1 # Deep insight into the minor fraction of virgin olive oil by using LC-MS and GC-
2 MS multi-class methodologies

3 Abbreviated running title: Multianalyte LC and GC methods to determine minor olive oil components

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18 **Abstract**

19 Several analytical methods are available to evaluate virgin olive oil (VOO) minor compounds;
20 however, multi-class methodologies are yet rarely studied. Herewith, LC-MS and GC-MS platforms
21 were used to develop two methods capable of simultaneously determine more than 40 compounds
22 belonging to different VOO minor chemical classes within a single run. A non-selective and highly
23 efficient liquid-liquid extraction protocol was optimized for VOO minor components isolation. The
24 separation and detection conditions were adjusted for determining phenolic and triterpenic
25 compounds, free fatty acids and tocopherols by LC-MS, plus sterols and hydrocarbons by GC-MS.
26 Chromatographic analysis times were 31 and 50 min, respectively. A comparative assessment of
27 both methods in terms of analytical performance, easiness, cost and adequacy to the analysis of
28 each class was carried out. The emergence of this kind of multi-class analytical methodology
29 greatly increases throughput and reduces cost, while avoiding the complexity and redundancy of
30 single-chemical class determinations.

31

32 **Keywords:** virgin olive oil; liquid chromatography; gas chromatography; mass spectrometry;
33 multi-class methodologies; phenolic compounds; pentacyclic triterpenes; tocopherols, sterols, fatty
34 acids.

35

36 **1. Introduction**

37 Apart from being one of the three main macronutrients (together with carbohydrates and proteins)
38 with structural and metabolic functions, fat plays an important role in cooking, since it has different
39 culinary uses (emulsifiers, texturizers, flavorings…) and improves food appearance and
40 acceptability. Virgin olive oil (VOO) has been the main source of lipids in the Mediterranean diet
41 for thousands of years. Over the last decades, its consumption has increased in popularity outside
42 the Mediterranean basin due to its unique sensory characteristics and the health benefits associated
43 with its intake (Piroddi et al., 2016).

44 VOO has a plethora of minor components of undeniable significance that remain in the oil due to
45 the lack of chemical refining. This minor fraction (2–5%) includes phenolic and triterpenic
46 compounds, tocopherols, sterols, hydrocarbons and pigments (chlorophylls and carotenoids), among
47 others (Velasco & Dobarganes, 2002). Their concentration in VOO is strongly affected by different
48 agro-technological parameters (pedoclimatic conditions, cultivar, fruits' maturity, extraction
49 procedures, etc.), which determine their biosynthesis and degradation (Fregapane & Salvador,
50 2013). Therefore, the determination of VOO minor components can be used for the assessment of
51 VOO quality, purity, authenticity and/or typicity (Tena, Wang, Aparicio-Ruiz, García-González, &
52 Aparicio, 2015).

53 Over the last 20 years, extensive studies have been conducted to elucidate the causes of greater
54 longevity and low-incidence of nutrition related-diseases in Mediterranean countries (Serra-Majem,
55 Roman, & Estruch, 2006). It has been shown that several health-promoting effects of VOO are
56 linked to its fatty acids profile and minor components (Hashmi, Khan, Hanif, Farooq, & Perveen,
57 2015; Piroddi et al., 2017). Regarding the latter ones, some reviews have compiled all the available
58 scientific evidences concerning the biological activities of phenolic compounds (Martín-Peláez,
59 Covas, Fitó, Kušar, & Pravst, 2013; Parkinson & Cicerale, 2016; Rigacci & Stefani, 2016; Servili et
60 al., 2013), tocopherols (Sayago, Marín, Aparicio, & Morales, 2007), triterpenic compounds

61 (Rodríguez-Rodríguez & Ruiz-Gutiérrez, 2010; Sánchez-Quesada et al., 2013) and phytosterols
62 (Jones & AbuMweis, 2009), predominantly. As a result, and promoted by health claims regulations,
63 producers and consumers have shown great interest in knowing the content of the main bioactive
64 compounds in VOO.
65 The determination of VOO minor compounds has undoubtedly been a real challenge in the field of
66 food analysis over the last years. Technological advances have led to the proliferation of analytical
67 methods based on advanced instrumental techniques capable of (qualitatively and quantitatively)
68 characterize the formerly unknown analytes of this VOO fraction. Each family of metabolites has
69 been traditionally studied separately because of their chemical heterogeneity (i.e. using specific
70 methods for phenolic compounds, triterpenic substances, sterols, fatty acids, etc., respectively).
71 Some spectroscopic methods of analysis can be found in literature (Christophoridou & Dais, 2009;
72 Escuderos, Sayago, Morales, & Aparicio, 2009; Mora-Ruiz et al., 2017), although the complexity of
73 the matrix requires the use of separative techniques (such as liquid/gas chromatography (LC/GC) or
74 capillary electrophoresis) to facilitate the subsequent determination of the individual components
75 (Cerretani, Lerma-García, Herrero-Martínez, Gallina-Toschi, & Simó-Alfonso, 2010; García,
76 Brenes, Dobarganes, Romero, & Ruíz-Méndez, 2008; Ríos, Gil, & Gutiérrez-Rosales, 2005; Rocco
77 & Fanali, 2009). Table 1a SM (Supplementary Materials) provides a general overview of the
78 available methods for the determination of the main families of VOO minor compounds; five
79 examples per family have been selected among all the published protocols in order to show
80 different sample treatments, separation techniques and detection systems.
81 In contrast to the great number of published methodologies for specific and independent
82 determinations, robust and high-throughput multi-class methodologies capable of monitoring
83 compounds from different chemical classes within a single run (and using just one sample
84 treatment) are very difficult to find. Several researchers have put great efforts trying to develop
85 multi-class methods, being aware of their potential and looking for robust, powerful and high-

86 throughput alternatives (Seppanen, Rahmani, & Csallany, 2003; Tasioula-Margari & Okogeri,
87 2001). Table 1b SM includes some attempts to simultaneously determine compounds belonging to
88 different chemical classes of VOO minor fraction. It is also worth mentioning that different non-
89 targeted metabolomic approaches have been reported too. In this regard, Purcaro et al. (Purcaro,
90 Barp, Beccaria, & Conte, 2015) showed the potential of a multidimensional GC×GC–FID/MS
91 (flame ionization and mass spectrometry detectors) method for the generation of a VOO chemical
92 fingerprint, including sterols, terpenic alcohols, tocopherols, fatty acids and waxes. In another
93 interesting work, a LC-MS method was used to monitor some sterols, triterpenic compounds,
94 tocopherols, carotenoids and fatty acid derivatives, when comparing four saponification methods
95 for the characterization of the VOO unsaponifiable fraction (Sánchez de Medina, Priego-Capote, &
96 Luque de Castro, 2013). Another methodology capable of determining several minor components in
97 edible oils (involving solid phase micro extraction and GC-MS) was recently reported (Alberdi-
98 Cedeño, Ibargoitia, Cristillo, Sopelana, & Guillén, 2017). Although the method was not applied to
99 VOO samples, it showed its capability to monitor sterols and derivatives, tocols, hydrocarbons,
100 aromatic esters, lactones, monoglycerides and fatty amides in a single run without using solvents or
101 reagents for sample preparation. ^{1}H NMR spectra obtained directly from VOO samples
102 (suppressing the main lipid signals) can also be considered as a very useful approach to characterize
103 several VOO minor components (acyl groups, squalene, sterols, triterpenes, fatty alcohols, wax
104 esters and phenols) (Ruiz-Aracama, Goicoechea, & Guillén, 2017).
105 In the current work, LC-MS and GC-MS platforms were used to develop two multi-class
106 methodologies. As working in the context of non-targeted approaches, a non-selective and highly
107 reproducible and effective extraction protocol was adequately optimized. The chromatographic and
108 detection conditions were assessed for LC-MS and GC-MS to achieve a larger number of analytes
109 within a shorter run as well as appropriate analytical performance. This was a challenging task
110 bearing in mind the heterogeneity regarding the physicochemical properties of the analytes under

111 study. These methods represent tangible alternatives to traditional single-class methods and
112 definitely stand for interesting additions to the non-targeted protocols of any laboratory working in
113 the evaluation of oil quality, purity and/or typicity.
114

