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Elucidation of the composition of bovine milk fat triacylglycerols using high-performance liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry

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Abstract

Bovine milk fat triacylglycerols (TAGs) have been characterised using high-performance liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry (HPLC–APCI-MS) and high-temperature gas chromatography–mass spectrometry (GC–MS). The complex nature of the fat meant that prefractionation was necessary to provide simpler fractions for more detailed molecular analyses. Silica thin-layer chromatography gave rise to two fractions, one of which contained predominantly butyric acid containing TAGs. Gel permeation chromatography (GPC) gave rise to 16 fractions, which were subsequently analysed using HPLC–APCI-MS. Twelve of the GPC fractions were also analysed by high-temperature GC–MS using a capillary column coated with a polarisable stationary phase. TAGs present in the fractions were correlated with those in the chromatogram of the whole milk fat through retention time comparison and the use of mass chromatograms. In total, 120 TAGs were identified.

Keywords: Fats; Milk; Food analysis; Triacylglycerols; Glycerols; Fatty acids

1. Introduction

Milk fat represents a particularly challenging problem to the analytical chemist. The acyl lipids that comprise the fat contain a large number of different fatty acids, with chain lengths ranging from four to 20 carbon atoms. Significant proportions of branched chain compounds are present, as well as a range of geometric and positional isomers of mono-unsaturated acids [1,2]. The large number of fatty acyl moieties give rise to one of the most complex naturally occurring fats, with thousands of possible triacylglycerol (TAG) species.

One approach to milk fat analysis has been to employ normal-phase or silver ion thin-layer chromatography (TLC) to pre-fractionate the TAGs using TLC or HPLC prior to more detailed molecular analysis. For example, Fraga et al. used silver-ion thin-layer chromatography (Ag-TLC) to obtain six fractions, which were subsequently analysed by GC [3]. Robinson and MacGibbon analysed Ag-TLC fractions by HPLC and identified 97 TAG species [4]. A similar approach has been to obtain fractions corresponding to peaks in a reversed-phase HPLC profile then to use GC to establish the fatty acid composition for each peak [5,6]. Without pre-frac-

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tionation, deconvolution of co-eluting TAGs is difficult, however Ruiz-Sala et al. identified over 180 TAG species in sheep milk fat through equivalent carbon numbers, retention times and analysis of fractions by GC–MS [7]. An alternative approach has been to fractionate the fat by silver-ion HPLC (Ag-HPLC) followed by reversed-phase HPLC (RP-HPLC), then to perform a fatty acid analysis on each fraction [8]. Winter et al. used this method to identify over 160 TAG species [9]. Other references to the analysis of TAGs are included in a review by Laakso [10].

Mass spectrometry can be used to identify the fatty acyl moieties present in TAGs at the level of carbon number and degree of unsaturation. Using capillary GC with a polarisable stationary phase in conjunction with negative ion chemical ionisation MS to determine which fatty acyl moieties were present in the TAGs comprising each of the chromatographic peaks, some TAG molecular species were identified and a number of structural features influencing retention were identified [11]. Laakso and Manninen obtained similar information using capillary supercritical fluid chromatography (SFC)–atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) [12]. TLC on both normal-phase silica gel and silver nitrate impregnated plates was used by Myher et al. to prefractionate milk fat prior to analysis by GC–MS [13]. This allowed absolute identification of over 100 molecular species. Similarly, Spanos et al. used desorption chemical ionisation tandem mass spectrometry (DCI-MS–MS) to identify TAGs in fractions resulting from HPLC analysis [14]. Currie and Kallio identified 58 TAG species in human milk using negative ion tandem mass spectrometry [15]. Deprotonated molecular ions, $[M\text{-}H]^-$, focused from the first quadrupole were fragmented by collision-induced decomposition in the second quadrupole, giving rise to product ion spectra exhibiting RCO_2^- and $[M\text{-}H\text{-}RCO_2H\text{-}100]^-$ ions, which enabled identification of each TAG.

The efficient separation achieved using RP-HPLC and the structural information provided by MS suggest that HPLC–MS has considerable potential for elucidation of bovine milk fat composition. However, very little work has been reported in this area. Using RP-HPLC with both positive and negative chemical ionisation MS, Kuksis et al. were able to analyse the TAGs present in a butteroil fraction [16]. Of the 30 peaks observed in the HPLC profile, five were chosen for further detailed analysis. Chloride attachment negative ion chemical ionisation mass spectrometry (NICI-MS) gave rise to $[M+Cl]^-$ ions which enabled the molecular mass of each TAG species to be determined. Analysis of the same fractions by positive CI resulted in both protonated molecular ions and diacylglycerol ions, which allowed the fatty acids present in each TAG to be determined. Fifteen molecular species were identified in the five peaks studied.

More recently, the technique of HPLC in conjunction with APCI-MS has been shown to be a powerful tool for the analysis of TAGs in both vegetable oils and animal fats [17–22]. APCI-MS spectra of TAGs typically give rise to protonated molecular ions, $[M+H]^+$, diacylglycerol ions, $[M\text{-}RCO_2]^+$ or DG^+, resulting from the loss of a fatty acyl moiety, and acylium ions, RCO^+, corresponding to the acid itself. Whilst the protonated molecular ion is of low abundance in highly saturated TAG species, the DG^+ ions allow identification of the fatty acids present in each molecule and the relative abundances of these DG^+ ions allow the fatty acid in the 2-position to be distinguished from those in the 1- and 3-positions [23]. The aim of this study was to investigate the composition of bovine milk fat using a combination of prefractionation techniques followed by molecular species analysis by means of HPLC–APCI-MS and high-temperature GC–MS.

2. Experimental

2.1. Reagents

All solvents were HPLC grade and were obtained from Rathburn, Walkerburn, UK. Milk fat samples were given by Reading Scientific Services Ltd. (Reading, UK).

2.2. HPLC–APCI-MS

HPLC–MS analyses were performed on a Waters 600MS (Waters, Milford, MA, USA) quaternary solvent delivery system, coupled to a Finnigan MAT (San Jose, CA, USA) TSQ700 triple sector mass

spectrometer fitted with an APCI source and operated in single analyzer mode. This was typically operated with a vaporiser temperature of 450°C, capillary temperature of 280°C and corona current of 5 μA. High-purity nitrogen was used for the sheath and auxiliary gases, at 60 p.s.i. and 20 ml min^{-1}, respectively (1 p.s.i.=6894.76 Pa). Spectra were obtained over the range m/z 200–1000, with a scan time of 2 s. Commercial milk fat samples were analysed using two Supelcosil LC-18 columns (octadecylsilyl bonded phase, 25 cm×10 mm I.D., 5 μm particle size, 100 Å pore size, Supelco, Poole, UK) in series, with propionitrile as the mobile phase at a flow-rate of 0.8 ml min^{-1}. Samples were dissolved in propionitrile to a concentration of 5% (v/v) for injection (20 μl) on to the HPLC column. An isocratic solvent system was chosen to aid stability and tuning of the mass spectrometer. The mass spectral data were expressed as base peak chromatograms, in which the intensity of the base peak of each scan was plotted against time. Since APCI-MS spectra exhibit little fragmentation, base peak chromatograms assisted in removing background noise.

