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Improved NARP-HPLC method for separating triglycerides of palm olein and its solid fractions obtained at low temperature storage

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Non-aqueous reversed-phase high-performance liquid chromatography (NARP-HPLC) with refractive index (RI) detection is described and used for palm olein and its fractions obtained at 12.5°C for 12–24 h. The calculation formula for fatty acid methyl esters (FAMEs) and carbon number (CN) from the data obtained by NARP-HPLC is described and correction factors for all carbon numbers and fatty acids are tabulated. The results were compared with those obtained from FAMEs analysed on 10% SP 2330 Supelco packed column gas–liquid chromatography (GLC) and CN analysed on 3% OV-1 Supelco packed column GLC. The results were found to agree well for C48, C50, C52, C16:0, C18:0 and C18:1 (correction factor ≈ 1.0); however, a slight variation was observed for components C54, C14:0 and C18:2 (correction factor 1.0 ± 0.37). Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Triglyceride analysis is important in the natural oil industry for process and product quality control purposes. At the research and development level, detailed triglyceride data might facilitate the understanding of triglyceride biosynthesis and deposition in plants and animal cells (El-Hamdy & Perkins, 1981). El-Hamdy & Perkins (1981) reported that the pattern of triglyceride elution sequence within each triglyceride category with the same equivalent carbon number (ECN) starts with the triglyceride with the highest number of double bonds and terminates with those with the lowest number of double bonds, with the lower ECN triglycerides eluting ahead of those with higher ECN.

The liquid chromatography system most commonly employed in triglyceride analysis consists of an alkyl bonded-phase column and a refractive index (RI) detector. Although aqueous mobile phases are generally used with these columns (Dong & Dicesare, 1983), because of the lipophilicity of triglycerides, water cannot be used in the mobile phase for this particular application. Therefore, the mobile phases generally employed consists of mixtures of acetone and acetonitrile, and occasionally tetrahydrofuran, methylene chloride or hexane (Dong & Dicesare, 1983). The absence of water in the mobile phase, prompted the term non-aqueous reversed-phase (NARP) to describe the above system (Dong & Dicesare, 1983).

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Petersson *et al.* (1981) used a 100-cm long column packed with Nucleosil 5 C18 and the mobile phase was acetonitrile–acetone, 60:40 (v/v). It was found that using this system, the oils could be separated directly into fractions specified by having the same carbon number (CN) and number of double bonds. The fractions were found to be very pure and to have simple triglyceride composition with only one totally dominating triglyceride type in almost all cases.

Podlaha & Töregard (1982) had presented a system for identification of triglycerides in reversed-phase HPLC chromatograms based on ECN. A graphical system for tentative identification and prediction of peaks in reversed-phase HPLC chromatograms of triglycerides was presented. Straight, parallel lines were found for different homologous series of triglycerides when plotting CN against ECN.

Advances in column and instrument technology have significantly enhanced liquid chromatography (Dicesare *et al.*, 1981). Dong & Dicesare (1983) separated the natural oil triglycerides by liquid chromatography using columns packed with 3-μm alkyl bonded-phase particles. The analysis times range from 8 to 16 min with the mobile phase acetone–acetonitrile (7:3) at the flow rate of 2.5 ml/min.

Geeraert & Schepper (1983) reported the structure detection of triglycerides by RP-HPLC and the importance of temperature and mobile phase composition. RP-HPLC analysis of triglycerides was best performed on 5 μm particles at 14°C with 100% propionitrile as the mobile phase. Lozano (1983)

analysed the triglycerides in avocado oil by RP-HPLC on C18 column, eluted with acetonitrile–acetone–tetrahydrofuran (58:38:4) solvent system at 30°C, and detection by differential refractometry. Ritchie & Jee (1985) developed a HPLC system which separates and quantifies monoglycerides in addition to other lipid components. Analysis was carried out using a 25 cm×4.6 mm Zorbax silica column with the mobile phase isooctane–tetrahydrofuran–formic acid (90:10:0.5) and at ambient temperature and initial flow rate of 1.5 ml/min; or (80:20:0.5) at column temperature of 35°C and initial flow rate of 1.0 ml/min. The latter solvent mixture resulted in monoglycerides eluting in about 30 min (vs 1 h) with considerable improvement in peak shape of both mono- and diglycerides.

