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New CE–ESI-MS analytical method for the separation, identification and quantification of seven phenolic acids including three isomer compounds in virgin olive oil

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ABSTRACT

A sensitive and expeditious CE–ESI-MS analytical method for the separation, identification and determination of seven selected antioxidants (cinnamic and benzoic acids), including three isomers of coumaric acid (ortho-, meta- and para-) has been developed. In order to obtain the analytical separation, capillary electrophoresis and CE–MS interface parameters (e.g., buffer pH and composition, sheath liquid and gas flow rates, sheath liquid composition, electrospray voltage, etc.) were carefully optimized.

The polar fraction containing the selected phenolic acids was obtained using a previously optimized SPE pretreatment. An MS detector in order to extract structural information about the target compounds and facilitate their qualitative analysis was used in the negative ion mode. The proposed off-line SPE CE–ESI-MS method was validated by assessing its precision, LODs and LOQs, linearity range and accuracy.

The optimized and validated method was used in order to quantify the selected antioxidants in various samples of virgin olive oil and extra-virgin olive oil obtained from the main olive varieties cropped in Castilla-La Mancha, Spain. Salicylic acid was used as internal standard throughout in order to ensure reproducibility in the quantitative analysis of the oil samples.

The results confirmed the presence of hydroxyphenyl acetic, *p*-coumaric, ferulic and vanillic acids in substantial amounts ($\mu g\,g^{-1}$ level) in all samples.

1. Introduction

Although olive oil has been produced in Spain for a long time, it has experienced a dramatic rise in importance as available knowledge on Mediterranean foods and diet has grown and the healthy properties of the oil for cooking have become increasingly appreciated [1].

Virgin olive oil (VOO) is a juice obtained by exclusively mechanical means (pressing) from the fruit of the olive tree (*Olea europaea* L.). This is one of the few oil types requiring no refining, but merely washing, filtration, decantation or centrifugation, prior to consumption [2].

Chemically, olive oil consists mainly of glycerols, which account for more than 98% of its total weight. In addition, it contains about 2% of other, nearly 250 minor components including aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants [3]. The main antioxidants in VOO are carotenes, and phenolic compounds including fat-soluble and water-soluble phenols. While the fat-soluble phenols (tocopherols) of VOO can be found in other vegetable oils, some of its water-soluble phenols are rarely present in other oils or fats [4].

Phenolic compounds, which are generally acknowledged to be of considerable importance [5] comprise various chemical categories as phenolic acids, phenyl ethyl alcohols, hydroxyl-isochromans, flavonoids, lignans and secoiridoids [6] of which that of phenolic acids constitutes the one of the most important group into VOO [7]; chemically, such acids are derivatives of benzoic acid and cinnamic acid (e.g., vanillic, coumaric and hydroxyphenyl acetic).

Because VOO is a natural product, its chemical composition obviously varies among samples. For example, the phenolic content of VOO is influenced by olive variety, location, environmental conditions and degree of ripeness, and also by the oil extraction procedure utilized, since using refining process the antioxidants from oil are removed. Phenolic acids have received considerable attention in recent years because they are essential to its quality and nutritional properties of the olive oil. Thus, they extend its shelf life by delaying oxidation reactions and improve some sensory properties including pungency, astringency, bitterness and flavour [8–10].

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Moreover, they play a prominent role in human nutrition as preventive agents against various conditions including cardiovascular disease [3,11], stroke and cancers [12]. As a result, the determination of phenolic acids in VOO has aroused increasing attention from researchers and has been the subject of a growing amount of literature in recent times.

A number of extraction procedures and analytical methods for indentifying and qualifying these compounds in VOO have so far been reported. The phenolic fraction of olive oil has traditionally been isolated by liquid–liquid extraction (LLE) [8,13,14]. This technique, however, is usually labour-intensive and sometimes more expensive; also, it often requires using large amounts of organic solvents. Some authors have used solid phase extraction (SPE) with C_{18}, C_8 [15,16] or -diol [17,18] to isolate phenolic compounds. Others have compared LLE and SPE for this purpose [19,20], and they found LLE with hexane and methanol–water as mixture solvents and SPE with a normal phase to provide the best recoveries for these polar compounds.

Some authors have addressed the separation and quantitation of specific phenols by GC with various detection techniques including MS [21] and NMR [22]; however, the GC technique is less widely used for this purpose since the analytes are unstable at high temperatures and require a derivatization reaction for their determination.

Although optical detectors are most common for this purpose [13,14,20,23], mass spectrometry is a powerful detector in some respects thus, they require using no chromophore or fluorophore, provide lower LODs than UV in most cases [24] and allow structural information to be extracted and co-eluting peaks to be easily resolved from mass information as a second dimension. In addition tandem MS/MS affords structural elucidation and enhanced selectivity with a view to reducing chemical noise through an increased sensitivity [25].

The HPLC–MS couple has been used for the characterization of phenolic compounds in olive oil samples. The MS technique was used with electrospray ionization (ESI) [17,26,27] and atmospheric pressure chemical ionization (APCI) [28]; and the MS analyser was a quadrupole (Q) or triple quadrupole (TQ) [17,27], ion trap (IT) [26], or time-of-flight (TOF) type [29,30].

Capillary electrophoresis (CE) has become one of the major choices for the separation of charged analytes and a solid alternative to LC, especially if we are speaking of polar or charged compounds using its different modalities (CZE, CEC, etc.) [31–34]. Mass spectrometers have gained increasing acceptance as supplements or replacements for conventional detectors in CE. CE–MS coupling combines the high efficiency and resolution power of CE with the high selectivity and sensitivity inherent in MS, thus providing a powerful, highly attractive analytical tool. While CE–MS is mostly performed with electrospray ionization, the soft-ionization technique can be used to obtain ions even from thermally labile, non-volatile, polar compounds [25,35,36]; however, CE–ESI-MS appears to have only been used very few times [29,37,38] to determine phenolic compounds in oils, using IT and TOF MS as analysers. Time ago, Lafont et al. [37] carried out a sensitive method for qualitative and quantitative analysis of several phenolic compounds (including ferulic and vanillic acids) in olive mill wastewater by CE–ESI-MS. More currently, Carrasco-Pancorbo et al. developed several methods for just the qualitative and semi-quantitative determination of phenolic fraction (phenyl alcohols, phenyl acids, lignans, flavonoid and secoiridoids) of extra-VOO using CE–ESI-MS and CE–TOF-MS respectively [38,29].

