Determination of triacylglycerol regioisomers using differential mobility spectrometry

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RATIONALITY: Triacylglycerols (TG) contain three fatty acyls attached to the glycerol backbone in stereochemically numbered positions sn-1, 2 and 3. Isobaric TG with exchanged fatty acyl chains in positions sn-1/3 vs. sn-2 are referred to as regioisomers and the determination of their regiosomeric ratios is important for nutrition purposes.

METHODS: Differential mobility spectrometry (DMS) coupled to electrospray ionization mass spectrometry (ESI-MS) is applied for the separation of simple unsaturated TG regioisomers extracted from porcine adipose tissue using their silver-ion molecular adducts.

RESULTS: Four pairs of TG regioisomers containing combinations of unsaturated and saturated fatty acyl chains are successfully separated using DMS with 1-butanol or 1-propanol as the chemical modifier. Various experimental parameters are carefully optimized, such as the separation and compensation voltages applied to DMS electrodes, the type and flow rate of chemical modifier and the dwell time of analyte ions in the DMS cell. The optimized DMS approach is used for the characterization of TG regioisomers in less than one minute, compared to tens of minutes typical for silver-ion or reversed-phase high-performance liquid chromatography/mass spectrometry approaches.

CONCLUSIONS: The application of this method for the characterization of TG regioisomers in porcine adipose tissue shows the method suitability for analyses of other animal fats. Copyright © 2015 John Wiley & Sons, Ltd.
acyl in the sn-2 position, because the neutral loss of the acyl chain from the sn-2 position is less preferred.\cite{12,17} Another possibility for the differentiation of the sn-2 position is based on the formation of regiospecific ‘G’ and ‘J’ product ions using high-energy collision-induced dissociation MS/MS.\cite{13,14}

Various analytical methods have been explored to improve the identification of TG regioisomers, including (1) silver-ion high-performance liquid chromatography (HPLC),\cite{14,15,17,18} (2) the ratio of $[\text{M} + \text{H} - \text{RCOOH}]^+$ fragment ions in APCI mass spectra,\cite{2,19} (3) the stereospecific enzymatic hydrolysis followed by another suitable analytical technique,\cite{22} and (4) nonaqueous reversed-phase (NARP)-HPLC using two columns with very long retention times (100–500 min).\cite{20,21,22}

The silver-ion HPLC provides accurate results due to the separation of most TG regioisomers and it can be considered as a reference method, but analysis times range from 60 to 120 min. The fragment ion ratios of $[\text{M} + \text{H} - \text{RCOOH}]^+$ are typically used in coupling with NARP-HPLC, with analysis times in the range of tens of minutes; however, the accuracy and robustness is lower than with the silver-ion HPLC determination of TG regioisomers. NARP is a frequently employed method due to its potential to separate numerous TG according to the equivalent carbon number, which is defined as the number of carbon atoms in fatty acyl chains minus two times the number of double bonds (DB).\cite{12,15,17} It also enables the regiosomeric separation of TG differing in the DB distributions between the sn-1/3 and sn-2 positions.\cite{12,16,22} TG enantiomers and also many TG regioisomers can be also distinguished by chiral HPLC in normal-phase systems.\cite{23,24} Silver ions have also been doped into LC effluent to produce adducts with TG,\cite{25} with regiosomeric analyses derived from MS$^3$ level fragmentation of $[\text{TG} + \text{Ag} + \text{AgNO}_3]^+$ adduct ions.

More recently, various ion mobility spectrometry (IMS) techniques have been employed instead of or in addition to HPLC for the improvement of TG analyses by MS. At present, different types of IMS systems are commercially available and have been applied for the analysis of lipids, i.e., drift tube IMS,\cite{26} traveling wave IMS (TWIMS),\cite{27} high-field asymmetric waveform IMS (FAIMS)\cite{28} and differential mobility spectrometry (DMS).\cite{29} The drift tube IMS and TWIMS systems are typically mounted within the vacuum manifold (low-pressure region) of a mass spectrometer, while FAIMS and DMS techniques are positioned at an elevated pressure (atmospheric pressure) between the ionization source and the MS orifice.

