DOI:10.1021/jf900435y

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Vitamin E Profile as a Reliable Authenticity Discrimination Factor between Chestnut (*Castanea sativa* Mill.) Cultivars

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In this study, the profile of tocopherols and tocotrienols in chestnut (*Castanea sativa* Mill.) kernel oil was evaluated. Four Portuguese chestnut varieties were selected: Aveleira, Boaventura, Judia, and Longal. The vitamin E determination had already been applied to similar matrices, but, to the authors' knowledge, it is the first time that chestnut kernel oil has been evaluated. The prevalent vitamer was γ-tocopherol, often present in trace amounts in other natural products. Due to the high commercial value of chestnut, a statistical analysis of the obtained results was also conducted to define the tocopherol and tocotrienol profile as a reliable indicator of a specific chestnut variety. To achieve this objective, an analysis of variance was performed to evaluate the accuracy of the method as well as the uniformity of results for each variety. A discriminant analysis was also carried out revealing quite satisfactory results. Four varieties were clustered in four individual groups through the definition of two discriminant analysis dimensions.

KEYWORDS: Chestnut kernel oil; HPLC; vitamin E; discriminant analysis

INTRODUCTION

According to the FAO, chestnut worldwide production is estimated at 1.1 million tons, distributed by surface with nearly 340000 ha. China is the major producer with 800000 tons per year. Europe is responsible for about 12% of worldwide production, with relevance for Italy and Portugal, corresponding to 4 and 3%, respectively. The Trás-os-Montes region represents 75.8% of Portuguese chestnut crops and 84.9% of chestnut orchards area (23338 ha). In 1994 three protected designations of origin (PDO), Castanha da Terra Fria (all of the cultivars in this study belong to this PDO), Castanha dos Soutos da Lapa, and Castanha da Padrela, were created (*1*). The best development conditions are found at altitudes above 500 m and low temperatures, as in the Trás-os-Montes region (northeastern Portugal) (*2*).

Previous studies on the chemical composition of chestnut kernels focused on starch, fiber, fat and fatty acid, protein and amino acids, ash, and mineral contents (*3*−*7*). There are some studies on the phenolic composition of chestnut flowers, leaves, skins, and kernels (*8*, *9*). However, the vitamin E composition present in the lipidic fraction had not yet been analyzed. Vitamin E is a term frequently used to designate a family of related compounds, namely, tocopherols and tocotrienols (*10*), which are important lipophilic antioxidants with essential effects in living systems against aging (*11*), strengthening the immune system and reducing the risk of cancer (*12*), reducing the viral load in HIV-infected patients (*13*), and aiding in the treatment of Parkinson's syndrome (*14*). There is also several evidence of its antioxidant activity (*15*, *16*). Tocopherols may also contribute to minimize the adverse effects of inflammatory diseases such as rheumatoid arthritis or hepatitis, which are among the leading causes of death and disability in the world. This becomes more important considering the contribution of chronic inflammation to the development of degenerative diseases, including cancer (*17*), cardiovascular diseases (*18*), and neurodegenerative disorders (*19*). Because of the central roles of prostaglandin E2 (PGE2) and leukotriene B4 (LTB4), the inducible form of cyclooxygenase (COX-2) and 5-lipoxygenase have been recognized as key targets for drug therapy in inflammation-associated diseases. In particular, COX-2 inhibitors, which are classified as nonsteroidal anti-inflammatory drugs (NSAIDs), have proved to be effective in attenuating inflammatory response and are beneficial for certain inflammation-associated diseases (*20*). It had recently been found that γ-tocopherol (γ-T) and its physiological metabolite, 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC), inhibit COX-2-catalyzed formation of PGE2 (*20*). This indicates that γ-T and its metabolite may have anti-inflammatory properties similar to those of NSAIDs. In contrast, α-tocopherol (α-T), the predominant form of vitamin E in the tissues and most supplements, is much less effective in this regard (*21*).

