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Isolation of Palm Tocols Using Supercritical Fluid Chromatography

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Abstract

Crude palm oil contains 600 to 1000 ppm of tocols in the form of tocopherols and tocotrienols. These palm tocols have been isolated and analyzed in the past by various chromatographic techniques such as open column chromatography, high-performance liquid chromatography, as well as thin-layer chromatography. Supercritical fluid chromatography (SFC) has emerged as a more advanced chromatographic technique in recent years. The tocols present in palm oil are successfully isolated using SFC. Identification of these tocols is supported by various spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR, and mass spectrometry.

Introduction

Palm oil consists of mainly glycerides with approximately 1% minor components. Some of these minor components are the carotenes, tocols (better known as vitamin E), or sterols, as well as hydrocarbons such as squalene (1). Palm tocols consist of α-tocopherol (α-T), α-tocotrienol (α-T_3), γ-tocotrienol (γ-T_3), and δ-tocotrienol (δ-T_3). The molecular structures of these tocols are as depicted in Figure 1. The palm tocotrienols and α-tocopherol have been successfully analyzed in the past using chromatographic methods such as high-performance liquid chromatography (HPLC) (2–5). Detectors, such as UV–variable wavelength, as well as fluorescence detection, were used for the detection of palm tocols analyzed by HPLC. Analysis carried out by HPLC generally uses large amount of organic solvents. A more advanced method, supercritical fluid chromatography (SFC), has been developed for the isolation of palm tocols. SFC is a powerful tool because it combines the advantages of both HPLC and gas chromatography in terms of efficiency, sensitivity, and the capability to analyze thermally labile compounds.

Several attempts have been made for the separation of tocols; in particular, tocopherol using SFC. Synder et al. and Ibanez et al. demonstrated the separation of tocopherols by capillary SFC (6,7). Separation of tocopherol isomers by packed SFC has also been reported (8). The only successful attempt at separating the tocopherol and tocotrienol isomers in a mixture was reported by Choo et al. (9), in which α-T, α-T_3, γ-T_3, and

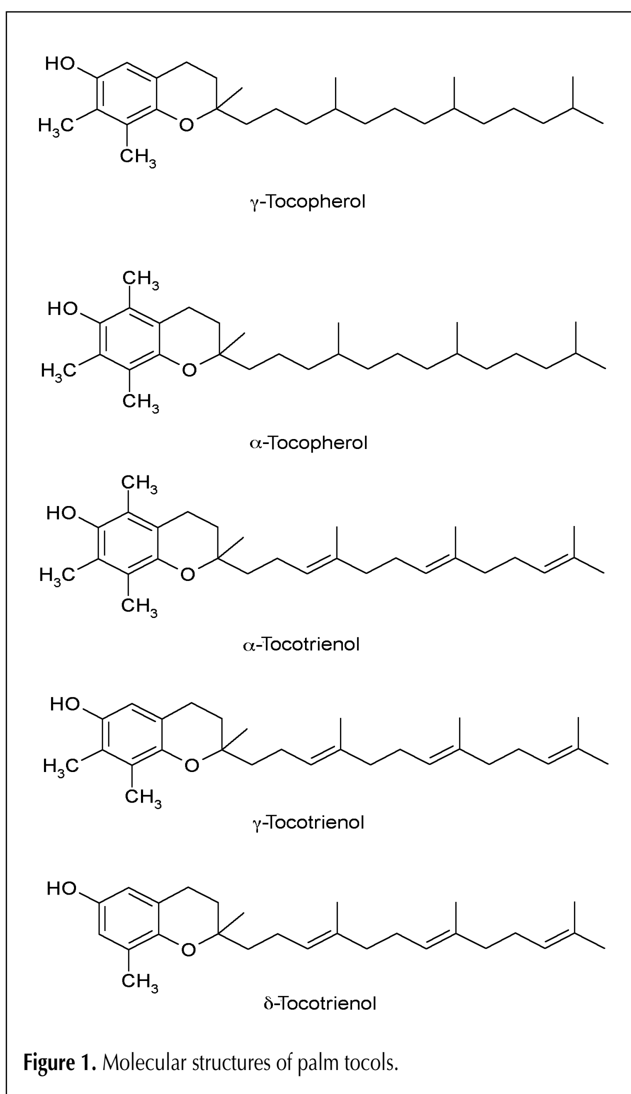

Figure 1. Molecular structures of palm tocols.

