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ORIGINAL PAPER

Regiospecific Analysis of Mono- and Diglycerides in Glycerolysis Products by GC × GC-TOF-MS

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Abstract Comprehensive bidimensional gas chromatography coupled with time-of-flight mass spectrometry (GC × GC-TOF-MS) was used for the characterization of regiospecific mono- and diglycerides (MG-DG) content in the glycerolysis products derived from five different lipids included lard (LA), sun flower seed oil (SF), corn oil (CO), butter (BU), and palm oil (PA). The combination of fast and high temperature non-orthogonal column set namely DB17ht (6 m × 0.10 mm × 0.10 μm) as the primary column and SLB-5 ms (60 cm × 0.10 mm × 0.10 μm) as the secondary column was applied in this work. System configuration involved high oven ramp temperature to obtain precise mass spectral identification and highest effluent's resolution. 3-Monopalmitoyl-*sn*-glycerol (MG 3-C16) was the highest concentration in LA, BU and PA while monostearoyl-*sn*-glycerol (MG C18) in CO and 1,3-dilinoleol-*rac*-glycerol (DG C18:2c) in SF. Principal component analysis accounted 82% of variance using combination of PC1 and PC2. The presence of monostearoyl-*sn*-glycerol (MG C18), 3-Monopalmitoyl-*sn*-glycerol (MG 3-C16), 1,3-dilinoleol-*rac*-glycerol (DG C18:2c), 1,3-dipalmitoyl-glycerol (DG 1,3-C16), and 1,3-dielaidin (DG C18:1t) caused differentiation of the samples tested.

Keywords Monoglycerides · Diglycerides · Comprehensive two-dimensional gas chromatography · GC × GC · Time-of-flight · Mass spectrometry · PCA

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Introduction

Monoglycerides (MG) and diglycerides (DG) are widely used as emulsifying agents for product stabilization in food, cosmetic and pharmaceutical for several decades [1] as well as the intermediate in the biosynthesis of triglycerides (TG). Currently, the manufacturing process of MG- and DG compounds (MG-DG) involves interesterification of glycerines with TG obtained from plants or animals at 200–260 °C in the presence of an inorganic alkali as a catalyst [2]; or through enzymatic glycerolysis [3]. As the product of the glycerolysis process, MG-DG comprise a varied fatty acid distribution coupling to glycerin either in the α- or *β*- position [4, 5]. Furthermore, the product with a higher α-mono content is always demanded due to its greater emulsifying behavior and functionality as the intermediate for organic synthesis of structured lipids, phospholipids, glycolipids as well as enzyme agonists and antagonists [1]. In the field of lipid chemistry, segregation of regiospecific isomers of MG-DG is also an important approach.

The general method for MG-DG analysis is the IUPAC-AOAC standard method which applies one dimensional gas chromatography (GC) after the trimethylsilyl ether (TMS) derivatization. The weakness of this method is that most of the saturated compounds cannot be separated from the unsaturated ones [6]. A pre-filtration process using solid phase extraction (SPE) has been employed to remove TG from the glyceride products [7], MG-DG samples purification, compound isolation and removal of excess reagent [8] prior to GC analysis. Deng et al. [9] recently described a direct separation method for both MG-DG compounds using a column system in high performance liquid chromatography without any derivatization step. However, the baseline resolution for molecular species with different acyl groups was difficult to achieve with such a technique.

Comprehensive two-dimensional gas chromatography (GC × GC) has been used widely in various fields namely petrochemical, environment, food and essential oil in the past few years to provide reliable analysis for complex specimens [10]. In GC × GC analysis, compounds are processed through combination of two different type columns operating simultaneously with enhanced sensitivity, peak capacity, and minimal detectable concentrations [11]. Nevertheless, the common drawback of this emerging technique is the need for a relatively longer second dimension run time which in turn imposes a slower elution condition for the primary dimension for proper modulated sampling [10]. Application of short length microbore columns is becoming popular to reduce the time of analysis. An enantioselective separation of several types of essential oil [12] and oxime compounds [13] has successfully achieved better peak performance than those in conventional method and in a shorter time.

Determination of glyceride mixtures remain a challenging prospect for the chemical analysis of foods, cosmetics, and pharmaceuticals. The approach of using a GC × GC technique incorporating fast GC microbore columns as the primary and secondary dimension was demonstrated in this study to rapidly unravel the composition of MG-DG in the products of the glycerolysis process from five different TG origins, which were oils of lard, sun flower seed, corn, butter and palm.

