SPECTROSCOPIC IDENTIFICATION OF GEOMETRICAL ISOMERS OF α- AND β-CAROTENES FROM PALM OIL

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ABSTRACT

To date, all identification of palm carotenoids are tentative based on electronic absorption spectra, comparison of the elution sequence with past studies and authentic standards. This study reports the isolation of individual major isomers of palm carotenes using a semi-preparative C_{30} column. The results of MS, ^{1}H NMR of four isolated peaks, assigned as Fraction 1 (a mixture of 13 and 13' cis α-*carotene), Fraction 2 (13* cis β-*carotene), Fraction 3 (all* trans α-*carotene) and Fraction 4 (*cis β-*carotene) supported the identification of their structures.*

Keywords: geometrical isomers, palm oil carotenes, ^{1}H NMR – MS, data of palm carotenoids.

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INTRODUCTION

Crude palm oil is one of the richest natural sources of carotenoids. The carotenoid concentration varies from 400-3500 ppm depending on the species of oil palm. The crude oil from *tenera*, the commercial planting material, contains 500-700 ppm carotenes. The early identification of palm carotenoids used the C_{18} column separation with HPLC with a photo diode array detector (DAD) (Ng and Tan, 1988) or UV-Vis detector (Yap *et al.*, 1991). Their findings showed that palm carotenoids comprised 11 types of hydrocarbon carotenes and oxygenated ones referred to as xanthophylls. Identification of the palm carotenoids from these findings were based on retention time comparison with authentic standards and UV-Vis spectral identification. However, complete separation of the geometric isomers of α- and β-carotene were not achieved. With the advent of the C_{30} column, the geometric isomers of these major carotenes were resolved. Analyses of carotenoids in crude palm oil, red palm olein and carotene concentrates have shown the presence of 13 *cis*, 13' *cis* and 9 *cis* isomers of the major carotenes, α- and β-carotenes (Tay *et al.*, 2002; Darnoko *et al.*, 2000; Puspitasari-Nienaber *et al.*, 2002). However, again identification of these geometric isomers were based on comparison with the elution sequences from past studies and electronic absorption spectra. No standards of *cis* isomers were available for comparison. This study was aimed at isolating the individual geometric isomers of the major carotenes from a preparative reverse phase C_{30} column and confirming their structures by ^{1}H NMR and MS.

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MATERIALS AND METHODS

Materials

A 30% palm-based carotene concentrate produced via the methyl esters route was obtained from a commercial source. Synthetic all *trans* β-carotene was obtained from Sigma Chemical Co. The HPLC solvents were methanol (certified ACS; Fisher Chemcial, Fairlawn, NJ, USA) and methyl-*tert*-butyl ether (MTBE) (HPLC grade; Fisher). Analytical grade acetone was obtained from Fisher Chemicals.

Methods

Instrumentation and chromatography. An analytical Develosil reverse phase (RP)-aqueous C_{30} (4.6 mm i.d. x 250 mm, 5 mm) and semi-preparative C_{30} (10 mm i.d. x 250 mm, 5 mm) columns were used and protected with guard columns (4.6 mm i.d x 50 mm) and (10 mm i.d x 50 mm). The HPLC system comprised of a Hewlett Packard Model 1100 with

quarternary solvent delivery system and a HP1100 series online degasser, and was connected to a HP1100 series diode array detector (DAD) covering the spectral range 190 –800 nm.

Analytical carotenoid profile analyses. The carotenoid isomer chromatogram from the carotene concentrate was obtained at analytical scale using the isocratic mobile phase of MeOH:MTBE (89:11) v/v at a flow rate of 1 ml min^{-1}. About 0.3 g of the concentrates was dissolved in 2 ml acetone and 20 ml were injected. The chromatogram was monitored at 444 nm with the column temperature at ambient laboratory (25ºC). The chromatogram is shown in *Figure 1*.

Isolation of Palm Carotenes Isomers

About 0.35 g of the 30% carotene concentrate was dissolved in 20 ml acetone. The mixture was injected into the HPLC-PDA attached to a semi-preparative C_{30} column with a sample injector loop of 2 ml. The solvent system was 89:11 methanol:MTBE (v/v) and at a flow rate of 4.6 ml min^{-1}. The chromatogram showed four major fractions as shown in *Figure 2*. These fractions were focused for isolation. In the case of Fractions 1 and 2, about 2-3 ml of the mobile phase were collected. For Fractions 3 and 4, 5-6 ml of the mobile phase were collected. The solvents were removed *in vacuo* at 35ºC for 30 min and the remaining solvent removed by nitrogen sparging. All the samples were kept under a head space of nitrogen at –30ºC prior to analysis. The collection of peaks was repeated till the pooled fractions of the isomers weighed 4-10 mg, sufficient for ^{1}H NMR and MS analyses.

Mass spectrometric analyses of isolated fractions. The mass spectra of the four isolated fractions from preparative HPLC were obtained by direct insertion (DI) into a Shimadzu QP5050A, Mass Spectrometer using electron impact (EI) ionization and analysed using GC-MS solution software.

