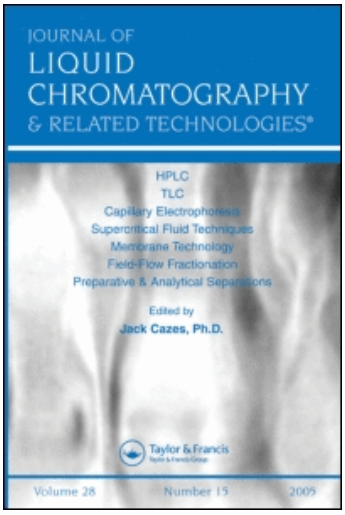

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Tocopherols and Tocotrienols in Foods and Tissues by High Performance Liquid Chromatography

J. N. Thompson[a]; G. Hatina[a]
[a] Nutrition Research Laboratories Food Directorate Health Protection Branch Tunney's Pasture, Ottawa, Ontario, Canada

To cite this Article Thompson, J. N. and Hatina, G.(1979) 'Determination of Tocopherols and Tocotrienols in Foods and Tissues by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 2: 3, 327 — 344
To link to this Article: DOI: 10.1080/01483917908060067
URL: http://dx.doi.org/10.1080/01483917908060067

JOURNAL OF LIQUID CHROMATOGRAPHY, 2(3), 327-344 (1979)

DETERMINATION OF TOCOPHEROLS AND TOCOTRIENOLS IN FOODS AND TISSUES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

J.N. Thompson and G. Hatina

Nutrition Research Laboratories
Food Directorate
Health Protection Branch
Tunney's Pasture
Ottawa, Ontario
Canada

ABSTRACT

Samples (10 g) of foods and tissues were homogenized in isopropanol and acetone. Low polarity lipids were obtained in the epiphase after the addition of hexane and water to the filtered extract. After separation, the hypophase was extracted twice with hexane and the combined epiphases were evaporated. The residue, which contained 97% of the vitamin E in the sample, was dissolved in hexane and aliquots (20 µl) were chromatographed on a 25 cm column of 5µ LiChrosorb Si60 with 0.2% isopropanol or 5% diethyl ether in moist hexane. A spectrofluorometer set at 290 nm excitation and 330 nm emission was used as a detector. Up to 2 mg lipid could be injected and 4 ng tocopherol produced a detectable peak. Four tocopherols (α,β,γ and δ) and three tocotrienols (α,β and γ) were measured in a variety of tissues and foods. Plastochromanol-8 and the antioxidant BHA were also detected in some samples. A preparative (1 cm diam) column was used to isolate tocotrienols from rubber latex and wheat flour and the purity and identify of the specimens, which were used as standards, were confirmed by mass spectroscopy. As esters of tocopherol are not fluorescent, foods containing added tocopheryl acetate were analysed after saponification.

INTRODUCTION

The earliest methods for determining naturally occurring forms of vitamin E, which consist of eight tocols (four tocopherols and four tocotrienols), involved simple colorimetric tests. Individual tocols were not distinquished. More sophisticated procedures have since been developed, involving GLC, TLC, paper and column chromatography, which can be used to separate and measure all forms of the vitamin (1). These methods, however, are difficult and tedious to perform.

Recently, HPLC has been used to measure tocols in specific foods or tissues (2-8). We now describe a selective and sensitive HPLC method which can be applied to all types of sample.

MATERIALS

Standards

Tocopherols (α-, β-, γ- and δ-) were gifts and purchases from Hoffman-La Roche Ltd, and Distillation Products Industries. Each tocopherol was purified, before use, by preparative HPLC. Tocotrienols were isolated from natural sources as described below.

Solvents

Acetone and hexane were glass-distilled. Moist hexane for HPLC was prepared by mixing equal parts of dry hexane and water-saturated hexane. Anhydrous diethyl ether was distilled over reduced iron immediately before use.

Analytical HPLC

A Perkin-Elmer Model 601 system was fitted with a Waters U6K loop injector and a 25 cm x 3.2 mm column of 5μ LiChrosorb Si60. A Perkin-Elmer MPF-3 spectrophotofluorometer, equipped with a flow cell and a LC55 absorption detector (set at 295 nm), were used to monitor the effluent. A Perkin-Elmer calculating integrator (Model M2) was used with the fluorometer during quantitative analysis. The excitation was set at 290 nm (slit 9 nm) and the emission at 330 nm (slit 18 nm); a high-pass filter (310 nm) was placed in the emission beam.

