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Isolation of four tocopherols and four tocotrienols from a variety of natural sources by semi-preparative high-performance liquid chromatography☆

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Abstract

Quantitative measurements of tocopherols and tocotrienols required isolation of four tocopherols from a mixture of soybean oil and wheat germ and four tocotrienols from a mixture of wheat bran and rubber latex. Semi-preparative HPLC was accomplished using a 250 mm × 10 mm I.D. column packed with 10-μm silica gel in hexane containing 2–15% tetrahydrofuran. Identification of isolated and purified tocopherols and tocotrienols was confirmed from mass spectra, and concentrations of identified vitamers were determined by absorption coefficients. Recovery of tocopherols and tocotrienols ranged from 54 to 83%. Isomer purities were found to be above 99% by capillary GC and HPLC.

1. Introduction

There are four known naturally occurring tocopherols termed α-, β-, γ- and δ-tocopherol, as well as four closely related compounds termed tocotrienols, which have three double bonds in the isoprenoid side chain (Fig. 1). Tocopherols are widely distributed in animals, cereals, fruits, vegetable oil, nuts and vegetables. Tocotrienols are mostly absent in nuts, fruits and vegetables. Small amounts of tocotrienols are found in carrots, sweetcorn and cereal bran and germ oils. These endogenous antioxidants each have a different specific activity, necessitating a separate determination.

Thin-layer chromatography (TLC) has been used widely to prepare tocopherol and tocotrienol standards, which were not available commercially. Natural sources were soybean oil for α-, γ- and δ-tocopherol, whole ground barley for α- and β-tocotrienol, corn for α-tocotrienol, and barley germ oil for γ-tocotrienol [1–5]. The primary disadvantage of the TLC procedure is its inability to distinguish between γ-tocopherol and β-tocotrienol in a one-dimensional system. Also, since tocopherols are antioxidants and are known to be light-sensitive, the TLC system has the disadvantage of leaving them vulnerable to oxidation during the time they are on the plate [1]. In 1979, Thompson and Hatina [6] used a preparative column to isolate α- and γ-toco-

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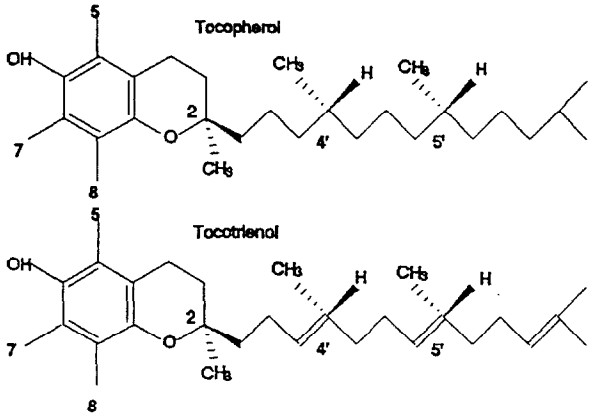

Position of methyl group	Tocopherols	Tocotrienols
5,7,8-Trimethyl	α-T	α-T3
5,8-Dimethyl	ß-T	ß-T3
7,8-Dimethyl	γ-T	γ-T3
8-Monomethyl	δ-T	δ-T3

Fig. 1. Structures of tocopherols and tocotrienols.

trienol as standards from wheat flour and rubber latex. More recently, Bruns et al. [7,8] applied technical-scale preparative liquid chromatography using a silica column to isolate tocopherols from vegetable oil. Saito and Yamauchi [15] isolated α-and β-tocopherol from wheat germ oil by recycle, semi-preparative supercritical fluid chromatography. For this isolation, special equipment is needed, such as a CO_2 pump, pre-heating coil and air-circulating oven, normally unavailable in typical laboratories.

