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Sorting olive oil based on alpha-tocopherol and total tocopherol content using Near-Infra-Red Spectroscopy (NIRS) analysis

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Abstract

Olive oil is an important vitamin E source, which shows a wide variation range. Therefore the interest on distinguish classes. In this study, we assessed models based on partial least squares (PLS) and discriminant analysis (PLS-DA), using near-infrared spectroscopy (NIRS). Estimating the α–tocopherol and total tocopherols contents by using the PLS models were suitable according to the predicting exercises, which gave residual predictive deviations 2.37 and 2.01. Sorting test of olive oil in two classes by α-tocopherol with the PLS model provided 99.9% success. The PLS-DA assessment for the same purpose gave coefficients of predictive specificity and sensitivity for the high α-tocopherol class 0.96 and 0.84, respectively. The data proves the feasibility of estimating the olive oil α-tocopherol or total tocopherols contents by using NIRS. Besides, these techniques can be helpful rapid methods in the industry for sorting olive oils according to their vitamin E content. They are friendly to the environment, which is important.

Key words: α-tocopherol, classification, olive oil, NIRS, tocopherols, vitamin E.

Abbreviations: AOTF, acousto-optic tunable filter; CSIC, Spanish Council for Scientific Research; 1DSG, first Savitzsky-Golay derivative; 2DSG, second Savitzsky-Golay derivative; EVOO, extra virgin olive oil; FCV, full cross validation; HαT, High α-Tocopherol class; InGaAs, detector using In, Ga and As chemical elements; iPLS, interval partial least squares; LαT, Low α-Tocopherol class; MN, mean normalization; NIR, near infrared; NIRS, near infrared spectroscopy; PCA, principal component analysis; PLS, partial least squares; PLS-DA, partial least squares discriminant analysis; PLS_f, PLS factors; *r*, regression coefficient between the predicted and reference values; R, calibration coefficient; $R_{CV,}$ calibration coefficient from the internal cross validation; RPD, residual predictive deviation; SEC, standard error of calibration; Vis/NIR, visible and near infrared; Vis/NIRS, visible and near infrared spectroscopy; Vit E, vitamin E.

1. Introduction

Olive oil is a prominent source of vitamin E (Vit E), since it provides between 10 and 150 mg of α-tocopherol each 100 g. In fact, vegetable oils are the richest source of Vit E according to food composition tables (Souci, Fachman & Kraut, 1994; USDA, 2007). Pomace olive oil shows higher quantities than those above indicated (Perona, Arcemis, Ruíz, & Catalá, 2005). A daily consumption of 25 g Virgin Olive Oil (VOO) would provide almost 30% of the recommended intake of this vitamin (EFSA, 2015, www.nal.usda.gov/fnic/foodcomp/ search).

Tocopherols are present in variable quantities within the minor olive oil fraction. As an example, Beltrán et al. (2010) reported total tocopherols content ranging between 8.4 and 46.3 mg/100 g within thirty olive cultivars, which were monitored during fruit ripening for three consecutive crop seasons. Other leading sources of Vit E as sunflower peeled seed and almond, contribute around 37 mg/100 g and 26 mg/100 g (Souci, Fachman & Kraut, 1994).

Tocopherols are methylated phenols many of which have Vit E activity. In nature, Vit E exists as at least eight naturally occurring compounds, including α-, β-, δ- and γ-tocopherol and α-, β-, δ- and γ-tocotrienol. The α-tocopherol is the most biologically active and occurs naturally as one isomer (Dutta & Dutta, 2003). It is the main isomer in Europe, where the main dietary sources are olive and sunflower oils (Wagner, Kamal-

53 Eldin, & Elmadfa, 2004). In contrast, γ-tocopherol is the most common form in the
54 American diet because of a higher intake of soybean and corn oil (Jiang, Christen,
55 Shigenaga, & Ames, 2001; Wagner, Kamal-Eldin, & Elmadfa, 2004).

56 Vit E is antioxidant and it has regulatory cellular and molecular roles. As antioxidant,
57 Vit E inhibits lipid oxidation in food and organisms (Lodge, Traber, Elsner, &
58 Brigelius-Flohé, 2000) by stopping the radical oxygen species (ROS) chain reaction
59 (Wiseman, Tijburg, & Van de Put, 2002). As a result, it prevents the peroxidation of
60 polyunsaturated fatty acids from cellular and subcellular membranes. Also, α-
61 tocopherol plays an important role as antioxidant in olive oil stability (Deiana et al.,
62 2002). The biological activity of tocopherols makes them compounds that play an
63 essential role against aging (Shaidi, 2004; Jessup, Horne, Yarandi, & Quindry, 2004).

64 Improving the olive oil industry and its economy, continuously stimulates the search for
65 new technologies. The main goals are the product's quality, for which tocopherols
66 content is important. The olive oil industry has great interest on checking the quality
67 using fast and reliable techniques, both to simplify the product handling, as well as for
68 the classification and labeling. Among the various nondestructive solutions to these
69 needs, near-infrared spectroscopy (NIRS) has made major achievements. The ability of
70 NIRS for analyzing the major quality features of olive oil, such as free acidity or
71 peroxides value, has been reported (Mailer, 2004; Bendini et al., 2007, Conte et al.,
72 2008, among others). In fact, NIRS techniques are methods for these routine analyses in
73 a growing number of laboratories. NIRS is based on correlating the spectral data with
74 the analyzed feature, using multivariate models. It has several important advantages, as
75 NIRS does not need solvents or reagent, thus avoiding a major expense. It protects the
76 environment, increasingly important. Besides, NIRS techniques are rapid and can be

multi-parameter. Must note that using NIRS techniques needs calibrating for a defined purpose, using a specific spectrometer. As well, it needs a procedure for periodic validation.

The more usual methods for olive oil tocopherols analysis is HPLC, according to standards ISO 9936 or IUPAC 2432, although there are not official methods. Analyzing tocopherols in animal feeds by using NIRS (González-Martín et al., 2006a; González-Martín et al., 2006b) has been reported. As well, Ayerdi-Gotor et al. (2007) suggested that NIRS could be useful to discriminate sunflower seeds according to their tocopherol levels. Besides, Szłyk et al. (2005) reported NIRS determination of α-tocopherol in several edible oils, after extraction with ethanol. Measuring olive oil tocopherols directly without sample preparation by using NIRS has not been studied up to date at the best of our knowledge.

Besides, from a consumer's perspective there is increasing interest in information about food bioactive compounds content. Its satisfaction is a major target for the food industry according to official rules (EU, 2011). The industry may wish distinguishing olive oil according to tocopherols content, rather than standing a detailed tocopherols analysis. This technique can provide sorting the produce, avoiding the mix of batches with different tocopherols content. Later, it allows labeling the olive oil according to their Vit E, as well as diversify the product. This labeling holds a potential added value, either in economic terms or in trade competitiveness.

