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Atmospheric pressure photoionization mass spectrometry for analysis of fatty acid and acylglycerol lipids

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Abstract

In this work, we optimize parameters and conditions for analysis of fatty acid ester and acylglycerol lipids by atmospheric pressure photoionization-mass spectrometry (APPI-MS). The investigated parameters include atmospheric pressure chemical ionization (APCI) nebulizer/vaporizer physical orientation and APPI lamp face position, solvent selections, mobile phase compositions and flow rates, cone voltages and probe temperatures. APPI sensitivity is found to be highly dependent on mobile phase compositions. Normal phase solvents offer much higher sensitivity and better peak shape than reversed phase for nonpolar lipids. Hexane and isooctane are found to be two solvents generating highest S/N for eicosapentaenoic acid (EPA) methyl ester. The effects of mobile phase flow rates on sensitivity are found to be target analytes and target ions specific. However, the flow rate changes do not significantly affect the sensitivity of three out of four tested analytes under normal phase conditions over tested flow rates of 50–500 μL/min. Cone voltage is found to be one of key parameters affecting sensitivity. Optimum probe temperature is found to be more dependent on mobile phase compositions than on the specific target analytes. Aqueous reversed-phase mobile phase requires higher probe temperature than normal phase for better sensitivity. More volatile mobile phase solvents require lower probe temperature for analyte desolvation. APPI offers four to five decades of linear ranges under normal phase condition. Full scan mass spectra of individual lipid standards, custom lipid mixtures and natural fish oil show that APPI spectra are clean and very easy to interpret. APPI also gives stable, reproducible peak responses with good peak shape. Limits of detection (LODs) by FIA (S/N = 3) are estimated to be 12 pg for EPA methyl ester and monoarachidin, 19 pg for diarachidin and 7 pg for trielaidin. LODs on-column are estimated to be 94 pg for EPA methyl ester, 90 pg for monoarachidin and diarachidin and 24 pg for trielaidin.
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1. Introduction

Lipids comprise a family of biomolecules that play prominent roles in many critical metabolic and biochemical processes such as energy production and storage, the formation and functioning of cellular membranes, signal transduction, and steroidgenesis. These diverse ranges of molecules include waxes, fatty acids, fatty-acid derived phospholipids, sphingolipids, glycolipids and terpenoids, such as retinoids and steroids. Routine lipid analytical methods utilize instruments such as high performance liquid chromatography with UV detector (HPLC/UV) and evaporative light scattering detector (HPLC/ELSD), silver ion HPLC/ELSD or UV, and gas chromatography with flame ionization detector (GC/FID). These methods require complete chromatographic separation for target analyte identification. None of these methods provides chemical structure information. GC methods also require derivatization for analysis of non-volatile and thermal labile lipids.

Lipidomics is a large scale analysis of non-water-soluble lipid molecules and metabolites. The detection of these biologically active compounds requires sensitive and specific technique to describe the detailed biochemical events that take place within a cell because these lipids are complex and usually present at very low concentrations. It is preferable to analyze these compounds intact. The major technologies used for analysis of non-volatile lipids are liquid chromatography coupled with mass spectrometry (LC/MS). Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are two popular ionization techniques used in LC/MS for qualitative and quantitative analysis of these lipids [1–10,42–44,46,47]. Based on their ionization

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mechanisms, ESI is more suitable for ionization of polar and ionic compounds and is capable of ionizing both small and large biomolecules. APCI can ionize less polar and neutral compounds more efficiently than ESI. However, ionization of nonpolar compounds can sometimes be difficult by ESI and APCI.

Atmospheric pressure photoionization (APPI) is a newly introduced ionization technique which extends the range of ionizable compounds [11–13]. APPI can ionize both polar and nonpolar small molecules simultaneously with high sensitivity. This is especially true when appropriate non-aqueous reversed-phase mobile phase or normal phase solvent systems are used. APPI has been gaining its polularity mainly due to its ability to ionize those compounds which are poorly ionized or cannot be ionized by APCI and ESI. APPI has been reported to analyze such chemicals as drug discovery compounds, pharmaceauticals and metabolites, peptides, polyaromatic hydrocarbons, steroids, mycotoxin, perflurochemicals, microbial respiratory quinines, aldehyde and ketones, pesticides and antibiotics [14–32].

In our previous work [33], we compared the quantitative accuracy and sensitivity of analyzing lipids by APPI, APCI and ESI. The target analytes included free fatty acids, fatty acid esters, acylglycerols (monoacylglycerol, diacylglycerol and triacylglycerol) and natural fish oil containing unknown triacylglycerols. Our results indicated that APPI offered four to five decades of linear dynamic range. APPI was approximately two to four times more sensitive than APCI and much more sensitive than ESI when mobile phase did not contain a modifier. ESI sensitivity could be dramatically enhanced by using mobile phase modifier or additive (i.e., 10 mM ammonium formate). However, these lipid ammonium adduct responses were either nonlinear (i.e., monoarachidin) or had very narrow linear ranges (i.e., diarachidin and trielaidin). Furthermore, APPI offered the most stable signal responses among the three ionization sources tested. In this paper, we describe the optimization of operating parameters and conditions for lipid analysis by APPI and discuss how major parameters (i.e., mobile phase compositions and flow rates, and desolvation temperatures) interact and how they affect the sensitivity of APPI.

2. Experimental

2.1. Chemicals

Methyl ester of eicosapentaenoic acid (EPA methyl ester, C20:5, MW = 316) was purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). Monoarachidin (monoacylglycerol, C20:0, MW = 386), diarachidin (diacylglycerol, C20:0, MW = 680) and trielaidin (triacylglycerol, C18:1, MW = 884) were all purchased from AccuStandard, Inc. (New Haven, CT, USA). All individual lipid standards are of ≥99% purity. *n*-Hexane (95+%) and isooctane (99.7%), HPLC grade, were purchased from Alfa Aesar (Ward Hill, MA, USA). Isopropyl alcohol (IPA), residue grade, was purchased from EM Science (Gibbstown, NJ, USA). Ammonium formate (99.995+%) and sodium acetate (99%) were purchased from Aldrich Chemicals Co., Inc. (Milwaukee, WI, USA). The chemical structures of EPA methyl ester and acylglycerol analytes are presented in Fig. 1.

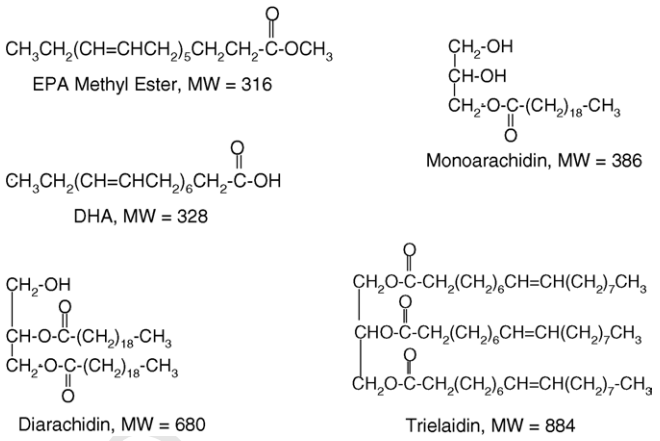

Fig. 1. Chemical structure of lipid target analytes.

2.2. Custom lipid mixture and natural fish oil samples

Three lipid samples were provided by Ocean Nutrition Canada. Sample ONC-3 is a custom mix of free fatty acids containing EPA, docosahexaenoic acid (DHA, Fig. 1), C18:1, C18:0 and C16:0. Sample ONC-2 is a custom mix of fatty acids in ethyl ester form containing major components of EPA and DHA. Sample ONC-1 is natural fish oil with an unknown acylglycerol mixture with MW ranging from ~700 to 1100.

2.3. Instruments

Waters Micromass ZQ LC-MS (Waters Corporation, Milford, MA, USA) was used for this work. The instrument is equipped with APPI (Syagen Technology, Inc), APCI and ESI with the data processing software Masslynx v4.0.

