JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Subcritical Water Extraction of Antioxidant Compounds from Rosemary Plants

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Subcritical water extraction at several temperatures ranging from 25 to 200 °C has been studied to selectively extract antioxidant compounds from rosemary leaves. An exhaustive characterization of the fractions obtained using subcritical water at different temperatures has been carried out by LC-MS, and the antioxidant activities of the extracts have been measured by a free radical method (DPPH). Results indicate high selectivity of the subcritical water toward the most active compounds of rosemary such as carnosol, rosmanol, carnosic acid, methyl carnosate, and some flavonoids such as cirsimaritin and genkwanin. The antioxidant activity of the fractions obtained by extraction at different water temperatures was very high, with values around 11.3 μg/mL, comparable to those achieved by SFE of rosemary leaves. A study of the effect of the temperature on the extraction efficiency of the most typical rosemary antioxidant compounds has been performed.

KEYWORDS: Antioxidant activity; antioxidant compounds; rosemary plants; SFE; subcritical water extraction

INTRODUCTION

The growing interest in natural food has raised the demand for natural antioxidants, that is, products that have a nonsynthetic origin and are able to prevent or retard oxidation of fats and oils. Antioxidants are important in the food industry not only because of their usefulness as a preservation method but also because of their beneficial effects on human health (*1*). Among the natural antioxidants, rosemary has been widely accepted as one of the spices with highest antioxidant activity (*2*). Several studies about the antioxidative constituents of rosemary indicate that the most active compounds are the phenolic diterpenes such as carnosol, rosmanol, 7-methyl-*epi*-rosmanol, isorosmanol, rosmadial, carnosic acid, methyl carnosate, and other phenolic acids such as rosmarinic and caffeic. These compounds are described in the literature and have been isolated and identified by numerous authors (*3*−*6*).

Several methods have been used to extract antioxidants from aromatic plants, such as solid−liquid extraction, aqueous alkaline extraction, extraction with vegetable oils, extraction with aqueous solutions (*7*−*9*), and supercritical fluid extraction (SFE) (*10*−*14*). Products obtained by SFE from rosemary leaves have, in general, a higher antioxidant activity than extracts obtained by using solvent extraction with organic solvents (*6*), probably due to a difference in composition deriving from the extraction conditions applied. Therefore, as it has been previously suggested by some authors, extracting parameters (*15*, *16*) as well as other factors related to the quality of the original plant, its geographic origin, the harvesting date, its storage and its processing prior to extraction (*15*, *17*, *18*) directly influence the final composition of the extracts obtained.

Subcritical water extraction, that is, extraction using hot water under pressure sufficient to maintain water in the liquid state, has demonstrated its ability to selectively extract different classes of compounds depending on the temperature used, with the more polar extracted at lower temperatures and the less polar compounds extracted at higher temperatures. The selectivity of subcritical water extraction allows for manipulation of the composition of the extracts by changing the operating parameters and has been used for essential oil isolation (*19*−*25*). Subcritical water extraction has been suggested to extract valuable compounds from plant materials, but, to our knowledge, there is no report of the use of subcritical water to isolate antioxidant compounds from plants.

The goal of the present investigation was to study the selectivity of subcritical water extraction at several temperatures to extract antioxidant compounds from rosemary leaves. In the present study, an exhaustive characterization of the fractions obtained using water at different temperatures was carried out. An LC-MS system equipped with electrospray (positive ioniza-

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10.1021/jf025878j CCC: $25.00
Published on Web 12/07/2002

tion method) was used, along with a diode array detector to characterize the extracts in terms of chemical composition. A study of the effect of the temperature on the extraction efficiency of the most typical rosemary antioxidant compounds was also performed.

MATERIALS AND METHODS

Samples and Chemicals. The rosemary sample (*Rosmarinus officinalis* L.) consisted of 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) (*15*). 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. All solvents were purchased from Lab Scan (Dublin, Ireland) except ethanol (99.5%), which was from Panreac (Barcelona, Spain). Milli-Q water to perform the HPLC analysis was obtained from a purification system (Millipore, Bedford, MA). CO_2 (SFC quality) was kindly donated by AL Air Liquide España S.A. (Madrid, Spain). The water used to carry out the subcritical water experiments was of HPLC grade (Fisher Scientific, Pittsburgh, PA).

