Pigments in Extra Virgin Olive Oils produced in different Mediterranean Countries in 2014: near UV-vis spectroscopy *versus* HPLC-DAD.

Cristina Lazzerini[1], Mario Cifelli[1] and Valentina Domenici[1]*

1. Dipartimento di Chimica e Chimica Industriale, Università di Pisa, via Moruzzi, 13 – 56124 Pisa (Italy).

* Corresponding author:
Prof. Valentina Domenici, Dipartimento di Chimica e Chimica Industriale, Università di Pisa, via Moruzzi, 13 – 56124 Pisa (Italy). Tel: 0039-0502219215/267; FAX: 0039-0502219260. E-mail: valentina.domenici@unipi.it

Keywords: EVOO, UV-vis spectroscopy, HPLC, carotenoids, pheophytins.

Abstract

Carotenoids and chlorophyll derivatives play a key role in Extra Virgin Olive Oils (EVOOs). Many factors, such as cultivar, geographic origin, maturity of olives, climate and storage conditions, influence the pigments' content. The quantification of pigments is usually done by chromatographic techniques. However, recent works evidenced the potentialities of UV-visible-related methodologies. In this research, a selection of EVOO samples produced from olives harvested at the beginning of November 2014 in Greece, Tunisia, Italy and Spain, was investigated in terms of pigments by means of two methods. The first one is a recent approach based on the mathematical treatment of near UV-vis absorption spectra of olive oils to quantify in a fast, cheap and non-destructive way four main pigments, namely β-carotene, lutein, pheophytin A and pheophytin B.

The second one is a more standard HPLC-DAD method. From the comparison between the two methods, we can conclude that the new near UV-vis approach gives reliable results, with good precision and high reproducibility. Pigments quantified by these two methods in EVOOs produced in four countries from different cultivars are analyzed by principal component analysis (PCA). Results indicate that pigments can be correlated to several factors such as ripeness stage, geographic origin and cultivars.

1. Introduction

Extra Virgin Olive Oil (EVOO) is one of the main agricultural product in Mediterranean countries for its role in diet and, more generally, in Culture. The benefits of using EVOO as condiment for the human health have been widely demonstrated (Parkinson & Cicerale, 2016 and reference therein) and this is mainly related to the amount of bioactive components present in EVOOs. Among bioactive constituents in olive oils, it's worth mentioning minor components, such as hydrocarbons, tocopherols, pigments, sterols, terpene acids, phenolic acids and their derivatives (Boskou, 2015).

Pigments, constituted by carotenoids and chlorophyll derivatives (Mínguez-Mosquera, Gandul-Rojas, Garrido-Fernández, & Gallardo-Guerrero 1990), play a key role in EVOO general aspect, since they determine the colour (Mínguez-Mosquera et al., 1991). Moreover, pigments are related to EVOO quality, due to their relationship with freshness, nutritional and antioxidant properties. Among pigments, chlorophylls are usually present in the form of pheophytins; pheophytin A is the most predominant one, while chlorophylls can be found in fresh olive oils. Carotenoid fraction is dominated by β-carotene and lutein, while other carotenoids, namely the xanthophylls, such as β-criptoxanthin, violaxanthin, neoxanthin and others, can be found in smaller amount (Gandul-Rojas, Roca & Gallardo-Guerrero, 2016).

Quantification of pigments in EVOOs and their relationship with quality parameters, however, is not an easy task (Lazzerini, Cifelli, & Domenici, 2016). The amount of a single pigment and the relative content in carotenoids and chlorophyll derivatives strongly depend on the olive varieties

and geographic origin (Gandul-Rojas & Minguez-Mosquera, 1996; Giuffrida et al., 2007; Aparicio-Ruiz, Gandul-Rojas & Roca, 2009; Pizarro et al., 2013), the maturation of olives at the time of harvesting (Cevik et al, 2014), the extraction process (Gallardo-Guerrero, Roca & Minguez-Mosquera, 2002) and the storage conditions (Gambacorta et al., 2009). Recent works have shown a correlation between the amount of pigments and the authenticity of EVOOs (Gandul-Rojas, Cepero & Minguez-Mosquera, 2000). For all these reasons, it is not surprising that a great effort is dedicated to develop standardized methods to quantify pigments and possibly correlating them to specific features of EVOO production and quality. Several analytical methods based on high performance liquid chromatography (HPLC) have been proposed to identify and quantify carotenoids (Cortes et al, 2004), chlorophyll derivatives (Watanabe, 1984) or both (Seppanen, Rahmani, & Csallany, 2003; Minguez-Mosquera, Gandul-Rojas, & Gallardo-Guerrero, 1992). Most of these methods require a preventive solvent extraction of pigments from the olive oil lipid matrix. The chosen analytical methods, temperature and sequence of the extractions can cause significant differences in the final pigments' content (Cert., Alba, & Pérez-Camino, 1999). On the other hand, the analysis of the pigments' content obtained from a spectrum directly acquired on the EVOO sample requires no sample pre-treatment.

