Analysis of Unsaturated Compounds by Ag^+ Coordination Ionspray Mass Spectrometry: Studies of the Formation of the Ag^+/Lipid Complex

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Coordination ionspray mass spectrometry (CIS-MS) is a useful tool in the detection and identification of cholesterol ester and phospholipid hydroperoxides and diacyl peroxides. Extensive studies of a series of cholesterol esters using CIS-MS revealed the following: (1) Cholesterol esters with equal number of double bonds as the internal standard showed a linear relative response in the mass spectrometer while compounds with non-equal numbers of double bonds gave a nonlinear relative response. (2) Complex adducts containing cholesterol ester, silver ion, AgF, $AgBF_4$, and 2-propanoxide form when silver is in molar excess of cholesterol esters, reducing the $[M + Ag]^+$ signal. (3) In a mixture of cholesterol esters where silver is limiting, Ch22:6 and Ch20:4 bind to silver at the expense of Ch18:2 and have a higher signal in the mass spectrometer. (4) In a mixture of cholesterol esters where silver concentration is twofold greater than total cholesterol ester concentration, Ch22:6 and Ch20:4 form large complex adducts more frequently than Ch18:2 and have a lower signal in the mass spectrometer. (J Am Soc Mass Spectrom 2003, 14, 872–880) © 2003 American Society for Mass Spectrometry

The study of free radical-mediated lipid peroxidation has been of interest for decades because of its perceived role in the pathogenesis of a number of degenerative diseases including cancer [1], neurodegenerative disorders [2], and atherosclerosis [3]. In one example of interest, the oxidative modification of low-density lipoproteins (LDL), the major carrier of cholesterol esters in human blood, has been suggested to play an important role in the development of atherosclerosis [3]. Cholesteryl linoleate and cholesteryl arachidonate are the main LDL components that undergo free radical oxidation but a complex mixture of products including primary hydroperoxides, monocyclic peroxides, serial cyclic peroxides, and isoprostanes are present in the oxidation mixture [4, 5]. Techniques for identifying lipid peroxidation products usually involve several chromatography steps and conversion to known compounds, a procedure that is tedious and prohibitively time consuming for complex lipid peroxidation mixtures [6, 7].

Mass spectrometry has proven to be a useful tool in the analysis of oxidized lipids. Recently, Murphy and colleagues reported the use of negative ion electrospray mass spectrometry for the analysis of lipid hydroperoxides and long-chain keto acids [8]. This technique allows for the coupling of reverse phase HPLC and electrospray ionization mass spectrometry (ESI-MS), thus providing a powerful separation and structure elucidation tool. However, to generate negative ions, free acids must be used instead of cholesterol esters [8, 9]. Our interest lies in the study of intact lipids.

Organic molecules containing hard or soft Lewis basic sites can be ionized by cations such as Li^+, Na^+, and Ag^+ by a technique known as coordination ion spray mass spectrometry (CIS-MS) [4, 10–12]. The addition of a suitable coordinating ion to an analyte forms a positively charged analyte complex that is detectable by the mass spectrometer. When coupled with high pressure liquid chromatography (HPLC), CIS-MS can be used to elucidate structures of complex mixtures.

We have previously shown the application of this technique to the analysis of cholesteryl linoleate and cholesteryl arachidonate peroxidation products [4, 13], phospholipids peroxidation products [14], and diacyl

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peroxides [15]. Identification of lipid peroxidation classes and their regioisomers as well as their relative abundance in vitro and in vivo is important in assessing oxidative injury and CIS-MS is particularly powerful because it allows for the study of intact lipid hydroperoxides without the prior derivatization steps needed for other mass spectrometric methods.

Traditional approaches to quantitation of lipid peroxidation products rely on the use of gas chromatography mass spectrometry (GC-MS). Direct analysis of hydroperoxides by this technique is difficult due to the thermal instability of the peroxide bond. Therefore, hydroperoxides must be reduced to alcohols and derivatized to compounds less polar and more volatile, typically through the use of methyl esters and trimethylsilyl ethers.

ESI-MS has proven useful in the quantitation of lipid peroxidation products in recent years. Murphy and colleagues have reported the quantitation of 5-hydroperoxyeicosatetraenoic acid generated by lipid peroxidation of red blood cell ghost phospholipids [9]. The quantitation of F_2-isoprostane regioisomers by ESI-MS has also been reported by Waugh et al. [16]. The disadvantage of this technique is that the phospholipid hydroperoxides must be hydrolyzed to the free acids in order to be ionized. CIS-MS offers the advantage of studying intact cholesterol esters and phospholipids but the introduction of a coordinating silver ion provides additional complications because its binding efficiency is expected to be related to the degree of unsaturation in the molecule. Herein we report our attempts at adapting CIS-MS for quantitation of lipids and their oxidation products.

Materials and Methods

General Methods

All HPLC solvents were filtered through Whatman Nylon membrane filters (0.45-μM pore size) prior to use.

Materials

The lipids and glycerides were purchased from Nu-Chek Prep (Elysian, MN). Cholesteryl 10,13-nonadecadienoate was prepared according to a literature procedure [17]. Organic solvents such as 2-propanol (IPA) and benzene were HPLC quality and purchased from Fisher Chemical (Phillipsburg, NJ). Hexanes was purchased from Burdick and Jackson (Muskegon, MI). All other reagents were purchased from Aldrich Chemical Company (Milwaukee, WI). Reagents, except for monolinolein and 1,3-dilinolein which were purchased as a mixture of regioisomers, were used without further purification.