Pure standard grade tocopherols (α, γ, δ) are available commercially, but tocotrienol standards are not. Recently released standards for γ- and δ-tocopherol (ICN Biochemicals, Division, CA, USA) are expensive and/or of lower purity. Research in our laboratory on rice bran composition and oxidative stability required analysis of endogenous tocopherols and tocotrienols. The purpose of the present study was to develop a procedure for isolation of highly purified tocopherols and tocotrienols as analytical standards from natural sources using semi-preparative liquid chromatography.

2. Experimental

2.1. Chemicals and materials

All solvents were HPLC grade from Mallinckrodt (Paris, KY, U.S.A). L-Ascorbic acid was from Sigma (St. Louis, MO, USA). Rubber latex was obtained from Malaysia, and soybean oil, wheat bran and wheat germ were purchased from a local grocery.

2.2. Extraction of crude oil

Rubber latex was used as a source for α-, γ- and δ-tocotrienols, soybean oil for α-, γ- and δ-tocopherol, wheat bran for β-tocotrienol, and wheat germ for β-tocopherol. To facilitate isolation, a mixture of soybean oil and wheat germ oil was used for tocopherols, as was a mixture of rubber latex lipid and wheat bran oil for tocotrienols.

For extraction of crude wheat germ and bran oil, 20 g of wheat germ or 40 g of wheat bran were placed in a 500-ml Erlenmeyer flask with 200 ml ethanol and 5 g ascorbic acid. The mouth of the flask was covered with a beaker and placed in a 60°C water bath for 10 min. Then 1.2 ml of 80% KOH were quickly added and mixed by vortexing. The sample was saponified for 10 min at 80°C. During saponification, the sample was agitated using a wrist-type shaker. After saponification, the flask was placed in an ice bath, and 30 ml water and 50 ml hexane were added. The mixture was vortexed, transferred to centrifuge bottles, and centrifuged at 120 *g* for 1 min. The upper layer was transferred to a 500-ml separatory funnel. Extraction of the sample with 50 ml hexane was repeated twice. The pooled hexane layer was washed three times with 30 ml water to remove residual KOH, filtered through Na_2SO_4, and then evaporated to dryness on a rotary evaporator. The crude oil sample was diluted with 10 ml methanol and the mixture was allowed to stand overnight at −20°C. The mixture was centrifuged, 12 000 *g* at −20°C for 30 min, and the supernatant filtered through a 0.45-μm filter. The filtrate was diluted with 20 ml water and extracted with 20 ml hexane twice.

Solvent was evaporated to dryness under a stream of nitrogen and sample diluted with a known amount of hexane and placed under nitrogen at −20°C.

For extraction of latex oil, the method of Whittle et al. [9] was employed with modification. Samples of latex (50 ml) were added to 500 ml chloroform with stirring and then homogenized in a Tissumizer (Tekmar, Cincinnati, OH, USA) for 5 min. To this mixture 250 ml methanol were added and stirred until the rubber coagulated. The coagulated rubber was filtered, then dried on a rotary evaporator under reduced pressure.

Saponification and crystallization [9] of latex oil (4 g) and commercial soybean oil (4 g) were as described previously.

