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Determination of Tocopherols and Tocotrienols in Vegetable Oils by Nanoliquid Chromatography with Ultraviolet−Visible Detection Using a Silica Monolithic Column

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A method for the determination of tocopherols and tocotrienols in vegetable oils by nanoliquid chromatography with UV−vis detection has been developed. The separation of tocopherols was optimized in terms of mobile phase composition on the basis of the best compromise between efficiency, resolution, and analysis time. The optimal conditions were achieved using a C18 silica monolithic column (150 mm × 0.1 mm) with an isocratic elution of acetonitrile/methanol/water (acidified with 0.2% acetic acid) at a flow rate of 0.5 μL min^{-1}, giving a total analysis time below 18 min. The method has been applied to the quantification of tocopherols and tocotrienols present in several vegetable oils with different botanical origins.

KEYWORDS: Botanical origin; nano-LC; silica monolithic columns; tocopherols; tocotrienols; vegetable oils

INTRODUCTION

Vegetable oils contain vitamin E, which is a mixture of tocopherols (Ts) and tocotrienols (T_3s). These important lipid antioxidants are characterized by a two-ring structure known as chromanol (a substituted phenol with a cyclic ether), which is attached to a branched hydrocarbon side chain having 16 carbon atoms. According to the number and position of the methyl substituents in the phenol ring, four Ts (α-, β-, γ-, and δ-T) and four T_3s (α-, β-, γ-, and δ-T_3) are distinguished. Essentially, the family of vitamin E compounds is a major lipid-soluble chain-breaking antioxidant that protects natural oils from oxidation (*1*−*3*) and also prevents lipid peroxidation in biological membranes (*4*, *5*). Their positive influence to retard the development of precancer lesions and tumors (*6*) and to combat free radical reactions that can cause DNA mutations (*7*) has been demonstrated.

Ts are found in variable proportions in vegetable oils, while T_3s are present especially in palm oil (*8*) and in cereal seed oils (*9*). The relative concentrations of Ts and T_3s vary widely from one oil to another, which can be used to distinguish the oils according to their botanical origin. Thus, α-T is the most representative antioxidant in olive oil (*10*, *11*), while γ- and δ-T contents are high in soybean and sunflower oils, with soybean oil particularly rich in γ-T (*12*). Ts and/or T_3s concentrations have been used to detect the adulteration of olive oil with red palm (*13*) and hazelnut oils (*14*) and soybean oil with linseed oil (*15*). Therefore, rapid and reliable analytical methods, capable of assessing the composition of these compounds in vegetable oils, are important in food quality control and in relation to human health studies.

Vitamin E components have usually been determined in vegetable oils by gas chromatography (*16*) and high-performance liquid chromatography (HPLC) (*17*−*20*). Although normal phase columns are more efficient in separating the β- and γ-isomers of Ts and T_3s (*17*), reversed phase columns show higher column stability, better reproducibility, and shorter analysis times (*11*, *17*, *21*). Other techniques, such as Fourier transform infrared spectroscopy (*22*), synchronous fluorescence spectroscopy (*19*), and capillary electrochromatography (CEC) (*23*, *24*) have also been applied to the determination of Ts in vegetable oils.

Miniaturization is one of the present trends in science and technology, especially in the field of analytical chemistry. The use of these miniaturized techniques, such as nanoliquid chromatography (nano-LC), offers several advantages over classical techniques, such as better separation efficiencies, increase in sensitivity, shorter analysis time, and lower sample and reagent consumption (*25*, *26*).

Nano-LC has been applied to the separation of a wide number of compounds in different areas, mainly in proteomics, but also in pharmaceutical or environmental fields (*25*); however, the application of this technique appears very useful in food analysis (*25*, *27*−*32*), especially in quality control or to highlight contamination and/or adulteration. Nano-LC has been applied to the separation of Ts in serum and pharmaceutical preparations using a packed column (*33*); however, as far as we are concerned, it has not been applied to determine Ts in vegetable oils.

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Among the common stationary phases used in nano-LC, silica-modified particles of 3−5 μm, bed monoliths (*34*), or walls coated with appropriate materials (*35*, *36*) have been used. In particular, monolithic materials exhibited important advantages in comparison to packed columns. Some of these benefits are simple preparation, absence of retaining frits, high permeability, adjustable porosity, and pore size and functionality of columns (*37*−*40*).

In this work, a nano-LC-UV−vis method using a C18 silica monolithic column has been developed. After optimization of mobile phase composition, this method has been applied to determine Ts and T_3s in vegetable oils with different botanical origins.

