Analysis of Tocopherols in Vegetable Oils by High-Performance Liquid Chromatography: Comparison of Fluorescence and Evaporative Light-Scattering Detection

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A comparison of the responses of an evaporative light-scattering detector (ELSD) and a fluorescence detector for tocopherols in vegetable oils by high-performance liquid chromatography is presented. The tocopherols were separated from acylglycerols by gel-permeation chromatography (GPC). The tocopherol fraction was collected off a set of four GPC columns with a mobile phase of methylene chloride before separation on a normal-phase silica column with a mobile phase of hexane/isopropanol, 99.7:0.3 (vol/vol). An internal standard of 5,7 dimethyltocol, which was detected by both the ELSD and fluorescence detector, was used to obtain quantitative data. The fluorescence detector was ten times more sensitive than the ELSD. γ-Tocopherol was the major tocopherol detected in the vegetable oils studied and ranged from 24.1–93.3 mg/100 g. The amounts of tocopherols found in the vegetable oils agreed favorably with the literature values.

KEY WORDS: ELSD, evaporative light-scattering detector, fluorescence, GPC, HPLC, internal standard, normal-phase, tocol, tocopherols.

Over the past decade, the tocopherol content of foods has been determined by a wide range of techniques, including high-performance liquid chromatography (HPLC) (1-3). The HPLC techniques have been used mainly either with ultraviolet (UV) or fluorescence detection. Yao *et al.* (4) adapted methods of Hakansson *et al.* (2) for normal-phase chromatography with a silica column in conjunction with a fluorescence detector to quantitate tocopherols in pecans. Excellent chromatograms were obtained with a mobile phase of 1% isopropanol in hexane. Normal-phase chromatography is advantageous for resolving tocopherols because β- and γ-tocopherol cannot be resolved by reverse-phase chromatography (5). Recently, however, Warner and Mounts (6) developed a reverse-phase chromatographic method to assay tocopherols in vegetable oils. Fairly good separation was achieved for α-, β-, γ- and δ-tocopherols with a C-18 column and a mobile phase of $CH_3CN/THF/H_2O$ (60:25:15, vol/vol/vol) in conjunction with an evaporative light-scattering detector (ELSD). The oils were dissolved in hexane with no prior extraction or clean-up. The fact that hexane is immiscible with the mobile phase of $CH_3CN/THF/H_2O$ led Warner and Mounts (6) to speculate that the tocopherols are deposited on the head of the column rather than immediately flowing with the mobile phase, thus enhancing the peak separation. This, in fact, may be true. However, injecting a solvent that is immiscible and incompatible with the mobile phase can only lead to shortened column life as more compounds become trapped on the column.

The ELSD is a fairly new type of HPLC detector. The ELSD is considered a universal detector due to its ability to detect every compound eluting off the column with or without a chromophore or fluorophore. The HPLC column effluent is converted to a fine mist by passage through a nebulizer assisted by a carrier gas. The fine mist droplets are carried through a temperature-controlled drift tube where vaporization occurs, and the less volatile droplets pass through a laser light beam. The droplets scatter the light, which is then detected by a sensitive photodiode system. The detector response is thus a function of the mass of the solute particles (7).

The objective of this study was to compare the responses and detection limits between fluorescence and ELSD connected in series for four tocopherols, α, β, γ and δ, in edible oils.

MATERIALS AND METHODS

Materials. Four of the five samples of commercially available oils, namely crude soy oil, refined bleached and deodorized (RBD) soy oil, crude peanut oil and crude canola oil, were obtained from Archer Daniels Midland Company (Decatur, IL). The fifth sample, RBD palm oil was obtained from Misr Gulf Oil Processing Company (Cairo, Egypt). Tocopherol standards (α, β, γ and δ) were obtained from Henkel Corporation (LaGrange, IL). 5,7-Dimethyltocol internal standard was obtained from Matreya Inc. (Pleasant Gap, PA). The purity of each standard was established by measuring the E1% (extinction coefficient in a 1-cm cuvette) values spectrophotometrically. All solvents were HPLC-grade and obtained from Fisher Scientific (Norcross, GA).

HPLC. A Hewlett-Packard (Avondale, PA) 1090 Win liquid chromatograph, equipped with a 1046A programmable fluorescence detector (set at 290 nm excitation and 330 nm emission) coupled to a Vectra 486 computer, was used to analyze the samples. A Sedex 45 evaporative light-scattering mass detector (Richard Scientific, Novato, CA) was connected in series with the fluorescence detector. The ELSD was set at a gain of 7 at 40°C with a nebulizer gas pressure of 2.1 psi. A Hewlett-Packard 35900 digital A/D analog interface connected the mass detector electronically to the Vectra 486 computer.