2.4. Preparation of standards

Ten thousand micrograms per milliliter (ppm) of EPA methyl ester stock solution was prepared in hexane. Five thousand micrograms per milliliter of monoarachidin, diarachidin and trielaidin stock solutions were prepared in chloroform. Working solutions were prepared from these stock solutions using corresponding mobile phases as solvents. For evaluation of linear dynamic ranges, the linearity standards were prepared with the used mobile phase by a series dilution method. For EPA methyl ester, the following concentrations were prepared: 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.781, 0.391, 0.195 and 0.0977 ng/μL (ppm). For acylglycerols, the following concentrations were prepared: 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 ng/μL.

3. Results and discussion

3.1. Instrument tuning

For instrument tuning, 10 ng/μL EPA methyl ester in hexane was infused into the source by a syringe pump at a flow rate of

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100 μL/min. A general set of parameters (i.e., repeller = 0.5 kV; APCI probe = 450 °C; cone = 25 V; desolvation gas = 400 L/h; extractor = 3 V; RF lens = 0.2 V; source = 150 °C) was used to optimize the physical orientation of the APCI probe (shared by APPI) and the position of the APPI lamp face. The protonated molecule (*m*/*z* 317) of EPA methyl ester was used for tuning on the Masslynx tune page. For maximum sensitivity, the APCI probe nebulizer is tilted toward the cone face as close as possible but far enough to minimize the nebulized mobile phase spraying directly on the cone face. The APPI lamp face should be set as close to the APCI probe as possible but sufficiently far away to avoid electrical arcing between the repeller (mounted around the lamp face) and the metal wall of the APCI probe. As a general rule of thumb, the optimum vertical distance from the center of the APPI lamp face to the closest point of the APCI probe wall is about 2 mm. After setup of the APCI probe and the lamp face position, other parameters were further adjusted on the tune page.

3.2. Full scan analysis

Ten microliters of 10 ng/μL of EPA methyl ester and 31.3 ng/μL of each acylglycerol standard were analyzed in APPI positive-ion full scan mode. The full scan spectra first presented in a previous work [33] in comparison to APCI and ESI, are shown in Fig. 2 for reference. EPA methyl ester gives a base peak of a protonated molecule *m*/*z* 317. Monoarachidin gives a major ion of *m*/*z* 369, which corresponds to $[M + H - H_2O]^+$, a protonated molecule with loss of water. Diarachidin gives a major ion of *m*/*z* 663 ($[M + H - H_2O]^+$) and a second major ion of *m*/*z* 369 $[M + H - 312]^+$. The neutral moiety of mass 312 corresponds to the saturated fatty acid $C_{19}H_{39}COOH$ (C20:0). The second major ion of diarachidin coincides with the major ion of monoarachidin. Trielaidin gives a major ion of *m*/*z* 603 ($[M + H - 282]^+$) and a second major ion of *m*/*z* 885 ($[M + H]^+$). The neutral moiety of 282 corresponds to the monounsaturated fatty acid $C_{17}H_{33}COOH$ (C18:1). The APPI spectra are clean and easy to interpret. In contrast to the complex ESI lipids spectra reported in our previous work [33], no cluster, dimer or adduct ions are observed in these APPI generated spectra. This implies that APPI is an ionization source which is more selective and less susceptible to the effects of matrix due to less competition for charge or proton. This in turn translates to lower background noise intensity than that of ESI. Full scan analyses were also performed in APPI negative-ion scan mode, but no major ions were observed.

3.3. Selection of mobile phase

Since long chain fatty acids and acylglycerols have limited solubility in aqueous reversed-phase mobile phase solvent systems, we mainly focused on the use of normal phase solvents for analysis of these hydrophobic target analytes. We also notice in the literature that LC separations of many lipids [41–46] are usually achieved under normal phase or nonaqueous reversed-phase conditions. One of the major factors affecting APPI sensitivity is the solvent composition of the mobile phase used. By using EPA methyl ester as a model compound, we tested some commonly used normal phase solvents available in our laboratory. Fig. 3a–c, respectively, show the peak area response, baseline noise intensity and signal to noise (S/N) ratio of EPA methyl ester versus the corresponding used mobile phase. A few solvents or their mixtures (i.e., hexane/chloroform, isooctane, hexane/IPA, dichloromethane, hexane and IPA) offer comparable high peak

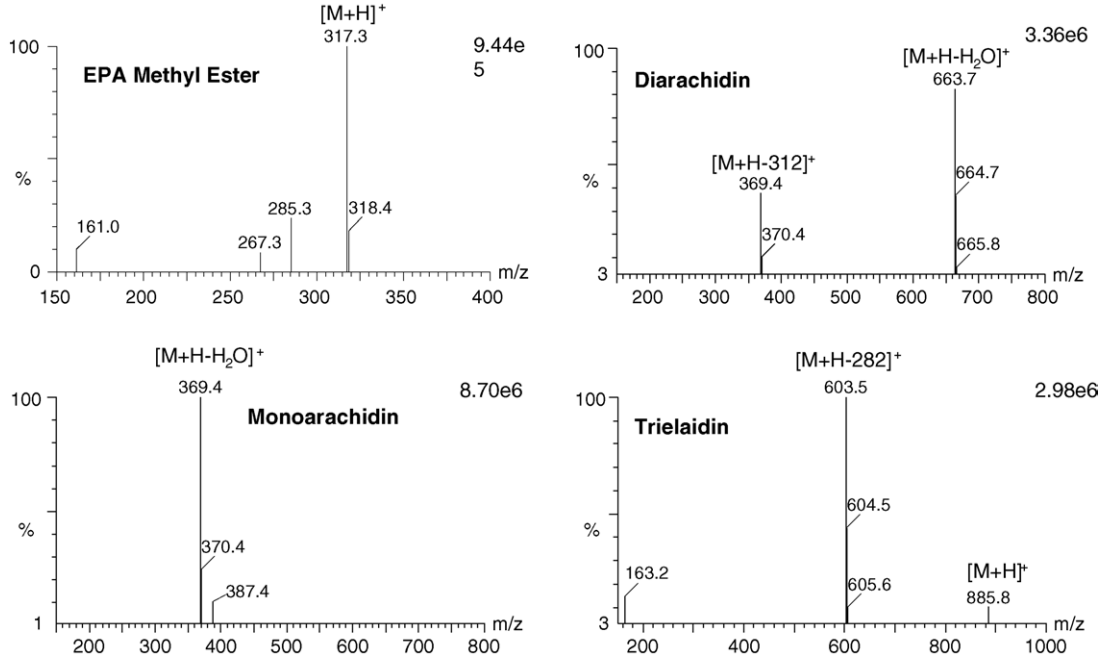

Fig. 2. APPI full scan mass spectra of lipids. MW: EPA methyl ester = 316, monoarachidin = 386, diarachidin = 680, trielaidin = 884. Injection amount: EPA = 100 ng, acylglycerols = 312.5 ng each. Cone: EPA = 30 V, acylglycerols = 25 V. Mobile phase: EPA methyl ester = hexane, acylglycerols = 1:1 isooctane/IPA.

