1 ml water to the other. After incubation at about 25° for 20 minutes, the viscosity of the pullulanase-treated solution is visibly lower than that of the untreated solution.

PURITY

Loss on drying (Vol. 4)

Not more than 6% (90°, pressure not more than 50 mm Hg, 6 h)

<u>Mono-, di- and oligosaccharides</u>	Not more than 10% (expressed as glucose) See description under TESTS
<u>Viscosity</u>	100-180 mm ² /s (10% w/w aqueous solution at 30°) See description under TESTS
<u>Lead</u> (Vol. 4)	Not more than 1 mg/kg Determine using an atomic absorption technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, "Instrumental Methods".
<u>Microbiological criteria</u> (Vol. 4)	Yeast and moulds: Not more than 100 CFU/g Coliforms: Negative in 25 g Salmonella: Negative in 25 g

TESTS

PURITY TESTS

<u>Mono-, di- and oligosaccharides</u>	<u>Principle</u> The soluble mono-, di- and oligosaccharides of pullulan are measured using the anthrone-sulfuric acid method after pullulan has been precipitated with methanol and KCl.
	<u>Equipment</u> Spectrophotometer capable of measuring absorbance at 620 nm
	<u>Procedure</u> <i>Preparation of standard:</i> Weigh accurately 0.2 g glucose, dissolve in water and make up to 1 l. <i>Measurement of mono-, di- and oligosaccharides:</i> Weigh accurately 0.8 g sample and dissolve in water to make 100 ml (stock solution). Place 1 ml of the stock solution in a centrifuge tube. Add 0.1 ml saturated potassium chloride solution. Add 3 ml methanol and mix vigorously for 20 sec. Centrifuge at 11000 rpm for 10 minutes. Add 0.2 ml of the supernatant to 5 ml modified anthrone solution (0.2 g anthrone in 100 g 75% (v/v) sulfuric acid, freshly prepared). Add 0.2 ml of glucose standard solution and 0.2 ml water (blank control) to separate 5 ml portions of modified anthrone solution. Mix rapidly. Place samples in a 90° water bath and incubate for 15 min. Measure absorbance of the test solution at 620 nm.
	Calculate the percent of mono-, di- and oligosaccharides expressed as glucose, C, in the sample:

$$C(\%) = [(A_t - A_b) \times 0.41 \times G \times 100] / (A_s - A_b) \times W$$

where

A_t is absorbance of the test solution
 A_b is absorbance of the water blank
 A_s is absorbance of the standard solution
 G is weight of the glucose (g)
 W is weight of the sample (g)

Viscosity

Dry the sample for 6 h at 90° under reduced pressure (50 mm Hg). Weigh 10.0 g of the sample and dissolve in water to yield 100 g of solution.

Use an Ubbelohde-type (falling-ball) viscometer. Charge the viscometer with sample in the manner dictated by the design of the instrument. Immerse the viscometer vertically in the thermostatic tank at 30 ± 0.1° and allow to stand for 20 min so that the sample equilibrates with the temperature in the tank. Adjust the meniscus of the column of liquid in the capillary tube to a position about 5 mm above of the first mark. With the sample flowing freely, measure, in seconds, the time required for the meniscus to pass from the first to the second mark. Calculate the viscosity, V :

$$V (\text{mm}^2/\text{s}) = C \times t$$

where

C = calibration constant of the viscometer (mm^2/s^2)
 t = flow time (s)

METHOD OF ASSAY

Calculate the percentage of pullulan on dried basis, P , as the difference between 100% and the sum of the percentages of known impurities (mono-, di- and oligosaccharides and water).

$$P(\%) = 100 - (L+C)$$

where

L is loss on drying
 C is taken from the calculation for mono-, di- and oligosaccharides

COCHINEAL EXTRACT

Prepared at the 55th JECFA (2000), published in FNP 52 Add 8 (2000) superseding specifications prepared at the 51st JECFA (1998), published in FNP 52 Add 6 (1998). No ADI allocated at the 21st JECFA in 1977.

SYNONYMS

CI Natural Red 4, CI (1975) No. 75470; INS No. 120

DEFINITION

Cochineal consists of the dried bodies of the female insect *Dactylopius coccus* Costa; Cochineal extract is the concentrated solution obtained after removing the alcohol (ethanol and/or methanol) from an aqueous, aqueous alcoholic or alcoholic extract of cochineal; the colouring principle is chiefly carminic acid; commercial products may also contain proteinaceous material derived from the source insect.

In commercial products the colouring principle may also be present in association with ammonium, potassium or sodium cations, singly or in combination, and these cations may also be present in excess.

Chemical names

7-beta-D-glucopyranosyl-3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxoanthracene-2-carboxylic acid

C.A.S. number

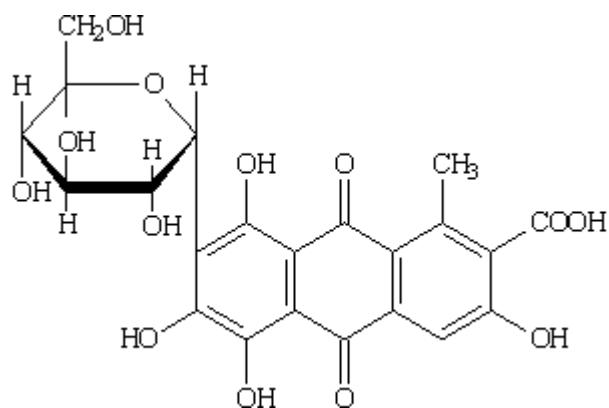
1343-78-8 (cochineal)
1260-17-9 (carminic acid)

Chemical formula

C₂₂H₂₀O₁₃ (Carminic acid)

Structural formula

Carminic acid:



Formula weight

Carminic acid: 492.39

Assay

Not less than 2.0% C₂₂H₂₀O₁₃

DESCRIPTION

Dark red liquid

FUNCTIONAL USES

Colour

CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Freely soluble in water
<u>Colour reactions</u>	<p>Make a solution of the sample slightly alkaline by adding 1 drop of 10% sodium hydroxide or potassium hydroxide solution. A violet colour is produced.</p> <p>Add a small sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) crystal to acid, neutral or alkaline solutions of the sample. The solutions are not decolourized.</p> <p>Dry a small quantity of the sample in a porcelain dish. Cool thoroughly and treat the dry residue with 1 or 2 drops of cold sulfuric acid TS. No colour change occurs.</p>
	<p>Acidify a dispersion of the sample in water with 1/3 volume of hydrochloric acid TS and shake it with amyl alcohol. Wash the amyl alcohol solution 2-4 times with an equal volume of water to remove hydrochloric acid. Dilute the amyl alcohol solution with 1-2 volumes of petroleum ether (40-60°) and shake with a few small portions of water to remove colour. Add, dropwise, 5% uranium acetate, shaking thoroughly after each addition. A characteristic emerald-green colour is produced.</p>

PURITY

<u>Protein</u> (Vol. 4)	Not more than 2.2% Proceed as directed under Nitrogen Determination (non-ammonia N x 6.25)
<u>Ethanol</u> (Vol. 4)	Not more than 150 mg/kg Proceed as directed under Residual solvent
<u>Methanol</u> (Vol.4)	Not more than 150 mg/kg Proceed as directed under Residual solvent
<u>Microbiological criteria</u> (Vol. 4)	<i>Salmonella</i> : Negative per test
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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 about 1 g of the sample, dissolve in 30 ml of boiling 2N hydrochloric acid, and cool. Transfer quantitatively to a 1000 ml volumetric flask, dilute to volume with water, and mix. Determine the absorbance of the solution in a 1 cm cell at the wavelength of maximum absorbance (about 494 nm) using water as the blank. Calculate the percentage of carminic acid in the sample using the formula:

$$\frac{100 \times A \times 100}{1.39 \times W}$$

where

A = absorbance of the sample solution;

W = weight, in mg, of the sample taken; and

1.39 = absorbance of a solution of carminic acid having a concentration of 100

mg per 1000 ml

If the measured absorbance is not within the range 0.650 to 0.750, prepare another sample and adjust the weight accordingly.

THIODIPROPIONIC ACID

Prepared at the 17th JECFA (1973), published in FNP 4 (1978) and in FNP 52 (1992). Metals and arsenic specifications revised at the 61st JECFA (2003). An ADI of 0-3 mg/kg bw was established at the 17th JECFA (1973)

SYNOMYS INS No. 388

DEFINITION

Chemical names 3,3'-Thiodipropionic acid, diethyl sulfide 2,2'-dicarboxylic acid, thioldihydracrylic acid, β,β' -thiodipropionic acid

C.A.S. number 111-17-1

Chemical formula $C_6H_{10}O_4S$

Structural formula

$$\begin{array}{c} \text{CH}_2-\text{CH}_2-\text{COOH} \\ | \\ \text{S} \\ | \\ \text{CH}_2-\text{CH}_2-\text{COOH} \end{array}$$

Formula weight 178.21

Assay Not less than 98.5%

DESCRIPTION White crystalline solid having a slight characteristic odour

FUNCTIONAL USES Antioxidant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water; freely soluble in ethanol

Melting range (Vol. 4) 130 - 134°

Sulfur Between 17.5% and 18.5%
See description under TESTS

PURITY

Sulfated ash (Vol. 4) Not more than 0.2%

Selenium (Vol. 4) Not more than 30 mg/kg

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an atomic absorption technique 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."

TESTS

IDENTIFICATION TESTS

Sulfur

Weigh 0.700 g of the sample and add 100 ml of acetic acid and 50 ml of ethanol and heat the mixture gently until the sample dissolves completely. Add 3 ml of hydrochloric acid and add 4 drops of p-ethoxychrysoidin TS and immediately titrate with 0.1 N bromide-bromate TS. As the end point is approached (pink colour) add 4 more drops of the indicator solution and continue the titration dropwise, to a colour change from red to pale yellow. Perform a blank determination and make any necessary correction. Each ml of 0.1 N bromide-bromine TS is equivalent to 1.603 mg of S.

METHOD OF ASSAY

Dissolve 0.350 g of the sample in 40 ml of water, add phenolphthalein TS and titrate with 0.1 N sodium hydroxide to the first appearance of a faint pink colour that persists for at least 30 sec. Each ml of 0.1 N sodium hydroxide is equivalent to 8.910 mg of $C_6H_{10}O_4S$.

DESOXYCHOLIC ACID

Prepared at the 17th JECFA (1973), published in FNP 4 (1978) and in FNP 52 (1992). Metals and arsenic specifications revised at the 55th JECFA (2000). An ADI of 0-1.25 mg/kg bw was established at the 17th JECFA (1973)

SYNONYMS Deoxycholic acid

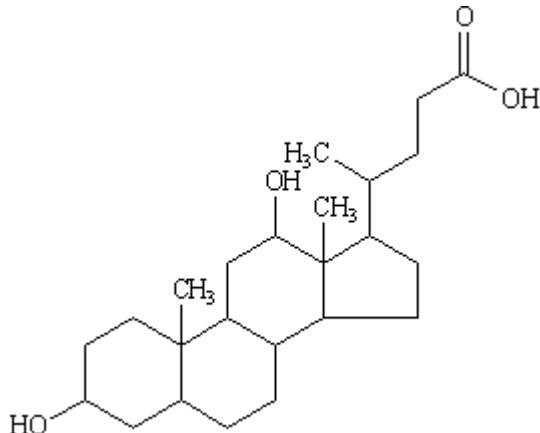
DEFINITION

Chemical names Deoxycholic acid, 3alpha,12alpha-dihydroxy-5beta-cholan-24-oic acid, 3,12-dihydroxycholanic acid

C.A.S. number 83-44-3

Chemical formula C₂₄H₄₀O₄

Structural formula



Formula weight 392.58

Assay Not less than 98% and not more than the equivalent of 102% after drying. The article of commerce may be further specified as to cholic acid content.

DESCRIPTION White crystalline powder

FUNCTIONAL USES Emulsifier

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Very slightly soluble in water, soluble in ethanol

Melting range (Vol. 4) 172 - 176°

Colour reactions To 1 ml of a 0.02% solution of the sample in 50% acetic acid add 1 ml of a 1% solution of furfural in water, 6 ml water and 5 ml concentrated sulfuric acid. The mixture turns rose and then violet blue within 5 min. (The same colour is produced by cholic acid).

To about 10 mg of the sample add 2 drops of benzaldehyde and 3 drops of 75% sulfuric acid and heat at 50° for 5 min. Then add about 10 ml of glacial acetic acid; a green colour is produced (cholic acid gives a brown colour).

PURITY

Loss on drying (Vol. 4) Not more than 1% (140°, 4 h, pressure not exceeding 5 mm of mercury)

Specific rotation (Vol. 4) [alpha] 25, D: Not less than + 55° (1% (w/v) solution in ethanol)

Sulfated ash (Vol. 4) Not more than 0.2%
Test 1 g of the sample

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an atomic absorption technique 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 Transfer about 500 mg of the dried sample, accurately weighed, into a 250-ml flask, add 20 ml of water and 40 ml of ethanol, cover with a watch glass, heat gently on a steam bath until dissolved and cool. Add 5 drops of phenolphthalein TS and titrate with 0.1 N sodium hydroxide, to the first pink colour that persists for 15 sec. Perform a blank determination and make any necessary corrections. Each ml of 0.1 N sodium hydroxide is equivalent to 39.26 mg of C₂₄H₄₀O₄.

PATENT BLUE V

Prepared at the 69th JECFA (2008), published in FAO JECFA Monographs 5 (2008), superseding specifications prepared at the 31st JECFA (1987), published in the combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). No ADI could be allocated at the 26th JECFA (1982).

SYNONYMS

CI Food Blue 5, Patent Blue 5; CI (1975) No. 42051; INS No. 131

DEFINITION

Patent Blue V consists essentially of the calcium or sodium salt of 2-[(4-diethylaminophenyl)(4-diethylimino-2,5-cyclohexadien-1-ylidene)methyl]-4-hydroxy-1,5-benzenedisulfonate and subsidiary colouring matters. Water, sodium chloride, sodium sulfate, calcium chloride, and calcium sulfate can be present as the principal uncoloured components.

Patent Blue V may be converted to the corresponding aluminium lake, in which case only the *General Specifications for Aluminium Lakes of Colouring Matters* applies.

Chemical names

Calcium or sodium salt of 2-[(4-diethylaminophenyl)(4-diethylimino-2,5-cyclohexadien-1-ylidene)methyl]-4-hydroxy-1,5-benzene-disulfonate; Calcium or sodium salt of [4-[*alpha*-(4-diethylaminophenyl)-5-hydroxy-2,4-disulfonatophenylmethylidene]-2,5-cyclohexadien-1-ylidene] diethylammonium hydroxide inner salt

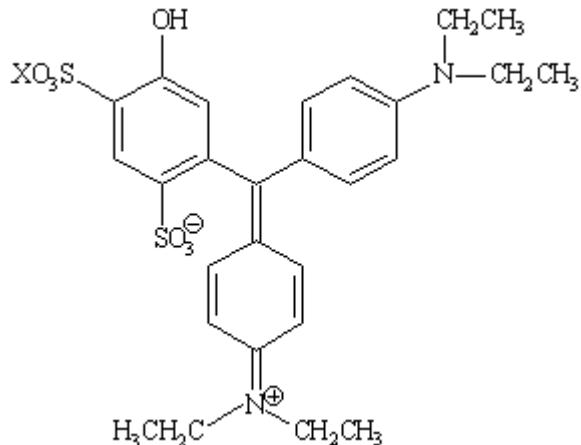
C.A.S. number

3536-49-0

Chemical formula

Calcium salt: C₂₇H₃₁N₂O₇S₂½Ca
Sodium salt: C₂₇H₃₁N₂O₇S₂Na

Structural formula



where

X = ½Ca for the calcium salt

X = Na for the sodium salt

Formula weight	$\frac{1}{2}$ Calcium salt: 579.14 Sodium salt: 582.15
Assay	Not less than 85% total colouring matter
DESCRIPTION	Blue powder or granules
FUNCTIONAL USES	Colour
CHARACTERISTICS	
IDENTIFICATION	
<u>Solubility</u> (Vol. 4)	Soluble in water; slightly soluble in ethanol
<u>Colouring matters, Identification</u> (Vol. 4)	Passes test
PURITY	
<u>Water content (Loss on drying)</u> (Vol. 4)	Not more than 15% together with chloride and sulfate calculated as sodium salts
<u>Water-insoluble matter</u> (Vol. 4)	Not more than 0.5%
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under "General Methods, Metallic Impurities").
<u>Chromium</u> (Vol. 4)	Not more than 50 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under "General Methods, Metallic Impurities")
<u>Subsidiary colouring matter content</u> (Vol. 4)	Not more than 2% Use the following conditions: Chromatography solvent: n-butanol:water:ethanol:ammonia (s.g. 0.880) (600:264:135:6) Height of ascent of solvent front: approximately 17 cm
<u>Organic compounds other than colouring matters</u>	Not more than 0.5% (Sum of 3-hydroxybenzaldehyde, 3-hydroxybenzoic acid, 3-hydroxy-4-sulfonatobenzoic acid and <i>N,N</i> -diethylaminobenzenesulfonic acids) See description under TESTS

Leuco base (Vol. 4)

Not more than 4%

Proceed as directed in Volume 4 using the following parameters:

- Sample: 110 mg
- Ratio of the formula weight of the colouring matter to the formula weight of its leuco base:
Sodium salt: $582.15/606.66 = 0.95960$
 $\frac{1}{2}$ Calcium salt: $579.14/600.76 = 0.96401$
- Absorptivity: 0.200 l/(mg·cm) at 638 nm

Unsulfonated primary aromatic amines (Vol. 4)

Not more than 0.01%, calculated as aniline

Ether-extractable matter (Vol. 4)

Not more than 0.2%

TESTS**PURITY TESTS****Organic compounds other than colouring matters (Vol. 4)**Proceed as directed under *Determination by High Performance Liquid Chromatography* using the following conditions:

Instrument: High Performance Liquid Chromatograph fitted with a gradient elution accessory

Detector: A UV detector monitored at 254 nm

Column: 250 x 4 mm (Kartusche). LiChrosorb RP 18, 7 µm or equivalent.

Mobile phase:

- (A) Acetate buffer pH 4.6: water (10% w/v) - prepared using 1 M sodium hydroxide, 1 M acetic acid and water (5:10:35)
- (B) Acetonitrile

Gradient

<i>Min</i>	<i>% (A)</i>	<i>% (B)</i>	<i>Flow rate (ml/min)</i>
0	85	15	1
12	85	15	1
25	20	80	2
28	20	80	2
40	85	15	1

METHOD OF ASSAYProceed as directed under *Colouring Matters Content by Titration with Titanous Chloride* (Volume 4), under *Food Colours, Colouring Matters*), using the following:

Weight of sample: 1.3-1.4 g

Buffer: 15 g sodium hydrogen tartrate

Weight (*D*) of colouring matters equivalent to 1.00 ml of 0.1 N TiCl₃:

28.98 mg of the calcium salt

29.13 mg of the sodium salt.

CALCIUM HYDROGEN PHOSPHATE

Prepared at the 19th JECFA (1975), published in NMRS 55B (1976) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). A group MTDI of 70 mg/kg bw, as phosphorus from all food sources, was established at the 26th JECFA (1982)

SYNONYMS Dibasic calcium phosphate, dicalcium phosphate, INS No. 341 (ii)

DEFINITION

Chemical names Calcium monohydrogen phosphate, calcium hydrogen orthophosphate, secondary calcium phosphate

C.A.S. number 7757-93-9

Chemical formula Anhydrous: CaHPO_4
Dihydrate: $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$

Formula weight Anhydrous: 136.06
Dihydrate: 172.09

Assay Not less than 98.0% and not more than the equivalent of 102.0% after drying

DESCRIPTION White crystals or granules, granular powder or powder

FUNCTIONAL USES Dough conditioner, yeast food

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Sparingly soluble in water; insoluble in ethanol

Test for calcium (Vol. 4) Passes test

Test for phosphate (Vol. 4) Passes test

PURITY

Loss on drying (Vol. 4) Anhydrous: Not more than 2% (200° , 3 h)
Dihydrate: Not less than 18% and not more than 22% (200° , 3 h)

Fluoride (Vol. 4) Not more than 50 mg/kg (Method I or III)

Arsenic (Vol. 4) Not more than 3 mg/kg (Method II).

Lead (Vol. 4) Not more than 4 mg/kg
Determine using an atomic absorption technique 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 about 0.3 g of the sample, previously dried for 3 h at 200°. Dissolve in 10 ml of dilute hydrochloric acid TS, add about 120 ml of water and a few drops of methyl orange TS, and boil for 5 min, keeping the volume and pH of the solution in the beaker constant during the boiling period by adding hydrochloric acid or water as necessary. Add 2 drops of methyl red TS and 30 ml of ammonium oxalate TS. Then add dropwise, with constant stirring, a mixture of equal volumes of ammonia TS and water until the pink colour of the indicator just disappears.

Digest on a steam bath for 30 min, cool to room temperature, allow the precipitate to settle, and filter the supernatant liquid through an asbestos mat in a Gooch crucible, using gentle suction. Swirl the precipitate in the beaker with about 30 ml of a cold (below 20°) wash solution prepared by diluting 10 ml of ammonium oxalate TS to 1000 ml. Allow the precipitate to settle, and pass the supernatant through the filter. Repeat this washing by decantation three more times. Using the wash solution, transfer the precipitate as completely as possible to the filter. Finally, wash the beaker and the filter with to 10 ml portions of cold (below 20°) water. Place the Gooch crucible in the beaker, and add 100 ml of water and 50 ml of cold dilute sulfuric acid (1 in 6). Add from a buret 35 ml of 0.1 N potassium permanganate, and stir until the colour disappears. Heat to about 70°, and complete the titration with 0.1 N potassium permanganate. Each ml of 0.1 N potassium permanganate is equivalent to 6.803 mg of CaHPO₄.

MAGNESIUM CHLORIDE

Prepared at the 27th JECFA (1983), published in FNP 28 (1983) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). An ADI 'Not limited' was established at the 23rd JECFA (1979).

SYNONYMS

INS No. 511

DEFINITION

Chemical names Magnesium chloride hexahydrate

C.A.S. number 7786-30-3

Chemical formula $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

Formula weight 203.30

Assay Not less than 99.0% and not more than 105.0%

DESCRIPTION Colourless, odourless flakes, granules, lumps or crystals; it is very deliquescent

FUNCTIONAL USES Firming agent, colour retention agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Very soluble in water; freely soluble in ethanol

Test for chloride (Vol. 4) Passes test

Test for magnesium (Vol. 4) Passes test

PURITY

Ammonium Not more than 50 mg/kg
Dissolve 1 g of the sample in 90 ml of water, and slowly add 10 ml of a freshly boiled and cooled solution of sodium hydroxide (1 in 10 soln). Allow to settle, then decant 20 ml of the supernatant liquid into a colour comparison tube, dilute to 50 ml with water, and add 2 ml of Nessler's TS. Any colour does not exceed that produced by 10 µg of ammonium (NH_4^+) ion in 48 ml of water and 2 ml of the sodium hydroxide solution.

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an atomic absorption technique 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

Dissolve about 450 mg of the sample, accurately weighed, in 25 ml of water, add 5 ml of ammonia/ammonium chloride buffer TS and 0.1 ml of eriochrome black TS and titrate with 0.05 M disodium ethylenediaminetetraacetate until the solution is blue in colour. Each ml of 0.05 M disodium ethylenediaminetetra-acetate is equivalent to 10.16 mg of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

CELLULASE from *TRICHODERMA LONGIBRACHIATUM*

Prepared at the 39th JECFA (1992), published in FNP 52 Add 1 (1992) superseding specifications prepared at the 31st JECFA (1987), published in FNP 38 (1988) in FNP 52 (1992) under the name Cellulase from Trichoderma reesei. An ADI 'not specified' was established at the 39th JECFA (1992)

SOURCES

Commercial enzyme preparations are produced extracellularly by the controlled fermentation of *Trichoderma longibrachiatum* and isolated from the medium. The name of the organism used previously was *Trichoderma reesei*. In 1986, the International Commission on the Taxonomy of Fungi (ICTF) recommended use of the name *Trichoderma longibrachiatum*.

Active principles

1. Cellulase (endo-1,4- β -glucanase)
2. Exo-1,4- β -D-glucosidase (glucan-1,4- β -glucosidase)
3. Exo-cellobiohydrolase (cellulose 1,4- β -cellobiosidase)
4. β -glucanase

Systematic names and numbers

1. 1,4-(1,3; 1,4)- β -D-Glucan-4-glucanohydrolase (EC 3.2.1.4)
2. 1,4- β -D-Glucoside glucohydrolase (EC 3.2.1.74)
3. 1,4- β -D-Glucan cellobiohydrolase (EC 3.2.1.91)
4. 1,3-(1,3; 1,4)- β -D-glucan-3(4)-glucanohydrolase (EC 3.2.1.6)

Reactions catalyzed

The enzyme preparations hydrolyze 1,4- β -glucan linkages in such polysaccharides as cellulose, yielding β -dextrans.

Secondary enzyme activities

Amyloglucosidase (EC 3.2.1.3); Cellobiase
Xylanase (EC 3.2.1.32); Proteinase
Hemicellulase; Lipase (ED 3.1.1.3)
Pectinase (EC 3.2.1.15)

DESCRIPTION

Off-white to tan amorphous powders, or liquids that may be dispersed in food-grade diluents and carriers; soluble in water but practically insoluble in ethanol, chloroform and ether

FUNCTIONAL USES

Enzyme preparation
Used in the preparation of fruit juices, wine, beer and vegetable oils

GENERAL SPECIFICATIONS

Must conform to the *General Specifications for Enzyme Preparations used in Food Processing* (see Volume Introduction)

CHARACTERISTICS

IDENTIFICATION

Cellulase activity (Vol. 4) The sample shows cellulase activity

EDIBLE GELATIN

Prepared at the 14th JECFA (1970), published in NMRS 48B (1971) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). An ADI 'not limited' was established at the 14th JECFA (1970)

SYNONYMS	Gelatin edible
DEFINITION	A protein produced by partial hydrolysis of collagen in skin, tendons, ligaments, bones, etc. of animals. The article of commerce may further specify special requirements for criteria such as gel strength and limits of iron, calcium, lactose or other chemical or microbiological requirements, such as requirements concerning selected pathogenic organisms including <i>Salmonella</i> , <i>Staphylococcus aureus</i> , <i>Clostridium</i> spp. and mould spores. Information is requested on the microbiological criteria given below, which are tentative only.
C.A.S. number	9000-70-8
DESCRIPTION	Sheets, flakes, or shreds, or coarse to fine powder, faintly yellow or amber in colour, the shade varying in depth according to the particle size and with a slight characteristic bouillon-like odour; stable in air when dry, but is subject to microbial decomposition when moist or in solution.
FUNCTIONAL USES	Stabilizer, gelling agent, emulsifying agent, crystallization inhibitor
CHARACTERISTICS	
IDENTIFICATION	
<u>Solubility</u> (Vol. 4)	Insoluble in cold water, but swells and softens when immersed, gradually absorbing from 5 to 10 times its own weight of water; soluble in hot water, forming a jelly on cooling; soluble in acetic acid; insoluble in ethanol, chloroform and ether
<u>Precipitate formation</u>	To a solution (1 in 100) add trinitrophenol TS or a solution of potassium dichromate (1 in 15) previously mixed with about one-fourth its volume of dilute hydrochloric acid: a yellow precipitate is formed To a solution (1 in 100) add mercuric nitrate solution; a white precipitate is formed which develops a brick red colour on warming.
<u>Development of turbidity</u>	To a solution (1 in 5,000) add tannic acid TS; the solution becomes turbid
<u>Evolution of ammonia</u>	When heated with soda lime, ammonia is evolved
PURITY	
<u>Loss on drying</u> (Vol. 4)	Not more than 18% (100-105°, 6 h)
<u>Odour and water insoluble substances</u>	A hot solution (1 in 40) is free from any disagreeable odour; when viewed in a layer 2 cm thick, shows not more than a slight opalescence
<u>Sulfur dioxide</u> (Vol. 4)	Not more than 40 mg/kg

<u>Ash</u> (Vol. 4)	Not more than 2%
<u>Arsenic</u> (Vol. 4)	Not more than 1 mg/kg (Method II)
<u>Lead</u> (Vol. 4)	Not more than 1.5 mg/kg Determine using an atomic absorption technique 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."
<u>Cadmium</u> (Vol. 4)	Not more than 0.5 mg/kg
<u>Mercury</u> (Vol. 4)	Not more than 0.15 mg/kg
<u>Microbiological criteria</u> (Vol. 4)	Standard plate count: $<10^4$ /g <i>Enterobacteriaceae</i> or bacteria of the <i>coli-aerogenes</i> group: <10 /g Lancefield group D <i>streptococci</i> : $<10^2$ /g

SODIUM o-PHENYLPHENOL

Prepared at the 8th JECFA (1964), published in NMRS 38A and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). An ADI of 0-0.2mg/kg bw was established at the 8th JECFA (1964)

SYNONYMS INS No. 232

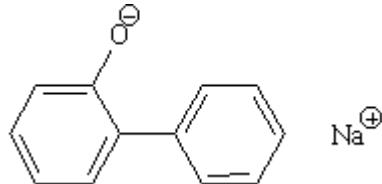
DEFINITION

Chemical names Sodium (1,1'-biphenyl)-2-olate, sodium o-phenylphenate, sodium o-phenylphenolate

C.A.S. number 132-27-4

Chemical formula $C_{12}H_9NaO \cdot 4H_2O$

Structural formula



Formula weight 264.26

Assay Not less than 97.0%

DESCRIPTION White powder of flakes; absorbs carbon dioxide and releases free o-phenylphenol which slowly sublimes when exposed to air

FUNCTIONAL USES For the post-harvest treatment of fruits and vegetables to protect against microbial damage

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) 122 g dissolve in 100 ml water, 138 g dissolve in 100 ml methanol, and very soluble in ethanol

pH (Vol. 4) About 12.7 (aqueous soln)

Test for phenylphenolate When neutralized, a precipitate of o-phenylphenol forms and, when filtered and dried, this material melts at about 57° and its ethanolic solution (1 g in 10 ml) produces a green colour upon addition of 10% ferric chloride solution.

PURITY

Excess alkalinity Not more than 1% as sodium hydroxide
Weigh 5.0 g into a 250-ml beaker, dissolve in 50 ml of water and titrate with 1 N hydrochloric acid to a pH of 11.0 using a suitable pH meter. Each ml of

1 N hydrochloric acid is equivalent to 40 mg of sodium hydroxide.

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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 3.100 g of sodium o-phenylphenol, dissolve in water, adding a few drops of 10% sodium hydroxide solution if necessary to clear any turbidity, and dilute to 500.0 ml with water. Pipette 25.0 ml into a 250- ml iodine flask, and add 30.0 ml of 0.1 N bromide-bromate TS and 50 ml of anhydrous methanol. Place stopper in the flask and add 10 ml of dilute (1 to 1) hydrochloric acid to the well. Raise the stopper slightly to allow the acid to flow down the sides of the flask, but retain a small amount of the acid in the well to act as a seal. Mix the contents by swirling and allow it to react for exactly 30 sec at $25\pm5^\circ$. Immediately add 10 ml of 20% potassium iodide solution to the well and allow it to drain into the flask. Mix well and allow the solution to stand for 5 min, shaking occasionally. Wash the stopper and the sides of the flask with water and titrate the liberated iodine with 0.1 N sodium thiosulfate adding starch TS as the endpoint is approached. Each ml of 0.1 N bromide-bromate TS consumed is equivalent to 6.608 mg of $C_{12}H_9ONa \cdot 4H_2O$.

POTASSIUM ACETATE

Prepared at the 18th JECFA (1974), published in NMRS 54B (1975) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). A group ADI 'Not limited' for acetic acid and its K & Na salts was established at the 17th JECFA (1973).

SYNONYMS

INS No. 261(i)

DEFINITION

Chemical names Potassium acetate

C.A.S. number 127-08-2

Chemical formula $\text{C}_2\text{H}_3\text{KO}_2$

Structural formula $\text{CH}_3\text{-COOK}$

Formula weight 98.14

Assay Not less than 99.0% after drying

DESCRIPTION Colourless, deliquescent crystals or a white, crystalline powder, odourless or with a faint acetic odour

FUNCTIONAL USES Buffer, antimicrobial preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Very soluble in water; freely soluble in ethanol

pH (Vol. 4) 7.5 - 9.0 (1 in 20 soln)

Test for potassium (Vol. 4) Passes test

Test for acetate (Vol. 4) Passes test

PURITY

Loss on drying (Vol. 4) Not more than 8.0% (150°, 2 h)

Alkalinity Dissolve 1 g of the sample in 20 ml of freshly boiled and cooled water, and add 3 drops of phenolphthalein TS. If a pink colour is produced, not more than 0.5 ml of 0.1 N hydrochloric acid should be required to discharge it.

Test for sodium (Vol. 4) Negative test

Lead (Vol. 4) Not more than 2 mg/kg

Determine using an atomic absorption technique 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**

Dissolve about 200 mg of the dried sample, accurately weighed, in 25 ml of glacial acetic acid. Add 2 drops of crystal violet TS, and titrate with 0.1 N perchloric acid in glacial acetic acid. Perform a blank determination, and make any necessary correction. Each ml of 0.1 N perchloric acid is equivalent to 9.814 mg of $C_2H_3KO_2$

GLUCOAMYLASE FROM *TRICHODERMA REESEI* EXPRESSED IN *TRICHODERMA REESEI*

New specifications prepared at the 77th JECFA (2013) and published in FAO JECFA Monographs 14 (2013). An ADI "not specified" was established at the 77th JECFA (2013).

SYNOMYS

Amyloglucosidase; γ -amylase; lysosomal α -glucosidase; acid maltase; exo-1,4- α -glucosidase; glucose amylase; γ -1,4-glucan glucohydrolase; acid maltase; and 1,4- α -D-glucan

SOURCES

Produced by submerged straight-batch or fed-batch fermentation of a genetically modified non-pathogenic, non-toxigenic strain of *Trichoderma reesei* which contains a gene coding for glucoamylase from *T. reesei*. The enzyme is secreted to the fermentation broth. The cell mass along with the solid waste slurry carrying the residual microorganism is separated from the enzyme by centrifugation and/or filtration. The liquid enzyme filtrate is concentrated by ultrafiltration followed by diafiltration to remove colour. The product is further polish-filtered and formulated using food-grade stabilizing agents and standardized to the desired activity.

Active principles

Glucoamylase

Systematic names and numbers

Glucan 1,4- α -glucosidase; EC 3.2.1.3; CAS No. 9032-08-0

Reactions catalysed

Hydrolysis of terminal (1 \rightarrow 4)-linked α -D-glucose residues successively from non-reducing ends of the chains with release of β -D-glucose.

Secondary enzyme activities

No significant levels of secondary enzyme activities

DESCRIPTION

Amber liquid

FUNCTIONAL USES

Enzyme preparation.
Used in the manufacture of corn sweeteners such as high fructose corn syrup, baking, brewing and potable alcohol manufacture

GENERAL SPECIFICATIONS

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

CHARACTERISTICS

IDENTIFICATION

Glucoamylase activity

The sample shows glucoamylase activity
See description under TESTS.

TESTS

Glucoamylase activity

Principle

Glucoamylase hydrolyses the substrate *p*-nitrophenyl-alpha-D-glucopyranoside (PNPG) to glucose and *p*-nitrophenol (PNP) at alkaline pH. The released PNP is proportional to enzyme activity and measured at 400 nm. Enzyme activity is expressed in GlucoAmylase Units (GAU). One GAU is defined as the amount of glucoamylase that releases one gram of glucose per hour (= 5.6 mmol of glucose per hour) from soluble starch substrate at pH 4.3 and temperature of 30 °.

Apparatus

Spectrophotometer (400 nm)

Water bath with thermostatic control (40 ° ± 1)

Water bath with thermostatic control (30 ° ± 1)

Vortex mixer

Magnetic stir plate and stir bars

Positive displacement and repeater pipettes

Reagents and solutions

Preparation of Sodium Acetate buffer (0.1 M, pH 4.3):

Weigh 4.4 g of sodium acetate trihydrate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) and transfer to a 1-litre volumetric flask. Add 800 ml of deionised water and mix until dissolved. Adjust the pH to 4.30 ± 0.05 with glacial acetic acid. Make up the volume to 1000 ml with water and mix. This solution can be stored at 4° for two weeks.

Preparation of Borax Solution (0.1 M, pH 9.2):

Weigh and transfer 19.04 g of sodium borate decahydrate to a 500 ml volumetric flask. Add 400 ml of water and mix until dissolved. Measure pH. Adjust pH, if needed, to 9.20 ± 0.05 . Bring the volume to 500 ml with water and mix. The solution can be stored at room temperature for six weeks.

Preparation of PNPG substrate (1.1 mg/ml) (light sensitive):

Weigh 55.0 ± 0.5 mg of PNPG substrate (Sigma) in a beaker. Add 40 ml of Sodium Acetate buffer (0.1 M, pH 4.3) and stir on a magnetic stirrer until dissolved (may require heating in a water bath at 30 °). Transfer contents into a 50 ml volumetric flask. Make up the volume to 50 ml. Transfer the solution to an amber bottle for storage. This solution can be stored at 4 ° for two weeks.

Preparation of Standard and Sample Solutions:

Preparation of Stock Standard Solution:

Heat a 1 ml aliquot of glucoamylase standard solution with the activity expressed in GAU/ml as reported on the Certificate of Analysis, in a water bath at 40 ° for 10 min. Label as Stock Standard Solution.

Preparation of Working Standard Solutions:

Dilute different aliquots of the stock standard with the 0.1M Sodium Acetate Buffer to obtain at least three working standards with concentrations that fall within the linear range of the assay (the linear range of the assay is ~0.5 - 2.4 GAU/ml) after subtracting blank. The

prepared solutions should be kept at room temperature and must be used within 2 h of their preparation.

Preparation of sample:

Heat a 1 ml aliquot of glucoamylase sample in a water bath at 40 ° for 10 min. Dilute the glucoamylase sample with the Sodium Acetate buffer. The solution should be kept at room temperature and used within 2 h of its preparation.

Procedure

Preheat water bath to 30 °. Prepare duplicate tubes in a rack and label as Working Standard, Sample and Blank. Using a positive displacement pipette, dispense 250 µl of 0.1M Sodium Acetate buffer, pH 4.3 to each labeled tube.

Using a positive displacement pipette, add 200 µl of each Working Standard and Sample to the respective labeled tubes. Add an additional 200 µl of Sodium Acetate buffer to the blank tube. Place the tubes in the 30 ° water bath for 5 min. Add to each set of duplicate tubes, 500 µl of 1.1 mg/ml PNPG substrate solution with a repeater pipette at timed intervals and vortex for 3 sec each. Incubate all tubes in a water bath for 10 ± 0.1 min from the time of addition of the substrate. Remove from the water bath and immediately add 1.0 ml of 0.1M Borax solution at the same time interval used for the substrate addition using a repeater pipette to each tube. Vortex each tube for 3 sec and place on a second rack. Transfer the standards, samples and blanks to 1.5 ml cuvettes. Measure the absorbances at 400 nm, after appropriately zeroing with blank. Prepare the standard curve using linear regression. The correlation coefficient must be ≥ 0.99 . Determine the glucoamylase concentration of each sample from the standard curve.

Weigh sample. Record the value as density in g/ml, up to two significant figures.

Calculate the glucoamylase activity for each sample of the glucoamylase preparation in GAU/g as follows:

$$\text{Glucoamylase activity, GAU/g} = \frac{C \times D}{\text{Sample density}}$$

Where:

C is the glucoamylase concentration of the sample determined from the standard curve (GAU/ml), and

D is the dilution factor of the sample divided by the sample density (g/ml)

AZORUBINE

Prepared at the 28th JECFA (1984), published in FNP 31/1 (1984) and in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002). An ADI of 0.4 mg/kg bw was established at the 27th JECFA (1983)

SYNONYMS

CI Food Red 3, Carmoisine, CI (1975) No. 14720, INS No. 122

DEFINITION

Consists essentially of disodium 4-hydroxy-3-(4-sulfonato-1-naphthylazo)-1-naphthalenesulfonate and subsidiary colouring matters together with sodium chloride and/or sodium sulfate as the principal uncoloured components.

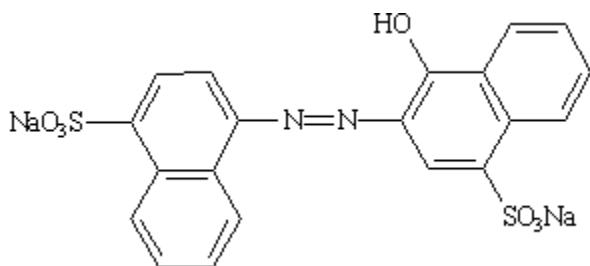
May be converted to the corresponding aluminium lake in which case only the *General Specifications for Aluminium Lakes of Colouring Matters* shall apply.

Chemical names Disodium 4-hydroxy-3-(4-sulfonato-1-naphthylazo)-1-naphthalenesulfonate

C.A.S. number 3567-69-9

Chemical formula C₂₀H₁₂N₂Na₂O₇S₂

Structural formula



Formula weight 502.44

Assay Not less than 85% total colouring matters

DESCRIPTION Red powder or granules

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water, sparingly soluble in ethanol

Identification of colouring matters
(Vol. 4) Passes test

PURITY

<u>Loss on drying</u> (Vol. 4)	Not more than 15% at 135° together with chloride and sulfate calculated as sodium salts
<u>Water insoluble matter</u> (Vol. 4)	Not more than 0.2%
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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."
<u>Subsidiary colouring matters</u> (Vol. 4)	Not more than 1% Use the following conditions: Developing solvent: No. 4 Height of ascent of solvent front: approximately 17 cm
<u>Organic compounds other than colouring matters</u> (Vol. 4)	Not more than 0.5% of 4-Amino-1-naphthalenesulfonic acid and 4-Hydroxy-1-naphthalenesulfonic acid together Use HPLC under the following conditions: HPLC elution gradient: 1 to 100% at 2.0% per min (exponential)
<u>Unsulfonated primary aromatic amines</u> (Vol. 4)	Not more than 0.01% calculated as aniline of
<u>Ether extractable matter</u> (Vol. 4)	Not more than 0.2%

METHOD OF ASSAY

Proceed as directed under *Total Content by Titration with Titanous Chloride* in Volume 4, using the following:
Weight of sample: 0.5 - 0.6 g
Buffer: 15 g sodium hydrogen tartrate
Weight (D) of colouring matters equivalent to 1.00 ml of 0.1 N TiCl₃: 12.56 mg

CHYMOSIN A from *ESCHERICHIA COLI* K-12 containing the PROCHYMOSEN A GENE

Prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7 (1999), superseding tentative specifications prepared at the 37th JECFA (1990), published in FNP 52 (1992). ADI "Not specified" established at the 37th JECFA in 1990.

SYNOMYS	Rennin, milk-clotting enzyme, chymosin, chymosin A, aspartyl protease
C.A.S. number	84484-18-4
SOURCES	Produced intracellularly by the controlled fermentation of <i>Escherichia coli</i> K-12 containing the bovine prochymosin A gene. The strain is non-pathogenic and non-toxicogenic (for example, JA198). Prochymosin is liberated by cell disruption followed by harvesting of the prochymosin by centrifugation or membrane concentration and washing with buffer solution. The residual production cells are inactivated by acid treatment, then the prochymosin is dissolved in buffer solution and after pH adjustment the solution is filtered. Prochymosin is activated to chymosin by acid treatment, followed by final purification via anion-exchange chromatography and elution with a buffered salt solution.
Active principles	Chymosin
Systematic names and numbers	None (EC 3.4.23.4)
Reactions catalyzed	Cleaves a single bond in kappa-casein
DESCRIPTION	Clear, colourless or slightly coloured aqueous solution containing the active enzyme; preparations may contain caramel colour to facilitate their identification in cheese manufacture.
FUNCTIONAL USES	Enzyme preparation Used in clotting of milk for cheese production
GENERAL SPECIFICATIONS	Must conform to the General Specifications for Enzyme Preparations used in Food Processing (see Volume Introduction)
CHARACTERISTICS	
IDENTIFICATION	
<u>Milk clotting activity</u> (Vol. 4)	The sample shows milk clotting activity

ALUMINIUM POWDER

Prepared at the 63rd JECFA (2004) and published in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). A PTWI of 2 mg/kg bw for aluminium was established at the 74th JECFA (2011).

SYNONYMS

CI Pigment Metal; CI (1975) No. 77000; INS No. 173

DEFINITION

Produced by grinding aluminium that may be carried out in the presence of edible vegetable oils and/or food grade fatty acids.

Chemical names

Aluminium

C.A.S. number

7429-90-5

Chemical formula

Al

Atomic weight

26.98

Assay

Not less than 99.0 %

DESCRIPTION

Silvery grey powder

FUNCTIONAL USES

Colour (for surface only)

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Insoluble in water and in organic solvents, soluble in dilute hydrochloric acid.

Test for aluminium (Vol. 4)

A sample dissolved in dilute hydrochloric acid passes test.

PURITY

Loss on drying (Vol. 4)

Not more than 0.5 % (105°)

Arsenic (Vol. 4)

Not more than 3 mg/kg (Method II)

Lead (Vol. 4)

Not more than 20 mg/kg

Weigh 5 g of sample and transfer to a beaker. Add 50 ml concentrated hydrochloric acid and heat on a hot plate until totally dissolved. Dilute with water to 100 ml in a volumetric flask. Determine using an atomic absorption technique appropriate to the specified level.

METHOD OF ASSAY

Wash a small sample in hexane, repeating to remove traces of any associated oil or fatty acid. Transfer about 0.2 g of the sample, accurately weighed, to a 500 ml flask fitted with a rubber stopper carrying a 150 ml separating funnel, an inlet tube connected to a cylinder of carbon dioxide and an outlet tube dipping into a water-trap. Add 60 ml of freshly boiled and cooled water and disperse the sample, replace the air by carbon dioxide and add, by the separating funnel, 100 ml of a solution containing 56 g of ferric ammonium sulfate and 7.5 ml of sulfuric acid in freshly boiled and cooled water.

While maintaining an atmosphere of carbon dioxide in the flask, heat to boiling and boil for 5 min. After the sample has dissolved, cool rapidly to 20°, and dilute to 250 ml with freshly boiled and cooled water. To 50 ml of this solution, add 15 ml of phosphoric acid and titrate with 0.1 N potassium permanganate. 1 ml of 0.1 N potassium permanganate is equivalent to 0.8994 mg of Al.

TETRASODIUM PYROPHOSPHATE

Prepared at the 41st JECFA (1993), published in FNP 52 Add 2 (1993) superseding specifications prepared at the 37th JECFA (1990), published in FNP 52 (1992). Metals and arsenic specifications revised at the 55th JECFA (2000). A group MTDI of 70 mg/kg bw, as phosphorus from all food sources, was established at the 26th JECFA (1982)

SYNOMYS

Tetrasodium diphosphate, sodium pyrophosphate; INS No 450 (iii)

DEFINITION

Chemical names Tetrasodium diphosphate, tetrasodium pyrophosphate

C.A.S. number 7722-88-5

Chemical formula Anhydrous: Na₄P₂O₇
 Decahydrate: Na₄P₂O₇ · 10H₂O

Formula weight Anhydrous: 265.94
 Decahydrate: 446.09

Assay Not less than 95.0% on the ignited basis

DESCRIPTION Colourless or white crystals, or a white crystalline or granular powder; the decahydrate effloresces slightly in dry air

FUNCTIONAL USES Emulsifier, buffering agent, emulsifying agent, sequestrant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water; insoluble in ethanol

pH (Vol. 4) 9.9 - 10.8 (1 in 100 soln)

Test for phosphate
(Vol. 4) Passes test

Test for sodium (Vol. 4) Passes test

PURITY

Loss on ignition (Vol. 4) Not more than 0.5% for anhydrous, 38-42% for decahydrate (105°, 4h then 550°, 30 min)

Water insoluble matter Not more than 0.2%
 Dissolve 10 g of the sample in 100 ml of hot water, and filter through a tared filtering crucible. Wash the insoluble residue with hot water, dry at 105° for 2 h, cool and weigh.

Fluoride Not more than 10 mg/kg (Method I or III)

<u>Arsenic</u> (Vol. 4)	Not more than 3 mg/kg
<u>Lead</u> (Vol. 4)	Not more than 4 mg/kg Determine using an atomic absorption technique 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	Dissolve an accurately weighed quantity of the sample, equivalent to about 500 mg of anhydrous $\text{Na}_4\text{P}_2\text{O}_7$, in 100 ml of water in a 400-ml beaker. Adjust the pH of the solution to 3.8 with hydrochloric acid, using a pH meter, then add 50 ml of a 1 in 8 solution of zinc sulfate ($125 \text{ g of } \text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in water, diluted to 1000 ml, filtered, and adjusted to pH 3.8) and allow to stand for 2 min. Titrate the liberated acid with 0.1 N sodium hydroxide until a pH of 3.8 is again reached. After each addition of sodium hydroxide near the end-point, time should be allowed for any precipitated zinc hydroxide to redissolve. Each ml of 0.1 N sodium hydroxide is equivalent to 13.30 mg of $\text{Na}_4\text{P}_2\text{O}_7$.

α -AMYLASE from *BACILLUS MEGATERIUM* expressed in *BACILLUS SUBTILIS*

Prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7 (1999), superseding tentative specifications prepared at the 37th JECFA (1990), published in FNP 52 (1992). An ADI 'not specified' was established at the 37th JECFA (1990)

SYNOMYS	Glycogenase
SOURCES	Produced by the controlled fermentation of <i>Bacillus subtilis</i> containing the gene for alpha-amylase from <i>Bacillus megaterium</i> . The strain of <i>Bacillus subtilis</i> is non-pathogenic and non-toxicogenic (for example ATCC 39,701). When fermentation is complete, the broth is clarified by centrifugation or filtration. The clarified broth containing the soluble enzyme is ultrafiltered to produce the desired activity.
Active principles	alpha-Amylase
Systematic names and numbers	1,4-alpha-D-Glucan glucanohydrolase (EC 3.2.1.1)
Reactions catalysed	Endohydrolysis of 1,4-alpha-D-glucosidic linkages in polysaccharides, containing three or more 1,4-alpha-linked D-glucose units
Secondary enzyme activities	Glycosyl transferase, protease
DESCRIPTION	Typically tan to dark brown liquid containing the active enzyme
FUNCTIONAL USES	Enzyme preparation Used in starch hydrolysis
GENERAL SPECIFICATIONS	Must conform to the General Specifications for Enzyme Preparations Used in Food Processing (See Volume Introduction)
CHARACTERISTICS	
IDENTIFICATION	
alpha-Amylase activity (Vol. 4)	The sample shows bacterial alpha-amylase activity

MICROCRYSTALLINE WAX

Prepared at the 55th JECFA (2000) and published in FNP 52 Add 8 (2000), superseding specifications prepared at the 49th JECFA (1997) and published in FNP 52 Add 5 (1997). A group ADI of 0-20 mg/kg bw was established at the 44th JECFA (1995).

SYNONYMS

Petroleum wax; INS No. 905c(i)

DEFINITION

Microcrystalline Wax is a refined mixture of solid, saturated hydrocarbons, mainly branched paraffin, obtained from petroleum

DESCRIPTION

Colourless or white, somewhat translucent, tasteless and odourless wax

FUNCTIONAL USES

Chewing gum base, protective coating, defoaming agent, surface finishing agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in water, very slightly soluble in ethanol, sparingly soluble in diethyl ether and hexane

Refractive index (Vol. 4) n (100, D): 1.434 - 1.448

Infrared absorption The infrared absorbance spectrum of the sample melted and prepared on a caesium or potassium bromide plate corresponds to the spectrum in the Appendix

PURITY

Viscosity, 100° Not less than 11 mm²/sec
See description under TESTS

Carbon number at 5% distillation point Not more than 5% of molecules with carbon number less than 25
See description under TESTS

Average molecular weight Not less than 500
See description under TESTS

Residue on ignition Not more than 0.1%
See description under TESTS

Colour Passes test
See description under TESTS

Sulfur Not more than 0.4%
See description under TESTS

Lead (Vol. 4)

Not more than 3 mg/kg

Determine using an atomic absorption technique 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 on Volume 4, "Instrumental methods".

Polycyclic aromatic hydrocarbons

The sample shall meet the following ultraviolet absorbance limits when subjected to the analytical procedure described under the TESTS.

nm	max. absorbance per cm path length
280 - 289	0.15
290 - 299	0.12
300 - 359	0.08
360 - 400	0.02

TESTS

PURITY TESTS

Viscosity, 100°

(ASTM D 445 Adopted with permission from the Annual Book of ASTM Standards copyright American Society for Testing and Materials, 100 Harbor Drive, West Conshohocken, PA 19428. Copies of the complete ASTM standard may be purchased direct from ASTM, phone 610-832-9585, fax: 610-832-9555, e-mail: service@astm.org , website: <http://www.astm.org>)

Use a viscometer of the glass capillary type, calibrated and capable of measuring kinematic viscosity with a repeatability exceeding 0.35% only in one case in twenty. Immerse the viscometer in a liquid bath at the temperature required for the test $\pm 0.1^\circ$ ensuring that at no time of the measurement will any portion of the sample in the viscometer be less than 20 mm below the surface of the bath. Charge the viscometer with sample in a manner directed by the design of the instrument. Allow the sample to remain in the bath for about 30 min. Where the design of the viscometer requires it, adjust the volume of sample to the mark. Use pressure to adjust the head level of the sample to a position in the capillary arm of the instrument about 5 mm ahead of the first mark. With the sample flowing freely, measure, in seconds (± 0.2 sec), the time required for the meniscus to pass from the first to the second timing mark. If the time is less than 200 s, select a viscometer with a capillary of smaller diameter and repeat the operation. Make a second measurement of the flow time. If two measurements agree within 0.2%, use the average for calculating the kinematic viscosity. If the measurements do not agree, repeat the determination after thorough cleaning and drying the viscometer.

$$\text{Viscosity, } 100^\circ \text{ (mm}^2/\text{sec)} = C \times t$$

where

C = calibration constant of the viscometer (mm²/s)

t = flow time (s)

Carbon number at 5% distillation point

(ASTM D 5442 See TEST for Viscosity, 100° for Copyright permission)
"Carbon number" is number of carbon atoms in a molecule. Determine the carbon number distribution of the sample by gas chromatography. Below is shown some typical working conditions for determination of up to carbon number 45.

Column length (m)	25	30	15
inside diameter (mm)	0.32	0.53	0.25
stationary phase	DB-1 methyl silicone	RTX-1 methyl silicone	DB-5 5% phenyl methyl silicone
film thickness (μm)	0.25	0.25	0.25
Carrier gas	helium	helium	helium
flow (ml/min)	1.56	5.0	2.3
Linear velocity (cm/sec)	33	35	60
Temperature program			
initial temperature	80°	80°	80°
rate (°/min)	10	8	5
final temperature	380°	340°	350°
Injection technique	cool on-column	cool on-column	cool on-column
Detector	FID	FID	FID
temperature	380°	340°	375°
Sample size (μl)	1.0	1.0	1.0

NOTE: By optimizing the length of separation column and/or column temperature, waxes with carbon number higher than 45 can also be included.

Standards for calibration and identification

Standard samples of normal paraffins covering the carbon number range of the sample of purity greater than 95%.

Linearity standard

Prepare a weighed mixture of n-paraffins covering the range between C₁₆ to C₆₅ and dissolve the mixture in cyclohexane. Use approximately equal amounts of each of the paraffins weighed with 1% accuracy. Solutions of 0.01 % (w/w) may be used. It is not necessary to include every n-paraffin in this mixture so long as it contains C₁₆, C₄₄, (C₆₀ if determination of higher carbon numbers is relevant) and at least one of every fourth n-paraffin. This standard must be capped tightly to prevent solvent loss.

Internal standard solution

Prepare a stock solution containing 0.5% (w/w) n-C₁₆ in n-hexane. (minimum purity of 98%) by accurately weighing approximately 0.4 g n-C₁₆ into a 100 ml volumetric flask. Add 100 ml of cyclohexane and reweigh. Record the mass of n-C₁₆ (W_{ISTD}) to within 0.001 g and the mass of the

stock solution (Ws) to within 0.1 g. Prepare a dilute solution of internal standard by diluting one part of stock solution with 99 parts of cyclohexane. Calculate the concentration of internal standard using the following equation:

$$C_{INST} = \frac{W_{ISTD}}{Ws} \times \frac{100}{100} \% \text{ (w/w)}$$

Where

C_{INST} = concentration of n-C₁₆ in the internal standard dilute solution in % (w/w)

W_{ISTD} = weight of n-C₁₆ used for the stock solution in g.

Ws = Weight of the stock solution in g

Check of solvent blank

Inject 1 µl of the solvent. No peaks must be detected within the retention time range over which the wax elutes.

Column resolution

Inject 1 µl of a solution of 0.05 % each of n-C₂₀ and n-C₂₄ in cyclohexane. The column resolution R not be less than 30 as calculated by the following equation:

$$R = \frac{2d}{1.699 (W1 + W2)}$$

Where

d = the distance in mm between the peak maxima of n-C₂₀ and n-C₂₄

W1 = the peak width in mm of half height of n-C₂₀

W2 = the peak width in mm of half height of n-C₂₄

Linearity

Analyze the linearity standard. The calculated mass response factors relative to hexadecane must be between 0.90 and 1.10.

Retention time repeatability

Analyze the linearity standard in duplicate. The retention times for duplicate analysis must not differ more than 0.10 min between duplicate runs.

Calibration for n-paraffin identification

Determine the retention time of each n-paraffin in the range from C₁₆ to C₄₄ (or C₆₀ if determination of higher carbon numbers is relevant) by injecting small amounts of each paraffin either separately or in known mixtures.

Sampling

Heat the sample to 10° above the temperature at which the wax is completely molten. Mix well by stirring.. Using a clean eyedropper, transfer a few drops to the surface of a clean sheet of aluminium foil, allow to solidify and break into pieces. Aluminium foil usually contains a thin film of oil from processing. This oil must be removed by rinsing the foil with solvents such as hexane or mineral spirits, prior to use.

Procedure

Accurately weigh about 0.0100 g of the sample (W_{sample}) obtained as described under sampling into a glass vial of approximately 15 ml capacity. Add approximately 12 ml of dilute internal standard solution, cap the vial and determine the exact weight of the added dilute internal standard solution (W_{ISTD}). Agitate the vial until the wax is completely dissolved using gentle heating if necessary.

Inject 0.5 to 1.0 μl of the sample solution. Record the chromatogram and store the detector signal. The peak from the internal standard must be completely resolved from the wax sample area. Based on the retention time as obtained under Calibration for n-paraffin identification, identify the normal paraffin peaks. Using a vertical drop to a horizontal baseline construction (see Figure 1), integrate the detector signal. Sum the area of all the peaks of each carbon number. By convention, the peaks assigned the carbon number n are those that elute between the valley immediately following the normal paraffin peak (C_{n-1}) and the corresponding valley following the next normal paraffin peak (C_n).

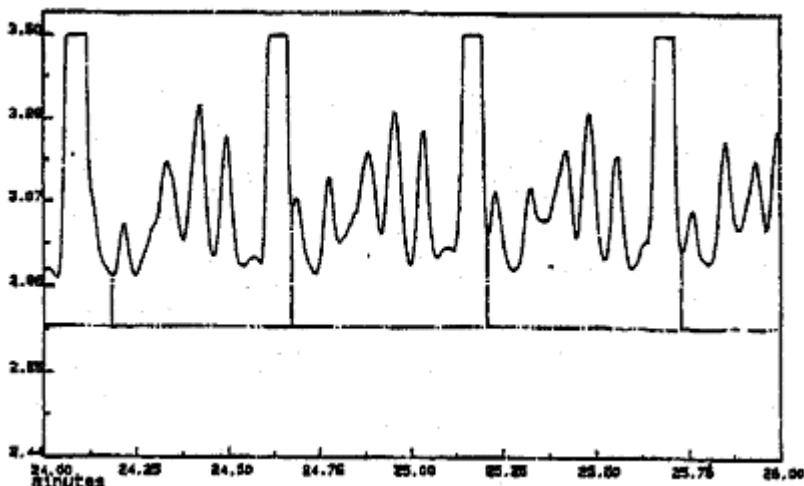


Figure 1. Carbon number summation (vertical drop to horizontal baseline)

Calculation

For each carbon number, calculate the content in % (w/w) by using the following equation:

$$C_i = \frac{A_i}{A_{\text{ISTD}}} \times RRF_i \times \frac{W_{\text{ISTD}}}{W_{\text{sample}}} \times C_{\text{ISTD}}$$

Where

C_i = content in % (w/w) of hydrocarbons with carbon number i

A_i = area sum of hydrocarbons with carbon number i

A_{ISTD} = the area of n-C₁₆ internal standard peak

RRF_i = the response factor relative to n-C₁₆

W_{ISTD} = the weight of dilute internal standard solution

W_{sample} = the weight of wax sample

C_{ISTD} = the concentration of n-C₁₆ in the dilute internal standard solution

The combined contents of components with carbon number less than 25 is not more than 5%.

Average molecular weight Using the carbon number distribution obtained in the test for "Carbon number at 5% distillation point" calculate the average molecular weight by the following formula:
Average molecular weight =

$$\frac{\sum_{i=1}^{i=i} C_i (14i+2)}{100}$$

where

i = the carbon number

C_i = the content in % of components having a carbon number of i

Residue on ignition Accurately weigh about 2 g of the sample in a tared porcelain or platinum dish and heat over a flame. The sample volatilizes without emitting an acrid odour. Ignite to not exceeding a very dull redness until free from carbon. Cool in a desiccator and weigh.

Colour Melt about 10 g of the sample on a steam bath, and pour 5 ml of the liquid into a clear-glass, 16 x 150-mm bacteriological test tube: the warm, melted liquid is not darker than a solution made by mixing 3.8 ml of ferric chloride TS(FNP 5) and 1.2 ml of cobaltous chloride TS (FNP 5) in a similar tube, the comparison of the two being made in reflected light against a white background, the tubes being held directly against the background at such an angle that there is no fluorescence.

Sulfur (ASTM D 2622 See TEST for Viscosity, 100° for Copyright permission)
Determine by X-ray spectrometry using the following conditions:

Apparatus

- X-ray spectrograph, equipped for soft ray detection in the 537 Å range
- Optical path of helium

- Pulse height analyzer or other means of energy discrimination

- Detector designed for detection of long wavelength X-rays

Analyzing crystal suitable for the dispersion of sulfur K_α X-rays within the angular range of the spectrometer employed. Pentaerythritol and germanium are the most popular although less reflective materials such as EDDT, ADP and quartz may be used.

X-ray tube of exiting sulfur K_α radiation.

X-ray tube with tungsten, platinum or chromium target

Sensitivity standards

Liquid petroleum materials containing sulfur in concentrations approximately in the middle of the calibration graph used for the test.

Calibration standards

Prepare calibration standards by careful weight dilution of di-n-butyl sulfide (high purity standard with a certified analysis, manufactured especially as a

calibration material for this method, available from Philips Petroleum Co., Bartlesville, OK, USA) with white oil (containing less than 5 mg/kg). Exact standards of approximately 0.100, 0.250, 0.500 and 1.0 % (w/w) should be prepared.

Calibration curve

Measure the net emitted sulfur radiation from each of the calibration standards. Plot the intensity, in terms of net counts per sec, against sulfur concentration.

To maintain the validity of the calibration curve with slight changes in the instruments sensitivity, measure the sensitivity standard at frequent intervals during the course of the days run. Establish the counting rate of this standard by measuring its intensity at frequent intervals during the preparation of the calibration curve. Calculate the correction factor for changes in daily instrument sensitivity by using the following equation:

$$F = \frac{A}{B}$$

Where

A = the counting rate of the sensitivity standard determined at the time of calibration

B = the counting rate of the sensitivity standard determined at the time of analysis

Procedure

Place the sample in an appropriate cell. Although sulfur radiation will penetrate through only a small distance in the sample, scatter from the sample cup and the sample may vary to such an extent that a specific amount or a minimum amount of the sample must be used.

Place the sample in the X-ray beam and allow the X-ray optical atmosphere to come to equilibrium. Determine the intensity of the sulfur Ka radiation at 5.373 Å by making counting rate measurements at the precise angular settings for this wavelength. Measure background count-rate at 5.190 Å.

Calculation

Calculate the content of sulfur in the sample using the following equation:

$$R = \left[\frac{C_K}{S_1} - \frac{C_B \times F'}{S_2} \right] \times F$$

R = the corrected net counting rate

C_K = total counts collected at 5.373 Å for the sample

S₁ = the time in sec required to collect C_K counts

C_B = total counts collected at 5.190 Å for background

S₂ = the time in sec required to collect C_B counts

F' = count-rate at 5.373 Å / count-rate at 5.190 Å for a sample not containing sulfur

F = the correction factor for changes in daily instrument sensitivity

Using the corrected net counting rate, read the sulfur concentration from the calibration curve.

Polycyclic aromatic hydrocarbons

General Instructions

Because of the sensitivity of the test, the possibility of errors arising from contamination is great. It is of the greatest importance that all glassware be scrupulously cleaned to remove all organic matter such as oil, grease, detergent residues, etc. Examine all glassware, including stoppers and stopcocks, under ultraviolet light to detect any residual fluorescent contamination. As a precautionary measure it is a recommended practice to rinse all glassware with purified isoctane immediately before use. No grease is to be used on stopcocks or joints. Great care to avoid contamination of wax samples in handling and to assure absence of any extraneous material arising from inadequate packaging is essential. Because some of the polynuclear hydrocarbons sought in this test are very susceptible to photo-oxidation, the entire procedure is to be carried out under subdued light.

Apparatus

- Separatory funnels: 250-ml, 500-ml, 1,000-ml, and preferably 2000-ml capacity, equipped with tetrafluoroethylene polymer stopcocks.
- Reservoir: 500 ml capacity, equipped with a 24/40 standard taper male fitting at the bottom and a suitable balljoint at the top for connecting to the nitrogen supply. The male fitting should be equipped with glass hooks.
- Chromatographic tube: 180 mm in length, inside diameter to be 15.7 mm \pm 0.1 mm, equipped with a coarse, fritted-glass disc, a tetrafluoroethylene polymer stopcock, and a female 24/40 standard tapered fitting at the opposite end. (Overall length of the column with the female joint is 235 mm). The female 24/40 standard tapered fitting at the opposite end.
- Disc: Tetrafluoroethylene polymer 2-inch diameter disc approximately 3/16-inch thick with a hole bored in the center to closely fit the stem of the chromatographic tube.
- Heating jacket: Conical, for 500-ml separatory funnel. (Used with variable transformer heat control).
- Suction flask: 250-ml or 500-ml filter flask.
- Condenser: 24/40 joints, fitted with a drying tube, length optional.
- Evaporation flask (optional): 250-ml or 500-ml capacity all-glass flask equipped with standard taper stopper having inlet and outlet tubes permitting passage of nitrogen across the surface of the liquid to be evaporated.
- Vacuum distillation assembly: All glass (for purification of dimethyl sulfoxide); 2 litre distillation flask with heating mantle; Vigreux vacuum-jacketed condenser (or equivalent) about 45 cm in length and distilling head with separable cold finger condenser. Use of tetrafluoroethylene polymer sleeves on the glass joints will prevent freezing. Do not use grease on stopcocks or joints.
- Spectrophotometric cells: Fused quartz cells, optical path length in the range of 5.000 ± 0.005 cm; also for checking spectrophotometer performance only, optical path length in the range 1.000 ± 0.005 cm. With distilled water in the cells, determine any absorbance differences.
- Spectrophotometer: Spectral range 250-400 nm with spectral slit width of 2 nm or less, under instrument operating conditions for these absorbance measurements, the spectrophotometer shall also meet the following

performance requirements:

Absorbance repeatability: ± 0.01 at 0.4 absorbance

Absorbance accuracy: ± 0.05 at 0.4 absorbance

Wavelength repeatability: ± 0.2 nm

Wavelength accuracy: ± 1.0 nm

- Nitrogen cylinder: Water-pumped or equivalent purity nitrogen in cylinder equipped with regulator and valve to control flow at 5 p.s.i.g.

