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IDENTIFICATION OF LUTEIN IN CRUDE PALM OIL AND EVALUATION OF CAROTENOIDS AT VARIOUS RIPENING STAGES OF THE OIL PALM FRUIT

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ABSTRACT

The xanthophylls in crude palm oil (CPO) were identified as lutein, α-carotene 5,6-epoxide, α-carotene 5,8-epoxide, and ß-carotene 5,6-epoxide and/or ß-carotene 5',6'-epoxide based on their electronic absorption maxima from HPLC-PDA. Lutein, a dihydroxy carotenoid, was isolated from CPO extract and its structure confirmed by co-elution with an authentic standard using HPLC-photodiode array (PDA) and mass spectra. At 7 DAA (days after anthesis), lutein was predominant with only trace amounts of α- and ß-carotenes. At 21, 84 and 126 DAA, the levels of α- and ß-carotenes increased although lutein still predominated. However, when the palm fruit ripens at 140 DAA, the level of lutein decreased dramatically to a minor carotene while α- and ß-carotenes predominated with other minor carotenes, i.e. phytoene, lycopene, ß-zeacarotene, γ-carotene, ς-carotene, formed.

Keywords: α-carotene 5,6 epoxide and α-carotene 5,8 epoxide; β-carotene 5,6 epoxide; crude palm oil, mass spectra of lutein, unripe palm fruits.

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INTRODUCTION

Neoxanthin, violaxanthin, lutein, zeaxanthin, b-cryptoxanthin, and α- and β-carotene epoxides are oxygenated carotenes/xanthophylls usually associated with higher plants. These xanthophylls are derived from α- and β-carotenes which, incidentally, are the major carotenoids in palm oil. Some xanthophylls, *i.e.* 3-dehydroretinal, aurochrome, ξ-carotene 1, 2-epoxide, citroxanthin, α-carotene 5,8 and 5,6 epoxides, β-carotene 5,8 and 5,6 epoxides, were tentatively identified in crude palm oil (CPO) (Ng and Tan, 1998). Lutein was tentatively identified in red palm olein when a more polar solvent mixture, *i.e.* diethyl ether/petroleum ether, was used (Darnoko *et al.*, 2000; Lietz and Henry, 1997). This suggested that lutein may be present in oil palm fruits and palm oil.

Lutein, together with zeaxanthin, usually accumulates at high levels in the macular region of the human retina (Bone *et al.*, 1997). Epidemiological and experimental studies suggest that lutein can help treat macular degeneration by reducing the amount of harmful short wavelength blue light between 400-475 nm (Landrum *et al.*, 1997; Landrum and Bone, 2001). A study by (Park *et al.*, 1998) suggested the anti-cancer properties of lutein. They found that with laboratory mice injected with breast cancer cells, upon feeding a diet with lutein, their incidence of tumors was lower compared to that of the untreated ones. It has also been linked to the prevention of epithelial cancers (Yang *et al.*, 1996).

The α-carotene, having both β and ε rings, is believed to be the immediate precursor of lutein. In red pepper (*Capsicum anuum*. L), lutein together with β-carotene were found in the unripe green fruit and leaves but not in the ripe fruit (Goodwin, 1971). The presence of lutein has also been reported in palm fruits but not confirmed (Hunter *et al.*, 1944). This study is to confirm the presence of lutein in CPO by mass and electronic absorption spectra and co-

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elution with an authentic standard. The points of synthesis of lutein and other hydrocarbons carotenes were also investigated by examining the palm fruits at various days after anthesis (DAA).

EXPERIMENTAL PROCEDURES

Materials

Oil palm (*Elaeis guineensis* var *tenera*) fruits of various ages, *i.e.* 7, 21, 84, 126 and 140 DAA were obtained from two plantations in Selangor, Malaysia. CPO was obtained from the Malaysian Palm Oil Board (MPOB) Experimental Mill in Labu, Negeri Sembilan, Malaysia. Lutein standard was purchased from Sigma Chemicals Co., St. Louis, Missouri, USA. HPLC grade methanol and *tert*-butyl methyl ether (TMBE) were supplied by Fisher Scientific. HPLC grade acetonitrile and analytical grade dichoromethane were from Merck. Diethyl ether, hexane and ethanol used during saponification were of analytical grade from Merck.

