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Determination of mono- and di-acylglycerols in milk lipids

Peter Fagan, Chakra Wijesundera*, Peter Watkins

CSIRO Food Science Australia, 671 Sneydes Road, Werribee, Vic. 3030, Australia

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Abstract

A simple and rapid method based on solid phase extraction and gas chromatography has been developed for the determination of monoacylglycerols (MAG) and diacylglycerols (DAG) at low-concentration levels typically found in milk and dairy ingredients. The method enabled measurement of individual milk MAG (including isomeric forms) with the exception of glycerol, monobutyrate. The DAG were separated and quantified as groups according to their carbon numbers. However, it was possible to identify the major DAG components within a group. The minimum detection limits for MAG and DAG were in the range of 5–8 and 10–17 μg/ml, respectively. The corresponding R.S.D. values were 1.7–3.9 and 0.2–9.9%, respectively. C8–C18 MAG and C26–C36 DAG were present in the lipids extracted from whole milk, anhydrous milk fat and buttermilk. The concentrations of MAG and DAG in buttermilk were, respectively, thirteen- and three-folds higher than that in whole milk or anhydrous milk fat. In dairy lipids, 1,2(2,3)-DAG isomers predominated almost to the exclusion of 1,3-isomers.

Keywords: Milk; Food analysis; Solid-phase extraction; Lipids; Monoacylglycerols; Diacylglycerols

1. Introduction

Triacylglycerols (TAG) are by far the main lipid class in bovine milk, accounting for 97–98% of the total lipid [1]. The milk TAG are invariably accompanied by small amounts of minor lipids, e.g. phospholipids (PL), diacylglycerols (DAG) and monoacylglycerols (MAG), free fatty acids (FFA) and glycolipids (GL). The DAG and MAG in milk can result from lipolysis of milk TAG due to inappropriate handling and storage of milk. However, DAG have been shown to occur naturally in freshly secreted milk suggesting that some of the DAG occurring in milk are intermediates in the biosynthesis of TAG rather than degradation products [1].

It has been shown that, despite their relatively low concentrations, the minor lipids in milk can exert significant effects on the functional properties of dairy products and ingredients. In particular, PL, MAG and DAG are surface active, and can alter the interfacial properties of emulsions. Minor lipids have been shown to influence dairy product functional properties such as foaming [2]; gelling [3–6], emulsion stability [7–10], and heat stability [11–13].

Minor lipids also impact on the crystallisation properties of fats. Hernqvist and Anjou [14] demonstrated that addition of small amounts of DAG to margarines helped to stabilise the β crystal polymorph and delayed its transformation to the less desirable β^1 form. Although DAG did not alter the structure and mechanical properties of milk fat [15], they changed the kinetics of milk fat crystallisation by delaying the onset of crystallisation at low degrees of super cooling [16]. Wright and Marangoni [17] showed that racemic purity was an important factor in the ability of DAG to influence TAG crystallisation, with *sn*-1,2 isomers being more effective that *sn*-1,3 isomers. Tietz and Hartel [18] reported that minor lipids from milk fat, even at the low concentrations typically found in nature, affected the crystallisation of milk fat–cocoa butter blends, impacted on chocolate microstructure, and affected bloom development in chocolate.

TLC, HPLC and GC have all been used to determine MAG and DAG in fats and oils. In TLC, the MAG and DAG are separated from each other and then quantified by gravimetry or densitometry. TLC can be performed using simple equipment, but the procedure is time-consuming and does not measure individual MAG and DAG. Although the technique has been automated by conducting the separation on adsorbent-coated micro rods followed by detection and quantification by flame ionisation detection, it still does not allow the determination of individual MAG and DAG.

* Corresponding author. Fax: +61-3-9731-3250.
E-mail address: chakra.wijesundera@csiro.au (C. Wijesundera).

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HPLC with evaporative light scattering detection (ELSD) or UV/fluorescence detection after suitable derivatisation has been successfully used to determine MAG and DAG (including molecular species and positional isomers) in vegetable oils [19–24]. However, HPLC methods are not readily applicable to the determination of MAG and DAG in milk lipids. Milk lipid MAG, DAG and TAG are very complex as they contain fatty acids ranging from C4–C18. This causes co-elution of components, for example, lower molecular weight TAG with higher molecular weight DAG. Resolution of isomeric forms of MAG and DAG by HPLC is also difficult to accomplish.

GC, on the other hand, does not have the drawbacks mentioned above. Indeed, a GC-based method has been adopted as a standard method for the determination of MAG and DAG in vegetable oils and emulsifiers [25,26]. However, a reliable method currently is not available for the determination of individual MAG and DAG in milk lipids. Here, we report on the development of a GC method for the determination of individual components of MAG and DAG at the low concentration levels typically occurring in milk lipids.