Aside from its beneficial health effects, vitamin E could also work as a reliable authenticity indicator, allowing the identification of chestnut varieties according to their tocopherol and tocotrienol profile. This is an important feature, with chestnuts gaining economical importance each year.

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 Published on Web 06/02/2009

Table 1. Chromatographic Characteristics of the Reported Method

	t_R (retention time)				limits	
compound	min	CV (%) (n = 4)	correlation coefficient (R^2)	linearity range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
α-tocopherol	5.24	0.09	0.9943	0.065−2.619	0.007	0.020
α-tocotrienol	6.44	0.10	0.9902	0.064−2.565	0.006	0.018
β-tocopherol	7.96	0.12	0.9990	0.067−2.676	0.013	0.039
γ-tocopherol	8.73	0.14	0.9929	0.677−13.538	0.005	0.016
β-tocotrienol	10.12	0.12	0.9976	0.079−3.165	0.023	0.068
γ-tocotrienol	11.23	0.11	0.9988	0.081−3.228	0.017	0.051
δ-tocopherol	13.17	0.12	0.9988	0.063−12.602	0.010	0.030
I.S. (tocol)	15.04	0.12				
δ-tocotrienol	17.19	0.13	1.0000	0.082−3.283	0.018	0.054

MATERIALS AND METHODS

Standards and Reagents. Tocopherols and tocotrienols (α, β, γ, and δ) were purchased from Calbiochem (La Jolla, San Diego, CA). 2-Methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol (tocol) (Matreya Inc., Pleasant Gap, PA) was used as internal standard (IS). Butylated hydroxytoluene (BHT) was obtained from Aldrich (Madrid, Spain), hexane was of HPLC grade from Merck (Darmstadt, 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. Working standard mixtures with concentrations in the expected ranges were prepared from this standard stock solution. A stock solution of tocol at 10 mg/mL in hexane was kept at −20 °C, protected from light, and diluted to work solutions (500 μg/mL) as necessary. BHT was prepared in hexane at a concentration of 1%.

Samples and Sample Preparation. Four of the most important Portuguese cultivars were chosen: Longal, Judia, Aveleira, and Boaventura, all of them belonging to Castanha da Terra Fria PDO. Longal was used during method development and validation procedures. Five trees were selected in each orchard, collecting 50 fruits from each tree, according with the tree phenological cycle (chestnuts from Aveleira cultivar were collected in October; chestnuts from the other cultivars were collected in November) during the crop year of 2006. Orchards are located in Vinhais (Aveleira, 41° 49′ N, 7° 01′ W; Boaventura, 41° 51′ N, 7° 01′ W; Judia, 41° 50′ N, 7° 01′ W; Longal, 41° 50 ′N, 7° 00′ W), Trás-os-Montes, in northeastern Portugal.

Chestnut fruits were kept at −20 °C and protected from light during 3 months. Immediately before the extraction procedure, each sample was manually peeled (inner and outer skins), incubated at 50 °C until constant weight (≈ 24 h), and then chopped to obtain a fine dried powder (20 mesh).

Extraction Procedure. Crude lipidic fraction was obtained from finely chopped chestnuts (≈50 g with anhydrous sodium sulfate) extracted with light petroleum ether (bp 40−60 °C) during 1.5 h (for the determination of total fat content the extraction time was 24 h) in a Universal extraction system B-811 (Büchi, Switzerland); the residual solvent was removed by flushing with nitrogen. An accurately weighed oil sample plus the internal standard was diluted in hexane, ultracentrifuged at 9000*g*, and injected in the HPLC system. The extraction procedure was carefully conducted due to the high sensitivity of the studied compounds to light, heat, and oxygen (*22*). Whenever it was possible, samples were kept on ice and the extraction procedure was performed with low light conditions. The oxidation of vitamins was partially overcome through the addition of BHT, which proved to be innocuous for all of the eluting compounds with no chromatographic interferences.

