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Cubero Herrera, Lisandra; Potvin, Michael A.; Melanson, Jeremy E.

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Quantitative analysis of positional isomers of triacylglycerols *via* electrospray ionization-tandem mass spectrometry of sodiated adducts

Lisandra Cubero Herrera and Jeremy E. Melanson*

*** To whom correspondence should be addressed**

Jeremy E. Melanson
National Research Council
Institute for Marine Biosciences
1411 Oxford St.
Halifax, Nova Scotia, Canada B3H 3Z1
tel 902.426.6357 fax 902.426.9413
jeremy.melanson@nrc-cnrc.gc.ca

Abstract

Herein we report a reversed phase-high performance liquid chromatography tandem mass spectrometry (RP-HPLC-MS/MS) method for the analysis of triacylglycerols (TAGs) positional isomers in vegetable oils. The fragmentation behavior of $[M + X]^+$ ions (X= NH_4, Li, Na or Ag) was studied on a quadrupole-time-of-flight (Q-TOF) mass spectrometer under low-energy collision-induced dissociation (CID) conditions. Mass spectra that were dependent on the X^+ ion and the nature and position of the acyl substituents were observed for four pairs of 'AAB/ABA'-type TAGs, namely PPO/POP, OOP/OPO, LLO/LOL and OOL/OLO (where P is 16:0, palmitic acid; O is 18:1, oleic acid; and L is 18:2, linoleic acid). For the majority of $[M + X]^+$ adducts, the loss of the fatty acid in the outer positions (*sn*-1 or *sn*-3) was favored over the loss in the central position (*sn*-2), which enabled the determination of the fractional abundance of the isomers. Ratios of the intensity of fragment ions at various AAB/ABA compositions produced linear calibration curves with positive slopes, comparable to those obtained traditionally by ESI-MS/MS of $[M + NH_4]^+$ adducts. The only exceptions were the $[M + Ag]^+$ adducts of the PPO/POP system, which produced calibration curves with negative slopes. Sodium adducts provided the most consistent level of isomeric discrimination for the TAGs studied and also offered the most convenience in that they required no additive to the mobile phase. Therefore, calibration curve data derived from $[M + Na]^+$ adducts were applied to the quantification of TAG regioisomers in sunflower and olive oils. The regiospecific analysis showed that palmitic acid was typically located at positions *sn*-1 or *sn*-3, whereas unsaturated fatty acids, oleic and linoleic acids, were mostly found at the *sn*-2 position.

Introduction

Triacylglycerols (TAGs) are the primary components of natural fats and oils, and consist of three fatty acids on a glycerol backbone. Clinical studies have shown that the type and position of the fatty acyl substituents of TAGs play an essential role in lipid digestion, absorption and metabolism.[1-4] Therefore, the development of methods for the positional

analysis of individual TAG species can provide valuable information for the planning of dietary, nutritional, and metabolic studies.

As natural oils tend to be complex mixtures of TAGs, analytical methods offering high-specificity are generally required for their analysis. Typically, reversed phase-high performance liquid chromatography (RP-HPLC) with on-line mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) is employed for comprehensive TAG analysis. This approach has been successfully employed to profile TAGs in a variety of vegetable oils[5-7] and animal fats.[7,8] However, RP-HPLC typically cannot separate positional isomers of TAGs (*ie.* AAB and ABA). Therefore, the role of the mass spectrometer is not simply for identification of TAGs, but also to provide information on the position of fatty acyl substituents within TAGs. In general, this is accomplished by exploiting the differential fragmentation of the fatty acid at the *sn*-2 position relative to the loss of the fatty acids at the *sn*-1 and *sn*-3 positions.

A variety of ionization techniques have been reported for the determination of the position of fatty acids in TAGs. Relying on in-source fragmentation for the generation of diacylglycerol (DAG) fragments, atmospheric pressure chemical ionization (APCI) has been implemented successfully for the differentiation of TAG positional isomers .[5-10] Mottram and Evershed observed in APCI-MS that the least abundant DAG ion generated corresponded to the loss of the fatty acid from position *sn*-2, which enabled determination of the positional distribution of fatty acids.[9] Despite the success of this approach, implementation of the methodology for complex mixtures with potentially co-eluting TAGs is problematic for both TAG identification and positional analysis. APCI mass spectra of TAGs typically yield predominantly DAG fragment ions $[M + H - RCOOH]^+$ with some level of intact $[M + H]^+$ ions observed, the intensity of which is inversely related to the degree of saturation.[5,11] Attempts to enhance specificity of this approach by performing tandem MS on the $[M + H]^+$ ions generated by APCI have not been successful.[6] Therefore, APCI is well suited for positional analysis of simple mixtures operating in single-stage MS mode but is generally not useful for complex samples that require enhanced specificity offered by tandem MS.