Clean-up HPLC. The tocopherols were separated from the oil in each sample by gel permeation chromatography (GPC) with a mobile phase of CH_2Cl_2, an injection volume of 250 μL and a flow rate of 1.0 mL/min. Four GPC columns were connected in series and consisted of a Beckman (San Ramon, CA) Ultrasphere 1000 Å (7.7 mm × 30 cm), a Beckman Ultrasphere 500 Å (7.7 mm × 30 cm), a Waters (Milford, MA) μStyragel 100 Å (7.8 mm × 30 cm) and a Waters μStyragel 500 Å (7-8 mm × 30 cm).

Analytical HPLC. A Beckman Ultrasphere 5 μm silica column (4.6 mm × 25 cm) was used with a mobile phase of 99.3:0.7 hexane/isopropanol with an injection volume of 100 μL and a flow rate of 1.0 mL/min.

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Analysis. Approximately 1 g of each oil was accurately weighed and diluted to 10 mL in CH_2Cl_2. Upon mixing, 250 μL was injected into the liquid chromatograph system with the four GPC columns installed in series. The tocopherol fraction was collected at a predetermined retention time window *via* a three-way valve installed after the fluorescence detector. The collected fraction was evaporated to dryness with nitrogen and reconstituted to 0.5 mL with the hexane/isopropanol mobile phase.

The vials containing the collected fractions off the GPC from each oil and the standard dilutions were loaded into the autoinjector for quantitation with the silica column. The tocopherol peaks were identified by predetermining the retention times of individual tocopherol standards. The amount of sample was quantitated by an on-line computer in the presence of tocol as an internal standard.

RESULTS AND DISCUSSION

Unlike a UV detector of fluorescence detector, the ELSD detector is universal. It does not require a chromophore or a fluorophore in the analyte. Any molecule present in the injection volume, if the concentration is adequate, will be detected by the ELSD. Initially, the vegetable oils were injected directly onto the silica column. The tocopherols were easily detected by fluorescence (Fig. 1a) with no interfering peaks. The tocopherols were not clearly separated or detected by the ELSD (Fig. 1b) due to other compounds in the oil, such as acylglycerols, creating interference peaks in the chromatogram.

An earlier method, developed by Landen and Eitenmiller (8) for the separation of retinyl palmitate and β-carotene from oils and margarines, was adapted to this study. Landen used three GPC columns in his initial work (8,9) and four GPC columns later on in the work with vitamin D (10) for resolution of the acylglycerol from the nutrients of interest. The elution of the acylglycerol was then monitored by refractive index (RI) detection. This study used four GPC columns in series with an ELSD in place of an RI detector to monitor the elution of the acylglycerol peak. The tocopherol fraction was then collected from the GPC columns and re-injected onto the analytical column. The chromatograms from the fluorescence detector (Fig. 2a) and ELSD (Fig. 2b) were now free from interference. Although this study did not compare the responses of an RI detector to an ELSD detector, Hopia and Ollilainen (11) have shown that the ELSD is in fact more sensitive than an RI detector. Their work in lipid analysis showed that the detection limit of triolein is 50 ng per injection by RI detection and 30 ng per injection by ELSD. Incorporation of an ELSD is a promising modification to Landen's methods (8–10), not only because the ELSD is more sensitive than RI, but because the ELSD vaporizes to exhaust the column effluent which, in this case, is methylene chloride, and eliminates costly hazardous waste disposal.

The findings in this study seem to be in contradiction to the work of Warner and Mounts (6), who reported baseline resolution of the four tocopherols after direct injection onto a reverse-phase C-18 column connected to an ELSD. It is unclear, however, whether the chromatogram they presented represents a standard or a sample. However, an unsuccessful attempt was made in this laboratory to use their methodology with a similar C-18 column to

