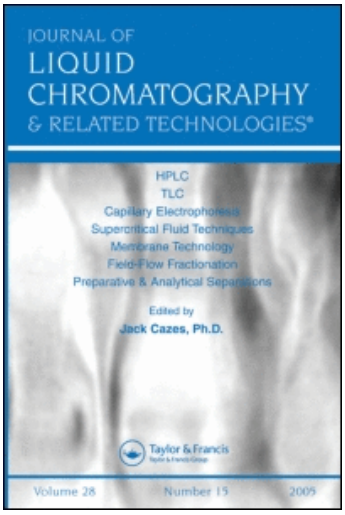

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VITAMIN E CONTENT OF MARGARINE AND REDUCED FAT PRODUCTS USING A SIMPLIFIED EXTRACTION PROCEDURE AND HPLC DETERMINATION

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ABSTRACT

A liquid chromatographic method is described for the analysis of vitamin E in margarine and vegetable oil products (spreads). The tocopherol homologs (α-, γ-, δ-tocopherol) are extracted in hexane with anhydrous $MgSO_4$ added to remove water. Complete resolution of the homologs is achieved on a normal phase column and a mobile phase of 0.9% isopropanol in hexane with fluorescence detection. Based on five repetitive assays the mean recoveries were 99.0±1.4, 97.4±2.2%, and 99.5±2.6% for α-, γ-, and δ-tocopherol, respectively. The method is rapid, specific, and accurate for the measurement of vitamin E in margarine and vegetable oil spreads. Further, the method avoids: (1) saponification techniques (2) emulsion forming organic solvent extractions and (3) the use of chlorinated solvents. The limit of detection for α - , γ - , and δ - tocopherol were 23.2, 2.96, and 1.98 μg/100g , respectively. The limit of quantitation for α - , γ, and δ - tocopherol were 39.8, 5.00, and 3.02 μg/100g, respectively. The vitamin E content of the margarine and spreads ranged from 5.58 to 24.2 α-TE/100g.

INTRODUCTION

The increased public interest in the health benefits of vitamin E is warranted. Vitamin E prevents cell damage by preventing *in vivo* peroxidations and plays an important role in preventing inflammation, cardiovascular disease and cancer.[1-8] Vitamin E content of margarine includes tocopherols (T) and tocotrienols (T3) that possess the biological activity of RRR α - T and include α-, β- γ-, and δ-T and the corresponding T3. In order to assess the vitamin E activity of a specific food, the individual homologs must be quantitated and the level converted to RRR α-T equivalents (α-TE). One α-TE is equal to 1 mg of α-T.[9] Biological activity of other homologs are β-T, 0.5; γ-T, 0.1; δ-T, 0.03 mg.

The most significant source of vitamin E activity in the diet is vegetable oils and their products. Recent reports on products providing a significant source of vitamin E in the American diet rate margarine second at 6.8%[10] of the available vitamin E.

Preparation of the vitamin E fraction usually requires saponification and organic solvent extraction.[11-14] In a recent report,[14] Rader et al[14] used saponification and reverse - phase LC to determine only α - tocopherol content in margarine and margarine-like products, γ-T and δ-T content were not determined in this report. Saponification procedures can cause analyte degradation if conditions are improperly controlled and emulsions, formed during solvent extraction, can lead to low recoveries. Eitenmiller and Landen[15] used extraction with methylene chloride and gel permeation chromatography for lipid removal followed by reverse phase LC for quantitation. This assay requires the use of two LC systems. Chase et al[16] used a direct extraction procedure for the determination of α - tocopherol acetate, tocopherols, and retinyl palmitate in infant formulas. The vitamins are extracted in isopropanol and hexane/ethyl acetate mixture and quantitated by normal phase chromatography with fluorescence detection. The method also eliminates the use of chlorinated solvents. The Thompson et al[17] procedure did not require saponification; however, the procedure used excessive amount of organic solvent. For oils, direct injection on silica column, has been utilized by several researchers.[11,17-20] The silica column can accommodate up to 2 mg of fat per injection.[20] The triacylglycerols remain soluble in the mobile phase and do not interfere with the chromatography. Both reverse phase and normal phase LC can be used to quantitate the vitamin E homologs. Reverse phase systems cannot resolve β- and γ-T while normal phase LC gives complete resolution of the homologs.