Although not considered the ideal ionization mode for non-polar compounds, electrospray ionization (ESI) has been successfully employed for the determination of TAG positional isomers. The analysis of TAGs by ESI-MS/MS was first reported by Duffin *et al.* who observed DAG fragments after collision-induced dissociation (CID) of $[M + NH_4]^+$ ions in a triple quadrupole (QqQ) mass spectrometer.[12] However, their method was not applicable to the quantification of positional isomers because the formation of DAG ions was not clearly dependent on the location of fatty acids within TAGs. More recently, Hvattum[13] was able to distinguish the positional isomers of ammoniated TAGs in a QqQ instrument, and observed that the neutral loss of the fatty acid on *sn*-2 was less favorable than the loss of the fatty acid from the *sn*-1 or *sn*-3 positions. Malone and Evans, [7] and Marzilli *et al.*[14] also used the relative abundance of DAG fragments from $[M + NH_4]^+$ ions to differentiate between primary and secondary positions by ion-trap MS. In addition to $[M + NH_4]^+$ ions, the use of $[M + Li]^+$ adducts for the positional analysis of TAGs has also been examined. Hsu and Turk observed that CID of $[M + Li]^+$ adducts in a QqQ instrument produced mainly $[M + Li - RCOOH]^+$, $[M + Li - RCOOLi]^+$, and RCO^+ ions which permitted assigning the position of the fatty acids within a TAG.[15] However, in another ESI-MS/MS study with a QqQ instrument, CID spectra of $[M + Li]^+$ adducts from positional isomers were indistinguishable.[16]

Due to the various adducts that have been previously employed for the determination of TAG regioisomers,[7,14-15] and some apparent inconsistencies in the literature [12,16] a comprehensive investigation of various adducts for the determination of TAG isomers was carried out. TAGs that are positional isomers (*sn*-AAB/*sn*-ABA) PPO/POP, OOP/OPO, LLO/LOL and OOL/OLO were used as standards in the study. Low-energy CID tandem spectra of their $[M + X]^+$ adducts contained product ions that identify each fatty acyl substituent, and their relative intensity allows assignment of the location of the fatty acids on the glycerol backbone. Standard mixtures of positional isomers were analyzed, and linear calibration curves were obtained for each set of $[M + X]^+$ adducts. Using this approach, calibration plots derived from $[M + Na]^+$ adducts were applied to the quantification of PPO/POP, OOP/OPO, LLO/LOL and OOL/OLO in sunflower and olive oils *via* RP-HPLC-ESI-MS/MS. To the best of our knowledge, this is the first report

of the use of low-energy CID tandem mass spectra of ESI-generated $[M + Na]^+$ ions for positional isomer differentiation of TAGs.

EXPERIMENTAL

Materials

Positional isomers are denoted throughout the text as AAB and ABA, where TAGs of the ABA-type contain fatty acid A at positions *sn*-1 and *sn*-3, and fatty acid B at position *sn*-2. TAGs such as AAB and BAA, where A and B denote different fatty acids, exist as pairs of enantiomers and cannot be distinguished by this approach. Therefore, for our purposes, positions *sn*-1 and *sn*-3 are indistinguishable. DAG ions $[M + X - RCOOH]^+$ and $[M + X - RCOOX]^+$, where X can be H, Li, Na or Ag, are defined also as $[AA]^+$ and $[AB]^+$ throughout the text. One-letter abbreviations used are: P, palmitic acid (16:0); O, oleic acid (18:1 *cis*-9) and L, linoleic acid (18:2 *cis*,*cis*-9,12). TAG standards POP, PPO, LOL, LLO, OOL and OLO were purchased from Larodan Fine Chemicals AB (Malmö, Sweden) while OPO and OOP were acquired from Sigma-Aldrich (St. Louis, MO, USA). Isomeric purity of TAG standards was confirmed by ^1H and ^{13}C NMR on a 700 MHz Bruker Avance III NMR spectrometer using $CDCl_3$.

The solvents methanol, 2-propanol and dichloromethane (distilled in glass) were purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada). The salts ammonium formate (≥99.995%), sodium acetate (TraceSelect), lithium acetate dihydrate (SigmaUltra) and silver trifluoromethane sulfonate (≥99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and solvents were used without further purification. Sunflower oil (Organic, Compliments, Mississauga, ON, Canada) and extra virgin olive oil (Originale, Bertolli, London, UK) were purchased from a local supermarket. Nitrogen and argon (UHP) for the source and collision gases of the mass spectrometer, respectively, were obtained from Praxair (Halifax, NS, Canada).

Solutions

The ammonium, lithium, and silver salts were dissolved in methanol to yield solutions with *ca.* 1×10^{-4} mol L^{-1}. Stock solutions of all TAG standards (1.0×10^{-3} mol L^{-1}) were prepared in a dichloromethane/2-propanol/methanol solvent system (2:1:7, v/v/v).

Binary mixtures of the four pairs of positional isomers (PPO/POP, LLO/LOL, OOL/OLO, OOP/OPO) were prepared in methanol, 1×10^{-4} mol L^{-1} ammonium formate, 1×10^{-4} mol L^{-1} lithium acetate and 1×10^{-4} mol L^{-1} silver trifluoromethane sulfonate. Pairs of TAG positional isomers AAB and ABA were mixed to form solutions with final molar ratios (AAB:ABA) of 0:100, 25:75, 50:50, 75:25 and 100:0. The total concentration of TAG isomers in each mixture was 4.0×10^{-6} mol L^{-1}. Calibration curves were constructed by plotting the relative molar quantity (%) of the ABA isomer in a mixture of ABA and AAB, [ABA/(ABA+AAB)]×100, *versus* the relative counts of $[AB]^+$-type ions (%), $(i_{AB}/(i_{AB}+i_{AA}))\times100$, where i_{AB} is the intensity of $[AB]^+$ ions, etc. Vegetable oils (sunflower and olive oil) were dissolved in a dichloromethane/2-propanol/methanol solvent system (2:1:7, v/v/v) to yield solutions with *ca.* 10 mg mL^{-1}.

