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Supercritical Fluid Extraction of Natural Antioxidants from Rosemary: Comparison with Liquid Solvent Sonication

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Supercritical fluid extraction (SFE) and liquid solvent sonication, in combination with two different sample treatments, were compared for the extraction of natural antioxidants from rosemary leaves. Dried, ground, and sieved rosemary leaves (20 mg) were subjected to SFE with CO_2 at 355 bar at 100 °C (CO_2 density 0.72 g/mL) for 20 min at a liquid flow rate of 4 mL/min. The analytes were concentrated on an ODS trap and subsequently eluted with acetone. Antioxidants in the SF and liquid solvent extract were analyzed by HPLC. Compounds of known antioxidant activity such as carnosol, carnosic acid, and methyl carnosate were identified by mass spectrometry of the HPLC fractions collected. Freezing and grinding the samples in liquid nitrogen resulted in decreased carnosic acid recoveries. Supercritical CO_2 extraction provided the highest recovery of carnosic acid from rosemary leaves (35.7 mg/g), the lowest relative standard deviation (4.4%), and the cleanest extract—no cleanup prior to HPLC was required. Among the liquid solvents studies, only acetone provided comparable results (73% recovery relative to SC-CO_2 extraction); however, it required decoloration with active carbon prior to HPLC analysis.

Antioxidants are added to fat-containing foods to prevent the formation of off-flavor and toxic compounds resulting from lipid oxidation.[1,2] Rosemary and sage leaves are well-known for their antioxidant properties. Their extracts in vegetable oils are sold as a natural alternative to synthetic antioxidants (e.g., BHT and BHA) as they possess similar or even higher antioxidant activity.[3–5] The antioxidative properties of plant extracts are attributed to tocopherols, flavonoids, and polyphenols. Recently, Cuvelier et al.[3] reported the purification and identification of six major antioxidant compounds of sage oleoresin by IR, MS, and ^{1}H NMR spectrometry as phenolic diterpenes: carnosol, carnosic acid, rosmadial, rosmanol, epirosmanol and methyl carnosate. Carnosic acid and carnosol have been determined in rosemary and sage leaves by reversed-phase HPLC with UV[6] and electrochemical[7] detection. These polyphenols have been proposed as substitutes for other, less effective natural antioxidants such as tocopherols, use of which is constrained by toxicological reasons.

An increasing number of liquid extraction methods are being superseded by supercritical fluid extraction (SFE) methods that are rapid, automatable, and selective and avoid the use of large amounts of toxic solvents. CO_2 is the most commonly used supercritical fluid because it has modest critical conditions, can readily be separated from solutes, poses no environmental problems, and is nonflammable and inexpensive.[8,9] The intrinsic features of SFE are ideal for the extraction of natural products from plant materials.[10] SFE is especially indicated for thermolabile compounds, as extractions are carried out at a low temperature, and provides cleaner plant extracts because the degradation of certain compounds by lengthy exposure to high temperatures or oxygen is avoided and chlorophylls are insoluble in SC-CO_2. SFE extracts are also more concentrated (even solventless extracts can readily be obtained). In addition, the nontoxic character of CO_2 is a strong reason to choose SFE for food applications. In most cases, SFE is more efficient than conventional extraction methods; also, it allows different classes of compounds to be fractionated by changing the extraction temperature and/or pressure. Verschuere[11] reported the class-selective extraction of hops with SC-CO_2; 90% of the essential oils were extracted at a density of

