CHAPTER 2
RESULTS AND DISCUSSION

2.1 DETERMINATION OF CAROTENES IN VARIOUS OIL PALM SPECIES

2.1.1 Analysis of Palm Oil Carotenes by Non Aqueous Reversed-Phase HPLC

Recent epidemiological evidence has suggested that an inverse relationship between the β-carotene content and the incidence of several types of human cancers,[58] and different types of carotene isomers have also been shown to have different antioxidant capacities and bioactivities.[60,201] These studies have resulted in the development of advanced analytical techniques that are able to separate, identify and quantify the mixtures of carotenes present from various sources.[83]

Generally, it has been the practice to determine the total carotenoids in palm oil by a UV-visible spectrophotometer at 446 nm as ppm of β-carotene.[202] However, carotenes present in palm oil are known to be a mixture of different carotene isomers, and the increased awareness of the high carotene content in palm oil has led to increased research using palm oil as a source of carotene (or pro-vitamin A) for various nutritional studies.[203-205] Therefore, it is important to know in detail the carotene constituents of palm oil and palm oil products.

Most of the earlier work carried out on palm oil carotenes using column chromatography and TLC were not comprehensive as only the major carotenes were reported.[206] Recently, a more detailed analysis was carried out by Tan[207] using column chromatography on various palm oil products. However, separation of the carotene isomers

was not adequate for precise quantification, especially for the minor carotenes present in palm oil. Similar work using column chromatography was also carried out by Jose[208] on carotenes from different oil palm species. In general, column chromatography has been shown to be time consuming, insensitive and poorly reproducible. Thus, the method is not practical for routine quantitative analysis.[62]

Recently, modern liquid chromatography (HPLC) has emerged as a method of choice for the analysis of various carotenoids. HPLC has advantages of effectiveness, rapid separation, being non-destructive and more importantly, high resolution. However, due to differences in the nature of the carotene isomers and differences in carotene distribution from various sources, diverse HPLC systems have been developed for specific applications, each of which may be of limited scope.[83] Analysis of palm oil carotenoids using HPLC has been carried out by Ng[194] using a photodiode array (PDA) detector. However, the carotene separation was not adequate to carry out a full range quantification of the carotenes present in palm oil as only the two major carotenes have been quantified, while certain minor carotenes present in palm oil, e.g. lycopene and phytofluene, were not detected.

Since there is an increased awareness in the usage of palm oil carotenes, it is important to have an accurate and relatively fast method to determine the carotene profiles of the palm oil and palm products. In this respect, a rapid analytical method needs to be developed to detail the palm carotenes. This could be achieved using Non Aqueous Reversed-Phase High-Performance Liquid Chromatography (NARP-HPLC) using a C18 column and eluting with a solvent system of acetonitrile (89%) and dichloromethane (11%). A typical experimental chromatogram depicting the separation of a complex mixture of carotenes present in palm oil using a variable UV-Vis detector is shown in Fig. 2.1. The two major carotenes present in palm oil, α- and β-carotenes, were well separated from each other and the other nine minor carotene constituents were also well resolved. In the present study, the nine *cis*-carotenes identified include 3 *cis*-lycopenes, 2 *cis*-ζ-carotenes and one *cis*-isomer each for phytofluene, α-, β- and δ-carotenes.

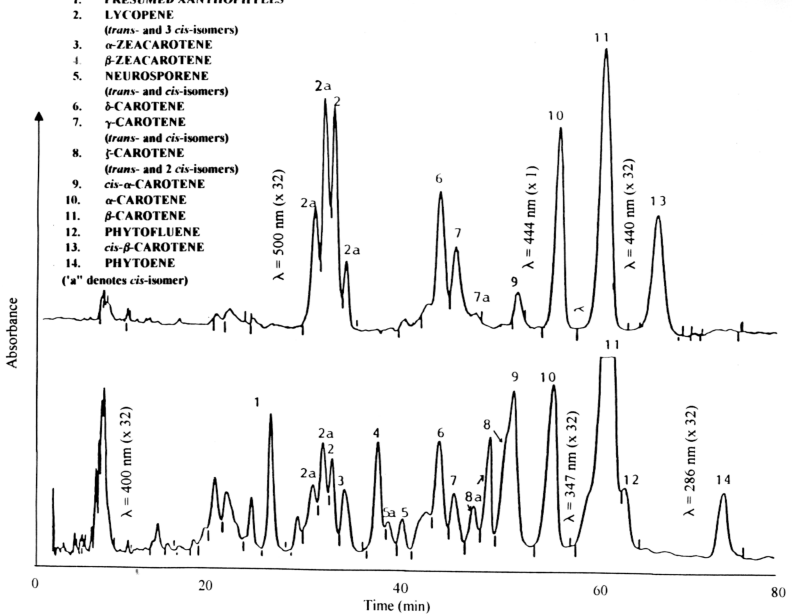


Figure 2.1 HPLC of carotenoids of palm oil

The major characteristic of carotenoids is the presence of highly conjugated polyene chains which normally give absorptions in the visible region. This is advantageous for carotenoid detection and the interference from other non-carotenoid components can be eliminated when the appropriate wavelengths are selected. This is particularly important since apart from carotenes, the non-saponifiable fraction of palm oil contains other minor constituents (e.g. tocols) which can absorb in the UV-region.

Identification of carotenes was carried out by co-chromatography with the few available authentic carotenes purchased from Sigma; in many instances identification of the resolved carotene peaks were mainly based on their characteristic UV-Vis absorption spectra obtained from the pure carotenes collected by HPLC. The number of conjugated double bonds as well as differences in end groups of the carotene components determine the nature of UV-Vis spectra and absorption maxima (λ_{max}). The spectral maxima (normally three) for the identified carotenes obtained in this study together with those of previously published data, given in order of their elution, are shown in Table 2.1.

Figs. 2.2, 2.3 and 2.4 show the UV-Vis spectra of the carotenes found in the study. As expected Fig. 2.2 shows that as the number of conjugated double bonds for the acyclic carotenes increases, the absorption maxima also shifts to longer wavelength. The effects of the ring closure of the ψ-end group to form ε- and β-end groups as described elsewhere[46] are also clearly shown in Figs 2.3 and 2.4. The displacement of the absorption maxima to shorter wavelength with a concomitant loss of persistence (i.e. less defined λ_{min}) of spectra is clearly shown for the β-carotene spectrum in Fig. 2.3, whereas for α-carotene there is no loss in persistence, merely a shift to lower wavelength because there is one conjugated double bond less and the formation of an ε-cyclic end group. These characteristics allow for the detection of some partially resolved carotene components (using HPLC) by selecting the UV-Vis wavelength of the detector as described below. Structural differences such as conjugation of double bonds and end groups cause polarity or partition/absorption differences among the carotenes and lead to the characteristic elution profile observed for the carotenes.

Table 2.1 Main Absorption Maxima (nm) of Carotenes in Hexane

		This Study				Reference[46]		
Carotenoid	*cis* peak							
1. Xanthophylls		not determined				-		
2. Lycopene	*cis*	362	438	464	495			
	cis	362	442	477	497			
	trans		444	470	500	448	473	504
	cis	362	438	464	495			
3. α-Zeacarotene			398	420	448	398	421	449
4. β-Zeacarotene			404	426	452	407	427	454
5. Neurosporene	*cis*	330	414	436	467			
	trans		416	438	468	416	440	470
6. δ-Carotene			431	456	484	428	458	490
7. γ-Carotene	*trans*		435	462	490	437	462	492
	cis	348	434	459	487			
8. ζ-Carotene	*cis*	295	376	397	423			
	cis	295	378	399	424			
	trans		380	401	426	380	400	425
9. *cis*-α-Carotene		330	415	438	470			
10. α-Carotene			420	440	471	420	442	472
11. β-Carotene			426	449	477	425	450	477
12. Phytofluene			331	347	366	331	347	366
13. *cis*-β-Carotene		334	420	444	472			
14. Phytoene			276	286	297	276	286	297

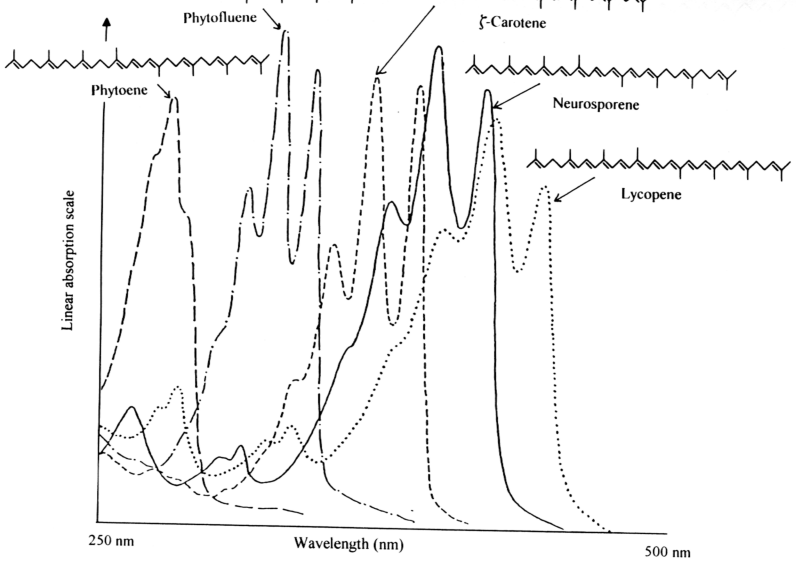


Figure 2.2 UV-Vis spectra of acyclic carotenes

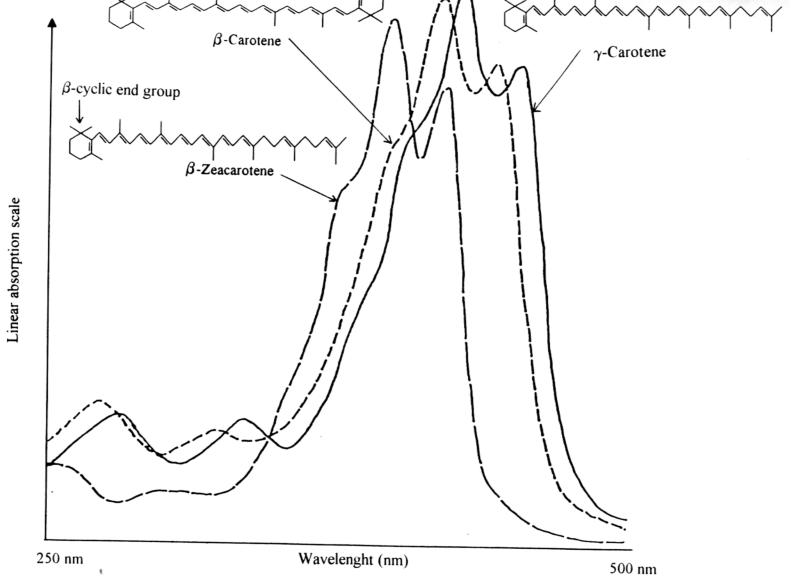


Figure 2.3 UV-Vis spectra of carotenes with β-cyclic end groups

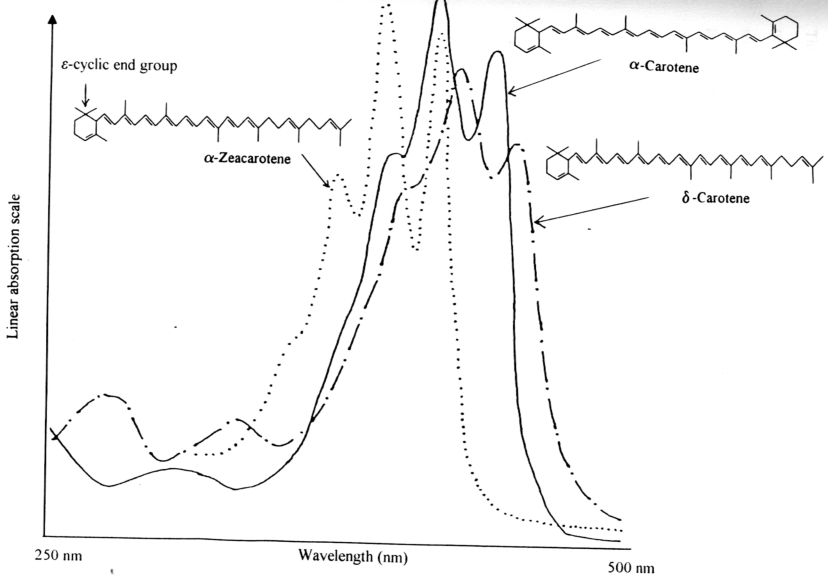


Figure 2.4 UV-Vis spectra of carotenes with ε-cyclic end groups

The appearance of an absorption maximum in the UV region (the '*cis* peak') of the spectrum of most of the *cis* isomers and chromatographic analysis of the iodine isomerised products of selected HPLC separated carotenes further assist in the confirmation of some carotenes particularly those with *cis*-double bonds.

As reported earlier,[209] the most "polar" hydrocarbon carotene, i.e. lycopene, was the first carotene to be eluted out from the reversed-phase (C18) column. Oxygenated carotenes (xanthophylls) were eluted much faster and were well separated from the hydrocarbon carotenes. The small amount of the highly conjugated carotene, i.e. lycopene, which was not detected in the palm oil samples by Ng and Tan,[209] can now be quantified in palm oil from the present commercial oil palm species (Tenera) in Malaysia. Lycopene was also found to be comparatively higher in Dura and Pisifera varieties of the *E. guineensis* species. Besides *trans*-lycopene, three other *cis*-lycopenes (which have spectra with lower absorption maxima and further confirmed by their iodine-catalysed photoisomerisation to the *trans*-isomer) were also detected in which two were eluted before but one after the *trans*-lycopene peak; all three *cis*-isomers show the "*cis* peak" at 362 nm but the position of the *cis*-double bonds are unknown.

The least polar carotene, i.e. phytoene (peak 14), with seven conjugated double bonds was the last to elute out and it was well separated from the preceding peak 13 (*cis*-β-carotene) as shown in Fig. 2.1. The spectral maxima of phytoene obtained in this study was identical to the published data.[46] Identification of *cis*-β-carotene (peak 13) and *cis*-α-carotene (peak 9) which eluted after the *trans*-β-carotene (peak 11) and before *trans*-α-carotene (peak 10) respectively, were based on the characteristic *cis* peaks at 338 nm and 332 nm respectively and also the hypsochromic shift of the spectral bands of the *cis*-isomers. Further confirmation was based on the re-chromatography of the iodine isomerised products from the respective pure *cis* carotenes collected by HPLC. Based on the reported UV-Vis spectrum and the elution order,[210] the *cis*-β-carotene (peak 13) found in this study is most likely to be 9-*cis*-β-carotene.

The two major carotenes in palm oil, i.e. α- and β-carotenes (peaks 10 and 11 respectively), were determined by co-chromatography with standards as well as spectral comparison. Phytofluene (peak 12) which was only observed when the chromatogram was run at λ_{max} 347 nm was not well resolved due to the comparatively large β-carotene peak; phytofluene gives a characteristic greenish fluorescence on thin layer chromatography (TLC) on exposure to long-wavelength (360 nm) UV radiation.

trans-ζ-Carotene (peak 8) was eluted just before but not well separated from *cis*-α-carotene. However, when the UV-Vis detector was set at 375 nm, a better resolution from *cis*-α-carotene, which has a low absorptivity at this wavelength, can be observed. Two *cis*-ζ-carotenes (8a) which showed a shift of the spectral bands to shorter wavelength and the extra peak at 296 nm (*cis* peak) not present in the *trans*-isomer, were eluted before *trans*-ζ-carotene. However, the positions of the *cis* double bond were not determined.

Peaks 5, 6 and 7 (Fig. 2.1) have been identified as neurosporene, δ- and γ-carotenes respectively based on the UV-Vis spectral data as shown in Table 2.3. The elution order was in accordance with that published earlier.[209] Peaks 5a and 7a were tentatively assigned as the *cis*-isomers of neurosporene and γ-carotene respectively. These two carotenes show lower absorption maxima than their respective *trans* carotenes and the *cis* peaks for the two *cis*-isomers were observed at the near UV region as has been reported.[39,46]

α-Zeacarotene (peak 3) and β-zeacarotene (peak 4) which were not reported by Ng and Tan[209] in their HPLC chromatogram of the palm oil samples were eluted after lycopene. The elution order of α- and β-zeacarotenes was in agreement with the theory that the end group for α-zeacarotene is more "polar" than the end group for the β-zeacarotene. Hence, α-zeacarotene elutes before β-zeacarotene.

In this study, the complex mixture of carotenes present in palm oil were well-separated, so that detailed quantitative analysis of the carotenes can be carried out by measuring the peak heights of the individual carotene peaks in the chromatograms and using extinction coefficients. Individual carotene peaks were recorded at different UV-Vis

wavelengths. The wavelength chosen (normally λ_{max} of each carotene) and the published extinction coefficients (Ex) used in this study are shown in Table 2.2. It was reported that *cis*-carotenoids exhibit lower extinction coefficients than their corresponding *trans* isomers.[46] However, because of the uncertainty of the position of the *cis* double bond and the lack of data on the extinction coefficients for the different types of *cis* carotenes, the extinction coefficients for the corresponding *trans* isomers were used in the quantitation of cis carotene isomers; it is expected that the actual values will be slightly lower, for example if the extinction coefficient for *trans*-β-carotene is used for the quantification of 9-*cis*-β-carotene, about 5% less of the 9-*cis*-β-carotene will be reported.[211]

Table 2.2 Extinction Coefficients of Various Carotenes at the Chosen Wavelengths[46]

Carotene	Absorption Maxima (nm)	Extinction Coefficient (Ex)
Lycopene	472	3450
α-Zeacarotene	421	2450
β-Zeacarotene	427	2520
Neurosporene	440	2918
δ-Carotene	456	3290
γ-Carotene	462	3100
ζ-Carotene	400	2555
α-Carotene	444	2800
β-Carotene	453	2592
Phytofluene	347	1577
Phytoene	286	915

Figure 2.5 Plot of the standard curves for **α**− and **β**−carotenes

For calibration, α- and β-carotenes were used as the external standards in the present study. The peak heights (H) of these carotenes recorded at the chosen wavelengths were linearly correlated with their concentrations. By plotting the H/Ex of both α- and β-carotenes versus their respective concentrations, a linear plot was obtained over the range of concentrations of HPLC analysis (Fig. 2.5).

It is noted from the present study that the extinction (Ex) for α- and β-carotenes in petroleum ether can be applied to the mobile phase solvent used (11% dichloromethane in acetonitrile), and it is assumed that the Ex value chosen for other carotenes (in petroleum ether or hexane) can also be applied to the quantification of the peaks recorded in the present chromatograms.

Based on the HPLC system described above, detailed analyses of the carotene profiles of the palm oil from various oil palm species have been carried out and is described below.

2.1.2 Carotenes in Various Oil Palm Species

Oil palm breeding has been actively carried out in Malaysia and elsewhere primarily to improve the oil yield by intercrossing within the *E. guineensis* species; more recently the possibility to alter the fatty acid composition of palm oil to meet some of the present day market requirements has also been vigorously pursued. The main consideration in agronomic research is to increase the level of unsaturated fatty acids in palm oil, and this has been made possible by the availability of another oil palm species, *Elaeis oleifera* (or Melanococca).[1] This particular species has no commercial value because of the extremely low oil yields. However, *E. oleifera* yield oil with higher level of unsaturated fatty acids and the oil palm tree have slow yearly height increments and also show great resistance to certain oil palm diseases. Palm oil from *E. oleifera* has also been shown to have a higher carotene content compared to oil from *E. guineensis* species.

Hybridisation of the *E. oleifera* and *E. guineensis* palms has been shown to yield hybrid palms which have retained the characteristics of the *E. oleifera* palm in terms of height, fruit shape and fruit colour. The fatty acid composition is reported to be intermediate between those found in the two parent species.[212] Similar results are also observed in palms selected for this study as regards to the fatty acid composition of these hybrids and their parent species (Table 2.3).

Table 2.3 Fatty Acid Composition of Oils from Various Oil Palm Species*

	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0
O	-	0.2	18.5	1.7	1.0	55.9	21.2	1.1	tr
OP	-	0.4	32.4	0.3	3.1	52.4	10.3	0.4	0.4
OD	-	0.4	35.6	0.1	4.4	44.8	13.5	0.6	0.4
OD x P	-	1.5	42.9	0.2	3.8	34.2	16.8	0.5	tr
P	-	1.1	42.1	-	4.9	39.6	11.8	0.3	-
D	-	2.1	54.0	-	2.7	29.7	10.9	0.3	0.1
Te[a]	0.3	1.2	44.3	-	4.3	39.3	10.0	0.4	0.3

* O = *Elaeis oleifera;* P = Pisifera; D = Dura; Te = Tenera. The fatty acid composition was determined according to Section 3.5.2.

[a] Siew and Tan, (1988).

The total carotene contents based on the spectrophotometric measurement shown in Table 2.4 clearly show that each hybrid and the backcross in the study provide carotene of intermediate level between those found in the parent species. Because of differences in colour intensity of the exocarp (skin) of oil palm fruits from different oil palm species (the *E. oleifera*

have orangy colour, whereas *E. guineensis* have dark red colour), as well as differences in pro-vitamin A activities and anticancer property of various carotenes, it is of interest to obtain the carotene profiles in *E. oleifera*, the hybrids and the backcross by using the NARP-HPLC method described in Section 2.1.1. Albescens, an oil palm variety characterised by its low carotene content in the oil has also been included in this study.

Table 2.4 Total Carotenoid Concentrations of Oils from Various Oil Palm Species

Oil Palm	Total Carotenoids* (ppm)
Elaeis oleifera (O)	4347
Elaeis oleifera x Dura (OD)	1846
Elaeis oleifera x Pisifera (OP)	1289
OD x Pisifera	864
Pisifera	380
Dura	948
Albescens	100

*Total carotenoids estimated at 446 nm

Table 2.5 shows the detailed carotene profiles of oils from the various oil palm varieties/species studied. α-and β-Carotenes are the major carotene constituents found in all these oils with β-carotene and α-carotene ranging from 54 to 61% and 24 to 40% respectively. No significant variation in the nature of carotenes was found among the *E. oleifera*, *E. guineensis*, their hybrids, the backcross and the Albescens varieties/species. However, in the case of *E. oleifera*, carotenes, other than α- and β-carotenes, were found in

relatively smaller amounts as compared to those of *E. guineensis* species (i.e. Dura, Pisifera or Tenera varieties). The most significant difference between the *E. oleifera* and *E. guineensis* species is the amount of lycopene; *E. guineensis* species provide a relatively high content of lycopene whereas only trace amounts of lycopene were found in the oils of *E. oleifera* palm and the hybrids from *E. oleifera* and *E. guineensis*. This may be the cause of differences in the shades of colour of the various oil palm fruits among the different species; lycopene imparts dark red colour to the oil. The fruits of *E. guineensis* are dark red when they are ripe, but the palm fruits of *E. oleifera*, their hybrids and backcross varieties remain orange when they are ripe, in spite of much higher total carotene contents.

The results in this study show that although there are differences in total carotene content of oils from different oil palm species, there is no significant change in the carotene profiles; α- and β-carotenes are still the major carotenes present in all the oil palm species and varieties which account for a total of 87-94% of the total carotenes present in the oils. All the hybrids with *E. oleifera* are shown to inherit the nature of *E. oleifera* (or Melanococca) in terms of the colour of the fruits, e.g. the fruits become orange when ripe (Fig. 2.6), whereas Dura, Pisifera and Tenera varieties are dark red.

The high carotene content present in *E. oleifera* and hybrids (esp. OD) could provide a good natural source of carotenes. However, due to the low oil yield in the *E. oleifera*, it is difficult to extract the oil using the normal milling process and the palms is not economical for oil production. The OD and OP varieties, although having higher oil yields compared to the *E. oleifera* species, have thin mesocarps but thick kernels (especially the OD hybrids), as well as showing the existence of parthenocarpy (sterile fruits) and thin cell fruitlets (OP hybrids), all of which affect the commercial planting of the two varieties.

Table 2.5 Carotene Profiles of Palm Oils Extracted from *Elaeis guineensis*, *Elaeis oleifera* and their Hybrids

Carotene	Carotene Composition (%)							
	Alb.	O	P	D	OP	OD	ODxP	Te
Phytoene	1.1	1.12	1.68	2.49	1.83	2.45	1.30	1.27
cis-β-Carotene	0.9	0.48	0.10	0.15	0.38	0.55	0.42	0.68
Phytofluene	0.3	tr	0.90	1.24	tr	0.15	tr	0.06
β-Carotene	61.1	54.08	54.39	56.02	60.53	56.42	51.64	56.02
α-Carotene	29.8	40.38	33.11	24.39	32.70	36.40	36.50	35.16
cis-α-Carotene	3.1	2.30	1.64	0.86	1.37	1.38	2.29	2.49
ζ-Carotene[a]	0.7	0.36	1.12	2.31	1.13	0.70	0.36	0.69
γ-Carotene	0.3	0.09	0.48	1.10	0.23	0.26	0.19	0.33
δ-Carotene	0.2	0.09	0.27	2.00	0.24	0.22	0.14	0.83
Neurosporene[b]	0.3	0.04	0.63	0.77	0.23	0.08	0.08	0.29
β-Zeacarotene	1.0	0.57	0.97	0.56	1.03	0.96	1.53	0.74
α-Zeacarotene	0.2	0.43	0.21	0.30	0.35	0.40	0.52	0.23
Lycopene[c]	1.0	0.07	4.50	7.81	0.05	0.04	0.02	1.30
Total Carotene (ppm)	98	4592	428	997	1430	2324	896	673

* O = *Elaeis oleifera* (Melanococca) ; P = Pisifera; D = Dura; Te = Tenera = DxP; Alb. = Albescens.

[a] one *trans* and two *cis* isomers, [b]one *trans* and one *cis* isomers, [c]one *trans* and three *cis* isomers.

E. oleifera

Hybrid (*E. oleifera* x Dura)

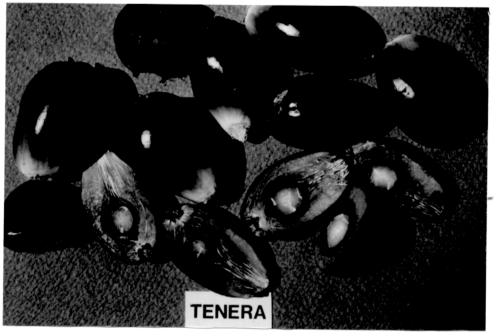


Figure 2.6 Oil palm fruits of various oil palm species

2.2 OTHER MINOR CONSTITUENTS IN VARIOUS OIL PALM SPECIES

Besides carotenoids, crude palm oil is known to provide relatively higher contents of other important minor components such as vitamin E (tocopherol and tocotrienols) and sterols[10] (Table 2.6).

