Supercritical Fluid Extraction and Fractionation of Different Preprocessed Rosemary Plants

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Two-step supercritical fluid extraction of rosemary leaves at selected conditions of pressure and temperature is proposed to divide the oleoresin into two fractions with different antioxidant activities and essential oil compositions. Rosemary leaves obtained from different sources have been extracted and evaluated in terms of antioxidant activity and essential oil yield and composition. Also, a new device is proposed to improve the performance of the technique in terms of sample collection after SFE.

Keywords: *Rosemary antioxidant; rosemary essential oil; supercritical fluid extraction; fractionation*

INTRODUCTION

Antioxidants are used in food products containing fats and oils to prevent or retard the development of oxidative rancidity. Oxidation of unsaturated bonds to form hydroperoxides can lead to changes in color, odor, and aroma that can lower the product quality. Antioxidants, along with packaging and storage conditions, are important factors in extending the shelf life of a food product (Maestro and Borja, 1993).

The use of synthetic antioxidants in the food industry is severely restricted as to both application and level of use. Among the natural antioxidants, rosemary has been widely accepted as one of the spices, along with sage, with highest antioxidant activity (Chipault et al., 1952). Several studies about the antioxidative constituents of rosemary indicate that the major antioxidant active compounds are the phenolic diterpenes carnosic acid, carnosol, rosmanol, and epi- and isorosmanol (Inatani et al., 1983; Schwarz and Ternes, 1992; Schwarz et al., 1992).

Rosemary essential oil is also of high interest as a flavor and fragance ingredient in the food, flavoring, and pharmaceutical industries. Essential oil obtained from the plant must have a high resemblance to the original material and, at the same time, has to be obtained free of residues.

One important trend in the food industry is the demand of natural food additives, free of chemicals. Therefore, special attention has been paid in the study of new processes directed toward the obtention of ingredients, to be used in food industry, with both GRAS and GMP labels, that is, manufactured using safe solvents and by good elaboration processes (Sanders, 1993).

An important contribution to this objective has been the introduction of supercritical fluid extraction (SFE) technology as an alternative to conventional procedures such as solid−liquid extraction, steam distillation, and molecular distillation. The use of supercritical solvents, mainly CO_2, has several advantages related to its solvent power and ease of solvent removal. Carbon dioxide has a low latent heat of evaporation and a high volatility, which allow one to obtain extracts with very low residual solvent levels without the use of high temperatures and, therefore, without the possibility of degradation of thermal labile components.

SFE has been proposed for antioxidant (Nguyen et al., 1991, 1994; Muehlnikel, 1992; Gerard et al., 1995) and essential oil extraction (Stahl and Gerard, 1985; Reverchon and Senatore, 1992; Moyler, 1993; Coehlo, 1997) from rosemary leaves. Antioxidant extracts originated by SFE have shown a higher activity than extracts obtained by using solvent extraction with organic solvents (Schwarz et al., 1992), 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. As suggested by Schwarz et al. (1992), by using CO_2 extraction, carnosic acid is the major diterpene component found in the extract.

Some work has been done related to fractionation of rosemary (Reverchon et al., 1992); nevertheless, exhaustive characterization of the different extracts obtained, in terms of essential oil content and composition and antioxidant activity, has not been performed.

To study the importance of the raw material, as well as the drying technique, rosemary from three different sources has been used. Taking into account that drying has been recognized as a technique that can greatly influence the behavior of the plant in the SFE process (Reverchon et al., 1992), it seemed interesting to study the effect of the different drying techniques on the quality of the final product obtained (antioxidant activ-

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ity and essential oil composition). In the present work, a two-step SFE process has been applied to the sequential fractionation of the rosemary oleoresin; the resulting extracts from the different raw materials used showed properties clearly differentiated.

To be able to perform the studies mentioned above, a new device has been developed to improve the performance of the technique in terms of sample collection after SFE. The homemade device consisted of a refrigerated reservoir designed to avoid extract losses after CO_2 decompression.

