APCI–MS for lipid analysis

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Atmospheric pressure chemical ionization (APCI) is a soft ionization technique that allows the direct coupling of the effluent from a liquid or supercritical fluid chromatographic system to a mass spectrometer operating at high vacuum. Very simple in its design, an APCI source allows the desolvation and ionization of analytes at ambient pressure followed by sampling through a capillary orifice into an area of intermediate vacuum, followed by introduction into the high-vacuum region of the mass analyzer. An APCI source is capable of accommodating high flow rates, up to 2 mL/min, and thus does not require the use of specialized microflow chromatographic systems or splitting of the effluent stream. For this and other reasons, APCI–MS (mass spectrometry) has found many applications in diverse areas. This review will focus on applications of interest to those engaged in lipid analysis.

Mechanism of ionization

As the name atmospheric pressure chemical ionization implies, chemical ionization occurs by interaction of analyte molecules with gaseous ionizing molecules present at atmospheric pressure in the source. The first reports of a source that produced chemical ionization at atmospheric pressure used a Ni-63 foil as a source of electrons (1,2). The Ni-63 foil was later replaced by a corona discharge needle, and the modern incarnation of the APCI source was born. These sources are now commonly available commercially. Several manufacturers offer APCI sources on new mass spectrometer systems, but they all have similar designs. One such design is shown in Figure 1. Generally, the chromatographic effluent is connected to a piece of capillary tubing from which the effluent is sprayed down the center of a heated cylinder (vaporiz-

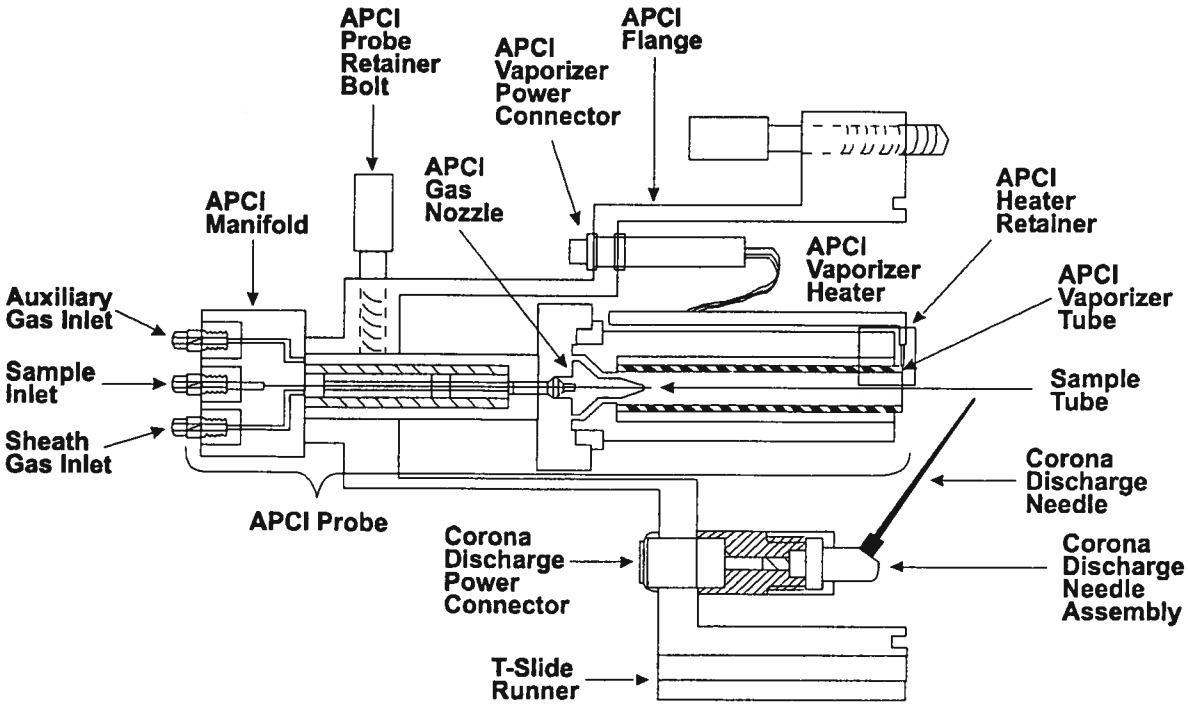

Figure 1. APCI probe assembly includes the APCI flange, APCI probe, and the corona-discharge needle assembly (Figure reprinted with permission of Finnigan MAT Inc.).