Materials and Methods

Materials

Oils of sun flower seed (SF), corn (CO), butter (BU) and palm (PA) were purchased from local markets in Selangor, Malaysia. Lard (LA) was extracted from the subcutaneous tissue of pigs using microwave extraction as described by De Pedro et al. [14]. Chromatographic grade pyridine, Sylon derivatization reagent, and the following MG-DG standards were obtained from Sigma–Aldrich (St. Louis, MO, USA): 1-Monooctanoyl-*rac*-glyceryl (MG C8), 3-Monopalmitoyl-*sn*-glycerol (MG 3-C16), 1-*cis*-13-decosenoyl-*rac*-glycerol (MG C22), 1,2-dipalmitoyl-*sn*-glycerol (DG 1,2-C16), 1,3-dipalmitoyl-glycerol (DG 1,3-C16), 1,3-distearoyl-glycerol (DG 1,3-C18), 1,3(2)-dioleoyl-glycerol (DG C18), 1,2-dioleoyl-*sn*-glycerol (DG 1,2-C18), 1,3-dilinoleol-*rac*-glycerol (DG C18:2c), and 1,3-dielaidin (*trans*-9-c18:1, DG C18:1t). All MG-DG standards had a purity of >98%. Analytical grade solvents of glycerine, hexane, diethyl ether, chloroform, methanol, dichloromethane and sodium hydroxide pellets were obtained from Fisher Scientific (Fair Lawn, NJ, USA). SPE cartridge with 500 mg/3 ml diol content was purchased from Agilent (Palo Alto, CA, USA).

Preparation of Standard Solutions and Samples

The MG-DG standard solutions (with a concentration range of 1–10 mg/ml) were prepared by dissolving appropriate amounts of MG-DG in pyridine. For the preparation of the samples, a 35-g oil sample was mixed with 15 g glycerine solution and 0.2 g sodium hydroxide catalyst, the mixture was then heated at 250 °C with vigorous mixing for 60 min. Excessive glycerine was then washed away with distilled water from the resulting glyceride product prior to SPE filtration of the TG compounds.

Since TG compounds remaining in the mixture would cause difficulties in the time-of-flight mass spectrometry (TOF-MS) operation due to their high molecular weight, SPE filtration with diol-phase was applied to restrict the elution of TG in the analysis as described previously [7]. The cartridge was first conditioned with 4 ml hexane solution, and then a 500-µl sample (50 mg/ml hexane) was loaded onto the cartridge. Then 6 ml of a mixture of hexane, dichloromethane and diethyl ether at a ratio of 78:20:2 was used to flush the TG compound from the cartridge followed by a 4-ml mixture of chloroform and methanol at a ratio of 1:1 to obtain the remaining fractions of MG-DG compounds. The last eluted fraction was dried under vacuum, dissolved in 200 µl pyridine and subjected to TMS derivatization with 200 µl Sylon reagent. The 0.1 µl TMS-derivatized sample was then injected into the GC × GC-TOF-MS system.

GC × GC–TOF-MS Configuration

An Agilent model 6890 N GC with a Leco dual-stage Thermal Modulator coupled to a TOF-MS (LECO Pegasus 4D, USA) was used to analyze TMS MG-DG compounds. The injector was heated at 350 °C under a split ratio of 100:1. A DB17ht capillary column (6 m × 0.10 mm × 0.10 µm) from J & W Scientific (Agilent technologies, Palo Alto, CA, USA) and a SLB-5 ms capillary column (60 cm × 0.10 mm × 0.10 µm) from Supelco (Bellefonte, PA, USA) were used as the primary and secondary dimensions, respectively, in the GC × GC analysis at a constant flow-rate of 0.5 ml/min. The column used in the first dimension was a mid-polar column coated with 50%-phenyl-methylpolysiloxane. It can be used at temperatures up to 365 °C with excellent peak shape and faster elution times for high boilers [15]. The apolar microbore column with 5% diphenyl/95% methyl siloxane coating was used for the second dimension separation. This column offers less bleeding, inertness, durability, and consistency [16]. The primary oven was initially programmed at 120 °C for 1 min, ramped to 220 °C at a rate of 50 °C/min, then increased to 290 °C at 10 °C/min rate, and finally increased to 330 °C at 5 °C/min and put on hold for 1 min.

