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Pressurized liquid extraction–capillary electrophoresis–mass spectrometry for the analysis of polar antioxidants in rosemary extracts

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Abstract

A method based on capillary electrophoresis–electrospray–mass spectrometry (CE–ESI–MS) was developed to qualitatively characterize natural antioxidants from rosemary (*Rosmarinus officinalis* L.) in different fractions obtained by pressurized liquid extraction (PLE) using subcritical water. The parameters of CE–ESI–MS were adjusted allowing the separation and characterization of different compounds from rosemary in the PLE fractions. These parameters for CE are kind, pH and concentration of the separation buffer, parameters for ESI–MS are dry gas temperature and flow, nebulizing gas pressure, and make-up flow. The following analytical conditions were found most favorable: aqueous CE buffer (40 mM ammonium acetate/ammonium hydroxide, pH 9); sheath liquid containing 2-propanol–water (60:40, v/v) and 0.1% (v/v) triethylamine at a flow rate of 0.24 mL/h; drying gas flow rate equal to 7 L/min at 350 °C, nebulizing gas pressure of 13.8 kPa (2 psi), using a compound stability of 50%. Different antioxidant compounds (e.g., rosmarinic acid and carnosic acid) could be detected in the rosemary extracts by CE–ESI–MS without any additional treatment, enabling the determination of variations in the extract composition caused by the different PLE conditions (i.e., 60 and 100 °C). The results provide complementary information to HPLC analysis.

Keywords: Antioxidants; Rosemary; Pressurized liquid extraction; Capillary electrophoresis; Electrospray mass spectrometry

1. Introduction

The demand for natural antioxidants has risen notably because of the growing interest paid to natural food. Such antioxidants, able to prevent or retard oxidation of fats and oils, are usually applied by food industry not only because of their value for preservation, but also because of their beneficial effects on human health as recently described [1]. Among the antioxidants of non-synthetic origin, rosemary turned out being one of the spices with highest antioxidant activity [2]. Several previous studies have described these constituents of rosemary [3–6]. They have been isolated and identified as phenolic diterpenes, such as carnosol, rosmanol, 7-methyl-epirosmanol, isorosmanol, rosmadial, carnosic acid, methyl carnosate, and other phenolic acids, such as rosmarinic acid.

Several methods exist to extract antioxidants from aromatic plants; those that use environmentally friendly solvents are supercritical fluid extraction (SFE) [7–11] and subcritical water extraction (SWE) [12]. The latter method is based on the extraction with hot water under a pressure sufficient to maintain water in the liquid state; it has been commonly employed to extract soil samples and plant material, but recently, also its possibility to extract antioxidant compounds from rosemary has been demonstrated [12]. With small changes in water temperature different extracts were obtained that contain different compounds enriched therein.

To characterize the isolated fractions attained using the mentioned extraction methods from different samples, high-performance liquid chromatography (HPLC) has been

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applied [11,13]. In previous work, we have demonstrated the suitability of this technique to identify the antioxidant fraction of rosemary obtained by SFE [14] or SWE [12]. Nevertheless, under typical reversed-phase conditions, the most polar compounds (e.g., rosmarinic acid) might be hardly determinable because they elute with the dead volume, i.e., they are unretained. In addition to the known antioxidant activity from carnosic acid and carnosol, the biological activity from other polar compounds has been pointed out [15–17]. The presence of these polar compounds can reduce considerably the information about the antioxidant composition of the extracts derived from RP–HPLC, especially when relatively polar conditions are applied, as is the case in SWE.

In order to overcome this limitation, the use of capillary electrophoresis (CE) is an interesting alternative [18–22]. Its utility is greatly enhanced by mass spectrometry (MS)detection and particularly, electrospray ionization mass spectrometry (ESI–MS). This soft-ionization technique allows the production of ions even of labile compounds, as natural antioxidants are. MS provides the high sensitivity and detectability often required for CE, in addition to its compound identification capability. Together it makes CE–ESI–MS to one of the most powerful analytical methodologies. CE–ESI–MS procedures have already been described for the analysis of phenolic compounds from different samples [23,24]. Although various methods have been previously developed to analyze rosemary extracts by CE [25–26], none of these procedures is compatible with ESI–MS. Therefore, the goal of the present paper focuses on the development of CE–MS conditions allowing the qualitative analysis of fractions from rosemary obtained from pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction) with subcritical water, containing natural antioxidants.

