Chemical components influencing oxidative stability and sensorial properties of extra virgin olive oil and effect of genotype and location on their expression

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Abstract: Extra virgin olive oil (EVOO) chemical composition is characterized by high content of monounsaturated fatty acids and minor compounds including phenols, sterols, tocopherols, squalene and volatile compounds. These components are related to EVOO quality in terms of healthy properties, shelf life alteration due to susceptibility to oxidative degeneration and sensory properties. In this work, the variability of 66 different chemical compounds, oxidative stability and sensory attributes of EVOO was analyzed in order to study the relationships among them and the effect of cultivar, growing location and their interaction on their expression. Partial least squares (PLS) regression models allowed accurate prediction for EVOO stability on the basis of the chemical composition of the oils, with marked positive influence of oleic acid and 3,4-DHPEA-EA phenol content on stability

values, while poor prediction results were obtained for sensory attributes. Cultivar and location showed limited effect on the sensory properties of EVOO, even though the same factors provide significant effect for the rest of chemical compounds and stability. These results should be taken into account in breeding programs aimed to obtain new cultivars with improved EVOO characteristics and to determine the best cultivar to be planted in each environment.

Keywords: fatty acid composition; minor components; oil quality; *Olea europaea*; olive breeding

1. Introduction

Extra virgin olive oil (EVOO) contributes to the healthy and nutritional properties of the Mediterranean diet inscribed in 2013 on the Representative List of the Intangible Cultural Heritage of Humanity by UNESCO (Radd-Vagenas et al., 2017). Its fatty acid composition, mainly composed of monounsaturated fatty acids, as well as a myriad of minor components including phenols, sterols, tocopherols and squalene, are the main responsible for the healthy properties of EVOO, particularly regarding cardiovascular diseases, inflammation, cancer and a general increase in life expectancy (Francisco et al., 2019; Gouvinhas et al., 2017). These components are also responsible for EVOO quality in terms of shelf life, mainly related to alteration due to susceptibility to oxidative degeneration (Aparicio et al., 1999; Mateos et al., 2003). The EVOO sensory profile is the result of a combination of taste, odor and chemical responses produced by different compounds. Among these sensorial properties, three main positive attributes (fruity, bitter and pungent) are used for classification of EVOO (IOC, 2018).

Several associations between individual components or groups of components and oxidative stability have been attempted (Aparicio et al., 1999; Mateos et al., 2003). Similarly, correlations among several phenolic compounds and EVOO sensorial attributes bitterness

52 and pungency as well as several volatile compounds and fruity sensorial attribute have been
53 reported (Andrewes et al., 2003; Campestre et al., 2017; Cerretani et al., 2008; Mateos et al.,
54 2004). However, comprehensive studies including proper experimental design able to
55 identify the main factors affecting the chemical composition of EVOO have not been carried
56 out. Also, the potential effects of these factors on the association between chemical
57 composition and oxidative stability and sensorial properties are poorly understood.

58 Recent works indicate that the genetic effect is the main source of variation for most
59 EVOO chemical components and a high variability for oil composition has been reported in
60 different olive plant materials (Cerretani et al., 2008; de la Rosa et al., 2016; García-Vico et
61 al., 2017; León et al., 2018). This genetic influence is also claimed regarding both oxidative
62 stability and sensorial properties. In fact, the peculiarity of certain local cultivars is
63 considered one of the main singularities for EVOO Protected Denomination of Origin
64 declarations. Moreover, environmental influence on chemical components, oxidative
65 stability and sensorial properties of EVOO has also been reported, particularly from studies
66 of single cultivars grown in different locations (Ben Mansour et al., 2017; Issaoui et al.,
67 2010).

68 However, genotype by location studies on EVOO quality are very scarce and necessaries,
69 as recent works indicate a differential performance of cultivars under different environments
70 for olive fruit traits (Navas-Lopez et al., 2019). Particularly, as far as we know, the combined
71 effect of genotype and location on the potential associations among chemical components,
72 oxidative stability and sensorial properties of EVOO is completely unknown. Therefore, the
73 present work aims to determine the genetic and location effects and their interaction on the
74 variability of 66 chemical components of EVOO, and in its stability and sensory profile. For
75 that, four different cultivars were evaluated in this work. ‘Picual’ is the most widely grown
76 cultivar in Spain and (Barranco et al., 2000). It shows many favorable agronomic
77 characteristics, such as early bearing, high productivity and easy mechanical harvesting, and

78 also produces highly appreciated EVOO characterized by high oleic acid content and
79 stability. However, its high susceptibility to Verticillium wilt caused by the soil fungus
80 *Verticillium dahliae* hindered its cultivation in some areas, which promotes the development
81 of breeding programs for Verticillium wilt resistance (Arias-Calderón et al., 2015). EVOO
82 from three advanced selections of this breeding program were also evaluated in this work.
83 Data gathered were also used to investigate how the variability of EVOO chemical
84 composition is influencing both its stability and sensory profile.

85

86 **2. Materials and Methods**

87 *2.1. Plant materials*

88 Three advanced selections of the breeding program for Verticillium wilt resistance
89 developed at IFAPA were evaluated, together with 'Picual' as a reference cultivar. One of
90 the selections (Sel1) comes from open pollination of 'Koroneiki' and the other two (Sel2 and
91 Sel3) from crosses between 'Frantoio' and 'Arbosana'. All four genotypes were planted in
92 comparative trials in spring of 2015 in three locations in Jaén province, Arjona, Begíjar and
93 Úbeda, hereafter named as Loc1, Loc2 and Loc4 respectively. In 2016, the four genotypes
94 were also planted in experimental microplots at IFAPA research Centre, Córdoba (Loc3). In
95 all these comparative trials, the genotypes were distributed in three randomized blocks with
96 4 to 6 plants per elementary plot. Olive fruit samples of 4 kg were randomly picked by hand
97 from each elementary plot in November 2018. An almost complete set of samples from 4
98 genotypes x 4 locations x 3 replicates was collected, with only one missing sample of Sel3
99 in Loc4. After harvesting, olive fruit samples were immediately transported to the laboratory
100 and stored at 4°C until olive oil extraction within 24h.

101 *2.2. EVOO extraction*

102 Only healthy fruits, without visible damage, were processed. EVOO was extracted using
103 the Abencor system (Comercial Abengoa, S.A., Seville, Spain), which is a laboratory set for

104 olive extraction composed by stainless hammer mill, thermo-mixer and centrifugal machine,
105 reproducing the industrial process of mechanical extraction. Firstly, olive fruits were milled
106 at 3000 rpm with a 5 mm sieve. 2.5 g/100 g of talc was added to the resulting olive paste that
107 then was malaxed at 28°C for 30 min, adding 100 ml of water at room temperature for the
108 last 10 minutes of malaxation. Then, the olive paste was centrifuged for 1 min at 1372 g
109 relative centrifugal force. The EVOO obtained was decanted, filtered through paper,
110 transferred into dark glass bottles and stored in the dark at 4°C until analysis. As expected
111 from healthy fruit samples without damage, all the extracted oils were classified as EVOO,
112 meeting the regulatory values established for quality criteria. For instance, only two samples
113 showed free acidity values higher than 0.4, and all of them lower than the 0.8 value regulated
114 for classification as EVOO (data not shown).