HPLC Analysis. The HPLC equipment consisted of an integrated system with a PU-980 pump, an AS-950 autosampler, and an MD-910 multiwavelenght 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, with a gain of 10. 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, The Netherlands) operating at room temperature. The mobile phase used was a mixture of hexane and dioxane (97:3 v/v) at a flow rate of 0.7 mL/min, and the injection volume was 20 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. The concentrations used for each vitamer (**Table 1**) were selected according to preliminary chromatograms obtained with the lipid fraction of Longal chestnut.

Statistical Analysis. Fifty fruits were randomly collected from each of the five trees of each cultivar. The assays were carried out in duplicate, obtaining 10 results per variety. The results are expressed as mean values ± standard deviation (SD). The inter- (**Figure 1**) and intravarietal (**Table 2**) influences on the tocopherol composition were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test with α = 0.05, coupled with Welch's statistic.

Discriminant function analysis was done following a stepwise method, aiming to determine which variables discriminate between the four naturally occurring groups. The values of F to enter and F to remove are the guidelines of the stepwise procedure. The F value for a variable indicates its statistical significance in the discrimination between groups. Discriminant analysis defines an optimal combination of varieties in a way that the first function furnishes the most general discrimination between groups, the second provides the second most, and so on (*23*).

These treatments were carried out using SPSS v. 16.0 program.

RESULTS AND DISCUSSION

The extraction procedure was carefully conducted due to the high sensitivity of the studied compounds to light, heat, and oxygen (*22*). Whenever possible, samples were kept on ice and the extraction procedure was performed with low light conditions. The problem of the oxidation of vitamins was overcome through the addition of BHT, which proved to be innocuous for all of the eluting compounds.

The internal standard method was followed to enhance the accuracy of the assay. According to some previous results, tocol was selected as I.S. because it minimizes the interfering impurities presented by alternative compounds such as α-tocopherol acetate.

The selected chromatographic method (NP-HPLC) is generally chosen due to its capacity of resolution of all vitamin E vitamers (*10*). Besides, NP-HPLC has the advantage of allowing the use of organic solvents, thus achieving higher lipid solubility and higher loading capacity. Several columns and solvents have been used with NP-HPLC. On the basis of the work of Kamal-Eldin et al. (*24*), we adopted the Inertsil 5 SI NP-column along with hexane/1,4-dioxane (97:3) as mobile phase. The organic polar modifier (1,4-dioxane) was tested in different proportions, with better results using 3%, allowing good separation of all isomers in a short period of time. With the chosen column and mobile phase, there were no problems of irreversible adsorption of tocopherols and tocotrienols.

