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FAO JECFA Monographs

23

COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives

87th Meeting 2019

Food and Agriculture Organization of the United Nations

World Health Organization

FAO JECFA Monographs 23

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Food and Agriculture Organization of the United Nations
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Rome, 4 – 13 June 2019

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INTRODUCTION

This volume of FAO JECFA Monographs contains specifications of identity and purity prepared at the 87th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), held in Rome, 4 – 13 June 2019. The specifications monographs are one of the outputs of JECFA's risk assessment of food additives, and should be read in conjunction with the safety evaluation, reference to which is made in the section at the head of each specifications monograph. Further information on the meeting discussions can be found in the summary report of the meeting (see Annex 1), and in the full report which will be published in the WHO Technical Report series. Toxicological monographs of the substances considered at the meeting will be published in the WHO Food Additive Series.

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

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

Chemical and Technical Assessments (CTAs) for some of the food additives have been prepared as background documentation for the meeting. These documents are available online at: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/technical-assessments/en/.

Contact and Feedback

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

jecfa@fao.org

SPECIFICATIONS FOR CERTAIN FOOD ADDITIVES

New and revised specifications

New (N) or revised (R) specifications monographs were prepared for 11 food additives and these are presented in this publication.

Black Carrot Extract (N, T)
Brilliant Black (R)
Cassia Gum (R, T)
Citric and fatty acid esters of glycerol
Gellan gum (R, T)
Mannoproteins from yeast cell walls
Metatartaric Acid
Potassium polyaspartate (N)
Rosemary extract (R)
(Framework for) Steviol glycosides (R, T)
β-apo-8'-carotenal (R)
β-Carotene from blakeslea trispora (R)
β-Carotene, synthetic (R)
β-Carotene-rich extract from dunaliella salina (R)

In the specifications monographs that have been assigned a tentative status (T), there is information on the outstanding data and a timeline by which this information should be submitted to the FAO JECFA Secretariat.

BLACK CARROT EXTRACT

(TENTATIVE)

New tentative specifications for black carrot extract as the powder form were prepared at the 87th JECFA and published in JECFA Monograph 23 (2019). The 87th JECFA did not conclude on the safety of black carrot extract or establish an ADI.

Information required:
Data regarding a full characterization of the protein, carbohydrate, lipid, fibre, mineral, and non-anthocyanin polyphenol components in five lots each of the liquid and powder forms of black carrot extract.

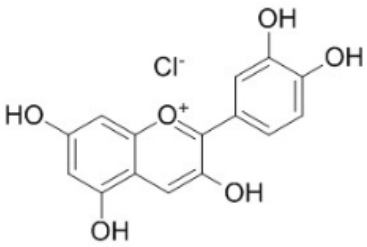
SYNONYMS INS No. 163(vi), purple carrot extract, black carrot colour, purple carrot colour, black carrot anthocyanins, purple carrot anthocyanins

DEFINITION Black carrot extract is a food colour obtained from black, purple, or red carrot (Daucus carota L. ssp. Sativus). The principal colouring components are five anthocyanins formed from the aglycone cyanidin substituted at the central hydroxyl position with a sugar moiety consisting of galactose, glucose, and/or xylose. Three of the five anthocyanins are acylated with p-coumaric, ferulic, or sinapinic acids. Anthocyanins formed from other aglycones (malvidin, pelargonidin, and peonidin) are present in minor amounts along with other polyphenols. Other components include proteins, carbohydrates, lipids, fibre, minerals, and water. Black carrot extract is produced by aqueous acidic extraction of crushed, ground, or milled carrot roots followed by fermentation to reduce sugars. Methanol or ethanol may be produced during the fermentation step. The pigments may be concentrated by ultrafiltration, reverse osmosis, or adsorption onto a polymeric resin followed by desorption with ethanol, isopropyl alcohol, and/or water. The concentrate is spray-dried with a carrier such as maltodextrin, dextrin, or gum to produce the powdered product.

Chemical names, formulas, and C.A.S. numbers

Cyanidin 3-p-coumaroylxylosylglucosylgalactoside, $C_{41}H_{45}O_{22}^+$
C.A.S. 142506-21-6
Cyanidin 3-feruloylxylosylglucosylgalactoside, $C_{42}H_{47}O_{23}^+$
C.A.S. 142561-99-7
Cyanidin 3-xylosylgalactoside, $C_{26}H_{29}O_{15}^+$
C.A.S. 142506-19-2
Cyanidin 3-xylosylglucosylgalactoside, $C_{32}H_{39}O_{20}^+$
C.A.S. 142561-98-6
Cyanidin 3-sinapoylxylosylglucosylgalactoside, $C_{43}H_{49}O_{24}^+$
C.A.S. 142630-71-5

Structural formula



Cyanidin chloride

Assay — Anthocyanin content not less than 3%.

DESCRIPTION — Red or purplish-red powder with characteristic odour.

FUNCTIONAL USES — Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) — Soluble in water, ethanol, and isopropyl alcohol.

Spectrophotometry (Vol. 4) — Maximum wavelength approximately 518 nm
Determine the UV-visible absorption spectrum of the sample dissolved in water, pH 3.

Colour reaction — Add 0.1 g of sample to 50 ml of acidified water (pH 3 – 3.5) and shake to mix. Filter if necessary. The red or purplish-red solution will turn blue or dark green upon addition of sodium hydroxide TS.

PURITY

Residual solvents (Vol. 4) — Methanol, not more than 50 mg/kg
Ethanol, not more than 50 mg/kg
Isopropyl alcohol, not more than 50 mg/kg

Arsenic (Vol. 4) — Not more than 3 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”).

Cadmium (Vol. 4) — Not more than 1 mg/kg
Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the method described in Volume 4 (under “General Methods, Metallic Impurities”).

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 (under “General Methods, Metallic Impurities”).

Mercury (Vol. 4)

Not more than 1 mg/kg
Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the method described in Volume 4 (under “General Methods, Metallic Impurities”).

Colouring matters

The major colouring principals shall correspond to the five anthocyanins derived from cyanidin as defined above. Determine by liquid chromatography.
See description under TESTS

TESTS

PURITY TESTS

Colouring matters

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

- Column: C18 Hypersil ODS, 250 mm x 3 mm i.d., 5 µm particle size or equivalent
- Eluent A: Formic acid/water (10:90)
- Eluent B: Formic acid/water/acetonitrile (10:40:50)
- Injection volume: 20 µl
- Column temperature: 40°
- Detector: UV-visible/diode array at 518 nm
- Flow rate: 1.0 ml/min

Gradient:

Elution time (min)	Eluent A (%)	Eluent B (%)
0	88	12
1.0	88	12
26.0	70	30
35.0	0	100
38.0	0	100
43.0	88	12
46.0	88	12

Reagents: HPLC grade

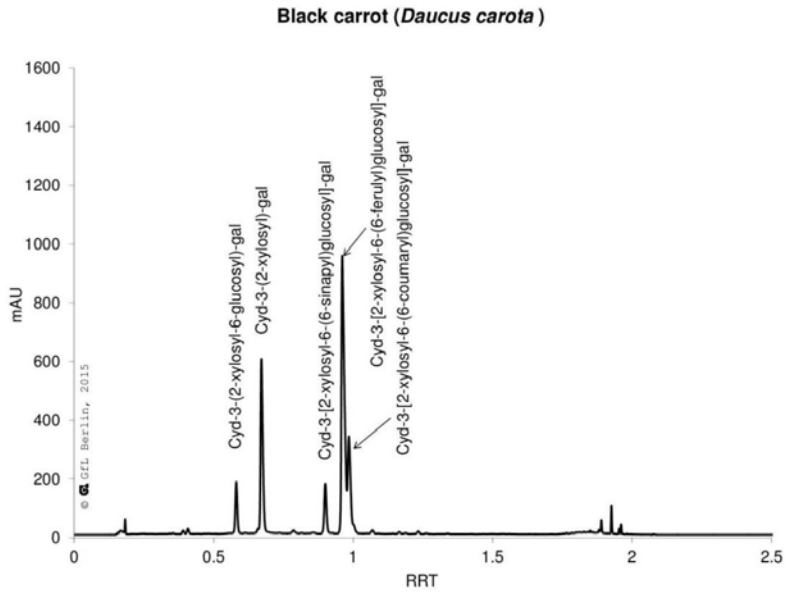
Internal standard:

- Pelargonidin-3-glycoside chloride (C.A.S. 18466-51-8) – BOC Sciences, Cat. No. 18466-51-8 or equivalent

Preparation of internal standard solution:
Weigh accurately about 2 mg of standard and dissolve in 2 ml of 1.2 M hydrochloric acid. Store at -20° for up to two months. Thaw shortly before analysis.

Sample preparation:
Weigh accurately 1.0±0.02 g of sample and dissolve in 5 ml of water. Centrifuge if necessary and filter through a 0.45 µm filter. Dilute the solution if necessary to avoid saturating the HPLC detector. Transfer 500 µl to an HPLC vial and mix with 10 µl of internal standard solution.

Procedure:
Inject the sample solution. Obtain the retention times of the colouring matters components relative to the pelargonidin-3-glycoside chloride internal standard. Identify the individual components by comparing the retention times to the reference chromatogram.



Reference chromatogram for colouring matters in Black carrot extract.

METHOD OF ASSAY

Determine total colouring matters content by spectrophotometry using Procedure 1 in Volume 4 (under “Specific Methods, Food Colours”) and water acidified to pH 3.0 with citric acid as the solvent.

Absorptivity (a) = 30.0 l/(g·cm) and wavelength of maximum absorbance = 518 nm.

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

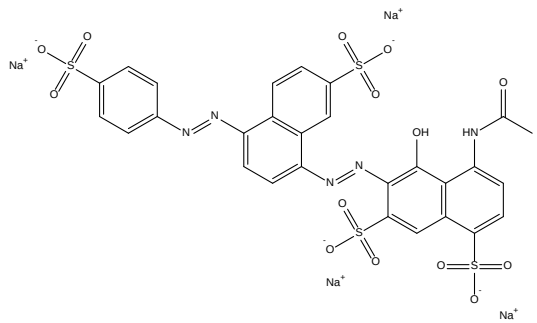
DEFINITION Brilliant Black PN consists of tetrasodium 4-(acetylamino)-5-hydroxy-6-[2-[7-sulfo-4-[2-(4-sulfophenyl)9pprox.9]-1-naphthalenyl]9pprox.9]-1,7-naphthalenedisulfonate and subsidiary colouring matters, as well as sodium chloride and/or sodium sulfate as the principal uncoloured components. Brilliant 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).
Brilliant Black PN may be converted to the corresponding aluminium lake in which case only the requirements in the *General Specifications for Aluminium Lakes of Colouring Matters* apply.

Chemical name 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)9pprox.9]naphthalen-1-yl]hydrazinylidene]naphthalene-1,7-disulfonate

C.A.S. number 2519-30-4

Chemical formula $C_{28}H_{17}N_5Na_4O_{14}S_4$

Structural formula



Formula weight	867.69
Assay	Not less than 80% total colouring matters
DESCRIPTION	Black powder or granules
FUNCTIONAL USES	Colour
CHARACTERISTICS	
IDENTIFICATION	
Solubility (Vol. 4)	Soluble in water, sparingly soluble in ethanol.
Spectrophotometry (Vol. 4)	Maximum wavelength approximately 572 nm Determine the UV-visible absorption spectrum of the sample dissolved in water.
PURITY	
Loss on drying, chloride and sulfate as sodium salts (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”).
Water insoluble matter (Vol. 4)	Not more than 0.2%
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 (under “General Methods, Metallic Impurities”).
Subsidiary colouring matters	Not more than 4% See description under TESTS
Organic compounds other than colouring matters	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’-(diazoamino)dibenzenesulfonic acid See description under TESTS
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

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·cm) and wavelength of maximum absorbance = 572 nm.

B-apo-8'-CAROTENAL

Prepared at the 87th JECFA (2019) and published in FAO Monographs 23 (2019), superseding specifications prepared at the 74thJECFA (2011) and published in FAO Monographs 11 (2011). 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'-carotenoic acid was established at the 18th JECFA (1974).

SYNONYMS CI Food Orange 6; CI (1975) No. 40820; INS No. 160e

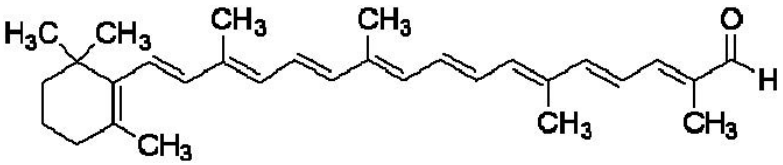
DEFINITION These specifications apply to β-apo-8'-carotenal which consists predominantly of all-trans- β-apo-8'-carotenal and may also contain minor quantities of other carotenoids such as all-trans-crocetindialdehyde, all-trans-β-apo-12'-carotenal and all-trans-β-carotene. Commercial preparations of β-apo-8'-carotenal intended for use in food are prepared from β-apo-8'-carotenal 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 ß-Apo-8'-carotenal, 8'-apo-ß-carotene-al
2E,4E,6E,8E,10E,12E,14E,16E)-2,6,11,15-tetramethyl-17-(2,6,6-trimethyl-1-cyclohexenyl)heptadeca-2,4,6,8,10,12,14,16-octaenal

C.A.S. number 1107-26-2

Chemical formula $C_{40}H_{40}O$

Structural formula All-*trans*-β-apo-8'-carotenal (main compound)



Formula weight 416.65

Assay Not less than 96% total colouring matters

DESCRIPTION Deep violet crystals with metallic lustre 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

Solubility (Vol. 4)	Insoluble in water; slightly soluble in ethanol; sparingly soluble in vegetable oils.
Spectrophotometry (Vol. 4)	Determine the absorbance of the diluted sample solution used in the Method of Assay at 460 nm and 488 nm. Determine the absorbance at 332 nm of a solution containing a ten-fold higher concentration as that of the diluted sample solution used in the Method of Assay. The ratio A_{488}/A_{460} is between 0.77 and 0.85. The ratio of A_{332}/A_{460} is between 0.63 and 0.75.

PURITY

Sulfated ash (Vol. 4)	Not more than 0.1% Test 2 g of the sample (Method I)
Carotenoids other than ß-apo-8'-carotenal	Not more than 3% of total colouring matters. See description under TESTS
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 methods described in Volume 4 (under "General Methods, Metallic Impurities").

TESTS

PURITY TESTS

Carotenoids other than ß-apo-8'-carotenal

Chromatographic system

- HPLC equipped with a UV/Vis detector or a photodiode array detector, refrigerated auto sampler and integrator
- Detector wavelength: 463 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 15pprox.. 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: (15pprox.. 15°)
- Run time: 15pprox.. 35 min

Reagents

- Butylated hydroxytoluene (BHT), reagent grade
- 2-Propanol, HPLC grade
- N-ethyldiisopropyl-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 times for all-*trans*-β-apo-8'-carotenal is in the range of 7-9 min and corresponds to the largest peak in the chromatogram. The relative retention times of minor carotenoids with respect to the retention time of all-*trans*-β-apo-8'-carotenal are: all-*trans*-crocetindialdehyde (0.54); all-*trans*-β-apo-12'-carotenal (0.84); all-*trans*-β-carotene (2.55).

Integrate the areas of the peaks in the chromatogram.

Calculation

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

$$\text{Carotenoids other than } \beta-\text{apo}-8'-\text{carotenal } \left(\%, \frac{\text{w}}{\text{w}}\right) = \frac{\text{A}_{\text{total}} - \text{A}_{\beta-\text{apo}-8'-\text{carotenal}}}{\text{A}_{\text{total}}}$$

where

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

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

METHOD OF ASSAY **Total colouring matters content by spectrophotometry**

Proceed as directed under Total Colouring Matters Content – Colouring Matters Content 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: $A_{1cm}^{1\%} = 2640$;

Wavelength of maximum absorption: λ_{max} about 461 nm.

Calculation

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

$$\text{Total colouring matters } (\%, \text{w/w}) = \frac{\text{A} \times \text{V}_1 \times \text{D}}{\text{A}_{1\text{cm}}^{1\%} \times \text{W}}$$

where

A is the absorbance of the twice-diluted sample solution at 461 nm; and

D is the dilution factor $(V_2 x V_3)/(v_1/v_2)$.

B-CAROTENE from *BLAKESLEA TRISPORA*

Prepared at the 87th JECFA (2019) published in FAO Monographs 23 (2019). Superseding specifications prepared at the 61st JECFA (2003), published in FNP 52 Add 11 (2003). A group ADI with β -carotene (synthetic) of 0 – 5 mg/kg bw was established at the 57th JECFA (2001).

SYNONYMS CI Food Orange 5; INS No. 160a(iii)

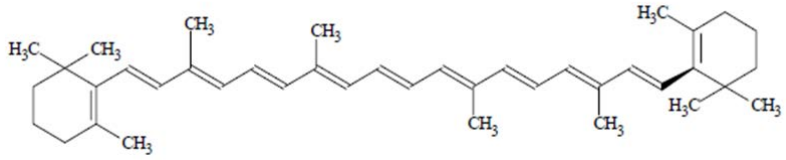
DEFINITION Obtained by a fermentation process using the two sexual mating types (+) and (-) of the fungus Blakeslea trispora. The colour is isolated from the biomass by solvent extraction and crystallised. The colouring principal consists predominantly of trans β-carotene together with variable amounts of cis isomers of β-carotene. Minor amounts of other carotenoids of which γ-carotene accounts for the major part may also be present. The only organic solvents used in the extraction and purification are ethanol, isopropanol, ethyl acetate and isobutyl acetate. The main articles of commerce are suspensions in food grade vegetable/plant oil and water dispersible powders.

Chemical names ß-Carotene, ß,ß-carotene

C.A.S. number 7235-40-7

Chemical formula $C_{40}H_{56}$

Structural formula



Formula weight 536.88

Assay Not less than 96.0% total colouring matter (expressed as ß-carotene).

DESCRIPTION Red to brownish-red crystals or crystalline powder.

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility	Insoluble in water; practically insoluble in ethanol; slightly soluble in vegetable oils.
Spectrophotometry (Vol. 4)	Determine the absorbance of the diluted sample solution used in the Method of Assay from 300 – 600nm. The spectrum shows a shoulder at about 427 nm, an absorption maximum at about 455 nm, and another maximum at about 483 nm. The ratio A_{455}/A_{483} is between 1.14 and 1.18. Determine the absorbance of the diluted sample solution used in the Method of Assay at 455 nm and 340 nm. The ratio A_{455}/A_{340} is not less than 15.
PURITY	
Sulfated Ash (Vol. 4)	Not more than 0.2%
Carotenoids other than ß-carotene	Carotenoids other than ß-Carotene: Not more than 3% of total colouring matters. See description under TESTS
Residual solvents (Vol. 4)	Ethanol and Ethyl acetate: Not more than 0.8% singly or in combination Isopropanol: Not more than 0.1% Isobutyl acetate: Not more than 1.0% See description in Volume 4
Lead (Vol. 4)	Not more than 2 mg/kg. Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, under “General Methods, Metallic Impurities”.

