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Isolation and Identification of Individual Palm Carotenes using Supercritical Fluid Chromatography

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Abstract A method for the isolation of individual carotenes in palm oil using supercritical fluid chromatography (SFC) is reported. While the isolation of individual carotenes in palm oil has been carried out in the past using high performance liquid chromatography (HPLC), SFC nevertheless offers more powerful and efficient separating properties as well as being a clean, hygienic and non-toxic method. In the past, the identification of individual carotenes was carried out based on the wavelength where they absorb maximum UV (λ_{max}). With the new SFC method developed, it was found that the data obtained in the past for identification of individual carotenes dissolved in hexane solution can not be applied for their identifications in a supercritical fluid environment. Thus, the study on the behaviour of λ_{max} of the individual carotenes in supercritical carbon dioxide has been carried out and reported. The data collected on the λ_{max} of the individual carotenes in supercritical carbon dioxide environment will serve a useful purpose in their analyses and identification in the future.

Abstrak Satu kaedah menggunakan kromatografi cecair superkritikal (SFC) bagi pengasingan karoten individu di dalam minyak sawit dilaporkan. Walaupun pengasingan karoten individu di dalam minyak sawit menggunakan kromatografi cecair prestasi tinggi (HPLC) telah dijalankan pada masa lalu, SFC menawarkan sifat-sifat pengasingan yang lebih baik dan efisien di samping merupakan kaedah yang bersih, selamat dan tidak toksik. Pada masa lalu, pengenalpastian identiti karoten individu dilakukan dengan mengenalpasti jarak gelombang di mana mereka menyerap sinar ultra ungu secara maksima (λ_{max}). Dengan penemuan kaedah SFC yang baru ini, didapati bahawa data λ_{max} yang telah dikumpul pada masa lalu untuk pengenalpastian identiti karoten individu yang terlarut dalam heksana tidak boleh digunakan untuk pengenalpastian di dalam keadaan cecair superkritikal. Oleh yang demikian, kajian mengenai kelakuan λ_{max} karoten di dalam superkritikal karbon dioksida telah dijalan dan dilaporkan. Data λ_{max} karoten individu di dalam keadaan superkritikal karbon dioksida akan berguna di dalam analsis dan pengenalpastian identity karoten pada masa akan dating.

(Carotenes, palm oil, supercritical fluid chromatography, λ_{max})

INTRODUCTION

The emergence of supercritical fluid chromatography (SFC) in separation science has grown rapidly in recent years. This is due to the fact that separation using a substance at its supercritical state is advantageous as it possesses dual behavior, the permeability of a gas and viscosity of a liquid [1, 2, 3, 4, 5, 6]. In combination, this offers the supercritical fluid a powerful and efficient separating property. Although GC and HPLC are good separating tools in their own ways, the SFC is a more powerful technique in comparison. In addition, it offers a clean, hygienic and healthy working environment which is equally as important as the results of an analysis. While SFC has been applied to the separation of α- and β-carotene [7,

8], much has yet to be studied on the separation of these compounds when in mixture with other structurally similar compounds, in other words, other members of the carotenes' family. In this study, palm oil carotenes were separated using SFC with carbon dioxide (SC-CO_2) as the carrier phase.

Palm oil is unique as it is the richest source of natural carotenes amounted 500 – 700ppm in crude palm oil (CPO) [9, 10, 11, 12]. The orangey color of the oil is attributed to β-carotene which is the major carotene found in palm oil followed by α-carotene [9, 10, 11, 12]. These two types of carotenes have been known to possess nutritional benefits such as being pro-vitamin A, antioxidant, anti-cancer as well as preventing xerophtalmia [13, 14, 15]. Lycopene, another powerful anti-cancer agent is also found in palm oil [16]. Table 1 depicts the composition of individual carotenes found to be present in palm oil.

While the identification of individual carotenes was carried out in the past based on wavelength where they absorbed maximum UV (Table 2) [17], it was observed that this method cannot be readily adopted in the SFC. This is due to the fact that λ_{max} values changed with different polarity of the dissolving solvents. In other words, the λ_{max} of the carotenes in hexane are not the same as in supercritical fluid. In the course of identifying the individual palm carotenes separated by SFC, different percentage of modifier; in this case, ethanol was added.