Table 1. Botanical Origin, Number of Samples, and Brand of the Oil Samples Used in This Work

origin	no. of samples	brand
avocado	1	Guinama
	1	Marnys
corn	1	Guinama
	1	Gloria
extra virgin olive	1	Intercoop Oliva[a]
	1	Tenuta Pennita[b]
grapeseed	1	Guinama
	1	Coosur
hazelnut	1	Guinama
	1	Percheron
peanut	1	Guinama
	1	Maurel
red palm	2	Blue Bay
soybean	1	Guinama
	1	Coosur

[a] Oil produced in Spain from the Serrana cultivar. [b] Oil produced in Italy from the Brisighella cultivar.

MATERIALS AND METHODS

Reagents. Used were the following analytical grade reagents: acetonitrile (ACN), methanol (MeOH), 2-propanol (Scharlau, Barcelona, Spain), acetic acid (Panreac, Barcelona), and butylated hydroxytoluene (BHT, Fluka, Buchs, Switzerland). Deionized water was obtained with a Barnstead deionizer (Sybron, Boston, MA). Standards of α-, γ-, and δ-T were from Sigma (St. Louis, MO). Stock solutions of the analytes (ca. 1500 μg mL^{-1}) were prepared in MeOH with 0.1% BHT (w/v) and stored at −20 °C in amber vials. Working solutions were prepared daily by dilution of the stock solutions with the mobile phase. The vegetable oils employed in this study (**Table 1**) were either purchased in the local market or kindly donated by the manufacturers. The botanical origin and quality grade of all of the samples were guaranteed by the suppliers.

Instrumentation and Working Conditions. A 1200 series liquid chromatograph provided with a degasser, a nanopump, and a diode array detector with a micro flow cell (Agilent Technologies, Palo Alto, CA) was used. The column was directly coupled to a 10 nL injector equipped with a microelectric actuator (Valco, Schenkon, Switzerland). Separation was carried out with a Chromolith CapRod RP-18 capillary column (150 mm × 0.1 mm, Merck, Darmstadt, Germany). Elution was performed isocratically with 75:8:17 (ACN/MeOH/water, v/v/v) containing 0.2% acetic acid. UV−vis detection was performed at 295 ± 16 nm (360 ± 100 nm as reference), with a flow rate of 0.5 μL min^{-1}.

Sample Preparation. According to Aturki et al. (*23*), vegetable oil samples (4 g) were extracted twice with 10 mL of MeOH containing 0.1% BHT (w/v) as an antioxidant and once again with 10 mL of a MeOH/2-propanol mixture 80:20 (v/v). All extractions were performed during shaking for 30 s followed by centrifugation at 8000*g* for 10 min. The combined extracts were evaporated to dryness using a rotary evaporator at 40 °C. The residue was dissolved in 1 mL of MeOH and stored at −20 °C in amber vials. For each sample, three extracts were performed. Each extract was properly diluted with the mobile phase and injected three times.

RESULTS AND DISCUSSION

Optimization of Tocopherol Separation. To optimize tocopherol separation in terms of mobile phase composition, a test mixture

