Practical Guide to Establishing Palm Carotenoids Profiles by HPLC with Three Dimensional Diode Array Detector

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INTRODUCTION

Crude palm oil is one of the richest plant source of carotenoids in terms of retinol (pro-vitamin A) equivalents. Carotenoids are naturally occurring C-40 plant pigments. Their conjugated polyenic chromophore is responsible for the characteristic light absorbing properties. Hydrocarbon and oxygenated carotenoids are termed carotene and xanthophyll respectively. Carotene, in particular α-carotene and β-carotene have long been associated with pro-vitamin A activity. Other minor carotene such as γ-carotene and β-zeacarotene also possess vitamin A activity (Choo, 1995).

Aside from pro-vitamin A activity, carotene such as β-carotene, α-carotene and lycopene are effective antioxidant and singlet oxygen quenchers (Dimascio *et al.,* 1989). Serbinova *et al.* (1992) found that the order of inhibition by palm-based carotene in *in vitro* lipid peroxidation was α-carotene > lycopene > β-carotene. There are considerable evidence from epidemiological and animal data linking β-carotene and other carotene to decrease risk of some cancers (Ziegler, 1989; Ziegler *et al.,* 1996a,b). Another interesting finding is that α-carotene is 10 times more potent as an anti-cancer agent than β-carotene (Murakoshi *et al.,* 1989; 1992). Manorama *et al.* (1993) showed that red palm oil (RedPO) was more effective in preventing chemical carcinogenesis in rats in comparison to refined bleached deodorized palm olein (RBDPOo), and attributed this effect to the carotenoids present in the former. The *in vivo* and *in vitro* chemical carcinogenesis studies by Tan and Chu (1991) in the rat hepatic cytochrome-P450-mediated monoxygenase system showed the order of anti-tumour reactivity was palm oil (with carotenoids) > β-carotene > cantha-xanthin > palm oil (without carotenoids).

The various physiological potential of palm carotenoids warrants identification of the carotenoids present in palm oil. The quantification of carotene content by the PORIM Test Method using UV-Visible spectrophotometer, sums all the carotene as β-carotene. The use of high performance liquid chromatography (HPLC) is very useful in the qualitative identification of palm carotenoids because it allows the display of the various carotenes in the chromatograms. This paper describes a step by step guide to analyse crude palm oil carotenoids using a HPLC-three dimensional photodiode array detector (PDA).

PREPARATION OF SAMPLES

The oil sample was saponified according to PORIM Test Method, but with slight changes. About 5 g of oil and 5 ml of 50% ethanolic KOH were heated at 50°C in a water bath

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under a stream of nitrogen for 30 min. The saponified sample was then cooled to room temperature and extracted with 50 ml portions of petroleum ether until the supernatant became colourless. The pooled petroleum ether extracts were washed four times with 50 ml portions of distilled water and dried over anhydrous sodium sulphate. Then the extract was dried in a rotary evaporator at 50°C. The dried extract was dissolved in a known volume of mobile phase containing an antioxidant before it was injected into the HPLC.

HPLC CONDITIONS

The carotene profiles analyses was performed using a HPLC with Waters 990 series PDA. The PDA was set at spectral range of 222-800 nm. The isocratic non-aqueous separation system developed by Yap *et al.* (1991) was used. The analyses used a Metaphase reverse phase C18 column (4.6 mm i.d. x 25 cm, stainless steel, 5 μm spherical particles), and the solvent system was acetonitrile:dichloromethane (89:11 v/v) at a flow rate of 1.0 ml min^{-1}.

ESTABLISHING CAROTENE PROFILE USING THREE DIMENSIONAL PDA

A concentrated extract was prepared by saponification of the crude palm oil prior injection as described. The extract was dissolved in small amount of mobile phase and injected into the HPLC-PDA system. The wavelength at 286 was chosen to monitor the profile. After analysis, the different chromatograms at the wavelength maxima of the various carotene were retrieved from the scanned data file. *Figure 1* shows the HPLC chromatogram at 444 nm. *Figure 2* shows the three dimensional (3D) chromatogram at 444 nm, where the UV spectra and chromatogram can be monitored simultaneously (*Figure 2*). The 3D chromatogram here shows that the peaks designated for α-carotene and β-carotene is not properly resolved using the chromatographic conditions. There are several spectra hidden behind the α-carotene and β-carotene peaks respectively, which may be the isomers of these carotenes.

