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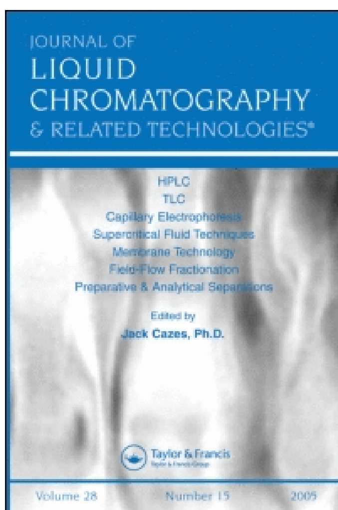

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SIMULTANEOUS HPLC ANALYSIS OF PALM CAROTENOIDS AND TOCOPHEROLS USING A C-30 COLUMN AND PHOTODIODE ARRAY DETECTOR

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SIMULTANEOUS HPLC ANALYSIS OF PALM CAROTENOIDS AND TOCOPHEROLS USING A C-30 COLUMN AND PHOTODIODE ARRAY DETECTOR

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ABSTRACT

An HPLC method for simultaneous identification and quantitation of tocopherols and carotenoids in red palm oil was developed. A C-30 column was used in a HPLC equipped with a photodiode detector and with methyl tertiary-butyl ether/methanol/water as the mobile phase. Tocopherols and carotenoids were monitored at 295 nm and 450 nm, respectively. Gradient elution resulted in complete separation of five tocopherols such as α-, δ-, γ-tocopherols and tocotrienols in palm oil in less than 10 minutes. Several (13) carotenoids were also detected and quantitated in red palm oil within 45 minutes.

INTRODUCTION

Crude palm oil contains an unusually high concentration of carotenoids and tocopherols. As many as 13 carotenoids and its isomers have been detected in crude palm oil, with β-carotene and α–carotene being the major components.[1] The tocopherols in palm oil consists mainly of γ-tocotrienol, α-tocotrienol and α-tocopherol.[2,3] These components contribute significantly to

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the oxidative stability and shelf life of palm oil by acting as free radical scavengers. Carotenoids contain conjugated double bonds that can act as singlet oxygen quenchers. When oxidation occurs, oxygen is removed from the oil.and degradation of fatty acids is minimized.[4] Maximum stability of the oil is achieved when the tocopherols level are 400-600 ppm. Tocopherols and carotenoids are also important nutritionally due to their vitamin E and provitamin A activities.

The analysis of tocopherols and carotenoids is usually done by HPLC with silica or C-18 columns. Simultaneous analysis of both groups of compounds has been done using a photodiode array detector or dual UV/Vis and fluorescence detectors on C-18 columns.[5-7] However, for samples containing several isomers of tocopherols and carotenoids, this column would not separate the isomers completely. The recently developed C-30 carotenoid column provides longer retention times and higher selectivity than C-18 columns resulting in better resolution for both polar and nonpolar carotenes.[8] Different classes of carotenoids and their geometric isomers can be separated completely by this column with different solvent systems.

To date, the C-30 column has been used primarily for carotenoid analysis. The objective of this study was to develop a method for simultaneous separation and quantitation of all major carotenoids and tocopherols in palm oil using the C-30 column and a photodiode array detector.

EXPERIMENTAL

Materials

Red palm oil (Nutrolein, produced in Malaysia) was provided by Malaysian Palm Oil Council in Chicago. Standards such as α-carotene, β-carotene, α-tocopherol, δ-tocopherol and γ-tocopherol were obtained from Sigma Chemical Co., St. Louis, MO. Lutein and β-cryptoxanthin standards were obtained from Extrasynthese, Genay, France.

Sample Preparation

Samples were first saponified by adding 5 mL methanol to 400 mg of the oil sample in a 25 mL test tube. This was followed by 0.3 mL of 60% aqueous potassium hydroxide and one mL methanolic pyrogallol solution (containing 20mg/100mL) as an antioxidant. The mixture was sealed under nitrogen and heated at 100°C for 30 minutes with occasional shaking. After cooling, 10 mL of water and 10 mL diethyl ether were added and the mixture was shaken for one minute. The ether layer was then transferred to a 125 mL separatory funnel containing 10 mL water. The soap solution was extracted two more times

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with 10 mL ether. The separatory funnel was gently swirled to prevent the formation of an emulsion.

