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Identification of phenolic compounds in rosemary honey using solid-phase extraction by capillary electrophoresis–electrospray ionization-mass spectrometry

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Abstract

Complex extracts of rosemary honey constituents often require very effective separation techniques to allow the identification of different compounds. Capillary electrophoresis (CE) coupled to mass spectrometry (MS) detection can provide structure-selective information about the analytes in such matrices and has turned out to be an attractive alternative to HPLC methods. A simple and cost-effective analytical method involving solid-phase extraction (SPE) and capillary zone electrophoresis coupled to electrospray ionization-ion trap mass spectrometry (CZE-ESI-MS) to identify and characterize phenolic compounds in rosemary honey is described. The SPE, CE and ESI-MS parameters were optimized in order to maximize the number of phenolic compounds detected and the sensitivity of their determination. All CE–ESI-MS experiments were performed with uncoated fused-silica capillaries and an alkaline volatile buffer system consisting of 100 mM NH_4Oac with 10% of 2-propanol at pH 10. Since sheath liquids can made significant effects on the sensitivity in typical CE–ESI-MS application, the effect of type and flow rate of the sheath liquid on the sensitivity of phenolic compounds were investigated. As result, the best sensitivity was obtained with a sheath liquid containing 2-propanol/water 60:40 (v/v) and 0.1% (v/v) of triethylamine at 3 μL/min in the negative ion mode. We describe the first method for the analysis of phenolic compounds in rosemary honey at mg/L levels by using a simple SPE before CE–ESI-MS analysis.

Keywords: Capillary electrophoresis; Electrospray ionisation; Mass spectrometry; Phenolic compounds; Rosemary honey

1. Introduction

In the past several years, there has been increasing evidence of the antioxidant capacity of honey. Honey can prevent deteriorative oxidation reactions in foods, such as lipids oxidation in meat [1,2] and enzymatic browning of fruits and vegetables [3,4]. Honey has therefore great potential to serve as a natural food antioxidant. In a previous study, it was demonstrated that honey is similar in antioxidant capacity to many fruits and vegetables on a fresh weight basis, as measured by the oxygen radical absorbance capacity assay [5]. The antioxidant activity of honey, however, varies greatly depending on the honey floral source [5,6]. There is a lack of knowledge about the profiles of antioxidant substances in honey from various floral sources. The variation in these profiles might be responsible for the widely varying abilities of honeys to protect against oxidative reactions [7].

Honey is a remarkably complex natural liquid that is reported to contain at least 181 substances [8]. The composition of honey is rather variable and primarily depends on the floral source; however, certain external factors also play a role, such as seasonal and environmental factors and processing. Honey is a supersaturated solution of sugars and a wide range of minor constituents is also present in honey, many of which are known to have antioxidant properties [7]. The antioxidant activity of phenolic compounds might significantly contribute to the human health benefits of plant foods [9,10] and beverages such as red wine and tea [10–12]. Honey contains a great number of phenolic

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compounds, which are generally acknowledged to be of considerable importance because of its chemoprotective effect in human beings.

Rosemary honey is produced from *Rosmarinus officinalis* (Lamiaceae). This honey has a very good consumer acceptance and commercial value in European countries because of its mild flavor and light color [13].

Even though the characterization of phenolic compounds from honey has been succesfully carried out using GC [14,15] and HPLC [16–19], CE [20–22] has become an alternative or complement to chromatographic separations because it needs no derivatization step, requires only small amounts of sample and buffer and has proved to be a high-resolution technique, so the technique has emerged as a good alternative in pharmaceutical, forensic and food research laboratories.

The hyphenation of CE to MS combines the high speed and efficiency of CE with the selectivity and sensitivity inherent to MS. The use of CE as analytical separation technique coupled to MS as detection method can provide important advantages in food analysis because of the combination of the high separation capabilities of CE and the power of MS as identification and confirmation method [23]. In general, if a separation technique is coupled with MS the interpretation of the analytical results can be more straightforward [24–26]. In this sense, ESI has emerged as a highly useful technique which allows direct coupling with electrophoretic separation techniques [27]. Furthermore, collisionally induced dissociation can be used to obtain fragment ions of structural relevance for identifying target compounds in a highly complex matrix.

The aim of the present work has been to develop the first simple SPE-CE–ESI-MS method for the identification and characterization of phenolic compounds in rosemary honey samples.

2. Experimental

2.1. Chemical and samples

Rosemary honey samples were collected from a commercial centre market (Apisol, S.A. Montroy (Valencia)).

All chemicals were of analytical reagent grade and used as received. Ammonium acetate was from Panreac (Barcelona, Spain) and ammonia from Merck (Darmstadt, Germany) were used to prepare CE running buffers at different concentrations and pH values. Buffers were prepared weighting the appropriate amount of ammonium acetate at the concentrations indicated and adding ammonium hydroxide (0.5 M) to adjust the pH. The buffers were prepared with doubly deionized water, stored at 4 °C and brought to room temperature before use.