This study had as a first purpose to prove a new NIRS technique using PLS models, for estimating α-tocopherol and total tocopherols content in olive oil. A second objective was to test two alternative techniques for sorting olive oils according to their α-tocopherol content, based on quantitative PLS and qualitative PLS-DA. These

techniques can be helpful as rapid methods for sorting olive oils in the industry according to Vit E content. Thus they may be useful to the competitiveness of the product.

2. Material and Methods

2.1. Olive Oils

An important reason for the robustness of NIRS calibrations is the statistical range of the analyte. Olive oil samples were taken from different sources to guarantee that their characteristics were sufficiently broad ~~enough~~. High quality Extra Virgin Olive Oils (EVOO) were acquired on special markets, this group contributing with 16 samples, 11 of which were varietal and the remaining 5 were coupages from different varieties. Olive oils normally found in the market were included, this group composed by 80 EVOO and 80 not virgin olive oils. Olive oil samples were provided also from a collaborator industry, contributing with 20 EVOO and 25 virgin olive oils. Additionally, 28 lampante and 30 pomace olive oils were used to increase the diversity of –tocopherols (α, β, or γ –tocopherol). Besides, a research project provided 48 EVOO samples which were used for the optical configuration transflectance measurements, described later. These were extracted in the Instituto de la Grasa (CSIC) from the olives using a laboratory mill (MC2, Seville, Spain) based on the Abencor system (Martínez et al., 1975). The samples in total used were 327.

2.2. Spectral Acquisition

The spectrum of every sample were acquired using two spectrometers with different features, for standing results with their optical configurations. The spectrometers used

were Labspec (Analytical Spectral Devices Inc., Boulder) and Luminar 5030 (Brimrose Corp., Maryland).

The temperature of a body has an important influence on the NIR radiation it reflects and absorbs, thus it is decisive in NIRS (Jiang et al., 2008). Therefore, the samples were taken from 4 °C storage and placed in the laboratory 18 h before processing. Before recording spectra with both spectrometers, a water bath (Nahita, London, United Kingdom) fixed at 33 °C for 30 min. held the 20 mL sample containers, assuring the temperature stability.

Wavelengths in the visible spectrum can carry useful information related to the analyzed parameter, since the olive oil tocopherols are pale yellow color (Budavari, 1989). This was the purpose for using Labspec (Analytical Spectral Devices Inc., Boulder, Colorado, USA), a Visible/NIR (Vis/NIR) spectrometer equipped with three detectors. The detector for the visible range (350-1000 nm) is a fixed reflective holographic diode array with a sensitivity of 512 pixels. A holographic fast scanner InGaAs detector cooled at -25 ºC covers the wavelength range of 1000-1800 nm. The same coupled with a high order blocking filter runs for the 1800-2500 nm interval. The instrument equips internal shutters and automatic offset correction, the scanning speed is 100 ms. The repeatability of the instrument, expressed as standard deviation on the average absorbance of five measures of a white tile between 350 and 2500 nm, is 6.00 10^{-4} cm^{-1} mol^{-1}.With the Labspec, the spectra were registered by transmittance from each sample of EVOO directly, without any other treatment. A Hellma quartz spectrophotometric cuvette with 10 mm path length held the samples while their averaged spectra were acquired. The whole spectrum Vis/NIR (350−2500 nm) was registered, each spectral variable matching to a 1 nm interval. Configuration for 50 spectra in continuous acquisition was used, each spectral variable matching to 1 nm interval. Indico Pro

148 software (Analytical Spectral Devices Inc., Boulder, Colorado, USA) was used for this
149 purpose. The registering time is less than a minute for each sample spectrum, all steps
150 included.

151 Luminar 5030 (Brimrose Corp., Baltimore, Mayland, USA)is an AOTF (acousto-optic
152 tunable filter) NIR spectrophotometer, equipped with a transflectance post dispersive
153 optical configuration and InGaAs (1100-2300 nm) detector. The reference spectrum is
154 taken automatically by the instrument, similarly to an UV-Vis spectrophotometer dual
155 beam. The beam divides before leaving the instrument, and a small portion is sent to a
156 second detector that makes the reference. The scanning speed in Luminar 5030 is 60 ms.
157 The spectrometer has a hand-held unit, equipped with a base for optional use in the
158 laboratory. The instrument's liquid probe accessory was used. The liquid probe is in
159 stainless steel, with a threaded interchangeable optical path.The NIR radiation coming
160 from the optical fiber goes through the liquid sample, reflects in the polished stainless
161 steel surface of the optical path's cylinder. Then, it back through the liquid sample,
162 heading spectrometer detector. Thus, this is transflectance. The spectrometer set is
163 complete with its computer unit. The whole spectrum was registered, each spectral
164 variable matching to a 2 nm interval. The repeatability of the instrument, expressed as
165 standard deviation on the average absorbance of 1100 to 2300 nm of five measures of a
166 white tile, is 6.76 10^{-4} cm^{-1} mol^{-1}. The signals are acquired with software Acquire
167 (Brimrose Corp., Baltimore, Maryland, USA). Averaged spectrum were obtained for
168 each sample, resulting from a total 100 spectra matching to two measures of 50 spectra
169 each, it for both spectrometers.

170 *2.3. Reference Analysis*

The analysis of α, β, and γ tocopherols were carried out by HPLC according to standards ISO 9936 and IUPAC 2432, in the Instituto de la Grasa (CSIC). Briefly, 100 mg of oil was diluted with hexane to 10 mL final volume. The prepared solution was filtered with Minisart RC15 (0.45 µm pore size, Hannover, Germany), and then a portion (20 µL) of the final solution was subjected to the FLD-normal Phase HPLC. The HPLC system consisted of a HP 1100 series (Hewlett-Packard, strasse 8, 76337 Waldbrown, Germany), G 1311A Quat pump, G1316A column oven, G1313A ALS injector. Silice 5 µm, 250x4 mm (Lichrospher 5 µ, sil 60 A, 626077-1) was used as the HPLC column. The mobile phase it was hexane/isopropilic alcohol (99:1). The flow rate was adjusted to 1.0 mL/min and the temperature was maintained at 25ºC. Tocopherols were detected by HP-FLD detector (excitation 290 nm, emission 330 nm, HP1100 series, G1321-95002, Germany). All peak areas were registered using HP Chemistation program 2010.

Calibration curves were established from patterns with different concentrations of *α–, β–, γ–* and total tocopherols between 1 and 11.5 mg/mL in n-hexane. The standards curves were linear, with a concentration range of 1-10 mg/L. These were admitted when $r^2 \geq 0.999$, respectively. The -tocopherols concentrations in olive oil were calculated by extrapolating the peak areas within the calibration curves. The results are expressed as ppm, with one decimal.