2. Experimental

2.1. Chemicals and samples

All chemicals were of analytical reagent grade and used as received. Ammonium acetate was from Panreac (Barcelona, Spain), ammonium hydroxide from E. Merck (Darmstadt, Germany), both were used for the CE running background electrolyte (BGE) at different concentrations and pH values. BGEs were prepared by weighting ammonium acetate at the concentrations indicated and adding ammonium hydroxide (0.5 M) to adjust the pH. The BGEs were stored at 4 °C and brought to room temperature before use. Distilled water was further deionized by a Milli-Q system (Millipore, Bedford, MA, USA). Sodium hydroxide was purchased from E. Merck. Triethylamine (Sigma, St. Louis, MO, USA) and 2-propanol (HPLC grade, Scharlau, Barcelona, Spain) were used in the sheath flow.

The rosemary samples consisted of dried rosemary (*Rosmarinus officinalis* L.) leaves obtained from an herbalist's shop (Murcia, Spain), dried using a traditional method as described previously [27]. Samples were ground under cryogenic carbon dioxide and stored (for two months maximum) in amber flasks at −20 °C until use.

2.2. Pressurized liquid extraction

SWE was performed in an ASE 200 system (Dionex, Sunnyvale, CA, USA) equipped with a solvent controller, carried out at two different temperatures (60 and 100 °C) for 25 min. Previous to each extraction, an extraction cell heat-up time was completed for 5 min. Likewise, all extractions were performed in 11 mL extraction cells, containing 2.0 g of sample. Extraction procedure was as follows: (i) sample was loaded into cell, (ii) cell was filled with water up to 10.3 MPa (1500 psi), (iii) heat-up time was applied, (iv) static extraction was undertaken, in which all system valves were closed, (v) cell was rinsed with 60% of cell volume with water, (vi) water was purged from cell with gaseous N_2 and (vii) depressurization took place. A rinse of the complete system was made between extractions. The extracts obtained were immediately protected from light and stored under refrigeration until dried. For this purpose, a freeze-dryer (Unitop 400 SL, Virtis, Gardiner, NY, USA) was used. After freeze–drying, 10 mg of each dry extract were freshly dissolved in 1 ml of water and immediately injected into CE–MS without further purification. Water was deoxygenated by purging with He for 15 min prior its use as extraction solvent.

2.3. Capillary electrophoresis

Analyses were carried out in a CE apparatus (P/ACE 5500, Beckman Instruments, Fullerton, CA, USA) equipped with a UV–vis detector working at 200 nm and coupled with an orthogonal electrospray interface (ESI) to the MS detector. The CE instrument was controlled by a personal computer running System Gold software from Beckman. A commercial coaxial sheath-flow interface was used (see below). The bare fused-silica capillary with 50 μm i.d. was purchased from Composite Metal Services (Worcester, UK). The detection length to the UV detector was 20 cm, the total length (to MS detection) was 87 cm. Injections were made at the anodic end using N_2 at a pressure of 3450 Pa (0.5 psi) for 10 s. All separations were at 20 kV as running voltage.

Capillary conditioning was carried out by flushing for 1 min with 0.1 M sodium hydroxide, then for 2 min with water, and finally for 3 min with the separation buffer. Before first use, capillaries were conditioned by rinsing for 20 min with 0.1 M NaOH followed by water for 10 min. At the end of the day, the capillary was rinsed for 10 min with water, and flushed with air for 5 min.

2.4. Mass spectrometry

MS experiments were performed with an ion-trap mass spectrometer (Esquire 2000, Bruker Daltonik, Bremen,

Germany) equipped with an orthogonal ESI (model G1607A, Agilent Technologies, Palo Alto, CA, USA). Electrical contact at the electrospray needle tip was established via a sheath liquid composed of 2-propanol–water (60:40, v/v) containing 0.1% (v/v) trietylamine and was delivered at a flow rate of 0.24 mL/h by a syringe pump (74900-00-05, Cole Palmer, Vernon Hills, IL, USA). The mass spectrometer was operated in the negative ion mode. The spectrometer was scanned at *m*/*z* 200–500 range at *m*/*z* 13,000 per second during separation and detection (target mass *m*/*z* 350). Electrospray operating conditions were optimized as described under Section 3 (dry and nebulizer gas was N_2). The instrument was controlled by a personal computer running Esquire NT software from Bruker Daltonics.

