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FAO JECFA Monographs 31

COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives

96th Meeting
Geneva, 27 June – 6 July 2023

Food and Agriculture Organization of the United Nations

World Health Organization

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WORLD HEALTH ORGANIZATION

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SPECIAL NOTE

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Geneva, 27 June – 6 July 2023

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Introduction

This volume of FAO JECFA Monographs contains specifications prepared at the ninety-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), held in Geneva, 27 June – 6 July 2023. The specifications monographs are one of the outputs of JECFA's risk assessment of food additives, and should be read in conjunction with the safety evaluation, reference to which is made in the section at the head of each specifications monograph. Further information on the meeting discussions can be found in the summary report of the meeting (see Annex 1), and in the full report which will be published in the WHO Technical Report series. Toxicological monographs of the substances considered at the meeting will be published in the WHO Food Additive Series.

Specifications monographs prepared by JECFA up to the 65th meeting, other than specifications for flavouring agents, have been published in consolidated form in the Combined Compendium of Food Additive Specifications which is the first publication in the series FAO JECFA Monographs. This publication consists of four volumes, the first three of which contain the specifications monographs on the identity and purity of the food additives and the fourth volume contains the analytical methods, test procedures and laboratory solutions required and referenced in the specifications monographs. FAO maintains an online searchable database of all JECFA specifications monographs from the FAO JECFA Monographs, which is available at: 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 Arabic, Chinese, English, French and Spanish. Information about analytical methods referred to in the specifications is available in the Combined Compendium of Food Additive Specifications (Volume 4), which can be accessed from the query pages.

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

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

Contact and Feedback

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

Specifications for certain food additives

New and revised specifications

The Committee evaluated the safety of one (1) food additive (R) and fifteen flavouring agents (N) and revised the specifications (R) for three food additives and eight flavouring agents.[1]

Aspartame (R)
Lycopene (synthetic); and lycopene from *Blakeslea trispora* (R)
Pentasodium triphosphate (R)
Steviol glycosides (R)

Esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids
Structural class I
4-Methylpentyl 4-methylvalerate 2280 (N)
5-Methylhexyl acetate 2281 (N)
4-Methylpentyl isovalerate 2282 (N)
Ethyl 4-methylpentanoate 2283 (N)
Ethyl 2-ethylbutyrate 2284 (N)
Ethyl 2-ethylhexanoate 2285 (N)

Hydroxy- and alkoxy-substituted benzyl derivatives
Structural class I
2-Ethoxy-4-(hydroxymethyl)phenol 2271 (N)
2-Phenoxyethyl 2-(4-hydroxy-3-methoxyphenyl)acetate 2272 (N)
3-Phenylpropyl 2-(4-hydroxy-3-methoxyphenyl)acetate 2273 (N)
Ethyl-2-(4-hydroxy-3-methoxyphenyl)acetate 2274 (N)
cis-3-Hexenyl salicylate 2275 (N)
4-Formyl-2-methoxyphenyl 2-hydroxypropanoate 2276 (N)
2-Hydroxy-4-methoxybenzaldehyde 2277 (N)
3,4-Dihydroxybenzoic acid 2278 (N)
3-Hydroxybenzoic acid 2279 (N)

(E)-2-hexenal diethyl acetal 1383 (R)
3-Butylidenephthalide 1170 (R)
1,4-Cineole 1233 (R)
Octahydrocoumarin 1166 (R)
3-(l-Methoxy)-2-Methylpropane-1,2-diol 1411 (R)
p-Methane-3,8-diol 1416 (R)
p-Isopropylacetophenone 808 (R)
Acetanisole 810 (R)

[1] N: new specifications, R: revised specifictions.

Aspartame

Revised specifications prepared at the 96th JECFA (2023) and published in FAO JECFA Monograph 31 (2023) superseding specifications prepared at the 82nd JECFA (2016). Metals and arsenic specifications revised at the 57th JECFA (2001). An ADI of 0-40 mg/kg bw was established at the 25th JECFA (1981).

SYNONYMS Aspartyl phenylalanine methyl ester, α-aspartame, L-aspartyl-L-phenylalanine methyl ester, N-L-α-aspartyl-L-phenylalanine-1-methyl ester, APM

DEFINITION Aspartame is a dipeptide methyl ester of L-aspartic acid and L-phenylalanine. It is produced chemically or enzymatically. Chemical synthesis of aspartame is accomplished by reacting L-phenylalanine or L-phenylalanine methyl ester with N-protected L-aspartic anhydride. This is followed by separation and crystallization of the major component, α-aspartame, from its non-sweet isomer, β-aspartame.

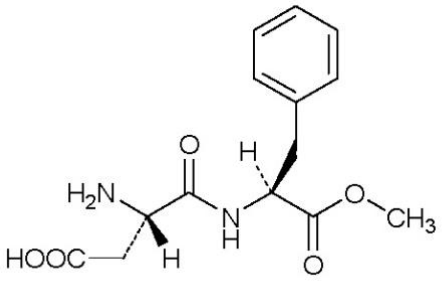
Chemical names 3-Amino-N-(alpha-carboxy-phenethyl)-succinamic acid, L-alpha-aspartyl-L-phenylalanine-1-methyl ester

C.A.S. number 22839-47-0

INS number 951

Chemical formula $C_{14}H_{18}N_2O_5$

Structural formula



Formula weight 294.30

Assay Not less than 98.0% and not more than 102.0% on the dried basis

See description under TESTS

DESCRIPTION White, odourless, crystalline powder

FUNCTIONAL USES Sweetener, flavour enhancer

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Slightly soluble in water and practically insoluble or insoluble in ethanol
Test for amine group	Dissolve 2 g of ninhydrin in 75 ml of dimethylsulfoxide, add 62 mg of hydrindantin, dilute to 100 ml with 4 M lithium acetate buffer solution (pH 9), and filter. Transfer about 10 mg of the sample to a test tube, add 2 ml of the reagent solution, and heat. A dark purple colour is formed.
Test for ester	Dissolve about 20 mg in 1 ml of methanol, add 0.5 ml of methanol saturated with hydroxylamine hydrochloride, mix, then add 0.3 ml of 5 M potassium hydroxide in methanol. Heat the mixture to boiling, then cool, adjust the pH to between 1 and 1.5 with hydrochloric acid TS, and add 0.1 ml of ferric chloride TS. A burgundy colour is produced.

PURITY

Loss on drying (Vol. 4)	Not more than 4.5% (105 °C, 4 h)
pH (Vol. 4)	4.5 - 6.0 (1 g in 125 ml solution)
Specific rotation (Vol. 4)	$[\alpha]_D^{20}$: Between + 14.5° and + 16.5° (4% solution in 15 M formic acid; determine within 30 min after preparation of the sample solution)
Sulfated ash (Vol. 4)	Not more than 0.2% Test 5 g of the sample (Method I)
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 method described in Volume 4, “Instrumental Methods.”
5-Benzyl-3,6-dioxo-2-piperazineacetic acid (diketopiperazine or DKP)	Not more than 1.5% See description under TESTS
Other related impurities	Not more than 2.0% See description under TESTS

TESTS

PURITY TESTS

DKP

Apparatus

High performance liquid chromatography system equipped with a UV detector.

Reagents and solutions

Mobile phase: Dissolve 5.6 g of potassium dihydrogen phosphate in 820 ml of water before adjusting the pH to 4.3 with 10% phosphoric acid solution. Add 180 ml of methanol to 820 ml of this solution and mix well.

Standard Preparation

Standard stock solution: Dissolve 25 mg DKP Reference Standard (Aspartame Related Compound A from The United States Pharmacopeial Convention, or equivalent) in 10 ml of methanol and dilute to 100 ml with water.

Standard solutions: Dilute the standard stock solution with 10% methanol to concentrations of 100, 75, 50, 25 and 5 µg/ml.

Sample Preparation

Accurately weigh 100 mg of the sample and dissolve in 10% methanol to make exactly 20 ml (5 mg/ml).

Procedure
HPLC conditions:
Column: L-column2 ODS (4.6 mm I.D. × 150 mm, particle size: 5 µm, Chemical Evaluation and Research Institute, Japan) or equivalent
Column temperature: 40 °C
Mobile phase: Mixture of phosphate buffer solution (0.05 mol/l, pH 4.3) and methanol (82:18 v/v)
Flow rate: 1.0 ml/min
Injection volume: 20 µl
Detector: UV at 210 nm
Run Time: 50 min

Inject the sample and read the concentration of the sample from the standard curve.

Calculation
Calculate the content (%) of DKP using the following formula:

$$\%DKP = \frac{C \times V \times 0.1}{W}$$

where
C is the concentration of DKP in the sample solution (µg/ml)
V is the volume of the sample solution (20 ml)

W is the weight of the sample (mg)

Other related impurities

Apparatus

High performance liquid chromatograph equipped with a UV detector.

Reagents and Solutions:
Use the Reagents and Solutions, Diluent, System suitability solution, and HPLC Conditions described in the METHOD OF ASSAY.

Sample stock solution: 5 mg/ml in Diluent. [Prepare immediately before injection.]
Sample solution: Using the *Sample stock solution*, prepare a 0.1 mg/ml solution in Diluent.

System suitability
Suitability requirement 1: The resolution, R, between L-phenylalanine and DKP in the *System suitability solution* is not less than 8.

Analysis: Separately inject equal volumes of the *Sample stock solution* and the *Sample solution* into the chromatograph, record the chromatograms, and measure the sum of responses for the major peaks on the resulting chromatograms.

The sum of all peak responses of the *Sample stock solution*, excluding DKP and aspartame, is not more than the aspartame peak response of the *Sample solution* (not more than 2.0%).

METHOD OF ASSAY

Apparatus

High performance liquid chromatograph equipped with a UV detector.

Reagents and Solutions:
Buffer solution: 0.05 M monobasic potassium phosphate adjusted with phosphoric acid to pH 4.3
Mobile phase: Methanol and Buffer solution (18:82)
System suitability, Standard and Sample Preparation:
Diluent: Methanol and water (1:9)
System suitability solution: 0.1 mg/ml each of DKP Reference Standard and L-phenylalanine Reference Standard in Diluent
Standard solution: 0.5 mg/ml of aspartame Reference Standard in Diluent.
Sample solution: 0.5 mg/ml in Diluent.

[NOTE— Prepare Standard and Sample solutions immediately before injection, avoiding exposure to heat. Use aspartame Reference Standard and DKP Reference Standard (Aspartame related compound A) from the United States Pharmacopeial Convention, or equivalent.]

Procedure:
HPLC Conditions:

Column: 250-mm × 4.6-mm column that contains 5-µm octadecylsilane chemically bonded to porous silica or ceramic micro-particles (Lichrospher 100RP-18 (Merck KGaA), or equivalent)
Column temperature: 40 °C
Flow rate: 2 ml/min
Injection volume: 20 µl
Run time: 45 min

System suitability
Suitability requirement 1: The relative standard deviation, RSD, for the aspartame peak area is not more than 1.0% for five replicate injections of the *Standard solution*.

Suitability requirement 2: The resolution, R, between L-phenylalanine and DKP in the *System suitability solution* is not less than 8.
[NOTE: The relative retention times for L-phenylalanine and DKP are 0.6 and 1.0, respectively.]

Suitability requirement 3: The tailing factor for the aspartame peaks in the *Standard solution* is not more than 1.5.

Analysis: Separately inject equal volumes of the *Standard solution* and the *Sample solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks on the resulting chromatograms.

Calculation:

$$Percentage\ of\ Aspartame\ = \frac{r_U}{r_S} \times \frac{C_S}{C_U} \times 100$$

where
r_U = peak response of aspartame in the Sample solution
r_S = peak response of aspartame in the Standard solution
C_S = concentration of in the Standard solution (mg/ml)
C_U = concentration of the Sample solution (mg/ml)

Lycopene (synthetic)

Specifications revised at the 96th JECFA (2023) and published in FAO JECFA Monographs 31 (2023) superseding specifications prepared at the 67th JECFA (2006). A group ADI "not specified" for lycopene from all sources was established at the 71st JECFA (2009).

SYNONYMS INS 160d(i)

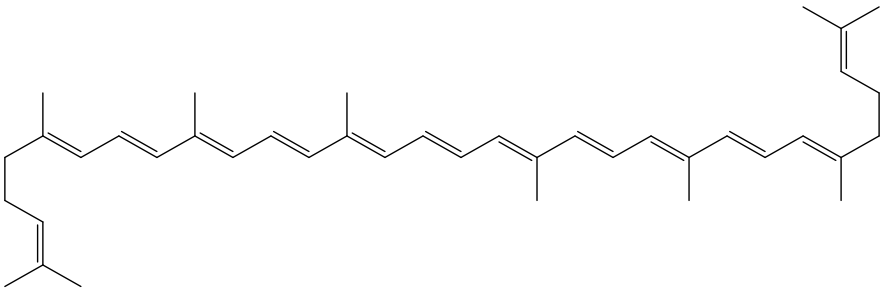
DEFINITION Synthetic lycopene is produced by the Wittig condensation of synthetic intermediates commonly used in the production of other carotenoids used in food. Synthetic lycopene consists predominantly of all-trans-lycopene together with 5-cis-lycopene and minor quantities of other isomers. Commercial lycopene preparations intended for use in food are formulated as suspensions in edible oils or water-dispersible powders and are stabilised with antioxidants.

Chemical names Ψ,Ψ-carotene
all-trans-lycopene (all-E)-lycopene
(all-E)-2,6,10,14,19,23,27,31-octamethyl-
2,6,8,10,12,14,16,18,20,22,24,26,30-dotriacontatridecaene

C.A.S. number 502-65-8

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

Structural formula



Formula weight 536.9

Assay Not less than 96% total lycopenes; not less than 70% all-*trans*-lycopene

DESCRIPTION Red crystalline powder

FUNCTIONAL USES Colour, nutrient supplement

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Insoluble in water, sparingly soluble in tetrahydrofuran (THF)
Test for carotenoids	The colour of the solution of the sample in acetone disappears after successive additions of a 5% solution of sodium nitrite and 1 N sulfuric acid
Solution in THF	A 1% solution is clear and has intensive red-orange colour
Spectrophotometry (Vol. 4)	A solution in hexane shows an absorption maximum at approximately 470 nm

PURITY

Loss on drying (Vol. 4)	Not more than 0.5% (40 °C, 4 h at 10 mm Hg)
Lead (Vol. 4)	Not more than 1 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on principles of methods described in Volume 4 (under "General Methods, Metallic Impurities").
Apo-12'-lycopenal	Not more than 0.15% See description under TESTS
Triphenyl phosphine oxide (TPPO) (Vol. 4)	Not more than 0.01%

TESTS

PURITY TESTS

Apo-12'-lycopenal

Determine by HPLC using the following conditions:

Reagents (Note: all solvents should be HPLC-grade)
Hexane
Triethylamine (TEA)
Tetrahydrofuran (THF)
Toluene stabilised with BHT (0.5 g BHT in 1000 ml toluene)
Apo-12'-lycopenal (also known as lycopene C_{25}-aldehyde) standard (available from DSM Nutritional Products)

Apparatus
HPLC system with a suitable pump, injector, and

integrator Column: Stainless steel (200x4.0 mm)
Stationary phase: Nucleosil Si 100 3 µm (Macherey-Nagel or equivalent)
Detector: UV-Vis

HPLC conditions
Flow: 2.0 ml/min
Injection volume: 5.0 µl
Pressure: approx. 135 bar
Detection: 435 nm
Mobile phase: A – Hexane
B – Hexane: TEA (99.9:0.1) (v/v)
C – Hexane: THF (80:20) (v/v)

Gradient

Time, min	A%	B%	C%
0	80	20	0
16	60	20	20
22	40	20	40
24.5	80	20	0

Run time
Approximately 25 min.