METHODS

Isolation of Lutein from CPO

Preparation of carotenoid concentrate of CPO via saponification. CPO (10 g) was dissolved in 60 ml absolute ethanol and 0.5 g pyrogallol added. Ten millilitres aqueous KOH (50% wt/v) were added to the homogenized mixture. The mixture was refluxed in a water bath at 100°C for 1 hr. Then 100 ml diethyl ether/hexane (50:50 by volume) were added to extract the carotenoids. The extraction was repeated until all the non-saponified materials were recovered. The extract was washed with water until neutral, and dried over anhydrous sodium sulphate. Sample solutions were concentrated *in vacuo*. The above process was repeated until 200 g concentrated carotenoids were accumulated.

HPLC-photodiode array (PDA) analytical scale detection of lutein and other xanthophylls in the unsaponifiable samples. The carotenoids profile of unsaponifiable CPO was obtained using an analytical reverse phase Genesis C_{18} column (4.6 mm i.d. x 25 cm; 5 µm particle size) protected by a guard column of the same material using an isocratic mobile phase of methanol/TMBE (89:11 v/v), at a flow rate of 1 ml min^{-1}.

HPLC-PDA procedures for analysis of unsaponifable CPO sample spiked with authentic lutein standard. The samples were analysed using a silica-based nitrile bonded (4.6 mm i.d. x 25 cm; 5 µm particle size) column (Regis Chemical Co.) with an isocratic mixture of hexane/dichloromethane/methanol/N,N-diisopropylethylamine (74.65:25:0.25:0.10 v/v)(Khachik *et al.*, 1992), at a flow rate of 1 ml min^{-1}.

Purification of carotenoids extracts from saponification by preparative thin layer chromatography (PTLC). The extracts were separated by a PTLC (20 x 20 cm) plate coated with silica gel of 1 mm thickness. Standard lutein was spotted on the plate beside the extract. Repeated development of up to four times with heptane/acetone (70:30 v/v) allowed separation of the lutein from the carotenes. Lutein was detected as a light yellow band having the same R_f as that of standard lutein. The band of interest was then scrapped, extracted with acetone/hexane (50:50 v/v) and the collected mixture dried by nitrogen sparging. The 150 PTLC plates were used for isolation of lutein from the 200 g of carotenoids extracts. All the purified mixtures were combined and kept at -30°C under nitrogen until use.

Semi-preparative purification of extracts from PTLC. The combined extracts were dissolved in acetone and injected into HPLC-PDA through a semi-preparative C_{30} column (25 cm x 10.0 mm i.d; 5 µ particle size) with methanol/TBME (80:20 v/v) at a flow rate of 4.6 ml min^{-1}. This condition allows the best separation of the lutein peak from interfering peaks at the UV region (monitored at 286 nm). The authentic lutein standard was also injected to ascertain the retention time for peak collection from the PTLC carotenoid extracts.

Mass spectrometric analysis of carotenes. The EI mass spectra of carotenoids purified by semi-preparative C_{30} column were recorded by Shimadzu QP5050A by the direct insertion method and analysed using the GC-MS solution software.

Carotenoids Profiles of Palm Fruits at Various Stages of Ripening

Sampling procedure. Fruits were picked randomly from four bunches weighing between 15-20 kg at different stages of ripening.

Fruits treatment. The unripe fruits harvested at 7, 21, 84 and 126 DAA were not sterilized. The ripe fruits at 140 DAA were sterilized. Mesocarp from fruits were cut from the nuts, and ground to pulp. The wet mesocarp pulp was first soaked in methanol and followed by hexane, acetone and hexane/acetone (50:50 v/v) until the solvent was almost colourless to indicate full extraction. The methanol, hexane, acetone and hexane/acetone fractions were combined and designated as the crude extract.

Purification of crude extract. Only the crude extracts from the unripe fruits (7 to 84 DAA) were further purified prior to injection into the HPLC-PDA. The cherry red crude extract was first suspended in distilled water and poured into a separating funnel. Then 50 ml portions of hexane/diethyl ether (50:50 v/v) were added until no more oil was extracted. The combined solvent extracts were dried *in vacuo* at 35°C, and a greenish paste obtained containing a high level of chlorophyll.