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δ-T_3 were separated. Researchers have always been faced with the problem of resolving the β/γ pair, but as of yet there has not been a successful attempt at separating the eight isomers of tocols using SFC. This study reports on the separation of all the eight tocols isomers using packed SFC.

The supercritical fluid used in this study is the supercritical carbon dioxide (SC-CO_2) (10,11). Being non-toxic, SC-CO_2 is the eluent of choice for the chromatography of food products such as the palm tocols (11–14). The presence of these palm tocols isomers were identified using various spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR , and also mass spectra (MS).

Experimental

Apparatus

A JASCO Model SUPER-200 SFC system with a UV-970 variable wavelength UV–vis detector equipped with high-pressure flow cells was used (JASCO, Easton, MD). The ^{1}H and ^{13}C NMR spectra were recorded on a JEOL GSX270 spectrometer (JEOL, Peabody, MA).

Materials

Crude palm oil (CPO) was obtained from MPOB Experimental Palm Oil Mill (Labu, Negri Sembilan). Solvents used for SFC were of chromatographic grade purchased from Merck (Darmstadt, Germany) and degassed with nitrogen. Tocopherols and tocotrienols standards were purchased from Calbiochem (San Diego, CA).

SFC

Columns used consist of a Lichrosorb silica 4.6- × 250-mm (Agilent Technologies, Palo Alto, CA) and Macherey-Nagel EC 250/4.6 Nucleosil 100-5OH diol column (Macherey-Nagel, Duren, Germany). SFC conditions using silica column included a temperature of 60°C, pressure of 180 kg/cm^2, and flow rate at 3.0 mL/min for CO_2 and 0.12 mL/min for ethanol.

Separating conditions for the diol column included a temperature 40°C, pressure of 190 kg/cm^2, and flow rate at 3.0 mL/min for CO_2 and 0.18 mL/min for methyl-tert-butyl-ether. Five grams of CPO was saponified in the dark under nitrogen atmosphere for 1 h with 30 mL absolute ethanol, 5 mL 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 was 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. Initial identifications and calibrations of the individual tocols were carried out using authentic standards.

Results and Discussion

Using silica as stationary phase, the tocols isomers were separated following the sequence: α-T, α-T_3, β-T/β-T_3/γ-T, γ-T_3, and δ-T_3. It was not possible to resolve β-T, β-T_3, and γ-T. Injection of a sample of palm oil resulted in peaks that correspond to all standards' peaks, except for δ-T. Thus, it was concluded that δ-T was not present in palm oil. Figure 2 shows the separation of palm tocols by SFC using a silica column. As γ-T and the β-tocols coelute, identity of the isomer in palm tocol that gave rise to the third peak remained a mystery until further characterization.

An improved method for the separation of tocols using SFC was achieved using a diol stationary phase. With this method, all the eight isomers of the tocols were separated. The sequence of the elution was α-T followed by α-T_3, β-T, δ-T, β-T_3, γ-T, and γ-T_3, and δ-T_3 was the strongest to be retained in the stationary phase as shown in Figure 3. With this improved method, all the possible isomers of the tocols can be separated and analyzed. An injection of palm oil samples showed that the tocols isomers present were α-T, α-T_3, γ-T, γ-T_3, and δ-T_3 (shown in Figure 4). The concentration of the tocols isomers in CPO were calibrated using authentic standards, and the results are depicted in Table I.