The determination was made twice in each sample, and these repeats averaged. The values in which there was an error of ± 10% were eliminated. The tocopherols contents are reported in mg/kg of olive oil (ppm). Analytical sensitivity of tocopherols was 2 ppm. The time needed for the analysis was about 20 min.

2.4. Chemometrics and Calibration Procedure

Principal Component Analysis (PCA) was carried out from the olive oil spectra using The Unscrumbler 9.7 (CAMO Software AS, Oslo, Norway). It was used both for the NIR and Vis/NIR wavelengths for analyzing the possible bundling of olive oils and detecting possible spectral outliers. The outliers were detected in PCA as those samples with high residuals, according to the procedure described by the software previously referred. Quantitative tocopherols models were built from the spectral variables visible (Vis) and near infrared (NIR), by Partial Least Squares (PLS). The data from analysis of virgin olive oils were used as reference. Transmittance spectral data were reduced to 8 nm intervals by average, transformed to absorbance and mean normalized (MN). Then, treatments by first (1DSG) and second (2DSG) Savitzsky−Golay derivatives were tested. These derivatives were both carried out with 3 smoothing points, and their polynomial order was 2. The full cross internal validation (FCV) procedure was used. The calibration set for models development excluded an external validation set. We took one out of each three olive oil samples from the third one, to stand one third of the total samples. Therefore, this external validation set does not engage in the multivariate models. The calibration and validation sample sets at beginning were the same for all the tests. Calibration models for predicting α–, β–, γ– and total tocopherols were established from the spectra gained with Luminar and Labspec. The models' PLS factors were set after tests, using 15 at first. These treatments were made by using The Unscrumbler 9.7.

Selecting the spectral variables involved in the models was made by consecutive cycles removing those which the contribution were closer to zero. Variable selection ended in the last cycle that improved the statistical model R^2_{CV}. Model fitness was assessed by the closeness between their R^2 and R^2_{CV} and by the standard error of calibration (SEC).

2.5. PLS Model Performance Assessment

Assessing the PLS model performance was mainly according to the *r* from external validation exercises. This is the correlation coefficient of the simple linear regression between the analyzed and predicted values. The residual predictive deviation (RPD) was considered also to aid interpreting the calibrations performance. The RPD is defined according to [1] (Williams and Sobering, 1996).

$$RPD = \frac{SD}{SEP} \qquad [1]$$

Where SD is the standard deviation of the reference data from the validation set, and SEP is the standard error of model performance in the validation.

As well, contrasting the calibration statistics from the different optical configurations used allows confirming their predictive abilities. These calibrations were assessed independently.

2.6. Sorting Tests

The spectral data from the optical analysis providing the best yields for predicting α-tocopherol, were used in the sorting tests. These tests were carried out in the validation set described previously. The olive oil *α*-tocopherol content was classified according to the classes High *α*-Tocopherol (H*α*T), with α-tocopherol higher to 200 ppm, and Low α-Tocopherol (LαT), lower or equal to 200 ppm. The α-tocopherol statistical mean of the ~~of the~~ olive oils used in this study was about 180 ppm (Table 1). Thus, the threshold was set at 200 ppm as it was above this mean~~Thus, it was fixed the threshold at 200 ppm for be above this mean~~. This two way classification may be useful in the industry, since the labeling would need simplicity. This separation was carried out by using the

quantitative α-tocopherol PLS model (M_1). The ~~assessing of~~ sorting performance ~~was by~~ success was assessed by~~, expressed as~~ the percentage of samples in which the classifications according to the predicted values were ~~coincident with~~equivalent to the classes ~~on~~of the reference values.

Partial Least Squares Discriminant Analysis (PLS-DA) models were established and assessed as an alternatively to PLS. Classification exercises in the two classes before described were carried out by using this qualitative technique. These models were developed from the olive oils spectra previously classified in two classes (HαT and LαT), which were used as class variables. Interval Partial Least Squares (iPLS) procedure was tested for spectral variable selection in the PLS-DA models. The same calibration and validation sets previously employed with the quantitative PLS model were used, therefore allowing their comparison. PLS-DA analysis was made with PLS-Toolbox 8.0 (Eigenvector Research Inc., Manson, USA), and its results were assessed according to the sensitivity (S_n) and specificity (S_p) statistics. S_n, also called the true positive rate, measures the proportion of correct positives. Sp, also called the true negative rate, measures the proportion of correct negatives (Altman & Bland, 1994).

3. Results and Discussion

3.1. Olive Oil Spectrum

Near-infrared spectra show various overlapping bands, because of the first and second overtones and fundamental vibrations combinations, mainly carbon–hydrogen. Shenk, Workman, & Westerhaus (2001), among others, assigned the major near-infrared absorption bands of agricultural products, and on olive oil and other plant oils, it has

been done by Harwood & Aparicio (2000). Assigning the major visible absorption bands of olive oil was made by Moyano, Meléndez, Alba, & Heredia (2008).

Olive oil spectra from the samples analyzed in this work, shown in Figure 1, agree with the previous reports. A first minor peak occurs next to 415 nm. This area suits to the wavelengths of oil absorption which are dark blue colored light. It could be due mainly to carotenoids, as well to pheophytin a, pheophorbide a and pyropheophytin a (Moyano, Meléndez, Alba, & Heredia, 2008). A second minor peak is near 450 nm, matching to blue light absorption, characteristic of carotenoids (Moyano, Meléndez, Alba, & Heredia, 2008). The wavelength suiting to absorption of pale yellow, the color of the olive oil tocopherols, is at 610 nm where is a third minor peak, not described. A fourth major peak appears around at 670 nm, which coincides with chlorophylls absorption. There are bands of high intensity, related to the strong water absorption that exists from its first overtone at 1400 to 1500 nm and a combination band at 1880–2100 nm. They link with the first overtone of the C-H vibration of several chemical groups (–CH3, –CH2). About the latter spectral band, should note the main triglyceride and the major part of olive oil is triolein. The maximum absorption peak in the triolein spectrum is at 1725 nm (García, Baeten, Fernández, & Tena, 2013). This maximum absorption band is characteristic of olive oils, as previously reported (García, Baeten, Fernández, & Tena, 2013, García, 2015). A broad absorbance band exists around 1220 nm, probably from oil and due to second overtones of C–H and CH=CH– stretching vibrations. A high intensity absorbance peak occurs about 2300 nm caused by a combination of fundamental vibrations from the C-H groups (Hourant, Baeten, Morales, Meurens, & Aparicio, 2000, Moyano, Meléndez, Alba, & Heredia, 2008).

