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(54) Titre : PROCEDE DE PREPARATION DE L'ACIDE CARNOSIQUE ET UTILISATION DE CET ACIDE POUR SES PROPRIETES ANTICANCEROGENES ET ANTIVIRALES
(54) Title: PROCESS FOR THE PREPARATION OF CARNOSIC ACID AND THE USE OF THE ACID FOR ITS ANTICARCINOGENIC AND ANTIVIRAL PROPERTIES

(57) **Abrégé/Abstract:**

The invention relates to a process for the preparation of carnosic acid from rosemary or sage, in which the spice is extracted with a non-polar solvent or with a mixture of highly apolar solvents, the extract obtained is subjected to a selective adsorption treatment on a solid support, the carnosic acid is desorbed with a polar solvent or with a mixture of highly polar solvents and the solvent is evaporated.

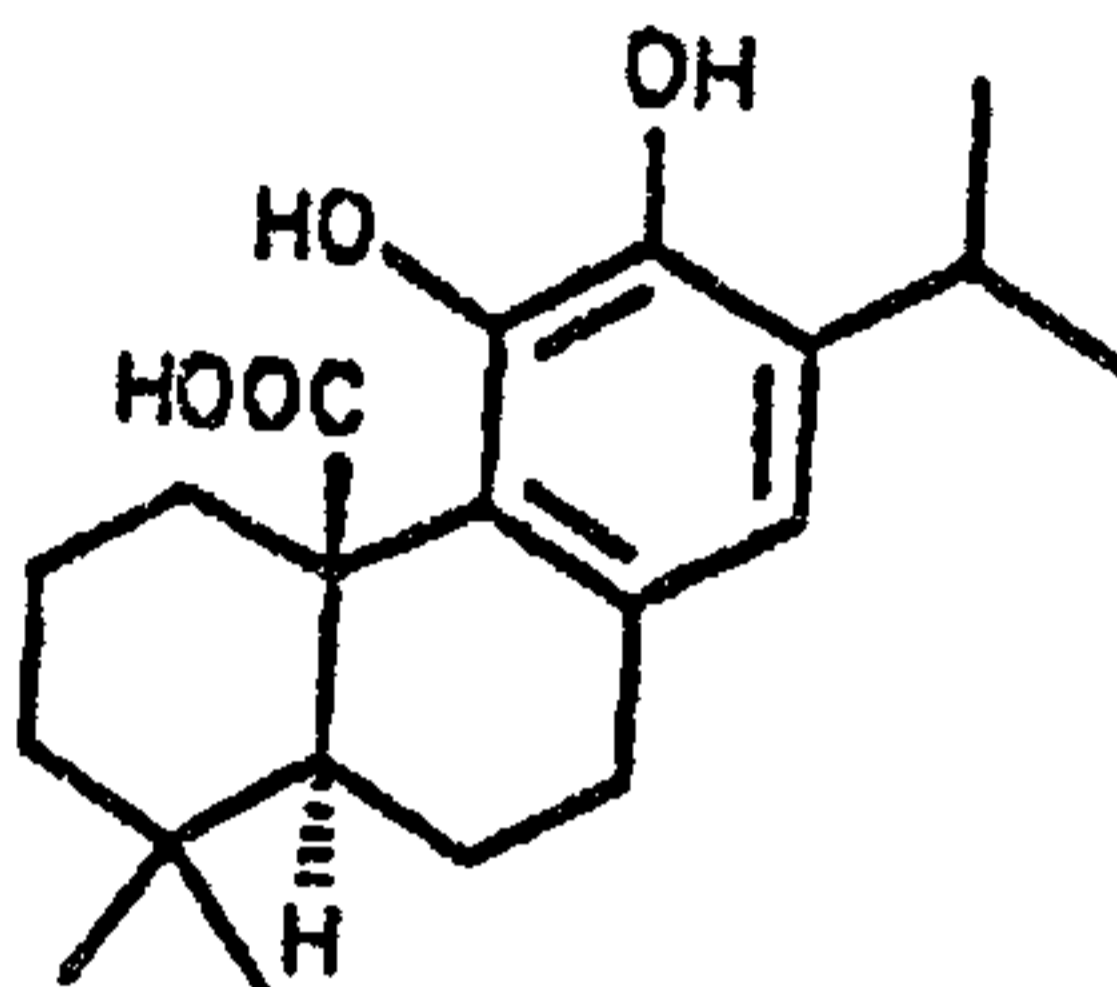
Abstract

A process for the preparation of carnosic acid and the use of the acid for its anticarcinogenic and antiviral properties

The invention relates to a process for the preparation of carnosic acid from rosemary or sage, in which the spice is extracted with a non-polar solvent or with a mixture of highly apolar solvents, the extract obtained is subjected to a selective adsorption treatment on a solid support, the carnosic acid is desorbed with a polar solvent or with a mixture of highly polar solvents and the solvent is evaporated.

This invention relates to a process for the preparation of carnosic acid from rosemary or sage. The invention also relates to the use of carnosic acid for its anticarcinogenic and antiviral properties.