Instrumentation

Mass spectra were obtained with a Waters Q-TOF Premier mass spectrometer (Waters, Milford, MA, USA) running under MassLynx ver. 4.1 software and equipped with either an ESI or an IonSABRE APCI probe. The mass spectrometer was used in ESI(+) or APCI(+) modes with nitrogen as source gas. Ionization conditions were optimized using a solution of OOL in methanol (1.0×10^{-6} mol L^{-1}). Regular operating parameters were: corona voltage (APCI) = 5.0 kV, capillary voltage (ESI) = 3.5 kV, cone voltage = 50 V, source temperature = 100 °C, probe temperature (APCI) = 500 °C, desolvation temperature (ESI) = 500 °C, cone gas flow = 50 L h^{-1} and desolvation gas flow = 500 L h^{-1}. Low-energy CID tandem mass spectra of $[M + X]^+$ species (X= H, NH_4, Li, Ag or Na) were acquired using argon collision gas at a gas flow rate of 0.45 ml min^{-1}, and collision energies between 10 and 45 eV. Product-ion spectra and full-spectrum data were obtained over a 50–1500 *m/z* range.

All solutions were introduced into the ion source of the mass spectrometer by flow injection using a model 1100 HPLC System (Agilent Technologies, Mississauga, ON, Canada) comprised of a binary pump and an autosampler. For the analysis of standard solutions of positional isomers (25 μL), methanol was delivered at a flow rate of 0.2 mL min^{-1}. For the purpose of constructing the calibration curves, an average background was subtracted from the average analytical signal (ten scans) and the responses (ion counts) at the observed *m/z* values of the product ions resulting from the dissociation of the fatty acyl chains from the TAGs, were recorded.

TAGs in vegetable oil solutions (1 μL) were separated on a C18 column (Nova-pak, 150 mm × 3.9 mm i.d., particle size 4 μm, Waters, Milford, MA, USA) using the following methanol / 2-propanol gradient at a flow rate of 0.5 mL min^{-1}: initial methanol/2-propanol (90:10); linear from 1 to 35 min to methanol/2-propanol (20:80), and held isocratic for 5 min. The column was returned to its original condition using a linear program from 40 to 45 min, and was equilibrated for 5 min before starting the next run. A post-column split directed a flow of 0.15 mL min^{-1} to the mass spectrometer.

RESULTS AND DISCUSSION

Selection of ionization mode - Electrospray ionization of TAG standards

A brief study was performed to determine the optimal ionization mode configuration for our particular mass spectrometer. Without any additive present, the electrospray mass spectra of TAG standards in methanol yielded sodiated adducts $[M + Na]^+$ as base peaks, as has been previously reported.[17] Addition of a sodium salt to the mobile phase did not significantly increase the intensity of $[M + Na]^+$ adducts indicating that sufficient sodium ions were present in methanol for efficient ionization at the range of TAG concentrations employed in this study (0.04 μM – 4 μM). Upon addition of the appropriate salt to the mobile phase, $[M + NH_4]^+$,$[M + Li]^+$, and $[M + Ag]^+$ ions were detected as the base peaks in their respective spectra. Without adding any sodium salt, nearly equivalent signals were observed for a given $[M + X]^+$ adduct of the TAG standards, with differences in intensity measured at less than 5 %. Although minor variations among TAG adducts were detected for a given X^+ ion, the signal of $[M + X]^+$ species was

observed to increase with the degree of unsaturation. Finally, the sensitivity for ESI-generated $[M + X]^+$ adducts was at least ten times higher than that observed for $[M + H]^+$ ions generated by APCI.

In contrast to APCI-MS, which produced abundant DAG fragment ions even when operated under the mildest possible conditions, ESI mass spectra of the TAG standards generated minimal in-source fragmentation. In addition, quantification of positional isomers by APCI-MS was challenging due to the presence of co-eluting TAGs with common DAG fragments. To reduce this limitation we targeted the analysis to TAGs of a particular molecular weight (M.W.) using CID. However, test studies at our laboratory showed that CID mass spectra of APCI-generated $[M + H]^+$ ions were indistinguishable, making our APCI-MS/MS method incapable of quantifying TAG regioisomers. Kallio and colleagues also observed no differentiation between TAG positional isomers in APCI-MS/MS.[6] Due to the difficulties associated with the use of APCI-MS and APCI-MS/MS for the regiospecific analysis of TAGs, electrospray ionization was employed for all further studies described below.