Reagents and materials

- Organic solvents: All solvents used throughout the procedure shall meet the specifications and tests described in this specification. The isoctane, benzene, acetone, and methyl alcohol designated in the list following this paragraph shall pass the following test:

To the specified quantity of solvent in a 250-ml Erlenmeyer flask, add 1 ml of purified n-hexadecane and evaporate on the steam bath under a stream of nitrogen (a loose aluminium foil jacket around the flask will speed evaporation). Discontinue evaporation when not over 1 ml of residue remains. (To the residue from benzene add a 10 ml portion of purified isoctane, reevaporate, and repeat once to insure complete removal of benzene).

Alternatively, the evaporation time can be reduced by using the optional evaporation flask. In this case the solvent and n-hexadecane are placed in the flask on the steam bath, the tube assembly is inserted, and a stream of nitrogen is fed through the inlet tube while the outlet tube is connected to a solvent trap and vacuum line in such a way as to prevent any flow-back of condensate into the flask.

Dissolve the 1 ml of hexadecane residue in isoctane and make to 25 ml volume. Determine the absorbance in the 5 cm path length cells compared to isoactane as reference. The absorbance of the solution of the solvent residue (except for methyl alcohol) shall not exceed 0.01 per cm path length between 280 and 400 nm. For methyl alcohol this absorbance value shall be 0.00.

- Isooctane (2,2,4-trimethylpentane): Use 180 ml for the test described in the preceding paragraph. Purify, if necessary, by passage through a column of activated silica gel (Grade 12, Davison Chemical Company, Baltimore, Maryland, or equivalent) about 90 cm in length and 5 cm to 8 cm in diameter.

- Benzene, reagent grade: Use 150 ml for the test. Purify, if necessary, by distillation or otherwise.

- Acetone, reagent grade: Use 200 ml for the test. Purify, if necessary, by distillation.

- Eluting mixtures:

1. 10% benzene in isoctane: Pipet 50 ml of benzene into a 500-ml glass-stoppered volumetric flask and adjust to volume with isoctane, with mixing.

2. 20% benzene in isoctane: Pipet 50 ml of benzene into a 250-ml glass-stoppered volumetric flask, and adjust to volume with isoctane, with mixing.

3. Acetone-benzene-water mixture: Add 20 ml of water to 380 ml of

acetone and 200 ml of benzene, and mix.

- n-Hexadecane, 99% olefin-free: Dilute 1.0 ml of n-hexadecane to 25 ml with isoctane and determine the absorbance in a 5-cm cell compared to isoctane as reference point between 280-400 nm. The absorbance per cm path length shall not exceed 0.00 in this range. Purify, if necessary, by percolation through activated silica gel or by distillation.

- Methyl alcohol, reagent grade: Use 10.0 ml of methyl alcohol. Purify, if necessary, by distillation.

- Dimethyl sulfoxide: Pure grade, clear, water-white, m.p. 18° minimum. Dilute 120 ml of dimethyl sulfoxide with 240 ml of distilled water in a 500-ml separatory funnel, mix and allow to cool for 5-10 min. Add 40 ml of isoctane to the solution and extract by shaking the funnel vigorously for 2 min. Draw off the lower aqueous layer into a second 500 ml separatory funnel and repeat the extraction with 40 ml of isoctane. Draw off and discard the aqueous layer. Wash each of the 40 ml extractives three times with 50 ml portions of distilled water. Shaking time for each wash is 1 min. Discard the aqueous layers. Filter the first extractive through anhydrous sodium sulfate prewashed with isoctane (see Sodium sulfate under "Reagents and Materials" for preparation of filter), into a 250-ml Erlenmeyer flask, or optionally into the evaporating flask. Wash the first separatory funnel with the second 40 ml isoctane extractive, and pass through the sodium sulfate into the flask. Then wash the second and first separatory funnels successively with a 10 ml portion of isoctane, and pass the solvent through the sodium sulfate into the flask. Add 1 ml of n-hexadecane and evaporate the isoctane on the steam bath under nitrogen.

Discontinue evaporation when not over 1 ml of residue remains. To the residue, add a 10 ml portion of isoctane and reevaporate to 1 ml of hexadecane. Again, add 10 ml of isoctane to the residue and evaporate to 1 ml of hexadecane to insure complete removal of all volatile materials. Dissolve the 1 ml of hexadecane in isoctane and make to 25 ml volume. Determine the absorbance in 5 cm path length cells compared to isoctane as reference. The absorbance of the solution should not exceed 0.02 per cm path length in the 280-400 nm range. (Note - Difficulty in meeting this absorbance specification may be due to organic impurities in the distilled water. Repetition of the test omitting the dimethyl sulfoxide will disclose their presence. If necessary to meet the specification, purify the water by redistillation, passage through an ion-exchange resin, or otherwise).

Purify, if necessary, by the following procedure: To 1.5 L of dimethyl sulfoxide in a 2 l glass-stoppered flask, add 6.0 ml of phosphoric acid and 50 g of Norit A (decolorizing carbon, alkaline) or equivalent. Stopper the flask, and with the use of a magnetic stirrer (tetrafluoroethylene polymer coated bar) stir the solvent for 15 min. Filter the dimethyl sulfoxide through four thicknesses of fluted paper (18.5 cm) (Schleicher & Schuell No. 597, or equivalent). If the initial filtrate contains carbon fines, refilter through the same filter until a clear filtrate is obtained. Protect the sulfoxide from air and moisture during this operation by covering the solvent in the funnel and collection flask with a layer of isoctane. Transfer the filtrate to a 2-l separatory funnel and draw off the dimethyl sulfoxide into the 2-l distillation flask of the vacuum distillation assembly and distil at approximately 3 mm Hg pressure or less. Discard the first 200 ml fraction of the distillate and replace the distillate collection flask with a clean one. Continue the distillation until approximately 1 litre of the sulfoxide has been collected. At completion of the distillation, the reagent should be stored in glass-

stoppered bottles since it is very hygroscopic and will react with some metal containers in the presence of air.

- Phosphoric acid, 85% reagent grade
- Sodium borohydride, 98%
- Magnesium oxide (Sea Sorb 43, Food Machinery Company, Westvaco Division, distributed by chemical supplier firms, or equivalent): Place 100 g of the magnesium oxide in a large beaker, add 700 ml of distilled water to make a thin slurry, and heat on a steam bath for 30 min with intermittent stirring. Stir well initially to insure that all the absorbent is completely wetted. Using a Buchner funnel and a filter paper* of suitable diameter, filter with suction. Continue suction until water no longer drips from the funnel. Transfer the absorbent to a glass trough lined with aluminium foil (free from rolling oil). Break up the magnesia with a clean spatula and spread out the absorbent on the aluminium foil in a layer about 1-2 cm thick. Dry at $160\pm 1^\circ$ for 24 h. Pulverize the magnesia with mortar and pestle. Sieve the pulverized absorbent between 60-180 mesh. Use the magnesia retained on the 180-mesh sieve.
- Celite 545 (Johns-Manville Company, diatomaceous earth, or equivalent)
- Magnesium oxide-Celite 545 mixture (2+1) by weight: Place the magnesium oxide (60-180 mesh) and the Celite 545 in 2 to 1 proportions, respectively, by weight in a glass-stoppered flask large enough for adequate mixing. Shake vigorously for 10 min. Transfer the mixture to a glass trough lined with aluminium foil (free from rolling oil) and spread it out on a layer about 1 to 2 cm thick. Reheat the mixture at $160\pm 1^\circ$ for 2 h, and store in a tightly closed flask.
- Sodium sulfate, anhydrous, reagent grade, preferably in granular form: For each bottle of sodium sulfate reagent used, establish as follows the necessary sodium sulfate prewash to provide such filters required in the method: Place approximately 35 g of anhydrous sodium sulfate in a 30 ml coarse, fritted-glass funnel or in a 65 ml filter funnel with glass wool plug; wash with successive 15 ml portions of the indicated solvent until a 15 ml portion of the wash shows 0.00 absorbance per cm path length between 280 nm and 400 nm when tested as prescribed under "Organic solvents." Usually three portions of wash solvent are sufficient.

Procedure

Before proceeding with the analysis of a sample, determine the absorbance in a 5 cm path cell between 250 nm and 400 nm for the reagent blank by carrying out the procedure, without a wax sample, at room temperature, recording the spectra after the extraction stage and after the complete procedure as prescribed. The absorbance per centimeter path length following the extraction stage should not exceed 0.040 in the wavelength range from 250 to 400 nm; the absorbance per cm path length following the complete procedure should not exceed 0.070 in the wavelength range from 250 to 299 nm, inclusive, nor 0.045 in the wavelength range from 300 nm to 400 nm. If in either spectrum the characteristic benzene peaks in the 250-260 nm region are present, remove the benzene by the procedure under "Organic solvents" and record absorbance again.

Place 300 ml of dimethyl sulfoxide in a 1 liter separatory funnel and add 75 ml of phosphoric acid. Mix the contents of the funnel and allow to stand for 10 min. (The reaction between the sulfoxide and the acid is exothermic.

Release pressure after mixing, then keep funnel stoppered). Add 150 ml of isoocane and shake to preequilibrate the solvents. Draw off the individual layers and store in glass-stoppered flasks.

Place a representative 1 kg sample of wax, or if this amount is not available, the entire sample, in a beaker of a capacity about three times the volume of the sample and heat with occasional stirring on a steam bath until the wax is completely melted and homogenous. Weigh four 25 ± 0.2 g portions of the melted wax in separate 100 ml beakers. Reserve three of the portions for later replicate analyses as necessary. Pour one weighed portion immediately after remelting (on the steam bath) into a 500 ml separatory funnel containing 100 ml of the preequilibrated sulfoxide-phosphoric acid mixture that has been heated in the heating jacket at a temperature just high enough to keep the wax melted. (Note: In preheating the sulfoxide-acid mixture, remove the stopper of the separatory funnel at intervals to release the pressure).

Promptly complete the transfer of the sample to the funnel in the jacket with portions of the preequilibrated isoocane, warming the beaker, if necessary, and using a total volume of just 50 ml of the solvent. If the wax comes out of solution during these operations, let the stoppered funnel remain in the jacket until the wax redissolves. (Remove stopper from the funnel at intervals to release pressure).

When the wax is in solution, remove the funnel from the jacket and shake it vigorously for 2 min. Set up three 250 ml separatory funnels with each containing 30 ml of preequilibrated isoocane. After separation of the liquid phases, allow to cool until the main portion of the wax-isoocane solution begins to show a precipitate. Gently swirl the funnel when precipitation first occurs on the inside surface of the funnel to accelerate this process. Carefully draw off the lower layer, filter it slowly through a thin layer of glass wool fitted loosely in a filter funnel into the first 250 ml separatory funnel, and wash in tandem with the 30 ml portions of isoocane contained in the 250 ml separatory funnels. Shaking time for each wash is 1 min. Repeat the extraction operation with two additional portions of the sulfoxide-acid mixture, replacing the funnel in the jacket after each extraction to keep the wax in solution and washing each extractive in tandem through the same three portions of isoocane.

Collect the successive extractives (300 ml total) in a separatory funnel (preferably 2 liter), containing 480 ml of distilled water, mix, and allow to cool for a few minutes after the last extractive has been added. Add 80 ml of isoocane to the solution and extract by shaking the funnel vigorously for 2 min. Draw off the lower aqueous layer into a second separatory funnel (preferably 2 litre) and repeat the extraction with 80 ml of isoocane. Draw off and discard the aqueous layer. Wash each of the 80 ml extractives three times with 100 ml portions of distilled water. Shaking time for each wash is 1 min. Discard the aqueous layers. Filter the first extractive through anhydrous sodium sulfate prewashed with isoocane (see Sodium Sulfate under "Reagents and Materials" for preparation of filter) into a 250-ml Erlenmeyer flask (or optionally into the evaporation flask). Wash the first separatory funnel with the second 80 ml isoocane extractive and pass through the sodium sulfate. Then wash the second and first separatory

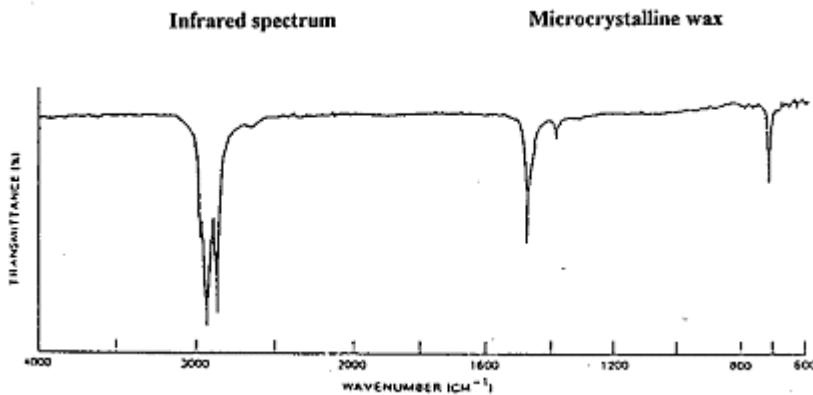
funnels successively with a 20 ml portion of isoctane and pass the solvent through the sodium sulfate into the flask. Add 1 ml of n-hexadecane and evaporate the isoctane on the steam bath under nitrogen. Discontinue evaporation when not over 1 ml of residue remains. To the residue, add a 10 ml portions of isoctane, reevaporate to 1 ml of hexadecane, and repeat this operation once more.

Quantitatively transfer the residue with isoctane to a 25 ml volumetric flask, make to volume, and mix. Determine the absorbance of the solution in the 5 cm path length cells compared to isoctane as reference between 280-400 nm (take care to lose none of the solution in filling the sample cell). Correct the absorbance values for any absorbance derived from reagents as determined by carrying out the procedure without a wax sample. If the corrected absorbance does not exceed the limits prescribed in the Characteristics, the wax meets the ultraviolet absorbance specifications. If the corrected absorbance per centimeter path length exceeds the limits prescribed in the Characteristics, proceed as follows: Quantitatively transfer the isoctane solution to a 125 ml flask equipped with 24/40 joint and evaporate the isoctane on the steam bath under a stream of nitrogen to a volume of 1 ml of hexadecane. Add 10 ml of methyl alcohol and approximately 0.3 g of sodium borohydride (Minimize exposure of the borohydride to the atmosphere. A measuring dipper may be used). Immediately fit a water-cooled condenser equipped with a 24/40 joint and with a drying tube into the flask, mix until the borohydride is dissolved, and allow to stand for 30 min at room temperature, with intermittent swirling. At the end of this period, disconnect the flask and evaporate the methyl alcohol on the steam bath under nitrogen until the sodium borohydride begins to come out of the solution. Then add 10 ml of isoctane and evaporate to a volume of about 2-3 ml. Again, add 10 ml of isoctane and concentrate to a volume of approximately 5 ml. Swirl the flask repeatedly to assure adequate washing of the sodium borohydride residues.

Fit the tetrafluoroethylene polymer disc on the upper part of the stem of the chromatographic tube, then place the tube with the disc on the suction flask and apply the vacuum (approximately 135 mm Hg pressure). Weigh out 14 g of the 2+1 magnesium oxide-Celite 545 mixture and pour the adsorbent mixture into the chromatographic tube in approximately 3 cm layers. After the addition of each layer, level off the top of the adsorbent with a flat glass rod or metal plunger by pressing down firmly until the adsorbent is well packed. Loosen the topmost few ml of each adsorbent layer with the end of a metal rod before the addition of the next layer. Continue packing in this manner until all the 14 g of the adsorbent is added to the tube. Level off the top of the adsorbent by pressing down firmly with a flat glass rod or metal plunger to make the depth of the adsorbent bed approximately 12.5 cm in depth. Turn off the vacuum and remove the suction flask. Fit the 500 ml reservoir onto the top of the chromatographic column and prewet the column by passing 100 ml of isoctane through the column. Adjust the nitrogen pressure so that the rate of descent of the isoctane coming off of the column is between 2-3 ml per min. Discontinue pressure just before the last of the isoctane reaches the level of the adsorbent. (Caution: Do not allow the liquid level to recede below the adsorbent level at any time). Remove the reservoir and decant the 5 ml isoctane concentrate solution onto the column and with slight pressure again allow the liquid level to

recede to barely above the adsorbent level. Rapidly complete the transfer similarly with two 5 ml portions of isoctane, swirling the flask repeatedly each time to assure adequate washing of the residue. Just before the final 5 ml wash reaches the top of the adsorbent, add 100 ml of isoctane to the reservoir and continue the percolation at the 2-3 ml per minute rate. Just before the last of the isoctane reaches the adsorbent level, add 100 ml of 10% benzene in isoctane to the reservoir and continue the percolation at the aforementioned rate. Just before the solvent mixture reaches adsorbent level, add 25 ml of 20% benzene in isoctane to the reservoir and continue the percolation at 2-3 ml per minute until all this solvent mixture has been removed from the column. Discard all the elution solvents collected up to this point. Add 300 ml of the acetone-benzene-water mixture to the reservoir and percolate through the column to elute the polynuclear compounds. Collect the eluate in a clean 1-l separatory funnel. Allow the column to drain until most of the solvent mixture is removed. Wash the eluate three times with 300 ml portions of distilled water, shaking well for each wash. (The addition of small amounts of sodium chloride facilitates separation). Discard the aqueous layer after each wash. After the final separation, filter the residual benzene through anhydrous sodium sulfate prewashed with benzene (see *Sodium sulfate* under "Reagents and Materials" for preparation of filter) into a 250-ml Erlenmeyer flask (or optionally into the evaporation flask). Wash the separatory funnel with two additional 20 ml portions of benzene which are also filtered through the sodium sulfate. Add 1 ml of n-hexadecane and completely remove the benzene by evaporation under nitrogen, using the special procedure to eliminate benzene as previously described under "Organic Solvents". Quantitatively transfer the residue with isoctane to a 25 ml volumetric flask and adjust the volume. Determine the absorbance of the solution in the 5 cm path length cells compared to isoctane as reference between 250 - 400 nm. Correct for any absorbance derived from the reagents as determined by carrying out the procedure without a wax sample. If either spectrum shows the characteristic benzene peaks in the 250 - 260 nm region, evaporate the solution to remove benzene by the procedure under "Organic Solvents". Dissolve the residue, transfer quantitatively, and adjust to volume in isoctane in a 25 ml volumetric flask. Record the absorbance again. If the corrected absorbance does not exceed the limits prescribed in the Characteristics the wax meets the ultraviolet absorbance specifications.

Appendix



TOLUENE

Prepared at the 25th JECFA (1981), published in FNP 19 (1981) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). An ADI 'not specified' was established at the 25th JECFA (1981) (No toxicological problems from residues as a food solvent in accordance with GMP)

SYNOMYS

Toluol, phenylmethane

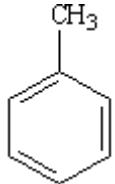
DEFINITION

Chemical names Toluene, methylbenzene

C.A.S. number 108-88-3

Chemical formula C₇H₈

Structural formula



Formula weight 92.13

Assay Not less than 99%

DESCRIPTION Clear, colourless liquid with a characteristic odour

FUNCTIONAL USES Extraction solvent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Very slightly miscible with water; miscible with ethanol

Specific gravity (Vol. 4) 0.864 - 0.870

PURITY

Distillation range (Vol. 4) Not more than 1° including 110.6°

Colour Not more than Colour Standard No. 20

Non-volatile residue (Vol. 4) Not more than 5 mg/100 ml

Sulfur compounds Passes test
See description under TESTS

Non-aromatic substances Not more than 0.2% v/v

Proceed as directed in the *Aromatic Hydrocarbons Determination* and calculate the content of non-aromatic substances by the method of area percentage (area normalization)

Benzene

Not more than 0.5% v/v

Proceed as directed under the *Aromatic Hydrocarbons Determination*

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

Sulfur compounds Negative for hydrogen sulfide and sulfur dioxide by the following test:

Reagents

- Lead acetate solution (saturated)
- Potassium Iodate Solution (10 g of KI₀₃/100 ml)
- Starch Paper: Dip strips of filter paper in starch solution and dry.

Procedure

Make a qualitative test for hydrogen sulfide (H₂S) and sulfur dioxide (SO₂) at the time of performing the distillation test. This is done by hanging a strip of filter paper moisten with the lead acetate solution and a strip of starch paper moistened with the potassium iodate solution on the end of the condenser tube. The strips are so placed that they are suspended in the upper part of the receiving cylinder so that drops of condensate pass between the strips without touching them. If, at the end of the test, the lead acetate paper shows discolouration, H₂S is present, but not SO₂. If the lead acetate paper shows no discolouration but the starch iodate paper develops a blue colour, SO₂ is present but not H₂S. If neither paper shows discolouration, neither H₂S nor SO₂ is present.

MANNITOL

Prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996) superseding specifications prepared at the 33rd JECFA (1988), published in FNP 38 (1988). Metals and arsenic specifications revised at the 57th JECFA (2001). An ADI 'not specified' was established at the 30th JECFA (1986)

SYNOMYS D-Mannitol, mannite, INS No. 421

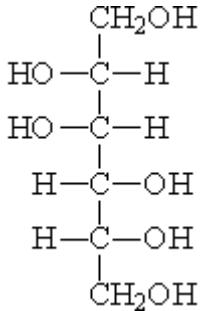
DEFINITION

Chemical names D-Mannitol

C.A.S. number 69-65-8

Chemical formula C₆H₁₄O₆

Structural formula



Formula weight 182.17

Assay Not less than 96.0% and not more than 102.0% on the dried basis

DESCRIPTION White, odourless, crystalline powder

FUNCTIONAL USES Sweetener, humectant, texturizer, stabilizer, bulking agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water, very slightly soluble in ethanol; practically insoluble in ether

Melting range (Vol. 4) 164 - 169°

Thin layer chromatography (Vol. 4) Passes test
Proceed as directed under *Thin Layer Chromatography of Polyols*

Use the following:

Standard solution

Dissolve 50 mg of reference standard mannitol (available from US Pharmacopeial Convention, Inc. 12601 Twinbrook Parkway, Rockville, MD 20852, USA) in 20 ml water

Test solution

Dissolve 50 mg of the sample in 20 ml of water

PURITY

Loss on drying (Vol. 4)

Not more than 0.3% (105°, 4 h)

Specific rotation (Vol. 4)

[alpha] 20, D: Between +23 and +25°

Accurately weigh and dissolve 2.0 g of sample and 2.6 g of disodium tetraborate in about 20 ml of water previously heated to about 30°, shake continuously for 15-30 min without further heating. Dilute the resulting clear solution to 25 ml with water.

pH (Vol. 4)

Between 5 and 8

Add 0.5 ml of a saturated solution of potassium chloride to 10 ml of a 10% w/v solution of the sample, then measure the pH.

Sulfated ash (Vol. 4)

Not more than 0.1%

Test 2 g of sample (Method I)

Chlorides (Vol. 4)

Not more than 70 mg/kg

Test 10 g of sample by the Limit Test using 2.0 ml of 0.01N hydrochloric acid in the control

Sulfates (Vol. 4)

Not more than 100 mg/kg

Test 10 g of sample by the Limit Test using 2.0 ml of 0.01N sulfuric acid in the control

Nickel (Vol. 4)

Not more than 2 mg/kg

Proceed as directed under *Nickel in Polyols*

Reducing sugars(Vol. 4)

Not more than 0.3%

Proceed as directed under *Reducing Substances (as glucose)*, Method II. The weight of cuprous oxide shall not exceed 50 mg

Total sugars(Vol. 4)

Not more than 1.0% (as glucose)

Transfer 2.1 g of the sample into a 250 ml flask fitted with a ground glass joint, add 40 ml of 0.1N hydrochloric acid, attach a reflux condenser, and reflux for 4 h. Transfer the solution to a 400 ml beaker, rinsing the flask with about 10 ml of water, neutralize with 6N sodium hydroxide and proceed as directed in the *General Method for Reducing Substances (as glucose)* Method II. The weight of the cuprous oxide shall not exceed 50 mg.

Lead (Vol. 4)

Not more than 1 mg/kg

Determine using an atomic absorption technique 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

Determine the mannitol content of the sample using *liquid chromatography*

ASSAY

(see Volume 4)

Apparatus

Liquid chromatograph (HPLC)

Detection: differential refractometer maintained at constant temperature

Integrator recorder

Column: AMINEX HPX 87 C (resin in calcium form), length 30 cm, internal diameter 9 mm

Eluent: double distilled degassed water (filtered through Millipore membrane filter 0.45 µm)

Chromatographic conditions

Column temperature: 85±0.5°

Eluent flow rate: 0.5 ml/min

Standard preparation

Dissolve an accurately weighed quantity of standard reference mannitol in water to obtain a solution having known concentration of about 10.0 mg of mannitol per ml.

Sample preparation

Transfer about 1 g of the sample accurately weighed to a 50 ml volumetric flask, dilute with water to volume and mix.

Procedure

Separately inject equal volumes (about 20 µl) of the sample preparation and the standard preparation into the chromatograph. Record the chromatograms and measure the response of the mannitol peak.

Calculate the quantity, in mg, of mannitol in the portion of sample taken by the following formula:

$$50 \times C \times \frac{R_U}{R_S}$$

where

C = the concentration, in mg per ml, of mannitol in the standard preparation

R_U = the peak response of the sample preparation

R_S = the peak response of the standard preparation.

GELLAN GUM

Prepared at the 79th JECFA (2014), published in FAO JECFA Monographs 16 (2014), superseding specifications prepared at the 49th JECFA (1997), published in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). An ADI 'not specified' was established at the 37th JECFA (1990)

SYNONYM	INS No. 418
DEFINITION	Gellan gum is a high molecular weight polysaccharide gum produced by a pure culture fermentation of a carbohydrate by <i>Pseudomonas elodea</i> , purified by recovery with ethanol or 2-propanol, dried, and milled. The high molecular weight polysaccharide is principally composed of a tetrasaccharide repeating unit of one rhamnose, one glucuronic acid, and two glucose units, and is substituted with acyl (glyceryl and acetyl) groups as the O-glycosidically-linked esters. The glucuronic acid is neutralized to a mixed potassium, sodium, calcium, and magnesium salt. It usually contains a small amount of nitrogen containing compounds resulting from the fermentation procedures.
C.A.S. number	71010-52-1
Formula weight	Approximately 500,000
Assay	Yields, on the dried basis, not less than 3.3% and not more than 6.8% of carbon dioxide (CO ₂).

DESCRIPTION	Off-white powder
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FUNCTIONAL USES	Thickener, gelling agent, stabilizer
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CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Soluble in water, forming a viscous solution; insoluble in ethanol
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<u>Gel test with calcium ion</u>	Add 1.0 g of the sample to 99 ml of water, and stir for about 2 h, using a motorized stirrer having a propeller-type stirring blade. Draw a small amount of this solution into a wide bore pipet and transfer into a 10% solution of calcium chloride. A tough worm-like gel will be formed immediately.
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<u>Gel test with sodium ion</u>	Add 1.0 g of the sample to 99 ml of water, and stir for about 2 h, using a motorized stirrer having a propeller-type stirring blade. Add 0.50 g of sodium chloride, heat to 80° with stirring, and hold at 80° for 1 min. Allow the solution to cool to room temperature. A firm gel is formed.
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PURITY

<u>Loss on drying</u> (Vol. 4)	Not more than 15% (105°, 2½ h)
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<u>Nitrogen</u> (Vol. 4)	Not more than 3%
<u>Residual solvents</u>	Not more than 50 mg/kg of ethanol; not more than 750 mg/kg of 2-propanol See description under TESTS
<u>Microbiological criteria</u>	Total plate count: Not more than 10,000 cfu/g <i>E. coli</i> : Negative by test <i>Salmonella</i> : Negative by test Yeasts and moulds: Not more than 400 cfu/g See description under TESTS
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an AAS (Electrothermal atomization technique) appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under "General Methods, Metallic Impurities").

TESTS

PURITY TESTS

<u>Residual solvents</u>	<u>Standard solutions</u> Transfer 100.0 mg of chromatographic quality ethanol into a 200-ml volumetric flask and 150.0 mg of 2-propanol into a 20-ml volumetric flask, then dilute to volume with water. Pipet each 1 ml of solutions into a 100-ml volumetric flask and dilute to volume with water as standard solution A. Pipet 10 and 5 ml of standard A into two separate 20-ml volumetric flasks and dilute to volume with water as standard solution B and standard solution C.
	<u>Chromatography conditions</u> Column: 25% diphenyl-75% dimethylpolysiloxane (60 m x 0.25 mm i.d. with 1.4 µm-film) [Aquatic-2 (GL-Sciences Inc.) or equivalent] Carrier gas: Helium Flow rate: 1.8 ml/min Detector: Flame ionization detector (FID) Temperatures: - Injection port: 250° - Oven: Hold for 5 min at 40°, then 40° to 92° at 4°/min - Detector: 260° The retention times of ethanol and 2-propanol are about 6.5 and 7.5 min, respectively.

Samples

Weigh accurately 0.10 g of the sample into each of four 20 ml head-space vials. Add a magnetic stirring bar and 10 ml of either water, standard solution A, B or C into each vial and seal. After standing vials overnight at room temperature, stir the solution in the vials for 1 min.

Procedure

Place the sample vial in the sample tray on head-space gas chromatograph. Heat vials at 60° for 40 min with continuous agitation. Inject 1.0 ml of the head space gas (Syringe temperature: 100°,

Transfer temperature: 120°) in the vial into the chromatograph and measure the peak area for ethanol and 2-propanol. Plot the relationship between the added amount against the peak area for ethanol or 2-propanol. Extrapolate the x-intercept for ethanol and 2-propanol (w_e and w_p). Calculate the concentration of ethanol and 2-propanol from;

$$\text{Ethanol (mg/kg)} = w_E / W$$

$$\text{2-Propanol (mg/kg)} = w_P / W$$

Where

W is weight of sample (g).

Microbiological criteria

Total plate count

Using aseptic technique, disperse 1 g of sample into 99 ml of phosphate buffer and use a Stomacher, shaker or stirrer to fully dissolve. Limit dissolving time to about 10 min and then pipette 1 ml of the solution into separate, duplicate, appropriately marked petri dishes. Pour over the aliquot of sample in each petri dish 12-15 ml of Plate Count Agar previously tempered to 44-46°. Mix well by alternate rotation and back and forth motion of the plates, allow the agar to solidify. Invert the plates and incubate for 48±2 h at 35±1°.

After incubation count the growing colonies visible on each plate and record the number of colonies. Take the average of both plates, and multiply by the sample dilution factor, 100. Where no colonies are visible, express the result as less than 100 cfu/g.

E. coli

Using aseptic technique, disperse 1 g of sample in 99 ml of Lactose broth using either a Stomacher, shaker or stirrer to fully dissolve the sample. Limit the dissolving time to about 15 min and then lightly seal the container and incubate the broth for 18-24 h at 35±1°. Using a sterile pipette, inoculate 1 ml of the incubate into a tube containing 10 ml GN broth. Incubate for 18-24 h and then streak any GN broths showing positive growth or gas production onto duplicate plates of Levine EMB agar. Incubate the plates for 24±2 h at 35±1° and then examine for colonies typical of *E. coli* i.e. showing strong purple growth with dark centre and a green metallic sheen sometimes spreading onto the agar. Record any typical *E. coli* colonies as presumptive positive, otherwise negative.

Streak any well isolated suspect colonies onto a plate of PCA and incubate for 18-24 h at 35±1°. Perform a Gram stain on any growth to confirm it is Gram negative. If so, disperse any colony growth into a small volume of 0.85% saline and perform chemical tests to confirm the identity of the bacterial growth. This can most conveniently be done by using API 20E or Micro ID strips or equivalent systems.

After completion of the tests, identify the organism from the Identification manual of the system used and record the final result.

Media

GN Broth (Gram Negative Broth)

Peptone 20.0 g

Dextrose 1.0 g

Mannitol 2.0 g

Sodium citrate 5.0 g

Sodium deoxycholate 0.5 g

Potassium phosphate (dibasic) 4.0 g
Potassium phosphate (monobasic) 1.5 g
Sodium chloride 5.0 g
Make up to 1 litre with distilled or de-ionised water, pH 7.0±0.2 at 25°.

Salmonella

Using aseptic technique, disperse 5 g of sample into 200 ml of sterile lactose broth using either a Stomacher, shaker or stirrer to maximise dissolution over a 15 min period. Loosely seal the container and incubate at 35±1° for 24±2 h.

Continue as per method on page 104 in Volume 4 (under "General methods, *Salmonella*"). Identification can be more conveniently done using API or Micro ID systems or equivalent.

Yeasts and moulds

Using aseptic technique, disperse 1 g of sample into 99 ml of phosphate buffer and use a Stomacher, shaker or stirrer to fully dissolve. Limit dissolving time to about 10 min and then pipette 1 ml of the solution into separate, duplicate, appropriately marked petri dishes. Pour over the aliquot of sample in each petri dish 15-20 ml of Potato dextrose Agar (either acidified or containing antibiotic) previously tempered to 44-46°. Mix well by alternate rotation and back and forth motion of the plates, and allow the agar to solidify. Invert the plates and incubate for 5 days at 20-25°.

After incubation, count the growing colonies visible on each plate using a colony counter and record the number of colonies. Separate the yeasts from the moulds according to their morphology and count them separately. Take the average of both plates and multiply by the sample dilution factor, 100. Where no colonies are visible, express the result as less than 100 cfu/g.

METHOD OF ASSAY Proceed as directed in the test for Alginates Assay (Carbon Dioxide Determination by Decarboxylation) in Volume 4 (under "Assay Methods"), using 1.2 g of the sample.

ERYTHROSINE

Prepared at the 41st JECFA (1993), published in FNP 52 Add 2 (1993) superseding specifications prepared at the 37th JECFA (1990), published in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002). An ADI of 0-0.1 mg/kg bw was established at the 37th JECFA (1991)

SYNOMYS

CI Food Red 14, FD&C Red No. 3; C.I. (1975) No. 45430 INS No. 127

DEFINITION

Consists essentially of disodium salt of 9-(o-carboxyphenyl)-6-hydroxy-2,4,5,7-tetraiodo-3-isoxanthone monohydrate and subsidiary colouring matters together with water, sodium chloride and/or sodium sulfate as the principal uncoloured components.

May be converted to the corresponding aluminium lake in which case only the *General Specifications for Aluminium Lakes of Colouring Matters* shall apply.

Chemical names

Disodium salt of 9-(o-carboxyphenyl)-6-hydroxy-2,4,5,7-tetraiodo-3-isoxanthone monohydrate

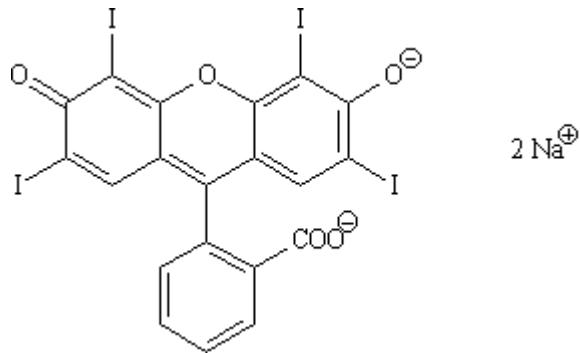
C.A.S. number

16423-68-0

Chemical formula

$C_{20}H_6I_4Na_2O_5 \cdot H_2O$

Structural formula



Formula weight

897.88

Assay

Not less than 87% total colouring matters

DESCRIPTION

Red powder or granules

FUNCTIONAL USES

Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water and in ethanol

Identification of colouring matters (Vol. 4) Passes test

PURITY

<u>Loss on drying at 135°</u> (Vol. 4)	Not more than 13% together with chloride and sulfate calculated as sodium salts
<u>Inorganic iodides</u>	Not more than 0.1% calculated as sodium iodide See description under TESTS
<u>Water insoluble matter</u> (Vol. 4)	Not more than 0.2%
<u>Zinc</u> (Vol. 4)	Not more than 50 mg/kg
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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."
<u>Subsidiary colouring matters</u> (Vol. 4)	Not more than 4% (except fluorescein) Use the following conditions: Developing solvent: No. 5 Height of ascent of solvent front: 17 cm Note: Take special care not to allow the chromatograms to be exposed to direct sunlight.
<u>Fluorescein</u>	Not more than 20 mg/kg See description under TESTS
<u>Organic compounds other than colouring matters</u> (Vol. 4)	Tri-iodoresorcinol: Not more than 0.2% 2-(2,4-dihydroxy-3,5-di-iodobenzoyl) benzoic acid: Not more than 0.2% Proceed as directed under <i>Column chromatography</i> , using, for example, the following absorptivities: 2(2,4-dihydroxy-3,5-di-iodobenzoyl)benzoic acid: $0.047 \text{ mg L}^{-1}\text{cm}^{-1}$ at 348 nm (alkaline) Tri-iodoresorcinol: $0.079 \text{ mg L}^{-1}\text{cm}^{-1}$ at 223 nm (acidic).
<u>Ether extractable matter</u> (Vol. 4)	From a solution of pH not less than 7, not more than 0.2%

TESTS

PURITY TESTS

<u>Inorganic iodides</u>	Weigh 1.0 g of the sample into a 100-ml beaker. Add 75 ml distilled water and the magnetic follower. Stir to dissolve. Immerse a iodide specific electrode and a reference electrode in the solution and set a suitable millivoltmeter to read the potential of the system in millivolts. Add 0.001 M silver nitrate solution from a burette initially in 0.5 ml aliquots, reducing these to 0.1 ml as the end-point approaches as indicated by an
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increasing change in potential for each addition. After allowing time for the reading to stabilize, record the millivolt readings after each addition. Continue the titration until further additions make little change in the potential.

Plot the millivolt readings against the volume of silver nitrate solution added. The equivalent point is the volume corresponding to the maximum slope of the curve. The percentage of sodium iodide in sample is: Titre x 0.015%

Fluorescein

Principle

The fluorescein is separated from the sample by TLC and compared with a standard chromatogram prepared from fluorescein at the concentration corresponding to the limit figure.

Solvent

Methanol+water+ammonia (s.g. 0.890) (500 ml+400 ml+100 ml)

Sample

Weigh 1.0 g of the sample, dissolve in about 50 ml solvent and dilute to 100 ml in a volumetric flask.

Standard

Weigh an amount of fluorescein, previously purified by recrystallisation from ethanol, equal to 1 g x the colouring matter content of the sample as determined under Assay. Dissolve in water (or in water with 10 ml ammonia s.g. 0.890 if fluorescein-free acid is being used) and dilute to 100 ml. Make further sequential dilutions as follows:

- 1 ml to 100 ml with water
- 1 ml to 100 ml with water
- 20 ml to 100 ml with solvent

Chromatography solvent

n-Butanol+water+ammonia (s.g. 0.890)+ethanol (100 ml+44 ml+1 ml+22.5 ml)

Procedure

Spot 25 μ l of the sample and standard solutions side by side on a cellulose plate. Develop for 16 h in the chromatography solvent. Allow the plate to dry. View under a UV light source and compare the fluorescence of the standard with the fluorescence of the corresponding area on the chromatogram of the sample. The intensity of the latter shall not be greater than that of the former.

Note: Take special care not to allow the chromatograms to be exposed to direct sunlight.

METHOD OF ASSAY

Dissolve about 1 g of the sample, accurately weighed, in 250 ml of water, transfer to a clean 500-ml beaker, add 8.0 ml of 1.5 N nitric acid and stir well. Filter through a sintered glass crucible (porosity 3, diameter 5 cm) which has been weighed containing a small glass stirring rod. Wash thoroughly with 0.5% nitric acid until the filtrate gives no turbidity with silver nitrate TS, and then wash with 30 ml water. Dry to constant weight at $135\pm 5^\circ$, carefully breaking up the precipitate by means of the glass rod.

Cool in a desiccator and weigh.

$$\text{Total colouring matters} = \frac{\text{weight of residue} \times 107.4}{\text{weight of sample}} \%$$

Determination of Hydrochloric Acid-insoluble Matter in Erythrosine Lake

Reagents

- Concentrated hydrochloric acid
- Hydrochloric acid 0.5% v/v
- Dilute ammonia solution (dilute 10 ml ammonia, s.g. 0.890 to 100 ml with water).

Procedure

Accurately weigh approximately 5 g of the lake into a 500-ml beaker. Add 250 ml water and 60 ml concentrated hydrochloric acid. Boil to dissolve the alumina while the Erythrosine converts to its "free acid" form, which is insoluble in acid. Filter through a tared No. 4 sintered glass crucible. Wash the crucible with a small amount of hot 0.5% hydrochloric acid and then with some hot distilled water. Remove the acid filtrate from the filter flask, replace the crucible and wash with hot dilute ammonia solution until the washings are colourless. Dry the crucible to constant weight at 135°. Express the residue as a percentage of the weight taken.



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Residue Monograph prepared by the meeting of the Joint FAO/WHO Expert
Committee on Food Additives (JECFA), 31st Meeting 1987

MONOSODIUM L-GLUTAMATE

This monograph was also published in FAO FNP 38 (1988) and FNP 52 (1992)

MONOSODIUM L-GLUTAMATE

Prepared at the 31st JECFA (1987), published in FNP 38 (1988) and in FNP 52 (1992). Metals and arsenic specifications revised at the 57th JECFA (2001). A group ADI 'not specified' for glutamic acid and its Ammonium, Ca, K, Mg & Na salts, was established at the 31st JECFA (1987)

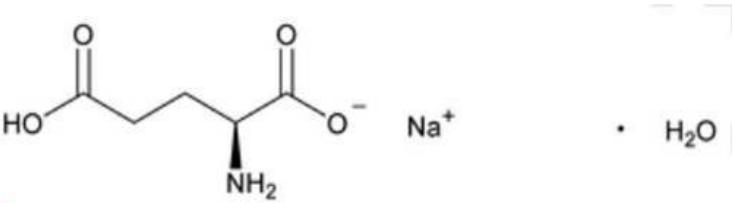
SYNONYMS	Sodium glutamate, MSG, INS No. 621
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DEFINITION

Chemical names	Monosodium L-glutamate monohydrate, glutamic acid monosodium salt monohydrate
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C.A.S. number	6106-04-3
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Chemical formula	$\text{C}_5\text{H}_8\text{NNaO}_4 \cdot \text{H}_2\text{O}$
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Structural formula	
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Formula weight	187.13
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Assay	Not less than 99.0% on the dried basis
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DESCRIPTION	White, practically odourless crystals or crystalline powder
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FUNCTIONAL USES	Flavour enhancer
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CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Freely soluble in water; sparingly soluble in ethanol; practically insoluble in ether
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<u>Test for glutamate</u> (Vol. 4)	Passes test
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<u>Test for sodium</u> (Vol. 4)	Passes test
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PURITY

<u>Loss on drying</u> (Vol. 4)	Not more than 0.5% (98°, 5 h)
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<u>pH</u> (Vol. 4)	6.7 - 7.2 (1 in 20 soln)
<u>Specific rotation</u> (Vol. 4)	[alpha] 20, D: : Between +24.8 and +25.3° (10% (w/v) solution in 2N hydrochloric acid)
<u>Chlorides</u> (Vol. 4)	Not more than 0.2% Test 0.07 g of the sample as directed in the Limit Test using 0.4 ml of 0.01 N hydrochloric acid in the control
<u>Pyrrolidone carboxylic acid</u> (Vol. 4)	Passes test
<u>Lead</u> (Vol. 4)	Not more than 1 mg/kg Determine using an atomic absorption technique 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	Dissolve about 200 mg of the sample, previously dried and weighed accurately, in 6 ml of formic acid, and add 100 ml of glacial acetic acid. Titrate with 0.1 N perchloric acid determining the end-point potentiometrically. Run a blank determination in the same manner and correct for the blank. Each ml of 0.1 N perchloric acid is equivalent to 9.356 mg of $C_5H_8NNaO_4 \cdot H_2O$



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Residue Monograph prepared by the meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), 84th meeting 2017

Steviol Glycosides from *Stevia rebaudiana* Bertoni

This monograph was also published in: *Compendium of Food Additive Specifications. Joint FAO/WHO Expert Committee on Food Additives (JECFA), 84th meeting 2017. FAO JECFA Monographs 20*

STEViol GLYCOSIDES FROM STEVIA REBAUDIANA BERTONI

Prepared at the 84th JECFA (2017) and published in FAO JECFA Monographs 20 (2017), superseding tentative specifications prepared at the 82nd JECFA (2016) and published in FAO JECFA Monographs 19 (2016). An ADI of 0 - 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

SYNONYMS

INS No. 960

DEFINITION

Steviol glycosides consist of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni. The product is obtained from the leaves of *Stevia rebaudiana* Bertoni. The leaves are extracted with hot water and the aqueous extract is passed through an adsorption resin to trap and concentrate the component steviol glycosides. The resin is washed with a solvent alcohol to release the glycosides and the product is recrystallized from methanol or aqueous ethanol. Ion exchange resins may be used in the purification process. The final product may be spray-dried.

Chemical name

See Appendix 1

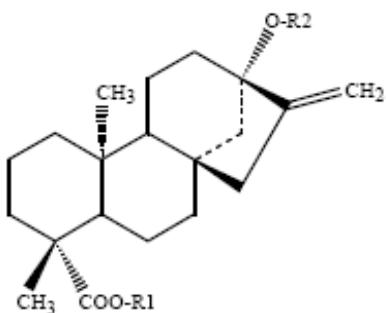
C.A.S. number

See Appendix 1

Chemical formula

See Appendix 1

Structural formula



Steviol (R1 = R2 = H) is the aglycone of the steviol glycosides.

Glc, Rha, Fru, deoxyGlc, Gal, Ara and Xyl represent, respectively, glucose, rhamnose, fructose, deoxyglucose xylose, galactose, arabinose and xylose sugar moieties.

Assay Not less than 95% of total of steviol glycosides, on the dried basis, determined as the sum of all compounds containing a steviol backbone conjugated to any number, combination or orientation of saccharides (glucose, rhamnose, fructose, deoxyglucose xylose, galactose, arabinose and xylose) occurring in the leaves of *Stevia rebaudiana* Bertoni.

DESCRIPTION White to light yellow powder, odourless or having a slight characteristic odour. About 200 - 300 times sweeter than sucrose.

FUNCTIONAL USES Sweetener

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in a mixture of ethanol and water (50:50)

HPLC chromatographic profile The main peaks in a chromatogram obtained by analysing a sample following the procedure in METHOD OF ASSAY correspond to steviol glycosides

pH (Vol. 4) Between 4.5 and 7.0 (1 in 100 solution)

PURITY

Total ash (Vol. 4) Not more than 1%

Loss on drying (Vol. 4) Not more than 6% (105°, 2 h)

Residual solvents (Vol. 4) Not more than 200 mg/kg methanol and not more than 5000 mg/kg ethanol (Method I, General Methods, Organic Components, Residual Solvents)

Arsenic (Vol. 4) Not more than 1 mg/kg
Determine using a method appropriate to the specified level (Use Method II to prepare sample solution). The selection of sample size and method of sample preparation may be based

on the principles of the methods described in Vol. 4 (under “General Methods, Metallic Impurities”).

Lead (Vol. 4)

Not more than 1 mg/kg

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

Microbiological criteria
(Vol. 4)

Total (aerobic) plate count: Not more than 1,000 CFU/g

Yeasts and moulds: Not more than 200 CFCU/g

E. coli: Negative in 1 g

Salmonella: Negative in 25 g

METHOD OF ASSAY

Determine the percentages of major steviol glycosides (those with analytical standards) using Method A (HPLC, Vol. 4). Confirm the presence of each minor steviol glycoside (compounds where analytical standards are not available) using Method B (HPLC-MS). Calculate the concentration of the minor compounds using respective molecular mass corrected UV peak area against the rebaudioside A UV standard curve. Calculate their sum and express the content on the dried basis.

Method A: Determination of Major Steviol Glycosides by HPLC:

Reagents:

- Acetonitrile: HPLC grade with transmittance more than 95% at 210 nm.
- Deionized water: HPLC grade
- Standards (Reference and Quality Control Standards): Stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, rebaudioside M, rebaudioside N, rebaudioside O, dulcoside A, rubusoside and steviolbioside. Chromadex, USA; Wako Pure Chemical Industries Ltd., Japan; Sigma-Aldrich; US Pharmacopeia or equivalent.

Note: Standards of other steviol glycosides, which may become commercially available in the future, may also be included. The analyst should consider that the inclusion of additional standards will lower the concentration of the mixed standards described below.

Preparation of Steviol Glycosides Standard Solutions:

Prepare individual stock standard solutions (1.5 mg/mL) in water:acetonitrile (7:3)

Prepare mixed standard solution (115 µg/mL) by mixing 1.0 mL each individual stock standard solutions.

Prepare Peak Identification Standard Solutions (0.1 mg/mL) from individual stock standard solutions in water:acetonitrile (7:3).

Prepare mixed working standard solutions in the range of 20 – 100 µg/mL by following appropriate dilution of mixed standard solution (b) with water:acetonitrile (7:3).

Prepare quality control and system suitability individual stock standard solutions (1.5 mg/mL) as well as mixed standard solution (115 µg/mL) using standards from a different batch /manufacturer (if available).

Prepare quality control mixed working standard solutions (40 and 80 µg/mL) and system suitability standard (52 µg/mL) by following appropriate dilutions of mixed standard solution

Preparation Sample Solution:

Accurately weigh 50 mg of sample and quantitatively transfer into a 50-mL volumetric flask. Add about 20 mL of water:acetonitrile (7:3), sonicate and shake well to dissolve the sample and make up to volume.

Procedure:

Use a HPLC consisting of a high precision binary pump and an auto sampler (capable of operating at 2 -8°); Diode-Array detector @ UV at 210 nm; and Mass Spectrometric Detector (Electrospray Negative Ionisation over a mass range from 50 to 1500 m/z using a unit mass resolution, For use in Method B below) connected in series. Agilent 1200 with Waters Quattro or equivalent:

- Column: Luna 5µ C18(2), 100A, (150 mm x 4.6 mm, 5µm, Phenomenex) or Capcell pak C₁₈ MG II (250 mm x 4.6 mm, 5µm, Shiseido Co. Ltd) or equiv.
- Column temperature: 50°
- Autosampler temperature: 2 – 8°
- Injection volume: 10 µl
- Mobile phase A: Deionised or LC-MS grade water (0.2 µm filtered)
- Mobile phase B: LC-MS grade Acetonitrile (0.2 µm filtered)

HPLC Gradient Time table:

Time (min)	% Solvent A	% Solvent B	Flow Rate (mL/min)
0.00	85.0	15.0	0.3
40.0	70.0	30.0	0.3
60.0	55.0	45.0	0.3
70.0	55.0	45.0	0.3
70.1	85.0	15.0	0.3
80.0	85.0	15.0	0.3

Inject peak identification standard solutions (c), identify peaks and calculate relative retention times (RRT) with respect to rebaudioside A (Typical RRT values are given in Appendix-3). See Appendix 2 for an example of a chromatogram obtained using the method.

Inject working mixed standard solutions (d) and construct standard curves for each steviol glycoside. Inject quality control and system suitability standard solutions (f) to ensure a satisfactory working system.

Inject prepared samples. Dilute sample solution, if required, to bring the concentration of each analyte within the standard curve range. Make duplicate injections. Deduce concentration of each steviol glycoside from its corresponding standard curve and obtain average concentration in sample solution ($\mu\text{g/mL}$).

Calculation of major steviol glycosides content:

Calculate the concentration of each steviol glycoside in the sample solution using the following formula:

$$\text{Conc } (\%w/w) = c_{\text{sample}} \times \frac{100}{W_{\text{sample}}}$$

Where:

- C_{sample} is the average concentration ($\mu\text{g/mL}$) in the sample solution
- W_{sample} is the weight of sample (μg) in 1 mL of sample solution (~1000 $\mu\text{g/mL}$)

Note: Above calculation will change if additional dilutions were done prior to LC injection. Analyst shall account such dilutions in the calculation.

Calculate the percentage of major steviol glycosides in the sample by summation of percentages of individual steviol glycosides in the sample (A).

Note: If the concentration of major steviol glycosides in the sample is <95%, then analyst should perform Method B.

Method B: Determination of Minor Steviol Glycosides by HPLC-MS:

HPLC-MS conditions may vary based on the manufacturer and model of the system used. Analyst should set the conditions following the manufacturer's instructions. Typical HPLC-MS Conditions for Waters Quattro Micro mass spectrometer are shown in the Annexure.

The mass spectrometer is connected to the HPLC-UV system used in method A. Analyse the mass spectral data of the minor peaks (major steviol glycoside peaks are identified from RRT in method A). Confirm the presence of each minor steviol glycoside from the observed molecular mass ion (Typical molecular mass ions of steviol glycosides are given in Appendix-3) and one or more of the following mass spectral diagnostic ions:

Mass spectral diagnostic ions observed during in-source fragmentation of steviol glycosides

[Fragment-H] - m/z	Identity
317	Steviol
427	Related Steviol glycoside #3
479	Steviol-GLC
625	Steviol-2GLC [M-16]
641	Steviol-2GLC
787	Steviol-3GLC deoxyglucose [M-16]
803	Steviol-3GLC
819	-
965	Steviol-4GLC

Note: The example chromatogram of minor steviol glycosides shown in Appendix 2 is obtained from the purified in-house standards.

After confirming the presence of a minor steviol glycoside, correct its mean peak area (obtained from the UV chromatogram) as described below.

Calculation of minor steviol glycosides content:

Calculate the molecular mass corrected peak area abundance for each minor steviol glycoside using the formula:

$$\text{Molecular mass corrected peak area} = \frac{M_x \times \text{MPA}}{M_{\text{RebA}}}$$

Where:

- M_x is the molecular mass of the minor steviol glycoside
- M_{RebA} is the molecular mas of Rebaudioside A (967 amu)
- MPA is the mean peak area

Deduce the concentration ($\mu\text{g/mL}$) of each minor steviol glycoside using from the UV standard curve of rebaudioside A. Calculate the concentration of each minor steviol glycoside in the sample solution using the following formula:

$$\text{Minor Steviol Glycoside Conc. (\%w/w)} = \frac{\text{Conc}_{\text{sample}} \times 100}{\text{Weight}_{\text{sample}}}$$

Where

- $\text{Conc}_{\text{sample}}$ is the assayed concentration ($\mu\text{g/mL}$) in the test sample
- $\text{Weight}_{\text{sample}}$ is the sample weight in 1 mL solution ($\mu\text{g/mL}$)

Note: Above calculation will change if additional dilutions were done prior to LC injection. Analyst shall account such dilutions in the calculation.

Calculate the percentage of minor steviol glycosides in the sample by summation of percentages of individual minor steviol glycosides in the sample (B).

Determine the total amount of steviol glycoside content using the following formula:

$$TSG = \frac{(A + B) \times 100}{(100 - M)}$$

Where:

- TSG is the Total steviol glycosides content (%w/w, on the dried basis)
- A is the percent major steviol glycosides
- B is the percent minor steviol glycosides
- M is the percent loss on drying

Annex Typical LCMS Conditions

Instrumentation	Waters Quattro Micro mass spectrometer
Ionization:	Electrospray negative polarity
Capillary voltage:	4.0 kV
Cone voltage:	35 V (low) and 60 V (high)
Extractor voltage:	5.0 V
RF lens voltage:	1.0 V
Source temperature:	90 °
Desolvation temperature:	350 °
Desolvation flow rate:	400 L/h
Collisional pressure:	Not applicable
Collisional voltage:	Not applicable
Collision gas:	Not applicable
Resolution:	1 amu
Data acquisition	Scanning from 50 to 1500 m/z using Mass Lynx

Appendix 1: Chemical Information of Some Steviol Glycosides

Common Name	Trivial Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Group 1: Steviol + Glucose (SvGn)							
<u>Steviolmonoside</u>	SvG1	H	Glcβ1-	13-[(β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	60129-60-4	C ₂₈ H ₄₀ O ₈	481
<u>Steviolmonoside A</u>	SvG1	Glcβ1-	H	13-[(hydroxyl)kaur-16-en-18-oic acid, β -D-glucopyranosyl ester	64977-89-5	C ₂₈ H ₄₀ O ₈	481
<u>Rubusoside</u>	SvG2	Glcβ1-	Glcβ1-	13-[(β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β -D-glucopyranosyl ester	64849-39-4	C ₃₂ H ₅₀ O ₁₃	643
<u>Steviolbioside</u>	SvG2	H	Glcβ(1-2)Glcβ1-	13-[(2-O- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	41093-60-1	C ₃₂ H ₅₀ O ₁₃	643
<u>Stevioside</u>	SvG3	Glcβ1-	Glcβ(1-2)Glcβ1-	13-[(2-O- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β -D-glucopyranosyl ester	57817-89-7	C ₃₈ H ₆₀ O ₁₈	805
<u>Stevioside A Or Rebaudioside KA</u>	SvG3	Glcβ(1-2)Glcβ1-	Glcβ1-	13-[(2-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid 4'-O- β -D-glucopyranosyl-deoxy-(1,2)-O-[β -(d-glucopyranosyl)ester	127345-20-4	C ₃₈ H ₆₀ O ₁₈	805
<u>Stevioside B</u>	SvG3	Glcβ(1-3)Glcβ1-	Glcβ1-	13-[(2-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, O- β -D-glucopyranosyl-deoxy-(1,3)-O-[β -D-glucopyranosyl]ester	-	C ₃₈ H ₆₀ O ₁₈	805
<u>Rebaudioside B</u>	SvG3	H	Glcβ(1-2)Glcβ(1-3)Glcβ1-	13-[(2-O- β -D-glucopyranosyl-3-O- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	58543-17-2	C ₃₈ H ₆₀ O ₁₈	805

Common Name	Trivial Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside G	SvG3	Glcβ1-	Glcβ(1-3)Glcβ1	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid(4')-O-β-D-glucopyranosyl ester	127345-21-5	C ₃₈ H ₆₀ O ₁₈	805
Rebaudioside E	SvG4	Glcβ(1-2)Glcβ1-		13-[(O-β-D-glucopyranosyl-(1,2)-O-[β-D-glucopyranosyl]-oxy)-kaur-16-en-18-oic acid (4')-O-β-D-glucopyranosyl-deoxy-(1,2)-O-[β-D-glucopyranosyl] ester	63279-14-1	C ₄₄ H ₇₀ O ₂₃	967
Rebaudioside A	SvG4	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	58543-16-1	C ₄₄ H ₇₀ O ₂₃	967
Rebaudioside A2	SvG4	Glcβ1-	Glcβ(1-6)[Glcβ(1-2)]Glcβ1-	13-[(6-O-β-D-glucopyranosyl-2-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl ester	1326217-29-1	C ₄₄ H ₇₀ O ₂₃	967
Rebaudioside D	SvG5	Glcβ(1-2)Glcβ1-		13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl ester	63279-13-0	C ₅₀ H ₈₀ O ₂₈	1129
Rebaudioside L	SvG5	Glcβ1-	Glcβ(1-6)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(6-O-β-D-glucopyranosyl-2-O-β-D-glucopyranosyl-3-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl ester	1220616-38-5	C ₅₆ H ₈₈ O ₂₈	1129

Common Name	Trivial Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
<u>Steviolmonoside</u>	SvG1	H	Glcβ1-	13-[(β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	60129-60-4	C ₂₈ H ₄₀ O ₃	481
<u>Steviolmonoside A</u>	SvG1	Glcβ1-	H	13-[(hydroxyl)kaur-16-en-18-oic acid, β -D-glucopyranosyl ester	64977-89-5	C ₂₈ H ₄₀ O ₃	481
<u>Rubusoside</u>	SvG2	Glcβ1-	Glcβ1-	13-[(β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β -D-glucopyranosyl ester	64849-39-4	C ₃₂ H ₅₀ O ₁₃	643
<u>Steviolbioside</u>	SvG2	H	Glcβ(1-2)Glcβ1-	13-[(2-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	41093-60-1	C ₃₂ H ₅₀ O ₁₃	643
<u>Stevioside</u>	SvG3	Glcβ1-	Glcβ(1-2)Glcβ1-	13-[(2-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β -D-glucopyranosyl ester	57817-89-7	C ₃₈ H ₆₀ O ₁₈	805
<u>Stevioside A Or Rebaudioside KA</u>	SvG3	Glcβ(1-2)Glcβ1-	Glcβ1-	13-[(2-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid 4'-O- β -D-glucopyranosyl-deoxy-(1,2)-O-[β (-D-glucopyranosyl)ester	127345-20-4	C ₃₈ H ₆₀ O ₁₈	805
<u>Stevioside B</u>	SvG3	Glcβ(1-3)Glcβ1-	Glcβ1-	13-[(2-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, O- β -D-glucopyranosyl-deoxy-(1,3)-O-[β -D-glucopyranosyl ester	-	C ₃₈ H ₆₀ O ₁₈	805
<u>Rebaudioside B</u>	SvG3	H	Glcβ(1-2)[Glcβ(1-3)Glcβ1-	13-[(2-O- β -D-glucopyranosyl-3-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	58543-17-2	C ₃₈ H ₆₀ O ₁₈	805

Common Name	Trivial Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside I	SvG5	Glc β (1-3) Glc β 1-	Glc β (1-2) [Glc β (1-3)]Glc β 1-	13-[(2-O- β -D-glucopyranosyl-3-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 3-O- β -D-glucopyranosyl- β -D-glucopyranosyl ester	-	C ₅₀ H ₈₀ O ₂₈	1129
Rebaudioside I2	SvG5	Glc β 1-	Glc α (1-3) Glc β (1-2) [Glc β (1-3)]Glc β 1-	13-[(3-O- β -D-glucopyranosyl-2-O- β -D-glucopyranosyl-3-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O- β -D-glucopyranosyl ester	-	C ₅₀ H ₈₀ O ₂₈	1129
Rebaudioside I3	SvG5	[Glc β (1-2) Glc β (1-6)]Glc β 1-	Glc β (1-2) Glc β 1-	13-[(2-O- β -D-glucopyranosyl-1-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O- β -D-glucopyranosyl-1-6-O- β -D-glucopyranosyl-1- β -D-glucopyranosyl ester	-	C ₅₀ H ₈₀ O ₂₈	1129
Rebaudioside Q	SvG5	Glc β 1-	Glc α (1-4) Glc β (1-2)[Glc β (1-3)]Glc β 1-	13-[(4-O- β -D-glucopyranosyl-2-O- β -D-glucopyranosyl-3-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O- β -D-glucopyranosyl ester	-	C ₅₀ H ₈₀ O ₂₈	1129
Rebaudioside Q2	SvG5	[Glc α (1-2) Glc α (1-4)] Glc β 1-	Glc β (1-2) Glc β 1-	13-[(2-O- β -D-glucopyranosyl-1-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O- β -D-glucopyranosyl-4-O- β -D-glucopyranosyl- β -D-glucopyranosyl ester	-	C ₅₀ H ₈₀ O ₂₈	1129
Rebaudioside Q3	SvG5	Glc β 1-	Glc α (1-4) Glc β (1-3)[Glc β (1-2)]Glc β 1-	13-[(4-O- β -D-glucopyranosyl-3-O- β -D-glucopyranosyl-2-O- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O- β -D-glucopyranosyl ester	-	C ₅₀ H ₈₀ O ₂₈	1129

Common Name	Trivial Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside M	SvG6	Glc β (1-2)[Glc β (1-3)]Glc β 1-	Glc β (1-2)[Glc β (1-3)]Glc β 1-	13-[(O- β -D-glucopyranosyl-(1,2)-O- β -D-glucopyranosyl-(1,3)]- β -D-glucopyranosyl-18-oic acid (4')-O- β -D-glucopyranosyl-(1,2)-O-[β -D-glucopyranosyl-(1,3)]- β -D-glucopyranosyl ester	1220616-44-3	C ₅₈ H ₁₀₀ O ₃₃	1291
Related SvGn#1		-	-	-	-	C ₂₁ H ₃₀ O ₁₁	458
Related SvGn#2		-	-	-	-	C ₄₀ H ₇₀ O ₂₄	982
Related SvGn#3		-	-	-	-	C ₃₂ H ₅₂ O ₁₅	676
Related SvGn#4		-	-	-	-	C ₅₀ H ₈₀ O ₂₈	1129
Related SvGn#5		-	-	-	-	C ₄₀ H ₇₀ O ₂₄	982
Group 2: Steviol + Rhamnose + Glucose (SvR1Gn)							
Dulcoside A	SvR1G2	Glc β 1-Rha α (1-2)Glc β 1-	Rha α (1-2)Glc β 1-	13-[(2-O- α -L-rhamnopyranosyl- β -D-glucopyranosyloxy]kaur-16-en-18-oic acid, β -D-glucopyranosyl ester	64432-06-0	C ₃₈ H ₆₀ O ₁₇	789
Dulcoside C	SvR1G2	H	Rha α (1-2)[Glc β (1-3)]Glc β 1-	13-[(2-O- β -D-rhamnopyranosyl-3- β -D-glucopyranosyl- β -D-glucopyranosyl-oxy]kaur-16-en-18-oic acid		C ₃₈ H ₆₀ O ₁₇	789
Rebaudioside C	SvR1G3	Glc β 1-Rha α (1-2)[Glc β (1-3)]Glc β 1-	13-[(2-O- α -L-rhamnopyranosyl-3-O- β -D-glucopyranosyl- β -D-glucopyranosyloxy]kaur-16-en-18-oic acid, β -D-glucopyranosyl ester	63550-99-2	C ₄₄ H ₇₀ O ₂₂	951	

Common Name	Trivial Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside C2	SvR1G3	Rha ^Q (1-2)Glc β 1-	Glc β (1-2)Glc β 1-	13-[(2-O- β -D-glucopyranosyl)- β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O- β -rhamnopyranosyl- β -D-glucopyranosyl ester	-	C ₄₄ H ₇₀ O ₂₂	951
Rebaudioside N	SvR1G5	Rha ^Q (1-2)[Glc β (1-3)]Glc β 1-	Glc β (1-2)[Glc β (1-3)]Glc β 1-	13-[(2-O- β -D-glucopyranosyl)-(1,2-O-[β -D-glucopyranosyl](1,3)) β -D-glucopyranosyl)oxy]kaur-16-en-18-oic acid (4')-O-2-deoxy-L-rhamnopyranosyl-3-O- β -D-glucopyranosyl- β -D-glucopyranosyl ester	1220616-46-5	C ₄₈ H ₆₀ O ₃₂	1274
Rebaudioside O	SvR1G6	Glc β (1-3)Rha ^Q (1-2)[Glc β (1-3)]Glc β 1-	Glc β (1-2)[Glc β (1-3)]Glc β 1-	13-[(2-O- β -D-glucopyranosyl)-3-O- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]ent-kaur-16-en-19-oic acid-[(2-O-(3-O- β -D-glucopyranosyl)- α -L-rhamnopyranosyl)-3-O- β -D-glucopyranosyl- β -D-glucopyranosyl ester]	1220616-48-7	C ₆₂ H ₁₀₀ O ₃₇	1436
Rebaudioside O2	SvR1G6	Glc β (1-4*)Rha ^Q (1-2)[Glc β (1-3)]Glc β 1-	Glc β (1-2)[Glc β (1-3)]Glc β 1-	13-[(O- β -D-glucopyranosyl)-(1,2-O-[β -D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-O- β -D-glucopyranosyl-(1,4)-O-6-deoxy-L-rhamnopyranosyl-(1,2)-O-[β -D-glucopyranosyl(1,3)]- β -D-glucopyranosyl ester	-	C ₆₂ H ₁₀₀ O ₃₇	1436