The greenish paste was then subjected to saponification at room temperature (25 ± 1°C) to remove the chlorophyll. Few grammes of the paste were dissolved in 50 ml hexane in a 100 ml conical flask. The 5 ml KOH (50% by weight) and a few milligrams of pyrogallol acting as an antioxidant were added, and the mixture stirred for 4 hr under a headspace of nitrogen. Another 100 ml hexane/diethyl ether were added to the mixture into a separating funnel. The 100 ml of distilled water: ethanol (70:30 v/v) was used to wash the organic phase until it became neutral in the conical flask. The mixture from the conical flask were then transfered into a separating funnel. The ethanol fraction collected was dried *in vacuo*.

Analysis of free fatty acid (FFA) and triglyceride (TG) by gas chromatography-flame ionization detector (FID). The method of Lau *et al.* (2003) was used. The oily sample was silylated by dissolving in dichloromethane and a few drops of *bis* silytrifluoroacetamide in a vial and immersing the vial in a water bath at 60°C for 2 hr. A 1 µl sample was injected into a Hewlett Packard 5890 GC-FID with a capillary column, BPX5 (0.32 mm i.d. x 15 m), with helium as the carrier gas. The oven temperature was programmed to hold at 100°C, increasing at 10°C min^{-1} to 360°C where it was maintained for 16 min. Quantification was by external standard calibration with standard mixtures of TG.

HPLC-PDA carotenoid profiles of palm fruits extracts. The carotenoid profiles of the palm fruits extracts at various ripening stages were obtained on an analytical reverse phase Develosil C_{30} column (4.6 mm i.d. x 25 cm; 5 µm particle size) protected by a guard column of the same material using an isocratic mobile phase of methanol/TMBE (89:11 v/v), and at a flow rate of 1 ml min^{-1}. The purified crude extracts containing carotenes were dissolved in acetone prior to injection. The chromatograms were monitored at 286, 347 and 444 nm with the column maintained at ambient laboratory temperature (25 ± 1°C).

RESULTS AND DISCUSSION

Lutein and Other Xanthophylls in CPO

Figure 1 shows the carotenoid profile of an unsaponifiable extract from CPO. Lutein, a dihydroxy carotenoid, and other oxygenated xanthophylls, 5,8 and 5,6 epoxides of α- and β-carotenes, were identified based on their absorption spectra (*Table 1*).

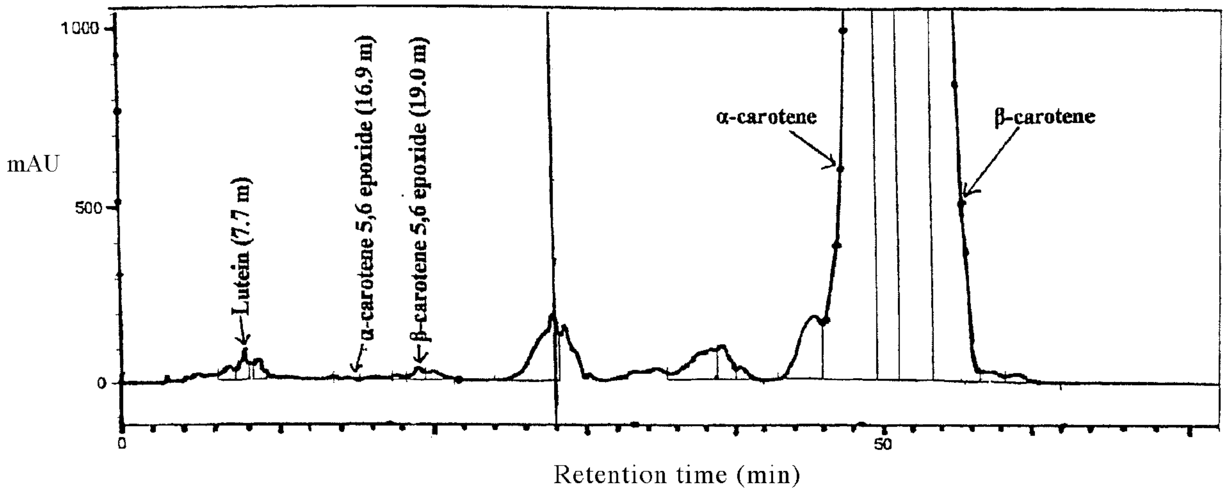


Figure 1. HPLC-PDA chromatogram of concentrated carotenoid extract from crude palm oil at 444 nm detection using a reversed phase C_{18} column. Mobile phase was acetonitrile:dichloromethane 89:11 (v/v) and at a flow rate of 1 ml min^{-1}.

