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Effect of geographic origin, variety and storage on tocopherol concentrations in walnuts by HPLC

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Retinol, tocopherols and carotenes were extracted from walnut kernels. Three walnut extraction procedures were compared. Direct hexane extraction was selected. The fat-soluble vitamin separation was achieved by reversed-phase high performance liquid chromatography equipped with a ternary mobile phase gradient (methanol, butanol, water), and wavelength programming. The chromatographic run was under 35 minutes. This technique allowed a good resolution of α, γ, δ tocopherols, α and β carotenes and retinol.

Two walnut varieties (Franquette and Hartley) from two geographical origins (France, USA) were analyzed. Retinol, and carotenes were not identified in walnuts. The amounts of α, γ, δ tocopherols ranged respectively from 1.08 to 4.05, 21.78 to 26.46, and 2.51 to 4.23 (mg/100 g).

Significant differences related to variety and geographical origin were observed. A decrease in tocopherols (about 30%) was observed after 3 month refrigerated storage. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The present study is part of a large determination on walnut (Nux Regia L.) composition. Walnut kernels contain about 60% lipids. Most of them (67%) are unsaturated (Kawecki & Jaworski, 1975). Their oxidation is linked to the apparition of unpleasant flavours. The well-known antioxidant properties of alpha and beta carotenes, and tocopherol isomers suggest a protective effect against oxidation (Bhagavan & Nair, 1992). Due to their prevention of oxidative degradation, and to their nutritional interest, quantification of antioxidant vitamins in walnut is important. Curiously, the levels of tocopherols in walnuts are not well documented. No recent HPLC determination of walnut tocopherol composition has been published. Most of the data come from food composition tables in which the processing methods are not described neither are the varieties specified.

Gamma-tocopherol has been identified as the major homologue in walnut but it had been quantified fourteen years ago from data on optical densities according to Emmerie-Engel reaction (Lambertsen *et al.*, 1962; Rikhter, 1981). No information is available on the complete fat-soluble vitamin profile of walnut depending on cultivar variation and geographic origin.

So, the specific objectives of our study were to quantify tocopherols (α, γ, δ), and carotenes (α, β), to verify the absence of retinol (unusual component of plant materials) in walnut, to determine the specificity of French walnut composition in comparison to Californian walnut and to monitor possible changes during storage.

Simultaneous determinations of retinol, tocopherol isomers and carotenes by reversed-phase HPLC are well documented, mostly in plasma (Epler *et al.*, 1993; Bui, 1994; Chuang *et al.*, 1994). Recent detailed reports on separate vitamin analysis in foods have also been published (Olmedilla *et al.*, 1990; Rizzolo & Polesello, 1992; Hollman *et al.*, 1993; Lumley, 1993; Panfili *et al.*, 1994).

The HPLC method described here involves a chromatographic programming. It is suitable for walnut analysis. Different extraction procedures were compared, based on direct solvent extraction of vitamins or alkaline hydrolysis or acid hydrolysis of walnut. Alkaline hydrolysis is the most usual process used to hydrolyse ester forms of vitamins. However acid hydrolysis has also been used as it is one of the official methods for lipid determination in foods (AFNOR, 1980). It permits the extraction of fatty acids linked to other components.

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MATERIALS AND METHODS

Sample description

The study was carried out during two consecutive years.

In 1993, one variety of walnut (Franquette) was collected in Isere (France). Vitamin levels and influence of storage (12 months) on vitamin levels were studied.

Then, we were interested in the study of geographical and varietal influence on walnut vitamin levels. For this purpose two varieties of walnuts, Franquette and Hartley were purchased in 1994. Each of them came from two geographic areas, California (USA), and Dauphiné (France).

Unshelled walnuts were stored at +4°C during all the experiment until analysis. Walnut samples (200 g) were removed randomly from 5 kg packages for extraction. Walnut kernels (about 100 g) from each sample were ground into a fine powder as briefly as possible with a robot (BÜHLER H04) food processor.

Reagents and chemicals

Butanol, diethyl ether, cyclohexane, hexane, methanol were HPLC grade (SDS France). Tridistilled water was prepared for chromatographic use. Analytical grade pyrogallol, potassium hydroxide, sodium sulphate and chloride were obtained from MERCK (Germany). Carotenes (α and β) were gift from ROCHE (France). Trans-retinol acetate, trans-retinol palmitate, trans-retinol, α-tocopherol acetate were purchased from SIGMA (USA), and tocopherol isomers from MERCK (Germany).

