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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quantification of Tocopherols and Tocotrienols in Portuguese Olive Oils Using HPLC with Three Different Detection Systems

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Three different HPLC detection systems were compared for the determination of tocopherols and tocotrienols in olive oil: fluorescence and diode array connected in series, ultraviolet, and evaporative light scattering. The best results were obtained with the fluorescence detector, which was successfully applied in the quantification of tocopherols and tocotrienols in 18 samples of Portuguese olive oils. To support the validity of the method, the parameters evaluated were linearity, detection limits, repeatability, and recovery. All of the studied samples showed similar qualitative profiles with six identified compounds: α-T, *β*-T, *γ*-T, *δ*-T, α-T_3, and *γ*-T_3. α-Tocopherol (α-T) was the main vitamin E isomer in all samples ranging from 93 to 260 mg/kg. The total tocopherols and tocotrienols ranged from 100 to 270 mg/kg. Geographic origin did not seem to influence the tocopherol and tocotrienol composition of the olive oils under evaluation.

KEYWORDS: Tocopherols; tocotrienols; olive oil; NP-HPLC

INTRODUCTION

"Virgin" olive oil is a high-priced product obtained from the fruit of the olive tree (*Olea europaea* L.) exclusively by mechanical means without any further treatment. Olive oil contains several bioactive substances related with beneficial effects on human health. This is the case of the high mono-unsaturated-to-polyunsaturated fatty acid ratio and a pool of minor compounds with a powerful antioxidant activity, namely, vitamin E, phytosterols, carotenoids, and phenols (*1*).

Vitamin E is the collective name of eight naturally occurring forms encompassing four tocopherols (α-T, *β*-T, *γ*-T, and *δ*-T] and four tocotrienols (α-T_3, *β*-T_3, *γ*-T_3, and *δ*-T_3). All of them contain a chromanol ring and a hydrophobic side chain, a phytyl in tocopherols and an isoprenyl with three double bonds in tocotrienols (*2*). Tocopherols are the major group of primary antioxidants occurring in vegetable oils and fats. In virgin olive oils nearly 95% of the total tocopherols content is α-T (*3*, *4*).

The compounds included in the designation vitamin E are correlated with preventive action against reactive oxygen species (ROS) in biological systems such as plasma, membranes, and tissues (*5*, *6*). In the past, α-T was the most studied isomer due to the idea that it exhibits the highest biological activity, and several methods have been exclusively developed for its quantification (*7*). Nevertheless, recent studies suggest that the other vitamin E isoforms have also important roles in the human organism. For example, *γ*-T has considered as a cancer chemopreventive agent (*8*) and as a potent and effective agent in the prevention of cerebral infarction induced by middle cerebral artery occlusion (*9*). Tocotrienols seem to inhibit cholesterol synthesis, reducing plasma cholesterol levels as well as other risk factors for cardiovascular disease (*1*, *8*, *9*), and suppress tumor-cell proliferation (*9*). For these reasons, it is essential to have a notion of the vitamin E profile of a determined food to estimate and understand their possible antioxidant and other biological activities.

Due to the structural similarity of these compounds it is very interesting to develop analytical techniques that allow the extraction, separation, identification, and quantification of the individual isomers, instead of the global vitamin E activity or just α-T content. Besides, the referred profile may also be useful in the assessment of identity and quality of olive oils, a food matrix in which they are usually present (*10*, *11*). However, the tocopherol and tocotrienol profiles are not constant, depending frequently on the cultivar, fruit ripening stage, edafoclimatic conditions, and olive-growing techniques (*12*).

