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# Determination and Characterization of Glycerophospholipids in Olive Fruit and Oil by Nonaqueous Capillary Electrophoresis with Electrospray-Mass Spectrometric Detection

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**ABSTRACT:** A nonaqueous capillary electrophoresis method with electrospray-mass spectrometric detection was developed to study the glycerophospholipid fraction in olive fruit and olive oil samples. In olive fruits, where the information available about the phospholipid fraction was very scarce, results obtained in this work allowed us to complete and improve this knowledge. The glycerophospholipid fraction of the olive fruit samples analyzed was composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (lyso-PE), phosphatidylinositol (PI), phosphatidic acid (PA), lysophosphatidic acid (lyso-PA), and phosphatidylglycerol (PG). Differences in the relative abundance of the glycerophospholipid classes determined were observed as a function of the botanical and geographical origin of the olive fruits analyzed. Interestingly, the olive stone and pulp analyzed also showed different glycerophospholipid compositions. For olive oil, five glycerophospholipids (lyso-PA, PC, PE, lyso-PE, and PG) were detected. Finally, identification of the main molecular species in the different glycerophospholipid classes for the olive fruit samples analyzed was accomplished by tandem mass spectrometric experiments and information from the literature.

**KEYWORDS:** *nonaqueous capillary electrophoresis, mass spectrometry, glycerophospholipid, olive fruit, olive oil*

## INTRODUCTION

Phospholipids constitute the major components of biological membranes and participate in activities involving cell signaling and substrate transport.[1] Due to their important biological activity, they have been associated with the prevention or decrease of certain diseases such as cardiovascular, inflammatory processes, diabetes, cognitive and memory problems, and cancer.[1,2] Valuable effects of dietary phospholipids have been demonstrated in human health.[3] Moreover, phospholipids present many industrial applications based on their surfactant properties as emulsifiers or emulsion stabilizers.[4]

There are two main classes of phospholipids, those that contain sphingosine (sphingolipids) and those with a glycerol backbone (glycerophospholipids). Sphingomyelin is the dominant species in the first group and does not appear to occur in plants or microorganisms. Glycerophospholipids are the class of polar lipids having a glycerol backbone esterified to fatty acids in positions *sn*-1and *sn*-2, and with a phosphate group in position *sn*-3. As Table 1 shows, this phosphate group is also esterified by the hydroxyl group of various alcohols such as ethanolamine, choline, inositol, and glycerol to form different families of phospholipids. Apart from this, lysophospholipids are glycerophospholipids in which one acyl chain is lacking and only one hydroxyl group of the glycerol backbone is acylated.[5]

Analysis of phospholipids involves some problems, such as their low abundance with respect to the nonpolar triglycerides and the high variety of fatty acids that can be found in each individual phospholipid class.[3] Additionally, due to the lack of chromophores and the consequent low UV absorption of phospholipids, different detection systems have been also considered for their analysis, such as laser-induced fluorescence (LIF),[4,6] or indirect UV detection,[4,6] evaporative light scattering detection (ELSD),[4,6] refractive index detectors,[4] or mass spectrometry (MS).[4,6,7]

The phospholipid composition of olive fruits has scarcely been studied in spite of the biological importance of this polar fraction as mentioned above. There is one article dealing with the analysis of Russian olives, called wild olives, belonging to another botanical family, Elaeagnaceae, although with similar appearance as olives.[8] Concerning olive fruits from *Olea europaea* L., there are only two articles published that were carried out in the 1970s,[9,10] highlighting the limited and poor information available. In the first one, phosphatidylcholine (PC) (48.4%), phosphatidylinositol (PI) (29.3%), phosphatidic acid (PA) (16.3%), and phosphatidylethanolamine (PE) (6.0%) were determined in olive seed as the main phospholipids by use of thin-layer chromatography (TLC).[9] In this work, the phospholipid fatty acids were also determined by gas chromatography with flame ionization detection (GC-FID). The results showed that oleic (C18:1), palmitic (C16:0), linoleic (C18:2), and stearic (C18:0) acids were the main fatty acids present in the olive seed samples analyzed. Also via TLC and GC-FID, the analysis of olive pulp was achieved in the second article.[10] Only PC and PI were determined and the phospholipid fatty acid composition obtained was very similar to that found for the olive seed.[9]

Regarding olive oils, there is some evidence on the significant biological activity of phospholipids in vegetable oils,[11] such as some influence on their antioxidant capacity and their oxidative

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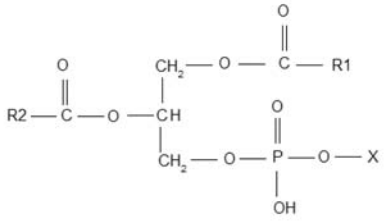
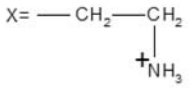
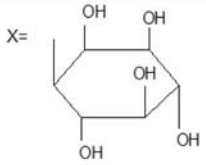
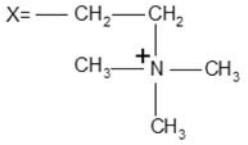
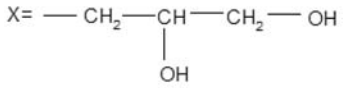
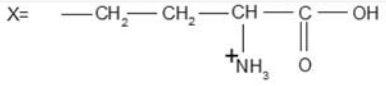
Table 1. General Structure of Glycerophospholipids and Main Phospholipid Classes

| *General structure (R1 and R2 correspond with two fatty acids)* | |
|---|---|
|  | |
| ***Main phospholipids*** | |
| Phosphatidic acid (PA) | X= —H |
| Phosphatidylethanolamine (PE) |  |
| Phosphatidylinositol (PI) |  |
| Phosphatidylcholine (PC) |  |
| Phosphatidylglycerol (PG) |  |
| Phosphatidylserine (PS) |  |

stability.[12,13] Phospholipids are considered synergistic since they regenerate other antioxidant as phenols[14] by donation of the hydrogen atom from amino groups, with the main antioxidant effect observed between amino alcohol phospholipids [PC, PE, and phosphatidylserine (PS)] and $\gamma$- and $\delta$-tocopherols.[15] In spite of these properties, the information available to date about the phospholipid composition in olive oils is scarce. In the articles published on this subject, TLC,[16] high-performance liquid chromatography (HPLC) with UV detection[11] or MS/MS detection,[17] micellar liquid chromatography with UV detection,[18] and nuclear magnetic resonance ($^{31}$P NMR)[19] were employed as analytical techniques. A content of phospholipids of 21−124 mg/kg was determined in different olive oils by analyzing the total phosphorus content by the colorimetric IUPAC method.[13] It has been established that this content is influenced by oil filtration[13] and refining treatments.[19]