Table 2.6 Important Minor Components in Palm Oil

Minor component	Concentration (ppm)
Tocopherols and tocotrienols	600-1000
Sterols	326-527
Carotenoids	500-700

Because of the importance of these minor components in physiological processes, it is of interest to study the contents of these components in various oil palm species and their hybrids and to determine if these component profiles are similar to those from commercially planted oil palm species.

2.2.1 Vitamin E Compositions

There are eight compounds possessing vitamin E activity that occur in nature, *viz.* four tocopherols and four tocotrienols (collectively referred as tocols). The basic structure of tocol

consists of a 4-hydroxylchroman ring to which is attached a long hydrocarbon side chain containing 16 carbon atoms as shown in Fig. 2.7. The tocotrienols differ from the corresponding tocopherols by the presence of three unconjugated double bonds in the isoprenoid side chain.

Unlike most of vegetable oils which contain tocopherols (mostly α-tocopherol) as major components of their vitamin-E contents, palm oil contains only about 20% of α-tocopherol. But palm oil has relatively higher concentrations of tocotrienols; α-tocotrienol (22%), γ-tocotrienol (46%) and δ-tocotrienol (12%), which together account for about 80% of the total vitamin-E content.[12] Tocopherol and tocotrienols have been shown to be excellent chain-breaking antioxidants.[213] In fact, they are important for the protection (against peroxidation) of unsaturated lipids particularly in biomembranes.[214] Numerous studies have also demonstrated that the tocopherols and tocotrienols have potential protective effects against some diseases[215-218] such as cancer, ageing and cardiovascular diseases. For instance, α-tocopherol and α-tocotrienol have been reported to have anti-cancer properties in experimental animals.[215,217] α-Tocotrienol has also been shown to suppress the elevation of cholesterol levels in blood in some hypercholesterolemic subjects,[216] and δ-tocotrienol has been reported as able to prevent aggregation of platelets in blood as well.[219]

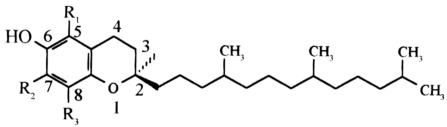
Vitamin-E isomers of palm oil can be easily separated and quantified by normal phase HPLC using a fluorescence detector. The fluorescence detector was found to be more sensitive and selective than a UV detector because of its advantage of having little interferences by other components, which means that no pretreatment of the oil is needed for the direct analysis of palm oil samples. Fig. 2.8 shows a typical chromatogram of the palm-oil vitamin-E isomers using fluorescence detection set at 295 nm excitation and 325 nm for the emission (or fluorescence).

Table 2.7 shows the total vitamin-E compounds (tocopherol and tocotrienols) and the vitamin-E isomer profiles of various oil palm species and varieties. No significant differences were observed in terms of the total vitamin-E contents of the *E. guineensis, E. oleifera* species and their hybrids. The variations of the total vitamin-E contents (624-920 ppm) are mainly due to variations among the individual plants. The vitamin-E isomer profiles of various oil palm varities/species and hybrids were also quite similar to palm oil samples from commercially planted Tenera variety, the major vitamin-E isomer being γ-tocotrienol with total tocotrienols of more than 70%.

Table 2.7 Tocopherol and Tocotrienols (%) of Palm Oils Derived from *E. guineensis, E. oleifera* and their Hybrids

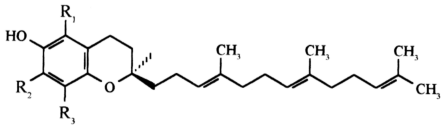
	Alb.	O	P	D	OP	OD	DOxP	Te
α-Tocopherol	25	16	25	30	18	23	10	20
α-Tocotrienol	20	26	38	23	22	20	32	22
γ-Tocotrienol	40	55	31	40	47	52	50	46
δ-Tocotrienol	15	3	6	7	13	5	8	12
Total (ppm)	709	872	641	920	701	628	788	800-1000

* Alb.= Albescens, O = *E.oleifera* (Melanococca); *E. guineensis* varieties :- P = Pisifera, D = Dura, Te = Tenera.



Tocopherols

α-Tocopherol, R_1=CH_3, R_2=CH_3, R_3=CH_3;
β-Tocopherol, R_1=CH_3, R_2=H, R_3=CH_3;
γ-Tocopherol, R_1=H, R_2=CH_3, R_3=CH_3;
δ-Tocopherol, R_1=H, R_2=H, R_3=CH_3.



Tocotrienols

α-Tocotrienol, R_1=CH_3, R_2=CH_3, R_3=CH_3;
β-Tocotrienol, R_1=CH_3, R_2=H, R_3=CH_3;
γ-Tocotrienol, R_1=H, R_2=CH_3, R_3=CH_3;
δ-Tocotrienol, R_1=H, R_2=H, R_3=CH_3.

Figure 2.7 Tocopherols and tocotrienols

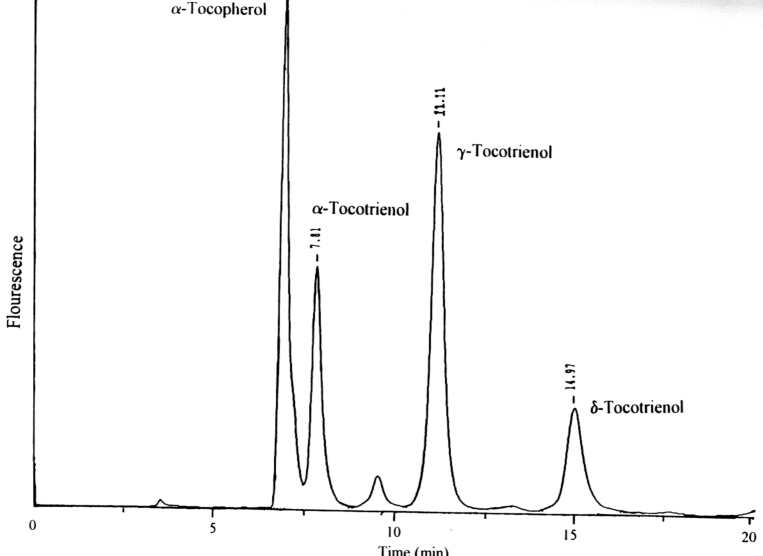


Figure 2 8 HPLC chromatogram of tocopherol and tocotrienols

2.2.2 Sterol Compositions

Crude palm oil contains about 326-527 ppm of total sterols with β-sitosterol (61%), stigmasterol (23%) and campesterol (12%) as major components. Cholesterol (4%) is only present in a very small amount. Most of the sterols (>70) were removed during the commercial refining process. The sterols do not seem to serve any useful function on palm oil, neither do they have any detrimental effect on it. Sterols, if recovered, will have potential uses in the pharmaceutical industry for conversion into steroid derivatives.[220] β-Sitosterol has been reported to be beneficial as a hypocholesterolemic agent.[221] The structures of the sterols present in palm oil are shown in Fig. 2.9.

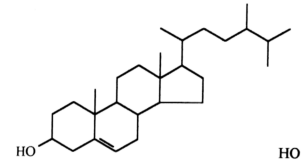
The detailed gas chromatography (GC) profile was obtained after saponification and purification by preparative TLC. Fig. 2.10 shows a typical GC chromatogram of sterols in a palm oil sample; quantification was carried out using cholesterol as an external standard.

Table 2.8 shows the sterol compositions of oils from various oil palm species. As observed for tocopherol and tocotrienol contents (Table 2.8), no significant difference was found on the sterol profile among the oil palm species/varieties and hybrids. The sterol levels for the O and D species were relatively higher as compared to the other species/varieties; it was first suspected that this could be due to a higher content of sterols in the exocarp but this was proven not to be so at the later stage of the study (Section 2.4). β-Sitosterol is still the major sterol present in all the oils and the relative cholesterol content (~ 4%) is lowest in all the oil palm species.

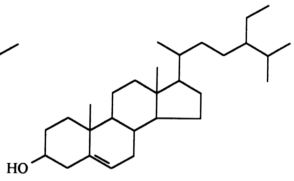
Table 2.8 Sterol Compositions (%) of Palm Oils Derived from *E. guineensis*, *E. oleifera* and their Hybrids.

	Alb.	O	P	D	OD	OP	DOxP	Te
β-Sitosterol	70	66	52	56	60	59	61	61
Campesterol	21	18	16	26	20	24	21	12
Stigmasterol	7	14	24	9	15	13	15	23
Cholesterol	2	2	8	7	5	4	3	4
Total (ppm)	556	3840	1735	2321	1320	1186	728	326-527

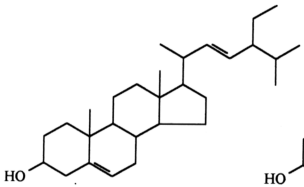
* Alb. = Albescens, O = *E. oleifera* (Melanococca); *E. guineensis* varieties :-
P = Pisifera, D = Dura, Te = Tenera.



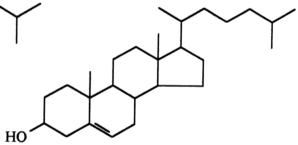
Campesterol



β-Sitosterol



Stigmasterol



Cholesterol

Figure 2.9 Sterols in palm oil

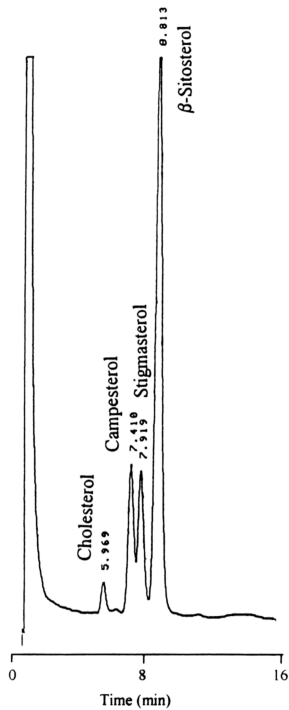


Figure 2.10 Gas chromatogram of sterols in palm oil

2.3 EXTRACTION OF CAROTENE ENRICHED OIL

2.3.1 Residual Oil from Pressed Palm-Fruit Fibres

An oil palm fruit bunch consists of two main parts *viz.* the stalk and the fruitlets (Fig. 2.11). The fruitlets are made up of the pericarp (i.e. mesocarp and the exocarp) and the nut. Two types of oil can be obtained, *viz.* palm oil from the oil cells in the mesocarp and kernel oil from the inner part of the nut (kernel). These oils are "extracted" and recovered separately in palm oil mills and kernel crusher plants. In general, a palm oil mill extracts 20% of oil from the fresh fruit bunch, produces 23% empty bunch, 15% pressed fibre and 12% nut. The remaining materials are mainly moisture and oil losses in various by-products including fibres. The mesocarp oil is extracted by a screw press in the oil mill. The pressed fibres contain 5-6% residual oil but is normally burnt as fuel to provide energy for the mill which is normally self-sufficient in energy supply.

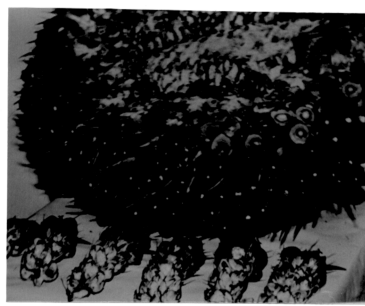


Figure 2.11 Stalk and the fruitlets of a fresh fruit bunch (FFB).

Numerous attempts have been made towards the recovery of the residue oil in the pressed fibres, but due to the low oil content and poor oil quality, it is presently not economical to recover the oil. However, in this study, it was found that the oil extracted from the pressed fibre contains some valuable components which may have good potential to make the recovery of the oil economically viable.

(a) Organic Solvent Extraction

Different solvents have been used to study the efficiency and selectivity of oil extraction. Among the solvents used were *n*-hexane, chloroform and liquid CO_2.

Table 2.9 shows the carotene content from the fibre oil obtained by Soxhlet extraction (with hexane) of the fibres collected from various palm oil mills. The total carotene content was in the range of 3,600-5,050 ppm which is 6-8 times higher than the carotene content obtained from commercial crude palm oil. Because of the instability of carotenes, it was found that in order to maximise the carotene content, it was necessary to collect and dry the fibres immediately after the fibre/nut separation. It was also found that drying is necessary in order to facilitate the extraction of residual oil and carotenes.

Table 2.9 Carotenoid Content of Residual Oils of Fibres Collected from Various Palm Oil Mills

Palm Oil Mill*	Carotenoids (ppm)
Mill A	4070
Mill B	4100-4520
Mill C	4000-5000
Mill D	4000-5500
Mill E	3600-4770
Mill F	4440-5050

* A-F = Different palm oil mills in Malaysia.

(b) Liquid CO_2 Extraction

Liquid CO_2 was one of the solvents used in this study using a high pressure Soxhlet extractor (HPSE). The apparatus used to extract the dry fibres is shown in Fig. 2.12; HPSE extraction can be carried out at low temperature in the absence of oxygen and light. This is particularly useful for the extraction of components sensitive to light, heat and oxygen, such as carotenes and vitamin E. Besides these advantages, no solvent residues are left in the products as the CO_2 will be released to the atmosphere. Liquid CO_2 is completely miscible with low molecular weight hydrocarbons and oxygenated organics and it has solvent characteristics similar to hexane, but the liquid has been found to be better especially for food products as it is non-toxic, non-flammable and inert to most materials, and it is inexpensive when compared to a number of organic solvents.[222]

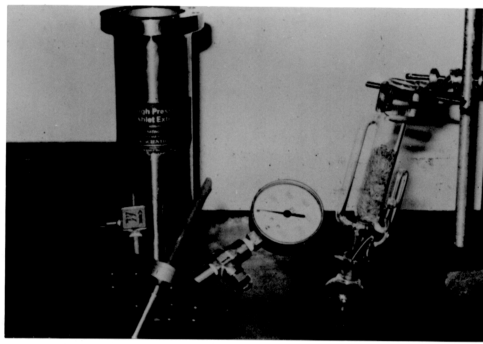


Figure 2.12 High pressure Soxhlet extractor and pressed palm-fruit fibres

Table 2.10 Percentage of Oil Extracted using a High Pressure Soxhlet Extractor under Various Extraction Conditions

Pressure/psi	Water bath temp./°C	Time/hrs	% of oil extracted
660	40	2.0	2.3
700	40	2.0	2.8
750	40	2.0	3.4
800	40	2.0	1.2
750	45	2.0	4.2
750	45	3.0	4.0
750	50	2.0	0.5
750	45	1.5	3.5

Different conditions have been used for the extraction of fibre oil using liquid CO_2 and Table 2.10 shows the results. The extractor was unable to operate at pressures higher than 800 psi, as under such high pressure, too much liquid CO_2 will condense in the extractor, whereas higher water bath temperatures will result in insufficient amount of liquid CO_2 for extraction. It was found that the optimum condition for the extraction of fibre oil is at a pressure of 750 psi and water bath temperature at 45±1°C . Most of the oil was found to be extracted within 2 hours.

(c) Chemical Composition of Residual Fibre Oil

Table 2.11 shows the percentage of oil extracted from fibre and the variation of oil quality using different solvents. It was found that a higher percentage of oil was extracted using chloroform but the PV, FFA and phosphorus content were also found to be relatively higher.

The liquid CO_2 extracts provide a lower oil yield as compared to those extracts from the use of organic solvents, but the CO_2-extracts were shown to be cleaner (better quality) as the phospholipids and other impurities were not extracted.

The concentrations of the minor components present in the residual oil extracted by different solvents are shown in Table 2.12. The total carotenes, vitamin E and sterols present in the oil extracted using different solvents did not show any significant difference, but it was clearly shown that the residual oil extracted by liquid CO_2 contained a higher concentration of vitamin E. This could be due to lower extraction temperature (40-45°C) and the inert atmosphere.

Special arrangement was made to carry out a large scale extraction for a batch of hybrid oil (which was from a mixture of OD and OP palms) in palm oil mills in Kluang. A batch of pressed fibres from the hybrid oil palm fruits was collected and the data of the minor constituents in the residual oil extracted by Soxhlet extraction with hexane is shown in Table 2.12. The carotene content was found to be even higher (5,000 - 7,000 ppm) than normal residual fibre oil; this is not surprising as the hybrid oil palm fruits have relatively higher carotene content compared to commercial Tenera fruit and the thinner mesocarp also increases the exocarp to mesocarp ratio.

Table 2.11 Quality of Oil Extracts from Fibres using Different Solvents

Sample	% of oil extracted	FFA (%)	PV (meq/kg)	AV	Total Phosphorus (ppm)
Hexane extract	5.5-6.5	9-11	4-6	15-17	572-880
Chloroform extract	6-7	9-15	6-8	15-17	700-1200
Liquid CO_2	4-5	7-8	5-6	9-11	5-10

FFA = free fatty acids, PV = peroxides value, AV = anisidine value.

Table 2.12 Minor Components from Pressed Fibres and Crude Palm Oil

Samples	Carotenoids (ppm)	Tocopherols & Tocotrienols (ppm)	Sterols (ppm)
Pressed fibre oil			
Chloroform extract	3800-5300	1650-2600	6906-8200
Hexane extract	4000-5500	1200-2400	7050-8490
Liquid CO_2 extract	4100-6000	2500-3000	4509-5200
Fibre from hybrid* fruits	5500-7000	1400-2400	6030
Crude palm oil	500-700	600-1000	250-650

* Mixture of OD and OP palms

2.3.2 Oil from Second Pressings of the Mesocarp Fibres

The oil from the mesocarp is conventionally extracted by single-stage screw press. However, some mills have employed what is known as a double pressing process,[223] in which the initial pressing is carried out at a relatively lower pressure to avoid cracking of the nuts. After removal of the nuts, the fibres are then subjected to the second pressing. The benefits of double pressing realised by these mills are as follows :-

- o Lower oil loss in the fibre.
- o Minimisation of broken nuts and broken kernels.
- o Better consistency in press operation in terms of broken nuts and oil losses in fibre.
- o Less wear on screw worms and cages.
- o Minimise contamination of palm oil by lauric oil of kernels.
- o Possibility of producing palm oil with higher iodine value (IV) and carotenoid content.

Fig. 2.13 shows the flowchart of the double pressing process. Conventionaly, the second stage pressed oil was mixed together with first stage pressed oil. In this study, to obtain the "second-pressed" oil, the pressed oil from second stage pressing was clarified separately from first stage oil; where the hot water-diluted oil from the second stage pressing was screened to remove coarse fibrous materials and the oil was separated by gravity settling, centrifugation and vacuum drying.

Table 2.13 shows that the total carotene content of "second-pressed" oil collected from 3 different oil palm mills. It was found that the carotene content was 3-4 times higher (ranging from 1,800 to 2,400 ppm) than those in the normal palm oil.

Table 2.13 Total Carotene Contents from "Second-Pressed" Oil Collected from Different Palm Oil Mills

Palm oil mills*	Carotenoids (ppm)
Mill A	1800-2020
Mill B	1900-2300
Mill C	1950-2400

* A-C = Different palm oil mills in Malaysia.

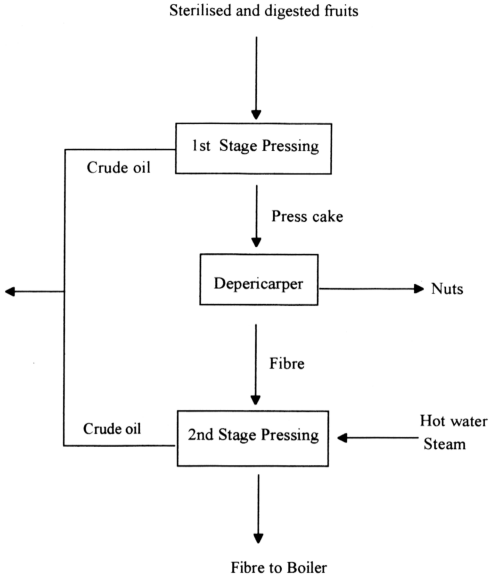


Figure 2.13 Flowchart of the double pressing process

2.3.3 Oils Extracted from Exocarp and Mesocarp

The higher carotene content found in the fibre and "second-pressed" oils led to the investigation of carotene distribution in the oil palm fruits; in the dried fibres, it was observed that the exocarp of the fruits still remained red in colour; on examining the fresh fruits, it was also observed that the pigment content of the skin was relatively higher as compared to the mesocarp (Fig.2.14).

Figure 2.14 Exocarp and mesocarp of oil palm fruits

Table 2.14 shows the percentage oil and the carotene concentration of the oils extracted from the skin and the mesocarp from both fresh and sterilised fruits. It is obvious that more carotenes were extractable from the sterilised fruits; in fact, the exocarp from the sterilised fruits are easier to peel off as compared to fresh fruits where some of mesocarp oil cells tend to peel off together with the exocarp.

The oil extracted from the exocarp in the fresh fruits was mainly from part of the mesocarp and it has caused a dilution of carotenes in the oil extracted from the skin of the fresh fruits.

Table 2.14 Carotene Distribution in Oil Palm Fruits

	% of oil extracted	Carotene concentration (ppm)	% of oil in pericarp	% of carotene in pericarp
Fresh fruits				
Exocarp	69.7	3568	11.9	42.9
Mesocarp	66.3	642	88.1	57.7
Sterilised fruits				
Exocarp	66.6	5796	6.6	33.0
Mesocarp	77.4	810	93.4	67.0
Pericarp	68.1	860	-	-

Large amounts of carotenes were therefore distributed in the exocarp of the fruits, constituting about 33% of the total carotenes in the oil palm fruit, although the exocarp only contributed about 7-8% of the total weight of the pericarp. The carotene concentration found in the exocarp oil is close to 6,000 ppm, which is 6-7 times higher than the carotene concentration in the mesocarp oil. The carotene concentration in the total oil extracts from the pericarp (mesocarp plus exocarp) was therefore slightly higher than that of the mesocarp oil.

From the above observations, it is clearly shown that there is some relationship in the carotene contents in the residual oil from fibre, "second-pressed" oil and oil from the exocarp of the oil palm fruits. The higher carotene present in fibre oil and "second-pressed" oil could have been contributed from carotenes present in the exocarp of the oil palm fruits. This is further supported by the observed chemical composition among these oils as described in the following section.

2.4 RELATIVE DISTRIBUTION OF MINOR CONSTITUENTS IN FIBRE, "SECOND-PRESSED" AND EXOCARP OILS

2.4.1 Carotene Profiles of Oil Extracts

The carotene profiles of various oil extracts were determined by high-performance liquid chromatography (HPLC) using a binary solvent system (acetonitrile and dichloromethane) on a C18 reversed-phase column with variable wavelength detector as described earlier (Section 2.1). The carotene profiles of the "second-pressed" and fibre oils, oils from the exocarp and mesocarp of the Tenera oil palm fruit and the oil from the commercial Tenera palm are summarised in Table 2.15.

It is clear that there is a similarity in the carotene profiles among the oils extracted from pressed fibre, exocarp and the "second-pressed" oil. The major carotenes present in commercial palm oil from the Tenera palm are α- and β-carotenes which constitute about 90% of the total carotenes present. This is quite similar to the oil extracted from the mesocarp which contributed more than 60% of the total carotenes in oil palm fruit. However, in the case of fibre, exocarp and "second-pressed" oils, the major carotenes are still α- and β-carotenes but these constitute only about 50% of the total carotenes present in the oils. A relatively higher percentage of lycopene (13-26%), phytoene (6.5-12%) and ζ-carotene (4.6-7.6%) were found in these oils. Other minor carotenes such as phytofluene, γ-carotene and δ-carotene were also found to be in relatively higher amounts as compared to oil from Tenera palm and oil from the mesocarp. These results showed that the higher carotene concentration found in the residual oil from fibres and the "second-pressed" oil are mainly contributed by the exocarp (skin) of the oil palm fruits. In conventional oil palm mills, most of the carotenes present in the "skin" remained together with the pressed fibre, possibly because

the contact time during screw-pressing was insufficient for the oil to dissolve all the carotenes; however the carotene retained in the exocarp can be easily extracted by solvent extraction (for about 1-2 hours). With regard to the "second-pressed" oil, it is observed that most of the mesocarp oil was extracted during the first pressing under low pressure and after the separation of nuts and fibre from pressed cake, the second pressing at higher pressure on the fibre caused more carotenes in the exocarp to be extracted into the residual oil, thus, the observed increase in carotene concentration of the "second-pressed" oil.

Table 2.15 Composition (%) of Carotenes from Various Parts of the Palm Fruit

Carotene	Fibre oil	"Second-pressed"oil	Exocarp oil	Mesocarp oil	CPO
Phytoene	11.87	6.50	10.61	1.12	1.27
cis-β-Carotene	0.49	0.28	tr	0.09	0.68
Phytofluene	0.40	1.63	2.14	0.17	0.06
β-Carotene	30.95	31.10	30.52	63.21	56.02
α-Carotene	19.45	20.68	21.76	31.41	35.06
cis-α-Carotene	1.17	1.70	1.09	1.47	2.49
ζ-Carotene[a]	7.56	4.62	6.87	0.20	0.69
γ-Carotene	2.70	2.48	1.71	0.30	0.33
δ-Carotene	6.94	2.13	6.76	0.23	0.83
Neurosporene[b]	3.38	1.88	1.49	tr	0.29
β-Zeacarotene	0.37	0.58	3.18	1.69	0.74
α-Zeacarotene	tr	0.15	1.07	0.42	0.23
Lycopene[c]	14.13	26.45	13.01	0.30	1.30
Total (ppm)	5162	2510	5028	423	673

[a] one *trans* and two *cis* isomers, [b] one *trans* and one *cis* isomers, [c] one *trans* and three *cis* isomers; CPO = Crude palm oil (commercial mesocarp oil).

The higher lycopene content in the oil of the exocarp was verified from the previous observation that lycopene contributed to the dark red colour of the skin of *E. guineensis* oil palm fruits. Most of the lycopene (13% of the total carotenes in exocarp oil)was determined to be in the exocarp layer while only 0.3% of the total carotenes in mesocarp oil is lycopene.

2.4.2 Other Minor Constituents

Amongst the many minor constituents found in palm oil, only vitamin E and sterols were examined.