^{1}H NMR spectroscopy. The ^{1}H NMR spectra were obtained on a JEOL JMM ECA-400 Mhz NMR spectrometer. All the spectra were measured in $CDCl_3$ at ≈ 25°C. The internal standard was tetramethylsilane.

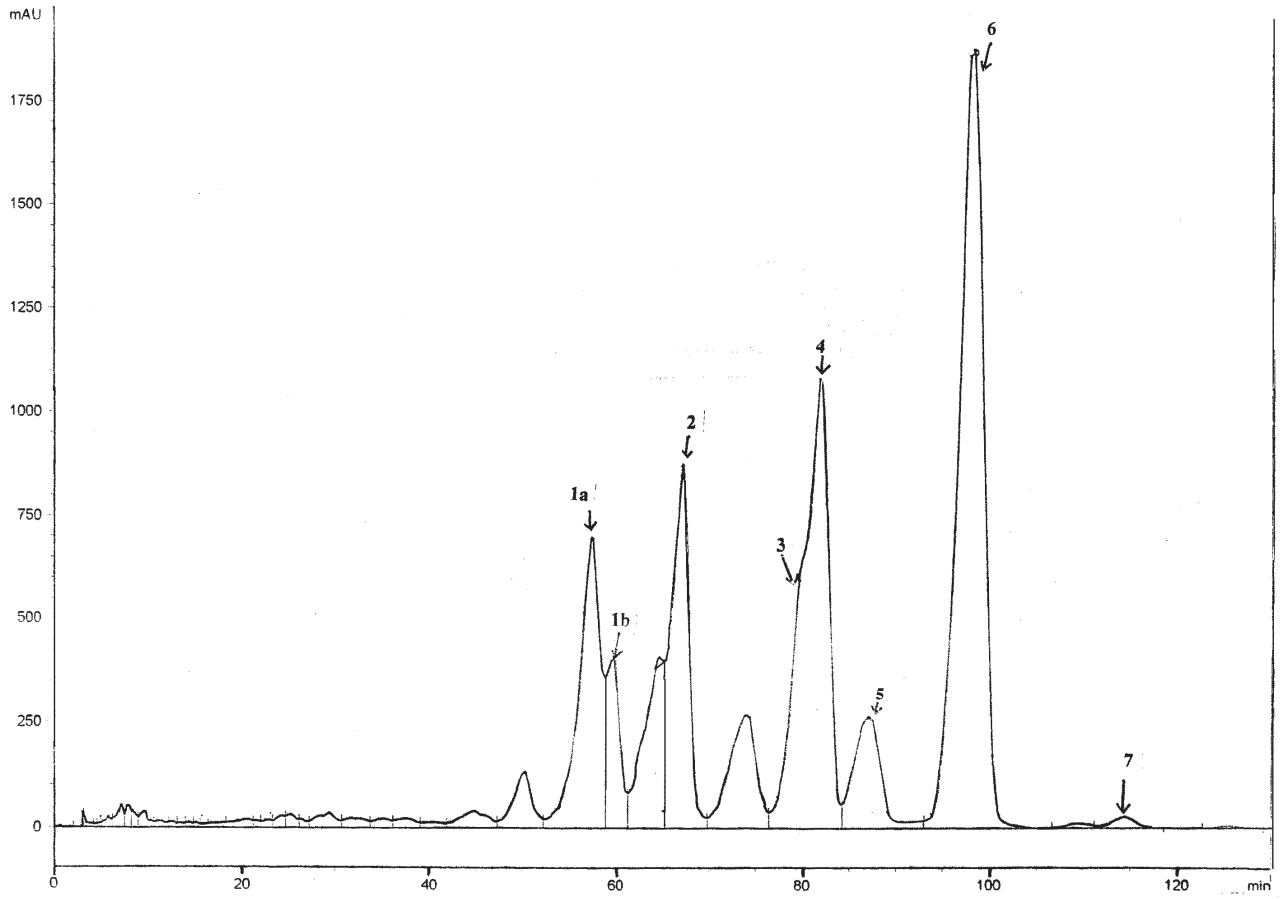

Figure 1. HPLC profile at 444 nm of the carotene concentrate using an analytical column with reverse phase C_{30} stationary phase, MTBE-methanol (11:89, v/v) mobile phase; at a flow rate of 1 ml min^{-1}. 1a) 13 cis *α-carotene 1b) 13' cis α-carotene 2) 13* cis *β-carotene 3)* cis *α-carotene 4) all* trans *α-carotene 5) 9* cis *α-carotene 6) all* trans *β-carotene and 7) 9* cis *β-carotene.*