In particular, while Ultra-violet (*UV*) absorption of extra-virgin olive oils ($\lambda < 400$ nm) is mostly determined by the presence of phenolic components (Fuentes et al., 2012), near UV-visible (vis) light absorption of EVOOs is associated to pigments ($400 \text{ nm} < \lambda < 800$ nm) (Lazzerini, Cifelli, & Domenici, 2016) and this specificity offers the possibility to overcome the above mentioned chromatography's limitations. Based on this EVOO's spectral characteristic, several spectroscopic approaches have been developed to extract pigments' information from visible absorption spectra of EVOOs. For instance, a recent method (Cayuela et al., 2014) associates the absorbance measured at specific wavelengths in the visible region, namely the K_{470} and K_{670} indexes, to the amount of carotenoids and chlorophyll derivatives, respectively. However, the sole absorbance values at specific wavelengths in the visible absorption spectrum of EVOOs do not allow a reliable and

unambiguous quantification of single the pigments' content. Another approach is based on the mathematical deconvolution of near UV-vis-absorption spectra to quantify specific pigments present in EVOOs (Ayuso, Haro, & Escolar, 2004; Domenici et al., 2014). Other methods recently developed use intelligent systems and chemiometric tools to analyze UV-vis absorption spectra of EVOOs (Aroca-Santos et al, 2016) in order to characterize olive oils offering a valid instrument against potential frauds (Torrecilla et al, 2010).

In the present work, we used a recently proposed spectroscopic method (Domenici et al., 2014) and a High Pressure Liquid Chromatographic with Diode Array Detector (HPLC-DAD) method, modified from Hornero-Mendez, Gandul-Rojas, & Minguez-Mosquera (2005), to quantify main pigments in several EVOOs. The spectroscopic approach allowed us to quantify β-carotene, lutein, pheophytin A and pheophytin B, while the HPLC-DAD method was here optimized to quantify β-carotene, β-cryptoxanthin, lutein, chlorophyll A and pheophytin A. These techniques are here validated and compared in order to evaluate advantages and disadvantages. Both methods are applied to a selection of monovarietal EVOOs produced in different geographical areas located in four countries (Italy, Greece, Tunisia and Spain) and obtained from different olive varieties (*Leccino*, from Italy, *Koroneiki*, from Greece, *Chemlali*, from Tunisia, and *Cornicabra*, *Verdial de Huévar*, *Hojiblanca*, *Poniente de Granada* and *Arbequina*, from Spain). These olive oil samples were produced from olives harvested in the same period and a particular care was taken to select the samples: ripeness stage of harvested olives was known and storage conditions were controlled. Pigments' concentrations and other relevant parameters are analyzed by Principal Component Analysis (PCA). The differences among EVOOs produced in different geographic areas are discussed and compared with the literature in order to evaluate the correlation between pigments' content in olive oils and factors such as ripeness stage, geographic origin and cultivars.

2. Materials and methods

2.1 EVOO samples

EVOO's samples, provided by private producers, were obtained from olives (fruits of *Olea europaea, L.* trees) harvested in the same period (beginning of November 2014). In particular, we had 3 Italian, 2 Greek, 6 Spanish and 6 Tunisian EVOO samples, whose details about specific geographical origin, cultivar and the ripeness stage of olives at harvesting are reported in **Table 1**. The ripeness stage is characterized through the colour of the olive fruits at the moment of harvesting, as follows: G, green; LG, light green; SRS, small reddish spots; TC, turning color; P, purple; B, black (Loudiyi et al, 1984). All EVOO samples were classified as "extra virgin olive oils" by sensorial characteristics (International Regulations, Reg. CE 640/2008) and analytical indices (European Regulation, Reg. CE 1234/2007, annex XVI). EVOO samples were stored in dark glass bottles in the dark at 5 °C.