TESTS

PURITY TESTS

Carotenoids other than β-carotene

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

Chromatographic system

- HPLC equipped with a UV/Vis detector or a photodiode array detector, refrigerated auto sampler and integrator
- Detector wavelength: 445 nm
- Column: Reverse phase C18; Vydac 218 TP54 (250 x 4.6 mm, 5 µm) or equivalent
- Mobile phase: 99% methanol and 1% tetrahydrofuran containing 50 mg/l of L-ascorbic acid
- Isocratic elution
- Column temperature: 30°
- Flow rate: 0.6 ml/min
- Injection volume: 10 µl

- Temperature of the autosampler: (20pprox.. 15°)
- Run time: 20pprox.. 25 min

Sample preparation

Weigh 25 mg of the sample and dissolve in tetrahydrofuran. Transfer to a 100 ml volumetric flask and bring to volume with tetrahydrofuran. Dilute 1 ml of the solution to 25 ml in a volumetric flask with mobile phase. Use freshly prepared solutions.

Results

The retention time for β-carotene (all trans isomer) is about 19 minutes corresponding to the largest peak in the chromatogram. The retention time for γ-carotene is about 20 minutes and the peak at about 22 minutes corresponds to the 13-cis isomer.

Γ-Carotene as a % of total β-carotene:

$$= \frac{A_1 \times 100}{A_1 + A_2 + A_3}$$

where

A_1 is the area of the γ-carotene peak

A_2 is the is the area of the all-trans β-carotene peak

A_3 is the combined area of the peaks from the isomers of all-trans β-carotene

METHOD OF ASSAY

Total colouring matters content by spectrophotometry

Proceed as directed under Total Colouring Matters Content – Colouring Matters Content 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: $A_{1cm}^{1\%} = 2500$;

Wavelength of maximum absorption: λ_{max} about 455 nm.

Calculation

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

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

where

A is the absorbance of the twice-diluted sample solution at 455 nm; and

D is the dilution factor $(V_2 x V_3)/(v_1/v_2)$.

β-CAROTENE, SYNTHETIC

Prepared at the 87th JECFA (2019) and published in FAO Monographs 23 (2019). Superseding specifications prepared at the 74thJECFA (2011) and published in FAO Monographs 11 (2011). A group ADI of 0-5 mg/kg bw for beta carotene, synthetic and from Blakeslea trispora, was established at the 57th JECFA (2001).

SYNONYMS CI Food Orange 5; INS No. 160°(i); CI (1975) No. 40800

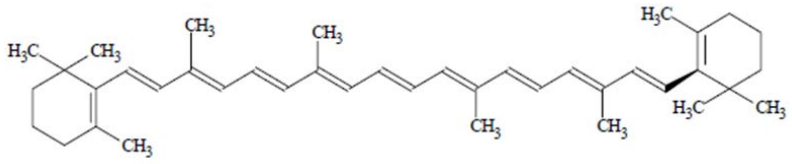
DEFINITION These specifications apply to synthetic β-carotene which consists predominantly of all-trans-β-carotene. Synthetic β-carotene may also contain minor amounts of cis-isomers and other carotenoids such as all- trans-retinal, β-apo-12'-carotenal, and β-apo-10'-carotenal. Commercial preparations of β-carotene intended for use in food are prepared from β-carotene meeting these specifications and are formulated as suspensions in edible oils or water-dispersible powders. These preparations may have different ratio of trans/cis isomers.

Chemical names ß-Carotene, ß,ß-carotene 1,1'-(3,7,12,16-tetramethyl-1,3,5,7,9,11,13,15,17-octadecanonaene-1,18- diyl)bis[2,6,6-trimethylcyclohexene]

C.A.S. number 7235-40-7

Chemical formula $C_{40}H_{56}$

Structural formula All-*trans*-β-carotene (main compound)



Formula weight 536.88

Assay Not less than 96% total colouring matters, expressed as ß-carotene.

DESCRIPTION Red to brownish-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

Solubility	Insoluble in water; practically insoluble in ethanol; slightly soluble in vegetable oils.
Spectrophotometry (Vol. 4)	Determine the absorbance of the diluted sample solution used in the Method of Assay from 300 – 600nm. The spectrum shows a shoulder at about 427 nm, an absorption maximum at about 455 nm, and another maximum at about 483 nm. The ratio A_{455}/A_{483} is between 1.14 and 1.18. Determine the absorbance of the diluted sample solution used in the Method of Assay at 455 nm and 340 nm. The ratio A_{455}/A_{340} is not less than 15.

PURITY

Sulfated Ash (Vol. 4)	Not more than 0.1% Test 2 g of the sample (Method I).
Carotenoids other than ß-carotene	Not more than 3% of total colouring matters. See description under TESTS
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 methods described in Volume 4, under “General Methods, Metallic Impurities”.

TESTS

PURITY TESTS

Carotenoids other than β-carotene

Chromatographic system

- HPLC equipped with a UV/Vis detector or a photodiode array detector, refrigerated auto sampler and integrator
- Detector wavelength: 453 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 23pprox.. 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: (24pprox.. 15°)
- Run time: 24pprox.. 35 min

Reagents

- Butylated hydroxytoluene (BHT), reagent grade
- 2-Propanol, HPLC grade
- N-ethyldiisopropyl-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 times for all-*trans*-β-carotene and cis-isomers are in the range of 20-25 min. The largest peak in the chromatogram corresponds to all-*trans*-β-carotene. The relative retention times of minor carotenoids and cis-isomers of β-carotene with respect to the retention time of all-*trans*-β-carotene are: all-*trans*-retinal (0.26), all- *trans*-β-apo-12'-carotenal (0.33), all-*trans*-β-apo-10'carotenal (0.34), all-*trans*-□-carotene (0.85), all-*trans*-α-carotene (0.95), 9-cis-β-carotene (1.05), 13-cis-β-carotene (1.15) and 15-cis-β-carotene (1.18). Integrate the areas of the peaks in the chromatogram.

Calculation

Calculate the percentage of carotenoids other than β-carotenes (%, w/w) using the following formula:

$$Carotenoids\ other\ than\ \beta\text{-}carotenes\ \left(\%, \frac{w}{w}\right) = \frac{A_{total} \times A_{\beta-carotenes}}{A_{total}} \times 100$$

where

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

$A_{\beta\text{-carotene}}$ is the sum of the areas of the peaks of all β-carotenes (all- *trans*-β-carotene, 9-cis-β-carotene, 13-cis-β-carotene and 15-cis-β- carotene) in the chromatogram (area units).

METHOD OF ASSAY

Total colouring matters content by spectrophotometry

Proceed as directed under Total Colouring Matters Content – Colouring Matters Content 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: $A_{1cm}^{1\%} = 2500$;
Wavelength of maximum absorption: λ_{max} about 455 nm.

Calculation

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

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

where

A is the absorbance of the twice-diluted sample solution at 455 nm; and
D is the dilution factor $(V_2 x V_3)/(v_1/v_2)$.

β-CAROTENE-RICH EXTRACT FROM *DUNALIELLA SALINA*

Prepared at the 87th JECFA and published in FAO JECFA Monographs 23 (2019), superseding specifications prepared at the 84th JECFA (2017) and published in JECFA Monograph 20 (2017). The 84th JECFA (2017) concluded that there was no health concern for the use of β-carotene-rich extract from D. salina when used as a food colour in accordance with the specifications established at 84th meeting. The Committee emphasized that this conclusion applies to the use of this extract as food colour, not as food or food supplement.

SYNONYMS Natural ß-carotene, carotenes-natural; CI (1975) No. 75130; CI (1975) No. 40800 (ß-Carotene)

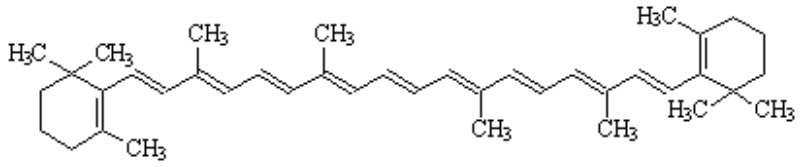
DEFINITION β-Carotene-Rich Extract from *Dunaliella salina* is obtained by extraction from strains of the algae *Dunaliella salina* (syn. *D. bardawil* and *D. Kone*) using the essential oil d-limonene. The extract is then prepared as a suspension in vegetable oil after removal of the essential oil. The main colouring principles are trans- and cis–isomers of ß-carotene together with minor amounts of other carotenes including α-carotene, lutein, zeaxanthin and cryptoxanthin. The isomers of β-carotene account for approximately 90% of the carotenes in the product. Besides the colour pigments and vegetable oil, β-Carotene-Rich Extract from *Dunaliella salina* contains lipids and other fat-soluble components naturally occurring in the source material such as fatty acids typically found in food oils, long-chain alcohols, alkenes, and waxes. The main articles of commerce may be further blended with vegetable oil to standardize the colour content.

Class Carotenoid

C.A.S. number 7235-40-7

Chemical formula $C_{40}H_{56}$ (ß-Carotene)

Structural formula All-trans-ß-Carotene



Formula weight 536.88 (ß-Carotene)

Assay Content of carotenes (calculated as ß-carotene) is not less than declared
See description under TESTS

DESCRIPTION Opaque, deep-red, viscous suspension

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility — Insoluble in water; practically insoluble in ethanol; soluble in hexane

Spectrophotometry (Vol. 4) — A cyclohexane solution of the sample shows maximum absorptions at 448 – 457 and 474 – 486 nm

Colour reaction — A spot of a solution of the sample in toluene (about 400 µg /mL of ß-carotene) on a filter paper turns blue 2-3 min after application of a spray or drop of 20% solution of antimony trichloride solution in toluene.

PURITY

Arsenic (Vol. 4) — Not more than 3 mg/kg
Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under “General Methods, Metallic Impurities”).

Lead (Vol. 4) — Not more than 2 mg/kg

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

TESTS

PURITY TESTS

d-Limonene — Principle

Determination of essential oil limonene by gas chromatography (Vol. 4) under the following conditions:

Chromatography conditions

- Column: polyethylene glycol Agilent DB-WAX, or equivalent, 30m x 0.53mm x 1.0 microns
- Carrier Gas: High purity Nitrogen, 12kPa head pressure, 5:1 split or splitless
- Detector: Flame ionization detector, 200°
- Injection: 2.0 µL (split) or 0.5µl (splitless), 150°

- Temp. Program:
  - Initial: 80°, 5.0 minute
  - Rate: 10°/minute
  - Final: 150°, 3 minutes
  - Total run time: 15 minutes
- Quantitation:
  - Calculate by: Area
  - Method: Internal standard
  - Curve Fit Type: Linear
- Internal standard solution: Prepare a 10.0 mg/mL solution of terpinene in n-hexane.

Sample solution:

Accurately weigh a sample based on the expected quantity of limonene (1.0-2.5g) into a 25mL volumetric flask. Pipette 1 mL of the Internal standard solution into the flask and vortex 15 seconds. Add about 10mL dichloromethane and vortex 15 seconds. Make up to volume with dichloromethane. Mix thoroughly

System Suitability Check

- Perform 2 injections of the blank. No limonene peak should be detected.
- Perform 5 injections of the standard solution containing
- 1.0 mg/mL of limonene and 0.4 mg/mL of terpinene in n- hexane. The coefficient of variation of 5 injections is
- <1.00%. Separation between the limonene and terpinene peaks should be not less than 0.5 min.

Calculations:

$$RF_1 = \frac{A_{istd} \times [STD]}{A_{std} \times [ISTD]}$$

$$[LIM] = \frac{A_{lim} \times DIL \times REF}{A_{istd} \times m}$$

$$[ISTD] = \frac{m_{istd}}{12.5}$$

Where:

RF_1 = Response factor calculated from the calibration standard
[STD] = Concentration of the standard as % w/w [ISTD] = Concentration internal standard as % w/w [LIM] = Concentration of limonene in the sample as % w/w
A_{istd} = Area of the Internal standard peak
A_{std} = Area of the limonene peak in the calibration standard

A_{lim} = Area of the limonene peak in the sample
DIL = Dilution factor, mL
m = Sample mass, g
m_{istd} = Mass of internal standard, g

METHOD OF ASSAY Proceed as directed under Colouring Matters, Total Content by Spectrophotometry (procedure 2) in Volume 4, using the following conditions:

W = amount to obtain adequate absorbance, g

V1 = V2 = V3 = 100 mL
v1 = v2 = 5 mL Solvent: cyclohexane
$A_{1cm}^{1\%} = 2500$;
lambda max = 448 – 457 nm

CASSIA GUM

(TENTATIVE)

At the 86th JECFA the Committee decided to set the specification of Cassia gum to „Final“, because a new HPLC-method for the determination of anthraquinones in Cassia gum had been evaluated during the meeting. Based on user complaints about method criteria and performance of the method the Committee started to review the method again at 87th meeting. It was decided to set the specification on “tentative”, while the investigation is ongoing. At a later stage a call for data might be started.

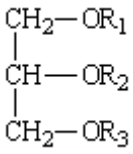
CITRIC and FATTY ACID ESTERS of GLYCEROL

Prepared at the 87th JECFA (2019) and published in FAO JECFA Monographs 23 (2019), superseding specifications prepared at the 86th JECFA (2018) and published in FAO JECFA Monographs 22 (2018). An ADI 'not limited' was established at the 17th JECFA (1973).

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. 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. It may contain minor amounts of free fatty acids, free glycerol, free citric acid and mono- and diglycerides. The mono- and diglycerides may include either one or two edible fatty acids from C12:0 to C18:0, mainly 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 (C20:4). CITREM may be partially or wholly neutralized with sodium hydroxide or potassium hydroxide or by using sodium, potassium or calcium salts of weak acids such as acetic, lactic, propionic or carbonic acids.

Structural formula



Where at least one of R_1, R_2 or R_3 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 (Vol. 4)	Passes test
Test for glycerol (Vol. 4)	Passes test

PURITY

Sulfated ash (Vol. 4)	Non-neutralized products: not more than 0.5% Partially neutralized products: not more than 3% 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% See description under TESTS
Total fatty acid	37-81% See description under TESTS
Lead (Vol. 4)	Not more than 2 mg/kg. (Not more than 0.5 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	Total glycerol, after hydrolysis with KOH, is determined by HPLC (Vol. 4) Equipment and Reagents: HPLC consisting of a Refractive Index (RI) detector, autosampler and column thermostat. Column: Aquasil C18 (250 mm x 4.6 mm x 5 µm) or equivalent Glycerol standard: > 99.5% Phosphoric acid: 85% Potassium hydroxide: reagent grade Potassium dihydrogen phosphate: reagent grade Methanol, acetonitrile and water: HPLC grade

Preparation of standard solutions:
Prepare five solutions of glycerol by weighing 20 – 100 mg, to the nearest 0.01 mg, dissolving in water and making up to 100 ml in separate volumetric flasks.
Sample preparation:

Weigh, to the nearest 0.01 mg, about 50 mg of sample into a 25 ml screw cap vial. Add 2 ml of methanolic KOH (0.5 mol/l, prepared by weighing 33 g KOH into a 1-liter volumetric flask and diluting to volume with methanol). Hydrolyze for 2 hours in a heating block at 110º. Allow to cool, add 20 ml of dilute phosphoric acid (prepared by dissolving 5 ml phosphoric acid in 1 l of water) and shake well. Remove 500 µl of the aqueous phase and filter through 0.45 µm membrane filter for HPLC analysis.

Procedure:

Mobile phase: Dissolve 6.8 g KH_2O_4 in a 1 l volumetric flask with water, add 5 ml phosphoric acid (85%) and make up to volume with water.

Column temperature: 35º
RI detector temperature: 35º
Flow rate: 1.0 ml/min
Injection volume: 10 µl

Inject separately 10 µl of each standard solution and construct the standard curve. The retention time for glycerol is about 2.6 min. Inject 10 µl sample solution and calculate the percentage of glycerol in the sample as below:

Calculation
Determine the content of glycerol using the standard curve. Calculate the total glycerol by the following equation.

Total glycerol, %w/w = (CU (mg/ml) * 22 (ml)* 100) / W

W = weight of sample, mg

CU = concentration of glycerol determined from the standard curve (mg/ml).

Total citric acid

Total citric acid after hydrolysis is determined by HPLC.

Equipment and Reagents:
HPLC consisting of an Ultraviolet (UV) detector, autosampler and a column thermostat.
Column: Synergi 4 µm Hydro RP 80 A (4.6 mm x 250 mm) or equivalent.
Citric acid standard: > 99.5%
Phosphoric acid: 85%
Potassium hydroxide: reagent grade
Methanol, acetonitrile, KH_2PO_4 and water: HPLC grade

Preparation of standard solutions:
Prepare six solutions of citric acid by weighing 10-85 mg, to the nearest 0.01 mg, dissolving in neutralization solution (5 ml phosphoric acid, 85% in 1 l of HPLC water) and making up to 100 ml in separate volumetric flasks.

Sample preparation:

Weigh to the nearest 0.01 mg about 50 mg of sample into a 25 ml screw cap vial. Add 2 ml of methanolic KOH 0.5 mol/l (prepared by weighing 33 g KOH into a 1-liter volumetric flask and making to volume with methanol). Hydrolyze for 2 h in a heating block at 110º. Allow to cool, add 20 ml of dilute phosphoric acid (prepared by dissolving 5 ml phosphoric acid, in 1 l of water) and shake well. Filter 500 µl of the aqueous phase through a 0.45 µm membrane filter.

Procedure:

Mobile phase: Dissolve 6.8 g KH_2PO_4 in a 1 l volumetric flask with water, add 5 ml phosphoric acid (85%) and make up to volume with water.

Colum temperature: 25º
UV detector at 205 nm
Flow rate: 1.0 ml/min
Injection volume: 20 µl

Inject separately 10 µl of each standard solution and construct the standard curve. The retention time of citric acid is about 6.9 min. Inject 10 µl of sample solution and calculate the percentage of citric acid in the sample as below:

Calculation
Determine the content of citric acid using the standard curve. Calculate the total citric acid by the following equation.

Total Citric acid, %w/w= (CU (mg/ml) * 22 (ml)* 100%) / W

W = weight of the sample, mg

CU = concentration of the citric acid determined from the standard curve (mg/ml)

Total fatty acid

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

Procedure

Weigh accurately 5.000 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 water bath.

Quantitatively transfer the content 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 a red methyl orange end point. Add 1 ml of excess acid after the end point is reached. 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:

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

GELLAN GUM

(TENTATIVE)

Tentative specifications prepared at the 87th JECFA (2019), published in FAO JECFA Monographs 23 (2019), superseding specifications prepared at the 79th JECFA (2014). An ADI 'not specified' was established at the 37th JECFA (1990).

Information is required on:

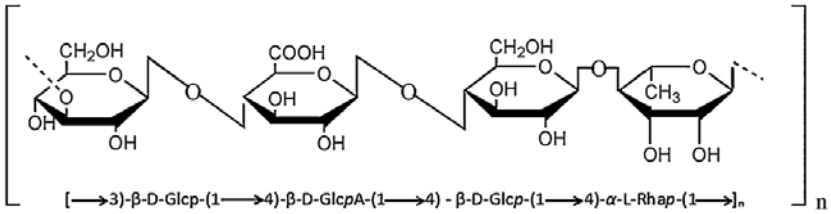
- *A method to differentiate the three commercial forms of gellan gum, i.e., high-acyl, low-acyl, and low-acyl clarified.*
- *A method to determine the degree of acylation.*
- *Validation data for the above methods including detailed description of the sample preparation.*
- *Data from 5 non-consecutive commercial batches of material using the proposed validated methods.*

SYNONYMS INS No. 418

DEFINITION Gellan gum is a high molecular weight (greater than 500,000 Daltons) extracellular, anionic polysaccharide. In its native form, gellan gum is made up of a tetra-saccharide backbone repeating unit consisting of D-glucose, L-rhamnose, and D-glucuronic acid in molar ratios of 2:1:1; this is also called high-acyl gellan gum. Native gellan gum also contains an acetyl and a glyceryl group bound to the glucose adjacent to the glucuronic acid residues.