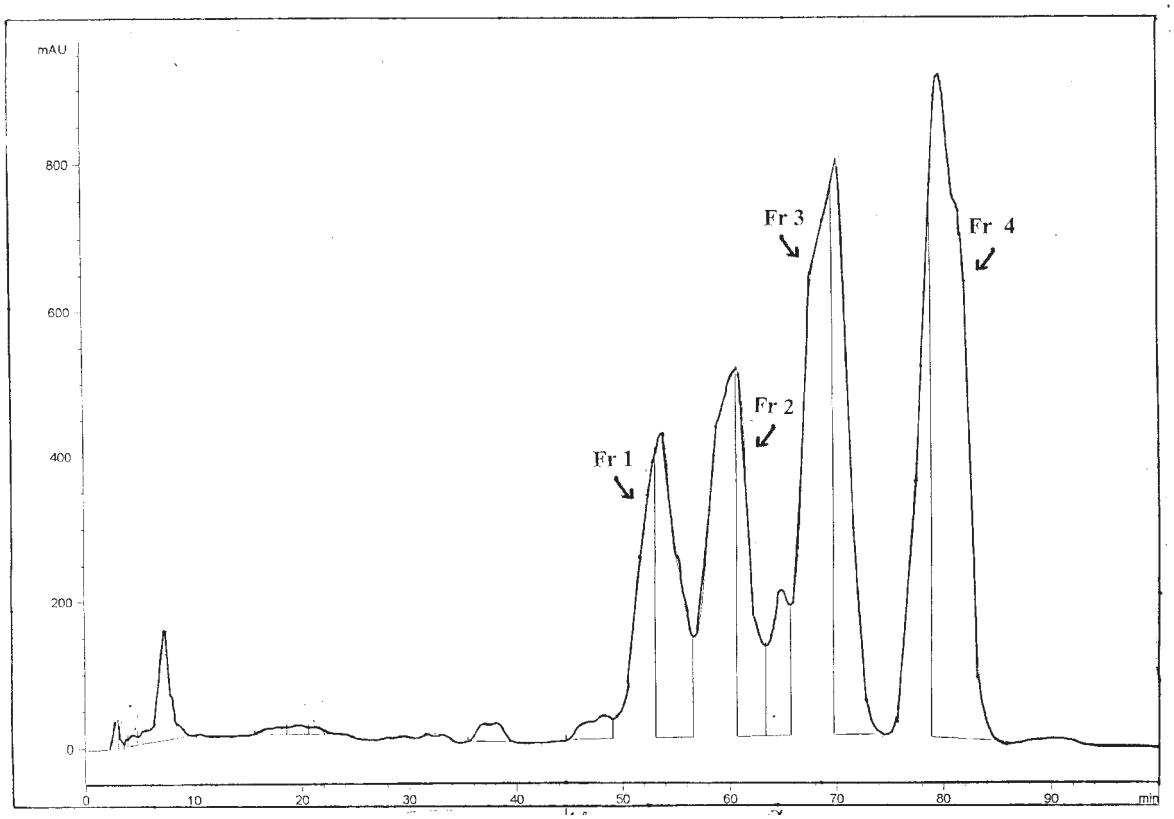

Figure 2. HPLC profile at 444 nm of the carotene concentrate using a semi-preparative column with RP C_{30} stationary phase, MTBE-methanol (11:89,v/v) mobile phase; flow rate of 4.6 ml min^{-1}. (a) Fraction 1 (b) Fraction 2 (c) Fraction 3 and (d) Fraction 4.

RESULTS AND DISCUSSION

The four major fractions between 40-100 min in *Figure 2* were collected individually and repeatedly until about 4 mg for Fractions 1 and 2 and 5-10 mg for Fractions 3 and 4. Peak purity checks were performed on the four fractions using HP Chemstation and it was found that the fractions contained some impurities which could be (1) an overlapping carotene isomer, and/or (2) traces of esters or grease. Based on the electronic absorption spectra (*Table 1*) and the apparent split in peak 1 (*Figure 1*), it was deduced to contain mono *cis* geometric isomers of α-carotene, in which the *cis* bonds are present at carbons 13 and 13′, *i.e.* at opposite ends of the molecule. Peak 2 was assigned as 13 *cis* β-carotene, peak 3 *(Table 1)* as all *trans* α-carotene and peak 6 *(Table 1)* as all *trans* β-carotene.

TABLE 1. ELECTRONIC ABSORPTION MAXIMA OF PEAKS FROM *Figure 1*

Peak	Absorption maxima (nm)	
	This study	Previous studies[b]
1a (13 *cis* α-carotene)	332 410 (440)[a] 463	331 nd (438) 466
1b (13′ *cis* α-carotene)	331 410 (440) 464	331 nd (438) 465
2 (13 *cis* β-carotene)	338 421 (444) 470	(443)
4 (*trans* α-carotene)	423 (445) 476	nd (445) 474
6 (*trans* β-carotene)	422 (452) 478	(450)

Notes:
[a] Values in parentheses represent the main absorption maxima.
[b] Absorption maxima for α-carotene isomers in MTBE (Emenhiser *et al.*, 1996) and main absorption maxima for β-carotene isomers (Emenhiser *et al.*, 1995).
nd - no maxima detected.