Common Name	Trivial Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside K	SvR1G4	Glcβ(1-2)Glcβ1-	Rhaα(1-2)[Glcβ(1-3)Glcβ1-	13-[(2-O-β-D-rhamnopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	1220616-40-9	C ₅₀ H ₈₆ O ₂₇	1112
Rebaudioside S	SvR1G3	Rhaα(1-2)Glcβ1-	Glcα (1-2)Glcβ1-	13-[(2-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-glucopyranosyl ester, O-2-deoxy-L-rhamnopyranosyl β-D-glucopyranosyl ester	1931085-11-8	C ₄₄ H ₇₀ O ₂₂	951
Rebaudioside K2	SvR1G4	Glcβ(1-6)Glcβ1-	Rhaα(1-2)[Glcβ(1-3)Glcβ1-	13-[(2-O-β-D-rhamnopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 6-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	-	C ₅₀ H ₈₆ O ₂₇	1112
Rebaudioside H	SvR1G4	Glcβ1-	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)Glcβ1-	13-[(3-O-β-D-glucopyranosyl-2-O-β-D-rhamnopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	1220616-36-3	C ₅₀ H ₈₆ O ₂₇	1112
Rebaudioside J	SvR1G4	Rhaα(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)Glcβ1-	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	1313049-59-0	C ₅₀ H ₈₆ O ₂₇	1112
Group 3: Steviol + Xylose + Glucose (SvX1Gn)							
Stevioside F	SvX1G2	Glcβ1-2)Glcβ1-	Xylβ(1-2)Glcβ1-	13-[(2-O-β-D-xylopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	-	C ₃₇ H ₅₉ O ₁₇	775

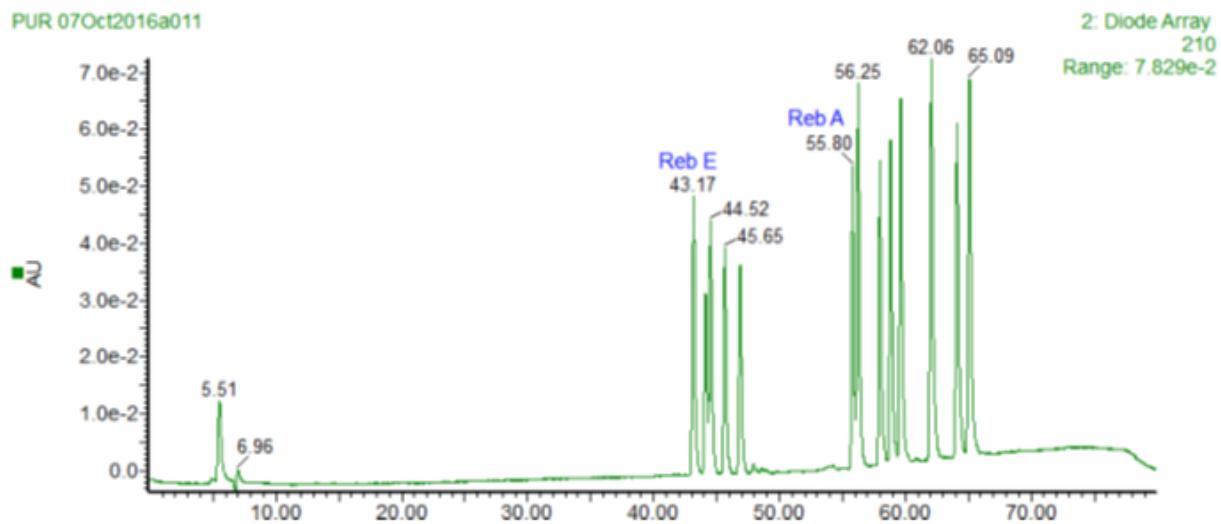
Common Name	Trivial Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside F	SvX1G3	Glcβ1-	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-O-β-D-xylopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyloxy)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	438045-89-7	C ₄₃ H ₆₈ O ₂₂	937
Rebaudioside F2	SvX1G3	Glcβ1-	Glcβ(1-2)[Xylβ(1-3)]Glcβ1-	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-xylopyranosyl-β-D-glucopyranosyloxy)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	-	C ₄₃ H ₆₈ O ₂₂	937
Rebaudioside F3	SvX1G3	Xylβ(1-6)Glcβ1-	Glcβ(1-2)[Glcβ1-Xylβ1]	13-[(2-O-β-D-glucopyranosyl-β-D-xylopyranosyl-β-D-glucopyranosyloxy)oxy]kaur-16-en-18-oic acid, 6-O-β-D-xylopyranosyl-β-D-glucopyranosyl ester	-	C ₄₃ H ₆₈ O ₂₂	937
Rebaudioside R	SvX1G3	Glcβ1-	Glcβ(1-2)[Glcβ1-Xylβ1]	13-[(2-O-β-D-glucopyranosyl-β-D-xylopyranosyl-3-O-β-D-glucopyranosyloxy)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	1931083-53-2	C ₄₃ H ₆₈ O ₂₂	937
Rebaudioside U2	SvX1G4	Xylβ(1-2*)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ1-Xylβ1]	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl-β-D-xylopyranosyl-β-D-glucopyranosyloxy)oxy]kaur-16-en-18-oic acid, 2-O-β-D-xylopyranosyl-3-O-β-D-glucopyranosyl ester	-	C ₅₀ H ₈₂ O ₂₈	1099
Rebaudioside T	SvX1G4	Xylβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-xylopyranosyl-β-D-glucopyranosyloxy)oxy]kaur-16-en-18-oic acid, 2-O-β-D-xylopyranosyl-β-D-glucopyranosyl ester		C ₅₀ H ₈₂ O ₂₈	1099
Rebaudioside V2	SvX1G5	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-xylopyranosyl-β-D-glucopyranosyloxy)oxy]kaur-16-en-18-oic acid, 2-O-β-D-xylopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	-	C ₅₈ H ₉₂ O ₃₁	1261

Common Name	Trivial Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Rebaudioside V	SvX1G5	<u>Glcβ(1-2)[Glcβ(1-2*)]Glcβ(1-3)Glcβ1-</u>		13-[(2-O-β-D-xylopyranosyl-3-O-β-D-glucopyranosyl)-β-D-oxo acid, 2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl]-β-D-glucopyranosyl ester	-	C ₅₈ H ₉₂ O ₃₁	1261
Group 4: Steviol + Arabinose + Glucose (SvA1Gn)							
Rebaudioside U	SvA1G4	Ara(1-2*)Glcβ1	<u>Glcβ(1-3)Glcβ1-</u>	13-[(2-O-β-D-glucopyranosyl-3-O β-D-glucopyranosyl)-β-D-glucopyranosyl]oxygent-kaur-16-en-19-oic acid-(6-O-α-L-Arabinopyranosyl-β-D-glucopyranosyl) ester		C ₅₀ H ₈₂ O ₂₈	1098
Rebaudioside W	SvA1G4	<u>Glcβ(1-2)[Araβ(1-3*)]Glcβ1</u>	<u>Glcβ(1-2)Glcβ1-</u>	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-Dglucopyranosyl-3-O-β-D-Arabinopyranosyl-β-D-glucopyranosyl ester	-	C ₅₀ H ₈₂ O ₂₈	1098
Rebaudioside W2	SvA1G4	<u>Araβ(1-2*)Glcβ1</u>	<u>Glcβ(1-3)Glcβ1-</u>	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-Arabinopyranosyl-β-D-glucopyranosyl ester	-	C ₅₀ H ₈₂ O ₂₈	1098
Rebaudioside W3	SvA1G4	<u>Araβ(1-6)Glcβ1-</u>	<u>Glcβ(1-2)[Glcβ(1-3)Glcβ1-</u>	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 6-O-β-D-Arabinopyranosyl-β-D-glucopyranosyl ester	-	C ₅₀ H ₈₂ O ₂₈	1098
Rebaudioside Y	SvA1G5	<u>Glcβ(1-2)[Araβ(1-3*)]Glcβ1-</u>		13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-3-O-β-D-Arabinopyranosyl-β-D-glucopyranosyl ester	-	C ₅₀ H ₈₂ O ₂₈	1098
Rebaudioside Y	SvA1G5	<u>Glcβ(1-2)[Glcβ(1-3)Glcβ1-</u>		13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-3-O-β-D-Arabinopyranosyl-β-D-glucopyranosyl ester	C ₅₀ H ₈₂ O ₂₈	1260	

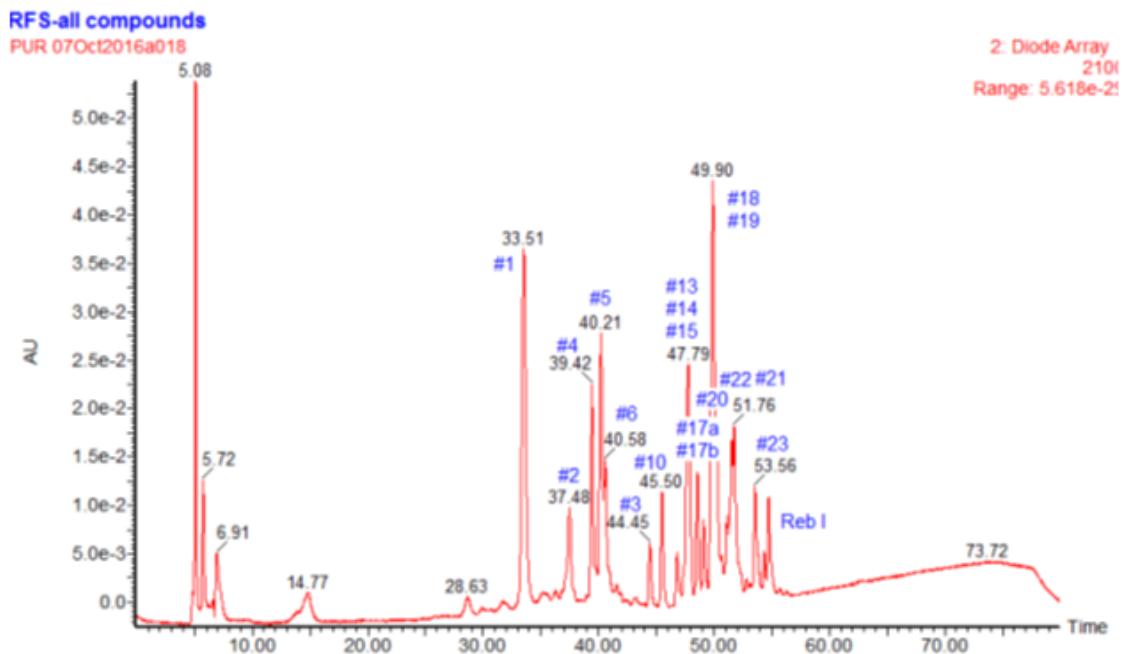
Common Name	Trivial Name	R ₁	R ₂	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Group 5: Steviol + Galactose + Glucose (SvGα1Gn)							
Rebaudioside T1	SvGa1G4	Galβ(1-2*)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-galactopyranosyl-β-D-glucopyranosyl ester	-	C ₅₀ H ₈₀ O ₂₈	1128
Group 6: Steviol + Fructose + Glucose (SvFrGn)							
Rebaudioside A ₃	SbF1G3	Glcβ1-	Glcβ(1-2)[Fruβ(1-3)]Glcβ1-	13-[(2-O-β-D-fructofuranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	-	C ₄₄ H ₇₀ O ₂₂	951
Group 7: Steviol + -de-oxy glucose + Glucose (SvdG1Gn)							
Stevioside D	SvDg1G2	Glcβ1-	6-deoxyGlcβ(1-2)Glcβ1-	13-[(2-O-β-D-6-deoxyglucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	-	C ₃₈ H ₆₀ O ₁₇	789
Stevioside E	SvDg1G3	Glcβ1-	6-deoxyGlcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-O-β-D-6-deoxyglucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	-	C ₄₄ H ₇₀ O ₂₂	951
Stevioside E2	SvDg1G3	6-deoxyGlcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-6-deoxyglucopyranosyl ester	-	C ₄₄ H ₇₀ O ₂₂	951

Steviol (R₁ = R₂ = H) is the aglycone of the steviol glycosides. Glc, Rha, Fru, deoxyGlc, Gal, Ara and Xyl represent, respectively, glucose, rhamnose, fructose, deoxysugars, arabinose and xylose sugar moieties.

Note: This list is not exhaustive. More steviol glycosides may have been identified in stevia leaf extracts in the literature

Appendix- 2: Representative chromatograms for steviol glycosides using Method of Assay


Example Chromatogram of Representative Steviol Glycoside Standards from a Phenomenex Luna C18 (150 mm x 4.6 mm, 5 μ m). Order of retention times from left to right: rebaudioside E, rebaudioside O, rebaudioside D, rebaudioside N, rebaudioside M, rebaudioside A, stevioside, rebaudioside F, rebaudioside C, dulcoside A, rubusoside, rebaudioside B and steviolbioside.



Example Chromatogram from a Phenomenex Luna C18 (150 mm x 4.6 mm, 5 μ m) of Minor Steviol Glycosides using in-house purified reference standards.

Appendix-3: Typical Retention Time (RT), Relative Retention Time (RRT) and Mass Ions of Steviol Glycosides

Compound Name	Typical Retention Time (RT)*	Relative Retention Time to Rebaudioside A (RRT)*	Molecular Mass Ion [M-H]
Related steviol glycoside #1	32.6	0.58	517 or 427
Related steviol glycoside #2	33.6	0.60	981
Related steviol glycoside #3	34.3	0.61	427 or 735
Related steviol glycoside #4	38.1	0.68	675 or 1127
Related steviol glycoside #5	40.8	0.73	981
Rebaudioside V	43.0	0.77	1259
Rebaudioside T	42.0	0.75	1127
Rebaudioside E	43.7	0.78	965
Rebaudioside O	44.6	0.79	1435
Rebaudioside D	45.1	0.80	1127
Rebaudioside K	45.8	0.81	1111
Rebaudioside N	46.1	0.82	1273
Rebaudioside M	47.5	0.84	1289
Rebaudioside S	48.3	0.86	949
Rebaudioside J	48.4	0.86	1111
Rebaudioside W	49.1	0.87	1097
Rebaudioside U2	49.1	0.87	1097
Rebaudioside W2	49.7	0.88	1097
Rebaudioside W3	50.3	0.89	1097
Rebaudioside U	50.7	0.90	1097
Rebaudioside O2	50.6	0.90	965
Rebaudioside Y	50.8	0.90	1259
Rebaudioside I	50.7	0.90	1127
Rebaudioside V2	52.2	0.93	1259
Rebaudioside K2	51.7	0.93	1111
Rebaudioside H	53.7	0.96	1111
Rebaudioside A	56.2	1.00	965
Stevioside	56.6	1.01	803
Rebaudioside F	58.3	1.04	935
Rebaudioside C	59.2	1.05	949
Dulcoside A	60.0	1.07	787
Rubusoside	62.4	1.11	641
Rebaudioside B	64.5	1.15	803
Steviolbioside	65.5	1.17	641

*RT and RRT values given in the above table are for information purpose only. They may vary based on the chromatographic system and conditions used. Analyst needs to establish during method validation.

ANNATTO EXTRACTS (SOLVENT-EXTRACTED NORBIXIN)

Prepared at the 80th JECFA and published in FAO JECFA Monographs 17 (2015) superseding specifications prepared at the 67th JECFA (2006) published in FAO JECFA Monographs 3 (2006). A group ADI for norbixin and its disodium and dipotassium salts of 0 – 0.6 mg/kg bw expressed as norbixin was established at the 67th JECFA (2006).

SYNONYMS

Annatto B, Orlean, Terre orellana, L. Orange, CI (1975) 75120 (Natural Orange 4), INS 160b(ii)

DEFINITION

Solvent-extracted norbixin is obtained from the outer coating of the seeds of the annatto tree (*Bixa orellana L.*) by washing with one or more of the following food grade solvents: acetone, methanol, hexane, ethanol, isopropyl alcohol, ethyl acetate, alkaline alcohol or supercritical carbon dioxide followed by solvent removal, crystallization and drying. Aqueous alkali is added to the resultant powder, which is then heated to hydrolyse the colouring matter and cooled. The aqueous solution is filtered, and acidified to precipitate the norbixin. The precipitate is filtered, washed, dried and milled, to give a granular powder.

Solvent-extracted norbixin contains several coloured components; the major colouring principle is *cis*-norbixin, a minor colouring principle is *trans*-norbixin; thermal degradation products of norbixin may also be present as a result of processing.

Products supplied to the food industry may be formulated with appropriate carriers of food grade quality.

Chemical name

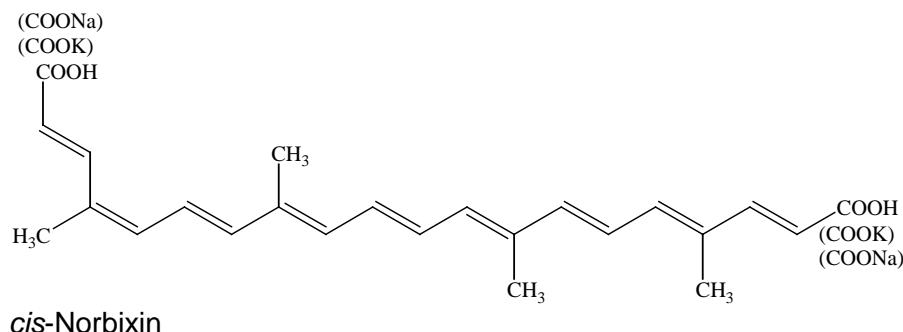
cis-Norbixin: 6,6'-Diapo-Ψ,Ψ-carotenedioic acid
cis-Norbixin dipotassium salt: Dipotassium 6,6'-diapo-Ψ,Ψ-carotenedioate
cis-Norbixin disodium salt: Disodium 6,6'-diapo-Ψ,Ψ-carotenedioate

C.A.S. number

cis-Norbixin: 542-40-5
cis-Norbixin dipotassium salt: 33261-80-2
cis-Norbixin disodium salt: 33261-81-3

Chemical formula

cis-Norbixin: C₂₄H₂₈O₄, *cis*-Norbixin dipotassium salt: C₂₄H₂₆K₂O₄, *cis*-Norbixin disodium salt: C₂₄H₂₆Na₂O₄

Structural formula

Formula weight

380.5 (acid), 456.7 (dipotassium salt), 424.5 (disodium salt)

Assay Not less than 85 % colouring matter (expressed as norbixin)
DESCRIPTION Dark red-brown to red-purple powder

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in alkaline water, slightly soluble in ethanol

UV/VIS absorption (Vol. 4) The sample in 0.5% potassium hydroxide solution shows absorbance maxima at about 453 nm and 482 nm.

Thin Layer Chromatography Activate a TLC plate (e.g. LK6D SILICA GEL 60 A (layer thickness: 250 µm, size: 5 x 20 cm)) for 1 h at 110°. Prepare a 5% solution of the sample in 95% ethanol and apply 10 µl to the plate. Allow to dry and develop using a mixture of n-butanol, methyl ethyl ketone and 10% aqueous ammonia (3:2:2 by volume) until the solvent front has ascended about 10 cm. Allow to dry. Bixin and norbixin appear as yellow spots with R_f values of about 0.50 to 0.45, respectively. Spray with 5% sodium nitrite solution and then with 0.5 mol/l sulfuric acid and the spots immediately decolourise.

PURITY

<u>Residual Solvents</u>	Acetone: Not more than 30 mg/kg Methanol: Not more than 50 mg/kg Hexane: Not more than 25 mg/kg Ethanol: Isopropyl alcohol: Ethyl acetate:	Not more than 50 mg/kg, singly or in combination
		See Description under TEST
<u>Arsenic</u> (Vol. 4)	Not more than 3 mg/kg Determine using an AAS (Hydride generation technique) 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").	
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an AAS (Electrothermal atomization technique) 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").	
<u>Mercury</u> (Vol. 4)	Not more than 1 mg/kg Determine using AAS (Cold vapour generation technique). 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").	
METHOD OF ASSAY	Proceed as directed in Food Colours, Colouring Matters Content by Spectrophotometry (Vol. 4), procedure 1, using 0.5 % potassium	

hydroxide as solvent. Measure the absorbance at the A_{\max} of about 482 nm. The specific absorbance ($A_{1\text{cm}}^{1\%}$) is 2870.

TESTS

Residual solvents

Proceed as directed in Residual Solvents by Headspace Gas Chromatography (Vol. 4) using the following:

Stock standard solution

Add 10 ml dimethylformamide to a 20 ml volumetric flasks. Accurately weigh, to within 0.01 mg, each flask. Pipet 250 μl each of chromatography grade methanol, ethanol, isopropanol, and ethyl acetate, and 150 μl each of acetone and hexane into each of the flask. Reweigh accurately and then fill the flask with dimethylformamide. Mix well.

Standard mixture solution A: Pipet each 3.0 ml of stock standard solution into a 20 ml volumetric flask and fill the flask with dimethylformamide.

Standard mixture solution B: Pipet 4.0 ml solution A into a 10 ml volumetric flask and fill the flask with dimethylformamide.

Standard mixture solution C: Pipet 2.0 ml solution A into a 20 ml volumetric flask and fill the flask with dimethylformamide.

Standard mixture solution D: Pipet 1.0 ml solution A into a 20 ml volumetric flask and fill the flask with dimethylformamide.

Samples

Weigh accurately 0.2 g sample into a 20 ml injection vial. Add 2.5 ml dimethylformamide and seal.

Standard solutions

Introduce 0.1 ml of the each standard mixture solution (A, B, C and D) into each 20 ml injection vial. Add 2.4 ml dimethylformamide and seal.

Standard curves

Place the four standard solutions in the sample tray on head-space gas chromatography. Heat vials at 60° for 20 min with continuous agitation. Analyze using the analytical condition as described above. Measure the peak area for each solvent. Construct the standard curves by plotting the ratios of the peak areas of each solvent against the concentrations of each solvent (mg/ml) in the standards solutions.

Procedure

Place the sample solution in the sample tray on head-space gas chromatograph. Heat vials at 60° for 20 min with continuous agitation.

Analyze using the analytical conditions for Residual Solvents by Headspace Gas Chromatography as described in Vol. 4.

Measure the peak area for each solvent and obtain the concentration of each solvent (C, mg/ml) from the standard curves.

Calculation

Calculate the concentration of each residual solvent in samples from;

$$\text{Residual solvent (mg/kg)} = C \times 2.5/W \times 1000$$

Where:

W is weight of sample (g).

BONE PHOSPHATE

Prepared at the 33rd JECFA (1988), published in FNP 38 (1988) and in FNP 52 (1992). Metals and arsenic specifications revised at the 61st JECFA (2003)
A group MTDI of 70 mg/kg bw, as phosphorus from all food sources, was established at the 29th JECFA (1985)

SYNONYMS

Edible bone phosphate, INS No. 542

DEFINITION

A heterogeneous residual mixture of calcium phosphates, principally $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{Ca}(\text{OH})_2$, obtained by the grinding of bones which have been treated with hot water and steam under pressure; may contain unextracted fat and proteins.

Assay

Not less than 30% and not more than 40% of Ca, and not less than 32% of P_2O_5 .

DESCRIPTION

White to pale cream coloured, odourless powder

FUNCTIONAL USES

Emulsifier, moisture retaining agent, sequestrant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in ethanol and water

Test for phosphate (Vol. 4) Passes test
Use a solution obtained by dissolving 1 g of the sample by warming in 50 ml diluted hydrochloric acid.

Test for calcium (Vol. 4) Passes test
Use a solution obtained by dissolving 1 g of the sample by warming in 50 ml diluted hydrochloric acid.

PURITY

Loss on drying (Vol. 4) Not more than 2%

Loss on ignition (Vol. 4) Not more than 20%

Fluoride (Vol. 4) Total not more than 1000 mg/kg
Dissolve an amount of ash (obtained from the test for Loss on ignition) equal to 0.1 g of the sample, and proceed as described under the Limit Test, Method IV using buffer solution C.

Copper Not more than 25 mg/kg
See description under TESTS

Zinc Not more than 250 mg/kg
See description under TESTS

<u>Arsenic</u> (Vol. 4)	Not more than 3 mg/kg
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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."
<u>Fat residue</u>	Not more than 2% Accurately weigh 5-10 g sample. Without previous drying, extract in soxhlet or other suitable container with petroleum ether (40-60°) for about 6 h. Filter extract through small hardened paper into weighed vessel, washing paper into weighed vessel, washing paper finally with small portion of hot fresh solvent. Distil or evaporate solvent at temperature ca 100° and dry vessel containing residue in air oven for 1h at 100-105°C. Weigh the dried residue and calculate percentage of the sample.
<u>Protein residue</u> (Vol. 4)	Not more than 10% (N x 6.25) Proceed as directed under <i>Nitrogen Determination (Kjeldahl Method)</i> Method II
<u>Microbiological criteria</u> (Vol. 4)	Total aerobic microbial count: Max 1000 in 1 g <i>Salmonella</i> : Absent in 50 g <i>E. coli</i> : Absent in 10 g

TESTS

PURITY TESTS

Copper and zinc

General precautions

Because of the min amounts of metals involved special care must be taken to reduce the reagent blanks to as low a value as possible and to avoid contamination during the test. All apparatus should be thoroughly cleaned with a mixture of hot dilute acids (1 part hydrochloric acid, 1 part concentrated nitric acid, and 3 parts water) followed by thorough washing with water immediately before use. The methods of preparation described should be followed exactly.

Apparatus

Atomic absorption spectrophotometer equipped with air/acetylene flame and lamps for copper and zinc determination.

Reagents

Reagents shall be of an order of purity higher than accepted analytical reagent grade quality. Metal-free water (see below) shall be used throughout.

- Sulfuric acid, 98% H₂SO₄
- Nitric acid, sp.gr. 1.42
- Hydrochloric acid, sp. gr. 1.16-1.18 (conc.)
- Hydrochloric acid 5 M solution prepared by dilution of hydrochloric acid (conc.) with water
- Hydrochloric acid 0.5 M solution prepared by dilution of hydrochloric acid 5 M with water
- Water, metal free. Distilled water may be re-distilled from an all glass

apparatus or may be passed down a column of cation exchange resin, e.g., Amberlite IR 120 (H).

Standards

Standard copper solution: Dissolve 3.928 g of pure copper sulfate CuSO₄·5H₂O in distilled water, dilute to 1000 ml at 20° with distilled water in a one-mark graduated flask. Dilute 10 ml to 100 ml with water in a one-mark graduated flask as required. 1 ml = 100 µg Cu.

Standard zinc solution: Dissolve 1.000 g of pure zinc powder in a mixture of 10 ml distilled water and 5 ml hydrochloric acid special reagent (d) and dilute to 1000 ml at 20° with distilled water, in a one-mark graduated flask. Dilute 10 ml to 100 ml with water in a one-mark graduated flask as required. 1 ml = 100 µg Zn.

Sample preparation

Place about 2.5 g of the sample, accurately weighed, in a suitable crucible, add sufficient sulfuric acid to wet the sample, and carefully ignite at a low temperature until thoroughly charred, covering the crucible loosely with a suitable lid during the ignition. After the substance is thoroughly carbonized, add 2 ml of nitric acid and 5 drops of sulfuric acid, and cautiously heat until white fumes are evolved, then ignite, preferably in a muffle furnace, at 500° to 600° until all the carbon is burned off. Cool, add 4 ml of hydrochloric acid 5 M, cover, and digest on a steam bath for 10 to 15 min. Uncover, and slowly evaporate on a steam bath to dryness. Finally cool, add 10 ml 5 M hydrochloric acid and boil gently for a few min. Cool and transfer the solution to a 50-ml one-mark graduated flask washing out the Kjeldahl flask with small portions of water. Add the washings to the graduated flask and dilute to the mark with water (Solution A). To a 100 ml one mark volumetric flask pipet 10 ml of solution A and dilute to the mark with hydrochloric acid 0.5 M (Solution B). Prepare a reagent blanks using the same quantities of reagents as used in the sample preparation for obtaining solutions A and B (Blank A and Blank B).

Preparation of standard curve solutions

To a series of 100-ml one-mark volumetric flasks pipet 0, 1, 2, 3, 4 and 5 ml of each of the two standard solutions to (e) and dilute to about 50 ml. Add 20 ml of hydrochloric acid 5 M and dilute to the mark with metal-free water. These solutions then contain 0, 1.0, 2.0, 3.0, 4.0 and 5.0 µg per ml of copper and zinc.

Instrumental Conditions

Select the wavelength to be used for the particular element under consideration 324.7 nm for copper; 213.9 for zinc. The recommended settings for the various instrumental parameters vary from model to model, and certain parameters require optimization at the time of use to obtain the best results. Instruments should therefore be adjusted as described in the manufacturer's instructions using wavelength settings specified above.

Set the atomic absorption spectrophotometer to the appropriate conditions. Aspirate the strongest standard containing the element to be determined and optimize the instrument settings to give full-scale or maximum deflection on the chart recorder. Measure the absorbances of the other standards and plot a graph showing the net absorbance against the concentration of the element in

the standard solutions. Aspirate Solution A and the corresponding Blank A for determination of copper or Solution B and the corresponding Blank B for determination of zinc and determine the net absorbance. Using the graph prepared above, determine the concentration of the element in the sample solution.

Calculate the content of copper and zinc, respectively from:

$$\text{Copper (mg / kg)} = \frac{c \times 50}{w}$$

$$\text{Zinc (mg / kg)} = \frac{c \times 50}{w}$$

where

c = concentration of element ($\mu\text{g/ml}$) in the sample solution

w = the weight (g) of sample taken

METHOD OF ASSAY

Calcium:

Weigh accurately about 0.150 g of the sample. Dissolve, with the aid of gentle heat if necessary, in a mixture of 5 ml of hydrochloric acid and 3 ml of water contained in a 250 ml beaker equipped with a magnetic stirrer, and cautiously add 125 ml of water. With constant stirring, add, in the following order, 0.5 ml of triethanolamine, 300 mg of hydroxynaphthol blue indicator, and, from a 50 ml buret, about 23 ml of 0.05 M disodium ethylenediamine tetraacetate. Add sodium hydroxide solution (45 in 100) until the initial red colour changes to clear blue, then continue to add it drop wise until the colour changes to violet, then add an additional 0.5 ml. The pH is between 12.3 and 12.5. Continue the titration drop wise with the 0.05 M disodium ethylenediamine tetraacetate until the appearance of a clear blue endpoint that persists for not less than 60 sec. Each ml of 0.05 M disodium ethylenediaminetetraacetate is equivalent to 2.004 mg of Ca.

P₂O₅: Proceed as directed in the *Phosphate Determination as P₂O₅*, Method II (see Volume 4).

MALTOTETRAOHYDROLASE FROM *PSEUDOMONAS STUTZERI* EXPRESSED IN *BACILLUS LICHENIFORMIS*

New specifications prepared at the 80th JECFA (2015) and published in FAO JECFA Monographs 17 (2015). An ADI “not specified” was established at the 80th JECFA (2015).

SYNONYMS Exo-maltotetraohydrolase; 1,4-alpha-D-glucan maltotetraohydrolase

SOURCES Produced by straight-batch or fed-batch fermentation of a genetically modified non-pathogenic, non-toxigenic strain of *Bacillus licheniformis* containing the gene from *Pseudomonas stutzeri*. The enzyme is recovered from the fermentation broth. The recovery process includes the separation of cell mass along with the solid waste slurry carrying the residual microorganism from the enzyme by centrifugation and/or filtration. The liquid enzyme filtrate is concentrated by ultrafiltration followed by polish filtration. The final product is standardized with food-grade materials to the desired activity.

Active principles Maltotetraohydrolase

Systematic names and numbers 4-alpha-D-glucan maltotetraohydrolase; EC 3.2.1.60

Reactions catalyzed Hydrolysis of (1→4)-alpha-D-glucosidic linkages in amylaceous polysaccharides, to remove successive maltotetraose residues from the non-reducing chain ends, using amylaceous polysaccharides like starch as a substrate.

Secondary enzyme activities No significant levels of secondary enzyme activities

DESCRIPTION Amber liquid or off-white granulate

FUNCTIONAL USES Enzyme preparation.
Used in the manufacture of baked goods, and in starch processing.

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

CHARACTERISTICS

IDENTIFICATION

Maltotetraohydrolase activity The sample shows maltotetraohydrolase activity. See description under TESTS.

TESTS

Enzyme Activity

Principle

Maltotetraohydrolase activity is measured by a method using end-blocked *p*-nitrophenyl-maltoheptaoside substrate in the presence of 2 exo-acting enzymes, namely amyloglucosidase and α -glucosidase (maltase). When the oligosaccharide is hydrolyzed by maltotetraohydrolase, the non-blocked nitrophenyl-linked maltooligosaccharide that is produced is hydrolysed to glucose and free *p*-nitrophenol by the combined action of excess amyloglucosidase and α -glucosidase present in the reaction mixture. The amyloglucosidase hydrolyses *p*-nitrophenyl maltooligosaccharide to glucose and *p*-nitrophenyl α -D-glucoside, and the α -glucosidase hydrolyses the *p*-nitrophenyl α -D-glucoside to glucose and yellow coloured *p*-nitrophenol. The reaction is terminated by the addition of an alkaline solution which assists in optimal colour development.

The *p*-nitrophenol release is proportional to maltotetraohydrolase activity, and is monitored at 410 nm.

One BMU refers to an internal standard with a defined activity of the enzyme. One BMU is defined as the activity degrading 0.0351 mmole per min of blocked *p*-nitrophenyl- α -D-maltoheptaoside in the presence of amyloglucosidase and α -glucosidase at 25°, in a specific assay mix for 5 min.

Apparatus

Spectrophotometer

Water bath with thermostatic control

Positive displacement pipettes

Reagents and solutions

MilliQ water, or equivalent

Blocked *p*-Nitrophenyl- α -D-Maltoheptaoside (BPNG7), (Sekisui Diagnostics, US# 70-3685-01/International# BLMN-70-3685 or equivalent)

α -Glucosidase (maltase), (Sekisui Diagnostics, US# 70-1235-01/International # MALT-70-1235 or equivalent)

Glucoamylase, (Sekisui Diagnostics: # GLUC-70-6881 or equivalent)

DL-malic acid, (Sigma # M0875 or equivalent)

Sodium Chloride, reagent grade, (Sigma # S9888 or equivalent)

Calcium Chloride, anhydrous, (Sigma # C1016 or equivalent)

Bovine Serum Albumin, (Sigma # A3294 or equivalent)

1N Sodium Hydroxide TS (from J.T. Baker #3728-01 or equivalent)

Boric Acid, (Sigma # B0394 or equivalent)

1,2-Propanediol (Propylene glycol), (Sigma # P4347 or equivalent)

Enzyme standard (standard solution with certified activity expressed in BMU/ml, available from DuPont (Danisco US Inc.), Rochester, NY, USA).

50 mM Malate buffer, pH 5.6 (For Assay buffer)

Dissolve 6.7 g DL-malic acid in 800 ml MilliQ water. Add 2.92 g NaCl and 0.29 g CaCl₂ and dissolve. Adjust pH to 5.6 with 1N NaOH. Make up to 1.0 l with MilliQ water.

Assay buffer

1% Bovine Serum Albumin (BSA) in Malate Buffer, pH 5.6:

Weigh an amount of BSA and dissolve in the appropriate volume of 50 mM Malate Buffer, pH 5.6, in order to obtain a 1% (w/v) solution.

(Example: 1.0 g BSA dissolved in 100 ml 50 mM Malate Buffer, pH 5.6)

Stop solution (200mM Borate solution)

Dissolve 6.18 g Boric Acid in 400 ml MilliQ water. Adjust pH to 10.2 with 1N NaOH TS. Make up to 500 ml with MilliQ water.

Working substrate solution

Add 54.5 mg BPNPG7, 300 Units α -Glucosidase (maltase), and 120 Units glucoamylase in 10 ml Assay Buffer. Label as Working Substrate Solution.

Standard solutions

Weigh out an amount of enzyme standard, and dilute with Assay buffer to make three dilutions to set up a three point standard curve with a linear range of ~0.2 to 1.6 BMU/ml, at 410 nm. Label accordingly.

Prepare a blank by pipetting 50 μ l Assay buffer into a test tube. Label accordingly.

Store at room temperature.

Samples

Liquid sample: Heat liquid samples to 37 - 40° in a water bath for 15 - 30 min to dissolve any precipitate. Dilute the samples with Assay buffer to obtain a final absorbance within the linear range of the assay. Heat the final dilutions of samples to 37 - 40° in a water bath for 15 - 30 min before assaying. Store diluted samples at room temperature.

Granular sample: Weigh out duplicate granular samples (0.5 g-10 g) in a 100 ml beaker. Add approximately 80 ml of assay buffer to each. Stir on a magnetic plate for 20 min. Transfer to a 100 ml volumetric flask, and adjust to volume with Assay buffer. Prepare additional dilutions if necessary, using Assay Buffer, to obtain a final absorbance within the linear range of the standard curve, at 410 nm. Store diluted samples at room temperature.

Procedure

Preheat water bath to 30°.

Prepare tubes, in duplicates, on a rack. Label as Working Standard, Sample and Blank. Using a positive displacement pipette, dispense 50 μ l standards and working sample dilutions to each labeled tube. Place the rack in a water bath at 25°. Also place the Working Substrate Solution in the water bath to equilibrate for 5 min.

Using a positive displacement pipette, dispense 400 μ l aliquots of Working Substrate Solution into the labelled test tubes, at timed intervals. Incubate all test tubes at 25° for exactly 5 min, in a water bath. Add 600 μ l Stop Solution to each test tube and vortex. Transfer the content of each tube to a plastic cuvette. Measure the absorbance at 410 nm, after appropriately zeroing with blank.

Prepare the standard curve using linear regression. The correlation coefficient must be ≥ 0.99 . Determine the maltotetrahydrolase

concentration of each enzyme standard and sample from the standard curve.

Weigh sample. Record the value as density in g/ml, up to two significant figures.

Calculations

Calculate the activity for each sample in BMU/g as follows:

$$\text{Maltotetraohydrolase Activity, BMU per gram} = \frac{C \times DF}{W}$$

Where

C is concentration of maltotetraohydrolase from the standard curve in BMU/ml

DF is Dilution Factor of sample

W is sample density in g/ml

SERINE PROTEASE WITH CHYMOTRYPSIN SPECIFICITY FROM NOCARDIOPSIS PRASINA EXPRESSED IN BACILLUS LICHENIFORMIS

New specifications prepared at the 76th JECFA (2012) and published in FAO JECFA Monographs 13 (2012). An ADI “not specified” was established at the 76th JECFA (2012)

SYNOMYS	Chymotrypsins A and B; α-chymar ophth; avazyme; chymar; chymotest; enzeon; quimar; quimotrase; α-chymar; α-chymotrypsin A; α-chymotrypsin
SOURCES	Produced by submerged fermentation of a genetically modified non-pathogenic and non-toxigenic strain of <i>Bacillus licheniformis</i> which contains a gene coding for serine protease with chymotrypsin specificity from <i>Nocardiopsis prasina</i> . The enzyme is secreted to the broth. The cell mass and other solids are separated from the broth by vacuum drum filtration or centrifugation. Ultrafiltration and/or evaporation are applied for concentration and further purification. Residual production strain microorganisms are removed by germ filtration. The final product is formulated using food-grade stabilizing and preserving agents and is standardized to the desired activity.
Active principles	Serine protease with chymotrypsin specificity
Systematic names and numbers	EC 3.4.21.1, CAS number: 9004-07-3
Reactions catalysed	Preferential cleavage: Tyr, Trp, Phe, Leu
Secondary enzyme activities	None
DESCRIPTION	Brown liquid
FUNCTIONAL USES	Enzyme preparation. Used in the hydrolysis of proteins like casein, whey, soy isolate, soy concentrate, wheat gluten and corn gluten in the production of partially or extensively hydrolyzed proteins of vegetable and animal origin.
GENERAL SPECIFICATIONS	Must conform to the current edition of JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Serine protease activity with chymotrypsin specificity

The sample shows serine protease activity with chymotrypsin specificity.

See descriptions under TESTS

TESTS

Serine protease activity with chymotrypsin specificity

Principle:

Serine protease hydrolyses the substrate Suc-Ala-Ala-Pro-Phe-pNA. The release of p-nitroaniline (pNA) results in an increase of absorbance at 405 nm and is proportional to the enzyme activity. Enzyme activity is measured in PROT units. One PROT unit is the amount of enzyme that releases 1 μ mol of p-nitroaniline from 1 mM substrate (Suc-Ala-Ala-Pro-Phe-pNA) per minute at pH 9.0 and temperature 37°.

Reagents and Solutions:

0.1M Tris buffer, pH 9.0:

Weigh 12.11 g of Tris (tris(hydroxymethyl)aminomethane) and transfer it to a 1L beaker. Weigh out 8.77 g of sodium chloride and transfer to the beaker. Add 900 ml deionized water. Add 3 drops of Triton X-100 while stirring. Maintain buffer temperature between 23 and 25° prior to next step. Measure and adjust pH after all the Triton X-100 has dissolved. Adjust to pH 9.0 \pm 0.1 using 4M HCl. Transfer to a 1L volumetric flask and make up to volume with deionized water. Solution can be stored at room temperature for up to 24 h. Ensure that the buffer is stirred prior to withdrawing for testing.

Suc-Ala-Ala-Pro-Phe-pNA (Substrate) Stock Solution:

Weigh 50 mg of the Suc-Ala-Ala-Pro-Phe-pNA substrate in a small beaker. Add 1 ml of DMSO to the substrate. Mix well. Transfer solution to an appropriate container, cover with aluminum foil and store away from light. Solution can be stored at room temperature for up to 1 day.

Suc-Ala-Ala-Pro-Phe-pNA (Substrate) Working Solution:

Transfer 350 μ l of Suc-Ala-Ala-Pro-Phe-pNA (Substrate) Stock Solution into a 25 ml volumetric flask. Make up to volume with 0.1M Tris Buffer, pH 9.0. Mix well, and wrap the flask immediately with foil to avoid light. Solution can be stored at room temperature in the dark, up to 6 h.

10 mM citrate buffer, pH 3.40:

Fill a 1000 ml volumetric with about 500ml deionized water. Weigh

1.56 g of citric acid monohydrate, 0.76 g tri-sodium citrate-dihydrate and 8.77 g sodium chloride and transfer to the volumetric flask. Fill the flask to about 900 ml with deionized water. Stir. Add 3 drops of Triton X-100, and continue to stir. Adjust pH to 3.40 ± 0.03 . if necessary, after the Triton X-100 has dissolved. Make up to volume with deionized water and stir. Solution is stable at room temperature for up to 3 days.

Preparation of Standards and Samples:

Preparation of stock standard: Weigh the PROT standard corresponding to 750.1 PROT (± 0.7 PROT) in a 250 ml volumetric flask Dissolve and make up to volume with 10 mM citrate buffer. Stir for 15 min at room temperature. This solution can be stored at room temperature for up to 6 h.

Preparation of samples: All samples, liquid or frozen, must be brought to room temperature, and be thoroughly mixed before weighing.

Weigh a known quantity of sample within ± 1 mg, transfer to an appropriate volumetric flask and make up the volume with 10 mM citrate buffer. The activity of the final dilution(s) of the sample(s) must be around 200 mPROT/ml. Dilute further with citrate buffer, if this concentration is not observed. Solutions can be stored up to 6 h at room temperature.

Procedure

Preparation of Standard Curve: Prepare a standard curve using the stock standard and 10 mM citrate buffer as shown in the table below. The solutions can be stored up to 6 h at room temperature.

Standard No.	Dilution Ratio	Example		Concentration (mPROT/ml)
		Stock Standard, μ l	10 mM citrate buffer, μ l	
1	50	30	1470	60.0
2	30	50	1450	100.0
3	25	60	1440	120.0
4	20	75	1425	150.0
5	15	100	1400	200.0
6	12	125	1375	250.0
7	10	150	1350	300.0

Place the Suc-Ala-Ala-Pro-Phe-pNA (Substrate) Working Solution in a water bath set to $37.0 \pm 1.0^\circ$. Set the spectrophotometer at 405 nm and the temperature of the cuvette holder at $37.0 \pm 0.5^\circ$. Pipette 2.4 ml of the working substrate solution into a cuvette. Add 600 μ l of each standard and sample to the cuvette. Place the cuvette in the spectrophotometer set to $37.0 \pm 0.5^\circ$. Set and start stopwatch to 1

min. Read absorbance at 20 sec intervals for 3 min.

Calculations

Calculate the average absorbance per minute for each standard via linear regression. Plot the standard curve using the average absorbance per minute calculated against activity of the standards (mPROT/ml). Read the absorbance of the sample(s) from the standard curve generated and calculate enzyme activity as shown below:

$$\text{Activity, PROT / g} = \frac{S \times V \times F}{W \times 1000}$$

Where, S is reading in mPROT/ml, from the standard curve, V is Volume of the volumetric flask used for the preparation of the sample for the standard curve in ml, F is Dilution Factor (including the 2nd dilution, if needed during sample preparation), W is weight of the sample in grams and 1000 is the Conversion Factor from mPROT to PROT.

HYDROCHLORIC ACID

Prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996) superseding specifications prepared at the 20th JECFA (1976), published in FNS 1B (1977) and in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002). An ADI not limited' was established at the 9th JECFA (1965)

SYNOMYS Muriatic acid, INS No. 507

DEFINITION

Chemical names	Hydrochloric acid
C.A.S. number	7647-01-0
Chemical formula	HCl
Formula weight	36.46
Assay	Not less than 97.0% and not more than 103.0% of the labelled amount

DESCRIPTION Clear colourless or slightly yellowish liquid with a pungent odour. Various concentrations are supplied as products of commerce.

FUNCTIONAL USES Acid

CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Soluble in water and in ethanol
<u>Test for acid</u>	A 1 in 100 solution of the sample is acid to litmus paper
<u>Test for chloride</u> (Vol. 4)	Passes test

PURITY

<u>Non-volatile residue</u>	Not more than 0.5% Transfer 1 g into a tared glass dish, evaporate to dryness on a steam bath, dry at 110° for 1 h, cool in a desiccator and weigh. The weight of the residue does not exceed 5 mg.
<u>Reducing substances</u>	Not more than 70 mg/kg as sulfur dioxide ; Transfer 1 ml of reagent grade hydrochloric acid into a 30-ml test tube, dilute to 20 ml with freshly boiled and cooled water, and add 1 ml of potassium iodide TS, 1 ml of starch TS and 2 ml of 0.001N iodine. Stopper the test tube and mix thoroughly. The blue colour produced is not discharged by 1 ml of the sample.
<u>Oxidizing substances</u>	Not more than 30 mg/kg as chlorine Transfer 1 ml of the sample into a 30-ml test tube, dilute to 20 ml with freshly boiled and cooled water, and add 1 ml of potassium iodide TS and 1 ml of starch TS. Stopper the test tube and mix thoroughly. The intensity of any blue colour developed does not exceed that produced in a control

prepared similarly but containing 1 ml of 0.001N iodine (instead of potassium iodide TS) and 1 ml of reagent grade concentrated hydrochloric acid (instead of sample).

Sulfate

Not more than 0.5%

Dilute 1 g of the sample to 100 ml with water, transfer 5 ml of this dilution to a 50-ml tall-form Nessler tube and dilute to 20 ml with water. Add a drop of phenolphthalein TS, neutralize the solution with ammonia TS, and then add 1 ml of hydrochloric acid TS prepared from reagent grade hydrochloric acid. To the clear solution (filtered if necessary) add 3 ml of barium chloride TS, dilute to 50 ml with water and mix. Prepare a control consisting of 1 ml of reagent grade concentrated hydrochloric acid and 250 µg of sulfate (SO_4^{2-}) and the same quantities of the reagents as used for the sample. Any turbidity shown in the sample does not exceed that in the control.

Total organic compounds Total organic compounds (non-fluorine): Not more than 5 mg/kg

Benzene: Not more than 0.05 mg/kg

Fluorinated organic compounds (total): Not more than 25 mg/kg

See description under TESTS

Iron

Not more than 5 mg/kg

Dilute 5 g (4.3 ml) of the sample to 40 ml and add about 40 mg of ammonium persulfate and 10 ml of ammonium thiocyanate TS. Any red colour developed does not exceed that in a control prepared by mixing 2.5 ml of Iron standard solution in an equal volume of a solution containing the same quantities of reagent grade hydrochloric acid and the reagents as used in the test.

Lead (Vol. 4)

Not more than 1 mg/kg

Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

Organic compounds

Carry out analyses by gas chromatography employing Vapour Partitioning or Solvent Extraction, depending upon the characteristics of the compound being determined. It is necessary, however, to use the Vapour Partitioning method for the determination of benzene.

Vapour Partitioning Method

This method is suitable for the determination of extractable organic compounds at 0.05 to 100 mg/kg but is most appropriate for organic compounds with a vapour pressure greater than 10 mm Hg at 25°. Use a gas chromatograph equipped with a flame ionization detector and a 4-m x 2-mm (id) stainless-steel column packed with 15%, by weight, methyl trifluoropropyl silicone (DCFS 1265, or QF-1, or OV-210, or SP-2401) stationary phase on 80/100 mesh Gas Chrom R or the equivalent. A newly packed column should be conditioned at 120° and 30 ml/min helium flow for at least 2 h (preferably overnight) before it is attached to the detector. For

analysis, the column is maintained isothermally at 105°, the injection port and detector are maintained at 250°, the carrier gas flow rate is set at 11 ml/min, fuel gas flows should be optimized for the gas chromatograph and detector in use. The experimental conditions may be changed as necessary for optimal resolution and sensitivity. The signal-to-noise ratio should be at least 10:1.

Preparation of Standard Solutions

Prepare a standard solution of the organic compounds to be quantitated in Hydrochloric Acid (known to be free of interfering impurities) at approximate concentrations of 5 mg/kg, or within ±50% of the concentrations in the samples to be analyzed.

Place a stirring bar in a one-litre volumetric flask equipped with a ground-glass stopper, and tare the combination. Fill the flask with reagent-grade hydrochloric acid so that no air space is present when the flask is stoppered, and determine the weight of the Hydrochloric Acid. Calculate the volume (V) in 1l of each organic component to be added from the formula

$$V = (C \times W)/(D \times 1000)$$

where C is the desired concentration, in mg/kg; W is weight, in g, of the Hydrochloric Acid; D is the density, in mg/1l, of the organic compound; and 1000 is a conversion factor with the units g/kg. Add the calculated amount of each component to the Hydrochloric Acid with a syringe (ensure that the syringe tip is under the solution surface), stopper the flask, and stir the solution for at least 2 h using a magnetic stirrer.

Calibration

Treat the standard in the same way as described for the sample under Procedure (below). Determine a blank for each lot of reagent-grade Hydrochloric Acid, and calculate a response factor (R) by dividing the concentration (C) in mg/kg for each component by the peak area (A) for that component (subtract any area obtained from the blank sample):
 $R = C/(A - \text{area of blank})$

Gaseous compounds present special problems in the preparation of standards. Therefore, to determine response factors for gaseous compounds use the following method, which will be referred to as the Method of Multiple Extractions. Dilute a sample of Hydrochloric Acid known to contain the gaseous compound of interest with an equal volume of water. Draw 20 ml of this solution into a 50-ml glass syringe; then draw 20 ml of air into the syringe, cap with a rubber septum, and place the syringe on a shaker for 5 min. Withdraw 1 ml of the vapour through the septum, and inject it into the chromatograph. Expel the vapour phase from the 50-ml syringe, draw in another 20 ml of air, repeat the extraction, and inject another 1-ml vapour sample into the gas chromatograph. Repeat the extraction, and GC analysis on the same sample of acid a total of six times. For each impurity, plot the area (A_N) determined for extraction N against the difference between A_N and the area determined for extraction ($N + 1$); that is, plot A_N against $[A_N - A_{N+1}]$. The slope of this line is the extraction efficiency (E) for that impurity into the air.

Inject into the chromatograph 1 ml of 0.1% (by volume) standard gas sample of each impurity in air and determine the absolute factor (F_A) in g, per peak area (A) by the following formula:

$$F_A = (M \times 4.0816 \times 10^{-8})/A,$$

where M is the molecular weight of the compound.

The concentration (C), in mg/kg, of the component in the original sample is calculated by the formula

$$C = (A \times F_A \times 1.6949 \times 10^6)/E.$$

where A is the peak area corresponding to the compound (as above), F_A is the absolute factor, and E is extraction efficiency. The response factor is then calculated as $R = C/A$

Procedure

Dilute a 10-ml sample of Hydrochloric Acid to be analyzed with an equal volume of water. Draw this solution into a 50-ml glass syringe. Then draw 20 ml of air into the syringe, cap with a rubber septum, and place the syringe on a shaker for 5 min. Draw 1 ml of the vapour through the septum, and inject it into the gas chromatograph. Approximate elution times in min for some specific organic compounds are as follows:

Methane and acetylene:	1.70
Methyl chloride:	2.21
Vinyl chloride:	2.29
1,1,1-Trichlorofluoromethane:	2.62
Ethyl chloride:	2.90
Vinyldene chloride:	3.20
Methylene chloride:	3.64
Chloroform:	4.49
1,1-Dichloroethane:	4.53
Carbon tetrachloride:	4.86
1,1,1-Trichloroethane:	5.50
Benzene:	6.00
Trichloroethylene:	6.22
Ethylene dichloride:	6.61
Propylenedichloride:	8.41
Perchloroethylene:	9.73

Alternate columns may be required to resolve some combinations of components. Methyl chloride and vinyl chloride are resolved by a 3.7-m x 3-mm (id) squalene column at 45° and a helium flow of 10 ml/min. Chloroform and 1,1-dichloroethane are resolved by a 4-m x 3-mm (id) DC 550R column at 110° and a helium flow of 12 ml/min.

Calculation

Calculate the concentration (C) in mg/kg of each compound by multiplying its corresponding peak area (A) by the appropriate response factor (R) determined in the Calibration protocol:

$$C = R \times A$$

Precision

The relative standard deviation at 5 mg/kg should not exceed 15% for five analyses.

Solvent Extraction Method

The solvent extraction technique is suitable for the determination of extractable organic compounds at 0.3 to 100 mg/kg, but is most appropriate for organic compounds with vapour pressures less than 10 mm Hg at 25°. The conditions for the gas chromatograph are the same as for the Vapour Partitioning method, except that the column temperature is 120°, and the carrier-gas flow is 21 ml/min.

Preparation of Standards

Prepare the Standard Solution as described under Vapour Partitioning.

Calibration

Extract a sample of the Standard Solution as directed under Procedure (below) and inject it into the gas chromatograph. Determine a blank for each lot of reagent-grade Hydrochloric Acid and perchloroethylene by extracting the Hydrochloric Acid in the same way as the standard. Calculate a response factor (R) by dividing the concentration (C) in mg/kg for each component by the peak area (A) for that component (subtract any area obtained from the blank sample):

$$R = C/(A - \text{area of blank})$$

Procedure

Accurately transfer 90 ml of the Hydrochloric Acid sample and 10 ml of perchloroethylene (free of interfering impurities) into a narrow-mouth, 125-ml bottle. Place the bottle in a mechanical shaker for 30 min. Separate the two phases (perchloroethylene on the bottom) and inject 3 µl of the perchloroethylene extract into the gas chromatograph. Approximate elution times in min for some chlorinated organic compounds are as follows:

Vinylidene chloride:	2.94
Methylene chloride:	3.27
Chloroform:	3.83
Carbon tetrachloride:	4.07
1,1,1-Trichloroethane:	4.50
Trichloroethylene:	4.97
Ethylene dichloride:	5.26
Propylene dichloride:	6.36
Perchloroethylene:	6.95
1,1,1,2-Tetrachloroethane:	10.12
1,1,2,2-Tetrachloroethane:	13.70
Pentachloroethane:	16.19

To determine perchloroethylene and higher-boiling impurities, substitute methylene chloride (free of interfering impurities) for perchloroethylene in the extraction step. For higher-boiling impurities such as monochlorobenzene and the three dichloro- benzenes, use a 2.74-m x 2.1-mm (id) stainless steel column packed with 10% Carbowax 20M/20% KOH on 80/100 mesh Chromasorb W (acid washed) at 150° and a nitrogen flow of 35 ml/min.

METHOD OF ASSAY

Calculation

Calculate the concentration (C), in mg/kg, of each compound by multiplying the corresponding peak area (A) (subtracted any area obtained from the blank sample) by the appropriate response factor (R) determined in the Calibration protocol:

$$C = R \times (A - \text{area of blank})$$

Precision

The relative standard deviation at 5 mg/kg should not exceed 15% for five analyses.

Tare accurately a 125-ml glass-stoppered conical flask containing 50 ml of 1N sodium hydroxide. Partially fill, without the use of vacuum, a 10-ml serological pipet from near the bottom of a representative sample, remove any acid adhering to the outside and discard the first ml flowing from the pipet. Hold the tip of the pipet just above the surface of the sodium hydroxide solution, and transfer between 2.5 and 3 ml of the sample into the flask, mix the contents, and weigh accurately to obtain the weight of the sample. Add methyl orange TS and titrate the excess of sodium hydroxide with 1N hydrochloric acid. Each ml of 1N sodium hydroxide is equivalent to 36.46 mg of HCl.

NEOTAME

New specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add 11(2003). An ADI of 0 – 2 mg/kg bw was established at the 61st JECFA (2003).

SYNONYMS

INS No. 961

DEFINITION

Neotame is manufactured in single process in which aspartame and 3,3-dimethylbutyraldehyde are reacted together in a methanol solution in the presence of hydrogen. Neotame is isolated by removal of methanol, followed by washing and drying.

Chemical names

N-[N-(3,3-Dimethylbutyl)-L- α -aspartyl]- L-phenylalanine 1-methyl ester

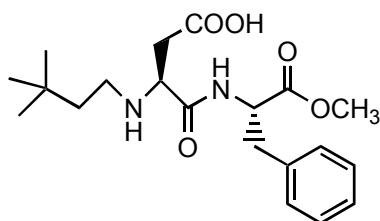
C.A.S. number

165450-17-9

Chemical formula

C₂₀H₃₀N₂O₅

Structural formula



Formula weight

378.47

Assay

Not less than 97.0% and not more than 102.0% on the anhydrous basis

DESCRIPTION

White to off-white powder

FUNCTIONAL USES

Sweetener, flavour enhancer

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Sparingly soluble in water, very soluble in ethanol

Infrared spectrum

The infrared spectrum of a potassium bromide dispersion of the sample corresponds to the standard infrared spectrum in Appendix A.

PURITY

pH (Vol. 4)

5.0 – 7.0 (0.5 % solution)

Melting range (Vol. 4)

81° - 84°

Water (Vol. 4)

Not more than 5.0% in a sample size of 25±5 mg (Karl Fischer)

<u>N-[N-(3,3-Dimethylbutyl)-α-aspartyl]-L-phenylalanine</u>	Not more than 1.5% See under METHOD OF ASSAY
<u>Other related substances</u>	Not more than 2.0% based on the results of the Method of Assay using the following formula:
	100 x A/(A+B)
	where
	A = the sum of the peak areas for all secondary peaks other than those for neotame and N-[N-(3,3-dimethylbutyl)-L- α -aspartyl]-L-phenylalanine and
	B = the sum of the peak areas for neotame and N-[N-(3,3-dimethylbutyl)-L- α -aspartyl]-L-phenylalanine.
<u>Sulfated ash</u> (Vol. 4)	Not more than 0.2%
<u>Specific rotation</u> (Vol. 4)	$[\alpha]_D^{20}$: Between -40.0° and -43.3° (0.5 % solution) calculated on the anhydrous basis
<u>Lead</u> (Vol. 4)	Not more than 1 mg/kg Determine using an atomic absorption technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles described in Volume 4, "Instrumental Methods".

METHOD OF ASSAY

Neotame

Determine by HPLC using the following conditions:
Mobile phase: 25% acetonitrile and 75% buffer (final pH of 3.7). The buffer is composed of 0.02 M heptanesulfonic acid sodium salt and 0.5% v/v triethylamine at pH 3.5.
Preparation of sample: Dissolve the sample in mobile phase solution to a concentration of 1 mg/ml.
Preparation of standard: Dissolve the neotame standard (NutraSweet Kelco) in mobile phase solution to a concentration of 1 mg/ml
HPLC Conditions:
 Column: Partisil 5 ODS3 (4.6 x 100 mm length) or equivalent.
 Column temperature: 45°
 Pump: Isocratic
 Solvent: 25% acetonitrile and 75% buffer adjusted to a pH of 3.7.
 Flow rate: 1.5 ml/min
 Injection: 25 μ l
 Detection: UV 210 nm
 Run Time: approximately 18 min.
Calculation: Compare the area of the neotame peak in the sample (A_{sample}) to that in the standard (A_{standard}). Calculate the percentage content of the sample, on the dry basis, from the formula

$$\% \text{ neotame} = (A_{\text{sample}}/A_{\text{standard}}) \times 100 \times F$$

Where
 $F = 100 / (100 - \% \text{ water in sample})$

N-[N-(3,3-Dimethylbutyl)- α -aspartyl]-L-phenylalanine

This is determined using the same HPLC method:

Preparation of sample: Dissolve the sample in mobile phase solution to a concentration of 2 mg/ml.

Preparation of standard: Dissolve the N-[N-(3,3-dimethylbutyl)- α -aspartyl]-L-phenylalanine standard (NutraSweet Kelco) in mobile phase solution to concentrations of 75, 45, 15, 3 and 0.9 $\mu\text{g}/\text{ml}$.

Calculation: The retention time for N-[N-(3,3-dimethylbutyl)- α -aspartyl]-L-phenylalanine is approximately 4.4 min compared with approximately 12.2 min for neotame.

Determine the area response of N-[N-(3,3-dimethylbutyl)-L- α -aspartyl]-L-phenylalanine from the sample preparation. Prepare a full fit linear regression standard curve by plotting the area response of N-[N-(3,3-dimethylbutyl)-L- α -aspartyl]-L-phenylalanine in the standard solution on the ordinate scale versus its respective concentration in $\mu\text{g}/\text{ml}$. From the slope and intercept of the standard curve, calculate the concentration, C_1 ($\mu\text{g}/\text{ml}$), of N-[N-(3,3-dimethylbutyl)-L- α -aspartyl]-L-phenylalanine in the sample using the equation:

$$C_1 = (A_{\text{sample}} - \text{intercept}) / \text{slope of curve}$$

Calculate the percentage of N-[N-(3,3-dimethylbutyl)-L- α -aspartyl]-L-phenylalanine in the sample using the equation

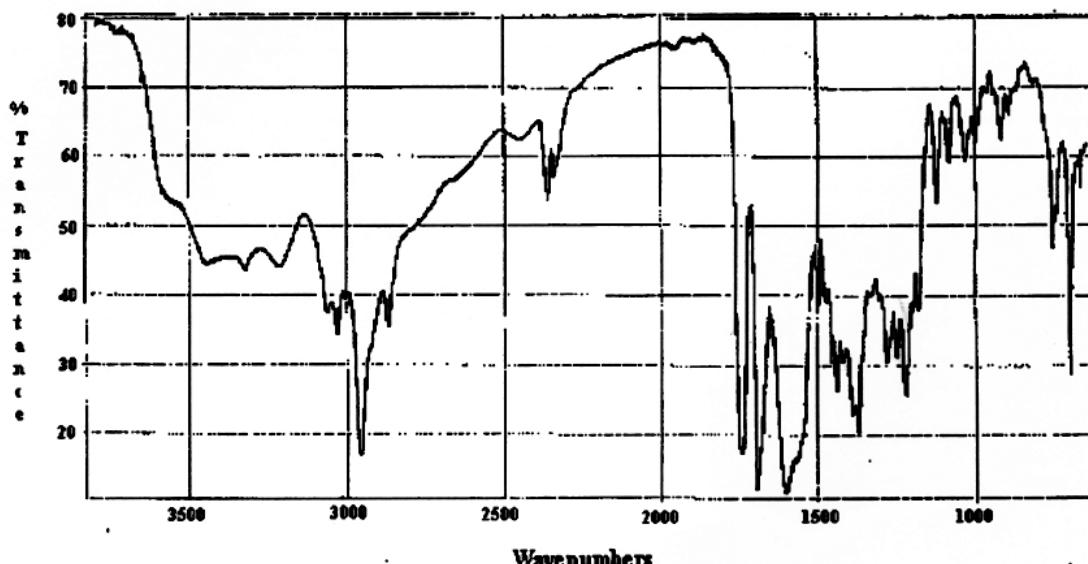
$$\% \text{ N-[N-(3,3-dimethylbutyl)-L-}\alpha\text{-aspartyl]-L-phenylalanine} = (C_1/C_2) \times 100$$

where C_2 is the concentration of the sample.

See Appendix B for examples of chromatograms obtained using the method.

