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# Polar lipid profiling of olive oils as a useful tool in helping to decipher their unique fingerprint

Eliana Alves [a, *], Tânia Melo [a], Felisa Rey [b], Ana S.P. Moreira [a], Pedro Domingues [a], Maria Rosário Domingues [a]

[a] Mass Spectrometry Centre, Department of Chemistry & QOPNA, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[b] Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

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ABSTRACT

Olive oil (OO), a key ingredient of the Mediterranean diet, is frequently subjected to adulteration. A lipidomic approach has been used to study the lipid composition of Portuguese commercial OOs, to find out differences in their profile or a unique fingerprint, namely in the polar lipid pool. Extra virgin (EVOO, $n = 3$) and virgin olive oils (VOO, $n = 3$) were analysed. The fatty acids (FA) were analysed by GC-MS and the triacylglycerols by MALDI-TOF-MS. The polar lipid fraction was obtained through solid phase extraction and analysed by hydrophilic interaction liquid chromatography-electrospray ionization-MS. The amount of FA $C_{16:0}$ and $C_{18:1}$ was significantly different between the EVOOs and the VOOs. Both categories of OO were discriminated by the FAs $C_{18:3}$, $C_{18:2}$ and $C_{16:1}$. Five classes of phospholipids were identified in the polar lipid fraction, with significant differences in phosphatidylcholines. Besides FA, it is important to study the polar lipidome to identify minor components that could help to reveal a fingerprint associated with each OO.

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## 1. Introduction

In the Mediterranean countries, olive oil (OO) is a key component of the diet. The increase in world-wide OO consumption is due not only to a greater availability, but also due to the recognition of its positive impact on nutrition and health (Aparicio & García-González, 2013).

Despite the current standards of OO quality, that include sensory and chemical analysis, there are still neither accurate established molecular markers nor fast and unambiguous chemical indices that could be correlated with the sensory characteristics to allow proper authentication and qualification of OO (Montealegre, Marina Alegre, & García-Ruiz, 2010). Due to the importance that OO has in the human diet and in the global economy, the establishment of a well-documented traceability system for its quality control in the food chain has become a requirement.

While fatty acids (FAs) and triacylglycerols (TAGs), the most abundant components of OO, have been extensively used as markers of botanical origin (Montealegre et al., 2010), it is recognized that minor components, that include polar lipids, have greater discriminatory power than major components to decipher the botanical origin of OO. As such, their study has been suggested as useful for establishing the identity of an OO (Montealegre et al., 2010). Consequently, in recent years, the use of genomics (Agrimonti, Vietina, Pafundo, & Marmiroli, 2011) and lipidomics (European Commission Directorate General Agriculture & Rural Development, European Commission Joint Research Centre Institute for Reference Materials and Measurements, & Council, 2013) have been proposed as valuable tools for determining the genuineness of OO.

Lipidomics is useful to reveal the lipid profile of olives and OO at the molecular level, informing not only about their origin but also about their nutritional value, and the meteorological conditions to which they have been exposed (Cozzolino & De Giulio, 2011).

The polar lipids, namely phospholipids (PL), are found in small quantity in OO, around 21–124 mg $kg^{-1}$, which represents 0.01% (g/kg) (Koidis & Boskou, 2006). Their analysis is a complex task that requires efficient extraction and sensitive detection methods. The most representative classes of PL in OO have been reported but it was not possible to assign a characteristic profile (Boukhchina, Sebai, Cherif, Kallel, & Mayer, 2004). PL are key structural components of membranes and important regulatory and signalling

* Corresponding author.
*E-mail address:* elianaalves@ua.pt (E. Alves).

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molecules. Additionally, they are sources of polyunsaturated fatty acids (PUFAs), in contrast to TAG, rich in monounsaturated fatty acids (MUFAs), and have demonstrated therapeutic properties (Küllenberg, Taylor, Schneider, & Massing, 2012). On the other hand, only very recently some attempts to optimize the extraction of PL from OO using different lipidomic approaches have been reported (Shen et al., 2013; Verardo et al., 2013). Up until now, mass spectrometry has proven to be the best method for the analysis of lipids in biological samples but also in OO (Montealegre, Sánchez-Hernández, Crego, & Marina, 2013; Shen et al., 2013; Verardo et al., 2013). The polar lipid fraction of OO, in addition to containing PL, also contains glycolipids and phenolic compounds (Nomikos, Karantonis, Fragopoulou, & Demopoulos, 2002) that possess bioactive properties (Tsantila et al., 2007). The aim of this study was to establish the lipid profile of Portuguese commercial OOs, in particular, their major components (TAGs and total FAs), and their minor components (PL and glycolipids), using a lipidomic approach. With this approach we intended to find out differences in their composition that would allow unravelling a unique lipid fingerprint.

## 2. Materials and methods

### 2.1. Samples

Six samples of OO were obtained from a local supermarket. The selection criteria were based on the choice of Portuguese OOs from different brands and geographical origins (Alentejo and Trás-os-Montes), of two categories: extra virgin OO (EVOO, $n = 3$) and virgin OO (VOO, $n = 3$). For the experiments of GC-MS and MALDI-TOF-MS (section 2.3 and 2.4, respectively), samples were used directly without being subjected to extraction processes. For those experiments, stock solutions of 10 mg of OO were prepared to be re-suspended in 1 mL of chloroform before analysis. The polar lipid fraction, obtained after extraction, was analysed by HILIC-ESI-MS, as described below (sections 2.5 and 2.6).