Instruments

Analytical HPLC was conducted on a Waters model 610 HPLC instrument with a Hewlett-Packard 1050 Multiwavelength detector and a Hewlett-Packard 3396 Series III integrator. For glycerides analysis, the HPLC was equipped with a single Beckman Ultrasphere 5-μM silica columns (4.6 mm $\times$ 25 cm), with a mobile phase of 8% IPA in hexanes at delivery rate of 1 mL/min and ultraviolet (UV) detection at 205 nm.

Mass Spectrometry

CIS-MS was performed using a Finnigan TSQ-7000 (San Jose, CA) triple quadrupole mass spectrometer equipped with a standard API-1 electrospray ionization source outfitted with a 100 μM deactivated fused Si capillary. Data acquisition and spectral analysis were conducted with ICIS software, version 8.3.2, running on a Digital Equipment Alpha Station 200 4/166. Data collected for selected reaction monitoring (SRM) experiments was also processed using Xcalibur, version 1.2 (Finnigan, San Jose, CA). Nitrogen gas served both as the sheath and auxiliary gas, and argon served as the collision gas. The heated capillary temperature was maintained at 250 °C and the sheath gas was 35 psi for direct liquid injection (DLI) analysis and 70 psi for LC/MS analysis. In both cases, the auxillary gas was maintained at 5 units.

Samples were introduced either by direct liquid infusion (DLI) or HPLC. For DLI experiments, samples were introduced to the ESI source with a Harvard Apparatus (Cambridge, MA) syringe pump at a flow rate of 20 μL/min. For HPLC sample introduction, a Hewlett-Packard 1090 HPLC system was used. For glyceride analysis, a Beckman Ultrasphere 5-μm silca column (2.0 mm $\times$ 25 cm) was used with a mobile phase of 8% IPA in hexanes with a flow rate of 150 μL/min. Column effluent was passed through an Applied Biosystems 785A programmable absorbance UV detector. An Upchurch PEEK high-pressure mixing tee was connected next in series for the postcolumn addition of the silver salts. The silver tetrafluroborate ($AgBF_4$) solution of variable concentration was added via a Harvard Apparatus (Cambridge, MA) syringe pump at a flow rate of 75 μL/min. A section of PEEK tubing (1.04 m, 0.25 mm i.d.) allowed approximately 30 s of time for the complexation of the silver to the lipid while delivering effluent to the mass spectrometer.

Silver Ion Complexation with a Series of Cholesterol Esters

Stock lipid solutions (150 μM) of **1**–**6** were prepared in 1% IPA in hexane. These solutions were mixed 1:1 with $AgBF_4$ solution (260 μM in IPA). These samples were introduced to the MS through DLI (10 μL/min). The stability of the silver complex as a function of the degree of unsaturation was evaluated by increasing the tube

lens voltage to cause in-source collision induced dissociation (CID) fragmentation. The typical range of the tube lens values was 20 to 160 V. The electrospray needle was maintained at 4.6 kV, and the heated capillary temperature was 200 °C.

Degree of Instrument Response versus Unsaturation

Stock solutions (50 mM) of **7**–**10** were prepared. Cholesterol esters **7**–**9** were added to separate vials containing the internal standard **10** (50 μM) at concentrations of 0.1 μM, 0.4 μM, 1 μM, 2 μM, 10 μM, 20 μM, 40 μM, 100 μM, and 200 μM in hexanes. Furthermore, for CEs **7**–**9**, two separate 50 mM stock solutions were prepared to give two independent sets of serial dilutions. The samples were mixed 1:1 by volume with $AgBF_4$ (600 μM) in IPA. Samples were introduced into the mass spectrometer by DLI. Prior to selected ion monitoring (SIM) analysis, an averaged spectrum of ~10 scans was acquired in a range encompassing the CEs but not exceeding 30 aμ to determine the precise molecular ion value. Each sample was analyzed three times in SIM mode monitoring the cholesterol esters (CEs) complexed to both the ^{107}Ag and ^{109}Ag isotopes at a scan rate of 1 scan per s for 60 scans. The signal ratios of CE to internal standard for both sets of serial dilutions were averaged and then plotted against the molar ratio of analyte to internal standard. The electrospray needle was maintained at 4.7 kV, the capillary voltage was 20 V, the tube lens voltage was 70 V, the capillary temperature was 250 °C, and the sheath gas was 37 psi.

$AgBF_4$ CIS/MS Analysis of Lipid Mixtures

Equimolar mixtures of **2**, **4**, **8**, **10**, and **11** were prepared to give total lipid concentrations of 50 μM, 100 μM, and 150 μM. The mixtures were analyzed by DLI and pseudo LC/MS in triplicate. For the DLI experiments, the lipids were mixed 1:1 with $AgBF_4$ (300 μM) to give a silver to lipid ratio of 6:1, 4:1, and 2:1 respectively. For the pseudo LC/MS experiments, 30 μL of each mixture was injected into the HPLC effluent and $AgBF_4$ (300 μM) was added via a mixing tee. The HPLC was coupled directly to the mass spectrometer without use of a chromatography column. The resulting peak in the total ion chromatogram was averaged for analysis.

To identify the presence of multi-component complexes, a cholesteryl arachidonate standard (30 μM) was mixed 1:1 with $AgBF_4$ (200 μM) in 40:60 hexanes:IPA to simulate the solvent system used in LC-MS experiments previously reported [4, 13]. The mixture was introduced to the mass spectrometer by DLI.

To investigate the effect of silver ion concentration on the formation of silver ion/lipid adducts, an equimolar mixture of **8**, **6**, **11** was prepared to give a total lipid concentration of 300 μM. The CEs were mixed 1:1 with limiting (50 mM) and excess (1500 mM) amounts of $AgBF_4$ and were introduced into the mass spectrometer by DLI.