ESI(+)-MS/MS of $[M + X]^+$ adducts (X = NH_4, Li, Na or Ag)

A comprehensive investigation of various adducts for the determination of TAG isomers was carried out to determine the optimal adduct for use on our specific instrumental configuration. Given the well-known affinity of silver ions to double bonds through the advent of silver-ion chromatography [20] and the recent use of $[M + Ag]^+$ adducts for regioisomer differentiation by ESI-MS/MS,[21] silver was also investigated as a potential metal ion that might offer unique selectivity for the determination of TAG regioisomers. Therefore, the fragmentation behavior of TAG adducts $[M + X]^+$ (where X is NH_4, Li, Na or Ag) was studied to compare the degree of isomeric differentiation offered by each adduct type.

In general, based on observation of fragmentation as a function of collision energy, the relative ease of generating DAG ions from $[M + X]^+$ adducts followed the order $[M + Na]^+ < [M + Ag]^+ < [M + Li]^+ < [M + NH_4]^+$, with ammoniated species being the most labile. The product-ion mass spectra of $[M + X]^+$ adducts of PPO and POP are shown in

Fig. 1 . In general, dissociation of all adducts produced DAG ions with relative intensities that were dependent on the position of the fatty acyl substituents. Ammoniated species generated uniquely DAG ions of the type $[M + NH_4 - NH_3 - RCOOH]^+$, presumably by the loss of a neutral fatty acid and ammonia as reported in previous studies.[7] Adducts of TAGs with lithium, sodium and silver ions fragmented in the collision cell producing two major ions, $[M + X - RCOOH]^+$ and $[M + X - RCOOX]^+$, that allowed the identification of positional isomers. This can be observed from the different signal intensities of $[AA]^+$ and $[AB]^+$ ions of either the $[M + X - RCOOH]^+$ or $[M + X - RCOOX]^+$ type generated from PPO and POP. In all cases, preferential cleavage of the fatty acyl substituents on positions *sn*-1 and *sn*-3 show increased formation of both $[M + X - RCOOH]^+$ and $[M + X - RCOOX]^+$ product ions. Therefore, the relative abundance of these DAG ions can be used to distinguish between fatty acids on positions *sn*-1/3 and *sn*-2. A similar behavior was observed for the OOP/OPO, OOL/OLO and LLO/LOL systems used in the study.

The use of $[M + NH_4]^+$, $[M + Li]^+$ and $[M + Ag]^+$ adducts for the determination of positional isomers of TAGs is well documented.[7,15,19] However, differentiation of regioisomers by low-energy CID of $[M + Na]^+$ ions has not been reported to date. Duffin *et al.*[12] studied the fragmentation of $[M + Na]^+$ adducts of TAGs on a triple quadrupole instrument using argon as collision gas. The authors found that interpretation of their MS/MS mass spectra was hindered by the low abundance of structurally informative product ions even under extreme collisional activation conditions. Segall *et al.*[17] did observe both $[M + Na - RCOOH]^+$ and $[M + Na - RCOONa]^+$ type DAG ions on an ion trap mass spectrometer using helium as collision gas, yet the relative abundances of DAG product ions were not significantly different, making differentiation of positional isomers impossible. The most complete structural analysis of TAGs has been performed by high-energy (4 to 5 keV) CID of $[M + Na]^+$ adducts utilizing a four-sector tandem mass spectrometer, [20,21] and more recently, a TOF/TOF mass spectrometer.[22] Interpretation of the patterns observed in these spectra allows the determination of the total number of carbon atoms in the fatty acyl chains, the number of double bonds and their location, as well as the position of the fatty acyl groups on the glycerol backbone. Although these results are significant, application of high-energy CID for routine analysis of TAGs is

impractical as tandem sector instruments are generally not available in most modern laboratories. A current limitation of the use TOF/TOF-MS for TAG analysis is the fact that precursor ion selection is limited to a mass window of 4 *m/z* in most TOF/TOF instruments, making the analysis of TAGs separated by one double bond problematic. In addition, as commercial TOF/TOF systems rely on matrix assisted laser desorption ionization (MALDI), the technological challenges of performing LC-MALDI for analyzing complex TAG mixtures would be a significant drawback.

CID tandem mass spectra of AAB/ABA standards show that in almost all cases, the loss of the fatty acid at position *sn*-2 was least favored, and differentiation of positional isomers was possible. The preferential loss of the fatty acid at positions *sn*-1/3 seems to be a general phenomenon for TAG molecules since it has been observed by several authors, independent of the instrumentation employed.[5-7, 23] However, CID mass spectra of $[M + Ag]^+$ adducts of PPO and POP (Fig. 1) at different collision energies did not follow the generally accepted theory. For this system, we observed unexpectedly higher $[PO]^+/ [PP]^+$ ratios for PPO than for POP (2.5 *vs.* 1.6), even though one of the two possible $[PO]^+$ ions derived from PPO is formed by loss of palmitic acid (or its neutral salt) from position *sn*-2. Theoretical calculations are currently being performed in our laboratory in an attempt to explain this unusual behavior, which has not been previously reported.