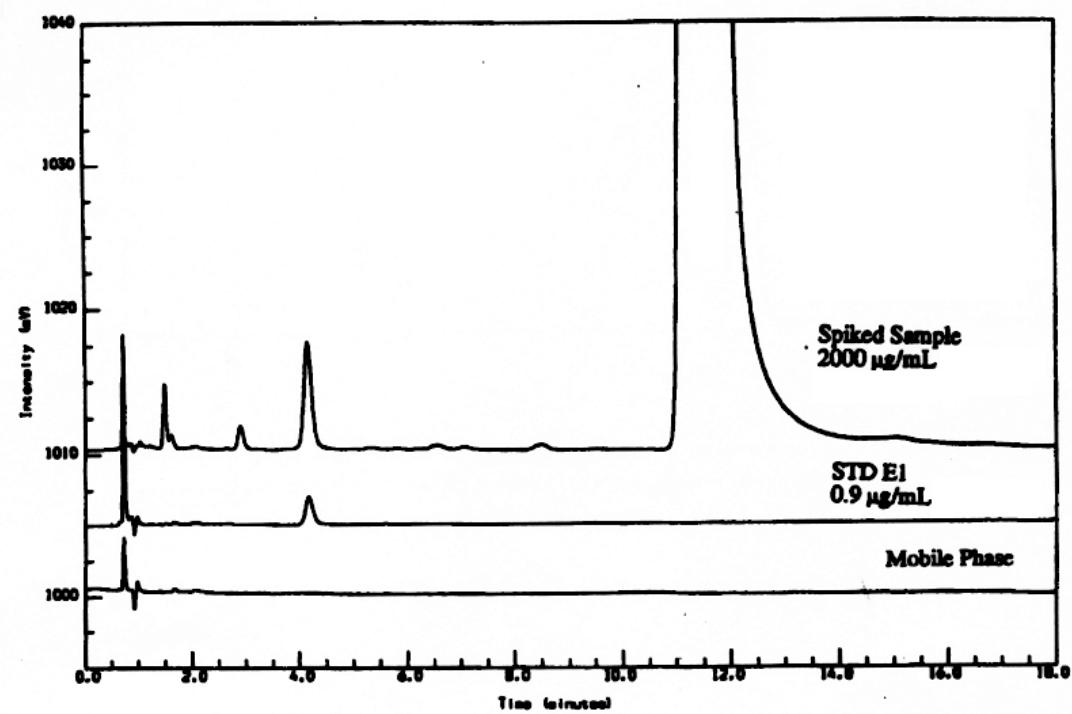
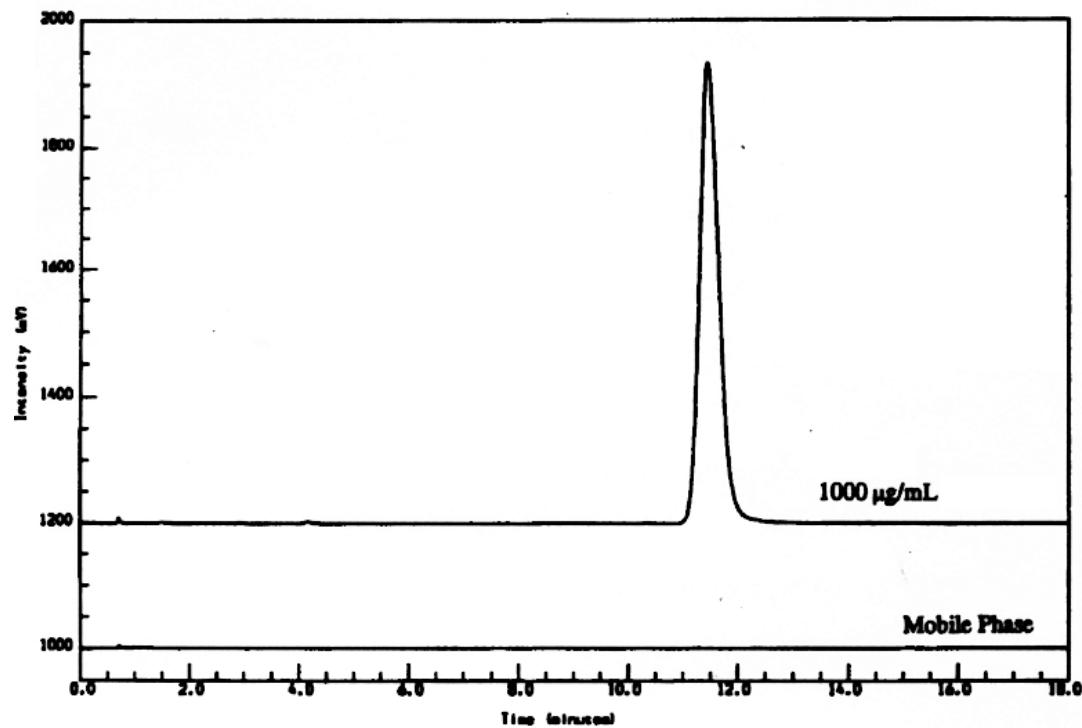
Appendix A

IR Spectrum of neotame standard



Appendix B

Chromatograms for neotame and N-[N-(3,3-dimethylbutyl)-L- α -aspartyl]-L-phenylalanine (Std E1)



MONOAMMONIUM L-GLUTAMATE

Prepared at the 31st JECFA (1987), published in FNP 38 (1988) and in FNP 52 (1992). Metals and arsenic specifications revised at the 57th JECFA (2001). A group ADI 'not specified' for glutamic acid and its ammonium, Ca, K, Mg & Na salts, was established at the 31st JECFA (1987)

SYNONYMS

Ammonium glutamate, INS No. 624

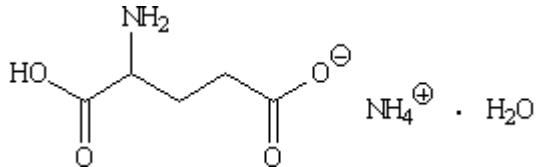
DEFINITION

Chemical names Monoammonium L-glutamate monohydrate

C.A.S. number 7558-63-6

Chemical formula C₅H₁₂N₂O₄ · H₂O

Structural formula



Formula weight 182.18

Assay Not less than 99.0% on the dried basis

DESCRIPTION

White, practically odourless crystals or crystalline powder

FUNCTIONAL USES Flavour enhancer, salt substitute

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in water

Test for glutamate (Vol. 4) Passes test

Test for ammonium (Vol. 4) Passes test

PURITY

Loss on drying (Vol. 4) Not more than 0.5% (50° , 4 h)

pH (Vol. 4) 6.0 - 7.0 (1 in 20 soln)

Specific rotation (Vol. 4) [alpha] 20, D: Between +25.4 and +26.4° (10% (w/v) solution in 2N hydrochloric acid)

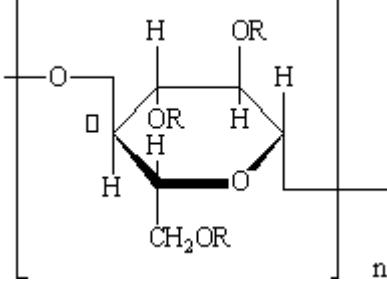
Sulfated ash (Vol. 4) Not more than 0.1%.
Test 1 g of the sample (Method I)

Pyrrolidone carboxylic acid Passes test
(Vol. 4)

<u>Lead</u> (Vol. 4)	Not more than 1 mg/kg Determine using an atomic absorption technique 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	Dissolve about 200 mg of the sample, previously dried and weighed accurately, in 6 ml of formic acid, and add 100 ml of glacial acetic acid. Titrate with 0.1 N perchloric acid determining the end-point potentiometrically. Run a blank determination in the same manner and correct for the blank. Each ml of 0.1 N perchloric acid is equivalent to 9.106 mg of $C_5H_{12}N_2O_4 \cdot H_2O$.

METHYL ETHYL CELLULOSE

Prepared at the 17th JECFA (1973), published in FNP 4 (1978) and in FNP 52 (1992). Metals and arsenic specifications revised at the 57th JECFA (2001). A group ADI 'not specified' for modified celluloses was established at the 35th JECFA (1989)

SYNONYMS	MEC; INS No. 465
DEFINITION	A mixed ether of cellulose, prepared from cellulose by treatment with alkali, dimethyl sulfate and ethyl chloride; both the methyl and ethyl groups are attached to the anhydroglucosamine units by ether linkages. The article of commerce can be specified further by viscosity.
Chemical names	Ethyl methyl ether of cellulose
C.A.S. number	9004-69-7
Chemical formula	$[C_6H_{7O_2(OH)}_x(OCH_3)_y(OC_2H_5)_z]_n$ where $z = 0.57 \text{ to } 0.8$ $y = 0.2 \text{ to } 0.4$ $x = 3 - (x + y)$ ($y + z = \text{degree of substitution}$)
Structural formula	
	where $R = H \text{ or } CH_3 \text{ or } C_2H_5$
Formula weight	Unsubstituted structural unit: 162.14 Structural unit with a total degree of substitution of 0.77: 181 Structural unit with a total degree of substitution of 1.2: 190 Macromolecules: 30 000 - 40 000 (n about 200)
Assay	Methyl Ethyl Cellulose contains, on the dried basis, not less than 3.5% and not more than 6.5% of methoxyl groups ($-OCH_3$), not less than 14.5% and not more than 19.0% of ethoxyl groups ($-OCH_2CH_3$), and not less than 13.2% and not more than 19.6% of total alkoxy groups, calculated as methoxyl (on the dry basis).
DESCRIPTION	Hygroscopic and slightly yellowish odourless fibre or powder
FUNCTIONAL USES	Emulsifier, stabilizer, thickening agent, foaming agent
CHARACTERISTICS	

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Swelling in water, producing a clear to opalescent, viscous, colloidal solution; insoluble in ethanol.
<u>Foam test</u>	A 0.1% solution of the sample is shaken vigorously. A layer of foam appears. (This test permits the distinction of sodium carboxymethyl cellulose from other cellulose ether and alginates and natural gums).
<u>Precipitate formation</u>	To 5 ml of an 0.5% solution of the sample add 5 ml of a 5% solution of copper sulfate or of aluminium sulfate. No precipitate appears. (This test permits the distinction of cellulose ethers from sodium carboxymethyl cellulose, gelatine, carob bean gum and tragacanth gum).
<u>Substituents</u>	Determine the substituents by <i>Gas Chromatography</i>

PURITY

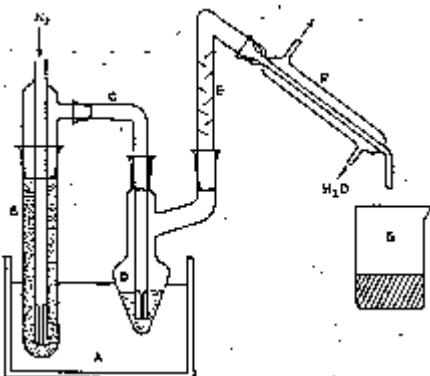
<u>Loss on drying</u> (Vol. 4)	Not more than 15% for the fibrous form, and not more than 10% for the powdered form, after drying to constant weight
<u>Sulfated ash</u> (Vol. 4)	Not more than 0.6% Test 1 g of the sample (Method I)
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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

Determination of the Ethoxyl group (Ethoxyl and methoxyl can be separately determined by gas chromatography (Cobler, Samsel and Beaver, *Talanta*, 9, 473, 1962)).

Apparatus

The apparatus for ethoxyl group determination is shown in the accompanying diagram. The boiling flask, D, is fitted with an aluminium foil-covered Vigreux column, E, on the sidearm and with a bleeder tube through the neck and to the bottom of the flask for the introduction of steam and nitrogen. A steam generator, B, is attached to the bleeder tube through tube C, and a condenser, F, is attached to the Vigreux column. The boiling flask and steam generator are immersed in an oil bath, A, equipped with a thermoregulator such that a temperature of 155° and the desired heating rate may be maintained. The distillate is collected in a 150-ml beaker, G, or other suitable container.



Procedure

Transfer about 100 mg, weighed to the nearest 0.1 mg, of the sample, previously dried at 105° for 2 h, into the boiling flask, and add 10 ml of chromium trioxide solution (60 g in 140 ml of water). Immerse the steam generator and the boiling flask in the oil bath (at room temperature) to the level of the top of the chromium trioxide solution. Start cooling water through the condenser and pass nitrogen gas through the boiling flask at the rate of one bubble per second. Starting at room temperature, raise the temperature of the oil bath to 155° over a period of not less than 30 min, and maintain this temperature until the end of the determination. Distil until 50 ml of distillate is collected. Detach the condenser from the Vigreux column, and wash it with water, collecting the washings in the distillate container. Titrate the combined washings and distillate with 0.02 N sodium hydroxide to a pH of 7.0, using a pH meter set at the expanded scale. (Note: Phenolphthalein TS may be used for this titration if it is also used for all standards and blanks.)

Record the volume, V_a , of the 0.02 N sodium hydroxide used. Add 500 mg of sodium bicarbonate and 10 ml of dilute sulfuric acid TS, and then, after evolution of carbon dioxide has ceased, add 1 g of potassium iodide. Stopper the flask, shake the mixture and allow it to stand in the dark for 5 min. Titrate the liberated iodine with 0.02 N sodium thiosulfate to the sharp disappearance of the yellow colour, confirming the end-point by the addition of a few drops of starch TS. Record the volume of 0.02 N sodium thiosulfate required as Y_a .

Make several reagent blank determinations, using only the chromium trioxide solution in the above procedure. The ratio of the sodium hydroxide titration (V_b), corrected for variation in normalities, will give the acidity-to-oxidizing ratio $V_b/Y_b = K$, for the chromium trioxide carried over in the distillation. The factor K should be constant for all determinations.

Make a series of blank determinations using 100 mg of methyl cellulose (containing no foreign material) in place of the sample, recording the average volume of 0.02 N sodium hydroxide required as V_m and the average volume of 0.02 N sodium thiosulfate required as Y_m .

Calculate the ethoxyl content of the sample, in mg, by the formula:

$$45.0 \times [N_1(V_a - V_m) - kN_2(Y_a - Y_m)]$$

where

N_1 = exact normality of the 0.02 N sodium hydroxide solution,
 N_2 = exact normality of the 0.02 N sodium thiosulfate solution, and
 $k = V_b N_1 / Y_b N_2$
Record the percentage of ethoxyl as B%.

Determination of the methoxyl content

Determine the methoxyl plus methoxyl content (Total alkoxy content) as directed under *Ethoxyl and Methoxyl Group Determinations*. Then calculate the methoxyl content as follows:

$$\% \text{Methoxyl} = \frac{31}{45} \times (A - B)$$

where

A = the total alkoxy content expressed as % ethoxyl

B = the ethoxyl content expressed as %, as determined above.

Determination of total alkoxy content (as methoxyl)

Each ml of 0.1 N sodium thiosulfate required in the determination of total alkoxy content is equivalent to 0.517 mg of alkoxy expressed as methoxyl.

CHYMOSIN B from *ASPERGILLUS NIGER* var. AWAMORI containing the PROCHYMOSEN B GENE

Prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7 (1999), superseding tentative specifications prepared at the 37th JECFA (1990), published in FNP 52 (1992). ADI "Not specified" established at the 37th JECFA in 1990.

SYNOMYS Rennin, milk-clotting enzyme, chymosin, chymosin B, aspartyl protease

C.A.S. number 85713-24-2

SOURCES Produced extracellularly by the controlled fermentation of *Aspergillus niger* var. *awamori* containing the bovine prochymosin B gene. The strain is non-pathogenic and non-toxicogenic (for example, NRRL 3112). After inactivation of the production organism, the cellular material is removed by centrifugation or filtration. The enzyme is purified by extraction with polyethylene glycol, followed by clarification with activated carbon and separation from the solvent by cation exchange chromatography.

Active principles Chymosin

Systematic names and numbers None (EC 3.4.23.4)

Reactions catalyzed Cleaves a single bond in kappa-casein

DESCRIPTION Clear, colourless or slightly coloured aqueous solutions containing the active enzyme

FUNCTIONAL USES Enzyme preparation
Used in clotting of milk for cheese production

GENERAL SPECIFICATIONS Must conform to the General Specifications for Enzyme Preparations used in Food Processing (see Volume Introduction)

CHARACTERISTICS

IDENTIFICATION

Milk clotting activity
(Vol. 4) The sample shows milk clotting activity

HYDROXYPROPYL CELLULOSE

Revised specification prepared at the 63rd JECFA (2004) and published in FNP52 Add 12 (2004) superseding specifications prepared at the 29th JECFA (1985) and published in FNP 52. An ADI 'not specified' was established for modified celluloses at the 35th JECFA (1989).

SYNONYMS

Cellulose hydroxypropyl ether; modified cellulose; INS No. 463

DEFINITION

An ether of cellulose containing hydroxypropyl substitution prepared from cellulose by treatment with alkali and propylene oxide. The article of commerce can be specified further by viscosity.

Chemical names

Hydroxypropyl ether of cellulose, cellulose hydroxypropyl ether

C.A.S. number

9004-64-2

Chemical formula

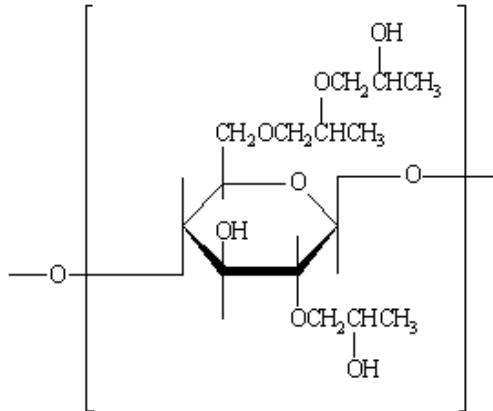
[C₆H₇O₂(OH)_x(OCH₂CHOHCH₃)_y(OCH₂CH[R_w]CH₃)_z]_n
where

$$x + y + z = 3$$

$$y + z (1+w) = \text{not greater than } 4.6$$

R = A substituent comprising "w" hydroxypropoxy groups

Structural formula



One of many possible structural formulae for the repeating unit of a hydroxypropyl cellulose with molar substitution of 3.0 and a degree of polymerization of n, showing a monomeric hydroxypropyl substitution at C₂ and a dimeric hydroxypropyl substitution at C₆.

Formula weight

Unsubstituted structural unit: 162.14

Trisubstituted structural unit: 336.37

Macromolecules: from about 30 000 (n about 100) up to about 1 million (n about 2500)

Assay

Not more than 80.5% of hydroxypropoxy groups equivalent to not more than 4.6 hydroxypropyl groups per anhydroglucose unit on the dried basis

DESCRIPTION

Slightly hygroscopic, white or off-white, almost odourless, granular or fibrous powder

FUNCTIONAL USES

Emulsifier, thickener, stabiliser, binder, suspension agent, film coating

CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Swells in water, producing a clear to opalescent, viscous colloidal solution; insoluble in ethanol; insoluble in ether
<u>Foam formation</u>	A 0.1% solution of the sample is shaken vigorously. A layer of foam appears. This test permits the distinction of sodium carboxymethyl cellulose from other cellulose ethers.
<u>Precipitate formation</u>	To 5 ml of a 0.5% solution of the sample, add 5 ml of a 5% solution of copper sulfate or of aluminium sulfate. No precipitate appears. This test permits the distinction of sodium carboxymethyl cellulose from other cellulose ethers.
<u>Substituents</u>	See description under METHOD OF ASSAY

PURITY

<u>Loss on drying</u> (Vol. 4)	Not more than 10.0% (105° to constant weight)
<u>pH</u> (Vol. 4)	Not less than 5.0 and not more than 8.0 (1 in 100 soln)
<u>Sulfated ash</u> (Vol. 4)	Not more than 0.5%. Test 1 g of the sample
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg. Determine using an atomic absorption technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, "Instrumental Methods"

Propylene chlorohydrins

Not more than 0.1 mg/kg
See description under TESTS

TESTS

PURITY TESTS

Propylene chlorohydrins

Determine by gas liquid chromatography (see Volume 4) using the following procedure:

Preparation of Standards

Stock Standard Solution: Weigh 0.1 g propylene chlorohydrin (C.A.S. No. 127-00-4, mixture of 1-Chloro-2-propanol-70% and 2-Chloro-1-propanol-30%) to the nearest 0.0001g and bring to a final volume of 100 ml with diethyl ether.

Working Standard Solution: Perform serial dilutions (in diethyl ether) of stock standard to achieve a working calibration range of 6-25 ng/ml.

Note: All standard solutions should be prepared with diethyl ether of the highest purity

Gas Chromatography

Gas Chromatograph with a Halogen Specific Detector, on-column injector, and linear column temperature programming.

Column: 30 m x 0.53 mm x 1 µm DB-WAX or equivalent.

Temperature programming:

Initial Temperature	35°
Initial Hold Time	7.0 min
Ramp Rate	8.0°/min
Final Temperature	200°
Final Hold Time	5.0 min
Inlet	200°
Detector (XSD)	1000°

Flow rates:

Helium (carrier gas) 5 psi (column head pressure at 35°)

Detector Make-up Gas (air) 40 psi

Retention times (min):

1-Chloro-2-propanol	~11.7
2-Chloro-1-propanol	~12.5

Procedure:

Weigh ~1 g of sample into a centrifuge tube and record weight to the nearest 0.01 g. Quantitatively add 5.0 ml diethyl ether to the sample and sonicate for 10 minutes. Centrifuge the sample to separate the mixture. Remove a portion of the diethyl ether extract for GC analysis.

Calculations:

Prepare a calibration curve by plotting the concentration (ng/ml) versus detector response (in a linear range of 6-25 ng/ml). From the linear regression of this curve, calculate ng/g using the following equation:

$$\text{ng/g} = (V \times (R-b)/m)/W$$

where:

- R= detector response for the sample
- b = y-intercept of the linear regression curve
- m = slope of the linear regression curve
- V= final volume (5.0 ml)
- W= weight of the sample in grams

METHOD OF ASSAY

Determination of the hydroxypropoxy group content

Apparatus

The apparatus for hydroxypropoxy group determination is shown in the accompanying diagram. The boiling flask, D, is fitted with an aluminium foil-covered Vigreux column, E, on the sidearm and with a bleeder tube through the neck and to the bottom of the flask for the introduction of steam and nitrogen. A steam generator, B, is attached to the bleeder tube through Tube C, and a condenser, F, is attached to the Vigreux column. The boiling flask and steam generator are immersed in an oil bath, A, equipped with a thermo-regulator such that a temperature of 155° and the desired heating rate may be maintained. The distillate is collected in a 150 ml beaker, G, or other suitable container.

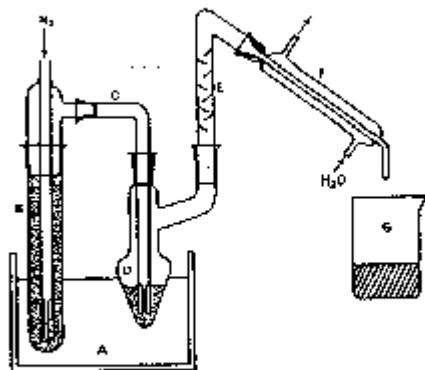


Figure Apparatus for Hydroxypropyl Determination

Procedure

Transfer about 100 mg of the sample, previously dried at 105° for 2 h and accurately weighed, into the boiling flask, and add 10 ml of chromium trioxide solution (60 g in 140 ml of water). Immerse the steam generator and the boiling flask in the oil bath (at room temperature) to the level of the top of the chromium trioxide solution. Start cooling water through the condenser and pass nitrogen gas through the boiling flask at the rate of one bubble per sec. Starting at room temperature, raise the temperature of the oil bath to 155° over a period of not less than 30 min, and maintain this temperature until the end of the determination. Distil until 50 ml of the distillate is collected. Detach the condenser from the Vigreux column, and wash it with water, collecting the washings in the distillate container. Titrate the combined washings and distillate with 0.02 N sodium hydroxide to a pH of 7.0, using a pH meter set at the expanded scale.

NOTE: Phenolphthalein TS may be used for this titration, if it is also used for all standards and blanks.

Record the volume, V_a of the 0.02 N sodium hydroxide used. Add 500 mg of sodium bicarbonate and 10 ml of dilute sulfuric acid TS, and then after evolution of carbon dioxide has ceased, add 1 g of potassium iodide. Stopper the flask, shake the mixture, and allow it to stand in the dark for 5 min. Titrate the liberated iodine with 0.02 N sodium thiosulfate to the sharp disappearance of the yellow colour, confirming the end-point by the addition of a few drops of starch TS. Record the volume of 0.02 N sodium thiosulfate required as Y_a . Make several reagent blank determinations, using only the chromium trioxide solution in the above procedure. The ratio of the sodium hydroxide titration (V_b) to the sodium thiosulfate titration (Y_b), corrected for variation in normalities, will give the acidity-to-oxidizing ratio, $V_b/Y_b = K$, for the chromium trioxide carried over in the distillation. The factor K should be constant for all determinations. Make a series of blank determinations using 100 mg of methyl cellulose (containing no foreign material) in place of the sample, recording the average volume of 0.02 N sodium hydroxide required as V_m and the average volume of 0.02 N sodium thiosulfate required as Y_m .

Calculate the hydroxypropoxy group content of the sample, in mg, by the formula:

$$75.0 \times [N_1 (V_a - V_m) - k N_2 (Y_a - Y_m)]$$

where

N_1 = the exact normality of the 0.02 N sodium hydroxide solution

N_2 = the exact normality of the 0.02 N sodium thiosulfate solution

$$k = V_b N_1 / Y_b N_2$$

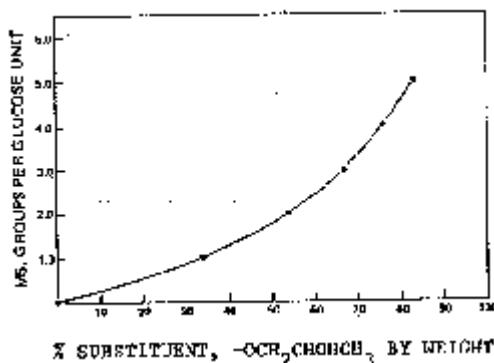


Chart for converting percentage of substitution, by weight, of hydroxypropoxy groups to molecular substitution per glucose unit.

Determination of the methoxy group

See Apparatus and Procedure in *Ethoxy and Methoxy Group Determination* and determine the content of methoxy group (-OCH₃).

Calculation

Calculate as percentage. Correct the % of methoxy groups thus determined by the formula:

$$A - (B \times 0.93 \times 31 / 75)$$

where

A = the total % of -OCH₃ groups determined

B = the % of -OCH₂CHOHCH₃ determined in the Method of Assay for Hydroxypropoxy group content

0.93 = an average obtained by determining, on a large number of samples, the propylene produced from the reaction of hydriodic acid with hydroxypropoxy groups during the Method of Assay for methoxy groups (-OCH₃).

PROPYLENE GLYCOL

Prepared at the 49th JECFA (1997), published in FNP 52 Add 5 (1997) superseding specifications prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996). Metals and arsenic specifications revised at the 63rd JECFA (2004). An ADI of 0-25 mg/kg bw was established at the 17th JECFA (1973)

SYNOMYS Propanediol, Methyl glycol, INS No. 1520

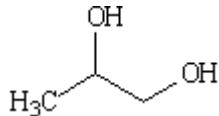
DEFINITION

Chemical names Propane-1,2-diol, 1,2-dihydroxypropane

C.A.S. number 57-55-6

Chemical formula C₃H₈O₂

Structural formula



Molecular weight 76.10

Assay Not less than 99.5% on the anhydrous basis

DESCRIPTION Clear, colourless, hygroscopic, viscous liquid

FUNCTIONAL USES Solvent, glazing agent, humectant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water, ethanol and acetone

Infrared absorption The infrared spectrum of a potassium bromide dispersion of the sample corresponds with the infrared spectrum below

PURITY

Water (Vol. 4) Not more than 1.0% (Karl Fischer)

Distillation range (Vol. 4) 99% v/v distils between 185-189°

Specific gravity (Vol. 4) d (20, 20): 1.035 - 1.040

Sulfated ash (Vol. 4) Not more than 0.07%
Test 5 g of the sample

Free acid Add 3-6 drops of phenol red TS to 50 ml water, then add 0.1N sodium hydroxide until solution remains red for 30 sec. To this solution add about

50 g of the sample accurately weighed. Titrate with 0.01N sodium hydroxide until the original red colour returns and remains for 15 sec. Not more than 1.67 ml of 0.01N sodium hydroxide are consumed by a sample of 50.0 g.

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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."

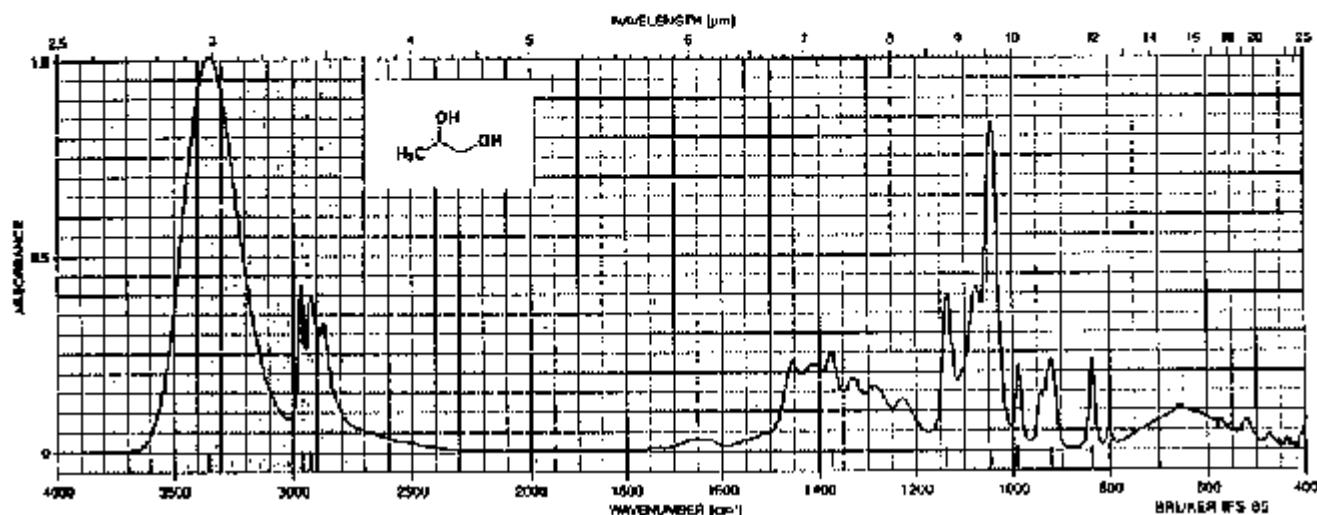
METHOD OF ASSAY

Inject a 10- μ l portion of the sample into a suitable gas chromatograph equipped with a thermal conductivity detector and a stainless steel column, 1-m x 1/4-in, packed with 4% Carbowax 20 M on 40/60-mesh Chromosorb T, or equivalent materials. The carrier gas is helium flowing at 75 ml/min. The injection port temperature is 240°, the column temperature 120 to 200°, programmed at a rate of 5° per min, and the block temperature 250°. Under the conditions described, the approximate retention time for propylene glycol is 5.7 min, and 8.2, 9.0, and 10.2 min for the three isomers of dipropylene glycol, respectively. Measure the area under all peaks by any convenient means, calculate the normalized area percentage of propylene glycol, and report as weight percentage.

Infrared spectrum:

Propylene glycol

Infrared spectrum from Merck FT-IR Atlas through courtesy of Dr. K.G.K. Pachler, Mr. F. Matlok and Dr. H-U. Grenlich, c/o Merck, Darmstadt, and VCH Verlagsgesellschaft GmbH, Weinheim, Germany.



PAPAIN

Prepared at the 15th JECFA (1971), published in NMRS 50B (1972) and in FNP 52 (1992). An ADI 'not limited' was established at the 15th JECFA (1971)

SYNONYMS	INS No.1101(ii)
SOURCES	Purified proteolytic substances derived from the fruit of <i>Carica papaya</i> (L) (Fam. <i>Caricaceae</i>).
Active principles	1. Papain (papaya peptidase I, cystein proteinase) 2. Chymopapain (cystein proteinase)
Systematic names and numbers	1. None (EC 3.4.22.2) 2. None (EC 3.4.22.6)
Reactions catalyzed	These enzymes hydrolyze polypeptides, amides and esters, especially at linkages involving basic amino acids, or leucine or glycine, yielding peptides of lower molecular weight.
DESCRIPTION	White to light tan amorphous powder or liquids; soluble in water, the solutions being colourless to light yellow and somewhat opalescent; practically insoluble in alcohol, chlorform and ether
FUNCTIONAL USES	Enzyme preparation Used in the chillproofing of beer, tenderizing of meat, preparation of precooked cereals, and production of protein hydrolysates
GENERAL SPECIFICATIONS	Must conform to the <i>General Specifications for Enzyme Preparations used in Food Processing</i> (see Volume 1, Introduction)
CHARACTERISTICS	
IDENTIFICATION	
<u>Papain activity</u> (Vol. 4)	The sample shows plant proteolytic activity

TURMERIC OLEORESIN

Prepared at the 35th JECFA (1989), published in FNP 49 (1990) and in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002). A temporary ADI established at the 30th JECFA (1986) was not maintained at the 35th JECFA (1989)

DEFINITION

Obtained by solvent extraction of turmeric (*Curcuma longa* L.). Only the following solvents may be used in the extraction: acetone, dichloromethane, 1,2-dichloroethane, methanol, ethanol, isopropanol and light petroleum (hexanes).

The selection of a turmeric oleoresin of a particular composition is based on the intended use in food. In general, all turmeric oleoresins contain colouring matter and most contain flavouring matter but some oleoresins are processed to remove aromatic compounds. Commercial products include oleoresins (*per se*) and formulations in which oleoresin is diluted in carrier solvents and which may contain emulsifiers and antioxidants. Purified extracts of turmeric containing more than 90% total colouring matter are subject to specifications for "Curcumin".

Turmeric Oleoresins are sold on the basis of "colour value" or "curcumin content", which generally means the total content of the curcuminoid substances: (I) curcumin, (II) demethoxycurcumin and (III) bis-demethoxycurcumin.

Chemical names

The principle colouring components are:

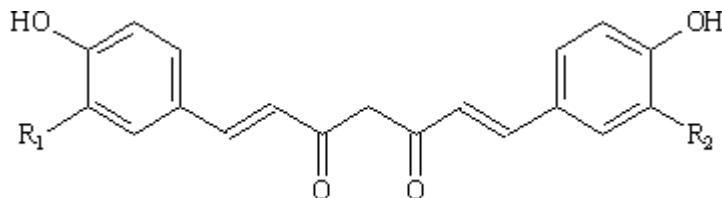
- I. 1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene- 3,5-dione
- II. 1-(4-Hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene- 3,5-dione
- III. 1,7-bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione

Chemical formula

Chemical formula

- I. C₂₁H₂₀O₆
- II. C₂₀H₁₈O₅
- III. C₁₉H₁₆O₄

Structural formula



- I. R₁ = R₂ = -OCH₃
- II. R₁ = -OCH₃, R₂ = H
- III. R₁ = R₂ = H

Formula weight

I. 368.39

II. 338.39

III. 308.39

Assay

Content of total colouring matter (curcuminoid content) not less than declared.

DESCRIPTION Turmeric Oleoresins, *per se*, are deep brownish-orange viscous oily fluids, pasty semisolids or hard amorphous solids containing 37–55% curcuminoids and up to 25% volatile oil. Diluted turmeric oleoresin formulations are, generally yellow solutions containing 6–15% curcuminoids and nil to 10% volatile oil.

FUNCTIONAL USES Colour, flavouring agent

CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Insoluble in water
<u>Colour in ethanol</u>	The ethanol-soluble fraction of the sample is characterized by its pure yellow colour and light green fluorescence; if this ethanol extract is added to concentrated sulfuric acid, a deep crimson is produced.
<u>Boric acid test</u>	Treat an aqueous or dilute ethanolic suspension of the sample with hydrochloric acid until a slightly orange colour begins to appear. Divide mixture into 2 parts and add some boric acid powder or crystals to one portion. Marked reddening will be quickly apparent, best seen by comparison with the portion to which the boric acid has not been added. The test may also be made by dipping pieces of filter paper into an ethanolic suspension of the sample, drying at 100°, and then moistening with a weak solution of boric acid to which a few drops of hydrochloric acid have been added. On drying, a cherry red colour will develop.

PURITY

<u>Residual solvents</u> (Vol. 4)	Acetone : Not more than 30 mg/kg Methanol: Not more than 50 mg/kg Ethanol: Not more than 50 mg/kg Isopropanol: Not more than 50 mg/kg Dichloromethane and 1,2-dichloroethane: Not more than 30 mg/kg, singly or in combination Light petroleum (hexanes): Not more than 25 mg/kg
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<u>Arsenic</u> (Vol. 4)	Not more than 3 mg/kg
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<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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."
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METHOD OF ASSAY

Method I

Standard Preparation

Transfer about 250 mg of purified curcumin, accurately weighed, into a 100-ml volumetric flask, and record the weight as W, in mg. Dissolve in acetone, dilute to volume with acetone, and mix. Pipet a 1-ml portion of this solution into a second 100-ml volumetric flask, dilute to volume with acetone, and

mix. Finally, pipet a 5-ml portion of the last solution into a 50-ml volumetric flask, dilute to volume with acetone, and mix.

Sample Preparation

Transfer an accurately weighed amount of the sample, equivalent to about 250 mg of curcumin, into a 100-ml volumetric flask, and record the weight as w, in mg. Dissolve in acetone, dilute to volume with acetone, and mix. Pipet a 1-ml portion of this solution into a second 100-ml volumetric flask, dilute to volume with acetone, and mix. Finally, pipet a 5-ml portion of the last solution into a 50-ml volumetric flask, dilute to volume with acetone, and mix.

Procedure

Determine the absorbance of each solution in 1-cm cells at the wavelength of maximum absorption at about 421 nm with a suitable spectrophotometer, using acetone as the blank.

Calculate the percentage of curcumin in the sample by the formula:

$$100 \times \frac{W}{w} \times \frac{A_u}{A_s}$$

where

A_u = the absorbance of the Sample Preparation

A_s = the absorbance of the standard preparation.

(NOTE: The absorbance readings should be made as soon as possible after the solutions are prepared to avoid colour loss).

Method II

Accurately weigh (W) about 0.1 g of the sample in a 100-ml beaker. Add 50 ml of ethanol and extract the colour by vigorously stirring. Filter the solution into a 200-ml volumetric flask. Make up to volume with ethanol.

Take an aliquot of the colour solution and dilute with additional ethanol according to the estimated colouring matters content as follows:

Colouring matter content	Dilution factor
Less than 20%	20
Between 20 and 40%	50
More than 40%	100

Determine the absorbance (A) at 425 nm in a 1-cm cell.

Calculate the total colouring matters content of the sample by the formula:

$$\frac{A \times 10000 \times D}{W \times 1607}$$

where

D = 0.4, 1 and 2 for dilution factors of 20, 50, and 100, respectively.

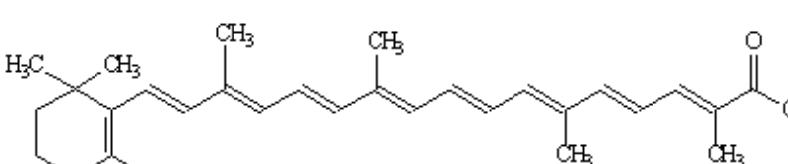
CATALASE from BOVINE LIVER

Prepared at the 15th JECFA (1971), published in NMRS 50B (1972) and in FNP 52 (1992). An ADI 'not limited' was established at the 15th JECFA (1971)

SOURCES	Commercial enzyme preparations are partially purified extracts from bovine liver
Active principles	Catalase
Systematic names and numbers	Hydrogen-peroxide: hydrogen-peroxide oxidoreductase (EC 1.11.1.6)
Reactions catalyzed	$\text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \Rightarrow \text{H}_2\text{O} + \text{O}_2$
DESCRIPTION	Powders or liquids
FUNCTIONAL USES	Enzyme preparation Used in the manufacture of certain cheeses
GENERAL SPECIFICATIONS	Must conform to the <i>General Specifications for Enzyme Preparations used in Food Processing</i> (see Volume Introduction)
CHARACTERISTICS	
IDENTIFICATION	
<u>Catalase activity</u> (Vol. 4)	The sample shows catalase activity

β-apo-8'-CAROTENOIC ACID ETHYL ESTER

Prepared at the 74th JECFA (2011) and published in FAO Monographs 11 (2011), superseding specifications prepared at the 28th JECFA (1984), published in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). A group ADI of 0·5 mg/kg bw expressed as the sum of carotenoids including β-carotene, β-apo-8'-carotenal, and the methyl and ethyl esters of β-apo-8'-carrenoic acid was established at the 18th JECFA (1974).

SYNONYMS	CI Food Orange 7; CI (1975) No. 40825; INS No. 160f
DEFINITION	These specifications apply to β -apo-8'-carotenoic acid ethyl ester which consists predominantly of all-trans- β -apo-8'-carotenoic acid ethyl ester and may also contain minor quantities of all-trans- β -apo-12'-carotenal, methyl-all-trans- β -apo-8'-carotenoate, all-trans-ethyl 4'-apo- β -carotenate and all-trans- β -carotene. Commercial preparations of β -apo-8'-carotenoic acid ethyl ester intended for use in food are prepared from β -apo-8'-carotenoic acid ethyl ester meeting these specifications and are formulated as suspensions in edible oil, emulsions and water dispersible powders. These preparations may also contain cis isomers.
Chemical names	All-trans- β -apo-8'-carotenoic acid ethyl ester, ethyl 8'-apo- β -caroten-8'-oate, ethyl (2E,4E,6E,8E,10E,12E,14E)-2,6,11,15-tetramethyl-17-(2,6,6-trimethylcyclohexen-1-yl)heptadeca-2,4,6,8,10,12,14,16-octaenoate
C.A.S. number	1109-11-1
Chemical formula	C ₃₂ H ₄₄ O ₂
Structural formula	All-trans- β -Apo-8'-carotenoic acid ethyl ester (main compound)
	
Formula weight	460.70
Assay	Not less than 96% of total colouring matters
DESCRIPTION	Red to violet-red crystals or crystalline powder; sensitive to oxygen and light and should therefore be kept in a light-resistant container under inert gas.
FUNCTIONAL USES	Colour
CHARACTERISTICS	

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Insoluble in water, very slightly soluble in ethanol, slightly soluble in vegetable oils.
<u>Spectrophotometry</u> (Vol. 4)	Determine the absorbance of the diluted sample solution used in the Method of Assay at 449 nm and 475 nm. The ratio A_{475}/A_{449} is between 0.82 and 0.86.
<u>Test for carotenoid</u>	The colour of a solution of the sample in acetone disappears after successive additions of a 5% solution of sodium nitrite and 0.5 M sulfuric acid.

PURITY

<u>Sulfated ash</u> (Vol. 4)	Not more than 0.1% Test 2 g of the sample (Method I)
<u>Subsidiary colouring matters</u>	Not more than 3% of total colouring matters See description under TESTS
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique 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, under "General Methods, Metallic Impurities."

TESTS

PURITY TESTS

<u>Subsidiary colouring matters</u>	Carotenoids other than β-apo-8'-carotenoic acid ethyl ester Subsidiary colouring matters (carotenoids other than β -apo-8'-carotenoic acid ethyl ester) are determined by high performance liquid chromatography (HPLC) using the following conditions: <u>Chromatographic system</u> <ul style="list-style-type: none">– HPLC system equipped with a UV/Vis detector or a photodiode array detector, refrigerated auto sampler– Detector wavelength: 446 nm– Column: reverse phase C18, Suplex pkb-100 (250 x 4.6 mm, 5 μm) from Supelco or equivalent– Mobile phase: In a 1000 ml volumetric flask, dissolve 50 mg BHT in 20 ml 2-propanol and add 0.2 ml N-ethyldiisopropyl-amine, 25 ml 0.2% aqueous ammonium acetate solution, 455 ml acetonitrile, and approx. 450 ml methanol. Mixture cools and contracts. Allow to reach room temperature and dilute to volume with methanol. Discard after 2 days.– Isocratic elution– Column temperature: 30°– Flow rate: 0.6 ml/min– Injection volume: 10 μl– Temperature of the autosampler: (approx. 15°)– Run time: approx. 35 min
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Reagents

- Butylated hydroxytoluene (BHT), reagent grade
- 2-Propanol, HPLC grade
- N-ethyl-diisopropyl-amine, reagent grade
- Ammonium acetate, reagent grade
- Acetonitrile, HPLC grade
- Methanol, HPLC grade
- Ethanol, HPLC grade
- Tetrahydrofuran, HPLC grade

Sample solution

Weigh accurately (to ± 0.1 mg) 0.010 g of the sample and dissolve in tetrahydrofuran (stabilized with 0.025% BHT). Transfer to a 100 ml volumetric flask and bring to volume with tetrahydrofuran. Dilute to the ratio of 1:10 with ethanol.

Procedure

Inject the sample solution using the conditions detailed under *Chromatographic system*. The retention time for all-trans-apo-8'-carotenoic acid ethyl ester is in the range of 9-11 min and corresponds to the largest peak in the chromatogram. The relative retention times of carotenoids with respect to the retention time of all-trans- β -apo-8'-carotenoic acid ethyl ester are: all-trans- β -apo-12'-carotenal (0.73); methyl all-trans- β -apo-8'-carotenoate (0.97); all-trans-ethyl 4'-apo- β -carotenate (1.22), all-trans- β -carotene (2.23). Integrate the areas of the peaks in the chromatogram.

Calculation

Calculate the percentage of carotenoids other than β -apo-8'-carotenoic acid ethyl ester (%), w/w) using the following formula:

Carotenoids other than β -apo-8'-carotenoic acid ethyl ester (%), w/w)

$$= \left(\frac{A_{\text{total}} - A_{\beta\text{-apo ester}}}{A_{\text{total}}} \right) \times 100$$

where

A_{total} is the sum of the area of all the peaks in the chromatogram, excluding the solvent peak (area units); and

$A_{\beta\text{-apo-ester}}$ is the area of the peak of β -apo-8'-carotenoic acid ethyl ester in the chromatogram (area units).

METHOD OF ASSAY (Vol. 4)

Total colouring matters content by spectrophotometry

Proceed as directed under Total Colouring Matters Content – Colouring Matters Contents by Spectrophotometry, Procedure 2, using the following conditions:

Sample weight (W): 0.08 g (± 0.01 g)

Volume of the three volumetric flasks: $V_1 = V_2 = V_3 = 100$ ml

Volume of the two pipets: $v_1 = v_2 = 5$ ml

Specific absorbance of the standard: $A^{1\%}_{1\text{ cm}} = 2550$

Wavelength of maximum absorption: λ_{max} about 449 nm

Calculation

Calculate the percentage of total colouring matters using the following formula:

$$\text{Total colouring matters (\%, w / w)} = \frac{A \times V_1 \times D}{A_{1\text{cm}}^{1\%} \times W}$$

where

A is the absorbance of the twice-diluted sample solution at 449 nm; and
D is the dilution factor $(V_2 \times V_3) / (V_1 \times V_2)$.



**Food and Agriculture
Organization of the
United Nations**



**World Health
Organization**

Residue Monograph prepared by the meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), 86th Meeting 2018

CITRIC AND FATTY ACID ESTERS OF GLYCEROL (TENTATIVE)

This monograph was also published in: Compendium of Food Additive Specifications. Joint FAO/WHO Expert Committee on Food Additives (JECFA), 86th meeting 2018. FAO JECFA Monographs 22

CITRIC AND FATTY ACID ESTERS OF GLYCEROL (TENTATIVE)

Prepared at the 86th JECFA (2018) and published in FAO JECFA Monographs 22 (2018), superseding specifications prepared at the 82nd JECFA (2016), and published in FAO JECFA Monographs 19 (2016). An ADI 'not limited' was established at the 17th JECFA (1973)

Information required:

- A validated method for the determination of total citric acid content
- Performance characteristics (method validation data) of the citric acid determination method
- Data on the total citric acid content, in at least five batches of products currently available in commerce, determined using the above method.

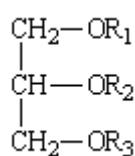
SYNONYMS

Citric acid esters of mono- and di-glycerides, citroglycerides, CITREM; INS No. 472c

DEFINITION

Citric and fatty acid esters of glycerol (CITREM) consists of mixed esters of citric acid and edible fatty acids with glycerol. It may contain free fatty acids, glycerol, citric acid and mono- and diglycerides, in minor quantities. The mono- and di- glycerides may include either one or two edible fatty acids from C12:0 to C18:0, mainly the saturated palmitic (C16:0) and stearic (C18:0) acids. It may also contain minor amounts of other fatty acids such as myristic (C14:0), oleic (C18:1), linoleic (C18:2) and arachidic acid (C20:0). CITREM is obtained by esterification of glycerol with citric acid and edible fatty acids, or by reaction of a mixture of mono- and diglycerides of edible fatty acids, with citric acid. CITREM may be partially or wholly neutralized with sodium hydroxide or potassium hydroxide.

Structural formula



Where at least one of R₁, R₂ or R₃ represents a citric acid moiety, one represents a fatty acid moiety and the remainder may represent citric acid, fatty acid or hydrogen.

DESCRIPTION	White to ivory coloured, oily to waxy material.
FUNCTIONAL USES	Stabilizer, emulsifier, dough conditioner, antioxidant synergist

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in water; soluble in oils and fats; insoluble in ethanol

Test for fatty acids
(Vol. 4) Passes test

Test for citric acid Information required

Test for glycerol
(Vol. 4) Passes test

PURITY

Sulfated ash (Vol. 4) Non-neutralized products: not more than 0.5%
Partially or wholly neutralized products: not more than 10%; test 2 g of the sample (Method I)

Free glycerol (Vol. 4) Not more than 4%

Total glycerol 8-33%
See description under TESTS

Total citric acid 13-50%
(Information required)

Total fatty acid 37-81%
See description under TESTS

<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg. (Not more than 0.1 mg/kg for use in infant formula and formula for special medical purposes intended for infants)
	Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under "General Methods, Metallic Impurities").

TESTS

PURITY TESTS

Total glycerol CITREM is hydrolyzed, glycerol in the aqueous phase is oxidized using known excess of sodium periodate in a strongly acid medium and the unreacted periodate is back titrated using standard sodium thiosulfate solution.

Procedure:

Accurately weigh about 2 g of the sample into a saponification flask, add 50 ml of 0.5 M ethanolic potassium hydroxide, and reflux for 30 min.

To a 1-L volumetric flask add 99 ml \pm 0.2 ml of chloroform using a burette and add 25 ml of glacial acetic acid using a graduated cylinder. Quantitatively transfer the content of the saponification flask to the volumetric flask, using three 25 ml portions of water. Add about 500 ml of water further, and shake vigorously for about 1 min. Dilute to volume with water, stopper, mix thoroughly and set aside for separation of layers.

Pipet 50 ml of acetic periodic acid TS into a series of 400 ml beakers. Prepare two blanks by adding 50 ml of water to each. Pipet 50 ml of the aqueous layer into one of the 400 ml beakers containing 50 ml of acetic periodic acid TS; shake gently to mix; cover with watch glass,

and allow to stand 30 min but not longer than 1.5 h. Add 20 ml of 15% potassium iodide solution, shake gently to mix, and allow to stand at least 1 min. but not more than 5 min. Do not allow to stand in bright or direct sunlight. Add 200 ml of water and titrate with 0.1 N sodium thiosulfate. Use a variable speed electric stirrer to keep the solution thoroughly mixed. Continue the titration to the disappearance of the brown iodine colour from the aqueous layer. Add 2 ml of starch TS and continue the titration to the disappearance of iodine from the tiny chloroform layer separated during titration and the disappearance of the blue iodine-starch complex colour from the aqueous layer. Read the burette to the nearest 0.01 ml. Treat the blanks in the same way as the sample.

Calculation

$$\% \text{ total glycerol} = [(B - S) \times N \times 2.302 \times 900]/(W \times 50)$$

where

- B volume of 0.1 N sodium thiosulfate used for the blank, ml
- S volume of 0.1 N sodium thiosulfate used for the sample, ml
- N exact normality of 0.1 N sodium thiosulfate
- W mass of sample, g

Total citric acid

Information required

Total fatty acid

Principle: This method measures total fatty acids by extracting with diethyl ether.

Procedure

Weigh accurately 5 g of the sample into a 250-ml round-bottomed flask, add 50 ml of potassium hydroxide, ethanolic, TS, and reflux for 1 h on a boiling water bath.

Quantitatively transfer the contents of the saponification flask to a 1,000 ml separating funnel, using three 25 ml portions of water, and add 5 drops of methyl orange indicator solution.

Cautiously add 50% hydrochloric acid until the colour of solution changes to orange red.t. Add 1 ml of excess acid. Shake well to mix the contents and separate the fatty acids.

Cool to room temperature and extract the separated fatty acids with three 100 ml portions of diethyl ether. Combine the extracts, and

wash with 50 ml portions of 10% sodium chloride solution until the washed sodium chloride solution becomes neutral.

Dry the ether solution with anhydrous sodium sulfate. Then evaporate off ether on a steam bath, leave additional 10 min on the steam bath, and weigh the residue. This is the weight of the total fatty acids.

Calculation:

$$\text{Total Fatty acids \%} = \frac{\text{mass of fatty acids g} \times 100}{\text{mass of sample g}}$$

GLUCONO- δ -LACTONE

Prepared at the 51st JECFA (1998), published in FNP 52 Add 6 (1998) superseding specifications prepared at the 30th JECFA (1986), published in FNP 37 (1986) and republished in FNP 52 (1992). Group ADI "not specified" for glucono-delta-lactone and gluconates, excluding ferrous gluconate, established at the 51st JECFA in 1998.

SYNOMYS

Glucono-delta-lactone, gluconolactone, delta-gluconolactone, GDL; INS No. 575

DEFINITION

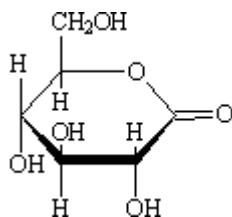
Glucono-delta-lactone is the cyclic 1,5-intramolecular ester of D-gluconic acid. In aqueous media it is hydrolyzed to an equilibrium mixture of D-gluconic acid (55-66%) and the delta- and gamma-lactones.

Chemical names D-Glucono-1,5-lactone, D-gluconic acid delta-lactone

C.A.S. number 90-80-2

Chemical formula C₆H₁₀O₆

Structural formula



Formula weight 178.14

Assay Not less than 99.0% on the dried basis

DESCRIPTION White, odourless or nearly odourless crystals or crystalline powder

FUNCTIONAL USES Acidifier, raising agent, sequestrant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in water; sparingly soluble in ethanol

Colour reaction To 1 ml of a 1 in 50 solution, add 1 drop of ferric chloride TS. A deep yellow colour is produced

Test for gluconate (Vol. 4) Passes test

PURITY

Loss on drying (Vol. 4) Not more than 1% (105°, 2 h)

Sulfated ash (Vol. 4) Not more than 0.1%

Test 2 g of the sample (Method I)

<u>Reducing substances</u>	Not more than 0.5% (as D-glucose) See description under TESTS
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

<u>Reducing substances</u>	Weigh accurately 10 g into a 400-ml beaker, dissolve the sample in 40 ml of water, add phenolphthalein TS, and neutralize with sodium hydroxide solution (1 in 2). Dilute to 50 ml with water, and add 50 ml of alkaline cupric tartrate TS. Heat the mixture on an asbestos gauze over a Bunsen burner, regulating the flame so that boiling begins in 4 min, and continue the boiling for exactly 2 min. Filter through a Gooch crucible, wash the filter with 3 ml or more small portions of water, and place the crucible in an upright position in the original beaker. Add 5 ml of water and 3 ml of nitric acid to the crucible, mix with a stirring rod to ensure complete solution of the cuprous oxide, and wash the solution into a beaker with several ml of water. To the beaker add sufficient bromine TS (5 to 10 ml) until the colour becomes yellow, and dilute with water to about 75 ml. Add a few glass beads, boil over a Bunsen burner until the bromine is completely removed, and cool. Slowly add ammonium hydroxide until a deep blue colour appears, then adjust the pH to approximately 4 with glacial acetic acid, and dilute to about 100 ml with water. Add 4 g of potassium iodide, and titrate with 0.1 N sodium thiosulfate, adding starch TS just before the endpoint is reached. Not more than 16.1 ml is required.
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METHOD OF ASSAY

Dissolve about 0.6 g of the dried sample, accurately weighed, in 50 ml of 0.1 N sodium hydroxide, and allow to stand for 20 min. Add 3 drops of phenolphthalein TS, and titrate the excess sodium hydroxide with 0.1 N sulfuric acid. Perform a blank determination, and make any necessary correction. Each ml of 0.1 N sodium hydroxide is equivalent to 17.81 mg of C₆H₁₀O₆.

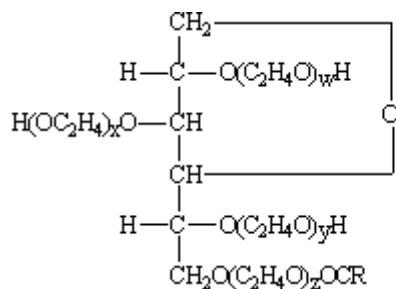
POLYOXYETHYLENE (20) SORBITAN TRISTEARATE

Prepared at the 25th JECFA (1981), published in FNP 19 (1981) and in FNP 52 (1992). Metals and arsenic specifications revised at the 55th JECFA (2000). An ADI of 0.25 mg/kg bw was established at the 17th JECFA (1973)

SYNONYMS Polysorbate 65; INS No. 436

DEFINITION Consists of a mixture of the partial esters of sorbitol and its mono- and dianhydrides (which have an acid value below 15 and a water content below 0.2%) with edible commercial stearic acid and condensed with approximately 20 moles of ethylene oxide per mole of sorbitol and its anhydrides.

Structural formula Nominal formula and approximate composition:



where $w + x + y + z = \text{approx. } 20$ and RCO^- is the fatty acid moiety

Assay Not less than 46.0 and not more than 50.0% of oxyethylene groups, equivalent to not less than 96.0 and not more than 104.0% of polyoxyethylene (20) sorbitan tristearate on the anhydrous basis

DESCRIPTION Tan coloured, waxy solid at 25° , with a faint characteristic odour

FUNCTIONAL USES Emulsifier, dispersing agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Dispersible in water; soluble in mineral oil, vegetable oils, petroleum ether, acetone, ether, dioxane, ethanol and methanol

Congealing range (Vol. 4) $29 - 33^\circ$

Infrared absorption The infrared spectrum of the sample is characteristic of a partial fatty acid ester of a polyoxyethylated polyol

Colour reaction To 5 ml of a 5% (w/v) aqueous solution of the sample add 10 ml of ammonium cobaltothiocyanate solution and 5 ml of chloroform, shake well and allow to separate; a blue colour is produced in the chloroform layer. (Ammonium cobaltothiocyanate solution: 37.5 g of cobalt nitrate and 150 g

of ammonium thiocyanate made up to 100 ml with water - freshly prepared).

Test for fatty acids

To 5 ml of a 5% (w/v) aqueous solution of the sample add 5 ml sodium hydroxide TS. Boil for a few min, cool, and acidify with dilute hydrochloric acid. The solution is strongly opalescent, owing to the fatty acids liberated.

Saponification (Vol. 4)

100 g of the sample yields approximately 43 g of fatty acids and 56 g of polyols

PURITY

Water (Vol. 4)

Not more than 3% (Karl Fischer Method)

Sulfated ash (Vol. 4)

Not more than 0.25%
Test 2 g of the sample (Method I)

Acid value (Vol. 4)

Not more than 2

Saponification value
(Vol. 4)

Not less than 88 and not more than 98

Hydroxyl value (Vol. 4)

Not less than 40 and not more than 60

1,4-Dioxane (Vol. 4)

Not more than 10 mg/kg

Lead (Vol. 4)

Not more than 2 mg/kg
Determine using an atomic absorption technique 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**

Determine the content of *Oxyethylene groups*.

POLYDEXTROSES

Prepared at the 51st JECFA (1998) and published in FNP 52 Add 6 (1998) superseding specifications prepared at the 44th JECFA (1995), published in FNP 52 Add 3 (1995). An ADI "not specified" was established at the 31st JECFA in 1987.

SYNONYMS

Modified polydextroses; INS No. 1200

DEFINITION

Randomly bonded condensation polymers of glucose with some sorbitol end-groups, and with citric acid or phosphoric acid residues attached to the polymers by mono or diester bonds. They are obtained by melting and condensation of the ingredients which consist of approximately 90 parts D-glucose, 10 parts sorbitol and up to 1 part citric acid or 0.1 part phosphoric acid. The 1,6-glucosidic linkage predominates in the polymers but other linkages are present. The products contain small quantities of free glucose, sorbitol, levoglucosan (1,6-anhydro-D-glucose) and citric acid and may be neutralized with any food-grade base and/or decolourized and deionized for further purification. The products may also be partially hydrogenated with Raney nickel catalyst to reduce residual glucose. Polydextrose-N is neutralized Polydextrose.

C.A.S. number

68424-04-4

Assay

Not less than 90.0% of polymer on the ash-free and water-free bases

DESCRIPTION

White to light tan-coloured solid. Polydextroses dissolve in water to give clear, colourless to straw-coloured solutions

FUNCTIONAL USES

Bulking agent, humectant, stabilizer, thickener

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Very soluble in water

Test for sugar

To 1 drop of 1 in 10 solution of the sample, add 4 drops of 5% phenol solution, then rapidly add 15 drops of sulfuric acid TS. A deep yellow to orange colour is produced.

Solubility in acetone

With vigorous swirling add 1 ml of acetone to 1 ml of a 1 in 10 solution of the sample. The solution remains clear. With vigorous swirling add 2 ml of acetone to the solution. A heavy, milky turbidity develops immediately.

Test for reducing sugar

To 1 ml of a 1 in 50 solution of the sample, add 4 ml of alkaline cupric citrate TS. Boil vigorously 2-4 min. Remove from heat and let precipitate (if any) settle. The supernatant is blue or blue-green.

PURITY

Water (Vol. 4)

Not more than 4.0% (Karl Fischer Method)

<u>pH</u> (Vol. 4)	2.5 - 7.0 (for Polydextrose) (1 in 10 soln) 5.0 - 6.0 (for Polydextrose-N) (1 in 10 soln)
<u>Sulfated ash</u> (Vol. 4)	Not more than 0.3% (for Polydextrose) Not more than 2.0% (for Polydextrose-N)
<u>Nickel</u> (Vol. 4)	Not more than 2 mg/kg for hydrogenated polydextroses Use method <i>Nickel</i> for polyols
<u>1,6-Anhydro-D-glucose</u>	Not more than 4.0% on the ash-free and the dried bases See description under TESTS
<u>Glucose and sorbitol</u>	Not more than 6.0% combined on the ash-free and the dried bases; glucose and sorbitol are determined separately See description under TESTS
<u>Molecular weight limit</u>	Negative to test for polymer of molecular weight greater than 22,000 See description under TESTS
<u>5-Hydroxymethylfurfural</u>	Not more than 0.1% in Polydextrose Not more than 0.05% in Polydextrose-N See description under TESTS
<u>Lead</u> (Vol. 4)	Not more than 0.5 mg/kg Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

Lead (Vol. 4)

Apparatus:

Use a suitable atomic absorption spectrophotometer (Perkin-Elmer Model 6000, or equivalent), a graphite furnace containing a L'vov platform (Perkin-Elmer Model HGA-500, or equivalent), and an autosampler (Perkin-Elmer Model AS-40, or equivalent).

Use a lead hollow cathode lamp (lamp current of 10 mA), a slit width of 0.7 mm (set low), the wavelength set at 283.3 nm, and a deuterium arc lamp for background correction. Use argon as the carrier gas. (Note: For this test use reagent-grade chemicals with as low a lead content as practicable, as well as high-purity water and gases. Before use, rinse all glassware and plasticware twice with 10% nitric acid and twice with 10% hydrochloric acid, and then rinse thoroughly with high-purity water.)

Lead Nitrate Stock Solution:

Dissolve 159.8 mg of reagent-grade lead nitrate in 1000.0 ml of water. Prepare and store this solution in glass containers that are free from lead salts. Each ml of this solution contains the equivalent of 100 µg of lead ion.

Standard Lead Solution:

On the day of use, dilute 10.0 ml of Lead Nitrate Stock Solution with water to 100.0 ml, and mix. Each ml of Standard Lead Solution contains the equivalent of 10 µg of lead ion.

Standard Solutions:

Prepare a series of lead standard solutions serially diluted from the Standard Lead Solution. Into separate 100 ml volumetric flasks, pipet 0.2, 0.5, 1, 2 ml, and 5 ml, respectively, of Standard Lead Solution, dilute to volume with water, and mix. The Standard Solutions contain, respectively, 0.02, 0.05, 0.1, 0.2, and 0.5 µg of lead per ml.

Matrix Modifier:

Transfer 100.0 mg of ammonium phosphate, dibasic ($(\text{NH}_4)_2\text{HPO}_4$) to a 10 ml volumetric flask, dilute to volume with water, and mix.

Sample Solution: Transfer about 1 g of the sample, accurately weighed, to a 10 ml volumetric flask, add 5 ml of water, and mix. Dilute to volume with water, and mix.

Spiked (fortified) Sample Solution:

Prepare a solution as directed under Sample Solution, but add 100 µl of the Standard Lead Solution, dilute to volume with water, and mix. This solution contains 0.1 µg/ml of added lead.

Procedure:

With the use of an autosampler, atomize 10-µl aliquots of the five Standard Solutions, using the following sequence of conditions: step (1) dry at 130° with a 20-sec ramp period, a 40-sec hold time, and a 300 ml/min argon flow rate; step (2) char at 800° with a 20-sec ramp period, a 40-sec hold time, and a 300 ml/min argon flow rate; step (3) atomize at 2400° for 6 sec with a 50 ml/min argon flow rate, and read; step (4) clean at 2600° with a 1-sec ramp period, a 5-sec hold time, and a 300 ml/min argon flow rate; and step (5) recharge at 20° with a 2-sec ramp period, a 20-sec hold time, and a 300 ml/min argon flow rate. Atomize 10 µl of the Matrix Modifier in combination with 10 µl of the Sample Solution under identical conditions used for the Standard Solutions. Repeat with 10 µl of the Matrix Modifier in combination with 10 µl of the Spiked Sample Solution.

Plot a standard curve using the concentration, in µg/ml, of each Standard Solution versus its maximum absorbance value compensated for background correction, and draw the best straight line. From the Standard Curve, determine the concentrations C_S and C_A in µg/ml, of the Sample Solution and the Spiked Sample Solution, respectively. Calculate the quantity, in mg/kg, of lead in the sample by the formula:

$$\frac{10 \times C_S}{W}$$

where

W = the weight, in g, of the sample taken.

Calculate the recovery by the formula:

$$\frac{C_s - C_A}{0.1} \times 100$$

where

0.1 = the amount of lead, in $\mu\text{g}/\text{ml}$, added to the Spiked Sample Solution.

1,6-Anhydro-D-glucose, glucose and sorbitol

Gas chromatography

- Octadecane Solution: Accurately weigh 50 mg of n-octadecane into a 100-ml volumetric flask and make up to volume with pyridine.
- Monomer Standard Solution: Weigh accurately 50 mg reagent grade alpha-D-glucose, 40 mg anhydrous D-sorbitol (min. 97% purity), and 35 mg of reagent grade (1,6-anhydro-D-glucose), into a 100-ml volumetric flask and make up to volume with pyridine.

Silylation of Monomer Standard Solution

Transfer 1.0 ml of Monomer Standard Solution to a screw-cap vial and add 1 ml of Octadecane Solution and 0.5 ml of N-trimethylsilylimidazole. Cap the vial and immerse in an ultrasonic bath at 70° for 60 min.

Gas Chromatograph conditions

Glass column, 2.44 m by 2 mm i.d. packed with 3% OV-1 on Gas Chrom Q 100/120 mesh. Flame ionization detector. Temperatures: column 175° ; injection port 210° ; detector 230° . Retention times (min): 1,6-anhydro-D-glucose, pyranose form 3.7; 1,6-anhydro-D-glucose furanose form (not present in standard) 4.3; n-octadecane 5.1; alpha-D-glucose 8.7; D-sorbitol 11.3; beta-D-glucose 13.3.