Standard solution
Accurately weigh between 14.5 and 15.5 mg of the *apo*-12'-lycopenal standard into a 50-ml volumetric flask. Dissolve in toluene stabilised with BHT and make up to volume. Transfer 2 ml of the solution into 100-ml volumetric flask and add toluene stabilised with BHT to volume.

Sample solution
Accurately weigh between 29.0 and 31.0 mg of the sample into a 10-ml volumetric flask and dissolve and dilute to volume with toluene stabilised with BHT. Put the solution in an ultrasonic bath for 10 min.

Results
The retention time of a*po*-12'-lycopenal is approximately 14 min. The relative retention time of *apo*-12'-lycopenal with respect to all-*trans*-lycopene is 1.6.

Calculation

$$\text{Apo-12'-lycopene (\%)} = \frac{A_S \times W_{St} \times 10}{A_{St} \times W_{St} \times 2500} \times 100$$

where
A_S is the peak area of the sample;

A_{St} is the peak area of the standard;
W_{St} is the weight of the standard (mg);
W_S is the weight of the sample (mg);
10 is the volume of the volumetric flask in which the sample was dissolved (ml); and
2500 is the volume of the volumetric flask in which the standard was dissolved (50 ml) multiplied by dilution (50).

METHOD OF ASSAY Determine total lycopenes and all-*trans*-lycopene by HPLC using the following conditions:

Reagents (Note: all solvents should be HPLC-grade)
Hexane
Tetrahydrofuran stabilised with 0.025% BHT N-Ethyl-diisopropylamine
Lycopene standard (purity 95% or higher; available from CaroteNature GmbH)

Apparatus
Spectrophotometer with a 1-cm cuvette
HPLC system with a suitable pump, injector, thermostated column compartment, and integrator
Column: Two serially-connected stainless steel columns (250x4.0 mm)
Stationary phase: Nucleosil 300-5, 5 µm (Macherey-Nagel or equivalent)
Detector: UV-Vis

HPLC conditions

Flow rate:	0.8 ml/min
Injection volume:	20 µl
Pressure:	approx. 80 bar
Column temperature:	20 °C
Detection:	470 nm
Mobile phase:	0.15% solution of N-ethyl-diisopropylamine in hexane (v/v)
Run time:	30 min

HPLC standard solution
Accurately weigh between 5.5 and 6.5 mg of the lycopene standard into a 100-ml volumetric flask. Dissolve in 5 ml of tetrahydrofuran stabilised with BHT and make up to volume with hexane. This is a standard solution for the HPLC assay.

Spectrophotometric standard solution
Transfer 5.0 ml of the HPLC standard solution into a 100-ml volumetric flask and make up to volume with hexane. This is a standard solution for the spectrophotometric determination of lycopene in the lycopene standard.

Sample solution
Accurately weigh between 4.5 and 5.5 mg of the sample into a

100-ml volumetric flask. Dissolve in 5 ml of tetrahydrofuran stabilised with BHT and make up to volume with hexane.

Spectrophotometric determination of lycopene

Measure the absorbance of the spectrophotometric standard solution in a 1-cm cuvette at the wavelength of maximum absorption (approximately 470 nm). Use hexane as the blank.

Calculation

$$C_{St}(mg/l) = \frac{A \times 10000}{3450}$$

where

C_{St} is the lycopene concentration in the spectrophotometric standard solution (mg/l)

A is absorbance at the wavelength of maximum absorption;

3450 is the specific absorbance $A_{1\,cm}^{1\%}$ of all trans-lycopene in hexane; and

10000 is the scaling factor.

HPLC analysis

Repeatedly inject 20 µl of the HPLC standard solution. Record the total peak area of all detected lycopene isomers (exclude the solvent peak). Calculate the mean peak area from repeated injections and calculate the lycopene response factor (RF) according to the formula:

$$RF = \frac{A_{St}}{C_{St}} \times 20$$

where

RF is the response factor of lycopene (AU x l/mg);

A_{St} is the mean peak area of all lycopene peaks (AU);

C_{St} is the concentration of lycopene in the spectrophotometric standard solution (mg/l); and

20 is the dilution factor used in the preparation of the spectrophotometric standard solution from the HPLC standard solution.

Inject the sample solution and record the peak areas of lycopene isomers.

Results

Retention times

Lycopene isomer	Relative retention time*	Absolute retention time (approx.)
13-*cis*-lycopene	0.6	14 min
9-*cis*-lycopene	0.8	19 min
All-*trans*-lycopene	1.0	22 min
5-*cis*-lycopene	1.1	24 min

* relative to all-*trans*-lycopene

Calculations

Calculate the content of total lycopenes according to the formula:

$$Total\ lycopenes\ (\%) = \frac{A_{trans}+A_{5cis}+A_{9cis}+A_{13cis}+A_{xcis}\times 0.1}{RF\times W_S} \times 100$$

Where:

A_{trans} is the peak area of all-*trans*-lycopene (AU);

A_{5cis}, A_{9cis}, and A_{13cis} are the peak areas of 5*cis*-, 9*cis*-, and 13-*cis*-lycopene (AU);

A_{xcis} is the peak area of other *cis* isomers, if detected (AU);

0.1 is the volume, in litres, of the flask in which the sample was dissolved;

RF is the response factor of lycopene (AU x l/mg); and

W_S is the weight of the sample (mg).

Calculate the content of all-*trans*-lycopene as follow

$$\text{All-}trans\text{-lycopene}\ (\%) = \frac{A_{trans} \times 0.1}{RF \times W_S} \times 100$$

Lycopene from *Blakeslea trispora*

Specifications revised at the 96th JECFA (2023) and published in FAO JECFA Monographs 31 (2023) superseding specifications prepared at the 67th JECFA (2006). A group ADI "not specified" for lycopene from all sources was established at the 71st JECFA (2009).

SYNONYMS INS 160d(iii)

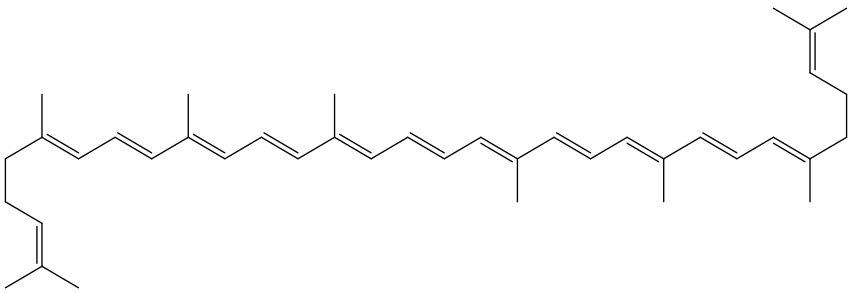
DEFINITION Lycopene from *Blakeslea trispora* is extracted from the fungal biomass and purified by crystallization and filtration using only Isopropanol and isobutyl acetate. It consists predominantly of all-*trans*-lycopene. It also contains minor quantities of other carotenoids. Commercial lycopene preparations intended for use in food are formulated either as suspensions in edible oils or as water-dispersible powders and are stabilised with antioxidants.

Chemical names
Ψ,Ψ-carotene
all-*trans*-lycopene (all-E)-lycopene
(all-E)-2,6,10,14,19,23,27,31-octamethyl-2,6,8,10,12,14,16,18,20,22,24,26,30-dotriacontatridecaene

CAS number 502-65-8

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

Structural formula



Formula weight 536.9

Assay Not less than 95% total lycopenes; not less than 90% all-*trans*-lycopene

DESCRIPTION Red crystalline powder

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION
Solubility (Vol. 4) Insoluble in water, sparingly soluble in tetrahydrofuran (THF)

Test for carotenoids	The colour of the solution of the sample in acetone disappears after successive additions of a 5% solution of sodium nitrite and 1N sulfuric acid.
Solution in THF	A 1% solution is clear and has intensive red-orange colour
Spectrophotometry (Vol. 4)	A solution in hexane shows an absorption maximum at approximately 470 nm

PURITY

Other carotenoids	Not more than 5% See description under METHOD OF ASSAY
Loss on drying (Vol. 4)	Not more than 0.5% (40 °C, 4 h at 20 mm Hg)
Lead (Vol. 4)	Not more than 1 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on principles of methods described in Volume 4 (under "General Methods, Metallic Impurities").
Residual solvents (Vol. 4)	Isopropanol: Not more than 0.1% Isobutyl acetate: Not more than 1.0%

METHOD OF ASSAY

The HPLC method of assay is suitable for determination of total lycopenes (all-*trans*-lycopene and *cis*-lycopene isomers), all-*trans*- lycopene, and other carotenoids. (Note: the predominant *cis* isomer detected in lycopene from *B. trispora* is 13-*cis*-lycopene.)

Reagents (Note: all solvents should be HPLC-grade)
Acetonitrile
Methanol Acetone Hexane
Methylene chloride
Lycopene standard (purity 95% or higher; available from Vitatene S.A.)

Apparatus
VIS or UV-Vis spectrophotometer with a 1-cm light path optical cell HPLC system with either a VIS or UV-Vis detector or a suitable diode array detector, injector, column oven, and integrator
Column: Vydac 218 TP54 5 µm (4.6x250 mm) or equivalent

HPLC conditions

Mobile phase:	acetonitrile/methanol (40:60)
Flow rate:	1 ml/min
Detection:	470 nm
Injection volume:	10 µl
Column temperature:	30 °C
Injector temperature:	10 °C
Run time:	15 min

Standard solution
Weigh accurately about 25 mg lycopene standard into a 100-ml volumetric flask. Dissolve in 10 ml of methylene chloride and add hexane to volume. Pipet 1 ml of the above solution into a 50-ml volumetric flask and add acetone to volume.

Sample solution
Prepare in the same manner as the standard solution.

HPLC analysis
Inject the standard solution into the chromatograph and record the resulting chromatogram. The retention time of all-*trans*-lycopene is approximately 11.5 to 12.5 min. The relative retention time of 13-*cis*-lycopene with respect to all-*trans*-lycopene is 1.25. The relative retention times for other carotenoids with respect to all-*trans*- lycopene are 1.2 for β-carotene and 1.1 for γ-carotene.

Record the total peak area of all-*trans*-lycopene and *cis*-lycopene isomers and calculate the response factor (RF) for lycopene as follows:

$$RF = \frac{A_{st} \times 5000}{W_{st} \times P_{st}}$$

Where

RF is the response factor for lycopene (AU ml/mg);
A_{st} is the total lycopene (all-*trans*-lycopene and *cis*-lycopene isomers) peak area;
5000 is the volume of the volumetric flask in which the standard was dissolved (100 ml) multiplied by dilution (50);
W_{st} is the weight of the standard (mg); and
P_{st} is the purity of the standard expressed as a proportion of lycopene in the lycopene standard (determined as described under Standard purity determination).

Inject the sample solution into the chromatograph and record the following peak areas:
A_1 – all-*trans* lycopene
A_2 – total lycopene (all-*trans*-lycopene + *cis*-lycopene isomers)
A_3 – other carotenoids
A_4 – all carotenoids (all-*trans*-lycopene + *cis*-lycopene isomers + other carotenoids)

Results
Calculate the % of total lycopenes, all-*trans*-lycopene, and other carotenoids as follows:

$$Total\ lycopenes\ \% = \frac{A_2 \times 5000}{W \times \frac{RF}{}} \times 100$$

$$All - trans - lycopene\ (\%) = \frac{A_1}{A_2} \times 100$$

$$Other\ carotenoids\ \%\ = \frac{A_3}{A_4} \times 100$$

W is the sample weight (mg);
RF is the response factor (AU ml/mg); and
5000 is the volume of the volumetric flask in which the standard was dissolved (100 ml) multiplied by dilution (50).

Standard purity determination

Accurately weigh about 20 mg of the lycopene standard into a 100-ml volumetric flask. Dissolve in 10 ml of methylene chloride and add hexane to volume. Pipet 1 ml of the solution into a 100-ml volumetric flask and add hexane to volume. Measure the absorbance in a 1-cm optical cell at the wavelength of maximum absorption (approximately 470 nm). Use hexane as the blank.

Calculation

$$P_{st} = \frac{A_{max}}{345 \times W_{st}} \times 10000$$

where

P_{st} is the purity of the lycopene standard calculated as a proportion of lycopene in the lycopene standard (NOTE: P_{st} equals 1 for a 100% pure standard and is less than 1 for a standard with purity below 100%);
A_{max} is the absorbance at 470 nm;
W_{st} is the weight of the standard (mg);
10000 is the volume of the volumetric flask in which lycopene was dissolved (100 ml) multiplied by dilution (100); and
345 is the absorptivity of lycopene in hexane.

Pentasodium triphosphate

Revised specifications prepared at the 96th JECFA (2023) and published in FAO monograph 31 (2023) superseding specifications prepared at the 55th JECFA (2000). No ADI was established, but a group MTDI of 70 mg/kg bw, expressed as phosphorus from all food sources, was established at the 26th JECFA (1982).