Gellan gum is produced by a controlled pure culture fermentation of the non-pathogenic Gram-negative bacterium *Pseudomonas elodea* (reclassified as *Sphingomonas elodea)* in the presence of a carbon source, a nitrogen source, and inorganic salts. Fermentation is stopped by heat step; this also kills viable cells. Precipitation of the gellan gum from the broth with food-grade isopropanol or ethanol yields the high acyl form. Treatment with hot alkali prior to alcohol precipitation results in deacylation and yields gellan gum with varying degrees of acylation including the low acyl form.

Low acyl gellan gum can be further filtered to obtain low acyl clarified gellan gum to remove cell protein residue. This is the form used in infant formula applications. By-products of fermentation include polyhydroxybutyrate, enzymes, and viable cells of the production organism, which are removed and/or inactivated during processing. Gellan gum may contain a small amount of nitrogen containing compounds from fermentation. The resulting gellan gum is separated, dried and milled. The gelling properties of the article of commerce are controlled by the addition of metal ions such as sodium, potassium and calcium to neutralize the glucuronic acid.



C.A.S. number 71010-52-1

Assay Yields, on the dried basis, not less than 3.3% and not more than 6.8% of carbon dioxide (CO_2); this corresponds to not less than 85% and not more than 108% of gellan gum

DESCRIPTION Off-white powder.

FUNCTIONAL USES Thickener, gelling agent, stabilizer

CHARACTERISTICS

IDENTIFICATION

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

Gel test with calcium ion 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.

Gel test with sodium ion 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.

PURITY

Loss on drying (Vol. 4) Not more than 15% (105°, 2½ h)

Nitrogen (Vol. 4) Not more than 3%

Residual solvents (Vol. 4) 2-propanol: not more than 750 mg/kg
Determine using the method described for solvent extracted bixin and norbixin in Vol. 4.

Microbiological criteria (Vol. 4) Total plate count: Not more than 10,000 cfu/g
E. coli: Negative by test
Salmonella: Negative by test
Yeasts and moulds: Not more than 400 cfu/g

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 methods described in Volume 4 (under "General Methods, Metallic Impurities").

TESTS

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.

MANNOPROTEINS FROM YEAST CELL WALLS

Prepared at the 87th JECFA (2019) and published in FAO JECFA Monographs 23 (2019), superseding specifications prepared at the 84th JECFA (2017) and published in FAO JECFA Monographs 20 (2017). No ADI was established at the 84th JECFA (2017) as the use of this substance is not of health concern when used for oenological uses at maximum use levels up to 400 mg/L for the stabilization of wine.

SYNONYMS INS.No. 455

DEFINITION Mannoproteins from Yeast Cell Walls are a large family of natural compounds from *Saccharomyces cerevisiae* in which polysaccharides are connected to proteins and peptides by covalent and non-covalent bonds. The structures and molecular weights of mannoproteins vary, depending on the degree and type of glycosylation. The polysaccharide chains consist almost exclusively of mannose units linked together by α-links, with a long α-1→6 linked backbone containing short α-1→2- and α-1→3 linked side chains. Several of the side chains may have phosphodiester linkages to other mannosyl residues. Mannoproteins are extracted from purified yeast cell walls by enzymatic extraction using glucan 1,3- β-glucosidase (EC 3.2.1.58) or by thermal treatment. The enzyme hydrolyses the yeast cell wall allowing the mannoproteins to be solubilized. The thermal treatment breaks the links with β-glucans. The mannoproteins thus solubilized by either treatment are then separated from the insoluble cell wall material and concentrated by micro- or ultra-filtration. Mannoproteins have molecular weights ranging from below 20 kDa to more than 450 kDa.

Assay Total polysaccharides: Not less than 60% expressed as mannose on the dried basis.
Mannose: Not less than 70% of the total polysaccharides.

Nitrogen content: 0.5-7.5% on the dried basis

DESCRIPTION White or beige, odourless powder, or yellow, translucent colloidal solution

FUNCTIONAL USES Wine stabilizer

CHARACTERISTICS

IDENTIFICATION

Solubility Soluble in water and insoluble in ethanol

PURITY

Loss on drying (Vol.4) Powder form: Not more than 15% (105°, 5h)

Specific rotation (Vol.4) $[\alpha]_D^{20}$: between +80 and +150°,

Test solution: 1.0 g of dried sample in 100 ml of water, using an optical cell with 100-mm path length.

Total Ash (Vol. 4) Not more than 8%, on dried basis

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

Determine using 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
METHOD OF ASSAY

Total polysaccharides

Reagents:
Mannose, >99 % pure
Sulfuric acid, Concentrated
Phenol solution (50 mg/ml): Dissolve 5 g of phenol in 100 ml of deionized water

Preparation of mannose standard solution (0.1mg/ml): Accurately weigh 100 mg of mannose, dissolve in deionized water and make up to 100 ml in a volumetric flask. Pipette 5 mL of solution into a 50 ml volumetric flask and make up to volume with deionized water (0.1 mg/ml).

Preparation of sample solution (15 mg/l): Accurately weigh 150 mg (W) of sample, dissolve in deionized water and make up to 100 ml in a volumetric flask.

Procedure:
Add 200 µl of phenol solution and 1 ml of concentrated sulfuric acid to 200 µl of the sample solution and mix immediately. Prepare a reference solution by adding 200 µl of phenol solution and 1 ml of concentrated sulphuric acid to 200 µl of a 0.1 mg/ml solution of mannose in water and mix immediately. Heat both solutions to 100° in a water bath for 5 min then cool to reach room temperature and measure the absorbance values at 490 nm in a spectrophotometer against a blank solution prepared similarly omitting the standard.

Total polysaccharides, %w/w (Expressed as mannose on the dried basis) = $\frac{A_{Sample}}{A_{STD} \times (100 - \%M)}$

Where: A_{Sample} is the absorbance of the sample solution

A_{Std} is the absorbance of the standard solution (0.1 mg/ml)
W is the weight of sample, g

Mannose

Instrumentation and reagents:
Spectrophotometer: 340 nm
Stop-watch
Triethanolamine: >99 % pure
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$): AR grade
Sodium hydroxide: AR grade
Disodium nicotinamide adenine dinucleotide phosphate: AR grade
Adenosine-5'-triphosphate (ATP): AR grade
Sodium hydrogen carbonate: AR grade
Hexokinase solution: 2 mg of protein/ml or 280 U/ml
Glucose-6-phosphate(G-6-P)-dehydrogenase solution: 1 mg of protein/ml
Phosphoglucose-isomerase (PGI): 2 mg of protein/mL or 700 U/ml
Phosphomannose isomerase: 616 U/ml
Sulfuric acid: 5 M
Potassium hydroxide: 10 M

Buffer solution (0.3 M triethanolamine, 0.004 M Mg^{2+}, pH 7.6): Dissolve 11.2 g of triethanolamine hydrochloride, and 0.2 g magnesium sulfate in 150 ml deionized water, adjust the pH 7.6 with about 4 ml of 5 mol/l sodium hydroxide solution and make up to 200 ml.

Nicotinamide adenine dinucleotide phosphate (NADP) solution (10 mg/ml): Dissolve 50 mg disodium nicotinamide adenine dinucleotide phosphate in 5 ml of deionized water.

Adenosine-5'-triphosphate (ATP) solution (0.08 M): Dissolve 250 mg disodium adenosine-5'-triphosphate and 250 mg sodium hydrogen carbonate in 5 ml of deionized water.

Hexokinase/glucose-6-phosphate(G-6-P)-dehydrogenase solution: Mix 0.5 ml hexokinase solution with 0.5 ml G-6-P-dehydrogenase solution.

Preparation of sample solution (5g/l):
Dissolve 0.500 g (W) of sample in 100 ml of deionized water. Place 100 µl of the sample solution in airtight sealed tubes and add 1 ml of 5M sulphuric acid solution. Cap the tubes, heat at 100° in a water bath for 30 min, remove tubes and quickly cool to 0° in ice. Take out the tubes from ice and allow tubes to reach room temperature. Neutralise the acid by adding 1 ml of 10 M potassium hydroxide solution to each tube.

Procedure:

Set the spectrophotometer at 340 nm wavelength. Using matched cells, zero the instrument (according to the manufacturer's instructions).
Into two cells with 1 cm path length, place the following:

	Reference cell	Sample cell
Buffer solution (@20°)	2.50 ml	2.50 ml
NADP solution	0.10 ml	0.10 ml
ATP solution	0.10 ml	0.10 ml
Sample to be measured	--	0.20 ml
Deionized water	0.20 ml	--

Start the stop-watch and mix the solution in the cell. Add 0.02 ml of G-6-P-dehydrogenase solution to both cells after three minutes and mix. Add 0.02 ml of PGI Solution to both cells after 17 min and mix. Read the absorbance of the solution in reference as well as sample cells, after 10 min. After two more minutes, read the absorbance (A_1) of the solution l to ensure that the reaction has stopped (indicated by no increase in absorbance).

Add 0.02 ml each of phosphomannose isomerase solution (616 U/ml) and mix. Read the absorbance after 30 min. Check absorbance (A_2) after two more minutes to ensure that the reaction has stopped (indicated by no increase in absorbance).

Note: The time needed for the completion of enzyme activity may vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

Calculation:
Calculate the differences in absorbance between A_1 and A_2 ($A_2 – A_1$ corresponds to mannose) for the reference cell (ΔA_T) and the sample cell (ΔA_D), and then obtain $\Delta A_M = \Delta A_D - \Delta A_T$

Calculate mannose by the following expression.

$$Cg/l = 0.423 \times \Delta A_M$$

$$\text{Mannose, \% w/w (on the dried basis)} = \frac{0.423 \times \Delta A_M \times 100}{W \times (100-\%M)}$$

Where: W is the weight of sample, g
%M is the loss on drying, g

$$\text{\% Mannose in total polysaccharides} = \frac{\%\text{Mannose on DB} \times 100}{\%\text{Polysaccharides DB}}$$

(DB = Dried basis)

Nitrogen Content
Weigh accurately 1.0 g of yeast mannoprotein, and proceed as directed under Nitrogen determination (Kjeldahl Method, Method 1) in Volume 4 (under "General Methods, Inorganic components").

METATARTARIC ACID

Prepared at the 87th JECFA and published in FAO JECFA Monographs 23 (2019), superseding specifications prepared at the 84th JECFA (2017) and published in FAO JECFA Monographs 20 (2017). The 84th JECFA concluded that metatartaric acid (when used in winemaking) is included in the group ADI of 0–30 mg/kg bw for L(+)-tartaric acid and its sodium, potassium, potassium–sodium salts, expressed as L(+)- tartaric acid.

SYNONYMS	INS No. 353
DEFINITION	Metatartaric acid is a polydisperse polymer of tartaric acid with a degree of esterification above 32%. It is manufactured by heating L- tartaric acid from natural sources at temperatures of 150-170º under atmospheric or under a reduced pressure. The product contains di- tartaric monoester and diester, other polyester acids of variable chain length, as well as free tartaric acid.
Chemical name	Metatartaric acid
C.A.S. number	56959-20-7/ 39469-81-3
Chemical formula	$(C_4H_4O_5)_n$
Assay	Not less than 105% as total tartaric acid
DESCRIPTION	Crystalline or powder form with an off-white colour. Very deliquescent with a faint odour of caramel
FUNCTIONAL USES	Stabilizer (prevents growth and precipitation of potassium bitartrate and calcium tartrate crystals in wine)
CHARACTERISTICS	
IDENTIFICATION	
Solubility	Freely soluble in water and soluble in ethanol
Infra-red spectrum (Vol.4)	The solid state transmission spectrum of a sample, obtained by using a FT-IR spectrophotometer (with an internal ATR reflectance module and a zinc-selenide crystal), corresponds to the reference spectrum given in the Annex.
Test for tartrate (Vol. 4)	Passes test

PURITY

pH (Vol. 4)	1.4 - 2.2 (1% solution)
Loss on drying	Not more than 5% at 105º, 2h
Free tartaric acid	Not more than 73% See description under METHOD OF ASSAY
Degree of esterification	Not less than 32% See description under METHOD OF ASSAY
Optical rotation (Vol.4)	Between -34º and -41º (5% solution, 20º)
Molecular weight distribution and polydispersity index	Medium molecular weight range is between 2 – 9 kDa Polydispersity index (Mz/Mn): Not less than 10 See description under TESTS
Arsenic (Vol. 4)	Not more than 3 g/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 principles of methods described in Volume 4 (under “General Methods, Metallic Impurities”).
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 principles of methods described in Volume 4 (under “General Methods, Metallic Impurities”).

TESTS

Molecular weight distribution	Determine by size-exclusion chromatography with UV, refractive index and multiangle light scattering detection (MALS) using dextran standards of known molecular weights for the system calibration. Equipment and reagents: A size-exclusion chromatograph (ÄKTA 10 Purifier system), consisting of an autosampler capable of injecting up to 1000 µl, UV (UPC-900, GE Healthcare), Refractive Index (Metrohm/ Bischoff 8120, Filderstadt, Germany) and Multiangle Light Scattering Detector (MALS, miniDawn TREOS 591-TS, Wyatt, Dernbach, Germany) or equivalent. Detectors are connected in series. Column: Superose 12 column (300 x 10 mm, GE Healthcare /Pharmacia) or equivalent.

Detector: UV at λ=280 nm

RI Detector

MALS: The basic calibration of the MALS detector is done with filtered toluene as an isotropic scattering agent (calibration constant 4.9644 x 10^{-5} V/cm). The instrument is normalized using bovine serum albumin.

System calibration standards: Standard dextrans -T2000, T500, T70, T40, T10 (GE Healthcare/Pharmacia) and stachyose -738 Da or equivalent.

Mobile phase: sodium chloride solution, 0.1M

Flow rate: 0.5 ml/min

Preparation of system calibration solutions (2 mg/ml): Accurately weigh about 100 mg each of system calibration standards, dissolve in mobile phase and make up to 50 ml in a volumetric flask. Filter through a 0.1 µm membrane-filter.

Preparation of sample solution (4 mg/ml): Accurately weigh about 200 mg of the sample into a 50-ml volumetric flask and dilute to volume with mobile phase. Filter through a 0.1 µm membrane-filter.

Procedure:

System calibration: Inject 500 µl of system calibration standard solutions into the pre-stabilized chromatograph. During the run, the refractive index signal is fed into the light scattering detector providing the essential concentration information for MALS detector.

Determination of absolute molecular masses with MALS using an appropriate software (Astra 6.0.5.software, Wyatt Corp., Santa Barbara, Ca or equivalent). The absolute molecular masses requires the input of refractive index increment (dn/dc value) the test material.

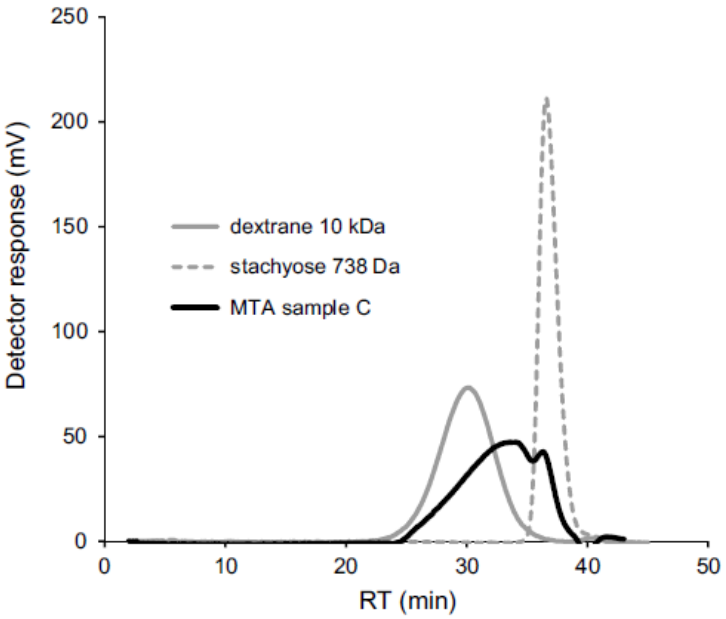
<u>Determination of the refractive index increment (dn/dc)</u>

Dissolve the sample in concentration series of 0.1-1.0 mg/ml in the mobile phase and inject directly into the refractive index detector with a syringe and get the dn/dc value. The mean value of dn/dc for MTA is 0.116 ml/g.

Input UV and RI data following the procedure detailed (Sprenger, S., Hirn, S., Dietrich, H and Will, F. (2015) Metatartaric acid: physicochemical characterization and analytical detection in wines and grape juices. Eur.Food.Res. Technol., 241:785-791.), to calculate of dn/dc values and deduce the medium molecular weight (Mw), low (Mn) and high molecular weights (Mz) using Astra software (Wyatt).

(https://www.wyatt.com/products/software/astra.html). Calculate the polydispersity index (Mz/Mn).

Typical SEC chromatogram of MTA with RI detector



Treating metatartaric acid with sodium hydroxide will cause de- esterification of metatartaric acid resulting in tartaric acid. This allows calculation of the degree of esterification. Addition of a known excess of sodium hydroxide solution followed by back titration with standard sulfuric acid to ~ pH=7 (bromothymol blue indicator) will allow calculation of the total free and esterified acid present in the sample.

Reagents:
Standard sodium hydroxide solution, 1 M
Standard sulfuric acid solution, 0.5 M
Bromothymol blue TS

Procedure:

Accurately weigh about 20 g of metatartaric acid (W), dissolve in deionized water and make up to volume in a 1 l volumetric flask, and mix well. Pipette 50 ml of this solution into an Erlenmeyer flask

Add about 10 drops of bromothymol blue TS and mix well.

Titrate with 1 M sodium hydroxide solution until the indicator turns bluish-green (pH=7). Record the titer value, ml (n).

Pipette 20 ml of 1 M sodium hydroxide into the Erlenmeyer flask, stopper and allow to stand for 2 hours at ambient temperature.

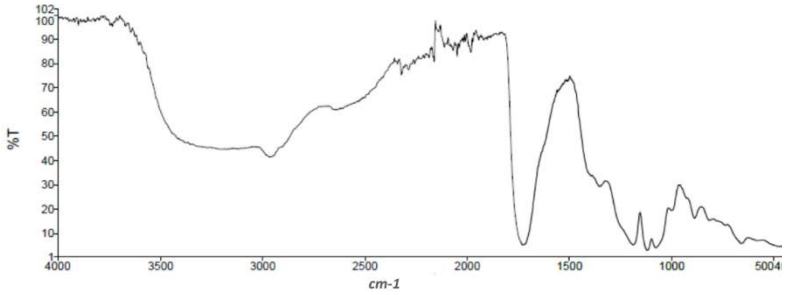
Titrate with 0.5 M sulfuric acid until the indicator turns bluish- green (pH=7). Record the titer value, ml (n').