AgOTf CIS/MS Analysis of Lipid Mixtures

Silver triflate (AgOTf) was compared to $AgBF_4$ as a source of silver ion. To investigate the effect of silver ion concentration on the formation of silver ion/lipid adducts, an equimolar mixture of **8**, **6**, **11** was prepared to give a total lipid concentration of 300 μM. The CEs were mixed 1:1 with limiting (50 mM) and excess (4500 mM) amounts of AgOTf and were introduced into the mass spectrometer by DLI.

LC-MS Detection of Linoleate Glycerides

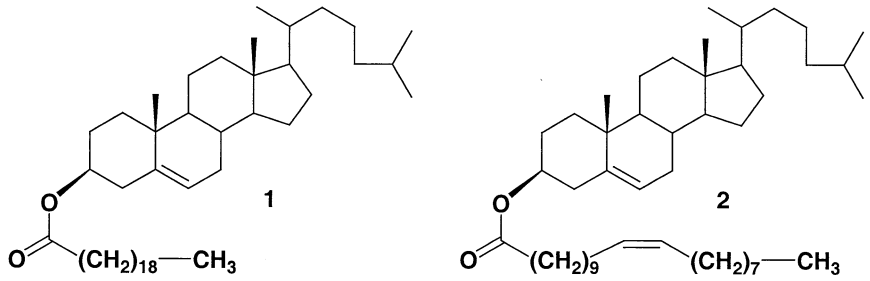
Monolinolein and 1,3-dilinolein were purchased as a mixture of regioisomers and purified by analytical HPLC prior to use (Si column, 8% IPA in hexanes, λ = 205). An equimolar mixture of monolinolein **12**, 1,3-dilinolein **13**, and trilinolein **14** was prepared to give a total glyceride concentration of 300 μM. The glycerides were separated by HPLC (Si column, mobile phase 8% IPA in hexanes, λ = 205 nm) coupled to a mass spectrometer operating in SIM mode. Silver (300 μM) was introduced via a mixing tee. The peaks in the MS chromatogram were integrated and the signal areas were plotted.

Results and Discussion

A series of cholesterol esters of unsaturated fatty acids was examined by CIS-MS in order to show the range of utility of the CIS-MS method. Most of these esters are important lipid natural products that are very prone to undergo free radical oxidation. Some CIS-MS studies of lipid peroxide products have been published elsewhere 4, 13, 14].

Silver Ion Complexation with a Series of Cholesterol Esters

Silver ion is a soft Lewis acid and carbon-carbon double bonds are soft Lewis bases and serve as likely sites for complex formation in unsaturated cholesterol esters (CEs). The two silver isotopes ^{107}Ag and ^{109}Ag are present in a ratio of ~1:1 and the adducts provide distinctive doublets in the mass spectrometer if one silver ion is present and triplets if two ions are present in a complex. In order to probe the relationship between structure and complexation, a series of CEs, **1**–**6**, with 20 carbon fatty acid chains having varying degrees of unsaturation (Ch20:*X*, where X = 0–4) was examined. Each lipid was prepared (see Structure **1**–**6**) as a 75 μM solution with 130 μM $AgBF_4$ in 50% IPA in hexanes. Each sample was infused into the ESI source at 10 μL/min. Data was collected for 60 scans over the mass range 100–1000 aμ. The tube lens voltage was incre-



1, cholesteryl arachidate (Ch20:0)
2, cholesteryl eicosa-11-endoate (Ch20:1)
3, cholesteryl eicosa-11,14-dienoate (Ch20:2)
4, cholesteryl eicosa-11,14,17-trienoate (ω-3 Ch20:3)
5, cholesteryl eicosa-8,11,14-trienoate (ω-6 Ch20:3)
6, cholesteryl eicosa-5,8,11,14-tetraenoate (ω-6 Ch20:4)
7, cholesteryl octadeca-9-dienoate (Ch18:1)
8, cholesteryl octadeca-9,12-dienoate (ω-6 Ch18:2)
9, cholesteryl octadeca-9,12,15-trienoate (ω-3 Ch18:3)
10, cholesteryl nonadeca-10,13-dienoate (Ch19:2)
11, cholesteryl docosa-4,7,10,13,16,19-hexaenoate (Ch22:6)

Structure

mentally increased from 20 to 150 V to induce fragmentation of the CE-silver complex in the ion source. The major ions observed were the positively charged parent ion and fatty acyl fragment after CE cleavage. The energy-resolved CID spectra for each ion was plotted as the ratio of the intensity of either the parent ion $[CE + Ag^{107}]^+$ or the fatty acyl fragment $[RCOOH + Ag^{107}]^+$ over the sum of the intensities of both ions ($I/\Sigma I$) versus tube lens potential. This ratio predicts the stability of these complexes at a given tube lens voltage. As the tube lens voltage is increased, more energy is deposited into the silver ion/lipid adduct, thus the greater the chance for fragmentation. The results of these studies are presented in Figure 1.

As expected, the compound with the fewest double bonds, cholesterol ester **1** (Ch20:0) which contains only the steroid double bond, was observed to form silver ion adducts with the greatest difficulty. Only a low abundance of $[CE + Ag]^+$ was observed at tube lens voltages less than 60 V. Instead the dominant ions at low voltages were intense silver-solvent clusters $[2\ IPA + Ag]^+$ at $m/z = 227/229$. As the tube lens voltage was incrementally increased, the abundance of the silver-solvent clusters decreased, freeing silver ion to coordinate with **1**. The cholesterol ester bond fragmented at tube lens voltages >100 V giving lipid fragment-silver ion complexes in low abundance. The fatty acid fragments of **1** formed silver ion adducts even though there was no unsaturation in the chain. This suggests the silver ion may bind to the ester moiety through coordination of the oxygen lone pairs [18].

