

1 **Quality classification of Spanish olive oils by untargeted gas chromatography coupled**  
2 **to hybrid quadrupole–time of flight mass spectrometry with atmospheric pressure**  
3 **chemical ionization and metabolomics-based statistical approach**

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8 **ABSTRACT**

9 The novel atmospheric pressure chemical ionization (APCI) source has been used in  
10 combination with gas chromatography (GC) coupled to hybrid quadrupole time-of-flight  
11 (QTOF) mass spectrometry (MS) for determination of volatile components of olive oil,  
12 enhancing its potential for classification of olive oil samples according to their quality  
13 using a metabolomics-based approach. The full-spectrum acquisition has allowed the  
14 detection of volatile organic compounds (VOCs) in olive oil samples, including extra  
15 virgin, virgin and lampante qualities. A dynamic headspace extraction with cartridge  
16 solvent elution was applied. The metabolomics strategy consisted of three different steps: a  
17 full mass spectral alignment of GC-MS data using MzMine 2.0, a multivariate analysis  
18 using Ez-Info and the creation of the statistical model with combinations of responses for  
19 molecular fragments. The model was finally validated using blind samples, obtaining an  
20 accuracy in oil classification of 70 % taking the official established method, “PANEL  
21 TEST” as reference.

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27 **1. INTRODUCTION**

28 Food quality is a matter of concern for consumers and producers. This quality is, in most  
29 cases, connected to the origin of the products, as it is the case for French wine  
30 (Arvanitoyannis, Katsota, Psarra, Soufleros, & Kallithraka, 1999) or Spanish ham  
31 (Sánchez-Peña, Luna, García-González, & Aparicio, 2005), and contributes to rise their  
32 prizes. For this reason and to avoid fraud, guarantee of the genuine quality is a critical step  
33 from the economical point of view. Thus, the characterization of this kind of products is an  
34 important aim where analytical chemists can be of great support.

35 In vegetable products, qualitative and quantitative analysis of volatile organic compounds  
36 (VOCs) has been an important issue of scientific interest for their organoleptic  
37 characterization. Thus, the determination of VOCs and characterization of volatile profiles  
38 in agricultural or feeding products, among others, are interesting work fields involved in  
39 assuring food quality (Dutta, Kashwan, Bhuyan, Hines, & Gardner, 2003; Ribes, Carrera,  
40 Gallego, Roca, Berenguer & Guardino, 2007). One of the most interesting studies is related  
41 to the determination of olive oil quality, as its classification is typically performed by  
42 testers who establish if an olive oil must be labelled as extra virgin, virgin or lampante (not  
43 recommended for consumption) (Council, 2016). This strategy is known as “PANEL  
44 TEST”, which classifies the oils according to two main properties: defects and goods. The  
45 classification requirements are established by the International Council of Olive Oil  
46 (Council, 2016), regarding their characteristics. These defects can be fusty, mouldy, sour  
47 and woody, and the goods (positive factors) can be fruity (specifying green attribute), bitter  
48 and spicy. According to the literature (Kalua, Allen, Bedgood Jr, Bishop, Prenzler &  
49 Robards, 2007; Luna, Morales, & Aparicio, 2006), the organic compounds responsible of

50 these flavours are typically esters, ketones, aldehydes, alcohols, terpenes, phenols and their  
51 derivatives, in concentrations ranging from few ng L<sup>-1</sup> to hundreds of mg L<sup>-1</sup> and with  
52 different odour thresholds. PANEL TEST methodology could be considered slightly  
53 subjective, as the opinion of testers may vary in such a significant way that the same  
54 sample can sometimes be designated as belonging to two different qualities at a time. These  
55 discrepancies can lead to a quality misclassification of an oil, causing considerable  
56 economical loses, commercial problems and fraud. A more objective alternative could be  
57 based in the use of chromatographic techniques coupled to mass spectrometry (MS), which  
58 could allow the determination of chemical composition of the volatile fraction of olive oil  
59 samples, even at really low concentration levels.

60 In this sense, the most adequate analysis technique is gas chromatography coupled to mass  
61 spectrometry (GC-MS) (Angerosa, Servili, Selvaggini, Taticchi, Esposto, Montedoro,  
62 2004; Flath, Forrey, & Guadagni, 1973).

