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Fast separation and determination of tyrosol, hydroxytyrosol and other phenolic compounds in extra-virgin olive oil by capillary zone electrophoresis with ultraviolet-diode array detection

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Abstract

Olive oil is the main source of fat in the Mediterranean diet, and its consumption has been related to a low incidence of coronary heart disease and certain cancers. Recent findings demonstrate that olive oil phenolics are powerful in vitro and in vivo antioxidants and display other biological activities that could partially account for the observed healthful effects of the Mediterranean diet. A detailed method optimization plan was carried out to separate the most popular phenols in olive oil for four separation parameters: buffer concentration, buffer pH, applied voltage and temperature. Consequently, an analytical method capable of separating 21 different phenols and polyphenols by capillary zone electrophoresis was developed; the separation was performed within 10 min, using a 40 cm×50 μm capillary, with a 45 m*M* sodium tetraborate buffer (pH 9.60), at 27 kV and 30 °C. The optimized method was applied to methanolic extracts of several Italian extra-virgin olive oils obtained by different technologies in order to characterize and to compare their antioxidant profile. Positive correlations of phenolic compounds found by capillary zone electrophoresis (CZE) and two colorimetric indexes (total polyphenols and *o*-diphenols) were found and discussed.

Keywords: Olive oil; Food analysis; Phenols; Polyphenols; Tyrosols

1. Introduction

Virgin olive oil is a fundamental ingredient of the Mediterranean diet and, over the past few years, its diffusion and consumption has spread remarkably outside the Mediterranean basin. The growing interest in olive oil may be partly related to its unique taste; however, particular interest is due to its nutritional properties. In fact, the saturated-to-unsaturated fatty acid ratio and the presence of natural antioxidants could prevent certain human diseases. In most cases, health and dietary benefits induced by consumption of virgin olive oil are due to the synergistic activity among the several minor constituents of virgin olive oil, such as vitamins (α- and γ-tocopherols and β-carotene), phytosterols, pigments, terpenic acids, flavonoids (such as luteolin and quercetin), squalene, and phenolic compounds

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(phenols and polyphenols) [1–5]. In particular, phenolic compounds are strong antioxidants and are also responsible for the astringency and bitterness of olive oils.

Hydroxytyrosol and tyrosol are the main phenolic compounds in extra-virgin olive oil. According to several authors, the antioxidant activity of virgin olive oil is directly related to their concentration [5–7]. To extract the polar fraction, namely the phenolic and polyphenolic compounds, a simple and fast methanol–*n*-hexane-based liquid–liquid extraction is usually followed [8]. Several extraction methods of the polar fraction from virgin olive oil have been compared in a previous study [9].

Several methods have been developed to analyze phenolic species in olive oil. TLC, NMR and, especially, HPLC have been used for this purpose; the latter technique is able to provide the phenolic profile of virgin olive oil [8,10–16]. These analytical techniques often need general complex sample preparation protocols, and HPLC analysis is time-consuming. On the other hand, capillary electrophoresis has proven to be a fast, valid and reliable tool for food analysis, especially for analysis of phenolic compounds [17–20].

The main advantages of the use of capillary electrophoresis even in its basic mode, namely capillary zone electrophoresis (CZE), for phenol analysis are its high separation power, which leads to fast and well-resolved separations of similar compounds, and the possibility to use a low and specific detection wavelength that leads to high sensitivity. Despite these characteristics, CZE has been rarely used for the separation of phenols and polyphenols in olive oils. The first goal of this work was, therefore, to develop the fastest and the simplest CZE method that allowed to separate simultaneously hydroxytyrosol and tyrosol, as well as the main and the most common antioxidant compounds of virgin olive oil. The second aim of this work was to compare the amount of phenolic compounds in several Italian virgin olive oil samples differing by their technology, in order to characterize and to compare their antioxidant contents. Statistical correlations among phenolic contents found by CZE and two colorimetric indexes (total polyphenols and *o*-diphenols) are also reported and discussed.

2. Experimental

2.1. Reagents and chemicals

HPCE-grade water, HPCE-grade 0.1 *M* NaOH, HPCE-grade 1 *M* NaOH, HPCE-grade 0.1 *M* HCl, sodium tetraborate, and HPLC-grade water were from Fluka (Buchs, Switzerland). All HPLC-grade organic solvents were from Merck (Darmstadt, Germany).