The oven ramping program in the secondary dimension was set at 15 °C higher than the primary dimension for each oven ramping step. The thermal modulator was controlled at an offset temperature of 55 °C and a 2-s modulation period with a hot pulse at 0.5 s. TOF-MS was connected to the GC through a transfer line heated at 350 °C. The electron impact was operated at 230 °C with a mass scan ranging from 50 to 800 *m*/*z* at 1,700 V detector voltage.

Data were collected using 150 spectra per second acquisition rate and performed automatically using automated peak find and true signal deconvolution of the ChromaTOF software version 3.34 (Leco, St. Joseph, MI, USA). The data was screened for 50–800 molecular weights and under a threshold of 100 signals per noise. Peaks identification and library search was accomplished by comparing their mass spectra to the NIST library version 2.0, authentic standards, as well as published data [17]. Normalized percentages of MG-DG compounds were analyzed by analysis of variance (ANOVA) to identify differences among means using SAS statistical computer package software version 6.12 (SAS Cary, NC) and principal component analysis (PCA) using Unscramble software version 9.6 (CAMO Software AS, Oslo, Norway).

Results and Discussion

Configuration of Instrument Setting

Fats and oils used in this study were composed of complex mixtures of molecular species; varying from simple free fatty acids, MG, DG, TG, phospholipids to other minor components. Direct injection of oil or fat to the GC system should be avoided since large amounts of TG content would enter the column and this would require higher an oven ramp temperature to be eluted over a longer time [18]. For effective isolation of MG-DG, all other components from the fats and oils should be removed prior to GC analysis. Previous studies [7, 8, 19, 20] have reported that an SPE diol cartridge served as support for isolating MG/DG and showed negligible isomerization of 1,2 (2,3)-isomer converted to 1,3-isomer, or vice versa, compared to other SPE bonded phases and absorbents. The passage of an admixture of dipalmitin and oleic acid through diol-phase column proved that the presence of free fatty acids does not produce isomerization during the isolation of the corresponding fraction [18].

The determination of MG-DG by gas chromatography involves the conversion of MG-DG into more volatile trimethylsilyl ether derivatives by using a silylation agent before injecting into the GC system. Individual MG-DG compounds were separated under the non-orthogonal separation condition. The MG group could be distinguished from the DG grouping based on their boiling points and molecular masses along the primary dimension and the degree of saturation of fatty acids at the glycerol backbone on the secondary dimension. The separation in the first dimension was based on both the volatility and polarity of the compounds. The separations in the first and second dimensions were not independent of each other since the volatility plays a role in the second dimension as well [10]. This particular column combination allows separation of the DG on the basis of their carbon number, the 1,2- and 1,3- isomeric structure and the number of double bonds [7]. The fast GC × GC-TOF-MS system in this work employed the short microbore column combination in order to achieve the desired resolution of the compounds of a mixture in the shortest possible analysis time and minimize the band broadening. Relatively low flow rates and a high level of chromatographic separation was able to be achieved by using that column combination. In a previous work, Chin et al. [21] demonstrated that the utilization of fast GC × GC approach in analysis of fatty acid gave a three-fold shorter analysis time compared to that needed by conventional GC × GC analysis.

The optimization of GC × GC separations was done on the basis of a modulation setting and oven temperature program which were crucial in improving the resolution, sensitivity and peak structure. Modulation becomes the key bottleneck factor and thus limiting the performance of the bidimensional GC [11]. The modulator temperature offset was limited by the secondary column maximum temperature setting. That was the reason why the temperature setting for the secondary column in the GC method becomes very important. In this study, by maintaining the modulation offset at 55 °C, the release of the cold-trapped effluent could be achieved without the degradation of analytes. Three different initial temperature programs with the same oven ramp temperature were applied to find the best separation of mixture. The temperature of the second column was programmed at 15 °C higher than temperature of the primary column. Figure 1 shows that a high initial temperature gave better separation in MG (α, β)-C18:1. The peak shape of the modulated MG C18:1 cluster became inappropriate under lower initial temperature of 60 °C. Increasing the temperature up to double had the effect that MG α-C18:1 was clearly separate from MG β-C18:1. As the reliable method for the analysis of volatile and semivolatile compounds, initial temperature and oven temperature control contribute most to the analysis time, especially in fast GC × GC separation. Klee and Blumberg [22] mentioned that some important theoretical concepts could be used in routine GC method to optimize the time of analysis, which increased the carrier gas flow-rate and temperature-heating rate; used a faster carrier gas