63 According to the volatile characteristics of the compounds of interest, a specific extraction  
64 technique is also an important issue in order to perform a suitable separation from the  
65 matrix. From several studies on determination of VOCs in different matrices (Barco-  
66 Bonilla, Plaza-bolaños, Fernández-Moreno, Romero-González, Garrido, Martínez, 2011;  
67 Jiménez, Aguilera, Beltrán, & Uceda, 2006; Lam & Proctor, 2003; Salemi, Lacorte,  
68 Bagheri, & Barceló, 2006; Serrano, Beltrán, & Hernández, 2009), there is an evidence of  
69 the extended use of trapping processes of the compounds in some kind of sorbent; either by  
70 forcing them to pass through the sorbent bed (P&T) (Barco-Bonilla et al., 2011; Salemi et  
71 al., 2006) or letting them to establish an equilibrium between the vapour phase and the  
72 adsorbent in a closed place (SPME) (Pouliarekou, Badeka, Tasioula-margari, Kontakos,

73 Longobardi, & Kontominas, 2011; Serrano et al., 2009). Other techniques also used for  
74 VOCs extraction include: direct headspace (HS) injection (Hu et al., 2014), stir bar sorptive  
75 extraction (SBSE) (Bicchi, Iori, Rubiolo, & Sandra, 2002), or liquid phase micro extraction  
76 (LPME) (Lee, Lee, Rasmussen, & Pedersen-Bjergaard, 2008).

77 The determination of the chemical fingerprint of food samples is an interesting well-known  
78 approach for characterization of food products, for example in oil (Reboredo-Rodríguez, et  
79 al 2016, Reboredo-Rodríguez, González-Barreiro, Cancho-Grande, & Simal-Gándara,  
80 2012) or tomato samples (Vaz-Freire, Da Silva, & Freitas, 2009). Chemical fingerprint,  
81 normally obtained using targeted analyses, can be further used to classify unknown  
82 samples, but due to the fact that a limited number of compounds must be selected a priori,  
83 some information regarding the samples is lost. In this context, metabolomics, which is  
84 defined as "the unbiased, global screening approach to classify samples based on metabolite  
85 patterns or fingerprints that change in response to disease, environmental or genetic  
86 perturbations with the ultimate goal to identify discriminating metabolites" (Cevallos-  
87 Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009), can be an  
88 interesting approach to solve complex classification problems. One of the main drawbacks  
89 of metabolomics occurs when the relations or discrepancies between samples are just  
90 determined by a compound or group of compounds which are present at very low levels in  
91 complex matrices and can be easily dismissed. High resolution (HR) MS systems, such as  
92 time of flight (TOF)(Kind, Tolstikov, Fiehn, & Weiss, 2007) or magnetic sector (Kieken et  
93 al., 2009), enhance the detection of molecules in complex matrices at very low levels  
94 (Salihovic, Nilsson, Hagberg, & Lindström, 2013) due to their accurate mass measurements  
95 and extraordinary sensitivity, providing good results for the determination of non-target

96 compounds. Furthermore, the use of atmospheric pressure chemical ionization (APCI), a  
97 very promising ionization source in GC, which is softer than the common electron  
98 ionization (EI), allows to obtain high intensity peaks for the molecular ion ( $M^+$ ) and/or the  
99 protonated molecule ( $[M+H]^+$ ), depending on the nature of the compounds (Portolés,  
100 Sancho, Hernández, Newton, & Hancock, 2010). This information is really convenient as in  
101 some cases the molecular peak is absent in EI due to its high fragmentation and in terms of  
102 sensitivity, the peak intensity can be reduced. GC coupled to hybrid quadrupole time of  
103 flight (QTOF) MS equipped with APCI allows the acquisition of the accurate-mass full  
104 scan spectra at low and at high collision energy ( $MS^E$  mode) being a very useful tool for  
105 elucidation purposes.

106 Data processing, together with data acquisition, are the mainstays of metabolomics. The  
107 direct observation of sample chromatograms does not give significant information about the  
108 difference between samples quality, and thus specialized software is required to obtain  
109 chromatographic peaks and masses from raw data. In literature, metabolomics studies use  
110 different software to get the information needed from the chromatograms, such as XCMS  
111 package of R (Díaz, Pozo, Sancho, & Hernández, 2014), MetAlign (Tikunov et al., 2005)  
112 or MzMine 2.0 (Kind et al., 2007).

113 The aim of this work has been the development of a GC-(APCI)QTOF MS methodology to  
114 obtain chemical profile/fingerprint of olive oil volatile compounds in order to establish  
115 differences between virgin olive oil qualities using P&T extraction and through the use of  
116 metabolomics techniques in order to give a more objective decision in their classification  
117 compared with that provided by the “PANEL TEST”.

