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Comparison of Atmospheric Pressure Photoionization, Atmospheric Pressure Chemical Ionization, and Electrospray Ionization Mass Spectrometry for Analysis of Lipids

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In this work, we compare the quantitative accuracy and sensitivity of analyzing lipids by atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI) LC/MS. The target analytes include free fatty acids and their esters, monoglyceride, diglyceride, and triglyceride. The results demonstrate the benefits of using LC/APPI-MS for lipid analysis. Analyses were performed on a Waters ZQ LC/MS. Normal-phase solvent systems were used due to low solubility of these compounds in aqueous reversed-phase solvent systems. By comparison, APPI offers lower detection limits, generally highest signal intensities, and the highest S/N ratio. APPI is 2−4 times more sensitive than APCI and much more sensitive than ESI without mobile-phase modifiers. APPI and APCI offer comparable linear range (i.e., 4−5 decades). ESI sensitivity is dramatically enhanced by use of mobile phase modifiers (i.e., ammonium formate or sodium acetate); however, these ESI adduct signals are less stable and either are nonlinear or have dramatically reduced linear ranges. Analysis of fish oils by APPI shows significantly enhanced target analyte intensities in comparison with APCI and ESI.

Lipid compounds are ubiquitous in nature, comprising, for example, fatty acids, cholesterol, and triacylglycerols. They are characteristically long-chained, oily or fatty compounds that are generally insoluble in water. The study presented here uses examples of lipid compounds found in fish oils; however, the results pertain to lipid analysis in general. There is a tremendous chemical diversity of lipids in biological systems. This diversity is responsible for specific functions relating to cellular responses. Many investigators seek identification of these lipids and their alterations as indicators of diseases. LC/MS figures prominently in these analyses.[1] A rapidly growing field is lipidomics, which is a branch of metabolomics that seeks to understand how lipids interact with genes and proteins to regulate cellular functions. Lipidomics involves the study of non-water-soluble metabolites, which can present challenges for analysis by electrospray ionization (ESI). On an entirely different front, the lipid content in bacterial cells is being used in homeland security applications as a means to identify toxic bacterial substances, such as biological weapons. Several groups are using MS for the lipid analysis.[2,3]

Lipid analysis has been traditionally difficult by gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), and high performance liquid chromatography (HPLC). GC or GC/MS requires tedious sample pretreatment and derivatization due to low volatility and thermal instability of lipid compounds. HPLC with a UV detector or evaporative light scattering detector (ELSD) avoids analyte derivatization, but it lacks sensitivity and specificity. The technique of liquid chromatography coupling with mass spectrometry (LC/MS) simplifies sample preparation procedure by requiring fewer cleanups and avoiding derivatization. Furthermore, LC/MS offers higher sensitivity and provides analyte chemical structure information. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are two widely used ionization techniques for analysis of polar and less polar compounds by LC/MS, and these methods have been used for lipid analysis.[4] However, analysis of nonpolar compounds can often be difficult by ESI and APCI. Atmospheric pressure photoionization (APPI) is a recently introduced technique that extends the range of ionizable compounds. APPI is gaining its popularity mainly due to its ability to ionize those compounds that are often missed or are not readily ionized by ESI and APCI. A recent paper reported on the application of APPI to lipid analysis.[5]

APPI has been rapidly adopted by LC/MS users since its commercial introduction in the year 2000.[6−8] APPI has been used for analysis of many classes of compounds, including pharma-

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ceutical drugs and metabolites,[9−12] hydrophobic peptides,[13] polyaromatic hydrocarbons,[14] steroids,[15−19] mycotoxin,[20] perfluorooctane sulfonate,[21] microbial respiratory quinones,[22] aldehydes and ketones,[23] pesticides,[24,25] and antibiotics.[26] Among many of these applications, APPI has been shown to be a superior technique by offering wider linear dynamic ranges,[10] higher sensitivity and lower detection limits,[14−17,19−23,25] and less or no matrix suppression[20,26] and, therefore, requires minimal or no off-line sample cleanups.[15,20] APPI has also been reported to require less heat for desolvation than APCI,[16] allowing analysis of thermally labile compounds with less concern for thermal chemistry and thermal degradation in the ionization source. In this work, we compare the quantitative accuracy and sensitivity of analyzing lipids by APPI, APCI, and ESI.

EXPERIMENTAL SECTION

Chemicals and Reagents. EPA methyl ester (C20:5, MW = 316) is the methyl ester of eicosapentaenoic acid (EPA). This compound was selected to represent unsaturated fatty acid series of target analytes. EPA methyl ester (99%) was purchased from Nu-Chek Prep, Inc. (Elysian, MN). Monoarachidin is a saturated monoacylglycerol, (C20:0, MW = 386). Diarachidin is a saturated diacylglycerol, (C20:0, MW = 680), and trielaidin is monounsaturated triacylglycerol, (C18:1, MW = 884). These three compounds were selected to represent saturated and unsaturated acylglycerols. All acylglycerol standards (99%) were purchased from AccuStandard, Inc. (New Haven, CT). *n*-Hexane (95+%) and isooctane (99.7%), HPLC grade, were purchased from Alfa Aesar (Ward Hill, MA); isopropyl alcohol (IPA), residue grade, was purchased from EM Science (Gibbstown, NJ). Ammonium formate (99.995+%) and sodium acetate (99%) were purchased from Aldrich Chemicals Co., Inc. (Milwaukee, WI).

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Fish Oil Samples. Three fish oil mixtures were provided by Ocean Nutrition Canada. Sample ONC-3 contains fish oil components in free fatty acid form. Sample ONC-2 contains fish oil components with fatty acids in ethyl ester form. ONC-3 and ONC-2 are custom mixtures with major components of EPA and DHA (docosahexaenoic acid). Sample ONC-1 is natural fish oil with an unknown triacylglycerol mixture with MW ranging from ∼700 to 1100.

Instrument, Parameters, and Conditions. A Waters Micromass ZQ LC/MS was used for these experiments. This instrument was equipped with APPI (Syagen Technology, Inc., Tustin, CA), APCI and ESI sources, and the data acquisition and processing software Masslynx V4.0. Instrument parameters were tuned with 5 ng/μL EPA methyl ester standard in hexane by flow injection analysis. Various parameters were adjusted to obtain optimum operating conditions on the Masslynx tune page for a maximum intensity of the m/z 317 ion $[M + H]^+$. The parameters and conditions were further optimized using each individual standard. The general parameters were set as follows:

APPI. Repeller voltage was set at 0.5 kV. APCI probe temperature was set at 450 °C. Cone voltage was set at 25 V. Desolvation gas flow rate was set at 400 L/h. Mobile phase was either pure hexane or 1:1 isooctane/IPA.

