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Isolation of functional ingredients from rosemary by preparative-supercritical fluid chromatography (Prep-SFC)

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Abstract

A supercritical fluid extract of rosemary has been fractionated under supercritical conditions by using a preparative-SFC system. In this work, the optimum conditions have been evaluated to achieve a selective isolation of the compounds responsible for both, antioxidant and antimicrobial activities. A 25 cm × 10 mm i.d. LC-Diol packed column (dp = 5 μm) has been used and the separation took place at 80 °C of column temperature, 130 bar of pressure, and 10% of ethanol as modifier of the mobile phase (CO_2). Two cyclones were employed to collect the fractions which were subsequently characterized by HPLC-DAD, GC, and in vitro antioxidant and antimicrobial assays. By a careful selection of the separation conditions it is possible to obtain two different fractions, one enriched with antioxidant and antimicrobial compounds (with an improvement of about 20% and 40% of antioxidant and antimicrobial activity, respectively, compared to the original extract) collected in cyclone 2 and with no residual rosemary aroma and another one containing the essential oil.

Keywords: Rosemary extracts; Functional ingredients; Preparative-SFC; Antioxidants; Antimicrobials

1. Introduction

The growing interest in functional foods has fostered research on new natural sources of active ingredients. Vegetables are by far the most well studied natural sources since they contain a huge variety of active compounds that could be used in the food industry as functional compounds or nutraceuticals.

Spices and herbs have been added to foods since ancient times, mainly to modify or improve their flavors. Since 1952 [1], the antioxidant properties of some of these species have been recognized. Antioxidants are compounds that when present in foods at low concentrations, compared to that of an oxidizable substrate, markedly delay or prevent oxidation of the substrate [2,3]. Moreover, and even more important, the beneficial effects of antioxidants on human health have also been described [4]. It is also a well-known fact that aromatic plants and spices as well as their essential oils have varying degrees of antimicrobial activity [5–7]. For this reason extracts from these plants can be used to delay or inhibit the growth of pathogenic or spoilage microorganisms [8]. Besides, the majority of the essential oils are classified as Generally Recognized As Safe (GRAS) ingredients [9].

Among herbs and spices, rosemary (*Rosmarinus officinalis* L.) is a common household plant grown in many parts of the world. It is used for flavoring food, in cosmetics and in traditional medicine for its choreretics, hepatoprotective and antitumorigenic activity [10]. Rosemary is also known to exhibits antioxidant [11–14], and antimicrobial activities [15–18]. The potent antioxidant and antibacterial properties of rosemary extracts have been mainly attributed to its major diterpene, carnosic acid [19–22] and some compounds of the essential oil. Carnosic acid is quite unstable and, usually, is converted to carnosol upon heating. Carnosol can degrade further to produce other compounds such as rosmanol, epirosmanol and metoxyepirosmanol which still possess antioxidant activity.

Thus, rosemary extracts have a great interest for the food industry as a source of active compounds but to obtain a use-

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ful natural food additive is necessary to improve the functional activities while eliminating the interfering compounds that produce odour, taste and colour.

Supercritical fluids have useful physical properties such as low viscosity and high diffusivity into the sample matrix. This results in remarkably faster mass transport than in common organic solvents. Furthermore, SFE operates at low temperatures, which makes it a very suitable technique for the extraction of thermolabile compounds, such as antioxidants. Also because of these properties, supercritical fluids are uniquely suited to preparative separations [23] in which the supercritical solvent is used as mobile phase. Among the different methods available to separate complex extracts for the isolation of one or more compounds of the mixture, preparative-scale chromatographic techniques on both polar and non-polar stationary phases are a useful alternative. Most of the separations have been performed using HPLC but the disadvantages of these methods are the sample dilution and the high consumption of organic solvents. In supercritical fluid chromatography (SFC), the lower operating temperatures, the higher diffusivities of the solutes and the lower viscosity of the eluent also provide advantages compared to liquid chromatography (LC) [24] such as: fast analysis time, yielding at least a three-fold increase in the throughput, easy recovery of products by simple decompression, low consumption of organic solvents and wider range of applicability [25,26].

The objective of the present work was the separation of a complex supercritical fluid rosemary extract by semi-preparative SFC to obtain fractions with improved functional activities to be used as food ingredients and/or nutraceuticals. The isolation of biologically active fractions can lead to obtaining ingredients and/or nutraceuticals more active at lower concentrations. The isolated fractions have been chemically and functionally characterized using HPLC, GC, and antioxidant and antimicrobial in vitro assays.

