1 **Degradation of the polar lipid and fatty acid molecular species in extra virgin olive**
2 **oil during storage based on shotgun lipidomics**
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4 Anna Laura Capriotti[1], Andrea Cerrato[1], Sara Elsa Aita[1], Carmela Maria Montone[1], Susy Piovesana[1], Aldo
5 Laganà[1,2,*], Chiara Cavaliere[1]
6
7 [1] Department of Chemistry, Università di Roma “La Sapienza”, Piazzale Aldo Moro 5, 00185
8 Rome, Italy
9 [2] CNR NANOTEC, Campus Ecotekne, University of Salento, Via Monteroni, 73100 Lecce, Italy
10
11
12 ***Corresponding author**
13 Aldo Laganà
14 Department of Chemistry, Università di Roma “La Sapienza” Piazzale Aldo Moro 5 00185 Rome,
15 Italy
16 E-mail: aldo.lagana@uniroma1.it
17 tel: +39 06 4991 3679
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27 **Abstract**
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29 Among the bioactive compounds present in extra-virgin olive oil, polar lipids and free fatty acids are
30 minor compounds with well-known nutritional values and have been studied for traceability and
31 adulteration investigations as well. In the present paper, the simultaneous characterization of polar
32 lipids and free fatty acids in a pool of fifteen EVOO samples was achieved by means of reversed
33 phase C18 analysis coupled to negative polarity high-resolution mass spectrometry. A total of 24
34 polar lipids, comprising 19 phospholipids and 5 sulfolipids, and 27 free fatty acids were tentatively
35 identified, including several odd-chain and very long-chain fatty acids at trace levels. Moreover, a
36 one-month study of lipid degradation on simulated storage conditions was carried out thanks to the
37 set-up of a dedicated approach for degradation product analysis which was implemented of
38 Compound Discoverer software. By virtue of the customized data processing workflow, more than
39 forty compounds were tentatively identified, including compounds deriving from hydrolysis and
40 oxidation reactions. Finally, by analysis of peak area trends, phosphoester hydrolyses of polar heads
41 of phospholipids emerged as the fastest reactions, followed by glycerol ester hydrolyses and oxidative
42 processes.
43
44 **Keywords**
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46 Extravirgin olive oil; phospholipids; free fatty acids; lipid degradation; lipidomics; Compound
47 Discoverer
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1. Introduction

Extra-virgin olive oil (EVOO) is one of the most distinctive food products in the Mediterranean and Middle East regions, where it still represents one of the main sources of vegetable fat. However, due to olive oil well-recognized benefits on human health and sensorial properties, nowadays its consumption is expanding to other European Union (EU) and extra-EU countries [1]. According to EU law definition, EVOO is a superior category olive oil obtained directly from olives (*Olea europea* l. drupes) and solely by mechanical means, satisfying specific organoleptic and acidity (< 0.8%) characteristics. This process allows to maintain in the final product most of the original fruit components. Indeed, EVOO differs from the other vegetable oils for its unique composition, including antioxidants, monounsaturated fatty acids (MUFAs) and volatile substances [2]. This high MUFA component, mainly due to oleic acid, makes EVOO less prone to oxidation than other vegetable oils containing higher amount of polyunsaturated fatty acids (PUFAs) [1].

Besides the major component (ca. 98%) constituted by triacylglycerols (TAGs), some minor compounds, such as chlorophylls, carotenoids, phenolic compounds (mainly hydroxytyrosol, tyrosol, oleuropein and their derivatives), terpenoids, phospholipids, and diacylglycerols (DAGs), are also considered important for EVOO authentication and quality assessment. The quality and quantity of these minor compounds can differ by olive variety, growth conditions, and storage conditions [3]. In particular, phospholipids (PLs) are a wide class of amphiphilic substances characterized by a polar head and one or two fatty acyl chains. According to the hydrophilic part of the molecule, PLs are, in turn, divided into phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidylserines (PSs), phosphatidylinositols (PIs), phosphatidylglycerols (PGs), and phosphatidic acids (PAs)[4]. They show important healthy properties, such as antithrombotic[5], anti-atherosclerotic and anti-inflammatory functions, as well as the role in reducing the risk of cardiovascular disease and blood cholesterol levels[6]. Moreover, the composition of the fatty acyl chains in the PL structure plays a key role in several biological functions, as antioxidant activity, memory increase and immunological