3. Results and discussion

3.1. Development of CE–ESI–MS method for rosemary fraction analyses

The two published CE procedures for the separation of rosemary compounds [25,26] are not suitable for CE–ESI–MS. The first one uses a micellar electrokinetic approach with sodium dodecyl sulfate (SDS), the second applies a running buffer composed of sodium borate, substances that are known to be incompatible with ESI–MS [28]. Moreover, these two methods deal with rosemary extracts obtained with supercritical CO_2 or methanol, resulting in a composition expected being different from our fractions obtained by subcritical water. This can be deduced taking into account both, the different extraction procedures (i.e., supercritical extraction, direct extraction and subcritical extraction) and the different properties of the three fluids involved (i.e., CO_2, methanol and water, see Table 1). It can be deduced from their respective dielectric constants, that compounds of low polarity are better extracted with CO_2, those of higher polarity with subcritical water, and intermediate polarity compounds with methanol. Therefore, the appropriate CE–MS method had to be developed for the extracts under consideration.

Initially, different BGEs at high pH values, which arecompatible with CE–ESI–MS were tested. Under such basic conditions, the compounds expected to be present in the subcritical water extracts (mainly polyphenols) can acquire negative charges that could favor their separation by CE. Ammonium acetate concentrations from 10 to 100 mM (in steps of 30 mM) and pH values from 8 to 10 (in steps of 0.5 pH units) were tested. The sample was a rosemary extract using subcritical water at 100 °C. The best conditions in terms of peak resolution and analysis speed were found with 40 mM ammonium acetate at pH 9.0. Therefore, these conditions were chosen for the subsequent optimization of the ESI parameters.

Table 1
Working parameters for the extraction of rosemary (for methanol and CO_2, see [25,26])

Solvent	Temperature (°C)	Pressure (atm)	Dielectric constant
Methanol	25	1	32.6
CO_2	0–100	1	1.00–.60
Water	100	100	58.5
Water	60	100	∼65.0

1 atm = 10,1325 Pa.

It is obvious that optimization of the parameters plays a key role in the achievement of adequate MS signals for any analyte [29,30]. Optimization for the detection of the compounds of interest was carried out by a univariate method; output parameter was the sum of the peak intensities of the four major compounds from the PLE extract obtained at 100 °C. Initially, four different compositions of the sheath flow liquid were tested, namely, 2-propanol–water (60:40, v/v), or 2-propanol–water (80:20, v/v), each with and without 0.1% (v/v) triethylamine. It was observed that with 2-propanol–water (80:20, v/v) (independent of the addition of triethylamine) the current broke down very frequently indicating a poor electrical contact between the CE and ESI electrical circuits, probably caused by the high organic content of this solution. The solution, containing 2-propanol–water (60:40) provided higher stability, and addition of triethylamine resulted in a higher MS signal. Therefore, 2-propanol–water (60:40, v/v) with 0.1% (v/v) triethylamine was selected as sheath liquid. Next, other ESI–MS parameters were optimized with the height of the MS signal for the main compounds detected in the rosemary extract as the criterion: dry gas temperature and flow, nebulizing gas pressure, compound stability and sheath liquid flow (Fig. 1). The highest temperature at which the instrument can work (i.e., 350 °C) provided the best signal (Fig. 1A), and the optimum of the nebulizer gas pressure was obtained at 13.8 kPa (2 psi) (i.e., the minimum required in order to obtain a stable spray, see Fig. 1B). Optimum dry gas flow was achieved at 7 L/min as can be seen in Fig. 1C. This optimum dry gas flow results from the balance between the lower peak efficiencies (and therefore MS signals observed at flows higher than 7 L/min) and the lower ionization yields (and therefore, MS signals obtained at flows lower than 7 L/min). It was also observed that the compound stability played an important role for this type of analytes (Fig. 1D). Thus, considering a low stability of compounds (i.e., 25%) the number of molecules that were transferred into the MS analyzer was too low due to the low electric field applied by the MS instrument into the capillary skimmer. However, if the compounds are considered 100% stable, a higher electric field is then used by the MS instrument to force the entrance of ions from the gas phase into the capillary skimmer and, under these conditions, some of the compounds (mostly the compound marked as 4, see below) become unstable, indicated by a decrease of their peak intensity. A balance was found for a compound stability percentage of 50% (Fig. 1D) and this value was used for all subsequent experiments.