2. Experimental

2.1. Materials

The following MAG and DAG standards were obtained from Sigma–Aldrich (Milwaukee, WI, USA): 1-monooctanoyl-*rac*-glycerol (C8 MAG); 1-monodecanoyl-*rac*-glycerol (C10 MAG); 1-monododecanoyl-*rac*-glycerol (C12 MAG); 1-monotetradecanoyl-*rac*-glycerol (C14 MAG); 1-monohexadecanoyl-*rac*-glycerol (1-C16 MAG); 2-monohexadecanoylglycerol (2-C16 MAG); 1-monooctadecanoyl-*rac*-glycerol (C18 MAG); 1,3-dioctanoyl-*rac*-glycerol (C16 DAG); 1,2(3)-didecanoyl-*rac*-glycerol (C20 DAG); 1,2(3)-didodecanoyl-*rac*-glycerol (C24 DAG); 1,2(3)-ditetradecanoyl-*rac*-glycerol (C28 DAG); 1,2(3)-dihexadecanoyl-*rac*-glycerol (C32 DAG); 1,2-dihexadecanoyl-*sn*-glycerol (C32 DAG); 1,2(3)-Dioctadecanoyl-*rac*-glycerol (C36 DAG). 1-Monotridecanoyl-*rac*-glycerol (C13 MAG) was obtained from Indofine Chemical Company (NJ, USA). 1,3-Dinonadecanoyl-*rac*-glycerol (C38 DAG) was obtained from Nu-Chek Prep, Inc. (MN, USA). All the MAG and DAG standards had purity >98%. The MAG and DAG standards were dissolved in dichloromethane (ACS/HPLC grade, Burdick & Jackson Muskegon, MI, USA). Methanol (HiperSolv HPLC grade) was obtained from BDH (Poole, England) and *t*-butylmethylether (Chromasolv grade) was obtained from Riedel-de Hahn (Sigma–Aldrich; Milwaukee, WI, USA). Trimethyl chlorosilane (TMCS), bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine were obtained from Sigma–Aldrich (Milwaukee, WI, USA). Bovine whole milk (pasteurised, WM) was obtained from a retail shop in Melbourne. Anhydrous milk fat (AMF) and buttermilk (BM) serum were obtained from a dairy factory in Melbourne (Bonlac Foods Ltd).

2.2. Extraction of lipids

The milk lipids were extracted from WM and BM using a modification of the Bligh and Dyer method [27]. In brief, a sample of milk (10 g) was mixed (Vortex, 2 min) with methanol (10 ml) and dichloromethane (5 ml) in a polyethylene centrifuge tube (50 ml, BioCorp Aust., Huntingdale, Vic., Australia). Dichloromethane (5 ml) and NaCl (0.1 g) was then added and mixed (Vortex, 30 s). The mixture was centrifuged (1780 × *g*, 20 min, 0 °C) to partition into two distinct solvent layers separated by a white gelatinous layer. The bottom layer (dichloromethane) was collected. A dichloromethane wash (5 ml) was added to the gelatinous layer, mixed (Vortex, 1 min) and centrifuged (1780 × *g*, 10 min, 0 °C). Dichloromethane wash was combined with the original extract. The lipid samples were stored in amber-coloured glass vials with screw-top lids fitted with PTFE/silicone inserts at −20 °C until analysed.

2.3. Pre-concentration of milk lipid MAG and DAG by solid phase extraction (SPE)

The lipid sample (approximately 4.0 mg dissolved in 0.5 ml dichloromethane) was spiked with appropriate amounts of 1-monotridecanoyl-*rac*-glycerol (C13 MAG) and 1,2(3)-didecanoyl-*rac*-glycerol (C20 DAG) internal standards. The mixture was separated on a Mega BE-Si (silica) or a Mega BE-2OH (diol) (1 g stationary phase) SPE cartridge (Varian Inc., Harbour City, CA, USA) conditioned with dichloromethane (15 ml). Elution with dichloromethane (25 ml) extracted the TAG, while elution with a mixture of dichloromethane/*t*-butylmethylether (1:1 (v/v), 5.0 ml) eluted the DAG. Further elution with dichloromethane/methanol (2:1 (v/v), 7.0 ml) furnished the MAG.

2.4. Derivatisation

The MAG and DAG were silylated prior to GC using the following procedure. The solvents for the MAG or DAG standards or each fraction separated by SPE from the milk lipids were removed under a stream of N_2 gas. The lipids (7–30 μg) were subsequently dissolved in pyridine (200 μl) by vortex mixing for 30 s, followed by addition of BSTFA (200 μl) and TMCS (100 μl) and mixing for further 30 s. The mixture was heated at 70 °C for 20 min in a 8 ml glass vial sealed with Teflon-lined screw caps, and analysed by GC as follows.