Capillary electrophoresis (CE) has scarcely been employed to analyze phospholipids. Due to the poor aqueous solubility of these compounds, micellar electrokinetic chromatography (MEKC) and nonaqueous capillary electrophoresis (NACE) have been the most used electrophoretic modes to accomplish phospholipid analysis by CE. Thus, the methodologies used for phospholipid analysis have been NACE-UV, to differentiate between oxidized and nonoxidized PC[20] and to study the phospholipid composition of some plant seeds;[21] NACE with indirect UV detection, for phospholipid determination in human sera[22] or human blood[23] or to separate standard phospholipids;[24] MEKC-UV, to study the phospholipid composition of different lecithins[25,26] or human blood plasma;[27] and capillary zone electrophoresis (CZE)-LIF[28,29] and MEKC-LIF[30] in standards. In the case of MS detection, two articles have been developed that use NACE-MS methodologies for the separation and determination of standard phospholipids[31] and for the analysis of phospholipid composition in biological samples.[32] In addition, a capillary electrochromatography (CEC)-ESI-MS method for the determination of phospholipids in human urine, using an open tubular column, has recently been reported.[33]

The aim of this work was to provide valuable information about the composition of important food components as glycerophospholipids in olive fruit and olive oil samples. For that purpose, an analytical methodology by NACE-ESI-MS was developed. The composition of glycerophospholipids in olive stone and pulp was studied, and possible differences according to the variety and the geographical origin of the olives analyzed were also investigated. The developed method was also applied to study the glycerophospholipid fraction in an extra virgin olive oil sample. MS/MS experiments were also carried out to

enable identification of the main molecular species in the different glycerophospholipid classes.

## MATERIALS AND METHODS

**Reagents and Materials.** HPLC-grade acetonitrile (ACN) and methanol (for sheath liquid and buffer preparations), chloroform, methanol, hexane, and ethanol (all for sample preparation), and acetic acid were obtained from Scharlau Chemie (Barcelona, Spain). Sodium hydroxide pellets and ammonium acetate were purchased from Merck (Darmstadt, Germany). Water employed was from a Milli-Q system (Millipore, Bedford, MA). All samples were filtered before use through 0.45 $\mu$m pore size disposable nylon filters from Titan (Titan 2, Eatontown, NJ).

Glycerophospholipid standards [phosphatidylglycerol (PG) from egg yolk lecithin, PC from egg yolk, PE from egg yolk, PA sodium salt from egg yolk lecithin, PI sodium salt from *Glycine max* soybean, oleoyl-lyso-PA sodium salt, lyso-PC from egg yolk, lyso-PE from egg yolk, and PS sodium salt from bovine brain] were purchased from Sigma (St. Louis, MO) and used without further purification.

Arbequina from Córdoba, Empeltre, and Lechín de Sevilla olives analyzed in this work were kindly supplied by the Olive World Germplasm Bank of IFAPA (Junta de Andalucía, Córdoba, Spain). Arbequina from Toledo and Arbequina from Jaén were collected in the same harvest and at the same stage of maturity. Monovarietal extra virgin olive oil from Arbequina variety analyzed in this work was purchased in a local market.

**Sample Preparation.** Lipid extraction from olive fruits was carried out by the method proposed by Folch et al.[34] with some modifications. First of all, olive fruits were washed with water to neutralize phospholipase activity.[17] Sample homogenization was made in different phases to ensure a uniform mixture. Fruits were manually depulped and olive stones were ground in a domestic mill (Kenwood Ibérica, Barcelona, Spain) to obtain a fine powder. Subsequently, olive pulp was added to the stone powder and was also ground. For the samples in which the pulp and stone were analyzed separately, the olive pulp was ground individually. Thirty milliliters of 2:1 (v/v) chloroform/methanol was added to 2.5 g of homogenized olive and the mixture was vigorously vortexed for 2 min. Additionally, 10 mL of water was added and the sample was vortexed again for 2 min. Then a centrifugation step (Heraeus Instrument, Hanau, Germany) at 4000*g* for 15 min was performed. After centrifugation, the lower phase was collected and evaporated in a rotary evaporator Laborota 4001 (Heidolph Instruments, Viertrieb, Germany) at 45 °C. The polar lipids fraction was reconstituted in 500 $\mu$L of methanol containing 0.5% (v/v) acetic acid and centrifuged at 4000*g* for 5 min, and the upper soluble phase was collected.

The extraction of glycerophospholipids from olive oil was carried out according to Hatzakis et al.[19] Briefly, 50 g of olive oil was dissolved in 200 mL of hexane, and the solution was extracted with 3 × 50 mL of 87:13 (v/v) ethanol/water. The ethanolic extracts were washed with 50 mL of hexane, and the glycerophospholipids were obtained upon removal of the solvent under vacuum. After solvent evaporation, glycerophospholipids were dissolved in 500 $\mu$L of methanol containing 0.5% (v/v) acetic acid and centrifuged at 4000*g* for 5 min. Finally, the upper soluble phase was collected.