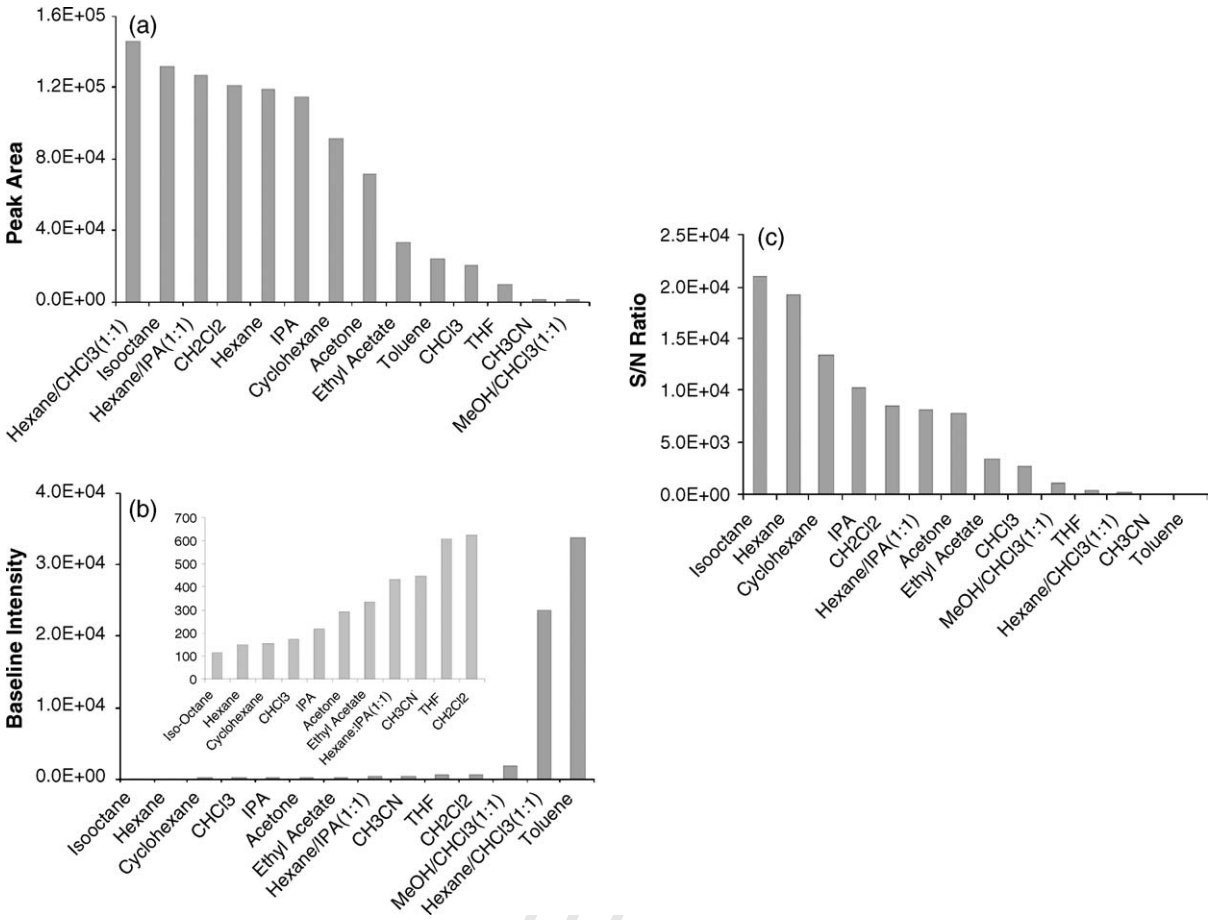

Fig. 3. Effects of mobile phase on (a) peak area, (b) baseline intensity, and (c) S/N ratio of EPA methyl ester (m/z 317). Injection amount = 100 ng (10 ng/μL × 10 μL), mobile phase flow rate = 100 μL/min, cone voltage = 25 V, probe temperature = 450 °C.

area responses for EPA methyl ester (Fig. 3a). However, their S/N ratios (Fig. 3c) are extremely different due to their significantly different background noise intensity (Fig. 3b). One extreme example is the 1:1 hexane/chloroform mixture as a mobile phase, which generated the highest peak area for EPA methyl ester (Fig. 3a), but it gives almost the lowest S/N ratio among all the solvent systems tested (Fig. 3c). By comparison, isooctane and hexane are identified to be two solvents which generate relatively high peak intensities and the lowest background noise, which in turn translates to the highest S/N ratio. It appears that isooctane and hexane exhibit similar behavior as normal phase solvent components except that isooctane gives slightly different solubility for the target analytes.

APPI sensitivity can be affected by many properties of mobile phase components and analytes including volatility, polarity, proton affinity (PA) and ionization energy (IE), and also photon absorption and impurity of mobile phase. We note that the peak area dependence in Fig. 3a is not related to solvent proton affinity. For example the three highest PA solvents, IPA, acetone, and THF give very different analyte ion yields as measured by peak area. A more likely explanation is the differing absorption cross sections of the solvents to the photons. Several researchers have explored these ionization mechanisms of APPI [35–38].

3.4. Effect of mobile phase flow rate

The effects of mobile phase flow rates on the sensitivity of target analytes were examined by injecting 5 or 10 ng/μL of an individual standard at the same injection volume of 10 μL each over a flow rate range of 50–500 μL/min. The major ions of target analytes were acquired to record peak area intensities. For easy relative comparison and graphing purpose, the peak areas were converted to % peak area using the maximum peak area intensity of a particular analyte as a base peak value. The % peak area versus mobile phase flow rate plots are presented in Fig. 4. The results show that the effects of mobile phase flow rate on the sensitivity are not only compound and ion specific but also mobile phase composition dependent. In general, APPI offers higher sensitivity at lower flow rate (i.e., 50–100 μL/min). The sensitivity declines at varying extents as the flow rate increases, depending on target analytes, the specific ions acquired and the compositions of solvent system used.

The peak area intensities of EPA methyl ester $[M+H]^+$ with hexane as mobile phase and diarachidin $[M+H-H_2O]^+$ and trielaidin $[M+H]^+$ with 1:1 isooctane/IPA as mobile phase remain relatively constant over the tested flow rate range of 50–500 μL/min. However, the flow rate effect on the peak area intensity of trielaidin fragment ion $[M+H-282]^+$ behaves

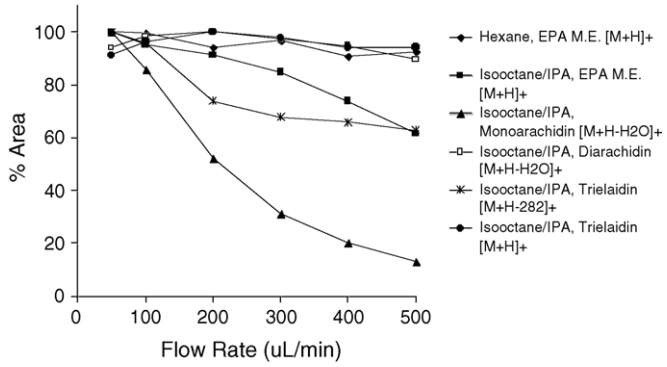

Fig. 4. Effects of mobile phase flow rate on target analyte sensitivity. The syringe pump is not capable of handling a flow rate higher than 500 μL/min.

somewhat differently from its protonated molecule when the same mobile phase of 1:1 isooctane/IPA is used. The area intensity drops by 26% at 200 μL/min and 32% at 300 μL/min relative to its value at 50 μL/min, then it remains relatively constant as the flow rate continues to increase. A somewhat similar situation applies to the case of EPA methyl ester when comparing hexane and 1:1 isooctane/IPA as mobile phases. Unlike with hexane as mobile phase where the sensitivity remains relatively constant, the sensitivity of EPA methyl ester decreases slowly and gradually over the entire tested flow rate range of 50–500 μL/min. In this case, the sensitivity drops by 39% once the 1:1 isooctane/IPA flow rate increases to 500 μL/min. This trend has also been noted by Hsieh et al. [14] and Yang and Henion [17] in their application papers. The possible mechanism behind this phenomenon has been studied by Robb and Blades [39], who believed that decrease of analyte sensitivity with an increased mobile flow rate was partially due to the growth in size of the ion-solvent clusters within the source. They claimed that the larger protonated solvent clusters have higher solvation energies and are less likely to undergo proton transfer reactions with target analytes and that larger clusters are more likely to be neutralized through recombination. The effect of mobile phase flow rate has also been studied by Kauppila and Bruins who believe that the photon adsorption of solvent at higher flow rate is responsible for sensitivity decrease of those analytes which undergo proton transfer [40]. The latter mechanism is plausible and even expected based on estimated absorption cross sections of these solvent molecules at the ionizing photon energy of about 10 eV and is supported by measurements comparing APPI and APCI as a function of flow rates [13].