Extraction

During all the steps that follow, care was taken to protect the assays from light and atmospheric oxygen. Six replicates of each sample were run.

Method 1

One gram of finely ground walnut was weighed and introduced into a Soxhlet cartridge with 50 mg pyrogallol as antioxidant. The cartridge was placed into a 150 ml Soxhlet extractor. The extraction unit was wrapped in foil and extracted twice for 4 hours with 200 ml hexane. Extracts were evaporated until dried in a vacuum rotoevaporator (BIOBLOCK, France) at a temperature not exceeding 40°C. The residue was dissolved in 5 ml methanol–butanol (50/50), filtered (0.5 μm MILLIPORE) and placed in non-actinic vials. They were overlayered with nitrogen and stored for up to 24 hours at +4°C.

Method 2

One gram of finely ground walnut was saponified with 5 ml 50% aqueous KOH (w/v), 15 ml methanol, and 50 mg pyrogallol for 30 minutes in a water bath at 56°C under continuous nitrogen stream and magnetic stirring. Samples were then cooled for 20 minutes at -20°C. Forty ml 25% aqueous NaCl (w/v) were added and vitamins were extracted three times following 10 minute of magnetic stirring with 20 ml diethyl ether.

The ether extracts were pooled, washed with water to neutrality to remove the fatty acid soaps, and desiccated on anhydrous sodium sulphate.

The extract was evaporated until dried in a vacuum rotoevaporator. The residue was dissolved in 5 ml methanol-butanol (50/50), filtered (0.5 μm MILLIPORE) and placed in non actinic vials. They were overlayered with nitrogen and stored, up to 24 hours at +4°C.

Standard solutions were processed as walnut.

Method 3

One gram of finely ground walnut was introduced in flasks protected from light, heated under back-flow for one hour with 20 ml 6N HCl, and 50 mg pyrogallol. The residue was filtered on two wet filters (DURIEUX 111 without fats) and washed with distilled water until neutrality. After drying under vacuum at 40°C, the residue was extracted by hexane as described in method 1.

HPLC apparatus and conditions

The chromatograph was supplied by VARIAN instruments and consisted of a solvent delivery pump (VARIAN 9010), a Valco injector valve equipped with a 20 μl loop, and a variable wavelength UV-visible detector (VARIAN 9050). The analytical column was a Lichrocart cartridge (125×4 mm) filled with Spherisorb ODS_2 (5 μm), pore size 80Å, fitted with a Lichrosorb 18 precolumn (MERCK).

Compounds were quantified using the peak area with a MERCK D 2500 integrator. Retinol was monitored at 325 nm, tocopherols at 292 nm, and carotenes at 450 nm. Eluent and wavelength program are reported in Table 1.

Table 1. Eluent composition (%) and flow program (A) and wavelength program (B)

	Time (min)	Methanol %	Butanol %	Water %	Flow (ml/min)
A					
1	0.00	85	5	10	0.8
2	2.00	89	5	6	1.2
3	19.00	89	5	6	1.2
4	20.00	89	11	0	1.2
5	32.00	89	11	0	1.2
6	35.00	85	5	10	0.8
		Time (min)	λ (nm)	A.U.F.S.	
B					
Retinol		0	325	0.050	
Tocopherols		7	292	0.050	
Carotenes		19	450	0.050	

All solvents and solutions were filtered (0.5 μm) and degassed by sonication before use.

Standard preparation and storage

Individual stock standards were prepared in appropriate solvents (retinol 25 mg/100 ml methanol, tocopherols 50 mg/50 ml methanol, carotenes 10 mg/100 ml cyclohexane) and stored at –20°C under nitrogen in dark bottles. The exact concentrations were verified spectrophotometrically by using the following extinction coefficients: retinol in methanol: 1950 (max 325 nm), tocopherols in methanol, α: 72.0 (max 292 nm), γ: 91.5 (max 298 nm), δ: 91.2 (max 297 nm), carotenes in cyclohexane, α: 2800 (max 450 nm) β: 2592 (max 450 nm).

A mixed working solution (retinol 0.5 mg/ml, α, γ, δ tocopherols 10 μg/ml, α carotene 0.5 μg/ml, and β carotene 1 μg/ml) was prepared every two weeks and stored at –20°C.

Standard curve and recovery determination

The compounds were identified by their retention times and by adding standards to samples before Soxhlet extraction and verifying retention time of specific peaks. Recovery studies were performed by spiking tocopherols into the one gram sample before Soxhlet extraction (method 1).

Limits of detection were determined with standards.