Sample preparation for chromatographic determination of tocopherols and tocotrienols in vegetable oils can include liquid−liquid extraction without saponification or solvent extraction after saponification (*7*). These different procedures are conditioned by the food matrix under evaluation. The analysis can be carried out with high-pressure liquid chromatography (HPLC) or gas chromatography (GC) (*11*, *13*). However, GC is normally disregarded due to the nonvolatile nature of these compounds, requiring derivatization prior to the quantification step. Both normal (NP) and reversed phase (RP) HPLC (*14*, *15*) are the most common methodologies used for

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Table 1. Comparison of Validation Results Obtained with the Three Detectors under Evaluation

	ELSD			UV			fluorescence/DAD			
	linearity range (μg/mL)	R^2	limit of detection (μg/mL)	linearity range (μg/mL)	R^2	limit of detection (μg/mL)	linearity range (μg/mL)	R^2	limit of detection (μg/mL)	detector gain
α-T	20–75	0.9912	5.4	10–75	0.9961	2.69	2.5–75	0.9981	0.0002	10
β-T	0.5–2	0.8800	0.25	0.3–2	0.8731	0.13	0.065–2	0.9997	0.0004	100
γ-T	0.8–3	0.8575	0.38	0.4–3	0.8877	0.19	0.1–3	0.9972	0.0007	100
δ-T	0.5–2	0.8674	0.35	0.3–2	0.8623	0.13	0.065–2	0.9997	0.0009	10
α-T_3	4–15	0.7685	2.8	2–15	0.8341	0.94	0.25–15	0.9994	0.0032	10
β-T_3	0.5–2	0.7782	0.25	0.3–2	0.7888	0.13	0.033–2	0.9998	0.0004	100
γ-T_3	1.2–5	0.9820	0.63	0.6–5	0.9999	0.32	0.0825–5	0.9993	0.0001	100
δ-T_3	6.6–25	0.9493	1.13	3.3–25	0.9559	0.56	0.4–25	0.9998	0.0072	100

the analysis of tocopherols and tocotrienols. Comparing RP and NP columns for separation, the latter show the main advantage, allowing a good separation of all isomers (*7*, *14*, *16*, *17*).

HPLC detectors used in the analysis of these compounds include ultraviolet (UV), fluorescence, evaporative light scattering (ELSD), electrochemical, and amperometric detection (*3*, *7*, *18*, *19*). Fluorescence detection is described as more sensitive and selective than UV. (*3*). ELSD has been already successfully applied in the analysis of different compounds and consequently is increasingly used in many analytical laboratories (*20*, *21*). Although there are several methods reported allowing the simultaneous determination of tocopherols and tocotrienols (*16*, *18*, *22*−*25*), data concerning the tocotrienol composition of olive oils are still scarce (*26*, *27*).

The aim of this study was the evaluation of three detection systems (fluorescence and diode array detector connected in series, UV, and ELSD) based on the separation efficiency and limits of detection of a NP-HPLC method for the determination of tocopherols and tocotrienols. Moreover, it was also an objective to validate and optimize a method with both simple and fast sample preparation, allowing the simultaneous determination of the referred compounds in olive oils.

MATERIALS AND METHODS

Instrumentation. Chromatographic analyses were performed in a Jasco chromatographic equipment (Jasco) consisting of a PU-980 pump and a model AS-950 autosampler. The detection systems tested were a Jasco model MD-910 multiwavelength diode array detector (DAD) and a Jasco model FP-920 fluorescence detector connected in series; a Jasco model 970 ultraviolet (UV) and a model 75-Sedere (ELSD). Data were analyzed using Borwin-PDA Controller software (JMBS). An Inertsil 5 SI normal-phase column (250 mm × 3 mm i.d.) operating at room temperature was used for separation. The mobile phase consisted of 1,4-dioxane/*n*-hexane (3.5:96.5, v/v). The flow rate was 0.7 mL/min and the injection volume 10 μL. The effluent was monitored with an ELSD (evaporator temperature, 40 °C; air pressure, 3 bar; and photomultiplier sensitivity, 4), a UV (at 295 nm) and a DAD connected in series with a fluorescence detector (excitation, 290 nm; and emission, 330 nm). The compounds were identified by chromatographic comparisons with authentic standards and by their UV spectra. Sample contents of tocopherols and tocotrienols were quantified by fluorescence detection based on the internal standard method. The results were obtained from triplicate measurements.