Figure 1

3.2. *Population Characterization*

The ~~reference~~ statistical analysis results~~statistics from~~ of the α, β, and γ-tocopherol and total tocopherol contents for the calibration and external validation sets ~~gathers~~ are shown in Table 1. ~~The same statistics arithmetically calculated (sum of α, β, and γ-tocopherols) are included for total tocopherols.~~ Olive oils from all commercial types were analyzed. As shown, a wide tocopherols variation range integrates into the validations and calibrations, this last ranging from 64.2 ppm to 1078.0 ppm for total tocopherols. This range reflects the extent of the tocopherols variation in olive oil depending on the produce. Pomace olive oils were included since they show the higher content in tocopherols among the olive oil products. In fact, the total tocopherols maximum in the present study was similar to that reported in pomace olive oil (Perona, Arcemis, Ruíz, & Catalá, 2005). The α-tocopherol range in the current study (54.5-755.9 ppm) was wider than reported previously by several authors. For example, Cimato et al. (1991) reported α-tocopherol contents of 74 to 454 ppm depending on the variety and region, while Sayago, Marín, Aparicio, & Morales (2007) pointed out levels in olive from 93 to 354 ppm for the same compound. Similarly, total tocopherols ranging from 100 to 420 ppm has been reported, depending mainly on variety and harvest date (Uceda & Hermoso, 2001; Velasco & Dobarganes, 2002). The statistical mean of α, γ, and β-tocopherols in the analyzed calibrations sets were 179.7, 17.8 and 2.4 ppm, while it was 209.0 ppm for total tocopherol.

Table 1

3.3. *Spectral Variable Analysis*

Many spectral variables did not contribute to the PLS models, since their removal allowed improving calibrations. Uninformative spectral variables can provide false

contribution in the calibration models, leading to inappropriate PLS methods, thus reducing their predictive reliability. Beebe & Kowalski (1987) showed this effect by adding new columns of NIR wavelength data that had no useful information for describing their protein and moisture PLS models. Therefore, spectral variable selection is of major importance. The contributing wavelengths in this study were established by the procedure previously described for removing uninformative spectral variables. The spectral windows and single wavelengths from the Luminar spectrometer contributing for the α-tocopherol model (M_1) are shown in the Figure 2. In these graphs, wavelengths within the horizontal line have zero contribution to the model.

Figure 2

The iPLS method for selecting spectral variables did not operate better than the whole spectrum when ~~elaborating~~ developing the PLS-DA model, ~~according~~ based on their sensitivity and specificity statistical values.

3.4. PCA Analysis

The PCA analysis of olive oil spectra from both NIR and Vis/NIR showed the absence of sample groups. No outliers were detected in either PCA analysis from spectra NIR nor Vis/NIR. The PCA from 1100-2300 nm is shown in Figure 3*a*, where the PC1 explained 99% and the PC2 explained 1% of variability. It highlights ~~for~~ the ~~appearing~~ appearance of several samples separated from the major group, which ~~agreeing~~ agrees with the α-tocopherol values. However, ~~T~~these values are not the cause for the separation of these samples from the group. In fact, their α-tocopherol contents are intermediate, as deduced from the statistical analysis results listed in Table 1. The same applies to the other species of tocopherols. This segregation did not suit to a specific

cultivar, since there were different olive oil varieties among the separated samples (data not shown). However, we checked that all samples with values higher ~~to~~than 0.025 for PC2 in Figure 3*a* match to the EVOO class (data not shown).

The PCA from Vis/NIR spectra obtained from~~of~~ Labspec equipment is shown in Figure 3*b*, the PC1 explaining 76% and PC2 15% of variability. Several samples appear clearly separated, this segregation differing from that shown in Figure 3*a*. These samples suit to EVOO as in the PCA previously discussed, their α-tocopherol contents showing also intermediate values. In this case, however, excepting one with α-tocopherol value 188.1 ppm, they are olive oils provided from the industry or extracted in the research center. These samples have in common a fresh extraction, while the exception before signaled is an EVOO sample also. Therefore, the tocopherols contents do not have any relation with the segregation in this PCA analysis. From these results, we can assume that PCA analysis is uninformative for classifying olive oils according to their tocopherols content.

Figure 3

3.5. Tocopherols PLS Quantitative Models

The treatments mean normalization and second Savitzsky-Golay mostly provided the best performance, with both optical configurations. Their statistics gather in Table 2. There were exceptions in the calibrations for γ-tocopherol with 1100-2300 nm from the NIR spectra, and in α– and β-tocopherols with the Vis/NIR spectra, for which the first Savitzsky-Golay derivative treatment fitted better.

Some lampante and pomace olive oils showed significant differences between their ~~values~~ analyzed and predicted values when calibrating for α-tocopherol with NIRS and

Vis/NIRS. As mentioned previously, the purpose for using these olive oils types was to improve the robustness of the calibrations. Those samples showing relation of predicted to analyzed values higher to 2, as well as the same for the inverse, were separated in these calibrations only from such classes. The same happened for the NIRS and Vis/NIRS calibrations of total tocopherols, since α-tocopherol is the major in them, applying the same treatment. On the contrary, in the β-tocopherol and γ-tocopherol calibrations, some EVOO samples showed bad fit while the fact before described with lampante and pomace olives oils did not occurs. This probably is because of the higher β-tocopherol and γ-tocopherol values in some pomace and lampante samples, which we did check. These facts explained the different sizes of the calibration sets for both optical modes and types of tocopherols shown in Table 2.

Table 2

The NIRS model for α-tocopherol (M_1) provided R = 0.95, R_{CV} = 0.94 and SEC = 36.14. The calibration statistics from the model for α-tocopherol held with Vis/NIR provided R = 0.94, R_{CV} = 0.92 and SEC = 33.90.

On total tocopherols, the model from NIRS (M_2) yields R = 0.92, R_{CV} = 0.88 and SEC = 57.15. Besides, the statistics from the Vis/NIR were R = 0.91, R_{CV} = 0.89 and SEC = 43.83.

The general mathematical equation of M_1 and M_2 models are [2]:

$$Y_p = B_0 + \sum (B_{\lambda i} \cdot X_{\lambda i}) \qquad [2]$$

Where

Y_p = values predicted of α-tocopherol or total tocopherols respectively in M_1 and M_2 (ppm).

B_0 = B_0 coefficient.

$B_{\lambda i}$ = Coefficient of math treated spectrum for each contributing wavelength.

$X_{\lambda i}$ = Absorbance of the sample for each contributing wavelength (cm^{-1} mol^{-1} nm^{-2}).

The B_0 and $B_{\lambda i}$ values are given by the software.

