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Olive oil mixtures. Part one: decisional trees or how to verify the olive oil percentage in declared blends.

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ABSTRACT

The commercialization of declared blends of olive oil and seed oil is something long approved by the European Union. There, the olive oil percentage must be at least 50 % if the producer aims to advertise its presence on the front label, i.e., somewhere other than in the ingredients list. However, the Regulation did not propose any method to verify such proportion. For this purpose, we recommend the use of decisional trees, being the parameters under study those in which the greatest differences between olive and seed oils are shown: triacylglycerols, acyclic saturated hydrocarbons, free sterols, and tocopherols. In this way, to guarantee the presence of olive oil at 50 % : i) palmitodiolein must be above 11-15 %; ii) the β/γ -tocopherol ratio must be below 2.4; iii) the alkane sum C21-C25 should be higher than 3.5-6 %; and iv) the total sterol content cannot surpass 2400 mg/kg.

Keywords: decisional tree, declared blends, OLEUM Project, olive oil percentage, oil labelling, seed oil.

Chemical compounds studied in this article:

Beta-tocopherol (PubChem CID: 6857447); Gamma-tocopherol (PubChem CID: 92729);
Heneicosane (PubChem CID: 12403); Palmitodiolein (PubChem CID: 25240174); Pentacosane
(PubChem CID: 12406); Sterols (PubChem CID: 12303662); Tricosane (PubChem CID: 12534)

37

38 **1. Introduction**

39 According to the International Olive Council (IOC) ‘virgin olive oils are oils obtained from the fruit
40 of the olive tree (*Olea europaea* L.) solely by mechanical or other physical means under conditions,
41 particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone
42 any treatment other than washing, decantation, centrifugation, and filtration’ (IOC, 2016).

43 The paramount importance of olive oil in the global market derives from three phenomena: First of
44 all, the role of the European Union as the most important producer and consumer of this kind of oil
45 in the world. Actually, it accounts for 66 % of the production according to the IOC predictions for
46 season 2017-18 (IOC, 2018). Secondly, the fact that more than 20 non-EU countries (many of them
47 out of the Mediterranean area) are developing a certain olive oil culture, increasing their domestic
48 production and rising the competitiveness of the global olive oil market. Thirdly, the role of olive oil
49 as a pool of healthy constituents which has no comparison with other edible fats and oils thanks to
50 both, its beneficial fatty acid composition, where oleic acid can be present at concentrations as high
51 as 83 % (IOC, 2016), and to its minor compound profile responsible for its antioxidant activity,
52 sensory characteristics, and overall complexity (Gómez-Coca, Pérez-Camino, and Moreda, 2015).

53 It is then justified that olive oil price and reputation had boosted over the years and so had its
54 attractiveness as a target for adulteration, either by illegal blending with vegetable oils other than
55 olive oil or by deliberate mislabeling. In this context, the European Parliament pointed out that olive
56 oil was one of the foods which was most at risk of suffering fraudulent activities (European
57 Parliament, 2014). This situation was of high concern due to the potential impact on the market’s
58 confidence. As a consequence, the European Commission was requested to give it full attention which
59 finally ended up in the so-called OLEUM Project (2016). The Project’s global goal evolves around

olive oil fraud detection by both improving the existing analytical methods and developing new strategies of analysis. It has been organized in seven work packages distributed in a number of tasks. This work focuses on Work Package 4 ('Analytical solution addressing olive oil authentication issues'), specifically on the study of legal blends between olive oils and other vegetable oils.

The concept of 'legal blends' arises from the authorization of the European Commission to market blends of olive oil with other vegetable oils and to highlight the presence of olive oil on the labelling, and not just on the ingredient list, only if it accounts for at least 50 % of the blend (European Commission, 2012). This last requirement evidenced a major problem: The lack of analytical methods to control the percentage of olive oil in declared mixtures. Truly, when conducting a bibliographical search through the SciFinder database using terms like 'olive oil legal blends', and 'olive oil commercial blends' as research topics, it is unmistakable that most of the work focuses on the detection of olive oil adulterations with low quality olive oils or with vegetable oils other than olive (Ou, Hu, Zhang, Li, Luo, and Zhang, 2015; Santos, Kock, Santos, Lobo, Carvalho, and Colnago, 2017), and on authentication issues such as that of monovarietal oils (Agrimonti, Vietina, Pafundo, and Marmiloli, 2011; Da Ros, Masuero, Riccadonna, Brkic Bubola, Mulinacci, Mattivi, Lukic, and Vrhovsek, 2019) or geographical origin confirmation (Gertz, Gertz, Matthäus, and Willenberg, 2019; Vera, Jiménez-Carvelo, Cuadros-Rodríguez, Ruisánchez, and Callao, 2019; Quintanilla-Casas, Bertin, Leik, Bustamante, Guardiola, Valli, Bendini, Gallina Toschi, Tres, and Vichi, 2020). Although some of these approaches can be applied with semi-quantitative purposes, like the use of DNA-based methodologies for the detection of olive oil in commercial products and plant oils (Ramos-Gómez, Busto, Albillos, and Ortega, 2016; Alonso-Rebollo, Ramos-Gómez, Busto, and Ortega, 2017), very few of them really focus on verifying the percentage of olive oil in fraudulent blends with other vegetable oils. Such is the case of Santos and coworkers (Santos et al., 2017) who used Time-Domain NMR Relaxometry (TD-NMR) to detect olive oil adulteration with seed oils present at high concentrations. Specifically, they tested this approach on olive oil samples mixed with soybean oil at different concentrations and were able to separate them according to the adulteration