TABLE 1. ABSORPTION SPECTRA OF XANTHOPHYLL PEAKS IN (*Figure 1*)

Retention time (min)	Carotenoids	Absorbtion maxima (nm), this study	Absorption maxima (nm), references
7.7	Lutein	418, 444, 471	420, 444, 475[a]
17.7	α-carotene 5, 6-epoxide	420, 444, 468	416, 440, 468[b]
19.0	β-carotene 5, 6 epoxide	420, 444, 473	422, 444, 472[b]

Notes: [a]Absorption maxima from analysis of a 98% pure lutein standard on a C_{18} column attached to a HPLC-PDA with mobile phase of acetonitrile/dichloromethane (89:11 v/v).
Source: [b]Ng and Tan (1988).

The same CPO unsaponifiable extract, authentic lutein standard and spiked sample were analysed using a silica-based nitrile bonded column which allowed better separation of the dihydroxy carotenoids. The results are shown in *Figures 2a* to *2c*. The retention times for the peak of interest in the CPO extract (*Figure 2a*) were near to that of the lutein standard (*Figure 2b*). There was only one peak at 5.8 min for the spiked sample (*Figure 2c*). This tentatively established the similarity between the peak at 5.7 min for the CPO crude extract and the lutein standard.

MS Identification of Lutein in CPO

Figures 3a and *3b* show the MS spectra of a lutein standard and the purified peaks obtained from a C_{30} semi-preparative column. The molecular ion at 568 $[M^+]$ and fragment ion at 550 $[M^+-H_20]^+$, and a dehydrated fragment ion from protonated molecules were observed for both the standard and isolated pure sample which are characteristic of hydroxylated carotenoids with a $C_{40}H_{56}0_2$ molecular formula, in this case lutein.

Figures 4a to *4d* show the carotene profiles of the crude extracts from unripe palm fruits at 7, 21, 84 and 126 DAA. At 7 DAA, lutein was the major carotene with only trace amounts of α- and β-carotenes. However, at 21 DAA, 84 DAA and 126 DAA, α- and β-carotenes were detected at higher levels. *Table 2* shows the percentages of TG and FFA at various DAA. Fruits at 126 DAA and below with TG content below 10% were considered as unripe. Other carotenes, *i.e.* phytoene, lycopene, α-zeacarotene, β-zeacarotene, neurosporene, δ-carotene, γ-carotene, ξ-carotene and phytofluene found in CPO were not detected. At this stage, the oxygenated carotene, lutein, was only the major product of carotegenesis. The presence of lutein in unripe fruits was also observed in the red pepper (*Capsicum anuum*. L) (Goodwin, 1971). *Figure 5* shows the carotene profile of the oil extract at 140 DAA, when oil synthesis was at its peak. The lutein level had dropped drastically to minority level and the level of α- and β-carotenes rise rapidly. The active syntheses of α- and β-carotenes and other carotenes, *i.e.* phytoene, lycopene, β-zeacarotene, γ-carotene, ς-carotene, were detected (*Figure 5*) at 140 DAA coinciding with the active synthesis of TG. *Table 3* shows the absorption maxima of carotenoids for the sample at 140 DAA.

TABLE 2. PERCENTAGE COMPOSITION TRIGLYCERIDE (TG%) AND FREE FATTY ACID (FFA %) OF OILY CRUDE EXTRACTS FROM PALM FRUITS AT VARIOUS DAYS AFTER ANTHESIS (DAA)

DAA	TG[a] (%)	FFA[a] (%)
7	-	-
21	0.15	2.2
84	0.11	2.2
126	3.3	9.7
140	25.4	12.1

Note: [a]Analyses of TG and FFA were by GC-FID.

TABLE 3. ABSORBANCE MAXIMA (NM) OF CAROTENOIDS PEAKS FOR THE SAMPLE AT 140 DAA AS SHOWN IN *Figure 4* EXTRACTED FROM ACCUMULATED SPECTRA DATA IN HPLC-PDA

Carotenoid	This study absorption maxima (nm)	Davies [a] 1976
Lutein	421, 446, 475	423, 446, 476[b]
Lycopene	450, 475, 500	448, 473, 504
β-zeacarotene	398, 424, 452	407, 427 ,454
γ-carotene	434, 462, 490	437, 462, 492
ς-carotene	382, 402, 429	380, 400, 425
cis a-carotene	330, 415, 442, 474	-
α-carotene	422, 447, 475	420, 442, 472
β-carotene	436, 453, 490	425, 450, 477
phytoene	278, 287, 300	276, 286, 297

Sources: [a]Davies (1976); [b]Maoka (2003).

