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Evaluation of α-tocopherol in virgin olive oil by a luminiscent method

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RESUMEN

Evaluación de α-tocoferol en aceite de oliva virgen mediante un método luminiscente.

Los fluoróforos naturales tales como los tocoferoles son de gran importancia para la caracterización y autenticación del aceite de oliva virgen. Se detectó la banda del espectro luminiscente que mejor se asociaba con la presencia de α-tocoferol (380-420 nm) at λ_{exc} = 350 nm y se evaluó la precisión una vez que el proceso analítico fue optimizado. Se añadieron diferentes cantidades de α-tocopherol, de 25 mg/Kg a 1200mg/Kg, a un aceite de oliva virgen, *cv.* Cornicabra, para construir un modelo de regresión robusta (R^2 ajustada = 0,99) basado en cinco longitudes de onda (370, 371, 378, 414 and 417 nm) que son atribuidas a la fluorescencia de este compuesto. El modelo tentativo fue validado (R^2 ajustada = 0,87) con 8 muestras de un aceite de oliva virgen, *cv.* Picual, a las que se añadieron entre 25 mg/kg y 250mg/kg de α-tocoferol. Finalmente, el modelo fue también validado con éxito (R^2 ajustada = 0,92) utilizando 7 aceites de oliva vírgenes monovarietales de diversos países productores.

PALABRAS CLAVE: Aceite de oliva virgen – Fluorescencia – Luminiscencia – Quimiometría – Tocoferoles.

SUMMARY

Evaluation of α-tocopherol in virgin olive oil by a luminescent method

Natural fluorophores such as tocopherols are of great importance for the characterization and authentication of virgin olive oil. The band of the luminescent spectrum which is most accurately associated with the presence of α-tocopherol (380-420 nm) at λ_{exc} = 350 nm was detected and its precision was evaluated once the analytical process was optimized. A virgin olive oil, *cv.* Cornicabra, was spiked with several quantities of α-tocopherol, from 25 mg/Kg to 1200mg/Kg, to build a ridge regression model (adjusted-R^2 = 0.99) based on five wavelengths (370, 371, 378, 414 and 417 nm) which are attributed to the fluorescence of this compound. The tentative model was validated (adjusted-R^2 = 0.87) with 8 samples of a virgin olive oil, *cv.* Picual, spiked with amounts of α-tocopherols ranging from 25 mg/kg to 250mg/kg. Finally, the model was successfully validated with 7 monovarietal virgin olive oils from various olive producing countries (adjusted – R^2 = 0.92).

KEY-WORDS: Chemometrics – Fluorescence – Luminescence – Tocopherols – Virgin olive oil.

INTRODUCTION

Vitamin E is a general term employed for the designation of tocopherols and tocotrienols, including α, β, γ, and δ species. The structure of tocotrienols differs from tocopherols by the presence of three *trans* double bonds in the hydrocarbon tail but both series contain a polar chromanol ring linked to an isoprenoid derived hydrocarbon chain. Thus, α, β, γ and δ species of both tocopherols and tocotrienols differ with regard to the number and position of methyl groups on the chromanol ring (Sayago *et al.*, 2007). Tocotrienols are not present in virgin olive oil while only the first three tocopherols have been quantified in percentages that vary from 52-87% for α-tocopherol to 15-20% and 7-23% for β- and γ-tocopherols respectively. Tocopherols contribute to the antioxidant properties of virgin olive oil and its profile and composition are often criteria of purity. The importance of these compounds is supported by verifiable evidence of their role in the quality and authenticity of virgin olive oil as well as their contribution to human health (Sayago *et al.*, 2007; García-González *et al.*, 2008).

A variety of methods has been described for the determination of vitamin E in virgin olive oils from the initial analytical procedures based on colorimetric or UV spectrophotometric determination and preparative chromatographic methods to purify tocopherols (Gracián and Arévalo, 1965). Since the eighties the standard method for the determination of tocopherols and tocotrienols in vegetable oils and fats has been based on liquid chromatography (IUPAC, 1987). Several alternatives to the standard, though based on HPLC, have been developed. Normal phase columns have been widely used to achieve the separation of tocopherols and tocotrienols (Rastrelli *et al.*, 2002; Seppanen *et al.*, 2003) although HPLC reversed-phase columns, mostly with UV and/or fluorescence detections, have gained more interest nowadays. (Gimeno *et al.*, 2000; Gliszczynska-Swigło and Sikorska, 2004)

Such methods are successful but are also time consuming and require access to laboratory facilities. An alternative for tocopherol determination is the

application of luminiscent methods which are much cheaper, more simple, faster and can also be applied on site, demands which have increased significantly in the last few years (Aparicio *et al.*, 2007).

