

Tocopherol Content in Vegetable Oils Using a Rapid HPLC Fluorescence Detection Method

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Abstract

A quick and direct method based on reversed phase high performance liquid chromatography with fluorescence detector for measuring tocopherols (α , $\beta + \gamma$ and δ) has been developed. Oils are diluted in methanol: hexane: tetrahydrofuran (neither previous extraction of tocopherols nor saponification procedure are required) and after being vortexed and centrifuged, an aliquot of the overlay was injected directly into an Alltima C 18 column. Acetonitrile and methanol (50: 50) mixture was used as a mobile phase with a flow rate of 1 mL min⁻¹. Quantification of tocopherols was performed by fluorescence detector at 290 nm excitation wavelength and 325 nm emission wavelength. Tocopherols were separated at 25°C in less than 10 min after injection. The method has good limit of detection (9 ng g⁻¹ for α -tocopherol and 8 ng g⁻¹ for β -, γ - and δ - tocopherols) and reproducibility (CV < 2.9 %). This method can be used to assess the influence of genetic modification of oil seeds on the distribution of tocopherols or the effect of tocopherols on the oxidative stability of edible plant oils.

Keywords: food analysis, oils, RP-HPLC, tocopherols

Introduction

Vegetable oils are probably the major dietary source of vitamin E (Hammond, 2003), with a variable isomer profile according to the oil identity (Amaral *et al.*, 2005). Tocopherols protect food from oxidation by protecting the stability of oils and fats (Tangolar *et al.*, 2011). Tocopherols are a group of fat soluble antioxidants with a chromanol ring and a hydrophobic side chain and exist in four different congeners (vitamers) called alpha, beta, gamma and delta, which differ in the methylation pattern of the benzopyran ring (Boschin and Arnoldi, 2011; Schwarz *et al.*, 2008). Alpha tocopherol has three methyl groups, beta and gamma forms have two methyl groups and the delta has one methyl group. The biological activities of these compounds are mainly attributed to their radical-chain-breaking antioxidant in membranes and lipoproteins, as well as in foods (Kamal-Eldin and Appelqvist, 1996). The most active form of vitamin E is α -tocopherol which is believed to protect the body against degenerative malfunction, particularly cancer and cardiovascular disease (Hasani *et al.*, 2008; Zingg, 2007). γ -Tocopherol has been reported to be more potent than α -tocopherol in decreasing platelet aggregation, delaying intra-arterial thrombus aggregation and LDL oxidation (Li *et al.*, 1999; Saldeen *et al.*, 1999).

Several methods have been described for the analysis of tocopherols by gas chromatography (GC) and high performance liquid chromatography (HPLC) (Habib *et al.*, 2013; Lee *et al.*, 2012; Slavin and Yu 2012). Analysis of

tocopherols in vegetable oils by HPLC can employ either normal- or reversed phase columns, as well in isocratic as in gradient elution, with fluorescent, electrochemical and UV detection (Jedlicka and Klimes, 2005). The normal phase columns provide separation of all tocopherols, while reversed phase columns (usually C18) are unable to separate the β - and γ - tocopherols (Andres *et al.*, 2011). RP-HPLC is preferred over normal-phase systems due to the reproducibility of retention times, fast equilibration, and robustness of reversed-phase columns over other stationary phases (Cert *et al.*, 2000). When the separation of β - and γ -tocopherols is not the point of analysis, reversed phase columns are preferred (Gliszczynska-Swiglo and Sikorska, 2004). Fluorescence detection permits to get lower detection limits (Gimeno *et al.*, 2000).

In this work we optimized a quick and simple method for routine analysis of tocopherols in vegetable oils by RP-HPLC with fluorescence detection. The oil was diluted in methanol / hexane/ tetrahydrofuran and after being vortexed and centrifuged, an aliquot of the overlay was injected directly into an Alltima C18 column.

Materials and methods

Reagents and standard solutions

Acetonitrile, methanol, tetrahydrofuran and hexane were HPLC grade and were purchased from Merck (Darmstadt, Germany). α -, β -, γ - and δ -Tocopherols standards were obtained from Calbiochem (Merck Biosciences, Darmstadt, Germany). Standard stock solutions of

tocopherols were prepared in ethanol and stored at -20°C in dark bottles for up to a month. The exact concentrations were determined spectrophotometrically (Ryyanen *et al.*, 2004). Working standard solutions of analytes were prepared from these solutions directly before analysis.

High-performance liquid chromatography (HPLC)

Separation by HPLC was carried out using a Shimadzu liquid chromatograph system equipped with two delivery pumps (LC-10 AD), a FL detector (FR-10 AXL), a degasser and a model 7725i manual injector valve with a $20\mu\text{L}$ sample loop. The column was an Alltima RP C-18 ($250\times 4.6\text{ mm}$, $5\mu\text{m}$, Alltech Associates Inc.).