EXPERIMENTAL PROCEDURES

Sample. Rosemary (*Rosmarinus officinalis* L.) was obtained from three different sources: (1) commercial rosemary spice (McCormick, Madrid, Spain) obtained in a local market; (2) dried rosemary leaves obtained from an herbalist's shop (dried using the traditional method, as follows: once collected, the plant is ventilated to remove humidity, covered with a blanket to avoid sunlight, and allowed to dry in a ventilated place for 20−30 days, depending on the season (Murcia, Spain); (3) fresh rosemary leaves collected from a back garden in Madrid, Spain. Samples 1 and 2 were subjected to extraction without further preparation, whereas sample 3 was dried by using the following procedures: (a) freeze-drying; (b) oven at 45 °C until constant weight; (c) vacuum rotary evaporator at 35 °C until constant weight.

Samples were ground and stored in amber flasks in the refrigerator until use.

Reactants. 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95% purity) was obtained from Sigma-Aldrich (Madrid, Spain), acetone from Quimicen (Madrid, Spain), ethanol from Ferosa (Barcelona, Spain), and methanol (HPLC quality) from Lab Scan (Dublin, Ireland). CO_2 (N-48) was kindly donated by AL Air Liquide España S.A. (Madrid, Spain).

Extraction Method. A Suprex PrepMaster (Suprex Corp., Pittsburgh, PA) supercritical fluid extractor was used to perform all of the experiments. Sample [0.85 g (dry weight basis)] was placed into a 5 mL stainless steel extraction cell. Supercritical CO_2 flow rate was controlled using a needle valve as variable restrictor; flow rates between 3 and 4 mL/min were obtained at the experimental conditions tested.

Sample was extracted by using a two-step method; the first fraction was obtained at 10 MPa and 40 °C and the second fraction at 40 MPa and 60 °C. Extraction time was 5 min static extraction followed by 30 min dynamic extraction for each step.

In a previous paper (López-Sebastián et al., 1998), conditions for essential oil extraction were optimized, and because results showed a good agreement with those provided by other authors (Stahl and Gerard, 1985; Reverchon et al., 1992), optimal conditions were selected for essential oil obtention (fraction 1, 10 MPa, 40 °C).

Residue obtained after the first extraction was re-extracted at conditions selected for fraction 2. This fraction, which contains the antioxidant compounds, was obtained by extraction at conditions previously suggested by other authors (Djarmati et al., 1991; Nguyen et al., 1994).

Supercritical fluid extracts were collected in a specially designed device that provided two main advantages compared to conventional collecting devices, that is, minimization of losses of material and retention of volatile constituents of the essential oil in fraction 1. Figure 1 shows a scheme of the homemade device. It is composed of a two-piece chamber; the lower part contains a replaceable glass test vial (2 cm × 0.5 cm) where the extract is deposited during the extraction. The vial is surrounded by a cooling jacket that can be refrigerated at a target temperature by using a convenient gas or liquid. Yield of the process can be obtained by weighing the vial; other operations can be also performed directly. After 5 min of static extraction, the needle valve was opened and the dynamic extraction started. By using this device, yields ranging from

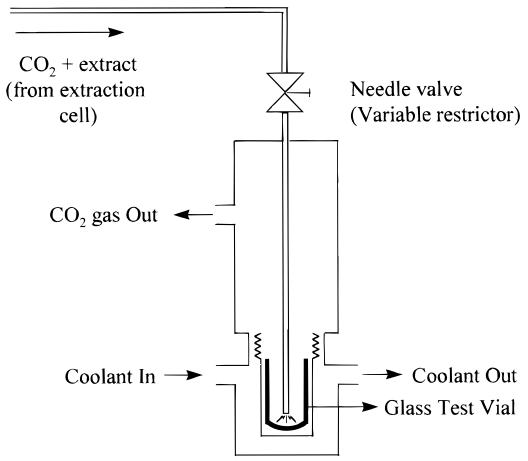

Figure 1. Scheme of the homemade device designed to collect extracts after SFE.