The ability of virgin olive oil to emit fluorescent radiation is partially due to the presence of chlorophylls, pheophytins and tocopherols (Galano Diaz *et al.*, 2003; Sikorska *et al.*, 2004). Thus, spectrofluorimetric methods have been used to distinguish diverse edible oils (Sikorska *et al.*, 2005) to characterize olive oil designations (Nicoletti, 1990) and to detect the presence of hazelnut oil in olive oil (Sayago *et al.*, 2004). Studies based on virgin olive oil fluorescence have associated several regions of the spectrum with different parameters or variables of virgin olive oil: (i) the region of 380-400 nm seems to concern tocopherols; (ii) the region of 420-490 nm shows good correlations with conjugated dienes (K_{232}) and trienes (K_{270}); (iii) vitamin E is also associated with the region of 490-600 nm; and (iv) the bands of the region 650-700 nm are associated with chlorophylls and pheophytins. An emission band in the range of 300-350 nm has also been associated with the tocopherols dissolved in n-hexane (Sikorska *et al.*, 2005) although other authors (Zandomeneghi *et al.*, 2005) have associated the tocopherols dissolved in methanol with a unique band that goes from 300 nm to 400 nm.

The aim of this work was to determine the wavelengths that can explain the presence and relative amounts of tocopherols in virgin olive oil. An experimental design was planned to determine the optimal variables which affected the tocopherol evaluation while the stepwise linear regression procedure was used to relate the content of tocopherols with selected bands of the spectra, later validated with an external set of samples.

1. MATERIALS AND METHODS

1.1. Reagents and Samples

Two solvents, acetone (Merck, 99.5%) and ethanol (Panreac, 96%), and two α-tocopherol (Aldrich, 97%) solutions, in acetone (45.45 mg/Kg) and ethanol (47.61 mg/Kg), were used for the pattern recognition of the vitamin-E spectrum. In order to obtain the complete dissolution of α-tocopherol in the solvents, the samples were shaken for 15 min by magnetic stirring and then left for 30 min in the dark at room temperature to avoid the presence of bubbles.

A virgin olive oil *cv.* Cornicabra (Toledo, Spain) was used for the experimental design while a *cv.* Picual (Jaén, Spain) was used for determining the precision (RSD%) of the method. The calibration was carried out by spiking a virgin olive oil *cv.* Cornicabra with α-tocopherol, and was later validated with virgin olive oil of the *cv.* Picual. The additives ranged from 25 mg/Kg to 1200 mg/Kg for the training samples and from 25 mg/kg to 250mg/kg for the test samples. The order of the analyses was randomized to minimize bias.

Another set of varietal virgin olive oils (Hojiblanca, Picual, Royal, Coratina, Frantoio, Koroneiki and Chemlali) from several geographical origins and different designations, from lampante to extra-virgin, was also used to validate the regression model. They were characterized by quality parameters (acidity and peroxide values, K_{232} and K_{270}), the percentage of fatty acids, and the total amount of phenols, carotenoids and chlorophylls together with the α-tocopherol content (Table 1).

Table 1
Values of chemical compounds quantified in the varietal virgin olive oils.

