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Capillary electrophoretic separation of phenolic diterpenes from rosemary

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Abstract

The major phenolic diterpenes responsible for the antioxidant properties of rosemary extracts, namely carnosol and carnosic acid, were separated by capillary zone electrophoresis (CZE) using a 56 cm long uncoated fused-silica capillary and a 50 m*M* disodium tetraborate buffer of pH 10.1. The effect of the buffer type, pH and concentration, and the capillary length on the separation, was studied. Carnosol and carnosic acid were identified in the electrophoregrams of rosemary extracts through their migration times and UV spectra obtained by CZE analysis of pure compounds isolated from a rosemary extract by HPLC fractionation. The CZE method had good reproducibility (relative standard deviation less than 5%) and was applied to compare the contents of carnosol and carnosic acid in solid and oil-dispersed commercial extracts of rosemary and in rosemary leaves. The separation of carnosol and carnosic acid was accomplished in less than 11 min.

Keywords: Rosemary; Plant materials; Phenolic compounds; Diterpenes; Terpenes; Carnosol; Carnosic acid; Antioxidants

1. Introduction

Rosemary extracts exhibit a potent antioxidant activity and therefore they are commonly used as food and cosmetic additives to prevent the oxidation of fats and the formation of off-flavor-responsive compounds. The antioxidant activity of rosemary extracts is related to the presence of phenolic diterpenes such as carnosic acid, carnosol, methyl carnosate, rosmanol, epirosmanol and 7-methyl-epirosmanol. The compound mainly responsible for the antioxidant properties of rosemary and the major phenolic diterpene present in fresh rosemary leaves has been found to be carnosic acid [1–3]. Carnosic acid is converted into carnosol by oxidation and carnosol can degrade further to produce other phenolic diterpenes with γ-lactone structure [4]. Fig. 1

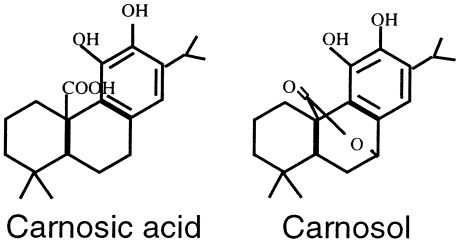

Fig. 1. Chemical structures of the major phenolic diterpenes found in rosemary extracts: carnosic acid and carnosol.

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shows the chemical structures of carnosic acid and carnosol.

The determination of phenolic diterpenes in rosemary leaves was carried out by high-performance liquid chromatography (HPLC) after extraction with an organic solvent [4,5] and supercritical fluid extraction [6]. Cuvelier et al. [7] have identified 22 compounds, including phenolic acids, carnosol derivates and flavonoids, in commercial extracts of sage and rosemary by high-performance liquid chromatography coupled with mass spectrometry (MS).

The determination of the profile and the content of these compounds is quite important to evaluate the oxidation and degradation of carnosic acid during the manufacturing process of commercial extracts, the quality of rosemary leaves as raw material and the optimum for harvesting the plant [8].

Capillary zone electrophoresis (CZE) is a highly efficient separation technique for charged compounds and partially charged organics such as phenolic compounds at a high pH. It provides faster analysis times and better separation efficiency than HPLC, and consumes only small amounts of aqueous solvents. The latter has environmental and economic advantages, however, HPLC is better in terms of accuracy, sensitivity and precision. Therefore, it has been proposed as a complementary technique to HPLC for the separation of phenolic compounds present in wines such as phenolic acids and flavonoids [9–13]. In addition, micellar electrokinetic capillary chromatography has been proposed to separate synthetic and natural food-grade antioxidants, such as gallates, butylated hydroxyanisole, butylated hydroxytoluene, *tert*.-butylhydroquinone, ascorbic and isoascorbic acids, and α-tocopherol [14,15]. This paper reports for the first time the use of capillary zone electrophoresis for separating the rosemary antioxidants.

The aim of this work was to study the experimental conditions for separating the main components of rosemary extracts, namely carnosol and carnosic acid by CZE, as well as, to identify these compounds in the electropherograms obtained for rosemary extracts. Finally, the profile of different rosemary extracts and samples were determined using CZE in order to demonstrate the usefulness of the proposed method.