Calculation:
Free tartaric acid: F (%w/w) = 150.09 × n/W

Esterified tartaric acid: P (%w/w) = 150.09 (20-n')/W

Where:
W is the weight of sample
Total tartaric acid, %w/w = F+P
Degree of Esterification (%) = 100 (20-n')/[n+(20-n')]

Annex FTIR spectrum of metatartaric acid



POTASSIUM POLYASPARTATE

Prepared at the 87th JECFA (2019) and published in FAO JECFA Monographs 23 (2019). The use of potassium polyaspartate in wine at the maximum proposed use level of 300 mg/l was determined to not be a safety concern at the 87th JECFA (2019).

SYNONYMS INS 456, Aspartic Acid, homopolymer potassium salt; E 456, KPA; polyaspartate macromonomer (A-5D K/SD; A-5D K SD; A-5DK/SD; A-5DK).

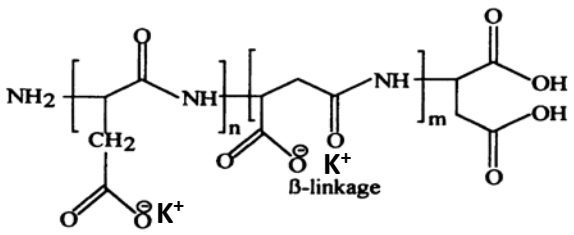
DEFINITION Potassium polyaspartate is produced from L-aspartic acid in a two-step process. During the first step, heating of solid L-aspartic acid leads to solid phase polycondensation. Racemization occurs during this step. The water insoluble polysuccinimide obtained is subsequently treated with aqueous potassium hydroxide under controlled conditions which leads to hydrolysis, opening of the succinimide rings and production of the water soluble potassium salt of poly-D,L-aspartic acid. The final spray-dried product contains approximately 70% β-peptide bonds and 30% α-peptide bonds.

Chemical names

C.A.S. number

Chemical formula $[C_4H_4NO_3K]_n$

Chemical structure



(The structural formula above is for illustrative purposes only. The molecule will contain a random distribution of α- and β-peptide bonds).

Molecular weight Approximately 5000 Daltons (weight average)

Assay Potassium polyaspartate: Not less than 98% on the dried basis
See description under TESTS

Potassium: Not less than 23% on the dried basis.

DESCRIPTION Light tan to brown powder.

FUNCTIONAL USES Stabilizer (in wine)

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Very soluble in water; slightly soluble in methanol, acetone, ethyl acetate and hexane.
Infrared Spectrum (Vol. 4)	The solid state transmission spectrum of a sample, obtained by using a FT-IR spectrophotometer equipped with an internal ATR reflectance module and a diamond crystal, corresponds to the reference spectrum given in the Annex.
pH (Vol. 4)	7.5 - 8.5 (40 % aqueous solution).

PURITY

Loss on drying (Vol. 4)	Not more than 11% (24h, 105^{o})
Free aspartic acid	Not more than 1% on the dried basis See description under TESTS
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 Vol. 4.
Molecular weight distribution and polydispersity index	Fraction below 1000 Daltons: Not more than 20% Fraction above 7000 Daltons: Not more than 27% Polydispersity index: Less than 5.7 See description under TESTS

PURITY TESTS

Free aspartic acid

Free aspartic acid in the sample is derivatized with o-phthalaldehyde (OPA) and analysed by HPLC-FLD (Vol. 4) using aminocaproic acid as internal standard.

Equipment and reagents

HPLC system consisting of a quaternary pump, auto sampler consisting of on-line derivatization module, column thermostat and fluorescence detector (FLD)
HPLC Column: Syncronis aQ 4.6x250mm; 5 µm or equivalent
DL-Aspartic acid: ≥ 99% (CAS number: 617-45-8)
Aminocaproic acid: ≥ 99% (CAS number: 60-32-2)
Orthophtalaldehyde (OPA): Analytical grade
2-Mercaptoethanol: Analytical grade
Sodium tetra borate decahydrate, Analytical grade
Sodium acetate, anhydrous: Analytical grade
Acetonitrile, methanol, tetrahydrofuran and water: HPLC grade

Preparation of solutions
Standard solutions:
Stock standard solution (1 mg/ml): Accurately weigh 100 mg of aspartic acid, dissolve in 100 ml water in a volumetric flask.
Standard solution-1 (0.2 mg/ml): Pipette 10 ml of stock standard solution and dilute to 50 ml with water in a volumetric flask.

Working standard solutions: Prepare 20 ml each of working standard solutions of 2, 10, 50, 100, 250 and 500 µg/ml in water by diluting either stock standard solution or standard solution-1 as required.

Internal standard: Accurately weigh 100 mg of aminocaproic acid, dissolve in 100 ml water in a volumetric flask.

Into six 20 ml volumetric flasks pipette 5,0 ml of each working standard solution, 0.2 ml of internal standard and bring to volume with water.

Derivatization solution: In a 10 ml volumetric flask introduce 100 mg of OPA, 200 µl of mercaptoethanol, 1 ml of methanol and bring to volume with a buffer solution of pH 10.5 of sodium tetraborate decahydrate (0.1M). The solution shall be prepared just before the use as it degrades within one day after preparation.

Sample preparation:
Weigh about 0.5 g of sample, with an accuracy of 0.1 mg, in a 100 ml volumetric flask, dissolve in water and make up to volume. Into a 20 ml volumetric flask, pipette 5.0 ml of the sample solution and 0.2 ml of internal standard and make up to volume with water.

HPLC mobile phases
A: Water
B: Sodium acetate buffer (0.05M): tetrahydrofuran (96:4)
C: Methanol
D: Acetonitrile

HPLC Analysis:
Mobile phase flow rate: 1.1 ml/min.
Column oven Temperature 40°C
Detector wavelength (FLD): Ex 340 nm, Em 450 nm
Gradient conditions:

Time (min)	% B	%C	% D
0.0	100	0	0
3.0	100	0	0
15.0	50	25	25
17.0	84	8	8
18.0	100	0	0
Stop time 21 min + 2 min post time			

Start the HPLC system using mobile phase B at 100% and wait until the system is stabilized indicated by a linear baseline.

Autosampler: Vial 1: methanol, Vial 2: Derivatization solution, Vial 3: empty, Vial 4-9: Standard solutions, Vial 10: Sample solution and Vial 11: water

On-line derivatization step:
- draw 2.0 µl from air
- draw 20.0 µl from vial 1
- eject 20.0 µl into vial 3
- draw 5.0 µl from standard (from vials 4 -9)
- eject 5.0 µl into seat
- draw 0.0 µl from vial 1
- draw 5.0 µl from vial 3
- eject 5.0 µl into seat
- mix 10.0 µl in seat, 10 times
- wait 0.50 min
- inject

Construct standard curve using the original concentrations of the standards and ensure that R^2 is >99. Inject sample solution (dilute sample solution, if the concentration is higher than the upper limit of the standard curve, as required and repeat sample injections) and deduce the concentration of aspartic acid in sample solution C (mg/l).

Calculate the percent free D,L aspartic acid impurity in the sample using the following equation

Free Aspartic acid on the dried basis, %w/w
= 100*100(ml)* C(mg/l) /[1000(ml/l)*W(mg)*(100-M)/100]

Where :
W = amount of sample dissolved in 100 ml, mg
C = concentration of aspartic acid deduced from standard curve, mg/l
M = Loss on drying, %w/w

Molecular weight and molecular weight distribution

The average molecular weight and molecular weight distribution is determined by gel permeation chromatography (GPC) using polyacrylic acid standards of known molecular weights for the system calibration.

Instrumentation:
GPC consisting of a binary pump, column oven, an autosampler capable of injecting up to 1000 µl and UV detector. Agilent HP1100 Series or equivalent
Column: Ultrahydrogel Linear (Waters, part #11545) or equivalent.
Flow rate: 0.7 ml/min
Column Temperature: 25°
Detection: 220 nm UV
Injection Volume: 200 µl
Run time: 40 min

Mobile Phase (Buffer: 0.1M Na_2SO_4 and 0.01M KH_2PO_4 containing 50 µg/ml sodium azide): Weigh 56.8 g Na_2SO_4, 5.44 g KH_2PO_4 and

0.2 g sodium azide, dissolve in water and make up to 4 l. Store in an amber glass bottle in the refrigerator. Filter using a 0.22 µm filter prior to use.

Sample Preparation: Weigh 50 mg of sample, dissolve in mobile phase and make up to 50 ml with mobile phase. Filter through a 0.22 µm filter. Prepare fresh samples just before the analysis.

System Calibration:
Use polyacrylic acid standards ranging in molecular weights from 585,400 to 1250 Daltons. Add aspartic acid monomer as a standard. Prepare calibration standard solutions following the procedure shown under sample preparation and inject to GPC. Typical calibration order is shown below:

Typical Calibration Order
585400 + 4100 Mp
130500 + 2925 Mp
47500 + 1250 Mp
28000 Mp
16000 Mp
7500 Mp
133 L-aspartic acid

Treat data based on the instructions of the instrument.
Report the weight average molecular weight, percent molecular weight fraction above 7000 Daltons, percent molecular weight fraction below 1000 Daltons and the polydispersity index in the sample.

Potassium polyaspartate:
Total aspartic acid content in the sample is determined after acid hydrolysis by HPLC following the procedure given under the determination of free aspartic acid.

Apparatus and reagents (for acid hydrolysis)
Oven, temperature controlled
Screw cap reaction vial with teflon liner, 4 ml
Potassium metabisulphite solution ($Na_2S_2O_2$): 10 g/l
Hydrochloric acid solution: 6M
Sodium hydroxide solution: 5M
Water, HPLC grade

Procedure:
Accurately weigh 50 mg of sample to the nearest 0.1 mg and quantitatively transfer into a 4 ml reaction vial. Add 0.2 ml of 10 g/l sodium metabisulphite solution, 2 ml of 6 M HCl. Heat to 108 ± 2° for 72 hours. Cool to room temperature and transfer to a 100 ml volumetric flask, add 2.4 ml of 5 M NaOH and make up to volume with water. Filter 5 ml of the solution through a 0.20 µm filter into a 20 ml volumetric flask. Add 0.2 ml internal standard solution (refer to free aspartic acid procedure). Make up to volume with water.

Follow the HPLC analysis given under the determination of free aspartic acid and determine the concentration aspartic acid in the sample solution.

Determine the concentration C (mg/l) of the total aspartic acid in the hydrolysed solution from the standard curve. Calculate the percent aspartic acid (total) on the dried basis using the formula below:

Total Aspartic acid on the dried basis, %w/w
= 100*100(ml)* C/[1000(ml/l)*W*(100-M)/100]

Where :
W = amount of sample dissolved in 100 ml, mg
C = concentration of aspartic acid determined from standard curve, mg/l
M = loss on drying, %w/w

Calculation:
Potassium polyaspartate (KPA), %w/w on the dried basis =
= (% total aspartic acid - % free aspartic acid) * f_{KPA}

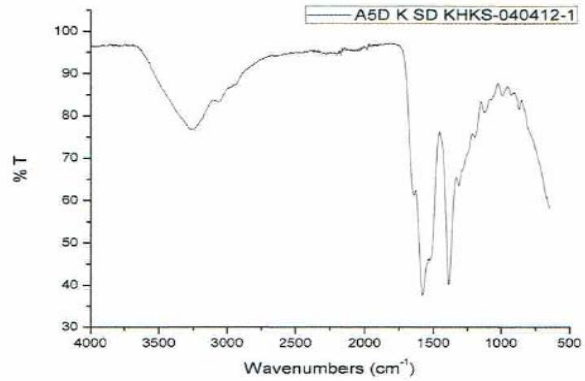
f_{KPA} = 1.15, is the conversion factor of aspartic acid to KPA [calculated as the ratio of the molar mass of the KPA repeat unit (153) and the molecular mass of aspartic acid (133)].

% free aspartic acid = Obtained under the determination of free aspartic acid

Potassium content:
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 Vol. 4

Annex: Infrared spectrum

Main Peaks:
3254 cm^{-1}: N-H stretching
3062 cm^{-1}: Overtone of Amide II (C–N and N–H)
2930 cm^{-1}: C-H stretching
1641 cm^{-1}: Amide C=O sym stretching
1522 cm^{-1}: COO stretching
1385 cm^{-1}: C-H bending vibration



ROSEMARY EXTRACT

Prepared at the 87th JECFA (2019), published in FAO JECFA Monograph 23 (2019) superseding specifications prepared at the 82nd JECFA (2016). A temporary ADI of 0-0.3 mg/kg bw was established at the 82nd JECFA (2016).

SYNONYMS INS No. 392

DEFINITION Rosemary extract consists of phenolic diterpenes, carnosic acid and carnosol as principal antioxidants. Other components present include triterpenes and triterpenic acids. Rosemary extract is obtained from ground dried leaves of Rosmarinus officinalis L using food-grade solvents, namely, acetone or ethanol. Solvent extraction is followed by filtration, solvent removal, drying and sieving to obtain a fine powder. Additional concentration and/or precipitation steps followed by deodorisation, decolourisation and standardisation using diluents and carriers of food grade quality may be included to produce the final product.

The product of commerce can be standardized to a total carnosic acid and carnosol content up to 33%.

Chemical names Carnosic acid: 4a(2H)-Phenanthrenecarboxylic acid, 1,3,4,9,10,10a- hexahydro-5,6-dihydroxy-1,1-dimethyl-7-(1-methylethyl)-, (4aR-trans)-

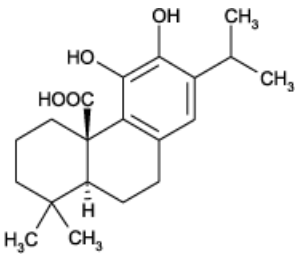
Carnosol: 2H-9,4a-(Epoxymethano)phenanthren-12-one, 1,3,4,9,10,10a- hexahydro-5,6-dihydroxy-1,1-dimethyl-7(1-methylethyl), (4aR- (4aα,9α,10aβ))-

C.A.S. number Extract of rosemary: 84604-14-8

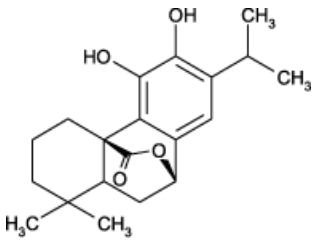
Carnosic acid: 3650-09-7
Carnosol: 5957-80-2

Chemical formula Carnosic acid: $C_{20}H_{28}O_4$
Carnosol: $C_{20}H_{26}O_4$

Structural formula



Carnosic acid



Carnosol

Formula weight Carnosic acid: 332.43
Carnosol: 330.42

Assay Not less than 5% (total of carnosic acid and carnosol)
See description under TESTS

DESCRIPTION Beige to light brown powder.

FUNCTIONAL USES Antioxidant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in water; soluble in vegetable and animal fats and oils.

Antioxidants/Reference Volatiles Ratio % Total of carnosic acid and carnosol/%Total of reference volatiles: not less than 15
See description under TESTS

PURITY

Loss on drying (Vol. 4) Not more than 5% (80° under vacuum, 4 h, 1 g).

Residual solvents (Vol. 4) Acetone: Not more than 50 mg/kg
See description under TESTS

Arsenic (Vol. 4) Not more than 3 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 Volume 4 (under “General Methods, Metallic Impurities”).

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 methods described in Volume 4 (under “General Methods, Metallic Impurities”).

TESTS

IDENTITY TESTS

Antioxidant/Reference Volatiles Ratio % Antioxidants (total of carnosic acid and carnosol) is determined by the Method of Assay.
Reference Volatiles [(-)-borneol, (-)-bornyl acetate, (-)-camphor, 1,8-cineole and verbenone] is determined using GC-MS.

Equipment and Standards:
GC-MS consisting an autosampler and FactorFour VF-5MS (30m x 0.25 mm x 0.25 µm) capillary column, or equivalent.
(-)-Borneol, Supelco. 15598 or equivalent
(-)-Bornyl acetate,Sigma-Aldrich 45855 or equivalent

(-)-Camphor, Supelco 21293 or equivalent
1,8-Cineole (Eucalyptol), Sigma-Aldrich C80601 or equivalent
Verbenone, Supelco 94882 or equivalent

Internal Standard: 4-Heptanone, Sigma-Aldrich. 43570 or equivalent
Tetrahydrofuran (THF), HPLC grade

Preparation of Mixed Standard Solution (SS, 400 µg/ml):
Accurately weigh 20 mg of each Standard into a 50 ml volumetric flask. Dissolve in THF and dilute to volume.

Preparation of Internal Standard Solution (ISS, 400 µg/ml):
Accurately weigh 20 mg of 4-heptanone in a 50 ml volumetric flask. Dissolve in THF and dilute to volume.

Preparation of Sample Solution:
Accurately weigh 2.5 g of the sample in a 10 ml volumetric flask. Add 500 µl of the Internal Standard Solution and dilute to volume with THF. Sonicate for10 min. Filter an aliquot through 0.45 µm filter.

Preparation of working standard solutions (WSS):

Standard	WSS Conc, µg/ml	SS, µl	ISS, µl	THF, µl	Total Volume, µl
Level 0	0	0	100	1900	2000
Level 1	Approx. 4	20	100	1880	2000
Level 2	Approx. 20	100	100	1800	2000
Level 3	Approx. 40	200	100	1700	2000
Level 4	Approx. 100	500	100	1400	2000
Level 5	Approx. 200	1000	100	900	2000

Procedure: Load the WSS and the Sample Solution, onto the autosampler and inject using following conditions.

GC conditions:
Carrier gas: Helium
Flow rate 1 ml/min with constant flow
Injection volume: 1 µl
Split: 100:1
Injector: 250°
Temperature: Ion source: 150°, Transfer line: 240°, Quadrupole: 230°

Temperature Program:

Temperature [°]	Rate [°/min]	Hold [min]	Total [min]
70	0.0	1.00	1.00
130	5.0	0.00	13.00
240	10.0	1.00	25.00

MS Acquisition:

Segments / Names	Ionization Scan type	Running Time [min]	Ion [m/z]
1.	Off	0.00 – 3.00	-
2. 4-Heptanone (IS)	EI - SIM	3.00 – 3.50	43 71 114
3.	Off	3.50 – 5.00	-
4. 1,8-Cineole	EI - SIM	5.00-6.50	43 139 154
5.	Off	6.50 – 8.00	-
6. Camphor, Borneol, Verbenone	EI - SIM	8.00 – 11.00	95 107 110 135 152
7. Bornyl acetate	EI - SIM	11.00 – 13.00	95 154 196

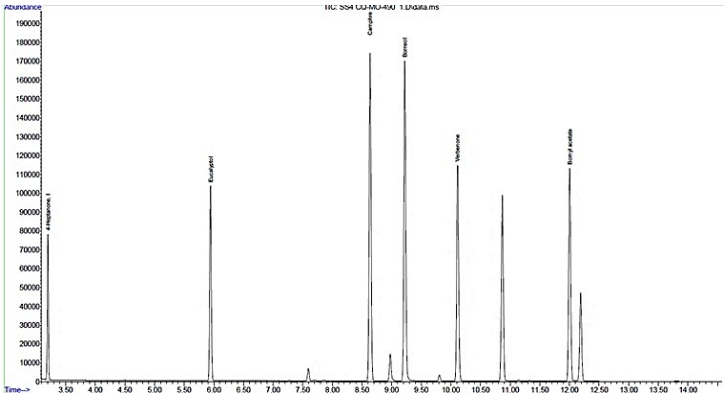
Analyze using the analytical condition as described above. Measure the peak area of each standard and 4-heptanone (IS). Construct internal standard curves by linear regression analysis for each standard. Calculate the concentration of each volatile as follows:

[Compound], mg/kg = A/a × V/W

A is peak ratio of individual volatile to internal standard (IS) in Sample Solution
a is slope of the regression line
V is volume [ml] of Sample Solution
W is weight [g] of sample

Calculate the sum of 5 volatiles and report.