Doubly deionized water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA). Triethylamine from Aldrich (Steinheim, Germany), sodium hydroxide, 2-propanol used in the sheath flow and on the buffer, methanol used on the buffer and in the extraction procedure and diethyl ether all HPLC grade were obtained from Panreac (Barcelona, Spain). All solutions were filtered through a 0.45 μm Millipore (Bedford, MA, USA) membrane filters before injection into the capillary.

2.2. CE–ESI-MS

The analyses were made in a P/ACE™ System MDQ (Beckman Instruments, Fullerton, CA, USA), CE apparatus equipped with an UV–vis detector working at 214 nm and coupled to the MS detector by an orthogonal electrospray interface (ESI).

A commercial coaxial sheath-flow interface was used. Bare fused-silica capillary of 50 μm i.d. came from Beckman Coulter Inc. (Fullerton, CA, USA). A detection window was created at 10 cm for the UV detector and 100 cm was the total length (corresponding to the MS detection length).

Before first use, the bare capillaries were conditioned with 0.1 M sodium hydroxide during 20 min followed by a water rinse for other 10 min. At the end of the day the capillary was flushed with water for 10 min and air for 5 min.

Capillary conditioning of the columns was done by flushing for 2 min sodium hydroxide, 4 min with water, and then for 10 min with the separation buffer (during all the capillary conditioning was used a pressure of 20 psi).

The instrument was controlled by a PC running the 32 Karat System software from Beckman.

MS experiments were performed on a Bruker Daltonics Esquire 2000™ ion trap mass spectrometer (Bruker Daltonik GmgH, Bremen, Germany) equipped with an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). Electrical contact at the electrospray needle tip was established via a sheath liquid by a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA). For the connection between the CE system and the electrospray ion source of the mass spectrometer, the outlet of the separation capillary was fitted into the electrospray needle of the ion source and a flow of conductive sheath liquid established the electrical contact between capillary effluent and water for electrospray needle. The mass spectrometer was run in the negative ion mode and the capillary voltage was set at 4000 V. The ion trap scanned at 50–650 *m/z* range at 13,000 u/s during the separation and detection. The maximum accumulation time for the ion trap was set at 5.00 ms, the target count at 20,000 and the trap drive level at 100%. Electrospray operating conditions were optimized as described in Results and Discussion. The instrument was controlled by a PC running the Esquire NT software from Bruker Daltonics.

2.3. Solid-phase extraction procedure

Extraction was performed according to methods described previously [28–30] with some modifications. About 30 g of rosemary honey samples were thoroughly mixed with five parts (150 mL) of distilled water, adjusted to pH 2 with concentrated HCL, until completely fluid by stirring with a magnetic stirrer at room temperature. The fluid samples were then filtered through cotton wool to remove solid particles. The filtrate was mixed with 40 g Amberlite XAD-2 (pore size 9 nm, particles size 0.3–1.2 mm) and stirred in a magnetic stirrer for 10 min, which was considered enough to adsorb honey phenolics with a recovery rate more than 80% [28,31]. The Amberlite particles were then packed in a glass column (42 cm × 3.2 cm) and the column

was washed with acidified water (pH 2 with HCl, 100 mL) and subsequently rinsed with distilled water (300 mL) to remove all sugars and other polar constituents of honey. The phenolic compounds remained adsorbed on the column [32] and were eluted with methanol (300 mL). The methanolic extract was concentrated to dryness under reduced pressure in a rotary evaporator at 50 °C. The residue was resuspended in distilled water (5 mL) and extracted with diethyl ether (5 mL × 3). The diethyl ether extracts were combined and the ether was removed to dryness under reduced pressure in a rotavapory evaporator at 30 °C. The dried residue was then redisolved in 0.5 mL of methanol, filtered through a 0.45 μm membrane filter. Finally distilled water was adding to the extract until obtaining a 50:50 methanol:water solution and analysed by CE–ESI-MS.

3. Results and discussion

3.1. Development of CE–ESI-MS method

The methanol–water extracts of rosemary honey were obtained as described in the Section 2.3. Extracts were used to optimize the electrophoretic and MS conditions.

