Electrospray and Tandem Mass Spectrometric Characterization of Acylglycerol Mixtures That Are Dissolved in Nonpolar Solvents

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This paper presents electrospray mass spectrometric analysis of mixtures containing monoglycerides, diglycerides, and triglycerides. Sample compounds were dissolved in concentrations of 1–50 pmol/μL in chloroform:methanol (70:30, v:v), which was modified by the addition of alkali-metal or ammonium salts or by addition of formic acid to favor the addition of a cationic species to the sample molecules. Electrospray mass spectrometric analysis of acylglycerol standards yielded positive-ion current signals for $(M + Na)^+$ or $(M + NH_4)^+$ of all the species that were present at low picomole per microliter concentrations with no fragmentation. For equimolar concentrations of these sample compounds, there was a general decrease in ion current response as the analyte polarity decreased. Therefore, acylglycerols that contained unsaturated fatty acid chains were observed to exhibit a response in the mass spectrum greater than those with saturated chains, and ion signals resulting from the molecular adduct ions of monoglycerides were more abundant than those of diglycerides, which were more abundant than those of triglycerides in the mass spectrum. Electrospray mass spectrometric analysis of an unknown lipid material recovered from a mammalian cell culture reactor revealed a mixture of triglycerides containing mostly C_{14}, C_{16}, and C_{18} fatty acids with varying degrees of unsaturation. The results obtained by electrospray mass spectrometry compared favorably to those obtained by gas chromatography after saponification and methylation of fatty acid components of the triglycerides. MS/MS fragmentation of sodiated acylglycerols required a dissociation energy significantly greater than that required for fragmentation of ammoniated acylglycerols, so MS/MS characterization of acylglycerols was generally performed on the ammoniated compounds. The most abundant product ions that were formed from the ammoniated acylglycerols resulted from loss of fatty acids, and the acylium ions of the fatty acids were also present in the MS/MS spectra. If the MS/MS collision energy was set at greater than 100 eV, fragmentation of the carbon–carbon bonds of the fatty acid chains was evident in the low-mass region of the mass spectrum, but locations of double bonds could not be distinguished because the site of unsaturation apparently migrated during the collisions. Application of nonpolar solvents to the dissolution and electrospray mass spectrometric analysis of other nonpolar compound classes should follow from the methodology presented in this paper.

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INTRODUCTION

Electrospray (ES) produces highly charged small droplets from which gas-phase organic analyte ions may be formed by ion evaporation (*1*). Ion spray or pneumatically assisted electrospray (*2*) is similar except a coaxial nebulizing gas is used to aid droplet formation. This nebulizing gas helps stabilize the spray and facilitates nebulization of highly aqueous solutions that have high surface tension and therefore will not undergo sufficient nebulization solely under the influence of the electrospray potential difference. Because mass spectra obtained under electrospray and ion spray conditions are essentially identical, no further distinction will be made between these two modes of ion formation in this article. In our laboratory we prefer to use ion spray for the aforementioned reasons, and all the data presented in this paper were acquired under ion spray conditions, but the term electrospray will be used throughout this paper to avoid confusing nomenclature.

In ES, sample compounds are usually dissolved in water, methanol, acetonitrile, or some combination of these solvents and the solutions are nebulized under the influence of an electric field to form electrically charged microdroplets that evaporate at atmospheric pressure to form gas-phase ions, which are sampled into a mass spectrometer vacuum system, mass-analyzed, and detected. To date, applications of electrospray mass spectrometry have included proteins and peptides (*3–5*), oligonucleotides (*4, 6*), oligosaccharides (*7*), drugs (*8, 9*), dyes (*10*), surfactants (*11*), and other polar classes of compounds. Application of ES to relatively nonpolar compounds has received little attention because these compounds are typically insoluble in the polar solvents that are used for ES. There have been only a couple reports of the use of nonpolar solvents for electrospray. McNeal et al. investigated acetone, benzene, pyridine, tetrahydrofuran, carbon tetrachloride, and octane as solvents for electrospray deposition of various oligopeptides, oligonucleotides, and antibiotics onto a solid support for characterization by plasma desorption mass spectrometry (*12*). More recently, Van Berkel et al. have reported the use of toluene and dichloromethane as solvents for electrospray mass spectrometric analysis of porphyrins (*13*).

Acylglycerols, including monoglycerides, diglycerides, and triglycerides, have proven difficult to characterize by common analytical methods (*14*). Despite the large number of publications describing their importance in nutrition (*15, 16*), cosmetics (*17*), biochemistry (*18, 19*), and many other areas of science, only a few of which have been referenced here, few reports have described progress in the separation and analysis of this compound class. The general procedure for characterizing acylglycerols requires isolation of individual compounds by thin-layer chromatography or HPLC, saponification

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of the fatty acid substituents of each acylglycerol, derivatization of the fatty acids to their methyl esters or some other volatile derivative, and separation of individual fatty acids by gas chromatography (GC) with detection by flame ionization or mass spectrometry (*20*).

Mass spectrometry has been applied to the analysis of acylglycerol mixtures with some success. Electron ionization (EI) (*21*), chemical ionization (CI) (*22*), desorption chemical ionization (DCI) (*23*), and field desorption (FD) (*24*) mass spectra of triglycerides have been reported. However, each of these ionization methods requires volatilization of the triglyceride sample through application of heat, and each of the reports describes a fractionation effect where more volatile triglycerides (those with shorter hydrocarbon chains) volatize first, making quantitation of triglyceride mixtures difficult. Desorption chemical ionization produced the smallest degree of fractionation (*23*) because the sample mixtures were rapidly heated.

In those reported cases where the mass spectra contained molecular ions, the total number of carbons and the degree of unsaturation of the combined fatty acid chains could be determined from the molecular weight of the triglyceride. Prior separation by chromatography was unnecessary because individual triglycerides could be separated by their mass-to-charge ratio. Mass spectra of pure triglycerides obtained by EI, CI, and DCI also contained fragment ions resulting from cleavage of fatty acid chains, so that the chain length and number of unsaturated sites of individual fatty acids of the triglyceride could be differentiated. However, fragmentation of the triglycerides precluded mass spectrometric analysis of triglycerides in the presence of monoglycerides, diglycerides, or fatty acids because fragment ions of the triglycerides have the same mass-to-charge ratio as intact protonated molecules and fragment ions of the monoglycerides, diglycerides, and fatty acids. Also, the fatty acid composition of individual triglycerides could not be determined in the presence of other triglycerides under these ionization conditions because all the triglycerides of a mixture dissociate, yielding overlapping fragment ion signals in the mass spectra.