For a given TAG, the abundance of DAG fragments $[M + X - RCOOH]^+$ was relatively much higher than the abundance of $[M + X - RCOOX]^+$ ions if these originated from $[M + Ag]^+$ adducts as opposed to $[M + Na]^+$ or $[M + Li]^+$ species, and this difference was more apparent in TAGs with a higher degree of unsaturation. This is in accordance with the results obtained by Kallio *et al.* in a recent study.[19] The authors developed an ESI-MS/MS method for the positional analysis of highly unsaturated TAGs in the form of $[M + Ag]^+$ adducts. CID tandem mass spectra of $[M + Ag]^+$ ions derived from the LnLL/LLnL system (where Ln is linolenic acid, 18:3) showed both $[M + Ag - RCOOH]^+$ and $[M + Ag - RCOOAg]^+$ ions, with the $[M + Ag - RCOOAg]^+$ DAG fragments being observed in much lower abundance than the $[M + Ag - RCOOH]^+$ ions. For this system,

the authors also observed that fatty acids on positions *sn*-1 or *sn*-3 were preferentially lost, as is generally observed with other adducts.

Lithium, sodium and silver ions can bind to several sites of a TAG molecule, for example, the carboxyl groups or the double bonds of unsaturated fatty acyl residues. Given that MS/MS mass spectra of $[M + X]^+$ adducts showed higher abundance ratios of $[M + X - RCOOH]^+$ relative to $[M + X - RCOOX]^+$ ions for Ag^+ than for Na^+ or Li^+ ions, we assume that Ag^+, unlike Na^+ or Li^+, prefers to bind to the carbon-carbon double bonds as opposed to binding to the oxygen atoms in the carboxyl groups of the fatty acyl moieties. Therefore, the difference in the fragmentation patterns observed between $[M + Ag]^+$ adducts of the PPO/POP pair and their $[M + Na]^+$ or $[M + Li]^+$ species could be attributed to differences in the binding sites of Ag^+, Na^+ and Li^+ in the TAG molecule.

Calibration curves for regioisomer quantification

The abundance of DAG product ions was determined from the ESI-MS/MS mass spectra of standard solutions of TAG positional isomers at different AAB to ABA ratios. Two sets of calibration curves were obtained for the $[M + X]^+$ species (X = Na, Li or Ag) of a given AAB/ABA pair, one set for the $[M + X - RCOOH]^+$ ions and the other, for $[M + X - RCOOX]^+$ ions (however, only data obtained for $[M + X - RCOOH]^+$ ions are shown in Table 1, see below). For the $[M + NH_4]^+$ adducts, only one type of calibration curve was plotted since only DAG product ions of the type $[M + NH_4 - NH_3 - RCOOH]^+$ were detected in their MS/MS mass spectra.

Calibration curve data and correlation coefficients (r^2) obtained for the systems studied are shown in Table 1. In all cases, regression lines were linear with r^2 values ranging from 0.96 to 0.99. The relative ratios of $[AA]^+$ and $[AB]^+$ ions at various isomeric compositions produced slopes (from –0.088 to 0.175) and y-axis intercepts (54.1 – 76.1) comparable to those obtained traditionally by ESI-MS/MS of $[M + NH_4]^+$ adducts.[6] More recently, a negative ion APCI-MS/MS method based on the formation of $[M - H - RCOOH]^-$ ions from $[M - H]^-$ ions was developed that produces calibration curves with higher slope values than those reported here (0.295 – 0.427) allowing for potentially more precise regioisomer quantification.[24] In our study, reproducibility was demonstrated

over five days (n = 5), and relative standard deviations were on average 5% and 1%, for the slope and intercept, respectively. As shown in Table 1, calibration curve data did not differ significantly, except for the silver adducts of the PPO/POP pair. In general, positive slopes for the calibration curves were observed for the systems studied. However, for the $[M + Ag]^+$ adducts of the TAGs containing one unsaturated fatty acid and two saturated fatty acids, PPO and POP, negative slopes were obtained for both sets of DAG product ions, $[M + Ag - RCOOH]^+$ and $[M + Ag - RCOOAg]^+$. These data demonstrate that not only the location of the fatty acids on the glycerol backbone (*sn*-1/3 or *sn*-2) has an effect on the fragmentation of the TAG adducts, but also the number of double bonds and the nature of the complexing ion X^+ have a major effect on fragmentation and thus on the relative abundance of DAG product ions. The identities of the acyl substituents of TAGs have also been reported to affect ion intensities by other authors studying the fragmentation behavior of $[M - H]^-$ ions[6] and $[M + NH_4]^+$ adducts[13] of TAGs by CID.

Due to the marginally higher and more consistent slope values obtained for DAG ions generated from the sodiated adducts, which potentially offer better quantitative performance, sodium adducts were selected for further investigation. Moreover, since production of $[M + Na]^+$ species of TAGs did not require the addition of any salt to the mobile phase or any instrumental modification, sodium ions provided the best combination of performance and convenience. The effect of adding sodium acetate to methanol on the ionization efficiency of TAG standards was studied. However, we did not observe a significant increase of the ion counts for the $[M + Na]^+$ adducts in a concentration range from 1×10^{-8} to 1×10^{-3} M for sodium acetate. Therefore, we concluded that the addition of a sodium salt to facilitate the ionization of TAGs was not required for the range of TAG concentrations used in this study.