Sample analysis

Each sample from the extraction procedures previously described was filtered (0.5 μm MILLIPORE) and injected into the HPLC system in triplicate.

Statistical analysis

Data were analysed by two-way analysis of variance and Student test.

RESULTS AND DISCUSSION

Quantification

The HPLC method described here required wavelength and gradient mobile phase programming. Vitamins were eluted as a series of well resolved peaks (Fig. 1). Beta and gamma-tocopherols were not separated.

The areas of vitamin peaks were linearly related to the amount injected over the entire range examined (0.01–5 μg/ml for retinol, 0.1–50 μg/ml for tocopherols, and 0.01–10 μg/ml for carotenes).

Linear relationships were observed for all vitamins (regression factor 0.9999). Assuming that the signal to noise ratio should be 3, and using an injection volume of 20 μl, the detection limits of the method are 0.01 ng for retinol, 2 ng for tocopherols, and 0.1 ng for carotenes. So quantification limits for walnuts are 1.5 μg/100 g, 300 μg/100 g and 15 μg/100g, for retinol, tocopherols and carotenes respectively. This range of linearity and the high sensitivity were consistent with walnut analysis.

The overall precision of the method was evaluated through repetitive analysis of a standard mixture and of walnut extracts. The relative standard deviation for repeatability (rSD) (6 injections of the same solution on the same day) and reproducibility (RSD) (6 injections of different solutions three days in a row) are reported for standards in Table 2A and for walnut extract in Table 2B. Extraction and analysis of the samples of walnut were repeated six times. RSD and rSD values of the whole assay were greater than those observed for standards. This may reflect a possible heterogeneity of the samples.

Figure 2 shows a representative chromatogram of a walnut (Franquette, France) extract (Method of extraction 1). In each cultivar, retinol, carotenes, and esterified forms of vitamins (retinol acetate, retinol palmitate, and α-tocopherol acetate) were not shown.

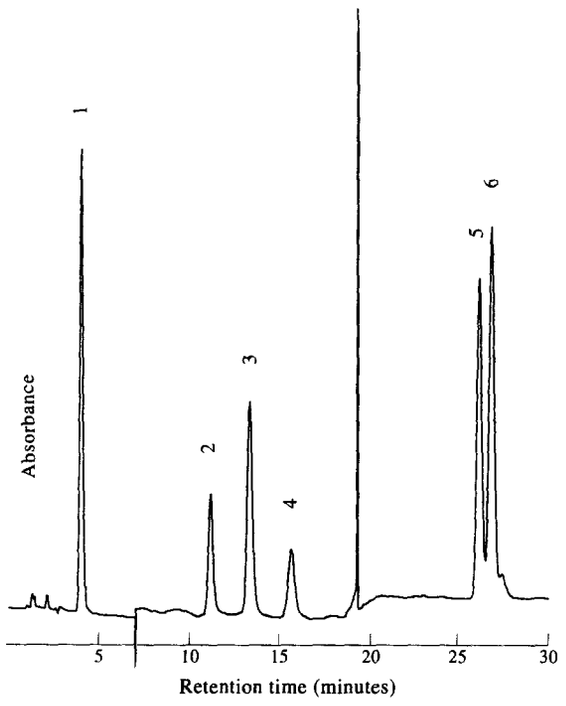

Fig. 1. HPLC chromatogram of a standard mixture of vitamins. 1: retinol; 2: δ-tocopherol; 3: γ-tocopherol; 4: α-tocopherol; 5: α-carotene; 6: β-carotene. HPLC Model 9010 (Varian) was used with ternary gradient program (Table 1A). The determination of vitamins was effected with a Valco injector (20 μl loop) on a reversed phase Lichrocart cartridge (125 mm ×4 mm) filled with Spherisorb ODS_2 RP 18 (5 μm) pore size 80 A and a pre column fitted with lichrosorb RP18 (Merck), a UV detector Model 9050 (Varian) with Wavelength program, (Table 1B), an integrator and recorder Model D 2500 (Merck).

Table 2. Repeatability and reproducibility coefficients of variation for standards (A) and walnut vitamins (B)

	Retinol	α-tocopherol	γ-tocopherol	δ-tocopherol	α-carotene	β-carotene
A						
rSD	0.73%	1.95%	2.84%	3.36%	2.28%	2.36%
RSD	1.41%	2.22%	3.38%	3.09%	4.40%	3.72%
B						
rSD		8.21%	1.33%	8.19%		
RSD		9.83%	5.14%	6.92%		

The identity of the vitamin peaks was confirmed by spiking samples with known amounts of standard vitamin solutions. Attempts were made to confirm peak identities with spectra of eluted peaks (diode array detector). However the sensitivity of the apparatus was not adequate. Vitamin recoveries were quantified for tocopherols only. They ranged respectively 95.5% for α-, 98.5% for γ- and 98% for δ-tocopherol.