The optimum sheath liquid flow was at 0.24 mL/h as can be deduced from Fig. 1E. This has been mentioned in the

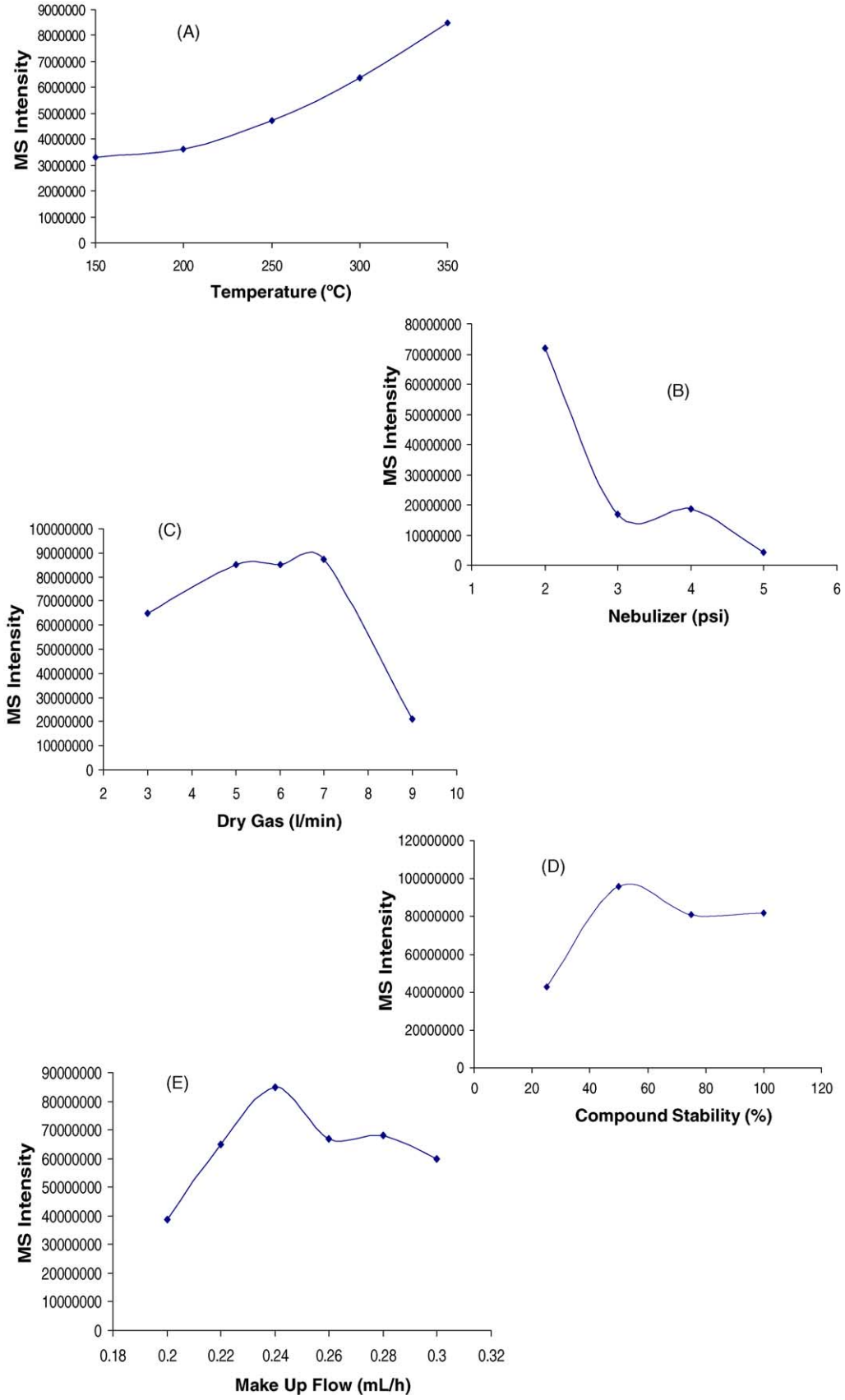


Fig. 1. Optimization of ESI–MS parameters: temperature (A), nebulizer gas pressure (B), dry gas flow (C), compound stability (D) and sheath liquid flow (E). For details see text.