Plasma desorption (PD) mass spectra of triglyceride mixtures also have been reported (*25*), but ionization by plasma desorption generated fragmentation, and intact triglycerides were not observed in the mass spectra. Also, relative abundances of diagnostic ions of the triglycerides were found to vary with sample preparation and handling using this ionization method. Another report does present mass spectra that contain intact triglyceride ions, but fragmentation is also apparent (*26*). Fast atom bombardment (FAB) mass spectrometry has been used to characterize lipid compound classes, including fatty acids (*27*, *28*) and phospholipids (*29*, *30*), but like plasma desorption, FAB analysis of triglycerides yields mass spectra that contain mostly fragment ions of the triglycerides (*31*, *32*), so unknown triglyceride compounds cannot be positively differentiated from diglycerides, monoglycerides, and fatty acids.

This paper describes the electrospray and tandem mass spectrometric analysis of synthetic mixtures containing monoglycerides, diglycerides, and triglycerides. The use of nonpolar solvents for dissolution and electrospray mass spectrometric characterization of acylglycerols and the addition of ionic modifiers to these solvents for increased ionization efficiency of acylglycerol compounds are described. Methodology developed on the standards was then applied to an unknown lipid material recovered from a mammalian cell culture reactor, and the analytical results are presented. Because electrospray is an extremely soft means of forming gas-phase ions, fragmentation does not occur, and molecular adduct ions of all the species present at low picomole per microliter concentrations are obtained without interference from fragment ions. MS/MS analysis of the acylglycerol $(M + NH_4)^+$ ions was performed to determine the fatty acid compositions of the individual acylglycerol species after they had been separated by their mass-to-charge ratio. Chromatographic separation of acylglycerol mixtures was not necessary when MS/MS was combined with electrospray mass spectrometry. The ability to form gas-phase ions from the aforementioned nonpolar compounds provides methodology that may be applied to other nonpolar and involatile compound classes that previously were intractible to mass analysis using other ionization methods.

EXPERIMENTAL SECTION

Standards. Acylglycerol standards were purchased as synthetic mixtures from Sigma Chemical Co. (St. Louis, Mo; product numbers 178-3, 178-8, 178-9, 178-12, D4907, P2290) and used without further purification. Sample mixtures were dissolved in chloroform:methanol (70:30, v:v) that contained 10 mM ammonium acetate, 10 mM sodium acetate, or 2% formic acid. The concentration of sample solutions was adjusted from 1 to 1000 pmol/μL to study dilution effects and determine detection limits.

TLC Analysis of Lipids. Lipid samples were collected from a 100-L mammalian cell culture reactor over the period of 3 months. Samples collected 2 weeks and 2 months apart were dissolved in hexane and in chloroform:methanol (2:1, v:v) in an approximate concentration of 1 mg/mL. Aliquots (5 μL) of each of the dissolved samples were spotted on silica gel G TLC plates (20 cm × 20 cm, 250-μm coating) and developed with hexane:diethyl ether:acetic acid (80:20:2, v:v:v). After drying, visualization of separated compounds was accomplished by reaction with iodine vapors, and the R_f values of the unknown lipid components were compared to those of lipid standards that were developed in adjacent lanes on the TLC plate.

Determination of Fatty Acid Composition of Lipids. Lipid samples collected over a period of 3 months were homogenized and dissolved in a 90% ethanol solution containing 1% saturated KOH solution, and the resulting solutions were incubated at 80 °C for 2 h. Free fatty acids released by the saponification reaction were extracted into hexane. This mixture was dried under a gentle stream of nitrogen at room temperature, and residual fatty acids were methylated in BF_3:methanol (14:86, v:v) (Pierce, Rockford, IL) at 60 °C for 10 min. The fatty acid methyl esters were extracted into hexane and subjected to gas chromatographic analysis with flame ionization detection (GC/FID). The GC analysis was performed on a HP 5890 system with a 25 m × 0.2 mm i.d. silica capillary column. The carrier gas was air:H_2:N_2 (4:3:2). The injection volume was 10 μL, and the column temperature was stepped from 200 to 270 °C in 20 min. Identification and quantitation of fatty acid components of the original lipid sample were based on the elution time and integrated peak areas of their methyl esters.

Electrospray Mass Spectrometry. A Sciex TAGA 6000E triple-quadrupole mass spectrometer equipped with an atmospheric pressure ion source was used to sample positive ions produced from an ion spray interface (*2*), which was maintained at 3–5 kV with respect to the ion entrance of the mass spectrometer. The ion spray interface is similar to an electrospray interface except a coaxial nebulizing gas aids in solvent dispersal at the sprayer tip. Sample mixtures of the acylglycerols were introduced continuously through the ion spray interface at a rate of 2 μL/min. Positive gas-phase ions, which were created during nebulization and desolvation of analyte solutions, were sampled through a 100-μm-i.d. conical orifice into the vacuum chamber for mass analysis. The atmospheric side of the conical orifice was bathed with a curtain of high-purity, dry nitrogen gas, which prevented contamination of the vacuum with solvent vapors and atmospheric gases and helped desolvate ions formed during nebulization of sample solutions. Ions entering the mass spectrometer were focused with a Brubaker lens (*33*) into the mass analyzer and separated from neutral nitrogen molecules, which were frozen onto cryogenically cooled surfaces (15–20 K) that surround the first and second quadrupoles. In this first region of the mass spectrometer, sample ions encounter an area of declining pressure as neutral molecules are pumped away. Increasing

the potential difference in this region causes greater desolvation and/or fragmentation of analytes. In this study, the potential difference was maintained at approximately 40 V, which resulted in full desolvation of analyte ions without dissociating the covalent bonds of the analyte. Fragmentation of sample compounds could be accomplished by increasing this potential difference to 50–80 V. A working pressure of 2×10^{-5} Torr was maintained in the analyzer chamber during routine operation of the instrument. Mass analysis of sample ions was accomplished by scanning the first quadrupole in 0.1-amu increments from 200 to 1200 amu in approximately 10 s and passing mass-selected ions through the second and third quadrupoles, operated in the rf-only mode, to the multiplier, which was operated in the pulse-counting mode. Mass spectra were averaged over five scans for all results presented in this study.

