Combined Use of Supercritical Fluid Extraction, Micellar Electrokinetic Chromatography, and Reverse Phase High Performance Liquid Chromatography for the Analysis of Antioxidants from Rosemary (*Rosmarinus officinalis* L.)

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Antioxidants from rosemary were determined by the combined use of supercritical fluid extraction (SFE) prior to reverse-phase high-performance liquid chromatography (RP-HPLC) or micellar electrokinetic chromatography (MEKC). The separation of antioxidants found in the SFE fractions was achieved by using a new MEKC method and a published HPLC procedure, both with diode array detection. The characterization of the different antioxidants was further done by HPLC−mass spectrometry. Advantages and drawbacks of HPLC and MEKC for analyzing the antioxidants found in the different extracts are discussed. From the results it is concluded that HPLC renders higher peak area and is better in its reproducibility than MEKC; both techniques provide similar analysis time reproducibility. The main advantage of MEKC is its much higher separation speed, which is demonstrated to be useful for the quick adjustment of SFE conditions, allowing rosemary fractions of higher antioxidative power to be obtained quickly. Moreover, the possibilities of this approach for following the degradation of antioxidants are discussed.

Keywords: *Rosemary; antioxidants; supercritical fluid extraction; capillary electrophoresis; micellar electrokinetic chromatography; HPLC-MS*

INTRODUCTION

Free radicals are common metabolites in biological and food systems. Both normal metabolism of oxygen and extraction of energy are processes usually involved in the generation of free radicals. In food systems, free radicals may attack unsaturated bonds of lipid molecules, proteins, carbohydrates, and nucleotides, causing the development of rancidity, off-flavors, and loss of the nutritive value and shelf life of products. Also, it is increasingly evident that biological oxidation reactions are implicated in several disease conditions such as cancer, coronary heart disease, arteriosclerosis, and the aging process (Deshpande et al., 1996). Antioxidants are compounds that effectively retard the onset of lipid oxidation without changing the sensory qualities of the food product. Therefore, antioxidants have become an indispensable group of additives in the food industry. Moreover, several epidemiological studies are being undertaken worldwide to determine if dietary antioxidants can be used in preventive as well as therapeutic medicine in many diseases.

Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are two powerful synthetic antioxidants that have been used for decades. However, recent studies about the possible toxicity of BHA and BHT have brought about a new interest in natural antioxidants. Among the plants with known antioxidant properties, rosemary is one of the most used and commercialized. In rosemary there has been reported the presence of at least six phenolic diterpenoids with antioxidant activity: carnosol, carnosic acid, rosmadial, rosmanol, epirosmanol, and methyl carnosate (Inatani et al., 1983; Schwarz and Ternes, 1992; Schwarz et al., 1992). The antioxidant activity of several flavonoids found in rosemary such as genkwanin and cirsimaritin has also been reported (Cuvelier et al., 1994). In the phenolic diterpenoid fraction of *Rosmarinus officinalis*, carnosic acid is the major component (Schwarz and Ternes, 1992; Okamura et al., 1994; Richheimer et al., 1996) and also the one exhibiting the highest antioxidant activity (Aruoma et al., 1992; Cuvelier et al., 1994; Frankel et al., 1996a,b), although other antioxidative compounds have also been described (Wu et al., 1982; Wei et al., 1999).

Several methods have been reported for the extraction of antioxidants from plant material, including solid−liquid extraction, aqueous alkaline extraction, and supercritical fluid extraction (SFE). Extracts obtained by SFE usually possess higher antioxidant activity than those obtained by solvent extraction with organic solvents (Schwarz et al., 1992; Tena et al., 1997), although, as has been previously suggested by some authors, antioxidative performance depends on the extraction parameters (Chen et al., 1992; Ibañez et al., 1999) as

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well as on other factors such as the quality of the original plant, its geographic origin, the harvesting date, and its storage and processing prior to extraction (Reverchon et al., 1992; Cuvelier et al., 1996; Hidalgo et al., 1998). It has been suggested that the lower activity of the extracts obtained with organic solvents may be due to the degradation of carnosic acid to its derivatives. In fact, some authors (Richheimer et al., 1996) have already indicated that heating converts carnosic acid into carnosol, rosmanol, and 7-oxy derivatives, which may explain the differences found among the extracts.