79 properties, as described in recent studies[7]. For these reasons, there has been a growing interest in
80 the study of PLs in food matrices[8]. During manufacturing and storage, PLs, particularly PCs and
81 PGs may potentially be degraded when exposed to excess moisture, heat, and light. Hydrolysis of
82 ester bonds due to the moisture releases long-chain free fatty acids (FAs). For instance, PCs can be
83 hydrolyzed to lysophosphatidylcholines (LPCs) and free fatty acids (FFAs), whereas
84 lysophosphatidylglycerols (LPGs) and FFAs are formed by the hydrolysis of PGs[9]. It is also known
85 that lipids are susceptible to oxidation, which alters both the sensory and health-promoting qualities,
86 leading to the generation of low-molecular-weight off-flavor substances, loss of antioxidants, and
87 accumulation of free radicals[2]. Oxygen, temperature and light are the main factors that can affect
88 PL oxidative stability, while natural antioxidants and oleic acid can preserve it[2]. Studies on PL
89 degradation during processing and storage have been reported in the literature, i.e. in poultry[10],
90 livestock[11], salted duck[12], fish[13], milk[14], egg yolk[15], and krill[16]. However, while several
91 studies have been addressed to meat and derivatives, there are few studies investigating the changes
92 in the PL molecular species in vegetable matrices. Similarly, while several EVOO profiling of FAs
93 have been reported, their degradation has not been extensively studied[17–19]. At present, the are in
94 fact no studies specifically focused on the degradation of PLs and FAs in EVOO, whereas some
95 studies have been carried out on degradation phenomena after frying processing in oils[20,21].
96 Moreover, the evolution of EVOO quality and shelf-life parameters has been investigated under
97 storage conditions, mainly for long periods[22,23]. Even if the results of these studies have
98 contributed to a greater understanding of the autoxidation process, the prediction of the shelf life of
99 EVOO and the exact role of its PL components are still a prime research objective.
100 The aim of this work was therefore to characterize PLs and FAs and their degradation products in
101 EVOO samples during simulated storage condition. Lipids were extracted by liquid-liquid extraction
102 (LLE) and their analysis was carried out by means of ultra-high-performance liquid chromatography
103 (UHPLC) in combination with high resolution mass spectrometry (HRMS). Subsequent identification
104 of PLs was performed using the recently developed software Lipostar[24,25], by exploiting a proper

105 database for vegetable lipids. FAs were identified by Compound Discoverer software using a
106 predefined workflow for food analysis. Finally, hydrolysis and oxidation products were also
107 putatively identified by Compound Discoverer thanks to a customized workflow specifically
108 perfected for the purpose.
109
110 **2. Materials and methods**
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112 **2.1. Chemicals and materials**
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114 HPLC-grade chloroform and methanol (MeOH) were acquired from VWR International (Milan,
115 Italy), whereas dichloromethane (DCM) and ethanol (EtOH) were from Carlo Erba Reagents (Milan,
116 Italy). Ultra-pure water and methanol of LC-MS grade were purchased from Thermo Fisher Scientific
117 (Waltham, MA, USA) and Romil Pure Chemistry (Pozzuoli, NA, Italy), respectively. Ammonium
118 formate, formic acid, trifluoroacetic acid (TFA), hydrochloric acid and tetramethylammonium
119 chloride (TMACl) were provided by Sigma (St. Louis, MO, USA). 1-pentadecanoyl-2-oleoyl(d7)-sn-
120 glycero-3-phosphate (sodium salt) was provided by Avanti Polar Lipids (Alabama, USA).
121
122 **2.2. EVOO sampling and storage**
123
124 Fifteen EVOO samples from various Italian regions were collected within few days from production,
125 and a pooled sample was obtained by an aliquot of each sample. To simulate standard storage
126 conditions, the pooled sample was stored in an amber glass bottle at room temperature for 28 days.
127 Aliquots of the pooled samples were analyzed on day 1 (T1), day 2 (T2), day 3 (T3), day 6 (T4), day
128 10 (T5), day 15 (T6), day 21 (T7) and day 28 (T8) of storage.
129

130 **2.3. Polar lipid extraction**

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132 EVOO samples were handled as previously described [4]. Briefly, 1 g of EVOO was dissolved in 4
133 mL of n-hexane and then added with 1 mL of EtOH/H_2O (80:20, *v/v*). Then, the sample was vortex-
134 mixed for 15 min, put in an ultrasound bath at room temperature for 15 min and finally centrifuged
135 at 3000 ×g for 15 min. The upper hydro-alcoholic layer containing polar lipids and fatty acids was
136 collected, the whole extraction was repeated twice and eventually dried up for solvent removal to 50
137 µL. Finally, the extracted PLs were reconstituted at 100 µL to obtain a final solvent composition of
138 MeOH/H_2O/$CHCl_3$, 80:15:5 (*v/v/v*). For peak areas normalization, 1-pentadecanoyl-2-oleoyl(d7)-sn-
139 glycero-3-phosphate was added to all samples at concentration of 1 mg L^{-1}.