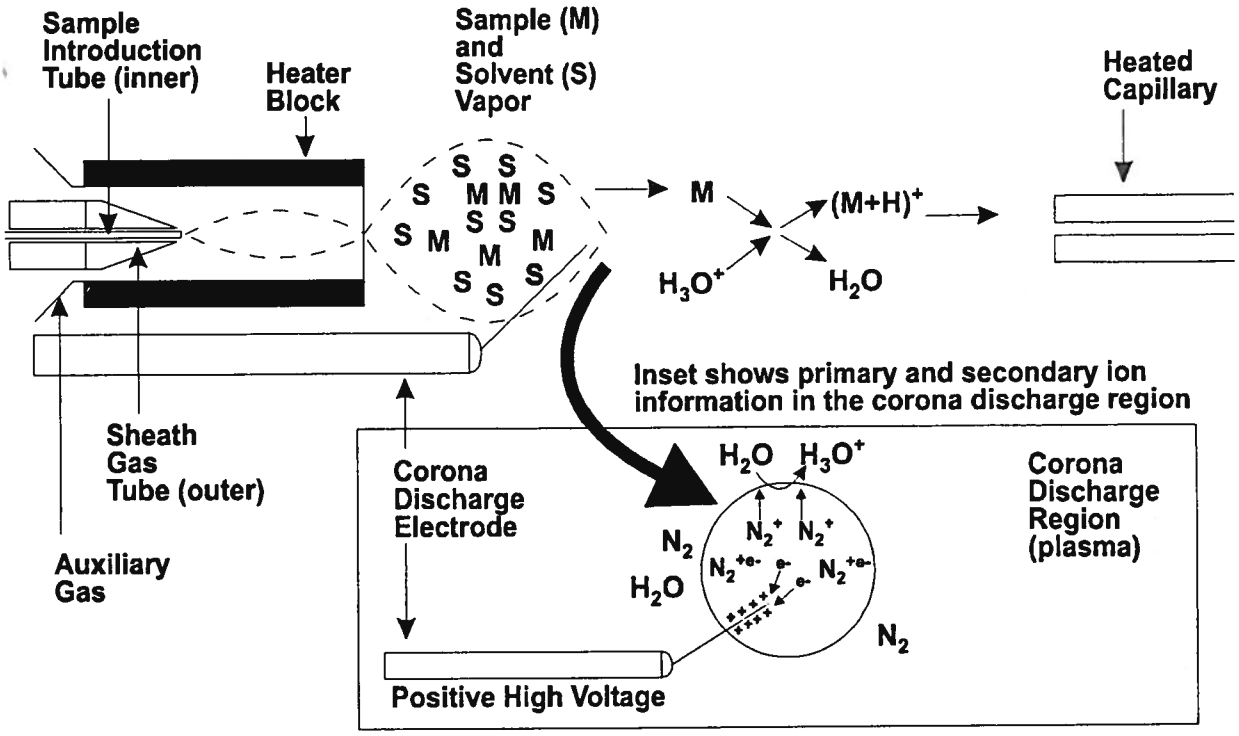

Figure 2. Basic process for APCI; inset shows primary and secondary ion formation in the corona discharge region (Figure reprinted with permission of Finnigan MAT Inc.).

er), with a sheath gas (usually high-purity N_2) flowing coaxially around the capillary tubing. This heated sheath gas acts to desolvate the effluent and produces a molecular mist which then passes by a corona discharge needle held at ~2 to 6 kV. A plasma of ionizing molecules from atmospheric gases surrounds the corona discharge needle and these interact with passing analyte molecules to produce analyte ions. These analyte ions are then sampled into the high-vacuum region of the mass spectrometer through a small heated capillary. Some source designs use an additional "curtain gas" flowing in the opposite direction of the analyte ions to help break up ion–molecule clusters formed with atmospheric components. Also, a coaxial auxiliary gas often is used to help focus the effluent down the center of the heated vaporizer during desolvation. The use of coaxial gases means analyte molecules do not contact the surface of the vaporizer cylinder, so even molecules normally considered to be thermally labile can be ionized intact.

The ionization that occurs in the APCI source in positive ion-mode involves two sets of processes. The first process is a set of reactions which result in formation of hydronium ions, H_3O^+. The second process is protonation of analyte molecules by the hydronium ions. These processes are summarized in Figure 2. The set of reactions leading to formation of hydronium ions and related clusters was described by Horning *et al.* (1). In short, they involve formation of nitrogen ions which undergo charge transfer to atmospheric water molecules to form hydronium ions. These hydronium ions most often act as the primary source for the ionization of analyte molecules, but this is not always the case. Since chromatographic effluent vapor is also present in the APCI source, components therein also can produce ionic adducts. Thus, some consideration must be given to the choice of a solvent system that will minimize undesirable adduct formation. Conversely, examples will be shown in which components in the flow system produce diagnostically useful adducts and in which modifiers are added to produce beneficial effects. Nevertheless, one of the strongest benefits of APCI as an ionization source for MS is its flexibility and the broad range of effluent systems that can be employed. Ionic modifiers (e.g., NH_4OH or CH_3COOH) and buffers (e.g., NH_4OOCCH_3) which are used to optimize chromatographic performance often are incorporated without deleterious effects. Only inorganic salts which might block the inlet capillary present a difficulty.

Applications

Fatty acid derivatives

APCI–MS has been available commercially since the mid-1980s and has been applied to many analytical problems. Applications have mostly centered on different types of drug analysis, pesticide analysis, and other types of molecules.

Lipid analysis using liquid chromatography (LC)/APCI–MS appears to have started with the reversed-phase (RP) high-performance liquid chromatography (HPLC)/APCI–MS analysis of fatty acids (FA) as their anilide derivatives, reported in 1988