Influence of injection solvent on the RP-HPLC of triglycerides was reported by Tsimidou & Macrae (1984). It was found that the injection solvent and its volume had a large effect on the entire chromatogram. Chloroform as an injection solvent produced inferior resolution under all conditions and this was accentuated when large injection volumes were used. Acetone was recommended as a reliable alternative. However, acetone is not suitable for high molecular weight saturated lipids (Tsimidou & Macrae, 1985).

The choice of mobile phase and temperature for separating palm oil triglycerides by RP-HPLC was studied by Deffense (1984). Best results were achieved with a mobile phase of acetone–acetonitrile (62.5:37.5) at 50°C. The objective of this research is to find the optimum separation for analysis of palm olein triglycerides by NARP-HPLC, and to find a correction factor to be used in calculating CN and fatty acid composition (FAC).

MATERIALS AND METHODS

Malaysian unblended RBD palm (*Elaeis guineensis* var *Tenera*) olein was obtained from local refinery. All chemicals used were either of analytical or high-performance liquid chromatography (HPLC) grade. Standard methyl esters and triglycerides were purchased from Sigma Chemical Co. (St Louis, MO, USA).

The test methods adopted in this study were gas–liquid chromatography (GLC) for the analysis of triglyceride carbon number (CN) (IUPAC 2.322) (IUPAC, 1979) and fatty acid methyl esters (FAMEs) (IUPAC 2.301) (IUPAC, 1979) as used by the Palm Oil Research Institute of Malaysia (PORIM, 1988). The non-aqueous reversed-phased high-performance liquid chromatography (NARP-HPLC) method used to determine the triglyceride composition was modified from the method of Dong & Dicesare (1983). All the analyses were done in replicates of four, and the results are expressed as mean weight percentage.

Crystallization

Palm olein was completely melted at 70°C in an oven for 30 min prior to crystallization. Crystallization was carried out in standard 115 ml (4 oz) cloud point determination bottles (Beatson bottle) with a diameter of 42 mm and a wall thickness of 2 mm as described in the AOCS Official Method Cc 6-25 (AOCS, 1984). An oil sample weighing 50±0.005 g was placed in each bottle and crystallized in a water bath which was placed in cold room with the temperature set at 12.5°C. Crystals that formed were collected between 12 and 24 h at 3 h intervals.

Crystals were separated from liquid oil by centrifugation at 3000 rpm for 3 min at 12.5°C. The liquid portion was decanted without disturbing the crystals which were retained at the bottom of the bottle. The wall of the bottle was washed with cold acetone (12.5°C). Special attention was given to maintain the temperature consistently at 12.5°C. The crystallization and separation processes were done in four replications.

GLC analysis of triglycerides carbon number (CN) and methyl esters (FAME)

A Pye Unicam (Cambridge, UK) Series 204 Gas Chromatograph system equipped with flame-ionization detector (FID) and a Hewlett-Packard (Palo Alto, CA, USA) data integrator (Model 3380A) was used for both CN and FAME analyses. Triglycerides were separated according to their CN on a glass column (0.5 m×3 mm i.d.) packed with 3% OV-1 on Gas Chrom Q 100/120 mesh size (Supelco, Belle Forte, PA, USA). The column temperature was programmed from 280 to 350°C at a rate of 5°C/min. The nitrogen carrier gas flow rate was set at 80 ml/min. The injector and detector temperatures were both set at 400°C. The injection volume was 1 μl and oils or fat crystals were first dissolved in hexane at a concentration of 5% (w/v). A secondary calibration standard (palm olein, PORIM) was used to identify the components. Fatty acid methyl esters were analysed on a glass column (1.8 m×2.5 mm i.d.) packed with 10% SP2330 on 100/120 mesh supelcoport (Supelco, Belle Forte, PA, USA). An isothermal column temperature was set at 190°C. The nitrogen carrier gas flow rate was set at 40 ml/min. The injector and detector temperatures were both set at 220°C. The injection volume was 1 μl where a 5% (w/v) oil or fat crystal after conversion to the methyl esters in petroleum ether was used. A mixture of standard methyl esters (Supelco) was used to identify the components. Integrator-produced peak area per cents were used to quantify the components after calibration with the known standards.

Non-aqueous reversed-phase high-performance liquid chromatography (NARP-HPLC)

The HPLC system used was equipped with a Waters (Milford, MA, USA) HPLC pump (Model 501), a column oven, a loop injector with a 20 μl sample loop, Waters RI detector (Model M401) and a Waters system interface module connected to a computer and a printer.