A representative GC-MS chromatogram of the volatile standards is shown below



Residual solvents

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

Equipment and Standards:
GC/FID with dynamic headspace auto-sampler
Acetone >99.5% (Sigma-Aldrich Cat. 32201) or equivalent

Preparation of stock standard solution (1mg/ml):
Accurately weigh 0.1 g of acetone in to a 100 ml volumetric flask and make up to volume with N, N-dimethylformamide (DMF).

Preparation of working standard solution:
Dilute stock standard solution with DMF to get 1, 2, 5, 10, 20, 40 µg/ml of working standard solutions.

Pipette 1.0 ml each of working standard solutions into 20 ml glass autosampler vials and seal.

Preparation of Sample Solution:

Accurately weigh 200 mg sample into a 20 ml glass autosampler vial. Add 1.0 ml dimethylformamide, sonicate it for several minutes and seal.

GC conditions:
Column: Capillary column DB-624 (30 m x 0.53 mm x 3 µm) or equivalent
Column temperature program: 40°, 5 min →10°/min→200°, 9 min
Carrier gas: He at a flow rate of 6 ml/min with constant flow
Split ratio: 1.2:1
Injector temperature: 260°
Detector: FID; Detector temperature: 300°
Hydrogen flow: 30 ml/min
Air flow: 400 ml/min
Nitrogen (detector makeup gas) flow: 25 ml/min
Head space volume for injection: 1000 µl

Headspace conditions:
Heating temperature: 70°
Heating time: 60 min
Syringe temperature: 95°
Transfer line temperature: 95°

Procedure:
Place the vials of sample solutions and standard solutions in the sample tray on head-space gas autosampler. Inject head-space and record areas. Construct standard curve and deduce the concentration of acetone using the formula:

Calculation:

$$\text{Acetone (mg/kg)} = \frac{\left(\frac{A_S - y}{a} \times \frac{P_{std}}{100} \times 1000000\right)}{W}$$

where
A_S is peak area of acetone in Sample Solution
y is y-intercept of acetone standard curve
a is slope of standard curve
P_{std} is purity of standard (%)
W is sample weight (mg)
1000000 is multiple concentration conversion in mg/kg

METHOD OF ASSAY

Determine carnosic acid and carnosol content by HPLC using the following conditions:

Equipment and Reagents:
HPLC consisting of a UV detector and Autosampler.
Column: Chemically bonded octadecylsilane column: ZORBAX SB-C18 (250 mm x 4.6 mm ID x 5-µm), Agilent Technologies or equivalent;
Detector Wavelength: 230 nm
Flow rate: 1.5 ml/min
Temperature: 25°
Injection volume: 5 µl
Reference Standard: USP Powdered Extract of Rosemary RS
Phosphoric acid, ACS grade
Methanol, HPLC grade
Water, HPLC grade

Preparation of mobile phase:
Combine acetonitrile with 0.5% phosphoric acid in water (v/v) at a ratio of 65:35.

Preparation of phosphoric acid solution: Dilute 0.5 ml of phosphoric acid with 100 ml of methanol.

Preparation of Reference Standard Solution:

Prepare 200-500 µg/ml of USP Powdered Extract of Rosemary RS in phosphoric acid solution. Sonicate for 5 min; filter through a 0.45-µm filter.

System Suitability Standard Solution:

Accurately prepare 100 µg/ml of USP Carnosic acid RS (or equivalent) in phosphoric acid solution. Sonicate for 5 min; filter through a 0.45-µm filter.

Sample Solution:

Prepare 500 µg/ml of the sample in phosphoric acid solution. Sonicate for 5 min; filter through a 0.45-µm filter.

Procedure:

Separately inject in duplicate the System Suitability Standard Solution, Reference Standard Solution and Sample Solution, and record the HPLC UV outputs. Identify the peaks present in the chromatograms from the sample by comparison to the peaks from the Reference Standard chromatograms.

Calculations:

System Suitability Requirements:

Tailing Factor for the carnosic acid peak in the chromatogram is 0.90 to 1.30.

The RSD for the carnosic acid peak response on replicate injections is not more than 2%.

% Carnosic acid or Carnosol in sample:

$$\% \text{ Carnosic acid} = \frac{A_{Analyte}}{A_{Std}} \times \frac{C_{Std}}{C_u} \times 100$$

$$\% \text{ Carnosol} = \frac{A_{Analyte}}{A_{Std}} \times \frac{C_{Std}}{C_u} \times \frac{1}{F} \times 100$$

where

AAnalyte is peak area of the analyte of interest (carnosic acid or carnosol) obtained from the chromatogram of the Sample Solution

AStd is peak area of carnosic acid obtained from the chromatogram of System Suitability Standard Solution

CStd is concentration of carnosic acid in the System

Suitability Standard Solution (µg/ml)
CU is concentration of Sample Solution (µg/ml)
F is Relative Response Factor of the analyte of interest (1.00 for carnosic acid; 0.92 for carnosol).

(Framework for) STEVIOL GLYCOSIDES

Prepared at the 87th JECFA (2019) and published in FAO JECFA Monograph 23 (2019), superseding specifications included in "Steviol glycoside from Stevia rebaudiana Bertoni" prepared at the 84th JECFA (2017), published in FAO JECFA Monographs 20(2017).

Introduction

The functional use of steviol glycosides in food is as a general purpose sweetener. They are 100 to 300 times sweeter than sucrose. Steviol glycosides are the constituents of the leaves of the plant, Stevia rebaudiana Bertoni and are responsible for the sweet taste. They share a similar molecular structure in that the same steviol aglycone is bound to different type and number of glycoside units (e.g., glucose, rhamnose, xylose, fructose, or deoxyglucose). More than forty steviol glycosides have been identified to date (see Annex A).

Background

Steviol glycosides produced by the extraction from the leaves of Stevia rebaudiana Bertoni were reviewed by the Committee at its fifty-first, sixty-third, and sixty-eighth meetings. At the sixty eighth meeting the Committee extended the temporary ADI of 0–2 mg/kg bw for steviol glycosides, expressed as steviol, pending submission of the results of the ongoing studies by the end of 2008. The sixty-eighth meeting also removed the 'tentative' designation on the specifications for steviol glycosides. At the sixty-ninth meeting, the Committee evaluated steviol glycosides extracted from the leaves of the plant Stevia rebaudiana Bertoni for use as a sweetener. After reviewing all the information, the Committee concluded that the data was sufficient to establish an ADI for steviol glycosides of 0–4 mg/kg bw, expressed as steviol equivalents. A specifications monograph was prepared.

At the eighty-second meeting, the Committee evaluated steviol glycosides produced by fermentation of a strain of Yarrowia lipolytica, genetically modified to express the Stevia rebaudiana metabolic pathway. The primary steviol glycoside from this process is rebaudioside A. Based on its chemical structure and toxicological studies, the Committee considered it to be as safe as steviol glycosides extracted from the leaves of the plant Stevia rebaudiana Bertoni; an ADI of 0–4 mg/kg bw, expressed as steviol equivalents was applied to this ingredient as well. A new specifications monograph was prepared to reflect additional considerations resulting from the new source material. (Steviol Glycosides from *Stevia rebaudiana* Bertoni).

The Definition and Assay specification was expanded from nine named leaf-derived steviol glycosides to include any mixture of steviol glycoside compounds derived from Stevia rebaudiana Bertoni, provided that the total percentage of steviol glycosides is not less than 95%. The specifications for steviol glycosides were established as tentative pending method of assay to replace the existing method and including as many steviol glycosides as possible (at least those listed in Appendix 1 of the specifications) in steviol glycoside mixtures.

At the eighty-fourth meeting, the Committee revised the specifications for steviol glycosides from *Stevia rebaudiana* Bertoni and removed the tentative status.

Explanation for the framework approach

The two existing specification monographs for steviol glycosides produced either from leaf extracts or by fermentation require that the products consist of at least 95% steviol

glycosides on a dried basis. The major glycosides present in the extract of the leaves from the Stevia rebaudiana Bertoni plant are stevioside and rebaudioside A, and the minor glycosides include rebaudioside M and rebaudioside D. According to industry, several minor glycosides have more favourable sensory characteristics than the major glycosides. Consequently, technologies were developed to produce steviol glycosides, which enhance the proportion of minor glycosides to modify the sensory profile of steviol glycosides, the article of commerce.

Apart from the extraction of the leaves (see annex 1 for further information and specifications), three other technologies were presented by industry for evaluation:

a) Fermentation; a process in which a genetically engineered microorganism is used to produce specific steviol glycosides from simple sugars (see annex 2 for further specifications and information).

b) Bioconversion; a process in which steviol glycosides that have been extracted from the leaves of Stevia rebaudiana Bertoni undergo enzymatic conversion of major steviol glycosides to minor ones (see annex 3 for further information and specifications).

c) Glucosylation; a process in which steviol glycosides that have been extracted from the leaves of *Stevia rebaudiana* Bertonti undergo enzymatically catalyzed reactions to add glucose units to the steviol glycosides via α-linkages (see annex 4 for further information and specifications).

After reviewing the data 87th JECFA consented that as long as the reviewed products consist of 95% steviol glycosides and the remaining 5% can be explained by varying amounts of starting material, dietary sugars, water and residues of food-grade processing aids there is the possibility to include all four different ways of production into this presented framework document. For this reason, the annexes, each a specification monograph for one production pathway, were included in this document. Although the annexes are including redundant information about specifications or methods that the different production pathways share, it was the opinion of 87th JECFA to keep the annexes apart for clarity.

Methods section

Method of assay

METHOD OF ASSAY (for annexes 1-4)

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 and an example chromatogram are provided below 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 (µg/mL).

Calculation of major steviol glycosides content:
Calculate the concentration of each steviol glycoside in the sample solution using the following formula:

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

Where:
- C_{sample} is the average concentration (µg/mL) in the sample solution
- W_{sample} is the weight of sample (µg) in 1 mL of sample solution (~1000 µg/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 at the end of this document.

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 provided at the end of this document) 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 at the end of this document was 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:

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

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

Deduce the concentration (µg/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:

Minor steviol glycosides conc. (%w/w) = $C_{sample} \times 100 / W_{sample}$

Where:
- C_{sample} is the assayed concentration (µg/mL) in the test sample
- W_{sample} is the sample weight in 1 mL of solution (µg/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).

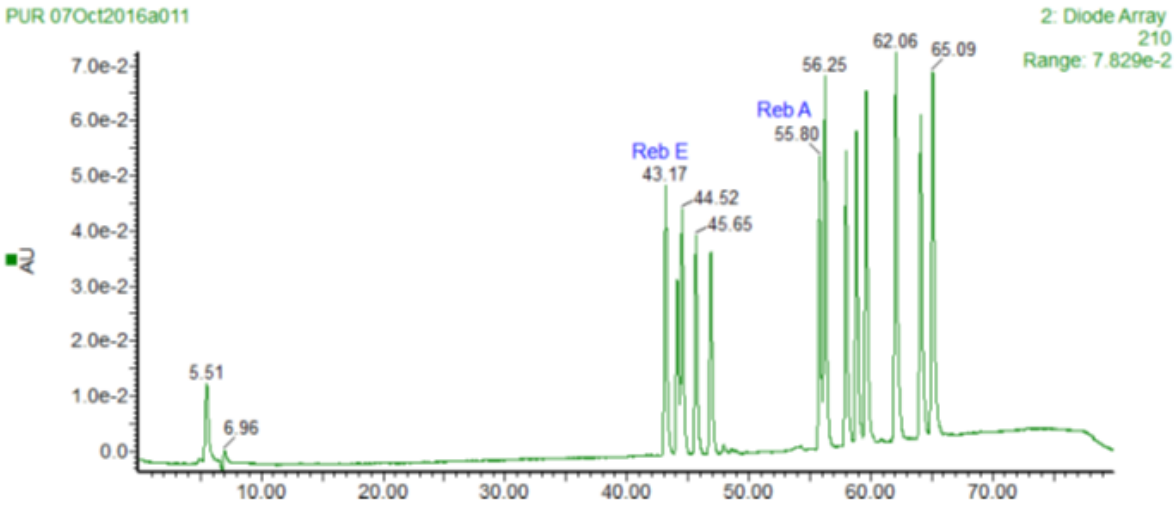
Determine the total amount of steviol glycoside content using the following formula:

$$TSG = (A + B) \times 100 / (100 - M)$$

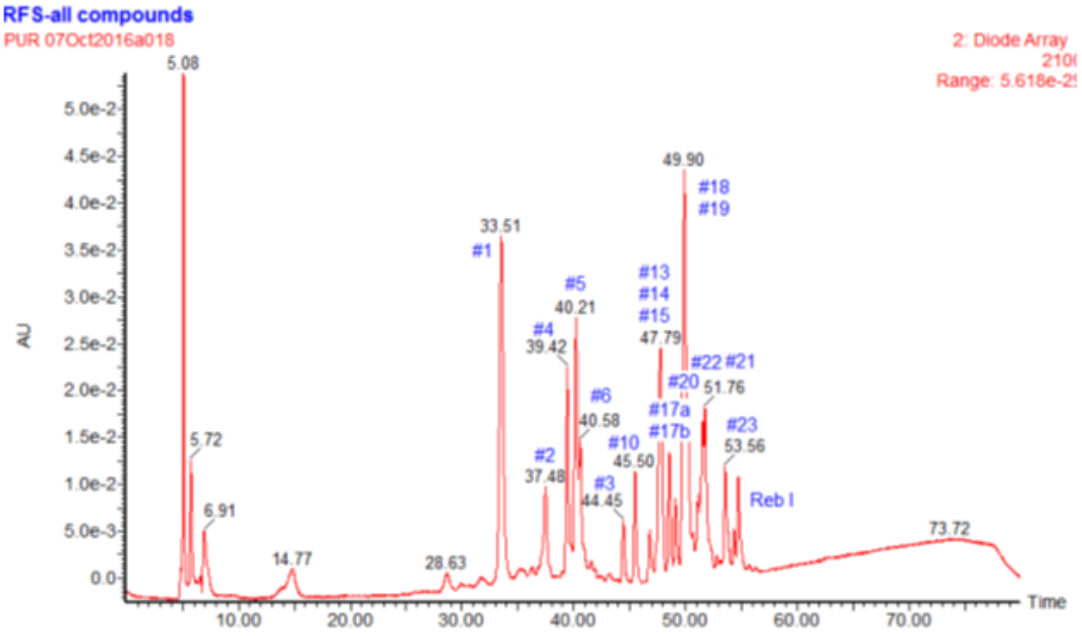
Where:
- TSG is the Total steviol glycosides content (%w/w, on the dried basis)
- A is the percent major steviol glycosides
- B is the percent minor steviol glycosides
- M is the percent loss on drying

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



Example Chromatogram of Representative Steviol Glycoside Standards from a Phenomenex Luna C18 (150 mm x 4.6 mm, 5µm). 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, 5µm) of Minor Steviol Glycosides using in-house purified referencce standards.

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 or1127
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.

Appendix A: Chemical Information for Some Steviol Glycosides

Note: This list is not exhaustive - at least 30 steviol glycosides have been identified in stevia leaf extracts in literature.

Common Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Group 1: Steviol + Glucose (SvGn)						
Rubusoside	Glcβ1-	Glcβ1-	13-[(β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	64849-39-4	$C_{32}H_{50}O_{13}$	642.73
Steviolbioside	H	Glcβ(1-2)Glcβ1-	13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid	41093-60-1	$C_{32}H_{50}O_{13}$	642.73
Stevioside	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	$C_{38}H_{60}O_{18}$	804.87
Rebaudioside *B*	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	$C_{38}H_{60}O_{18}$	804.87
Rebaudioside *E*	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-	13-[(*O*-β- D-Glucoopyranosyl-(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	$C_{44}H_{70}O_{23}$	967.01
Rebaudioside *A*	Glcβ1-	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, β-D-glucopyranosyl ester	58543-16-1	$C_{44}H_{70}O_{23}$	967.01
Rebaudioside *D*	Glcβ(1-2)Glcβ1-	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, 2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl ester	63279-13-0	$C_{50}H_{80}O_{28}$	1129.15
Rebaudioside *M*	Glcβ(1-2)[Glcβ (1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(*O*-β- D-Glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-*O*-β- D-glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl ester	1220616-44-3	$C_{56}H_{90}O_{33}$	1291.29

Common Name	R_1	R_2	Chemical Name	CAS Number	Chemical Formula	Formula Weight
Group 2:Steviol + Rhamnose + Glucose (SvR1Gn)						
Dulcoside *A*	Glcβ1-	Rhaα(1-2)Glcβ1-	13-[(2-*O*-β-D-rhamnopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	64432-06-0	$C_{38}H_{60}O_{17}$	788.87
Rebaudioside *C*	Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-*O*-β-D-rhamnopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	63550-99-2	$C_{44}H_{70}O_{22}$	951.01
Rebaudioside *N*	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(*O*-β- D-Glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-*O*-6-deoxy-L-mannopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl ester	1220616-46-5	$C_{56}H_{90}O_{32}$	1275.29
Rebaudioside *O*	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(*O*-β-D-Glucopyranosyl-(1,2)-*O*-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-*O*-β-D-glucopyranosyl-(1,3)-*O*-6- deoxy-L-mannopyranosyl-(1,2)-*O*-[β-D-glucopyranosyl-(1,3)]-β-D-glucopyranosyl ester	1220616-48-7	$C_{62}H_{100}O_{37}$	1437.44
Group 3: Steviol + Xylose + Glucose (SvX1Gn)						
Rebaudioside *F*	Glcβ1-	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	13-[(2-*O*-β-D-xylopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester	438045-89-7	$C_{43}H_{68}O_{22}$	936.99

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

ANNEX 1: STEVIOL GLYCOSIDES FROM STEVIA REBAUDIANA BERTONI

Prepared at the 87th JECFA (2019) and published in FAO Monographs 23 (2019), superseding specifications prepared at the 84th JECFA (2017) and published in FAO JECFA Monographs 20 (2017). An ADI of 0 - 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

SYNONYMS	INS No. 960a
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 names	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK
C.A.S number	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK
Chemical formula	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK
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 Volume 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 CFU/g *E. coli*: Negative in 1 g *Salmonella*: Negative in 25 g
METHOD OF ASSAY	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

ANNEX 2: STEVIOL GLYCOSIDES FROM FERMENTATION

Prepared at the 87th JECFA (2019) and published in FAO Monographs 23 (2019), superseding 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. 960b

DEFINITION Steviol glycosides from fermentation consist of a mixture of compounds containing a steviol backbone conjugated to various sugar moieties (e.g. glucose or sucrose) depending on the specific production organism and fermentation conditions used.

Steviol glycosides from fermentation are obtained from the fermentation of non-toxigenic non-pathogenic strains of *Yarrowia lipolytica* and *Saccharomyces cerevisiae* that have been genetically modified with heterologous genes from multiple donor organisms to overexpress steviol glycosides. After removal of the biomass by solid-liquid separation and heat treatment, the process involves concentration of the steviol glycosides (e.g. by resin adsorption), followed by purification of the desired steviol glycosides by crystallization and drying. Ion exchange resins may be used in the purification process. The final product may be spray-dried. Commercial products are primarily composed of either rebaudioside A, rebaudioside M, or a combination of rebaudioside M and rebaudioside D; additional minor steviol glycosides may be present.