In this work, we developed a sensitive, reliable, off-line SPE CE–ESI-MS method for the analytical separation and determination for first time of phenolic acids such as hydroxyphenylacetic acid (HFA), gentisic (GEN), ferulic (FER) and vanillic (VAN) acids, including three isomer (*o*-, *m*-, and *p*-) of coumaric acid (COU) in extra and VOO samples. The factors governing the performance of CE and the ESI-MS interface were carefully optimized, and a validation procedure in terms of precision, limits of detection (LODs) and quantitation (LOQs), linearity and recoveries were studied, in order to improve the qualitative and quantitative determination of the target compounds in six natural oil samples.

2. Materials and methods

2.1. Reagents

All antioxidants studied (both benzoic and cinnamic acid derivatives, Table 1) were purchased from Sigma–Aldrich (St. Louis, MO).

Ammonium acetate, ammonium hydroxide and acetic acid, all analytical grade, were obtained from Panreac (Barcelona, Spain). The organic solvents (*viz.*, 2-propanol for instrumental analysis and *n*-hexane in analytical reagent grade) were also supplied by Panreac.

Ultrapure water from a Milli-Q apparatus (Millipore, Milford, MA) was used to prepare all solutions including the background electrolyte, and a 1 M solution of sodium hydroxide (Panreac, Barcelona, Spain) was used to rinse the capillary.

2.2. Solutions and samples

2.2.1. Standard solutions

Standard stock solutions of the analytes were prepared by dissolving and appropriate amount of each pure substance in 25 mL of ethanol (analytical grade) to obtain a final concentration of 1000 mg L^{-1}. The resulting solutions were stored at 5 °C in topaz glasses.

Working standard solutions of 5 mg L^{-1} were prepared on a daily basis by diluting appropriate aliquots of the previous standard stock solutions in Milli-Q water.

When some variable related to the CE–MS interface was altered, appropriate *tuning standard solutions* were prepared. These solutions contained a mixture of 20 mg L^{-1} of concentration of each antioxidant diluted in running buffer.

2.2.2. Background electrolyte

Phenolic acids were separated in a background electrolyte (BGE) consisting of a 10 mM NH_4/NH_3 buffer solution at pH 10.0 that was prepared by weighing the required amount of ammonium acetate and adjusting its pH with a few drops of ammonium hydroxide. The running buffer was prepared on a daily basis because stored solutions result in unstable CE and ESI currents, and hence in irreproducible migration times (MTs). In addition, all solutions and buffers were degassed by sonication for 5 min before use in order to avoid changes during the ionization process and ensure acceptable reproducibility.

pH measurements were made with a Crison model 2002 pH meter furnished with a combined glass electrode.

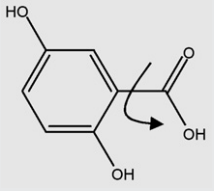
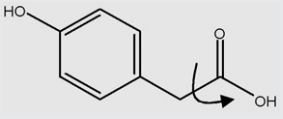
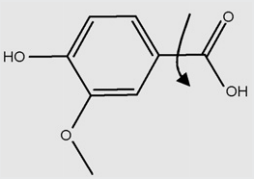
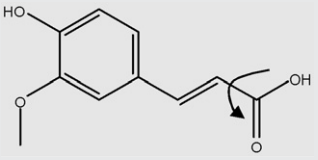
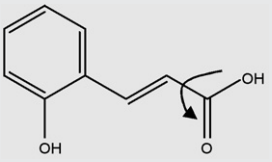
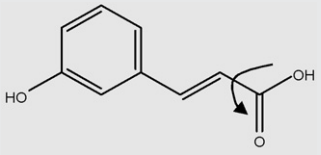
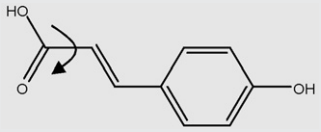
2.2.3. Sheath liquid

The sheath liquid used for detection in the ESI-negative mode was a solution consisting of 75% 2-propanol and 5 mM running buffer (NH_4/NH_3, pH 10). This solution was also freshly prepared each day and degassed by sonication for 5 min prior to use in order to ensure proper, reproducible ionization.

2.2.4. Olive oil samples and SPE procedure

Analyses were done on six olive oil samples obtained from the same area in the Castilla–La Mancha region (central Spain). Three were VOO samples obtained from olives of the Cornicabra (1), Picual (2) and Arbequina (3) varieties, and the other three extra-VOO samples from the same olive varieties (4–6). All samples had been bottled at the same time of year (2006).

Table 1
Structure, mass spectrometric (ESI-MS and MS/MS) and pK_a data for the phenolic compounds studied.

Compound	Structure	Molecular weight (g mol^{-1})	[M−H]$^-$ (m/z)	MS/MS ions	Collis. energy (%)	pK_{a1}	pK_{a2}
Benzoic acid derivates							
Gentisic acid (GEN)		154	153	109	30	3.0	11.0
Hydroxypheny acetic acid (HFA)		152	151	107	28	4.5	9.2
Vanillic acid (VAN)		168	167	123	30	4.4	9.3
Cinnamic acid derivates							
Ferulic acid (FER)		194	193	149	31	4.0	10.2
o-Coumaric acid (*o*-COU)		164	163	119	31	3.9	10.6
m-Coumaric acid (*m*-COU)		164	163	119	31	3.8	10.1
p-Coumaric acid (*p*-COU)		164	163	119	31	4.1	10.2

We studied and optimized to use an SPE procedure involving -diol cartridges (Vac 3cc, 500 mg, Waters, Milford, Ireland) in order to isolate the phenolic fraction, clean-up and concentrate the natural samples, based on the good recoveries thus obtained for all studied compounds. The SPE system consisted of a water manifold (Supelco Visiprep^{TM} Sep-Pack system, Madrid, Spain) coupled to a Millipore XF 54 23050 Vacuum pump (Milford, MA).