Subcritical Extraction with Water. Subcritical water extraction was performed in a home-built apparatus previously described in detail (*26*, *27*). The extraction system consisted of two ISCO model 100D syringe pumps (ISCO, Lincoln, NE) delivering water at a constant flow rate to an HP 5890 gas chromatograph oven (Hewlett-Packard, Wilmington, DE), where the extraction cell was mounted. The water was purged with nitrogen to remove dissolved oxygen prior to the extraction and supplied to the system with the pump at a constant flow rate of 1 mL/min. To preheat the extractant water to the required temperature, a 3-m preheating coil was installed in the oven before the extraction cell. A miniature back-pressure regulator (Upchurch Scientific, Oak Harbor, WA) was placed at the outlet of the extraction system (outside the oven) to maintain the system pressure between 60 and 70 bar, thus ensuring that the water was in the liquid state at all of the temperatures tested.

All extractions were carried out in a 3.47-mL SFE cell (9.4 mm i.d., 50 mm long, Keystone Scientific, Bellefonte, PA) equipped with a 0.5 μm frit at the inlet and a 2 μm frit at the outlet. The extraction cell was completely filled with plant material (1 g) and mounted vertically in the oven with water flowing from top to bottom.

Two different extraction procedures have been used depending on whether individual extractions (at a chosen temperature) or sequential experiments were performed. All of the experiments have been performed in quadruplicate.

For extraction at individual temperatures, each extraction was started at 25 °C at 1 mL/min; after 2 min, when the cell was filled, heating was started. At minute 3 the system was pressurized and, first, water was collected, at around 3.3 and 6 min (depending on the temperature selected to perform extraction); the system achieved the required temperature, and the extraction time was set to zero. During extraction, the pressure was maintained between 40 and 70 bar. Individual temperatures considered were 25, 100, 150, and 200 °C. Extractions were performed for 30 min.

For sequential experiments, the extraction procedure started by pressurizing the system with water to ∼60 bar at a flow of 1 mL/min and heating the oven to the required temperature. After 3 min, the back-pressure regulator (set to 60 bar) opened and collection of the eluent began. Each temperature was held for 15 min, and then the collection vial was replaced and the system heated to the next higher temperature. In the present study, sequential experiments were carried out at 100, 150, and 200 °C.

Samples obtained were freeze-dried and stored in amber flasks until use.

LC-MS Analysis of the Extracts. Samples to be analyzed were prepared by dissolving 1 mg of freeze-dried rosemary extract into 100 μL of Milli-Q water. Analyses were performed with a quadrupole 1100 MSD by using an electrospray interface. The separation was carried out in an HPLC apparatus (HP series 1100) with an autosampler (injection volume = 20 μL) equipped with a Nova-Pak C_{18} column (Waters, Madrid, Spain), 4-μm particle, 3.9 × 150 mm. The mobile phase was a mixture of solvent A (1% acetic acid in water) and solvent B (1% acetic acid in acetonitrile) according to a step gradient, lasting 40 min, changing from 50% B at 5 min to 70% B at 15 min and to 100% B at 40 min, at a flow rate of 0.7 mL/min. Detection was accomplished by using a diode array detector 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 API-ES method, the eluted compounds were mixed with nitrogen in the heated nebulizer interface and polarity was tuned to positive. Adequate calibration of ES parameters (needle potential, gas temperature, nebulizer pressure, and fragmentator voltage) was required to optimize the response and to obtain a high sensitivity of the molecular ion. The selected values were as follows: needle potential, 4000 V; gas temperature, 320 °C; drying gas, 10.0 mL/min; nebulizer pressure, 40 psig; fragmentator voltage, 60 V.

Determination of Antioxidant Activity. Antioxidant activity was measured in all fractions obtained. The method used was based on a procedure described by Lamaison et al. (*28*), modified as previously described (*29*). The method consists of the neutralization of free radicals of 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 (corresponding to 212 μg). The 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 to zero and ascorbic acid to calibrate the method. The equation described by Lamaison et al. (*28*) 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

To develop the subcritical water extraction conditions, two different sets of experiments have been studied, as follows: for one set sequential extractions are performed, whereas for the other set extractions of fresh samples at different temperatures are performed. The first approach consists of the sequential extraction of rosemary leaves at different temperatures from 100 to 200 °C for 15 min. In this approach, the same material is extracted sequentially at higher temperatures, providing information about selectivity. The second approach allows direct comparison of the total antioxidant activity obtained with a single temperature of extraction, as might be used in an industrial process, thus allowing the different compounds to be determined at each extraction temperature; the temperatures selected covered a wide range of water polarities and ranged from 25 to 200 °C.