SYNONYMS Pentasodium tripolyphosphate, Sodium triphosphate, Sodium tripolyphosphate, Triphosphate; INS No. 451(i)

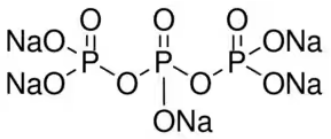
DEFINITION

Chemical names: Pentasodium triphosphate, pentasodium tripolyphosphate

C.A.S. number: 7758-29-4 (anhydrous); 15091-98-2 (hexahydrate)

Chemical formula: $Na_5P_3O_{10} \times H_2O$ (x = 0 or 6)

Structural formula



Formula weight: Anhydrous: 367.86
Hexahydrate: 475.94

Assay: Anhydrous: not less than 85% of $Na_5P_3O_{10}$; not less than 56% and not more than 59% of P_2O_5

Hexahydrate: not less than 65% of $Na_5P_3O_{10}$; not less than 43% and not more than 45% of P_2O_5

DESCRIPTION White, slightly hygroscopic granules or powder

FUNCTIONAL USES Sequestrant, texturizer

CHARACTERISTICS

IDENTIFICATION

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

pH (Vol. 4): 9.1-10.2 (1% solution)

Test for phosphate (Vol. 4): Passes test

Test for sodium (Vol. 4): Passes test

PURITY

Loss on drying (Vol. 4)	Anhydrous: not more than 0.7% (105 °C, 1 h) Hexahydrate: not more than 23.5% (60 °C, 1 h, followed by 105 °C, 4 h)
Water-insoluble matter (Vol. 4)	Not more than 0.1%
Higher polyphosphates	Not detectable See description under TESTS
Fluoride (Vol. 4)	Not more than 50 mg/kg (Method I or III)
Arsenic (Vol. 4)	Not more than 3 mg/kg (Method II)
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the method described in Volume 4, “Instrumental Methods”.

TESTS

Higher polyphosphates

Chromatographic solvent
Mix 75 ml of isopropanol, 10 ml of water, 20 ml of 20% trichloroacetic acid and 0.3 ml of 20% ammonia. Make fresh on a weekly basis.

Chromatographic spray
Dissolve 1 g of ammonium molybdate in 85 ml of water, 10 ml of 1 N hydrochloric acid and 5 ml of 60% perchloric acid.

Sample solution
Dissolve 1 g of the sample in 50 ml of water.

Reference solution
Dissolve 1 g of the pentasodium triphosphate standard in 50 ml of water.

Procedure
Place 0.01 ml of the sample solution and 0.01 ml of reference solution on the starting line of the chromatographic paper and allow to dry in a stream of warm air. Use ascending chromatography at 18-20 °C until the solvent has ascended about 25 cm from the starting line (12-15 h). Dry at 60 °C in an oven and spray with the chromatographic spray. Place the paper under an ultraviolet lamp and irradiate until the phosphates are visible as blue spots (about 2 min).

Three spots (one from the monophosphate (R_f = 0.69), a second from the diphosphate (R_f = 0.44) and the third from the triphosphate (R_f = 0.29) are observed, and no other spot is observed.

METHOD OF ASSAY

1.Determination of $Na_5P_3O_{10}$

Reagents and solutions

- Potassium acetate buffer (pH 5.0): Dissolve 78.5 g of potassium acetate for 15 to 20 min. Add methyl orange TS and neutralize the solution with stronger ammonia TS. Add 1 g of ammonium nitrate crystals, stir to dissolve, and cool. Add 15 ml of ammonium molybdate TS, with stirring, and stir vigorously for 3 min or allow to stand with occasional stirring for 10 to 15 min. Filter the contents of the beaker with suction through a 6-7 mm paper pulp filter pad supported in a 25 mm porcelain disk. The filter pad should be covered with a suspension of a filtering aid. After the contents of the beaker have been transferred to the filter, wash the beaker with five 10 ml portions of a 1% solution of sodium or potassium nitrate, passing the washings through the filter, then wash the filter with five 5-ml portions of the wash solution. Return the filter pad and the precipitate to the beaker, wash the funnel thoroughly with water into the beaker, and dilute to about 150 ml. Add 0.1 N sodium hydroxide from a buret until the yellow precipitate is dissolved, then add 5 to 8 ml in excess. Add phenolphthalein TS and titrate the excess alkali with 0.1 N nitric acid. Finally, titrate with 0.1 N sodium hydroxide to the first appearance of the pink colour. The difference between the total volume of 0.1 N sodium hydroxide added and the volume of nitric acid required represents the volume, V, in ml, of 0.1 N sodium hydroxide consumed by the phosphomolybdate complex.

Calculate the $Na_5P_3O_{10}$ content of the sample in % by the formula

$$\%Na_5P_3O_{10} = \frac{0.533 \times 25 \times V}{\mathrm{a}} \times 100$$

where

a = the weight of the sample (mg)

V= Volume of 0.1 N sodium hydroxide consumed by the phosphomolybdate complex

2.Determination of P_2O_5

Accurately weigh about 20 g of the sample into a beaker. Add 150 ml water and 20 ml concentrated nitric acid. Introduce anti-bumping granules, cover the beaker with a watch glass and boil gently for 1 h. Cool to room temperature. Quantitatively transfer the solution to a 500-ml volumetric flask, dilute with water, mix well and dilute to the mark with water. Transfer 20.0 ml of the solution to a plastic beaker, dilute to about 50 ml with water and place the beaker in an automatic titrator equipped with a pH meter. Adjust the pH of the solution to between 2.5 and 2.8 with 5 mol/l sodium hydroxide. Titrate the solution with 0.5 mol/l sodium hydroxide. Record the consumed volumes at the inflection points at about pH 4 (V1) and about pH 9 (V2).

Calculate the P_2O_5 content of the sample in % by the formula

$$\% P_2O_5 = \left[\frac{V2-V1}{2000}\right] x\ f\ x\ 70.97\ x\ \left(\frac{500}{20}\right) x\ (\frac{100}{w}) = \left[\frac{V2-V1}{w}\right] x\ f\ x\ 88.7$$

where
w = weight of the sample (g)
f = factor of 0.5 mol/l sodium hydroxide (= actual molarity/0.5)

Steviol glycosides

Revised at 96th JECFA (2023) and published in FAO Monographs 31 (2023), superseding specifications prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021). An ADI of 0 – 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

Introduction

Steviol glycosides are constituents of the leaves of the plant, *Stevia rebaudiana* Bertoni and have a sweet taste. Steviol glycosides have the same steviol aglycone bound to different types and numbers of glycoside units (e.g., glucose, rhamnose, xylose, fructose, or deoxyglucose). More than forty steviol glycosides have been identified to date (see Appendix A). The functional use of steviol glycosides in food is as a sweetener. They are 100 to 300 times sweeter than sucrose.

Background

Steviol glycosides produced by extraction from the leaves of *Stevia rebaudiana* Bertoni were reviewed by the Committee at its fifty-first, sixty-third, sixty-eighth, eighty-second, eighty-fourth, eighty-seventh and ninety-first meetings. At the sixty-eighth meeting the Committee extended the previously designated temporary ADI of 0–2 mg/kg bw for steviol glycosides, expressed as steviol, pending submission of the results of then-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 received additional data and reevaluated steviol glycosides from *S. rebaudiana* Bertoni. The Committee at that meeting 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 for *Steviol glycosides* was prepared.

At the eighty-second meeting, the Committee evaluated steviol glycosides produced by fermentation of a strain of *Yarrowia lipolytica*, genetically modified to simulate the *S. 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 *S. rebaudiana* Bertoni; an ADI of 0–4 mg/kg bw, expressed as steviol equivalents was applied. A new specifications monograph was prepared for *Rebaudioside A from multiple gene donors expressed in Yarrowia lipolytica* to reflect considerations resulting from this method of manufacture. The existing specifications monograph for *Steviol glycosides* was revised with new tentative specifications and the new title of *Steviol glycosides from Stevia rebaudiana Bertoni*. The Definition and Assay were expanded from nine named leaf-derived steviol glycosides to include additional steviol glycoside compounds derived from *S. rebaudiana* Bertoni, provided that the total percentage of steviol glycosides is not less than 95%. The specifications for *Steviol glycosides from S. rebaudiana Bertoni* were established as tentative, pending receipt of a method of assay that was also capable of measuring minor steviol glycosides. At the eighty-fourth meeting, the Committee revised the specifications for *Steviol glycosides from Stevia rebaudiana Bertoni* and removed the tentative status.

At the eighty-seventh 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 remainder consists of residues of starting material and food-grade processing aids depending on the method of production. The Committee recognized that the < 5% residues may contain impurities other than those listed above, and it is the responsibility of manufacturers to address these issues. The *(Framework*

for) steviol glycosides combined specifications monograph was prepared with four Annexes describing steviol glycosides based on the method of manufacture.

At the present meeting, the Committee revised chemical information for steviol glycosides (Appendix A), the steviol glycosides assay method (Appendix B), the glucosylated steviol glycosides assay method (Annex 4) and the solubility for all steviol glycosides (Annexes 1-3) in the (Framework for) Steviol Glycosides. The Committee also removed the tentative status from Enzyme modified glucosylated steviol glycosides (Annex 4).

Explanation for the framework approach

The two previously existing specification monographs for steviol glycosides required 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 *S. rebaudiana* Bertoni plant are stevioside and rebaudioside A, and the minor glycosides include rebaudioside M and rebaudioside D. Several minor glycosides have been determined to have more favourable sensory characteristics than the major glycosides. This has prompted development of new technologies to produce steviol glycosides with higher proportions of minor glycosides to modify the sensory profile of the articles of commerce. The current framework was developed to address steviol glycosides manufactured using the four existing methods.

The Annexes include the method of production as well as assay and impurity specifications and shall be used in conjunction with information contained elsewhere in the framework including Appendix A and Appendix B. To meet the requirements of the present monograph, steviol glycosides should be produced as described in one of the Annexes (described below) and meet the corresponding specifications. Modifications in the production method will require revisions to an existing Annex or the development of a new Annex. An Annex could have a tentative status if more information is required to complete it. The tentative status of one Annex does not affect the status of the other Annexes.

- Annex 1 - Steviol glycosides from *Stevia rebaudiana* Bertoni: extraction of the leaves of *Stevia rebaudiana* Bertoni.
- Annex 2 - Steviol glycosides from fermentation: a process in which a genetically modified microorganism is used to produce specific steviol glycosides.
- Annex 3 - Enzyme modified steviol glycosides: 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.
- Annex 4 - Enzyme modified glucosylated steviol glycosides: a process in which steviol glycosides that have been extracted from the leaves of *Stevia rebaudiana* Bertoni undergo enzyme catalyzed reactions to add glucose units to the steviol glycosides via α-(1-4) linkages.

Table A.
Summary of Formula and R-Groups of Identified Steviol Glycosides from the Leaves of *Stevia rebaudiana* Bertoni (see Figure A for Backbone Structure)
[Adapted from Purkayastha & Kwok (2020)]

#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
1. Steviol + Glucose (SvGn)								
1.01	Steviolmonoside		SvG1	481	0.66	H	Glcβ1-	Ohta et al. (2010)
1.02	Steviolmonoside A		SvG1	481	0.66	Glcβ1-	H	Gardana et al. (2010)
1.03	Rubusoside	64849-39-4	SvG2	643	0.49	Glcβ1-	Glcβ1-	Ohta et al. (2010)
1.04	Steviolbioside	41093-60-1	SvG2	643	0.49	H	Glcβ(1-2)Glcβ1-	Kohda et al. (1976)
1.05	Stevioside	57817-89-7	SvG3	805	0.40	Glcβ1-	Glcβ(1-2)Glcβ1-	Bridel & Lavielle (1931)
1.06	Stevioside A		SvG3	805	0.40	Glcβ(1-2)Glcβ1-	Glcβ1-	Wu et al. (2012)
1.07	Rebaudioside B	58543-17-2	SvG3	805	0.40	H	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Kohda et al. (1976)
1.08	Rebaudioside G		SvG3	805	0.40	Glcβ1-	Glcβ(1-3)Glcβ1-	Ohta et al. (2010)
1.09	Stevioside B		SvG3	805	0.40	Glcβ(1-3)Glcβ1-	Glcβ1-	Chaturvedula & Zamora (2014)
1.10	Rebaudioside E	63279-14-1	SvG4	967	0.33	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-	Sakamoto et al. (1977a)
1.11	Rebaudioside A	58543-16-1	SvG4	967	0.33	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Kohda et al. (1976)
1.12	Rebaudioside A2		SvG4	967	0.33	Glcβ1-	Glcβ(1-6)Glcβ(1-2)Glcβ1-	Chaturvedula & Prakash (2011a)
1.13	Rebaudioside D	63279-13-0	SvG5	1129	0.28	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Sakamoto et al. (1977a)

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[Adapted from Purkayastha & Kwok (2020)]

#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
1.14	Rebaudioside I		SvG5	1129	0.28	Glcβ(1-3)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.15	Rebaudioside L		SvG5	1129	0.28	Glcβ1-	Glcβ(1-6)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.16	Rebaudioside Q2		SvG5	1129	0.28	Glcα(1-2)Glcα(1-4)Glcβ1-	Glcβ(1-2)Glcβ1-	Chaturvedula & Prakash (2011b)
1.17	Rebaudioside Q		SvG5	1129	0.28	Glcβ1-	Glcα(1-4)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	-
1.18	Rebaudioside I2		SvG5	1129	0.28	Glcβ1-	Glcα(1-3)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Chaturvedula et al. (2011a)
1.19	Rebaudioside Q3		SvG5	1129	0.28	Glcβ1-	Glcα(1-4)Glcβ(1-3)[Glcβ(1-2)]Glcβ1-	Chaturvedula et al. (2011a)
1.20	Rebaudioside I3		SvG5	1129	0.28	Glcβ(1-2)[Glcβ(1-6)]Glcβ1-	Glcβ(1-2)Glcβ1-	Chaturvedula et al. (2011a)
1.21	Rebaudioside AM		SvG5	1129	0.28	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-	Prakash & Ma (2018)
1.22	Rebaudioside M	1220616-44-3	SvG6	1291	0.25	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.23	Rebaudioside 1h		SvG7	1453	0.22	Glcβ(1-3)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Ma (2018)
1.24	Rebaudioside IX		SvG9	1778	0.18	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-3){Glcβ(1-3)[Glcβ(1-2)]Glcα(1-6)Glcβ(1-2)}Glcβ1-	Prakash & Ma (2018)

Table A.
Summary of Formula and R-Groups of Identified Steviol Glycosides from the Leaves of *Stevia rebaudiana* Bertoni (see Figure A for Backbone Structure) [Adapted from Purkayastha & Kwok (2020)]