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141 **2.4. UHPLC-MS/MS analysis**

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143 The UHPLC apparatus consisted in a Vanquish chromatograph equipped with a binary pump,
144 equipped with thermostated column compartment and autosampler (Thermo Fisher Scientific,
145 Bremen, Germany). Analytes were separated by a C18 Kinetex EVO chromatographic column (100
146 × 2.1 mm, 1.7 µm particle size; Phenomenex, Torrance, CA, USA). Mobile phases were constituted
147 by water (A) and MeOH/isopropanol 80/20 (*v/v*) (B) both containing 3 mmol acetic acid and 5 mmol
148 L^{-1} ammonium acetate. The gradient was as follows: 60-70% phase B in 5 min, 70-99% phase B in
149 25 min; at the end of the gradient a washing step at 99% phase B for 5 min and a re-equilibration step
150 at 60% phase B for 10 min were performed. The chromatographic system was coupled to a Q Exactive
151 hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) via a
152 heated ESI (HESI) source. The HESI source was operated in negative polarity with the following
153 parameters: spray voltage 2500 V; capillary temperature 320 °C; auxiliary gas was set at 15 arbitrary
154 units (a.u.); auxiliary gas heater temperature 400 °C; sheath gas 35 a.u.; S-lens RF level was 100%.
155 Detection was conducted in TOP 5 data dependent acquisition mode, with a resolution of 140,000

156 and 70,000 for MS and MS/MS, respectively. Higher-energy collisional ionization was performed at
157 40% normalized collision energy (NCE). All samples were run in triplicate analysis and acquired by
158 Xcalibur software (version 3.1, Thermo Fisher Scientific).
159
160 **2.5. Polar lipid identification**
161
162 Lipid identification was accomplished by the software for lipidomics Lipostar[24]. Dealing with plant
163 polar lipids, Lipostar DB manager was used to build a customized database of vegetable polar lipids,
164 including PLs, glycolipids and sulfolipids, as described in a previous work[4]. More specifically,
165 Lipid Builder Tool was employed to build the lipid database by combining the polar head groups of
166 lipid classes reported for plant matrices with fatty acyl chains, including odd chains species[26,27].
167 Moreover, *in silico* simulated MS/MS spectra of all included polar lipids were obtained with the
168 implementation of the largely studied fragmentation rules[28,29]. A preprocessing of the
169 chromatograms by applying baseline and noise reduction, peak extraction, leveling, signal-to-noise
170 ratio filter, deisotoping and deconvolution, was carried out according to the parameters reported in
171 Table S1. Extracted features were eventually matched to the customized database with a tolerance of
172 5 ppm and 10 ppm respectively for precursor and product ions. Filtered features were finally manually
173 checked.
174
175 **2.6. Fatty acid identification**
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177 FA identification was carried out by Compound Discoverer™ 3.1 (Thermo Fisher Scientific,
178 Waltham, MA, USA) using the Food Research workflow template with slight modifications. After
179 spectra selection and alignment and compound detections, adducts are grouped and blank signals are
180 removed. *Fill Gaps* tool was also enabled, with the purpose of granting a better evaluation of peak
181 areas. Spectra matching was performed against FooDB and Lipid Maps databases with a mass

182 tolerance of 5 ppm. *Apply Spectral Distance* tool, which provides a ranking for compound annotation
183 based on isotopic pattern comparison, was also enabled. Finally, *Apply mzLogic* tool, which provides
184 a ranking for annotation for unknown compounds based on MS/MS data, was also set up. Detailed
185 Compound Discoverer workflows and parameters are available in Figure S1 and Table S2.
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187 **2.7. Identification of hydrolysis and oxidation products of polar lipids and fatty acids**

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189 With the purpose of gaining knowledge on the degradative processes of polar lipids and FAs during
190 storage, a customized data processing workflow was set up on Compound Discoverer based on the
191 *Expected Compounds* tool. For polar lipid degradation products, the ten most abundant polar lipid
192 species identified by Lipostar, i.e. 16:0-18:1 PA, 16:0-18:2 PA, 18:1-18:2 PA, 16:0-18:1 PG, 16:0-
193 18:2 PG, 18:1-18:1 PG, 16:0-18:1 PI, 16:0-18:2 PI, 16:0-18:1 sulfoquinovosyl diacylglycerol
194 (SQDG) and 18:1-18:1 SQDG, were selected and their structures were implemented in the *Generate*
195 *Expected Compounds* tool, while in the case of FAs, all the identified structures were implemented.
196 Some transformations were selected among the default ones, while others, such as loss of fatty acyl
197 chains, glycerol and inositol, were manually implemented. A maximum of three modifications were
198 considered and dealkylation and dearylation tools were not enabled. After spectra selection and
199 alignment from the raw data files, expected compounds were searched with a mass tolerance of 5
200 ppm and a minimum peak intensity of 20000. Regular detected compounds and found expected
201 compound were then merged, since some of the transformation products could have already been
202 reported in LipidMAPS database. FISh scoring tool, which provides a score for the detected expected
203 compounds and annotates related MS/MS scans by fragment ion search, was also enabled. Detailed
204 Compound Discoverer workflows and parameters are available in Figure S2 and Table S3.
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206 **3. Results and Discussion**