The DMS method employed in this work has shown potential in lipidomics as demonstrated by its ability to separate glycerophospholipid and sphingolipid classes\cite{30} and to separate phosphatidylcholine (PC) regioisomers.\cite{30} Other examples of the separation capabilities of DMS include differentiation of structural isomers,\cite{31–33} stereoisomers,\cite{34,35} or ions differing only in their protonation sites.\cite{36} In the DMS cell, ions are transmitted between two planar electrodes by a flow of gas at atmospheric pressure. A high-voltage radiofrequency asymmetric waveform is applied across these electrodes, where the difference between the mobility of ions during the high- and low-field portions of the waveform determines their trajectories. The separation is achieved as a function of the direct current voltage (the compensation voltage, CV) required to steer the trajectory of ions toward the MS inlet. In addition, vapors of organic chemical modifiers can be introduced into the DMS transport gas, which affects the solvation state of analyte ions and alters the apparent mobility of such species.\cite{37} This phenomenon is described by the dynamic cluster/decluster model, because analyte ions are clustered with modifier molecules in the lower field part and declustered in the higher field part of the radiofrequency waveform.\cite{34,39} Further improvements in the selectivity can be achieved with the use of so-called throttle gas, which is a variable flow of nitrogen gas added into the chamber that effectively reduces the transport gas flow through the DMS towards the mass spectrometer, increasing the residence time of ions in the DMS cell and improving the resolution.\cite{39}

The goal of this work is the development of a new DMS-MS method for the determination of TG regioisomers, specifically for the analysis of these regioisomers in porcine adipose tissue, where the most abundant TG regioisomers are combinations of monounsaturated and saturated fatty acyl chains. Different types and concentrations of chemical modifiers and also different types of molecular adducts are investigated with the goal of achieving the regiosomeric separation of these TG. This new DMS-MS method for the regiosomeric analysis is also compared with previously published results with silver-ion HPLC and ratios of fragment ions in mass spectra in terms of the regiosomeric resolution (RR), analysis times, limits of detection and quantitation, with favorable comparisons drawn.

**EXPERIMENTAL**

**Materials**

Methanol, ethanol, 2-propanol (all LC/MS grade), 1-propanol, 1-butanol, hexane (all HPLC grade), sodium methoxide and silver nitrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards of tristearin (SSS, C18:0), triolein (OOO, C18:1 (9Z)), trilinolein (LLL, C18:2 (9Z,12Z)) and tripalmitin (PPP, C16:0) used for the randomization reaction were purchased from Nu-ChekPrep (Elysian, MN, USA) with 99% purity. TG regioisomers (OSO, SOS, SSO, POP, OPP, OPO and OOP) were purchased from Larodan Fine Chemicals AB (Malmo, Sweden) with 99% purity. The stereospecific numbering (sn-1) of fatty acyls is reflected by TG abbreviation, e.g., OSO means 1-oleoyl-2-stearoyl-3-oleoyl-sn-glycerol. The porcine adipose tissue was purchased from a local butcher in Pardubice, Czech Republic.

**Standards and sample preparation**

Silver nitrate (AgNO$_3$) solution was prepared in 2-propanol/water (1:1, v/v) at the concentration of 1 mg/mL. Stock solutions of TG regioisomers standards were prepared in hexane at concentration of 1 mg/mL and then diluted to 25 µg/mL in hexane/2-propanol (1:1, v/v). A 2% AgNO$_3$ solution was added to this solution and left under ambient
conditions for 30 min to induce the formation of silver-ion adducts. The porcine adipose tissue sample was prepared according to an established method[9]. One mg of tissue was weighed and homogenized with 1 mL of hexane. The suspension was filtered through a 0.22 μm cellulose filter (Teknokroma, Barcelona, Spain). Then 20 μL of the filtrate was diluted with 1 mL of hexane/2-propanol (1:1, v/v) with the addition of a 2% AgNO₃ solution and left under ambient conditions for 30 min. The porcine adipose tissue extract was analyzed directly by DMS without any fractionation.