#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
1.14	Rebaudioside I		SvG5	1129	0.28	Glcβ(1-3)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.15	Rebaudioside L		SvG5	1129	0.28	Glcβ1-	Glcβ(1-6)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.16	Rebaudioside Q2		SvG5	1129	0.28	Glcα(1-2)Glcα(1-4)Glcβ1-	Glcβ(1-2)Glcβ1-	Chaturvedula & Prakash (2011b)
1.17	Rebaudioside Q		SvG5	1129	0.28	Glcβ1-	Glcα(1-4)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	-
1.18	Rebaudioside I2		SvG5	1129	0.28	Glcβ1-	Glcα(1-3)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Chaturvedula et al. (2011a)
1.19	Rebaudioside Q3		SvG5	1129	0.28	Glcβ1-	Glcα(1-4)Glcβ(1-3)[Glcβ(1-2)]Glcβ1-	Chaturvedula et al. (2011a)
1.20	Rebaudioside I3		SvG5	1129	0.28	Glcβ(1-2)[Glcβ(1-6)]Glcβ1-	Glcβ(1-2)Glcβ1-	Chaturvedula et al. (2011a)
1.21	Rebaudioside AM		SvG5	1129	0.28	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-	Prakash & Ma (2018)
1.22	Rebaudioside M	1220616-44-3	SvG6	1291	0.25	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
1.23	Rebaudioside 1h		SvG7	1453	0.22	Glcβ(1-3)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Ma (2018)
1.24	Rebaudioside IX		SvG9	1778	0.18	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-3){Glcβ(1-3)[Glcβ(1-2)]Glcα(1-6)Glcβ(1-2)}Glcβ1-	Prakash & Ma (2018)

Table A.
Summary of Formula and R-Groups of Identified Steviol Glycosides from the Leaves of *Stevia rebaudiana* Bertoni (see Figure A for Backbone Structure)
[Adapted from Purkayastha & Kwok (2020)]

#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
2.02	Dulcoside B		SvR1G2	789	0.40	H	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.03	Rebaudioside C	63550-99-2	SvR1G3	951	0.33	Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Sakamoto et al. (1977b)
2.04	Rebaudioside C2		SvR1G3	951	0.33	Rhaα(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-	Purkayastha et al. (2019)
2.05	Rebaudioside S		SvR1G3	951	0.33	Rhaα(1-2)Glcβ1-	Glcα(1-2)Glcβ1-	Ibrahim et al (2016)
2.06	Rebaudioside H		SvR1G4	1113	0.29	Glcβ1-	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.07	Rebaudioside K		SvR1G4	1113	0.29	Glcβ(1-2)Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.08	Rebaudioside K2		SvR1G4	1113	0.29	Glcβ(1-6)Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
2.09	Rebaudioside J		SvR1G4	1113	0.29	Rhaα(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.10	Rebaudioside N	1220616-46-5	SvR1G5	1275	0.25	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.11	Rebaudioside N2		SvR1G5	1275	0.25	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Ma (2018)
2.12	Rebaudioside N6		SvR1G5	1275	0.25	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-	Prakash & Ma (2018)
2.13	Rebaudioside O	1220616-48-7	SvR1G6	1437	0.22	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Ohta et al. (2010)
2.14	Rebaudioside O2		SvR1G6	1437	0.22	Glcβ(1-4)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2016)

Table A.
Summary of Formula and R-Groups of Identified Steviol Glycosides from the Leaves of *Stevia rebaudiana* Bertoni (see Figure A for Backbone Structure)
[Adapted from Purkayastha & Kwok (2020)]

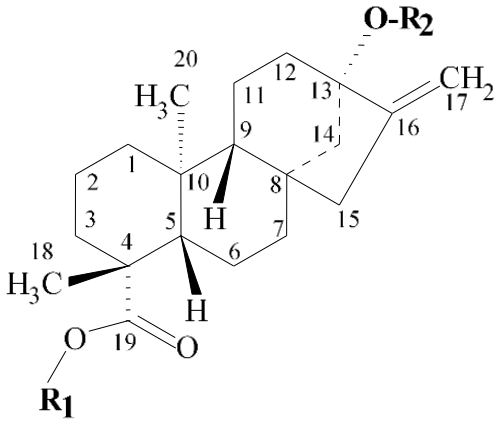
#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
2.16	Rebaudioside O6		SvR1G7	1600	0.20	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-6)Glcβ(1-3)[Glcβ(1-2)]Glcβ1-	Prakash & Ma (2018)
2.17	Rebaudioside O7		SvR2G6	1584	0.20	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Ma (2018)
3. Steviol + Xylose + Glucose (SvX1Gn)								
3.01	Stevioside F		SvX1G2	775	0.41	Glcβ1-	Xylβ(1-2)Glcβ1-	Chaturvedula & Prakash (2011c)
3.02	Rebaudioside F	438045-89-7	SvX1G3	937	0.34	Glcβ1-	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	Starratt et al. (2002)
3.03	Rebaudioside F2		SvX1G3	937	0.34	Glcβ1-	Glcβ(1-2)[Xylβ(1-3)]Glcβ1-	Chaturvedula & Prakash (2011c)
3.04	Rebaudioside F3		SvX1G3	937	0.34	Xylβ(1-6)Glcβ1-	Glcβ(1-2)Glcβ1-	Chaturvedula et al. (2011b)
3.05	Rebaudioside R		SvX1G3	937	0.34	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Xylβ1-	Ibrahim et al (2016)
3.06	Rebaudioside U		SvX1G4	1099	0.29	Xylβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
3.07	Rebaudioside U2		SvX1G4	1099	0.29	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)Glcβ1-	Purkayastha et al. (2016)
3.08	Rebaudioside U3		SvX1G4	1099	0.29	Xylβ(1-2)[Glcβ(1-4)]Glcβ1-	Glcβ(1-2)Glcβ1-	Purkayastha et al. (2019)
3.09	Rebaudioside V		SvX1G5	1261	0.25	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Xylβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
3.10	Rebaudioside V2		SvX1G5	1261	0.25	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Prakash & Chaturvedula (2013)

Table A.
Summary of Formula and R-Groups of Identified Steviol Glycosides from the Leaves of *Stevia rebaudiana* Bertoni (see Figure A for Backbone Structure)
[Adapted from Purkayastha & Kwok (2020)]

#	Common Name	CAS Number	Trivial Formula	Mol. Wt	Steviol Equivalent	R_1	R_2	Reference
4.02	Rebaudioside W2		SvA1G4	1099	0.29	Araα(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2016)
4.03	Rebaudioside W3		SvA1G4	1099	0.29	Araα(1-6)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
4.04	Rebaudioside Y		SvA1G5	1261	0.25	Glcβ(1-2)[Araα(1-6)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2019)
5. Steviol + Fructose + Glucose (SvF1Gn)								
5.01	Rebaudioside A3		SvF1G3	967	0.33	Glcβ1-	Glcβ(1-2)[Fruβ(2-3)]Glcβ1-	Chaturvedula et al. (2011c)
6. Steviol + Galactose + Glucose (SvGa1Gn)								
6.01	Rebaudioside T		SvGa1G4	1129	0.28	Glcβ(1-2)Glcβ1-	Galβ(1-2)[Glcβ(1-3)]Glcβ1-	Purkayastha et al. (2016)
7. Steviol + Deoxyglucose + Glucose (SvdG1Gn)								
7.01	Stevioside D		SvdG1G2	789	0.40	Glcβ1-	6-deoxyGlcβ(1-2)Glcβ1-	Chaturvedula & Prakash (2011d)
7.02	Stevioside E		SvdG1G3	951	0.33	Glcβ1-	6-deoxyGlcβ(1-2)[Glcβ(1-3)]Glcβ1-	Chaturvedula & Prakash (2011d)
7.03	Stevioside E2		SvdG1G3	951	0.33	6-deoxyGlcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	Chaturvedula et al. (2011d)

Figure A

Backbone structure for steviol glycosides



Appendix B: Method of Assay Details

Method of assay

METHOD OF ASSAY (for annexes 1-4)

Determine the percentages of major steviol glycosides (those with analytical standards, e.g. rebaudioside A, B, C, D, E, F, M, N, O; dulcoside A; rubusoside; stevioside; and steviolbioside) on the dry basis using an HPLC-UV technique (see Part 1 and HPLC, Vol. 4). If the sum of the major steviol glycosides in the sample is <95%, an optional HPLC-UV-MS based technique may be utilized to identify the minor steviol glycosides (see Part 2) and obtain their corresponding molecular weights. The minor steviol glycosides are quantified using either the standard curve of the commercially available minor steviol glycoside reference standards or the respective molecular weight-corrected UV peak area and the rebaudioside A standard curve (obtained using Part 1). Calculate the sum of the major and minor (if applicable) steviol glycosides and express the total glycoside content on the dried basis.

Reagents:

- Mobile phase A: Deionized water, HPLC or LC-MS grade, filtered using a 0.2-µm filter, with 0.1% formic acid or acetic acid. (Note: If only UV detection will be used, 20 mM sodium phosphate buffer at pH 2.6 or 0.01% trifluoroacetic acid may be used.)
- Mobile phase B: Acetonitrile, HPLC or LC-MS grade, filtered using a 0.2-µm filter
- Diluent: Water:acetonitrile (7:3)
- 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 may be included when they become commercially available.)

Equilibration:

Powdered samples should be equilibrated in the lab not less than 12 hours before assaying. Spread 1–2 g of sample into a thin layer not more than 6 mm thick in an open container, stirring occasionally to ensure uniform moisture absorption. The loss on drying of the equilibrated sample should be determined concurrently with performing the assay using the conditions in Annexes 1-4 (Vol. 4).

Preparation of Steviol Glycoside Standard Solutions:

If multiple commercially available reference standards are being used, prepare individual or mixed five point working standard solutions in the range of 5–500 µg/mL. Prepare all solutions in the Diluent.

If the only commercially available reference standard is rebaudioside A, prepare five working standard solutions of rebaudioside A in the range of 5–500 µg/mL. Prepare all solutions in the Diluent.

Prepare quality control solution(s) with concentrations that fall within the calibration range.

Preparation of Sample Solution:
Accurately weigh 40-50 mg of the equilibrated sample and quantitatively transfer into a 100-mL volumetric flask. Add about 80 mL of Diluent, sonicate and shake well to dissolve the sample. Allow to return to room temperature and dilute to volume with Diluent.

Part 1: Determination of Major Steviol Glycosides by HPLC-UV

Procedure:

- Column: C18 column (150 mm x 4.6 mm, 2.7µm), for example Agilent Poroshell 120 SB-C18, or equivalent.
- Column temperature: 45°
- Autosampler temperature:10 – 20°
- Detector: UV-Vis or DAD at 210 nm
- Flow rate: 0.5 mL/min
- Injection volume: 5 µl

Table 1. HPLC Gradient Timetable

Time (min)	%Solvent A	%Solvent B
0.0	75.0	25.0
8.0	75.0	25.0
13.0	68.0	32.0
16.0	68.0	32.0
19.0	60.0	40.0
23.0	60.0	40.0
23.5	40.0	60.0
25.0	40.0	60.0
25.5	75.0	25.0
35	75.0	25.0

Inject blank(s) and peak identification standard solutions.

Inject working mixed Standard Solutions and create standard curves for each steviol glycoside. If rebaudioside A is the only commercially available reference standard, derive a standard curve for rebaudioside A from the Standard Solutions. (Note: Use of 1/x weighting and not forcing the curve through 0 are recommended.)

Inject quality control and system suitability standard solutions to ensure the system performance is acceptable.

Inject prepared samples. Dilute the Sample Solution, if required, to bring the concentration of each analyte within the standard curve range. Make at least duplicate injections. Determine the concentration of each steviol glycoside from its corresponding standard curve and determine the average concentration in the Sample Solution (µg/mL).

Identification:
The retention times of each major peak in the chromatogram of the Sample Solution should be determined using primary reference standards. Example HPLC-UV chromatogram of major steviol glycosides obtained using commercially available quantitative reference standards, *Figure 1*.

Calculation:

1) Using individual steviol glycoside reference standards
Calculate the weight percentage of each steviol glycoside in the Sample Solution:

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

where:
C_{SG} is the average concentration of the steviol glycoside in the Sample Solution, as determined from the relevant standard curve (µg/mL)
C_{sample} is the concentration of the Sample Solution (µg/ml)

2) Using a rebaudioside A standard and relative response factors (RRF)
Calculate the percentage of each steviol glycoside in the Sample Solution:

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

where:
C_X is the average concentration of the steviol glycoside as rebaudioside A, as determined from the rebaudioside A standard curve (µg/mL)
F is the UV RRF for the steviol glycoside at 210 nm, from Table 2
C_{sample} is the concentration of the Sample Solution (µg/mL)

The RRF of a given steviol glycoside (reb X) to rebaudioside A may alternatively be calculated using experimental data obtained at 210 nm with reference standards.

Table 2. Relative Response Factors (RRFs) at 210 nm

	RRF against rebaudioside A	
Compounds	**Experimental ***	**Molecular Weight**
Rebaudioside A	---	1

Rebaudioside B	0.82	0.82
Rebaudioside C	1.03	0.98
Rebaudioside D	1.16	1.17
Rebaudioside E	0.98	1.00
Rebaudioside F	0.97	0.97
Rebaudioside M	1.36	1.34
Rebaudioside N	1.25	1.32
Rebaudioside O	1.56	1.49
Stevioside	0.80	0.83
Dulcoside A	0.83	0.82
Steviolbioside	0.76	0.83
Rubusoside	0.65	0.66

* All experimental RRFs were obtained using a 10 mm long UV/PDA flow cell. The RRF is calculated using the assigned purity values as provided by the reference standard manufacturer, including their corrections for moisture and solvents. Independent confirmation of the RRFs of major glycosides is suggested when adopting the method or when changing any operational parameters.

Calculate the percentage of major steviol glycosides in the sample by summation of percentages of individual steviol glycosides in the sample. If the sum of the concentrations of the major steviol glycosides in the sample is <95%, then proceed to Part 2.

Part 2: Determination of Minor Steviol Glycosides by LC-UV-MS

The mass spectrometer is connected to the LC-UV system used in Part 1. It is used to identify the minor peaks that do not match the retention times of the major steviol glycosides identified in Part 1. Quantification of minor glycosides is based on comparison to standard curves for the minor glycosides, where pure reference standards are available (see calculation 1, described in Part 1). If pure reference standards for the minor glycosides are not available, quantification is based on comparison to the rebaudioside A standard curve and the molecular weight-based relative response factor (see calculation 2, described in Part 1).

LC-UV-MS operating conditions may vary based on the manufacturer and model of the system used; conditions should be set following the manufacturer's instructions. Example MS conditions are provided below for a Waters Acquity SQD mass spectrometer. The MS can be operated in full scan mode or in selected ion monitoring mode (SIM) using the m/z values listed in Table 3. (Note: Table 3 m/z values encompass identified major and minor steviol glycosides from the leaves of *Stevia rebaudiana* Bertoni. See Appendix A, Table A for chemical information related to these identified steviol glycosides.)

Table 3. Example SIM m/z values and their possible identification if peak retention time differs from major steviol glycosides.