207

208 **3.1 Profiling of the polar lipid molecular species in EVOO samples**

209

210 Although PLs are minor components of EVOO, their presence may affect oil oxidative stability[30]
211 and, therefore, their identification represent a crucial issue in the characterization of such commonly
212 employed food. In an earlier study, in which UV was used for PL detection, PCs and PEs were
213 described as the most abundant subclasses[31]. However, more recent papers, in which lipid detection
214 was achieved thanks to higher performance instrumentation, such as NMR and HRMS, have shown
215 completely different results[4,32–34], with PAs emerging as the major components of EVOO
216 phospholipidome. In the present paper, polar lipids have been identified by means of UHPLC coupled
217 to ESI-HRMS in negative polarity, followed by raw data processing by Lipostar and manual
218 validation of the compounds. HRMS allows the discrimination of the polar heads thanks to specific
219 product ions. Moreover, negative polarity was chosen over positive polarity, since the former grants
220 the presence of fatty acyl chain fragments in the MS/MS spectra[35]. A total of 24 polar lipids have
221 been tentatively identified in the EVOO pool on T1 (day 1) and are reported in Table 1 and in
222 Supplementary Material Table S4. Tentative identification was achieved by matching the
223 experimental MS/MS product ions to the expected fragments predicted by the well-known
224 fragmentation rules for determining both the polar heads and the fatty acyl chains. In Figure 1, the
225 total peak areas of the major subclasses of polar lipids are reported. Total peak areas of each polar
226 lipid subclass were calculated by sum of the average normalized peak areas of each component of the
227 subclass. It must be noted that ionization efficiency is highly depending on the physico-chemical
228 properties of the polar heads; PAs and SQDGs, for example, present a free acid group, which is very
229 likely responsible for a boost in the ionization, while PCs, PEs and PSs possess a positively charged
230 amine group, which could hinder their ionization in negative polarity. Moreover, as several polar
231 lipids coelute at the same retention time, a certain degree of competition for ionization is expected
232 and ion suppression phenomena are also likely to occur in untargeted analyses. Given the above, the
233 results are still interesting, as they are in agreement with a previous work in which PLs in olive oil

234 were identified by means of ^{31}P NMR spectroscopy, which is not affected by the acid-base properties

235 of the polar heads[33]. PAs are by far the most abundant polar lipids in EVOO with over 60% of the

236 total peak area, followed by PGs and PIs. Sulfoquinovosyl diacylglycerols (SQDG), a subclasses of

237 non-phosphorus polar lipid that is unique to vegetable matrices, are often neglected, although are

238 present in high concentration and have shown anti-inflammatory, immunosuppressive, antiviral and

239 anti-carcinogenic activities[36–39].