2. Experimental

2.1. Reagents and samples

Boric acid, disodium tetraborate and tris(hydroxymethyl)aminomethane (Tris) were from Merck, sodium hydroxide from Prolabo and acetic acid from Carlo Erba. HPLC-grade methanol and acetonitrile from Merck and Milli-Q ultrapure water (Millipore) were used. All solutions were filtered through a 0.45 μm filter and sonicated for 15 min before use.

Phenolic diterpene standards were not commercially available so quantitative analysis of the extracts was not possible.

Rosemary leaves were sampled from a wild population in Clavijo (La Rioja, Northern Spain) and purchased in a local supermarket. Commercial rosemary extracts were provided by Bordas Chinchurreta (Seville, Spain). The technological process used to obtain the commercial extracts was unknown because it was not mentioned by the manufacturer. Two extracts were in powder form (S1 and S2) and two other were dispersed in a vegetable oil (L1 and L2).

2.2. Sample preparation

Commercial solid extracts of rosemary (150 mg) in methanol (2 ml) were sonicated in a 5510 Branston ultrasonic bath for 15 min and centrifuged using a 5804 Eppendorf centrifuge at 5000 rpm for 15 min at room temperature. The methanol supernatant was then transferred into a vial and filtered through a 0.45 μm filter before the HPLC or CZE analysis.

Commercial liquid extracts (150 mg) and powdered dried leaves (150 mg) of rosemary were processed following the same procedure.

The extraction procedure was repeated three times to ensure exhaustive extraction. The recovery rates of the first extraction were calculated for the three different samples. Carnosol recoveries of 93, 92 and 79% were obtained for commercial liquid and solid extracts, and rosemary leaves, respectively. Similar recoveries were found for carnosic acid in these samples: 94, 92 and 78%, respectively.

Methanolic solutions of the rosemary extracts

were stored protected from light at −40 °C until the HPLC or CZE analysis.

2.3. CZE separation

Capillary zone electrophoresis was carried out using an Agilent CE instrument equipped with a standard cassette containing an uncoated fused-silica capillary (50 μm I.D. and variable effective lengths of 26, 40, 56 and 93 cm) and a diode array detector.

The capillary was conditioned before injection by a first washing with 0.1 *M* sodium hydroxide for 3 min, then with ultrapure water for 2 min, and finally with the running buffer for 5 min. The buffer vials were replenished automatically after each run in order to use fresh buffer each time and so improving reproducibility of the migration times.

The running buffer was 50 m*M* sodium borate (pH 10.1) with a voltage of 30 kV and an average current of 100 μA. The capillary temperature was maintained at 25 °C and samples were injected by hydrodynamic injection at 50 mbar for 3 s (3 nl sample volume or 1.5 mm plug length). Phenolic compounds at pH 10.1 are expected to be negatively charged and to have electrophoretic mobilities towards the anode, but the separation was carried out from the anode to the cathode.

Electrophoregrams were recorded at 250 and 280 nm and the spectrum from 200 to 600 nm was also collected for each peak. All analysis were done in duplicate and the results expressed as mean values.

2.4. HPLC–UV spectrophotometry

HPLC was performed using a modular Waters liquid chromatograph consisting of two 515 HPLC pumps, an on-line degasser, a 717 plus autosampler and a photodiode array detector, and furnished with a Nucleosil 120 C_{18} column (20 cm×0.46 cm, 5 μm).

The mobile phase was a mixture of solvent A (water–acetonitrile–acetic acid, 84:15:1) and solvent B (methanol) according to a step gradient from 0% B to 100% B lasting 55 min, at a flow-rate of 1 ml/min. Injection volume was 20 μl. Chromatographic separation was carried out at room temperature and chromatograms were recorded at 250 nm. UV spectra from 200 to 650 nm were also recorded.

2.5. HPLC–mass spectrometry

The system consisted of an Engine 5989-B Hewlett-Packard quadrupole mass spectrometer equipped with a 59987 Hewlett-Packard electrospray ionization (ESI) source operated in the positive ion mode, and a 1100 Hewlett-Packard liquid chromatograph. Chromatographic separation was performed under the same conditions described above. Injection volume was 5 μl and mobile phase was split 35:965 μl/min between the electrospray interface and the UV detector at 250 nm. In the electrospray ionization interface the eluted compounds were mixed with nitrogen at a 30 l/min flow-rate at a source temperature of 250 °C. The cone voltage was a linear function of the molecular mass, starting at 40 V for m/z 100 and ending at 80 V for m/z 600. The compounds were chemically ionized by proton transfer, the positive ions generated were introduced into the mass spectrometer and the abundances of selected m/z (271, 315, 331, 333, 345, 347, 361, 375 and 463), corresponding to MH^+ ions of rosemary compounds, were recorded.