115 *2.3. Chemical composition*

116 A total of 66 chemical compounds of different groups were quantified (Table 1).

117 *2.3.1. Fatty acid composition*

118 Fatty acid composition was analyzed by gas chromatography (GC) on a Perkin Elmer
119 Clarus 600 GC (Perkin Elmer Inc, Waltham, MA, USA) equipped with a BPX70 30 m x 0.25
120 mm internal diameter x 0.25 μm film thickness capillary column (SGE Analytical Science
121 Pty Ltd, Ringwood, Australia). Hydrogen was used as carrier gas at a constant flow of 0.8
122 ml/min. A split injector and flame ionization detector were maintained at 300 ºC. The initial
123 oven temperature was 140 ºC maintained for 2 min, followed by a rate increase of 20 ºC /
124 min up to 250 ºC, maintained for 2 min.

125 *2.3.2. Analysis of Tocopherols*

126 Tocopherol extraction, separation by high-performance liquid chromatography (HPLC)
127 and quantification was done on around 100 mg of EVOO using a fluorescence detector
128 (Waters 474) at 295-nm excitation and 330-nm emission and *iso*-octane/*tert*-
129 butylmethylether (94:6) as eluent at an isocratic flow rate of 0.8 ml/min (Velasco et al., 2019).

130 Chromatographic separation of the tocopherols was performed on a LiChrospher 100 diol
131 column (250 mm 9 2 mm I.D.) with 5-lm spherical particles, connected to a silica guard
132 column (LiChrospher Si 60, 5 mm 9 4 mm I.D.). Quantitative determination of tocopherols
133 was done by using external calibration curves obtained for each of the tocopherol homologs
134 α-, β-, γ-, and δ-tocopherol using tocopherol standards (Calbiochem Tocopherol Set, catalog
135 no. 613424, Merck KGaA, Darmstadt, Germany). Total tocopherol content was calculated as
136 the sum of α-, β-, γ-, and δ-tocopherol contents.

137 *2.3.3. Analysis of Phytosterols and Squalene*

138 Sterols and squalene contents in EVOO were analyzed by GC of the unsaponifiable
139 fraction following silylation, without preliminary thin-layer chromatography (TLC)
140 fractionation. Alkaline hydrolysis was performed by adding 2 g/100 mL of a solution of
141 potassiumhydroxide dissolved in ethanol at a concentration of 2%. After vortexing, the tubes
142 were left in a water bath at 80 °C for 15 min. The unsaponifiable was extracted by vortexing
143 with 1 mL hexane and 1.5 mL water. The upper hexane layer was transferred to 2-mL glass
144 vials that were maintained in an oven at 37.5 °C overnight. Fifty microliter hexane and 50
145 µL silylating mixture composed of pyridine:hexamethyldisilazane:trimethylchlorosilane
146 9:3:1 by vol (Cat. No. 355650.0922, Panreac Química, Barcelona, Spain) were added and the
147 vials were left at room temperature for 15 min. The solution was transferred to 2-mL vials
148 containing 200 µL inserts and centrifuged at 4,000 rpm for 10 min. The vials were capped
149 and conserved at −20 °C until analysis, usually within 24 h of preparation. GC analyses were
150 performed on a Perkin Elmer Clarus 600 GC (Perkin Elmer Inc, Waltham, MA, USA)
151 equipped with a ZB-5 capillary column (id = 0.25 mm, length = 30 m, film thickness = 0.10
152 µm; Phenomenex, Torrance, CA, USA) using hydrogen as carrier gas at a pressure of 125
153 KPa. Split injector and flame ionization detector were maintained at 320 °C. The oven
154 thermal regime was the following: initial temperature of 240 °C was increased at 5 °C / min
155 to final temperature of 265 °C and held for 10 min. Total analytical time was 15 min. Total

156 phytosterol content was calculated as the sum of individual phytosterols and expressed as
157 mg/kg. Sterol peaks were identified by comparison with a sample analysed at the reference
158 laboratory of the Instituto de la Grasa (CSIC) at Sevilla, Spain. Squalene was identified using
159 a commercial standard (Cat. No. S3626, Sigma-Aldrich). 5α-cholestan-3β-ol (Cat. No.
160 D6128, Sigma- Aldrich, St. Louis, MO, USA) and squalene (Cat. No. S3626, Sigma-
161 Aldrich) were used as internal standard

162 *2.3.4. Analysis of volatile compounds*

163 Volatile compounds were extracted and analyzed by means of HS-SPME/GC-MS-FID.
164 EVOO samples (1g) were prepared in duplicate vials of 10mL and placed in a vial heater at
165 40ºC for a 10 min equilibration time. Volatile compounds from the headspace were adsorbed
166 onto SPME fiber DVB/Carboxen/PDMS 50/30 µm (Supelco Co., Bellefonte, PA, USA). The
167 sampling time was 50 min at 40ºC, and the desorption of volatile compounds was performed
168 directly into the GC injector. Volatile compounds were identified on a Bruker model Scion
169 456-GC-TQ MS system (Bruker, Massachusetts, USA) equipped with a Supelcowax 10
170 capillary column (30 m × 0.25 mm i.d.; thickness, 0.25 µm; Sigma-Aldrich Co. LLC)
171 working under the following conditions: helium (carrier gas) flow rate of 1mL/min;
172 injection by splitless method at 250 ºC; 5 min of column holding time at 50 ºC and then
173 ramped up at 4 ºC/min to 200 ºC; the mass detector operated in electronic impact mode at 70
174 eV, with the temperature source set at 250 ºC and the mass spectra were scanned at 7 scans/s
175 in the m/z 30–250 mass-to-charge ratio range. Volatile compounds were matched to the
176 Wiley/NBS and NIST libraries and by with GC retention time in comparison with standards.
177 For the quantification of volatile compounds, calibration curves were obtained for each one
178 by adding known amounts of the pure standards to deodorized olive oil at six level (Acesur,
179 Seville, Spain). The absence of target volatile compounds in the matrix was checked and this
180 olive oil was used to build calibration curves. As control of the extraction and analysis,

181 samples containing a mixture of volatile standards and blank samples (no oil) were run at the
182 beginning and during sample analysis.