86 level (i.e. 25, 50, and 75 % adulterant) applying Principal Component Analysis to the TD-NMR
87 results. Those results are promising but the technique is an expensive one and has not been tried with
88 controlled blends (e.g. blends prepared in a laboratory and whose composition is actually well
89 known). Other approaches to quantify EVOO in commercial blends have been proposed by Aroca-
90 Santos and colleagues, but the 50:50 proportion was not included (Aroca-Santos, Lastra-Mejías,
91 Cancilla, and Torrecilla, 2019). So far, and to the best of our knowledge, the only lines of research
92 developed *ad hoc* for declared blends were those of de la Mata (de la Mata, Domínguez-Vidal,
93 Bosque-Sendra, Ruiz-Medina, Cuadros-Rodríguez, and Ayora-Cañada, 2012) and of Monfreda
94 (Monfreda, Gobbi, and Grippa, 2012). De la Mata applied attenuated total reflection Fourier
95 transform infrared spectroscopy, together with chemometric analysis, and obtained very promising
96 results: They tested 76 mixed samples, some of them with olive oil at 50 %, and were able to classify
97 them correctly, although the model has some limitations -as the authors pointed out- that make it to
98 be considered as semi-quantitative. Monfreda used the fatty acid composition of the samples followed
99 by chemometric tools to classify the oil blends under study. The advantage of this proposal is clear,
100 since they used an official method for olive oil analysis highly established in laboratories devoted to
101 this matter. However, although they took into account the possible variability among olive oil
102 composition, their main limitation relies in the fact of having used a single sunflower oil sample,
103 whereas de la Mata took into account the unevenness encountered by the differences between olive
104 cultivars and by the dissimilar seed oils (although, again, including just one kind sunflower oil).
105 Accordingly, it is the purpose of the present work to design an analytical strategy in order to confirm
106 if the amount of olive oil in a label-claimed blend is at least 50 %, using two of the most representative
107 seed oils: normal type and high oleic sunflower oils. Our hypothesis is that there is actually no need
108 of developing new methods of analysis, neither of applying chemometric, metabolomic or genomic
109 strategies -the latter usually expensive-, all of them of great help either themselves or in combination
110 with other approaches in olive oil authentication and purity assessment (Agrimonti et al., 2011;
111 Bosque-Sendra, Cuadros-Rodríguez, Ruiz-Samblás, and de la Mata, 2012; de la Mata, et al. 2012;

112 Gómez-Caravaca, Maggio, and Cerretani, 2016; Avramidou, Doullis, and Petrakis, 2018; Da Ros et
113 al., 2019). Yet, it would be enough if one combines properly some of the official purity parameters
114 described in the legislation (IOC, 2016). In order to test it we decided to focus on the parameters in
115 which the greatest differences between olive and seed oils were to be observed and ended up in the
116 fact that not only one, but four different determinations were needed to discern olive oil concentration,
117 as was to be expected due to the complexity of olive oil chemical composition. In this sense, we
118 considered triacylglycerols (TAG), acyclic saturated hydrocarbons (SHC), free sterols (FS), and
119 tocopherols (TCPH), and we organized them in the form of decisional trees in a way that the blend
120 who claims to be composed of at least 50 % olive oil must comply not just with one but with four
121 terms. We must point out that we decided to use sunflower oils as representative of seed oils, since it
122 is the edible oil most used in this kind of mixtures. Actually, other studies pondering the same problem
123 than we consider here take sunflower as model seed oil too (Monfreda, et al., 2012).

124

125 **2. Materials and methods**

126 **2.1 Analytical materials and reagents**

127 All reagents and solvents were super purity or HPLC grade unless otherwise stated. Anhydrous
128 pyridine, 2',7'-dichlorofluorescein solution, dichloromethane, and hexamethyl disilazane were from
129 Honeywell (Fisher Scientific SL, Madrid, Spain). Acetone, ethanol, ethyl acetate, ethyl ether,
130 methanol, n-hexane, n-heptane, propan-2-ol, propionitrile and trimethylchlorosilane were supplied
131 by VWR International, LLC (West Chester, Pennsylvania, USA), anhydrous sodium sulfate and
132 potassium hydroxide by Panreac Quimica, S.A.U. (Castellar del Valles, Barcelona, Spain). The
133 standards 5 α -cholestan-3 β -ol, α -, β -, γ -, and δ -tocopherol, and n-eicosane (C20) were purchased at
134 Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Silica gel 60 for column chromatography, 70-
135 230 mesh (Merck KGaA, Darmstadt, Germany) was used directly from the container. Glass
136 chromatography columns (50 cm length x 1.5 cm i.d.) were supplied with Teflon stopcocks; a plug
137 of glass wool fiber was placed at the bottom and everything was washed with n-hexane before use.

138

139 **2.2 Instrumentation**

140 The TAG determination was carried out according to the IOC method (IOC 2017a) using 1 g SPE
141 silica gel cartridges (Varian, Inc., Harbor City, CA) for the intact analyte separation (i.e. TAG are
142 isolated as such and with no chemical transformation), and reverse phase high-resolution liquid
143 chromatography (RP-HPLC) with propionitrile as mobile phase at 0.6 mL/min flow rate in order to
144 get trilinolein elution at around 12.5 min. These RP-HPLC analyses were carried out by dissolving
145 the purified extract in 2 mL acetone and injecting 10 μ L onto an HPLC system equipped with a
146 Beckman Gold 508 autosampler (Beckman-Coulter, Fullerton, CA, USA) and a Lichrosphere 100
147 RP-18 phase column (4 μ m particle size, 25 cm x 4 mm i.d.). The system was set with a Beckman
148 Gold 126 pumping component (Beckman-Coulter, Fullerton, CA, USA), a Perkin Elmer 200
149 refractive index detector (RID) (Perkin Elmer, Norwalk, CT, USA), and a Beckman Mistral Peltier
150 thermostat oven (Beckman-Coulter, Fullerton, CA, USA) in order to keep the column temperature at
151 20 °C (Moreda, Pérez-Camino, and Cert, 2003). The peaks on the HPLC chromatogram were
152 integrated by the data acquisition system, grouped according to their equivalent carbon number
153 (ECN) and identified after the pattern given in the official method (IOC, 2017a).