115 ## 2. Materials and methods

116 ### *2.1. Reagents and standards*

117 Methanol (MeOH) and acetonitrile (ACN) LC-MS grade and ethanol (EtOH) 98%, v/v were
118 purchased from Prolabo (Paris, France). Water was daily deionized by using a Milli-Q system from
119 Millipore (Bedford, MA, USA). Acetic acid (AcH) for acidification of mobile phases in LC, and the
120 derivatization reagent for GC (N,O-bis(trimethylsilyl)trifluoroacetamide plus 1% of
121 trimethylchlorosilane, (BSTFA+TMCS, 99:1, v/v)), were acquired from Sigma-Aldrich (St. Louis,
122 MO, USA).
123 Pure standards of phenolic compounds (vanillin, *p*-coumaric, quinic and ferulic acids,
124 hydroxytyrosol, tyrosol, oleuropein, luteolin, apigenin and pinoresinol); tocopherols (α-, β-, γ- and
125 δ-tocopherols); sterols (β-sitosterol, campesterol and stigmasterol); pentacyclic triterpenes
126 (maslinic, betulinic and oleanolic acids; erythrodiol and uvaol); and fatty acids (palmitoleic, oleic,
127 linoleic and linolenic acids) were all supplied by Sigma-Aldrich. Stock solutions for each analyte
128 were prepared by dissolving the appropriate amount of each chemical standard in EtOH/H_2O
129 (80:20, v/v) and then, they were serially diluted to working concentrations. All the samples and
130 stock solutions were filtered through a Clarinert™ 0.22 µm nylon syringe filter from Agela
131 Technologies (Wilmington, DE, USA) and stored at −20 °C.

132 ### *2.2. Samples and sample treatment*

133 Monovarietal olive oil samples (cv. Carolea, Casaliva, Cayon, Frantoio, Kalamon, Maurino,
134 Moraiolo and Taggiasca) produced at laboratory scale at the UC Davis Olive Center (Davis, CA,

135 USA) by means of an Abencor® laboratory oil mill (MC2 Ingeniería y Sistemas, Seville, Spain)
136 were used in this study. A mixture of equivalent volumes of each sample (multi-varietal VOO
137 blend) was used for sample treatment optimization and chromatographic methods development.
138 A sample treatment pursuing the extraction of maximum number of compounds (belonging to
139 different chemical classes) from the VOO matrix was carried out by using LLE. A portion of 1 (±
140 0.01) g of VOO was weighed in a conical centrifuge tube and vortexed for 4 min with 6 mL of an
141 EtOH/H_2O mixture. Then, the tube was centrifuged at 7500 rpm for 6 min. These steps were
142 repeated four times, the first three stages with EtOH/H_2O (80:20, v/v) as extractant agent, and the
143 last one with EtOH/H_2O (60:40, v/v). All supernatants were combined, the solvent was evaporated
144 to dryness under reduced pressure at 35ºC and the obtained residue was reconstituted in 1 mL of
145 EtOH/H_2O (80:20, v/v). During the extraction protocol optimization, different solvents, volumes
146 and cycles number were tested as described in section 3.1. (Sample treatment optimization).
147 For GC analyses, aliquots of the extracts were preconcentrated and derivatized, following a protocol
148 previously described in some of our reports (García-Villalba et al., 2011; Olmo-García, Bajoub,
149 Monasterio, Fernández-Gutiérrez, & Carrasco-Pancorbo, 2018). Briefly, 200 µL of the extracts
150 were dried under N_2 flow, redissolved with 50 µL of BSTFA+TMCS (99:1, v/v) and kept at room
151 temperature for about 1 h to ensure the complete trimethylsilylation reaction before GC injection.
152 The same derivatization procedure was applied to the standards solutions.

153 ## *2.3. Separation and detection conditions*

154 ### *2.3.1. LC-MS methodology*

155 The LC-MS analyses were performed on an Agilent 1260 LC system (Agilent Technologies,
156 Waldbronn, Germany) coupled to a Bruker Daltonics Esquire 2000™ ion trap (IT) mass
157 spectrometer (Bruker Daltonik, Bremen, Germany) by an electrospray ionization (ESI) source. An
158 Acquity UPLC™ H-Class system (Waters, Manchester, UK) coupled to a high resolution mass

159 spectrometer (micrOTOF-QII™ (Bruker Daltonik)) by an ESI source was also used for obtaining
160 the accurate *m/z* signals of the compounds being studied.
161 The separation was carried out in a Zorbax Extend C18 column (4.6 × 100 mm, 1.8 µm particle
162 size) (Agilent Technologies), operating at 40 ºC. Analytes were eluted with acidified water (0.5% of
163 AcH) (Phase A) and acidified ACN (0.5% of AcH) (Phase B) as mobile phases, with the following
164 gradient: 0 to 2 min, 10%–25% B; 2 to 16 min, 25%–60% B; 16 to 18 min, 60%–80% B; 18 to 23
165 min, 80%–100% B (kept for 6.5 min); and finally, 29.5 to 31 min, 100%–10% B. Total run time
166 was 31 min with a post-run time for column equilibration between each run. The flow rate was set
167 at 1 mL/min from 0 to 23 min, increasing it to 1.5 mL/min during the isocratic part of the gradient,
168 and setting it again to the initial value afterwards (from 29.5 to 31 min). The injection volume was
169 10 µL.
170 The MS detection conditions were selected in accordance with previous works of our research
171 group involving the determination of analytes belonging to different chemical classes (phenolic
172 compounds, triterpenoids, tocopherols, sterols, etc.) (Bajoub et al., 2015; Olmo-García et al., 2018;
173 Zarrouk, Carrasco-Pancorbo, Zarrouk, Segura-Carretero, & Fernández-Gutiérrez, 2009). A flow
174 divisor (1:4) was used to reduce the flow delivered into the MS and ESI parameters were
175 accordingly chosen: nebulizer pressure was set at 30 psi, drying gas temperature at 300 ºC and
176 drying gas flow at 9 L/min. Acquisition of the spectra in the IT MS detector were made in full scan
177 (50-1000 *m/z*) using three different segments: 1-17 min, capillary voltage of +3200 V in negative
178 polarity; 17-22.5 min, capillary voltage set at +3500 V in negative ion mode; and 22.5-31 min,
179 capillary voltage of -3500 V in positive ion mode. The skimmers, octopoles and lenses voltages
180 were tuned considering the average mass which was set as target mass value for each segment.
181 Lastly, these voltages were transferred to the Q-TOF MS detector. Since switching polarity during a
182 run is not recommended in this system, two injections per sample (one for each polarity) were
183 needed. Data Analysis 4.0 (Bruker Daltonik) was used for LC-MS data treatment.

184 *2.3.2. GC-MS methodology*

185 GC-MS analyses were carried out on a Varian 450-GC coupled to a Varian 220-MS IT (Agilent
186 Technologies) through an electron impact (EI) ion source. A 5%-phenyl-methyl polysiloxane (HP-
187 5MS) capillary column (30 m × 0.25 mm i.d., 0.25 μm) (Agilent Technologies) was used to
188 separate the analytes, with He as a carrier gas at a flow rate of 1 mL/min. Oven temperature was
189 initially kept at 140 ºC for 5 min, ramped at 4 ºC/min to 310 ºC and held for 2.5 min. A sample
190 volume of 1 μL was injected at a split ratio of 1:25. Injector and transfer line temperatures were 240
191 ºC and 290 ºC, respectively. Spectra were recorded in full scan (from 50 to 600 *m/z)*, with the EI
192 source operating at a potential of 70 eV in positive ion mode, and a source temperature of 200 ºC.
193 Instrument control and data processing for GC-MS analyses were done with MS Workstation v6.9.3
194 (Agilent Technologies).