Monoarachidin is the compound where the flow rate changes have the most significant effect on sensitivity. The peak intensity drops by 48% at the flow rate of 200 μL/min and 69% at the flow rate of 300 μL/min when 1:1 isooctane/IPA is used as a mobile phase. We are unsure why the intensity for this compound drops so steeply with flow rate, but we do note that monoarachidin is the most polar compound of the compounds tested and perhaps requires higher probe temperature at high flow rates to effectively desolvate the compound. Another possible explanation is the proton affinity (PA) of monoarachidin which may be lower than the rest of tested compounds. Compounds with lower PAs or gas-phase basicities have been shown to be more susceptible to the effect of mobile phase flow rate [39].

Although the effect of aqueous reversed-phase mobile phase flow rate on target analyte sensitivity is not included for comparison, we point out that the flow rate increase under aqueous reversed phase condition has more significant adverse effect on the sensitivity of target analytes especially when the mobile phase contains high percentage of water. Since photoionization occurs in the vapor phase, a lower flow rate and a higher probe temperature are more favorable for higher sensitivity when the mobile phase contains high level of water content or when mobile phase contains a solvent with higher boiling point or lower volatility. Photon adsorption by water vapor and growth in size of protonated solvent-water clusters can also be explanations for sensitivity effect [13,39,40].

3.5. Effect of cone voltage

Cone voltage is one of the key parameters affecting the sensitivity of the target analytes. Cone voltage directly affects in-source fragmentation of the compounds. Change of cone voltage alters the relative ion ratios of the mass spectra. A major ion of a target analyte at one cone voltage may not be a major ion at another cone voltage. The degree of fragmentation increases as cone voltage increases. To identify optimum cone voltages for a major ion of a particular analyte, we created a Masslynx acquisition method for each acquired ion of the target analyte. The method contains multiple acquisition functions that enable simultaneously to acquire the target analyte response with a series of cone voltages by a single injection. Each function contains a single channel which has the acquired major ion of the same target analyte and the same dwell time (i.e., 0.02 s), and a cone voltage value that varies in increments of two volts from one function or one channel to the other. Fifty nanograms (i.e., 5 ng/μL × 10 uL) of each target analyte was analyzed for each injection. The mobile phase of 1:1 isooctane/IPA was delivered into the ionization source by a syringe pump at a flow rate of 50 μL/min. Fig. 5 shows the sensitivity of target analytes as

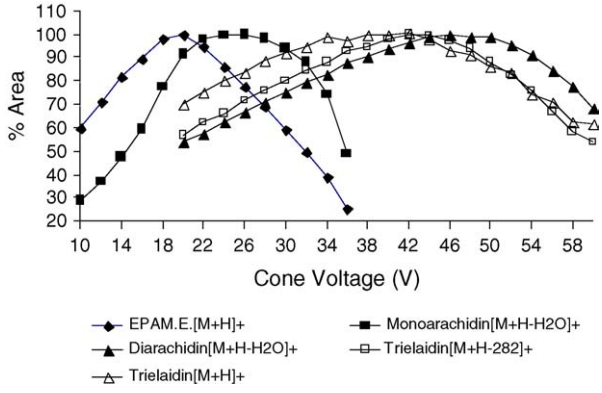

Fig. 5. Effects of cone voltage on sensitivity of analytes. Syringe pump injection, Probe temperature = 450 °C, mobile phase = 1:1 isooctane/IPA at 50 μL/min, injection amount = 50 ng each, optimum cone voltage: EPA methyl ester = 20 V, monoarachidin = 24–26 V, diarachidin = 46 V, trielaidin = 38 V for $[M + H]^+$ and 42 V for $[M + H - 282]^+$.

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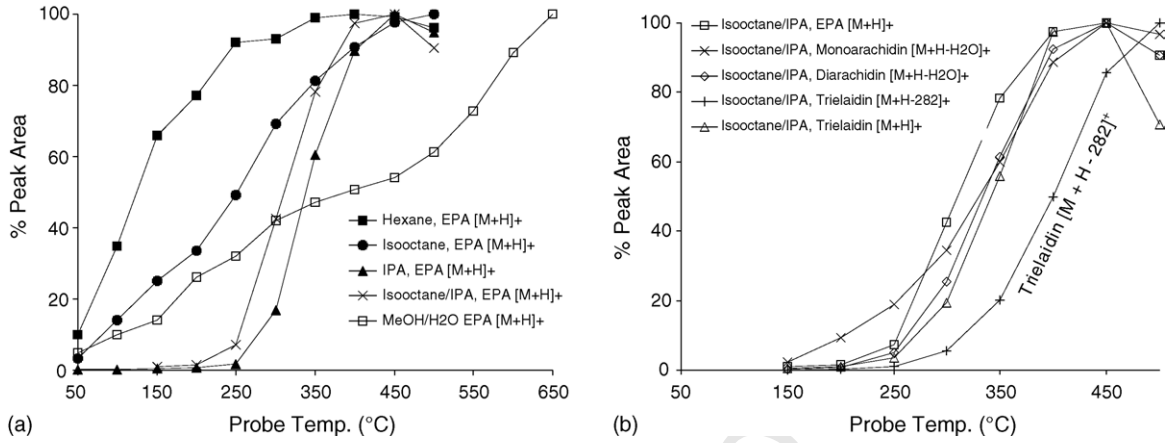

Fig. 6. Effect of mobile phase composition and probe temperature on the peak area of the analytes. (a) Different solvent systems for EPA methyl ester, (b) the same solvent system 1:1 isooctane/IPA for different lipid analytes.

a function of cone voltage. For easy comparison, the peak area intensities are converted to % area using the maximum peak area count as a base value. The maximum peak area of each analyte corresponds to the value of 100% on the vertical axis. The optimum cone voltages are found to be 20 V for EPA methyl ester $[M+H]^+$, 24–26 V for monoarachidin $[M+H-H_2O]^+$, 46 V for diarachidin $[M+H-H_2O]^+$, 38 V for trielaidin $[M+H]^+$ and 42 V for trielaidin fragment ion $[M+H-282]^+$.

3.6. Effect of mobile phase composition and probe temperature

To optimize operating conditions and parameters, we examine how the parameters of mobile phase compositions and probe temperatures interact and how these two variables affect the sensitivity of the target analytes. In one set of experiments (Fig. 6a), we compared the sensitivity of EPA methyl ester by using the commonly used reversed-phase solvent system, 1:1 MeOH/H_2O, and a few normal phase solvents including hexane, isooctane, IPA and 1:1 isooctane/IPA mixture at various APCI (shared by APPI) probe temperatures. In another set of experiments (Fig. 6b), we investigated how probe temperatures affect the sensitivity of different target analytes by using the same normal phase solvent system of 1:1 isooctane/IPA. The results suggest that, for maximum sensitivity, the optimum probe temperature is much more dependent on mobile phase compositions than on the particular compounds analyzed. When comparing different mobile phases used for analysis of the same target analyte EPA methyl ester (Fig. 6a), we observe that hexane requires significantly lower probe temperature than 1:1 MeOH:H_2O, pure IPA and 1:1 isooctane/IPA mixture as mobile phases. Sensitivity reaches almost maximum at about 250 °C with pure hexane versus over 650 °C with 1:1 MeOH/H_2O and 450 °C with pure IPA or 1:1 isooctane/IPA. The required optimum probe temperature follows the trend of 1:1 MeOH/H_2O > IPA > 1:1 isooctane/IPA > isooctane > hexane. The fact that 1:1 MeOH/H_2O mixture shows the highest dependency on probe temperature may also be due to the formation of solvent clusters, which have higher proton affinities than the individual solvent molecules. Lower probe temperature favors formation of these clusters, competing for protons more efficiently with the analytes. In a sense the two explanations are linked in that what makes for poorer evaporation are strong intermolecular bonds, particularly hydrogen bonds, which are due to high proton affinity.