1 to 1.5% (dry weight basis) for the first fraction and from 1 to 1.8% (dry weight basis) for the second fraction were obtained.

Aroma Analysis and Identification by GC/MS. Fractions 1 and 2 were characterized for aroma content and composition. Solutions of 500 mg/kg were prepared by dissolving the supercritical fluid extracts in acetone.

A Varian 3400 (Varian, Walnut Creek, CA) gas chromatograph equipped with a 10077 split/splitless injector (Varian) and coupled to a mass spectrometer Saturn 2000 (Varian) was used to perform the analysis. The system was coupled to a model Saturn 2000 chromatography/mass spectrometry 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 was used. Helium was the carrier gas at 10 psig. Three microliters was injected in a split mode (1:10 split ratio) at 200 °C. The oven temperature was programmed from 40 °C (10 min at constant temperature) to 240 °C at 5 °C/min and to 280 °C at 20 °C/min; final temperature was maintained for 5 min.

A mass spectrometer (EI 70 eV) was used with a 5 min solvent delay and with a mass range from 45 to 650. Compounds were tentatively identified by comparison of the spectra with those in a general library (NIST).

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. (1988), modified as previously described (López-Sebastián et al., 1998). The method consisted of the neutralization of free radicals of 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) by the antioxidant. The procedure used is as follows: 0.014 g of DPPH was 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. Ten grams of DPPH solution was placed in test tubes, and 30 μL of rosemary extract solution was added (which corresponds 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 Corp., Kyoto, Japan). Methanol was used to adjust zero. The equation described by Lamaison et al. (1988) was utilized 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 the oxidation index. Measurements were performed in triplicate.

RESULTS AND DISCUSSION

Two fractions of rosemary oleoresin were obtained by SFE at two different conditions of pressure and temperature; depending on the variables selection, the essential oil and the antioxidant fraction can be extracted without much contamination of nonvolatile and

Table 1. Normalized Areas of the Chromatograms Obtained by GC/MS of the Rosemary Extracts Corresponding to Fractions 1 and 2