(a) Vitamin E

Vitamin E profiles of the oil extracts from various oil palm sources are shown in Table 2.16. The total vitamin E content was found to be high in the oil extracted from fibres or 3-4 times higher than crude palm oil; the "second-pressed" oil was also found to contain two times more vitamin E than crude palm oil. The vitamin E contents in oils from the exocarp and mesocarp were also found to be relatively higher as compared to crude palm oil, but not much differences were observed between the solvent extracts of the exocarp and mesocarp.

As for the detailed vitamin E profile, the major vitamin E isomers present in the commercial crude palm oil (Tenera) are tocotrienols, which constitute about 70-80% of the total vitamin E present, and these were found to be similar to those in the oil extracted from mesocarp. However, in the case of fibre, "second-pressed" and the exocarp oils, it was found that the α-tocopherol contents were much higher, accounting for 46-67%; among these oils, the fibre oil provides a higher percentage of α-tocopherol than exocarp and "second-pressed" oils. The higher content of α-tocopherol present in the pressed fibre oil could be due to the higher stability of α-tocopherol as compared to the tocotrienols (γ and δ) which are known to be better antioxidants.[224] The γ-tocotrienol and δ-tocotrienol contents in these oils are lower as compared to crude palm oil. These results show that a substantial amount of vitamin E still remains in the fibre after the conventional oil extraction, and α-tocopherol, which is the usual commercial vitamin E, is the major component.

Table 2.16 Compositions (%) of Tocopherol (T) and Tocotrienols (T_3) from Various Sources

	α-T	α-T_3	γ-T_3	δ-T_3	T+T_3 (ppm)
$CHCl_3$ extract[a]	67.7	18.6	15.7	tr	2629
Hexane extract[a]	61.1	15.4	18.0	5.5	2412
Liquid CO_2 extract[a]	57.0	17.4	20.5	5.1	3005
"Second-pressed" oil	46.4	27.8	20.7	5.1	1649
Exocarp oil[b]	50.9	18.2	21.4	9.5	909
Mesocarp oil[b]	21.2	21.6	38.3	20.9	1130
Crude palm oil	22.0	20.0	46.0	12.0	792

[a] Oil extracted from pressed palm-fruit fibres using a Soxhlet extractor,

[b] Oil was extracted by Soxhlet extractor using *n*-hexane.

(b) Sterols

Table 2.17 shows the sterol profiles from various oil sources; as expected, the sterol content (concentrations ranging from 6,630 to 8,500 ppm) was found to be higher in the fibre extract than in CPO because of their low solubility in the oil and ease of extraction by organic solvents. The oil from second pressing of mesocarp was also found to be high in sterols, this is due to the higher press pressure causing membrane sterols to be extracted out together with the remaining oil, but their total concentration (2,149 ppm) is much lower as compared to the fibre oil extracted by solvents (> 4509 ppm).

The sterol contents in exocarp and mesocarp oils are similar to that of the oil from the solvent extract of the pericarp, which means that no specified distribution of sterols in the

exocarp and mesocarp exists. The higher sterol content in these oils as compared to the commercial crude palm oil is because the Soxhlet extractor was used for the extraction of these oils. As for the sterol composition profiles, all the oil extracts were found to have profiles similar to that of crude palm oil and mesocarp oil, in which β-sitosterol is the major component (56-67%), while campesterol (14-24%) and stigmasterol (14-20%) are found in lower amounts. Cholesterol is detectable at lower levels ranging from 2.3-5.2%.

Table 2.17 Sterol Compositions (%) of Various Oil Samples

Oil samples	β-Sitosterol	Stigmasterol	Campesterol	Cholesterol	Total sterols (ppm)
$CHCl_3$ extract[a]	58.4	18.7	19.9	3.0	6954
Hexane extract[a]	56.0	19.6	21.2	3.2	8490
Liquid CO_2 extract[a]	56.5	19.0	22.0	2.5	4509
"Second-Pressed" oil	66.4	17.6	18.3	5.2	2149
Exocarp oil[b]	58.0	20.1	18.4	3.5	657
Mesocarp oil[b]	63.1	14.2	21.0	2.3	987
Crude palm oil	57.0	15.0	24.0	4.0	250-620

[a] Oil extracted from pressed palm-fruit fibres using a Soxhlet extractor.

[b] Oil was extracted by Soxhlet using *n*-hexane.

2.4.3 Fatty Acid Compositions of Fibre and "Second-Pressed" Oils

The oil obtained from pressed fibres and "second-pressed" oil have been found to contain palm kernel oil as shown by a higher percentage of C12 and C14 fatty acids in Table 2.18, and the fibre oil was found to have higher contamination. This is not surprising as the

Table 2.18 Fatty Acid Compositions (%) of Various Oil Samples

	Fibre[@]			"Second-pressed"		
Fatty Acid	$CHCl_3$*	Hexane*	Liquid CO_2*	Oil**	CPO[#]	CPKO[#]
C6:0	0.1	tr	tr	-	-	0.3
C8:0	2.4	1.8	0.8	0.2	-	4.4
C10:0	1.8	1.3	0.6	0.3	-	3.7
C12:0	17.4	13.8	8.0	5.4	0.2	48.3
C14:0	6.9	6.2	4.2	2.7	1.1	15.6
C16:0	31.0	33.9	38.0	39.8	44.0	7.8
C16:1	-	-	-	0.2	0.1	-
C18:0	2.9	3.3	3.0	4.1	4.5	2.0
C18:1	29.7	31.7	36.8	37.6	39.2	15.1
C18:2	6.9	7.1	8.0	8.4	10.1	2.7
C18:3	0.8	0.6	0.3	0.3	0.4	-
C20:0	0.1	0.1	0.1	0.4	0.4	-

* Soxhlet extraction from pressed palm-fruit fibres using the solvent as indicated.

[#] CPO = Crude palm oil, CPKO = Crude palm kernel oil.

[@] Pressed palm-fruit fibres from a single-stage screw-press.

** "Second-pressed" oil refers to oil obtained by second pressings of the mesocarp.

relatively high pressure exerted in a single pressing has resulted in some broken nuts as well as broken kernels. The kernel debris were probably trapped and mixed with the fibre. Thus the kernel oil was extracted together with the fibre oil during solvent extraction. The higher press pressure in a second pressing must have pressed out the fibre oil together with the kernel oil from the broken kernels or nuts that were not removed after the first pressing.

2.4.4 Other quality Parameters of Fibre and "Second-Pressed" Oils

Quality parameters of fibre oil from single-pressed fibres and the "second-pressed" oil (i.e. oil pressed out again after the first stage pressing) have also been determined and the results are shown in Table 2.19. It was found that the quality of fibre oil and "second-pressed" oil are generally poorer as compared to the commercial crude palm oil, e.g. the phosphorus content in fibre oil is much higher because the solvent extraction tends to increase phospholipids extraction.

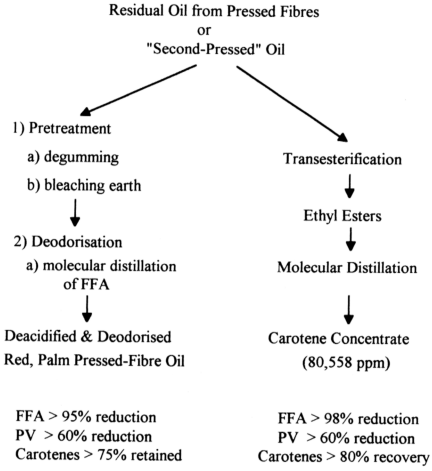
Due to the poorer quality of the "second-pressed" and fibre oils, these oils cannot be used directly for any food application requiring the oil, carotene or vitamin E nutrients. Hence, the oils need to be refined before they could be utilised as a source for food or minor components. In this respect, two processing methods have been investigated (which will be discussed in detail in Sections 2.5 and 2.6). The first method involved direct refining which included mild refining conditions and low temperature deodorisation and deacidification to produce refined, deacidified and deodorised palm fibre oil. The second method involved esterification and transesterification of the palm fibre oil into alkyl esters followed by molecular distillation of the esters. This latter method was able to produce a carotenoid concentrate of more than 80,000 ppm. Distilled alkyl esters are also important materials as

they can be used as oleochemical feedstocks. The schematic diagram of these two refining methods are shown in Fig. 2.15.

Table 2.19 Quality of the Oil Extracts*

	Fibre oil	"Second-pressed" oil	Crude palm oil
Free fatty acid (%)	7-11	5-11	3.6
Peroxide value (meq/kg)	4-6	7-8	2.4
Anisidine value	9-11	6-8	2.9
Phosphorus (ppm)	572-880	40-68	13.9
% of oil extracted	5-6.5	-	-

* Fibre oil was extracted by Soxhlet from pressed palm-fruit fibres using *n*-hexane; "Second-pressed" oil refers to oil obtained by second pressings of the mesocarp; Crude palm oil was obtained from conventional palm oil mill.



FFA = free fatty acid, PV = peroxide value

Figure 2.15 Refining of palm pressed fibre and "second-pressed" oils

2.5 RECOVERY OF CAROTENES FROM PALM OIL

2.5.1 Introduction

Of the vegetable oils which are widely produced, crude palm oil contains the highest known concentration of agro-derived carotenoids.[28] In fact, crude palm oil is the world's richest natural plant source of carotenes in terms of retinol (pro-vitamin A) equivalent. It contains about 15 to 300 times more retinol equivalents than carrots, green leafy vegetables and tomatoes, all of which are considered to have significant quantities of pro-vitamin A activities.[29] However, most of the carotenoids present in palm oil are destroyed in the present refining process to produce a light coloured oil used traditionally for cooking and for various food applications. In 1993, Malaysia produced about 7.4 million tonnes of palm oil and is estimated to reach 8 million tonnes by 2000. This represents a potential source of 4,000-5,000 tonnes of natural carotenoids which are lost during refining. Therefore, the recovery and the concentration of carotenoids from palm oil and palm oil products is of importance since carotenoids are growing in importance for nutritional and health applications beside being the ideal food colourant.[52-56]

Numerous extraction methods have been developed to recover carotenoids from crude palm oil, usually by either physical or chemical methods.

(a) Physical Recovery Methods

In the physical recovery methods, adsorbent materials such as activated carbon, silica gel, alumina and bonded reversed-phase materials are usually used.[109-113] The adsorbent used preferentially adsorbs the carotenes which were then desorbed by a solvent or solvents.

Several methods have reported the use of activated carbon or adsorbent earth but none of them has been proven to be satisfactory.[225] The extraction of carotenes using most of the carbon earth produced oxidised or isomerised carotene products and the recovery of carotenes was tedious. The recovered carotene concentration was usually only 4-10 times higher than that in crude palm oil. Todate, none of the existing physical methods has been put into commercial practice.

(b) Chemical Recovery Methods

In the chemical recovery methods, triglycerides are normally chemically transformed into esters or converted into soaps by saponification for various oleochemical applications.[116] For the saponified palm oil, the carotenes were solvent extracted as unsaponifiable materials. A carotene concentrate was then obtained after the removal of the solvent. Other processes involving the conversion of triglycerides to simple esters followed by solvent-solvent extraction and/or distillation of esters have also been developed.[106,119,120]

However, most of the reported methods of carotenes recovery from palm oil are difficult and costly, generally, carotene recovery is low when the carotene concentration in the recovered sample is high and the converse is also found. While it may be necessary to compromise on the two objectives, it is better to achieve high recovery and high concentration in the concentrate simultaneously.

In the pursuance of the dual objectives of achieving a relatively high carotenes concentrate as well as a higher recovery from palm oil, one particular method has been developed. This method was developed in connection with the production of volatile palm oil methyl esters that have been carried out on large scale for oleochemical applications or to use as diesel substitute.[117] In this method, a mild base-catalysed reaction converts triglycerides to volatile methyl esters leaving the valuable minor components unchanged. This allows for a unique opportunity for the recovery of carotenoids in palm oil as described in this study.

2.5.2 Molecular Distillation of Esters

This method involves the distillation of volatile alkyl esters using molecular distillation technique, where the volatile alkyl esters were distilled as distillate under high vacuum. Basically, the unique feature of the molecular distillation unit is the high operating vacuum that can be achieved. This is because of the design of the still body with an internal condenser, which can quickly condense the distilled components from the heated body within a short distance. Thus, a relatively lower temperature can be applied to distill high molecular weight components, which is very useful for the recovery of thermosensitive components (Fig. 2.16). An internal wiped-film system which will cause the feed material to form a thin layer on still body (where heating is evenly distributed to prevent any hot spots) also cause the feed material to have a very short retention time on the heated body to prevent thermosensitive components from being heated for long periods. Fig. 2.17 shows the laboratory-scale molecular distillation unit used in this study.

In this study, the volatile esters were first degassed and pumped free of volatile solvents which may be present in the crude esters. This could be achieved by applying vacuum to the feed material for an hour at a low heating temperature (80°C) or, alternatively by subjecting the crude esters to the molecular distillation system at 80°C at a fast flow rate under a vacuum of 150-200 mtorr. Normally, the latter procedure is preferred as it is faster and more efficient. This process will help the distillation system to achieve a better vacuum for the subsequent bulk distillation of esters.

Table 2.20 shows the carotene concentration, the percentage of distilled esters and residue obtained at different distillation cycles. The bulk of esters was first distilled at 130°C under a vacuum of 30-60 mtorr in which about 85-95% of esters were distilled over. Almost all the carotenes will remain as residue with a only small quantity of carotenes in the distillate as a result of spill-over.

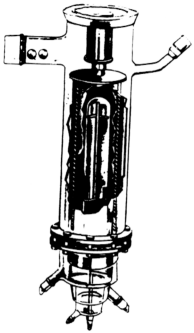


Figure 2.16 Internal view of a molecular distill body

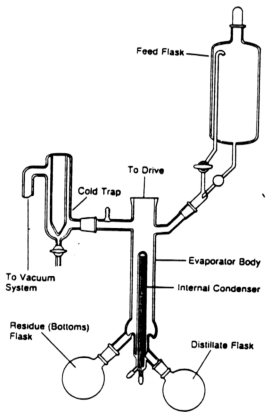


Figure 2.17 Laboratory scale molecular distillation unit

The residues containing carotenes collected at 130°C were pooled to undergo a second distillation. In order to increase recovery and to prevent the carotenes from being destroyed, a small quantity of oil (e.g. 5-10%, RBD olein) was added to the residue for dilution during the second distillation, so that the final carotene concentrate collected will be obtained as an oil paste. The remaining esters together with some monoglycerides will be distilled at 150°C temperature under a lower vacuum, i.e. 10-20 mtorr. The carotene-concentrated oil was collected as a residue.

As shown in Table 2.20, most of the carotenes remained in the residue after the second distillation and the carotene concentration increased to 20,000-40,000 ppm with the recovery of the carotenes at more than 80%.

Table 2.20. Carotenes and Vitamin E in the Distilled Esters and Residue*

Distillation temp.	Wt. of product (%)		Carotenes (ppm)		Vitamin E (ppm)	
	D	R	D	R	D	R
1st (130°C)	85-95	5-15	0.5-10	3,200-7,500	100-300	7,500-9,200
2nd (150°C)	75-85	15-25	30-150	20,000-40,000	7,500-12,000	2,000-3,400

* Crude esters: Carotene concentration = 500 ppm, vitamin E = 670 ppm.
Vitamin E = total tocopherol and tocotrienols, D = distillate, R = residue.

Vitamin-E compounds, which have lower molecular weights as compared to carotenes, could be partially distilled over together with the esters, at 130°C distillation temperature (at a vacuum of 30-60 mtorr) only about 1-6% of vitamin E was present in the distillate. When higher temperature (150°C) was applied during second distillation at lower vacuum (10-20 mtorr), more vitamin E as well as some monoglycerides were distilled over together with the remaining esters; only about 2,000-3,400 ppm of total vitamin-E content was remaining in the residue. The vitamin E was concentrated in the second distillate to about 7,500-12,000 ppm with about 10-20% of vitamin E being lost, probably due to oxidation during the distilling operations.

Table 2.21 shows the detailed tocopherol and tocotrienol compositions present in the carotene concentrate and distillate, but these are similar in compositional profile as the vitamin-E isomers in crude palm oil.

Table 2.21 Composition (%) of Tocopherol and Tocotrienols

	Carotene concentrate	Distillate	Crude palm oil
α-T	24.6	20.8	23.6
α-T_3	25.3	25.2	26.8
γ-T_3	40.2	41.0	39.8
δ-T_3	9.9	13.0	9.8
Total	3,194 ppm	10,542 ppm	670 ppm

T = Tocopherol, T_3 = Tocotrienol.

2.5.3 Carotene Profile by HPLC

The compositional profile of the carotene concentrate has also been monitored to determine any change of carotene composition due to this process. Table 2.22 shows the comparison of the compositions of carotene concentrate to those of commmercial crude palm oil. As shown clearly in the Table, no significant difference was observed for both the carotene profiles, there being only a slight decrease in the percentage of β-carotene. The percentage compositions of the other carotenes are quite similar to those of crude palm oil. The major carotenes are still α- and β-carotenes which constitute about 83% of the total carotenes.

Table 2.22 Carotene Compositions (%) of the Carotene Concentrate and Crude Palm Oil

Carotene	Carotene concentrate	Crude palm oil
Phytoene	1.5	1.3
Phytofluene	0.3	0.1
cis-β-Carotene	0.9	0.7
β-Carotene	49.9	56.0
α-Carotene	33.3	35.1
cis-α-Carotene	5.5	2.5
ζ-Carotene	1.7	0.7
γ-Carotene	1.3	0.3
δ-Carotene	0.6	0.8
Neurosporene	0.1	0.3
β-Zeacarotene	1.3	0.7
α-Zeacarotene	0.4	0.2
Lycopene	3.4	1.3
Total	40,560 ppm	673 ppm

2.5.4 Other Constituents

Tables 2.23 and 2.24 show the contents of sterols and fatty acid composition of the carotene concentrate prepared respectively. The high sterol content and their compositional profile (which is similar to the crude palm oil used for esterification) in the carotene concentrate showed that almost all the sterols remained in the residue. No detectable amount of sterols was detected in the distillate as the sterols have relatively higher molecular weight and thus remained as residue in the carotene concentrate.

The fatty acid composition was mainly influenced by the addition of RBD palm olein to the residue before the second distillation and in this case the palm olein has higher unsaturation in comparison to palm oil.

Table 2.23 Sterol Compositions (%) of the Carotene Concentrate and Crude Palm Oil

Sterol	Carotene concentrate	Crude palm oil
β-Sitosterol	60.5	62.2
Stigmasterol	15.2	12.2
Campesterol	21.9	23.3
Cholesterol	2.3	2.3
Total	21,842 ppm	442 ppm

Table 2.24 Fatty Acid Compositions (%) of the Carotene Concentrate and Crude Palm Oil

FAC	Carotene concentrate	Crude palm oil
C12:0	1.6	0.1
C14:0	1.8	0.9
C16:0	37.8	49.4
C18:0	4.9	3.0
C18:1	43.0	37.7
C18:2	10.3	8.1
C18:3	0.3	0.1
C20:0	tr	0.2

FAC = fatty acid composition

2.5.5 Applications of Carotene Concentrate

Presentation of the prepared carotene concentrate in powder form has been successfully formulated. It was found that 35% is the higher percentage of oil (carotene concentrate diluted with RBD palm olein) that can be used in order to obtain a dry powder with a fine structure. Samples with a higher oil percentage were quite difficult to homogenise and the powder obtained tends to be oily. Fig. 2.18 shows the carotene powder formulation containing 35% of oil, and this could be made into tablets or encapsulated in hard capsules for pharmaceutical purposes.

The storage stability of carotene powder has been carried out at 40°C, ambient temperature (27±2°C) and 4°C for a period of one year. Fig. 2.19 shows the carotene concentration plotted against time. Results show that carotenes in powder form were not very stable at ambient temperature and at 40°C, losing about 25% and 80% of its carotene respectively within one year. However, only a slight decline (<4%) in carotene content was observed for the powder kept in the refrigerator (4°C).

Figure 2.18 Presentation of carotene concentrate in powder form

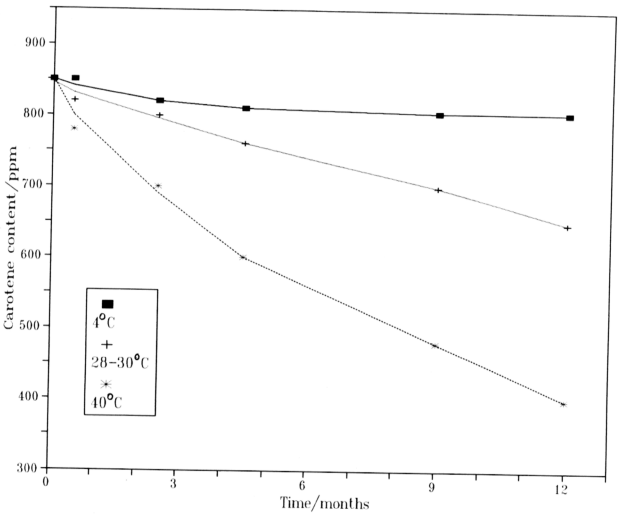


Figure 2.19 Storage stability of carotene powder at different temperatures

2.5.6 Toxicology Study

The carotenoid concentrate prepared by the molecular distillation method has been subjected to a toxicological study.[226] This study involved 4 groups of Sprague-Dawley rats (n=12 per group) which were fed on a semi-purified diet supplement with 0.2% palm oil based carotene concentrate (20,000 ppm), methyl esters, ethyl esters and a control diet for 16 weeks. Histopathological examinations of the major organs such as the heart, lungs, adrenals, kidneys, liver and spleen were found to be normal in all dietary groups. No extensive or significant amount of fat was deposited in the heart and the coronary vessels, and the aorta were found to be normal in all dietary groups. It can be concluded that the carotenoid concentrate and other dietary test groups do not have any toxicological effects on the major organs of the male rats.

2.6 PRODUCTION OF RED PALM OIL

2.6.1 Introduction

Carotenes, particularly β-carotene, have long been known for their pro-vitamin A activities as they are converted *in vivo*[152] to retinyl derivatives. The high levels of α- and β-carotenes make crude palm oil a rich source of vitamin A; this could be important to many developing countries where the inadequate intake of vitamin A can be one of the most urgent health problems, even though most of these countries may be located in areas with high production of palm oil or with a big potential for the cultivation of the crop. For this reason, palm oil has been targeted to be an important source of pro-vitamin A in some developing countries. However, presently, most of the palm oil and its products are consumed and used in refined, bleached and deodorised (RBD) forms which is light golden in colour and devoid of carotenes and it is difficult for most consumers to accept crude palm oil (CPO) as it contains up to 5% of free fatty acids as well as some odorous components.

The present conventional refining of CPO causes the thermal destruction of almost all the carotenes present during the bleaching and deodorisation/deacidification processes which are carried out at about 240-270°C. As a result, the final product is light golden in colour but devoid of carotenes. Malaysia has been the world's largest producer and exporter of palm oil and in 1993 Malaysia produced 7.4 million tonnes of CPO. This means that the present palm oil-refining process has allowed at least 4,000-5,000 tonnes of carotenes to be destroyed annually. Therefore, it is worthwhile to either recover the carotene before refining the oil or develop a refining process which can retain the carotenes for direct consumption.

As the methods of carotene recovery described in the previous section involved chemical transformation of the oil to esters and the isolation of carotenes directly from the oil

was also found to be difficult, it is perhaps useful to devise refining methods that retain the carotenes in the refined oil. In this connection, a process has been developed in this study which is able to yield refined, deodorised and deacidified palm oil of a similar quality to that of the normal RBD palm oil but retaining most of the carotenes originally present in the crude palm oil.

This process involves two stages. The first stage is a mild pretreatment, which involves degumming of the oil (i.e. removing phospholipids) with phosphoric acid followed by bleaching earth treatment (i.e. removing oxidative products, particulate and ionic impurities). The objective of this pretreatment is to remove impurities and some oxidative products in crude palm oil without removing or destroying the carotenes. The second stage of the process involves deacidification and deodorisation under vacuum.

2.6.2 Pretreatment of Crude Palm Oil

In the pretreatment process used by the palm oil refinery, different bleaching earths and varied conditions are used to reduce the oxidation products (based on peroxide value) and the colour, which usually means extreme conditions are used resulting in most of the carotenes being destroyed. In this study, a survey of the available commercial bleaching earths was carried out to find suitable materials and bleaching conditions so that carotenes losses during pretreatment can be reduced.

Pretreatment of the crude palm oil was carried out using eleven types of bleaching earths (all acid treated aluminium silicates) and also with Trisyl silica (a bleaching aid), the results are as shown in Table 2.25. Peroxides were removed most effectively by Trisyl silica which was used in combination with other bleaching earths. Some reduction (9-23%) of the carotene content was observed in almost all the treated samples. The five bleaching earth samples - Pureflo (activated), Tonsil Std. FF, Pureflo M65, Pureflo M80 and Pureflo M85 - were not effective in reducing the peroxide value in the absence of Trisyl silica. Furthermore,

Table 2.25 Preatreatment of Crude Palm Oil with Phosphoric Acid followed by Bleaching Earth Treatment

	Free fatty acids (%)							Peroxide value (meq/kg)								Carotenoids (ppm)							
Bleaching earth	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Control	2.20	2.96	2.96	2.96	2.96	2.31	2.31	2.31	1.00	5.58	5.58	5.58	5.58	0.75	0.75	0.75	648	622	622	622	622	647	647	647
Pureflo (Activated)	2.30	3.26	3.17	3.06	3.04				0.23	4.20	0.65	0.00	2.20				570	340	214	338	267			
Tonsil Std. FF	2.20	3.27	3.26	3.23	3.07				0.20	3.55	1.15	0.35	3.36				590	374	210	135	312			
Pureflo M65	2.25	3.27	3.19	3.02	3.07				0.20	4.10	0.40	0.10	1.85				555	289	123	159	228			
Pureflo M85	2.20	3.20	3.19	3.28	3.05				0.20	4.00	0.50	0.10	2.65				573	301	96	188	175			
Pureflo M80	2.20	3.00	3.23	3.07	3.05				0.20	3.00	1.90	0.10	2.30				516	288	91	227	212			
Siena	2.20					2.35	2.36	2.33	0.20					0.15	0.10	0.10	442					546	425	139
WAC Supreme	2.20					2.34	2.32	2.36	0.15					0.20	0.20	0.10	364					500	380	176
WAC 100	2.20					2.31	2.30	2.34	0.20					0.25	0.10	0.10	528					548	465	341
WAC100E	2.20					2.31	2.29	2.33	0.15					0.20	0.20	0.10	531					551	491	341
Fulmont AA	2.20					2.33	2.30	2.25	0.15					0.29	0.20	0.20	560					555	497	446
Tonsil Optimum FF	2.20					2.33	2.39	2.32	0.10					0.15	0.10	0.10	493					408	398	227

*In all cases, degumming were carried out by adding 0.5% phosphoric acid and heated for 10 minutes at 90°C

1. Bleaching earth treatment : 0.5% bleaching earth and 0.2% trisyl for 30 minutes at 110°C.
2. Bleaching earth treatment : 0.5% bleaching earth for 15 minutes at 105°C.
3. Bleaching earth treatment : 1.0% bleaching earth for 15 minutes at 105°C.
4. Bleaching earth treatment : 2.0% bleaching earth for 15 minutes at 105°C.
5. Bleaching earth treatment : 0.5% bleaching earth for 30 minutes at 105°C.
6. Bleaching earth treatment : 0.5% bleaching earth for 30 minutes at 110°C.
7. Bleaching earth treatment : 1.0% bleaching earth for 30 minutes at 110°C.
8. Bleaching earth treatment : 2.0% bleaching earth for 30 minutes at 110°C.

these earths also reduced the carotene content by 40% to 53%. Increasing amounts of bleaching earth and increase in the contact time improved the removal of peroxides but reduced the carotene content of the oil. Among other bleaching earths, Sienna, Tonsil Optimum FF, WAC 100, WAC 100 E, WAC Supreme and Fulmont AA were more effective in reducing the peroxide value. These earths cause less of carotenes removal (14% to 37%) as compared to the afore-mentioned group of bleaching earths. In general, increasing the amount of bleaching earth improves the removal of peroxides, but unfortunately it also reduces the carotene levels from the oil samples. As the bleaching earths are essentially acidic aluminium silicates, the use of about 2% of of these earths causes the removal or destruction of 31% to 78% of the carotenes.