Five measurements of the standards of the tocols isomers have been carried out using the previously described SFC conditions using a diol stationary phase to estimate the method precision and repeatability. The relative standard deviations (RSDs) for all tocol isomers were less than 5%, which indicates good stability and repeatability of the method. The regression

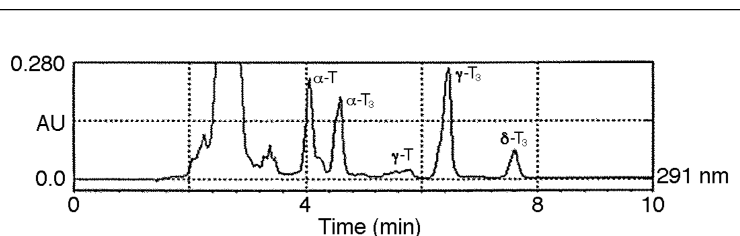

Figure 2. SFC profile of palm tocols. Tocols in CPO separated by SFC with Lichrosorb 4.6-mm i.d. × 250-mm length silica column at 180 kg/cm^2 and 60°C.

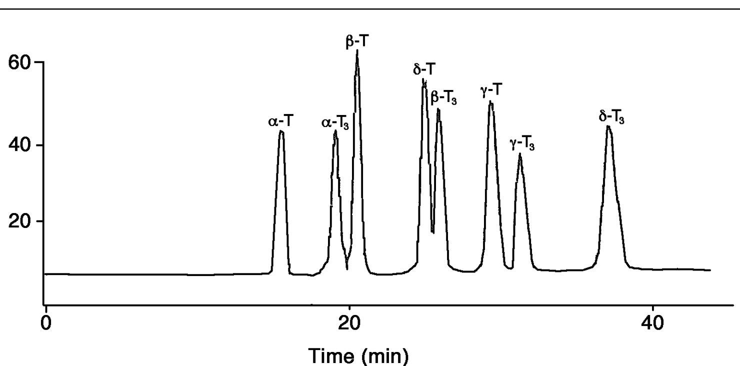

Figure 3. SFC separation of tocols standards using diol stationary phase. Tocols standards separated by SFC with Nucleosil 250/4.6 100-5OH column at 190 kg/cm^2 and 40°C.

equation and correlation coefficient between the content (*Y*-axis, ppm) and the peak area (*X*-axis, abs/ppm) of the standards calibrations were calculated for each of the tocol isomers. The results are depicted in Table II. It was found that each tocol isomers shows significant linearity. Also, the sensitivity of both SFC methods using silica and diol stationary phase were high enough to detect the presence of individual tocols in palm oil without a significant increase in the baseline noise.

Although the retention times of the palm tocols isomers are comparable with those of authentic standards, further characterizations were needed to support the identities of the individual tocols because some other compounds might have the same retention time as the tocols. Further identifications of these tocols were carried out and supported by various spectroscopic data such as MS, ^{1}H, and ^{13}C NMR.

The difference between a tocopherol and tocotrienol lies in their side chains. The side chain of tocopherol was saturated, and that of tocotrienol was three times unsaturated. Further difference in the structure of these tocols was the position in which they were protonated or methylated. The NMR showed

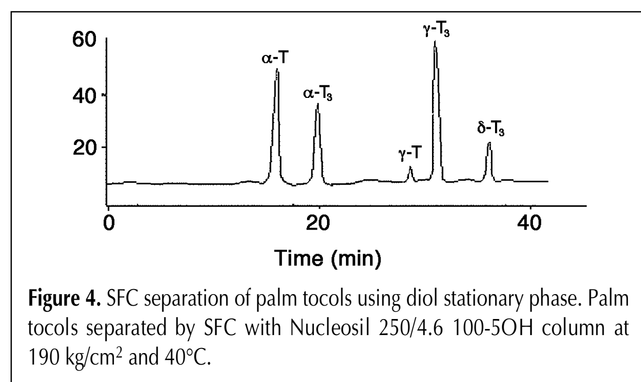

Figure 4. SFC separation of palm tocols using diol stationary phase. Palm tocols separated by SFC with Nucleosil 250/4.6 100-5OH column at 190 kg/cm^{2} and 40°C.

