APPLICATION OF ELECTROSPRAY MASS SPECTROMETRY TO THE ANALYSIS OF LIPIDS

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY IN FOOD TECHNOLOGY AT MASSEY UNIVERSITY

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Abstract

This work investigated the application of electrospray mass spectrometry (MS) to the elucidation of lipid structure, especially related to milkfat. This was the first time that the instrument in our laboratory had been used for this purpose.

Standard samples of triacylglycerols (TAGs) were prepared in 1,2 dichloroethane: acetonitrile:formic acid (63:35:2) at approximately 0.005 mg/mL and were then used to optimise and quantify the ion signal (response) generated by the Perkin Elmer Sciex API300 electrospray mass spectrometer. Both MS and tandem MS experiments were performed. In the spectra, the TAG molecule was seen as an ammonium ion adduct $(M+NH_4)^+$. It was found that the relative responses of diacylglycerol (DAG) ions, formed during front end fragmentation of the $(M+NH_4)^+$ ion, depended on the position of the 'lost' fatty acid on the glycerol backbone and its carbon number, with the former rather than the latter being the more critical. This information enabled the fatty acid esterified to the sn2 carbon of the glycerol backbone to be identified, and also demonstrated that it was possible to identify each of the fatty acids in a TAG molecule accurately by molecular and DAG ion identification. MS/MS experiments were performed on DAG ions, rather than parent ions, to identify and measure the response of acylium ions generated during collision-assisted dissociation (CAD). In contrast to the response of the DAG ions above, it was found that the response of these acylium ions was dependent on their carbon number and degree of saturation rather than the position that the fatty acid had held on the glycerol backbone. Optimal voltage settings for analysis of TAGs by infusion MS were obtained, which gave good quality spectra and ample amounts of molecular and DAG ions. With this information, a novel liquid chromatography-mass spectrometry (LC-MS) method, which was able to characterise the TAGs in a number of complex lipid samples, was developed. The method was used to elucidate differences in the TAG structure of different bovine milkfats and also differences in fat from milk of various mammalian species.

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Nomenclature

Common Names of Fatty Acids and their Carbon Numbers

Carbon number	*Systematic name*	*Common name*	*n*	*Molecular weight*
4:0(B)	butanoic	butyric		88.11
6:0	hexanoic	caproic		116.16
8:0	octanoic	caprylic		144.21
10:0	decanoic	capric		172.27
12:0	dodecanoic	lauric		200.32
14:0	tetradecanoic	myristic		228.38
16:0(P)	hexadecanoic	palmitic		256.43
16:1	hexadec-9-enoic	palmitoleic	7	254.41
17:0	heptadecanoic	margaric		270.46
18:0(S)	octadecanoic	stearic		284.48
18:1	octadec-6-enoic	petroselenic		282.47
18:1(O)	octadec-9-enoic (*cis*)	oleic	9	282.47
18:1(E)	octadec-9-enoic (*trans*)	elaidic	9	282.47
18:1	octadec-11-enoic (*trans*)	vaccenic	7	282.47
18:1	12-hydroxy-octadec-9-enoic	ricinoleic	9	298.47
18:2	octadeca-9,12-dienoic	linoleic	6	280.45
18:2c	octadeca-9,11-dienoic	conjugated linoleic		280.45
18:3c	octadeca-9,11,13-trienoic	elaeostearic		278.44
18:3	octadeca-9,12,15-trienoic	linolenic (alpha)	3	278.44
18:3	octadeca-6,9,12-trienoic	gamma linolenic	6	278.44
18:5	octadecapentaeonic			274.4
20:0	eicosanoic	arachidic		312.54
20:1	eicos-9-enoic	gadoleic		310.52
20:4	eicosa-5,8,11,14-tetraenoic	arachidonic	6	304.47
20:5	eicosa-5,8,11,14,17-pentaenoic	EPA	3	302.46
22:1	docos-11-enoic	cetoleic		338.58
22:1	docos-13-enoic	erucic	9	338.58
22:4	docosa-7,10,13,16-tetraenoic	adrenic	6	332.53
22:5	docosa-7,10,13,16,19-pentaenoic	clupadonic	3	330.51
22:6	docosa-4,7,10,13,16,19-hexaenioc	DHA	3	328.50
23:0	docosanoic			354.6
24:0	tetracosanoic	lignoceric		368.65
25:1	pentacosenoic			380.70

The triacylglycerols are abbreviated in the text as groups of individual fatty acids *e.g.* 16:0, 18:1, 18:0. Common triacylglycerols have been further abbreviated using the singly letter designations shown in the table above, *e.g.* POS for 1-palmitoyl-2-oleoyl-3-stearoylglycerol.

Diglycerols follow a similar pattern of abbreviation.

Unless stated no specific regioisomers are implied by the order of the nomenclature.

1 Introduction

1.1 Aim

The aim of this research project was to develop expertise in using electrospray mass spectrometry (MS) to measure and characterise the components of milkfat. This was an initial study of a complex area and the aim was to provide a basis for further detailed work.

The lipid fraction of bovine milk is made up largely of a complex mixture of triacylglycerols (TAGs) (98%) with minor contributions from diacylglycerols (DAGs), monoacylglycerols (MAGs), sterols (predominantly cholesterol), phospholipids, fat-soluble vitamins (A, D, E) and β-carotene (Creamer & MacGibbon, 1996).

As the performance of milkfat products is determined by both the detailed composition and the manufacturing conditions of the milkfat, it is becoming increasingly important to be able to accurately characterise and measure the components of this lipid fraction. With increased customer awareness of diet and diet-related health issues, the need to know not only the composition of milkfat, but also how we can manipulate these components to meet customer requirements, has never been more critical. It is essential therefore that analytical techniques that enable the detailed characterisation of lipid composition are available. Currently no one routine analytical technique with these capabilities exists. An analytical technique that does have these capabilities, although it has not been widely used in the analysis of TAGs, is electrospray MS.

Because milkfat is a complex mixture of TAGs, the initial optimisation of the electrospray mass spectrometer was carried out with pure TAG standards which have simple, well defined spectra. When the methods were optimised, simple oils such as coconut oil and cocoa butter were used to develop methods to characterise simple mixtures. Finally high-performance liquid chromatography (HPLC) was used in conjunction with MS to investigate the detailed composition of the complex mixtures of TAGs in milkfat samples.

1.2 Background

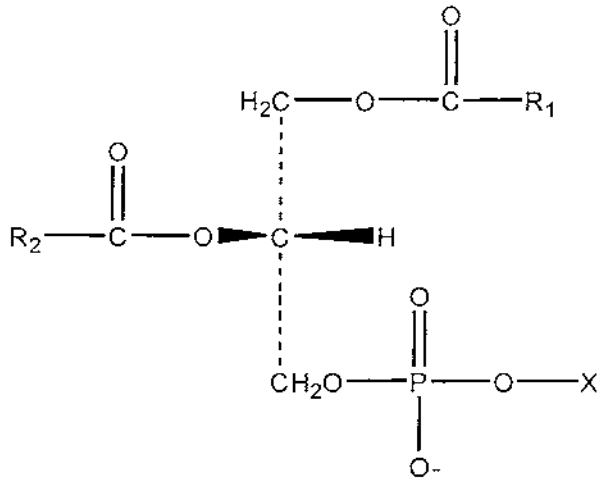
In this section, the more common lipids found in milkfat and existing methods used to characterise these lipids (particularly at the New Zealand Dairy Research Institute (NZDRI)) are summarised. MS is be discussed in general terms, with a more detailed review of the MS methods that have been used in the literature to characterise lipids (particularly, where possible, lipids from milkfat). Particular emphasis is placed, for obvious reasons, on electrospray MS in the characterisation of lipids.

1.2.1 Lipids in Milk

The lipid fraction of bovine milk is made up largely of TAGs (98%) with minor contributions from DAGs, MAGs, sterols (predominantly cholesterol), phospholipids, fat-soluble vitamins (A, D, E) and β-carotene (Creamer & MacGibbon, 1996).

Phospholipids

The glycerophospholipids phosphatidylcholine and phosphatidylethanolamine and the sphingophospholipid sphingomyelin are the main components of the phospholipid fraction of milkfat. Lysophospholipids are also present. Glycerophospholipids have an *sn* glycerol-3-phosphate as a backbone, which is esterified at carbon atoms 1 and 2 with fatty acid residues, and have a polar head group, *e.g.* choline and ethanolamine for phosphatidylcholine and phosphatidylethanolamine respectively. The hydroxyl groups of the polar head group are esterified to the phosphoric acid found esterified to carbon atom 3. Sphingomyelin contains phosphocholine esterified to the hydroxyl group of carbon atom 1 of ceramide (Christie, 1995).



where x is either choline or ethanolamine

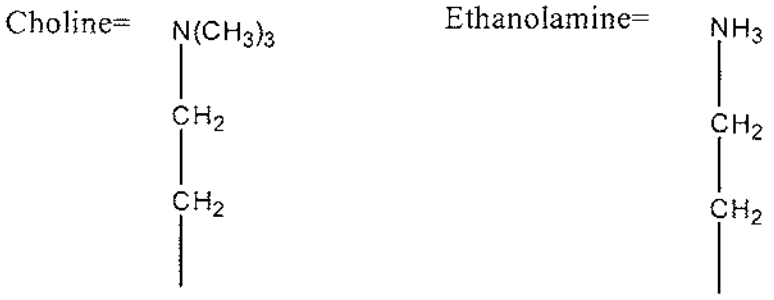


Figure 1. The structure of the principle glycerophospholipids found in bovine milkfat.

Neutral Lipids

A TAG consists of three fatty acids esterified to a glycerol backbone, whereas a DAG and a MAG consist of two and one fatty acid(s) respectively esterified to a glycerol backbone. The concentration of the various fatty acids present in the lipid fraction of milk depends largely on the stage of lactation and the diet of the cow (Creamer & MacGibbon, 1996). The major fatty acids in bovine milkfat are palmitic and oleic acids (50% by weight). Significant proportions of short chain fatty acids, most often esterified to the sn3 position of the glycerol backbone, together with minor fatty acids including branched chain fatty acids, odd-carbon-number fatty acids and trans-unsaturated fatty acids are also found in the lipid fraction of milk.

Both the position of the fatty acid on the glycerol backbone and the type of fatty acid (the length of the carbon chain and the degree of unsaturation) can influence physical

properties such as crystallisation and melting point (Christie, 1995). Variation in these physical properties has ramifications for both the sensory properties of milkfat (*i.e.* taste and texture) and the functional properties of milkfat (such as spreadability). Thus determination of the type of fatty acids present and their position on the glycerol backbone can give a valuable insight into the physical and functional properties of milkfat, and any given milkfat fraction.

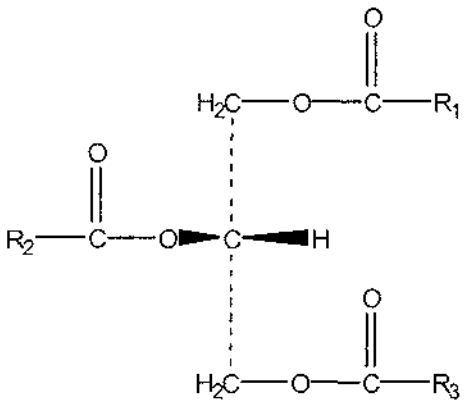


Figure 2. TAG structure

1.2.2 Lipid Separation Techniques

HPLC

HPLC and gas chromatography (GC) are two techniques that are commonly used in the separation and identification of the TAG content of milkfat, identification of the fatty acid content of these TAGs and identification of any minor components present in the fat (*e.g.* phospholipids and cholesterol).

In HPLC, lipids are solubilised in an organic solvent and are injected on to an HPLC column. The interaction of the lipid, the HPLC column and the solvent(s) flowing through the column determines the length of time each lipid component is retained by the column (retention time) before elution from it. This eluate then flows through a detection system, most commonly an evaporating light scattering detector (ELSD) (for lipids) although more recently mass spectrometers have been used. Changing the type of column, and/or the solvent system used, will change the retention time of the lipid components and hence the separation achieved, in some cases quite dramatically. Common separation techniques using HPLC include the following.

Silver Ion HPLC

Separation takes place on an HPLC column that contains either an ion exchange resin or silica packing on to which silver ions are bound. TAG components of milkfat are separated largely on the basis of the number and distribution of double bonds present within the fatty acids of each TAG. Silica-based columns also offer separation of regioisomeric TAGs.

Reversed phase HPLC

Separation takes place on a non-polar octadecylsilane bonded phase. Separation of TAGs is based on the combined chain lengths and the total number of double bonds of the fatty acids within each TAG.

Normal phase HPLC

Separation takes place on a silica column. This method is most commonly used for the separation of polar lipids and is not often used for TAG analysis (Laakso, 1996; Kuksis, 1998).

Gas Chromatography

In GC, the lipid sample is dissolved in a solvent. The sample is vaporised in a heated inlet port (injector). A carrier gas transfers the sample from the injector through the column and into the detector, most commonly a flame ionisation detector although mass spectrometers can also be used. Separation of each lipid component is determined by the distribution of each component between the carrier gas (mobile phase) and the stationary phase (the column). A component that spends little time in the stationary phase will elute quickly. Changing the column, the length of the column, the type of carrier gas or combinations of the three changes the elution pattern of the components in any given lipid sample (Kitson *et al.*, 1996).

A non-polar fused silica capillary column achieves separation of TAGs based on the total carbon number of each TAG. Polar capillary columns can give a different separation, based for example on the number of acyl carbons and double bonds. GC can also be used to detect and measure the fatty acid components of TAGs. Fatty acids are cleaved from the glycerol backbone, and are converted to esters, usually methyl esters, prior to analysis by GC.

"The technique of Gas Chromatography revolutionised the study of lipids by making it possible to determine the complete fatty acid composition of a lipid in a very short time" (Kitson *et al.*, 1996, p. 5).

1.2.3 Mass Spectrometry

MS is becoming an increasingly important tool in the analysis of a wide range of compounds, both as a stand-alone method of 'detection' and in conjunction with chromatographic techniques such as GC and HPLC.

A mass spectrometer is an analytical device that determines the molecular weight of chemical compounds by separating molecular ions according to their mass to charge (m/z) ratio. As a result, both molecular weight information and structural details (about these chemical compounds) can be obtained (Siuzdak, 1996). MS is used for the analysis of many compounds including proteins, peptides, carbohydrates, oligonucleotides, drug metabolites, lipids and lipid-related compounds.

"Mass Spectrometry can be considered as a universal detector for lipids and suitable for quantification" (Laakso, 1996, p. 214).

Simply put, a mass spectrometer consists of a sample inlet, an ion source, a high vacuum system, a mass analyser and an ion detector.

The Mass Analyser

"The function of the mass analyser is to separate molecules according to their mass to charge (m/z) ratio" (Siuzdak, 1996, p. 15).

This review is confined to work using mass spectrometers with quadrupole analysers. Quadrupoles are four precisely parallel rods with a direct current (DC) voltage and a superimposed radio frequency (RF potential). Once the voltages are applied, only ions of a certain m/z ratio pass through the quadrupole filter and all other ions are thrown out of their original path. A mass spectrum is obtained by monitoring the ions passing through the quadrupole filter as the voltages on the rods are varied.

Triple quadrupole MS utilises the second quadrupole as a collision cell, to generate fragment ions. An ion of interest is selected using the first quadrupole Q1. After selecting the MS/MS option, only this ion of interest is allowed into the collision cell

Q2. Here it collides with the collision-assisted dissociation (CAD) gas (usually argon) and fragments. These fragments can then be analysed by the third quadrupole Q3 (Siuzdak, 1996) (Figure 1).

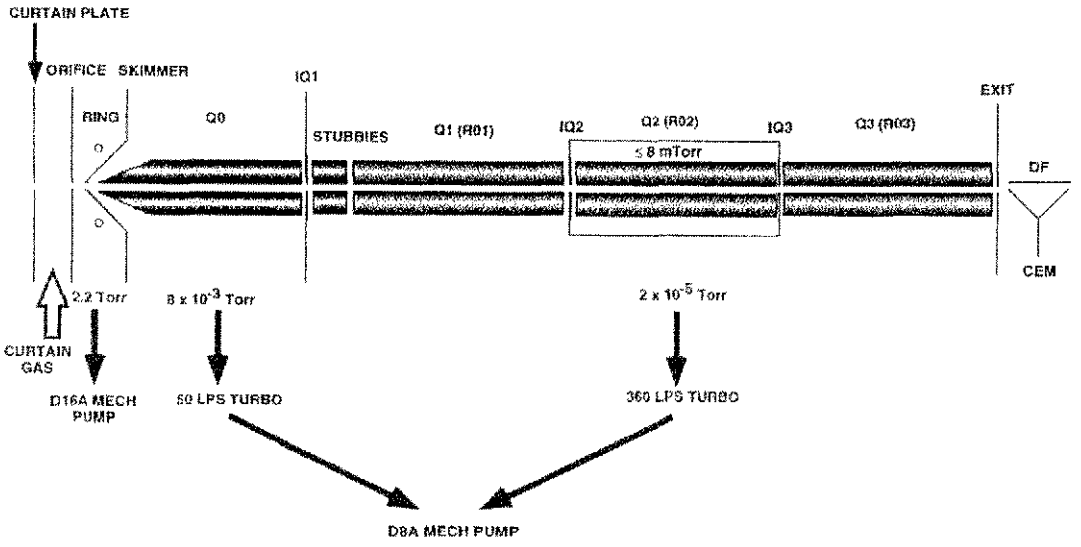


Figure 3. API300 ion optical path.

Sample Inlet

Samples are introduced into the ionisation region of the mass spectrometer via one of two methods.

Direct insertion probe

Direct insertion probe involves placing the sample on a probe, which is then inserted (through a vacuum lock) into the ionisation region. The sample can then undergo thermal or high energy desorption processes.

Direct infusion

A sample can be directly infused via a capillary into the ionisation region of the mass spectrometer. Depending on the ion source used, the capillary can also be connected to the outlet of a GC or HPLC instrument. In this way, GC-MS or LC-MS can be performed. Separation of a lipid sample on GC or HPLC prior to entry into the

analyser can simplify the spectra and make interpretation of structural data more straightforward.

The Ion Source

Much of this section of the review centres around those ion sources that have been used in the literature to successfully characterise lipids, with particular emphasis on electrospray ionisation.

Each of the ion sources described below produces ions by ionising a neutral molecule using one or more of the following:

- electron ejection or electron capture,
- protonation or deprotonation,
- cationisation, and
- transferral of a charged molecule from a condensed phase into a gas phase.

Electron ionisation (EI)

EI was the most common ionisation source for mass analysis until the 1980s (Siuzdak, 1996). The sample must be introduced into the ionisation source as a gas. If a direct probe is used as the sample introduction mechanism, the sample undergoes thermal desorption. Alternatively the effluent from a GC instrument can be directly infused into the ionisation source via a capillary (GC-MS). Further discussion of GC-MS as a tool to characterise lipids is outside the scope of this review. (The electrospray mass spectrometer used does not accommodate a GC inlet.)

Once in the ionisation source, the vaporised sample interacts with an electron beam, resulting in electron ejection (hence ionisation) and some degree of fragmentation. Often, excessive fragmentation of larger molecular weight samples makes interpretation of the spectra very difficult.

"The usefulness of electron ionisation decreases significantly for compounds above a molecular weight of 400Da" (Siuzdak, 1996, p. 15).

Thus, the use of EI as a means to determine structural information about molecules such as TAGs and phospholipids (which have a molecular weight higher than 400 Da) is limited.

Chemical Ionisation (CI)

Of the softer ionisation techniques applied to lipids, CI is the most common (Laakso, 1996, Table 2, p. 218).

In CI, a large excess of gas, *e.g.* methane, is introduced into the CI source at high pressure. The gaseous ions produced react with the gaseous analyte molecules to form ions by either a proton or hydride transfer. Components of an HPLC eluant or a sample solvent can also act as reactant gases. As with EI, sample can be introduced into the spectrometer via a direct exposure probe although CI is commonly used as an ion source for GC-MS.

Laakso & Kallio (1996) produced a method that uses negative ion CI with ammonia to assess the molecular weights of standard TAGs. Abundant $(M-H)^-$ ions (where M is the intact TAG) were produced in a very simple spectrum. The temperature and the pressure of the ion source were optimised so that very few cluster ions were produced as the presence of these complicated the spectrum. Laakso & Kallio (1996) noted that rapid desorption of the sample from the insertion probe was required. This minimised thermal degradation of unsaturated molecules, a common criticism of ionisation techniques that require an application of heat (Careri *et al.*, 1998).

Currie & Kaillio (1993) used ammonia negative ion tandem MS to analyse human milk and rapeseed oil TAGs. TAGs were introduced into the spectrometer via a direct exposure probe. $(M-H)^-$ ions were identified using the first quadrupole. Some of these ions were selected for MS/MS, *i.e.* collision in the second quadrupole. The ions formed were DAG ions $(M-H-RCOOH)^-$, $(M-H-RCOOH-74)^-$, $(M-H-RCOOH-100)^-$ and fatty acid ions $(RCOO)^-$. If a fatty acid was esterified at the sn2 position, the DAG ions $(M-H-RCOOH-74)^-$ and $(M-H-RCOOH-100)^-$ were not seen. It was therefore possible to determine the fatty acid components of the TAG and their position (1/3 or 2) on the glycerol backbone. However, it was not possible to gain positional information about the double bonds present in a fatty acid, or the possibility of branching.

Atmospheric pressure chemical ionisation (APCI)

In APCI, the sample enters the ionisation source as a liquid. The application of heat and a constant stream of gas nebulise the sample mixture. The solvent vapour is ionised by a 'corona discharge', which in turn ionises the sample molecules by proton transfer, reaction with solvated O_2^- or the formation of adduct ions. The sample ions pass into the high vacuum of the mass spectrometer via a series of orifices. APCI is useful for LC-MS applications as the ionisation source can handle liquid entering at atmospheric pressure at flowrates of 1-2 mL/min.

Byrdwell *et al.* (1996) used LC-APCI for both qualitative and quantitative analysis of TAGs. It was shown that the degree of fatty acid saturation determined the relative amounts of protonated molecular ions $(M+H)^+$ and DAG ions $(M-RCOO)^+$ found in the spectra. $(M+H)^+$ ions were observable from TAGs containing only saturated fatty acids. Each TAG produced up to three DAG fragments; however, positional isomers of TAGs were not distinguished.

Laakso & Manninen (1997) used capillary supercritical fluid chromatography-APCI to identify milkfat TAGs. They found that, although the introduction of methanol alone into the ionisation chamber yielded very few $(M+H)^+$ ions, it yielded many $(M-RCOO)^+$ ions. The use of ammonia and methanol produced abundant $(M+NH_4)^+$ ions as well as $(M-RCOO)^+$ fragment ions. The formation of the $(M+NH_4)^+$ ions of saturated TAGs was a new finding. If there were more than one species of TAG in a single chromatographic peak, identification of the fatty acid makeup of the different TAGs was not always straightforward.

Fast atom bombardment (FAB) ionisation

FAB ionisation most often requires the use of a direct insertion probe for sample introduction, although more recently LC/FAB-MS can be performed using continuous flow FAB. In FAB, the sample is prepared in what is known as a matrix, a non-volatile solvent (most often glycerol), and placed on a metal probe tip. A beam of fast moving xenon ions strikes the sample and 'sputters' the sample and matrix from the probe surface. In the vapour phase the matrix behaves like the reagent gas in CI, ionising the sample, *e.g.* by a proton transfer. FAB is generally regarded as a "technique for the analysis of relatively polar or surface active solvents" (Evershed, 1994, p. 130). As such, it is a useful tool for determining structural information about

phospholipids. FAB ionisation is most often used in conjunction with magnetic sector mass analysers, although more recently quadrupole analysers have been used.

Cole & Enke (1991) used FAB ionisation in conjunction with tandem MS to determine phospholipid structures in microorganisms. The authors found that MS/MS fragment ions in both positive and negative ion modes were common to all classes of phospholipid. MS/MS of $(M+H)^+$ ions produced the ion $(M\text{-}OPOOHOY)^+$ (where Y the polar head group was lost as a neutral). MS/MS of $(M-H)^-$ ions produced the ions $(R_1COO)^-$ and $(R_2COO)^-$ (where the rest of the parent ion was lost as a neutral). The authors were able to successfully identify phospholipids in two different types of bacteria, using a combination of positive and negative MS and MS/MS, along with neutral-loss scanning in the positive ion mode.

Electrospray ionisation

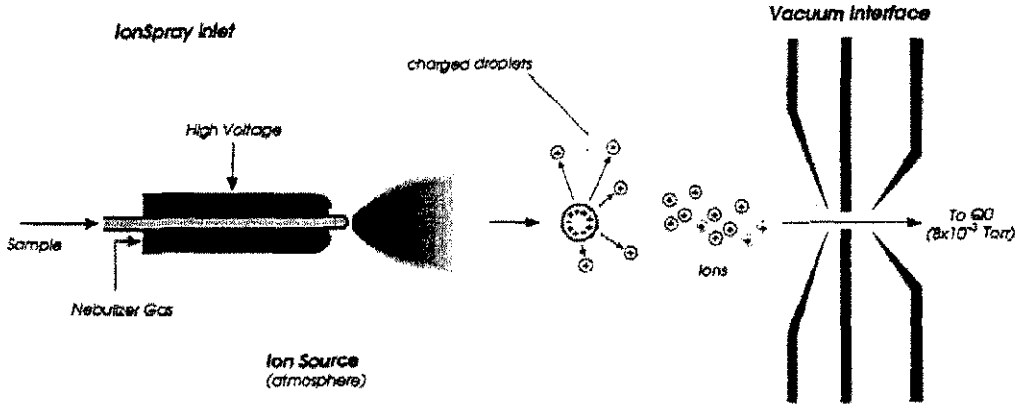


Figure 4. Electrospray ion source.

In electrospray ionisation, a sample solution is sprayed from a narrow bore metal capillary, which has a voltage of approximately 4000 V across it. The droplets 'spraying' from the capillary are thus highly charged and are electrostatically attracted to the mass spectrometer inlet. It is important to note here that this spraying takes place at atmospheric pressure. The solvent evaporates with the application of dry gas, heat or both, prior to entry to the mass spectrometer, causing ions to leave the droplet. It is these ions that are directed into the mass analyser through two cone plates. Most often, only molecular weight information is available from the spectra, as electrospray is a very mild process where fragmentation is kept to a minimum.

However, fragmentation can be induced by MS/MS techniques or by an increase in voltage difference between the cone plates. Fragmentation by MS/MS affects only the ion selected in Q1, the first quadrupole. On the other hand, fragmentation caused by increasing the voltage difference between the cone plates is not selective, and most of the ions passing through these cone plates will be affected.

As with FAB ionisation, electrospray ionisation is not generally regarded as a mass spectrometric technique that would be considered for the routine analysis of lipids.

" However there is an increasing body of evidence to suggest that most classes of lipid are amenable to ES-MS analysis" (Evershed, 1994, p. 132).

Electrospray is the ideal technique for the ionisation of phospholipids, which are easily ionised in solution (Laakso, 1996). However, TAGs are neutral molecules and require the addition of alkali metal or ammonium salts, or formic acid, to the solvent mixture to facilitate the formation of the required molecular ions. Electrospray is also an excellent way of interfacing LC with MS.

Kim *et al.* (1994) used LC-MS with electrospray ionisation and standard mixtures of phospholipids to develop a method to measure amounts of different phospholipids present in lipid samples. Initially standard solutions of phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS) phosphatidylethanolamine (PE) and sphingomyelin (SM) were infused into the electrospray ion source. It was noticed that, if the phospholipid was acidic, $(M+Na)^+$ was the most common ion formed. If the phospholipid was neutral, however, $(M+H)^+$ was the most common ion formed. Using these standard solutions, the capillary exit voltage that gave the smallest concentration of DAG ions was identified. Response curves of various phospholipids were created using selective ion monitoring and the detection limits for each phospholipid were established. LC-MS of standard phospholipids was achieved using reversed-phase HPLC and selective ion monitoring using the information obtained above. The elution time for each of the phospholipids was identified. It was noted that the method was 50 times more sensitive, and quantification was a lot more straightforward, than the existing technique.

Kerwin *et al.* (1995) used positive and negative electrospray MS/MS to characterise the fragmentation patterns of the polar lipids PE, PC, PS, PI and SM. Generally

positive ion MS/MS gave information about the polar head group, and negative ion MS/MS gave information about the acyl groups that were attached to the glycerol backbone. This was an exhaustive study, with detailed accounts of the mechanisms thought to take place in order for the various fragmentation patterns to occur. The authors noted the extreme sensitivity of the method, with positive ion MS being consistently more sensitive than negative ion MS. The authors noticed a linear relationship between peak intensity and sample concentration for a given lipid class, although this was not rigorously examined.

Smith *et al.* (1995) developed a technique using standard glycerophospholipids and negative ion electrospray MS/MS to successfully characterise four bacterial phospholipids. They found that MS/MS of the $(M-H)^-$ ion of each of the standard phospholipids used gave $(R_1CH_2COO)^-$ and $(R_2CH_2COO)^-$ ions. As the intensity of the $(R_1CH_2COO)^-$ ion was twice the intensity of the $(R_2CH_2COO)^-$ ion, it was possible to identify not only the fatty acid components of the phospholipid, but also their position on the glycerol backbone. The polar head group, and hence the phospholipid class, could be identified by subtraction of the weight of the groups identified above from the total molecular weight. Additional evidence for the identification of the phospholipid class was provided by low mass, class characteristic ions formed from the glycerol backbone or by fragmentation of the glycerophosphate bond.