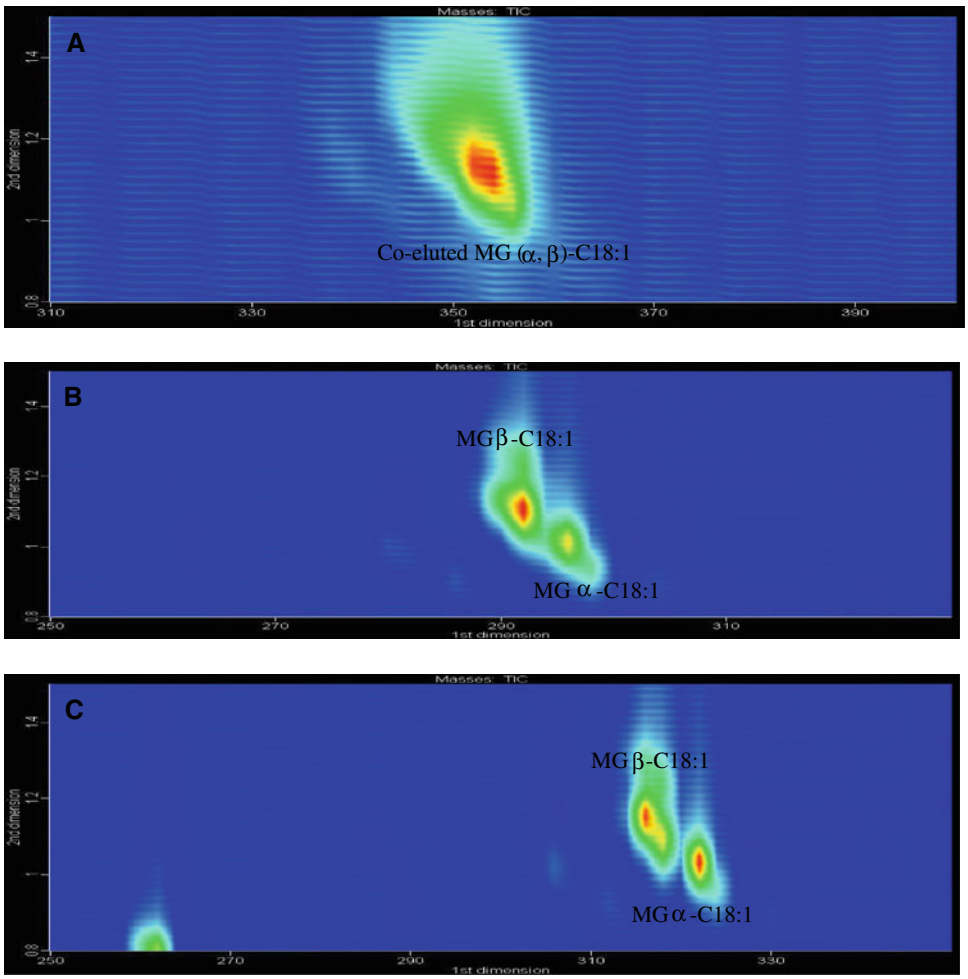

Fig. 1 GC × GC chromatograms of MG (α, β)-C18:1 separation using different primary oven temperature programs: **a** 60 °C (0.5 min)–80 °C/min to 220 °C–10 °C/min to 300 °C–5 °C/min to 335 °C (2 min), **b** 105 °C (0.5 min)–80 °C/min to 220 °C–10 °C/min to 300 °C–5 °C/min to 335 °C (2 min); **c** 120 °C (0.5 min)–50 °C/min to 220 °C–10 °C/min to 300 °C–5 °C/min to 335 °C (2 min); secondary oven temperatures were set 15 °C higher than primary oven temperature

(hydrogen); reduced the column length, the column internal diameter, and the thickness of the stationary phase; and used the detector that operates at a lower outlet pressure.