2.2. Standards and samples

The following commercial products were used: protocatechuic acid, 3,4-dihydroxyphenylacetic acid, tyrosol, 2,3-dihydroxyphenylethanol, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, caffeic acid, vanillic acid, dihydrocaffeic acid, siringic acid, *p*-coumaric acid, ferulic acid, *o*-coumaric acid, gentisic acid and cinnamic acid were from Fluka. Gallic acid, luteolin, taxifolin and quercetin were from Sigma (St. Louis, MO, USA); oleuropein glycoside was from Extrasynthèse (Genay, France).

One mg/ml standard stock solutions were prepared in HPLC-grade methanol. Appropriate dilutions (from 1 mg/ml to 0.001 mg/ml) for eight-point calibration curves were made. Two solutions at different concentration (100 μg/ml and 5 μg/ml) of each standard were prepared to perform the repeatability study.

The samples were defined as not filtered Protected Denomination of Origin (PDO) ‘‘Aprutino Pescarese’’ extra-virgin olive oils. The sampling was performed at two different harvesting periods (October 2001 and November 2001), in order to determine the effect of ripening time on the amount of phenolic compounds. The analyzed samples were from 95% *cv*. *Dritta* and 5% *cv*. *Leccino* olive fruits. Samples of virgin olive oil obtained by several processing systems were chosen, in order to investigate the relationship between phenolic amounts and type of processing. The processing systems were: two traditional pressure systems (Pressure1Oct and Pressure2Oct for October, and Pressure1Nov and Pressure2Nov for November), two continuous centrifugation systems (Centrifugation1Oct and Centrifugation2Oct for October, and

Centrifugation1Nov and Centrifugation2Nov for November) and the last one was an ''ECO'' continuous centrifugation system (EcoOct for October, and EcoNov for November), characterized by recycling of mill waste water during malaxation.

2.3. Synthesis of hydroxytyrosol

Hydroxytyrosol was prepared by chemical reduction of 3,4-dihydroxyphenylacetic acid, according to Baraldi et al. [21]. Briefly, to an ice-cooled and stirred slurry of $LiAlH_4$ (5.12 g) in dry THF (200 ml), 3,4-dihydroxyphenylacetic acid (7.6 g) was added portion wise during half an hour. After the addition was completed, the suspension was heated under reflux for 6 h, cooled in an ice bath, and the hydride excess eliminated by careful addition of water (100 ml) and 10% HCl (100 ml). The organic layer was separated and the aqueous acid phase was extracted with ethyl acetate (4×100 ml). The combined organic extracts were dried with magnesium sulfate and concentrated in vacuum. The oily residue was chromatographed on a silica gel column (1 cm diameter×20 cm height), eluting with ethyl acetate–light petroleum (b.p. 40–70 °C) (1:1, v/v) to give 3,4-dihydroxyphenylethanol (hydroxytyrosol), as a colorless oil (4.6 g, 66% yield).

2.4. Liquid–liquid extraction of phenolic from real samples

The extraction was performed according to the procedure described by Pirisi et al. [8]. Briefly, 2 g of oil were weighed in a centrifuge tube and added with 1 ml of *n*-hexane and 2.0 ml of CH_3OH–water (60:40, v/v). The mixture was stirred for 2 min in a vortex apparatus, and the tube was centrifuged at 3000 rev./min (30 cm diameter) for 5 min. The methanol layer was separated and the extraction repeated twice. The extracts were combined and evaporated to dryness under reduced pressure and low temperature (<35 °C). Samples were dissolved in 1 ml of CH_3OH–water (1:1, v/v) and filtered through a 0.45 μm nylon filter for capillary electrophoresis analysis.

2.5. Spectrophotometric determination of total phenols

The total phenol content of extracts was determined by the Folin-Ciocalteu spectrophotometric method at 750 nm [22], using a gallic acid calibration curve. The spectrophotometric analysis was repeated three times for each extract ($n=3$).

2.6. Spectrophotometric determination of o-diphenols

According to Mateos et al. [10], 0.5 ml of phenolic extract obtained from olive oil by liquid–liquid extraction was dissolved in 5 ml of CH_3OH–water (1:1, v/v); a mixture of 4 ml of the solution and 1 ml of a 5% solution of sodium molybdate dihydrate in CH_3CH_2OH–water (1:1, v/v) was shaken vigorously. After 15 min, the absorbance at 370 nm was measured using gallic acid for the calibration curve using a glass cuvette. The spectrophotometric analysis was repeated three times for each extract ($n=3$).