2. Experimental

2.1. Samples and chemicals

The rosemary sample (*Rosmarinus officinalis* L.) consisted of dried rosemary leaves obtained from Herboristeria Murciana (Murcia, Spain). Rosemary leaves were collected during September and dried using a traditional method previously described [27]. Cryogenic grinding of the sample was performed under carbon dioxide and particle size was determined by sieving the ground plant material to the appropriate size (between 999 and 500 μm). The whole sample was stored in amber flasks at −20 °C until use (a maximum of 2 months).

2,2-Diphenil-1-pycril hydrazyl hydrate (DPPH, 95% purity) and carnosic acid (93%) were purchased from Sigma–Aldrich (Madrid, Spain).

Chloroform, methanol, acetonitrile (ACN) and acetone were all HPLC grade from Lab Scan (Dublin, Ireland). Ethanol (99.5%) was obtained from Panreac (Spain) and acetic acid (99%) from Merck Schuchardt (Germany).

Milli-Q water was obtained from a purification system (Millipore). CO_2 (N-48 quality) was obtained from Air Liquide España S.A. (Madrid, Spain).

2.2. Supercritical fluid extraction of rosemary

The extraction of rosemary extract was carried out in a pilot-scale supercritical fluid extractor (Iberfluid, Spain) with a 285 ml extraction cell, previously described [28]. The extraction cell was made of 316 steel and was equipped with a 0.5 μm frit at the inlet and a 2 μm frit at the outlet. The extraction pressure was controlled by micrometering valves, and the carbon dioxide pump was from Bran + Luebbe (Germany). Fractionation was achieved in two different separators assembled in series, with independent control of temperature and pressure, by either a decrease in pressure, or in pressure and temperature.

The extraction cell was filled with 60 g of ground rosemary and 90 g of washed sea sand (Panreac). Dynamic extraction was performed at 150 bar and 40 °C, and fractionation pressures were set in a first stage at 50% of extraction pressure and in the second stage at a fixed value of 20 bar. Ethanol (7%) was used as modifier. The addition of ethanol started after the selected pressure had been reached half of the extraction time (60 min).

All extracts were kept under N_2, at −20 °C in the dark, and ethanol was eliminated at 35 °C in a vacuum rotary evaporator.

2.3. Supercritical fluid chromatography (SFC) system

Fig. 1 shows a schematic diagram of the preparative supercritical fluid chromatography pilot plant (Thar Designs, USA) employed. The separation was carried out in a 25 cm × 10 mm i.d. Supelco SIL LC-Diol packed column (5 μm particle diameter) purchased from Supelco (Bellefonte, PA, USA) witch is placed inside a water bath with temperature control. The column pressure is controlled by a back pressure regulator and the column is coupled to an UV/vis detector (UV 1000 model) purchased from SpectraSystem (San Jose, CA, USA). The CO_2 and modifier are pumped by high pressure pumps, and the CO_2 pump is cooled by a circulating bath at 5 °C. The sample is injected through a Rheodyne 6 port valve (700 μL injection loop). The pilot plant has three cyclonic separators, two in which the sample can be fractionated and a waste, with controlled temperature to collect different fractions from the injected sample. The plant has a computerized PLC-based instrumentation. The range of column pressures/temperatures tested was from 80 to 200 bar, and from 40 to 80 °C. The CO_2 flow rate was kept constant at 20 g/min. Ethanol was used as a modifier at different percentages between 5 and 15%.

2.4. Antioxidant activity assay

The antioxidant activity was determined by the DPPH scavenging assay based on a procedure described by Brand-Williams et al. [29]. This method consist in the neutralization of free radicals of DPPH (1,1-diphenyl-2-picryl hydrazyl) (Sigma–Aldrich, Spain) by the antioxidant extracts. For each fraction obtained in SFC and the original supercritical extract, different concen-