183 *2.3.5. Analysis of phenolic compounds*

184 EVOO phenolics were isolated by solid phase extraction (SPE) according to a previously
185 published methodology (Mateos et al., 2001). 0.5 ml of a methanol solution containing two
186 internal standards, p-hydroxyphenyl-acetic and o-coumaric acids (p-HPA and o-com) was
187 added to each oil sample (2.5 g) before the extraction. The solvent was evaporated in a rotary
188 evaporator at 40 °C under vacuum, and the residue was dissolved in 6 mL of hexane. This
189 oil solution was applied to a diol-bonded phase cartridge (Supelco, Bellefonte, PA)
190 previously conditioned. The column was washed twice with hexane (3 ml) and once with 4
191 mL of hexane/ethyl acetate (90:10, v/v). Finally, the column was eluted with 10 mL of
192 methanol, later evaporated until dryness in a rotary evaporator at room temperature and under
193 vacuum. The residue was extracted with 500 µL of methanol/water (1:1, v/v) at 40
194 °C..Phenolic extracts were analyzed by HPLC on a Beckman Coulter liquid chromatography
195 system equipped with a System Gold 168 detector, a solvent module 126, an autosampler
196 module 508 and a Waters column heater module. A Superspher RP 18 column (4.6mm i.d. ×
197 250mm, particle size 4 µm: Dr Maisch GmbH, Germany). Elution was performed at a flow
198 rate of 1.0 mL min□1, using water/phosphoric acid (99.5:0.5) (solvent A) and
199 methanol/acetonitrile (50:50) (solvent B) as the mobile phases and the following elution
200 program: (A) 0–25 min, 5–30% solvent B; (B) 25–35 min, 30–38% solvent B; 35–40 min,
201 38% solvent B; 40–45 min, 38–100% solvent B.. The quantification of phenolic components
202 was done at 280 nm. The identification of compounds was confirmed by HPLC/ESI-qTOF-
203 HRMS. The liquid chromatograph system was Dionex Ultimate 3000 RS UHPLC liquid
204 chromatograph system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a
205 similar Superspher RP 18 column but with formic acid (1%) instead of phosphoric acid
206 (0.5%) in solvent A. A split post-column of 0.4 mL/min was introduced directly on the mass

207 spectrometer electrospray ion source. The HPLC/ESI-qTOF operated for mass analysis using
208 a micrOTOF-QII High Resolution Time-of- Flight mass spectrometer (UHRTOF) with qQ-
209 TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an electrospray
210 ionization (ESI) interface. Mass spectra were acquired in MS fullscan mode and data were
211 processed using TargetAnalysis 1.2 software (Bruker Daltonics, Bremen, Germany).
212
213 *2.4. Oxidative stability*
214 Induction period was determined by Rancimat method. Oil samples (3.0 g) were heated
215 at 120 °C in a Rancimat equipment (Metrohm AG, Herisau, Switzerland), with a continuous
216 air flow of 20 L/h passing through the samples. Induction time (IT) was calculated as the
217 time needed (hours) for the appearance of a sudden water conductivity rise caused by the
218 adsorption of volatiles derived from oil oxidation.
219 *2.5. Sensory analysis*
220 Sensory analysis was carried out by the EVOO sensory panel of PDO Priego de Córdoba,
221 Andalucía, Spain, established in 1995. The panel was formed by 8 judges trained in the
222 method for the organoleptic assessment of EVOO according to the official method of the
223 IOC (2018). Positive attributes considered in the official methodology were: fruity (set of
224 olfactory sensations perceived directly and/or through the back of the nose), bitter
225 (characteristic primary taste of oil perceived in the circumvallate papillae of the tongue) and
226 pungent (biting tactile sensation perceived throughout the whole of the mouth cavity,
227 particularly in the throat). Sensory analysis was carried out in 41 out of the 47 EVOO samples
228 due to lack of enough amount for some of them, well balanced among cultivars and locations
229 and including all the combinations cultivar x location tested.
230 *2.6. Statistical analysis*
231 EVOO samples were obtained from three randomized blocks replicates for each cultivar
232 x location combination and all the chemical analyses were performed in duplicate. Principal

components analysis (PCA) was used to investigate the relationships among traits and the variability between and within the different groups of samples evaluated (by cultivar and location). Partial least squares (PLS) regression was used to study the associations of chemical components with oxidative stability and sensorial properties of EVOO. Full cross-validation (i.e. leave-one-out) was used for determining the performance of the models. Correlation between actual and predicted values (r), standard error of cross validation (RMSECV) and residual predictive deviation (RPD), defined as the ratio of the standard deviation for any given constituent to the standard error of cross validation or prediction for the same constituent, were determined to indicate the relative accuracy of each model, as previously described in PLS applications (Nicolaï et al., 2007). Analysis of variance was performed for the most important constituents to test differences between sources of variation (cultivar, location and interaction) and separation of means was carried out accordingly. Unscrambler (CAMO A/S, Trondheim, Norway) and Statistix (Analytical Software, Tallahassee, FL, United States) software were used for the statistical analysis.

3. Results

Descriptive statistics of the full data set showed wider variability for induction time compared to sensorial properties, with coefficient of variation (CV) of 43.98 % for IT vs. 15.86-19.94% for fruity, bitter and pungent sensorial traits (Table 2). Among the evaluated sensorial traits, fruity showed the highest range of variability (3.00-6.10) and pungent the lowest (2.00-3.50). As expected for EVOO, negative attributes were not detected in any of the evaluated samples. Regarding the main fatty acid (C18:1) and total amount of minor components, C18:1 showed the lowest CV, while Phenol, Volatile and Squalene contents showed much higher variability with CV 64.046, 43.30 and 38.90 and range of variation 1236.80-11882.00, 195.50-1079.30 and 11656-63797, respectively, much higher than C18:1 and tocopherol content.

259 Exploratory analysis by PCA showed a wide variability for both samples scores and
260 variables loadings in the model. The first two components of PCA carried out from the whole
261 dataset including 66 chemical components plus oxidative stability and sensorial data
262 evaluated in 47 EVOO samples, explained 22 and 15% of the total variability, respectively
263 (Figure 1). PC1 was positively correlated mainly with linoleic acid (C18:2), (*E*)-hex-2-enal
264 (V03) and β-Tocopherol (BToc) and negatively with stability (IT) and chemical compounds
265 such as oleic acid (C18:1), squalene and (*E*)+(*Z*)-hex-3-enal (V01). PC2 was associated
266 positively with volatiles such as hexyl acetate (V21) and arachidic acid (C20:0) and
267 negatively with palmitoleic acid (C16:1) and luteolin phenolic compound (Lut).