Chemical names See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

C.A.S number See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Chemical formula See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Assay Not less than 95% of total of steviol glycosides, on the dried basis.

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, sparingly soluble in water and sparingly soluble in ethanol.
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 Volume 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 CFU/g *E. coli*: Negative in 1 g *Salmonella*: Negative in 25 g
METHOD OF ASSAY	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

ANNEX 3: ENZYME MODIFIED STEVIOL GLYCOSIDES

Prepared at the 87th JECFA (2019) and published in FAO Monographs 23 (2019). An ADI of 0 - 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

SYNONYMS

DEFINITION

Enzyme modified 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 enzymatic treatment of purified steviol glycosides extracted from the leaves of *Stevia rebaudiana* Bertoni. The purified leaf extract is treated with enzymes produced by non-toxigenic non-pathogenic strains of *Pichia pastoris* and *Escherichia coli* that have been genetically modified with genes from multiple donor organisms (listed below) to produce glucosyltransferase (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13). The resulting material is heated and filtered to denature and remove the enzymes. The raw product is concentrated using resin adsorption/desorption or solid/liquid filtration, followed by purification and preparation of the product of commerce using processes that may include decolourization, crystallization, and spray drying.

This manufacturing technique maximizes the production of specific steviol glycosides that are not naturally present in high concentrations in the leaf extract, primarily rebaudioside M and rebaudioside D with minor amounts of other steviol glycosides.

Enzyme production organism	**Gene source**
Pichia pastoris	*Horedum vulgare* L *Stevia rebaudiana Bertoni* *Vigna radiate*
Escherichia coli	*Acidithiobacillus caldus* *Arapidopsis thaliana* *Solanum tuberosum* *Stevia rebaudiana*

Chemical names

See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

C.A.S number

See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

Chemical formula	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK
Assay	Not less than 95% of total of steviol glycosides, on the dried basis
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 Volume 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 CFU/g *E. coli*: Negative in 1 g *Salmonella*: Negative in 25 g
METHOD OF ASSAY	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK

ANNEX 4: ENZYME MODIFIED GLUCOSYLATED STEVIOL GLYCOSIDES (TENTATIVE)

Prepared at the 87th JECFA (2019) and published in FAO Monographs 23 (2019). An ADI of 0 - 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

SYNONYMS

DEFINITION

Enzyme modified glucosylated steviol glycosides are steviol glycoside mixtures composed predominantly of glucosylated steviol glycosides (e.g., mono-, di-, and tri-glucosylated glycosides) with small amounts of steviol glycosides from *Stevia rebaudiana* Bertoni. Glucosylated steviol glycosides are obtained through the enzymatic addition of glucose [1–20 additional subunits via α-(1-4) glucosyl linkages] to purified steviol glycosides obtained from the leaves of *Stevia rebaudiana* Bertoni. Cyclomaltodextrin glucanotransferase (EC 2.4.1.19) and α-amylase (EC 3.2.1.1) from non-toxigenic non-pathogenic strains of *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus subtilis* are used to facilitate the transfer of glucose to steviol glycosides. The resulting material is heated heating and treated with activated carbon to remove the enzymes. The raw product is concentrated using resin adsorption/desorption, followed by purification and preparation of the product of commerce using processes that may include decolourization, crystallization, and spray drying.

This manufacturing technique maximizes the production of enzyme modified glucosylated steviol glycosides that are not naturally present in the leaf extract.

[Note: Strains of *Bacillus stearothermophilus* and *Bacillus licheniformis* used to produce the enzymes used in this manufacturing technique may be products of genetic modification.]

Chemical names	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK
C.A.S number	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK
Chemical formula	See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK
Assay	Not less than 95% of total of steviol glycosides, on the dried , dextrin-free basis, determined as the sum of glucosylated steviol glycosides and steviol glycosides

DESCRIPTION White to light yellow powder, odourless or having a slight characteristic odour. About 100 - 167 times sweeter than sucrose.

FUNCTIONAL USES Sweetener

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Freely soluble in water
HPLC chromatographic profile	Following treatment with glucoamylase, the main peaks in a chromatogram obtained by analysing a sample following the procedure in METHOD OF ASSAY correspond to steviol glycosides from *Stevia rebaudiana* Bertoni
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 Volume 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 CFU/g *E. coli*: Negative in 1 g *Salmonella*: Negative in 25 g

METHOD OF ASSAY

Total steviol glycosides in enzyme modified glucosylated steviol glycosides are measured as the combined percentage of steviol glycosides and glucosylated steviol glycosides on the dried, dextrin-free basis. The percentage of steviol glycosides (those with analytical standards; these are present as unreacted steviol glycoside residues from the manufacturing process) is determined using a chromatographic method (See Appendix A to STEVIOL GLYCOSIDES SPECIFICATIONS FRAMEWORK). The percentage of glucosylated steviol glycosides as glucose after hydrolysis with glucoamylase using the colorimetric method described below. The content of dextrin is gravimetrically determined as indicated following the determination of glucosylated steviol glycosides.

Preparation of Sample Solution:
Weigh accurately about 1 g of sample and dissolve in 50 mL of water. Pass this solution through a glass column (25-mm internal diameter) packed with 50 mL of acrylic acid ester resin or styrene-divinyl-benzene resin at a rate of less than 3 mL/min. Wash the resin with 250 mL of water; collect the aqueous eluate and evaporate to dryness under vacuum for estimation of dextrin content (if necessary; Sample solution B). Pass 250 mL of 50% (v/v) ethanol through the column at a rate of not more than 3 mL/min. Evaporate the ethanolic eluate to about 100 mL, add 40 mL of acetate buffer (pH 4.5) and water to make about 180 mL. Allow this solution to stand at 55°C for about 5 min; add 20,000 units of glucoamylase, and allow to stand at 55°C for 45 min. Heat at 95°C for about 30 min, cool to room temperature, transfer to a 200-mL volumetric flask and dilute to volume with water (Sample solution A).

Procedure:
Proceed as directed under the METHOD OF ASSAY in the GENERAL SPECIFICATIONS FOR STEVIOL GLYCOSIDES using Sample solution A.

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

Determination of Glucosylated Steviol Glycosides as Glucose:

Prepartion of Standard solutions:
Transfer 1 g of glucose to a 100-mL volumetric flask and dilute to volume with water. Using this stock solution,

prepare a series of solutions containing 0.5 – 3.0 mg/mL of glucose in water.

Preparation of Sample solution:
Use Sample solution A as prepared for the Determination of Steviol Glycosides by HPLC.

Blank Sample solution:
Dilute 40 mL of acetate buffer (pH 4.5) to 180 mL with water and hold at 55° for about 5 min. Add 20,000 units of glucoamylase and allow to stand at 55° for 45 min. Heat the solution at 95° for 30 min, cool to room temperature, transfer to a 200-mL volumetric flask and dilute to volume with water.

Color fixing solution:
Dissolve 0.50 g of phenol, 130 units of galactose mutarotase, 9000 units of glucose oxidase, 650 units of peroxidase and 0.1 g of 4-aminoantipyrine in phosphate buffer (pH 7.1) and make to exactly 1L. Store at 2-10° and use within 1 month of preparation.

Procedure:
Add 20 µL of the sample solution to 3.0 mL of color fixing solution and allow to stand at 37° for exactly 5 min. Cool to room temperature and measure the absorbance of the resulting solution at a wavelength of 505 nm against a reference solution similarly prepared using 20 µL of water in place of the sample solution. Perform a blank test by measuring the absorbance of the blank sample solution prepared in the same manner as the sample solution and make any necessary correction.

Prepare a calibration curve by measuring the absorbances of the standard solutions prepared in the same manner as the sample solution.

Determine the concentration of d-glucose in the sample solution (corrected by subtracting the absorbance of the blank test solution) from the calibration curve and calculate the α-glucosyl residue content by the following formula:

$$\text{α-glucosyl residues (\%, w/w)} = C_{glucose} / (C_{sample} \times 0.900 \times 100)$$

Where:
- $C_{glucose}$ is the concentration of d-glucose in the sample solution as determined from the calibration curve (mg/mL)

- C_{sample} is the concentration of enzyme modified glucosylated steviol glycosides in the sample solution (mg/mL)

Total steviol glycosides content

Calculate the total steviol glycosides content on the dried basis by the following formula:

$$TSG = (A + B) \times 100 / (100 - M)$$

Where:
- TSG is the Total steviol glycosides content (%w/w, on the dried basis)
- A is the percent of steviol glycosides
- B is the percent of α-glucosyl residues
- M is the percent loss on drying

Where necessary, the content of residual dextrin can be determined as follows:

Evaporate Sample solution B (as prepared for the Determination of Steviol Glycosides by HPLC) to dryness. Further dry the residue in a vacuum oven at 105° for 2h and record the dry weight of the fraction. Calculate the content of residual dextrin by the following formula:

$$\text{Residual dextrin (\%, w/w)} = (W_{eluate} / W_{sample}) \times 100$$

Where:
- W_{eluate} is the weight of the dried aqueous fraction (Sample solution B, g)
- W_{sample} is the dry weight of the enzyme modified glucosylated steviol glycosides used to prepare Sample solution B (g)

If the content of residual dextrin is more than 3%, the adjusted TSG on the dextrin-free basis is calculated by the following formula:

$$\text{Adjusted TSG (\%, w/w)} = TSG \times (W_{sample}) / (W_{sample} - RD)$$

Where:
- W_{sample} is the dry weight of the enzyme modified glucosylated steviol glycosides used to prepare the Sample solutions (g)
- RD is the amount of residual dextrin present in the sample (g)

SPECIFICATIONS FOR CERTAIN FLAVOURING AGENTS

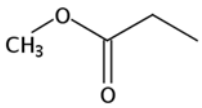
At the 87th meeting, the Committee prepared specifications of identity and purity of 10 flavourings for the following numbers: 141, 345, 547, 889, 893, 967, 979, 1029, 1236 and 1604.

Information on specifications for flavouring agents is given in the tables, most of which are self-explanatory: Name; Chemical name (Systematic name, normally IUPAC name); Synonyms; Flavour and Extract Manufacturers' Association of the United States (FEMA) No; FLAVIS (FL) No; Council of Europe (COE) No; Chemical Abstract Service Registry (CAS) No; Chemical formula (Formula); Molecular weight (MW); Physical form/Odour; Solubility; Solubility in ethanol, Boiling point (B.P. ºC – for information only); Identification test (ID) referring to type of test (NMR: Nuclear Magnetic Resonance spectrometry; IR: Infrared spectrometry; MS: Mass spectrometry); Assay min % (Gas chromatographic (GC) assay of flavouring agents); Acid value max; Refractive index (R.I.) (at 20º, if not otherwise stated); Specific gravity (S.G) (at 25º, if not otherwise stated). The field called "Other requirements" contains four types of entry:

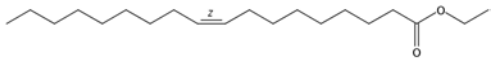
1. Items that are additional requirements, such as further purity criteria or other tests.
2. Items provided for information, for example the typical isomer composition of the flavouring agent. These are not considered to be requirements.
3. Substances which are listed as Secondary Constituents (SC) which have been taken into account in the safety evaluation of the named flavouring agent. If the commercial product contains less than 95% of the named compound, it is a requirement that the major part of the product (i.e. not less than 95% is accounted for by the sum of the named compound and one or more of the secondary constituents.
4. Information on the status of the safety evaluation.

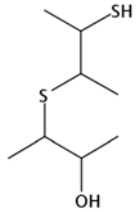
The fields named Session/Status contain the number of the meeting at which the specifications were prepared and the status of the specification. All specifications prepared at the 87th meeting were assigned full status.

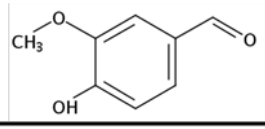
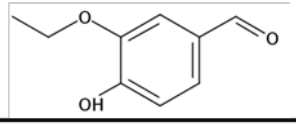
The flavouring agents were evaluated using the Procedure for the Safety Evaluation of Flavouring Agents and a list for conclusions in alphabetical order is given in Annex 5.

JECFA No. Status Session	Name Chemical Name Synonyms	FEMA FLAVIS COE CAS	Chemical Formula M.W Physical form; Odour	Solubility Solubility in ethanol B.P. °	ID test Assay min % Acid value	R.I. (20°) S.G. (25°)	Other requirements Information required
141	Methyl propionate	2742	$C_4H_8O_2$	miscible with alcohol, ether, propylene glycol; soluble 1 ml in 16 ml water	IR	1.373-1.380	
Full	Methyl propanoate		88.11	-	95.0%	0.912-0.918	
		415	colourless to pale yellow liquid with a fruity, rum odour				
87		554-12-1		79°	3		

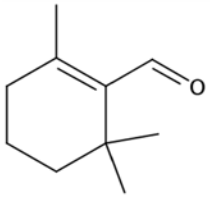
345	Ethyl oleate	2450	$C_{20}H_{38}O_2$	insoluble in water; soluble in ether	IR	1.448-1.453	SC: ethyl linoleate (3.4-11.5%), ethyl palmitate (0.4-5.1%), ethyl stearate (0.5-2.5%), ethyl laurate (1-2%) and other fatty acid ethyl esters; Saponification value: 175-190
Full	Ethyl octadic-9-enoate	-	310.5	Soluble	75%	0.868-0.873	
	Ethyl octadic-9-enoate	633	oily, slightly yellowish liquid with a floral odour	205-208	1		
87		111-62-6					

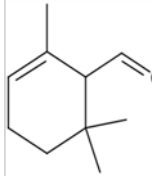


547	alpha-methyl-beta-hydroxypropyl alpha-methyl-beta-mercaptopropyl sulfide	3509	$C_8H_{18}OS_2$	Insoluble in water; soluble in alcohol; slightly soluble in fat	IR, NMR	1.512-1.522	
Full	3-[(2-Mercapto-1-methylpropyl)thio]-2-butanol	12.036	194.35	-	95%	1.040-1.050	
		2353		85-88° (0.4 mm Hg)			
87		54957-02-7					
889	Vanillin	3107	$C_8H_8O_3$	slightly soluble in water; soluble in organic solvents, oils	IR	N/A	Melting point: 81-84°
Full	4-Hydroxy-3-methoxybenzaldehyde	-	152.15	freely soluble	97%	N/A	
	Vanillaldehyde;Methylprotocatechuic aldehyde;Vanillic aldehyde	107	white or slightly yellow needles or crystalline powder with a sweet, creamy, vanilla odour				

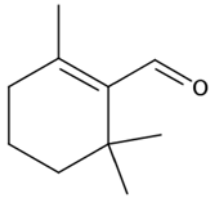
87		121-33-5		285°			
893	Ethyl vanillin	2464	$C_9H_{10}O_3$	insoluble in water; soluble in organic solvents, oils	IR	N/A	m.p. 76-79°
Full	3-Ethoxy-4-hydroxybenzaldehyde		166.18	very soluble (1g in 2ml)	98%	N/A	
	Bourbonal ethyl protal;3-Ethoxy protocatechualdehyde	108	colourless or slightly yellow crystal flakes with an intense vanilla odour				
87		121-32-4		285°			
967	2,2,3-Trimethylcyclopent-3-en-1-yl acetaldehyde	3592	$C_{10}H_{16}O$	Insoluble in water; soluble in oils	NMR	1.462-1.469	SC: Gamma-campholene, secondary component, not more than 2%
Full	2,2,3-Trimethylcyclopent-3-en-1-yl acetaldehyde	05.119	152.24	Miscible at room temperature	93%		

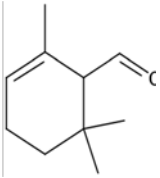
87	Campholenic aldehyde;(R)-2,2,3-Trimethylcyclopent-3-ene-1-acetaldehyde	10325 4501-58-0	Colourless liquid; refreshing sweet-woody odour	75° (105 mm Hg)		0.918-0.924	
979	2,6,6-Trimethyl-1&2-cyclohexen-1-carboxaldehyde	3639	$C_{10}H_{16}O$	Insoluble in water	IR, NMR	1.4986-1.4991	Other requirement [approximately 50% alpha-cyclocitral and 50% beta-cyclocitral]
Full	Mixture of 2,6,6-Trimethylcyclohex-1-ene-1-carboxaldehyde and 2,6,6-Trimethylcyclohex-2-ene-1-carboxaldehyde alpha- and beta-cyclocitral (50-50 mixture)	05.182 05.121 10326 11849 432-25-7 432-24-6 52844-21-0	152.23 Colourless liquid; camphoraceous odour	Miscible at room temperature 62° (3 mm Hg)	99%	0.950-0.957	

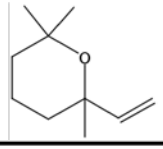




	Campholenic aldehyde;(R)-2,2,3-Trimethylcyclopent-3-ene-1-acetaldehyde	10325		75° (105 mm Hg)		0.918-0.924	
87		4501-58-0	Colourless liquid; refreshing sweet-woody odour				
979	2,6,6-Trimethyl-1&2-cyclohexen-1-carboxaldehyde	3639	$C_{10}H_{16}O$	Insoluble in water	IR, NMR	1.4986-1.4991	Other requirement [approximately 50% alpha-cyclocitral and 50% beta-cyclocitral]
Full	Mixture of 2,6,6-Trimethylcyclohex-1-ene-1-carboxaldehyde and 2,6,6-Trimethylcyclohex-2-ene-1-carboxaldehyde	05.182 05.121	152.23	Miscible at room temperature 62° (3 mm Hg)	99%	0.950-0.957	
	alpha- and beta-cyclocitral (50-50 mixture)	10326 11849	Colourless liquid; camphoraceous odour				
		432-25-7 432-24-6 52844-21-0					





Full	2,2,6-trimethyl-6-vinyltetrahydropyran	13.094	154.25	Soluble	95%	0.863-0.873
	Bois de rose oxide;2,6,6-trimethyl-2-vinyltetrahydropyran;Geranic oxide		Clear liquid with a musty, earthy, terpineolic note	22° (2.5 mm Hg)	1	
87		7392-19-0				

CORRIGENDUM

The following requests for corrections, reported to the Joint JECFA Secretariat, were evaluated by the 87th JECFA meeting and found to be necessary. These corrections, however, will only be made in the electronic versions and in the on-line database.