The cholesterol esters **2–6** form silver ion-lipid adducts more readily than does **1**. Cholesterol ester **2** (Ch20:1) binds silver ion slightly stronger than **1** but substantial energy was needed to fragment the CE bond as noted by an appearance potential crossover point of 130 V for the formation of ion $m/z = 417$. Intense silver ion-solvent clusters were also observed in the analysis of this cholesterol ester. Compounds **3–6** (Ch20:2; Ch20:3, *ω*-3; Ch20:3, *ω*-6, and Ch20:4) showed facile formation of $[CE + Ag]^+$ parent ions. At low tube lens voltages, silver-IPA solvent clusters were the predominate ions observed but as the tube lens voltage increased, the silver-solvent clusters decreased. In all cases, the optimal signal for intact cholesterol ester $[CE + Ag]^+$ adduct is obtained at tube lens voltages between 70–80 V, after which substantial fragmentation of the ester bond was observed.

Curiously, the position of the double bonds in the chain seems to affect the energy at which CE bond cleavage occurs. Comparing the cholesterol esters **4** and **5**, both having three double bonds in the chain, a difference was observed in the appearance energies for the CE fragmentation. More energy is required to cleave the CE bond for **5** than for **4**. Ester **4** has double bonds located further toward the end of the lipid chain, away from the ester bond than does **5**. The results indicate that the exact nature of the silver binding plays an important role in the energetics of cholesterol ester cleavage.

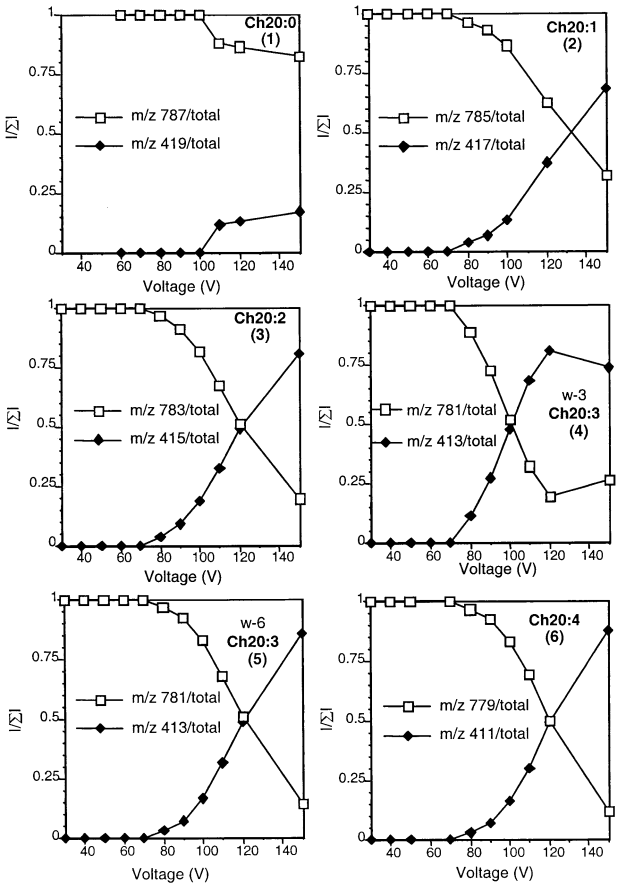


Figure 1. Appearance potential plots for formation of cholesterol ester fragments from in-source CID as a function of tube lens voltage.

Degree of Instrument Response versus Unsaturation

In order to develop an understanding of the nature of CIS-MS as applied to cholesterol esters, we examined the effect of acyl chain unsaturation of the CE on the propensity for formation of silver ion-adducts and thus the instrument response. Cholesteryl 10-13-nonadecadienoate (Ch19:2) **10**, an ester not found in nature, was used at a concentration of 25 μM as an internal standard. The three compounds studied were Ch18:1, **7**, Ch18:2, **8**, and cholesteryl linolenate ω-3 Ch18:3, **9**. The analyte/internal standard solutions were mixed with $AgBF_4$ immediately prior to analysis giving a final concentration of silver salt of 300 μM. Figure 2 shows the response of each CE relative to **10** as a function of concentration. Cholesteryl linoleate (Ch18:2) shows a linear response relative to the internal standard, a result that is expected since the esters have two double bonds and differ only by a methylene group. Cholesterol esters Ch20:1 and Ch18:3, however, do not show linear signal responses with respect to the standard. The relative response for compound Ch20:1 diminishes at higher concentrations relative to **10**. In contrast, Ch18:3 shows little response compared to **10** at low concentrations but at higher concentration, it approaches a response comparable to **10**.

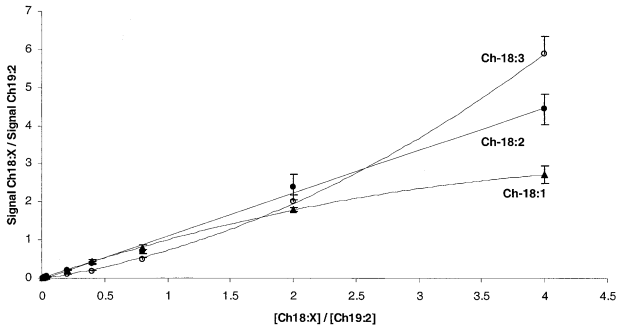


Figure 2. Effect of degree of unsaturation on instrument response at different concentrations. The Ch19:2 standard was 50 μM and the concentration of the Ch20:1 (closed triangles), Ch18:2 (open circles), and Ch18:3 (closed circles) was varied. The analyte/internal standard mixture was mixed 1:1 immediately prior to analysis with a $AgBF_4$ solution of 300 μM in IPA:hexanes 40:60. The final concentrations were 25 μM for the internal standard, 150 μM for $AgBF_4$ while the analyte concentration was varied.