APCI. Corona discharge needle current was set at 5.0 μA. APCI probe temperature was set at 450 °C. Cone voltage was set at 25 V. Desolvation gas flow rate was set at 400 L/h. Mobile phase was either pure hexane or 1:1 isooctane/IPA.

ESI. Capillary voltage was set at 3.5 kV. Desolvation temperature was set at 450 °C. Cone voltage was set at 10−25 V, depending on ions acquired. Desolvation gas flow rate was set at 250 L/h. Mobile phase was 1:1 isooctane/IPA with and without 10 mM ammonium formate and 10:15:1 isooctane/IPA/H_2O with 15.4 mM sodium acetate.

APPI, APCI, and ESI Shared Parameters. Extractor voltage was set at 3 V. RF lens voltage was set at 0.2 V. Acquisition dwell time was 0.2 s. Source temperature was set at 150 °C. Mobile phase was delivered into the ionization source with a syringe pump by flow injection at a flow rate of 100 μL/min. The injection volume was 10 μL per sample.

Preparation of Standards. A 10 000 μg/mL (i.e., 10 000 ppm) portion of EPA methyl ester stock solution was prepared in hexane. Stock solutions (5000 ppm of monoarachidin, diarachidin, and trielaidin) were prepared in chloroform. Working solutions were prepared from these stock solutions using corresponding mobile phases as solvents. For linearity establishment, 5000 ppm monoarachidin, diarachidin, and trielaidin individual stock solutions were first diluted to 1000 ppm using mobile phase. These solutions were diluted further by a series dilution method at a dilution factor of 2 each step, resulting in a final standard concentration (ppm) of 500, 250, 125, 62.5, 31.3, 15.6, 7.81, 3.91, 1.95, 0.977, 0.488, 0.244, 0.122, 0.0610, 0.0305, 0.0153, 0.00763, 0.00382 and 0.00191. For APPI and APCI analysis, the mobile phase used was either hexane or 1:1 isooctane/IPA. For ESI analysis, three sets of series dilution standards were prepared for each acylglycerol. The mobile phases were 1:1 isooctane/IPA, 1:1 isooctane/IPA with 10 mM ammonium formate, and 10:15:1 isooctane/IPA/H_2O with 15.4 mM sodium acetate, respectively.

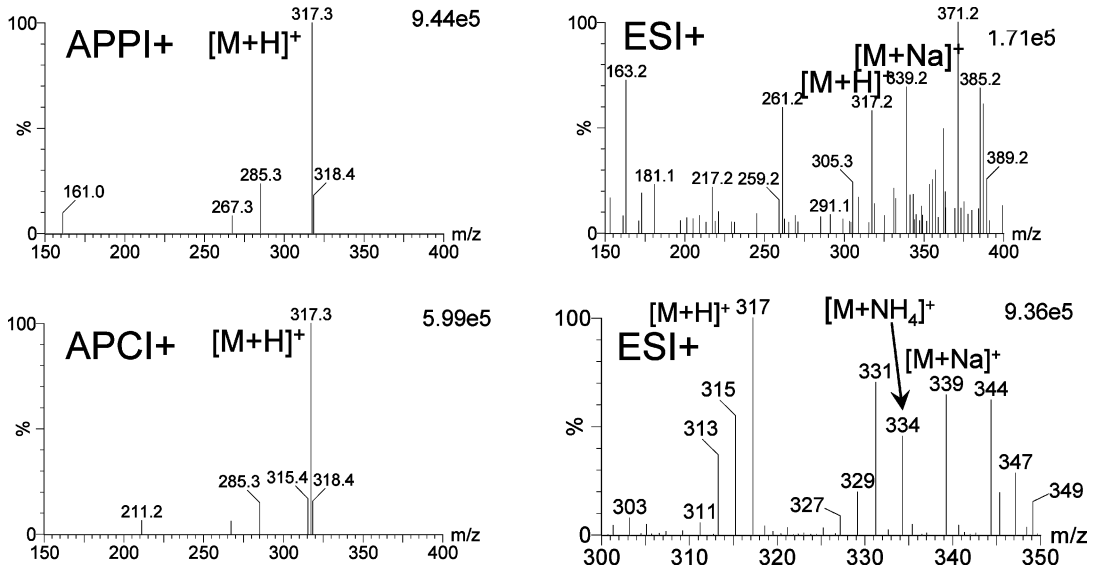

Figure 1. EPA methyl ester full scan mass spectra. APPI and APCI mobile phase was hexane, ESI mobile phase was 1:1 isooctane/IPA without or with 10 mM ammonium formate.

RESULTS AND DISCUSSION

Selection of Mobile Phase. The selected target analytes are nonpolar or neutral lipids, having limited solubility in methanol, IPA, and in aqueous reversed-phase solvent systems (e.g., methanol/water or acetonitrile/water mixtures). Our initial experiment was performed using an aqueous reversed-phase solvent system (i.e., 1:1 MeOH/water) as mobile phase and EPA methyl ester as a model test compound. We observed broad peak responses of EPA methyl ester with severe tailing. We also encountered difficulties preparing standard stock solutions by trying to dissolve target analytes in polar solvents, such as methanol. We found in the literature that chromatographic separation of many lipids was often achieved under normal-phase or nonaqueous reversed-phase conditions, possibly due to their poor solubility in an aqueous reversed-phase solvent system.[27] Therefore, we tested a list of organic solvents available in our lab and found that hexane and isooctane offered high sensitivity for EPA methyl ester by APPI and APCI. We added in the mobile phase 50% of isopropyl alcohol (IPA), a protic solvent expected to enhance the sensitivity of ESI. During our ESI full-scan experiments, we observed major sodium adduct molecular ions of these target analytes. We also notice in the literature that ammoniated adduct molecular ions are commonly acquired ions for analysis of lipids by ESI. Therefore, for further evaluation of ESI sensitivity, 1:1 isooctane/IPA with 10 mM ammonium formate and 10:15:1 isooctane/IPA/H_2O with 15.4 mM sodium acetate were also used. A small amount of water (~3.8%) was necessary to help dissolve the sodium acetate in the mobile phase. A detailed description for selection of mobile phase solvents and compositions for lipid analysis by APPI will be reported in a separate journal article.[28]

Full-Scan Analysis in Positive Ion Mode. The full scan analysis of four analytes was performed using the above-described instrumental parameters and conditions. The scans were first performed and compared by APPI, APCI, and ESI using the mobile phase without a modifier. The ESI full-scan analysis was further performed using a mobile phase containing additives of sodium acetate or ammonium formate due to low sensitivity of ESI relative to the other two ionization sources. The results show that APPI and APCI generate very clean, easy-to-interpret, almost identical spectra except that APPI offers a higher ion intensity than APCI does in most cases. For EPA methyl ester, both APPI and APCI give a base peak of protonated molecule m/z 317 $[M + H]^+$ (Figure 1a, b). The ESI spectra look more complex, showing $[M + H]^+$ and $[M + Na]^+$ and many other ions when the mobile phase does not contain any modifiers (Figure 1c). Addition of 15.4 mM sodium acetate in the mobile phase and in the standard solution significantly enhances the intensity of the molecular sodium adduct $[M + Na]^+$ (spectra not shown).