Sequential Extractions. The HPLC chromatographic profiles obtained at the different temperatures tested are shown in **Figure 1**. As can be seen, extractions performed at 100 °C allow the selective recovery of the compounds eluted at the beginning of the chromatogram (before 7 min, which corresponds to the more polar compounds considering that the analysis was performed in a reversed-phase column), whereas extraction at higher temperatures increases the solubility of less polar compounds that were poorly soluble at lower temperatures; these compounds, obviously, eluted later.

To exhaustively characterize the compounds extracted, several fractions were analyzed by LC-MS with ES (positive ionization). The conditions were selected on the basis of previous work done

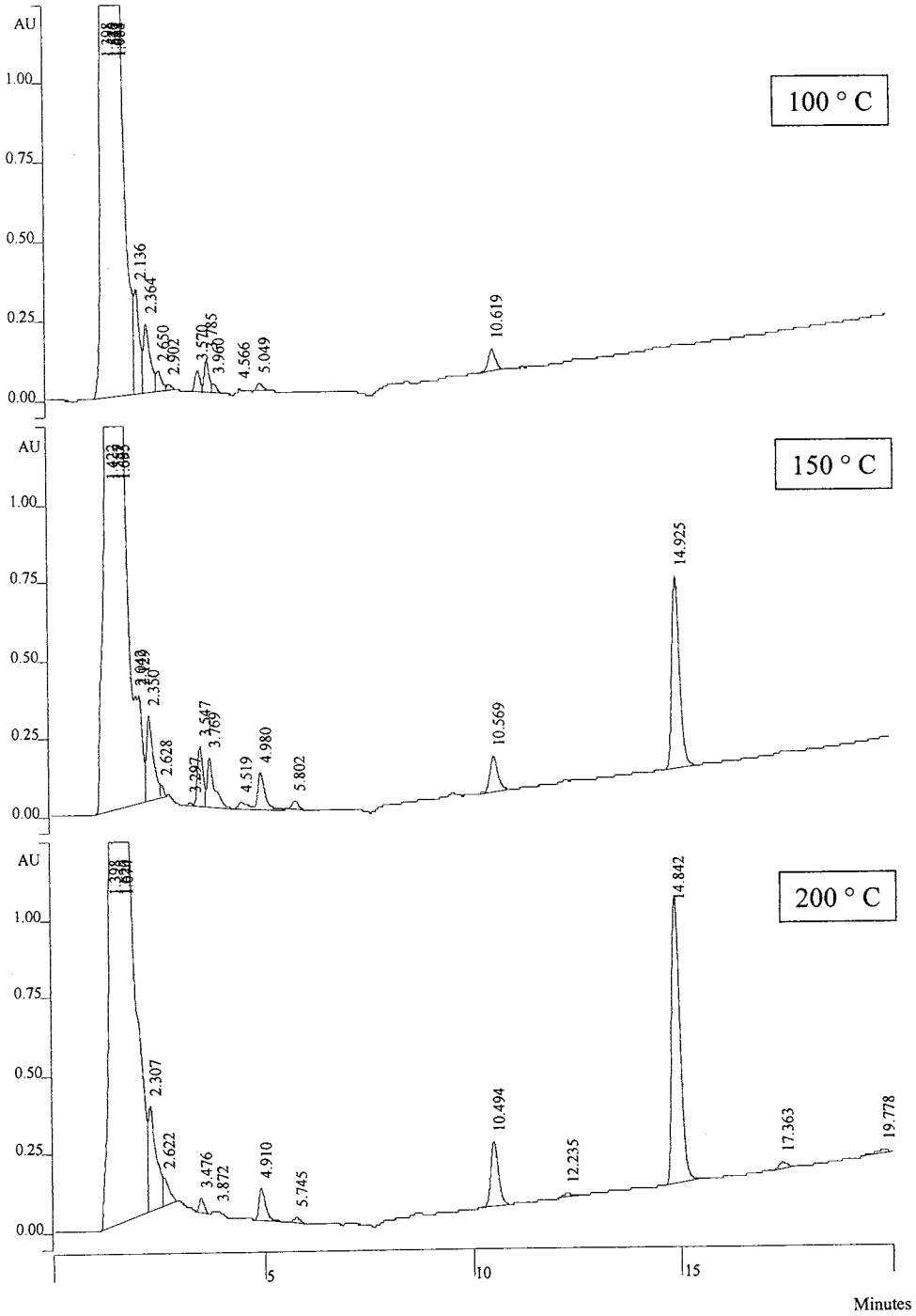

Figure 1. Chromatographic profiles obtained for the sequential extraction with subcritical water at different temperatures (from 100 to 200 °C). HPLC-DAD conditions are given under Experimental Procedures.