195 ***2.4. Method characterization***

196 The main analytical parameters of the developed LC-MS and GC-MS methods, were evaluated and
197 compared in a subsequent stage of the project. Both the multi-varietal VOO blend and solutions
198 containing standards belonging to different VOO minor chemical classes identified in the extracts
199 were used for this purpose.
200 First, external calibration curves for each individual standard were established to check the linearity
201 of the proposed methods. To that end, standard solutions at 8 concentration levels (using the
202 appropriate ranges for each compound considering the system response and the expected
203 concentration levels in VOO samples) were analyzed in triplicates and the resulting peak areas were
204 plotted as a function of their concentrations, performing a linear regression by the least-squares
205 method. The signal to noise (S/N) ratio of the analytes at the lowest concentration level was used
206 for the theoretical estimation of detection (LOD) and quantification (LOQ) limits, which were
207 calculated as the concentrations that generate an S/N ratio equal to 3 and 10, respectively.
208 Afterwards, the presence/absence of matrix effect was assessed in both platforms comparing the

209 slope of two calibration curves (the external one, prepared in EtOH/$H_2$0 (80:20 v/v), and another
210 one resulting from the standard addition of each compound (at 3 concentration levels) to an extract
211 of the multi-varietal VOO blend. Finally, accuracy was evaluated in terms of precision (*intra*- and
212 *inter*-day repeatability) and trueness. *Intra*-day repeatability was expressed as the relative standard
213 deviation (RSD) of peak area and retention time (Rt) of the targeted compounds for 4 injections of
214 the standard mixture carried out within the same sequence, and *inter*-day repeatability, as the RSD
215 of 4 injections (4 different sequences carried out over 4 days) of the same standard mixture.
216 Trueness was calculated based on the difference between the concentration of each analyte in the
217 sample extracted before and after the standard addition (at 3 concentration levels) and was
218 expressed as the found percentage of the spiked amount.
219

220 3. Results and discussion

221 *3.1. Sample treatment optimization*

222 The isolation of the targeted compounds is a key step in any analytical determination, so we paid
223 special attention to the optimization of the sample treatment in order to obtain extracts with the best
224 achievable recoveries containing as many compounds as possible. Considering the fact that a non-
225 targeted approach was selected, an unselective extraction protocol should be followed.
226 Saponification, SPE and LLE were considered as possible strategies to be used in the preliminary
227 tests, but after those assays, LLE was pointed out as the most suitable method taking into account
228 the following aspects: saponification was a tedious (and dispensable) process, SPE led to selective
229 extracts, and both strategies resulted to be more expensive (reagents and SPE cartridges) than LLE.
230 First of all, several pure solvents and mixtures of solvents -covering a broad range of polarities
231 (some of them traditionally used for the isolation of individual families)- were tested, intending to
232 extract as many compounds as possible. Therefore, 1 g of multi-varietal VOO blend was mixed in a
233 vortex with 10 mL of MeOH, ACN, EtOH, ACN/EtOH (50:50, v/v), MeOH/H_2O (60:40, v/v),

234 ACN/H_2O (60:40, v/v) and EtOH/H_2O (80:20, v/v) for 4 min; after centrifugation, evaporation of
235 the supernatants and reconstitution in 1 mL of the proper solvent, the obtained extracts were
236 analyzed by LC-MS. To facilitate the fair comparison among the different sample preparations,
237 EtOH/H_2O (80:20, v/v) was selected for redissolving the dried extracts (after corroborating in the
238 preliminary studies that it was the best possible option in terms of).
239 Fig. 1 SM shows the normalized areas of the peaks (grouped by chemical class) obtained after using
240 each tested extractant (solvent or mixture of solvents). When comparing the total area for each
241 family of compounds, the mixture of EtOH/H_2O (80:20, v/v) was noted as the best extractant agent
242 for tocopherols, triterpenic compounds and the less polar phenolic compounds (flavonoids and
243 lignans). This mixture was also the second best option for fatty acids extraction and gave high
244 recovery for secoiridoid derivatives (achieving the 95% of the total area accomplished when using
245 ACN, which was found as the optimal choice for complex phenols). With regard to simple phenols,
246 ACN/H_2O (60:40, v/v) gave the maximum recovery. Organic solvents without water gave, in
247 general, worse results for simple phenols and better recoveries for the less polar families.
248 Being EtOH/H_2O (80:20, v/v) the most promising mixture for the isolation of most families and
249 taking into account that the mixtures organic solvent/water (60:40, v/v) gave the best recoveries for
250 polar phenols, a new strategy implying the use of two mixtures of diverse polarity was designed
251 searching for a compromise solution. To that end, a first step with EtOH/H_2O (80:20, v/v) as
252 extractant mixture was followed by a second one with a more polar combination of solvents
253 (EtOH/H_2O (60:40, v/v)). The increase of the water percentage led to a remarkable improvement of
254 the recovery of the most polar phenols (achieving a value very close to 75% in the normalized area
255 axis).
256 After choosing the optimized combination of the extractant solvents, the potential of ultrasound-
257 assisted extraction was tested, aiming to facilitate the removal of the targeted compounds from the
258 VOO matrix. However, the use of UAE was discarded in the end, since it resulted in highly

259 emulsified and hardly separable solvent-oil mixtures. As a consequence, vortex shaking was
260 maintained for the last stage of the optimization process, where the extractant agent volume and
261 number of iterations with each ethanolic mixture was adjusted. 4, 6, 8 and 10 mL were the
262 evaluated volumes to be used merging cycles of EtOH/H_2O (80:20, v/v) (cycle/s a) and cycles of
263 EtOH/H_2O (60:40, v/v) (cycle/s b). The alternate use of both solvent polarities that we tried can be
264 summarized as follows: 1 cycle a + 1 cycle b, 2 cycles a + 1 cycle b, and 3 cycles a + 1 cycle b. As
265 a result of this study, the protocol including 3 cycles a + 1 cycle b with 6 mL (per cycle) was
266 selected. To estimate the yield of the optimized protocol, the samples' remnants after the 4-cycles
267 extraction, were subjected to two further consecutive extraction stages (consisting of 1 cycle a + 1
268 cycle b). Table 2 SM shows the amount of each compound extracted by using the optimized
269 protocol (recovery (%) of 4-cycles entire protocol, which is named in the table as 1st stage) and after
270 applying the two additional stages (2nd stage and 3rd stage). The results are expressed as a
271 percentage of the total amount extracted in all the stages. Keeping in mind the concentration ranges
272 of the analytes in VOO, and the fitness for purpose and detection limits of each methodology, we
273 decided that the recovery of phenolic and triterpenic compounds could be better studied using the
274 LC-IT MS platform, whilst the recovery of fatty acids, tocopherols and sterols could be properly
275 assessed with GC-MS. The percentage of the total amount extracted with the optimized protocol
276 was higher than 75% for most of the compounds except for two sterols (β–sitosterol and
277 methylencycloartanol), which exhibited a extraction yield of about 70% and are found at very high
278 concentration levels in VOO. The repeatability of the extraction was also checked, finding RSD
279 values (RSD) lower than 9.8 % in every case.

280 ### *3.2. Chromatographic methods optimization*

281 Due to the chemical complexity of the obtained extracts, chromatographic conditions (for both LC
282 and GC) where optimized to cover a wide range of polarities and volatilities, respectively, and to
283 monitor as many compounds as possible in a reasonable run time. Since commercially available

284 standards do not include some of the most abundant phenolic compounds in olive oil, several VOO
285 and multi-varietal VOO blend extracts were used for optimization purposes in both platforms. In
286 LC, a linear gradient ramp from 5% to 100% ACN (and 95% to 0% of acidified water) in 60 min
287 was firstly designed, adjusting the flow and temperature to 1 mL/min and 40 ºC, respectively (in
288 order to work under moderate pressure conditions). 10 min of extra time at 100% ACN (lengthening
289 the run time over 70 min) were needed to elute α-tocopherol, which was considered as the less polar
290 compound to be determined by using a RP-LC methodology. In order to reduce analysis time;
291 different solvents (MeOH, 2-propanol and tetrahydrofuran) were added to the organic mobile phase
292 to promote the elution of tocopherols. When the mixtures ACN/MeOH, ACN/2-propanol and
293 ACN/tetrahydrofuran (80:20, v/v; same proportion in the three cases) were used as Phase B, there
294 was a reduction in the α-tocopherol Rt of about 3%, 15% and 30%, respectively. Nevertheless, the
295 addition of these solvents presented a negative influence in peak resolution and shape for most of
296 the other analytes. Thus, the ACN/acidic water gradient was modified to decrease the run time. A 6
297 steps-gradient together with a flow gradient was designed giving rise to a 31 min total run time
298 (flow rate was set at 1.5 mL/min when pumping 100% ACN to speed up the elution of tocopherols).
299 Mobile phase composition influenced the analyte's response in the MS detector, which decreased
300 when pumping high percentages of Phase B (coinciding with tocopherols elution). MS signal
301 reduction coexists with the fact that tocopherols are *per se* hardly ionizable analytes in ESI, since
302 they lack strong protonation sites (Lanina, Toledo, Sampels, Kamal-Eldin, & Jastrebova, 2007).
303 When testing different solvent mixtures in Phase B, the intensity of the tocopherols MS signal
304 decreased in the following order: ACN/MeOH (80:20, v/v) > ACN/2-propanol (80:20, v/v) >
305 ACN/tetrahydrofuran (80:20, v/v) > ACN. Therefore, achieving an enhancement of tocopherols'
306 ionization in a mobile phase composed by 100% ACN was required. To that end, the strategy of
307 adding an organic acid to the mobile phase, proposed by other authors (Lanina et al., 2007), was
308 tested with good results. The acidification of ACN with 0.5% of AcH produced a more efficient

