Analysis of Tocopherols and Phytosterols in Vegetable Oils by HPLC with Evaporative Light-Scattering Detection[1]

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Methods were developed for the separation, detection, and quantification of tocopherols and phytosterols by high-performance liquid chromatography with an evaporative light-scattering detector. Four tocopherols—α, β, γ and δ—and four phytosterols—campesterol, β-sitosterol, brassicasterol, and stigmasterol—were analyzed in soybean, sunflower, low-erucic acid rapeseed (LEAR) and corn oils. The use of an evaporative light-scattering detector, in conjunction with modification of methods from the literature to prepare and analyze tocopherols and phytosterols by HPLC, showed consistent results between trials and levels of these minor constituents.

KEY WORDS: α-Tocopherol, β-sitosterol, β-tocopherol, brassicasterol, campesterol, δ-tocopherol, γ-tocopherol, mass detector, stigmasterol.

Tocopherol contents in oils and foods have been determined by gas chromatography (GC) (1-4), spectrophotometry (5), colorimetry (6) including the widely used Emmerie-Engel method (7), and thin-layer chromatography (TLC) (8). The first comprehensive compilation of the vitamin E content of foods was published in 1979 by the United States Department of Agriculture with data based on GC analysis (9). Measurement of tocopherols has been improved by application of high-performance liquid chromatography (HPLC). Van Niekerk (10) was the first to develop and publish an HPLC method that could separate the individual tocopherols rather than measure total tocopherols. Several groups of researchers have used HPLC with fluorometric detectors to measure tocopherols (11-15). Ultraviolet detection (UV) with HPLC has also been used (16-18). A collaborative study sponsored by the International Union of Pure and Applied Chemistry (IUPAC) determined the tocopherol contents of oil by HPLC with both fluorescence and UV detection techniques (19).

Phytosterols have been measured by gas chromatography (2, 3, 11), by thin-layer chromatography (20) and by HPLC (17, 21-23).

A new type of HPLC detector, the evaporative light-scattering detector (ELSD), has been used to detect several classes of lipids such as phospholipids and triglycerides (24,25) and methyl esters (26,27). However, no work has been published on measuring tocopherols or phytosterols by means of the ELSD. This paper reports the development of methods, in which HPLC is used with an evaporative light-scattering detector for separating, detecting, and quantifying four tocopherols α, β, γ and δ—and four phytosterols—campesterol, β-sitosterol, brassicasterol, and stigmasterol in 16 vegetable oils—four each of soybean, sunflower, low-erucic acid rapeseed (LEAR) and corn oil.

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EXPERIMENTAL

Materials—vegetable oils. Four samples each of commercially refined and bleached soybean, sunflower, low erucic acid rapeseed, and corn oil were obtained from oil processing plants over a 12-mon period. The oils were laboratory-deodorized and treated with citric acid as previously described (28).

Reference standards for tocopherols and sterols. Samples of α-tocopherol, γ-tocopherol, and δ-tocopherol were obtained from Eastman Kodak Company, Rochester, NY. The β-tocopherol and brassicasterol were obtained from Supelco, Inc., Bellefonte, PA. Stigmasterol, campesterol, and β-sitosterol were obtained from Sigma Chemical Co., St. Louis, MO. All standards were analyzed separately by HPLC to verify purity.

Solvents. All solvents were HPLC-grade, including hexane, acetonitrile, tetrahydrofuran, absolute ethanol, and methanol. Solvents were filtered before use to remove any small particles that might clog the column.

METHODS

High-performance liquid chromatography. Samples were analyzed for tocopherol and phytosterol levels by HPLC with a SP8700XR HPLC solvent delivery system (Spectra-Physics, San Jose, CA), an 100μL injection loop, an ELSD, (model ELSD II, Varex Corp., Rockville, MD), and a 5μ spherical reverse-phase C_{18} "Resolve" column (3.9 mm $\times$ 15 cm) (Waters Chromatographic Division, Milford, MA). The analog signal from the ELSD was integrated by a Mod-Comp digital computer (Mod-Comp, Inc., Fort Lauderdale, FL) programmed to calculate peak areas and relative percentage composition. Peaks in experimental samples were identified by comparison with elution times of standards.

RESULTS AND DISCUSSION

The development of oil analysis for tocopherol and phytosterol contents involved three phases. First, appropriate methods to prepare the oils for HPLC analysis were determined. Second, the conditions under which the compounds are separated and detected by the chromatographic system were developed. Third, reference standards for each of the tocopherols and phytosterols were chromatographed to determine retention times and integrator responses to quantify the levels of compounds in actual samples.