(1) Boyd, L. C.; Green, D. P.; Giesbrecht, F. B.; King, M. F. *J. Sci. Food Agric.* **1993**, *61*, 87.
(2) Huisman, M.; Madsen, H. L.; Skibsted, L. H.; Bertelsen, G. *Z. Lebensm. Unters. Forsch.* **1994**, *198*, 57.
(3) Cuvelier, M.-E.; Berset, C.; Richard, H. *J. Agric. Food Chem.* **1994**, *42*, 665.
(4) Hall, C., III; Cuppett, S.; Wheeler, D.; Fu, X. *J. Am. Oil Chem. Soc.* **1994**, *71*, 533.
(5) Chen, Q.; Shi, H.; Ho, C.-T. *J. Am. Oil Chem. Soc.* **1992**, *69*, 999.
(6) Okamura, N.; Fujimoto, Y.; Kuwabara, S.; Yagi, A. *J. Chromatogr.* **1994**, *679*, 381.
(7) Schwarz, K.; Ternes, W. *Z. Lebensm. Unters. Forsch.* **1992**, *195*, 95.
(8) Westwood, S. A., Ed. *Supercritical Fluid Extraction and its Use in Chromatographic Sample Prepration*; Chapman & Hall: Glasgow, 1993.
(9) Luque de Castro, M. D.; Valcárcel, M.; Tena, M. T. *Analytical Supercritical Fluid Extraction*; Springer: Heidelberg, 1994.
(10) Castioni, P.; Christen, P. Veuthey, J. L. *Analusis* **1995**, *23*, 95.
(11) Verschuere, M.; Sandra, P.; David, F. *J. Chromatogr. Sci.* **1992**, *30*, 388.

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0.3 g/mL, following isolation of bitter acids and triglycerides at a 0.7 g/mL density in a second step. On the other hand, after optimizing the SFE of volatile constituents (monoterpene, sesquiterpene, lactone, and phenolic representatives) from a model matrix plant, Smith and Burford[12] found that pressure and temperature changes were insufficient to selectively extract the test compounds selected. They proposed a fractionation technique using a silica column to obtain a highly purified sesquiterpene lactone fraction free from less polar terpenes.

The extraction of essential oils from rosemary and other spices[13−20] is well documented. Hyphenated SFE-GC systems[14−16] are particularly successful for the determination of these volatile compounds since they avoid analyte losses and provide maximal sensitivity with minimal handling.

Djarmati et al.[21] proposed the re-extraction of the bleached alcoholic extract of sage with SC-CO_2 to improve its antioxidant properties and reduce its odor, taste, and color as far as possible. They isolated rosmanol-9-ethyl ether, a phenolic diterpene and a more active antioxidant than BHT, from the SC-CO_2 extract. The preparative SC-CO_2 extraction of other phenolic lipids such as anacardic acids, cardols, and cardanols from cashew nut shell also yielded a better quality product than pentane extraction.[22]

Other major compounds processed by SFE in food and industrial food analyses include carotenes[23,24] and tocopherols.[25,26]

The preparation of plant extracts with antioxidative properties demanded by food, cosmetic, and pharmaceutical industries usually involves the use of organic liquid solvents.[3,5,6,27] Acetone has been reported to be a more efficient solvent for extracting rosemary antioxidants than are hexane and methanol.[5]

The aim of this work was to develop a rapid, automated, quantitative method for the determination of carnosic acid in order to characterize rosemary populations and the seasonal variations in carnosic acid content. SC-CO_2 has been used for the first time as extractant for isolating these compounds from rosemary leaves. The recovery thus achieved for the principal component, carnosic acid,[6] is compared with that obtained by using various organic solvents. In addition, the optimization study presented can be useful for implementing the preparative-scale isolation of rosemary antioxidants by SC-CO_2.

(12) Smith, R. M.; Burford, M. D. *J. Chromatogr.* **1992**, *600*, 175.
(13) Walker, D. F. G.; Bartle, K. D.; Breen, D. G. P. A.; Clifford, A. A.; Costiou, S. *Analyst* **1994**, *119*, 2789.
(14) Hawthorne, S. B.; Krieger, M. S.; Miller, D. J. *Anal. Chem.* **1988**, *60*, 472.
(15) Hawthorne, S. B.; Miller, D. J.; Krieger, M. S. *Fresenius Z. Anal. Chem.* **1988**, *330*, 211.
(16) Hawthorne, S. B.; Miller, D. J.; Krieger, M. S. *J. High Resolut. Chromatogr.* **1989**, *12*, 714.
(17) Blanch, G. P.; Ibáñez, E.; Herraiz, M.; Reglero, G. *Anal. Chem.* **1994**, *66*, 888.
(18) Huston, C. K.; Ji, H. *J. Agric. Food Chem.* **1991**, *39*, 1229.
(19) Reverchon, E.; Senatore, F. *Flavour Fragrance J.* **1992**, *7*, 227.
(20) Myler, D. A. *Flavour Fragrance J.* **1993**, *8*, 235.
(21) Djarmati, Z.; Jankov, R. M.; Schwirtlich, E.; Djulinac, B.; Djordjevic, A. *J. Am. Oil Chem. Soc.* **1991**, *68*, 731.
(22) Shobha, S. V.; Ravindranath, B. *J. Agric. Food Chem.* **1991**, *39*, 2214.
(23) Marsili, R.; Callahan, D. *J. Chromatogr. Sci.* **1993**, *31*, 422.
(24) Spanos, G. A.; Chen, H.; Schwartz, S. J. *J. Food Sci.* **1993**, *58*, 817.
(25) Lanças, F. M.; Queiroz, M. E. C.; da Silva, I. C. E. *Chromatographia* **1994**, *39*, 687.
(26) Saito, M.; Yamauchi, Y.; Inomata, K.; Kottkamp, W. *J. Chromatogr. Sci.* **1989**, *27*, 79.
(27) Chevolleau, S.; Mallet, J. F.; Ucciani, E.; Gamisans, J.; Gruber, M. *J. Am. Oil Chem. Soc.* **1992**, *69*, 1269.