As further confirmation of the assignment of peaks 1-4, the MS spectra were obtained and are shown in *Figures 3a* to *d*. The intensities of the characteristics peaks in the MS spectra of Fractions 1 to 4 from *Figure 3* is shown in *Table 2*. All the four peaks gave the strong (M+1) fragment peak indicating the same molecular formula ($C_{40}H_{56}$) and provided almost similar fragmentation patterns. However, the intensities of the characteristics peaks for Fractions 1, 4 for α-carotene and 3, 4 for β-carotene differed, enabling them to be distinguished from each other. Also, the MS spectra of peaks 1 and 3, showed that the two characteristics peaks due to losses of 56 and 123 mass units from the precursor

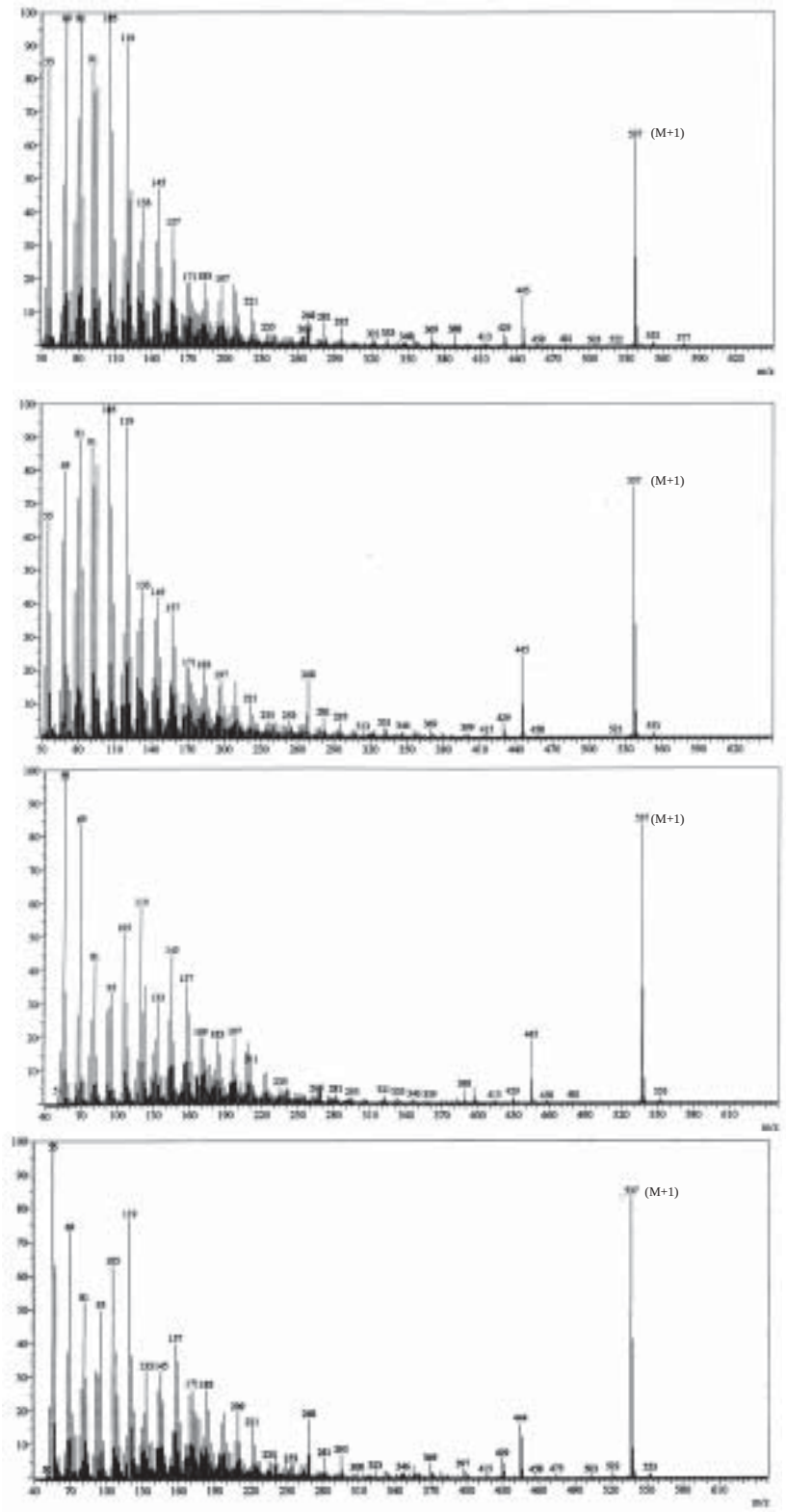

Figure 3. Mass spectra of (a) Fraction 1 (b) Fraction 2 (c) Fraction 3 and (d) Fraction 4.

ion attributed to the unconjugated 4,5 double bond in the terminal ring (alpha ionone ring) of alpha carotene $[M - 56]^+$ at m/z 481 resulting from retro-Diels Alder (RDA) fragmentation of the α-ionone ring, and $[M-123]^+$ at m/z 413 due to loss of the ring at the doubly allylic position. A combination of elimination of toluene and retro-Diels Alder fragmentation ($[M-92-56]^+$) at m/z 388 was detectable, but not in the β-carotene MS spectrum. For Fractions 2 and 4, an abundant fragment ion was observed at 444, from the apparent elimination of methyl toluene. No specific fragmentation due to the end groups was observed as shown in *Figures 3b* and *d*. The base peak for the *cis* form of α- and β-carotenes was at (M+1) 537 and m/z 105. The base peaks for the *trans* form of α- and β-carotenes were at (M+1)537 and m/z 55, respectively. There were some impurities in the four fractions as indicated by the peaks at m/z 552 (Fractions 1 and 3) corresponding to β-carotene 5,6 epoxide and at m/z 553 (Fractions 2 and 4).

