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Journal of Chromatography A, 870 (2000) 491–499

JOURNAL OF CHROMATOGRAPHY A

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Liquid chromatographic–mass spectrometric analysis of supercritical-fluid extracts of rosemary plants

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Abstract

A two-step supercritical fluid extraction process of rosemary leaves, on a pilot plant scale, is proposed to divide the oleoresin into two fractions with different antioxidant activities and essential oil composition. Rosemary leaves were extracted by using different conditions of pressure and temperature as well as different conditions for fractionation of the extracts. Conditions can be tuned to selectively extract one antioxidant fraction with almost no residual aroma. In the present investigation, the antioxidant fraction was exhaustively studied in terms of antioxidant activity measurements as well as of chemical composition. An LC–MS method was adapted to perform the analysis and identification of the compounds responsible for the antioxidant activity of the extracts. Different extraction and fractionation conditions were studied in order to correlate the process conditions with the antioxidant activities obtained.

Keywords: Plant material; Rosemary; Extraction methods; Antioxidants; Terpenes; Phenolic compounds; Flavanoids

1. Introduction

Antioxidants are widely used in food products to prevent or retard oxidation of fats and oils. In the last few years there has been a growing interest in the use of antioxidants in the food industry [1] not only for their usefulness as a preservation method but also because of their beneficial effects on human health.

The use of synthetic antioxidants in the food industry is severely restricted as to both application and level. Among the natural antioxidants, rosemary has been widely accepted as one of the spices, along with sage, with the highest antioxidant activity [2]. Several studies on the antioxidative constituents of rosemary indicate that the most active compounds are the phenolic diterpenes carnosic acid, carnosol, rosmanol, and epi- and iso-rosmanol [3–5].

Several methods have been used to extract antioxidants from aromatic plants, such as solid–liquid extraction, aqueous alkaline extraction, extraction with vegetable oils and supercritical fluid extraction (SFE) [6–10]. Products obtained by SFE from rosemary leaves have in general a higher antioxidant activity than extracts obtained by using solvent extraction with organic solvents [5], probably due to a difference in composition deriving from the extraction conditions applied under which carnosic acid is degraded to different extents and other phenolic diterpenes, with lower activity, are formed.

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)00941-3

As has been previously suggested by some authors, antioxidative performance depends on the extraction parameters [11,12] as well as on the quality of the original plant, its geographic origin, the harvesting date, its storage and its processing prior to extraction [11,13,14]. All of the above mentioned parameters directly influence the final composition of the extracts obtained.

Some work has been done on the fractionation of rosemary [11,15]. Based on previous work done in our laboratory, a two-step SFE of rosemary leaves is proposed to divide the oleoresin into two fractions with different antioxidant activities and essential oil compositions [11].

Exhaustive characterization of the fractions obtained with the aid of a pilot plant supercritical fluid extraction unit is carried out by LC–MS.

To study the influence of the process conditions at a large scale on the antioxidant activity of the products obtained, extraction conditions were selected based on the basis of earlier studies [11] and antioxidant activities were measured by a free radical method (DPPH). This allowed us to study both the effectiveness of the fractionation and the correlation between chemical composition and antioxidant activity.

LC–MS with electrospray (positive ionization method) has been used, along with a diode array detector to characterize the extracts in terms of chemical composition. The results obtained have been correlated with the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) measurements in order to identify the compounds or group of compounds responsible for the antioxidative properties of supercritical fluid rosemary extracts.

The correlation study has been suggested previously by other authors [13] but only qualitative data about the type of compound that greatly influence the antioxidant activity has been presented. In the present investigation, correlation studies have been performed using all the compounds identified in the samples obtained, that is, phenolic diterpenes such as carnosic acid, carnosol, rosmanol, rosmadial, carnosol isomer and methyl carnosate, and flavonoids such as cirsimaritin and scutellarein. The mentioned compounds have been considered both singly and in pairs.