Schuyl *et al.* (1998) used silver phase HPLC-electrospray MS to quantify relative amounts of TAGs found in modified vegetable oil. As this type of chromatography separates TAGs on the basis of the degree of saturation, it is important to note that a number of TAGs (with similar amounts of saturation) will elute as one HPLC peak. The authors used the mass spectrometer to both identify and quantify these TAGs, something that is not possible with standard methods of detection. Initially the authors used standard TAGs and infused solutions of these standards directly into the ion source. They found that sodium acetate was an appropriate salt to add (post column, at 0.1 mM/L in methanol) for quantification as the $(M+Na)^+$ ions formed were resistant to fragmentation. Dynamic ranges of three differently saturated TAGs, infused at 100 μL/min, were found and the TAG with the lowest dynamic range was then infused at different rates. It was found that infusing at 30 μL/min using a clean ion source could greatly improve the sensitivity. TAGs that would co-elute on the HPLC system used above were infused separately and together. The authors found

that when peaks co-eluted the dynamic range for each TAG was smaller. An appropriate cone voltage (95 V) was also established. Using this information, samples of vegetable oil were run through the LC-MS system. Peak areas of various TAGs were measured using the reconstructed ion chromatograms of particular ions. The results were found to be in agreement with another established method and the theoretical composition of the sample. The authors noted that the sum of the concentrations of the TAGs in the highest HPLC peak had to be well below the upper limit of the dynamic range (100 pmol/μL) for the results to be reliable.

Duffin *et al.* (1991) used electrospray MS to characterise both a series of lipid standards (MAG, DAG and TAG) and an unknown lipid sample. The authors dissolved the lipid standard in chloroform/methanol and initially tried three different modifiers (formic acid, ammonium actetate and sodium acetate) in the mix, to facilitate the formation of the molecular ion. Ammonium acetate and sodium acetate were found to be much more effective than formic acid at promoting ionisation in the lipid standards. As the polarity of the standards increased, the authors found that the ion current response increased. Although the response of different TAGs was not necessarily linear, the response of the same TAG varied linearly with its concentration in solution. As $(M+Na)^+$ ions were very difficult to fragment, MS/MS experiments were carried out using the $(M+NH_4)^+$ ions (where M is the intact MAG, DAG or TAG). Common 'daughter ions' found in the CAD mass spectra were $(DAG-H_2O-NH_4)^+$, $(MAG+H)^+$ and acylium ions resulting from carbon-carbon cleavages of the fatty acid chain and the ammonium ion. From this information, it was possible to determine the fatty acid makeup of the acylglycerol, but not their relative positions. It was also not possible, using $(M+NH_4)^+$ ions where M is a MAG, to determine the position of the double bonds in the unsaturated fatty acids. The authors used the information above to characterise an unknown lipid sample. A complex spectrum containing mainly TAGs with an ammonium or sodium ion attached was obtained. MS/MS was carried out on selected $(M+NH_4)^+$ ions primarily to show that with a complex sample the probability of two structurally different TAGs having the same m/z ratio is high.

Very recently, Hsu & Turk (1999) used electrospray MS to characterise a series of TAG standards. The authors prepared these standards in chloroform:methanol (70:30) and 2 mM lithium acetate. Standards were infused into the mass spectrometer and

prominent $(M+Li)^+$ ions were seen. By increasing the skimmer potential or selecting these $(M+Li)^+$ ions for CAD, the most prominent daughter ions identified in the spectra (in both cases) were $(M+Li\text{-}R_nCO_2Li)^+$ and $(M+Li\text{-}R_nCO_2H)^+$. The relative response of these two fragment ions depended on the degree of saturation of the fatty acids esterified to each of the glycerol backbones. Fatty acids esterified to the sn2 position of the glycerol backbone could be identified by the relative response of the DAG fragments (the DAG fragment containing the 1,3 fatty acids always has the lowest response) and the presence of two unidentified but characteristic ions with the same mass difference as the mass difference between the fatty acids esterified to the sn1 and sn3 positions of the glycerol backbone.

When the $(M+Li\text{-}R_nCO_2Li)^+$ and $(M+Li\text{-}R_nCO_2H)^+$ ions were selected for CAD, different fragment ions were seen. Acylium ions as reported by Duffin *et al.* (1991) were seen only in the latter case, and $(M+Li\text{-}(R_nCO_2H)\text{-}R_2CH{=}CHCO_2H)^+$ ions were the most prominent fragment ions in the former case.

Perhaps most importantly, and in contrast to the work reported by Duffin *et al.* (1991), the ability to predict double bond positions was reported. $(RCO_2Li_2)^+$ ions were generated in the Q1 quadrupole, when a high skimmer potential was selected. These ions were selected for CAD and it was the resulting fragment ions that enabled the positional identification of double bonds in fatty acids. As this paper was published during the course of this work, this technique could not be tested. However, it will be investigated in other studies.

1.3 Summary

The above discussion clearly indicates that MS is a powerful tool in the analysis of lipids. Both molecular weight and structural information can be generated quickly and relatively easily using comparatively small amounts of sample. No other analytical technique currently used in lipid analysis, by itself, is capable of generating such information. MS can also be used in conjunction with other separation techniques. Because of the number of components in lipid samples such as milkfat there are obvious benefits to using LC-MS, for example, as a way of simplifying the spectra obtained (Laakso & Manninen, 1997; Careri *et al.*, 1998; Schuyl *et al.*, 1998).

Quantification using MS has been approached in different ways and has met with some success (Byrdwell *et al.*, 1996; Schuyl *et al.*, 1998). The generation of response curves for different TAGs seems labour intensive, and not very practical for quantifying lipid components in such complex samples as milkfat. However, quantification of a single component of interest, using single ion monitoring, may well prove useful in the analysis of minor components of milkfat with special nutritional significance.

Fragmentation patterns using the different ionisation sources (excluding EI) mentioned above were very similar. All these ionisation sources were able to identify and partially characterise the acylglycerol and phospholipid components of milkfat. However, there were varying reports of positive positional identification of fatty acids in TAGs and phospholipids.

Electrospray ionisation appeared to be the softest ionisation technique reviewed with almost no fragmentation of the intact acylglycerols occurring in the ionisation source (Careri *et al.*, 1998). This is important when measuring complex samples of lipids containing mixtures of TAGs, DAGs and MAGs. It may also prove very useful for quantification purposes. In fact, there are many advantages to using electrospray ionisation for the analysis of lipids, despite its apparent lack of popularity (particularly in the analysis of TAGs) compared with other ionisation techniques (Laakso, 1996). As electrospray does not require the application of heat in order for the TAGs to become volatile, the so-called 'fractionation' effect amongst TAGs with different compositions (making analysis of TAG mixtures difficult) can be avoided (Careri *et al.*, 1998). Futhermore electrospray is more sensitive than many other methods of ionisation (Kim *et al.*, 1994; Kerwin *et al.*, 1995; Careri *et al.*, 1998) and is thought to be more sensitive than other routine techniques used to characterise lipids (Duffin *et al.*, 1991). Electrospray ionization is also an excellent way of interfacing HPLC with MS, which for the analysis of complex lipids such as milkfat is a powerful combination.

This review suggests that there are advantages in using electrospray MS for the analysis of complex lipids such as milkfat and that very little investigation into this type of analysis of complex mixtures of TAGs has been carried out.

2 Research Methodology

The major piece of instrumentation used throughout this project was a Perkin Elmer Sciex API300 mass spectrometer. Samples were introduced into the mass spectrometer via a syringe and pump and responses were assessed for many different lipid standards and samples. As the composition of the lipid samples became more complex, it became apparent that separation of the lipid samples prior to entry into the mass spectrometer would be required. Hence an existing HPLC method, developed to separate complex TAG mixtures found in milkfat, was used to separate the more complex lipid samples and the eluant stream from HPLC was directed into the mass spectrometer. The compositional results obtained from the mass spectral data were compared with those obtained by more traditional analyses, *i.e.* solid fat content (SFC) and GC.

2.1 Instrumentation

2.1.1 Mass Spectrometry

The mass spectrometer used for the duration of the experiments was a Perkin Elmer Sciex Triple Quadrupole API300 LC/MS/MS system (Foster City, CA, USA). The operation of the mass spectrometer is described in detail in Appendix 1. Samples were introduced into the mass spectrometer via the electrospray API PE Sciex Ion spray inlet interface either using a Hamilton syringe connected to a Harvard pump (So. Natick, MA, USA) or by HPLC. The mass spectrometer was calibrated weekly by infusing a polypropyleneglycol (PPG) standard and analysing the spectra using Mass Chrom software as described in detail in the PESCIEX operator's manual (1997).

2.1.2 HPLC

Reversed-phase chromatography was performed on an LC Module 1 Plus (Waters Associates, Milford, MA, USA) that included a quaternary gradient pumping system and a low pressure mixer. The chromatography column was a Waters Nova-Pak™ C18, 4 μm, 3.9 mm x 150 mm, with a pre-column module that contained inserts of

the same solid phase. The column temperature was maintained at 20°C by water circulating from a thermostatted water bath to the column jacket.

2.2 Methodology

2.2.1 Analysis of Lipid Standards and Fats and Oils by Infusion MS and Infusion MS/MS

Samples were pumped into the interface at 0.3 mL/h using a 100 μL syringe connected to a Harvard pump.

Analysis of TAGs

All standard TAGs analysed were synthesised by Robert Norris (NZDRI). Commercial coconut oil and cocoa butter were supplied by Yvonne van der Does (NZDRI). Generally, TAGs were prepared at a concentration of approximately 0.005 mg/mL in 1,2 dichloroethane:acetonitrile:formic acid (63:35:2) and 10 mM ammonium acetate. A small number of TAG standards were prepared at 1.5 x 10^{-6} M. All solvents and acids used were HPLC grade or better. Ammonium acetate and any other salts used were Analar grade.

TAG ions were focused using a positive ion spray (IS) voltage of 5500 V.

MS experiments were carried out with orifice (OR) and ring (RNG) voltages set at either 25 and 150 V, or 45 and 250 V respectively. Coconut oil and cocoa butter were analysed with an OR voltage of 25 V and a RNG voltage of 50 V. Other relevant MS settings are shown in Appendix 1.

MS/MS experiments were carried out using OR and RNG voltages of 45 and 250 V respectively. The voltage across the second quadrupole, RO2, was set at -45 V unless the carbon number of the DAG ion to be fragmented was particularly low, when it was lowered to –35 V. The CAD gas was originally set at 6 but was later reoptimised to 4 partway through the study. Other relevant MS settings are shown in Appendix 1.

Analysis of Fatty Acids

Equimolar concentrations of fatty acids (10 x 10^{-6} M) were prepared in 1,2 dichloroethane:acetonitrile (65:35).

Fatty acid ions were focused using a negative IS voltage of -4500 V.

Initially, all MS experiments were carried out with OR, RNG and IQ2 voltages of -50, -210 and 20 V respectively. Some lower molecular weight fatty acids (below C10) were reanalysed with lower OR and RNG voltages.

Analysis of Cholesterol

Both cholesterol and cholesteryl palmitate were purchased from Nu-Check-Prep, Inc. (Elysian, MN, USA). Both samples were prepared at 0.01 mg/mL in 1,2 dichloroethane:acetonitrile:formic acid (63:35:2) and 10 mM ammonium acetate.

Cholesterol and cholesteryl palmitate ions were focused using a positive IS voltage (IS) of 5500 V.

MS experiments were carried out with OR and RNG voltages set at 25 and 150 V respectively. Other relevant MS settings are shown in Appendix 1.

MS/MS experiments were carried out using OR and RNG voltages of 25 and 150 V respectively. The voltage across the second quadruple, RO2, was set at -30 V. The CAD gas was set at 4. Other relevant MS settings are shown in Appendix 1.

Analysis of Standard Phospholipids

All phospholipid standards used were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

For positive ion MS and MS/MS

All phospholipid standards were prepared in two solvent systems at 0.005 mg/mL. These were chloroform:methanol (70:30) and 1,2 dichloroethane:acetonitrile:formic acid (63:35:2) and 10 mM ammonium acetate. Phospholipid ions were focused using a positive IS voltage of 5500 V and OR and RNG voltages of 25 and 150 V respectively.

For negative ion MS and MS/MS

All phospholipid standards were prepared at 0.005 mg/mL in 1,2 dichloroethane: acetonitrile:formic acid (63:35:2) and 10 mM ammonium acetate. Phospholipid ions were focused using a negative IS voltage of -4500 V and OR and RNG voltages of -50 and -210 V respectively.

MS/MS experiments were done in the positive mode with OR, RNG, and RO2 voltages of 45 and 250 and -45 V respectively. OR and RNG voltages used in MS/MS carried out in the negative mode were typically set at -50 and -210 V respectively.

Other relevant MS settings are shown in Appendix 1.

Analysis of Sphingomyelin and Sphingosine

Both sphingomyelin (from bovine brain) and sphingosine (from bovine brain) standards were purchased from Sigma (St Louis, MO, USA).

Sphingomyelin was prepared at 0.005 mg/mL and at 0.05 mg/mL in 1,2 dichloroethane:acetonitrile:formic acid (63:35:2) and 10 mM ammonium acetate for positive ion MS and in chloroform:methanol (70:30) for negative ion MS. The voltages used for MS and MS/MS experiments were the same as those used for the phospholipid experiments above.

Sphingosine was prepared at 0.005 mg/mL in 1,2 dichloroethane:acetonitrile:formic acid (63:35:2) and 10 mM ammonium acetate. Sphingosine ions were focused using a positive IS voltage of 5500 V. MS experiments were performed using OR and RNG voltages of 25 and 150 V and 50 and 200 V respectively. No MS/MS experiments were carried out.

Analysis of Gangliosides

The ganglioside standards used originated from Matreya Inc. (Pleasant Gap, PA, USA). Stock solutions were prepared in chloroform:methanol (70:30) and small amounts were a gift from Gill Norris from the Institute of Molecular Biosciences, Massey University, Palmerston North.

Three stock solutions were supplied. One, a ganglioside mix, contained GM1 (asialo), GM1, GD1 and Gt1b. The second contained GM3 and the third contained GD3. The concentrations of each stock solution were unknown.

Ganglioside ions were focused using a negative IS voltage of -4500 V. Typically the OR and RNG voltages were set at -50 and -210 V respectively, although these were varied from experiment to experiment. With the exception of the ganglioside mix, not enough sample was supplied to allow MS/MS experiments to be undertaken.

2.2.2 Analysis of Natural Fats and Oils by LC-MS and LC-MS/MS

Samples of fats and oils were prepared at either 5 or 10 mg/mL in dichloroethane. These samples consisted of the following.

- Cocoa butter supplied by Anchor products Ltd (Edgcumbe, New Zealand).
- Hydrogenated coconut oil from commercial Kremelta supplied by Yvonne van der Does (NZDRI).
- Two bovine milkfats: one Friesian milkfat and one Jersey milkfat. These were from a group of 10 cows of each breed in the same herd grazing on the same pasture.
- Four bovine milkfats from an NZDRI Food Science Section seasonal survey, extracted from commercial butter supplied from the same dairy farm, specifically: early spring (MF 1050.1), late spring (MF 1050.3), summer (MF 1050.6) and autumn (MF 1050.8).
- Samples of milkfat extracted from milk of different mammalian species, specifically: horse, gifted by Mrs Robyn Hirst (NZDRI), seal and pygmy sperm whale, gifted by Dr Padraig Duignan, human, gifted by Mrs Jessica Harris, and possum, gifted by Dr Murray Grigor.

Typically, between 5 and 20 μL of sample was injected on to a reversed phase C_{18} HPLC column. The HPLC method used to separate the TAGs was based on one developed by Robinson & MacGibbon (1998). The mobile phase gradient programme is shown in Table 1.

Table 1. Mobile phase gradient programme used for TAG separation

Time (min)	Flow (mL/min)	%A	%B
0	1	90	10
20	1	90	10
100	1	65	35
110	1	50	50
115	1	50	50
115.1	1	90	10
125	1	90	10

A = acetonitrile:formic acid (98:2) and 10 mM ammonium acetate.
or acetonitrile:MilliQ water (98:2) and 10 mM ammonium acetate.
B = dichoromethane.

The eluant stream from the HPLC was split such that approximately 30 µL/mL was introduced into the mass spectrometer using an electrospray API PE Sciex ion spray inlet interface.

Ions were generated and focused using a positive IS voltage of 5500 V. The step size and the dwell time were set at 0.1 amu and 0.7 ms respectively. The detection range was between m/z 100 and m/z 1000.

LC-MS experiments were carried out using OR and RNG voltages set at 25 and 200 V respectively, although a few experiments were carried out with OR and RNG voltages set at 45 and 250 V.

LC-MS/MS experiments were carried out using OR, RNG and RO2 voltages set at 25, 200 and -45 V respectively.

2.3 Other Analytical methods

2.3.1 SFC

The SFC of lipids was determined using pulsed nuclear magnetic resonance (NMR) according to the method published by MacGibbon & McLennan (1987).

2.3.2 HPLC

HPLC was carried out according to the method published by Robinson & MacGibbon (1998).

2.3.3 Fatty Acid Methyl Ester (FAME) Analysis

FAME analysis was carried out according to the method published by MacGibbon (1988).

Analysis of spectra using the CTC spreadsheet

From the molecular ion, the carbon number and degree of unsaturation could be uniquely identified, as changes in carbon number resulted in mass ion changes of 14 (including odd numbered species), and the mass of the double bonds (2 for each double bond) did not reach this mass to any significant extent and certainly never reached 28 for solely examining the even carbon numbered species.

As the masses of each species were tabulated in a spreadsheet, the CTC spreadsheet, for any mass the corresponding carbon number and double bonds could be determined.

3 Results and Discussion

3.1 Neutral Lipid Standards

As TAGs are by far the biggest lipid group found in bovine milkfat, much of the thrust of this research was aimed at successful analysis of TAGs using electrospray MS. In order to perform successful electrospray MS, the TAGs had to be prepared in such a way that they would form a charge prior to entry into the mass spectrometer. Thus the first step in the research project was identification of an effective solvent system, using a standard TAG. Once this solvent system was identified, appropriate voltage settings for the mass spectrometer were established so that a strong molecular ion signal was seen in the spectra. Using the same solvent system and voltage settings, other standard TAGs were analysed and their responses were examined using both infusion MS and infusion MS/MS.

3.1.1 Method Development

Infusion MS and MS/MS of TAG Standards

Solvent selection

Initially four solvent systems were assessed for their ability to promote the formation of molecular ions. These solvent systems were selected based around the work of Duffin *et al.* (1996) and Robinson & MacGibbon (1998). A standard TAG was prepared in each of the first four solvent systems described below, at approximately 0.005 mg/mL, and infused into the mass spectrometer using a positive IS voltage of 5500 V, and OR and RNG voltages of 50 and 200 V respectively.

The first of the solvent systems trialed was chloroform:methanol:formic acid (70:28:2) and 10 mM ammonium acetate. This solvent system provided both abundant molecular ions seen as ammonium adducts $(M+NH_4)^+$, where M represents the TAG molecule. The formic acid was thought to promote the formation of the molecular ion.

The second solvent system trialed was 1,2 dichloroethane:acetonitrile:formic acid (63:35:2) and 10 mM ammonium acetate. This solvent system was closely aligned to that used in the separation of TAGs by reversed-phase HPLC as outlined by Robinson & MacGibbon (1998). As for the system trialed above, both TAG ions (seen as ammonium adducts $(M+NH_4)^+$) and DAG ions were seen. The 'noise' in the spectra, attributed to the solvent system, was noticeably less than that seen with the chloroform:methanol system.

The third solvent system trialed was 1,2 dichloroethane:acetonitrile:formic acid (63:35:2) and 10 mM sodium acetate. Although both molecular ions (seen as sodium adducts $(M+Na)^+$) and DAG ions were seen, the spectra were very busy with prominent ions seen every 23 mass units (sodium adducts). This made the spectra difficult to interpret. In addition, $(M+Na)^+$ ions were more difficult to fragment than $(M+NH_4)^+$ ions, thus giving less structural information than corresponding $(M+NH_4)^+$ ions.

The fourth solvent system trialed was 1,2 dichloroethane:acetonitrile:formic acid (63:35:2). Only very low levels of molecular ions $(M+H)^+$ were seen in the spectra.

The fifth and final solvent system trialed was 1,2 dichloroethane:acetonitrile (65:35) and10 mM tetramethylammonium chloride. Tetramethylammonium chloride was used, firstly because it is soluble in the 1,2 dichloroethane:acetonitrile mix without the need for an aqueous component (such as formic acid as used above) and secondly in the hope that $(M+Cl)^-$ molecular ions would form. Unlike the solvent systems used above, ions were focused using a negative IS voltage of -5500 V. OR and RNG voltages were not recorded. No molecular ions were seen.

As a result of the screening above, all further TAG samples analysed by infusion MS and MS/MS were prepared in 1,2 dichloroethane:acetonitrile:formic acid (63:35:2), 10 mM ammonium acetate (the second solvent system).

Maximising the response of the molecular ion

State files were generated for infusion MS and infusion MS/MS experiments. IS, NEB, CUR, OR, RNG, IQ2 and RO2 (in the case of the MS/MS state file) voltages were optimised using the TAG standard POS (16:0, 18:1, 18:0). These voltages were stored in what is called a state file and a copy of the state file as well as a definition of

each of the voltages can be found in Appendix 1. It was assumed that these values would give reasonable responses for any other TAG sample and thus these state files were used with very little alteration for the duration of these experiments. This enabled a direct comparison of responses for a number of TAG standards (and samples).

Infusion MS of Fatty Acids

Solvent selection

Equimolar concentrations of five free fatty acids (24:0, 20:0, 14:0, 10:0 and 4:0) were prepared in both 1,2 dichloroethane:acetonitrile:formic acid (63:35:2), 10 mM ammonium acetate and 1,2 dichloroethane:acetonitrile (65:35).

Whereas no 'satisfactory results' were obtained using a combination of the first of the solvent systems above and positive ion MS, a combination of the second solvent system and negative ion MS gave good results. With negative ion MS, an $RCOO^-$ ion was generated and it was the response of this negative ion that was assessed for each of the fatty acids above.

Maximising the response of the molecular ion

State files were generated for infusion MS experiments. IS, NEB, CUR, OR, RNG, and IQ2 voltages were optimised using the C14:0 fatty acid. It was initially assumed that these values would give reasonable responses for any other fatty acid analysed, although this turned out not to be the case. These state files were used in the analysis of all the fatty acid standards analysed and this enabled a direct comparison of responses. Because of the extremely poor response of the short chain fatty acids, the OR and RNG voltages were decreased in an attempt to obtain better signals.

Infusion MS and MS/MS of Cholesterol and Cholesteryl Palmitate

Both cholesterol and cholesteryl-palmitate were prepared in 1,2 dichloroethane: acetonitrile:formic acid (63:35:2), 10 mM ammonium acetate (the second solvent system). As both cholesterol and cholesteryl palmitate fragmented easily, relatively low voltages were chosen to analyse both compounds by both infusion MS and infusion MS/MS.

3.1.2 Analysis of Non-polar Lipid Standards by Infusion MS and Infusion MS/MS

TAG Standards by Infusion MS

TAGs were identified in the spectra as TAG-ammonium ions $(M+NH_4)^+$ (where M is the intact TAG). These molecular ions were seen clearly in the spectra as one major and two minor peaks. These minor peaks, one and two atomic mass units (amu) greater than the mass assigned to the major peak, were identified as carbon 13 isotopes which constitute approximately 1.11% of terrestrial carbon. The contribution of these isotopes may be given by the following binomial equation

$$b(x,n,p) = \binom{n}{x} p^x (1-p)^{n-x}$$

where x = the number of carbon 13 isotopes in the molecule, n = the total number of carbon atoms in the molecule and p is the probability of carbon 13 (0.011).

For example, in the case of tristearin (n = 57), for x = 0, 1 or 2 the percentage probabilities are 52, 33 and 10% respectively. It can be seen that, whereas the possibility of a carbon 13 isotope in a single carbon atom is small, the possibility in a 57 carbon TAG is much greater as shown in the trace below (Figure 5). The molecular ions have amu values of 908.8, 909.8 and 910.8 for 0, 1 and 2 carbon 13 atoms respectively.

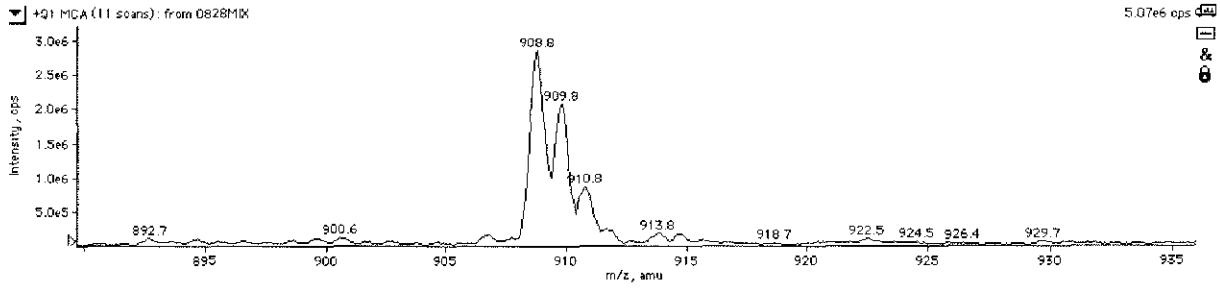


Figure 5. Isotope pattern of tristearin.

Sometimes, a small quantity of $(M+Na)^+$ ions was also seen in the spectra. The presence of these sodium ions was minimised by preparing samples in plastic rather than glass vials.

The analysis of equimolar concentrations of monoacid TAGs C22:0, C18:0, C15:0, C11:0 and C4:0 showed a response difference based on carbon number. It was found that, the greater the carbon number, the less was the total response of the sum of the molecular ions plus derivative fragments.

It is worth noting that the response also depended to some extent on the RNG voltage selected (and to a lesser extent on the OR and IQ2 voltages). In cases where the carbon number of the TAG was low (*e.g.* tributyrin), it was possible (if the RNG, OR and IQ2 voltages were too high) to shatter the TAG, leaving no trace of the molecular ion. Tributyrin is an extreme example, but even so it proved to be good practice, when analysing mixtures of TAGs, to perform these analyses at a number of different voltage settings. These results also indicated the ease with which TAG-ammonium ions fragmented to form DAG ions (and in some cases acylium ions) upon entry into the mass spectrometer. This is referred to as front end fragmentation and is, with respect to TAG-ammonium ions, quite difficult to avoid.

There can be up to three DAG ions in the spectrum for every parent TAG ion, *i.e.* fatty acid groups present on carbon numbers 1 and 2, 2 and 3 or 1 and 3 of the glycerol backbone. Figure 6 shows the front end fragmentation pattern of the TAG standard POS. Present in the spectrum is the molecular ion at m/z 878.8, and the three DAG ions at m/z 605.5 (OS), 579.4 (PS) and 577.4 (PO).

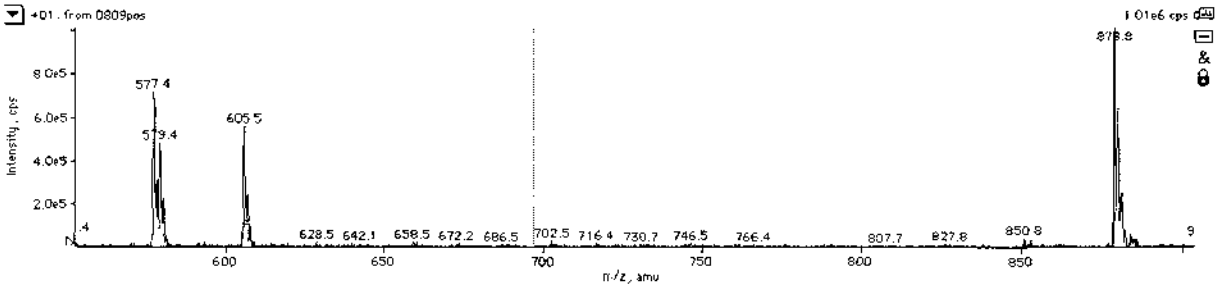


Figure 6. Front end fragmentation pattern of the TAG standard POS.

The schematic in Figure 7 illustrates the source of the ions found in figure 6 and the further fragmentation of these ions by MS/MS (which is described further in the text).