Product ion MS/MS spectra of acylglycerol ions were acquired by passing the $(M + NH_4)^+$ ions of individual acylglycerol compounds, which had been mass-selected with the first quadrupole, into the second quadrupole, where they were dissociated by collision with ultrapure argon gas. Fragment ions generated by these collisions then were mass-analyzed by the third quadrupole and detected. The third quadrupole was scanned in 0.1-amu increments from 10 amu up to and including the mass of the parent ion in less than 10 s, and five scans were averaged for each MS/MS spectrum. The argon target gas thickness was maintained at 2.3×10^{14} atoms/cm^2 for this study, and collision energies were chosen by adjusting the dc voltage offset of the collision quadrupole. For this study collision energies of 50–130 eV with respect to the laboratory reference were used; higher collision energies were necessary to dissociate carbon–carbon bonds contained in the fatty acid hydrocarbon chains. The first and third quadrupoles were adjusted to obtain unit mass resolution for all mass spectra and MS/MS product ion spectra obtained in this study. However, high collision energies (>100 eV) caused some broadening of product ions in the MS/MS spectra due to translational energy spread during collisional activation.

RESULTS AND DISCUSSION

Electrospray Mass Spectra of Standards. Acylglycerol standards were dissolved in a chloroform:methanol (70:30, v:v) solvent, which was modified by 10 mM sodium acetate, 10 mM ammonium acetate, or formic acid (2%). Background ions that were detected when these solvents were subjected to electrospray mass analysis were limited to sodium acetate clusters, which were present in electrospray mass spectra at positive mass values of $Na_n CH_3COO_{n-1}$ and to low-mass solvent clusters. Ammonium acetate clusters were not detected. Chloroform:methanol (70:30, v:v) was chosen as the electrospray solvent because it is a common solvent used for extraction of lipid components. The addition of methanol to chloroform also helped solubilize the sodium and ammonium salts, which were necessary for increased ES mass spectrometric sensitivity of the acylglycerols.

For electrospray mass analysis of acylglycerols, addition of sodium acetate or ammonium acetate to the solvent resulted in abundant $(M + Na)^+$ or $(M + NH_4)^+$ ions, respectively, in the electrospray mass spectra. Here, M represents the intact individual acylglycerol compounds. Approximately equal sensitivities were achieved when either Na^+ or NH_4^+ was used as the ionic modifier. However, electrospray mass analysis of acylglycerols dissolved in an acidic solvent yielded only a weak ion current for the protonated molecules. Under these experimental conditions, sodium or ammonium adduct ions, which resulted because these cations were present as low-level impurities in the samples, often were more abundant than the protonated molecules, even though the sample solution was 2% formic acid. Preferential addition of specific cations including ammonium and sodium to sample molecules also has been observed for many compound classes studied by thermospray combined with mass spectrometry (*34*). In the present study, abundant protonated molecules only could be generated by subjecting $(M + NH_4)^+$ to collision-induced

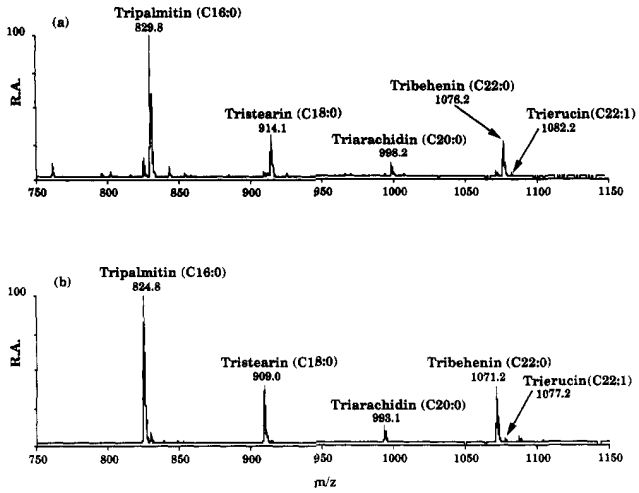


Figure 1. Electrospray mass spectrum of five triglycerides present in chloroform:methanol (70:30, v:v) at a concentration of 40 ng/μL each. The sample solution was modified with 10 mM sodium acetate (a) or 10 mM ammonium acetate (b) to form $(M + Na)^+$ or $(M + NH_4)^+$, respectively.

dissociation (CID) in the free-jet-expansion region of the API interface (see Experimental Section for details). Ammonium adduct ions then lost ammonia to yield protonated molecules. If the CID energy was adjusted to deposit only a small amount of internal energy into the ammoniated molecule, loss of ammonia to form the protonated molecule was the predominant dissociation pathway and other fragment ions were not observed. For all data presented in this paper, though, electrospray mass spectra were acquired under instrumental conditions that generated only $(M + NH_4)^+$ ions. Preventing fragment ion formation is especially important, when mixtures of similar compounds are analyzed, to prevent spectral overlap of fragment ions of one compound with the molecular ion or fragment ions of another compound in the mass spectra. Also, because all sample ions are known to originate from intact molecules in solution, mass spectral interpretation is simplified.

Quantitative determination of acylglycerols by electrospray mass spectrometry was complicated by a nonlinear ion current response that appears to correlate with the polarity of individual acylglycerol compounds. Because polarity is an intrinsic property of a compound that correlates with other properties (i.e. boiling point, solubility, etc.), the property that changes ion current response in this case cannot be directly determined. For approximately equimolar concentrations of these sample compounds, however, there was a general decrease in ion current response as the analyte polarity decreased. This general trend was observed for monoglycerides, diglycerides, and triglycerides that were associated with sodium or with ammonium ions. Therefore, for approximately equimolar concentrations of sample components, the $(M + Na)^+$ or $(M + NH_4)^+$ ions of acylglycerols that contained short or unsaturated fatty acid hydrocarbon chains were observed in greater abundance in the mass spectrum than those of acylglycerols with long or saturated hydrocarbon chains (Figure 1), and ion current signals resulting from the molecular adduct ions of monoglycerides were more abundant than those of diglycerides, which were more abundant than those of triglycerides in electrospray mass spectra.