2.7. Instrumentation

A Beckman capillary electrophoresis instrument P/ACE 5500 (Beckman Instruments, Fullerton, CA, USA), equipped with a diode array detector, was used. Data acquisition and processing were accomplished using a PC equipped with Beckman P/ACE Station software. The capillary cartridge containing uncoated fused-silica tubing (50 μm I.D.×375 μm O.D.) was supplied from Beckman. Total capillary length was 47 cm, whereas effective length was 40 cm. UV detection was performed at 200 nm. Peak identification was performed by spiking the samples with standard compounds and by spectral analysis.

2.8. CZE conditions

New capillaries were conditioned by flushing 1 *M* sodium hydroxide solution (5 min), 0.1 *M* sodium hydroxide (5 min), HPCE-grade water (5 min) and running buffer (5 min). The capillary not in use was stored in water to prevent buffer crystallization.

The optimized running buffer was 45 m*M* sodium

tetraborate (pH 9.6), prepared by dissolving an appropriate amount of solid salt in HPLC-grade water. The buffer was sonicated for 10 min and, then, filtered through a 0.2 μm cellulose acetate syringe filter (Orange Scientific, Waterloo, Belgium). Samples were injected hydrodynamically at the anodic end in low pressure mode (0.5 p.s.i.) for 3 s (1 p.s.i. = 6894.76 Pa).

Electrophoretic separations were carried out at positive power supply of 27 kV for 10 min, maintaining the capillary temperature at 30 °C; this resulted in a current of ~110 μA. Before each injection, the capillary was rinsed in high pressure mode (20 p.s.i.) with 0.1 *M* HCl (2 min), HPCE-grade water (2 min) and re-equilibrated with running buffer (2 min). After each electrophoretic cycle, the capillary was rinsed with HPCE-grade water (2 min). All washing steps were performed at 30 °C. The running buffer was changed after three runs.

All samples were injected in capillary electrophoresis seven times ($n = 7$).

3. Results and discussion

3.1. Optimization of CZE method

A detailed optimization plan was carried out because specific references about separation and determination of phenols and polyphenols in virgin olive oil by capillary electrophoresis have not been published yet [23–30].

Four separation parameters were investigated during the optimization study in order to verify the behavior of the analyzed phenolic compounds: buffer concentration, buffer pH, applied voltage and temperature during the electrophoretic run.

A phenolic compound mixture was prepared: tyrosol, 2,3-dihydroxyphenylethanol, oleuropein glycoside, hydroxytyrosol, dihydrocaffeic acid, cinnamic acid, 4-hydroxyphenylacetic acid, gentisic acid, syringic acid, ferulic acid, luteolin, taxifolin, *o*-coumaric acid, *p*-coumaric acid, quercetin, vanillic acid, 4-hydroxybenzoic acid, caffeic acid, 3,4-dihydroxyphenylacetic acid, gallic acid and protocatechuic acid. Hydroxytyrosol was synthesized as described previously [21]. Taxifolin was added to the mixture only at the end of the optimization plan. These compounds were chosen because they have been found to be the main phenolic compounds in virgin olive oil.

The optimization of the CZE method was evaluated in terms of resolution of five critical pairs (tyrosol/2,3-dihydroxyphenylethanol, syringic acid/ferulic acid, *p*-coumaric acid/quercetin, quercetin/vanillic acid, 3,4-dihydroxyphenylacetic acid/gallic acid) and total analysis time. Coelution of other phenolic compounds was also verified by spiking technique.

3.2. Effect of buffer concentration, pH, voltage and temperature on phenol migration time and peak resolution

First of all, in order to define the operative mode to adopt, the types of electrolytes and, eventually, the surfactants to mix were chosen. In fact, in the literature, the most efficient operative mode to separate phenolic compounds has been found to be borate-based CZE, but borate–phosphate-based micellar electrokinetic chromatography (MEKC) methods with sodium dodecylsulfate (SDS) as micellar agent have been also used [23–30]. Therefore, in order to simplify operative conditions, a borate-based CZE method was developed.