154 The FS, profile and content were determined as it is described by Cert, Moreda, and García-
155 Moreno (1997). In short: after sample saponification with a 2 M ethanolic potassium hydroxide
156 solution and extraction of the unsaponifiable matter with diethyl ether, the analytes are separated
157 using a Beckman Coulter HPLC system (Beckman Coulter Inc., CA, USA) provided with a System
158 Gold 125P Solvent Module, a System Gold 166P Detector, and a System Gold 508 Autosampler (200
159 μ L injection). The separation was done through a LiChroCART 250-4 Superspher Si 60 cartridge (5
160 μ m particle size; Merck KGaA, Darmstadt, Germany), using as mobile phase a hexane:diethyl ether
161 50:50 (v/v) mixture at 1 mL/min. The FS fraction was collected using a Gilson FC 203B fraction
162 collector (Gilson Inc., WI, USA). The FS were analyzed as trimethylsilyl ethers by capillary column
163 gas chromatography (GC) with a flame ionization detector (FID). The GC analyses were carried out

164 with an Agilent 6890N Gas Chromatograph (Agilent Technologies, Santa Clara, California) equipped
165 with an Agilent 7683B Automatic Liquid Sampler and a FID. Acquisition of data was done with the
166 Agilent ChemStation for GC System program. The conditions for the GC assays were: TRB-5HT
167 column (5 % diphenyl-95 % dimethylpolysiloxane; 30 m x 0.25 mm ID x 0.25 μ m film; Teknokroma,
168 Sant Cugat del Vallés, Barcelona, Spain), 1.0 μ L injection volume, hydrogen carrier gas at 1.9
169 mL/min, and 10:1 split injection. The oven worked isothermally at 255 °C during 40 min. The detector
170 temperature was 300 °C. The quantitative evaluation was carried out using α -cholestanol as internal
171 standard considering that the response factor for all sterols equaled 1.

172 The method of analysis of the SHC fractions was based on the official method for the analysis of
173 stigmastadienes in vegetable oils (Cert and Moreda, 1998; IOC 2017b) which uses column
174 chromatography (CC) on silica gel to separate alkanes from steroidal hydrocarbons. Summarizing,
175 this arrangement required 15 g of silica gel to be suspended in a container with 40 mL n-hexane, and
176 then poured into a column prepared with a plug of glass wool fiber at the bottom. The packing was
177 allowed to settle, a small amount of anhydrous sodium sulfate was added on top, and the excess of n-
178 hexane eluted. The silica bed was cleaned with 60 mL n-hexane and the oil together with the C20 IS
179 transferred to the top of the column. Linear SHC were then eluted with 35 mL n-hexane. The
180 equipment for the GC analysis was identical to that described for FS analysis. The GC conditions
181 were: DB-5HT column (5 % diphenyl-95 % dimethylpolysiloxane; 15 m x 0.32 mm ID x 0.10 μ m
182 film; Agilent Technologies, Santa Clara, California), 1.0 μ L injection volume, hydrogen carrier gas
183 at 5 mL/min and cool on column injection. The initial oven temperature was set at 70 °C for 2 min,
184 then raised at 12 °C/min until 280 °C, and finally at 7 °C/min until 340 °C, holding for 5 min. The
185 detector temperature was 380 °C. The quantitative evaluation was carried out using C20 as internal
186 standard considering that all alkanes had the same response coefficient.

187 Finally, TCPH measurements were carried out by HPLC analysis on silica gel column and
188 fluorescence detection (FLD) setting the excitation and emission wavelengths at 290 and 330 nm,
189 respectively. The analytical procedure consists on dissolving 10 mg oil in 1 mL n-hexane and

190 injecting it into an HPLC system set with a Superspher Si 60 column (4 μm particle size, 25 cm x 4
191 mm i.d.) and equipped with a Hewlett Packard 1050 Series pumping component and a RF-10AXL
192 Shimadzu detector. The quantitative evaluation was carried out by external standardization using each
193 standard to build the respective calibration curve (concentrations from 4 to 6 $\mu\text{g/mL}$ in n-hexane).
194 This procedure is based on the IUPAC Standard Method 2432 (IUPAC, 1988).

195

196 **2.3 Samples**

197 Samples were provided by Fera (Fera Science Ltd, Sand Hutton, York) within the frame of the
198 OLEUM Project. Individual (not blended) oils arrived at the laboratory in August 2017; these were:
199 extra virgin olive oil (EVOO), olive oil (OO), refined high oleic sunflower oil (HOSO) and refined
200 normal type sunflower oil (NTSO). In this way, we would prepare our own blends after the
201 instructions depicted on the Project's analytical plan (e.g. controlled temperature conditions). Those
202 blends consisted on binary mixtures of each of the olive oils with every sunflower oil at 60:40, 50:50,
203 and 40:60 v/v proportions. Lastly, in November 2018 we were provided with a batch of blind
204 commercial samples collected in the Swedish market (#1-#8) which consisted of EVOO, virgin olive
205 oil (VOO), or OO, and one oil other than olive such as rapeseed oil (RSO), NTSO, or a non-identified
206 vegetable oil (VGO). The composition of sample #8 was not given.

207 All samples had been prepared and bottled under a headspace of nitrogen to increase their stability,
208 and stored at 4 °C until their dispatch. Once in the laboratory, they were kept at analogous conditions
209 protected from light, until we were ready to perform the experimental work. At that moment, samples
210 were taken from the cold storage, left to equilibrate at room temperature a minimum of 6 hours and
211 shaken vigorously before extracting any aliquot.