The isolated fraction is usually composed of active substances plus some other impurities; therefore, a separation−identification step is then used. Thus, HPLC has been widely used for this purpose, that is, the characterization of SFE extracts from different samples [see, e.g., Cuvelier et al. (1996) and Tena et al. (1997)]. With regard to this point, in a previous work we have demonstrated the suitability of this methodology to fully identify the antioxidant fraction of rosemary extracted by SFE (Señoráns et al., 2000).

Capillary electrophoresis (CE) has emerged as a powerful analytical technique complementary in many features to HPLC (Kuhr, 1990; Kuhr and Monnig, 1992; Monnig and Kennedy, 1994; St. Claire, 1996; Beale, 1998). CE provides high efficiencies in short migration times in the separation of ionic and neutral compounds of very different nature. Thus, although the use of capillary electrochromatography (CEC) (Knox, 1994) is now increasing, micellar electrokinetic chromatography (MEKC) (Terabe, 1989) continues to be the preferred CE mode for analyzing neutral compounds such as food antioxidants (Hall et al., 1994; Abrantes et al., 1997). The main feature of MEKC is its high separation power, which brings about fast and well-resolved separations of very similar compounds. Moreover, the sample volume consumption is minimal because each injection needs only a few nanoliters.

Despite its good capabilities, MEKC has been scarcely applied in combination with SFE (Li and Li, 1995; Dunford et al., 1997; John et al., 1997; Buskov et al., 1998; Lancas et al., 1999). This low number of applications is certainly surprising because SFE and MEKC have in common a huge number of applications and a remarkable isolation power for nonpolar substances. Thus, it can be helpful to further study the great possibilities of combining both techniques to take advantage of their complementary natures. For instance, it should be possible, from a chosen SFE condition starting point, to extract a given group of substances from a complex matrix and, after development of an adequate MEKC method, to carry out the individual characterization of such substances. Moreover, with the data from MEKC employed in a feedback-like procedure, the conditions from SFE can be further optimized.

The first goal of this work was, therefore, to demonstrate the possibilities of SFE-MEKC when applied in combination to the analysis of rosemary extracts to obtain fractions of higher antioxidative power. The second goal was to carry out a comparative study of the capabilities of HPLC and MEKC when used to characterize rosemary SFE extracts.

EXPERIMENTAL PROCEDURES

Sample and Reagents. The rosemary sample (*Rosmarinus officinalis* L.) consisted of dried rosemary leaves obtained from an herbalist's shop (Murcia, Spain) dried using a traditional method as described previously (Ibañez et al., 1999). Samples were ground under cryogenic carbon dioxide and stored in amber flasks at −20 °C until use (a maximum of 2 months).

Table 1. Conditions Used for the SFE Experiments Performed at Pilot Plant Scale[a]

expt	% EtOH	P_{ext} (bar)	T_{ext} (°C)	ρ_{ext} (g/mL)	P_{s1} (bar)	T_{s1} (°C)	ρ_{s1} (g/mL)	P_{s2} (bar)	T_{s2} (°C)
1	0	350	50	0.9	200	50	0.78	20	25
2	2	300	40	0.91	150	60	0.6	20	25
3	2	350	60	0.87	150	40	0.78	55	25

[a] P_{ext}, extraction pressure; T_{ext}, extraction temperature; ρ_{ext}, extraction density; P_{s1}, pressure in separator 1; T_{s1}, temperature in separator 1; ρ_{s1}, density in separator 1; P_{s2}, pressure in separator 2; T_{s2}, temperature in separator 2.

All chemicals were of analytical reagent grade and used as received. Sodium deoxycholate (SDC) from Sigma (St. Louis, MO), sodium dodecyl sulfate (SDS) from Merck (Darmstadt, Germany), and boric acid and sodium tetraborate hydrate from Aldrich (Milwaukee, WI) were used for the CE running buffers at the different concentrations and pH values indicated below. The organic solvents methanol (MeOH) and acetonitrile (ACN) from Merck were of HPLC grade, ethanol (99.5%) was from Panreac, and acetone was from Quimicen (Madrid, Spain). Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA). CO_2 (SFC quality) was kindly donated by AL Air Liquide España S.A. (Madrid, Spain).