2.3. Apparatus

Semi-preparative HPLC

The semi-preparative HPLC system that was used consisted of Waters (Milford, MA, USA) M-45 and 510 pumps, a Waters 680 automated gradient controller, a Waters 470 scanning fluorescence detector with 18 nm spectral bandwidth for excitation and emission, a Hewlett-Packard (San Fernando, CA, USA) UV–Vis diode-array detector (series 1050) and a Waters 715 Ultra WISP injector equipped with a 2000-μl loop and 200-μl syringe. Chromatograms were recorded and peaks determined using a Baseline 810 Chromatography workstation (Waters). Concentrated extracts were injected into a 25 cm × 10 mm diameter column of 10-μm Alltech Econosil silica (Deerfield, IL, USA). The column was used with 5 cm × 4.6 mm I.D. guard column packed with 40-μm Supelco pellicular silica (Bellefonte, PA, USA). The mobile phase consisted of a gradient of 0–15% tetrahydrofuran (THF) in hexane at a flow-rate of 8–9 ml/min, and the eluate was monitored from the fluorescence detector at 290 nm excitation and 330 nm emission. Eluates considered as α-, β-, γ- and δ-tocopherols and tocotrienols in each experiment were collected into 250-ml amber bottles with PTFE cap using a Gilson Model 202 fraction collector (Beltline-Middleton, WI, USA). Collection bottles were placed in a 50°C water bath and solvent evaporated in the dark using ultra-high-purity nitrogen. The pooled specimens of α-, β-, γ- and δ-tocopherols and tocotrienols were rechromatographed until the specimens were pure chromatographically and spectrophotometrically. The pooled specimens were also concentrated. To check purity of each isomer, the absorption ratio of 295 nm to 245 nm wavelength with 4 nm bandwidth and 390 nm reference wavelength were compared using a diode array detector. The concentrations of fractionated vitamin E vitamers were determined using a Gilford UV–Vis spectrophotometer (Oberlin, OH, USA).

For semi-preparative HPLC, solvent A was 40% (v/v) THF in hexane, and solvent B was 100% hexane. The solvents were filtered through Millipore 0.45-μm membranes prior to use. THF was distilled [10] to remove peroxides that might form during storage prior to mobile phase incorporation.

Analytical HPLC

The analytical HPLC system was similar to the semi-preparative system with modifications as follow. Samples (0.5–5 μl) were injected using a 200-μl loop and 25-μl syringes into a 25 cm × 4.6 mm diameter column of 5-μm Sulpelcosil LC-Si (Supelco). The column was preceded by a 5 cm × 4.6 mm I.D. guard column packed with 40-μm pellicular silica. The mobile phase consisted of 2.4% ethyl acetate in isooctane at a flow-rate of 2.0 ml/min, and the eluate was monitored from the fluorescence detector. Isolated and purified tocopherols and tocotrienols were used as standards.

Gas chromatography (GC) and mass spectrometry (MS)

Purities of isomers were determined using a J & W Scientific (Folsom, CA, USA) 95% dimethyl-/5% diphenylpolysiloxane capillary column (0.25-μm stationary phase thickness, 30 m × 0.25 mm I.D.) on a Hewlett-Packard 5890 gas chromatograph equipped with a split/splitless capillary inlet system and a flame ionization detector. Column oven temperature was pro-

grammed to increase from an initial temperature of 40°C to a final temperature of 280°C. Oven temperature was maintained at the initial temperature for 3 min after injection and then increased at 20°C/min to a temperature of 280°C for 40 min. Other operation parameters were as follows: injector temperature, 300°C; detector temperature, 350°C; helium carrier gas flow, 30 cm/s; split ratio, 1/50. A Maxima 820 Chromatography workstation was used to determine peak areas (Waters).

Mass spectra of tocopherols and tocotrienols were obtained by a Hewlett-Packard 5890 series II gas chromatograph/5971A mass spectrometer with splitless (holding time, 0.75 min) injection. GC column and temperature program were as described previously. Additional conditions were as follows: ionization voltage, 70 eV; electron multiplier voltage, 1800; scan range, 40–450 u; interface temperature, 280°C; injector temperature, 250°C.

3. Results and discussion

3.1. Sample preparation

Saponification of oils concentrated vitamin E vitamers and removed interfering glycerides and other hydrolyzable materials. At the same time, saponification liberated tocopherols and tocotrienols from esters that may have been present. Chow et al. [11] reported that rubber latex lipid contains about 68% esterified tocotrienol. The unsaponifiable matter contains higher aliphatic alcohols (waxes), sterols, pigments, and hydrocarbons. In wheat germ and soybean oil, the major components of the unsaponifiable matter are sterols [12]. Some sterols may be removed by precipitation at low temperature and filtration [6,11,13,14]. During sample preparation it is important to reduce mass of sample as much as possible to increase sample loading, reduce analysis time, and improve column stability. Table 1 shows changes in mass balance of sample during separation steps. With saponification and crystallization, over 95% of sample mass could be reduced from commercial soybean oil and extracted oils.