2.2 Chemicals

Acetone, hexane, methanol, diethyl ether and water (HPLC purity) were used. The following chemical standards were used: β-carotene (C4582: 1mg. Type II, synthetic, ≥95% HPLC), β-cryptoxanthin (C6368: 1mg ≥ 97% TLC), chlorophyll A (C5753: 1mg. From spinach) and lutein (C7168: 1mg ≥95% (HPLC). All the above chemicals were purchased from Sigma Aldrich. Other analytes, such as pheophytins, were prepared or extracted as described below.

2.3 HPLC-DAD method

2.3.1 Solid Phase Extraction

About 0.3g of oil sample were weighted in a Solid Phase Extraction column (SPE-LC-Si of 300 mg from Supelco) until complete absorption by applying a vacuum pump (pressure ~1.33 kPa). One mL of hexane/diethyl ether mixture (87 mL / 13 mL) was added and then it was eluted with 9mL of the same mixture at a constant rate. This solution was dried under a gentle stream of nitrogen at room temperature, and the residual was redissolved in 700μL of acetone (first fraction). After the first extraction, rich in β-carotene, a second fraction, rich of all other pigments, was obtained by washing the residual in the SPE-LC with 10mL of acetone, drying it and redissolving it in 1mL of acetone (second fraction).

2.3.2. Optimization of the HPLC method

The analyses were carried out using a Perkin Elmer HPLC system, equipped with auto-sampling, a binary pump and a LC C18 column 18-DB Supelco, 3µ, 150mm x 4.6mm. The column was connected with a pre-column. A Perkin Elmer Flexar PDA plus Detector has been used. The injection volume was 10µL, the temperature of operation was kept constant at 25°C, and the run time was 30 minutes. The eluent were: (A) a mixture of 60% of acetone and 40% of methanol and (B) methanol. The flow was 1mL/min and the quantification of chromatogram peaks was carried out integrating signal detected at λ =410nm. The following linear gradient was used for all experiments: t=0 min (90% of eluent B); t=20 min (100% of eluent B); t=30 min (100% of eluent B) followed by an equilibration time of 5 minute (90% of eluent B). Data were processed with Total Chrom Navigator software (PDA). Chromatograms of the first and second fractions are reported in **Figure 1**. The quantification of pigments was done by using the calibration method, as described in the Supporting Information. The evaluation of main validation parameters of the analytical method, such as selectivity, limit of detection as well as intra-day precision and accuracy of the method are reported in **Tables S1**, **S2** and **S3**.

2.4 Near Uv-vis spectroscopic method

Near UV-vis absorbance spectra were measured with a Jasco V-550 spectrophotometer using quartz cells with 0.2cm optical path length. Oil samples were inserted in the quartz cell without any treatment and the spectra were acquired in the spectral range 390-720nm, with a band-with spectral resolution both fixed to 1nm. The measured absorbance was normalized to 1cm of optical path before analysis. The mathematical treatment of the EVOO absorption spectrum was performed following the method developed by us (Domenici et al., 2014) to obtain the concentration of four main pigments: β-carotene, lutein, pheophytin A and pheophytin B. This procedure consists of the following steps: 1. Acquisition of the experimental UV-vis spectrum of the sample; 2. Fitting of the normalized to 1cm optical path experimental spectrum; 3. Calculations of pigments concentrations and relevant statistical parameters, such as R^2 (coefficient of determination). Steps 2 and 3 are done,

automatically, by a home-made program compatible with excel (Domenici et al., 2014). Further details of the method used for the deconvolution of the near UV-vis spectrum of olive oils are also provided in the Supporting Information. The concentration of pigments is reported as average value ($\bar{X}$) ± standard deviation (SD) on three replicates for each sample. The limits of detection and limits of quantification are reported in the **Table S4**.

2.5 Statistical analysis

Data analysis was performed with XLSTAT software for EXCEL.