2.5. Gas chromatography

GC was performed on a DB-17ht fused silica capillary column (30.0 m, 0.32 mm i.d., 0.1 μm film thickness) using

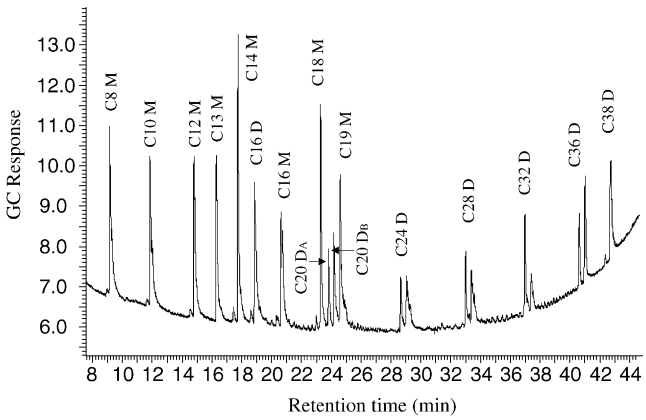

Fig. 1. GC separation of a mixture of C8–C18 monoacylglycerol (C8–C18M) and diacylglycerol (C16D–C38D) standards (as trimethylsilyl ether derivatives). The monoacylglycerols were primarily 1(3)-isomers, while most of the diacylglycerols contained both 1,2- and 1,3-isomers (denoted by A and B, respectively). The C16 and C38 diacylglycerols were mainly 1,3-isomers.

a Perkin-Elmer Model XL instrument fitted with a flame ionisation detector and a programmable temperature vaporiser (PTV) injector (Perkin-Elmer Corporation, Wellesley, MA, USA). Using an autosampler, samples (1.0 μl) of the silylation reaction mixture were injected at the split ratio of 10:1. The injector temperature was rapidly increased from 70 to 340 °C at the rate of 999 °C/min and held at that temperature for 20.0 min before cooling back to 70 °C at the rate of 20 °C/min. The column oven temperature was initially held at 65 °C for 1.0 min, and increased first to 140 °C at the rate of 20.0 °C/m and then to 340 °C at the rate of 5.0 °C/min, where it was held for 15.0 min. Helium was used as the carrier gas at a average linear velocity of 40.0 cm/s. Data acquisition and peak integration were performed using Turbochrom software (version 6.1.1, Perkin-Elmer Corporation, Wellesley, MA, USA).

To determine the detection limits for the mono- and di-acylglycerols, known amounts of each compound (standard) were silyated and analysed by GC using the same protocol applied to unknown samples. The minimum detection limit for each compound was calculated from the GC response for the known amount, assuming that a compound is detectable if the GC signal to noise ratio >10.

2.6. Gas chromatography–mass spectrometry (GC–MS)

GC–MS was performed on a system comprising an Agilent Model 6890 GC fitted with a PTV injector and 5973 MSD (Agilent, Palo Alto, CA, USA). The analytical column was the same type as that used for GC (DB-17ht fused silica capillary column, 0.1 μm film thickness) except that it was shorter (15 m) and narrower (0.25 mm i.d.). An average linear velocity of 41 cm/s of helium was maintained through the column. Samples (1.0 μl) were injected using an autosampler at a split ratio of 20:1. The PTV injector was initially held at 120 °C for 0.1 min, then ramped to 350 °C at the rate of 500 °C/min and held for 10 min before allowing to cool back to 120 °C at the rate of 20 °C/min. The GC oven temperature was increased from 120 to 340 °C at the rate of 10 C/min and held at that temperature for 30 min. The MS quadrapole and source temperatures were maintained at 150 and 230 °C, respectively. The MS acquired data in the electronic ionisation mode for the range 29–800 amu. Data was analysed using Chemstation software (Agilent, Palo Alto, CA, USA).

3. Results and discussion

3.1. GC separation of MAG and DAG standards

Fig. 1 shows a gas chromatogram of the trimethylsilyl ether (TMS) derivatives of a mixture of C8–C18 (C8,

Table 1
Response factors, precision of measurement (R.S.D. (%)) and detection limits for MAG and DAG standards under different GC operating conditions

Analyte	GC–FID/PTV		GC–FID/on-column		GC–MSD/PTV		GC–FID/PTV
	Response factor	R.S.D. (%)	Response factor	R.S.D. (%)	Response factor	R.S.D. (%)	Detection limit (μg/ml)
C8 MAG	0.99	3.1	1.01	5.4	1.29	5.5	7.7
C10 MAG	1.01	2.4	1.09	1.9	1.24	3.7	7.9
C12 MAG	0.99	1.7	1.12	0.4	1.13	2.0	5.5
C14 MAG	1.02	3.0	1.10	0.5	1.06	1.5	5.0
C16 MAG	0.92	2.4	1.05	2.3	0.92	3.5	7.2
C18 MAG	0.98	3.9	1.10	2.8	0.87	5.7	4.5
C19 MAG	0.87	1.6	1.01	4.1	0.92	3.5	7.9
C16 DAG	1.17	7.8	1.13	2.3	1.35	7.4	9.5
C24 DAG	0.96	2.5	0.97	2.3	0.79	6.6	17
C28 DAG	0.95	9.9	0.89	5.6	0.55	13	15
C32 DAG	1.04	12	0.70	11	0.38	8.5	9.5
C36 DAG	1.01	3.8	0.60	18	0.28	7.7	11
C38 DAG	0.89	0.2	0.58	21	0.26	7.8	10