2. Experimental

2.1. Samples and chemicals

The rosemary sample (*Rosmarinus officinalis L.*) consisted of dried rosemary leaves obtained from an herbalist's shop (Murcia, Spain) dried using the traditional method [11]. Samples were ground under cryogenic carbon dioxide and stored in amber flasks at −20°C until use.

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95% purity) was obtained from Sigma-Aldrich (Madrid, Spain). Acetonitrile was of HPLC grade, while acetone was of analytical-reagent grade. All solvents were purchased from Lab Scan (Dublin, Ireland), except ethanol (99.5%) from Panreac (Spain) and acetone from Quimicen (Madrid, Spain). Milli-Q water was obtained from a purification system (Millipore). CO_2 (SFC quality) was kindly donated by AL Air Liquide España (Madrid, Spain).

2.2. Instrumentation — extraction method

In this study, a pilot-scale supercritical fluid extractor (Iberfluid, Spain) was used (see Fig. 1). For all the experiments the extraction cell was of 316 stainless steel with a volume of 285 ml with a stainless steel frit. The extraction pressure was controlled by micrometering valves, and the carbon dioxide pump was from Braun-Luebbe.

2.3. Extraction procedure

For each experiment, the extraction cell was filled with 60 g of ground rosemary and 75 g of washed sea-sand (Panreac, Spain), and the cell was sealed in the extractor. Dynamic extractions were performed at the experimental conditions shown in Table 1. The range of extraction pressures tested was 300 to 350 bar. In the extractions with ethanol as modifier, the addition started after having reached the selected pressure during half of the extraction time. The extracts were fractionated by using two separation cells with an independent control of temperature and pressure.

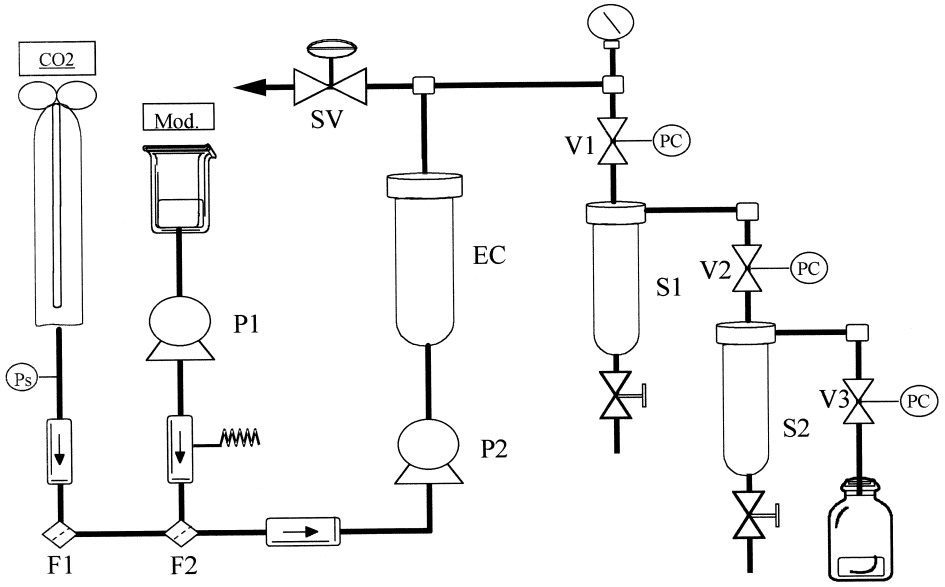

Fig. 1. Scheme of the pilot-scale supercritical fluid extractor used in the present study. P1, modifier pump, P2, CO_2 pump, V1, V2, V3, micrometering valves, S1, separator 1, S2, separator 2, EC, extraction cell, F1, F2, filters, SV, security valve.