3.2. Isolation of tocopherols and tocotrienols

Fig. 2 shows a chromatogram of vitamin E vitamers from soybean and wheat germ oil. Before sample injection, the column was flushed with a gradient system (Table 2) for 25 min. The mixture had a concentration of 479 mg/ml in 2.5% THF in hexane, and 60 μl (about 28.8 mg)

Table 1
Mass balance of sample preparation

Sample	Mass (g)			
	Soybean oil	Wheat germ	Wheat bran	Latex
Sample before extraction	2	20	40	20
Sample after extraction	–	0.0471[a]	0.0395[a]	0.2194[b]
Sample after saponification	0.0165	–	–	0.07635
Sample after crystallization[c]	0.00907	0.0164	0.0146	0.05008

[a] Extracted with saponification.
[b] Extracted without saponification.
[c] Crystallized at −20°C for 12 h and centrifuged at −20°C.

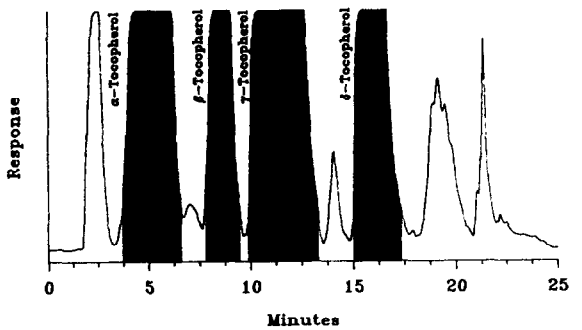

Fig. 2. Chromatogram of a mixture of a soybean oil and wheat germ oil; solid peaks represent fraction cuts. Chromatographic conditions are described in text and in Table 2.

were injected. Injection of over 35 mg of the mixture decreased vitamers' resolution. A flow-rate between 8 and 9.99 ml/min, which was the maximum flow-rate of our controller, had no influence on resolution. Peaks of α-, β-, γ- and δ-tocopherol in Fig. 2 represent amounts of 0.68, 0.29, 2.02 and 0.84 mg per injection, respectively. α-Tocotrienol eluted between α-tocopherol and β-tocopherol, and β-tocotrienol eluted between γ-tocopherol and δ-tocopherol. After 17 min, more polar compounds such as sterols eluted. Tocotrienols and most late eluates came from wheat germ oil. An injection of soybean oil alone produced level base line after δ-tocopherol eluted. Sterols could not be removed completely with precipitation at low temperature. To remove sterols completely, crystallization and digitonin precipitation were used [9].

The column required cleaning between injections to obtain constant retention time and pure fractions. Hexane with 14% THF (Table 2, from 13.7 to 14.5 min) was used to remove late-eluting compounds and stabilize retention time. A higher concentration of THF could be applied to accelerate elution of late compounds, but analysis time would be similar because the column would require reequilibration to the 12% THF mobile phase. Solvent A was prepared with 40% THF in hexane rather than pure 100% THF to improve control of the gradient system.

Fig. 3 shows the chromatogram of a mixture of a latex oil and wheat bran oil (gradient in Table 3). The mixture of latex oil and wheat bran oil with concentration of 485 mg/ml in hexane containing 2.5% THF was prepared, and 180 μl (about 87.3 mg) were injected. Injection of over 95 mg decreased resolution of tocopherols and tocotrienols. The eluate between α-tocotrienol and β-tocotrienol was β-tocopherol. The peak between γ-tocotrienol and δ-tocotrienol was presumably δ-tocopherol. But δ-tocopherol was not detected in wheat bran oil and latex lipid using an analytical column. Peaks of α-, β-, γ- and δ-tocotrienol in Fig. 3 represent amounts of 0.78, 0.55, 3.37 and 0.77 mg per injection, respectively. The sample matrix of wheat germ oil and wheat bran oil was complex, so isolation of β-