2.3. Gel permeation chromatography (GPC)

Preparative GPC was carried out using a LDC Model III Constametric HPLC pump (Milton Roy Company, Ivyland, PA, USA) with a PL Gel column (polystyrene–divinylbenzene, 60 cm×10 mm I.D., 10-μm particle size, 50-Å pore size, Polymer Labs, Amherst, MA, USA). Dichloromethane–methanol (1:2, v/v) was used as a mobile phase at a flow-rate of 3 ml min^{-1}. A 5:1 postcolumn split allowed approximately 12.5% of the column effluent to enter an Applied Chromatography Systems (Macclesfield, UK) 750/14 Evaporative Analyser. This was operated with an evaporator temperature of 140°C using air as nebuliser gas at a pressure of 20 p.s.i. The photomultiplier sensitivity was set at 2 and the time constant was 5 s. The detector output was linked to a chart recorder, which was used with a chart speed of 600 mm h^{-1}.

2.4. Thin-layer chromatography

Milk fat (25 mg) was applied to a silica TLC plate (LK6F, 250 μm layer thickness, 60 Å particle size, 20×20 cm, Whatman, Maidstone, UK) and developed using hexane–diethyl ether–acetic acid (94:4:2, v/v/v). The plate was sprayed with fluorescein and visualised under UV light. The bright band observed at R_F=0.3 was divided into two fractions: 1 (upper) and 2 (lower). These were scraped off the plate and the TAGs removed from the silica by addition of dichloromethane–methanol (2:1, v/v) followed by filtration through cotton wool. The solvent was removed under a gentle stream of nitrogen and the samples dissolved in propionitrile for analysis by HPLC.

2.5. Gas chromatography

Following derivatisation with *N,O*-bis-(trimethylsilyl) trifluoracetamide (BSTFA, 80°C, 1 h), the fractions resulting from preparative GPC of milk fat were analysed on a Hewlett-Packard (Palo Alto, CA, USA) 5890A gas chromatograph using on-column injection (1 μl) with a Restek Rtx-65TG capillary column (65% diphenyl–35% dimethylpolysiloxane, 30 m×0.32 mm I.D., 0.1 μm film thickness, Restek Corp., Bellefonte, PA, USA). The temperature program was 50°C (2 min) to 240°C at 20°C min^{-1} then to 360°C at 4°C min^{-1}. The temperature was held at 360°C for 20 min.

2.6. Gas chromatography–mass spectrometry

Analyses were performed on a Carlo Erba Mega Series gas chromatograph (Milan, Italy) coupled to a Finnigan MAT 4500 mass spectrometer which was operated in the electron ionisation (EI) mode. Scans were performed from m/z 50 to 850 at a frequency of two scans s^{-1}. Data were collected using an INCOS data system and processed using the Interactive Chemical Information Software (ICIS) package. The chromatographic conditions employed were identical to those described above.

3. Results and discussion

3.1. Milk fat analysis using HPLC–APCI-MS

An initial HPLC–APCI-MS analysis of milk fat

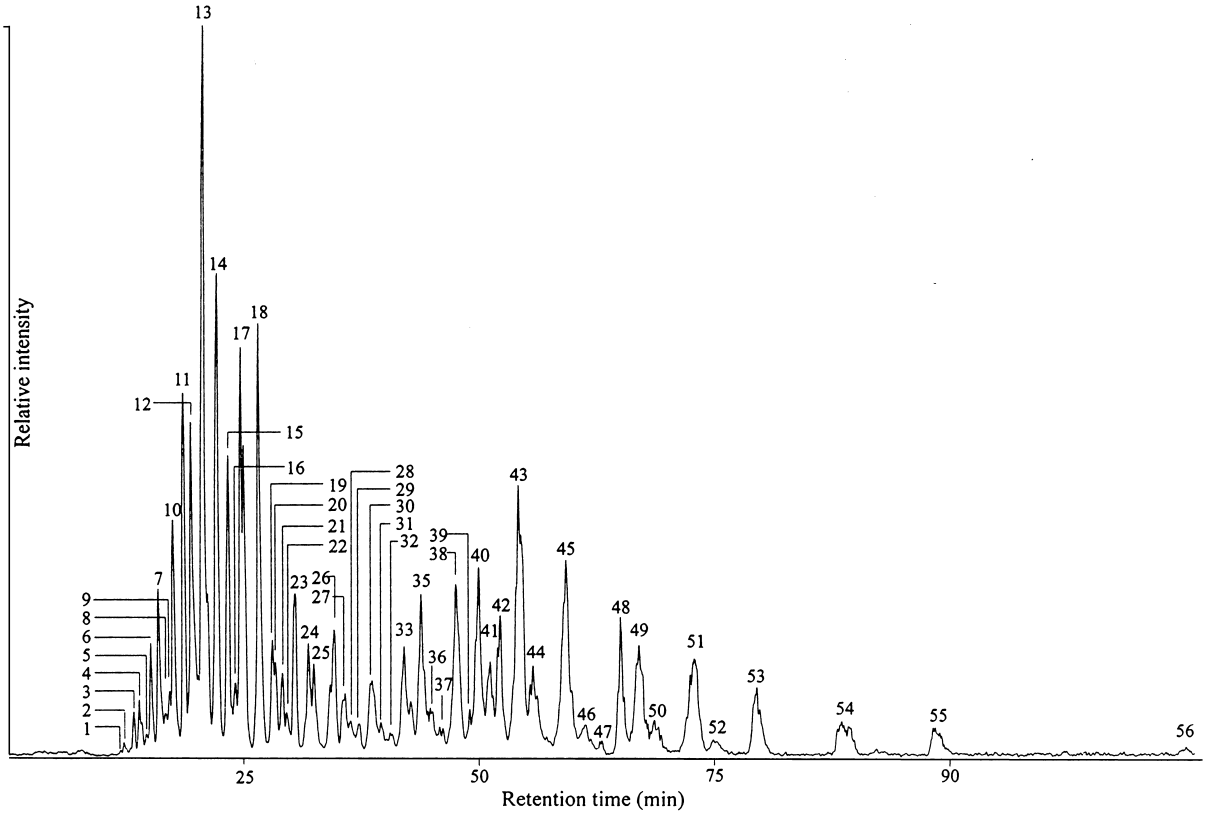

Fig. 1. The HPLC–APCI-MS profile of whole milk fat.