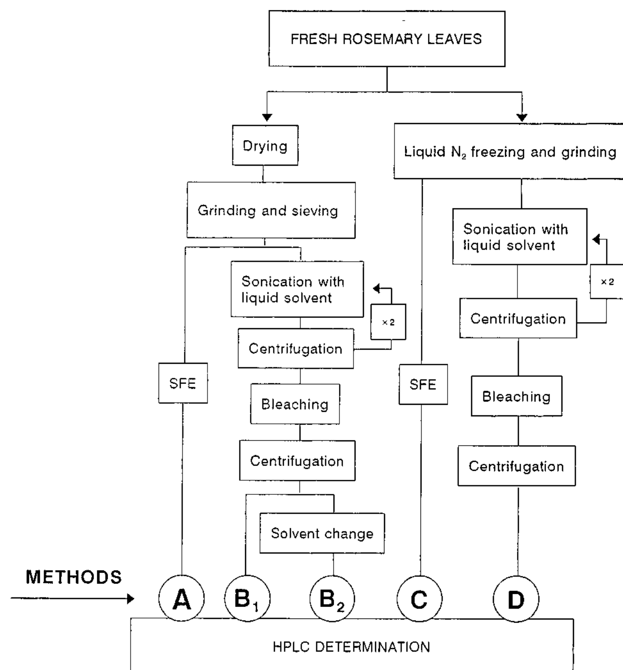

Figure 1. Schematic diagram of the steps involved in the five extraction methods proposed for the determination of natural antioxidants in rosemary leaves by HPLC.

EXPERIMENTAL SECTION

Instrument and Apparatus. All SFE experiments were performed on a 7680A Hewlett-Packard supercritical fluid extractor furnished with a 7-mL extraction vessel, a variable restrictor, and a solid-phase trap packed with stainless steel beads or Hypersil octadecylsilica (ODS) material. A 1050 Hewlett-Packard liquid chromatograph equipped with a 20-μL loop injector, an Ultrabase-C_{18} (250 × 4.6 mm, 5 μm) column, and an HP 1040A diode array detector were used for the separation/detection of antioxidants in the extracts. A Selecta ultrasonic bath was used for liquid solvent extractions. Finally, a Fisons VG Platform electrospray and a Fisons VG Autospec mass spectrometer were used to identify compounds in the extracts.

Reagents and Samples. Rosemary (*Rosmarinus officinalis*) leaves were sampled from a single plant of a wild population. Carnosic acid was a gift from Prof. N. Okamura (Fukuyama University, Fukuyama, Japan). A 1 g/mL carnosic acid stock solution in acetone was made from which calibration solutions were prepared by dilution with acetone. Extracts and solutions were stored at −40 °C in the dark.

SFE/SFC-grade CO_2 (Air Products), HPLC-grade dichloromethane (Romil Chemicals), *n*-hexane, methanol (Scharlau), and Normapur acetone (Prolabo) were used. Decolorizing active carbon (Panreac) was used to remove pigments from liquid solvent extracts.