Food additive	Original text	New text	Additional information
Copper sulfate (INS 519)	CAS: 7758-98-7	CAS: 7758-99-8	Original CAS number is for anhydrous form; however, the specifications are for the pentahydrate
Magnesium dihydrogen diphosphate (INS 450(ix))	METHOD OF ASSAY The determination of phosphorus contains the following formula: P2O5, %w/w = P% × 4.983	METHOD OF ASSAY The determination of phosphorus contains the following formula: P2O5, %w/w = P% × 2.2921	Original formula did not account for the presence of two phosphorus atoms per molecule
Basic methacrylate copolymer (INS 1205) **Will also be applied to anionic methacrylate copolymer (INS 1207) and neutral methacrylate copolymer (INS 1206)**	In section Definition: "Basic methacrylate copolymer is used as a coating and glazing agent for food supplements and foods for special medical purposes."	Sentence deleted.	Deletion requested by the Fifty-first Session of the Codex Committee on Food Additives1; sentence provided only marginal information.
2-Acetyl-1-pyrroline (JECFA No. 1604)	CAS: 99583-29-6	CAS: 85213-22-5	Correction to CAS number

- *The following name was missing from the List of participants in the meeting report of the eighty-sixth meeting of JECFA (WHO Technical Report Series, No. 1014, 2019):*

Dr E. Dessipri, European Directorate for the Quality of Medicines & HealthCare, Council of Europe, Strasbourg, France (*Member*)

- *The following participants were indicated as not attending the eighty-sixth meeting, but actually participated in the meeting by video conference:*

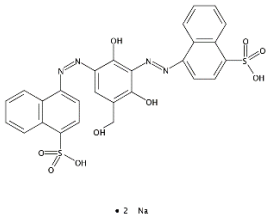
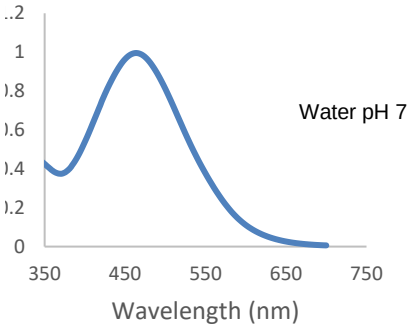
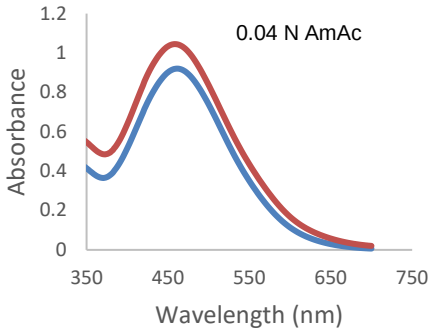
Dr M. DiNovi, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, Maryland, USA (*WHO Temporary Adviser*)

Dr J.R. Srinivasan, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, Maryland, USA (*FAO Expert*)

[1] http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FMeetings%252FCX-711-51%252FReport%252FREP19_FAe.pdf

Table 1

Replacement of the text for the spectrophotometric data for Brown HT and its aluminium lake originally published in "Table 1. Values for synthetic colours for use in performing tests for Colouring Matters Content by Spectrophotometry" (FAO JECFA Monographs 19, 82nd meeting, 2016)

JECFA name	Sample weight	Structure	Spectral data	Visible absorption spectrum
Brown HT	245.6 mg		Water, pH 7 λ_{max} = 464 A = 0.9957 Spec abs = 403 a = 40.3 Water λ_{max} = 464 A = 0.9804 Spec abs = 397 a = 39.7 0.04 N AmAc λ_{max} = 461 A = 0.9206 Spec abs = 373 a = 37.3	
Brown HT Aluminium Lake	53.3 mg		Straight colour (blue) 0.04 N AmAc λ_{max} = 461 A = 0.9206 Lake (red) 0.04 N AmAc λ_{max} = 458 A = 1.0451	

ANNEX 1: SUMMARY OF RECOMMENDATIONS FROM THE 86th JECFA

Food and Agriculture Organization of the United Nations

World Health Organization

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES
Eighty-seventh meeting
Rome, 4–13 June 2019

SUMMARY AND CONCLUSIONS
Issued 25 June 2018

A meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was held in Rome, Italy, from 4 to 13 June 2019. The purpose of the meeting was to evaluate certain food additives.

Dr R. Cantrill served as Chairperson, and Dr A. Mattia, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, served as Vice-Chairperson.

Dr M. Lipp, Agriculture and Consumer Protection Department, Food and Agriculture Organization of the United Nations (FAO), and Mr K. Petersen, Department of Food Safety and Zoonoses, World Health Organization (WHO), served as Joint Secretaries.

The present meeting was the eighty-seventh in a series of similar meetings. The tasks before the Committee were (a) to elaborate principles governing the evaluation of food additives, (b) to undertake safety evaluations of certain food additives, (c) to review and prepare specifications for certain food additives and (d) to establish specifications for certain flavouring agents.

The Committee evaluated the safety of six food additives (including one group of food additives) and revised the specifications for five other food additives (including one group of food additives). The Committee also revised the specifications for nine flavouring agents.

The report of the meeting will be published in the WHO Technical Report Series. Its presentation will be similar to that of previous reports – namely, general considerations, comments on specific substances and recommendations for future work. An annex will include detailed tables (similar to the tables in this report) summarizing the main conclusions of the Committee in terms of acceptable daily intakes and other toxicological, dietary exposure and safety recommendations. Information on the specifications for the identity and purity of certain food additives examined by the Committee and on the specifications for the nine flavouring agents will also be included.

The participants in the meeting are listed in Annex 1. Items of a general nature that the Committee would like to disseminate quickly are included in Annex 2. Future work and recommendations are listed in Annex 3.

Toxicological and dietary exposure monographs or monograph addenda on most of the substances that were considered will be published in WHO Food Additives Series No. 78. New and revised specifications for the identity and purity of the compounds will be published in FAO JECFA Monographs 23.

More information on the work of JECFA is available at:

http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/en/

and

http://www.who.int/foodsafety/areas_work/chemical-risks/jecfa/en/

Toxicological and dietary exposure information and information on specifications

Food additives evaluated toxicologically and assessed for dietary exposure

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
Black carrot extract	N[a], T[b]	The Committee concluded that the effects observed with one anthocyanin-containing test material cannot be extrapolated to another anthocyanin-containing test material. This is because the test articles used in metabolism and toxicity studies are very heterogeneous and often not fully described and/or the anthocyanin content of the test material is too low and variable. Only one genotoxicity study was available for black carrot extract. **Owing to the lack of toxicological data on black carrot extract, the Committee was not able to draw conclusions on its safety.** To proceed with its assessment, at least a 90-day toxicological study on a well-characterized extract representative of the material of commerce would be required. The Committee concluded that the total mean dietary exposure to anthocyanins from naturally occurring sources and added black carrot extract ranges from 0.1 to 1.9 mg/kg body weight (bw) per day for adults (18+ years) and from 0.1 to 5.3 mg/kg bw per day for children (<18 years). The Committee noted that the contribution of the use of the food colour itself to the total mean dietary exposure to anthocyanins including from naturally occurring sources is as high as 25%. The Committee noted that the ADI for grape skin extract established by the previous Committee in 1982 was not reconsidered as part of this assessment and remains unchanged.
Brilliant Black PN	R[c]	The Committee concluded that the newly available information does not give reason to revise the previously established ADI of 0–1 mg/kg bw based on a short-term toxicity study in pigs. **The Committee therefore retained the ADI for Brilliant Black PN.** The Committee noted that the range of estimated dietary exposures for Brilliant Black PN was below the upper end of the ADI and concluded that **dietary exposure to Brilliant Black PN does not present a safety concern**.
Carotenoids (provitamin A)	R[d]	The Committee reaffirmed the conclusion from the eighty-fourth meeting that rats are not an appropriate model for deriving an ADI for β-carotene due to the relatively low bioavailability of β-carotene in rats compared with humans. Therefore, **the Committee withdrew the two group ADIs of 0–5 mg/kg bw for (1) the sum of the synthetic carotenoids β-carotene, β-apo-8′-carotenal and β-apo-8′-carotenoic acid methyl and ethyl esters and (2) synthetic β-carotene and β-carotene derived from *Blakeslea trispora*[e],** which were based on a no-observed-adverse-effect level (NOAEL) from a rat study.

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
		The Committee considered that no adverse health effects were observed in the general population in large, well-conducted human intervention studies in which healthy participants were administered 20–50 mg β-carotene per day for up to 12 years, in addition to background exposure from the diet. An additional elevated risk of lung cancer and total mortality was seen in heavy smokers (at least one pack per day) and asbestos workers in intervention studies in which participants were administered 20 mg β-carotene per day for 5–8 years or 30 mg β-carotene per day and 25 000 IU vitamin A for 5 years. The Committee noted that a generally accepted explanation for the cause of these effects has not been identified. **The Committee was unable to reach any conclusion about risk from β-carotene exposure in heavy smokers.** **For the remainder of the general population, the Committee concluded that the estimated high exposure to β-carotene of 9 mg/day for a 30 kg child and 6 mg/day for a 60 kg adult from its current uses as a food additive, in addition to background exposure from the diet, would not be expected to be a safety concern.** This conclusion includes synthetic β-carotene, β-carotene derived from *B. trispora* and β-carotene-rich extract from *Dunaliella salina.* **The Committee was unable to establish a group ADI for synthetic β-carotene, β-carotene derived from *B. trispora*, β-carotene-rich extract from *D. salina*, and β-apo-8′-carotenoic acid methyl and ethyl esters** because a group ADI is applicable to the general population, which includes heavy smokers. The Committee noted that it is very unlikely that it will ever be possible to establish a group ADI because further data from the population of heavy smokers cannot be gathered ethically. Because β-apo-8′-carotenoic acid methyl and ethyl esters were previously evaluated on the basis of β-carotene and because no new data were submitted, **the Committee was unable to complete an evaluation on β-apo-8′-carotenoic acid methyl and ethyl esters**. **The present Committee established an ADI of 0–0.3 mg/kg bw for β-apo-8′-carotenal** on the basis of a NOAEL of 30 mg/kg bw per day in a 13-week study in rats and application of an uncertainty factor of 100. An additional uncertainty factor to take into account the short duration of the study was not considered necessary because kidney and liver effects observed in the 13-week study at 100 mg/kg bw per day were not observed in a 2-year study at 40 mg/kg bw per day, the single dose tested. Estimated dietary exposure to β-apo-8′-carotenal of 0.3 mg/kg bw per day was at the upper end of the ADI established by the Committee (i.e. 0–0.3 mg/kg bw per day). The Committee noted that the estimated dietary exposure is overestimated and concluded that the **current**

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
		use of β-apo-8′-carotenal as a food additive will not pose a safety concern.
Gellan gum	R[f], T[g]	Available studies confirm the absence of any adverse effects arising from exposure to gellan gum. **The Committee retained the previously established ADI "not specified"[h] for gellan gum.** The Committee evaluated low-acyl clarified gellan gum for use in formulas for special medical purposes for infants. Based on a NOAEL of 100 mg/kg bw per day, the highest dose of low-acyl clarified gellan gum tested in a 21-day neonatal pig study, which modelled the 0- to 12-week period of development in human infants, and the high estimate of dietary exposure of infants to gellan gum of 13 mg/kg bw per day (based on the requested maximum concentration of gellan gum of 50 mg/L and the high level of consumption of infant formula of 260 mL/kg bw per day), a margin of exposure of 7.7 was calculated. The Committee concluded on the basis of several considerations (e.g. the low toxicity of gellan gum, the NOAEL being the highest dose tested, clinical studies in preterm infants and post-marketing surveillance data showing that gellan gum is well tolerated) that the margin of exposure of 7.7 calculated for the use of gellan gum in formulas for special medical purposes for infants and liquid fortification products for addition to human milk or infant formula at a maximum level of 50 mg/L in the fed product indicates low risk for the health of infants, including preterm infants, and that **its proposed use is therefore of no safety concern**. **This conclusion applies only to the use of low-acyl clarified gellan gum.** The Committee recognizes that there is variability in medical conditions among infants requiring these products and that these infants would normally be under medical supervision.
Potassium polyaspartate	N	In vitro data suggest that the systemic bioavailability of potassium polyaspartate is low and that potassium polyaspartate would not be cleaved in the stomach or the intestine. The NOAEL in a 90-day rat study on potassium polyaspartate was 1000 mg/kg bw per day, the highest dose tested. There was no concern for genotoxicity. Potassium has been evaluated by the Committee in the course of its previous evaluation of potassium hydroxide, and the result of the evaluation was an ADI "not limited"[i]. Exposure to potassium that results from the use of potassium polyaspartate in wine would be within normal daily variation of background potassium exposure from the diet. Should microbial fermentation in the human colon occur, there would be potential exposure to L- and D-aspartic acid. L-Aspartic acid is a normal constituent of dietary protein, and systemic exposure to L-aspartic acid from the diet is much higher than potential exposure from the use of potassium polyaspartate in wine.

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological and dietary exposure conclusions
		There are no relevant toxicological data on D-aspartic acid. In three studies, rats exposed to around 130 mg/kg bw per day showed effects on sex hormone levels. However, NOAELs have not been identified in these studies due to the use of single doses. The Committee noted that there is a margin of exposure of more than 100-fold between the potential human dietary exposure to D-aspartic acid of up to 0.8 mg/kg bw per day and the effect level of 130 mg/kg bw per day. The estimated dietary exposure to D-aspartic acid from typical use of potassium polyaspartate in wine (up to 0.8 mg/kg bw per day) would be expected to be lower than the exposure from non-added sources in the diet. The Committee noted that it had limited data on concentrations of D-aspartic acid in food, but that food processing (e.g. heat treatment of protein, fermentation) will result in partial conversion of L-aspartic acid to D-aspartic acid. **The Committee concluded that the use of potassium polyaspartate in wine at the maximum proposed use level of 300 mg/L is not of safety concern.**
Rosemary extract	R[j]	The Committee concluded that the new studies provided evidence for the absence of reproductive toxicity, but not for the absence of developmental toxicity. **The Committee retained the temporary ADI of 0–0.3 mg/kg bw, pending the submission of studies on the developmental toxicity of rosemary extract and studies to elucidate whether the effects noted on rodent pup thyroid hormone levels can be replicated**. The temporary ADI will be withdrawn if the requested studies are not submitted by the end of 2021. Estimated mean and high-percentile dietary exposures to carnosic acid plus carnosol from use of rosemary extract as an additive for all countries assessed based on typical use levels did not exceed the upper end of the temporary ADI (0–0.3 mg/kg bw per day). The Committee noted that when dietary exposures from naturally occurring sources are combined with dietary exposures from added sources at typical use levels, the estimated dietary exposures for children were up to 0.42 mg/kg bw per day, which exceeds the ADI. The Committee also noted that the temporary ADI is based on the highest dose tested in a short-term toxicity study in rats and that in the newly submitted reproductive/developmental toxicity screening study, no effects on reproductive toxicity or on parental animals were observed at 316 mg/kg bw per day, the highest dose tested. **Therefore, the Committee does not consider the slight exceedance of the ADI to be a safety concern.**

N: new specifications; R: existing specifications revised; T: tentative specifications

[a] For the spray-dried powder form of black carrot extract.

[b] The specifications were made tentative pending further information on the material of commerce, including a full characterization of the proteins, carbohydrates, lipids, fibre, minerals and non-anthocyanin polyphenol components in five lots each of the liquid and powder forms of black carrot extract.

[c] Analytical methods for determining subsidiary colouring matters and organic compounds other than colouring matters were replaced with more specific and sensitive high-performance liquid chromatography methods. The existing titrimetric method for the assay of Brilliant Black PN was replaced with a visible spectrophotometric method.

[d] The specifications for synthetic β-carotene, β-carotene from *B. trispora* and β-apo-8'-carotenal were revised to replace an identification test for carotenoids with additional spectrophotometric requirements. Based on the arsenic levels from several batches of the product of commerce for β-carotene-rich extract from *D. salina*, the existing specifications for arsenic were revised from 1 mg/kg to 3 mg/kg.

[e] The Committee was aware that two group ADIs for carotenoids had been established at previous meetings and that synthetic β-carotene had been included in both group ADIs. The Committee speculated that the Committee at the fifty-seventh meeting did not recognize that synthetic β-carotene was already part of a group ADI and included it in a new group ADI.

[f] The Committee concluded that the use of ethanol in the manufacturing of gellan gum is not a safety concern when used according to good manufacturing practice. The specification for ethanol was removed.

[g] The specifications were made tentative, pending submission of new methods for characterizing the three forms of gellan gum in commerce by 2021.

[h] ADI "not specified" is used to refer to a food substance of very low toxicity that, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary exposure to the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice – i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect; it should not conceal food of inferior quality or adulterated food; and it should not create a nutritional imbalance.

[i] Now called an ADI "not specified" (see table note h).

[j] The Committee removed the specification for ethanol, and the tentative status of the specifications for rosemary extract was removed.

Food additives considered for specifications only

Food additive	Specifications
Cassia gum	T[a]
Citric and fatty acid esters of glycerol (CITREM)	R[b]
Metatartaric acid	R[c]
Mannoproteins from yeast cell walls	R[d]

R: existing specifications revised; T: tentative specifications

[a] At the eighty-sixth meeting, the Committee updated the specifications for cassia gum by including the high-performance liquid chromatographic method received and removed their tentative status. Based on comments received about the method performance, the present Committee reviewed the method again and noted that additional investigations were required. Therefore, the Committee decided to make the specifications tentative until ongoing investigations are completed.

[b] The Committee received a suitable validated replacement method for an obsolete packed column gas chromatographic method for the determination of total citric acid content, along with performance characteristics of the method and data on the total citric acid content in products currently available in commerce, determined using that method. The Committee included the new method in the specifications and deleted the previous method. A new high-performance liquid chromatography method for the analysis of glycerol, supported by validation data, was provided and included in the revised specifications. The limit for glycerol was maintained. Data on the use of additional neutralizing salts in CITREM manufacture were received and added to the specifications. The lead limit for use of CITREM in infant formula was corrected to 0.5 mg/kg according to the previous evaluation. Data on the sulfated ash levels and the content of minerals in neutralized CITREM products were provided. The limit for sulfated ash was maintained for non-

neutralized CITREM, and new limits were set for partially neutralized and for wholly neutralized CITREM. The tentative status of the specifications was removed.

[c] The Committee received information on optical rotation, infrared identification, free tartaric acid content, degree of esterification and molecular weight distribution, together with the analytical methods. The Committee revised the specifications for free tartaric acid, optical rotation, molecular weight and molecular weight distribution and included a specification for polydispersity index. The tentative status of the specifications for metatartaric acid was removed.

[d] The Committee revised the specifications monograph and noted that a change in the name of the additive from “Yeast extracts containing mannoproteins” to “Mannoproteins from yeast cell walls” was appropriate. The Committee noted that all mannoproteins, regardless of the range of molecular weights, were included in the same specifications monograph and therefore specifying a range of average molecular weight and a method for measuring it was not essential. Data were also received for metallic impurities. The Committee reviewed the information received and decided that only a limit for lead was required. The tentative status of the specifications was removed.

[e] A framework was adopted for developing specifications for steviol glycosides by four different methods of production. Specifications for steviol glycosides produced by different production methods were included as annexes, as below:

- Annex 1: Steviol Glycosides from *Stevia rebaudiana* Bertoni (**revised** from the specifications monograph for Steviol glycosides from *Stevia rebaudiana* Bertoni prepared at the eighty-fourth meeting of JECFA (INS 960a)).
- Annex 2: Steviol Glycosides from Fermentation (specifications for Rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica* (INS 960b(i)) prepared at the eighty-second meeting of JECFA were **revised** to include other steviol glycosides from *Saccharomyces cerevisiae* and *Yarrowia lipolytica*).
- Annex 3: Enzyme Modified Steviol Glycosides (new specifications).
- Annex 4: Enzyme Modified Glucosylated Steviol Glycosides (**new** specifications, **tentative** pending further information concerning the analytical methods).

For more information, see General considerations below.