Reagents. α-, β-, γ-, and δ-tocopherols (T) and α-, β-, γ-, and δ-tocotrienols (T_3) were purchased from Calbiochem (La Jolla, CA), and tocol (internal standard) was from Matreya (Pleasant Gap, PA). 1,4-Dioxane was from Fluka (Madrid, Spain), and *n*-hexane HPLC grade was from Merck (Darmstadt, Germany).

Standard Solutions. Stock standard solutions, α-T (150 mg/L), β-T, β-T_3, and δ-T (4 mg/L), γ-T (6 mg/L), α-T_3 (30 mg/L), γ-T_3 (10 mg/L), and δ-T_3 (50 mg/L), were prepared in *n*-hexane, flushed with nitrogen, and stored at −20 °C protected from light. Combined working standard mixtures, with concentrations similar to those present in the samples, were prepared daily from the stock standard solutions.

Samples. Samples of commercial Protected Designation of Origin (PDO) olive oils from two different geographical regions of Portugal, Trás-os-Montes (northeast) and Alentejo-Norte (south), were randomly purchased in the local market. A total of 18 samples were analyzed.

Sample Preparation. A representative sample of 0.5 g was weighed in a 4 mL amber vial, and 1 mL of *n*-hexane was added. An aliquot of the homogenized mixture (1 mL) was transferred into a 1.5 mL amber vial, 300 μL of the internal standard solution (250 mg/L tocol) was added, and then 200 μL of *n*-hexane was added to make up the volume. The tubes were homogenized for 30 s. All preparation steps were performed in a dark room and the samples kept in the darkness and refrigerated until their use, due to the characteristics of the analytes.

Statistical Analysis. Data were analyzed by ANOVA, with significant differences for $p < 0.05$. Data were also processed by principal component analysis (PCA). Bartlett's shericity and Kaiser−Meyer−Olkin tests were used to check that PCA might be applied to data set. PCA was applied under the following conditions: Kaiser's normalization and varimax rotation. Statistical analyses were carried out with Statistical Package for the Social Sciences for Windows version 11.5 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

The comparative study of the detectors was performed using the same sample and the same chromatographic conditions, namely, column, eluent, flow rate, and volume of injection. To support the validity of the study, the parameters evaluated were linearity, detection limits, and resolution in the samples. Linearity was performed with standard solutions conveniently diluted to cover the expected concentration ranges of the analytes in samples. The detection limit was estimated on the basis of signal-to-noise ratio (3:1).

In the ELSD the three instrumental parameters affecting the sensitivity, evaporation chamber temperature, nebulizing gas pressure, and photomultiplier gain, were first optimized. The evaporation chamber temperature must be selected as a compromise between uniformity of the generated particle size and complete solvent evaporation without analyte losses. Temperatures between 80 and 40 °C were tested, the best results being obtained at 55 °C. The nebulizing gas pressure (air flow rate) affects the uniformity and size of the droplets formed; a pressure of 3.0 bar was selected as optimal. The photomultiplier gain was studied in the range from 2 to 6. A gain of 4 was selected as it provided an optimal sensitivity with an adequate signal-to-noise ratio. Although satisfactory limits of detection and linearity were obtained for the standards (**Table 1**), the quantification in the sample was very difficult due to the presence of other compounds coeluting with the analytes of interest. This is in accordance with Rupérez et al. (*7*), who reported a poor sensitivity and selectivity of the ELSD detector for this kind of compound independent of the presence of a chromophore group in their structure.