After drawing off the water layer, the ether layer was washed three more times with 10 mL water each. Then 10 mL aqueous 0.5 N KOH solution was added followed by 10 mL water for washing. This KOH addition and water washing was repeated two more times followed by 5 more times of water washing or until the water wash was neutral. The ether layer was dried by adding anhydrous Na_2SO_4 and then filtered through 0.2 µm filter. The solvent was evaporated to dryness under nitrogen and the extract was kept at –20°C until analysis. Upon analysis, the extract was dissolved in the HPLC mobile phase and 20 µL of sample was injected.

HPLC System

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The HPLC system consisted of an HP-1050 unit with the HP-1050 photodiode array detector equipped with HP ChemStation software (Hewlett Packard, Wilmington, DE). Two columns were studied. One was a 150 x 4.6 mm, 5 µm Prodigy 100A silica column (Phenomenex, Torrance, CA). The mobile phase with this column was iso-octane:THF (97.5:2.5) used at room temperature and at a flow rate of 1.5 mL/min. The second column was a 250 x 4.6 mm, 5 µm C-30 YMC Carotenoid column (YMC, Wilmington, NC) protected by a guard column. The mobile phase for this column was 81:15:4 methanol:methyl tertiary butyl ether (MTBE):H_2O (solvent A) and 91:9 MTBE: methanol (solvent B). The gradient elution was 100% A to 50%/50% A/B in 45 minutes followed by 100% B in the next 10 minutes and 100% A in the next 5 minutes at a flow rate of 1 mL/min. Tocopherols were monitored at 295 nm and carotenoids were monitored at 450 nm.

RESULTS AND DISCUSSION

Tocopherols can be separated by both normal or reversed phase columns, but the latter usually does not separate β-tocopherol from γ-tocopherol very well. Cort et al.[9] reported a baseline separation of all tocopherols (α-, β-, δ-, γ-tocopherols and tocotrienols) using a normal phase column (silica) with 2.5% tetrahydrofuran in isooctane as the mobile phase. Figure 1 shows the separation of a standard mixture containing α-, β-, δ-, γ-tocopherol and β-carotene in a silica column. The mobile phase was isooctane: THF (97.5:2.5). The detector was set at 295 nm and 450 nm. Excellent resolution was obtained for tocopherols but β-carotene eluted too early. When a saponified palm oil sample was analyzed, all tocopherols/tocotrienols (Figure 2A) were resolved but poor separation was obtained for carotenoids (Figure 2B). This indicates that this system cannot be used for simultaneous analysis of tocopherols and carotenoids.