When the same mobile phase of 1:1 isooctane/IPA was used for different analytes, very similar dependences (sensitivity versus probe temperature) were observed regardless of the specific compounds analyzed except that of trielaidin fragment ion $[M+H-282]^+$, which requires higher probe temperature for maximum sensitivity than the rest of the tested compounds (Fig. 6b). The formation and intensity of this fragment ion seems to be related to thermal decomposition. The intensity of this fragment ion is not only a function of cone voltage as discussed above, but also a function of the probe temperature due to thermal degradation. Higher probe temperature is required to break down the parent compound, enhancing the sensitivity of this fragment ion.

The above observations indicate that the required probe temperature decreases as the volatility of the mobile phase increases. These findings are useful for analysis of thermal labile compounds where lower probe temperatures may be required to reduce or minimize thermal degradation. This may be accomplished by either using an appropriate mobile phase or by doing post column addition of a particular solvent (i.e., hexane or pentane) requiring lower desolvation temperatures. Although pentane, a lighter hydrocarbon than hexane, is not included in this study, we expect this normal phase solvent to be a good candidate for analysis of thermal unstable compounds. During our experiments, we also found that normal phase solvent systems offer much better results than reversed phase solvent system in terms of higher peak area count, lower background noise, smoother, narrower and more symmetric peak shapes. The peak responses of nonpolar lipids, generated by an aqueous reversed phase solvent system (i.e., 1:1 MeOH/H_2O), are usually broader and tend to tail more due to poor solubility of these analytes in the reversed phase solvent system. Fig. 7 shows a comparison of EPA methyl ester sensitivity by using the reversed phase solvent system of 1:1 MeOH/H_2O and the normal phase solvent

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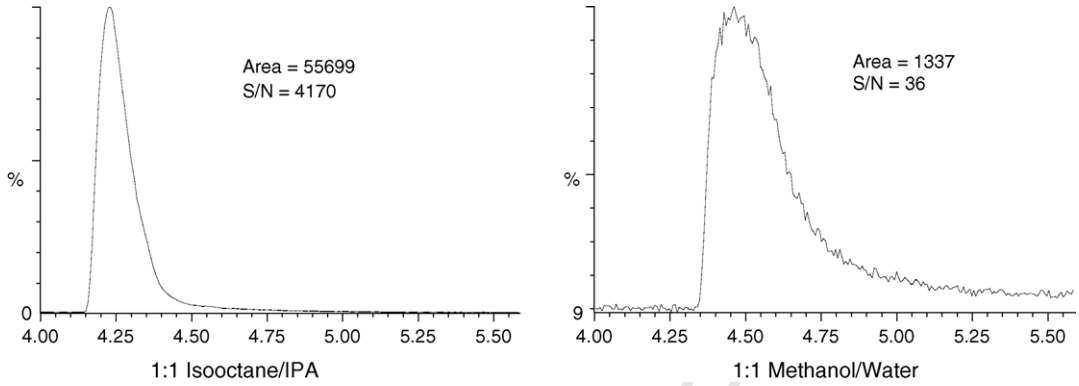

Fig. 7. Comparison of EPA methyl ester peak shape and sensitivity between normal and reversed-phase mobile phase. Syringe injection, injection amount = 50 ng, mobile phase flow rate = 100 μL/min, cone voltage = 25 V, probe temperature = 450 °C.

system of 1:1 isooctane/IPA. The results show that 1:1 isooctane/IPA gives approximately a 40-fold higher peak area count and 120 fold higher S/N ratio than 1:1 MeOH/H_2O. Fig. 7 also compares the peak shapes of EPA methyl ester by using normal and reversed-phase mobile phases. In comparison with the reversed-phase mobile phase, normal phase generates a peak response that is much less noisy, sharper and narrower and more symmetric with less tailing. Furthermore, it is worth mentioning that, based on our experience, acetonitrile/water reversed phase solvent system is sometimes an unfavorable mobile phase for analysis of many compounds by APPI. We observed, in many cases, that acetonitrile offered lower analyte responses than methanol (results not shown). This phenomenon has also been recorded by Robb and Blades [39]. We are not sure what contributes to this phenomenon. One possible reason is due to photon adsorption of acetonitrile, which may be more significant than that of methanol [40]. We advise that users avoid using acetonitrile as a solvent component in the reversed-phase mobile phase for APPI analysis whenever this solvent can be substituted by methanol.

3.7. Linear dynamic range

The series of calibration standards described in Section 2.4 were analyzed to evaluate the dynamic linear range of target analytes. Hexane was used as a mobile phase for EPA methyl ester. 1:1 isooctane/IPA was used as mobile phase for acylglycerol analytes. Single injections were performed for EPA methyl ester and triplicate analyses were performed for the acylglycerol analytes. The mobile phases were delivered into the ionization source by a syringe pump at a flow rate of 100 μL/min. The injection volume was 10 μL. Figs. 8 and 9 show the linearity plots of target analytes with their corresponding correlation coefficient (R^2). EPA methyl ester gave R^2 of 0.9964 for an injected mass range of 0.98–1000 ng (Fig. 8). Analysis was not performed for an injection amount below 0.98 ng or over 1000 ng for EPA methyl ester. The acylglycerol results are presented in Fig. 9. These plots are similar or the same to those presented in our previous work [33], with some differences owing to including data for lower and higher mass injections in this work. Monoarachidin gave a linear range of 19 pg to 2500 ng with R^2 of 0.9986 and a linear range of 19 pg to 5000 ng with R^2 of 0.9919 (Fig. 9a). Diarachidin gave a linear range of 19 pg to 1250 ng with an excellent R^2 of 0.9999 and 19 pg to 2500 ng with R^2 of 0.9960 (Fig. 9b). Two ions were acquired for trielaidin. Trielaidin $[M + H - 282]^+$ generates a linear range of 19 pg to 625 ng with R^2 of 0.9955 and 19 pg to 1250 ng with R^2 of 0.9935 (Fig. 9c). Trielaidin $[M + H]^+$ generates a linear range of 19 pg to 1250 ng with R^2 of 0.9969 and 19 pg to 2500 ng with R^2 of 0.9920 (Fig. 9d). These linearity results show that APPI offers linear dynamic ranges with four to five orders of magnitude for these lipid analytes under normal phase conditions.

3.8. Limits of detection

Limits of detection (LODs) are defined as the amount of a specific target analyte being able to give a response equivalent to three times the background noise (i.e., S/N = 3). The LODs were estimated by both FIA and on-column analysis. For FIA, 0.000977 ng/μL of EPA methyl ester, 0.00191 ng/μL of monoarachidin, diarachidin and trielaidin individual acylglycerol standards were analyzed at an injection volume of 10 μL. A 0.0153 ng/μL of trielaidin was also analyzed for the acquisition

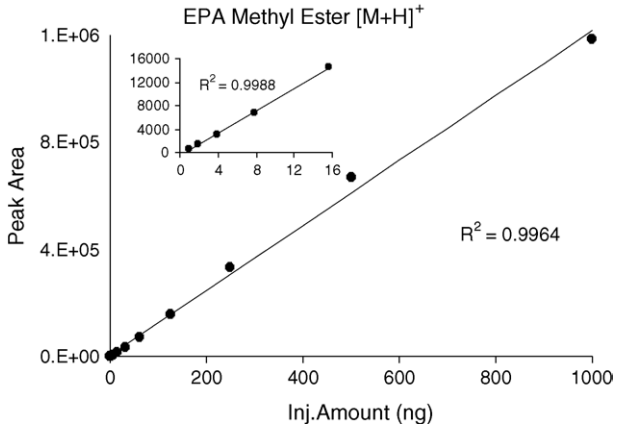

Fig. 8. EPA methyl ester linearity plot. Single-point analysis. Calibration range: 0.98–1000 ng. Injection amount was not analyzed below 0.98 ng and over 1000 ng. Syringe pump injection. Injection volume = 10 μL, mobile phase = hexane at 100 μL/min, probe temperature = 450 °C, cone voltage = 25 V.