Extraction Method. A pilot-scale supercritical fluid extractor (Iberfluid) was used, as previously described (Señoráns et al., 2000). The extraction cell was made of 316 stainless steel with a volume of 285 mL with a stainless steel frit. The extraction pressure was controlled by micrometering valves, and the carbon dioxide pump was from Braun-Luebbe.

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

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

In the HPLC-ESI-MS method, the eluted compounds were mixed with nitrogen in the heated nebulizer interface and polarity was tuned to positive. Adequate calibration of ESI parameters (needle potential, gas temperature, and nebulizer pressure) 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, 335 °C; drying gas, 10.0 mL/min; and nebulizer pressure, 50 psig.

CE Conditions. The analyses were carried out in a P/ACE 5510 (Beckman Instruments, Fullerton, CA) CE apparatus, equipped with a DAD. The fused-silica capillary used was 27 cm total length (20 cm effective length) × 50 μm i.d. purchased from Composite Metal Services (Worcester, U.K.). Injections were made at the anodic end using an N_2 pressure of 0.5 psi for 1 s (1 psi = 6894.76 Pa). The instrument was controlled by a Compact Deskpro PC running System GOLD software

from Beckman. All measurements were carried out at 25 °C. Voltages between 8 and 15 kV were tested for separation, and the detection took place at 230 nm.

The separation electrolytes were prepared by adding appropriate aliquots of 0.1 M SDS or 0.1 M SDC and 0.1 M borate buffer (pH 9.0) into water or into mixtures of water and organic solvents. The borate buffer pH was adjusted as required by adding aliquots of 0.1 M boric acid into 0.1 M sodium tetraborate hydrate and measured by a pH meter (Titriprocessor, model 670, Metrohm, Herisau, Switzerland), with glass and thermal compensation electrodes.

Before first use, a new capillary was preconditioned by rinsing with 1 M NaOH for 15 min, followed by a 15 min rinse with deionized water. At the start of each day, the capillary was conditioned with the separation electrolyte for 15 min. Between introductions of samples, the capillary was rinsed with the separation electrolyte for 2 min. At the end of each day, the capillary was rinsed with deionized water for 5 min.

RESULTS AND DISCUSSION

Different extraction and fractionation conditions were selected to perform the SFE experiments at pilot plant scale, as shown in Table 1. A maximum extraction density of 0.9 g/mL (350 bar and 50 °C) has been chosen followed by fractional separation in two separation vessels. Separation conditions tested covered a density range between 0.6 and 0.8 g/mL in the first separator, whereas a total decompression stage was achieved in the second separator. The differences will be observed in the selective precipitation of the compounds in the first separator (Señorans et al., 2000), containing the compounds with antioxidant properties.

The two fractions were analyzed by HPLC-MS (electrospray in positive ionization mode) using a method based on a previous work done in our laboratory (Señoráns et al., 2000). Semiquantitative data were obtained using primary detection wavelength at 230 nm, and a DAD was used over the range of 215−450 nm to achieve spectral data. Figure 1 shows the chromatographic profiles obtained by DAD at 230 nm [top profiles in (A) and (B)] for experiment 2, fractions 1 and 2. Along with these profiles, the signal for HPLC-ESI-MS in positive mode is also shown in the bottom profiles of (A) and (B). Peak assignment was done as described in a previous work (Señoráns et al., 2000).