in our laboratory (*30*). Considering the possibility of extracting antioxidant compounds from rosemary using subcritical water and, therefore, taking into account the type of compounds that can be obtained, a mass spectrometer with electrospray in positive ionization mode was selected, providing an increase in sensitivity for almost all of the compounds present in the sample except for acidic compounds, for which the signal was smaller in positive ionization mode.

To obtain semiquantitative data, the primary detection wavelength used was 230 nm. Simultaneously, spectral data were obtained over the range of 215−450 nm by using a diode array detector. These data can be very useful in the identification of compounds of interest. **Figure 2** shows the chromatographic profiles obtained by DAD at 230 nm (top) for experiments performed with subcritical water at 100 and 200 °C. Along with these profiles, a signal for API-ES in positive mode is also shown (bottom).

Compounds were characterized for their retention time, UV spectra, and mass spectra and were tentatively identified on the basis of previous data published by different authors (*17*, *30*, *31*). Different compounds have been identified corresponding to diterpenes such as carnosol, rosmadial, carnosic acid, methyl carnosate, rosmanol, epirosmanol, epirosmanol methyl ether, carnosol isomer, rosmanol isomer, and some flavonoids such as cirsimaritin, scutellarein, and genkwanin. Other compounds were also detected, but their complete identification was not possible. **Table 1** shows retention time, molecular ion (MH^+), and UV maximum absorbance for all of the compounds detected