Chemical compounds	Picual[a]	Cornicabra[a]	Hojiblanca	Picual	Royal	Coratina	Frantoio	Koroneiki	Chemlali
C16:0 (%)	10.10	8.73	12.42	12.85	9.61	13.34	14.4	12.11	16.98
C16:1 (%)	0.78	0.68	0.98	1.13	0.97	1.1	1.27	0.71	2.23
C17:0 (%)	0.04	0.06	0.14	0.07	0.25	0.08	0.19	0.06	0.09
C17:1 (%)	0.09	0.09	0.21	0.09	0.12	0.12	0.19	0.11	0.12
C18:0 (%)	2.91	3.71	3.18	2.75	2.65	2.32	1.93	2.71	2.22
C18:1 (%)	79.46	80.33	75.88	78.27	78.19	73.31	72.8	77.1	62.75
C18:2 (%)	5.26	4.76	5.79	3.55	6.46	8.52	7.91	5.68	14.34
C18:3 (%)	0.65	0.60	0.61	0.6	0.8	0.47	0.55	0.56	0.55
C20:0 (%)	0.34	0.48	0.41	0.4	0.42	0.36	0.39	0.48	0.39
C20:1 (%)	0.32	0.34	0.28	0.27	0.43	0.28	0.31	0.29	0.21
C22:0 (%)	0.04	0.13	0.11	0.03	0.12	0.11	0.10	0.18	0.12
Total phenols (mg/kg)	151.5	181.3	291.5	208.1	46.6	166.6	231.1	107.5	174.2
Total carotenoids (mg/kg)	10.00	5.64	7.35	6.91	5.00	3.65	7.09	3.46	6.62
Total chlorophylls (mg/kg)	13.21	8.75	9.48	5.99	3.86	6.83	6.28	8.58	9.42
Peroxide index (meq O_2/kg)	10.32	9.11	12.08	9.48	11.72	25.58	15.95	19.69	11.92
Acidity (%)	0.37	0.63	0.19	0.14	0.45	0.31	0.31	0.34	0.40
K_{232}	1.43	1.85	1.70	1.70	1.72	2.60	1.83	2.35	2.07
K_{270}	0.13	0.17	0.12	0.14	0.11	0.15	0.11	0.14	0.1
α-tocopherol (HPLC) (mg/kg)	173	142	206	202	173	316	159	137	272
α-tocopherol[b] (SLRA) (mg/kg)	182 ± 29	142 ± 30	216 ± 19	193 ± 20	182 ± 29	329 ± 25	184 ± 24	147 ± 25	273 ± 25

[a] virgin olive oils of the training and test sets that were spiked with several amounts of α-tocopherol.
[b] mean values of five determinations.

1.2. Analytical Procedures

All luminiscent measurements were performed with an RF-1501 Shimadzu spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with a continuous 150 watt xenon lamp, excitation and emission monochromators, and a photomultiplier. Right-angle geometry was used for all the samples in 10 mm fused-quartz cuvette. Emission spectra (1–nm interval) were collected at 290 nm and 350 nm excitation wavelengths for the objective of this work. Excitation and emission slits were both set at 10 nm.

Samples were kept in a topaz bottle at 4 °C while they were not in use, and were analyzed without any pre-treatment to avoid potential interferences. Each sample was analyzed in quintuplicate. The cuvette was cleaned after each measurement with detergent, followed by a rinse with deionized water and hexane in order to dry it and eliminate any fat. The spectrofluorophotometer was interfaced to a computer for spectral acquisition and data processing.

Samples were also characterized by analyzing fatty acids by gas chromatography (Varian 3900) equipped with an autosampler (Varian CP8410). Fatty acid methyl esters (FAMEs) were prepared by trans-esterification with cold methanolic solution of potassium hydroxide as described in the EU official method (EC, 1991). The results were expressed as relative area percent of the total.

Total phenols were isolated by extraction with a water:methanol (60:40) mixture three times, from an oil-in-hexane solution (Vázquez-Roncero *et al.*, 1973). The concentration of total polyphenols was estimated using the Folin–Ciocalteau method, the absorption of the solution was measured at 725 nm on a spectrophotometer Hewlett-Packard 8450 A UV/Vis, the spectrophotometric measurement was repeated three times for each extract. Results were given as mg/kg of caffeic acid.

Quality parameters (peroxide value, acidity value, K_{232} and K_{270}) were evaluated following the methodology proposed by EC 2568/91 regulation (EC, 1991).

Pigments were extracted from the virgin olive oil samples and the total contents of chloropylls and carotenoids were determined spectrophotometrically according to the method proposed by Mínguez-Mosquera *et al.* (1990).

