

A Method of Chromatographic Isolation for Non-Glyceride Components

Field of the Invention

The invention relates to a method of chromatographic isolation of squalenes, carotenes, vitamin E (sometimes abbreviated as Vit. E) and sterols from crude palm oil, palm oils products and/or by-products, vegetable oils and/or the like non-glyceride components-comprising compounds.

Background of the Invention

Crude palm oil contains about 5% of "**non-glyceride components**" which include carotenoids, tocots (tocopherols and tocotrienols), sterols and squalene. The carotenoids, present at 500-700ppm and comprising mainly α and β carotenes, are important constituents with pro-vitamin A activity, possible antitumor formation properties, and other physiological activities. The tocots which are Vitamin E and also natural anti-oxidants, are present at approximately 600 to 1000 ppm in crude palm oil; the major component being the gamma-tocotrienol which has recently been found to have anti-cancer properties besides its known anti-oxidant activity. Tocotrienol has been found to lower blood cholesterol. The sterols consisting mainly of β -sitosterol, stigmasterol and campesterol, provide raw materials for steroid intermediates and drugs, β -Sitosterol also possesses hypocholesterolemic effect. Squalene is an important ingredient for cosmetics. It also shows beneficial physiological properties.

Several methods have been developed to extract these valuable compounds. In the case of the carotenoids, the known methods can be classified as follows:-

- (i) Extraction by saponification e.g. British Patent 567,682; U.S. Patent 2,460,796; U.S. Patent 2,440,029; U.S. Patent 2,572,467; U.S. Patent 2,652,433.
- (ii) Iodine method
- (iii) Urea process



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- (iv) Extraction using Fuller's earth or activated carbon, e.g. British Patent 691,924; British Patent 1,562,794; U.S. Patent 2,484,040.
- (v) Extraction by selective solvents e.g. U.S. Patent 2,432,021.
- (vi) Molecular Distillation.

In the saponification method (i) the oil is saponified to give soap, glycerol and a non-saponifiable fraction containing carotenes.

In the iodine method (ii), the iodine is added to a solution of palm oil in petroleum ether, an insoluble precipitate of carotene di-iodide is formed. The iodo-compound when treated with sodium thiosulphate however yields iso-carotene or dehydro-carotene which has no biological activity.

With the urea method (iii), the triglycerides are broken down to fatty acids and methyl esters which then form insoluble compounds with urea and thiourea, leaving the carotenoids in the remaining liquid.

Extraction of carotenes using adsorbents has been carried out using Fuller's earth and activated carbon (method i v) . However, the extraction of the carotenes from the earth gives oxidized or isomerised products of carotenes. Carotene is concentrated six times in the extract.

Extraction of carotenes by selective solvents (method v) has been carried out using propane or furfural. The carotene is concentrated (three times that of the original oil) in the furfural phase.

By method (vi) carotenes can also be obtained by molecular distillation [0.13- 0.013 Pa (10^3 - 10^4 mm Hg)]. Fractions collected at 230°C have a carotene content of about five times that of the original oil.

Choo Y M et al "Separation of Crude Palm Oil Components by Semi-Preparation

Supercritical Fluid Chromatography", Journal of the American Oil Chemists' Society, US, American Oil Chemists' Society Champaign, vol 73, no. 4, 1 April 1996 (1996-04-01) describes a process for separating crude palm oil components using supercritical fluid chromatography. The document describes a process which employs more than one column to separate the palm oil components.

Lee H. Chung B.H., and Park, Y.H. "Concentration of Tocopherols from Soybean Sludge by Supercritical Carbon Dioxide", Journal of the American Oil Chemists' Society, vol 68, no. 8, 1991, pages 571-573 describes a supercritical fluid extraction method to test the feasibility of tocopherol concentration from soybean sludge.

GB 2,218,989 describes a process for recovery of carotenoids, tocopherols, tocotrienols and sterols from esterified palm oil using conventional solvent extraction techniques.

To-date, there has not been any method which discloses the use of supercritical fluid in the adsorption/desorption chromatography isolation/separation of the non-glyceride components (i.e. carotenoids, tocopherols, sterols and squalene) from plant source such as CPO (crude palm oil), palm oil products and/or by-products, vegetable oils and fats and/or the like non-glyceride components-comprising compounds.

All known method previously disclosed in the adsorption/desorption chromatographic separation solely involve the use of solvents (which are costly and hazardous). Thus, there is a need to provide a separation and/or isolation process, which avoids or discourages the sole use of solvents and consequently rendering it "non-hazardous" for recovering these non-glyceride components.

Summary to the Invention

The present invention provides a method of chromatographic isolation of squalenes, sterols, vitamin E and carotenes from a compound comprising squalenes, sterols, vitamin E and carotenes, said method comprising the steps of:

- i) esterifying the free fatty acid components of the compound with one or more monohydric alcohols to form an esterified

- composition having a very low fatty acid content,
- ii) transesterifying the glyceride components of the compound into monoesters by employing one or more monohydric alcohols,
 - iii) subsequently introducing the product mixture from steps i) and ii) onto a selective adsorbent to allow adsorption of the product mixture, and
 - iv) isolating the squalenes, sterols, vitamin E and carotenes from the adsorbent by desorption,

wherein the adsorption and/or desorption steps are carried out under a supercritical fluid environment.

