Simultaneous Detection of Tocopherols, Carotenoids, and Chlorophylls in Vegetable Oils by Direct Injection C_{30} RP-HPLC with Coulometric Electrochemical Array Detection

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ABSTRACT: Detection and quantification of tocopherols, carotenoids, and chlorophylls in vegetable oil have been used to aid their authentication. Their importance in influencing the oxidative stability of vegetable oils and their possible health benefits have been shown in numerous studies. Therefore, the need for a rapid and reliable analysis method has become increasingly important. This study demonstrates the application of C_{30} RP-HPLC with electrochemical detection for the simultaneous analysis of tocopherols, carotenoids, and chlorophylls in vegetable oils. Aliquots of vegetable oils were dissolved in appropriate solvents and injected directly without saponification, thus preventing sample loss or component degradation. Effective separation of tocopherols, carotenoids, and chlorophylls was achieved. Detection was performed using a coulometric electrochemical array detector set between 200 and 620 mV. For a 25-µL injection, the respective detection limits for carotenoids, tocopherols, and chlorophylls were 1 fmol, 0.15 pmol, and 0.5 pmol, representing 1000-, 25-, and 5-fold enhancement over the UV-vis methodology. The detector response was linear between 0.01 and 2.00 µg/mL for all compounds studied. Within-day variations (CV) were between 2.0 and 6.3%, whereas between-day variations were between 2.7 and 7.4%. This method can be applied for rapid and sensitive analysis in the study of oil quality and adulteration.

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KEY WORDS: Carotenoids, chlorophylls, C_{30} column, electrochemical detection, HPLC, tocopherols, tocotrienols,

Minor constituents of vegetable oils such as tocopherols, carotenoids, sterols, and chlorophylls have been widely utilized as authentication aids. Olive oil adulteration can be traced based on its tocopherol and tocotrienol contents (1,2) as well as chlorophyll and carotenoid composition (3). Carotenoids, tocopherols, and tocotrienols are abundant and characteristic of red palm oil (4). The presence of tocotrienols in olive oil can be attributed to contamination or adulteration with palm oil (1). More recently, the importance of these minor constituents to the oxidative stability of vegetable oils (5,6) and their possible health benefits (7,8) have prompted the need for rapid and reliable analytical methods.

Typically, analysis of minor compounds of vegetable oils involves their enrichment into the unsaponifiable fraction followed by separate analyses with GC and/or HPLC, utilizing various modes of detection. Saponification is often employed to remove lipids and chlorophylls and to release esterified xanthophylls (9). However, this step has been reported to result in a significant loss of carotenoids, especially the epoxycarotenoids and xanthophylls (10,11). When applied to red palm oil, saponification significantly decreased the amount of α- and β-carotene and α-tocopherol extracted and facilitated isomerization of the carotenoids (9).

HPLC with electrochemical detection (ECD) has been used in the analysis of tocopherols in olive oil (1), vegetable oils (12), and neonatal plasma samples (13) and of tocopherols and carotenoids in human blood plasma and various biological microsamples (14–16). The coulometric array detector employed in the latter two studies offers several advantages, including high sensitivity, selective detection, and ease of implementation. The limit of detection for γ-tocotrienol analyzed by RP-HPLC with amperometric detection was reported to be 10 times lower than normal-phase (NP) HPLC with fluorometric detection (1). Detection limits for all-*trans* β-carotene and lycopene were reported to be 10 and 50 fmol, respectively, which was 10 to 100 times lower than the conventional UV-vis detection (15,16). The application of a multichannel electrochemical detector allowed for enhanced selectivity because interfering compounds could be eliminated prior to detection of the compound of interest. In addition, current–voltage curves (CVC) may be constructed based on the analyte's response across the electrochemical array and used as an aid in peak identification (15,16).

In this study, tocopherols, tocotrienols, carotenoids, and chlorophylls (Scheme 1) were simultaneously determined in vegetable oils by C_{30} RP-HPLC with coulometric electrochemical array detection. Oil samples were dissolved in the mobile phase and directly injected without extraction and saponification. Limit of detection, linearity range, and reproducibility of the method were tested. This method was then applied to red palm oil, virgin olive oil, and various vegetable