309 ionization, leading to an increment in the signals of more than a 50% when compared with the
310 responses obtained with ACN/MeOH (80:20, v/v).
311 The chromatogram resulting from the final optimized conditions is shown in Fig. 2 SM (part A)
312 together with the flow and mobile phase gradients. The visual inspection of this illustration drives
313 us to observe that the steepest ramps in the Phase B gradient correspond to the less crowded parts of
314 the chromatogram. In those parts, a faster elution was logically pursued. In contrast, a slower
315 increment of Phase B percentage was needed for the appropriate separation within the crowded
316 chromatographic area of phenolic compounds. Moreover, 6 min of isocratic pumping of Phase B at
317 a higher flow (1.5 mL/min instead of 1 mL/min) were needed to elute the last compound of interest
318 (α-tocopherol).
319 In GC, a temperature ramp from 120 ºC to 320 ºC at 3 ºC/min was initially tested. Good peak
320 resolution was found by using these conditions, but the potential for shortening the
321 chromatographic run was evident. After testing different possibilities, a 4 ºC/min ramp from 140 ºC
322 to 310 ºC resulted in chromatograms with the best resolution/analysis time ratio, as shown in the
323 part B of Fig. 2 SM. Injection volume and split ratio were two crucial parameters when looking for
324 a compromise solution between desirable sensibility and low background noise. Injections of 0.1,
325 0.5 and 1 μL of sample, in both splitless and split modes (using 1:10, 1:25 1:50 and 1:75 as split
326 ratios) were carried out in the last stage of the optimization process. 1 μL was the optimum injection
327 volume with a split of 1:25; this decision was made considering that the selected combination of
328 volume and split ratio drove to appropriate S/N values for most of the compounds under study (and
329 therefore, proper LODs), preventing at the same time column contamination. A reduction of the
330 split ratio (1:10) caused a drastic soiling of the column after the injection of 15-20 extracts,
331 producing a broad solvent front.

332 ### *3.3. Compounds identification*

333 Preliminary studies in LC-IT MS and GC-MS showed the presence of compounds belonging to 6
334 VOO minor chemical classes in the extracts. A mixture of 26 pure standards as well as 8 different
335 monovarietal VOO extracts were analyzed under the optimal conditions. The use of pure standards
336 was logically very useful to assign the identity of some of the analytes under study in both
337 platforms (on the basis of their Rt and MS signals). Moreover, relative Rt and MS data, together
338 with databases and previously published reports were thoughtfully studied in order to identify some
339 other compounds within the detected profiles.

340 At this point, LC-MS analyses were carried out using a QTOF platform, which allowed the
341 prediction of the molecular formula for the compounds under study from their exact mass. Table 1a
342 shows Rt of the identified compounds, their high resolution MS data including experimental and
343 theoretical (calculated by the software) *m/z* values, error (difference between both values),
344 mSigma™ (value which indicates the similarity between the measured and the theoretical isotopic
345 pattern of the compound) and the predicted molecular formula of the *pseudo*-molecular ion in
346 negative or positive ion mode ($[M-H]^-$ or $[M+H]^+$, respectively) depending on the compound.
347 Tocopherols -detected in positive mode- produced MS signals not corresponding with the expected
348 $[M+H]^+$; this fact had been previously observed by other authors (Lanina et al., 2007; Lauridsen et
349 al., 2001).

350 9 simple phenols, 15 secoiridoids (with 4 isomers for each one of the two aglycone derivatives), 3
351 flavonoids, 3 lignans, 5 triterpenic compounds, 3 free fatty acids and 3 tocopherols (46 peaks in
352 total) were identified in, at least, one of the studied VOOs; the identity of 22 of them was
353 corroborated with their pure standards. Part A of Fig. 1 shows the LC-IT MS Extracted Ion
354 Chromatograms (EICs) of the identified compounds in a Cayon VOO extract (same sample in Fig. 2
355 SM). The positive ion mode was preferred for triterpenic alcohols and tocopherols detection. As a
356 result, oleic acid (peak number 41 at 23.0 min) is shown in this polarity in the chromatogram;
357 however, it was significantly better detected in negative ion mode.

358 Some unknown or tentatively identified compounds were also detected in the extracts by using the
359 LC-MS optimized method; they are reported in Table 1b. For instance, *m/z* 405.1555 and 389.1696
360 could correspond to the dimethyl oleuropein aglycone and dimethyl ligstroside aglycone,
361 respectively. The compounds at Rt 19.9 and 21.0 min were proposed as terpene-glucosides
362 (arjulonic acid-glucoside and maslinic acid-glucoside, respectively). Ongoing experiments are being
363 conducted in our lab to corroborate the identity of the compounds included in Table 1b.
364 In GC-MS, the identification of the peaks corresponding to compounds whose standard was not
365 commercially available was not as straightforward as in the case of LC-ESI-QTOF MS due to two
366 main reasons. On the one hand, most of the compounds under study were not in the GC-EI MS
367 databases. On the other hand, as EI is a harsh ionization method (i.e. which produces high
368 fragmentation in-source), the *m/z* signal of the molecular ion (or *pseudo*-molecular ion) was not
369 found in the MS spectra of some peaks (when the molecular ion signal was found, the intensity was
370 very low). As a consequence, in this platform, peak identification was mainly achieved bearing in
371 mind the relative Rt of the analytes, studying the fragmentation patterns and taking into account the
372 previously published results (Angerosa, D'Alessandro, Konstantinou, & Di Giacinto, 1995; García-
373 Villalba et al., 2011; Ríos et al., 2005; Saitta, Lo Curto, Salvo, Di Bella, & Dugo, 2002). In some of
374 the mentioned contributions, isolated pure standards were used to corroborate the identity of some
375 of the compounds under study, what means that even if the GC-EI-MS identification was more
376 intricate than in LC-MS, the final result was very reliable too. 47 peaks in total were identified: 9
377 simple phenols, 5 secoiridoids (with 3 isomers of oleuropein and ligstroside aglycones and elenolic
378 acid), 2 flavonoids, 2 lignans, 5 triterpenic compounds, 5 free fatty acids (with two isomers of oleic
379 acid), 4 tocopherols, 7 sterols and a hydrocarbon (squalene). Table 2a shows Rt, the most relevant
380 MS signals found in the spectrum of each peak (the relative intensity of each one is written between
381 brackets) and the formula assigned to the signal used for identification purposes (MS signal which
382 is presented in bold letters).