2.4. LC–MS analysis of the extracts

Analyses were performed with a quadrupole Hewlett-Packard 1100 MSD by using an electrospray interface. The separation was carried out in an HPLC apparatus (HP Series 1100) with an autosampler (injection volume 25 μl) equipped with a Zorbax C_{18} column 150×4.6 mm, 3.5-μm particle size. The mobile phase was a mixture of solvent A (50% acetonitrile in water) and solvent B (10 m*M* acetic acid in water) according to a step gradient, lasting 35 min, changing from 50% B at 5 min to 30% B at 15 min and to 0% B at 30 min, at a flow-rate of 0.6 ml/min. Detection was accomplished by using a diode array detection system (DAD) Series 1100 (Hewlett-Packard), storing the signal at a wavelength of 230 nm. A personal computer system running Hewlett-Packard software was used for data acquisition and processing.

In the atmospheric pressure electrospray ionisation (ESI) method, the eluted compounds were mixed with nitrogen in the heated nebulizer interface and polarity was tuned to positive. Adequate calibration of ESI parameters (needle potential, gas temperature, nebulizer pressure) was required to optimize the response and to obtain a high sensitivity of the molecular ion. The selected values were: needle potential 4000 V, gas temperature 335°C, drying gas

Table 1
Conditions used for the experiments performed on a pilot plant scale[a]

Exp.	EtOH (%)	P_{ext} (bar)	T_{ext} (°C)	ρ_{ext} (g/ml)	P_{s_1} (bar)	T_{s_1} (°C)	ρ_{s_1} (g/ml)	Antioxidant activity (μg/ml)	P_{s_2} (bar)	T_{s_2} (°C)	Antioxidant activity (μg/ml)
1	0	350	50	0.9	200	50	0.78	9.7	20	25	21.0
2	2	300	40	0.91	150	60	0.6	16.8	20	25	175.1
3	2	350	60	0.87	150	40	0.78	34.9	55	25	115.2

[a] P_{ext}, extraction pressure; T_{ext}, extraction temperature; ρ_{ext}, extraction density; P_{s_1}, pressure in separator 1; T_{s_1}, temperature in separator 1; ρ_{s_1}, density in separator 1; P_{s_2}, pressure in separator 2; T_{s_2}, temperature in separator 2.

10.0 ml/min, nebulizer pressure 50 p.s.i.g (1 p.s.i.= 6894.76 Pa). When negative ion mode was used, the polarity was tuned to negative and ammonium acetate was added instead of acetic acid to favor negative ionization of the compounds.

2.5. Determination of antioxidant activity

Antioxidant activity was measured in both fractions 1 and 2. The method used was based on a procedure described by Lamaison et al. [16], modified as previously described [17]. The method consists on the neutralization of free radicals of DPPH by the antioxidant. The procedure used is as follows: 0.014 g of DPPH were weighed and brought to 100 ml with methanol, sonicated for 10 min and diluted 1:5 with methanol; rosemary extract solutions were prepared by weighing 0.05 g and adding 7 g of ethanol. 10 g of DPPH solution were placed in test tubes, 30 μl of rosemary extract solution were added (that correspond to 212 μg). Reaction was complete after 3 h at room temperature and absorbance was measured at 516 nm in a Shimadzu UV-120-01 spectrophotometer (Shimadzu, Kyoto, Japan). Methanol was used to adjust zero, and ascorbic acid to calibrate the method. The equation described by Lamaison et al. [16], was utilized in order to determine the amount of antioxidant extract needed to reduce by 50% the initial DPPH concentration, this value provides a measure of the EC_{50} or efficient concentration, also called oxidation index. Measurements were performed in triplicate.

3. Results and discussion

The raw material and drying process applied to the rosemary plants were selected based on previous work [11]. As has been previously suggested, drying conditions had a great influence in the final antioxidant activity of the extracts obtained by SFE, aggressive conditions showing a significant loss of antioxidant activity.