Fluorescence detection provides greater sensitivity, specificity, and cleaner chromatograms compared to UV detection. Simultaneous quantitation of complete vitamin E profile can be difficult due to differences in concentration and differences in fluorescence intensity of the homologs and may require multiple injections at various detector sensitivity settings.

Current AOAC Official methods[21] do not provide a specific method for the analysis of vitamin E in margarine or low fat vegetable oil spreads.

The objective of the present study was to develop a method based upon direct solvent extraction to simplify vitamin E assay of margarine and reduced fat spread products. The developed method can be easily incorporated into routine laboratory operations.

EXPERIMENTAL

Reagents

All reagents are of analytical purity. n-Hexane, LC grade (J.T.Baker, Phillipsburg, NJ, USA); Isopropanol, LC grade (Fisher Scientific, Pittsburgh, PA, USA); 2,6-Di-tert-butyl-4-methyl phenol (BHT) (Sigma, St. Louis, MO, USA); Polyoxyethylene sorbitan monooleate (Tween 80) (J.T.Baker); Magnesium sulfate (anhydrous, powder) (J.T.Baker); D,L-α-tocopherol (USP), D,L-γ-tocopherol (Sigma), D,L-δ-tocopherol (Sigma); Hexane-BHT solution (0.1%BHT). Dissolve 1g BHT in 1 liter n-hexane.

Tocopherol standard solutions

Tocopherol pre-solutions (10 mg/5 mL).

Accurately weigh 20 mg α-tocopherol and dissolved in n-hexane. Transfer to a 10 mL volumetric flask and dilute to volume with hexane. Similarly, prepare tocopherol pre-solutions for γ-tocopherol, δ-tocopherol.

Stock solution (0.6 mg/mL).

Pipet 8.0 mL tocopherol pre-solution into 25 mL volumetric flask and dilute to volume with hexane-BHT solution. This solution is stable for ≥6 months if stored at -20^{0}C. Similarly prepare stock solution for the other homologs.

Table 1

Specific Absorption Coefficients ($E^{1\%}_{1cm}$) and Maximum Wavelengths (λmax) for Tocopherols in 96% (v/v) Ethanol Solutions[a]

Vitamer	λ_{max} nm	$E^{1\%}_{1cm}$
α - T	292	70.8
γ - T	298	92.8
δ - T	298	91.2

[a] From ref. 9.

Standard working solutions (I, 2 mg/25 mL).

Pipet 1.0 mL tocopherol pre-solution into 25 mL volumetric flask and dilute to volume with ethanol. Similarly prepare working solutions for the other standards. Determine absorbance difference (A - A^0) for each standard working solution with spectrophotometer at suitable wavelengths, using settings given in Table 1. A is the absorbance of the standard solution and A^0 is the absorbance of the blank (ethanol). Calculate concentration of each standard working solution from $E^{1\%}_{1cm}$ data.

Standard intermediate solution (1.2 mg/100 mL).

Prepare mixed standard solution containing α-, γ-, and δ-T. Pipet 2.0 mL of each stock solution (e. I) into 100 mL volumetric flask and dilute to volume with hexane-BHT solution. Prepare solution monthly and store at -20°C.

Standard working solutions (II, containing 14, 48 and 1200 ng tocopherol/mL).

Pipet 1.0 mL intermediate solution into 10 mL volumetric flask and dilute to volume with hexane-BHT solution to obtain std. 1 (1200 ng tocopherol/mL). Pipet 1.0 mL std. 1 into 25 volumetric flask and dilute to volume with hexane-BHT solution to obtain std. 2 (48 ng tocopherol/mL). Pipet 3.0 mL std. 2 into 10 mL volumetric flask and dilute to volume with hexane-BHT to obtain std. 3 (14 ng tocopherol/mL). Prepare fresh on day of use.