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## 119 **2. MATERIALS AND METHODS**

### 120 **2.1. Chemicals and reagents**

121 Internal standard triphenyl phosphate (TPP)  $\geq 99\%$  was purchased from Sigma Aldrich  
122 (Germany). Diethyl ether (residue analysis quality GC) and hexane (trace analysis quality  
123 (AT) GC) were provided by Scharlau (Barcelona, Spain).

124 Supelclean ENVI-Carb® SPE tubes 500 mg, volume 6 mL, 120-400 mesh, surface area 100  
125  $\text{m}^2 \text{g}^{-1}$ , used as traps were purchased from Supelco (Barcelona, Spain).

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### 127 **2.2. Olive oil samples**

128 A total of 425 olive oil samples were provided by the “Interprofesional del Aceite de Oliva  
129 Español” Organization (INTERPRO, Spain), the “Agencia para el Aceite de oliva del  
130 Ministerio de Agricultura, Alimentación y Medio Ambiente” and the oficial control  
131 services from the “Consejería de Agricultura, Pesca y Desarrollo Rural de la Junta de  
132 Andalucía”.

133 Oil samples were taken from different regions of Spain and included 300 quality  
134 characterized samples (120 extra virgin, 120 virgin and 60 lampante) and 125 blind samples  
135 (the quality was unknown during analysis). Samples were stored in freezer at  $-22\text{ }^\circ\text{C}$  until  
136 their use. Samples were characterized by means of pH measurements and physicochemical  
137 and organoleptic properties by the official participating laboratories (Laboratorio Arbitral  
138 Agroalimentario del MAGRAMA, Laboratorios Agroalimentarios de Córdoba y Atarfe de  
139 la Junta de Andalucía) and their corresponding certified “PANEL TESTs”.

140

141 **2.3. Sample treatment**

142 Olive oil samples were allowed to defrost at room temperature before analysis. Then, they  
143 were aliquoted in 4 different 10 mL vials. One aliquot was used to perform the extraction  
144 and the remaining ones were stored at 4 °C.

145 5 g of olive oil were weighted directly into a 150 mL flask before inserting a magnetic  
146 stirrer. The flask was rapidly closed with a glass tap with a nitrogen entrance and the exit  
147 connected to the sorbent trap (Envi-Carb cartridge). The cartridge was conditioned with 2 x  
148 5 mL of a mixture of hexane:diethyl ether (50/50; v/v) and vacuum dried for 10 min.  
149 Sample extraction was then carried out for 60 min at 40 °C with a nitrogen flow of 1 L  
150 min<sup>-1</sup> and with stirring at 300 rpm. After extraction, Envi-carb® cartridges were eluted by  
151 gravity with 5 mL of the hexane:diethyl ether mixture (50/50; v/v), into a glass tube  
152 previously weighted. 50 µL of TPP solution at 5 mg L<sup>-1</sup> (in hexane) were added as internal  
153 standard. The entire extract was then concentrated until total removal of diethyl ether, *i.e.*  
154 until approximately a final volume of 0.5 mL, under vacuum conditions with a MiVac Duo  
155 Concentrator (Genevac, Italy). Finally, in order to adjust the final volume of the extract (0.5  
156 mL of hexane), several drops of hexane were added until adjusted mass (0.3274 g). An  
157 aliquot of 100 µL of this extract was transferred to a 20 mL vial in order to generate a pull  
158 of extracts to prepare the quality control (QC) sample. The remaining extract was divided  
159 into two different vials with 200 µL inserts, sealed and stored in freezer at -20 °C until their  
160 analysis by GC-QTOF MS. In each extraction batch, 2 extra virgin, 2 virgin and 2 lampante  
161 oil samples were processed simultaneously.