383 As shown in the table, most of the substances under study showed a MS signal (with low intensity
384 in some cases) which corresponded to the completely trimethylsilylated molecule (total substitution
385 of active hydrogen by TMS groups). Nevertheless, some exceptions to that rule can be listed. For
386 example, in the case of quinic acid (Rt of 15.34 min), the *pseudo*-molecular ion of the complete
387 silylated structure (with 5 positions to be replaced by TMS groups, giving a *m/z* signal of 552 (M-
388 5H+5TMS)) was not observed. The main MS signal was 346, which corresponds to the loss of
389 TMSO (*m/z* 89) together with the loss of TMSO-CO (*m/z* 117). The first loss is characteristic of
390 hydroxyl group and the second is typical of the carboxylic group. The absence of the MS signal
391 corresponding to the completely trimethylsilylated molecule was also observed for the third isomer
392 of elenolic acid (Rt of 18.29). This substance generates a MS signal (with very low intensity, but
393 perfectly observable) which can be assigned to M-2H+2TMS-OCH_3 (*m/z* 355) as previously
394 reported (García-Villalba et al., 2011). As far as decarboxymethyl ligstroside aglycone (Rt of 29.91
395 min) is concerned, it gave the characteristic signal of ligstroside aglycone derivatives (*m/z* 192),
396 resulting from the McLafferty rearrangement (Angerosa et al., 1995), apart from a signal of very
397 low intensity with *m/z* 361, which was assigned to M-H+TMS-CH_3. Ligstroside aglycone presented
398 different isomers (Rt of 34.89, 36.15 and 36.98 min). None of them exhibited as main MS signal the
399 complete silylated molecule, being the fragment 192 the most intense one in every case. Moreover,
400 the *m/z* signal 177 was detected for the three isomers (as reported by other authors (García-Villalba
401 et al., 2011; Saitta et al., 2002)); additionally, for the third one, we found *m/z* 417 corresponding to
402 M-2H+2TMS-TMSO. Concerning the three oleuropein aglycone isomers, all of them presented the
403 *m/z* 280 as major feature in their spectra. This fragment is the main product of the above mentioned
404 McLafferty rearrangement of secoiridoid aglycons containing a hydroxytyrosol moiety in their
405 structures. The 2nd isomer (Rt of 38.67) did not show either the *m/z* signals 522 or 594 (molecule
406 with 2 or 3 hydrogens substituted by TMS groups) but the loss of TMSO from the complete
407 silylated chemical entity (*m/z* 505). Squalene is suitable for being analyzed by GC without the need

408 of derivatization (indeed, it is a dehydrotriterpenic hydrocarbon which has no active hydrogens to
409 be replaced by TMS groups); its identification was done by comparison with its reference NIST
410 spectrum. It is worth noting that all fatty acids, apart from the trimethylsilylated molecule, showed
411 the cluster series with consecutive losses of CH_2 (14 *m/z)* (Hurtado-Fernández et al., 2013). Sterols
412 exhibited some peculiarities too: cycloartenol did not present the *pseudo*-molecular ion but the loss
413 of the trimethylsilanol group (90 *m/z)*, which is common to most sterols. Methylencycloartanol
414 showed the silylated *pseudo*-molecular ion (with low intensity), but also the M-H+TMS-TMSOH
415 (*m/z* 422) and the further loss of CH_2 (*m/z* 408). Citrostadienol´s spectrum had *m/z* 358 and 400 as
416 distinctive signals, which have been also reported by other authors (Harrabi et al., 2007). The *m/z*
417 signal 394 was also detectable in its spectrum, corresponding to M-H+TMS-TMSOH-CH_2.
418 Triterpenic dialcohols instead of producing the MS signal corresponding to the totally
419 trimethylsilylated molecule had a predominant fragment (*m/z* 497) coming from the loss of one
420 TMSO. All pentacyclic triterpenoids that contain a C-12–C-13 double bond undergo a retro-Diels-
421 Alder cleavage of the C-ring into the EI source, leading to dienophile and diene fragments (Pollier
422 & Goossens, 2012). Accordingly, fragmentation of trimethylsilylated oleanolic acid, for instance,
423 led to an ion of *m/z* 320 (with relatively high intensity in the spectra). This ion underwent a
424 subsequent fragmentation, losing its TMSO-CO, and leading to a signal of *m/z* 203 (Razboršek,
425 Vončina, Doleček, & Vončina, 2008). The double silylated $C_{30}H_{48}O_3$ mass isomers also suffered a
426 loss of 117 Da, which, as stated above, is characteristic of the carboxylic groups (*m/z* 483).
427 Betulinic acid was the only triterpenoid which showed the *m/z* 189 in MS (indeed, it was the major
428 feature in its spectrum); that is a fragmentation pattern typical of a saturated lupane skeleton,
429 involving ring C cleavage (Razboršek et al., 2008). Maslinic acid had a MS spectrum defined by
430 *m/z* signals at 203, 73, 571 and 320 (in decreasing order of intensity). *m/z* 571 could be assigned to
431 the completed silylated molecule after losing TMSO-CO.

432 Table 2b shows the *m/z* signals of the major unknown peaks detected in GC-MS. Two ligstroside
433 derivatives were detected with Rt of 16.68 and 17.66 min, respectively. Moreover, the MS signals
434 detected at 27.72 min were assigned to *cis*-vaccenic acid, which is a positional isomer of oleic acid.
435 Apart from them, hydroxy decarboxymethyl ligstroside aglycone appeared in the profile (Rt 33.23
436 min) and one oleuropein derivative was detected at 33.69 min. Some other unknown peaks were
437 detected with a considerable intensity; however, with the EI-MS signals, it was not possible to find
438 a plausible identity for them.

439 ### *3.4. Methods characterization and comparison*

440 The performance of both LC-MS and GC-MS methods was compared considering some illustrative
441 analytical parameters. The main results of the characterization study are shown in Table 3; the pure
442 standards of some relevant compounds (which belong to different chemical categories and can be
443 easily found in VOO extracts) were considered.
444 Good linearity was achieved for all the calibration curves within the working concentration ranges,
445 with correlation coefficients (r^2) higher than 0.9927 (for maslinic acid) in LC-MS and 0.9926 (for
446 β-tocopherol) in GC-MS, respectively. LOD and LOQ in LC-MS were lower than those achieved
447 by GC-MS in every case. Regarding repeatability, Rt RSD (%) was lower than 2.7 and 3.1% for
448 *intra-* and *inter*-day, respectively, in LC-MS, and lower than 0.03% and 0.05% for *intra-* and *inter-*
449 day, respectively, in GC-MS (data not included to contain the size of the table). These values were
450 logically higher for peak area repeatability (they can be seen in the table), but not exceeding 12% in
451 any case. Trueness, expressed as recovery (%), presented values within the range from 75.1 to
452 113.4% in LC-MS, and between 81.0 and 108.3% in the case of GC-MS. Matrix effect was also
453 evaluated in both platforms, calculating the corresponding coefficients. Most of them were between
454 −20% to +20%, considered as the range in which there is a mild signal suppression or enhancement
455 effect. Only two analytes in LC-MS (pinoresinol and uvaol) and luteolin in GC-MS showed a
456 slightly more significant matrix effect.

457 To go even further into the comparison, we decided to consider some other aspects apart from those
458 clearly stated in Table 3, such as analysis time, number of compounds to be determined, easiness,
459 relative cost and facilities and reagents needed in the lab. We have also tried to point out the
460 chemical classes which are better covered by LC-MS or GC-MS, respectively. Table 4 shows this
461 critical comparison in view of different aspects other than those strictly related to the analytical
462 performance of the methods. In an attempt to summarize the info from the table in few sentences, it
463 is possible to say that the LC-MS methodology is more convenient in terms of analysis time,
464 sensitivity, and simplicity for identifying the analytes under study. GC-MS requires cheaper
465 instrumentation and allows the determination of sterols and squalene; but its main drawbacks are
466 the necessity of derivatization and the intricacy of identification. Considering the number of
467 compounds covered by each method, the two options were quite similar, although GC-MS fit better
468 for fatty acids, hydrocarbon, tocopherols, sterols and triterpenic dialcohols, whereas LC-MS was
469 more suitable for phenolic compounds and triterpenic acids.
470

471 ## 4. Conclusion

472 Two multi-class methodologies -LC-MS and GC-MS- were developed in an attempt to
473 simultaneously determine relevant minor components of VOO (different subclasses of phenolic
474 compounds, triterpenoids, free fatty acids, tocopherols, sterols and one hydrocarbon) within a single
475 run.
476 Few previous reports have dealt with the development of multi-class methods with application in
477 the field of olive oil, but to the best of our knowledge, the methodologies presented herewith cover
478 a significant number of analytes. These methodologies could represent a good chance to evaluate
479 (including but not limited to): the effect of technological parameters on the final composition of
480 olive oil minor fraction; the typicity and genuineness of different olive oil samples; the potential

481 healthful properties of an oil; and the profiling of olive oil-related matrices to get a comprehensive
482 characterization of their minor components.