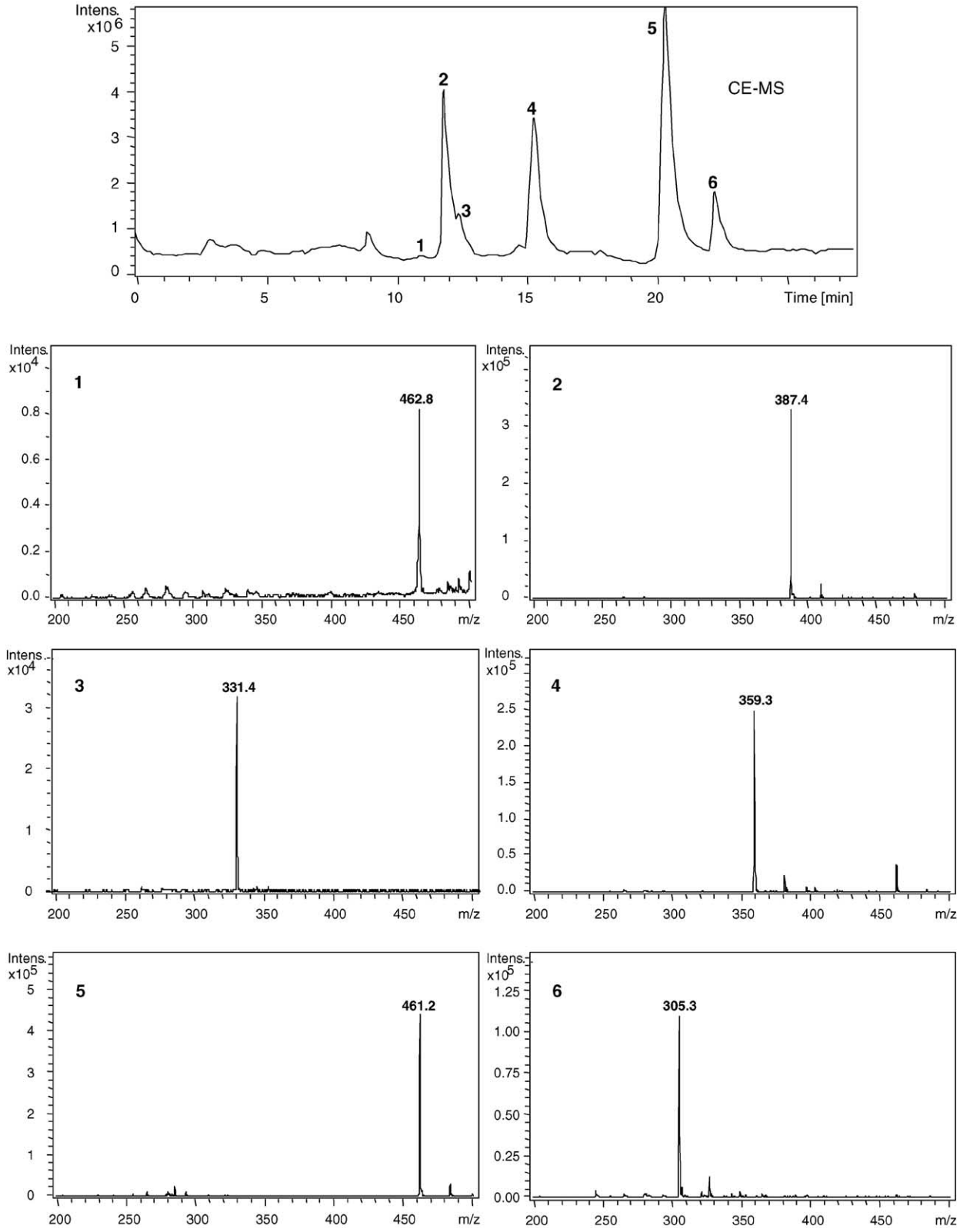
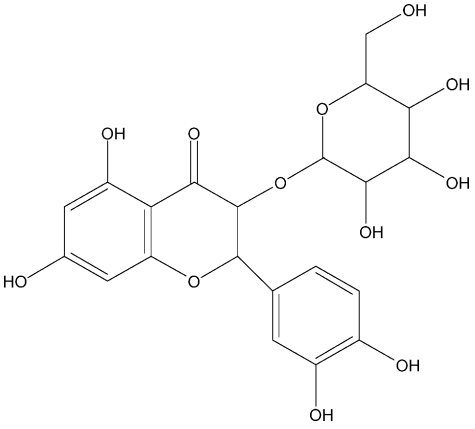
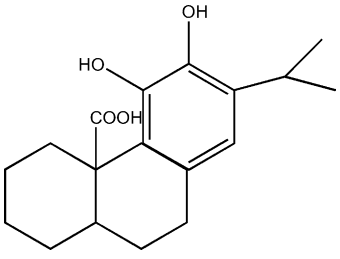
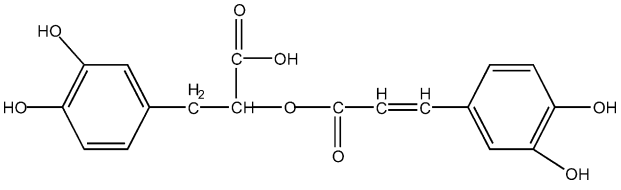
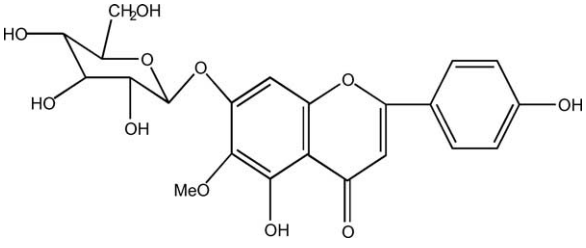
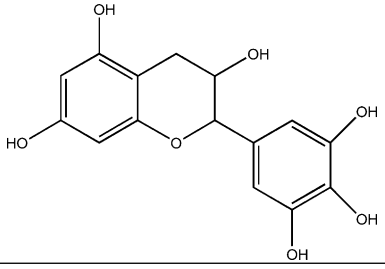