LC mobile phase.

n-Hexane-isopropyl alcohol (99.1+0.9). Dilute 991 mL n-hexane with 9 mL isopropyl alcohol and degas.

Apparatus

The LC System is equipped with a Shimadzu LC-6A pump (Shimadzu Scientific Instruments, Inc, Columbia, ML, USA), a 20μL injection loop, a SpectraSeries AS100 autosampler (Thermo Separation Products, Riviera Beach, FL, USA), a Shimadzu RF-10A fluorescence detector capable of excitation at 290nm and emission at 330 nm, and a Hewlett Packard (Avondale, PA, USA) 3392A integrator. The LC mobile phase flow rate was 1.0 mL/min.

LC column: (1) Guard column, LiChrosorb Si 60 (5μm) (E. Merck, Darmstadt, Germany); (2) Analytical column, 250x4.6mm id. Lichrosorb Si 60 (5μm)(E. Merck).

Sonicator, FS30 (Fisher Scientific); Bell Jar Filtration Apparatus, Kontes (Vineland, NJ, USA).

Sample Preparation and Extraction

Samples used in this study were three margarine brands from three different lots, and the spread samples were three brands from three different lots.

Gently melt two sticks of margarine at ~ 45°C before analysis and homogenize melted margarine. Homogenize spreads before analysis without warming.

Accurately weigh 5.0g margarine or spread into three individual 125 mL erlenmeyer flasks for duplicate determinations and a spike recovery. Add 40 mL of hexane-BHT solution, sonicate with intermittent mixing until the sample material has dissolved. Rinse sides of flask with 10 mL hexane-BHT solution and add 3 drops of Tween 80 and 3g anhydrous $MgSO_4$ (the amount of $MgSO_4$ added depends on water content, 1g for each mL of water plus 1g extra) and mix, then let stand for ≥ 2 hr.

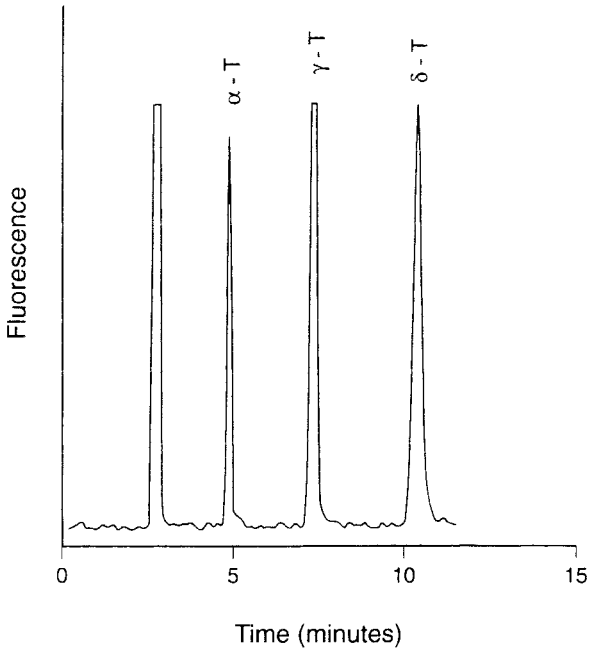

Figure 1. Chromatogram of std. mixture of tocopherol (~24 ng/mL) in hexane-BHT.

Filter the solution by using medium porosity fritted glass filter and bell jar filtration apparatus. Wash the filter with hexane-BHT solution and transfer filtrate to 100 mL volumetric flask and dilute to volume with hexane-BHT solution. Pipet 1.0 mL to 50 mL volumetric flask and dilute to volume with hexane-BHT solution.

Determination

Inject 20 μL of the sample extract (prefiltered 0.45 μm) in duplicate (appropriate dilutions were made to avoid injection of more than 2 mg fat). The concentration of the vitamin E homologs were calculated from the peak-area. T3 concentrations were calculated from the fluorescence responses of the respective tocopherols.[17] T3 retention times were verified by chromatography of a palm oil sample containing the representative T3 homologs (α-, γ-, δ-T3; not shown). Using standards, construct linear regression plot for each homolog and calculate concentration of each homolog in sample.