The applied voltage and the temperature were set at 20 kV and 30 °C, respectively, and the following concentrations of tetraborate were used: 20, 30, 40, 45, 50, 75, 100, 150 and 200 m*M*. When the tetraborate concentration was increased, it led to longer analysis times and improved peak resolution due to its specific complexing effect on the polyhydroxylated species (phenols and polyphenols) [29,30]. In fact, tetraborate complexes vicinal hydroxyl groups on the polyphenol ring resulting in a new charged species, which will be electrophorized by their differences in the charge-to-mass ratio. However, over a concentration of 75 m*M*, several compounds coeluted and analysis time was considerably increased; in addition, over 150 m*M*, current problems occurred. It was found that 45 m*M* tetraborate concentration represented the best com-

promise among resolution of the five critical pairs and other phenolic compounds at a reasonable analysis time.

Subsequently, the effect of buffer pH on peak resolution was evaluated, by adjusting the buffer pH at the following values: 8.0, 8.5, 9.0, 9.3, 9.5, 9.6, 9.7, 9.8 and 10.0. Desired pH values were obtained by adding a proper amount of a 0.1 *M* NaOH solution or a 0.1 *M* HCl solution to the optimized running buffer (namely, a 45 m*M* sodium tetraborate buffer). An increase in pH values caused higher migration times; in fact, higher pH values led to a higher ionization state of the species and at the selected basic pH, the phenolic compounds are negatively charged and would migrate towards the anode, thus away from the detector. However, due to the large electrosmotic flow in the system, polyphenols are propelled together with the bulk solution towards the cathode, but at a much lower rate. Therefore, phenols and polyphenols less dissociated and those with a higher molecular mass are first detected since they are less able to migrate upstream [23]. Moreover, the lower analyte velocities observed at higher pH values could be explained by the increase in ionic strength of the running buffer, which determines lower electroosmotic flow (EOF) [31].

The best electrophoretic separation in terms of resolution of the five phenolic pairs was obtained at higher pH values, but most of the total species overlapped. Therefore, the best compromise in terms of resolution of all phenolic compounds and total analysis time was obtained with a pH 9.60 buffer.

The effect of the applied voltage on resolution of the five phenolic pairs was studied using the optimized buffer composition. When the applied voltage increased from +15 to +30 kV, shorter analysis times and higher separation efficiencies were obtained. Moreover, when temperature rose from 20 to 40 °C, it led to shorter analysis times, but several species overlapped at temperatures over 32.5 °C. The best compromise among peak resolution of the five pairs, analysis time and separation of the other polyphenols in the mixture was found at +27 kV and 30 °C.

At the optimized conditions, the elution order of the analyzed phenols and polyphenols was: tyrosol, 2,3-dihydroxyphenylethanol, oleuropein glycoside, hydroxytyrosol, dihydrocaffeic acid, cinnamic acid, 4-hydroxyphenylacetic acid, gentisic acid, taxifolin, syringic acid, ferulic acid, luteolin, *o*-coumaric acid, *p*-coumaric acid, quercetin, vanillic acid, 4-hydroxybenzoic acid, caffeic acid, 3,4-dihydroxyphenylacetic acid, gallic acid and protocatechuic acid. The electropherogram obtained applying the best separation conditions is reported in Fig. 1A; the peaks are baseline separated and the last peak (protocatechuic acid) was detected within 10 min. The total analysis time, including rinse steps, was about 18 min. Fig. 1B shows that tyrosol and 2,3-dihydroxyphenylethanol were also satisfactorily resolved and separated from the solvent peak as well.

3.3. Repeatability study

Repeatability was assessed for each standard compound at two concentration levels (100 μg/ml and 5 μl/ml). The two solutions containing all the analytes were prepared and analyzed on the same day (intraday precision, $n=12$) and on 3 consecutive days (interday precision, $n=36$). The relative standard deviations of peak areas and the relative standard deviations of migration times were determined for each analyte in the two standard mixtures.

The intraday repeatability on the migration time (expressed as relative standard deviation) was within 0.8 and 0.5% for the 100 μg/ml and 5 μl/ml, respectively, whereas the interday repeatability on migration time was within 1.3 and 0.6% for the 100 μg/ml and 5 μl/ml, respectively. The intraday repeatability on the total peak area (expressed as relative standard deviation) was 1.7 and 2.1% for the 100 μg/ml and 5 μl/ml, respectively, while the interday repeatability on total peak area was 5.1 and 3.1% for the 100 μg/ml and 5 μl/ml, respectively. Only for three compounds, namely tyrosol, 2,3-dihydroxyphenylethanol and quercetin, the precision (expressed as relative standard deviation) was around 10%. For tyrosol and 2,3-dihydroxyphenylethanol this fact might be due to the closeness to the solvent peak, while in the case of quercetin the precision on peak area might be affected by the large closeness to *p*-coumaric acid and vanillic acid.