3. Results and discussion

3.1 Comparison between HPLC-DAD and near UV-vis spectroscopic methods

Specific validation tests for the two methods were performed following the Manual UNI/CHEM 190/1 (http://www.unichim.it/). The repeatability of both analytical methods was evaluated in a single laboratory, by a single operator, on a single instrument, in a short interval of time. This validation procedure, schematically shown in **Figure 2**, is referred to a single level of concentration of the analytes. The verification of the normal distribution of the collected data was performed by the *Shapiro-Wilk* test, while the verification of the presence of anomalous data was done by the *Dixon* test. The repeatability of both methods was evaluated by calculating the limits of repeatability, *r*, defined as:

$$r = ts_r\sqrt{2} \qquad (1)$$

Here *t* is the *t-student* parameter for a probability level of 95% and 9 degrees of freedom, s_r is the standard deviation calculated over ten replicates:

$$s_r = \sqrt{\frac{\sum_{i=1}^{n}(x_i-\bar{x})^2}{n-1}} \qquad (2)$$

With $\bar{x}$ being the average value over ten replicates.

The percent variation coefficient (CV%), the average values ($\bar{x}$) and the limits of repeatability, *r*, for all the analytes under investigations by the two methods are reported in **Tables 2** and **3**. The

validation test was successfully passed for all analytes determined by both methods, except for the chlorophyll A (HPLC-DAD), for which 1 data over 10 resulted anomalous. The precision of the HPLC-DAD resulted very high for β-carotene, pheophytin A and lutein (CV% < 6) and a bit worse in case of β-cryptoxantin and chlorophyll A (see **Table 2**). The recovery percentage (R) is excellent in case of all analytes investigated by HPLC-DAD, except than β-carotene (R=87%) (see Supporting Information). The spectroscopic approach shows a very high precision in the quantification of the four main pigments (CV% < 5). Moreover, the coefficient of determination (R^2) of the mathematical treatment of the spectra ranges between 0.991 and 0.998, showing a very good reproduction of the experimental near UV-vis absorption spectra by the fitting approach. It should be noted that the HPLC-DAD method here optimized was not able to quantify the pheophytin B pigment with sufficient accuracy and precision; for this reason, it is not reported. On the contrary, pheophytin B, could be determined with good precision and accuracy by the near UV-vis spectroscopic approach. A direct quantitative comparison between the two methods can be performed on the three main pigments: β-carotene, lutein and pheophytin A. To this purpose, the two approaches were used to investigate a set of selected EVOOs (see **Section 2.1** and **Table 1**). Pigments quantification was performed in the same days (end of January / beginning of February 2015). The concentrations of pigments quantified by the two methods are reported in **Tables 4** and **5**. In **Figures 3A**, **3B** and **3C**, the regression curves corresponding to pheophytin A, β-carotene and lutein determined by the two methods are reported, respectively. In the case of pheophytin A and β-carotene, the correlation between data obtained by near UV-vis spectroscopy and HPLC-DAD methods is high (R^2 equal to 0.800 and 0.782, respectively), all data are in the confidence interval at 95% (see dashed curves in **Figures 3A** and **3B**). Moreover, the residuals (not shown here) are homogeneously scattered. In the case of lutein, data (near UV-vis *versus* HPLC-DAD) show a lower correlation, with R^2 equal to 0.430. As seen in **Figure 3C**, among the seventeen EVOO samples, there is an outlier. Moreover, when plotting the residuals against the lutein measured by

Near UV-vis spectroscopy a positive trend is identified, thus indicating that the simple linear correlation model is not satisfactory in the case of lutein. This aspect could be related to the presence of other minor carotenoids having an absorption spectrum similar to that of lutein, not considered in the actual spectroscopic method. In **Figure 3D**, the regression curve of the chlorophyll derivatives *versus* carotenoids ratio (P/C) determined by near Uv-vis spectroscopic (Y) and by HPLC-DAD (X) is reported. The correlation between the two variables is good (R^2 equal to 0.776), without outliers. The residuals, not displayed here, are scattered with respect to both variables (X and Y), thus indicating the validity of the regression model, with p-value of <0.0001 (see **Table S5**).