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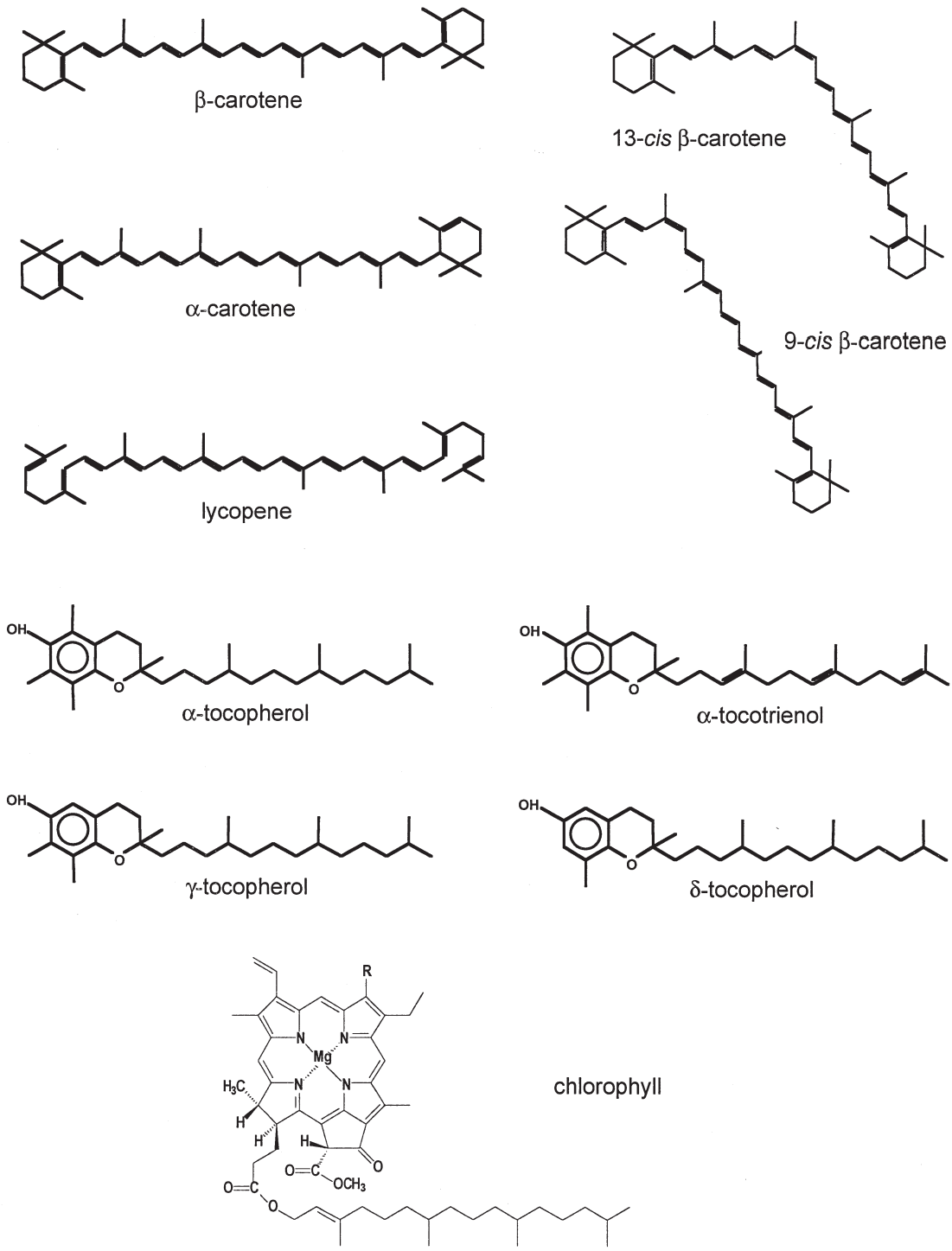

SCHEME 1

oil mixtures to illustrate the potential usefulness in the detection of oil adulteration.

EXPERIMENTAL PROCEDURES

Materials. HPLC-grade solvents were used throughout the experiments. Tocopherols (α, δ, and γ), chlorophyll α, all-*trans* β-carotene, and lycopene standards were purchased from Sigma-Aldrich (St. Louis, MO). All-*trans* α-carotene was obtained from Fluka Chemie AG (Buchs, Switzerland). Red palm oil, virgin olive oil, and soybean oil were purchased from a local grocery store, and wheat germ oil was from a local health food store.

Standard preparation. Stock solutions of carotenoids, tocopherols, and chlorophyll *a* were prepared in hexane, methanol, and diethyl ether, respectively. Their concentrations were determined spectrophotometrically using published extinction coefficients (17–19). Working solutions were made

between 0.005 and 0.5 μg/mL by serial dilutions using methyl-*tert*-butyl ether (MTBE) + methanol (1:1) followed by filtration (0.2 μm nylon filter) prior to injection.

Sample preparation. Vegetable oils (10–20 mg) were solubilized in 6 mL of MTBE + methanol (1:1), filtered through a 0.2 μm nylon filter, and analyzed immediately.