212

213 **3. Results and discussion**

214 TAG, FS, SHC, and TCPH were investigated in order to use them as markers for the determination
215 of the correct proportion of olive oil in legal blends. They were chosen because it is there where the

216 greatest differences between seed oils and olive oils appear, and have been often selected to detect
217 small amounts of vegetable oils other than olive oil in genuine olive oils (Christopoulou et al., 2004).
218 TAG are the main components of olive oil and they are mostly responsible for its principal features.
219 In fact, their usefulness becomes patent when approaches such as that of Chemometrics in
220 combination with TAG composition are used for authentication purposes (Bosque-Sendra et al., 2012;
221 Gertz et al., 2019) or in the discrimination between oil blends according to their olive oil percentage,
222 this latter one actually through the use of spectroscopic techniques (de la Mata et al., 2012). From a
223 more classical point of view, chromatography methods are utilized in TAG analysis and even if
224 traditionally the only compounds considered in an HPLC chromatogram were trilinolein (LLL) first,
225 and the equivalent carbon number (ECN) 42 TAG group later (Christopoulou et al., 2004; Aparicio,
226 Conte, and Fiebig, 2013), we decided to study the whole TAG profile. Table 1 shows the results of
227 the analysis of the four different mixtures of olive and sunflower oils at the three different proportions
228 under study. Although sunflower oil and olive oil normally exhibit considerably different fatty acid
229 and TAG composition (Christopoulou et al., 2004), if one compares the content of the various TAG
230 among the different oil proportions within a given blend it is clear that, on the one hand, except for
231 the OOO+PoPP pair (eluted as one chromatographic peak within the ECN48 group) and for POO, all
232 the species can be considered to maintain a constant value within the error limits -i.e. mean+standard
233 deviation (SD)- regardless the percentage of (EV)OO added. Besides, the SD is too high for some of
234 the TAG present at the lowest concentration such as OLLn or PLLn, which is usual when small
235 chromatographic peaks have to be -manually- integrated. On the other hand, the OOO+PoPP pair
236 showed one of the highest shifts from one blend to another, but such changes were not consistent,
237 that is to say, the OOO+PoPP concentration does not just decrease by decreasing the presence of olive
238 oil in the mixture, but it is also highly influence by the kind of sunflower oil added, in a way that in
239 blends with NTSO the lower the amount of olive oil, the lower the percentage of the OOO+PoPP
240 pair, whereas this trend reverses if HOSO is included. Actually, one can observe that in those latter
241 blends the lower the amount of olive oil, the *higher* the OOO+PoPP concentration, which is due to

242 the fact that HOSO can form more OOO than NTSO, presumably, thanks to the oleic acid availability.
243 However, if we observe the results in the case of POO (emphasized in bold letters), significant
244 differences among mixtures at dissimilar proportions appeared, indicating that such species is actually
245 affected by the presence of (EV)OO at different quantities and by the kind of sunflower oil (normal
246 type or high oleic) used, although in the same sense. In fact, in every case the higher the amount of
247 (EV)OO in the sample, the higher the percentage of POO, and on each blend the POO concentration
248 is higher if HOSO, and not NTSO, is the second component of the mixture. One could argue that in
249 the TAG chromatograms POO and SOL elute together in a single peak, but the amount of SOL in
250 olive oil is generally very low (below 0.4 %), since the presence of stearic acid is also scarce (between
251 0.5 and 5 %).

252 A more clear effect of the blend composition on the POO concentration may be observed in Table
253 S1 (Supplementary material), where the differences (Δ) between the mean values corresponding to
254 the 50:50 and 40:60 (EV)OO:(NT/HO)SO blends are given, together with the respective SD.
255 According to our results, a 10 % increment in olive oil (i.e., from 40:60 to 50:50 olive oil:sunflower
256 oil mixtures) rises the percentage of POO around 8-9 % in mixtures with HOSO, whereas the increase
257 is around three times higher (23 % and 30 %) in blends with NTSO. These obvious differences
258 demonstrate that even if HOSO can form much more POO than NTSO, it is not able to form as much
259 as olive oils, probably due to the differences in the substrate specificity and selectivity of the
260 acyltransferase enzymes involved in the TAG the biosynthetic pathway. Besides, according to our
261 experience, low POO concentrations are to be expected for seed oils. In the case of POO, such
262 differences are clearly above the method's uncertainty (i.e. there is a clear differentiation regardless
263 the error limit) for each of the cases under study. In view of such results, we may postulate that in
264 order to ascertain that (EV)OO is at least at 50 % in a mixture with sunflower oil, the percentage of
265 POO must be above 11 % if NTSO has been added, whereas it must go beyond 15 % in the case of
266 HOSO. Really, if we calculate the lower limit of the possible percentage range applying one SD (the
267 most conservative option in this case), the POO concentration for both 50:50 blends (with NTSO and

268 HOSO) are still above 11 % (actually it would be 11.6 % and 11.12 %, respectively), whereas the
269 calculation of the upper range for the 40:60 mixtures considering this time 3SD (9.96 % and 14.5 %,
270 respectively) would still be below the set limits, supporting our decision of taking 11 % and 15 % as
271 cut-off limits.

272 The second group of analytes under considerations were sterols (4-desmethyl sterols). To this
273 respect our results are shown in Table 2. Sterols are of utmost importance when analyzing vegetable
274 oils since they are known to be their analytical fingerprint. Countless efforts have been made to
275 unravel the many forms of sterols that one may encountered in vegetable oils in general, and in olive
276 oil in particular (Gómez-Coca, Fernandes, Aguila-Sánchez, Pérez-Camino, and Moreda, 2014).
277 Actually, these species are of special importance in the case of olive oil (Barjol, 2013) since their
278 application in its authentication goes back to the eighties (Brumley, Sheppard, Ridolf, Yasaei, and
279 Sphon, 1985). It was demonstrated, for instance, that the addition of sunflower or soybean oils boosts
280 the stigmasterol, campesterol and, in the case of sunflower oil, Δ^7 -stigmastenol percentages above
281 those found in pure olive oil, whereas brassicasterol is often useful to detect the addition of rape seed
282 oil (Grob, Giuffré, Leuzzi, and Mincione, 1994; Alonso, Fontecha, Lozada, and Juarez, 1997).
283 According to these statements, when we determined the sterol composition and content of the blends
284 under study the first hint of the presence of seed oil was the high campesterol concentration which,
285 as expected, was above the legal limit for any virgin olive oil (IOC, 2016). Actually, the higher the
286 share of sunflower oil in the blend, the higher the campesterol level. Nonetheless, when pondering
287 on taking this free sterol as indication for the presence of seed oil above the 50 % limit (and therefore,
288 that of olive oil below the threshold set by the European authorities), its concentrations for the 50:50
289 and 40:60 olive oil:seed oil samples (v/v) were the same within the error limits (one SD). The
290 respective ranges overlapped with each other, evidencing that a 10 % difference in the (EV)OO
291 presence did not have a clear enough effect on the campesterol percentage. The same held for other
292 seed-oil-characteristic sterols like stigmasterol and Δ^7 -stigmastenol. Apparent β -sitosterol, on the
293 other hand, was *below* the expected limit for virgin olive oils but again, not in a way that allowed us