Procedure

Accurately weigh 20 mg of the sample into a screw-cap vial and add 1.0 ml of Octadecane Solution, 1 ml of pyridine, and 0.5 ml of N-trimethylsilylimidazole. Cap the vial and immerse in an ultrasonic bath at 70° for 60 min. Prior to sample analysis, inject 3 μl of the silylated Monomer Standard Solution into the gas chromatograph. Repeat two times, then inject 3 μl of the sample solution. Calculate the percentage of each monomer by the formula:

$$\frac{R \times W_s}{R_s \times W}$$

where

W = the weight of the sample in mg, adjusted for ash and moisture

W_s = the weight in mg of the monomer in the Monomer Standard Solution

R = the ratio of the area of the monomer peak to the area of the octadecane peak in the sample injection

R_s = the mean ratio of the area of the monomer peak to the area of the octadecane peak in the standard injections.

In the case of glucose, the peak areas for the alpha- and beta-epimers and in the case of 1,6-anhydro-D-glucose the peak areas for the pyranose form and furanose form are combined.

Molecular weight limit

Apparatus

Use a suitable high-pressure liquid chromatograph (HPLC) equipped with a differential refractometer, either a loop injector or suitable autosampler,

a column heating block or oven and a computing integrator, or computer data handling system with molecular weight determination capabilities. Use a Waters Ultrahydrogel 250 A size exclusion column, or equivalent. The column is maintained at 45°, and the HPLC pump supplies eluent to it at 0.8 ml/min reproducible to 0.5%. The differential refractometer should be set at a sensitivity of 4×10^{-6} refractive index units full scale, and the plotter of the integrator should be set to 64 millivolts full scale. Maintain the detector cell at $35 \pm 0.1^\circ$. Noise attributable to the detector and electronics should be less than 0.1% full scale.

Eluent

The eluent is 0.1 N sodium nitrate containing 0.025% sodium azide. Dissolve 35.0 g of sodium nitrate and 1.0 g of sodium azide in 100 ml of HPLC-grade water. Filter through a 0.45- μm filter into a 4-l flask. Dilute to volume with HPLC-grade water. De-gas by applying an aspirator vacuum for 30 min.

Standard Solution

Transfer 20 mg each of dextrose, stachyose, 5800, 23,700, and 100,000 MW pullulan standards into a 10-ml volumetric flask. Dissolve in and dilute to volume with Eluent. Filter through a 0.45 μm syringe filter into a suitable autosampler vial, and seal (All components of the Standard Solution are available from Polymer Laboratories, Inc., Technical Center, Amherst Fields Research Park, 160 Old Farm Road, Amherst, MA 01002, USA).

Column Equilibration

After installation of a new column in the HPLC, pump Eluent through it overnight at 0.3 ml/min. Before calibration or analysis, increase the flow slowly to 0.8 ml/min over a 1-min period, then pump at 0.8 ml/min for at least 1 h before the first injection. Check the flow gravimetrically, and adjust it if necessary. Reduce the flow to 0.1 ml/min when the system is not in use.

Data System Setup

Set the integrator or computerized data handling system as their respective manuals instruct for normal gel permeation chromatographic determinations. Set the integration time to 15 min.

Column Standardization

After the HPLC system has been equilibrated at a flow rate of 0.8 ml/min for at least 1 h, inject 50 μl of the Standard Solution five times, allowing 15 min between injections. Record the retention times of the various components in the Standard Solution. Retention times for each component should agree within ± 2 sec. Insert the average retention time along with the molecular weight of each component into the calibration table of the molecular weight distribution software.

System Suitability

Check the regression results for a cubic fit of the calibration points. They should have an R^2 value of 0.9999+. Dextrose and stachyose should be baseline-resolved from one another and from the 5800 MW pullulan standard. Elevated valleys are usually observed between the 5800,

23,700 and 100,000 MW pullulan standards.

Sample Preparation

Transfer 50 mg of sample, accurately weighed, into a 10-ml volumetric flask. Dissolve in and dilute to volume with Eluent. Filter through a 0.45- μm syringe filter into a suitable autosampler vial.

Procedure

Inject 50 μl of the Sample Preparation, following the same conditions and procedure as described under Column Standardization. Using the Formula weight Distribution software of the data reduction system, generate a molecular weight distribution curve of the sample. There is no measurable peak above a molecular weight of 22,000.

5-Hydroxymethylfurfural (HMF)

Principle

HMF solutions absorb light in the ultraviolet region. The maximum absorption occurs at 283 nm and the molar extinction coefficient is 16,830 at that wavelength. The HMF concentration in polydextrose solutions is determined from the optical density at 283 nm and the application of the Beer-Lambert law.

Apparatus

- Standard laboratory equipment
- Ultraviolet spectrophotometer
- Spectrophotometer cells (quartz), 1.00 cm path length

Procedure

Accurately weigh 1.00 ± 0.01 g of the sample into a 100-ml volumetric flask and make up to volume with distilled water (for polydextrose-N 70% solution use 1.43 ± 0.01 g sample). Read the optical density of this solution against a water blank at 283 nm in a 1.00 cm quartz cell in the spectrophotometer according to the directions supplied with the instrument. Under these conditions, the % HMF in the original sample is $0.0749 \times$ optical density, on the dried basis.

Calculation

$$C = \frac{100 \times M \times D}{10 \times L \times E}$$

where

C = % HMF in the original polydextrose sample

M = HMF molecular weight

D = optical density of the solution

L = the path length of the spectrophotometer cell

E = the molar extinction coefficient for HMF

The numbers 100 and 10 are factors to convert solution concentration in mg/l to % HMF in the original sample, on the dried basis.

METHOD OF ASSAY

Phenol Solution

Add 20 ml of water to 80 g of phenol.

Glucose Standard Solutions

Weigh accurately 100 mg of alpha-D-glucose (minimum 97% purity) into a 500-ml volumetric flask and make up to volume with distilled water. Dilute five aliquots of the solution with distilled water to obtain the following concentrations of standard: 50, 40, 20, 10 and 5 µg/ml.

Standard Curve

Run each analysis in triplicate. On a daily basis, pipet 2.0 ml of each of the Glucose Standard Solutions into 4-dram (14.8 ml) acetone-free screw-cap vials. Add 0.12 ml of the phenol solution and mix gently. Uncap vials and add rapidly 5 ml of sulfuric acid TS. Immediately recap the vials and shake vigorously.

CAUTION: Rubber gloves and safety shield should be used in the sulfuric acid addition step.

Let the vials stand at room temperature for 45 min. Determine absorbances at 490 nm in a suitable spectrophotometer, using a Phenol Solution-sulfuric acid mixture as a blank in the reference cell. Plot mean absorbances versus concentrations in µg/ml.

Procedure

In triplicate, weigh accurately about 250 mg of the sample into a 250-ml volumetric flask and make up to volume with distilled water. Transfer a 10 ml aliquot to a 250-ml volumetric flask and make up to volume with distilled water. Proceed as in Standard Curve. Calculate the percentage of polymer by the formula:

$$\text{Polymer (\%)} = 1.05 \times \frac{100 \times A}{S \times C} - P_G - 1.11 \times P_1$$

where

A = the sample absorbance

S = the slope of absorbance versus glucose concentration in µg/ml obtained from the Standard Curve

C = the concentration of the sample solution in µg/ml (adjusted for ash and moisture)

P_G and P₁ = the percentages of glucose and 1,6-anhydro-D-glucose, respectively, determined by the tests for monomers (see Purity tests for 1,6-Anhydro-D-glucose, glucose and sorbitol as described above).

1-HYDROXYETHYLIDENE-1,1-DIPHOSPHONIC ACID

New specifications prepared at 63rd JECFA (2004) and published in FNP 52 Add 12 (2004). Levels of residue that are expected to remain on foods do not pose a safety concern (63rd JECFA, 2004).

SYNONYMS

HEDP, ethane-1-hydroxy-1,1-diphosphonic acid, EHDP, etidronic acid

DEFINITION

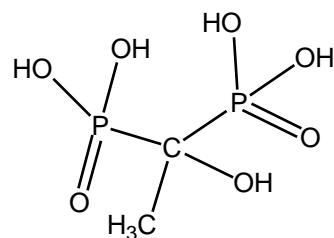
1-Hydroxyethylidene-1,1-diphosphonic acid (HEDP) is manufactured commercially by the reaction of phosphorous acid with one or more acetylating agents; specifically acetic anhydride, acetyl chloride and/or acetic acid. The final product is typically a 60% solution of HEDP in water.

Chemical name 1-hydroxyethylidene-1,1-diphosphonic acid

C.A.S. number 2809-21-4

Chemical formula $\text{CH}_3\text{C}(\text{OH})[\text{PO}(\text{OH})_2]_2$

Structural formula



Empirical formula $\text{C}_2\text{H}_8\text{O}_7\text{P}_2$

Formula weight 205.02

Assay Total active acid 58 – 62%

DESCRIPTION

Clear pale yellow liquid, free of suspended matter

FUNCTIONAL USES Sequestrant (for use in antimicrobial washing solutions)

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Miscible with water, phosphoric acid and ethylene glycol; soluble in most organic solvents

pH (Vol. 4) Not more than 2.0 (1% soln)

Specific gravity (Vol. 4) 1.430- 1.471 at 20°

Freezing point -25°

PURITY

Chloride Not more than 40 mg/kg
See description under TESTS

<u>Phosphorous acid</u>	Not more than 4.0% See description under TESTS
<u>Acetic acid</u>	Not more than 1.0% See description under TESTS
<u>Iron</u> (Vol. 4)	Not more than 10 mg/kg Determine using an atomic absorption technique appropriate to the specified level
<u>Arsenic</u> ((Vol. 4)	Not more than 5 mg/kg
<u>Lead</u> (Vol. 4)	Not more than 5 mg/kg Determine using an atomic absorption technique appropriate to the specified level. The selection of the sample size and method of sample preparation may be based on the principles of the method described in Volume 4, "Instrumental methods"

TESTS

PURITY TESTS

<u>Chloride</u>	Determine by potentiometric titration by placing 25 g of the sample, accurately weighed, into a titration vessel and adding sufficient water to cover the electrodes. Add 3 ml of concentrated nitric acid. Titrate with 0.005 mol/l silver nitrate to first inflection point and record the titre in ml (A). Calculate the chloride content (mg/kg) from: $\text{Chloride (mg/kg)} = [A \times M \times 3.55 \times 10000] / W$ <p>where M = concentration of silver nitrate solution (mol/l) W = weight of sample taken (g)</p>
<u>Phosphorous acid</u>	Determined by iodometric titration. Iodine oxidizes the phosphorous acid present to phosphate, excess iodine is determined and the Phosphorous acid calculated. Buffer solution pH 7.3: Dissolve 138 g of sodium dihydrogen phosphate in 800 ml of water and adjust pH to 7.3 with 50% sodium hydroxide solution. Make up to 1000 ml with water. Add 1.5 g of the sample to 20 ml of water in a 250 ml beaker. Add 50 ml of pH 7.3 phosphate buffer. Adjust pH to 7.3 using 50% sodium hydroxide. Transfer the solution to an iodine flask and add 25.0 ml of 0.1 N iodine. Stopper and swirl the solution and place in the dark immediately. After 15 min, remove the flask and add 5 ml acetic acid to flask. Titrate with 0.1 N sodium thiosulfate until a light straw yellow colour. Add starch indicator and continue titration until the end point "black to colourless" is observed and record the titre in ml (B). Repeat titration with a reagent blank determination omitting the sample and record the titre in ml (A).

Calculate the percentage of phosphorus acid from:

$$\text{Phosphorous acid (\%)} = [(A-B) \times N \times E \times 100] / [w \times 1000]$$

where

- N = normality of sodium thiosulfate solution
E = equivalent weight of H_3PO_3 (40.99)
w = weight of sample taken (g)

The accuracy has been determined as +/- 0.01% at phosphorous acid level of 1.28%

Acetic acid

Determine by ion chromatography using a Dionex ICE-ASI column with weak acid eluent. Set up the system in line with the instrument manufacturer's operation procedure.

The signal from the acetate ion is quantified against a calibration standard using Formic acid as the internal standard.

Equipment: Dionex ICE-AS1 column

Reagents: Acetic acid (analytical grade) and formic acid (analytical grade)

Procedure: Carry out the determination according to the instrument manufacturer's operation procedure

METHOD OF ASSAY

Place about 3 g of the sample, accurately weighed (w) into a beaker and add 100-150 ml of water. Stir the solution with a magnetic stirrer (maintain throughout titration). Insert pH electrode(s) and record the pH value. Titrate with 1 mol/l sodium hydroxide and record pH (or millivolts) after every 1ml added. Stop the titration at pH 10. Plot the pH as a function of added sodium hydroxide and manually draw the titration curve. Two inflection points will be observed at around pH 3 and pH 8. Take only into account the inflection point at around pH 8. Trace the tangent to this inflection point in order to determine the end-point. Calculate the total active acid from

$$\text{Total active acid (\%)} = [A \times 206 \times N] / [30 \times w] - [1.676 \times P]$$

Where

- A = ml of N NaOH from start of titration to end point at pH 8-85
N = concentration of sodium hydroxide used
P = concentration of phosphorous acid (%) (Determined as above)
1.676 = $[MW \text{ of HEDP} \times 2] / [MW \text{ of phosphorous acid} \times 3]$

Using auto-titration for end-point detection, accuracy has been determined as +/- 0.2% at total active acid level of 63.5%

GLUCOSE ISOMERASE from *ACTINOPLANES MISSOURIENSIS*

Prepared at the 28th JECFA (1984), published in FNP 31/2 (1984) and in FNP 52 (1992). An ADI 'acceptable' was established at the 29th JECFA (1985)

SYNONYMS	Xylose isomerase
SOURCES	Produced by the controlled fermentation of <i>Actinoplanes missouriensis</i>
Active principles	Xylose isomerase (glucose isomerase)
Systematic names and numbers	D-Xylose ketol-isomerase (EC 5.3.1.5)
Reactions catalyzed	D-Xylose and D-glucose are converted to D-xylulose and D-fructose, respectively
DESCRIPTION	Off-white to brown granules (immobilized preparation) or liquids, insoluble in water (granules), ethanol, chloroform and ether The immobilized preparations are rendered insoluble in water by treatment with gelatine (carrier) and glutaraldehyde (immobilization agent).
FUNCTIONAL USES	Enzyme preparation Used in the preparation of high fructose corn syrup and other fructose starch syrups.
GENERAL SPECIFICATIONS	Must conform to the <i>General Specifications for Enzyme Preparations used in Food Processing</i> (see Volume Introduction)
CHARACTERISTICS	
IDENTIFICATION	
<u>Glucose isomerase activity</u> (Vol. 4)	The sample shows glucose isomerase activity
PURITY	
<u>Glutaraldehyde</u> (Vol. 4)	Passes Limit Test for Glutaraldehyde from Immobilized Glucose Isomerases crosslinked with Glutaraldehyde

DICHLOROMETHANE

Prepared at the 51st JECFA (1998), published in FNP 52 Add 6 (1998) superseding specifications prepared at the 39th JECFA (1992), published in FNP 52 Add 1 (1992). ADI "should be limited to current uses", established at the 39th JECFA in 1992.

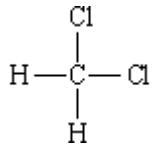
SYNONYMS	Methylene chloride, methylene dichloride
DEFINITION	Dichloromethane (DCM) is derived from the chlorination of methane during which other chlorinated methane derivatives may be formed. Propylene oxide, cyclohexane, and/or 2-methyl-2-butene are added as stabilizers. Purity depends on the amount of C ₂ and higher hydrocarbons in the methane and the extent of chlorination. Small amounts of several other chlorinated compounds may be present. Dichloromethane is commonly recovered from extraction processes and several grades are commonly found in commerce. Dichloromethane is stable when dry but hydrolyzes in the presence of water.

Chemical names Dichloromethane

C.A.S. number 75-09-2

Chemical formula CH₂Cl₂

Structural formula



Formula weight 84.93

Assay Not less than 99.0%

DESCRIPTION Clear colourless non-flammable liquid

FUNCTIONAL USES Extraction solvent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Sparingly soluble in water; miscible with ethanol and ether

Refractive index (Vol. 4) n^{20}_{D} : 1.423 - 1.425

Specific gravity (Vol. 4) d^{25}_{25} : 1.323 - 1.327

PURITY

Water (Vol. 4) Not more than 0.02% (Karl Fischer Method)

Distillation range (Vol. 4) 39 - 41°

<u>Non-volatile residue</u> (Vol. 4)	Not more than 2 mg/100 ml
<u>Free chlorine</u>	Shake 10 ml of the sample vigorously for 2 min with 10 ml of 10% potassium iodide solution and 1 ml of starch TS. A blue colour does not appear in the water layer.
<u>Acidity</u>	Not more than 0.002% w/w (as HCl) Place 100 ml of freshly boiled and cooled distilled water (neutralized to phenolphthalein TS) in a 500-ml glass-stoppered conical flask. Add 100 ml of the sample and shake vigorously. Allow the layers to separate, transfer the aqueous phase into an Erlenmeyer flask, add 0.5 ml of phenolphthalein TS and titrate with 0.1 N sodium hydroxide to a red endpoint using a microburette. Calculate any acidity thus found as hydrochloric acid, HCl, per cent by weight of sample. 1 ml 0.1 N NaOH = 0.00365 g HCl
<u>Alkalinity</u>	Not more than 0.01% w/w (as NaOH) Place 100 ml of freshly boiled and cooled distilled water (neutralized to phenolphthalein TS) in a 500-ml glass-stoppered conical flask. Add 100 ml of the sample and shake vigorously. Allow the layers to separate, transfer the aqueous phase into an Erlenmeyer flask, add 0.5 ml of phenolphthalein TS and titrate with 0.1 N hydrochloric acid using a microburette. Calculate any alkalinity thus found as sodium hydroxide, NaOH, per cent by weight of sample. 1 ml 0.1 N HCl = 0.0040 g NaOH.
<u>Lead</u> (Volume 4)	Not more than 1 mg/kg Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

<u>Lead</u>	<u>Principle</u> The sample is treated with bromine, heated on a steam bath to decompose the alkyl lead and alkyl lead salts, and then extracted with dilute nitric acid. The pH of the aqueous extract is adjusted by means of a buffer and the lead is extracted with a chloroform solution of dithizone. The absorbance of the chloroform extract is measured and the lead content is determined from a previously prepared calibration curve.
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Apparatus

- The glassware should be borosilicate and confirmed to be lead-free.
- Spectrophotometer, fitted with covered absorption cells having a 1-cm light path.
- Shaking machine (optional), capable of approximately 250 rpm.
- Separatory funnels, Squibb-type, 125-ml volume.

Reagents

- Purity of reagents: Reagent grade chemicals shall be used in all tests.
- Purity of water: Unless otherwise indicated, references to water shall be understood to mean distilled water or other water of equivalent purity.
- Bromine Solution: Dilute 300 ml of bromine to 1000 ml with chloroform. Filter through a sintered-glass filter before using. (Do in fume hood).
- Buffer solution: Dissolve 20 g of potassium cyanide (KCN) (Caution), 6 g of ammonium citrate, and 6 g of sodium sulfite (Na_2SO_3), separately in water. Mix the solutions, add 150 ml of concentrated ammonium hydroxide (NH_4OH , sp gr 0.90), and dilute to 1,000 with water. This solution is stable for 3 months (in refrigerator).
- Dithizone solution: Dissolve 30 mg of dithizone in 1000 ml of chloroform. This solution is stable for only 4 weeks.
- Lead standard solution: Dissolve 1.5985 g of lead nitrate ($\text{Pb}(\text{NO}_3)_2$) in 250 ml of water contained in a liter volumetric flask. Add 8 ml of concentrated HNO_3 (sp gr 1.42), dilute to volume with water, and mix thoroughly. For calibration purposes, pipet 5.0 ml of this solution into a 1-liter volumetric flask, add 8 ml of concentrated HNO_3 (sp gr 1.42), and dilute to volume with water (1 ml = 0.005 mg Pb).
- Nitric acid (sp gr 1.42): Concentrated nitric acid (HNO_3).
- Nitric acid (8:992): Mix 8 ml of concentrated HNO_3 (sp gr 1.42) with 992 ml of water.

Calibration

Prepare a calibration curve using the lead solution (1 ml = 0.005 mg Pb) as follows: Pipet 0.0, 2.0, 5.0, 10.0, and 15.0 ml of the solution respectively into each of five separatory funnels and dilute each solution to 50 ml with HNO_3 (8:992). Treat these solutions as described in paragraphs 3 and 4, under Procedure, using the one not containing lead as the reagent blank. Construct a calibration curve by plotting the absorbance of the solutions against the mg of lead per 25 ml of dithizone solution.

Procedure

Pipet 50 g of the sample into a 250 ml-beaker. Add bromine solution until the bromine colour persists for at least 2 min, then allow the beaker to stand for an additional 5 min. At the same time prepare a reagent blank by adding the same amount of bromine solution to 25.0 ml of HNO_3 (8:992) in a 250 ml-beaker. Place the beakers on a steam bath and heat until the bromine colour disappears. Place the beakers on a hot plate and bring the solutions to a vigorous boil. Cool to room temperature and transfer each solution quantitatively into separatory funnels using 25.0 ml of HNO_3 (8:992). Shake the test sample for 2 min.

Drain the aqueous extract of the test sample into another separatory funnel. Repeat the extraction of the test sample using 25.0 ml of HNO_3 (8:992). Drain the aqueous layer into the separatory funnel containing the initial extract. Transfer the reagent blank solution quantitatively to a 50 ml volumetric flask using 25.0 ml of HNO_3 (8:992). Dilute to the mark with water and drain the contents of the flask into a separatory funnel. Add 120 ml of the buffer solution to both separatory funnels to adjust the pH to a point between 9.5 and 11.0. Pipet 25 ml of dithizone solution and shake for 2 min.

Drain and discard a small portion of the chloroform layer from the funnel to

remove any water or lead that may have accumulated in the stem. Transfer a portion of each chloroform layer into separate absorption cells. Adjust the spectrophotometer to read zero absorbance for the reagent blank and then measure the absorbance of the sample with respect to the blank at a wavelength of 510 nm.

Convert the absorbance measurement to concentration of lead in milligrams of lead per 25 ml of dithizone solution by means of the previously prepared calibration curve.

Calculate the lead content in milligrams per kilogram as follows:

$$\text{Lead, mg/kg} = \frac{1000A}{50} = 20A$$

where A is the lead concentration, in mg per 25 ml of dithizone solution corresponding to the measured absorbance.

METHOD OF ASSAY

Principle

Test material is injected into a suitable gas chromatograph equipped with two capillary gas chromatographic columns connected in series and a flame ionization detector. Quantification of contaminants and added stabilizers is made by comparing peak areas against external standards.

Apparatus

A gas chromatograph equipped with a flame ionization detector and capable of split and splitless capillary column injection. Peak areas of unknowns are compared to external standard solutions by electronic integration. A 25 m by 0.53 mm i.d. fused silica capillary column coated with a 2.0 μm film of 5% phenyl/95% methylsilicone liquid phase (or equivalent) and a 30 m by 0.32 mm i.d. fused silica capillary column coated with 1.8 μm film of (6% cyanopropyl-phenyl)-methylpolysiloxane liquid phase or equivalent are connected in series with the 0.32 mm i.d. column placed ahead of the 0.53 mm i.d. column.

Instrumental conditions:

Temperatures:

Injector: 150°

Detector: 250°

Oven: 40° isothermal

Carrier gas: He 4.4 ml/min

Split flow: 98 ml/min

Standard

A standard solution containing appropriate concentrations of methyl chloride, chloroform, methylene chloride, vinyl chloride, ethyl chloride, vinylidene chloride, 2-methyl-2-butene, trans-1,2-dichloroethylene, cyclohexane, and propylene oxide is prepared in high purity DCM by adding each reagent to DCM in a glass bottle fitted with a silicone rubber septum. Sufficient amounts of each standard analyte are added to make the approximate concentrations as given below:

Methyl chloride: 0.014% (w/w)

Vinyl chloride: 0.007

Ethyl chloride: 0.0084
Propylene oxide: 2.4
Vinylidene chloride: 0.0098
trans-1,2-Dichloroethylene: 0.017
Chloroform: 0.012
Cyclohexane: 0.047
2-Methyl-2-butene: 0.009

Addition of the analytes to the high purity DCM is made by accurately weighing an appropriate syringe containing the analyte, injecting the analyte into the standard DCM through the septum and re-weighing the syringe to determine the amount of analyte added. The DCM used to make standards must be assayed without added analytes to determine the possible presence of the analytes.

Procedure

The standard solution prepared above is diluted to a series of standards in the range of approximately 10 to 300 ppm (mg/kg) except for propylene oxide which is made in the range of 0.06 to 2.4 (w/w%). Standards and unknowns are injected into the gas chromatograph in the range of 1 to 5 μ l (using split injection mode) and the peak areas determined by electronic integration. A standard curve is constructed from these dilutions by plotting peak area against concentration for each analyte. The concentration of additives and by-products are determined by comparison to the standard curve. The sum of the concentrations of the impurities and stabilizers must be less than 1.0%. The order of elution and approximate retention times (min) are:

Methyl chloride: 2.8
Vinyl chloride: 3.0
Ethyl chloride: 3.5
Propylene oxide: 4.1
2-Methyl-2-butene: 4.5
Vinylidene chloride: 4.6
Dichloromethane: 5.3
trans-1,2-Dichloroethylene: 5.9
Chloroform: 8.7
Cyclohexane: 10.5
Carbon tetrachloride: 12.0

MAGNESIUM HYDROXIDE

Prepared at the 19th JECFA (1975), published in NMRS 55B (1976) and in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002). An ADI not limited' was established at the 9th JECFA (1965)

SYNONYMS INS No. 528

DEFINITION

Chemical names Magnesium hydroxide

C.A.S. number 1309-42-8

Chemical formula Mg(OH)_2

Formula weight 58.32

Assay Not less than 95.0%

DESCRIPTION Odourless, white bulky powder

FUNCTIONAL USES Alkali, colour adjunct

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Practically insoluble in water and in ethanol

Test for alkali The sample is alkaline to moistened litmus paper

Test for magnesium (Vol. 4) Passes test

PURITY

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

Loss on ignition (Vol. 4) Not more than 30 - 33% (approx. 800° to constant weight)

Alkalies (free) and soluble salts Boil 2 g of the sample with 100 ml of water for 5 min in a covered beaker and filter while hot. Add methyl red TS and titrate 50 ml of the cooled filtrate with 0.1 N sulfuric acid. Not more than 2 ml of the acid is required to reach the endpoint. Evaporate 25 ml of the filtrate to dryness and dry at 105° for 3 h. Not more than 10 mg of residue remains.

Calcium oxide Not more than 1.5%

Dissolve about 500 mg of the sample, accurately weighed, in a mixture of 3 ml of concentrated sulfuric acid and 22 ml of water. Add 50 ml of ethanol and allow the mixture to stand overnight. If crystals of magnesium sulfate separate, warm the mixture to about 50° to dissolve. Filter through a Gooch

crucible containing an asbestos mat previously washed with dilute sulfuric acid TS, water, and ethanol and ignited and weighed. Wash the crystals on the mat several times with a mixture of 3 volumes of ethanol and 1 volume of water. Ignite the crucible and contents at a dull red heat, cool and weigh. The weight of calcium sulfate obtained, multiplied by 0.4119, gives the equivalent of calcium oxide in the sample taken for the test.

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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

Transfer about 400 mg of the sample, previously dried at 105° for 2 h and accurately weighed, into a conical flask. Add 25 ml of 1 N sulfuric acid and, after solution is complete, add methyl red TS and titrate the excess acid with 1 N sodium hydroxide. Subtract from the volume of 1 N sulfuric acid consumed in the assay the volume of 1 N sulfuric acid corresponding to the weight of CaO in the sample taken for the assay using as a factor 28.04 mg of CaO for each ml of 1 N sulfuric acid. Each ml of 1 N sulfuric acid used to neutralize the magnesium hydroxide is equivalent to 29.16 mg of Mg(OH)₂.

CALCIUM CHLORIDE

Prepared at the 19th JECFA (1975), published in NMRS 55B (1976) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). An ADI 'not limited' was established at the 17th JECFA (1973)

SYNONYMS INS No. 509

DEFINITION

Chemical names	Calcium chloride
C.A.S. number	10043-52-4
Chemical formula	Anhydrous: CaCl_2 Dihydrate: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ Hexahydrate: $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$
Formula weight	Anhydrous: 110.99 Dihydrate: 147.02 Hexahydrate: 219.08
Assay	Anhydrous: Not less than 93% Dihydrate: Not less than 99.0% and not more than the equivalent of 107.0% of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ Hexahydrate: Not less than 98.0% and not more than the equivalent of 110% of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$

DESCRIPTION Anhydrous: White, deliquescent lumps or porous masses
Dihydrate: White, hard, deliquescent fragments or granules
Hexahydrate: Colourless, very deliquescent crystals

FUNCTIONAL USES Firming agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Anhydrous: Freely soluble in water and ethanol
Dihydrate: Freely soluble in water; soluble in ethanol
Hexahydrate: Very soluble in water and ethanol

Test for chloride (Vol. 4) Passes test

Test for calcium (Vol. 4) Passes test

PURITY

Free alkali Not more than 0.15% as $\text{Ca}(\text{OH})_2$
Dissolve 1 g of the sample in 20 ml of freshly boiled and cooled water, and add 2 drops of phenolphthalein TS. If the solution is pink, the pink colour is discharged by adding 2 ml of 0.02 N hydrochloric acid.

<u>Magnesium and alkali salts</u>	Not more than 5% Dissolve 1 g of anhydrous calcium chloride, or the corresponding weight of a hydrate, in about 50 ml of water, add 500 mg of ammonium chloride, mix and boil for about 1 min. Quickly add 40 ml of oxalic acid TS, and stir vigorously until precipitation is well established. Immediately add 2 drops of methyl red TS, then add ammonia TS dropwise until the mixture is just alkaline, and cool. Transfer the mixture into a 100-ml cylinder, dilute with water to 100 ml, let stand for 4 h or overnight, and then decant the clear, supernatant liquid through a dry filter paper. To 50 ml of the clear filtrate in a platinum dish add 0.5 ml of sulfuric acid and evaporate the mixture on a steam bath to a small volume. Carefully evaporate the remaining liquid to dryness over a free flame, and continue heating until the ammonium salts have been completely decomposed and volatilized. Finally, ignite the residue to constant weight. The weight of the residue does not exceed 25 mg.
<u>Fluoride</u> (Vol. 4)	Not more than 40 mg/kg (Method III)
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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 about 1 g of anhydrous calcium chloride, or the corresponding weight of a hydrate, transfer to a 250-ml beaker, and dissolve in a mixture of 100 ml of water and 5 ml of dilute hydrochloric acid TS. Transfer the solution to a 250-ml volumetric flask, dilute with water to volume and mix. Pipet 50 ml of the solution into a suitable container, add 100 ml of water, 15 ml of sodium hydroxide TS, 40 mg of murexide indicator (amm. purpurate) and 3 ml of naphthol green TS, and titrate with 0.05 M disodium ethylenediaminetetra-acetate until the solution is deep blue in colour. Each ml of 0.05 M disodium ethylenediaminetetraacetate is equivalent to 5.55 mg of CaCl_2 ; 7.35 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; or 10.95 mg of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$.

ETHYL p-HYDROXYBENZOATE

Prepared at the 51st JECFA (1998), published in FNP 52 Add 6 (1998) superseding specifications prepared at the 44th JECFA (1995), published in FNP 52 Add 3 (1995). Group ADI 0-10 mg/kg bw for ethyl, methyl and propyl p-hydroxybenzoate, established at the 17th JECFA in 1973.

SYNONYMS

Ethylparaben, ethyl p-oxybenzoate, INS No. 214

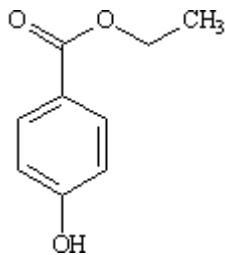
DEFINITION

Chemical names Ethyl p-hydroxybenzoate, ethyl ester of p-hydroxybenzoic acid

C.A.S. number 120-47-8

Chemical formula C₉H₁₀O₃

Structural formula



Formula weight 166.18

Assay Not less than 99.0% on the dried basis

DESCRIPTION Almost odourless, small, colourless crystals or a white, crystalline powder

FUNCTIONAL USES Preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in ethanol, ether and propylene glycol.

Melting range (Vol. 4) 115 - 118°

Test for p-hydroxybenzoate Melting range of p-hydroxybenzoic acid derived from the sample is 212-217°

To 0.5 g of the sample add 10 ml of sodium hydroxide TS. Boil for 30 min and concentrate to about 5 ml. Cool, acidify with dilute sulfuric acid TS, collect the precipitate on a filter, and wash thoroughly with water. Dry in a desiccator over sulfuric acid. Determine the melting range of p-hydroxybenzoic acid so obtained.

PURITY

Loss on drying (Vol. 4) Not more than 0.5% (80°, 2 h)

<u>Sulfated ash</u> (Vol. 4)	Not more than 0.05% Test 2 g of the sample (Method I)
<u>Acidity</u>	Heat 750 mg of the sample with 15 ml of water at 80° for 1 min, cool, and filter. The filtrate should be acid or neutral to litmus. To 10 ml of the filtrate add 0.2 ml of 0.1 N sodium hydroxide and 2 drops of methyl red TS. The solution should be yellow without even a light cast of pink.
<u>p-Hydroxybenzoic acid and salicylic acid</u>	Dissolve 0.5 g of the sample, accurately weighed, in 30 ml of ether, add 20 ml of a 1 in 100 sodium hydrogen carbonate solution, shake, and separate the water layer. Wash the water layer with two 20 ml portions of ether, add 5 ml of dilute sulfuric acid and 30 ml of ether, and shake. Separate the ether layer, and shake with about 10 ml of water. Filter the ether layer, and wash the vessel and the filter with a small amount of ether. Combine the washings and the filtrate, evaporate ether on a water bath, and dry the residue over sulfuric acid to constant weight. The weight of the residue should not exceed 5 mg. Dissolve any residue in 25 ml of water, heat to about 70°, filter, and add a few drops of dilute ferric chloride TS. No violet to reddish violet colour should be produced.
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg. Determine using an atomic absorption technique 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 g of the dried sample and transfer into a flask. Add 40 ml of N sodium hydroxide and rinse the sides of the flask with water. Cover with a watch glass, boil gently for 1 h and cool. Add 5 drops of bromothymol blue TS and titrate the excess sodium hydroxide with N sulfuric acid, comparing the colour with a buffer solution (pH 6.5) containing the same proportion of indicator. Perform a blank determination with the reagents and make any necessary correction. Each ml of N sodium hydroxide is equivalent to 166.18 mg of C ₉ H ₁₀ O ₃ .

MONO- AND DIGLYCERIDES

Prepared at the 17th JECFA (1973), published in FNP 4 (1978) and in FNP 52 (1992). Metals and arsenic specifications revised at the 55th JECFA (2000). An ADI not limited' was established at the 17th JECFA (1973)

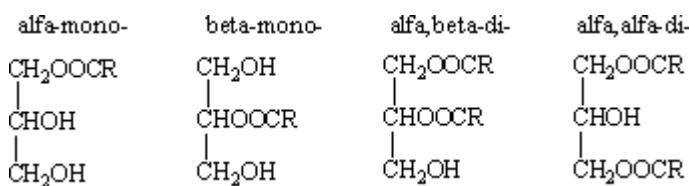
SYNONYMS

Glyceryl monostearate, glyceryl monopalmitate, glyceryl monooleate, etc; monostearin, monopalmitin, monoolein, etc.; GMS (for glyceryl monostearate); INS No. 471

DEFINITION

A mixture of mono- and diglyceryl esters of long chain, saturated and unsaturated fatty acids that occur in food fats; contain not less than 30% of alpha-monoglycerides and may also contain other isomeric monoglycerides, as well as di- and triglycerides, free glycerol, free fatty acids, soap and moisture; usually manufactured by the glycerolysis of edible fats and oils, but may also be prepared by esterification of fatty acids with glycerol, with or without molecular distillation of the product.

Structural formula



where -OCR represents the fatty acid moiety

Formula weight

Glyceryl monostearate: 358.6

Glyceryl distearate: 625.0

These are two major components of commercial products

DESCRIPTION

White or cream coloured hard fats of waxy appearance, plastic products or viscous liquids

FUNCTIONAL USES

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in water; soluble in ethanol, chloroform and benzene

Infrared absorption The infrared spectrum of the sample is characteristic of a partial fatty acid ester of a polyol

Tests for fatty acids (Vol. 4) Passes tests

Test for glycerol (Vol. 4) Passes tests

PURITY

Water (Vol. 4) Not more than 2.0% (Karl Fischer Method)

<u>Acid value</u> (Vol. 4)	Not more than 6
<u>Free glycerol</u> (Vol. 4)	Not more than 7%
<u>Soap</u>	Not more than 6%, calculated as a sodium oleate Add 10.00 g of the sample to a mixture of 60 ml of acetone and 0.15 ml of bromophenol blue solution (0.5%), previously neutralized with 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. Warm gently on a water bath until solution is complete, and titrate with 0.1 N hydrochloric acid until the blue colour is discharged. Allow to stand for 20 min, warm until any solidified matter has re-dissolved and, if the blue colour reappears, continue the titration. Each ml of 0.1 N hydrochloric acid is equivalent to 0.0304 g of C ₁₈ H ₃₃ O ₂ Na.
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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."
METHODS OF ASSAY	Determine as described under <i>alpha</i> -Monoglyceride and Free Glycerol Contents in Volume 4

NORDIHYDROGUAIARETIC ACID

Prepared at the 17th JECFA (1973), published in FNP 4 (1978) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). No ADI was allocated at the 17th JECFA (1973)

SYNONYMS NDGA

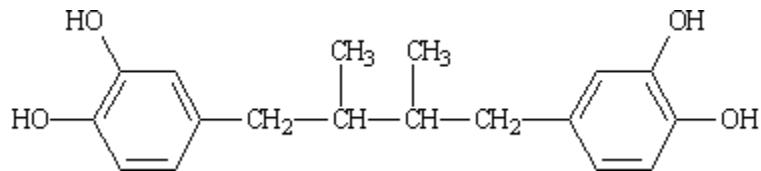
DEFINITION

Chemical names 4,4'-(2,3-Dimethyltetramethylene)-dipyro-catechol; 1,4-dipyro-catechol-2,3-dimethyl-butane; nordihydroguaiaretic acid; β,γ -dimethyl- α,δ -bis(3,4-dihydroxyphenyl) butane

C.A.S. number 500-38-9

Chemical formula $C_{18}H_{22}O_4$

Structural formula



Formula weight 302.36

Assay Not less than 95% and not more than 102%

DESCRIPTION White to greyish-white crystalline solid and may be prepared from the evergreen desert shrub, *Larrea divaricata*, (Fam. Zygophyllaceae)

FUNCTIONAL USES Antioxidant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in ethanol and ether, and in propylene glycol at 116°

Melting point (Vol. 4) About 184°

Colour reactions Passes test
See description under TESTS

PURITY

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an atomic absorption technique 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."

TESTS

IDENTIFICATION TESTS

- Colour reactions
- Add 2 ml of ferric chloride TS, ethanolic and 2 ml of 0.2% of 2,2'-bipyridine in absolute ethanol to 5 ml of 0.5% solution of the sample in 50% ethanol. A deep cherry-red colour appears
- To 5 ml of 1% solution of the sample in 75% ethanol, add 1 ml of strong ammonia TS. A yellow colour develops
- To 10 ml of 0.5% solution of the sample in 50% ethanol, add 1.5 ml of 1% barium hydroxide ($\text{Ba}(\text{OH})_2 \cdot \text{H}_2\text{O}$) in boiled water. A deep blue colour develops which is stable for approximately 1 h
- To 10 ml of 10% sodium hydroxide, add 1 ml of 0.5% solution of the sample in 50% ethanol. A rose-red colour develops

METHOD OF ASSAY

Weigh 1.00 g of the sample. Dilute with methanol so that the final concentration will be 1 mg of the sample per 100 ml of solution. Read the absorbance at 284 nm in a 1 cm quartz cell.

Calculate the % nordihydroguaiaretic acid from:

$$\frac{\text{Absorbance} - 0,008}{\text{weight of sample} \times 0.21} \times 100$$

where

a = the obtained absorbance

W = the weight of the sample

SORBITOL SYRUP

Prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996) superseding specifications prepared at the 33rd JECFA (1988), published in FNP 38 (1988) and in FNP 52 (1992). Metals and arsenic specifications revised at the 57th JECFA (2001). No ADI was allocated at the 33rd JECFA (1988)

SYNOMYS

D-Glucitol syrup, INS No. 420(ii)

DEFINITION

Formed by hydrogenation of glucose syrup; composed of D-sorbitol, D-mannitol and other hydrogenated saccharides
The part of the product which is not D-sorbitol is composed mainly of hydrogenated oligosaccharides formed by the hydrogenation of glucose syrup used as raw material (in which case the syrup is non-crystallizing) or mannitol; minor quantities of hydrogenated di-, tri- and tetrasaccharides may be present

Assay

Not less than 99.0% hydrogenated saccharides and not less than 50.0% of D-sorbitol on the anhydrous basis

DESCRIPTION

Clear colourless aqueous solution

FUNCTIONAL USES

Sweetener, humectant, sequestrant, texturizer, bulking agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water, glycerol and propan-1,2-diol

Thin layer chromatography (Vol. 4) Passes test
Proceed as directed under *Thin Layer Chromatography of Polyols*
Use the following:

Standard solution:

Dissolve 50 mg of reference standard sorbitol (available from US Pharmacopeial Convention, Inc. 12601 Twinbrook Parkway, Rockville, MD 20852, USA) in 20 ml water

Test solution:

Dissolve 50 mg of the sample in 20 ml of water

PURITY

Water (Vol. 4) Not more than 31% (Karl Fischer Method)

Sulfated ash (Vol. 4) Not more than 0.1%
Test 3 g of sample (Method I)

<u>Chlorides</u> (Vol. 4)	Not more than 50 mg/kg Test 10 g of sample by the Limit Test using 1.5 ml of 0.01N hydrochloric acid in the control
<u>Sulfates</u> (Vol. 4)	Not more than 100 mg/kg Test 10 g of sample by the Limit Test using 2.0 ml of 0.01N sulfuric acid in the control
<u>Nickel</u> (Vol. 4)	Not more than 2 mg/kg Proceed as directed under <i>Nickel in Polyols</i>
<u>Reducing sugars</u>	Not more than 0.3% Proceed as directed under <i>Reducing Substances (as Glucose)</i> , Method II. The weight of cuprous oxide shall not exceed 50 mg
<u>Lead</u> (Vol. 4)	Not more than 1 mg/kg Determine using an atomic absorption technique 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

Total hydrogenated saccharides (%):

$$\frac{100 - (\text{Water\%} + \text{Sulfated ash\%} + \text{Reducing sugars\%})}{100 - \text{Water\%}} \times 100$$

Determine the sorbitol content of the sample using *liquid chromatography*.

Apparatus

Liquid chromatograph (HPLC)

Detection: differential refractometer maintained at constant temperature

Integrator recorder

Column: AMINEX HPX 87 C (or equivalent resin in calcium form), length 30 cm, internal diameter 9 mm

Eluent: double distilled degassed water (filtered through Millipore membrane filter 0.45 µm)

Chromatographic conditions

Column temperature: 85±0.5°

Eluent flow rate: 0.5 ml/min

Standard preparation

Dissolve an accurately weighed quantity of sorbitol in water to obtain a solution having known concentration of about 10.0 mg of sorbitol per ml.

Sample preparation

Transfer about 1 g of the sample accurately weighed to a 50 ml volumetric flask, dilute with water to volume and mix.

Procedure

Separately inject equal volumes (about 20 µl) of the sample preparation

and the standard preparation into the chromatograph. Record the chromatograms and measure the responses of each polyol peak. Calculate separately the quantity, in mg, of sorbitol in the portion of sample taken by the following formula:

$$50 \times C \times \frac{R_u}{R_s}$$

where

C = concentration, in mg per ml, of sorbitol in the standard preparation

R_u = the peak response of the sample preparation

R_s = the peak response of the standard preparation.

TRISODIUM DIPHOSPHATE

Prepared at the 69th JECFA (2008), published in FAO JECFA Monographs 5 (2008), based on the previously withdrawn tentative specifications prepared at the 61st JECFA and published in FNP 52, Add 11 (2003). A group MTDI of 70 mg/kg bw, expressed as phosphorus from all food sources, was established at the 26th JECFA (1982).

SYNONYMS

Acid trisodium pyrophosphate, trisodium monohydrogen diphosphate; INS No. 450(ii)

DEFINITION

Trisodium diphosphate is manufactured by calcining sodium orthophosphate having a Na₂O:P₂O₅ ratio of 3:2

Chemical names Trisodium monohydrogen diphosphate

C.A.S. number
14691-80-6 (Anhydrous)
26573-04-6 (Monohydrate)

Chemical formula Na₃HP₂O₇ · x H₂O (x = 0 or 1)

Formula weight
243.93 (Anhydrous)
261.95 (Monohydrate)

Assay Not less than 57% and not more than 59% expressed as P₂O₅ on the dried basis

DESCRIPTION

White powder or grains

FUNCTIONAL USES

Stabilizer, leavening agent, emulsifier, nutrient

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water

Sodium (Vol. 4) Passes test

Phosphate (Vol. 4) Passes test

PURITY

Loss on drying (Vol. 4) Anhydrous: Not more than 0.5 % (105°, 4 h)
Monohydrate: Not more than 1.0 % (105°, 4 h)

Loss on ignition (Vol. 4) Anhydrous: Not more than 4.5%
Monohydrate: Not more than 11.5%

Water-insoluble matter (Vol. 4) Not more than 0.2 %

Fluoride (Vol. 4) Not more than 10 mg/kg
See description under TESTS

<u>Arsenic</u> (Vol. 4)	Not more than 3 mg/kg Determine by the atomic absorption hydride technique. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under "General Methods, Metallic Impurities").
<u>Lead</u> (Vol. 4)	Not more than 4 mg/kg Determine using an atomic absorption/ICP technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under "General Methods, Metallic Impurities").

TESTS

PURITY TESTS

<u>Fluoride</u> (Vol. 4)	Use Method III. The standard curve constructed in Method III may not be suitable for samples containing low fluoride levels. Therefore, it will be necessary to prepare standard solutions with concentrations other than those specified in Method III for the construction of the standard curve and to choose a sample size that will bring the fluoride concentration within the standard curve.
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METHOD OF ASSAY	Using a previously dried sample, proceed as directed under <i>Phosphate Determination as P₂O₅, Method I</i> , Inorganic components (Volume 4). Each ml of 1N sodium hydroxide consumed is equivalent to 3.088 mg of P ₂ O ₅ or 5.307 mg of trisodium monohydrogen diphosphate on the dried basis.
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CARBOHYDRASE from *BACILLUS LICHENIFORMIS*

Prepared at the 29th JECFA (1986), published in FNP 34 (1986) and in FNP 52 (1992) An ADI 'not specified' was established at the 29th JECFA (1985)

SYNOMYS	Diastase, ptyalin, glycogenase; INS No.1100
SOURCES	Commercial enzyme preparations are produced by the controlled fermentation of <i>Bacillus licheniformis</i> .
Active principles	alpha-Amylase
Systematic names and numbers	1,4-alpha-D-Glucan glucanohydrolase (EC 3.2.1.1)
Reactions catalyzed	The enzyme preparations hydrolyze 1,4-alpha-glucosidic linkages in polysaccharides, yielding dextrans and oligo- and monosaccharides.
Secondary enzyme activities	Microbial serine proteinase (EC 3.4.21.14)
DESCRIPTION	Off-white to tan amorphous powders or as brown liquids; soluble in water, but practically insoluble in ethanol, chloroform and ether
FUNCTIONAL USES	Enzyme preparation Used in the preparation of and/or in cereals and starch, fruits and vegetables, beverages, sugar and honey, confectionery and bakery.
GENERAL SPECIFICATIONS	Must conform to the <i>General Specifications for Enzyme Preparations used in Food Processing</i> (see Volume Introduction)
CHARACTERISTICS	
IDENTIFICATION	
<u>Alpha-Amylase activity</u> (Vol. 4)	The sample shows bacterial alpha-amylase activity

CALCIUM 5'-GUANYLATE

Prepared at the 18th JECFA (1974), published in NMRS 54B (1975) and in FNP 52 (1992). Metals and arsenic specifications revised at the 57th JECFA (2001). A group ADI 'not specified' for 5'guanylic acid and its Ca & Na salts was established at the 18th JECFA (1974)

SYNONYMS

Calcium guanylate, INS No. 629

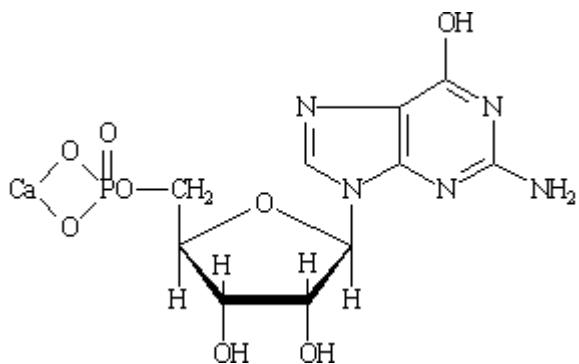
DEFINITION

Chemical names Calcium guanosine-5'-monophosphate

C.A.S. number 38966-30-2

Chemical formula $C_{10}H_{12}CaN_5O_8P \cdot xH_2O$

Structural formula



Formula weight 401.20 (anhydrous)

Assay Not less than 97.0% and not more than 102.0% of after drying

DESCRIPTION

Odourless, white or off-white crystals, or powder

FUNCTIONAL USES

Flavour enhancer

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Sparingly soluble in water

Spectrophotometry (Vol. 4) A 1 in 50,000 solution of the sample in 0.01 N hydrochloric acid exhibits an absorbance maximum at $256\pm2\text{nm}$. The ratio A_{250}/A_{260} is between 0.95 and 1.03, and the ratio A_{280}/A_{260} is between 0.63 and 0.71.

Test for calcium (Vol. 4) Passes test

Test for ribose (Vol. 4) Passes test

Test for organic Passes test

phosphate (Vol. 4) Test 5 ml of a 1 in 2000 solution

PURITY

Loss on drying (Vol. 4) Not more than 23% (120°, 4 h)

pH (Vol. 4) 7.0 - 8.0 (1 in 2,000 soln)

Water soluble matter To 1 g of the sample, add 50 ml of water, allow to stand for 10 min with occasional shaking, filter through analytical grade filter paper (Whatman No. 42 or equivalent). Evaporate a 25 ml portion of the solution to dryness on a water bath and dry the residue at 105° for 1 h. Residue weighs less than 80 mg.

Amino acids Not detectable by the following test: To 5 ml of a 1 in 2,000 solution add 1 ml of ninhydrin TS and heat for 3 min. No colour is produced.

Related foreign substances Chromatographically not detectable
Test 10 µl of a 1 in 2,000 solution of the sample.

Lead (Vol. 4) Not more than 1 mg/kg
Determine using an atomic absorption technique 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 about 500 mg of the sample, dissolve in and make to 1,000 ml with 0.01 N hydrochloric acid. Take 10.0 ml of this solution and dilute with 0.01 N hydrochloric acid to 250 ml. Determine the absorbance A of the solution in a 1-cm cell at the wave length of 260 nm using 0.01 N hydrochloric acid as the reference. Calculate the content of C₁₀H₁₂CaN₅O₈P, in % in the sample by the formula:

$$\frac{A}{294.1} \times \frac{250,000}{\text{weight of sample (mg)}} \times \frac{100}{100 - \text{loss on drying (\%)}} \times 100$$

α -AMYLASE from *BACILLUS SUBTILIS*

Prepared at the 41st JECFA (1993), published in FNP 52 Add 2 (1993) superseding specifications prepared at the 37th JECFA (1990), published in FNP 52 (1992). An ADI 'not specified' was established at the 37th JECFA (1990)

SYNONYMS	Glycogenase; INS No. 1100
SOURCES	Produced extracellularly by the controlled fermentation of <i>Bacillus subtilis</i>
Active principles	alpha-Amylase
Systematic names and numbers	1,4-alpha-D-Glucan glucanohydrolase - EC 3.2.1.1
Reactions catalyzed	Endohydrolysis of 1,4-alpha-D-glucosidic linkages in polysaccharides containing three or more 1,4-alpha-linked D-glucose units
DESCRIPTION	Typically brown liquids, granules or powders
FUNCTIONAL USES	Enzyme preparation Used in starch hydrolysis
GENERAL SPECIFICATIONS	Must conform to the <i>General Specifications for Enzyme Preparations used in Food Processing</i> (See Volume Introduction)
CHARACTERISTICS	
IDENTIFICATION	
<u>alpha-Amylase activity</u> (Vol. 4)	The sample shows bacterial alpha-amylase activity

TARA GUM

Prepared at the 30th JECFA (1986), published in FNP 37 (1986) and in FNP 52 (1992). Metals and arsenic specifications revised at the 57th JECFA (2001). An ADI 'not specified' was established at the 30th JECFA (1986)

SYNONYMS	Peruvian carob; INS No. 417
DEFINITION	Obtained by grinding the endosperm of the seeds of <i>Caesalpinia spinosa</i> (Fam. <i>Leguminosae</i>); consists chiefly of polysaccharides of high molecular weight composed mainly of galactomannans. The principal component consists of a linear chain of (1,4)-beta-D-mannopyranose units with alpha-D-galacto- pyranose units attached by (1 6) linkages; the ratio of mannose to galactose in tara gum is 3:1. (In carob bean gum this ratio is 4:1 and in guar gum 2:1.) The article of commerce may be further specified as to viscosity and loss on drying.
DESCRIPTION	White to white-yellow, nearly odourless powder
FUNCTIONAL USES	Thickening agent, stabilizer
CHARACTERISTICS	
IDENTIFICATION	
<u>Solubility</u> (Vol. 4)	Soluble in water; insoluble in ethanol
<u>Gel test</u>	To an aqueous solution of the sample add small amounts of sodium borate; a gel is formed
<u>Viscosity</u>	Transfer 2 g of the sample into a 400-ml beaker and moisten it thoroughly with about 4 ml of isopropanol. Add, with vigorous stirring, 200 ml of water and continue stirring until the gum is completely and uniformly dispersed. An opalescent, moderately viscous solution is formed. (This solution is less viscous than a guar gum solution, but more viscous than a carob bean gum solution when prepared and tested as indicated in the above described test). Transfer 100 ml of this solution into another 400-ml beaker, heat the mixture in a boiling water-bath for about 10 min and cool to room temperature. The solution shows a marked increase in viscosity.
<u>Gum constituents</u> (Vol. 4)	Proceed as directed under <i>Gum Constituents Identification</i> , using galactose and mannose as standards. Galactose and mannose should be present
<u>Microscopic examination</u>	Place some ground sample in an aqueous solution containing 0.5% iodine and 1% potassium iodide on a glass slide and examine under a microscope. Tara gum contains groups of round to pear-shaped cells; their contents are yellow to brown.

(Guar gum cells are similar in form but markedly larger in size. Carob bean gum shows long, stretched tubiform cells, separate or slightly interspaced and can be easily distinguished from tara gum.)

PURITY

<u>Loss on drying</u> (Vol. 4)	Not more than 15%
<u>Ash</u> (Vol. 4)	Not more than 1.5%
<u>Acid insoluble matter</u> (Vol. 4)	Not more than 2%
<u>Protein</u>	Not more than 3.5% Proceed as directed under <i>Nitrogen Determination (Kjeldahl method)</i> (see Volume 4). The percentage of nitrogen determined multiplied by 5.7 gives the percentage of protein in the sample.
<u>Starch</u>	Not detectable To a 1 in 10 solution of the sample, add a few drops of iodine TS. No blue colour is produced.
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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."

SODIUM METAPHOSPHATE, INSOLUBLE

Prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996). Metals and arsenic specifications revised at the 55th JECFA (2000). A group MTDI of 70 mg/kg bw, as phosphorus from all food sources, was established at the 26th JECFA (1982)

SYNONYMS	Insoluble sodium polyphosphate; IMP; Maddrell's salt
DEFINITION	A high molecular weight sodium polyphosphate composed of two long metaphosphate chains $(\text{NaPO}_3)_x$ that spiral in opposite directions about a common axis; the $\text{Na}_2\text{O}/\text{P}_2\text{O}_5$ ratio is about 1.0; the pH of a 1 in 3 slurry in water is about 6.5.
Chemical names	Sodium metaphosphate
C.A.S. number	50813-16-6
Structural formula	$\text{Na}_2\text{O}_3\text{PO} \left[\begin{array}{c} \text{Na} \\ \\ \text{O} \\ \\ \text{P} \text{ O} \\ \\ \text{O} \\]_x \text{ PO}_3\text{Na}_2 \end{array} \right]$ <p>where $x \geq 20$</p>
Assay	Not less than 68.7% and not more than 70.0% of P_2O_5
DESCRIPTION	White crystalline powder
FUNCTIONAL USES	Emulsifier, sequestrant, texturizer
CHARACTERISTICS	
IDENTIFICATION	
<u>Solubility</u> (Vol. 4)	Insoluble in water, soluble in mineral acids and in solutions of potassium and ammonium (but not sodium) chlorides
<u>Gel test</u>	Finely powder about 1 g of the sample, and add slowly to 100 ml of a 1 in 20 solution of potassium chloride while stirring vigorously. A gelatinous mass is formed.
<u>Test for sodium</u> (Vol. 4)	Prepare the test solution by mixing 500 mg of the sample with 10 ml of nitric acid and 50 ml of water. Boil for about 30 min and cool
<u>Test for phosphate</u> (Vol. 4)	Prepare the test solution by mixing 500 mg of the sample with 10 ml of nitric acid and 50 ml of water. Boil for about 30 min and cool
PURITY	
<u>Fluoride</u> (Vol. 4)	Not more than 10 mg/kg (Method I or III)

<u>Arsenic</u> (Vol. 4)	Not more than 3 mg/kg (Method II)
<u>Lead</u> (Vol. 4)	Not more than 4 mg/kg Determine using an atomic absorption technique 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	Transfer about 800 mg of the sample, accurately weighed, into a 400-ml beaker. Add 100 ml of water and 25 ml of nitric acid, cover with a watch glass, and boil for 10 min on a hot plate. Rinse any condensate from the watch glass into the beaker; cool the solution to room temperature; transfer it quantitatively to a 500-ml volumetric flask; dilute to volume with water; and mix thoroughly. Pipet 20.0 ml of this solution into a 500-ml Erlenmeyer flask, add 100 ml of water, and heat just to boiling. Add with stirring 50 ml of quimociac TS, then cover with a watch glass, and boil for 1 min in a well-ventilated hood. Cool to room temperature, swirling occasionally while cooling, then filter through a tared, sintered-glass filter crucible of medium porosity, and wash with five 25-ml portions of water. Dry at about 225° for 30 min, cool, and weigh. Each mg of precipitate thus obtained is equivalent to 32.074 µg of P ₂ O ₅ .

SORBITAN MONOOLEATE

Prepared at the 33rd JECFA (1988), published in FNP 38 (1988) and in FNP 52 (1992). Metals and arsenic specifications revised at the 55th JECFA (2000) A group ADI of 0-25 mg/kg bw as the sum of sorbitan esters of lauric, oleic, palmitic and stearic acids was established at the 26th JECFA (1982)

SYNOMYS

INS No. 494

DEFINITION

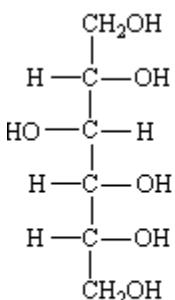
A mixture of the partial esters of sorbitol and its mono- and dianhydrides with edible oleic acid (R). The constituent in greatest abundance is 1,4-sorbitan monooleate, with lesser abundance of isosorbide monooleate, sorbitan dioleate and sorbitan trioleate.

C.A.S. number

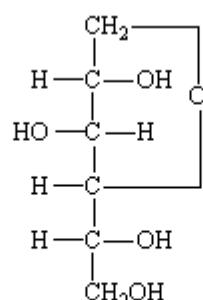
1338-43-8

Structural formula

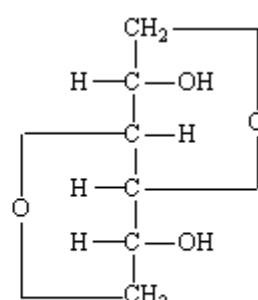
Contains oleic acid esterified with polyols derived from sorbitol including the following types:



Sorbitol



1,4-Sorbitan



Isosorbide

Assay

Saponification of 100 g of the sample yields not less than 28 g and not more than 32 g of polyols, and not less than 73 g and not more than 77 g of fatty acids. The polyol content shall be not less than 95% of a mixture of sorbitol, 1,4-sorbitan and isosorbide.

DESCRIPTION

Amber-coloured oily viscous liquid, light cream to tan beads or flakes or a hard, waxy solid with a slight odour

FUNCTIONAL USES

Emulsifier, stabilizer

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Soluble at temperatures above its melting point in ethanol, ether, ethylacetate, aniline, toluene, dioxane, petroleum ether and carbon tetrachloride; insoluble in cold water, dispersible in warm water

Iodine value (Vol. 4)

The residue of oleic acid, obtained from the saponification of the sorbitan monooleate in assay, has a iodine value between 80 and 100

PURITY

<u>Water</u> (Vol. 4)	Not more than 2% (Karl Fischer Method)
<u>Sulfated ash</u> (Vol. 4)	Not more than 0.5%
<u>Acid value</u> (Vol. 4)	Not more than 8
<u>Saponification value</u> (Vol. 4)	Not less than 145 and not more than 160
<u>Hydroxyl value</u> (Vol. 4)	Not less than 193 and not more than 210
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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	Transfer about 25 g of the sample, accurately weighed, into a 500-ml round-bottom flask, add 250 ml of alcohol and 7.5 g of potassium hydroxide, and mix. Connect a suitable condenser to the flask, reflux the mixture for 1 to 2 h, and then transfer to an 800-ml beaker, rinsing the flask with about 100 ml of water and adding it to the beaker. Heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol, and evaporate until the odour of alcohol can no longer be detected. Adjust the final volume to about 250 ml with hot water. Neutralize the soap solution with dilute sulfuric acid (1 in 2), add 10% in excess, and heat, while stirring, until the fatty acid layer separates. Transfer the fatty acids to a 500-ml separator, wash with three or four 20-ml portions of hot water to remove polyols, and combine the washings with the original aqueous polyol layer from the saponification. Extract the combined aqueous layer with three 20-ml portions of petroleum ether, add the extracts to the fatty acid layer, evaporate to dryness in a tared dish, cool, and weigh.

Neutralize the polyol solution with a 1 in 10 solution of potassium hydroxide to pH 7 using a suitable pH meter. Evaporate this solution to a moist residue, and separate the polyols from the salts by several extractions with hot alcohol. Evaporate the alcohol extracts on a steam bath to dryness in a tared dish, cool, and weigh. Avoid excessive drying and heating. Assay another 25 g sample by the *Sorbitan Ester Content* procedure to determine percent sorbitan ester.

NATAMYCIN

Prepared at the 61st JECFA (2003) and published in FNP 52 Add 11 (2003) superseding specifications prepared at the 57th JECFA (2001) and published in FNP 52 Add 9 (2001) superseding specifications for pimaricin prepared at the 20th JECFA (1976), published in FNP 52 (1992). An ADI 0-0.3mg/kg bw was established at the 20th JECFA (1976).

SYNONYMS

DEFINITION

Chemical names

Pimaricin; INS No. 235

A fungicidal antimycotic of the polyene macrolide group. It is produced by several species of *Streptomyces*. The commercial product may contain up to three moles of water.

C.A.S. number

A stereoisomer of 22-(3-Amino-3,6-dideoxy-β-D-mannopyranosyloxy)-1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28-trioxatricyclo[22.3.1.0^{5,7}]octacosa-8,14,16,18,20-pentaene-25-carboxylic acid

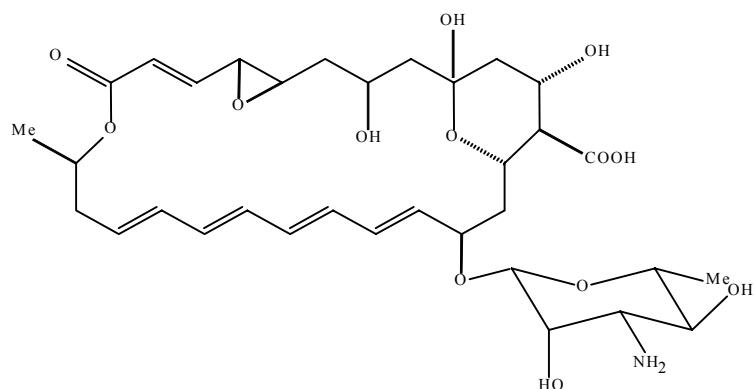
C.A.S. number

7681-93-8

Chemical formula

C₃₃H₄₇NO₁₃

Structural formula



Formula weight

665.74

Assay

Not less than 95.0% calculated on the dried basis

DESCRIPTION

White to creamy-white, almost odourless, crystalline powder

FUNCTIONAL USES

Fungicidal preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Practically insoluble in water, in lipid and in mineral oils; slightly soluble in methanol; soluble in glacial acetic acid and dimethylformamide.

Colour reaction

On adding a few crystals of the sample, on a spot plate, to a drop of - concentrated hydrochloric acid, a blue colour develops;

- concentrated phosphoric acid, a green colour develops, which changes into pale-red after a few minutes

Infrared absorption

The infrared spectrum of a potassium bromide dispersion of the sample corresponds with the reference infrared spectrum in Appendix A.

Ultraviolet absorption

A solution of 5mg/l of the sample in 0.1% glacial acetic acid in methanol has absorption maxima at about 290, 303 and 318 nm, a shoulder at about 280 nm and exhibits minima at about 250, 295.5 and 311 nm. See Appendix B.

PURITY

Loss on drying (Vol. 4) Not more than 8.0% (60°, over P₂O₅, pressure less than 5 mm Hg)

Specific rotation (Vol. 4) [α]_D²⁰ : + 250° to + 295° (1% w/v solution in glacial acetic acid)

pH (Vol. 4) 5.0 - 7.5 (1.0% w/v suspension in demineralised water)

Sulfated ash (Vol. 4) Not more than 0.5%
Test 2 g of the sample (Method I)

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an atomic absorption technique 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

High Performance Liquid Chromatography

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

Mobile phase: Dissolve 3.0 g of ammonium acetate and 1.0 g of ammonium chloride in 760 ml of water, and mix. Add 5.0 ml of tetrahydrofuran and 240 ml of acetonitrile, mix, and filter through a 0.5-μm or finer porosity filter. Make adjustments if necessary to meet the system suitability requirements.

Standard preparation: Transfer about 20 mg of natamycin Reference Standard, accurately weighed, to a 100-ml volumetric flask. Add 5.0 ml of tetrahydrofuran, and sonicate for 10 min. Add 60 ml of methanol, and swirl to dissolve. Add 25 ml of water, and mix. Allow to cool to room temperature. Dilute with water to volume, mix, and filter through a membrane filter of 5-μm or finer porosity.

Resolution solution: To prepare a mixture of natamycin and natamycin methyl ester, dissolve 20 mg of natamycin in a mixture of 99 ml of methanol and 1 ml of 0.1 N hydrochloric acid, and allow to stand for 2 h.

Note: use this solution within 1 h.

Assay preparation: Transfer about 20 mg of natamycin, accurately weighed, to a 100-ml volumetric flask. Proceed as directed under "Standard preparation", beginning with "add 5.0 ml of tetrahydrofuran...."

