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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF 1,3- AND 1,2(2,3)-POSITIONAL ISOMERS OF PALM-BASED DIACYLGLYCEROLS

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ABSTRACT

Reversed-phase high-performance liquid chromatography method using charged aerosol detector was developed for separation of 1,3- and 1,2(2,3)-positional isomers of palm oil- and palm kernel oil-based diacylglycerols (PO-DAG and PKO-DAG, respectively) with different equivalent carbon numbers (ECN) and without the need of sample derivatisation. In this method, step-wise gradient of acetone and acetonitrile was used and a total retention time of 28 min was attained. Identification of PKO- and PO-DAG molecular species was accomplished using synthetic DAG standards. Completeness of separation as well as identification of PKO- and PO-DAG molecular species including positional isomers with different ECN values were verified where similar elution patterns as well as the same number of identified peaks were observed for the chromatograms of PKO-DAG and PKO-DAG standards, as well as the PO-DAG and PO-DAG standards. Among the 1,3-DAG species and/or 1,2-DAG species, as ECN value of their fatty acid constituents increased, their corresponding retention time always increased. However, among the PKO- as well as PO-based synthetic DAG with the same ECN values, 1,3-DAG were always found to elute earlier than the respective 1,2-DAG. Furthermore, some exceptional examples were observed among the PKO- as well as PO-based synthetic DAG standards with the different ECN values where a few of 1,3-DAG with the relatively higher ECN values eluted earlier than 1,2-DAG with relatively lower ECN values. The last two observations evidence the defect of ECN-based prediction for identification of DAG positional isomers with the same ECN as well as for different ECN values.

Keywords: reversed-phase HPLC, CAD, palm-based diacylglycerol.

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INTRODUCTION

Diacylglycerols are esters of glycerol where two of the hydroxyl groups are esterified with fatty acids. Diacylglycerol (DAG) is naturally found as a minor component of different fats and oils at levels up to 10% (w/w). They exist in two structural isomers namely, 1,2-DAG and 1,3-DAG with a natural isomeric ratios of approximately 3:7. DAG is also used in small quantities in food as an emulsifier

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(Yasukawa and Katsuragi, 2004; Lo *et al*., 2008). Over the past two decades, DAG (specially 1,3-DAG) has gained much interest due to having nutritionally beneficial effects such as the ability to reduce serum triacylglycerol (TAG) levels (Murata *et al*., 1994; Taguchi *et al*., 2000) and body fat accumulation as well as liver TAG levels (Maki *et al*., 2002), in spite of having a comparable energy value and digestibility as known for TAG (Taguchi *et al*., 2000). DAG are also formed as the intermediate products in interesterification (Mu *et al*., 2000) and enzymatic hydrolysis of TAG (Gökmen *et al*., 2011). Therefore, the separation, identification and quantitative analysis of DAG molecular species are of particular interest in the food industry.

Several chromatographic methods for analysis of DAG have been published. In the past, gas chromatographic method with flame-ionisation detector was one of the methods usually used but it needed derivatisation of DAG (Goh and Timms, 1985) which was not only time-consuming but also unable to completely resolve the components in the complex mixtures (Marcato and Cecchin, 1996). Separation and/or quantitative analysis of underivatised acylglycerols including DAG using high-performance liquid chromatography-ultraviolet (HPLC-UV) (Riisom and Hoffmeyer, 1978; Bauza *et al*., 1992; Lin *et al*., 1997) has also been reported. However, the utilisation of an UV detector is limited by the chemical structure as well as the absorption characteristics of individual components where UV radiation absorption of unsaturated DAG and their saturated counterparts are not the same (Liu *et al*., 1993).

Extensive improvement was made using the evaporative light scattering detector (ELSD) as an universal detector whose response is the function of mass of vaporised analyte but also because it is compatible with gradient elution (Marcato and Cecchin, 1996; Liu *et al*., 1993; Lísa *et al*., 2007). Several attempts have been made for the separation of acylglycerol compositions (including 1,3- and 1,2-DAG positional isomers) using either normal-phase HPLC-ELSD or reverse-phase HPLC-ELSD (RP-HPLC-ELSD) in enzymatically glycerolised olive oil (Yang and Chen, 1991), olive oil and peanut oil containing DAG as a minor component (Liu *et al*., 1993), standard mixtures of different acylglycerols including positional isomers of dilaurin, dimyristin, dipalmitin, distearin and diarachidin (Marcato and Cecchin, 1996), different interesterified products containing DAG as the intermediate products (Mu *et al*., 2000), and in human very-low-density lipoproteins comprising positional isomers of dilinolein, dipalmitin, oleoyl-palmitoyl-glycerol and linoleoyl-oleoyl-glycerol (Perona and Ruiz-Gutierrez, 2003). Nevertheless, these studies have covered identification of only few types of DAG molecular species. This could be due to the limited availability of DAG reference standards which caused many challenging problems in the identification of DAG species.