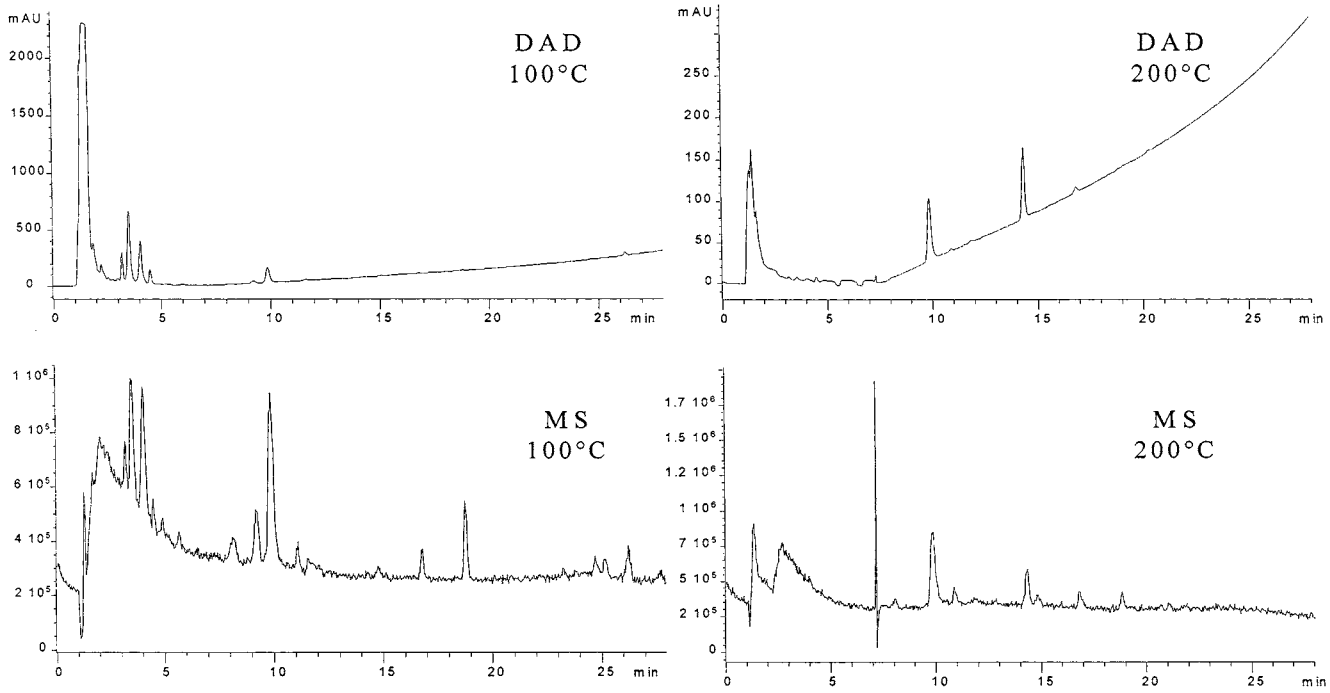

Figure 2. Chromatographic profiles obtained for subcritical water extraction at 100 °C, DAD signal at 230 nm (top) and API-ES positive ionization signal (bottom), and subcritical water extraction at 200 °C, DAD signal at 230 nm (top) and API-ES positive ionization signal (bottom). For peak assignment, see **Table 1**.

Table 1. Characteristic Parameters of the Compounds Detected in the Extracts Analyzed by LC-MS

compound[a]	retention time (min)	mass ions (ES+) MH^+	major fragments	UV absorbance max	extract temp (°C)[b]
epirosmanol	3.03	347	301	290	traces
scutellarein	3.48	287		268, 335	all
NI 1	3.70	151		258	all
rosmanol	3.90	347	301	284	all
rosmanol isomer	4.43	347	364	288, 334	100, 150
genkwanin	5.02	285		266, 336	100, 150, 200
NI 2	8.94	329		276, 330	200
epirosmanol methyl ether	9.97	361		288	150
carnosol	10.60	331		284	100, 150, 200
carnosol isomer	11.80	331	348	270	traces
NI 5	12.28	345		235, 286	200
carnosic acid	14.70	333		284	100, 150, 200
rosmadial	14.98	345		290	traces
methyl carnosate	17.46	347	301	282	200
cirsimaritin	19.47	315		248, 334	200
NI 7	27.10	319		286	traces

[a] NI 1, NI 2, NI 5, and NI 7 had been previously described, as mentioned in the text. [b] Extracts corresponding to those of water extraction temperature where the compound had been detected.

in the samples; also, additional data about the major fragments obtained using electrospray with positive ionization are presented. **Table 1** also includes the fractions where the compounds had been detected. Carnosol and rosmanol isomers had mass ions (MH^+) equal to 331 and 347, respectively, but they also presented peaks at *m*/*z* 348 and 364, respectively, that could be due to the addition of water to the corresponding molecule (that is, carnosol and rosmanol).

The unidentified compounds have been previously described by other authors, as was suggested in a previous work done in our laboratory. Therefore, NI 1, NI 2, NI 5, and NI 7 have been detected in supercritical fluid rosemary extracts (*30*). Other compounds that have been detected in the subcritical water extracts were genkwanin and epirosmanol methyl ether; these compounds were not previously found in supercritical fluid rosemary extracts (see ref *30*). **Figure 3** shows the chemical structures of some of the most important compounds from rosemary.

To perform the study of the semiquantitative composition of the extracts, some compounds were selected and their relative percentages (referred to as the total area of the selected components based on DAD peak area at 230 nm) are shown in **Table 2**, along with their variation coefficients (CV, $n = 4$) and antioxidant activities (oxidation index, μg/mL) obtained for the different extracts. No quantitation of the components could be performed due to the lack of standards. The variation coefficients were obtained with data of four independent extractions, each of them analyzed twice by HPLC. As can be observed, CV were, in most cases, <15% and for the major compounds even <5%.