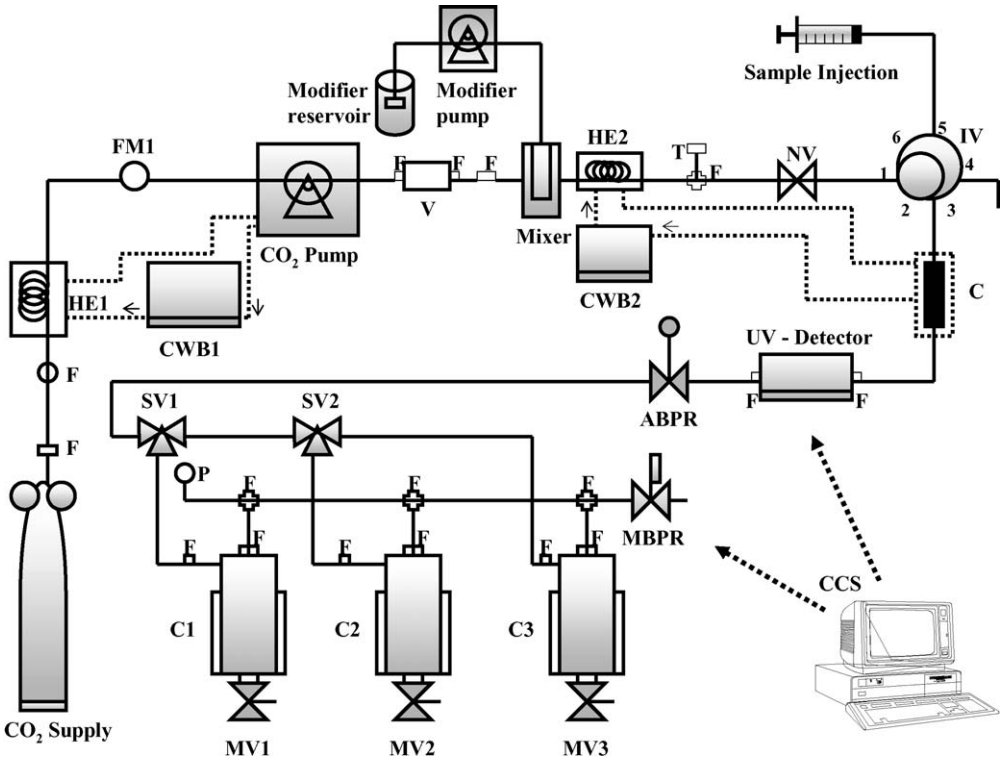

Fig. 1. Schematic diagram of the preparative-supercritical fluid chromatograph (Prep-SFC) used in the present study. F: filters; HE1 and HE2: heat exchangers; FM1: mass flow meter; CWB1: CO_2 pump cryogenic bath; CO_2 pump; V: pressure dampener; modifier pump; mixer: high pressure mixer; CWB2: column temperature heat exchanger; T: thermocouple; NV: needle valve; IV: Rheodyne 6 port valve; C: column; ABPR: back pressure regulator; SV1 and SV2: low pressure solenoid valves; C1, C2 and C3: cyclonic separators; MV1, MV2 and MV3: separators out valves; MBPR: manual restrictor; CCS: computer control system; P: pressure meter; Modifier reservoir; sample injection; CO_2 supply.

trations were tested (from 1 to 10 μg/mL in DPPH–methanol solution). One thousand nine hundred and fifty microlitres of DPPH solution (23.5 μg/L in ethanol) were placed in test tubes and 50 μL of the different concentrations of samples were added. Reaction was completed after 3 h at room temperature and absorbance was measured at 516 nm in a Shimazdu UV-120-01 spectrophotometer (Shimazdu, Kyoto, Japan). Ethanol was used to adjust zero and DHHP–ethanol solution as a reference sample. The DPPH concentration in the reaction medium was calculated from the following calibration curve, determined by linear regression ($r = 0.999$): $Y = 0.0247X - 0.0029$.

The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% or EC_{50}. Thus, the lower the EC_{50}, the higher the antioxidant power. Each determination was repeated twice.

2.5. Determination of antimicrobial activity

The extracts were individually tested against a panel of microorganisms including *Staphyloccocus aureus* ATCC 25923, *Escherichia coli* ATCC 11775 and *Candida albicans* ATCC 60193. Bacterial strains stock cultures were kept on nutrient agar at 4 °C. *Candida albicans* was kept on Sabouraud dextrose agar at 4 °C.

A broth microdilution method was used, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS), for determination of the minimum inhibitory concentration [30]. All tests were performed in Mueller-Hinton broth supplemented with 0.5% tween 20, with the exception of yeasts (Sabouraud dextrose broth + 0.5% tween 20). The inocula of bacterial strains were prepared from overnight Mueller-Hinton broth cultures at 37 °C. Yeasts were cultured overnight at 25 °C in Sabouraud dextrose broth. Test strains were suspended in Mueller-Hinton (bacteria) or Sabouraud dextrose (yeasts) broth to give a final density 10^7 cfu/mL. The rosemary extracts dilutions in ethanol ranging from 50 to 1 mg/mL.