To the best of our knowledge, very little information has so far been published concerning the separation and identification of a broad range of DAG molecular species including critical 1,2- and 1,3-isomers of DAG present in DAG oils produced from natural edible oils (Lo *et al*., 2004). In the former study, DAG molecular species of several enzymatically modified edible oils were analysed using RP-HPLC-UV. Identification of 1,2- and 1,3-positional isomers were conducted either through comparison with retention time of those commercially available DAG standards or through the prediction of elution order. The latter was performed based on the calculation of equivalent carbon number (ECN) of DAG molecular species from enzymatically-synthesised palm, soyabean, canola and corn oils as well as their binary blends (Lo *et al*., 2004). Besides the aforementioned disadvantage of UV detector, identification of 1,2- and 1,3-positional isomers of DAG species through ECN-based prediction cannot be achieved due to having the same ECN value (Lo *et al*., 2004).

Alternatively, universal charged aerosol detector (CAD) is not only a mass-dependent detector, but also does not depend on the physico-chemical properties of non-volatile compounds (like ELSD). Furthermore, CAD has a wide dynamic range of responses with high sensitivity from low ng to high μg of analytes and great accuracy for a various range of analytes. It is also more sensitive than ELSD (Vehovec and Obreza, 2010).

On the other hand, in order to obtain the precise elution order of unknown chromatographic DAG peaks, the probable corresponding DAG molecular species which were not commercially available as the standard, can be synthesised using lipase-catalysed esterification (Lo *et al*., 2007). By doing so, 1,2- and 1,3-positional isomers of individual DAG molecular species with the same ECN values would also be identifiable.

Based on this framework, the objective of this study was to develop a new method for the separation and identification of DAG molecular species (including positional isomers) of palm kernel oil- and palm oil-based DAG (as the sources of short- and medium-, and long-chain DAG, respectively) using RP-HPLC-CAD with gradient mode, and also to precisely provide the wide range of corresponding elution orders using synthetic DAG standards.

MATERIAL AND METHODS

Materials

Palm oil (PO) and palm kernel oil (PKO) were provided by Sime Darby Sdn Bhd (Banting, Selangor, Malaysia). Commercial immobilised lipase from *Candida antarctica* (*Novozym* 435) and

Rhizomucor miehei (*Lipozym RM IM*) were purchased from *Novozymes* A/S (Bagsvaerd, Denmark). Caprylic acid, capric acid, lauric acid, myrestic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, dilaurin, dimyrestin, dipalmitin, distearin, diolein, dilinolein, and glycerol were purchased from Sigma (St. Louis, USA). Sodium methoxide was purchased from Merck KgaA (Darmstadt, Germany). Hexane, acetone and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). All chemicals and solvents used were of analytical and HPLC grade, respectively.

Production of Diacylglycerol (DAG) Oils

DAG synthesis was conducted in 16 litres packed bed bioreactor consisting of a 10-litre reaction vessel and a 6-litre filtration vessel. The immobilised enzyme, *Novozym* 435 (10% of oil mass) was packed in the filtration vessel. Glycerolysis reaction was conducted through the stepwise addition of glycerol where the molar ratio of glycerol/oil was 1:1. PO (6 kg) and one-seventh of total mass of glycerol was added to the reactor vessel and heated up to 65°C while stirring using the impeller stirrer. After temperature equilibration, the mixture of oil and glycerol was passed through the packed enzyme employing the centrifugal pump at a flow rate of 850 ml min^{-1}. The rest of the glycerol was added at the following times: 0.5, 1, 1.5, 2, 2.5 and 3 hr and the reaction was continued for 7 hr. Then, the reaction mixture was removed from the bottom of the packed bed vessel and stored at -18°C for purification.

Purification of Diacylglycerol (DAG) Oils

Reaction mixture comprised glycerol, free fatty acid (FFA), monoacylglycerol (MAG), DAG and, TAG. Therefore, purification of DAG was carried out in two steps using short path distillation, KD6 system (UIC, Alzenau-Hoerstein, Germany). The separation of glycerol, FFA and MAG from the reaction mixture was done in the first step. Mixture of DAG and TAG collected from the vessel of residue was used for second purification step where the DAG was segregated from the TAG and collected from the distillate vessel. The following invariable conditions for both purification steps were used: evaporator vacuum, 0.001 mbar; feeding rate, 1.3 litres hr^{-1}; condenser temperature, 85°C; feeding tank temperature, 75°C; and roller speed, 280 rpm. The variable condition was evaporation temperature of 200°C and 250°C and they were applied in first and second steps, respectively.

