ANALYTICAL SCIENCES DECEMBER 2005, VOL. 21

Notes

Simultaneous Determination of Tocopherols and Tocotrienols in Hazelnuts by a Normal Phase Liquid Chromatographic Method

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A normal-phase high-performance liquid chromatography (NP-HPLC) method for the determination of tocopherols and tocotrienols in hazelnuts is reported. Three extraction procedures (with and without saponification) were assayed; the best results were obtained with a simple solid–liquid extraction procedure. Chromatographic separation was achieved using an Inertsil 5 SI column using isocratic elution with hexane/1,4-dioxane (95.5:4.5, v/v) at a flow rate of 0.7 mL/min. The effluent was monitored by a series arrangement of a diode-array followed by a fluorescence detector. All compounds were separated in a short period of time (17 min). The method proved to be rapid, sensitive, reproducible and accurate, allowing the simultaneous determination of all vitamin E homologues.

(Received December 8, 2004; Accepted May 23, 2005)

Vitamin E is a term frequently used to designate a family of related compounds, namely tocopherols and tocotrienols, which share a common structure with a chromanol head and an isoprenic side chain.[1–3] Tocotrienols are distinguished from tocopherols by the presence of three unsaturations in the isoprenic side chain. They both have four naturally occurring forms (α-, β-, γ- and δ-) that differ in the number and position of methyl groups attached to the chromanol head.[1,3,4]

The biological activities of these compounds are mainly attributed to their antioxidant activity in inhibiting lipid peroxidation in biological membranes.[4–6] In the past, α-tocopherol was the most studied vitamer; it was reported to exhibit the highest biological activity. Several methods were developed exclusively for the determination of this compound. However, many studies focusing on the health effects of the other vitamin E isoforms have been recently published.[5–8] Due to the structural similarity of these compounds and because of the large variation in their biological activities, it is of major interest to develop analytical techniques that allow the quantification of the individual vitamers, instead of the evaluation of the global content of vitamin E or the quantification of the single isomer α-tocopherol. Besides, the generated profile is more or less characteristic of each food product, and consequently, may be useful in the assessment of the identity and quality of vegetable oils,[9,10] a food matrix are usually present.

Vegetable oils are probably the main dietary source of vitamin E, with nuts, cereals, green vegetables, and fruits being other valuable sources.[2] In what concerns hazelnuts, bibliographic data generally refer only to α-tocopherol content, with the exception of the data reported from Alasalvar *et al.*,[11] who quantified the four tocopherol isomers, and the data from Delgado-Zamarreño *et al.*[12] who quantified α and (β + γ) tocopherols. As far as we know, there are few data concerning the simultaneous analysis of tocopherols and tocotrienols in foodstuffs,[4,13–15] and no such reports on hazelnuts. Owing to the nuts' richness in vitamin E, and since hazelnuts are widely used as raw material in a large range of products,[16] the objective of this work was to optimize and validate a simple and fast NP-HPLC analytical method for the simultaneous quantification of tocopherols and tocotrienols in hazelnuts.

Experimental

Standards and reagents

Tocopherols and tocotrienols (both α, β, γ and δ) were purchased from Calbiochem (La Jolla, CA, USA). 2-Methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol (tocol) (Matreya Inc., PA, USA) was used as internal standard (IS). Butylated hydroxytoluene (BHT) was obtained from Aldrich (Madrid, Spain), hexane was of HPLC grade from Merck (Darmstad, Germany) and 1,4-dioxane was from Fluka (Madrid, Spain). All other reagents were of analytical grade.