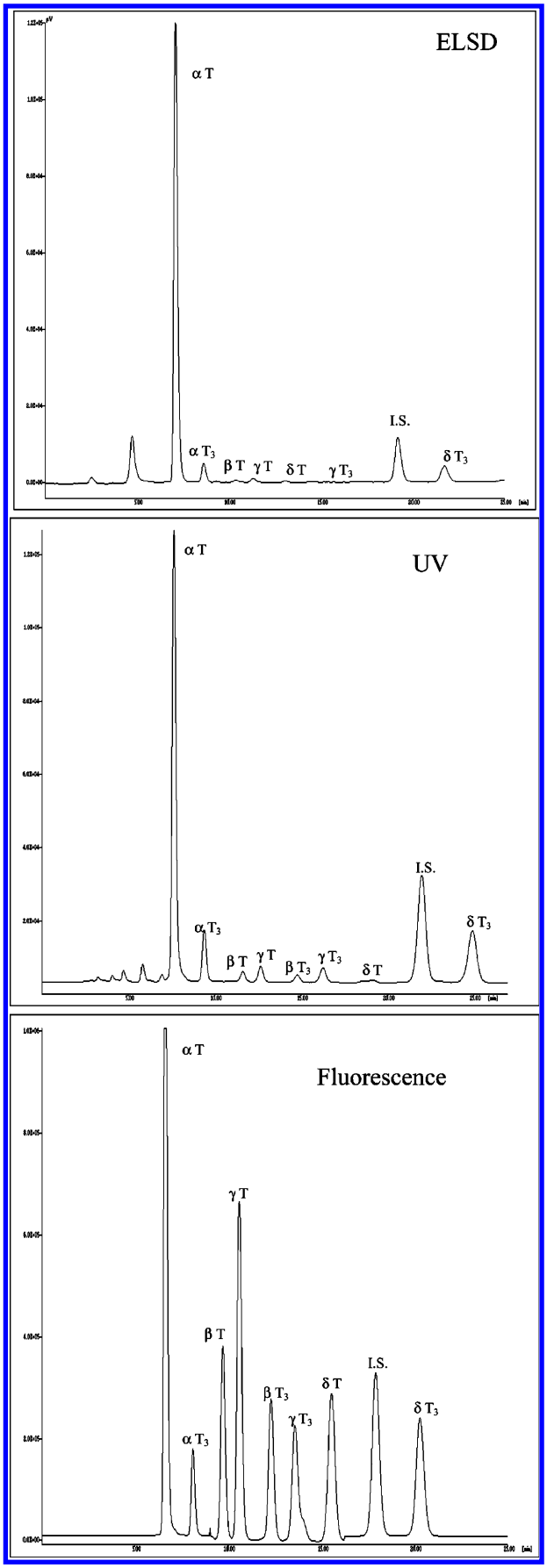

Figure 1. Chromatograms of a working standard mixture containing α-T (65 μg/mL), β-T, δ-T, and α-T_3 (1 μg/mL), γ-T (1.5 μg/mL), γ-T_3 (2.5 μg/mL), and δ-T (12.5 μg/mL) obtained with ELSD, UV, and fluorescence/DAD detectors, respectively.

Table 2. Validation Parameters of Tocopherol and Tocotrienol Determination in Olive Oil with Fluorescence and Diode Array Detectors Connected in Series

	olive oil sample (mg/kg)	accuracy[a] (recovery %)	repeatability CV % ($n = 6$)
α-T	256.07	89.36	1.81
β-T	2.08	80.71	1.20
γ-T	5.29	73.80	1.26
δ-T	0.45	78.24	3.31
α-T_3	0.67	83.70	1.90
β-T_3		103.02	
γ-T_3	0.49	83.88	6.59
δ-T_3		107.05	

[a] Mean values of three different concentrations of a spiked olive oil sample (μg/mL) (α-T, 5, 2.5, 1.25; β-T, β-T_3, and δ-T, 0.133, 0.065, 0.03; α-T_3, 1, 0.5, 0.25; γ-T, 0.2, 0.1, 0.05; δ-T_3, 1.6, 0.8, 0.4; γ-T_3, 0.33, 0.15, 0.075).

The UV is the most common detector used for chromatographic determination of tocopherols and tocotrienols (*5*). However, UV detectors present a loss in sensitivity of ~10^3-fold (*7*). In the present study, limits of detection and linearity obtained with standards (**Table 1**) were very satisfactory. The disadvantage of this detection procedure is the potential presence in samples of other compounds that can interfere, decreasing the separation efficiency (*7*).