3. Results and discussion

3.1. Optimization of CZE separation

A detailed study of the variables affecting CZE separation was performed by using the univariate method.

Three different 20 m*M* buffer solutions were tested: boric acid (pH 9.2), Tris (pH 8.4) and disodium tetraborate buffer (pH 9.2), the latter yielding the best resolution.

The effect of pH was studied between 8.4 and 10.1. The pH of the disodium tetraborate buffer had a marked influence on the peak resolution and the migration time as the number of peaks and the migration time increased with increased pH. With pH values lower than 10.1, some of the compounds give rise to broad and overlapped peaks or even only to a bulge in the baseline and are not detected as peaks. However, if we increase the pH to 10.1, the compounds produce sharp and well-resolved peaks. The

best separation for a rosemary extract was obtained at pH 10.1.

The electroosmotic flow can be modified by adjusting the concentration and ionic strength of the buffer solution, so the effect of the buffer concentration was also studied. The increased buffer concentration decreased the electroosmotic flow, which enhances the differences of mobility between the compounds. The concentration was studied over the range 10–50 m*M*. The increasing concentration resulted in an increased resolution but also in increased migration times. A buffer concentration of 50 m*M* was selected as optimum.

The capillary length was studied within the range 26–93 cm. The optimum value was found to be 56 cm. Shorter capillaries led to an insufficient resolution and overlapped peaks, whereas the longer capillary gave rise to too long migration times and peak broadening.

3.2. Identification of rosemary components in the electropherogram

The identification of the rosemary components in the electrophoregrams was not possible by using migration time values because of the lack of a standard for them. In addition, the UV spectral data were only available for acidic medium and the UV spectra were recorded at pH 10.1.

Nine components of rosemary extract were identified by HPLC–MS. The sample was a commercial extract (S2) processed as described in the Experimental section. The HPLC chromatogram is shown in Fig. 2. The identification of the peaks in the chromatogram was based on the abundance signal of MH^+ ions and the UV spectrum at the retention time, and the elution order according to Cuvelier et al. [7]. Table 1 lists the results of the HPLC–MS chromatograms.

The main peaks in the chromatogram were identified as carnosol and carnosic acid. Fractions of the HPLC effluent were collected at the retention time of both compounds and were analyzed by capillary electrophoresis in order to identify carnosol and carnosic acid in the electropherogram. The CZE separation was carried out using a 26 cm capillary under the conditions described in the Experimental section but with a hydrodynamic injection of 100 mbar s.

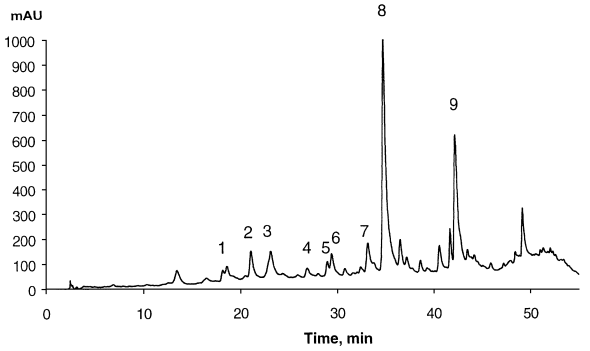

Fig. 2. HPLC chromatogram of a methanolic solution of a rosemary extract. For HPLC conditions, see the Experimental section. Peak identifications are listed in Table 1.

The volume of each HPLC fraction collected was lower than 100 μl, and fractions were analyzed by flow injection ESI-MS in order to check the accuracy of the fraction collection. Migration times for carnosol and carnosic acid were 2.78 and 4.21 min, respectively. The UV spectra for the peaks at 2.78 and 4.21 min in the electropherogram of the rosemary extract and the spectrum of carnosol and carnosic acid were well-matched, respectively.

3.3. Application of the CZE method to rosemary samples and extracts

In order to check the reproducibility of the CZE method, the relative standard deviation was calculated for the migration times and the peak areas of carnosol and carnosic acid in a rosemary extract. The migration time reproducibility expressed as a relative standard deviation ($n=11$) was 0.30 and 0.48%, and the peak-area reproducibility was 2.9 and 4.2% for carnosol and carnosic acid, respectively.