The Figure 4 represents the multivariate regressions for α-tocopherol (*a*) and total tocopherols (*b*).

According to the data above, both optical configurations showed similar calibration fitness, with small differences in their statistics. Therefore it is reasonable to expect similar working from both.

Figure 4

3.6. External Validation Exercises

The statistical~~s~~ results obtained from the external validations tests ~~by~~ to predict~~ing~~ *α*–, *γ*–, *β*– and total- tocopherols on the reserved set of 107 samples ~~reserved~~ are shown in Table 3. As these statistical~~s~~ results have shown, the models with the transflectance optical configuration ~~from~~ with NIR wavelengths ~~NIR~~ only, performed better than the Vis/NIR transmittance model. This fact happened for *α*, *γ* and total tocopherols. For *β*-tocopherols, both configurations provided low performance, Vis/NIRS showing r = 0.41 versus r = 0.26 with NIR. It is reasonable to think this last is because *β*–tocopherol is a minor compound in olive oil.

399 The validation using M_1 model (V_1) for α-tocopherol is plotted in Figure 5*a*. The
400 model performance is defined by the *r* value 0.91 and RPD = 2.37 from this external
401 validation exercise. The NIRS model for total tocopherols was tested for predicting the
402 validation set (V_2). This external validation, depicted in Figure 5*b*, gave *r* = 0.90 and
403 RPD = 2.01.

404 According to several authors (Williams, 2014; Esbensen, Geladi, & Larsen, 2014), RPD
405 interpretation must consider the material analyzed. There is general agreement that
406 models with RPD higher to 10 are excellent (Fearn, 2002, Williams, 2014, Fearn, 2015,
407 Esbensen, Geladi, & Larsen, 2014). RPD between 5 and 10 holds high accuracy and
408 aptness for analysis analogous to that of the reference methods. Those with values
409 between 2 and 5 offer the accuracy wanted for providing good estimations (Williams,
410 2014). Other authors consider good RPD greater than two (Barlocco et al., 2006). One
411 ~~S~~should note RPD is essentially the same statistic *r* (Fearn, 2002; Minasny &
412 McBratney, 2013). The prediction exercise for α-tocopherol, V_1 (Figure 8), provided *r* =
413 0.91 and RPD = 2.37, while for total tocopherols V_2 (Figure 9) gave *r* = 0.90 and RPD =
414 2.00. These statistics prove that referred models are useful for estimating *α*- and total
415 tocopherols in olive oils.

416

417 Table 3

418 Figure 5

419 *3.7. Sorting Tests*

420 The olive oil *α*-tocopherol content was classified by using the M_1 PLS model on the
421 validation set previously reserved. This classification was according to the classes High

α-Tocopherol (HαT), with α-tocopherol higher to 200 ppm, and Low α-Tocopherol (LαT), lower or equal to 200 ppm. The result ~~it~~ was 91 samples correctly sorted out of the 104 samples and a success of 99.9%. This procedure has been registered in the Spanish Patent and Trademark Office as the patent P201531729.~~, currently in priority year.~~ The sorting test results ~~and~~ for predicted and reference values of α-tocopherol are in the Appendix.

The iPLS method did select four spectral windows for the PLS-DA model, which gave the best calibration statistics, shown in Table 4. We must note in the case of PLS-DA models there is not a simple regression vector to use, as in PLS. The sorting test two olive oil classes according to α-tocopherol, by using this PLS-DA model with threshold at 200 ppm, gave Sn = 0.85 and Sp = 0.96. The results from the validation exercises carried out with PLS-DA models both from the whole spectrum and from the iPLS variable selection~~,~~ are gathered in Table 4. One can notice in these data, based on the statistical analysis results, that these models perform better ~~Can note in these data the statistics points out a better working~~ when using the whole spectrum. Therefore, the validation exercises ~~did~~ not prove ~~the seeming~~ a considerable improvement by iPLS method. The PLS-DA method for distinguishing the proposed classes of olive oils according to their α-tocopherol content was successful. PLS-DA achievement is similar, although, it is ~~in whole~~ overall slightly lower than that from PLS. PLS yields a continuous quantitative variable ~~continuous~~, which we use to set a discrete variable of two classes, while PLS-DA classifies using directly this discrete class variable. Thus, comparing results from PLS-DA ~~against~~ with PLS quantitative classification is difficult. Should note that more reference information involves in the quantitative PLS model than in PLS-DA, this last built directly from the discrete variable. Therefore, PLS

supplies more information, implying that PLS can provide greater accuracy and robustness. The results of this study agree with that.

Table 4

4. Conclusions

The models using only NIR wavelengths predicted better the total tocopherols, α-tocopherol and γ-tocopherol than that using wavelengths from the visible spectrum. Predicting α-tocopherol with the NIRS model provided a valid estimate of the Vit E in the olive oils. The validation exercises of sorting olive oils according to α-tocopherol by the quantitative PLS model affords success higher to 99%. The same purpose carried out by PLS-DA yields similar, although slightly lower. The data proves the feasibility of estimating the olive oil α-tocopherol or total tocopherols contents by using NIRS. It may provide useful information on product labeling. At the same time, these rapid techniques can be helpful in the industry for sorting olive oils according to their Vit E. The use of these techniques needs a suitable software, and calibrating for a specific spectrometer. As well, it requires a procedure for periodic validation.

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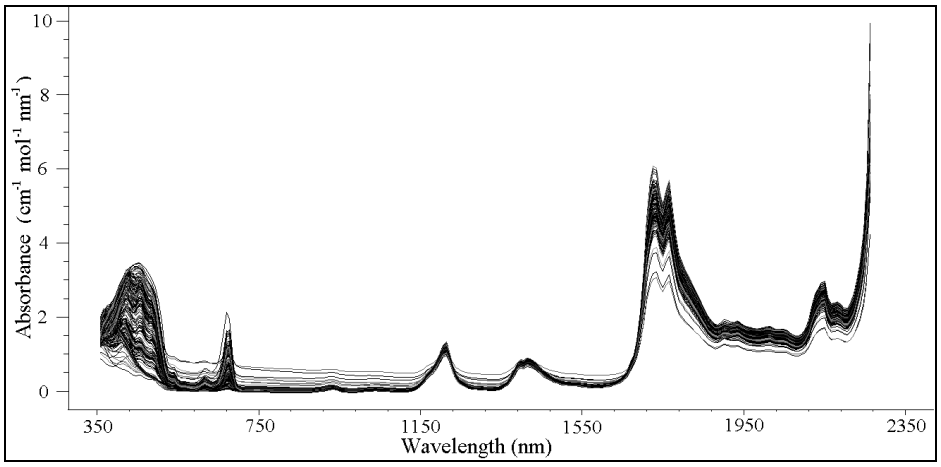
Figure 1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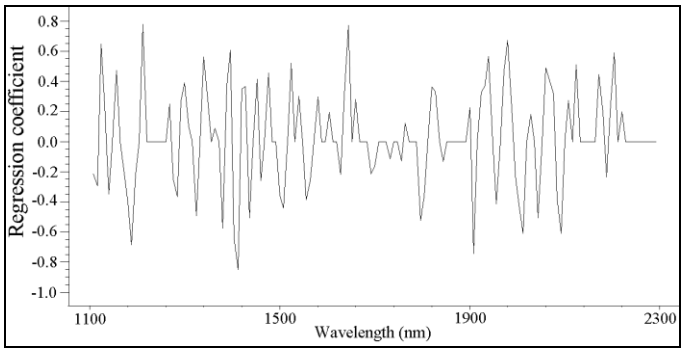
2
4
6
8
10
350
750
1150
1550
1950
2350
Wavelength (nm)