Table 2.26 Pretreatment of Crude Palm Oil with Phosphoric Acid and Bleaching Earth (WAC 100)*

Time minutes	FFA %	PV meq/kg	Carotenes ppm
Control	2.20	1.00	646
5	2.20	0.20	588
15	2.20	0.15	576
30	2.20	0.10	575
45	2.20	0.15	560
60	2.20	0.45	552

FFA = free fatty acids, PV = peroxide value.

* 0.5% H_3PO_4 for 10 minutes at 90°C, followed by 0.7% bleaching earth at 110°C.

Among the bleaching earths, WAC 100 is widely used by the palm oil refining industry in Malaysia. Experiments conducted using WAC 100 showed that the peroxides can be removed effectively without appreciably reducing the carotene content of the oil as can be seen in Table 2.25. Increasing the time and amount of WAC 100 bleaching earth did not improve the removal of peroxide value but it significantly reduced the carotene content in the range of 6% to 33% (Tables 2.26 and 2.27). Increasing the temperature from 90°C to 130°C during bleaching proses did not improve the removal of peroxides but it caused a slight reduction (8% to 10%) in the carotene content of the oil (Table 2.28). The results show that the most suitable pretreatment condition is by treating the crude palm oil with 0.2% WAC 100 bleaching earth for 30 minutes at 110°C.

Table 2.27 Pretreatment of Crude Palm Oil with Phosphoric Acid and Various Amounts of Bleaching Earth (WAC 100)*

Percentage of WAC 100	FFA %	PV meq/kg	Carotenes ppm
Control	2.20	0.90	652
0.2	2.20	0.10	612
0.4	2.20	0.15	619
0.6	2.20	0.20	577
0.8	2.20	0.10	535
1.0	2.20	0.10	542
1.5	2.20	0.05	466
2.0	2.20	0.05	433

FFA = free fatty acids, PV = peroxide value.

* 0.5% H_3PO_4 for 10 minutes at 90°C followed by the bleaching earth for 30 minutes at 110°C.

Table 2.28 Pretreatment of Crude Palm Oil with Phosphoric Acid and Bleaching Earth (WAC 100) at Various Temperatures*

Temperature °C	FFA %	PV meq/kg	Carotenes ppm
Control	2.20	0.75	646
90	2.20	0.40	601
100	2.20	0.20	596
110	2.25	0.20	593
120	2.30	0.25	594
130	2.30	0.25	584

FFA = free fatty acids, PV = peroxide value.

* 0.5% H_3PO_4 for 10 minutes at 90°C, followed by 0.2% bleaching earth for 30 minutes.

2.6.3 Deacidification and Deodorisation

The deacidification and deodorisation (i.e. removing volatile fatty acids and their fragmentation products) of the pretreated palm oil were conducted at 130-200°C under a vacuum of 20-60 mtorr, using a laboratory scale molecular distillation unit. The results showed that this process was able to reduce the free fatty acids (FFA) to 0.02% without significantly reducing the carotene content (Table 2.29). From a crude palm oil sample with an initial carotene content of 663 ppm and FFA of 2.40%, a deacidified and deodorised red palm oil was obtained having a carotene content of 612-622 ppm and FFA of 0.03% using a vacuum of 20-25 mtorr and temperatures of 130-150°C.

At the vacuum of 20-60 mtorr and temperature between 130-170°C, the red palm oil could retain at least 80% of the carotenes. However, at a higher temperature of 200°C, the carotene content was reduced by more than 50%. The results showed that increasing the temperature and reducing the pressure will remove the FFA more effectively while higher temperatures (>170°) would destroy more carotenes. It was also observed that the FFA in crude palm oil were not effectively removed at 130°C under a vacuum of 60 mtorr, but required at least a temperature of 150°C. The results of these experiments showed that the most suitable conditions to produce red palm oil while retaining most of the carotenes were deodorisation/deacidification at temperatures of 150-170°C and vacuum of 20-30 mtorr.

This process has also been successfully applied to hybrid palm oil which contains considerably more carotenes (800-1,400 ppm) than normal crude palm oil. The results are shown in Table 2.30

The deacidified and deodorised red palm oil retained substantial amount of tocopherol and tocotrienols which are important as antioxidants and vitamin-E compounds (Table 2.31). However, the levels of these compounds were very much reduced when the palm oil was processed at higher temperatures (above 170°C) under a vacuum of 20-25 mtorr. At a temperature of 130°C and a vacuum of 20-60 mtorr, red palm oil retained more than 78% of the tocopherol and tocotrienols. However, at 170°C and 20-25 mtorr, more than 77% of the tocopherol and tocotrienols were distilled off. Similarly with hybrid palm oil samples, the deacidified and deodorised hybrid palm oil obtained retained more than 84% of the tocopherol and tocotrienols when processed at 130-150°C and a vacuum of 20-60 mtorr (Table 2.32). At higher temperatures (170-200°C) and a vacuum of 20-25 mtorr, it appears that most of the tocopherol and tocotrienols have been removed.

Table 2.29 Deacidification and Deodorisation of Pretreated Palm Oil* at Various Temperatures and Pressures

Pressure	Temperature	Analyses	
mtorr	°C	FFA/%	Carotenes/ppm
20-25	130	0.03	622
	150	0.03	612
	170	0.03	536
	200	0.02	315
35-40	130	0.15	617
	150	0.15	619
	170	0.04	562
	200	0.03	249
60	130	1.60	607
	150	0.75	607
	170	0.65	609
	200	0.04	417
Control Sample*		2.40	663
Pretreated Sample**		2.40	605

* Crude palm oil for pretreatment; FFA = free fatty acids.

**Pretreated with 0.5% H_3PO_4 for 10 minutes at 90°C, followed by 0.2% bleaching earth (WAC 100) for 30 minutes at 110°C.

Table 2.30 Deacidification and Deodorisation of Pretreated Hybrid Palm Oil* at Various Temperatures and Pressures

Pressure	Temperature	Analyses	
mtorr	°C	FFA/%	Carotenes/ppm
20-25	130	0.06	830
	150	0.05	821
	170	0.04	787
	200	0.04	753
35-40	130	0.63	825
	150	0.10	812
	170	0.04	785
	200	0.02	764
60	130	1.60	814
	150	0.95	809
	170	0.45	802
	200	0.25	782
Control Sample*		1.65	912
Pretreated Sample**		1.70	815

* Crude palm oil for pretreatment; FFA = free fatty acids

**Pretreated with 0.5% of H_3PO_4 for 10 minutes at 90°C, followed by 0.2% of bleaching earth (WAC 100) for 30 minutes.

Table 2.31 Properties of Refined Red Palm Oil Processed by Molecular Distillation

Temp. °C	Pressure mtorr	Analyses					
		PV meq/kg	FFA %	Fe ppm	P ppm	Carotenes ppm	T & T_3 ppm
130	20-25	0.35	0.03	0.2	2.7	622	720
	35-40	0.30	0.15	0.3	2.7	617	897
	60	0.60	1.60	0.3	2.9	605	908
170	20-25	0.20	0.04	0.3	2.0	531	216
	35-40	0.45	0.04	0.3	1.5	562	642
	60	0.60	0.65	0.3	2.4	610	906
Control Sample*		0.80	2.40	3.3	7.8	660	923
Pretreated Sample**		0.20	2.40	0.4	2.7	598	909

PV = peroxide value; FFA = free fatty acids; Fe = iron content; P = phosphorus content; T & T_3 = total content of tocopherol and tocotrienols.

* Crude palm oil for pretreatment.

**Pretreated with 0.5% of H_3PO_4 for 10 minutes at 90°C, followed by 0.2% of bleaching earth (WAC 100) for 30 minutes.

Table 2.32 Refined, Red Hybrid-Palm Oil Processed by Molecular Distillation

Temp.	Pressure	Analyses					
°C	mtorr	PV meq/kg	FFA %	Fe ppm	P ppm	Carotenes ppm	T & T_3 ppm
130	20-25	2.1	0.05	0.4	0.7	842	707
	35-40	2.8	0.61	0.5	0.6	821	785
	60	2.8	1.60	0.3	1.0	814	814
150	20-25	2.6	0.05	0.4	0.5	826	754
	35-40	2.8	0.10	0.4	0.6	813	781
	60	3.3	1.00	0.5	1.0	810	847
170	20-25	2.9	0.05	0.4	0.6	790	163
	35-40	3.2	0.03	0.5	0.8	786	641
	60	3.2	0.40	0.5	0.4	804	786
200	20-25	2.4	0.03	0.3	0.3	756	NIL
	35-40	3.0	0.02	0.3	0.5	765	402
	60	3.3	0.30	0.4	0.4	783	600
Control Sample*		5.90	1.70	1.0	2.3	910	840
Pretreated Sample**		3.00	1.69	0.4	0.7	825	821

PV = peroxide value; FFA = free fatty acids; Fe = iron content; P = phosphorus content; T & T_3 = total content of tocopherol and tocotrienols.

* Crude palm oil for pretreatment.

**Pretreated with 0.5% of H_3PO_4 for 10 minutes at 90°C, followed by 0.2% of bleaching earth (WAC 100) for 30 minutes.

2.6.4 Pilot Plant Production of Red Palm Oil

The production of red palm oil has been scaled up to pilot plant scale in order to study the technical and economical feasibility. This was carried out using a 6" short path distillation unit with a flow rate capacity of 10-20 kg an hour as shown in Fig. 2.20 below.

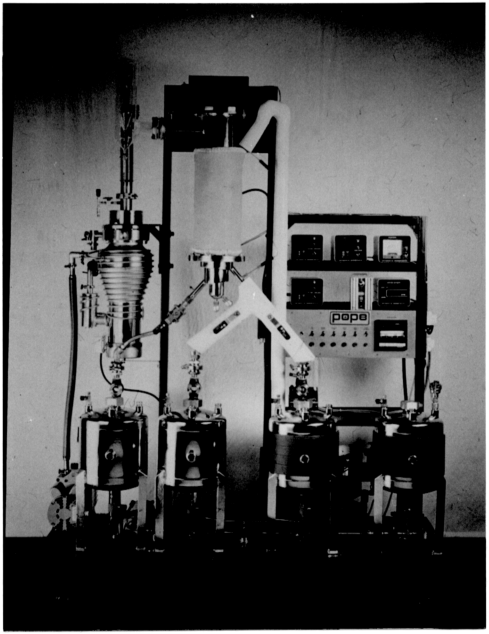


Figure 2.20 Pilot plant molecular distillation unit

The pretreated oil was obtained from a local refinery using conventional physical refining with controlled levels of bleaching earth, where about 80-90% of the original carotene content of crude palm oil were retained (Table 2.33). The PV was reduced to 0.4 meq/kg without much reduction in the vitamin E (T &T_3) contents.

Table 2.33 Quality Parameters of Red Palm Oil and Other Commercial Oils

Samples	FFA %	PV meq/kg	Carotenes ppm	T&T_3 ppm	M&I %	P ppm	Fe ppm
Crude palm oil[a]	3.53	2.32	643	864	-	5.0	12
P&D palm oil[a]	3.53	0.44	514	869	-	-	-
Red palm oil	0.04	0.10	513	707	0.00	1.6	0.2
RBD palm oil[a]	0.04	0.10	nil	561	0.03	1.6	0.2

[a] Samples from Keck Seng (M) Bhd.; PV = peroxide value; FFA = free fatty acids; Fe = iron content; P = phosphorus content; T & T_3 = total tocopherols and tocotrienols, M&I = moisture and impurities, P&D = pretreated and degummed.

Basically, the deodorisation process was similar to that of the laboratory scale experiment, where the pretreated oil (with FFA about 3.0 - 4.5%) was degassed at 80°C at a flow rate of about 10-15 kg an hour in order to obtain a better vacuum. Using this system, it was found that the free fatty acids can be reduced to less than 0.1% by using two consecutive distillations; first at 150°C and followed by a recycle at 160°C. Most of the free fatty acids and volatile components were first distilled over at 150°C still body temperature at a flow rate of about 7-10 kg an hour, the vacuum being maintained at a range of about 10-12 mtorr by

controlling the oil flow rate. This will normally decrease the FFA level to about 0.2-0.5%, and it is necessary to recycle the oil for distillation to reduce the FFA level to less than 0.1%. This was carried out at 160°C still body temperature using a better vacuum, i.e. 6-8 mtorr at a similar flow rate; a better vacuum can be obtained in the second cycle because most of the volatile components (including FFA) have been removed during the first cycle. The carotene concentration was found to remain unchanged (Table 2.33) but the FFA was reduced to 0.04%. In contrast, practically no carotene can be found in the RBD palm oil obtained from the conventional refinery. The total vitamin E content was reduced to about 80% of the original concentration in crude palm oil with not much changes in the vitamin E compositional profile (Table 2.34). The present process has been shown to be able to retain more vitamin E as compared to RBD palm oil processed at the refinery using the same batch of crude palm oil. Other quality parameters are comparable with RBD palm oil.

Table 2.34 Vitamin E Contents of Red Palm Oil and Other Commercial Samples of Palm Oil

Samples	Vitamin E (ppm)				Total Vit. E	% of Vit. E
	α-T	α-T_3	δ-T_3	γ-T_3	ppm	retained
Crude Palm oil[a]	187	208	376	98	869	100
P&D Palm Oil[a]	220	212	351	81	864	100
Red Palm Oil	166	202	275	64	707	81.4
RBD Palm Oil[a]	139	163	205	54	561	64.6

[a] Samples from Keck Seng Sdn. Bhd.; T = tocopherol, T_3 = tocotrienol, P&D = pretreated and degummed, RBD = refined, bleached and degummed.

Table 2.35 shows the material balance of oil, distilled FFA and vitamin E at each process cycle. Most of the FFA were removed during the first cycle, and about 6-10% of vitamin E co-distilled off with FFA as distillate at every distillation cycle, but it was found to be mostly concentrated in the second distillate, the vitamin E level of which is almost 20 times higher than that of crude palm oil. In practice, there are some losses of the oil as some oil will remain in the distillation system. In the present case, about 0.6 % of oil was remained in the system. The total of the vitamin E in the distillate and red palm oil obtained was less (~0.5%) than the original vitamin E in the pretreated and degummed oil, probably because of the losses due to some of the vitamin E have been oxidised during the process.

Table 2.35 Material Balance of Free Fatty Acids and Vitamin E after Processing

Sample	Vitamin E (ppm)	Oil weight (kg)	FFA (%)
P&D palm oil	1201	17.00	3.62
RPO 1st	1101	16.40	0.49
RPO 2nd	971	16.30	0.07
1st Distillate	3509	0.53	ND
2nd Distillate	22685	0.07	ND

P&D = pretreated and degummed; RPO 1st = red palm oil obtained after first distillation; RPO 2nd = red palm oil obtained after second distillation; FFA = free fatty acid; ND = not determined.

2.6.5 Carotene Composition of Deacidified and Deodorised Red Palm Oil

Analysis by HPLC shows that the carotene HPLC profile of deacidified and deodorised red palm oil is similar to that of the pretreated palm oil used as raw material, indicating that

carotenes were not selectively destroyed or significantly isomerised during the deodorisation process (Table 2.36). The major carotenes were still the α- and β-carotenes which constitute about 85% of the total carotenes present. Other carotenes determined are also shown in Table 2.36. However, it was observed that the carotene profile of the CPO was changed slightly by the pretreatment process, the β-carotene concentration was reduced relatively to the

Table 2.36 Carotene Compositions (%) of Deacidified and Deodorised Red Palm Oil and Crude Palm Oil

Carotene	Crude palm oil	P&D palm oil*	Red palm oil**
Phytoene	1.4	3.6	3.0
Phytofluene	0.1	0.3	0.3
cis-β-Carotene	0.6	0.7	0.6
β-Carotene	53.7	35.2	37.2
α-Carotene	35.6	47.6	47.5
cis-α-Carotene	3.3	5.5	5.1
ζ-Carotene	2.9	1.2	1.0
γ-Carotene	0.2	0.3	0.3
δ-Carotene	0.3	0.6	0.4
Neurosporene	tr	2.6	2.6
β-Zeacarotene	0.8	1.0	0.8
α-Zeacarotene	0.3	0.9	0.7
Lycopene	0.9	0.6	0.6
Total (ppm)	643	513	514

* P&D = pretreated and degummed.

** Pretreated, degummed, deodorised and deacidified.

α-carotene; the α-carotene level was found to be higher than β-carotene in the pretreated oil. This indicates that β-carotene is more easily adsorbed (or reacted) by the bleaching earth during the pretreatment process as compared to the α-carotene. Thus, more β-carotene was lost during the pretreatment, but this does not occur during the deacidifying and deodorising process.

2.6.6 Oxidative Stability of Red Palm Oil

Autoxidation of fat or oil can be described as occurring in two distinct phases. During the first phase, the oxidation goes slowly at an almost uniform rate. After the oxidation has proceeded to a certain point, the reaction enters a second phase, which is characterised by a rapidly accelerating rate of oxidation and eventually the oxidation rate at second phase is many times faster than that observed in the initial phase. The initial phase is called the induction period. It corresponds closely to the period before the onset of rapid oxidation causing flavour deterioration. Thus, based on the measurement of induction period, it will determine the oxidative stability of the fat or oil. In general, at ambient temperature, induction period is measured in weeks or even in months and this is too long for practical determination. Thus accelerated methods of testing which involve the use of elevated temperatures have therefore been developed and these technique are preferred.

The red palm oil produced in this study was subjected to accelerated oxidative stability tests based on the Rancimate method proposed by Zurcher and Hadorn,[227] the determination of the induction period is based on the measurement of conductivity. As some of the secondary oxidation products of autoxidation are volatile and ionic, especially the major hydroperoxide decomposition product being formic acid, they can be very easily determined by conductivity measurements. The oxidative stability of red palm oil was compared to commercial RBD palm oil by accelerated oxidation at 120°C.

Fig. 2.21 shows typical charts recorded in the Rancimate tests for various palm oil samples and the results are summarised in Table 2.37. The induction period for red palm oil is comparable if not better than the RBD palm oil, which means the red palm oil produced would have a similar oxidative stability (which usually means shelf life), if not better, than commercial RBD palm oil. Crude palm oil tends to show a shorter induction period, as most of the metal ions and other impurities which will act as pro-oxidants are only removed during the earth treatment and degumming process. The results also showed that the carotene present in the red palm oil does not improve the oxidation stability of red palm oil, in fact our study[228] has shown that higher carotene concentration will decrease the induction period as measured by Rancimate tests.

Table 2. 37 Rancimate Test on Red Palm Oil and Other Palm Oil Samples

Samples	Induction period (hours)
Crude palm oil	12.5-14.0
Pretreated & degummed palm oil	13.5-15.0
RBD palm oil	15.0-18.0
Red palm oil	12.0-18.0

RBD = Refined, bleached and degummed.

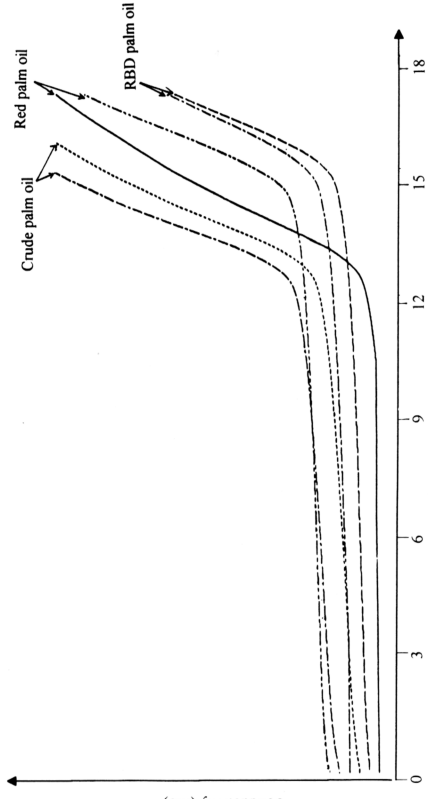


Figure 2.21 Rancimate oxidative-stability tests for red palm oil and other palm oil samples

2.6.7 Applications of Red Palm Oil

The red palm oil produced have been sent for sensory evaluation. It was shown that the red palm oil is of very good quality and is comparable to freshly prepared crude palm oil in the laboratory. It is of better quality as compared to the normal crude palm oil samples obtained from mills. The red palm oil is bland in flavour and therefore is shown by its low flavour intensity rating (Table 2.38). Fresh crude palm oil prepared in the laboratory has a sweet, pleasant caramel-like flavour.

The red palm oil samples produced from this process are shown in Fig. 2.22; the red palm oil has been demonstrated for the cooking of various local dishes, such as curry, satay sauce and "sambal". It can also be used for margarine formulation to give the required colouration. The red palm oil can be expected to be applicable to other dishes which are reddish in colour. It can also be used in salad dressing, cake making and ice-cream formulation (Fig. 2.22).

Table 2.38 Sensory Evaluation of Deacidified and Deodorised Red Palm Oil and Crude Palm Oil Samples**

Sample description	Sensory rating	
	Flavour intensity[@]	Quality*
Deacidified and deodorised red palm oil	1	5
Fresh CPO	4	5
Average CPO	4.5	3
Poor quality CPO	5	1

[@] Flavour intensity rating is from 1 to 5, 1 being bland and 5 being extreme.

* Quality rating is from 1 to 5, 1 being very poor and 5 being very good.

**Performed by PORIM Sensory Evaluation Group.

Figure 2.22 Red palm oil and its applications

2.7 PHOTOPROTECTIVE AND ANTIOXIDANT ACTIVITIES OF PALM CAROTENES

Free radical and singlet oxygen attack of vital cell components, mutations, and immunosuppression have been implicated in the progression of various chronic diseases.[229,230] The ability of β-carotene to suppress or counteract these potentially harmful processes may have health implications unrelated to its pro-vitamin A function.[44]

Specific structural features enable carotenes to be one of the most effective naturally occurring quenchers of singlet oxygen ($^1O_2$), a highly reactive chemical species. The carotenes (Car) are well known to be able to quench, by an energy transfer process, either triplet sensitisers (^3S) or $^1O_2$ generated *via* energy from ^3S or $^1O_2$ to ground state sensitiser (S) or oxygen ($^3O_2$).[121]

$$^3S + Car \text{ -------> } S + {}^3Car$$

$$^1O_2 + Car \text{ --------> } {}^3O_2 + {}^3Car$$

In both reactions, the resulting carotene triplet readily loses its energy to the environment and return to its original form, as depicted below:

$$^3Car \text{ -------> } Car + HEAT$$

Carotenes have also been shown to be capable of behaving as an antioxidant and can scavenge free radicals.[123,135]

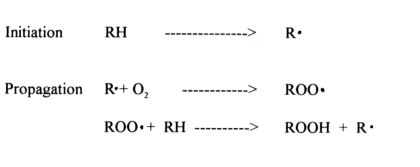
In the present study, the possible photoprotective and antioxidant activities of palm carotenes in the protection of fatty acid methyl esters and low density lipoproteins (LDL) were investigated.

2.7.1 Effect of Palm Carotenes on the Photoxidation of Fatty Acids

One of the properties of polyunsaturated fatty acids (PUFAs), particularly those with three or more unsaturated bonds, is their susceptibility to peroxidation, a radical chain reaction involving molecular oxygen as a reactant in one of the steps. In fact, the autoxidation of polyunsaturated fatty acid moieties is the cause of the rapid deterioration of foods containing PUFAs. Such oxidation is responsible for the development of rancidity due to the production of low molecular weight fission products that impart undesirable flavours.

In biological systems, enzymatic oxidation produces materials that are structurally related to those formed by non-enzymatic autoxidation, considerable evidence points to the possible involvement of lipid oxidation products in many diseases such as cancer, atherosclerosis and asthma and also the ageing process[231-234]

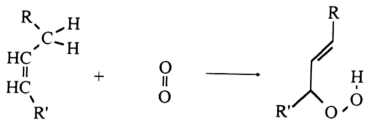
There are two types of non-enzymatic oxidation reactions in the production of peroxides and their degradation products and these are autoxidation and photoxidation. Autoxidation is often defined as oxidation by molecular oxygen under normal conditions of temperature and pressure; it is a radical chain reaction involving molecular oxygen and could be described in terms of initiation, propagation and termination reactions as shown below:-



Another important process for the oxidation of unsaturated lipids involves singlet oxygen ($^1O_2$) which can be produced by triplet sensitisers such as chlorophyll, rose bengal and methylene blue.[235,236]

$$\text{Sens} + h\nu \text{ --------> } {}^1\text{Sens} \text{ --------> } {}^3\text{Sens} \xrightarrow{{}^3O_2} {}^1O_2$$

Singlet oxidation occurs by the ene mechanism in which oxygen becomes attached to either of the olefin carbon atoms with attendant migration and stereomutation of the double bond.