The present invention concerns a method of chromatographic isolation for non-glyceride components (carotenes, vitamin E [sometimes abbreviated as Vit. E], sterols, squalene) from natural oils and fats, wherein the method comprises the use of supercritical fluid (such as SC-CO₂) in combination with adsorbents (such as silica gel or C18 reverse phase silica gel). The present invention may be subjected directly and/or indirectly to

- (a) crude vegetable oil esters (via catalytic alcoholic esterification/transesterification of vegetable oils) or
- (b) concentrate (through removal of the bulk of the esters from (a) via vacuum distillation including molecular distillation)

or

- (c) the unsaponifiable matters of the concentrate in (b) (through saponification process)

High concentration of carotenes, vitamin E, sterols and squalene can be obtained by monitoring supercritical fluid process conditions such as temperature between 30°C-100°C, pressure from 50kg/cm² to 600kg/cm², with and without an entrainer (co-solvent such as alcohols).

A main advantage of the invention lies in the use of liquified gas at supercritical conditions, hence avoiding or reducing the prior requirements for hazardous solvents.

Detailed description of the invention

According to the present invention there is provided a method of chromatographic isolation of carotenoids, Vitamin E sterols and squalene from CPO (crude palm oil), palm oil products and/or by-products, vegetable oil and/or the like non-glyceride components-comprising compounds, said method comprising the following steps for three different routes as described below:

Route (a)

- (i) Esterifying the free fatty acid component of the non-glyceride components-comprising compounds with one or more monohydric alcohols to form an esterified compound with a very low free fatty acid content;
- (ii) Trans-esterifying the glyceride components with one or more monohydric alcohols to convert into monoesters;
- (iii) Introducing the non-glyceride components-comprising compounds (i.e. esters from steps (i) and (ii) above) to allow the adsorption of carotenoids, Vitamin E, sterols and squalene onto a selective adsorbent; and subsequently
- (iv) Desorbing the non-glyceride components from the adsorbent, wherein the adsorption/desorption of the non-glyceride components in steps (iii) - (iv) is carried out under a supercritical fluid environment.

Route (b)

- (i) Esterifying the free fatty acid components of the non-glyceride components-comprising compounds with one or more monohydric alcohols to form an esterified compound with a very low free fatty acid content;
- (ii) Trans-esterifying the glyceride components with one or more monohydric alcohols to convert into monoesters;
- (iii) Removing bulk of the esters in step (i) and (ii) by vacuum distillation including molecular distillation to yield carotene concentrate of 1-8%;
- (iii) Introducing the non-glyceride components-comprising compounds (i.e. the carotene concentrate from step (iii) above) to allow the adsorption of non-glyceride

components (carotenoids, tocopherols, sterols and squalene) onto a selective adsorbent; and subsequently

- (iv) Desorbing the non-glyceride components from the adsorbent, wherein the adsorption/desorption of the non-glyceride components in steps (iv) – (v) is carried out under a supercritical fluid environment.

Route (c)

- (i) Esterifying the free fatty acid component of the non-glyceride components-comprising compounds with one or more monohydric alcohols to form an esterified compound with a very low free fatty acid content;
- (ii) Trans-esterifying the glyceride components with one or more monohydric alcohols to convert into monoesters;
- (iii) Removing bulk of the esters in step (i) and (ii) by vacuum distillation including molecular distillation to yield carotene concentrate of 1-8%.
- (iv) Saponifying the carotene concentrate in step (iii) to yield unsaponifiable matters;
- (v) Introducing the non-glyceride components-comprising compounds to allow the adsorption of carotenoids, Vitamin E, sterols and squalene onto a selective adsorbent; and subsequently
- (vi) Desorbing the non-glyceride components from the adsorbent, wherein the adsorption/desorption of the non-glyceride components in steps (v)-(vi) is carried out under a supercritical fluid environment.

Preferably, the esterification of step (i) above is carried out employing

- (a) a solid alkali metal bisulphate
- (b) a sulphate acid and/or strongly-acidic ion-exchange resin.

Preferably, the transesterification of step (ii) is carried out employing a basic catalyst .

Alternatively, both the esterification and transesterification are carried out using an enzyme; for example *candida rugosa* and lipase. 1 to 20% by weight of catalyst may be employed depending upon the weight of the free fatty carboxylic to acid moiety present in the non-glyceride components-comprising compounds.

Preferably, the monohydric alcohols used in the esterification of step(i) or transesterification of step (ii) comprise one or more C_1 to C_8 alcohols, most preferably methanol.

Preferably, the selective adsorbent for the adsorption of the non-glyceride components comprise silica gel and/or C18 reverse phase silica gel. The adsorbents may be subjected to adsorption of the non-glyceride components present within anyone of the following;

- (i) the esterified palm oil; or
- (ii) the carotene concentrate (1-8%) wherein bulk of the esters have been removed through vacuum distillation or molecular distillation (short path distillation); or
- (iii) the unsaponifiable materials by saponifying the carotene concentrate of (ii).