Table 2
Mobile phase gradient program for mixture of soybean oil and wheat germ oil

Time (min)	Solvent A (%)[a]	Solvent B (%)[b]	Curve[c]
Initial	30	70	*
0.5	6	94	5
2.0	10	90	5
9	10	90	6
13.7	35	65	6
14.5	35	65	6
20	30	70	6

Flow-rate 8 ml/min.
[a] Hexane–THF (60:40).
[b] Hexane (100%).
[c] Pre-programmed gradient curve in Waters 680 automated gradient controller. * = No curve.

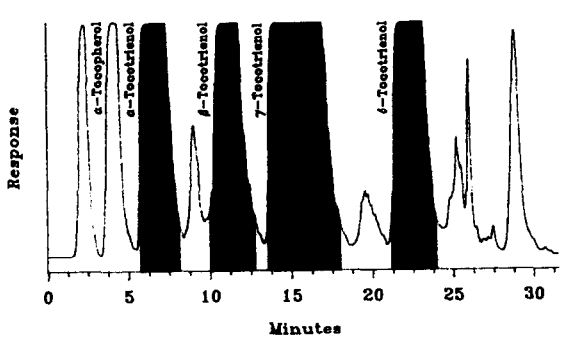

Fig. 3. Chromatogram of a mixture of a wheat bran oil and latex lipid; solid peaks represent fraction cuts. Chromatographic conditions are described in text and in Table 3.

Table 3
Mobile phase gradient program for latex lipid and wheat bran oil

Time (min)	Flow (ml/min)	Solvent A (%)[a]	Solvent B (%)[b]	Curve[c]
Initial	8	30	70	*
0.7	8	8	92	6
1.1	8	5	95	5
5	8	5	95	6
13	8	7	93	5
14.5	8	8	92	4
16.5	8	10	90	9
20	8	15	85	8
22	9	35	65	10
25	9	35	65	6
27	8	30	70	8

[a] Hexane–THF (60:40).
[b] Hexane (100%).
[c] Pre-programmed gradient curve in Waters 680 automated gradient controller. * = No curve.

tocopherol or β-tocotrienol from wheat germ oil took over 20 min.

3.3. Fraction purification

Purities of first fraction were over 88% and those of second fraction over 97% by analytical HPLC. Isocratic mobile phase systems were used during purification steps. The concentrations of THF in hexane as a modifier solvent ranged from 7% for α-tocopherol to 18% for δ-tocotrienol. To reduce the purification time in the last steps, the amounts of THF in hexane were increased.

3.4. Purity checks and concentration determination

To characterize fraction purity to a greater extent, the ratio of UV absorptions (295/245 nm) was determined using a UV diode array detector (Fig. 4). Saito and Yamauchi [15] used the ratio of 230 to 295 nm to check purities of α- and β-tocopherol fraction. Absorption at 230 nm represents tocopherols, fatty acids, and their esters, and the absorption at 295 represents only tocopherols. Presumably, our samples were free from fatty acids and triglycerides, so we used 245 nm to check for sterols in isomer fractions. Eluates from the fluorescence detector were