The oxidations (both photo- and auto-oxidation) of polyunsaturated fatty acids are known to be accompanied by the formation of a complex mixture of secondary oxidation products including aldehydes such as alkanals, alk-2-enals, 4-hydroxyalkenals and malonaldehyde.[237,238]

In this study, the protective effect of palm oil carotenes against photoxidation was studied on fatty acid esters with sensitisation by methylene blue, the extent of lipid oxidation being monitored by the carbonyl compounds formed as secondary peroxidation products.[239] Determination of the 2,4-dinitrophenylhydrazone derivatives (DNPs) of various groups of carbonyl compounds was made, after TLC separation of the complex mixture of the carbonyl derivatives as three different groups, i.e., non-polar, osazone and 4-hydroxyalkenals. The DNPs of carbonyl compounds have distinct absorption maxima at about UV 365 nm. The UV spectrum of one of the carbonyl derivatives is shown in Fig. 2.23.

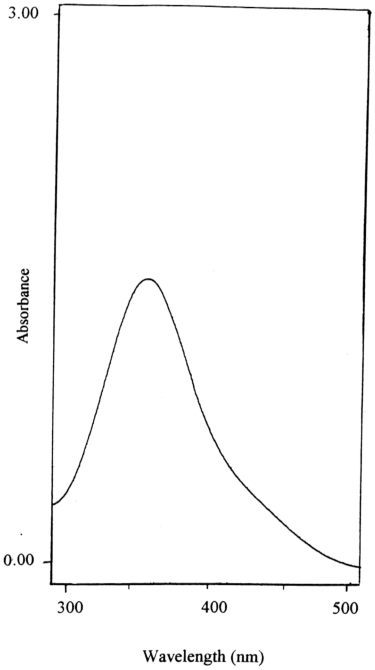


Figure 2.23 Typical UV spectra of the 2,4-dinitrophenylhydrazone derivatives of the 4-hydroxylalkenals in methanol

The absorbances for various carbonyl compounds formed during the photoxidation in the presence and absence of carotene are shown in the Tables 2.39 and 2.40 respectively. Data on the kinetic behaviour of the photoxidation of fatty acid esters in the presence and absence of palm oil carotenes are shown in Figs. 2.24, 2.25 and 2.26.

Table 2.39 Photosensitised Oxidation of Fatty Acid Methyl Esters[@] : Absorbance Values *vs* Time

Carbonyl derivatives*	Time (min)							
	0	15	45	75	135	195	255	375
Non-polar	1.039	1.535	1.921	3.735	3.105	2.801	1.842	1.268
Osazone	0.346	0.517	0.786	2.152	1.654	0.646	0.604	0.280
4-Hydroxylalkenals	0.219	0.199	0.322	3.176	2.550	0.970	0.706	0.775

[@] Methyl esters from soybean oil (4% w/v in dichloromethane) were photosensitised by methylene blue at 0 - 4°C.

* Carbonyl derivative classes were recorded at 365 nm. Each point represents the mean of two experiments.

Table 2.40 Photosensitised Oxidation of Fatty Acid Methyl Esters in the Presence of Carotenes[@] : Absorbance Values *vs* Time

Carbonyl derivatives*	Time (min)						
	30	60	90	120	180	240	360
Non-polar	1.278	1.204	1.139	0.804	0.820	0.915	1.026
Osazone	0.349	0.320	0.313	0.341	0.458	0.520	0.733
4-Hydroxylalkenals	0.189	0.275	0.230	0.230	0.242	0.342	0.354

[@] Methyl esters from soybean oil (4% w/v in dichloromethane) were photosensitised by methylene blue at 0 - 4 °C in the presence of 200 ppm of palm carotenes.

* Carbonyl derivative classes were recorded at 365 nm. Each point represents the mean of two experiments.

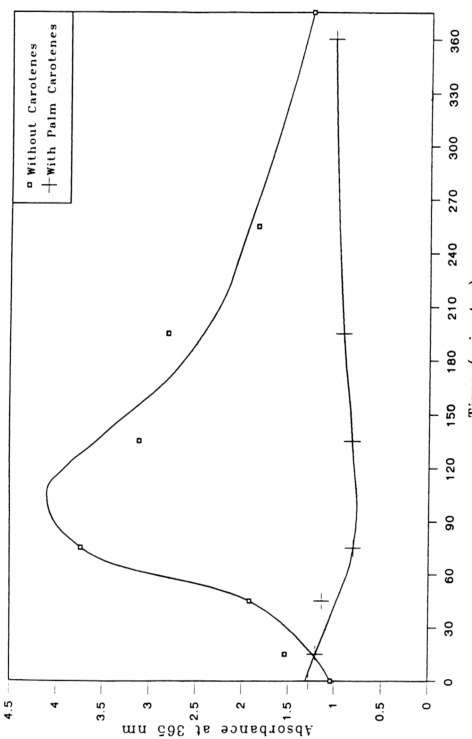


Figure 2.24 Absorbance at 365 nm for the 2,4-dinitrophenylhydrazones of the non polar group. Each point represents the mean of two experiments.

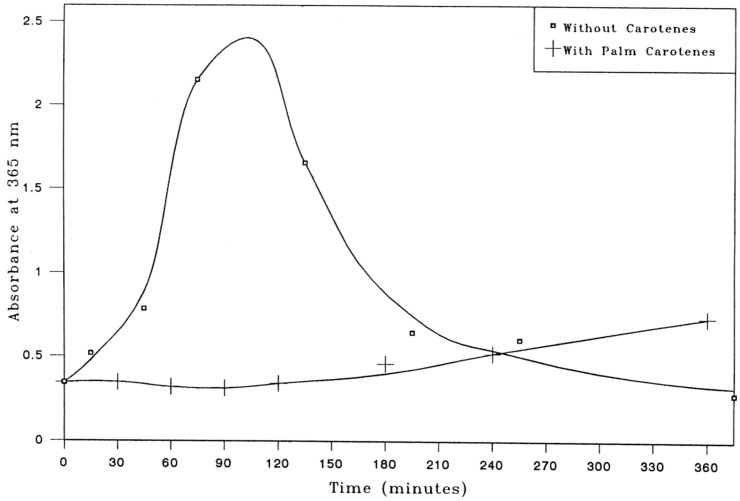


Figure 2.25 Absorbance at 365 nm for the 2,4-dinitrophenylhydrazones of the osazone group. Each point represents the mean of two experiments.

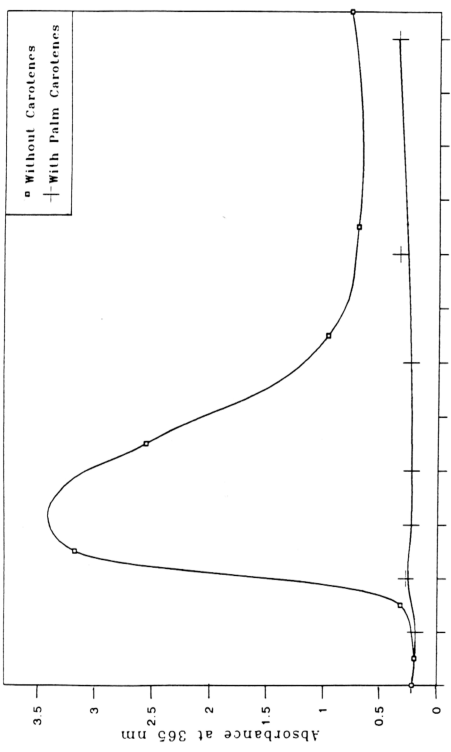


Figure 2.26 Absorbance at 365 nm for the 2,4-dinitrophenylhydrazones of the 4-hydroxylalkenals. Each point represents the mean of two experiments.

Generally, carbonyl compounds formed in the absence of carotene increased rapidly after about 50 minutes and reached a maximum value at about 100 minutes, followed by a slow decrease to a lower value over a longer reaction time. This indicated that the fatty acids were only rapidly oxidised after an induction period of about 50 minutes and after about 100 minutes, the decrease of carbonyl content was due to the formation of volatile carbonyl compounds.

When the photoxidation was carried out in the presence of palm carotenes, no formation of carbonyl compounds were observed in the first 300 minutes. The 3 different groups of carbonyl compounds, appeared slowly only after about 400 minutes of the photoxidation reaction have transpired. These observations clearly demonstrate that palm oil carotenes have a protective effect against the photoxidation of polyunsaturated fatty acids. The carbonyl compound formation also correlated well with the total carotene contents during the course of photoxidation (Table 2.41). There was a sharp decline in carotene content during the first 300 minutes indicating that the carotenes were consumed (Fig. 2.27).

Table 2.41 Carotene Absorbance Levels (446 nm) During the Photosensitised Reaction

Time (min)	Absorbance
0	0.720
30	0.638
90	0.541
150	0.494
210	0.454
270	0.371
360	0.263

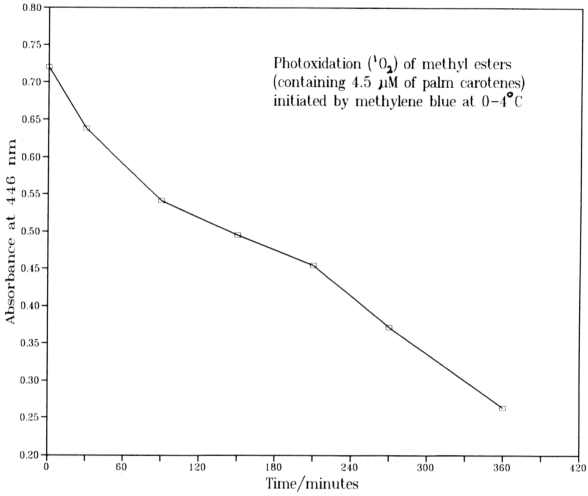


Figure 2.27 Loss of carotenes in the photoxidation of methyl esters

After the carotenes were almost all consumed after 300 minutes, the formation of carbonyl compounds were then observed to increase slowly. Palm carotenes are therefore useful triplet quenchers and can provide photoprotection for polyunsaturated fatty acids palm oil.

The extent of photoxidation has also been monitored by the analysis of fatty acid composition (FAC, as methyl esters), C17 being added as internal standard. Table 2.42 shows the FAC at different time intervals after being normalised to the C17 reference. The saturated fatty acids (e.g. 16:0 and 18:0), as expected, were not affected by photoxidation, the polyunsaturated fatty acids C18:2 and C18:3, however, were rapidly consumed.

Figure 2.28 shows the kinetic behaviour of the photoxidation of unsaturated fatty acids *vs* reaction time. The triunsaturated fatty acid, C18:3 is more susceptible to oxidation, followed by C18:2 with oleic acid (C18:1) being only slightly affected.

Table 2.42 Photosensitised Oxidation of Methyl Esters[@]

Time (min)	C14:0	C16:0	C17:0*	C18:0	C18:1	C18:2	C18:3	C20.0	Others
0	-	10.8	8.8	4.0	28.3	59.8	7.1	1.2	0.5
15	-	10.3	8.8	3.5	25.4	52.0	6.0	1.2	-
45	-	9.9	8.8	3.9	25.3	45.5	4.8	1.0	0.5
75	0.2	9.2	8.8	3.5	21.8	40.4	4.2	0.9	0.4
135	-	9.0	8.8	3.3	20.0	37.8	4.1	0.8	-
195	-	9.3	8.8	3.4	19.1	33.7	3.4	0.9	-
255	-	9.0	8.8	3.3	17.2	29.0	2.8	0.8	-
375	0.2	9.3	8.8	3.7	14.9	17.0	1.2	0.6	-

[@] Methyl esters from soybean oil (4% w/v in dichloromethane) were photosensitised by methylene blue at 0 - 4°C.

* Ester composition was normalised with the internal standard, C17:0.

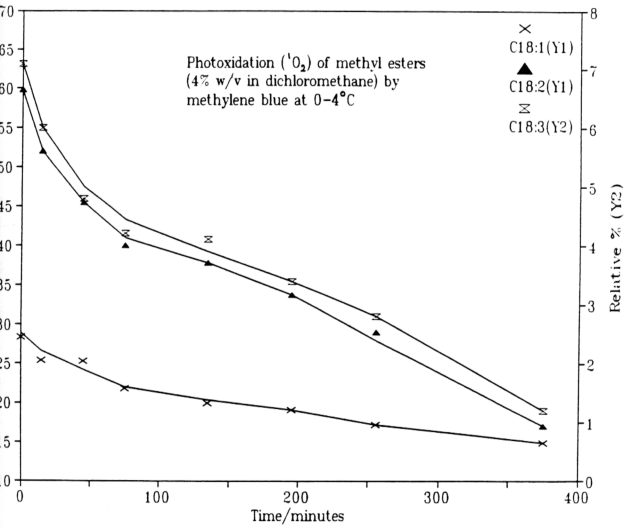


Figure 2.28 Reactivity of unsaturated fatty acids towards photoxidation

2.7.4 Effect of Palm Carotenes on LDL Oxidation

Several studies have provided evidence to indicate that atherosclerosis may be partly caused by free radical processes involving lipid peroxidation and oxidative modification of low density lipoprotein (LDL).[240-242] LDL consists of a glycoprotein (apo-B), free and esterified cholesterols, phospholipids, triglycerides, and lipid soluble vitamins. LDL delivers cholesterol to the peripheral cells by means of a receptor-mediated endocytosis. Oxidative modification of LDL whether induced by incubation with cells or as a result of autoxidation in the presence of transition metal ions, is linked to oxidation of its polyunsaturated fatty acids (PUFA). These fatty acids undergo extensive breakdown during LDL oxidation, yielding an array of low molecular weight fragments (ketones, aldehydes, alcohols, etc.),[243,244] some of which react to alter the LDL apolipoprotein-B such that they are recognised by macrophage scavenger receptors.[240,245] Enhanced uptake by the scavenger macrophages will affect cholesterol metabolism and lead to a conversion of macrophages into the lipid-laden foam cells that are constituents characteristic of fatty streaks and which later lead to atherosclerotic plaques.[241]

It has been shown that oxidative modification of LDL does indeed occur *in vivo*[246,247] and this can be protected by antioxidants such vitamin E and carotenoids.[241] We have carried out experiments to study the protective effect of palm oil carotenes against autoxidation and photoxidation of LDL isolated from rabbit plasma. Several methods are available for the measurement of the oxidation of LDL, e.g. determination of aldehydes, lipid hydroperoxides, conjugated dienes and fluorescence of apolipoprotein-B.[248] Presently, the measurement of the oxidation of LDL was carried out by continuously monitoring conjugated dienes formed through the oxidation of PUFA to conjugated PUFA-hydroperoxides.

The conjugated dienes for fatty acids have a distinct UV-absorption maximum at 234 nm as shown in Fig. 2.29. Since LDL is fully soluble in the aqueous phase and remains in solution during oxidation, the above measurement did not require extraction of the lipids but can be performed directly in an aqueous medium.

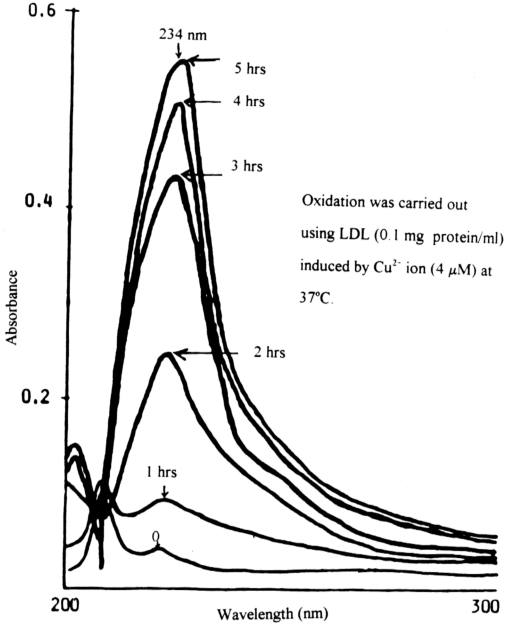


Figure 2.29 Formation of conjugated dienes (234 nm) from the free radical oxidation of *LDL* induced by Cu^{2+} ions

(a) Cu^{2+}-Catalysed Autoxidation of LDL

Palm carotenes were introduced into LDL *in vitro* from an ethanolic solution and autoxidation was initiated by Cu^{2+} ions. Vitamin E which is known to be an effective antioxidant, was also introduced into LDL *in vitro* and oxidation was carried out for comparative purposes.

Fig. 2.30 shows the kinetic plot of the conjugated diene content *vs* reaction time for LDL oxidation in the presence or absence of added carotenes and in the presence of added vitamin E. It was clearly demonstrated that in the oxidation of LDL supplemented with vitamin E, the lag phase is longer than those of LDL with or without palm carotenes.

By incorporation of palm vitamin E (mixture of α-tocopherol and tocotrienols) to a final concentration of 4.5 μM in LDL (0.1 mg protein/ml of phosphate buffer solution), the lag phase increased from about 0.5 hours to about 1.7 hours. This shows that the vitamin E introduced externally into LDL was effective in slowing down LDL oxidation. This corroborates other studies which have shown that the oxidative stability of LDL is highly correlated with the vitamin E content.

Although carotenoids have been reported to function as an antioxidant by a number of *in vitro* studies, including lipids in homogeneous solution, liposomes, isolated membranes and intact cell,[125-131] the present study shows that LDL supplemented with palm carotenes was not significantly protected from oxidation. It was possible that the *in vitro* incorporation of the carotenes into LDL was ineffective because of a solubility problem, as the final carotene concentration in LDL was only about 4.5 μM. There may be another problem, that is under Cu^{2+}-induced oxidation conditions, where there is an excess of oxygen, the palm carotenes would not act as an inhibitor. In fact it has been reported that potency of β-carotene as free radical scavenger is strongly increased at low oxygen tension but under higher oxygen pressures, β-carotene loses its antioxidant activity and shows instead an autocatalytic, pro-oxidant effect particularly at higher concentration.[148] It is evident that unlike tocopherols which terminates chain carriers, carotenes form carbon-based radical intermediates which can subsequently form reactive peroxyl radicals with oxygen.

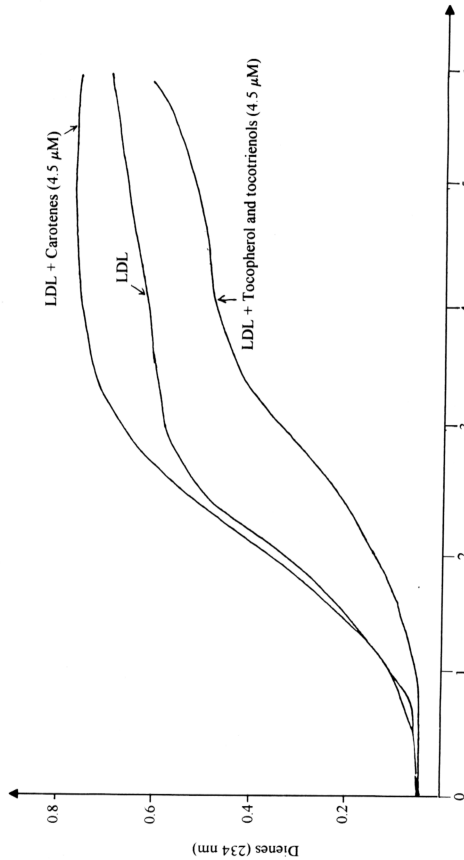


Figure 2.30 Kinetics of the formation conjugated dienes (234 nm) from the free radical oxidation of LDL induced by Cu^{2+} ions

(b) Photoxidation of LDL

Photoxidation of LDL was carried out using methylene blue as sensitiser (as described in Section 2.7.1). Oxidation was continuously monitored by the formation of conjugated dienes. Palm carotenes were introduced into LDL as an ethanolic solution to monitor their photoprotective effect on LDL; however due to the low solubility of carotene in ethanol, the final carotene concentration in LDL was only about 4.5 μM. The LDL sample was maintained at about 0-4°C to prevent any thermal-oxidation due to the heat generated by the UV lamp.

Fig. 2.31 shows the kinetics of the photoxidation (initiated by methylene blue) of LDL in the presence and absence of palm carotenes. An LDL sample without methylene blue was also included as a control, the lipid peroxidation rates did not rapidly accelerate in an exponential manner throughout the study as shown by the other two LDL samples.

Table 2.43 shows the lag time and the propagation rates for the photoxidation of LDL with/without carotenes. There was a significantly longer lag time shown by the LDL supplemented with carotene (25 minutes) as compared to LDL without carotene (12 minutes), and the propagation rates for the LDL without palm carotene was also shown to be higher, which means it will reach the maxima at a shorter time as clearly shown in Fig. 2.31.

Table 2.43 Effect of Carotenes on the Oxidative Susceptibility of LDL[@]

LDL Samples*	Lag time (min)	Lag phase gradient ($x10^{-4}$ min)	Propagation phase gradient ($x10^{-3}$min)
With Carotene	23±2	0.49	38.4
Without Carotene	15±3	1.03	60.0

* All samples were in duplicate
[@] Palm oil carotenes (to a final concentration of 4.5 μM) were used.

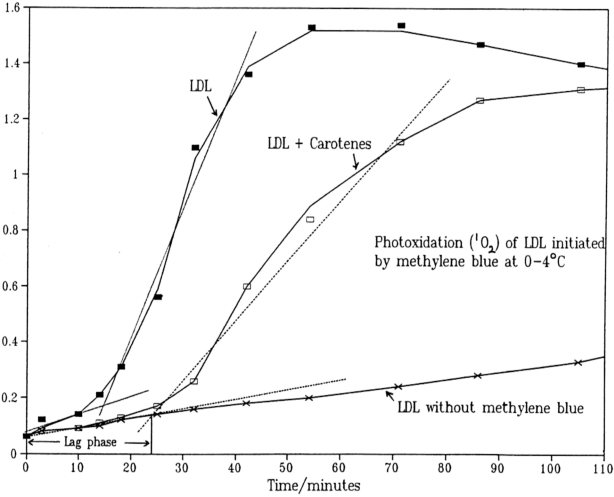


Figure 2.31 Photoxidation of LDL containing palm oil carotenes. Each point represents the mean of two experiments.

Fig. 2.32 shows the carotene concentration monitored throughout the LDL photoxidation. There was a sharp decrease in carotene concentration after 25 minutes, which then continued to decrease to almost zero after 110 minutes. The fairly constant value from 0-25 minutes shows inhibitory effect of carotenes together with endogenous tocopherols against singlet oxidation by $^1O_2$, and this initial period (induction period) is also reflected clearly in the lag phase of the kinetic plot for the formation of conjugated dienes during the photoxidation (Fig. 2.31), but after this stage there is a rapid loss of carotenes probably due to the free radical reactions of the carotenes.

The observations above show that palm carotenes have a protective effect against the photoxidation of LDL by $^1O_2$, it prolongs the lag time before the onset of accelerated free radical lipid peroxidation. The propagation rate as compared to that of LDL without carotene was also slowed, as the remaining carotene still provide a protective effect against photoxidation after the depletion of phenolic antioxidants. However, when compared to the results in Section 2.7.1 for the photoxidation of fatty acid methyl esters, the photoprotective effect of palm carotenes in LDL oxidation seems to be less effective. This result could be due to the problem of a low incorporation of carotene into the LDL particles in "solution".

In conclusion, it is conceivable that carotenes are useful for the protection of LDL against both autoxidation as well as photoxidation by $^1O_2$ due to their triplet quenching activity and radical scavenging capability. While experiments were not done under physiological conditions, the data complements observations that β-carotene is useful as an antioxidant either at the low partial pressures of oxygen found in mammalian tissues or by way of its singlet oxygen-quenching potential, thereby capable of retarding LDL oxidation and by implication, the atherosclerotic process. It is noted that the results are supported by a study among American physicians with cardiovascular disease who took 5.0 mg β-carotene or placebo on alternate days; those who were prescribed β-carotene had a 49% reduction in cardiovascular morbidity and mortality.[249]

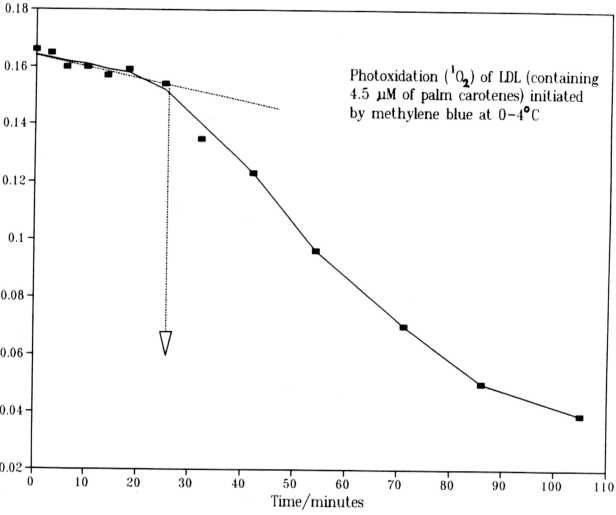


Figure 2.32 Loss of carotenes during photoxidation of LDL

2.8 EFFECT OF VARIOUS ANTIOXIDANTS ON THE AAPH-INDUCED OXIDATIONS OF LDL AND PLASMA

2.8.1 Introduction

Vitamins E and C are both well known as lipophilic and hydrophilic chain-breaking antioxidants. A co-operative and synergistic inhibition of oxidation by the combination of vitamin E and vitamin C has also long been suggested.[250-253] Recent studies have shown that oxidative modification of low density lipoproteins (LDL) result in several important changes in their properties and is believed to be responsible for a number of degenerative diseases.[240-242] During LDL oxidation, rapid depletion of endogenous antioxidants has been observed and this has been established by studies which showed that exogenously added vitamin E can prevent the oxidative modification of LDL.[234,235,241]

As described in Section 2.7, among the several mechanisms suggested for the protection by carotenes of biological systems from harmful oxidative reactions are the quenching of electronically activated species such as singlet oxygen ($^1O_2$) and the deactivation of reactive chemical species, such as peroxyl or alkoxyl radicals.[146,148] Carotenoid antioxidant activity is well documented in a number of *in vitro* studies, including lipids on homogeneous solutions, liposomes, isolated membranes and intact cells. This antioxidant activity is related to the chemical structure of the carotenoids (as radical sinks) but is only effective at low O_2 tension[148] when there is less likelihood of further formation of peroxy radicals.

In this study, the effects of exogenously added vitamin C and carotenes on the lipid peroxidation of LDLs induced by 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) were

examined. At the same time, the effects of the dihydrolipoic acid (reduced thiols) and it admixtures with vitamin C or carotene were also investigated.