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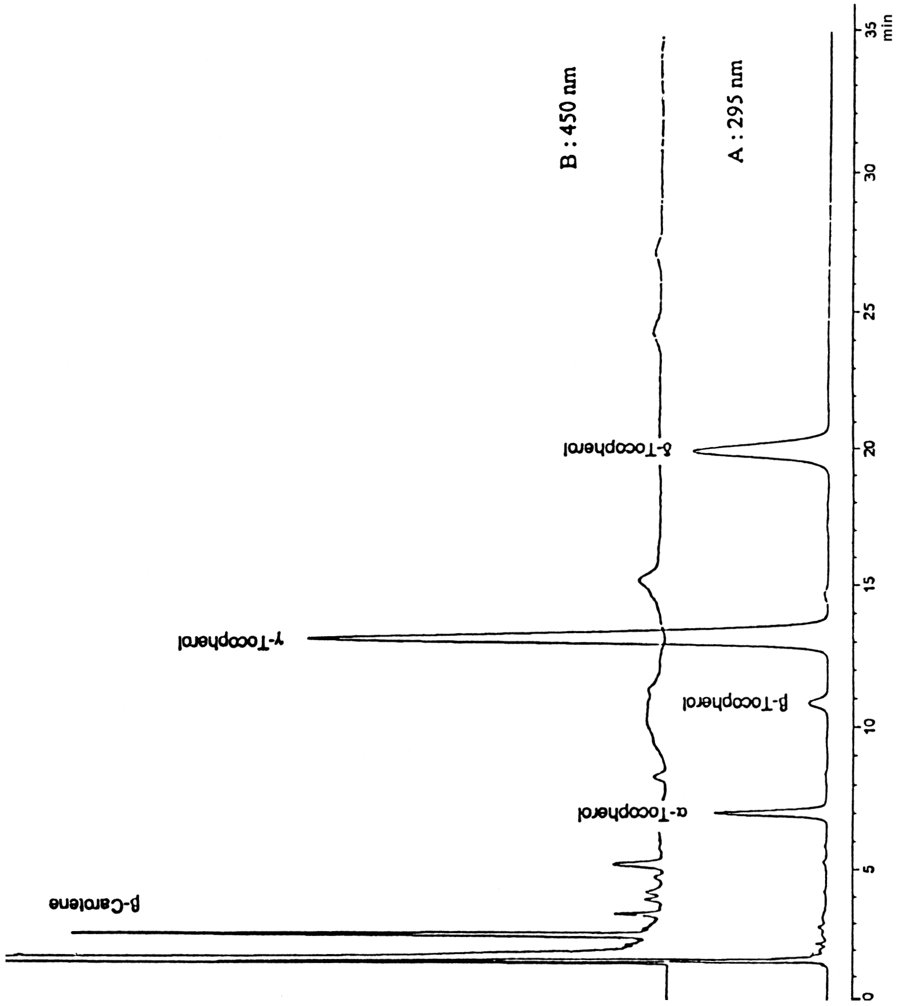

Figure 1. Chromatogram of standard compound mixture using a silica column.

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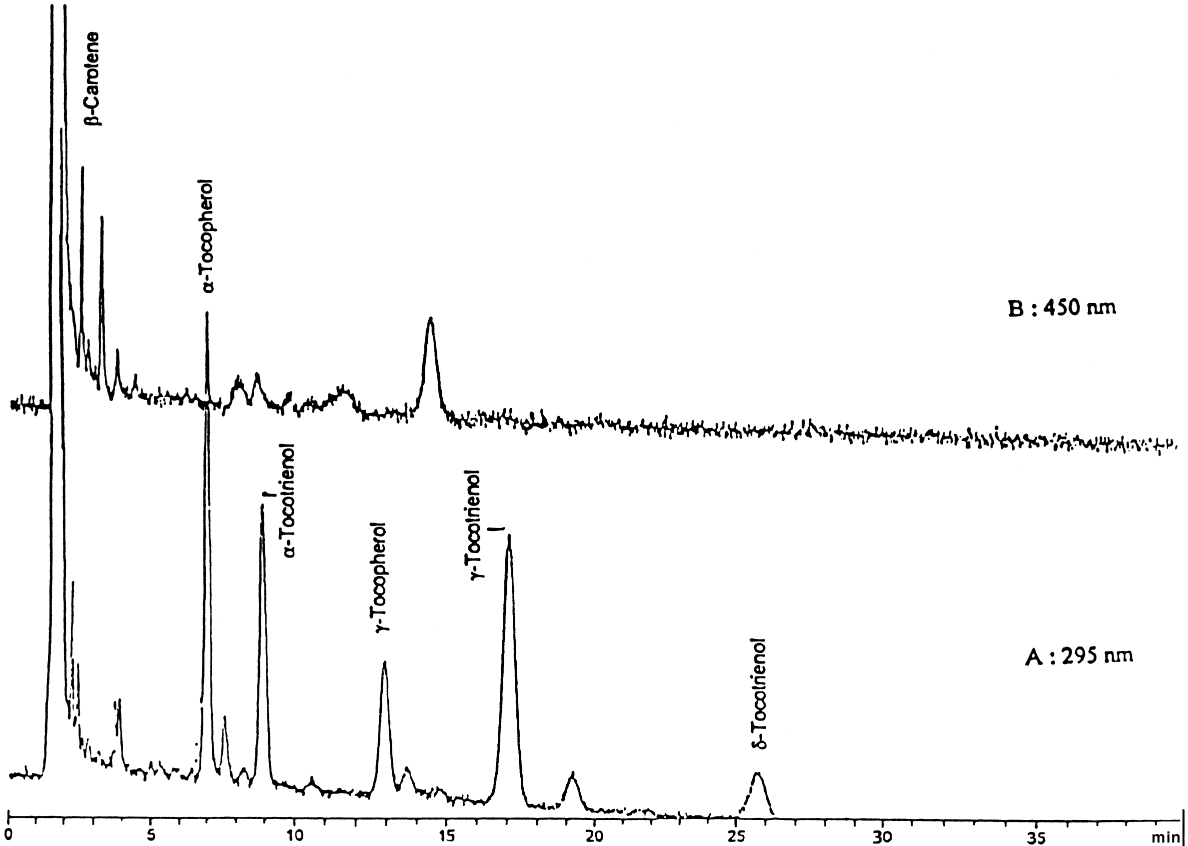