was carried out using two C_{18} columns in series with propionitrile as a mobile phase. The resulting chromatogram, shown in Fig. 1, contains 55 resolved or partially resolved chromatographic peaks. Whilst the identification of TAGs eluting at longer retention times was reasonably straightforward, identification of the majority of components was extremely complicated due to co-elutions, in particular of saturated TAG species. In these cases, interpretation of the mass spectra becomes especially difficult as an ion at a particular mass could correspond to one or more of a number of diacylglycerol fragments. For example, m/z 439 could be due to one of five different DG^+ ions, namely $[4{:}0\text{-}20{:}0]^+$, $[6{:}0\text{-}18{:}0]^+$, $[8{:}0\text{-}16{:}0]^+$, $[10{:}0\text{-}14{:}0]^+$ or $[12{:}0\text{-}12{:}0]^+$. In order to demonstrate the difficulties involved in deconvoluting the

Table 1
The ions resulting from coeluting 10:0-12:0-12:0, 10:0-10:0-14:0, 8:0-12:0-14:0, 8:0-8:0-18:0 and 8:0-10:0-16:0 TAGs in milk fat and other ions implied by those masses

Mass (m/z)	DG^+ ions resulting from TAGs present	Other DG^+ ions corresponding to mass
327	$[8{:}0\text{-}8{:}0]^+$	$[4{:}0\text{-}12{:}0]^+$, $[6{:}0\text{-}10{:}0]^+$
355	$[8{:}0\text{-}10{:}0]^+$	$[4{:}0\text{-}14{:}0]^+$, $[6{:}0\text{-}12{:}0]^+$
383	$[10{:}0\text{-}18{:}0]^+$, $[8{:}0\text{-}12{:}0]^+$	$[4{:}0\text{-}16{:}0]^+$, $[6{:}0\text{-}14{:}0]^+$
411	$[10{:}0\text{-}12{:}0]^+$, $[8{:}0\text{-}14{:}0]^+$	$[4{:}0\text{-}18{:}0]^+$, $[6{:}0\text{-}16{:}0]^+$
439	$[12{:}0\text{-}12{:}0]^+$, $[10{:}0\text{-}14{:}0]^+$, $[8{:}0\text{-}16{:}0]^+$	$[4{:}0\text{-}20{:}0]^+$, $[6{:}0\text{-}18{:}0]^+$
467	$[12{:}0\text{-}14{:}0]^+$, $[8{:}0\text{-}18{:}0]^+$, $[10{:}0\text{-}16{:}0]^+$	$[6{:}0\text{-}20{:}0]^+$

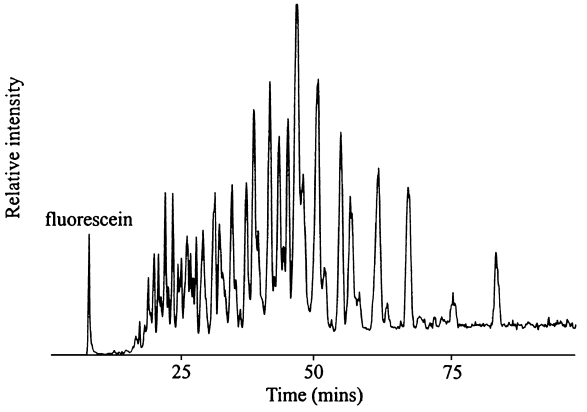

Fig. 2. The HPLC–APCI-MS profile of milk fat TLC fraction 1.

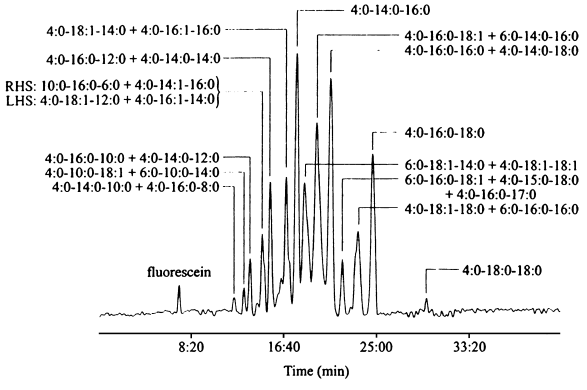

Fig. 3. The HPLC–APCI-MS profile of milk fat TLC fraction 2.

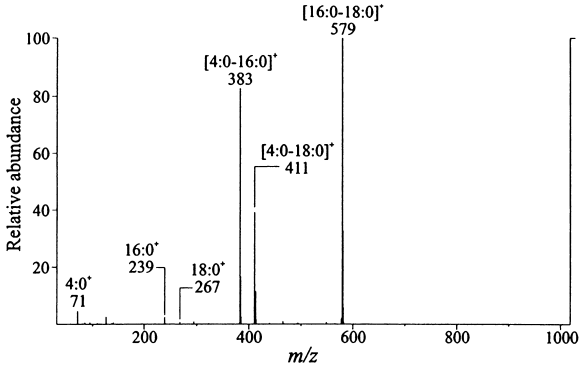

Fig. 4. The APCI mass spectrum of 1(3)-butyroyl-2-palmitoyl-(3)1-stearoyl glycerol (4:0-16:0-18:0).

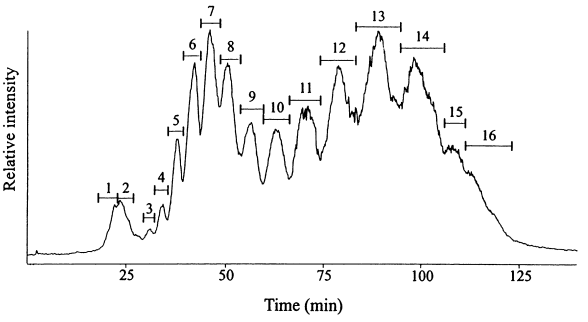

Fig. 5. The GPC profile of milk fat. The numbers refer to the fractions collected for subsequent RP-HPLC and GC analysis.

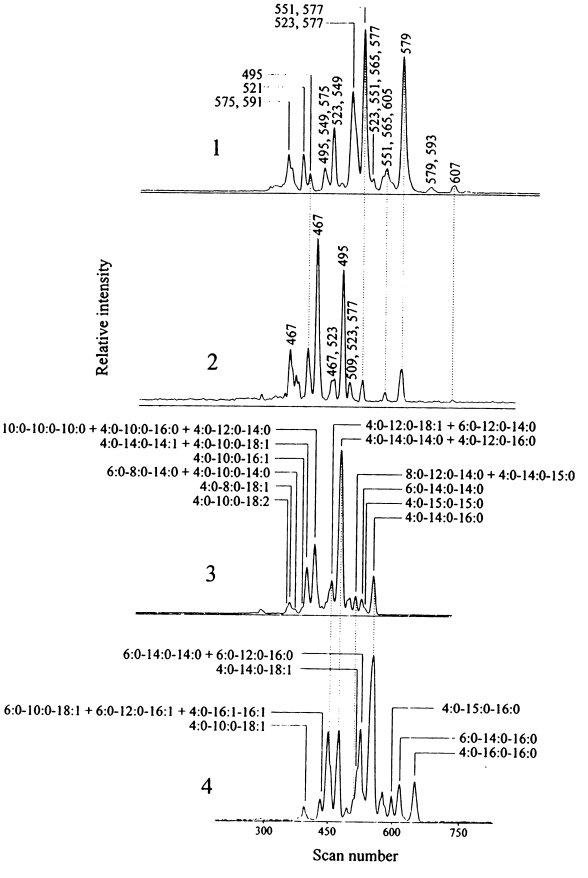

Fig. 6. The HPLC–APCI-MS profiles of GPC fractions 1–4 of milk fat. The numbers used to annotate the peaks in fractions 1 and 2 refer to the major ions seen in the APCI mass spectra.