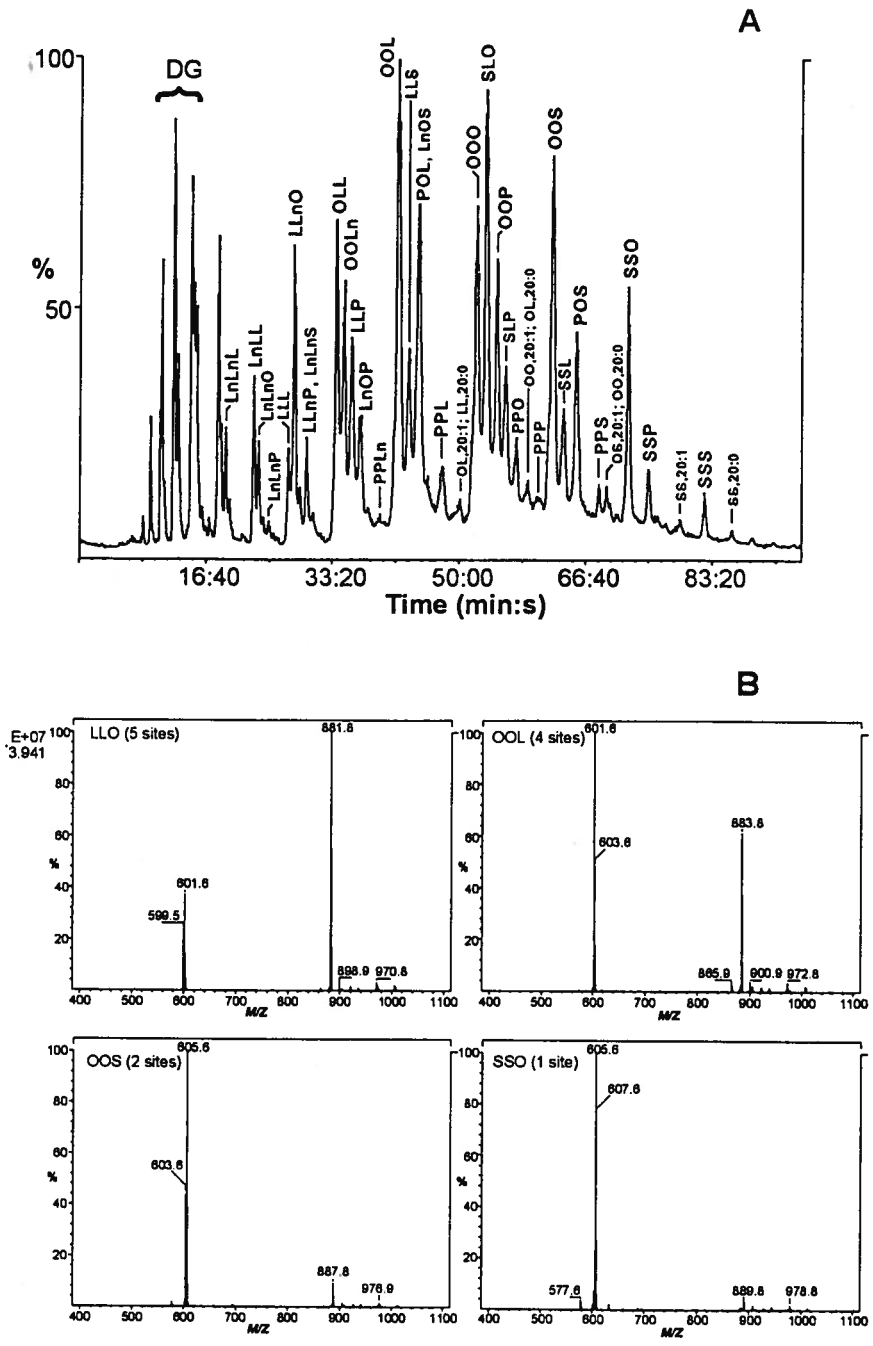

Figure 3. A: Reconstructed ion chromatogram for interesterified canola oil/trisaturates blend (80:20). B: Mass spectra of triacylglycerols containing 5, 4, 2, and 1 unsaturation sites, respectively.

by Kusaka *et al.* (3). They found that the anilide derivatives provided improved sensitivity under APCI conditions compared to the fatty acid methyl esters or free acids. Prominent $[M + H]^+$ ions allowed ready identification of numerous short- and long-chain, saturated and unsaturated fatty acid anilides. Later work published in 1992 by Ikeda and Kusaka (4) compared six different amide derivatives of palmitic acid or a saturated fatty acid mixture. The sensitivity of the six amide derivatives was found to decrease in the following order: *N-n*-propylamide (most sensitive and facile) > anilide > *N,N*-diethylamide ≈ amide > *N,N*-diphenylamide > *N*-1-naphthylamide (least sensitive). *N-n*-Propylamide derivatives also were made from hydroxy fatty acid standards and from FA (nonhydroxy and hydroxy) in rat brain extracts. Similarly, work published in 1993 by Kusaka and Ikeda (5) used RP-HPLC/APCI–MS for analysis of FA as their 3-methyl-7-methoxy-1,4-benzoxazin-2-one derivatives. These fluorescent derivatives were monitored using an ultraviolet-visible (UV-Vis) detector at 355 nm, as well as the APCI–MS detector. Derivatized hydroxy and hydroperoxy standards were run for comparison to oxidation products formed by photooxidation of FA and bovine liver lecithin. The oxygen-containing FA gave distinct and diagnostic near-molecular ions caused by loss of either H_2O or H_2O_2.

Triacylglycerols

Around this same time, Tyrefors *et al.* (6) reported examples of APCI–MS spectra obtained for several classes of lipids separated using supercritical fluid (SFC)/APCI–MS with an open tubular column. They reported the separation of methyl stearate, trilaurin, cholesterol, and cholesterol palmitate. This first report demonstrated that large neutral molecules such as triacylglycerols (TAG) could be ionized intact using APCI and that structurally useful fragments resulted. Still, it was not until 1995 that Byrdwell and Emken (7) reported the first separation of a mixture of TAG using RP-HPLC coupled to APCI–MS and started to characterize the fragmentation behavior that different TAG structures exhibited under APCI–MS conditions. It was observed that the fragmentation behavior of TAG was directly dependent on the amount of unsaturation present in the molecules. Mass spectra, such as those in Figure 3, of TAG with more than four sites of unsaturation were found to exhibit protonated molecular ions, $[M + H]^+$, as the base peaks, while those with fewer than three sites of unsaturation were found to exhibit diacylglycerol fragment ions, $[M - RCOO]^+$, as the base

peaks. The base peaks in mass spectra of TAG having three or four sites of unsaturation could be either of these ions, as seen in spectra of LLP vs. OOL (both with four sites, where L = linoleic acid; P = palmitic acid; and O = oleic acid) in Reference 17. This behavior appears to depend to some extent on the specific design of the APCI source employed, however, because the spectrum of trilaurin shown by Tyrefors *et al.* (6) exhibited a much stronger protonated molecular ion than has been reported by others. Spectra of trisaturated TAG reported by others have shown little or no protonated molecular ion, unless special measures were taken, as described below.