In view of the SFC fast becoming a favourable analytical tool and its potential in replacing other analytical methods such as the HPLC for the analyses of carotenes, the λ_{max} of the carotenes in SC-CO_2 environment needs to be identified. This set of data will serve to be the tool for rapid identification of carotenes in just the way their λ_{max} in non-polar solution has done before.

Table 1. Composition of Individual Carotenes in Crude Palm Oil

CAROTENES	CPO (%)
Phytoene	1.27
Phytofluene	0.06
β-Carotene	56.02
α-Carotene	35.06
cis-α-Carotene	2.49
ξ-Carotene	0.69
γ-Carotene	0.33
δ-Carotene	0.83
Neurosporene	0.29
β-Zeacarotene	0.74
α-Zeacarotene	0.23
Lycopene	1.30

Table 2. λ_{max} of Palm Carotenes in Hexane

CAROTENE	Yap, 1991			Tay, 2000		
Phytoene	276	286	297	276	287	299
Phytofluene	331	347	366	330	343	360
β-Carotene	426	429	477	430	444	480
α-Carotene	420	440	471	425	444	475
Lycopene	444	470	500	446	470	503

Source: Yap *et al.*, 1991, [8]
Tay and Choo, 2000

MATERIALS AND METHODS

Materials

Crude palm oil was obtained from Malaysian Palm Oil Board Experimental Mill in Labu, Negri Sembilan, Malaysia. All solvents used were of analytical or chromatography grades purchased either from Merck or J.T. Baker. 99.995% CO_2 was obtained from Malaysian Oxygen, Malaysia.

Saponification

5g CPO was saponified in the dark under nitrogen atmosphere for 1 hour with 30ml absolute ethanol, 5ml 50% w/v KOH and 1g pyrogallol. The unsaponifiable matter was extracted using hexane until the upper layer becomes colorless. The extract was later washed with distilled water and ethanol (9 : 1) until the washing water becomes neutral. Solvents in the extracts were distilled and pumped to dryness.

The dried extract containing unsaponifiable matter was then dissolved in dichloromethane and injected into SFC.

Supercritical Fluid Chromatograph

A JASCO Model SUPER-200 SFC system with a UV-970 variable wavelength UV/VIS detector equipped with high pressure flow cells was used. Column used was Silica 20mm I.D. x 250mm length. Temperature and pressure were set at 50^0C and 180 kg/cm^2. Mobile phase was CO_2 and ethanol. CO_2 flowrate was 5.0 ml/min with varying percentage of ethanol (5%, 18% and 35%)

Spectroscopic Characterization

Individual carotenes isolated from SFC were dried under vacuum for 24 hours. Thereafter, it was dissolved in d-choloroform ($CDCl_3$) for ^{1}H and ^{13}C NMR analyses.

RESULTS AND DISCUSSION

A total of five types of carotenes from palm oil were successfully separated using supercritical fluid chromatography (SFC) (Figure 1). Under the conditions used, phytoene was the first of the palm carotenes to be eluted followed by phytofluene. A large peak separated the initial two carotenes from the rest; namely lycopene, β-carotene and α-carotene. This large peak is suspected to contain a mixture of residual triglycerides and esters as well as other carotenes that may be present. While the other four individual carotenes isolated were easily detected, the detection of lycopene proves to be a challenge due to its low concentration. Lycopene was easier to be detected from the UV-visible contour plot rather than the maximum plot of the chromatogram.

λ_{max} of carotenes in supercritical carbon dioxide (SC-CO_2) in the presence of varying amount of modifier is as depicted in Table 3. It is observed that the λ_{max} shifted to a longer wavelength with the increase of modifier's percentage. This result is true for other temperatures and pressures or in other words, the density of the SC-CO_2. Thus, it is concluded that in the supercritical fluid environment, the λ_{max} determining factor of carotenes is solely depended on the amount of modifier present. This shift of λ_{max} to longer wavelength can be explained through the bathochromic effect of the molecules whereby polar solutions give longer λ_{max} than non-polar solutions. With the increased percentage of entrainer, the polarity of the whole SC-CO_2 and entrainer mixture was thus increased. Thus, it is observed that with bigger percentage of entrainer, the λ_{max} shifted to longer wavelength.