Molecular mass ion $[M-H]^-$	Identity
317	Steviol or its isomers
479	Steviol +1 glucoside
625	Dulcoside A1
641	Isomers of steviolbioside or rubusoside
774	Stevioside F or isomers
787	Isomers of dulcoside A
803	Isomers of reb B or stevioside
935	Isomers of reb F
949	Isomers of reb C
965	Isomers of reb A
1097	Steviol + 4 Glc + 1 Arabinose or isomers
1111	Steviol + 4 Glc +1 Rhamnose or isomers
1127	Isomers of reb D
1259	Steviol + 5 Glc + Xyl or isomers
1273	Isomers of reb N
1289	Isomers of reb M
1435	Isomers of reb O

This list does not include every possible steviol glycoside; additional m/z may be evaluated.

In the absence of available reference standards for the minor steviol glycosides, one or more of the in-source fragmentation ions in Table 4 should be used along with the molecular ions for identification purposes:

Table 4. Example diagnostic fragmentation ions

Fragmentation ion $[M-H]^-$	Identity
317	Steviol
479	Steviol+1 glucoside
625	Steviol+2 glucoside-oxygen [M-16]
641	Steviol +2 glucoside
787	Steviol +2 glucoside +1 rhamnoside
803	Steviol +3 glucoside
949	(Deoxy)-Steviol +4 glucoside
965	Steviol+4 glucoside

This list does not include every possible diagnostic ion; additional m/z may be evaluated.

Example MS Conditions

Instrumentation:	Waters Acuity SQD MS
Ionization:	Electrospray negative polarity
Cone voltage:	35 V (low – m/z ≤ 900 amu) and 80 V (high – m/z >900 amu)
Resolution:	1 amu
Data acquisition:	Scanning 50 to 2000 m/z

(Note: See example scans in *Figures 2* and *3*.)

Calculation:

Calculate the concentration of minor glycosides using the rebaudioside A standard curve:

$$\text{Conc. (\% w/w)} = C_X \times M_X \times 100 / M_A \times C_{sample}$$

where:

C_X is the average concentration of the minor steviol glycoside as rebaudioside A, as determined from the rebaudioside A standard curve (µg/mL)

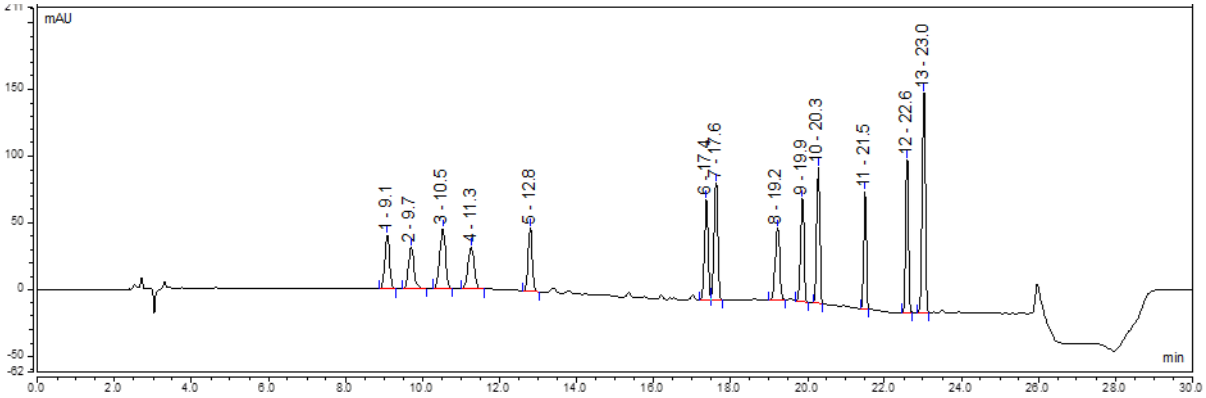
M_X is the molecular weight of the minor steviol glycoside obtained by mass spectrometry

M_A is the molecular weight of rebaudioside A

C_{sample} is the concentration of the Sample Solution (µg/mL)

(Note – Calculate the concentration of minor steviol glycosides using commercially available minor steviol glycoside reference standards when available.)

Figure 1. Example LC-UV chromatogram and retention times of major steviol glycosides with commercially available quantitative reference standards



Peak Number	Analyte	Approximate Retention Time (min)
1	Rebaudioside E	9.1
2	Rebaudioside O	9.7
3	Rebaudioside D	10.5
4	Rebaudioside N	11.3
5	Rebaudioside M	12.8
6	Rebaudioside A	17.4
7	Stevioside	17.6
8	Rebaudioside F	19.2
9	Rebaudioside C	19.9
10	Dulcoside A	20.3
11	Rubusoside	21.5
12	Rebaudioside B	22.6
13	Steviolbioside	23.0

Figure 2. Top trace: Full scan m/z 50 to 900amu; Bottom trace: Full scan m/z 901 to 2000 amu

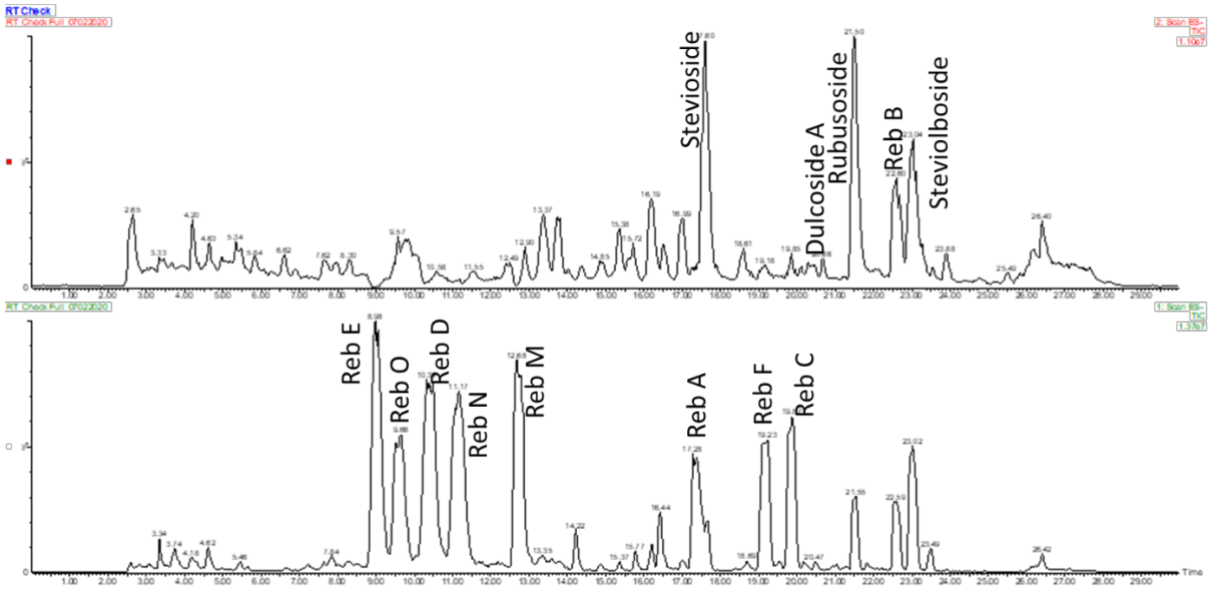
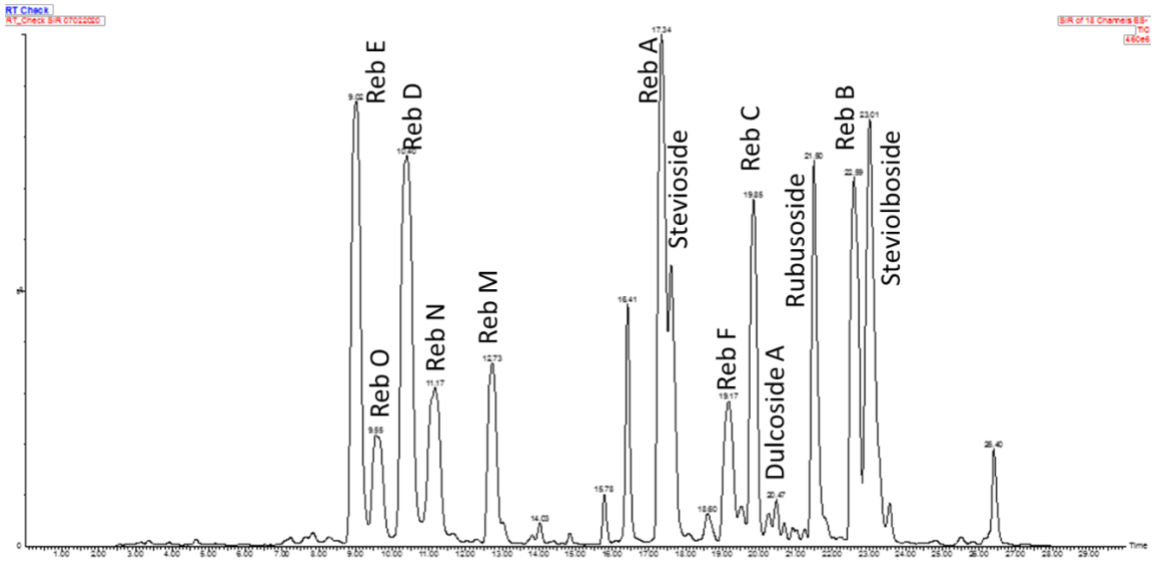


Figure 3. Example SIM scan using the m/z values listed in Table 3



ANNEX 1: STEVIOL GLYCOSIDES FROM STEVIA REBAUDIANA BERTONI

Revised at 96th JECFA (2023) and published in FAO Monographs 31 (2023), superseding specifications prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021). 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 of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
C.A.S number	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
Chemical formula	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
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)	Very slightly soluble to freely soluble in water; slightly soluble to freely soluble in a mixture of ethanol and water (50:50 v/v)

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 B of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES

ANNEX 2: STEVIOL GLYCOSIDES FROM FERMENTATION

Revised at 96th JECFA (2023) and published in FAO Monographs 31 (2023), superseding specifications prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021). 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 of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
C.A.S number	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
Chemical formula	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
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)	Very slightly soluble to freely soluble in water; slightly soluble to freely soluble in a mixture of ethanol and water (50:50 v/v)
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 B of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES

ANNEX 3: ENZYME MODIFIED STEVIOL GLYCOSIDES

Revised at 96th JECFA (2023) and published in FAO Monographs 31 (2023), superseding specifications prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021). 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* Bertoni

Chemical names

See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES

C.A.S number

See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES

Chemical formula	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
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)	Very slightly soluble to freely soluble in water; slightly soluble to freely soluble in a mixture of ethanol and water (50:50 v/v)
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 B of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES

ANNEX 4: ENZYME MODIFIED GLUCOSYLATED STEVIOL GLYCOSIDES

Revised at 96th JECFA (2023) and published in FAO Monographs 31 (2023), superseding specifications prepared at the 91st JECFA (2021) and published in FAO Monographs 26 (2021). 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 *Anoxybacillus caldiproteoliticus*, *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.
Chemical names	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
C.A.S number	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
Chemical formula	See Appendix A of the (FRAMEWORK FOR) STEVIOL GLYCOSIDES
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 dextrin and steviol glycoside fractions are separated using an adsorption column and elution with water and ethanol. The two fractions are dried and weighed to obtain the relative percentages of dextrin and total steviol glycosides (step 1). The percentage of glucosylated and unreacted parent steviol glycosides are determined using the HPLC method below (step 2).

Reagents:

- Ethanol
- Mobile phase A: Acetonitrile, HPLC grade
- Mobile phase B: Water, HPLC grade
- Reference Standards: Stevioside, rebaudioside A, rebaudioside C, rebaudioside F, rubusoside, and steviolbioside. Chromadex, USA; Wako Pure Chemical Industries Ltd., Japan; Sigma-Aldrich; US Pharmacopeia or equivalent.

Step 1: Column Adsorption

Weigh accurately 4 g of glucosylated steviol glycosides sample and dissolve with 100 mL water in a graduated cylinder. Record the exact weight and volume; the concentration of the Sample Solution is approximately 4%. Load the solution onto a glass column (25-mm ID) packed with 200 mL of Sigma Amberlite XAD 7 HP resin, or equivalent, at a rate of < 3 ml/min. Elute with 1000 ml of water to remove the dextrin. Next, elute with 1000 mL of 70% ethanol at a rate of 3 ml/min or less to remove the steviol glycosides. Evaporate the two eluted fractions to dryness, then dry in a vacuum oven at 105° for 2 hours. Weigh and record the dry weight of each fraction.

Calculate the percent of dextrin and of total steviol glycosides (TSG):

Dry weight initial sample (g) = wet weight initial sample (g) x [(100 – loss on drying %) / 100]

Dextrin (%) = [weight of dried aqueous fraction (g) / dry weight of initial sample (g)] X 100

TSG (%) = [weight of dried ethanol fraction (g) / dry weight of initial sample (g)] X 100

If the content of residual dextrin is more than 4%, the adjusted TSG on the dextrin-free basis is calculated by the following formula:

Adjusted TSG (%) = TSG (%) x dry weight initial sample (g) / [dry weight initial sample (g) – weight of dextrin (g)]

Step 2: HPLC Assay

Reagents:

- Diluent: 50% (v/v) ethanol in water

- Mixed Marker Solution: Prepare a solution containing 100 mg/l each of rubusoside, dulcoside A, stevioside, rebaudioside C, rebaudioside F and rebaudioside A solution in Diluent.

Equilibration:
Powdered samples should be equilibrated in the lab not less than 12 hours before assaying. Spread 4–5 g of sample into a thin layer not more than 6 mm thick in an open container, stirring occasionally to ensure uniform moisture absorption. The loss on drying of the equilibrated sample should be determined concurrently with the HPLC analysis.

Preparation of Rebaudioside A Standard Solution:
Weigh approximately 125 mg of rebaudioside A reference standard into a 25-ml volumetric flask and dilute to volume with the Diluent. Prepare in duplicate. The approximate concentration of each solution is 5000 mg/l.

Preparation of Sample Solution:
Weigh approximately 2500 mg of the sample into a 50 ml volumetric flask and dilute to volume with the Diluent. Prepare in duplicate. The approximate concentration of each solution is 50 000 mg/l.