		fraction 1					fraction 2				
t_r (min)	compound	rosemary 1[a]	rosemary 2	rosemary 3a	rosemary 3b	rosemary 3c	rosemary 1	rosemary 2	rosemary 3a	rosemary 3b	rosemary 3c
7.4	3-octanol	6.78	2.34	16.78	19.19	24.81	11.92	10.05	48.35	25.51	54.05
13.4	γ-terpinene	0.38	0.41	0.57	2.28	2.77	3.07	8.99	3.57	2.61	1.15
14.3	camphene	0.25	0.23	0.26	0.78	0.91	1.71	3.86	0.57	0.67	
15.2	1,2,3-trimethylbenzene				0.51				2.31		
15.4	1,2,4-trimethylbenzene				0.46						
16.3	1-octen-3-ol		0.16								
16.5	3-octanone		0.67					5.49			
16.6	β-pinene	0.15	0.44	0.29	1.94	0.77	0.10	1.00	8.09	0.23	
17.3	sabinene		0.01	0.08	0.26				0.52	0.31	
17.7	α-terpinene	0.11	0.08					0.20	0.71		
18.1	β-cimene	0.14	1.06	0.18	0.57	0.24	0.52	1.27	0.50	0.53	0.02
18.3	limonene	0.11	1.03	0.26	0.53	0.23	0.62	2.00	1.30	0.85	0.43
18.5	1,8-cineole	3.28	12.29	1.62	3.95	2.52	11.09	18.18	4.84	3.15	1.79
20.6	α-terpinene		0.91								
21.3	linalool	0.99	1.61	2.90	3.28	2.01	0.72	1.01	0.98		2.11
21.5	fenchol			0.15	0.22	0.44	0.08			1.38	0.77
22.0	bicyclo(3.1.0)hexane, 6-isopropylidene		0.35	0.64	0.92	0.46	0.13	0.19		0.38	0.42
22.9	camphor	9.43	40.85	7.02	10.25	6.64	13.12	21.16	5.74	4.81	5.52
23.2	isopulegol	0.30	1.49		0.60					0.21	
23.4	isocamphopinone	0.29	0.14	-	0.57	0.30			0.36	3.87	
23.9	borneol	21.27	7.62	9.59	12.72	11.39	14.32	5.21	2.22	0.39	7.58
24.2	terpinen-4-ol	1.69	1.51	1.24	0.78	0.71	1.46	1.03	0.44		1.23
24.4	*p*-cymen-9-ol	0.65	0.23	0.55	0.42	0.29	0.36	0.15		1.17	
24.7	α-terpineol	7.64	4.01	3.27	2.44	2.42	5.04	3.06	0.98	42.87	1.71
25.0	verbenone	15.80	9.85	27.24	18.85	13.72	11.75	7.74	7.47	0.22	12.77
26.1	unknown	0.09		0.53	0.37	0.36			0.08	0.29	
26.3	unknown			0.96	0.57	0.68			0.28		0.66
26.4	unknown			0.18	0.15	0.11					
27.3	thymol	0.17	0.06	0.12			0.24	0.09		0.21	
27.4	bornyl acetate	2.65	2.47	0.79	0.46	0.27	1.70	1.25	0.21		
27.6	*p*-cymen-2-ol	2.81	0.03				2.45	0.10			
29.0	unknown	0.19	0.15	0.21	0.26	0.26		0.14		0.10	
29.9	α-cubebene	0.80	0.44	0.43	0.23	0.31		0.24	0.35	0.42	
30.1	copaene	0.40	0.37	1.44	0.85	1.04	0.57	0.21		0.11	0.69
30.8	methyleugenol	0.12	0.12	0.38	0.86	0.67	0.24		0.36	1.46	
31.4	*trans*-caryophyllene	0.97	3.31	0.39	3.46	4.69	1.10	2.24	1.74		2.79
31.9	γ-gurjunene	0.18	0.13	5.29	0.17	0.31			0.11		
32.1	unknown	0.19	0.11	0.37	0.35			0.05		0.24	
32.4	α-caryophyllene	0.47	0.00	1.00	0.66	1.00	0.50	1.49	0.41	0.37	0.57
32.8	γ-cadidene	0.87	0.33	1.92	1.18	2.06	0.55	0.27	0.61	0.07	0.77
33.0	cuparene	0.92	0.26	0.39	0.10	0.27	0.54	0.17	0.34	0.13	
33.3	β-cubebene	0.25	0.16	0.44	0.34			0.04			
33.5	α-muurolene	0.30	0.25	1.09	0.64	1.20			0.36	0.16	0.54
33.7	β-bisobolene	0.54	0.32	0.69	0.45	0.69	0.35		0.34	0.37	
33.9	γ-muurolene	0.51	0.20	1.51	1.02	1.77	0.29	0.12	0.25	6.14	0.68
34.0	δ-cadidene	1.13	0.48	2.81	1.77	3.35	0.70	0.35	0.60	0.19	0.99
34.1	calamenene	0.84	0.12	1.10	0.80	1.34	0.34	0.07	0.45		0.23
34.5	unknown	0.08	0.02	0.15		0.17					
34.6	unknown	0.26	0.18	0.27	0.10	0.28	0.21	0.10		0.14	
35.7	unknown	3.02	0.15	1.61	0.29	0.96	2.33		1.36		0.42
36.0	unknown	0.51	0.36	0.37	0.61	0.44	0.46				
36.3	unknown	1.41	0.29	0.27	0.13	0.47	1.28	0.17	0.62		
36.9	β-cedrene	0.72	0.17	0.08			0.53	0.28			
37.4	β-guaiene	2.51	0.35	0.68	0.43	0.73	3.16	0.50	0.29		
37.8	cadalene	1.50	0.24	0.32	0.09	0.32	1.24		0.58		
38.1	unknown	0.49	0.19	0.44	0.20	0.42	0.45	0.31			
43.2	unknown	1.63	0.44	0.16		0.21	1.21	0.35	0.62		
43.7	unknown	0.91	0.30			0.15	0.75	0.27	0.42		
45.9	unknown	0.38					0.35				
46.1	abietatriene	2.74	0.70	1.26	1.52	2.54	2.07	0.60	0.95	0.45	1.39
46.9	unknown	0.15	0.01	0.36	0.38	0.75	0.20				
	total area (area counts)	6,062,572	26,069,446	5,099,370	4,628,341	3,130,910	4,009,004	3,381,368	1,359,738	2,119,125	740,369