Fatty Acid Composition (FAC) Analysis

FAC was determined as fatty acid methyl esters (FAME). The samples (0.05 g) were weighed and dissolved in 1 ml of hexane. The mixtures were then added with sodium methoxide solution [0.2 ml of $NaOCH_3$ (2 M) in anhydrous methanol] and then mixed for 1 min using a vortex mixer. After sedimentation of sodium glycerolate, 1 μl of the clear supernatant was injected into a Supelco, sp™ 2340 fused silica capillary column (60 m × 0.25 mm × 0.2 μ) and analysed using a Perkin Elmer Auto System excel gas chromatograph (Perkin Elmer Auto System XL, USA), equipped with a flame-ionisation detector. Injection and detection temperatures were 250°C. The oven temperature was programmed as follows: heat from 130°C to 170°C (20°C min^{-1}), heat from 170°C to 230°C (10°C min^{-1}), hold at 230°C for 10 min, heat from 230°C to 250°C (30°C min^{-1}) and hold for 1 min at 250°C. The carrier gas (nitrogen) flow rate was 50 ml min^{-1}. The peaks were identified by comparing retention times with FAME standards and quantified using peak area normalisation method. Determination was carried out in triplicate.

Synthesis of Diacylglycerol (DAG) Standards

Since the number of commercially available reference standards of DAG isomers were limited and did not cover the whole range of retention times wherein PKO- and PO-DAG isomers eluted, those inaccessible DAG reference standards determined based on possible combinations of the main FAC of DAG oils, were produced using esterification method. Lipase-catalysed esterification method was carried out according to the method of Lo *et al*. (2007) with some modifications. Synthesis was carried out in 5 ml round bottom volumetric flask. Two types of fatty acids (1:1, w/w) were added to the glycerol with the substrate molar ratios of 2.5 $litres^{-1}$ (fatty acid/glycerol) and total substrate weight of 2 g. The substrate mixture was then mixed with *Lipozym* RM IM (10% w/w) and reaction mixture was magnetically stirred at 800 rpm and incubated at 70°C for 3.5 hr. The separation of enzyme from the reaction mixture was conducted through centrifuge and impure DAG standards were kept in -20°C for further analysis.

High-performance Liquid Chromatography (HPLC) System

The DAG composition was determined using HPLC. The synthesised DAG standards (which were previously separated from the enzyme through the centrifuge) as well as PKO- and PO-DAG were individually dissolved in acetone at the concentration of 5% (v/v). They were then filtered through a 0.2 μm nylon membrane filter to remove impurities. Samples (2 μl) were then injected into LC column Shimadzu (Shim-Pack XR-ODS II, Shimadzu, Japan), a RP-C18 column with 2.2 μm particle size (150 mm × 2.0 mm) at an oven temperature of 35°C

using a Shimadzu ultra-fast liquid chromatography 2010 (Kyoto, Japan) equipped with a Shimadzu auto injector (Kyoto, Japan) and Corona Plus detector (ESA, Chelmsford, MA, USA) at a gas pressure of 35 psi within the 500 pA detection range. The following elution gradient of acetone (A) and acetonitrile (B) was used for each analysis: 0 min- 10%A+90%B, 15min- 15%A+85%B, 21 min- 93%A+7%B, 25 min- 86%A+14%B, 26 min- 10%A+90%B, 28 min- 10%A+90%B. The flow rate of 0.5 ml min^{-1} for mobile phase with the total run time of 28 min was applied. The chromatographic peaks were identified through comparison of their retention time with that of commercial and synthetic standards and quantified using peak area normalisation method.

RESULTS AND DISCUSSION

Fatty Acid Composition (FAC) Analysis

FAC of PKO-DAG and PO-DAG were displayed in *Table 1*. PKO-DAG was characterised by high contents of lauric acid (12:0, 51.3%) followed by myristic (14:0, 17.1%), oleic (18:1, 13.1%) and palmitic (16:0, 8.0%) acids as the major FA. It was also found to have appreciable amounts of caprylic (8:0, 2.9%) and capric (10:0, 3.3%) acids as the medium-chain FA. PO-DAG was characterised by high contents of palmitic (16:0, 45.8%) and oleic (18:1, 38.4%) acids followed by linoleic (18:2, 9.2%) and stearic acids (18:0, 4.3%). These FA were considered for determination of all possible DAG molecular species which must be produced as the synthetic DAG standards.

Separation and Identification of Diacylglycerol (DAG) Molecular Species

Under the RP-HPLC conditions developed through gradient elution, direct separation of PKO- and PO-DAG molecular species including 1,3- and 1,2-DAG positional isomers (with different ECN values) without the need of sample derivatisation could be obtained. This method also provided very short total retention time (RT) of 28 min for PKO-DAG and PO-DAG using a single column. PKO- and PO-DAG chromatograms are shown in *Figures 1* and *2*, respectively. Identifications of the chromatographic peaks were performed by matching the corresponding RT with those of synthesised DAG standards. Nevertheless, 1,3-dipalmitin and 1,2-diolein were observed to overlap and eluted as one peak (peak 19 in *Figure 1*, and peak 9 in *Figure 2*), and the attempts made to modify the gradient elution for their further separation were not successful.