5 Carnosic acid is a phenolic diterpene which corresponds to the empirical formula $C_{20}H_{28}O_4$ and which has the following structure



It is a constituent of the species *Salvia* and *Rosmarinus* where it is mainly to be found in the leaves. It was discovered for the first time by Linde in *Salvia officinalis* [Helv. Chim. Acta 47, 1234 (1962)] and by Wenkert et al. in *Rosmarinus officinalis* [J. Org. Chem. 30, 2931 (1965)]. It was then positively identified in various other species of sage, such as for example *Salvia canariensis* [Savona and Bruno, J. Nat. Prod. 46, 594 (1983)] or *Salvia willeana* [de la Torre et al., Phytochemistry 29, 668 (1990)]. It is also present in *Salvia triloba* and *Salvia sclarea*.

30 Carnosic acid is a powerful antioxidant [Brieskorn and Dömling, Z. Lebensm. Unters. Forsch. 141, 10 (1969)] and, according to a number of Russian works where it bears the name salvine, an antibiotic against *Staphylococcus aureus* [CA 86, 117602r; 90, 49011b; 97, 67513r, 69163a, 69164b; 104, 221930w; 111, 130594t] and against certain microorganisms responsible for dental caries and bad breath [CA 35 97, 84835q]. In connection with this latter property, it

is mentioned in the prior art for the production of dentifrices and mouthwashes [JP 59 103 665, Lion Corp.].

Despite this large number of references, the isolation on a preparative scale of carnosic acid from rosemary or sage has never been described either by Linde
5 or Wenkert, where its existence is indirectly proved, or by those who subsequently identified it in various species of sage.

By contrast, many other phenolic diterpenes similar in
10 structure to carnosic acid have been isolated from one or other of the two species *Salvia* and *Rosmarinus*. They include carnosol [Brieskorn et al., Chem. Ber. 95, 3034 (1962); J. Org. Chem. 29, 2293 (1964)] and, more recently, rosmanol [Inatani et al., Agric. Biol. Chem. 46, 1661
15 (1982)] or rosmaridiphenol [Houlihan et al., J. Am. Oil Chem. Soc. 61, 1036 (1984)], the last two having been patented as new antioxidant principles of rosemary [US-PS 4,450,097, Nakatani et al., Lion Corp., Tokyo; US-PS 4,638,095, Chang et al., Research Corp., N.Y.].

20 Although all these compounds are worthy of interest as antioxidants, it nevertheless remains that, compared with carnosic acid, they have certain unfavourable features: their content in rosemary or sage is much lower than that of carnosic acid.

25 Dried leaves of rosemary or sage (species *Salvia officinalis*) contain between 1.5 and 2.5% carnosic acid and only about 0.3-0.4% carnosol. Rosmanol and rosmaridiphenol are present in undetectable concentrations. Accordingly, from the point of view of the economy of a production
30 process, carnosic acid has an indisputable advantage. According to the data disclosed in US-PS 4,450,097 for example, it may be calculated that the yield of rosmanol isolated from rosemary is only 0.01%.

35 As was demonstrated by Wenkert et al., carnosol is an oxidative artefact of carnosic acid. This oxidation takes

place in the presence of oxygen both after the harvesting
of rosemary or sage in the leaves left to dry in air (it
can incidentally be demonstrated that the freshly cut
leaves of rosemary do not contain carnosol) and when the
5 leaves are subjected to extraction with solvents or when
the extracts themselves are subjected to conventional
operations of fractionation, enrichment and purification.
There is every reason to assume that rosmanol, which has
been identified in a rosemary fraction subjected to an
10 alkaline treatment, is itself a subsequent product of the
oxidation of carnosic acid, as Wenkert et al. already
suggested; the same may also be reasonably assumed of
rosmaridiphenol. Carnosic acid is therefore the only
phenolic diterpene present in the native state in rosemary
and sage and, accordingly, has the sole right to be called
15 a natural product.

Some methods for the preparation of carnosic acid by
chemical synthesis have also been proposed in the litera-
ture by W.L. Meyer et al. [Tetrahedron Letters 1966, 4261;
20 1968, 2963; J. Org. Chem. 41, 1005 (1976)]. However, the
syntheses involved are long and complex and, for economic
reasons, cannot be applied to an industrial process. In
addition, these syntheses lead to racemic mixtures of
carnosic acid precursors and not to the pure enantiomers.
25 It should also be pointed out that these works stop at the
preparation of carnosic acid precursors and omit to de-
scribe the final preparation step(s). Another method of
obtaining carnosic acid has been described in the litera-
ture by Brieskorn and Dömling [Arch. Pharm. 302, 641
30 (1969)], comprising the catalytic reduction of carnosol.
Once again, the application of this process on a large
scale could not be envisaged on account of the non-avail-
ability of carnosol.

The problem addressed by the present invention was to
35 provide a process for the extraction of carnosic acid from

rosemary or sage which would be economical, workable on an industrial scale and would enable pure carnosic acid to be obtained in a high yield.

5 The present invention relates to a process for the preparation of carnosic acid from rosemary or sage, in which the spice is extracted with a non-polar solvent or with a mixture of highly apolar solvents, the extract obtained is subjected to a selective adsorption treatment on a solid support, the carnosic acid is desorbed with a
10 polar solvent or with a mixture of highly polar solvents and the solvent is evaporated.