The SPE procedure previously optimized is showed to follow: olive oil samples (8 g) were dissolved in 8 mL of *n*-hexane and loaded through a pre-conditioned cartridge. The column was activated with 6 mL of *n*-hexane, 6 mL of 20:80 (v/v) methanol:water and 3 mL of acetonitrile. Then, the sample was percolated into the cartridge (a washing step with 4× 5 mL of *n*-hexane was needed to remove the non-polar fraction and obtain a clean extract). Next, phenolic acids were slowly eluted with 8 mL of 20:80 (v/v) methanol:water under vacuum (less than −40 kPa) in order to ensure efficient recovery of the polar fraction. This *first aqueous extract* was filtered through a 3 mm nylon membrane filter of 0.45 μm pore size from Millipore and directly injected into the CE–ESI-MS system. The remainder eluent was evaporated to dryness in a JOUAN RC10.09 centrifugal evaporator coupled to a refrigerated RCT90 Aspirator (Shimadzu, Madrid, Spain)—and a nitrogen stream, if needed. The dry residue thus obtained was dissolved in 300 μL of 20:80 (v/v) methanol:water the resulting *second pre-concentrated extract* injected into the CE–ESI-MS system as well.

Refined sunflower oil was used as glyceride matrix for validation since refined oil contains no natural antioxidants. Spiked sunflower oil solutions were subjected to the same SPE procedure in order to evaluate precision, linearity, limits of detection and quantification, and recoveries. Different concentrations of each phenolic acid were

added to spiked sunflower oil solutions and mentioned amounts have been presented in validation procedure (Section 3.4).

All oil samples were stored in topaz glass bottles at 4 °C until analysis.

2.3. Electrophoretic procedure

A P/ACE System MDQ capillary electrophoresis instrument equipped with a diode-array detection (DAD) system governed by dedicated capillary electrophoresis software, all from Beckman (Fullerton, CA, USA), was modified to facilitate coupling with the mass spectrometer. Separations were done on 80 cm fused-silica capillaries (75 μm i.d., 375 μm o.d.), using a 10 mM ammonium acetate/ammonium hydroxide buffer solution at pH 10.0 that was previously degassed by sonication as running electrolyte. Prior to first use, the untreated capillary was conditioned by consecutive flushing with 1.0 M NaOH at 20 psi for 10 min and water at 20 psi for 10 min, followed by electroconditioning with BGE at 20 psi for 5 min.

Then, samples were injected in the hydrodynamic mode (15 s at 1.0 psi) and separation effected by applying a constant voltage of 25 kV (a voltage ramp of 1.30 kV s^{-1}), which resulted in a generated current of approximately 30 μA. The electrophoretic medium was kept at 25 °C throughout.

Different electrolyte vials were used for rinsing and separation in order to maintain a constant electrolyte level on the anode side. Each set of separation vials was changed after 4 separation runs.

Prior to overnight storage, the capillary was flushed with 1.0 M NaOH and water for consecutive periods of 10 min, followed by drying with air for 3 min.

The quantitative results for the validation process and those for the analysis of the olive oil samples were calculated from relative peak areas (RPA, analyte peak area/internal standard peak area) in all cases.

2.4. Interface and MS

Capillary electrophoresis can be coupled to different types of MS analysers (Q, TQ, IT, and TOF); also, it can be used with various ionization methods (ESI, APCI, and MALDI). In this work, we used an LCQ DECA XP Plus mass spectrometer from ThermoFinnigan (San Jose, CA) equipped with a tricoaxial pneumatically assisted electrospray ionization (ESI) source designed for the CE–MS coupling and with an IT. The sheath liquid was a solution consisting of 75% isopropanol and 5 mM running buffer that was passed at a flow rate of 3 μL min^{-1}. The sheath gas flow was set to 40 on the scale of arbitrary units provided by the instrument software and the auxiliary gas to 0. Nitrogen gas for the LCQ™ and helium damping gas for the ion-trap were both supplied by Air Liquide (Madrid, Spain). The heated capillary temperature was held at 200 °C and the electrospray needle set at 3.75 kV. Around 5 cm of the poly-imide coating was removed from the fused-silica capillary in order to avoid dissolution by the sheath liquid and minimize contamination of the electrospray source as a result. We designated the length between the electrospray needle and heated capillary, which was 1.00 cm, "second position". A distance of 0 mm between the protruding portion of the CE capillary and the electrospray needle was selected as optimal.

Mass spectrometry data were processed by using Xcalibur 1.4 software. CE–MS data were acquired throughout the m/z range (135–195), using the centroid mode, negative polarity, and an injection time of 300 ms; scans were done at 3 μs intervals.

The MS detector was calibrated and a new method involving *tuning standard solutions* was developed by using a solution containing a mixture of 20 mg L^{-1} of each phenolic acid compounds and internal standard in running buffer that was infused through capillary to the detector at an injection pressure of 0.5 psi under a constant voltage of 3 kV.

The identification of the selected phenolic acids compounds was realized by comparing both migration time and MS data obtained from standard solutions of antioxidants under study.

3. Results and discussion

3.1. Preliminary tests with CE–DAD

Preliminary tests were performed by using the above-described CE apparatus, but coupled to an optical (diode array) detector in order to facilitate optimization of the operating conditions.

Because MS detection will be used in the subsequent, final analyses, the CE–DAD tests were conducted with provision for some chemical aspects such as the need for the running buffer to be volatile, which essentially restricted the choices of buffer to ammonia, acetate or formate. Based on the results of the preliminary tests, a 20 mM ammonium acetate buffer solution at pH 9.3 was selected as BGE and used at a constant voltage of 25 kV. Under these conditions, the migration and total analysis times were less than 10 min, and resolution between peaks, R_S, was 1 for all analytes except to isomers (*o*-, *m*- and *p*-) of coumaric acid, which had $R_S < 1$. Although complete separation of individual compounds is not required with an ion trap and an ESI-MS interface, it is crucial with a view to obtaining quantitative results since the ionization efficiency of the ESI is strongly influenced by changes in the sample matrix and relevant to the resolution of co-eluting compounds, which can considerably diminish the analytical response through ionic suppression in the electrospray source. Therefore, good resolution between peaks—particularly between the isomers (*o*-, *m*-, and *p*-) of coumaric acid, which exhibited an identical m/z value for their $[M-H]^-$ ion (163)—was mandatory.