483

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490

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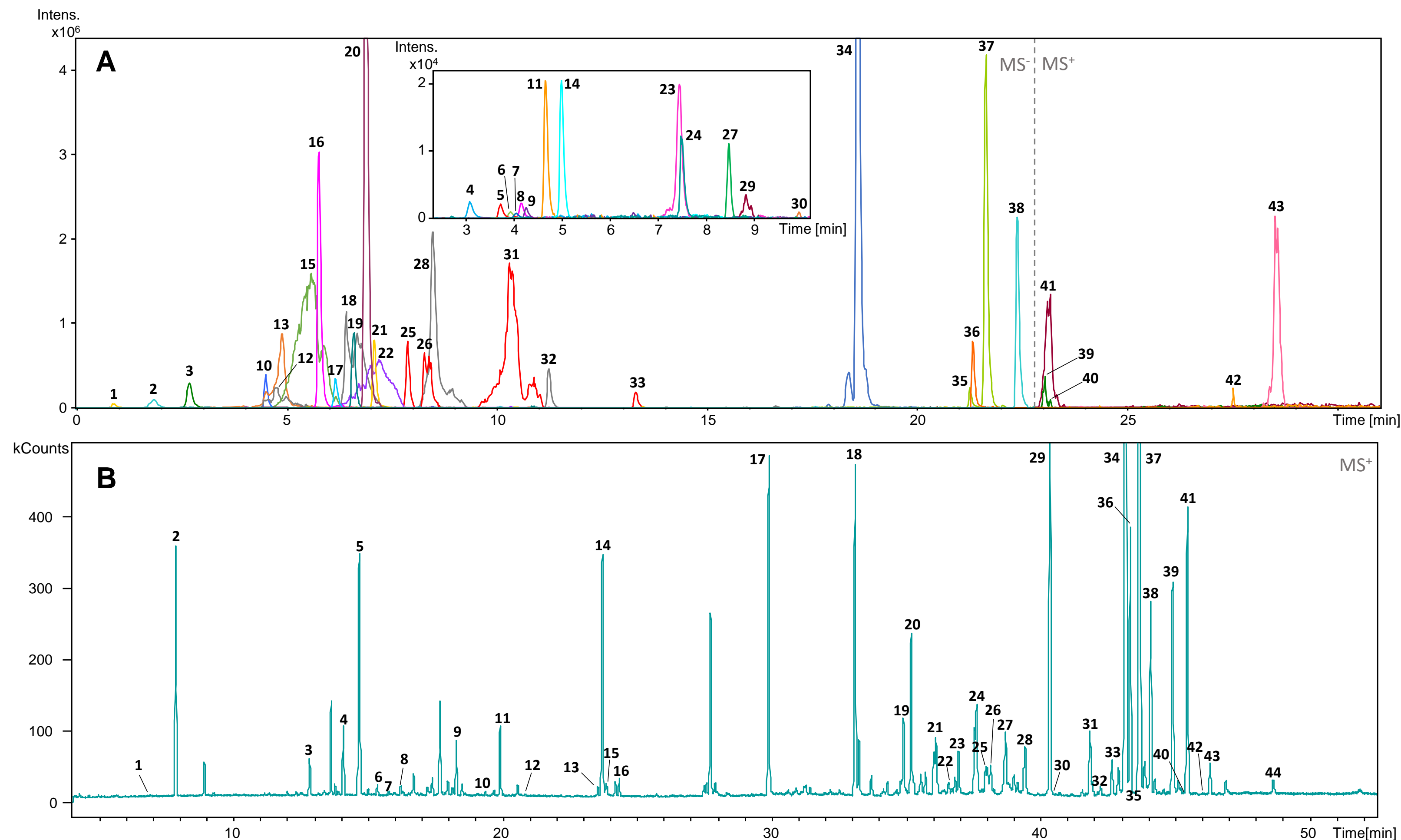

635

636 **Fig. 1**. A) LC-MS extracted ion chromatograms (EICs) of the identified compounds in a Cayon monovarietal oil extract. Peak number identification
637 can be found in Table 1a. A vertical line demarcates the time in which the MS system changed from negative polarity to positive mode. B) GC-MS
638 base peak chromatogram (BPC) of the same Cayon extract. Peak identification numbers as in Table 2a.

Table 1a. Compounds identified using LC-QTOF MS.

Rt (min)	Peak	Experimental *m/z*	Calculated *m/z*	Calculated formula	Error (ppm)	mSigma	MS Polarity	Compound
0.9	1	191.0563	191.0561	$C_7H_{11}O_6$	1.0	6.6	-	quinic acid
1.1		169.0505	169.0506	$C_8H_9O_4$	0.9	15.1	-	3,4-dihydroxyphenylglycol
1.8	2	153.0554	153.0557	$C_8H_9O_3$	2.1	4.7	-	hydroxytyrosol
2.7	3	137.0604	137.0608	$C_8H_9O_2$	3.1	11.5	-	tyrosol
3.1	4	167.0339	167.0344	$C_8H_7O_4$	3.2	9.6	-	vanillic acid
3.7	5	163.0400	163.0401	$C_9H_7O_3$	0.5	6.6	-	*p*-coumaric acid
3.9	6	151.0401	151.0401	$C_8H_7O_3$	0.1	17.3	-	vanillin
4.0	7	193.0506	193.0501	$C_{10}H_9O_4$	1.1	16.2	-	ferulic acid
4.1	8	225.0766	225.0768	$C_{11}H_{13}O_5$	1.2	4.0	-	desoxy elenolic acid
4.2	9	257.0668	257.0667	$C_{11}H_{13}O_7$	0.4	12.4	-	hydroxy elenolic acid
4.5	10	195.0665	195.0663	$C_{10}H_{11}O_4$	1.1	8.1	-	hydroxytyrosol acetate
4.7	11	381.1546	381.1555	$C_{19}H_{25}O_8$	2.4	7.4	-	hydroxytytosol acyclodihydroelenolate
4.8		539.1765	539.1770	$C_{25}H_{31}O_{13}$	1.0	19.1	-	oleuropein
4.8	12	377.1214	377.1242	$C_{19}H_{21}O_8$	0.3	1.7	-	oleuropein aglycone I
4.9	13	241.0717	241.0718	$C_{11}H_{13}O_6$	0.1	9.4	-	elenolic acid
5.0	14	335.1132	335.1136	$C_{17}H_{19}O_7$	1.4	26.3	-	hydroxy decarboxymethyl oleuropein aglycone
5.5	15	319.1188	319.1187	$C_{17}H_{19}O_6$	0.3	6.4	-	decarboxymethyl oleuropein aglycone
5.7	16	285.0412	285.0405	$C_{15}H_9O_6$	2.7	9.1	-	luteolin
6.2	17	417.1548	417.1555	$C_{22}H_{25}O_8$	1.7	2.3	-	syringaresinol
6.5	18	377.1255	377.1242	$C_{19}H_{21}O_8$	3.6	2.7	-	oleuropein aglycone II
6.6	19	357.1346	357.1344	$C_{20}H_{21}O_6$	0.5	1.6	-	pinoresinol
6.8		393.1203	393.1191	$C_{19}H_{21}O_9$	2.9	22.2	-	hydroxy oleuropein aglycone
6.9	20	415.1406	415.1398	$C_{22}H_{23}O_8$	2	12.2	-	acetoxypinoresinol
7.1	21	269.0460	269.0455	$C_{15}H_9O_5$	1.5	23.9	-	apigenin
7.2	22	303.1239	303.1238	$C_{17}H_{19}O_5$	0.4	18.8	-	decarboxymethyl ligstroside aglycone
7.4	23	199.0614	199.0612	$C_9H_7O_3$	1.2	1.4	-	hydroxy decarboxymethyl elenolic acid
7.4	24	299.0556	299.0561	$C_{16}H_{11}O_6$	1.7	27.8	-	diosmetin
7.9	25	361.1296	361.1293	$C_{19}H_{21}O_7$	1.0	3.6	-	ligstroside aglycone I
8.3	26	361.1293	361.1293	$C_{19}H_{21}O_7$	0.0	2.4	-	ligstroside aglycone II
8.4	27	375.1094	375.1085	$C_{19}H_{19}O_8$	2.3	18.5	-	dehydro oleuropein aglycone
8.6	28	377.1251	377.1242	$C_{19}H_{21}O_8$	2.5	11.5	-	oleuropein aglycone III
9.8	29	391.1398	391.1402	$C_{20}H_{23}O_8$	1.0	30.0	-	methyl oleuropein aglycone
9.9	30	359.1118	359.1131	$C_{19}H_{19}O_7$	3.6	26.9	-	dehydro ligstroside aglycone
10.3	31	361.1294	361.1293	$C_{19}H_{21}O_7$	0.4	7.3	-	ligstroside aglycone III
11.3	32	377.1245	377.1242	$C_{19}H_{21}O_8$	0.7	1.2	-	oleuropein aglycone IV
13.3	33	361.1294	361.1293	$C_{19}H_{21}O_7$	0.2	4.8	-	ligstroside aglycone IV
18.5	34	471.3484	471.3480	$C_{30}H_{47}O_4$	1.0	2.2	-	maslinic acid
21.2	35	455.3533	455.3531	$C_{30}H_{47}O_3$	0.4	16.1	-	betulinic acid
21.3	36	277.2159	277.2167	$C_{18}H_{29}O_2$	3.1	17.7	-	linolenic acid
21.5	37	455.3537	455.3531	$C_{30}H_{47}O_3$	1.4	26.7	-	oleanolic acid