Table I. Concentrations of Individual Tocols in CPO

	Concentrations (ppm)
α-Tocopherol	360 ± 30
α-Tocotrienol	240 ± 20
γ-Tocopherol	30 ± 10
γ-Tocotrienol	440 ± 50
δ-Tocotrienol	90 ± 15
Total	1160 ± 100

Table II. Linear Correlation of Tocols Standards

Standard	Regression equation	Coefficient
α-T	$Y = (0.314 + 1.242)\ 10^{3}\ X$	0.9984
α-T_3	$Y = (0.228 + 1.301)\ 10^{3}\ X$	0.9976
β-T	$Y = (0.336 + 1.614)\ 10^{3}\ X$	0.9897
β-T_3	$Y = (0.288 + 2.500)\ 10^{3}\ X$	0.9899
γ-T	$Y = (0.367 + 2.122)\ 10^{3}\ X$	0.9912
γ-T_3	$Y = (0.198 + 1.322)\ 10^{3}\ X$	0.9964
δ-T	$Y = (0.312 + 2.131)\ 10^{3}\ X$	0.9957
δ-T_3	$Y = (0.247 + 1.648)\ 10^{3}\ X$	0.9947

the protons and carbons coupling of the molecule and their chemical shifts revealed the exact locations of the side chain and methyl groups that differentiated between the α, γ, and δ isomers of tocols. The ^{1}H and ^{13}C NMR chemical shifts data of palm tocols are depicted in Tables III and IV, respectively.

The presence of two protons signals at the aromatic region of δ 6.6–6.8 ppm denotes the presence of only one methyl group that was attached to the aromatic ring. This corresponded to the structure of the δ-isomer. On the other hand, presence of two methyl groups attached to the aromatic ring resulted in only one aromatic proton signal as in the β- and γ-isomer. The aromatic proton at C5 and C7 resonated at δ 6.2–6.4 and δ 6.7–6.8 ppm, respectively. Thus, by recognizing the aromatic C5 proton, the γ-isomer can be distinguished from the β-isomer. The α-isomer being trimethylated can be recognized by the absence of proton signal at the aromatic region.

The presence of olefinic proton signals at δ 4.6–5.1 ppm was used to distinguish between the tocopherols and tocotrienols. These signals are absent in tocopherols, which do not have any olefinic protons because of its saturated side chain. The tocotrienols were also recognized by the olefinic carbons that resonate at the low field region of δ 131–135 ppm in the ^{13}C NMR spectrum. The corresponding carbon of C4',8', and 12' of the tocopherol resonates at much higher field of δ 31–33 ppm.

The MS confirmed the identities of the isomers by way of the fragmentations of the molecule that gave rise to the peaks in the spectra. In addition, it provides information about the molecular weight of the tocols, which is an important feature in their characterizations. The *m/z* of the molecular ions of α-T, α-T_3, γ-T, γ-T_3, and δ-T_3 are 430, 424, 416, 410, and 396, respectively. The tocols fragmentate to give a stable tropylium ion. The *m/z* of tropylium ion of trimethylated tocols such as α-T and α-T_3 is 165. The *m/z* of the tropylium ion of γ-T and γ-T_3, which are dimethylated, was 151, but δ-T_3 being monomethylated has a tropylium ion of *m/z* 137.