Figure 2. Chromatogram of palm oil tocopherols and carotenoids using a silica column.

Figure 3 shows a C-30 chromatogram of a standard mixture containing α-, δ-, γ-tocopherol, and α- and β-carotenes monitored at 295 nm and 450 nm. Baseline separation was obtained for tocopherols (Figure 3A) and carotenes (Figure 3B) with a large difference in retention times (almost 20 minutes). Sander et al.[8] reported that the first carotenoid eluted using the C-30 column and a similar mobile phase was the polar carotene (astaxanthin) with a retention time of 11 minutes. The large difference in retention time we obtained, together with the large difference in absorbance maxima between tocopherols and carotenoids, prevented overlapping of peaks of both compounds.

Identification of Palm Tocopherols and Carotenoids

The chromatogram of palm tocopherols at 295 nm (Figure 4A) shows that five tocopherol peaks eluted with baseline separation. These peaks were identified as δ-tocotrienol, γ-tocotrienol, α-tocotrienol, γ-tocopherol, and α-tocotrienol, respectively, based on a comparison with standard compounds and the order of elution. In contrast, Lietz and Henry[10] reported that no tocotrienol was detected during simultaneous HPLC analysis of red palm oil using dual UV/Vis and fluorescence detectors and a reversed phase column.

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The carotenoid chromatogram at 450 nm (Figure 4B) shows as many as 13 peaks in red palm oil. Identification was based on the order of elution, retention time, and spectra of absorbance maxima of a particular peak. These data were compared with information in the literature. Some of the peaks can only be tentatively identified due to limited information for comparison. The peaks identified in this analysis are shown in Table 1.

Lutein was identified at a retention time of 12.16 minutes with absorbance maxima at 421, 443, and 473 nm. This peak was confirmed by comparison with the peak of a lutein standard. Lietz and Henry[10] also identified lutein in red palm oil analyzed by HPLC with dual UV/Vis and fluorescence detectors using a reversed phase column. However other researchers[1,11,12] did not observe lutein in palm oil, perhaps due to differences in extraction and saponification methods. Lutein is a polar carotenoid which has limited solubility in non-polar solvents.

The other polar carotenoids identified were α-carotene-5,6-epoxide and β-cryptoxanthin at a retention time of 16.01 and 23.27 minutes, respectively. Ng and Tan[11] also identified α-carotene-5,6-epoxide. It is not clear whether this compound occurs naturally in palm oil or if it is a product of carotenoid oxidation that occurs during sample preparation or storage. The peak of β-cryptoxanthin was confirmed by comparison with the pure compound. Only Lietz and Henry[10] have previously reported the presence of β-cryptoxanthin in palm oil.