Instrumentation and chromatography. An analytical (4.6 mm i.d. × 250 mm, 5 μm) polymeric YMC™ C_{30} column (Waters, Milford, MA) was used and protected by a C_{18} guard (4.6 mm i.d. × 50 mm) column (Vydac, Hisperia, CA) and a precolumn filter. Column temperature was held constant at 25°C to control for temperature fluctuations in the laboratory at different times. The HPLC system consisted of a Hewlett-Packard model 1050 (Santa Clara, CA) solvent delivery system. Injection volume was 25 μL.

An eight-channel 5600 Coularray™ electrochemical detector (ESA, Chelmsford, MA) with potentials set between 200 and 620 mV in 60-mV increments from channel 1 to 8, respectively, was used. At the end of each day, the analytical cells were cleaned by applying oxidation and reduction potentials of 800 and –600 mV, respectively, for 60 s. This step aids in elimination of deposited compounds present on the cell surface that are unreactive at the applied analytical potentials. Data collection and integration were performed using the ESA Coularray™ version 1.01 software and data management system.

The method of Ferruzzi *et al.* (15) was modified to achieve baseline separation of tocopherols and tocotrienols. Ammonium acetate (1.0 M, pH 4.6) was prepared with C_{18} Sep-Pak® (Waters) solid phase extraction-purified HPLC-grade water. Solvent A consisted of methanol/MTBE/ammonium acetate/water (88:5:5:2), whereas solvent B consisted of methanol/MTBE/ammonium acetate (20:78:2). The following linear gradient was used: 0 to 5 min, 100% solvent A; 5 to 45 min, 15% solvent A, 85% solvent B; 45 to 50 min 100% solvent B.

Identification. When available, authentic standards were used for the identification of carotenoids, tocopherols, and chlorophylls. Identification of carotenoid *cis* isomers was aided by comparison to previously reported C_{30} UV-vis and electrochemical methods (15,16,20,21). Tocotrienols were identified by cochromatography using tocotrienol-containing oils and comparison to reported data (22,23).

Detection limit. We defined the detection limit as a signal-to-noise ratio equaling 3. Standard solutions were diluted in fivefold series and analyzed until the detection limits were reached.

RESULTS AND DISCUSSION

HPLC separation. Figure 1 depicts a typical separation of major red palm oil carotenoids, tocopherols, and tocotrienols. The C_{30} stationary phase allows for efficient separation of α-carotene (all-*trans*, 13-*cis*, 13′-*cis*, and 9-*cis* isomers), β-carotene (all-*trans*, 9-*cis*, and 13-*cis* isomers) and all-*trans* lycopene. The 9′-*cis* isomer of α-carotene coeluted with all-*trans* β-carotene, and further modification of the gradient elution was not able to completely resolve the two peaks while maintaining a reasonable analysis time. Lessin *et al.* (24) faced the same problem when analyzing thermally processed carrot using the same stationary phase. The amount of 9′-*cis* isomer typically is comparable to the corresponding 9-*cis* α-carotene (21,24). In the case of red palm oil, where β-carotene constitutes more than 60% of the total carotenoids (4), the quantity of 9′-*cis* α-carotene is minor compared to the all-*trans* β-carotene and most likely does not affect the quantification of all-*trans* β-carotene. In addition to the carotenoids, baseline separation of α-, δ-, and γ-tocopherols, and α- and δ-tocotrienols present in red palm oil was achieved. Using a C_{30} column coupled with a diode array detector, Darnoko *et al.* (22) achieved a