Fig. 2. CE–MS base peak electropherogram and MS spectra of the main peaks detected in a rosemary extract obtained using subcritical water at 100 °C. CE–MS conditions: 50 μm i.d. fused-silica capillary, 87 cm total length. BGE: 40 mM ammonium acetate, adjusted at pH 9.0 with ammonium hydroxide. Voltage: 20 kV. Injections: 10 s at 0.5 psi (3450 Pa). Sheath liquid: 2-propanol–water (60:40, v/v) containing 0.1% (v/v) triethylamine, flow rate 0.24 mL/h. Drying gas (N_2): 7 L/min, 350 °C. Nebulizing gas (N_2): pressure 13.8 kPa (2 psi). MS analyses were carried out using negative polarity. Compound stability: 50%. MS scan *m/z* 200–500 (target mass = *m/z* 350). Sample: rosemary extract, 10 mg/ml concentration. For other conditions see text.

literature [30], indicating that at low sheath liquid flows the ionization yield is reduced due to the instability of the spray, while at higher flows the high dilution of the electrophoretic bands emerging from the capillary can be too high and the intensity of the MS signal for these compounds is therefore reduced. As a result, the following CE–ESI–MS conditions were finally selected: running BGE, 40 mM ammonium acetate/ammonium hydroxide, pH 9; sheath liquid consisting of 2-propanol–water (60:40, v/v) and 0.1% (v/v) triethylamine delivered at a flow rate of 0.24 ml/h; a drying gas flow rate at 7 L/min and at 350 °C, nebulizing gas pressure of 13.8 kPa (2 psi); MS analyses were carried out using a compound stability of 50%.