Sample Treatment. Two different strategies were tested to reduce particle size; these, in combination with the extraction procedures, gave rise to the five methods described in Figure 1. Fresh rosemary leaves were dried at 50 °C, ground, sieved (≤500 μm; methods A and B) or ground immediately after freezing with liquid nitrogen (methods C and D).

SFE Procedure. The CO_2 was aspirated from a cylinder furnished with a dip tube, pressurized to 77−383 bar, and passed through the extraction vessel at a flow rate from 1 to 4 mL/min (liquid) by means of a dual-piston pump. The extraction vessel

was loaded with 20 mg of rosemary sample and was placed inside the extraction chamber, which was maintained at 40−120 °C throughout the experiment. Samples were subjected to static SFE for 0−10 min, depending on the particular experiment, followed by dynamic extraction for 0.5−60 min; the leached analytes were driven to a stainless steel bead or an ODS trap through a variable restrictor that avoided plugging to a great extent and provided a constant flow rate during the extraction process. In a subsequent step, following system depressurization the trap was rinsed with a liquid solvent (1.3 mL of acetone or acetonitrile) that was pumped through it at a flow rate of 0.5−1 mL/min by means of a syringe pump; the analyte solution was collected in a 2-mL vial. The trap was maintained at 5−20 and 10−20 °C during the extraction and rinsing steps, respectively. The nozzle temperature during extraction was 50 °C in all the experiments, but identical with that in the trap during the rinsing step.

Liquid Solvent Extraction/Sonication Procedure. The ultrasound-assisted extraction procedure used was based on that reported by Okamura et al,[6] with some modifications. Thus, 3 mL of a liquid solvent (acetone, methanol, hexane, or dichloromethane) was added to the sample (1.3 g of fresh rosemary leaves cryogenically ground using liquid nitrogen and 0.2 g of dried, ground, and sieved leaves in methods D and B, respectively); the mixture was sonicated in an ultrasonic bath for 5 min and then centrifuged at 3500*g* for 5 min. The solvent supernatant was transferred to a test tube and the residue subjected to the same procedure twice. The highly colored extracts obtained were combined and bleached by adding 0.3 g of active carbon. The mixture was centrifuged and the bleached supernatant transferred to a volumetric flask and made to 10 mL. A portion of the acetone and methanol extracts was directly injected into the HPLC column (methods B_1 and D); the hexane and dichloromethane extracts required solvent changeover before injection into the aqueous chromatographic mobile phase (method B_2). Thus, 1 mL of such extracts was evaporated under nitrogen and reconstituted with the same volume of acetone.

Chromatographic Separation/Detection. The natural antioxidants in the liquid extracts obtained by SFE and liquid solvent sonication were determined on an Ultrabase-C_{18} (250 × 4.6 mm, 5 μm) column. The injected volume was 20 μL, and the flow rate of the mobile phase was 1 mL/min. An acetonitrile/10 mM acetic acid solution gradient from 70:30 (for the first 8 min) to 100% acetonitrile in 5 min was used to separate the analytes in less than 17 min and to ensure that no compounds remained in the column. Separation was also accomplished by using a 0.1% phosphoric acid solution instead of acetic acid; however, the latter was selected because it is more suitable for mass spectometric detection. Resolution improved as the acetic acid concentration in the mobile phase was increased, through decreased peak width. Marked peak broadening was observed for carnosic acid at acetic acid concentrations below 10 mM. However, lower acetic acid concentrations decreased baseline drift (caused by the mobile-phase composition changing during the gradient programmed). Chromatograms were recorded at 230 nm. The main peak in the chromatogram (Figure 2) was identified as carnosic acid, the precursor for other polyphenolic diterpenes with high antioxidant activity.[3,6,28] The calibration curve obtained for carnosic acid from the peak area was linear (r = 0.997) throughout the range studied (100−1000 μg/mL).

(28) Geoffroy, M.; Lambelet, P.; Richert, P. *Free Radical Res.* **1994**, *21*, 247.