Carnosic acid, like any molecule of the catechol (ortho-diphenol) type, is a reactive compound which is highly sensitive to oxidation and, hence, to all the
15 operations typically carried out to isolate natural substances (extraction, liquid-liquid separation, chromatographic fractionation, etc.). By contrast, it has been found that, in its purified and crystallized form, carnosic acid is stable and can be handled without excessive precau-
20 tions, although its production in crystallized form can only be carried out from a preparation of vegetable material already enriched with carnosic acid.

The process according to the present invention enables the chemical integrity of carnosic acid to be preserved
25 because it only comprises two treatment steps which are sparing of the basic product and which are selective with respect to the carnosic acid.

The process according to the present invention utilizes two physico-chemical properties of carnosic acid. On
30 the one hand, the molecule comprises reputedly polar functions, such as the carboxylic acid and phenol function. On the other hand, the remainder of its skeleton, made up essentially of hydrocarbons, provides with a relatively apolar character by comparison with all other phenolic
35 compounds, such as the flavonoids or hydroxybenzoic or

hydroxycinnamic acids which are present in abundance in plants such as sage or rosemary. Thus, the process comprises only two steps, namely:

- 5 1. Extraction of the vegetable material (sage or rosemary) in an essentially apolar solvent, so that the carnosic acid and the apolar compounds of the vegetable material enter the extract;
- 10 2. Selective adsorption of the carnosic acid present in the extract onto a solid support selected for its affinity and for its selectivity with respect to the polar functions of carnosic acid, followed by desorption of the carnosic acid from the support with a
15 polar solvent.

In the first step, leaves of rosemary or sage are extracted with an essentially apolar solvent so that, besides all the other apolar or substantially apolar
20 compounds in the leaves of these plants, such as the components of the essential oil, lipids, waxes, chlorophyll-containing pigments and certain triterpenes, the extract obtained contains carnosic acid as virtually the only phenolic compound entering the extract. The degree of
25 extraction of the carnosic acid is between 70 and 100% while its content in the extract is between 13 and 25%.

In the second step, the extract obtained in the first step is treated with an adsorbent solid material which has an affinity for the compounds containing polar functions or
30 a particular selectivity with respect to phenolic compounds, such as for example silica gel, aluminium oxide as inorganic absorbent materials or polyamide, polyvinyl pyrrolidone as examples of organic absorbent materials. During this treatment, the carnosic acid is adsorbed with
35 a high affinity or selectivity on the adsorbent, the other

constituents of the extract essentially remaining in the liquid phase. After removal of the liquid phase, the carnosic acid is desorbed from the adsorbent by contact with a polar solvent which, after evaporation, gives a residue containing between 65 and 95% carnosic acid which, if necessary, may be further purified by recrystallization.

The yield of carnosic acid in the process according to the invention, based on the content of this acid in the vegetable starting material, is between 60 and 90%.

The vegetable starting material and the size of the vegetable particles play a part in the extraction yield of the carnosic acid. In principle, it is preferred to use finely ground rosemary or sage, fine grinding generally giving better yields. Although it is of greater advantage to start from the whole spice, the residues obtained after distillation of the essential oil with water may also be used although it is pointed out that, since this operation is accompanied by significant losses of carnosic acid in the form of its oxidation product, the process for obtaining carnosic acid would be economically less favourable. The residues obtained after extraction of the essential oil with a solvent in the supercritical phase, such as supercritical CO₂ for example, may also be used as starting material. In this case, the residues will be of better quality from the point of view of their carnosic acid content because this method of obtaining the essential oil provides for better management the valuable compounds of the vegetable material.

Generally, carnosic acid can be extracted more easily from sage than from rosemary. The reason for this is undoubtedly the fact that the sage leaves are less fibrous than the rosemary leaves. However, rosemary is far more widespread than sage and therefore represents a less onerous starting material which is more readily available in large quantities.

Irrespective of the method of extraction or the vegetable material used, it is important for the reasons mentioned above that the extraction solvent should be as apolar as possible. The extraction solvent will be selected from the solvents typically used as extraction solvents, i.e. solvents of relatively low boiling point, such as saturated, optionally branched hydrocarbons, for example hexane, pentane, heptane, 2-methyl butane, 2-methyl hexane and cyclohexane or mixtures of saturated hydrocarbons (petroleum ethers), or aromatic hydrocarbons such as toluene, or even binary mixtures of one of the above-mentioned solvents in a large excess with a chlorine-containing solvent (for example methylene chloride, chloroform, chloroethylenes) or oxygen-containing solvent, such as an ether (for example diethyl ether), or a ketone (for example acetone) or an ester (for example ethyl acetate) or an alcohol (for example ethanol, methanol, etc.). For example, it is possible to use a mixture of petroleum ethers, toluene or hexane with dichloromethane or ethanol in a ratio of 99:1 to 90:10. In the case of sage for example, the carnosic acid can be quantitatively extracted with a solvent of the saturated hydrocarbon type. With all the other combinations of vegetable material and extraction variant, it will be necessary to use a solvent of slightly increased polarity if a high degree of extraction of carnosic acid is to be obtained.

The step in which the spice is extracted may be carried out either by solvent extraction of the vegetable material, for example in an extractor of the Soxhlet type (variant I) or by charging, i.e. by immersion of the vegetable material in the solvent (variant II), or by percolation or by extraction in a pulsed column or by any other known solvent-based solid extraction technique. However, it is the first two variants that are exemplified in the following. Variant I gives better carnosic acid

extraction yields than variant II although variant II is more convenient to use where large quantities of vegetable material have to be extracted.

