ISOPRENOIDS COMPOSITION AND COLOUR TO DIFFERENTIATE VIRGIN OLIVE OILS FROM A SPECIFIC MILL

Running title: Colour and isoprenoids in olive oils

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ABSTRACT

The objective of this work was verify if, based on the concentration of some key isoprenoids and the colour, olive oils from a specific mill could be differentiated from those from other mills by linear discriminant analysis. The isoprenoids studied were β-carotene, lutein, α-tocopherol, β-sitosterol, pheophytin *a* and squalene, which are isoprenoids of interest in food science and nutrition. 88 % of correct classification was obtained by linear discriminant analysis and, therefore, a good differentiation was achieved. This fact can reveal the impact of the conditions of elaboration of each particular mill on the isoprenoid content of the olive oil and therefore on its colour, which is a characteristic of foods with a high effect on consumers.

KEYWORDS

Carotenoids; β-sitosterol; squalene; tocopherol.

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE

β-carotene (PubChem CID: 5280489); lutein (PubChem CID: 5281243); pheophytin *a* (PubChem CID: 459387); β-sitosterol (PubChem CID: 222284); squalene (PubChem CID: 638072); α-tocopherol (PubChem CID: 14985).

1. Introduction

The nutritional quality of olive oil concerns increasingly to both consumers and health systems. Olive oil consumption has been associated with a lower risk of developing diseases like myocardial infarction, atherosclerosis and certain cancers, among others (Fitó, De la Torre, & Covas, 2007; Pérez-Jiménez, Ruano, Pérez-Martínez, López-Segura, & López-Miranda, 2007). Until recently, most of the protective effects were associated almost exclusively to the high proportion of unsaturated fatty acids (Pérez-Jiménez *et al.*, 2007). However, in recent years it is becoming increasingly evident the importance that minor constituents of the unsaponifiable fraction, such as isoprenoids and phenolic compounds, have on the benefits associated with olive oil consumption (Covas *et al.*, 2006). Within the isoprenoid fraction, there are a series of compounds like α-tocopherol, β-sitosterol, squalene, carotenoids and chlorophylls, which are of great importance. Indeed, altogether, they are related to the nutritional and sensory quality of the product.

Tocopherols exhibit vitamin E activity. They are considered potent lipophilic antioxidants that can protect membrane lipids from oxidation, although non-antioxidant biological actions of these compounds have also been postulated (Azzi, 2007). They are commonly found in vegetable oils, fats and some vegetables like broccoli, celery and tomatoes (Monge-Rojas & Campos, 2011). The main tocopherol in virgin olive oil (VOO) is α-tocopherol (Haddam *et al*., 2014). Among phytosterols, the most abundant in olive oil is β-Sitosterol (Haddam *et al.,* 2014). It has a recognized effect on lowering cholesterol concentrations by interfering in the absorption of cholesterol in the intestinal tract (Trautwein *et al.*, 2003). Some studies suggest that the high concentration of squalene in olive oil could be one of the factors contributing to the anti-carcinogenic effect associated to the intake of it (Reddy & Couvreur, 2009). The major olive oil carotenoids are β-carotene and lutein (Giuffrida, Salvo, Salvo, Cossignani, & Dugo, 2011). β-carotene is a provitamin A carotenoid and continues eliciting interest as a health-promoting carotenoid. The study of the health

benefits of lutein is particularly important for its roles in the macula lutea and the brain (Johnson, 2004; Krinsky & Johnson, 2005). During the extraction of olive oil, chlorophylls lose the coordinated magnesium and are transformed into the respective pheophytin derivatives, which are consequently the major chlorophyll pigments in them (Giuffrida *et al.*, 2011). Unlike carotenoids, there are not many studies on the possible health benefits of chlorophylls (Moyano, Heredia, & Meléndez-Martínez, 2010).

The colour of foods is an attribute closely related to their acceptability or rejection by consumers, and therefore is very important in economic terms. Generally, the consumers associate the observed colours with possible aromas and tastes of the product (Moyano *et al*., 2010). The characteristic colour of an olive oil depends on the concentration and the ratio of the different pigments. These are classified in chlorophyll pigments, responsible for greenish hues, and carotenoids, responsible for yellow hues (Giuffrida *et al.*, 2011). Autooxidation of olive oil results in losses of pigments with the concomitant decolouration (Aparicio-Ruiz & Gandul-Rojas, 2014).

In this study, both some key isoprenoids and the colour of different VOO marketed in Andalusia were determined. The main objective was to evaluate if the concentration of these isoprenoids and the colour parameters can be useful to differentiate the samples from a specific mill (SM) from those from other mills (OM).

2. Materials and methods

2.1. *Reagents and standards*

HPLC-grade reagents, i.e. methanol (MeOH) and methyl tert-butyl ether (MTBE), and α-tocopherol, β-sitosterol, β-carotene, squalene, chlorophyll *a* and *b* standards were supplied by Sigma-Aldrich (Steinheim, Germany). Pheophytin *a* and *b* were obtained from chlorophylls *a* and *b* respectively by acidification with 0.1 mol/L methanolic hydrochloric acid (Sievers & Hynninen, 1977). Lutein was isolated from a saponified extract of green

leaves by open column chromatography according to recommended procedures (Rodríguez-Amaya, 2001).

2.2. *Samples*

Thirty samples of extra virgin olive oil (EVOO) produced in different parts of Andalusia were analysed. Fifteen of them (OM) were EVOO corresponding to common brands retailed in Spain and were purchased at a local supermarket. The rest (SM) were EVOO obtained with different olive varieties in an oil mill located in Palma del Rio (Oleopalma S.A.T., Palma del Río, Córdoba, Spain) from 2015 harvest. SM samples were obtained at an industrial scale. They were taken from the deposits from where the samples that are eventually bottled and marketed are kept. Both, OM and SM sets includes samples from different olive cultivars and geographical origins, within Andalucía. However, while OM were processed in different mills, the SM set includes oils obtained according to the same technological procedures. Information on the olive variety, geographic origin and type of packing as well as the companies of both groups of samples is summarised in Supplementary Table 1. Although in most cases the conditions of production of olive oil, i.e. temperature, extraction time, etc., were not indicated, it seems sensible to assume that these conditions vary between the different mills.