Figure 2


Regression coefficient
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0.6
0.4
0.2
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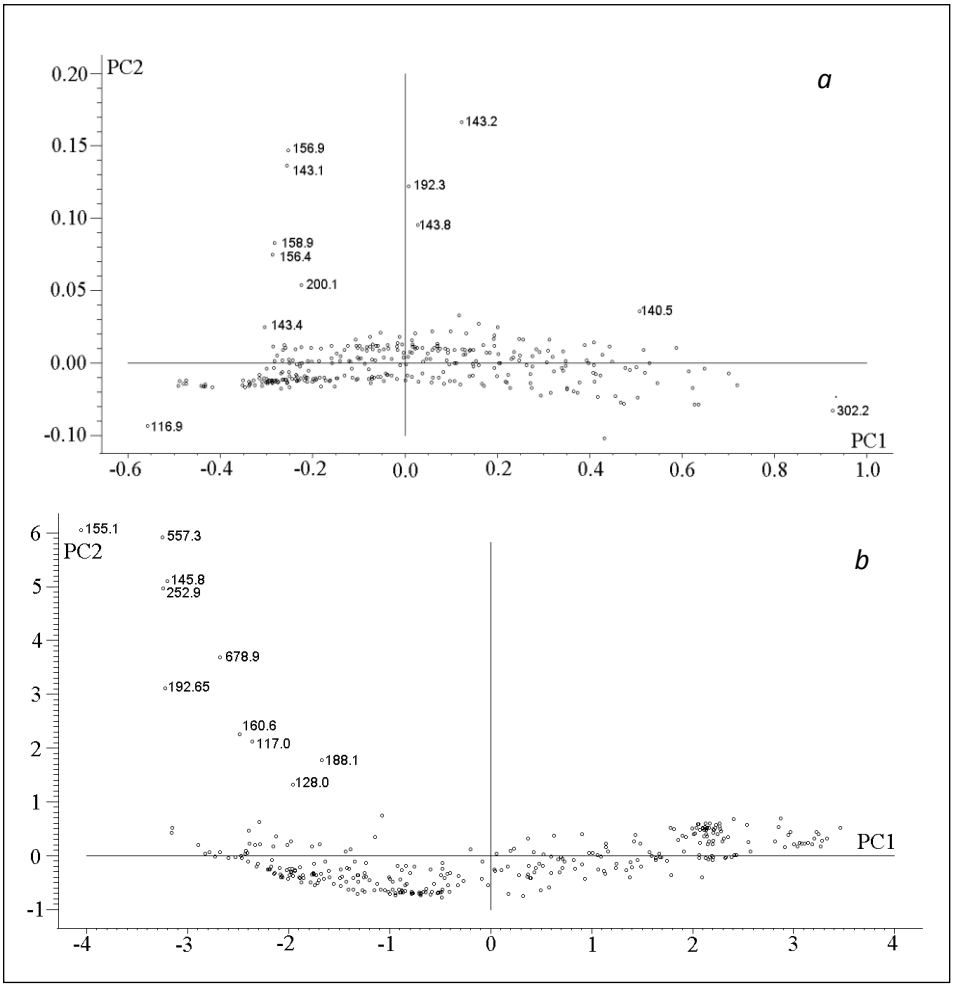
-0.6
-0.8
-1.0
1100
1500
1900
2300
Wavelength (nm)

Figure 3


a
PC2
PC1
0.20
0.15
0.10
0.05
0.00
-0.10
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
143.2
156.9
143.1
192.3
143.8
158.9
156.4
200.1
140.5
143.4
302.2
116.9
b
PC2
PC1
6
5
4
3
2
1
0
-1
-4
-3
-2
-1
0
1
2
3
4
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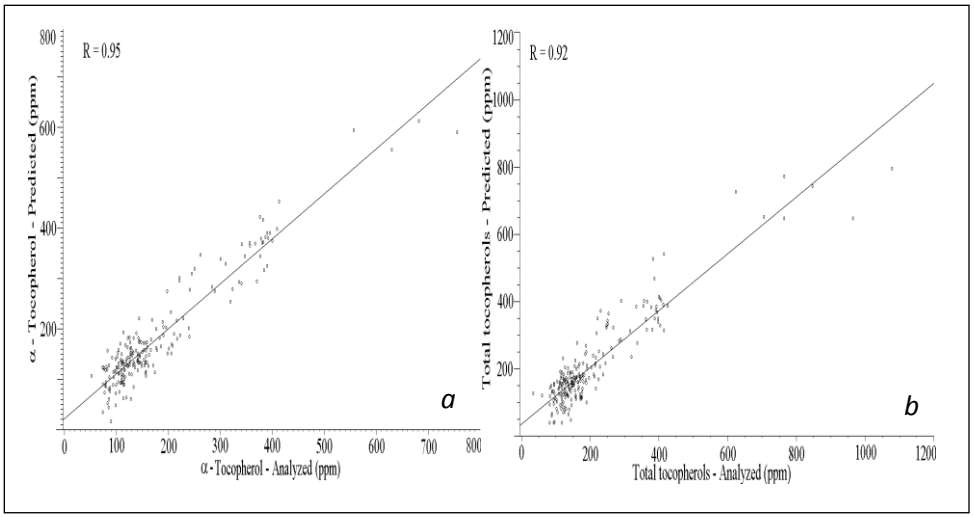
192.65
160.6
117.0
188.1
128.0