**Nonaqueous Capillary Electrophoresis–UV Procedure.** An HP$^{3D}$CE instrument (Agilent Technologies, Palo Alto, CA) equipped with an on-column diode array detector (DAD) and spectral collection was employed. The experiments were performed in fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) of 50 $\mu$m i.d. and effective length of 60 cm. A 60:40 (v/v) methanol/ACN mixture with 100 mM ammonium acetate buffer and 0.5% (v/v) acetic acid was employed as separation buffer. Capillary conditioning between sample injections was performed with methanol for 2 min, Milli-Q water for 1 min, 0.1 M NaOH for 1 min, Milli-Q water for 1 min, and separation buffer for 4 min. The selected CE conditions were as follows: capillary temperature 25 °C, applied voltage 30 kV, and UV detection at 200 nm with a bandwidth of 5 nm in all cases. The sample injection was performed by pressure at 50 mbar for 5 s.

**Nonaqueous Capillary Electrophoresis–Mass Spectrometry Procedure.** CE-MS experiments were performed in a HP$^{3D}$CE instrument (Agilent Technologies, Palo Alto, CA) coupled through an orthogonal electrospray interface (ESI, model G1607A from Agilent Technologies, Palo Alto, CA) to an ion trap mass spectrometer (model amaZon SL from Bruker Daltonics, Bremen, Germany). Uncoated fused-silica capillaries of 50 $\mu$m i.d. and 60 cm length were used. Electrophoretic conditions were as follows: injection at 50 mbar for 5 s, voltage 25 kV, and temperature of the capillary 25 °C. Sheath liquid composition was 80:20 (v/v) methanol/water with 0.5% acetic acid at a flow rate of 6.00 $\mu$L/min by a syringe pump (Hamilton). Nebulizer and drying gas optimized conditions were 8 psi and 5 L/min at 180 °C. The mass spectrometer operated with the ESI source in the positive ion mode (−4.5 kV) with an end plate offset of −1 kV. The ion charge control (ICC) was activated with a target up to 300 000 ions and maximum accumulation time of 200 ms, and seven averages, in the UltraScan mode. The $m/z$ range scanned was from 350 to 1000 $m/z$. Glycerophospholipid fragmentation was performed in the AutoMS$^n$ mode. The parameters of MS$^2$ experiments were as follows: eight precursor ions and absolute threshold of 15 000. The acquisition parameters in this mode were 1.00 V of fragmentation amplitude, isolation width of 2.0 $m/z$, and a range scanned of 200–900 $m/z$. For MS$^2$ experiments in negative mode, all the conditions were the same except the use of +2.8 kV.

## RESULTS AND DISCUSSION

**Development of Nonaqueous Capillary Electrophoresis–UV Methodology.** First experiments were carried out in order to achieve the separation of standard glycerophospholipids by NACE. This CE mode is the best choice for glycerophospholipid separation due to its great potential in the separation of analytes insoluble in water and with similar electrophoretic mobilities. In this separation mode, the proper selection of organic solvents and additives has great importance. For this reason, different mixtures of methanol with ACN at different proportions (20:80, 40:60, 60:40 and 80:20 v/v), ACN with 2-propanol (3:2 v/v),[21] and ACN with 2-propanol plus hexane (57:38:5 v/v/v)[31] were tested as organic solvents. Better results with methanol/ACN mixtures were obtained, due to the fact that the use of 2-propanol reduced the separation efficiency. Moreover, ammonium acetate was chosen as electrolyte salt and different concentrations from 20 to 100 mM were investigated. In order to prevent frequent breakage of the capillary due to the low solubility of ammonium salt in ACN, the addition of acetic acid (from 0 to 1% v/v) in the buffer solution was performed. With decreasing acetic acid percentage, higher migration times of the glycerophospholipids were observed, with 0.5% (v/v) selected as the optimum value.

Under CE conditions of 30 kV, 25 °C, and 60 cm total length, results showed lower migration times with enough separation efficiency with 100 mM ammonium acetate in methanol/ACN 60:40 (v/v) and 0.5% (v/v) acetic acid. An optimization of capillary conditioning was revealed to be critical to maintain the reproducibility of the capillary and to eliminate possible salt precipitation. Thus, it was shown that different washing steps with methanol, 0.1 M NaOH, and buffer solution between injections were required.

The glycerophospholipid standards separation obtained under these conditions is shown in Figure 1. As can be seen, separation of six glycerophospholipid classes was obtained in less than 25 min with adequate resolution. Due to the lack of chromophores, 200 nm is commonly used for phospholipid detection. Absorbance at this wavelength is possible due to the presence of unsaturated groups in the fatty acids as well as the carboxyl or phosphate groups.[21] However, the lysophospholi-

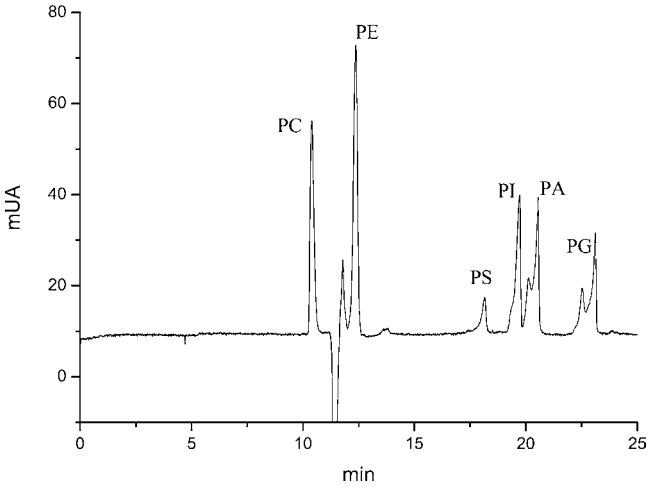



**Figure 1.** Electropherogram showing separation of the glycerophospholipid standards by NACE-UV. Experimental conditions: concentration of each phospholipid standard was 500 $\mu$g/mL; separation buffer was 100 mM ammonium acetate in 60:40 (v/v) methanol/ACN with 0.5% acetic acid; internal diameter 50 $\mu$m; total length 68.5 cm; injection by pressure at 50 mbar during 5 s; applied voltage 30 kV; temperature 25 °C; and UV detection at 200 nm. Peaks: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol.

pids forms were not detected by this methodology, probably due to their smaller UV absorption in comparison with phospholipid forms, caused by the loss of one carbonyl group[22] and one fatty acid with possible unsaturated groups.