Procedure:

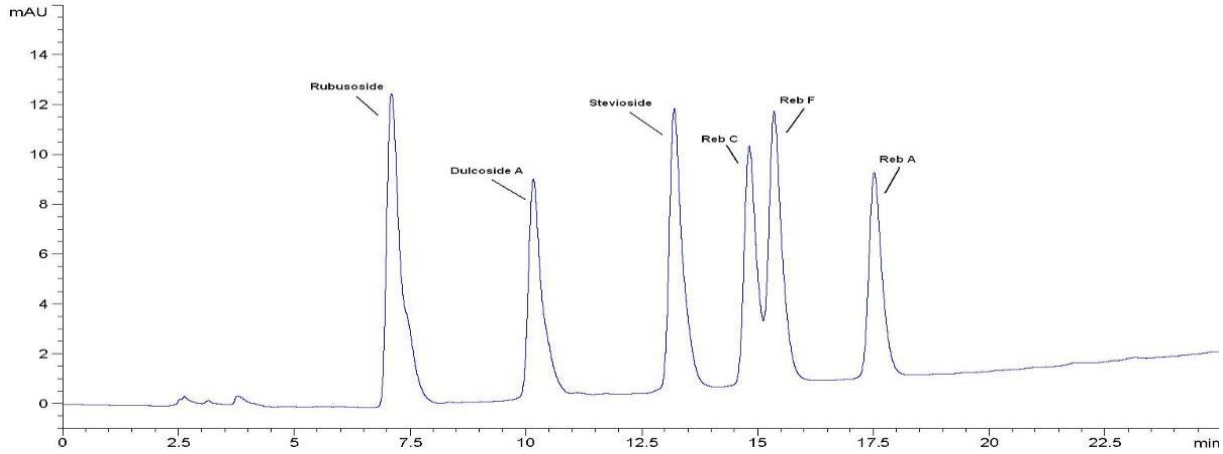
- Column: Zorbax NH2, or equivalent; 250 x 4.6 mm, 5-μm
- Column temperature: 40°
- Flow rate: 1.0 ml/minute
- Injection volume: 12 µl
- Detection: UV at 210 nm (4 nm bw); Reference: 360 nm (100 nm bw)
- Run time: 90 minutes
- Post time: 10 minutes

Table 1. HPLC Gradient Timetable

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
2	80	20
90	50	50
91	80	20
100	80	20

Analysis of the Parent Steviol Glycosides:
Inject the Mixed Marker Solution into the chromatograph and identify the retention time of each steviol glycoside on the resulting chromatogram by comparison to the following chromatogram (Annex 4 Figure 1):

Annex 4 Figure 1. HPLC chromatogram of Mixed Marker Solution



Inject 8.0-, 10.0- and 12.0-µl aliquots of the first (rebaudioside A) Standard Solution into the chromatograph and record the resulting chromatograms. Prepare a 3-point standard curve of peak area vs. concentration of rebaudioside A (mg/l).

Inject 12 µl of the duplicate (rebaudioside A) Standard Solution; its recovery should be within 98 -102% when the peak area response is compared to the 3-point standard curve.

Inject 12 µl each of the duplicate Sample Solutions and report the average of their responses. The % RSD for rebaudioside A and stevioside content in the duplicate Sample Solutions should be less than 2.0%.

Calculate the concentration of the steviol glycosides in the Sample Solution using following formula:

$$SG\ (mg/l) = A \times m + b$$

where:

A is the peak area of the steviol glycoside
m is the slope of the rebaudioside A standard curve
b is the y-intercept of the rebaudioside A standard curve

Multiply the concentration of other steviol glycosides present by their respective correction factors to correct for the differences in molecular weight. The correction factors for rubusoside, dulcoside A, stevioside, rebaudioside C, rebaudioside F are 0.665, 0.815, 0.832, 0.983, and 0.969, respectively (as compared to rebaudioside A).

Calculate the percentage of each steviol glycoside in the Sample Solution using the following formula:

$$\text{Conc. (w/w) \%} = SG \times 100/C_{Sample}$$

Where:

SG is the concentration of the steviol glycoside determined above (mg/l)

C_{Sample} is the concentration of the Sample Solution (mg/l)

Sum the steviol glycoside components to determine the "Total Parent Steviol Glycosides" (TPSG%).

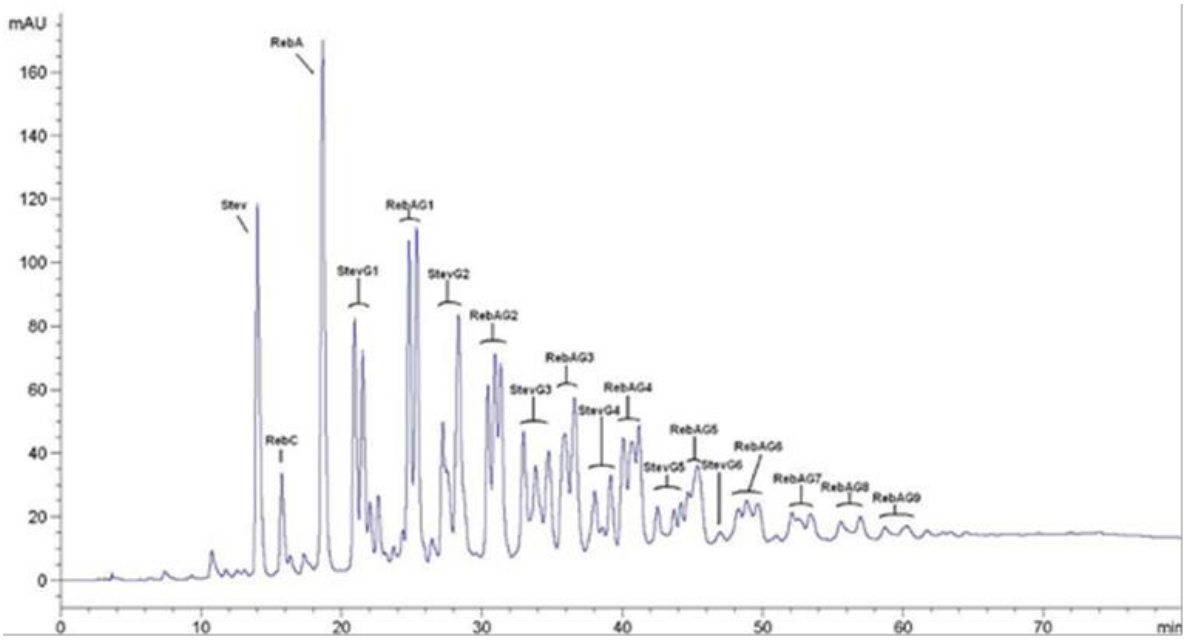
Analysis of the α-Glucosyl Steviol Glycosides:

Use the "Total Parent Steviol Glycosides" data obtained from the HPLC assay and the following formula to calculate the total content of α-glucosyl steviol glycosides:

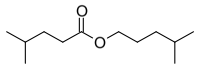
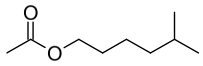
$$\text{Total α-glucosyl steviol glycosides \%} = \text{TSG \%} - \text{TPSG \%}$$

Each individual α-glucosyl steviol glycoside component is identified by comparison to the following chromatogram (Annex 4 Figure 2):

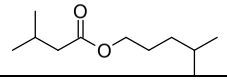
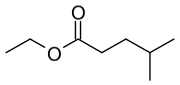
Annex 4 Figure 2. HPLC chromatogram of an Enzyme Modified Glucosylated Steviol Glycoside sample solution



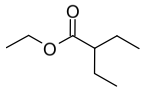
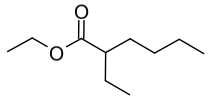
Esters of aliphatic acrylic primary alcohols with branched-chain aliphatic acrylic acids

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	Chemical Name	FLAVIS	M.W.	Solubility in ethanol	Assay min %	S.G. (25º)	
	Synonyms	COE	Physical form; Odour	B.P.º	Acid value		Information required
Session		CAS					
2280	**4-Methylpentyl 4-methylvalerate**	4749	$C_{12}H_{24}O_2$	Insoluble	HNMR		
Full	4-methylpentyl 4-methylpentanoate		200.32	Soluble	95%	0.861-0.873 (20 °C)	
	4-Methylpentyl 4-methylpentanoate; Pentanoic acid, 4-methyl-, 4-methylpentyl ester; 4-Methylpentanoic acid, 4-methylpentyl ester; isohexyl		Clear to pale yellow liquid	214-230 °C (760 Torr)			
96		35852-42-7					
2281	**5-Methylhexyl acetate**		$C_9H_{18}O_2$	Practically insoluble or insoluble	MS	1.411-1.421	sc: 5-6% hexyl acetate (JECFA No. 128), 3-4% heptyl acetate (JECFA No. 129)
Full	5-Methylhexyl acetate		158.24	Insoluble	87%	0.869-0.878	
	Isoheptyl acetate; Acetic acid, isoheptyl ester; 1-Hexanol, 5-methyl-, 1-acetate		Clear to pale yellow liquid; Liquid; sweet fruity aroma	183-184 °C			
96		72246-17-4; 180348-60-1					

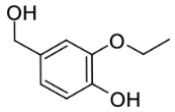
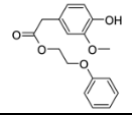
Esters of aliphatic acrylic primary alcohols with branched-chain aliphatic acrylic acids

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirement
Status	Chemical Name	FLAVIS	M.W.	Solubility in ethanol	Assay min %	S.G. (25º)	
	Synonyms	COE	Physical form; Odour	B.P.º	Acid value		Information required
Session		CAS					
2282	**4-Methylpentyl isovalerate**	4347	$C_{11}H_{22}O_2$	Practically insoluble or	HNMR, IR, MS	1.414-1.420	
Full	4- Methylpentyl 3-methylbutanoate		186.29	Insoluble	98%	0.853-0.859	
	4-Methylpentyl 3-methylbutanoate; Isohexyl 3-methylbutanoate; Butanoic acid 3-methyl-, 4-methylpentyl ester		Clear to pale yellow liquid; Sour apple-like aroma	61-63 °C			
96		850309-45-4					
2283	**Ethyl 4-methylpentanoate**	4343	$C_8H_{16}O_2$	Practically insoluble or	IR, MS	1.402-1.409	
Full	Ethyl 4-methylpentanoate		144.21	Insoluble	98%	0.865-0.875	
	Ethyl isohexanoate; Ethyl 4-methylvalerate; Ethyl isocaproate; Pentanoic acid, 4-methyl-, ethyl ester; 4-Methylvaleric Acid Ethyl Ester		Clear to pale yellow liquid; Fruity tropical aroma	153-155 °C	1		
96		25415-67-2					

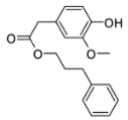
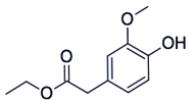
Esters of aliphatic acrylic primary alcohols with branched-chain aliphatic acrylic acids

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	Chemical Name	FLAVIS	M.W.	Solubility in ethanol	Assay min %	S.G. (25º)	
	Synonyms	COE	Physical form; Odour	B.P.º	Acid value		Information required
Session		CAS					
2284	**Ethyl 2-ethylbutyrate**	4344	$C_8H_{16}O_2$	Practically insoluble or insoluble	IR, MS	1.401-1.407	
Full	Ethyl 2-ethylbutanoate		144.21	Insoluble	98%	0.865-0.871	
	Ethyl 2-ethylbutanoate; Ethyl alpha-ethylbutyrate; 2-Ethyl-n-butyric acid ethyl ester; Butanoic acid, 2-ethyl-, ethyl ester		Clear to pale yellow liquid; Fruity tropical aroma	132-133 °C	1		
96		2983-38-2					
2285	**Ethyl 2-ethylhexanoate**	4345	$C_{10}H_{20}O_2$	Practically insoluble or insoluble	IR, MS	1.411-1.417	
Full	Ethyl 2-ethylhexanoate		172.26	Insoluble	95%	0.860-0.867	
	Ethyl 2-ethylcaproate; Ethyl alpha-ethylcaproate; Hexanoic acid, 2-ethyl-, ethyl ester		Clear to pale yellow liquid; Fruity tropical aroma	210-211 °C	1		
96		2983-37-1					

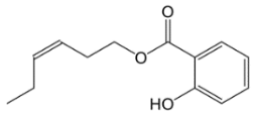
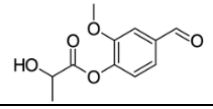
Hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	**Chemical Name**	**FLAVIS**	**M.W.**	**Solubility in ethanol**	**Assay min %**	**S.G. (25º)**	
	Synonyms	**COE**	**Physical form; Odour**	**B.P.º**	**Acid value**		**Information required**
Session		**CAS**					
2271	**2-Ethoxy-4-(hydroxymethyl)phenol**	4893	$C_9H_{12}O_3$	Soluble	HNMR, CNMR, IR, MS, UV/Vis		M.P. 61-64 °C
Full	2-Ethoxy-4-(hydroxymethyl)phenol		168.19	Soluble	95%		
	3-Ethoxy-4-hydroxybenzyl alcohol; Ethyl vanillyl alcohol; p-Hydroxymethyl)guethol; 2-Hydroxy-4-methoxybenzaldehyde; benzenemethanol,3-ethoxy 4 hydroxy		Off white solid; Vanilla aroma				
96		4912-58-7					
2272	**2-Phenoxyethyl 2-(4-hydroxy-3-methoxyphenyl) acetate**	4871	$C_{17}H_{18}O_5$	Slightly soluble	HNMR, CNMR, MS		M.P. 56 °C
Full	2-Phenoxyethyl 2-(4-hydroxy-3-methoxyphenyl) acetate		302.32	Soluble	95%	1.211 (20 °C)	
	2-phenoxyethyl homovanilate; Benzeneacetic acid, 4-hydroxy-3-methoxy-, 2-phenoxyethyl ester		Off white solid; Vanilla aroma				
96		1962956-83-7					

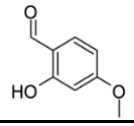
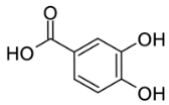
Hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	Chemical Name	FLAVIS	M.W.	Solubility in ethanol	Assay min %	S.G. (25º)	
	Synonyms	COE	Physical form;	B.P.º	Acid value		Information
Session		CAS					
2273	**3-Phenylpropyl 2-(4-hydroxy-3-methoxy-phenyl)acetate**	4826	$C_{18}H_{20}O_4$	Insoluble	HNMR, IR, MS	1.577-1.559	
Full	3-Phenylpropyl 2-(4-hydroxy-3-methoxy-phenyl)acetate		300.35	Slightly soluble	95%	1.153-1.155	
	3-Phenylpropyl homovanillate; 4-hydroxy-3-methoxy-3-phenylpropyl ester; benzene acetic acid, 4-hydroxy-3-methoxy-, 3-phenylpropyl ester		Slightly yellow liquid; Mint	178 °C (0.30 mbar)			
96		105025-99-8					
2274	**Ethyl-2-(4-hydroxy-3-methoxy-phenyl) acetate**	4810	$C_{11}H_{14}O_4$	Insoluble	HNMR, IR, MS		M.P. 44-47 °C
Full	Ethyl-2-(4-hydroxy-3-methoxy-phenyl) acetate		210.23		95%		
	Ethyl homovanillate; Ethyl 4-hydroxy-3-methoxyphenylacetate; Benzeneacetic acid, 4-hydroxy-3-methoxy-, ethyl ester; 4-hydroxy-3-methoxyphenylacetic acid ethyl ester		Solid				
96		60563-13-5					