The 96-microwell plates were prepared by dispensing into each well 185 μL of culture broth, 5 μL of the inoculums and 10 μL of the different extracts dilutions. The final volume of each well was 200 μL. Plates were incubated at 37 °C for 24 h for bacteria and at 24 °C for 48 h for yeasts. Negative controls were prepared using 10 μL of ethanol, the solvent used to dissolve the rosemary extracts. Chloranphenicol and amphotericin B (Sigma, Madrid) were used as positive reference standards to determine the sensitivity of the microbial species used. After incubation, the MIC of each extract was determined by visual inspection of the wells bottom, since bacterial growth was indicated by the presence of a white "pellet" on the well bottom. The lowest concentration of the extract that inhibited growth of the microorganism, as detected as lack of the white "pellet", was designated the minimum inhibitory concentration (MIC). The minimum bactericidal and fungicidal concentration (MBC) was determined by making subcultures from the clear wells which

did not show any growth. Each test was performed in triplicate and repeated twice.

2.6. HPLC-DAD analysis

The analysis of the original extract and the isolated fractions was carried out in an HPLC (Varian Pro-star) equipped with a Nova Pack C_{18} column (Waters) of 15 mm × 4.6 mm and 3.5 μm particle size. The mobile phase consisted of 1% acetic acid in acetonitrile (solvent A) and 1% acetic acid in water (solvent B) applying the following gradient: 0–5 min, 50% B; 5–15 min, 50–30% B; 15–40 min, 30–0% B. The flow rate was constant at 0.7 mL/min. Injection volume was 20 μL and the detection was accomplished by using a diode array detection system Varian storing the signal at a wavelength of 230 nm.

2.7. GC-FID analysis

SFE original rosemary extract and SFC fractions were analyzed by using a Varian 3400 (Varian, Walnut Creek, CA, USA) gas chromatograph equipped with a 1177 split/splitless injector (Varian). The system was coupled to a Saturn 2000 chromatography software system (Varian). A 30 m × 250 μm i.d. fused silica capillary column coated with a 0.25 μm layer of SE-54 stationary phase (Agilent Tecnologies) was used. Helium was the carrier gas at 18 psig.

Solutions of 10 mg/mL were prepared by dissolving the supercritical fluid extract and supercritical fluid chromatographic fractions in acetone. Three microliters of each solution were injected in a split mode (1:10 split ratio) at 200 °C. The oven temperature was programmed from 100 °C (10 min constant temperature) to 250 °C at 5 °C/min (10 min constant temperature); then to 300 °C at 15 °C/min. Final temperature was maintained for 10 min. Compounds were tentatively identified by comparison of retention time of those of standards injected at the same conditions and previous data.

3. Results and discussion

3.1. Retention behavior of carnosic acid in SFC

In order to select the best conditions for the fractionation of supercritical rosemary extracts by Prep-SFC in a diol column, different factors have been studied including mobile phase pressure, temperature and composition (in terms of concentration of modifier). The range of conditions selected to carry out the optimization at preparative scale were chosen based on previous results of our research group studying the solubility and separation of carnosic acid at analytical scale [31–33]. Following is a detailed description of the step-by-step optimization.

Fist of all, column temperature was optimized; three temperatures were tested, 40, 60 and 80 °C, while keeping the pressure and the modifier percentage constant at 200 bar and 10% ethanol, respectively. As expected, increasing the column temperature results in longer retention times of the solutes due to the decrease in density of the mobile phase, thus, 80 °C were selected to have a stronger retention of carnosic acid thus favoring the separation of similar compounds that can be found in the complex supercritical rosemary sample. Also, at 80 °C, carnosic acid integrity was maintained and no degradation was observed.

Once selected the column temperature, experiments were carried out to study the combination of pressure and modifier in the elution of carnosic acid. As expected, at isothermal conditions the density increases with the pressure thus increasing the solvating power. This results in shorter retention times and narrower peak widths as the pressure goes high. Similar behavior is found with the modifiers: the addition of a modifier shortens the retention mainly because the solvating power of the mobile phase increases; however, the modifier can also influence the retention by deactivation of the active sites of the packing material. Table 1 shows the results of carnosic acid behavior in terms of retention time and peak width at different chromatographic conditions (pressure and percentages of ethanol in the mobile phase). It is important to consider that no elution was obtained using pure CO_2 as mobile phase at the experimental conditions tested. This is in agreement with previous data published by our group for the elution of carnosic acid at analytical scale [33] where it was demonstrated that very high pressures (370 bar) were needed in order to elute carnosic acid from the chromatographic column when neat CO_2 was used. Considering that the semi-preparative SFC plant does not allow pressures higher than 300 bar, no elution was then possible. As can be seen, the best combination pressure/modifier can be found at intermediate percentage of ethanol, that is, 10%, since separations carried out using 5% or 15% of modifier provide too long and too short retention times, respectively. By using 10%, it is possible to select the best pressure conditions able to provide both, enough retention and narrower peak. Thus, the conditions selected as optimum for the fractionation of the SFE rosemary extract were: column temperature, 80 °C; column pressure, 130 bar; 10% ethanol as modifier; total mass flow 20 g/min being the CO_2 mass flow 18 g/min. The temperature of the three cyclones was set to 50 °C.