CONCLUSION

CPO was found to contain the physiologically important oxycarotene, lutein, ubiquitous in the leaves of higher plants. Its presence was confirmed via coelution with an authentic standard and mass spectra. Lutein predominated during early fruit development at 7 DAA. However, as the fruits matured (84-126 DAA) α- and α-carotenes began to be formed and their levels continually increased. However, lutein remained the major carotenoid at these stages until 140 DAA when oil synthesis was at its peak, then α- and β-carotenes began to predominate and the level of lutein dropped drastically for it to become the minor component.

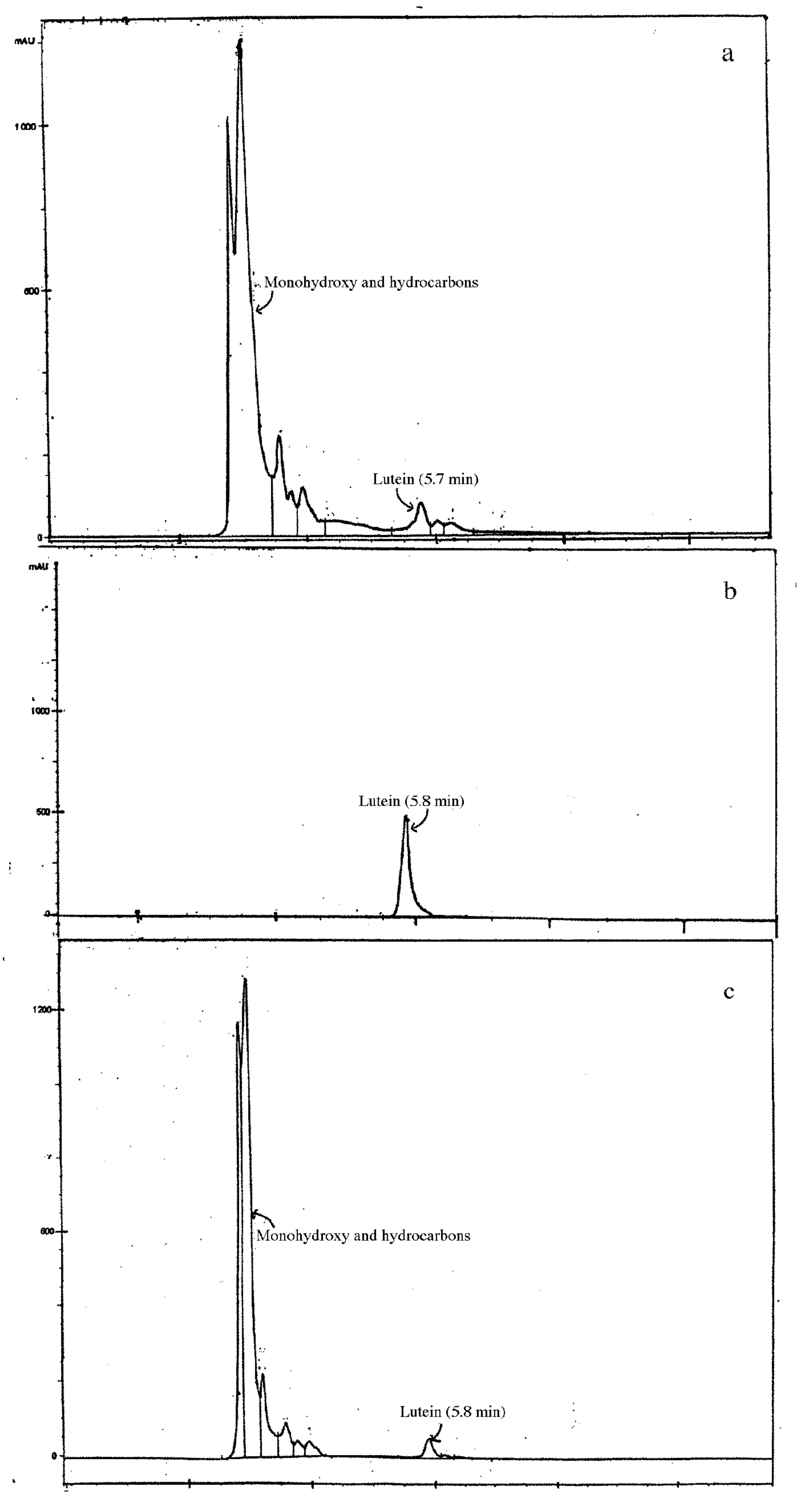
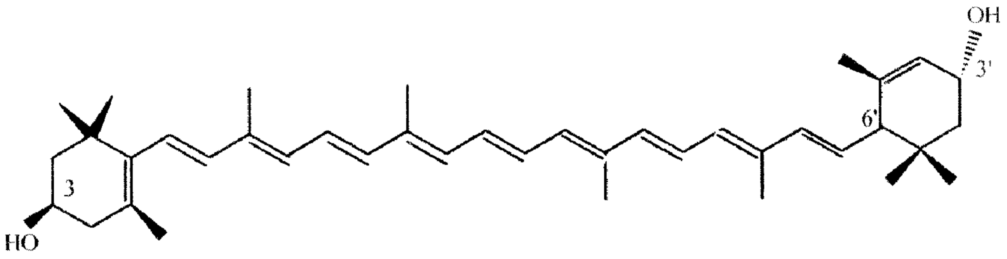