The MAG and DAG response factors are relative to C13 MAG and C20 DAG, respectively. Average values from six determinations are shown.

etc. refer to the number of carbon atoms in the fatty acid component) even-carbon MAG standards obtained by PTV injection. All the MAG standards, including the odd-carbon C13 and C19 members were well resolved from each other. The MAG standards that we used were predominantly 1(3)-isomers and contained only relatively small amounts of the 2-isomers as confirmed by GC–MS analysis. On the DB-17ht GC column used, the 2-MAG isomers were baseline-resolved from the corresponding 1(3)-MAG isomers with the former eluting first. Similar baseline separation was obtained for a mixture of C16, C20, C24, C28, C32, C36 and C38 DAG standards (C16, etc. refer to the total number of carbon atoms in the two fatty acid chains; the fatty acids were the same for a given DAG). Except for the C16 and C38 DAG, each of the other DAG standards consisted of a mixture 1,2(2,3)- and 1,3-isomers. The GC conditions we used eluted the 1,2(2,3)-isomer just ahead of the corresponding 1,3-isomer. When chromatographed together, a mixture of the above MAG and DAG standards were also baseline separated with only the C16 DAG or lower eluting within the MAG region of the chromatogram (Fig. 1). Based on its fatty acid composition, milk lipids can be expected to contain C4–C18 MAG and C8–C36 DAG. Under the GC conditions used in the present study, the C4 MAG eluted with the solvent peak and was not measurable. However, the method enabled the separation of all the other MAG and DAG components potentially occurring in milk lipids in a single GC run.

Whilst split, splitless and on-column injection techniques have been used for GC analysis of MAG and DAG, on-column injection has been preferred as it gives more consistent responses factors [25,28]. The main disadvantage of on-column injection is the contamination of the stationary phase that inevitably occurs, leading to peak broadening [29]. This is particularly so for relatively non-volatile compounds such as high molecular weight DAG. We found that repeated on-column injection of milk DAG led to rapid deterioration of the GC column performance. Although the use of an inert pre-column (retention gap) helped to alleviate the problem, in our experience, PTV injection is a better option. PTV is easy to use, and gave consistent GC performance over the period of this study.

The GC responses shown in Fig. 1 are for column loadings of approximately 20–30 ng each of MAG and DAG. The GC–FID detection limits for MAG and DAG were in the range of 5–8 and 10–17 μg/ml, respectively, i.e. column loadings of 5–8 and 10–17 ng for 1 μl injection (Table 1). The GC–FID and GC–MSD responses were linear for both the MAG (C8–C19) and DAG (C16–C38) standards for column loadings in the range 5–60 ng. For MAG, the precision of the measurement by GC–FID was excellent, the R.S.D. values being in the range of 1.6–3.9% for PTV injection and 0.4–5.4% for on-column injection. The precision was also good for the DAG when PTV injection was used either in GC–FID (R.S.D., 0.2–12.1%) or GC–MSD modes (R.S.D., 6.6–13.0%). However, the precision was rather unsatisfactory for C36 and C38 when on-column injection was used. Response factors for the C8–C18 MAG standards (calculated by reference to C13 MAG = 1.0) were close to unity irrespective of the injection or detection method used. Those for the C16–C38 DAG standards (calculated by reference to C20 DAG = 1.0) showed somewhat higher deviation from unity when on-column injection was used. The same was observed with GC–MSD even with PTV injection. Response