$AgBF_4$ CIS/MS Analysis of Lipid Mixtures

Silver ion forms adducts with unsaturated compounds to form highly stable π complexes [19]. It was therefore expected that the strength of the silver ion-lipid complex would increase with the unsaturation in the lipid. To test this, we examined equimolar mixtures of **2**, **4**, **8**, **10**, and cholesteryl docosa-4,7,10,13,16,19-hexadienoate **11** where total lipid concentrations were 50 μM, 100 μM, and 150 μM. The mixtures were analyzed by DLI and pseudo LC/MS in triplicate. For DLI introduction to the MS, the mixtures of lipids were mixed 1:1 with a 300 μM solution of $AgBF_4$ in IPA:hexanes 40:60. Silver was added to the lipid mixture immediately prior to analysis. Also, since the CEs are not easily separable by normal phase HPLC, pseudo LC/MS was used in which an HPLC pump was directly coupled to the mass spectrometer inlet without a column in order to simulate an LC-CIS-MS experiment without effecting an HPLC separation of the lipid mixture. The silver salt was added to the lipid mixture by the use of a mixing tee. The total lipid injected in 0.5% IPA/hexanes over the course of one min was 1.75 nmoles, 3.5 nmoles, or 5.25 nmoles while the amount of $AgBF_4$ in IPA injected over the course of one min was 22.5 nmoles.

Figure 3 shows the results of the DLI and pseudo LC/MS analysis of a lipid mixture of 150 μM and 5.35 nmoles respectively. Contrary to our expectations, **2**, having one double bond, was found to have the most intense signal both in the DLI and the pseudo LC/MS experiments. Highly unsaturated lipids gave poorer responses than more saturated analogs.

The difference in intensities is most apparent in the DLI experiment, Figure 3a. The sample better resembles an equimolar mixture when introduced by pseudo LC/MS, Figure 3b. Since silver is added online in the

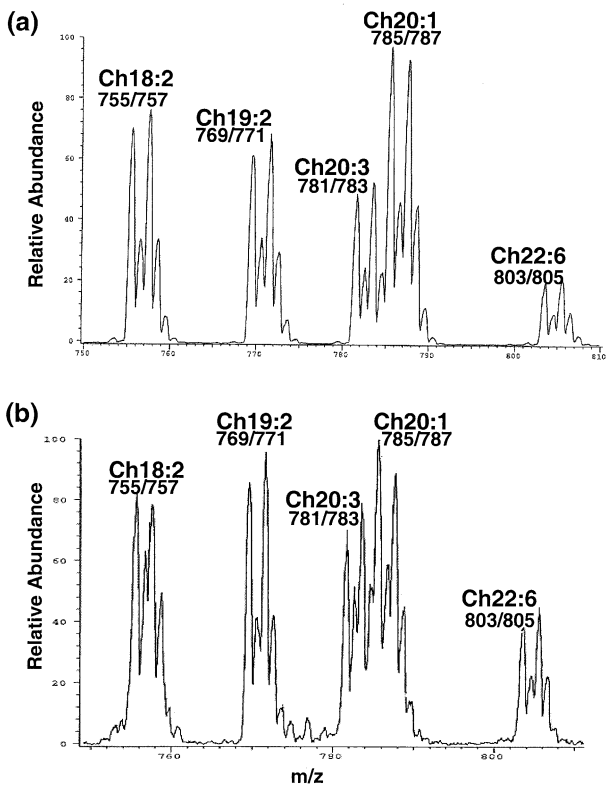


Figure 3. Analysis of an equimolar mixture of **2**, **4**, **7**, **9**, and **10** by DLI and pseudo LC/MS. (**a**) DLI: Total lipid was 75 μM, $AgBF_4$ was 150 μM in IPA:hexanes 40:60. $AgBF_4$ in IPA was added to the lipid mixture in hexanes immediately prior to analysis. (**b**) Pseudo LC/MS: HPLC pump coupled directly to the MS inlet without a column. The silver salt was mixed into the lipid mixture by the use of a mixing tee. Total lipid injected in 0.5% IPA over the course of one min was 5.25 nmoles, while the amount of $AgBF_4$ in IPA injected over the course of one min was 22.5 nmoles.

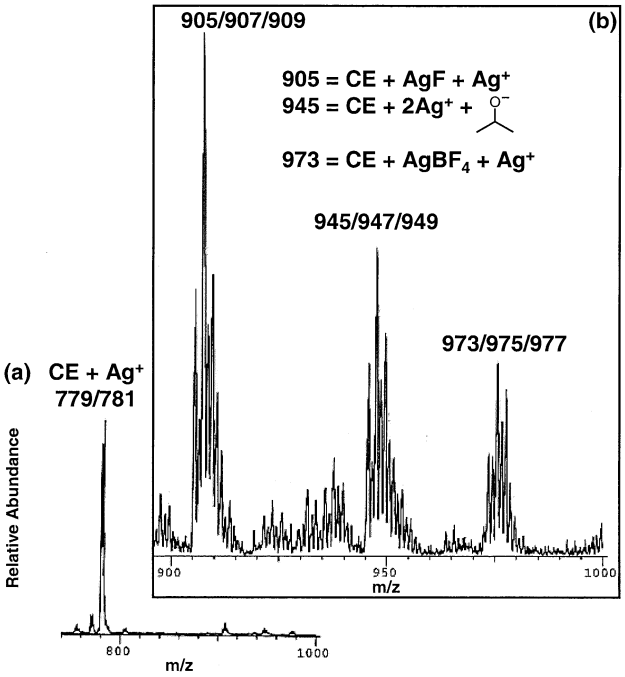


Figure 4. Formation of adducts containing multiple silver ions using $AgBF_4$. (**a**) Full DLI scan of a cholesteryl arachidonate standard (15 μM) in 60:40 hexanes:IPA with 100 μM $AgBF_4$. (**b**) Expansion of the 900 to 1000 aμ region of spectrum from (**a**).