Distribution of MG-DG of Glycerolysis Products

Chromatograms of the TMS derivatives of a mixture of even-carbon MG-DG from five types of common oils and fats (PA, LA, SF, CO, and BU) are illustrated in Fig. 2. Regardless of this, complete separation of all MG-DG groups was achieved using the fast GC × GC-TOF-MS approach, thus illustrating an even distribution of complex mixture compounds along the contour plots under the non-orthogonal system. TMS components were clustered orderly by shifting the modulation offset accordingly with a different carbon chain length and level of unsaturated bonds. The MG groups were eluted separately earlier than the DG groups; both were eluted gradually according to the number of carbon atoms in the fatty acids component and unsaturated degree or number of double bonds. MG clusters left the column before 500 s and DG came up after 600 s. In general, there were several distinct groups representing even-carbon C12–C18 in the MG–DG profile. Within each group, they were further divided into sub-groups based on the positional distribution of the fatty acid chains. The 1,2-isomer of DG was eluted just ahead of the corresponding 1,3-isomers. Compounds with short carbon chain had a higher volatility level than those compounds with a long carbon chain. The contour plot chromatograms illustrated a detail distribution of MG-DG for each sample tested and in comparison with the standards shown in Fig. 2. Since the products during the GC analysis were the TMS derivatives of glyceride compounds, there would not be any matching mass spectra in the current library. However, the mass spectra were built from the TMS derivatives of the authentic standards instead as well as published data [17].

LA and PA contain relatively complete MG, from C11 to C18 as well as DG (Table 1). MG 3-C16 and MG C18 compounds were found to be present in abundance in all tested samples. There were a few other components where the level was noticeably high of certain specific species. They were DG C18:1t in LA; DG C18:2c and DG C18:1t in SF; DG C18:2c and DG C18:1 in CO; DG 1,3-C16 in BU; DG 1,3-C18 and DG C18:1t in PA. SF contains only