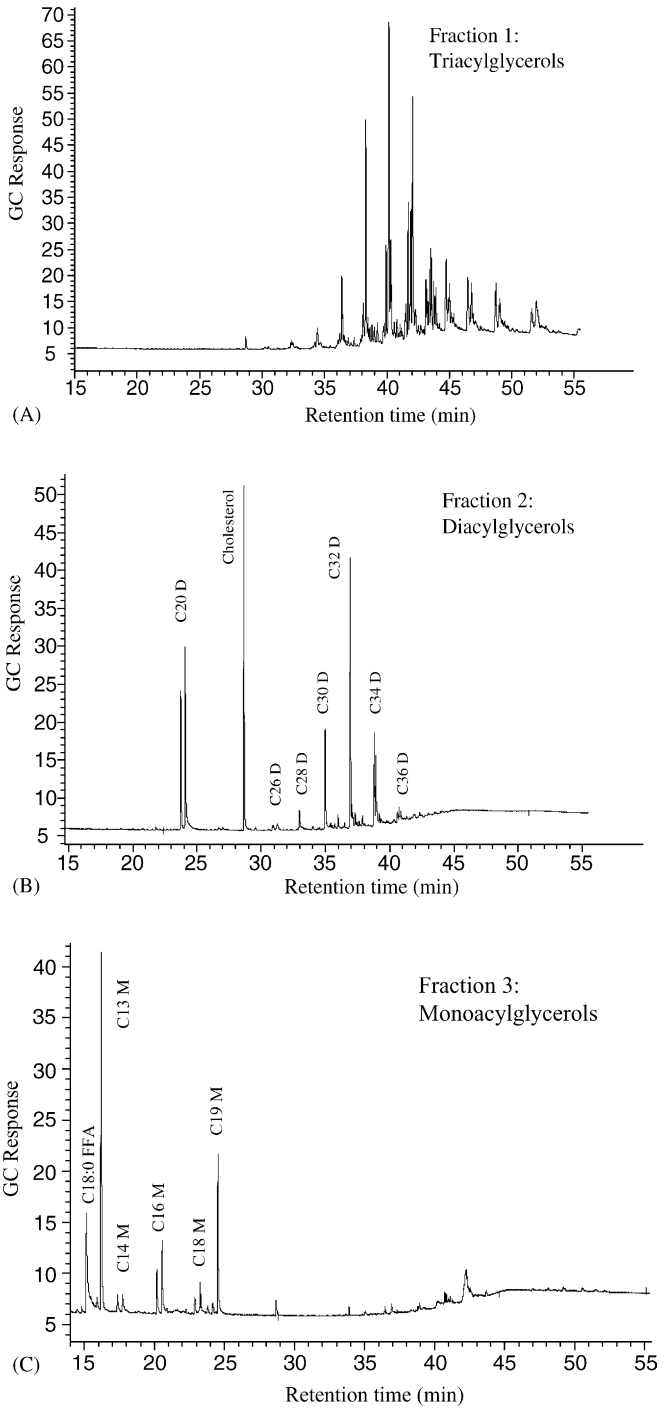

Fig. 2. GC of: (A) triacylglycerol, (B) diacylglycerol and (C) monoacylglycerol fractions separated from milk lipids by solid phase extraction on a silica cartridge.

factors for each MAG and DAG determined as above were used for the calculation of MAG and DAG concentrations in milk and milk products.

3.2. Separation of MAG and DAG by solid phase extraction

The predominant component of milk lipids, TAG, is composed of a complex mixture of molecular species. Some of the lower molecular weight components of the milk TAG tend to elute in the GC region where DAG are eluted. This makes it difficult to detect and measure MAG and DAG in milk and milk products, especially at the low concentration levels usually encountered. It is, therefore, necessary to remove all or most of the TAG from the milk lipids prior to GC analysis for the MAG and DAG. We used SPE to separate MAG and DAG from TAG and other milk lipids as discussed below.

Fig. 2 shows the separation of milk lipids in to fractions containing TAG, DAG and MAG, respectively, on silica SPE cartridges. Typically, 4 mg of lipids were separated on a single cartridge. Elution with 25 ml dichloromethane removed all of the TAG from buttermilk lipids (Fraction 1, Fig. 2A). Further elution with dichloromethane/*t*-butylmethylether (1:1 (v/v)) and dichloromethane/methanol (2:1 (v/v)) furnished the DAG (Fraction 2, Fig. 2B) and MAG (Fraction 3, Fig. 2C), respectively. Cholesterol eluted in the DAG fraction and co-eluted with C24 DAG in GC under the condition we used in this study. The MAG fraction (Fraction 3) contained free fatty acids (FFA). However, they were baseline separated from the MAG peaks (Fig. 2C) and did not interfere with the detection and quantification of the milk MAG. Indeed this method can potentially be used for the determination of FFA in milk lipids.

As discussed above, the GC conditions used in this study separated isomeric forms of MAG and DAG making it possible to determine the isomer ratios for each MAG and DAG. However, working with authentic standards, we found that MAG and DAG underwent a degree of isomerisation during pre-concentration on silica SPE cartridges (Table 2). Koprivnjak et al. [30] and Conte et al. [31] reported a similar effect when DAG were chromatographed on an aminopropyl phase. They found that the DAG 1,2(2,3)-isomer partially converted to the 1,3-isomer on the aminopropyl phase but not on a diol phase. For glycerol, dihexadecanoate, isomerisation from the 1,2- to 1,3-form was reported to be as high as 33% on the aminopropyl phase. We did not observe such marked isomerisation on the silica phase that we used, the corresponding isomerisation for glycerol-1,2-dioctadecanoate being approximately 6%. Isomerisation from the 1,3- to 1,2-form was observed also, percentage conversions for glycerol, dihexadecanoate and glycerol, dinonadecanoate being 9.5 and 8.4%, respectively. Isomerisation was more pronounced for MAG than for DAG with nearly 30% of 2-monohexadecanoin converting to the 1(3)-isomer. We observed virtually no isomerisation of all MAG and DAG isomers when a silica–diol SPE was used,

Table 2
Effect of SPE stationary phase on the isomerisation of selected DAG and MAG standards

	Isomer distribution (%)		
	No SPE	Silica SPE	Silica–diol SPE
C16-MAG			
1(3)-Isomer	0.4	28.9	0.6
2-Isomer	99.6	71.1	99.4
C16-DAG			
1(3)-Isomer	100.0	90.5	100.0
1,2(2,3)-Isomer	0.0	9.5	0.0
C20-DAG			
1(3)-Isomer	59.7	27.8	60.0
1,2(2,3)-Isomer	40.3	72.2	40.0
C32-DAG			
1(3)-Isomer	0.2	6.0	0.6
1,2(2,3)-Isomer	99.8	94.0	99.4
C38-DAG			
1(3)-Isomer	100.0	91.6	100.0
1,2(2,3)-Isomer	0.0	8.4	0.0