Table 1 lists the chromatographic retention time and absorbance maxima of the carotenes obtained from the PDA detector. Identification was done by comparison with the published spectral data.

Peak area normalization was used in this case for quantification. *Table 2* shows the crude palm oil carotene profiles expressed as percentage composition. The peak area is based on the peak from the carotene absorbance maxima. Quantification using external or internal standard calibration is

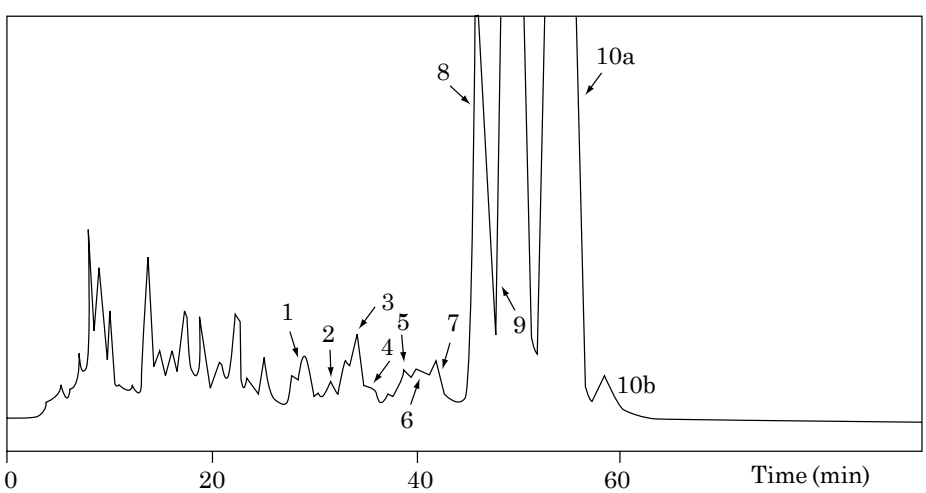

Figure 1. Crude palm oil carotenoids scanned at 444 nm. The peak numbers here are the same as those in Table 1.
Note: phytoene and phytofluene are not shown in this chromatogram because they are detected only at 286 and 347 nm respectively.

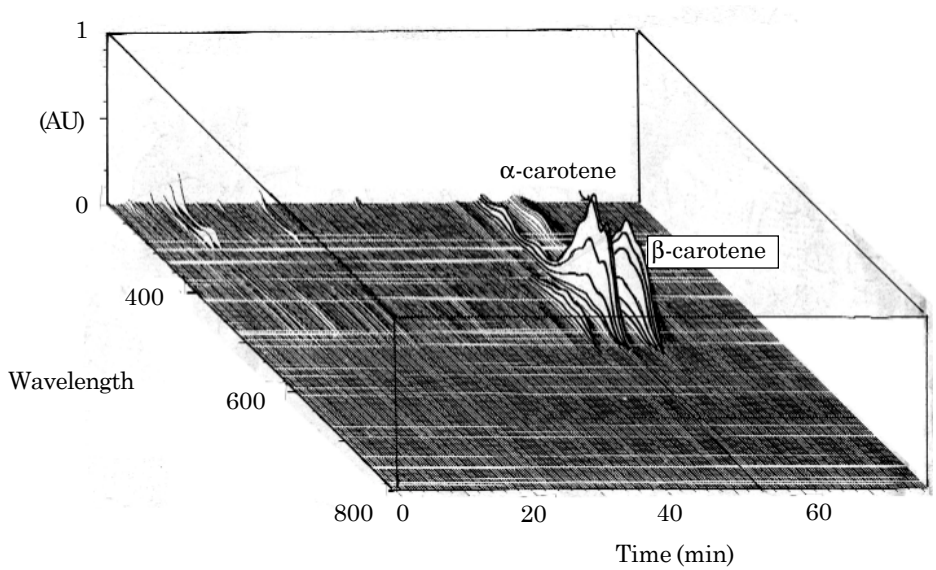

Figure 2. A 3D chromatogram of palm carotenoids.