5 Extraction by solvent extraction is carried out in an extractor of the Soxhlet type consisting of a reflux apparatus (flask + condenser) which contains the extraction solvent and between the two parts of which is arranged a siphon extractor equipped with a porous cartridge containing the vegetable material to be extracted. The vapours of
10 the solvent heated to the boiling temperature in the flask pass along the extractor into a tube provided for this purpose and condense into liquid form on arrival in the condenser. The condensed solvent drops back into the extraction cartridge which it fills. When the liquid level
15 in the extractor reaches the level of the siphoning tube, the extractor discharges its liquid contents which return to the flask, entraining a certain quantity of dissolved vegetable material. The process as a whole may be defined as an extraction cycle.

20 In this method of extraction, the preferred solvents for sage are light acyclic hydrocarbons, for example petroleum ether, preferably having a boiling temperature in the range from 40 to 60°C, and for rosemary the same solvents or a binary combination of one of them with a
25 chlorine-containing solvent, for example dichloromethane, in a ratio by volume of 99:1 to 9:1. The number of extraction cycles applied to the material to be extracted is of the order of 2 to 20.

Where extraction is carried out in batches, the
30 vegetable material and the extraction solvent are contacted in a conventional reactor and the mixture is stirred throughout the operation. The proportions of solvent and vegetable material are preferably in a volume/weight ratio of 5:1 in the case of rosemary and 10:1 in the case of sage
35 (the largest quantity of solvent in the case of sage is

determined by the much lower mass by volume of sage compared with that of rosemary). Generally, the vegetable material will be subjected to two or three successive extractions, the liquid phase and the solid phase being separated by filtration or centrifugation between each operation. The duration of each extraction is of the order of 30 minutes to 2 hours and generally 1 hour. The preferred solvents in this method of extraction for both the vegetable materials sage and rosemary are aromatic hydrocarbons, preferably toluene, or a binary combination of a light acyclic hydrocarbon, for example petroleum ether or hexane, with an oxygen-containing solvent, preferably ethanol or methanol, in ratios by volume of 99:1 to 9:1.

The first extraction step is carried out at a temperature in the range from 20 to 50°C.

In the second step, the vegetable extract obtained in the first step is treated with a solid adsorbent material. The carnosic acid of the extract is selectively adsorbed onto the solid material and, after the liquid phase has been removed, is recovered in concentrated form by desorption with a pure polar solvent, such as acetone, methanol, ethanol or ethyl acetate, or a mixture of one of these solvents in a large excess with an apolar or weakly polar solvent.

In principle, the liquid extract may be treated with any solid adsorbent material having an affinity or selectivity for compounds of this type. A list - by no means complete - of the adsorbent materials which may be used for this purpose has already been given in the foregoing. These materials are essentially those which are typically used in separation techniques based on liquid chromatography.

Among the adsorbent materials of economic interest, polyamide or any similar polymer, such as polyvinyl pyrrolidone, are preferred materials for adsorption of the

carnosic acid because they show a remarkable affinity for phenolic compounds (cf. for example "The Flavonoids" , Harbone et al., eds., Chapman and Hall, 1975, Chap. 1, p. 11). In addition, they are chemically inert supports which are in no danger of significantly altering the compounds with which they are contacted.

In practice, the liquid vegetable extract of the first step of the process may be contacted as such with the adsorbent material. If necessary, the extract may be filtered beforehand to remove small quantities of precipitated solid materials which may have formed during or after extraction. In practice, however, it is of advantage to concentrate the liquid extract before it is contacted with the adsorbing agent to promote the passage of the carnosic acid from the dissolved state into the adsorbed state. In numerous cases when the concentration step is accompanied by the formation of a solid precipitate, the solvent is preferably removed completely from the extract and the residue taken up in a second solvent selected for its ability readily and completely to dissolve the carnosic acid of the extract. In practice, it has been found that solvents of the aromatic hydrocarbon or chlorine-containing type are suitable for this operation, toluene and dichloromethane being the preferred solvents.

The liquid extract may be contacted with the adsorbent material by immersion or by passage of the extract through a column filled with the adsorbent material. The second alternative is more effective and may be carried out as follows: The liquid extract is introduced at the head of a column filled with the adsorbent material conditioned with the same solvent as that of the extract. When the extract is in contact with the adsorbent material, the column is washed with fresh solvent until all the materials of the extract have been eliminated except for the carnosic acid which remains adsorbed on the support. The carnosic

acid is then desorbed from the adsorbent material by passage through the column of a medium-polarity to polar solvent, for example a mixture of dichloromethane or toluene with ethanol or methanol. The solvent is removed from the eluate and the residue may even be subsequently purified by recrystallization to achieve the desired degree of purity of carnosic acid.

The present invention also relates to the use of carnosic acid for the preparation of a composition or diet intended for the prevention or treatment of cancer.

Certain chemical compounds have properties which, directly or indirectly, reduce or suppress the mutagenic activity induced by other chemical products. Thus, it has been shown that free radicals are capable of inducing a large number of different lesions in DNA and that they are also involved in the process of cancer, ageing and cardiovascular disease. Carnosic acid has an inhibiting effect on the degradation of DNA caused by the free radicals so that it may be considered for use in the prevention and treatment of cancerous or cardiovascular diseases.

