Accelerated solvent 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 characterize natural antioxidants from rosemary (*Rosmarinus officinalis L.*) in different fractions obtained by accelerated solvent extraction (ASE) using subcritical water. The parameters of CE-ESI-MS were adjusted allowing the separation and characterization of different compounds from rosemary in the ASE 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 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 rosemary extract composition caused by the different ASE conditions (i.e., 60 and 100 °C). The results provide complementary information to HPLC analysis.

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, HPLC has been 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 (as 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 detection (CE-MS) and particularly, electrospray ionization mass spectrometry (CE-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 analysis of fractions from rosemary obtained from accelerated solvent extraction (ASE) 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 pHs. 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. Accelerated solvent extraction

Subcritical water extraction was performed in an Accelerated Solvent Extraction system (ASE 200, Dionex Corporation, Sunnyvale, CA, USA) equipped with a solvent controller, carried out at two different temperatures (60°C and 100°C) for 25 minutes. Previous to each extraction, an extraction cell heat-up time was completed for 5 minutes. 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 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 N₂ 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 PC running System GOLD software from Beckman. A commercial coaxial sheath-flow interface was used (*vide infra*). The bare fused-silica capillary with 50 μm I.D. was purchased from Composite Metal Services (Worcester, U.K.). 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 pressure of 0.5 psi (3448 Pa) 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 5 min flushed with air.

2.4. Mass spectrometry

MS experiments were performed with an ion-trap mass spectrometer (Esquire 2000TM, Bruker Daltonik GmbH, Bremen, Germany) equipped with an orthogonal electrospray interface (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) triethylamine and was delivered at a flow rate of 0.24 mL/h by a syringe pump (74900-00-05, Cole Palmer, Vernon Hills, Illinois, USA). The mass spectrometer was operated in the negative ion mode. The spectrometer was scanned at 200-500 m/z range at 13000 m/z per s during separation and detection (target mass 350 m/z). Electrospray operating conditions were optimized as described under Results and Discussion (dry and nebulizer gas was N_2). The instrument was controlled by a PC 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 SDS, the second applies a running buffer composed of sodium tetraborate, substances that are known to be incompatible with ESI-MS [28]. Moreover, these two methods deal with rosemary extracts obtained using supercritical CO₂ or methanol, resulting in an expected composition 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., CO₂, methanol and water, see Table 1). Therefore, the appropriate CE-MS method had to be developed for the extracts under consideration.

Initially, different BGEs at high pH values compatible 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) and pH values from 8 to 10 (in steps of 0.5) 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.

It is obvious that optimization of the ESI 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 ASE 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) of 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 a ratio of 60:40 of 2-propanol/water 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. The highest temperature at which the instrument can work (i.e., 350 °C) provided the best signal, and the optimum of the nebulizer gas pressure was obtained at 2 psi (i.e., the minimum required in order to obtain a stable spray). Optimum dry gas flow was achieved at 7 L/min. 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. 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, *vide infra*) become unstable, indicated by a decrease of their peak intensity. A balance was found for a compound stability percentage of 50%. and this value was used for all subsequent experiments.

The optimum sheath liquid flow was at 0.24 mL/h. This has been mentioned in the literature [30] indicating that at low sheath liquid flows the ionization yield is reduced due to 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 2 psi; MS analyses were carried out using a compound stability of 50 %.

Under these conditions, CE-ESI-MS separations as the one given in Figure 1 were obtained for these rosemary ASE-fractions. Moreover, in the same Figure it is demonstrated that the selected conditions provide adequate MS spectra for all analytes. The reproducibility of the CE-ESI-MS analysis, expressed by the RSD% 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.

3.2 Comparison of CE-ESI-MS and HPLC-UV

The present approach was compared with two published procedures using LC-UV and LC-MS [12] for the same ASE extract. Reversed phase LC 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-LC 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 (see Figure 1) one of them being rosmarinic acid (*vide infra*). It should be mentioned that carnosol, rosmanol and epirosmanol were not detected by CE-MS, in contrast to the LC-MS method. The reason of this is not fully clear; most probably it is due to the low ASE extraction of these compounds at low temperatures, together with the lower sensitivity achieved by CE due to the small volume of sample injected compared with LC [12].

Also, it is worth mentioning that LC-MS and CE-MS require similar analysis time (about 20 min) for these experiments.

From the MS spectra shown in Figure 1 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 ($[M-H]^- = 462.8$ m/z), peak 5 as homoplantagenin ($[M-H]^- = 461.2$ m/z) and peak 6 as gallocatechin ($[M-H]^- = 305.3$ m/z). Peak 2 could not be identified.

3.3 Characterization of ASE-extracts from rosemary by CE-ESI-MS

Combined ASE and CE-ESI-MS was applied analyzing the extracts obtained at two different extraction conditions. Subcritical water was used at 60 and 100 °C, keeping constant the other parameters. Figure 2 shows the extracted ion electropherograms of the two fractions. 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 Figure 2). These results corroborate previous studies performed in our laboratory [12], where the ability of subcritical water to selectively extract and isolate different antioxidant compounds from plants depending on the temperature used was demonstrated. It follows that the more polar compounds (such as rosmarinic acid) are preferentially extracted at lower temperatures while the less polar compounds (e.g., carnosic acid) were extracted at higher temperatures. The result given in Figure 2 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 ASE-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 ASE-CE-MS to investigate natural compounds have been demonstrated.

ACKNOWLEDGEMENTS

The authors would like to thank to Dr. Gerard Bruin and Novartis Pharma AG (Basel, Switzerland) for the gift of the P/ACE 5500 instrument used in this work. Financial support from a bilateral project Spanish MCYT-University of Vienna (HU2002-0042) and a CICYT-AGL2002-04621-C02-02 project is acknowledged. M.H. thanks MCYT for an FPI grant. The grant for B.G. from ERASMUS is acknowledged.

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FIGURE LEGENDS

Figure 1. 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 (3448 Pa). Sheath liquid: 2-propanol/water 60:40 (v/v) containing 0.1 % (v/v) triethylamine, flow rate 0.24 mL/h. Drying gas (N₂): 7 L/min, 350 °C. Nebulizing gas (N₂) : pressure 2 psi. MS analyses were carried out using negative polarity. Compound stability: 50 %. MS scan 200-500 m/z (target mass= 350 m/z). Sample: rosemary extract, 10 mg/ml concentration. For other conditions see text.

Figure 2. Extracted ion electropherograms from two different rosemary extracts obtained using subcritical water at 60 °C and 100 °C, respectively. Extracted ions: 462.8 m/z (isoquercitrin); 387.4 m/z (non-assigned compound); 331.4 m/z (carnosic acid); 359.3 m/z (rosmarinic acid); 461.2 m/z (homoplantagenin); 305.3 m/z (gallocatechin); (all +/- 0.5 m/z). Conditions as in Figure 1.

Table 1. Working parameters for the extraction of rosemary (for methanol and CO₂, see refs. [25,26]).

Solvent	Temperature (°C)	Pressure (atm)	Dielectric Constant
Methanol	25	1	32.6
CO ₂	0 -100	1	1.00 - 1.60
Water	100	100	58.5
Water	60	100	~ 65.0

Figure 1

Figure 2