[a] Codes as described under Experimental Procedures.

volatile matter, respectively, as is shown in the following discussion. Extracts obtained from different pretreated raw materials were evaluated, at the conditions mentioned under Experimental Procedures, in terms of intensity of rosemary aroma, essential oil composition, and antioxidant activity.

Essential Oil Extraction. Rosemary oleoresin was extracted in a two-step process, and fractions 1 and 2 were obtained containing, respectively, essential oil and antioxidant compounds. Both fractions were analyzed for volatile composition. Table 1 shows the results obtained for fractions 1 and 2 of the five different types of rosemary studied, that is, three different types and one dried by using three different drying techniques, as described under Experimental Procedures. Total area of the chromatograms corresponding to the first fraction

ranged from 3 million area counts, for rosemary type 3 (dried by using vacuum rotary evaporation) to 26 million area counts for rosemary type 2. Analyzing the results for rosemary 3 dried by using different procedures, it can be seen that the treatment that provided the highest quantity of rosemary essential oil was freeze-drying followed by drying in oven at 45 °C and vacuum rotary evaporation. Freeze-drying is the mildest temperature treatment; therefore, less aroma loss is expected to be obtained. Drying in oven at 45 °C implied a higher temperature treatment than rotary evaporation (35 °C), but the first method is faster than the second and is performed in the absence of light. The vacuum treatment was a very slow process, in daylight, and artifacts could easily be formed; in fact, olfactory tests showed development of a noncharacteristic rosemary aroma.

Around 45 compounds were identified and quantitated by GC/MS; most of them had been previously described by other authors as typical constituents of rosemary essential oil. As can be seen in Table 1, comparing the normalized areas of the compounds detected, differences in essential oil composition were found, due to both type of rosemary and drying method.

Essential oil composition closest to the fresh rosemary, as compared with previous results obtained in our laboratory (Reglero et al., 1989), corresponded to rosemary 2 (herbalist's shop; dried at ambient temperature); its major components were those typically described for fresh rosemary, that is, camphor (40%), 1,8-cineole (12%), verbenone (9%), borneol (7%), and bornyl acetate (2.5%). Among the different types of rosemary studied, some quantitative differences can be detected. Rosemary 1 (rosemary spice), probably due to both the long storage time and conditions, showed a composition very different from the fresh rosemary leaves; the main components were borneol, verbenone, and camphor (21, 16, and 9% respectively). Fresh collected rosemary, dried by using different techniques 3a, 3b, and 3c, had a compositions really different from those of the other two types of rosemary; this was probably due to both type of rosemary and type of drying method used prior to SFE.

As it has been previously suggested by other authors (Reverchon et al., 1992), drying techniques can significantly affect the final product obtained by SFE because they can induce selective elimination and/or decomposition of some compounds. As can be seen, major components of rosemary 3a, 3b, and 3c were 3-octanol (which ranged from 17% for rosemary 3a to 25% for rosemary 3c), verbenone (which ranged from 14% for rosemary 3c to 27% for rosemary 3a), borneol (which ranged from 10% for rosemary 3a to 13% for rosemary 3b), and camphor (which ranged from 7% for rosemary 3c and 3a to 10% for rosemary 3b); their concentrations are different from those that can be found in fresh rosemary (Reglero et al., 1989), that is, around 40% camphor, 3% verbenone, and 6% borneol. There are two main factors that have to be considered: the drying process, which influences the essential oil composition and, therefore, extract quality; and the effect of the drying process on the plant cells. Damage to plant cell walls can produce selective releasing of compounds that can be more easily extracted in the SFE conditions used.