Initially, the electrophoretic conditions were optimized according to the following criteria: migration behaviour, sensitivity, analysis time and peak shape. First, buffers containing different concentrations of ammonium acetate at basic pH values were tested. Due to the simple composition of volatile buffer solution it is essential to decrease the background noise and not to suppress the ionization efficiency in ESI. Amonium acetate concentration was varied from 20 to 120 mM (in steps of 20) in an attempt to improve the resolution and minimize the analysis time; pH values from 8.5 to 10.5 (in steps of 0.5) and concentrations of methanol or 2-propanol from 0 to 10% (in steps of 5%) were assayed to obtain the best peak shape, resolution and efficiency among the phenolic compounds. Finally, the addition of 2-propanol as organic modifier raised the best resolution (Fig. 1). The best parameters turned out to be 100 mM ammonium acetate at pH 10 and 10% 2-propanol. The voltage applied was varied between 10 and 30 kV; a voltage of 25 kV was finally chosen in order to afford the best resolution together with satisfactory current and analysis time. The injections were made at the anodic end using a N_2 pressure of 0.5 psi for 20 s (1 psi = 6894.76 Pa). These conditions were chosen for the subsequent optimization of the ESI parameters. During buffer optimization we used the best values for the ESI parameters obtained in the preliminary studies: a sheath liquid containing 2-propanol/water 60:40 (v/v) and 0.1% (v/v) of triethylamine at a flow rate of 3 μL/min, a drying gas flow rate of 7 L/min at 350 °C, nebulizer gas pressure of 6 psi and a compound stability of 25%.

It has often been demonstrated that optimization of the ESI parameters plays a key role in the achievement of adequate MS signals for any analyte. To optimize the detection of the

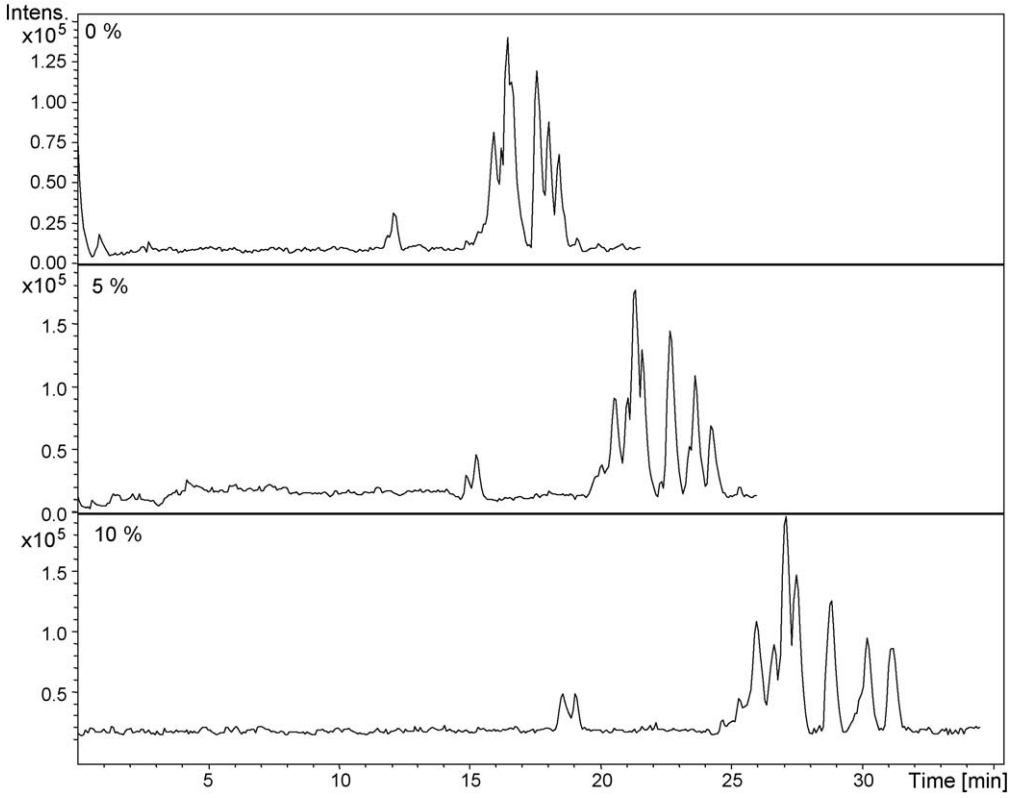

Fig. 1. Optimization of percentages of 2-propanol added to the running buffer. Initial conditions: 50 μm i.d. fused silica capillary, 100 cm total length; buffer: 100 mM ammonium acetate pH 10; voltage: 20 kV; injection time: 20 s at 0.5 psi; sheath liquid: 2-propanol/water 50:50 (v/v) at flow rate 0.20 mL/h; drying gas: 5 L/min; temperature: 300 °C; nebulizing gas pressure: 4 psi. MS analyses were carried out using negative polarity. Compound stability: 100%. MS scan 50–650 *m/z* (target mass 350 *m/z*). Sample: rosemary honey extract.

compounds extracted from rosemary honey we applied a univariant method.

It is also well known that the choice of sheath liquid has a significant effect on the sensitivity and electrical contact between CE and ESI [33,34]. Generally, small amounts of volatile triethylamine (TEA) or ammonium hydroxide can be used for ESI-negative detection [35].