For lipid extraction from olive fruits, we used, with slight modification, the procedure developed by Folch et al.[34] in which more than 95% recoveries were achieved. Information about the methodology employed in our work is collected in the Materials and Methods section. After the centrifugation step, different strategies were tested to isolate the polar fraction of this lipid extract, such as precipitation of glycerophospholipids with cold acetone[21] and solid-phase extraction (SPE) using silica columns with the methods employed by Avalli and Contarini[35] and Narvaéz-Rivas et al.[36] However, some glycerophospholipid losses were observed when the three above-mentioned strategies were used. For this reason, a simple redissolution step of the polar lipid fraction in 500 $\mu$L of methanol containing 0.5% (v/v) acetic acid was performed.

In olive oil, extraction of glycerophospholipids consisted of isolating the polar fraction of the oil matrix. Because the SPE and precipitation strategies tested for olive samples were not satisfactory, an extraction procedure from the literature was employed.[19]

When the extracted glycerophospholipids were injected in the NACE-UV system, similar profiles were observed for olives and olive oil, although complete identification could not be performed. In all cases, the profiles showed some matrix peaks in the same time range and no complete identification for PG, PI, and PA was possible. Thus, in order to provide proper identification of all glycerophospholipids, including the lysophospholipid forms, coupling of NACE with MS detection was proposed.

**Optimization of Nonaqueous Capillary Electrophoresis–Electrospray Mass Spectrometry Methodology.** In order to optimize MS conditions, some modifications in the NACE-UV conditions were performed. Capillary total length was 60 cm to maintain the electrophoretic separation to the detection window in CE. The applied voltage was reduced from 30 to 25 kV to avoid getting to the maximum allowed current in the CE-MS coupling (50 $\mu$A).

The ESI conditions optimized were composition and flow rate of sheath liquid, temperature and flow of the drying gas, and nebulizer gas pressure. For the sheath liquid, the same electrophoretic buffer with low salt concentration (50 mM ammonium acetate) was tested in a first instance, as reported by Gao et al.[32] However, due to the fact that a high salt concentration caused frequent capillary breakage and salt formation in the ESI interface, this sheath liquid was rejected. In fact, better signal-to-noise ratio (S/N) in the total ion electropherogram (TIE) and less frequent breakage were obtained with a sheath liquid previously employed by Wang et al.[37] based on the use of 80:20 (v/v) methanol/water containing 0.5% acetic acid. The sheath liquid flow was optimized for values between 3 and 7 $\mu$L/min, and 6 $\mu$L/min was chosen because it enabled better current stability.

The influence of positive- and negative-ion modes was also studied. Despite the possible variation of ionization efficiency for each glycerophospholipid produced by the different adducts formed, positive-ion mode resulted in higher intensities and S/N ratios for all glycerophospholipids except PE, as was expected according to Zink and Mangelsdorf.[38] Moreover, due to the better current stability in the CE-MS system, the positive-ion mode was selected in this work. Nebulizer gas pressure (2−15 psi) and drying gas flow (3−7 L/min) were other important parameters optimized. Elevated gas pressure was preferred because high signal intensity was achieved. However, different migration times with respect to NACE-UV were obtained. This fact could be explained by the Venturi effect produced with the MS coupling due to the low viscosity of organic solvents used. Low temperature for drying gas was selected (180 °C) based on the high volatility of organic solvents employed. With 8 psi dry gas pressure and 5 L/min flow, the final separation for standard glycerophospholipids was achieved in less than 14 min.

In the ion trap parameters, ion charge control (ICC) and number of scans averaged were optimized to reach a compromise between the highest S/N and enough points to define the peak. The best results were observed with ICC activated (with a target up to 300 000 ions and maximum accumulation time of 200 ms) and seven averages. Figure 2 shows the TIE for a glycerophospholipid standard mixture and the corresponding extracted ion electropherograms (EIE) for each glycerophospholipid attained under optimal NACE-ESI-MS conditions. To identify the characteristic ions of each class of standard glycerophospholipid, individual direct infusions were performed. The profile obtained was comparable with the NACE-UV results, with differences in the retention time due to the different voltage used and the Venturi effect previously discussed. However, the migration order was equivalent. Note that, by use of this NACE-ESI-MS method, the lysophospholipids forms were also detected. They coeluted with their corresponding glycerophospholipid forms with the exception of lyso-PA, which showed an anomalous migration order. Thus, for the first time, the separation and identification of nine glycerophospholipid classes was achieved in less than 14 min. This fact represents a considerable advantage with respect to the other two works available in the literature focused on phospholipid analysis by NACE-ESI-MS.[31,32]