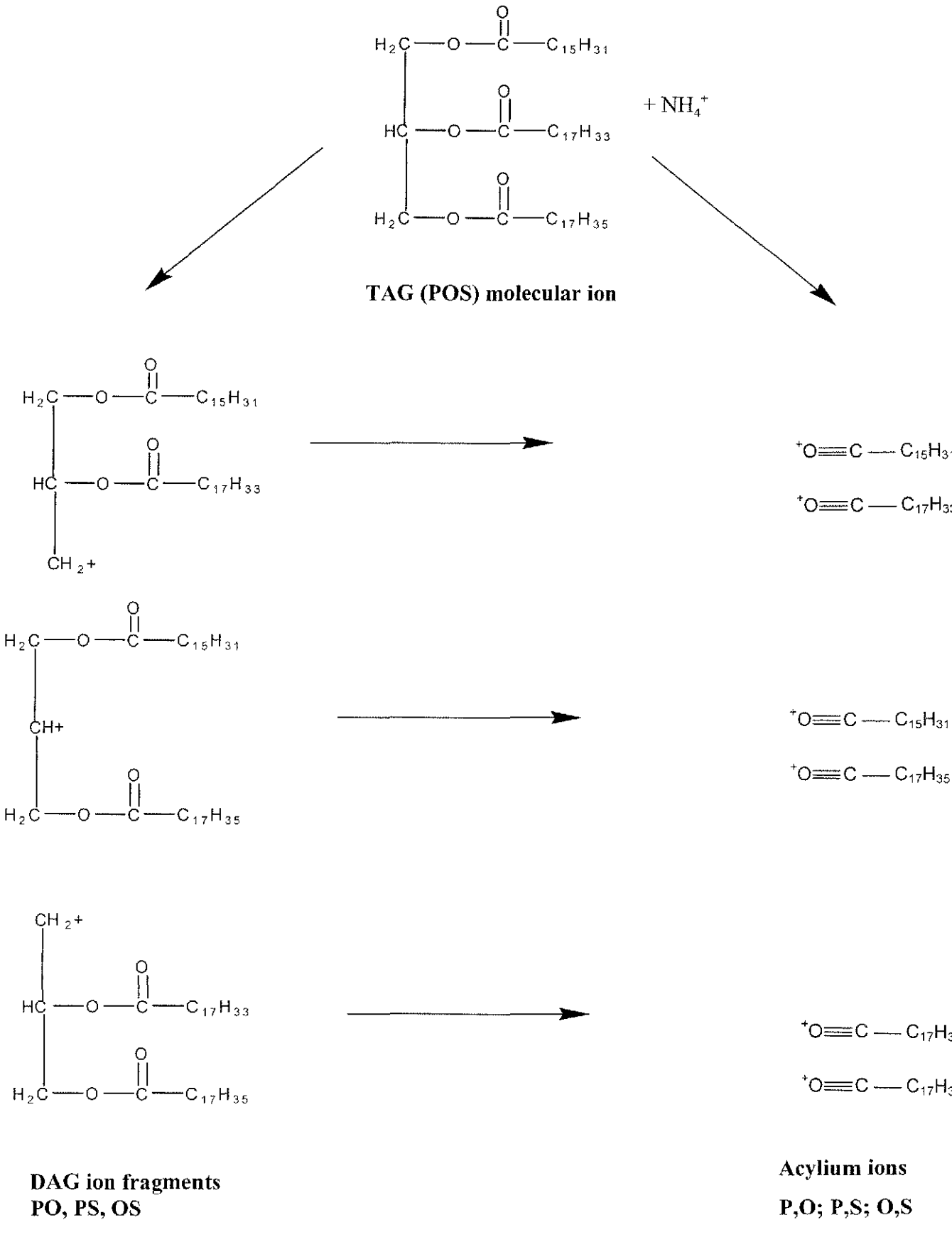


Figure 7. Fragment ions derived from the TAG (POS) molecular ion

The relative amounts of DAG ions formed during this front end fragmentation were found to depend on both the positions of the two remaining fatty acids on the glycerol backbone and the carbon number (and perhaps the degree of saturation) of the fatty acid that was lost. Figures 8, 9 and 10 show that, the lower the carbon number of each monoacid TAG, the less energy was required to form DAG ions (and in some cases acylium ions) and the greater was the total signal. Also as the voltage was increased, the response increased but the relative height of the molecular ion to the DAG ion decreased. In Figure 9 the higher voltage 'shattered' the TAG before it entered the mass spectrometer.

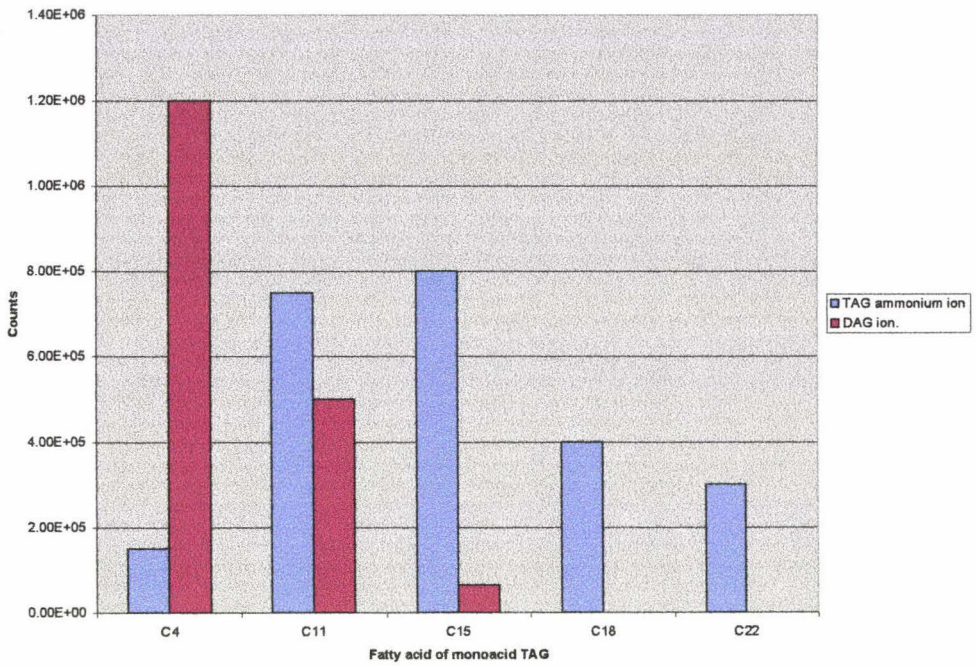


Figure 8. Monoacid TAG front end fragmentation patterns. OR = 25 V, RNG = 50 V

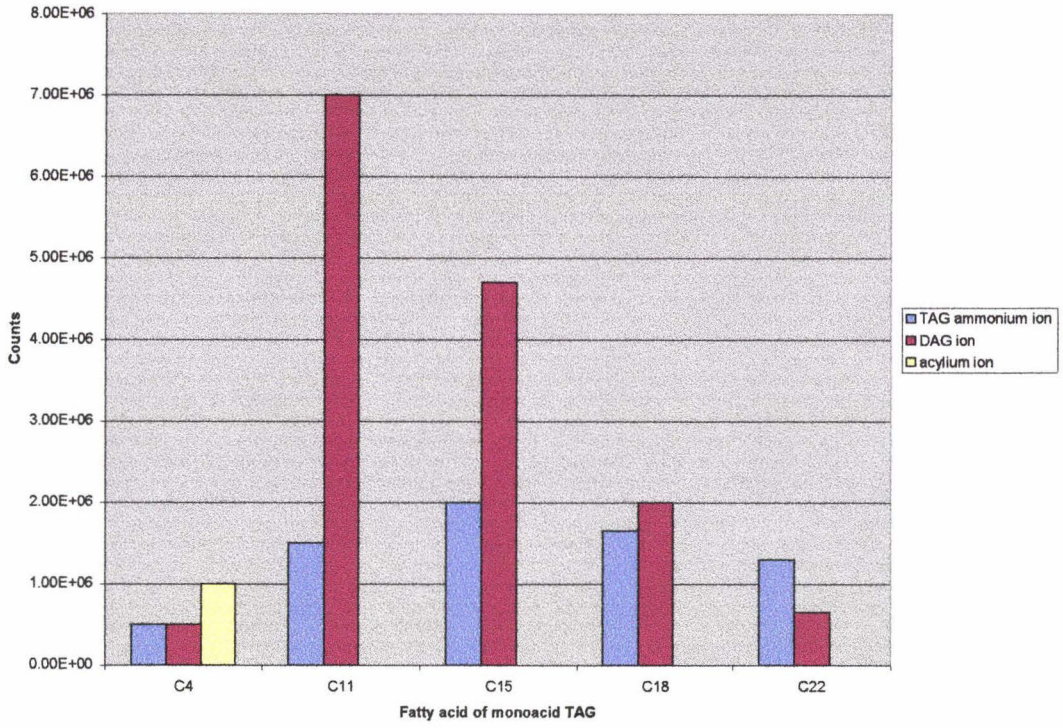


Figure 9. Monoacid TAG front end fragmentation patterns. OR = 25 V, RNG = 200 V.

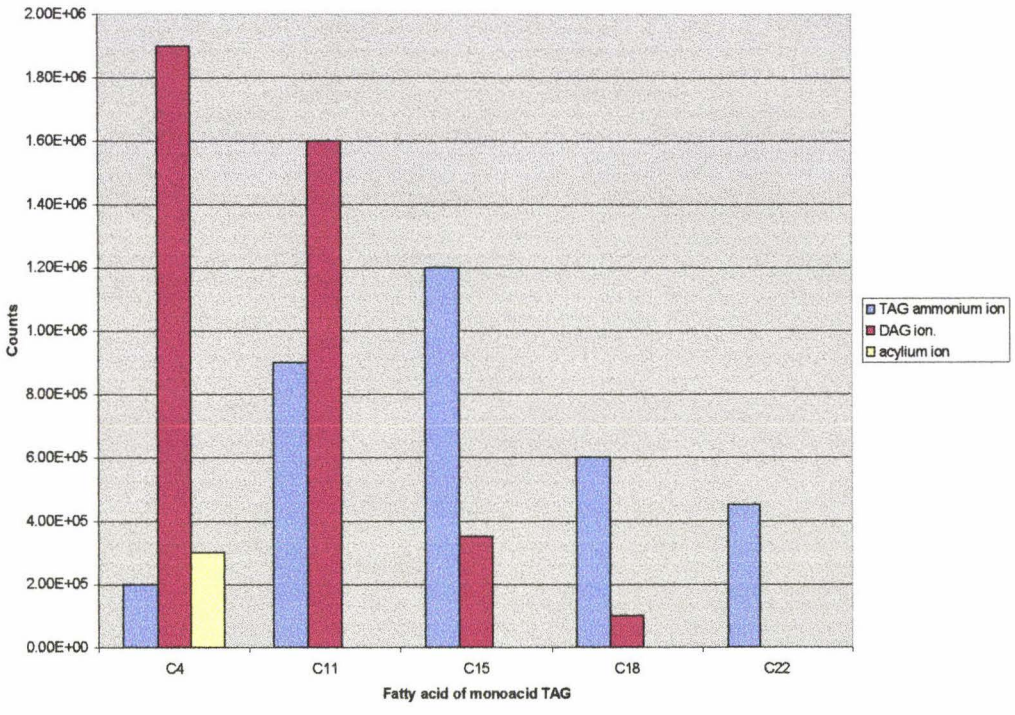


Figure10. Monoacid TAG front end fragmentation patterns. OR = 25 V, RNG = 100 V

However, Figures 11 and 12 show that the relative responses of DAG ions formed during front end fragmentation were related to the position of fatty acid groups on the glycerol backbone. The response of DAG ions with fatty acid groups on either positions 1 and 2, or 2 and 3 of the glycerol backbone was always found to be greater than the response of DAG ions with fatty acid groups on positions 1 and 3 of the glycerol backbone. In other words, fatty acids were fragmented more easily from positions 1 and 3 than position 2 of the glycerol backbone.

For example, Figure 11 shows fragmentation patterns of TAGs that have three different fatty acids esterified to the glycerol backbone (and therefore three different DAG ions can be identified in the spectra). It can be seen that the DAG response seen in the front end fragmentation of the two TAGs POS and SPO are different. In the case of the TAG POS, the DAG ions PO and OS are seen in greater abundance in the spectra than the DAG ion PS. In the case of the TAG SPO, similarly, the DAG ions SP and PO are seen in greater abundance than the DAG ion SO.

Another example can be seen in Figure 12, which shows fragmentation patterns of TAGs that have only two different fatty acids esterified to the glycerol backbone (and therefore only two DAG ions can be identified in the spectra). Like the TAGs POS and SPO above, it can be seen that the DAG responses in the front end fragmentation of the two TAGs PPS and PSP are different. The DAG ion PS has a greater response (approximately two times) in the spectra when the parent TAG is PPS (where S is esterfied to the third carbon of the glycerol backbone) rather than PSP (where S is esterified to the second carbon of the glycerol backbone). That is, the 1,3 DAG has a greater response.

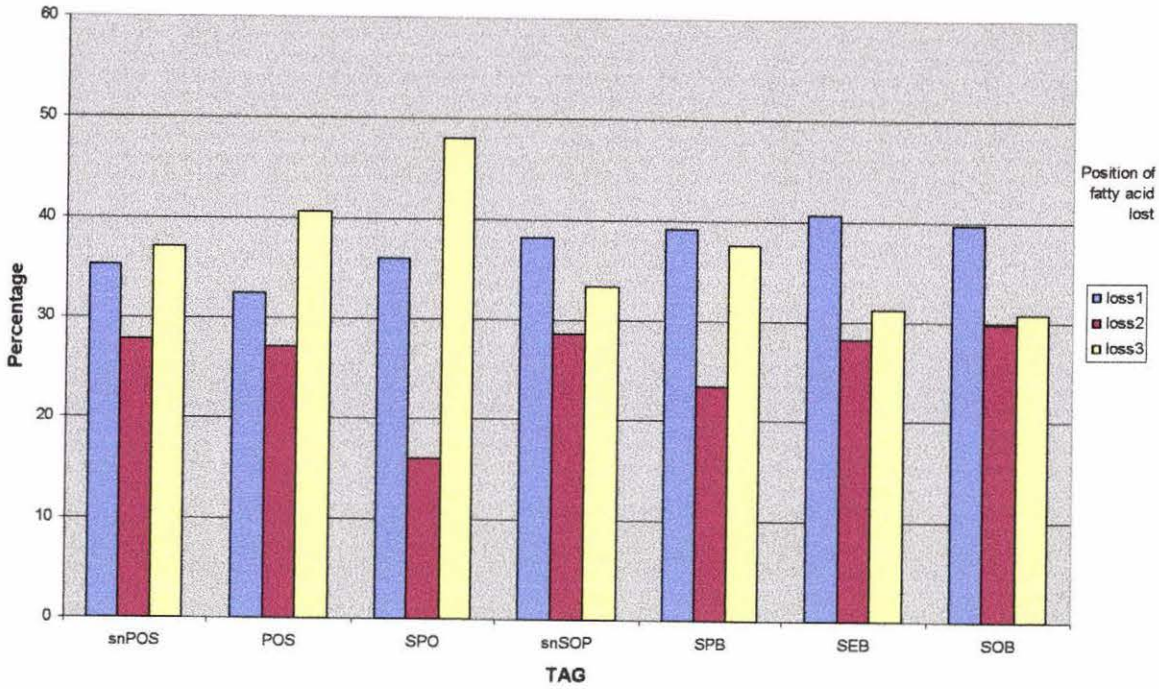


Figure 11. Front end fragmentation of TAGs.

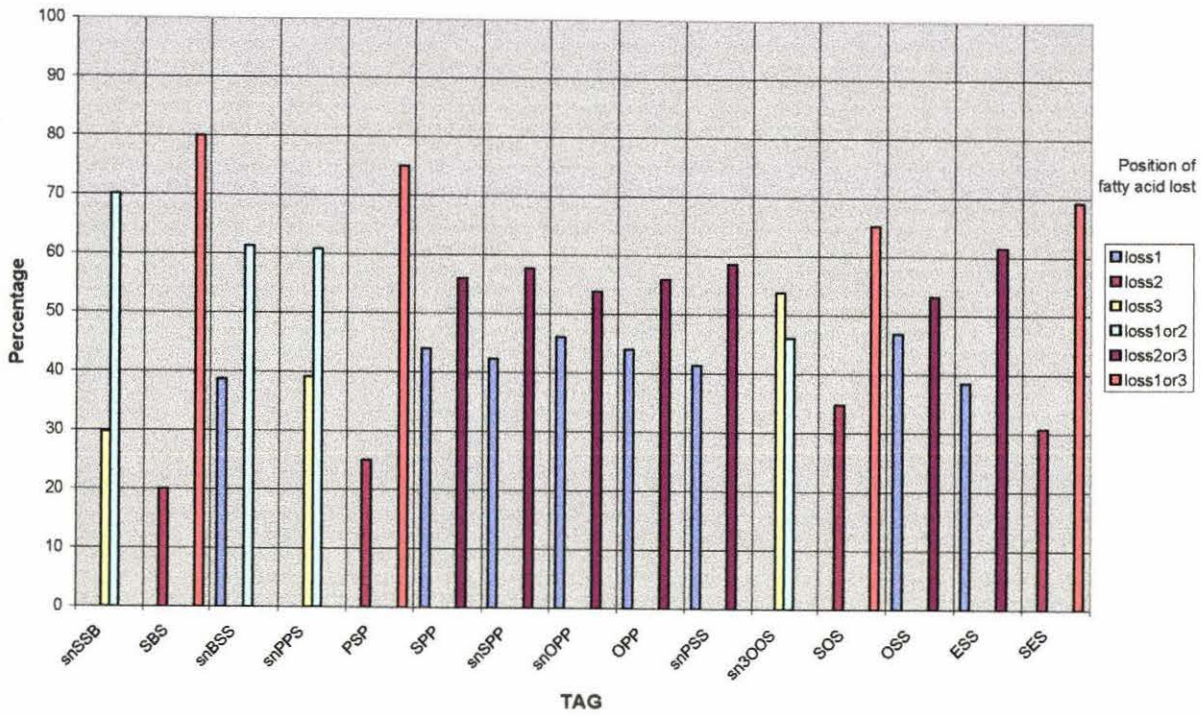


Figure 12. Front end fragmentation of TAGs.

It should be noted that, although this relative response (*i.e.* one DAG response in relation to another within the same TAG molecule) stayed constant, the absolute response varied from day to day. This may have been due to the cleanliness of the instrument and might necessitate the need for an internal standard if quantitative analysis, using the mass spectrometer, is to be explored.

Analysis of TAG Standards by Infusion MS/MS

Each TAG molecule has up to three different fatty acid groups attached to its glycerol backbone. When these groups are sheared from the glycerol backbone and maintain a positive charge, they are termed acylium ions. Thus the identification of acylium ions was important, because it allowed unequivocal identification of the fatty acids, that make up a TAG molecule.

Acylium ions were not readily identified during infusion MS even at relatively high OR and RNG voltages. As a result, the acylium ion response was examined using infusion MS/MS. Acylium ions were most clearly seen in the spectra when a DAG ion, rather than its parent TAG-ammonium ion, was selected for CAD.

It is interesting to note that MAG ions, which have been reported by Duffin *et al.* (1991), were seen only in tiny amounts, if at all, in the spectra generated during these experiments.

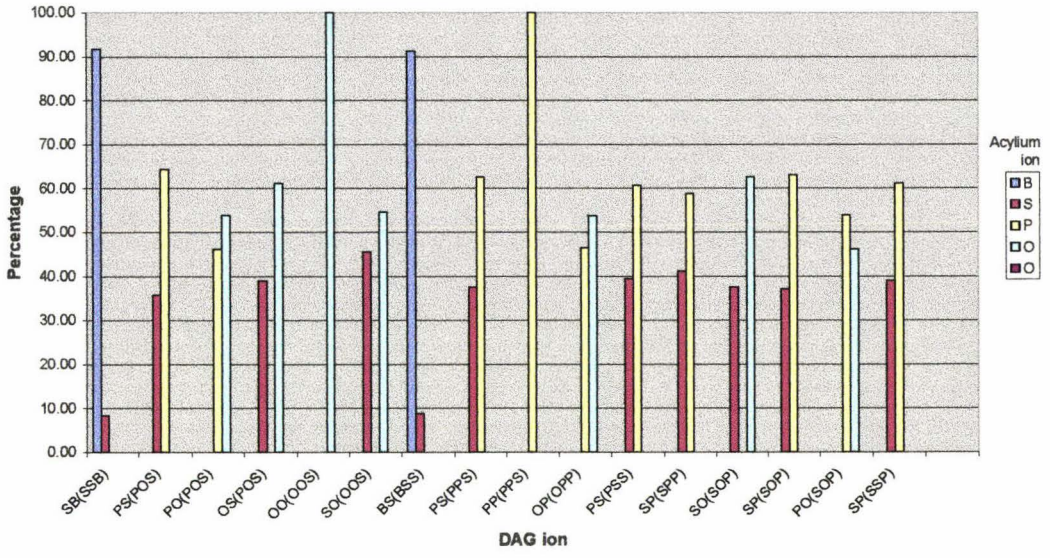


Figure 13. MS/MS fragmentation patterns of DAG ions.

The results showed that the response of the acylium ions in the spectra was based on their carbon number and degree of saturation rather than their relative positions on the glycerol backbone. Small quantities of acylium ions minus 18 amu were also seen in the spectra.

Figure 13 shows that the response of a given acylium ion increased with a decrease in carbon number. An example is SB (derived from SSB). The response of 'B' (four carbons) was many times greater than the response of 'S' (16 carbons). An exception to this rule appeared to be if one of the fatty acids esterified to the glycerol backbone was unsaturated. An example is SO (OOS) where both fatty acids have the same carbon number but the response of 'O' (which is unsaturated) was greater than the response of 'S' (which is saturated).

Summary of TAG analysis by MS

Front end fragmentation was used to look at relative responses of DAG ions seen in the spectra. These responses depended on the position of the 'lost' fatty acid on the glycerol backbone and its carbon number, with the former rather than the latter being the more critical.

MS/MS experiments were performed on DAG ions, rather than parent ions, to identify and measure the response of acylium ions generated during CAD.

The response of these acylium ions was dependent on their carbon number and degree of unsaturation.

Analysis of Free Fatty Acids by Infusion MS

The response of the free fatty acids in the spectra proved to be quite unusual in that the response peaked at C14 and declined sharply on either side of this mass (Figure 14). Although fatty acids above C14 followed the same trends (with regard to response) as the TAGs, it was difficult to explain the lower response of the fatty acids below C14. C4 could not be measured as the free fatty acid (or as a sodium salt in positive mode). It was hypothesised that the fatty acids below C14 were breaking up upon entry into the mass spectrometer under the conditions used above (as was seen with low molecular weight TAG standards). However, lowering the OR, RNG and IQ2 voltages did not produce an increase in the response of these acids.

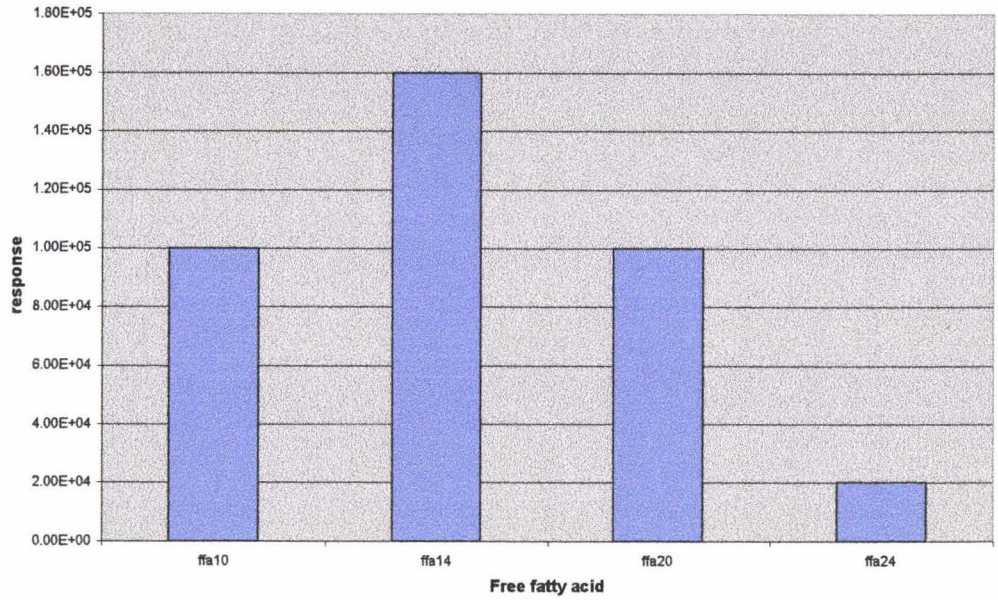


Figure 14. Free fatty acids. OR = -50 V, RNG = -210 V.

Analysis of Cholesterol and Cholesteryl Palmitate by Infusion MS

Cholesterol

Cholesterol was analysed, as it is found in milkfat and therefore could appear in milkfat spectra analysed later in the study.

Infusion MS of ch lesterol (MW=386.7) gave four prominent ions at m/z 432.4, 404.4, 369.3 and 338.3. The ions at m/z 404.4 and 369.3 were identified as cholesterol plus an ammonium ion $(NH_4)^+$ adduct and cholesterol minus an OH group respectively. It was possible that the ion at m/z 432.4 was cholesterol plus a formic acid adduct. The ion at m/z 338.3 was thought to be cholesterol minus both an OH group and two methyl moieties. MS/MS of ions at m/z 432.4 and 404.4 gave a product ion at m/z 369.3 (cholesterol minus an OH group). MS/MS of the parent ion at m/z 369.3 gave a range of product ions all less than m/z 300 with the most prominent product ion at m/z 147.1 (this ion has yet to be identified).

Cholesteryl palmitate

Infusion MS of cholesteryl palmitate (MW= 625.1) gave ions at m/z 642.7 (the ammonium adduct of cholesteryl palmitate) and m/z 663 (yet to be identified), a less prominent ion at m/z 647.7 (the sodium adduct of cholesteryl palmitate) and a very prominent ion at m/z 369.2 (cholesterol minus an OH group). MS/MS of ions at m/z 663 and 642.7 gave similar spectra, with a prominent product ion at m/z 369.2 (cholesterol minus an OH group). Thus the fingerprints of cholesterol and cholesterol ester have been established.

3.2 Polar Lipids

The term polar lipids covers a range of many different compounds all found in small quantities in milkfat. Unlike non-polar lipids, these compounds already carry charge, and because of this are ideally suited for analysis by electrospray MS. Many of these components have nutritional value and/or may be biologically active. Although these components are found in only minor quantities in milkfat, technologies that are used to extract and enrich minor components such as these are becoming increasingly more advanced. As such, and with the increasing interest in nutraceutical applications within the dairy industry, the ability to characterise and measure these components is becoming more important.

The selection of polar lipid standards, to be characterised by electrospray MS, was based on both their abundance in milkfat and their availability.

3.2.1 Method Development

Infusion MS and MS/MS of Polar Lipids

In the analysis of polar lipids, both positive ion and negative ion MS were performed. The selection of solvent systems for these analyses was based on work completed above on the mass spectral analysis of TAGs and work done by Kerwin *et al.* (1995) on electrospray mass spectral analysis of phospholipids. These solvents were chloroform:methanol (70:30) and the standard solvent system used for the analysis of TAGs above (1,2 dichloroethane:acetonitrile:formic acid (68:35:2) and 10 mM ammonium acetate). In one particular case, negative ion MS of sphingomyelin, chloroform:methanol (70:30) and 10 mM ammonium acetate was used. The success

or failure of each solvent system, with respect to each polar lipid analysed, is discussed in detail below.

3.2.2 Analysis of Phospholipids by Infusion MS and Infusion MS/MS

Phospholipids

(a) Analysis of 1,2 dipalmitoyl sn glycero-3-phosphocholine (MW 734)

Positive ion MS

Mass spectra for this phospholipid standard are presented in Appendix 2.

Positive ion MS was carried out only with the chloroform:methanol solvent system. The parent ion $(M+H)^+$ was identified at m/z 734.5, as expected from the known structure. Prominent peaks were seen at m/z 337.2 and 365.2. These peaks have not been identified, but may have been impurities in the sample.

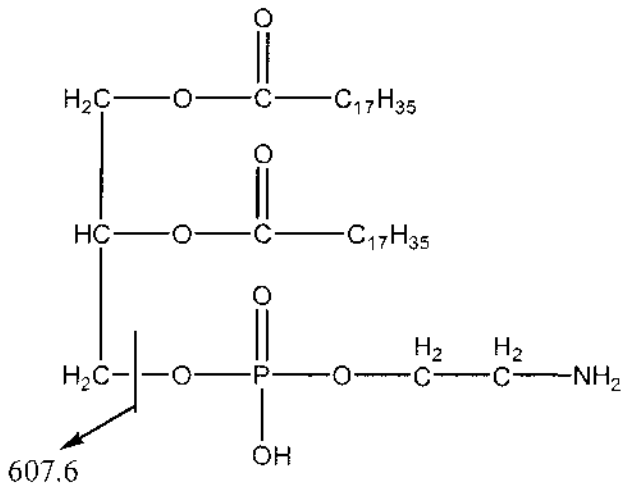
The molecular ion $(M+H)^+$ was selected for CAD. Fragments at m/z 496.5 and 183.8 were identified as the molecular ion minus a fatty acid group and the head group respectively.

Negative ion MS

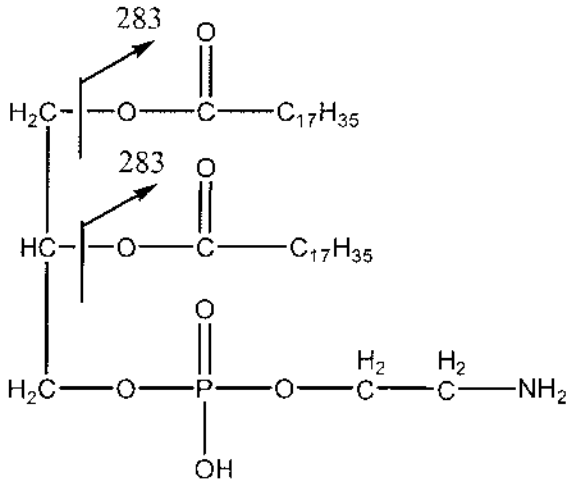
When the standard was prepared in the chloroform:methanol solvent system, no molecular ions were identified. This was probably due to the positive charge on the head group needing to be neutralised by a group such as formate before analysis in the negative mode could take place.

In fact, once this solvent system was replaced with the 1,2 dichloroethane:acetonitrile: formic acid and 10 mM ammonium acetate system, molecular ions were identified at m/z 778.6 $(M+HCOO)^-$, and fragment ions were seen at m/z 768.5 (not identified), 718.5 (a major ion, identified as the molecular ion minus a CH_2 group) and 255.1 (a smaller peak, identified as the $RCOO^-$ group). Increasing the RNG and OR voltages easily increased the response of this last peak.