240

241 **Table 1**

242 Tentatively identified polar lipids in EVOO samples at T1 (day 1)

Polar lipid	Molecular Formula	RT	Adduct	Major product ions
16:0-18:3 PA	$C_{37}H_{67}O_8P$	18.4	$[M-H]^-$	78.9587, 96.9695, 152.9959, 171.0065, 255.2329, 277.2172
16:1-18:2 PA	$C_{37}H_{67}O_8P$	18.0	$[M-H]^-$	78.9587, 96.9695, 152.9959, 171.0065, 253.2172, 279.2329
16:0-18:2 PA	$C_{37}H_{69}O_8P$	19.4	$[M-H]^-$	78.9586, 96.9694, 152.9958, 171.0064, 255.2329, 279.2329
16:0-18:1 PA	$C_{37}H_{71}O_8P$	20.6	$[M-H]^-$	78.9586, 96.9695, 152.9959, 171.0065, 255.2329, 281.2485
18:2-18:3 PA	$C_{39}H_{67}O_8P$	17.7	$[M-H]^-$	78.9587, 96.9695, 152.9959, 171.0066, 277.2172, 279.2329
18:2-18:2 PA	$C_{39}H_{69}O_8P$	18.7	$[M-H]^-$	78.9586, 96.9694, 152.9958, 171.0063, 279.2329
18:1-18:2 PA	$C_{39}H_{71}O_8P$	19.7	$[M-H]^-$	78.9586, 96.9695, 152.9959, 171.0064, 279.2329, 281.2485
16:0-16:0 PA	$C_{38}H_{75}O_{10}P$	19.4	$[M-H]^-$	78.9586, 152.9959, 171.0065, 227.0331, 255.2329
16:0-18:2 PC	$C_{42}H_{80}NO_8P$	20.2	$[M-CH_3]^-$	78.9586, 96.9694, 152.9958, 168.0431, 224.0696, 255.2329, 279.2329
18:1-18:1 PE	$C_{41}H_{78}NO_8P$	18.7	$[M-H]^-$	78.9586, 152.9958, 281.2485
16:0-18:2 PG	$C_{40}H_{75}O_{10}P$	18.9	$[M-H]^-$	78.9586, 152.9959, 171.0065, 209.0222, 227.0329, 255.2329, 279.2329
16:0-18:1 PG	$C_{40}H_{77}O_{10}P$	19.9	$[M-H]^-$	78.9586, 152.9959, 171.0065, 227.0329, 255.2329, 281.2485
18:1-18:2 PG	$C_{42}H_{77}O_{10}P$	19.3	$[M-H]^-$	78.9586, 96.9694, 152.9958, 171.0065, 227.0329, 279.2329, 281.2485
18:1-18:1 PG	$C_{42}H_{79}O_{10}P$	20.0	$[M-H]^-$	78.9587, 96.9695, 152.9959, 171.0065, 227.0330, 281.2485
16:0-18:3 SQDG	$C_{43}H_{76}O_{12}S$	17.4	$[M-H]^-$	80.9648, 164.9863, 206.9970, 225.0077, 255.2329, 277.2172
16:0-18:2 SQDG	$C_{43}H_{78}O_{12}S$	18.8	$[M-H]^-$	80.9648, 164.9862, 206.9969, 225.0076, 255.2329, 279.2329
16:0-18:1 SQDG	$C_{43}H_{80}O_{12}S$	19.9	$[M-H]^-$	80.9647, 164.9862, 206.9969, 225.0075, 255.2329, 281.2485
16:0-18:3 PI	$C_{43}H_{77}O_{13}P$	17.3	$[M-H]^-$	78.9586, 96.9695, 152.9959, 223.0015, 241.0122, 259.0229, 297.0387, 315.0493, 255.2329, 277.2172
16:0-18:2 PI	$C_{43}H_{79}O_{13}P$	18.4	$[M-H]^-$	78.9587, 96.9695, 152.9959, 223.0016, 241.0123, 259.0229, 297.0386, 315.0492, 255.2329, 279.2329
16:0-18:1 PI	$C_{43}H_{81}O_{13}P$	19.4	$[M-H]^-$	78.9586, 96.9695, 152.9959, 223.0016, 241.0122, 259.0228, 297.0385, 315.0493, 255.2329, 281.2485
18:1-18:1 SQDG	$C_{45}H_{80}O_{12}S$	18.7	$[M-H]^-$	80.9648, 164.9863, 206.9970, 225.0076, 281.2485
16:0-20:1 SQDG	$C_{45}H_{82}O_{12}S$	19.7	$[M-H]^-$	80.9648, 164.9863, 206.9970, 225.0076, 255.2329, 309.2798

18:1-18:2 PI	$C_{45}H_{81}O_{13}P$	19.2	$[M-H]^-$	78.9586, 96.9694, 152.9959, 223.0015, 241.0122, 259.0229, 297.0388, 315.0493, 281.2485, 279.2329
18:1-18:1 PI	$C_{45}H_{83}O_{13}P$	20.5	$[M-H]^-$	78.9586, 96.9694, 152.9958, 223.0014, 241.0121, 259.0227, 297.0383, 315.0491, 281.2485

243

244 As regards the fatty acyl chains in the tentatively identified polar lipids, palmitic (16:0), oleic
245 (18:1), linoleic (18:2) and linolenic (18:3) acids were the most common, in agreement with what
246 was already reported for olive oil both in terms of free and bound FAs[34,40].

247

248 **3.2 Profiling of free fatty acids in EVOO samples**

249

250 FFAs are major components of all vegetable oils, which constitute a fingerprint of the oil origin[41],
251 and are therefore used for testing oil adulteration[42]. The chemical nature of FFA has made them
252 simultaneously appealing and tricky for both gas-chromatography (GC) and LC. FFA are, in fact,
253 extremely non-polar, due to their long aliphatic chains, but also non-volatile, due to the carboxyl
254 group. Silylation prior to GC analysis, which highly enhances their volatility, has long been the
255 foremost technique for FFA analysis[43,44], while some derivatization-free GC platforms have been
256 more recently reported[45]. On the other hand, despite granting derivatization-free analyses[46], LC
257 platforms have to deal with the extreme hydrophobicity of these compounds. Kamphorst at al.[47]
258 have demonstrated that the use of less hydrophobic RP-C8 stationary phase assured the elution of
259 FAs up to 36 carbon atoms. In the present paper, the use of isopropanol/methanol solvent mixture,
260 together with the addition of both acetic acid and ammonium acetate has granted the separation of
261 endogenous FAs up to 30 carbon atoms by RP-C18 chromatography (Table 2 and S5). Moreover, our
262 HPLC approach has the advantage of simultaneously analyzing polar lipids and FFAs, with the result
263 of faster and less expensive analyses. As regards MS detections of these compounds, metalated
264 adducts obtained by fast atom bombardment MS in positive polarity are known to generate the richest
265 MS/MS spectra, to the extent that even the position of double bonds in the hydrocarbon skeleton
266 could be correctly attributed[48]. However, since such extreme conditions are impossible to replicate