Table 1 shows the results of experiments performed on a pilot plant scale. Different extraction and fractionation conditions were selected based on previous data obtained in our laboratory [11] where a two-step SFE process was found to provide two fractions with different properties. By using the pilot plant described in the Section 2, a maximum pressure of 350 bar can be used, considering that good results in terms of antioxidant extraction had been previously obtained at 400 bar and 60°C (density equal 0.9 g/ml), similar conditions were chosen. Use of ethanol as modifier was also tested in one of the conditions studied. The pilot plant used allowed the possibility of fractional separation in the two separation vessels. Fractional separation conditions tested covered a density range between 0.6 and 0.8 g/ml in the first separator, whereas a total decompression stage was achieved in the second separator. The differences are observed in the selective precipitation of the compounds in the first separator where compounds non-soluble at densities higher than the corresponding to the density of the separator precipitate. This fraction contains the compounds with antioxidant properties. In this study, the selectivity of the fractional separation was studied changing the density from 0.9 g/ml (extraction density) to 0.78 g/ml (conditions corresponding to experiment 1 with ethanol as modifier), decreasing the density from 0.91 to 0.6 g/ml (experiment 2) and from 0.87 to 0.78 (experiment 3).

To evaluate the selectivity of the fractionation, extracts corresponding to both fractions 1 and 2 were characterized in terms of antioxidant activity (oxidation index, μg/ml).

The experimental extraction conditions were selected among those that provided the most different antioxidant activities. Results obtained are shown in Table 1 along with the extraction and fractionation conditions tested. Conditions corresponding to a higher decrease in density (experiment 2) provide the highest difference in antioxidant activity between the two fractions (16.8 and 175.1, respectively, for fractions 1 and 2) and therefore the best fractionation between separator 1 and 2. Nevertheless, the highest extraction efficiency, in terms of obtaining the best antioxidant activity, was achieved under the conditions of experiment 1 (fraction 1, 9.7 μg/ml), that is, with a density reduction from 0.9 to 0.78 g/ml and no modifier added.

Fractions 1 and 2 were analyzed by LC–MS to identify the compounds responsible for the anti-

oxidant properties of the extract, and to subsequently correlate the antioxidant activity with the composition of the extracts.

An LC–MS method based on previous work done by other authors [10,13] was adapted in terms of tuning the HPLC conditions of analysis of the extracts. Taking into account the type of compounds that can be obtained in the different fractions analyzed, a mass spectrometer with electrospray in positive ionization mode was selected; the signal thus obtained for most of the compounds was higher than when using negative ionization mode, therefore, an increase in sensitivity was obtained for almost all the compounds present in the sample, except for acidic compounds where the signal was smaller in the positive ionization mode. This ionization favors the signal for compounds able to protonate, like phenolic diterpenes such as carnosol, rosmanol, etc. Fig. 2 shows the signal obtained for the same extract analyzed using the same HPLC conditions and different ionization mode. The signal decreases for carnosic acid but increases for the rest of the compounds in the sample.

In order to obtain semi-quantitative data, the primary detection wavelength used was 230 nm. Simultaneously, spectral data was obtained over the range of 215 to 450 nm by using a DAD. This data can be very useful to identify compounds of interest. Fig. 3 shows the chromatographic profiles obtained by DAD at 230 nm (a) and (b, top) for experiment 3, fractions 1 and 2. Along with these profiles, signal for ESI in the positive mode is also shown (a) and (b, bottom).

Compounds were characterized for their retention times (t_R), UV spectra and mass spectra, and were tentatively identified based on previous data published by other authors [13,18]. Two groups of phenolic compounds have been identified: diterpenes such as carnosol, rosmadial, carnosic acid, methyl carnosate, rosmanol, carnosol isomer, and flavonoids, such as cirsimaritin and scutellarein. Other compounds were also detected but their complete identification was not possible. Table 2 shows retention times, molecular ion (MH+), and UV maximum absorbance for all the compounds detected in the samples, also, additional data about the major fragments obtained using electrospray with positive ionization is presented for all the compounds found in the sample in substantial amounts. Table 2 also includes the fractions where the compounds had been detected. Carnosol isomer had a mass ion (MH+) equal to 331 but presented also a peak at m/z 348, that could be due to the addition of water to the carnosol molecule.