Although the relative ion current responses of acylglycerols with different polarities varied in the electrospray mass spectra, the absolute abundance of the same acylglycerol compound varied linearly with its concentration in solution. In Figure 2 a graph of ion current vs concentration for the same mixture of five triglycerides shows the abundance of $(M + NH_4)^+$ to be approximately linear over 4 orders of magnitude

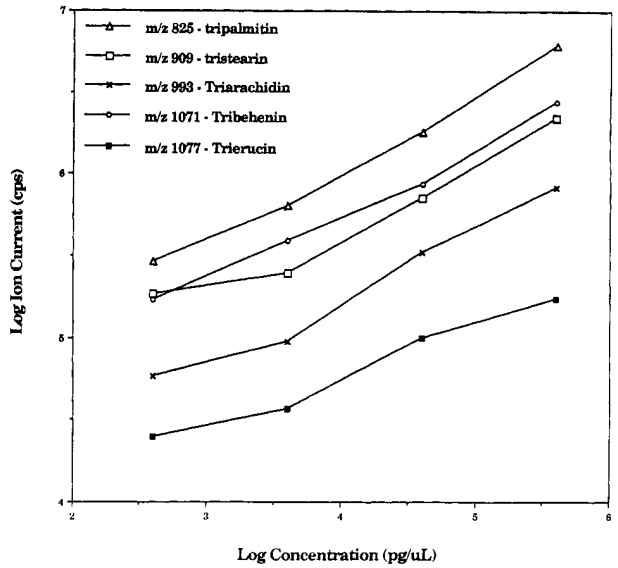


Figure 2. Plot of concentration versus ion current for the $(M + NH_4)^+$ ion of each of the five triglycerides present in the electrospray mass spectrum of Figure 1b. The ion current response of each triglyceride approaches linearity over 4 orders of magnitude of concentration.

of sample concentration for each triglyceride. The concentration of the most nonpolar triglyceride in the standard mixture ($C_{22:0}$) that was necessary for full-scan mass spectrometric detection with a signal-to-noise ratio of 3 was approximately 500 pg/μL, and less than 1 μL was consumed during the mass spectral acquisition. However, good-quality mass spectra required sample concentrations of 1–10 ng/μL and easy sample handling required 5–10 μL of sample solution.

MS/MS Spectra of Standards. Because protonated molecules of the acylglycerols were not generated in great abundance by electrospray, MS/MS experiments were conducted on the sodiated and ammoniated molecules of the acylglycerols. Dissociation of $(M + NH_4)^+$ was preferred to dissociation of $(M + Na)^+$ in this study because acquisition and interpretation of MS/MS spectra of acylglycerol $(M + Na)^+$ proved to be more difficult. Good-quality MS/MS spectra of $(M + Na)^+$ were difficult to obtain because the sodiated acylglycerols were very stable and structurally informative fragment ions could only be generated in low abundance under extreme collisional activation conditions. In this study, fragmentation of $(M + Na)^+$ in the rf-only collision quadrupole required high collision energies and required "preheating" ions through deposition of internal energy in the free-jet-expansion region of the mass spectrometer. Mass spectral interpretation of $(M + Na)^+$ MS/MS spectra was hindered by the low abundance of structurally informative fragment ions (sodium was the most abundant product ion) and by the presence of both sodiated and protonated product ions, which complicated structural assignments.

In contrast, MS/MS of $(M + NH_4)^+$ yielded good-quality CID mass spectra that contained abundant structural information for the acylglycerols. Collision-induced dissociation of the $(M + NH_4)^+$ ions resulted in loss of NH_3 as the most facile loss, and only protonated product ion signals were present in MS/MS spectra. For MS/MS experiments, collision energies were varied to obtain the greatest amount of structural information from the acylglycerols. If the MS/MS collision energy was set at 50 eV, dissociation of fatty acid chains from the acylglycerol $(M + NH_4)^+$ yielded the most abundant fragment ions in the mass spectrum. An increase in the MS/MS collision energy to 130 eV resulted in dissociation of the carbon–carbon bonds of the fatty acid chains to yield fragment ions in the low-mass region of the mass spectrum, and this collision energy was chosen for most

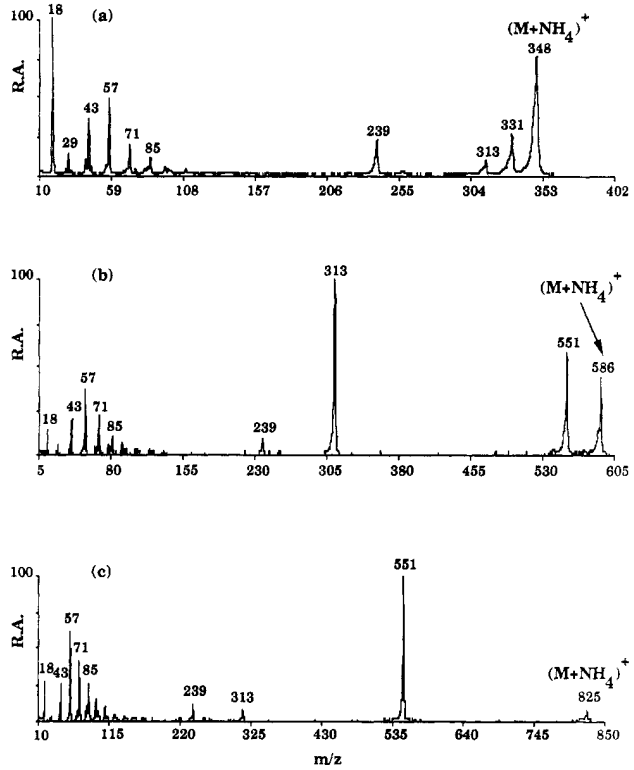


Figure 3. Comparison of the MS/MS product ion spectra of ammoniated monopalmitin (a), dipalmitin (b), and tripalmitin (c) acquired at 130-eV collision energy.

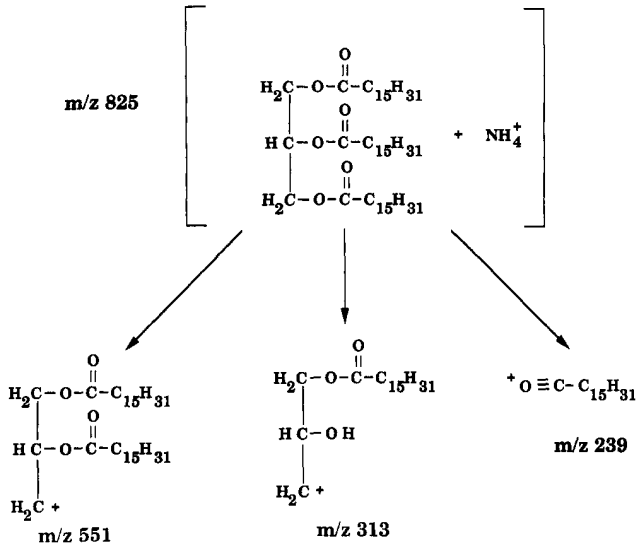
acylglycerol MS/MS analyses in this study (vide infra), except where otherwise noted.