For monoarachidin and diarachidin, both APPI and APCI give a major protonated molecule with a neutral loss of water. Monoarachidin gives m/z 369 $[M + H - H_2O]^+$ (Figure 2) and diarachidin gives m/z 663 $[M + H - H_2O]^+$ (Figure 3). ESI gives a sodiated molecular ion of m/z 409 for monoarachidin (Figure 2) and m/z 703 for diarachitin (Figure 3), even if the mobile phase is not spiked with a sodium-ion-containing modifier, apparently due to the ubiquitous presence of sodium ion in the solvents. When the standard and the mobile phase are fortified with 10 mM ammonium formate, APPI and APCI give major ions of $[M + NH_4]^+$ and $[M + Na]^+$ for monoarachidin and $[M + NH_4]^+$ and $[M + H - H_2O]^+$ for diarachidin (Figures 2, 3).

For trielaidin, both APPI and APCI give m/z 603, $[M + H - 282]^+$, a protonated molecule with loss of a fatty acid moiety ($C_{17}H_{33}COOH$). APPI and APCI also give a $[M + H]^+$ ion of m/z 885 for trielaidin but with a much lower intensity (Figure 4). Once again, ESI gives a sodiated molecular ion of m/z 907, $[M + Na]^+$ (Figure 4), even when the mobile phase, 1:1 isooctane/IPA, does not contain a sodium additive. Addition of 10 mM ammonium formate in the trielaidin standard and in the mobile phase gives a single ammoniated molecular ion $[M + NH_4]^+$ (Figure 4). It is worth pointing out that for ESI, the use of ammonium formate in the mobile phase enhances the formation of $[M + H]^+$ for EPA

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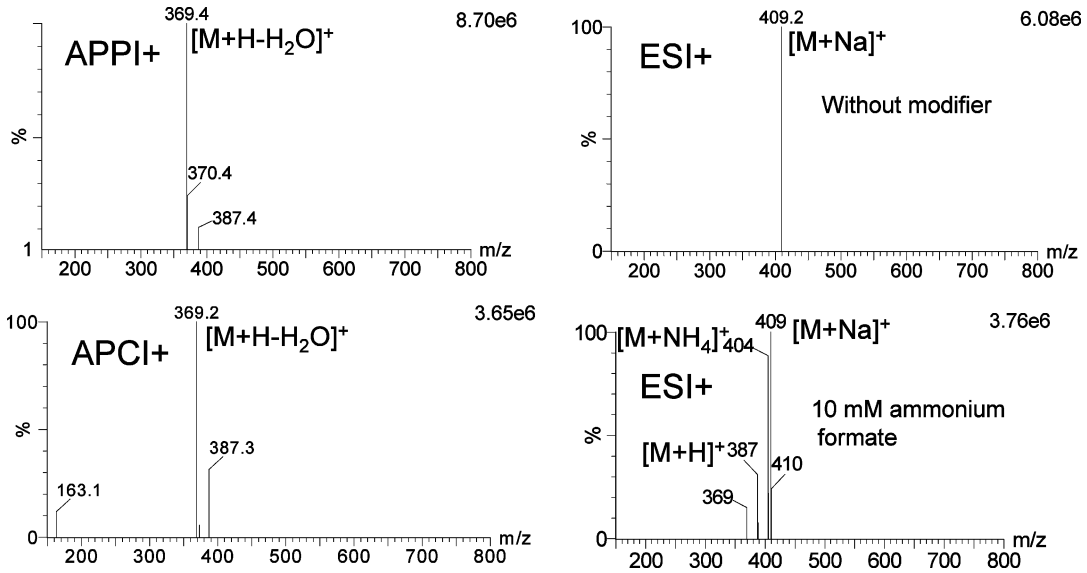

Figure 2. Monoarachidin full-scan mass spectra. See Figure 1 caption.

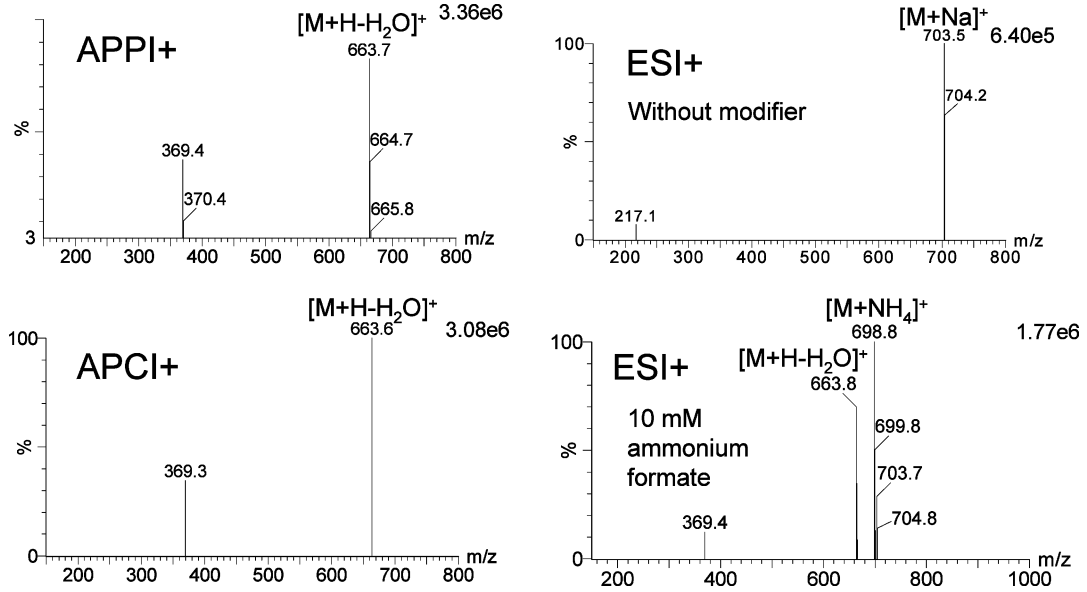

Figure 3. Diarachidin full-scan mass spectra. See Figure 1 caption.

methyl ester and monoarachidin (Figures 1 and 2) and $[M + H - H_2O]^+$ for diarachidin (Figure 3); however, this does not apply to trielaidin, which shows only a single peak of ammonium adduct molecular ion on the spectra.