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#### 164 2.4. GC-(APCI)QTOF MS

165 The chromatographic analysis were performed using an Agilent 7890A gas chromatograph,  
166 equipped with an Agilent 7693 autosampler, coupled to a quadrupole-TOF mass  
167 spectrometer, Xevo G2 QTOF MS (Waters Corporation, Manchester, UK), with APCI  
168 source. The GC separation was performed using a fused silica HP-5MS capillary column  
169 with a length of 30 m x 0.25 mm ID and a film thickness of 0.25  $\mu\text{m}$  (J&W Scientific,  
170 Folsom, CA, USA). The oven program was set as follows: 40  $^{\circ}\text{C}$  (3 min); 5  $^{\circ}\text{C}/\text{min}$  to 160  
171  $^{\circ}\text{C}$  (1 min); 50  $^{\circ}\text{C}/\text{min}$  to 300  $^{\circ}\text{C}$  (2.2 min) (total runtime 33 min). Injections of 1  $\mu\text{L}$  of  
172 sample extracts were performed using pulsed splitless mode (50 psi) at a temperature of  
173 270  $^{\circ}\text{C}$  with a pulse time of 0.90 min. Helium (99.999 %; Praxair, Valencia, Spain) was  
174 used as the carrier gas at a constant flow rate of 3  $\text{mL min}^{-1}$ .

175 The interface temperature was set to 310  $^{\circ}\text{C}$  using  $\text{N}_2$  as auxiliary gas at a flow rate of 150  
176  $\text{L h}^{-1}$ , the make-up gas flow rate was set at 300  $\text{mL min}^{-1}$  and the cone gas flow rate at 16  $\text{L}$   
177  $\text{h}^{-1}$ . The APCI corona pin was operated at 1.7  $\mu\text{A}$  with a cone voltage of 20 V. The  
178 ionization process occurred within an enclosed ion volume, which enabled control over the  
179 protonation/charge transfer processes. Water placed in an uncapped vial, which was located  
180 within a special designed holder placed in the source door, was used as modifier. Xevo  
181 QTOF MS was operated with a scan time of 0.4 s, acquiring the mass range  $m/z$  50–650.  
182 TOF MS resolution was approximately 18,000 (FWHM) at  $m/z$  614. For  $\text{MS}^E$   
183 measurements, two alternating acquisition functions were used applying different collision  
184 energies; 4 eV for low energy function (LE), and a collision energy ramp (10–40 eV) for  
185 high energy function (HE). Heptacose (PFTBA) was used for the daily mass calibration.  
186 Mass accuracy calibration was continuously performed using a background ion coming

187 from the GC-column bleed as lock mass (protonated molecule of  
188 octamethylcyclotetrasiloxane,  $m/z$  297.0830).

189 In order to avoid bias in the methodology, samples were analyzed in batches of 60 (20  
190 pertaining to each class, extra virgin, virgin and lampante) randomly distributed. In  
191 addition, in each analysis batch, 10 QCs were injected along the sequence (one QC every  
192 10 samples and 2 QCs at the beginning and at the end of the sequence).

193

## 194 2.5. Data processing

195 GC-QTOF MS data were converted from “.raw” to “.cdf” format using Databridge, a  
196 software provided by MassLynx v4.1 (Waters Corporation). Data mining was carried out  
197 using MzMine 2.0 software with the conditions indicated in Figure 1. The first part of the  
198 procedure (including cropping, mass detection, building chromatograms and baseline cut-  
199 off) led to a data matrix of areas corresponding to the markers detected in the form of  
200 combination of  $m/z$  value and retention time ( $m(m/z \text{ in Da})t(\text{time in sec})$ ). ~~Data mining was~~  
201 ~~performed by MzMine 2.0 software following the procedure described in Figure 1.~~

202 ~~Data were saved in a “.csv” file, exporting the row identification, the retention time, the~~  
203  ~~$m/z$ , the number of detected peaks and the peak area.~~ This matrix was then filtered to  
204 remove too low intensity peaks (could be considered noise) and unpaired markers (which  
205 were only present in a low proportion -less than 20%- of samples). The last step consisted  
206 in a “forced integration” of the considered markers in all samples, in order to eliminate the  
207 presence of zeroes in the final data matrix. The forced-integrated area was established as  
208 the 1 % of the background absolute signal at the considered RT, as this was the

209 normalization step which led to best results. Finally a last filter was applied to eliminate  
210 duplicated signals that corresponds to the same marker.

211 Data matrix obtained after applying MzMine 2.0 was exported to a text file (.csv). Using  
212 Microsoft Excel absolute areas were converted to relative areas using the area of one of the  
213 TPP m/z ions (m/z 327), which was quantified in each sample using TargetLynx®  
214 software. The relative areas were corrected with the nearest QC and then pareto scaled to  
215 minimize the impact of the peaks with higher standard deviation. In order to carry out the  
216 statistical analysis the EzInfo software (U-Metrics) was applied as it is easy to use and,  
217 from our previous experience, it allows to obtain well founded results (Díaz et al., 2014).