Sample preparation. In this study, no prior extraction of the tocopherols from the oil was required because preliminary tests showed that other minor constituents of the oil, such as carotenoids or phytosterols, did not elute at the same retention times as the tocopherols. Dilution of the oil in hexane, in a 1:10 ratio, produced chromatograms with all tocopherol compounds eluting separately. Selection of a dilution ratio of 1:10 was based on trial runs which showed the detector response within range for the tocopherols at the highest levels, while the tocopherols in the lowest amount were still detectable. Much of the published research on

tocopherol measurements used direct analysis of tocopherols after only diluting the oil in an organic solvent. A collaborative study of tocopherol determinations in vegetable oils by HPLC, with 19 laboratories participating, utilized a dilution technique of 1:10, oil in propan-2-ol (19). Carpenter (16) used a 1:20 dilution of oil in 1.5% isopropyl alcohol in hexane; whereas, Van Niekerk and Burger (11) used a 1:5 dilution of oil in hexane. Dilution factors of 1:100 or 1:20 in hexane were utilized by Syvaoja *et al.* (15), depending on the level of tocopherols in the oil samples tested. Ludwicki, Tayeb, and Dillion (29) saponified and extracted their oil samples before analyzing for tocopherols. The main advantage of direct analysis after dilution, compared with saponifying and extracting the tocopherols, is to simplify and shorten the time for the procedure.

Oils were initially analyzed for phytosterols by the same dilution technique as used for tocopherols. However, the phytosterols did not appear as distinct peaks as did the tocopherols, indicating that other unsaponifiable compounds in the oil could be interfering with the elution of the phytosterol compounds. Therefore, oils were analyzed for phytosterols after saponification and fractionation of the oils based on a method modified from that of Holen (17). One gram oil and 100 mL 0.8 M ethanolic KOH were placed in a 250-mL round-bottom flask and heated at 80°C for 30 min. The mixture was placed in a 250-mL separatory funnel with 100 mL of ethyl ether and shaken, then washed 3 times with 50 mL of distilled water to remove the water-soluble compounds. The ether layer containing the unsaponifiables was dried over anhydrous magnesium sulfate and evaporated completely in a rotary evaporator at 25°C. All flasks were flushed with nitrogen, and analyses were performed rapidly to minimize possibility of phytosterol oxidation.

The lipid residues containing the sterols were dissolved in 1 mL absolute ethanol. Reproducibility of the extraction method was tested by repeating an extraction of the same soybean oil five times. The error in yield among all runs was 3%. Previous reports indicate that sterols are usually measured only after they are extracted from the oil (2-4, 30).

Chromatographic conditions. Analysis of lipids by HPLC depends on sufficient appropriate separation and detection of the compounds. Separation of the compounds by HPLC requires a specific ratio of solvents at an appropriate flow rate through a column containing a nonpolar stationary phase. Therefore, the HPLC conditions tested were column type, solvents and ratios, and flow rate. Published HPLC methods for tocopherols included the use of either C_{18} normal or reversed-phase column. The reversed-phase column separates most lipids according to chain length as well as the number and location of double bonds and functional groups. Figures 1 and 2 present the chemical structures of the four tocopherol compounds and the four phytosterols tested. The more polar compounds elute first when analyzed with a reversed-phase column, which is opposite of normal-phase chromatographic columns that retain the more polar compounds and elute the least polar compounds first. Of the four tocopherols, δ-tocopherol eluted first, followed in order by β, γ, and α-tocopherol. The only difference in the chemical structures of the tocopherols that would cause different elution times is the number of methyl groups and their location. The addition of each methyl group makes the compound less polar which explains why δ-tocopherol with only one methyl group eluted first and α-tocopherol with three methyl groups eluted last. Both γ- and β-tocopherol contain two methyl groups but at different locations: on carbons 5 and 8 for β-tocopherol and on carbons 7 and 8 for γ-tocopherol. This similarity in chemical structure accounts for the difficulty that some researchers report in separating these two tocopherols.