Full Scan Analysis in Negative Ion Mode. In an effort to examine possible formation of major negative ions, full-scan analyses were also performed in negative ion mode by ESI, APCI, and APPI. Individual target analyte standards (10 ppm) were prepared in and analyzed with ammonium-formate- and chloroform-containing mobile phases. In ESI mode, when the mobile phase, 1:1 isooctane/IPA, contains 10 mM ammonium formate additive, EPA methyl ester gives major ions of m/z 361 $[M + HCOO]^-$ and 379 $[M + HCOO + H_2O]^-$ (spectra not shown); monoarachidin gives a major ion m/z 431 $[M + HCOO]^-$; diarachidin gives a molecular formate adduct ion of m/z 725 $[M + HCOO]^-$. No molecular formate adduct or other major negative ions are observed for the spectra of trielaidin.

For monitoring of chlorine adducts, EPA methyl ester standards were prepared in 1:1 hexane/$CHCl_3$ and 1:1 MeOH/$CHCl_3$, respectively. Monoarachitin, diarachitin, and trielaidin (10 ppm) were prepared in pure $CHCl_3$. Three mobile phases were used, which included pure chloroform, 1:1 hexane/$CHCl_3$, and 1:1 MeOH/$CHCl_3$ mixture. Full-scan spectra show that EPA methyl ester and trielaidin do not give major chlorine adduct ions or other major negative ions using any of the three mobile phases by ESI. Monoarachidin and diarachidin give molecular chlorine adducts, $[M + Cl]^-$. It was noticed that the presence of hexane and methanol in the mobile phase enhances the formation of chlorine adducts. APCI shows no major ion of formate adducts and a very weak signal of chlorine adducts. APPI does not provide any major negative ions for these tested analytes. By comparing the full-scan mass spectra for positive and negative ion mode, we found that positive ion mode was more sensitive than negative ion mode by at least 2 orders of magnitude for these tested target analytes. As a result, all further analyses were performed in positive ion mode only.

Linear Dynamic Range. Acylglycerol calibration standard solutions were analyzed in selective ion monitoring (SIM) mode

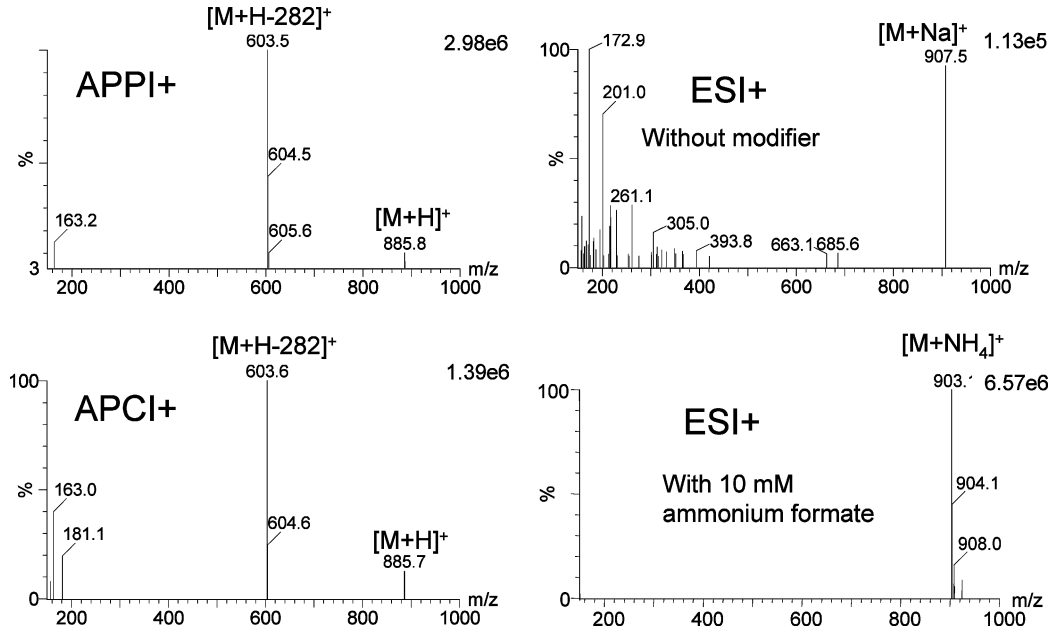

Figure 4. Trielaidin full-scan mass spectra. See Figure 1 caption.

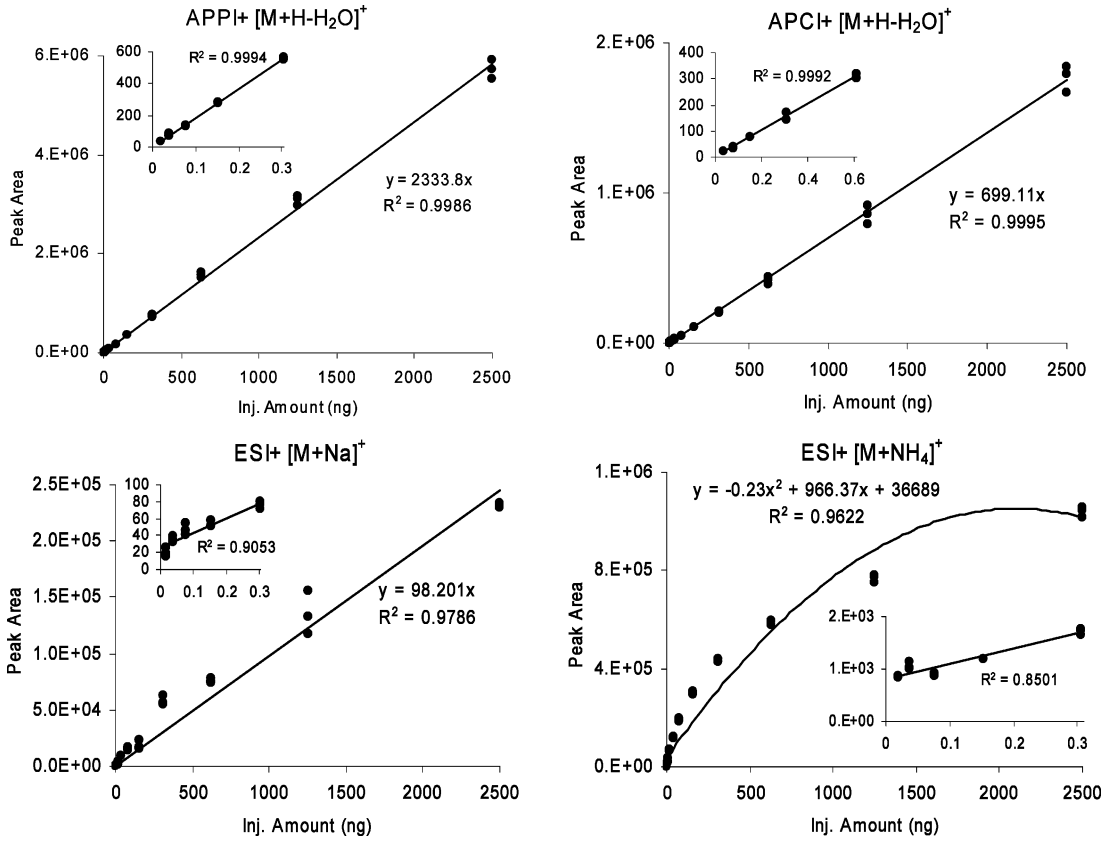

Figure 5. Monoarachidin linearity plots. Mobile phase: 1:1 isooctane/IPA (APPI and APCI). 10:15:1 isooctane/IPA/water with 15.4 mM sodium acetate (ESI sodium adduct) and 1:1 isooctane/IPA with 10 mM ammonium formate (ESI ammonium adduct).

with three replicate analyses by APPI, APCI, and ESI, beginning with the lowest concentrations. In general, linearity plots were generated by acquiring the most abundant ions on the full-scan spectra.