Figure 2. HPLC-PDA chromatograms at 444 nm detection using a silica-based nitrile bonded column of concentrated caroteniod samples of (a) crude palm oil, (b) lutein standard and (c) concentrated caroteniod samples mixed with lutein standard.



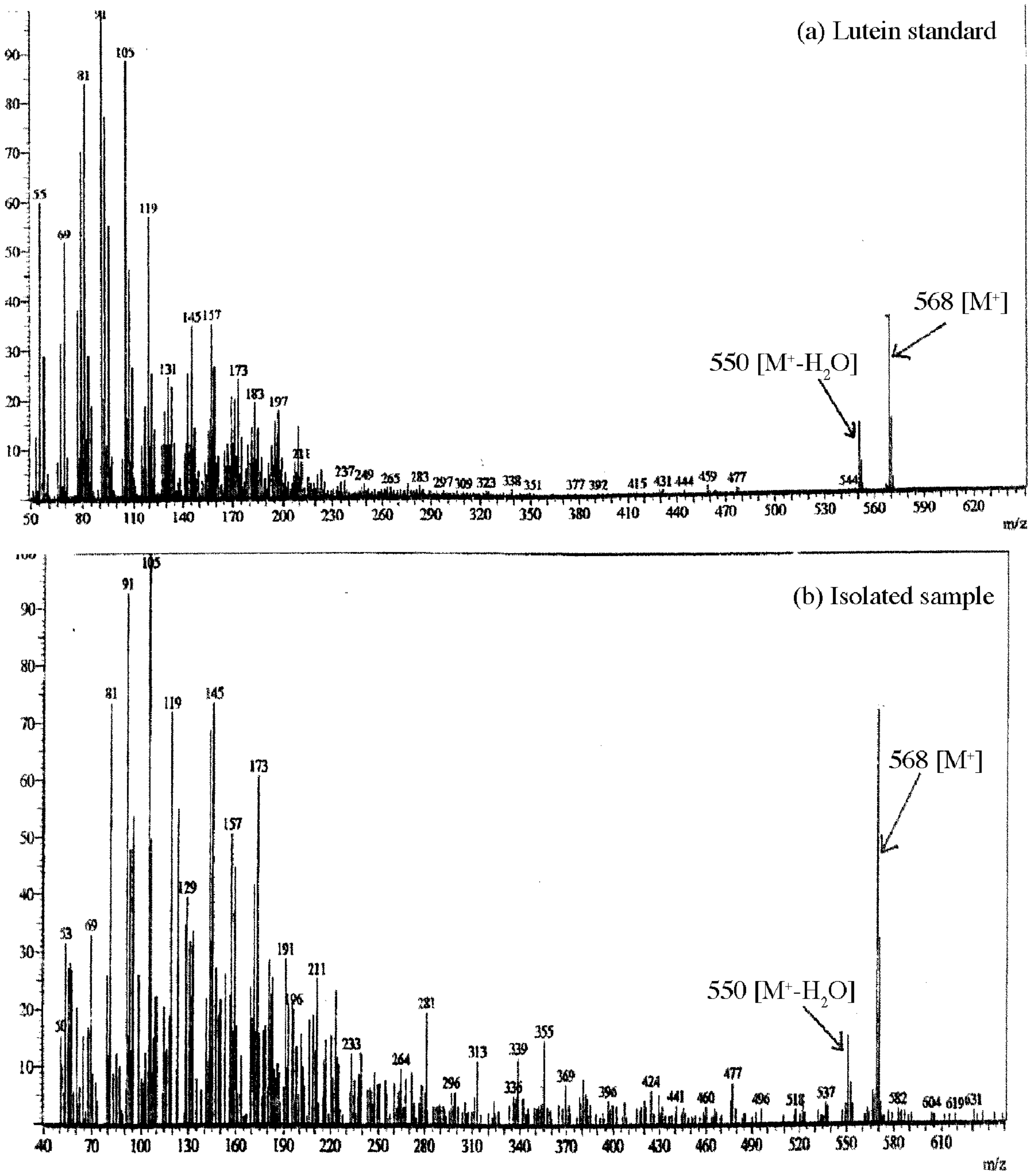


Figure 3. MS spectra of: (a) lutein standard (b) sample extract from crude palm oil.