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219  
220

### 221 **3. RESULTS AND DISCUSSION**

#### 222 **3.1. GC-(APCI)QTOF MS**

223 Chromatographic analysis of sample extracts was carried out by GC-(APCI)QTOF MS  
224 working in MS<sup>E</sup> mode in order to get both maximum sensitivity and full information. From  
225 previous experiences in our laboratory (Cervera, Portolés, López, Beltrán, & Hernández,  
226 2014) it was expected that the soft ionization provided by the APCI source combined with  
227 low energy acquisition function, would allow to see the molecular ion or the protonated  
228 molecule (M<sup>+</sup> or [M+H]<sup>+</sup>), as only low fragmentation occurs. Additionally, the information  
229 acquired at high energy function (collision energy ramp of 10-40 eV) allowed obtaining the  
230 fragmentation pattern, which resulted very useful for elucidation purposes.

231 | As an example, **Figure S12** shows the accurate-mass full spectrum obtained for a  
232 | sesquiterpene, the  $\alpha$ -farnesene, acquired by GC-APCI-QTOF MS (at low and high energy)  
233 | comparing them with its corresponding EI (70 eV) spectrum from a commercial library  
234 | (NIST). The information about the protonated molecule in the low energy function together  
235 | with the fragmentation obtained in the high energy one was of great help in the elucidation  
236 | process, which followed the steps detailed in a previous work (Portolés, Pitarch, López,  
237 | Hernández, & Niessen, 2011). All instrumental parameters were selected according to  
238 | previous screening research works (Cervera et al., 2014; Portolés, Mol, Sancho, López, &  
239 | Hernández, 2014), which have been found to be reproducible and robust, basic  
240 | requirements of metabolomics. An example of the instrument performance is shown in Fig  
241 | S2, a total ion chromatogram obtained for the injection of a QC using the detailed  
242 | parameters.

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### 244 | 3.2. Extraction procedure optimization

245 | Taking into account the properties of the potential analytes, especially their volatility and  
246 | considering the advantages of obtaining a final liquid extract (that can be injected several  
247 | times, even in different chromatographic instruments) purge and trap (P&T) with solvent  
248 | trap elution was selected as extraction technique. This approach requires low economical  
249 | investment and provides high reproducibility with the advantage of keeping a second  
250 | extract vial for further analysis. In previous works a similar P&T method with subsequent  
251 | GC-MS analysis was developed in our research group for the determination of volatiles in  
252 | tomato and melon samples (Beltran, Serrano, López, Peruga, Valcárcel, & Roselló, 2006;  
253 | Fredes, Sales, Barreda, Valcárcel, Roselló, Beltrán, 2016), and it has been used as a starting

254 point for method development, although some changes were considered to improve its  
255 performance in application to olive oil samples.

256 Efficiency of two different sorbent materials was tested in the P&T step: Tenax TA® SPE  
257 and non-porous carbon Envi-Carb cartridges. For this purpose, aliquots of an extra virgin  
258 olive oil sample were extracted using Tenax (n=3) and Envi-Carb (n=3) with the same  
259 extraction conditions. The comparison of peak areas of some selected analytes in these  
260 chromatograms showed that both cartridges provided almost the same performance, as seen  
261 previously in melon samples (Fredes et al, 2016). Although Tenax can be a bit more  
262 efficient for some compounds (20% more area) both sorbents allow to extract quantitatively  
263 the majority of the volatiles from the sample. Moreover, compared to Tenax, Envi-Carb  
264 cartridges are commercially available (while Tenax cartridges are produced on demand,  
265 with longer supply times) and have an analysis cost of almost 3 times lower. Finally, Envi-  
266 Carb was selected to proceed with further development.

267

### 268 **3.3. Metabolomics optimization**

269 After optimization of extraction and chromatographic procedures, 425 oil samples  
270 (including 300 samples for model generation and 125 samples for model validation) were  
271 analyzed in several batches, both for extraction (6 samples each extraction batch) and  
272 chromatographic analysis (including samples, 10 QC and 10 blank samples in each  
273 sequence). Firstly considering a completely unbiased analysis, a Principal Component  
274 Analysis (PCA) was applied over the training data matrix corresponding to 300 olive oil  
275 samples. **Figure 2a** illustrates the **PCA** obtained.