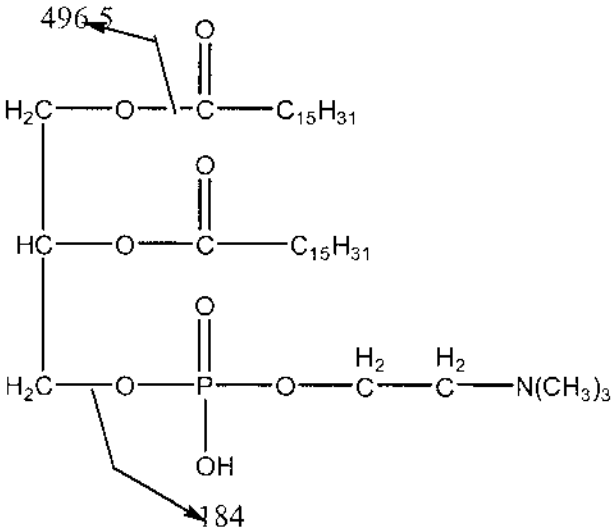
The schematic in Figure 15 illustrates the source of the fragment ions identified above.



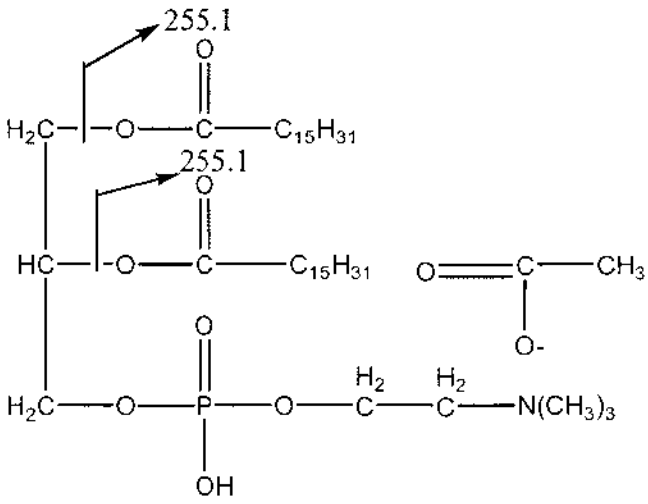
1,2 distearoyl sn glycero 3 phosphoethanolamine (+ve ion)



1,2 distearoyl sn glycero 3phosphoethanolamine (-ve ion)



1,2 dipalmitoyl sn glycero-3-phosphocholine (+ve ion)



1,2 dipalmitoyl sn glycero-3-phosphocholine (-ve ion)

Figure 15. Fragmentation patterns of 1,2 distearoyl sn glycero 3 phosphoethanolamine and 1,2 dipalmitoyl sn glycero 3 phosphocholine

(b) Analysis of L α-lysophosphatidoyl ethanolamine

Mass spectra for this phospholipid standard are presented in Appendix 3.

Positive ion MS

This phospholipid was prepared in both chloroform:methanol and 1,2 dichloroethane: acetonitrile:formic acid and 10 mM ammonium acetate solvent systems. The resulting spectra were very similar; however, the background noise in the spectra was less in the latter solvent system.

The standard was a mixture of two different lysophosphatidoyl ethanolamine phospholipids, one with stearic acid (MW 481.7) and the other with palmitic acid (MW 453.6) esterified to the glycerol backbone, and hence molecular ions $(M+H)^+$ were identified at m/z 482.3 and 454.2. Fragment ions were also seen in the spectra at m/z 341.3 and 313.4. These were identified as the $(M+H)^+$ ions minus their ethanolamine head groups.

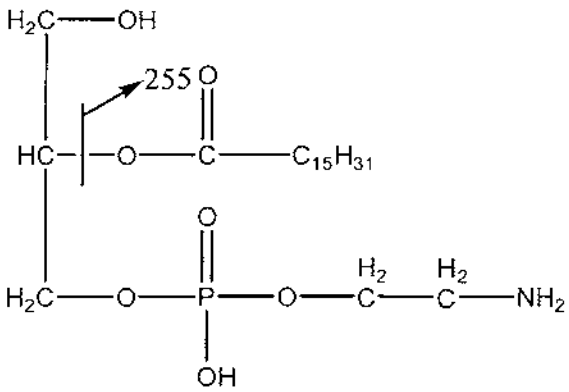
The molecular ions 482.3 and 454.2 were selected for CAD. The fragment ions seen were the same as those identified above, with Q1 scanning (m/z 341.1 and 313.2 respectively). It is interesting to note that with these experiments the protonated head group was not identified (unlike the phosphocholine analysis above).

Negative ion MS

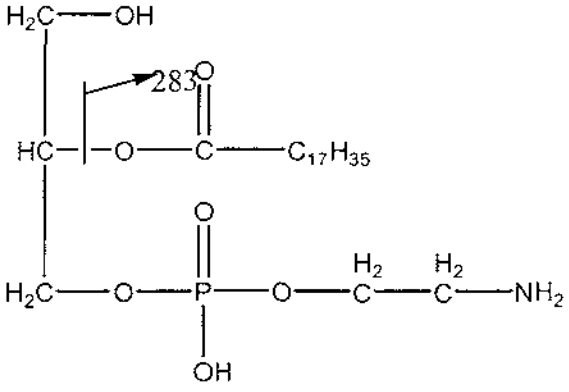
Samples were prepared in the 1,2 dichloroethane:acetonitrile:formic acid and 10 mM ammonium acetate solvent system, for negative ion MS. Parent $(M-H)^-$ ions were identified at m/z 480.1 and 452.1, along with fragment ions at m/z 283 and 255 respectively ($RCOO^-$ group of stearic and palmitic acids respectively).

There was no need to perform negative ion MS/MS.

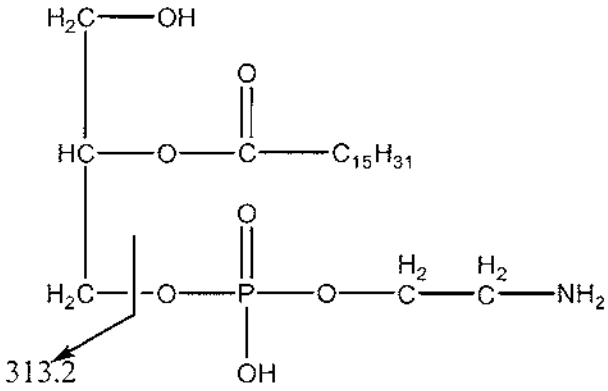
The schematic in Figure 16 identifies some of the fragment ions identified above.



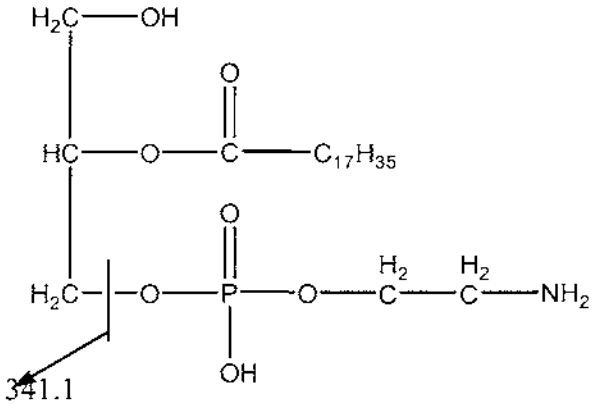
L α-lysophosphatidoyl ethanolamine negative ion



L α-lysophosphatidoyl ethanolamine negative ion



L α-lysophosphatidoyl ethanolamine positive ion



L α-lysophosphatidoyl ethanolamine positive ion

Figure 16. Fragmentation patterns of L α- lysophosphatidoyl ethanolamine

(c) Analysis of 1,2 distearoyl sn glycero-3-phosphoethanolamine

Mass spectra for this phospholipid standard are presented in Appendix 4.

Positive ion MS

This phospholipid was prepared in both chloroform:methanol and 1,2 dichloroethane: acetonitrile:formic acid and 10 mM ammonium acetate solvent systems. The resulting spectra were very similar; however, the background noise in the spectra was lower in the latter solvent system.

$(M+H)^+$ ions were identified at m/z 748.6 as expected from the known structure. Fragment ions were identified at m/z 607.6 (molecular ion minus the ethanolamine head group) and 550.6 (a smaller peak, not yet identified, but possibly $(M-RCOOH)^+$).

The molecular ion at m/z 748.6 was selected for CAD. The only peak identified was one at m/z 607.7. Once again, the head group was not seen in the spectra.

Negative ion MS

Samples were prepared in the 1,2 dichloroethane:acetonitrile:formic acid and 10 mM ammonium acetate solvent system, for negative ion MS. The parent ion $(M-H)^-$ at m/z 746.5 was identified, along with fragment ions at m/z 283.1 ($RCOO^-$ group) and 338.9, 325 and 311 (not identified).

The molecular ion (at m/z 746.5) was selected for CAD. A major peak was seen at m/z 283.3 (and identified as $RCOO^-$ ions) and minor peaks were seen at m/z 480.3 and 462.2. These results suggest that the ions at m/z 338, 325 and 311 seen above may have not been related to the parent ion and may in fact have been contaminants.

The schematic in Figure 15 illustrates the source of the fragment ions identified above.

Summary of analysis of phospholipid standards by infusion MS

These results show that, in the mass spectral analysis of phosphatidylcholines and phosphatidylethanolamines, negative ion MS can be used to identify the fatty acid groups attached to the glycerol backbone and, by subtraction, the type of phospholipid group being analysed. The only extra information that could be

provided by positive ion MS (and this was only in the case of phosphatidylcholine) was identification of the head group in the spectra. MS/MS spectra generated in both the positive and negative modes gave no extra information to that gained in the MS experiments (with increased OR and RNG voltages), although any impurities in the sample could be identified and thus eliminated.

It was always possible to use both positive and negative ion MS to generate molecular and fragment ions when the phospholipids were prepared in the 1,2 dichloroethane: acetonitrile:formic acid and 10 mM ammonium acetate solvent system (as opposed to the chloroform:methanol solvent system).

Masses identified and trends seen agreed with those reported in the literature by Kim *et al.* (1994) and Kerwin *et al.* (1995).

Sphingomyelin

Mass spectra for sphingomyelin are presented in Appendix 5.

Positive ion MS

Sphingomyelin from bovine brain was initially prepared at 0.005 mg/mL and then at 0.05 mg/mL in 1,2 dichloroethane:acetonitrile:formic acid (65:35:2) and 10 mM ammonium acetate. $(M+H)^+$ ions correlating to sphingomyelin containing the fatty acids 16:0, 18:0, 20:0, 22:0, 24:0, 24:1, 26:1 were identified. These ions were the same as those identified by Kerwin *et al.* (1995). The two major ions seen in the spectra were m/z 731.5 and 813.7 and these were the two major ions identified by Kerwin *et al.* (1995).

A number of the $(M+H)^+$ ions were selected for CAD. In all cases, the only ion seen was at m/z 184, and this was identified as the phosphocholine head group that was also seen in the MS experiments but at low levels.

Negative ion MS

Initially, sphingomyelin (at 0.05 mg/mL) was prepared in both chloroform:methanol, and the 1,2 dichloroethane:acetonitrile:formic acid mix used above for positive ion MS. However, as no molecular ions were seen in the spectra, the solvent systems trialed above were replaced with chloroform:methanol 70:30 and 10 mM ammonium acetate. (A counter ion such as acetate was obviously required to neutralise the

positive charge on the molecule, but, unlike the phospholipids analysed above, acid conditions were not conducive to the formation of negative ions.) With this solvent system molecular ions were seen, but at very low intensities. The molecular ions identified corresponded to the mass predicted by positive ion MS minus 16 amu, *i.e.* $(M-CH_4)^-$. These results differed from those reported by Kerwin *et al.* (1995) who identified molecular ions corresponding to $(M+acetate-CH_4)^-$.

MS/MS spectra were difficult to generate because of poor 'parent' ion intensity. MS/MS of m/z 716 gave a prominent ion at m/z 168, which was identified as the phosphoryl choline head group minus one methyl moiety.

Sphingosine

Mass spectra for sphingosine are presented in Appendix 5.

Sphingosine was prepared in the 1,2 dichloroethane:acetonitrile:formic acid solvent mix used above and was analysed by positive ion MS and MS/MS. A major ion at m/z 282.3 and two minor peaks at m/z 300.2 and 149.1 were seen in the MS spectra. The peak at m/z 300.2 was identified as the molecular ion $(M+H)^+$, and that at m/z 282.3 was identified as the molecular ion minus a water molecule.

The parent ion (at m/z 300.2) was selected for CAD. Ions seen and identified in the resulting spectra were at m/z 282.3 (loss of H_2O), 183 (loss of $H_3C-(CH_2)_{12}$) and 252.1 (possibly loss of CH_2O).

Gangliosides

Mass spectra for gangliosides are presented in Appendix 6.

All ganglioside samples were prepared in chloroform:methanol as mentioned previously. Only negative ion MS and MS/MS were performed.

Analysis of ganglioside mix

Analysis of the ganglioside mix gave a complex spectrum. The two most prominent ions seen were at m/z 917.7 and 931.7. These were identified as the doubly charged ions of GD1a. A small ion seen at m/z 1063 was identified as the singly charged ion of GM1. Other minor peaks at m/z 1317.8, 1290.0 and 1572.9 were observed.

MS/MS of ions at m/z 917.7 and 931.7 gave one major ion at m/z 289.8, which was identified as a sialic acid residue.

Analysis of the GM3 sample

Analysis of the GM3 sample gave a prominent ion at m/z 1262. Both Ghardashkhani *et al.* (1995) and Ohashi (1997) suggested that GM3 gangliosides form singly charged ions upon entry into the mass spectrometer with a mass of 1182 (an 80 amu difference). It is possible that sulphate esters can attach at position three of a galactosyl moiety in gangliosides derived from brain. If these gangliosides are derived from brain, this may explain the 80 amu difference. Unfortunately there was not enough sample to carry out MS/MS experiments.

Analysis of the GD3 sample

Analysis of the GD3 sample gave prominent ions at m/z 290.1 and 581.1. Clusters of ions were identified around m/z 770, m/z 944.9 and m/z 1235.8. These clusters disappeared when the OR and RNG voltages were increased to -100 and -300 V respectively.

MS/MS of the ion at m/z 581.1 gave a major ion at m/z 290.0.

Both Ghardashkhani & Gustavsson (1995) and Ohashi (1997) suggested that the GD3 ganglioside has a mass of 1473, and upon entry into the mass spectrometer it forms a doubly charged ion with an approximate mass of 736.5. The mass discrepancy between 581.1 and 736.5 could not be explained.

3.3 Simple Fats and Oils

Because they are simpler TAG mixtures than milkfat, the analysis of cocoa butter and coconut oil enabled the identification of the procedures required to analyse 'real lipid samples' by infusion electrospray MS. However, it also became obvious during the course of this last set of work that very complex lipid samples (*i.e.* milkfat) would need separation prior to entry into the mass spectrometer in order for accurate (and timely) interpretation of the spectra to occur. Thus an existing reversed-phase HPLC method used in the analysis of TAGs from milkfat was modified to incorporate the additives required for successful MS of lipids. LC-MS was performed (with cocoa

butter and coconut oil followed by milkfat) using the same voltage settings that had been identified previously.

3.3.1 Method development

Infusion MS and Infusion MS/MS of Fats and Oils

Solvent selection

The solvent system used in the analysis of fats and oils was the same as that used in the analysis of the TAG standards.

Maximising the response of the molecular ion

The state files used in the analysis of fats and oils were the same as those used in the analysis of TAG standards. In addition, samples were run with OR and RNG voltages of 25 and 50 V respectively. Although the overall response of the $(M+NH_4)^+$ ion was low, setting the RNG voltage at 50 V meant that, very little, if any, front end fragmentation occurred and thus low molecular weight ammoniated TAG ions were easily discernible from high molecular weight DAG ions. This extra step in the analysis of these samples made interpretation of the spectra more straightforward.

LC-MS of Simple Fats and Oils

Solvent selection

As has been mentioned previously, it was found that complex lipids required separation prior to entry into the mass spectrometer, in order to make interpretation of the spectra manageable. HPLC was selected to carry out this separation because it interfaces well with electrospray ionisation. The HPLC method used was developed by Robinson & MacGibbon (1998).

Initially solvent A was altered by the addition of 2% formic acid and 10 mM ammonium acetate, as it had been established previously that these additives were required to provide sufficient ionisation of the parent TAGs. Using this solvent system, LC-MS spectra generated using both coconut oil and cocoa butter showed not only $(M+NH_4)^+$ adducts as seen in infusion MS but also significant quantities of $(M+46)^+$ adducts. Although the concentration of the lipid sample loaded on to the column was quite high (10 µL at 10 mg/mL), the $(M+NH_4)^+$ ion was the adduct seen in the greater intensity. It was interesting to observe however, that as the

concentration of lipid sample injected onto the column was decreased, the relative proportions of the two ions changed, with the $(M+46)^+$ adduct becoming the ion seen with the greatest intensity.

A number of experiments were carried out to try to identify this $(M+46)^+$ adduct, using coconut oil, all without success. Firstly, fractions that were thought to contain (as identified by previous MS experiments) an ample quantity of both adducts were collected off the HPLC column. These fractions were infused back into the mass spectrometer and, although the $(M+NH_4)^+$ ions were seen in great abundance, only trace amounts of the other adduct were seen. It was also noted that the DAG patterns of both the infused MS and LC-MS experiments were identical, suggesting (at the very least) that the $(M+46)^+$ ion does not fragment under the same conditions as the $(M+NH_4)^+$ ions. Secondly, when LC-MS/MS experiments were carried out, with the $(M+NH_4)^+$ ions selected for fragmentation in the first instance, excellent DAG ions were generated as each $(M+NH_4)^+$ ion underwent CAD. The experiment was repeated with the $(M+46)^+$ ions. In sharp contrast to the results achieved above, no DAG ions were generated. This occurred because the parent ions selected for CAD were not identified 'as present' by the mass spectrometer. A possible explanation may be that the $(M+46)^+$ ion was so unstable that it completely disintegrated upon entry into the second quadrupole.

However, when solvent A was replaced with acetonitrile:water (98:2) and 10 mM ammonium acetate, the only molecular ions seen in the spectra were $(M+NH_4)^+$ ions. In the absence of formic acid, the peak shape on the total ion current (TIC) was a lot broader and the response, as the carbon number increased above about 42, was less. This has implications for samples such as coconut oil, where TAGs with a carbon number of 42 or greater are seen at relatively low levels in the sample anyway. With this sample, the response of some of the longer chain ammonium-TAGs seen in the spectra was so low that the DAG pattern was not as clear and thus identification of the TAGs present under each peak was not easy.

Replacing the water with acetic acid improved both the peak shape and the response at the higher carbon numbers (although it was still lower than that seen with formic acid). However, the $(M+46)^+$ adduct reappeared, but at much lower levels than seen previously. This suggested that a combination of ammonium acetate, acid pH and the

increased flowrate of sample into the mass spectrometer when performing LC-MS (as opposed to the relatively low flowrate when infusing a sample) all contributed to the formation of this adduct.

Figure 17 shows the difference in normalised peak areas when using the three different solvent A systems referred to above. Although the trends for all three systems were very similar, formic acid gave a slightly better 'total response' for carbon numbers 44 and greater (which were seen at low levels in the spectra anyway). As a result, and despite the presence of the $(M+46)^+$ ion, acetonitrile:formic acid (98:2) and 10 mM ammonium acetate was used as solvent A to analyse all further samples of simple fats and oils.

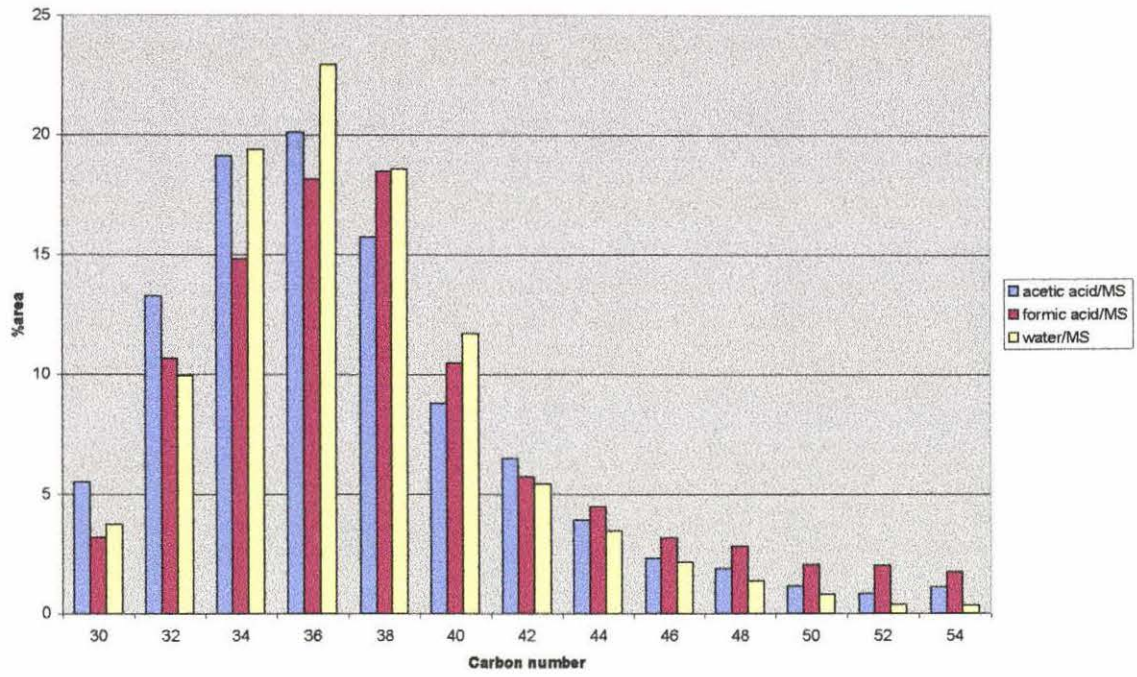


Figure 17. Comparison of normalised peak areas found for coconut oil using three different solvent A systems.

Maximising the response of the molecular ion

The state files used for infusion-MS and infusion MS/MS of TAG standards were found to be satisfactory for LC-MS and for LC-MS/MS.

3.3.2 Analysis of Simple Fats and Oils

Analysis of Cocoa Butter and Coconut Oil by Infusion MS and Infusion MS/MS

Cocoa butter and hydrogenated coconut oil were chosen for analysis because, although they had a relatively simple TAG composition when compared with milkfat, they were far more complex than the standards analysed above. This would allow development of techniques to study more complex fats and oils.

Appendices 7a and 7b show the spectra obtained from infusion MS of coconut oil with RNG voltages of 150 and 50 V respectively. It can be seen from these appendices that it was difficult to assign DAG ions to their parent molecular ions, because the large number of parent ions in the spectra made TAG identification difficult. It was found that TAGs could really only be identified unambiguously by selecting each molecular ion for CAD and using the masses identified from the resulting DAG ions to identify each TAG by difference. Figure 18 shows the MS/MS spectrum of the molecular ion m/z 769.4.

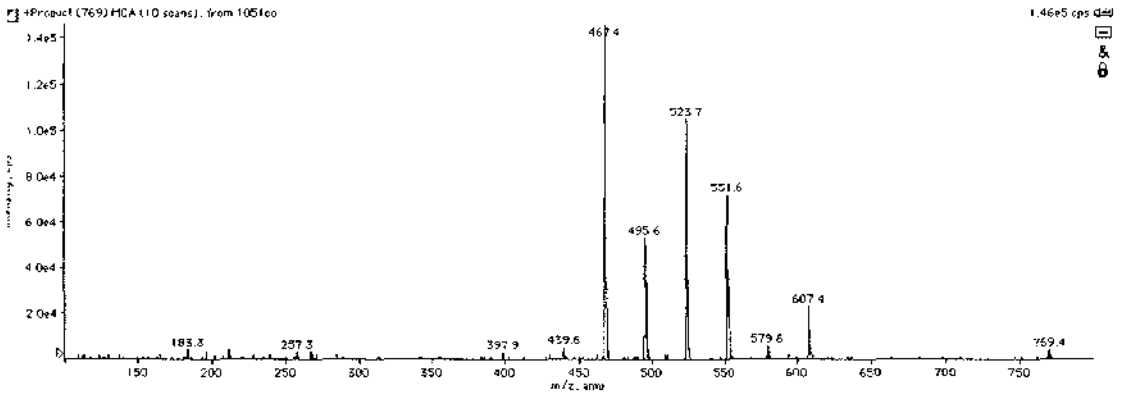


Figure 18. MS/MS spectrum of a molecular ion present in coconut oil.

In Table 2, the 'fatty acids lost' column represents all the possible fatty acids in the molecular ion. The 'remaining DAG' column represents the possible combination of these fatty acids in a DAG. From the DAG pattern, the TAGs shown in the 'possible TAG' column could be identified.

Table 2. Analysis of the MS/MS spectrum of the molecular ion m/z 769.4

DAG ions	Fatty acids lost	Remaining DAG	Possible TAG
439.6	20:0	24:0	20:0/12:0/12:0 20:0/14:0/10:0 20:0/16:0/8:0
467.4	18:0	26:0	18:0/14:0/12:0 18:0/16:0/10:0 18:0/18:0/8:0
495.6	16:0	28:0	12:0/16:0/16:0 16:0/14:0/14:0 18:0/16:0/10:0 20:0/16:0/ 8 :0
523.7	14:0	30:0	14:0/14:0/16:0 18:0/14:0/12:0 20:0/14:0/10:0
551.6	12:0	32:0	12:0/16:0/16:0 18:0/14:0/12:0 20:0/12:0/12:0
579.6	10:0	34:0	20:0/14:0/10:0 18:0/16:0/10:0
607.4	8:0	36:0	20:0/16:0/8:0 18:0/18:0/8:0

It can be seen from the relative heights of the DAG ions in the spectrum (specifically 439.6, 579.6 and 607.4) that TAG molecules containing fatty acids 20:0, 10:0 and 8:0 are going to be minority components of the 769.4 molecular ion. Similarly, it can be determined that the majority components of the 769.4 molecular ion are going to be TAG molecules containing fatty acids 18:0, 16:0, 14:0 and 12:0. As a result, of the eight different TAG species identified in Table 2, three major TAG species, specifically 16:0/16:0/12:0, 16:0/14:0/14:0 and18:0/14:0/12:0, can be determined.

Thus the MS/MS option was used to filter the mass of information obtained from the electrospray MS experiments and made accurate interpretation of the spectra possible. As a result most, if not all, of the MS/MS experiments carried out with both cocoa

butter and coconut oil involved selecting the molecular ions for CAD. This approach was in contrast to the MS/MS experiments carried out with TAG standards where DAG ions were selected for CAD. A useful check was MS/MS of the DAG ions (and hence identification of acylium ions and therefore each fatty acid group) although this was very time consuming and often unnecessary in complex samples such as these.

An additional technique, which was used to help with the interpretation of the spectra, was the use of the 'import spectra' command in the peptide map program, part of the proprietary software from Sciex. In samples such as cocoa butter, unsaturated TAGs are the major components and saturated TAGs, which are just 2 amu greater than these unsaturated TAGs, are minor components. In this situation it is difficult to differentiate whether a peak is an isotope effect or another, minor TAG. The peptide map software can assist greatly with this interpretation (by estimating the isotope responses).

Summary: analysis of fats and oils by infusion MS and MS/MS

1 Cocoa butter and coconut oil were infused with OR and RNG voltages of 25 and 150 V and 25 and 50 V respectively.

2 The peptide map software was used to differentiate an isotope effect from a low concentration saturated TAG if necessary.

3 Each ammonium-TAG ion in the spectrum was selected for CAD.

4 The resulting DAG patterns were analysed.

- Each parent ion upon CAD produced a number of different DAG ions. By difference, *i.e.* subtracting the mass of each DAG ion from the known mass of the molecular ion, identification of each 'lost' fatty acid was possible.
- From the mass of each DAG ion, the sum of the two constituent fatty acids could be determined.
- By identifying all the fatty acids 'lost' upon formation of the DAG ions and knowing the molecular weight of their parent TAGs, it was possible to identify most if not all of the TAGs that made up a particular ammonium-TAG ion.

- By looking at the relative response of the DAG ions, it was possible to ascertain the relative quantities of each of the TAGs present (of course also dependent on the fatty acid composition).

Analysis of Cocoa Butter and Coconut Oil by LC-MS

The HPLC separated TAGs of cocoa butter and coconut oil into groups or peaks based on their carbon number and degree of saturation. It was then possible to ascertain, from the MS data, the identity of each of the TAG species within a peak.

The TIC trace of coconut oil (Appendix 7d) showed well-resolved, well-separated peaks that closely resembled the ELSD trace (Appendix 7e), making correlation very straightforward. Analysis of the extracted ion chromatograms (XICs), taken from under each of the peaks on the TIC, showed that all of the $(M+NH_4)^+$ ions present under each peak had the same mass, *i.e.* many TAGs with different fatty acid combinations might be present under each peak, but they all had the same total mass. As a result, identification of these TAGs was relatively straightforward and done by analysing the DAG pattern corresponding to each $(M+NH_4)^+$ ion and identifying each fatty acid by difference. The results from this analysis are presented in Appendix 7. Table 3 is an exerpt from Appendix 7 and shows the analysis of the molecular and DAG ions present in the XIC taken from under the peak at 52.05 min on the TIC. It can be seen from Table 3 that in this instance the molecular ion (seen at m/z 712.8 in the spectrum) represented four different species of TAGs.

Table 3. Analysis of the molecular ions present under a peak seen in the TIC trace of coconut oil

Molecular ion	DAG ions	Fatty acids lost	Possible TAGs
712.8	523.4	10:0	18/10/12 16/10/14
	495.5	12:0	16/12/12 12/14/14
	467.3	14:0	12/14/14 16/10/14
	439.7	16:0	16/10/14 16/12/12
	411.3	18:0	18/10/12

(For a description of how TAGs are identified by their DAG patterns, see Section 4.3.2.)