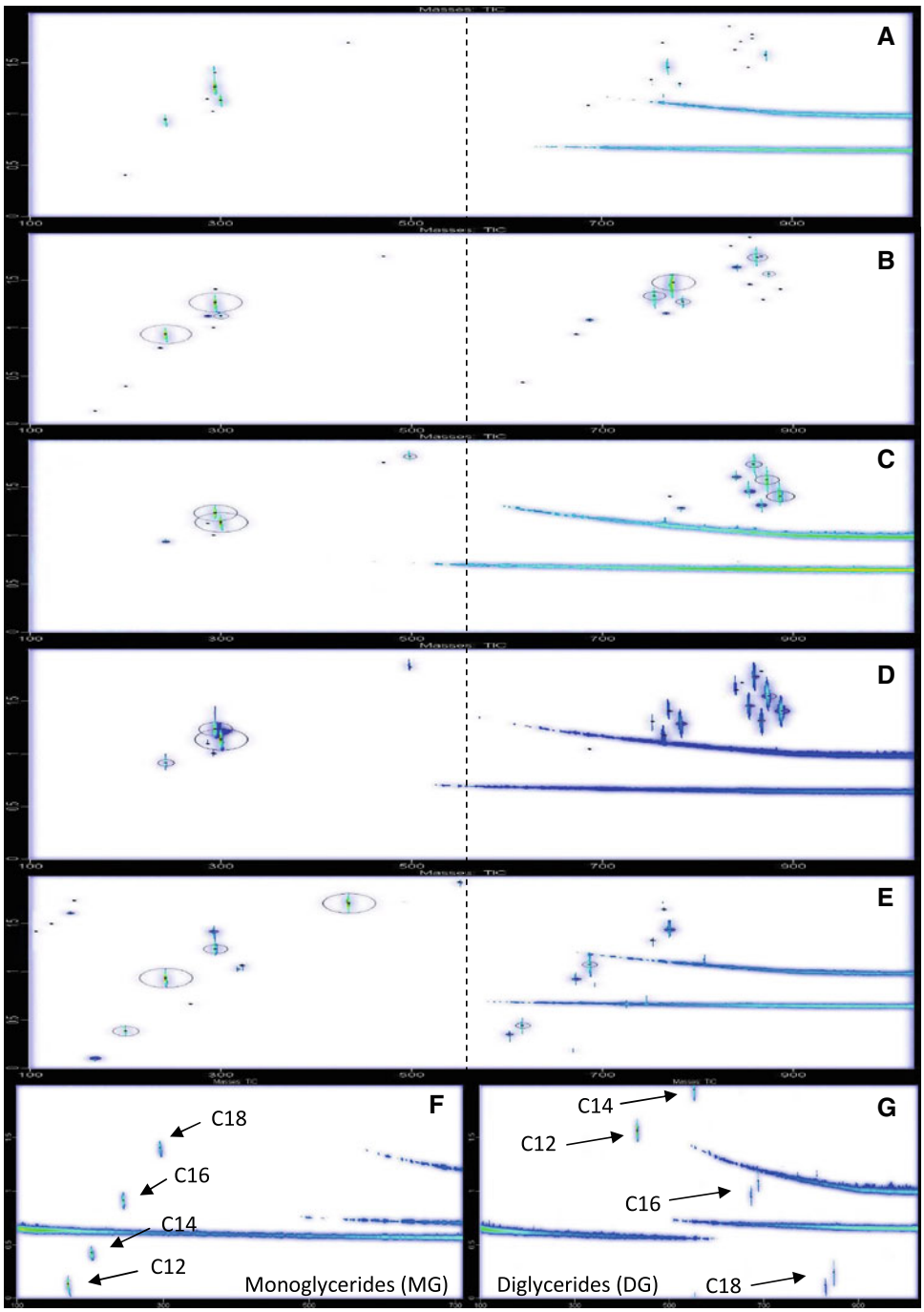

Fig. 2 Contour plot of overall TMS of MG (*left*) and DG (*right*) distribution in samples derived from: **a** palm oil, **b** lard, **c** sun flower seed oil, **d** corn oil, **e** butter, **f** MG C12-C18 standards, **g** DG C12-C18 standards

long-chain MG-DG, in contrast to BU which has short-chain MG and DG. The results presented here were in agreement with a previous study done by Peres-Camino et al. [18] which stated that sun flower seed oil does not have dipalmitine in its DG profiles. SF has only a few types of MG and DG, which are palmitine in the *sn*-3 position and stearine for MG, while C18:1t and C18:2c for DG and does not contain any short-chain fatty acids in the glycerol backbone. SF and CO contain only long-chain DG while 3-C16 and C18 in their MG groups, which CO has higher amount than those of SF. C18:1t and C18:2c were the main DG groups present in SF.

Using a combination of a non-orthogonal column set, DG with C18 fatty acid does not come out in BU. Fagan et al. [7] demonstrated the presence of C18 in DG structure from butterfat samples using a BD 17ht column. The same result was found by Bareth et al. [19] using a BD5 column. The existence of MG C10 and DG 1,3-C14 is not found in any species tested except BU. Hexadecanoic acid or C16 is the fatty acid attached in the MG-DG structure in LA and

Table 1 Concentration of MG-DG in lard (LA), sun flower seed oil (SF), corn oil (CO), butter (BU), and palm oil (PA)

Analyte	Concentration (%)				
	LA	SF	CO	BU	PA
Monodecanoyl-*sn*-glycerol (MG C10)	nd	nd	nd	0.88 (0.31)	nd
Monoundecanoyl-*sn*-glycerol (MG C11)	nd	nd	nd	0.53^{a} (0.04)	0.06^{b} (0.08)
Monododecanoyl-*sn*-glycerol (MG C12)	0.23^{b} (0.31)	nd	nd	0.88^{a} (0.45)	0.04^{b} (0.06)
Monotetradecanoyl-*sn*-glycerol (MG C14)	0.09^{b} (0.03)	nd	nd	6.03^{a} (0.83)	0.04^{b} (0.02)
2-Monopalmitoyl-*sn*-glycerol (MG 2-C16)	0.56^{a} (0.07)	nd	nd	0.45^{a} (0.63)	0.22^{a} (0.30)
3-Monopalmitoyl-*sn*-glycerol (MG 3-C16)	12.04^{b} (0.77)	1.78^{d} (0.71)	5.99^{c} (2.13)	16.94^{a} (0.69)	11.99^{b} (0.44)
Monostearoyl-*sn*-glycerol (MG C18)	12.00^{a} (6.69)	0.30^{b} (0.42)	12.35^{a} (1.14)	7.08^{ab} (1.51)	0.36^{b} (0.04)
1,3-Ditetradecanoyl-*sn*-glycerol (DG 1,3-C14)	nd	nd	nd	1.41 (0.76)	nd
1,2-Dipalmitoyl-*sn*-glycerol (DG 1,2-C16)	0.32^{b} (0.38)	nd	nd	5.78^{a} (1.28)	0.61^{b} (0.86)
1,3-Dipalmitoyl-glycerol (DG 1,3-C16)	1.08^{b} (0.66)	nd	0.03^{b} (0.05)	10.34^{a} (0.70)	1.77^{b} (2.50)
1,3-Distearoyl-glycerol (DG 1,3-C18)	1.59^{b} (0.62)	nd	2.33^{b} (0.31)	nd	4.26^{a} (0.23)
Dioleoyl-glycerol (DG C18:1)	1.58^{b} (0.05)	nd	6.13^{a} (0.28)	nd	0.70^{c} (0.01)
1,3-Dielaidin (DG C18:1t)	3.25^{ab} (1.56)	8.87^{a} (2.81)	2.51^{ab} (3.55)	nd	3.86^{ab} (3.55)
1,3-Dilinoleol-*rac*-glycerol (DG C18:2c)	0.30^{b} (0.05)	11.53^{a} (5.93)	14.31^{a} (0.26)	nd	0.02^{b} (0.02)