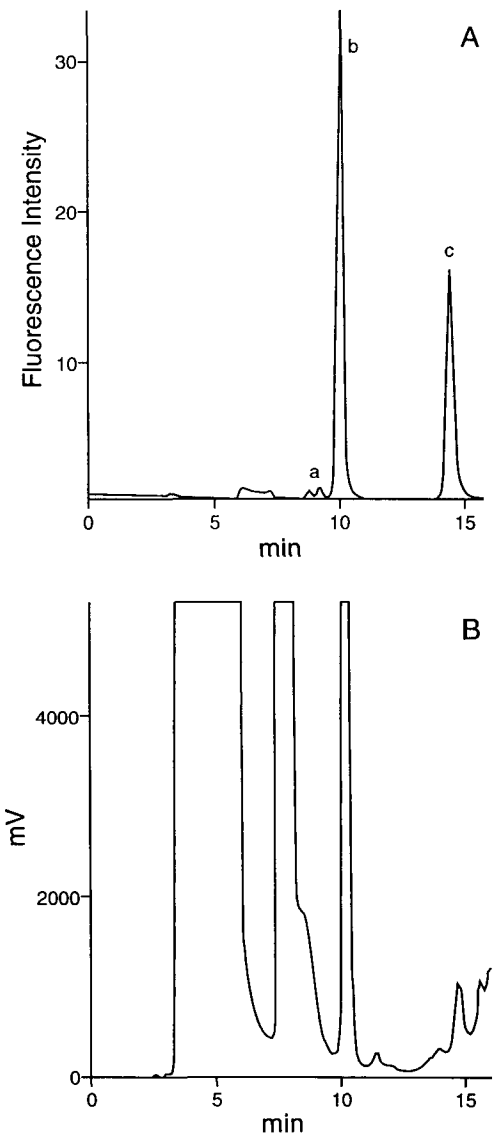

FIG. 1. High-performance liquid chromatography chromatogram of crude soy oil on a silica column without prior cleanup. (A) Fluorescence detection (excitation 290 nm, emission 330 nm), (B) evaporative light-scattering detector (gain 7, 40°C, nebulizer pressure 2.1 psi.), where a = β-tocopherol, b = γ-tocopherol and c = δ-tocopherol.

resolve β- and γ-tocopherols. Reverse-phase chromatography is normally incapable of resolving these tocopherols. An aliquot of the stock standard tocopherol solution was dissolved in hexane and then diluted in the mobile phase used by Warner and Mounts (6). Separation occurred immediately and two phases were observed in the flask. The standard was vigorously shaken prior to injecting the mixture of α-, β-, γ- and δ-tocopherols. The resulting chromatogram had only three peaks in place of four, indicating that two of the tocopherols (β- and γ-tocopherol) were co-eluting. This method was abandoned for fear of having components from the sample solution precipitate on the column and destroy it. Therefore, the earlier method of Yao *et al.* (4) that had been performed in this laboratory was adopted.

To avoid the use of a linear regression for quantitation of each tocopherol, this study successfully explored the

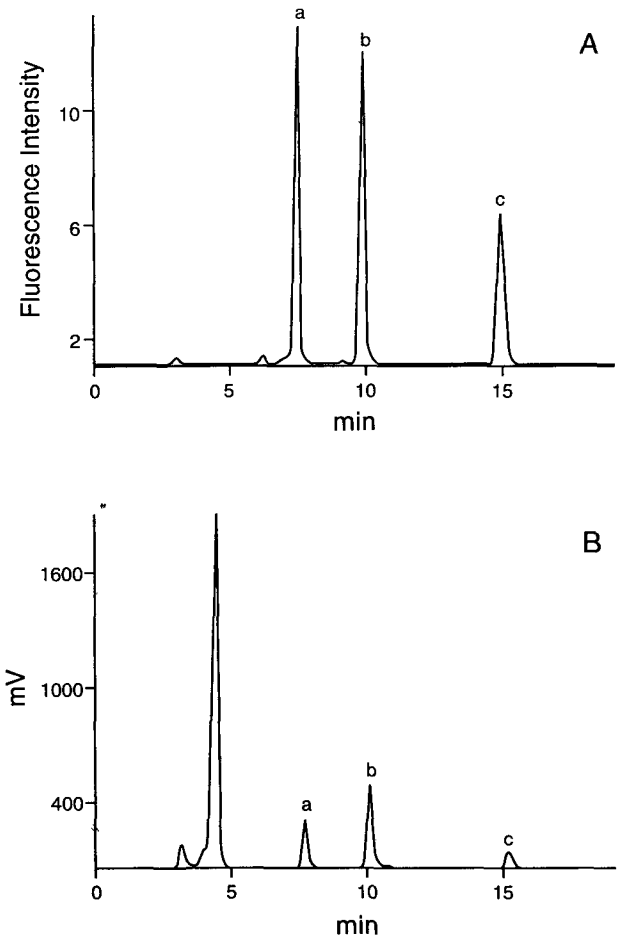

FIG. 2. High-performance liquid chromatography chromatogram of crude soy oil on a silica column with prior gel-permeation chromatography cleanup. (A) Fluorescence detection, (B) evaporative light-scattering detector (see Fig. 1 legend for conditions), where a = α-tocopherol, b = γ-tocopherol and c = δ-tocopherol.