The TIC trace of cocoa butter showed peaks that were less well resolved than the peaks seen in the TIC trace of coconut oil. This was because of the presence of both saturated and unsaturated TAGs. The ELSD and TIC traces of cocoa butter (Appendices 8c and 8b) were not as similar to each other as the corresponding ELSD and TIC traces of coconut oil. Peaks in the TIC trace of cocoa butter with XICs that contained molecular ions with two or more double bonds (specifically 50:2, 52:2, 54:2) were greatly amplified when compared with the corresponding peaks in the ELSD trace (which were seen as small bumps in the baseline). It was concluded that the degree of saturation of a TAG molecule impacted greatly on the response seen from the mass spectrometer.

It was seen from the analysis of the XICs taken from under the peaks present in the TIC that, although most of the major peaks contained $(M+NH_4)^+$ ions of one mass, some of the minor peaks had up to four groups of $(M+NH_4)^+$ ions of different mass. Identification of the different TAG groups present in the XICs taken from under each of the peaks in the TIC trace was carried out in the same manner as before. It was not always possible, however, due to the relatively low concentration of DAG ions in the XICs taken from under many of these minor peaks, to identify all of the TAG species present.

For example, Table 4 shows the analysis of the molecular and DAG ions present in the XIC taken from under the minor peak seen in the TIC trace at 103 min. It can be seen that, of the four molecular ions identified, only the fatty acid compositions of the two most prominent ions seen in the XIC (852.8 and 892.9) could be ascertained.

Table 4. Analysis of the molecular ions present under a minor peak seen in the TIC trace of cocoa butter

Molecular ion	DAG ions	Fatty acids lost	Possible TAG
852.8	551.3	18:0	
	579.5	16:0	18:0/16:0/16:0
892.9	605.4	17:0	
	593.5	18:1	
	591.5	18:0	17:0/18:0/18:1
904.9	not observed		
932.8	not observed		

As a result, identification of the different TAGs present, although still carried out in the same manner as before, was more difficult because of the relatively low concentration of DAG ions under these minor peaks. The results from this analysis are presented in their entirety in Appendix 8. For a description of how TAGs are identified by their DAG patterns, see Section 4.3.2.

For both cocoa butter and coconut oil, the HPLC separation prior to entry to the mass spectrometer proved to be a very effective way of analysing these lipid samples. More often than not, there was no need to perform LC-MS/MS on each ammonium-TAG ion (as had to be done with infusion MS in order to simplify the spectra) and as such a substantial amount of time was saved.

Summary: LC-MS of simple lipids

- LC-MS proved to be an effective way of analysing mixtures of lipids.
- The number of unsaturated fatty acids in a TAG molecule impacted greatly on the response seen in the TIC.
- An M+46 adduct was generated in addition to the ammonium-TAG ion seen in the spectra. This adduct, not seen in the infusion MS experiments, was only seen when performing LC-MS under acid conditions. The M+46 adduct did not contribute, under the conditions examined, to the DAG pattern seen in the spectra.
- Coconut oil could be easily analysed.
- In cocoa butter, the significant concentrations of the fatty acids led to significant response differences of the TIC trace relative to the ELSD trace.

3.4 Milkfat

Milkfat is a complex lipid, containing large numbers of TAGs with different compositions. The LC-MS method used in the analysis of cocoa butter and coconut oil was used to analyse different samples of milkfat. These analyses involved the following:

- Analysis of a bovine milkfat sample and identification of the carbon number and degree of saturation of TAGs under each peak resolved by HPLC.

- A comparison of Jersey and Friesian milkfat.
- A comparison of bovine milkfat collected at different times of the season.
- A comparison of milkfat from different mammalian species.

The last three analyses focused on identifying differences rather than complete characterisation of the TAG profile.

3.4.1 Method Development

Solvent Selection

As has been stated previously, the HPLC method used was one based on a method published by Robinson & MacGibbon (1998). Initially, as with the LC-MS separations of cocoa butter and coconut oil, solvent A contained acetonitrile:formic acid (98:2) and 10 mM ammonium acetate to facilitate the formation of molecular ions. Subsequently the formic acid was replaced by water. A sample of bovine milkfat was analysed by LC-MS using both solvent systems. The elution pattern of TAGs in bovine milkfat was different from that seen in either coconut oil or cocoa butter in that a large number of closely eluting peaks were seen in the TIC trace. Because many of these closely eluting peaks were only partially resolved in the TIC trace, optimal peak definition was critical to accurate interpretation of the TIC. The peak definition using formic acid was far superior to that given by water and therefore 2% formic acid, 10 mM ammonium acetate and 98% acetonitrile was used as 'solvent A' to analyse all further milkfat samples, despite the complicating factor of $(M+46)^+$ ions being present in the spectra. The traces below show the improvement found with the replacement of water with formic acid.

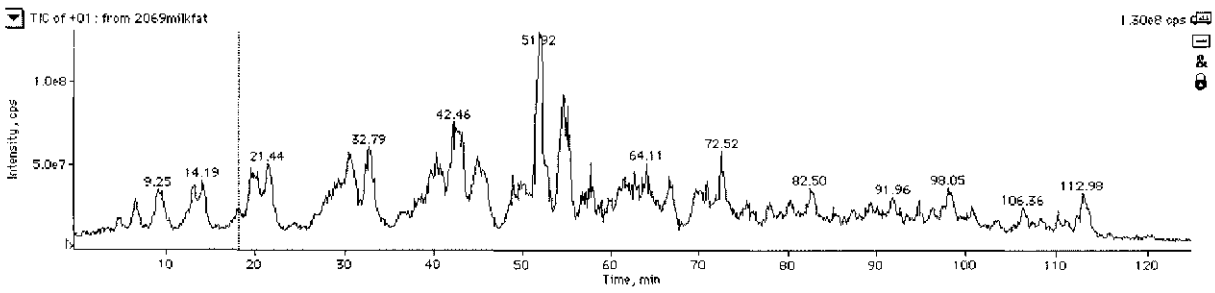


Figure 19. TIC trace of milkfat with solvent system containing water.

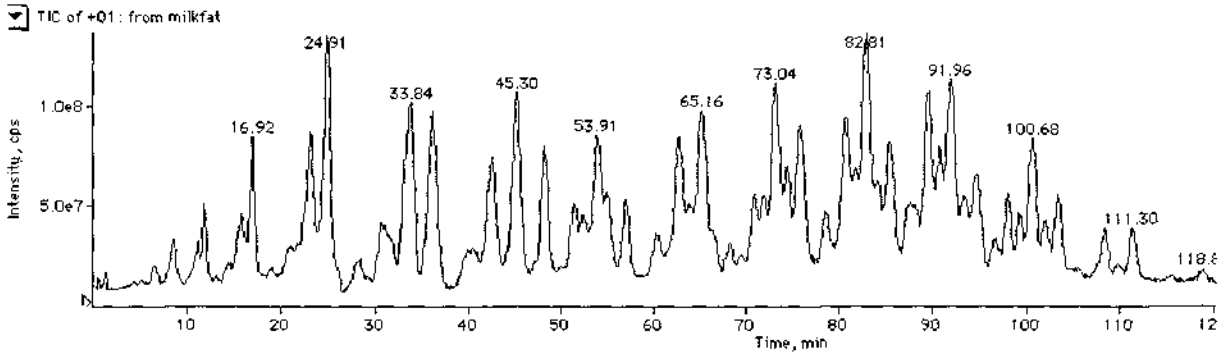


Figure 20. TIC trace of milkfat with solvent system containing formic acid.

Maximising the Response of the Molecular Ion

The state files used for LC-MS of milkfat were the same as those used for LC-MS of cocoa butter and coconut oil.

3.4.2 Analysis of a Range of Milkfat Samples

Analysis of Bovine Milkfat by LC-MS

A summer milkfat sample was analysed by LC-MS. The carbon number and degree of saturation for each TAG under every peak on the TIC trace was identified, using the CTC spreadsheet. This allowed the TIC trace to be compared with the ELSD trace from HPLC and peaks in the TIC trace to be correlated with peaks in the ELSD trace. The elution pattern of TAGs from milkfat had been characterised extensively by Robinson & MacGibbon (1998) using the same HPLC method. These authors had identified most of the peaks by the use of standards. The TIC trace (Appendix 9a) resembled the ELSD trace (Appendix 9b), making correlation between the two reasonably straightforward.

There were two minor complicating factors in making the correlation. Firstly, some closely eluting peaks were poorly resolved on the TIC trace. Secondly, the difference in the ELSD and TIC peak responses was quite marked. This may have been due to the type of TAG under a given peak: for example, it has been shown in the analysis of TAG standards by infusion MS that the TIC response of a given TAG decreases as the carbon chain increases. Thus it can be seen from the TIC trace (Appendix 9a) that the peak response decreased as the retention time (*i.e.* carbon number) increased. It has also been shown (in the analysis of cocoa butter) that peaks containing TAGs

with more than one double bond appear to be greatly amplified (when comparing with an ELSD trace).

Appendix 9 identifies both the carbon number and degree of saturation of TAGs under each peak resolved by HPLC, and gives an indication of how well the peak identities postulated by both the LC-MS and HPLC (Robinson & MacGibbon, 1998) methods agree. In general, the two sets of results compared well. However, the TAG component of a minority of peaks identified by LC-MS did not agree with the identities of the peaks as postulated by Robinson & MacGibbon (1998). For some other peaks it was seen that, whereas one species of TAG within the peak had been correctly 'assigned' by Robinson & MacGibbon (1998), there were other species of TAGs present within that same peak that had not been assigned. For example, peak 34 was identified by the mass spectrometer as containing TAGs 50:3, 48:2 and 43:0, whereas only the 48:2 TAGs had been identified as present in peak 34 by Robinson & MacGibbon (1998) using HPLC. This demonstrates the importance of MS in the absolute identification of complex species where standards of all the possible combinations of TAGs would be impossible to run.

It was also interesting to note that the composition of some peaks changed across the peaks, *i.e.* peak number 19 contained two species of TAG, 38:0 and 40:0. It was possible to see, by extracting small areas of the TIC at a time, across the width of the peak, that the 38:0 TAGs (16:0, 16:0 and 6:0) and small amounts of the 40:1 TAGs (16:0, 18:1, 6:0) eluted in the front half of the peak, whereas the 40:1 TAGs (18:1, 18:0, 4:0) eluted in the second half of the peak. This demonstrates the advantage of the MS technique in the analysis of complex mixtures.

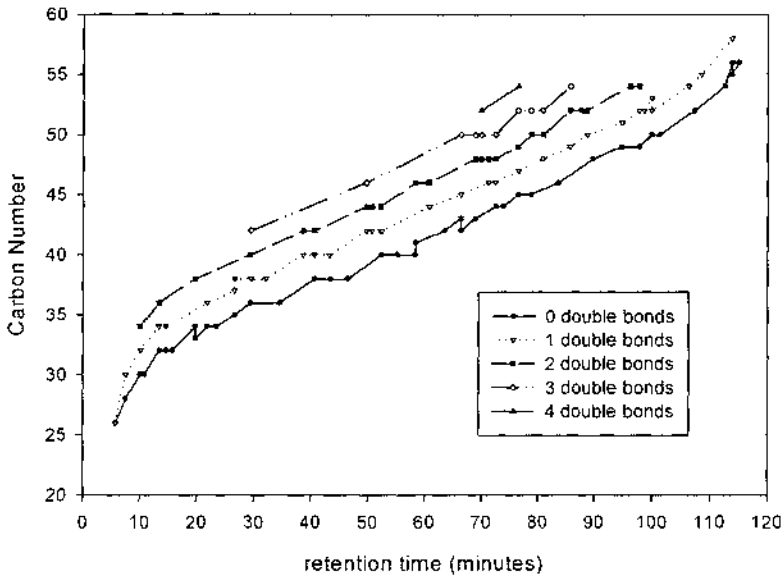


Figure 21. HPLC carbon number groups.

Figure 21 shows the relationship between retention time and carbon number, as determined from the LC-MS of milkfat, for the chromatography of milkfat. The peaks are grouped by the number of double bonds in the TAG. The linearity of each of these groups demonstrates the effect that both chain length and degree of unsaturation have on the HPLC retention time of a given TAG. The kinks seen in each of the four 'lines' show the fine separation of TAGs with a similar carbon number but different fatty acid constituent. The results presented in Figure 21 and Appendix 9 confirm that

- within a double bond group, carbon number governs elution order,
- TAGs of identical carbon number but higher double bond elute considerably faster, and
- TAGs of a peak contain a variety of different double bond groups, but most conform to PN = CN - 2DB where PN is partition number, CN is carbon number and DB is the number of double bonds.

Analysis of Friesian and Jersey Milkfat by LC-MS

Milkfat samples from Friesian and Jersey cows were analysed by LC-MS. For each sample, peaks 12, 13/14, 15, 19, 24, 29, 57 and 59/60 from the TIC were selected for examination. The relative intensities of the molecular ions under each peak and their associated DAG patterns were determined and a comparison between the two milkfats was carried out.

Molecular ions and their associated DAG ions were identified in the XICs taken from under each of the above peaks in both samples. These results showed that, for each peak, both the molecular ion(s) and the associated DAG ions were identical, *i.e.* in the Jersey and Friesian milkfats analysed, the fatty acid components of the TAGs present remained unchanged in each of the peaks analysed. However, there was a noticeable difference in response of the molecular ion between the two samples. Figure 22 shows (and confirms) that milkfat from Jersey cows is higher in trisaturated TAGs than milkfat from Friesian cows, and that milkfat from Friesian cows is higher in TAGs containing two 18:1 fatty acids than milkfat from Jersey cows.

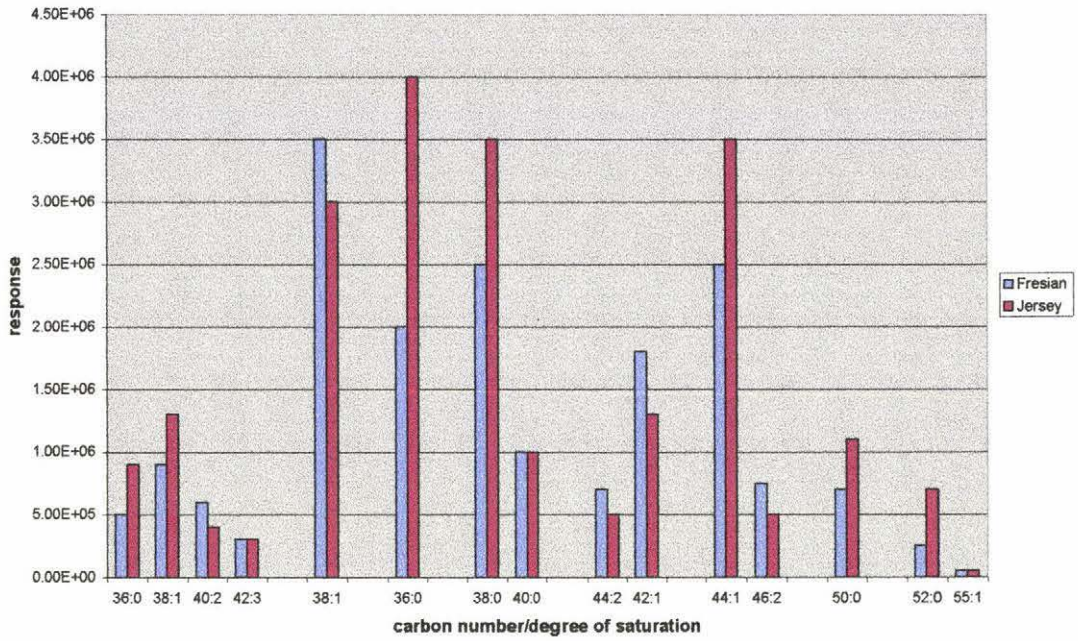


Figure 22. Comparison of Friesian and Jersey milkfats.

Analysis of Milkfats Collected at Different Times During the Season by LC-MS

Four samples of milkfat collected in early spring, spring, summer and autumn were analysed by LC-MS. For each sample, peaks 12, 13/14, 15, 19, 24, 29, 57 and 59/60 from the TIC were selected for examination. Molecular ions and their associated DAG ions were identified in the XICs taken from under each of the above peaks in all four samples. It was found that, for each of the peaks of the four samples of 'seasonal' milkfat analysed, the fatty acid identity of the TAGs present remained unchanged. However, the relative proportions of each of the molecular ions identified under each of the peaks changed in intensity throughout the season (Figure 23).

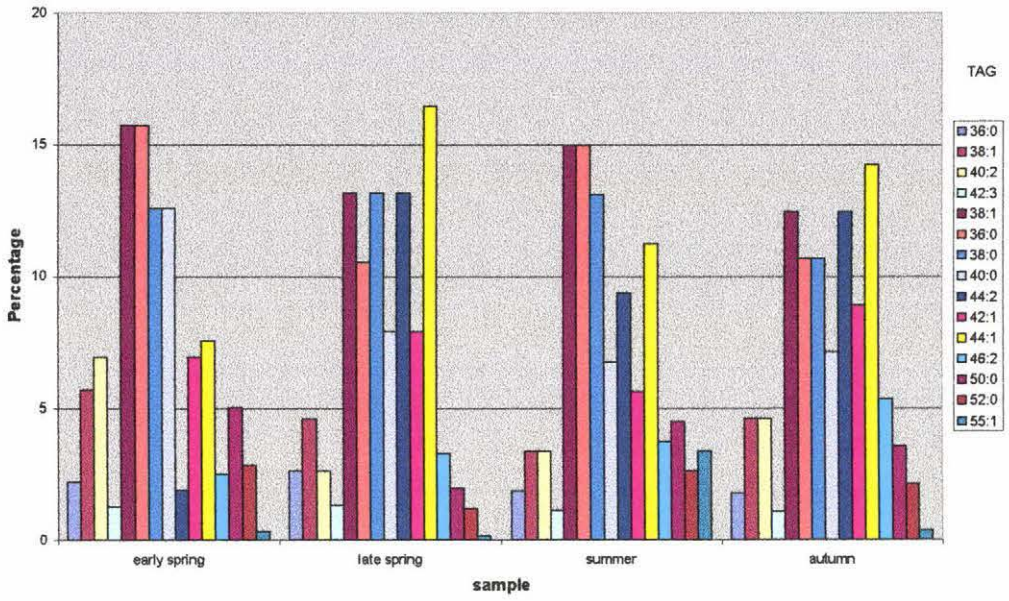


Figure 23. Comparison of milkfats collected at different times of the season.

Generally speaking, the greater the proportion of unsaturated to saturated TAGs in a given fat sample, the 'softer' is the fat. This degree of 'hardness or softness' in a milkfat sample is a very important functional property, and has been traditionally assessed by methods such as SFC by pulsed NMR. The SFC results of the four samples at 10°C (Appendix 10) show that the early spring milkfat is the 'softest', whereas both the late spring and the autumn milkfats, which have very similar SFC results, are 'harder' than the early spring milkfat. The summer milkfat is the 'hardest' of the four samples.

Theoretically electrospray MS has the potential to predict relative degrees of hardness of any given fat sample based on the relative proportions of saturated to unsaturated TAGs found. Figure 24 shows the relative response of the saturated and unsaturated TAGs found under each of the peaks mentioned above, for each of the four samples. The results, compared with those proposed by the SFC data, are mixed.

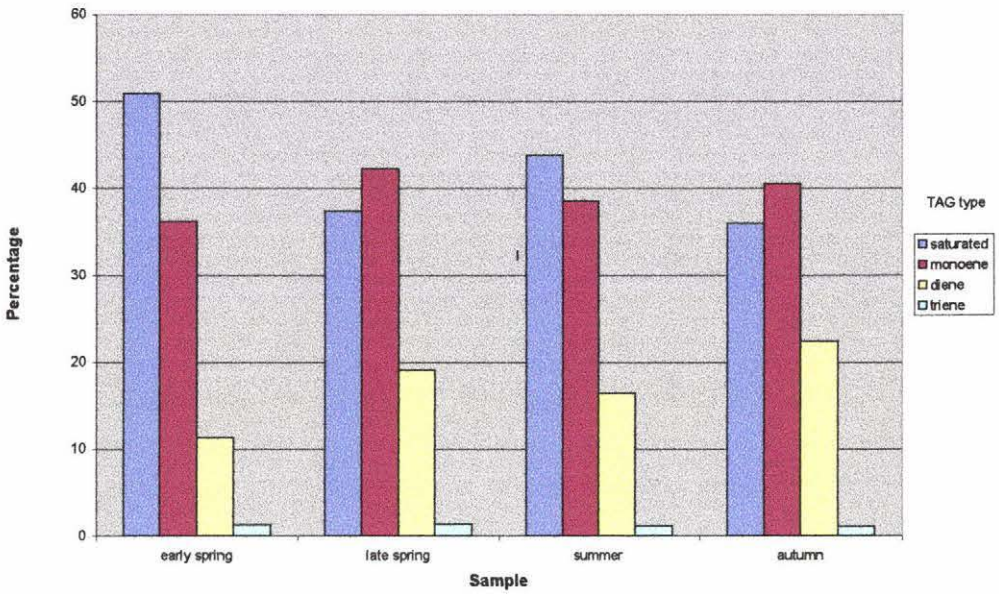


Figure 24. Comparison of milkfat collected at different times of the season.

On the one hand, Figure 24 shows that the early spring milkfat has the greatest proportion of saturated TAGs relative to unsaturated TAGs in the peaks analysed, compared with the other three samples. This suggests that out of the four samples analysed the spring milkfat has the 'hardest' fat whereas the SFC results say the reverse. On the other hand, Figure 24 suggests that the late spring and autumn milkfats have similar hardness properties and are softer than the summer milkfat. These trends agree with those predicted by the SFC results. Of course in this instance the saturated TAGs include those with C4:0 and C6:0 fatty acids, which would contribute to relative softness.

It has to be remembered that, out of the 65 odd peaks seen in the TIC, only nine were analysed for the purposes of this work. It may be that an in-depth analysis of all these peaks may give overall results that agree with SFC and other methods of this type and that this work, although outside the scope of this study, should be done in the future.

However, these results do suggest that, although the potential is there, all peaks need to be considered in assessing the effectiveness and reliability of LC-MS of milkfat to predict functional trends in milkfat.

Analysis of Milkfat from Different Species by LC-MS

Milkfat from different species (human, seal, horse, whale, possum) was analysed by LC-MS. As the TAGs from seal and whale milkfat were thought to contain longer chain fatty acids than those seen with bovine milkfat, the elution gradient on HPLC was extended from 110 min to 132 min in order to ensure a better separation of all the TAGs present. For the sake of consistency, this extended gradient was used to analyse all the milkfats above. Bovine milkfat was also reanalysed using this method, to ensure that the elution pattern of the TAGs was the same as had been obtained previously using the shorter gradient. However, apart from the extended gradient on HPLC, the LC-MS method used previously remained unchanged.

The ELSD (and associated TIC traces) (Appendix 11) obtained from each of the six milkfats analysed were distinctly different. Bovine milkfat gave the most complicated spectra, with TAGs eluting throughout the entire range of the gradient (5-100 min); for the other five milkfats analysed, the majority of TAGs eluted between (in the main) 40 and 100 min.

Although the peak heights of the eluting peaks were distinctly different, the focus of this study was on identifying changes in TAG composition within a single peak. To this end, the TAG composition of six peaks in the TIC traces of all six milkfat samples was identified and these results are presented in Table 5. The identities of the TAGs eluting at 80.14 min were all quite similar if not the same in five species. However, the seal milkfat differed a little, with the presence of an 18:3 fatty acid in one of the TAG species present (which was not seen in the TAGs of other species eluting at this time). Once again, the TAGs eluting at 83.18 min were similar in five species. However, the presence of the $(M+NH_4)^+$ ion 888.8 in the whale milkfat (containing an odd-numbered fatty acid) was particular only to this species. Like the TAGs eluting at 80.14 min, the TAGs eluting at 89.33 min were similar with the exception of seal milkfat, where the presence of a 20:1 fatty acid on one of the TAGs present was not seen in any of the TAGs eluting at 80.33 min in the other milkfats examined. This was seen again in the TAGs eluting at 92.12 min, as well as the

presence of an odd-numbered fatty acid in one of the TAG species in whale milkfat. The TAG species present in the whale and seal milkfats were the 'odd ones out' when comparing the TAGs eluting at 100.5 min. This was due to the presence of long chain (both whale and seal) and odd-numbered fatty acids (whale) which were not seen in the other milkfats analysed. Finally, the TAGs eluting at 41.5 min were quite different when comparing the six different milkfats analysed. Although the human, horse and bovine milkfats all had TAGs eluting at 41.5 min with similar $(M+NH_4)^+$ ions, the fatty acid components of these TAGs were different. Whereas bovine and horse milkfats contained TAGs with fatty acids with relatively low carbon numbers (6:0 and/or 8:0), the corresponding short chain fatty acids seen in human milkfat were either 10:0 or 12:0. The most prominent molecular ion (762.6) seen in the horse milkfat was not identified in either the bovine or the human (or the seal) milkfat and contained 18:3 fatty acids. 18:3 fatty acids were also identified in some of the species of TAGs eluting at 41.5 min in the seal milkfat. The $(M+NH_4)^+$ ions (916.7 and 968.8) eluting at 41.5 min in seal milkfat were not seen in any of the other milkfats. Three different species of TAGs were identified, and they contained some very long chain fatty acids (25:1) and some very long chain and unsaturated fatty acids (20:4, 18:5) not seen in TAGs eluting at this time in the other milkfats analysed.

These results are supported by the FAME results (Appendix 10). However, once again, the importance of the MS was shown by the ability to identify fatty acids not routinely identified using standard bovine milkfat analysis (*e.g.* 18:5) assisted by the increased response due to the presence of multiple double bonds. These results also indicate levels of odd-numbered fatty acids in whale milkfat (not seen in the other milkfats examined), and long chain fatty acids in seal (and to a lesser extent whale) milkfat not seen in human, possum, horse or bovine milkfat. Similarly, short chain fatty acids present in the bovine and horse milkfats were not present in any of the other milkfats examined. However, the MS analysis allowed identification of TAGs.

The analysis of these different milkfats by LC-MS demonstrates the power of this method in lipid analysis. Not only could the carbon number and degree of saturation of the TAGs present in each of the milkfats analysed be identified, but also the fatty acid constituents of each of these TAGs could be identified, and in a timely manner. The analysis of these samples by HPLC alone would not have indicated the TAG differences observed (in the six peaks analysed) between the different milkfats

examined. Similarly, it was difficult to assign an identity to all of the peaks seen on the FAME GC traces (these unidentified peaks presumably being contributed by unusual fatty acids such as 18:5, seen in seal milkfat).

The milkfat of ruminants is characterised by the presence of the short chain fatty acids (4:0, 6:0) and significant saturated fatty acids from biohydrogenation in the rumen. However, most non-ruminants absorb unchanged significant amounts of polyunsaturated fatty acids from the diet. For example, horse milkfat reflects the high c18 polyunsaturated fatty acid of the grass, whereas marine animals subsist on a diet of fish and other sea life and hence have higher proportions of very long chain fatty acids.

Table 5. Significant TAGs within specific peaks from HPLC as analysed by electrospray MS/MS*

Fat	Human		Seal		Horse		Whale		Cow		Possum	
Time (min)	$(M+NH_4)$	TAG identity	$(M+NH_4)$	TAG identity	$(M+NH_4)$	TAG identity	$(M+NH_4)$	TAG identity	$(M+NH_4)$	TAG identity	$(M+NH_4)$	TAG identity
Peak at 80.14	848.7 874.7	18:1/16:0/16:1 18:1/16:0/18:2	848.7 874.8 *900.8* *924.8*	18:1/14:0/18:1 18:1/16:0/16:1 18:1/16:0/18:2 18:0/16:0/18:3 18:1/16:1/18:1	848.7 874.7	18:1/16:0/16:1 18:1/18:2/16:0	848.7 874.8	18:1/16:0/16:1 18:1/18:1/14:0 18:1/18:1/16:1 18:1/18:2/16:0	**848.8** 874.7 *900.8*	18:1/16:0/16:1 18:1/18:1/14:0 18:1/18:1/16:1 18:1/18:2/16:0	848.7 **874.7** *857.7*	18:1/16:0/16:1 18:1/18:2/16:0
Peak at 83.18	822.7 848.7	16:0/16:0/16:1 14:0/16:0/18:1 16:0/16:0/18:2 16:1/16:0/18:1	**822.7** 848.7 *850.7*	14:0/16:0/18:1 18:1/16:1/16:0	822.7 848.7	14:0/16:0/18:1 16:0/16:0/16:1 16:0/16:0/18:2 16:1/16:0/18:1	**822.7** **888.8** *848.7*	15:0/15:0/18:1 14:0/16:0/18:1 18:1/17:1/18:1	**822.7** 848.7 *874.7*	16:0/16:0/16:1 14:0/16:0/18:1 16:0/16:0/18:2 16:1/16:0/18:1	822.7 **848.7** *874.8*	16:0/16:0/16:1 18:2/16:0/16:0
Peak at 89.33	876.7	18:1/18:1/16:0	876.7	18:1/18:1/16:0 20:1/16:1/16:0	876.7	16:0/18:1/18:1	**876.7** *902.8*	16:0/18:1/18:1	**876.7** 902.8	18:1/18:1/16:0 18:1/18:1/18:1	876.7	16:0/18:1/18:1
Peak at 92.12	850.7 876.7	18:1/16:0/16:0 18:1/16:0/18:1	850.7 876.7	18:1/16:0/16:0 20:1/14:0/16:0 18:1/16:0/18:1	850.7	18:1/16:0/16:0	850.7 876.7 890.7	18:1/18:0/14:0 18:1/16:0/16:0 18:1/16:0/18:1 19:1/18:1/16:0	**850.7** *876.7*	18:1/18:0/14:0 18:1/16:0/16:0	**850.7** 876.7 *878.7*	18:1/16:0/16:0 18:1/18:1/16:0
Peak at 100.5	878.8	18:0/18:1/16:0	878.8	20:1/16:0/16:0 18:1/18:0/16:0	878.8	18:0/18:1/16:0	**878.8** *904.8* *906.8*	20:1/16:0/16:0 17:0/17:0/18:1 15:0/17:0/20:1 19:0/18:1/15:0	**878.8**	18:0/18:1/16:0	Not present	
Peak at 41.5	**736.6** 710.5	18:2/12:0/12:0 18:1/10:0/12:0 16:1/12:0/12:0	**916.7** **968.8**	16:0/18:3/21:0 23:0/18:5/18:0 25:1/20:4/14:0	**762.6** 710.6 736.6 840.6	18:3/18:0/6:0 18:1/16:0/6:0 18:1/18:1/6:0 18:3/18:3/14:0	Not present		**710.6** 684.7 736.5	18:1/16:0/6:0 16:0/14:0/8:0 16:0/12:0/10:0 18:1/18:1/6:0	Not present	

* Note, no positional information should be assumed unless specifically stated.