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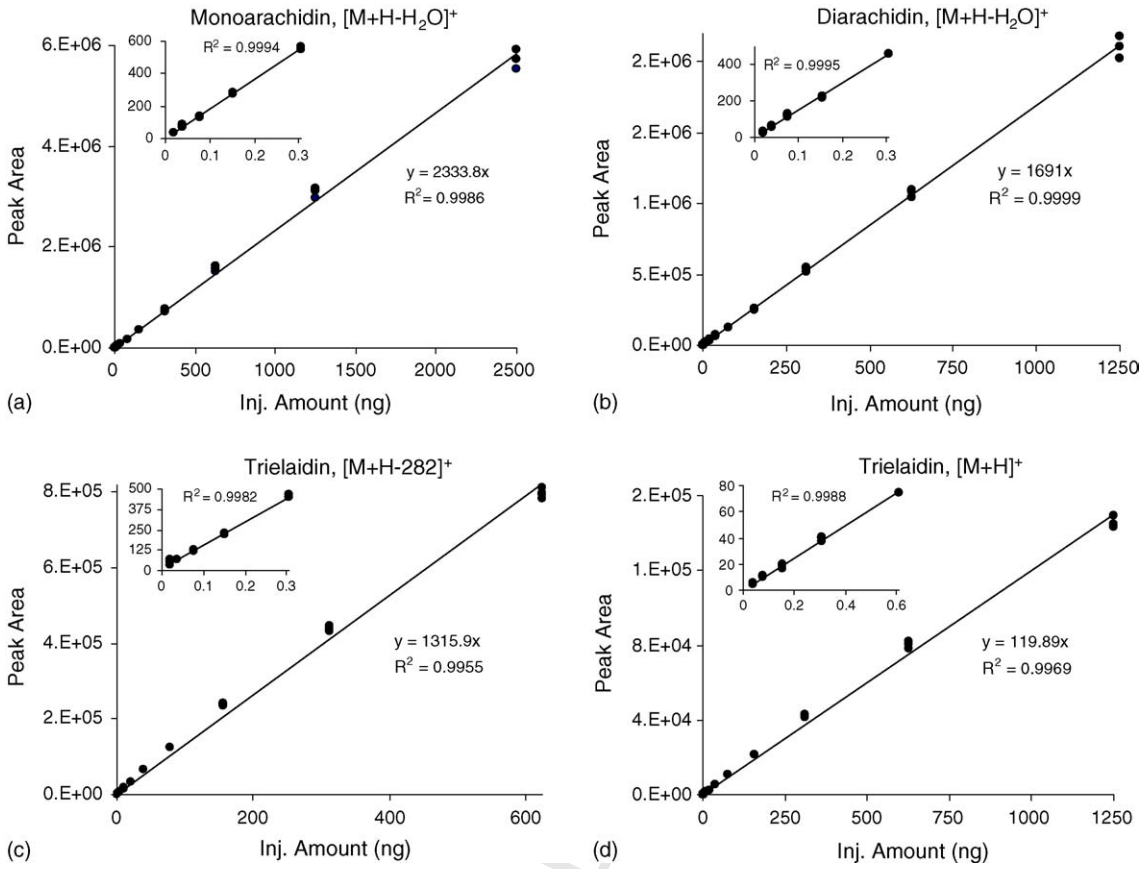

Fig. 9. Linearity plots. Syringe pump injection. Triplicate analyses. Injection volume = 10 μL. Mobile phase = 1:1 isooctane/IPA at 100 μL/min. Probe temperature = 450 °C, cone = 25 V.

of trielaidin protonated molecule which offers lower sensitivity than its fragment ion. These injection amounts were near FIA LOD levels. Analyses were performed by syringe flow injection analysis with the mobile phase of 1:1 isooctane/IPA at a flow rate of 100 μL/min. Blank injection analyses were performed using mobile phase to ensure that no responses of analytes were present prior to analyses of target analyte samples. Other operating parameters were as follows: repeller voltage was set at 0.5 kV; cone voltages were set using the optimum cone voltage values presented in Fig. 5; extractor was set at 3 V; RF lens was set at 0.2 V; APCI probe temperature was set at 450 °C; desolvation gas flow rate was set at 400 L/h and cone gas flow rate was set at 0 L/h (though the readback said 4 L/min). The FIA LODs were estimated to be 12 pg for EPA methyl ester $[M+H]^+$, 12 pg for monoarachidin $[M+H-H_2O]^+$, 19 pg for diarachidin $[M+H-H_2O]^+$, 7 pg for trielaidin $[M+H-282]^+$ and 170 pg for trielaidin $[M+H]^+$. Fig. 10 shows FIA selected ion chromatograms of three replicate analyses of each target analyte at the injection amount described above. On-column analysis was performed on a 50 mm × 2 mm Luna 3 μ Silica (2) (Phenomenex, Torrance, CA, USA) by triplicate injections of 10 μL of the standard mixture containing 0.1 ng/μL each of EPA methyl ester, monoarachidin, diarachidin and trielaidin at a flow rate of 200 μL/min using 1:1 isooctane/IPA as a mobile phase. The on-column LODs were estimated to be 94 pg for EPA methyl ester $[M+H]^+$, 90 pg for monoarachidin $[M+H-H_2O]^+$ and diarachidin $[M+H-H_2O]^+$, 24 pg for trielaidin $[M+H-282]^+$ and 341 pg for trielaidin $[M+H]^+$. Fig. 13 shows chromatographic separation of monoarachidin and diarachidin which generates a common ion of *m/z* 369. Other compounds are separated by their specific *m/z* ratios.

3.9. Analysis of custom lipid mixtures and fish oil

Samples ONC-3, ONC-2 and ONC-1 were diluted by a factor of 10,000 with the mobile phase of 1:1 isooctane/IPA. The resulting diluted samples each contain 100 ng/μL custom lipid mixtures or fish oil by total sample volume. The 100 ng/μL samples were analyzed by APPI in positive-ion full scan mode with a cone voltage of 25 V. Fig. 11 shows the full scan mass spectra of these three samples. ONC-3 gives major ions of *m/z* 303 (EPA) and *m/z* 329 (DHA), and a few other recognizable ions with less intensity including *m/z* 285 (C18:0), *m/z* 283 (C18:1) and *m/z* 257 (C16:0). These are protonated molecules of free fatty acids. ONC-2 gives major ions of *m/z* 331 (EPA), *m/z* 357 (DHA), *m/z* 311 (C18:1) and *m/z* 285 (C16:0). These are protonated molecules of fatty acids in ethyl ester form. The identified compounds in the above custom lipid mixtures (ONC-3 and ONC-2) are consistent with the analyte list provided on the information sheet obtained from Ocean Nutrition Canada. However, some