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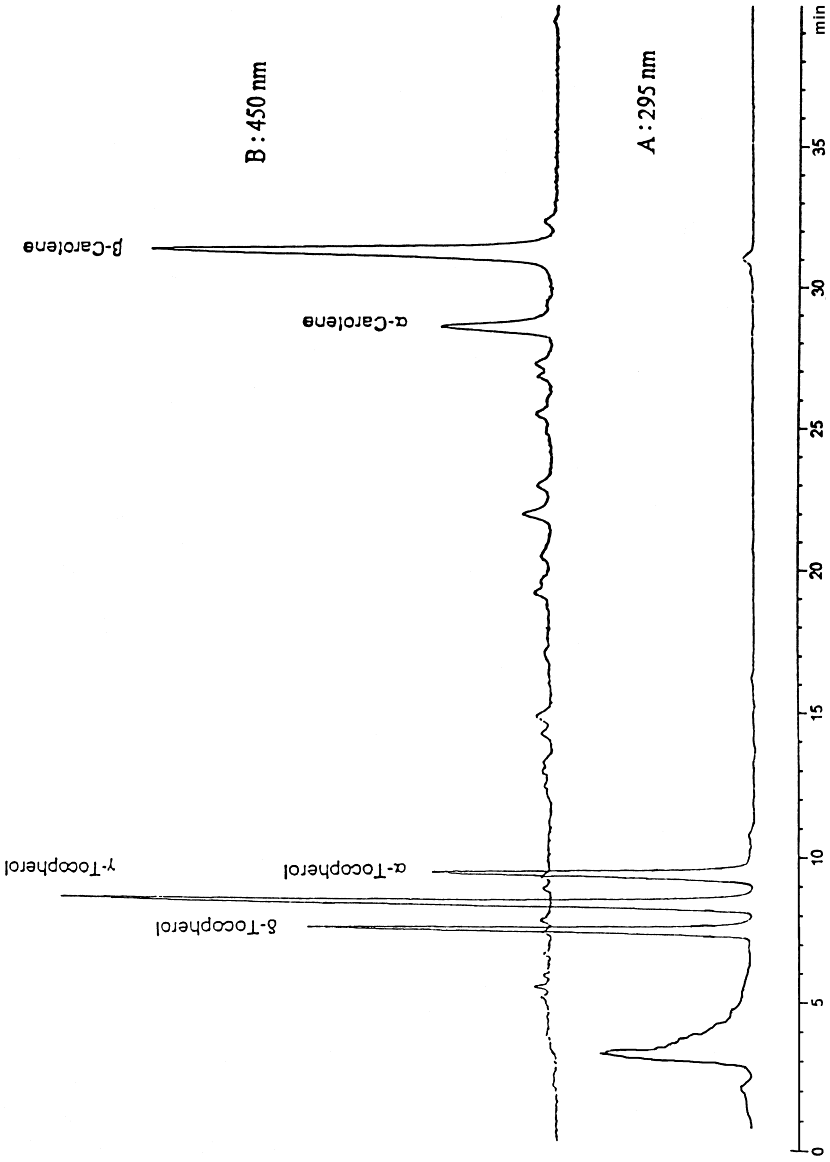

Figure 3. Chromatogram of standard compound mixture using C-30 column.

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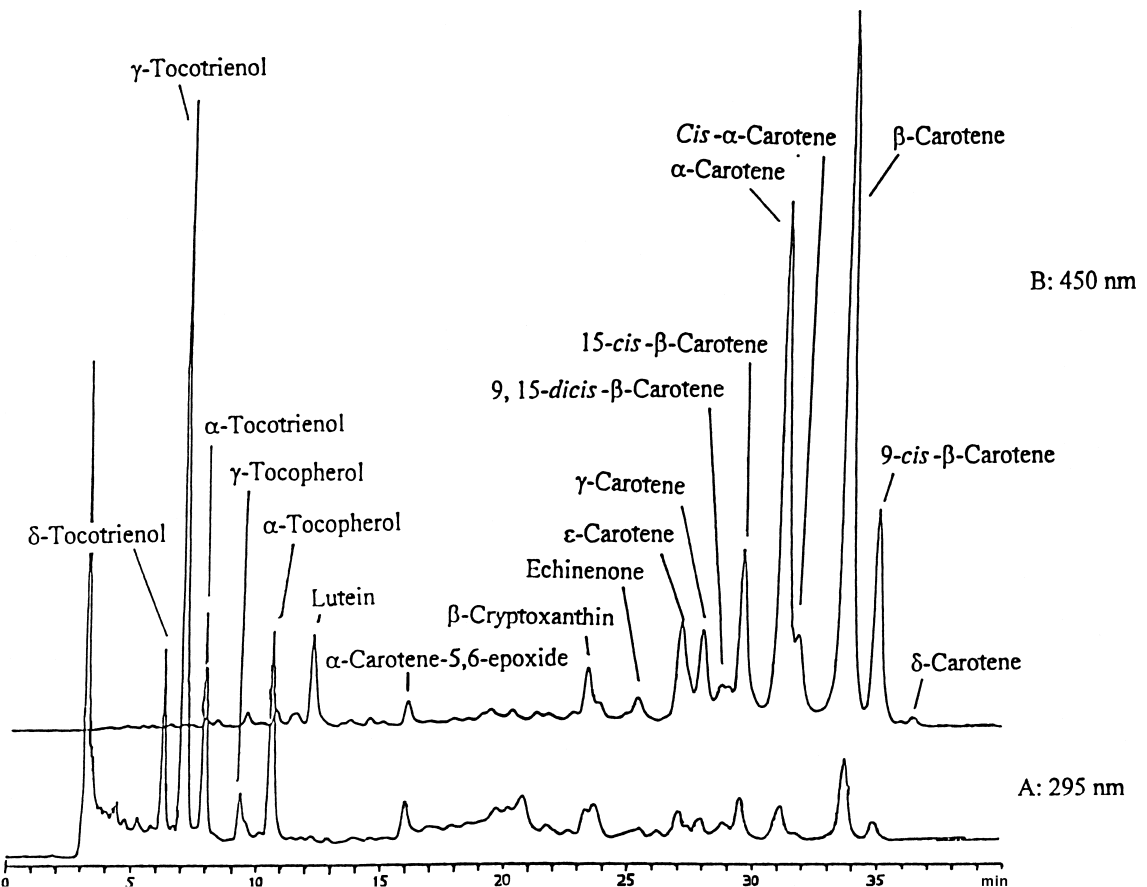