pseudo LC/MS experiment, approximately 30 s are allowed for complexation of the silver and lipid. On the other hand, DLI involves a longer time from sample preparation to analysis since in this experiment, the lipid is mixed with the silver solution, loaded into a syringe, and introduced to the mass spectrometer. The relative intensities of lipids **2**, **4**, **8**, **10**, and **11** for the 50 and 100 μM total lipid mixtures were comparable to those shown here.

One possibility that may account for the results of the studies of lipid mixtures is that compounds with a high degree of unsaturation form larger, complex adducts with silver, thus reducing the $[CE + Ag]^+$ signal observed. To investigate the possibility that multi-component complexes are formed, each CE was examined individually by DLI scanning (100 μM $AgBF_4$ and 15 μM CE) and each showed a group of triplets in the 900–1100 aμ region of the spectrum. Figure 4 shows the spectrum for **6**, which is typical of all the CEs. Adducts containing silver ion, AgF, $AgBF_4$, and 2-propanoxide (generated from abstraction of a proton from 2-propanol) were detected as shown in 4b. It is interesting to note that incremental increasing of the tube lens voltage from 30 to 150 V had no effect the abundance of the signal for these multiple silver adducts (data not shown). This illustrates that at even high energies, these complexes are extremely stable evidenced by the fact that they do not break apart into their components.

To investigate the effect of silver ion concentration on the formation of the silver ion/lipid adducts, the concentration of the silver salt was varied and an equimolar mixture of **6**, **8**, and **11** was analyzed by DLI. The experiments were carried out such $AgBF_4$ was both limiting (25 μM) and in excess (750 μM) of the total CE concentration (75 μM). When silver was in excess, cholesterol esters with more unsaturation in the molecule gave low intensity signals, Figure 5a. When silver was limiting, the opposite was true, Figure 5b. In this case, the greater the unsaturation, the greater the signal.

AgOTf CIS/MS Analysis of Lipid Mixtures

The formation of a $[CE + AgF + Ag]^+$ adduct suggests that BF_3 may be forming in the gas phase and may be the source of the phenomenon described above. Boron trifluoride is a hard Lewis acid and may cause the decomposition of the cholesterol esters reducing the signal for the $[CE + Ag]^+$ adduct. For this reason, we examined an equimolar mixture of **6**, **8**, and **11** by DLI using silver triflate (AgOTf) as a source of silver ion. The triflate anion is inert and should not decompose in the mass spectrometer. In addition to the $[CE + Ag]^+$ adducts, when AgOTf was used in excess of CE, large multi-component complexes appeared in the 1000–1800

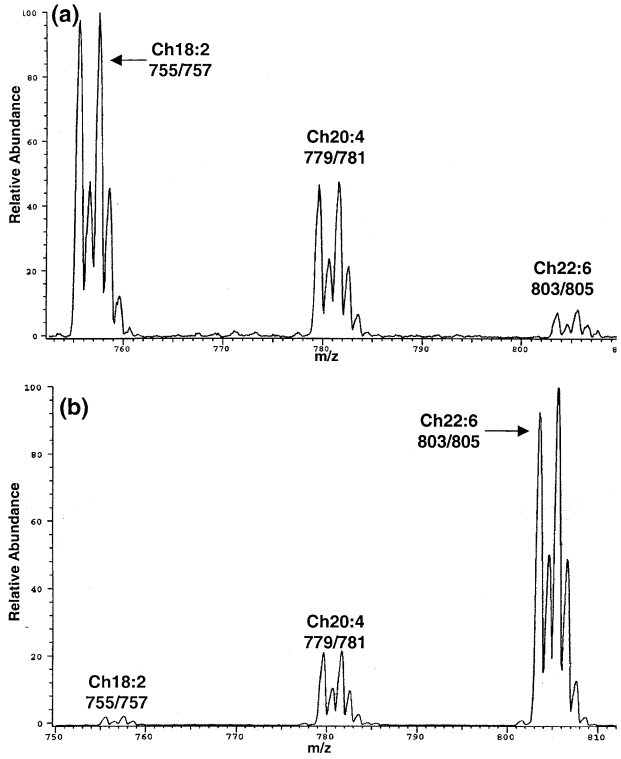


Figure 5. Variation of the silver to lipid ratio using $AgBF_4$. An equimolar mixture of cholesteryl linoleate, 5,8,11,14-cholesteryl eicosatetraenoate, and cholesteryl docosahexaenoate where the total lipid concentration was 75 μM was introduced into the mass spectrometer by DLI. (**a**) Silver concentration was 750 μM. (**b**) Silver concentration was 25 μM.

aμ region of the spectrum. Each CE formed adducts containing 1 to 3 AgOTfs and one silver ion, Figure 6. Also, $[n\text{AgOTf} + \text{Ag}]^+$ adducts where n = 1–6 were observed in both the Ch20:4 spectrum and a blank of AgOTf in IPA:hexanes 40:60.