Standards' preparation

All solutions were prepared in a dark room with subdued red light. Individual stock solutions (~5 mg/mL) of the eight isomers were prepared in hexane, flushed with nitrogen and stored protected from light, at –20°C. A stock standard mixture, with the different isomers in relative proportions similar to those presented by the samples analyzed, was prepared in hexane with the following final concentrations: α-tocopherol (150 μg/mL), β-tocopherol (50 μg/mL), γ-tocopherol (30 μg/mL), δ-tocopherol (4 μg/mL), α-tocotrienol (30 μg/mL), β-tocotrienol (4 μg/mL), γ-tocotrienol (10 μg/mL) and δ-tocotrienol (50 μg/mL). Working standard mixtures with

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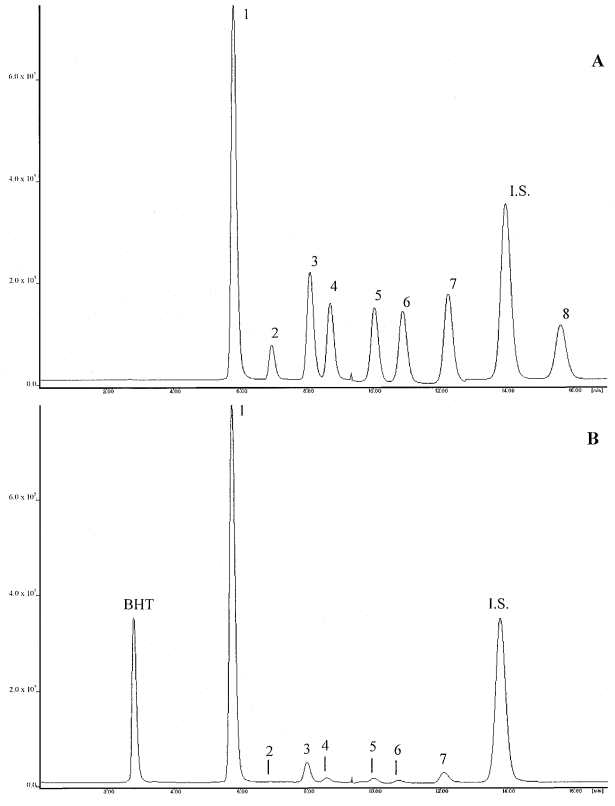

Fig. 1 HPLC fluorescence chromatogram of a working standard mixture (A) and of a hazelnut sample prepared using extraction method II (B). Peaks: I.S., internal standard (tocol); 1, α-tocopherol; 2, α-tocotrienol; 3, β-tocopherol; 4, γ-tocopherol; 5, β-tocotrienol; 6, γ-tocotrienol; 7, δ-tocopherol; 8, δ-tocotrienol; BHT, butylated hydroxytoluene. HPLC conditions as described in Experimental.

concentrations in the expected ranges were prepared from this stock standard solution. Figure 1 A shows a chromatogram of a standards' mixture. A stock solution of tocol at 10 mg/mL in hexane was kept at –4°C, protected from light, and diluted to working solutions (500 µg/mL) as necessary. BHT was prepared in hexane at a concentration of 10 mg/mL.

Samples

Six different cultivars (*cvs*. Butler, Campanica, Cosford, Couplat, Ennis and Lansing) were analyzed. *Cv*. Butler was used during method development and validation procedures. All cultivars were collected in Vila Real, in the North-eastern region of Portugal, during the crop year of 2003. They were kept frozen until analyses were performed. Immediately before the extraction procedure, each sample was manually cracked, shelled, and then chopped in a coffee mill to pass through a sieve of 0.7 mm.

Extraction procedures

To establish the best conditions for the determination of tocopherols and tocotrienols in hazelnuts, we compared different extractions (Table 1). All included the use of 150 µL of IS solution, and all were assayed with and without antioxidant (100 µL of BHT solution). BHT and tocol were added to the sample prior to the extraction procedures.

Method I. Chopped hazelnuts were extracted on a Soxhlet apparatus (Büchi, Switzerland) with light petroleum ether (b.p. 40 – 60°C) during 1.5 h, and the solvent that remained was removed under a stream of nitrogen. An accurately weighted sample of the obtained oil was diluted in hexane, filtered through a 0.22 µm disposable LC filter disk and then directly injected in the HPLC system.