### 2.2. Reagents

HPLC grade chloroform and methanol were purchased from Fisher Scientific Ltd. (Loughborough, UK). All other reagents (*n*-hexane, potassium hydroxide, sodium chloride, 2,5-dihydroxybenzoic acid, formic acid, ammonium hydroxide, acetonitrile and ammonium acetate) were purchased from major commercial sources. Purified water was used (Synergy®, Millipore Corporation, Billerica, MA, USA) whenever necessary.

### 2.3. Fatty acid analysis by gas chromatography-mass spectrometry (GC-MS)

The FAs in OO were analysed after transesterification. The FA methyl esters (FAMEs) (in 20 μL of the stock solution) were prepared using a methanolic solution of potassium hydroxide (2.0 mol $L^{-1}$) (Aued-Pimentel, Lago, Chaves, & Kumagai, 2004). After esterification, volumes of 2.0 μL of the hexane solution containing the FAMEs were submitted to analysis by GC–MS on an Agilent Technologies 6890 N Network (Santa Clara, CA, USA) equipped with a DB-1 column with 30 m of length, 0.25 mm of internal diameter, and 0.1 μm of film thickness (J&W Scientific, Folsom, CA, USA). This equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 40–500 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 90 °C, standing at this temperature for 1 min, a linear increase to 180 °C at 25 °C $min^{-1}$, followed by linear increase at 14.4 °C $min^{-1}$ to 220 °C, and then at 6 °C $min^{-1}$ to 250 °C. The injector and detector temperatures were 220 and 280 °C, respectively. Helium was used as carrier gas at a flow rate of 4.2 mL $min^{-1}$. The FAs were quantified according to their relative percentage by calculating the area of each peak of the chromatogram with appropriate normalization to the sum of all areas of the identified FAs.

### 2.4. Triacylglycerols analysis by matrix assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS)

For the analysis of TAGs by MALDI-TOF-MS, samples were prepared by mixing 2 μL of each stock solution of OO with 8 μL of matrix. The matrix consisted of 2,5-dihydroxybenzoic acid (DHB) dissolved in methanol:water (95:5) to reach a final concentration of 10 mg $mL^{-1}$. From each mixture of sample/matrix, 0.5 μL were deposited on a spot of the MALDI plate and allowed to dry at room temperature. The mass spectra were acquired in positive-ion full scan reflectron mode using a MALDI-TOF/TOF instrument (Applied Biosystems 4800 Proteomics Analyser, Framingham, MA, USA) equipped with a nitrogen laser emitting at 337 nm.

### 2.5. Polar lipids extraction

The polar lipid fraction was obtained by solid phase extraction (SPE) using aminopropyl columns (HybridSPE®-Phospholipid 30 mg, ref. 55261-U SUPELCO, Sigma-Aldrich). The columns were conditioned with 1 mL of acetonitrile (ACN), loaded with 1 g of OO dissolved in *n*-hexane (1:1, g/mL), washed with 3 mL of ACN:formic acid (99:1, mL/mL) and 3 mL of ACN. The elution of polar lipids was done with 5 mL of ACN:ammonium hydroxide (95:5, mL/mL) (Pinto, Maciel, Melo, Domingues, Galhano, Pita, et al., 2014). This fraction was collected, dried under vacuum and stored at −20 °C prior to analysis by HILIC-ESI-MS.

### 2.6. Polar lipids analysis by hydrophilic interaction liquid chromatography electrospray ionization mass spectrometry (HILIC-ESI-MS)

The lipid extracts obtained in the previous step were analysed on a Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA, USA) coupled to a LXQ electrospray linear ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA), as previously reported (Schwalbe-Herrmann, Willmann, & Leibfritz, 2010).

Polar lipids analysis was carried out both in positive- and in negative-ion electrospray ionization mass spectrometry (ESI-MS). The ESI-MS conditions were the following: electrospray voltage 4.7 kV in the negative-ion mode and 5.0 kV in the positive-ion mode; capillary temperature 275 °C, and the sheath gas (He) flow rate 25 (arbitrary units). A precursor ion isolation width of 0.5 *m/z* units was used, with a 30 ms activation time for $MS^2$ experiments. Full scan MS spectra and $MS^2$ spectra were acquired with a maximum ionization time of 50 ms and 200 ms, respectively. The normalized collision energy (CE) varied between 17 and 20 (arbitrary units) for $MS^2$. Data acquisition and treatment of results were carried out with the Xcalibur™ Data System 2.0 (Thermo Scientific, San Jose, CA, USA).

### 2.7. Statistical analysis

One-way analyses of variance (ANOVA) were performed using OO categories (two levels: EVOO and VOO) as fixed factor, in order to compare the relative abundance of the FA profile. Prior to analysis, normal distributions were assessed by the Shapiro test and homogeneity of variances by the Levene's test. A value of $p < 0.05$

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