The solvents employed for the present method comprises;

- (i) Supercritical fluid, e.g. SC- CO_2 (100%), and/or
- (ii) Supercritical fluid (e.g. SC- CO_2) in combination with an entrainer (solvent such as alcohol)

Carotene, tocopherols, sterols and squalene can be isolated with > 50% purity in a single run by varying pressure from 50 kg/cm²-600kg/cm² at temperatures 30°C-100°C.

It shall therefore be illustrated, by means of the following examples, that supercritical fluid, applied to solid adsorbents such as silica gel and/or C18 reverse phase silica gel, provides a very satisfactory means for isolating carotenoids, Vitamin E, sterols and squalene from CPO (crude palm oil), palm oil products and/or by-products, vegetable oil and/or the like non-glyceride components-comprising compounds.

Example 1

Crude palm oil methyl esters containing "minor" non-glyceride components (including carotenes, vitamin E, squalene and sterols), derived from alcoholic esterification/transesterification of crude palm oil with alkali bisulphate or sulphonated ion exchange resin (for esterification) and with 0.3-2% based catalyst and methanol was passed through molecular distillation at a temperature of 90°C-180°C and pressure of less than 13.3 Pa (100m Torr) to give a carotene concentrate of 1-8%. The carotene concentrate was subjected to saponification process to obtain unsaponifiable matters containing carotenes, vitamin E, sterols and squalene.

A typical sample of the unsaponifiable matter of palm carotene concentrate (0.0368g) containing carotenes (21.6%), vitamin E (2.7%), sterols (56.5%) and squalene (8.2%) was dissolved in an organic solvent, such as dichloromethane (2ml) and loaded onto a column (silica gel, with internal diameter 20mm and length: 250mm). The sample mixture was eluted using a mixture of supercritical carbon dioxide (3.0ml, 98.4%) and entrainer, ethanol (0.05ml, 1.6%) with a flowrate of 3.05ml/min. The pressure of the supercritical fluid chromatography system was monitored from $P=100\text{kg/cm}^2$ to 300kg/cm^2 in 9 hours, keeping temperature constant at 80°C. Various fractions at different time intervals were collected and results are shown in Table 1. Squalene was collected at Pressure 100-180 kg/cm^2 , Carotene at $P=180\text{-}220\text{kg/cm}^2$; Vitamin E at $P=220\text{-}280\text{kg/cm}^2$ and sterols at $>280\text{ kg/cm}^2$. Total carotenes, vitamin E, sterols and squalene collected in each fraction was determined by UV-visible spectrophotometer at 446nm, HPLC (fluorescent detector, excitation 323nm, and emission 296nm), and GC (for sterols and squalene) respectively.

Example 2

The procedure of example 1 was repeated except that the palm carotene concentrate (4% concentration) was used instead of unsaponifiable matters of the concentrate to recover carotenes, vitamin E, sterols and squalene separately in different fractions. Results are shown in Table 2.

Example 3

The procedure of Example 1 was repeated except that crude palm oil methyl esters were used directly to load onto supercritical fluid chromatography to recover carotenes, vitamin E, sterols and squalene separately in different fractions. The results are shown in Table 3.

Example 4

The procedure of Example 1 using unsaponifiable matters of the carotene concentrate was repeated except that only 100% SC-CO₂ (without entrainer) was used for adsorbing/desorbing carotenes, vitamin E, sterols and squalene. Results are shown in Table 4.

Example 5

The procedure of Example 2 using palm carotene concentrate (4% concentration) was repeated except that only 100% SC-CO₂ (without entrainer) was used for adsorbing/desorbing carotenes, vitamin E, sterols and squalene. Results are shown in Table 5.

Example 6

The procedure of Example 3 using crude palm oil methyl esters was repeated except that 100% of SC-CO₂ (without entrainer) was used. Results are shown in Table 6.

Example 7

The procedure of Example 1 using unsaponifiable matters of the carotene concentrate was repeated except that the adsorbent used was C18 reverse phase silica gel instead of silica gel. Results are shown in Table 7.

Example 8

The procedure of Example 2 using palm carotene concentrate (4% concentration) was repeated except that the adsorbent used was C18 reverse phase silica gel instead of silica gel. Results are shown in Table 8.

Example 9

The procedure of example 3 was repeated except that crude palm oil methyl esters were used directly to load onto supercritical fluid chromatography with C18 reverse phase silica gel to recover carotenes, vitamin E, sterols and squalene. Results are shown in Table 9.

Example 10

The procedure of example 7 using unsaponifiable matters of the carotene concentrate was repeated except that only 100% SC-CO₂ (without entrainer) was used for adsorbing/describing carotenes, vitamin E, sterols and squalene onto C18 reverse phase silica gel adsorbent. Results are shown in Table 10.

Example 11

The procedure of example 8 using palm carotene concentrate (4% concentration) was repeated except that only 100% SC-CO₂ (without entrainer) was used for adsorbing/desorbing carotenes, vitamin E, sterols and squalene onto C18 reverse phase silica gel adsorbent. Results are shown in Table 11.