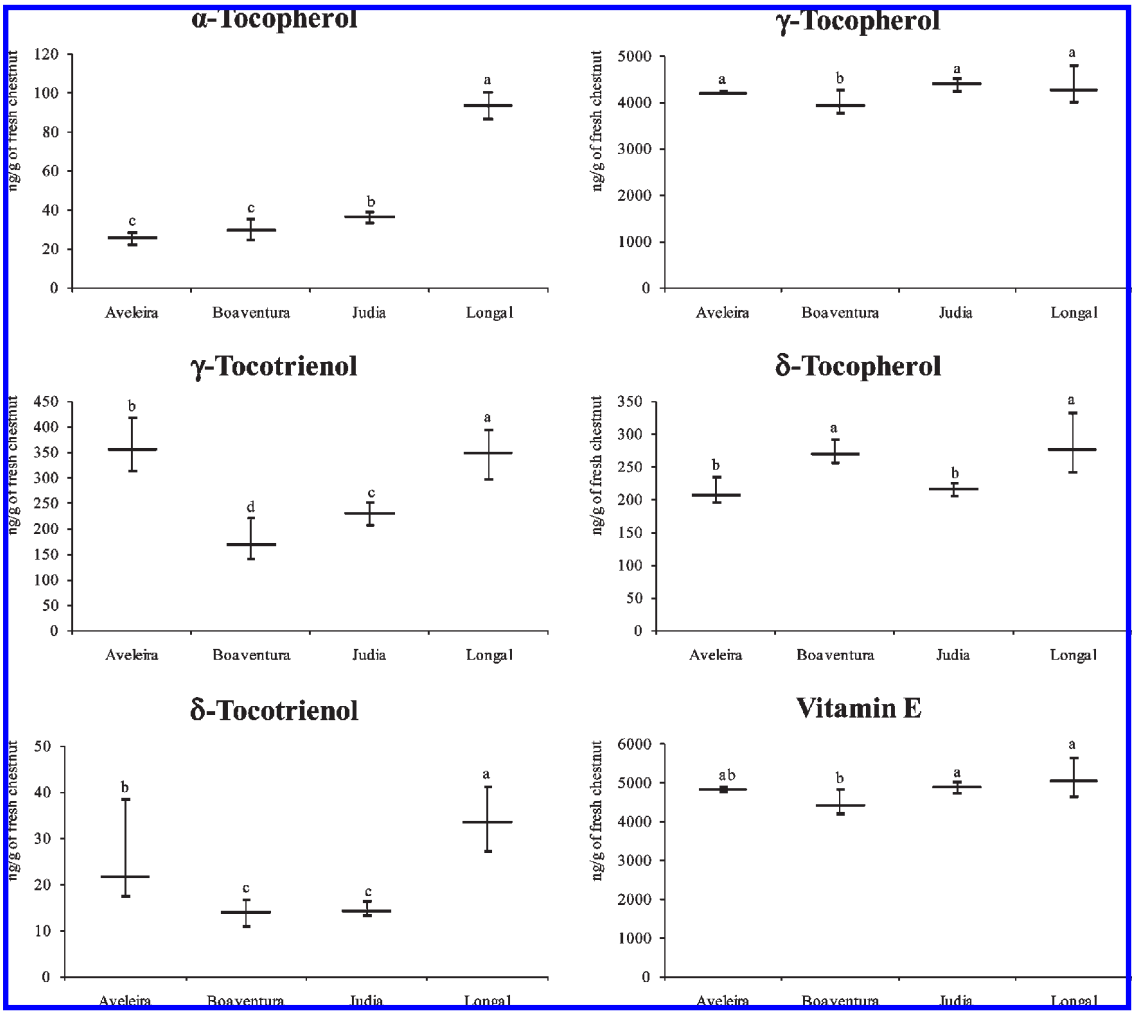

Figure 1. Intervarietal differences in vitamin E composition. Different letters indicate significant differences ($p < 0.05$). The vertical lines represent the maximal and minimal values.

Table 1 presents the linearity and the limits of detection (LOD) and quantification (LOQ). A four-level calibration curve was made for each compound using the peak/area ratio between the vitamer and tocol versus concentration of the standard (μg/mL). The LOD and LOQ were calculated.

The precision of the extraction method was determined by repeatability (intraday) and intermediate precision (interday). Intraday precision was evaluated by assaying a sample extracted six times during the same day (coefficients of variation ranged between 2.1 and 3.2%, for all vitamers). The interday precision was performed by analyzing the same sample in six different and subsequent days (coefficients of variation found varied between 4.5 and 5.5%, for all vitamers).

In the absence of a reference matrix, the method accuracy was evaluated by the standard addition procedure (percentage of recovery). The standards were added to the samples in three concentration levels before the extraction. The method showed good recovery values, with mean percentages ranging between 96 ± 2 and 100 ± 3%.

The crude fat content, obtained after a Soxhlet extraction conducted during 24 h, was also very similar for the studied cultivars (fresh weight contents: Aveleira, 0.84 ± 0.06%; Boaventura, 0.78 ± 0.07%; Judia, 0.81 ± 0.01%; Longal, 0.79 ± 0.07%). All of the cultivars reveal similar moisture contents (Aveleira, 52.14 ± 1.31%; Boaventura, 54.65 ± 1.04%; Judia, 53.26 ± 1.79%; Longal, 51.88 ± 1.05%).