It is also observed that all individual carotenes show greater λ_{max} values in hexane than in SC-CO_2 or its mixture with entrainer. However, as the percentage of entrainer increases, the λ_{max} values increases concomitantly. Thus, it is anticipated that at a certain percentage of entrainer, in this case, ethanol, the mixture of SC-CO_2 and entrainer will adopt a hexane-like behavior.

The palm carotenes isolated by the SFC were further characterized by ^{1}H NMR to confirm their identities. While λ_{max} alone is sufficient in identifying the carotenes in non-polar solutions such as hexane; the characterization of these compounds by ^{1}H NMR serves as added confirmation due to the complications that arise from varying λ_{max} in SC-CO_2. As such, the identities of the palm carotenes isolated by SFC were confirmed by ^{1}H NMR to be indeed phytoene, phytofluene, lycopene, α-carotene and β-carotene. As examples, the ^{1}H NMR of β-carotene; the most abundant carotene in palm oil and lycopene; a powerful anti-cancer agent are shown in Figures 2 and 3. The chemical shifts

assignment for these two compounds are as depicted in Tables 4 and 5.

The ^{1}H NMR further confirmed the identity of the individual carotenes. As such, the identity of the carotenes can be established through their λ_{max} without having to go through the hassle of further characterization by NMR or other spectroscopic technique in the future.

Although standard reference material (SRM) can be used to identify the content of certain samples, not all of these individual carotenes are available in the form of SRM. Moreover, the cost of SRM tends to be an important factor to consider, not to mention the hassle and time consumed in spiking the sample with the SRM every time an analyses is carried out. Thus, this set of data (Table 3) will serve as a valuable reference in future analyses.

Table 3. λ_{max} of Individual Palm Carotenes in Supercritical CO_2 with Different Percentage of Ethanol

INDIVIDUAL CAROTENE	PERCENTAGE OF ETHANOL		
	35%	18%	5%
Lycopene	436 462 490	432 458 488	428 452 480
Phytoene	275 287 297	273 285 295	273 285 295
β-Carotene	419 446 468	413 440 462	411 436 460
α-Carotene	413 438 462	409 434 460	405 430 456

Table 4. ^{1}H NMR Chemical Shifts of Lycopene

	δ/ppm
1, 1' – CH_3	1.5 – 1.7
5, 5' – CH_3	1.8
9, 9', 13, 13' – CH_3	1.95
3, 3', 4, 4' – CH_2	2.05 – 2.02
2, 2' – H	5.1
6, 6' – H	5.95
10, 10' – H	6.20 – 6.25
8, 8', 14, 14' – H	6.30 – 6.35
12, 12' – H	6.40
7, 7' – H	6.45 – 6.50
11, 11', 15, 15' - H	6.6

Table 5. ^{1}H NMR Chemical Shifts of β-carotene

	δ/ppm
2, 2' – CH_2	1.25
3, 3' – CH_2	1.45
5, 5' – CH_3	1.59
9, 9', 13, 13' – CH_3	1.95
4, 4' – CH_2	2.0
10, 10' – H	6.15 – 6.20
7, 7', 8, 8' – H	6.17
14, 14' – H	6.25
12, 12' – H	6.35
11, 11', 15, 15' – H	6.65