Monoarachidin. Figure 5 shows the linearity plots of monoarachidin by APPI, APCI, and ESI. The major ion $[M + H - H_2O]^+$ was acquired for APPI and APCI. APPI is linear over an injection amount range of 19 pg to 2500 ng with a correlation coefficient (R^2) of 0.9986, a linear range which covers over 5 orders of magnitude. APCI is linear over a 38 pg to 2500 ng range with an R^2 of 0.9995. Injection amounts of 19 pg were not detectable by APCI. By comparing the monoarachidin slopes of APPI and APCI linearity plots, it is evident that APPI is ~3.3 times more sensitive than APCI. For ESI linearity plots, sodiated and ammoniated molecular ions were acquired. The mobile phases used were 10:15:1 isooctane/IPA/H_2O with 15.4 mM sodium acetate and 1:1

Table 1. Comparison of Linearity Plots[a]

analyte	upper calibration range	APPI$^+$	APCI$^+$	ESI$^+$	ESI$^+$
monoarachidin	2500 ng	$[M + H - H_2O]^+$ $y = 2333.8x$ $r^2 = 0.9986$	$[M + H - H_2O]^+$ $y = 699.11x$ $r^2 = 0.9995$	$[M + Na]^+$ $y = 98.201x$ $r^2 = 0.9786$	$[M + NH_4]^+$ $y = -0.23x^2 + 966.37x + 36689$ $r^2 = 0.9622$
diarachidin	1250 ng	$[M + H - H_2O]^+$ $y = 1691x$ $r^2 = 0.9999$	$[M + H - H_2O]^+$ $y = 745.03x$ $r^2 = 0.9984$	$[M + H - H_2O]^+$ $y = -1.6261x^2 + 2989.6x + 44080$ $r^2 = 0.9678$	$[M + NH_4]^+$ $y = -0.65x^2 + 1197.7x + 22405$ $r^2 = 0.9514$
trielaidin	625 ng	$[M + H - 282]^+$ $y = 1315.9x$ $r^2 = 0.9955$	$[M + H - 282]^+$ $y = 622.45x$ $r^2 = 0.9922$		
trielaidin	1250 ng	$[M + H]^+$ $y = 119.89x$ $r^2 = 0.9969$	$[M + H]^+$ $y = 43.16x$ $r^2 = 0.9886$		$[M + NH_4]^+$ $y = -1.5707x^2 + 2777.7x + 56349$ $r^2 = 0.9458$

[a] Mobile phase: 1:1 isooctane/IPA (APPI and APCI), 10:15:1 isooctane/IPA/water with 15.4 mM sodium acetate (ESI sodium adduct), and 1:1 isooctane/IPA with 10 mM ammonium formate (ESI ammonium adduct).

Table 2. Comparison of Signal to Noise Ratio[a]

	without modifier			with modifier	
analyte	APPI$^+$	APCI$^+$	ESI$^+$	ESI$^+$	ESI$^+$
EPA M. E.	132 $[M + H]^+$	52 $[M + H]^+$	38 $[M + H]^+$	84 $[M + NH_4]^+$	20 $[M + Na]^+$
monoarachidin	121 $[M + H - 18]^+$	44 $[M + H - 18]^+$	19 $[M + H - 18]^+$	34 $[M + NH_4]^+$	78 $[M + Na]^+$
diarachidin	83 $[M + H - 18]^+$	18 $[M + H - 18]^+$	0 $[M + H - 18]^+$	106 $[M + NH_4]^+$	44 $[M + Na]^+$
trielaidin	153 $[M + H - 282]^+$	90 $[M + H - 282]^+$	0 $[M + H - 282]^+$	205 $[M + NH_4]^+$	69 $[M + Na]^+$

[a] Injection amount: 610 pg for each compound except EPA methyl ester, which is 1000 pg. Mobile phase: 1:1 isooctane/IPA with and without 10 mM ammonium formate.

isooctane/IPA with 10 mM ammonium formate. The 15.4 mM concentration of sodium acetate in the mobile phase was chosen to satisfy the target analyte concentrations corresponding to the upper linear ranges achieved by APPI and APCI. Similarly for acquisition of ammonium adducts, 10 mM was used to cover the upper concentration limits for possible maximum linear ranges by ESI (2−10 mM ammonium formate or ammonium acetate in the mobile phase being more typical for LC/MS analysis). The results in Figure 5 show that the monoarachidin ESI sodium and ammonium adduct ion signals vs concentration are poorly fit by either first- or second-order curves at both low and high concentrations. Sodium acetate is a nonvolatile salt that is incompatible with LC/MS. This mobile-phase modifier tends to foul the MS ionization source, causing the sensitivity to drop dramatically over time. No further analyses were performed using this modifier for the linearity plots of other analytes.

Diarachidin. Similar linearity plots were recorded for diarachidin, and the results are summarized in Table 1. APPI is linear over 19 pg to 1250 ng, with an excellent R^2 of 0.9999, and APCI is linear up to 1250 ng, with an R^2 of 0.9984. APCI cannot detect an injection amount below 76 pg due to lower sensitivity. On the basis of the comparison of the linearity slope, APPI is ∼2.3 times more sensitive than APCI. The diarachidin ESI linear range was first measured by acquiring the ammonium adduct ion. We observed an upper linear range by ESI of only 9.8 ng for diarachidin. Plotting the peak area response over the entire injection mass range up to 1250 ng using the second-order quadratic model also failed to give a good fit, yielding instead a curve with an R^2 of 0.9514. Since ESI offers such a narrow linear range in comparison with APPI and APCI, we sought an alternative ion for further examination. As mentioned earlier, ammonium formate additive in the mobile phase enhances the sensitivity of $[M + H - H_2O]^+$ ion for diarachidin (Figure 3). This ion was acquired to create a second linearity plot for diarachidin and gave an upper linear range of 9.8 ng, with an R^2 of 0.9963 by ESI, comparable to that of its ammonium adduct ion, but with much lower sensitivity. An injection amount below 1.22 ng cannot be detected by acquiring this ion.

