Food and Agriculture Organization of the United Nations

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Residue Monograph prepared by the meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), 82nd meeting 2016

Rosemary Extract
(Tentative)

This monograph was also published in: *Compendium of Food Additive Specifications. Joint FAO/WHO Expert Committee on Food Additives (JECFA), 82nd meeting 2016. FAO JECFA Monographs 19*

ROSEMARY EXTRACT
(TENTATIVE)

New specifications prepared at the 82nd JECFA (2016), published in FAO JECFA Monograph 19 (2016). A temporary ADI of 0-0.3 mg/kg bw was established at the 82nd JECFA (2016). .
Information required:
Validation data for residual solvents using Vol 4 Method "Determination of residual solvents in annatto extracts (solvent extracted bixin and norbixin), tentative method (June 2013)".

SYNONYMS INS No. 392

DEFINITION Rosemary extract is obtained from ground dried leaves of *Rosmarinus officinalis* L using food-grade solvents, namely, acetone or ethanol. Solvent extraction is followed by filtration, solvent evaporation, drying and sieving to obtain a fine powder. Additional concentration and/or precipitation steps followed by deodorisation, decolourisation and standardisation using diluents and carriers of food grade quality maybe included to produce the final product. Rosemary extract is characterised by its content of phenolic diterpenes, carnosic acid and carnosol, the principal antioxidative agents. Other antioxidant components present include triterpenes and triterpenic acids. Rosemary extract is identified by the total content of carnosol and carnosic acid as a ratio of reference volatile compounds which are responsible for flavour.

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

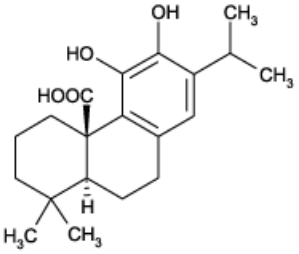
Chemical names Carnosic acid: 4a(2H)-Phenanthrenecarboxylic acid, 1,3,4,9,10,10a-hexahydro-5,6-dihydroxy-1,1-dimethyl-7-(1-methylethyl)-, (4aR-trans)-

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

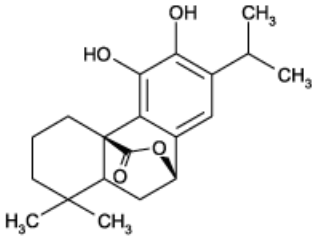
C.A.S. numbers Extract of rosemary: 84604-14-8
Carnosic Acid: 3650-09-7
Carnosol: 5957-80-2

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

Structural formula



Carnosic Acid



Carnosol

Formula weight	Carnosic acid: 332.43 Carnosol: 330.42
Assay	Not less than 5% of the total carnosic acid and carnosol.

DESCRIPTION Beige to light brown powder.

FUNCTIONAL USES Antioxidant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Insoluble in water; soluble in oil
Antioxidants/Reference Volatiles Ratio	Total % of carnosic acid and carnosol /Total % of reference volatiles: (-)-borneol, (-)-bornyl acetate, (-)-camphor, 1,8-Cineole (eucalyptol) and verbenone: not less than 15 See description under TESTS

PURITY

Loss on drying (Vol. 4)	Not more than 5% (80° under vacuum, 4 hours). Test 1 g of sample
Residual solvents (Vol. 4)	Acetone: Not more than 50 mg/kg Ethanol: Not more than 500 mg/kg Determine residual solvents following the method “Determination of residual solvents in annatto extracts (solvent extracted bixin and norbixin), tentative method (June 2013)”, or any other suitable method with similar performance characteristics.
Arsenic (Vol. 4)	Not more than 3 mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under “General Methods, Metallic Impurities”).
Lead (Vol. 4)	Not more than 2 mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under “General Methods, Metallic Impurities”).

TESTS

IDENTITY TESTS

Antioxidant/Reference Volatiles Ratio	Antioxidant: Total % of carnosic acid and carnosol (comes from METHOD OF ASSAY)

Reference Volatile Ratio: Total % w/w of (-)-borneol, (-)-bornyl acetate, (-)-camphor, 1,8-Cineole (eucalyptol) and verbenone is determined using GC-MSD

Equipment and Reagents:

Equipment
GC/MS chromatograph with autosampler

Solvent: Tetrahydrofuran (THF) from Carlo Erba, HPLC grade ref. 412452000 or equivalent