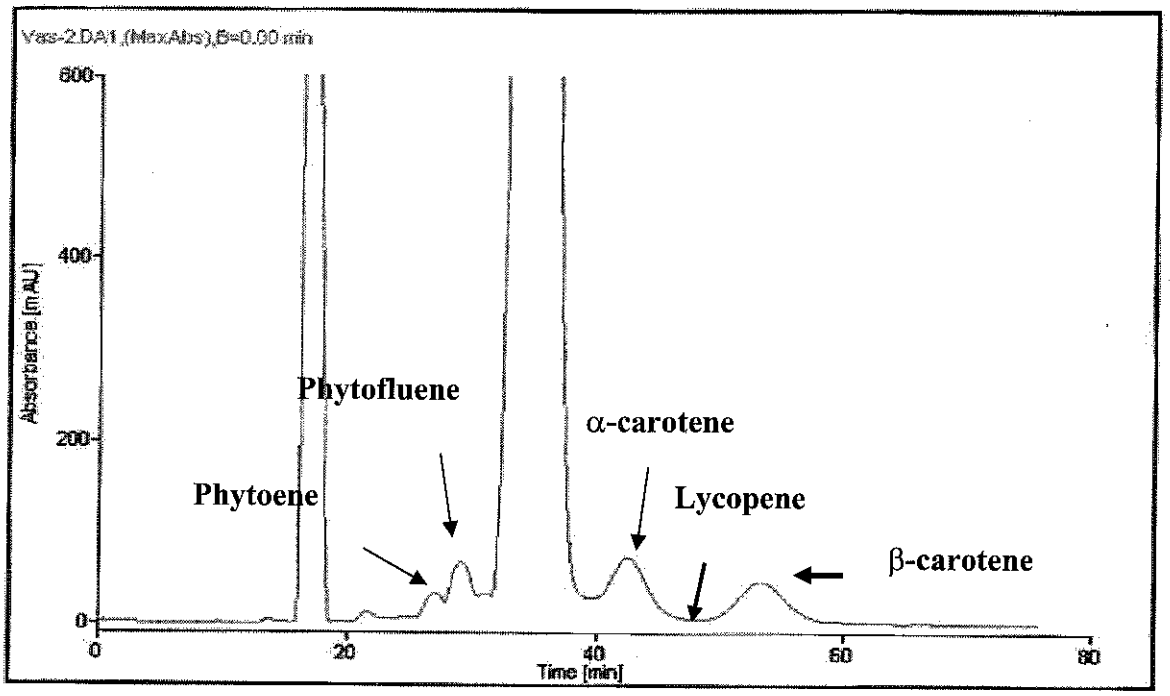

Figure 1. Separation of Palm Carotenes by SFC

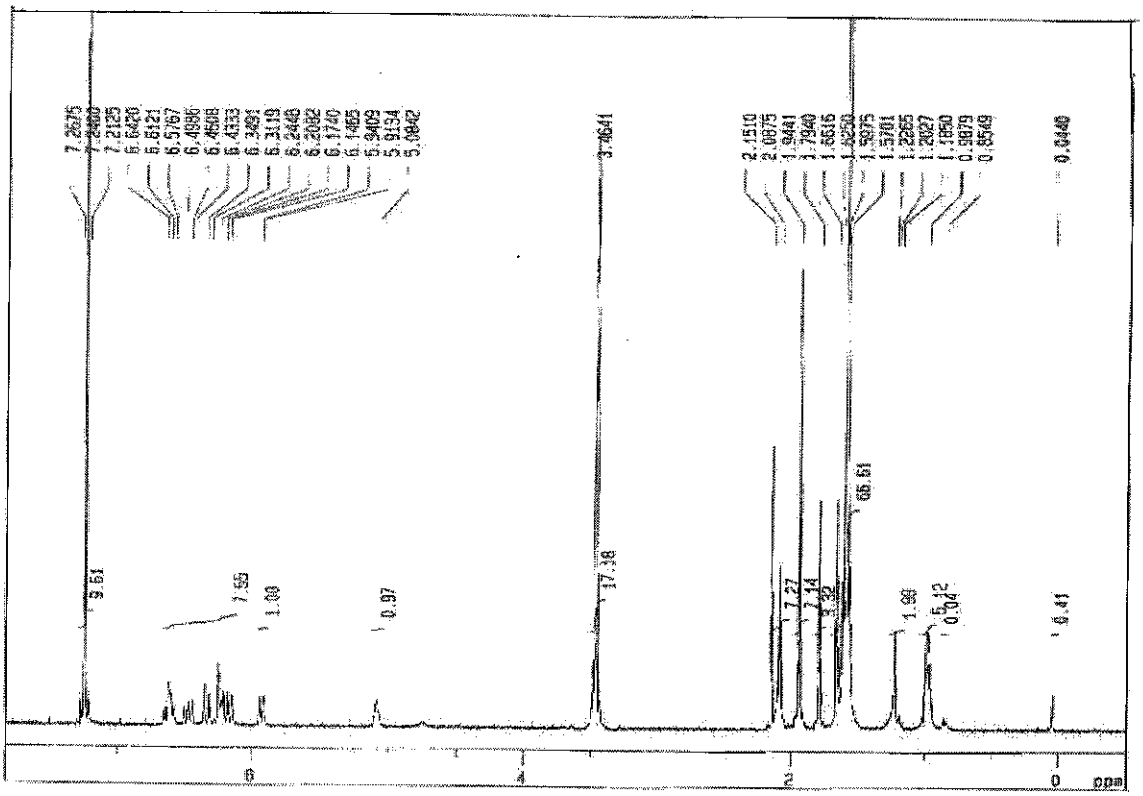