268 The position of oxidative stability on the loading biplot, located nearby chemical
269 compounds such as oleic acid (C18:1), Squalene and (*E*)+(*Z*)-hex-3-enal (V01), suggest a
270 positive correlation among them. Fruity, bitter and pungent sensorial traits were on the
271 contrary located closer to the loading plot center, which indicate low weight for these
272 components on the general variability of the dataset. Besides, these results suggest no
273 correlation among stability and sensorial data. The score biplot showed clear separation of
274 EVOO samples according to cultivars, while no grouping could be observed regarding
275 location of the trials (Figure 2). Main separation between cultivars was obtained through PC1,
276 with Sel2 and 3 occupying the right (positive) side and the opposite for 'Picual' and Sel1.
277 Therefore, higher values for stability and C18:1/C18:2 ratio can be expected for 'Picual' and
278 Sel1 compared with Sel2 and Sel3.

279 PLS models developed from 66 chemical compounds for stability (IT) showed high
280 correlation and RPD values, while the opposite was obtained for the three sensorial traits
281 (Table 3, Figure 3). In all cases, only one or two components were included in the models.
282 Scores plot of PLS model developed for stability reflects the same grouping by cultivar and
283 location previously described for PCA model (data not shown). Regression coefficients of
284 this PLS model showed the highest positive values for C18:1 and 3,4-DHPEA-EA, while

285 negative for C18:2 and 3,4-DHPEA-EDA (Figure 4). Total phenolic and squalene content
286 (positive) and sterols content (negative) play also important role in the model.
287 Analysis of variance showed significant differences by cultivar and location for all the
288 main chemical compounds of EVOO except location for oleic acid (C18:1). In all cases, non-
289 significant differences were obtained for cultivar x location interaction. Cultivar effect was
290 the main contributor of sums of squares for C18:1, total tocopherol, squalene and sterols
291 content, while location was higher for total phenols and volatile contents (Table 4).
292 Comparison of means showed similar chemical composition in 'Picual' and Sel1 on the one
293 hand (high C18:1 and squalene content and low sterols content) and Sel2 and Sel3 (both
294 coming from crosses between 'Frantoio' and 'Arbosana') on the other hand. Comparison of
295 means among locations showed different trends for the different evaluated traits. For
296 example, Loc1 differed from other locations in the lowest amount of squalene, the highest
297 phenols content was quantified in EVOO samples from Loc3 and higher volatile contents
298 were observed for Loc2 and Loc4.
299 Regarding stability (IT) and sensorial traits of EVOO, analysis of variance showed
300 significant differences by cultivar only for IT and bitter, and location effect for IT. No
301 significant differences for fruity and pungent sensorial traits were found neither by genotype
302 nor by location. Like for chemical compounds, non-significant differences were obtained for
303 cultivar x location interaction. Cultivar effect was the main contributor of sums of squares
304 only for IT, while error sums of squares was predominant for sensorial traits (Table 5). Again,
305 comparison of means suggests a general higher similitude between 'Picual' and Sel1
306 compared to Sel2 and Sel3, mainly due to higher oxidative stability.
307

308 **4. Discussion**

309 A wide variability has been observed for stability (induction time, IT), sensorial traits
310 and main chemical compounds of the set of EVOO samples. The average values observed

311 for 'Picual' are in general comparable to previous references. Thus, EVOO from 'Picual'
312 have been traditionally characterized by a high C18:1, phenol content and oil stability, being
313 its EVOO chemical composition one of the main reason for its widespread use as a genitor
314 in breeding programs (León et al., 2011). However, it should be noted that wide variability
315 for some of these components such as total phenol content has also been reported in some
316 works, as high as from 133–1295 mg/kg (Beltrán et al., 2007). High bitterness is also
317 characteristic of 'Picual' (Mateos et al., 2004). Similar values for total volatiles content have
318 been reported for 'Picual' in previous works (Pérez et al., 2016), although much lower values,
319 around 8,000-9,000 mg/kg have been also observed (Sánchez-Ortiz et al 2007). The average
320 values obtained in 'Picual' for others minor components such as squalene, phytosterol and
321 tocopherols are also similar than previously reported for this cultivar (Aparicio et al., 1999;
322 Velasco et al., 2015).. No significant correlation was observed among the different stability
323 and sensorial traits evaluated. On the contrary, significant correlations among fruity, bitter
324 and pungent sensorial traits from 0.60 to 0.77 were obtained in a previous work from a set of
325 100 samples from an annual competition (Pedan et al., 2019). It is unknown to what extent
326 the origin of samples could have affected these results.

327 *4.1. Chemical components influencing oxidative stability and sensorial properties*

328 Accurate predictive PLS model was obtained only for EVOO stability (induction time)
329 using the data of 66 chemical components analyzed, with high correlation between actual and
330 predicted values (around 0.9). This value could be considered accurate enough for ranking
331 and selection of genotypes and discrimination into high, medium and low values. Similarly,
332 RPD values near 2 indicates that coarse quantitative predictions are possible, although values
333 around 3 are recommended for excellent prediction accuracy (Nicolaï et al., 2007). However,
334 poor prediction results were obtained in the models developed for positive sensorial
335 properties (fruity, bitter and pungent).

336 The effect of various compounds on EVOO stability measured by Rancimat has been
337 reported in previous studies. A good correlation (R^2=0.91) has been previously found, using
338 stepwise linear regression analysis, between stability and both the oleic/linoleic ratio and the
339 contents of phenols and tocopherols (Aparicio et al., 1999). However, that study was
340 performed with only two cultivars with contrasting behaviors in terms of stability ('Picual'
341 and 'Hojiblanca') in a single environment. Grouping of samples can be inferred also in
342 correlations reported from other works (Bendini, 2007). In the present work, the PLS model
343 developed for stability showed highly significant correlation with only two latent variables.
344 The model showed high and positive regression coefficients values for C18:1 and 3,4-
345 DHPEA-EA and negative for C18:2 and 3,4-DHPEA-EDA. It is well established the negative
346 correlation between oleic and linoleic fatty acids in all vegetable oils including EVOO.
347 Regarding secoiridoid derivatives, all of them are produced by β-glucosidase hydrolysis of
348 olive fruits glycosides during crushing and malaxation (Bendini, 2007). Similar relationships
349 among individual phenols and IT measured by Rancimat were also reported from the analysis
350 of EVOOs obtained from a wide variability of malaxation conditions, suggesting the use of
351 the ratio (3,4-DHPEA-EA + *p*-HPEA-EA)/(3,4-DHPEA-EDA + *p*-HPEA-EDA) as a good
352 estimator of EVOO stability (Miho et al., 2020). Comparison of the antioxidant capacity of
353 isolated individual phenolic compounds using a similar accelerated oxidation test showed
354 high antioxidant activity for deacetoxy oleuropein aglycon and oleuropein aglycon, while
355 pro-oxidant effect was found for ligstroside aglycon (Carrasco-Pancorbo et al., 2005).
356 Stability was therefore related to the amount and composition of individual phenols rather
357 than to the total phenolic content.