Standards
(-)-Borneol from Fluka (Sigma-Aldrich) ref. 15598 or equivalent
(-)-Bornyl acetate from Fluka (Sigma-Aldrich) ref. 45855 or equivalent
(-)-Camphor from Fluka (Sigma-Aldrich) ref. 21293 or equivalent
1,8-Cineole (Eucalyptol) (from Aldrich (Sigma-Aldrich) ref. C80601 or equivalent
Verbenone from Fluka (Sigma-Aldrich) ref. 94882

Internal Standard: Heptanon-4 from Fluka (Sigma-Aldrich) ref. 43570 or equivalent

Preparation of Internal Standard Solution (ISS)
Accurately weigh 20 mg of 4-heptanon in a 50 ml volumetric flask. Dilute to volume with THF and homogenise the solution. The concentration of the Internal Standard Solution is approximately 400 µg/ml.

Preparation of Sample Solution:
Accurately weigh 2.5 g of the sample in a 10 ml volumetric flask. Add 500 µl of the Internal Standard Solution, and dilute to volume with THF. Sonicate 5 min for liquid samples or 10 min for powder extracts. Filter an aliquot through 0.45 µm filter.

Preparation of Standard Solutions (SS): Accurately weigh 20 mg of each Standard into a 50 ml volumetric flask. Dilute to volume with THF and homogenise the solution. The concentration of each Standard in the Standard Solution is approximately 400 µg/ml.

Preparation of Standard solutions for standard Curve (WSS):

Standard	WSS, µg each/ml	SS, µl	ISS, µl	THF, µl	Total Volume, µl
Level 0	0	0	100	1900	2000
Level 1	Approx. 5	20	100	1880	2000
Level 2	Approx. 20	100	100	1800	2000
Level 3	Approx. 50	200	100	1700	2000
Level 4	Approx. 100	500	100	1400	2000
Level 5	Approx. 200	1000	100	900	2000

Procedure: Load the WSS and the Sample solution, onto the autosampler of the GC/MS using following conditions. Inject in duplicate 1 µl of WSS.

GC conditions:

Column: FactorFour Capillary column VF-5ms 30M x 0.25 mm Ft = 0.25.
Carrier gas: He; flow rate 1 mL/min with constant flow
Split: 100/1

Temperature Program:

Temperature [°]	Rate [°/min]	Hold [min]	Total [min]
70	0.0	1.00	1.00
130	5.0	0.00	12.00
240	10.0	1.00	25.00

Injector: 250°
Temperature: Manifold: 150°, Transfer line: 240°, Quad: 230°

Auto sampler specifications
Syringe: 10 µl
Injection volume: 1 µl
Rinse: pre-clean solvent: 5 times, pre-clean sample: 5 times, post-clean solvent: 5 times
Washing solvent: Tetrahydrofuran

MS Acquisition:

Segments / Names	Ionization Scan type	Target TIC [counts]	Running Time [min]	Ion [m/z]
1	Off	-	0.00 – 3.00	-
2 / Heptanon (IS)	EI - SIS	10000	3.00 – 3.50	43 71 114
3	Off	-	3.50 – 5.00	-
4 / 1,8-Cineole (Eucalyptol)	EI - SIS	10000	5.00-6.50	43 139 154
5	Off	-	6.50 – 8.00	-
6 / Camphor, Borneol, Verbenone	EI - SIS	10000	8.00 – 11.00	95 107 110 135 152
7 / Bornyl acetate	EI - SIS	10000	11.00 – 13.00	95 154 196

Calculation:

Calculate the calibration curve by linear regression analysis for each individual volatile standard using the equation

$$Area = a \times (c \times P) + b$$

where:

Area is Individual Volatile Standard peak area in WSS chromatogram
c is Concentration [µg/ml] of Individual Volatile Standard
a is Slope of the regression line for Individual Volatile Standard
b is y-intercept of the regression line for Individual Volatile Standard
P is Purity of Individual Volatile Standard given by certificate of analysis from Supplier