The degree of the lipid oxidation was monitored from the formation of conjugated dienes and lipid hyroperoxides, and contents of various antioxidants were also analysed at various time intervals.

Similar oxidation studies were also carried out using plasma samples, and the degree of oxidation was studied based on the analysis of carbonyl compounds, lipid hydroperoxides and the antioxidant contents in the plasma at different time intervals.

2.8.2 AAPH Oxidation of LDL Isolated from Human Plasma

In this study, the effect of various antioxidants (including palm carotenes) added exogenously or present endogenously in the LDL isolated from human plasma was carried out using AAPH-induced oxidation, the extent of oxidation was being monitored by the formation of conjugated dienes and lipid hydroperoxides.

Results as compiled in Table 2.44 and Fig. 2.33 show that with the addition of ascorbic acid (ASC) and dihydrolipoic acid (DHLA) to LDL, the conjugated diene formation from the oxidation induced by AAPH was significantly reduced as compared to the control group. Oxidations were comparatively much lower for the LDL samples incorporating a mixture of ascorbic and dihydrolipoic acids, and a mixture of ascorbic acid, dihydrolipoic acid and palm carotenes (PAD), in comparison to experiments using either ascorbic or dihydrolipoic acids separately; however, no significant difference was observed between these two acid groups. The LDL samples incorporating palm carotenes was shown to have a similar oxidative susceptibility as that of the control sample, indicating that no significant protective effect was shown by the carotene against AAPH-induced oxidation. These results confirm

those reported that β-carotene does not show antioxidant activity at high oxygen pressure as under our reaction conditions.[148]

The results, however, show that there are some protective effects shown by the ascorbic and dihydrolipoic acids, and there was a tendency that the protective effect was slightly enhanced with the mixture of these two acids. The effectiveness of ascorbic and dihydrolipoic acids is mainly due to their hydrophilic nature of these antioxidants which can effectively inhibit radicals generated by 2,2'-azobis(2-amidinopropane)-hydrochloride.

Table 2.44 Formation of Conjugated Diene-Compounds (nmol/mg protein) in the AAPH-Induced Oxidation of LDL

Time/hrs	Control	POC	PAD	DHLA	ASC	D&A
0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.0	0.8	1.0	0.0	0.6	0.4	0.3
2.0	1.1	1.4	0.2	1.0	0.8	0.4
3.0	2.4	3.0	0.3	1.0	1.2	0.5
4.0	3.4	4.2	0.8	1.6	1.8	1.0
5.0	5.6	6.0	1.3	3.0	3.2	1.8
5.5	7.2	8.2	1.4	4.0	4.0	2.1

Each point represents the mean of two experiments.
AAPH = 2,2'-azobis(2-amidinopropane) hydrochloride (5 mM).
Control = pure LDL sample, the other experiments contain added antioxidant; POC = palm carotenes; PAD = palm carotenes + DHLA + ASC; DHLA = dihydrolipoic acid; ASC = ascorbic acid; D&A = DHLA plus ASC.

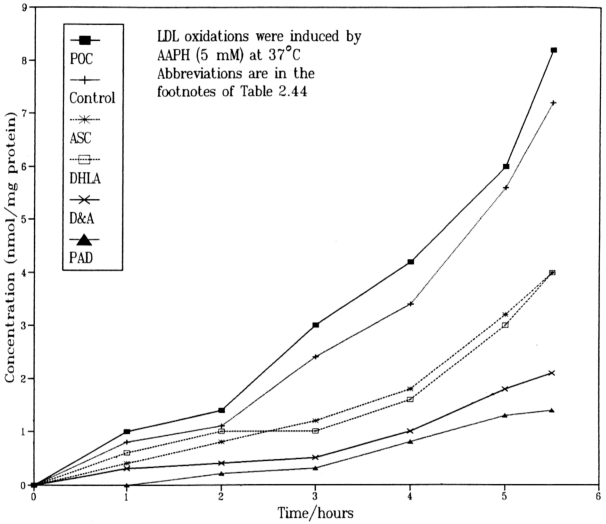


Figure 2.33 Formation of conjugated diene-compounds in the AAPH-induced oxidation of LDL. Each point represents the mean of two experiments.

In this study, no additional vitamin E was added exogenously. However, the endogeneous vitamin E, mainly α-tocopherol, in the LDL was monitored at different time intervals during the AAPH-induced oxidation (Table 2.45). The α-tocopherol contents for control experiment and LDL-supplemented with palm oil carotene were reduced sharply within 1.5 hours, whereas for other samples, the α-tocopherol contents decreased slowly to about 1/2 of the original values after about 6 hours as shown in Fig. 2.34. No significant differences were observed among the other four groups and this demonstrate the antioxidant effects of the hydrophilic supplements.

Table 2.45 LDL α-Tocopherol Levels (μM) in the AAPH-Induced Oxidation

Time/hrs	Control	POC	PAD	DHLA	ASC	D&A
0	14.6	11.1	15.1	14.0	14.6	14.6
1	14.1	7.8	15.6	-	14.4	14.2
2	9.1	4.2	14.0	13.9	12.0	14.0
3	4.6	0.6	12.6	12.5	13.4	14.0
4	1.7	-	12.3	11.1	13.3	11.6
5	0.2	0.2	-	11.4	11.9	11.4
6	0.2	0.0	-	10.9	-	8.0
7	0.0	0.0	10.9	7.9	8.5	7.1

Each point represents the mean of two experiments.

AAPH = 2,2'-azobis(2-amidinopropane) hydrochloride (5 mM).

Control = pure LDL sample, the other experiments contain added antioxidant; POC = palm carotenes; PAD = palm carotenes + DHLA + ASC; DHLA = dihydrolipoic acid; ASC = ascorbic acid; D&A = DHLA plus ASC.

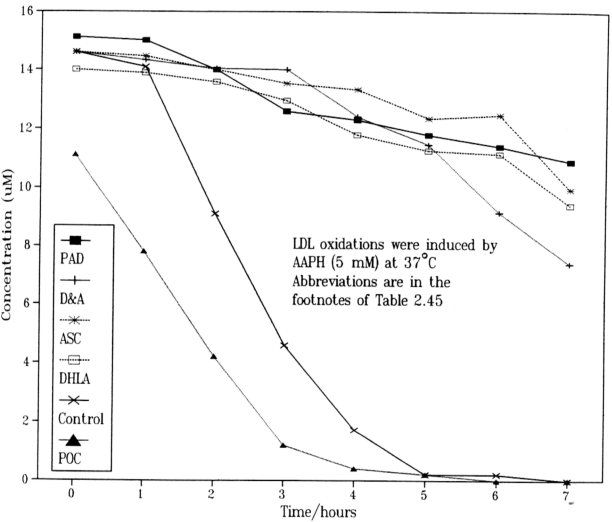


Figure 2.34 α-Tocopherol levels in the AAPH-induced oxidation of LDL. Each point represents the mean of two experiments.

The degree of LDL oxidation was also being monitored from the lipid hydroperoxide formation (hydroperoxide of the linoleic acid) as measured by chemiluminesence. The results are tabulated in Table 2.46 and a plot of lipid peroxide *vs* time is shown in Fig. 2.35. No lipid hydroperoxide was detected throughout the experiments for groups with added ASC, DHLA, A&D and PAD. The lipid hydroperoxide levels for the control experiment and that of LDL with added POC were increased significantly after 2 hours of oxidation induced by AAPH.

Table 2.46 Formation of Lipid Hydroperoxide* (μM) in the AAPH-Induced Oxidation of LDL

Time/hrs	Control	POC	PAD	DHLA	ASC	D&A
0.0	0.0	5.6	5.2	2.9	3.3	17.2
0.5	20.4	30.8	4.7	1.3	3.2	10.8
1.0	53.7	61.3	2.9	4.9	4.4	11.6
1.5	41.4	28.3	1.4	5.1	3.9	3.2
2.5	35.2	55.5	0.2	1.2	5.7	-
3.5	40.6	128.7	0.1	1.3	1.9	8.5
4.5	122.1	-	-	1.4	3.6	6.9
5.5	140.8	139.3	0.1	0.5	4.2	5.5

* Measured by HPLC with a chemiluminesence detector. Each point represents the mean of two experiments. AAPH = 2,2'-azobis(2-amidinopropane) hydrochloride (5 mM).

Control = pure LDL sample, the other experiments contain added antioxidant; POC = palm carotenes; PAD = palm carotenes + DHLA + ASC; DHLA = dihydrolipoic acid; ASC = ascorbic acid; D&A = DHLA plus ASC.

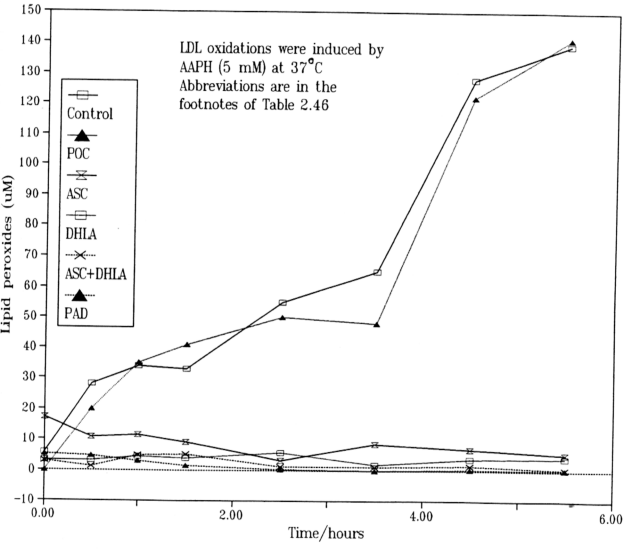


Figure 2.35 Formation of lipid peroxides in the AAPH-induced oxidation of LDL. Each point represents the mean of two experiments.

The results show that the incorporation of ascorbic acid or dihydrolipoic acid or both into LDL effectively inhibits AAPH-induced oxidations. In the control and POC groups, inhibition by the endogenous α-tocopherol (Fig. 2.34) provides a limited protective effect but could not completely inhibit lipid peroxidation; after the endogenous α-tocopherol was consumed, the lipid hydroperoxide levels (Fig. 2.35) were observed to increase significantly. The vitamin E level in LDL with added ascorbic acid and/or dihydrolipoic acid decreased only slightly throughout the experiment, indicating that ascorbic and dihydrolipoic acids are able to inhibit the lipid peroxidation of LDL caused by free radicals generated from AAPH in the aqueous medium, and it is noted that vitamin C showed a synergetic effect with vitamin E against oxidation.[250-253]

2.8.3 AAPH Oxidation on Human Plasma

Most studies of antioxidants in human body fluids have been focused on protecting lipids against peroxidation.[254,255] However, reactive oxygen species can also damage many other target molecules such as proteins and DNA.[256-258] Indeed, damage to proteins and DNA may often be more important than damage to lipids under oxidative stress situations *in vivo*.[255,259] In this connection, a study of the effect of various antioxidants on the AAPH oxidation of plasma has been carried out. The degree of oxidation was monitored by the formations of carbonyl compounds and lipid hydroperoxides; plasma samples were collected at different time intervals and the various antioxidants were determined.

The formation of carbonyl compounds during the AAPH-induced oxidation of plasma samples monitors the protein-derived carbonyl compounds and the results are shown in Table 2.47 and Fig. 2.36. The carbonyl compound levels for samples with palm carotenes (POC) and without palm oil carotenes (control) were found to increase rapidly as soon as the

experiments commenced, whereas for other four experiments, inhibition was observed for the first 4 hours of the experiments, increases in protein carbonyl contents were only observed after the fifth hour of oxidation.

Table 2.47 Formation of Protein-Derived Carbonyl Compounds* (nmol/mg protein) in the AAPH-Induced Oxidation of Plasma

Time/hrs	Control	POC	PAD	DHLA	ASC	D&A
0	3.5	6.2	2.5	1.1	0.5	0.9
2	14.0	7.8	4.2	1.6	1.6	1.4
4	17.0	18.6	4.4	2.5	2.3	3.0
6	23.0	19.4	4.9	5.0	3.7	3.6
8	30.6	26.8	6.6	11.1	6.1	3.2

* Measured at 320-400 nm. Each point represents the mean of two experiments.
AAPH = 2,2'-azobis(2-amidinopropane) hydrochloride (5 mM).
Control = pure plasma sample, the other experiments contain added antioxidants; POC = palm carotenes; PAD = palm carotenes + DHLA + ASC; DHLA = dihydrolipoic acid; ASC = ascorbic acid; D&A = DHLA plus ASC.

Table 2.48 shows the formation of hydroperoxides in the AAPH-induced oxidation of plasma. The data show that there were some lipid hydroperoxides already present in the original plasma samples; however, the levels of hydroperoxide for experiments with added D&A, PAD, ASC and DHLA decreased throughout the studies, which means that no lipid hydroperoxide was formed during the oxidation due to an effective inhibition by the

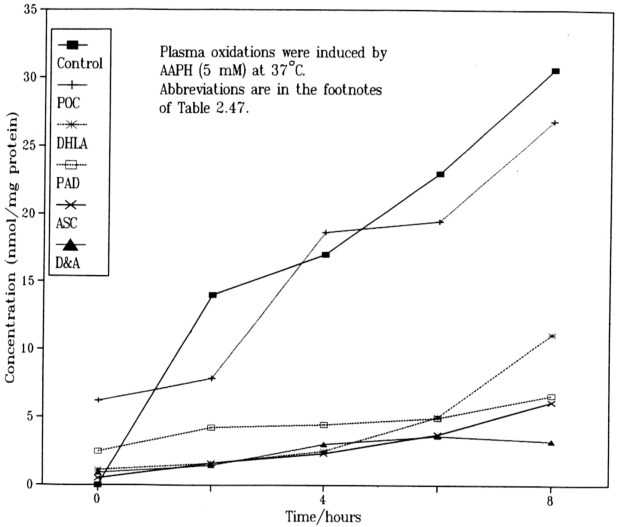


Figure 2.36 Formation of protein-derived carbonyl compounds in the AAPH-induced oxidation of plasma. Each point represents the mean of two experiments.

antioxidants. For the control and samples with POC, although there were slight decreases in lipid hydroperoxide levels at the early stages of the experiments as depicted in Fig. 2.37, the levels, however, increased after 4-5 hours.

Table 2.48 Formation of Lipid Peroxides* (μM) in the AAPH-Induced Oxidation of Plasma

Time/hrs	Control	POC	PAD	DHLA	ASC	D&A
0	0.40	0.60	0.51	0.27	0.38	0.43
2	0.33	0.57	0.61	0.26	0.25	0.36
3	0.18	0.53	0.39	0.13	0.22	0.27
4	0.12	0.31	0.25	0.12	0.06	0.23
5	0.44	0.17	0.16	0.02	0.06	0.00
6	0.70	0.56	0.13	0.00	0.03	0.00
7	1.20	0.72	0.00	0.00	0.00	0.00

* Measured by HPLC with a chemiluminesence detector. Each point represents the mean of two experiments. AAPH = 2,2'-azobis(2-amidinopropane) hydrochloride (5 mM).

Control = pure plasma sample, the other experiments contain added antioxidants; POC = palm carotenes; PAD = palm carotenes + DHLA + ASC; DHLA = dihydrolipoic acid; ASC = ascorbic acid; D&A = DHLA plus ASC.

The results show that an initial oxidation of the plasma-proteins had taken place (Fig. 2.36), especially for the control and samples with POC. No significant oxidation of plasma proteins was observed when inhibitors D&A and PAD were added, while some oxidation was observed only after 4 hours with added ASC and DHLA. And as shown in Fig. 2.37, oxidation subsequently extended to plasma-lipids, where the lipid hydroperoxides for the control and samples with POC are seen to increase significantly (after 4-5 hours of the

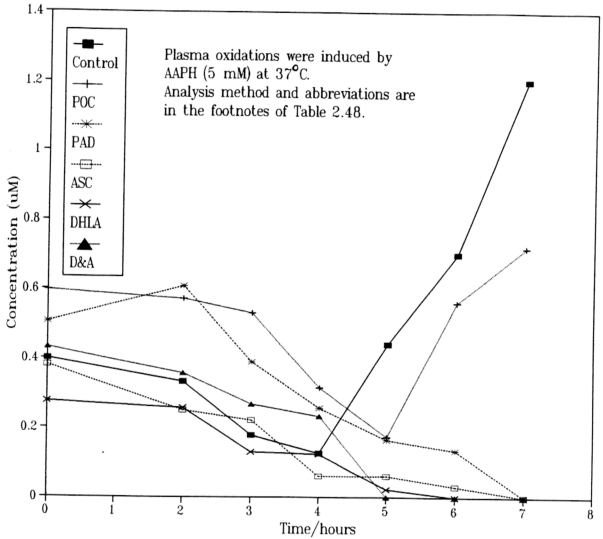


Figure 2.37 Formation of lipid peroxide in the AAPH-induced oxidation of plasma. Each point represents the mean of two experiments.

experiments); these occurred when the concentrations of protein oxidation products (protein carbonyls) were about 20-25 nmol/mg of protein (Fig. 2.36). No increase of lipid hydroperoxides was observed in other experimental groups indicating the useful effects of the respective antioxidants.

From the results of protein-derived, carbonyl compound formation of the various experiments, it was observed that a mixture of DHLA and ASC was able to protect the protein oxidation better than ASC or DHLA alone. In this study, the DHLA content was not monitored, but based on the results obtained, it can be infered that DHLA was just as effective as ASC in preventing protein oxidation.

Table 2.49 Ascorbic Acid levels* (μM) in the AAPH-Induced Oxidation of Plasma

Time/hrs	Control	POC	PAD	DHLA	ASC	D&A
0	48	44	760	62	523	814
1	36	34	752	50	374	814
2	24	24	549	35	428	811
3	14	13	448	31	277	561
4	1	1	282	12	247	448
5	-	1	37	4	167	304
6	-	1	-	1	75	147

* Measured by HPLC with an electrochemical detector. Each point represents the mean of two experiments. AAPH = 2,2'-azobis(2-amidinopropane) hydrochloride (5 mM).
Control = pure plasma sample, the other experiments contain added antioxidants; POC = palm carotenes; PAD = palm carotenes + DHLA + ASC; DHLA = dihydrolipoic acid; ASC = ascorbic acid; D&A = DHLA plus ASC.

Table 2.49 shows ascorbic acid levels from various experiments on the oxidation of plasma measured at different time intervals throughout the oxidation. About 700 μM of ascorbic acid were added exogenously for experiments with antioxidants (i.e. ASC, D&A and PAD). All plasma samples, with or without added ascorbic acid, contain some ascorbic acid (about 80 μM) endogenously present in the plasma (Fig. 2.38). Ascorbic acid was found to be quickly consumed during the oxidation of all plasma samples except when high levels of ascorbic acid were present, whereas for plasma samples without added ascorbic acid, its levels were almost undetectable after 4 hours of oxidation.

This result together with the results of monitoring protein-derived carbonyl compounds show that, the original ascorbic acid content in these samples were too low to protect against protein oxidation induced by AAPH in plasma. However, high concentrations (i.e., >100 μM) of ascorbic acid were able to prevent the protein oxidation. Lipid oxidation was only observed after ascorbic acid was consumed or after about 4-5 hours of oxidation as shown in experiments of the control and POC samples.

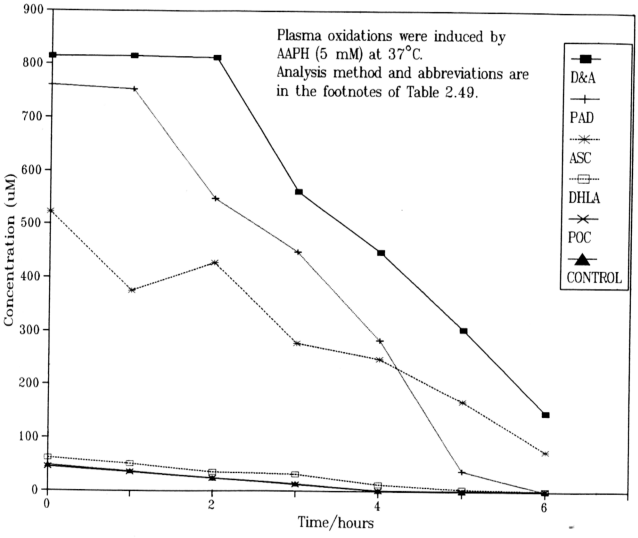


Figure 2.38 Ascorbic acid levels in the AAPH-induced oxidation of plasma. Each point represents the mean of two experiments.

The β-carotene concentrations in these samples have also been monitored throughout the study (Table 2.50). A final concentration of 18 μM of β-carotene was added exogenously to the plasma for the POC and PAD groups. The endogenous β-carotene content in ASC and D&A groups were maintained at about 0.45 μM throughout the study (Fig. 2.39) whereas the carotene content in the DHLA group showed a decrease at the later stage. There was a sharp decrease shown by the experiments of the control and POC groups after 6 hours of oxidation. Initially, a slight decrease in the carotene concentrations for both PAD and POC samples at the earlier stage of oxidation was observed, and for the PAD sample, it remained at about 18 μM. However, for the experiments of POC and control samples, there was a sharp decrease after 6 hours of oxidation. POC and PAD samples show decreases indicating that the carotenes introduced exogenously were incorporated with the protein particles.

Table 2.50 β-Carotene Concentration (μM) in the AAPH-Induced Oxidation of Plasma

Time/hrs	Control	POC	PAD	DHLA	ASC	D&A
0	0.48	19.10	18.33	0.50	0.50	0.46
2	0.47	20.00	17.03	0.47	0.42	0.47
4	0.47	18.02	16.45	0.51	0.47	0.53
6	0.45	17.50	15.84	0.59	0.48	0.47
8	0.23	17.59	12.56	0.33	0.47	0.47

* Measured by HPLC using a UV-Vis detector at 450 nm. Each point represents the mean of two experiments. AAPH = 2,2'-azobis(2-amidinopropane) hydrochloride (5 mM).

Control = pure plasma sample, the other experiments contain added antioxidants; POC = palm carotenes; PAD = palm carotenes + DHLA + ASC; DHLA = dihydrolipoic acid; ASC = ascorbic acid; D&A = DHLA plus ASC.

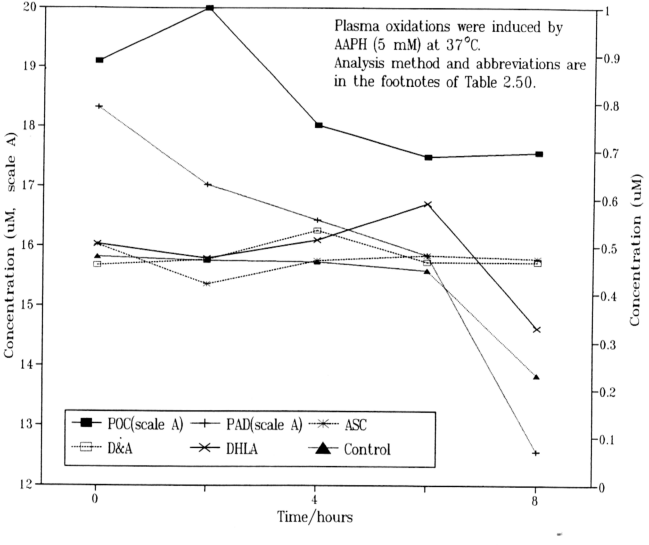


Figure 2.39 β-Carotene levels in the AAPH-induced oxidation of plasma. Each point represents the mean of two experiments.

The α-tocopherol contents in the plasma as shown in Table 2.51 were not much different for the D&A and PAD groups throughout the study. As shown in the Fig. 2.40, α-tocopherol contents in the experiments of the DHLA, ASC, POC and Control samples decreased slowly after 4 hours of oxidation, but the losses of α-tocopherol were faster in both the POC and Control groups. However, there were still significant amounts of α-tocopherol which remaining in the plasma at the end of the oxidation.

Table 2.51 α-Tocopherol Concentration* (μM) in the AAPH-Induced Oxidation of Plasma

Time/hrs	Control	POC	PAD	DHLA	ASC	D&A
0	8.3	8.2	8.2	8.0	8.2	8.3
2	8.2	7.8	8.1	7.7	8.0	8.2
4	8.0	7.4	8.1	7.8	8.0	8.2
5	7.4	7.5	8.0	7.5	7.9	8.1
6	7.0	7.1	8.0	7.3	7.6	8.1

* Measured by HPLC using an electrochemical detector. Each point represents the mean of two experiments. AAPH = 2,2'-azobis(2-amidinopropane) hydrochloride (5 mM). Control = pure plasma sample, the other experiments contain added antioxidants; POC = palm carotenes; PAD = palm carotenes + DHLA + ASC; DHLA = dihydrolipoic acid; ASC = ascorbic acid; D&A = DHLA plus ASC.

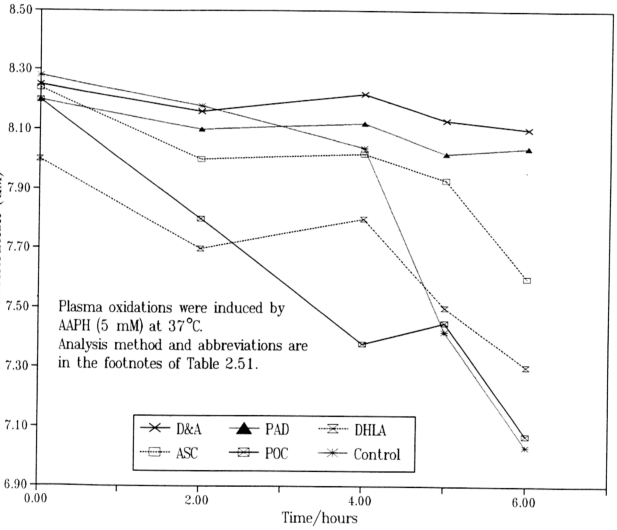


Figure 2.40 α-Tocopherol levels in the AAPH-induced oxidation of plasma. Each point represents the mean of two experiments.

From the results shown in this study, it is clear that ASC and DHLA have shown strong protective effects on both protein and lipid oxidation. However, in comparison, α-tocopherol present in plasma was unable to protect the protein against oxidation and, it was not effective in protecting all the lipid in the plasma against oxidation, as some lipid peroxide was observed to form during the oxidation although the α-tocopherol content still comparatively high at the end of the experiment. In other words, the lipid peroxide formed for the Control and POC samples were from the lipid components that was not protected or without the presence of α-tocopherol. The highly protective effect shown by ascorbic and dihyrolipoic acids could be due to its nature as water-soluble antioxidant, as AAPH is also water-soluble. This study has shown that ascorbic acid was able to trap the radicals generated in the aqueous phase before they could diffused into the plasma lipids.[253]

No significant protective effect was shown by palm carotene added exogenously or present endogenously. Also, to prevent protein and lipid peroxidation in the plasma, the hydrophilic antioxidants such as ascorbic acid and dihydrolipoic acid play more important roles as compared to other lipophilic antioxidants (vitamin E and β-carotene).