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277 ~~Data mining was carried out using MzMine 2.0 software with the conditions indicated in~~  
278 ~~Figure 1. The first part of the procedure (including cropping, mass detection, building~~  
279 ~~chromatograms and baseline cut off) led to a data matrix of areas corresponding to the~~  
280 ~~markers detected in the form of combination of m/z value and retention time ( $m(m/z \text{ in}$~~   
281  ~~$Da)/(time \text{ in sec})$ ). This matrix was then filtered to remove too low intensity peaks (could~~  
282 ~~be considered noise) and unpaired markers (which were only present in a low proportion—~~  
283 ~~less than 20% of samples). The last step consisted in a “forced integration” of the~~  
284 ~~considered markers in all samples, in order to eliminate the presence of zeroes in the final~~  
285 ~~data matrix. The forced integrated area was established as the 1 % of the background~~  
286 ~~absolute signal at the considered RT, as this was the normalization step which led to best~~  
287 ~~results. Finally a last filter was applied to eliminate duplicated signals that corresponds to~~  
288 ~~the same marker.~~

289 ~~Data matrix obtained after applying MzMine 2.0 was exported to a text file (.csv). Using~~  
290 ~~Microsoft Excel absolute areas were converted to relative areas using the area of one of the~~  
291 ~~TPP m/z ions (m/z 327), which was quantified in each sample using TargetLynx® software.~~  
292 ~~The relative areas were corrected with the nearest QC and then pareto scaled to minimize~~  
293 ~~the impact of the peaks with higher standard deviation. In order to carry out the statistical~~  
294 ~~analysis the EzInfo software (U Metrics) was applied as it is easy to use and, from our~~  
295 ~~previous experience, it allows to obtain well founded results (Díaz et al., 2014).~~

296 ~~Firstly considering a completely unbiased analysis, a Principal Component Analysis (PCA)~~  
297 ~~was applied over the training data matrix corresponding to 300 olive oil samples. **Figure 3a**~~  
298 ~~illustrates an example of a PCA corresponding to analysis subset of 60 olive oil samples.~~

299 This PCA analysis seemed to be at least promising, as at first sight a rough distinction  
300 between “extra” and “lampante” can be seen, although “virgin” samples appeared in the  
301 middle and overlapped the two other groups. Additionally, this step allows to check the  
302 correct normalization of the samples by analyzing the behavior of the QC samples, ideally  
303 clustered in the center of the Score Plot. This type of analysis is employed for dimension  
304 reduction; as it is not guided, the QC samples joining in the center of the plot implies the  
305 correct normalization of the batch.

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306 The next step consisted in performing a Partial Least Squares-Discriminant Analysis (PLS-  
307 DA), which considers additional information about groups during classification process. It  
308 is a biased PCA which provides a better separation between groups based on some  
309 parameters. Thus, a PLS-DA was applied to construct a statistical model to find differences  
310 in volatile compounds composition between samples (see **Figure 3b2b**). As it can be seen,  
311 the PLS-DA analysis separated the three groups of samples slightly better, though some  
312 group overlapping was still present.

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313 Finally, in order to get information relative to the relevant compounds allowing sample  
314 classification, an Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) was  
315 applied to highlight the differences between groups facing two different groups at a time,  
316 regarding different compound concentration in samples. For this purpose, S-Plot graphics  
317 for all two-classes comparisons, Virgin vs Extra, Extra vs Lampante and Virgin vs  
318 Lampante, were obtained (**Figure 43**) and studied looking for endpoints. Theoretically, in  
319 an S-Plot the points with a p-corr value between 0.9 and 1 are closely related to the  
320 compounds characterizing the samples of one group, which constitute important class  
321 markers. Thus the most significant ions, *i.e.* the further from the center in the S-Plot and

322 with a p-corr close to 1, were selected as tentative markers. According to this, a total of 15  
323 markers (corresponding to combination of an m/z ion and retention time) were selected and  
324 the corresponding chromatographic peaks, processed with TargetLynx® for a better  
325 precision of peak areas. **Table 1** shows the data for the 15 markers, together with the  
326 assigned elemental compositions for the protonated molecule and for the main fragment  
327 ions. In most cases they are labelled as *markers* as the high number of possible compounds  
328 with the same fragments made unfeasible to unequivocally identify them. We have been  
329 able to completely identify some of the markers even with the confirmation through  
330 injection of reference standards,

331 | An example of the elucidation process of a marker can be found in **Figure S14**, with the  
332 experimental APCI accurate-mass spectra obtained by GC-QTOF MS for the high and low  
333 collision energy functions and with the possible chemical structures proposed for the most  
334 abundant fragment ions together with the experimental mass errors (in mDa). The structure  
335 was assigned to ethyl-2-methylbutanoate and was then confirmed by the injection of its  
336 standard. This compound has been already identified by some authors in olive oil samples  
337 (Dierkes, Bongartz, Guth, & Hayen, 2012; Kiralan, Ozkan, Koyluoglu, Ugurlu, Bayrak, &  
338 Kiritsakis, 2012) and has been reported as related to the fruity, sweet and green apple  
339 flavour.