358 Up to one hundred and eighty different volatile compounds belonging to several
359 chemical groups (carbonyl, ester, alcohol, hydrocarbon) have been found in EVOO aromas
360 (Angerosa, 2002). Among them, those produced enzymatically from the lipoxygenase (LOX)
361 pathway have been generally considered the main responsible in the formation of EVOO

362 positive aroma attributes, while many others responsible for negative attributes (defects),
363 such as rancid, winey-vinegary, fusty, muddy sediment, musty, are not present in EVOO
364 (Angerosa, 2002; Campestre et al., 2017). EVOO fruitiness has been previously correlated
365 positively with the content of individual volatiles such as Z-2-penten-1-ol; 3,5-dimethyl-1,6-
366 heptadiene; and sum of aldehydes C6, and negatively with 3-methyl-1-butanol; 2-methyl-1-
367 butanol; 2,4-dimethylheptane; hexyl acetate; nonanal; decanal; Z-2-decenal, although the
368 extent of these correlations was not reported (Cerretani et al., 2008). However, associations
369 between individual volatile concentration and specific EVOO aromas such as fruity could be
370 hindered by different odor thresholds, the complex interactions between volatiles and
371 receptors responsible of EVOO smell, the existence of multiple volatiles responsible for a
372 flavor sensation, and the combinations of volatiles yielding flavors different to those expected
373 from individual compounds (Campestre et al., 2017; Chambers & Koppel, 2013; Genovese
374 et al., 2019). PLS results obtained in this work confirm these difficulties as the model
375 developed was not able to accurately predict the level of fruitiness, that is the main positive
376 odor attribute of EVOO. Using a similar PLS approach, good predictions were previously
377 achieved for some negative attributes such as vinegar, not detected in our work as we were
378 working only with EVOO samples, but satisfactory cross-validation was not obtained for
379 prediction of other sensory attributes (Servili et al., 1995). Similarly, PLS models based on
380 volatile fingerprint have been reported to be able to discriminate between olive oil categories,
381 i.e. extra virgin vs. non-extra virgin samples; virgin vs. lampante categories with 97% correct
382 classification in cross-validation (Quintanilla-Casas et al., 2020).

383 The secoiridoid derivatives resulting from the enzymatic hydrolysis of oleuropein,
384 ligstroside and demethyloleuropein, identified as the dialdehydic forms of
385 decarboxymethyloleuropein and decarboxymethylligstroside aglycones (3,4-DHPEA-EDA
386 and *p*-HPEA-EDA, respectively) and the aldehydic forms of oleuropein and ligstroside
387 aglycones (3,4-DHPEA-EA and *p*-HPEA-EA, respectively) are the most abundant phenolic

388 components found in EVOO. These compounds have been suggested to underlay the bitter
389 and pungent sensory attributes of EVOO. In fact, the absorbance of the phenolic extract
390 obtained from EVOO measured at 225 nm was proposed as a simple method for bitterness
391 evaluation, although comparison of samples from cultivars with very different phenolic
392 profiles was considered non accurate (Gutiérrez Rosales et al., 1992; Mateos et al., 2004).
393 Total phenol content, measured as the absorbance at 726 nm after reaction with the Folin-
394 Ciocalteau reagent, was also suggested as an easy tool for bitterness assessment without
395 sensory evaluation (Beltrán et al., 2007). More specifically, *p*-HPEA-EDA (oleocanthal) was
396 described as the main phenolic responsible for the EVOO pungency (Andrewes et al., 2003),
397 while 3,4-DHPEA-EA was suggested as the main responsible for bitterness attribute (Mateos
398 et al., 2004), even though the magnitude of these relationships is discussed (Campestre et al.,
399 2017; Cerretani et al., 2008; Pedan et al., 2019). Literature reviews show different results,
400 relating bitterness intensity to the presence of oleuropein derivatives, to both oleuropein and
401 ligstroside aglycons, or only to ligstroside derivatives (Campestre et al., 2017). Our results
402 indicate that prediction of positive sensorial properties (fruity, bitter and pungent) was not
403 possible from chemical constituents.

404 It should be noted that, unlike previous studies, our work was conducted using a wide
405 EVOO sample set with combined effects of genotype and location, and cross-validation was
406 carried out for testing the results. Generalization of results obtained from simple pair
407 comparison of highly different EVOO could have occurred in previous works (Bendini, 2007;
408 Lukić et al., 2018). The use of commercial EVOO samples without controlling the potential
409 effects of other factors such as harvest time or extraction system could also difficult the
410 analysis of results (Beltrán et al., 2007; Gutiérrez Rosales et al., 1992; Mateos et al., 2004).
411 Finally, it cannot be excluded some differences in determination and identification of the
412 different phenolic compounds among works, as a wide variability of methodologies are used
413 for these analyses.

414 *4.2. Cultivar and location effects*

415 Significant differences among cultivars and locations have been obtained in this work
416 for the main chemical components of EVOO. These differences among chemical components
417 led to subsequent differences regarding EVOO stability. This was expected based on the
418 relationships between oil composition and stability discussed above. A stronger effect of
419 cultivar, compared to some environmental factors such as year of harvest and ripening stage,
420 has been previously reported for some compositional and antioxidant properties of EVOO
421 (Borges et al., 2019). In our work, Loc3 showed the highest stability, probably due to its
422 higher phenol content. The geographical area of origin has been also found to play a role in
423 the qualitative and quantitative characteristics of EVOO in previous works (Ben Mansour et
424 al., 2017).

425 On the contrary, significant differences among cultivars and locations for chemical
426 components of EVOO were not translated into significant differences in sensory attributes.
427 Previous studies indicate the importance of the genetic effect on the volatile composition of
428 EVOO. Comparison of contrasting cultivars such as 'Arbequina' and 'Picual' showed clear
429 genotypic effect for both the availability of nonesterified polyunsaturated fatty acids,
430 especially linolenic acid, and the enzymatic activity of the LOX system responsible of the
431 biosynthesis of VOO aroma compounds and therefore its sensorial characterization
432 (Sánchez-Ortiz et al., 2007). Consequently, the effects of cultivar on the sensorial properties
433 of EVOO has been underlined, linking these sensorial differences to the activities of the
434 different enzymes involved in the different pathways (Campestre et al., 2017; Sánchez-Ortiz
435 et al., 2007). However, similar to our work, no significant differences in sensory parameters
436 were observed between Italian and Spanish EVOOs in relation to their area origin and olive
437 cultivar and, therefore, the inclusion of additional positive sensory notes was recommended
438 for regulations of some PDO-EVOOs (Genovese et al., 2019). Fruitiness was also found to
439 be poor inter-cultivar but potent intra-cultivar typicity discriminator for Istrian cultivars, even

440 though significant differences for many volatile compounds were observed (Lukić et al.,
441 2018).