3.2 Pigments' content in EVOOs produced in different European Countries in 2014

As already known (Gandul-Rojas, Roca, & Gallardo-Guerrero, 2016 and reference therein), the amount of pigments in olive oils is affected by the ripeness stage of olives. The maturity of olives before oil production is also related to the climate conditions. The year 2014 was peculiar for some geographical areas, such as Italy and Spain, due to unusual weather during Summer, which was at the basis of the known drastic reduction of olive oil production in 2014/2015. The relationship between the ripeness stage and pigments content in EVOO is indeed very important. From data reported in **Tables 4** and **5**, a progressive decrease of both carotenoids and chlorophylls, and their derivatives, through ripeness stage, can be observed. In **Figure 4** the ratio between total amount of chlorophylls' derivatives and the total amount of carotenoids (P/C) is reported as a function of the ripeness stage. Previous studies (Roca, & Minguez-Mosquera, 2001) reported that this ratio decreases, depending on the ripeness stage, in table olives, but in olive oils it remains constant around the value of 1.14, shifting in the range 0.53 ÷ 1.40. Our study, however, shows that this ratio can assume values in a much larger range, in agreement with other works (Psomiadou & Tsimidou, 2001; Criado et al, 2007; Aparicio-Ruiz, Gandul-Rojas & Roca, 2009; Lazzerini, Cifelli & Domenici, 2016; Lazzerini & Domenici 2017).

231 Differences among EVOO samples are also associated to the geographic origin of the harvested
232 olives. Italian EVOOs analyzed in this work were produced in a restricted area close to Lucca
233 (Tuscany) from olives of *Leccino* variety, a typical Tuscan cultivar. These samples differ only for
234 the maturity of olives (TC or P) at harvesting (see **Table 1**). As seen in **Table 4**, the chlorophyll A
235 content is low, as expected due to the ripeness stage, and the overall mean pigment content is low:
236 7.98 ppm (HPLC-DAD) and 13.01 ppm (near UV-vis). A direct comparison with the literature is
237 difficult since, to our knowledge, pigments were never quantified in EVOOs produced from
238 *Leccino* monocultivar in Tuscany. Moreover, sensible differences between 2014 and previous
239 harvesting years could be related to the unlucky climate conditions in 2014, especially in Italy.
240 Tunisian EVOO samples studied in this work have a very homogeneous distribution of pigments,
241 with a mean total content of about 11.0 ppm (HPLC-DAD) and 17.7 ppm (near UV-vis). They were
242 produced from olives of the same cultivar, *Chemlali*, harvested in two regions (Sousse and Sfax).
243 The total amount of carotenoids and chlorophylls is similar to that reported for *Chemlali* EVOOs
244 produced in 2012-2013 (Gargouri et al., 2016). As observed in **Tables 4** and **5**, Tunisian *Chemlali*
245 olive oil samples are characterized by a lutein percentage, over carotenoids, rather high (similar to
246 Spanish EVOOs). Moreover, the ratio between chlorophylls and carotenoids ranges from 1.8 to 3.5
247 (HPLC-DAD) and from 1.4 to 1.7 (near UV-vis). Also in the case of Tunisian EVOOs, a direct
248 comparison with the literature is not possible, since other works (see, for instance, Rigane et al.,
249 2013; Gargouri et al., 2016) focus on the total amount of carotenoids and total amount of
250 chlorophyll derivatives, and not to single pigments. Spanish EVOO samples analyzed in this work
251 were produced from different cultivars (*Verdial de Huévar*, *Hojiblanca*, *Poniente de Granada*,
252 *Arbequina* and *Cornicabra*) and in different geographical areas (**Table 1**). The variability of this set
253 of Spanish samples is also related to the ripeness stage, starting from green (G) to black (B) olives.
254 This variability reflects in the concentration of chlorophyll derivatives, higher for oils produced
255 from light green (S 1) or small reddish spots olives (S 2) and lower for other cases, with sensible
256 differences due to cultivars. Differently from EVOOs produced in other countries, almost all