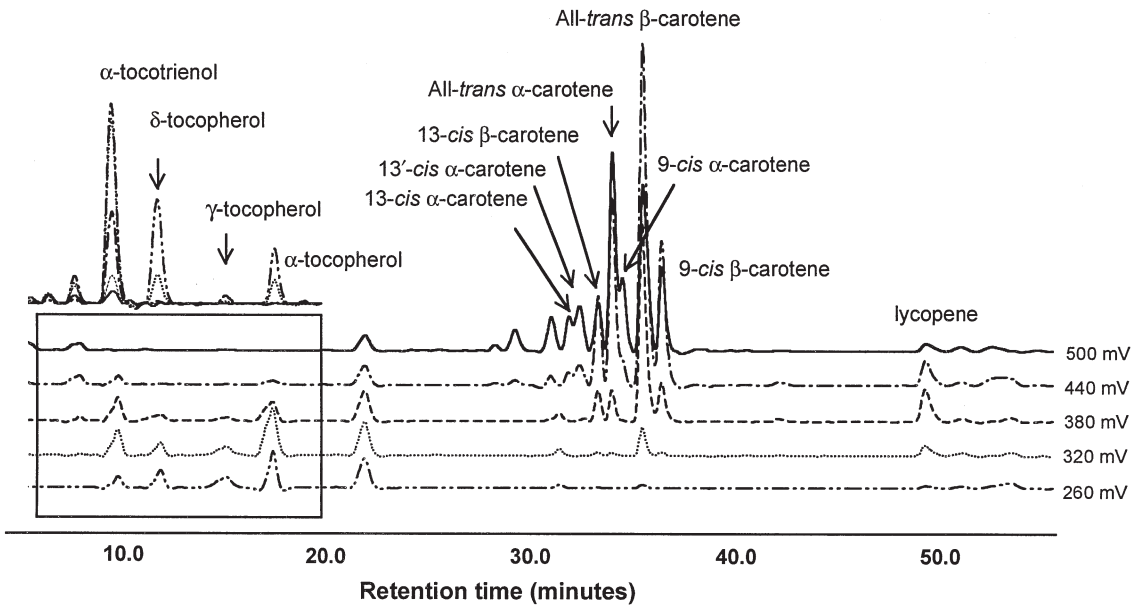

FIG. 1. C_{30} HPLC separation of tocopherols, tocotrienols, and carotenoid geometrical isomers in red palm oil.