Under these conditions, CE–ESI–MS records as the one given in Fig. 2 were obtained for these rosemary PLE fractions. Moreover, in the same Figure it is demonstrated that

Table 2
Chemical structures of compounds 1, 3–6 from Fig. 2 (see text for details)

Compound	Chemical structure	Theoretical mass	$[M-H]^-$
(1) Isoquercitrin		464.4	462.8
(3) Carnosic acid		332.4	331.4
(4) Rosmarinic acid		360.3	359.3
(5) Homoplantaginin		462.4	461.2
(6) Gallocatechin		306.2	305.3

the selected conditions provide adequate MS spectra for all analytes. From these spectra shown and the MS–MS spectra recorded (data not shown), peaks 3 and 4 could be unequivocally identified as carnosic and rosmarinic acid. Their assignment was additionally confirmed by co-injection with standards. Moreover, based on the mass spectra, peak 1 could tentatively be assigned as isoquercitrin ($[M-H]^- = m/z$ 462.8), peak 5 as homoplantagenin ($[M-H]^- = m/z$ 461.2) and peak 6 as gallocatechin ($[M-H]^- = m/z$ 305.3). Peak 2 could not be identified. Chemical structures, molecular masses and obtained $[M-H]^-$ values are given in Table 2.

The reproducibility of the CE–ESI–MS analysis, expressed by the R.S.D. of five consecutive injections was 1.0% for the analysis time and 5.9% for the peak area, adequate for the goal of the present work.