Example 12

The procedure of example 9 was repeated except that crude palm oil methyl esters (instead of concentrate), 100% SC-CO₂ (without entrainer) and C18 reverse phase silica gel (instead of silica gel) were used. Results are shown in Table 12.

Example 13

The procedure of Example 1, 2 and 3 were repeated except that other palm oil products such as neutralized palm oil, crude palm olein, crude palm stearin, palm fibre oil and palm fatty acid distillate were used. The results are shown in Table 13. Squalene was collected at pressure 100-180kg/cm²; carotene at P= 180-220 kg/cm²; vitamin E at P=220-280 kg/cm² and sterols at P> 280 kg /cm².

Example 14

The procedure of Example 1, 2 and 3 were repeated except other vegetable oils such as soybean, canola, rapeseed, coconut, sunflower seed, corn oil, olive oil were used. The results are tabulated in Table 14. Squalene was collected at pressure 100-180kg/cm²; carotene at P=180-220 kg/cm² (if any); vitamin E at P=220 280 kg /cm² and sterols at P> 280 kg/cm².

Example 15

The procedure of Examples 7, 8, and 9 using the same starting materials as described therein were repeated and the results are presented in Table 15. Some variations to the operating conditions are also presented in Table 15 .

Example 16 (not according to the invention): Isolation of Palm Vitamin E isomers

Vitamin E concentrate (0.01 g; concentration: -50%) was dissolved in organic solvent, such as dichloromethane or ethanol and loaded onto a column (silica gel, with internal diameter 20mm and length 250mm). The Vitamin E concentrate mixture was eluted using a mixture of supercritical carbon dioxide (5ml, 95.2%) and an entrainer, ethanol (0.25ml, 4.8%) with a flowrate of 5.25ml/min. The pressure of the supercritical fluid chromatography system was kept constant right through the run at P=180kg/cm², keeping temperature constant at 70°C. The four vitamin E isomers were collected at different time interval and are shown in Table 16. The presence and concentration of Vitamin E isomer

were confirmed by HPLC as in Example 1.

Example 17: Isolation of Carotenes, Vitamin E, sterols and squalene (Fixed pressure and temperature)

The procedure of Example 16 was repeated except the unsaponifiable matter of palm carotene concentrate (0.01 g) containing carotenes (21.6%), Vit E (2.7%), sterols (56.5%) and squalene (8.2%) was used, Carotenes, Vit E, sterols and squalene were separated into different fractions and the results are shown in Table 17. The difference between Example 17 and that of Example 1 is that carotenes, Vit E, sterols and squalene can be isolated at fixed temperature (70°C) and pressure (180 kg/cm²).

Example 18

The procedures of Examples 16 and 17 were repeated. Unsaponifiable matter of palm carotene/Vit E concentrate (0.01g) was used and loaded onto a column (silica gel, with internal diameter 20mm and length 200mm). The results are shown in Table 18. Carotenes, Vit E., sterols and squalene were isolated in different fractions.

Example 19

The procedures of Examples 16, 17 and 18 were repeated except the temperature: 40°C, pressure: 160kg/cm² and SC-CO₂: ethanol (v/v=93.8%:6.2%). Unsaponifiable matter of palm carotene/vit. E concentrate (0.005g) was used and loaded onto a column (silica gel, with internal diameter: 4.6mm and length: 250mm). Carotenes, Vit. E, sterols and squalene were isolated in different fractions and results are presented in Table 19.

Table 1: Unsaponifiable Matters (with entrainer)

Fractions	Time (min)	Pressure (kg/cm ²)	Squalene (%)	Carotenes (%)	Vitamin E (%)	Sterols (%)
SM	-	-	8.2	21.6	7.7	59.5
F1	0-135	100	86	-	-	-
F2	135-195	180	9.4	83.4	-	-
F3	195-240	220	-	9.2	60.1	-
F4	240-260	250	-	4.4	20.2	-
F5	260-300	250	-	1.5	11.1	-
F6	300-360	280	-	0.8	8.8	5.0
F7	360-500	280	-	-	-	95.5

SM: Starting Materials

Table 2: Carotene Concentrate (with entrainer)

Fraction's	Time (min)	Pressure (kg/cm ²)	Squalene (%)	Carotenes (%)	Vitamin E (%)	Sterols (%)
SM	-	-	9.8	4.1	2.3	5.3
F1	0-135	100	92	-	-	-
F2	135-195	180	4.8	70.6	-	-
F3	195-240	220	-	8.2	13.8	-
F4	240-260	250	..	3.1	57.3	-
F5	260-300	250	-	3.2	22.5	-
F6	300-360	280	-	1.4	6.6	2.4
F7	360-500	280	-	-	-	96.6

SM: Starting Materials

Table 3: Crude Palm Oil Methyl Esters (with entrainer)