The connection of a more universal detector (diode array) with a more specific one (fluorescence detector) gives the ability to get more information from one analysis and directly confirm the identity of tocopherols and tocotrienols. As can be seen in **Table 1** the best limits of detection were obtained with the fluorescence detector. The possibility to change, during the run acquisition, the gain settled in the fluorescence detector allows a better detection and, consequently, the quantification of minor compounds present in olive oil, such as γ- and δ-T and α- and γ-T_3. The gain selected for each analyte is indicated in **Table 1**. Chromatograms of a standard mixture of tocopherols and tocotrienols obtained by NP-HPLC/ELSD, UV, and fluorescence/DAD were compared and are presented in **Figure 1**.

To study the accuracy of the proposed method, an olive oil sample was spiked with three different concentration levels of tocopherols and tocotrienols.The results, given in **Table 2**, demonstrate the acceptable quantitative recoveries of all analytes (from 74 to 107%). The repeatability of the method, determined by the coefficient of variation (CV) of six consecutive preparations and analyses of the same olive oil sample (**Table 2**), indicate that the assayed method provides a good repeatability range.

The European Union dominates world production of olive oil, >70%, and is its major consumer (*28*). Portugal is the fourth largest producer of olive oil in Europe, Portuguese olive oils being recognized as high quality oils. Besides, olive oil is considered one of the most important Portuguese products, and a great breeding effort has been made in recent years to increase production. Nevertheless, data concerning the Portuguese olive oils are still scarce (*4*, *23*). Although olive oil is an important source of tocopherols, few works mention their contents in tocotrienols (*26*, *27*).

All of the studied samples showed similar qualitative profiles with six identified isomers: α-T, β-T, γ-T, δ-T, α-T_3, and γ-T_3. The contents are depicted in **Table 3**. Some general trends of the data can be inferred. With respect to tocopherols, the major constituent is α-T followed by γ-T, β-T, and δ-T. α-T amounts herein reported range from 93 to 260 mg/kg. These contents are generally in agreement with data previously reported for

Table 3. Tocopherol and Tocotrienol Contents (Milligrams per Kilogram) of 18 PDO Olive Oils from Two Regions of Portugal (Trás-os-Montes and Alentejo-Norte)[a]

sample	α-T	β-T	γ-T	δ-T	α-T_3	γ-T_3	total
			Trás-os-Montes				
1	108.97abcd ± 0.002	1.67bcd ± 0.017	4.07cde ± 0.058	0.20bcde ± 0.001	0.35ab ± 0.006	0.69bcde ± 0.006	115.99
2	95.91ab ± 0.001	1.79cde ± 0.020	2.83a ± 0.123	0.23cde ± 0.003	0.57abc ± 0.029	0.89ef ± 0.019	102.26
3	102.11ab ± 0.001	1.16ab ± 0.043	2.69a ± 0.151	0.20bcd ± 0.002	0.33ab ± 0.020	0.77cdef ± 0.010	107.34
4	112.57bcd ± 0.001	1.83cde ± 0.031	2.61a ± 0.079	0.17bc ± 0.003	0.50abc ± 0.006	0.69bcde ± 0.006	118.43
5	112.14bcd ± 0.000	1.47bcd ± 0.008	3.78cd ± 0.021	0.11a ± 0.003	0.54abc ± 0.039	0.59abcd ± 0.009	118.63
6	125.39de ± 0.001	1.50bcd ± 0.025	4.29def ± 0.115	0.16b ± 0.004	0.82bc ± 0.005	0.61abcd ± 0.005	132.89
7	123.73cd ± 0.003	1.60bcd ± 0.012	3.05ab ± 0.070	0.16b ± 0.003	0.63bc ± 0.038	0.68bcd ± 0.018	129.88
8	105.33abc ± 0.000	1.68bcde ± 0.005	2.53a ± 0.168	0.20bcde ± 0.006	0.47abc ± 0.009	0.76cdef ± 0.009	110.97
9	163.26ed ± 0.004	2.95f ± 0.015	4.81fg ± 0.075	0.27defg ± 0.004	0.96c ± 0.004	0.77def ± 0.007	173.05
10	142.81e ± 0.009	3.37f ± 0.020	4.39def ± 0.002	0.25def ± 0.030	0.89bc ± 0.003	0.44f ± 0.001	152.20
11	256.07i ± 0.006	2.08de ± 0.001	5.29g ± 0.002	0.45l ± 0.002	0.67a ± 0.006	0.49abcd ± 0.001	265.06
12	259.94i ± 0.002	2.31e ± 0.020	5.37g ± 0.001	0.44jl ± 0.002	0.79bc ± 0.000	0.65dfe ± 0.001	269.54
13	164.98f ± 0.002	1.45bcd ± 0.004	5.34g ± 0.001	0.31fgh ± 0.031	0.50abc ± 0.004	0.45ab ± 0.001	173.05
14	204.76h ± 0.007	1.67bcd ± 0.036	4.90cd ± 0.023	0.37hlj ± 0.100	0.58ab ± 0.000	0.77abc ± 0.002	213.12
			Alentejo-Norte				
15	103.37ab ± 0.000	1.94a ± 0.010	3.53bc ± 0.004	0.28efg ± 0.022	0.61bc ± 0.000	0.52abcd ± 0.004	110.26
16	198.40gh ± 0.002	2.07de ± 0.000	9.77h ± 0.002	0.41m ± 0.190	0.66bc ± 0.002	0.61abcd ± 0.002	211.93
17	185.53g ± 0.019	1.74bcde ± 0.010	5.17g ± 0.002	0.39ijl ± 0.024	0.41abc ± 0.006	0.43a ± 0.008	193.70
18	92.57a ± 0.007	1.24abc ± 0.040	4.47ef ± 0.020	0.34ghi ± 0.035	0.51abc ± 0.015	0.35b ± 0.002	99.53