In order to confirm complete separation of PKO- and PO-DAG molecular species (including positional isomers with different ECN values) as well as their corresponding identifications, the mixtures of synthetic PKO- (*Figure 3*) and PO-DAG standards (*Figure 4*) were run separately in the RP-HPLC system. Similar elution patterns as well as the same number of identified peak were observed for the chromatograms of PKO-DAG and PKO-DAG standards (*Figures 1* and *3*, respectively) as well as PO-DAG and PO-DAG standards (*Figures 2* and *4*, respectively). These results suggested the completeness of separation as well as identification of PKO- and PO-DAG molecular species including positional isomers with different ECN values. The FA concentrations of C8:0, C10:0 and C18:0 for PKO-DAG are relatively quite low (2.9%, 3.3% and 1.8%, respectively). Therefore, it can be concluded that among the DAG molecular species which are possibly present in chromatographic peaks 5 and 6, 7 and 8, and 14 and 16 (*Table 2* and *Figure 1*), 1,3- and 1,2-dilaurin, 1-lauroyl-3-myristoyl-glycerol and 1-lauroyl-2-myristoyl-glycerol, and 1-myristoyl-3-palmitoyl-glycerol and 1-myristoyl-2-palmitoyl-glycerol, respectively are the main DAG.

Elution Order of Synthetic Standards

RT as well as ECN value of synthetic PKO- and PO-DAG standards are shown in *Tables 2* and *3*, respectively. RT of 38 synthetic DAG standards (namely, 1-capryloyl-3-lauroyl-glycerol, 1(2)-capryloyl-2(3)-lauroyl-glycerol, 1,3-dicaprin, 1,2-dicaprin, 1-caprioyl-3-lauroyl-glycerol, 1(2)-caprioyl-2(3)-lauroyl-glycerol, 1-capryloyl-3-myristoyl-glycerol, 1(2)-capryloyl-2(3)-myristoyl-glycerol,1-caprioyl-3-myristoyl-glycerol, 1(2)-caprioyl-2(3)-myritoyl-glycerol,1-capryloyl-3-palmitoyl-glycerol, 1(2)-capryloyl-2(3)-palmitoyl-glycerol, 1-lauroyl-3-myristoyl-glycerol, 1(2)-lauroyl-2(3)-myristoyl-glycerol, 1-palmitoyl-3-caprioyl-glycerol,1(2)-palmitoyl-2(3)-caprioyl-glycerol, 1-lauroyl-3-oleoyl-glycerol, 1(2)-lauroyl-2(3)-oleoyl-glycerol, 1-lauroyl-3-palmitoyl-glycerol, 1(2)-lauroyl-2(3)-palmitoyl-glycerol, 1-myristoyl-3-oleoyl-glycerol, 1(2)-myristoyl-2(3)-oleoyl-glycerol, 1-myristoyl-3-palmitoyl-glycerol, 1(2)-myristoyl-2(3)-palmitoyl-glycerol,1-lauroyl-3-stearoyl-glycerol, 1(2)-lauroyl-2(3)-stearoyl-glycerol, 1-palmitoyl-3-oleoyl-glycerol, 1(2)-palmitoyl-2(3)-oleoyl-glycerol, 1-oleoyl-3-linoleoyl-glycerol, 1(2)-oleoyl-2(3)-linoleoyl-glycerol, 1-palmitoyl-3-linoleoyl-glycerol, 1(2)-palmitoyl-2(3)-linoleoyl-glycerol, 1-palmitoyl-3-oleoyl-glycerol, 1(2)-palmitoyl-2(3)-oleoyl-glycerol, 1-oleoyl-3-stearoyl-glycerol, 1(2)-oleoyl-2(3)-stearoyl-glycerol, 1-palmitoyl-3-stearoyl-glycerol, 1(2)-palmitoyl-2(3)-stearoyl-glycerol) tabulated in *Tables 2* and *3* have not been previously reported (Lin *et al*., 1997, Marcato and Cecchin, 1996, Lo *et al*., 2004). The DAG were arranged in *Table 2* and *3* in order of elution (decreasing polarity). The elution orders of DAG species in RP-HPLC systems were determined by their polarities where the low-

TABLE 1. FATTY ACID COMPOSITION (FAC) OF PALM KERNEL OIL-BASED DIACYLGLYCEROL (PKO-DAG) AND PALM OIL-BASED DIACYLGLYCEROL (PO-DAG)[a]

	PKO-DAG	PO-DAG
C6:0	0.2 ± 0.01	0.0
C8:0	2.9 ± 0.01	0.0
C10:0	3.3 ± 0.01	0.0
C12:0	51.3 ± 0.01	0.5 ± 0.02
C14:0	17.1 ± 0.03	1.2 ± 0.01
C16:0	8.0 ± 0.07	45.8 ± 0.03
C18:0	1.8 ± 0.01	4.3 ± 0.01
C18:1	13.1 ± 0.06	38.4 ± 0.04
C18:2	2.2 ± 0.02	9.2 ± 0.01
C20:0	0.1 ± 0.01	0.3 ± 0.01
C18:3	0.1 ± 0.01	0.3 ± 0.00

Note: [a]FAC values show the means ± standard deviation of triplicate analysis.