Fractions	Time (min)	Pressure (kg/cm ²)	Squalene (%)	Carotenes (%)	Vitamin E (%)	Sterols (%)
SM	-	-	0.03	0.06	0.06	0.04
F1	0-135	100	3.6	-	-	-
F2	135-195	180	-	10.2	-	-
F3	195-240	220	-	3.2	1.2	-
F4	240-260	250	-	1.2	12.6	-
FS	260-300	250	-	12.0	10.8	-
F6	300-360	280	-	-	2.8	0.8
F7	360-500	280	-	-	-	3.6

SM: Starting Materials

Table 4: Unsaponifiable matters (without entrainer)

Fractions	Time (min)	Pressure (kg/cm ²)	Squalene (%)	Carotenes (%)	Vitamin E (%)	Sterols
SM	-	-	8.2	21.6	7.7	59.5
F1	0-100	100	36	-	-	
F2	100-160	180	88	2	-	-
F3	160-200	220	-	81.3	0.8	
F4	200-240	250	-	11.7	12.7	-
F5	240-260	250	-	6.6	73.6	0.9
F6	260-300	300	-	-	8.8	8.2
F7	300-450	300	-	-	-	90.2

SM: Starting Materials

Table 5: Carotenes Concentrate (without entrainer)

Fractions	Time (min)	Pressure (kg/cm ²)	Squalene (%)	Carotenes (%)	Vitamin E (%)	Sterols (%)
SM	-	-	9.8	4.1	2.3	5.3
F1	0-135	100	24	-	-	-
F2	135-195	180	76	-	-	-
F3	195-240	220	-	67.8	18.7	-
F4	240-260	250	-	18.9	58.9	..
F5	260-300	250	-	8.7	20.3	-
F6	300-360	280	-	0.2	6.1	1.1.4
F7	360-500	280	-	-	-	83.3

SM: Starting Materials

Table 6: Crude Palm Oil 'Methyl Esters (without entrainer

Fractions	Time (min)	Pressure (kg/cm ²)	Squalene (%)	Carotenes (%)	Vitamin E (%)	Sterols (%)
SM	-	-	0.03	0.06	0.06	0.04
F1	0-135	100	-	-	-	-
P2	135-195	180	8.6	-	-	-
F3	195-240	220	-	10.8	-	-
F4	240-260	250	-	8.7	20.8	-
F5	260-300	250	..	6.8	16.7	-
F6	300-360	280	-	-	6.6	1.5
F7	360-500	280	-	-	-	8.6

SM: Starting Materials

Table 7: Unsaponifiable matter (with entrainer)

Fractions	Time (min)	Pressure (kg/cm)	Compounds		Carotenes	Sterols
			Squalene	Vitamin E		
Starting materials	—	—	6.8	9.8	20.8	42.8
F1	0 — 70	0 — 10min — 100 kg/cm ² 10 — 20min - 140 kg/cm ²	83.8	—	—	—
F2	70 — 100	20 — 55min—160 kg/cm ² 55 — 65min—180 kg/cm ² 65 — 90min--200 kg/cm ²	12.3	8.3	—	—
F3	100 - 140	90 — 300min -250 kg/cm ²	—	16	0.3	—
F4	140 — 170	> 250 kg/cm ²	—	1.8	8.6	—
F5	170 — 200	> 250 kg/cm ²	—	0.5	22.8	—
F6	200 — 250	> 250 kg/cm ²	—	—	99.4	10.2
F7	250 — 450	> 250 kg/cm ²	—	—	—	88.1

Temperature: 80°C

Flowrate: 6.0/0.2 ml/min (SC-CO₂ : ethanol)

Table 8: Carotene Concentrate (with - entrainer : ethanol)

Fractions	Time (min)	Pressure (kg/cm ²)	Compounds (%)		
			Squalene	Vitamin E	Carotenes
Starting materials	—	—	0.8	2.15	8.06
F 1	0 — 70	0 — 10min - 1 0 0 kg/cm ² 10 — 20min - 1 4 0 kg/cm ²	66.8	—	—
F 2	70 — 100	20 — S 5min - 1 6 0 kg/cm ² 55 — 65min — 180 kg/cm ² 65 — 90min — 200 kg/cm ²	7.5	76	0.2
F 3	100 — 140	90 — 300min — 250 kg/cm ²	—	18	0.2
F 4	140 — 170	> 250 kg/cm ²	—	2	9.3
F 5	170 — 200	> 250 kg/cm ²	—	0.6	32.2
F 6	200 — 250	> 250 kg/cm ²	—	—	98.1
F 7	250 - 450	> 250 kg/cm ²	—	—	—
					83

Temperature: 80°C

Flowrate: 6.0/0.2 ml/min (SC-CO₂ : ethanol)

Table 9: Crude Palm Oil Methyl Esters (with entrainer)