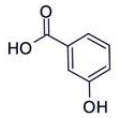
Hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	**Chemical Name**	**FLAVIS**	**M.W.**	**Solubility in ethanol**	**Assay min %**	**S.G. (25º)**	
	Synonyms	**COE**	**Physical form;**	**B.P.º**	**Acid value**		**Information**
Session		**CAS**					
2275	***cis*-3-Hexenyl salicylate**	4750	$C_{13}H_{16}O_3$	Practically insoluble or	HNMR, IR	1.517-1.525	
Full	[(Z)-hex-3-enyl]2-hydroxybenzoate	09.570	220.26	Freely soluble	96%	1.052-1.067	
	2-Hydroxybenzoic acid 3Z-hexenyl ester; (Z)-3-Hexenyl salicylate; 3Z-Hexenyl 2-hydroxybenzoate; Hex-3-en-1-yl salicylate	10685	Clear colorless liquid; Green balsamic	271 °C			
96		65405-77-8			2		
2276	**4-Formyl-2-methoxyphenyl 2-hydroxypropanoate**	4606	$C_{11}H_{12}O_5$	Slightly soluble	CNMR, HNMR, IR, MS		SC: 3% lactic acid (JECFA No. 930)
Full	4-Formyl-2-methoxyphenyl 2-hydroxypropanoate		224.21	Soluble	93%		M.P. 85.5 °C
	Vanillyl lactate; propanoic acid, 2-hydroxy-, 4-formyl-2-methoxy phenyl ester		Colorless powder; Vanilla, creamy, harmonizing	271-274 °C			
96		930587-76-1					

Hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	**Chemical Name**	**FLAVIS**	**M.W.**	**Solubility in ethanol**	**Assay min %**	**S.G. (25º)**	
	Synonyms	**COE**	**Physical form;**	**B.P.º**	**Acid value**		**Information**
Session		**CAS**					
2277	**2-Hydroxy-4-methoxybenzaldehyde**	4435	$C_8H_8O_3$	Slightly soluble	HNMR, CNMR, IR, MS		M.P. 39-42 °C
Full	2-Hydroxy-4-methoxybenzaldehyde		152.15	Soluble	97%	1.231	
	2-Formyl-5-methoxyphenol; 2-Hydroxy-p-anisaldehyde; 4-Methoxysalicylaldehyde		White or yellow to beige crystal	271-274 °C			
96		673-22-3					
2278	3,4-Dihydroxybenzoic acid	4430	$C_7H_6O_4$	Soluble	IR		M.P. 210-221 °C
Full	3,4-Dihydroxybenzoic acid	08.133	154.12	Soluble	99%		
	Protocatehuic acid		Light brown solid				
96		99-50-3					

Hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	**Chemical Name**	**FLAVIS**	**M.W.**	**Solubility in ethanol**	**Assay min %**	**S.G. (25º)**	
	Synonyms	**COE**	**Physical form; Odour**	**B.P.º**	**Acid value**		**Information required**
Session		**CAS**					
2279	**3-Hydroxybenzoic acid**	4431	$C_7H_6O_3$	Soluble	HNMR, CNMR, IR		M.P. 201-205 °C
Full	3-Hydroxybenzoic acid	08.132	138.12	Soluble	99%		
	m-Hydroxybenzoic acid; m-Salicylic acid		White crystalline powder				
96		99-06-9					

Revisions to existing flavour specifications

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	Chemical Name	FLAVIS	M.W.	Solubility in ethanol	Assay min %	S.G. (25º)	
	Synonyms	COE	Physical form; Odour	B.P.º	Acid value		Information required
Session		CAS					
1383	**(E)-2-Hexenal diethyl acetal**	4047	$C_{10}H_{20}O_2$	Soluble in most fixed oils; insoluble in water	IR, MS	1.418-1.426	Isomers: 94-99% (E); 0.4-3.2% (Z)
Full	(2E)-1,1-Diethoxy-2-hexene	06.031	172.26	Soluble	97% (sum of isomer)	0.843-0.849	
	(E)-1,1-Diethoxy-2-hexene; Leafaldehyde diethyl acetal		Colourless liquid; fruity aroma	76-77 °C (15 mm Hg)			
96		67746-30-9					
1170	**3-Butylidenephthalide**	3333	$C_{12}H_{12}O_2$	Insoluble in water; Soluble in oils	HNMR	1.554-1.592	Isomers: 86-96% (Z); 2-14% (E)
Full	3-Butylidene-1(3H)-isobenzofuranone	10.024	188.22	Soluble	95% (sum of isomers)	1.080-1.117	
	3-n-Butylidene-phthalide; n-Butylidenephthalide; Ligusticum lactone		Yellow liquid; pervasive warm spicy aroma	114-116 °C (0.05 mm Hg)	1		
96		551-08-6					

Revisions to existing flavour specifications

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	**Chemical Name**	**FLAVIS**	**M.W.**	**Solubility in ethanol**	**Assay min %**	**S.G. (25º)**	
	Synonyms	**COE**	**Physical form; Odour**	**B.P.º**	**Acid value**		**Information required**
Session		**CAS**					
1233	**1,4-Cineole**	3658	$C_{10}H_{18}O$	Insoluble in water; Soluble in oils	HNMR	1.400-1.500	Isomers: not less than 75% 1,4-cineole with the remainder as 1,8-cineole (JECFA No. 1234)
Full	1-Methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]heptane	03.007	154.25	Soluble	95% (sum of isomers with not less than 75%	0.850-0.908	
	1,4-Epoxy-p-menthane; Isocineole		Colourless mobile liquid; camphor like aroma	172-174 °C	1		
96		470-67-7					
1166	**Octahydrocoumarin**	3791	$C_9H_{14}O_2$	Insoluble in water;	HNMR	1.489-1.493	
Full	Octahydro-2H-1-benzopyran-2-one	13.161	154.21	Soluble	99%	1.089-1.096	
	Cyclohexyl lactone; Bicyclononalactone		Colourless liquid; slight spicy note	293-298 °C	2		
96		4430-31-3					

Revisions to existing flavour specifications

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	**Chemical Name**	**FLAVIS**	**M.W.**	**Solubility in ethanol**	**Assay min %**	**S.G. (25º)**	
	Synonyms	**COE**	**Physical form; Odour**	**B.P.º**	**Acid value**		**Information required**
Session		**CAS**					
1411	**3-(l-Methoxy)-2-methylpropane-1,2-diol**	3849	$C_{14}H_{28}O_3$	Soluble in hexane; Slightly	HNMR	1.468-1.474	
Full	(l)-2-Methyl-3-[5-methyl-2-(1-methylethyl)cyclohexyl]oxy -1,2-propanediol		244.37	Soluble	99%	0.978-0.987 (20 °C)	
			Colourless liquid; minty, cool aroma	124 °C (0.4 mm Hg)	1		
96		195863-84-4					
1416	**p-Methane-3,8-diol**	4053	$C_{10}H_{20}O_2$	Slightly soluble in water	IR		M.P. 34-35 °C; Isomers: 55-61% (1S,2S,4R) (CAS No. 107133-84-6); 34-39% (1S,2R,4R) (CAS No. 238748-68-0); 1.5-3.5% (1R,2S,4R) (CAS No. 92471-23-3, 3564-95-2); 1-2% (1R,2R,4R) (CAS No. 91739-72-9, 3564-98-5)
Full	p-Menthane-3,8-diol		172.26	Soluble	95% (sum of isomers)		
	2-Hydroxy-alpha,alpha, 4-trimethylcyclohexane methanol		Opaque white crystals; minty, herbaceous, eucalyptus-like aroma				
96		42822-86-6					

Revisions to existing flavour specifications

JECFA No.	Name	FEMA	Chemical Formula	Solubility	ID test	R.I. (20º)	Other requirements
Status	**Chemical Name**	**FLAVIS**	**M.W.**	**Solubility in ethanol**	**Assay min %**	**S.G. (25º)**	
	Synonyms	**COE**	**Physical form; Odour**	**B.P.º**	**Acid value**		**Information required**
Session		**CAS**					
808	**p-Isopropylacetophenone**	2927	$C_{11}H_{14}O$	Insoluble in water; soluble in organic solvents, oils	IR	1.520-1.527	
Full	1-(4-Isopropylphenyl)ethanone		162.23	Miscible at room temperature	95%	0.967-0.975	
	p-Acetylcumene; p-Isopropylacetylbenzene; Methyl p-isopropylphenyl ketone; Acetocumene		Colourless liquid; powerful spicy, woody, herbaceous odour	252 °C			
96		645-13-6					
810	**Acetanisole**	2005	$C_9H_{10}O_2$	Insoluble in water; soluble in organic solvents, oils	IR		M.P. 36-38 °C; Isomers: 97% (o-, m-, p-isomers); >95% (p-isomer)
Full	1-(4-Methoxyphenyl)ethanone		150.17	Very soluble	97% (sum of isomers)		
	Methyl 4-methoxyphenyl ketone; 4-Acetylanisole; 4-Methoxyacetophenone; p-Acetylanisole; p-Methoxyacetophenone		Colourless to pale yellow fused solid; floral, powdery, vanillic, balsamic odour	153 °C (26 mm Hg)			
96		100-06-1					

Annex 1: Summary and conclusions from 96th JECFA[2]

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES
Ninety-sixth meeting (Safety evaluation of certain food additives)
27 June–6 July 2023

SUMMARY AND CONCLUSIONS
Issued on 14 July 2023

The ninety-sixth meeting of the Joint FAO/WHO Executive Committee on Food Additives was held in Geneva from 27 June to 6 July 2023. The purpose of the meeting was to evaluate the safety of certain food additives and flavourings. The present meeting was the Ninety-sixth in a series of similar meetings. The tasks before the Committee were to (a) further elaborate principles governing the evaluation of food additives; (b) undertake safety evaluations of certain food additives; (c) review and prepare specifications for certain food additives; and (d) establish specifications for certain flavouring agents.

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

The Committee evaluated the safety of one food additive, revised the specifications for three food additives, evaluated the safety of two groups of flavouring agents and revised the specifications for eight flavouring agents.

The report of the meeting will be published in the WHO Technical Report Series (No. 1050). The report will summarize the main conclusions of the Committee in terms of acceptable daily intakes (ADIs) and other toxicological, dietary exposure and safety recommendations. Information on deliberations and conclusions with regards to the specifications for the identity and purity of certain food additives examined by the Committee and on specifications for the flavouring agents will also be included.

The participants are listed in Annex 1. Future work and recommendations arising from the summary report of the Ninety-sixth JECFA meeting are summarized in Annex 2. Finally, Annex 3 includes requests for corrections that were reported to the JECFA Secretariat, evaluated by the Committee and found to be necessary (note that these corrections will only be made in the electronic versions available in the online database).

Toxicological monographs summarizing the data that were considered by the Committee in establishing ADIs will be published in WHO Food Additives Series No. 87. New and revised specifications for the identity and purity of the compounds will be published in FAO JECFA Monographs No. 31.

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

[2] Please note that the annexes referred to in this document are to be found in the original summary of the 95th meeting and are not those in this volume of the FAO JECFA Monographs series.

Toxicological and dietary exposure information and conclusions

Food additive evaluated toxicologically, assessed for dietary exposure and specifications revised

Aspartame

At its twenty-fifth meeting, the Committee established an ADI of 0–40 mg/kg bodyweight (bw) for aspartame *(1)*. This ADI was based on the no-observed-adverse-effect limit (NOAEL) of 4000 mg/kg bw per day, the highest dose tested, in a 104-week study in rats exposed to aspartame in the diet reported by Ishii et al. *(2)*, and the application of a 100-fold uncertainty factor. At the present meeting, the Committee evaluated biochemical, toxicological and epidemiological studies on aspartame, its metabolites and degradation products that had become available since the previous Committee's evaluation. The Committee also assessed estimates of dietary exposure to aspartame for the first time.

Following oral exposure, aspartame is fully hydrolysed in the gastrointestinal tract of humans and animals into three metabolites: phenylalanine, aspartic acid and methanol. The Committee therefore reaffirmed that there is no systemic exposure to aspartame after dietary exposure. Phenylalanine, aspartic acid and methanol are also released from commonly consumed foods by enzymatically catalysed hydrolysis.

After the pre-systemic hydrolysis of aspartame, these substances enter the systemic circulation at levels lower than those derived from consumption of common foods. The Committee noted that in oral aspartame exposure studies in humans at doses up to the current ADI, there were no increases in the plasma concentrations of the metabolites of aspartame.

The Committee concluded that there was no concern for genotoxicity of oral exposure to aspartame.

The Committee evaluated data from twelve oral carcinogenicity studies of aspartame and identified deficiencies with all of them. The Committee noted that all the studies apart from those by Soffritti et al. *(3–6)* showed negative results. The Committee considered the positive findings of Soffritti and colleagues, noting that there were limitations in the study design, execution, reporting and interpretation of these studies. In particular, this was because of the use of a test protocol in which most animals were allowed to reach natural death. As a result, the interpretation of these studies was complicated by the known increases in cancer occurrence with ageing. The Committee reached the view that the results of the Soffritti et al. studies are of uncertain relevance and therefore cannot be used for the risk assessment of aspartame. The Committee concluded that the carcinogenicity study by Ishii et al. *(2)* was close to meeting the current testing guidelines and showed negative results. The Committee reviewed several recently published studies that investigated possible mechanisms that may be relevant to the induction of cancer, including oxidative stress. The studies that reported changes in markers of oxidative stress had limitations in their design. The Committee noted that histopathological changes that would be expected from prolonged oxidative stress were not observed in other short- and long-term toxicity studies of aspartame.

Based on the negative results of the Ishii et al. study as well as the other negative carcinogenicity studies, no concern of genotoxicity, and a lack of a plausible mechanism by which oral exposure to aspartame could induce cancer, the Committee concluded that there was no concern for carcinogenicity in animals from oral exposure to aspartame.

The NOAEL in one- or two-generation reproductive and developmental toxicity studies in rats was 4000 mg/kg bw per day, the highest dose tested. The NOAEL for developmental toxicity in mice was 5700 mg/kg bw per day, the highest dose tested. The Committee therefore concluded that aspartame was not a reproductive or developmental toxicant in animals.

The Committee evaluated data from randomized controlled trials (RCTs) and epidemiological studies to examine the association between aspartame consumption and certain health effects, such as cancer, type 2 diabetes (T2D) and other non-cancer health end-points in humans.

The Committee noted that statistically significant increases were reported for some cancers, such as hepatocellular, breast and haematological (non-Hodgkin lymphoma and

multiple myeloma) cancers, in some cohort studies conducted with aspartame or beverages containing aspartame as an intense sweetener. However, a consistent association between aspartame consumption and a specific cancer type was not observed. All studies have limitations with respect to their assessment of exposure and, in many studies, particularly with respect to aspartame versus intense sweeteners in general. Reverse causality, chance, bias and confounding by socioeconomic or lifestyle factors, or consumption of other dietary components cannot be ruled out. Overall, the Committee concluded that the evidence of an association between aspartame consumption and cancer in humans is not convincing.