Value in the same row with different letter that indicate there are significant varies among the species ($p < 0.05$ under Duncan test)

The concentrations shown are the average value for six replicate analyses of single lipid extracts. Standard deviation values are shown in parentheses

nd not detected

PA, which is present in a significant amount. Monoglyceride with palmitin in the third position of acylglycerol backbone appeared to have the highest concentration in comparison to others. Similar configuration of fatty acids in MG–DG structure of LA and PA could present the possibility of them substituting each other.

In the pharmaceutical industry, mixtures of MG, DG and TG of caprylic (C8) and caproic (C10) acids are widely utilized to maximize the oral bioavailability of poorly soluble drugs [23]. In the respect, there is the need for a further investigation of the effect of differing ratios of MG, DG and TG should be done in the next work, as well as the ratio of C8 and C10 in increasing the degree of absorption and also the functionalities of pharmaceuticals and drugs. The GC × GC-TOF-MS and in combination with various column sets could be used for this purpose.

Principle Component Analysis of Glycerolysis Products

Principal component analysis (PCA) was used to evaluate clusters of the normalized MG-DG from different samples. PCA is a well known pattern recognition technique which visualizes data in a reduced hyperspace defined by the principal component. It is an unsupervised data projection method that manipulates the data in a way that the points can be displayed on an *x, y*-coordinate system. Figure 3a shows the PCA score plot of MG-DG of LA, PA, SF, CO and BU, which represent the projection of samples defined by principal component 1 (PC1) and principal component 2 (PC2). PC1 is the linear combination of variables that explain the highest variation among the samples, while PC2 is orthogonal to PC1 and exhibited the second largest variation. The score plot will give a better visualization of the MG-DG data profile shown in Table 1. Figure 3a showed that variation occurred for each sample, although they were well-separated along the PC coordinate. PC1 accounted for 57% of the variation while PC2 described 25% of the variation, making up of 82% variance. BU and CO clusters were well-separated along the PC1 axis while PC2 displaced the BU and CO clusters along its positive side and the PA cluster along the negative side. LA and SF clusters were laid in between the positive and negative side of the PC1 axis. Whilst, LA was on the negative side of PC2 and the SF cluster was on the opposite. The LA cluster was clearly distinguished from the other samples which was placed in the middle of the PC1 and PC2 coordinate.

Analysis of the loading plot of the principal components was done to find out which variables influence the separation of samples. The PCA loading plot (Fig. 3b) represents the visualization of variables in the same type as the score plot. The important contribution of a particular component is described by the absolute value of the loading in a component. Variables with a greater contribution to the model will be positioned further away from the origin. Based on Fig. 3b, the main compounds that form the basis separation of LA clustering were components MG C18, MG 3-C16, DG C18:2c, DG 1,3-C16, and DG C18:1t. This result was in agreement with our previous report [19] explaining that