22.3	38	279.2334	279.2324	$C_{18}H_{31}O_2$	3.6	22.1	-	linoleic acid
22.9	39	443.3879	443.3889	$C_{30}H_{51}O_2$	2.3	24.4	+	erythrodiol
23.0	40	443.3876	443.3889	$C_{30}H_{51}O_2$	2.9	21.2	+	uvaol
23.0	41	281.2484	281.2486	$C_{18}H_{33}O_2$	0.7	24.6	-	oleic acid
27.2	42	415.3574	415.3571	$C_{28}H_{47}O_2$	0.8	35.3	+	β+γ-tocopherol
28.2	43	429.3740	429.3727	$C_{29}H_{49}O_2$	3.0	37.7	+	α-tocopherol

The compounds which have no number peak assigned were not found in the VOO sample chosen to illustrate the figures 1 and 2 (SM). Table 1a only includes the compounds recurrently found in most of the oils.

Table 1b. Compounds tentatively identified using LC-QTOF MS.

Rt (min)	Experimental *m/z*	Calculated *m/z*	Calculated formula	Error (ppm)	mSigma	MS/MS fragmentation pattern	Compound	Chemical class / Other comments
12.8	405.1552	405.1555	$C_{21}H_{25}O_8$	0.7	5.2	377.1312 (100)	dimethyl oleuropein aglycone	secoiridoid
13.3/13.6	451.1976	451.1974	$C_{23}H_{31}O_9$	0.5	6.1	147.0435 (100); 149.0989 (96); 121.0255 (60); 119.0506 (56); 223.0601 (53)	oleuropein aglycone-related compound	secoiridoid
14.9	389.1596	389.1606	$C_{21}H_{25}O_7$	2.4	3.6	269.1049 (100); 361.1295 (12)	dimethyl ligstroside aglycone	secoiridoid
15.2	567.3179	567.3175	$C_{30}H_{47}O_{10}$	0.8	25.4	368.2435 (100); 308.2233 (32)	di-O-acetyldarutoside	terpene glycoside
15.4/15.7	435.2026	435.2024	$C_{23}H_{31}O_8$	0.4	21.0	315.1455 (100)	ligstroside aglycone -related compound	secoiridoid
16.2	529.2352	529.2384	$C_{36}H_{33}O_4$	6.2	20.5	469.2153 (100)	-	
17.8	547.3633	547.3640	$C_{32}H_{51}O_7$	1.3	9.3	296.0723 (100); 180.0657 (87)	methoxyl-passifloic acid	cycloartane triterpenoid
19.4	563.3226	563.3226	$C_{31}H_{47}O_9$	0.1	14.7	223.1329 (100)	-	
19.9	649.3924	649.3957	$C_{36}H_{57}O_{10}$	5.2	20.6	205.1220 (100); 306.1612 (72)	arjulonic acid-glucoside	terpene-glucoside / triterpene saponin
21.0	633.3982	633.4008	$C_{36}H_{57}O_9$	4.1	24.2	285.0478 (100); 392.0990 (83)	maslinic acid-glucoside	terpene-glucoside / triterpene saponin

MS negative polarity was used to achieve the data included in this table.
In the MS/MS fragmentation pattern-column the relative intensity of each fragment is indicated between brackets.

Table 2a. Identified compounds in GC-MS

Rt	Peak	MS Signals (Relative abundance)	Identifier	Compound
6.93	1	194 (100)/209 (65)/**224** (38)/45 (11)	M-H+TMS	vanillin
7.84	2	179 (100)/267 (34)/**282** (31)/180 (29)	M-2H+2TMS	tyrosol
9.26		267 (100)/73 (62)/223 (60)/**282** (5)	M-2H+2TMS	4-hydroxybenzoic acid
12.83	3	267 (100)/**370** (85)/73 (28)/193 (14)	M-3H+3TMS	hydroxytyrosol
14.07	4	280 (100)/73 (55)/193 (20)/**340** (1)	M-2H+2TMS	hydroxytyrosol acetate
14.66	5	153 (100)/299 (76)/196 (74)/**314** (2)	M-H+TMS	elenolic acid I
15.34	6	**346** (100)/256 (41)/73 (39)/419 (8)	M-5H+5TMS-2TMSO-CO	quinic acid
16.26	7	73 (100)/178 (77)/165 (59)/**314** (2)	M-H+TMS	elenolic acid II
17.20	8	294 (100)/73 (40)/**308** (38)/250 (29)	M-2H+2TMS	*p*-coumaric acid
18.29	9	73 (100)/249 (36)/193 (30)/**355** (15)	M-2H+2TMS-OCH_3	elenolic acid III
19.30	10	312 (100)/129 (66)/117 (54)/**326** (7)	M-H+TMS	palmitoleic acid
19.92	11	117 (100)/314 (86)/129 (50)/**328** (14)	M-H+TMS	palmitic acid
20.83	12	**338** (100)/324 (31)/294 (16)/73 (12)	M-2H+2TMS	ferulic acid
21.94		**396** (100)/220 (36)/73 (17)/382 (8)	M-3H+3TMS	caffeic acid
23.54	13	338 (100)/73 (60)/129 (52)/**352** (8)	M-H+TMS	linoleic acid
23.76	14	**354** (100)/117 (79)/129 (76)/340 (22)	M-H+TMS	oleic acid I
23.87	15	340 (100)/129 (71)/117 (64)/**354** (11)	M-H+TMS	oleic acid II
24.34	16	342 (100)/117 (78)/129 (44)/**356** (13)	M-H+TMS	stearic acid
29.91	17	192 (100)/177 (6)/73 (4)/**361** (1)	M-H+TMS-CH_3	decarboxymethyl ligstroside aglycone
33.12	18	280 (100)/73 (14)/193 (8)/**464** (1)	M-2H+2TMS	decarboxymethyl oleuropein aglycone
34.89	19	**192** (100)/73 (11)/280 (11)/**177** (8)	*	ligstroside aglycone I
35.17	20	69 (100)/81 (85)/41 (66)/95 (32)	NIST Ref. spectrum	squalene
36.15	21	**192** (100)/73 (19)/**177** (10)/297 (8)	*	ligstroside aglycone II
36.56	22	**475** (100)/209 (6)/73 (6)/249 (3)	M-H+TMS	δ-tocopherol
36.98	23	193 (100)/73 (22)/**417** (20)/177 (10)	M-2H+2TMS-TMSO	ligstroside aglycone III
37.62	24	280 (100)/73 (16)/193 (8)/**522** (3)	M-2H+2TMS	oleuropein aglycone I
37.95	25	**489** (100)/223 (10)/73 (6)/41 (3)	M-H+TMS	β-tocopherol
38.13	26	**489** (100)/223 (13)/73 (6)/43 (3)	M-H+TMS	γ-tocopherol
38.67	27	280 (100)/73 (18)/45 (4)/**505** (4)	M-3H+3TMS-TMSO	oleuropein aglycone II
39.46	28	280 (100)/73 (14)/193 (6)/**594** (1)	M-3H+3TMS	oleuropein aglycone III
40.33	29	**503** (100)/238 (10)/73 (9)/43 (4)	M-H+TMS	α-tocopherol
40.35	30	472 (100)/45 (4)/399 (3)/**486** (2)	M-3H+3TMS	apigenin
41.82	31	503 (100)/**472** (70)/73 (68)/383 (55)	M-H+TMS	campesterol
42.21	32	395 (100)/**485** (74)/83 (63)/256 (62)	M-H+TMS	stigmasterol
42.61	33	560 (100)/45 (3)/472 (3)/**574** (1)	M-4H+4TMS	luteolin
43.16	34	397 (100)/358 (41)/**486** (40)/381 (28)	M-H+TMS	β-sitosterol
43.20	35	**502** (100)/223 (69)/235 (33)/488 (24)	M-2H+2TMS	pinoresinol
43.34	36	386 (100)/297 (75)/282 (49)/**484**(10)	M-H+TMS	Δ^5-avenasterol
43.67	37	276 (100)/246 (40)/546 (14)/**560** (2)	M-2H+2TMS	acetoxy pinoresinol
44.07	38	393 (100)/366 (53)/**408** (37)/69 (28)	M-H+TMS-TMSOH	cycloartenol
44.89	39	408 (100)/380 (96)/422 (45)/**512** (1)	M-H+TMS	methylencycloartanol
45.41	40	**497** (100)/216 (68)/73 (38)/203 (25)	M-2H+2TMS-TMSO	eythrodiol
45.44	41	358 (100)/400 (33)//268 (17)/**394** (8)	M-H+TMS-TMSOH-CH_2	citrostadienol
45.96	42	**497** (100)/73 (38)/216 (32)/203 (23)	M-2H+2TMS-TMSO	uvaol
46.29	43	203 (100)/**483** (53)/73 (43)/320 (40)	M-2H+2TMS-TMSO-CO	oleanolic acid
46.58		189 (100)/73 (90)/203 (40)/**483** (21)	M-2H+2TMS-TMSO-CO	betulinic acid
48.63	44	203 (100)/73 (62)/**571** (50)/320 (42)	M-3H+3TMS-TMSO-CO	maslinic acid