Trielaidin: APPI and APCI yielded two major ions, a base peak of $[M + H - 282]^+$ and a second major ion of $[M + H]^+$ at lower intensity. For the ion $[M + H - 282]^+$, APPI is linear over the injection amount of 19 pg to 625 ng, with an R^2 of 0.9955, and APCI is linear over the same injection amount range, with an R^2 of 0.9922 (Table 1). The acquired ion $[M + H - 282]^+$ from APPI and APCI is so sensitive that its peak intensity saturates the MS detector when the injection amount exceeds 625 ng. For the ion $[M + H]^+$, the correlation coefficient (R^2) of the APPI linearity plot is 0.9969 over the injection amount range of 38 pg to 1250 ng, and the R^2 of the APCI plot is 0.9886 over a range of 153 pg to 1250 ng (Table 1). APCI cannot detect an injection amount below 153 pg due to lower sensitivity than APPI. By comparing the slopes of APPI and APCI linearity plots, APPI is more sensitive than APCI by a factor of ∼2.1 for the ion $[M + H - 282]^+$ and ∼2.8 for the ion $[M + H]^+$. The ESI linearity plot was obtained by acquiring the ammoniated molecular ion. The ESI response is linear over 19 pg to 4.9 ng with an R^2 of 0.9909 (Table 1). Once again, the linear range of ESI is much narrower that that of APPI and APCI.

Comparison of Signal to Noise Ratio. The sensitivity of APPI, APCI, and ESI was compared by injecting EPA methyl ester at 1000 pg and monoarachidin, diarachidin, and trielaidin at 610 pg each. In the first set of experiments, the target analytes were

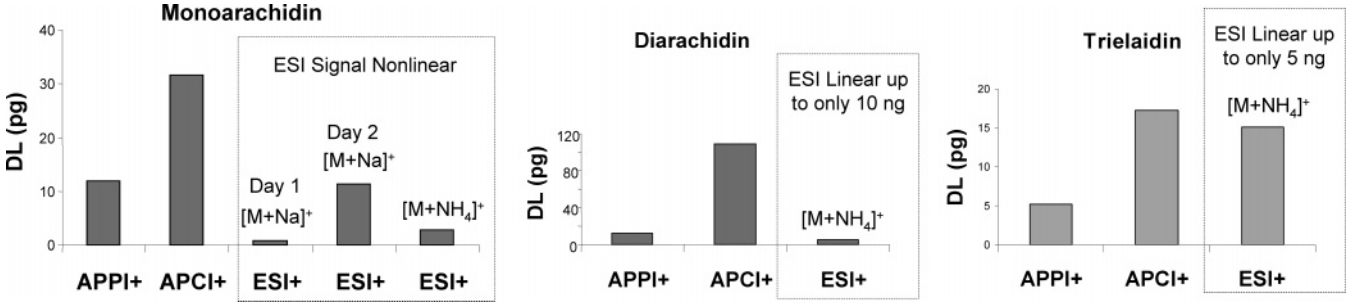

Figure 6. Comparison of detection limits. ESI $[M + Na]^+$ responses are unstable. ESI $[M + NH_4]^+$ responses are either nonlinear or offer a very narrow linear range.

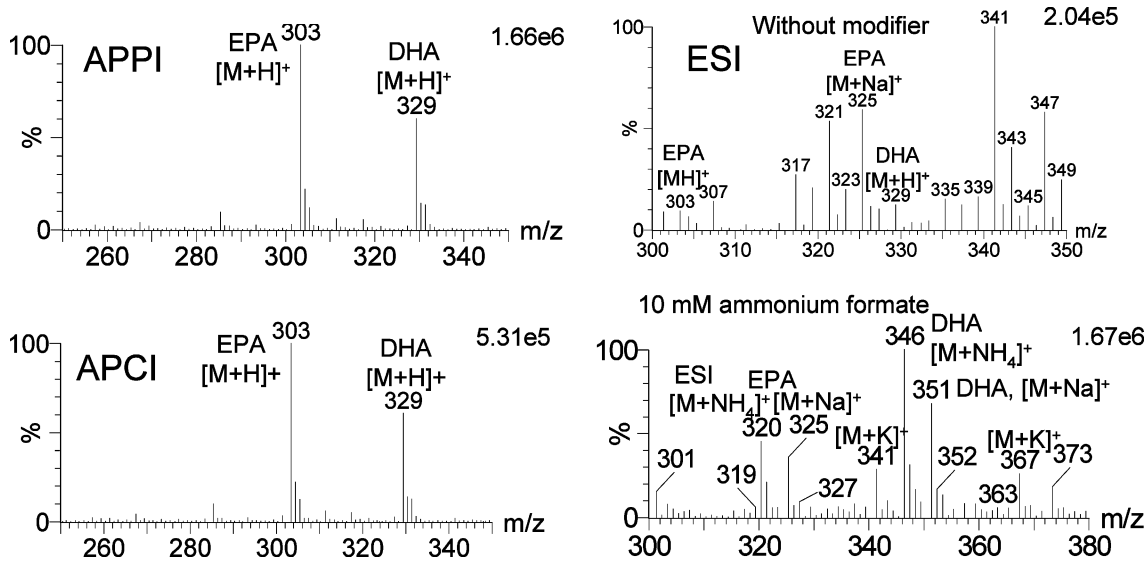

Figure 7. Full-scan mass spectra of sample ONC-3, a custom fatty acid mix. APPI and APCI give similar spectra with major ion $[M + H]^+$. ESI gives complex spectra, showing $[M + H]^+$, $[M + H + H_2O]^+$, $[M + Na]^+$, $[M + NH_4]^+$, $[M + K]^+$. EPA *m/z*: 303 = $[M + H]^+$, 321 = $[M + H + H_2O]^+$, 325 = $[M + Na]^+$, 320 = $[M + NH_4]^+$. DHA *m/z*: 329 = $[M + H]^+$, 351 = $[M + Na]^+$, 346 = $[M + NH_4]^+$, 367 = $[M + K]^+$.

analyzed in SIM mode by APPI, APCI, and ESI, respectively, using the mobile phase of 1:1 isooctane/IPA without a modifier. The results show that APPI offers the highest sensitivity in terms of S/N ratio for all the tested compounds (Table 2). The ESI experiments were further performed by spiking ammonium formate both in the analyte standards and in the mobile phase. Ammoniated and sodiated molecular ions were acquired. The results show that use of 10 mM ammonium formate additive in the mobile phase dramatically enhances the sensitivity of ESI, especially for diarachidin and trielaidin. The sensitivity of ESI ammoniated EPA methyl ester and sodiated monoarachidin exceeds that of APCI, but APPI sensitivity still remains the best among the three ionization sources for these two compounds (Table 2). In the case of diarachidin and trielaidin, ESI ammonium adducts offer the highest sensitivity; however, the linear ranges of diarachidin and trielaidin presented earlier are only up to about 10 and 5 ng, respectively, as compared to an upper limit of at least 1250 ng by APPI as discussed above.