2.3. Isoprenoid analysis

2.3.1. Extraction and saponification

The samples were extracted in triplicate by using the protocol described by Mínguez-Mosquera, Gandul-Rojas, & Gallardo-Guerrero (1992) with some modifications. The method is based on a liquid-liquid distribution so that there is a partition of the compounds between N,N-dimethylformamide (DMF) and hexane. In the hexane phase, which is subsequently saponified, triglycerides, carotenes (mainly β-carotene), squalene and β-sitosterol are retained and in the DMF phase, xanthophylls, tocopherols and chlorophyll pigments are extracted.

Three-gram aliquots of the EVOO were dissolved directly in 30 mL of DMF saturated with $MgCO_3$ and treated with five successive 10 mL portions of hexane. The mixtures were centrifuged at 3000 rpm for 5 min. at 4 °C. The two phases were transferred to different separatory funnels.

Eighty mL of Na_2SO_4 solution (2 g/100 mL in water at approximately 0 °C), 15 mL of hexane and 15 mL of diethyl ether were added to the separatory funnel containing the DMF phase. This solution was shaken and kept approximately 20 min. Then, the lower aqueous phase was discarded. Afterward, the ether phase was washed three times with the Na_2SO_4 solution and was concentrated to dryness in a rotary evaporator at 30 ºC.

The hexane phase was saponified by adding 20 mL of methanolic KOH (35 g/100 mL) and 30 mL diethyl ether. The reaction mixture was maintained with mechanical shake overnight, in darkness and at room temperature. Then, the organic phase was washed with water and a solution of 10 g/100 mL NaCl until neutrality of the waste water. Finally, the remaining water was removed with the Na_2SO_4 solution. The organic phase was concentrated to dryness by rotary evaporation at 30 ºC.

The two extracts obtained from each sample (DMF and hexane extracts) were kept in the freezer under a nitrogen atmosphere. Prior to injection in the HPLC system, the two extracts were re-dissolved in 300 µL of ethyl acetate and filtered through a nylon membrane (13 mm × 0.45 µm) (Billerica, MA, USA). The injection volume was 20-µL. In samples where the concentrations of squalene and β-sitosterol were out of the linearity range of the detector, the hexane phase was further diluted with approximately 1.5 mL of ethyl acetate and 20 µL were injected.

2.3.2. Qualitative and quantitative analysis

The analysis of isoprenoids was carried out by High Performance Liquid Chromatography (HPLC) with external calibration. The concentrations of the standards were determined spectrophotometrically using the specific extinction coefficients in ethanol

(Britton, 1995). To prepare the stock solutions the standards were dissolved in ethyl acetate. HPLC analyses were carried out on an Agilent Technologies 1100 system (Agilent Technologies, Palo Alto, CA, USA). A C_{30} YMC column (3 µm, 150 cm × 4.6 mm) (Wilmington, NC, USA) kept at 20 ºC and a flow rate of 1 mL/min were used. The diode array detector was set at 450, 410, 290, 215 and 210 nm for the detection of carotenoids, pheophytins, tocopherols, squalene and β-sitosterol, respectively. The gradient was: 0 min: 85 % MeOH + 15 % MTBE; 20 min: 68 % MeOH + 32 % MTBE; 25 min: 85 % MeOH + 15 % MTBE. MeOH contained a small proportion of ammonium acetate (0.1 g/100 mL) in order to protect the carotenoids during the chromatographic analysis and to improve the recovery of them from the column.

The identification of the isoprenoids was made by comparison of their spectroscopic and chromatographic features with those of standards. For chlorophyll pigments and derivatives, the characteristic peak ratio was that between the absorbance of the Soret band (I) and the absorption maximum in the red region (II). For carotenoids, the height of the largest wavelength absorption band (III) was expressed as a percentage of that of the middle absorption band (II) (Rodríguez-Amaya, 2001). To identify the *cis* isomers of carotenoids the hypsochromic shifts of the absorption maxima relative to those of the corresponding all-*trans* isomers and the intensity of the *cis* peak (measured as D_B/D_{II}) have been taken into account (Rodríguez-Amaya, 2001).

2.4. Colour measurement

Colour measurements were made on a Hewlett Packard 8453 UV–Vis diode array spectrophotometer (Palo Alto, CA). Samples, without dilution, were filtered through paper filter. The entire visible spectra of the filtered samples in 5-mm pathlength quartz cuvettes were recorded. n-Hexane was used as blank reference due to its transparency and lipophilicity (Moyano, Meléndez-Martínez, Alba, & Heredia, 2008a). The colour parameters under CIE Illuminant D65 and 1964 Standard Colourimetric Observer were obtained by means of the

166 software CromaLab© (Heredia, Álvarez, González-Miret, & Ramírez, 2004). The colour
167 parameters of the CIELAB space (CIE, 1978), i.e. L^* (lightness), a^* (ranging from green to
168 red), and b^* (ranging from blue to yellow), and the angular coordinates, i.e. C^*_{ab} (chroma, the
169 quantitative expression of colourfulness) and h_{ab} (hue angle, the qualitative expression of
170 colour), which are more related to the psychophysical characteristics of colour, were
171 calculated.