The mobile phase was a mixture of acetonitrile and methanol (50:50, v/v) and eluted at a flow rate of 1.0 mL min^{-1} . The analytical column was kept at 25°C . The fluorescence detector was set at 290 nm excitation wavelength and 325 nm emission wavelength. The total separation time was 10 min. The injection volume was $20\mu\text{L}$. The tocopherols were identified by comparison of the retention times with standards of the α -, β -, γ - and δ -tocopherols.

Tocopherols quantification

An external calibration was performed prior to analyses of edible plant oils, by injecting different volumes (10 and $20\mu\text{L}$) of tocopherol working solutions (0.2 - 5 mg L^{-1}) on column. Standard curves (concentration versus peak area) were calculated by linear regression analysis. Injections in triplicate were made at each concentration for both standards and samples. The calibration curves were constructed using standard solutions of α -, β -, γ - and δ -tocopherol and used for quantification. The total tocopherol content is expressed as milligrams per gram oil.

Sample preparation

Corn, walnut, grape seed, rice, virgin olive, sesame, peanut, sunflower oils were purchased in a local market. The samples were stored in the dark at $(21\pm 2)^{\circ}\text{C}$ until

the measurements were performed. Pure oil samples were weighted (about 50 mg) and diluted 10 times in hexane. Thereafter, $50\mu\text{L}$ of above solution was taken into a screw-capped tube and diluted with 1 mL of a mixture of methanol: hexane: tetrahydrofuran (80:10:10, v/v/v). The sample was vortexed for 5 min and centrifuged for 10 min. at 5000 rpm. After that, the sample was filtered through a $0.45\mu\text{m}$ pore size filter and an aliquot of the clear liquid was directly injected into HPLC column.

Results and discussion

Method assessment and validation

Typical chromatograms obtained for the oils tested are presented below (Fig. 1). A good resolution and acceptable retention times were obtained. Tocopherols were further identified by comparing retention times with those of authentic standards. The retention times of δ -, ($\beta+\gamma$)- and α -tocopherols were about 7.85 min, 8.65 min and 9.72 min., respectively and were determined from their authentic standards which were injected both individually and as a mixture. The analysis time is lower than found in the literature (Breithaupt and Kraut, 2006; Delgado-Zanarreno *et al.*, 2006; Kuhn *et al.*, 2008). Similar results were found by other authors with different protocols for sample preparation and HPLC separation of tocopherols (Chen *et al.*, 2011; Gliszczynska-Swiglo and Sikorska, 2004).

Validation results are summarized in Tab. 1. Calibration curves ($n = 8$ points) were linear between 0.05 - $10\mu\text{g g}^{-1}$. Linear correlation coefficient (r) for all standard curves were not lower than 0.995. The reproducibility of the analysis (sample dilution, injection and chromatographic run) was measured by CV% of α - and ($\beta+\gamma$)-tocopherol (Chen *et al.*, 2011) in three replicates of olive oil and of δ -tocopherol in three replicates of walnut oil. Accuracy was tested by the standard addition procedure (% of recovery). The standard mixture was added to aliquots of grape seed oil at three concentration levels ($n=3$ replicates). The

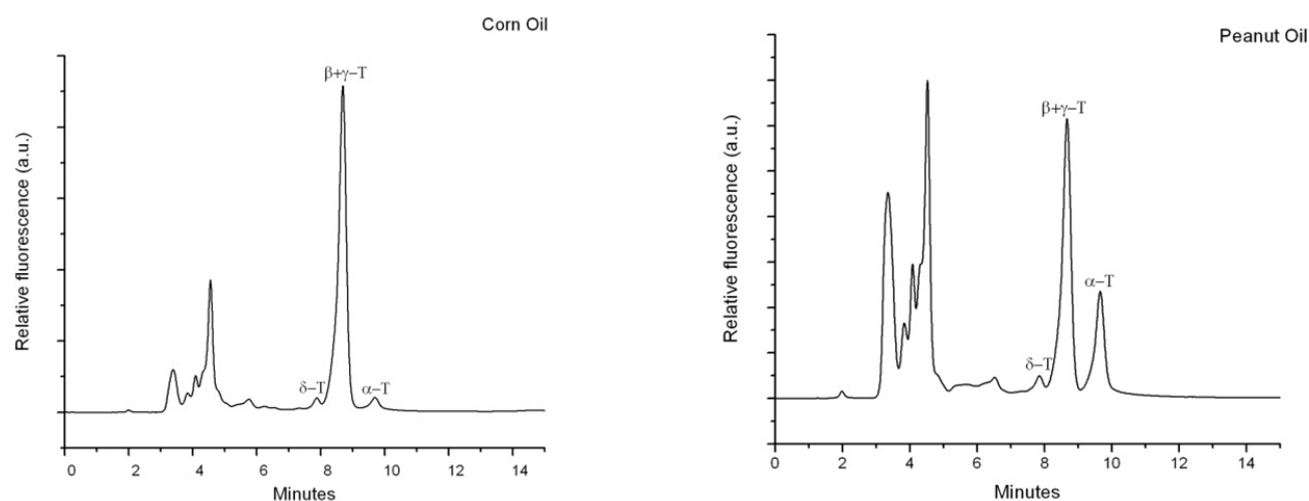


Fig. 1. Typical chromatograms of corn and peanut oils

results demonstrate good recovery for the tocopherols (ranging from 97.5% to 101.0%). The limits of detection (LOD) calculated as the concentration corresponding to three times the standard deviation of the baseline noise were not higher than 9 ng mL⁻¹. The limits of quantification (LOQ) were investigated by sample dilution and were not higher than 28 ng mL⁻¹.