442 **5. Conclusions**

443 Our results suggest that other parameters, apart from cultivar and location, provide
444 significant variation for sensory properties of EVOO together with the inherent difficulties
445 associated to sensory evaluation. A deeper knowledge of these additional factors could open
446 up the possibilities of modulating sensory attributes regardless cultivar and location of origin.
447 The implication of these results regarding current PDOs regulation should be further studied
448 in future works. On the other hand, the lack of accuracy in the models developed for
449 prediction of sensory attributes underline the need for maintaining sensory evaluation panel
450 test as a tool of paramount importance for evaluating EVOO sensory quality. On the contrary,
451 EVOO stability seems to be easy to predict based on the chemical composition. For this trait,
452 the influence of genotype and location conditions could be quantified. This is of paramount
453 importance in breeding programs aimed to obtain new cultivars with improved EVOO
454 characteristics and to determine the best cultivar to be planted in each growing area.

455

456 **Declaration of competing interest**

457 Authors declare no conflict of interest.

458 **Acknowledgments:** This work was supported by projects of Andalusian Institute of
459 Agricultural Research and Training (AVA201601.2, AVA2019.027), partly funded by
460 European Regional Development Fund (ERDF). AS acknowledges Spanish National
461 Institute of Agricultural Research for financial support. Field and lab staff assistance is highly
462 acknowledged. Thank also to the members of the Tasting Panel from the Protected
463 Denomination of Origin "Priego de Córdoba" (Priego de Córdoba, Córdoba, Spain) who
464 carried out the sensory analysis of EVOO samples.

465 **Credit Author Statement**

Conceptualization, R.R. and L.L.; Investigation and methodology, all authors; data curation, A.S. and L.L.; writing—original draft preparation, A.S. and L.L; writing—review and editing, all authors; project administration, L.L.; funding acquisition, A.S, R.R., A.S., J.C., L.V., L.L.

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Table 1. Traits evaluated in EVOO samples

Group	Compound	Abbreviation	Units
Oxidative Stabililty		IT	h
Sensory properties		Fruity	(0-10)
		Bitter	(0-10)
		Pungent	(0-10)
Fatty acids	Palmitic	C160	%
	Palmitoleic	C161	%
	Stearic	C180	%
	Oleic	C181	%
	Linoleic	C182	%
	Linolenic	C183	%
	Arachidic	C200	%
	Eicosenoic	C201	%
	Behenic	C220	%
Tocopherols	Total	Tocopherols	mg/kg
	α-Tocopherol	aToc	%
	β-Tocopherol	BToc	%
	γ-Tocopherol	γToc	%
Squalene		Squalene	mg/kg
Phenols	Total	Phenols	mg/kg
	Hydroxytyrosol	HTyr	%
	Tyrosol	Tyr	%
	Vanillic acid	Van	%
	Vanillin	Vani	%
	pCumaric acid	pCum	%
	Hydroxytyrosol acetate	AcHTyr	%
	Oleacein	3,4-DHPEA-EDA	%
	Oleocanthal	p-HPEA-EDA	%
	Pinoresinol	Pino	%
	Cinnamic acid	Cin	%
	Acetoxypinoresinol	AcPino	%
	Oleuropein aglycone	3,4-DHPEA-EA	%
	Ligstroside aglycone	p-HPEA-EA	%
	Ferulic acid	Fer	%
	Luteolin	Lut	%
	Apigenin	Api	%

Group	Compound	Abbreviation	Units
Sterols	Total	Sterols	mg/kg
	Campesterol	Camp	%
	Stigmasterol	Stig	%
	Δ7-Campesterol	Δ7Camp	%
	Clerosterol	Clero	%
	β-sitosterol	Sito	%
	Δ5-avenasterol	Δ5Av	%
	Δ5-24-stigmastadienol	Δ524Stig	%
	Δ7-stigmastenol	Δ7Stig	%
	Δ7-avenasterol	Δ7Av	%
Volatiles	Total	Volatiles	µg/kg
	(E)+ (Z)-hex-3-enal	V01	%
	(Z)-hex-2-enal	V02	%
	(E)-hex-2-enal	V03	%
	(Z)-hex-3-enol	V04	%
	(E)-hex-2-enol	V05	%
	Hexanal	V06	%
	Hexan-1-ol	V07	%
	(Z)-pent-2-enal	V08	%
	(E)-pent-2-enal	V09	%
	Pent-1-en-3-ol	V10	%
	(Z)-pent-2-en-1-ol	V11	%
	(E)-pent-2-en-1-ol	V12	%
	Penten dimer-1	V13	%
	Penten dimer-2	V14	%
	Penten dimer-3	V15	%
	Penten dimer-4	V16	%
	Penten dimer-5+6	V17	%
	Penten dimer-7	V18	%
	Pentan-3-one	V19	%
	Pentanal	V20	%
	Hexyl acetate	V21	%
	(Z)-hex-3-en-1-yl acetate	V22	%
	Limonene	V23	%
	Ocimene	V24	%

Table 3. Cross-validation results for PLS models developed for stability (induction time, IT) and sensorial traits of EVOO samples (n=47).

	nPLS	**r**	**RMSECV**	**RPD**
IT (h)	2	0.88	3.44	1.89
Fruity (0-10)	1	0.21	0.85	0.98
Bitter (0-10)	1	0.29	0.54	1.02
Pungent (0-10)	1	0.11	0.45	0.96

[1]Number of latent variables (nPLS), Correlation between actual and predicted constituent values (r), Standard error of cross validation (RMSECV), Residual predictive deviation (RPD), Range Error Ratio (RER).

Table5

Table 5. Percentage of sums of squares for each source of variation and comparison of means by Cultivar and Location for stability (induction time, IT) and sensorial traits of EVOO.

Source	df	IT (h)		Fruity (0-10)	Bitter (0-10)		Pungent (0-10)
Cultivar	3	69.9		24.0	16.8		3.9
Location	3	10.5		0.5	15.9		5.3
C x L	9	5.2		6.1	25.8		20.7
Error	31	14.4		69.3	41.6		70.1
‘Picual’		21.83	a	4.69	3.10	a	2.81
Sel1		18.11	b	5.24	2.71	ab	2.75
Sel2		7.73	d	4.18	2.40	b	2.48
Sel3		12.45	c	5.25	2.79	ab	2.75
Loc1		12.81	b	4.82	2.61		2.54
Loc2		15.22	ab	4.97	2.61		2.82
Loc3		17.80	a	4.93	3.09		2.78
Loc4		13.32	b	4.84	2.60		2.66

Different letter by Cultivar or Location indicates significant differences at $P<0.05$.