TABLE 1. CRUDE PALM OIL CAROTENOIDS ORDER OF ELUTION AND ABSORBANCE MAXIMA

Peak	Retention time	Carotenes	Absorbance maxima (nm)						
			This study				Davies, 1976		
1	29.1	Lycopene	444	468	500		448	473	504
2	31.5	α-Zeacarotene	400	423	448		398	421	449
3	34.0	β-Zeacarotene	400	430	449		407	427	454
4	35.0	Neurosporene	415	440	462		416	440	470
5	38.8	δ-Carotene	425	459	487		428	458	490
6	40.0	γ-Carotene	436	461	488		437	462	492
7	41.8	ξ-Carotene	381	402	426		380	401	426
8	46.2	*cis*-α-Carotene	333	415	442	468		-	
9	49.2	α-Carotene	420	445	474		420	442	472
10a	53.2	β-Carotene	430	452	477		425	450	477
10b	58.5	*cis*-β-Carotene	330	420	440	480		-	
11	64.3	Phytoene	275	287	298		276	286	297
12	57.8	Phytofluene	332	345	362		331	347	366

TABLE 2. CAROTENE COMPOSITION (%) BASED ON PEAK AREA

Carotenes	%
Lycopene	0.9
α-Zeacarotene	0.2
β-Zeacarotene	1.1
Neurosporene	0.3
δ-Carotene	1.2
γ-Carotene	1.2
ξ-Carotene	0.2
cis-α-Carotene	6.2
α-Carotene	39
β-Carotene	48
cis-β-Carotene	0.6
Phytoene	0.03
Phytofluene	0.2

not practical for analysis using the HPLC-PDA method. This is because the pure carotene standard may degrade during the long separation time of 65 min. The insolubility of the carotene standard in the mobile phase may also affect quantification.

VARIABLE WAVELENGTH UV-VISIBLE DETECTOR (VWUV-Vis) DETECTOR *vs.* PDA

Yap *et al.* (1991) were the first researchers to report on the method to identify palm carotenoids using HPLC with a VW UV-Vis detector. However, there are certain limita-

tions to this method. The VW UV-Vis detector only allows any wavelength to be monitored sequentially. Palm carotenoids absorb at different wavelength maxima, *i.e.* phytoene (286), phytofluene (347), lycopene (472), α-zeacarotene (421), β-zeacarotene (427), neurosporene (440), δ-carotene (456), γ-carotene (462), ξ-carotene (400), *cis*-α-carotene (444), α-carotene (444) and β-carotene (453). Up to 11 time injections is needed during analysis of a sample. The analysis time is usually long at about 80 min. Lycopene, α-carotene and β-carotene were identified using the Sigma standards. For the other minor palm carotene where standards are unavailable, such as α-zeacarotene, β-zeacarotene, phytoene, phytofluene, δ-carotene, γ-carotene and ξ-carotene, they were collected individually. Their spectra was then recorded on a UV-Visible spectrophotometer. This method is cumbersome and not practical for routine analysis of palm carotenoids. Besides, the peaks have to be well resolved prior collection.

The PDA offers an easier and quicker way to identify carotenoids qualitatively. This is because, unlike the VW-UV Vis detector, the sample needs to be injected once only at any chosen wavelength. The chromatograms at multiple wavelengths can be retrieved from the data files after analysis. The PDA also allows all the carotenes to be identified by their elution sequence and absorption spectra. The PDA is able to accumulate spectral data rapidly without disturbing the chromatographic process. After analysis, the spectrum of the individual carotene peak is extracted from the spectral data and compared with the spectra of carotenes in literature. Identification of carotenes can be achieved tentatively based on their characteristic spectral maxima and shape. However, to identify the structure absolutely, other methods such as MS , NMR and IR are required.

CONCLUSION

The analyses of palm carotenoids by three dimensional HPLC-PDA was found to be more convenient for routine analyses of carotene profiles compared to a VW-UV Vis detector. No multiple injections of a sample were needed and this saved time. Furthermore, the 3D chromatogram enables the unresolved peaks to be detected. The diode array detector accumulates the spectra which can be retrieved after the analysis making identification easier. The minor carotenes can be tentatively identified even though no commercial standards are available. Therefore, the HPLC-PDA can be used as a routine method to qualitatively identify palm carotenoids.

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