294 to use it as a marker (data not shown). Nevertheless, the fact of not being able to use individual sterols
295 as indicators to assess the presence of olive oil does not turn the total sterol content in a useless
296 parameter. On the contrary, a maximum limit of 2400 ppm can be set to assure that the proportion of
297 (EV)OO in a blend with sunflower oil is at least 50 %. This is supported by the data shown in Table
298 2. According to our results, the lower limit for the FS concentration to be found in 40:60 olive oil:seed
299 oil blends is always above 2400 ppm, whereas the upper one for 50 % olive oil blends is in half of
300 the cases below 2400 ppm. The fact that the OO mixtures display upper limits slightly above the
301 proposed cut-off values is a clear hint that more determinations are needed. In the worst case, this
302 would drive to discard some of the correctly declared 50:50 blends but never to admit as good those
303 blends in which olive oil is below 50 %.

304 Our third set of compounds was that of tocopherols, which comprise four different forms: α -, β -
305 , γ -, and δ -TCPH-, and whose separation and analysis has been long ago described (Kofler, 1947).
306 Table 3 shows the results on their analysis. Lines 3 to 6 shows data on the individual oils, whereas
307 from lines 7 to the end the outcomes of the different mixtures are displayed. All four oil types gave
308 results according to our expectations: Absence of δ -TCPH, olive oil α -TCPH (vitamin E) content
309 lower than that of sunflower oils, and β -TCPH concentration lower than γ -TCPH (the one that exerts
310 the highest antioxidant activity in vitro) in the cases of olive oils, whereas it is in the other way round
311 for sunflower samples. If we analyze the individual TCPH profiles for the blends under study the first
312 hint of the presence of seed oil is that the β - vs γ -TCPH relationship typical of olive oils (β -TCPH <
313 γ -TCPH) flips in all of them regardless the kind of seed oil. Besides, the β -TCPH / γ -TCPH ratio
314 increases in direct relationship with the sunflower oil presence. Also, the average value for the total
315 TCPH concentration in all twelve mixtures affected by with three times the SD (3SD) displays an
316 upper limit equal to or below 2.5 in 75 % of the 50:50 blends, whereas the lower limit for the 40:50
317 olive oil:seed oil blends is always above the 2.5 cut-off value. Again, just one of the blends (i.e.
318 OO:NTSO, 50:50 v/v) behaves in an unexpected way. Therefore, a β -TCPH / γ -TCPH ratio equal to
319 or lower than 2.4 will assure the presence of olive oil at least at 50 %.

320 The last parameter under consideration was the alkane fraction (Table 4). Generally speaking,
321 hydrocarbons have been used as fingerprint of certain seed oils, to characterize varietal virgin olive
322 oils, in geographical traceability, to detect irradiated food, or in the study of mineral oil
323 contaminations (Lesgards, Raffi, Pouliquen, Chaouch, Giamarchi, and Prost, 1993; Moreda, Pérez-
324 Camino, and Cert, 2001; Ju, Huynh, Gunawan, Chern, and Kasim, 2012; Gómez-Coca, Pérez-
325 Camino, and Moreda, 2016; Quintanilla-Casas et al., 2020). In our case, the analysis of the n-alkane
326 series showed natural hydrocarbon profiles consisting of odd C-atom number compounds mainly,
327 between C21-C35 in the cases of olive oils, and between C27-C41 (there is a very small amount of
328 C25) for both types of sunflower oils, although in all circumstances centered at C29. Since the n-
329 alkane range from C21-C25 was virtually absent in sunflower oils, we decided to check on that in our
330 quest for markers since it was clear than in any mixture practically all C21-C25 linear saturated
331 hydrocarbons would come from olive oil. Subsequently, according to our results in mixtures with
332 EVOO, percentages of Σ C21-C25 above 6 % would guaranty the presence of at least 50 % olive oil,
333 whereas in mixtures with OO this must be above 3.5 %.

334 All these limits and their application have been summarized in the form of two decision trees
335 combined in one (Figure 1), differentiating EVOO mixtures from mixtures with OO, and at the same
336 time considering the possibility of using NTSO or HOSO.

337 Additionally, in order to test the effectiveness of this method a number of declared blind commercial
338 samples collected in the Swedish market were tested and the decision trees applied (Table 5). The
339 identity and composition of these samples were initially unknown, being disclosed once the test
340 results have been reported. On the front label of each of the original bottles it was clearly revealed
341 the fact that they contained olive oil, therefore one would expect to detect it at least at 50 %. However,
342 the results of applying the decision trees let us conclude that none of these packers have complied
343 with the official regulation: POO was below the minimum percentage in all cases. The limit for such
344 TAG had been set at 11 % when NTSO was mixed with olive oils. Strictly speaking only samples #1,
345 #2, and #8 contained that kind of oil, but the same limit was considered for every one of them since

346 it was set on the basis of a non-high oleic acid oil such as those at hand. Thus, when observing the
347 analysis outcomes, results on the POO concentration ranged between 1.70 and 8.71 %, showing one
348 of the lowest values in the case of sample #4 which turned out to be the one with the highest declared
349 EVOO content. Regarding the global FS content, just one out of six determinations was below the
350 2400 mg/kg maximum threshold. In the case of the SHC percentages (which include species with odd
351 C-atom number from C21-C25) two minimum limits were established depending on the kind of olive
352 oil used: 3.5 % for OO (exclusively in sample #7) and 6 % for EVOO (for the purpose of the present
353 study, VOO and EVOO can be considered to be of the same kind since, generally speaking, from a
354 chemical point of view the main difference between them and OO is the presence of refined oil in the
355 sample). Results showed that 50 % of the samples (#2, #3, #5, and #7) would fulfill this limit. Lastly,
356 the β -/ γ -TCPH ratio was calculated, being results below the 2.4 threshold in five out of eight samples,
357 and surprisingly high in the case of sample #5. Therefore, according to our results none of these
358 commercial blends contained olive oil in percentages equal or above 50 %, hence, their highlighting
359 of the presence of olive oil on the label was against Regulation No 29/2012 (European Commission,
360 2012). Actually, checking over the list of ingredients samples #1-#4 contained between 20 and 35 %
361 EVOO, samples #5 and #6 contained 20 % VOO, and sample #7 contained 25 % OO. The characters
362 on sample #8's list of ingredients were too small to be readable and on the front label it was just
363 indicated that the blend consisted of EVOO and NTSO. This means that in no case the fact of
364 containing olive oil should have been highlighted on the main label.