The ESI-MS operating conditions were optimized by adjusting the needle–counter electrode distance, sheath liquid composition, nebulizer gas flow rate and applied electrospray potentials while a sample solution was injected and separated in the CE–ESI-MS system. For the optimization of the ESI parameters was used the signals corresponding to the high peak, because this family has the same behaviour in this optimization.

Initially we tested different types of sheath-flow liquids (after checking in the preliminary studies that the best results were obtained with 2-propanol as organic modifier): 2-propanol/water (50:50, v/v); 2-propanol/water (60:40, v/v) and 2-propanol/water (80:20, v/v), with and without 0.1 and 0.2% (v/v) TEA. Using 80:20 sheath liquid with and without TEA, the current broke down after 10 min, possibly due to poor electrical contact between the CE and ESI, which may have been due to the high organic content of the solution. However, the use of a sheath liquid of 60:40 (v/v) 2-propanol/water plus 0.1% (v/v) TEA provided higher current stability and MS signal. Therefore, 60:40 (v/v) 2-propanol/water with 0.1% (v/v) TEA was selected as sheath liquid.

We then optimized the other ESI-MS parameters, drying gas temperature and flow, nebulizing gas pressure, compound stability and sheath liquid flow (Fig. 2A–E) using the height of the MS signal. Initially the value for each parameter was the best found in the preliminary studies; after re-optimizing each parameter we then used the new value to complete the optimization of the other parameters.

As can be seen, a temperature of 350 °C (Fig. 2A), drying gas flow at 7 L/min (Fig. 2B), and nebulizer gas pressure at 6 psi (Fig. 2C) provided the best signals.

It can also be seen in Fig. 2D that compound stability plays an important role in detecting rosemary honey compounds. Thus at higher percentages of compound stability the MS signal decreases due to the low number of molecules transferred into MS, whilst at lower percentages most of the compounds become