mass spectral data obtained, we take as an example a single peak reported in the analysis of ovine milk fat by HPLC–evaporative light scattering detection (ELSD) [7]. This single chromatographic peak was found to contain no less than five TAG species, namely 10:0-12:0-12:0, 10:0-10:0-14:0, 8:0-12:0-14:0, 8:0-8:0-18:0 and 8:0-10:0-16:0. If this same peak were to be analysed by HPLC–APCI-MS, although 12 DG^+ ions would result from ionisation of these TAGs, these would correspond to only six different masses (Table 1). When identifying unknown TAGs, it is impossible to know to which specific DG^+ ions a particular mass corresponds and consequently, in order to unambiguously identify the TAG species present in such a peak, it is clear that pre-fractionation is needed prior to HPLC–MS.

Two types of pre-fractionation were chosen. The first employed silica TLC to separate the mixture into two fractions, which were analysed by HPLC–APCI-MS. The second approach involved the use of GPC, which separates molecules on the basis of their effective molecular size in solution, by forcing the sample molecules through a rigid, highly porous, polymeric gel. Fractions obtained from GPC were analysed by both HPLC–APCI-MS and by GC using a capillary column with a polarisable stationary phase.

3.2. Prefractionation with silica TLC

Separation of milk fat by silica TLC gave two fractions which were analysed by RP-HPLC–APCI-

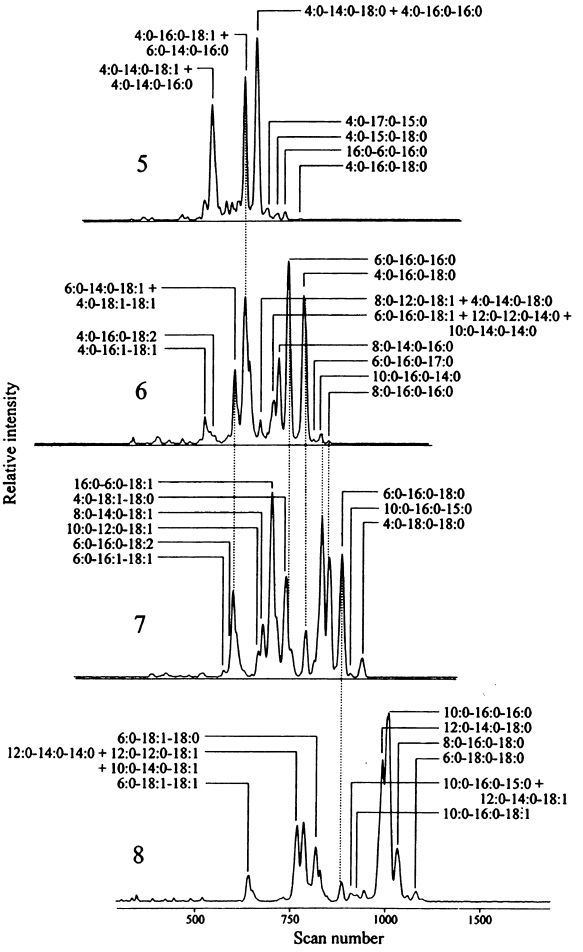

Fig. 7. The HPLC–APCI-MS profiles of GPC fractions 5–8 of milk fat.

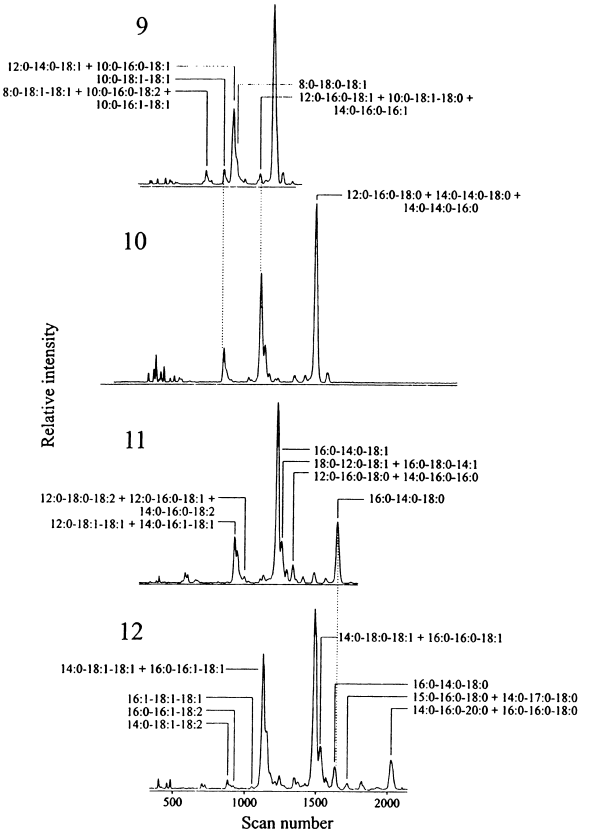

Fig. 8. The HPLC–APCI-MS profiles of GPC fractions 9–12 of milk fat.

MS. Band 1 was found to contain the majority of the TAG species present, but the early part of the chromatogram was still too complex to allow identification of peaks (Fig. 2). However, band 2 contained fewer peaks (ca. 15) and it was possible to identify 28 TAG species with certainty (Fig. 3), the majority of which were butyric acid (4:0) containing species. Since there were few co-elutions, it was possible to use the relative abundances of the DG^+ ions to identify the fatty acid at the 2-position of the TAG [23]. In general, the butyric acid was not esterified at the 2-position; this is consistent with previous reports [24–27]. It was noted that TAGs containing butyric acid, such as 1(3)-butyroyl-2-palmitoyl-3(1)-stearoyl glycerol (4:0-16:0-18:0), typically give rise to APCI-MS spectra with very little background noise (Fig. 4).