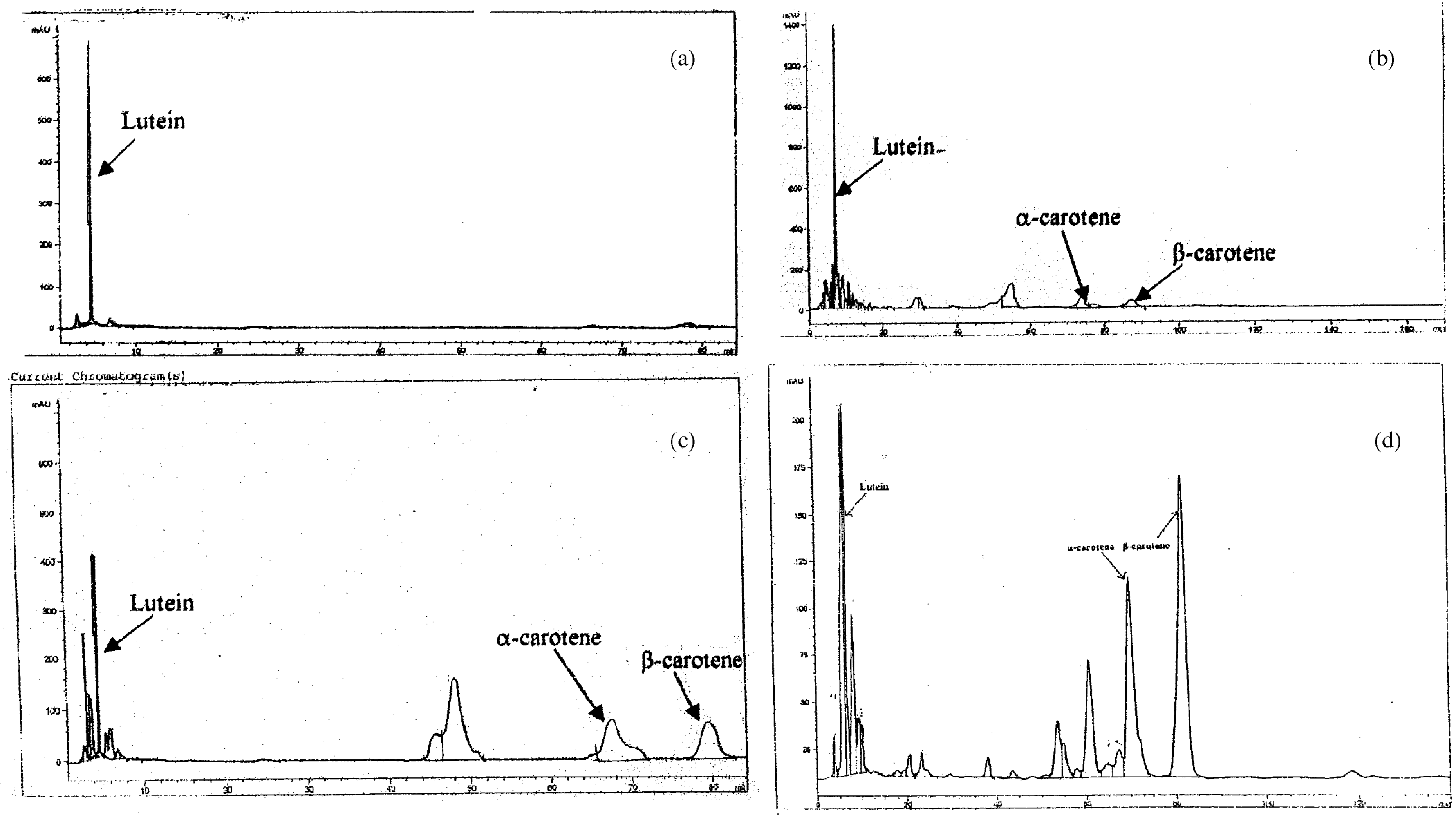


Figure 4. HPLC-chromatograms of extracts of palm fruits at (a) 7 DAA, (b) 56 DAA, (c) 84 DAA and (d) 126 DAA obtained on an analytical RP Develosil C_{30} column (4.6 mm I.d x 25 cm), using isocratic mobile phase of methanol/TBME (89:11 v/v) at a flow rate of 1 ml min^{-1}.