Table 1
Retention time (t_R) and peak width at half height ($w_{1/2}$) of carnosic acid standard in SFC at different pressures and percentages of modifier

Pressure (bar)	5% Ethanol		10% Ethanol		15% Ethanol	
	t_R (min)	$w_{1/2}$ (mm)	t_R (min)	$w_{1/2}$ (mm)	t_R (min)	$w_{1/2}$ (mm)
130	39.8	43.6	8.2	9.6	3.3	4.8
150	17.0	24.0	5.3	8.9	2.8	4.2
200	12.2	14.2	3.5	3.5	1.6	2.2
250	11.1	12.4	1.5	1.5	1.5	1.8

All the experiments were performed at constant temperature equal to 80 °C.

Table 2
HPLC identification, neat peak area and peak area contribution (%) of compounds found in the supercritical rosemary extract and in the different cyclones (C1, C2, C3) after preparative SFC in diol column

Compounds	t_R (min)	UV Mx Abs (nm)	Total extract area (%)	C1 area	C1 area (%)	C2 area	C2 area (%)	C3 area	C3 area (%)
Scutellarein	3.68	230, 275, 332	3.5	15.158.594	5.25	11053113	2.51	2197803	1.59
NI 1	3.81	255	5.7	144.544.448	50.04	3360688	0.76	3211081	2.33
Rosmanol isomer	4.42	230, 273	1.0	n.d.[a]	n.d.	2396716	0.54	3112626	2.26
Genkwanin	4.92	230, 266, 334	4.2	49.534.156	17.15	6494556	1.48	2964488	2.15
Carnosol	10.20	232, 282	25.4	6.541.069	2.26	116525424	26.49	17360018	12.58
NI	11.21	231, 424	3.8	n.d.	n.d.	64670284	14.70	12551332	9.09
NI 5	11.94	230, 284	1.7	n.d.	n.d.	6013212	1.37	11780574	8.54
NI	12.24	230, 266, 329	2.0	n.d.	n.d.	17048856	3.88	n.d.	n.d.
NI	12.56	230, 267, 329	1.7	n.d.	n.d.	18354632	4.17	9870840	7.15
Carnosic acid	14.40	239, 283	40.7	n.d.	n.d.	172270368	39.16	15126071	10.96
Methyl carnosate	17.12	231, 281	5.1	n.d.	n.d.	15302235	3.48	20571826	14.91
NI	19.45	230	2.6	n.d.	n.d.	4895318	1.11	15771393	11.43
NI	19.65	230, 284	1.5	n.d.	n.d.	n.d.	n.d.	5586948	4.05
NI 7	26.06	230, 282	1.2	n.d.	n.d.	n.d.	n.d.	6756526	4.67

[a]n.d.: compound not detected.

3.2. Characterization of SFE rosemary extract

Previous to its separation by Prep-SFC, the supercritical rosemary extract was analyzed by HPLC-DAD using a method based on a previous work done in our laboratory [28]. Compounds were characterized for their retention times (t_R) and UV spectra (see Table 2). As commercial availability of reference standards was limited, tentative identification of the compounds was made based on previous data published by other authors [34,35] and previous experience of our research group [28]. Two groups of phenolic compounds have been identified: diterpenes such as carnosic acid, carnosol and methyl carnosate, and flavonoids, such as genkwanin and scutellarein. Some compounds were also detected but could not be identified or completely identified although others, such as NI 1 or NI 5 (non-identified 1 and 5, respectively), had been previously described [34]. To perform the study of the semiquantitative composition of the original SFE extract and SFC fractions, the detected compounds' relative percentages (referred to the total area of the selected components based on DAD peak area at 230 nm) were calculated, as shown in Table 2.

The essential oil composition of the extract was analyzed by GC using a method based on a previous work done in our laboratory [18]. Results are reported in Table 3. The main constituents of this SFE extract were verbenone (26.7%), camphor (24%), borneol (11.9%) and 1,8-cineole (10.2%). This chemical composition showed no big differences with the ones previously reported for rosemary essential oil [17,36] and for a supercritical rosemary extract [18].