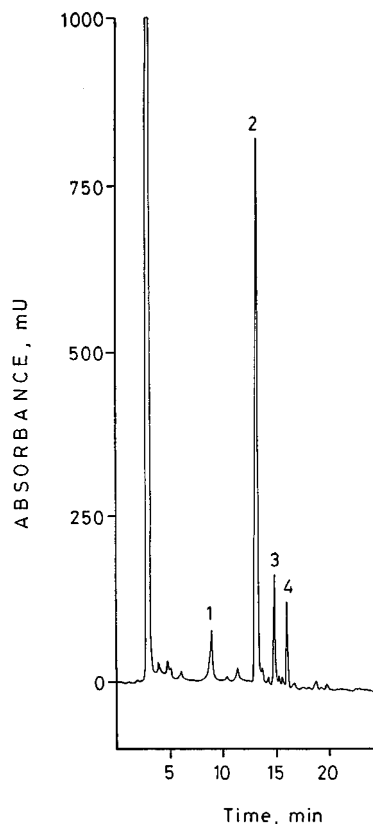

Figure 2. HPLC chromatogram for a SC-CO_2 extract from rosemary leaves (20 mg). SFE conditions: 355 bar, 100 °C, and 4 mL/min (liquid) for 20 min. Peak identification: 1, carnosol; 2, carnosic acid; 3, methyl carnosate; 4, unknown compound(s). For HPLC conditions, see text.

Mass Spectrometric Identification. Fractions of the chromatographic effluent (0.2−0.4 mL) for the major peaks in the chromatogram were collected individually (and concentrated under nitrogen when necessary) in order to identify components in the sample extracts by mass spectrometry. Seventeen fractions were screened by electrospray MS (negative ionization mode) in order to detect acid compounds such as carboxylic acids and phenolics from their M − 1 peaks. Ammonia was added to the solutions, and 10 μL of the mixture was injected into a 50:50 methanol/water carrier solution at 10 μL/min. The source temperature and cone voltage were 60 °C and 25 V, respectively. Electrospray MS for chromatographic peaks 2 and 3 exhibited M − 1 peaks at 331 and 345, respectively. A higher cone voltage (60 V) caused the loss of a CO_2CH_3 group in compound 3, corresponding to methyl carnosate. In addition, MS peaks at 393 (M − 1) and 456 (M − 1) were found in the nonfractionated extracts. The former suggested the presence of a rosmanol or epirosmanol ethyl ether (M = 394), but this could not be confirmed (even though this type of compound has only been identified in sage extracts[21]).

Mass spectra (electronic impact) for the compounds were recorded on a Fisons VG Autospec instrument (70 eV) by direct insertion of 1 μL of solution at a ionization chamber temperature of 225 °C. Following the m/z data and the relative abundances reported by Cuvelier,[3] peaks 1 and 3 in the chromatograms were identified as carnosol and methyl carnosate, respectively. Carnosol m/z (rel intensity): 330 (M^+, 19), 287 (19), 286 (100), 271 (8), 215 (17), 204 (11), 202 (11). Methyl carnosate m/z (rel intensity): 346 (M^+, 13), 301 (23), 300 (100), 285 (11), 257 (8),

Table 1. Optimization of SFE Variables

variable	range studied	optimum value
pressure, bar	77–383	383
temperature, °C	40–120	120
flow rate, mL/min	1–4	4
equilibration time, min	0–10	0
extraction time, min	0.5–60	20
rinsing solvent	acetone, acetonitrile	acetone
trap type	stainless steel, ODS	ODS
trap temperature, °C		
extraction step	5–20	10
rinsing step	10–20	20
rinsing flow rate, mL/min	0.5–1	1

244 (19), 232 (8), 231 (8), 229 (8). The spectrum obtained for peak 4 was impossible to interpret because it was probably impure.

RESULTS AND DISCUSSION

Optimization of SFE Variables. The effect of experimental variables was evaluated in order to develop a rapid, quantitative SFE method. The ranges over variables were studied, and the optimum values found are listed in Table 1 by way of summary of the optimization process. Trapping and rinse variables werestudied first in order to ensure quantitative collection of the extracted analytes. The peak area at 230 nm was used to study the extraction under different conditions. Area values (as shown in the graphs) were corrected by multiplication by the extract volume obtained and division into the sample amount used.