3.2. Optimization of the separation by CE as coupled to MS

A number of CE separation variables including capillary length, buffer ionic strength, pH and viscosity have a direct influence on the intensity of electroosmotic flow (EOF) and hence on the fluxes of solution reaching an ESI source. An uneven EOF can perturb the solution flux and instabilize the electrospray as a result. Therefore, monitoring these factors can facilitate effective control and adjustment of a stable spray [39].

Electrophoretic conditions were optimized in terms of separation selectivity (R_S between peaks, mainly isomers of coumaric acid), sensitivity (high areas and signal/noise ratio), analysis time (short migration times) and peak shape.

3.2.1. Effect of electrolyte pH

The influence of the electrolyte pH on the CE separation was assessed by using ammonium buffer solutions spanning the pH range 6.0–10.0. As can be seen from the pK_a values of Table 1 and expected for anionic compounds, MTs decreased, and peak areas and signal/noise ratios increased, with increasing pH up to 9.0; however, coumaric acid isomers were poorly resolved. This led us to conduct a more precise study over the pH range 9.0–10.0 with a view to improving resolution between peaks and reducing the total analysis time. Raising the pH above 9.0 increased MT for all analytes through an increased negative charge—mainly by effect of their pK_{a2} values—and improved resolution between *o*-, *m*- and *p*-coumaric acids as a result.

Based on the foregoing, we chose to use an ammonium buffer solution at pH 10.0 for separation as it provided the maximum possible R_S between peaks, large peak areas and acceptable signal/noise ratios.

3.2.2. Effect of ionic strength and BGE composition

The influence of the ammonium buffer concentration on MTs and R_S between analytes was examined over the range 5–30 mM. A buffer concentration of 5 mM precluded resolution of coumaric acid isomers. On the other hand, buffer concentrations above 10 mM provided good R_S values—as high as 1.5—for all peaks, but rather long total analysis times (more than 20 min). We thus adopted a 10 mM concentration of ammonium buffer at pH 10 as optimal in order to ensure good peak shapes, short migration times, acceptable R_S values and low currents (28–30 μA). Besides the lowest concentration as you can of buffer is required in order to avoid the accumulation of excess of these salts on the entrance of the MS detector.

An attempt was made at enhancing electrophoretic separation by using two organic modifiers (methanol and 2-propanol) in variable proportions (5, 10 and 15%) as additives. However, neither improved on the previous results in terms of MT nor peak area were reported.

3.2.3. Optimization of CE instrumental parameters

The effects of instrumental parameters such as electrophoretic separation voltage, temperature and injection-related variables (*viz.*, pressure and duration) were examined with a view to establishing the best possible compromise between sensitivity, resolution between isomers and analysis time in the separation of all analytes.

The effect of the applied voltage was studied over the range 5–30 kV. A potential of 25 kV was found to provide the best results in terms of run time and resolution between peaks.

The effect of temperature on electrophoretic separation was examined over the range 15–30 °C. A temperature of 25 °C was selected as optimal because it provided the best compromise between MT and R_S. In fact, raising the capillary temperature reduced migration times through a decreased electrolyte viscosity, but also led to lower R_S values.

In order to improve sensitivity, we studied the influence of the amount of injected sample, which was adjusted via the injection time (8–17 s) and pressure (0.3–1.5 psi). We tested long times and high pressures since the size of the capillary (80 cm long × 75 μm i.d.) afforded them. An injection time of 15 s and a pressure of 1.0 psi were selected as optimal since longer times and higher pressures resulted in distorted peaks and poor resolution.

3.3. Optimization of interface and electrospray related parameters

On-line coupled CE–MS systems are often inadequately robust for quantitative determinations; this shortcoming can be circumvented by carefully adjusting the most common CE interface and electrospray related parameters in order to ensure stable CE–ESI-MS conditions [36,40,41].

So in this work, we assessed the effect of the most relevant CE–MS interface and electrospray variables through the relative standard deviation (RSD) obtained in replicate injections ($n = 6$), in order to obtaine a robust quantitative result.

3.3.1. Sheath liquid composition and flow rate

The sheath liquid, which is typically coaxially delivered by a capillary surrounding the metal needle, provides both electrical contact and a constant (electrolyte-independent) flow [25]. This requires optimizing its composition and flow rate in order to maximize ionization efficiency and spray stability.

Proportions of 2-propanol from 25 to 90% in the mixture and variable concentrations of running buffer from 2 to 10 mM in the sheath liquid were tested. Increasing the proportion of 2-propanol and the concentration of ammonium buffer up to 75% and 5 mM respectively, resulted in increased signals for the phenolic compounds. A sheath liquid consisting of 75% 2-propanol and 5 mM running buffer was thus selected.

The optimum sheath liquid flow rate would be that minimizing dilution of the analytes in order to ensure acceptable sensitivity and a stable spray. The effect of the flow rate was studied over the range 2–6 μL min^{-1}. Raising it up to 3 μL min^{-1} increased the analytical response; above that level, however, a dilution effect was observed. Therefore, 3 μL min^{-1} was selected as the optimum sheath liquid flow rate since, as can be seen from Fig. 1, this value provided the best signals for most of the analytes in addition to high signal/noise ratios and low relative standard deviations (RSDs) in peak area measurements ($n = 6$).

3.3.2. Sheath gas auxiliary flow rate

The effect of the sheath gas auxiliary flow rate [42] was studied over the range 20–60 (arbitrary units) and a value of 40 chosen as it provided the largest peak areas and signal/noise ratios, thus affording a high sensitivity and good resolution between peaks.

3.3.3. Spray voltage

Electrospray voltages between 2 and 5 kV were tested in order to obtain high signals. As expected, increasing the voltage up to 4 kV increased the analytical response. Values above 4 kV, however, considerably increased the current or even caused the formation of a discharge arch between the end of CE capillary and MS inlet. A voltage of 3.75 kV was thus selected as optimal.

3.3.4. Heated capillary temperature

The effect of the MS heated capillary temperature was examined from 150 to 300 °C.

The signal/noise ratio was affected slightly differently by the heated capillary temperature; thus, S/N rose up to 200 °C for the less sensitive analytes (HFA and GEN), and levelled off or decreased above 200 °C. A temperature of 200 °C was thus adopted for the heated capillary as it provided good signals, high S/N ratios and low RSD values for peak areas.