3.3. Separation of SFE rosemary extracts by Prep-SFC

Fig. 2 shows the retention behavior of the rosemary SFE extract and the collection windows for the isolation in different cyclones. The fractionation was as follows: cyclone 1 (C1), from 0 to 3.20 min; cyclone 3 (waste, C3), from 3.20 to 6.45 min and cyclone 2 (C2), from 6.45 to 15 min. At these conditions, a good separation was obtained in a reasonable time, 15 min.

The isolation and enrichment of specific compounds in the cyclones is done following a protocol consisting in the injection of the sample up to 12 times and collection in the cyclones at the selected times, as mentioned above.

3.4. Chemical characterization of the isolated fractions

The fractions collected in the three different cyclones were chemically characterized to know their composition and to evaluate the enrichment or purification achieved during the SFC separation step.

Table 2 shows the composition of the three fractions (cyclones 1–3) by RP-HPLC for the identification and quantification of antioxidant compounds (carnosic acid and by-products). As can be seen, cyclone 1 contains mainly flavonoids and NI 1 and only a small concentration of carnosol. Cyclone 3, which is actually the waste, shows no real fractionation containing almost all the compounds detected in the original extract; among them, small quantities of carnosic acid and carnosol were found while methyl carnosate was preferentially fractionated in this cyclone. In contrast, cyclon 2 is mainly enriched in carnosic acid and carnosol. Actually, 92% of total carnosic acid and 83% of total

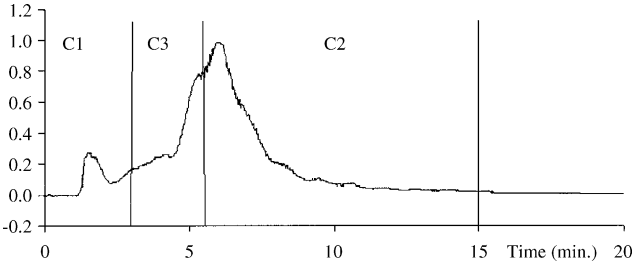

Fig. 2. SFC fractionation of a SFE rosemary extract at 130 bar, 80 °C and 10% ethanol in three different cyclones (C1, C2, C3).

Table 3
GC identification, peak area contribution (%) and concentration (when standards were available) of compounds found in the original SFE extract of *Rosmarinus officinalis* L.

Compounds	t_R (min)	Total extract area (%)	Total extract concentration (mg/ml)	C1 area	C1 area (%) (concentration, mg/ml)	C2 area	C2 area (%) (concentration, mg/ml)	C3 area	C3 area (%) (concentration, mg/ml)
α-Pinene	5.35	n.d.	n.d.	1.628	0.4 (0.06)	n.d.	n.d.	n.d.	n.d.
1,8-Cineole	6.71	10.2	0.06	7.645	1.9 (0.07)	n.d.	n.d.	n.d.	n.d.
Linanool	8.54	1.7	n.d.	10.664	2.7	n.d.	n.d.	n.d.	n.d.
Camphor	10.27	24.0	0.14	72.042	182 (0.63)	n.d.	n.d.	1.638	19.88 (0.01)
Borneol	11.51	11.9	0.06	56.420	14.2 (0.42)	n.d.	n.d.	1.792	21.74 (0.01)
Terpinen-4-ol	11.99	2.4	n.d.	13.828	3.5	n.d.	n.d.	n.d.	n.d.
α-Terpineol	12.47	7.1	n.d.	39.594	10.0	n.d.	n.d.	n.d.	n.d.
Verbenone	12.89	26.7	0.15	121.286	30.6 (0.88)	1.235	100 (0.03)	3.341	40.54 (0.04)
Trans-caryophyllene	21.62	7.2	n.d.	37.123	9.4	n.d.	n.d.	n.d.	n.d.
NI	22.67	2.8	n.d.	15.923	4.0	n.d.	n.d.	n.d.	n.d.
NI	26.22	1.5	n.d.	6.641	1.7	n.d.	n.d.	n.d.	n.d.
NI	27.57	1.5	n.d.	5.328	1.3	n.d.	n.d.	n.d.	n.d.
NI	28.10	2.8	n.d.	8.173	2.1	n.d.	n.d.	1.470	17.84

Neat peak area and peak area contribution (%) is included for compounds found in the different cyclones after preparative SFC in diol column (C1, C2, C3).

Table 4
Concentration of carnosic acid (ppm) and efficient concentration EC_{50} (μg/ml), obtained in the DPPH test

Sample	EC_{50} (μg/ml)	LC-DAD (ppm carnosic acid)
Original extract	6.52	670
Cyclone 2	5.34	740
Cyclone 3	19.1	74

carnosol are detected in cyclone 2. Also, 43% of the total methyl carnosate is found in this cyclone. If we compare the original extract with cyclone 2, in terms of carnosic acid concentration, an increase from 670 to 740 ppm is obtained.