Initially, APCI–MS for intact TAG analysis was applied to normal, genetically modified, and specialty seed oils. Neff and Byrdwell (8) reported the analysis of normal, high-stearic and high-palmitic soybean oils using APCI–MS, an evaporative light-scattering detector (ELSD), and a flame-ionization detector (FID). The APCI–MS data were obtained using a RP separation with a propionitrile/hexane solvent system. Spectra therein exhibited adducts formed from addition of the propionitrile solvent to the TAG molecules. A small peak arising from addition of water to diacylglycerol fragment ions and protonated molecular ions is also common under APCI–MS conditions.

Neff and Byrdwell (9) then reported the analysis of the specialty seed oils from *Crepis alpina* and *Vernonia galamensis*, which contain crepenynic acid (*cis*-9-octadecen-12-ynoic acid) and vernolic acid (*cis*-12,13-epoxy-*cis*-9-octadecenoic acid), respectively. Spectra of TAG that contained crepenynic acid were very similar to normal TAG, exhibiting mostly protonated molecular ions, since most of the TAG contained several sites of unsaturation. Spectra of vernolic acid-containing TAG exhibited more complex fragmentation patterns than normal TAG. The epoxy group at the Δ12 position underwent intra-annular cleavage to yield a 12:2 chain on the remaining TAG and an oxygen-containing leaving group. The leaving group then added to other intact TAG molecules to produce adducts. Diacylglycerol fragments also underwent the intra-annular cleavage to produce a 12:2 remnant on the diacylglycerol fragment. These samples demonstrated the capability of APCI–MS to identify, in one chromatographic run, numerous TAG molecular species for which commercially available standards were not available.

Byrdwell and Neff (10) recently reported the RP-HPLC/APCI–MS analysis of intact hydroxy-containing TAG. Hydroxy-containing TAG have traditionally been very difficult to analyze using mass spectrometric techniques because the hydroxy groups are so easily lost to yield ions that are identical to normal TAG. However, APCI–MS was able to directly detect numerous mono-, di-, and trihydroxy-containing TAG in castor oil and in two species of *Lesquerella*. APCI–MS mass spectra showed sequential loss of each of the hydroxy groups in those containing more than one group, and a set of diagnostically useful adduct ions was formed from acetonitrile in the APCI source.

Argentation chromatography

Laakso and Voutilainen (11) were the first to combine another popular method of TAG separation, silver-ion (argentation) (AG) chromatography, with APCI–MS. They demonstrated the separation of α- and γ-linolenic acid-rich seed oils with detection by both ELSD and APCI–MS. This chromatographic system provided separation based primarily on the amount of unsaturation within each TAG, with further separation dependent on the position of the double bonds in the acyl chains. The distribution of the unsaturation within the TAG species also provided a degree of separation, so that a TAG with three monounsaturated FA eluted before a TAG containing a combination of saturated, monounsaturated, and diunsaturated FA. α-Linolenic acid-containing TAG were retained slightly longer than γ-linolenic acid-containing TAG on the AG column, so the chromatographic separation provided valuable information for differentiation of TAG having identical masses. Within each chromatographic peak having the same amount of unsaturation, carbon chain lengths differed, so mass spectral data were capable of identification of the individual TAG molecular species. Quantitative analysis was performed by using the areas under peaks in the reconstructed ion chromatograms, but overlapped TAG having the same number of sites of unsaturation were not distinguished in the percentage composition. The AG chromatography/APCI–MS technique identified 43 molecular species in cloudberry oil, 39 species in evening primrose oil, 79 species in borage oil, 44 species in alpine currant oil, and 56 species in blackcurrant seed oil.

A more recent report by Laakso (12) utilized RP-HPLC with APCI–MS detection for analysis of these same α- and γ-linolenic acid-rich seed oils. This separation, based on an entirely different retention mechanism from that of AG chromatography described above, resulted in a greater number of separate peaks, with less overlap of coeluting species. Nevertheless, the complexity of the natural TAG samples precluded complete separation of all molecular species. Still, different masses arising from fragments of overlapped TAG allowed differentiation of most overlapped TAG, and allowed assignment of most fragments to specific molecular species. Some intractable overlaps still remained, however, which simply highlighted the need for powerful instrumental techniques for analysis of TAG mixtures. The ability of APCI–MS to distinguish overlapped TAG far exceeds the capabilities of two-dimensional detectors commonly used. The RP-HPLC/APCI–MS method was able to identify 52 molecular species in cloudberry seed oil, 40 molecular species in evening primrose oil, 80 molecular species in borage oil, and 64 molecular species in blackcurrant seed oil. In blackcurrant seed oil, identification was complicated by the presence of both α- and γ-linolenic acids having identical masses. Fortunately, the α-linolenic acid-containing TAG eluted before the corresponding γ-linolenic acid-containing TAG in this seed oil. The increased number of

molecular species identified by RP-HPLC/APCI–MS compared to Ag-HPLC/APCI–MS was attributed to minor components detected at low levels.

Triacylglycerol positional isomers

A very important aspect of TAG structural characterization is the identification of the specific position of individual fatty acyl chains on the glycerol backbone. Laakso and Voutilainen (11) and Mottram and Evershed (13) both described the preferential loss of fatty acyl chains in the *sn*-1(3) positions over loss of the fatty acyl chain from the *sn*-2 position under APCI–MS conditions. Laakso and Voutilainen (11) presented data for three isomeric pairs of TAG standards that demonstrated differences in the fragmentation patterns. Dipalmitoyl-oleoyl glycerol (POP and PPO) theoretically would be expected statistically to produce $[PO]^+$ and $[PP]^+$ fragments in a ratio of 2:1, or have an abundance of 100% (base peak) and 50%, respectively. They showed that POP gave abundances of 100% and 33.5% for the diacylglycerol fragments, respectively, while PPO gave abundances of 100% and 88.7%, respectively. Similarly, OPO and OOP theoretically would be expected to give $[PO]^+$ and $[OO]^+$ diacylglycerol fragments having abundances of 100% and 50%, respectively. OPO yielded abundances of 100% and 8.5% for the diacylglycerol fragments, respectively, while OOP gave abundances of 100% and 69.9%, respectively. Data for OLnO and OOLn (Ln = linolenic acid) similarly showed that the loss of *sn*-1(3) fatty acyl chains was favored over loss of the *sn*-2 chain. Mottram and Evershed (13) presented data for the isomeric pairs of SSO/SOS and PPO/POP, as well as for a TAG containing three different FA. As mentioned, statistically PPO and POP would be expected to produce $[PP]^+$ and $[PO]^+$ fragments in a ratio of 1:2 or 0.5. They showed that PPO gave diacylglycerol fragments in a ratio of 0.95, while POP produced these fragments in a ratio of 0.20. This compares to ratios of 0.89 and 0.34 given by the data of Laakso and Voutilainen (11), when calculated in the same way.