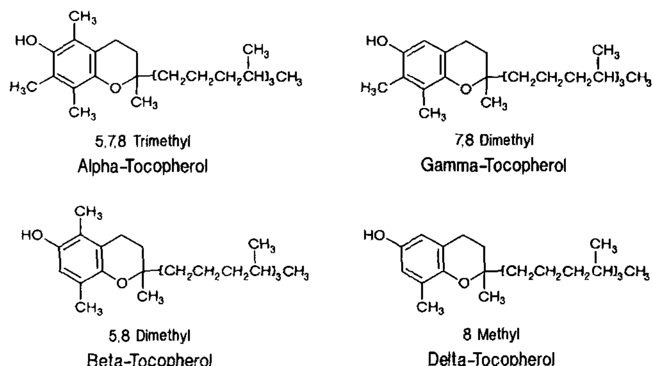

FIG. 1. Chemical structures of α-, β-, γ-, and δ-tocopherols.

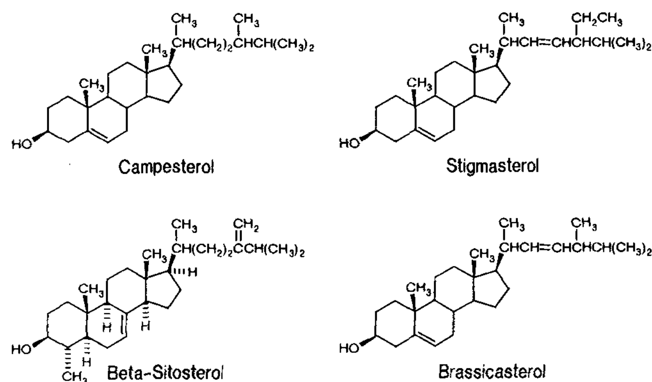

FIG. 2. Chemical structures of four phytosterols: brassicasterol, stigmasterol, campesterol, and β-sitosterol.

The initial conditions for the isocratic separation of the tocopherols included a ternary solvent mixture of acetonitrile: tetrahydrofuran:water at a ratio of 50:35:15 and a flow rate of 1 mL/min. Preliminary runs showed poor peak separation, therefore the conditions were modified to a solvent ratio of 60:25:15 with a flow rate of 0.7 mL/min. The resolution of peaks for β- and γ-tocopherol (Fig. 3) is not commonly reported and may be influenced by the use of hexane as an injection solvent which is immiscible in the acetonitrile/tetrahydrofuran (THF)/water mobile phase. It may be speculated that the tocopherols are deposited on the head of the column rather than immediately flowing with the mobile phase, therefore enhancing peak separation.

Both C_8 and C_{18} columns have been used for the HPLC separation of phytosterols, although Holen (17) found that the phytosterols were retained to a greater degree on the C_{18} column than on the C_8 column. The C_{18} column was

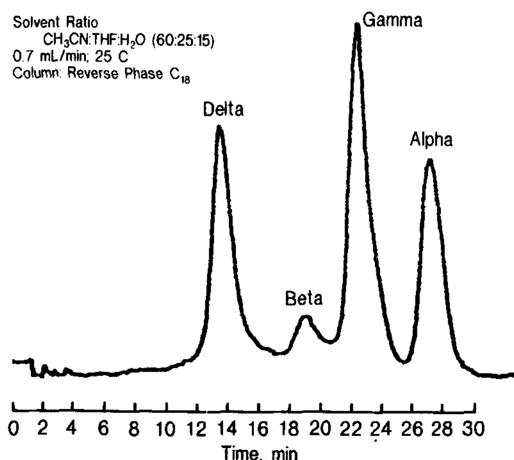

FIG. 3. Chromatogram of high-performance liquid chromatographic analysis of tocopherols.

typically the column of choice as indicated in the literature and was therefore chosen for this study.

For the phytosterol analysis, preliminary runs showed poor peak separation with a binary solvent ratio of 99:1 methanol to water; however, additional tests with a ratio of 98:2 methanol:water produced better separation. Flow rates of 1 mL/min and 0.7 mL/min were tested with the 0.7 mL/min providing better separation. The effect of column temperature on resolution of phytosterols was also investigated. Tests at both 25°C and 30°C showed little difference in resolution of the peaks; therefore, the analyses were performed at room temperature, 25°C (Fig. 4).

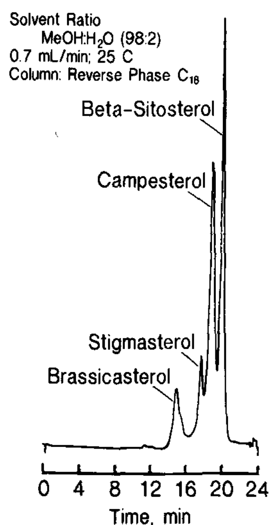

FIG. 4. Chromatogram of high-performance liquid chromatographic analysis of phytosterols.