As for the essential oil composition of the isolated fractions, Table 3 shows the results obtained for the main constituents of rosemary essential oil [17,18,36]. As can be seen, the conditions for the supercritical rosemary extract fractionation allow the selective isolation of the essential oil in the first cyclone while only verbenone was collected in separator 2 and in a very small amount. This fractionation can improve the characteristics of the functional ingredient since it is able to improve the quality by removing the residual aroma in the fraction collected in cyclone 2. Fig. 3 shows the GC chromatograms of the fractions collected after SFC separation.

3.5. Functional characterization of the isolated fractions

3.5.1. Antioxidant activity

Antioxidant activity of the fractions isolated by Prep-SFC was measured using the DPPH method. Table 4 shows the EC_{50} (μg/mL) values of the original SFE rosemary extract along with those of fractions collected from cyclone 2 and cyclone 3, where antioxidant compounds were found. Results show that all samples have an important antioxidant activity demonstrated by the low EC_{50} values (less than 20 μg/mL in all cases), although with some important differences among them. As for the original extract and the fraction collected in cyclone 2, the higher the concentration of carnosic acid, the lower the EC_{50} obtained. These results are in agreement with data reported by other authors where carnosic acid has been described as the most active rosemary's antioxidant compound [19,33]. Nevertheless, results achieved in cyclone 3 (with a carnosic acid content of 74 ppm) do not follow the same trend, thus, further studies of synergy effects to predict EC_{50} values through the chemical composition of the samples were carried out. An estimated model based on previous results of our group [37] was used to consider the effect of other compounds in the antioxidant activity of rosemary extracts. Three compounds, in decreasing order of importance to predict the mentioned activity, were selected: carnosic acid, methyl carnosate and carnosol. The estimated model was:

$$EC_{50} = 64.422 - 0.501\,(\text{carnosic acid}) - 3.998\,(\text{methyl carnosate}) - 0.694\,(\text{carnosol})$$

with values of 0.95 for the coefficient of determination (R^2), and 3.02 for the standard error of estimate. As can be seen, all of them contribute negatively to the equation, that is, decreasing

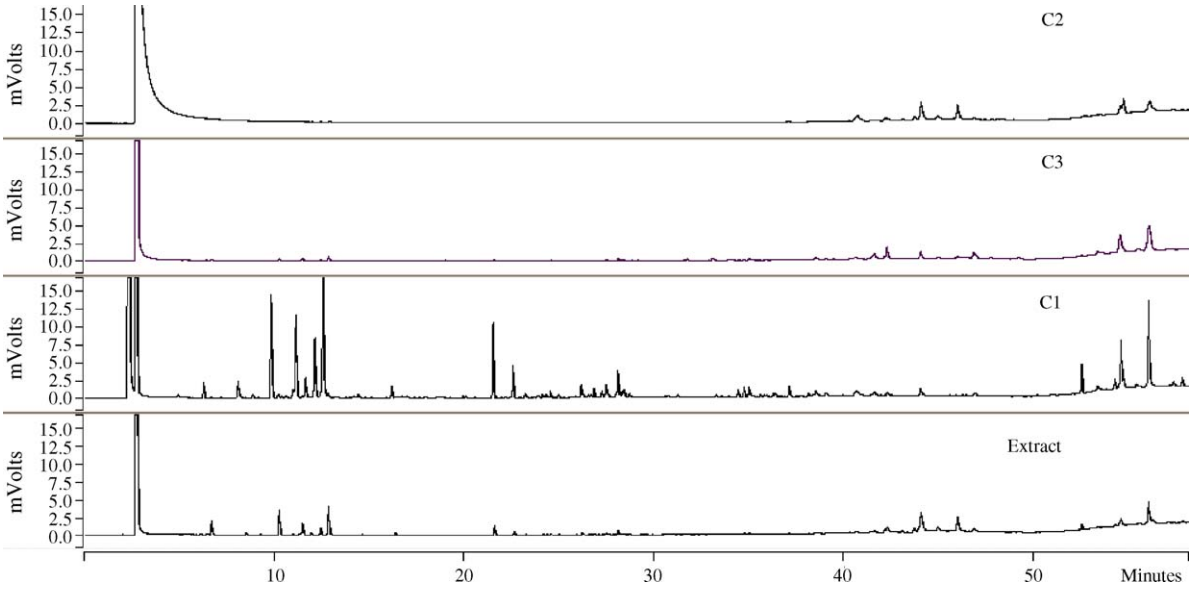

Fig. 3. GC chromatograms of the different fractions collected after the SFC separation at 130 bar, 80 °C and 10% ethanol.

the EC_{50} value when increasing their content in the composition of the extracts. The possible explanation of our results is the important contribution of methyl carnosate in the fraction of cyclone 3, where the normalized area corresponded to 15% of the total sample.