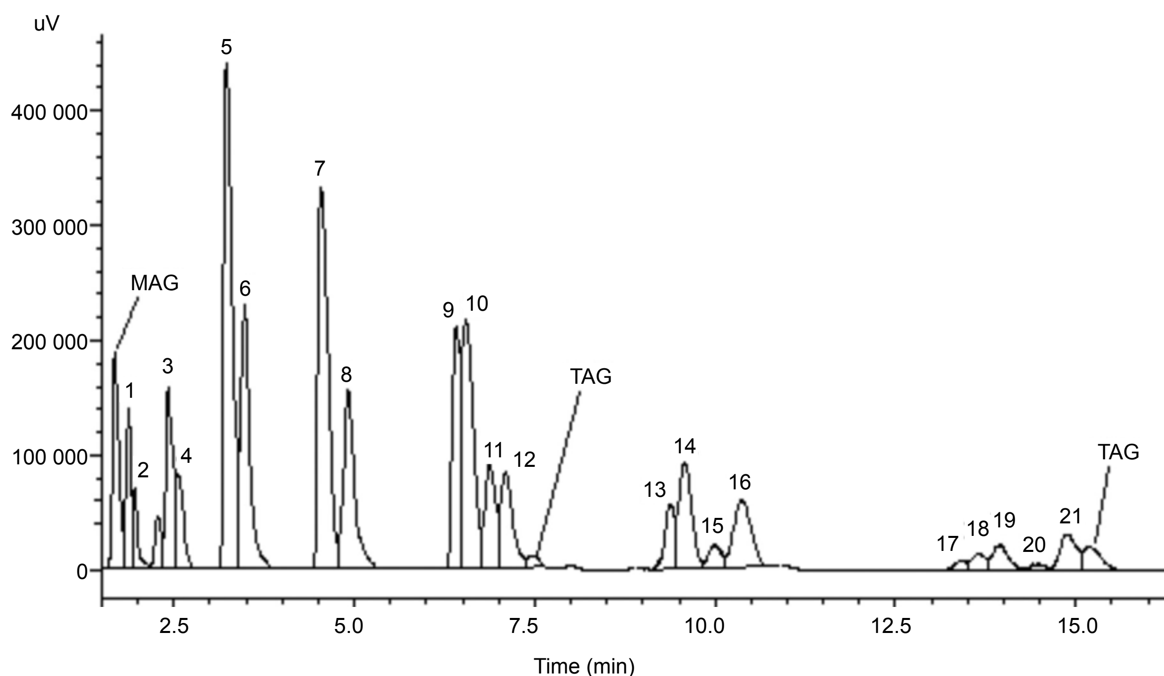

Figure 1. Reverse-phase high-performance liquid chromatography (RP-HPLC) separation of palm kernel oil-based diacylglycerol (PKO-DAG). See text for HPLC conditions and Table 2 *for peak identification.*

er polarity led to the longer RT. Elution order and thereby polarity order of DAG species with identical and/or mixed fatty acids directly depended on their FA constituents. The presence of double bond increased polarity of FA and thus decreased the RT of DAG species. Furthermore, the presence of FA with the longer chain length decreases the polarity and thereby increased the RT of DAG (Lin *et al*., 1997; 1995). Accordingly, among the 1,3-DAG species and/or 1,2-DAG species, as ECN value of their FA constituents increased, their corresponding RT always increased (*Tables 2* and *3*). This was not surprising, since the ECN value of acylglycerols are the sum of ECN values of their FA constituents (Podlaha and Töregård, 1989), and it increased by increasing the chain length of the FA constituent of DAG and decreased as the number of double bond increased (Lo *et al*., 2004).

Among the PKO- as well as PO-based synthetic DAG with the same ECN value, 1,3-DAG were always found to elute earlier than the respective 1,2-DAG (*Tables 2* and *3*). This result was in line with Lin *et al*. (1997) and Lo *et al*. (2004) suggesting that DAG with a hydroxy group at *sn*-2 position of the glycerol

TABLE 2. EQUIVALENT CARBON NUMBER (ECN), PEAK NUMBER AND RETENTION TIME OF SYNTHETIC PALM KERNEL OIL-BASED DIACYLGLYCEROL (PKO-DAG) STANDARDS