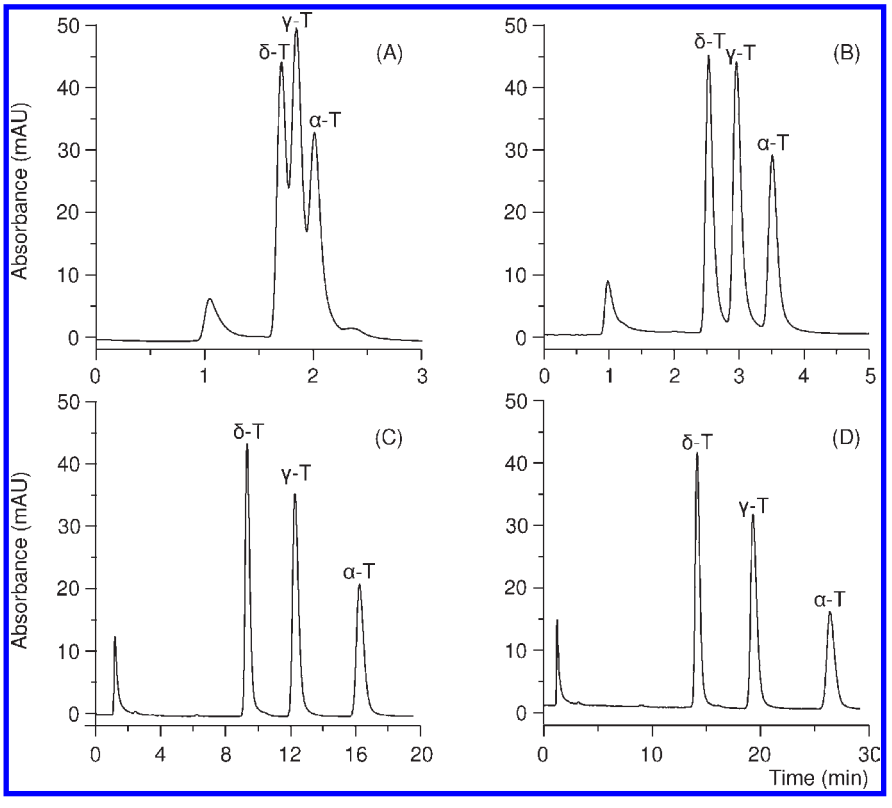

Figure 1. Influence of water content in the mobile phase composition on the separation of tocopherol standards: (**A**) 89:10:1, (**B**) 84:9:7, (**C**) 75:8:17, and (**D**) 71:8:21 ACN/MeOH/water (v/v/v) with 0.2% acetic acid. Chromatographic conditions: flow rate, 0.5 μL min^{-1}; wavelength detection, 295 nm.

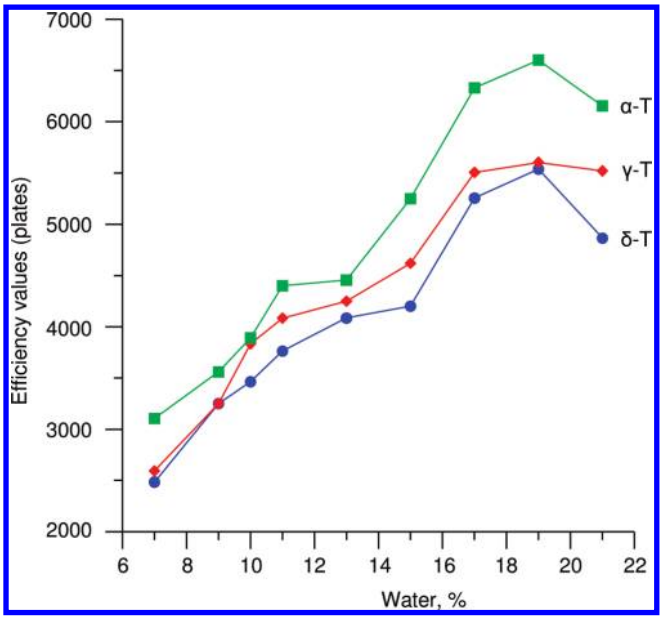

Figure 2. Efficiency values of Ts at several water contents in the mobile phase.

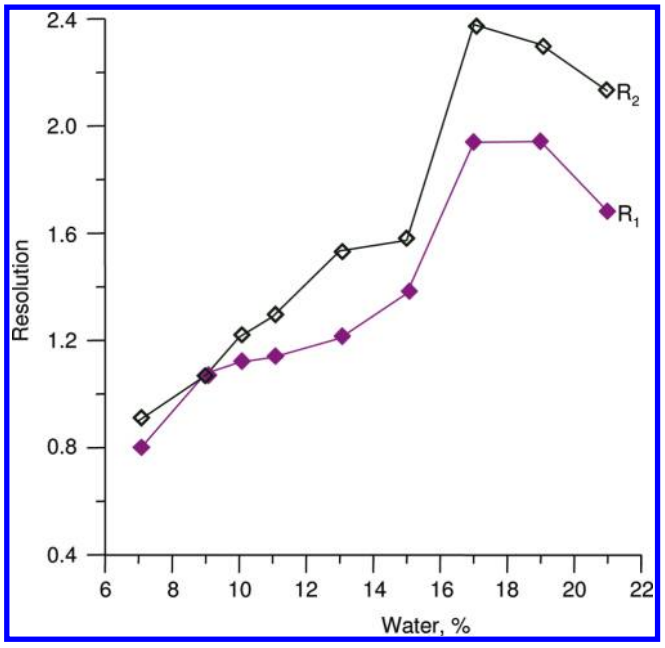

Figure 3. Resolution of Ts at several water contents in the mobile phase (R_1 = resolution between δ- and γ-T; R_2 = resolution between γ- and α-T).

composed of the α-, γ-, and δ-T (ca. 300 μg mL^{-1}) standards was used. According to literature (*20*), an 89:10:1 ACN/MeOH/water mixture (v/v/v) containing 0.2% acetic acid was first tried as a mobile phase in isocratic elution mode using a flow rate of 0.5 μL min^{-1}. As observed in **Figure 1A**, this mobile phase composition was unable to resolve all analyte pairs. To improve this separation, water content was progressively increased from 1 to 21% keeping constant the acetic acid percentage. As shown in **Figure 1**, an increase of analysis time was observed when the water content increased.