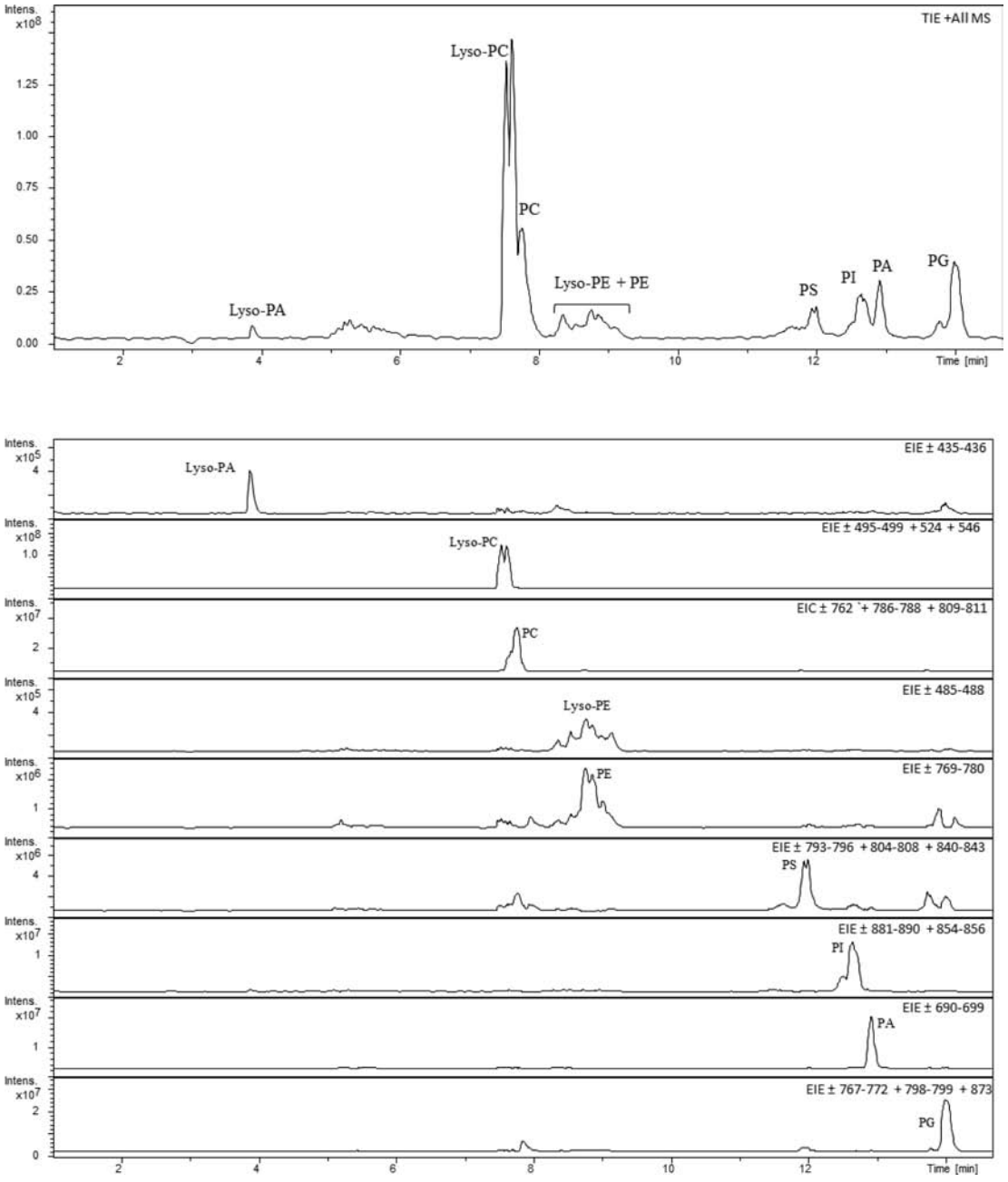



**Figure 2.** Total ion electropherogram (TIE) of glycerophospholipid standards in NACE-ESI-MS and corresponding EIE for each phospholipid (concentration 500 $\mu$g/mL). CE conditions: 100 mM ammonium acetate in 60:40 (v/v) methanol/ACN with 0.5% acetic acid as separation buffer; internal diameter 50 $\mu$m; total length 60 cm; injection by pressure at 50 mbar during 5 s; applied voltage 25 kV; temperature 25 °C. ESI conditions: positive-ion mode −4.5 kV; end plate −1 kV; sheath liquid, 80:20 (v/v) methanol/water with 0.5% (v/v) acetic acid at 6 $\mu$L/min; drying gas flow 5 L/min; drying temperature 180 °C; nebulizer pressure 8 psi. Ion trap conditions: ICC activated (maximum accumulation time 200 and 300 000 ions); seven averages; scan 350−1000 $m/z$. Peaks: lyso-PA, lysophosphatidic acid; lyso-PC, lysophosphatidylcholine; PC, phosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol.

Analytical characteristics of the developed method were evaluated for the EIEs in terms of precision, limit of detection (LOD), and limit of quantitation (LOQ). To assess precision, instrumental and method repeatability, and intermediate precision were assessed as shown in Table 2. The values of relative standard deviations (RSDs) were acceptable for the coupled NACE-MS, being less than 7% for migration times and less than 16% for corrected areas in all cases except lyso-PA, where some abnormal variations were observed. The LODs and LOQs were calculated as the minimum concentration yielding an S/N ratio equal to 3 and 10 times, respectively. As can be observed in Table 2, in positive-ion mode, LODs were in the microgram per milliliter range, with the lowest LOD for PC (2.2 $\mu$g/mL) and the highest for PE (40.9 $\mu$g/mL).

**Olive Fruit Analysis by Nonaqueous Capillary Electrophoresis−Electrospray Mass Spectrometry.** The NACE-ESI-MS method developed was applied to study the glycerophospholipid composition in olives. For that purpose, five different olive types picked in the same harvest were analyzed. Arbequina variety collected in three different


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**Table 2. Analytical Characteristics of NACE-ESI-MS**

| | precision (RT and Ac in RSD, %)[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | instrumental repeatability[b] | | method repeatability[c] | | intermediate precision[d] | | | |
| phospholipid | RT | Ac | RT | Ac | RT | Ac | LOQ ($\mu$g/mL) | LOD ($\mu$g/mL) |
| lyso-PA | 1.5 | 14.2 | 17.4 | 1.3 | 26.0 | 1.1 | 106.3 | 32.2 |
| lyso-PC | 2.3 | 5.8 | 4.7 | 0.6 | 3.6 | 2.2 | 19.4 | 5.8 |
| PC | 1.5 | 3.1 | 8.7 | 1.6 | 4.7 | 2.7 | 7.4 | 2.2 |
| PE | 1.7 | 8.7 | 4.6 | 0.7 | 9.6 | 2.0 | 136.5 | 40.9 |
| lyso-PE | 1.7 | 13.0 | 6.0 | 1.1 | 5.5 | 0.1 | 122.2 | 36.7 |
| PS | 2.5 | 4.9 | ND | ND | 6.1 | 0.9 | 127.8 | 38.3 |
| PI | 2.8 | 2.2 | 11.1 | 5.4 | 16.3 | 0.6 | 9.4 | 2.8 |
| PA | 2.7 | 10.7 | 6.6 | 5.3 | 8.8 | 1.1 | 73.2 | 21.9 |
| PG | 2.9 | 12.2 | 5.7 | 7.0 | 3.5 | 1.8 | 10.5 | 3.1 |