Synthetic standards	ECN[a]	Peak number	Retention time (min)
1-Capryloyl-3-lauroyl-glycerol & 1,3-Dicaprin	20	1	1.86
1-Capryloyl-2-lauroyl-glycerol & 1,2-Dicaprin	20	2	1.95
1-Caprioyl-3-lauroyl-glycerol & 1-Capryloyl-3-myristoyl-glycerol	22	3	2.41
1-Caprioyl-2-lauroyl-glycerol & 1-Capryloyl-2-myristoyl-glycerol	22	4	2.55
1,3-Dilaurin, 1-Caprioyl-3-myristoyl-glycerol & 1-Capryloyl-3-palmitoyl-glycerol	24	5	3.28
1,2-Dilaurin, 1-Caprioyl-2-myristoyl-glycerol & 1-Capryloyl-2-palmitoyl-glycerol	24	6	3.51
1-Lauroyl-3-myristoyl-glycerol & 1-Palmitoyl-3-Caprioyl-glycerol	26	7	4.62
1-Lauroyl-2-myristoyl-glycerol & 1-Palmitoyl-2-Caprioyl-glycerol	26	8	4.96
1-Lauroyl-3-oleoyl-glycerol	27.05	9	6.48
1-Lauroyl-3-palmitoyl-glycerol & 1,3-Dimyristin	28	10	6.58
1-Lauroyl-2-oleoyl-glycerol	27.05	11	6.92
1-Lauroyl-2-palmitoyl-glycerol & 1,2-Dimyristin	28	12	7.09
1-Myristoyl-3-oleoyl-glycerol	29.05	13	9.42
1-Myristoyl-3-palmitoyl-glycerol & 1-Lauroyl-3-stearoyl-glycerol	30	14	9.57
1-Myristoyl-2-oleoyl-glycerol	29.05	15	10.05
1-Myristoyl-2-palmitoyl-glycerol & 1-Lauroyl-2-stearoyl-glycerol	30	16	10.36
1,3-Diolein	30.1	17	13.25
1-Palmitoyl-3-oleoyl-glycerol	31.05	18	13.57
1,3-Dipalmitin & 1,2-Diolein	32 & 30.1	19	13.90
1-Palmitoyl-2-oleoyl-glycerol	31.05	20	14.47
1,2-Dipalmitin	32	21	14.95

Note: [a] ECN values of DAG were calculated as sum of their fatty acids ECN values (Lo *et al.*, 2004).

backbone was slightly more polar than the hydroxy groups at *sn*-1 and/or *sn*-3. Stereospecific isomers of 1,2-DAG (*sn*-1,2-DAG and *sn*-2,3-DAG) may coexist in the DAG sample and may have slightly different polarity and thereby slightly different elution order (Lo *et al*., 2004). However, such a small discrepancy between polarity of 1,2- and 2,3-DAG was too close to be resolved with RP-HPLC condition used.

As previously mentioned, the prediction of elution order of 1,2- and 1,3-DAG using ECN value was not possible, which was due to the fact that the location of fatty acyl groups on the acylglycerol molecule had no measurable effect on the ECN value (Lo *et al*., 2004). Several examples were found among the PKO- as well as PO-DAG species showing the deficiency of ECN-based prediction for identification of DAG positional isomers with different ECN values which have not been previously reported using synthetic DAG standards (*Tables 2* and *3*). Among the PKO-DAG, 1-lauroyl-3-palmitoyl-glycerol and/or 1, 3-dimyristin (ECN=28) were observed to elute earlier than 1-lauroyl-2-oleoyl-glycerol (ECN=27.05), and 1-myristoyl-3-palmitoyl-glycerol and/or 1-lauroyl-3-stearoyl-glycerol showed lower RT (9.57 min) than 1-myristoyl-2-oleoyl-glycerol (10.05 min) despite having higher ECN value (30 and 29.05, respectively). Also, 1,3-dipalmitin (ECN=32) eluted earlier than 1-palmitoyl-2-oleoyl-glycerol (31.05) (*Table 2*). The PO-DAG positional isomers were also observed to have some exceptional