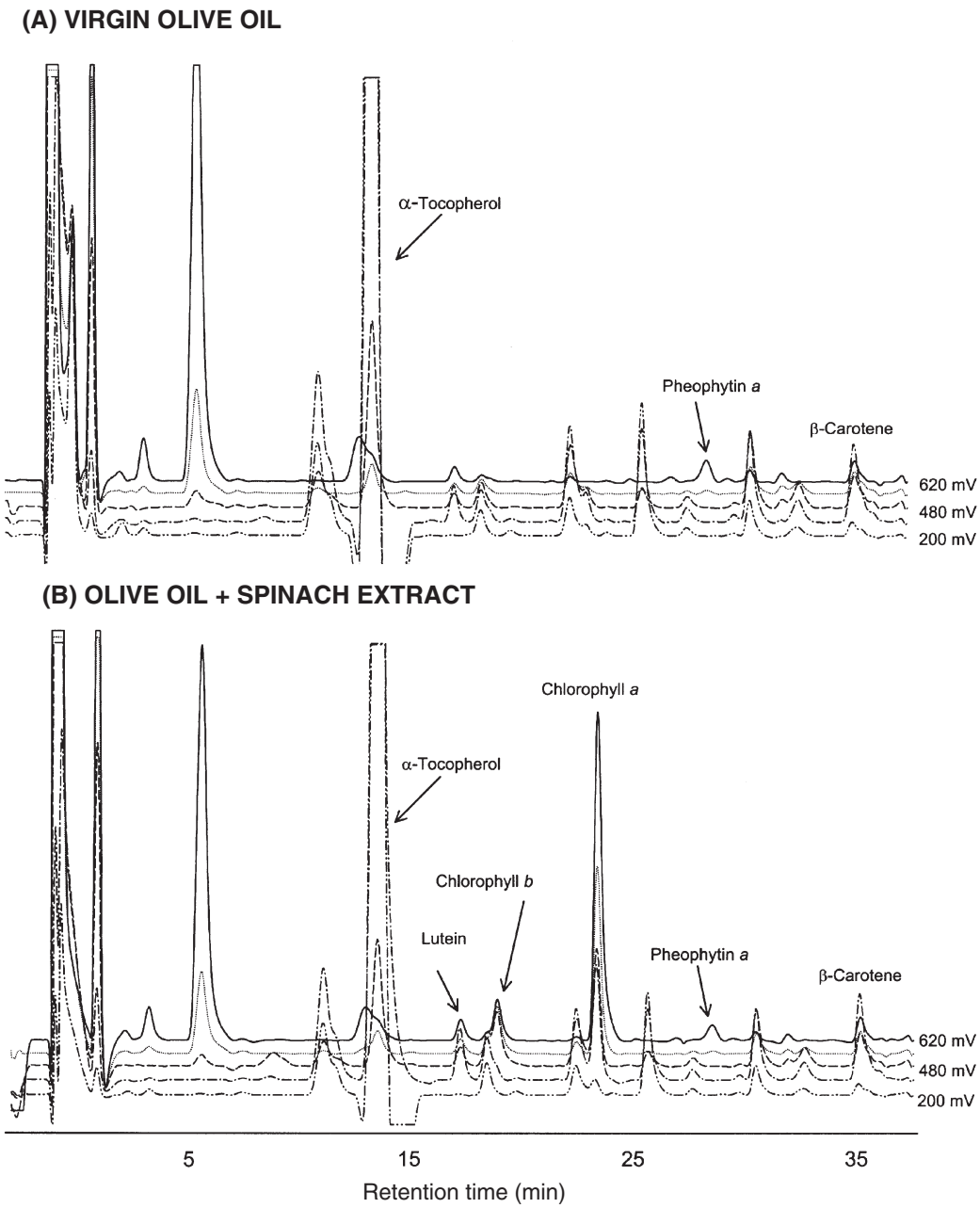

FIG. 2. C_{30} HPLC separation of tocopherols, carotenoids, and chlorophylls in (A) virgin olive oil and (B) virgin olive oil spiked with spinach leaves.

similar baseline separation of tocopherols and carotenoids in red palm oil.

A typical electrochemical chromatogram of virgin olive oil is presented in Figure 2A. Major tocopherols in olive oil were well resolved, although baseline separation of β- and γ-tocopherol was not achieved. The two tocopherols differ only in the position of the two methyl groups in their structures, and most normal as well as RP-HPLC methods (1,5,17) failed to completely separate this pair. Balz *et al.* (25) used a diol column to achieve baseline separation of β- and γ-tocopherols and β- and γ-tocotrienols. The only detectable chlorophyll derivative present in our virgin olive oil sample was pheophytin *a*. To test our method further, we spiked the oil with fresh spinach extract (Fig. 2B) and were able to detect chlorophyll *a* and *b* in addition to pheophytin *a*. Gandul-Rojas *et al.* (3) detected chlorophylls and pheophytins in their virgin olive oil samples. The discrepancy most likely comes from the age of the oil. Gandul-Rojas *et al.* (3) analyzed freshly extracted virgin olive oil, whereas our sample was purchased from a local store and may have been exposed to light for a period of time. Studying the pigments present in virgin olive oil, Mínguez-Mosquera *et al.* (26) found a decrease in chlorophyll concentration during storage, and pheophytin *a* was found to be the major chlorophyll derivative after 8 wk of storage.

In 1998, Psomiadou and Tsimidou (5) developed a C_{18} HPLC method coupled with a photodiode array detector that was able to detect tocopherols, carotenoids, and chlorophylls simultaneously in virgin olive oil. Their method, however,

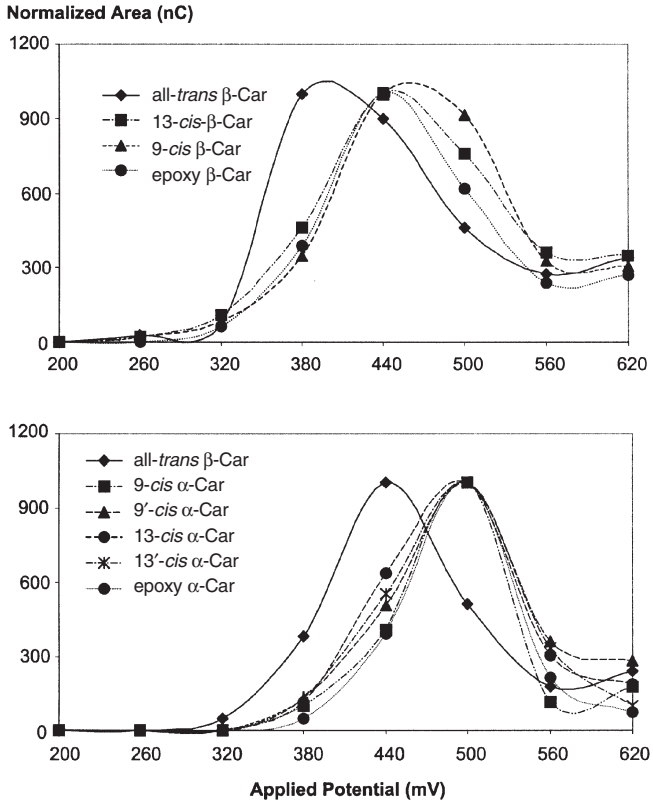

FIG. 3. Current-voltage curves of (A) β-carotene and its geometrical isomers and (B) α-carotene and its geometrical isomers. Car = carotene; nC, nanocoulombs.

was not able to separate the *cis*-isomers of carotenoids. With a C_{30} stationary phase and gradient elution, analysis of tocopherols, geometrical isomers of carotenoids, and chlorophylls in vegetable oil was completed within 50 min. For the study of major palm oil carotenoids (i.e., α- and β-carotene), virgin olive oil, or samples devoid of lycopene, analysis can be terminated after the elution of 9-*cis* β-carotene in 37 min. Furthermore, analysis specifically applied for tocopherols and tocotrienols can be completed in 20 min.