Some of the non-identified compounds have been previously described by other authors, such as NI 1 and NI 2 (non-identified 1 and 2, as proposed by Cuvelier et al. [13]). Nevertheless, other non-identified compounds described in the present paper have not been, to our knowledge, reported previously. Even though these compounds have not been identified completely, it is possible to know, for some of them, the family of compounds to which they belong, i.e., flavones 1 and 2. The compound labeled NI 7 might be a carnosol derivative based on its UV spectra and characteristic mass spectra.

To perform the study of the semi-quantitative composition of the extracts, some compounds were selected and their relative percentages (referred to the total area of the selected components based on DAD peak area at 230 nm) is shown in Table 3, where profiles of the different extracts can be observed. Unfortunately, the lack of standards for most of the components and their unknown molecular absorption coefficient did not allow quantitation of their content.

Rosmanol and scutellarein appeared mainly in fraction 1 while NI 1, NI 2, rosmadial and cirsimaritin can be found, when extracted, in fraction 2. Carnosol, carnosol isomer, carnosic acid and methyl carnosate were found in both fractions but at different relative composition depending on the extraction and fractionation conditions, these compounds, considered the most active in terms of antioxidant activity, can be preferentially observed in fraction 1.

Compound NI 7 was also found in both fractions but its highest relative contribution to the total composition of the sample was observed in fraction 2 in all the conditions tested.

In terms of relative composition of the extracts, the greatest difference among fractions 1 and 2 was achieved in experiment 2. This experiment showed the largest difference in density between the two separators, leading to a bigger difference among the two fractions mainly observed in the carnosic acid, carnosol and methyl carnosate content. This can