Several studies assessing the effects of aspartame consumption on T2D and other non-cancer health end-points in humans showed inconsistent results. For example, RCTs showed reduced glycaemic responses after aspartame consumption, whereas in epidemiological studies aspartame consumption was associated with a greater T2D risk. The Committee noted that the results of the epidemiological studies may be biased by how T2D cases were identified (either specific medications and self-reported physician diagnosis). The Committee therefore concluded that the evidence of an association between aspartame consumption and the evaluated non-cancer health end-points is not convincing.

Overall, the Committee concluded that there was no convincing evidence from experimental animal or human data that aspartame has adverse effects after ingestion. This conclusion is underpinned by the information that aspartame is fully hydrolysed in the gastrointestinal tract into metabolites that are identical to those absorbed after consumption of common foods, and that no aspartame enters the systemic circulation. The Committee concluded that the data evaluated at the present meeting indicated no reason to change the previously established ADI of 0–40 mg/kg bw for aspartame. The Committee therefore reaffirmed the ADI of 0–40 mg/kg bw for aspartame at the present meeting.

The Committee determined that dietary exposure estimates to aspartame at the mean of up to 10 mg/kg bw per day for children and 5 mg/kg bw per day for adults, and for high dietary exposures up to 20 mg/kg bw per day for children and 12 mg/kg bw per day for adults, were appropriate for the present assessment.

The Committee noted that these dietary exposure estimates do not exceed the ADI. The Committee therefore concluded that dietary exposure to aspartame does not pose a health concern.

After review of the data submitted, the Committee made the following modifications to the specifications monograph for aspartame that was previously revised at the Eighty-second meeting *(7)*: updated the description to include details on manufacturing; added flavour enhancer to the functional uses; replaced the method of assay with a high-performance liquid chromatography method; added a test and specification for “other related impurities”; and removed the test and specification for “other optical isomers”.

An addendum to the toxicology and dietary exposure monograph was prepared. The specifications were revised.

Aspartame references

1. Evaluation of certain food additives (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). Geneva: World Health Organization; 1981. WHO Technical Report Series, No. 669.

2. Ishii H, Koshimizu T, Usami S, Fujimoto T. Toxicity of aspartame and its diketopiperazine for Wistar rats by dietary administration for 104 weeks. Toxicology. 1981;21(2):91–4. doi:10.1016/0300-483x(81)90119-0

3. Soffritti M, Belpoggi F, Degli Esposti D, Lambertini L. Aspartame induces lymphomas and leukaemias in rats. Eur J Oncol. 2005;10:107–16.

4. Soffritti M, Belpoggi F, Degli Esposti D, Lambertini L, Tibaldi E, Rigano A. First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed to Sprague-Dawley rats. Environ Health Perspect. 2006;114:379–85. doi:10.1289/ehp.8711

5. Soffritti M, Belpoggi F, Tibaldi E, Esposti DD, Lauriola M. Life-span exposure to low doses of aspartame beginning during prenatal life increases cancer effects in rats. Environ Health Perspect. 2007;115:1293–7. doi:10.1289/ehp.10271

6. Soffritti M, Belpoggi F, Manservigi M, Tibaldi E, Lauriola M, Falcioni L, Bua L. Aspartame administered in feed, beginning prenatally through life span, induces cancers of the liver and lung in male Swiss mice. Am J Ind Med. 2010;53:1197–206. doi:10.1002/ajim.20896
7. Safety evaluation of certain food additives. Geneva: World Health Organization; 2017. WHO Food Additives Series, No. 73.

Food additives considered for specifications only

Food additive	Specification	Details
Lycopene (synthetic); and lycopene from *Blakeslea trispora*	R	Upon request from the CCFA, the Committee revised the specifications for lycopene (synthetic) (INS 160d(i)) and lycopene from *Blakeslea trispora* (INS 160d(iii)) by replacing "freely soluble in chloroform" with "sparingly soluble in tetrahydrofuran (THF)" in the solubility test, and replacing the "solution in chloroform" test with a "solution in THF" test requirement.
Pentasodium triphosphate	R	At the request of the CCFA, the Committee revised the specifications for pentasodium triphosphate (INS 451(i)) by revising: the assay value for P_2O_5 to not less than 56% and not more than 59% of P_2O_5; the pH value to 9.1–10.2 (1% solution); and the level of lead from 4 mg/kg to not more than 2 mg/kg.
Steviol glycosides	R	The Committee was requested to change the list of non-toxigenic nonpathogenic strains used to facilitate the transfer of glucose to steviol glycosides to: *Anoxybacillus caldiproteoliticus*, *Bacillus licheniformis* and *Bacillus subtilis* in Annex 4: Enzyme Modified Glucosylated Steviol Glycosides of the Ninety-fifth JECFA meeting report. The following text was also added: "The production strain of the enzyme used to facilitate the transfer of glucose to steviol glycosides was incorrectly identified as *Bacillus stearothermophilus.* The revised identification is *Anoxybacillus caldiproteoliticus*."

CCFA: Codex Committee on Food Additives; R: revised specification.

Flavouring agents evaluated by the revised Procedure for the Safety of Evaluation of Flavouring Agents

A. Esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
4-Methylpentyl 4-methylvalerate	2280	N	No safety concern
5-Methylhexyl acetate	2281	N	No safety concern
4-Methylpentyl isovalerate	2282	N	No safety concern
Ethyl 4-methylpentanoate	2283	N	No safety concern
Ethyl 2-ethylbutyrate	2284	N	No safety concern
Ethyl 2-ethylhexanoate	2285	N	No safety concern

N: new specifications.

B. Hydroxy- and alkoxy-substituted benzyl derivatives

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
2-Ethoxy-4-(hydroxymethyl)phenol	2271	N	No safety concern
2-Phenoxyethyl 2-(4-hydroxy-3-methoxyphenyl)acetate	2272	N	No safety concern
3-Phenylpropyl 2-(4-hydroxy-3-methoxyphenyl)acetate	2273	N	No safety concern
Ethyl-2-(4-hydroxy-3-methoxyphenyl)acetate	2274	N	No safety concern
cis-3-Hexenyl salicylate	2275	N	No safety concern

4-Formyl-2-methoxyphenyl 2-hydroxypropanoate	2276	N	No safety concern
2-Hydroxy-4-methoxybenzaldehyde	2277	N	No safety concern
3,4-Dihydroxybenzoic acid	2278	N	No safety concern
3-Hydroxybenzoic acid	2279	N	No safety concern

N: new specifications.

Favouring agents considered for specifications only

Food additive	No.	Specification
(*E*)-2-hexenal diethyl acetal	1383	R
3-Butylidenephthalide	1170	R
1,4-Cineole	1233	R
Octahydrocoumarin	1166	R
3-(*l*-Methoxy)-2-Methylpropane-1,2-diol	1411	R
p-Methane-3,8-diol	1416	R
p-Isopropylacetophenone	808	R
Acetanisole	810	R

R: revised specification.

Annex 2. Recommendations and future work

Withdrawal of specifications without full safety review

The Committee noted that several flavourings have full specifications but are not accompanied by a full safety evaluation. The ninety-sixth Committee recommends the compilation of a list of such flavourings with a view to withdrawing their specifications.

Annex 3. Corrigenda

The Committee discussed the tentative errata. One request was for the amendment of the CAS number for the flavouring agent ethyl levulinate propyleneglycol ketal (No. 1973) for which specifications were prepared at the Seventy-third JECFA meeting, but a full safety evaluation was not completed. The Committee did not consider the request to revise the CAS number and instead withdrew the specifications for No. 1973 as information to allow the completion of the safety review of the flavouring agent has not been provided to the Committee in a timely manner. A recommendation for future work was made to compile a list of flavourings for which a full safety evaluation has not been completed with a view to withdraw such specifications.

The following requests for corrections, submitted to the CCFA, were evaluated at the ninety-sixth meeting of JECFA and found to be necessary. Corrections will be made only in the online database for flavouring specifications.

Flavouring	Original text	Revised text	Additional information
S-Methyl hexanethioate (No. 489)	CAS No.: 20756-86-9 Chemical formula: $C_7H_{14}O_2S$ Molecular weight: 162.24	CAS No.: 2432-77-1 Chemical formula: $C_7H_{14}OS$ Molecular weight: 146.25	Correction to CAS number, chemical formula and molecular weight.
Isopulegol (No. 755)	CAS No.: 89-79-2	CAS No.: 7786-67-6 and CAS No.: 89-79-2	According to the specifications from the Fifty-fifth JECFA meeting,[a] No. 755 is a mixture of isomers. CAS No. 89-79-2 is specifically for the L isomer. CAS No. 7786-67-6 does not specify stereochemistry, and represents the mixture of isomers. Both CAS numbers will be included in the updated specification.
Farnesene (α and β) (No. 1343)	CAS No.: 502-61-4	CAS Nos: 502-61-4 (alpha); 18794-84-8 (beta); 688330-26-9 (mixture)	According to specifications from the Sixty-third JECFA meeting,[b] No. 1343 is a mixture of 3,7,11-trimethyldodeca-1,3,6,10-tetraene and 3-methylene-7,11-dimethyldodeca-1,6,10-triene. CAS No. 688330-26-9 is for a mixture of the two compounds. CAS No. 502-61-4 only represents 3,7,11-trimethyldodeca-1,3,6,10-tetraene. CAS No. 18794-84-8 represents 3-methylene-7,11-dimethyldodeca-1,6,10-triene. All three CAS numbers will be included in the updated specification.
1-Butanethiol (No. 511)	CAS No. 61122-71-2	CAS No. 109-79-5	Original CAS number is incorrect and not related to 1-butanethiol. The correct CAS number is 109-79-5.
8-Ocimenyl acetate (No. 1226)	Missing CAS number	CAS No. 197098-61-6	CAS number missing from specifications. Correct CAS number (197098-61-6) was originally included in table 4 of the report from the Sixty-first JECFA meeting.[c]
Methylthio 2-(propionyloxy) propionate (No. 493)	Missing CAS number	CAS No.: 827024-53-3	Added missing CAS number.
2, 3, or 10-Mercaptopinane (No. 520)	Missing CAS number	CAS Nos: 23832-18-0, 72361-41-2 and 6588-78-9	CAS No. 23832-18-0 corresponds to 2-mercaptopinane; CAS No. 72361-41-2 corresponds to 3-mercaptopinane; CAS No. 6588-78-9 corresponds to 10-mercaptopinane
Methyl 3-methyl-1-butenyl disulfide (No. 571)	Missing CAS number	CAS No.: 233666-09-6	Added missing CAS number.
Potassium 2-(1'-ethoxy) ethoxypropanoate (No. 933)	Missing CAS number Chemical formula: $C_7H_{13}O_4$	CAS No.: 100743-68-8 Chemical formula: $C_7H_{13}O_4K$	Added missing CAS number and revised formula to include potassium.

(-)-Menthol 1- and 2-propylene glycol carbonate (No. 444)	CAS No.: 156329-82-2	CAS No.:	The original CAS No. (156329-82-2) is no longer in the CAS registry. A proposal was made to JECFA to replace it with CAS No. 30304-82-6. However, CAS No. 30304-82-6 does not match the flavouring reviewed by JECFA.
Lactic acid (No. 930)	CAS No.: 598-82-3	CAS Nos: 10326-41-7, 79-33-4 and 50-21-5	The original CAS No. (598-82-3) is no longer valid. The following CAS numbers have been added: CAS No. 10326-41-7 for D-lactic acid; CAS No. 79-33-4 for L-lactic acid; CAS No. 50-21-5 for the mixture of isomers.
Allyl 10-undecenoate (No. 9)	CAS No.: 7439-76-7	CAS No.: 7493-76-7	Typographical error
Geranyl formate (No. 54)	CAS No.: 1005-86-2	CAS No.: 105-86-2	Typographical error
Allyl heptanoate (No. 4)	CAS No.: 142-91-8	CAS No.: 142-19-8	Typographical error
Allyl propionate (No. 1)	CAS No.: 2408-70-0	CAS No.: 2408-20-0	Typographical error
3-Hexenyl formate (*cis* and *trans* mixture) (No. 1272)	CAS No.: 151824	CAS Nos: 33467-73-1, 56922-80-6 and 2315-09-5	The original CAS number is no longer valid. The following CAS numbers were added: CAS No. 33467-73-1 for the *cis* isomer; CAS No. 56922-80-6 for the *trans* isomer; and CAS No. 2315-09-5, which is not specific to double bond geometry.
trans-3-Heptenyl acetate (No. 135)	CAS No.: 34942-91-1	CAS No.: 1576-77-8	The original CAS number is not specific to the double bond geometry. CAS number 1576-77-8 is specific for the *trans* isomer.
Methyl 4-methylvalerate (No. 216)	CAS No.: 2412-24-1	CAS No.: 2412-80-8	Typographical error
2,6-Dimethyloctanal (No. 273)	CAS No.: 1321-89-7 Synonyms: I isodecylaldehyde; isodecanal; 2,6-dimethyl octanoic aldehyde	CAS No.: 7779-07-9 Synonyms: 2,6-dimethyl octanoic aldehyde	Replacement of incorrect CAS number. Removal of two incorrect synonyms.
Menthone-8-thioacetate (No. 506)	Flavouring name: menthone-8-thioacetate CAS No.: 109-79-5	Flavouring name: menthone-8-thioacetate (*cis*- and *trans-)* CAS No.: 94293-57-9	Revision of name to match the flavouring evaluated at the Fifty-third JECFA meeting[d] and replacement of incorrect CAS number.

[a] Evaluation of certain food additives and contaminants (Fifty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). Geneva: World Health Organization; 2001. WHO Technical Report Series, No. 901.

[b] Evaluation of certain food additives (Sixty-third report of the Joint FAO/WHO Expert Committee on Food Additives). Geneva: World Health Organization; 2005. WHO Technical Report Series, No. 928.

[c] Evaluation of certain food additives and contaminants (Sixty-first report of the Joint FAO/WHO Expert Committee on Food Additives). Geneva: World Health Organization; 2004. WHO Technical Report Series, No. 922.

[d] Evaluation of certain food additives and contaminants (Fifty-third report of the Joint FAO/WHO Expert Committee on Food Additives). Geneva: World Health Organization; 2000. WHO Technical Report Series, No. 896.

COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives
96th Meeting, Geneva, 27 June – 6 July 2023

This document contains food additive specification monographs, analytical methods, and other information prepared at the ninety-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which was held in Geneva from 27 June to 6 July 2023. 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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