Since the total concentration of positional isomers in natural mixtures of TAGs is unknown, we examined the effect of the concentration of TAGs on the calibration curve data. This study was carried out by preparing mixtures of AAB and ABA isomers in methanol at three different total concentrations, c_{total} = [AAB] + [ABA] = 4.0×10^{-6} M, 4.0×10^{-7} M or 4.0×10^{-8} M. Under these conditions, calibration plots derived from relative abundances of DAG ions $[M + Na - RCOOH]^+$ showed approximately the same

sensitivity and linearity. This demonstrates that MS/MS of $[M + Na]^+$ adducts can be used to quantify positional isomers in unresolved mixtures of unknown total concentration of TAGs.

Quantification of positional isomers in vegetable oils

Calibration plots derived from $[M + Na]^+$ adducts were applied to the quantification of regioisomers in olive and sunflower oils *via* RP-HPLC-MS/MS. Preliminary experiments were performed with TAG standards to ensure that the isomer pairs perfectly co-eluted as anticipated and to obtain retention times for the TAG pairs for peak confirmation in subsequent oil analyses. CID mass spectra along with retention time data were used to identify the four pairs of positional isomers in the vegetable-based oils. Selected-ion chromatograms and CID tandem mass spectra of ions at *m/z* 855.7, 881.7, 903.7 or 905.7 are shown in Fig. 2. These correspond to the masses of $[M + Na]^+$ adducts for PPO/POP, OOP/OPO, OOL/OLO and LLO/LOL, respectively. TAGs that differed by one carbon-carbon double bond were completely separated on our RP-LC system, as demonstrated by the separation of LLO/LOLand OOL/OLO. Otherwise, the presence of ^{13}C isotope peaks from the LLO/LOL pair could have interfered with OOL/OLO, potentially compromising results. The DAG product ions were used to evaluate peak purity to determine if other TAGs of the same mass were co-eluting with any of the TAGs of interest. For all TAGs studied, CID tandem mass spectra yielded only four DAG peaks as observed in the analysis of pure TAG standards. Therefore, we concluded that no other TAGs with the same ECN and M.W. eluted along with any of the four AAB/ABA systems studied here.

The relative amount of ABA isomer in sunflower and olive oils calculated using the current method is shown in Table 2. The dominance of unsaturated fatty acids (L and O) in position *sn*-2 is apparent from these data. Palmitic acid is preferentially incorporated into positions *sn*-1/3, with POP and OOP being present as single regioisomers. Results also show that quantification of positional isomers according to our method was consistent with fractional composition determined from other previously reported data. Major differences were observed for the LLO/LOL pair. In this case, relative abundances

of positional isomers are presumably more sample-dependent than for other isomeric mixtures.

CONCLUSIONS

A comprehensive investigation of various adducts for the quantification of triacylglycerol regiosomers was carried out. In general, CID mass spectra of $[M + X]^+$ ions (X= NH_4, Li, Na or Ag) generated preferential loss of the fatty acid at the *sn*-1/3 positions compared to the *sn*-2 position, allowing for the determination of TAG regioisomers. Sodium adducts of TAGs were demonstrated for the first time to be useful for the quantitation of positional isomers by low-energy CID in tandem mass spectrometry, and offered marginally better quantitative performance while not requiring the addition of a salt to the mobile phase. These results suggest that these extremely precise measurements are highly instrument-specific, as previous similar studies have reported limited success with sodium adducts for the determination of TAG regiosiomers. Factors such as choice of collision gas (*ie*. argon *vs* nitrogen) and differences in collision cell configurations between different MS manufacturers are likely the cause of apparent inconsistencies in the literature in this field.[12,17,20-22] However, it should be noted that on a given mass spectrometer configuration these measurements can be performed very reproducibly and can provide reliable results.

Application of calibration plots derived from $[M + Na]^+$ adducts to the regiospecific analysis of olive and sunflower oils showed that TAGs were asymmetrical (AAB) more often than symmetrical (ABA), with unsaturated fatty acids, L and O, preferentially esterified at the *sn*-2 position. Further studies are currently underway in our laboratory to extend the application of this work to other isomeric systems, specifically those containing omega-3 polyunsaturated fatty acids such as docosahexaenoic (DHA, 22:6) and eicosapentaenoic (EPA, 20:5) acids. Additional investigations are also focusing on evaluating the LC separation of EPA- and DHA-containing TAGs and the CID analysis of $[M + X]^+$ adducts for their future identification and quantification in marine oils.

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Table 1. Calibration curve data for [M + X – RCOOH]+ (X = Li, Na, Ag) and [M + NH4 – NH3 – RCOOH]+ ions derived from adducts of AAB/ABA isomers.

AAB/ABA	$[M + Na]^+$	$[M + Li]^+$	$[M + Ag]^+$	$[M + NH_4]^+$
PPO/POP	$y = 0.156x + 61.6$ r^2=0.99	$y = 0.147x + 60.1$ r^2=0.99	$y = -0.088x + 76.1$ r^2=0.97	$y = 0.129x + 54.1$ r^2=0.99
OOP/OPO	$y = 0.136x + 63.9$ r^2=0.99	$y = 0.113x + 65.1$ r^2=0.98	$y = 0.149x + 59.5$ r^2=0.99	$y = 0.103x + 59.6$ r^2=0.98
LLO/LOL	$y = 0.159x + 58.5$ r^2=0.99	$y = 0.139x + 61.5$ r^2=0.99	$y = 0.175x + 58.1$ r^2=0.98	$y = 0.117x + 55.0$ r^2=0.99
OOL/OLO	$y = 0.148x + 63.4$ r^2=0.98	$y = 0.143x + 62.9$ r^2=0.96	$y = 0.107x + 66.0$ r^2=0.96	$y = 0.135x + 61.1$ r^2=0.97

Table 2. Amount (%) of ABA-type isomer relative to (ABA + AAB) in olive and sunflower oils measured using the current procedure (n=5).