340 Finally, considering the 15 markers selected, a series of linear combinations of these ions  
341 were studied in order to achieve the best separation possible between groups, and a model  
342 for the classification of the three groups was built. This model was based in a combined  
343 double classification, first differentiating between Extra/Virgin oils and Virgin/Lampante  
344 oils and then distinguishing among the three different classes.



345 As an efficiency pre-test, the samples used to build the model were classified using it,  
346 comparing the model classification results with the classes assigned by the corresponding  
347 “PANEL TEST”, which was considered as reference. After the first classification, which  
348 distinguishes between only two classes, “extra” and “lampante” oils, nearly 90 % of oils  
349 were correctly classified, considering that “virgin” oils can be present in both groups. Then,  
350 a second classification was applied to both groups, allowing a final distinction between the  
351 three different classes. After the application of these series of binary classifications, around  
352 70 % of the samples were correctly classified as extra, virgin or lampante. In all cases, the  
353 worst classification efficiency corresponded to virgin samples, which are always the most  
354 difficult to assign.

355 In order to take the maximum advantage possible from the samples, PLS-DA and OPLS-  
356 DA analysis considering the defects perceived by the PANEL TEST were created. As it can  
357 be seen in **Fig S3a**, the samples cannot be easily separated by defects with a PLS-DA, as  
358 the overlapping of samples with different defects is quite important. From **Fig S3b-S3e**  
359 obtained facing samples with each kind of defect with the flawless extras in several OPLS-  
360 DA analyses, a clearer distinction is observed, which points out the fact that, from an  
361 analytical point of view, the extra samples are easier to classify from the rest by the absence  
362 of defect rather than the presence of a good attribute. This preliminary approach to the  
363 study of the defects shows a promising potential for the refining of the classification  
364 methodology, and will be further studied in depth with an appropriate design to maximize  
365 the outputs of the research, but they won't be analysed in more detail here.

366

### 367 **3.4. Model Validation**

368 Once the model was created with the 300 training samples, it was validated using the 125  
369 blind samples to evaluate its efficiency. Results obtained for the ternary classification for  
370 the blind samples were sent to the project coordinating centre in order to evaluate the  
371 accuracy and the performance of the model. As it can be seen in **Figure 5**, extra samples  
372 were always correctly classified as extra or, in some few cases, as virgin, but never as  
373 lampante, which would lead them to be discarded with a high economic loss. In addition,  
374 there was a low portion of virgin oils which got classified as lampantes and a small quantity  
375 of lampantes which got overrated. It is important to highlight that the method is able to  
376 distinguish the samples which have clear defects and poor fruity properties, as the match in  
377 the first binary classification is around 90%. It is also remarkable that almost none of the  
378 lampantes (a residual 5%) are overclassified as extras. This can be very useful in discarding  
379 clear lampante samples and filtering the amount of samples which have to be tested by the  
380 test panel, being an important complement to it. The distinction between lampantes and  
381 virgins, as they usually share defects, has shown to be the most difficult, with a  
382 considerable number of lampantes classified as virgin. This is also agreement with the  
383 preliminary results of the PLSDA and OPLS-DAs obtained considering the defects found  
384 in the samples (**Fig S3**), and the efforts of future works will focus on the refining of the  
385 methodology from those two perspectives.

386

#### 387 **4. CONCLUSIONS**

388 A simple and efficient methodology has been developed for the analysis of volatile  
389 compounds in olive oil samples, with a prior extraction by P&T technique and subsequent  
390 analysis by GC-(APCI)QTOF MS.

391 The use of GC-(APCI)QTOF MS has allowed to work in MS<sup>E</sup> mode, which provides  
392 information at low and high collision energies which has been proved to be an important  
393 added benefit in order to elucidate the markers. The developed method has demonstrated to  
394 be sensitive enough and capable of obtaining a considerable number of resolved  
395 chromatographic peaks for volatile compounds. The use of APCI source with water as  
396 modifier has favoured the formation of the protonated molecules ( $[M+H]^+$ ) which, added to  
397 the soft in source fragmentation, enhanced the sensitivity for most of the compounds when  
398 compared to the traditional EI source.