Unsaturated FA generally were esterified in the sn-*2 position, while saturates were incorporated into the* sn-*1(3) positions.*

Mottram and Evershed (13) showed that SSO and SOS, which should yield $[SS]^+$ and $[SO]^+$ fragments having abundances in the ratio of 1:2 (= 0.5) gave ratios of 1.07 and 0.29 for these two TAG isomers, respectively. The mass spectrum of 1-myristoyl-2-oleoyl-3-palmitoyl glycerol (MyOP) standard exhibited the smallest diacylglycerol ion abundance for the [MyP]+ fragment, showing that the fragment representing loss of the FA in the *sn*-2 position was the least favored in TAG having three different FA. The differences in the diacylglycerol ratios obtained by the different authors for PPO/POP indicate that, while the diacylglycerol fragment ratio can be used qualitatively to identify the most likely isomer, some work remains to be done before quantification of the relative amounts of the isomers may be performed with confidence using these abundances. Mottram *et al.* (14) extended the determination of positional isomers by APCI–MS to the analysis of several vegetable oils: blackcurrant, blue poppy seed, evening primrose, extra-virgin olive, hazelnut, maize, rapeseed, and soybean oils. They presented a list of the most likely positional isomers present for an extensive list of TAGs from the various oil samples, with a confidence rating for each of the assignments. Their results agreed with results obtained by other methods that indicated unsaturated FA, generally, were esterified in the *sn*-2 position, while saturates were incorporated into the *sn*-1(3) positions. Their results also presented the possibility that when both positional isomers are present, they may be slightly chromatographically resolved so that the front of a chromatographic peak contains mostly one isomer while the tail of the peak contains the other isomer.

Triacylglycerol hydroperoxides

Kusaka *et al.* (15) also mentioned the ability of LC/APCI–MS to discriminate between the *sn*-2 and *sn*-1(3) positions of FA on the glycerol backbone. Their report also described the first application of APCI–MS to analysis of TAG hydroperoxides produced by irradiation of a TAG standard with a tungsten lamp for 20 hr at 15°C. The primary fragments reported for the resultant hydroperoxides were $[M - H_2O + H]^+$ and $[M - H_2O_2 + H]^+$. There is uncertainty, however, why the masses reported by these authors were 2 amu larger than those given by other groups. A report by Herderich *et al.* (16) showed APCI–MS mass spectra for the hydroperoxides of the fatty acid linoleic acid. These authors used APCI–MS/MS to produce daughter ion spectra of $[M + NH_4]^+$ precursors, which gave diagnostic fragments that allowed identification of the regioisomeric hydroperoxide isomers.

Triacylglycerol quantitation

The dependence of the fragmentation patterns of TAG on the degree of unsaturation had distinct implications for quantification. The first systematic treatment of the use of APCI–MS for quantitative analysis of TAG molecular species was presented by Byrdwell *et al.* (17). Several alternative methods for quantification of TAG were presented and compared. Conventional calibration curves were constructed for a set of mono-acid TAG standards, using deuterated d_{12}-tripalmitin as an internal standard. It was shown that

calibration curves with good correlation coefficients could be constructed for individual TAG molecular species. Thus, if one or a few TAG molecular species are of interest, these may be quantified by comparison to standards of known composition. However, most TAG samples of natural origin contain 40 to 100 or more individual TAG species for which quantitative estimation is sought. Construction of calibration curves for every one of these TAG was considered impractical, so several alternative options were investigated.

The use of a synthetic mixture of 35 TAG to produce response factors for application to natural mixtures proved to be unsatisfactory. Two methods that did provide useful results were demonstrated. The quality of the quantitative estimation was determined by comparison of the TAG composition determined by APCI–MS to the known composition for a TAG mixture, when possible, and by comparison of the FA composition calculated from the TAG composition determined by APCI–MS to the FA composition determined by calibrated gas chromatography–FID (GC–FID). By both of these measures, quantification was performed by APCI–MS which showed less error in the TAG composition and FA composition than was obtained by HPLC with FID detection, which is generally accepted without response factors.

Some of the decrease in the amount of error associated with the use of APCI–MS was undoubtedly due to the ability of APCI–MS to differentiate, by mass, completely overlapped TAG peaks which are not resolved by liquid chromatography (LC)–FID. The first method for quantification utilized application of response factors calculated for a randomized mixture to a corresponding nonrandomized TAG mixture. The composition of a completely randomized sample was readily calculated from the random distribution of the component FA (determined by GC–FID). The known percentage of each TAG in the randomized sample was then divided by the raw area percentage determined by APCI–MS to produce a response factor for each TAG. These response factors were applied to a nonrandomized sample of similar composition. This approach was used to produce response factors for randomized soybean oil which were then applied to normal soybean oil, and for randomized lard which were then applied to normal lard. This method produced less average relative error than the data obtained by LC–FID for both sets of samples.