Method II. A sample of chopped hazelnuts (~300 mg) was accurately weighted in glass screw cap tubes (Supelco, Bellefonte, PA, USA) and homogenized with 2 mL of ethanol by vortex mixing (1 min). Subsequently, 4 mL of hexane were added and again vortex mixed for 1 min. After that, 2 mL of saturated NaCl aqueous solution were added and the mixture was homogenized (1 min), centrifuged (2 min, 5000*g*) and the clear upper layer was carefully transferred to another glass screw cap tube. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, at room temperature, on a Reacti-Therm module (Pierce, Rockford, IL, USA), transferred to microcentrifuge tubes with 1.5 mL of hexane and, finally, dehydrated with anhydrous sodium sulfate. The extract was centrifuged (10000*g*, 20 s), transferred into a dark injection vial and analyzed by HPLC.

Method III. This method involved an alkaline digestion prior to extraction of the unsaponifiable compounds with hexane. Ethanol (2.5 mL), water (2.5 mL) and 10 M NaOH (0.5 mL) were added to each sample of chopped hazelnuts accurately weighted in a glass screw cap tube and homogenized for 1 min, by vortex mixing. After that, the tubes were flushed with nitrogen and closed. Saponification was performed at 60°C during 20 min on a Reacti-Therm module. After the addition of water (2.5 mL) and hexane (5.0 mL) and vortex mixed for 1 min the tubes were centrifuged (5 min, 5000*g*) and the clear upper layer was carefully transferred to another glass screw cap tube. The sample was re-extracted twice with hexane and processed as described in method II.

Table 1 Comparison of the three extraction methods (relative %)

Extraction method	α-Toc	β-Toc	γ-Toc	δ-Toc	α-TTR	β-TTR	γ-TTR
I	76	74	100	100	100	60	54
II	100	100	88	99	58	100	73
III	81	92	95	66	39	52	100

I, Soxtec extraction; II, solid-liquid extraction; III, saponification. Toc, tocopherol; TTR, tocotrienol. Relative % assuming 100% for the highest areas obtained.

HPLC analysis

The HPLC equipment consisted of an integrated system with a PU-980 pump, an AS-950 auto-sampler, an MD-910 multiwavelength diode array detector (DAD) connected in series with an FP-920 fluorescence detector (Jasco, Japan) programmed for excitation at 290 nm and emission at 330 nm. Data were analyzed using Borwin-PDA Controller Software (JMBS, France). The chromatographic separation was achieved with an Inertsil 5 SI (250 × 3 mm) normal-phase column from Varian (Middelburg, Netherlands) operating at room temperature. The mobile phase used was a mixture of hexane and dioxane (95.5:4.5, v/v) at a flow rate of 0.7 mL/min, and the injection volume was 10 µL. The compounds were identified by chromatographic comparisons with authentic standards and by their UV spectra. Quantification was based on the fluorescence signal response, using the internal standard method.

Results and Discussion

Optimization of chromatographic conditions

Column and mobile phase. Although there are several reports

Table 2 Analytical characteristics of the reported method

Compound	R_t (retention time)		Correlation coefficient (r^2)	Linearity range/μg mL^{-1}	Dectector gain	Limit	
	min	CV, % (n = 6)				LOD/μg mL^{-1}	LOQ/μg g^{-1}
α-Tocopherol	5.69	0.89	0.9995	1.00 - 40.0	10	0.30	0.83
α-Tocotrienol	6.79	1.10	0.9992	0.30 - 15.0	10	0.22	0.72
β-Tocopherol	7.96	0.37	0.9997	0.25 - 25.0	10	0.16	0.47
γ-Tocopherol	8.56	0.40	0.9997	0.15 - 15.0	10	0.11	0.34
β-Tocotrienol	9.84	0.52	0.9995	0.04 - 2.0	100	0.01	0.02
γ-Tocotrienol	10.67	0.56	0.9997	0.10 - 5.0	100	0.03	0.08
δ-Tocopherol	12.02	0.58	0.9996	0.04 - 2.0	100	0.03	0.07
I.S. (tocol)	13.71	0.50	—	—	10	—	—
δ-Tocotrienol	15.28	0.79	0.9999	0.50 - 25.0	10	0.22	0.65

mentioning the determination of tocopherols by RP-HPLC,[3,12,17-20] NP-HPLC is generally preferred[4,11,13-15,21-24] because RP systems do not completely resolve β and γ isomers.[2,3,23-25] Besides, NP-HPLC has the advantage of allowing the use of organic solvents, thus achieving higher lipid solubility and higher loading capacity.[3,23] Although some researchers claim that RP-HPLC presents better retention time reproducibility, the coefficients of variation achieved in the present work were low (less than 1.1% for all the standard compounds) (Table 2).