Triglycerides were separated on a single commercially packed (250×4.0 mm) RP-18 (Merk, Damn Stadt,

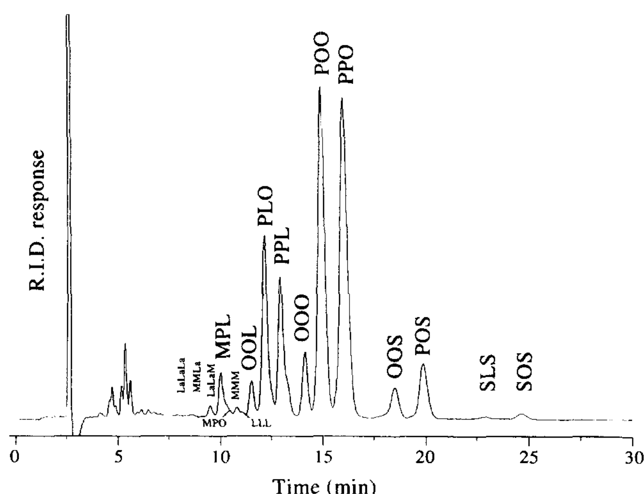

Fig. 1. NARP-HPLC chromatogram of typical palm olein at room temperature (about 27°C): LaLaLa, lauric–lauric–lauric; LaLaM, lauric–lauric–myristic; MMLa, myristic–myristic–lauric; MMM, myristic–myristic–myristic; MPO, myristic–palmitic–oleic; MPL, myristic–palmitic–linoleic; PPO, palmitic–palmitic–oleic; PPL, palmitic–palmitic–linoleic; LLL, linoleic–linoleic–linoleic; POS, palmitic–oleic–stearic; POO, palmitic–oleic–oleic; PLO, palmitic–linoleic–oleic; OOS, oleic–oleic–stearic; SOS, stearic–oleic–stearic; SLS, stearic–linoleic–stearic; OOO, oleic–oleic–oleic; and OOL, oleic–oleic–linoleic. RID, refractive index detector.

Germany) column with a particle size of 5 μm. The mobile phase was a mixture of acetone–acetonitrile (63.5:36.5) and the flow rate was set at 1 ml/min. The column temperature was set at 35°C. The injection volume was 10 μl of 5% (w/v) oil in acetone. Sensitivity was adjusted to 16×10^4 RI units full-scale deflection. Peaks were identified from 17 triglyceride standards (Supelco) injected individually and by matching retention times. Peak area per cent produced by the data integrator was used to quantify the components after calibration with the known standards.

Methyl ester preparation for FAME

Samples were melted thoroughly in an oven at 70°C for 30 min before being esterified in 2 ml glass vial fitted with a screw cap. The sample (0.05 ± 0.005 g) was placed in a vial and 0.95 ml petroleum ether was added. The vial was shaken well to dissolve the oil. Fifty microlitres of sodium methoxide (1 M) was then added and the mixture was shaken quickly with the help of a vortex mixer. The mixture was allowed to stand for 5 min or until separation of phases occurred. One microlitre of the upper layer was injected into the GLC.

Sample preparation for GLC-CN and NARP-HPLC

Samples were melted thoroughly in an oven at 70°C for 30 min, of which 0.05 ± 0.005 g was transferred into 2 ml glass crimp seal vials (Supelco) fitted with a Teflon-lined septum. For GLC analysis, 0.95 ml of hexane was added to dilute the sample while 0.95 ml acetone was used for NARP-HPLC analysis.