4 Conclusions

Electrospray MS proved to be an effective method for the analysis of polar and non-polar lipids.

The analysis of phospholipid, sphingomyelin and sphingosine standards by infusion MS gave good results. Identification of the phospholipid species and the fatty acids esterified to the glycerol backbone was straightforward, and could be achieved by negative ion MS with the solvent system 1,2 dichloroethane:acetonitrile:formic acid (63:35:2), 10 mM ammonium acetate. However, it must be pointed out that this work was carried out only with standards. Although these initial results look promising, more work needs to be carried out with 'real' phospholipid fractions to fully assess the effectiveness of the method. It may also be possible to analyse the phospholipid concentration in milkfat fractions quantitatively, although an LC-MS method may well need to be developed to do this. This may well prove very useful, particularly as current quantitative methods used to analyse phospholipids at the NZDRI do not give consistent results.

The analysis of ganglioside standards gave mixed results. The response of many of the ganglioside molecular ions was low, and the molecular weights obtained were not always those expected from either the known structure or results reported in the literature. As this area looks as if it will become important in the future, more work should be carried out, in the first instance with ganglioside standards preferably from a bovine source.

Electrospray MS proved to be a very effective way of analysing TAGs. Both TAG standards and simple mixtures of TAGs could be analysed very effectively using the solvent system 1,2 dichloroethane:acetonitrile:formic acid (63:35:2), 10 mM ammonium acetate and infusion MS. The fatty acid component of ammoniated standard TAG ions could be determined by front end fragmentation (which gave ample DAG ions, and therefore the fatty acid components could be determined by difference), or by MS/MS of DAG ions which resulted in the formation of acylium ions (and hence the fatty acid components could be categorically identified). However, the fatty acid components of ammonium-TAG ions found in simple

mixtures had to be identified by MS/MS of each of the parent ions, which gave ample quantities of DAG ions (and the fatty acid components could be determined by difference). On some occasions, for low molecular weight TAGs, the acylium ions could also be identified in the spectra.

Response differences of ammoniated TAG ions, based on carbon length and degree of saturation were observed. It was found that, the greater the carbon number, the lower was the response. Similarly, response differences of DAG ions (produced during front end fragmentation of a TAG molecule) based on the relative positions of fatty acids on the glycerol backbone were also observed. The response of DAG ions with fatty acid groups on either positions 1 and 2, or 2 and 3 of the glycerol backbone was always found to be greater than that of DAG ions with fatty acid groups on positions 1 and 3 of the glycerol backbone. It was possible, therefore, to determine which fatty acid was esterified to the second carbon of the glycerol backbone.

Although it was possible to analyse complex lipid mixtures by infusion MS, it was far easier and a lot quicker to intepret the spectra if the TAG mix was separated chromatographically prior to entry into the mass spectrometer (LC-MS). Like infusion MS, the TAG response seen in the TIC of a given milkfat sample varied depending on the length of the eluting TAG and its degree of saturation.

This work is the first describing electrospray MS and LC-MS of bovine milkfat. It will prove useful in

- identifying of TAGs within peaks,
- identifying the proportion of different carbon numbers within the same peak, and
- initial positional information.

As such, this work has shown the usefulness of electrospray MS in the analysis of lipids, has laid a quantitative basis for future investigations and has initiated specific structure determination of milkfats from a wide range of sources.

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Appendix 1 Instrument

Operation of the Mass Spectrometer

In order to ensure accurate and efficient data collection, a sample specific-state file and an experiment file must be generated. The machine must also be calibrated prior to sample infusion. The generation of a state file, an experiment file and a calibration file is described below.

Components of the PE Sciex API300 Mass Spectrometer and Generation of a 'State File'

Components of the PE Sciex API300 mass spectrometer are described below. This desciption gives an overview of the ion path through the mass spectrometer as well as parameters (voltages and gases) that need to be defined, often according to both the mass and the type of sample being analysed. Once defined, these voltages (specifically IS, OR, RNG, Q0, IQ1, ST, RO1, IQ2, RO2, IQ3, RO3, DF and CEM) and gases (NEB, CUR and CAD) are stored in what is called a state file, which can remain largely unchanged throughout the duration of the experiments.

Sample inlet

The sample is dissolved in an appropriate solvent, sometimes with other additives prior to infusion. Sample is directly infused via a capillary into the ionisation region of the mass spectrometer. This capillary can also be connected to an HPLC instrument.

Ion source

The electrospray source converts liquid phase into gas phase ions.

Ion spray performance depends on the following factors:

- Sprayer position
- Ion spray voltage (this value should be ramped between 3500 and 6000 V in positive ion mode and between -3000 and -5000 V in negative ion mode to obtain an optimum value)
- Nebuliser gas
- Curtain gas (discussed below)
- Orifice and ring voltages (discussed below)

- Sprayer fused silica line (this needs to be cut daily, and replaced on an 'as-required basis')
- Sample solvent composition / flowrate (if sample is flowing into the ionisation chamber via an HPLC instrument)
- Source exhaust pump

The vacuum interface

This interface separates the low pressure vacuum chamber from the atmospheric ion source. The purpose of this interface is to allow the transfer of ions into this vacuum chamber, while restricting the entry of sample, solvent and ambient air. This is accomplished using a gas curtain of dry nitrogen (CUR), which assists in breaking ion clusters and separating the sample ions from the solvent molecules. Nebuliser gas (NEB) and curtain gas (CUR) are optimised 6 monthly by the instrument engineer.

The operator can adjust the ion flow by varying the voltages applied to the orifice plate (containing a small orifice through which the ions and a small volume of curtain gas must pass before entering the vacuum chamber) and the focusing ring. The orifice plate and focusing ring voltages (OR and RNG respectively) need to be optimised according to the type and the mass of sample being analysed respectively, *i.e.* OR is compound dependent and RNG is mass dependent (the voltage must be increased over the mass range scanned). Ideally these voltages should be set high enough to reduce chemical noise, but low enough to avoid fragmentation. (Note: sometimes 'front end' fragmentation can be useful for structural determination.)

High pressure entrance quadrupole

The high pressure entrance quadrupole is an RF-only quadrupole, which has no filtering effect. This quadrupole focuses and transfers ions from the vacuum interface to the stubbies. Q0 is the voltage applied to this high pressure entrance quadrupole. Q0 is typically set at -10 V and almost all other lens parameters are set relative to Q0. It is possible to link Q0 to IQ1, ST and RO1 (discussed further on) using the parameter configuration table under the menu heading 'view' in the PE SCIEX API300 mass spectrometer software.

Inter-quadrupole lens

This lens is used to focus the ion stream from the high pressure entrance quadrupole to Q1

(discussed below), and also helps maintain peak shape. IQ1 is the voltage applied to this lens and typically IQ1= Q0 - 1 V.

Prefilters (stubbies)

A stubby is the prefilter to the first resolving quadrupole, and affects the focusing and transmission of ions into this quadrupole. ST is the voltage applied to the stubbies and typically ST = Q0 - 3 V. ST is independent of ion mass and energy.

First resolving quadrupole (Q1)

Q1 is a DC and RF mass filter, which selectively filters ions, based on their m/z ratio. RO1 is the voltage applied to the first resolving quadrupole and typically RO1 = Q0 - 1 V, in both single and MS/MS mode. This offset determines the ion energy (in this quadrupole) which affects resolution and sensitivity.

Inter-quadrupole lens 2

The inter-quadrupole lens 2 focuses the ions into the second quadrupole (Q2). IQ2, the voltage applied to the inter-quadrupole lens 2, is mass dependent.

In MS/MS mode, generally IQ2 = RO2 - 5 V (RO2 is discussed below). Setting IQ2 more positive than RO2 will increase the sensitivity, but will also induce 'cross talk', which should be avoided.

Collision cell quad (Q2)

Q2 is an RF-only quadrupole, which has no filtering effect. RO2 is the voltage applied to Q2.

In single MS mode, Q2 simply transmits the ions from Q1 towards the detector. In these circumstances, RO2 is usually fixed between -20 and -40 V.

In MS/MS mode, the potential difference between RO2 and Q0 is the collision energy (which is compound dependent). RO2 should be linked to IQ2, IQ3 and RO3 using the parameter configuration table in the mass spectrometer software. This will reduce 'cross talk' and maintain proper ion transmission through Q3.

Inter-quadrupole lens 3

In single MS mode, the inter-quadrupole lens 3 has no effect on peak shape or sensitivity, as long as the voltage applied to the lens, IQ3, it is kept between -100 and -200 V.

In MS/MS mode, IQ3 is mass dependent and, although IQ3 is set relative to RO2, its offset is dependent on the fragment ion mass. In general, IQ3 = RO2 - 25 V to -30 V is said to provide good sensitivity for all fragments when acquiring MS/MS fragment ion spectra.

Last resolving quadrupole (Q3)

Q3 is a DC and RF mass filter, which selectively filters ions based on their m/z ratio.

In single MS mode, Q3 has no effect on peak shape or sensitivity, as long as RO3, the voltage applied to Q3, is kept between -100 and -200 V.

In MS/MS mode, RO3 is set relative to RO2, and its offset determines the ion energy in the third quadrupole, which affects the resolution and sensitivity of the fragment ions. In general, RO3 = RO2 - 2 V to -5 V.

Deflector

The voltage applied to the deflector (DF) has a preset optimum which should not be changed.

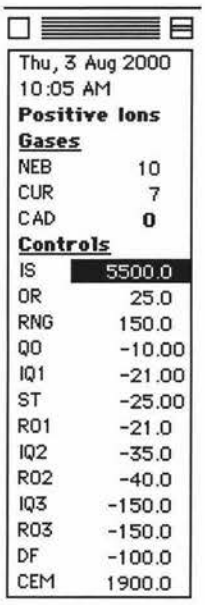
Channel electron multiplier (CEM)

Ions are drawn from the mass filters into the ion detector (CEM). When struck by an ion, the CEM emits an electron pulse. The CEM signal is converted to a digital signal, which is processed at the system controller to provide an absolute ion count. The CEM degrades with time and continued use, and must be reoptimised periodically. The service engineer, on a 6 monthly basis, usually does this.

CAD gas

The CAD gas is the target gas in the collision cell. Collisions between the ions speeding along the ion path and the CAD gas molecules in the collision cell provide the energy for ion dissociation.

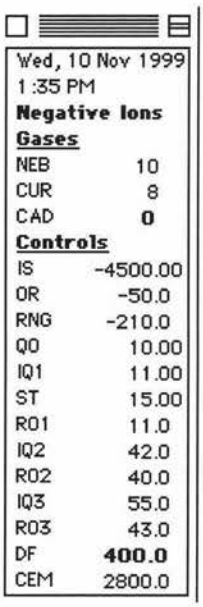
The CAD gas is set at zero for MS applications. For MS/MS applications, the CAD gas must be optimised according to the type of compound being fragmented.



Thu, 3 Aug 2000
10:05 AM
Positive Ions

Gases	
NEB	10
CUR	7
CAD	**0**
Controls	
IS	5500.0
OR	25.0
RNG	150.0
QO	-10.00
IQ1	-21.00
ST	-25.00
RO1	-21.0
IQ2	-35.0
RO2	-40.0
IQ3	-150.0
RO3	-150.0
DF	-100.0
CEM	1900.0

TAG state file positive ion



Wed, 10 Nov 1999
1:35 PM
Negative Ions

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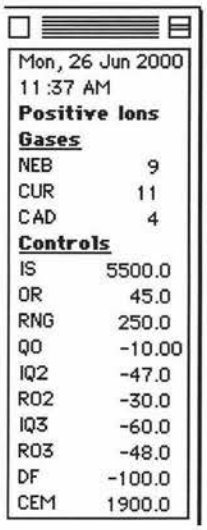
NEB	10
CUR	8
CAD	**0**
Controls	
IS	-4500.00
OR	-50.0
RNG	-210.0
QO	10.00
IQ1	11.00
ST	15.00
RO1	11.0
IQ2	42.0
RO2	40.0
IQ3	55.0
RO3	43.0
DF	**400.0**
CEM	2800.0

Phospholipid statefile negative ion



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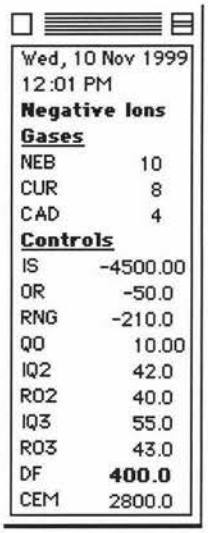
11:37 AM
Positive Ions

Gases	
NEB	9
CUR	11
CAD	4
Controls	
IS	5500.0
OR	45.0
RNG	250.0
QO	-10.00
IQ2	-47.0
RO2	-30.0
IQ3	-60.0
RO3	-48.0
DF	-100.0
CEM	1900.0

MS/MS statefile positive ion



Wed, 10 Nov 1999
12:01 PM
Negative Ions

Gases	
NEB	10
CUR	8
CAD	4
Controls	
IS	-4500.00
OR	-50.0
RNG	-210.0
QO	10.00
IQ2	42.0
RO2	40.0
IQ3	55.0
RO3	43.0
DF	**400.0**
CEM	2800.0

MS/MS statefile negative ion

Calibration of the Mass Analysers Q1 and Q3, and Generation of a Calibration File(s)

The mass filters should be calibrated on a regular basis by calling up the appropriate state and experiment files (as set up by the instrument engineer) and infusing a standard solution, the PPG standard solution, using the ion spray source. The PPG standard solution can be used to calibrate the mass spectrometer in both positive and negative ion mode (in general, a positive ion calibration will be sufficient for negative ion mode).

Eight PPG ions, of known mass, are used for Q1 and Q3 calibration in the positive and negative ion mode respectively. Usually a difference between known mass and mass obtained, for each PPG ion, of 0.05 or less is not considered to be significant.

The peak widths of all the ions specified in the experiment file (below) should be 0.7 or less. In order to ensure this, it is may be necessary to change the offset values of one or more of the four mass regions specified in the resolution window of the PPG state file. (The offset value is a setting for the mass filter DC voltage adjustment, needed to resolve ions and achieve a constant peak width over the entire mass range.) It is important that these offset values are transferred across to the resolution window of any other state file that is to be used while the calibration remains valid (unchanged).

Once the machine is calibrated, the command 'add to Q1/Q3 calibration' is used to update the calibration files. The frequency of instrument calibration will depend on the mass accuracy required. Generally for accurate mass measurements, smaller molecules such as TAGs require less frequent calibration (weekly) than proteins and peptides (daily).

Generation of an Experiment File

An experiment file contains the information required by the instrument to perform a scan of a single sample (see Figure 4). The experiment file is where the type of scan required, *e.g.* a Q1 scan or a product ion scan (MS/MS of an ion of known mass), is selected. Seven parameters are also defined in the experiment file.

Mass range

This defines the start and stop masses to be scanned. The mass range can be entered as a mass range, or as a centre mass with a specified mass width.

Parameter scan range

This can be used to increment mass dependent parameters (*e.g.* RNG and IQ2 voltages) as a function of mass. The parameter is incremented linearly over the mass range. This is particularly useful when scanning a wide mass range. Values for any parameters defined here overide those same values defined in the state file.

Step size

The step size is the increment in atomic mass units by which the mass is incremented for each scan in the specified mass range.

Mass defect

Mass defect allows the user to increment the step size by a non-integer value.

Duration

The scan duration is expressed in seconds and is related directly to the number of scans indicated in the scan field, to the dwell time and to the step size.

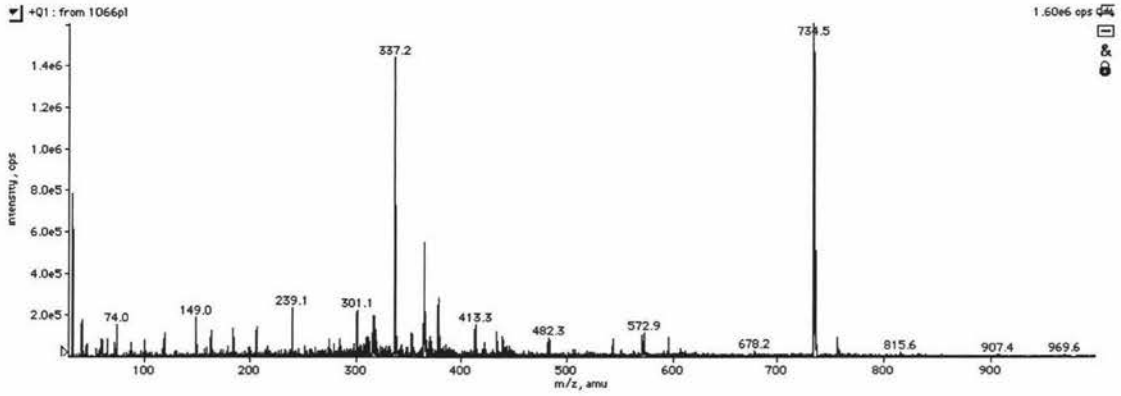
Dwell time

The dwell time is the time allotted for acquiring data at each step over the mass ranges, expressed in milliseconds.

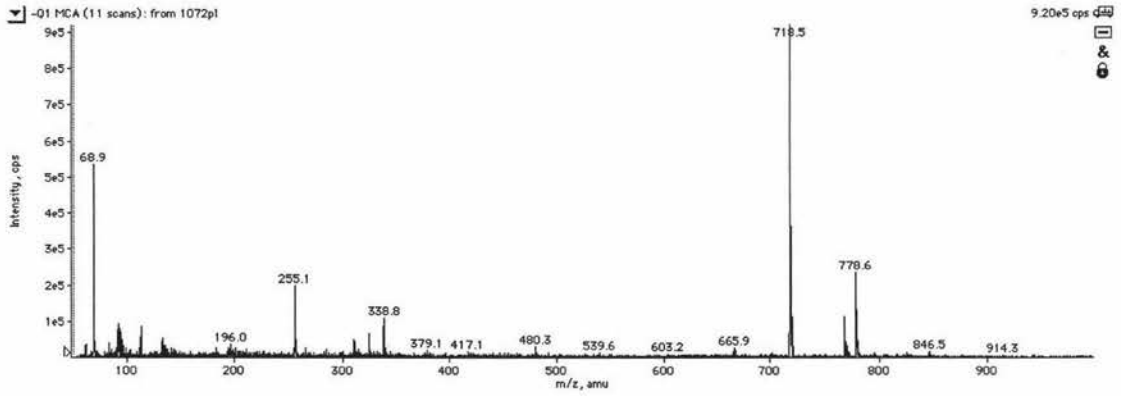
Pause time

The pause time is the time, specified in milliseconds, that is added at the beginning of each mass range specified in the experiment.

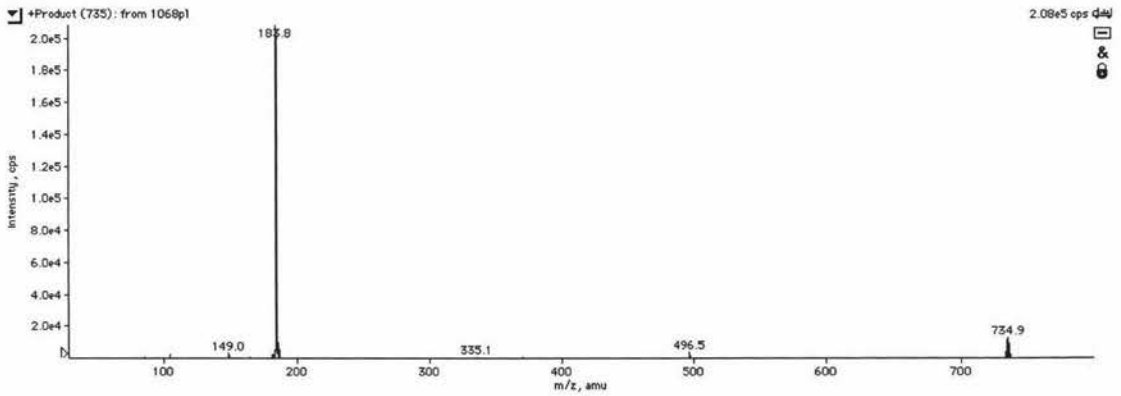
Appendix 2 Mass Spectra of 1,2 Dipalmitoyl sn Glycero-3-phosphocholine



a. 1,2 Dipalmitoyl sn glycero-3-phosphocholine positive ion MS.

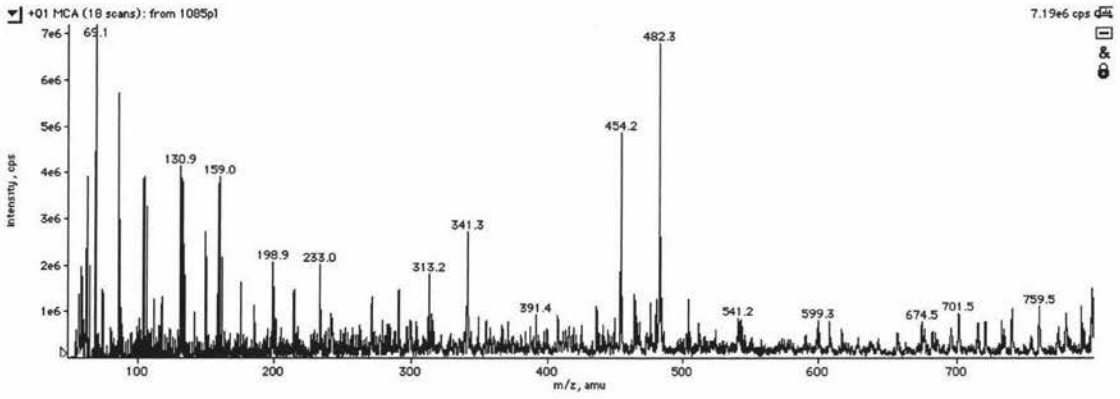


b. 1,2 Dipalmitoyl sn glycero-3-phosphocholine negative ion MS.

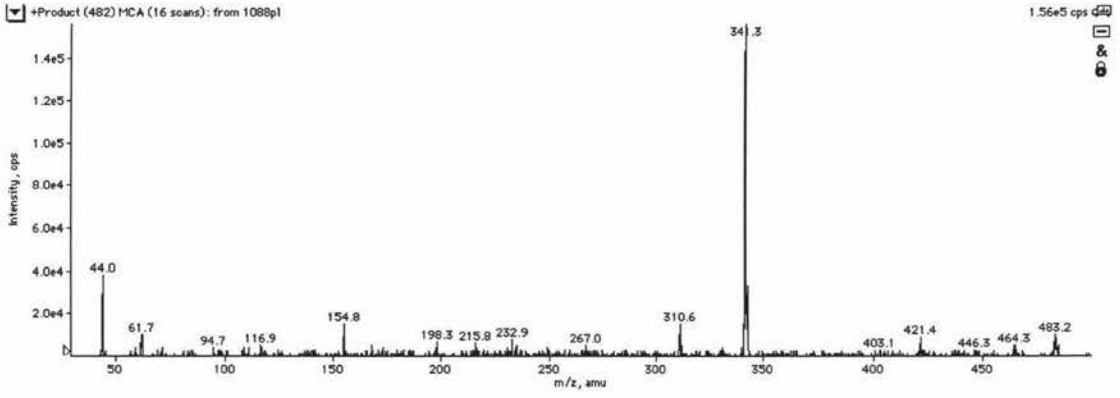


c. 1,2 Dipalmitoyl sn glycero-3-phosphocholine positive ion MS/MS (734.5).

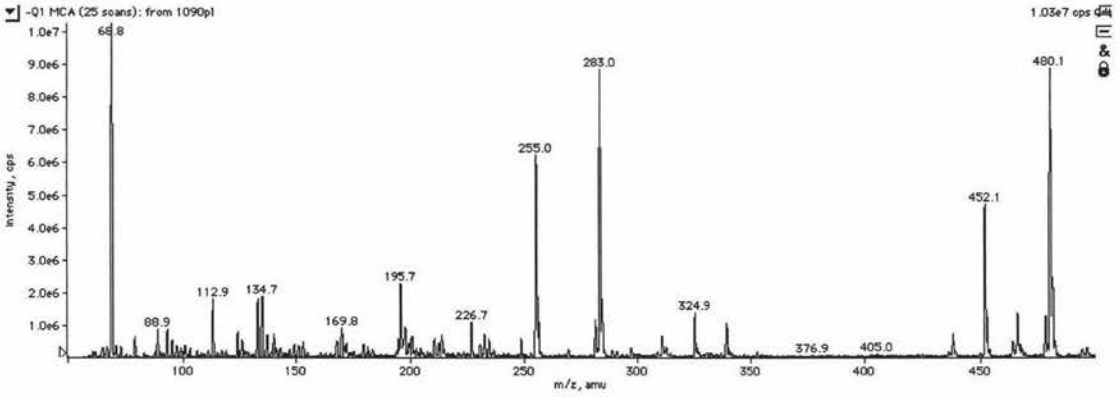
Appendix 3 Mass Spectra of L-α-Lysophosphatidoyl Ethanolamine



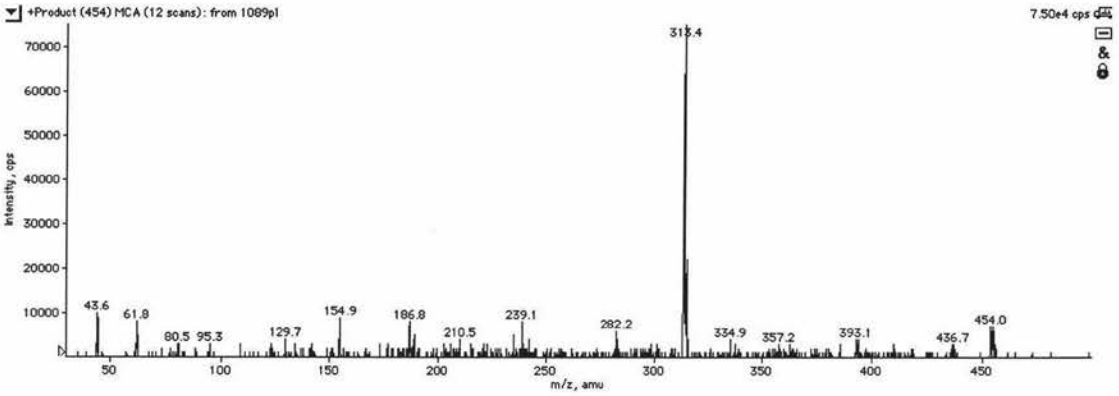
a. L α-Lysophosphatidoyl ethanolamine positive ion MS.



b. L α-Lysophosphatidoyl ethanolamine positive ion MS/MS (482.3).

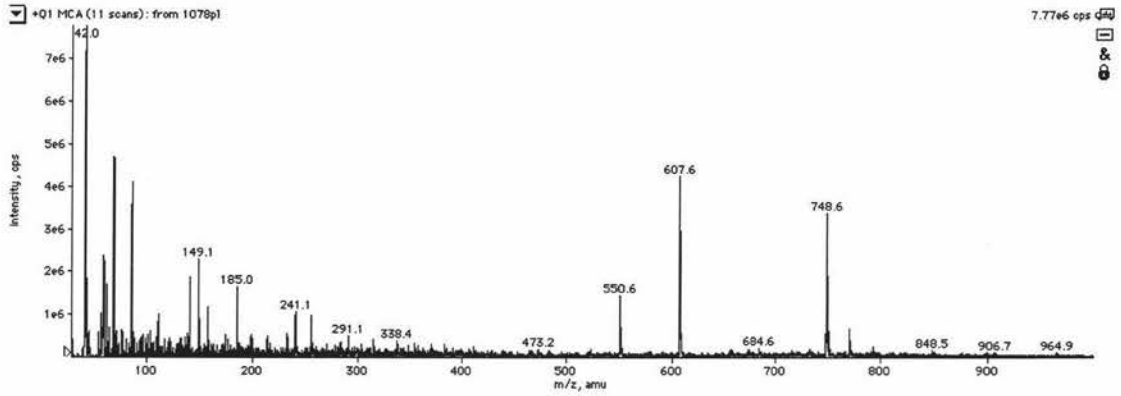


c. L α-Lysophosphatidoyl ethanolamine negative ion MS.