DMS-MS conditions

Experiments were performed on a 6500 QTRAP mass spectrometer equipped with a SelexION™ DMS cell (SCIEX, Concord, ON, Canada) using the positive-ion ESI mode. The systematic optimization of all relevant parameters was performed with the goal of achieving the best regioisomeric separation, sensitivity and robustness. These parameters included the type of chemical modifier and its flow rate (120–290 μL/min), the separation voltage (SV) (1500–4100 V), the pressure of throttle gas (30–46 psi) and the DMS temperature (DT) (150–300 °C). After the initial screening of several chemical modifiers commonly used in DMS (acetonitrile, acetone and alcohols), only alcohols (methanol, ethanol, 1-propanol, 2-propanol and 1-butanol) were further investigated due to the best results for TG regioisomers. Two selected reaction monitoring (SRM) transitions were used for monitoring of TG: m/z 937–655 and 937–653 for OPP/POP, m/z 963–681 and 963–679 for OPO/OOP, m/z 995–713 and 995–711 for SSO/SOS and m/z 993–709 for OSO/SOO. The optimized conditions used for final measurements were as follows: flow rate of analyte of 10 μL/min, 1-butanol as the modifier at the flow rate of 120 μL/min in nitrogen (10 or 20 psi) as the curtain gas, SV of 4100 V, electrospray voltage 5500 V, DT of 225 °C, DMS offset of -5 V, and source temperature of 50 °C. The pressure of the throttle gas (differential resolution, DR) was adjusted for the DMS resolution of each regioisomeric pair (see Table 1). SRM data were acquired for CV ramped from 0 to +10 V in 0.10 V steps.

RESULTS AND DISCUSSION

Optimization of DMS separation using OSO/SOO as a model TG regioisomeric mixture

The resolution of TG regioisomers is a very demanding analytical task in lipidomics analyses, so the careful optimization of all relevant experimental parameters is essential to achieve successful separation. The initial optimization of the DMS-MS system began with the selection of the TG molecular adduct. For [M + Na]⁺ or [M + NH₄]⁺ adducts, no separation was observed for the equimolar mixture of the regioisomeric pair OSO and SOO. However, the separation of [M + Ag]⁺ adducts of those same TG regioisomers was next attempted. This choice is based on the analogy with silver-ion HPLC of TG regioisomers[12,22] and in light of earlier DMS-based separation of silver-ion adducts of PC 16:0/18:1 and PC 18:1/16:0 regioisomers.[30] Recently, drift-time ion mobility spectrometry has shown promise in separating the same regioisomeric ions,[30] albeit with initial lower overall sensitivity.

Figures 1–4 illustrate the optimization of key parameters affecting the DMS separation of [OSO + Ag]⁺ and [SOO + Ag]⁺ ions, such as the type (Fig. 1) and the flow rate (Fig. 2) of the chemical modifier, the separation voltage (Fig. 3), and the DR (Fig. 4). Several parameters are mutually interconnected, so optimization was done iteratively with the best settings of other parameters; however, these figures should illustrate the effects of individual parameters on the regioisomeric resolution (RR) and the signal intensity. SRM transitions representing neutral losses of oleic (m/z 993–711) or stearic (m/z 993–709) acid from these [M + Ag]⁺ ions were used for the method development. Different ratios in SRM traces also confirm the separation of regioisomers, because TG regioisomers have a lower preference for neutral losses of fatty acids from the sn-2 position and therefore [M + H–R₂COOH]⁺ fragment ions have lower relative abundances than [M + H–R₁COOH]⁺ and [M + H–R₂COOH]⁺ ions.