2.9 CAROTENE DISTRIBUTION AND OXIDATIVE STABILITY OF LDL

2.9.1 Introduction

Crude palm oil is known to be the richest source of carotenoids in higher plants in terms of pro-vitamin A equivalent.[28] Besides β-carotene which constitutes about 50% of the total carotenoids, about 30-40% α-carotene are also present. Other carotenes such as phytoene, phytofluene, ζ-carotene, γ-carotene, lycopene, β- and α-zeacarotenes are also present as minor constituents. There are few reports on the accumulation of other carotenoids besides β-carotene in animal tissues,[260,261] but rodents such as rabbits are known to be very efficient in converting β-carotene to retinol and they normally accumulate little or no carotenes in their tissues.[262] At a higher dosage of carotenes, however, some carotenes will be absorbed into certain organ tissues.[263,264]

Previous studies also showed no direct toxic effects on humans and animals fed with excessive amounts of carotenes, and there were no clinical signs of vitamin A toxicity.[265,266] A yellow pigmentation of the skin (carotenemia) was observed with elevated carotenoid intake from foods and β-carotene supplements.[267]

In the present study, the carotene distribution in the organs of rabbits fed with a diet enriched with palm oil carotenes for 12 weeks was investigated. The possible antioxidant effect of carotenes on the low density lipoproteins (LDL) from rabbits fed with a diet containing palm carotenes was also evaluated. For comparison, the effects of different dietary fats on the oxidative susceptibility of LDL were studied.

2.9.2 Carotene Distribution

For the analysis of carotene compositions, a non-aqueous reversed-phase HPLC system was used essentially as described earlier in Section 2.1 but with some modifications. By using a photodiode array (PDA) detector, the concentration of retinol and retinyl esters were also determined simultaneously with the carotenes present in rabbit plasma and organ tissues. All the components of interest with the absorption in the range 280 to 550 nm can be readily recorded by the PDA detector, which permits the on-line recording of the complete absorption spectrum of a chromatographic peak in less than 1 second. Quantitation was carried out based on the peak area of the individual components recorded from the stored chromatogram monitored at the absorption maxima. The extinction coefficient for the individual components used in this study are shown in Tables 2.11 (for carotenes) and 2.52 (for retinyl esters).[267a,267b] The concentration of an individual component was calculated essentially following the methods described in Section 2.2.1 using β-carotene as an external standard. The carotene composition in the rabbit's feed (added with palm carotene concentrate) as compared to that of palm carotene concentrate is shown in Table 2.53.

Table 2.52 Absorption Maxima and Extinction Coefficients for Retinol and Retinyl Esters

	$\lambda_{max.}$	$E^{1\%}/_{1cm}$
Retinol	325	1820
β-Retinyl ester	325	960
ε-Retinyl ester	325	1500

Table 2.53 Composition of the Carotene Concentrate and Rabbit Feed (mg/kg)

Carotene	Carotene concentrate[@]	Rabbit feed*
Lycopene	689	1
α-Zeacarotene	81	2
β-Zeacarotene	264	1
Neurosporene	20	-
δ-Carotene	122	1
γ-Carotene	264	-
ξ-Carotene	345	9
cis-α-Carotene	1,115	15
α-Carotene	6,753	127
β-Carotene	10,120	133
cis-β-Carotene	183	-
Phytofluene	61	3
Phytoene	304	17
Total Carotene	20,321	309

[@] Palm oil carotene concentrate (Section 2.5)

* For palm carotenes dietary groups (CE and CX).

In the analysis of organ and blood samples, a higher percentage of dichloromethane was used (20% dichloromethane in acetonitrile) to reduce the HPLC running time. However, all the carotenes present could still be quantified by the use of an advanced computer-data collecting system; a lower concentration of α- and β-carotenes present in plasma and tissues (as compared to the palm oil samples) also reduced the peak overlapping of these two compounds.

Fig. 2.41 shows the typical chromatogram from post-run analysis using selected wavelengths in the range 280-550 nm. Wavelengths were selected to enable optimal quantification of the different carotenes present.

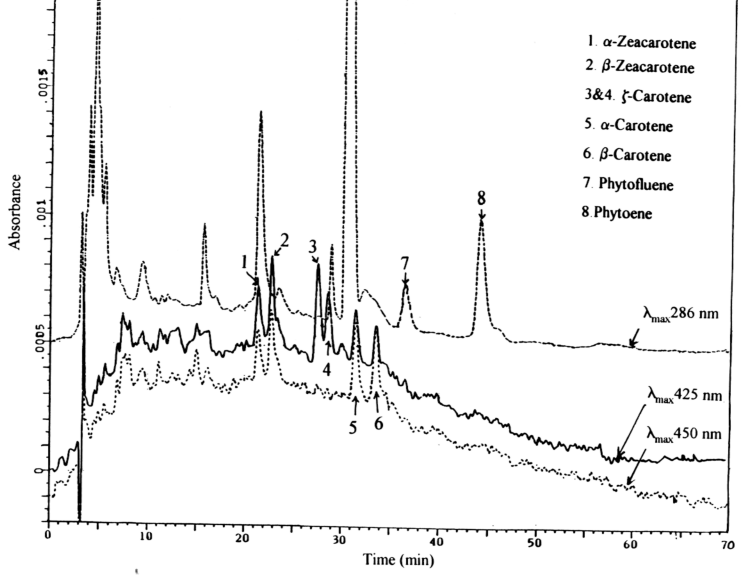


Figure 2.41 HPLC Chromatograms of the liver extract recorded at three wavelengths

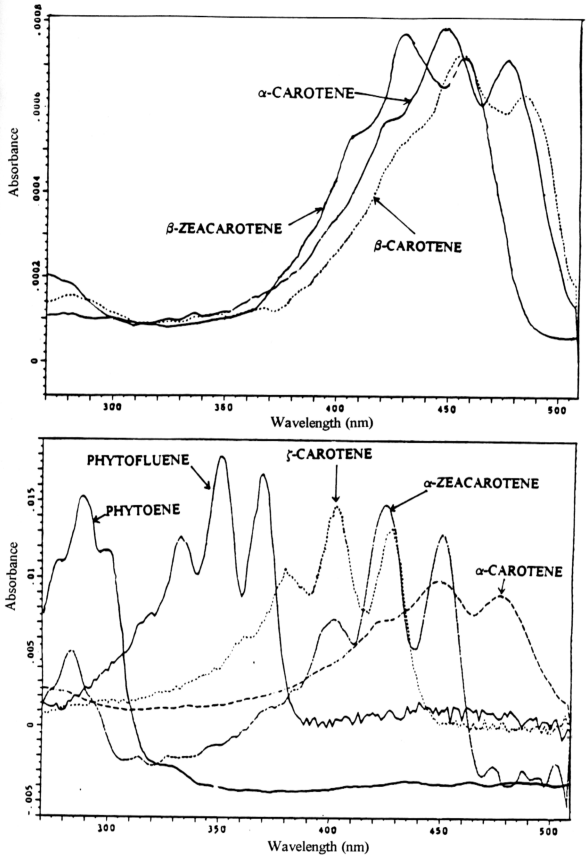


Figure 2.42 Spectra of carotenes obtained from the data recorded by a photodiode array detector

For identification purposes, each individual spectrum from the peak of interest was output from the stored chromatogram and compared to reference spectra. Typical spectra of the carotenes present in a typical organ (liver) extract are shown in Fig. 2.42.

Table 2.54 shows the carotene contents present in the plasma and organs of the rabbits fed with a diet containing refined, bleached and deodorised palm olein (RBDPOL) plus carotenes (CE group); no detectable amounts of carotenes were found in the heart, muscle and adipose tissues. The carotenes found in other organ-parts are α- and β-zeacarotenes, ζ-carotene, α- and β-carotenes, phytofluene, phytoene and lycopene. Phytoene is the major component found in the plasma as well as in all the organs. A relatively higher content of various carotenes was found to be stored in plasma, liver, adrenal and spleen as compared to other organ tissues. Only small amounts of lycopene were detected in plasma and liver.

Table 2.54 Carotenes in Plasma and Organs of Rabbits Fed with the CE* Diet (ng/g tissue)

	Plasma[@]	Liver	Spleen	Adrenal	Pancreas	Lung	Kidney
α-Zeacarotene	1.3	1.8	0.4	0.0	0.4	0.0	0.0
β-Zeacarotene	4.6	1.7	1.3	1.6	0.2	0.7	0.2
ζ-Carotene	24.4	5.6	5.6	7.1	0.3	3.3	2.9
α-Carotene	12.4	1.6	1.6	2.6	0.7	1.1	1.2
β-Carotene	14.0	1.1	1.1	2.1	0.4	1.3	1.3
Phytofluene	21.0	12.6	12.6	9.4	1.6	3.8	3.2
Phytoene	57.0	82.3	82.3	51.9	14.1	21.4	16.0
Lycopene	3.0	0.1	0.1	0.0	0.0	0.0	0.0

* CE = RBDPOL containing palm vitamin E (500 ppm) plus palm carotenes (2000 ppm).
[@] Values in ng/ml, no carotenes were detected in the heart, muscle and adipose tissues.

Although the animals were fed with diets containing relatively high percentages of α- and β-carotenes (Table 2.53), it was found that only small percentages of α- and β-carotenes were stored in the plasma and organs. However, substantial amounts of the α- and β-carotenes apparently were converted to retinol and stored as retinol esters. On the other hand, phytoene, phytofluene and ζ-carotene, which do not have any pro-vitamin A activity, were found to remain at relatively higher percentages in contrast to their relative compositions in the feed material.

For the diet containing RBDPOL plus palm carotenes but without palm vitamin E (CX group), carotenes were detected only in plasma, liver and adrenal gland (Table 2.55). The major carotenes are similar to the CE group, which are phytoene, phytofluene and ζ-carotene.

Table 2.55 Carotenes in Plasma and Organs of Rabbits Fed with the CX* Diet (ng/g tissues)

	Plasma[@]	Liver	Adrenal
α-Zeacarotene	18.5	0.6	3.0
β-Zeacarotene	28.7	0.4	3.1
ζ-Carotene	18.9	1.1	3.8
α-Carotene	13.7	0.2	4.0
β-Carotene	13.7	trace	1.1
Phytofluene	14.3	0.7	15.4
Phytoene	88.4	13.7	84.7
Lycopene	3.0	0.0	0.0

* CX = RBDPOL plus palm carotenes (2000 ppm) but without palm vitamin E, about 10 ppm of vitamin E from the added commercial chow was present in the diet.

[@] Values in ng/ml, no carotenes were detected in other organ parts.

In rabbits fed diets containing RBDPOL with vitamin E (PO group) or without vitamin E (PX group), only phytoene was detectable in plasma and liver as shown in Table 2.56. No other carotenes was detected in other organ parts.

Table 2.56 Phytoene Contents in Plasma and Liver of Rabbits Fed with PO and PX Diets*

	PO	PX
Plasma (ng/ml)	10	28
Liver (ng/g)	13	55

* PO = RBDPOL containing palm vitamin E (500 ppm); PX = RBDPOL without palm vitamin E, about 10 ppm of vitamin E from the added commercial chow was present in the diet.

These results show that, in spite of the high carotene diets, a small quantities of carotenes remain in the plasma, and some are stored in rabbit organs, especially in the liver, adrenal grand, lung and kidney. A relatively higher percentage of carotenes without pro-vitamin A activity (e.g. phytoene, phytofluene and ζ-carotene) was found in these organs as compared to the composition present in the feed; most of these carotenes have fewer conjugated double bonds (<10) as compared to the β-carotene (11 conjugated double bonds). δ-Carotene (which have 10 conjugated double bonds), although present in significant quantities in the palm carotene diets, was not detected in the plasma or organ tissues. These observations show that, most of the highly conjugated carotenes (>9) were being metabolised more effectively than carotenes with lower numbers of conjugated double bonds (<10). Therefore relatively higher amounts of phytoene, phytofluene and ζ-carotene were found in the plasma and organ tissues as compared to α- and β-carotenes, although the latter carotenes

were predominant in the diet. β-Zeacarotene (9 conjugated double bonds) is known to have pro-vitamin A activity and it was only present in small quantities in the feed (Table 2.53). However, it was readily detectable in the plasma and various other organs, showing that it was less effectively metabolised. In fact, β-zeacarotene was known to have less pro-vitamin A activity as compared to γ- and α- carotenes (which have 10 conjugated double bonds), although all these carotenes have one β-ionone end group in their structures.[268]

A relatively higher concentration of carotenes was found in various parts of the rabbit which was fed with palm carotenes plus palm vitamin E as compared to rabbits fed with palm carotene without vitamin E. This is not supprising as previous results have shown that vitamin E exerts a sparing action on carotenes at certain dietary intakes; vitamin E was believed to function as an antioxidant in the gastrointestinal tract, protecting carotenes from oxidation.[269-271]

2.9.3 Distribution of Retinyl Esters

Retinyl esters could be easily detected at λ_{max} 325 nm, and the retinyl ester chromatogram shown in Fig. 2.43, contained eleven retinyl esters which were identified based on UV spectral data and the retention times compared to the available standards and previously published data.[272,273]

The major retinyl esters found in this study are β-retinyl esters (with a β-ionone group, mainly metabolised from β-carotene) of fatty acid including C_{12}, C_{14}, C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ acids; retinyl myristate (C_{14}) was found to be eluted after retinyl linoleate ($C_{18:2}$) as an overlapping shoulder as shown in Fig. 2.43. All these retinyl esters contained a β-ionone group as part of their structures and have a similar characteristic absorption at 325 nm (Fig.2.44). Four other components (one of which overlapped with retinyl oleate) have absorption maxima at about 315 nm with a shoulder at each side of the main absorption band (Fig. 2.44); these were identified as ε-retinyl esters with an ε-ionone group attached to the ester. These groups of retinyl esters were metabolised from α-carotene (or β,ε-carotene) which constituted the second largest amount in the palm carotene diet; two types of retinyl esters were formed from α-carotene, i.e. β-retinyl esters and ε-retinyl esters. The ε-ionone group is known to have greater persistence as compared to the β-ionone group, which means ε-retinyl ester has a higher degree of fine structure in the spectrum as compared to the β-retinyl ester as shown in Fig. 2.44. The ε-retinyl ester is also known to have an absorption maximum at a lower wavelength than the β-compound and it is eluted in the C_{18} reversed-phase before the β-retinyl ester (Fig. 2.43).

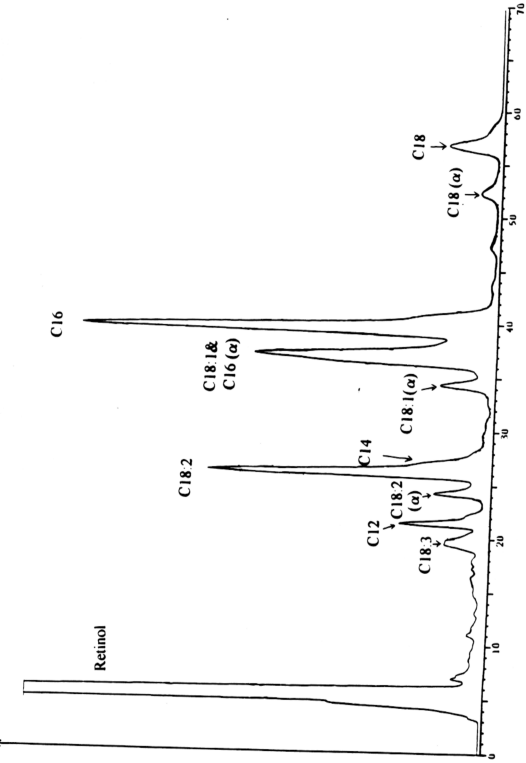


Figure 2.43 HPLC chromatogram of the liver extract recorded at λ_{max} 325 nm for retinol and retinyl esters

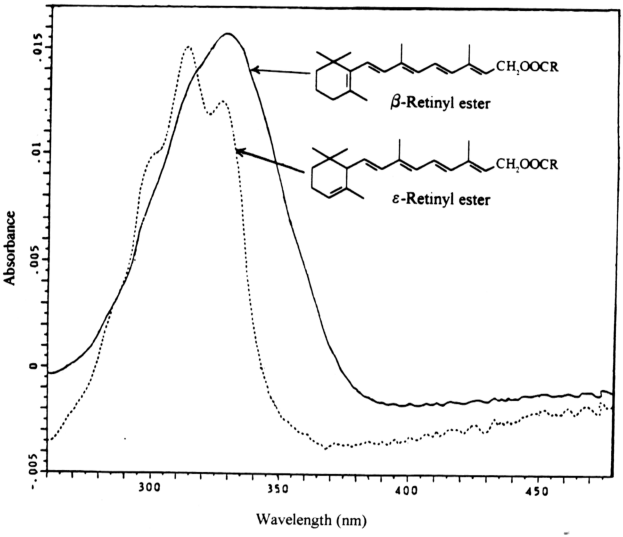


Figure 2.44 UV Spectra of ε- and β-retinyl esters

Table 2.57 shows the distribution of various retinyl esters in the plasma and organs from CE group of rabbits fed with palm carotenes. No retinyl esters were detected in the muscle and spleen. The major retinyl esters found in other organs are retinyl palmitate, oleate, linoleate and stearate; other common retinyl esters being retinyl laurate, myristate and linolenate. Liver contained the highest concentration of retinyl esters followed by pancreas and adipose tissue. ε-Retinyl esters were only detected in the liver and pancreas.

Table 2.57 Retinyl Esters in Plasma and Various Organs of Rabbits Fed with the CE* Diet (ng/g tissues)

	C18:3	C12	C18:2(α)[#]	C18:2& C14	C18:1(α)	C18:1& C16(α)	C16	C18(α)	C18
Plasma[@]	0	0	0	0	0	0	120	0	110
Liver	38	37	288	2,122	332	3,331	3,562	180	644
Adrenal	0	2	0	2	0	10	7	0	0
Pancreas	3	6	0	20	40	140	280	0	12
Adipose	6	4	0	2	0	52	160	0	0
Kidney	0	12	0	14	0	14	15	0	21
Heart	0	0	0	7	0	7	14	0	0
Lung	0	3	0	13	0	19	41	0	18

* CE = RBDPOL containing palm vitamin E (500 ppm) plus palm carotenes (2000 ppm).
[#] α in parenthesis refers to ε-retinyl esters, the rest are β-retinyl esters.
[@] Values in ng/ml, no retinyl esters were detectable in spleen and muscle.

For the CX group of rabbits fed with a diet supplemented of RBDPOL plus palm carotenes but without palm vitamin E (Table 2.58), a complete retinyl ester profile could only be found in the liver, where retinyl oleate, retinyl palmitate and retinyl linoleate were still the major components. As observed in the previous CE group, only retinyl palmitate and retinyl

stearate were detected in the plasma of the CX group animals. However, no detectable amount of retinyl esters was found in other organ parts from the present group of rabbits, except for traces of retinyl palmitate in the pancreas. The importance of antioxidants such as vitamin E in the diet is demonstrated.

Table 2.58 Retinyl Esters in Plasma and Various Organs of Rabbbits Fed with the CX* Diet (ng/g tissues)

	C18:3	C12	C18:2(α)#	C18:2 &C14	C18:1(α)	C18:1& C16(α)	C16	C18(α)	C18
Plasma@	0	0	0	0	0	0	228	0	160
Liver	9	61	20	181	17	184	245	11	57
Pancreas	0	0	0	0	0	6	0	0	0

* CX = RBDPOL plus palm carotenes (2000 ppm) but without palm vitamin E, about 10 ppm of vitamin E from the added commercial chow was present in the diet.

α in parenthesis refers to ε-retinyl esters, the rest are β-retinyl esters.

@ Values in ng/ml, no retinyl esters were detectable in the adrenal gland, pancreas, adipose, kidney, heart, lung muscle and spleen.

Table 2.59 shows the retinyl ester profile in organs for the PO and PX dietary groups. No ε-retinyl esters were detected, as the oils were depleted of carotenes and no palm oil carotenes were added into the feeds. Retinyl esters present in the plasma, liver and pancreas were derived from the dietary vitamin mixture which provided about 7.56 mg of retinyl palmitate (as a source of vitamin A) per kg of rabbit diet. Retinyl esters are assumed to be absorbed into the intestinal cells and transported to the liver, the retinyl esters being hydrolysed prior to transport into the liver cells, where the vitamin A is re-esterified for storage. The plasma retinyl esters found in these groups are still mainly retinyl palmitate and

retinyl stearate. Most of the retinyl esters were still found in the liver, where retinyl palmitate, retinyl oleate and retinyl linoleate are still the major components. No retinyl esters were detected in other organs except the pancreas, where retinyl palmitate was the major component with small amounts of retinyl oleate, linoleate and stearate.

Table 2.59 Retinyl Esters in Plasma and Various Organs of Rabbits Fed with PO and PX* Diets (ng/g tissues)

	C18:3	C12	C18:2&C14	C18:1	C16	C18
PO						
plasma[@]	0	0	0	0	18	7
liver	4	20	79	42	102	18
pancreas	0	0	0	6	17	0
PX						
plasma[@]	0	0	0	3	23	7
liver	5	18	62	49	199	41
pancreas	0	0	10	8	28	4

* PO = RBDPOL containing palm vitamin E (500 ppm); PX= RBDPOL without palm vitamin E, about 10 ppm of vitamin E from the added commercial chow was present in the diet.

[@] Values in ng/ml, no retinyl esters were detectable in other organ parts.

In general, the retinyl esters circulating in rabbit plasma are primarily retinyl palmitate and partly retinyl stearate, no other retinyl esters were detected. Previous study[274] on Ferret have also shown that retinyl palmitate and retinyl stearate are the major retinyl esters found in the plasma but with retinyl stearate as primary component. The higher percentage of retinyl

palmitate in rabbits plasma found in this study could be due to the partial effect from the dietary fats (RBDPOL) which is high in palmitate (36%) and low in stearate (4%) as shown in Table 2.61 (Section 2.9.5). Present results also show that in all the rabbit organs, retinyl palmitate was the major component present followed by lesser amounts of retinyl oleate, linoleate and stearate. Previous results[275] on other animals have also shown that retinyl palmitate was the predominant ester of vitamin A in the liver, with smaller amounts of retinyl stearate and traces of oleate, linoleate and myristate present. It has also been reported that, in the rat experiment, palmitate is the predominant liver retinyl ester under conditions favouring *de novo* synthesis of palmitate (including elongation of shorter chain fatty acid precursors) or when preformed palmitate is available. However, in the presence of large amounts of long-chain dietary fatty acids, the composition of liver retinyl esters reflects the nature of dietary fat, especially for linoleate acid. In this study, the relatively higher percentage of retinyl oleate and retinyl linoleate found in the liver and other organ tissues could be partially affected by the nature of the dietary fats, which contained about 47% oleic and 11% linoleic acids; however, palmitate is still the predominant retinyl ester in all the rabbit organ tissues being analysed, as the dietary fats also contain about 36% of palmitic acid.

In rabbits, it is clearly seen that most of the α- and β-carotenes were transformed and stored as retinyl esters in the liver and pancreas, with only a small quantity of unmetabolised carotenes being stored in the organs. The majority from the pro-vitamin A carotenes absorbed from the feed are, therefore, not retained but converted to vitamin A ester. A high quantity of both carotenes and retinyl esters was found in organs of rabbits fed with supplementary palm vitamin E (CE group) in contrast to rabbits without supplementary palm vitamin E (CX group). This could be due to the similar sparing (protective) effect of vitamin E as discussed earlier in Section 2.9.2. Results from this study also confirmed previous studies that retinyl

palmitate is the predominant retinyl ester from metabolism and the retinyl ester composition was partially modulated by the dietary fats.[275]

However, for rabbits fed with diets of palm olein without palm carotenes (PO & PX dietary groups), no significant differences were observed among the total retinyl esters and the effect of palm vitamin E was not obvious probably because the retinyl ester contents in these groups were too low.

2.9.4 Retinol Distribution

Retinol identification was based on the UV spectrum and co-chromatography with a standard compound. Among the components of interest, retinol was eluted first as shown in the HPLC trace (Fig. 2.43). The UV spectrum is similar to those of β-retinol esters which also absorb at λ_{max} 325nm (Fig. 2.45). The retinol contents in the plasma and various organs of different groups of rabbits quantified using HPLC are shown in Table 2.60.

Retinol was found to be present in all of the organs analysed, being present at high levels in the liver and plasma, relatively smaller amounts were present in the pancreas, adipose tissue, kidney and lung and trace amounts in other organ tissues (Table 2.60). In general, the retinol content determined for the CE group (supplemented with palm carotenes and vitamin E) was found to be higher than the other three groups of rabbits; in the CX group (palm carotene without palm vitamin E) retinol content was only found to be relatively higher in the liver as compared to the groups without carotenes (PO and PX). Other organs have retinol concentrations similar to the groups not fed with palm carotenes. Of all the organs analysed, the liver contains more than 70 % of the total retinol present. Relatively small amounts of retinol were found in the pancreas, adipose tissue, lung and kidney, and only trace amounts (<3 %) was found in other organ tissues. This is because most of the retinol is stored as retinyl esters in the liver and these are being transported to various organs through the

plasma; on the other hand, the plasma retinol levels for all the different dietary groups in this study were found to be not much different, as the plasma retinol concentration is known to be homeostatically controlled *via* a retinol-binding protein, thus the retinol content in most of the organs remained relatively low and constant.

Table 2.60 Retinol Contents in Plasma and Various Organs of Rabbits Fed Different Diets (ng/g)

	CE	CX	PO	PX
Plasma[@]	490	227	206	362
Liver	1,150	1,072	303	358
Adrenal gland	65	10	3	4
Pancreas	229	76	43	86
Adipose tissue	69	15	20	12
Kidney	38	12	27	34
Heart	34	6	9	7
Lung	27	8	18	8
Muscle	12	7	10	12
Spleen	17	9	5	3

[@] Values in ng/ml. Abbreviations for various dietary groups : CE = RBDPOL containing palm vitamin E (500 ppm) plus palm carotenes (2000 ppm); CX = RBDPOL plus palm carotenes (2000 ppm) but without palm vitamin E, about 10 ppm of vitamin E from the added commercial chow was present in the diet; PO = RBDPOL containing palm vitamin E (500 ppm); PX = RBDPOL without palm vitamin E, about 10 ppm of vitamin E from the added commercial chow was present in the diet.