Fractions	Time (min)	Pressure (kg/cm ²)	Compounds (%)			
			Squalene	<i>Vitamin E</i>	Carotenes	Sterols
Starting materials	—	—	0.03	0.06	0.06	0.04
F 1	0 — 70	0-10min — 100kg/cm ² 10 — 20min — 140 kg/cm ²	44.2	—	—	—
F2	70 — 100.	20 — 55min - 160 kg/cm ² 55 — 65min — 180 kg/cm ² 65 — 90min - 200 k&/cm ²	3.3	58.2	—	—
F3	100 — 140	90 — 300min — 250 kg/cm ²	—	16.6	0.1	—
F4	140 — 170	> 250 kg/cm ²	—	4.8	—	—
F5	170 — 200	> 250 kg/cm ²	—	1.0	16.8	—
F6	200 — 250	> 250 kg/cm ²	—	—	46.6	5.7
F7	250 — 450	> 250 kg/cm ²	—	—	—	57.8

Temperature: 80°C

Flowrate: . 6.0/0.2 ml/min (SG-CO₂ : ethanol)

Table 10: Unsaponifiable matter (without entrainer)

Fractions	Time (min)	Pressure (kg/cm ²)	Squalene	Compounds (%)		
				Vitamin E	Carotenes	Sterols
Starting materials	—		6.8	9.8	20.8	42.8
F 1	0 – 50	0 – 10min – 1 0 0 kg/cm ² 10 – 20min – 1 4 0 kg/cm ²	GO	—	—	—
F2	50 – 70	20 – 55min – 160 kg/cm ² 55 – 100min – 1 8 0 kg/cm ² 100 – 140min – 200 kg/cm ²	89	7.6	0.01	
F3	70 — 90	140 – 180min – 220 kg/cm ² 180 — 280min — 240 kg/cm ² 280 — 300min – 300 kg/cm ²		83	0,04	—
F4	90 — 110	300 kg/cm ²	—	59	0.02	—
F5	110 — 130	300 kg/cm ²	—	52	0.02	—
F6	130 - 145	300 kg/cm ²	—	4	0.2	2.3
F7	145 - 170	300 kg/cm ²	—	8	0.4	6.8
F8	170 — 300	300 kg/cm ²	—	—	99.1	8.6
F9	300 — 450	300 kg/cm ²	—	—	--	80.3

Temperature: 80°C

Flowrate: : 6.0 ml/min

Table 11 Carotene Concentrate (without entrainer)

Fractions	Time (min)	Pressure (kg/cm ²)	Squalene	Compounds (%)	
				Vitamin F	Sterols
Starting materials	—	—	0.8	2.15	9.22
F1	0 — 50	0 - 10min—100 kg/cm ² 10 - 20min - 140 kg/cm ²	5.3	—	—
F2	50 — 70	20 — 55min—160 kg/cm ² 55 — 100min —180 kg/cm ² 100 —140min - 200 kg/cm ²	81	9.8	0.03
F3	70 — 90	140 -180min— 220 kg/cm ² 180 — 280min — 240 kg/cm ² 280 — 300min - 300 kg/cm ²	—	72	0.2
F4	90 — 110	300 kg/cm ²	—	59	0.08
F5	110 — 130	300 kg/cm ²	—	51	0.03
F6	130 — 145	300 kg/cm ²	—	6	0.8
F7	145 — 170	300 kg/cm ²	—	10	1.7
F8	170 — 300	300 kg/cm ²	—	—	98.3
F9	300 —450	300 kg/cm ²	—	—	70.3

Temperature: 80°C

Flowrate: 6.0 ml/min

Table 12: Crude Palm Oil Methyl Esters (without entrainer)

Fractions	Time (min)	Pressure (kg/cm)	Compounds (%)			
			Squalene	Vitamin E	Carotenes	Sterols
Starting materials	—	—	0.03	0.06	0.06	0.04
F1	0-50	0-10min — 100 kg/cm ² 10 — 20min — 140 kg/cm ²	20	-	-	-
F2	50 — 70	20 — 55min — 160 kg/cm ² 55 — 100min — 180 kg/cm ² 100-140min — 200 kg/cm ²	61	-	—	—
F3	70 — 90	140 — 180min — 220 kg/cm ² 180 — 280min — 240 kg/cm ² 280 — 300min — 300 kg/cm ²	—	56.7	—	—
F4	90 — 110	300 kg/cm ²	—	32.2	0.8	-
F5	110 — 130	300 kg/cm ²	—	31.2	1.6	—
F6	130 — 145	300 kg/cm ²	—	1.3	1.8	-
F7	145 — 170	300 kg/cm ²	-	-	12.2	3.2
F8	170 — 300	300 kg/cm ²	-	—	57.8	4.6
F9	300 — 450	300 kg/cm ²	—	-	—	58.3

Temperature: 80°C

Flowrate: 6.0 ml/min

Table 13 (Palm oil products and by-products)