A representative MS/MS spectrum of a monoglyceride that contains a single $C_{16:0}$ fatty acid, monopalmitin, is shown in Figure 3a. Collision-induced dissociation of ammoniated monoglycerides yields product ions corresponding to the protonated monoglyceride (m/z 331), loss of water and ammonia from the ammoniated monoglyceride (m/z 313), the acylium ion of the fatty acid (m/z 239), low-mass hydrocarbon or acylium ions resulting from carbon–carbon cleavages of the fatty acid chain, and the ammonium ion (m/z 18). The MS/MS spectrum of a representative diglyceride (Figure 3b) composed of two $C_{16:0}$ fatty acids, dipalmitin, contains a product ion corresponding to loss of ammonia and water (m/z 551), and the remainder of the product ions are observed at the same mass/charge values as those of the monoglyceride. The MS/MS spectrum of a triglyceride composed of three $C_{16:0}$ fatty acids, tripalmitin (Figure 3c), contains the same product ions that are present in the MS/MS spectrum of dipalmitin (Figure 3b).

Scheme I displays the proposed structures of product ions formed from the dissociation of ammoniated tripalmitin. Product ions in the MS/MS spectra of ammoniated monopalmitin and dipalmitin with the same m/z value as those in Scheme I would be expected to have the same structure. Preferential cleavages of fatty acid chains due to their position on a triglyceride were not observed (vide infra), so proposed product ions can be drawn with the fatty acid chain at any position of the triglyceride.

Acylglycerols that contain unsaturated fatty acids dissociate at the same location as acylglycerols that contain saturated fatty acids, so the degree of unsaturation of individual fatty acids that comprise an acylglycerol can be determined by the mass of the product ions in the MS/MS spectrum (vide infra). Gross et al. (*27, 28*) have shown that fragmentation of free fatty acids by high-energy collisions (8 keV) on a sector mass spectrometer dissociates carbon–carbon bonds of the hydro-

Scheme I. Proposed Product Ions Produced from the Dissociation of Tripalmitin $(M + NH_4)^+$



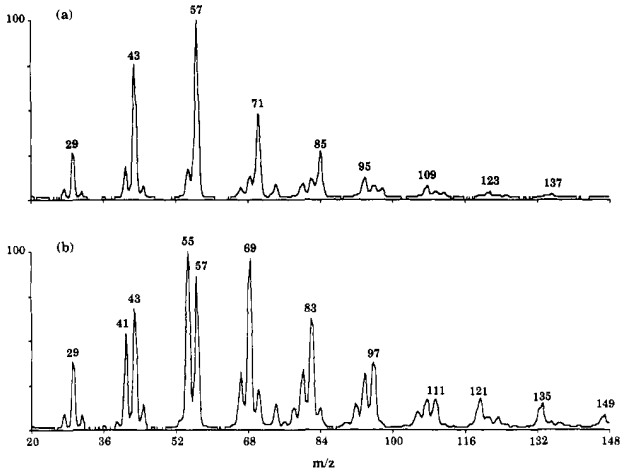


Figure 4. Comparison of the MS/MS product ion spectra from 20 to 150 amu of monostearin and monoolein, which contain a saturated (a) and unsaturated (b) C_{18} fatty acid, respectively.

carbon chain, which allows determination of branch points and sites of unsaturation of the hydrocarbon chain. In the present study, conducted on a quadrupole instrument, a collision energy of 130 eV was chosen to dissociate carbon–carbon bonds of the fatty acids to determine whether sites of unsaturation could be distinguished on the hydrocarbon chains of fatty acids that comprise acylglycerols. Figure 4 shows a comparison of the low-mass region of an MS/MS product ion spectrum of a monoglyceride that contains a $C_{18:0}$ fatty acid, monostearin, to the low-mass region of an MS/MS product ion spectrum of a monoglyceride that contains a $C_{18:1}$ fatty acid, monoolein. Although the product ion distribution is considerably different in the two MS/MS spectra, the site of unsaturation, which occurs between C_9 and C_{10} of the unsaturated fatty acid chain of monoolein, cannot be distinguished because the site of unsaturation apparently migrates during collision, so mass differences of 2 amu are observed across the entire mass range. It has been shown that fatty acid alcohol $(M + H)^+$ ions do not decompose analogously to the $(M + Li)^+$ ions (27) and do not result in structurally informative CAD spectra because of double-bond migration (35), so by analogy, ionization by NH_4^+ addition may not favor production of informative product ions for determination of unsaturated hydrocarbon sites in the MS/MS product ion spectra.

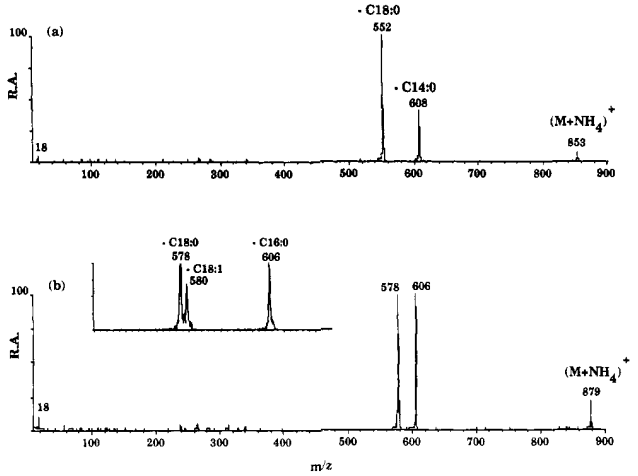


Figure 5. Positive ion MS/MS product ion spectra of two ammoniated triglycerides that contain mixed fatty acid components. The top spectrum results from 1,2-distearoyl-3-myristoylglycerol, and the bottom results from 1-palmitoyl-2-oleoyl-3-stearoylglycerol. Both MS/MS spectra were acquired at 50-eV collision energy.