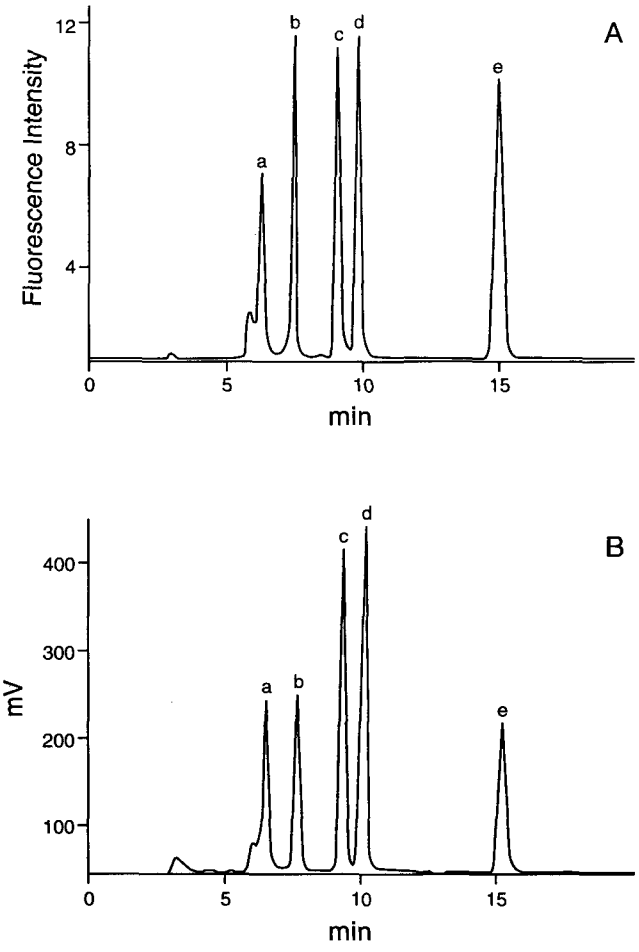

FIG. 3. High-performance liquid chromatography chromatogram of a mixed tocopherol standard on a silica column with tocol added as an internal standard. (A) Fluorescence detection, (B) evaporative light-scattering detector (see Fig. 1 legend for conditions), where a = internal standard, b = α-tocopherol, c = β-tocopherol, d = γ-tocopherol and e = δ-tocopherol.

use of an internal standard for quantitation of the tocopherols by both an ELSD and a fluorescence detector. Tocol is unique in that it contains a fluorophore for fluorescence detection and can also be detected by the ELSD. Figure 3 shows mixed tocopherol standard solutions and illustrates that 5,7-dimethyltocol elutes between the α- and β-tocopherol with baseline resolution. Therefore, four tocopherols and an internal standard were chromatographed in 15 min. Warner and Mounts method (6) required up to 27.7 min for complete elution. This is almost twice the time necessary for elution by normal-phase chromatography.

The fluorescence detector was linear over an approximate range of 25–0.25 μg/mL for each tocopherol with an injection volume of 100 μL (Fig. 4a). The correlation coefficients (r) for the fluorescence detector were 0.994, 0.997, 0.997 and 0.998 for α-, β-, γ- and δ-tocopherol, respectively. The same standards were used with the ELSD, but it was linear over a range of 25–2.5 μg/mL with an injection volume of 100 μL (Fig. 4b). The correlation coefficients (r) for the ELSD were 0.987, 0.990, 0.990 and 0.995, for α-, β-, γ- and δ-tocopherol, respectively. Below an injection amount of 250 ng the ELSD baseline became noisy, and the tocopherols could not be discerned. This level of 250 ng is a 40-fold increase in sensitivity over that illustrated in the work of Warner and Mounts with a Varex ELSD (Varex Corp., Burtonsville, MD) (6). The lowest point on the calibration curve in the study by Warner and Mounts (6) was 10 μg. A comparison between the fluorescence detector and the Sedex 45 ELSD indicated that the fluorescence detector was ten times more sensitive than the ELSD.