172 2.5. *Statistical analysis*

173 Data processing was performed using the IBM SPSS Statistics 20® software (SPSS
174 Inc., 2012). Analysis of variance (ANOVA) was performed in order to determine significant
175 differences ($P < 0.05$) in the concentrations of compounds between OM and SM. Linear
176 discriminant analysis (LDA) was performed to explore the possibility of classification of the
177 two groups of samples according to their isoprenoid composition and colour. The forward
178 stepwise and the "leave one out" methods were employed.

179

180 **3. Results and discussion**

181 *3.1. Isoprenoid analysis*

182 The main isoprenoids of interest in food science and nutrition present in olive oils, i.e.
183 β-carotene, lutein, α-tocopherol, β-sitosterol, pheophytin *a* and squalene were analysed.
184 Considering the retention time of each compound and the phase in which it was extracted
185 (hexane or DMF), it can be readily inferred that there were no co-elution problems (Table 1
186 and Supplementary Fig. 1). The HPLC method used is also appropriate for the analysis of
187 some carotenoid isomers, such as two *cis* isomers of β-carotene that were detected in almost
188 all the samples. These isomers have been identified as the 9-*cis* isomer and the 13-*cis* or 15-
189 *cis* isomer. 9-*cis*-Lutein has also been detected in some samples. Although some *cis* isomers
190 of carotenoids have also been detected by other authors, they are not reported in the majority
191 of studies. The study of the presence of *cis* isomers of carotenoids in olive oil should be

192 encouraged as they could provide interesting information for the comparison of different
193 processing conditions or to compare the age of different oils. Zeaxanthin and antheraxanthin,
194 which are common olive oil carotenoids (Aparicio-Ruiz, Mínguez-Mosquera, & Gandul-
195 Rojas, 2011; Gandul-Rojas, Cepero, & Mínguez-Mosquera, 2000), were also detected.
196 However, they were not analysed as their concentrations were very low.
197 There are many studies (Criado, Motilva, Goñi, & Romero, 2007; Giuffrida *et al.*,
198 2011) aiming specifically at analysing the pigments in VOO by using the extraction method
199 developed by Mínguez-Mosquera *et al.* (1992). On the other hand, there are also studies that
200 focus on some of the isoprenoids analysed in this study by using other techniques and
201 methodologies such as GC, capillary electrophoresis, spectrophotometry, and direct injection
202 in HPLC, among others (Cañabate Díaz *et al*., 2007; Galeano-Díaz, Acedo-Valenzuela, &
203 Silva-Rodríguez, 2012; Karabagias *et al.*, 2013; Manzi, Panfili, Esti, & Pizzoferrato, 1998;
204 Puspitasari-Nienaber, Ferruzzi, & Schwartz, 2002; Sivakumar, Bati, Perri, & Uccella, 2006).
205 However, extraction and HPLC methods appropriate for the determination of all the
206 isoprenoids determined in the present study are not well-described in the literature. Some of
207 the methods mentioned above could be useful for the simultaneous analysis of all these
208 isoprenoids but it would require studies to confirm it. According to a study conducted by
209 Lietz and Henry (1997), an alternative to the basic hydrolysis could be an enzymatic
210 hydrolysis using the non-specific *Candida cylindracea* lipase such that losses of palm oil
211 carotenoids and tocopherols due to alkaline hydrolyses are reduced. In this work the
212 enzymatic hydrolysis is performed directly without prior separation of chlorophylls. In this
213 sense, it is not clear if this approach is also appropriate for the analysis of chlorophylls and its
214 derivatives, as the phytol group in them may also be hydrolysed by the enzyme.

215 *3.2. Isoprenoids content*

216 The mean concentrations and the calibration curves are presented in Table 2 and
217 Supplementary Table 2, respectively. The results of the ANOVA indicated that OM had

218 significantly higher concentrations of lutein and squalene and a significantly lower
219 concentration of α-tocopherol than those of SM ($P < 0.05$) (Table 3). The concentrations of α-
220 tocopherol in the samples ranged between 10.2 and 29.1 mg/kg. Other authors have reported
221 similar and higher concentrations in EVOO (Gimeno, Castellote, Lamuela-Raventós, De la
222 Torre, & López-Sabater, 2002). The squalene contents fluctuated between 544.6 and 1706.9
223 mg/kg. Concentrations of squalene in VOO ranging from 500 to 6000 mg/kg have been found
224 in the literature (Abdalle, El-Difrawy, & Abdelneem, 2008; Contiñas, Martínez, Carballo, &
225 Franco, 2008; Manzi *et al*., 1998). On the other hand, β-sitosterol showed ranges between
226 530.2 and 2638.6 mg/kg while reported concentrations ranged from 667 to 5491 mg/kg
227 (Cañabate Díaz *et al*., 2007; Sivakumar *et al*., 2006). In the case of pigments, β-carotene
228 concentrations oscillated between 0.15 and 0.67 mg/kg, those of lutein between 0.65 and 3.60
229 mg/kg, and the pheophytin *a* contents between 0.08 and 0.49 mg/kg. Concentrations of β-
230 carotene ranging from 0.5 to 4 mg/kg, and of lutein between 1 and 20 mg/kg have been
231 reported in a recent review (Fernández Gutiérrez & Segura Carretero, 2010). However, in
232 many studies the reported values of lutein do not exceed 4 mg/kg (Giuffrida *et al*., 2011;
233 Motilva & Romero, 2010; Ranalli, Malfatti, Lucera, Contento, & Sotiriou, 2005). Moreover,
234 large differences in the concentration of pheophytin *a* have been reported, from traces in VOO
235 from Arbequina olives (Criado *et al.*, 2007) to 26 mg/kg in samples from Picholine Marocaine
236 olives (Csallany & Rahmani, 1991). Considering these data, it can be concluded that the
237 average concentrations of the isoprenoid under study (Table 2) are comparable to those
238 reported for VOO of different origins. Taking into account that some of the samples have
239 been bought in supermarkets, it is necessary to point out the possibility of adulteration or
240 contamination of these oils. In recent years many studies have focused on this topic because
241 VOO is a relatively expensive and popular food which makes it a target product for
242 adulterations (Jabeur *et al*., 2017; Li *et al*., 2017; Tavares Melo Milanez *et al*., 2017).