Quantification of tocopherols in edible plant oils

The distribution of individual tocopherols and their total content in assessed oils are reported in Tab. 2. All values are arithmetic mean of at least three separate determinations.

α -Tocopherol dominates in olive, grape seed, peanut and sunflower oils. In corn, walnut and sesame oils ($\beta + \gamma$)-tocopherols dominate. The concentration of δ -tocopherol in all oils is not higher than 0.032 mg g⁻¹. α -Tocopherol is absent in sesame oil and δ -tocopherol in olive oil. The results obtained are generally in agreement with the literature data (Berasategi *et al.*, 2012; Rammell and Hoogenboom, 1985; Sanchez-Perez *et al.*, 2000). Lack of separation of β - and γ - tocopherols in the case of edible oils introduce rather small error in quantification of these isomers (Gliszczynska-Swiglo and Sikorska, 2004). This is because, according to literature (Crawley, 1993; Gregory, 1996) plant oils contain small quantities of β -tocopherol as compared with γ - tocopherol. When necessary, all to-

copherols can be fully separated using normal phase chromatography.

Conclusions

This study presents a simple, fast and precise method for determination of tocopherols in edible oils. The method proposed a new fast sample preparation which avoids quantification errors (dilution of the oil in methanol/hexane/tetrahydrofuran mixture, no saponification procedures, then the sample was vortexed-mixed and centrifuged), direct injection of the overlay in a low-cost RP- HPLC column and is based on a high sensitivity and selectivity of the fluorimetric detector. Tocopherols can preserve their stability by using the protocol proposed for sample preparation. Lack of separation of β - and γ - tocopherols by RP-HPLC did not introduce major error in the determination of latter one because vegetable oils contain small quantities of β - tocopherol as compared to γ - tocopherol. Therefore, the method proposed can be useful for the routine analysis of α -, ($\beta + \gamma$)- and δ - tocopherols in edible vegetable oils and is comparable only to a few published methods (Chen *et al.*, 2011; Gliszczynska-Swiglo and Sikorska, 2004). This method can be used to assess the influence of genetic modification of oil seeds on the distribution of tocopherols or the effect of tocopherols on the oxidative stability of edible plant oils.

Tab. 1. Method validation parameters for determination of tocopherols in edible plant oils

Parameter	α -Tocopherol $t_r = 9.72 \pm 0.1$ min	β -Tocopherol $t_r = 8.65 \pm 0.1$ min	γ -Tocopherol $t_r = 8.65 \pm 0.1$ min	δ -Tocopherol $t_r = 7.85 \pm 0.1$ min
Standard linearity				
Range ($\mu\text{g g}^{-1}$)	0.05-10	0.05-10	0.05-10	0.05-10
R ²	0.9956	0.9967	0.9974	0.9979
Precision (CV%) (N=3)	1.6%	2.9%	2.7%	2.3%
Sensitivity				
LOD (ng g ⁻¹) ^a	9	8	8	8
LOQ (ng g ⁻¹) ^b	28	23	23	23
Accuracy				
Mean recovery (%)	98.2	97.5	98.7	101.0

^a Calculated based on a S/N ratio of three.

^b Calculated as 3x LOD

Tab. 2. Distribution of α -, ($\beta + \gamma$)-, δ - tocopherols and total tocopherol content in assessed oils

Oil sample	α -Tocopherol (mg per 100g)	($\beta + \gamma$)-Tocopherols (mg per 100g)	δ -Tocopherol (mg per 100g)	Total Tocopherols (mg per 100g)
Olive	19.53±0.1	0.91±0.01	-	20.44
Grape seed	12.45±0.01	2.14±0.01	0.71±0.04	15.3
Corn	5.60±0.02	41.12±0.4	1.82±0.04	48.54
Walnut	1.47±0.01	19.13±0.07	3.23±0.04	23.83
Sesame	-	29.37±0.4	0.57±0.07	29.84
Peanut	19.22±0.2	9.32±0.2	0.91±0.03	29.45
Rice	3.31±0.05	3.56±0.03	0.62±0.05	7.49
Sunflower	73.02±0.3	1.98±0.06	0.82±0.04	75.82

Data are calculated from three replicated analysis of each sample \pm SD

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