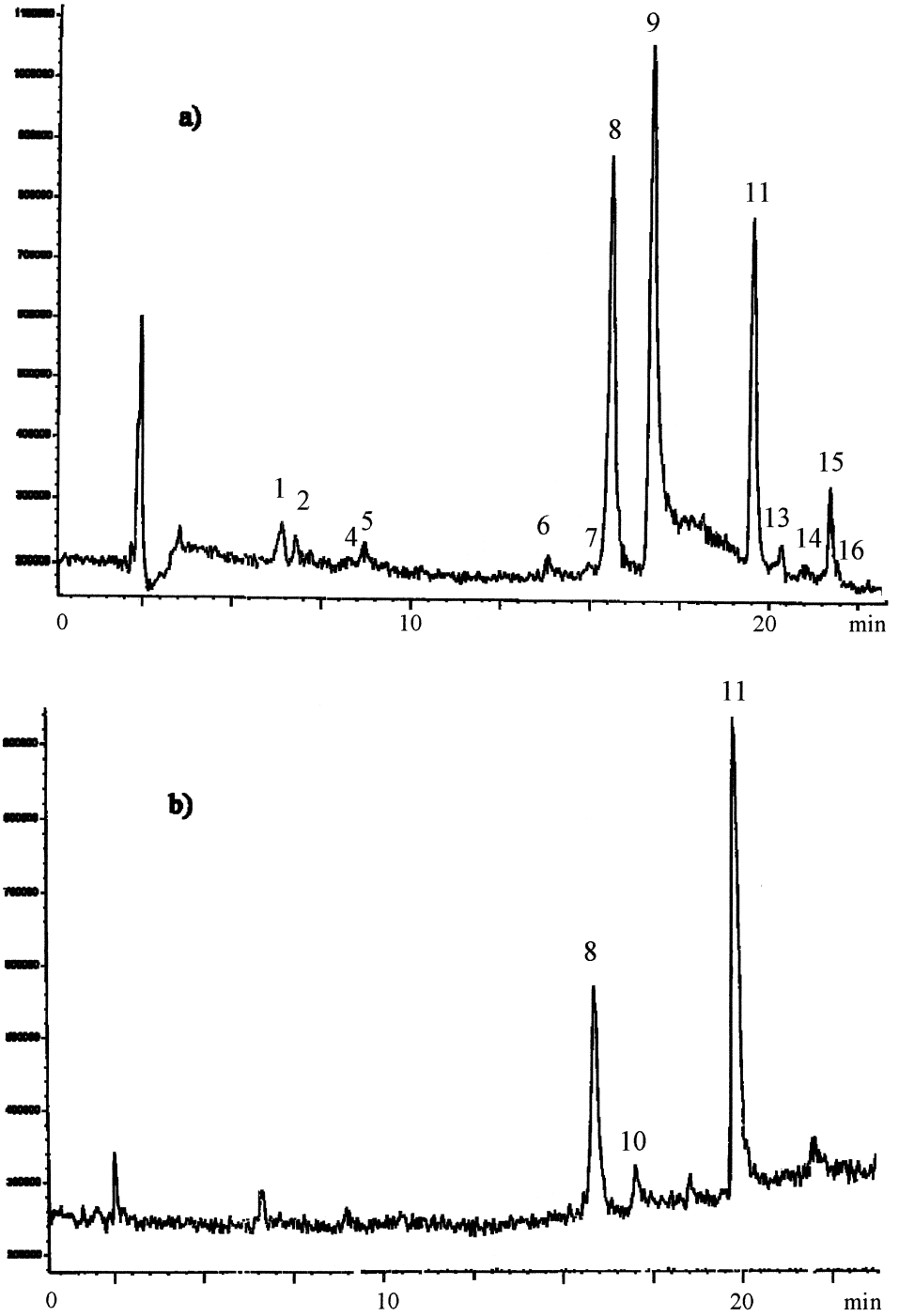

Fig. 2. Signal obtained for a supercritical extract (experiment 3) using the same HPLC conditions and different ionization mode. (a) Positive ionization, (b) negative ionization. For peak assignment, see Table 2.