Table 1 illustrates the total and individual tocopherol contents found in each of the five oils studied by fluorescence detection. RBD palm oil was rich in α-tocopherols and γ-tocotrienols, while crude canola oil was rich in α- and γ-tocopherols. γ-Tocopherol was the major tocopherol detected in the vegetable oils studied (21.3–93.3 mg/100 g). Each of the values in the table falls either within or close to the range listed by Machlin (12), with the exception of canola oil, which was not listed. The oils could not be injected directly onto the silica column after being diluted with hexane, as previously discussed, but first required a GPC clean-up step. This step resulted in a dilution of the sample, which pushed the tocopherols either to or below the detection limit of the ELSD. To bring the

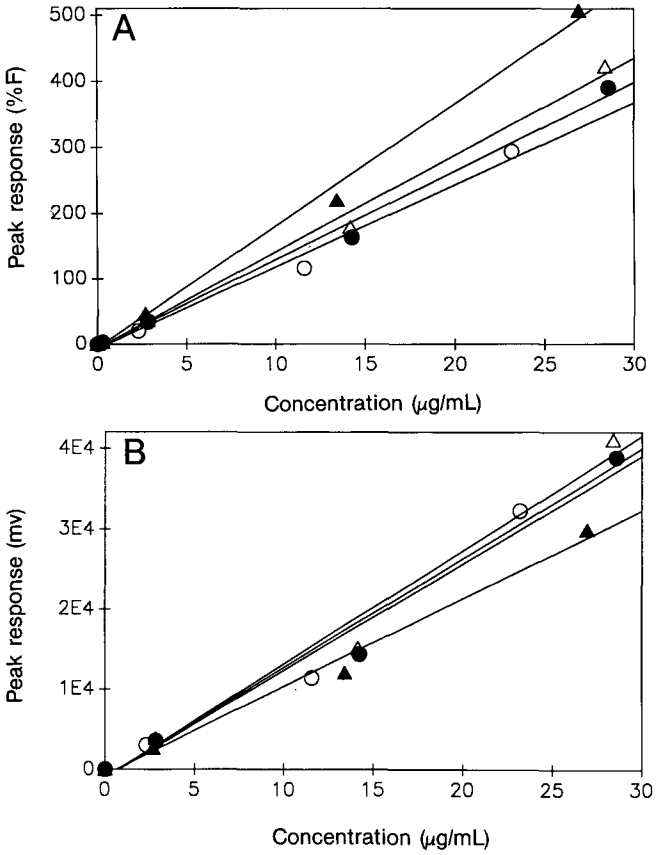

FIG. 4. Calibration curves for mixed tocopherol standards for (A) fluorescence detection and (B) evaporative light-scattering detector, where %F is the fluorescence intensity and mv is millivolts. ○, α-tocopherols; ●, β-tocopherols; △, γ-tocopherols; ▲, δ-tocopherols.

TABLE 1

Tocopherol Content of Vegetable Oils by Fluorescence Detection in the Presence of an Internal Standard

	Tocopherols (mg/100 g)				
Vegetable oil	α	β	γ	δ	Total content
RBD[a] soy	6.92	3.29	81.8	43.6	136
Crude soy	7.22	3.39	93.3	45.3	149
Crude peanut	7.74	1.62	24.1	4.05	37.5
Crude canola	17.2	—	21.3	2.03	40.5
RBD palm	26.3	4.7	32.5[b]	8.89[c]	72.4

[a]RBD, refined, bleached and deodorized.
[b]γ-Tocotrienol.
[c]δ-Tocotrienol.

level of tocopherols above the detection limit, multiple injections on the GPC columns would be necessary. These multiple injections have to be done individually with the collected fractions being combined because the four GPC columns have a maximum limit of about 26 mg of lipid per injection volume. The fluorescence detector was used in this particular experiment (Table 1) to quantitate the tocopherols in the oil samples due to its increased sensitivity over the ELSD. For tocopherol standards, the ELSD was equally good for quantitative work in the presence of the internal standard (Fig. 3b).

This study has successfully shown that an ELSD can be substituted for an RI detector to monitor the elution of the oil peak prior to collection of the tocopherols off GPC columns. In addition, an internal standard was introduced to aid in the quantitation of tocopherols by either ELSD or fluorescence detection.

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