Comparison of Instrument Detection Limit. The instrument detection limit (IDL) is defined as the amount (i.e., picograms) of target analyte that is able to generate a signal-to-noise ratio of 3 (i.e., S/N = 3). The IDLs were determined from the analyses at the lowest concentrations of the calibration standards. The results are presented in Figure 6 and show that APPI offers lower detection limits than APCI for all three compounds. Use of sodium acetate and ammonium formate in the mobile phase enhances the sensitivity of ESI. For monoarachidin, the ESI sodium adduct detection limits changed over time (day 1 vs day 2) due to signal instability and source contamination caused by the nonvolatile sodium acetate mobile phase additive. The ammonium adduct provides the lowest detection limit for monoarachidin by ESI, but unfortunately, the responses are nonlinear at both low and high concentrations (Figure 5 and Table 1), and therefore, the responses are not quantifiable. For diarachidin, ESI ammonium adduct gives the lowest detection limit, but the signal linear range is as narrow as a 10-ng injection quantity. In the case of trielaidin, the ESI ammonium adduct detection limit is slightly lower than that for APCI but higher than that for APPI. In addition, this ESI ammonium adduct response is linear up to only 5 ng.

Full-Scan Spectra of Fish Oil Samples. The samples ONC-3, ONC-2, and ONC-1 were diluted with the mobile phase to 100 ppm and analyzed in full-scan mode. Analyses were performed by APPI, APCI, and ESI with the mobile phase of 1:1 isooctane/IPA without a modifier. ESI analyses were further performed by using the mobile phase containing 10 mM ammonium formate. Figures 7, 8, and 9 show full-scan mass spectra of samples ONC-3, ONC-2, and ONC-1, respectively. For samples ONC-3 and ONC-2, APPI and APCI give major protonated molecules of EPA (*m/z* 303) and DHA (*m/z* 329) in free fatty acid form and EPA (*m/z* 331) and DHA (*m/z* 357) in ethyl ester form. For ONC-1, APPI

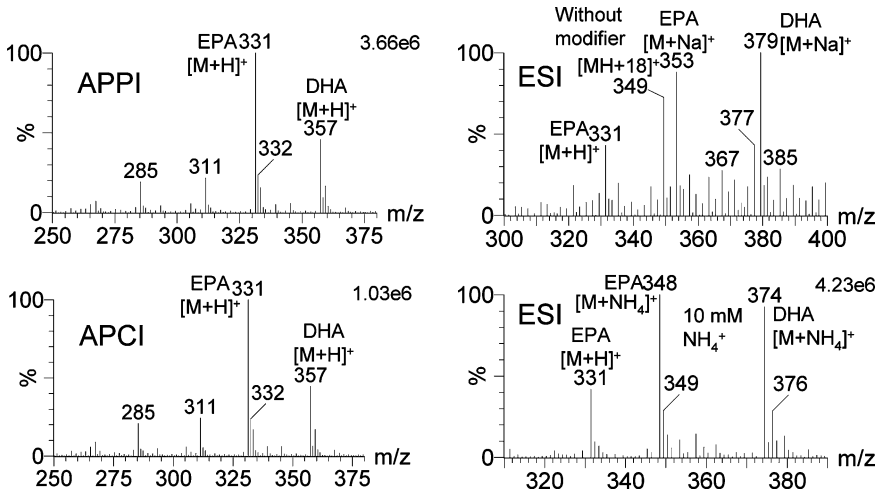

Figure 8. Full-scan mass spectra of sample ONC-2, an ethyl ester of fatty acid mix. APPI and APCI give similar spectra with major ion $[M + H]^+$. ESI gives complex spectra, showing $[M + H]^+$, $[M + H + H_2O]^+$, $[M + Na]^+$, $[M + NH_4]^+$, $[M + K]^+$. EPA *m/z*: 331 = $[M + H]^+$, 349 = $[M + H + H_2O]^+$, 353 = $[M + Na]^+$, 348 = $[M + NH_4]^+$. DHA *m/z*: 357 = $[M + H]^+$, 379 = $[M + Na]^+$, 374 = $[M + NH_4]^+$.

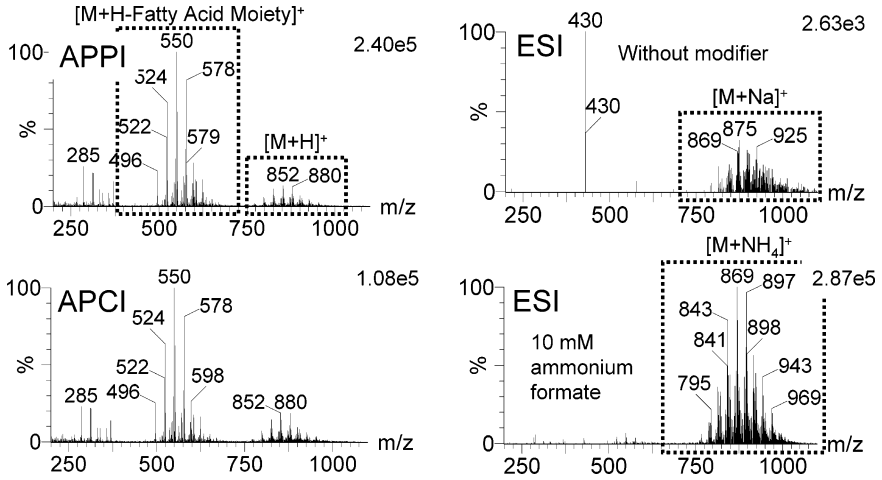

Figure 9. Full-scan mass spectra of sample ONC-1, an unknown triacylglycerol mix with MW ranging from ~700 to 1100. APPI and APCI give similar spectra, offering $[M + H]^+$ and $[M + H -$ fatty acid moiety$]^+$. ESI gives $[M + Na]^+$ when mobile phase does not contain modifier and gives $[M + NH_4]^+$ with enhanced sensitivity when mobile phase contains 10 mM ammonium formate.

and APCI give a complex triacylglycerol pattern with protonated molecule $[M + H]^+$ and a complex triacylglycerol fragment pattern with higher intensity. These major fragments are protonated molecules with each losing a fatty acid moiety. This fragmentation pattern matches well with that of trielaidin, the individual triacylglycerole compound tested (Figure 4). For all three samples, APPI and APCI offer almost identical mass spectra, except that APPI intensity is higher than that of APCI. ESI mass spectra look much more complex than those of APPI and APCI for ONC-3 and ONC-2, showing major ions of $[M + H]^+$, $[M + H + H_2O]^+$, $[M + Na]^+$, $[M + NH_4]^+$, and $[M + K]^+$. Use of ammonium formate additive in the mobile phase dramatically enhances the intensity of some of the major ions by ESI. For ONC-1, ESI gives a pattern of sodium adduct molecular ions with the mobile phase without modifier. When the mobile phase contains 10 mM ammonium formate, the spectra show major ions of ammonium adducts, with a sensitivity increase of ~2 orders of magnitude.