365 We are aware that even if these decision trees have been designed on the bases of results obtained
366 from blends with two types of sunflower oils (normal type and high oleic kinds), this is a pilot
367 approach, therefore it will be interesting to observe how they also work with mixtures with other
368 relevant seed oils such as rapeseed and soybean which are the first and third most produced seed oils
369 in the last ten years in the EU (FEDIOL, 2019). For both matrices a comprehensive characterization
370 is being carried out.

371 Finally, our use of sunflower oil is justified, on the one hand, by the place that it keeps within the
372 ranking of seed oil production in the EU, which drives to understand why it is the most commonly
373 utilized oil in blends with olive oil: According to FEDIOL, sunflower oil has been the second most
374 consumed oil in the European Union in the last eleven years. Besides, it plays an important role
375 regarding exports, imports and, as pointed out, production, this latter one amounting to more than
376 39000000 tones in 2018 (FEDIOL, 2019). On the other hand, sunflower gave us the possibility of
377 getting both normal type and high oleic oils easily and thus of including this possibility in the study.

378

379 **4. Conclusions**

380 Our hypothesis has been confirmed: The combination of four of the official purity parameters,
381 arranged as decision trees, may be enough to check if olive oil is at least at 50 % in commercial
382 mixtures with seed oils. The effectiveness of the decision trees has been tested with positive results
383 by applying them to blends prepared ad hoc and composed of olive oils and sunflower oils.

384 So far, we can state that to make sure that olive oil is present at least at 50 %:

385 a) The concentration of POO must be at least 11 % for mixtures with NTSO or 15 % for blends with
386 HOSO.

387 b) The total sterol content must lie below 2400 ppm.

388 c) The β -TCPH/ γ -TCPH ratio must not exceed 2.4.

389 d) The percentages of the n-alkanes of the Σ C21-C25 series must be above 6.0 % or 3.5 % in mixtures
390 with EVOO or OO, respectively.

391 This is a preliminary study where data were generated from a limited number of samples (two olive
392 oils and two sunflower oils), all corresponding to the first stages of the project. However,
393 determinations with blind samples were later on carried out and the utility of the decision trees
394 ratified. Further studies will consider olive oil climate and seasonal variability and its dependence on
395 olive variety, specific soil and climatic conditions, geographical origin, possibility of mixing with
396 edible vegetable oils other than sunflower oils, etc.

397 Presently, we have decided to treat all four parameters as complementary of each other in a way that
398 all together will assure the presence of olive oil at least at 50 %, even if by doing so some genuine
399 50:50 OO:seed oil samples may not be considered as compliant. We think that this could be taken as
400 a ‘worse-case scenario’ and that such situation would be better than open the margins to
401 counterfeiters. In any case, it remains to be seen if a certain flexibility regarding the application of
402 the decision trees (e.g. allowing one of the four parameters to fall outside the established interval in
403 the cases of blends with OO, but being more restrictive with another) will increase the likelihood of
404 fraud by permitting OO:sunflower oil mixtures at proportions of 40:60 v/v to be labelled as containing
405 OO at 50 %. This is something that will also be addressed in the future.

406

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419

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TABLE 1 Triacylglycerol (TAG) composition of the oil blends under study, consisting of extra virgin olive oil (EVOO) and normal type or high oleic sunflower oil (NTSO and HOSO, respectively), at three different proportions (60:40, 50:50, and 40:60, v/v). Measurements were done in duplicate. The standard deviation (SD) is also given; italics have been used when the SD is higher than the mean value, and bold to emphasize the usefulness of POO in the decision trees.

Blend	EVOO:NTSO						EVOO:HOSO					
Proportion, v/v	60:40		50:50		40:60		60:40		50:50		40:60	
TAG*	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD
LLL	10.41	1.45	13.45	1.44	17.04	0.35	0.66	0.01	0.81	0.01	1.08	0.03
OLLn			0.06	<i>0.09</i>	0.07	<i>0.10</i>	0.13	0.04	0.12	0.04	0.08	<i>0.11</i>
PoLL												
PLLn	0.10	<i>0.10</i>	0.06	<i>0.09</i>	0.08	<i>0.11</i>	0.03	0.01	0.03	0.01	0.03	0.01
OLL	10.95	1.37	13.76	1.37	17.46	0.45	1.45	0.01	1.52	0.01	1.62	0.01
OOLn+PoOL	0.98	0.10	0.82	0.08	3.32	<i>3.64</i>	0.90	0.03	0.75	0.03	0.64	0.01
PLLn+PoPoO	3.74	0.44	4.70	0.44	2.95	<i>4.17</i>	0.46	0.01	0.46	0.01	0.49	0.01
POLn+PPoPL+PPoL	0.49	0.10	0.42	0.01	0.35	0.03	0.44	0.01	0.36	0.01	0.30	0.01
OOL+LnPP	9.47	0.06	9.54	0.06	9.61	0.18	7.72	0.01	7.33	0.01	6.98	0.02
PoOO	3.13	0.06	3.51	0.06	3.97	0.17	1.29	0.02	1.19	0.02	1.14	0.08
SLL+PLO	4.66	0.07	4.75	0.07	4.96	0.07	2.85	0.01	2.51	0.01	2.23	0.08
PoOP+SPoL+SOLn+SPoPo	0.63	0.10	0.48	0.09	0.35	0.02	0.69	0.03	0.59	0.03	0.61	0.16
PLP	0.51	0.08	0.55	0.08	0.62	0.07						
OOO+PoPP	27.68	1.68	23.83	1.68	19.14	0.01	51.20	0.22	53.40	0.22	55.76	0.33
SOL	1.74	0.10	1.94	0.10	2.12	0.06	0.94	0.01	0.86	0.01	0.87	0.11
POO	14.98	1.00	12.58	1.00	9.69	0.09	17.75	0.04	16.30	0.04	14.90	0.02
POP	2.68	0.24	2.38	0.24	2.01	0.04	2.43	0.06	2.11	0.06	1.78	0.01
SOO	4.64	0.30	3.95	0.32	3.22	0.05	7.14	0.02	7.19	0.02	7.23	0.04
POS+SLS	1.25	0.05	1.12	0.05	0.94	0.04	1.22	0.07	1.10	0.07	1.04	0.04
SSO	0.57	0.04	0.52	0.04	0.41	0.02	0.79	0.04	0.86	0.04	0.81	0.01