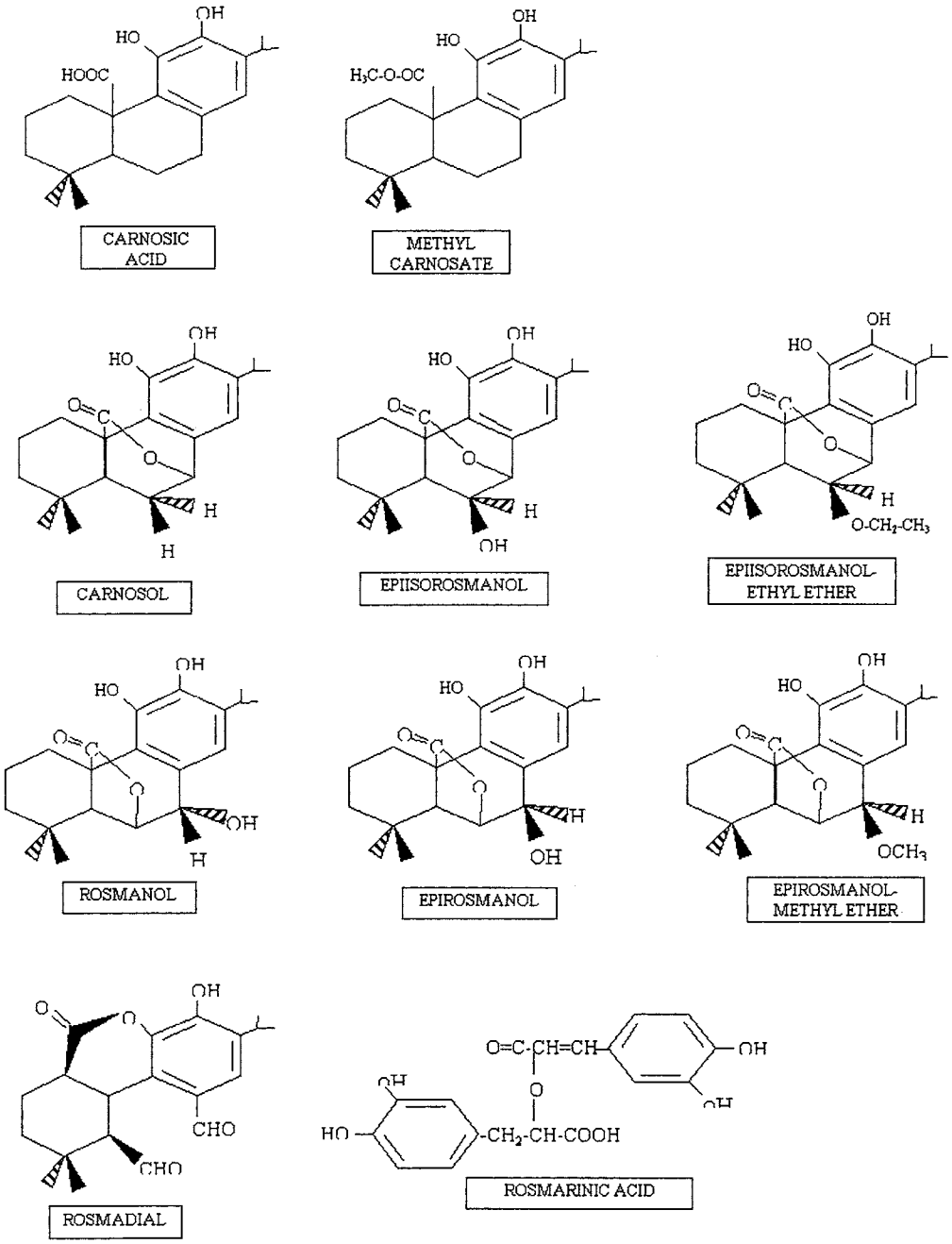

Figure 3. Chemical structures of some of the most important compounds from rosemary.

The difference in selectivity of the subcritical water at different extraction temperatures (from 100 to 200 °C) can be easily observed by analyzing both the chromatographic profiles (**Figure 1**) and the semiquantitative composition of the extracts (**Table 2**).

Subcritical water shows compound class selectivity that exists because extraction depends on solvation of the target compounds in the liquid state of the water. As a polar fluid, water normally solvates more polar compounds more readily than nonpolar compounds. Higher temperatures reduce the polarity of water, thus increasing its ability to solvate nonpolar compounds. At the lowest temperature, the more polar compounds, such as scutellarein, rosmanol, rosmanol isomer, and genkwanin, were extracted. When water was heated to 200 °C, the dielectric constant of water was reduced to values similar to those of methanol or acetonitrile, which increased the solubilities of less polar compounds such as carnosol, carnosic acid, and methyl carnosate by several orders of magnitude, resulting in such compounds being the major constituents of these fractions.