Palm Oil Products	Compounds (%)			
	Squalene	Carotene	Vitamin E	Sterols
1. CPOs	0.012	0.032	0.042	0.026
ME CPOs	28.7	24.4	18.2	26.7
Concentrate CPOs	56.2	48.8	44.3	56.8
Unsap. matters CPOs	76.8	63.3	60.5	80.1
2. CPOo	0.022	0.092	0.076	0.033
ME CPOo	30.6	38.6	36.4	44.5
Concentrate CPOo	63.1	78.2	64.3	79.9
Unsap. matters CPOo	85.7	95.2	89.1	98.1
3. NPO	0.034	0.065	0.066	0.044
MENPO	32.1	40.4	41.1	55.4
Concentrate NPO	65.0	62.5	60.6	88.2
Unsap. matters NPO	88.1	81.8	80.7	96.9
4. PFAD	0.8	-	0.78	1.12
ME PFAD	36.7	-	45.5	50.8
Concentrate PFAD	73.3	-	79.4	80.6
Unsap. matters PFAD	92.6	-	88.7	91.3
5. Residual fibre oil	0.056	0.52	0.23	0.48
ME R fibre oil	30.2	38.9	38.8	57.7
Concentrate R fibre oil	76.3	60.1	58.6	78.8
Unsap. matters R fibre oil	90.7	88.3	82.1	93.3

CPOs crude palm stearin

CPOo crude palm olein

NPO neutralised palm oil

PFAD palm fatty acid distillate

R residual

ME methyl esters

Table 14: Vegetable oils

Vegetable Oils	Compounds (%)		
	Squalene	Vitamin E	Sterols
1. Soyabean Oil (SO)		0,116	0.320
ME SO	—	27.7	32.2
Concentrate SO		52.9	60.7
Unsap. matter SO '		72.8	90.1
2. Sunflower seed (SU)		0.064	0.3 62
ME SU	—	20.1	33.3
Concentrate SU		50.3	61.8
Unsap. matter SU		63.9	91.5
3. Rapeseed oil (Ra)		0,027	0.582
ME Ra	—	21.0	35.7
Concentrate Ra		43.7	65.4
Unsap. matter Ra		58.5	89.5
4. Rice bran (Ri)		0.098	1.52
ME Ri	—	21.6	46.3
Concentrate Ri		48.2	68.2
Unsap. matter Ri		65.8	96.1
5. Olive oil (OL)	0.688	0.01	0.11
ME OL	26.3	16.6	32.1
Concentrate OL	62.0	38.8	60.6
Unsap. matter OL	89.6	58.1	88.6
6. Corn Oil		0.060	1.02
ME corn oil	—	21.2	44.4
Concentrate corn oil		41.8	65.8
Unsap. matter corn oil		60.5	92.0
7. Coconut oil			0.10
ME coconut oil	—	—	31.3
Concentrate coconut oil			58.5
Unsap. matter coconut oil			84.7

Table 15

Fractions	Coll ctn time (min)	Eluent CO ₂ /ent. ml/min	Cpds %	(A) Unsap. Matters	(B) Carotene Conc.	(C) Crude Palm Oil Methyl Esters
1	0-90	6ml/0rnl	Methyl Esters Squalene	- 95	20 80	99.9 0.02
2	90-190	6ml/0ml & 6ml/0.2ml	Vit. E Sterols	81 18.9	76.3 14.2	68.9 6.2%
3	190-210	6ml/0.2ml & 6rnl/0.4ml	Sterols Vit. E	80.2 18.E	54.2 16.8	26.1 10.9
4	210-270	6ml/1ml	Carotenes	96	82.3	54.7

Operating pressure: 180 lcg/cm²

Table 16: Isolation of Palm Vit. E Isomers (α -T, α -T3, γ -T3 and δ -T3)

Fractions	Collection Time (min)	Vit. E α -T	Vit. E α -T3	Vit. E γ -T3	Vit. E δ -T3
S/M	-	12.7	11.4	19	5.8
1					
2	41-47	100			
3	47-54		100		
4	54-61			100	
5	61-77			99	1
6	77-145			33	97
	145-				

- SIM : Starting material (Vit E concentrate -50%)
- T: tocopherol
- T3: tocotrienol
- Conditions: P= 180kg/cm², T = 70°C
- SC-CO₂ : ethanol = 5ml:0.25ml(95.2% : 4.8%)

Table 17: Unsaponifiable matters (with entrained)

Fractions	Time (min)	Squalene (%)	Carotenes (%)	Vit. E (%)	Sterols (%)
S/M		8.2	21.6	7.7	59.5
F1	0-34	98.0			
F2	34-41		90.0		
F3	41-47		20.0	(α -T) 80.0	
F4	47-54			(α -T3) 100.0	
F5	54-61			(α -T3) 100.0	
F6	61-72			(γ -T3) 100.0	
F7	72-77			(γ -T3) 10.0	90.0
F8	77-145			(δ -T3) 100.0	

Conditions: P= 180kg/cm², T=70°C

SC-CO₂: ethanol = 5ml:0.25ml(95.2%:4 v/v)

Table 18: Carotene and Vit. E Concentrate

Fractions	Time (min)	Squalene (%)	Carotenes (%)	Vit. E (%)	Sterols (%)
S/M		8.2	21.6	48.8	21.3
F1	0-7	96			
F2	7-11		90	(α -T) 10.0	
F3	11-13		15	(α -T) 85.0	
F4	13-14			(α -T3) 98.0	
F5	15-20			(γ -T3) 98.0	
F6	20-25			(δ T3) 98.0	
F7	25-40				98