Figure 4. Chromatogram of palm oil tocopherols and carotenoids using C-30 column.

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Table 1

Identification of Carotenoids in Red Palm Oil*

Peak No.	Cartenoid	Retention Time (Min)	Spectral Maxima (nm) This Work	Spectral Maxima (nm) Literature	Ref.	MTBE	MEOH	Water
1	Lutein	12.16	(421), 443, 473	422, 443, 470	14	25.27	71.27	3.46
2	α-Carotene-5,6-epoxide	16.01	391, 441, 467	416, 440, 468	11	28.52	68.19	3.29
3	β-Cryptoxanthin	23.27	(387), 409, 431	426.8, 450.8, 480.1	6	34.65	62.39	2.97
4	Echinenone	25.32	333, 421, 455	299.4, 465.5	11	36.38	60.74	2.87
5	γ-Carotene	27.00	(417), 439, 463	435-43, 460-66	13	37.80	59.40	2.80
6	ε-Carotene	27.87	331, 439, 465	418, 438, 466	11	38.53	58.70	2.76
7	9, 15-*dicis*-β-Carotene	28.93	341, 441, 465	336.6, 446, 465	6	39.43	57.86	2.71
8	15-*cis*-β-Carotene	29.45	339, 445, 471	336.6, 450.9, 470.3	6	39.86	57.44	2.69
9	α-Carotene	31.06	s421, 447, 475	(424.5), 445.5, 473.5	11	41.23	56.15	2.62
10	*Cis*-α-Carotene	31.68	417, 441, 469	418, 438, 466	11	41.75	55.66	2.59
11	β-Carotene	33.60	449, 473	450.5, 478.5	15	43.47	54.12	2.51
12	9-*cis*-β-Carotene	34.83	432, 447, 473	436.4, 446, 472.7	15	44.41	53.14	2.45
13	δ-Carotene	36.34	423, 441, 469	419-20, 439-44, 464-72	11	53.84	45.15	2.01

* Chromatogram is in Figure 4B.

Echinenone (23.27 minutes) was identified based on the order of elution and comparison of absorbance maxima (which were at 387, 409, and 431 nm) with the literature. However, spectral maxima reported in the literature are based on absorbance in pure solvents. If a gradient elution is used, such as in this work, the solvent composition changes continuously which may cause a shift in the spectral maxima. Table 1 shows the calculated solvent composition for each compound at its retention time. γ-Carotene and ε-carotene were tentatively identified at retention times of 27.00 and 27.87 minutes, respectively, based on their spectral maxima.[16] These carotenoids have also been reported by others[11,13] in palm oil.