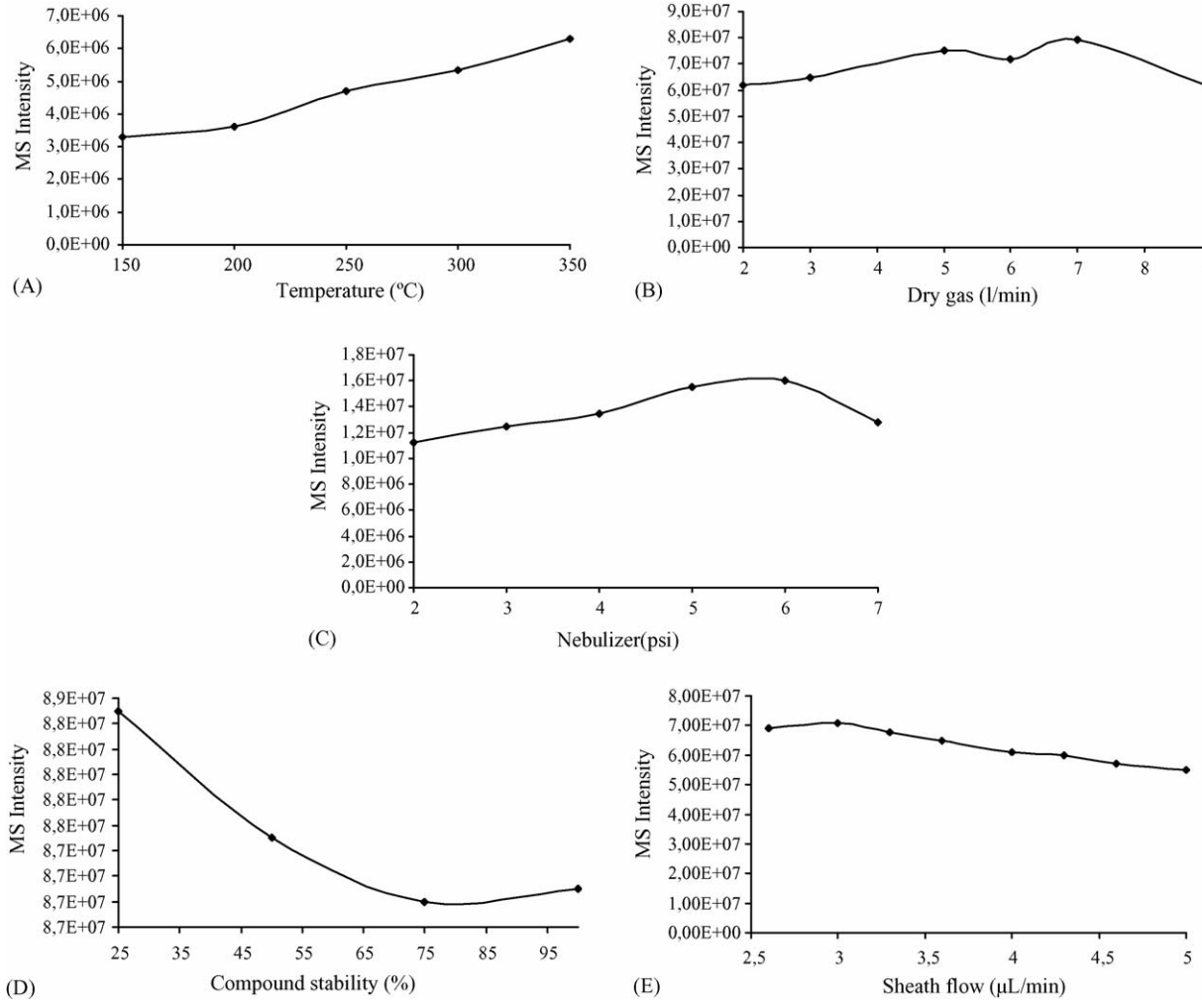

Fig. 2. Optimization of ESI-MS parameters. Conditions: buffer: 100 mM ammonium acetate and 10% 2-propanol at pH 10; voltage: 25 kV; injection time: 20 s at 0.5 psi. MS analyses were carried out using negative polarity. MS scan 50–650 *m/z* (target mass 350 *m/z*). Sample: rosemary honey extract.

more stable, as indicated by an increase in the MS signal. This parameter is related to the voltage used in the capillary placed at the MS entrance; thus, the higher the voltage applied by the MS instrument and, therefore, the higher the solute fragmentation that can take place at that point. We chosen 25% as the best value.

The best sheath-liquid flow was one of 3 μL/min (Fig. 2E). This effect has also been mentioned in the literature [36]; at low sheath-liquid flows the ionization yield is reduced because of the instability of the spray whilst at higher flows the increased dilution of the electrophoretic bands emerging from the capillary may be excessive and the intensity of the MS signal for these compounds is therefore reduced.

Under these conditions CE–ESI-MS separations such as the one shown in Fig. 3 were obtained for methanol–water extracts of rosemary honey. The prolonged analysis time is a consequence of the long capillary lengths that are needed to couple a CE instrument to the MS. This is not a problem as such, but counteracts one of the main advantages of CE, namely its speed.

The repeatability of the CE–ESI-MS analysis, expressed by the RSD of five consecutive injections was 0.89% for the analysis time and 2.8% for the high peak area, adequate for the goal of the present work.

3.2. Characterization of rosemary honey phenolic compounds by CE–ESI-MS

The potential of the CE–ESI-MS method was checked by characterizing the SPE extracts obtained from a rosemary honey. In the first time a qualitative analysis is demonstrated in order to identify phenolic compounds in rosemary honeys. The extracts of rosemary honey were analyzed according to the procedure described in the Section 3.1. When honey extracts were analyzed, some coexistent substances tended to affect on the inner surface of capillary, which would decrease the electro-osmotic flow (EOF) and the peak height gradually. In order to improve the reproducibility of this method, when the capillary was used for analysis of honey samples, it was flushed sequentially with 0.2 M sodium hydroxide for 1 min, water for 3 min, and finally equilibrated with background electrolyte solution for 8 min before each injection.

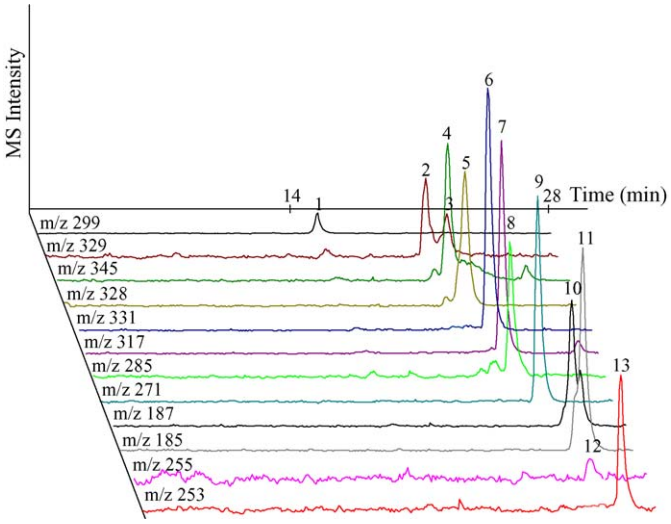

Fig. 4. Extracted ion electropherogram of the compound detected. (1) Kaempferid, (2) Quercetin 3′,3′-dimethyl eter, (3) Quercetin 7,3′-dimethyl eter, (4) N.I., (5) N.I., (6) Monogalloyl, (7) Myricetin, (8) Kaempferol, (9) Pinobanksin, (10) N.I., (11) N.I., (12) Pinocembrin, (13) Chrysin. All conditions as in Fig. 3. N.I: not identified.