Electrochemical behavior. The use of a coulometric electrochemical array detector enabled us to construct the characteristic CVC of carotenoids (Fig. 3A,B), tocopherols (Fig. 4) and chlorophylls (Fig. 5). Dominant responses for β- and α-carotene were detected at the applied potentials of 380 and 440 mV, respectively, and were similar to an earlier report (15). Using static systems, Liu *et al.* (27) demonstrated that the oxidation potential of β-carotene was lower than that of α-carotene, indicating that the latter is more difficult to oxidize. The observed results most likely were caused by the difference in the double bond position between β- and α-carotene in their β-ionone rings. As a consequence, β-carotene has 11 conjugated double bond systems, one more than α-carotene. Extension of the conjugated double bond system, and not the oxygenation in the carotenoid structures as with xanthophylls, was found to affect their electrochemical behavior. The observed dominant oxidation potentials for zeaxanthin and lutein (dihydroxy β- and α-carotene, respectively) were very similar to those of β- and α-carotene (15,27). Our data also indicated that geometrical isomers of carotenoids oxidized at a higher potential than their parent compounds, which was in agreement with previous studies (15,16). Therefore, in addition to their structures, molecular configuration appeared to be an important aspect of carotenoid electrochemical behavior. 5,6-Epoxy β-carotene, an oxidation product of β-carotene, displayed a slightly higher maximal response oxidation potential than its parent compound and one that was similar to the 9-*cis* and 13-*cis* β-carotene isomers.

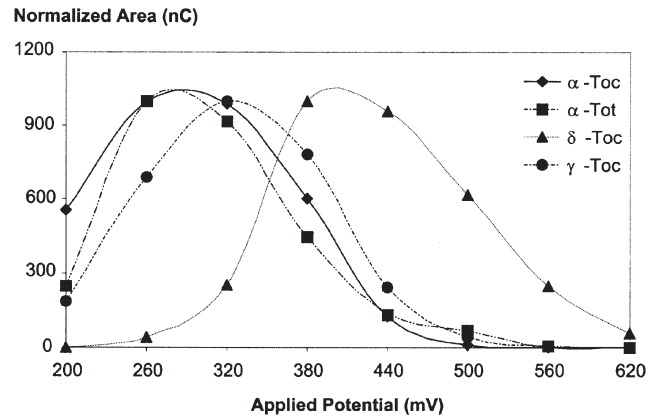

FIG. 4. Current-voltage curves of α-tocopherol (α-Toc), α-tocotrienol (α-Tot), γ-tocopherol (γ-Toc), and δ-tocopherol (δ-Toc). For other abbreviation see Figure 3.

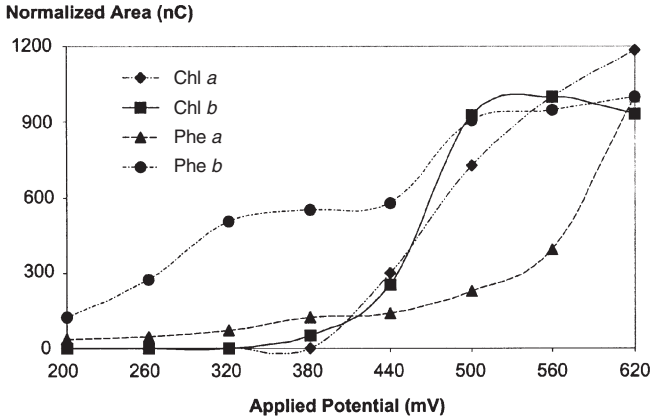

FIG. 5. Current-voltage curves of chlorophyll and its derivatives. Chl, chlorophyll; Phe, pheophytin; for other abbreviation see Figure 3.

The dominant response oxidation potentials of tocopherols are lower than those of carotenoids (Fig. 4). α-Tocopherol exhibited a dominant response oxidation potential at around 260 mV and γ- and δ-tocopherol at around 320 and 380 mV, respectively. A similar observation was made by Finckh *et al.* (13) and Litescu and Radu (28). α-Tocotrienol behaved similarly to its corresponding tocopherol, indicating that the presence of three double bonds in its structure did not affect its electrochemical properties. This observation reaffirms that electrochemical behavior is dependent on the oxidation and reduction properties of a compound (15).