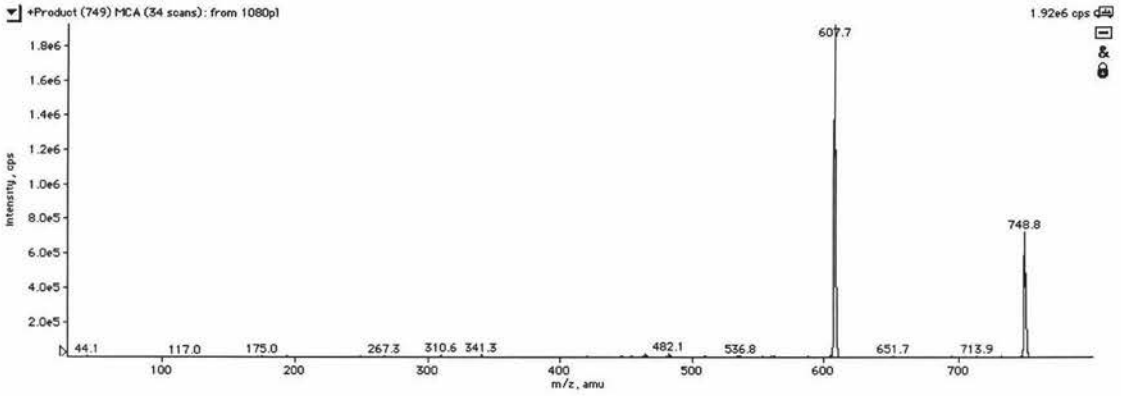


d. L α-Lysophosphatidoyl ethanolamine positive ion MS/MS (454.0).

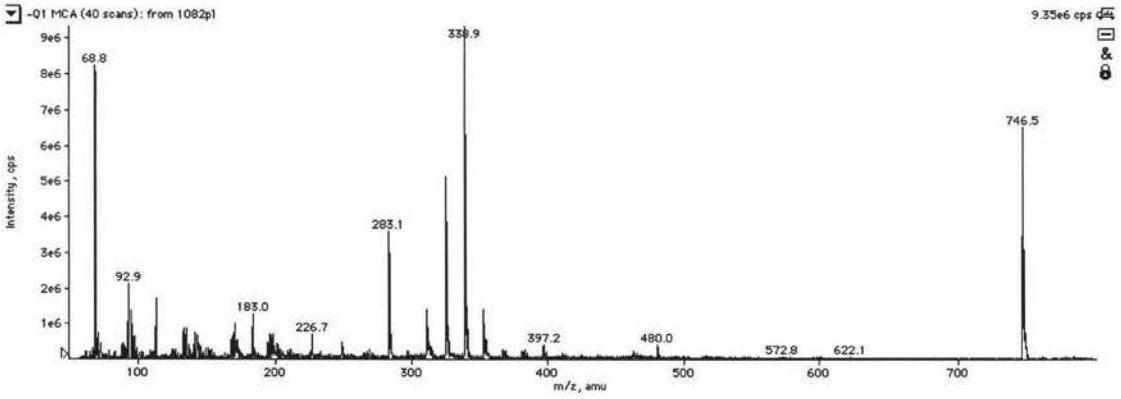
Appendix 4 Mass Spectra of 1,2 Distearoyl sn Glycero-3-phosphoethanolamine



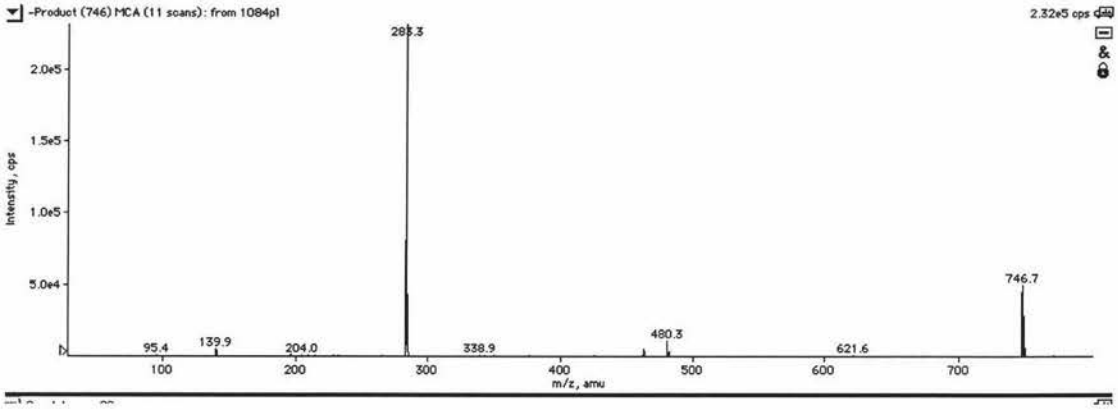
a.1,2 Distearoyl sn glycero-3-phosphoethanolamine positive ion MS.



b.1,2 Distearoyl sn glycero-3-phosphoethanolamine positive ion MS/MS (748.6).

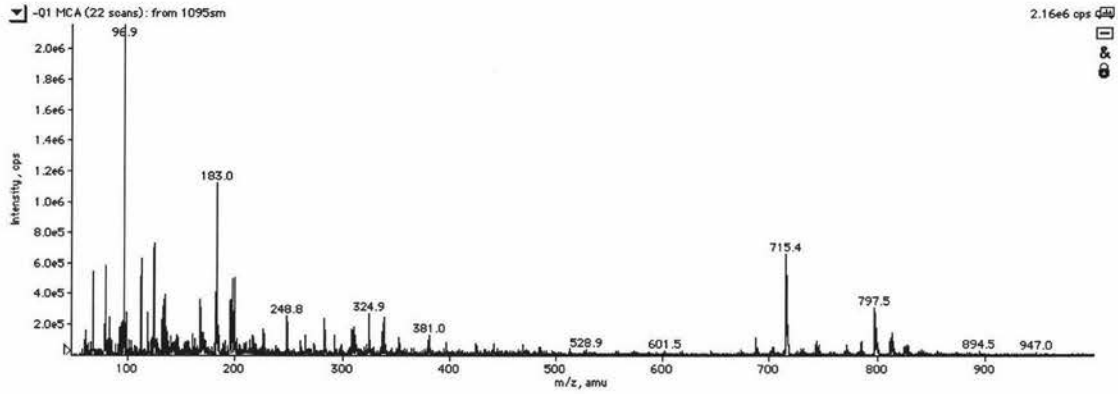


c. 1,2 Distearoyl sn glycero-3-phosphoethanolamine negative ion MS.

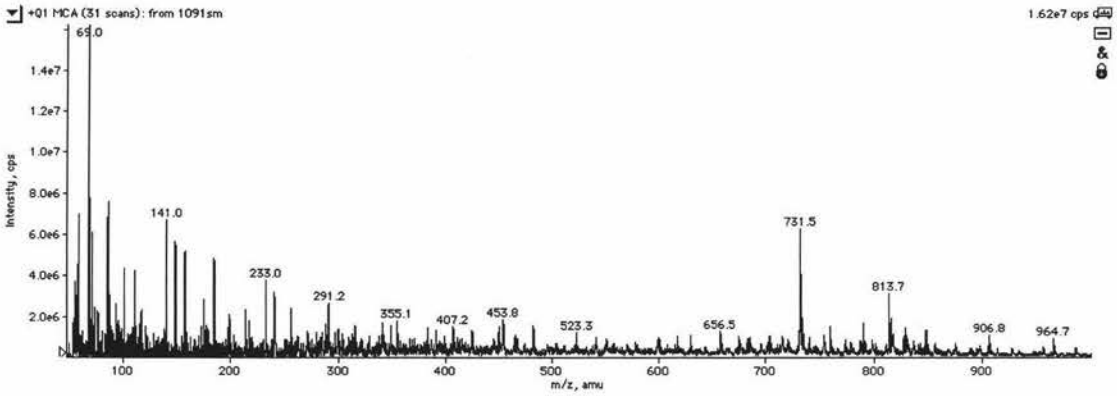


d. 1,2 Distearoyl sn glycero-3-phosphoethanolamine negative ion MS/MS (746.7).

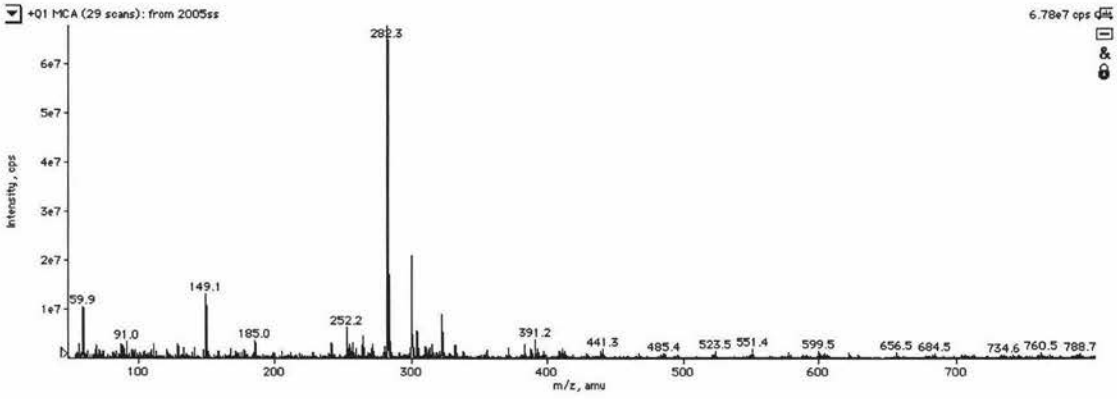
Appendix 5 Mass Spectra of Sphingomyelin/Sphingosine



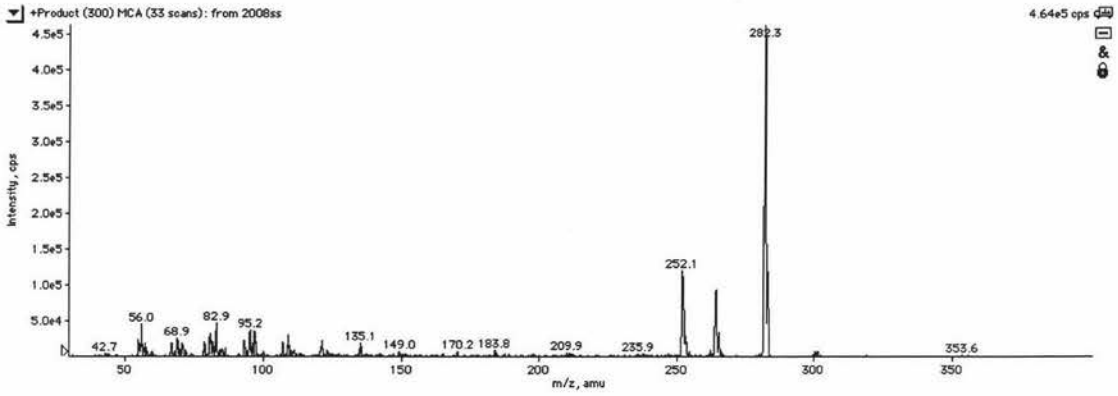
a. Sphingomyelin negative ion MS.



b. Sphingomyelin positive ion MS.

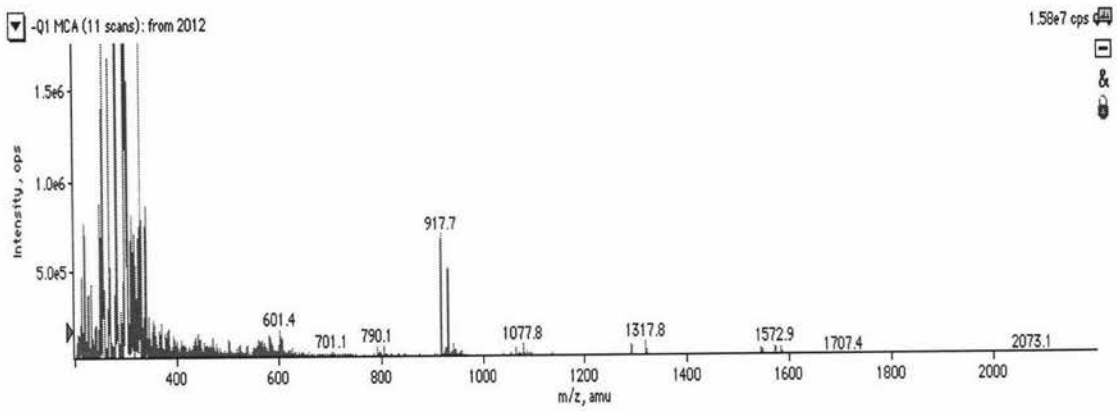


c. Sphingosine positive ion MS.

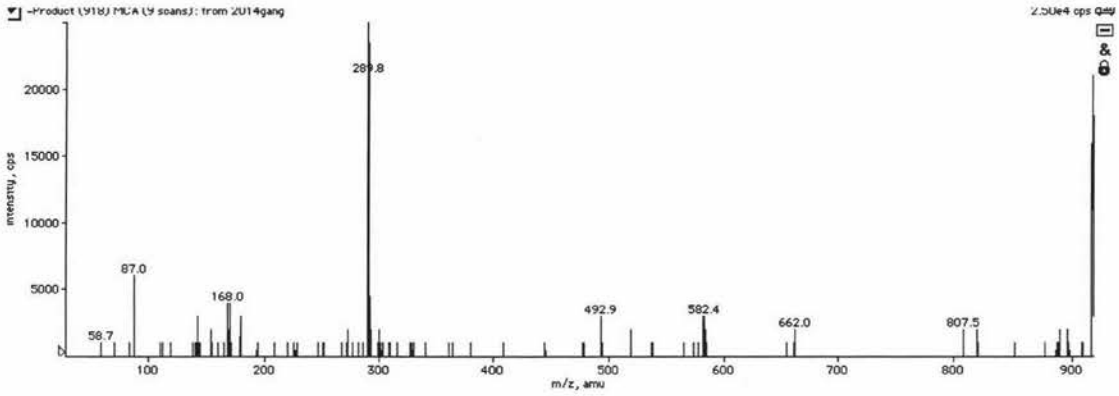


d. Sphingosine positive ion MS/MS (300).

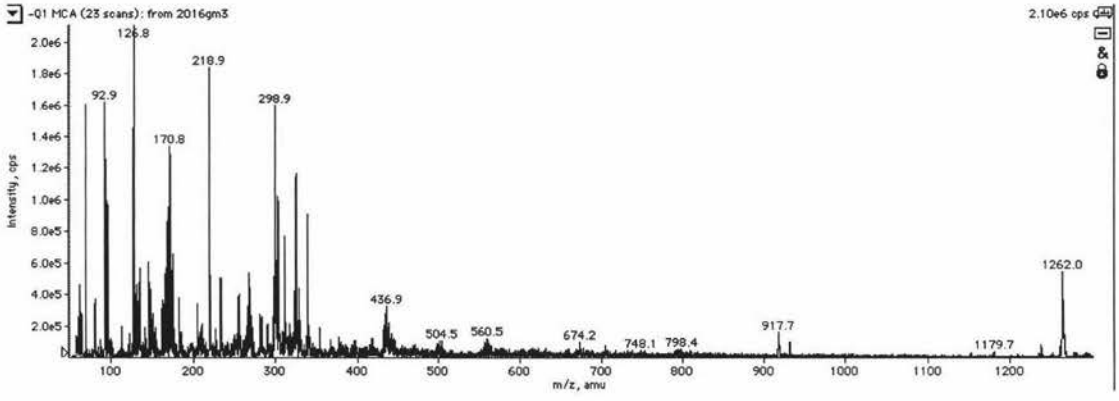
Appendix 6 Mass Spectra of Gangliosides



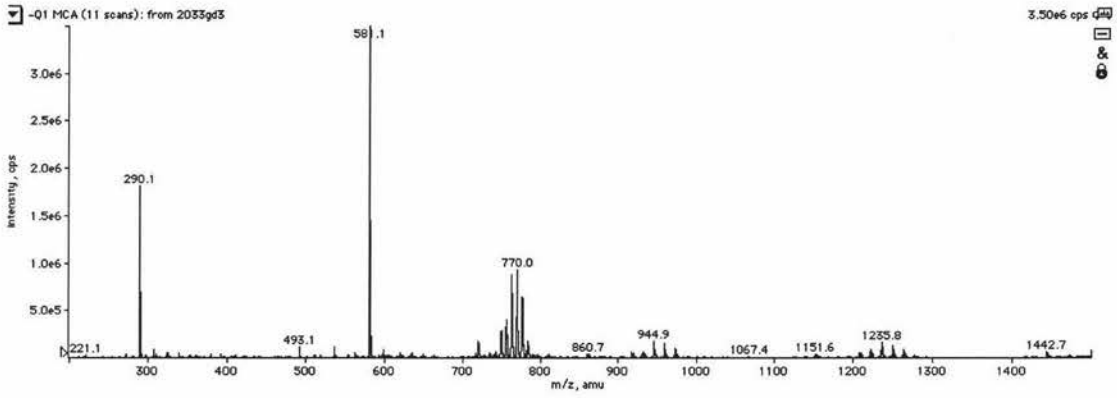
a. Ganglioside mix negative ion MS.



b.Ganglioside mix negative ion MS/MS (917.7).

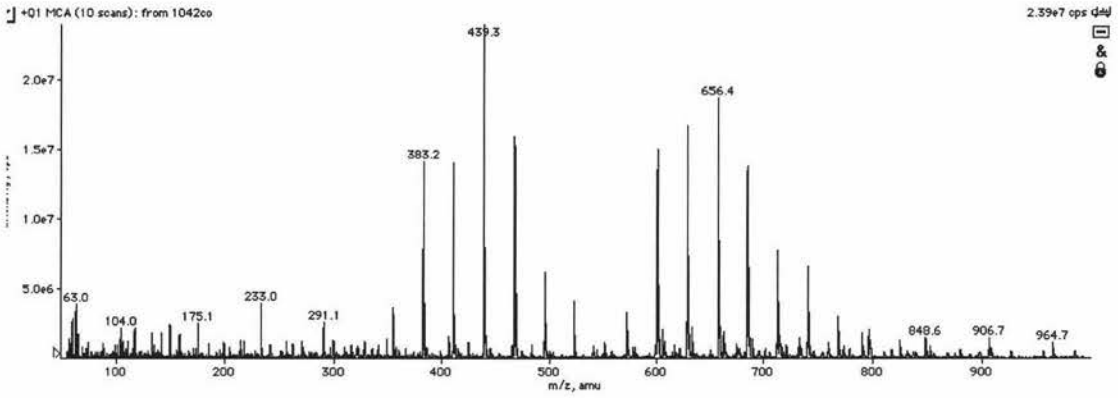


c. GM3 negative ion MS.

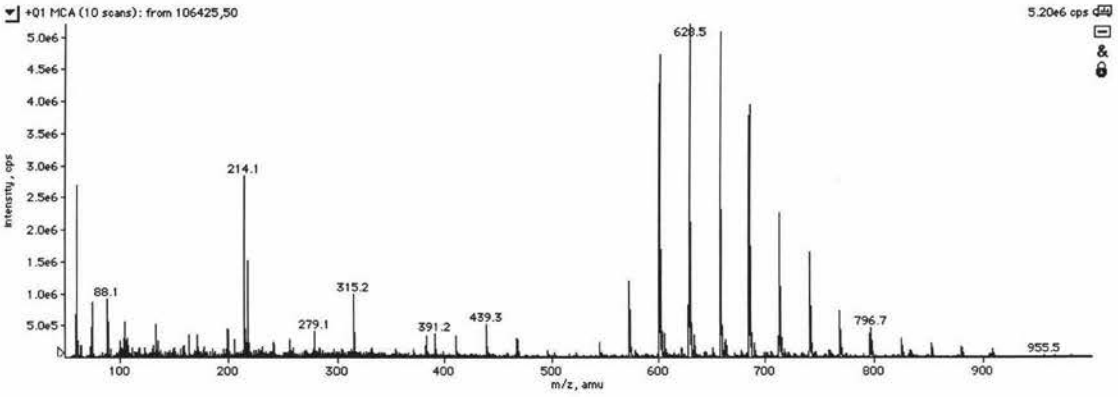


d. GD3 negative ion MS.

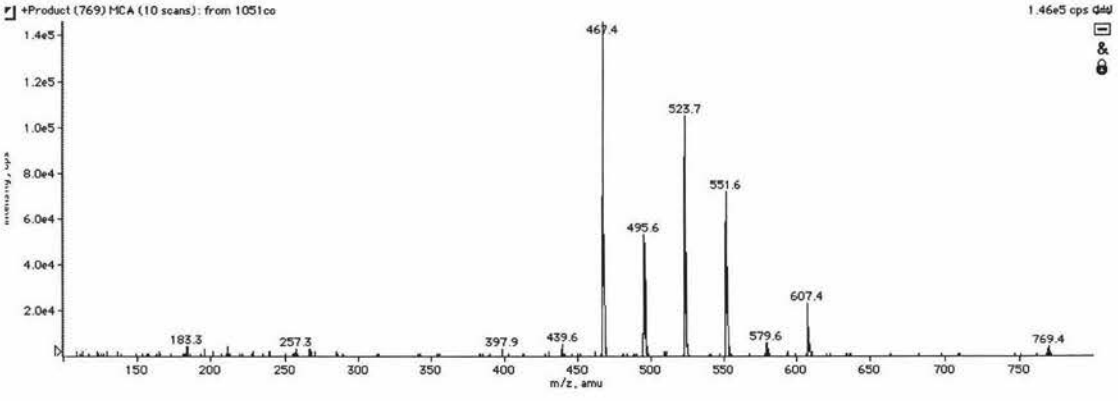
Appendix 7 Mass Spectra of Coconut Oil and ELSD Trace of Coconut Oil



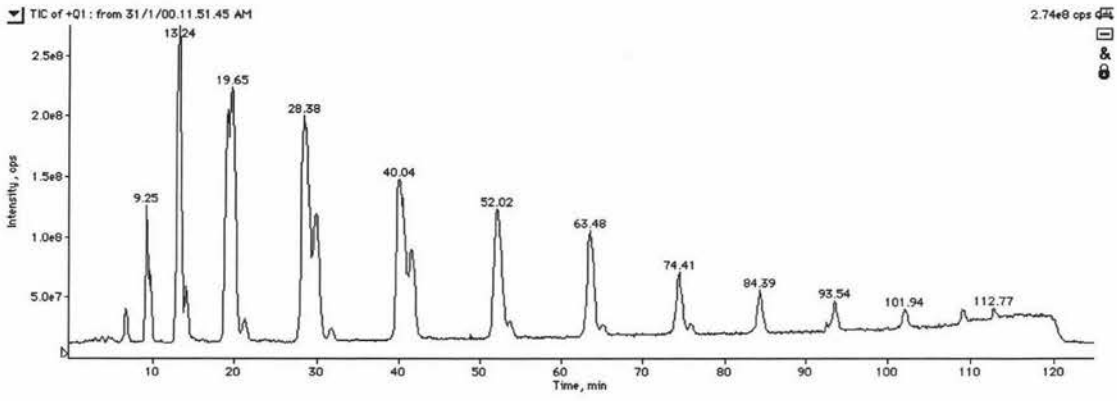
a. Coconut oil, infused, positive ion MS. OR = 25 V, RNG = 150 V.



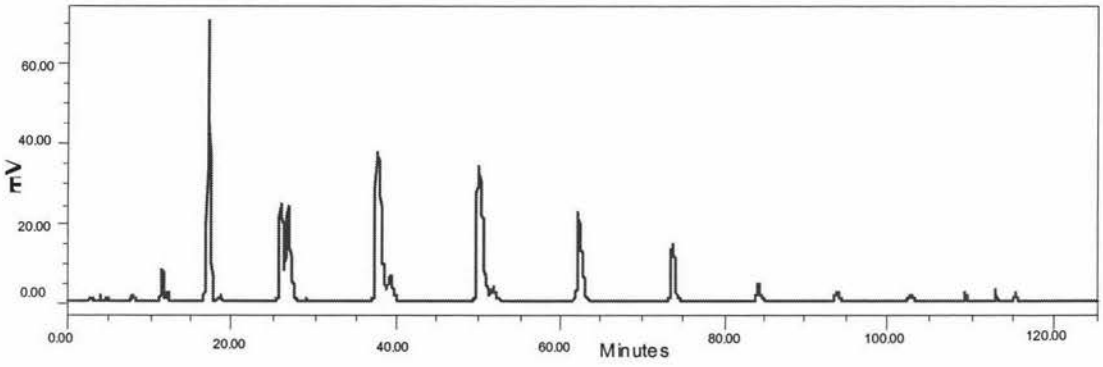
b. Coconut oil, infused, positive ion MS. OR = 25 V, RNG = 50 V.



c. Coconut oil, infused, positive ion MS/MS (769.4).



d. Coconut oil, positive ion LC-MS.

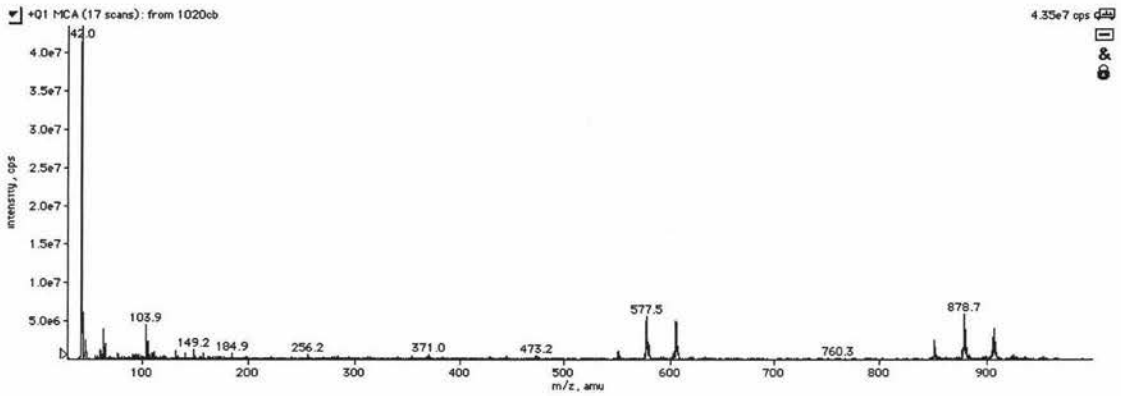


e. Coconut oil ELSD trace.

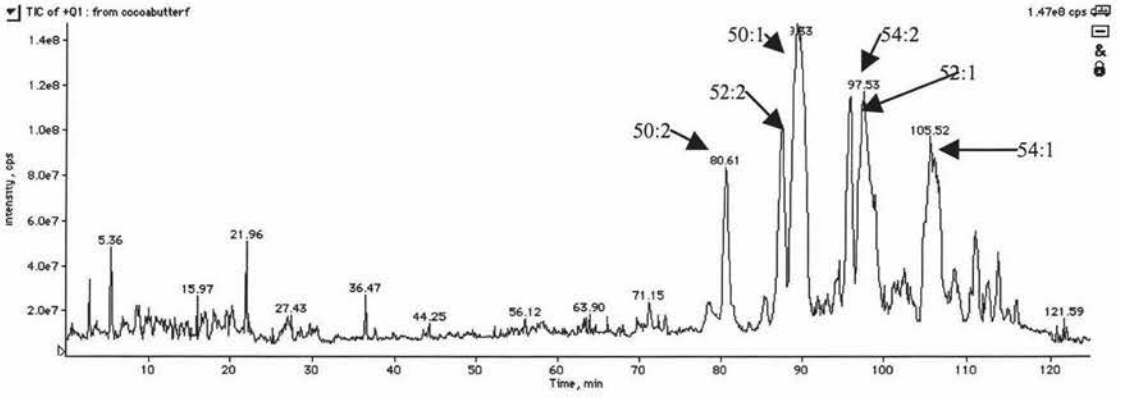
Molecular ion	DAG ions seen	Carbon no		Fatty acid loss (by difference)	Suggested TAG present
572.5	453.7	29:00			
	411.3			8:0	12/08/10
	383.3			10:0	
	355			12:0	
600.5	383.2	32:00		12:0	12/10/10
	439.2			8:0	12/08/12
	411.3		min	10:0	14/06/12
	453.7				
	467.3		min	6:0	
	355.1				
628.6a	411.3	34:00		12:0	14/10/10 min
	439.4			10:0	12/10/12
	383.2		min	14:0	14/8/12 min
	453.7		min		
	467.4		min	8:0	
628.6b	383.2	34:00		14:0	14/08/12
	411.3			12:0	10/12/12
	467.5			8:0	
	439.4		min	10:0	
656.5a	411.3	36:00	min	14:0	12/10/14
	439.4			12:0	12/12/12
	453.7				8/14/14
	467.3			10:0	
	495.6		min	8:0	
656.5b	383.3	36:00	min	16:0	8/14/14
	411.3			14:0	8/12/16
	439.3			12:0	14/12/10
	453.7				10/16/10
	495.5			8:0	
	449.7				
	467.5		min	10:0	
684.7a	411.3	38:00	min	16:0	12/12/14
	439.4			14:0	10/14/14
	453.7				16/12/10
	467.4			12:0	
	495.5		min	10:0	
	523.5		min	8:0	
684.7b	383.2	38:00		18:0	12/18/08
	453.7				8/16/14
	467.5			12:0	10/14/14
	523.5			8:0	
	411.3		min	16:0	
	439.4		min	14:0	
	449.7				
	495.5		min	10:0	18/10/10
712.8	523.4	40:00	min	10:0	12/14/14
	495.5			12:0	16/12/12
	467.3			14:0	18/10/12
	453.7		min		16/10/14
	449.7		min		
	439.4			16:0	
	411.3		min	18:0	

Molecular ion	DAG ions seen	Carbon no		Fatty acid loss (by difference)	Suggested TAG present
740.7	523.4	42:00		12:0	18/12/12
	495.5			14:0	16/14/12
	467.5			16:0	14/14/14
	453.8				
	449.7		min		
	439.3			18:0	
768.7	607.2	44:00	min	8:0	8/18/18
	579.4		min	10:0	18/16/10
	551.5			12:0	14/14/16
	523.4			14:0	18/14/12
	495.5			16:0	16/16/12
	467.5			18:0	18/16/10
	439.7			20:0	20/12/12
					20/16/8
796.8	607.6	46:00		10:0	20/12/14
	579.6			12:0	18/14/14
	551.4			14:0	16/16/14
	523.5			16:0	18/16/12
	495.5			18:0	18/18/10
	471.8				20/16/10
	467.8			20:0	
	453.7		min		
	449.7		min		
	451.7		min		
824.8	607.6	48:00		12:0	18/20/10
	580.7			14:0	18/16/14
	551.5			16/0	18/18/12
	523.5			18:0	
	471.9		min		
	467.8		min	22:0?	
	494.8		min	20:0	
	485.8		min		
	453.7		min		
	449.7		min		
	451.7		min		
852.8	551.6	50:00		18:0	18/18/14
	579.6			16:0	18/16/16
	607.6			14:0	22/14/14
	521.6		min		
	494.8		min	22:0	
	499.8		min		
	467.8		min		
	453.7				
880.9	579.6	52:00		18:0	18/18/16
	607.6			16:0	
	656.7		min		
	683.7		min		
	700.7		min		
	712.7		min		
	728.7		min		
908.9	607.6	54:00		18:0	18/18/18
	699.7		min		
	683.6		min		
	728.8		min		

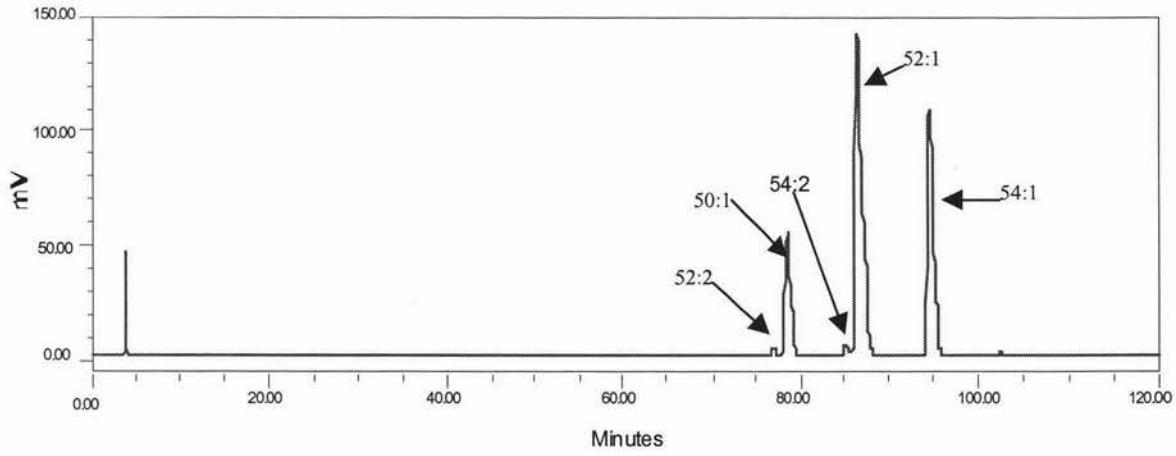
Appendix 8 Mass Spectra and ELSD Trace of Cocoa Butter



a. Cocoa butter, infused, positive ion MS.