Table 3 shows the ^{1}H signals for the α-carotene isomers, Fractions 1 and 3, obtained on 400 MHz NMR. The chemical shifts (δ in ppm) of Fractions 1 and 3 were compared with those reported by Emenhiser *et al.* (1996) from a pure mixture of standard α-carotene.

Figures 4 and *6* show the ^{1}H NMR spectra for Fractions 1 and 3. Fraction 1 was confirmed to be a mixture of two isomers, 13 *cis* and 13'*cis*. The chemical shifts at H-2, H-3, H-4, H-16 to H-20 for Fraction 1 correlated well with the published data for 13 *cis* and 13'*cis* α-carotenes. In Fraction 1, the downfield isomerization shift of Δδ = 0.52 observed at H-12 (6.89), H-15 (6.79) and upfield at H-14 (6.10) supported the presence of the 13 *cis* form. At the same spectral, downfield signals at H-12'(6.86), H-10' (6.17), H- 15 (6.55) and upfield at H-14 (6.24) are characteristic of a 13' *cis* form. Peaks at C15' were not detected.

The chemical shifts of Fraction 3 data showed good correlation with published data for all *trans* α-carotene. However, not all the signals were assigned, *i.e.* methylene protons, C-H eq (2') and olefinic protons C-H (7,10', 11 and 15'). The ^{1}H NMR spectra (*Figure 6*) showed impurities in Fraction 3 at δ 0.06, δ 0.80, δ 0.79, δ 1.25. ~ δ 3.6, δ 4.72, δ 5.11 and δ 5.4 ppm. However, these impurities did not obscure the characteristic peaks for structure assignment.

Table 4 shows the ^{1}H signals for β-carotene isomers, Fraction 2, synthetic all *trans* β-carotene and published data for *trans* β-carotene. The chemicals shift of Fraction 4, assigned as *trans* β-carotene correlated well with those of synthetic all *trans* β-carotene and published data.

Fraction 2 was previously tentatively assigned as the 13 *cis* form of β-carotene and reconfirmed by MS to be β-carotene. Assignment of the peaks at H-12,12' at 6.98 and H-14,14' at 6.117 was based on the isomerization shifts Δδ = δ *cis* - δ *trans* (in ppm) of different protons based on the average Δδ values by Englert, (1982) at H-12 of 0.62 and H-14 of 0.12 for the 13 *cis* isomer. The methylene protons, H-7, H-8, H-10, H-11, and all the methyl protons correlated well with published data (Vetter et al., 1971) and synthetic all *trans* β-carotene. This supported the presence of 13 *cis* β-carotene in this fraction. In the ^{1}H NMR spectra (*Figure 5*), there was some on-column reisomerization in Fraction 2, as evidenced by the olefinic protons of the *trans* form at δ 6.24, δ 6.33 and δ 6.64.

Impurities were detected at the chemical shifts between δ 3.0 and δ 5.0 ppm for peaks 2 and 4. However, these impurities did not have any major impact on the main characteristic proton shifts essential for identification.

TABLE 2. INTENSITIES OF CHARACTERISTIC PEAKS IN THE MASS SPECTRA OF FRACTIONS 1 – 4, SYNTHETIC ALL *trans* β-CAROTENE, AND FROM PUBLISHED DATA FOR β- AND α-CAROTENE*

Carotene	Relative intensity at m/z								
	43	59	69	73	83	91	109	133	(M+1)
β-Carotene*	30	0	100	0	23	45	22	34	44
Synthetic all *trans* β-carotene	15	0	100	0	20	43	25	33.6	70
Peak 2	nm	0	80	14	50	87	40	44	75
Peak 4	nm	0	73	13	30	32	25	32	83
α-Carotene*	38	0	92	2	20	94	29	35	100
Peak 1	nm	0	98	16	18	84	32	41	98
Peak 3	nm	0	84	4	11	27	16	30	87

Notes: nm - not measured; M^+ = molecular ion.
* from Vetter *et al*. (1971).