RESULTS AND DISCUSSION

A typical triglyceride chromatogram for palm olein is shown in Fig. 1. Based on the NARP-HPLC chromatogram of palm olein, it can be estimated that there are altogether 17 identifiable triglycerides found in palm olein. They are lauric–lauric–lauric [LaLaLa (0.08%)], lauric–lauric–myristic [LaLaM (0.43%)], myristic–myristic–lauric [MMLa (0.16%)], myristic–myristic–myristic [MMM (0.59%)], myristic–palmitic–oleic [MPO (0.69%)], myristic–palmetic–linoleic [MPL (3.01%)], palmitic–palmitic–oleic [PPO (27.95%)], palmitic–palmitic–linoleic [PPL (10.65%)], linoleic–linoleic–linoleic [LLL (0.007%)], palmitic–oleic–stearic [POS (5.12%)], palmitic–oleic–oleic [POO (27.49%)], palmitic–linoleic–oleic [PLO (12.64%)], oleic–oleic–stearic [OOS (3.82%)], stearic–oleic–stearic [SOS (0.58%)], stearic–linoleic–stearic [SLS (0.20%)], oleic–oleic–oleic [OOO (4.57%)] and oleic–oleic–linoleic [OOL (2.19%)]. Triglyceride chromatogram for palm olein solid fractions collected after 12, 15, 18, 21 and 24 h at low temperature (12.5°C) storage were very similar. The unidentified peaks appeared in the early region of the chromatograms were expected to be of partial glycerides. Evidence of a stable and straight baseline, sharp and symmetrically defined peaks show the quality of separation using the improved NARP-HPLC method. According to Frede (1986) the baseline could be stabilized by indirect thermostating of the detector. The increase of selectivity with decreasing temperature and the increasing sharpness of peaks at higher temperature were also observed. A high temperature was indispensible for saturated long-chain compounds which tend to crystallize on the column. To fulfill this requirement, and to avoid loss of selectivity, temperature programming was investigated. At higher temperature and additional axial temperature gradient up to 0.05K/cm was applied to enhance peak symmetry. However, in the case of palm olein, preset temperature at 35°C was found to be sufficient. The analysis time for each injection was found to be 30 min at flow rate of 1.0 ml/min. Table 1 tabulates the results of triglyceride composition and content. An increase was observed in PPO and POS, and a decline was found in the composition of POO, PLO, OOO, OOL and MPL during the first 18 h of crystallization. The rest of the components did not show obvious changes.

It is possible to use the NARP-HPLC results to determine triglycerides according to the unsaturation degree in that it allows the calculation of CN as well as FAC to be made according to the following formula:

$$\text{Calculated composition of CN} = \sum \text{Individual triglycerides with the same CN}$$

$$\text{e.g. } C52 = POO + POS + PLO$$

$$\text{Calculated FAC} = \sum \text{Individual fatty acid found in total glycerides}$$

$$\text{e.g. } C14 = MPO/3 + MPL/3$$

Table 1. Triglyceride composition based on HPLC analysis during low temperature storage (wt %)

Hours	Triglyceride*																
	MPO	MPL	PPO	PPL	POS	POO	POL	OOS	SOS	SLS	OOO	LaLaLa	LaLaM	LLL	MMLa	MMM	OOL
Control	0.69^{abc}	3.01^{a}	27.95^{c}	10.65^{d}	5.12^{c}	27.49^{a}	12.64^{a}	3.28^{ab}	0.58^{b}	0.20^{c}	4.57	0.08^{a}	0.43^{b}	0.007^{ab}	0.16^{b}	0.59^{b}	2.19^{ab}
12	0.66^{d}	2.96^{a}	29.32^{b}	10.72^{cd}	5.42^{b}	26.42^{b}	12.31^{a}	3.36^{a}	0.62^{ab}	0.31^{a}	4.41	0.11^{a}	0.50^{a}	0.04^{ab}	0.17^{ab}	0.56^{b}	2.08^{bc}
15	0.66^{cd}	2.72^{b}	32.92^{a}	10.86^{bc}	6.04^{a}	24.05^{d}	11.17^{b}	2.69^{c}	0.67^{a}	0.20^{c}	4.07	0.06^{ab}	0.10^{c}	0.03^{b}	0.167^{b}	0.52^{b}	1.9^{c}
18	0.68^{bcd}	2.69^{b}	33.53^{a}	10.95^{b}	6.04^{a}	23.98^{d}	11.13^{b}	3.04^{b}	0.67^{a}	0.28^{b}	3.98	0.08^{a}	0.10^{c}	0.009^{a}	0.195^{a}	0.52^{b}	1.89^{c}
21	0.72^{a}	3.14^{a}	29.82^{b}	11.15^{a}	5.29^{bc}	25.54^{c}	12.83^{a}	2.74^{c}	0.57^{b}	0.16^{d}	4.61	0.0^{b}	0.0^{d}	0.04^{ab}	0.16^{b}	0.70^{a}	2.39^{bc}
24	0.70^{ab}	2.96^{a}	30.10^{b}	10.96^{b}	5.48^{b}	26.32^{b}	12.22^{a}	3.17^{ab}	0.62^{ab}	0.17^{d}	4.36	0.0^{b}	0.07^{c}	0.04^{ab}	0.165^{b}	0.57^{b}	2.08^{bc}
$LSD_{0.05}$	2.96×10^{-2}	0.19	1.21	0.15	0.29	0.16	0.72	0.24	5.3×10^{-2}	1.16×10^{-2}	0.31	6.11×10^{-2}	3.70×10^{-2}	5.54×10^{-2}	2.64×10^{-2}	0.102	0.28

La, lauric; M, myristic; P, palmitic; S, stearic; O, oleic; and L, linoleic acid.