The peaks of the main phenolic compounds of rosemary honey were identified by comparing both migration time and MS data obtained from rosemary honey samples with standards.

Fig. 4 shows the extracted ion electropherograms of several compounds detected in the methanol-water extract of a rosemary honey and the Fig. 5A and B the mass spectra of the identified compounds; the following compounds were identified: (1) Kaempferid ($[M-H]^-$ 299 *m/z*), (2) Quercetin 3′,3′-dimethyl eter ($[M-H]^-$ 329 *m/z*), (3) Quercetin 7,3′-dimethyl eter ($[M-H]^-$ 329 *m/z*), (6) Monogalloyl-glucose ($[M-H]^-$ 331 *m/z*), (7) Myricetin ($[M-H]^-$ 317 *m/z*), (8) Kaempferol ($[M-H]^-$ 285 *m/z*), (9) Pinobanksin ($[M-H]^-$ 271 *m/z*), (12) Pinocembrin ($[M-H]^-$ 255 *m/z*), (13) Chrysin

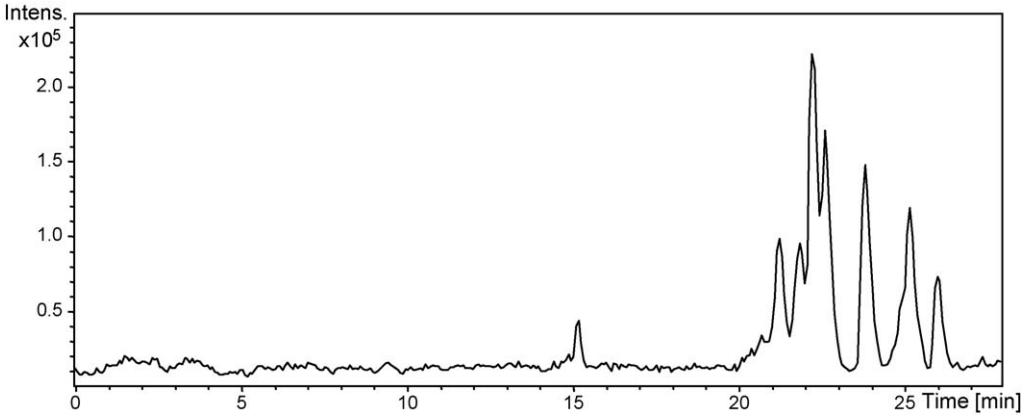

Fig. 3. Base peak electropherogram of rosemary honey sample using the optimal conditions. CE-MS conditions: buffer: 100 mM ammonium acetate 10% 2-propanol at pH 10; voltage: 25 kV; injection time: 20 s at 0.5 psi; sheath liquid: 2-propanol/water 60:40 (v/v) containing 0.1% (v/v) triethylamine; flow rate: 0.18 mL/h; drying gas: 7 L/min, 350 °C; nebulizing gas pressure: 6 psi. MS analyses were carried out using negative polarity. Compound stability: 25%. MS scan 50–650 *m/z* (target mass 350 *m/z*). Sample: rosemary honey extract.