Table 3
Mass spectra data for the MAG isomers in buttermilk lipids

MAG	t_R (min)	$M-15$	$M-90$	*a*	*b*	*c*-1	RCO	RCO + 74
1(3)-C8	4.64	347	–	205	259	–	127	202
1(3)-C10	6.22	375	–	205	287	–	155	229
2-C12	7.62	403	–	–	–	218	183	257
1(3)-C12	7.79	403	–	205	315	–	183	257
2-C14	9.11	431	356	–	–	218	211	285
1(3)-C14	9.29	431	356	205	343	–	211	285
2-C16	10.53	459	384	–	–	218	239	313
1(3)-C16	10.71	459	384	205	371	–	239	313
2-C18:0	11.86	487	–	–	–	218	267	341
2-C18:1	11.86	485	–	–	–	218	265	339
1(3)-C18:0	12.06	487	412	205	399	–	267	341
1(3)-C18:1	12.06	485	410	203	397	–	265	339

The letters *a*, *b* and *c* refer to mass spectral fragmentations shown in Fig. 3-1 and 3-2.

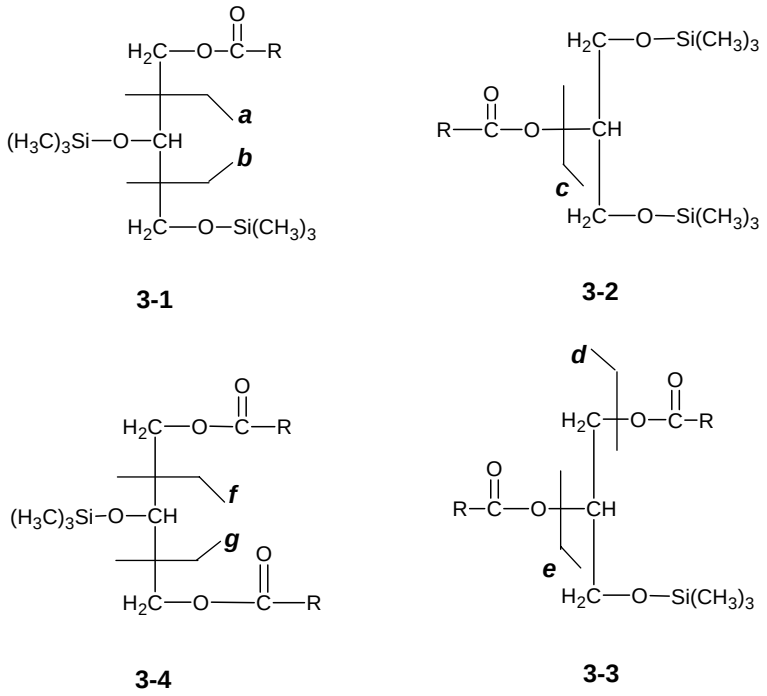

Fig. 3. Mass spectral fragmentation patterns for: (3-1) 1-monoacylglycerol, (3-2) 2-monoacylglycerol, (3-3) 1,2-diacylglycerol and (3-4) 1,3-diacylglycerol.

which is in agreement with Koprivnjak et al. [30] and Conte et al. [31] (Table 2). Aminopropyl and silica phases can be used for the pre-concentration of MAG and DAG by SPE when determination of their isomeric ratios is not required. Otherwise, the use of a diol phase is recommended.

3.3. Characterisation of milk MAG and DAG by GC–MS

We analysed the lipids extracted from a commercial sample of buttermilk powder for MAG and DAG by GC–MS. Table 3 shows the characteristic mass spectral fragment ions for the bis-TMS ether derivatives of the MAG fraction separated from milk lipids. The molecular ion (M) was either not present or occurred in very low abundance, but $(M - 15)^+$ ions corresponding to loss of a TMS methyl radical were generally present enabling determination of the molecular weight. The 1(3)-MAG components produced relatively strong fragment ions due to the elimination of a (CH_2OSiCH_3) group (Fig. 3-1, *b*) producing a homologous series of strong ions differing by 28 amu from 259 to 399 amu for the C8–C18 MAG. They also gave a relatively weak but common ion at 205 amu (Fig. 3-1, *a*). The presence of distinct fragment ions at 218 amu (Fig. 3-2, *c*) in the spectra of 2-MAG isomers, corresponding to $[M - \text{RCOOH}]^+$ easily distinguished them from the 1(3)-MAG isomers as previously reported by Curstedt [32]. The presence of fragment ions corresponding to $[\text{RCO}]^+$ and $[\text{RCO} + 74]^+$ in the spectra of both 1(3)- and 2-MAG; (Table 3) facilitated the identification of the fatty acid moieties.