267 in HPLC coupled to orbitrap-MS instrumentation, positive adducts are seldom generated and negative
268 polarity must be employed for higher ionization efficiency. Saturated FAs (SFAs) present rather
269 scarce fragmentation, since the extreme instability of saturated carbanions hinders further
270 fragmentations than water loss; MUFAs and PUFAs generate richer spectra due to the stabilizing
271 effect of double bonds[49]. Other than MS, chromatographic data can also help the attribution, since
272 FFA retention times in RP-C18 analysis increase with the number of C atoms in the aliphatic chain
273 (14:0 < 16:0 < 18:0) and decrease with the number of insaturations, given the same number of atoms
274 in the chain (18:2 < 18:1 < 18:0).
275 A total of 27 FFAs was tentatively identified in the EVOO pool on T1, comprising 15 SFAs, 7
276 MUFAs and 5 PUFAs. Relative abundances of the reported compounds are perfectly in line with the
277 reference values formulated by the International Olive Council for EVOO (COI/T.15.2013). even
278 though validation was not performed and matrix effects were not calculated for the tentatively
279 identified FFAs. This phenomenon could be easily attributed to the extreme structural consistency of
280 FFAs, which present only ionizable carboxylic group. Compared to previous papers[17–19], a larger
281 number of minor components was reported, including several odd numbered and very long chain
282 FFAs. Although their contribution to EVOO nutritional value and organoleptic properties is
283 negligible, such minor components assume critical importance for evaluating adulteration of EVOO
284 with cheaper vegetable oil[42].
285 An exemplary base peak chromatogram is reported in Supplementary Material Figure S3.
286
287 **Table 2**
288 Tentatively identified free fatty acids (FFAs) in the EVOO pool at T1 (day 1). Relative abundances
289 are calculated on peak areas.
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291
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FFA (systematic name)	FFA (trivial name)	Molecular Formula	RT	Abundance	EVOO[a]
Octadecenoic acid (18:1)	Oleic acid	$C_{18}H_{34}O_2$	9.4	63.5%	55.0-83.0%
Hexadecanoic acid (16:0)	Palmitic acid	$C_{16}H_{32}O_2$	8.7	18.6%	7.5-20.0%
Octadecadienoic acid (18:2)	Linoleic acid	$C_{18}H_{32}O_2$	7.7	8.9%	3.5-21.0%
Octadecanoic acid (18:0)	Stearic acid	$C_{18}H_{36}O_2$	11.9	3.5%	0.5-5.0%
Hexadecenoic acid (16:1)	Palmitoleic acid	$C_{16}H_{30}O_2$	6.7	2.4%	0.3-3.5%
9,12,15-octadecatrienoic acid (18:3)	α-Linolenic acid	$C_{18}H_{30}O_2$	5.9	1.0%	≤1.0%
Icosenoic acid (20:1)	Gadoleic acid	$C_{20}H_{38}O_2$	12.5	0.5%	≤0.5%
Eicosanoic acid (20:0)	Arachidic acid	$C_{20}H_{40}O_2$	15.0	0.3%	≤0.6%
Docosenoic acid (22:1)	Brassidic acid	$C_{22}H_{42}O_2$	15.5	0.2%	
Tetracosanoic acid (24:0)	Lignoceric acid	$C_{24}H_{48}O_2$	20.2	0.1%	≤0.2%
6,9,12-octadecatrienoic acid (18:3)	γ-Linolenic acid	$C_{18}H_{30}O_2$	6.7	0.1%	
Docosanoic acid (22:0)	Behenic acid	$C_{22}H_{44}O_2$	17.8	0.1%	≤0.2%
Heptadecanoic acid (17:0)	Margaric acid	$C_{17}H_{34}O_2$	10.3	0.1%	≤0.4%
Octadecatraenoic acid (18:4)	Stereidonic acid	$C_{18}H_{28}O_2$	5.3	0.1%	
Octacosanoic acid (28:0)	Montanic acid	$C_{28}H_{56}O_2$	23.9	0.1%	
Hexadecadienoic acid (16:2)		$C_{16}H_{28}O_2$	5.3	0.1%	
Hexacosanoic acid (26:0)	Cerotic acid	$C_{26}H_{52}O_2$	22.3	0.1%	
Pentadecenoic acid (15:1)		$C_{15}H_{28}O_2$	5.8	<0.1%	
Tetradecanoic acid (14:0)	Myristic acid	$C_{14}H_{28}O_2$	5.9	<0.1%	≤0.03%
Heptadecenoic acid (17:1)		$C_{17}H_{32}O_2$	7.8	<0.1%	≤0.6%
Nonadecanoic acid (19:0)		$C_{19}H_{38}O_2$	13.4	<0.1%	
Pentacosanoic acid (25:0)	Pentacosylic acid	$C_{25}H_{50}O_2$	21.3	<0.1%	
Tricosanoic acid (23:0)	Tricosylic acid	$C_{23}H_{46}O_2$	19.0	<0.1%	
Henicosanoic acid (21:0)	Heneicosylic acid	$C_{21}H_{42}O_2$	16.3	<0.1%	
Heptacosanoic acid (27:0)	Heptacosylic acid	$C_{27}H_{54}O_2$	23.1	<0.1%	
Triacontanoic acid (30:0)	Melissic acid	$C_{30}H_{60}O_2$	25.6	<0.1%	
Nonadecenoic acid (19:1)		$C_{19}H_{36}O_2$	10.9	<0.1%	