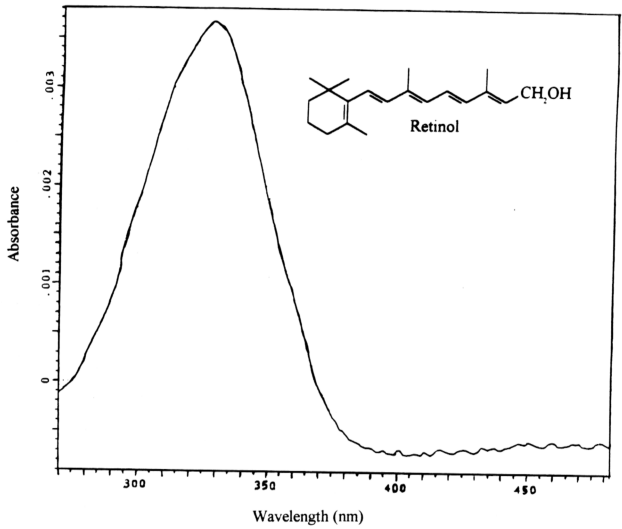


Figure 2.45 Spectrum of Retinol

2.9.5 Susceptibility of LDL to Oxidation

An *in vitro* oxidative stability study of the low density lipoproteins (LDL) isolated from four groups of rabbits on different palm-olein diets (CE, CX, PO and PX) have been carried out using copper ion-induced oxidation as described in Section 2.7. A comparative study to complement this series was also carried out on the oxidative stability of LDL isolated from groups of rabbits fed with other dietary fats including crude palm olein (CPOL), coconut oil with 13.3% corn oil (CNO) or soybean oil (SO).

The fatty acid compositions of the various dietary fats are given in Table 2.61. The CNO-based diet is rich in saturated fatty acids [lauric (12:0) and myristic (14:0); corn oil was added for its essential fatty acids], palm-olein diets (PO, CPOL, CE, CX and PX) are rich in oleic (18:1) and palmitic (16:0) acids, and the SO diet is rich in polyunsaturated fatty acids, mainly linoleic acid (18:2).

In this study, atherogenic diets (supplemented with 0.5 % cholesterol) were used, as this is a part of another study on the effects of dietary fats and antioxidants on atherosclerosis which, however, will not be presented in detail in this study. A group of animals fed RBDPOL without cholesterol (PO_{wc}) was also included in this study.

Table 2.61 Fatty Acid Compositions (%) of the Dietary Fats of Various Dietary Groups*

Dietary group	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0
CNO	3.4	5.1	4.6	40.4	16.4	8.8	-	3.0	9.7	8.6	-	-	-
palm olein	-	-	-	0.2	0.9	36.1	0.2	3.8	46.8	11.2	0.5	0.2	-
CPOL	-	-	-	0.2	1.0	38.3	0.2	4.1	45.0	11.0	0.4	0.3	-
SO	-	-	-	0.1	0.1	11.0	0.1	3.7	19.8	55.9	8.6	0.2	0.4

* Abbreviations: CNO = coconut oil with 13.3% corn oil dietary group; palm olein = refined, bleached and deodorised palm olein for palm-olein dietary groups (PO, PX, CE, CX); CPOL = crude palm-olein dietary group; SO = soybean oil dietary group.

(a) Fatty Acid Compositions of Plasma and LDL

Fatty acid profiles of plasma and LDL samples derived from rabbits fed various diets are given in Tables 2.62 and 2.63. Significant changes in the composition of long chain fatty acids were observed in the plasma. The levels of oleic acid from all rabbits fed diets supplemented with cholesterol increased remarkably after a 12-week dietary treatment, particularly for all palm-olein diets which have a comparatively higher oleic acid content than the others. The oleic acid in the plasma of rabbits fed with palm olein without cholesterol (PO_{wc}) also increased slightly but was not as high as in the cholesterol-supplemented groups. A major effect of the added cholesterol is to increase the levels of oleic acid in the plasma. There appears to be a selectivity for plasma (and LDL) cholesterol esters to be predominant as oleate.

Plasma "palmitic acid" was found to decrease from the initial relative percentage of 46.7% (commercial pellets as feed) to about 30% for all rabbits fed with cholesterol-supplemented diets, except for the soybean group (20%), which showed a greater relative decrease of palmitic acid. Plasma linoleic acid in the SO-fed diet of high polyunsaturation showed a relative increase (two-fold), whereas plasma linoleic acid in the CNO-fed diet group increased only slightly (~4%); however, plasma linoleic acid levels from palm-olein diets decreased (2.5%) in relative terms. Palmitoleic acid levels were 2-3 times higher than the initial plasma fatty acid composition for all the rabbits fed cholesterol-supplemented diets. Other saturated fatty acids (lauric, myristic and stearic) decreased considerably for all the cholesterol-supplemented groups, with the exception of the CNO-fed group, which showed relatively higher lauric and myristic acids in the plasma because of the saturated fatty acids from coconut oil.

Generally, among the different dietary fat groups supplemented with cholesterol, palm olein-fed rabbits have relatively higher levels of plasma oleic acid but not linoleic acid; CNO-fed rabbits have higher levels of plasma lauric and myristic acids; SO-fed rabbits have

high linoleic acid but low oleic and palmitic acid levels. No drastic change was found in the fatty acid composition of the plasma of rabbits fed palm olein without cholesterol; only a slight increase in oleic and decreases in linoleic and linolenic acids were observed. No significant differences were observed among various palm-olein groups fed with cholesterol-supplemented diets (CPOL, PO, PX, CE, CX), as all these groups were fed with a similar dietary fat. Quantitatively, these results show modulation by the nature of dietary fats.

Table 2.62 Fatty Acid Composition (%) of Plasma Lipids

Group	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Initial*	0.8±0.4	6.3±2.1	46.7±6.0	1.4±1.0	10.3±2.0	18.4±4.1	14.2±4.0	1.3±1.0	0.1±0.1
CNO	1.5±0.6	6.3±3.5	29.5±3.2	3.7±1.2	6.8±1.2	33.7±3.3	18.9±1.4	0.4±0.2	0.2±0.1
SO	0.2±0.1	2.1±0.9	20.8±2.8	3.4±1.1	5.9±1.3	32.5±5.1	33.2±2.4	1.0±0.5	0.3±0.2
CPO	0.1±0.1	1.2±1.0	31.6±3.9	3.7±0.8	5.8±1.5	44.5±5.4	12.7±2.4	0.2±0.1	0.2±0.1
PO	0.1±0.1	2.2±0.2	30.2±2.8	4.2±1.7	5.7±1.2	43.7±3.9	13.6±6.4	0.3±0.1	0.2±0.1
PX	0.1±0.1	2.7±0.8	28.5±1.6	3.9±1.2	3.1±0.4	52.7±1.9	8.7±0.9	0.2±0.1	0.1±0.1
CE	0.1±0.1	2.9±0.8	31.4±0.8	3.3±0.1	4.9±0.9	46.3±1.2	10.9±2.0	0.2±0.1	0.1±0.1
CX	0.1±0.1	3.5±0.5	32.1±0.6	2.7±0.2	5.4±1.4	45.1±2.8	10.9±1.4	0.2±0.2	0.1±0.1
PO_{wc}	0.6±0.3	9.7±3.2	44.8±8.8	0.6±0.1	5.3±1.4	27.8±3.0	10.8±1.9	0.3±0.1	0.1±0.1

* Initially composition (fed on commercial rabbit-feed pellets) before switching to various diets with different dietary fats. All dietary groups except PO_{wc} containing 0.5% cholesterol. Abbreviations for various dietary groups: CNO = coconut oil with 13.3% corn oil; SO = soybean oil; CPOL = crude palm olein; PO = refined, bleached and deodorised palm olein; PX = RBDPOL without palm vitamin E; CE = RBDPOL plus palm carotenes (2000 ppm); CX = RBDPOL plus palm carotenes (2000 ppm) without added palm vitamin E; PO_{wc} = RBDPOL-based diet but without cholesterol.

Almost similar trends are also shown in the LDL fatty acid compositions from various dietary groups. As expected, SO-fed rabbits have a higher linoleic acid content; the short chain fatty acids (lauric and myristic) were found to be relatively higher from CNO-fed diet; whereas palm olein-fed diets show relatively higher oleic acid levels. The rabbits fed with palm olein without cholesterol also showed LDL fatty acid composition quite similar to their plasma fatty acid composition.

Table 2.63 Fatty Acid Composition (%) of LDL Lipids

Group*	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
CNO	1.7±0.4	7.0±0.7	39.8±2.3	2.2±1.8	7.7±1.1	28.6±2.6	11.9±1.1	0.4±0.5	1.4±0.8
SO	0.6±0.4	1.2±0.1	37.4±2.5	2.6±2.3	8.6±1.3	29.8±3.5	21.2±4.5	0.6±0.1	0.6±0.1
CPOL	0.3±0.1	4.5±1.0	33.5±2.9	5.4±0.8	4.3±0.3	43.7±2.5	8.0±1.9	0.2±0.1	0.2±0.1
PO	1.0±0.6	3.7±0.6	37.7±2.4	3.4±1.7	5.9±1.1	36.9±0.8	12.2±2.1	0.3±0.4	0.8±0.2
PX	0.6±0.2	3.2±0.7	28.2±3.0	4.2±1.3	3.1±0.4	51.7±1.8	8.0±0.7	0.2±0.1	0.8±0.2
CE	0.4±0.1	4.6±1.0	36.2±1.2	3.7±1.2	3.0±0.3	44.2±1.2	7.2±0.8	0.3±0.1	0.4±0.3
CX	0.3±0.1	4.0±1.2	35.5±1.0	3.0±0.6	4.2±1.0	43.1±2.5	9.3±1.0	0.4±0.4	0.3±0.1
PO_{wc}	0.3±0.1	7.2±2.9	46.3±5.3	3.7±1.3	4.0±1.0	27.7±1.5	10.3±0.7	0.2±0.1	0.2±0.2

* Abbreviations are the same as in footnotes of Table 2.62. Mean values ± standard deviations; n = 6 for each dietary group.

The dietary regimes used provided three diverse types of fats, i.e. highly saturated (coconut), moderately saturated/monounsaturated (palm) and highly polyunsaturated (soybean). The fatty acid distributions of the diets have undergone considerable modulation by the time they are incorporated into the plasma or LDL particles, so that their distributions in plasma and LDL only partially reflect the nature of fatty acids of the diets (Tables 2.61,

2.62 and 2.63). The PUFA (reflected as 18:2) levels of the LDL particles only increased noticeably with the SO-diet; those of CNO- and palm olein (i.e. CPOL, PO, PO_{wc}, PX, CE and CX)-diets remained virtually unchanged. The CNO-diet contains almost exclusively of short and medium chain fatty acids, and corn oil was added for its essential fatty acids, which are not available from coconut oil. The incorporation of short and medium chain fatty acids in LDL of the CNO-diet group was however minimal, a reflection that the metabolic (portal) pathway for these fatty acids is different from those of long chain fatty acids.

(b) Oxidative Stability of LDL

Although the actual initiation processes of the peroxidation of LDL *in vivo* are not precisely known, accumulated evidences indicate that the end products are from lipid peroxidation.[240-242] Many oxidation studies using *in vitro* experiments have demonstrated that many of the properties of oxidised LDL particles have been observed to be similar to those from *in vivo* studies.[276] Such *in vitro* studies, although not exactly simulating the physiological conditions of oxidative stress, provide a fairly reliable index for comparing the relative susceptibility of LDL toward oxidative stress *in vivo*. Oxidative stability of LDL particles can be conveniently studied using Cu^{2+}-catalysed oxidation as established by Esterbaur.[239] The main targets of this oxidation are the polyunsaturated fatty acids (mainly linoleic, but also include, to a small extent, linolenic and arachidonic acids) and the formation of conjugated diene hydroperoxides (absorption at 234 nm) provides a profile containing lag, propagation and decomposition phases as shown in a typical plot (Fig. 2.46). It is noted that because this is a lipid peroxidation process, the inhibition of autoxidation in LDL occurs by antioxidants such as α-tocopherol, ubiquinol, carotenoids, etc. It is assumed that after the depletion of the antioxidants, a rapid oxidation occurs (propagation phase) and finally, a plateau is reached when the breakdown of peroxides becomes significant. Typical results on the oxidation of

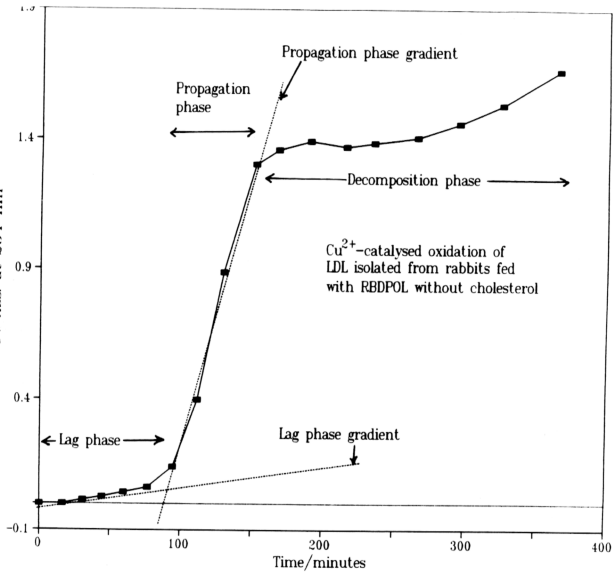


Figure 2.46 Kinetic plot of conjugated diene formation in the Cu^{2+}-catalysed oxidation of LDL

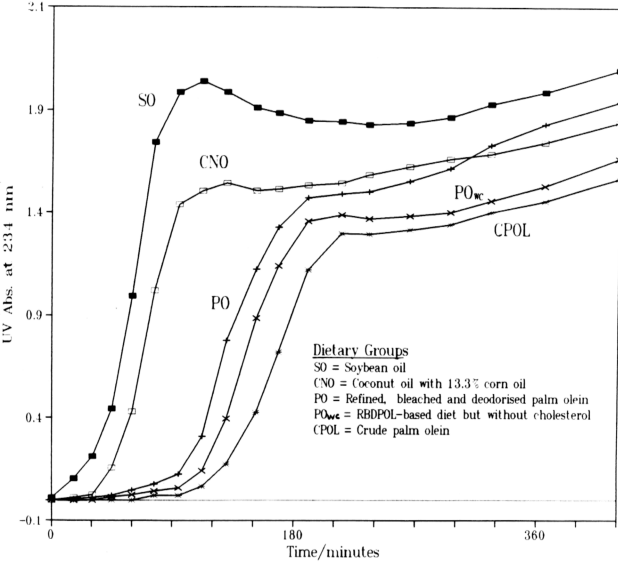


Figure 2.47 Cu^{2+}-catalysed oxidations of LDL isolated from rabbits fed with different dietary fats

LDL from different dietary fats are shown in Fig. 2.47. Data on lag times, lag phase and propagation gradients, etc. are given in Table 2.64. The lag time (and also the lag phase gradient) which precedes the onset of rapid lipid autoxidation is important and is an indicator of the oxidative susceptibility of the LDL.

Table 2.64 Effect of Different Dietary Fats on the Oxidative Susceptibility of LDL

Dietary group	Lag time (min.)	Lag phase gradient ($x10^{-4}$/min.)	Propagation phase gradient ($x10^{-3}$/min.)	Maximum absorbance at 234 nm	Total PUFA in LDL (%)	Plasma vitamin E (μg/ml)
CNO	56±6	20.0±9.0	15±4	1.52±0.35	12.7±2.4	17.7±4.3
SO	54±15	18.0±7.0	45±4	2.18±0.01	22.4±4.7	12.2±5.1
CPOL	103±25	2.3±1.3	15±6	1.19±0.02	8.4±2.1	25.4±10.8
PO	111±23	2.9±0.9	19±4	1.35±0.08	13.3±2.7	25.2±5.6
PX	104±9	4.0±0.4	17±3	1.21±0.20	9.0±1.0	18.0±9.5
CE	96±6	2.1±0.5	8±1	1.02±0.12	7.9±1.2	28.1±1.9
CX	63±29	2.7±0.9	9±4	1.26±0.15	9.9±1.5	21.7±5.9
PO_{WC}	110±8	3.5±0.5	19±5	1.26±0.17	10.7±0.4	3.8±1.6

* Values are mean ± standard deviations; n=6 for each dietary group. PUFA = polyunsaturated fatty acids. Abbreviations for various dietary groups: CNO = coconut oil with 13.3% corn oil; SO = soybean oil; CPOL = crude palm olein; PO = refined, bleached and deodorised palm olein; PX = PO without palm vitamin E; CE = RBDPOL plus palm carotenes (2000 ppm); CX = RBDPOL plus palm carotenes (2000 ppm) without added palm vitamin E; PO_{wc} = RBDPOL-based diet but without cholesterol.

The results shown in Fig. 2.47 and Table 2.64 provide comparisons of the lag times and lag phase gradients for the oxidations of LDL resulting from the three common dietary

fats and four different palm-based products. No significant differences were observed on the data of lag times during the oxidation of LDL from diets of the various palm oleins. Lag times from these groups are almost two times longer than those of the LDL isolated from CNO- and SO-fed rabbits. The LDL from the CX group of rabbits fed RBDPOL plus palm carotenes but without added palm vitamin E tend to have shorter lag times, which could be due to lower vitamin-E contents as compared to other palm olein-diets. However, the differences were not significant as a high variation was observed among these LDL samples perhaps reflecting the instability of carotenes or their metabolites present.

Both the LDL samples from CNO- and SO-fed rabbits have relatively higher (almost 6 times) lag phase gradients than those of the LDL samples from palm olein-fed rabbits, SO-fed rabbits also showed higher propagation phase gradients (2-5 times) and maximum absorbance values (almost 2 times) as compared to other dietary groups. Not much differences were observed among the palm olein-fed rabbits in terms of lag phase gradients and maximum absorbance values. Lower propagation gradients (2 times) were shown by the CE- and CX-fed (palm olein supplemented with palm carotene-diets) groups as compared to other palm olein groups, this could be due to some protective effect by the carotenes present in these groups. The CPOL-diet (containing about 500 ppm of palm carotenes in the oil) also tends to show a slightly lower propagation phase gradient as compared to those from other palm olein diet without carotenes. However, the differences were not pronounced as the carotene concentration in CPOL was not as high as in other carotene-supplemented groups.

Plasma vitamin-E contents (α-tocopherol) in the palm olein groups were relatively high as compared to other dietary fat groups, except for the PO_{wc} group fed palm olein without cholesterol. Slightly lower levels of plasma vitamin-E were shown by the PX- and CX-dietary groups (fed palm olein without palm vitamin-E) as compared to other palm olein groups. However, these levels were still considerably high as the diets contained less than 10 ppm of vitamin E (α-tocopherol); these results indicate the accumulative effect for α-tocopherol especially in cholesterol-supplemented diets, as the total plasma vitamin-E in

PO_{wc}-fed rabbits was comparatively low (6-9 times lower). In fact, a study[277] has shown that the vitamin E content increases with increasing cholesterol in diets, an α-tocopherol selective protein in the liver may have a determining role in LDL formation.

No significant differences were observed in the total percentage of LDL polyunsaturated fatty acids (PUFA) among the palm olein groups, as all the rabbits consumed the same dietary fat; similarly, the maximum absorbance values in the LDL oxidations were comparable since the absorbance is directly related to the hydroperoxidation of PUFA. Higher absorbance maxima were shown by samples from the SO-fed rabbits, as the percentage of PUFA is almost two times higher than the other dietary fat groups.

The lag times and lag phase gradients were reflective of the dietary fats, with the LDL samples from palm-olein diets having the highest oxidative stability and all the palm olein-fed rabbits showing longer lag times (than CNO and SO groups). During the initial stage of oxidation or lag phase, a slow increase in absorption at 234 nm is indicative of the inhibition oxidation by antioxidants (Fig. 2.47). As indicated by the gradients of the lag phase, the rates of oxidation of LDL derived from CNO and SO groups were found to be about 6-fold faster than LDL from palm olein groups. However, during the propagation phase, the oxidation rate for the LDL from the SO group was about 3-fold higher compared to the other groups. No significant differences were observed in the lag time and lag phase gradients among the various palm olein diets, but there was a tendency for lower propagation phase gradients in groups (particularly CE and CX) fed with palm carotene supplemented diets as compared to other palm olein groups. This result shows that carotenes and their metabolites could have some protective effect against the LDL oxidation especially during the propagation phase. While the lag phase is due to inhibition by tocopherol, the propagation phase is attributed to the breakdown of peroxides and further uninhibited (by tocopherol) oxidation of polyunsaturated fatty acids; an involvement of poorer antioxidants such as carotenes or their metabolites is indicated.

Previous work[278] has indicated that increasing levels of antioxidants (especially vitamin E and ubiquinols) can effectively prolong lag times. Oxidative stability of LDL will be dependent on vitamin E, which is the major antioxidant in LDL, and this is demonstrated in the present data. The coconut-oil diet provides low levels of vitamin E, whereas soybean oil with a high PUFA content places a greater demand on the antioxidants as a result of which a lower amount of vitamin E was observed in the LDL particles. Whereas levels of antioxidants may be an important factor in oxidative stability, it is noted that the degree of polyunsaturation of the LDL is also important. The stability of the LDL derived from PO_{wc}-fed rabbits, despite a low vitamin-E content, is indicative of this fact. However, there may be other factors which have not been investigated. A low-cholesterol diet allows the LDL to be continually removed by the liver LDL-receptors. A high-cholesterol diet causes various degrees of the shut-down of the LDL-receptors resulting in the accumulation of LDL. It is possible that such LDL may have certain degrees of peroxidation. There may be other reasons, e.g. LDL particles may be of different sizes and compositions for different diets. There may be other antioxidant components (e.g. ubiquinols)[279] in palm oil, of which tocotrienols may not be included since these are minimal in LDL as they are not, in contrast to α-tocopherol, incorporated by the liver into LDL.[280] Not much is known of the oxidative protection provided by tocotrienols, but in view of the possibility of their higher *in vivo* activity,[281,282] significant protection of LDL or more likely its preformed intermediates [intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL) and chylomicrons] may be possible. Antioxidant α-tocopherol levels are generally low in LDL and curiously increases with cholesterol levels,[277] possibly due to control by an α-tocopherol binding protein in the liver LDL; vitamin E levels as present in LDL are probably insufficient to effectively protect against oxidation of all the PUFAs.[244] The results from rabbits fed CPOL (which is unrefined liquid palm oil containing carotenes) and palm carotene enriched diets (CE and CX) are also noteworthy, in that although the oils are rich in carotenes in addition to vitamin E (in contrast to PO), this did not confer increased stability to the LDL in terms of

the length of the lag phase observed. Although many studies have focused on the *in vitro* oxidation of LDL, not much is known on the actual chemistry of oxidation of lipoproteins under *in vivo* conditions. Recent work[283] has indicated that without vitamin C, vitamin E is not only insufficient to protect LDL against oxidation but may even be pro-oxidant under certain conditions. Beneficial effects of vitamin E can be realised if the radicals formed are regenerated by ascorbic acid reduction. Other minor antioxidants, notably ubiquinols, are also effective antioxidants in aqueous lipid emulsions. Furthermore, there is considerable heterogeneity among lipoproteins and recent evidence indicates that high density forms of LDL and Lp(a) may be more atherogenic.[284] The observed behaviour between the oxidative stability of LDL and percentage of PUFAs is indicative of preformed or endogenous peroxides in the LDL. It is likely that oxidative damage may already have occurred in its earlier intermediates (e.g. VLDL and IDL) to give rise to the poorer oxidative stability of LDL from coconut oil diet. The shorter lag times and higher lag phase gradients for LDL from coconut oil and soybean oil diets are the result of facile oxidation from a combination of low vitamin E, PUFA levels and possibly endogenous peroxides.

The propagation phase for the oxidation of LDL from the SO-diet is much more rapid and is attributed to the direct effect of polyunsaturation. The CNO-diet is essentially similar to palm olein diets in terms of the degree of polyunsaturation (if the medium and short chain fatty acids are neglected) and show essentially similar propagation phase gradients. Monounsaturated oleic acid, like saturated fatty acids, also confers oxidative resistance to LDL.[285] A lower oxidation rate during the propagation phase is shown by LDL from the palm carotene diets, especially in the carotene-enriched diets (CE and CX), which could be the effect of the antioxidant properties of the carotenes present in the LDL. These observations show that although the palm carotene was unable to increase the lag times of LDL oxidation, it was able to slow down the LDL oxidation during the propagation phase due to its poorer antioxidant activity. However, this has been poorly understood as carotenes are not peroxyl radical chain-breaking antioxidants, but rather they act as radical 'sinks' providing less reactive

addition products which would give rise again to hydroperoxy radicals in the presence of oxygen . Among the range of dietary oils used, the tendency for rapid and extensive oxidation of LDL from the SO-diet would apparently be an undesirable feature since LDL has been demonstrated to be taken up by macrophages only after extensive oxidation and in subsequent stages, progress to atherosclerosis.

Although coronary heart disease and atherosclerosis have generally been taken to be linked to excessive consumption of diets containing high levels of cholesterol and some saturated fats (e.g. myristic acid), it is noted that oxidative resistance of LDL is likely to be an equally important factor for consideration in dietary recommendations. For example, extensive epidemiological studies[286] have shown that dietary antioxidants found in the plasma are inferred to be protective of cardiovascular diseases. In many countries, when highly atherogenic diets are not correlated with high mortality from cardiovascular diseases, there is usually a high consumption of vegetables and fruits which apparently provides antioxidants. The "French paradox", an apparent compatibility of a high fat diet with low incidence of coronary atherosclerosis has been attributed to antioxidants from fruits and vegetables, and to phenolic antioxidants from red wine[287] or even to the presence of natural salicylates. It may also be noted that hydrogenation (i.e. possible introduction of *trans* fatty acids) is not allowed in France. Willet's finding[288] of the detrimental effects of *trans* fatty acids in American diets indicates the operation of multi-factorial effects. It is apparent that in view of the importance of maintaining oxidative stability of LDL, provision of dietary antioxidants is essential while a high monounsaturated fat diet with moderate amounts of saturates and polyunsaturates would be desirable.