	Olive Oil		Sunflower Oil	
ABA	Observed	Literature	Observed	Literature
POP	97 ± 5	98[a]	99 ± 5	100[c]
OPO	2 ± 4	5[a]	0 ± 4	(1 ± 3)[d] 2[c] (9 ± 5)[e]
OLO	49 ± 4	39[a]	33 ± 5	(34 ± 5)[d] 32[c] (39 ± 2)[e]
LOL	15 ± 4	0[b] 33[a]	25 ± 5	(27 ± 3)[b] (12 ± 7)[d] 23[c] (7 ± 4)[e]

[a] From Ref. 26
[b] From Ref. 25
[c] From Ref. 27
[d] From Ref. 6
[e] From Ref. 24

Figure captions

Fig. 1. MS/MS mass spectra of [M + X]+ adducts (X = NH4, Li, Na and Ag) of POP and PPO. The unlabelled peaks correspond to [M + X – RCOOH]+ ions.

Fig. 2. Selected ion chromatograms and product-ion mass spectra of *m/z* 855.7 (POP/PPO), 881.7 (OOP/OPO), 903.7 (LLO/LOL) or 905.7 (OOL/OLO) from olive oil and sunflower oils. The minor peaks (●) in the selected ion chromatograms arise from isotopic peaks of TAGs having a mass 2 u less than the ion selected for fragmentation. Other TAGs observed at *m/z* 881.7 and 905.7 were identified as: 16:0/18:2/18:0 (○), 18:2/18:2/18:0 (■), and 18:3/18:1/18:0 (♦) (positional distribution was not studied in these cases).

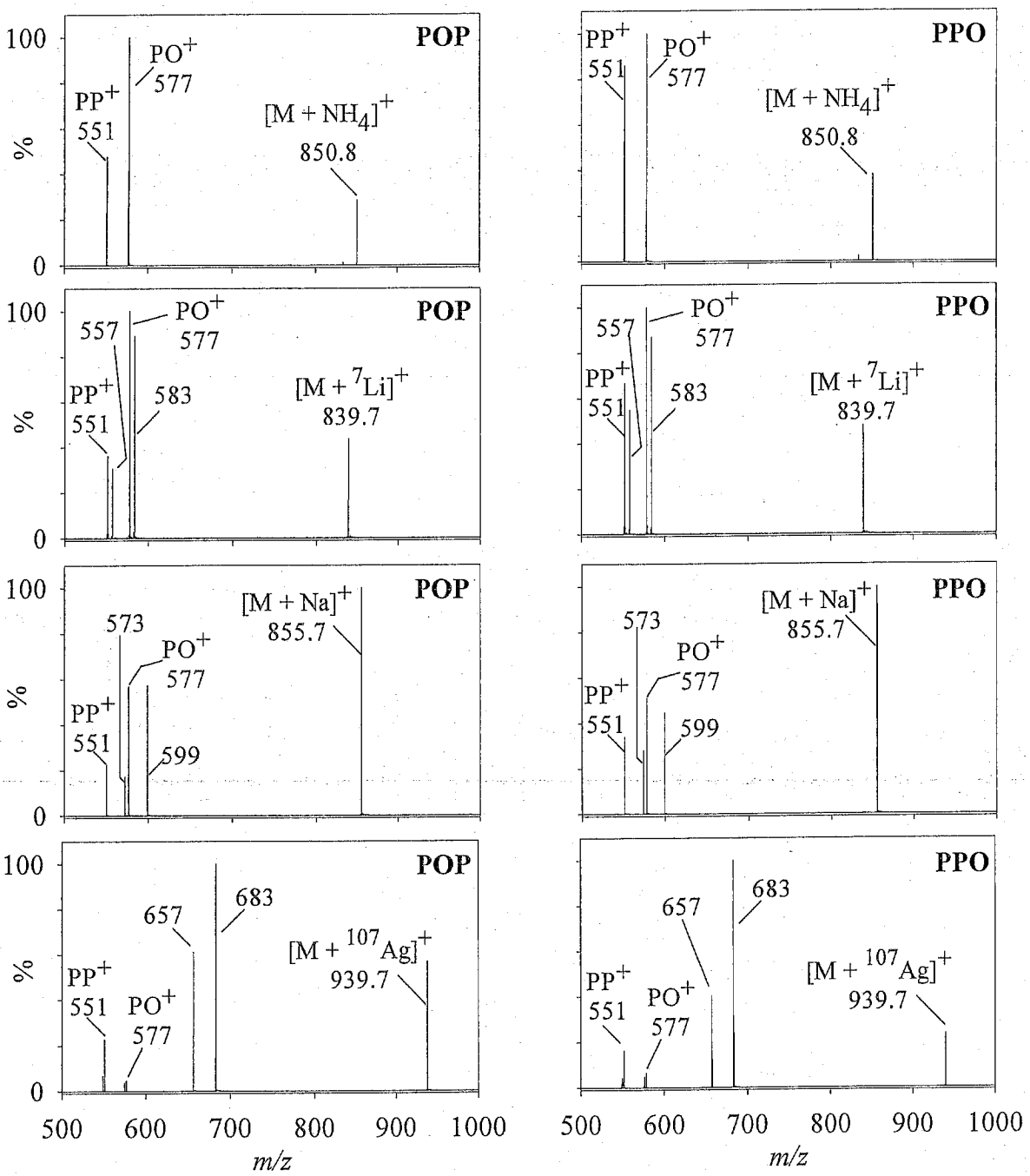
POP
PPO
100
%
0
PP+ 551
PO+ 577
[M + NH4]+ 850.8
557
583
[M + 7Li]+ 839.7
573
599
[M + Na]+ 855.7
657
683
[M + 107Ag]+ 939.7
500
600
700
800
900
1000
m/z