The dietetic or pharmaceutical compositions may be made up in various forms adapted to the method of administration, for example oral, enteral or parenteral. For example, they may be made up as capsules, gelatin-coated tablets or syrups. In the case of enteral or parenteral administration, the compositions will be made up in the form of physically and chemically stabilized solutions or emulsions.

Physiological doses may be administered in the prevention or, optionally, the treatment of certain forms of cancer and cardiovascular disease.

In addition, the carnosic acid may be used for the preparation of a composition intended for the treatment of herpes which is a viral disease. This composition may be presented in various forms adapted to the method of admin-

istration, for example oral or topical application. For example, the composition may be presented in the form of capsules, gelatin-coated tablets or ointments. Physiological doses are administered for the treatment of this disease.

The invention is illustrated by the following Examples.

Examples 1 to 21

Table 1 below illustrates the results obtained from a series of tests on the extraction of rosemary and sage by extraction variants I and II using the solvents described above. It can be seen that the higher the polarity of the solvent (column 5), the better the extraction yield of carnosic acid (column 8), but the lower the concentration of carnosic acid in the extract (column 7) and the larger the amount of ballast in the extract, the ballast being formed in particular by other phenolic compounds than carnosic acid which are therefore capable of interfering negatively with the carnosic acid in the second step of the process.

1	2	3	4	5	6	7	8	9
Ex-ample	Start. mat.	CA con- tent (%)	Extrac- tion variant	Extrac- tion solvent	Extrac- tion yield (%)	CA con- tent in extract (%)	CA ex- tract- ion- yield (%)	QI of extr. (max = 100)
1	Rosemary	1.85	I	P	9.1	16	79	68
2	Rosemary	1.85	I	P/D 9/1	10.1	16	87	76
3	Rosemary	1.85	I	P/D 9/1	11.1	15	91	74
4	Sage	2.50	I	P	10.0	25	100	100
5	Rosemary	1.80	II	P	5.8	13	42	30
6	Rosemary	1.80	II	T	9.6	14	75	58
7	Rosemary	1.80	II	P/E 98/2	8.2	16	73	65
8	Rosemary	1.80	II	P/E 99/1	7.2	15	60	50
9	Rosemary	1.80	II	P/E 98/2	8.2	15	68	57
10	Rosemary	1.80	II	P/E 95/5	11.4	13	82	59
11	Rosemary	1.80	II	P/A 98/2	6.4	13	46	33
12	Rosemary	1.80	II	P/A 95/5	7.6	14	59	46
13	Rosemary	1.80	II	P/A 9/1	10.0	12	67	44
14	Sage	1.80	II	P	6.0	16	53	47
15	Sage	1.80	II	P/E 99/1	9.0	15	75	63
16	Sage	1.80	II	P/E 98/2	9.0	15	75	63
17	Sage	1.80	II	P/E 95/5	13.4	11	82	50
18	Sage	1.80	II	T	10.2	16	91	82
19	Sage	1.80	II	P/A 98/2	6.8	14	53	41
20	Sage	1.80	II	P/A 95/5	8.4	16	75	66
21	Sage	1.80	II	P/A 9/1	11.6	13	84	61

Legend:

CA: Carnosic acid

QI: Quality index

Solvents: P = petroleum ether E = ethanol A = acetone
 D = dichloromethane T = toluene

On the basis of the measured data set out in the Table, it is possible to define a quality index (QI) of the extract which is determined from the criteria of effectiveness and selectivity of the extraction solvent. The effectiveness (E) of the solvent is measured by the degree of extraction of the carnosic acid (column 8). The more effective the solvent, the better will be the yield of carnosic acid recovered at the end of the process. The selectivity (S) of the extraction solvent is measured by the content of carnosic acid in the extract (column 7). The more selective the solvent, the better will be the purity of the carnosic acid isolated at the end of the process. The quality index (QI) may thus be defined as the product of the factors E and S weighted by the content of carnosic acid (T) in the vegetable starting material (column 3).

$$QI = (E \times S) / T$$

To facilitate comparison of the various Examples in Table 1, the quality index (column 9) was relativized to a scale of 100, the data of Example 4 being arbitrarily considered as optimal.

Examination of the data in the Table enables the following observations to be made:

- 1) The quality index is the expression of a compromise between selectivity and effectiveness. The ideal solvent would be that which extracted all, and only, the active material required. The compromise to be made is to find a balance between the yield of the process for obtaining the active material and the economy of that process.
- 2) Given a comparable extraction variant and solvent, the

carnosic acid is extracted more easily from sage than from rosemary.

5 3) Given a comparable solvent and irrespective of the vegetable starting material, extraction variant I is more effective than variant II.

10 4) The use of binary mixtures of extraction solvents with an increasing proportion of the more polar solvent enables an optimal concentration of the more polar solvent to be defined; this optimal concentration is around 5% in the case of the Examples given in the Table (Examples 7-9, 10-12, 15-17, 18-19).

15 Specific examples of the extraction of carnosic acid in accordance with the Examples 4, 1, 2, 7 and 18 of Table 1 above are given in the following.