Previous studies showed strong effects of chemical modifiers used in the DMS analysis on the resolution[32–37,40–43] but these effects greatly depend on the type of analyte. Alcohols provide the most promising results based on our initial tests with acetonitrile, acetone, methanol, ethanol, 1-propanol, 2-propanol and 1-butanol. A general trend is observed that the RR is improved with the increasing length of alkyl chain

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**Table 1.** Parameters used for measurements of calibration curves \( y = ax + b \) for individual TG regioisomers: compensation voltage (CV), slope (a), intercept (b), regression coefficient \( r² \), differential resolution (DR), scanned m/z range, limit of detection (LOD) at signal-to-noise \( S/N = 3 \) and limit of quantitation (LOQ) at \( S/N = 10 \)

<table>
<thead>
<tr>
<th>TG regioisomer</th>
<th>CV (V)</th>
<th>a ( \times 10^3 )</th>
<th>b ( \times 10^3 )</th>
<th>( r² )</th>
<th>DR (psi)</th>
<th>m/z range</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POP</td>
<td>3.7</td>
<td>0.41</td>
<td>0.24</td>
<td>0.977</td>
<td>45</td>
<td>937–947</td>
<td>4.1</td>
<td>13.8</td>
</tr>
<tr>
<td>OPP</td>
<td>2.6</td>
<td>1.7</td>
<td>2.5</td>
<td>0.947</td>
<td>42</td>
<td>962–972</td>
<td>0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>OPO</td>
<td>5.8</td>
<td>0.6</td>
<td>0.74</td>
<td>0.946</td>
<td>42</td>
<td>962–972</td>
<td>0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>OOP</td>
<td>6.7</td>
<td>1.7</td>
<td>0.68</td>
<td>0.967</td>
<td>45</td>
<td>992–1002</td>
<td>2.5</td>
<td>8.3</td>
</tr>
<tr>
<td>SOS</td>
<td>3.9</td>
<td>0.64</td>
<td>0.66</td>
<td>0.989</td>
<td>45</td>
<td>992–1002</td>
<td>1.5</td>
<td>4.9</td>
</tr>
<tr>
<td>SSO</td>
<td>3.3</td>
<td>0.86</td>
<td>1.6</td>
<td>0.992</td>
<td>42</td>
<td>992–1002</td>
<td>3.4</td>
<td>11.6</td>
</tr>
<tr>
<td>OSO</td>
<td>5.2</td>
<td>3.8</td>
<td>3.2</td>
<td>0.973</td>
<td>42</td>
<td>992–1002</td>
<td>3.4</td>
<td>11.6</td>
</tr>
<tr>
<td>OOS</td>
<td>6.2</td>
<td>9.3</td>
<td>8.2</td>
<td>0.951</td>
<td>42</td>
<td>992–1002</td>
<td>1.9</td>
<td>6.4</td>
</tr>
</tbody>
</table>
in the alcohol (butanol > propanol > ethanol > methanol), albeit with the loss of signal intensity accompanying improvements in the resolution (Fig. 1). The comparison between the primary (1-propanol) and secondary (2-propanol) alcohols shows slightly better resolution and sensitivity for the primary alcohol. The compromise between the resolution and the sensitivity is the selection of 1-butanol (better resolution and lower sensitivity) or 1-propanol (lower resolution and higher sensitivity). More importantly, in cases where the most abundant TG in animal fats and plant oils are being analyzed, or when sample availability is not a limiting factor, TG regioisomer separation and time savings – not sensitivity – are the critical parameters for optimization. Hence, 1-butanol was selected in the final method (see Experimental section).

Figures 2–4 show the optimization of the DMS separation for OSO and SOO using 1-propanol as the chemical modifier. When the modifier flow rate was increased, the resolution was improved, to the detriment of the signal intensity (see Fig. 2). SV is also a critical parameter for the optimization of DMS separation (Fig. 3), since no regioisomeric separation is observed at SV below 3100 V, with the best resolution being achieved at the maximum SV value of the system (4100 V). At this maximum SV, the signal intensity is somewhat reduced and an increased probability of discharges is observed, but almost baseline separation of these TG regioisomers is achieved. To mitigate these deleterious effects, the flow rate of the modifier can be reduced. In the previous study of Blagojevic et al.,[43] various multicomponent modifiers were tested to overcome these issues. Attempts of separations with higher potentials have already been reported in the literature and yielded promising results.[44–46]