As expected, the intraday precision was higher

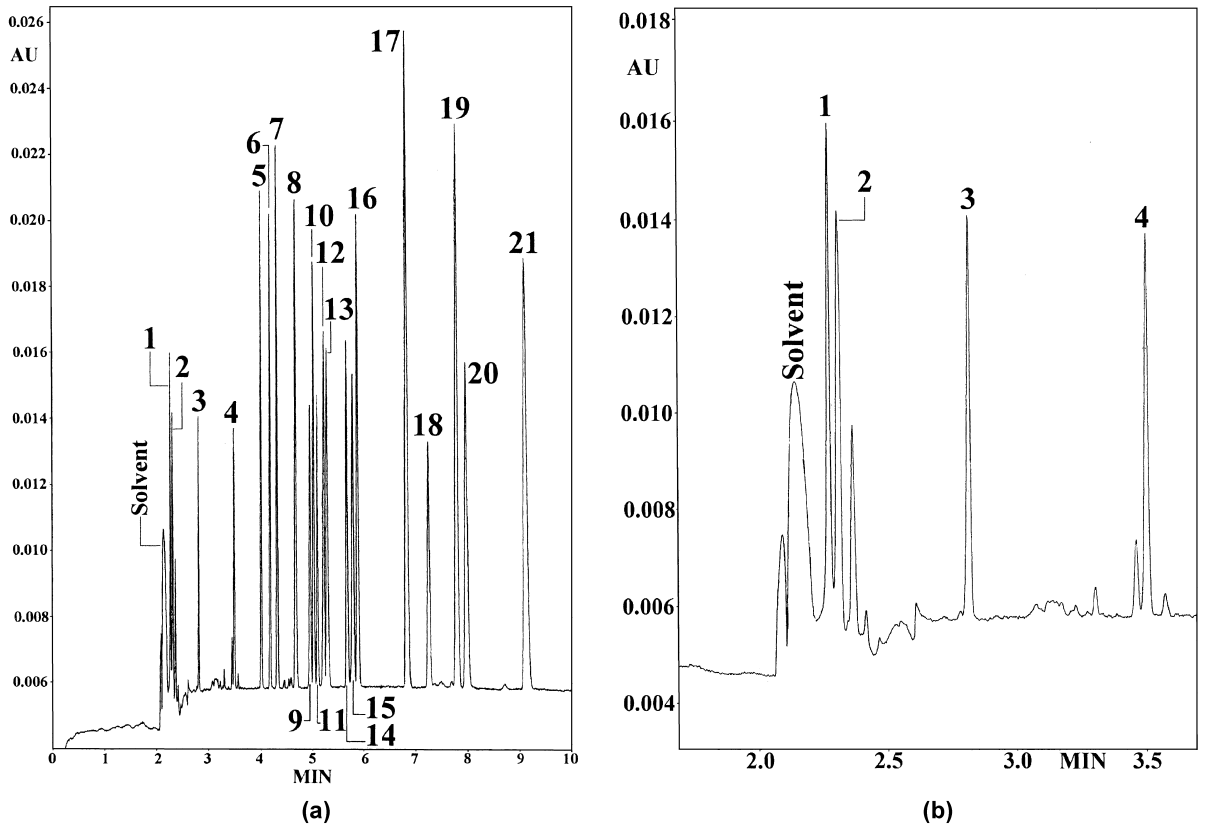

Fig. 1. (A) Separation of the standard mixture of the 21 phenolic and polyphenolic compounds by CZE under optimized conditions. Separation conditions: capillary, 40 cm×50 μm; applied voltage, 27 kV; applied temperature, 30 °C; buffer, 45 m*M* sodium tetraborate (pH 9.60); UV detection, 200 nm; hydrodynamic injection, 0.5 p.s.i., for 3 s. Peak identification numbers: 1, tyrosol (10 μg/ml); 2, 2,3-dihydroxyphenylethanol (20 μg/ml); 3, oleuropein glycoside (20 μg/ml); 4, hydroxytyrosol (10 μg/ml); 5, dihydrocaffeic acid (20 μg/ml); 6, cinnamic acid (20 μg/ml); 7, 4-hydroxyphenylacetic acid (20 μg/ml); 8, gentisic acid (20 μg/ml); 9, taxifolin (15 μg/ml); 10, syringic acid (20 μg/ml); 11, ferulic acid (20 μg/ml); 12, luteolin (100 μg/ml); 13, *o*-coumaric acid (20 μg/ml); 14, *p*-coumaric acid (20 μg/ml); 15, quercetin (50 μg/ml); 16, vanillic acid (20 μg/ml); 17, 4-hydroxybenzoic acid (20 μg/ml); 18, caffeic acid (20 μg/ml); 19, 3,4-dihydroxyphenylacetic acid (20 μg/ml); 20, gallic acid (50 μg/ml); 21, protocatechuic acid (20 μg/ml). (B) Enlargement of the first 3.5 min of (A). Separation conditions: capillary, 40 cm×50 μm; applied voltage, 27 kV; applied temperature, 30 °C; buffer, 45 m*M* sodium tetraborate (pH 9.60); UV detection, 200 nm; hydrodynamic injection, 0.5 p.s.i., for 3 s. See (A) for analyte identification numbers.