Although this method proved useful, the disadvantages of the method

were that chemical randomization of a sample was required and that the thorough randomization of the sample had to be confirmed independently by a method such as lipase hydrolysis of the sample. Both of these steps required additional effort and expertise.

A second method for quantification proved superior. Response factors were calculated for each FA by dividing the FA composition determined by calibrated GC–FID by the FA composition calculated from the TAG composition. These FA response factors were then multiplied together to produce response factors for each TAG. This method provided the least error in both the TAG composition and in the FA composition. This method had the advantages of being applicable to both normal and randomized samples alike, and of requiring very little extra effort (most laboratories engaged in lipid analysis determine fatty acid methyl ester composition routinely). Both of the methods for quantification compensated for such things as isotope contributions (^{13}C and ^{18}O) to extracted ion chromatographic peaks, which could cause some peaks to be overrepresented. This and other factors involved in quantification of TAG, as well as a more thorough treatment of the characteristics of the fragmentation behavior, have been discussed elsewhere in greater detail by Byrdwell and Neff (18). Among the important considerations discussed was the treatment of intractably overlapped diacylglycerol fragment ion peaks. Byrdwell and Neff (18) presented two methods for apportionment of overlapped areas; both worked well and gave virtually identical composition results.

The method for quantitative analysis developed by Byrdwell *et al.* (17) was applied to analysis of genetically modified canola varieties by Byrdwell and Neff (19). Normal canola oil, high-stearic acid and high-lauric acid canola oils were analyzed. As with the other publications on quantification (17,18), the results provided by APCI–MS showed less average error than the results provided by LC–FID. This report highlighted the utility of APCI–MS, because the high-lauric canola oil contained numerous TAG molecular species for which commercial standards were not available. Thus, the LC–FID analysis could not be performed without prior qualitative identification, using APCI–MS, of the numerous lauric acid-containing TAG.

Supercritical fluid chromatography

As mentioned, Tyrefors *et al.* (6) first demonstrated the coupling of SFC with APCI–MS for lipid analysis. Later, Schmeer *et al.* (20) used SFC coupled with APCI–MS for analysis of an extract from seed arils of a tree species from the dry forest of western Madagascar. The study was focused on identification of the diacylglycerol 1,2-dioleoylglycerol which has been identified as an ant attractant that induces ants to collect the seeds and carry them into their colonies. A homemade interface was used to couple the SFC column to the APCI mass spectrometer. Collision-induced dissociation (CID) caused by increased skimmer (conical orifice between intermediate- and high-vacuum regions) voltages was used to produce diacylglycerol fragments from TAG in positive-ion mode, while fatty acyl chains were observed using CID in negative-ion mode.

Manninen and Laakso (21,22) and Laakso and Manninen (23) have extensively used APCI–MS in combination with SFC for analysis of samples of natural origin. They demonstrated the coupling of capillary SFC (cSFC) with a commercially available APCI source with only slight modification. It was necessary to preheat the sheath and auxiliary gases, and this was accomplished by simply passing the gas lines between the SFC outer wall and the oven cover. Also, these authors passed the sheath gas through several solvents to produce chemical ionization reactant ions. Methanol, isopropanol, water, and 0.5% ammonium hydroxide in methanol were compared based on their effects on sensitivity, stability, and the nature of the ions formed. Isopropanol produced the lowest ion abundances, while methanol produced the best overall sensitivity and stability, and therefore was selected for the further analyses. Ammonium hydroxide in methanol produced $[M + 18]^+$ ions instead of protonated molecular ions. The initial report (21) described the application of cSFC/APCI–MS to cloudberry and sea buckthorn seed oils, while a subsequent report (22) applied the technique to blackcurrant and alpine currant seed oils. The retention of TAG on this column was based on increasing ACN + 2*n*, where ACN is the total acyl carbon number and *n* is the number of double bonds in the TAG, with further separation provided by the position of the double bonds (γ-linolenic acid-containing TAG eluted before the corresponding α-linolenic acid-containing isomers).

The cSFC separations, based on the different retention mechanism, produced fewer chromatographic peaks for the seed oil mixtures compared to the RP-HPLC/APCI–MS results presented for the same seed oils (12). Thus, most peaks contained several overlapped TAG molecular species, with some peaks containing up to seven molecular species. In many cases, these were differentiable by the presence of different diacylglycerol fragment ions or a different protonated molecular ion, although the TAG often shared common diacylglycerol fragments. For this reason, quantitative estimation of composition was performed using only the $[M + H]^+$ ion abundances. Nevertheless, the authors proposed that cSFC could have advantages over LC/APCI–MS applications in which a gradient elution resulted in a continuous change in the amounts of reactant ions coming from the LC effluent. The authors also demonstrated that, in addition to the other factors which affect the proportions of diacylglycerol fragment ions and protonated molecular ions discussed above, the distance of the double bonds from the glycerol backbone had an effect on the fragment proportions in APCI–MS. The γ-isomer-containing OLnO TAG gave a higher abundance of the *sn*-1(3) fragment than did the α-isomer, indicating that the closer proximity of the double bond in the γ-isomer made it a better leaving group.