Chromatographic analysis revealed the presence of five of the eight possible vitamin E isoforms; α-tocotrienol, β-tocopherol, and β-tocotrienol were not detected (**Table 2**). γ-Tocopherol was the major compound in a very high amount, and the vitamin E profiles were similar for all of the assayed varieties: γ-tocopherol $\gg$ γ-tocotrienol > δ-tocopherol > α-tocopherol > δ-tocotrienol for Aveleira, Judia, and Longal and γ-tocopherol $\gg$ δ-tocopherol > γ-tocotrienol > α-tocopherol > δ-tocotrienol for Boaventura.

Figure 1 shows the intervarietal differences in vitamin E composition. The Longal variety proved to have higher amounts of the detected vitamers. Considering total vitamin E, Longal and Judia showed statistical dominance when compared with the other varieties.

From a qualitative point of view, chestnuts present a vitamin E profile quite dissimilar from other related foods such as almond or hazelnut, but very related to others such as walnut, pistachios, or pecans (*25*, *26*).

Table 3 shows the results from applying the algorithm for selecting variables according to a specific vitamer. In **Table 4** the correlations between discriminating variables and standardized canonical discriminant functions are presented. The discriminant analysis (DA) defined three dimensions, but once the first two explained 97.8% of the observed variance, only those were considered in the canonical analysis (**Figure 2**). The first DA dimension separates mainly Longal from the other varieties (means of the canonical variance: Longal, 17.928; Aveleira, −5.061; Boaventura, −7.629; and Judia, −5.238), on the basis

Table 2. Tocopherols Content (Nanograms per Gram of Fresh Fruit) in Chestnut Samples

sample[a]	α-tocopherol	γ-tocopherol	γ-tocotrienol	δ-tocopherol	δ-tocotrienol
A1	26 ± 1	4245 ± 10	378 ± 27	212 ± 3	18 ± 3
A2	27 ± 0	4221 ± 56	325 ± 4	195 ± 0	18 ± 0
A3	22 ± 0	4173 ± 128	418 ± 3	235 ± 1	20 ± 1
A4	28 ± 1	4196 ± 73	314 ± 3	197 ± 0	17 ± 1
A5	25 ± 0	4219 ± 1	348 ± 5	198 ± 2	18 ± 0
B1	29 ± 0	3843 ± 71	165 ± 3	292 ± 0	14 ± 0
B2	32 ± 0	4261 ± 89	220 ± 3	280 ± 1	16 ± 1
B3	35 ± 0	3770 ± 9	154 ± 2	263 ± 3	15 ± 1
B4	29 ± 0	4053 ± 60	167 ± 1	262 ± 1	14 ± 0
B5	25 ± 1	3771 ± 96	141 ± 6	257 ± 2	11 ± 0
J1	37 ± 2	4506 ± 65	231 ± 1	219 ± 1	14 ± 0
J2	33 ± 0	4246 ± 55	235 ± 3	204 ± 1	14 ± 1
J3	39 ± 1	4466 ± 48	228 ± 2	224 ± 2	13 ± 0
J4	39 ± 1	4378 ± 74	206 ± 0	219 ± 0	16 ± 1
J5	35 ± 1	4427 ± 13	251 ± 4	215 ± 0	14 ± 0
L1	97 ± 1	4307 ± 70	373 ± 5	266 ± 1	32 ± 0
L2	100 ± 1	4784 ± 59	393 ± 2	332 ± 5	38 ± 1
L3	91 ± 1	3990 ± 28	297 ± 2	242 ± 0	27 ± 1
L4	94 ± 0	4281 ± 48	351 ± 7	291 ± 3	41 ± 0
L5	86 ± 0	4080 ± 11	331 ± 9	256 ± 3	29 ± 1
	46	4210	276	243	71
σ	28	251	86	38	5
max	100	4784	420	332	83
min	22	3770	137	195	64

[a] A, Aveleira; B, Boaventura; J, Judia; L, Longal.