Sage, extraction variant I, Example 4

20 297 g ground officinal sage containing 2.5% carnosic acid are introduced into an extractor of the Soxhlet type equipped with a cellulose cartridge. The sage is extracted with petroleum ether (2.5 l; Bp. 40-60°C) for 48 h in the
25 absence of air (nitrogen atmosphere). On completion of extraction, the solvent is removed in a rotary evaporator and 30 g of a coloured oily extract (yield 10%) containing 7.4 g carnosic acid (yield 100%) are collected.

30 The extract is dissolved in dichloromethane (150 ml) and, after this solution has been filtered to eliminate a small proportion of insoluble material, the solution is poured into a column filled with polyamide and prepared from a suspension of 150 g of that material in 1 l dichloromethane. The column is then eluted with the same solvent
35 to eliminate those materials from the extract which are not

retained on the polyamide and which correspond to a strongly coloured fraction (fraction 1, 700 ml, 18 g residue without solvent). Elution is continued with an 8:2 (v/v) of dichloromethane and methanol. The transition zone
5 between the two solvents shows up on the column in the form of a ring-shaped yellow zone which corresponds to the carnosic acid. An intermediate fraction (fraction 2, 700 ml, 2 g residue without solvent) and then the ring zone (fraction 3, 100 ml, 6.1 g residue after removal of the
10 solvent) are collected.

After trituration in petroleum ether, the semi-crystalline residue of fraction 3 gives 6.0 g of a light yellow solid (Mp. 170-195°C) containing 95% carnosic acid. Yield: 82%.

15

Rosemary, extraction variant I, Example 1

A stocking of thin cloth containing 383 g ground rosemary containing 1.85% by weight carnosic acid is placed
20 in the extraction container of a Soxhlet extractor. The height of the vegetable mass in the extractor measures 30 cm. The extractor is placed under an inert atmosphere and the rosemary is extracted with petroleum ether (2.5 l; Bp. 40-60°C) in a total of 4 filling and siphoning cycles each
25 lasting 75 minutes. The solvent is removed in a rotary evaporator and 35 g of a dark oily extract (yield 9.1%) containing 5.6 g carnosic acid (yield 79%) are collected.

The extract is dissolved in 240 ml dichloromethane and the solution is poured onto a column of polyamide. The
30 column is eluted in the same way as before, 3 fractions being collected, namely: fraction 1, 700 ml; fraction 2, 550 ml; fraction 3, 500 ml.

The solvent is removed from the last fraction and 7.3 g of a deep yellow solid mass containing 5.6 g (77%)
35 carnosic acid, i.e. the entire extracted fraction of that

acid, are obtained. Yield: 79%.

Rosemary, extraction variant I, Example 2

5 The procedure is as in preceding Example, except that
the rosemary is extracted with a 9/1 (v/v) mixture of pet-
roleum ether and dichloromethane. The extract weighs 38 g
(yield 10.1%) and contains 6.2 g carnosic acid (yield 87%).
Part of the extract (4 g) is insoluble in dichloromethane
10 and care has to be taken to filter this solid material
before applying the extract to the column of polyamide.
The residue of fraction 3 obtained by elution weighs 7.7 g
and contains 5.3 g (59%) carnosic acid. Yield: 74%.

15 Rosemary, extraction variant II, Example 7

2.5 kg ground rosemary containing 1.8% carnosic acid
and 12.5 l of a 98/2 (v/v) mixture of hexane and ethanol
are introduced into a 20 litre reactor. The whole is
20 stirred for 1 hour at ambient temperature in a nitrogen
atmosphere. The liquid phase is separated from the solid
phase by vacuum filtration (Büchner). The solution of the
extract is placed on one side and the vegetable mass is
subjected to a second extraction similar to the first.
25 After filtration, the two extract solutions are combined
and the solvent is removed in a rotary evaporator. 206 g
(yield 8.2%) of a dark oily extract containing 33 g car-
nosic acid (yield 73%) are obtained.

The extract is dissolved in 1.6 l dichloromethane and
30 the solution is poured onto a column of polyamide. The
column is eluted as in Example 1, 3 fractions being col-
lected, namely: fraction 1, 3.36 l; fraction 2, 3.23 l;
fraction 3, 1.65 l. After evaporation of the solvent, the
last fraction gives a semi-oily residue (41.5 g) which is
35 triturated in petroleum ether to a solid consistency. The

yellow product thus obtained weighs 37 g and contains 73% carnosic acid. Yield: 27 g (60%).

Sage, extraction variant II, Example 18

5

A mixture of 50 g ground sage (carnosic acid content 1.8%) and 600 ml toluene is stirred for 1 hour at ambient temperature in a nitrogen atmosphere. After separation of the two phases (solid and liquid) by filtration, the vegetable mass is subjected to a second extraction. The 2 extract solutions are combined and the solvent is removed in a rotary evaporator. 5.1 g (10.2%) of an oily residue containing 0.82 g carnosic acid (yield 91%) are obtained.