The tocotrienols are distinguishable from the tocopherol by having an intense peak at *m/z* 69, which is a typical charac-

Table III. ^{1}H NMR Chemical Shifts (ppm) of Palm Tocols*

Proton	α-T	α-T_3	γ-T	γ-T_3	δ-T_3
5-H (aromatic)	–	–	6.41	6.29	6.72
7-H (aromatic)	–	–	–	–	6.79
3', 7', 11'-CH (olefinic)	–	5.06	–	5.06	4.63
6-OH (phenolic)	4.21	4.09	5.02	5.02	4.61
4-CH_2	2.53	2.55	2.54	2.59	–
5a-CH_3	2.08	2.04	–	–	–
7a-CH_3	2.00	2.04	2.04	2.18	–
8b-CH_3	2.00	2.04	2.04	2.18	2.21
1', 2', 5', 6', 9', 10'-CH_2	–	1.99	–	2.06	1.74
3-CH_2	1.75	1.75	1.74	1.89	1.52
13'-CH_3	–	1.69	–	1.75	1.17
4'a, 8'a, 12'a-CH_3	1.15	1.60	1.18	1.68	1.09
2a-CH_3	1.17	1.17	1.17	1.18	0.83

* In $CDCl_3$.

teristic of tocotrienols. This intense peak arises from the fragmentation of the tocotrienols isoprenoid side chain yielding a smaller ion of *m/z* 69. The MS data of palm tocols are shown in Table V.

Conclusion

SFC has been found to be a good tool to isolate palm tocols.

Table IV. ^{13}C NMR Chemical Shifts (ppm) of Palm Tocols*

Carbon	α-T	α-T_3	γ-T	γ-T_3	δ-T_3
6	145.8	144.68	137.50	146.25	147.70
8a	144.8	145.62	134.72	145.60	145.80
4'	32.79	135.05	31.94	135.07	135.12
8'	32.79	135.05	31.94	134.95	135.12
12'	27.97	131.34	30.55	131.23	132.00
4a	122.61	122.73	126.66	125.70	127.50
3'	37.48	31.68	37.50	124.40	124.39
7'	37.48	124.51	37.50	124.40	124.39
11	39.37	124.31	40.97	124.19	124.28
8	121.1	121.13	126.38	121.60	121.80
7	118.45	118.58	125.00	118.24	115.61
5	117.35	117.40	125.00	112.13	112.56
2	74.52	74.40	75.00	75.22	75.20
1'	39.83	39.79	40.27	39.70	39.69
5'	37.48	39.79	37.50	39.68	39.69
9'	37.48	39.79	37.50	39.68	39.69
3	31.56	31.68	33.33	31.41	31.35
10'	24.79	26.86	27.77	26.75	26.74
6'	24.43	26.70	26.38	26.59	26.58
13'	22.70	25.79	22.22	25.67	25.67
2a	23.79	23.82	24.30	23.99	24.02
2′	21.03	22.33	21.11	22.28	22.47
4	20.75	20.84	20.83	22.28	22.15
12'a	22.70	17.78	22.22	17.66	17.66
8'a	19.73	16.09	18.05	15.98	16.02
4'a	19.73	15.99	18.05	15.87	16.02
7a	12.18	12.30	15.97	11.88	–
8b	11.75	11.87	11.11	11.83	15.90
5a	11.25	11.36	–	–	–

* In $CDCl_3$.

Table V. Major Fragmentations of Palm Tocols

Tocols	Formula	Major Mass-spectrometric peaks (*m/z*)
α-T	$C_{29}H_{50}O_2$	430 (M^+), 205, 165, 164
α-T_3	$C_{29}H_{44}O_2$	424 (M^+), 205, 165, 164, 69
γ-T	$C_{28}H48O_2$	416 (M^+), 191,151, 81
γ-T_3	$C_{28}H42O_2$	410 (M^+), 191, 189, 151, 150, 81, 69
δ-T_3	$C_{27}H40O_2$	396 (M^+), 177, 137, 81, 69

The identities of the individual palm tocols, the α-T, α-T_3, γ-T, γ-T_3, and δ-T_3 can be confirmed by spectroscopic techniques such as ^1H and ^{13}C NMR, as well as through their MS.

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