Figure 5 contains the MS/MS spectra of two different triglycerides, which are composed of mixed fatty acid components. Both MS/MS spectra were collected at 50-eV collision energy. The triglyceride of Figure 5a contains $C_{18:0}$ fatty acid chains at the 1- and 2-positions and a $C_{14:0}$ fatty acid at the 3-position. The relative abundances of the product ions that correspond to loss of $C_{18:0}$ and $C_{14:0}$ fatty acids from the triglyceride are approximately 2:1, which is expected if the probability for dissociation of the fatty acid chains does not strongly depend on a small difference in hydrocarbon chain length or on the position of the fatty acid chain on the triglyceride. Figure 5b displays the MS/MS spectrum of a triglyceride that contains a $C_{18:0}$, $C_{18:1}$, and $C_{16:0}$ fatty acid at positions 1, 2, and 3, respectively. Again, the relative abundances of product ions resulting from dissociation of each of the fatty acid chains from the triglyceride are not significantly different, which infers that there is not selective dissociation of a certain fatty acid component when it is similar in length and unsaturation to the other fatty acid components.

Characterization of an Unknown Lipid Sample. Lipid samples were collected from a 100-L mammalian cell culture reactor over the period of 3 months. The identity of the lipids was of interest because they accumulated in the cell culture reactor with time and caused mechanical and operational problems for the production of a recombinant protein. TLC elution of the lipid samples showed the sample components to have an R_f value consistent with that of a triglyceride standard (Figure 6a, top). After saponification of the lipid samples, free fatty acids released by the saponification reaction were methylated, and the fatty acid methyl esters were characterized by GC/FID. Identification and quantitation of fatty acid components of the original lipid sample were based on the elution time and integrated peak areas of their methyl esters. The most abundant fatty acids, as determined by GC/FID, were $C_{14:0}$, $C_{16:1}$, $C_{16:0}$, $C_{18:1}$, and $C_{18:0}$ (Figure 6b, bottom).

Because electrospray mass spectrometry had successfully characterized the lipid standards discussed above, the unknown lipid sample was analyzed by using the same methodology. Electrospray coupled with tandem mass spectrometry complements GC analysis of methylated fatty acids of saponified acylglycerols because it can generate the molecular weight and fatty acid composition of individual acylglycerols directly, whereas GC/FID can determine the identity and relative amounts of fatty acids that constitute the entire acylglycerol population. Identification of individual acyl-

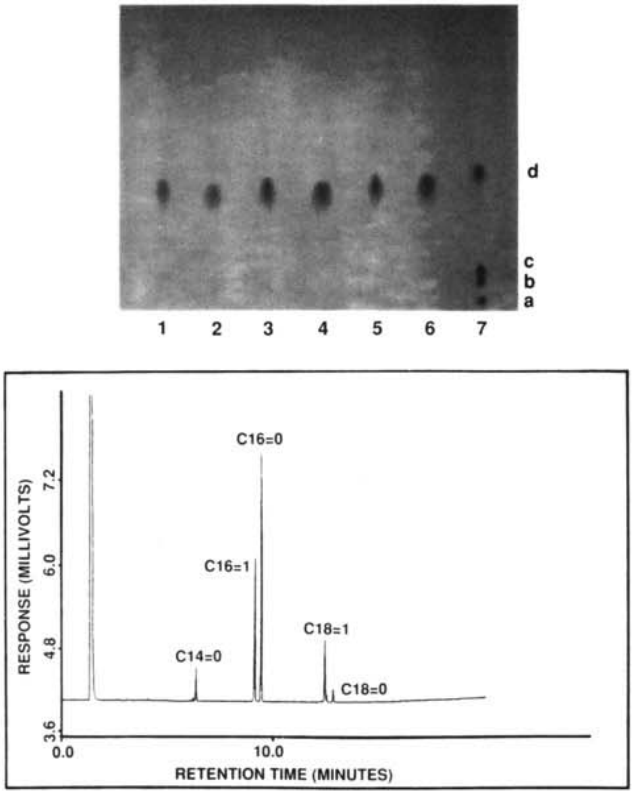


Figure 6. Thin-layer chromatogram of a mixture of unknown lipids that were collected from a mammalian cell culture reactor over a 3-month period. The R_f values of the unknown lipids are similar to that of a triglyceride standard (upper). Gas chromatogram of the methyl esters of fatty acids derived from the lipid mixture showing the most abundant species that are present (lower). The lipid sample characterized by gas chromatography was a composite of all the samples collected from the mammalian cell culture reactor. See Experimental Section for details. Key (upper): (1) 1:5:90 in hexane; (2) 1:5:90 in CHC_{13}/MeOH; (3) 11:13:89 in hexane; (4) 11:13:89 in CHC_{13}/MeOH; (5) 10:31:89 in hexane; (6) 10:31:89 in CHC_{13}/MeOH; (7) glyceride standards; (a) monoglyceride; (b) 1,2-diglyceride; (c) 1,3-diglyceride; (d) triglyceride.

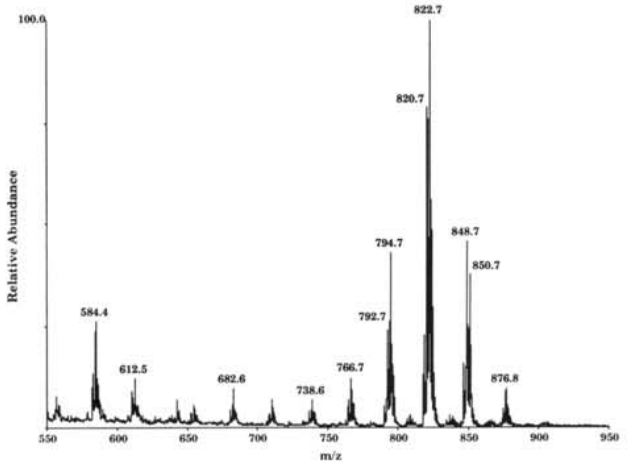


Figure 7. Electrospray mass spectrum of a mixture of unknown lipids dissolved in chloroform:methanol (70:30, v:v) containing 10 mM ammonium acetate. The masses of the most abundant ammoniated molecules suggest that the mixture contains mostly triglycerides.