TABLE 3. ^{1}H NMR DATA FOR FRACTIONS 1 AND 3

Position and type of proton	Fraction 1		Fraction 3
	13 *cis*	**13′ *cis***	***cis trans* α-carotene**
2 methylene	1.47 (≈ 1.47)	nd (≈ 1.47)	1.47 (≈ 1.47)
2′ methylene	nd (≈ 1.47 ax) nd (≈ 1.20 eq)	nd (≈ 1.47 ax) nd (≈ 1.19 eq)	nd (≈ 1.47 ax) nd (≈ 1.18 eq)
3 methylene	1.67 (≈ 1.62)	nd (≈ 1.61)	1.59 (≈ 1.61)
3′ methylene	2.03 (≈ 2.02)	nd (≈ 2.02)	2.02 (≈ 2.02)
4 methylene	2.03 (≈ 2.03)	nd (≈ 2.02)	2.02 (≈ 2.03)
4′ olefinic	5.41 (5.41)	nd (5.42)	5.40 (5.41)
6′ methine	nd (2.18)	nd (2.20)	2.19 (2.18)
7 olefinic	- (6.19)	6.17 (6.17)	6.14 (6.17)
7′ olefinic	5.51 (5.52)	5.55 (5.55)	5.53 (5.53)
8 olefinic	6.15 (6.15)	- (6.13)	6.14 (6.14)
8′ olefinic	6.11 (6.11)	- (6.12)	6.11 (6.11)
10 olefinic	- (6.19)	6.15 (6.15)	6.15 (6.15)
10′ olefinic	- (6.13)	6.17 (6.17)	nd (6.13)
11 olefinic	- (6.65)	6.64 (6.64)	6.64 (6.65)
11′olefinic	- (6.60)	6.61 (6.62)	6.61 (6.61)
12 olefinic	6.89 (6.88)	6.36 (6.36)	6.36 (6.35)
12′ olefinic	6.33 (6.34)	6.86 (6.87)	6.33 (6.34)
14 olefinic	6.10 (6.10)	6.24 (6.23)	6.25 (≈ 6.25)
14′ olefinic	6.24 (6.23)	6.10 (6.10)	-
15 olefinic	6.78 (6.79)	6.55 (6.55)	6.25 (≈ 6.25)
15′ olefinic	nd (≈- 0.07)	nd (≈0.17)	nd (6.55)
16 & 17 methyl	1.03 (1.03)	- (1.031)	1.02 (1.029)
16′ methyl	0.90 (0.904)	- (0.911)	0.90 (0.904)
17′ methyl	0.82 (0.822)	- (0.828)	0.82 (0.822)
18 methyl	1.72 (1.726)	- (1.719)	1.71 (1.718)
18′ methyl	1.59 (1.585)	- (1.592)	1.58 (1.584)
19 methyl	1.97 (1.977)	- (1.970)	1.97 (1.972)
19′ methyl	1.91 (1.909)	- (1.917)	1.91 (1.911)
20 methyl	1.99 (1.972)	- (1.963)	1.97 (1.972)
20 methyl	1.95 (1.952)	- (1.981)	1.96 (1.962)

Notes: nd - not detected.
(-) = peaks overlapped for 13 *cis* and 13′ *cis.*
Published results are in brackets.
Source: Emenhiser *et al.* (1996).