Trapping/Collection Variables. The trapping/collection efficiency of two types of trap (stainless steel beads, ODS) and two rinsing solvents tested (acetone, acetonitrile) was evaluated by comparing the peak areas obtained for the four major compounds in the extracts. Extractions were carried out with CO_2 at 299 bar, 50 °C (0.87 g/mL density) and a 1 mL/min flow rate for 15 min of dynamic extraction after 2 min of static extraction. The trap and nozzle temperatures were 10 and 50 °C, respectively, during extraction, and 10 °C during the rinsing step. The rinsing flow rate was 0.5 mL/min. The trappig efficiency of the stainless steel and ODS traps was very similar for carnosol, carnosic acid, and methyl carnosate; however the amount of unidentified compound(s) (peak 4) collected by the stainless steel bead trap was 3 times smaller than that collected by the ODS trap. The latter exhibited a higher volatility for this/these compound(s), which made it more prone to losses. Also, the ODS trap seemed to increase the stability of carnosic acid during extraction. The influence of the rinsing solvent on recovery was stronger than that of the trap type. The amounts recovered with acetone were 2–3 times those obtained with acetonitrile, depending on the particular compound and trap type. The effect was less marked for peak 4. An ODS trap and acetone as the rinsing solvent were thus selected for further experiments. Completeness of the rinsing step was confirmed by the fact that no compounds were obtained from a second rinse of the trap.

The influence of trap temperature during extraction was studied over the range 5–20 °C; differences in the amounts recovered at the different trap temperatures were within the standard deviation range. Similar results were obtained at the two nozzle and trap temperatures tested during the rinsing step (10 and 20 °C).

The amounts of analytes obtained by using the higher rinsing solvent flow rate tested were slightly greater (16% for carnosic acid). In addition, a higher flow rate shortened the rinse time, so 1 mL/min was chosen.

CO_2 Extraction Variables. The effect of extraction pressure on the extraction yield was studied at a constant temperature (40 °C). The solvent power of SC-CO_2 below 90 bar (0.5 g/mL) was too low to dissolve polar compounds such as carnosol, carnosic acid, and methyl carnosate; however, as expected, increased amounts of these compounds were extracted as the pressure was raised. On the other hand, this effect was not observed for compound 4, which was extracted at low densities and must thus be less polar than phenolic diterpenes.

The influence of extraction temperature on the extraction efficiency was studied at a constant pressure (350 bar). Although SC-CO_2 solvent power decreased with increasing temperatures (CO_2 densities varied from 0.93 to 0.65 g/mL for temperatures from 40 to 120 °C, respectively), the amount of carnosic acid increased as a result. This behavior reveled that the extraction rate was primarily controlled by analyte/matrix interaction rather than by the CO_2 solubility. Therefore, increased temperatures favored diffusion and/or desorption.[29] The largest amounts of carnosic acid were obtained at 120 °C; however, an extraction temperature of 100 °C was selected in order to lengthen the thimble cap lifetime. Moreover, no degradation of natural antioxidants was observed at high temperatures.

The effect of CO_2 flow rate on the extraction yield at a short extraction time (5 min), 355 bar, and 100 °C was studied. Increasing CO_2 flow rate resulted in increased yields of carnosic acid (the recoveries were 53, 67, and 81% at 1, 2, and 4 mL/min, respectively). Since no inert material was used to fill the almost empty extraction thimble (20 mg of sample in a 7-mL extraction thimble) a high flow rate was necessary in order to effectively flush the large void volume. The same influence was observed for carnosol and methyl carnosate; however, once again, the behavior of compound(s) 4 was different: high flow rates decreased the trapping efficiency.

Static extraction (5–10 min) prior to 5-min dynamic SFE did not improve recoveries. Therefore, a 0-min equilibration time was selected for further experiments.

Finally, the influence of the extraction time was studied under the previously selected conditions (355 bar, 100 °C, and 4 mL/min). The recovery of carnosic acid increased sharply during the first 2–3 min up to 20 min, beyond which it remained constant.

Reproducibility. The precision of the HPLC method for carnosic acid, expressed as percent relative standard deviation ($n = 7$), and that of the whole method (SFE + HPLC) was 2.3 and 3.6%, respectively.

Once the optimal SFE conditions were established, three successive extractions of the same rosemary sample (20 mg) were carried out in order to ensure completeness of the SFE. The amounts of carnosic acid found in the first, second, and third extractions were 95, 4, and 1%, respectively, of the total amount extracted.