Spanish EVOOs have a very high concentration of lutein, with a percentage over other carotenoids reaching the mean value of 70% (HPLC-DAD) and 60% (near UV-vis). In case of Spanish olive oils produced from olives having high maturity (P or B), the ratio between chlorophyll derivatives and carotenoids is close to 1.3 ÷ 1.5 (from both methods), in good agreement with the literature (Roca & Minguez-Mosquera, 2001; Gandul-Rojas, Cepero & Minquez-Mosquera, 2000). In case of early ripeness stages, our results indicate a higher ratio, reaching the value of 3.3 for sample S 2, which reflects the high concentration of pheophytin A with respect to all other pigments. The differences among Spanish EVOOs can be explained based on the known differences among cultivars (Gandul-Rojas, Roca, & Gallardo-Guerrero, 2016; and references therein; Domenici et al., 2014). Greek EVOOs were produced from olives at the first stages of maturity (G and LG) from *Koroneiki* variety, one of the most important cultivar in Greece for the production of olive oils. The amount of chlorophyll derivatives is the highest found in this set of samples. As reported in Aparicio-Ruiz, Gandul-Rojas & Roca (2009) the ratio P/C in *Koroneiki* EVOOs is higher than in Spanish EVOOs. The amounts of chlorophylls and carotenoids found in our two samples (G 1 and G 2) are very similar to those reported in the case of *Koroneiki 1* (Aparicio-Ruiz, Gandul-Rojas & Roca, 2009) and those reported for *Koroneiki* EVOOs produced in the same geographic area (Peloponnese, Greece) for similar ripeness stage (Psomiadou & Tsimidou, 2001). As in our case, in spite of the low maturation stage of the harvested olives, the amount of chlorophyll A is not very high with respect to pheophytin A due to the fast degradation of chlorophyll A to pheophytin A (Psomiadou & Tsimidou, 2001). Similarly to some Spanish samples, Greek EVOOs have the higher concentrations of total chlorophyll derivatives (Psomiadou et al., 2003).

To better visualize the differences among EVOOs from different European countries, a principal component analysis (PCA) was performed (**Figure 5**). Since HPLC-DAD and near UV-vis data are highly correlated, we used only parameters calculated from near UV-vis analysis (**Figure 5b**) and, to take into account the ripeness stage, the index of ripeness (*color index* in **Figure 5b**). PCA modelling gave 86.3% of explained variance by considering the first two PCs as shown in **Figure**

5a. The first component (PC1) explains 64.4% of the total variance and include six parameters (**Table S6**); lutein and the color index are associated with the second component (PC2), with 21.9% of the total variance. The only parameter not included in the first two factors is pheophytin B (**Table S6**). Chemometric elaboration permitted the pattern recognition and a satisfactory distinction among the EVOOs produced in different countries, in particular in Italy, Tunisia and Greece. The Spanish samples are not well distinguished by other samples, due to the not homogeneity of this set of samples, as previously discussed.

4. Conclusions

EVOOs produced in 2014 in four Mediterranean countries have been analyzed in terms of main pigments' content. Two methods have been exploited, namely the HPLC-DAD method and a recent near UV-vis spectroscopic approach. The two methods were validated and compared. Pheophytin A is determined with high precision and accuracy by both methods, while some discrepancies have been found for the two main carotenoids: β-carotene and lutein. The two approaches were used to quantify pigments in a selected set of EVOOs produced from different olive varieties and in two cases, namely *Leccino* cultivar from Tuscany (Italy) and *Chemlali* cultivar from Tunisia, single pigments were quantified for the first time. The ripeness stage of olives at harvesting was correlated to the amount of pigments in EVOOs. Interestingly, a decrease of the ratio between chlorophyll derivatives and carotenoids in olive oils was observed by increasing the maturity of olives at harvesting. PCA analysis on these samples allowed us a good pattern recognition and a satisfactory distinction among EVOOs produced in Greece, Italy and Tunisia in 2014, while Spanish samples turned to be too widely distributed to be clustered.

References

307 Aparicio-Ruiz, R., Gandul-Rojas, B., & Roca, M. (2009) Pigment Profile in Non-Spanish Olive
308 Varieties (Olea europaea L. Var. Coratina, Frantoio, and Koroneiki). *Journal of Agricultural and*
309 *Food Chemistry, 57*, 10831-10836.
310
311 Aroca-Santos, R., Cancilla, J.C., Pariente, E.S. & Torrecilla, J.S. (2016). Neural networks applied to
312 characterize blends containing refined and extra virgin olive oils. *Talanta, 161*, 304-308.
313
314 Ayuso, J., Haro, M. R., & Escolar, D. (2004). Simulation of the Visible Spectra for Edible Virgin
315 Olive Oils: Potential Uses. *Applied Spectroscopy, 58*, 474-480.
316
317 Bengana, M., Bakhouche, A., Lozano-Sánchez, L., Amir, Y., Youyou, A., Segura-Carretero, A.,
318 Fernández-Gutiérrez, A. (2013). Influence of olive ripeness on chemical properties and phenolic
319 composition of Chemlal extra-virgin olive oil. *Food Research International*, 54, 1868–1875.
320
321 Boskou, D. (2015). *Olive and Olive Oil Bioactive Constituents*. Urbana: AOCS Press, pp. 1-30.
322
323 Cayuela, J. A., Yousfi, K., Martinez, M. C., & Garcia, J. M. (2014). Rapid Determination of Olive
324 Oil Chlorophylls and carotenoids by Using Visible Spectroscopy. *Journal of the American Oil*
325 *Chemists Society, 91*, 1677-1684.
326
327 Cert., A., Alba, J., & Pérez-Camino, C. (1999). Influence of extraction methods on the
328 characteristics and minor components of extra virgin olive oil. *Olivae, 79*, 41-50.
329
330 Cevik, S., Ozkan, G., Kiralan, M., & Bayrak, A. (2014). Effect of harvest time on physicachemical
331 quality parameters, oxidation stability and volatile compounds of extra virgin olive oil. *Acta*
332 *Alimentaria, 43*, 526-537.