*Fragments previously reported in literature

The compounds which have no number peak assigned were not found in the VOO sample chosen to illustrate the figures 1 and 2 (SM).

Table 2b. Major unknown peaks in GC-MS

Rt	MS Signals (Relative abundance)	Compound
8.91	192 (100)/178 (25)/43 (12)/73 (10)/117 (3)/151 (3)/237 (1)/297 (1)	-
13.60	153 (100)/225 (91)/196 (62)/167(50)/239(45)/135(42)/163 (40)/270 (5)	-
16.68	73 (100)/192 (62)/45(41)/267 (39)/297 (32)/165 (31)/267 (30)/327 (30)	ligstroside aglycone-related compound
17.66	73 (100)/192 (56)/165 (38)/297 (37)/267 (35)/253 (27)/119 (26)/311 (25)	ligstroside aglycone-related compound
27.72	338 (100)/128 (78)/116 (75)/75 (74)/131 (66)/144 (59)/198 (46)/354 (30)	*cis*-vaccenic acid
33.23	192 (100)/73(12)/177 (8)/280 (5)/45 (3)/299 (1)/151 (1)/255 (1)/359 (1)	hydroxy decarboxymethyl ligstroside aglycone
33.69	73 (100)/280 (76)/129 (57)/147 (32)/103 (28)/257 (23)/203 (23)/339 (22)	oleuropein aglycone-related compound
46.86	563 (100)/147 (36)/240 (15)/73 (14)/253 (11)/266 (8)/225 (8)/45 (7)	-

Table 3. Analytical parameters of representative analytes belonging to different VOO families which can be determined by both LC-MS and GC-MS methodologies.

	LC-MS						**GC-MS**					
Compound	LOD (µg/L)	LOQ (µg/L)	Matrix Effect Coef. (%)[a]	Accuracy: Repeatability (area RSD(%))[b] *Intra-day*	Accuracy: Repeatability (area RSD(%))[b] *Inter-day*	Accuracy: Trueness[c]	LOD (µg/L)	LOQ (µg/L)	Matrix Effect Coef. (%)[a]	Accuracy: Repeatability (area RSD(%))[b] *Intra-day*	Accuracy: Repeatability (area RSD(%))[b] *Inter-day*	Accuracy: Trueness[c]
quinic acid	22	75	4.3	4.3	5.7	99.2	1000	3333	-2.7	1.1	5.2	98.2
hydroxytyrosol	40	133	-0.9	3.1	4.8	84.7	167	556	3.5	1.9	8.7	81.0
tyrosol	47	156	-3.8	5.3	5.8	100.6	167	556	6.1	0.7	5.2	99.0
p-coumaric acid	32	106	-1.9	5.5	6.9	80.6	446	1486	-2.1	0.9	5.9	99.0
vanillin	6	21	-7.4	6.3	7.6	107.9	94	313	-2.7	1.8	5.9	105.7
ferulic acid	36	118	-7.6	5.5	8.8	96.7	120	400	0.5	1.0	7.3	102.5
luteolin	1	3	2.9	1.8	3.8	84.4	323	1076	21.3	1.2	11.9	98.4
apigenin	2	7	4.9	3.0	5.3	86.7	443	1477	-11.3	3.8	8.8	99.5
pinoresinol	4	14	24.7	1.8	9.8	89.5	255	850	-0.3	6.6	8.0	111.0
maslinic acid	2	8	-3.5	5.9	7.5	100.6	1731	5769	-11.0	2.6	8.5	102.7
betulinic acid	2	6	0.9	4.3	8.7	91.2	833	2778	-14.2	1.8	7.9	108.3
oleanolic acid	2	7	3.4	3.3	4.3	83.5	1667	5556	-2.4	5.7	7.4	104.1
erythrodiol	109	362	2.3	8.4	10.6	75.1	594	1980	4.8	3.2	5.6	101.0
uvaol	113	377	23.3	9.7	11.1	107.8	750	2500	7.8	2.4	7.4	93.7
linoleic acid	1	3	15.3	3.2	4.7	113.4	195	650	-0.9	2.4	6.8	96.4
α-tocopherol	27	90	4.6	0.9	3.4	101.8	54	179	-4.5	1.7	7.1	103.1
β-tocopherol	87	289	9.5	0.7	8.1	110.4	100	333	5.9	1.3	5.2	96.4
γ-tocopherol							56	185	4.9	0.6	8.9	91.1

[a]Matrix effect coefficient (%)=(1−(slope matrix/slope solvent))×100.
[b]Repeatability is expressed as the RSD (%) of peak area for 4 injections (of the standard mixture at an intermediate concentration level) carried out within the same sequence (*intra*-day) or for 4 injections from different sequences carried out over 4 days (*inter*-day).
[c]Trueness is expressed as recovery (%), which was estimated by analyzing the multi-varietal VOO blend extracted before and after the standard addition and calculating the difference between the obtained results afterwards. The values included in this table are those achieved for the intermediate concentration level.

Table 4. Comparison between the two developed methodologies taking into account different aspects other than those strictly analytical.

	LC-MS	**GC-MS**
Relative cost. Facilites, materials and reagents needed	– LC-MS instrumentation more expensive than GC-MS – Higher cost in terms of mobile phases	– Need of derivation reagents
Easiness	– No need of any derivatization reaction – More straightforward identification	– Limited stability of extracts after being derivatized – Difficult identification of analytes when they are not available as pure standards or present in commercial databases – Harsh ionization sources in most of commercial equipments (*pseudo*-molecular ions not detected sometimes)
Analysis time	31 min	50 min
Analytical performance	– Better LODs – 2 compounds showing a slight matrix effect	– More robust methodology – 1 compound showing a slight matrix effect
Number of compounds	46 peaks in total: 9 simple phenols, 15 secoiridoids (with 4 isomers for each one of the two aglycone derivatives), 3 flavonoids, 3 lignans, 5 triterpenic compounds, 3 free fatty acids and 3 tocopherols (two coeluting)	47 peaks in total: 9 simple phenols, 5 secoiridoids (with 3 isomers of elenolic acid and the aglycones), 2 flavonoids, 2 lignans, 5 triterpenic compounds, 5 free fatty acids (with two isomers of oleic acid), 4 tocopherols, 7 sterols and a hydrocarbon
Chemical classes more suitable to be determined	– Simple phenols – Secoiridoids – Flavonoids and lignans – Triterpenic acids	– Fatty acids – Hydrocarbon – Tocopherols – Sterols – Triterpenic alcohols