The tocopherol content was analyzed in accordance with the IUPAC 2432 method (IUPAC, 1987). 1.5 g oil was dissolved in the mobile phase (10 ml) of 0.5% isopropanol in n-hexane. The chromatographic separation was performed using a Perkin–Elmer liquid chromatograph equipped with an isocratic pump LC200 and a UV–vis detector Lc295. A normal phase column Lichrosphere Si60 (250 mm length, 4.6 mm i.d. and 5 lm particle size) was used with an injection volume of 20 µl and a flow rate of 1.0 ml/min. The absorbance was measured at 295 nm. The results were expressed as mg of tocopherol per kg of oil.

1.3. Statistical analysis

Microsoft Excel 2003 and Statistica 7.0 (StatSoft, Tulsa, OK) were used for building the database and carrying out the statistical analyses. Stepwise linear regression analysis (SLDA) was applied to select the most remarkable wavelengths related to the α-tocopherol fluorescent emission. F-to-enter was used to select the wavelengths and results were expressed with the adjusted square regression coefficient. The value of F-to-enter was selected from the F-distribution table F (k, N-k-1) at 0.975 taking into account the numbers of samples (N) and wavelengths.

2. RESULTS AND DISCUSSION

The determination of tocopherols first required checking the bands from the whole spectrum of α-tocopherol. Therefore, α-tocopherol was dissolved in acetone (45.45 mg/Kg) and the spectra collected, a clear emission band centred around 320 nm (λ_{ex} = 290 nm) which has been associated with tocopherols (Sikorska *et al.*, 2004) was found. Another band appeared at 382 nm (λ_{ex} = 350nm) (Figure 1) which is also associated with tocopherols (Sayago *et al.*, 2004). Emission spectra from both excitation wavelengths were studied although only the results at λ_{ex} = 350 nm are presented as they showed the best results from a mathematical point of view.

Once having checked the band of interest (370-420 nm) for tocopherols evaluation at 350 nm of excitation wavelength, the next step was to spike a virgin olive oil with aliquots of α-tocopherol in order to get a calibration line. This simple analytical procedure, however, involves determining the experimental conditions that lead to the most homogeneous solution. Two variables influenced the results, the time of shaking by means of a magnetic stirrer (T_s) and the time that the sample should be left in the dark at room temperature to avoid the presence of bubbles (T_r). Although the greater the time of agitation the better the homogeneity, it might also mean a greater loss in the compound due to possible oxidation. A small experimental design was programmed with the band centred at 386 nm as the output variable to be maximized, and the range of the relative standard deviation (RSD) of the band as the variable to be minimized. The first variable means the amount of α-tocopherol present in the solution while the second indicates the level of homogeneity. Six levels of T_s (5, 10, 15, 20, 25 and 30 min) and three levels of T_r (10, 20 and 30 min) were programmed, and all the solutions were measured in quintuplicate. The experiments with a T_r lower than 30 min showed high values of %RSD (> 20%) and the information was discarded. The results of the experiments with T_r = 30 min showed high values of %RSD (ranging from 12%-18%) for T_s = 30 min, probably due to the presence of bubbles after a long

stirring time; while for T_s = 5 min, the shortest stirring time, the range of values of %RSD oscillated between 5% and 7%, probably due to the lack of homogeneity after such a short stirring time. The lowest values of RSD were for T_s = 25 min and T_s = 10 min (3%-4%), while the lowest were determined for T_s = 15 min and T_s = 20 min (< 2%). Thus, the experimental conditions were fixed at T_r = 30min and T_s = 15min.

The inter-day repeatability (or intermediate reproducibility), the next work to be performed, consisted of five analyses of a virgin olive oil *cv.* Picual carried out in five successive days by two different analysts (5 × 5 × 2). The relative standard deviation (%RSD) was lower than 10% (5.7%-9.7%) for all the wavelengths of the selected band (370-420 nm).

Once the band of α-tocopherol was detected, the optimal experimental conditions achieved and the intermediate repeatability calculated, the next objective was to determine the linearity of the instrument response to increasing amounts of α-tocopherol. Instead of a refined olive oil, a virgin olive oil was selected for diluting α-tocopherol in order to perform the experiments with the interferences from other fluorescent chemical compounds present in the virgin olive oil matrix.