Preparative HPLC

An Altex 100 pump, fitted with a preparative head (0-30 ml/ min), was used with a Waters U6K loop injector, a 25 cm x 1 cm column of 5μ LiChrosorb Si60, and a Perkin-Elmer LC55 absorption detector.

METHODS

Extraction of Lipids

Samples were mixed until uniform. An aliquot (10 g) was weighed and then was placed for 10 minutes in 100 ml boiling isopropanol in the cup of the Virtis homogenizer. The digest was homogenized for 1 minute. Acetone (50 ml) was added and the mixture was filtered through glass-fibre paper (Whatman GF/A) into a 500 ml separatory funnel. The residue was extracted with acetone (50 ml) and the filter paper and its contents were homogenized with another portion of acetone (100 ml). The extract was filtered as before into the separatory funnel and

the residue was washed with 50 ml acetone. Hexane (100 ml) was added to the pooled extracts and the funnel was swirled to mix the solvents. Water (100 ml) was added, the flask was swirled gently, and the phases were allowed to separate. The hexane epiphase was transferred to a second funnel and the aqueous hypophase was extracted twice with 100 ml portions of hexane. The pooled hexane extracts were washed twice with water (100 ml) and then evaporated under reduced pressure.

Saponification of Infant Formula Containing Tocopheryl Acetate

The formula was mixed until uniform; concentrated preparations were diluted with an equal volume of water. An aliquot (10 ml or 10 g) was placed in a 125 ml Erlenmeyer flask with 25 ml ethanol and 10 ml ethanolic pyrogallol. An air condensor, 16 cm long, was attached and the mixture was refluxed for 3 minutes. 50% aqueous KOH (5 ml) was then added slowly through the condensor and refluxing was continued for 5 minutes. The flask was cooled in ice and the contents were transferred to a 250 ml separatory funnel using 30 ml water as a rinse. The cooled, diluted saponification mixture was extraced with two 50 ml portions and one 100 ml portion of diethyl ether. The ether extracts were washed with water until the washings no longer turned phenolphthalein red. The ether was evaporated in a flash evaporator almost (but never completely) to dryness, ethanol (20 ml) and hexane were added, and evaporation was continued to remove traces of water. When the volume was again reduced to approximately 1 ml, the solution was transferred to

a 25 ml volumetric flask which was then filled to the mark with hexane.

High Performance Liquid Chromatography

The fluorometer lamp was ignited and left for 20 minutes to stabilize before samples were chromatographed. The flow rate was 2 ml/min. During routine analysis the solvent system was 5% diethyl ether in moist hexane. Other solvent systems were utilized occasionally as indicated. A mixture of standards (α-, β-, γ- and δ-tocopherols) was injected after every two samples. The volume injected was usually 20 μl. The results were calculated from the peak areas using factors derived by chromatography of standards.

RESULTS

Development of HPLC Separation of Tocopherols and Tocotrienols

The separation of tocols in various solvent systems was investigated using solutions of α-, β-, γ- and δ-tocopherols. A solution of barley oil (0.5%), which is a rich source of α-, β- and γ-tocotrienols (9), was used in conjunction with the tocopherol standards to develop a system that would separate the four tocopherols and the three tocotrienols. Good separations were achieved on 5μ LiChrosorb Si60 in moist hexane containing either 5% diethyl ether or 0.2% isopropanol.

Isolation of Tocotrienols Using Preparative HPLC.

In order to make quantitative measurements of the tocotrienols it was necessary to obtain pure specimens for use as standards. These were obtained by preparative HPLC of extracts

of rubber latex, a source of α- and γ-tocotrienols (10), and wheat flour, a source of β-tocotrienol (9).