Chromatographic system (see High-Performance Liquid Chromatography, (see Volume 4):

Use a high performance liquid chromatograph equipped with an ultraviolet detector measuring at 303 nm and a 4.6-mm x 25-cm column packed with octadecylsilanized silica (Supelcosil LC 18 or equivalent). The flow rate is about 3 ml/min. Chromatograph the "standard preparation", and record the peak responses. The column efficiency should not be less than 3000 theoretical plates and the tailing factor should be between 0.8 and 1.3. The relative standard deviation for three replicate injections of the standard preparation is not more than 1.0 %.

Chromatograph the "resolution solution". The relative retention times are about 0.7 for Natamycin and 1.0 for its methyl ester. The resolution (R) between Natamycin and its methyl ester is not less than 2.5:

$$R = 2(t_2 - t_1)/(W_2 + W_1)$$

where:

t_2 and t_1 are the retention times of natamycin methyl ester and natamycin, respectively

W_2 and W_1 are the width of the corresponding peaks at their bases extrapolated to the baseline.

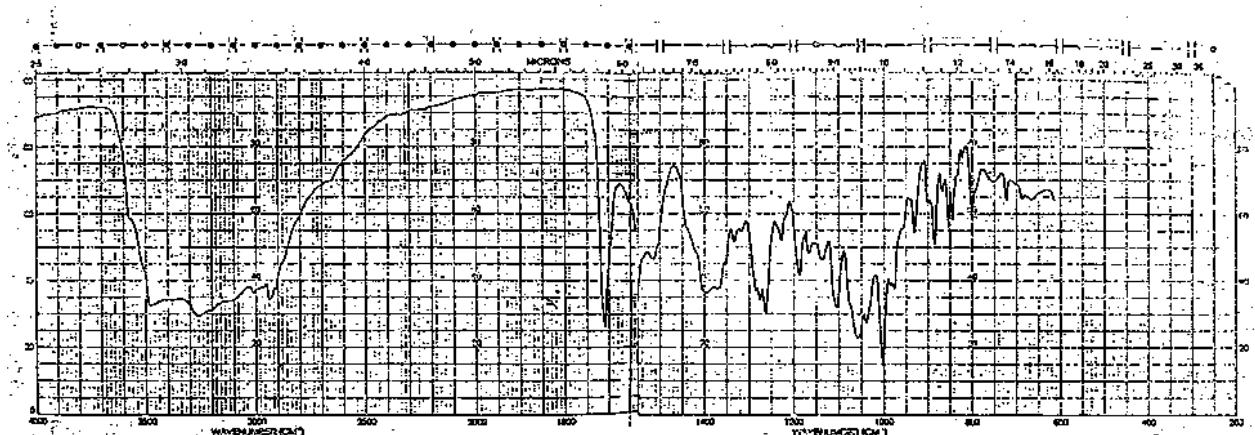
Procedure: Separately inject about 20 μ l for each of the "standard preparation" and the "assay preparation" into the chromatograph, and record the peak areas of the major peaks. Calculate the percentage of Natamycin in the portion taken by the formula:

$$0.1(W_s P_s / W_u)(r_u / r_s)$$

in which W_s is the weight, in mg, of Natamycin Reference Standard taken to prepare the "Standard preparation"; P_s is the stated content, in μ g/ml, of Natamycin Reference Standard; W_u is the weight, in mg, of Natamycin taken to prepare the "Assay preparation"; and r_u and r_s are the peak area responses obtained with the "Assay preparation" and the "Standard preparation", respectively.

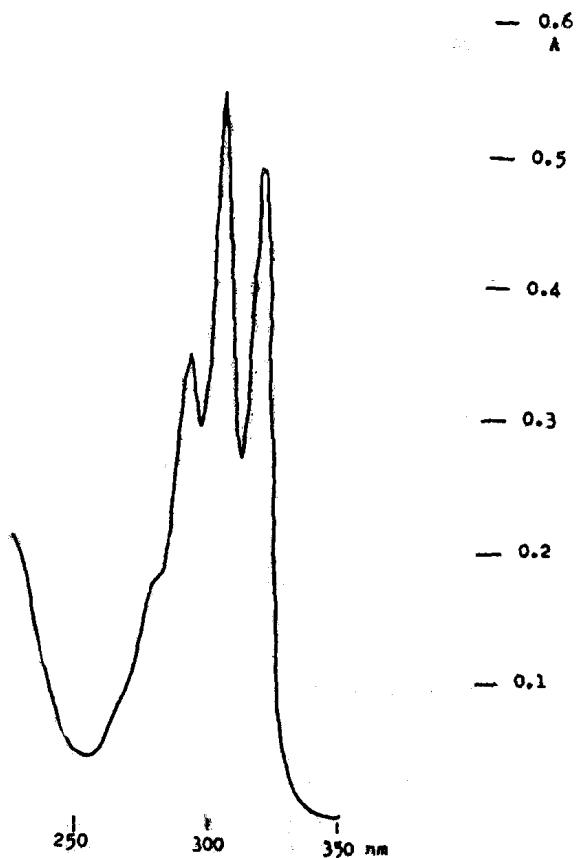
Appendix A

Reference Infrared Spectrum (1.3 mg solid in 300 mg potassium bromide) for natamycin



Appendix B

Ultraviolet absorption spectrum of natamycin
Concentration: 5 µg/ml in methanol/glacial acetic acid mixture



METHANOL

Prepared at the 28th JECFA (1984), published in FNP 31/2 (1984) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). An ADI 'limited by GMP' was established at the 14th JECFA (1970)

SYNONYMS Carbinol

DEFINITION

Chemical names Methanol, methyl alcohol

C.A.S. number 67-56-1

Chemical formula CH₃OH

Structural formula H₃C—OH

Formula weight 32.04

Assay Not less than 99.5%

DESCRIPTION Clear colourless, mobile liquid with a characteristic odour

FUNCTIONAL USES Extraction solvent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Miscible with water, ether and ethanol

Specific gravity (Vol. 4) 0.792 - 0.795

Refractive index (Vol. 4) n (20, D): 1.328 - 1.330

Boiling point (Vol. 4) About 65°

PURITY

Water (Vol. 4) Not more than 0.1% (Karl Fischer Method)

Distillation range (Vol. 4) 64.5 - 65.5°

Non-volatile residue (Vol. 4) Not more than 3 mg/100 ml

Acidity Not more than 15 mg/kg as formic acid
To a mixture of 10 ml of ethanol and 25 ml of water add 0.5 ml of phenolphthalein TS, and titrate with 0.02 N sodium hydroxide to the first pink colour that persists for at least 30 sec. Add 19 ml (about 15 g) of the sample, mix and titrate with 0.02 N sodium hydroxide until the pink colour is

restored. Not more than 0.25 ml is required.

Alkalinity

Not more than 3 mg/kg as ammonia

Add 1 drop of methyl red TS to 25 ml of water, add 0.02 N sulfuric acid until a red colour just appears, then add 29 ml (about 22.5 g) of the sample, and mix. Not more than 0.2 ml of 0.02 N sulfuric acid is required to restore the red colour.

Aldehydes and ketones

Not more than 0.015% w/v as acetone

See description under TESTS

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

Aldehydes and ketones

Principle

The aldehydes and ketones present are converted with 2,4-dinitrophenylhydrazine into the corresponding 2,4-dinitrophenylhydrazone. In alkaline medium these have a red colour, which is determined spectrophotometrically or visually.

Apparatus

- Photoelectric absorptionmeter or spectrophotometer, with 0.5-cm cells.
- Alternatively flat bottom tubes, capacity about 20 ml
- Water bath, controlled at $60 \pm 1^\circ$

Reagents

The reagents used shall be of a recognized analytical reagent quality.

Distilled water or water of at least equal purity shall be used throughout

- Carbonyl-free methanol: Reflux 1000 ml of methanol with 5 g of 2,4-dinitrophenylhydrazine and 5 drops of concentrated hydrochloric acid ($d = 1.18$) for 2-3 h. Distil off the methanol using a 300 mm by 25 mm diameter Widmer or other suitable distillation column. Reject the first 100 ml and collect the next 800 ml, rejecting the remainder. If, in spite of the precautions taken, the distillate is found to be coloured, then it should be re-distilled.

- 2,4-Dinitrophenylhydrazine solution: Dissolve 0.03 g of 2,4-dinitrophenylhydrazine in 40 ml of the carbonyl-free methanol containing 0.3 ml of concentrated hydrochloric acid ($d = 1.18$) and dilute to the mark in a 50-ml one-mark volumetric flask with the carbonyl-free methanol. Prepare this solution fresh each day.

- Potassium hydroxide solution: Dissolve 10 g of potassium hydroxide in 10 ml of water, cool and dilute to the mark in a 50-ml one-mark volumetric flask with carbonyl-free methanol. Prepare this solution fresh each day.

- Standard acetone solution: Weigh 1.00 g of acetone and dilute to the mark in a one-mark 100 ml volumetric flask with carbonyl-free methanol. Dilute 1.0 ml of this solution to 100 ml with the carbonyl-free methanol. 1 ml of the

diluted solution contains 0.1 mg of acetone.

Procedure

Prepare five solutions by diluting 1.0, 2.0, 4.0, 8.0 and 10.0 ml portions of the standard acetone solution to 25.0 ml with carbonyl-free methanol. To 1.0 ml of each of the solutions thus obtained (containing 0.004 - 0.04 mg of acetone/ml), contained in a test tube fitted with a ground glass stopper, add 1.0 ml of the 2,4-dinitrophenylhydrazine solution. Stopper the tube and heat for 50 min in the water bath at 60°, cool, add 8.0 ml of the potassium hydroxide solution and after 5 to 15 min measure the optical density of each solution at a wavelength of 430 nm using as a blank 1.0 ml of the carbonyl-free methanol treated in the same way. Prepare a calibration chart by plotting weights (in mg) of acetone against corresponding values of optical density.

Dilute 5.0 ml of the sample to 25.0 ml with the carbonyl-free methanol. Transfer 1.0 ml of this solution to a test tube fitted with a ground glass stopper and add 1.0 ml of the 2,4-dinitrophenylhydrazine solution. Stopper the tube and heat for 50 min in the water bath at 60°, cool, add 8.0 ml of the potassium hydroxide solution and after 5 to 15 min measure the optical density of the solution at the wavelength of 430 nm using as a blank 1.0 ml of the carbonyl-free methanol treated in the same way. By reference to the calibration chart prepared as described above, read the acetone content (in mg) of the solution.

The content is not more than 0.03 mg.

METHOD OF ASSAY

Using the procedures for *Gas chromatography* (see Volume 4), establish the following conditions:

Column

- length: 1.8 m
- diameter: 4 mm
- packing: 120-150 mesh Porapak R, or equivalent

Carrier gas: Nitrogen

Flow rate: 25 ml/min

Detector: FID

Temperatures

- injection port: 200°
- column: 160°
- detector: 210°

Prepare a standard solution of 0.4% (v/v) methanol in dioxane. Adjust column temperature and/or gas flow rate so that methanol retention time is about 5-7 min. Adjust detector so that 8 µl of standard solution provides at least one-half scale deflection. Inject 5-10 µl sample, obtain chromatogram and determine methanol content by the method of area normalization.

FERRIC AMMONIUM CITRATE

Prepared at the 28th JECFA (1984), published in FNP 31/2 (1984) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). A PMTDI of 0.8 mg/kg bw for iron was established at the 29th JECFA (1985).

SYNONYMS

Iron ammonium citrate, ammonium ferric citrate, ammonium iron citrate, ammonium iron (III) citrate, INS No. 381

DEFINITION

A complex salt of undetermined structure, composed of iron, ammonia and citric acid; there are two types of salts - brown and green - containing different amounts of iron

Chemical names

Ferric ammonium citrate, ammonium iron (III) citrate

Assay

Not less than 16.5% and not more than 22.5% of iron (Fe) for the brown salt, and not less than 14.5% and not more than 16.0% of iron (Fe) for the green salt.

DESCRIPTION

Brown salt: thin, transparent brown, reddish brown, or garnet red scales or granules, or a brownish yellow powder; odourless or has a slight ammoniacal odour

Green salt: thin, transparent green scales, granules, powder, or transparent green crystals; odourless

FUNCTIONAL USES

Nutrient, dietary supplement (brown salt)
Nutrient, dietary supplement, anticaking agent for sodium chloride(green salt)

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Very soluble in water; insoluble in ethanol

Test for iron and ferric salts Ignite 0.5 g of the sample gently, and dissolve the residue in 5 ml of dilute hydrochloric acid TS. The solution gives positive tests for *iron* and for *ferric salts*.

Test for citrate

To 5 ml of a 1-in-10 solution of the sample add 0.3 ml of potassium permanganate TS and 4 ml of mercuric sulfated TS and then heat the mixture to boiling. A white precipitated forms.

Test for ferric and ammonium salt

Dissolve 0.5 g of the sample in 5 ml of water, and add 5 ml of sodium hydroxide TS. A reddish brown precipitate forms and ammonia is evolved when the mixture is heated.

PURITY

Ferric citrate

Add potassium ferrocyanide TS to a 1 in 100 solution of the sample. No blue precipitation forms.

Oxalate

Transfer 1 g of the sample into a 125-ml-separator, dissolve in 10 ml of water, add 2 ml of hydrochloric acid, and extract successively with 50-ml portion and one 20-ml portion of ether. Transfer the combined ether extracts to a 150-ml beaker, add 10 ml of water, and remove the ether by evaporation on a steam bath. Add 1 drop of glacial acetic acid and 1 ml of calcium acetate solution (1 in 20) to the residual aqueous solution. No turbidity is produced within 5 min.

Sulfates

Not more than 0.3%

Dissolve a 100 mg sample in 1 ml of diluted hydrochloric acid TS, and dilute to 30 to 40 ml with water. Proceed as directed in the Limit Test for Sulfates, beginning with the addition of 3 ml of barium chloride TS. Any turbidity produced does not exceed that shown in a control containing 0.6 ml of 0.01 N sulfuric acid.

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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**

Transfer about 1 g of the sample, accurately weighed, into a 250 ml glass-stoppered Erlenmeyer flask, and dissolve in 25 ml of water and 5 ml of hydrochloric acid. Add 4 g of potassium iodide, stopper, and allow to stand protected from light for 15 min. Add 100 ml of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate, using starch TS as the indicator. Perform a blank determination and make any necessary correction. Each ml of 0.1 N sodium thiosulfate is equivalent to 5.585 mg of iron (Fe).

1,1,2-TRICHLOROTRIFLUOROETHANE

Prepared at the 26th JECFA (1982), published in FNP 25 (1982) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). No ADI was allocated at the 23rd JECFA (1979)

SYNONYMS Fluorocarbon 113

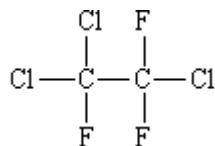
DEFINITION

Chemical names 1,1,2-Trichloro-1,2,2-trifluoroethane

C.A.S. number 76-13-1

Chemical formula $C_2Cl_3F_3$

Structural formula



Formula weight 187.4

Assay Not less than 99.8%

DESCRIPTION Colourless, volatile liquid with a faint characteristic odour

FUNCTIONAL USES Extraction solvent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Practically insoluble in water; miscible with ethanol, chloroform and ether

Refractive index (Vol. 4) $n(20, 20)$: about 1.359

Specific gravity (Vol. 4) 1.571 - 1.578

Boiling range (Vol. 4) $47.0 - 48.0^\circ$

PURITY

Non-volatile residue (Vol. 4) Not more than 2 mg/100 ml

Free chlorine Not more than 5 mg/kg (as hydrochloric acid)
Shake 10 ml of the sample vigorously for 2 min, with 10 ml of 10% potassium iodide solution and 1 ml of starch TS. A blue colour does not appear in the water layer

Acidity Not more than 0.5 mg/kg
Shake 20 ml with 20 ml of freshly boiled and cooled water for 3 min.

Separate the aqueous layer and add a few drops of bromocresol purple indicator solution. Not more than 0.3 ml of 0.01 N NaOH is required to change the colour of the indicator.

Other halogenated hydrocarbons

Not more than 0.2%
See description under TESTS

Lead (Vol. 4)

Not more than 2 mg/kg
Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

Other halogenated hydrocarbons

Examine by *gas chromatography* using the following solutions:

- Trichlorotrifluoroethane to be examined
- A 0.2% (v/v) solution of 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane) in ethanol as internal standard.

The chromatographic procedure is carried out under the following conditions:

Column

- length: 2.75 m
- diameter: 0.5 cm (i.d.)
- packing: the first 1.80 m: 30% PEG 400 supported on pink firebrik; the remainder with 30% dinonylphthalate on the same support.

Carrier gas: Nitrogen

Detector type: F.I.D.

Temperatures

- column: 50°

Record the chromatogram with the appropriate attenuation and measure the area of all peaks and summate. The sum of the total impurities is less than 0.2%.

The chromatographic procedure may also be carried out with a column prepared with Porapak Q 130.

METHOD OF ASSAY

Determine by *Gas-liquid chromatography* (see Volume 4): After determination of the total content of specified impurities, the balance consists of 1,1,2-trichloro-trifluoroethane together with any trace of other halogenated hydrocarbons that may be present. Calculate the percentage of 1,1,2-trichlorotrifluoroethane by the formula 100%-X, in which X is the percentage of other halogenated hydrocarbons determined as directed above.

SUCROSE ACETATE ISOBUTYRATE

Prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996) superseding specifications prepared at the 41st JECFA (1993), published in FNP 52 Add 2 (1993). Metals and arsenic specifications revised at the 61st JECFA (2003. An ADI of 0-20 mg/kg bw was established at the 46th JECFA (1996))

SYNONYMS

SAIB, INS No. 444

DEFINITION

A mixture of the reaction products formed by the esterification of food grade sucrose with acetic anhydride and isobutyric anhydride, followed by distillation. The mixture contains all possible combinations of esters in which the molar ratio of acetate to isobutyrate is about 2:6

Chemical names

Sucrose diacetate hexaisobutyrate (approximate)

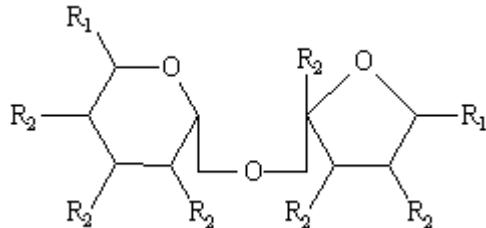
C.A.S. number

137204-24-1; 27216-37-1; 126-13-6

Chemical formula

$C_{40}H_{62}O_{19}$ for sucrose diacetate hexaisobutyrate

Structural formula



where

$R_1 = -CH_2OCOCH_3$, and

$R_2 = -CH_2OCOCH(CH_3)_2$, or $-OCOCH(CH_3)_2$

Formula weight

832 - 856 (approximate), $C_{40}H_{62}O_{19} = 846.9$

Assay

Not less than 98.8% and not more than 101.9% of $C_{40}H_{62}O_{19}$

DESCRIPTION

Pale straw coloured liquid, clear and free of sediment and having a bland odour

FUNCTIONAL USES Density adjusting agent, cloud-producing agent in non-alcoholic beverages

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in water, soluble in most organic solvents

Refractive index (Vol. 4) n (40, D): 1.4492 - 1.4504

<u>Specific gravity</u> (Vol. 4)	d (25, 25): 1.141 - 1.151
<u>Infrared absorption</u>	The infrared spectrum of a potassium bromide dispersion of the sample corresponds with the reference infrared spectrum in the Appendix

PURITY

<u>Acid value</u> (Vol. 4)	Not more than 0.2 Proceed as directed under <i>Acid Value</i> , using 50 g of the sample and a microburette
<u>Saponification value</u> (Vol. 4)	Between 524 and 540 Use 1 g of the sample
<u>Triacetin</u>	Not more than 0.1% See description under TESTS
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

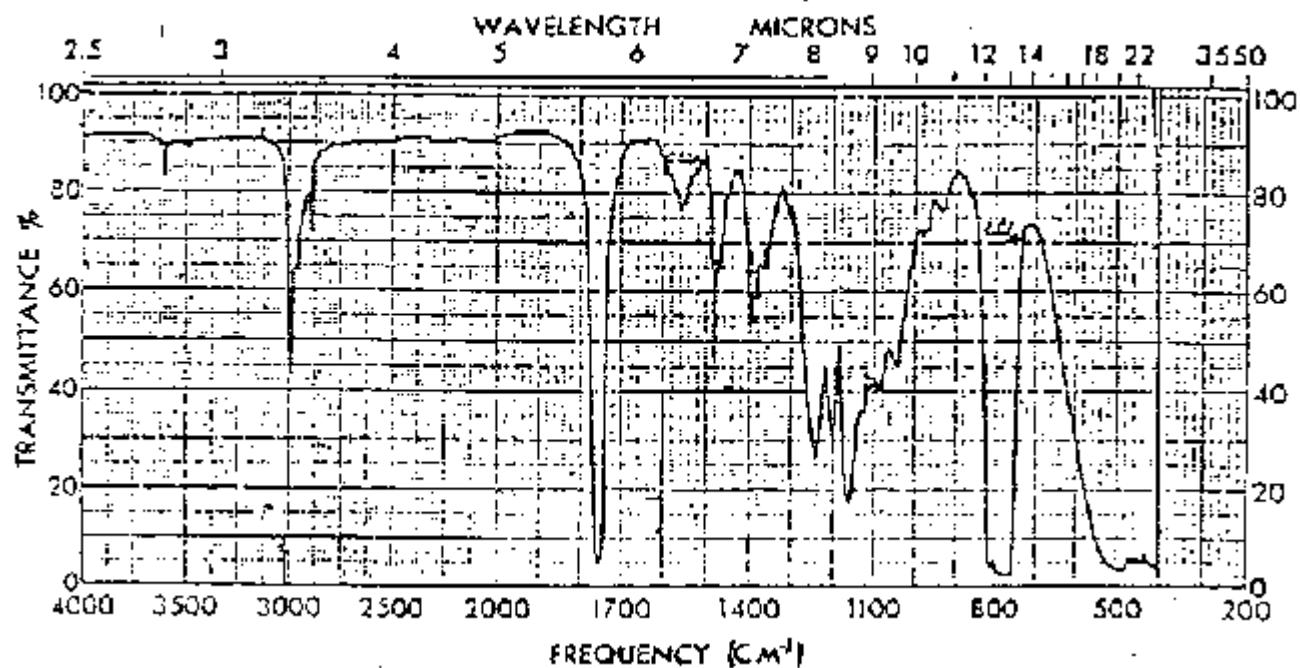
<u>Triacetin</u>	Test by the following <i>gas chromatographic</i> procedure <u>Apparatus:</u> Gas chromatograph equipped with a flame ionization detector Column: Stainless steel, 1.5 m, 3.2 mm i.d.
	<u>Preparation of sample:</u> Dilute the sample by adding an equal volume of carbon disulfide.

Conditions
 Stationary phase: SE-30, 3%
 Solid phase: Chromosorb AW-DMCS, 80-100 mesh
 Carrier gas: Helium
 Flow rate: 20 ml/min
 Temperatures
 - Column: Programmed at 10° per min from 100° to 300° immediately after injection of the sample
 - Injector: 300°
 Injected volume: 1 µl

METHOD OF ASSAY	Using the saponification value, calculate the percentage of C ₄₀ H ₆₂ O ₁₉ by the formula:
	$\frac{SV \times 10586}{56.1} \times 100$

where
SV = saponification value

Infrared spectrum Sucrose acetate isobutyrate



SALTS of FATTY ACIDS

Prepared at the 33rd JECFA (1988), published in FNP 38 (1988) and in FNP 52 (1992). Metals and arsenic specifications revised at the 55th JECFA (2000). An ADI 'not specified' was established at the 33rd JECFA (1988)

SYNONYMS

INS No. 470

DEFINITION

These products consist of calcium, potassium or sodium salts of commercial myristic, oleic, palmitic, stearic, acids or mixtures of these acids from edible fats and oils. The article of commerce can be further specified by:

- saponification value,
- solidification point for the fatty acids obtained from the salts,
- iodine value,
- residue on ignition including assay of the cation, and
- moisture content

Assay

Not less than 95% total fatty acid salts, dry weight basis

DESCRIPTION

Hard, white or faintly yellowish, somewhat glossy and crystalline solids or semi-solids or white or yellowish-white powder

FUNCTIONAL USES

Anticaking agent, emulsifier

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Potassium and sodium salts are soluble in water and ethanol; calcium salts are insoluble in water, ethanol and ether

Test for cations

Heat 1 g of the sample with a mixture of 25 ml of water and 5 ml of hydrochloric acid. Fatty acids are liberated, floating as a solid or oil layer on the surface which is soluble in hexane. After cooling, aqueous layer is decanted and evaporated to dryness. Dissolve the residue in water and *test for the appropriate cation*.

Fatty acid composition

Using the Method of Assay, identify the individual fatty sample. The fatty acid(s) in primary abundance should conform to those declared on the label of the product

PURITY

Free fatty acids

Not more than 3%

Measure free fatty acids as directed in the method *Free Fatty Acids*. Compute free fatty acid content using an equivalence factor (e) equal to 1/10th the molecular weight of the salt.

Unsaponifiable matter

Not more than 2%

See description under TESTS

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

Unsaponifiable matter

Unsaponifiable matter is the whole of the products present in a fatty substance which, after saponification thereof with an alkaline hydroxide and extraction by a specified solvent, remains non-volatile under defined conditions of the test.

It includes lipids of natural origin such as sterols, higher aliphatic alcohols, pigments and hydrocarbons as well as any foreign matter non-volatile at 103° which may be present.

Weigh to the nearest 0.01 g about 5 g of the well-mixed sample into a 250 ml round-bottomed flask. Add 50 ml of approximately 0.5N potassium hydroxide solution and some pumice, attach a reflux condenser, and boil gently for 1 h. Stop heating. Add 100 ml of distilled water through the top of the condenser and swirl.

After cooling, transfer the solution to a separatory funnel. Rinse the flask and the pumice several times with diethyl ether (100 ml in all) and pour this into the separatory funnel. Stopper and shake vigorously for 1 min, periodically releasing pressure by inverting the separating funnel and opening the stopcock.

Allow to stand until there is complete separation of the two phases. Then draw off the soap solution as completely as possible into a second separating funnel.

Extract the aqueous ethanolic soap solution twice more, each time in the same way with 100 ml of diethyl ether. Combine the three ether extracts in one separating funnel containing 40 ml of water.

Gently rotate the separating funnel containing the combined extracts and the 40 ml water. Violent agitation at this stage may result in troublesome emulsions. Allow the layers to separate completely and draw off the lower aqueous layer. Wash the ethereal solution twice more with 40 ml portions of water, shaking vigorously each time and discarding the lower aqueous layers after separation. Draw off each washing solution up to 2 ml, then rotate the separating funnel around its axis, wait some min to give the last remainders the opportunity for collection and draw off the collected remainders, close stopcock when ether starts to pass the bore of the stopcock.

Wash the ethereal solution successively with 40 ml of 0.5 N potassium hydroxide solution, 40 ml of water, and again with 40 ml of potassium hydroxide solution, then at least twice more with 40 ml of water. Continue to wash with water until the wash-water no longer gives a pink colour on the addition of a drop of phenolphthalein solution.

Transfer the ethereal solution quantitatively a little at a time through the top of the separating funnel into a flask previously dried and weighed to the nearest 0.0001 g.

Evaporate the solvent by distillation on a boiling-water bath.

Add 5 ml of acetone and remove the volatile solvent completely in a gentle current of air, holding the flask obliquely while turning it in a boiling-water bath.

Dry the residue at 103±2° for 15 min, placing the flask in an almost horizontal position. Cool in a desiccator and weigh to the nearest 0.0001 g. Repeat the drying for successive 15 min periods until the loss of weight between two successive weighings is less than 0.0015 g.

Note: If constant mass is not obtained after three periods of drying, the unsaponifiable matter is probably contaminated.

After weighing the residue dissolve it in 4 ml of diethyl ether and then add 20 ml of ethanol previously neutralized to a faint pink colour, using phenolphthalein TS as indicator. Titrate with accurately standardized 0.1N ethanolic potassium hydroxide solution (prepare an approx. 1 N ethanolic solution by dissolving 60 g of potassium hydroxide in 50 ml of water and making up to 1 liter with ethanol; dilute this solution 1:10 with ethanol) to the same final colour.

Correct the weight of the residue for the free acidity content of the blank. Calculate the unsaponifiable matter, in % (m/m) from:

$$\frac{100 \times (m_1 - 0.281 \times T \times V)}{m}$$

where

m = the mass, in g, of the test portion

m₁ = the mass, in g, of the residue

V = the number of ml of the standardized potassium hydroxide solution used

T = the exact normality of the potassium hydroxide solution used

METHOD OF ASSAY

Principle:

Saponification of the salts and esterification by methanol of the fatty acids in the presence of boron trifluoride, alkaline methanol. Gas liquid chromatography of the fatty acid methyl esters.

Part A - Preparation of the fatty acid methyl esters

Apparatus

- 50 and 100 ml ground-necked round-bottom flasks.
- Reflux condenser, 20 to 30 cm effective length, with ground joint appropriate to the flask.
- 250 ml separating funnels.
- Inlet tube for passing nitrogen.
- Test tubes with ground glass stoppers.
- Graduated pipette, capacity at least 10 ml, fitted with a rubber bulb or automatic pipette.

- Boiling chips (fat-free).

Reagents

- Heptane, chromatographic quality (Notes 2 and 4)
- Redistilled light petroleum (b.p. 40-60°), bromine value less than 1, residue free, or hexane (Note 2)
- Sodium sulfate, anhydrous
- Sodium hydroxide, appropriately 0.5 N methanolic solution: Dissolve 2 g of sodium hydroxide in 100 ml methanol containing not more than 0.5% (m/m) of water. When the solution has to be stored for a considerable time, a small amount of white precipitate of sodium may be formed; this has no effect on the preparation of the methyl esters.
- Boron trifluoride, 12 to 25% (m/m) methanolic solution. 14 and 50% solutions are available commercially (Note 2).
Caution: Boron trifluoride is poisonous. For this reason it is not recommended that the analyst prepare the methanolic solution of boron trifluoride from methanol and boron trifluoride (Note 3).
- Sodium chloride, saturated aqueous solution
- Methyl red, 1 g/l solution in 60% (v/v) ethanol
- Nitrogen, containing less than 5 mg/kg of oxygen

Procedure

Because of the toxic character of boron trifluoride, the following operations are best performed under a ventilated hood. All glassware must be washed with water immediately after use

Dry the sample at 105° to constant weight, using 2 hour increments of heating. Accurately weigh about 350 mg of dried sample. Sample sizes larger or smaller than 350 mg may be taken, however, the size of flask and quantities of reagents should conform to the following table:

<i>weight of sample (mg)</i>	<i>Flask capacity (ml)</i>	<i>0.5N NaOH (ml)</i>	<i>Methanolic soln. of BF₃ (ml)</i>
100-250	50	4	5
250-500	50	6	7
500-700	100	8	9
750-1000	100	10	12

Place the desired amount of prepared fatty acids in the appropriate flask. Add the appropriate amount of methanolic boron trifluoride solution. Boil for 2 min.

Add 2 to 5 ml of heptane (Note 4) (the precise amount does not affect the reaction) to the boiling mixture through the top of the condenser and continue boiling for 1 minute.

Withdraw the source of heat, and then remove the condenser. Add a small portion of saturated sodium chloride solution and shake the flask gently by rotating it several times.

Add more saturated sodium chloride solution to the flask in order to bring the level of liquid into the neck of the flask. Allow to separate and transfer about 1 ml of the upper layer (heptane solution) into a ground-necked test tube and add a little anhydrous sodium sulfate to remove any trace of water. If the sample taken was 350 mg, this solution contains about 7-17 percent

of methyl esters and may be injected directly onto the column for gas-liquid chromatography. In the other cases dilute the heptane solution to obtain a 5-10% concentration of methyl esters (Note 6).

In order to recover the whole of the dry esters, transfer the saline solution and the heptane layer into a separating funnel. Separate the layers. Extract the saline solution twice with 50 ml portions of light petroleum. Combine the heptane solution and the two extracts, and wash them with 20-ml portions of water until free from acid (methyl red indicator). Dry over anhydrous sodium sulfate, filter and evaporate the solvent over a boiling-water bath in a current of nitrogen (Notes 6 and 7). For samples under 500 mg it is desirable to reduce proportionately the volumes of solvent and water used.

Alternative methods which do not involve the use of boron trifluoride are available. In the general method the methylation reagents, 0.5 N methanolic sodium hydroxide and 12-25 % methanolic boron trifluoride, may be substituted with:

- 1 N Methanolic potassium hydroxide, (reacted with fatty substance in the presence of excess low moisture content methanol);
- Sodium methylate solution (prepared by dissolving 1 g of sodium metal in 100 ml of low-moisture content methanol).

Notes

1. If the unsaponifiable matter interferes, dilute the saponified solution with water and eliminate the unsaponifiable matter by extraction with diethyl ether or hexane. Acidify the aqueous soap solution and separate the fatty acids. Prepare the methyl esters from these as described.

2. In the course of the gas-liquid chromatography of the methyl esters, certain reagents, particularly the methanolic boron trifluoride solution may produce adventitious peaks on the graph (in the region of C₂₀-C₂₂ esters in the case of methanolic boron trifluoride solutions). Consequently any new batch of reagent should be checked by preparing the methyl esters of pure oleic acid, and chromatographing them; if an extraneous peak appears, the reagent should be rejected. The various reagents must not give peaks interfering with those of methyl esters of fatty acids during the gas-liquid chromatography.

The methanolic solutions of boron trifluoride must be stored in a refrigerator.

3. If it is absolutely unavoidable to prepare a solution of boron trifluoride from gaseous boron trifluoride, the recommended method is:

Weigh a 2 l flask containing 1 l of methanol. Cool in an ice bath, and with the flask still in bath, bubble BF₃ from a cylinder through a glass tube into the methanol until 125 g BF₃ is absorbed. Perform the operation in a fume-cupboard. BF₃ must be flowing through the glass tube before it is placed in and until it is removed from methanol to prevent liquid from being drawn into the gas cylinder valve system. Gas should not flow so fast that white fumes emerge from flask.

This reagent is stable for 2 years.

4. If fatty acids containing twenty or more carbon atoms are absent, hexane may be substituted for heptane (mixture of pure C₇ isomers tested by gas-liquid chromatography).

5. If the suggested amount of sample is not available, 10 mg, or even less, may be used, provided that the amounts of reagents and the size of the containers are reduced proportionally.

6. Preferably, the solutions of methyl esters should be analysed as soon as possible. If necessary, the heptane solution containing the methyl esters may be stored under an inert gas in a refrigerator. In the case of prolonged storage, it is desirable to protect the methyl esters from autoxidation by adding to the solution an antioxidant in such a concentration as will not interfere with the subsequent analysis, e.g. 0.05 g/l of EHT (2,6-di-tertbutyl 4-methyl phenol).

If necessary, the dry and solvent-free methyl esters may be stored 24 h under inert gas in a refrigerator, or longer in sealed tube under vacuum in a deep-freeze.

7. There is some risk of losing part of the most volatile methyl esters if the evaporation of the solvent is prolonged, or if the current of nitrogen is too vigorous.

For infrared spectroscopy, elimination of the solvent must be as complete as possible.

For gas liquid chromatography remove solvent.

Part B - Gas-liquid chromatography of fatty acid methyl esters

Apparatus

The instruction given relate to the ordinary equipment used for gas-liquid chromatography, employing a packed column and a flame-ionization detector (Note 1). Any apparatus giving efficiency and resolution for the specific fatty substance is suitable.

Gas liquid chromatography

Injection system: The injection system should have the least dead space possible. If possible, it should be heated to a temperature 20 to 50° higher than that of the column.

Oven: The oven should be capable of heating the column to at least 220° and of maintaining the desired temperature to within 1°.

If programmed heating is to be employed, an apparatus with a twin column is recommended.

Packed column:

- Column: The column must be constructed of a material inert to the substances to be analysed: glass or, failing this, stainless steel (Note 2); Length: 1 to 3 m, a relative short column should be used when long-chain acids (C_{20+}) are present. For the determination of C_4 and C_6 fatty acids, a 2-m column is recommended; Internal diameter: 2 to 4 mm.

Packing

- Support: Acid-washed and silanized diatomaceous earth, or other suitable inert support with a narrow range (25 µm) of grain size between the limits 125-200 µm, the average grain size being related to the internal diameter and length of the column.

- Stationary phase: Polyester type of polar liquid (e.g. diethylene glycol polysuccinate, butanediol polysuccinate, ethylene glycol polyadipate ...) or

any other liquid (e.g. cyanosilicones ...) meeting the requirements below. The stationary phase should amount to 5 to 20% of the packing. A non-polar stationary phase can be used for certain separations

- Conditioning the newly prepared column: The column being disconnected from the detector, if possible, heat the oven gradually to 185° and pass a current of inert gas through the freshly prepared column at a rate of 20 - 60 ml/min for at least 16 h at this temperature, and for 2 h more at 195°.

Detector : The manipulations described below relate to the use of a flame ionization detector (Note 1).

Syringe: Syringe, maximum capacity 10 µl, graduated in 0.1 µl.

Recorder

If the recorder curve is to be used to calculate the composition of the mixture analysed, an electronic recorder of high precision is required. It should be compatible with the apparatus used. The characteristics of the recorder should be:

- Rate of response below 1.5 sec, preferentially below 1 sec (the rate of response is the time taken for the recording pen to pass from 0 to 90% following the momentary introduction of a 100% signal)
- Breadth of the paper: 25 cm minimum
- Paper speed: 25-100 cm/h

Integrator or Calculator (Optional)

Rapid and accurate calculation can be performed with the help of an electronic integrator or calculator. This must give a linear response with adequate sensitivity, and the correction for deviation of the base-line must be satisfactory.

Reagents

- Carrier gas: Inert gas (nitrogen, helium, argon ...) thoroughly dried and containing less than 10 mg/kg of oxygen.
- Auxiliary gases: Hydrogen (99.9% min.) free from organic impurities, air or oxygen.
- Reference standards: A mixture of methyl esters, or the methyl esters of an oil, of known composition, preferably similar to that of the fatty matter to be analysed.

Procedure

Conditions of Test

Determining optimal operating conditions

As a rule, the figures shown in Table 1 and 2 below, will lead to the desired results.

Table 1

<u>Internal diameter of column</u>	<u>Carrier gas supply</u>
2 mm	15-25 ml/min
3 mm	20-40 ml/min
4 mm	40-60 ml/min

Table 2

<u>Concentration of stationary phase</u>	<u>Temperature</u>
--	--------------------

5 %	175°
10 %	180°
15 %	180°
20 %	185°

When the apparatus allows it, the injector should be at a temperature of about 200°C and the detector at a temperature equal to, or higher than, that of the column.

The flow of hydrogen to the flame-ionization detector is, as a rule, about half that of the carrier gas, and the flow of oxygen about 5 to 10 times that of the hydrogen.

Determining the efficiency and the resolution (Optional)

Carry out the analysis of a standard of methyl stearate. Choose the size of the sample, the temperature of the column and the carrier gas flow so that the maximum of the methyl stearate peak is recorded about 15 min after the solvent peak, and rises to three-quarters of the full scale

- Analysis

The sample for examination shall be 0.1 to 2 µl of the heptane solution of methyl esters obtained according to Part A. In the case of esters not in solution, prepare an approximate 10% solution in heptane and inject 0.1 to 1 µl of this.

As a rule, the operating conditions will be those defined above.

Nevertheless, it is possible to work with a lower column temperature where the determination of acids below C₁₂ is required, or at higher temperature when determining fatty acids above C₂₀.

On occasion, it is possible to employ temperature programming in both the previous cases. If the sample contains the methyl esters of fatty acids below C₁₂, it is necessary to inject the sample at 100° (or at 50-60° if butyric acid is present) and immediately to raise the temperature at a rate of 4-8°/min to the optimum. In some cases the two procedures can be combined: after the programmed heating, continue the elution at a constant temperature until all the components have been eluted. If the instrument does not operate with programme heating, work at two fixed temperatures between 100° and 195°.

Expression of the results

Qualitative Analysis

Analyse the reference standard mixture of known composition in the same operating conditions as those employed for the sample, and measure the retention distances (or retention times) for the constituent fatty esters. Using a semi-logarithmic paper, construct the graphs showing the logarithm of the retention distance (or retention time) as a function of the number of carbon atoms of the acids; in isothermal conditions the graphs for straight chain esters of the same degree of unsaturation should be straight lines. These straight lines are approximately parallel.

Identify the peaks for the sample from these graphs, if necessary by interpolation. It is necessary to avoid conditions such that "masked peaks" exist, i.e. where the resolution is insufficient to separate two components.

Quantitative Analysis

Determination of the composition

Apart from exceptional cases, use the method of area normalization, i.e. assume that the whole of the components of the sample are represented on the chromatogram, so that the total of the areas under the peaks represents 100 percent of the constituents (total elution).

By appropriate standardization procedures (using a reference standard mixture or an internal standard), determine the total weight of fatty acids in the dried sample. Calculate the content of Fatty Acid Salts for the specific cation(s) in the sample. the content of total Fatty Acid Salts shall be not less than 95% of the dried sample. In addition if there are specifications on the label of the Fatty Acid Salt for content of individual fatty acids, the sample must comply with these specifications.

Notes

1. A gas-liquid chromatograph employing a catharometer (working on thermal conductivity changes) may be used. Operating conditions must then be modified as follows:

Column

- length: 2 to 4 m
- internal diameter: 4 mm
- support: grain size between 160 and 200 µm
- stationary phase: 15 to 25%

Carrier gas: helium, or failing this, hydrogen, with a content of oxygen as low as possible. No auxiliary gases.

Flow rate: usually between 60 and 80 ml/min

Temperatures

- Injector: 40° to 60° above that of the column
- Column: 180° to 200°

Quantitative analysis: correction of factors derived from the analysis of a reference mixture of esters of known composition, determined under operating conditions identical with those used for the sample, must be used.

2. If polyunsaturated components with more than three double bonds are present, they may decompose in a stainless-steel column.

POTASSIUM SULFITE

Prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7 (1999), superseding tentative specifications prepared at the 51st JECFA (1998), published in FNP 52 Add 6 (1998). Group ADI 0-0.7 mg/kg bw as SO₂ for sulfites established at the 51st JECFA in 1998.

SYNOMYS INS No. 225

DEFINITION

Chemical names Potassium sulfite

C.A.S. number 10117-38-1

Chemical formula K₂SO₃

Formula weight 158.25

Assay Not less than 90.0%

DESCRIPTION White, odourless, granular powder

FUNCTIONAL USES Antibrowning agent, antioxidant, preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in water; slightly soluble in ethanol

Test for potassium (Vol. 4) Passes test

Test for sulfite (Vol. 4) Passes test

PURITY

Alkalinity Between 0.25 and 0.45% as K₂CO₃
Dissolve 1 g of the sample in 20 ml of water and add 25 ml of 3% hydrogen peroxide, previously neutralized to methyl red TS. Mix thoroughly, cool to room temperature, and titrate with 0.02 N hydrochloric acid. Perform a blank determination using 25 ml of neutralized hydrogen peroxide solution. Each ml of 0.02 N hydrochloric acid is equivalent to 1.38 mg of K₂CO₃.

Iron (Vol. 4) Not more than 10 mg/kg
Proceed as directed in the Limit Test using 0.5 ml of Iron Standard Solution (5 µg Fe) in the control

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an atomic absorption technique 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."

Selenium

Not more than 5 mg/kg

See description under TESTS

TESTS

PURITY TESTS

Selenium

Reagents:

Hydrochloric acid, hydrazinium sulfate, standard selenium solution (100 µg Se/ml)

Procedure

Weigh 2.0 ± 0.1 g of sample and transfer to a 50-ml beaker. Add 10 ml water, 5 ml hydrochloric acid and boil to remove SO_2 .

Into a second beaker, weigh 1.0 ± 0.1 g of sample, add 0.05 ml standard selenium solution and proceed as above.

To each beaker add 2 g hydrazinium sulfate and warm to dissolve. Let stand for 5 min. Dilute the contents of each beaker to 50 ml in a Nessler tube and compare the colour of the two solutions. The sample should be less pink than the sample with the added standard.

METHOD OF ASSAY

Weigh accurately about 0.75 g of the sample and dissolve in a mixture of 100 ml of 0.1 N iodine and 5 ml of dilute hydrochloric acid TS. Titrate the excess iodine with 0.1 N sodium thiosulfate, adding starch TS as the indicator. Each ml of 0.1 N iodine is equivalent to 7.912 mg of K_2SO_3 .

DISODIUM HYDROGEN PHOSPHATE

Prepared at the 19th JECFA (1975), published in NMRS 55B (1976) and in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002). A group MTDI of 70 mg/kg bw, as phosphorus from all food sources, was established at the 26th JECFA (1982)

SYNONYMS

Dibasic sodium phosphate, disodium phosphate, disodium acid phosphate, secondary sodium phosphate; INS No. 339(ii)

DEFINITION

Chemical names Disodium hydrogen phosphate, disodium hydrogen orthophosphate, disodium hydrogen monophosphate

C.A.S. number 7558-79-4

Chemical formula Anhydrous: Na₂HPO₄
Hydrated: Na₂HPO₄ · x H₂O

Formula weight 141.98 (anhydrous)

Assay Not less than 98.0% after drying

DESCRIPTION

Anhydrous: White, hygroscopic, odourless powder
Dihydrate: White crystalline, odourless solid
Heptahydrate: White, odourless, efflorescent crystals or granular powder
Dodecahydrate: White, efflorescent, odourless powder or crystals

FUNCTIONAL USES Emulsifier, texturizer, buffer

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in water; insoluble in ethanol

pH (Vol. 4) 9.0- 9.6 (1 in 100 soln)

Test for sodium (Vol. 4) Passes test

Test for phosphate (Vol. 4) Passes test

Test for orthophosphate (Vol. 4) Dissolve 0.1 g of the sample in 10 ml water, acidify slightly with dilute acetic acid TS, and add 1 ml of silver nitrate TS. A yellow precipitate is formed.

PURITY

Loss on drying (Vol. 4) Anhydrous: Not more than 5.0% (40°, 3 h, then 105°, 5 h)

Dihydrate: Not more than 22.0% (40°, 3 h, then 105°, 5 h)

Heptahydrate: Not more than 50.0% (40°, 3 h, then 105°, 5 h)

Dodecahydrate: Not more than 61.0% (40°, 3 h, then 105°, 5 h)

Water insoluble substances Not more than 0.2%
(Vol. 4)

Fluoride (Vol. 4) Not more than 50 mg/kg (Method I or III)

Arsenic (Vol. 4) Not more than 3 mg/kg (Method II)

Lead (Vol. 4) Not more than 4 mg/kg
Determine using an atomic absorption technique 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

Into a 250-ml beaker transfer about 6.5 g of the dried sample accurately weighed. Add 50 ml of 1N hydrochloric acid and 50 ml of water, and stir until the sample is completely dissolved. Place the electrodes of a suitable pH meter in the solution and titrate the excess acid with 1N sodium hydroxide to the inflection point occurring at about pH 4. Record the buret reading and calculate the volume (A) of 1N hydrochloric acid consumed by the sample. Continue the titration with 1N sodium hydroxide until the inflection point occurring at about pH 8.8 is reached, record the buret reading, and calculate the volume (B) of 1N sodium hydroxide required in the titration between the two inflection points (pH 4 to pH 8.8). When (A) is equal to, or less than, (B), each ml of the volume (A) of 1N hydrochloric acid is equivalent to 142.0 mg of Na_2HPO_4 . When (A) is greater than (B), each ml of the volume 2(B) -(A) of 1N sodium hydroxide is equivalent to 142.0 mg of Na_2HPO_4

QUILLAIA EXTRACT (TYPE 1)

Specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add 11 (2003). The previous tentative specifications for Quillaia extracts prepared at the 57th JECFA (2001), published in FNP 52 Add 9 (2001), are replaced by these and by separate specifications for "Quillaia extract (Type 2)". A group ADI of 0-1 mg quillaia saponins /kg bw for Quillaia Extracts Types 1 & 2 was established at 65th JECFA (2005)

SYNOMYS

Quillaia extract, Soapbark extract, Quillay bark extract, Bois de Panama, Panama bark extract, Quillai extract; INS No. 999(i)

DEFINITION

Quillaia extract (Type 1) is obtained by aqueous extraction of the milled inner bark or of the wood of pruned stems and branches of *Quillaia saponaria* Molina (family Rosaceae). It contains triterpenoid saponins (quillaia saponins, QS) consisting predominantly of glycosides of quillaic acid. Polyphenols and tannins are major components and some sugars and calcium oxalate will be present.

Quillaia extract (Type 1) is available commercially as liquid product or as spray-dried powder that may contain carriers such as lactose, maltitol or maltodextrin. The liquid product is usually preserved with sodium benzoate or ethanol.

C.A.S. number

68990-67-0

Formula weight

Monomeric saponins range from ca. 1800 to ca. 2300, consistent with a triterpene with 8-10 monosaccharide residues

Assay

Saponin content: not less than 20 % and not more than 26 % on the dried basis

DESCRIPTION

Red-brownish liquid or light brown powder with a pink tinge

FUNCTIONAL USES

Emulsifier, foaming agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Very soluble in water, insoluble in ethanol, acetone, methanol and butanol

Foam

Dissolve 0.5 g of powder extract in 9.5 g of water or 1 ml of liquid extract in 9 ml of water. Add 1 ml of this mixture to 350 ml of water in a 1000-ml graduated cylinder. Cover the cylinder, vigorously shake it 30 times, and allow settling. Record the foam level (ml) after 30 min. Typical values are 150 ml of foam

Chromatography

Determine as in METHOD OF ASSAY. The retention time of major peak of the sample corresponds to the major saponin peak (QS-18) of the standard.

Colour and turbidity

Powder form only: Dissolve 0.5 g in 9.5 g of water. The solution is not turbid. Determine the absorbance of the solution against water at 520 nm. The absorbance is less than 1.2.

PURITY

<u>Water</u> (Vol. 4)	Powder form: not more than 6% (Karl Fischer Method)
<u>Loss on drying</u> (Vol. 4)	Liquid form: 50 to 80% (2 g, 105°, 5 h)
<u>pH</u> (Vol. 4)	3.7 -5.5 (4 % solution)
<u>Ash</u> (Vol. 4)	Not more than 14% on a dried basis (use 1.0 g for powder samples; for liquid samples, use the residue from loss on drying)
<u>Tannins</u>	Not more than 8% on a dried basis See description under TESTS
<u>Lead</u> (Vol. 4)	Not more 2 mg/kg. Determine using an atomic absorption technique appropriate to the specified level. The selection of the sample size and method of sample preparation may be based on the principles of the method described in FNP 5, "Instrumental Methods".

TESTS

PURITY TESTS

Tannins Weigh either 3.0 g of the powder form or an equivalent amount of liquid sample, accounting for solids content determined from loss on drying. Dissolve in 250 ml of water. Adjust the pH to 3.5 with acetic acid. Dry 25 ml of this solution at 105° for 5 h and determine the weight of the dried solid, in g (W_i). Mix 50 ml of the solution with 360 mg of polyvinyl polypyrrolidone. Stir the solution for 30 min at room temperature; then centrifuge at 800 × g. Recover the supernatant and dry this solution at 105° (5 h). Weigh the recovered solid (W_f , in g). The percentage of tannins in the sample is:

$$\% \text{ tannins (dried basis)} = 100 \times (W_i - W_f/2) / W_i$$

METHOD OF ASSAY

Principle:

The saponins QS-7, QS-17, QS-18 and QS-21 are separated by reversed phase HPLC and their quantitation is used as an indicator for total saponins levels in Quillaia extract (Type 1).

Sample preparation:

Powders: Weigh 0.5 g of sample and dissolve in 9.5 g of water. Filter through a 0.2 µm filter.

Aqueous extracts (~ 550 mg solids/ml): Weigh 1 g of sample and dilute with 9 g of water. Filter through a 0.2 µm filter.

In either case, the sample volume is ca. 10 ml.

Standard preparation:

Weigh 1.5 g of purified saponins (SuperSap, Natural Response, Chile; Quil-A, Superfos, Denmark or similar, containing a known saponin content) and dissolve in 100 ml of water. Filter through a 0.2 µm filter.

High performance liquid chromatography (HPLC):

HPLC conditions:

Column: Vydac 214TP54 (4.6 x 250 mm length, 5 µm pore) or equivalent

Column temperature: room temperature

Pump: gradient

Solvent A: 0.15% trifluoroacetic acid in HPLC-grade water.

Solvent B: 0.15% trifluoroacetic acid in HPLC-grade acetonitrile.

Gradient: Time(min) % solvent A % solvent B

0	70	30
---	----	----

40	55	45
----	----	----

45	70	30
----	----	----

Flow rate: 1 ml/min

Detection wavelength: 220 nm

Injection volume: 20 µl

Calculation:

The concentration of saponins, C_{sap} , in mg/ml, in the solution prepared as directed under sample preparation is:

$$C_{\text{sap}} = (A_{\text{sample}}/A_{\text{standard}})C_{\text{Standard}}$$

where C_{Standard} (mg/ml) is the saponins concentration of the standard injected (e.g., $C_{\text{Standard}} = 13.5$ mg/ml if the saponin content of 1.5 g of standard sample is 90 %) and A_{sample} and A_{standard} are the sums of the peak areas attributed to the four principle saponins in the sample preparation and in the standard preparation, respectively, as noted in the figure. (Tannins and Polyphenols will elute before the saponins. The peaks due to the saponins will appear after the major peak due to the polyphenols - see figure.)

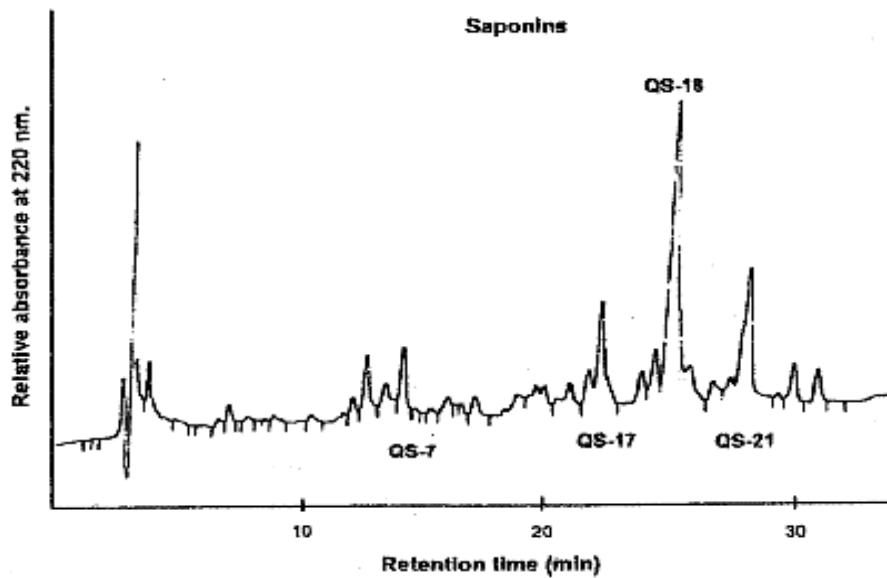
The percentage of saponins in the test sample is:

$$\% \text{ Saponins} = 100 \times C_{\text{sap}}/(0.1W_{\text{sample}})$$

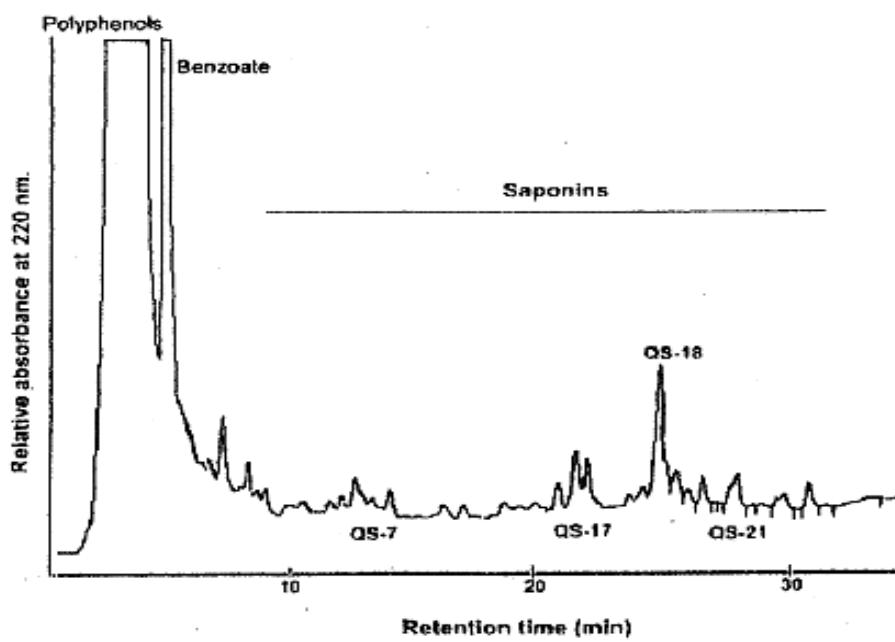
where W_{sample} is the weight of the sample (mg) taken for the sample preparation and 0.1 is the inverse of the sample volume, 10 ml.

Appendix

Chromatogram of Standard (15 mg solids/ml equivalent to 13.5 mg saponins/ml).



Chromatogram of Quillaia extract (Type 1) (55 mg solids/ml)



MONOGLYCERIDE CITRATE

Prepared at the 18th JECFA (1974), published in FNP 4 (1978) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). ADI not evaluated at the 18th JECFA (1974)

SYNONYMS	Citric acid ester of glyceryl monooleate
DEFINITION	A mixture of glyceryl monooleate and its citric acid monoester, manufactured by the reaction of glyceryl monooleate with citric acid under controlled conditions

Structural formula	$\begin{array}{c} \text{CH}_2-\text{OR}_1 \\ \\ \text{CH}-\text{OR}_2 \\ \\ \text{CH}_2-\text{OR}_3 \end{array}$
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where R₁ represents oleic acid moiety and R₂ and R₃ a citric acid moiety or hydrogen

DESCRIPTION	Soft, white to ivory coloured, waxy solid with a lard-like consistency and a bland odour
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FUNCTIONAL USES Synergist and solubilizer for antioxidants and flavours

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in water; soluble in ethanol

Tests for fatty acids (Vol. 4) Passes tests

Test for citrate (Vol. 4) Passes tests

Test for glycerol (Vol. 4) Passes tests

PURITY

Water (Vol. 4) Not more than 0.2% (Karl Fischer Method)

Sulfated ash (Vol. 4) Not more than 0.3%
Test 1 g of the sample

Acid value (Vol. 4) Not less than 70 and not more than 100

Saponification value (Vol. 4) Not less than 260 and not more than 265

Total citric acid Not less than 14.0 and not more than 17.0%
See description under TESTS

Lead (Vol. 4) Not more than 2 mg/kg

Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

Total citric acid

Standard Solution:

Transfer about 35 mg of sodium citrate dihydrate, accurately weighed, into a 100-ml volumetric flask. Dissolve and dilute to volume with water, and mix. Calculate the concentration (C) in μg per ml of citric acid in the final solution by the formula:

$$C = 1000 \times \frac{0.65333 W}{100}$$

where W is the weight, in mg, of the sodium citrate dihydrate taken, and 0.6533 is a factor converting sodium citrate dihydrate to citric acid.

Sample Solution:

Transfer about 150 mg of the sample accurately weighed, into a saponification flask. Add 50 ml of 4% alcoholic potassium hydroxide solution, and reflux for 1 h. Acidify the reaction mixture with hydrochloric acid to a pH of 2.8 - 3.2, transfer to a 400-ml beaker, and evaporate to dryness on a steam bath. Quantitatively transfer the contents of the beaker into a separator, using no more than 50 ml of water. Extract with three 50-ml portions of petroleum ether (b.p. 30-60°) discarding the extracts. Transfer the water layer to a 100-ml volumetric flask, dilute to volume with water, and mix.

Procedure:

Pipet 2.0 ml each of the Standard Solution and of the Sample Solution into separate 40-ml graduated centrifuge tubes. Add 2 ml of a 1 in 2 sulfuric acid solution and 11 ml of water to each tube. Boil for 3 min, cool, and add 5 ml of bromine TS to each tube. Dilute to the 20-ml mark, allow to stand for 10 min, and centrifuge. Transfer 4.0 ml of each solution into separate 19 x 110-mm test tubes. Add 1 ml of water, 0.5 ml of a 1 in 2 sulfuric acid solution, and 0.3 ml of 1 M potassium bromide, and shake. Add 0.3 ml of 1.5 N potassium permanganate, shake, and allow to stand for 2 min. Add 1 ml of a saturated solution of ferrous sulfate, shake, allow to stand for 2 min. Dilute to 10 ml with water. Add 10.0 ml of n-hexane (previously washed with sulfuric acid, followed by a water wash, and then dried over anhydrous sodium sulfate), shake vigorously for 2 min and centrifuge at a low speed for 1 min. Transfer 5.0 ml of the hexane extract into a 20 x 145-mm tube containing 10.0 ml of sodium sulfide solution (4 g of $\text{Na}_2\text{S} \cdot 9 \text{ H}_2\text{O}$ in 100 ml of water), and shake vigorously briefly (3 oscillations only). Centrifuge the mixture at low speed for 1 min.

Immediately determine the absorbance of each aqueous layer in a 1-cm cell at 450 nm with a suitable spectrophotometer, using a reagent blank in the reference cell. Calculate the quantity, in mg, of citric acid in the sample

taken by the formula:

$$0.1C \propto \frac{A_u}{A_s}$$

C is as defined under Standard Solution, A_u is the absorbance of the final solution from the Sample Solution, and A_s is that of the final solution from the Standard Solution.

CALCIUM DIHYDROGEN PHOSPHATE

Prepared at the 46th JECFA (1996), published in FNP 52 Add 4 (1996) superseding specifications prepared at the 9th JECFA (1965), published in NMRS 40ABC (1967) and in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002). A group MTDI of 70 mg/kg bw, as phosphorus from all food sources, was established at the 26th JECFA (1982)

SYNOMYS

Monobasic calcium phosphate, monocalcium orthophosphate, monocalcium phosphate, calcium biphosphate, acid calcium phosphate, INS No. 341(i)

DEFINITION

Chemical names Calcium dihydrogen phosphate

C.A.S. number Anhydrous: 7758-23-8
Monohydrate: 10031-30-8

Chemical formula Anhydrous: Ca(H₂PO₄)₂
Monohydrate: Ca(H₂PO₄)₂,H₂O

Formula weight Anhydrous: 234.05
Monohydrate: 252.07

Assay Anhydrous: Not less than 16.8% and not more than 18.3% of Ca
Monohydrate: Not less than 15.9% and not more than 17.7% of Ca

DESCRIPTION Hygroscopic white crystals or granules, or granular powder

FUNCTIONAL USES Buffering agent, firming agent, sequestrant, leavening agent, dough conditioner, texturizer, yeast food, and nutrient

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Sparingly soluble in water, insoluble in ethanol

Test for calcium (Vol. 4) Passes test

Test for phosphate (Vol. 4) Passes test

PURITY

Loss on drying (Vol. 4) Monohydrate: Not more than 1% (60°, 3 h)

Loss on ignition (Vol. 4) Anhydrous: Between 14.0 and 15.5% (800°, 30 min)

Fluoride (Vol. 4) Not more than 50 mg/kg
Anhydrous: Determine as directed in Method II
Monohydrate: Proceed as directed under Method IV

<u>Arsenic</u> (Vol. 4)	Not more than 3 mg/kg (Method II)
<u>Lead</u> (Vol. 4)	Not more than 4 mg/kg Determine using an atomic absorption technique 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 a portion of the sample equivalent to about 475 mg of the anhydrous salt and dissolve it in 10 ml of hydrochloric acid TS. Add a few drops of methyl orange TS, and boil for 5 min, keeping the volume and pH of the solution constant during the boiling period by adding hydrochloric acid or water, if necessary. Add 2 drops of methyl red TS and 30 ml of ammonium oxalate TS, then add dropwise, with constant stirring, a mixture of equal volumes of 6N ammonia solution and water until the pink colour of the indicator just disappears. Digest on a steam bath for 30 min, cool to room temperature, allow the precipitate to settle, and filter the supernatant liquid through a sintered-glass crucible, using gentle suction. Wash the precipitate in the beaker with about 30 ml of cold (below 200) wash solution, prepared by diluting 10 ml of ammonium oxalate TS to 1000 ml. Allow the precipitate to settle, and pour the supernatant liquid through the filter. Repeat this washing by decantation three more times. Using the wash solution, transfer the precipitate as completely as possible to the filter. Finally, wash the beaker and the filter with two 10 ml portions of cold (below 200C) water. Place the sintered-glass crucible in the beaker, and add 100 ml of water and 50 ml of cold dilute sulfuric acid (1 in 6). Add from a buret 35 ml of 0.1N potassium permanganate, and stir until the colour disappears. Heat to about 700, and complete the titration with 0.1N potassium permanganate. Each ml of 0.1N potassium permanganate is equivalent to 2.004 mg of Ca.

CARRAGEENAN

Prepared at the 79th JECFA (2014) and published in FAO JECFA Monographs 16 (2014), superseding specifications prepared at the 68th JECFA (2007), published in FAO JECFA Monographs 4 (2007). A group ADI “not specified” for carrageenan and processed Eucheuma seaweed was established at the 57th JECFA (2001).

SYNONYMS

Irish moss gelose (from *Chondrus* spp.); Eucheuman (from *Eucheuma* spp.); Iridophycan (from *Iridaea* spp.); Hypnean (from *Hypnea* spp.); Furcellaran or Danish agar (from *Furcellaria fastigiata*); INS No. 407.

DEFINITION

A substance with hydrocolloid properties obtained from certain members of the class *Rhodophyceae* (red seaweeds).

The principal commercial sources of carrageenans are the following families and genera of the class of *Rhodophyceae*:

Furcellariaceae such as *Furcellaria*
Gigartinaceae such as *Chondrus*, *Gigartina*, *Iridaea*
Hypnaeaceae such as *Hypnea*
Phyllophoraceae such as *Phyllophora*, *Gymnogongrus*, *Ahnfeltia*
Soliariaceae such as *Eucheuma*, *Anatheca*, *Meristotheca*.

Carrageenan is a hydrocolloid consisting mainly of the ammonium, calcium, magnesium, potassium and sodium sulfate esters of galactose and 3,6-anhydrogalactose polysaccharides. These hexoses are alternately linked α -1,3 and β -1,4 in the copolymer. The relative proportions of cations existing in carrageenan may be changed during processing to the extent that one may become predominant.

The prevalent polysaccharides in carrageenan are designated as kappa-, iota-, and lambda-carrageenan. Kappa-carrageenan is mostly the alternating polymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose; iota-carrageenan is similar, except that the 3,6-anhydrogalactose is sulfated at carbon 2. Between kappa-carrageenan and iota-carrageenan there is a continuum of intermediate compositions differing in degree of sulfation at carbon 2. In lambda-carrageenan, the alternating monomeric units are mostly D-galactose-2-sulfate (1,3-linked) and D-galactose-2,6-disulfate (1,4-linked).