In milk lipids, we detected both 1(3)- and 2-isomers for the C12–C18 MAG, whereas only the 1(3)-isomers were observed for the C8 and C10 MAG. Whilst some isomerisation to 1(3)-isomers could have occurred during pre-concentration on silica SPE, it is also known that the shorter-chain acids in milk are predominantly bound to the 1(3) position of the TAG [1]. On the DB-17ht GC column that we used in this study, the 2-MAG peaks corresponding to stearic (18:0) and oleic (18:1) acids co-eluted as did the corresponding 1,3-MAG peaks. Determination of 18:0 and 18:1 MAG was, therefore, not possible on this column when FID was used for peak detection. However, this co-elution does not pose a problem when GC–MS is used as the proportion of 18:0 to 18:1 MAG can be calculated by the relative intensities of the corresponding M-15 ions (487 and 485 Da, respectively).

Table 4 shows mass spectral data for the DAG fraction (as bis-TMS derivatives) extracted from milk lipids. It shows the main fragment ions and the probable structures for each DAG species that were sufficiently resolved to enable structure identification. Although the molecular ions (*M*) were barely detected, significant $M - 15$ and $M - 90$ ions enabled the determination of DAG molecular weights. Also, as observed by Horning et al. [33], the mass spectra of 1,2- and 1,3-DAG were not identical, and it was not difficult to distinguish one type of structure from the other. In the TMS derivatives of 1,2-DAG, the principal fragmentation occurs at CH–O bonds linking the fatty acids to the glycerol backbone (*d* and *e* in Fig. 3-3) giving ions *M*–RCOO and *M*–RCOO corresponding to the attached fatty acids. In 1,2-DAG, cleavage at the C–C bonds in the glycerol moiety is insignificant. In contrast, in the case of the TMS derivatives of 1,3-DAG, cleavage at the C–C bonds in the

Table 4
Mass spectra data for the main DAG isomers in buttermilk lipids

t_R (min)	Main species	$M-15$	$M-90$	d/e	f/g	RCO	RCO + 74
C26 DAG							
15.78	1,2-(10:0 + 16:0)	541	–	385, 301	–	155, 239	229, 313
C28 DAG							
16.81	1,2-(12:0 + 16:0)	569	494	385, 357	–	183, 211	257, 285
16.86	1,2-(14:0 + 14:0)			329	–	239	313
16.90	1,2-(10:0 + 18:1)	567	492	410, 301	–	155, 265	229, 339
C30 DAG							
17.80	1,2-(14:0 + 16:0)	597	522	385, 357	–	211, 239	285, 313
17.87	1,2-(12:0 + 18:1)	595	520	411, 329	–	183, 265	257, 339
17.99	1,3-(14:0 + 16:0)	597	522	–	371, 343	211, 239	285, 313
C32 DAG							
18.75	1,2-(16:0 + 16:0)	625	550	413, 385		239	313
18.82	1,2-(14:0 + 18:1)	623	548	411, 385		285, 265	285, 339
18.93	1,3-(16:0 + 16:0)	625	550		371	239	313
18.98	1,3-(14:0 + 18:0)	625	550		371, 343	239, 267	313, 341
C34 DAG							
19.69	1,2-(16:0 + 18:0)	653	578	413, 385		239, 267	313, 341
19.73	1,2-(16:0 + 18:1)	651	576	411, 385		239, 265	313, 339
19.84	1,3-(16:0 + 18:0)	653	578		399, 371	239, 267	313, 341
19.92	1,3-(16:0 + 18:1)	651	576		397, 371	239, 265	313, 339
C36 DAG							
20.53	1,2-(18:0 + 18:0)	681	606	413	–	267	341
20.59	1,2-(18:0 + 18:1)	679	604	413, 411	–	265, 267	339, 341
20.67	1,2-(18:1 + 18:1)	677	–	411	–	265	339

The letters *d*, *e*, *f* and *g* refer to mass spectral fragmentations shown in Fig. 3-3 and 3-4.

glycerol moiety (*f* and *g* in Fig. 3-4) predominates producing $[M–RCOOCH_2]^+$ as the major fragment ions [33]. Ions corresponding to RCO and RCO + 74 were also prominent in the spectra of both 1,2- and 1,3-DAG.

In the DAG fraction from milk lipids, six distinct groups representing even-carbon C26–C38 DAG were present (Fig. 4). Within each group, there was further resolution in to sub-components based on the degree of unsaturation as well as the positional distribution of the fatty acid chains. 1,2-DAG predominated almost to the exclusion of 1,3-isomers. This probably is a reflection of the fact that the glycerol-3-phosphate pathway is the primary route to