Lipids from three 10 ml aliquots of rubber latex were extracted as described above. The extracts were evaporated and saponified and the unsaponifiable lipid was chromatographed in two portions on a 25 cm x 1 cm diameter column of 5μ LiChrosorb Si60. The column was eluted with 5% diethyl ether in moist hexane (10 ml/min) and the eluate was monitored from the absorption at 295 nm. Two fractions, containing α- and γ-tocotrienols respectively, were collected. A third fraction, considered to contain δ-tocotrienol (10), was also investigated; however, as it was contaminated with an unidentified non-fluorescent substance with strong U.V. absorption, it was discarded. The pooled specimens of α- and γ-tocotrienols were rechromatographed to complete their purification. A specimen of β-tocotrienol was obtained by applying the same procedure to whole wheat flour.

The separation of the seven available tocols (i.e. the three tocotrienols and four tocopherols) by HPLC is shown in Figure 1 .

Properties of Tocotrienols Isolated from Rubber Latex and Wheat

The absorption spectra of the tocotrienols were measured in alcohol and hexane. The peaks were at the wavelengths specified in the literature (11) and the low absorption values at the minima at 260 nm indicated that absorbing impurities were unlikely to be present in significant quantities. The

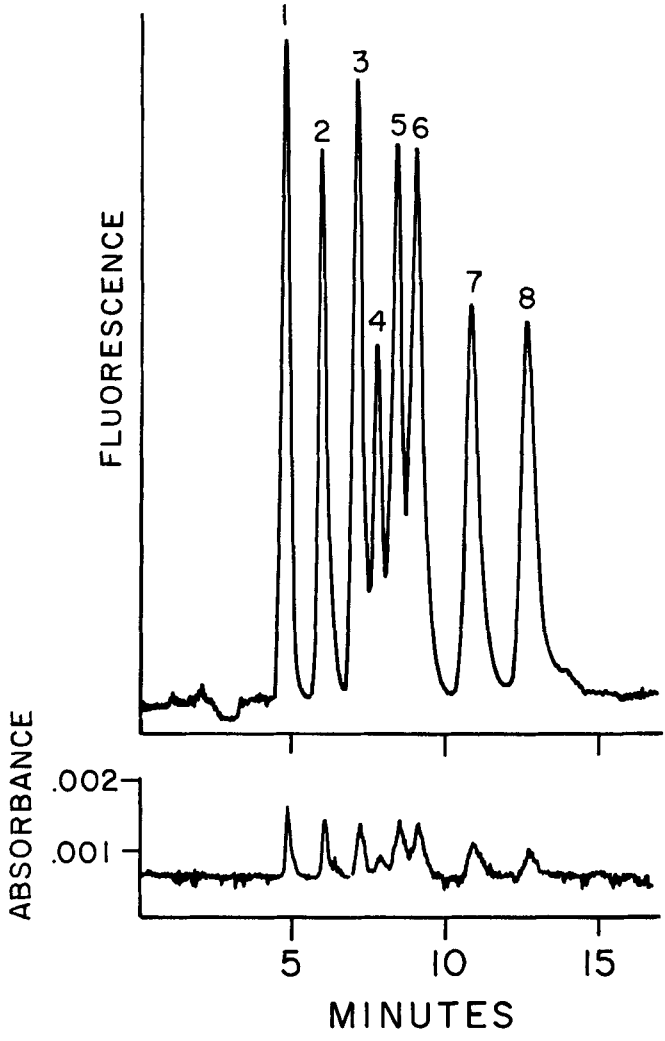

FIGURE 1. HPLC of standards on LiChrosorb Si60, 5 μ in 5% diehtyl ether in hexane, 2 ml/min. Passes first through fluorescence detector (upper) set at 290 nm excitation 330 nm emission and secondly through absorption detector (lower) set at 295 nm. Peaks as follows: 1. α-tocopherol; 2. α-tocotrienol; 3. β-tocopherol; 4. BHA; 5. γ-tocopherol; 6. β-tocotrienol; 7. γ-tocotrienol; 8. δ-tocopherol. Sample injected: 20 μl of solution containing (per ml) 5 μg each tocol + 1 μg BHA.

concentrations of the tocotrienols could therefore be calculated from published $E^{1\%}_{1cm}$ values (11). Fluorescence spectra were next recorded on solutions in hexane and ethanol, which contained according to calculations based on absorbtion measurements, 5 µg tocotrienol/ml. Fluorescence spectra were also recorded on solutions of similar quantities of the tocopherols and the intensities at the peaks were compared; the results indicated that the fluorescence intensity of each tocotrienol was identical to that of the corresponding tocopherol (Table 1). This suggested that the response of the fluorometric detector (i.e. the area under the peak) would be quantitatively similar for the tocopherols and tocotrienols; this was confirmed by chromatographing equal