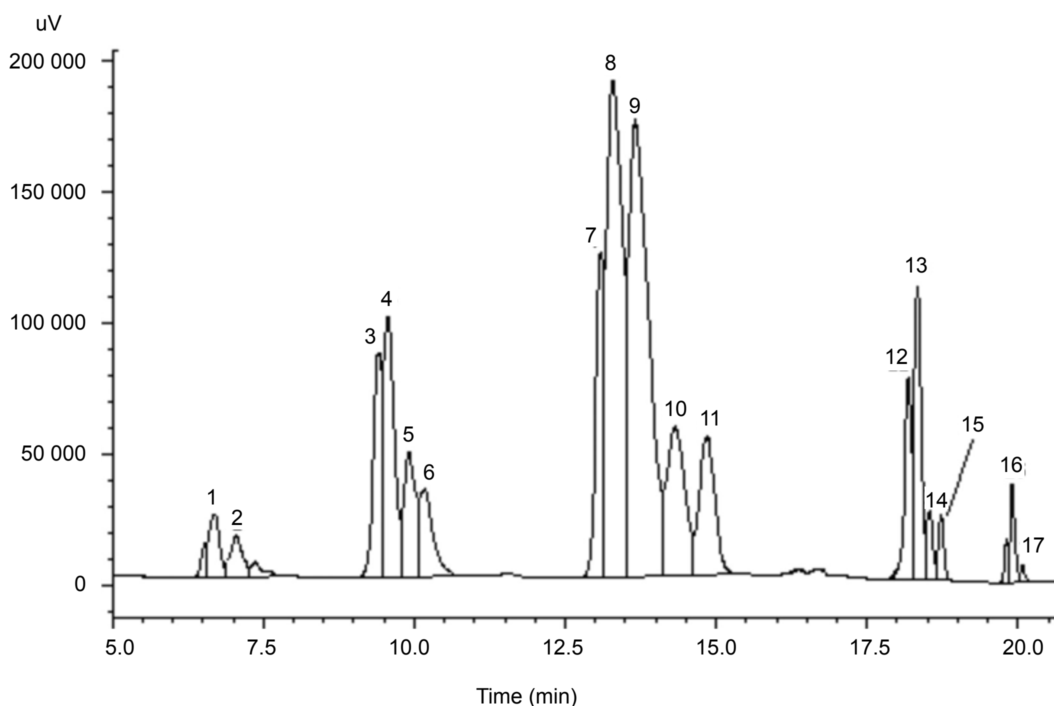

Figure 2. Reverse-phase high-performance liquid chromatography (RP-HPLC) separation of palm oil-based diacylglycerol (PO-DAG. See text for HPLC conditions and Table 3 *for peak identification.*

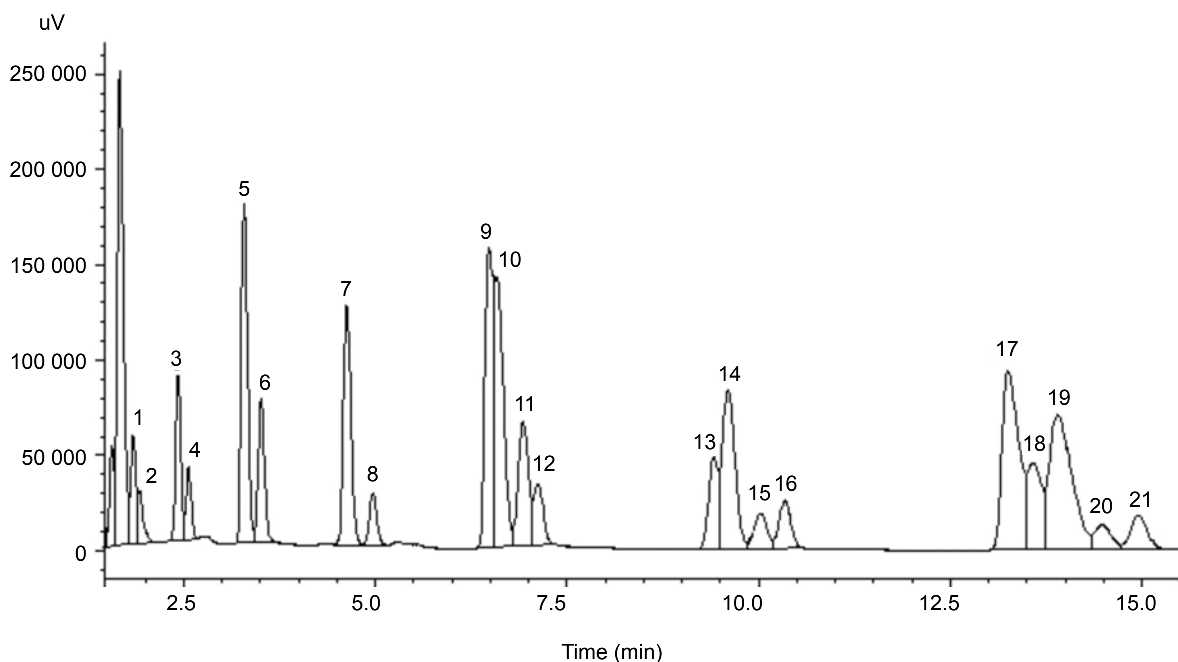

Figure 3. Reverse-phase high-performance liquid chromatography (RP-HPLC) separation of synthetic palm kernel oil-based diacylglycerol (PKO-DAG) standards. See text for HPLC conditions and Table 2 *for peak identification.*

elution orders as follow: 1-palmitoyl-3-linoleoyl-glycerol (ECN=28.73)<1-oleoyl-2-linoleoyl-glycerol (ECN=27.78), 1,3-dipalmitin (ECN=32)<1-palmitoyl-2-oleoyl-glycerol (ECN=31.05) and 1-palmitoyl-3-stearoyl-glycerol (ECN=34)<1-oleoyl-2-stearoyl-glycerol (ECN=33.05) (*Table 3*). These valuable and precise elution orders of DAG positional isomers for such complex mixtures of DAG oil could only be obtained using synthesis of a wide range of DAG standards.