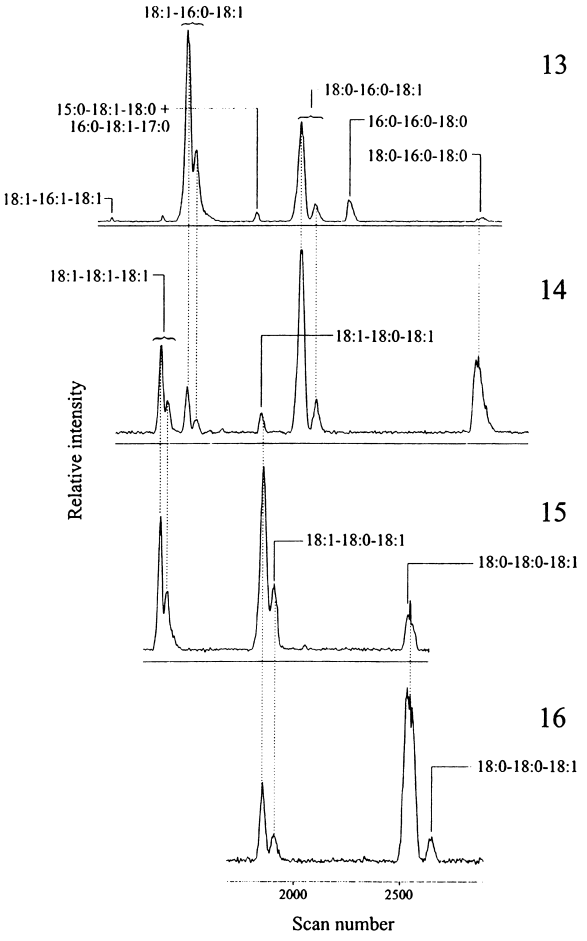

Fig. 9. The HPLC–APCI-MS profiles of GPC fractions 13–16 of milk fat.

3.3. Prefractionation with GPC

Analysis of whole milk fat by GPC, using a polystyrene–divinylbenzene gel, gave rise to the chromatogram shown in Fig. 5. As far as we are aware, this is the first time that GPC has been reported for separation of TAGs. Sixteen fractions (shown by numbers 1–16) were collected and subsequently analysed by RP-HPLC–APCI-MS. The RP-HPLC profiles obtained for the fractions are

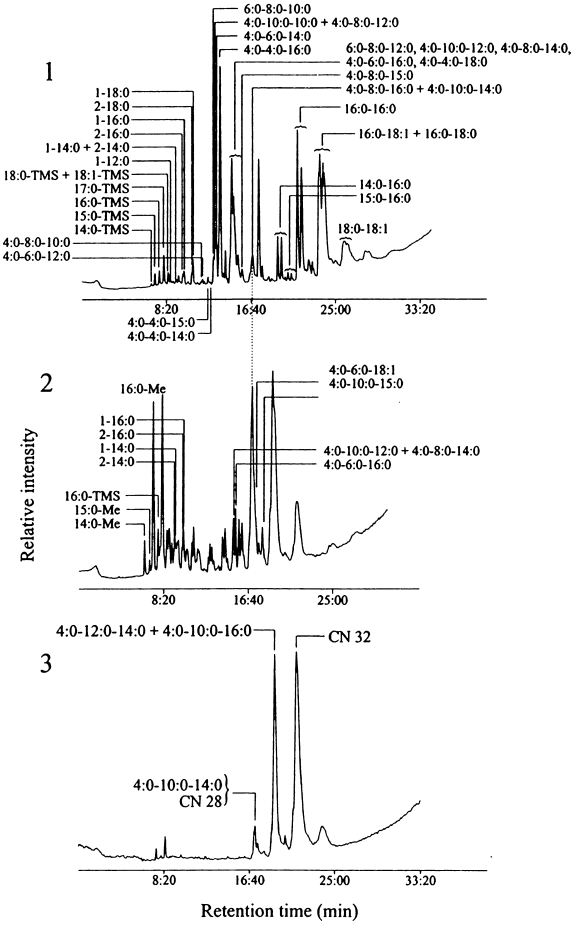

Fig. 10. GC–MS profiles of GPC fractions 1–3 of milk fat. Free fatty acids, present as trimethylsilyl (TMS) esters are shown as X-TMS, fatty acid methyl esters as X-Me, MAGs as X-MAG, where X=fatty acid. DAGs are shown as the two constituent fatty acids.

shown in Figs. 6–9 inclusive. Fractions 1 and 2 appeared to comprise mainly diacylglycerols (DAGs). However, it was not possible to determine which fatty acids were present in each DAG due to the extremely low abundance of the acylium ions (RCO^+) and consequently the peaks are annotated with the major ions seen in the mass spectra. Usually, separation by GPC would cause the low-molecular-mass species to elute after high-molecular-mass components. This does not appear to be the case in this analysis, suggesting that the mode of separation is more similar to reversed-phase chromatography than true size-exclusion chromatography.

Fractions 1–12 were also analysed by GC–MS, using a capillary column coated with a polarisable stationary phase specifically designed for the analysis of TAGs. The resulting chromatograms are shown in Figs. 10 and 11. Fractions 13–16 were not analysed by GC–MS since the TAGs present in these fractions had already been fully elucidated by HPLC–APCI-MS. The low column capacity meant that the best resolution was achieved with very dilute samples. The rising baseline observed in some of the chromatograms therefore reflects the small quantities of analytes applied to the column.

Analysis by HPLC separated TAGs according to the equivalent carbon number (i.e. carbon number−2×number of double bonds; ECN) as predicted by Plattner [28]. Within groups of TAGs of the same ECN, those TAGs containing more unsaturated fatty acyl residues, such as 18:1, tended to elute earlier, whereas the presence of long, saturated fatty acyl