[a] The absence of common letters in rows indicates significantly different values ($p < 0.05$).

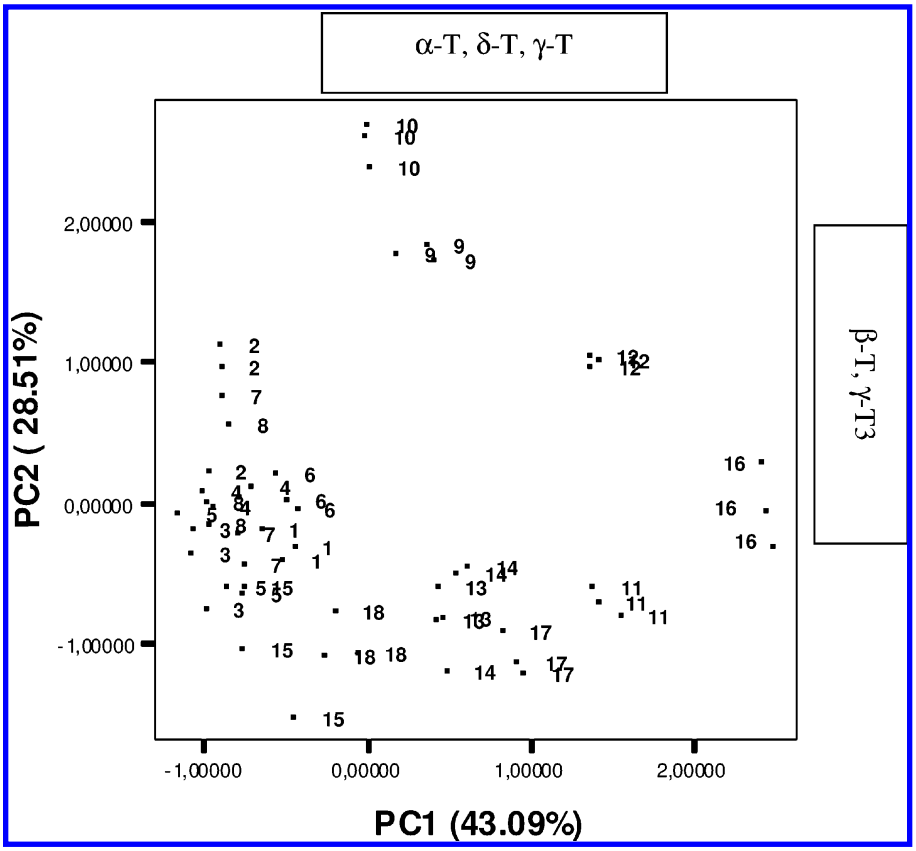

Figure 2. PCA of tocopherols and tocotrienols, from 18 PDO olive oils of Trás-os-Montes (1–14) and Alentejo-Norte (15–18).