Of the four phytosterols, brassicasterol with a chemical formula of $C_{28}H_{46}O$ and two double bonds eluted first, followed by stigmasterol, campesterol and β-sitosterol, which had the highest molecular weight and two double bonds.

Detector conditions. The last factor to be considered in the analyses of tocopherols and phytosterols was the detection of the eluted compounds after they were separated by the chromatographic conditions.

During the operation of an ELSD, the effluent from the chromatographic column is converted to a fine mist by passing through a nebulizer into a stream of pre-heated nitrogen (31). The fine droplets are carried through a temperature-controlled drift tube where the volatile solvent is vaporized, leaving very fine particles of solute in the nitrogen stream. These particles then pass through a helium/neon laser light beam. The light scattered by the particles is detected by a sensitive photodiode system and transformed into an electrical current used as a detector signal. Because of its unique design, response of the detector is a function of the mass of the solute particles (27).

The three conditions to be controlled for the ELSD are temperature of the nebulizer-drift tube, temperature of the exhaust, and rate of nitrogen gas flow. The selection of temperatures depends on the polarity of the solvents. Polar compounds such as water, methanol and acetonitrile are difficult to vaporize; therefore, the temperature and solvent flow conditions have to be optimized for these solvents (31).

Conditions for the nebulizer-drift tube were varied between temperatures of 115°C and 135°C and between flow rates of 45 to 75 mL/min (10-32 psi) for the tocopherol and phytosterol analyses. Analyses at 115°C showed a high signal to noise ratio, whereas the tests conducted above 130°C resulted in poorer sensitivity. The flow rate also affected sensitivity and noise, with the higher rates causing greater sensitivity but with more noise. Therefore, trials to optimize the conditions were conducted with the selection of the following parameters for tocopherols: nebulizer-drift tube temperature, 120°C; exhaust temperature, 77°C; nitrogen flow rate, 50 mL/min (14 psi); and for phytosterols: nebulizer-drift tube temperature, 125°C; exhaust temperature, 82°C; nitrogen flow rate, 70 mL/min (31 psi).

Reproducibility obtained at these chromatographic and detector conditions showed a 2% error among values for tocopherol levels in five runs and compared favorably to the error reported by Stolyhwo, Colin, Martin, and Guiochon (32) of 1% when the ELSD was used to measure methyl ester levels.

Reference standards. Reference standards for each of the four tocopherol compounds and the four phytosterols were diluted to various levels to develop calibration curves for HPLC detector responses and to determine retention times. Calibration curves for the tocopherols are shown in Figure 5. Detector responses as measured by integrator counts were similar for β-, γ-, and δ-tocopherols; however, the α-tocopherol curve had less. This may be due to a difference in the volatility of this homologue relative to the other tocopherols in the series. Calibration curves for the phytosterols (Fig. 6) showed similar slopes for β-sitosterol and campesterol. Slopes of the curves for brassicasterol and campesterol were less than for the other two phytosterols. Correlation coefficients calculated for the weight of the compounds versus the detector responses were either 0.98 or 0.99 ($P < 0.05$) for all standards (33). Reference standards were also used to determine the retention time of the tocopherols and phytosterols. Reference standards were injected singly and in mixtures to determine times of elution. Figure 3 presents retention times for the four tocopherols: δ, 14.0 min; β, 19.1 min; γ, 22.5 min; and α, 27.7 min. Retention times for the four phytosterols were 15.7 min, brassicasterol; 17.8 min, stigmasterol; 18.7 min, campesterol and 20.2 min, β-sitosterol (Fig. 4).

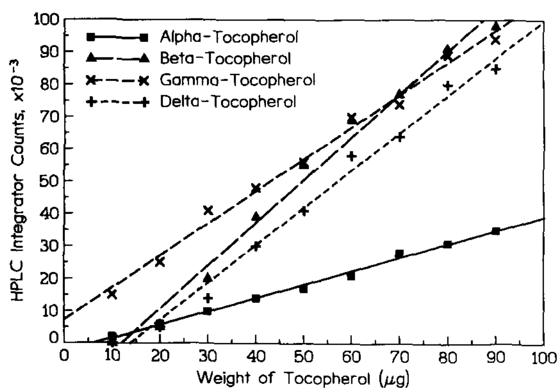

FIG. 5. Calibration curves for evaporative light-scattering detector responses for the high-performance liquid chromatographic analysis of tocopherols.