The ranges of concentration obtained for the different compounds were, in most cases, very wide. The percentage differences between the maximum and minimum concentrations were 96 %, 127 %, 133 %, 103 %, 146 % and 139 % for α-tocopherol, β-carotene, β-sitosterol, squalene, pheophytin *a* and lutein respectively. The narrowest dispersion was observed in the α-tocopherol. This may be due in part to the fact that, among the olive oil antioxidants, the concentrations of α-tocopherol have been reported to vary to a lesser extent during the ripening of the olives (Gimeno *et al.*, 2002). Therefore, it is expected that the variability of samples due to the degree of ripening of the olives had a lower influence on the concentration of α-tocopherol. Moreover, it appears that the concentration of α-tocopherol, unlike other minority compounds of olive oil, is not much influenced by the type of extraction system (Gimeno *et al*., 2002).

3.3.Colour

A typical visible spectrum of olive oil with the characteristic absorption maxima due to pheophytin *a* (around 670 nm) and carotenoids (between 430 and 480 nm) is shown in Fig. 1.

The mean colour parameters are shown in Supplementary Table 3. The average values of L^* for both groups of samples were near to 100 CIELAB units, which indicated that the samples were very light. SM and OM showed values of a^* positive and close to zero. This could be attributable in part to the low concentration of chlorophyll compounds since negative values of a^* correspond to greenish colours. Similar to the results obtained by Moyano *et al*. (2008a), the value of the chroma for the two groups was virtually identical to its corresponding value of b^*. In all the samples the value of b^* was close to 100 units, which indicates that the colour of them have an important yellow contribution. Since the yellow hue of the olive oils is mainly due to the contents of β-carotene and lutein (Giuffrida *et al.*, 2011), this statement is consistent with the fact that the carotenoid fraction has an important contribution to the colour of the samples analysed.

OM had significantly higher values of b^* and C^*_{ab} than SM ($P < 0.05$) (Table 3). It is interesting to note that, b^* and C^*_{ab} are thought to be the colour parameters best correlated to the carotenoids index (an approach to estimate the concentration of carotenoids), the correlations being positive (Moyano, Meléndez-Martínez, Alba, & Heredia, 2008b). This observation agrees well with the results of the present study, as OM had higher values of b^* and C^*_{ab} and also higher concentration of carotenoids.

3.4. LDA analysis

Taking into account that the SM were elaborated in the same mill with the same method of production while OM were elaborated in different mills (Supplementary Table 1) and considering that the processing has an important impact on the composition of the oils, it appears sensible to hypothesize that the SM could be differentiated from the OM according to their isoprenoids composition and colour.

The discriminatory power of the variables for differentiating the two sets of EVOO samples followed the decreasing order: a^*, C^*_{ab}, lutein, α-tocopherol, squalene and β-carotene (Supplementary Table 4). Discriminatory variables with positive contributions were a^*, α-tocopherol and squalene. 88.6 % of the SM and 88.9 % of the OM were grouped correctly (Table 4). The canonical discriminant function explained 88.7 % of the accumulated variance. This good classification achieved (Fig. 2 and Table 4) can be due to the fact that the concentrations of the isoprenoids analysed are dependent on the processing method. For example, the storage period of olives before the extraction appears to affect considerably the content of β-sitosterol, tocopherols and chlorophylls in the oil (Mínguez-Mosquera, 1997). Higher temperatures during malaxation process are thought to lead to higher extraction of the minor components of oil, albeit excessive temperatures can promote their degradation (Aparicio-Ruiz *et al*., 2011; Ranalli *et al*., 2005). Moreover, according to Gimeno *et al.* (2002), the oils extracted by a two-phase decanter have higher concentrations of tocopherols than those obtained by a three-phase decanter. It is to be noted though that there is certain

295 controversy in this respect (Di Giovacchino, Sestili, & Di Vincenzo, 2002). In this regard, it is
296 important to consider that the extraction of lutein and other xanthophylls, which are more
297 polar than carotenes, may be influenced by the addition of water in the three-phase system.
298 Besides, the use of sodium chloride as adjuvant seems to affect the pigment content and thus
299 the colour of the resulting oil (Pérez, Romero, Yousfi, & García, 2008). It has also been
300 reported that the filtered oils have a lower amount of chlorophylls than the unfiltered ones
301 (Bottino, Capannelli, Mattei, Rovellini, & Zunin, 2008). Research is needed in order to
302 evaluate if the amount of carotenoids also decreases.
303
304 **4. Conclusions**
305 Although the development of the chromatographic method was not the main objective
306 of this work, it is worth noting that with this HPLC method it have been able to analyse lutein,
307 β-carotene, pheophytin *a*, β-sitosterol, squalene and α-tocopherol, which are isoprenoids of
308 interest due to their nutritional and / or sensory importance. Moreover, the developed HPLC
309 method allows detection of some *cis* isomers of carotenoids. Overall, the concentrations of
310 lutein and squalene were significantly higher in the OM than in the SM ($P < 0.05$). The
311 comparison of pigments concentration data between the two groups of samples is consistent
312 with the results obtained by comparing colour parameters. Despite the chemical composition
313 of olive oils is dependent of numerous factors it has been found that some key isoprenoids and
314 colour parameters can be useful to differentiate the samples obtained in a particular mill from
315 those obtained in others. More specifically, 89 % of correct classifications were obtained by
316 LDA. These results highlight the importance that the processing conditions have in the
317 extraction of olive constituents and therefore in the composition of the virgin olive oils.
318
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CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest.