To evaluate the quality of tocopherol separation, efficiency (N) and resolution values were calculated (see **Figures 2** and **3**, respectively). It can be seen that both efficiency and resolution are highly dependent on the water content in the mobile phase. As shown in **Figure 2**, a progressive increase in the efficiency values of Ts was observed when the water content was increased, reaching a maximum at 19%. In all cases, theoretical plate values followed the next order: α-T > γ-T > δ-T. On the other hand, resolution also improved when the water content increased, reaching a maximum value at 17% (**Figure 3**). On the basis of these results, a mobile phase containing 75:8:17 (ACN/MeOH/water, v/v/v) was selected as the best compromise between efficiency, resolution, and analysis time (ca. 18 min; see **Figure 1C**).

Quantitation Studies and Application to Vegetable Oils. External calibration curves of peak areas were constructed by injecting six standard solutions in the range 5−500 μg mL^{-1}. Each solution contained the three tocopherol standards. In all cases, an excellent linearity with $r^2 > 0.998$ was obtained. Other analytical figures of merit are shown in **Table 2**. Repeatabilities of peak areas and retention times were obtained by injecting a mixture containing 50 μg mL^{-1} of each standard 10 times per day during 3 days. The limits of detection (LODs) and limits of quantification (LOQs) were estimated for signal-to-noise ratios of 3 and 10, respectively. In all cases, these values were lower than others reported in literature (*24*, *41*). Similar sensitivities were obtained for δ- and γ-T, this value being lower in α-T than in δ- and γ-T. Therefore, a higher LOD was obtained for α-T with respect to the other two Ts assayed. These results are in agreement with the literature (*33*).

The optimized method was applied to the determination of Ts in several oil samples. Peak identification was performed by

Table 2. Analytical Figures of Merit for the Nano-LC Method in the Determination of Ts

	repeatability[a] (%)		μg mL^{-1}		
analyte	area	t_R	LOD	LOQ	relative sensitivity[b]
δ-T	2.8	0.1	0.07	0.2	1.81
γ-T	3	0.1	0.07	0.2	1.92
α-T	4.2	0.2	0.16	0.5	1.00

[a] For a tocopherol concentration of 50 μg mL^{-1} (n = 30). [b] As the ratio of the slopes of calibration curves of Ts (respect to α-T).

comparing the retention times and absorption spectra with those of the standards. Representative chromatograms of corn, grapeseed, hazelnut, and soybean oil extracts are shown in **Figure 4**. As indicated above, each extract was injected three times. In all cases, the relative standard deviation was below 2.3%. Different fingerprints of Ts were obtained according to the botanical origin of the oil. The monolithic column used in this work had reversed-phased characteristics and was therefore unable to resolve the β-T and γ-T isomers present in the oil samples, which agrees with RP-HPLC studies (*11*, *17*, *21*). Consequently, the sum of the concentrations of both isomers was considered for quantification. The found concentrations of Ts, expressed as mg kg^{-1} oil, are reported in **Table 3**. These data are consistent with those previously reported for oils of the same botanical origins (*12*, *13*, *42*). As shown in **Table 3** and **Figure 4A**, large quantities of β-T + γ-T and small amounts of α-T and δ-T were present in corn oil. Hazelnut oil showed α-T concentrations higher than those of the other Ts (**Table 3** and **Figure 4C**). In soybean oil, large quantities of β-T + γ-T and δ-T were observed (**Table 3** and **Figure 4D**). Thus, these Ts could be used to detect the presence of soybean oil in virgin olive oil (*13*), whose major isomer was α-T (**Table 3**).