Comparison of EPA and DHA Response Intensity in Fish Oil. The response intensities of the EPA and DHA were obtained from the extracted ions of the target analytes from ONC-3 and ONC-2 full-scan spectra presented in Figures 7 and 8. These full scans were acquired at the same scan rate and scan range so that the data comparison could be done directly. The intensities were compared and are presented in Figures 10 and 11. In all cases, APPI provides the highest peak intensities and ESI, the poorest sensitivity for mobile phase without modifier. Ammonium formate enhances the sensitivity of ESI to varying extents, depending on the target analytes. For EPA in free fatty acid form, sensitivity ranks in the following order:

APPI > APCI ≥ ESI ($[M + NH_4]^+$) > ESI ($[MH]^+$). For DHA in free fatty acid form, sensitivity ranks in the following order:

APPI ≥ ESI ($[M + NH_4]^+$) > APCI > ESI ($[MH]^+$). For EPA in ethyl ester form, sensitivity ranks in the following order:

APPI ≈ ESI ($[M + NH_4]^+$) > APCI > ESI ($[MH]^+$). For DHA in ethyl ester form, sensitivity ranks in the following order:

ESI ($[M + NH_4]^+$) > APPI > APCI > ESI ($[MH]^+$).

We would like to point out that some polar lipid components present in fish oil may be analyzed by ESI^- with high sensitivity under reversed-phase conditions; however, the present work strikes to seek a common set of parameters to cover a wide range of lipid target analytes.

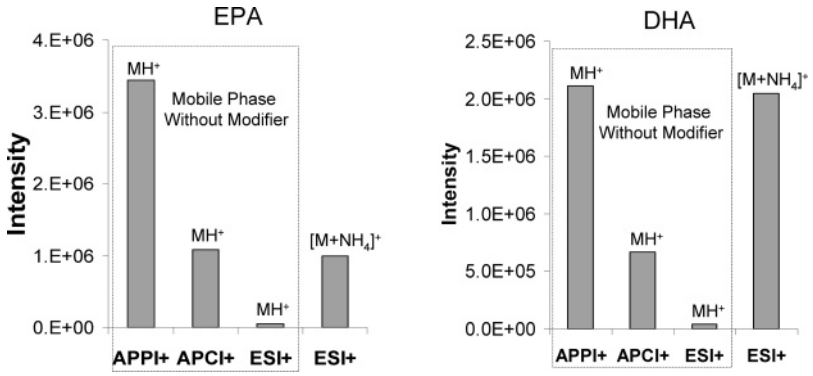

Figure 10. Response intensity comparison of fish oil EPA and DHA in free fatty acid form. APPI offers the highest intensity without mobile phase modifier. Ammonium formate enhances the ESI sensitivity but with dramatically reduced linear range. Extracted ion EPA $[M + H]^+$ $m/z = 303$. DHA $[M + H]^+$ $m/z = 329$. EPA $[M + NH_4]^+$ $m/z = 320$. DHA $[M + NH_4]^+$ $m/z = 346$.

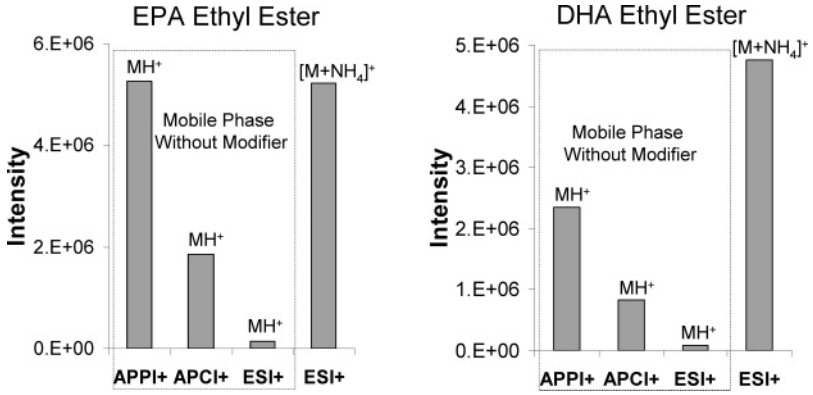

Figure 11. Response intensity comparison of fish oil EPA and DHA in ethyl ester form. APPI offers the highest intensity without mobile phase modifier. Ammonium formate enhances the ESI sensitivity but with dramatically reduced linear range. Extracted ion EPA $[M + H]^+$ $m/z = 331$. DHA $[M + H]^+$ $m/z = 357$. EPA $[M + NH_4]^+$ $m/z = 348$. DHA $[M + NH_4]^+$ $m/z = 374$.

CONCLUSION

APPI has proved to be a powerful analytical tool capable of ionizing small molecules which cannot be ionized efficiently by APCI and ESI. In the above work, we compared the quantitative accuracy and sensitivity of analyzing lipids by APPI, APCI, and ESI-LC/MS. APPI exhibited superior performance in analyzing the tested lipid compounds, including fatty acids, methyl and ethyl esters of fatty acids, monoacylglycerol, diacylglycerol, and triacylglycerol. APPI yielded 4−5 orders of linear dynamic range with upper limits of at least ~1250 to 2500 ng. APPI is ~ 2−4 times more sensitive than APCI and much more sensitive than ESI when the mobile phase does not contain an additive or a modifier. APPI offers better detection limits, higher peak area or intensity, and a higher S/N ratio. Use of ammonium formate as a mobile phase modifier enhances the sensitivity of ESI by varying extents, depending on the target analytes. Although the ESI ammonium adducts can sometimes be more sensitive than APPI, these adduct ion responses are either nonlinear or have significantly reduced linear ranges.

Although reversed-phase APCI and ESI in positive and negative ion mode may work best for some lipids, this work suggests that $APPI^+$ in normal phase may have greater generality over a wide range of lipid compounds. Furthermore, $APPI^{\pm}$ mode is compatible with reversed-phase conditions for those compounds amenable to an aqueous mobile phase. One of the major objections to normal phase is the discharge hazard of APCI and ESI in the presence of flammable organic solvents. It is important to note that APPI does not use a discharge (e.g., APCI), neither is it prone to discharge (e.g., ESI), making APPI in normal phase a safe alternative.

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