TABLE 1

	Fluorescence Intensity[a]		HPLC Peak Area[b]
	Hexane	Ethanol	
α-Tocopherol	100	146	100
α-Tocotrienol	100	154	97
β-Tocopherol	79	214	129
β-Tocotrienol	75	206	127
γ-Tocopherol	100	224	110
γ-Tocotrienol	95	225	108
δ-Tocopherol	171	201	122

[a]Measured at peaks with slits at 5 nm on solutions containing 5 µg tocol/ml; expressed relative to α-tocopherol in hexane.

[b]Area under peak during HPLC in 5% diethyl ether in hexane; expressed relative to α-tocopherol. Excitation 290 nm (slit 9 nm), emission 330 nm (slit 18 nm) with filter.

quantities (Table 1). Finally, the purity and identity of the isolated tocotrienols were confirmed by mass spectroscopy; the spectra were as described in the literature (12) and they were compatible with the calculated molecular weights (Table 2).

Isolation and Identification of Plastochromanol-8

In certain vegetable oils and plant extracts, a small peak was resolved from γ-tocopherol in the isopropanol system but not in the diethyl ether system. A concentrate of this material was prepared from rapeseed oil by first removing fat by low-temperature crystallization in acetone, secondly preparing unsaponifiable lipids from the residue, thirdly removing sterols by crystallization from methanol, and finally performing HPLC on a 1 cm diameter column of LiChrosorb Si60. The isolated fraction contained a substance related to the tocols: the absorption maximum in hexane was at 297 nm and the ratio of the absorption to the fluorescence (which was determined by comparing the responses of the two types of detector during HPLC) was similar to that of γ-tocopherol. The substance was further purified by thin layer chromatography in chloroform on silica gel. The main band, identified by the Emerie-Engel reaction, was eluted and the mass spectrum was determined. The molecular ion of 750.67 and details of the spectrum (Table 2) revealed that the substance was plastochromanol-8, which is structurally related to γ-tocotrienol (12). Confirmation of the identity of the substance was obtained by comparison of its migration rate with those of the tocols during thin layer chromatography in

TABLE 2

Molecular Weights and Major Peaks (m/e) in Mass Spectra

	M.W.	m/e							
α-Tocopherol[a]	430	43	55	57		165		205	430
α-Tocotrienol[b]	424	41	55	69	81	165	203	205	424
β-Tocopherol[a]	416	43	55	57		151		191	416
β-Tocotrienol[c]	410	41	55	69	81	151	189	191	410
γ-Tocopherol[a]	416	43	55	57		151		191	416
γ-Tocotrienol[b]	410	41	55	69	81	151	189	191	410
δ-Tocopherol[a]	402	43	55	57		137		177	402
Plastochromanol-8[d]	750		55	69	81	151	189	191	750

[a]Commercial source

[b]From rubber latex

[c]From whole wheat flour

[d]From rapeseed oil

two solvent systems: chloroform and 20% isopropyl ether in petroleum ether (13).

Analysis of Vegetable Oils and Extracts of Foods

The sensitivity of the fluorometric detector depended upon the width of the slits, the age of the lamp and the performance of the photomultiplier amplifier. These factors thus determined the detectability of tocols. When the slits were set at 9 nm for excitation and 18 nm for emission, a measurable peak was obtained with 4 ng α-tocopherol (i.e. 20 µl of a solution containing 0.2 µg/ml) which was approximately double the height of the "noise". The sensitivity of the fluorometric detector with the tocols was a least ten-times superior to that of the variable wavelength absorbance detector (Figure 1). Up to 2 mg oil could be injected without affecting the quality of the separation. The fluorescence detector was also more selective than the absorbance detector and it could be used to measure tocols in crude lipid extracts (Figure 2). The response obtained with common vegetable oils (rapeseed, soybean etc) was similar to that obtained with purified standards of their constituent tocols except that large peaks were obtained at the "front" of the chromatography. Extracts of many foods and tissues have been chromatographed and the tocols were readily identified and measured in all the samples (Figure 3). So far, the only substances in lipid extracts which have been confirmed to produce peaks near those of the tocols are plastochromanol-8 and the antioxidant, BHA (Figures 1 and 2).