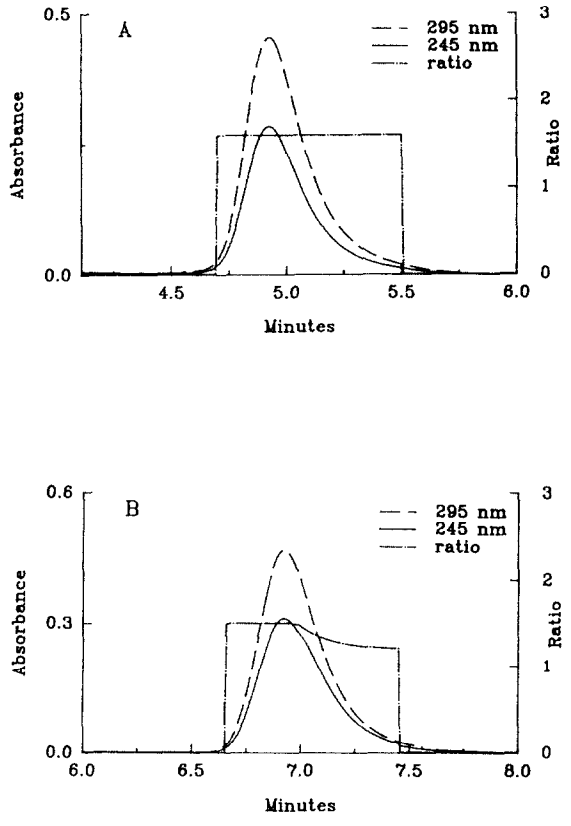

Fig. 4. Chromatograms at wavelengths 295 nm, 245 nm and the ratio of the two for (A) δ-tocopherol and (B) δ-tocotrienol.

Table 4
Analytical data for isolated and purified tocopherols and tocotrienols fractions from 6 g of a mixture of soybean oil and wheat germ oil and 8 g of a mixture of wheat bran oil and latex lipid

Sample	α-T	β-T	γ-T	δ-T	α-T3	β-T3	γ-T3	δ-T3
Purity (%)[a]	99	99	99	99	99	99	99	99
Recovery (%)	79	75	83	78	76	72	81	54
Yield (mg)	112.2	45.22	350.4	136.5	54.16	33.96	250.5	38.2

[a] By gas chromatography.

passed through the UV detector with HPLC conditions similar to the purification procedure, except injection quantity was reduced to 5–20 μl. As shown in Fig. 4A, the δ-tocopherol peak produced a constant signal ratio (295 nm/245 nm) throughout the peak's elution. However, the δ-tocotrienol peak evidenced a change in the signal ratio (295 nm/245 nm). Impurities in the δ-tocotrienol fraction eluted at a similar time, so purification with the column could not be obtained. The impurities were not identified.

Fractionated δ-tocotrienol were scanned at UV–Vis wavelengths from 200 to 350 nm. The chromatogram had high absorption values in the wavelength range around 260 nm (minimum wavelengths of δ-tocotrienol). The fraction of δ-tocotrienol after three HPLC passes still contained impurities, which were then chromatographed two additional times using TLC with silica gel G (250 μm) and 20% diisopropyl ether in light petroleum (b.p. 35–60°C) [9]. After TLC fractionation the purity was satisfactory as is shown in Table 4.

Each fractionated solution in the last purification steps was evaporated to dryness, weighed, and diluted with known amount of hexane. A known amount of each tocopherol and tocotrienol solution was evaporated to dryness and diluted with ethanol to determine the concentration of vitamin E vitamers in hexane solution by published molar absorbance values $E^{1\%}_{1\,cm}$ in Table 5. However, absorption maxima and molar absorbance values found in the literature differ. Absorption maxima that matched published $E^{1\%}_{1\,cm}$ were chosen in accordance with UV–Vis spectrum maxima of purified vitamers in Fig. 5. For β-tocotrienol, two $E^{1\%}_{1\,cm}$ values at 294 nm have been published, so the average value of

Table 5
UV absorption maxima and molar absorbance of tocopherols and tocotrienols in ethanol solution taken from the literature

Compound	λ_{max} (nm)[a]	λ_{max} (nm)	$E^{1\%}_{1\,cm}$
Tocopherol			
α-	292	*292* [16,20]	*75.8* [16,19]
β-	296	*296* [16]	*89.4* [16]
γ-	298	*298* [16]	*91.4* [16]
δ-	298	*298* [16]	*87.3* [16]
Tocotrienol			
α-	292	*292.5* [17], 290 [18]	*91* [17], 77.2 [18]
β-	294	*294* [16,18], 295.5 [20]	*87.3* [16], *85.5* [18], 87.5 [20]
γ-	296	*296* [19], 298 [20]	*90.5* [17], 103 [20]
δ-	297	*297* [9], 292 [20]	*88.1* [9], 83.0 [20]

Italicized values used to determine the concentration of vitamers.
[a] Isolated and purified vitamers in Fig. 5.