*L (linoleic acid), Ln (linolenic acid), O (oleic acid), P (palmitic acid), Po (palmitoleic acid), S (stearic acid)

TABLE 1 (cont.)

Blend	OO:NTSO						OO:HOSO					
Proportion, v/v	60:40		50:50		40:60		60:40		50:50		40:60	
TAG*	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD	Mean, %	SD
LLL	11.84	0.17	14.01	0.17	16.86	0.01	0.95	0.02	0.94	0.02	1.06	0.02
OLLn	0.53	0.01	0.17	0.01	0.08	0.12	0.21	0.01	0.19	0.01	0.17	0.02
Poll							0.03		0.02		0.05	
PLLn	0.12	0.01	0.15	0.01	0.08	0.12	0.07	0.01	0.05	0.01	0.05	0.01
OLL	13.34	0.19	15.05	0.19	17.57	0.06	2.54	0.05	2.30	0.05	2.27	0.06
OOLn+PoOL	0.99	0.05	0.85	0.05	0.70	0.03	0.95	0.03	0.82	0.03	0.68	0.04
PLL+PoPoO	4.74	3.40	2.93	3.40	6.13	0.01	0.98	0.02	0.87	0.02	0.81	0.02
POLn+PPoPL+PPoL	0.47	0.02	0.41	0.02	0.33	0.03	0.47	0.02	0.38	0.02	0.30	0.02
OOL+LnPP	11.31	0.10	11.09	0.10	10.76	0.06	9.61	0.20	8.87	0.20	8.29	0.23
PoOO	3.56	0.03	3.75	0.03	4.12	0.07	1.51	0.01	1.40	0.01	1.25	0.03
SLL+PLO	6.17	0.01	6.02	0.01	5.85	0.03	4.36	0.03	3.74	0.03	3.14	0.08
PoOP+SPoL+SOLn+SPoPo	0.53	0.03	0.42	0.03	0.33	0.02	0.64	0.09	0.62	0.09	0.46	0.02
PLP	0.80	0.04	0.80	0.04	0.79	0.01	0.47	0.07	0.42	0.07	0.00	
OOO+PoPP	23.22	0.22	21.10	0.22	17.71	0.08	48.24	0.59	51.67	0.59	54.85	0.35
SOL	1.78	0.02	1.98	0.02	2.16	0.03	0.98	0.08	0.89	0.08	0.78	0.03
POO	12.66	0.03	11.15	0.03	9.05	0.05	16.79	0.14	15.27	0.14	14.14	0.12
POP	2.49	0.01	2.28	0.01	2.03	0.05	2.51	0.05	2.10	0.05	1.69	0.03
SOO	3.00	0.02	2.80	0.02	2.46	0.01	5.68	0.05	6.07	0.05	6.43	0.03
POS+SLS	0.83	0.01	0.83	0.01	0.78	0.01	0.98	0.01	0.89	0.01	0.84	0.02
SSO	0.39	0.14	0.46	0.14	0.33	0.01	0.63	0.01	0.65	0.01	0.70	0.72

*L (linoleic acid), Ln (linolenic acid), O (oleic acid), P (palmitic acid), Po (palmitoleic acid), S (stearic acid)

TABLE 2 Total sterol content (mg/kg) in the oil blends under study, which consisted of either extra virgin olive oil (EVOO) or olive oil (OO), and normal type or high oleic sunflower oil (NTSO and HOSO, respectively), at three different proportions. Measurements were done in duplicate. The standard deviation (SD) and the corresponding lower (mean value – SD) and upper (mean value + SD) limits are also given.

Blend, proportion (v/v)	Mean	Sterol content (mg/kg)		
		SD	lower limit	upper limit
EVOO:NTSO, 60:40	2014	113		
EVOO:NTSO, 50:50	2296	104		2399
EVOO:NTSO, 40:60	2575	121	2454	
EVOO:HOSO, 60:40	2092	60		
EVOO:HOSO, 50:50	2259	79		2338
EVOO:HOSO, 40:60	2488	41	2447	
OO:NTSO, 60:40	2301	38		
OO:NTSO, 50:50	2409	68		2477
OO:NTSO, 40:60	2579	8	2572	
OO:HOSO, 60:40	2121	83		
OO:HOSO, 50:50	2330	133		2463
OO:HOSO, 40:60	2481	32	2450	

TABLE 3 Total tocopherol content and contents of α -, β -, and γ -Tocopherol (TCPH), in the initial oils and in the oil blends under study, which consisted of either extra virgin olive oil (EVOO) or olive oil (OO), and normal type or high oleic sunflower oil (NTSO and HOSO, respectively), at three different proportions. Measurements were done in duplicate. The three times the standard deviation (3SD) and the corresponding lower (mean value – 3SD) and upper (mean value + 3SD) limits are also given.