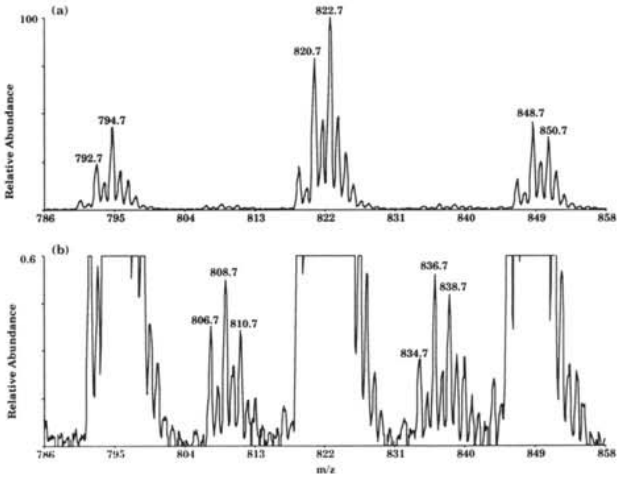


Figure 8. Expanded view of Figure 7 from m/z 780 to 860 (a) and the same mass spectrum multiplied by a factor of approximately 150 (b).

glycerols or fatty acids can be important in determining the mechanism of specific biochemical pathways (*36*). Because electrospray mass spectrometry can determine the molecular weights of individual acylglycerols that are contained in a lipid mixture, chromatographic separation of lipid components is not required to differentiate fatty acids, monoglycerides, diglycerides, and triglycerides from one another. Also, electrospray mass spectrometric analysis has an added benefit of requiring minimal sample handling and analysis time.

Figure 7 displays the electrospray mass spectrum of the unknown lipid sample dissolved in chloroform:methanol (70:30, v:v) containing 10 mM ammonium acetate. The masses of ammoniated molecules present in the mass spectrum indicate that the lipid sample is composed primarily of triglycerides and, to a lesser extent, of diglycerides. In mammalian tissues, triglycerides are present to the largest extent, but diglycerides and monoglycerides are also present in certain tissues (*37*). The same lipid sample was also dissolved in a chloroform:methanol (70:30, v:v) solvent that contained 10 mM sodium acetate, and its electrospray mass spectrum contained molecular adduct ions shifted 5 amu higher as a result of the mass difference of ammonium and sodium. Comparison of the electrospray mass spectrometric results to those obtained by thin-layer chromatography demonstrates a general agreement in compound class identification, but the increased sensitivity of electrospray mass spectrometry allowed determination of low amounts of diglycerides in the lipid mixture that could not be readily detected on the thin-layer chromatogram using iodine staining in this study.

From the mass-to-charge ratio of $(M + NH_4)^+$ or $(M + Na)^+$ for each of the acylglycerols in the mixture, the total number of carbons and the number of unsaturated sites contained on the fatty acids that comprise the acylglycerol can be calculated. Mass differences of 28 amu are evident in the electrospray mass spectra of the lipid sample, which arise from the mass difference of two methylene groups (i.e. C_{16} vs C_{18} fatty acid). Mass differences of 2 amu, which are more apparent in Figure 8a, indicate differences in the number of unsaturated sites on the fatty acids of the triglycerides. For example, ions present at m/z 819, 821, 823, and 825 in Figure 8a result from the $(M + NH_4)^+$ ions of triglycerides that contain fatty acids with a total of 54 carbons and 3, 2, 1, and 0 sites of unsaturation, respectively. The ion current at m/z 820, 822, 824, and 826 results from the ^{13}C isotope of each of these respective ions. If the ion current in the mass spectrum displayed in Figure 8a is multiplied by approximately 150 (Figure 8b) $(M + NH_4)^+$ ions resulting from triglycerides that contain a fatty acid with an odd number of carbons (i.e. C_{15} or C_{17}) are apparent in the electrospray mass spectrum (labeled peaks).

The complexity of the electrospray mass spectra shown in Figures 6 and 7 results from the statistical distribution of different fatty acids on the triglyceride and diglyceride molecules. Because only the molecular weights of the intact triglycerides or diglycerides are being measured, more infor-

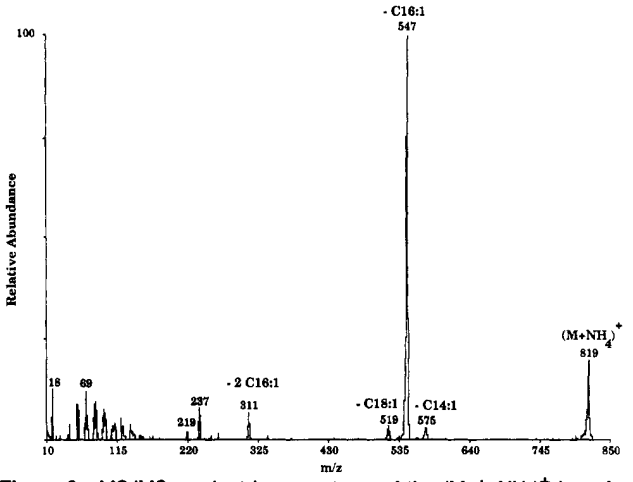


Figure 9. MS/MS product ion spectrum of the $(M + NH_4)^+$ ion of a triglyceride that is present in an unknown lipid mixture recovered from a mammalian cell culture reactor.

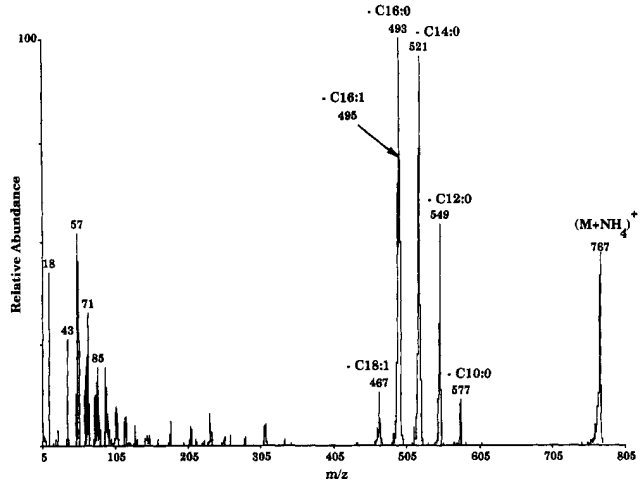


Figure 10. MS/MS product ion spectrum of the $(M + NH_4)^+$ ion of a triglyceride that was present in an unknown lipid mixture recovered from a mammalian cell culture reactor.

mation is required to determine the composition of each of the individual fatty acids. For example, a triglyceride containing three $C_{16:0}$ fatty acids, tripalmitin, will have the same molecular weight as a triglyceride containing a $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$ fatty acid. Because the sample mixture in this study is complex, the probability of two structurally different triglycerides having the same mass-to-charge ratio is high. Therefore, MS/MS analysis may be used to further elucidate the fatty acid composition of the triglycerides and diglycerides in this sample. Two examples are discussed.