Figure 4 shows the effect of temperature on the extraction efficiency of the major compounds found in the extracts, which also correspond to the most typical rosemary antioxidant compounds (rosmanol, rosmanol isomer, scutellarein, genkwanin, carnosol, and carnosic acid). At the lowest extraction temperature tested in the sequential extraction (100 °C), compounds such as carnosol, scutellarein, genkwanin, and NI 1 were preferentially extracted, whereas rosmanol and rosmanol isomer were found at lower concentrations. Carnosic acid, considered to be one of the most potent antioxidants from rosemary, started to be extracted at 100 °C, with increasing extraction yield obtained by increasing the temperature to 200 °C. At this temperature, carnosic acid was the major component of the extract (>70%), with >85% of the composition of the extract formed by carnosic acid and carnosol. At this temper-

Table 2. Relative Percentage [Normalized Areas (%)] of the Compounds Identified by LC-MS and Selected To Semiquantitatively Describe the Composition of the Extracts Obtained during Sequential Extraction with Subcritical Water at Different Temperatures, As Shown in **Table 1**

	100 °C		150 °C		200 °C	
compound	normalized areas[a] (%)	CV (%)	normalized areas (%)	CV (%)	normalized areas (%)	CV (%)
scutellarein	19.94	1.4	12.89	3.3	2.45	10.6
NI 1	29.03	4.7	10.41	15.2	0.08	0.5
rosmanol	8.35	10.4	2.30	26.3	0.38	20.0
rosmanol isomer	2.66	12.4	1.73	10.3		
genkwanin	9.79	3.3	8.80	5.9	6.60	5.1
NI 2					0.51	6.6
epirosmanol methyl ether			1.38	28.9		
carnosol	29.15	3.6	10.33	3.9	14.30	2.0
NI 5					1.60	4.4
carnosic acid	3.23		52.15	0.7	71.75	0.6
methyl carnosate					1.60	4.4
cirsimaritin					1.72	3.6
antioxidant activity (μg/mL)	11.3	0.2	11.4	0.3	11.3	0.2

[a] Areas normalized to 100% for each LC run.

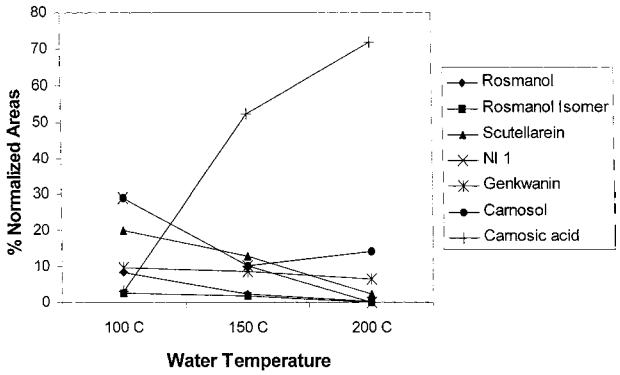

Figure 4. Graph representing the percentage of normalized areas vs water extraction temperature (sequential extraction) for some rosemary antioxidant compounds.

ature, almost no extraction of more polar compounds such as rosmanol and rosmanol isomer was observed.

With regard to the stability of carnosic acid, taking into account that this compound is the more easily oxidized and, at the same time, the major compound in the extracts obtained at 200 °C, it is not reasonable to consider a problem of decomposition or conversion induced by the high temperature of extraction. Oxidation of organics seems to be unlikely because the water used for subcritical water extraction is deoxygenated, and no exposure to air occurs during extraction. Molecules such carnosic acid can be much more sensitive to oxygen than to temperature (or also to temperature if oxygen is present).

These results show the high selectivity of the extraction process that can be obtained by simply tuning the subcritical water temperature. A tailor-made composition of the final extract can be achieved by performing the extraction at the desired water temperature obtaining extracts with a higher percentage of carnosic acid or even with a carnosol-rich composition.

As mentioned before, **Table 2** shows also the antioxidant activities of the extracts obtained, under sequential extraction, at different subcritical water temperatures, measured by a free radical method (DPPH). As can be observed, values obtained for the different fractions were very high, comparable to those achieved by supercritical fluid extracts of rosemary leaves (*30*). In the mentioned publication, antioxidant activities achieved ranged from 9.7 to 34.9 for the antioxidant fraction (the one obtained in separator 1, after cascade depressurization). For the subcritical water extracts, values of antioxidant activity were around 11.3 for all of the temperatures tested.