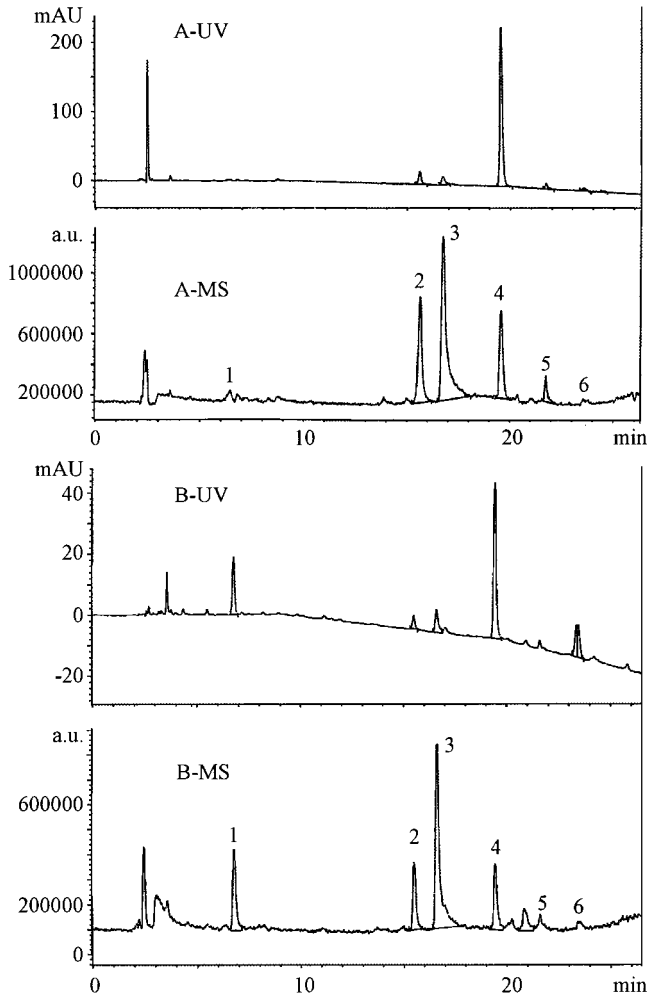

Figure 1. Liquid chromatographic profiles obtained for (A) experiment 2, fraction 1 [(top) DAD signal at 230 nm; (bottom) ESI-MS positive ionization signal], and (B) experiment 2, fraction 2 [(top) DAD signal at 230 nm; (bottom) ESI-MS positive ionization signal]. Peak assignment: 1, rosmanol; 2, carnosol; 3, carnosol isomer; 4, carnosic acid; 5, methyl carnosate; 6, nonidentified compound. See Table 1 for experimental conditions.

As stated above, some authors (Hall et al., 1994; Abrantes et al., 1997) have already demonstrated the possibilities of MEKC for the separation of food antioxidants. However, to our knowledge no CE method has been developed for the separation of antioxidants from rosemary. Therefore, we started using a typical MEKC buffer to try to separate the antioxidants found in a given SFE rosemary fraction. The initial MEKC buffer consisted of 20 mM boric acid/sodium tetraborate and 40 mM SDS at pH 9. An example of the MEKC separations obtained with this buffer is shown in Figure 2 A). As can be seen, the electrophoregram obtained shows a good resolution and analysis speed, with a separation time of <5 min. However, using the peak-purity capability of the DAD, it was found that the peak marked with an asterisk contained more than one substance. Therefore, a further optimization of the MEKC conditions was needed. To do so, two organic solvents were tested (i.e., MeOH and ACN) and added at different percentages to the separation buffer (from 3 to 25%, v/v), to modify the selectivity of the micellar system (Balchunas and Sepaniak, 1987; Cifuentes et al., 1998). Simultaneously, run voltages from 8 to 15 kV were tested. Although some improvement was observed in terms of separation of the substances under the peak marked with an asterisk, no baseline resolution was achieved in any case. Similar results were obtained when the initial buffer with SDS was substituted with others containing different concentrations of the bile salt SDC. Although it has been demonstrated that this type of surfactant introduces a selectivity different from that of SDS within the MEKC buffer (Crego et al., 1998), no baseline resolution was obtained either. A further study was carried out by combining the use of SDC and organic solvents (ACN and MeOH) at different percentages together with a variation of the run voltage from 8 to 15 kV. The best results in terms of resolution and separation speed were then obtained by using a buffer containing 50 mM SDC, 20 mM boric acid/sodium tetraborate at pH 9, and 15% ACN (v/v) together with 10 kV as the separation voltage. Under these conditions electrophoregrams such as the one shown in Figure 2B were achieved. As can be seen, it was possible to obtain well-resolved separation of the different antioxidants and impurities from rosemary with no detectable overlapping as demonstrated by using the peak-purity capability of the DAD. Moreover, the separation was completed in <7 min. Peaks in the MEKC electrophore-