3.5.2. Antimicrobial activity

Three different microbial species, including a gram positive bacteria (*Staphylococcus aureus*), a gram negative bacteria (*Escherichia coli*) and a yeast (*Candida albicans*), were used to screen the antimicrobial activity of the rosemary fractions isolated by Prep-SFC. The antimicrobial activity of these fractions was assessed by the determination of the minimal inhibitory concentration (MIC) and the bactericidal concentration (MBC). The results obtained are given in Table 5.

All the fractions collected from the different cyclones (1–3) showed antimicrobial activity against all the microorganism tested, with MBCs values in the range of 0.35–1.75 mg/mL. The most active fraction, in all cases, was the one collected in cyclone 2, followed by the fraction from cyclone 3, whereas the least active was fraction 1. Comparing these results with those obtained when using the original extract, only fraction collected in cyclone 2 presented a higher antimicrobial activity than original extract (with an improvement of about 40%). Meanwhile, fraction in cyclone 3 showed a similar antimicrobial activity to the original extract and this activity in cyclone 1 was clearly lower.

Staphylococcus aureus was the most sensitive microorganism, whereas the least susceptible was the yeast *Candida albicans*. These results were in agreement with those obtained with the rosemary original extract.

In order to explain the higher antimicrobial activity found in the fraction collected in cyclone 2 and knowing that this fraction is mainly composed by carnosic acid, a pure standard of this compound was also examined for antimicrobial activity under the same conditions (Table 5). The antimicrobial activity showed by carnosic acid was very similar to that reported for the fraction collected in cyclone 2, which seemed to indicate that the activity of this fraction could be associated with the presence of this compound. The small differences found in the antimicrobial activity between the standard and the fraction 2 could be attributed to the presence of small quantities of verbenone in the fraction 2, since this compound has been reported to have some antimicrobial activity [18].

The antimicrobial activity detected in the fraction collected in cyclone 3, similar to that found in the original extract, could be explained since cyclone 3 showed no real fractionation containing almost all the compounds detected in the original extract; with a lower quantity of carnosic acid respect to clyclone 2.

On the other hand, fraction collected in cyclone 1 presented the lowest antimicrobial activity, although the presence of oxygenated terpenes as camphor, borneol and verbenone, compounds that have been reported to posses a high antimicrobial activity [18,38,39] could induce to expect a higher activity in this fraction. However, this data could be explained by the small concentration of these compounds detected by GC analysis.

Table 5
Antimicrobial activities of different cyclones after preparative SFC in diol column from a supercritical fluid extract of *Rosmarinus officinalis* L. and carnosic acid

Sample	MBC[a]		
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*
Original extract	0.6	0.75	1.5
Cyclone 1	0.9	1.25	1.75
Cyclone 2	0.35	0.5	0.9
Cyclone 3	0.7	0.75	1.25
Carnosic acid	0.4	0.5	1
Reference antibiotics	10	10	100

[a] MBC, minimum bactericidal concentration. Values given as mg/mL for samples and μg/mL for antibiotics.

As a general comment, the level of enrichment achieved cannot be obtained by SFE either using stepwise extraction or fractionation, as has been demonstrated in previous work of our research group, at least using the systems and conditions studied and reported in [27,37].

4. Conclusions

In this work, the potential use of preparative-supercritical fluid chromatography has been demonstrated to fractionate complex supercritical rosemary extracts. By a careful selection of the separation conditions it is possible to obtain two different fractions, one collected in cyclone 2 with an improvement of about 20% and 40% of antioxidant and antimicrobial activities, respectively, compared to the original extract and with no residual rosemary aroma and another one containing the essential oil. With this approach, a better fractionation of the supercritical extracts can be achieved with an increase of functional properties and with a very low consumption of organic solvents (only 10% of ethanol in the mobile phase).

With the process developed in this work we have been able to improve both, the antimicrobial and antioxidant activity of the supercritical rosemary extract to a high extent; by increasing the biological activity of the isolated fraction, lower amounts can be used without loss in activity which can have several advantages in terms of both, industrial costs and possible side effects of the functional ingredients or nutraceuticals, which depend on the concentration used.

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