Cortes, C., Esteve, M. J., Frigola, A., & Torregrosa, F. (2004). Identification and quantification of carotenoids including geometrical isomers in fruit and vegetable juices by liquid chromatography with ultraviolet-diode array detection. *Journal of Agricultural and Food Chemistry, 52*, 2203-2212.

Criado, M.N., Motilva, M. J., Goni, M., Romero, M.P. (2007). Comparative study of the effect of the maturation process of the olive fruit on the chlorophyll and carotenoid fractions of drupes and virgin oils from *Arbequina* and *Farga* cultivars. *Food Chemistry*, *100*, 748-755.

Domenici, V., Ancora, D., Cifelli, M., Serani, A., Veracini, C. A. & Zandomeneghi, M. (2014). Extraction of Pigment Information from Near-UV Vis Absorption Spectra of Extra Virgin Olive Oils. *Journal of Agricultural and Food Chemistry, 62*, 9317−9325.

Fuentes, E., Báez, M. E., Bravo, M., Cid, C., & Labra, F. (2012). Determination of Total Phenolic Content in Olive Oil Samples by UV–visible Spectrometry and Multivariate Calibration. *Food Analytical Methods, 5*, 1311-1319.

Gallardo-Guerrero, L., Roca, M., & Minguez-Mosquera, M.I. (2002). Distribution of chlorophylls and carotenoids in ripening olives and between oil and alperujo when processed using a two-phase extraction system. *Journal of the American Oil Chemists Society*, *79*, 105-109.

Gambacorta, G., Baiano, A., Previtali, M.A., Terracone, C., & La Notte, E. (2009). Shelf life of some monovarietal extra virgin olive oils. *Italian Journal of Food Science, 21*, 208-211.

Gandul-Rojas, B., Cepero, M. R. L., & Minguez-Mosquera, M. I. (2000). Use of chlorophyll and carotenoid pigment composition to determine authenticity of virgin olive oil. *Journal of the American Oil Chemists Society, 77,* 853-858.

Gandul-Rojas, B., & Minguez-Mosquera, M.I. (1996). Chlorophyll and carotenoid composition in virgin olive oils from various Spanish olive varieties. *Journal of the Science of Food and Agriculture, 72*, 31-39.

Gandul-Rojas, B., Roca, M., & Gallardo-Guerrero, L. (2016). Chlorophylls and carotenoids in food products from olive tree. In Boskou, D., Clodoveo, M. L. (Eds.) *Products from Olive Tree*, (ISBN 978-953-51-4806-7) InTech Publisher, Rijeka, Croatia, chapter 5, pp. 67.

Gargouri, O.D., Rouina, Y. B., Mansour, A.B., Flamini, G., Rouina, B.B., Bouaziz, M. (2016). Comparative Study of Oil Quality and Aroma Profiles from Tunisian Olive Cultivars Growing in Saharian Oasis Using Chemometric Analysis. *Journal of Oleo Science*, *65*, 1033-1044.

Giuffrida, S., & La Pera, D. (2007). Pigments composition in monovarietal olive oils from various sicilian olive varieties. *Food Chemistry*, *101*, 833-837.

Giuffrida, D., Salvo, F., Salvo, A., Cossignani, L., & Dugo, G. (2011). Pigments profile in monovarietal virgin olive oils from various Italian olive varieties. *Food Chemistry*, *124*, 1119-1123.