Carrageenan is obtained by extraction from seaweed into water or aqueous dilute alkali. Carrageenan may be recovered by alcohol precipitation, by drum drying, or by precipitation in aqueous potassium chloride and subsequent freezing. The alcohols used during recovery and purification are restricted to methanol, ethanol, and isopropanol. Articles of commerce may include sugars for standardization purposes, salts to obtain specific gelling or thickening characteristics, or emulsifiers carried over from drum drying processes.

C.A.S. number

9000-07-1

DESCRIPTION

Yellowish or tan to white, coarse to fine powder that is practically odourless.

FUNCTIONAL USES Thickener, gelling agent, stabilizer, glazing agent.**CHARACTERISTICS****IDENTIFICATION**

<u>Solubility</u> (Vol. 4)	Insoluble in ethanol; soluble in water at a temperature of about 80°, forming a viscous clear or slightly opalescent solution that flows readily; disperses in water more readily if first moistened with alcohol, glycerol, or a saturated solution of glucose or sucrose in water.
<u>Test for sulfate</u>	Dissolve a 100-mg sample in 20 ml of water (with heating if necessary), and add 3 ml of barium chloride TS and 5 ml of hydrochloric acid, dilute TS; filter if a precipitate forms. Boil the solution or the filtrate for 5 min. A white, crystalline precipitate appears.
<u>Test for galactose and anhydrogalactose</u> (Vol. 4)	Proceed as directed in Vol.4 (under "General Methods, Organic Components, Gum Constituents Identification") using the following as reference standards: galactose, rhamnose, galacturonic acid, 3,6-anhydrogalactose, mannose, arabinose and xylose. Galactose and 3,6-anhydrogalactose should be present.
<u>Identification of hydrocolloid and predominant type of copolymer</u>	Add 4 g of sample to 200 ml of water, and heat the mixture in a water bath at 80°, with constant stirring, until dissolved. Replace any water lost by evaporation, and allow the solution to cool to room temperature. It becomes viscous and may form a gel. To 50 ml of the solution or gel add 200 mg of potassium chloride, then reheat, mix well, and cool. A short-textured ("brittle") gel indicates a carrageenan of a predominantly kappa type, and a compliant ("elastic") gel indicates a predominantly iota type. If the solution does not gel, the carrageenan is of a predominantly lambda type.
<u>Infrared absorption</u>	Passes test See description under TESTS
PURITY	
<u>Loss on drying</u> (Vol. 4)	Not more than 12% (105° to constant weight)
<u>pH</u> (Vol. 4)	Between 8 and 11 (1 in 100 suspension)
<u>Viscosity</u>	Not less than 5 cp at 75° (1.5% solution) See description under TESTS
<u>Sulfate</u>	Not less than 15% and not more than 40% (as SO ₄ ²⁻) on the dried basis See description under TESTS
<u>Total ash</u>	Not less than 15% and not more than 40% on the dried basis See description under TESTS.
<u>Acid-insoluble ash</u> (Vol. 4)	Not more than 1% Use the ash from the Total ash test

<u>Acid-insoluble matter</u> (Vol. 4)	Not more than 2% Use 2 g of sample obtained from part (a) of the procedure for sulfate determination.
<u>Residual solvents</u> (Vol. 4)	Not more than 0.1% of ethanol, isopropanol, or methanol, singly or in combination See description under TESTS
<u>Microbiological criteria</u> (Vol. 4)	Initially prepare a 10^{-1} dilution by adding a 50-g sample to 450 ml of Butterfield's phosphate-buffered dilution water and homogenising the mixture in a high-speed blender. Total (aerobic) plate count: Not more than 5000 cfu/g <i>Salmonella</i> spp.: Negative per test <i>E. coli</i> : Negative in 1 g
<u>Arsenic</u> (Vol. 4)	Not more than 3 mg/kg Determine using an AAS (Hydride generation technique) 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").
<u>Lead</u> (Vol. 4)	Not more than 5 mg/kg Determine using an AAS (Electrothermal atomization technique) 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").
<u>Cadmium</u> (Vol. 4)	Not more than 2 mg/kg Determine using an AAS (Electrothermal atomization technique) 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").
<u>Mercury</u> (Vol. 4)	Not more than 1 mg/kg Determine using AAS (Cold vapour generation technique). 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

IDENTIFICATION TESTS

Infrared absorption

Obtain infrared absorption spectra on the gelling and non-gelling fractions of the sample by the following procedure:

Disperse 2 g of the sample in 200 ml of 2.5% potassium chloride solution, and stir for 1 h. Let stand overnight, stir again for 1 h, and transfer into a centrifuge tube. (If the transfer cannot be made because the dispersion is too viscous, dilute with up to 200 ml of the potassium chloride solution.) Centrifuge for 15 min at approximately 1000 x g.

Remove the clear supernatant, resuspend the residue in 200 ml of 2.5% potassium chloride solution, and centrifuge again. Coagulate the

combined supernatants by adding 2 volumes of 85% ethanol or isopropanol (NOTE: Retain the sediment for use as directed below). Recover the coagulum, and wash it with 250 ml of the alcohol. Press the excess liquid from the coagulum, and dry it at 60° for 2 h. The product obtained is the non-gelling fraction (lambda-carrageenan).

Disperse the sediment (retained above) in 250 ml of cold water, heat at 90° for 10 min, and cool to 60°. Coagulate the mixture, and then recover, wash, and dry the coagulum as described above. The product obtained is the gelling fraction (kappa- and iota-carrageenan).

Prepare a 0.2% aqueous solution of each fraction, cast films 0.5 mm thick (when dry) on a suitable non-sticking surface such as Teflon, and obtain the infrared absorption spectrum of each film. (Alternatively, the spectra may be obtained using films cast on potassium bromide plates, if care is taken to avoid moisture).

Carrageenan has strong, broad absorption bands, typical of all polysaccharides, in the 1000 to 1100 cm⁻¹ region. Absorption maxima are 1065 and 1020 cm⁻¹ for gelling and non-gelling types, respectively. Other characteristic absorption bands and their intensities relative to the absorbance at 1050 cm⁻¹ are as follows:

Wave number (cm ⁻¹)	Molecular Assignment	Absorbance relative to 1050 (cm ⁻¹)		
		Kappa	Iota	Lambda
1220-1260	ester sulfate	0.3-1.4	1.2-1.7	1.4-2.0
928-933	3,6-anhydrogalactose	0.2-0.7	0.2-0.4	0-0.2
840-850	galactose-4-sulfate	0.2-0.5	0.2-0.4	-
825-830	galactose-2-sulfate	-	-	0.2-0.4
810-820	galactose-6-sulfate	-	-	0.1-0.3
800-805	3,6-anhydrogalactose-2-sulfate	0-0.2	0.2-0.4	-

PURITY TESTS

Sulfate

Principle

Hydrolysed sulfate groups are precipitated as barium sulfate.

Procedure

(a) Disperse an accurately weighed 15 g sample of commercial product into 500 ml of 60% w/w isopropanol/water at room temperature. Stir gently for 4 h. Filter through ash-free filter paper. Discard the filtrate. Wash the material remaining on the filter paper with two 15-ml portions of 60% isopropanol/water. Dry the material at 105° to constant weight. Approximately 1 g of the dried matter is to be used for part (b). The remainder should be retained for determination of Total ash, Acid-insoluble matter, and viscosity.

(b) Accurately weigh a 1 g sample (W_1) obtained from part (a). Transfer the sample to a 100-ml long-neck round-bottom flask. Add 50 ml of 0.2 N hydrochloric acid. Fit a condenser, preferably one with at least 5 condensing bulbs, to the flask and reflux for 1 h. Add 25 ml of a 10% (by volume) hydrogen peroxide solution and resume refluxing for about 5 h or until the solution becomes completely clear.

Transfer the solution to a 600-ml beaker, bring to a boil, and add dropwise 10 ml of a 10% barium chloride solution. Heat the reaction mixture for 2 h on a boiling water bath. Filter the mixture through ash-free slow-filtration filter paper. Wash with boiling distilled water until the filtrate is free from chloride. Dry the filter paper and contents in a drying oven. Gently burn and ash the paper at 800° in a tared porcelain or silica crucible until the ash is white. Cool in a desiccator.

Weigh the crucible containing the ash. Calculate the percentage sulfate from the weight in g (W_2) of the ash (barium sulfate) using the formula:

$$(W_2/W_1) \times 100 \times 0.4116$$

Total ash

Accurately weigh 2 g of the dried sample (W_1) obtained from part (a) under the procedure for sulfate determination above. Transfer to a previously ignited, tared silica or platinum crucible. Heat the sample with a suitable infrared lamp, increasing the intensity gradually, until the sample is completely charred; continue heating for an additional 30 min. Transfer the crucible with the charred sample into a muffle furnace and ignite at about 550° for 1 h. Cool in a desiccator and weigh. Repeat the ignition in the muffle furnace until a constant weight (W_2) is obtained. If a carbon-free ash is not obtained after the first ignition, moisten the charred spot with a 1-in-10 solution of ammonium nitrate and dry under an infrared lamp. Repeat the ignition step.

Calculate the percentage of total ash of the sample:

$$(W_2/W_1) \times 100$$

Retain the ash for the Acid-insoluble ash test.

Viscosity

Transfer 7.5 g of the dried sample obtained from part (a) under the procedure for sulfate determination into a tared, 600-ml tall-form (Berzelius) beaker, and disperse with agitation for 10 to 20 min in 450 ml of deionized water. Add sufficient water to bring the final weight to 500 g, and heat in a water bath with continuous agitation, until a temperature of 80° is reached (20 - 30 min). Add water to adjust for loss by evaporation, cool to 76-77°, and heat in a constant temperature bath at 75°.

Pre-heat the bob and guard of a Brookfield LVF or LVT viscometer to approximately 75° in water. Dry the bob and guard, and attach them to the viscometer, which should be equipped with a No. 1 spindle (19 mm in diameter, approximately 65 mm in length) and capable of rotating at 30 rpm. Adjust the height of the bob in the sample solution, start the viscometer rotating at 30 rpm and, after six complete revolutions of the viscometer, take the viscometer reading on the 0-100 scale.

If the viscosity is very low, increased precision may be obtained by using the Brookfield UL (ultra low) adapter or equivalent. (Note. Samples of some types of carrageenan may be too viscous to read when a No. 1 spindle is used. Such samples obviously pass the specification, but if a viscosity reading is desired for other reasons, use a No. 2 spindle and take the reading on the 0-100 scale or on the 0-500 scale.)

Record the results in centipoises, obtained by multiplying the reading on the scale by the factor given by the Brookfield manufacturer.

Residual solvents (Vol.4) See Method 1 under Vol. 4. General Methods, Organic Components, Residual Solvents.

Prepare standard, blank, and calibration solutions as directed under Method 1.

Sample Preparation

Disperse 1 ml of a suitable antifoam emulsion, such as Dow-Corning G-10 or equivalent, in 200 ml of water contained in a 1000-ml 24/40 round-bottom distilling flask. Add about 5 g of the sample, accurately weighed, and shake for 1 h on a wrist-action mechanical shaker. Connect the flask to a fractionating column and distil about 100 ml, adjusting the heat so that the foam does not enter the column. Quantitatively transfer the distillate to a 200-ml volumetric flask, fill to the mark with water and shake the flask to mix. Weigh accurately 8.0 g of this solution into an injection vial. Add 1.0 ml of the internal standard solution. Heat at 60° for 10 min and shake vigorously for 10 sec.

CAROB BEAN GUM (CLARIFIED)

Prepared at the 69th JECFA (2008), published in FAO JECFA Monographs 5 (2008), superseding tentative specifications prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006). An ADI "not specified" was established at the 25th JECFA (1981) for carob bean gum.

SYNOMYS

Locust bean gum clarified, INS No. 410

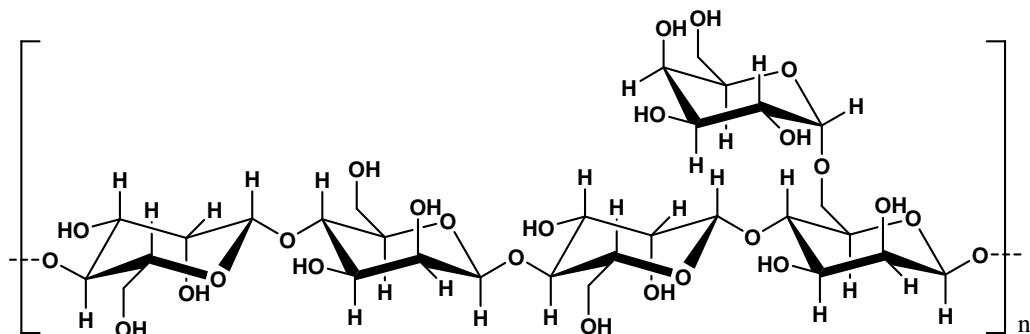
DEFINITION

Primarily the ground endosperm of the seeds from *Ceratonia siliqua* (L.) Taub. (Fam. Leguminosae) mainly consisting of high molecular weight (approximately 50,000-3,000,000) polysaccharides composed of galactomannans; the mannose:galactose ratio is about 4:1. The seeds are dehusked by treating the kernels with dilute sulfuric acid or with thermal mechanical treatments, elimination of the germ, followed by milling and screening of the endosperm to obtain native carob bean gum. The gum is clarified by dispersing in hot water, filtration and precipitation with ethanol or isopropanol, filtering, drying and milling. The clarified carob bean gum does not contain cell wall materials. Clarified carob bean gum in the market is normally standardized with sugars for viscosity and reactivity.

C.A.S. number

9000-40-2

Structural formula



DESCRIPTION

White to yellowish white, nearly odourless powder

FUNCTIONAL USES

Stabilizer, thickener, emulsifier, gelling agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in ethanol

Gel formation Add small amounts of sodium borate TS to an aqueous dispersion of the sample; a gel is formed.

<u>Viscosity</u>	Transfer 2 g of the sample into a 400-ml beaker and moisten thoroughly with about 4 ml of isopropanol. Add 200 ml of water with vigorous stirring until the gum is completely and uniformly dissolved. An opalescent, slightly viscous solution is formed. Transfer 100 ml of this solution into another 400-ml beaker. Heat the mixture in a boiling water bath for about 10 min and cool to room temperature. There is an appreciable increase in viscosity (differentiating carob bean gums from guar gums).
<u>Gum constituents</u> (Vol. 4)	Proceed as directed under Gum Constituents Identification using 100 mg of the sample instead of 200 mg and 1 to 10 µl of the hydrolysate instead of 1 to 5 µl. Use galactose and mannose as reference standards. These constituents should be present.
PURITY	
<u>Loss on drying</u> (Vol. 4)	Not more than 14% (105°, 5 h)
<u>Total ash</u> (Vol. 4)	Not more than 1.2% (800°, 3-4 h)
<u>Acid-insoluble matter</u> (Vol. 4)	Not more than 3.5%
<u>Protein</u> (Vol. 4)	Not more than 1.0% Proceed as directed under Nitrogen Determination (Kjeldahl Method) in Volume 4 (under "General Methods, Inorganic components"). The percentage of nitrogen determined multiplied by 6.25 gives the percentage of protein in the sample.
<u>Starch</u>	To a 1 in 10 solution of the sample add a few drops of iodine TS; no blue colour is produced
<u>Residual solvents</u>	Not more than 1% of ethanol or isopropanol, singly or in combination See description under TESTS
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under "General Methods, Metallic Impurities").
<u>Microbiological criteria</u> (Vol. 4)	Initially prepare a 10 ⁻¹ dilution by adding a 50 g sample to 450 ml of Butterfield's phosphate-buffered dilution water and homogenising the mixture in a high-speed blender. Total (aerobic) plate count: Not more than 5,000 CFU/g <i>E. coli</i> : Negative in 1 g <i>Salmonella</i> : Negative in 25 g Yeasts and moulds: Not more than 500 CFU/g

TESTS

PURITY TESTS

Residual solvents Determine by gas chromatography in Volume 4 (under "Analytical Techniques, Chromatography").

Chromatography conditions

Column: 25% Diphenyl-75% dimethylpolysiloxane (60 m x 0.25 mm i.d., 0.25 µm film) [Aquatic-2 (GL-Sciences Inc.) or equivalent]

Carrier gas: Helium

Flow rate: 1.5 ml/min

Detector: Flame-ionization detector (FID)

Temperatures:

- injector: 280°

- column: Hold for 6 min at 40°, then 40-110° at 4°/min, 110-250° at 25°/min, hold for 10 min at 250°

- detector: 250°

Standard solutions

Solvent standard solution: Transfer 100 mg each of chromatography grade ethanol and isopropanol into a 100-ml volumetric flask containing about 90 ml water and dilute to 100 ml with water.

TBA standard solution: Transfer 100 mg of chromatography grade tertiary-butyl alcohol (TBA) into a 100-ml volumetric flask containing about 90 ml water and dilute to 100 ml with water.

Mixed standard solutions: Transfer 1, 2, 3, 4 and 5 ml of Solvent standard solution into each of five 100-ml volumetric flasks. Add 4 ml of TBA standard solution to each flask and dilute to volume with water.

Sample preparation

Disperse 1 ml of a suitable antifoam emulsion, such as Dow-Corning G-10 or equivalent, in 200 ml of water contained in a 1000-ml 24/40 round-bottom distilling flask. Add about 4 g of the sample, accurately weighed, and shake for 1 h on a wrist-action mechanical shaker. Connect the flask to a fractionating column, and distil about 95 ml, adjusting the heat so that foam does not enter the column. Add 4 ml of TBA standard solution to the distillate and make up to 100 ml with water to obtain the Sample solution.

Standard curves

Inject 1 µl of each Mixed standard solution into the chromatograph. Measure the peak areas for each solvent and TBA. Construct the standard curves by plotting the ratios of the peak areas of each of the solvents/TBA against the concentrations of each solvent (mg/ml) in the Mixed standard solutions.

Procedure

Inject 1 µl of the Sample solution into the chromatograph. Measure the peak areas for each solvent and TBA. Calculate the ratios of the peak areas of each solvent/TBA, and obtain the concentration of each solvent from the standard curves.

Calculate the percentage of each solvent from:

$$\% \text{ Solvent} = (C \times 100/W \times 1000) \times 100$$

where C is the concentration of solvent (mg/ml)
W is weight of sample (g)

CARBOHYDRASE from *SACCHAROMYCES* species

Prepared at the 15th JECFA (1971), published in NMRS 50B (1972) and in FNP 52 (1992) An ADI 'not limited' was established at the 15th JECFA (1971)

SOURCES

Commercial enzyme preparations of carbohydrases (*Saccharomyces*) are produced by the controlled fermentation of a number of species of *Saccharomyces* traditionally used in the manufacture of food.

Active principles

1. β -Fructofuranosidase (invertase, saccharase)
2. β -Galactosidase (lactase)

Systematic names and numbers

1. β -D-Fructofuranoside fructohydrolase (EC 3.2.1.26)
2. β -D-Galactoside galactohydrolase (EC 3.2.1.23)

Reactions catalyzed

1. Hydrolyzes sucrose to a mixture of glucose and fructose.
2. Hydrolyzes lactose to a mixture of glucose and galactose.

DESCRIPTION

White to tan amorphous powders; soluble in water, the solutions usually being light yellow; practically insoluble in alcohol, chloroform and ether.

FUNCTIONAL USES

Enzyme preparation
Used in the manufacture of candy and ice cream and modification of dairy products

GENERAL SPECIFICATIONS

Must conform to the *General Specifications for Enzyme Preparations used in Food Processing* (see Volume Introduction)

CHARACTERISTICS

IDENTIFICATION

Invertase activity

The sample shows invertase activity
An example of determination of invertase: AOAC Official Methods of Analysis, 11th ed., 529 (1970)

β -Galactosidase activity (Vol.4)

The sample shows β -galactosidase activity

SORBITAN MONOLAURATE

Prepared at the 55th JECFA (2000) and published in FNP 52 Add 8 (2000), superseding specifications prepared at the 44th JECFA (1995) and published in FNP 52 Add 3 (1995). A group ADI of 0-25 mg/kg bw for sorbitan esters of lauric, oleic, palmitic, and stearic acid was established at the 26th JECFA (1982).

SYNOMYS

Sorbitan laurate; INS No. 493

DEFINITION

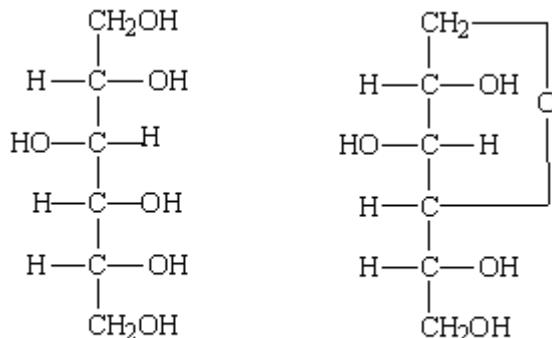
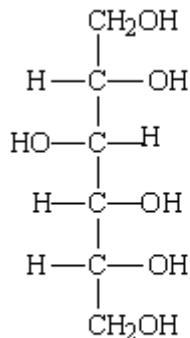
A mixture of the partial esters of sorbitol and its mono- and dianhydrides with edible lauric acid

C.A.S. number

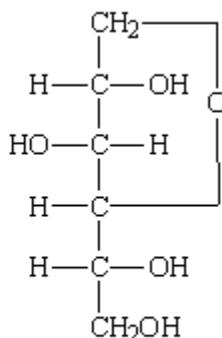
1338-39-2

Structural formula

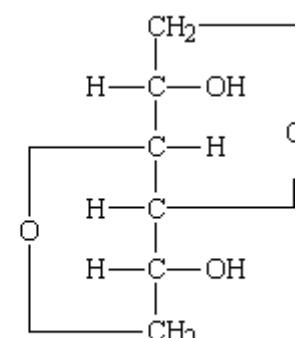
Contains lauric acid esterified with polyols derived from sorbitol including the following types:



Sorbitol



1,4-Sorbitan



Isosorbide

Assay

Saponification of 100 g of the sample yields not less than 36 g and not more than 49 g of polyols, and not less than 56 g and not more than 68 g of fatty acids. The polyol content shall be not less than 95% of a mixture of sorbitol, 1,4-sorbitan and isosorbide.

DESCRIPTION

Amber-coloured oily viscous liquid, light cream to tan beads or flakes or a hard, waxy solid with a slight odour

FUNCTIONAL USES

Emulsifier, stabilizer

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Dispersible in hot and cold water

PURITY

Water (Vol. 4)

Not more than 2% (Karl Fischer Method)

<u>Sulfated ash</u> (Vol. 4)	Not more than 0.5%
<u>Acid value</u> (Vol. 4)	Not more than 7
<u>Saponification value</u> (Vol. 4)	Not less than 155 and not more than 170
<u>Hydroxyl value</u> (Vol. 4)	Not less than 330 and not more than 358
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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

Transfer about 25 g of the sample, accurately weighed, into a 500-ml round-bottom flask, add 250 ml of alcohol and 7.5 g of potassium hydroxide, and mix. Connect a suitable condenser to the flask, reflux the mixture for 1 to 2 h, and then transfer to an 800-ml beaker, rinsing the flask with about 100 ml of water and adding it to the beaker. Heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol, and evaporate until the odour of alcohol can no longer be detected. Adjust the final volume to about 250 ml with hot water.

Neutralize the soap solution with dilute sulfuric acid (1 in 2), add 10% in excess, and heat, while stirring, until the fatty acid layer separates. Transfer the fatty acids to a 500-ml separator, wash with three or four 20-ml portions of hot water to remove polyols, and combine the washings with the original aqueous polyol layer from the saponification. Extract the combined aqueous layer with three 20-ml portions of petroleum ether, add the extracts to the fatty acid layer, evaporate to dryness in a tared dish, cool and weigh.

Neutralize the polyol solution with a 1 in 10 solution of potassium hydroxide to pH 7 using a suitable pH meter. Evaporate this solution to a moist residue, and separate the polyols from the salts by several extractions with hot alcohol. Evaporate the alcohol extracts on a steam bath to dryness in a tared dish, cool, and weigh. Avoid excessive drying and heating.

CALCIUM POLYPHOSPHATE

Prepared at the 26th JECFA (1982), published in FNP 25 (1982) and in FNP 52 (1992 Metals and arsenic specifications revised at the 55th JECFA (2000). A group MTDI of 70 mg/kg bw, as phosphorus from all food sources, was established at the 26th JECFA (1982)

SYNONYMS	INS No. 452(iv)
DEFINITION	A heterogeneous mixture of calcium salts of polyphosphoric acids of general formula $H_{n+2}P_nO_{n+1}$.
Assay	Not less than 50.0 and not more than 71.0% of P_2O_5 on the ignited basis
DESCRIPTION	Odourless, colourless crystals or powder
FUNCTIONAL USES	Emulsifier, moisture-retaining agent, sequestrant, texturizer

CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Usually incompletely soluble in water; soluble in acid medium
<u>Test for phosphate</u> (Vol. 4)	Mix 0.5 g of the sample with 10 ml of nitric acid and 50 ml of water, boil for about 30 min and cool. The resulting solution is used for the test
<u>Test for calcium</u> (Vol. 4)	The solution of the test for phosphate gives positive tests for calcium

PURITY

<u>Loss on ignition</u> (Vol. 4)	Not more than 2% after drying (105° , 4 h) followed by ignition (550° , 30 min)
<u>Cyclic phosphate</u> (Vol. 4)	Not more than 8% calculated on P_2O_5 content
<u>Fluoride</u> (Vol. 4)	Not more than 10 mg/kg
<u>Arsenic</u> (Vol. 4)	Not more than 3 mg/kg Dissolve 1 g of the sample in 15 ml dilute hydrochloric acid TS, add 20 ml of water. Test this solution as directed in the Limit Test (Method II).
<u>Lead</u> (Vol. 4)	Not more than 4 mg/kg. Determine using an atomic absorption technique 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	Mix about 300 mg of the sample, accurately weighed, with 15 ml of nitric acid and 30 ml of water, boil for 30 min and dilute with water to about 100 ml. Heat at 60° , and add excess of ammonium molybdate TS, and heat at 50° for 30 min. Filter, and wash the precipitate with dilute nitric acid (1 in 36), followed by potassium nitrate solution (1 in 100) until the filtrate is no longer acid to litmus.
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Dissolve the precipitate in 50 ml of 1 N sodium hydroxide, add phenolphthalein TS, and titrate the excess of sodium hydroxide with 1 N sulfuric acid. Each ml of 1 N sodium hydroxide is equivalent to 3.086 mg of P₂O₅.

ETHYL CELLULOSE

Revised specification prepared at the 76th JECFA (2012), published in FAO JECFA Monographs 13 (2012) superseding specifications prepared at the 26th JECFA (1982), published in FNP 25 (1982) and FNP 52 (1992). Metals and arsenic specifications revised at the 57th JECFA (2001). A group ADI 'not specified' was established at the 35th JECFA (1989).

SYNONYMS	INS No. 462
DEFINITION	Ethyl ether of cellulose, prepared from wood pulp or cotton by treatment with alkali and ethylation of the alkali cellulose with ethyl chloride. The article of commerce can be specified further by viscosity. Antioxidants permitted for use in food may be added for stabilizing purposes.
Chemical names	Cellulose ethyl ether, ethyl ether of cellulose
C.A.S. number	9004-57-3
Assay	Not less than 44% and not more than 50% of ethoxyl groups (-OC ₂ H ₅) on the dried basis (equivalent to not more than 2.6 ethoxyl groups per anhydroglucoside unit).
DESCRIPTION	Free-flowing, white to light tan powder
FUNCTIONAL USES	Tableting aid, binder, filler, diluent of colour and other food additives
CHARACTERISTICS	
IDENTIFICATION	
<u>Solubility</u> (Vol. 4)	Practically insoluble in water, in glycerol, and in propane-1,2-diol, but soluble in varying proportions in certain organic solvents, depending upon the ethoxyl content. Ethyl cellulose containing less than 46-48% of ethoxyl groups is freely soluble in tetrahydrofuran, methyl acetate and aromatic hydrocarbon ethanol mixtures. Ethyl cellulose containing 46-48% or more of ethoxyl groups is freely soluble in ethanol, methanol, toluene and ethyl acetate.
<u>Film forming test</u>	Dissolve 5 g of the sample in 95 g of an 80:20 (w/w) mixture of toluene-ethanol. A clear, stable, slightly yellow solution is formed. Pour a few ml of the solution onto a glass plate, and allow the solvent to evaporate. A thick, tough continuous, clear film remains. The film is flammable.

pH (Vol. 4) Neutral to litmus (1 in 20 suspension)

PURITY

Loss on drying (Vol. 4) Not more than 3% (105°, 2 h)

Sulfated ash (Vol. 4) Not more than 0.4%
Test 1 g of the sample (Method I)

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an AAS (Electrothermal atomization technique)
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").

METHOD OF ASSAY Determine the ethoxyl content as directed under *Ethoxyl and Methoxyl Group Determination* (see Volume 4).

SORBITAN MONOSTEARATE

Prepared at the 17th JECFA (1973), published in FNP 4 (1978) and in FNP 52 (1992). Metals and arsenic specifications revised at the 55th JECFA (2000). A group ADI of 0-25 mg/kg bw as the sum of sorbitan esters of lauric, oleic, palmitic and stearic acids was established at the 26th JECFA (1982)

SYNOMYS

INS No. 491

DEFINITION

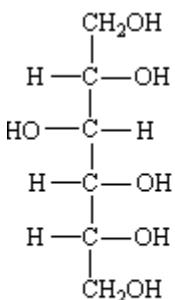
A mixture of the partial esters of sorbitol and its mono- and dianhydrides with edible stearic acid

C.A.S. number

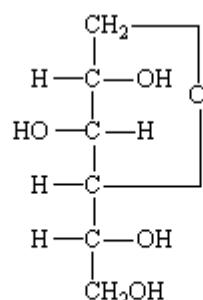
1338-41-6

Structural formula

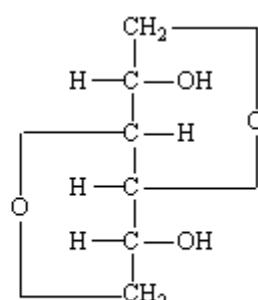
Contains palmitic acid esterified with polyols derived from sorbitol including the following types:



Sorbitol



1,4-Sorbitan



Isosorbide

Assay

Saponification of 100 g of the sample yields approximately 31.5 g of polyols and 73 g of fatty acid. The polyol content shall be not less than 95% of a mixture of sorbitol, 1,4-sorbitan and isosorbide

DESCRIPTION

Light cream to tan beads or flakes or hard, waxy solid with a slight characteristic odour

FUNCTIONAL USES

Emulsifier

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Soluble at temperatures above its melting point in toluene, dioxane, carbon tetrachloride, ether, methanol, ethanol and aniline; insoluble in petroleum ether and acetone; insoluble in cold water but dispersible in warm water; soluble with haze at temperatures above 50° in mineral oil and ethyl acetate.

Congealing range (Vol. 4) 50 - 52°

Infrared absorption

The infrared spectrum of the sample is characteristic of a partial fatty acid ester of a polyol

PURITY

<u>Water</u> (Vol. 4)	Not more than 1.5% (Karl Fischer Method)
<u>Acid value</u> (Vol. 4)	Not less than 5 and not more than 10
<u>Saponification value</u> (Vol. 4)	Not less than 147 and not more than 157
<u>Hydroxyl value</u> (Vol. 4)	Not less than 235 and not more than 260
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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**

Proceed as directed under the *Sorbitan Ester Content* (Volume 4)

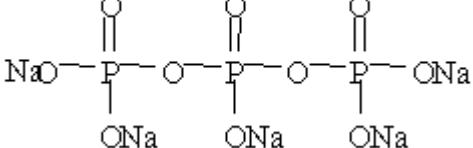
PENTASODIUM TRIPHOSPHATE

Prepared at the 55th JECFA (2000) and published in FNP 52 Add 8 (2000), superseding tentative specifications prepared at the 20th JECFA (1976) and published in FNS 1B (1977) and in FNP 52 (1992). No ADI was established, but a group MTDI of 70 mg/kg bw, expressed as phosphorus from all food sources, was established at the 26th JECFA (1982).

SYNOMYS

Pentasodium tripolyphosphate, Sodium triphosphate, Sodium tripolyphosphate, Triphosphate; INS No. 451(i)

DEFINITION

Chemical names	Pentasodium tripolyphosphate, pentasodium tripolyphosphate
C.A.S. number	7758-29-4
Chemical formula	$\text{Na}_5\text{O}_{10}\text{P}_3 \cdot x \text{H}_2\text{O}$ ($x = 0$ or 6)
Structural formula	
Formula weight	Anhydrous: 367.86 Hexahydrate: 475.94
Assay	Anhydrous: not less than 85.0% of $\text{Na}_5\text{O}_{10}\text{P}_3$ and not less than 56.0% and not more than 58.0% of P_2O_5 Hexahydrate: not less than 65.0% of $\text{Na}_5\text{O}_{10}\text{P}_3$ and not less than 43.0% and not more than 45.0% of P_2O_5

DESCRIPTION

White, slightly hygroscopic granules or powder

FUNCTIONAL USES

Sequestrant, texturizer

CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Freely soluble in water; insoluble in ethanol
<u>pH</u> (Vol. 4)	9.1 - 10.1 (1 % soln)
<u>Test for phosphate</u> (Vol. 4)	Passes test
<u>Test for sodium</u> (Vol. 4)	Passes test

PURITY

<u>Loss on drying</u> (Vol. 4)	Anhydrous: not more than 0.7% (105°, 1 h)
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	Hexahydrate: not more than 23.5% (60°, 1 h, followed by 105°, 4 h)
<u>Water-insoluble matter</u> (Vol. 4)	Not more than 0.1%
<u>Higher polyphosphates</u>	Not detectable See description under TESTS
<u>Fluoride</u> (Vol. 4)	Not more than 50 mg/kg (Method I or III)
<u>Arsenic</u> (Vol. 4)	Not more than 3 mg/kg (Method II)
<u>Lead</u> (Vol. 4)	Not more than 4 mg/kg Determine using an atomic absorption technique 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".

TESTS

PURITY TESTS

<u>Higher polyphosphates</u>	<u>Chromatographic solvent</u> Mix 75 ml of isopropanol, 10 ml of water, 20 ml of 20% trichloroacetic acid and 0.3 ml of 20% ammonia. Make fresh every week.
	<u>Chromatographic spray</u> Dissolve 1 g of ammonium molybdate in 85 ml of water, 10 ml of N hydrochloric acid and 5 ml of 60% perchloric acid.
	<u>Sample solution</u> Dissolve 1 g of the sample in 50 ml of water.
	<u>Reference solution</u> Dissolve 1 g of a standard sample of pentasodium triphosphate in 50 ml of water.
	<u>Procedure</u> Place 0.01 ml of the sample solution and 0.01 ml of reference solution on the starting line of the chromatographic paper and allow to dry in a stream of warm air. Use ascending chromatography at 18-20° until the solvent has ascended about 25 cm from the starting line (12 - 15 h). Dry at 60° in an oven and spray with the chromatographic spray. Place the paper under an ultraviolet lamp and irradiate until the phosphates are visible as blue spots (about 2 min).

Three spots (one from the monophosphate ($R_f = 0.69$), a second from the diphosphate ($R_f = 0.44$) and the third from the triphosphate ($R_f = 0.29$) are observed, and no other spot is observed.

METHOD OF ASSAY

1. Determination of $\text{Na}_5\text{O}_{10}\text{P}_3$

Reagents and solutions

- Potassium acetate buffer (pH 5.0): Dissolve 78.5 g of potassium acetate

in 1000 ml of water and adjust the pH of the solution to 5.0 with acetic acid. Add a few mg of mercuric iodide to inhibit mould growth.

- 0.3 M Potassium chloride: Dissolve 22.35 g of potassium chloride in water, add 5 ml of potassium acetate buffer, dilute with water to 1000 ml, and mix. Add a few mg of mercuric iodide.
- 0.6 M Potassium chloride: Dissolve 44.7 g of potassium chloride in water, add 5 ml of potassium acetate buffer, dilute with water to 1000 ml, and mix. Add a few mg of mercuric iodide.
- 1 M Potassium chloride: Dissolve 74.5 g of potassium chloride in water, add 5 ml of potassium acetate buffer, dilute to 1000 ml with water, and mix. Add a few mg of mercuric iodide.

Chromatographic Column

Use a standard chromatographic column 20 to 40 cm length, 20 to 28 cm inside diameter, with a sealed-in, coarse porosity fritted disk. If a stopcock is not provided, attach a stopcock having a 3 to 4 mm, diameter bore to the outlet of the column with a short length of flexible vinyl tubing.

Procedure

Close the column stopcock, fill the space between the fritted disk and the stopcock with water, and connect a vacuum line to the stopcock. Prepare a 1:1 water slurry of Dowex F x 8, or equivalent, chloride form, 100-200 or 200-400 mesh, styrenedivinylbenzene ion exchange resin, and decant off any fine particles and any foam. Do this two or three times or until no more finely suspended material or foaming is observed. Fill the column with the slurry, and open the stopcock to allow the vacuum to pack the resin bed until the water level is slightly above the top of the resin, then immediately close the stopcock. Do not allow the liquid level to fall below the resin level at any time. Repeat this procedure until the packed resin column is 15 cm above the fritted disk. Place one circle of tightly fitting fibre filter paper on top of the resin bed, then place a perforated polyethylene disk on top of the paper. Alternatively, a loosely packed plug of glass wool may be placed on top of the bed. Close the top of the column with a rubber stopper in which a 7.6 cm length of capillary tubing (1.5 mm i.d., 7 mm o.d.) has been inserted through the centre, so that about 12 mm of the tubing extends through the bottom of the stopper. Connect the top of the capillary tubing to the stem of a 500-ml separator with flexible vinyl tubing, and clamp the separator to a ring stand above the column. Wash the column by adding 100 ml of water to the separator with all stopcocks closed. First open the separator stopcock, then open the column stopcock. The rate of flow should be about 5 ml per min. When the separator is empty, close the stopcock on the column then close the separator stopcock.

Transfer about 500 mg of the sample (a), accurately weighed, into a 250 ml volumetric flask, dissolve and dilute to volume with water, and mix. Transfer 10 ml of this solution into the separator, open both stopcocks and allow the solution to drain into the column, rinsing the separator with 20 ml of water. Discard the eluate. Add 370 ml of 0.3 M Potassium Chloride to the separator, and allow this solution to pass through the column, discarding the eluate. Add 250 ml of 0.6 M Potassium Chloride to the column, allow the solution to pass through the column, and receive the eluate in a 400-ml beaker. (To ensure a clean column for the next run, pass 100 ml of 1 M Potassium Chloride through the column, followed by 100 ml of water. Discard all washings.) To the beaker add 15 ml of nitric acid, mix, and boil

for 15 to 20 min. Add methyl orange TS, and neutralize the solution with stronger ammonia TS. Add 1 g of ammonium nitrate crystals, stir to dissolve, and cool. Add 15 ml of ammonium molybdate TS, with stirring, and stir vigorously for 3 min or allow to stand with occasional stirring for 10 to 15 min. Filter the contents of the beaker with suction through a 6-7 mm paper pulp filter pad supported in a 25 mm porcelain disk. The filter pad should be covered with a suspension of a filtering aid. After the contents of the beaker have been transferred to the filter, wash the beaker with five 10 ml portions of a 1% solution of sodium or potassium nitrate, passing the washings through the filter, then wash the filter with five 5-ml portions of the wash solution. Return the filter pad and the precipitate to the beaker, wash the funnel thoroughly with water into the beaker, and dilute to about 150 ml. Add 0.1 N sodium hydroxide from a buret until the yellow precipitate is dissolved, then add 5 to 8 ml in excess. Add phenolphthalein TS, and titrate the excess alkali with 0.1 N nitric acid. Finally, titrate with 0.1 N sodium hydroxide to the first appearance of the pink colour. The difference between the total volume of 0.1 N sodium hydroxide added and the volume of nitric acid required represents the volume, V, in ml, of 0.1 N sodium hydroxide consumed by the phosphomolybdate complex.

Calculate the $\text{Na}_5\text{O}_{10}\text{P}_3$ content of the sample in % by the formula

$$\% \text{Na}_5\text{O}_{10}\text{P}_3 = \frac{0.533 \times 25 \times V}{a} \times 100$$

where

a = the weight of the sample (mg)

2. Determination of P_2O_5

Accurately weigh about 20 g of the sample into a beaker. Add 150 ml water and 20 ml concentrated nitric acid. Introduce anti-bumping granules, cover the beaker with a watch glass and boil gently for 1 h. Cool to room temperature. Quantitatively transfer the solution to a 500-ml volumetric flask, dilute with water, mix well and dilute to the mark with water. Transfer 20.0 ml of the solution to a plastic beaker, dilute to about 50 ml with water and place the beaker in an automatic titrator equipped with a pH meter. Adjust the pH of the solution to between 2.5 and 2.8 with 5 mol/l sodium hydroxide. Titrate the solution with 0.5 mol/l sodium hydroxide. Record the consumed volumes at the inflection points at about pH 4 (V1) and about pH 9 (V2).

Calculate the P_2O_5 content of the sample in % by the formula

$$\begin{aligned} \% \text{P}_2\text{O}_5 &= [(V_2 - V_1)/2000] \times f \times 70.97 \times (500/20) \times (100/w) \\ &= [(V_2 - V_1)/w] \times f \times 88.71 \end{aligned}$$

where

w = weight of the sample (g)

f = factor of 0.5 mol/l sodium hydroxide (= actual molarity/0.5)

SUNSET YELLOW FCF

Prepared at the 69th JECFA (2008) and published in FAO JECFA Monographs 5 (2008), superseding specifications prepared at the 28th JECFA (1984), published in combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). An ADI of 0-4 mg/kg bw was established at the 74th JECFA (2011).

SYNONYMS CI Food Yellow 3; Orange Yellow S; CI (1975) No. 15985; INS No. 110

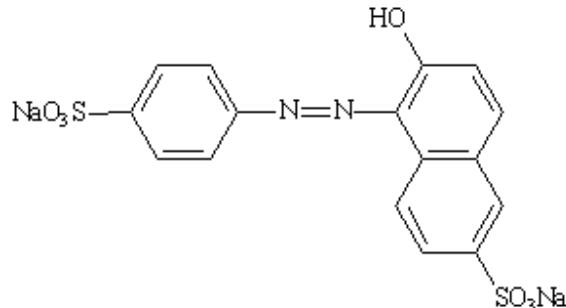
DEFINITION Sunset Yellow FCF consists principally of the disodium salt of 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonic acid and subsidiary colouring matters together with sodium chloride and/or sodium sulfate as the principal uncoloured components.
(NOTE: The colour may be converted to the corresponding aluminium lake, in which case only the *General Specifications for Aluminium Lakes of Colouring Matters* apply.)

Chemical names Principal component:
Disodium 6-hydroxy-5-(4-sulfonatophenylazo)-2-naphthalene-sulfonate

C.A.S. number 2783-94-0

Chemical formula C₁₆H₁₀N₂Na₂O₇S₂ (Principal component)

Structural formula



(Principal component)

Formula weight 452.38 (Principal component)

Assay Not less than 85% total colouring matters

DESCRIPTION Orange-red powder or granules

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Soluble in water; sparingly soluble in ethanol
<u>Colour test</u>	In water, neutral or acidic solutions of Sunset Yellow FCF are yellow-orange, whereas basic solutions are red-brown. When dissolved in concentrated sulfuric acid, the additive yields an orange solution that turns yellow when diluted with water.
<u>Colouring matters, identification</u> (Vol. 4)	Passes test

PURITY

<u>Water content (Loss on drying)</u> (Vol. 4)	Not more than 15% together with chloride and sulfate calculated as sodium salts
<u>Water-insoluble matter</u> (Vol. 4)	Not more than 0.2%
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique 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 (under "General Methods, Metallic Impurities").
<u>Subsidiary colouring matter content</u> (Vol. 4)	Not more than 5% Not more than 2% shall be colours other than trisodium 2-hydroxy-1-(4-sulfonatophenylazo)naphthalene-3,6-disulfonate Use the following conditions: Chromatography solvent: 2-Butanone:acetone:water:ammonia (s.g. 0.880) (700:300:300:2) Height of ascent of solvent front: approximately 17 cm
<u>Sudan I (1-(Phenylazo)-2-naphthalenol)</u>	Not more than 1 mg/kg See description under TESTS
<u>Organic compounds other than colouring matters</u> (Vol. 4)	Not more than 0.5%, sum of the: monosodium salt of 4-aminobenzenesulfonic acid, disodium salt of 3-hydroxy-2,7-naphthalenedisulfonic acid, monosodium salt of 6-hydroxy-2-naphthalenesulfonic acid, disodium salt of 7-hydroxy-1,3-naphthalenedisulfonic acid, disodium salt of 4,4'-diazoaminobis-benzenesulfonic acid, and disodium salt of 6,6'-oxybis-2-naphthalenesulfonic acid
	Proceed as directed under <i>Determination by High Performance Liquid Chromatography</i> using an elution gradient of 2 to 100% at 4% per min (linear) followed by elution at 100%.
<u>Unsulfonated primary aromatic amines</u> (Vol. 4)	Not more than 0.01%, calculated as aniline

Ether-extractable matter Not more than 0.2%
(Vol. 4)

TESTS

PURITY TESTS

Sudan I (1-(Phenylazo)-2-naphthalenol)

Principle

The additive is dissolved in water and methanol and filtered solutions are analysed by Reverse-Phase Liquid Chromatography (Volume 4 under "Analytical Techniques, Chromatography"), without extraction or concentration. (Based on *J.AOAC Intl* 90, 1373-1378 (2007).)

Mobile phase

Eluant A: Ammonium acetate (LC grade), 20 mM aqueous

Eluant B: Methanol (LC grade)

Sample solution

Accurately weigh 200 mg of Sunset yellow FCF and transfer it into a 10-ml volumetric flask. Dissolve the sample in 4 ml water via swirling or sonication. Add 5 ml of methanol and swirl. Allow the solution to cool for 5 min and adjust the volume to the mark with water. Filter a part of the solution for analysis through a 13 mm syringe filter with a 0.2 µm pore size PTFE membrane by using a 5 ml polypropylene/polyethylene syringe. (NOTE: Do not substitute a PVDF filter for the PTFE filter, as a PVDF filter adsorbs Sudan I.)

Standard

Sudan I (>97%, Sigma Aldrich, or equivalent), recrystallized from absolute ethanol (5g/150 ml)

Standard stock solution

Accurately weigh a sufficient quantity of the *Standard* to prepare a solution in methanol of 0.0100 mg/ml.

Standard solutions

Transfer 0, 20, 50, 100, 150, 200, and 250 µl aliquots of the *Standard stock solution* to seven 10-ml volumetric flasks. To each flask, add 5 ml of methanol, swirl to mix, and add 4 ml of water. Dilute to volume with water, mix, and filter each solution through a PTFE membrane syringe filter (see *Sample solution*, above) into LC vials for analysis. (NOTE: These solutions nominally contain 0, 0.02, 0.05, 0.10, 0.15, 0.20, and 0.25 µg of Sudan I/ml.)

Chromatographic system

Detector: Photodiode Array (485 nm)

Columns: 150 mm x 2.1 mm id, packed with 5 µm reversed-phase C18, or equivalent, with a guard column (10 mm x 2.1 mm i.d.) – Waters Corp., or equivalent

Column temperature: 25°

Flow rate: 0.25 ml/min

Injection volume: 50 µl

Elution: 50% *Eluant A*/50% *Eluant B* for 5 min; 50 to 100% *Eluant B* in 10 min; 100% *Eluant B* for 10 min. (NOTE: The column should be reequilibrated with 50% *Eluant A*/50% *Eluant B* for 10 min.)

System suitability: Inject three replicates of the *Standard solutions* with concentrations of 0.05 and 0.25 µg of Sudan I/ml. The responses for each set of three injections show relative standard deviations of not more than 2%.

Procedure

Separately inject the seven *Standard solutions* and the *Sample solution* into the chromatograph and measure the peak areas for Sudan I. From the chromatograms for the *Standard solutions*, prepare a standard curve of the concentration of Sudan I vs. the peak areas. (NOTE: The retention time for Sudan I is 19.0 min. Other peaks appearing at earlier retention times in the sample chromatograph are likely attributed to sulfonated subsidiary colours.) Determine the concentration of Sudan I in the *Sample solution* and convert it to mg/kg in the sample of Sunset Yellow FCF.

(NOTE: The limit of determination is 0.4 mg/kg.)

METHOD OF ASSAY Proceed as directed under *Colouring Matters Content by Titration with Titanous Chloride* (Volume 4, under "Food Colours, Colouring Matters"), using the following:

Weight of sample: 0.5-0.6 g

Buffer: 10 g sodium citrate

Weight (*D*) of colouring matters equivalent to 1.00 ml of 0.1 N TiCl_3 :
11.31 mg

PROPAN-2-OL

Prepared at the 51st JECFA (1998), published in FNP 52 Add 6 (1998) superseding earlier specifications prepared by the 28th JECFA (1984), published in FNP 31/2 (1984) and republished in FNP 52 (1992). No ADI allocated at the 25th JECFA in 1981.

SYNONYMS Isopropyl alcohol, isopropanol

DEFINITION

Chemical names 2-Propanol, propan-2-ol

C.A.S. number 67-63-0

Chemical formula C₃H₈O

Structural formula CH₃CHOHCH₃

Formula weight 60.10

Assay Not less than 99.5% of C₃H₈O

DESCRIPTION Clear, colourless, mobile liquid with a characteristic odour

FUNCTIONAL USES Extraction solvent, carrier solvent, flavouring agent (see "Flavouring agents" monograph)

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Miscible with water, ethanol, ether and other organic solvents

Specific gravity (Vol. 4) d²⁰₂₀: 0.784 - 0.788

Refractive index (Vol. 4) n²⁰_D: 1.377-1.380

PURITY

Water (Vol. 4) Not more than 0.2% (Karl Fischer Method)

Distillation range (Vol. 4) Within a range of 1° including 82.3°

Warning: Check first for *Peroxides*

Non-volatile residue (Vol. 4) Not more than 2 mg/100 ml

Acidity Not more than 0.002% (as acetic acid)
Add 2 drops of phenolphthalein TS to 100 ml of water, add 0.01N sodium hydroxide to the first pink colour that persists for at least 30 sec, then add 50 ml (about 39 g) of the sample and mix. Not more than 0.7 ml of 0.1N sodium hydroxide is required to restore the pink colour.

Other alcohols, ethers and Not more than 0.5% total, with not more than 0.1% of any single ethers.

<u>volatile impurities</u>	See under METHOD OF ASSAY
<u>Lead</u> (Vol. 4)	Not more than 1 mg/kg Determine using an atomic absorption technique 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	Determine the content of propan-2-ol and volatile impurities using the procedures for <i>gas chromatography</i> under the following conditions: <u>Column</u> - length: 1.8 m - diameter: 6 mm - material: steel - packing: 10% P.E.G. 400 on Chromosorb W (60/80 mesh), or equivalent Carrier gas: Helium Flow rate: 45 ml/min Detector type: FID <u>Temperatures</u> - injection port: 150° - column: 90° - detector: 150°

Inject 1 to 5 μ l of sample, obtain chromatogram and determine content of each constituent by the method of area normalization.

SODIUM CARBOXYMETHYL CELLULOSE

Prepared at the 74th JECFA (2011) and published in FAO JECFA Monographs 11 (2011), superseding specifications prepared at the 55th JECFA (2000), published in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). An ADI ‘not specified’ was established for modified celluloses at the 35th JECFA (1989).

SYNOMYS

Sodium cellulose glycolate; Na CMC; cellulose gum; sodium CMC; INS No. 466

DEFINITION

Prepared from cellulose by treatment with alkali and monochloro-acetic acid or its sodium salt. The article of commerce can be specified further by viscosity.

Chemical names

Sodium salt of carboxymethyl ether of cellulose

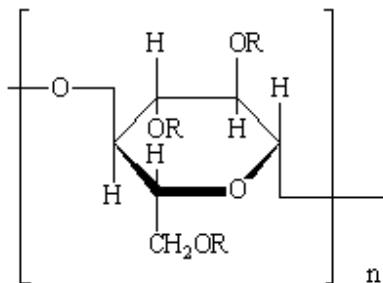
C.A.S. number

9004-32-4

Chemical formula

$[C_6H_{7-O_2(OH)_x(OCH_2COONa)_y]_n$
where
n is the degree of polymerization
x = 1.50 to 2.80
y = 0.2 to 1.50
x + y = 3.0
(y = degree of substitution)

Structural formula



where R = H or CH_2COONa

Formula weight

Structural unit with a degree of substitution of 0.20: 178.14
Structural unit with a degree of substitution of 1.50: 282.18
Macromolecules: greater than about 17,000 (n about 100)

Assay

Not less than 99.5% of sodium carboxymethyl cellulose, calculated on the dried basis

DESCRIPTION

White or slightly yellowish, almost odourless hygroscopic granules, powder or fine fibres

FUNCTIONAL USES Thickening agent, stabilizer, suspension agent

CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Yield viscous colloidal solution with water; insoluble in ethanol
Foam test	Vigorously shake a 0.1% solution of the sample. No layer of foam appears. This test distinguishes sodium carboxymethyl cellulose from other cellulose ethers and from alginates and natural gums.
Precipitate formation	To 5 ml of a 0.5% solution of the sample add 5 ml of a 5% solution of copper sulfate or of aluminium sulfate. A precipitate appears. (This test permits the distinction of sodium carboxymethyl cellulose from other cellulose ethers, and from gelatine, carob bean gum and tragacanth gum).
Colour reaction	Add 0.5 g of powdered carboxymethylcellulose sodium to 50 ml of water, while stirring to produce a uniform dispersion. Continue the stirring until a clear solution is produced. To 1 ml of the solution, diluted with an equal volume of water, in a small test tube, add 5 drops of 1-naphthol TS. Incline the test tube, and carefully introduce down the side of the tube 2 ml of sulfuric acid so that it forms a lower layer. A red-purple colour develops at the interface.

PURITY

<u>Loss on drying</u> (Vol. 4)	Not more than 12% after drying (105°, to constant weight)
<u>pH</u> (Vol. 4)	6.0 - 8.5 (1 in 100 solution)
<u>Sodium</u> (Vol. 4)	Not more than 12.4% on the dried basis Determine total sodium content by <i>Atomic Absorption Spectroscopy</i> or <i>Flame Photometry</i>
<u>Sodium chloride</u>	Not more than 0.5% on the dried basis See description under TESTS
<u>Free glycolate</u>	Not more than 0.4% calculated as sodium glycolate on the dried basis See description under TESTS
<u>Degree of substitution</u>	Not less than 0.20 and not more than 1.50 See description under TESTS
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under "General Methods, Metallic Impurities").

TESTS

PURITY TESTS

Sodium chloride

Heat 5 g of the sample, weighed to the nearest 0.1 mg, in a platinum or porcelain crucible, first with a small flame so that the sample does not ignite and then, when the charring is complete, heat further in an electric oven for 15 min at about 500°. After cooling, pulverize the ashes thus obtained and extract several times with warm water. Filter the extracts into a 500-ml volumetric flask, acidify with nitric acid and dilute to the mark. Determine the NaCl content of 100 ml of this extract by the method of Volhard, using 0.02 N silver nitrate and 0.02 N ammonium thiocyanate. Each ml of 0.02 N silver nitrate is equivalent to 1.169 mg of NaCl. Calculate the sodium chloride content by the formula:

$$\% \text{ NaCl} = \frac{a \times 0.001169 \times 5}{b} \times 100$$

where

a is ml of 0.02 N silver nitrate used; and
b is the dry weight of 5 g of the sample.

Free glycolate

Weigh 0.5 g of the sample to the nearest 0.1 mg, and transfer to a 100-ml beaker. Moisten the sample thoroughly with 5 ml of glacial acetic acid, followed by 5 ml of water, and stir with a glass rod until the solution is complete (usually about 15 min are required). Slowly add 50 ml of acetone while stirring and then approximately 1 g of sodium sulfate. Continue the stirring for several min to ensure complete precipitation of the carboxymethyl cellulose. Filter through a soft, open-texture paper, previously wetted with a small amount of acetone, and collect the filtrate in a 100-ml volumetric flask. Use 30 ml of acetone to facilitate the transfer of the solids and to wash the filter cake. Make up to volume with acetone and mix.

Prepare a blank solution containing 5 ml of water, 5 ml of glacial acetic acid and acetone in another 100-ml volumetric flask. Pipet 2 ml of the sample solution and 2 ml of the blank solution into two 25-ml volumetric flasks. Remove the acetone by heating the uncovered flasks upright in a boiling water bath for exactly 20 min. Cool to room temperature and add 5 ml of naphthalenediol TS, mix thoroughly, then add 15 ml more of the TS and mix. Cover the mouth of the flask with a small piece of aluminium foil and heat upright in the boiling water bath for 20 min. Cool to room temperature and make up to volume with naphthalenediol TS.

Measure the absorbance of sample solution against blank solution at 540 nm using 1-cm cells. Read the corresponding mg of glycolic acid from the calibration curve obtained as follows:

Introduce 0, 1, 2, 3 and 4-ml aliquots of standard glycolic acid solution (1 mg per ml, prepared by weighing accurately 0.100 g of glycolic acid, previously dried in a vacuum desiccator for at least 16 hours, and then dissolving in 100 ml of water; do not keep the solution longer than 30 days) into a series of five 100-ml volumetric flasks. Add water to each flask to a volume of 5 ml, then add 5 ml of glacial acetic acid and make up with

acetone to mark and mix. Pipet 2 ml of each solution (containing, respectively, 0, 1, 2, 3, and 4 mg of glycolic acid per 100 ml) into a series of five 25-ml volumetric flasks and proceed in the same manner as described for the Test Solution. Plot the mg of glycolic acid in the original 100 ml of solution against absorbance to give a calibration curve.

Calculate the sodium glycolate (free glycolate) content by the formula:

$$\% \text{ Sodium glycolate} = \frac{a \times 0.129}{b}$$

where

a is mg of glycolic acid read from the calibration curve; and
b is g of dry-weight of the sample.

Degree of substitution

Sample preparation

Weigh 5 g of the sample to the nearest 0.1 mg, and transfer into a 500-ml conical flask. Add 350 ml of methanol or ethanol (80% by volume). Shake the suspension mechanically for 30 min. Decant through a tared glass filtering crucible under gentle suction. Avoid, at the end of the decanting, suction of air through the crucible. Repeat the treatment with the extraction liquid until the test for chloride ions with a solution of silver nitrate TS is negative. Normally three treatments are sufficient. Transfer the sodium carboxymethyl cellulose into the same crucible. Displace the extraction liquid that adheres to the substance with acetone. Dry the crucible in an oven at 110° until constant in weight. Weigh the first time after 2 h. Cool the crucible every time in a desiccator and pay attention during weighing to the fact that sodium carboxymethyl cellulose is slightly hygroscopic.

Procedure

Weigh 2 g, to the nearest 0.1 mg, of the bone dry substance, obtained with the above-mentioned alcohol-extraction procedure, in a tared porcelain crucible. Initially, char carefully with a small flame and afterwards for 10 min, with a large flame. Cool and then moisten the residue with 3-5 ml of concentrate sulfuric acid. Heat cautiously until the fuming is finished. After some cooling add about 1 g of ammonium carbonate, distributing the powder over the whole contents of the crucible. Heat again, initially with a small flame until the fuming is finished and heat then at a dull red heat for 10 min. Repeat the treatment with sulfuric acid and ammonium carbonate if the residual sodium sulfate still contains some carbon. Cool the crucible in a desiccator and weigh. Instead of adding ammonium carbonate and heating further with a flame, the crucible can be placed for 1 h in an oven at about 600°.

Calculate the sodium content of the alcohol-extracted sample by the formula:

$$\% \text{ Sodium} = \frac{a \times 32.38}{b}$$

where

a is the weight of residual sodium sulfate; and

b is the weight of the alcohol-extracted dry sample.

Calculate the degree of substitution by the formula:

$$\text{Degree of substitution} = \frac{162 \times \% \text{ sodium}}{2300 - (80 \times \% \text{ sodium})}$$

METHOD OF ASSAY Calculate the percentage of sodium carboxymethyl cellulose in the sample by subtracting from 100% the sum of the percentages of sodium chloride and sodium glycolate (free glycolate), determined separately by the procedures above.

$$\text{Content \%} = 100 - (\% \text{NaCl} + \% \text{ sodium glycolate})$$

CALCIUM HYDROXIDE

Prepared at the 19th JECFA (1975), published in NMRS 55B (1976) and in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002). An ADI 'not limited' was established at the 9th JECFA (1965)

SYNONYMS Slaked lime; INS No. 526

DEFINITION

Chemical names Calcium hydroxide

C.A.S. number 1305-62-0

Chemical formula $\text{Ca}(\text{OH})_2$

Formula weight 74.09

Assay Not less than 92.0%

DESCRIPTION White powder

FUNCTIONAL USES Neutralizing agent, buffer, firming agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Slightly soluble in water, insoluble in ethanol, soluble in glycerol.

Test for alkali The sample is alkaline to moistened litmus paper

Test for calcium (Vol. 4) Passes test

PURITY

Barium Not more than 0.03%

Mix 1.5 g of the sample with 10 ml of water, add 15 ml of dilute hydrochloric acid TS and dilute to 30 ml with water and filter. To 20 ml of the filtrate, add 2 g of sodium acetate, 1 ml of dilute acetic acid TS and 0.5 ml of potassium chromate TS, and allow to stand for 15 min. The turbidity of the solution is not greater than that of a control prepared by adding water to 0.3 ml of barium standard solution (1.779 g barium chloride in 1000 ml of water) to make to 20 ml, adding 2 g of sodium acetate, 1 ml of dilute acetic acid TS and 0.5 ml of potassium chromate TS and allowing to stand for 15 min.

Magnesium and alkali salts Not more than 6%

Dissolve 500 mg of the sample in a mixture of 30 ml of water and 10 ml of dilute hydrochloric acid TS and boil for 1 min. Quickly add 40 ml of oxalic acid TS and stir vigorously until precipitation is well established. Immediately add 2 drops of methyl red TS, then add ammonia TS dropwise until the mixture is just alkaline and cool. Transfer the mixture to a 100-ml cylinder, dilute to volume with water, let stand for 4 h or overnight, then decant the clear,

supernatant liquid through a dry filter paper. To 50 ml of the clear filtrate in a platinum dish add 0.5 ml of sulfuric acid, and evaporate the mixture on a steam bath to a small volume. Carefully evaporate the remaining liquid to dryness over a flame, and continue the heating until the ammonium salts have been completely decomposed and volatilized. Finally, ignite the residue to constant weight. The weight of the residue does not exceed 15 mg.

Acid insoluble ash

Not more than 1.0%

Dissolve 2 g of the sample in 30 ml of dilute hydrochloric acid (1 in 3) and heat to boiling. Filter the mixture, wash the residue with hot water and ignite. The weight of the residue does not exceed 20 mg.

Fluoride (Vol. 4)

Not more than 50 mg/kg (Method I or III)

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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 about 1.5 g of the sample, transfer to a beaker, and gradually add 30 ml of dilute hydrochloric acid TS. When solution is complete, transfer to a 500-ml volumetric flask, rinse the beaker thoroughly, adding the rinsings to the flask, dilute to volume with water, and mix. Pipet 50 ml of the solution into a suitable container and add 50 ml of water and 15 ml of sodium hydroxide TS, 40 mg of murexide indicator (amm. purpurate) and 3 ml of naphthol green TS, and titrate with 0.05 M disodium ethylenediaminetetraacetate until the solution is deep blue in colour. Each ml of 0.05 M disodium ethylenediaminetetraacetate is equivalent to 3.705 mg of Ca(OH)_2 .

FERROCYANIDES of CALCIUM, POTASSIUM and SODIUM

Prepared at the 17th JECFA (1973), published in FNP 4 (1978) and in FNP 52 (1992). Metals and arsenic specifications revised at the 57th JECFA (2001). An ADI of 0-0.025 mg/kg bw was established at the 18th JECFA (1974)

SYNONYMS

Yellow prussiate of lime, potash or soda; hexacyanoferrate of calcium, potassium or sodium; INS No. Calcium salt 538, Potassium salt 536, Sodium salt 535

DEFINITION

Chemical names	Calcium (or Potassium or Sodium) ferrocyanide, Calcium (or Potassium or Sodium) hexacyanoferrate (II)
C.A.S. number	1327-39-5, Calcium salt 13943-58-3, Potassium salt 13601-19-9, Sodium salt
Chemical formula	$\text{Ca}_2\text{Fe}(\text{CN})_6 \cdot 12\text{H}_2\text{O}$ $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ $\text{Na}_4\text{Fe}(\text{CN})_6 \cdot 10\text{H}_2\text{O}$
Formula weight	Calcium salt 508.3 Potassium salt 422.4 Sodium salt 484.1
Assay	Not less than 99.0% of the respective ferrocyanide

DESCRIPTION

Yellow crystals or crystalline powder

FUNCTIONAL USES

Anticaking agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water; potassium and sodium salts are insoluble in ethanol

Test for ferrocyanide To 10 ml of a 1% solution of the sample add 1 ml of ferric chloride TS. A dark blue precipitate is formed. (Retain the mixture for the Test for calcium).

Test for calcium (Vol. 4) Passes test
Test the mixture from the Test for ferrocyanide

Test for potassium (Vol. 4) Passes test

Test for sodium (Vol. 4) Passes test

PURITY

Cyanide Not detectable

Dissolve 10 mg of copper sulfate in a mixture of 8 ml of water and 2 ml of ammonia TS. Wet a strip of filter paper with this solution, and place the wet paper in a stream of hydrogen sulfide. When one drop of a 1% solution of the sample is placed on the brown reagent paper, a white circle should not be produced.

Ferricyanide

Not detectable

Dissolve about 10 mg of the sample in 10 ml of water and place one drop of this solution on a spot plate. Add one drop of a 1% solution of lead nitrate, followed by a few drops of a solution prepared by saturating cold 2 N acetic acid with benzidine. No blue precipitate or blue coloration should appear.

Arsenic (Vol. 4)

Not more than 3 mg/kg (Method II)

Lead (Vol. 4)

Not more than 5mg/kg

Determine using an atomic absorption technique 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 3 g of the sample to the nearest 0.1 mg and transfer into a 400-ml beaker. Dissolve in 225 ml of water, and add cautiously about 25 ml of sulfuric acid TS. Add, with stirring, 1 drop of orthophenanthroline TS, and titrate with 0.1 N ceric sulfate until the colour changes sharply from orange to pure yellow. Each ml of 0.1 N ceric sulfate is equivalent to 50.83 mg of $\text{Ca}_2\text{Fe}(\text{CN})_6 \cdot 12\text{H}_2\text{O}$; 42.24 mg of $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ or 48.41 mg of $\text{Na}_4\text{Fe}(\text{CN})_6 \cdot 10\text{H}_2\text{O}$.

POTASSIUM METABISULFITE

Prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7 (1999), superseding tentative specifications prepared at the 51st JECFA (1998), published in FNP 52 Add 6 (1998). Group ADI 0-0.7 mg/kg bw as SO₂ for sulfite established at the 51st JECFA in 1998.