3.3.5. Length of the CE capillary protruding from the ES needle

The distance was changed from 0 to 0.5 mm by using a micrometric screw and the maximum signal was obtained with the CE capillary not protruding from the electrospray needle. Clearly, a protruding length greater than 0 mm resulted in lower signals for all analytes, and also in poorer electrospray stability—probably through instability in the formation of charged droplets. A length of 0 mm was therefore selected since it provided the highest, most stable signals.

3.3.6. Distance between the CE capillary and MS heated capillary

The influence of this variable was studied by positioning the interface between the greatest and smallest settings afforded by the instrument (*viz.*, positions 1–4, which were equivalent to 1 and 2.5 cm, respectively). As expected, placing the spray and the MS inlet at a short distance boosted the analytical signal by effect of the increased amount of ions being introduced into the mass spectrometer. A distance of 1.5 cm between CE capillary and the MS heated capillary was adopted as optimal since the smallest setting (1 cm) resulted in high instrumental noise and RSD, and also in poorer areas and S/N ratios.

3.3.7. Injection time for IT

Using the selected analyser (a Q-ion trap model) in the automatic mode requires a pre-scan before each analysis in order to calculate the optimum open injection time for the ion in order to maximize the amount of ions that are loaded into the trap. However, no pre-scan is needed in the manual mode and the best open injection time

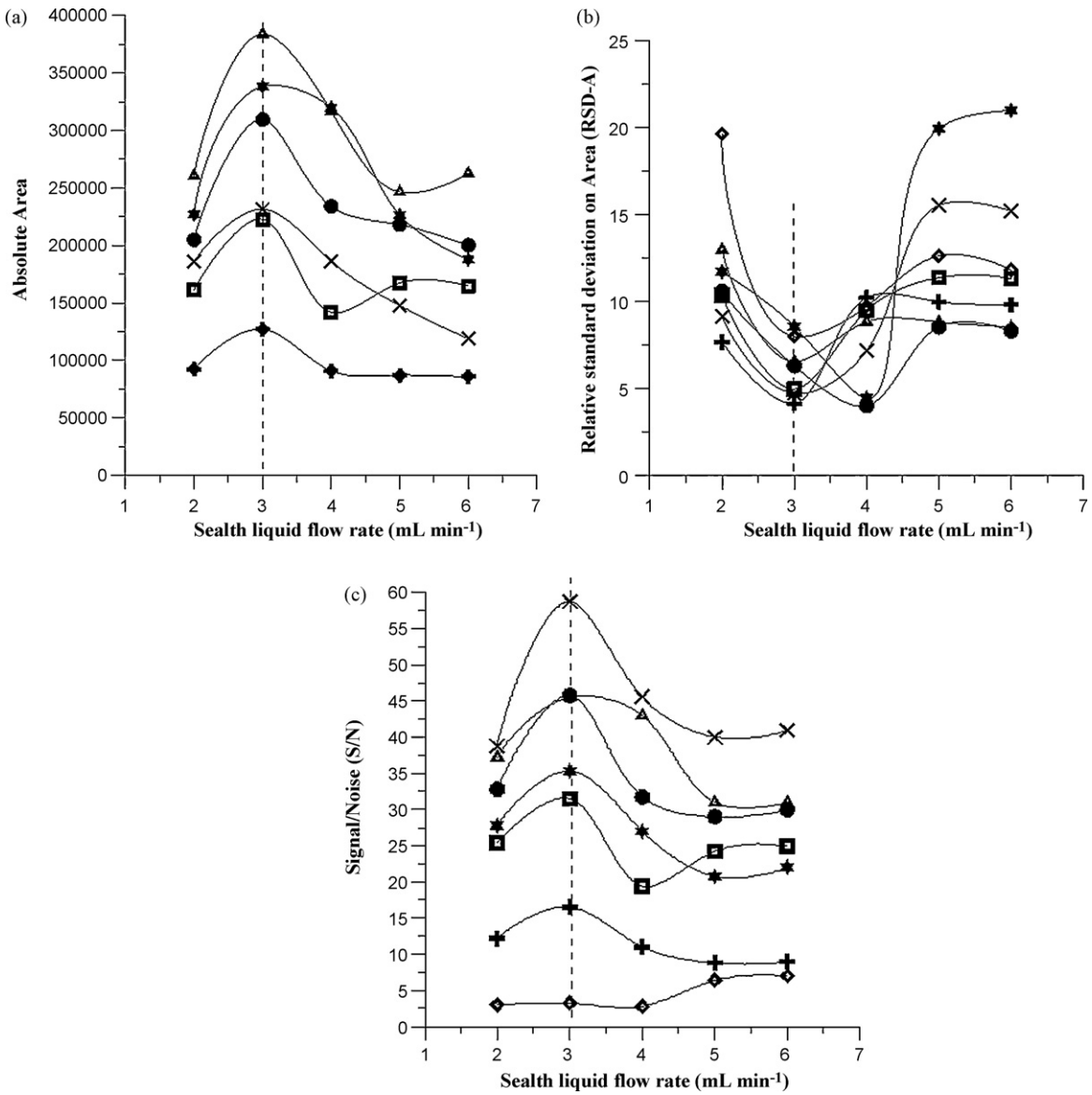


Fig. 1. Effect of sheath liquid rate flow on (a) absolute area, (b) relative standard deviation for the area ($n = 6$) and (c) signal/noise (S/N) ratio (+ HFA, ◊ GEN, □ *m*-COU, ✱ *p*-COU, ● *o*-COU, ▲ FER, × VAN).

is easy to determine; this allows the mass spectrometer to focus entirely on analysing the ions and boosts sensitivity as a result.

The effect of the injection time for IT was studied over the range 50–500 ms. The response was as expected. Thus, increasing the injection time resulted in increased peak areas and S/N ratios; beyond 350 ms, however, the ion trap saturated and results impoverished. A time of 300 ms was thus selected as optimal for filling the ion trap since longer ones led to asymmetric, distorted peaks that detracted from resolution.

Fig. 2 shows the extracted ion electropherograms of selected phenolic acids obtained when the capillary electrophoresis and ESI-MS interface parameters were optimized. As can be seen, the ratio m/z 163 belonging to isomers of coumaric acid is selected, R_S between peaks higher than 1.5 are obtained.