293 [a] Reference value of extra virgin olive oil formulated by International Olive Council (COI/T.15.2013).

294

295 **3.3 Dynamic changes in polar lipids and fatty acids molecular species during simulated storage**

296 **condition**

297

298 The study on the changes in lipid species during storage is essential to improve the knowledge of

299 EVOO and to further elucidate the complicated mechanisms of lipid degradation, flavor formation,

300 and eventual harmful substance formation[12]. However, while some recent papers have been

301 studying lipid degradation in meat processing[10–16], there are few papers dealing with this topic in

302 oil matrices and are all correlated to frying processes[20,21]. With the purpose of evaluating the

303 process of lipid degradation during storage, 15 EVOO samples were pooled, stored in an amber glass

304 bottle, and then analyzed on 8 time points during the course of a month (T1-T8). Peak area
305 normalization was achieved by use of a labeled PA as internal standard. As Lipostar software allows
306 automatic peak area normalization if retention time and *m/z* of the internal standard are implemented
307 in the data processing workflow, normalized peak areas were used for studying the trends over time.
308 The trends of the four main classes of identified polar lipids (PAs, PGs, PIs, and SQDGs) are shown
309 in Figure 2. PGs, PIs and SQDGs follow a similar trend, with a progressive decrease of the total peak
310 area from T1 (day 1) to T8 (day 28), with SQDG and PG molecular species being the most and least
311 stable over time, respectively. This is in agreement with a previous found[12], in which PGs are
312 proven to be less stable than PIs and PSs. PAs, instead, present a peculiar trend, with an earlier
313 increase (for T1 to T3) before following the path of the other classes. This phenomenon could be
314 easily explained by hydrolysis reactions occurring to PG and PI polar heads; phosphoester hydrolysis
315 and the consequential loss of glycerol or inositol, in fact, lead in both cases to PA molecular species.
316 The increase of PAs in the first time points can demonstrate that phosphoester hydrolyses are faster
317 than other degradation processes which those species undergo over time.
318
319 With the purpose of getting more knowledge on the degradation processes, the search of possible
320 degradation products was achieved thanks to a customized workflow specifically set up on Compound
321 Discoverer. The ten most abundant polar lipids that were tentatively identified on T1 along with the
322 tentatively identified FAs were implemented to the workflow by means of the *Generate Expected*
323 *Compounds* tool. Hydration, oxidation, saturation and desaturation were chosen among the default
324 transformation, while loss of glycerol, loss of inositol, loss of 16:0, 18:1 and 18:2 FAs were manually
325 implemented considering the structures of the compounds in study. Raw data files were therefore re-
326 processed, and the extracted expected compounds were manually validated. The list of the tentatively
327 identified expected compounds alongside the putative precursor molecule(s) is available in
328 Supplementary Material Table S6. Since several structures and reaction pathways were considered,
329 different reactions on different compounds could lead to the same degradation products. As the exact

330 reactions occurring are not known, all the different parent structures were reported in Table S6,
331 regardless of the likelihood of the reactions. Twenty molecular species deriving from polar lipids,
332 including three PAs and 21 compounds deriving from FAs, were tentatively identified thanks to our
333 approach.

334 Among the compounds deriving from polar lipids, lysphospholipids (LPL) and sulfoquinovosyl
335 monoacylglycerols (SQMG) represent the largest class both in terms of number of identifications and
336 peak areas. Hydrolysis of one of the two glycerol esters of diacyl lipid species generates lyso-forms
337 and FFAs.