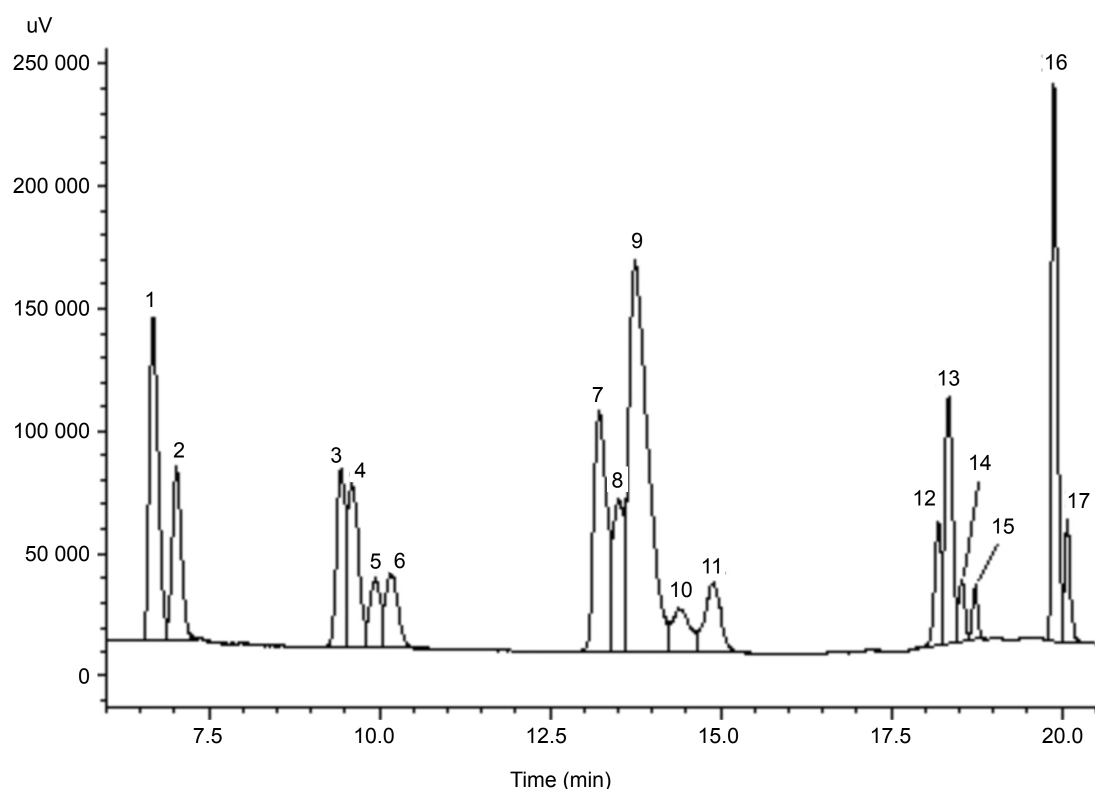

Figure 4. Reverse-phase high-performance liquid chromatography (RP-HPLC) separation of synthetic palm oil-based diacylglycerol (PO-DAG) standards. See text for HPLC conditions and Table 3 *for peak identification.*

TABLE 3. EQUIVALENT CARBON NUMBER (ECN), PEAK NUMBER AND RETENTION TIME OF SYNTHETIC PALM OIL-BASED DIACYLGLYCEROL (PO-DAG) STANDARDS

DAG species	ECN[a]	Peak number	Retention time (min)
1,3-Dilinolein	25.46	1	6.67
1,2-Dilinolein	25.46	2	7.04
1-Oleoyl-3-linoleoyl-glycerol	27.78	3	9.39
1-Palmitoyl-3-linoleoyl-glycerol	28.73	4	9.58
1-Oleoyl-2-linoleoyl-glycerol	27.78	5	9.93
1-Palmitoyl-2-linoleoyl-glycerol	28.73	6	10.16
1,3-Diolein	30.1	7	13.10
1-Palmitoyl-3-oleoyl-glycerol	31.05	8	13.49
1,3-Dipalmitin & 1,2-Diolein	32 & 30.1	9	13.73
1-Palmitoyl-2-oleoyl-glycerol	31.05	10	14.37
1,2-Dipalmitin	32	11	14.86
1-Oleoyl-3-stearoyl-glycerol	33.05	12	18.18
1-Palmitoyl-3-stearoyl-glycerol	34	13	18.34
1-Oleoyl-2-stearoyl-glycerol	33.05	14	18.53
1-Palmitoyl-2-stearoyl-glycerol	34	15	18.73
1,3-Distearin	36	16	19.91
1,2-Distearin	36	17	20.08

Note: [a] ECN values of DAG were calculated as sum of their fatty acids ECN values (Lo *et al.*, 2004).

CONCLUSION

The RP-HPLC-CAD method developed here allowed the direct separation of PKO- and PO-DAG molecular species including 1,3- and 1,2-isomers (with different ECN values) without the need for sample derivatisation. The separation of all components was accomplished within 28 min using a single run. The identification of DAG species was conducted using synthetic DAG standards. Furthermore, the elution characteristics of synthetic PKO- and PO-DAG standards reported in this article can be useful in identifying DAG molecular species when the standards are unavailable.

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