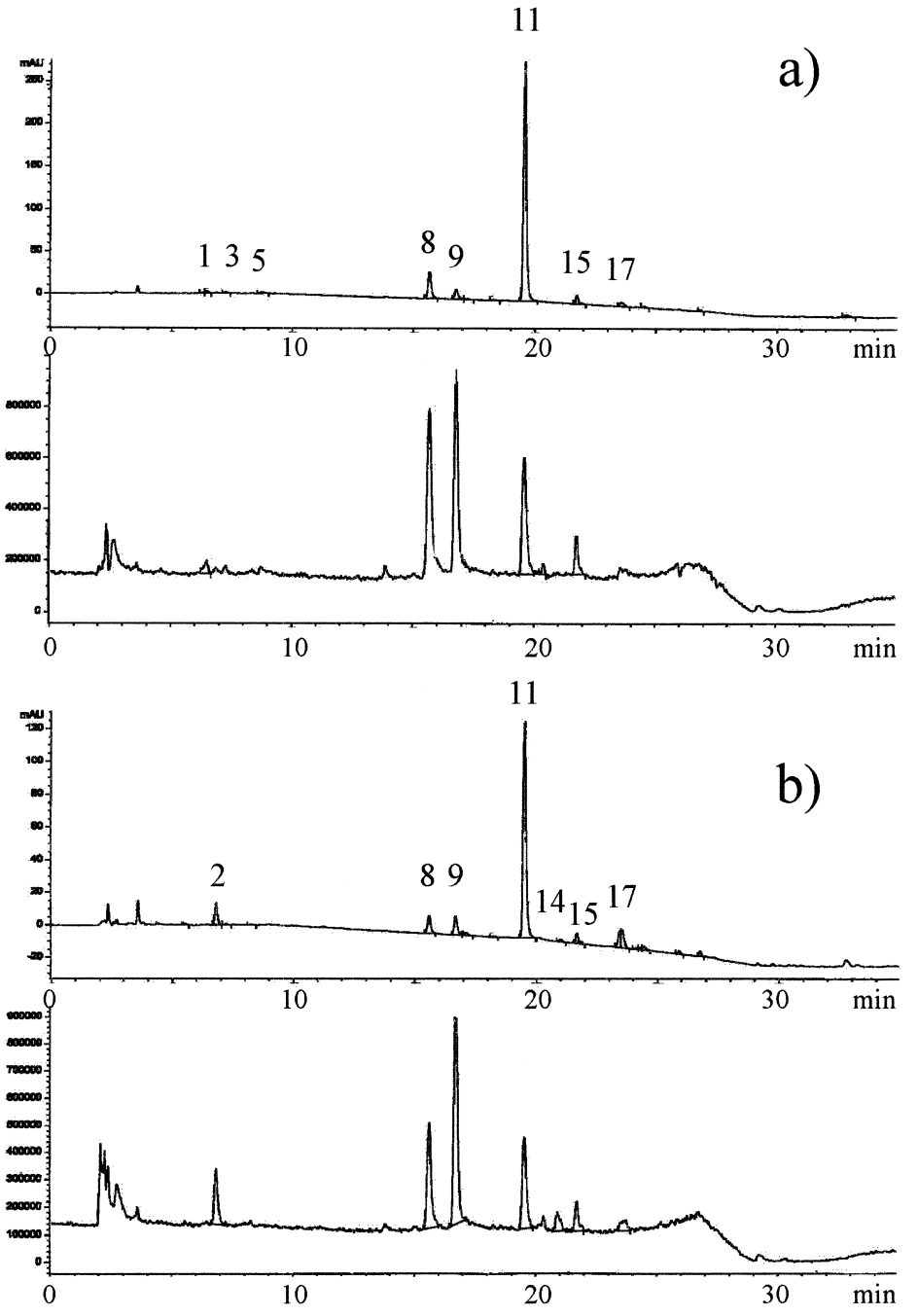

Fig. 3. Chromatographic profiles obtained for (a) experiment 3 fraction 1, top, DAD signal at 230 nm, bottom, ESI positive ionization signal and (b) experiment 3 fraction 2, top, DAD signal at 230 nm, bottom, ESI positive ionization signal. For peak assignment, see Table 2.

Table 2
Characteristic parameters of the compounds detected in the extracts analyzed by LC–MS

Peak no.	Compounds	t_R (min)	Mass ions (ES+) MH+	Major fragments	UV absorbance maximum (nm)	Fractions[a]
1	Rosmanol	6.3	347	301	284	Exp-1-F1, F2, Exp-2-F1, Exp-3-F1
2	NI 1[b]	6.8	151	109	258	Exp-1-F2, Exp-2-F1, F2, Exp-3-F2
3	Epirosmanol	7.3	347	301	290	Exp-3-F1
4	NI 3	8.2	151	–	242, 284	Exp-1-F2
5	Scutellarein	8.6	287	–	268, 335	Exp-1-F2, Exp-2-F1, Exp-3-F1
6	NI 2[b]	13.7	329	–	276, 330	Exp-1-F2
7	NI 4	14.9	333	315, 297	260	Exp-1-F2
8	Carnosol	15.5	331	–	284	Exp-1-F1, F2, Exp-2-F1, F2, Exp-3-F1, F2
9	Carnosol isomer	16.6	331	299	270	Exp-1-F1, F2, Exp-2-F1, F2, Exp-3-F1, F2
10	NI 5	17.0	345	362	235, 286	Exp-1-F2
11	Carnosic acid	19.4	333	287	284	Exp-1-F1, F2, Exp-2-F1, F2, Exp-3-F1, F2
12	Flavone 1	20.0	317	–	225, 270	Exp-1-F2
13	Rosmadial	20.2	345	–	290	Exp-1-F2
14	Cirsimaritin	20.9	315	301	248, 334	Exp-1-F2, Exp-3-F2
15	Methyl carnosate	21.6	347	301	282	Exp-1-F1, F2, Exp-2-F1, Exp-3-F1, F2
16	NI 6	21.8	337	301	260	Exp-1-F2
17	NI 7	23.4	319	301	286	Exp-1-F1, F2, Exp-2-F1, F2, Exp-3-F1, F2
18	Flavone 2	23.7	283	–	245, 295	Exp-1-F2

[a] Fractions correspond to those where the compound had been detected.
[b] NI 1 and NI 2 had been previously described, as mentioned in the text.

explain the large differences observed in the antioxidant activity of these two fractions.