Figure 9 shows the MS/MS spectrum from the $(M + NH_4)^+$ ion that is present at m/z 819 in the electrospray mass spectrum of the lipid sample (Figure 7). The fragmentation pattern is very similar to that in the MS/MS spectrum of the triglyceride standard (Figure 3c), in which protonated product ions are formed after the ammoniated parent ion expels neutral ammonia. The product ions at m/z 519, 547, and 575 result from loss of $C_{18:1}$, $C_{16:1}$, and $C_{14:1}$ fatty acids, respectively, from the protonated molecule. The product ion at m/z 311 results from subsequent loss of a $C_{16:1}$ fatty acid from the ion at m/z 547, and the product ion at m/z 237 corresponds to the acylium ion of the $C_{16:1}$ fatty acid. Because MS/MS spectra of ammoniated triglyceride standards did not exhibit selective dissociation of similar fatty acids, it can be inferred from the relative abundances of product ions in Figure 9 that $(M + NH_4)^+$ at m/z 819 is composed predominantly of a tri-$C_{16:1}$ triglyceride (tripalmitolein) with a minor component from a triglyceride containing $C_{14:1}$, $C_{16:1}$, and $C_{18:1}$ fatty acids. The ions that are present in the low-mass region of the MS/MS spectrum result from carbon–carbon cleavages of the fatty acid hydrocarbon chain and from the ammonium ion (m/z 18).

Figure 10 shows the MS/MS spectrum for the $(M + NH_4)^+$ ion that is present at m/z 767 in the electrospray mass spectrum of the lipid sample. The product ions at m/z 467, 493, 495, 521, 549, and 577 result from loss of $C_{18:1}$, $C_{16:0}$, $C_{16:1}$, $C_{14:0}$, $C_{12:0}$, and $C_{10:0}$ fatty acids, respectively, from the protonated triglyceride, which is formed after expulsion of ammonia from the ammoniated molecule. As expected for a triglyceride parent ion of lower molecular weight (compare to Figure 9), the product ion losses are shifted to fatty acids with shorter hydrocarbon chains. The product ion composition in Figure 10 also demonstrates that a site of unsaturation appears with greater statistical frequency in fatty acids with long hydrocarbon chains. This general result was apparent in all the MS/MS spectra that were generated of the lipid sample and agrees with gas chromatographic data of the fatty acid profile of the lipid sample (Figure 6b). In fact, characterization of the lipid sample by ES/MS/MS was able to reveal low-level fatty acid components that were not observed in the GC/FID chromatogram.

To obtain the fatty acid profile of the entire acylglycerol population, MS/MS spectra must be obtained of all the ions observed in the electrospray mass spectrum. For a complex lipid mixture the fatty acid profile is best obtained by saponification of the entire mixture followed by fatty acid characterization. However, electrospray and tandem mass spectrometry is a useful method for characterizing simple mixtures or specific lipids of a mixture. Also, mass spectrometry can characterize intact lipids with greater specificity than chromatographic techniques, which helps to identify the nature of the sample lipids.

CONCLUSIONS

The scope of compounds amenable to electrospray has been extended to compound classes that are soluble in nonpolar solvents (chloroform:methanol) and ionized by attachment of a cationic species to the sample molecules. Ammonium acetate and sodium acetate were dissolved in 10 mM concentrations into the solvent to facilitate cation attachment. Mixtures of monoglycerides, diglycerides, and triglycerides yielded positive-ion signals for $(M + NH_4)^+$ or $(M + Na)^+$ of all the species that were present at low picomole per microliter concentrations with no evidence of fragmentation. For equimolar concentrations of these sample compounds, the ion current response decreased as the analyte polarity decreased. Therefore, acylglycerols that contained unsaturated fatty acid chains were observed in greater abundance in the electrospray mass spectrum than the corresponding acylglycerols with saturated chains, and ion signals resulting from molecular adduct ions of monoglycerides gave a response higher than those of diglycerides, which gave a response higher than those of triglycerides in the mass spectrum. The concentration detection limit for the least sensitive triglyceride standard characterized in this study ($C_{22:0}$) was approximately 500 pg/μL. Routine analysis was performed on samples with concentrations of 1–10 n/μL per component.

Mass spectral interpretation of triglyceride $(M + Na)^+$ MS/MS spectra was hindered by the low abundance of structurally informative fragment ions and by the presence of both sodiated and protonated product ions, which complicated structural assignments. However, collision-induced dissociation of acylglycerol $(M + NH_4)^+$ resulted in loss of ammonia as the most facile loss, so MS/MS spectra contained only protonated product ions, which were present in high abundance. The most abundant product ions in the MS/MS

spectra of the acylglycerol $(M + NH_4)^+$ resulted from cleavage of fatty acid chains from the acylglycerol parent ion. The measured mass differences that resulted from cleavage of fatty acids indicated the number of carbons and the number of unsaturated sites of the individual fatty acids that comprised the acylglycerol compound. Sites of unsaturation on the fatty acid hydrocarbon chains could not be distinguished by MS/MS because the double bond apparently migrated during fragmentation of the acylglycerol ions. MS/MS product ion spectra of triglycerides composed of mixed fatty acid components contained product ion signals resulting from dissociation of each of the fatty acid esters from the triglyceride in the approximate proportion that they were present on the triglyceride.

Electrospray mass spectrometric characterization of an unknown lipid material isolated from a mammalian cell culture reactor yielded molecular adduct ions for a diverse mixture of triglycerides. MS/MS analysis of $(M + NH_4)^+$ ions that were produced from the lipid mixture revealed a high abundance of $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$ fatty acid components of the triglycerides. The MS/MS data were in good agreement with the fatty acid profile generated by GC/FID. However, relative amounts of individual triglycerides and the fatty acids that comprise the triglycerides were not deduced from the electrospray mass spectrometric data because of the demonstrated dependence of ion current abundance on analyte polarity.

ACKNOWLEDGMENT

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Registry No. Monopalmitin, 26657-96-5; dipalmitin, 26657-95-4; tripalmitin, 555-44-2; monostearin, 31566-31-1; monoolein, 25496-72-4; 1,2-distearoyl-3-myristoyl glycerol, 60138-19-4; 1-palmitoyl-2-oleoyl-3-stearoyl glycerol, 2190-27-4; tristearin, 555-43-1; triarachidin, 620-64-4; tribehenin, 18641-57-1; trierucin, 2752-99-0.

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