Flavouring agents considered for specifications only

Flavouring agent	No.	Specifications
Methyl propionate	141	Ra
Ethyl oleate	345	Rb
alpha-Methyl-beta-hydroxypropyl alpha-methyl-beta-mercaptopropyl sulfide	547	Rc
Vanillin	889	Rd
Ethyl vanillin	893	Re
2,2,3-Trimethylcyclopent-3-en-1-yl acetaldehyde	967	Rf
alpha- and beta-Cyclocitral (50:50 mixture)	979	Rg
Sodium 2-(4-methoxyphenoxy)propanoate	1029	Rh
2,2,6-Trimethyl-6-vinyltetrahydropyran	1236	Ri

R: existing specifications revised

[a] The Committee revised the specific gravity to 0.912–0.918.

[b] The Committee revised the assay minimum to not less than 75% ethyl oleate. Specifications for the secondary components were also established: ethyl linoleate (3.4–11.5%), ethyl palmitate (0.4–5.1%), ethyl stearate (0.5–2.5%), ethyl laurate (1–2%) and other fatty acid ethyl esters.

[c] The Committee revised the refractive index to 1.512–1.522, the specific gravity to 1.040–1.050 and the assay minimum to 95%.

[d] The Committee revised the melting point to 81–84 °C.

[e] The Committee revised the melting point to 76–79 °C

[f] The Committee revised the assay minimum to 93%, with a secondary component of up to 2% of gamma-campholenic aldehyde.

[g] The Committee revised the specifications to include the Chemical Abstracts Service (CAS) numbers for alpha-cyclocitral (CAS No. 432-24-6) and for the mixture of alpha- and beta-cyclocitral (CAS No. 52844-21-0). The Flavis and Council of Europe (COE) numbers for alpha- and beta-cyclocitral were also included. The refractive index range was revised to 1.4986–1.4991.

[h] The Committee revised the CAS number (150436-68-3) and Flavis number (08.127) to reflect the salt form. The melting point was revised to 184–190 ºC. Identifiers and synonyms associated with the free acid were removed.

[i] The Committee changed the minimum assay to 95%, the refractive index to 1.442–1.452 and the specific gravity to 0.863–0.873.

ANNEX 2. GENERAL CONSIDERATIONS

Application of group ADIs

At the Fiftieth Session of the Codex Committee on Food Additives (CCFA), the Codex Secretariat noted that some food additives – such as provitamin A carotenoids (i.e. synthetic β-carotenes, β-carotenes from *Blakeslea trispora,* β-apo-8'-carotenal and methyl and ethyl esters of β-apo-8'-carotenoic acid); chlorophylls and chlorophyllins, copper complexes; and polyoxyethylene sorbitan esters (i.e. polyoxyethylene (20) sorbitan esters of lauric, stearic, palmitic and oleic acids and triesters of stearic acid) – were listed under the same food additive heading in the Codex General Standard for Food Additives (GSFA), despite not being included in a group acceptable daily intake (ADI). The Codex Secretariat sought clarification from the present Committee on the application of group ADIs.

In making recommendations on the safety of food additives, the Committee takes into consideration the principles regarding group ADIs contained in the publication *Principles and methods for the risk assessment of chemicals in food* (Environmental Health Criteria No. 240 [EHC 240]).

The Committee noted that most of the food additives about which CCFA had sought advice had been last considered as groups at several meetings up to and including the twenty-third meeting in 1980 and that the Committee did not explicitly use the term group ADI at those early meetings. For these food additives, the Committee was able to confirm that the chlorophylls and chlorophyllins (copper complexes), polyoxyethylene sorbitan esters (polysorbates), ascorbyl esters, ethylenediaminetetraacetates, thiodipropionates, ferrocyanides, tartrates, stearoyl lactylates and iron oxide food additives should have been allocated group ADIs.

For nitrates and nitrites, the respective ADIs are expressed as the ions and therefore encompass the different salts. The group ADI for steviol glycosides, expressed as steviol, includes the whole family of steviol glycosides. The Committee was also able to confirm that the provisional tolerable weekly intake (PTWI) of 2 mg/kg body weight (bw) for aluminium and its salts, when expressed as aluminium, refers to all aluminium salts used in food additives, as well as other sources of aluminium.

An “unconditional” ADI of 0–0.2 mg/kg bw for 2-phenylphenol was first established by JECFA at its eighth meeting in 1964. According to FAO documents, 2-phenylphenol and sodium *o*-phenylphenate were first evaluated by the 1962 JECFA for their use as a post-harvest treatment of fruits and vegetables to protect against microbial damage during storage and distribution. The current FAO specifications still refer to this use. In 1999, the Joint FAO/WHO Expert Meeting on Pesticide Residues (JMPR) established an ADI of 0–0.4 mg/kg bw for 2-phenylphenol; an ADI was not established for the sodium salt because it rapidly dissociates to 2-phenylphenol. 2-Phenylphenol has a minor use as a flavouring agent, and, during its evaluation at the fifty-fifth meeting of JECFA, the Committee cited the most recent ADI established by JMPR for its risk assessment. In view of its major use as a post-harvest treatment of fruits and vegetables, the Committee is seeking advice from Codex on its current usage as a food additive.

The Committee noted that provitamin A carotenoids were evaluated at the current meeting (see above).

Clarification of ADI “not specified”

Codex requested clarification of the use of the term “ADI ‘not specified’” by JECFA, particularly with respect to the addition of food additives to Table 3 of the GSFA (Additives permitted for use in food in general, unless otherwise specified, in accordance with GMP).

The Committee confirmed its definition of “ADI ‘not specified’” (from EHC 240):

> A term applicable to a food substance of very low toxicity that, on the basis of the available chemical, biochemical and toxicological data as well as the total dietary intake of the

> substance (from its use at the levels necessary to achieve the desired effect and from its acceptable background in food), does not, in the opinion of the Joint FAO/WHO Expert Committee on Food Additives, represent a hazard to health. For that reason, and for reasons stated in individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of Good Manufacturing Practice: that is, it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal inferior food quality or adulteration, and it should not create a nutritional imbalance.

Thus, the definition is based upon information on both toxicity and dietary exposure. A conclusion that a substance is of very low toxicity could be based, for example, upon evidence that the substance did not show adverse effects at the highest doses tested in relevant toxicological studies, is poorly absorbed and does not bioaccumulate, and does not contain toxicologically relevant impurities. The estimate of total dietary exposure (intake) is based upon the uses proposed at the time of the evaluation.

The Committee noted that Guideline 2 (Food Additives with an ADI of "Not Specified") of the GSFA (CODEX STAN 192-1995) specifies:

> When an additive has been allocated an ADI "not specified" it could in principle, be allowed for use in foods in general with no limitation other than in accordance with Good Manufacturing Practices (GMP). It should, however, be born [*sic*] in mind that ADI not specified does not mean that unlimited intake is acceptable. The term is used by JECFA in case [*sic*] where "on the basis of the available data (chemical, biochemical, toxicological, and other) the total daily intake of the substance arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food does not, in the opinion of the Committee, represent a hazard to health.
>
> If, therefore, a substance is used in larger amounts and/or in a wider range of foods than originally envisaged by JECFA it may be necessary to consult JECFA to ensure that the new uses fall within the evaluation. For example a substance may have been evaluated as a humectant without including a later use as a bulk sweetener, which could give considerable [*sic*] higher intake.

The Committee endorses Guideline 2 of the GSFA and recommends that it be applied by addition of appropriate qualifications in Table 3 of the GSFA.

Update of guidance on evaluation of enzyme preparations (EHC 240)

The Committee was informed about activities of an expert working group established in 2018 to discuss available information on the safety of enzymes used in food and current practices of the food enzyme industry. This activity is being undertaken within the context of a joint FAO/WHO project to update various chapters of EHC 240.

The starting point of the discussion was a background document prepared from a review of the current literature and conversations with representatives of the food enzyme industry and their technical experts.

It was noted that the current JECFA guidance on the evaluation of enzyme preparations was designed to address the potential toxicity of secondary metabolites generated by some enzyme sources (e.g. *Aspergillus* species) under certain growth conditions. The guidance includes a requirement to conduct genotoxicity tests as well as 90-day oral toxicity tests in animals.

After nearly 15 years of using this guidance to assess the safety of enzyme preparations, JECFA has not identified any that were toxic. The expert working group has proposed that the safety of enzyme preparations could be assessed with methodologies using fewer animals (e.g. metabolic profiling of microbial fermentation products, genomic DNA sequencing identifying mycotoxin synthesis genes). The expert working group focused on enzymes from genetically modified microorganisms and the information requirements for their safety evaluation.

The expert working group will propose changes to the relevant sections of EHC 240 and produce a checklist of information required in enzyme submissions for future JECFA evaluations.

The Committee urges the expert working group to finalize its work and make the output available for public comment in time for the next JECFA meeting in 2020.

Update of guidance on evaluation of genotoxicity of chemical substances in food (section 4.5 of EHC 240)

The Committee was informed about activities of a joint FAO/WHO expert working group established in 2018 to update and extend the guidance on evaluation of genotoxicity of chemical substances in food. This activity is being undertaken within the context of a joint FAO/WHO project to update various chapters of EHC 240. The aim of the expert working group is to provide guidance on interpretation of test results, in addition to general descriptions of genotoxicity tests, special considerations for data-poor substances, and considerations for chemically related substances and mixtures. The expert working group will also address recent developments and future directions.

This work is ongoing. A public consultation is intended before finalization.

Update of guidance on dose–response assessment and derivation of health-based guidance values (Chapter 5 of EHC 240)

At the eighty-third meeting of the Committee (in 2016), some general considerations regarding dose–response modelling were discussed. The Committee recommended that an expert working group be established to develop detailed guidance for the application of the methods most suitable to its work, in particular for the use of the benchmark dose (BMD) approach. The Committee asked that the expert working group address several aspects, including the use of constraints when fitting models, the use of model averaging, the use of non-parametric methods as alternatives for dose–response risk assessment, the use of biological information for selection of models and transparent presentation of modelling outcomes in JECFA publications.

The Committee was informed that the recommended expert working group was established in 2017 to update and extend the guidance on dose–response assessment and derivation of health-based guidance values. This activity is being undertaken within the context of a joint FAO/WHO project to update various chapters of EHC 240.

The work was undertaken electronically and culminated in a meeting of the expert working group in March 2019 in Geneva to revise and update Chapter 5 of EHC 240, including the preparation of more detailed advice on the BMD approach. The draft revised chapter will include guidance on the use of the freely available BMD software (both the United States Environmental Protection Agency Benchmark Dose Software suite of models and PROAST, which was developed by the Dutch National Institute for Public Health and the Environment, now available through the European Food Safety Authority as a web tool). The draft guidance will encourage the use of the BMD approach wherever possible and appropriate, but will acknowledge that in some situations, use of the no-observed-adverse-effect level (NOAEL)/lowest-observed-adverse-effect level (LOAEL) approach may still be appropriate. The draft guidance will include a decision-tree to aid decision-making about which approach should be followed.

It is anticipated that a revised draft of Chapter 5 of EHC 240 will be ready in June 2019, to be reviewed by the expert working group. The draft will then go out for public consultation, will be revised if necessary and will be published online as a standalone chapter.

Update of guidance on assessing dietary exposure to chemical substances in food (Chapter 6 of EHC 240)

The Committee was informed about activities of a joint FAO/WHO expert working group established in 2018 to update and extend the guidance on assessing dietary exposure to chemical substances in food. This activity is being undertaken within the context of a joint FAO/WHO project to update various chapters of EHC 240.

A revision of the chapter was required to incorporate technological and methodological changes in dietary exposure assessments, including progress in the use of exposure models and more recently available data and databases.

WHO undertook an initial scoping exercise that identified areas of the current chapter that needed to be reviewed and new areas of work to be included and prepared a first draft of an updated

chapter. The draft chapter will be reviewed by a number of dietary exposure experts at a consultation in September 2019. A final draft will be prepared and then released for public comment.

Dietary exposure assessment reporting

In 1996, WHO held an expert consultation that introduced dietary exposure assessment in JECFA's risk assessments for food additives and contaminants. At a 2005 expert consultation to prepare a dietary exposure assessment chapter for what would become EHC 240, a tiered process for systematically preparing dietary exposure assessments was elucidated. This process includes 1) a budget or other screening method, 2) international and national dietary exposure assessments based on summary food consumption data (e.g. Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme [GEMS/Food] cluster diets, FAO/WHO Chronic Individual Food Consumption database – Summary statistics [CIFOCOss], national/regional surveys, published exposure assessments) and 3) refined dietary exposure assessment using food consumption data derived from individual consumers. In this last step, deterministic and probabilistic assessments could be completed as needed and appropriate. Guidance to JECFA monographers was prepared from these consultations.

At the current meeting, the Committee determined that not all steps of the tiered approach are needed in every case to complete the Committee's evaluations. When preparing monographs, JECFA experts comment on each of the steps as appropriate, but in the report of the meeting, only those assessments where sufficient data were available to produce reliable estimates of dietary exposure are described and used in the safety assessment. The Committee noted that lack of discussion of any of the steps in report items does not reflect a lack of consideration during the overall evaluation.

Framework for developing specifications for steviol glycosides by method of production

Steviol glycosides are constituents of the leaves of the plant *Stevia rebaudiana* Bertoni and have a sweet taste. The functional use of steviol glycosides in food is as a sweetener. Steviol glycosides are approximately 100–300 times sweeter than sucrose.

The major glycosides present in the extract of the leaves from the *Stevia rebaudiana* Bertoni plant are stevioside and rebaudioside A. The minor glycosides include rebaudioside M and rebaudioside D and about 40 other steviol glycosides that have been identified to date. Several minor glycosides have more favourable sensory characteristics than the major glycosides, prompting development of technologies that enhance the proportion of minor glycosides to modify the sensory profile of the articles of commerce. These technologies include the following:

a. Extraction: a process of hot water extraction from the leaves of *Stevia rebaudiana* Bertoni.
b. Fermentation: a process in which a genetically modified microorganism is used to produce specific steviol glycosides.
c. Enzymatic modification: a process in which steviol glycosides that have been extracted from the leaves of *Stevia rebaudiana* Bertoni undergo enzymatic conversion of major steviol glycosides to minor ones.
d. Enzymatic glucosylation: a process in which steviol glycosides that have been extracted from the leaves of *Stevia rebaudiana* Bertoni undergo enzyme-catalysed reactions to add glucose units to the steviol glycosides via α-(1-4) linkages.

The microorganisms used in the fermentation or in the production of enzymes used to modify steviol glycosides are of safe lineage. The inserted genes are isolated from non-toxigenic and non-pathogenic sources. Residues from manufacturing processes do not pose any concerns with respect to toxicity or allergenicity.

Steviol glycosides consist of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (e.g. glucose, rhamnose, xylose, fructose, arabinose, galactose, deoxyglucose). Existing specifications for steviol glycosides require that the product consists of ≥95% steviol glycosides on the dried basis.

At the present meeting, the Committee reviewed data on the methods of manufacture, identity and purity of steviol glycosides. The Committee noted that the reviewed products consist of

≥95% steviol glycosides on the dried basis; the remaining 5% or less consists of residues of starting material and food-grade processing aids, depending on the method of production.

A framework was adopted for developing specifications for steviol glycosides by four different methods of production. Specifications for steviol glycosides produced by different production methods were included as annexes, as below:

- Annex 1: Steviol Glycosides from *Stevia rebaudiana* Bertoni (revised from the specifications monograph for Steviol glycosides from *Stevia rebaudiana* Bertoni prepared at the eighty-fourth JECFA (INS 960a)).
- Annex 2: Steviol Glycosides from Fermentation (specifications for Rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica* (INS 960b(i)) prepared at the eighty-second JECFA were revised to include other steviol glycosides from *Saccharomyces cerevisiae* and *Yarrowia lipolytica*).
- Annex 3: Enzyme Modified Steviol Glycosides (new specifications).
- Annex 4: Enzyme Modified Glucosylated Steviol Glycosides (new specifications, tentative pending further information concerning the analytical methods).

At the present meeting, the Committee determined that no safety issues exist for steviol glycosides produced by any one of these methods resulting in products with ≥95% steviol glycosides as per existing specifications. The Committee indicated that the ADI of 0–4 mg/kg bw established at the sixty-ninth meeting of JECFA for steviol glycosides (expressed as steviol) applies to steviol glycosides produced by the four methods indicated in the annexes of the specifications monograph produced at the current meeting.

The Committee recognized that steviol glycosides could be produced via a new method or the modification or combination of the methods currently described in the annexes of the specifications monograph. If the final product meets the current specification of ≥95% steviol glycosides, the Committee will evaluate possible impurities from the method of manufacture. When appropriate, the modifications will be introduced into the relevant annex; alternatively, a new annex would be added.

ANNEX 3. FUTURE WORK AND RECOMMENDATIONS

Unsulfonated primary aromatic amines in food colours

The Committee requests analytical data on unsulfonated primary aromatic amines in the following synthetic food colours – Allura Red AC, Amaranth, Azorubine, Brilliant Black PN, Brilliant Blue FCF, Brown HT, Fast Green FCF, Fast Red E, Green S, Indigotine, Lithol Rubine BK, Patent Blue V, Ponceau 4R, Quinoline Yellow, Sunset Yellow FCF and Tartrazine – along with the analytical methods used, in order to update specifications.

Black carrot extract

To proceed with the assessment of black carrot extract, at least a 90-day toxicological study on a well-characterized extract representative of the material of commerce would be required.
The specifications were made tentative pending the submission of further information on the material of commerce, including a full characterization of the proteins, carbohydrates, lipids, fibre, minerals and non-anthocyanin polyphenol components in five lots each of the liquid and powder forms of black carrot extract.

Carotenoids (provitamin A)

The Committee noted that the use levels of β-carotene and β-apo-8'-carotenal provided by the sponsor were much lower than the corresponding maximum permitted levels as specified in the Codex General Standard for Food Additives (GSFA), and that the sponsor indicated that the majority of the maximum permitted levels are not justifiable from a technological point of view. Also, use levels were not provided for all authorized food categories. The Committee recommended that the Codex Alimentarius Commission should review current uses of β-carotene (synthetic β-carotene, β-carotene from *Blakeslea trispora* and β-carotene-rich extract from *Dunaliella salina*) and β-apo-8'-carotenal in the GSFA, including the maximum permitted levels and the food categories in which these additives may be used.

Gellan gum

The specifications were made tentative pending submission of new methods for characterizing the three forms of gellan gum in commerce by 2021. Specific information required is as follows:

- A method to differentiate the three commercial forms of gellan gum – i.e. high-acyl, low-acyl and low-acyl clarified.
- A method to determine the degree of acylation.
- Validation data for the above methods, including detailed description of the sample preparation.
- Data from five non-consecutive commercial batches of material using the proposed validated methods for all three forms of gellan gum.

Rosemary extract

Studies on the developmental toxicity of rosemary extract and studies to elucidate whether the effects noted on pup thyroid hormone levels can be replicated were identified as research needs to complete the evaluation. The Committee requests that this information be provided by the end of 2021.

COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives

87th Meeting 2019

This document contains food additive specification monographs, analytical methods, and other information prepared at the eighty-seventh meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which was held in Rome, 4-13 June 2019. The specification monographs provide information on the identity and purity of food additives used directly in foods or in food production. The main three objectives of these specifications are to identify the food additive that has been subjected to testing for safety, to ensure that the additives are of the quality required for use in food or in processing and to reflect and encourage good manufacturing practice. This publication and other documents produced by JECFA contain information that is useful to all those who work with or are interested in food additives and their safe use in food.

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