10

The extract is redissolved in 50 ml toluene and the solution obtained is filtered to eliminate some insoluble materials. The filtrate is poured onto a column of polyamide (30 g; 30 x 2 cm) conditioned in toluene. The column is eluted as in Example 1 with toluene and then with an 8/2 mixture of toluene and ethanol, 3 fractions being collected, namely: fraction 1, 200 ml (3.3 g vegetable matter); fraction 2, 160 ml (0.2 g); fraction 3, 50 ml (1.1 g). The last fraction contains 0.78 g (71%) carnosic acid. Yield: 87%.

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Purification of the carnosic acid

30 g of the yellow product obtained in Example 7 containing 73% carnosic acid are recrystallized twice in cyclohexane in the presence of active charcoal. 16.4 g carnosic acid are obtained in the form of colourless crystals with a purity of more than 95% (melting point: 193-199°C). Instead of cyclohexane, the product may also be recrystallized from benzene or toluene.

30

35

The physiological anticarcinogenic and antiviral properties of carnosic acid are illustrated in the follow-

ing tests:

Anticarcinogenic activity of carnosic acid

5 The antimutagenic activity of carnosic acid was
evaluated in an Ames test using the strain *Salmonella*
10 *typhimurium* TA 102 which is known to respond readily to
active oxygen-containing species. This strain is placed in
tert. butyl peroxide (tBOOH) which is known to produce
15 peroxyl radicals and of which the biological action is
considered to be particularly interesting because it
generates the oxygen radicals inside the cells. The tBOOH
produces a certain number of local alterations in the DNA
of the bacteria and the inhibition of these alterations
20 produced by the antioxidant when it is incorporated in the
culture medium is measured. The following antioxidants
were tested in a range of active doses: carnosic acid,
carnosol and ascorbic acid. The ascorbic acid, of which
the anticarcinogenic is well known, served as a positive
25 control. In order to dissociate the "true" antimutagenic
effect from a bactericidal effect, a pre-incubation test is
used and the results are expressed in the form of the
ratio: number of revertant colonies induced/number of
surviving colonies [Aeschbacher et al., Food Safety, 8,
30 167-177 (1987)].

 Description of the test: the incubation medium is pre-
pared by mixing 1 ml bacterial suspension (5×10^9 bact./ml)
of *Salmonella typhimurium* TA 102 prepared in accordance
with Maron and Ames [Mutation Research, 113, 175-215
30 (1983)], 50 μ l saline buffer, 0.95 ml 0.15 M KCl and 2.8 ml
Davis-Mingioli medium supplemented with 24 μ g histidine and
10 μ g biotine per ml. 0.5 ml tBOOH solution (final concen-
tration 2.5 mM) and 0.5 ml of a solution of antioxidant in
water for the ascorbic acid and in medium-chain triglycer-
35 ides for the water-insoluble antioxidants, carnosic acid

and carnosol, are then added. The medium is incubated for 1 hour at 37°C and, after addition of 9 ml 0.8% nutrient broth, for another 3 hours at 37°C to fix the mutation. The medium is then centrifuged and the washed bacteria are resuspended in 3.5 ml saline buffer.

Counting of the revertant and surviving colonies is carried out on plates of nutrient agar, which have been incubated for 3 days at 37°C and to which 0.1 ml of the above bacterial suspension has been applied, respectively in the absence and presence of histidine. Counting is carried out automatically using a Fisher Count-All 800 counter.

Table 2 expresses the results obtained in the form of the estimated concentrations of antioxidants tested which are capable of reducing by half the mutagenic effect induced by the tBOOH in a concentration of 2.5 mM in the absence of antioxidants (inhibiting concentration 50 = IC 50).

Table 2

Input	Compound	IC50 (mg)	IC50 (mM)	Relative activity, comparison of the IC50 (mM)
1	Carnosic acid	0.3	0.15	100
2	Carnosol	17.8	9.3	2
3	Ascorbic acid	2.8	2.7	6

It is clearly apparent from the results set out in the Table that, although the anticarcinogenic activity of carnosol is slightly weaker, but of an order comparable with that of ascorbic acid (positive control), the activity of carnosic acid is higher by a factor of approximately 15 than that of ascorbic acid and by a factor of 50 than that of carnosol.

Antiviral activity of carnosic acid

5 The antiviral activity of carnosic acid was tested in
 vitro at various concentrations (5; 2.5; 1.25; 0.62 $\mu\text{g/ml}$)
 against infectious batches of simple herpes of type 1
 (HSV1) and type 2 (HSV2) and of polio virus of type 3
 (polio 3) cultured on the VERO cell line. After incubation
 for 2 hours, the titers obtained are compared with that of
 the inhibitor-free controls (test 1). Aliquot portions of
 10 various test supernatants are then inoculated onto new
 cells. After incubation for 4 days, counting of the viral
 particles this time enables viral production in the pres-
 ence of the inhibitor to be evaluated (test 2). The
 results of these two tests are set out in Table 3 below.