Chlorophylls and pheophytins oxidized at much higher

TABLE 1
Linear Ranges and Detection Limits of Carotenoids, Tocopherols, and Chlorophyll *a* Analyzed on a C_{30} RP-HPLC with Electrochemical Array Detection

	Linear range (μg/mL)	R^2	$y = a + b\,x$ [a]	Detection limit[b]
α-Carotene	0.01–2.00	0.9993	$y = 3.3 + 4316.6\,x$	1 fmol
β-Carotene	0.01–2.00	0.9953	$y = 154.9 + 4271.0\,x$	1 fmol
Lycopene	0.01–2.00	0.9988	$y = -42.4 + 3877.4\,x$	1 fmol
α-Tocopherol	0.01–2.00	0.9988	$y = -18.5 + 2891.5\,x$	0.15 pmol
δ-Tocopherol	0.01–2.00	0.9988	$y = 19.3 + 1261.0\,x$	0.15 pmol
γ-Tocopherol	0.01–2.00	0.9959	$y = 30.4 + 1020.9\,x$	0.15 pmol
Chlorophyll *a*	0.01–2.00	0.9981	$y = -59.5 + 3130.3\,x$	0.5 pmol

[a] y = peak area (nanocoulombs) and x = concentration (μg/mL).
[b] For 25-μL injections.

potentials than tocopherols and carotenoids (Fig. 5), showing their dominant oxidation potentials between 560 and 620 mV. The response factors of pheophytins were notably lower than those of chlorophylls (data not shown), and this may affect the detection of pheophytins when present in low concentrations in vegetable oil.

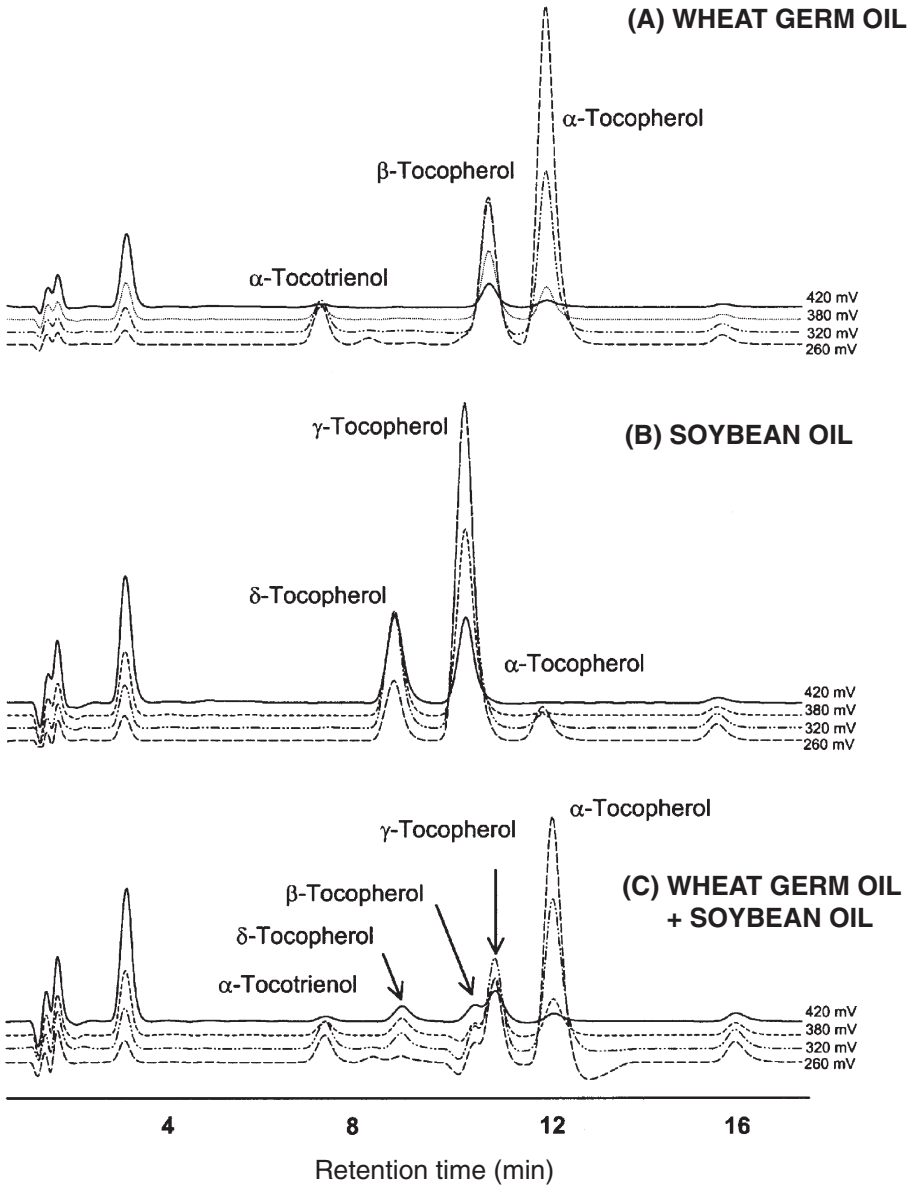

FIG. 6. C_{30} HPLC separation of tocopherols in (A) wheat germ oil, (B) soybean oil, and (C) mixture of wheat germ + soybean oil (2:1).