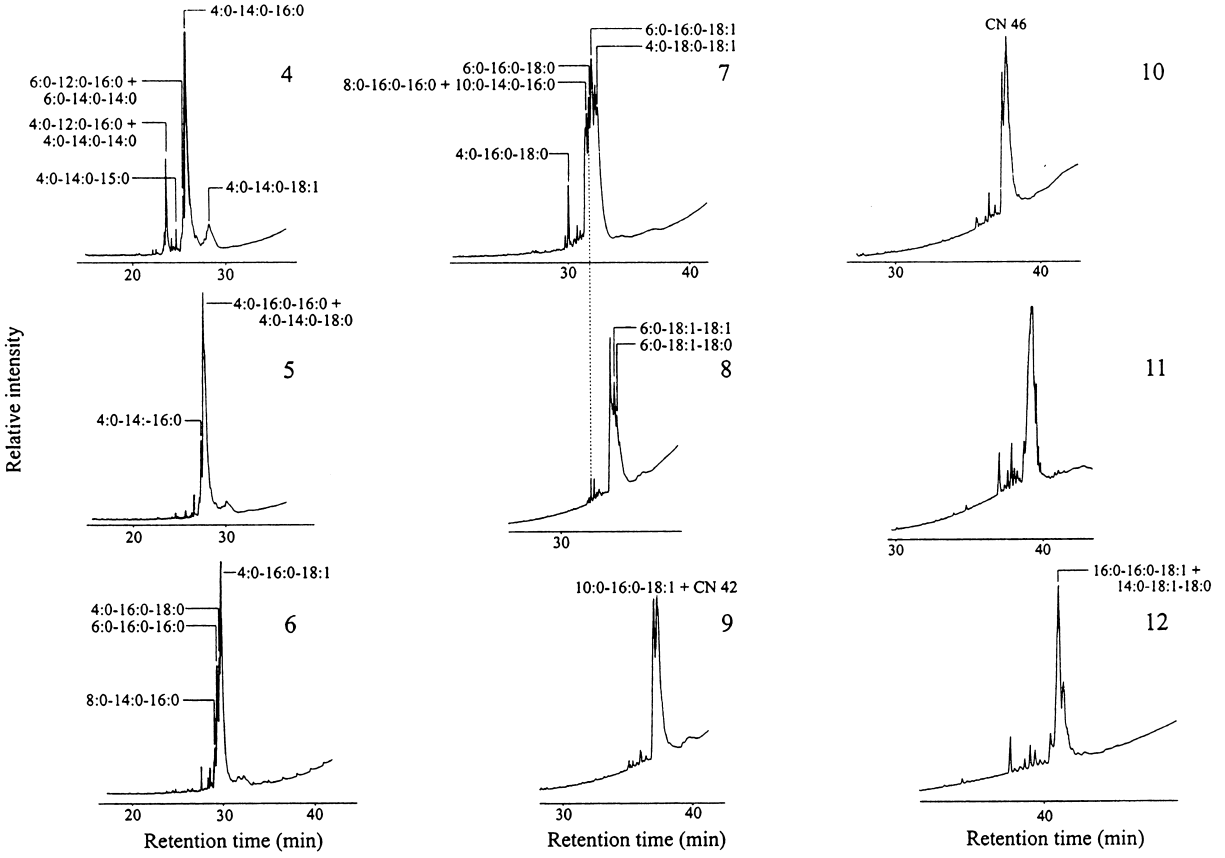

Fig. 11. Gas chromatograms resulting from analysis of GPC fractions 4–12 of milk fat. The numbers given refer to the fractions collected as shown in Fig. 5.

residues tended to have the opposite effect, leading to enhanced retention. In GC analyses, separation was according to carbon number.

Whilst the resolution of TAGs by GC was inferior to that achieved by HPLC, the higher ionisation energies involved in the electron ionisation gave more abundant $[RCO]^+$ ions than were observed in APCI-MS spectra, which helped in identification. Fig. 12 shows a comparison of the EI-MS spectrum obtained for butyroylmyristoylpalmitoyl glycerol (4:0-14:0-16:0) (Fig. 12a) with the spectrum obtained for the same TAG by APCI-MS (Fig. 12b). Whilst the GC–MS analyses did not always allow identification of all the TAG species in each fraction, they did provide information about the carbon number (CN) of the TAGs present, which aided in the interpretation of the HPLC–APCI-MS data. GC–MS showed that fractions 1 and 2 contained free fatty acids, monoacylglycerols (MAGs), DAGs and TAGs (Fig. 10). However, the TAG components were only present at low concentration and despite using mass chromatograms to search for them in the HPLC fractions, it was not possible to conclusively determine where they eluted.

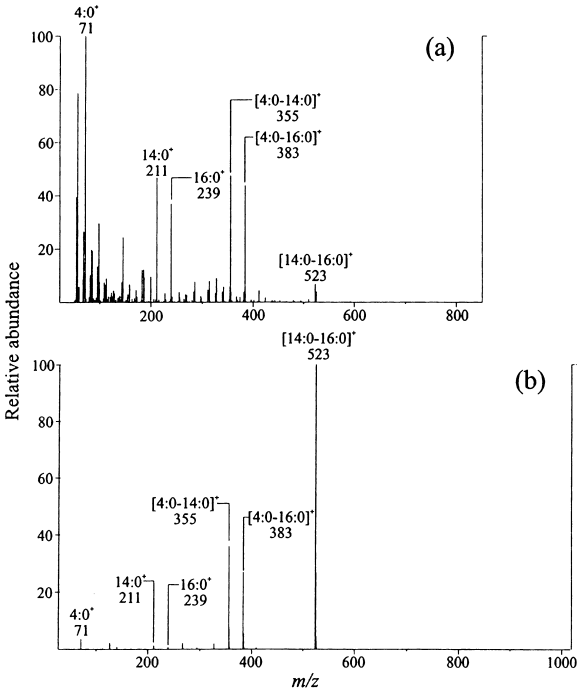

Fig. 12. The mass spectra of butyroylmyristoylpalmitoyl glycerol (4:0-14:0-16:0) resulting from (a) electron ionisation and (b) atmospheric pressure chemical ionisation.

3.4. Identification of components in RP-HPLC profile

Compounds which had been identified in the TLC and GPC fractions could be identified in the original RP-HPLC profile through retention time comparisons. Mass chromatograms were also used to search for specific compounds. Table 2 lists the TAGs identified in the HPLC analyses of the GPC fractions and the TLC band 2, as well as in the original profile. Compounds which do not have an asterisk in any of the fraction columns were identified in the original profile according to their mass spectra. Table 3 lists the identifications according to the peak numbers marked on the chromatogram in Fig. 1. The nomenclature used to describe the TAGs does not reflect the regioisomerism of the molecule. In some cases, the presence of a TAG which was suggested by retention time comparison could not be confirmed by the mass spectrum of the chromatographic peak. This was generally because the DG^+ ions involved were present in such low abundance relative to the background noise that identification was inconclusive. Consequently these compounds have been included in Tables 2 and 3, but are italicised.

Some overlap of ECN groups was observed, due to the presence of very short chain or unsaturated groups decreasing the effective ECN, or because long chain, saturated components caused the effective ECN to be increased. For example, 16:0-18:1-18:3 and 12:0-18:1-18:1 (ECN 44), eluted before 6:0-18:0-18:0 and 8:0-16:0-18:0 (ECN 42). Some of the fatty acyl residues, in particular those with odd carbon numbers, may have branched chains, which may also have caused overlap with other ECN groups. Some TAGs, such as 16:0-16:0-18:1 and 18:0-16:0-18:1, were present as more than one chromatographic peak, implying the presence of different geometric isomers of the 18:1 fatty acid. Comparison of retention times with those observed in vegetable oils suggested that the TAG containing the *cis*-isomer eluted before the *trans*-analogue [14]. Where the TAG contained only one 18:1 residue (e.g. 16:0-16:0-18:1), two distinct peaks were ob-

Table 2
The TAG species identified in GPC fractions 1-16, TLC band 2 and whole milk through analysis by HPLC–APCI-MS