3.4. Validation of the proposed off-line SPE CE–ESI-MS method

The proposed method (off-line SPE CE–ESI-MS) was validated by using extracts of refined sunflower oil, which thus contained no antioxidants. These extracts provided a suitable matrix for the whole validation procedure and were spiked with appropriate amounts of the analytes and internal standard.

The extracts were obtained by using the SPE procedure described in Section 2.2. Each variable involved in the procedure (*viz.*, organic and aqueous solvent volumes for the conditioning and washing steps, sample volume, eluent volume and extract final volume) was examined in order to ensure virtually complete extraction of all phenolic compounds. As shown by the validation results, which are discussed below, the optimized SPE procedure provided recoveries near 100%.

Analytical parameters such as precision, limits of detection (LODs) and quantitation (LOQs), linearity and recoveries were determined as a part of the validation process from triplicate injections of spiked oil extracts containing 2 mg L^{-1} salicylic acid as internal standard. Quantitation was based on average relative peak areas (RPA).

The precision of the proposed method in terms of repeatability was determined by replicate analysis ($n = 15$) of oil extracts spiked with a 2 mg L^{-1} concentration of each compound—internal standard included. The relative standard deviation for MT and RPA was

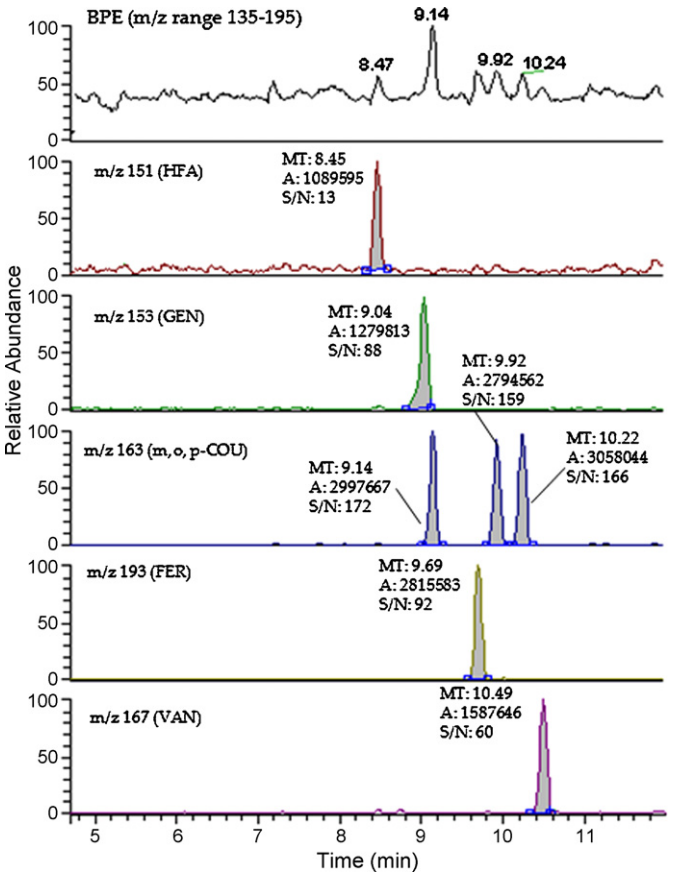


Fig. 2. Base peak electropherogram (BPE) and extracted ion electropherograms obtained during the separation of the studied phenolic compounds (2 mg L^{-1} each). Capillary electrophoresis conditions: capillary, 80 cm (75 μm i.d., 375 μm o.d.); BGE, 10 mM ammonium buffer pH 10.0; applied voltage, 25 kV; hydrodynamic injection, 1.0 psi for 15 s; and ESI-MS parameters: sheath liquid composition and low rate, 75% 2-propanol, 5 mM BGE at 3 μL min^{-1}; sheath gas auxiliary flow rate, 40 a.u.; spray voltage, 3.75 kV; heated capillary temperature, 200 °C; length of CE capillary, 0 mm; distance between CE–MS heated capillary, 1.0 cm and injection time for IT, 300 ms (MT migration time, *A* area, S/N signal/noise ratio).

less than 2% and 5–10%, respectively, for all analytes; the highest RSD values for RPA were those for compounds HFA and GEN—a result of their poor sensitivity and nearness to instrumental noise.

Limits of detection (LODs) were estimated by sequentially injecting increasingly low concentrations of the studied analytes until no detectable peak signal was obtained. Limits of quantitation (LOQs) were calculated by multiplying the LODs by 10/3 (*viz.*, ten and three times, respectively, the standard deviation of noise).

The linearity of the proposed method was evaluated by plotting the RPA for each compound against variable concentrations, and obtaining the corresponding linear regression equations, coefficients of determination (σ^2) and linearity ranges.

Table 2 summarizes the LOD, LOQ and linearity data obtained.

Recovery of the phenolic acids was assessed by comparing the mean RPA for oil samples spiked at three different concentration levels (high, medium and low) with the mean RPA for those spiked after SPE, which was taken to represent 100%. Both types of samples were obtained by subjecting refined sunflower oil to the sample pre-treatment procedure described in Section 2.2. Recoveries were found to range from 92.8 to 107.0% (Table 3).

3.5. Analysis of VOO and extra-VOO by off-line SPE CE–ESI-MS

Following careful optimization and validation of off-line SPE CE–ESI-MS for analytical separating a mixture of selected phenolic acids, including the isomers of coumaric acid, the ensuing method was validated by application to real samples.

To this end, six samples of monovarietal VOO and extra-VOO obtained from three different olive varieties named Picual, Cornicabra and Arbequina, were analysed after optimal SPE procedure. As described in Section 2.2, an amount of 8 g of olive oil was dissolved in 8 mL of *n*-hexane and loaded through a pre-conditioned -diol cartridge. A volume of 8 mL of *first aqueous extract*, 20:80 (v/v) methanol:water, was thus obtained for direct injection into the CE–ESI-MS system in order to quantify some compounds without pre-concentration. The remainder eluent was evaporated to dryness and dissolved in 300 μL of 20:80 (v/v) methanol:water to obtain the *second pre-concentrated extract* for final injection into the CE–ESI-MS system. Samples were analysed by using the standard addition method ($n = 3$) with salicylic acid as internal standard.

The analytes were identified by comparing their MTs, MS data obtained from standard solutions of antioxidants under study and by spiking the oil extracts with standards at variable concentration levels in order to confirm their identifications.