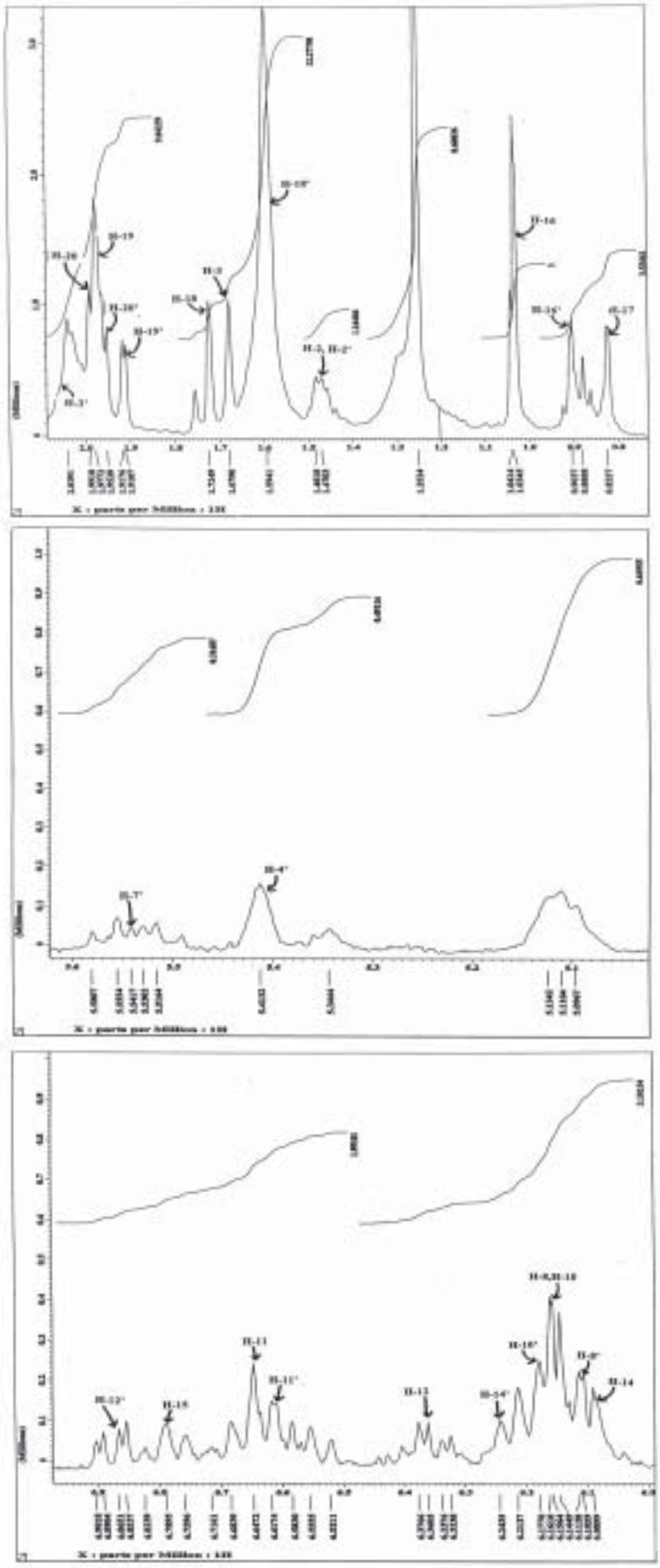

Figure 4. [1]H NMR spectra of Fraction 1.

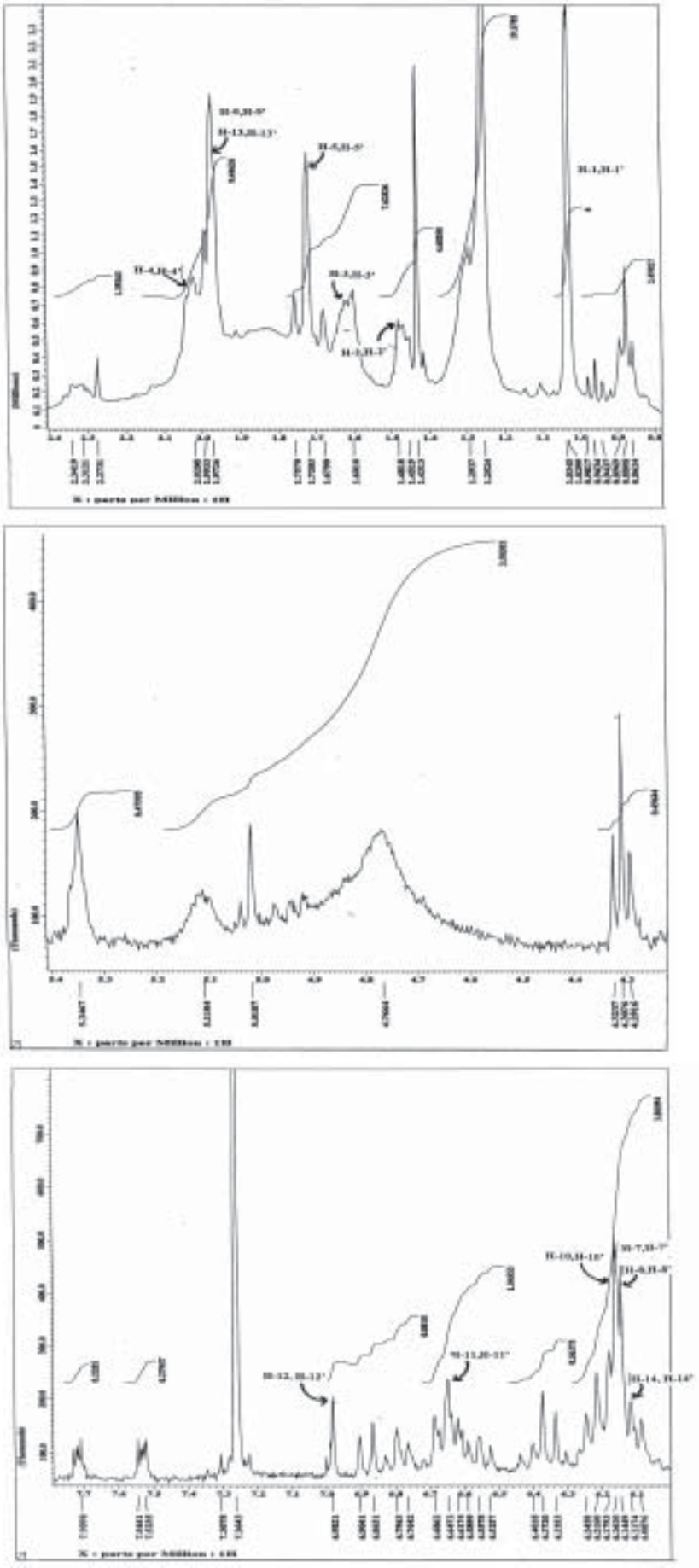

Figure 5. [1]H NMR spectra of Fraction 2.