As expected, γ-tocopherol was the predominant homologue (Klepping *et al.*, 1989). The inability of the HPLC method to separate beta- and gamma-tocopherol was not inconvenient as Alary *et al.* (1983) showed that walnut oil did not contain beta-tocopherol.

We observed some variations in the retention times of the tocopherols. These modifications in the chromatograms could be related to the variation of room temperature during the day.

Methods of extraction

Three extraction methods for the determination of fat-soluble vitamins were assayed (oil saponification or oil acid hydrolysis prior to extraction and direct solvent extraction). Results for the Franquette (France) walnut show very small differences between vitamin levels. The tocopherol concentrations expressed as mg/100 g (means ± SD; $n=6$) were:

- method 1: α-tocopherol 1.15 ± 0.09, γ-tocopherol = 31.12 ± 1.44, δ-tocopherol 4.11 ± 0.12; total tocopherols 36.38 ± 1.53
- method 2: α-tocopherol, 1.38 ± 0.26, γ-tocopherol = 29.87 ± 2.17, δ-tocopherol 3.78 ± 0.02; total tocopherols 35.03 ± 2.36
- method 3: α-tocopherol 1.87 ± 0.13, γ-tocopherol = 30.31 ± 1.87, δ-tocopherol 3.72 ± 0.27; total tocopherols 35.90 ± 1.28

The results obtained from extraction methods 1 and 2 were compared by Student test. No significant differences appeared between the δ and γ tocopherol levels ($p > 0.1$). Only a slight difference was observed for α-tocopherol ($p < 0.05$). As γ-tocopherol is the predominant homologue, and method 1 (direct solvent extraction of walnut) was easier to realize, it was selected.

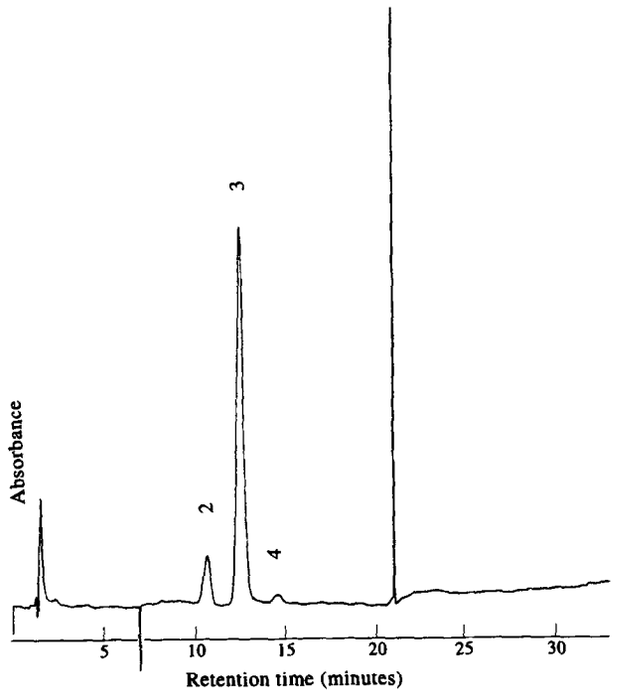

Fig. 2. HPLC chromatogram of a walnut extract. 2: δ-tocopherol; 3: γ-tocopherol; 4: α-tocopherol HPLC conditions: as in Fig. 1.

Storage influence (Table 3)

Tocopherol levels were also examined during refrigerated storage of Franquette (France) variety.

After a three month storage at +4°C (December 93–February 94), significant losses for all the tocopherols were observed ($p < 0.05\%$). Decreases were respectively 29%, 28% and 30% for α, γ, and δ tocopherol. Nine month later (February–November 94), the levels of α, γ, and δ tocopherol showed a slight increase. However it was not significant ($p > 0.1$). It could be related to a low desiccation of walnuts, although they were stored at +4°C.