Results for fraction 2 showed a great reduction, between 33 and 87% of aroma intensity, with respect to the first fraction. These results indicate that a selective extraction of essential oils was achieved at the conditions used for obtaining fraction 1.

Table 2. Oxidation Index (Micrograms per Milliliter) for Fractions 1 and 2 Obtained by SFE of Rosemary Oleoresin

	fraction 2	fraction 1
rosemary 1[a]	28.5	61.9
rosemary 2	33.9	50.7
rosemary 3a	43.9	148
rosemary 3b	153.8	177.7
rosemary 3c	128.4	5331

[a] Codes as described under Experimental Procedures.

Antioxidant Extraction. As was described under Experimental Procedures, material extracted at the mildest extraction pressure and temperature was re-extracted at 40 MPa and 60 °C to selectively obtain the antioxidant fraction. To evaluate the selectivity of the method, extracts corresponding to both fractions 1 and 2 were characterized in terms of antioxidant activity. Table 2 shows the oxidation index (micrograms per milliliter) (average of three replicates, RSD% ~2%) obtained for the two fractions (1 and 2) of the different types of rosemary tested in the study. As can be seen by comparing the results for fractions 1 and 2, the antioxidant activity of fraction 2 is, in all cases, higher than that of fraction 1. Results for extracts obtained at 40 MPa and 60 °C ranged from 28.5 (rosemary 1, spice) to 153 (rosemary 3, dried in the oven), whereas the oxidation index corresponding to fraction 1 (which contained the essential oil) ranged from 50 (rosemary 2, herbalist's shop) to 5330 (rosemary 3, dried in a vacuum rotary evaporator).

From a comparison of the results obtained for each fraction, it can be seen that there is a correlation between the antioxidant activity and the thermal treatment utilized because the rosemary leaves treated at the mildest thermal conditions provided a higher antioxidant activity than those dried at higher temperatures. Rosemary 3b dried at 45 °C in the oven showed the lowest activity. It has been demonstrated that heat treatment can cause decomposition of carnosic acid to other phenolic diterpenes with lowest antioxidant activity (Schwarz et al., 1992). The fraction corresponding to the essential oil (fraction 1) showed almost no antioxidant properties compared to fraction 2, no matter the type of rosemary tested. Among the results obtained for fraction 1, the worst corresponded to those dried in the oven or in a vacuum rotary evaporator.

Results accomplished in the present work demonstrate that a two-step SFE process provides an acceptable selectivity in terms of obtaining two fractions with properties clearly differentiated: fraction 1 contains the essential oil (characterized by GC/MS) and fraction 2 the extract with antioxidant properties (characterized by free radical method). Complete characterization of the two fractions allows one to conclude that the first is mainly formed by essential oil (with almost no antioxidant activity) and the second contains compounds with antioxidant properties (reduced rosemary aroma). It is important to consider that the methodology used in the present paper could be further improved by adopting a better selection of the extraction time and fractional separation of the extracts. Future research is directed toward the optimization, by means of an experimental design, of the extraction and fractionation conditions of rosemary plants.

The homemade device, especially designed to collect

SFE extracts, minimizes extract losses and avoids continuous cleaning of the collection reservoir. Yields attained were in the same range as those obtained by other authors (Reverchon et al., 1992); that is, they ranged from 1 to 1.5% (dry weight basis) for the first fraction and from 1 to 1.8% (dry weight basis) for the second fraction.

In terms of rosemary sample, the study shows a great influence of both type of rosemary and drying treatment on the final results. It seems clear that drying at ambient temperature, in a ventilated place, is the method that provides better results. Rosemary treated in more aggressive conditions shows an important antioxidant activity loss (higher with greater thermal treatment used) and a modification in essential oil composition with reference to fresh rosemary.

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