399 The methodology was evaluated in terms of accuracy, obtaining an overall accuracy near to  
400 70 % on 125 blind samples, discarding only lampante samples and a low percentage of  
401 virgin samples, which makes it perfect for being a complementary analysis together with  
402 the “PANEL TEST” method.

403 The metabolomics-based approach allowed the classification of the samples with a  
404 considerable performance and a minimal data treatment after the method was built. Among  
405 the different software used, the combination of MzMine 2.0 and Ez-Info dropped the best  
406 results. MzMine 2.0 allowed the inclusion of a large number of samples in order to build  
407 the model, and the addition of new samples to expand the population taken to create it. On  
408 the same way, Ez-Info provided reliable results with a really simple user interface and was  
409 preferred to other software due to the previous experience working with it.

410 In order to improve the classification method, a higher number of samples should be  
411 required to include every possible combination of defects and goods. Considering the huge  
412 variety of possible defects and goods than can give a specific classification to the samples,

413 the overall accuracy of 70 % achieved enable the developed methodology to be an  
414 important complement to the official PANEL TEST.

415

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424 del Aceite de Oliva Español”.

425

426

#### 427 **FIGURE CAPTIONS**

428 **Figure 1:** Workflow followed for chromatographic data treatment on MzMine 2.0.

429 **Figure 2:** PCA (a) and PLS-DA (b) figures corresponding to the samples analysed to create  
430 the model.

431 ~~Comparison between the MS spectrum of a farnesene obtained by (A) electron ionization~~  
432 ~~(NIST library), and by APCI-QTOF (MS<sup>E</sup> mode) (B) high energy function and (C) low~~  
433 ~~energy function. **Figure 3:** SPLoTs Extra vs Virgin, Virgin vs Lampante and Extra vs~~

434 Lampante. Tentative markers are enlightened in red squares and designated by their  
435 corresponding M(m/z)T(Rt). ~~Example of a PCA (a) and PLS DA (b) figures corresponding~~  
436 ~~to one batch analysis of 60 samples.~~

437 **Figure 4:** GC-(APCI) QTOF MS narrow-window extracted ion chromatogram (mass  
438 window 0.02 Da) showing the detection of a possible marker, tentatively identified as  
439 ethyl-2-methylbutanoate- Experimental APCI accurate-mass spectra for the high collision  
440 energy function and for the low collision energy function are also shown with the chemical  
441 structures proposed for the most abundant fragment ion together with the experimental  
442 mass errors (in mDa). ~~SPLoTs: Extra vs Virgin, Virgin vs Lampante and Extra vs~~  
443 ~~Lampante. Tentative markers are enlightened in red squares and designated by their~~  
444 ~~corresponding M(m/z)T(Rt).~~

445 **Figure 5:** Confusion matrix showing the Comparison-comparison between the objective  
446 (up) of getting every blind sample classified as “PANEL TEST” classification and the  
447 results (down) after processing these unknown samples through the entire developed  
448 procedure.

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449 **Figure S1:** Comparison between the MS spectrum of  $\alpha$ -farnesene obtained by (A) electron  
450 ionization (NIST library), and by APCI-QTOF (MS<sup>E</sup> mode) (B) high energy function and  
451 (C) low energy function. ~~GC-(APCI) QTOF MS narrow-window extracted ion~~  
452 ~~chromatogram (mass window 0.02 Da) showing the detection of a possible marker,~~  
453 ~~tentatively identified as ethyl 2-methylbutanoate- Experimental APCI accurate mass~~  
454 ~~spectra for the high collision energy function and for the low collision energy function are~~  
455 ~~also shown with the chemical structures proposed for the most abundant fragment ion~~  
456 ~~together with the experimental mass-errors (in mDa).~~

457 Figure S2: Full-scan total ion chromatogram (TIC) obtained by the injection of a QC.

458

459 Figure S3: (A) PLS-DA analysis based on the defects determined by the PANEL TEST in  
460 a subset of 150 samples used for the creation of the model. OPLS-DA analysis facing (B)  
461 “Atrojado” defect versus no defect, (C) “Agrio” defect versus no defect, (D) “Moho” defect  
462 versus no defect and (E) “Heno” defect versus no defect. Darkened area highlights the  
463 region of major predominance of the defect.

464

465

#### 466 **Conflict of Interest**

467 The authors declare no conflict of interest

468

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