The effect of food processing and storage on total tocopherol and specifically on alpha-tocopherol has

Table 3. Influence of storage on tocopherol concentrations (mg/100 g) of Franquette walnut (France) 1993 harvesting

	Dec 1993	Feb 1994	Nov 1994
α-tocopherol	1.15 ± 0.09	0.82 ± 0.02	1.05 ± 0.20
γ-tocopherol	31.12 ± 1.44	22.34 ± 1.21	23.30 ± 1.48
δ-tocopherol	4.11 ± 0.12	2.88 ± 0.19	3.26 ± 0.35
Total tocos	36.38 ± 1.53	26.04 ± 0.70	27.61 ± 1.86

Means ($n=6$) ± SD.

Table 4. Tocopherol concentrations of four walnut varieties. The results are expressed as mg/100 g

	Franquette, France	Franquette, USA	Hartley, France	Hartley, USA
α-tocopherol	1.87 ± 0.46	1.40 ± 0.35	4.05 ± 1.36	1.08 ± 0.09
γ-tocopherol	23.68 ± 0.84	21.79 ± 0.80	26.46 ± 1.25	24.78 ± 0.65
δ-tocopherol	4.07 ± 0.34	3.52 ± 0.64	4.23 ± 0.34	2.51 ± 0.18
Total tocopherols	29.62 ± 1.21	26.70 ± 0.80	34.74 ± 2.68	28.37 ± 0.60

Means ($n = 6$) ± SD.

Table 5. Tocopherol content (mg/100 g) reported in the literature

	α-tocopherol	γ-tocopherol	δ-tocopherol
Beringer & Dompert, 1975	0.92	36.94	2.84
Feinberg *et al.*, 1987	4.3–15.4	82.2–94.5	0.7–1.2
Klepping *et al.*, 1989	0.4	15.8	0.94
Lambertsen *et al.*, 1962	1.5	20.5	—
Souci *et al.*, 1994	1.90	41.4	0.20

been described in some detail. Tocopherols are unstable in the presence of unsaturated fats, oxygen, alkali and metal ions (Nelis *et al.*, 1985). The very high level of polyunsaturated fatty acids (linoleic acid > 60%; linolenic acid 10%) in walnut composition could explain the decrease of tocopherol amounts observed within three months. Such a decrease was not observed by Yao *et al.*, 1992 on pecan kernels stored at +4°C, probably because of their low percentage of polyunsaturated fatty acid, linoleic acid (25%) and linolenic acid (1.5%) compared to the high percentage of monounsaturated fatty acids (oleic acid > 60%).

Influence of geographic origin and variety (Table 4)

The comparison between the four walnut samples was realized on the 1994 harvesting. For technical reasons, it was carried out after a six month storage (at +4°C) of walnuts. This delay explains the lower levels of Franquette walnut tocopherols as compared to the first value from analysis of 1993 harvesting reported in Table 3.

Gamma-tocopherol was predominant in the four samples. It ranged from 21.79 mg/100 g (Franquette, USA) to 26.46 mg/100 g (Hartley, France). Delta-and alpha-tocopherol levels were about ten times lower. French Hartley had the highest levels for all tocopherols.

The effect of geographical and varietal influences were analyzed by two-way ANOVA. Tocopherols were significantly higher for French vs Californian walnuts ($p < 0.001$ for α, γ, δ tocopherol) and for Hartley vs Franquette variety ($p < 0.05$ for α, γ, δ tocopherol). So, geographical origin influence seems to be more important than the varietal one.

Very few papers have been published on walnut tocopherol composition and they are not recent (Lambertsen *et al.*, 1962; Beringer & Dompert, 1975). The literature values for walnut tocopherols are shown in Table 5. Wide ranges were observed for all the vitamins. However most of them are taken from food tables (Feinberg *et al.*, 1987; Klepping *et al.*, 1989; Souci *et al.*, 1994) and are determined by unknown methods. Our values are all near average.

The high level of gamma-tocopherol in the four species of walnuts is of particular interest. Although alpha-tocopherol has the highest vitaminic activity, gamma- and delta- tocopherols nevertheless act as more effective *in vitro* antioxidants to protect the stored fats (Lehmann *et al.*, 1994).

CONCLUSION

The HPLC method seems to be sufficiently sensitive and accurate in the determination of walnut tocopherols.

This method permitted a simultaneous separation of retinol, α, γ, and δ tocopherols, α and β carotenes in thirty five minutes. Gamma tocopherol level in walnuts is about 25 mg/100 g walnut kernels whereas those of δ and α-tocopherol are lower, within 1–5 mg/100 g.

Significant differences related to variety and geographical origin were observed. Highest levels were observed for French Hartley walnuts.

Storage at +4°C could not preserve tocopherol levels (about 30% losses within 3 months).

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