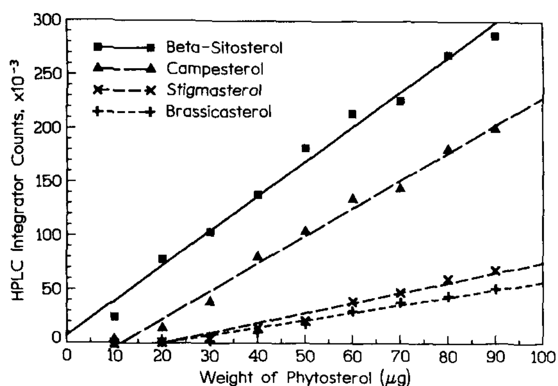

FIG. 6. Calibration curves for evaporative light-scattering detector responses for the high-performance liquid chromatographic analysis of phytosterols.

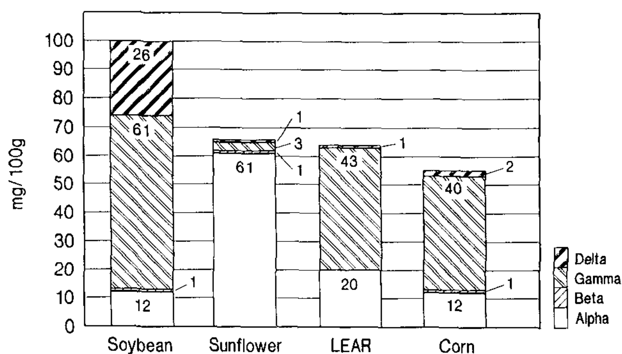

FIG. 7. Tocopherol contents (mg/100 g) of oils.

Tocopherol analysis. Tocopherols were detected in the four oils in distinct ratios (Fig. 7). Soybean, LEAR and corn oils contained low levels of α-tocopherol, from 12 to 20 mg/100 g oil; whereas, sunflower oil contained an average of 61 mg/100 g oil which is 92% of the total tocopherol. β-Tocopherol was present in only small amounts in soybean, sunflower and corn oils. No β-tocopherol was detected in LEAR oil. Corn and LEAR oils contained nearly equal amounts of γ-tocopherol. Sunflower oil had only small amounts of γ, whereas soybean oil had the greatest amount of γ-tocopherol: a mean level of 61 mg/100 g oil. δ-Tocopherol was detected at the 1-2 mg/100 g of oil level in sunflower, corn and LEAR oils. Soybean oil had higher levels of δ-tocopherol—26 mg/100 g oil—than the other three oils.

The total amounts of tocopherols in the oils did not vary much between the corn, LEAR, and sunflower oils with mean values of 55, 64, and 66 mg/100 g of oil respectively. However, soybean had a higher level of total tocopherols than the other oils with a mean of 100 mg/100 g oil.

Phytosterol analysis. The phytosterol contents of the four oils varied in amount as well as in composition. The LEAR oil contained higher levels of total phytosterols than the other three oils with a mean level of 673 mg/100 g of oil (Fig. 8). β-Sitosterol accounted for 57% of the total phytosterols in LEAR oil, followed by 34% campesterol, and 9% brassicasterol. The LEAR oil is one of the few vegetable oils that contain brassicasterol. Corn oil contains approximately 50% less total phytosterols than the LEAR oil, with β-sitosterol accounting for 69% of the total, followed by 22% campesterol and 6% stigmasterol. The soybean and sunflower oils had approximately 50% less total phytosterols than corn oil. Sunflower oil contained 78% β-sitosterol, 12% campesterol and 10% stigmasterol. Soybean oil had the lowest level of β-sitosterol (55%) of all four oil types. Levels of other phytosterols in soybean oil were 23% campesterol and 22% stigmasterol.

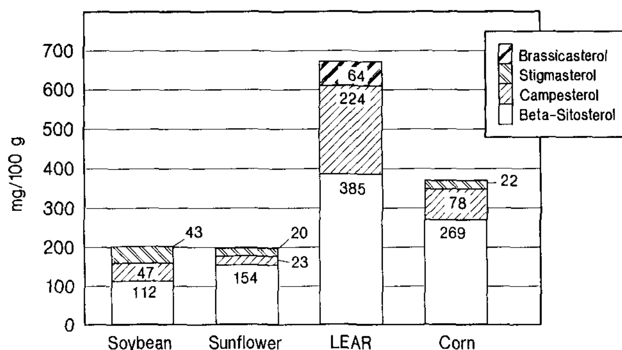

FIG. 8. Phytosterol contents (mg/100 g) of oils.

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