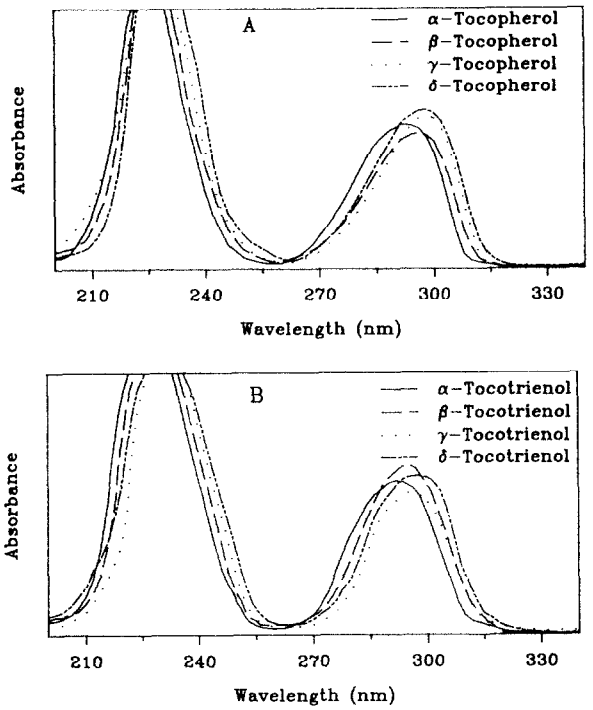

Fig. 5. UV–visible spectra of isolated and purified tocopherols and tocotrienols.

86.4 was used for calculation of β-tocotrienol concentration. The concentrations of purified vitamin E vitamers as determined were used as standards for HPLC assays.

3.5. GC–MS

Table 6 shows molecular masses and major peaks (m/z) of purified vitamin E vitamers in mass spectra. The major mass fragmentations of β- and γ-tocopherol and β- and γ-tocotrienol were the same, since these are positional vitamers having the same molecular masses. Also, they had a similar retention time on GC. However, using HPLC, differences in absorption maximum and retention time were noted (Table 5). The peaks of m/z 205, 191 or 177 ($M_r - 255$ or 219) indicates the loss of a side chain ($C_{16}H_{33}$ for tocopherols or $C_{16}H_{27}$ for tocotrienol), and peaks of m/z 165, 151 or 137 (205, 191 or 177 − 40) originated from the cleavage of the side chain accomplished by the breakdown of

Table 6
Molecular masses and major m/z ratios of vitamin E vitamers in mass spectrum

Compound	M_r	m/z
Tocopherol		
α-	430	43, 55, 57, 165, 205, 430
β-	416	43, 55, 57, 107, 151, 191, 416
γ-	416	43, 55, 57, 107, 151, 191, 416
δ-	402	43, 55, 69, 137, 163, 177, 402
Tocotrienol		
α-	424	41, 55, 69, 81, 165, 203, 205, 424
β-	410	41, 55, 69, 81, 151, 189, 191, 410
γ-	410	41, 55, 69, 81, 151, 189, 191, 410
δ-	396	41, 55, 69, 81, 137, 177, 189, 396

chroman structure with hydrogen rearrangement and loss of a methyl acetylene $CH_3-C\equiv CH$ fragment [21].