Sample Particle Size. Grinding is an effective method for samples where analyte extraction is restricted by diffusion through the matrix.[30] The effect of particle size on the extration rate of rosemary antioxidants was apparent from the kinetic extraction curves (amount extracted vs extraction time) obtained for ground

(29) Langenfeld, J. J.; Hawthorne, S. B.; Miller, D. J.; Pawliszyn, J. *Anal. Chem.* **1995**, *67*, 1727.

(30) Hawthorne, S. B.; Galy, A. B.; Schmitt, V. O.; Miller, D. J. *Anal. Chem.* **1995**, *67*, 2723.

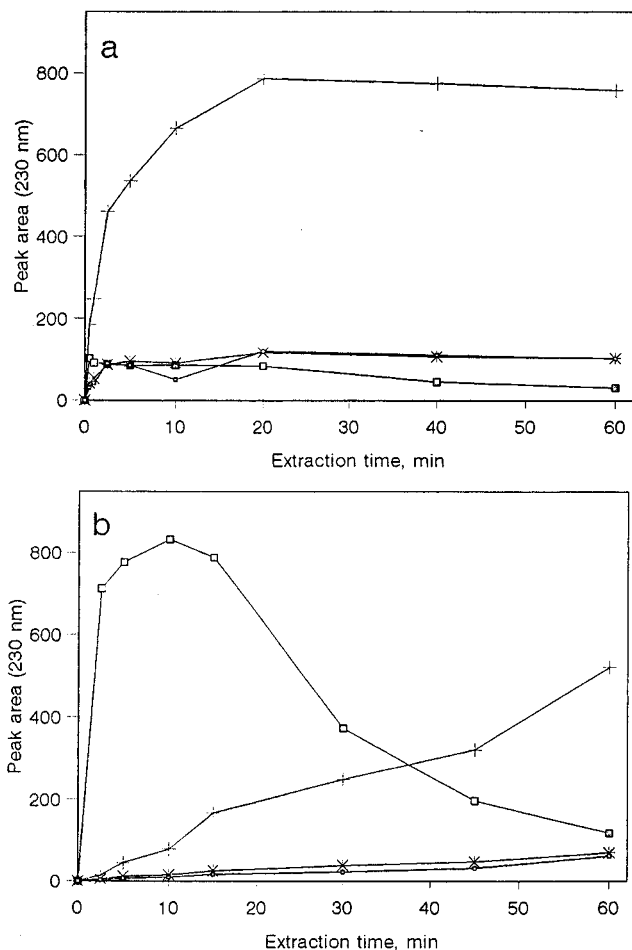

Figure 3. Effect of particle size reduction (grinding, method A) on the extraction profiles for carnosol (○), carnosic acid (+), methyl carnosate (*), and unidentified compound(s) (□): (a) ground, sieved sample; (b) whole leaves. SFE conditions: 4 mL/min CO_2 at 355 bar at 100 °C.

and sieved (particle size <500 μm) and unground rosemary leaves. The results are shown in Figure 3, where the peak areas of the four major chromatographic peaks are plotted as a function of extraction time for ground (a) and unground (b) samples. The extraction of carnosol, carnosic acid, and methyl carnosate from ground samples was faster (it was complete within 20 min, whereas 60 min was not long enough to achieve complete extraction of unground samples, which gave a 66% recovery relative to ground samples). Reducing particle size accelerated extraction, improved the extraction efficiency, and shortened the extraction time for natural antioxidants.

An interesting, surpsing behavior was observed for peak 4. The recovery decreased with the extraction time, so the trapping system was not efficient enough for this/these volatile compound(s). In addition, the amounts obtained from unground leaves were much greater than those extracted from ground leaves (only 12% of this compound was recovered from ground leaves). Such a large difference was the result of volatilization during grinding (a strong smell of rosemary was perceived during the process). The highest yield for this compound was achieved from unground leaves in 10 min; however, higher efficiency could be obtained by using a more efficient trapping system. In addition, the extraction of compound(s) 4 from unground sample was anomalously faster than that of carnosic acid. A surface distribution of compound(s) 4 such as that of rosemary essential oils may account for the higher extraction rate observed.