Overall, the DMS regioisomeric separation has the following prerequisites: (1) the formation of silver-ion adducts, (2) the alcoholic modifier at the optimal flow rate, (3) the highest possible SV, and (4) the optimized DR value. Figure 4 illustrates the difference between nonresolved peaks at 36 psi and well-resolved peaks at 45 psi. However, this improved resolution results in an overall decrease in sensitivity (Fig. 4). Again, such compromises in sensitivity and regioisomeric resolution in the SRM-based experiments could be offset by using linear ion trap based scans (e.g., accumulating ion signals for several ms).

Separation of other TG regioisomers common in animal fats

After the complete optimization of the DMS-MS separations of the OSO/SOO regioisomeric pair, these conditions were applied to the analysis of other combinations of TG containing...
unsaturated and saturated fatty acyl chains, which are common for animal adipose tissues. These analytes were measured at equimolar concentrations (25 μg/mL) using the Q1 scan in the appropriate m/z range for monitoring of [M + Ag]+ ions (Table 1). The data presented in Fig. 5 and Tables 1 and 2 for OPP/POP, OPO/OOP, SSO/SOS and OSO/SOO regioisomeric pairs suggest that this DMS-MS workflow could be generally applied for the regioisomeric determination of TG containing monounsaturated and saturated fatty acyl chains.

The best results obtained for this DMS-MS technique employed standards of TG regioisomers for the construction of calibration curves. This was due to the interesting observation that the relative intensities of TG regioisomers injected at identical concentrations (Fig. 5) yielded nonequivalent peak areas for each regioisomer in a pair. For example, one of the regioisomers (OPP, OOP, SSO and SOO) provided a higher peak intensity than its matched regioisomer (POP, OPO, SOS and OSO). Lower intensities are observed in two cases (POP and SOS) for species with higher CV while the situation is just reversed for another two cases (OPO and OSO). When compounds are infused separately with the DMS cell turned off, the relative abundances are identical, which is well known from silver-ion HPLC[12,22] and confirms that the ionization efficiencies of these TG regioisomers are identical. This behavior is under investigation as it differs from the DMS-MS separation observed for smaller lipid regioisomer pairs (e.g., PC 16:0/18:1 and PC 18:1/16:0).[28] However, the use of calibration curves fully eliminates this discrimination and ultimately provides accurate results (Table 2).

**Determination of TG regioisomers in the porcine adipose tissue extract**

To demonstrate the applicability of the optimized DMS-MS method for real biological sample analysis, porcine adipose tissue was chosen as a representative sample of animal fat (Fig. 6), in which unsaturated fatty acyl chains preferentially occupy the sn-1/3 positions.[4] In addition, the regioisomeric ratios of this tissue type of several TG containing combinations of saturated and monounsaturated fatty acyls have already been measured using silver-ion HPLC/MS[22] and fragment ion ratios in NARP-HPLC/MS[18] (Table 2). For DMS-MS results, the RR was calculated in a similar way as the chromatographic resolution, with the difference in CV values (instead of retention times) between two regioisomers being divided by the mean peak width (w) at the half maximum.
\[ RR = 2^4(CV_2-CV_1)/(w_1 + w_2) \]  

Note that the RR cannot be calculated for the fragment ratio method because the principle of this method does not enable this calculation.