We also compared the $[\text{CE} + \text{Ag}]^+$ signals of an

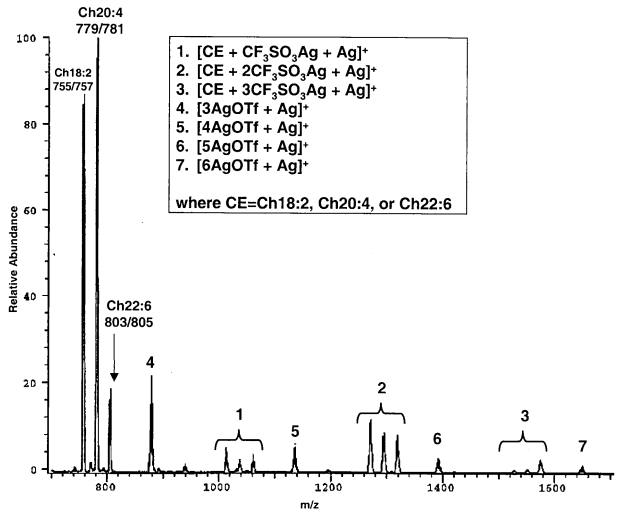


Figure 6. Formation of adducts containing multiple silver ions using AgOTf. Full DLI scan of a cholesteryl arachidonate standard (25 μM) in 60:40 hexanes:IPA with 2250 μM $AgBF_4$.

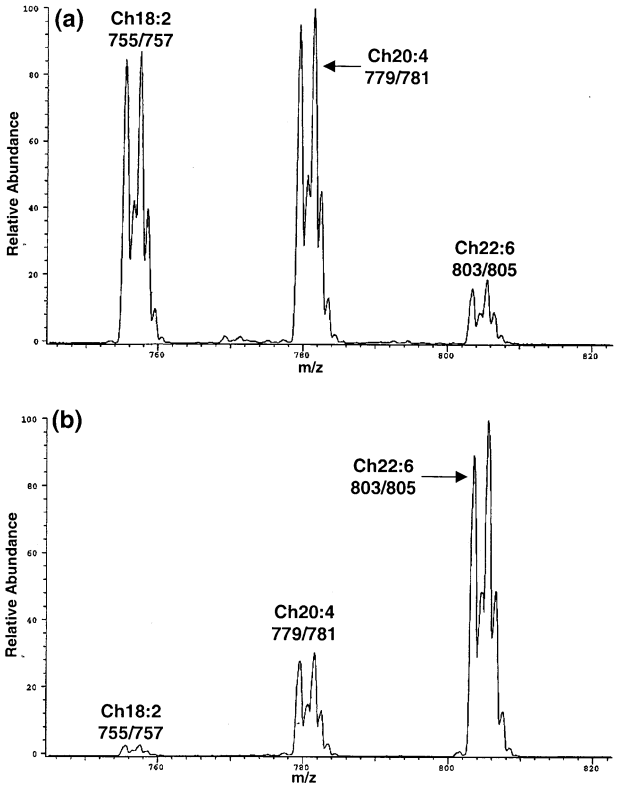


Figure 7. Variation of the silver to lipid ratio using AgOTf. An equimolar mixture of cholesteryl linoleate, 5,8,11,14-cholesteryl eicosatetraenoate, and cholesteryl docosahexaenoate where the total lipid concentration was 75 μM was introduced into the mass spectrometer by DLI. (**a**) Silver concentration was 1500 μM. (**b**) Silver concentration was 25 μM.

equimolar mixture **6**, **8**, **11** by DLI using AgOTf as the coordinating salt. Silver triflate was both limiting (25 μM) and in excess (2250 μM) of the total CE concentration (75 μM). It appears that the use of AgOTf as a source of silver ion gives a similar response in the mass spectrometer as $AgBF_4$. When silver was limiting, cholesterol esters with less unsaturation in the molecule gave low intensity signals, Figure 7b. When silver was in excess, the compound with the most unsaturation gave the lowest intensity signal, Figure 7a. The signals for Ch18:2 and Ch20:4, though, were nearly equal in intensity and a greater silver to lipid ratio was needed to effect significant coordination to Ch18:2. We believe this is because BF_4^- is a weaker coordinating anion than TfO^- and $AgBF_4$ is easily solvated whereas AgOTf is not [20–22]. Therefore, BF_4^- is more readily displaced by π-donors such as olefins than TfO^- and would give a greater $[\text{CE} + \text{Ag}]^+$ for CEs in the mass spectrometer. However, since the signals for the cholesterol esters show similar dependence upon the concentration of silver triflate as they do for $AgBF_4$, it suggests that this dependence has nothing to do with CE decomposition by BF_3.

Thus, we interpret the experiments described as follows: Compounds with higher degrees of unsaturation form stronger silver ion/lipid adducts than com-