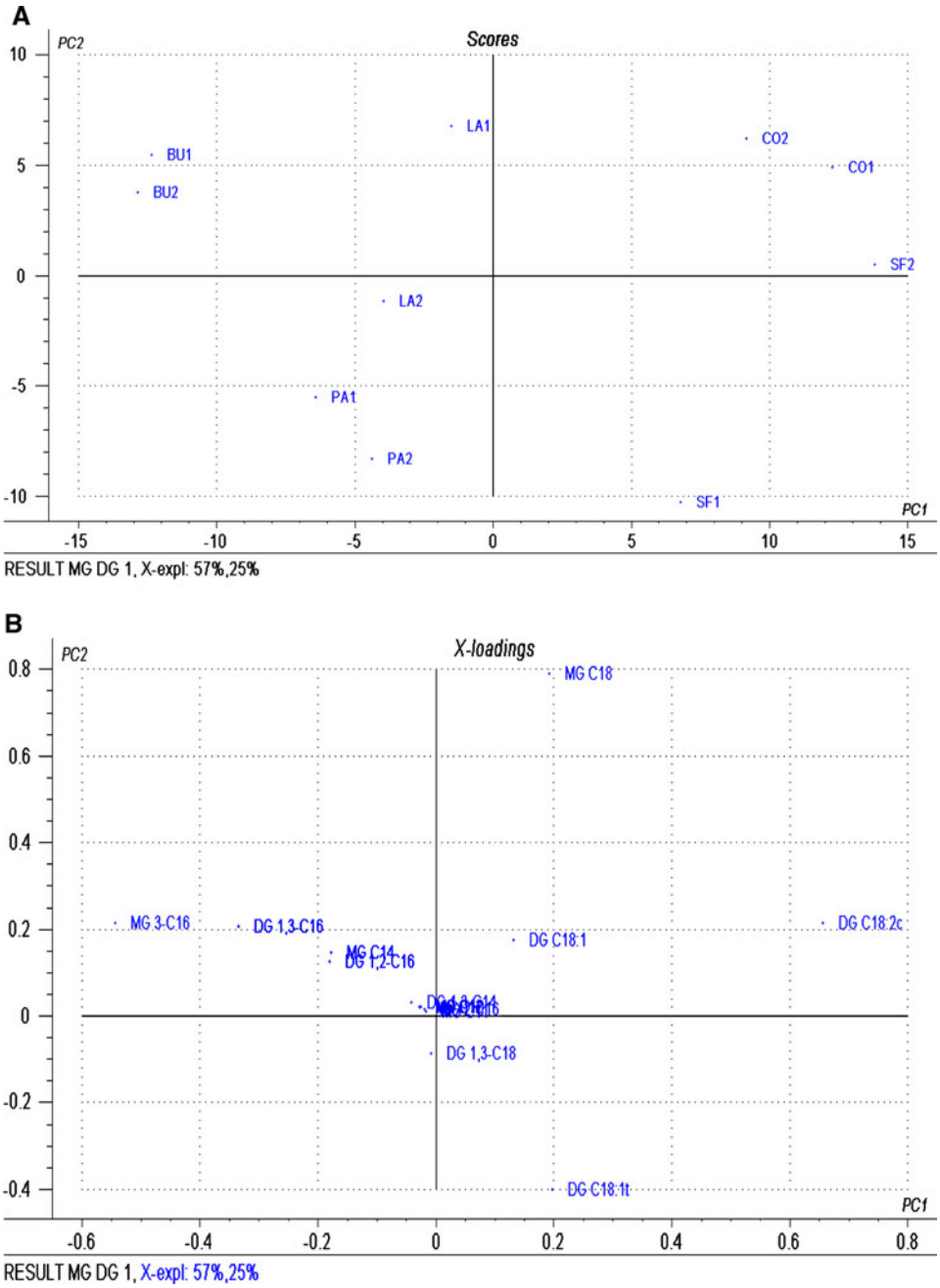

Fig. 3 Principal component analysis of overall TMS MG-DG: **a** score plot, **b** loading plot

C18:2 and C18:1 in profiling of LA fatty acids was used to distinguished LA from other animal fats.

The PCA score plot (Fig. 3a) shows that the points related to LA and PA were close and located in the lower left quadrant. This is due to the fact that they have quite similar compositions in terms of concentrations of MG-DG contents. However, when we compared PA and LA, the scores were mainly contributed by DG 1,3-C16, DG 1,2-C16 and MG C14, as can be seen in the PCA loading plot (Fig. 3b). CO and SF were grouped together in the right quadrant and were characterized by a high content of DG C18:2c. BU was located in the opposite quadrant of CO and SF, while discriminated from other oils based on its high content of MG 3-C16. The same result was found by Fagan et al. [7] when comparing concentrations of MG-DG in the lipids from whole milk, buttermilk, and anhydrous milk fat. With a concentration value of 56% from the total MG content, MG C16 became the highest MG type in buttermilk fat.

Conclusion

A simple and rapid GC × GC-TOF-MS method involving non-orthogonal columns combination was developed in

this work to analyze specific regioisomers of mono- and diglycerides in glycerolysis products. This method was capable of illustrating the glyceride profile within 20 min which is beneficial for product development and quality monitoring in various sectors. In addition, clusterization of different fats and oils was successfully achieved using this approach. Future work needs to be carried out to characterize the enantiomers of acylglycerol molecules from various sources using a chiral column set, as well as combination of orthogonal and non-orthogonal columns. This will greatly assist in the authentication of foods, pharmaceuticals, petrochemicals and other products.

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