Fig. 6 shows chromatograms of purified α-tocopherol and α-tocotrienol. As can be seen,

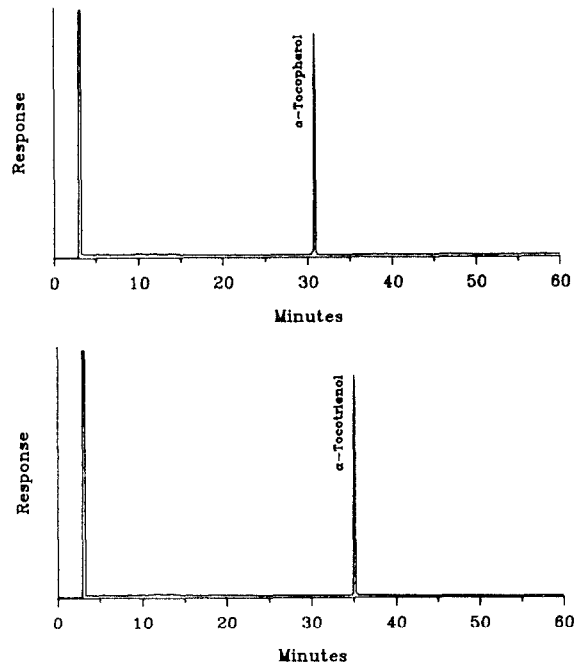

Fig. 6. Gas chromatograms of isolated and purified α-tocopherol and α-tocotrienol. Chromatographic conditions are given in the text.

the chromatograms have a single peak and clear baseline. Other chromatograms for β- + γ- and δ-tocopherols and tocotrienols had similar characteristics. Purities of vitamers were over 99% based on the peak area percentage.

3.6. Analytical HPLC analysis of isolated and purified vitamers

Baseline resolution was obtained for all vitamers in the mixtures of soybean oil and wheat germ oil, and wheat bran oil and latex. Table 7 shows the concentrations using analytical HPLC. Concentrations of tocopherols and tocotrienols were calculated from peak areas and corresponding standard curves ranging from 0.1 to 1.5 μg/ml. Table 4 shows recoveries, purities and yields of isolated and purified vitamers. Purities of all vitamers were over 99% by GC and HPLC. Analytical HPLC chromatograms were clearer than GC ones due to the high selectivity of fluorescence detection for vitamin E vitamers. γ-Tocopherol and tocotrienol had higher recovery than other vitamers because concentrations of these vitamers in source oils were higher than those of other vitamers. Recovery of δ-tocotrienol was lower than the other vitamers because it required additional TLC procedures to attain purification.

4. Conclusions

Four vitamers of tocopherol and tocotrienol were isolated from a mixture of natural sources by semi-preparative HPLC for use in analytical HPLC. Recovery was higher than by conventional TLC. The semi-preparative HPLC column is useful to obtain small amounts of pure tocopherols and tocotrienols as an alternative to conventional methods that tend to be tedious and time consuming, such as distillation, extraction, crystallization and TLC. Isolation also was more efficient because all tocopherols and tocotrienols can be obtained from a mixture of several natural sources simultaneously and each fraction collected and rechromatographed to obtain pure standards.

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Table 7
Contents of tocopherols and tocotrienols in a mixture of soybean and wheat germ oil and a mixture of wheat bran oil and latex lipid

Sample	Contents (mg/g)							
	α-T	β-T	γ-T	δ-T	α-T3	β-T3	γ-T3	δ-T3
Mixture of soybean oil and wheat germ oil[a]	23.66	10.05	70.29	29.171	0.114	0.491	–	–
Mixture of wheat bran oil and latex lipid[b]	4.164	0.612	–	–	8.914	5.901	38.66	8.846

[a] Soybean oil (1 g)–wheat germ oil (1 g) in 2.5% THF in hexane.
[b] Wheat bran oil (1 g)–latex lipid (2 g) in 2.5% THF in hexane.

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