Hornero-Mendez, A., Gandul-Rojas, B., & Minguez-Mosquera, M. I. (2005). Routine and sensitive SPE-HPLC method for quantitative determination of pheophytin a and pyropheophytin a in olive oils. *Food Research International*, *38*, 1067-1072.

Lazzerini, C., Cifelli, M., & Domenici, V. (2016). Extra virgin olive oil pigments: authenticity and quality. In Boskou, D., Clodoveo, M. L. (Eds.) *Products from Olive Tree*, (ISBN 978-953-51-4806-7) InTech Publisher, Rijeka, Croatia, chapter 6, pp. 99.

Lazzerini, C. & Domenici, V. (2017). Pigments in Extra-Virgin Olive Oils Produced in Tuscany (Italy) in Different Years. *Foods*, *6*, 25.

Loudiyi, W.D., Chmitah, M., Loussert, R., Mahhou, A., Boulouha, B. (1984). Morphologic and physiologic characters of olive clones from *Picholine Marroqui* variety. *Olivae*, *3*, 26-31.

Parkinson, L. & Cicerale, S. (2016). The Health Benefiting Mechanisms of Virgin Olive Oil Phenolic Compounds. *Molecules*. *21*, 1734.

Mínguez-Mosquera, M.I., Gandul-Rojas, B., Garrido-Fernández, J. & Gallardo-Guerrero, L. (1990). Pigments Present in Virgin Olive Oil. *Journal of the American Oil Chemists Society*, *67*, 192-196.

Minguez-Mosquera, M. I., Gandul-Rojas, B., & Gallardo-Guerrero, M. L. (1992). Rapid Method of Quantification of Chlorophylls and Carotenoids in Virgin Olive Oil by High-Performance Liquid Chromatography. *Journal of Agricultural and Food Chemistry*, *40*, 60-63.

Mínguez-Mosquera, M.I., Rejavo-Navarro, L., Gandul-Rojas, B., Sanchez-Gomez, A.H., & Garrido-Fernández, J. (1991). Color-Pigment correlation in Virgin Olive Oil. *Journal of the American Oil Chemists Society*, *68*, 332-336.

Pizarro, C., Rodriguez-Tecedor, S., Perez-del-Notario, N., Esteban-Diez, I. & Gonzalez-Saiz, J. M. (2013) Classification of Spanish extra virgin olive oils by data fusion of visible spectroscopic fingerprints and chemical descriptors. *Food Chemistry, 138,* 915-922.

Psomiadou, E., Tsimidou, M. (2001). Pigments in Greek virgin olive oils: occurrence and levels. *Journal of the Science of Food and Agriculture, 81,* 640-647.

Psomiadou, E., Karakostas, K., Blekas, G., Tsimidou, M., Boskou, D. (2003). Proposed parameters for monitoring quality of virgin olive oil (*Koroneiki* cv). *European Journal Lipid Science and Technology, 105,* 403–408.

Roca, M., & Minguez-Mosquera, M. I. (2001). Change in the natural ratio between chlorophylls and carotenoids in olive fruit during processing for virgin olive oil. *Journal of the American Oil Chemists Society, 78,* 133-138.

Rigane, G., Ayadi, Boukhris, M., Sayadi, S., Bouaziz, M. (2013). Characterization and phenolic profiles of two rare olive oils from souther Tunisia: Dhokar and Gemri-Dhokar cultivars. *Journal of Science and Food Agriculture, 93,* 527-534.

Seppanen, C. M., Rahmani, M., Csallany, A. S. (2003). Simultaneous determination of chlorophylls, pheophytins, beta-carotene, tocopherols, and tocotienols in olive and soybean oils by high-performance liquid chromatography. *Journal of Food Science, 68,* 1644-1647.

Torrecilla, J. S., Rojo, E., Dominguez, J. C., & Rodriguez, F. (2010). A novel method to quantify the adulteration of extra virgin olive oil with low-grade olive oils by UV-Vis. *Journal of Agricultural and Food Chemistry, 58,* 1679–1684.

Watanabe, T., Hongu, A., Honda, K., Nakazato, M., Konno, M., & Saitoh, S. (1984). Preparation of Chlorophylls and Pheophytins by Isocratic Liquid Chromatography. *Analytical Chemistry, 56*, 251-256.