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ISOPRENOIDS COMPOSITION AND COLOUR TO DIFFERENTIATE VIRGIN OLIVE OILS FROM A SPECIFIC MILL

Running title: Colour and isoprenoids in olive oils

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ABSTRACT

The objective of this work was verify if, based on the concentration of some key isoprenoids and the colour, olive oils from a specific mill could be differentiated from those from other mills by linear discriminant analysis. The isoprenoids studied were β-carotene, lutein, α-tocopherol, β-sitosterol, pheophytin *a* and squalene, which are isoprenoids of interest in food science and nutrition. 88 % of correct classification was obtained by linear discriminant analysis and, therefore, a good differentiation was achieved. This fact can reveal the impact of the conditions of elaboration of each particular mill on the isoprenoid content of the olive oil and therefore on its colour, which is a characteristic of foods with a high effect on consumers.

KEYWORDS

Carotenoids; β-sitosterol; squalene; tocopherol.

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE

β-carotene (PubChem CID: 5280489); lutein (PubChem CID: 5281243); pheophytin *a* (PubChem CID: 459387); β-sitosterol (PubChem CID: 222284); squalene (PubChem CID: 638072); α-tocopherol (PubChem CID: 14985).

1. Introduction

The nutritional quality of olive oil concerns increasingly to both consumers and health systems. Olive oil consumption has been associated with a lower risk of developing diseases like myocardial infarction, atherosclerosis and certain cancers, among others (Fitó, De la Torre, & Covas, 2007; Pérez-Jiménez, Ruano, Pérez-Martínez, López-Segura, & López-Miranda, 2007). Until recently, most of the protective effects were associated almost exclusively to the high proportion of unsaturated fatty acids (Pérez-Jiménez *et al.*, 2007). However, in recent years it is becoming increasingly evident the importance that minor constituents of the unsaponifiable fraction, such as isoprenoids and phenolic compounds, have on the benefits associated with olive oil consumption (Covas *et al.*, 2006). Within the isoprenoid fraction, there are a series of compounds like α-tocopherol, β-sitosterol, squalene, carotenoids and chlorophylls, which are of great importance. Indeed, altogether, they are related to the nutritional and sensory quality of the product.

Tocopherols exhibit vitamin E activity. They are considered potent lipophilic antioxidants that can protect membrane lipids from oxidation, although non-antioxidant biological actions of these compounds have also been postulated (Azzi, 2007). They are commonly found in vegetable oils, fats and some vegetables like broccoli, celery and tomatoes (Monge-Rojas & Campos, 2011). The main tocopherol in virgin olive oil (VOO) is α-tocopherol (Haddam *et al*., 2014). Among phytosterols, the most abundant in olive oil is β-Sitosterol (Haddam *et al.,* 2014). It has a recognized effect on lowering cholesterol concentrations by interfering in the absorption of cholesterol in the intestinal tract (Trautwein *et al.*, 2003). Some studies suggest that the high concentration of squalene in olive oil could be one of the factors contributing to the anti-carcinogenic effect associated to the intake of it (Reddy & Couvreur, 2009). The major olive oil carotenoids are β-carotene and lutein (Giuffrida, Salvo, Salvo, Cossignani, & Dugo, 2011). β-carotene is a provitamin A carotenoid and continues eliciting interest as a health-promoting carotenoid. The study of the health

benefits of lutein is particularly important for its roles in the macula lutea and the brain (Johnson, 2004; Krinsky & Johnson, 2005). During the extraction of olive oil, chlorophylls lose the coordinated magnesium and are transformed into the respective pheophytin derivatives, which are consequently the major chlorophyll pigments in them (Giuffrida *et al.*, 2011). Unlike carotenoids, there are not many studies on the possible health benefits of chlorophylls (Moyano, Heredia, & Meléndez-Martínez, 2010).

The colour of foods is an attribute closely related to their acceptability or rejection by consumers, and therefore is very important in economic terms. Generally, the consumers associate the observed colours with possible aromas and tastes of the product (Moyano *et al*., 2010). The characteristic colour of an olive oil depends on the concentration and the ratio of the different pigments. These are classified in chlorophyll pigments, responsible for greenish hues, and carotenoids, responsible for yellow hues (Giuffrida *et al.*, 2011). Autooxidation of olive oil results in losses of pigments with the concomitant decolouration (Aparicio-Ruiz & Gandul-Rojas, 2014).

In this study, both some key isoprenoids and the colour of different VOO marketed in Andalusia were determined. The main objective was to evaluate if the concentration of these isoprenoids and the colour parameters can be useful to differentiate the samples from a specific mill (SM) from those from other mills (OM).

2. Materials and methods

2.1. *Reagents and standards*

HPLC-grade reagents, i.e. methanol (MeOH) and methyl tert-butyl ether (MTBE), and α-tocopherol, β-sitosterol, β-carotene, squalene, chlorophyll *a* and *b* standards were supplied by Sigma-Aldrich (Steinheim, Germany). Pheophytin *a* and *b* were obtained from chlorophylls *a* and *b* respectively by acidification with 0.1 mol/L methanolic hydrochloric acid (Sievers & Hynninen, 1977). Lutein was isolated from a saponified extract of green

leaves by open column chromatography according to recommended procedures (Rodríguez-Amaya, 2001).

2.2. *Samples*

Thirty samples of extra virgin olive oil (EVOO) produced in different parts of Andalusia were analysed. Fifteen of them (OM) were EVOO corresponding to common brands retailed in Spain and were purchased at a local supermarket. The rest (SM) were EVOO obtained with different olive varieties in an oil mill located in Palma del Rio (Oleopalma S.A.T., Palma del Río, Córdoba, Spain) from 2015 harvest. SM samples were obtained at an industrial scale. They were taken from the deposits from where the samples that are eventually bottled and marketed are kept. Both, OM and SM sets includes samples from different olive cultivars and geographical origins, within Andalucía. However, while OM were processed in different mills, the SM set includes oils obtained according to the same technological procedures. Information on the olive variety, geographic origin and type of packing as well as the companies of both groups of samples is summarised in Supplementary Table 1. Although in most cases the conditions of production of olive oil, i.e. temperature, extraction time, etc., were not indicated, it seems sensible to assume that these conditions vary between the different mills.