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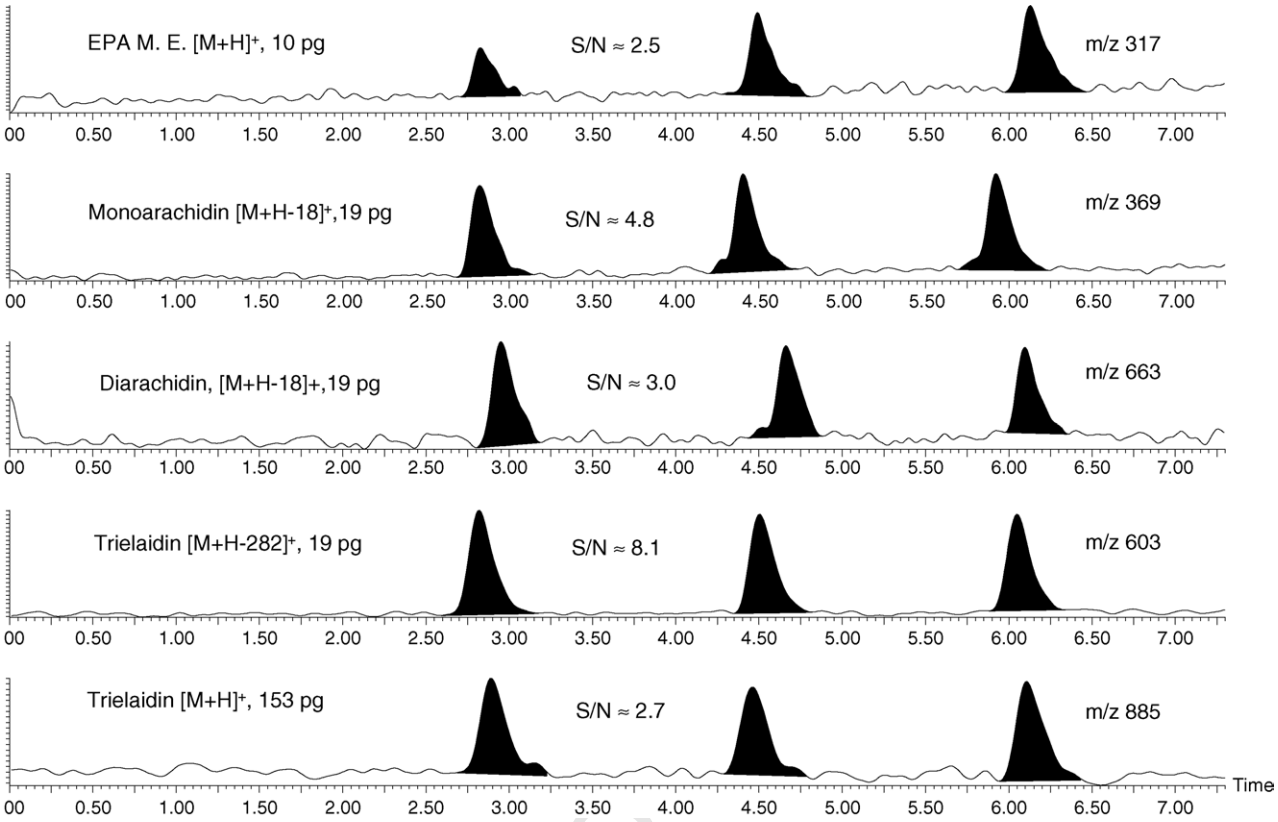

Fig. 10. Instrument detection limits. Standard concentration: EPA methyl ester = 0.977 pg/μL, monoarachidin and diarachidin = 1.91 pg/μL each, trielaidin = 1.91 pg/μL for $[M + H - 282]^+$ and 15.3 pg/μL for $[M + H]^+$. Injection volume = 10 μL each. Triplicate analyses performed by flow injection for each analyte with mobile phase of 1:1 isooctane/IPA at 100 μL/min, probe temperature of 450 °C and optimum cone voltage presented in Fig. 5.

of observed ions may overlap with other compound fragment ions, sharing the same *m/z* ratios. An appropriate LC separation coupled with MS would offer a better method to more accurately identify and quantify each compound in the mixtures. ONC-1 shows a pattern of protonated molecules of acylglycerols and a pattern of acylglycerol fragment ions $[M + H - \text{fatty acyl}]^+$ with higher intensities. These unknown acylglycerol compounds give APPI mass spectra that match very well with that of trielaidin, a known triacylglycerol with a molecular weight of 884. Trielaidin gives $[M + H]^+$ and a fragment ion of $[M + H - 282]^+$ (Fig. 2).

The 100 ng/μL samples were further diluted by a factor of 10 with the mobile phase and analyzed by FIA in selected ion

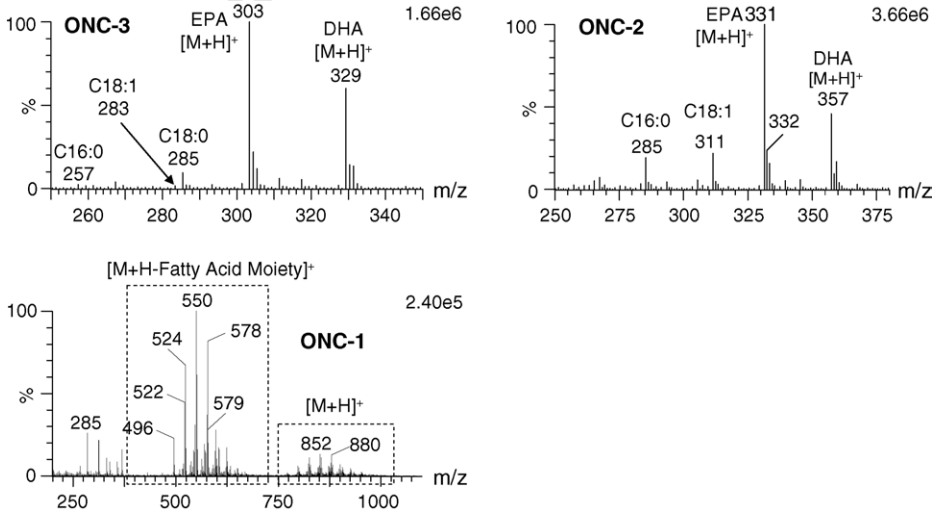

Fig. 11. Full scan mass spectra of 100 ng/μL ONC-3 (free fatty acid mixture), ONC-2 (fatty acid ethyl ester mixture) and ONC-1 (natural fish oil with unknown acylglycerols), injection volume = 10 μL, cone = 25 V, probe temperature = 450 °C. Mobile phase = 1:1 isooctane/IPA at 100 μL/min. These plots are similar or the same to those presented in Ref. [33].

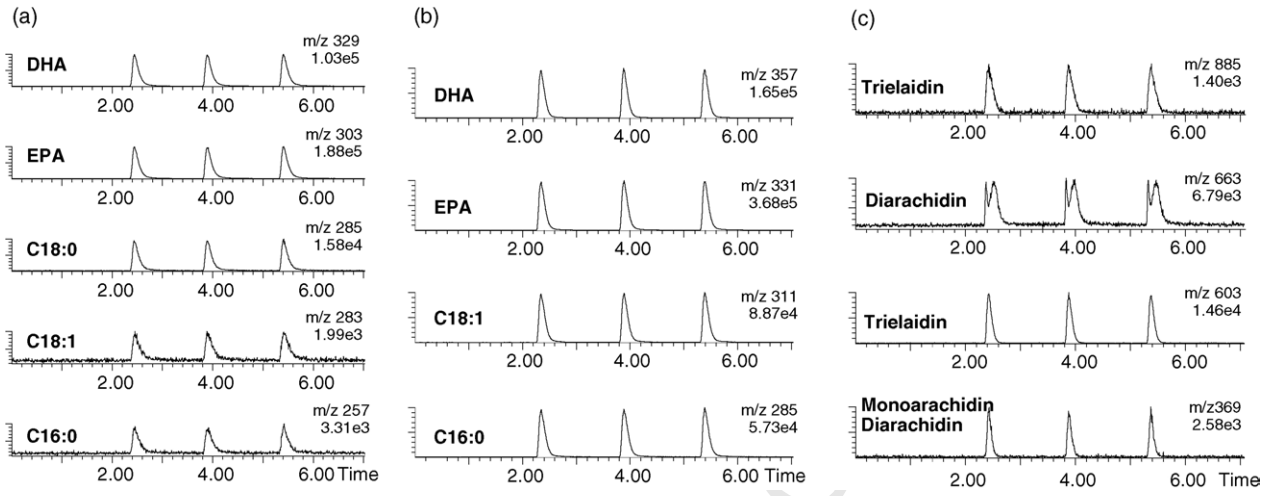

Fig. 12. Selected ion chromatograms. Flow injection analysis. Injection amount = 10 ng/μL fish oil × 10 μL, cone = 20 V except for (c), which used optimum voltages described in Fig 5. Probe temperature = 450 °C, mobile phase = 1:1 isooctane/IPA at 100 μL/min. For (c) *m/z* 663 may represent more than one components sharing the same *m/z* ratio. Diarachidin gives a major ion of *m/z* 663 and a second fragment ion of *m/z* 369 that coincides with the major ion of monoarachidin. For all chromatograms, other ions might also cover more than one component due to the complexity of natural fish oil. *m/z* 369 from monoarachidin and diarachidin can be separated by retention time on Luna silica(2) column (see Fig. 13). (a) ONC-3 fatty acid mixture; (b) ONC-3 fatty acid mixture in ethyl ester form; (c) ONC-1 fish oil in acylglycerol form.