RESULTS AND DISCUSSION

Typical chromatograms obtained from standard and margarine are shown in Figures 1 and 2. Retention time for α-, γ-, and δ-T were 4.8, 7.5, and 10.6 min, respectively. No interfering peaks were observed in the chromatograms of reagent blanks.

Fluorescence response for α-, γ-, and δ-T were linear (r^2 =0.9999) for the range 0.28 -23.2, 0.32 - 26.9, and 0.32 - 26.7 ng/20μL injected, respectively.

The limit of detection (LOD) and limit of quantitative (LOQ) were determined by measuring the magnitude of analytical background response by injecting a number of blank samples and calculating the mean and standard deviation of this response. The mean plus 3 times the standard deviation provides the limit of detection (LOD), and the mean plus 10 times the standard deviation provides the limit of quantitation (LQD). The LOD in μg/100g is 23.20 for α-T, 2.96 for γ-T, and 1.98 for δ-T. The LOQ in μg/100g is 39.84 for α-T, 5.00 for γ-T, and 3.02 for δ-T.

The LC suitability data are included in Table 2 as analytical figures of merit for each homolog. These include linearity (R^2), capacity factor (K'), theoretical plates (N), tailing factor (T), selectivity (a), and resolution (RS).

Table 3 contains recovery data based on 5 trials for each homolog. The % mean recoveries ± SD (n=5) were 99.0 ± 1.4% for α - T, 97.4 ± 2.2% for γ-T, and 99.5 ± 2.6% for δ-T. Table 4 shows the intra - and inter - day assay results for each homolog as determined on one brand of margarine. For intra-day, the mean results (n=5) range from 2.39 mg/100g and 3.5% RSD for δ-T to 42.46 mg/100g for γ-T and 0.8% RSD for α-T. β-T and β-T3 were not detected in the margarine and spread samples included in this study.

Peak purity was established using a peak ratio technique described by Haroon et al [22]. The emission wavelength was kept constant for the analytes while the fluorescence was measured at three different excitation wavelengths. The fluorescence emission of tocopherols at 330 nm was determined at excitation wavelengths of 270, 280, and 290 nm. Peak response ratios were calculated for 280/290, 280/270, and 290/270 nm. These ratios were compared for the standard and sample as illustrated in Table 5. Good agreement was obtained for the ratio of the standard and sample indicating good peak purity.

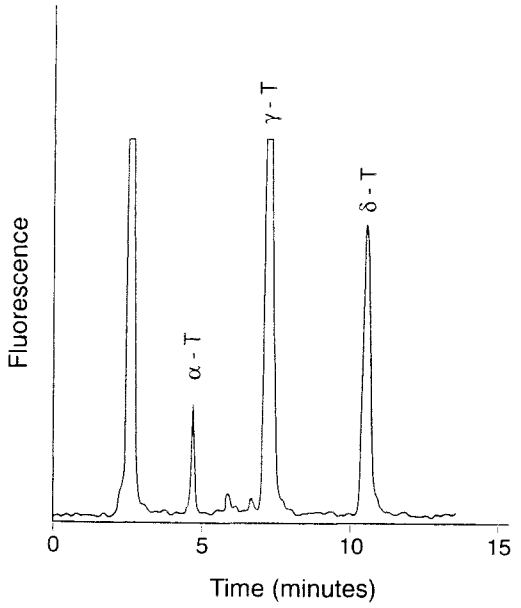

Figure 2. Chromatogram of a mixture of margarine in hexane-BHT.

Table 2

Analytical Figures of Merit for Vitamin E Assay

Homolog	Linearity[a] R^2	Capacity Factor K'	Theoretical Plates[b] N	Tailing Factor[c] T	Selectivity[d] a	Resolution RS
Alpha = T	0.9999	1.0	4956	1.1		
					1.5	7.9
Gamma - T	0.9999	2.1	6815	0.9		
					1.4	7.8
Delta - T	0.9999	3.4	7530	1.1		

[a] Range from 13.91 - 1159 ng/mL alpha - T (n=5), 16.15 - 1346 ng/mL gamma - T (n=5) and16.04 - 1337 ng/mL delta - T (n=5). [b] calculated as $n=16(t/w)^2$. [c]Calculated at 5% peak height. [d] $a = t_2/t_1$.