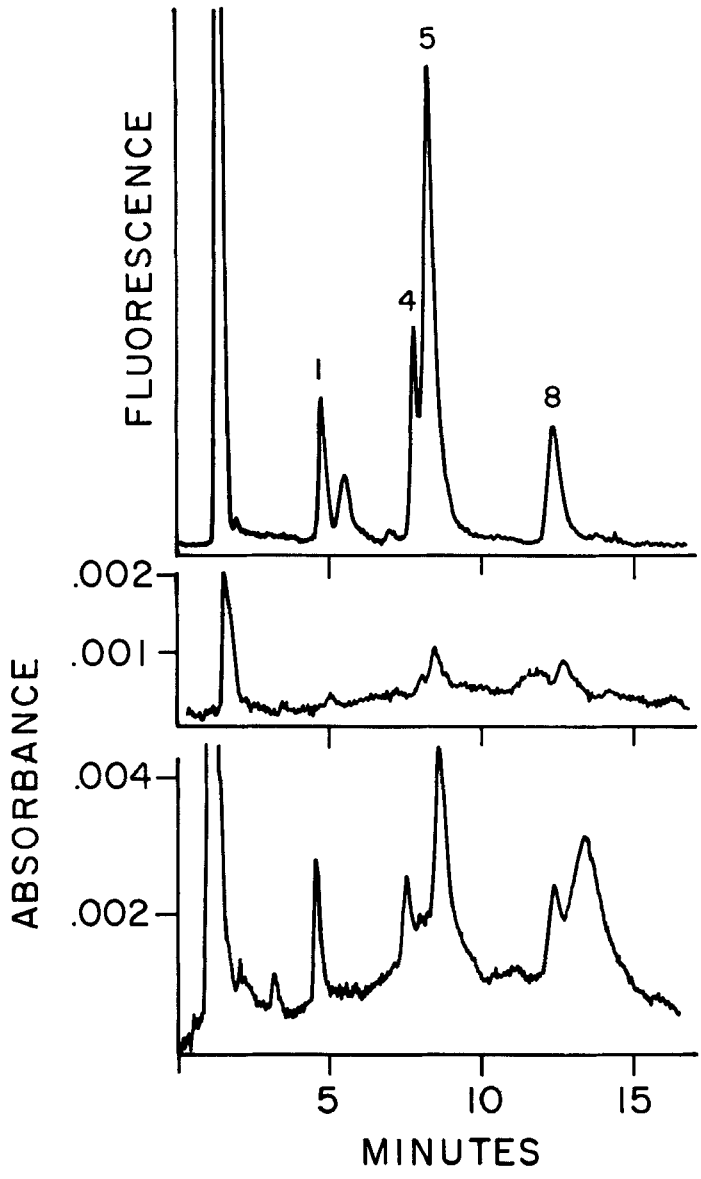

FIGURE 2. HPLC of extract of TV dinner (equivalent to 1 g dinner/ml) passed through fluorescence detector (upper) and secondly through adsorption detector (centre). HPLC of concentration extract (5g dinner/ml) using absorption detector (lower). HPLC system and identification of peaks as in Figure 1.