monitoring (SIM) mode. Ten nanograms per microliter ONC-3 and ONC-2 were analyzed using those major or recognizable ions present in full scan spectra and using a uniform cone voltage of 20 V for all analytes. Ten nanograms per microliter ONC-1 natural fish oil was analyzed by acquiring major ions of monoarachidin, diarachidin and trielaidin and by using their optimum cone voltages presented in Fig. 5. Three replicate analyses were performed for each sample using the mobile phase of 1:1 isooctane/IPA at a flow rate of 100 μL/min and injection volume of 10 μL by a syringe pump flow injection (Fig. 12a–c). The selected ion chromatograms show that APPI offers reproducible responses with good peak shapes except for that of

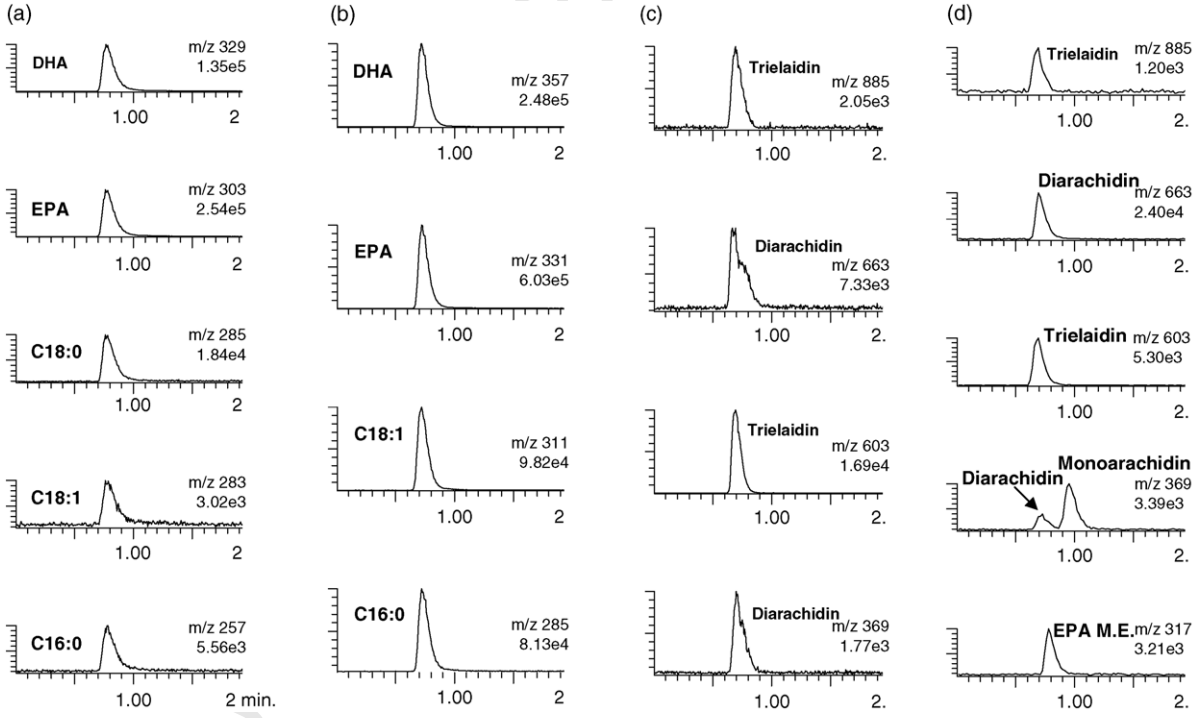

Fig. 13. On-column analysis selective ion chromatograms. Inj. amount = 10 ng/μL fish oil × 10 μL, cone = 20 V except for (c) and (d), which used optimum voltages described in Fig 5. Probe temperature = 450 °C. Column = 50 × 2 mm Luna 3 μ silica (2). Mobile phase = 1:1 isooctane/IPA at 200 μL/min, isocratic. For (c) *m/z* 663 may represent more than one component sharing the same *m/z* ratio. Diarachidin gives a major ion of *m/z* 663 and a second fragment ion of *m/z* 369 which coincides with the major ion of monoarachidin. *m/z* 369 generated from diarachidin and monoarachidin is chromatographically separated (d). All other analytes coelute by retention time but are separated by their *m/z* ratios. Monoarachidin is not detected in (c). (a) ONC-3 free fatty acid mixture; (b) ONC-2 fatty acid mixture in ethyl ester form; (c) ONC-1 fish oil in acylglycerol form; (d) standard mix 0.1 ng/μL × 10 μL.

diarachidin. Diarachidin gives doublet peak responses possibly due to co-elution of more than one component or analogue at the same *m*/*z* 663. Monoarachidin gives a major ion of *m*/*z* 369 which coincides with the second major ion of diarachidin (see also Fig. 2). Fig. 12c also show that the ion intensity ratio of trielaidin fragment ion *m*/*z* 603 $[M + H - 282]^+$ versus the protonated molecule *m*/*z* 885 $[M + H]^+$ is approximately 10 which matches well with that of the trielaidin individual standard full scan mass spectrum presented in Fig. 2. This result implies that it may be possible to perform fast fish oil analysis for some of the target analyte components (i.e., trielaidin) by APPI without chromatographic separation if the target analytes of interest generate unique ions. APPI is an ionization source which is less susceptible to matrix suppression than APCI [20,26] and ESI [34].

The above 10 ng/μL custom lipid mixtures and natural fish oil were also analyzed using the 50 mm × 2 mm Luna 3 μ silica (2) column. Analyses were performed using 1:1 isooctane/IPA as mobile phase with an injection volume of 10 μL and a flow rate of 200 μL/min under an isocratic condition. The acquired ions were the same as those used by FIA as shown in Fig. 12. The used LC condition fails to separate these analytes by their retention time but they can be separated by their *m*/*z* ratios (Fig. 13a–c). Realizing that the monitored ions may overlap with the coeluted fragment ions of other compounds, we did not make any effort to chromatographically separate these target analytes on column. A better method for analysis of these target analytes is to perform multiple reaction monitoring (MRM). However, this is not within the scope of this article. Acetonitrile/chloroform binary solvent system is the typical mobile phase used for nonaqueous reversed-phase HPLC separation of triacylglycerols (TAG) [41]. Hexane/1-propanol with additives as mobile phase has been used to separate many phospholipids compounds [42]. Methanol/ethanol mixture has been used to separate glycolipids on reversed-phase C18 column [43]. Methanol/acetonitrile [44,45] and methanol/IPA [46] have been used for separation of many oxidized lipids. Evaluation of these pre-established LC conditions and mobile phases for successful separation and sensitive analysis of lipid analytes by APPI will be the focus of our future work.

4. Summary and conclusions

In this work, we expand on a previous paper that compared the performance of APPI for the study of nonpolar lipids compared to APCI and ESI. Here, we show the optimum conditions for APPI analysis. More generally, this work shows that APPI is well suited to normal phase or nonaqueous reversed-phase solvent conditions with regard to sensitivity, dynamic range and uniform signal with flow rate.

Because normal phase or nonaqueous reversed-phase solvents tend to be more volatile than aqueous reversed-phase solvents, lower probe temperatures can be used to achieve desolvation. The lower probe temperature is shown to significantly reduce decomposition of analyte and improve detectability by giving a higher proportion of ion signal as the parent ion. A common characteristics of the mobile phases used for LC separations of many lipids is that these mobile phases contain little or no water [41–46]. APPI offers high sensitivity under normal phase or nonaqueous reversed-phase LC conditions. We believe that APPI-LC/MS will prove to be a very successful technique for analysis of lipids in the future.

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