Table2

Table 2. Descriptive statistics for stability (induction time, IT), sensorial traits and main chemical compounds of EVOO samples (n=47).

Trait/compound	Mean	SD	CV	Min	Max
IT (h)	14.79	6.50	43.98	4.75	27.03
Fruity (0-10)	4.89	0.83	17.00	3.00	6.10
Bitter (0-10)	2.75	0.55	19.94	1.90	4.00
Pungent (0-10)	2.70	0.43	15.86	2.00	3.50
C18:1 (%)	75.43	5.49	7.28	65.58	82.53
Tocopherol (mg/kg)	248.14	45.16	18.20	155.32	382.95
Squalene (mg/kg)	5,245.00	3,380.70	64.46	1,236.80	11,882.00
Sterols (mg/kg)	1,674.10	313.58	18.73	1,025.90	2,179.10
Phenol (mg/kg)	465.77	201.68	43.30	195.50	1,079.30
Volatile (µg/kg)	28,732.00	11,176.00	38.90	11,656.00	63,797.00

Table 4. Percentage of sums of squares for each source of variation and comparison of means by Cultivar and Location for main chemical compounds of EVOO.

Source	df	C18:1 (%)	Tocopherol (mg/kg)	Squalene (mg/kg)	Sterols (mg/kg)	Phenols (mg/kg)	Volatiles (µg/kg)
Cultivar	3	91.6	42.6	83.4	66.3	11.8	18.4
Location	3	1.2 NS [(1)]	15.7	7.8	11.9	40.5	60.0
C x L	9	3.1	11.8	5.5	8.4	11.9	7.7
Error	31	4.1	29.9	3.3	13.3	35.8	14.0
'Picual'		81.0 a [(2)]	257.7 a	7,765.6 b	1,426.6 b	460.7 ab	24,009 b
Sel1		78.5 b	256.3 a	8,669.4 a	1,428.2 b	491.3 ab	36,634 a
Sel2		67.3 d	276.3 a	1,969.2 c	1,934.4 a	362.0 b	27,116 b
Sel3		74.9 c	198.1 b	2,333.2 c	1,928.5 a	556.7 a	27,027 b
Loc1		74.7	266.0 a	3,658.1 b	1,808.8 a	425.2 b	24,796 b
Loc2		75.9	262.8 ab	6,117.4 a	1,754.9 ab	391.5 b	34,578 a
Loc3		75.0	231.6 bc	5,445.7 a	1,610.9 bc	681.8 a	17,100 c
Loc4		76.1	230.8 c	5,805.6 a	1,508.0 c	355.5 b	39,337 a

[(1)] NS: non-significant differences at $P<0.05$. [(2)] Different letter by Cultivar or Location indicates significant differences at $P<0.05$.

Figure1

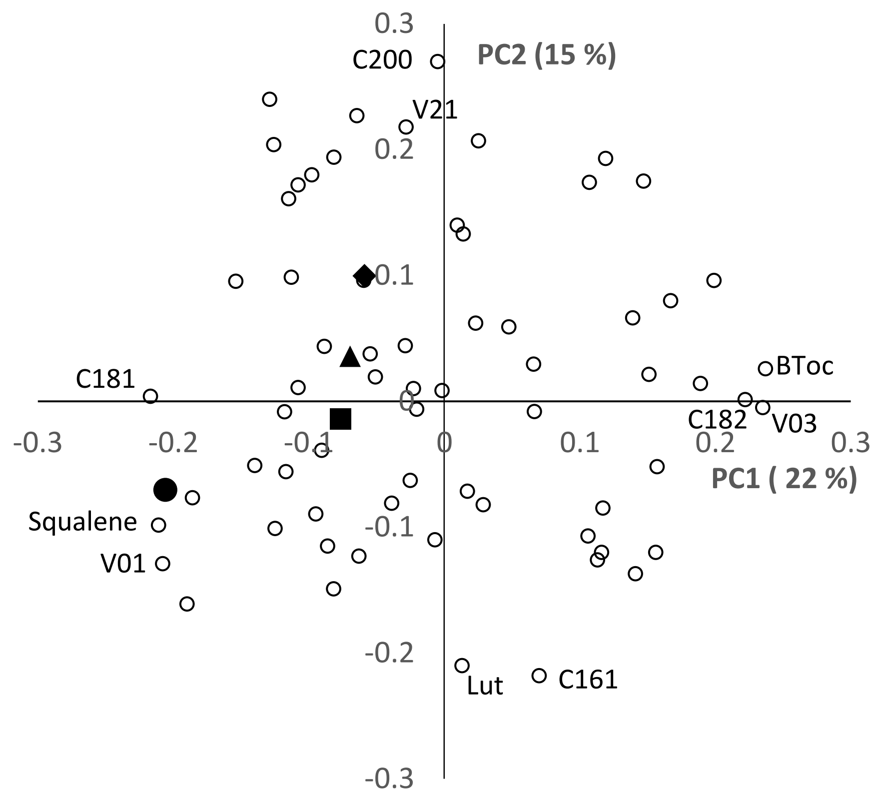

Figure 1. Loading plot of PCA model developed from 66 chemical compounds (white circles), stability (black circle) and sensorial traits including fruity (black diamond), bitter (black square) and pungent (black triangle) evaluated in EVOO samples. Compounds abbreviations are given in Table 1.

Figure2

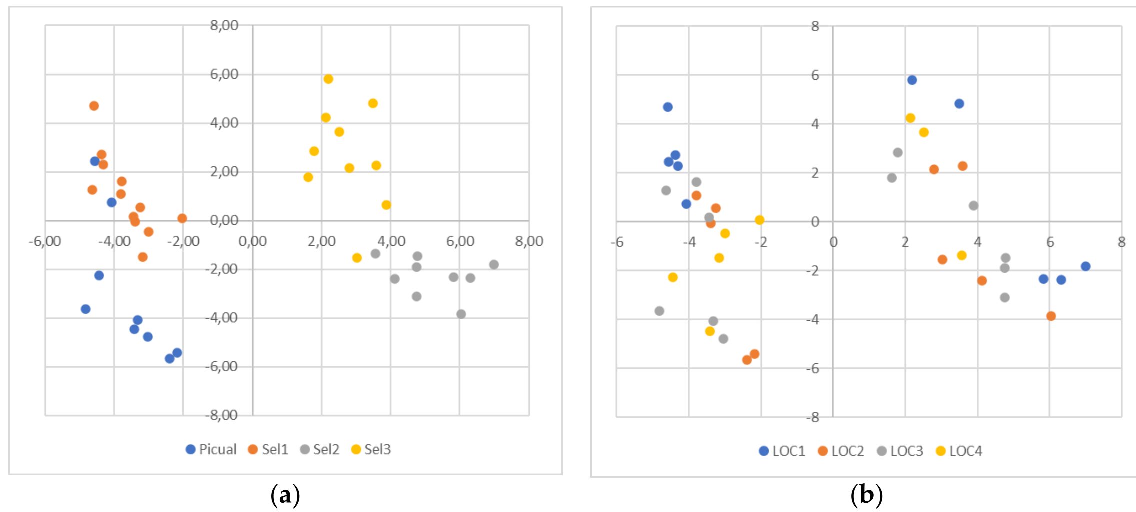

Figure 2. Scores plot of PCA model developed from 66 chemical compounds, stability and sensorial traits evaluated in EVOO samples. (**a**) Distribution by cultivar; (**b**) Distribution by location.