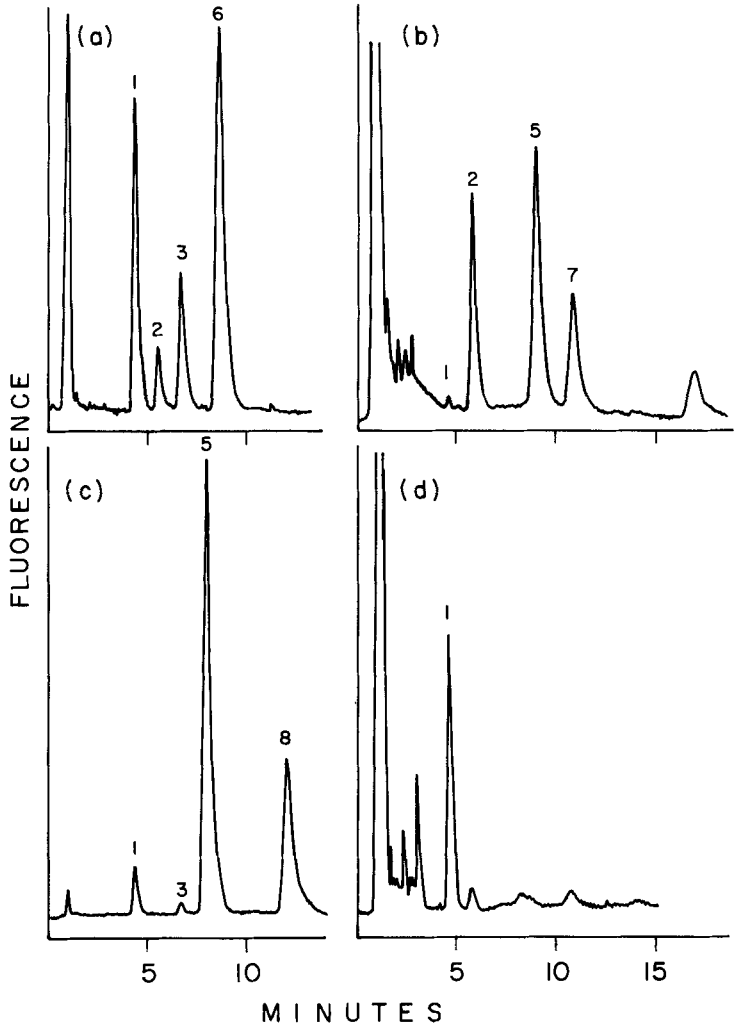

FIGURE 3. HPLC of lipids from (a) stone ground wheat flour (extract 0.5 g flour/ml) (b) barley (extract 2.5 g grain/ml); (c) soybean oil (10 mg oil/ml); and (d) milk (extract from 5 ml/ml).

Quantitative Analysis of Foods

Foods were analysed for individual tocols by extraction of lipids and chromatography of the crude lipid extracts.

Standards of α-, β- and γ-tocopherol were chromatographed after every two samples to correct for changes in the response of the fluorometric detector which dropped steadily from the maximum value obtained each day when the lamp was first ignited. The quantities of tocotrienol were calculated from the areas under the peaks using the factors obtained from the standards of corresponding tocopherols. Once the ratios of the responses of the different tocols had been obtained (Table 1), routine analysis could be speeded by using a standard of only α-tocopherol. The efficiency of the extraction procedure was tested during the analysis of spinach by reextracting the solid residue and filter paper and performing a second analysis. The second extract contained less than 1% of the α- and γ-tocopherols found in the first analysis. In a similar test with wheat flour, the second extract contained 2.5% of the α-tocopherol. Thus it was concluded that, even with grains, the efficiency of extraction was better than 97%.

The results of typical analyses are given in Table 3. The analysis of infant formula without saponification revealed the levels of tocols in the ingredients whereas analysis after saponification measured also the added α-tocopheryl acetate. The differnce was 11.5 μg α-tocopherol/g which was equivalent to 1.56 IU d-α-tocopherol/100 g or 1.27 IU dl-α-tocopherol/100 g. The label claimed 1.2 IU dl-α-tocopherol/100 ml. The standard deviations of replicate analysis of infant formula, spinach and beef (Table 3) give an indication of the reproduc-

TABLE 3

Tocols (μg/ml) in Foods[a]

	Number of Replicates	α	α_3	β	β_3	γ	γ_3	δ
Infant formula (not saponified)	4	0.84(0.08)	-	0.21(0.04)	-	15.80(0.79)	-	7.76(0.26)
Infant formula (saponified)	4	12.38(0.64)	-	0.24(0.05)	-	14.60(0.52)	-	7.41(0.65)
Spinach	3	26.05(0.31)	9.14(0.59)	-	-	-	-	-
Beef	3	2.24(0.05)	-	-	-	-	-	-
Wheat flour	1	8.2	1.7	4.0	16.4	-	-	-
Barley	1	0.02	7.0	-	6.9	-	2.8	-
Wheat breakfast cereal	1	4.4	3.3	2.5	8.7	-	-	-

[a]Standard deviation in parenthesis

ibility of the procedure. As an illustration of the specificity, the response of the detector to a variety of samples is shown in Figure 3.