As can be seen from the extracted ion electropherograms of Fig. 3, some phenolics such as HFA and *p*-COU were detected in the *first aqueous extract* from samples 1 and 5. Thus, sample 1 (VOO Cornicabra) contained 10.41 ± 1.03 μg g^{-1} of HFA; and sample 5 (extra-VOO Picual) contained 1.54 ± 0.33 μg g^{-1} of HFA and 0.13 ± 0.05 μg g^{-1} of *p*-COU.

The analysis of samples from the *second pre-concentrated extract* revealed the presence of all studied antioxidants at variable concentrations; by exception, *o*-COU was not detected in any sample (see Table 4). All samples were found to contain high levels of HFA – which made preconcentration unnecessary for samples 1 and 5 – however, sample 3 (Arbequina) contained no HFA. Interestingly, *p*-COU was the sole analyte detected in all samples. Also, GEN was found at a substantial concentration in sample 1 (VOO Cornicabra). Finally, FER and VAN exhibited their peak concentrations in samples 3, 4 and 6.

By way of example, Fig. 4 shows an extracted ion electropherogram obtained by using the proposed off-line SPE CE–ESI-MS method following optimization and validation for the determination of sample 3 (VOO Arbequina). As can be seen, all MTs were longer than in the preliminary and optimization tests; this was a result of continuous injection of sunflower or olive extracts from oil samples resulting in partial adsorption onto capillary walls. Ensur-

Table 2
Linear range, LOD and LOQ for the phenolic compounds as determined with the optimized CE–ESI-MS method.

Compound	Conc. range (mg L^{-1})	Regression equation	Coef. determ. (σ^2)	LOD (μg L^{-1})	LOQ (μg L^{-1})
Hydroxyphenyl acetic acid	0.4–16	$y = 4.49 \times 10^{-2}x + 1.00 \times 10^{-2}$	0.9993	60	200
Gentisic acid	2.0–40.0	$y = 6.64 \times 10^{-2}x - 27.86 \times 10^{-2}$	0.9562	0.5[a]	1.5[a]
m-Coumaric acid	0.05–2.0	$y = 10.86 \times 10^{-2}x + 11.31 \times 10^{-2}$	0.9889	5.5	18
o-Coumaric acid	0.3–1.3	$y = 0.13x + 0.26 \times 10^{-2}$	0.9852	3.5	12
p-Coumaric acid	0.05–2.0	$y = 0.12x + 0.35 \times 10^{-2}$	0.9919	6	20
Ferulic acid	0.1–4.0	$y = 0.11x + 0.43 \times 10^{-2}$	0.9979	14	45
Vanillic acid	0.1–4.0	$y = 6.73 \times 10^{-2}x + 0.36 \times 10^{-2}$	0.9939	11	35

[a] (mg L^{-1}).

Table 3
Recoveries of phenolic compounds as obtained with the optimized SPE procedure at three concentrations levels and the proposed CE–ESI-MS method for determination.

Compound	Concentration 1			Concentration 2			Concentration 3		
	[spk][a]	[fou][b]	R[c]	[spk][a]	[fou][b]	R[c]	[spk][a]	[fou][b]	R[c]
HFA	2.35	2.40	102.1	4.76	4.55	95.6	9.55	9.87	103.4
GEN	5.80	5.53	95.3	11.90	12.25	95.6	9.55	9.87	104.4
m-COU	0.29	0.27	97.6	0.57	0.56	99.2	1.15	1.18	103.7
o-COU	0.19	0.21	105.6	0.38	0.33	93.2	0.76	0.74	97.1
p-COU	0.29	0.28	99.6	0.57	0.61	107.0	1.14	1.21	106.6
FER	0.6	0.62	105.1	1.19	1.10	92.8	2.38	2.42	102.0
VAN	0.6	0.60	101.1	1.19	1.11	93.2	2.38	2.23	94.0

[a] Spiked after SPE concentration (mg L^{-1}).
[b] Found concentration (mg L^{-1}).
[c] Recovery (%).