2.3. Isoprenoid analysis

2.3.1. Extraction and saponification

The samples were extracted in triplicate by using the protocol described by Mínguez-Mosquera, Gandul-Rojas, & Gallardo-Guerrero (1992) with some modifications. The method is based on a liquid-liquid distribution so that there is a partition of the compounds between N,N-dimethylformamide (DMF) and hexane. In the hexane phase, which is subsequently saponified, triglycerides, carotenes (mainly β-carotene), squalene and β-sitosterol are retained and in the DMF phase, xanthophylls, tocopherols and chlorophyll pigments are extracted.

Three-gram aliquots of the EVOO were dissolved directly in 30 mL of DMF saturated with $MgCO_3$ and treated with five successive 10 mL portions of hexane. The mixtures were centrifuged at 3000 rpm for 5 min. at 4 °C. The two phases were transferred to different separatory funnels.

Eighty mL of Na_2SO_4 solution (2 g/100 mL in water at approximately 0 °C), 15 mL of hexane and 15 mL of diethyl ether were added to the separatory funnel containing the DMF phase. This solution was shaken and kept approximately 20 min. Then, the lower aqueous phase was discarded. Afterward, the ether phase was washed three times with the Na_2SO_4 solution and was concentrated to dryness in a rotary evaporator at 30 ºC.

The hexane phase was saponified by adding 20 mL of methanolic KOH (35 g/100 mL) and 30 mL diethyl ether. The reaction mixture was maintained with mechanical shake overnight, in darkness and at room temperature. Then, the organic phase was washed with water and a solution of 10 g/100 mL NaCl until neutrality of the waste water. Finally, the remaining water was removed with the Na_2SO_4 solution. The organic phase was concentrated to dryness by rotary evaporation at 30 ºC.

The two extracts obtained from each sample (DMF and hexane extracts) were kept in the freezer under a nitrogen atmosphere. Prior to injection in the HPLC system, the two extracts were re-dissolved in 300 µL of ethyl acetate and filtered through a nylon membrane (13 mm × 0.45 µm) (Billerica, MA, USA). The injection volume was 20-µL. In samples where the concentrations of squalene and β-sitosterol were out of the linearity range of the detector, the hexane phase was further diluted with approximately 1.5 mL of ethyl acetate and 20 µL were injected.

2.3.2. Qualitative and quantitative analysis

The analysis of isoprenoids was carried out by High Performance Liquid Chromatography (HPLC) with external calibration. The concentrations of the standards were determined spectrophotometrically using the specific extinction coefficients in ethanol

(Britton, 1995). To prepare the stock solutions the standards were dissolved in ethyl acetate. HPLC analyses were carried out on an Agilent Technologies 1100 system (Agilent Technologies, Palo Alto, CA, USA). A C_{30} YMC column (3 µm, 150 cm × 4.6 mm) (Wilmington, NC, USA) kept at 20 ºC and a flow rate of 1 mL/min were used. The diode array detector was set at 450, 410, 290, 215 and 210 nm for the detection of carotenoids, pheophytins, tocopherols, squalene and β-sitosterol, respectively. The gradient was: 0 min: 85 % MeOH + 15 % MTBE; 20 min: 68 % MeOH + 32 % MTBE; 25 min: 85 % MeOH + 15 % MTBE. MeOH contained a small proportion of ammonium acetate (0.1 g/100 mL) in order to protect the carotenoids during the chromatographic analysis and to improve the recovery of them from the column.

The identification of the isoprenoids was made by comparison of their spectroscopic and chromatographic features with those of standards. For chlorophyll pigments and derivatives, the characteristic peak ratio was that between the absorbance of the Soret band (I) and the absorption maximum in the red region (II). For carotenoids, the height of the largest wavelength absorption band (III) was expressed as a percentage of that of the middle absorption band (II) (Rodríguez-Amaya, 2001). To identify the *cis* isomers of carotenoids the hypsochromic shifts of the absorption maxima relative to those of the corresponding all-*trans* isomers and the intensity of the *cis* peak (measured as D_B/D_{II}) have been taken into account (Rodríguez-Amaya, 2001).

2.4. Colour measurement

Colour measurements were made on a Hewlett Packard 8453 UV–Vis diode array spectrophotometer (Palo Alto, CA). Samples, without dilution, were filtered through paper filter. The entire visible spectra of the filtered samples in 5-mm pathlength quartz cuvettes were recorded. n-Hexane was used as blank reference due to its transparency and lipophilicity (Moyano, Meléndez-Martínez, Alba, & Heredia, 2008a). The colour parameters under CIE Illuminant D65 and 1964 Standard Colourimetric Observer were obtained by means of the

166 software CromaLab© (Heredia, Álvarez, González-Miret, & Ramírez, 2004). The colour
167 parameters of the CIELAB space (CIE, 1978), i.e. L^* (lightness), a^* (ranging from green to
168 red), and b^* (ranging from blue to yellow), and the angular coordinates, i.e. C^*_{ab} (chroma, the
169 quantitative expression of colourfulness) and h_{ab} (hue angle, the qualitative expression of
170 colour), which are more related to the psychophysical characteristics of colour, were
171 calculated.