virgin olive oils ranging from 90 to 300 mg/kg (*4*, *26*, *29*−*33*). Notwithstanding some works that have reported a relationship of α-T contents with the geographic origin of the olives (*29*), significant differences were not observed among the PDO olive oils from the two regions evaluated. The reduced number of Alentejo-Norte samples can probably explain the discrepancy.

Even though different cultivars and geographical locations are considered, the results herein reported are in good agreement with other results previously reported for tocopherol contents of olive oils. According to García et al. (*30*), β- and γ-tocopherols of olive oils from different Spanish cultivars (Picual, Hojiblanca, Arbequina, and Cornicabra, all acquired in Spanish markets) presented contents similar to those described herein (0.8−1.7 mg/kg for β-T and 2.3−13.0 mg/kg for γ-T). However, Béltran et al. (*12*) obtained higher contents for β-T (3.3 mg/kg) in Hojiblanca virgin olive oils, and Aturki et al. (*17*) obtained higher contents for γ-T (22 mg/kg) in commercial Italian virgin olive oils. The obtained δ-T contents fit within the range (not detected−0.7 mg/kg) reported by Sanchez et al. (*27*), but are lower than those indicated by Gliszczyñska-Œwiglo and Sikorska (*15*), who reported contents of 1.3 mg/kg for Polish olive oil.

The quantitative differences verified can be explained by several reasons such as cultivar employed, ripening stage, agroclimatic conditions, olive-growing techniques, and storage conditions (*12*, *31*).

Most of the published papers concerning tocopherol and tocotrienol profiles described the presence of tocopherols, but not the presence of tocotrienols (*26*, *27*), probably as consequence of employed quantitative procedures. In this work two tocotrienols were quantified, α-T_3 and γ-T_3, the contents of which were in accordance with the ones reported by Sanchez et al. (*27*) (from not detected to 3.1 mg/kg and from not detected to 4.7 mg/kg for α-T_3 and γ-T_3, respectively). However, α-T_3 and γ-T_3 herein reported (mean values of 0.65 and 0.60 mg/kg, respectively) are slightly higher than the values described in some food composition tables, mean contents of 0.2 and 0.3 mg/kg for α-T_3 and γ-T_3, respectively (*26*).

In the evaluated PDO olive oil samples, total contents of tocopherols and tocotrienols ranged from 100 to 270 mg/kg, values similar to the ones reported in the literature for olive oils (*4*, *17*, *18*, *29*).

The one-way analysis of variance (ANOVA) verified the significant differences, if present, between the mean scores of each tocopherol and tocotrienol of PDO olive oils. In general, tocopherols and tocotrienols were normally distributed and homoscedasticity was observed. Some significant differences were observed by the aforementioned ANOVA. Tukey's post-hoc test was used to find where such differences were located (**Table 3**).

The differences between tocopherol and tocotrienol compositions of the two regions were evaluated by PCA. The results are depicted on a two-dimensional plot, explaining 71.6% of total variance. The first component (PC1) by itself condensed 43.1% and the second component (PC2) represented 28.5% of total information. PC1 is primarily responsible for the differences between the contents of α-T, δ-T, and γ-T. PC2 relates the contents of γ-T_3 and β-T. Olive oil samples, as a function of the two principal components, are plotted in **Figure 2**. Generally, all samples had a large dispersion. Geographic origin did not seem to influence the tocopherol and tocotrienol composition of the olive oils under evaluation.

This work emphasizes the importance of the detector used to obtain consistent and accurate results for all analytes of interest. The HPLC/diode array/fluorescence detection seems to be a useful tool in the evaluation of tocopherol and tocotrienol profiles of olive oils as a quality parameter and authenticity marker.

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