The content of α-tocopherol in a virgin olive oil varies from 40 to 400 mg/Kg according to several authors (Morales and León-Camacho, 2000; Agramont-Linas *et al.*, 1986; Fedeli and Cortesi, 1993; Uceda and Hermoso, 2001) which is inside the range of this study (25-1200 mg/kg) and agrees with the range of 70-1900 mg/kg for edible oils described by deMan (1999). Ten samples were prepared by adding increasing amounts of α-tocopherol (25, 50, 75, 100, 150, 200, 300, 500, 750 and 1200 mg/kg) to a virgin olive oil *cv.* Cornicabra (142 mg/kg of total tocopherols). These spiked virgin olive oil samples were used to build a regression equation by means of the statistical procedure of stepwise linear regression analysis (SLRA). They were the training samples.

Other nine samples were prepared by adding other amounts of α-tocopherol (25, 55, 85, 110, 130, 160, 220, 227, 250 mg/kg) to a virgin olive oil *cv*. Picual (202 mg/kg of total tocopherols). This set was used to test the results of the regression equation. They were the test samples. All the analyses were carried out in quintuplicate. Figure 2 shows the selected band of each one of the training samples. This figure shows an increment of the band when the amount of α-tocopherol is increased with a very small shift in the wavelength for the maximum intensity (382 nm for 25 mg/kg *vs*. 380 nm for 1200 mg/kg) and the band shape. The change in the band shape can be due to the increment in the band maximum or the interactions occurring in the most concentrated samples due to molecular interactions as well as inner filter effects (Sirkoska *et al.*, 2004).

Stepwise linear regression analysis (SLRA) was applied to the data of the selected band (370-420 nm) which is associated with the fluorescence emission of α-tocopherol. The spectrum of the genuine virgin olive oil was subtracted from the virgin olive oils spiked with α-tocopherol prior to building the regression model. SLRA was applied under the strictest conditions (F-to-Enter = 8.00 and Tolerance= 0.01; Ridge Regression with λ=

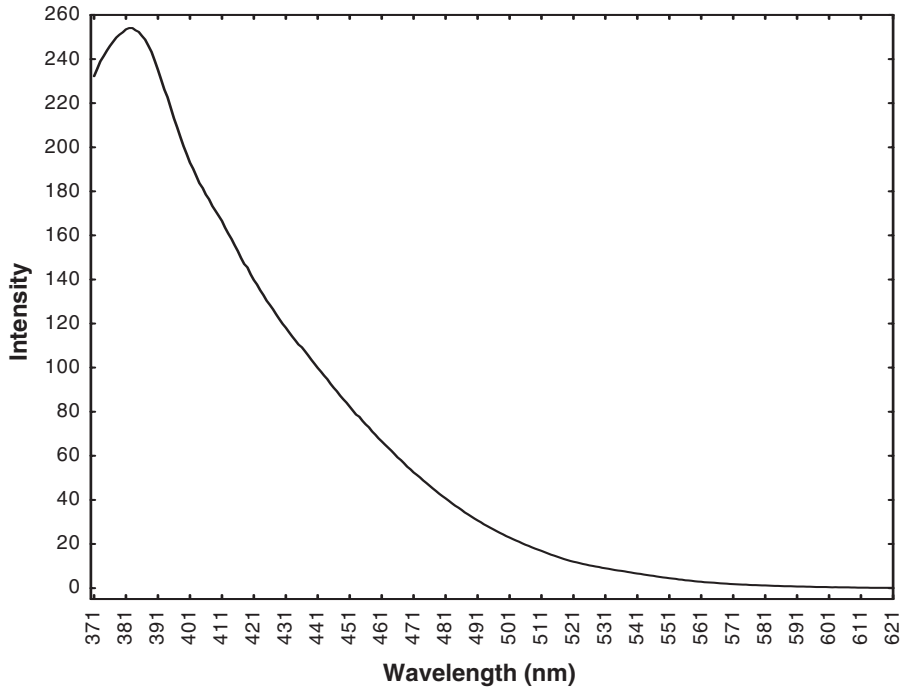

Figure 1
Emission spectrum of a-tocopherol (λex = 350 nm).