172 2.5. *Statistical analysis*

173 Data processing was performed using the IBM SPSS Statistics 20® software (SPSS
174 Inc., 2012). Analysis of variance (ANOVA) was performed in order to determine significant
175 differences ($P < 0.05$) in the concentrations of compounds between OM and SM. Linear
176 discriminant analysis (LDA) was performed to explore the possibility of classification of the
177 two groups of samples according to their isoprenoid composition and colour. The forward
178 stepwise and the "leave one out" methods were employed.

179

180 **3. Results and discussion**

181 *3.1. Isoprenoid analysis*

182 The main isoprenoids of interest in food science and nutrition present in olive oils, i.e.
183 β-carotene, lutein, α-tocopherol, β-sitosterol, pheophytin *a* and squalene were analysed.
184 Considering the retention time of each compound and the phase in which it was extracted
185 (hexane or DMF), it can be readily inferred that there were no co-elution problems (Table 1
186 and Supplementary Fig. 1). The HPLC method used is also appropriate for the analysis of
187 some carotenoid isomers, such as two *cis* isomers of β-carotene that were detected in almost
188 all the samples. These isomers have been identified as the 9-*cis* isomer and the 13-*cis* or 15-
189 *cis* isomer. 9-*cis*-Lutein has also been detected in some samples. Although some *cis* isomers
190 of carotenoids have also been detected by other authors, they are not reported in the majority
191 of studies. The study of the presence of *cis* isomers of carotenoids in olive oil should be

192 encouraged as they could provide interesting information for the comparison of different
193 processing conditions or to compare the age of different oils. Zeaxanthin and antheraxanthin,
194 which are common olive oil carotenoids (Aparicio-Ruiz, Mínguez-Mosquera, & Gandul-
195 Rojas, 2011; Gandul-Rojas, Cepero, & Mínguez-Mosquera, 2000), were also detected.
196 However, they were not analysed as their concentrations were very low.
197 There are many studies (Criado, Motilva, Goñi, & Romero, 2007; Giuffrida *et al.*,
198 2011) aiming specifically at analysing the pigments in VOO by using the extraction method
199 developed by Mínguez-Mosquera *et al.* (1992). On the other hand, there are also studies that
200 focus on some of the isoprenoids analysed in this study by using other techniques and
201 methodologies such as GC, capillary electrophoresis, spectrophotometry, and direct injection
202 in HPLC, among others (Cañabate Díaz *et al*., 2007; Galeano-Díaz, Acedo-Valenzuela, &
203 Silva-Rodríguez, 2012; Karabagias *et al.*, 2013; Manzi, Panfili, Esti, & Pizzoferrato, 1998;
204 Puspitasari-Nienaber, Ferruzzi, & Schwartz, 2002; Sivakumar, Bati, Perri, & Uccella, 2006).
205 However, extraction and HPLC methods appropriate for the determination of all the
206 isoprenoids determined in the present study are not well-described in the literature. Some of
207 the methods mentioned above could be useful for the simultaneous analysis of all these
208 isoprenoids but it would require studies to confirm it. According to a study conducted by
209 Lietz and Henry (1997), an alternative to the basic hydrolysis could be an enzymatic
210 hydrolysis using the non-specific *Candida cylindracea* lipase such that losses of palm oil
211 carotenoids and tocopherols due to alkaline hydrolyses are reduced. In this work the
212 enzymatic hydrolysis is performed directly without prior separation of chlorophylls. In this
213 sense, it is not clear if this approach is also appropriate for the analysis of chlorophylls and its
214 derivatives, as the phytol group in them may also be hydrolysed by the enzyme.

215 *3.2. Isoprenoids content*

216 The mean concentrations and the calibration curves are presented in Table 2 and
217 Supplementary Table 2, respectively. The results of the ANOVA indicated that OM had

218 significantly higher concentrations of lutein and squalene and a significantly lower
219 concentration of α-tocopherol than those of SM ($P < 0.05$) (Table 3). The concentrations of α-
220 tocopherol in the samples ranged between 10.2 and 29.1 mg/kg. Other authors have reported
221 similar and higher concentrations in EVOO (Gimeno, Castellote, Lamuela-Raventós, De la
222 Torre, & López-Sabater, 2002). The squalene contents fluctuated between 544.6 and 1706.9
223 mg/kg. Concentrations of squalene in VOO ranging from 500 to 6000 mg/kg have been found
224 in the literature (Abdalle, El-Difrawy, & Abdelneem, 2008; Contiñas, Martínez, Carballo, &
225 Franco, 2008; Manzi *et al*., 1998). On the other hand, β-sitosterol showed ranges between
226 530.2 and 2638.6 mg/kg while reported concentrations ranged from 667 to 5491 mg/kg
227 (Cañabate Díaz *et al*., 2007; Sivakumar *et al*., 2006). In the case of pigments, β-carotene
228 concentrations oscillated between 0.15 and 0.67 mg/kg, those of lutein between 0.65 and 3.60
229 mg/kg, and the pheophytin *a* contents between 0.08 and 0.49 mg/kg. Concentrations of β-
230 carotene ranging from 0.5 to 4 mg/kg, and of lutein between 1 and 20 mg/kg have been
231 reported in a recent review (Fernández Gutiérrez & Segura Carretero, 2010). However, in
232 many studies the reported values of lutein do not exceed 4 mg/kg (Giuffrida *et al*., 2011;
233 Motilva & Romero, 2010; Ranalli, Malfatti, Lucera, Contento, & Sotiriou, 2005). Moreover,
234 large differences in the concentration of pheophytin *a* have been reported, from traces in VOO
235 from Arbequina olives (Criado *et al.*, 2007) to 26 mg/kg in samples from Picholine Marocaine
236 olives (Csallany & Rahmani, 1991). Considering these data, it can be concluded that the
237 average concentrations of the isoprenoid under study (Table 2) are comparable to those
238 reported for VOO of different origins. Taking into account that some of the samples have
239 been bought in supermarkets, it is necessary to point out the possibility of adulteration or
240 contamination of these oils. In recent years many studies have focused on this topic because
241 VOO is a relatively expensive and popular food which makes it a target product for
242 adulterations (Jabeur *et al*., 2017; Li *et al*., 2017; Tavares Melo Milanez *et al*., 2017).