[a]RT, retention time; Ac, corrected area. [b]Five consecutive injections on the same day ($n$ = 5) of a standard mixture. [c]Obtained from three individual standard mixtures at the same concentration injected in triplicate on the same day ($n$ = 3). [d]Determined from three individual standard mixtures at the same concentration injected in triplicate on three consecutive days ($n$ = 9).

geographical locations (Córdoba, Jaén, and Toledo), and two olive varieties from different botanical origins (Lechín de Sevilla and Empeltre) were evaluated to study the influence of pedoclimatic conditions and variety on the glycerophospholipid fraction. An example of the separation obtained for the Arbequina variety from Jaén is shown in Figure 3. Glycerophospholipids found in each olive sample and their relative abundance can be observed in Table 3. Identification of each glycerophospholipid class was performed according to their retention time and their $m/z$. For integration purposes, data were obtained by injecting two individual samples in duplicate. Due to the different response factor observed for each glycerophospholipid, mainly related to the polar headgroup of each classes and the unavailability of standards for each component,[39] data collected in Table 3 were calculated as the area found divided by their response factor (relationship between signal and concentration of each glycerophospholipid standard). As Table 3 shows, PA was the most abundant glycerophospholipid in all olive samples analyzed (54−82%), followed by PE (4−16%), PC (3−9%), lyso-PE (1.3−18%), PI (4.4−8%), PG (3.7−6.3%), and lyso-PA (0.1−0.2%). On the other hand, PS and lyso-PC were not detected in any sample analyzed. Among the same Arbequina variety but collected in different geographical regions, similar relative abundances of the glycerophospholipids were observed except in the case of lyso-PE of the Arbequina from Toledo olives, where higher values (18%) were obtained. For PE, some variations were also observed; this glycerophospholipid was 2 times higher in Arbequina from Jaén than from the other two origins, Toledo and Córdoba. Moreover, the three olive varieties analyzed in this work also showed similar results with the exception of the high value determined for PA in Empeltre variety and the PE (16%) values found in Lechín de Sevilla, in comparison with the other varieties analyzed. Although these values need to be further validated with total quantitative methods, these results showed the main glycerophospholipids in the olive fruit.

To study the glycerophospholipid origin in the different parts of the olive fruit, stone and pulp (included the skin or exocarp) were analyzed separately. An amount of 2.5 g of each part was extracted by the same procedure employed for the complete olive fruit. Interestingly, in the Arbequina sample from Toledo, lyso-PE and PE were found only in the olive pulp and were not detected in the stone. The PI and PA values were slightly higher in olive stone compared with olive pulp.

**Olive Oil Analysis by Nonaqueous Capillary Electrophoresis−Electrospray Mass Spectrometry.** Due to the highly promising results obtained for olive fruits and as a preliminary analysis, a monovarietal extra virgin olive oil was analyzed by the NACE-ESI-MS method developed. As can be observed in Table 3, PE (42%), PG (37.9%), and PC (15%) were clearly observed with high relative abundance. Lyso-PA (0.2%) and lyso-PE (4.5%) were also found, although their presence was much less abundant. In this case, PS, PA, PI, and lyso-PC were not detected. These results are only partially in agreement with the two previous articles.[17,19] In the last one, using $^{31}$P NMR, lyso-PA and PG were found as the main glycerophospholipids in almost all the olive oil samples analyzed, with PC and PE detected only in one sample.[19] The other article fixed the relative abundance for each glycerophospholipid as PG (63%), PA (12%), PI (11%), PE (9%), and PC (5%).[14] In our work, PE, PG, and PC were the most abundant glycerophospholipids detected and PA was not found. However, as Table 3 shows for olive fruits, it is necessary to consider that glycerophospholipid abundance changes as a function of olive variety and geographical origin, and only one olive oil sample was analyzed in this work as preliminary data. For these reasons, a possible comparison of the results obtained for this olive oil with those found in olive fruits seems to be problematic. Moreover, the different extraction procedure used for solid samples of olive fruits compared to the one used for the olive oil could also influence the different composition found in both matrices.

**Study of Fatty Acid Composition by Nonaqueous Capillary Electrophoresis−Electrospray Tandem Mass Spectrometry.** In order to elucidate the molecular species of each glycerophospholipid found in the samples analyzed, NACE-ESI-MS$^2$ experiments were carried out. AutoMS$^n$ mode permitted us to perform MS$^2$ experiments where up to eight precursor ions could be selected to obtain their MS$^2$ spectra. Only precursor ions with a threshold intensity of 15 000 were fragmented. This threshold was evaluated as a function of the lowest LOD signal. The scan was modified from 200 to 900 $m/z$ due to the low $m/z$ of the fatty acids without the headgroup, and only two averages were used as the number of scans to generate the MS$^2$ spectra. These analyses were carried out for the five olive fruits and the olive oil analyzed. Table 4 collects the main molecular species found for each glycerophospholipid in all olive fruits analyzed. However, in the case of olive oil, maybe due to the low phospholipid