Table 3

Accuracy of Assay[a]

Homolog	Spike (mg/100g)	1	2	3	4	5	Mean	RSD (%)
		Recovery (100%)						
Alpha - T	23.76	100.3	100.3	97.1	98.8	98.3	99.0	1.4
Gamma - T	41.83	100.9	97.2	95.3	96.2	97.3	97.4	2.2
Delta - T	8.32	98.6	104.0	98.1	97.6	99.2	99.5	2.6

[a] Sample: Margarine (Brand B, 5.0g). Spiked with 1.1880 mg α - T, 2.0832 mg γ - T and 0.4160 mg δ - T.

Table 4

Precision of Assay[a]

Homolog	1	2	3	4	5	Mean	RSD(%)
				(mg/100g)			
Intra-day							
Alpha - T	17.65	17.84	17.48	17.49	17.58	17.61	0.8
Gamma - T	43.11	42.58	42.14	41.96	42.51	42.46	1.0
Delta - T	2.33	2.43	2.28	2.45	2.47	2.39	3.5
Inter - day							
Alpha - T	17.61	17.22	16.08	18.03	18.20	17.57	3.3
Gamma - T	42.46	43.69	43.84	42.92	42.84	43.15	1.4
Delta - T	2.39	2.40	2.34	2.18	2.41	2.34	4.1

* Sample: Margarine (Brand B, 5.0g).

Results of vitamin E content in margarine and spreads are listed in Table 6. Products containing corn and sunflower oils had the higher levels of vitamin E at similar fat contents.

Table 5

Specificity (Peak - Purity Test)[a,b]

Vary Excitation Wavelengths (nm)	Peak Emission Rates					
	Alpha - T		Gamma - T		Delta - T	
	Standard	Sample	Standard	Sample	Standard	Sample
280/290	1.51	1.54	1.14	1.09	1.12	1.08
280/270	1.38	1.40	1.60	1.53	1.62	1.68
290/270	0.91	0.90	1.40	1.40	1.45	1.55

[a] Constant emission wavelength: 330 min. [b] Fluorescence ratios shown were calculated by dividing the values for the two peak heights for each analyte (α-T, γ-T, δ-T) obtained from separate chromatographic runs at two different excitation wavelengths, with the emission wavelength constant at 330 nm.

Table 6

Determination of Vitamin E Content[a,b]

Sample Brand	Fat (%)	mg/100g			Mean Vitamin E IU/100g	Mean α - TE per 100g
		α - T	γ - T	δ - T		
A	80	4.43	54.26	20.00	15.74	10.46
B	80	19.34	47.99	2.40	36.14	24.21
C	64	1.90 (2.98)[c]	25.20 (2.21)[c]	9.06 (1.33)[c]	9.30[d]	5.58[d]
D	50	3.58	23.81	7.73	9.29	6.19
E	50	4.57	25.99	9.61	11.19	7.46
F	43	8.33	4.64	1.89	11.32	8.85

[a] Oil components in order of label listing: A:SB, PHSB; B: C, PHC; C: SB, PO, CA; D: SB, PHSB; F: SF, HSB, CA; where PH = partially hydrogenated; SB = soybean oil; C = corn oil; SF = sunflower oil; H = hydrogenated; CA = canola ; PO = palm oil. [b] n=3. [c] Corresponding T3. [d] Including α - T3.

In summary, the direct extraction with hexane avoids emulsion formation and direct LC injection of the extracted oil avoids saponification techniques to give a reliable and accurate LC method for margarine and low fat spreads. The vitamin E content of the margarine and spreads ranged from 5.58 - 24.21 α-E/100g. The method was applied to the analysis of margarine and vegetable oil spreads. The LC system utilizes normal phase chromatography with direct oil injection and fluorescence detection.(λ_{ex} : 290 nm, λ_{em} : 330 nm) for a rapid and accurate determination for the individual vitamin E homologs.

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