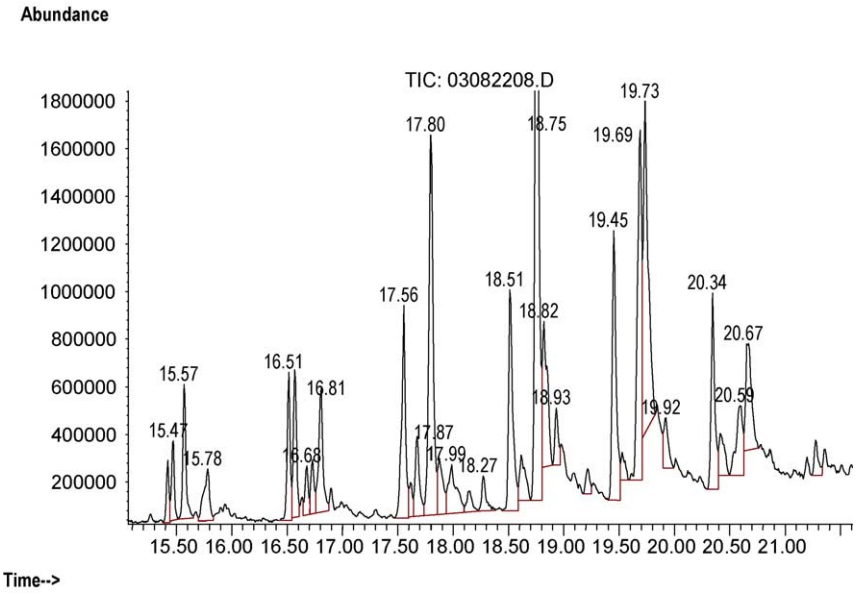

Fig. 4. GC–MS total ion chromatogram for the diacylglycerol fraction separated from milk lipids by solid phase extraction. See Table 4 peak identification.

Table 5
Concentration of MAG and DAG in the lipids from whole milk (WM), buttermilk (BM), and anhydrous milk fat (AMF)

	WM (mg/g)	AMF (mg/g)	BMP (mg/g)
MAG			
C10	Trace	Trace	0.32 (3.1)
C12	Trace	Trace	0.26 (1.6)
C14	0.14 (9.1)	0.066 (25)	1.87 (6.7)
C16	0.37 (13)	0.16 (25)	6.07 (0.7)
C18	0.26 (4.6)	0.18 (10)	2.24 (14.3)
Total MAG	0.78	0.40	10.8
DAG			
C26	0.14 (5.8)	0.36 (16)	0.51 (1.8)
C28	0.15 (2.2)	0.14 (4.1)	1.24 (3.4)
C30	1.27 (12)	1.04 (3.6)	5.58 (7.4)
C32	3.96 (15)	3.63 (3.7)	11.5 (3.2)
C34	3.13 (20)	4.67 (4.9)	9.83 (5.4)
C36	0.88 (29)	2.01 (6.9)	1.51 (25)
Total DAG	9.52	11.8	30.1

The concentrations shown are the average values for six replicate analysis of single lipid extracts. R.S.D. (%) values are shown in parenthesis.

TAG biosynthesis in the mammary gland [1]. Sambaiah and Lokesh [34] who examined fresh milk fat also reported that the small amounts of DAG present were mainly 1,2-isomers. The small amounts of DAG 1,3-isomers that we detected could have resulted from isomerisation during SPE fractionation on silica cartridges as alluded to previously.

C30, C32, C34 and C36 were the main DAG groups present in milk lipids. As previously observed by Mariani et al. [35], the C30–C36 DAG serve as a characteristic fingerprint for bovine milk lipids. The 1,2-(14:0/16:0) and 1,2-(16:0/16:0), respectively, were the predominant DAG species present within the C30 and C32 groups. Mass spectral data indicated that the amounts of 1,2-(12:0/18:0) and 1,2-(14:0/18:0) were relatively small. In the C34 group, 1,2-(16:0/18:0) and 1,2-(16:0/18:1) were the main components, which were present in approximately equal amounts. The C36 DAG group comprised of a mixture of 1,2-(18:0/18:0), 1,2-(18:0/18:1) and 1,2-(18:1/18:1).

3.4. MAG and DAG in milk and milk products

Table 5 shows the concentration of MAG and DAG in the lipids extracted from WM, AMF and BM. BM lipids contained significantly higher amounts of MAG (thirteen-fold higher) and DAG (three-fold higher) than did WM lipids or AMF. AMF and WM lipids were not significantly different in this respect. In the lipids from WM and BM, the predominant MAG and DAG species were C16, C18, C14, and C32, C34, C30, C36, respectively (in decreasing order of concentration). The order was different for AMF, being C18, C16, C14 for MAG and C34, C32, C36, C30 for the DAG. This difference probably reflects a difference in fatty acid composition rather than lipolysis.

4. Conclusions

This work has shown that GC can be used to measure low concentrations of MAG and DAG typically occurring in milk and milk products after pre-concentration by SPE. Significant isomerisation of MAG and DAG occurs during SPE using silica but not diol phase. The method allows separation and quantification of all individual MAG components. The DAG are only separated into groups on the basis of carbon number although identification of the main components within a group is also possible. For studies requiring analysis of a large number of samples, PTV injection is preferable to on-column injection to minimise problems associated with column deterioration resulting from injection of high boiling material.

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