Table 3. Four Most Important Vitamers for Discrimination between Variety Groups

	Wilks' lambda	*F*-remove (2.71)	*p* level	tolerance	1-tolerance (R^2)
α-tocopherol	0.0038	407.6477	0.0000	0.6514	0.3486
γ-tocopherol	0.0004	32.5554	0.0000	0.3886	0.6114
γ-tocotrienol	0.0012	120.8698	0.0000	0.6099	0.3901
δ-tocopherol	0.0004	34.8954	0.0000	0.4713	0.5287

Table 4. Correlations between Discriminating Variables and Standardized Canonical Discriminant Functions[a]

	function		
	1	2	3
α-tocopherol	0.746[b]	−0.253	−0.443
γ-tocotrienol	0.248	0.594[b]	0.370
δ-tocopherol	0.083	−0.293[b]	0.262
δ-tocotrienol[c]	0.097	−0.100	0.488[b]
γ-tocopherol	0.030	0.127	−0.405[b]

[a] Variables are ordered by absolute size of correlation within function. [b] Largest absolute correlation between each variable and any discriminant function. [c] This variable was not used in the analysis.

of the relatively high amounts of α-tocopherol, γ-tocotrienol, and δ-tocotrienol. The second DA dimension reveals the separation of the other three varieties (means of the canonical variance: Aveleira, 5.857; Boaventura, −5.876; and Judia, 0.571), on the basis of the differences of relative levels of γ-tocotrienol, δ-tocopherol, and δ-tocotrienol.

The fact that all of the samples cluster together in the respective groups signifies that there are not great differences among duplicates or between the fruits collected from each tree. This fact is also reflected in the small residual errors revealed by the analysis of variance (**Table 2**).

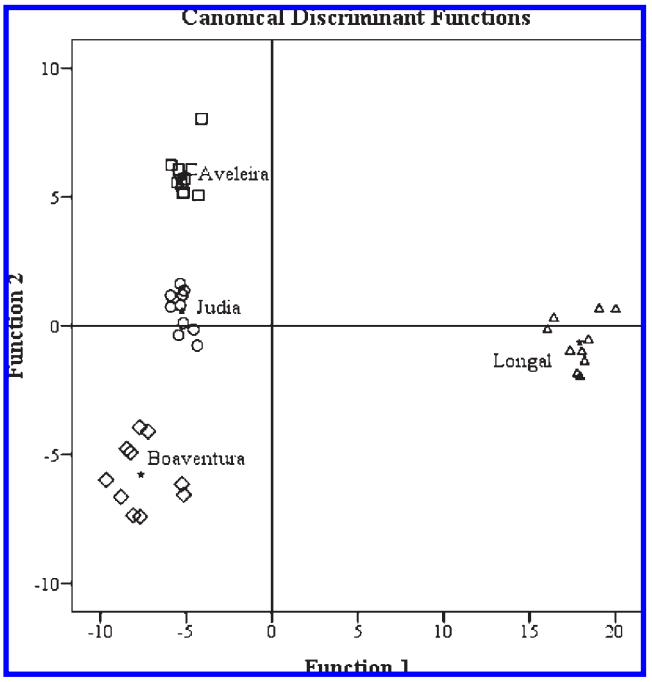

Figure 2. Canonical analysis of chestnut varieties based on the vitamin E profile.

In conclusion, after the obtained results, we conclude that chestnut kernel has a typical vitamin E profile with a high predominance of γ-tocopherol, bolstering its inclusion in the development of nutraceuticals, which can be applied therapeutically or prophylactically. The application of these new formulations may constitute an alternative to NSAIDs besides the health benefits of its consumption as a natural product.

Due to its economical importance, it is also valuable to accomplish methods that ensure the authenticity of a determined chestnut variety. Accordingly, the results were evaluated through a discriminant analysis to point out the vitamin E vitamers as a useful factor of discrimination among different chestnut varieties, making it possible to use them in authenticity studies.

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Received October 3, 2008. Revised manuscript received April 21, 2009. Accepted May 11, 2009. We are grateful to the Foundation for Science and Technology (Portugal) for financial support to J.C.M.B. (SFRH/BD/29060/2006) and INTERREG IIIA Project PIREFI.