SYNONYMS

INS No. 224

DEFINITION

Chemical names	Potassium disulfite, potassium pentaoxodisulfate, potassium pyrosulfite
C.A.S. number	16731-55-8
Chemical formula	K ₂ S ₂ O ₅
Formula weight	222.33
Assay	Not less than 90%

DESCRIPTION

Colourless free-flowing crystals, crystalline powder, or granules, usually having an odour of sulfur dioxide

FUNCTIONAL USES

Antibrowning agent, antioxidant, preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water; insoluble in ethanol

Test for potassium (Vol. 4) Passes test

Test for sulfite (Vol. 4) Passes test

PURITY

Water insolubles Dissolve 20 g of the sample in 200 ml of water. The solution should be clear with only a trace of suspended matter.

Thiosulfate Not more than 0.1%
A 10% solution of the sample should remain clear on acidification with sulfuric or hydrochloric acid

Iron (Vol. 4) Not more than 10 mg/kg
Determine as directed in the Limit Test using 0.5 ml of Iron Standard Solution (5 µg Fe) in the control

Lead (Vol. 4) Not more than 2 mg/kg

Determine using an atomic absorption technique 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."

Selenium

Not more than 5 mg/kg

See description under TESTS

TESTS

PURITY TESTS

Selenium

Reagents:

Hydrochloric acid, hydrazinium sulfate, standard selenium solution (100 µg Se/ml)

Procedure

Weigh 2.0 ± 0.1 g of sample and transfer to a 50-ml beaker. Add 10 ml water, 5 ml hydrochloric acid and boil to remove SO_2 .

Into a second beaker, weigh 1.0 ± 0.1 g of sample, add 0.05 ml standard selenium solution and proceed as above.

To each beaker add 2 g hydrazinium sulfate and warm to dissolve. Let stand for 5 min. Dilute the contents of each beaker to 50 ml in a Nessler tube and compare the colour of the two solutions. The sample should be less pink than the sample with the added standard.

METHOD OF ASSAY

Weigh 250 mg of the sample, add to 50.0 ml of 0.1 N iodine in a glass stoppered flask, and stopper the flask. Allow to stand for 5 min, add 1 ml of dilute hydrochloric acid TS and titrate the excess iodine with 0.1 N sodium thiosulfate, using starch TS as the indicator. Each ml of 0.1 N iodine is equivalent to 5.558 mg of $\text{K}_2\text{S}_2\text{O}_5$.

AMMONIUM CARBONATE

Prepared at the 26th JECFA (1982), published in FNP 25 (1982) and in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002)
An ADI 'not specified' was established at the 26th JECFA (1982)

SYNONYMS	INS No. 503(i)
DEFINITION	Consists of ammonium carbamate, ammonium carbonate and ammonium hydrogen carbonate in varying proportions
C.A.S. number	10361-29-2
Chemical formula	$\text{CH}_6\text{N}_2\text{O}_2$, $\text{CH}_8\text{N}_2\text{O}_3$ CH_5NO_3
Structural formula	$\text{NH}_2\text{COONH}_4$ $(\text{NH}_4)_2\text{HCO}_3$ NH_4HCO_3
Formula weight	Ammonium carbamate 78.06 Ammonium carbonate 98.73 Ammonium hydrogen carbonate 79.06
Assay	Not less than 30.0% and not more than 34.0% of NH_3
DESCRIPTION	White powder or hard, white or translucent masses of crystals with an odour of ammonia. On exposure to air it becomes opaque and is finally converted into white porous lumps or powder (of ammonium bicarbonate) due to loss of ammonia and carbon dioxide.
FUNCTIONAL USES	Acidity regulator, raising agent
CHARACTERISTICS	
IDENTIFICATION	
<u>Solubility</u>	Soluble in water
pH (Vol.4)	About 8.6 (1 in 20 solution)
<u>Test for carbonate</u> (Vol. 4)	Passes test
<u>Test for ammonia</u> (Vol. 4)	Passes test
<u>Heat test</u>	When heated, it volatilizes without charring and the vapour is alkaline to moist litmus
PURITY	

<u>Non-volatile residue</u> (Vol. 4)	Not more than 500 mg/kg Test 4 g of the sample in 10 ml of water
<u>Chlorides</u>	Not more than 30 mg/kg Dissolve 500 mg of the sample in 10 ml of hot water, add about 5 mg of sodium carbonate, and evaporate to dryness on a steam bath. Test the residue as directed under the Limit Test. Any turbidity produced does not exceed that shown in a control containing 15 µg of chloride ion (Cl^-).
<u>Sulfates</u>	Not more than 50 mg/kg Dissolve 4 g of the sample in 40 ml of water, add about 10mg of sodium carbonate and 1 ml of 30% hydrogen peroxide, and evaporate the solution to dryness on a steam bath. Treat the residue as directed under the Limit Test. Any turbidity produced does not exceed that shown in a control containing 200 µg of sulfate ion (SO_4^{2-}).
<u>Lead</u>	Not more than 2 mg/kg Determine using an atomic absorption technique 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	Place about 10 ml of water in a weighing bottle, tare the bottle and its contents, add about 2 g of the sample and weigh accurately. Transfer the contents of the bottle to a 250-ml flask and slowly add, with mixing, 50 ml of 1 N sulfuric acid. When solution has been effected, wash down the sides of the flask, add methyl orange TS, and titrate the excess acid with 1 N sodium hydroxide. Each ml of 1 N sulfuric acid is equivalent to 17.03 mg of NH_3 .

UREA

Prepared at the 41st JECFA (1993), published in FNP 52 Add 2 (1993). Metals and arsenic specifications revised at the 63rd JECFA (2004). Use level of up to 3% in chewing gum, of no toxicological concern, at 41st JECFA (1993)

SYNONYMS Carbamide

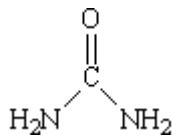
DEFINITION

Chemical names Urea

C.A.S. number 57-13-6

Chemical formula CH₄N₂O

Structural formula



Formula weight 60.06

Assay Not less than 99.0% and not more than 101.0% on the dried basis

DESCRIPTION Colourless or white small pellets or crystalline powder.

FUNCTIONAL USES Texturizer in chewing gum, yeast food

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Very soluble in water; soluble in ethanol

Precipitate formation Dissolve 0.1 g of the sample in water. Add 1 ml of concentrated nitric acid. A white, crystalline precipitate is formed.

Colour reaction Heat 1 g of the sample in a test tube until it liquefies and the liquid becomes turbid. Cool, dissolve in a mixture of 10 ml water and 1 ml of 2 N sodium hydroxide and add 0.05 ml of cupric sulfate TS. A reddish-violet colour is produced.

Melting range (Vol. 4) 132° - 135°

PURITY

Loss on drying (Vol. 4) Not more than 1.0% (105°, 1 h)

Sulfated ash (Vol. 4) Not more than 0.1%

Ethanol-insoluble matter Not more than 0.04%

Dissolve 5.0 g of the sample in 50 ml of warm ethanol. Filter the solution on a tared filter, wash the filter with 20 ml of warm ethanol and dry at 105° for 1

h. Cool in a desiccator and weigh. The difference between the total weight and the weight of the filter is the weight of the ethanol-insoluble matter. Calculate the percentage.

Alkalinity

To 10 ml of a 5.0% solution of the sample add 0.1 ml of methyl red TS and 0.4 ml of 0.01 N hydrochloric acid. The resulting solution is red to orange.

Ammonium ion

Not more than 500 mg/kg

Mix 0.1 ml of a 20% solution of the sample with 14 ml of water in a test tube, make alkaline if necessary by the addition of dilute sodium hydroxide TS and dilute to 15 ml with water. To the solution add 0.3 ml of alkaline potassium tetraiodomercurate solution (dissolve 11 g potassium iodide and 15 g of mercuric iodide in water and dilute to 100 ml. Immediately before use, mix 1 volume of this solution with an equal volume of a 25% solution of sodium hydroxide). Prepare a standard by mixing 10 ml of ammonium-ion solution ($1 \text{ mg NH}_4^+/\text{l}$) with water and add 0.3 ml of alkaline potassium tetraiodomercurate solution. Stopper the test tubes. After 5 min, any yellow colour in the test solution is not more intense than in the standard.

Biuret

Not more than 0.1%

To 10 ml of a 20% solution of the sample add 5 ml water, 0.5 ml of a 0.5 % w/v solution of cupric sulfate and 0.5 ml of 10 N sodium hydroxide. Allow to stand for 5 min. Any reddish-violet colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 10 ml of a 0.02% w/v of biuret in place of the sample solution being examined.

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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

Accurately weigh about 0.5 g of the sample and dissolve in a 10% sulfuric acid solution and dilute to 100 ml with the same acid. Introduce 5.0 ml of this solution into a long-necked combustion flask, add 10 ml of sulfuric acid TS and heat gently until gas is no longer evolved. Boil gently for 10 min, cool and add cautiously 40 ml of water. Cool again and place in a steam-distillation apparatus. Add 50 ml of 10 N sodium hydroxide solution and distil immediately by passing steam through the mixture. Distil for 1 hour, collecting about 50 ml of distillate in 40 ml of a 4% w/v solution of boric acid. Add 0.25 ml of methyl red/methylene blue TS and titrate with 0.1 N hydrochloric acid. Carry out a blank determination. Each ml of 0.1 N hydrochloric acid is equivalent to 3.003 mg of $\text{CH}_4\text{N}_2\text{O}$.

STANNOUS CHLORIDE

Prepared at the 22nd JECFA (1978), published in FNP 7 (1978) and in FNP 52 (1992). Heavy metals and arsenic specifications revised at the 61st JECFA (2003). A PTWI of 0·14 mg/kg bw for tin was established at the 33rd JECFA (1988).

SYNONYMS Tin dichloride; INS No. 512

DEFINITION

Chemical names Tin (II) chloride, stannous chloride dihydrate

C.A.S. number 7772-99-8

Chemical formula $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$

Formula weight 225.63

Assay Not less than 98.0% and not more than 102.0%

DESCRIPTION Colourless or white crystals, odourless or having slight odour of hydrochloric acid

FUNCTIONAL USES Reducer or antioxidant in some bottled or lacquered canned vegetables

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water in less than its own weight of water, but it forms an insoluble basic salt with excess water; soluble in ethanol

Test for stannous ion To a 1 in 20 solution of the sample in dilute hydrochloric acid TS add mercuric chloride TS dropwise. White or greyish precipitate forms

Test for chloride (Vol. 4) Passes test

PURITY

Hydrochloric acid insoluble matter Heat 5 g of the sample to 40° in a mixture of 5 ml of water and 5 ml of hydrochloric acid. The sample should dissolve completely, and the solution should be clear.

Sulfates Not more than 30 mg/kg
Dissolve 5 g of the sample in 5 ml of hydrochloric acid, dilute to 50 ml with water, filter if not clear and heat the filtrate or clear solution to boiling. Add 5 ml of barium chloride TS, digest in a covered beaker on a steam bath for

2 h, and allow to stand overnight. No precipitate forms.

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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**

Transfer about 2 g of sample accurately weighed, to a 250 ml volumetric flask, dissolve in 25 ml of hydrochloric acid, dilute to volume with water, and mix well. Transfer 50 ml of this solution to a 500 ml conical flask, and add 5 g of potassium sodium tartrate, and then a cold saturated solution of sodium bicarbonate until the solution is alkaline to litmus paper. Titrate at once with 0.1 N iodine using starch TS as the indicator. Each ml of 0.1N iodine consumed is equivalent to 11.28 mg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$.

(Note: Stannous salts are readily susceptible to oxidation, yet the method of assay does not take account of this. Distilled water contains dissolved oxygen, therefore water used in the method of assay should be "oxygen free"; this may be achieved by purging the water with nitrogen or carbon dioxide or by boiling the air out. In addition to this the iodine solution used in the determination should be free from dissolved oxygen; ideally the iodine solution should be stored in a self-filling apparatus under carbon dioxide.)

POTASSIUM CHLORIDE

Prepared at the 23rd JECFA (1979), published in FNP 12 (1979) and in FNP 52 (1992). Metals and arsenic specifications revised at the 63rd JECFA (2004). A group ADI 'not limited' for hydrochloric acid and its ammonium, Mg, K salts was established at the 23rd JECFA (1979)

SYNONYMS Sylvine, sylvite; INS No. 508

DEFINITION

Chemical names Potassium chloride

C.A.S. number 7447-40-7

Chemical formula KCl

Formula weight 74.56

Assay Not less than 99.0% on the dried basis

DESCRIPTION Colourless, elongated, prismatic, or cubical crystals, or white granular powder; odourless

FUNCTIONAL USES Seasoning agent, gelling agent, yeast food

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in water; insoluble in ethanol

Test for potassium (Vol. 4) Passes test

Test for chloride (Vol. 4) Passes test

PURITY

Loss on drying (Vol. 4) Not more than 1% (105°, 2 h)

Acidity or alkalinity To a solution of 5 g of the sample in 50 ml of recently boiled and cooled water add 3 drops of phenolphthalein TS. No pink colour is produced. Then add 0.3 ml of 0.02 N sodium hydroxide. A pink colour is produced.

Iodide or bromide Dissolve 2 g of the sample in 6 ml of water, add 1 ml of chloroform, and then add, dropwise and with constant agitation, 5 ml of a mixture of equal parts of chlorine TS and water. The chloroform is free from even a transient violet or permanent orange colour.

Test for sodium (Vol. 4) Negative test

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an atomic absorption technique 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

Dissolve about 250 mg of the dried sample, accurately weighed in 50 ml of water in a glass-stoppered flask. Add, while agitating, 50 ml of 0.1 N silver nitrate, 3 ml of nitric acid, and 5 ml of nitrobenzene, shake vigorously, add 2 ml of ferric ammonium sulfate TS, and titrate the excess silver nitrate with 0.1 N ammonium thiocyanate. Each ml of 0.1 N silver nitrate is equivalent to 7.456 mg of KCl.

DISODIUM ETHYLENEDIAMINETETRAACETATE

Prepared at the 17th JECFA (1973), published in FNP 4 (1978) and FNP 52 (1992). Metals and arsenic specifications revised at the 61st JECFA (2003). An ADI of 0-2.5 mg/kg bw was established at the 17th JECFA (1973)

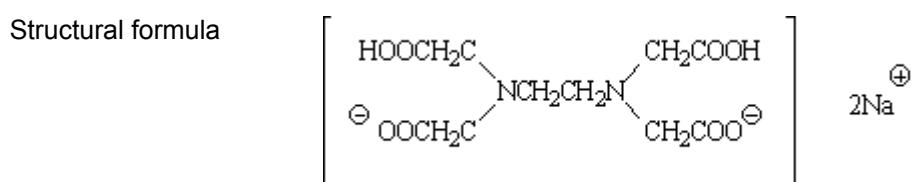
SYNONYMS Disodium EDTA, disodium edetate; INS No. 386

DEFINITION

Chemical names Disodium salt of N,N'-1,2-Ethanediylbis[N-(carboxymethyl)glycine]; disodium dihydrogen ethylenediaminetetraacetate; disodium dihydrogen (ethylene-dinitriilo)-tetraacetate

C.A.S. number 139-33-3

Chemical formula $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$



Formula weight 372.24

Assay Not less than 99%

DESCRIPTION White, odourless crystalline granules or a white to nearly white powder

FUNCTIONAL USES Sequestrant, antioxidant synergist, preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in water, practically insoluble in ethanol

Infrared absorption The infrared spectrum of the sample corresponds with that of a reference standard (a Reference standard may be obtained from the U.S. Pharmacopeia, 12601 Twin Brook Parkway, Rockville, Maryland 20852)

Test for sodium (Vol. 4) Passes test

Chelating activity to metal ions To 5 ml of water in a test tube add 2 drops of ammonium thiocyanate TS and 2 drops of ferric chloride TS. A deep red solution develops. Add about 50 mg of the sample and mix. The deep red colour disappears.

PURITY

pH (Vol. 4) 4.3 - 4.7 (1 in 100 soln)

Nitrilotriacetic acid Passes test
See description under TESTS

<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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."
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TESTS

PURITY TESTS

<u>Nitrilotriacetic acid</u>	<u>Stock test solution</u> Transfer 10 g of the sample into a 100-ml volumetric flask, dissolve in 40 ml of a (1 in 10) potassium hydroxide solution, dilute to volume with water, and mix.
	<u>Diluted stock test solution</u> Pipette 10 ml of the "stock test solution" into a 100-ml volumetric flask, dilute to volume with water, and mix.
	<u>Test preparation</u> Pipette 20 ml of the "diluted stock test solution" into a 150-ml beaker, add 1 ml of a (1 in 10) potassium hydroxide solution, 2 ml of a (1 in 10) ammonium nitrate solution, and about 50 mg of eriochrome black T indicator, and titrate with a (3 in 100) cadmium nitrate solution to a red endpoint. Record the volume, in ml, of the titrant required as V, and discard the solution.
	Pipette 20 ml of the "diluted stock test solution" into a 100-ml volumetric flask, and add the volume V of the (3 in 100) cadmium nitrate solution required in the initial titration, plus 0.05 ml in excess. Add 1.5 ml of a (1 in 10) potassium hydroxide solution, 10 ml of a (1 in 10) ammonium nitrate solution, and 0.5 ml of methyl red TS, then dilute to volume with water and mix.
	<u>Stock standard solution</u> Transfer 1.0 g of nitrilotriacetic acid into a 100-ml volumetric flask, dissolve in 10 ml of a (1 in 10) potassium hydroxide solution, dilute to volume with water, and mix.
	<u>Dilute stock standard solution</u> Pipette 1 ml of the "stock standard solution" and 10 ml of the "stock test solution" into a 100-ml volumetric flask, dilute to volume with water, and mix.
	<u>Standard preparation</u> Proceed as directed under "test preparation", using "dilute stock standard solution" where "diluted stock test solution" is specified.
	<u>Polarographic test</u> Rinse a polarographic cell with a portion of the "standard preparation", then add a suitable volume to the cell, immerse it in a constant-temperature bath maintained at $25 \pm 0.5^\circ$, and de-aerate by bubbling oxygen-free nitrogen

through the solution for 10 min. Insert the dropping mercury electrode of a suitable polarograph, and record the polarogram from -0.6 to -1.2 volts at a sensitivity of 0.006 microampere per mm, using a saturated calomel electrode as the reference electrode. In the same manner, obtain a polarogram for a portion of the "test preparation". The diffusion current observed with the "test preparation" is not greater than 10% of the difference between the diffusion currents observed with the "standard preparation" and the "test preparation", respectively. (Note: An extra polarographic wave appearing ahead of the nitrilotriacetic acid-cadmium complex wave is probably due to uncomplexed cadmium. This wave should be ignored in measuring the diffusion current).

METHOD OF ASSAY

Transfer about 5 g, accurately weighed, of the sample, into a 250-ml volumetric flask, dissolve in water, dilute to volume and mix, to give the assay preparation. Place about 200 mg, accurately weighed, of reagent grade calcium carbonate of known purity in a 400-ml beaker, add 10 ml of water and swirl to form a slurry. Cover the beaker with a watch glass and introduce 2 ml of dilute hydrochloric acid TS from a pipette inserted between the lip of the beaker and the edge of the watch glass. Swirl the contents of the beaker to dissolve the calcium carbonate. Wash down the outer surface of the pipette, the watch glass and the sides of the beaker, and dilute to about 100 ml with water. While stirring the solution, preferably with a magnetic stirrer, add about 30 ml of the assay preparation from a 50-ml burette. Add 15 ml of sodium hydroxide TS, 300 mg of hydroxynaphthol blue indicator and continue the titration with the assay preparation to a blue end point. Calculate the percentage of $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$ in the sample by the formula:

$$\% \text{ disodium EDTA} = \frac{92,980 \times W_{CaCO_3}}{V_{ASSAY} \times W_{SAMPLE}}$$

where

W_{CaCO_3} = the weight in grams of calcium carbonate;

V_{ASSAY} = the volume in ml of assay preparation; and

W_{SAMPLE} = the weight in gram of the sample taken.

ACTIVATED CARBON

Prepared at the 73rd JECFA (2010) and published in FAO JECFA Monographs 10 (2010), superseding specifications prepared at the 37th JECFA (1990) and published in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). No ADI was established at the 31st JECFA (1987).

SYNONYMS

Activated charcoal, decolourizing carbon

DEFINITION

A solid, porous, carbonaceous material prepared by carbonizing and activating organic substances. The raw materials, which include sawdust, peat, lignite, coal, cellulose residues, coconut shells, petroleum coke, etc., may be carbonized and activated at high temperature with or without the addition of inorganic salts in a stream of activating gases such as steam or carbon dioxide. Alternatively, carbonaceous matter may be treated with a chemical activating agent such as phosphoric acid or zinc chloride and the mixture carbonized at an elevated temperature, followed by removal of the chemical activating agent by water washing.

Chemical names Carbon

C.A.S. number 7440-44-0

Chemical formula C

Formula weight 12.01

DESCRIPTION Powder or granules, black, odourless

FUNCTIONAL USES Adsorbent, decolourizing agent

CHARACTERISTICS

IDENTIFICATION

Solubility Insoluble in water and organic solvents

Adsorption Place about 3 g of powdered sample in a glass-stoppered flask containing 10 ml of dilute hydrochloric acid (5%), boil for 30 s, and cool to room temperature. Add 100 ml of iodine TS, stopper, and shake vigorously for 30 sec. Filter through filter paper (Whatman No. 2 or equivalent), discarding the first portion of filtrate. Compare 50 ml of the filtrate with a reference solution prepared by diluting 10 ml of iodine to 50 ml with water, but not treated with carbon. The colour of the carbon treated iodine solution shall be lighter in colour than that of the reference solution, indicating the adsorptivity of the sample.

PURITY

<u>Adsorption power</u>	Not less than 90% and not more than 110% of the value stated on label. See description under TESTS
<u>Loss on drying</u> (Vol. 4)	Not more than 15% (120° , 4 h) (See Volume 4 under "GENERAL METHODS, Inorganic Components.")
<u>Sulfide compounds</u>	To 1.0 g of the sample in a conical flask add 5 ml of 1 N hydrochloric acid and 20 ml of water. Heat to boiling. The fumes released do not turn lead acetate paper brown. (Lead acetate paper is prepared by saturating filter paper with lead acetate TS and drying the paper at 100°).
<u>Acid soluble substances</u>	Not more than 3% To about 1 g of the sample, accurately weighed, add 25 ml of dilute nitric acid TS and boil for 5 min. Filter whilst hot through a sintered-glass filter (10) and wash with 10 ml of hot water. Evaporate the combined filtrate and washings to dryness on a water bath, add to the residue 1 ml of hydrochloric acid, evaporate to dryness again and dry the residue to constant weight at $103\pm2^{\circ}$.
<u>Sulfated ash</u>	Not more than 5% Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator and weigh. Accurately weigh about 1 g of sample in the crucible and add 2 ml of sulfuric acid TS. Heat at first on a water bath, then cautiously over a flame, then progressively to about 600° . Continue the incineration until all black particles have disappeared and allow the crucible to cool. Add a few drops of dilute sulfuric acid TS, heat and incinerate as before and allow to cool. Evaporate and incinerate carefully, allow to cool, weigh, and repeat the ignition for 15 min to constant weight.
<u>Water extractable substances</u>	Not more than 4% Transfer about 5 g of sample, accurately weighed, into a 250 ml flask provided with a reflux condenser and a Bunsen valve. Add 100 ml of water and several glass beads, and reflux for 1 h. Cool slightly, and filter through Whatman No 2 or equivalent filter paper, discarding the first 10 ml of filtrate. Cool the filtrate to room temperature, and pipet 25.0 ml into a tared dish. Evaporate the filtrate in the dish to incipient dryness on a hot plate never allowing the solution to boil. Dry for 1 h at $103\pm2^{\circ}$ in a vacuum oven, cool and weigh. Calculate the percentage of water extractables in the filtrate, based on the sample weight and volume of sample taken for gravimetric measurement.
<u>Alcohol soluble substances</u>	Not more than 0.5% To 2.0 g of sample add 50 ml of ethanol (96 per cent) and boil under a reflux condenser for 10 min. Filter immediately, wash residue with 10 ml of warm ethanol and filter. Quantitatively transfer the combined filtrate into a tared beaker containing a few

antibumping stones. Evaporate to dryness on a water bath and dry to a constant mass at $103\pm 2^\circ$. The residue on evaporation weighs not more than 10 mg.

Alkali soluble coloured substances

To 0.25 g of sample add 10 ml of 2 N sodium hydroxide and boil for 1 min. Cool, filter and dilute the filtrate to 10 ml with water. Prepare a reference solution by mixing 1.90 ml of solution A (1% hydrochloric acid) and 0.10 ml of a solution B (9.6 ml of ferric chloride TS + 0.2 ml of cobaltous chloride TS + 0.2 ml of cupric sulfate TS). The colour of sample solution shall not be more intense than that of the reference solution.

Cyanogen compounds

Mix 5 g of sample with 50 ml of water and 2 g of tartaric acid. Distil the mixture, collecting 25 ml of distillate below the surface of a mixture of 2 ml of sodium hydroxide TS and 10 ml of water contained in a small flask placed in an ice bath. Dilute the distillate to 50 ml with water, and mix. Add 12 drops of ferrous sulfate TS to 25 ml of the diluted distillate, heat almost to boiling, cool, and add 1 ml of hydrochloric acid. No blue colour is produced.

Higher aromatic hydrocarbons

Extract 5 g of the sample with about 45 ml of cyclohexane in a continuous extraction apparatus for 2 h. Collect the extract and dilute to 50 ml with cyclohexane. Examine under ultraviolet light at 365 nm. The colour or fluorescence of the solution is not more intense than that of a 83 ng/ml solution of quinine prepared in 0.01N sulfuric acid, examined under the same conditions.

Arsenic (Vol. 4)

Not more than 3 mg/kg
Accurately weigh about 4 g of the sample into a conical flask, add 80 ml of 2 N hydrochloric acid, extra pure, and boil gently under reflux for 1 h, filter and wash the filter with 2 N hydrochloric acid. Cool and quantitatively transfer the filtrate into 100 ml volumetric flask and make up to volume with the same acid. Determine arsenic using atomic absorption hydride generation technique.

Lead (Vol. 4)

Not more than 5 mg/kg
Determine using an AAS/ICP-AES technique appropriate to the specified level using the solution prepared under arsenic.

Zinc (Vol.4)

Not more than 25 mg/kg
Determine using an AAS/ICP-AES technique appropriate to the specified level using the solution prepared under arsenic.

TESTS

PURITY TESTS

Adsorption power

To about 0.3 g of dried sample, accurately weighed, in a 100 ml ground-glass-stoppered conical flask, add 25.0 ml of a freshly prepared solution of 0.5 g of phenazone in 50 ml of water. Shake thoroughly for 15 min. Filter and reject the first 5 ml of filtrate. Pipette 10.0 ml of the filtrate into a conical flask, add 1.0 g of potassium bromide and 20 ml of dilute hydrochloric acid TS. Using 0.1 ml of ethoxychrysoidine solution as indicator, titrate

with 0.1 N potassium bromate until the colour changes from reddish-pink to yellowish-pink. Titrate slowly (1 drop every 15 sec) towards the end of the titration. Carry out a blank titration using 10.0 ml of the phenazone solution.

Calculate adsorption power from:

$$[235.3 (a - b)]/[d \times m]$$

where

a is the volume (ml) of 0.1 N potassium bromate consumed by the blank;

b is the volume (ml) of 0.1 N potassium bromate consumed by the test solution;

m is the mass (g) of dried sample; and

d is the value stated on the label.

PROPYLENE OXIDE

Prepared at the 3rd JECFA (1958), published in Specifications for Identity and Purity of Food Additives, Vol. I, FAO (1962) and in FNP 52 (1992). No ADI was allocated at the 3rd JECFA (1958)

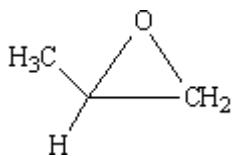
DEFINITION

Chemical names Propylene oxide, methyl oxirane, 1,2-epoxypropane

C.A.S. number 75-56-9

Chemical formula C₃H₆O

Structural formula



Formula weight 58.08

Assay Not less than 99%

DESCRIPTION A colourless liquid with a sweetish odour

FUNCTIONAL USES Antimicrobial preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) 1 g of the sample is soluble in 1.7 ml of water. Miscible in ethanol and in ether

Infrared absorption Infrared spectrum is recommended

PURITY

Water (Vol. 4) Not more than 0.1% (Karl Fischer Method)

Distillation range 32 - 37° (at 760 mm Hg)
See description under TESTS

Insoluble matter (Vol. 4) Substantially free from suspended matter

Non-volatile residue
(Vol. 4) Not more than 0.1%

Total acidity Not more than 0.05% (as acetic acid)
See description under TESTS

Total chlorides Not more than 0.1%
See description under TESTS

Aldehydes

Passes test
See description under TESTS

TESTS

PURITY TESTS

Distillation range

Measure a 100-ml sample in a 100-ml graduated cylinder at 10 to 15° and transfer directly to the distillation flask. Connect the flask to the condenser. Place the graduated cylinder used to measure the sample, without rinsing or drying, at the outlet of the condenser tube in such a position that the condenser tube extends into it for at least 2.5 cm but not below the 100-ml mark. Immerse the receiving cylinder up to the 100- ml mark in a transparent bath maintained between 10 and 15°. Cover the top of the graduated cylinder with a piece of blotting paper cut so as to fit tightly over the end of the condenser tube. Apply heat slowly with a micro-Bunsen burner and regulate (a good needle valve is best) so that the first drop of condensate falls from the condenser in not less than 5 or more than 10 min. Adjust the receiving cylinder so that the end of the condenser tube touches its side. Regulate the heat so that the distillation proceeds at a uniform rate of 4 to 5 ml per min. Maintain this rate and continue the distillation to dryness. Record the temperature when the first drop of distillation falls from the end of the condenser. Take readings when the level reaches 5 ml, 10 ml, and each additional 10 ml, including 90 ml, and at 95 ml. Take the final reading when the liquid disappears from the bottom of the flask. the boiling points observed may be corrected for barometric pressure by adding 0.044° to the observed boiling point for each mm Hg pressure under 760 mm or by subtracting and equal amount for each mm Hg pressure over 760 mm. The range of difference between the initial boiling point and the dry point should be 32-37°.

Total acidity

Measure 50 ml of the sample in a chilled 100-ml graudate cylinder and transfer to a cooled 250-ml conical flask. Add 1 ml of phenolphthalein TS and titrate with 0.01 N sodium hydroxide to a faint pink colour which persists for at least 15 sec. The proportion, expressed as acetic acid, should not exceed 0.05%.

$$\% \text{ acetic acid} = \frac{\text{ml of } 0.01 \text{ N NaOH} \times 0.060}{\text{ml of sample} \times \text{specific gravity}} \times 100$$

Total chlorides

Method A:

Connect a weighed stainless steel sample cylinder (30-ml capacity) to the sample container and flush the cylinder for a few sec with liquid ethylene oxide. Close the valve on the cylinder and allow it to fill to between 22 and 25 g of the sample. Weight the cylinder and contents to the nearest 0.1 g. Connect the cylinder to the burner assembly as shown in Figure 1. Pour 50±0.5 ml of freshly prepared 0.02 N sodium arsenite into the gas absorber. Connect the absorber to the air condenser. Connect the vacuum line to the gas absorber and apply a light vacuum to the system. Light the pilot burner and burn the sample in the following manner: allow a small flow of oxygen to the ring around the burner (Figure 1, valve A). Allow a small flow of hydrogen to the burner (Figure 1, valve B) and light it with a flint lighter.

Adjust the flame to a height of about 2.5 cm. Regulate the oxygen flow to the burner tip so as to obtain a flame cone about 1.3 cm high (Figure 1, valve C). Set the chimney in place secure the clamps. Place a beaker of hot water (50°) around the cylinder (replenish with warm water during the run) Open the Hoke valve very slowly and allow the sample to raise the flame height to about 6.5-7.5 cm. Adjust the oxygen flow to the burner tip to obtain a cone in the flame about 1.3 cm high.

Readjust the vacuum as necessary to ensure a slight vacuum in the system at all times. Allow the sample to burn until the flame gives evidence that the sample is almost burned out (20 to 25 min) then adjust the oxygen flow to the burner to keep the flame cone at about 1.3 cm. Adjust the Hoke valve on the sample cylinder during the run so that when the run is finished the valve is completely open. Shut off the oxygen flow to the tip of the burner when the sample is burned out, then shut off the hydrogen flow. Allow several min for the system to be purged with the oxygen flow from the ring around the burner. Then release the vacuum and shut off the oxygen flow to the ring. Allow the condenser and glass to cool to room temperature and then rinse with a minimum amount of water into the gas scrubber. Transfer the contents of the gas-washing bottle to a 250-ml beaker and chill to about 5° in a refrigerator or in crushed ice. Insert a magnetic agitator and the freshly washed silver chloride electrodes into the beaker. Acidify the solution with nitric acid using methyl red TS as indicator. Titrate the solution potentiometrically with 0.02 N silver nitrate solution. Prepare a blank solution of the reagents and titrate. The proportion of total chlorides should not exceed 0.1%.

$$\% \text{ total chlorides} = \frac{(V_1 - V_2) \times N \times 0.0355}{\text{weight of sample}} \times 100$$

where

V_1 = ml of silver nitrate required by the sample

V_2 = ml of silver nitrate required by the blank

N = normality of the silver nitrate

Method B (Alternative method):

Cool a 50-ml graduated cylinder by immersing in ice water or by placing in a refrigerator at 0° for about 15 min. Pour 50 ml of sodium ethoxide TS and 70 ml of methyl alcohol into a 250-ml conical flask and place in ice water or in refrigerator at 0° for 30 min. Pour as rapidly as possible 30 ml of the sample into the cooled cylinder and transfer the contents to the flask containing the cooled sodium ethoxide. Attach a water-cooled reflux condenser and gently boil the mixture for 1 h. Cool and transfer the mixture to a 600-ml beaker rinsing the flask with 100 ml of water. Make the liquid just acid with 1.5 N nitric acid (usually about 130 ml) using litmus paper as indicator and add 10 ml more. Keep the solution cool (below 30°) and stir constantly during the neutralization. Titrate the chloride present electrometrically using 0.025 N silver nitrate. Prepare a blank in the same way but omitting the sample.

$$\% \text{ total chlorine} = \frac{(V - V_b) \times N \times 35.5}{10V_s \times 0.89}$$

where

V = volume of silver nitrate used in titrating sample

V_b = volume of silver nitrate used in titrating blank

N = normality of silver nitrate

V_s = volume of sample.

Aldehydes

Measure 20 ml of the sample into a glass-stoppered conical flask containing 5 ml of chilled sodium bisulfite TS and 25 ml of water. Place the flask and its contents in a refrigerator for exactly 15 min. Prepare a reagent blank along with each set of samples and treat it in the same manner as the samples. Titrate the excess bisulfite solution in the blank and sample flasks with 0.1 N iodine solution until persistence of the yellow brown colour due to the liberated iodine. The proportion, expressed as propionic aldehyde, should not exceed 0.1%. Calculate the ion % propionic aldehyde from:

$$\frac{(V_b - V_s) \times N \times 0.029}{(ml \text{ of sample}) \times \text{specific gravity}} \times 100$$

where

V_s = ml of iodine solution required by the sample

V_b = ml of iodine solution required by the blank

N = normality of iodine solution.

METHOD OF ASSAY

Introduce 0.8000-1.3000 g of propylene oxide into a tared thin-walled glass bulb (15 mm in diameter with a neck 30 mm long) which is packed in dry ice. Seal the bulb with the flame from an oxygen-gas torch, remove the bulb from the dry ice, wash with acetone, dry in a 60° oven, cool and weigh.

From a pipette introduce 25 ml of pyridinium chloride-chloroform TS into a pressure bottle. Insert the bulb into the pressure bottle, seal the latter, and place it in an ice bath for 30 min. Remove the bottle from the ice bath, put it into a canvas bag and shake to break the bulb. After the bulb is broken, allow the pressure bottle to warm to room temperature and then put it into a steam bath for 20 min. Remove the bottle from the steam bath and cool.

After the bottle is cool remove the canvas bag, wash the stopper and neck of the bottle with methanol, add 10 drops of phenolphthalein TS and titrate with N sodium hydroxide in methanol (which should be freshly standardized before using). Two blanks should be run with the sample (A N aqueous solution of sodium hydroxide may be used if methanol is added to the chloroform solution in the pressure bottle.)

Calculate the % propylene oxide from:

$$\frac{ml \text{ of } N \text{ NaOH} \times 0.0581}{(weight \text{ of sample})} \times 100$$

SORBITAN MONOPALMITATE

Prepared at the 17th JECFA (1973), published in FNP 4 (1978) and in FNP 52 (1992). Metals and arsenic specifications revised at the 55th JECFA (2000). A group ADI of 0-25 mg/kg bw as the sum of sorbitan esters of lauric, oleic, palmitic and stearic acids was established at the 26th JECFA (1982)

SYNOMYS

INS No. 495

DEFINITION

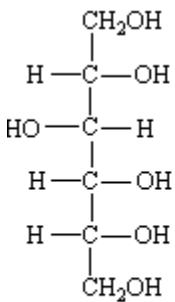
A mixture of the partial esters of sorbitol and its mono- and dianhydrides with edible commercial palmitic acid

C.A.S. number

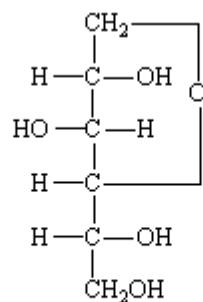
26266-57-9

Structural formula

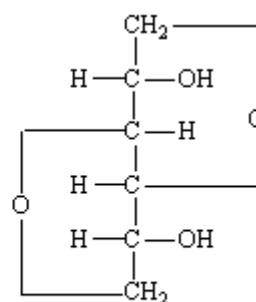
Contains palmitic acid esterified with polyols derived from sorbitol including the following types:



Sorbitol



1,4-Sorbitan



Isosorbide

Assay

Saponification of 100 g of the sample yields approximately 37 g of polyols and 65 g of fatty acid. The polyol content shall be approximately 95% of a mixture of sorbitol, 1,4-sorbitan and isosorbide

DESCRIPTION

Light cream to tan beads or flakes or hard, waxy solid with a characteristic odour

FUNCTIONAL USES

Emulsifier

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Soluble at temperatures above its melting point in ethanol, methanol, ether, ethylacetate, aniline, toluene, dioxane, petroleum ether and carbon tetrachloride; insoluble in cold water but dispersible in warm water.

Congealing range (Vol. 4) 45 - 47°

Infrared absorption

The infrared spectrum of the sample is characteristic of a partial fatty acid ester of a polyol

PURITY

<u>Water</u> (Vol. 4)	Not more than 1.5% (Karl Fischer Method)
<u>Acid value</u> (Vol. 4)	Not less than 4.0 and not more than 7.5
<u>Saponification value</u> (Vol. 4)	Not less than 140 and not more than 150
<u>Hydroxyl value</u> (Vol. 4)	Not less than 270 and not more than 305
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique 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**

Proceed as directed under the *Sorbitan Ester Content* (Volume 4)

β-CYCLODEXTRIN

Prepared at the 44th JECFA (1995), published in FNP 52 Add 3 (1995) superseding specifications prepared at the 41st JECFA (1993), published in FNP 52 Add 2 (1993). Metals and arsenic specifications revised at the 63rd JECFA (2004). An ADI of 0.5 mg/kg bw was established at the 44th JECFA (1995)

SYNOMYS

Beta-cyclodextrin, βCD, BCD, β-Schardinger dextrin, cyclodextrin B, INS No. 459

DEFINITION

A non-reducing cyclic saccharide consisting of seven alpha-1,4-linked D-glucopyranosyl units manufactured by the action of cyclodextrin transglycolase on hydrolysed starch followed by purification of the β-cyclodextrin; purification is by preparation of a β-cyclodextrin/solvent inclusion compound followed by steam-stripping of the solvent before final purification.

Chemical names

Cycloheptaamylose

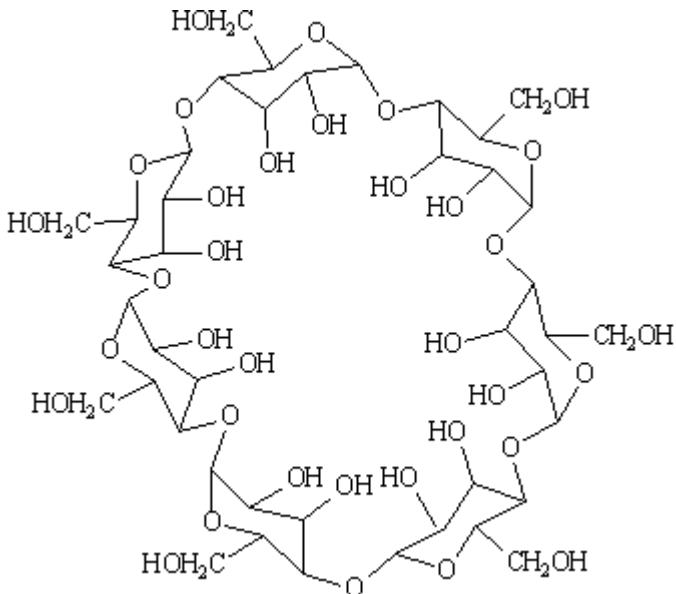
C.A.S. number

7585-39-9

Chemical formula

(C₆H₁₀O₅)₇

Structural formula



Formula weight

1135.00

Assay

Not less than 98.0% of (C₆H₁₀O₅)₇ on an anhydrous basis

DESCRIPTION

Virtually odourless, slightly sweet tasting white or almost white crystalline solid

FUNCTIONAL USES Encapsulation agent for food additives, flavouring and vitamins

CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Sparingly soluble in water; freely soluble in hot water; slightly soluble in ethanol
<u>Specific rotation</u> (Vol. 4)	[alpha] 25, D: Between +160 and +164° (1% solution)
<u>Infrared absorption</u>	The infrared spectrum of the sample corresponds with that of a reference standard.
<u>Chromatography</u>	The retention time for the major peak in the liquid chromatogram of the sample solution corresponds to that for β-cyclodextrin in the chromatograms of the standard solutions prepared as directed in the Method of Assay.

PURITY

<u>Water</u> (Vol. 4)	Not more than 14% (Karl Fischer Method)
<u>Other cyclodextrins</u>	Not more than 2% on an anhydrous basis See description under TESTS
<u>Residual solvents</u>	Not more than 1 mg/kg of each of toluene and trichloroethylene See description under TESTS
<u>Reducing substances</u> (Vol. 4)	Not more than 1% (as glucose)
<u>Sulfated ash</u> (Vol. 4)	Not more than 0.1%
<u>Lead</u> (Vol. 4)	Not more than 1 mg/kg Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

<u>Other cyclodextrins</u>	Analyses of alpha- and gamma-cyclodextrins are included in the Method of Assay. Adjust the attenuation of the instrument or adjust sample size to obtain a chromatogram in which the β-cyclodextrin peak height nearly reaches the top of the recording chart. Measure peak heights or peak areas of the alpha-, β- and gamma-cyclodextrin responses. Calculate % other cyclodextrins (CX) using the formula: $\% \text{ Other CX} = \frac{\alpha\text{peaks} + \gamma\text{peaks}}{\alpha\text{peaks} + \beta\text{peaks} + \gamma\text{peaks}} \times 100$
<u>Residual solvents</u>	A dynamic-headspace gas chromatographic technique is used for the following procedure. The organic volatile impurities are trapped on an absorbent trap and the purge gas is vented. The trapped organic volatile

impurities are desorbed from the trap by heating the trap, and carried into the gas chromatograph by back flushing the trap with the carrier gas. Quantitate each solvent by the technique of standard additions.

Purge and Trap Apparatus

(The apparatus is based on that described in the US Environmental Protection Agency Test Method for Purgeable Halocarbons - Method 601): The apparatus consists of three separate sections: the sample purge; the trap; and the desorber. The sample purge is designed to accept 5 ml samples with a water column at least 3 cm deep. The gaseous headspace between the water column and the trap has a total volume of less than 15 ml. The purge gas is passed through the water column as finely-divided bubbles with a diameter of less than 3 mm at the origin. The purge gas is introduced not more than 5 mm from the base of the water column. The trap is not less than 25 cm long and has an inside diameter of not less than 2.67 mm. The trap is packed to contain the indicated minimum lengths of adsorbents in the following order, beginning at the trap inlet:

7.7 cm of 2,6-diphenylene oxide polymer (the 2,6-diphenylene oxide polymer is commercially available as TENAX TA), 7.7 cm of silica gel, and 7.7 cm of coconut charcoal.

The desorber is capable of rapidly heating the trap at 2500. The trap should not be heated higher than 2500.

Condition the assembled trap, prior to initial use, at 2250 overnight with an inert gas at a flow rate of not less than 20 ml per min. Prior to use daily, condition the trap for 15 min at 225°.

Standard Solution

Accurately weigh 50 mg of trichloroethylene and 50 mg of toluene in a 50 ml volumetric flask. Dilute with methanol.

Calibration Solutions

Into five 50 ml volumetric flasks, accurately add 0.5, 1.0, 2.0, 3.0, and 5.0 ml of the Standard Solution and dilute with water. These calibration solutions correspond to the concentrations 10.2, 20.4, 40.8, 61.2 and 102 ng per for each solvent.

Chromatographic system

The purge and trap apparatus is connected to a gas chromatograph with a flame-ionisation detector.

Column: capillary column, 30 m, 0.32 mm diameter, 1 micron film thickness of dimethylpolysiloxane oil (such as DB-1, OV-1).

Temperature programme: 40° for 3 min, then raise to 220° at 40 per min.

Detector: 280°

Carrier gas: Helium

Purge gas: Nitrogen

Flow rate: 40 ml/min

Calibration

Introduce precisely 20 µl of each calibration solution on the wall (inner side)

of the sample purge. Desorb according to equipment instructions. Record the peak areas. Prepare calibration graphs of peak areas versus weight of each solvent introduced into the purge.

Procedure

Introduce on the fritted sparger of the sample purge an accurately weighed amount of sample (W), about 250 mg. Purge and desorb according to equipment instructions. Record the peak area of each solvent and read the corresponding weight (X) from the respective calibration curve.

Calculation

Calculate the amount of each residual solvent by the formula:

$$\text{Residual solvent (mg / kg)} = \frac{X \text{ (ng)}}{W \text{ (mg)}}$$

METHOD OF ASSAY

Principle

β -Cyclodextrin is identified by *liquid chromatography* and quantified by comparison to reference standards containing standard cyclodextrins.

Preparation of sample solution

Weigh accurately about 500 mg of sample. Add 50 ml of twice-distilled water. Heat and stir until the sample has completely dissolved. Cool, adjust the total volume to 100 ml. Filter on a Millex HA 0.45 μm filter.

Preparation of standard solutions

Use USP grade alpha- and β -cyclodextrin. Samples of gamma-cyclodextrin can be obtained from commercial suppliers such as Aldrich Chemical Co. or Sigma Chemical Co. Prepare three standard solutions (S_1 , S_2 and S_3) containing increasing amounts (mg/kg) of alpha-cyclodextrin, β -cyclodextrin and gamma-cyclodextrin as follows:

S_1 : 2.0 mg/kg A + 3.0 mg/kg B + 2.0 mg/kg G

S_2 : 3.5 mg/kg A + 5.0 mg/kg B + 3.5 mg/kg G

S_3 : 5.0 mg/kg A + 8.0 mg/kg B + 5.0 mg/kg G

where

A = alpha- cyclodextrin

B = β -cyclodextrin

G = gamma-cyclodextrin

Apparatus

Liquid chromatograph maintained at a constant temperature of 25° and equipped with a refractive index detector.

Conditions

Column

- length: 25 cm

- diameter: 4.6 mm

- packing: 5 μm octadecylsilane bonded to silica (Silica C18) with a guard column containing the same packing

Solvent: Water: methanol (94:6)

Flow rate: 0.7 ml/min

Procedure

Inject 10 µl of each of the 3 standard solutions. For each cyclodextrin draw a graph by plotting on the x axis the concentration in g/l and on the y axis the areas of the peaks. Inject 10 µl of the sample solution and determine the area of the eluted β-cyclodextrin peak. The concentration of β-cyclodextrin (L g/l) in the sample solution is then read from the graphs.

Calculation

Calculate the content of β-cyclodextrin in the sample using the formula:

$$B = \frac{L}{C} \times 100$$

where

B = percentage of β-cyclodextrin in the sample

L = the concentration of β-cyclodextrin in the sample solution as determined under "Procedure"

C = the concentration of sample in the sample solution in g/l.

HELIUM

New specifications prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7 (1999).

SYNOMYS INS No. 939

DEFINITION

Chemical name Helium

C.A.S. number 7440-59-7

Chemical formula He

Formula weight 4.0

Assay Not less than 99.0% by volume

DESCRIPTION Colourless and odourless gas

FUNCTIONAL USES Processing aid

CHARACTERISTICS

IDENTIFICATION

Flame test The flame of a burning splinter of wood is extinguished when inserted into an inverted test tube filled with helium.

GC test See Method of Assay

PURITY

Air Not more than 1.0%, by volume
See Method of Assay

Carbon monoxide Not more than 10 µl/l, by volume
Pass 1050 ± 50 ml of the gas sample through a carbon monoxide detector tube at the rate specified for the tube. The indicator change corresponds to not more than 10 µl/l.

Odour Carefully open the container valve to produce a moderate flow of gas. Do not direct the gas stream toward the face, but deflect portion of the stream toward the nose: no appreciable odour is discernible.

METHOD OF ASSAY Determine by Gas chromatography (see Volume 4) using the following conditions:

Column
- material: stainless steel

- length: 6 m
- internal diameter: 4 mm
- packing material: PoraPak Q, or equivalent

Carrier

- gas: Helium (99.99% (v/v))
- flow 40 ml/min

Detector: thermal conductivity detector

Injector: loop injector

Column temperature: 60°

Detector temperature: 130°

Procedure

Introduce a specimen of helium into a gas chromatograph by means of gas sampling valve. Select the operating conditions of the gas chromatograph so that the standard peak signal resulting from the following procedure corresponds to not less than 70% of the full-scale reading, and which permit complete separation of nitrogen and oxygen from helium, although nitrogen and oxygen may not be separated from each other. The peak response produced by the assay specimen exhibits a retention time corresponding to that produced by an air-helium certified standard (a mixture of 1.0% air in industrial-grade helium is available from most suppliers) and indicates not more than 1.0% air, by volume, when compared with the peak response of the air-helium certified standard, and not less than 99.0% of He, by volume.

ISOPROPYL CITRATE MIXTURE

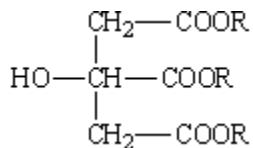
Prepared at the 17th JECFA (1973), published in FNP 4 (1978) and in FNP 52 (1992). Metals and arsenic specifications revised at the 61st JECFA (2003). An ADI of 0-14 mg/kg bw was established at the 17th JECFA (1973)

SYNOMYS INS No. 384

DEFINITION

Chemical names Citric acid mixed ester of 2-propanol. The article of commerce, monoisopropyl citrate mixture, is composed of approximately 38 parts by weight of isopropyl citrate in 62 pars by weight of mono- and diglycerides

Structural formula



where R is either hydrogen or a isopropyl group. The major component of the 38 parts of isopropyl citrate mixture is monoisopropyl citrate (approximately 25 parts), the remainder being diisopropyl citrate (approximately 9 parts) and triisopropyl citrate (approximately 4 parts)

Approximate composition:

Monoisopropyl citrate - 27 parts by weight
Diisopropyl citrate - 9 parts by weight
Triisopropyl citrate - 2 parts by weight

DESCRIPTION

Oil miscible semi-solid material. The commercial product, monoisopropyl citrate mixture, is a viscous, colourless syrup exhibiting some crystallization upon standing, and may be further specified as to saponification value, acid value, citric acid and isopropyl content.

FUNCTIONAL USES Antioxidant, sequestrant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Soluble in water and ethanol

Test for citrate Reflux 3 g of sample with 50 ml of sodium hydroxide TS for 1 h, and let stand to cool. This solution is used for the following tests:

(1) Neutralize the solution with a (1 in 20) sulfuric acid solution, add an excess of mercuric sulfate TS, heat to boil, and add potassium permanganate TS. The permanganate colour of the solution disappears, and a white precipitate forms.

(2) Neutralize the solution with hydrochloric acid, add an excess of calcium chloride TS, and boil. A white crystalline precipitate is formed which is insoluble in sodium hydroxide TS, but soluble in dilute hydrochloric acid

TS.

Test for isopropanol

Reflux 2 g of sample with 50 ml of sodium hydroxide TS for 1 h. Distil off 20 ml. Place 8 g of chromic oxide in a flask, add 15 ml water and 2 ml concentrated sulfuric acid. Provide the flask with a reflux condenser and add 5 ml distillate slowly through the condenser. Reflux for 30 min, then cool and distil off 2 ml. Add 3 ml water and 10 ml mercuric sulfate TS to the distillate. Heat in a boiling water bath for 3 min. A white or yellow precipitate within 3 min indicates the presence of isopropanol.

PURITY

Acids other than citric acid Should be absent

Alcohols other than isopropanol Should be absent

Sulfated ash (Vol. 4) Not more than 0.3%

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an atomic absorption technique 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."



Food and Agriculture
Organization of the
United Nations



World Health
Organization

Specification Monograph prepared by the meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), 53rd Meeting 1999

RIBOFLAVIN from *BACILLUS SUBTILIS*

This monograph was also published in: Compendium of Food Additive Specifications. Joint FAO/WHO Expert Committee on Food Additives (JECFA), 53rd meeting 1999. FAO JECFA Monograph 1 (2006)

RIBOFLAVIN from *BACILLUS SUBTILIS*

Prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7 (1999), superseding specifications prepared at the 51st JECFA (1998), published in FNP 52 Add 6. Group ADI 0-0.5 mg/kg bw for riboflavin from *Bacillus subtilis*, synthetic riboflavin and riboflavin-5-phosphate established at the 51st JECFA in 1998.

SYNONYMS

Vitamin B₂; lactoflavin; INS No. 101(iii)

SOURCE

Prepared by submerged fermentation by *Bacillus subtilis* genetically modified for riboflavin overproduction. The strain is non-pathogenic and non-toxicogenic.

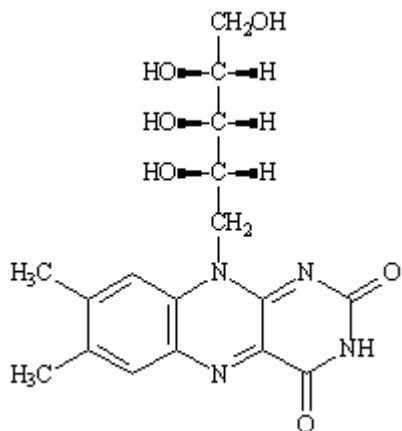
DEFINITION

Chemical names Riboflavin; 3,10-dihydro-7,8-dimethyl-10-[(2S,3S,4R)-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 C₁₇H₂₀N₄O₆

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, nutrient supplement

CHARACTERISTICS

IDENTIFICATION

<u>Solubility</u> (Vol. 4)	Practically insoluble in ethanol, acetone and diethyl ether; very soluble in dilute alkali solutions
<u>Spectrophotometry</u> (Vol. 4)	Using the aqueous solution from the Assay, determine the absorbance (A) 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.
<u>Specific rotation</u>	$[\alpha]^{20}_D$: Between -120 and -135° Dry the sample at 100° for 4 h. Dissolve 50.0 mg in 0.05 N sodium hydroxide free from carbonate and dilute to 10.0 ml with the same solvent. Measure the optical rotation within 30 min of dissolution.
<u>Colour reaction</u>	Dissolve about 1 mg of sample in 100 ml of water. The solution has a pale greenish-yellow colour by transmitted light, and by reflected light has an intense yellowish-green fluorescence, which disappears on the addition of mineral acids and alkalis.

PURITY

<u>Loss on drying</u> (Vol. 4)	Not more than 2.0% (105°, 4 h)
<u>Sulfated ash</u> (Vol. 4)	Not more than 0.1% Test 2 g of the sample (Method I)
<u>Lumiflavin</u> (Vol. 4)	Not more than 0.025% See description under TESTS
<u>Primary aromatic amines</u> (Vol. 4)	Not more than 100 mg/kg calculated as aniline
<u>Lead</u> (Vol. 4)	Not more than 1 mg/kg Determine using an atomic absorption technique 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."

TESTS

PURITY TESTS

<u>Lumiflavin</u> (Vol. 4)	Reference Solution: Dissolve 25 mg of lumiflavin in 50.0 ml of chloroform. Dilute 1.0 ml of this solution with chloroform to 20.0 ml, and dilute 2.5 ml of the resultant solution to 100 ml. This solution contains 0.625 µg lumiflavin per ml. Test Solution: Shake 25 mg of the sample with 10.0 ml chloroform for 5 min and filter.
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Thin Layer Chromatography:

Stationary phase: Precoated HPTLC plates of silica gel WRF₂₅₄, 10 x 20 cm, layer thickness 0.1 mm (Merck Cat No 1.12363)

Mobile phase: Water

Run length: approx. 6 cm

Elution time: approx. 20 min

Application volumes: 10 µl of Reference Solution and 10 µl of Test Solution

Detection: Dry the plate in a current of cold air and evaluate the fluorescence at 366 nm

Any spot in the chromatogram of the Test Solution, which corresponds to the main spot of the Reference Solution, shall not be larger or more intensely coloured than the Reference Solution spot.

**METHOD OF
ASSAY**

Carry out the assay in subdued light. In a brown-glass 500-ml volumetric flask, suspend 65.0 mg of the sample in 5 ml of water, ensuring that it is completely wetted, and dissolve in 5 ml of 2 N sodium hydroxide solution. As soon as dissolution is complete, add 100 ml of water and 2.5 ml of glacial acetic acid and dilute to 500.0 ml with water. Place 20.0 ml of this solution in a brown glass 200-ml volumetric flask, add 3.5 ml of a 1.4% w/v solution of sodium acetate and dilute to 200.0 ml with water. Measure the absorbance (A) at the maximum, 444 nm.

$$\% \text{ Riboflavin} = (A \times 5000) / (328 \times W)$$

where A = absorbance of the sample solution at 444 nm

W = weight of sample in g

CALCIUM OXIDE

Prepared at the 19th JECFA (1975), published in NMRS 55B (1976) and in FNP 52 (1992). Metals and arsenic specifications revised at the 59th JECFA (2002). An ADI 'Not limited' was established at the 9th JECFA (1965).

SYNONYMS Lime; INS No. 529

DEFINITION

Chemical names	Calcium oxide
C.A.S. number	1305-78-8
Chemical formula	CaO
Formula weight	56.08
Assay	Not less than 95.0% after ignition

DESCRIPTION Odourless, hard, white or greyish white masses or granules, or white to greyish white powder

FUNCTIONAL USES Alkali, dough conditioner, yeast food

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Slightly soluble in water, insoluble in ethanol, soluble in glycerol
(Caution: Protect eyes when adding water)

Reaction with water Moisten the sample with water; heat is generated
(Caution: Protect eyes when adding water).

Test for alkali The sample is alkaline to moistened litmus paper

Test for calcium (Vol. 4) Passes test

PURITY

Loss on ignition (Vol. 4) Not more than 10% (1 g, about 800° to constant weight)

Barium Not more than 0.03%
Cautiously mix 1.5 g of the sample with 10 ml water, add 15 ml of dilute hydrochloric acid TS, dilute to 30 ml with water and filter. To 20 ml of the filtrate add 2 g of sodium acetate, 1 ml of dilute acetic acid TS and 0.5 ml of potassium chromate TS and allow to stand for 15 min. The turbidity of the

solution is not greater than that of a control prepared by adding water to 0.3 ml of barium standard solution (1.779 g barium chloride in 1000 ml of water) to make to 20 ml, adding 2 g of sodium acetate, 1 ml of dilute acetic acid TS and 0.5 ml of potassium chromate TS and allowing to stand for 15 min.

Magnesium and alkali salts

Not more than 3.6%

Dissolve 500 mg of the sample in 30 ml of water and 15 ml of dilute hydrochloric acid TS. Heat the solution and boil for 1 min. Add rapidly 40 ml of oxalic acid TS and stir vigorously. Add 2 drops of methyl red TS and neutralize the solution with ammonia TS to precipitate the calcium completely. Heat the mixture on a steam bath for 1 h, cool, dilute to 100 ml with water, mix well and filter. To 50 ml of the filtrate carefully add 0.5 ml of concentrated sulfuric acid, evaporate to dryness and ignite to constant weight in a tared platinum crucible.

Acid insoluble matter

Not more than 1%

Slake 5 g of the sample, mix with 100 ml of water and sufficient hydrochloric acid, added dropwise, to effect solution. Boil the solution, cool, add hydrochloric acid, if necessary, to make the solution distinctly acid, and filter through a tared crucible. Wash the residue with water until free of chlorides, dry at 105° for 1 h, cool, and weigh.

Fluoride (Vol. 4)

Not more than 50 mg/kg (Method I or III)

Lead (Vol. 4)

Not more than 2 mg/kg

Determine using an atomic absorption technique 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

Ignite at approximately 800° about 1 g of the sample to constant weight, accurately weigh the residue and dissolve it in 20 ml of dilute hydrochloric acid TS. Cool the solution, dilute with water to 500 ml and mix. Pipet 50 ml of this solution into a suitable container and add 50 ml of water, then add 15 ml of sodium hydroxide TS, 40 mg of murexide indicator preparation and 3 ml of naphthol green TS, and titrate with 0.05 M disodium ethylenediamine tetraacetate until the solution is deep blue in colour. Each ml of 0.05 M disodium ethylenediamine tetraacetate is equivalent to 2.804 mg of CaO.

SODIUM HYDROGEN SULFITE

Prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7 (1999), superseding tentative specifications prepared at the 51st JECFA (1998), published in FNP 52 Add 6 (1998). Group ADI 0-0.7 mg/kg bw as SO₂ for sulfites established at the 51st JECFA in 1998.

SYNOMYS INS No. 222

DEFINITION

Chemical names	Sodium hydrogen sulfite, sodium bisulfite
C.A.S. number	7631-90-5
Chemical formula	NaHSO ₃
Formula weight	104.06
Assay	Not less than 58.5% and not more than 67.4% of SO ₂

DESCRIPTION White crystals or granular powder having an odour of sulfur dioxide

FUNCTIONAL USES Antibrowning agent, antioxidant, preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in water; slightly soluble in ethanol

Test for sodium (Vol. 4) Passes test

Test for sulfite (Vol. 4) Passes test

PURITY

Water insolubles Dissolve 20 g of the sample in 200 ml of water. The solution should be clear with only a trace of suspended matter

pH (Vol. 4) 2.5 - 4.5 (1 in 10 soln)

Iron (Vol. 4) Not more than 10 mg/kg
Proceed as directed in the Limit Test using 0.5 ml of Iron Standard Solution (5 µg Fe) in the control

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an atomic absorption technique 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."

Selenium Not more than 5 mg/kg

See description under TESTS

TESTS

PURITY TESTS

Selenium

Reagents:

Hydrochloric acid, hydrazinium sulfate, standard selenium solution (100 µg Se/ml)

Procedure

Weigh 2.0 ± 0.1 g of sample and transfer to a 50-ml beaker. Add 10 ml water, 5 ml hydrochloric acid and boil to remove SO_2 .

Into a second beaker, weigh 1.0 ± 0.1 g of sample, add 0.05 ml standard selenium solution and proceed as above.

To each beaker add 2 g hydrazinium sulfate and warm to dissolve. Let stand for 5 min. Dilute the contents of each beaker to 50 ml in a Nessler tube and compare the colour of the two solutions. The sample should be less pink than the sample with the added standard.

METHOD OF ASSAY

Weigh 0.2 g of the sample, to the nearest mg, add 50.0 ml of 0.1 N iodine in a glass-stoppered flask, and stopper the flask. Allow to stand for 5 min, add 1 ml of hydrochloric acid, and titrate the excess iodine with 0.1 N sodium thiosulfate, adding starch TS as the indicator. Each ml of 0.1 N iodine is equivalent to 3.203 mg of SO_2 .

SODIUM THIOSULFATE

Prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7 (1999), superseding tentative specifications prepared at the 51st JECFA (1998), published in FNP 52 Add 6 (1998). Group ADI 0-0.7 mg/kg bw as SO₂ for sulfites established at the 51st JECFA in 1998.

SYNONYMS Sodium hyposulfite; INS No. 539

DEFINITION

Chemical names Sodium thiosulfate

C.A.S. number 7772-98-7

Chemical formula Na₂S₂O₃ · 5H₂O

Formula weight 248.17

Assay Not less than 99.0% on the dried basis

DESCRIPTION Colourless crystals or coarse crystalline powder; deliquesces in moist air and effloresces in dry air above 33°

FUNCTIONAL USES Antibrowning agent, antioxidant, sequestrant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Very soluble in water; insoluble in ethanol

Reducing activity To a 1 in 10 solution of the sample add a few drops of iodine TS; the colour is discharged

Test for sodium (Vol. 4) Passes test

Test for thiosulfate (Vol. 4) Passes test

PURITY

Loss on drying (Vol. 4) 32-37% (40-45°, 16 h, under vacuum)

Lead (Vol. 4) Not more than 2 mg/kg
Determine using an atomic absorption technique 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."

Iron (Vol. 4) Not more than 10 mg/kg
Proceed as directed in the Limit Test using 0.5 ml of Iron Standard Solution (5 µg Fe) in the control

Selenium Not more than 5 mg/kg
See description under TESTS

TESTS

PURITY TESTS

Selenium Reagents:
Hydrochloric acid, hydrazinium sulfate, standard selenium solution (100 µg Se/ml)

Procedure

Weigh 2.0 ± 0.1 g of sample and transfer to a 50-ml beaker. Add 10 ml water, 5 ml hydrochloric acid and boil to remove SO_2 . Into a second beaker, weigh 1.0 ± 0.1 g of sample, add 0.05 ml standard selenium solution and proceed as above.

To each beaker add 2 g hydrazinium sulfate and warm to dissolve. Let stand for 5 min. Dilute the contents of each beaker to 50 ml in a Nessler tube and compare the colour of the two solutions. The sample should be less pink than the sample with the added standard.

METHOD OF ASSAY Dissolve about 0.5 g of the dried sample, accurately weighed, in 30 ml of water and titrate with 0.1 N iodine solution using starch TS as the indicator. Each ml of 0.1 N iodine is equivalent to 15.81 mg of $\text{Na}_2\text{S}_2\text{O}_3$.



**Food and Agriculture
Organization of the
United Nations**



**World Health
Organization**

Specifications Monograph prepared by the meeting of the Joint FAO/WHO
Expert Committee on Food Additives (JECFA), 92nd Meeting 2021

Sodium benzoate

This monograph was also published in: Compendium of Food Additive Specifications. Joint
FAO/WHO Expert Committee on Food Additives (JECFA), 92nd meeting 2021. FAO
JECFA Monographs 27

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 ninety-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

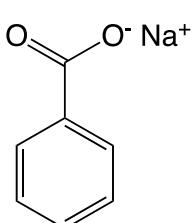
DEFINITION

Chemical names Sodium benzoate

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

Chemical formula C₇H₅NaO₂

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 Cl₂)
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 C₇H₅NaO₂.