DISCUSSION

Several reports describe the analysis of concentrates (6), grains (3), and serum (7,8) for vitamin E using HPLC and an absorbance detector. Only one system, however, was applied to the separation of tocopherols and tocotrienols (3) and it involved a run of 112 minutes on a 2m column. The use of a fluorometric detector to detect tocopherols during column chromatography was first reported for a low pressure chromatography on hydroxyalkoxypropyl Sephadex (14). Fluorometric detectors have since been used with modern HPLC systems to measure tocopherols in vegetable oils (2,4) and brain tissue (5).

The present procedure, which was developed from earlier work with columns of lipophilic Sephadex (14), represents an extension and refinement of the less sensitive method described by van Niekerk (2) for plants oils. It is rapid and it can be used to measure individual tocopherols and tocotrienols in serum, tissues and foods including complete meals. As the HPLC method is simple to perform and involves no preliminary steps of purification, it is superior in speed and reliability to older methods involving GLC or TLC. Moreover, it can be undertaken on a preparative scale. It is, therefore, an ideal method for the measurement and isolation of tocols in nutritional and biochemical research.

REFERENCES

(1) Bunnel, R.H., The Vitamins, Gyorgy, P and Pearson, W.N., eds., 2nd edit., Vol. VI, Academic Press, New York, 1967, p. 261.

(2) van Niekerk, P.J., The Direct Determination of Free Tocopherols in Plant Oils by Liquid-Solid Chromatography, Anal. Biochem. 52, 533 (1973).

(3) Cavins, J.F. and Inglett, G.E., High-Resolution Liquid Chromatography of Vitamin E Isomers, Cereal Chem. 51, 605 (1974).

(4) Abe, K., Yuguchi, Y. and Katsui, G., Quantitative Determination of Tocopherols by High-Speed Liquid Chromatography, J. Nutr. Sci. Vitaminol., 21, 183 (1975).

(5) Vatassary, G.T. and Hagen, D.F., A Liquid Chromatographic Method for Quantitative Determination of α-Tocopherol in Rat Brain, Anal. Biochem. 79, 129 (1977).

(6) Rückemann, H., and Raufft, K., Methoden zur Bestimmung von Vitaminen mittels Hochleistmgsflüssigkeitschromatographie, Z. Lebensm. Unters.-Forsch. 166, 151 (1978).

(7) Nilsson, B., Johansson, B., Jansson, L. and Holmberg, L., Determination of Plasma α-Tocopherol by High-Performance Liquid Chromatography, J. Chromatogr. 145, 169 (1978).

(8) De Leenheer, A.P., De Bevere, V.O., Cruyl, A.A. and Claeys, A.E., Determination of Serum α-Tocopherol (Vitamin E) by High-Performance Liquid Chromatography. Clin. Chem. 24, 585 (1978).

(9) Slover, H.T., Lehmann, J. and Valis, R.J., Vitamin E in Foods: Determination of Tocols and Tocotrienols, J. Amer. Chem. Soc. 46, 417 (1968).

(10) Whittle, K.J., Dunphy, P.J. and Pennock, J.F., The Isolation and Properties of δ-Tocotrienol from Hevea Latex, Biochem. J. 100, 138 (1966).

(11) Schudel, P., Mayer, H. and Isler, O., The Vitamins, Sebrell, W.H. and Harris, R.H., eds., 2nd edit., Vol. V, Academic Press, New York, 1972, p. 168.

(12) Mayer, H., Metzger, J. and Isler, O., Die Sterochemie von natürlichem γ-Tocotrienol (Plastochromanol-3, Platochromanol-8 und Plastochromenol-8), Helv. Chim. Acta 50, 1376 (1967).

(13) Whittle, K.J. and Pennock, J.F., The Examination of Tocopherols by Two-dimensional Thin-layer Chromatography and Subsequent Colorimetric Determination, Analyst 92, 423 (1967).

(14) Thompson, J.N., Erdody, P. and Maxwell, W.B., Chromatographic Separation and Spectrophotofluorometric Determination of Tocopherols Using Hydroxyalkoxypropyl Sephadex, Anal. Biochem. 50, 267 (1972).