Calculate the concentration of the volatiles in the sample using the following formula:

$$[\text{Compound}],\ mg/kg = \frac{A_S - y}{m \times A_{IS}} \times \frac{V}{W} \times C_{IS}$$

where

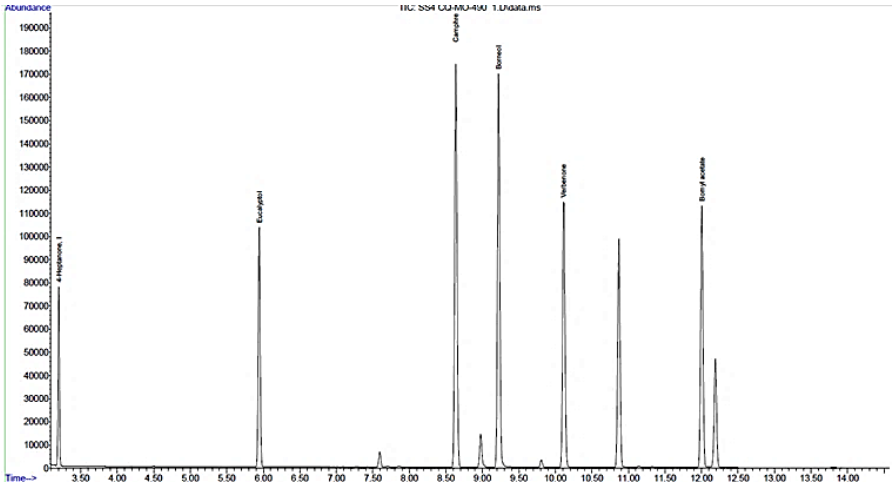
A_S is Individual Volatile peak area in Sample Solution chromatogram
y is y-intercept of Individual Volatile calibration curve
m is Slope of Individual Volatile calibration curve
A_{IS} is Peak area of Internal standard in Sample Solution chromatogram
V is Dilution volume (ml)
W is Weight of sample (g)
C_{IS} is Concentration of the Internal Standard Solution

With the software Varian MS Workstation version 6.9 Service Pack 1, report the following settings during the review:
Amount Standard is 1
Multiplier is Dilution volume [ml]
Divisor is Weight of sample [g]

The reported result, Total Volatiles, is the sum of each Individual Volatile result.

The limit of quantification (LOQ) is 20 ppm and the limit of detection (LOD) is 2 ppm.

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



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

HPLC conditions:
Detector: Ultraviolet (UV) 230 nm
Column: ZORBAX SB-C18 (Agilent Technologies) or equivalent; 4.6-mm x 250-mm containing 5-µm porous silica microparticles chemically bonded to octadecylsilane
Flow rate: 1.5 mL/min
Temperature: 25°
Injection size: 5 µl

Preparation of Mobile Phase:

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

Preparation of Solutions:

Preparation of Phosphoric Acid Solution: Dissolve 0.5 ml of phosphoric acid, ACS grade, in 100 ml of Methanol, HPLC grade.

Reference Standard Solution: Dissolve 200-500 µg/ml of USP Powdered Extract of Rosemary RS in Phosphoric Acid Solution. Sonicate for 5 min; filter through a 0.45-µm filter.

System Suitability Standard Solution: Dissolve 100 µg/ml of USP Carnosic Acid RS in Phosphoric Acid Solution. Sonicate for 5 min; filter through a 0.45-µm filter.

Sample Solution: Dissolve 500 µg/ml of the sample in Phosphoric Acid Solution. Sonicate for 5 min; filter through a 0.45-µm filter

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

Calculations:
System Suitability Requirements:

Tailing Factor for the Carnosic Acid Peak in the chromatogram is 0.90 to 1.30

The RSD for the Carnosic Acid peak response on replicate injections is not more than 2%

% Carnosic Acid or Carnosol in sample:

$$\%\ \text{Carnosic Acid or Carnosol} = \frac{A_{Analyte}}{A_{Std}} \times \frac{C_{Std}}{C_u} \times F \times \frac{MW1}{MW2} \times 100$$

where

$A_{analyte}$ is peak area of the analyte of interest (carnosic Acid or Carnosol) obtained from the chromatogram of the Sample Solution

A_{Std} is peak area of carnosic acid obtained from the chromatogram of System Suitability Standard Solution

$C_{Carnosic\ Acid\text{-}SS}$ is concentration of carnosic acid in the System Suitability Standard Solution (µg/mL)

$C_{Carnosic\ Acid}$ is concentration of carnosic acid in Sample Solution (µg/mL)

F is Relative Response Factor of the analyte of interest (1.00 for carnosic acid; 0.92 for carnosol)

MW1 is molar weight of Carnosic acid (332.4 g/mol)

MW2 is molar weight of Carnosol (330.4 g/mol)

Add the individual percentages of Carnosic acid and Carnosol calculated using the above formula, and report the result as the total content of Carnosic acid and Carnosol in the sample taken.