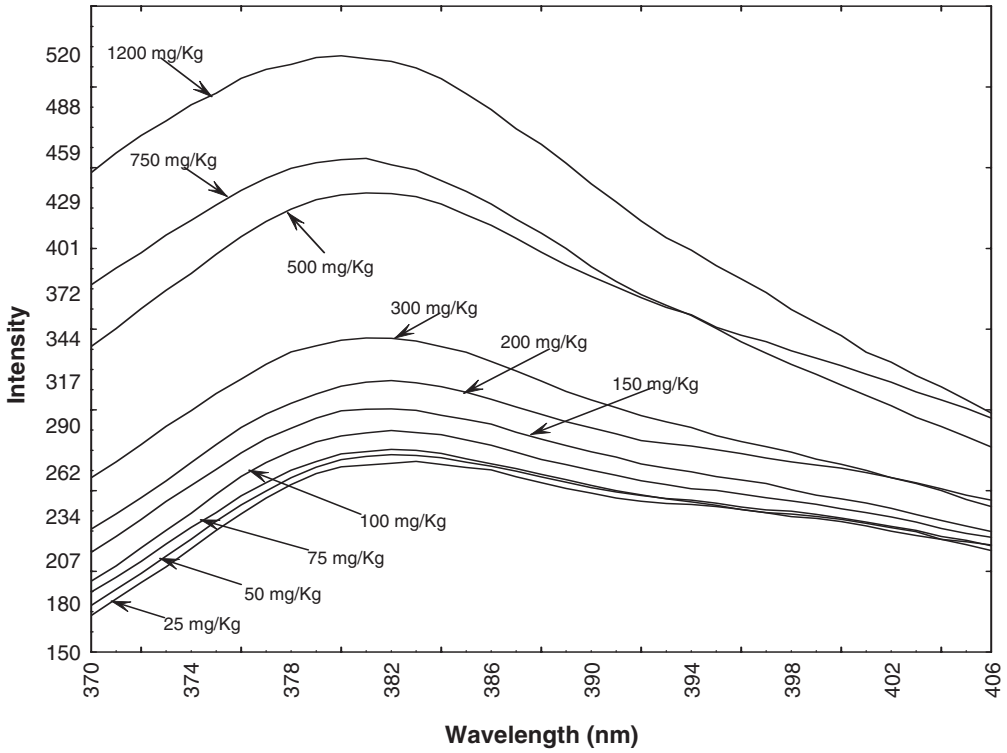

Figure 2
Emission spectrum (λex = 350 nm) of selected wavelengths of ten samples of virgin olive oil *cv.* Cornicabra spiked with increasing amounts of a-tocopherol, from 25 mg/kg to 1200mg/kg.

0.01). Five wavelengths were selected (370, 371, 378, 414 and 417 nm) to get an adjusted-R^2 of 0.994 (R^2= 0.990; p=0.0357; Durbin-Watson D statistic = 2.68) although the best wavelengths were three only; 370, 371 and 378 nm. The regression equation was validated with a set of 8 test samples made of a virgin olive oil *cv.* Picual spiked with amounts of α-tocopherol varying from 25 mg/kg to 250mg/kg. The results of the test set were slightly worse, adjusted-R^2=0.87 (R^2=0.90; p=0.075). Figure 3A shows the regression results with the training samples while Figure 3B shows the result of applying the model to the test set. The regression equation of the model was the following:

α-tocopherol (mg/kg) = *209.6-106.4*v370* + + *119.6*v371-13.997*v378* + *6.89*v414-6.41*v417*

vXXX being the intensity of the spectrum at a particular wavelength; e.g., v370 is the intensity of the spectrum at 370 nm.