Several columns and solvents have been used with NP-HPLC. Based on the work of Kamal-Eldin *et al*.,[23] who compared several NP-columns and mobile phases performances in the separation of the 8 vitamin E vitamers, we adopted the Inertsil 5 SI NP-column and hexane/1,4-dioxane as mobile phase. The organic polar modifier (1,4-dioxane) was tested in different proportions (2.5, 4, 4.5, 5 and 7.5%), with better results achieved using 4.5%, allowing good separation of all isomers in a short period of time.

Hewavitharana *et al*.[15,26] reported some problems due to irreversible adsorption of tocopherols and tocotrienols using another kind of silica column and a similar mobile phase. With the chosen column and mobile phase, these problems did not happen.

Internal standard. In order to improve the accuracy, precision and robustness, we did the quantification by an internal standard method. α-Tocopherol acetate and tocol are most frequently referred to as suitable for this purpose.[3] Although both materials exhibited different retention times when compared to the compounds under analysis, α-tocopherol acetate presented some interfering impurities that co-eluted with compounds in the samples, and so tocol was chosen (Fig. 1A).

Detector settings. The fluorescence detector was selected for quantification purposes since it provided a higher sensitivity than the DAD detector. Since all vitamers, with the exception of α-tocopherol, are expected to occur in low quantities, different fluorescence gains were tested, in order to allow the detection and quantification of all the eight isomers within the same chromatographic run. The best conditions achieved were: 0 min, gain 10; 9.4 min, gain 100; 12.7 min, gain 10.

Extraction procedure

Special precautions were taken when handling the compounds under study, due to their sensitivity to light, heat and oxygen.[1-3] All operations were carried out in a dark room with subdued red light and, as far as possible, during all the extraction procedures the samples were kept on ice. In order to overcome oxidation of the vitamins, several authors recommend the use of an antioxidant. BHT is often described for this purpose and under the conditions of this method it gives no interferences with any eluting compound. Three extraction methods were assayed with and without BHT, each one tested in duplicate. The areas obtained using BHT were always identical or higher than the ones without BHT, and are shown in Table 1. The presence of δ-tocotrienol was not confirmed with any method allowing us to think that this compound is not present in hazelnuts.

Although with other matrices some researchers reported that they could obtain their best results using saponification,[3,4] the results obtained with method III (with saponification) were in general lower (with the exception of γ-tocotrienol) than those obtained with method II (simplified solid-liquid extraction), probably due to oxidation and partial degradation losses. Besides, saponification is more time-consuming and laborious. When compared to method I (Soxhlet extraction), method II presents much lower values of α-tocotrienol but considerable higher values for α-tocopherol, β-tocopherol and β-tocotrienol. Considering that α-tocopherol is reported to be the major isomer present in hazelnuts and that in general method II seemed to be the one that gives best results, we have proceeded our studies using this extraction method. Figure 1B shows a chromatogram of a hazelnut sample obtained with extraction method II.

Linearity and sensitivity of the HPLC analysis

The linearity and the limits of detection and quantification were determined and are presented in Table 2. For each compound, a 6-level calibration curve was constructed using the peak-area ratio between the vitamin E isomer and tocol *versus* concentration of the standard (μg/mL). The average of triplicate determinations for each level was used. The correlation coefficients were always higher than 0.999 for all the compounds (Table 2).

The limits of detection (LOD), calculated as the concentration corresponding to three times the standard deviation of the baseline noise, ranged from 10 ng/mL to 0.3 μg/mL. The limits of quantification (LOQ) were investigated by sample dilution and ranged from 0.02 μg/g to 0.83 μg/g.