Figure 2. ^{1}H NMR of Lycopene

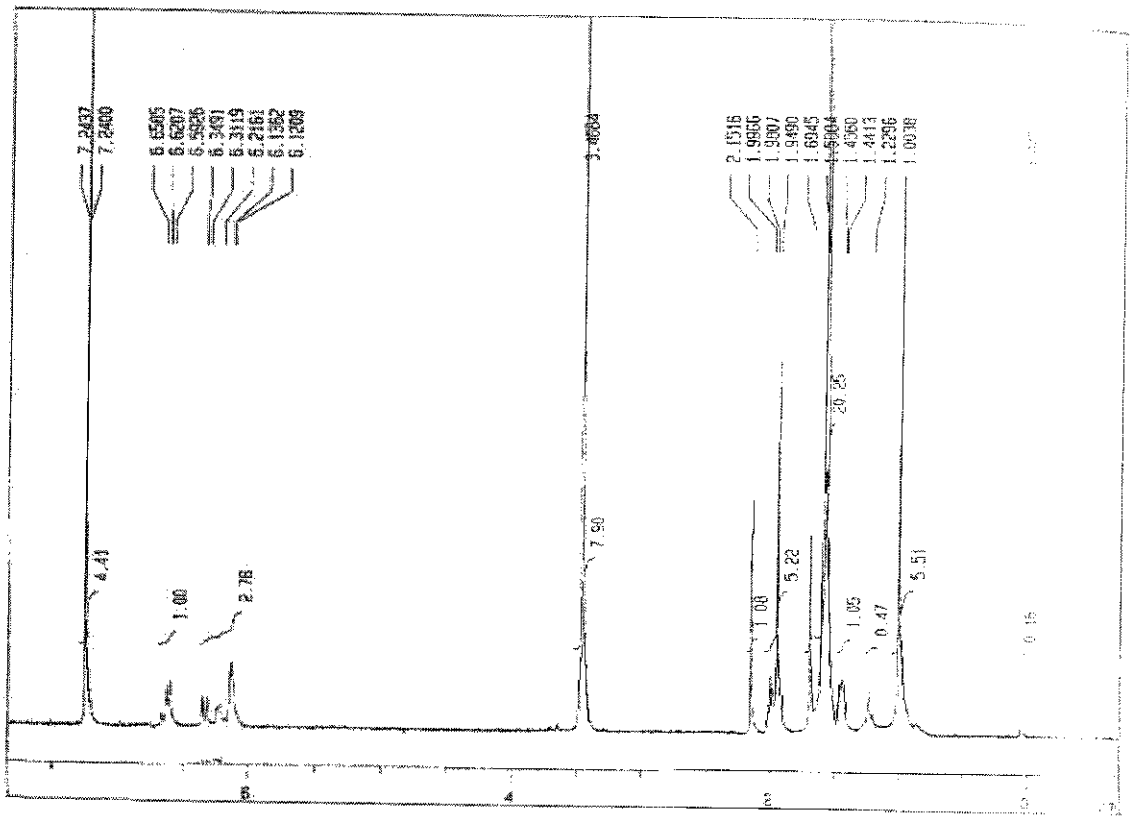

Figure 3. ^{1}H NMR of β-carotene

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