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ROSEMARY EXTRACT

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ROSEMARY EXTRACT

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

SYNONYMS INS No. 392

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

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

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

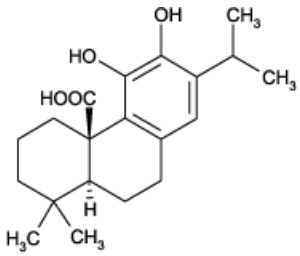
Carnosol: 2H-9,4a-(Epoxymethano)phenanthren-12-one, 1,3,4,9,10,10a- hexahydro-5,6-dihydroxy-1,1-dimethyl-7(1-methylethyl), (4aR- (4aα,9α,10aβ))-

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

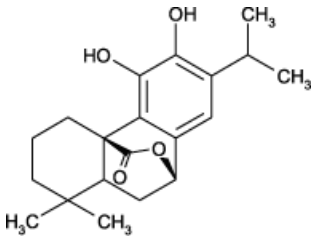
Carnosic acid: 3650-09-7
Carnosol: 5957-80-2

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

Structural formula



Carnosic acid



Carnosol

Formula weight Carnosic acid: 332.43
Carnosol: 330.42

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

DESCRIPTION Beige to light brown powder.

FUNCTIONAL USES Antioxidant

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Insoluble in water; soluble in vegetable and animal fats and oils.
Antioxidants/Reference Volatiles Ratio	% Total of carnosic acid and carnosol/%Total of reference volatiles: not less than 15 See description under TESTS

PURITY

Loss on drying (Vol. 4)	Not more than 5% (80° under vacuum, 4 h, 1 g).
Residual solvents (Vol. 4)	Acetone: Not more than 50 mg/kg See description under TESTS
Arsenic (Vol. 4)	Not more than 3 mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under "General Methods, Metallic Impurities").
Lead (Vol. 4)	Not more than 2 mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4 (under "General Methods, Metallic Impurities").

TESTS

IDENTITY TESTS

Antioxidant/Reference Volatiles Ratio	% Antioxidants (total of carnosic acid and carnosol) is determined by the Method of Assay.

Reference Volatiles [(-)-borneol, (-)-bornyl acetate, (-)-camphor, 1,8-cineole and verbenone] is determined using GC-MS.

Equipment and Standards:
GC-MS consisting an autosampler and FactorFour VF-5MS (30m x 0.25 mm x 0.25 µm) capillary column, or equivalent.
(-)-Borneol, Supelco. 15598 or equivalent
(-)-Bornyl acetate,Sigma-Aldrich 45855 or equivalent
(-)-Camphor, Supelco 21293 or equivalent
1,8-Cineole (Eucalyptol), Sigma-Aldrich C80601 or equivalent
Verbenone, Supelco 94882 or equivalent

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

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

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

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

Preparation of working standard solutions (WSS):

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

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

GC conditions:
Carrier gas: Helium
Flow rate 1 ml/min with constant flow
Injection volume: 1 µl
Split: 100:1

Injector: 250°
Temperature: Ion source: 150°, Transfer line: 240°, Quadrupole: 230°

Temperature Program:

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

MS Acquisition:

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

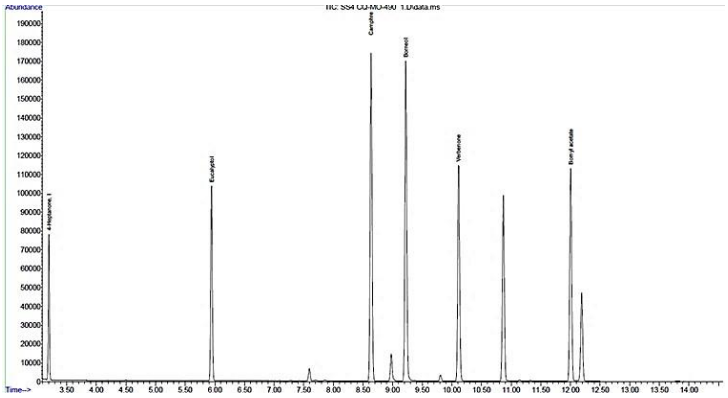
Analyze using the analytical condition as described above. Measure the peak area of each standard and 4-heptanone (IS). Construct internal standard curves by linear regression analysis for each standard. Calculate the concentration of each volatile as follows:

[Compound], mg/kg = A/a × V/W

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

Calculate the sum of 5 volatiles and report.

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



Residual solvents

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

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

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

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

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

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

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

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

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

Calculation:

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

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

METHOD OF ASSAY

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

Equipment and Reagents:
HPLC consisting of a UV detector and Autosampler.
Column: Chemically bonded octadecylsilane column: ZORBAX SB-C18 (250 mm x 4.6 mm ID x 5-µm), Agilent Technologies or

equivalent;
Detector Wavelength: 230 nm
Flow rate: 1.5 ml/min
Temperature: 25°
Injection volume: 5 µl
Reference Standard: USP Powdered Extract of Rosemary RS
Phosphoric acid, ACS grade
Methanol, HPLC grade
Water, HPLC grade

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

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

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

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

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

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

Calculations:
System Suitability Requirements:
Tailing Factor for the carnosic acid peak in the chromatogram is 0.90 to 1.30.

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

% Carnosic acid or Carnosol in sample:

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

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

where

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

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

CStd is concentration of carnosic acid in the System Suitability Standard Solution (µg/ml)

CU is concentration of Sample Solution (µg/ml)

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