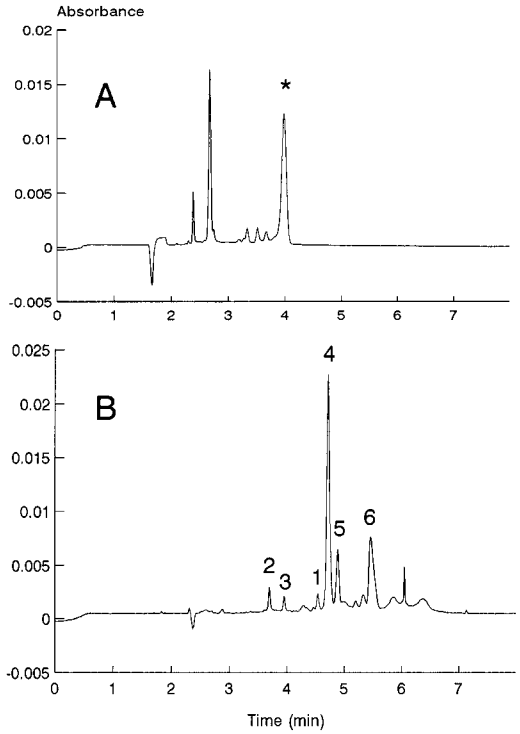

Figure 2. Optimization of MEKC conditions for the separation of antioxidants from an SFE extract of rosemary: (A) buffer, 40 mM SDS/20 mM boric acid/sodium tetraborate at pH 9, run voltage = 8 kV (asterisk indicates a peak constituted by comigration of various compounds); (B) buffer, 50 mM SDC/20 mM boric acid/sodium tetraborate at pH 9 and 15% ACN (v/v), run voltage = 10 kV. Other conditions: capillary, 27 cm of total length, 20 cm of detection length with 50 μm i.d; injection, 0.5 psi for 1 s of rosemary extract; temperature, 25 °C; detection at 230 nm. Peak assignment as in Figure 1 (see text).

Table 2. Percentage Determined by RP-HPLC and MEKC of Five Identified Compounds Found in Two Different Rosemary SFE Extracts

	SFE extract 1		SFE extract 2	
	MEKC	RP-HPLC	MEKC	RP-HPLC
rosmanol	19.18	20.02	1.58	0.55
carnosol	9.39	4.92	7.56	7.06
carnosol isomer	3.96	11.33	4.43	5.42
carnosic acid	64.36	62.37	80.90	83.79
methyl carnosate	3.11	1.36	5.53	3.18

grams were tentatively assigned by comparing their UV spectra obtained from the CE-diode array equipment with those ones obtained using the HPLC-diode array instrument. Moreover, as indicated above, the peaks obtained from HPLC were further characterized by using HPLC-MS (Señoráns et al., 2000).

Under these conditions, it is now possible to establish a comparison between the different features of MEKC and RP-HPLC for the analysis of antioxidants from rosemary SFE extracts.

Thus, both techniques allow a semiquantitative determination of the percentages of the different compounds found in SFE extracts to be carried out. Table 2 shows the results achieved by HPLC and MEKC from two rosemary extracts indicated as 1 and 2 corresponding to experiments 2, fraction 2, and experiment 1, fraction 2, respectively, obtained under the SFE conditions shown in Table 1. The agreement between the results from both techniques is relatively good, which

Table 3. Comparison of Peak Area Reproducibility and Analysis Time Reproducibility[a] Obtained by MEKC and HPLC in the Separation of Antioxidants from Rosemary SFE Extracts

	t_{av} (min)	% RSD_t	$area_{av}$ (au)	% RSD_{area}
MEKC	4.89	0.68	9.2	4.21
RP-HPLC	19.44	0.32	3476	2.99

[a] All data are referred to the major peak, i.e., carnosic acid, and for $n = 6$.

corroborates the usefulness of both procedures for this type of analysis.