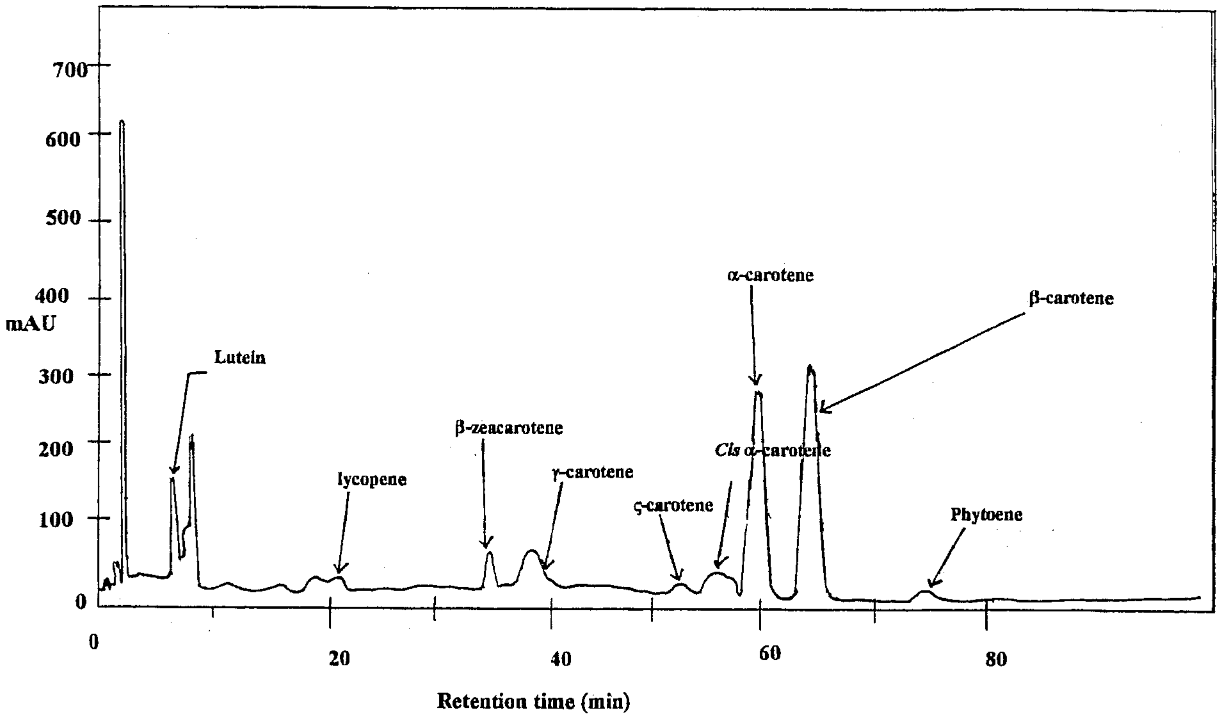


Figure 5. Attenuated HPLC-PDA chromatograms of extract from palm fruits at 20 DAA obtained on a reverse phase C_{18} column, mobile phase acetonitrile: dichloromethane 89:11(v/v).

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