Silica column:

20mm ID x 200 mm length

Condition:

P = 180 kg/cm², T = 70°C

SC-CO₂: ethanol = (95% : 5%, v/v)

Table 19

Fractions	Time (min)	Squalene (%)	Carotenes (%)	Vit. E (%)	Sterols (%)
S/M		8.2	21.6	48.8	21.3
1	0 -1.5	98			
2	1.5-2.5		92		
3	2.5-3.2			(α -T).96.0	
4	3.2-3.8			(α -T3) 98.0	
5	3.8-4.2			(γ -T3) 98.0	
6	4.2-5			(δ -T3) 98.0	
7	5-8				99

Conditions: P= 160 kg/cm², T=40°C

SC-CO₂: ethanol = 3.0:0.2 (mls/min) (93.75% : 6.25%, v/v)

CLAIMS

1. A method of chromatographic isolation of squalenes, sterols, vitamin E and carotenes from a compound comprising squalenes, sterols, vitamin E and carotenes, said method comprising the steps of:

- i) esterifying the free fatty acid components of the compound with one or more monohydric alcohols to form an esterified composition having a very low fatty acid content,
- ii) transesterifying the glyceride components of the compound into monoesters by employing one or more monohydric alcohols,
- iii) subsequently introducing the product mixture from steps i) and ii) onto a selective adsorbent to allow adsorption of the product mixture, and
- iv) isolating the squalenes, sterols, vitamin E and carotenes from the adsorbent by desorption,

wherein the adsorption and/or desorption steps are carried out under a supercritical fluid environment.

2. A method as claimed in claim 1, wherein the squalenes, sterols, vitamin E and carotenes are isolated in a single run.

3. A method as claimed in claim 1 or 2, wherein the squalenes, sterols, vitamin E and carotenes are isolated at fixed pressure.

4. A method as claimed in any preceding claim, wherein the squalenes, sterols, vitamin E and carotenes are isolated at fixed temperature.

5. A method as claimed in any preceding claim, wherein the selective adsorbent

comprises either silica gel or reverse phase C 1 8 silica gel.

6. A method as claimed in any preceding claim, wherein the adsorption/desorption are carried out under a supercritical fluid comprising an effective amount of CO₂ as solvent.

7. A method as claimed in any one of claims 1 to 5, wherein the adsorption/desorption are carried out under a supercritical fluid comprising an effective amount of a combination of CO₂ and an entrainer, as solvent.

8. A method as claimed in claim 7, wherein the entrainer comprises an organic solvent.

9. A method as claimed in claim 8, wherein the organic solvent comprises an alkyl alcohol.

10. A method as claimed in any preceding claim, wherein the adsorption/desorption are carried out under a supercritical fluid environment which employs a constant flow rate of solvent.

11. A method as claimed in any preceding claim, wherein the adsorption/desorption are carried out under a supercritical fluid environment which employs constant relative solvent proportions.

12. A method as claimed in any preceding claim, wherein the esterification is carried out employing a solid alkali metal bisulfate, a sulfate acid and/or a strongly acidic ion-exchange resin as a catalyst, and the transesterification is carried out using a basic catalyst.

13. A method as claimed in any preceding claim, wherein the esterification and/or transesterification comprises the use of one or more C1 to C8 alcohols.

14. A method as claimed in claim 13, wherein the esterification and/or transesterification comprises the use of methanol.

15. A method as claimed in any preceding claim, which further comprises the step of removing the bulk of the esters produced in step i) and ii) by vacuum distillation including molecular distillation prior to step iii).

16. A method as claimed in claim 15, said method comprising the additional step of saponifying the carotene concentrate to yield unsaponifiable matters, wherein this additional step is taken subsequent to the esterification and trans-esterification steps and the distillation step, and prior to the introduction step of part (iii) and wherein the product mixture of steps (i) and (ii) introduced onto the selective adsorbent contains or comprises the unsaponifiable matters.

17. A method as claimed in claim 15 or 16, wherein the molecular distillation is carried out at 90 to 190 degrees C and a pressure of less than 13.3 Pa (100mTorr).

18. A method as claimed in any preceding claim, wherein the compound comprising squalenes, sterols, vitamin E and carotenes is selected from the group consisting of crude palm oil, palm oil products and/or by-products, and vegetable oil.

19. A method as claimed in any preceding claim, which is employed to separate palm Vitamin E into the following palm Vitamin E isomers:

α -T₃, γ -T₃, δ -T₃ and α -T.

20. A method as claimed in claim 19, wherein the pressure is kept constant at 180 kg/cm², the temperature is kept constant at 70 degrees C, the solvent flow rate is 5.25 ml/min and the solvent used is a mixture of supercritical CO₂ and ethanol with relative proportions of 95.2%:48% by volume.

21. A method as claimed in claim 19, wherein the pressure is kept constant at 160 kg/cm², the temperature is kept constant at 40 degrees C, and the solvent used is a mixture of supercritical CO₂ and ethanol with relative proportions of 93.75%:6.25% by volume and flow rate of 3.0:0.2 ml/min.