338 In Figure 3, the trends of LPLs and SQMGs are shown. Lysophosphatidic acids (LPAs) dramatically
339 increase from T1 to T4 before more slowly decreasing. As a matter of fact, it is not until day 21 (T7)
340 that LPA peak areas fall again under that of T1. LPGs, lysophosphatidylinositol (LPI) and SQMG
341 total peak areas, instead, stayed pretty stable from T1 to T3 before eventually starting decreasing,
342 with LPG once again decreasing more rapidly than the other two classes.

343

344 For evaluating the contribution of both phosphoester and glycerol ester hydrolyses, the individual
345 trend of 16:0-18:1 PG was investigated (Figure 4). From T1 to T3, its PA analogous rapidly increases,
346 while equally quickly decreasing from T3 to T5. On the contrary, 18:1 LPG, one of the two lysoforms
347 deriving from the PG in study, slowly and continuously increases from T1 to T4 and just starts
348 decreasing from T4 to T5. Those results could indicate that phosphoester hydrolysis was, in fact,
349 faster than glycerol ester hydrolysis on PG species. Moreover, due to the fast degradation of PA,
350 alongside the rapid increase of LPAs, glycerol ester hydrolyses on PA molecular species could be
351 faster than those on PGs.

352

353 Oxidation products, on the other hand, were far less abundant, probably due to the scarce number of
354 insaturations on the fatty acyl aliphatic chains. However, these results could also indicate that
355 oxidative processes are slower than hydrolysis reactions during storage, since all oxidized polar lipids

356 were PA derivatives. Further studies should be performed for a better understanding of the kinetics
357 of these two main degradation pathways.
358 As regards FFA degradation products, 21 oxidized compounds were tentatively identified, all of
359 which but one were mono hydroxylated species (Table S6). FFA dynamic changes are summarized
360 in Figure S4. Their growth over time can be easily explained by hydrolysis reactions occurring to
361 high abundance triacylglycerols, similarly of what has been previously described for polar lipids.
362 However, the curve representing oxidized FAs grows noticeably more rapidly. This trend could be
363 explained by oxidation of FFA rather than hydrolysis of oxidized lipids, once again demonstrating
364 that oxidation processes on EVOO polar lipids are probably slower than hydrolysis reactions during
365 storage.
366
367 **4. Conclusions**
368
369 In the present paper, a simultaneous characterization of PLs and FFAs in EVOO was achieved thanks
370 to a dedicated approach based on RP-C18 HPLC coupled to negative polarity HRMS. For the first
371 time, the described approach allowed the tentative identification of several very long chain FFAs at
372 trace concentration, which are of great interest for studies on traceability and adulteration of EVOO
373 samples. A one-month study of PLs and FFAs degradation after simulated storage conditions was
374 carried out. PL and FFA trends over time were evaluated and their degradation products were
375 searched thanks to a dedicated data analysis approach implemented on Compound Discoverer
376 software, which allowed prediction of expected compounds deriving from the previously identified
377 PLs and FFAs. The results indicate that hydrolysis reactions are the main degradation pathways of
378 PLs during storage, while oxidative reactions assume a smaller role in early days storage. Among the
379 hydrolysis reactions, phosphoester hydrolyses seem to be faster than glycerol ester ones. Obviously,
380 further studies are needed to evaluate the kinetics of such reactions and compare PL degradation
381 pathway in storage conditions to others, such as frying processes.

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383

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386 VIOLIN"]

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554
555

556 **Figure 1.** Pie chart reporting the total peak areas of the major subclasses of polar lipids tentatively

557 identified in the EVOO pool at T1 (day 1).

558

559 **Figure 2.** Dynamic changes of phosphatidic acid (PA), phosphatidylglycerol (PG),

560 phosphatidylinositol (PI) and sulfoquinovosyl diacylglycerol (SQDG) molecular species during

561 storage. Normalized total peak areas of each class are reported in logarithmic scale with standard

562 deviation obtained by nine technical replicates from three experimental replicates.

563

564 **Figure 3.** Dynamic changes of lyso forms of lipidphosphatidic acid (LPA), phosphatidylglycerol

565 (LPG), phosphatidylinositol (LPI) and sulfoquinovosyl diacylglycerol (SQMG) polar lipid

566 molecular species during storage. Normalized total peak areas of each class are reported in

567 logarithmic scale with standard deviation obtained by nine technical replicates from three

568 experimental replicates.

569

570 **Figure 4.** Dynamic changes of 16:0-18:1 PG, 16:0-18:1 PA, 18:1 LPG, 16:0 LPA and 18:1 LPA from

571 T1 (day 1) to T5 (day 10). Normalized peak areas are reported with standard deviation obtained by

572 nine technical replicates from three experimental replicates.