From an analysis of the results, it is clear that not only carnosic acid and carnosol give a high antioxidant activity to the extracts but also rosmanol and other polar compounds belonging to the rosmanol family provide good activities. Haraguchi et al. (*32*) studied the inhibition of lipid peroxidation by four diterpenoids, carnosic acid, carnosol, rosmanol, and epirosmanol, and found that all of them were effective in protecting biological systems against oxidative stresses. Also, Djarmati et al. (*33*) isolated rosmanol-9-ethyl ether from sage, demonstrating the important antioxidant activity of that compound.

Extraction at Individual Temperatures. Fresh rosemary samples were extracted at 25, 100, 150, and 200 °C to compare the results obtained under sequential extraction with those achieved at a single extraction temperature. This process is more likely to be used at the industrial scale; therefore, we considered the interest of evaluating the ability of the subcritical water at the different temperatures toward the specific extraction of different compounds and how this composition will influence the total antioxidant activity. One important item to consider in this process is the extraction yield (% w/w) for the experiments performed at the different temperatures that, on average, were the following: 25 °C, 12.3%; 100 °C, 17.5%; 150 °C, 30.9%; and 200 °C, 48.6%.

Samples extracted were analyzed and characterized as mentioned above by using HPLC with DAD and LC-MS with ES (positive ionization); **Table 1** also includes the fractions where the compounds have been detected.

To obtain semiquantitative data, the primary detection wavelength used was 230 nm. With these data, the composition of the extracts (in terms of percent of normalized areas) can be obtained. **Figure 5** shows, as a bar diagram, the composition (percent normalized areas) of the extracts obtained at the four different individual temperatures tested. As can be seen, at 25 °C the more polar compound (rosmanol) was the major component of the extract (counting for >50% of the total composition of the extract), and only scutellarein and NI 1 were observed along with rosmanol. When the temperature was increased from 25 to 200 °C, a decrease in the extraction ability of subcritical water toward the more polar compounds was observed. Carnosic acid, carnosol, and genkwanin were first extracted at 100 °C, decreasing their content in the extract while

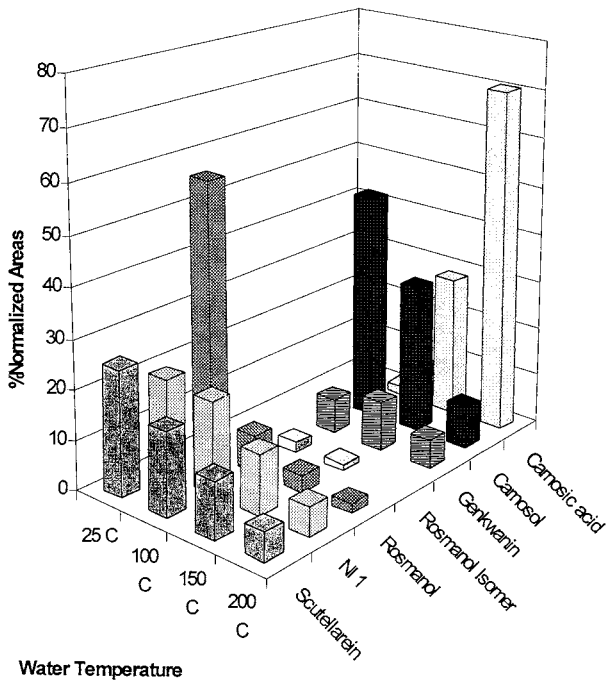

Figure 5. Bar diagram representing the percentage of normalized areas vs water extraction temperature (individual extraction) for some rosemary antioxidant compounds.

increasing temperatures for all compounds except carnosic acid, which is preferentially extracted at 200 °C. These results are in good agreement with those achieved by sequential extraction; the differences observed can be due to the different extraction times used in both sets of experiments, 15 min for sequential extraction and 30 min for extraction at individual temperatures. As for the antioxidant activity, all of the extracts tested have low values (corresponding to high antioxidant activities) equal to 11.3 μg/mL.

In the present study, the possibility of tuning the selectivity for antioxidant extraction by a small change in water temperature has been demonstrated for the first time. Therefore, by using the process described it is possible to obtain extracts enriched with different types of compounds with very important antioxidant activities.

ACKNOWLEDGMENT

We thank M. I. Jiménez Vacas for kind help in the acquisition of the MS spectra.

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Received for review August 7, 2002. Revised manuscript received October 22, 2002. Accepted October 27, 2002. This work was supported by the Ministerio de Ciencia y Tecnología (Project AGL2000-0448). S.C. thanks the Ministerio de Educación y Cultura for a grant.

JF025878J