On the other hand, HPLC shows a better reproducibility in terms of peak area than MEKC, and both techniques show similar analysis time reproducibility, as can be deduced from the results shown in Table 3, where these figures of merit are compared. However, the higher analysis speed of MEKC compared with that of HPLC has to be taken into account, because by using the MEKC procedure a 4-fold decrease in analysis time is obtained (e.g., 4.89 min in MEKC versus 19.44 min in HPLC for carnosic acid). Similar results have already been shown using other different food compounds (Cifuentes et al., 1993), concluding that both techniques, HPLC and CE, are complementary and, therefore, useful as simultaneous separation techniques in analytical laboratories.

One of the main goals in the isolation of natural antioxidants from plants is to gain the highest purity by using the most selective extraction and fractionation processes. This is also related to the interest of using the smallest amount of a pure antioxidant compound with the highest activity, improving in that way its usefulness to food commodities. The main capability of MEKC, that is, its high separation speed, can be favorably employed for optimizing in a fast way the SFE conditions to achieve such goals. An example of this is given in Figure 3, where the SFE parameters were refined to increase the purity of the carnosic acid extracted (peak 4 in electrophoregrams). This improvement was intended because carnosic acid has been demonstrated to possess the highest antioxidative power among different compounds extracted from rosemary (Schwarz et al., 1992). This connection has been further corroborated through the high correlation (96%) obtained between the percent of carnosic acid and antioxidant activity (micrograms per milliliter), showing a higher antioxidant activity with increasing percentages of carnosic acid (Señoráns et al., 2000). Besides, no other correlation could be clearly demonstrated, and no synergistic effects among the different compounds identified in the extracts could be observed. Thus, Figure 3A shows the MEKC profile from an extract of rosemary obtained using the following SFE parameters: P_{ext} = 350 bar, T_{ext} = 60 °C, ρ_{ext} = 0.87 g/mL, 2% ethanol, P_{s1} = 150 bar, T_{s1} = 40 °C, ρ_{s1} = 0.78 g/mL. Under these conditions and using the electrophoregram of Figure 3A provided by MEKC, it was possible to determine in 6 min that the purity of carnosic acid, calculated as the percentage of its peak area relative to the sum of areas from all of the peaks, is ~35%. After several modifications of the SFE conditions (data not shown) followed by MEKC analysis, it was possible to quickly achieve SFE conditions (i.e., P_{ext} = 350 bar, T_{ext} = 50 °C, ρ_{ext} = 0.9 g/mL, 0% ethanol, P_{s1} = 200 bar, T_{s1} = 50 °C, ρ_{s1} = 0.78 g/mL) that provide fractions with a content in carnosic acid >80%, as calculated from the electrophoregram shown in Figure 3B. Moreover, the two