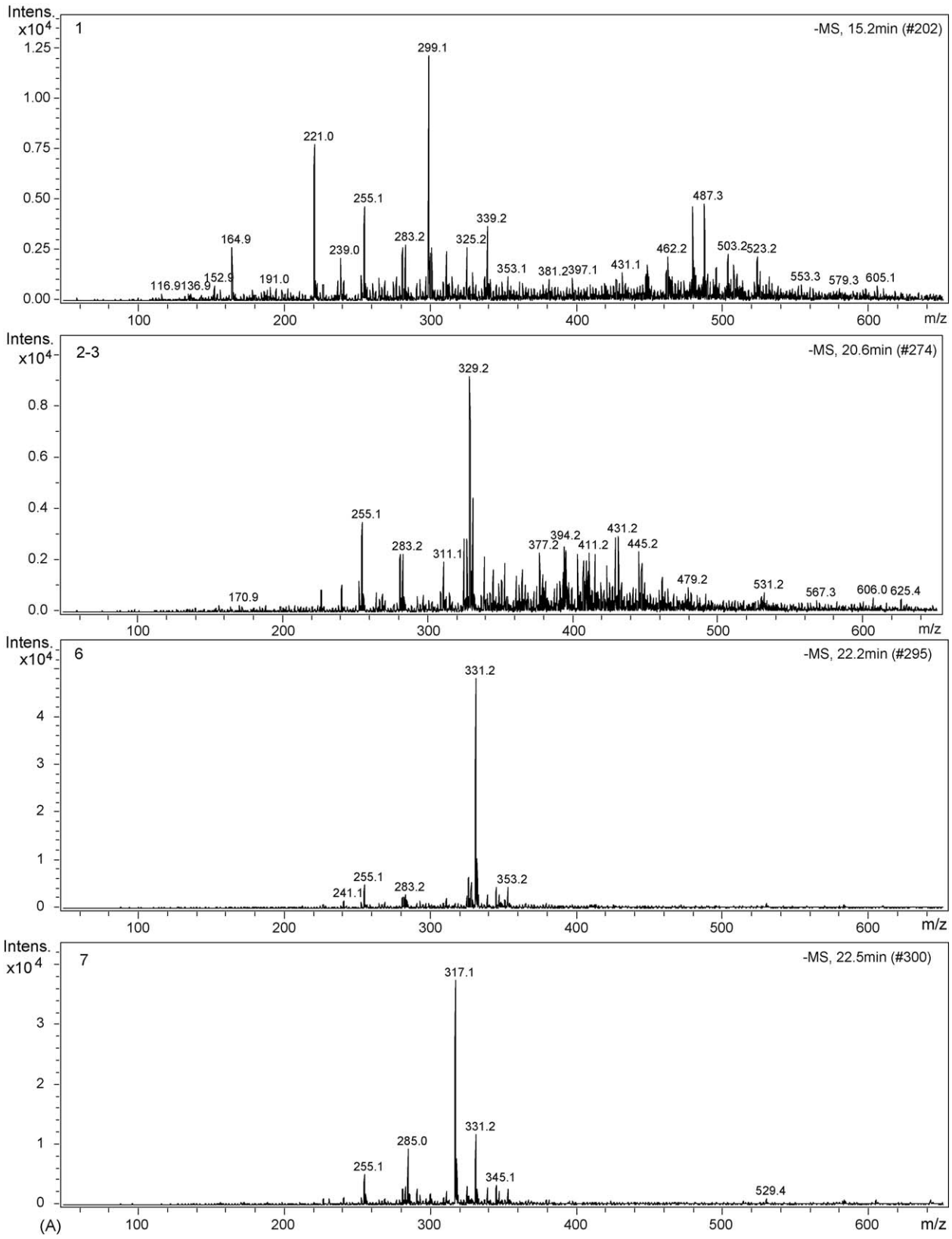

Fig. 5. (A and B) MS spectra of the identified peaks in a methanol–water rosemary honey extract. All conditions as in Fig. 3.