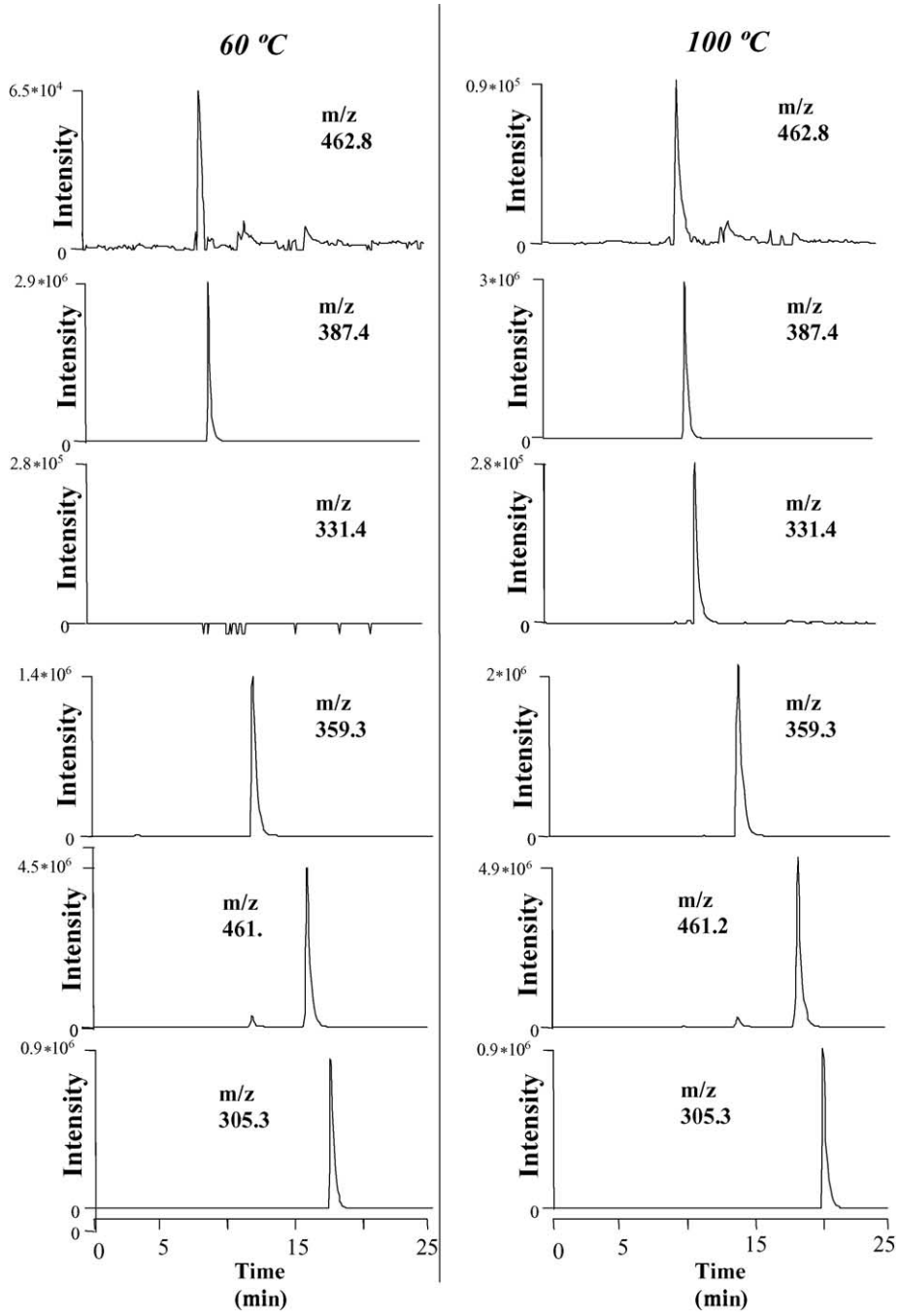


Fig. 3. Extracted ion electropherograms from two different rosemary extracts obtained using subcritical water at 60 and 100 °C, respectively. Extracted ions: *m/z* 462.8 (isoquercitrin); *m/z* 387.4 (non-assigned compound); *m/z* 331.4 (carnosic acid); *m/z* 359.3 (rosmarinic acid); *m/z* 461.2 (homoplantagenin); *m/z* 305.3 (gallocatechin); (all ± *m/z* 0.5). Conditions as in Fig. 2.

3.2. Comparison of CE–ESI–MS and HPLC analysis

For the same PLE extract, the present approach was compared with two published procedures using HPLC–UV and HPLC–MS [12]. Reversed-phase HPLC was not able to detect the most polar compounds [17], because they are unretained and elute with the dead volume, not being differentiable even using an MS instrument as detector. Under the RP–HPLC conditions used in this work, carnosic acid, a less polar compound, has a retention time of about 15 min, whereas rosmarinic acid, a higher polar compound, indeed elutes unretained [12,17]. In contrast, the CE–ESI–MS procedure clearly distinguishes at least six different compounds (Fig. 2) one of them being rosmarinic acid (see below). It should be mentioned that carnosol, rosmanol and epirosmanol were not detected by CE–MS, in contrast to the HPLC–MS method. The reason of this is not fully clear; most probably it is due to the low PLE of these compounds at low temperatures, together with the lower sensitivity achieved by CE due to the small volume of sample injected compared with HPLC [12]. It has been pointed out [31] that limits of detection achieved by CE–MS are worse (about 100–1000 times lower depending on the analytical conditions) than those obtained by HPLC–MS. Also, it is worth mentioning that HPLC–MS and CE–MS require similar analysis time (about 20 min) for these experiments.

3.3. Characterization of PLE extracts from rosemary by CE–ESI–MS

Fig. 3 shows the extracted ion electropherograms of the two fractions obtained at two different extraction conditions, namely, with subcritical water at 60 and 100 °C, keeping constant the other parameters. It can be seen that the extraction of carnosic acid (*m/z* 331.4) is favored at higher temperatures: in contrast to the 100 °C fraction, that at 60 °C does not contain carnosic acid (see the extracted ion electropherograms at *m/z* 331.4 in Fig. 3). These results corroborate previous studies performed in our laboratory [12] and other laboratories [32], in which the ability of subcritical water to selectively extract and isolate different antioxidant compounds from plants depending on the temperature used was shown. It follows that the more polar compounds (such as rosmarinic acid) are preferentially extracted at lower temperatures, while at higher temperatures the less polar compounds (e.g., carnosic acid) were better extracted. The result given in Fig. 3 demonstrates the possibility of tuning the selectivity for antioxidant extraction by using different water temperatures, being able to yield extracts of diverse composition, and thus, of different activity.

In conclusion, it was shown that PLE–CE–MS is a suitable technique to characterize antioxidants from natural sources in a fast and efficient way, providing complementary information to chromatographic procedures. Moreover, this work is one of the few applications of CE–MS to characterize antioxidants in real samples and, to our knowledge, the first one in which the possibilities of PLE–CE–MS to investigate natural compounds have been demonstrated.

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