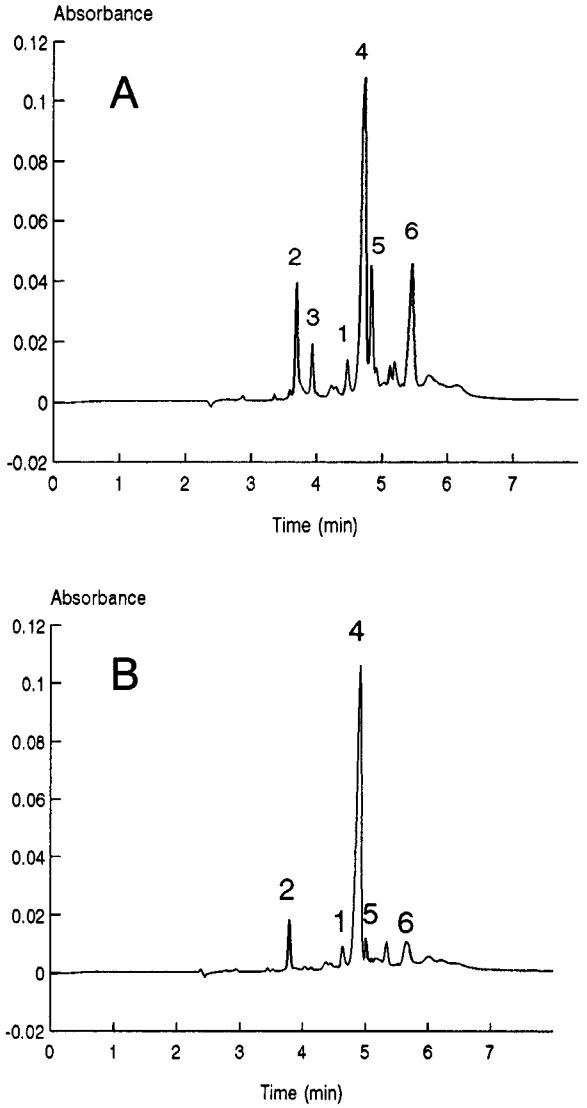

Figure 3. Effect of the SFE conditions on the purity achieved for carnosic acid in rosemary extracts monitored by MEKC. For SFE conditions (A and B), see text. All of the MEKC conditions were as in Figure 2B.

mentioned fractions provide very different antioxidant activities (measured with the DPPH test, 34.9 versus 9.7 μg/mL, respectively) (Señoráns et al., 2000), which agrees perfectly with the purity obtained using the electrophoregrams shown in Figure 3.

The MEKC method also allows the degradation of antioxidants to be followed, as can be deduced from the electrophoregrams of Figure 4. Thus, Figure 4A shows the MEKC separations obtained from a fresh SFE extract of rosemary; Figure 4B shows the electrophoregram obtained from the same extract after ~24 h at room temperature. As can be seen from a comparison of parts A and B of Figure 4, there is a clear decrease of carnosic acid (peak 4) together with an increase of carnosol isomer (peak 3). It has been mentioned in the literature that carnosic acid is degraded to different extents to other phenolic diterpenes, with lower antioxidant activity (Schwarz et al., 1992). Moreover, the possibilities of using fast procedures, such as the one shown here, to monitor transformations of food constituents following much faster kinetics or the importance of obtaining higher throughput monitoring of such reactions have to be kept in mind as important advantages of MEKC for other applications.

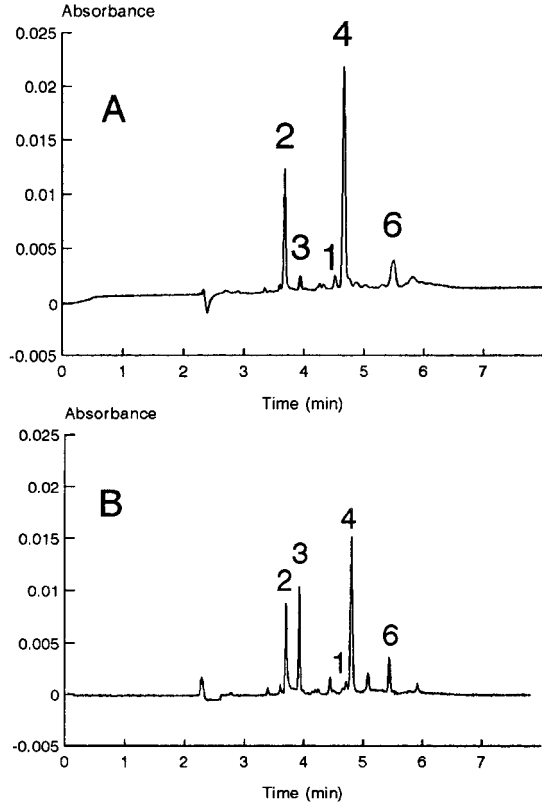

Figure 4. Degradation of antioxidants from an SFE rosemary extract followed by MEKC. Electrophoregrams of rosemary antioxidants from (A) a fresh SFE extract and (B) the same extract as in (A) but after being at room temperature for ~24 h. All of the MEKC conditions were as in Figure 2B.

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