The ranges of concentration obtained for the different compounds were, in most cases, very wide. The percentage differences between the maximum and minimum concentrations were 96 %, 127 %, 133 %, 103 %, 146 % and 139 % for α-tocopherol, β-carotene, β-sitosterol, squalene, pheophytin *a* and lutein respectively. The narrowest dispersion was observed in the α-tocopherol. This may be due in part to the fact that, among the olive oil antioxidants, the concentrations of α-tocopherol have been reported to vary to a lesser extent during the ripening of the olives (Gimeno *et al.*, 2002). Therefore, it is expected that the variability of samples due to the degree of ripening of the olives had a lower influence on the concentration of α-tocopherol. Moreover, it appears that the concentration of α-tocopherol, unlike other minority compounds of olive oil, is not much influenced by the type of extraction system (Gimeno *et al*., 2002).

3.3.Colour

A typical visible spectrum of olive oil with the characteristic absorption maxima due to pheophytin *a* (around 670 nm) and carotenoids (between 430 and 480 nm) is shown in Fig. 1.

The mean colour parameters are shown in Supplementary Table 3. The average values of L^* for both groups of samples were near to 100 CIELAB units, which indicated that the samples were very light. SM and OM showed values of a^* positive and close to zero. This could be attributable in part to the low concentration of chlorophyll compounds since negative values of a^* correspond to greenish colours. Similar to the results obtained by Moyano *et al*. (2008a), the value of the chroma for the two groups was virtually identical to its corresponding value of b^*. In all the samples the value of b^* was close to 100 units, which indicates that the colour of them have an important yellow contribution. Since the yellow hue of the olive oils is mainly due to the contents of β-carotene and lutein (Giuffrida *et al.*, 2011), this statement is consistent with the fact that the carotenoid fraction has an important contribution to the colour of the samples analysed.

OM had significantly higher values of b^* and C^*_{ab} than SM ($P < 0.05$) (Table 3). It is interesting to note that, b^* and C^*_{ab} are thought to be the colour parameters best correlated to the carotenoids index (an approach to estimate the concentration of carotenoids), the correlations being positive (Moyano, Meléndez-Martínez, Alba, & Heredia, 2008b). This observation agrees well with the results of the present study, as OM had higher values of b^* and C^*_{ab} and also higher concentration of carotenoids.

3.4. LDA analysis

Taking into account that the SM were elaborated in the same mill with the same method of production while OM were elaborated in different mills (Supplementary Table 1) and considering that the processing has an important impact on the composition of the oils, it appears sensible to hypothesize that the SM could be differentiated from the OM according to their isoprenoids composition and colour.

The discriminatory power of the variables for differentiating the two sets of EVOO samples followed the decreasing order: a^*, C^*_{ab}, lutein, α-tocopherol, squalene and β-carotene (Supplementary Table 4). Discriminatory variables with positive contributions were a^*, α-tocopherol and squalene. 88.6 % of the SM and 88.9 % of the OM were grouped correctly (Table 4). The canonical discriminant function explained 88.7 % of the accumulated variance. This good classification achieved (Fig. 2 and Table 4) can be due to the fact that the concentrations of the isoprenoids analysed are dependent on the processing method. For example, the storage period of olives before the extraction appears to affect considerably the content of β-sitosterol, tocopherols and chlorophylls in the oil (Mínguez-Mosquera, 1997). Higher temperatures during malaxation process are thought to lead to higher extraction of the minor components of oil, albeit excessive temperatures can promote their degradation (Aparicio-Ruiz *et al*., 2011; Ranalli *et al*., 2005). Moreover, according to Gimeno *et al.* (2002), the oils extracted by a two-phase decanter have higher concentrations of tocopherols than those obtained by a three-phase decanter. It is to be noted though that there is certain

295 controversy in this respect (Di Giovacchino, Sestili, & Di Vincenzo, 2002). In this regard, it is
296 important to consider that the extraction of lutein and other xanthophylls, which are more
297 polar than carotenes, may be influenced by the addition of water in the three-phase system.
298 Besides, the use of sodium chloride as adjuvant seems to affect the pigment content and thus
299 the colour of the resulting oil (Pérez, Romero, Yousfi, & García, 2008). It has also been
300 reported that the filtered oils have a lower amount of chlorophylls than the unfiltered ones
301 (Bottino, Capannelli, Mattei, Rovellini, & Zunin, 2008). Research is needed in order to
302 evaluate if the amount of carotenoids also decreases.
303
304 **4. Conclusions**
305 Although the development of the chromatographic method was not the main objective
306 of this work, it is worth noting that with this HPLC method it have been able to analyse lutein,
307 β-carotene, pheophytin *a*, β-sitosterol, squalene and α-tocopherol, which are isoprenoids of
308 interest due to their nutritional and / or sensory importance. Moreover, the developed HPLC
309 method allows detection of some *cis* isomers of carotenoids. Overall, the concentrations of
310 lutein and squalene were significantly higher in the OM than in the SM ($P < 0.05$). The
311 comparison of pigments concentration data between the two groups of samples is consistent
312 with the results obtained by comparing colour parameters. Despite the chemical composition
313 of olive oils is dependent of numerous factors it has been found that some key isoprenoids and
314 colour parameters can be useful to differentiate the samples obtained in a particular mill from
315 those obtained in others. More specifically, 89 % of correct classifications were obtained by
316 LDA. These results highlight the importance that the processing conditions have in the
317 extraction of olive constituents and therefore in the composition of the virgin olive oils.
318
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CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest.

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