Using the HPLC peak areas (DAD at 230 nm) of the compounds selected in the extracts, mathematical correlation was attempted between antioxidative activity and chromatographic profile. Linear regression by the least-squares method was used to perform the correlation studies between the phenolic diterpenes such as carnosic acid, carnosol, rosmanol, rosmadial, carnosol isomer, methyl carnosate or the flavonoids such as cirsimaritin and scutellarein and the antioxidant activity. These compounds were considered both singly and in pairs.

A high correlation (96%) was obtained for the percentage of carnosic acid vs. DPPH content (μg/ml) by means of a linear regression ($y = -6.6272x + 558.48$; $R^2 = 0.964$). The values of DPPH were lower (that is, higher antioxidant activi-

Table 3
Relative percentage (normalized areas (%)) of the compounds identified by LC–MS and selected to semi-quantitatively describe the composition of the extracts obtained at different conditions of extraction and fractionation, as shown in Table 1

Compound	Normalized areas					
	Exp-1-F1 (%)	Exp-1-F2 (%)	Exp-2-F1 (%)	Exp-2-F2 (%)	Exp-3-F1 (%)	Exp-3-F2 (%)
Rosmanol	0.55	0.26	0.68	0	0.69	0.00
NI 1	0.00	6.89	0.49	18.09	0	6.55
Scutellarein	0.00	0.28	0.97	0.00	0.82	0.00
NI 2	0.00	0.50	0.00	0.00	0.00	0.00
Carnosol	6.92	4.28	8.32	4.44	10.07	5.99
Carnosol isomer	5.32	4.62	5.15	10.23	4.10	6.92
Carnosic acid	82.16	59.88	80.26	56.35	79.14	70.38
Rosmadial	0.00	0.50	0.00	0.00	0.00	0.00
Cirsimaritin	0.00	1.26	0.00	0.00	0.00	0.76
Methyl carnosate	3.11	3.23	2.16	0.00	2.99	3.50
NI 7	1.94	18.30	1.97	10.88	2.19	5.91

ty) with increasing percentage of carnosic acid. One of the experimental results obtained (F2, experiment 1) did not correlate with the linear regression and probably the large difference can be due to the type of compounds extracted when no ethanol was used. As can be seen in Table 3, a large amount of non-identified compound NI 7 was found in this extract making the relative percentage of the rest of the compounds extracted erroneous. Therefore, this value was not considered in the linear regression.

Carnosol did not show any correlation with DPPH and carnosol isomer seemed to correlate (with a 87%) negatively with the antioxidant activity. One explanation might be the possibility of degradation of carnosol (with proved antioxidant activity) to carnosol isomer (with lower antioxidant activity). This can be also observed by the fact that the sum of both percentages remained almost constant (except for F2, experiment 1). No linear correlation were obtained for rosmanol, cirsimaritin, scutellarein and methyl carnosate.

Also, synergistic studies had been attempted by representing the sum of the relative percentages vs. DPPH content. No correlation was observed when carnosic acid was not involved in the calculations, that is, when the following combinations were been studied: rosmanol + carnosol isomer, methyl carnosate + carnosol isomer, rosmanol + methyl carnosate, carnosol + rosmanol, carnosol + methyl carnosate, carnosol + carnosol isomer. Carnosic acid showed a correlation when added to the other phenolic diterpenes tested. Light differences among the slopes obtained seemed to indicate that no synergistic effects can be observed.

Acknowledgements

The authors acknowledge the kind support provided by AL Air Liquide España, S.A. (Madrid, Spain) in this research. The authors thank Chubby Vicente for her kind help in the acquisition of the MS spectra.

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