A similar cSFC/APCI–MS method using a nonpolar column was then extended to a very complex natural

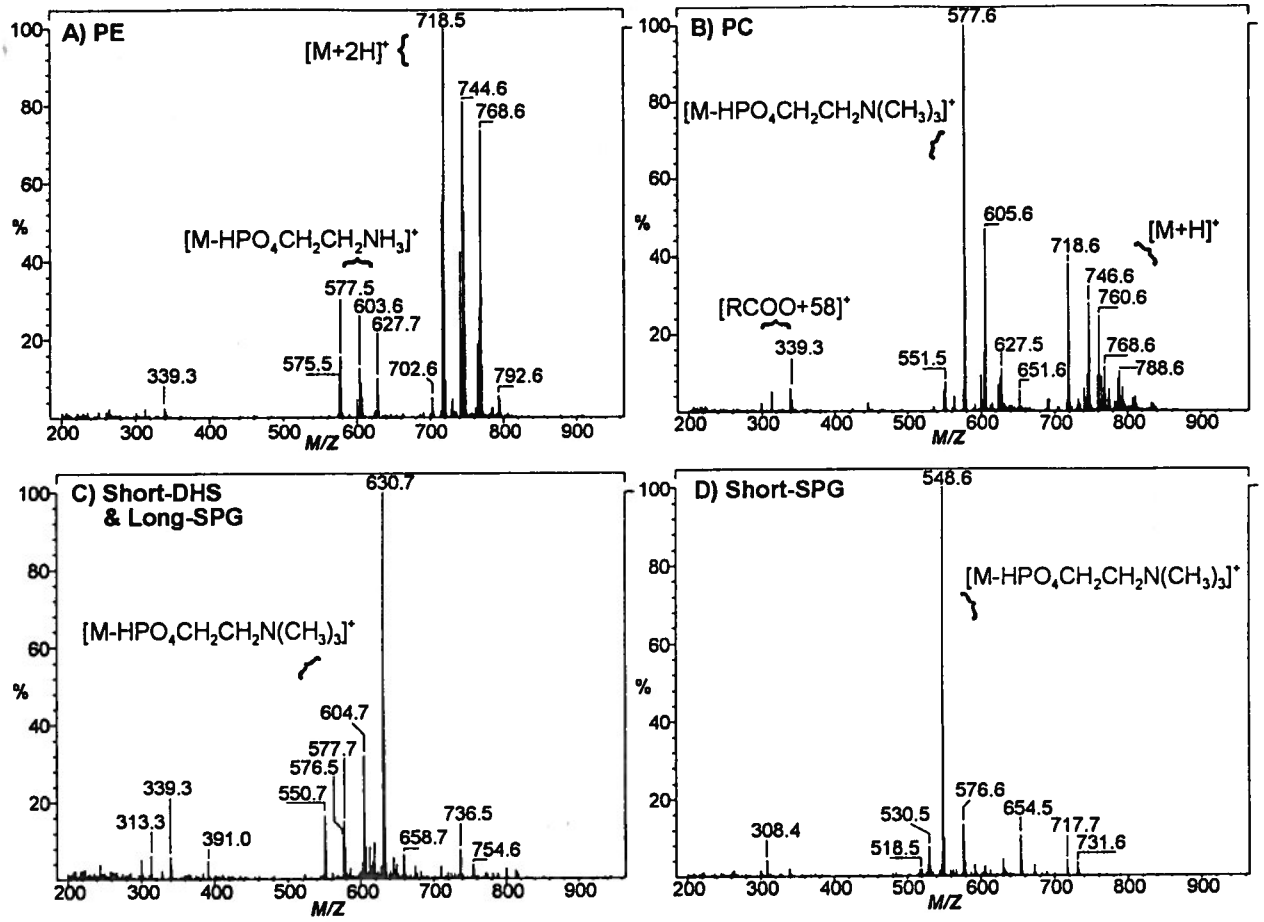

Figure 4. APCI mass spectra of phospholipid standards. A: egg yolk phosphatidylethanolamine. B: bovine brain phosphatidylcholine. C: bovine brain sphingomyelin (long-chain) and accompanying dihydrosphingomyelin (short-chain). D: bovine brain sphingomyelin (short-chain).

sample, milk fat TAG (23). The complexity of the sample resulted in so many coeluted TAG molecular species in each peak that it was difficult to identify the specific molecular species that produced each of the diacylglycerol fragments. Nevertheless, some trends could be identified, such as the propensity for short 4:0 FA to be associated with TAG having an ACN of 40 or less. The authors suggested the use of prefractionation to simplify interpretation of milk fat TAG data. An important observation was made in this study. The authors used 0.5% ammonium hydroxide in methanol as a chemical ionization reagent and found that saturated TAG, which normally gave no protonated molecular ion, produced useful abundances of ammonium adducts, $[M + 18]^+$, under these conditions.

A different SFC interface design has been reported by Sjoberg and Markides (24) which allowed easy changing between APCI and electrospray ionization modes. Its function was demonstrated using several disparate analyte classes, including a mixture containing methyl esters, a TAG, cholesterol, and cholesterol palmitate.

Phospholipids

Another important class of lipids is the phospholipids. Electrospray ionization mass spectroscopy (ESI–MS) has become the most common mass spectrometric method for phospholipid (PL) analysis, as was reviewed in these pages (25). Karlsson *et al.* (26) first demonstrated the application of APCI–MS to PL by showing positive- and negative-ion mass spectra of dioleoyl phosphatidylcholine (PC) standard. Byrdwell and Borchman (27) recently demonstrated the first application of HPLC/APCI–MS to analysis of PL extracts. In that report, molecular species of PC, sphingomyelin (SPM) and dihydrosphingomyelin (DHS) were identified in egg yolk and human eye lens PL extracts. Results obtained by APCI–MS were compared to results for the same samples obtained by ESI–MS. The two ionization techniques were complementary, with APCI–MS yielding mostly [M – headgroup]$^+$ fragments and small protonated molecular ions, $[M + H]^+$, while ESI–MS yielded exclusively $[M + H]^+$ ions.

Typical APCI–MS spectra are shown in Figure 4 for phos-

phatidylethanolamine (PE), PC, DHS and SPM. PE yielded protonated molecular ions as base peaks, PC gave both protonated molecular ions and diacylglycerol fragments, and the sphingolipids gave mostly only fragments arising from the loss of the choline headgroup. APCI–MS mass spectra of diacyl PL also gave $[RCOO + 58]^+$ ions which further characterized the fatty acyl chains. Quantification using the $[M - headgroup]^+$ fragments from APCI spectra gave results similar to those obtained by $[M + H]^+$ ions in ESI spectra. While the APCI–MS data provide invaluable fragment information for structural characterization, ESI–MS for analysis of PL has the advantage of its much lower limit of detection, having a 10- to 100-fold lower detection limit in many, but not all, cases.