Method validation

In order to evaluate the instrumental precision, we injected the same sample extract six times. The chromatographic method proved to be precise (CV% between 0.8% and 3.5%). Repeatability was evaluated by applying the whole extraction procedure 6 times to the same sample. All the obtained values were low (CV% ranging from 3.5% to 6.8%).

The accuracy of the method was evaluated by the standard addition procedure (% of recovery) with three addition levels (15%, 30% and 60% of the expected values, each one in duplicate). The standard mixture was added to the sample, and all the extraction procedures were carried out. The results demonstrate good recovery for the compounds under study

(ranging from 93.3% to 104.8%). Results are shown in Table 3.

Hazelnut samples

A total of six hazelnut samples were evaluated (Table 4). In all samples, α-tocopherol was the major compound, ranging from 110.2 to 177.5 μg/g. The four tocopherols (α, β, γ and α) and β-tocotrienol were present in all samples. α-and γ-tocotrienols were detected in minor amounts only in some of the studied samples.

Conclusion

The use of the fluorescence detector provided high sensitivity and selectivity, while the use of the DAD detector allowed the confirmation of the compounds' identity by its spectrum analysis. The proposed method allows the simultaneous determination of all tocopherol and tocotrienol vitamers, which is of great importance in the accurate determinations of vitamin E activities, since they are known to have different biological potencies. The results obtained demonstrate that the method is sensitive, precise, accurate and fast, being suitable for routine determinations. The results obtained in the analysis of hazelnut samples point to the existence of apparent differences in what concerns vitamin E composition among hazelnut cultivars. Nevertheless, more studies should be made in order to evaluate if the vitamin E profile can be useful to discriminate different cultivars, years of production and/or geographical origins.

Acknowledgements

J. S. Amaral is grateful to Programa para o Desenvolvimento Educativo para Portugal (PRODEP III) for financial support.

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Table 3 Method validation parameters

Compound	Precision CV, % (n = 6)	Repeatability CV, % (n = 6)	Accuracy (Recovery, %)
α-Tocopherol	0.8	4.6	104.8
α-Tocotrienol	3.5	5.4	93.3
β-Tocopherol	0.9	3.5	98.0
γ-Tocopherol	1.3	4.2	95.9
β-Tocotrienol	3.5	6.8	97.3
γ-Tocotrienol	2.0	5.5	93.8
δ-Tocopherol	2.5	5.1	97.4
δ-Tocotrienol	—	—	97.7

Table 4 Tocopherol and tocotrienol content of hazelnut samples (μg g^{-1})

Hazelnut cultivar	α-Toc	α-TTR	β-Toc	γ-Toc	β-TTR	γ-TTR	δ-Toc
Butler	156.29 ± 7.26	0.95 ± 0.05	5.53 ± 0.20	2.11 ± 0.09	0.23 ± 0.02	0.33 ± 0.02	0.40 ± 0.02
Campanica	165.50 ± 0.37	nd	4.82 ± 0.04	3.75 ± 0.03	0.35 ± 0.01	0.62 ± 0.04	0.40 ± 0.01
Cosford	177.47 ± 0.76	nd	8.97 ± 0.01	5.49 ± 0.01	0.17 ± 0.00	0.22 ± 0.01	1.32 ± 0.02
Couplat	158.79 ± 0.68	1.12 ± 0.03	7.60 ± 0.04	4.31 ± 0.06	0.09 ± 0.00	0.48 ± 0.01	1.09 ± 0.02
Ennis	126.78 ± 0.42	1.11 ± 0.04	7.29 ± 0.05	1.04 ± 0.02	0.29 ± 0.01	nd	0.25 ± 0.01
Lansing	110.15 ± 1.01	1.34 ± 0.03	3.29 ± 0.01	1.72 ± 0.02	0.18 ± 0.01	nd	0.23 ± 0.01

Mean values obtained for three determinations (mean ± standard deviation). nd, not detected; Toc, tocopherol; TTR, tocotrienol.