The most abundant carotenoids in palm oil are hydrocarbon carotenoids with their geometric isomers. α-Carotene and its *cis* isomer were identified at retention times of 31.06 and 31.68 minutes, respectively. The *trans*-β-carotene, the single major carotenoid in palm oil, was resolved at 33.60 minutes. The geometric isomers of this compound are 9,15-di*cis*-β-carotene, 15-*cis*-β-carotene and 9-*cis*-β-carotene, which were eluted at 28.93, 29.45, and 34.83 minutes, respectively. The separation of these hydrocarbon carotenoids was also reported by Sander et al.[8] The last carotene to be eluted was δ-carotene at 36.34 minutes.

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Quantitative Analysis

Due to limited availability of standard compounds, only α-tocopherol and β-carotene were used for developing standard curves. All other compounds were calculated based on these two standard curves.[16] Quantitative analysis of

Table 2

Quantitative Analysis of Tocopherols*

Peak No.	Tocopherols	Retention Time (Min)	Relative Std. Dev. of Retention Time (%)	Conc. (ppm)	Conc. (%)	Relative Std Dev. of Conc. (%)
1	δ-Tocotrienol	6.20	0.08	130.62	13.68	12.40
2	γ-Tocotrienol	6.97	0.01	444.80	46.59	5.11
3	α-Tocotrienol	7.84	0.05	162.39	17.01	8.05
4	γ-Tocopherol	9.32	0.11	55.70	5.83	20.45
5	α-Tocopherol	10.54	0.13	161.21	16.89	0.68
	Total			954.72	100.00	

* 3 replications.

tocopherols and carotenoids are shown in Tables 2 and 3, respectively. The relative standard deviation varied between 2% to 25% for carotenoids and between 0.6% to 20% for tocopherols. Large deviations were observed for compounds with low concentration. One explanation for this deviation is differences in solubility of each compound in mobile phase A due to different polarity. Hydrocarbon carotenoids were more soluble in solvent B. However, the use of solvent B as an injection solvent caused splitting of some polar carotenoids due to solvent interactions. Lietz and Henry[10] also mentioned the importance of the injecting solvent during HPLC analysis. They found that acetone did not result in chromatographic artifacts while dichloromethane produced peak distortion.

Table 3

Quantitative Analysis of Carotenoids*

Peak No.	Tocopherols	Retention Time (Min)	Relative Std. Dev. of Retention Time (%)	Conc. (ppm)	Conc. (%)	Relative Std. Dev. of Conc. (%)
1	Lutein	12.16	0.24	11.85	3.11	4.04
2	α-Carotene-5,6-epoxide	16.01	0.31	3.28	0.86	2.02
3	β-Cryptoxanthin	23.27	0.54	10.58	2.77	25.29
4	Echinenone	25.32	0.23	5.83	1.53	23.79
5	γ-Carotene	27.00	0.37	22.76	5.97	5.62
6	ε-Carotene	27.87	0.28	18.31	4.80	3.83
7	9,15-*dicis*-β-Carotene	28.93	0.20	10.31	2.70	7.61
8	15-*cis*-β-Carotene	29.45	0.23	28.21	7.40	19.25
9	α-Carotene	31.06	0.18	89.80	23.56	10.07
10	Cis-α-Carotene	31.68	0.18	16.80	4.41	7.53
11	β-Carotene	33.60	0.16	126.58	33.21	10.51
12	9-*cis*-β-Carotene	34.83	0.18	35.52	9.32	7.93
13	δ-Carotene	36.34	0.19	1.38	0.36	14.01
	Total			381.19	100.00	

* 3 replications.

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CONCLUSIONS

The method described in this paper could simultaneously identify and quantify different tocopherols and carotenoids in palm oil. Better baseline separation was obtained with most of these compounds that are commonly found in palm oil as compared to other methods. A complete analysis took about 45 minutes with gradient elution. This method could also be used for monitoring degradation products of tocopherols and carotenoids during processing. Extraction and saponification methods need to be studied further to determine optimum conditions for preventing degradation and losses of tocopherols and carotenoids during analysis. Injection solvents that can dissolve carotenoids and tocopherols without producing solvent interactions also need to be optimized.

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