Byrdwell (28) most recently employed a dual parallel mass spectrometer arrangement with both APCI and ESI sources for analysis of PL extracts. Single and triple quadrupole instruments were employed simultaneously, along with ELSD and UV detectors, to identify molecular species of PE and its plasmalogen (alkenyl, acyl PE), of PC and its plasmalogen (alkenyl, acyl PC), and of SPM and DHS in biological extracts. Bovine brain SPM, human plasma whole extract, and porcine lens extract were examined. Quantification was performed using the $[M - headgroup]^+$ fragments from APCI spectra. In the human plasma whole extract, TAG also were observed. Ammonium hydroxide used in the chromatographic system produced $[M + 18]^+$ ions from TAG with significantly enhanced abundances, similar to the enhancement reported by Laakso and Manninen (23). Quantification of the TAG by carbon number:sites of unsaturation, using the $[M + 18]^+$ ions, compared favorably to the composition determined by RP–HPLC/APCI–MS of the same sample.

An iron corona discharge needle enhanced reproducibility of APCI–MS spectra, as did use of an automatic switching valve which introduced only peak fractions into the source.

Ceramides, which are precursors in the formation of sphingolipids and also act as messengers in signal transduction controlling cell growth, have been analyzed by Couch *et al.* (29) using RP–HPLC/APCI–MS. Underivatized and also perbenzoylated ceramide standards and ceramides from cultured human leukemic cells were examined. Low skimmer cone voltages in the source were used to produce $[M + H]^+$ and $[M + H - H_2O]^+$ ions. Higher skimmer cone voltages caused in-source collision-induced dissociation to give fragments that identified the fatty chains. The combination of low- and high-skimmer voltages allowed characterization of ceramide bases on molecular ions and sphingosine/sphinganine-derived fragment ions with greater sensitivity than was previously reported.

Carotenoids

Other applications of APCI–MS have included analysis of carotenoids. This class of thermally labile, nonvolatile long-chain molecules has presented a number of difficulties for analysis. The most common method for analysis has been HPLC with UV detection, but coelution of numerous structurally similar molecules has led to ambiguities which have necessitated the use of mass spectrometric detection. Clarke *et al.* (30) reported the use of RP-HPLC coupled to APCI–MS for analysis of carotenoids.

Van Breeman *et al.* (31) demonstrated the use of RP-HPLC using a C_{30} column coupled to APCI–MS and a UV detector for the separation and identification of carotenoid standards and carotenoids in extracts of fresh vs. canned heat-processed sweet potatoes. The molecules were shown to exhibit molecular ions, $M^{+\cdot}$ and $M^{-\cdot}$, in positive- and negative-ion modes, respectively, in addition to protonated and deprotonated molecules, respectively, with little fragmentation. In-source CID was used to produce structurally diagnostic fragments, without loss of the higher mass peaks. Limits of detection determined by APCI–MS were similar to those reported using ESI–MS.

Hagiwara *et al.* (32) reported the quantitative determination of carotenoids in eight kinds of vegetable juices. They employed RP-HPLC/APCI–MS using selected-ion monitoring (SIM) of the protonated molecular ions in positive-ion mode, with cholesterol benzoate added as internal standard. They reported that an iron corona discharge needle enhanced reproducibility of APCI–MS spectra, as did use of an automatic switching valve which introduced only peak fractions into the source. These authors more recently reported an improved method for carotenoid analysis which was used for determination of serum lycopene, α-carotene, and β-carotene (33). They found that coeluting serum cholesterol species interfered with SIM of carotenoids, so modifications to the mobile phase were necessary. Flow-injection APCI–MS has been used by Tang *et al.* (34) to measure the enrichment of deuterated (d_8) β-carotene in the serum of a subject to whom it was orally administered. Van Breeman *et al.* (35) reported the development of an HPLC/APCI–MS method for analysis of two molecules in the vitamin A family: *trans*-retinol and *trans*-retinyl palmitate. They demonstrated a separation using a C_{30} RP column coupled directly to an APCI interface. APCI–MS was found to produce more linear calibration curves than a method using ESI–MS, to which it was compared.

Steroids

Other lipid classes such as cholesterol and cholesterol-related molecules have been mentioned briefly in the reports cited above (6,24,27,28,32). Also, mass spectra of numerous cholesterol oxidation products were reported recently (36). Other types of lipid-related molecules, such as mixtures of steroids (37,38), have been examined using APCI–MS. Analyses of several specific classes of steroid molecules using APCI–MS have been reported, including brassinosteroids (39), corticosteroids (40), ecdysteroids (41) and azasteroids (42), but discussion of each of these is beyond the scope of this review.

Conclusion

The advantages APCI–MS have been highlighted by the variety of applications to which it has been applied that incorporate very different chromatographic systems and conditions. Analyses that have proved difficult or impossible using other techniques have been amenable to this soft ionization method. The tendency for APCI–MS mass spectra to exhibit both quasimolecular ions and diagnostically useful fragments makes it an excellent intermediate between other techniques which produce only pseudomolecular ions (such as ESI–MS) and those which produce extensive fragmentation with few high-mass ions. The ease with which in-source CID spectra are obtained makes structural characterization of many molecules possible without the need for tandem instrumentation. The fragmentation patterns formed from functional groups on TAG and other lipids sometimes provide invaluable fragment data not available from other ionization sources. The interaction of reactant molecules present in the APCI source with analytes can make interpretation of mass spectra more complicated, but more often results in beneficial adducts which facilitate data interpretation or offer other benefits. For these and other reasons, the use of APCI–MS in the analysis of lipids is growing more widespread as researchers become aware of its potential.

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