than the interday precision and the method showed a good overall repeatability.

3.4. Identification and quantification of virgin olive oil components

Fig. 2A shows a typical CZE electropherogram of a virgin olive oil extract, while Fig. 2B shows the first 3 min of a run. Three main compounds were identified (tyrosol, 2,3-dihydroxyphenylethanol and hydroxytyrosol), by comparing UV spectra of unknown peaks with those of standards and by spiking the sample with standard compounds.

Moreover, several minor compounds were detected, which eluted between 2 and 4 min (marked with points in Fig. 2A); if their UV spectra were compared with those of the available standard compounds, these peaks showed the two highest UV-spectra similarity indexes with those of oleuropein glycoside and oleuropein aglycone. For qualitative assay oleuropein aglycone was obtained by acid hydrolysis of oleuropein glycoside with fuming HCl–water (1:1, v/v) at 70 °C for 30 min. It can be

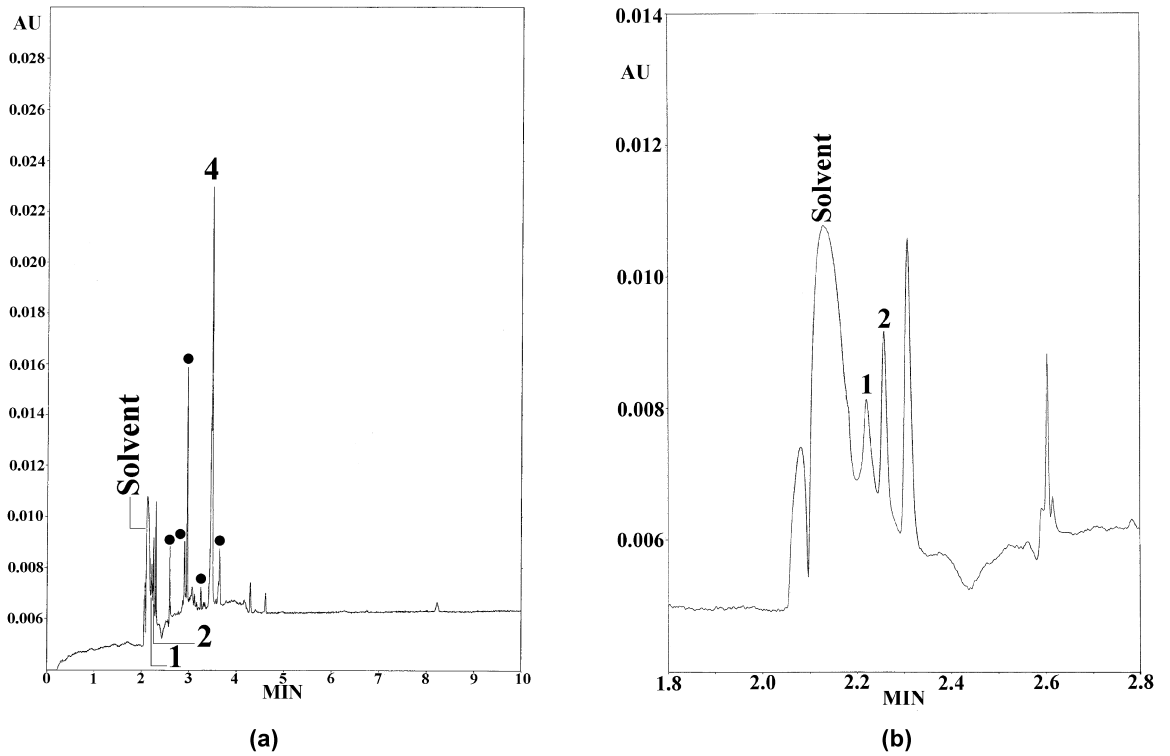

Fig. 2. (A) Typical CZE electropherogram of the phenolic fraction extracted from a virgin olive oil sample. Separation conditions: capillary, 40 cm×50 μm; applied voltage, 27 kV; applied temperature, 30 °C; buffer, 45 m*M* sodium tetraborate (pH 9.60); UV detection, 200 nm; hydrodynamic injection, 0.5 p.s.i., for 3 s. The point-marked peaks could be oleuropein derivatives. Peak identification: 1, tyrosol (about 50 μg/ml); 2, 2,3-dihydroxyphenylethanol (about 7 μg/ml); 4, hydroxytyrosol (about 60 μg/ml); •, oleuropein derivatives (to be confirmed). (B) Enlargement of the first 3 min of (A). Separation conditions: capillary, 40 cm×50 μm; applied voltage, 27 kV; applied temperature, 30 °C; buffer, 45 m*M* sodium tetraborate (pH 9.60); UV detection, 200 nm; hydrodynamic injection, 0.5 p.s.i., for 3 s. Peak identification: 1, tyrosol; 2, 2,3-dihydroxyphenylethanol.

assumed that these compounds may correspond to oleuropein aglycone and its derivatives rather than the oleuropein glycoside itself, because the latter one has been found only in traces in olive oils [8,10–14], while it is abundant in olive fruits, and it undergoes enzymatic and chemical modifications during the manufacturing process.

The reason why the spectra of the point-marked peaks showed a high spectral similarity index with the oleuropein glycoside could be explained by its UV-absorbance properties, since these are mainly due to the aglycone moiety rather than to the glucose moiety.