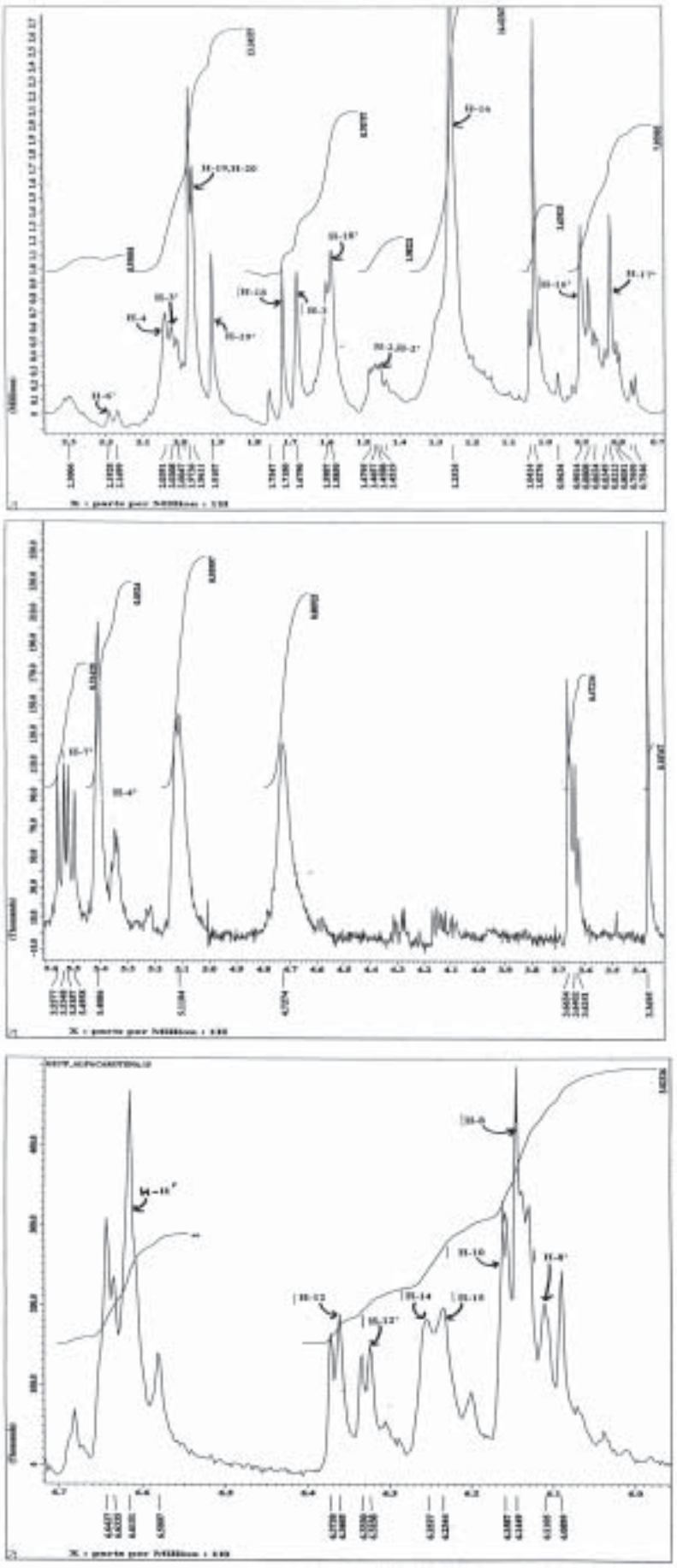

Figure 6. ^{1}H NMR spectra of Fraction 3.

TABLE 4. ^{1}H NMR DATA FOR FRACTION 2, FRACTION 4 AND SYNTHETIC ALL *trans* β-CAROTENE

Position and type of proton	Fraction 2 13 *cis* β-carotene	Fraction 4 all *trans* β-carotene	Synthetic all *trans* β-carotene
2 2′ methylene	1.48	1.48	1.48 (1.45)
3 3′methylene	1.60	1.60	1.60 (≈1.60)
4 4′ methylene	2.02	2.02	2.01 (2.02)
7 7′ olefinic	6.14	6.14	6.14 (≈6.14)
8 8′olefinic	6.14	6.14	6.14 (≈6.14)
10 10′ olefinic	6.14	6.14	6.14 (6.145)
11 11′ olefinic	6.64	6.64	6.64 (6.65)
12 12′ olefinic	6.98	6.33	6.33 (6.34)
14 14′ olefinic	6.11	6.24	6.24 (≈6.24)
15 15′olefinic	NA	6.63	6.62 (≈6.63)
1 1′ methyl	1.03	1.02	1.02 (1.03)
5 5′methyl	1.72	1.72	1.71 (1.72)
9 9′ methyl	1.97	1.97	1.97 (1.98)
13 13′ methyl	1.97	1.97	1.97 (1.98)

Notes: NA – not assigned.
Published data are in column 4 in brackets.
Source: Vetter *et al.* (1971).

CONCLUSION

Previous identifications of palm carotenoids were based on electronic absorption spectra obtained from analysis in a HPLC-PDA with a C_{30} column. By collecting the fractions individually and analysing them with ^{1}H NMR and MS the combined spectroscopic data supported the identification of the major palm carotenes. These data are the first ever published results for the major palm carotenoids. This information is important because of the increasing use of palm carotenoids as health supplements.

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