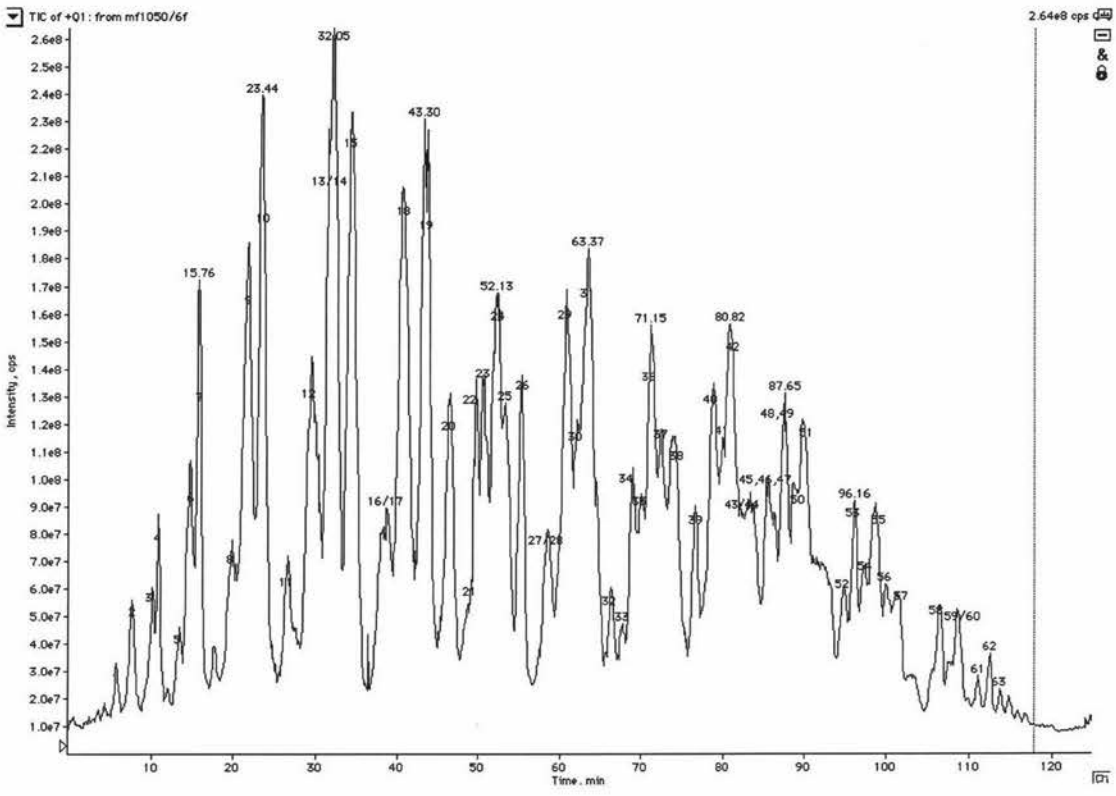
b. Cocoa butter, positive ion LC-MS.



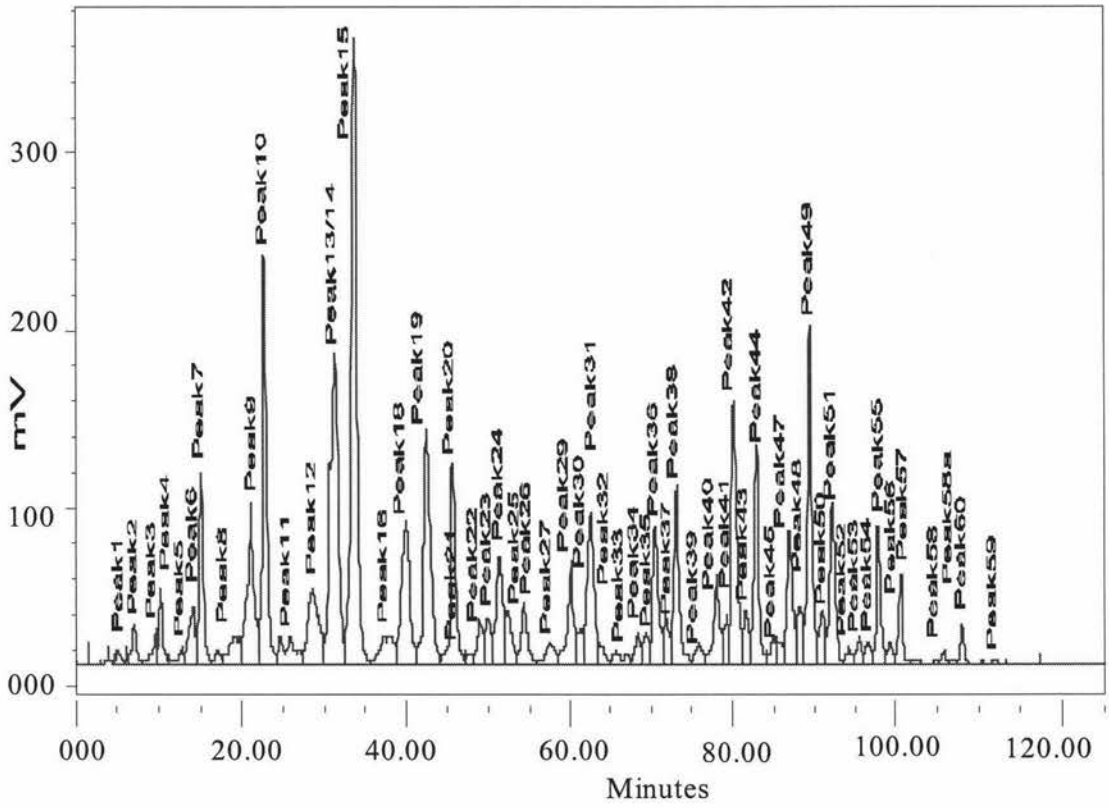
c. Cocoa butter ELSD trace.

Molecular ion	DAG ions seen	Carbon no	Selected molecular ion	Fatty acid loss (by difference)	suggested TAG present
874.7		52:3:			
**848.8	621.5	50:2	848.8		
822.5	603.5	48:1?		14:0	18:2/16:0/16:0
894.7?adduct	575.5			16:0	
	551.4			18:2	
902.9	578.9	54:3		18:1	18:1/18:1/18:1
	603.5				
	620.1				
877	577.5	52:2		18:1	18:1/18:1/16:0
	603.5			16:0	
	605.7			16:1	
	663.5				
850.8	577.6	50:1		16:0	16:0/18:1/16:0
	551.4			18:1	
824.8	520	48:0			
876.9	551.4	52:2			
890.9	578.6	53.2			
	619.9				
	663.4				
864.9	565.5	51:1		18:1	18:1/16:0/17:0
	577.5			17:0	
	591.7			16:0	
	520.1				
	537.6				
904.9	603.5	54:2:		18:0	18:0/18:1/18:1
	605.7			18:1	
878.8	577.5	52:1:		18:0	18:0/18:1/16:0
	579.5			18:1	
	605.5			16:0	
892.9	577.3	53:1	852.8		
852.8	551.3	50:0		18:0	18:0/16:0/16:0
878.8(carryov)	579.5			16:0	
904.9	605.4	54:2	892.9	17:0	17:0/18:0/18:1
932.8	620.2	56:2			
	664.3				
	591.5			18:00	
	593.5			18:01	
906.9	605.4	54:1		18:0	18:0/18:1/18:0
	607.4			18:1	
880.9	577.2	52:0	880.9		
906.9(carryov)	579.4			18:0	18:0/18:0/16:0
	605.3				
	607.5			16:0	
	663.4				
880.8	579.5				
	607.6				
	605.6				
935	577.1	56:1		22:0	18:0/18:1/20:0
	605.7			20:0	22:0/18:1/16:0
	633.6			18:0	
	635.6			18:1	
	663.4			16:0	
908.8	605.5	54:0			20:0/18:0/16:0
	607.6				
	679.3				
962.8		58:1			20:0/20:0/18:1
990.9		60:1			24:0/18:0/18:1

Appendix 9 Mass Spectrum and ELSD Trace of Bovine Milkfat



a. Bovine milkfat positive ion LC-MS.



b. Bovine milkfat ELSD trace.

Peak number	Ret time	$(M+NH_4)^+$	Carbon numbermass spec	Intensityof ion	Carbon number HPLC
1	5.68	516.5	26:0		26:0
	5.68	542.5	28:1		
2	7.57	570.4	30:1		
	7.57	544.4	28:0		28:0
3	10.09	572.5	30:0		
	10.09	598.5	32:1	major	32:1
	10.09	624.5	34:2	minor	
4	10.82	572.5	30:0		30:0
5	13.45	600.4	32:0		36:0
	13.45	626.5	34:1		
	13.45	652.4	36:2	minor	
6	14.71	600.4	32:0		
	14.71	626.5	34:1	major	34:1
7	15.76	600.5	32:0		32:0
8	19.76	614.5	33:0		
	19.76	628.6	34:0		
	19.76	680.5	38:2		38:2
9	21.86	654.6	36:1		36:1
	21.86	628.6	34:0	minor	34:0
10	23.44	628.5	34:0		34:0
11	26.69	682.5	38:1		42:0
	26.69	668.5	37:1		
	26.69	642.7	35:0		
12	29.43	656.5	36:0	minor	40:0
	29.43	682.6	38:1		
	29.43	708.6	40:2		
	29.43	734.6	42:3	minor	
13/14	32.05	682.5	38:0		38:0
15	34.47	656.4	36:0		36:0
16/17	38.68	710.6	40:1		40:1
	38.68	736.6	42:2		42:2
	38.68	670.6			
18	40.67	684.6	38:0		38:0
	40.67	736.6	42:2		40:1
	40.67	710.6	40:1	major	
19	43.3	684.6	38:0		38:0
	43.3	710.6	40:1		40:0
20	46.45	684.6	38:0		38:0
21/22	49.71	738.6	42:1		44:2/42:1
	49.71	764.6	44:2		
	49.71	790.7	46:3	min	
23	50.66	738.6	42:1		42:1
	50.66	764.6	44:2	min	
24	52.16	738.6	42:1		42:1
	52.16	712.7	44:2		44:2
25	52.44	738.6	42:1		42:1
	52.44	712.7	40:0		40:0
26	55.17	712.7	40:0		40:0
27/28	58.43	792.7	46:0		46:0
	58.43	726.7	41:0		41:0
	58.43	712.7	40:0		
29	60.74	792.7	46:2	min	

Peak number	Ret time	$(M+NH_4)^+$	Carbon numbermass spec	Intensityof ion	Carbon number HPLC
	60.74	766.6	44:1		44:1
30/31	63.37	740.6	42:0		42:0
					44:1
32/33	66.32	846.8	50:3		
	66.32	740.6	42:0		42:0
	66.32	754.7	43:0		
	66.32	780.7	45:1		
34	68.84	846.8	50:3		
	68.84	820.8	48:2		48:2
	68.84	754.7	43:0	minor	
35	69.89	820.8	48:2	major	48:2
	69.89	846.8	50:3	minor	
	69.89	872.7	52:4		
36	71.15	794.6	46:1	major	46:1
	71.15	820.7	48:2		
37	72.41	846.8	50:3		
	72.41	820.8	48:2		
	72.41	794.6	46:1	maj	46:1
	72.41	768.6	44:0		
38	73.78	768.6	44:1		44:0
39	76.51	782.7	45:0		
	76.51	808.7	47:1		47:1
	76.51	834.9	49:2	minor	
	76.51	874.8	52:3		
	76.51	900.8	54:4		
40	78.72	782.7	45:0	minor	
	78.72	848.7	50:2		50:2
	78.72	874.7	52:3		
41/42	80.82	822.7	48:2		48:2
	80.82	848.7	50:2		50:2
	80.82	874.7	52:3		52:3
43/44	83.55	796.7	46:0	major	46:00:00
	83.55	836.8	49:2		48:1t
	83.55	862.8	51:2		
45,46,47	85.65	810.8	47:0		52:2
	85.65	836.8	49:2		49:1
	85.65	902.8	54:3	major	54:3
48,49	87.65	876.7	52:2		52:2
50	88.7	850.8	50:1		50:1
	88.7	876.7	52:2		
51	89.75	824.7	48:0		48:0
52	94.8	838.7	49:0		
	94.8	864.9	51:1		51:1
53	96.16	904.8	54:2		54:2
54	97.72	838.7	49:0	minor	
	97.72	878.8	52:1	minor	
	97.72	904.8	54:2		54:2t
55	98.58	878.7	52:1		52:01:00
56	99.84	892.7	53:1		
	99.84	852.7	50:0		
	99.84	878.7	52:1	major	52:1t
57	101.21	852.8	50:0		50:0
58	106.36	906.8	54:1		54:1
59/60	107.41	880.8	52:0		52:0

Peak number	Ret time	$(M+NH_4)^+$	Carbon numbermass spec	Intensityof ion	Carbon number HPLC
	108.56	920.8	55:1		
61	112.56	908.9	54:0		54:0
62	113.82	922.9	55:0		
	113.82	962.9	58:1		
	113.82	936.8	56:0		
63	114.87	936.8	56:0		

Appendix 10 FAME Results: Milkfat from Different Mammalian Species. SFC Results: Seasonal Survey

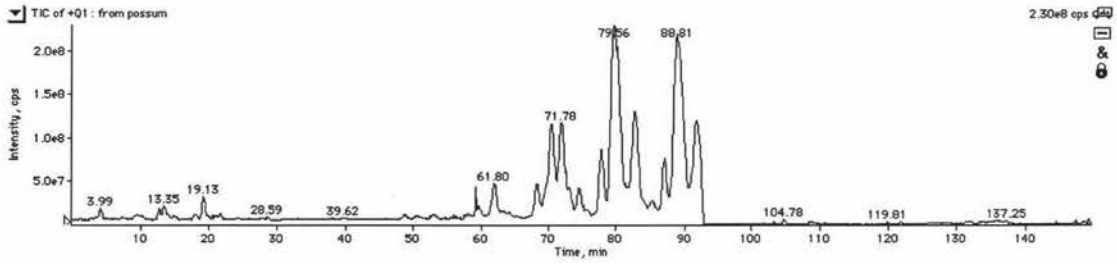
FAME results

Sample Id	Human	Horse	Possum	Whale	Seal
C4:0	0	0.219	0	0	0
C6:0	0	0.331	0	0	0
C8:0	0.161	1.953	0	0	0
C10:0	1.315	2.729	0	0.089	0
C10:1	0	0.894	0	0	0
C12:0	6.472	3.076	0	0.175	0.115
C12:1	0	0.16	0	0.066	0
C13:0 Br	0	0.138	0	0	0
C13:0	0	0	0	0	0
C14:0 Br	0	0	0	0	0
C14:0	7.482	4.564	1.303	2.935	5.513
C14:1	0.107	0.53	0	0.167	0.204
C15:0 iso Br	0.137	0.057	0	0.161	0.252
C15:0 ante-iso Br	0.132	0.076	0	0.068	0
C15:0	0.461	0.495	0.217	0.678	0.613
C16:0 Br	0.498	0.3	0	0.133	0.113
C16:0	31.246	24.488	35.73	20.057	19.251
C16:1	2.274	10.639	1.724	1.634	6.149
C17:0 iso Br	0.266	0.102	0	0.212	0.336
C17:0 ante-iso Br	0.2	0.282	0	0.09	0.133
C17:0	0.532	0.24	0.37	0.653	0.458
C17:1	0.212	0.973	0.225	1.405	0.499
C18:0	9.197	1.039	3.149	3.913	2.846
C18:1n-9	25.088	27.034	38.239	44.13	22.094
C18:1n-7	4.04	1.877	1.119	4.618	4.098
Unknown I	1.525	0.117	0	0.227	0.106
C18:2n-6 (Linoleic)	6.402	6.128	13.815	1.077	1.145
Unknown J	0.162	0	0.154	0.23	0.265
C18:3n-3	0.203	10.192	2.696	0.285	0.489
C18:2 conj.	0	0.083	0	0	0
C18:4n-3	0	0	0	0.081	0.928
C20:0	0.297	0.065	0	0.081	0.074
C20:1n-11	0	0	0	0.759	0.479
C20:1n-9	0.47	0.219	0.338	7.676	7.047
C20:2n-6	0.348	0	0.186	0.166	0.311
C20:3n-6	0.094	0	0.25	0	0.07
C20:4n-6 (AA)	0.089	0	0.098	0.869	0.98
C20:3n-3	0	0.328	0	0.111	0.212
C20:4n-3	0	0	0.124	0.44	1.387
C20:5n-3 (EPA)	0	0	0	1.561	7.23
C22:0	0.589	0.672	0.263	0	0
C22:1n-13	0	0	0	1.186	1.223
n-11	0	0	0	0.435	0.824
C22:1n-9	0	0	0	0	0
C22:4n-6	0	0	0	0	0
C22:5n-6	0	0	0	0.726	2.484
C22:5n-3	0	0	0	0	0
C24:0	0	0	0	2.796	11.661
C22:6n-3 (DHA)	0	0	0	0.109	0.414
C24:1	0	0	0	0	0

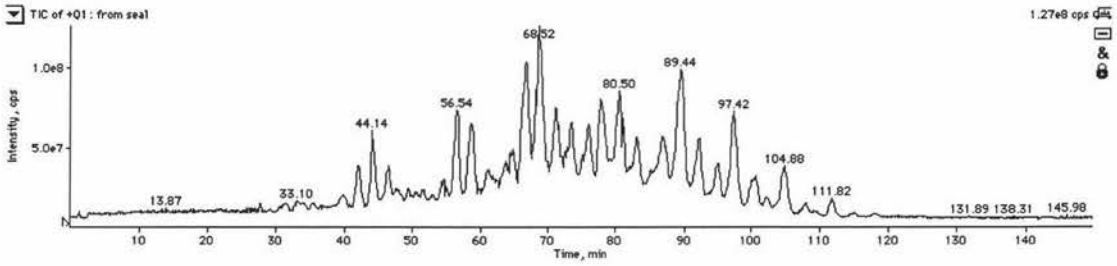
SFC results

Sample	SFC 10°C
Early spring	49.55
Spring	56.35
Summer	59.02
Autumn	56.41

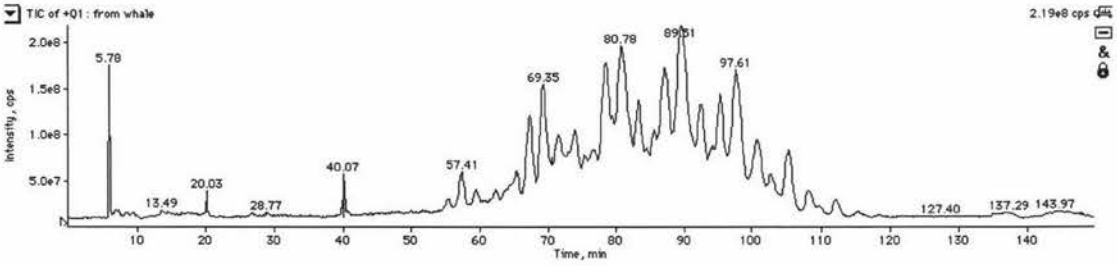
Appendix 11 Mass Spectra and ELSD Traces of Milkfat from Different Mammalian Species



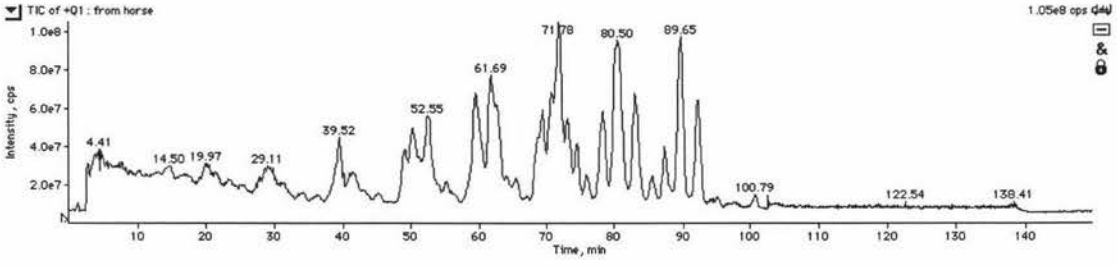
a. Possum milkfat, positive ion LC-MS.



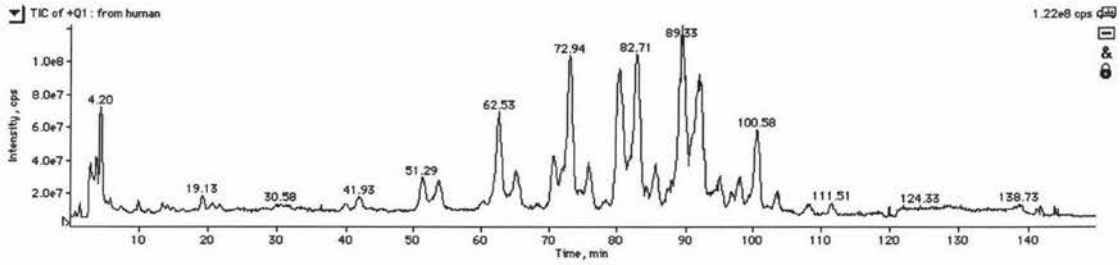
b. Seal milkfat, positive ion LC-MS.



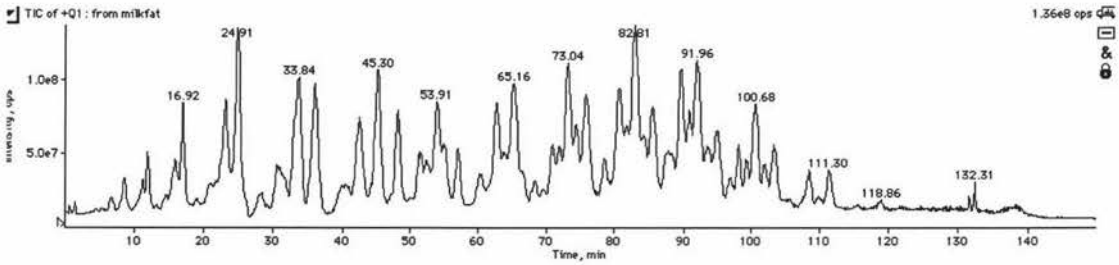
c. Whale milkfat, positive ion LC-MS.



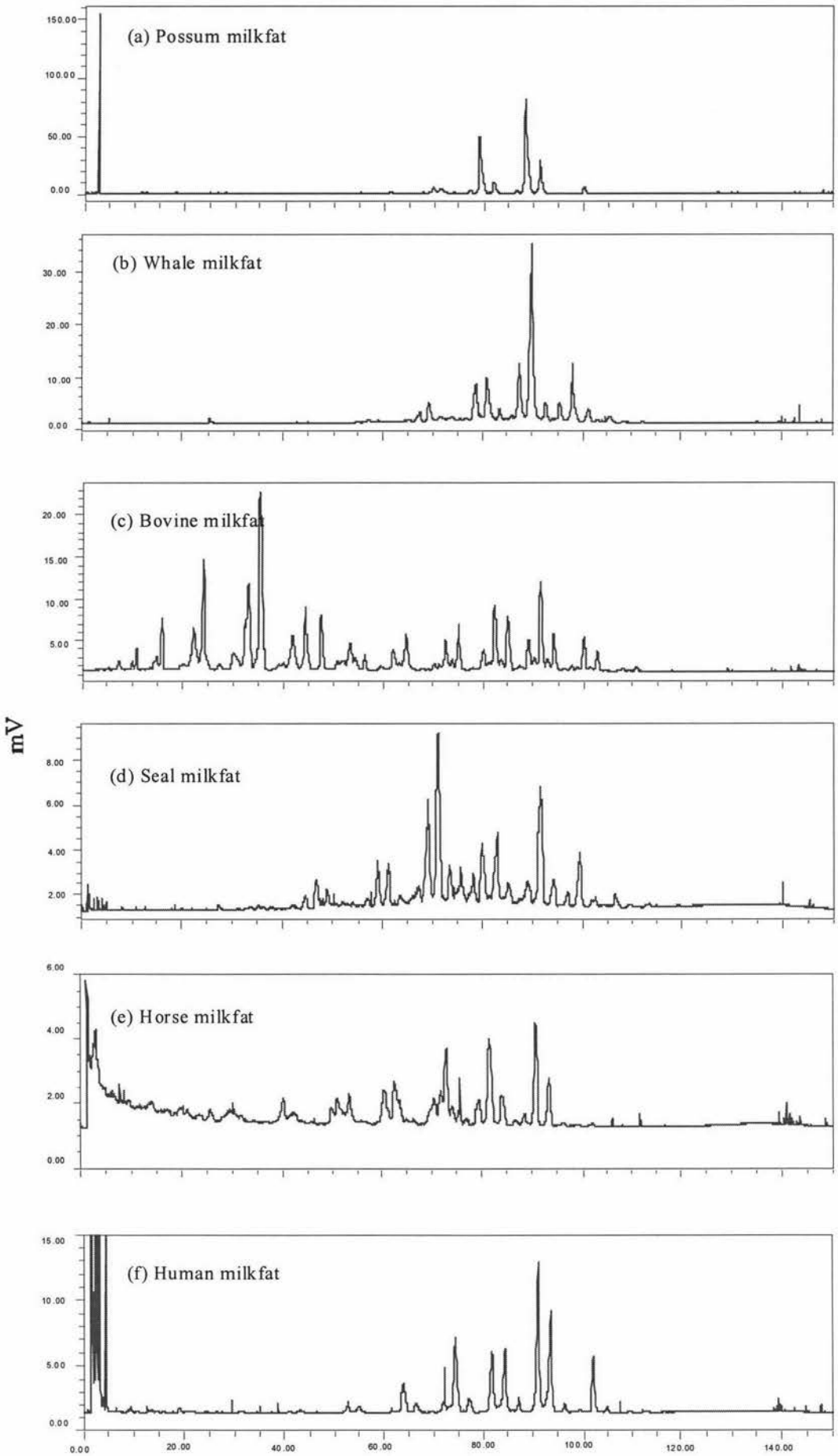
d. Horse milkfat, positive ion LC-MS.



e. Human milkfat, positive ion LC-MS.



f. Bovine milkfat, positive ion LC-MS.


(a) Possum milkfat
(b) Whale milkfat
(c) Bovine milkfat
(d) Seal milkfat
(e) Horse milkfat
(f) Human milkfat
mV
Minutes