Due to the lack of a more hyphenated detection system interfaced with the CE instrument, it is difficult to provide an exact identification of those compounds; but, from a previous work performed by HPLC–UV diode array detection (DAD) interfaced with a mass spectrum detector, three main substances were detected and identified by mass spectrum analysis in the same virgin olive oil sample [9]. A characteristic complex fraction was detected in that study. A qualitative analysis of this fraction was performed by MS and three main compounds were identified: oleuropein aglycone (m/z 377) and another two compounds chemically related to the oleuropein aglycone structure, such as ligstroside aglycone (m/z 361) and decarboxymethyl oleuropein aglycone (m/z 319).

Therefore, since the point-marked peaks presented a high spectral similarity index with oleuropein aglycone, they could correspond to the substances of

Table 1
CZE calibration curve parameters, $A = mc \pm q$, where A is the peak area (AU), c is the concentration (μg/ml), q is the y intercept and r^2 is the correlation coefficient. See Fig. 1A for analyte identification numbers

Analytes	Calibration range (μg/ml)	Calibration equations	r^2 areas[a]
1	1–1000[b]	$A = 232\,208c + 1227.2$	0.998
2	1–360[c]	$A = 384\,278c + 1137.1$	0.999
3	1–500[c]	$A = 147\,365c + 674.19$	0.998
4	1–500[c]	$A = 494\,109c + 2884.1$	0.999
5	1–500[c]	$A = 417\,922c + 4108.6$	0.997
6	1–500[c]	$A = 441\,854c + 2657.7$	0.999
7	1–500[c]	$A = 471\,992c + 8184.5$	0.990
8	1–500[c]	$A = 665\,495c + 4913.8$	0.998
9	1–500[c]	$A = 627\,694c + 4182$	0.998
10	1–500[c]	$A = 512\,060c + 3978.5$	0.998
11	1–500[c]	$A = 361\,382c + 2754.5$	0.997
12	3–200[b]	$A = 587\,396c + 198.65$	0.999
13	1–500[c]	$A = 450\,196c + 2231.2$	0.998
14	1–500[c]	$A = 518\,529c + 2857.3$	0.998
15	1–1000[b]	$A = 569\,169c + 1380.1$	0.996
16	1–500[c]	$A = 826\,062c + 4656.9$	0.998
17	1–500[c]	$A = 1\,000\,000c + 10\,049$	0.998
18	1–500[c]	$A = 557\,029c + 1931.2$	0.999
19	1–500[c]	$A = 1\,000\,000c + 6065.1$	0.999
20	1–1000[b]	$A = 853\,442c + 1152$	0.998
21	1–500[c]	$A = 1\,000\,000c + 7344.3$	0.998

[a] Correlation coefficients of the calibration curves using peak area.
[b] Eight concentration levels.
[c] Seven concentration levels.