Figure 4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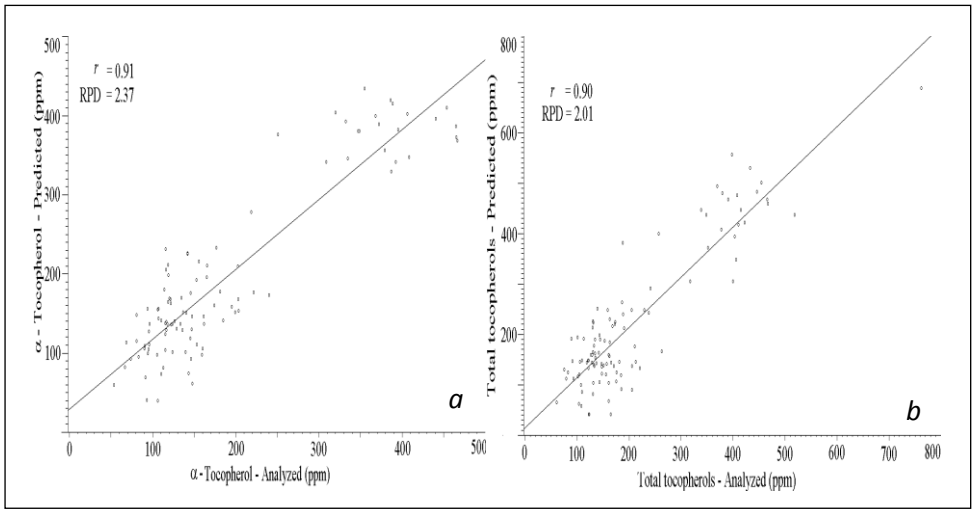
α - Tocopherol - Predicted (ppm)
α - Tocopherol - Analyzed (ppm)
a
R = 0.92
Total tocopherols - Predicted (ppm)
Total tocopherols - Analyzed (ppm)
b

Figure 5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RPD = 2.37
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α - Tocopherol - Analyzed (ppm)
a
r = 0.90
RPD = 2.01
Total tocopherols - Predicted (ppm)
Total tocopherols - Analyzed (ppm)
b

Table 1

Table 1. Statistics of the tocopherols contents (ppm) in the calibration and external validation sets.

	Calibration N = 218			*Validation* N = 109		
Tocopherols	$\overline{x}$	σ	Range	$\overline{x}$	σ	Range
Total	209.0	142.6	64.2 -1078.0	209.5	124.3	63.1 - 762.3
α	179.7	111.8	54.5 - 755.9	187.9	111.9	55.2 - 466.4
β	2.4	1.7	0.7 - 14.1	2.4	1.4	0.5 - 10.0
γ	17.8	13.4	2.5 - 103.8	17.7	12.1	1.8 - 73.4

α, α-tocopherol; β, β–tocopherol; γ, γ–tocopherol (α, β, and γ-tocopherols, HPLC analyzed). Total, Total tocopherols (arithmetically calculated by sum of α, β, and γ-tocopherols).

Table 2

Table 2. Statistics of PLS quantitative models.

	Total Tocopherols		***α-tocopherol***		***γ-tocopherol***		***β-tocopherol***	
	NIR	Vis/NIR	NIR	Vis/NIR	NIR	Vis/NIR	NIR	Vis/NIR
N	213	197	206	189	211	195	211	197
Tr.	D2SG	D2SG	D2SG	D1SG	D1SG	D2SG	D2SG	D1SG
PLS_f	15	5	15	9	14	10	10	10
R	0.92	0.91	0.95	0.94	0.92	0.87	0.64	0.66
R_{CV}	0.88	0.89	0.91	0.92	0.88	0.85	0.52	0.54
SEC	57.15	43.83	36.14	33.90	5.34	4.54	0.58	0.59

PLS, Partial Least Squares; N, samples number; Tr., treatment; PLS_f, PLS factors; R, calibration coefficient; $R_{CV,}$ calibration coefficient from the internal cross validation; SEC, standard error of calibration. NIR, spectra acquired with Luminar (1100-2300 nm); Vis/NIR, spectra acquired with Labspec (350-2500 nm); D2SG, Savitzsky-Golay second derivative; D1SG, Savitzsky-Golay first derivative.

Table 3

Table 3. Statistics of predicting exercises with PLS quantitative models.

	Total Tocopherols		***α-tocopherol***		***γ-tocopherol***		***β-tocopherol***	
	NIR	Vis/NIR	NIR	Vis/NIR	NIR	Vis/NIR	NIR	Vis/NIR
N	107	91	104	93	106	101	108	102
r	0.90	0.80	0.91	0.86	0.88	0.75	0.26	0.41
SEP	61.84	76.26	47.21	58.28	6.33	8.12	1.35	1.32
RPD	2.01	1.63	2.37	1.92	1.91	1.49	1.04	1.06

PLS, Partial Least Squares; N, samples number; *r*, regression coefficient between the predicted and reference values; SEP, standard error of prediction; RPD, residual predictive deviation; NIR, spectra acquired with Luminar (1100-2300 nm); Vis/NIR, spectra acquired with Labspec (350-2500 nm).

Table 4

Table 4. PLS-DA for α-tocopherol from NIR spectra.

	Calibration N = 218					*Validation* N = 109			
	HαT	*LαT*	*HαT*	*LαT*		*HαT*	*LαT*	*HαT*	*LαT*
	Spectrum whole		**iPLS variables[(1)]**			**Spectrum whole**		**iPLS variables[(1)]**	
$S_{n\text{-}Cal}$	0.85	0.96	0.86	0.95	$S_{n\text{-}Pred}$	0.84	0.96	0.84	0.94
$S_{p\text{-}Cal}$	0.96	0.85	0.95	0.86	$S_{p\text{-}Pred}$	0.96	0.84	0.94	0.84
$S_{n\text{-}CV}$	0.85	0.95	0.86	0.95					
$S_{p\text{-}CV}$	0.95	0.85	0.95	0.86					

PLS-DA, Partial Least Squares Discriminant Analysis; *HαT*, high α-tocopherol; *LαT*, low α-tocopherol. iPLS, interval Partial Least Squares; $S_{n\text{-}Cal}$, calibration sensitivity; $S_{p\text{-}Cal}$, calibration specifity; $S_{n\text{-}CV}$, sensitivity in the cross validation; $S_{p\text{-}CV}$, specifity in the cross validation; $S_{n\text{-}Pred}$, prediction sensitivity; $S_{p\text{-}Pred}$, prediction specifity. [(1)] iPLS variables: 1302-1342 nm; 1390-1422 nm; 1830-1862 nm; 2030-2062 nm.

Note: *The PLSDA classification can be represented as a PLS predicting between class A and B, by using y = 0 or y = 1. The threshold value where y predict greater than or less than the same, it determines to which class a sample belongs to:*

$$\text{Probability that a sample is class 1} = \frac{P(y,1)}{(P(y,1)+P(y,0))}$$

where y is the y value predicted from the PLSDA model for the sample in question, P(y,1) is the probability of measuring the given y value for a class 1 sample and P(y,0) is the probability of measuring the y value for a class 0 sample. The two probabilities used above (P(y,1), P(y,0)) are estimated from the y-values observed in the calibration data.