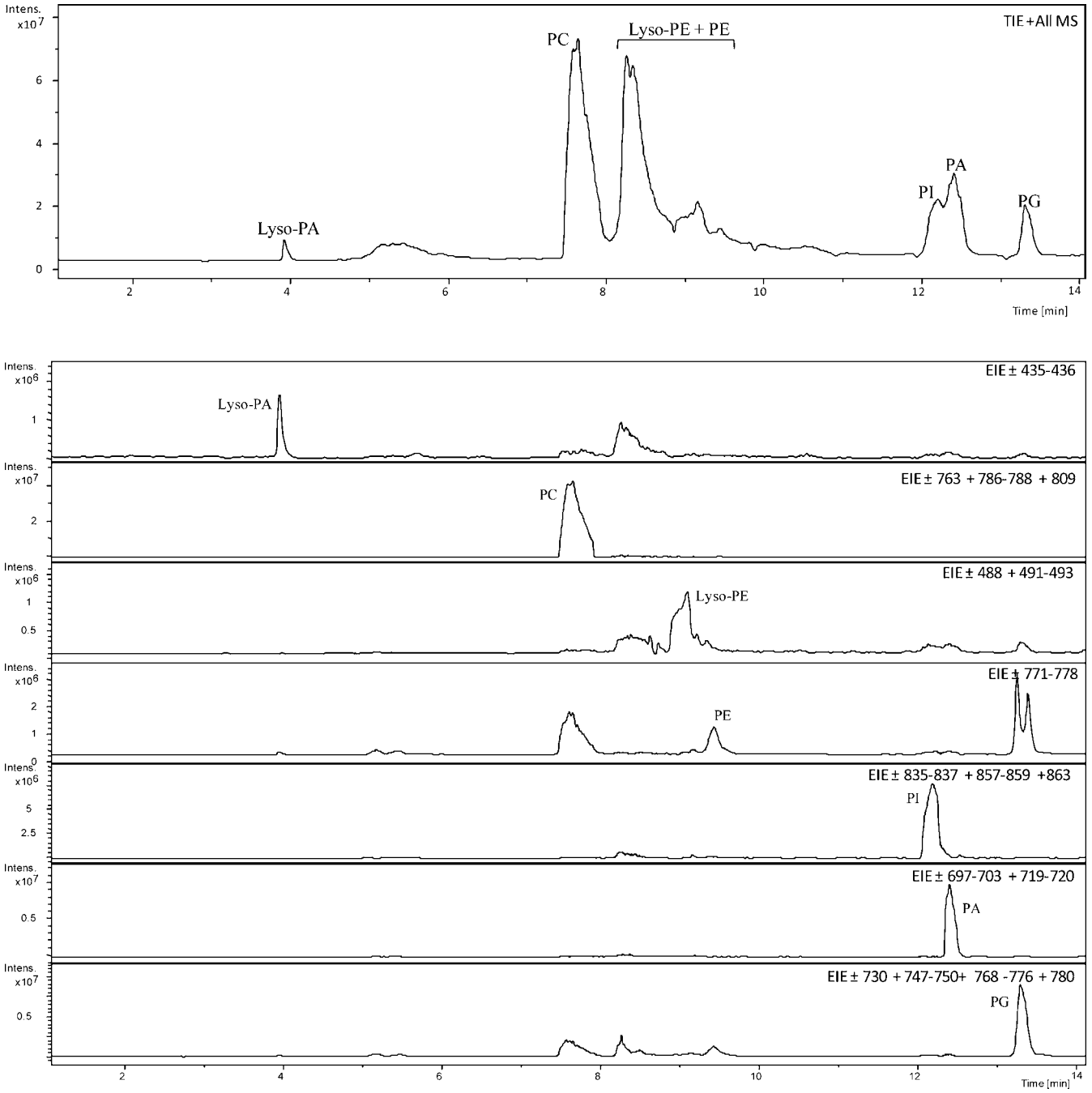


**Figure 3.** TIE of Arbequina variety from Jaén as an example of glycerophospholipid separation in the olive samples analyzed, and corresponding EIE for each phospholipid. Conditions were as in Figure 2. Peaks: lyso-PA, lysophosphatidic acid; PC, phosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol.

concentration and the complexity of the matrix, fragmentation results were not satisfactory and further investigations need to be performed.

As Table 4 shows, the results obtained for positive ionization mode showed that the protonated molecular ion, $[M + H]^+$, was the only one detected for PC and PE; other possible adducts as $[M + Na]^+$ or $[M + NH_4]^+$ were not found, as reported by Zink et al.[37] and Pulfer et al.[39] Fragment identification was carried out, taking into account the possible fragments in positive-ion mode as loss of a ketene, $[M + H - R_1CH{=}C{=}O]^+$, $[M + H - R_2CH{=}C{=}O]^+$, or loss of a fatty acid, $[M + H - R_1CH_2COOH]^+$ and $[M + H - R_2CH_2COOH]^+$.[39] In this work, only 2−5 ions for each glycerophospholipid were detected in a reliable way. PC only showed two main molecular species. The $m/z$ 788 was the most abundant ion, with a fatty acid distribution identified as 18:1/18:0, on the basis of the fragments mentioned above found among the fragmented ions and according to previous results.[4,38] The other ion, 809, was identified as 20:2/18:3, according to Lee.[40] Identification of PE and lyso-PE was more problematic due to their mentioned LOD. Only two ions could be assigned for PE: 773 to the fatty acids 20:2/18:0 and 777 to 20:1/18:2.[40]

Fragmentation in positive-ion mode was also performed for PI, PA, PG, and lyso-PA in the $MS^2$ experiments. PI and PA were observed as $[M + Na]^+$ and PG as $[M + NH_4]^+$, according


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**Table 3. Relative Abundance of Each Glycerophospholipid in Olive and Olive Oil Samples Analyzed[a]**

| | olive fruit[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| phospholipid | ATS | ATP | AT | AC | AJ | E | LS | olive oil |
| lyso-PA | 0.2 ± 0.1 | 0.3 ± 0.2 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | – | 0.1 ± 0.1 | 0.2 ± 0.2 |
| PC | 4.4 ± 0.5 | 2.0 ± 0.2 | 3 ± 1 | 6 ± 1 | 7 ± 2 | 3.1 ± 0.4 | 9 ± 2 | 15 ± 2 |
| lyso-PE | – | 29 ± 5 | 18 ± 4 | 4.1 ± 0.4 | 5 ± 1 | 1.3 ± 0.7 | 2.5 ± 0.6 | 4.5 ± 0.1 |
| PE | – | 6.0 ± 0.7 | 5.4 ± 0.7 | 5 ± 1 | 11 ± 1 | 4 ± 2 | 16 ± 1 | 42 ± 1 |
| PI | 6 ± 2 | 2.5 ± 0.6 | 4.4 ± 0.8 | 5 ± 2 | 8 ± 4 | 8 ± 1 | 6 ± 2 | – |
| PA | 88 ± 2 | 58 ± 4 | 71 ± 4 | 73 ± 4 | 65 ± 6 | 82 ± 4 | 60 ± 3 | – |
| PG | 1.2 ± 0.2 | 2.4 ± 0.4 | 3.7 ± 0.7 | 5.8 ± 0.5 | 4 ± 1 | 4.2 ± 0.3 | 6.3 ± 0.6 | 37.9 ± 0.3 |

[a]Determined as the area found divided by the response factor. [b]Obtained from two independent samples injected in duplicate ($n$ = 4). Abbreviations: ATS, stone of Arbequina variety from Toledo; ATP, pulp of Arbequina variety from Toledo; AT, Arbequina variety from Toledo; AC, Arbequina variety from Córdoba; AJ, Arbequina variety from Jaén; E, Empeltre variety from Córdoba; LS, Lechín de Sevilla variety from Córdoba.