Table 2
Amount of phenols (determined by the CZE method), content of total phenols (determined at 750 nm) and quantification of *o*-diphenols (determined at 370 nm) of virgin olive oils obtained with different processing conditions and at different olive ripening stages

	Mean±SD		
	Amount of phenols found by CZE ($n=7$)	Total phenols ($n=3$)[a]	*o*-Diphenols ($n=3$)[b]
Pressure1Oct	313.4±58.9	394.5±14.4	128.6±12.3
Pressure1Nov	159.9±13.5	170.2±10.3	58.7±6.5
Pressure2Oct	216.1±42.4	437.4±24.5	295.4±20.5
Pressure2Nov	65.8±5.2	90.1±9.9	91.6±7.0
Centrifugation1Oct	432.6±48.1	329.5±5.0	131.7±7.6
Centrifugation1Nov	78.7±16.0	81.0±9.3	48.7±2.3
Centrifugation2Oct	142.1±42.7[c]	102.3±9.9	50.4±2.0[d]
Centrifugation2Nov	149.3±35.3[c]	184.7±13.9	53.7±0.1[d]
Eco Oct	527.2±80.9	353.3±23.7	172.4±9.2
Eco Nov	454.1±101.7	239.7±21.7	136.6±6.6

Listed are the mean values of the reported quantifications of the two samples obtained from the same manufacturing system, but having different ripening stage. The same superscript letters indicate no-significant differences ($P<0.05$). Unless otherwise stated, mean values are significantly different.

[a] Expressed as mg of gallic acid/kg oil.
[b] Expressed as mg of gallic acid/kg oil.

the complex fraction analyzed by HPLC. Two of the unidentified peaks (with 2.6 min and 2.9 min migration times) were tentatively quantified, because they were particularly abundant in all analyzed samples. They were quantified using the calibration curve of oleuropein glycoside, due to the lack of oleuropein aglycone standard. The correlation among the total amount of phenols quantified by capillary electrophoresis (tyrosol, 2,3-dihydroxyphenylethanol, hydroxytyrosol, and the two unidentified peaks) and the two spectrophotometric determinations (total phenols at 750 nm and *o*-diphenols at 370 nm) was verified.

Table 1 shows the parameters of the calibration curves of the standard compounds, while Table 2 lists the amount of phenolic compounds determined by the CE method and by the two colorimetric assays.

Analytical results were evaluated by one-way analysis of variance (ANOVA) with Tukey's HSD multiple comparison (Statistica Software, StatSoft, 1999). All samples showed significant differences ($P<0.05$) among October and November values of the three analyzed parameters, which are related to the loss of phenolic compounds during the ripening of olive fruits. Only for the Centrifugation2 system, November's amount of total phenols and *o*-diphenols was higher than the October one, while the content of phenols determined by CE did not show significant differences.

Moreover, statistical analysis confirmed the existence of a linear correlation between the amount of phenols quantified by CE and total phenols determined by the Folin-Ciocalteau method ($r^2=0.8357$, $P<0.05$), as well as with the amount of *o*-diphenols ($r^2=0.9058$, $P<0.05$).

As Table 2 shows, it is clear that the manufacturing process can strongly influence the amount of phenols in virgin olive oils. In most cases, the phenol content was significantly different for the same ripening period samples, even if the raw material was homogeneous.

4. Conclusions

A CZE method for the quantification of the main phenolic compounds in olive oil extracts was developed and optimized. Separation and identification of three well-known olive oil antioxidants (tyrosol, hydroxytyrosol and 2,3-dihydroxyphenylethanol) was performed; other unidentified compounds were resolved, and could be oleuropein aglycone derivatives. The amount of these substances was determined in several extracts obtained from different olive oil manufacturing systems and olive ripening periods. In fact, the distribution of the phenolic compounds shows quantitative differences, which are related to the degree of olive ripening as well as to the manufacturing process.

The lack of a more specific detection system interfaced with the capillary electrophoresis instrument, such as a mass spectrum detector, did not allow to confirm the identification of some peaks.

Nevertheless, the CZE method developed here showed good repeatability and rapidity. Since capillary electrophoresis has less operative costs than HPLC, the use of this technique as an alternative or in addition to HPLC to quantify the antioxidant profile in virgin olive oil, has been demonstrated.

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