Figure4

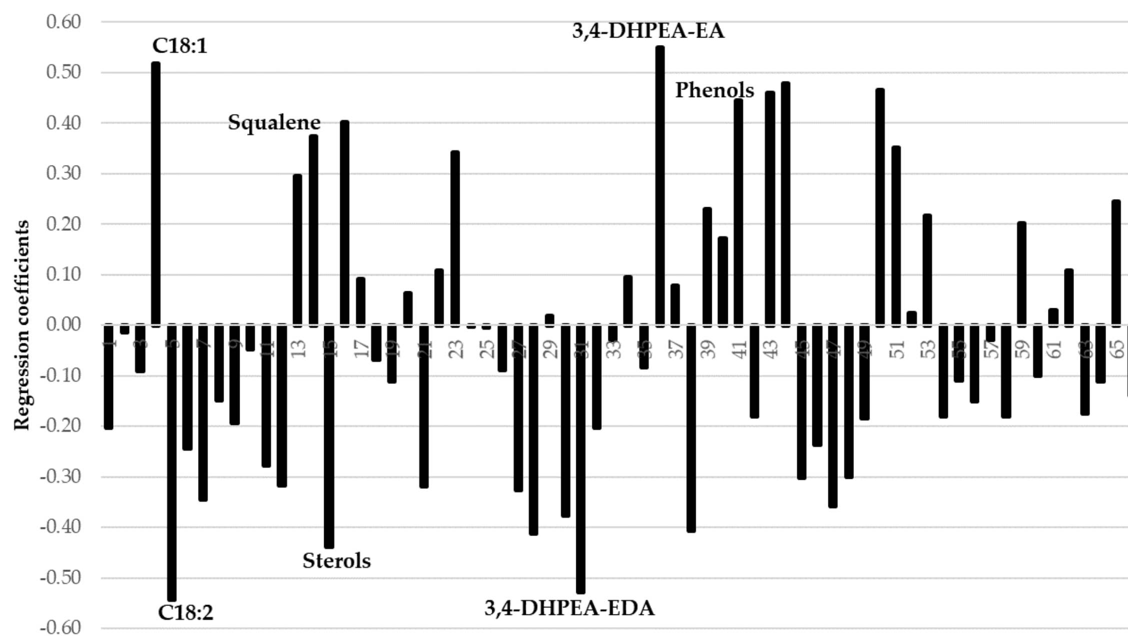

Figure 4. Regression coefficients of PLS model developed for stability (induction time, IT) from 66 chemical compounds evaluated in EVOO. Main components in the model are indicated.

Figure3

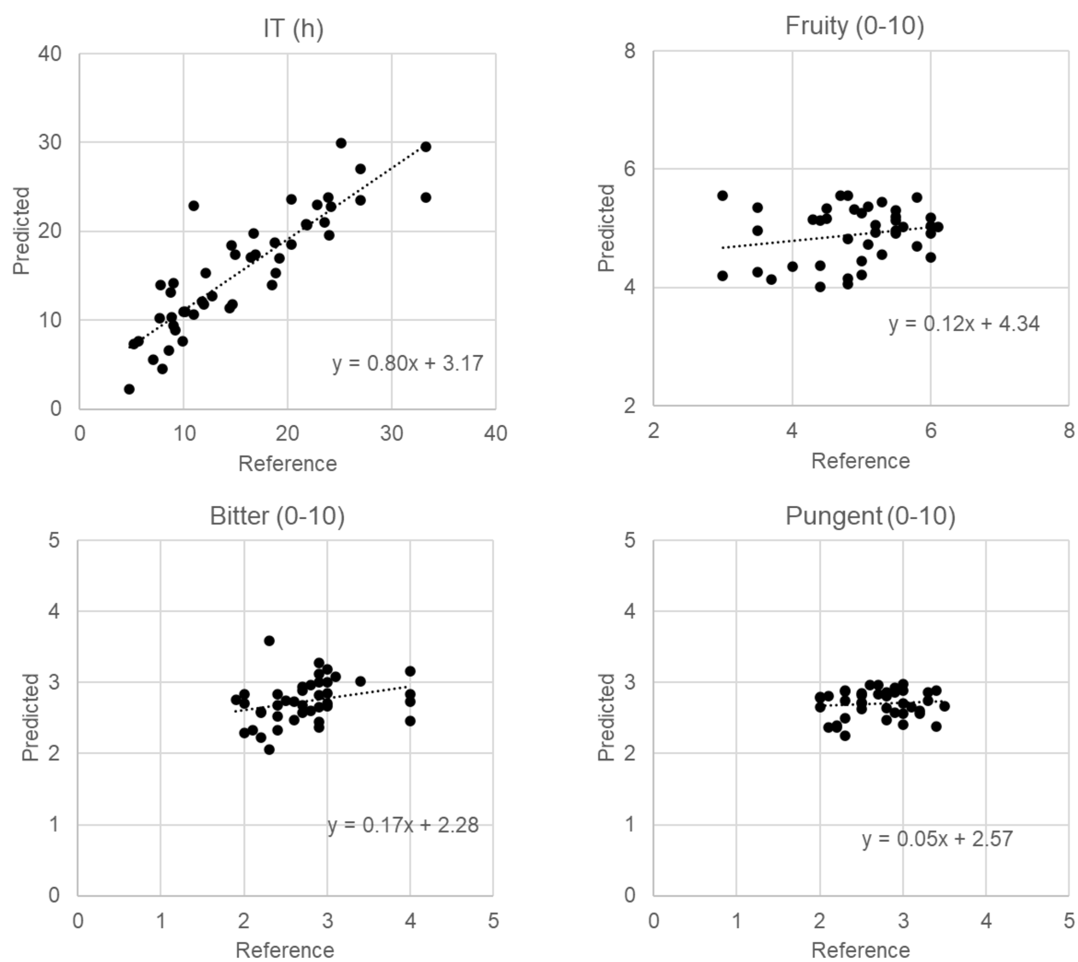

Figure 3. Predicted vs. reference values from PLS models developed for stability and sensorial traits based on values of 66 chemical compounds evaluated in EVOO.