The comparison between DMS-MS and silver-ion HPLC (Table 2) shows slightly better RR for POP/OPP (3.1 vs. 2.3) and OSO/SOO (2.1 vs. 1.7) pairs and slightly worse RR for OPO/OOP (1.8 vs. 2.1) and SOS/SSO (1.2 vs. 3.9) pairs. The RR for TG containing unsaturated and saturated fatty acyl chains can be considered as comparable for both methods. For TG containing polyunsaturated fatty acyl chains (e.g., LSL/SLL and SLS/SSL pairs), the DMS-MS method could not provide satisfactory RR, while silver-ion HPLC can be applied for the partial RR of polyunsaturated TG with fatty acyl chains differing at least by two DB, such as OLnO/OOLn and OLnLn/LnOLn.\(^{[22]}\) However, both analytical techniques are hindered for fully saturated TG regioisomers, because no DB are available for either gas-phase or liquid-phase binding of Ag\(^+\). The regioisomeric ratio of OSO/SOO cannot be determined in the porcine adipose samples, because their concentration is below the detection limit of our method, similarly to previous works.\(^{[18,22]}\)

The precision of different analytical methods used for the regioisomeric determination is also compared. Fragment ratios in mass spectra typically exhibit a certain level of fluctuation, so the accurate determination is rather difficult, with most papers reporting only the prevailing fatty acyl in the sn-2 position\(^{[2,3]}\) or at maximum values rounded to the nearest multiple of 10% with correlation coefficients in the range 0.970–0.986.\(^{[2,18]}\) Thus far, silver-ion HPLC/MS provides the most accurate results due to good chromatographic resolution of many TG regioisomers, but the serious drawback is the analysis time in the range of 60–120 min plus time needed for the system equilibration (typically overnight before the first injection) to achieve a good reproducibility of retention times. Thus, a new analytical method with higher throughput would be highly desirable, and the DMS-MS

![Figure 5](image-url) DMS separation of the regioisomeric pairs OSO/SOO, SOS/SSO, POP/OPP and OPO/OOP (the concentration is 12.5 μg/mL for all TG) monitored by Q1 scan. The flow rate of 1-butanol as the chemical modifier is 120 μL/min and DR values are listed in Table 1. Other experimental conditions are the same as for Fig. 3.

Table 2. Comparison of determined TG regioisomeric ratios in porcine adipose tissue using various analytical approaches together with their regioisomeric resolution (RR)

<table>
<thead>
<tr>
<th>TG regioisomeric pair</th>
<th>DMS method</th>
<th>Silver-ion HPLC/MS(^{[22]})</th>
<th>Fragment ion ratios in HPLC/MS(^{[18]})</th>
</tr>
</thead>
<tbody>
<tr>
<td>POP/OPP</td>
<td>5/95</td>
<td>8/92</td>
<td>0/100(^b)</td>
</tr>
<tr>
<td>OPO/OOP</td>
<td>90/10</td>
<td>88/12</td>
<td>100/0(^b)</td>
</tr>
<tr>
<td>SOS/SSO</td>
<td>30/70</td>
<td>n.d.(^c)</td>
<td>30/70</td>
</tr>
<tr>
<td>OSO/SOO</td>
<td>n.d.(^c)</td>
<td>n.d.(^c)</td>
<td>1.7</td>
</tr>
</tbody>
</table>

\(^a\)RR calculated from previously published data.\(^{[12,22]}\)

\(^b\)Only the prevailing fatty acyl in the sn-2 position is determined.

\(^c\)n.d.: not determined.

![Table 2](image-url)
The DMS-MS separation of TG regioisomers is reported for the first time using their silver adducts and 1-butanol or 1-propanol as the chemical modifier. This work serves as a proof-of-concept that DMS provides the selectivity for the regioisomeric resolution of several combinations of monounsaturated and saturated fatty acyl chains by separating regioisomeric TG. DMS-MS yields quantitative data for OSO/SOO, SOS/SSO, POP/OPP and OPO/OOP regioisomeric pairs determined in the porcine adipose tissue extract and compares well with previously published results obtained with established methods. This DMS-MS technique is suggested as a complementary approach to silver-ion HPLC with much higher throughput, because DMS-MS is accomplished in less than 1 min compared to 1 h and more for silver-ion HPLC. DMS-MS can be applied for fast determination of regioisomeric ratios of TG containing combinations of saturated and monounsaturated fatty acyls, which are typical for mammal adipose tissues and also occurring in plant oils.

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Determination of TG regioisomers using DMS