Table 2. Concentration of Carnosic Acid Found in Rosemary Leaves by HPLC after SFE or Liquid Solvent Extraction

solvent	carnosic acid ± SD, mg/g
SC-CO_2	35.7 ± 1.6
acetone	26.2 ± 1.5
methanol	15.9 ± 1.3
hexane	1.90 ± 0.08
dichloromethane	7.9 ± 1.1

Sample Treatment. Cryogenically ground samples should be introduced immediately into the extractor or added to the liquid solvent to avoid thawing and browning in the presence of oxygen. The oxidation of many plant components (polyphenols included) is accelerated when organel membranes are broken by freezing as they are exposed to oxygen and certain enzymes (e.g., polyphenoloxidases). The latter may account for the decreased amounts of carnosic acid recovered with method C. The amount of carnosol extracted was approximately the same as that obtained with method A; on the other hand, only 10% of the carnosic acid obtained with method A was recovered by method C. Also, the amount of methyl carnosate recovered was smaller (by up to 35%), but the area of peak 4 was 1.4 times that obtained with method A. Cryogenic grinding using liquid nitrogen is expected to reduced volatilization losses relative to alternative methods [e.g., compound(s) 4 during particle size reduction]; however, it seems to favor undesirable reactions of oxygen-labile compounds (e.g., carnosic acid).

One other drawback of method C arises from the presence of water during SFE. Although it caused no plugging problems during the SFE process because a variable restrictor was used, the high water content in the sample (~70%), which was not removed, could decrease the efficiency. Ionizable organics will be more difficult to extract in the presence of water as their solubility in CO_2 is decreased; however, the pH of water in contact with SC-CO_2 is acid (~2.9),[31] so weakly acidic species are not expected to become charged. In addition, the collection efficiency can be altered by the retention of water in the solid phase trap. Recoveries decreased, particularly for carnosol and peak 4, when water was removed from cryogenically ground samples by drying in a stove (80 °C) prior to extraction with SC-CO_2. The amounts of all of the compounds obtained using method D (acetone as liquid solvent gave rise to better results than methanol) were greater than those recovered with method C (4, 2, and 3 times for carnosic acid, methyl carnosate, and unidentified compound(s), respectively); however, they were half those obtained with method A, except for peak 4, the recovery of which was ~5 times higher. Once again, the behavior of compound 4 can be ascribed to its volatile nature. Since samples in method D are added to the liquid solvent while still frozen, the antioxidants are less prone to degradation than in method C.

Comparison with Ultrasound-Assisted Liquid Solvent Extraction. Methods A and B were compared by using four different liquid solvents. The results, shown in Table 2, revealed that the SFE method is more efficient than the conventional extraction method. The recovery of carnosic acid with SC-CO_2

(31) Toews, K. L.; Shroll, R. M.; Wai, C. M. *Anal. Chem.* **1995**, *67*, 4040.

was 136% relative to acetone extraction. The liquid extracts were highly green-colored (particularly the methanolic and acetone extracts), so a bleaching step (method B) was mandatory before injection into the HPLC column. Acetone was the most efficient liquid solvent among those studied. Its high polarity and hydrogen-bonding properties favor interactions with the phenolic diterpenes and facilitate their extraction. Nonpolar solvents such as hexane or dichloromethane, which are postulated to be better than polar solvents at preserving carnosic acid from oxidation,[6] were unsuitable for extracting phenolic diterpenes. The hydrogen-bonding ability of acetone and methanol seems to be crucial to leaching these phenolic compounds. The most important peak in the HPLC chromatogram for the hexane and dichloromethane extracts was that at 16 min, which once again suggests the less polar nature of this/these unidentified compound(s).

A second extraction of an acetone-extracted sample following the whole procedure again showed that the first extraction was exhaustive. Some degradation of carnosic acid can occur during acetone extraction, which may account for the yield difference.

FINAL REMARKS

The proposed SFE method (method A) is more rapid and simple to implement than the liquid solvent sonication method and offers higher efficiency and precision. Also, it replaces toxic organic solvents with an oxygen-free, more environmentally acceptable solvent. Moreover, human errors and personnel hazards are reduced as a consequence of the increased automation. One other significant advantage of SC-CO_2 for the extraction of rosemary leaves is that pigments such as chlorophylls are insoluble in the SF. The extracts obtained by using method A were cleaner than those provided by methods B and D, which required a decolorizing step.

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