Sample	α -TCPH	β -TCPH	γ -TCPH (mg/kg)	TOTAL	β -TCPH/ γ -TCPH ratio			
					Mean	3SD	Upper limit	Lower limit
OO	256.20	2.61	13.23	272.03	0.20			
EVOO	332.72	3.19	18.65	354.56	0.17			
HOSO	460.76	36.77	2.65	500.18	13.89			
NTSO	518.49	46.98	3.40	568.88	13.81			
EVOO:NTSO, 60:40 (v/v)	350.00	10.44	8.37	368.82	1.21	0.18		
EVOO:NTSO, 50:50 (v/v)	432.72	15.25	10.44	458.41	1.46	0.18	1.6	
EVOO:NTSO, 40:60 (v/v)	470.99	21.03	7.04	499.06	2.99	0.18		2.8
EVOO:HOSO, 60:40 (v/v)	441.47	13.68	9.86	465.01	1.36	0.10		
EVOO:HOSO, 50:50 (v/v)	481.96	16.81	9.16	507.92	1.87	0.14	2.0	
EVOO:HOSO, 40:60 (v/v)	521.51	19.70	7.46	548.67	2.64	0.01		2.6
OO:NTSO, 60:40 (v/v)	477.87	15.78	7.84	501.49	1.93	0.34		
OO:NTSO, 50:50 (v/v)	331.71	11.75	4.95	348.41	2.28	0.39	2.7	
OO:NTSO, 40:60 (v/v)	520.49	18.69	5.83	545.01	3.27	0.27		3.0
OO:HOSO, 60:40 (v/v)	443.82	13.61	8.14	465.57	1.66	0.07		
OO:HOSO, 50:50 (v/v)	515.82	16.84	7.13	539.79	2.31	0.20	2.5	
OO:HOSO, 40:60 (v/v)	526.60	18.99	5.92	551.51	3.15	0.24		2.9

TABLE 4 Acyclic saturated hydrocarbons or n-alkanes (SHC) composition (%) in the oil blends under study, consisting of either extra virgin olive oil (EVOO) or olive oil (OO), and normal type or high oleic sunflower oil (NTSO and HOSO, respectively), at three different proportions. SHC are named according to their C-atom number from C21 to C45.

					EVOO:NTSO (v/v)			EVOO:HOSO (v/v)			OO:NTSO (v/v)			OO:HOSO (v/v)		
	EVOO	OO	NTSO	HOSO	60:40	50:50	40:60	60:40	50:50	40:60	60:40	50:50	40:60	60:40	50:50	40:60
Alkanes,	%				%			%			%			%		
C21	0.84	0.20			0.35	0.32	0.15	0.38	0.39	0.19		0.71	0.08	0.11	0.11	0.06
C23	5.44	1.85			2.18	1.91	1.05	2.20	1.89	1.16	0.64	0.76	0.60	0.90	0.76	0.68
C25	13.57	6.37	0.66	0.56	5.65	4.96	3.06	5.01	4.22	3.14	3.05	2.94	2.21	3.33	2.79	2.44
C27	15.71	18.26	8.59	7.13	11.35	10.77	9.92	10.26	9.50	8.90	12.46	12.36	11.28	12.51	11.38	10.51
C29	21.33	25.70	44.44	41.89	35.10	35.88	39.87	34.08	35.29	37.49	34.83	36.45	38.26	33.75	35.22	36.23
C31	14.87	18.34	32.05	35.54	25.30	25.52	28.69	27.83	29.05	30.95	25.38	26.41	28.06	27.46	29.05	30.32
C33	6.91	11.03	1.93	2.15	3.86	3.48	2.87	3.79	3.41	3.07	5.96	5.62	4.42	6.24	5.50	4.82
C35	1.79	3.43	0.53	0.50	0.97	0.80	0.81	0.98	0.78	0.79	1.80	1.61	1.45	1.83	1.52	1.47
C37	0.28		0.50	0.65	0.32	0.36	0.60	0.36	0.23	0.29	0.18	0.76	0.38	0.22	0.30	0.29
C39			0.37	0.34	0.20		0.31	0.23	0.33	0.34	0.23	0.64	0.35	0.22	0.24	0.32
C41			0.20	0.27	0.13		0.24	0.20	0.37	0.27	0.17		0.20	0.16	0.31	0.29
C43			0.18				0.12	0.10		0.12	0.10		0.25	0.08	0.13	0.14
C45										0.12					0.09	0.11
ΣC21-C25	19.86	8.43	0.66	0.56	8.18	7.19	4.26	7.59	6.49	4.49	3.70	4.41	2.90	4.33	3.66	3.18

TABLE 5 Dioleoyl palmitin (POO) percentage, total sterol content, saturated aliphatic hydrocarbon sum from C21 to C25, and β/γ -tocopherol ratio in commercial blends. The original blend compositions are also given, where extra virgin olive oil (EVOO), virgin olive oil (VOO), olive oil (OO), and normal type or high oleic sunflower oil (NTSO and HOSO, respectively), unspecified vegetable oil (VGO), and rapeseed oil (RSO).

Sample No	Blend composition according to the label	POO (%)	Sterol content (mg/kg)	Σ C21-C25 (%)	β/γ -Tocopherol	Compliance with EU R 29/212
#1	25% EVOO:75% RSO	6.03	5837	2.00	0.01	No
#2	20% EVOO:80% RSO	6.08		9.60	0.00	No
#3	20% EVOO:80% RSO	5.83		9.20	0.01	No
#4	35% EVOO:65% NTSO	2.65	1904	4.20	2.81	No
#5	20% VOO:80% NTSO	3.29	2587	9.20	31.08	No
#6	20% VOO:80% VGO	3.41	3004	4.30	1.09	No
#7	25% OO:75% RSO	8.71	6536	4.20	0.01	No
#8	EVOO:NTSO	1.70	3262	3.50	4.31	No

Figure 1. Gómez-Coca, R. B. Decision tree for mixtures of extra virgin olive oil (EVOO) or olive oil (OO) with normal type or high oleic sunflower oils (NTSO or HOSO, respectively), where POO stands for dioleoyl palmitin, and $\Sigma C_{21}-C_{25}$ corresponds to the group of alkanes that includes those with odd C-atom number from C21 to 25 C.