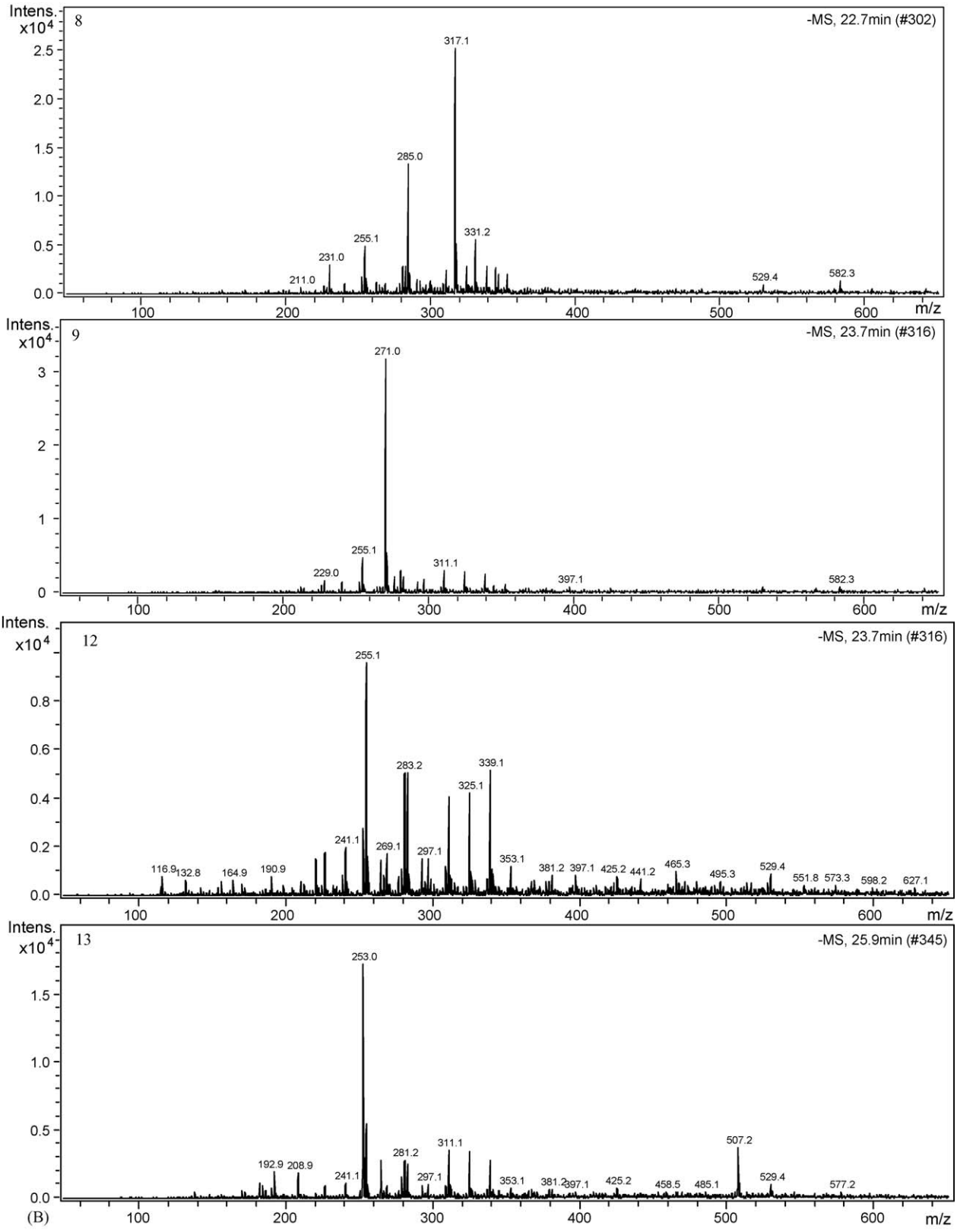

Fig. 5. (*Continued*).

($[M-H]^-$ 253 *m/z*). The compounds (1), (2), (3), (7), (8) are flavonols, (6) is a tannin compound, (9), (12) are flavanones and (13) is a flavone compound.

This demonstrates that it is possible to obtain satisfactory MS spectra for all the analytes. It is important to bear in mind that the detection in negative polarity is more selective than in positive mode, but we can also highlight the difficulty of ESI-MS analysis in the negative mode in terms of sensitivity. Some of the MS spectra given in the previous figure are quite noisy, fact that can be explained firstly, for the concentration of several of the compounds under study in the sample of rosemary honey and secondly, because of carrying out the detection in negative mode.

In any case, detection limits were calculated for several polyphenols and the values were around 1–25 mg/L with a RSD between 3 and 6% in all cases at these concentrations.

As has been reported in previous papers, the use of phenolic compound analysis generally using HPLC was used in the identification of honeys [37] and has been used as a tool for studying the floral and geographical origins of honeys. In these studies the authenticity of the floral origin of honey has been demonstrated [29,30]. The flavonol, Kaempferol ($[M-H]^-$ 285 *m/z*) has been used as marker for rosemary honey [38]. In addition Pinocembrin, Pinobanksin (flavanones) and Chrysin (flavone) ($[M-H]^-$ 255, 271, 253 *m/z*, respectively) are the characteristic flavonoids of propolis, and these flavonoid compounds have been found in most European honey samples [39].

In our research the extract ion electropherogram shows two peaks which correspond with $[M-H]^-$ 329 *m/z*. These peaks correspond at two compounds described by Tomás-Barberán and co-workers [40]. They claimed that the first peak $[M-H]^-$ 329 *m/z*, corresponds to Quercetin 3′,3′-dimethyl eter and the second one corresponds to Quercetin 7,3′-dimethyl eter. In addition, a third form (Quercetin 3,7-dimethyl eter) exits but is barely detected by UV detection. We could confirm that the third form was non-detected using CE–ESI-MS because the sheath liquid has a significant effect on the sensitivity (diluted of sample) of the compounds. These results confirm the developed study.

The other four compounds $[M-H]^-$ 345, 328, 185 and 187 *m/z* could not be identified, although some possible structures come out after carrying out the MS–MS experiments. A clearer assignment of these compounds is now being carried out in our lab.

4. Conclusions

In conclusion the present work describes the first qualitative SPE-CE–ESI-MS method to study phenolic compounds, in rosemary honey samples after their extraction by SPE. Different parameters were optimized and a successful CE–ESI-MS separation was obtained by using a running buffer consisting of 100 mM ammonium acetate and 10% of 2-propanol at pH 10. The sheath liquid used in the ESI-MS interface was a mixture of isopropanol/water 60:40 (v/v) in presence of 0.1% TEA obtaining a good repeatability of the method studied and a relative standard deviations (RSDs) of peak areas/migration time were 2.8 and 0.89%. Under these conditions, several phenolic compounds were identified in rosemary honey at mg/L levels. The compounds were identified using the electrophoretic results, the molecular weight and the structural information of MS obtained using CE.

In comparison to the chromatographic methods, the proposed method is a good alternative for simultaneous analysis of phenolic components in rosemary honey due to this technique provides fast and efficient separations in this type of analysis and used reduced sample and solvents consumption. Also, the hyphenation of CE to MS combines the advantages of CE with the selectivity and sensitivity inherent to MS.

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