15

Table 3

Concentration of carnosic acid ($\mu\text{g/ml}$)	Test 1			Test 2		
	HSV1	HSV2	POLIO 3	HSV1	HSV2	POLIO 3
5	10	10	10^{12}	10^2	<10	2×10^{23}
2.5	10	10	10^{12}	10^5	2×10^2	2×10^{23}
1.25	10^3	10	10^{12}	2.5×10^6	10^5	10^{23}
0.62	10^4	10	10^{12}	6×10^6	8×10^5	10^{22}
0 (control)	10^5	10^4	10^{12}	2×10^8	10^7	10^{23}

The results of test 1 show that the virus strain polio 3 is not inhibited by carnosic acid. By contrast, the anti-HSV1 and anti-HSV2 effect is altogether significant because reductions in titer of as much as 3 to 4 factors of 10 are observed. Carnosic acid therefore has a specific effect. The results of test 1 are confirmed by those of test 2. It is found that the production of polio 3 can be

considered as equivalent, irrespective of the concentration of carnosic acid. By contrast, the production of HSV1 and HSV2 is greatly affected. This confirms the specificity of the effect of the product and also the absence of cell toxicity because, at the highest concentration (5 $\mu\text{g/ml}$), there is no change in the production of polio 3.

CLAIMS:

1. A process for the preparation of carnosic acid from rosemary or sage, wherein the rosemary or sage is extracted with a non-polar solvent or with a solvent mixture of high apolarity, the extract obtained is subjected to a selective adsorption treatment on a solid support, the carnosic acid is desorbed with a polar solvent or with a mixture of highly polar solvents and the solvent is evaporated.
2. The process as claimed in claim 1, wherein the non-polar solvent is selected from petroleum ethers, toluene, hexane and a mixture thereof with dichloromethane or ethanol in a ratio of 99:1 to 90:10.
3. The process as claimed in claim 1 or 2, wherein the solid support is selected from silica gel, aluminium oxide, polyamide and polyvinyl pyrrolidone.
4. The process as claimed in any one of claims 1 to 3, wherein the polar solvent or the mixture of highly polar solvents is selected from methanol, ethanol and a mixture thereof with dichloromethane or toluene.
5. The process as claimed in any one of claims 1 to 4, wherein between 2 and 20 extractions are carried out.
6. The process as claimed in claim 5, wherein each extraction lasts between 30 minutes and 2 hours.
7. The process as claimed in claim 5 or 6, wherein the extractions are carried out at a temperature of 20 to 50°C.

8. A process for obtaining carnosic acid, comprising: extracting a vegetable material selected from the group consisting of sage and rosemary with an apolar solvent to obtain an extract comprising carnosic acid; contacting the extract with a solid support adsorbent material having an affinity for polar compounds for adsorbing the carnosic acid to separate the carnosic acid from the apolar compounds of the extract; desorbing the adsorbent material with a polar solvent to obtain the carnosic acid in the solvent; and then evaporating the polar solvent from the carnosic acid to obtain a residue containing the carnosic acid.

9. The process according to claim 8, further comprising crystallizing the carnosic acid from the residue.

10. The process according to claim 8, further comprising triturating the residue in petroleum ether.

11. The process according to any one of claims 8 to 10, further comprising concentrating the extract to obtain a concentrated extract and then contacting the concentrated extract with the adsorbent material.

12. The process according to any one of claims 8 to 11, wherein the vegetable material is extracted at a temperature of from 20°C to 50°C.

13. The process according to any one of claims 8 to 12, wherein the adsorbent material is contained in a column and wherein the extract is contacted with the adsorbent material by passing the extract through the material in the column.

14. The process according to any one of claims 8 to 13, wherein the apolar solvent is selected from the group consisting of saturated hydrocarbon solvents, aromatic hydrocarbon solvents and mixtures thereof.

15. The process according to claim 14, wherein the apolar solvent further comprises a solvent selected from the group consisting of a chlorine-containing solvent, an ether solvent, a ketone solvent, an ester solvent and an alcohol solvent.

16. The process according to any one of claims 1 to 13, wherein the apolar solvent is an acyclic hydrocarbon having a boiling point of from 40⁰C to 60⁰C.

17. The process according to claim 16, wherein the vegetable material is rosemary and the apolar solvent further comprises a chlorine-containing solvent in a ratio of acyclic hydrocarbon to chlorine-containing solvent of from 99:1 to 90:10.

18. The process according to claim 17, wherein the chlorine-containing solvent is dichloromethane.

19. The process according to any one of claims 1 to 13, wherein the apolar solvent is selected from the group consisting of pentane, hexane, heptane, 2-methylbutane, 2-methylhexane, cyclohexane, and toluene and mixtures thereof.

20. The process according to claim 19, wherein the apolar solvent further comprises a solvent selected from the group consisting of methylene chloride, chloroform, chloroethylenes, dichloromethane, diethyl ether, acetone, ethyl acetate, methanol and ethanol.

21. The process according to any one of claims 8 to 13, wherein the apolar solvent is selected from a group of saturated hydrocarbon solvents consisting of petroleum

ethers, toluene and hexane in combination with a further solvent selected from the group consisting of dichloromethane and ethanol, wherein the ratio of the saturated hydrocarbon solvent to further solvent is from 99:1 to 90:10.

22. The process according to any one of claims 8 to 21, wherein the adsorbent is selected from the group consisting of silica gel, aluminum oxide, polyamide and polyvinyl pyrrolidone.

23. The process according to any one of claims 8 to 22, wherein the polar solvent is selected from the group consisting of methanol, ethanol, acetone and ethyl acetate.

24. The process according to any one of claims 8 to 22, wherein the polar solvent is selected from the group consisting of methanol and ethanol in admixture with a further solvent selected from the group consisting of dichloromethane and toluene.