In addition to the model validation with the test set, the selected wavelengths were also validated with a set of varietal virgin olive oils cultivated in the main olive producing countries - Spain (Cornicabra, Hojiblanca, Picual, Royal), Italy (Coratina, Frantoio), Greece (Koroneiki) and Tunisia (Chemlali) – and whose concentrations of α-tocopherol determined by HPLC varied from 137 mg/kg and 316 mg/kg (Table 1). These varietal virgin olive oils were also characterized by their fatty acid content, total chlorophyll, phenol and carotenoid composition together with their basic quality parameters (acidity, peroxide index and K270 and K232) (Table 1) in order to know their chemical characteristics and to assure that the samples were distinct enough from a chemical point of view so as to validate the regression model. The percentage of fatty acids varied depending on the cultivars. Thus, virgin olive oil from the Chemlali variety had the lowest content of oleic acid and the highest level of linoleic and palmitic acids.Virgin olive oil from Royal variety, however, showed a surprisingly lower concentration of total phenols (46.6 mg/kg) while Hojiblanca, a varietal olive oil with a non bitter though pungent taste, presented the highest value (291.5 mg/kg). The lowest concentration of total carotenoids was detected in virgin olive oil from the Coratina variety while the lowest content of chlorophylls was also detected in virgin olive oil from the Royal variety. The results from the quality parameters showed that virgin olive oil from the Coratina variety was a lampante virgin olive oil due to the value of the peroxide index (25.58 meq O_2/kg), which was higher than 20 meq O_2/kg. Finally, Table 1 also shows the content of α-tocopherol as determined by HPLC and the ranges of total tocopherol (p= 0.95%) obtained by applying the regression model (adjusted-R^2 = 0.92). A good agreement was found between the results obtained from both methods.

3. CONCLUSIONS

These tentative results show that the luminescent spectra of virgin olive oils contain sufficient information so as to be used as base of

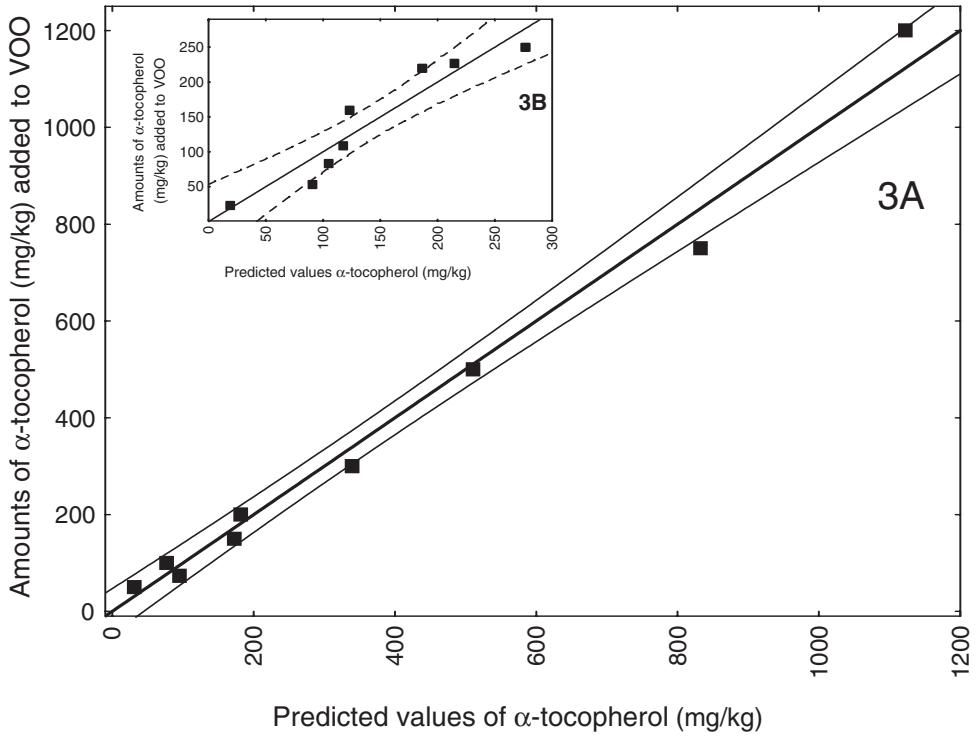

Figure 3
Regression equation built with the training samples (*cv*. Cornicabra spiked with amounts of α-tocopherol from 25 mg/kg to 1200mg/kg) (3A) and validated with the test samples (*cv*. Picual spiked with amounts of α-tocopherol from 25 mg/kg to 250mg/kg) (3B).

rapid methods to evaluate α-tocopherol in virgin olive oil samples. However, the differences in the spectra of the right-angle (RA) technique that can be due not only to the intrinsic fluorescence of α-tocopherol but also to the light absorption together with artifacts associated to the technique would explain the lack of linearity in the response of some spiked samples in some wavelengths of the band selected. These results, although satisfactory, are expected to be improved by future studies applying the front-face technique, which does not show potential artifacts.

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