TAG[a]	GPC fraction															ECN[b]	Peak number
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	TLC		
4:0-10:0-18:2	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*28*	*1*
4:0-8:0-18:1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*28*	*1*
6:0-8:0-14:0	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*28*	*2*
4:0-10:0-14:0	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	28	2
4:0-8:0-16:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	28	2
4:0-10:0-16:1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*28*	*3 LH shoulder*
4:0-14:0-14:1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*30*	*3*
4:0-10:0-18:1	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	30	3
6:0-10:0-14:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	30	3
10:0-10:0-10:0	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*30*	*4*
4:0-10:0-16:0	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	30	4
4:0-12:0-14:0	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	30	4
4:0-18:3-18:1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	32	4
6:0-10:0-18:1	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	*32*	*5*
6:0-12:0-16:1	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	*32*	*5*
4:0-16:1-16:1	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	*32*	*5*
10:0-16:0-6:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	32	6
4:0-14:1-16:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	32	6
4:0-12:0-18:1	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	32	6
4:0-16:1-14:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	32	6
6:0-12:0-14:0	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	*32*	*6*
4:0-14:0-14:0	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	32	7
4:0-12:0-16:0	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	32	7
4:0-14:0-15:0	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	33	9
8:0-12:0-14:0	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	34	9
4:0-14:0-18:1	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	34	10
6:0-14:0-14:0	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	34	10
6:0-12:0-16:0	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	34	10
4:0-16:1-16:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	34	10
4:0-16:1-18:1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*34*	*10*
4:0-16:0-18:2	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	*34*	*10 RH shoulder*
4:0-14:0-16:0	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	34	11
6:0-14:0-18:1	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	36	12
4:0-18:1-18:1	−	−	−	+	+	−	−	−	−	−	−	−	−	−	+	36	12
4:0-15:0-16:0	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	35	12
6:0-16:0-18:2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	36	12
6:0-14:0-16:0	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	36	13
4:0-16:0-18:1	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+	36	13
8:0-12:0-18:1	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	36	14
4:0-16:0-16:0	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	36	14
4:0-14:0-18:0	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+	36	14
10:0-12:0-18:1	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	38	14
6:0-18:1-18:1	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	38	14
4:0-17:0-15:0	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	*36*	*15*
6:0-16:0-18:1	−	−	−	+	+	+	−	−	−	−	−	−	−	−	+	38	15
12:0-12:0-14:0	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	38	15
10:0-14:0-14:0	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	38	15
8:0-14:0-16:0	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	38	16

Table 2. Continued

TAG[a]	GPC fraction															ECN[b]	Peak number
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	TLC		
4:0-15:0-18:0	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	*37*	*17*
4:0-16:0-17:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	*37*	*17*
4:0-18:1-18:0	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	38	17
6:0-16:0-16:0	−	−	+	+	+	−	−	−	−	−	−	−	−	−	+	38	17
8:0-18:1-18:1	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	*40*	*17 RH shoulder*
10:0-16:0-18:2	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	*40*	*17 RH shoulder*
10:0-16:1-18:1	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	*40*	*17 RH shoulder*
4:0-16:0-18:0	−	−	+	+	+	−	−	−	−	−	−	−	−	−	+	38	18
8:0-14:0-18:1	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	38	18
6:0-18:1-18:0	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	40	19
12:0-14:0-14:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	40	19
10:0-16:0-14:0	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	40	20
8:0-16:0-16:0	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	40	21
10:0-18:1-18:1	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	42	22
6:0-16:0-18:0	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	40	23
14:0-14:0-14:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	42	23
10:0-16:0-18:1	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	42	24
12:0-14:0-18:1	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	42	24
4:0-18:0-18:0	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	40	25
6:0-16:0-20:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	42	25
8:0-18:0-18:1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	42	25
12:0-14:0-16:0	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	42	26
10:0-16:0-16:0	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	42	26
16:0-18:1-18:3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	44	26
18:2-18:2-18:1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	44	26
12:0-18:1-18:1	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	44	27
14:0-16:1-18:1	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	44	27
8:0-16:0-18:0	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	42	27
14:0-16:0-18:1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	44	28
6:0-18:0-18:0	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	42	29
14:0-16:0-18:2	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	44	30
10:0-18:1-18:0	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	44	30
12:0-18:0-18:2	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	44	30
12:0-16:0-18:1	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	44	30
14:0-16:0-16:1	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	44	30
16:0-15:0-16:1	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	*45*	*31*
14:0-15:0-18:1	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	*45*	*31*
16:1-18:1-18:1	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	*46*	*32*
16:0-18:1-18:2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	46	33
12:0-14:0-20:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	46	33
12:0-16:0-18:0	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	46	33
14:0-14:0-18:0	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	46	33
14:0-16:0-16:0	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	46	33
12:0-14:0-14:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	46	34
14:0-18:1-18:1	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	46	35/36[c]
16:0-16:1-18:1	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	46	35/36[c]
16:0-16:0-18:2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	46	37
16:0-14:0-18:1	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	46	38
12:0-18:0-18:1	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	46	38
12:0-18:0-18:1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	46	39

Table 2. Continued

TAG[a]	GPC fraction															ECN[b]	Peak number
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	TLC		
xxxx16:0-14:1-18:0	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	46	39
18:1-18:1-18:1	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	48	40/41[c]
14:0-16:0-18:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	48	42
14:0-14:0-20:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	48	42
18:2-18:1-18:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	48	42
18:1-16:0-18:1	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	48	43/44[c]
14:0-18:1-18:0	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	48	45/46[c]
16:0-16:0-18:1	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	48	45/46[c]
18:1-15:0-18:0	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	49	47
16:0-17:0-17:1	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	*48*	*47*
16:0-18:1-17:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	49	47
14:0-18:0-18:0	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	50	48
14:0-16:0-20:0	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	50	48
18:1-18:1-18:0	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	50	49/50[c]
15:0-16:0-18:0	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	49	50
14:0-17:0-18:0	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	49	50
18:0-16:0-18:1	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	50	51/52[c]
16:0-16:0-18:0	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	50	53
14:0-16:0-20:0	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	*50*	*53*
18:0-18:0-18:1	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	52	54
18:0-16:0-18:0	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	52	55
18:0-18:0-18:0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	54	56

+, present in fraction; −, not detected in fraction; compounds in italics were identified by comparison of retention time, but not confirmed by mass spectrum.

[a] Positional isomer not distinguished.

[b] ECN, equivalent carbon number=carbon number−2×number of double bonds.

[c] Present as two peaks (i.e. different geometric isomers).

served (45 and 46). However, where the TAG comprised more than one 18:1 residue, such as 18:1-18:1-18:1, the number of possible combinations of *cis*- and *trans*- was such that complete resolution of all molecular species was not possible and two peaks are seen, each with several partially resolved shoulders (40 and 41).

The most abundant peak (peak 13) contained two TAG species, namely 4:0-16:0-18:1 and 6:0-14:0-16:0. Other abundant peaks contained 8:0-12:0-18:1, 4:0-16:0-16:0, 4:0-14:0-18:0, 10:0-12:0-18:1, 6:0-18:1-18:1, 6:0-14:0-18:1, 4:0-18:1-18:1, 4:0-15:0-16:0 and 6:0-16:0-18:2. Since the mass spectral response for different fatty acids varied considerably, it was difficult to ascertain which TAGs were most abundant. In addition, the effects of band broadening caused those components eluting at longer retention times to have very different peak shapes from those eluting in the early parts of the chromatogram, thereby complicating direct comparison.