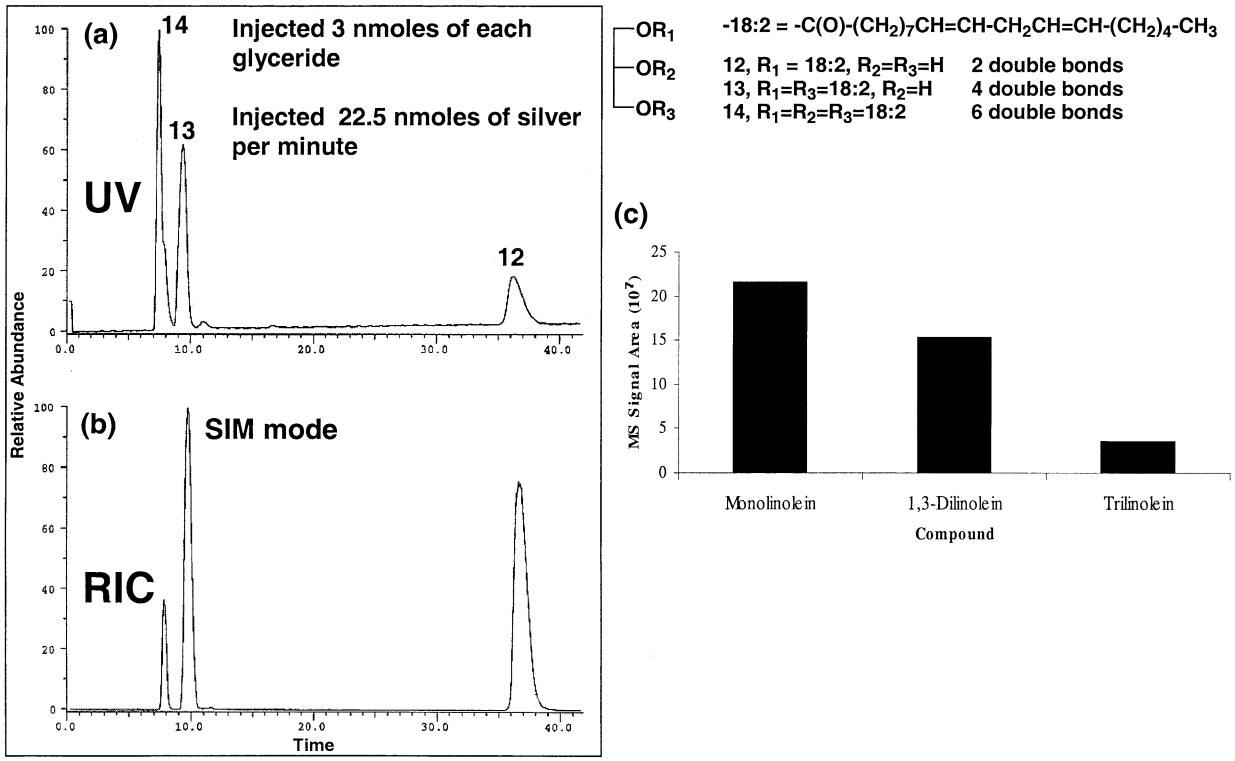


Figure 8. LC/MS detection of linoleate glycerides. **I**, trilinolein; **II**, 1,3-dilinolein; **III**, monolinolein. (**a**) Chromatograms of an equimolar mixture of 14,trilinolein; 13,1,3-dilinolein; 12,monolein with UV detection at 205 nm. (Mobile phase 8% IPA in hexanes); (**b**) LC/MS chromatogram of an equimolar mixture of monolinolein, 1,3-dilinolein, and trilinolein with the mass spectrometer operating in SIM mode; (**c**) Comparison of the areas of the individual $[M + Ag]^+$ peaks analyzed in SIM mode by LC-CIS-MS. The graph is a result of one analysis.

pounds with fewer double bonds. At high concentrations of silver salt, adducts containing multiple components, as seen in Figure 3b, form more readily, reducing the amount of the simple $[CE + Ag]^+$ adduct. Since **8** has only two double bonds, it forms simple silver ion/lipid complexes and is less likely to form the larger complexes involving multiple silver ions seen for **6** and **11**. As a result, a stronger $[CE + Ag]^+$ signal is observed. At concentrations of silver salt that are limiting, **11** binds to silver ion at the expense of **6** and **8** due to its higher degree of unsaturation and thus **11** has the largest response in the mass spectrometer.

This offers an explanation for the response curves seen in Figure 2. At low concentrations of lipid, silver salt is in large excess of lipid. A larger portion of Ch18:3 than Ch19:2 forms multi-silver complexes due to its greater propensity to bind to silver ions. This lowers the Ch18:3/Ch19:2 signal ratio. At higher concentrations of lipid, the ratio of silver to lipid is decreased lowering the percentage of Ch18:3 forming multi-silver complexes and increasing the abundance of the $[CE + Ag]^+$ signal so that the Ch18:3/Ch19:2 signal ratio is higher. This would account for non-linearity in the calibration curves for analytes with a greater number of double bonds than the internal standard. For analytes with fewer double bonds than the internal standard, the calibration curve also appears nonlinear but levels off at lower silver-lipid ratios.

LC/MS Detection of Linoleate Glycerides

Since the cholesterol esters **1**–**11** are not readily separable by normal phase HPLC, an equimolar mixture of monolinolein **12**, 1,3-dilinolein **13**, and trilinolein **14**, was examined, Figure 8. The compounds in this series have, respectively, 2, 4, and 6 double bonds. Figure 8a shows the HPLC and MS chromatogram for this mixture with UV detection at 205 nm. The areas of the glyceride peaks in the MS chromatogram operating in SIM mode were compared to assess the silver ion/glyceride binding efficiency. Similar to the results found with the cholesterol esters, when silver was in excess, **12** (2 double bonds) had the greatest signal in the mass spectrometer while **14** (6 double bonds) had the smallest signal, 8c.

Conclusions

CIS-MS is a useful technique in the identification of unsaturated organic compounds and, when coupled to an HPLC, it proves to be quite powerful in analyzing complex mixtures. The propensity of silver ion to bind

to cholesterol esters increases with the degree of CE unsaturation to the point where large multi-silver complexes are formed. Since the kinetics of the formation of these larger complexes is undetermined, direct quantitation by the use of CIS-MS will likely prove to be difficult if not impossible, due to the competitive nature of the silver ion complexation. Isotopic dilution techniques provide quantitative information, however, if standards are available and the results of studies using deuterated lipids as standards in biological oxidations will be reported in due course.

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