Figure Captions

Figure 1 - Olive oil Vis/NIR spectra from all samples analyzed.

Figure 2 - Spectral variables contributing to the α-tocopherol NIRS model (M_1).

Figure 3 – PCA from olive oils spectra *a*) 1100-2300 nm. ; *b*) 350-2500 nm. α-tocopherol (ppm).

Figure 4 - PLS models *a*) α-tocopherol (M_1); *b*) Total tocopherols (M_2).

Figure 5 - Prediction exercises *a*) α-tocopherol (V_1); *b*) Total tocopherols (V_2).

Sample	Com. Class	Predicted	Reference	Success
1	EVOO-Com.	178.06	182.28	*
2	EVOO-Com.	209.83	203.60	*
3	EVOO-Com.	232.84	177.54	
4	EVOO-Com.	176.70	222.43	
5	EVOO-Com.	158.90	196.05	*
6	EVOO-Com.	159.98	175.16	*
7	EVOO-Com.	141.16	185.89	*
8	EVOO-Com.	140.81	111.61	*
9	EVOO-Com.	231.50	116.96	
10	EVOO-Com.	170.19	135.92	*
11	EVOO-Com.	210.55	166.77	
12	Lamp.-Ind.	130.28	148.30	*
13	Lamp.-Ind.	136.50	162.89	*
14	Lamp.-Ind.	97.49	160.52	*
15	Lamp.-Ind.	162.68	123.11	*
16	Lamp.-Ind.	139.15	117.76	*
17	Lamp.-Ind.	128.84	136.89	*
18	Lamp.-Ind.	136.42	123.39	*
19	Lamp.-Ind.	173.68	241.04	
20	Lamp.-Ind.	278.16	219.58	*
21	OO-Com.	168.37	204.14	
22	OO-Com.	115.44	82.00	*
23	OO-Com.	74.50	144.60	*
24	OO-Com.	92.41	147.34	*
25	OO-Com.	118.78	146.56	*
26	OO-Com.	148.47	82.24	*
27	OO-Com.	105.22	96.62	*
28	OO-Com.	129.14	117.97	*
29	OO-Com.	136.58	125.34	*
30	OO-Com.	176.28	147.01	*
31	OO-Com.	136.71	134.45	*
32	OO-Com.	130.78	130.20	*
33	OO-Com.	140.61	127.82	*
34	OO-Com.	146.71	148.38	*
35	EVOO-Com.	111.01	154.41	*
36	EVOO-Com.	155.82	108.36	*
37	EVOO-Com.	101.54	140.74	*
38	EVOO-Com.	104.06	116.38	*
39	EVOO-Com.	106.08	91.64	*
40	EVOO-Com.	154.96	107.72	*
41	EVOO-Com.	150.62	141.78	*
42	EVOO-Com.	192.42	153.95	*
43	EVOO-Com.	226.64	143.22	
44	EVOO-Com.	151.92	200.12	
45	EVOO-Com.	153.47	204.50	
46	EVOO-Com.	215.50	156.86	
47	EVOO-Com.	225.43	143.39	
48	EVOO-Com.	81.19	114.21	*
49	EVOO-Com.	137.00	97.84	*
50	EVOO-Com.	99.55	95.83	*
51	EVOO-Com.	69.35	92.83	*
52	EVOO-Com.	198.85	120.29	*
53	EVOO-Com.	97.69	106.85	*
54	EVOO-Com.	39.37	107.68	*
55	EVOO-Com.	40.50	94.41	*
56	EVOO-Com.	108.78	91.97	*
57	EVOO-Com.	60.00	55.21	*
58	EVOO-Com.	82.09	68.24	*
59	EVOO-Com.	92.55	75.16	*
60	OO-Com.	211.54	120.00	
61	OO-Com.	164.76	119.72	*
62	OO-Com.	136.50	119.06	*
63	OO-Com.	169.23	121.55	*
64	OO-Com.	61.30	149.12	*
65	OO-Com.	110.94	97.18	*
66	OO-Com.	73.66	111.65	*
67	OO-Com.	95.45	84.72	*
68	OO-Com.	205.08	117.42	
69	OO-Com.	127.05	96.79	*
70	OO-Com.	124.06	116.71	*
71	OO-Com.	113.88	69.68	*
72	OO-Com.	144.07	108.46	*
73	POO-Com.	346.01	335.07	*
74	POO-Com.	403.76	320.42	*
75	POO-Com.	375.95	251.43	*
76	POO-Com.	400.16	368.52	*
77	POO-Com.	329.63	387.37	*
78	POO-Com.	341.90	392.60	*
79	POO-Com.	382.61	395.72	*
80	POO-Com.	415.17	389.16	*
81	POO-Com.	341.62	309.31	*
82	EVOO-R.P.	356.22	379.60	*
83	EVOO-R.P.	392.87	333.00	*
84	EVOO-R.P.	368.48	466.40	*
85	EVOO-R.P.	396.30	440.70	*
86	EVOO-R.P.	402.21	406.50	*
87	EVOO-R.P.	347.94	408.60	*
88	EVOO-R.P.	434.57	355.50	*
89	EVOO-R.P.	387.07	465.00	*
90	EVOO-R.P.	372.48	465.30	*
91	EVOO-R.P.	389.44	372.50	*
92	EVOO-R.P.	410.46	453.90	*
93	EVOO-R.P.	419.33	386.60	*
94	EVOO-R.P.	380.58	347.60	*
95	EVOO-R.P.	381.06	349.30	*
96	VOO-Ind.	195.72	166.30	*
97	VOO-Ind.	146.74	162.72	*
98	VOO-Ind.	151.62	137.75	*
99	VOO-Ind.	180.04	115.30	*
100	VOO-Ind.	155.90	95.41	*
101	VOO-Ind.	167.82	122.87	*
102	VOO-Ind.	101.46	125.06	*
103	VOO-Ind.	137.82	116.45	*
104	VOO-Ind.	105.70	161.13	*

Appendix. Sorting test using PLS model M_1.

Predicted and reference values of α-tocopherol, ppm; EVOO-Com., extra virgin olive oil commercial; Lamp.-Ind., lampante olive oil industrial; OO-Com., current olive oil commercial; POO-Com., pomace olive oil commercial; EVOO-R.P., extra virgin olive oil from research project; VOO-Ind., virgin olive oil industrial; Success (*), coincidence in sorting the olive oil as high or low in α-tocopherol. It is considered high α-tocopherol for contents greater than 200 ppm and low-tocopherol for less or equal to this value.