The performance of the method was tested by applying it to the determination of the peak area for the carnosol and the carnosic acid in six different rosemary extracts. The samples were prepared in triplicate as described in the Experimental section before CZE injection. The results obtained are listed in Table 2. Three electrophoregrams of methanolic solutions of these samples are shown in Fig. 3: (a) a commercial extract in solid form (S1), (b) a commer-

Table 1
Identification of nine phenolic compounds separated by HPLC in a commercial extract of rosemary

Peak number	Retention time (min)	MH^+	Fragment	Compound
1	18.3	463		Homoplantaginin
2	21.0	347		Rosmanol
3	23.0	347	287	Epirosmanol
4	27.0	271		Apigenin
5	29.0	347		Epirosmanol
6	29.5	315		Cirsimaritin
7	33.5	361		Epirosmanol methyl ether
8	35.5	331		Carnosol
9	42.5	333	287	Carnosic acid

Table 2
Peak areas of carnosol and carnosic acid obtained by CZE in different rosemary extracts

Sample	Peak area, mean±SD (n=3) (mAu s)	
	Carnosol	Carnosic acid
S1 (commercial, solid form)	49±1	238±8
S2 (commercial, solid form)	112±11	71±2
L1 (commercial, oil dispersion)	12±1	41±4
L2 (commercial, oil dispersion)	8±1	20±2
Leaves of commercial rosemary	11±2	113.8±0.5
Leaves of wild rosemary	14±3	175±9

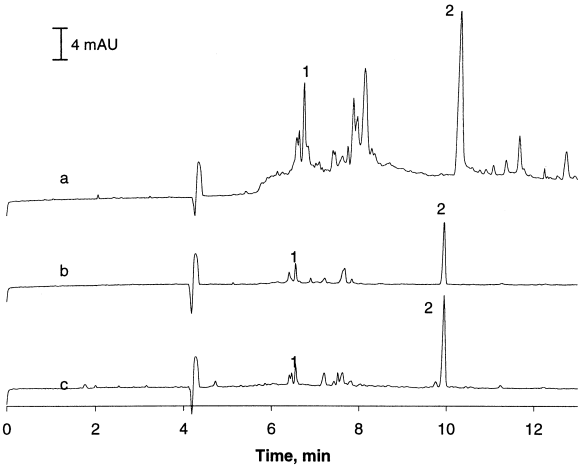

Fig. 3. CZE electrophoregrams of methanolic solutions of three different samples: (a) commercial extract of rosemary in solid form (S1), (b) commercial extract of rosemary dispersed in vegetable oil (L1), and (c) commercial leaves of rosemary. Peak identification: 1, carnosol; 2, carnosic acid. CZE conditions: capillary, 56 cm as effective capillary length and 50 μm I.D.; applied voltage, 30 kV; buffer, 50 mM disodium tetraborate (pH=10.1); temperature, 25 °C; hydrodynamic injection, 100 mbar×s; detection wavelength, 250 nm.

cial extract dispersed in vegetable oil and (c) commercial rosemary leaves.

As it can be seen, the content in carnosic acid of the extract S1 was more than three times higher than that of the extract S2, while its content of carnosol is more than twofold lower which indicates that the industrial process used to obtain extract S2 promoted the degradation of the carnosic acid. In addition, the commercial oil-dispersed extracts of rosemary (L1 and L2) had lower contents of phenolic diterpenes than the solids forms and the highest ratio carnosic acid–carnosol was found in the rosemary leaves.

4. Conclusions

The optimization of the CZE separation and the identification of carnosol and carnosic acid in rosemary extracts has been successfully performed. Therefore, CZE has proven to be an efficient technique to separate the phenolic compounds of rosemary extracts. Against HPLC, CZE presents three outstanding advantages: (a) the CZE separation is faster, (b) it does not require organic solvents as components of the mobile phase and consumes a much lower amount of reagents, and (c) capillaries are less expensive than HPLC columns. Therefore, CZE can be used as an alternative to HPLC or as a complementary technique in the determination of natural antioxidants in rosemary extracts.

Since the reproducibility of the CZE separation was demonstrated, the CZE method proposed was used to compare the contents of carnosol and carnosic acid of different commercial extracts of rosemary.

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