Red palm and grapeseed oils constitute important sources of T_3s (*8*, *42*); these compounds are also present in other oils, such as corn oil (*43*). Thus, T_3s were identified according to their UV spectra (which are closely similar to those of Ts due to the fact that the double bonds of the side chain of T_3s are not conjugated) and comparing the elution order with that previously reported in the literature for C18 reversed-phase analysis (*44*−*46*). These

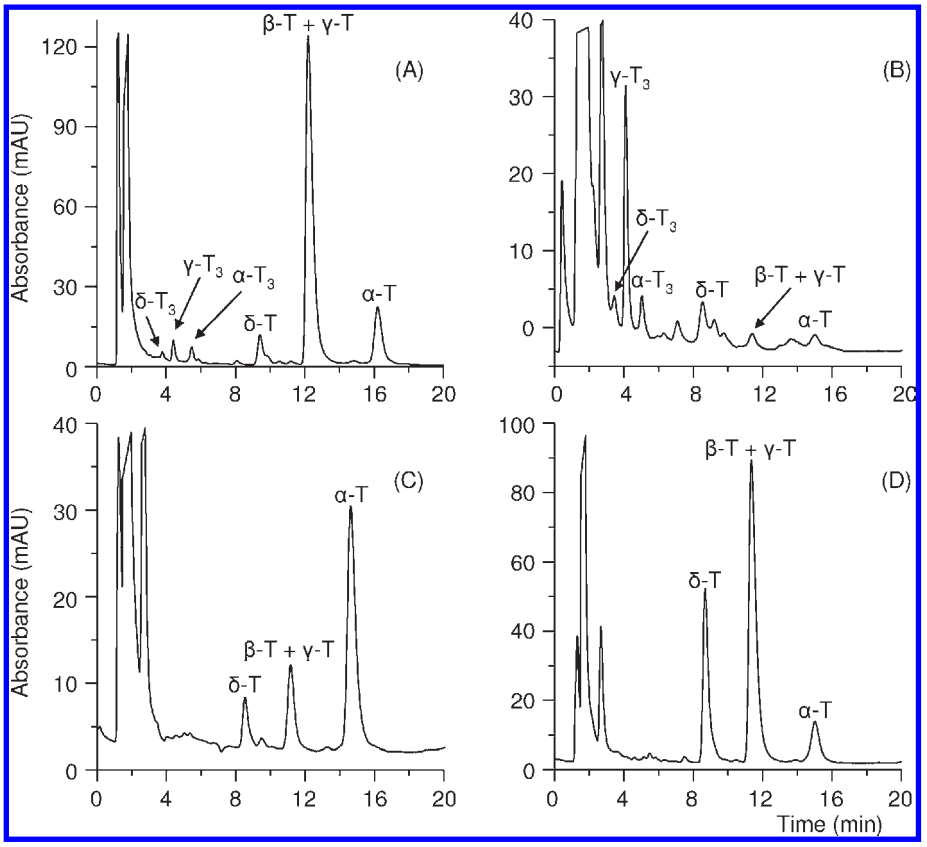

Figure 4. Chromatograms of (**A**) corn, (**B**) grapeseed, (**C**) hazelnut, and (**D**) soybean oil extracts. Chromatographic conditions: 75:8:17 ACN/MeOH/water (v/v/v) with 0.2% acetic acid. Other experimental conditions are as in **Figure 1**.

Table 3. Contents of Ts and T_3s in Vegetable Oils (mg kg^{-1})

oil	δ-T	β-T + γ-T	α-T	δ-T_3	β-T_3 + γ-T_3	α-T_3
avocado	12.2−23.9	6.3−67.6	34.2−55.1	ND[a]−7.9	ND[a]−9.3	ND[a]−5.3
corn	11.1−22.3	125.0−237.0	51.7−82.6	4.8−7.1	4.6−7.3	2.3−12.4
extra virgin olive	ND[a]−5.6	7.5−10.1	52.1−111.7	ND[a]	ND[a]	ND[a]
grapeseed	6.0−10.2	6.3−17.2	5.8−54.8	0−8.2	10.3−31.0	2.1−12.3
hazelnut	7.0−12.1	18.8−32.2	71.5−119.7	ND[a]	ND[a]	ND[a]
peanut	6.9−31.3	36.7−74.1	42.6−44.5	ND[a]	ND[a]	ND[a]
red palm	ND[a]	4.2−8.3	6.8−20.8	8.8−11.3	27.0−40.4	15.2−35.1
soybean	66.9−87.8	95.4−177.1	17.4−52.6	ND[a]	ND[a]	ND[a]

[a] ND, not detected (below the LOD value).

features were employed to assign the additional peaks observed in avocado, corn, grapeseed, and red palm oils. Each T_3 was quantified using the calibration curve of its corresponding tocopherol. Their concentration is also reported in **Table 3**. These T_3 contents were also consistent with those reported in literature (*9*, *42*). Because T_3s did not occur naturally in olive oil, their presence in a sample clearly indicates its adulteration with tocotrienol-rich oils.

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