Sample preparation. Our C_{30} RP-HPLC-ECD methodology allowed for direct injection of diluted oil samples (20–30 ng oil), therefore eliminating a saponification procedure and reducing the time for sample preparation. Psomiadou and Tsimidou (5) injected more than 20 times the amount of oil sample for their normal-phase-HPLC with UV-vis detection in order to obtain an acceptable detector response. Furthermore, saponification is known to affect carotenoids (9–11) and tocopherols (9) adversely. This step has been reported to induce carotenoid isomerization and degradation (9). Lietz and Henry (9) developed an enzymatic method that hydrolyzes TG in red palm oil as an alternative to saponification. No significant loss of carotenoids and tocopherols occurred. However, a 4-h sample preparation time was required for the enzymatic method. Sánchez-Pérez *et al.* (12) employed a continuous tocopherol extraction from vegetable oil by a silicone nonporous membrane coupled in-line with an RP high-performance liquid chromatograph and a coulometric detector. Although the system was fully automated, a washing step was required between successive analyses, and a surfactant was required to aid sample solubilization in injection solvents.

Detection limit, linearity range, and reproducibility. Detection limits for carotenoids, tocopherols, and chlorophylls were determined by serial dilutions of β-carotene, α-tocopherol, and chlorophyll *a* standards, respectively, and the results are shown in Table 1. The detection limits for carotenoids, tocopherols, and chlorophylls were found to be 1 fmol, 0.15 pmol, and 0.5 pmol, respectively, for a 25-μL injection and represent 1000-, 25-, and 5-fold enhancements over the reported UV-vis data (5). Values for β-carotene and α-tocopherol were similar to those found in previous studies (13,15, 16). The detector response was linear between 0.01 and 2.00 μg/mL for all the compounds studied, as indicated by the correlation coefficients (Table 1). Response factors for carotenoids (α-carotene, β-carotene, and lycopene) were in similar ranges, whereas tocopherols showed slightly lower responses by comparison. Chlorophyll *a* maintained a response factor between that of carotenoids and tocopherols.

The reproducibility of the method was tested using samples representative of red palm oil. Within-day variations (CV, $n = 5$) were 4.5% for α-tocopherol, 2.3% for α-tocotrienol, 6.3% for γ-tocopherol, 4.0% for α-carotene, and 2.0% for β-carotene. These values are lower than previous reports for similar compounds (13). Between-day variations were similar for the tocopherols (3.2% for α-tocopherol, 2.7% for α-tocotrienol, 7.2% for γ-tocopherol) and slightly higher for the carotenoids (5.2% for α-carotene and 7.4% for β-carotene). Finckh *et al.* (13) found the between-day variations for both tocopherols and carotenoids were significantly higher than the within-day variations. These differences can be attributed to sample preparation and baseline noise from the electrochemical detector. Motchnik *et al.* (14) and Ferruzzi *et al.* (15) suggested the use of a pulse dampener and/or low-pulse pumps to reduce background noise. Our solvent delivery system has a built-in pulse dampener. Although Darnoko *et al.* (22) were able to achieve similar separations of red palm oil tocopherols and carotenoids, their method exhibited larger variations (up to 25%) than the current methodology. They noted larger variations for compounds with low concentrations. This is most likely the result of harsh saponification conditions applied, where red palm oil was heated at 100°C for 30 min (22). The ability of our methodology to inject oil samples directly onto the HPLC system without introducing error and loss from extraction, saponification, and solvent removal may explain our lower variations.

Application. The current methodology was applied to detect adulteration of vegetable oils based on their tocopherol patterns. Figures 6A and 6B illustrate the distinct tocopherol patterns of wheat germ and soybean oils. Figure 6C demonstrates the change in the tocopherol pattern when wheat germ oil is adulterated with soybean oil in a 2:1 ratio. In addition, this method has been successfully applied in studying the oxidative stability of red palm oil (29). Tocopherols, tocotrienols, and carotenoids (including their geometrical isomers) were quantified simultaneously. This method also allowed for the identification of β-carotene oxidation products (i.e., epoxy β-carotene) not previously identified because of their loss during saponification. Thus, rapid and sensitive analyses of oil quality and adulteration can be achieved with this methodology.

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