*Means of four readings.

[a–d]Mean in a column followed by different letters are different ($P < 0.05$).

Table 2. Comparison between actual and calculated results of carbon number (wt %)

Hours	Carbon number*											
	C48			C50			C52			C54		
	Actual	Calc.	CF	Actual	Calc.	CF	Actual	Calc.	CF	Actual	Calc.	CF
Control	3.87	3.70	1.05	39.99	38.60	1.04	45.61	45.25	1.01	10.52	8.62	1.22
12	5.20	3.61	1.44	41.55	40.03	1.04	43.60	44.15	0.99	9.89	8.70	1.14
15	3.70	3.38	1.09	44.01	43.77	1.01	41.88	41.26	1.02	10.19	7.63	1.34
18	4.79	3.38	1.42	44.49	44.47	1.00	41.10	41.15	1.00	9.62	7.96	1.21
21	2.97	3.86	0.77	43.17	40.97	1.05	43.46	43.66	1.00	10.40	8.08	1.29
24	2.84	3.66	0.78	41.25	41.06	1.00	45.18	44.02	1.03	10.73	8.32	1.29

*Means of four readings.
CF, correction factor.

Table 3. Comparison between actual and calculated results of fatty acid (wt %)

Hours	Fatty acids*														
	C14			C16:0			C18:0			C18:1			C18:2		
	Actual	Calc.	CF	Actual	Calc.	CF	Actual	Calc.	CF	Actual	Calc.	CF	Actual	Calc.	CF
Control	0.78	1.23	0.63	38.59	42.05	0.92	3.53	3.32	1.06	43.79	40.74	1.07	12.63	8.83	1.43
12	0.79	1.20	0.66	40.16	42.61	0.94	3.64	3.55	1.03	42.61	40.37	1.06	12.13	8.76	1.38
15	0.75	1.13	0.66	41.67	44.06	0.95	3.72	3.49	1.07	41.52	39.05	1.06	11.70	8.31	1.41
18	0.73	1.13	0.65	41.53	44.49	0.93	3.54	3.66	0.97	41.97	39.34	1.07	11.60	8.35	1.39
21	0.74	1.29	0.57	40.53	43.15	0.94	3.56	3.17	1.12	42.57	39.87	1.07	11.90	9.09	1.31
24	0.70	1.22	0.57	39.53	43.26	0.91	3.58	3.41	1.05	43.21	40.39	1.07	11.49	8.77	1.31

*Means of four readings.
CF, correction factor.

Tables 2 and 3 show the comparison between actual findings and calculated result of CN and FAMEs along with the correction factors (CFs). The CFs can be calculated by using following equation:

$$\text{CF} = \text{actual result}/\text{calculated result}$$

The CFs for C48, C50, C52, C16:0, C18:0, and C18:1 were found to be ≈ 1.0 showing a good agreement between calculated and actual analyses results. Using the following equations, derived by incorporating the CF, the composition of CN and FAMEs can be calculated accurately from the NARP-HPLC results.

$$\text{Actual composition of CN} = \text{CF of CN} \times \sum \text{Individual triglycerides of CN with the same CN}$$

$$\text{e.g. C52} = \text{CF of C52} \times (\text{POO} + \text{POS} + \text{PLO})$$

$$\text{Actual FAC} = \text{CF of fatty acid} \times \sum \text{Individual fatty acid found in total glycerides}$$

$$\text{e.g. C14} = \text{CF of C14} \times (\text{MPO}/3 + \text{MPL}/3)$$

By applying these equations to NARP-HPLC results, one can acquire the composition of CN and FAMEs. The applicability of these equations to the other natural oils and fats requires further investigation. With some changes made such as mobile phase (acetone–acetonitrile, 36.5:63.5), column and detector temperature (35°C) made this NARP-HPLC method more reproducible. Modification on column and detector temperature can be adjusted for the analysis of solid fats triglycerides.

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