In total, 120 TAG species were identified in milk fat, eight of which were present as two chromatographic peaks due to the presence of different geometric isomers. These compared very well with the TAGs reported in bovine milk fat by Robinson and MacGibbon, using RP-HPLC [4]. The elution order was very similar, although there were slight differences within each ECN group. Fifty of the TAGS reported in this study were also reported by Myher et al. who used GC–negative ion CI-MS [13]. Most of the more abundant components reported by Myher et al., such as 4:0-16:0-18:1, 4:0-16:0-16:0, 10:0-12:0-18:1, 6:0-18:1-18:1, 12:0-14:0-14:0, 18:1-18:1-18:1, 18:1-18:1-16:0, 16:0-16:0-18:1 and 16:0-16:0-18:0, were seen in this study. Seventy components which were not observed in this study were mostly minor components, but included 22 acetyl-containing TAGs, which have not been reported in any other study on bovine milk fat. Fraga et al. used GC to analyse the TAGs present in six butter fat

Table 3
The TAGs identified in whole milk fat by HPLC–APCI-MS

Peak number	TAGs identified
1	*4:0-10:0-18:2, 4:0-8:0-18:1*
2	*6:0-8:0-14:0*, 4:0-10:0-14:0, 4:0-8:0-16:0
3	*4:0-10:0-16:1 (LH shoulder)*, *4:0-14:0-14:1*, 4:0-10:0-18:1, 6:0-10:0-14:0
4	*10:0-10:0-10:0*, 4:0-10:0-16:0, 4:0-12:0-14:0, 4:0-18:3-18:1
5	*6:0-10:0-18:1*, *6:0-12:0-16:1*, *4:0-16:1-16:1*
6	10:0-6:0-16:0, 4:0-14:1-16:0, 4:0-12:0-18:1, 4:0-16:1-14:0, *6:0-12:0-14:0*
7	4:0-14:0-14:0, 4:0-12:0-16:0
8	ni
9	4:0-14:0-15:0, 8:0-12:0-14:0
10	4:0-14:0-18:1, 6:0-14:0-14:0, 6:0-12:0-16:0, 4:0-16:1-16:0, *4:0-16:1-18:1*, *4:0-16:0-18:2*
11	4:0-14:0-16:0
12	6:0-14:0-18:1, 4:0-18:1-18:1, 4:0-15:0-16:0, 6:0-16:0-18:2
13	6:0-14:0-16:0, 4:0-16:0-18:1
14	8:0-12:0-18:1, 4:0-16:0-16:0, 4:0-14:0-18:0, 10:0-12:0-18:1, 6:0-18:1-18:1
15	*4:0-17:0-15:0*, 6:0-16:0-18:1, 12:0-12:0-14:0, 10:0-14:0-14:0
16	8:0-14:0-16:0
17	*4:0-15:0-18:0*, *4:0-16:0-17:0*, 4:0-18:1-18:0, 6:0-16:0-16:0
17 RH shoulder	*8:0-18:1-18:1*, *10:0-16:0-18:2*, *6:0-16:1-18:1*
18	4:0-16:0-18:0, 8:0-14:0-18:1
19	6:0-18:1-18:0, 12:0-14:0-14:0
20	10:0-16:0-14:0
21	8:0-18:1-18:1
22	10:0-18:1-18:1
23	6:0-16:0-18:0, 14:0-14:0-14:0
24	10:0-16:0-18:1, 12:0-14:0-18:1
25	4:0-18:0-18:0, 6:0-16:0-20:0, 8:0-18:0-18:1
26	12:0-14:0-16:0, 10:0-16:0-16:0, 16:0-18:1-18:3, 18:2-18:2-18:1
27	12:0-18:1-18:1, 14:0-16:1-18:1, 8:0-16:0-18:0
28	14:1-16:0-18:1
29	6:0-18:0-18:0
30	14:0-16:0-18:2, 10:0-18:1-18:0, 12:0-18:0-18:2, 12:0-16:0-18:1, 14:0-16:0-16:1
31	*16:0-15:0-16:1*, *14:0-15:0-18:1*
32	16:1-18:1-18:1
33	16:0-18:1-18:2, 12:0-14:0-20:0, 12:0-16:0-18:0, 14:0-14:0-18:0, 14:0-16:0-16:0
34	12:0-14:0-14:0
35	14:0-18:1-18:1, 16:0-16:1-18:1
36	14:0-18:1-18:1*t*, 16:0-16:1-18:1
37	16:0-16:0-18:2
38	16:0-14:0-18:1, 12:0-18:0-18:1
39	12:0-18:0-18:1, 16:0-14:1-18:0
40	18:1-18:1-18:1
41	18:1-18:1-18:1*t*
42	14:0-16:0-18:0, 14:0-14:0-20:0, 18:2-18:1-18:0
43	18:1-16:0-18:1
44	18:1-16:0-18:1*t*
45	14:0-18:1-18:0, 16:0-16:0-18:1
46	14:0-18:1-18:0*t*, 16:0-16:0-18:1*t*
47	18:1-15:0-18:0, *16:0-17:0-17:1*, 16:0-18:1-17:0
48	14:0-18:0-18:0, 14:0-16:0-20:0

Table 3. Continued

Peak number	TAGs identified
49	18:1-18:1-18:0
50	18:1-18:1-18:0*t*, 15:0-16:0-18:0, 14:0-17:0-18:0
51	18:0-16:0-18:1
52	18:0-16:0-18:1*t*
53	16:0-16:0-18:0, *14:0-16:0-20:0*
54	18:0-18:0-18:1
55	18:0-16:0-18:0
56	18:0-18:0-18:0

Compounds in italics were identified by comparison of retention time, but not confirmed by mass spectrum; RH, right hand; LH, left hand; ni, not identified; *t*, *trans*-isomer of 18:1.

fractions obtained from Ag-TLC and identified 80 components, 52 of which were found in the current study [3].

4. Conclusions

TLC and GPC were successfully used to pre-fractionate the milk fat prior to analysis by HPLC–APCI-MS. To our knowledge, this is the first time that GPC has been reported for the separation of TAGs. It was observed that separation was according to polarity rather than molecular size, suggesting that the mode of separation was more similar to reversed-phase chromatography than true size-exclusion chromatography. As expected, HPLC separated TAGs according to the equivalent carbon number (i.e. carbon number$-2\times$number of double bonds). Within ECN groups, TAGs containing residues of short chain or unsaturated fatty acids tended to elute earlier, whilst long, saturated fatty acyl residues tended to have the opposite effect, leading to enhanced retention. The GPC fractions were also analysed by GC–MS, using a capillary column coated with a polarisable stationary phase. Whilst the separation was inferior to that afforded by HPLC, the spectra exhibited acylium ions which were much more abundant than those observed in APCI-MS and which aided in mass spectral interpretation. Using the data from GC–MS and HPLC–APCI-MS of the fractions, it was possible to assign structures to peaks in the original chromatogram allowing 120 TAG species to be identified in total. This is the first time HPLC–APCI-MS has been used for the identification of intact TAGs in whole milk fat.

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