**Table 4. Main Phospholipid Fatty Acids Obtained in MS/MS Experiments for Each Glycerophospholipid in Olive Fruit Samples Analyzed**

| precursor ion | | |
|---|---|---|
| $m/z$ | pseudomolecular ions | assignment |
| | Phosphatidylcholine | |
| 788 | $[M + H]^+$ | 18:1/18:0 |
| 809 | $[M + H]^+$ | 20:2/18:3 |
| | Phosphatidylethanolamine | |
| 773 | $[M + H]^+$ | 20:2/18:0 |
| 777 | $[M + H]^+$ | 20:1/18:2 |
| | Phosphatidylinositol | |
| 835 | $[M - H]^-$ | 16:0/18:1 |
| 837 | $[M - H]^-$ | 16:0/18:0 |
| 859 | $[M - H]^-$ | 18:2/18:1 |
| 863 | $[M - H]^-$ | 18:0/18:1 |
| | Phosphatidic Acid | |
| 701 | $[M - H]^-$ | 18:0/18:0 |
| 703 | $[M - H]^-$ | 18:1/18:0 |
| | Phosphatidylglycerol | |
| 730 | $[M - H]^-$ | 18:1/14:1 |
| 747 | $[M - H]^-$ | 16:0/18:1 |
| 749 | $[M - H]^-$ | 18:0/16:0 |
| 773 | $[M - H]^-$ | 18:1/18:1 |
| 775 | $[M - H]^-$ | 18:1/18:0 |

to the fragments found and other identifications established in literature.[38] However, negative-ion mode was evaluated for fragmentation purposes in order to simplify the information by $[M - H]^-$ ions. Identification was carried out by taking into account that the main product ions in negative-ion mode were obtained from the loss of the fatty acids, with those in position C-2 lost preferentially and with higher intensity.[37] In this mode, the same conditions as for experiments in positive-ion mode were employed, except for the use of 2.8 kV.

For the $MS^2$ experiments in negative-ion mode for PI, PA, and PG, simpler spectra were obtained because, in most cases, only two fatty acids were observed. For PI, four molecular species were mainly identified in all the olives analyzed, 835 with the combination of fatty acids 16:0/18:1, 837 as 16:0/18:0, and 859 as 18:2/18:1, according to previous results found by Pelillo et al.;[41] and 863 as 18:0/18:1.[41] In the case of PA, only two main precursor ions were fragmented: 701, corresponding to 18:0/18:0, and 703 as 18:1/18:0.[33] For PG, five molecular species were identified: 730 as 18:1/14:1, 747 as 16:0/18:1,[38] 749 as 18:0/16:0,[38] 773 with the combination of fatty acids 18:1/18:1,[38] and 775 as 18:1/18:0. Lyso-PA was not clearly fractionated, perhaps due to its low concentration in olive samples. All the results obtained are collected in Table 4.

Analysis of fatty acids of the glycerophospholipids in the olives analyzed showed that the most repeated fatty acids were stearic (18:0), oleic (18:1), and palmitic (16:0) acids. These results are mostly in accordance with those previously found in literature, in which oleic acid was the most abundant fatty acid phospholipid, followed by linoleic (18:2), palmitic, and stearic acids in olive seed[9] and olive pulp.[10] It is interesting at this point to compare the fatty acid composition in phospholipids obtained from the olive fruit samples analyzed with the information existing on fatty acid profiles in triglycerides in olive fruits. As occurs in triglycerides, where it provided more than 50% of total fatty acids,[7] oleic acid was demonstrated to be one of the most predominant fatty acid in phospholipids. Our results also suggest a high degree of saturated fatty acids, such as stearic and palmitic acids, occurring in the triglycerides of olive fruits.[42] Moreover, the predominant pair of fatty acids in the molecular species determined was 18:1/18:0, as Table 4 shows. The mentioned preference of certain combinations of fatty acids could be related to a preferable biosynthesis in olive fruits of these fatty acids and these pair combination.

In summary, for glycerophospholipid determination by the NACE-ESI-MS method, methanol/ACN with 100 mM ammonium acetate and 0.5% (v/v) acetic acid buffer allowed us to obtain the best separation efficiency with short analysis times, less than 14 min in all cases. The optimal MS and NACE conditions enabled the determination of nine different standard glycerophospholipids, including the lysophospholipids of PC, PA, and PE.

The NACE-ESI-MS method developed was applied to the determination and identification of glycerophospholipids in olive fruits and olive oil. In olive fruits, where information available about phospholipid fractions was very scarce, results obtained in this work allowed us to contribute and improve this knowledge. Results showed that PA was the main glycerophospholipid in all olive samples analyzed, followed by PE, PC, lyso-PE, PI, PG, and lyso-PA. PS and lyso-PC were not detected in the olive samples analyzed. In olive oil, although preliminary results were shown, PC, PE, and PG were determined as the main glycerophospholipids in the oil analyzed.

The results obtained from the NACE-ESI-$MS^2$ experiments allowed us to identify the main molecular species of each glycerophospholipid found in the olive samples analyzed, for the first time. The results showed that stearic (18:0), oleic (18:1), and palmitic (16:0) acids were the most abundant fatty acids detected.

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### Notes

The authors declare no competing financial interest.

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