Figure. 1.

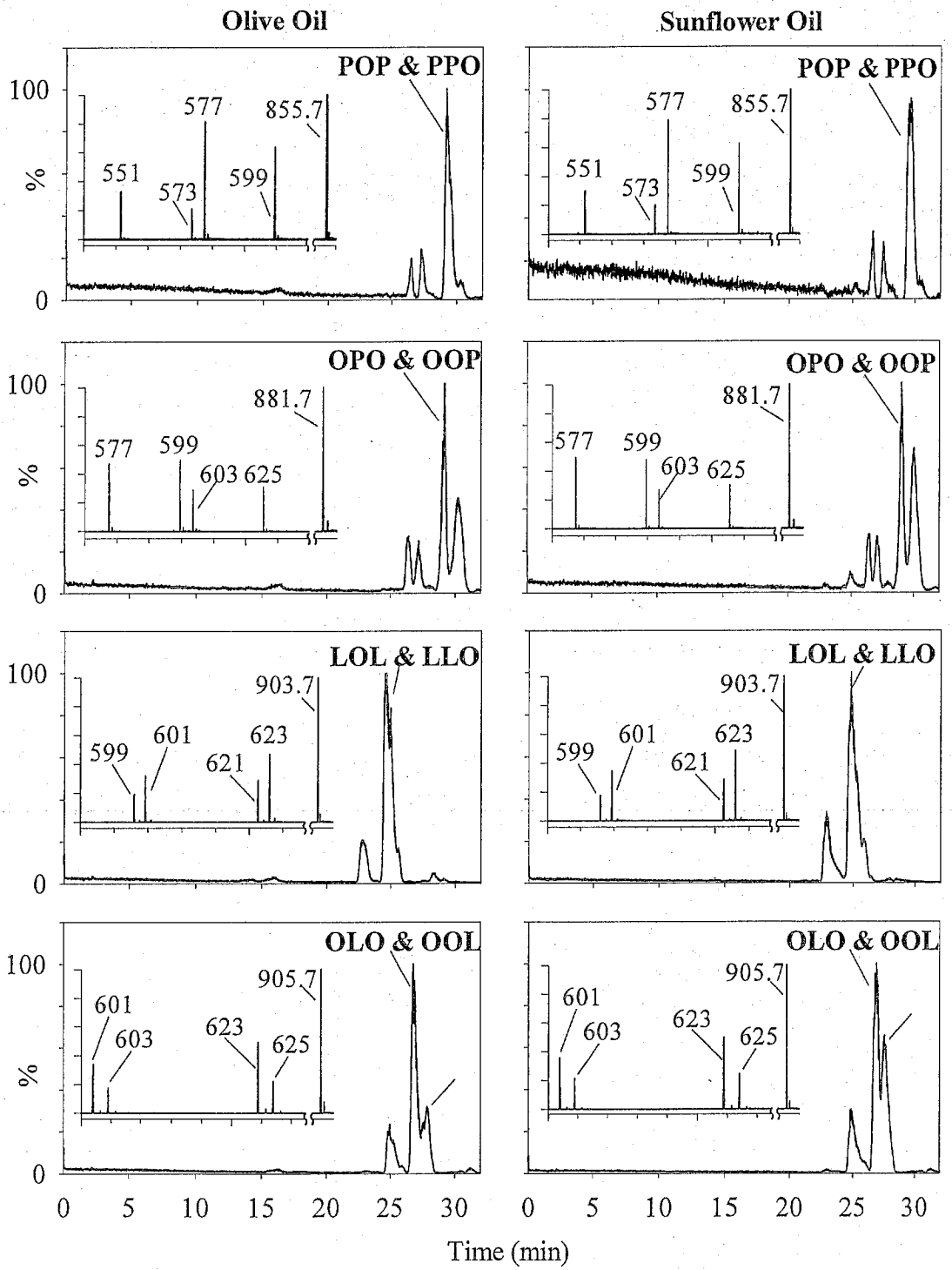
Olive Oil
Sunflower Oil
POP & PPO
OPO & OOP
LOL & LLO
OLO & OOL
100
%
0
551
573
577
599
855.7
881.7
601
603
623
625
621
903.7
905.7
0 5 10 15 20 25 30
Time (min)

Figure 2