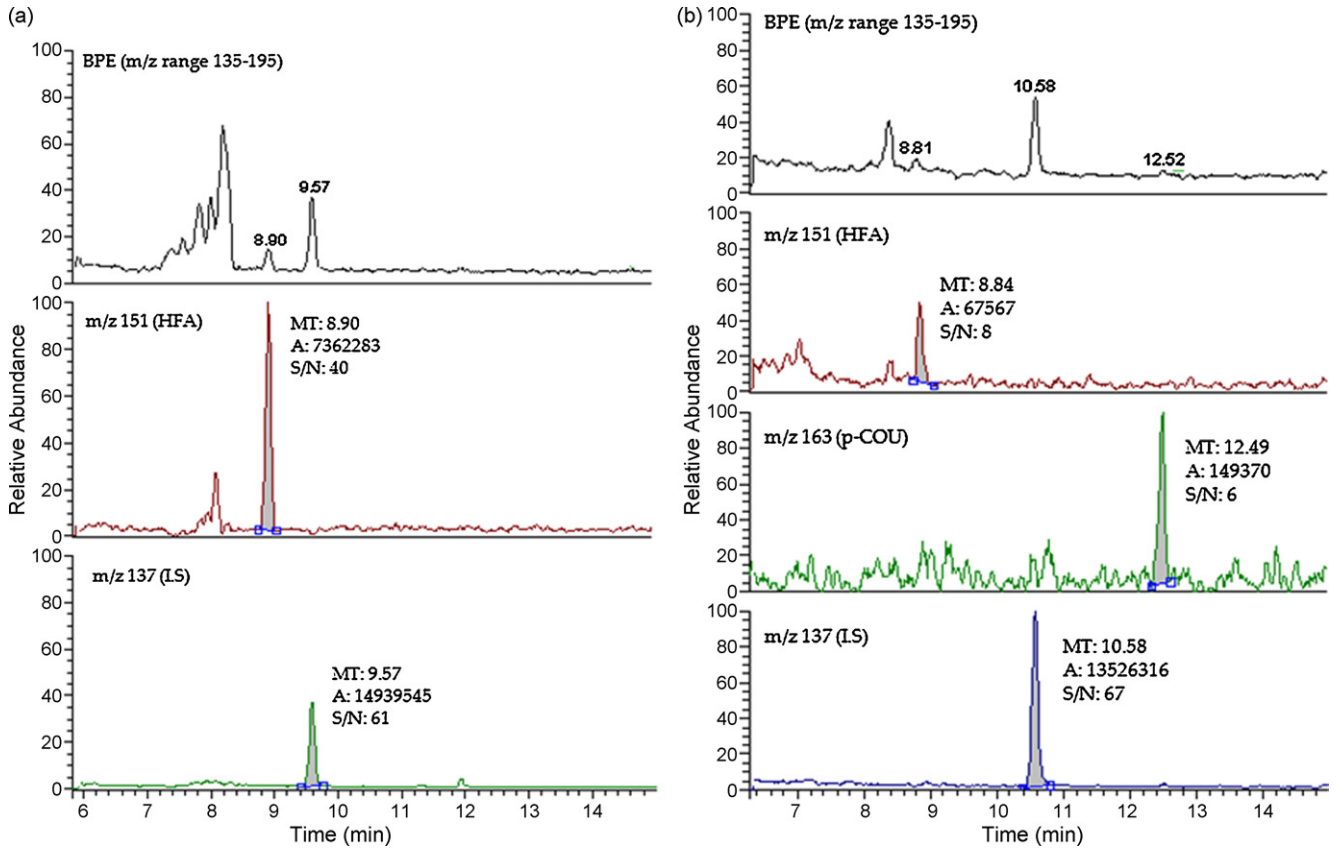


Fig. 3. Base peak electropherogram (BPE) and extracted ion electropherograms for the *first aqueous extract* (8 mL), (a) sample 1 (VOO Cornicabra) and (b) sample 5 (extra-VOO Picual), both containing 2 mg L^{-1} of I.S.

Table 4
Antioxidant concentrations found in the *second pre-concentrated extract* as determined with the proposed off-line SPE CE–ESI-MS method.

Compound	Virgin olive oil (ng g^{-1})			Extra-virgin olive oil (ng g^{-1})		
	Cornicabra (sample 1)	Picual (sample 2)	Arbequina (sample 3)	Cornicabra (sample 4)	Picual (sample 5)	Arbequina (sample 6)
HFA	10.41[a]	317.5	n.d	89.9	1.54[a]	51.8
GEN	104.6	n.d	n.d	n.d	n.d	n.d
m-COU	n.d	1.1	2.8	n.d	n.d	n.d
o-COU	n.d	n.d	n.d	n.d	n.d	n.d
p-COU	9.8	7.0	21.4	116.5	0.13[a]	30.4
FER	1.4	n.d	13.0	20.65	10.8	22.6
VAN	n.d	35.4	79.8	71.4	n.d	80.1

n.d.: not detected.
[a] Quantified in the first aqueous extract.

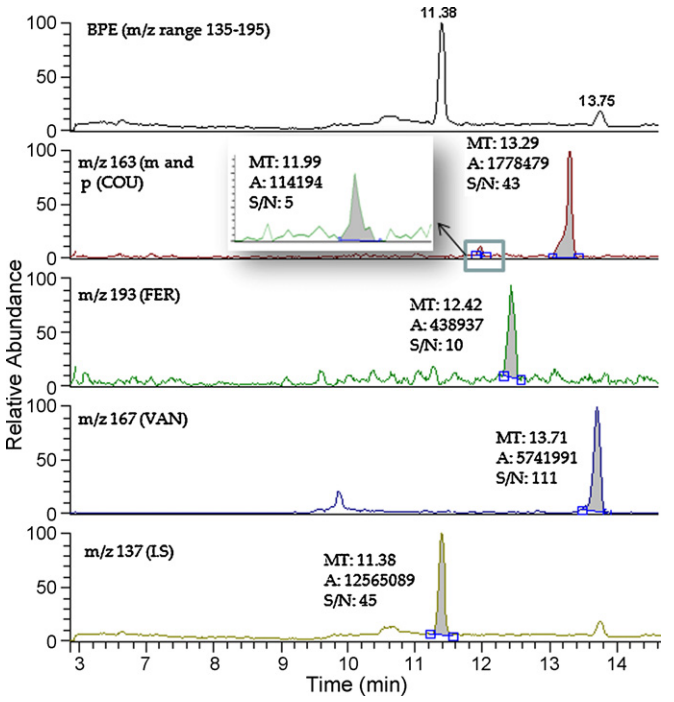


Fig. 4. Base peak electropherogram (BPE) and extracted ion electropherograms for the *second pre-concentrated extract* (300 μL) as obtained by using the proposed off-line SPE CE–ESI-MS method to determine seven phenolic compounds in sample 3 (VOO Arbequina).

ing maximal reproducibility entailed using an internal standard, opening the interface and carefully cleaning the capillary between oil sample injections.

4. Concluding remarks

The flexibility of capillary electrophoresis for food analysis was clearly shown here with the successful development of a new, reliable method for the determination of selected phenolic acids in extra-VOO.

For the first time a method has been developed for the analytical separation, identification and quantification of seven antioxidants phenolic acids such as hydroxyphenylacetic, gentisic, ferulic, vanillic acids and including the isomers (*o*-, *m*- and *p*-) of coumaric acid, using CE–ESI-MS in combination with SPE in commercial oil samples.

The optimum conditions for this determination were established by examining the effect of all factors potentially influencing the separation and quality of the signals for the compounds in the oil extracts. In this regard, e.g., the lowest concentration of BGE, volatile running buffer and sheath liquid composition and flow rate, among other parameters were carefully selected.

A whole validation procedure of proposed method (off-line SPE CE–ESI-MS) by using extracts of refined sunflower oil, was carried out including precision (<10%), limits of detection (LODs) and quantitation (LOQs) (around $\mu g\,L^{-1}$), linearity and recoveries (between 92.8 and 107.0%). The results obtained for validation procedure were good enough as to make final application to real oil samples of extra and VOO from the Cornicabra, Arbequina, and Picual olive varieties. The final analysis results confirmed the presence of substantial amounts of HFA, *p*-COU, FER and VAN in all studied samples.

Finally we can conclude that, the efficiency and expeditiousness of CE separation, in combination with the selectivity and sensitivity of MS detection, make the proposed method an attractive choice for food analysis laboratories.

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