Mapping the regioisomeric distribution of fatty acids in triacylglycerols by hybrid mass spectrometry

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ABSTRACT

This study describes the use of hybrid mass spectrometry for the mapping, identification and semi-quantitation of triacylglycerol regioisomers in fats and oils. The identification was performed based on the accurate mass and fragmentation pattern obtained by data-dependent fragmentation. Quantitation was based on the high resolution ion chromatograms, while relative proportion of sn-1(3)/sn-2 regioisomers was calculated based on generalized fragmentation models and the relative intensities observed in the product ion spectra. The key performance features of the developed method are: inter-batch mass accuracy < 1 ppm (n=10); lower limit of detection (triggering threshold) 0.1 μg/mL (equivalent to 0.2 weight % in oil); lower limit of quantitation 0.2 μg/mL (equivalent to 0.4 weight % in oil); peak area precision 6.5 % at 2 μg/mL concentration and 15 % at 0.2 μM concentration; inter-batch precision of fragment intensities < 1 % (n=10) independent of the investigated concentration; averaged accuracy using the generic calibration 3.8 % in the 1-10 μg/mL range and varies between 1-23 % depending on analytes. Inter-esterified fat, beef tallow, pork lard and butter fat samples were used to illustrate how well regioisomeric distribution of palmitic acid can be captured by this method.

Keywords: hybrid mass spectrometry, mapping, regioisomers, triacylglycerol
INTRODUCTION

The complexity of naturally occurring triacylglycerols (TAG) present in mammalian cells is well illustrated by previous investigations revealing multiple isobaric species having molecular weights at virtually every even mass between 600 and 900 Da(1). For example, the combination of only 30 different fatty acids (FA) would result in more than 25,000 different molecular species including positional isomers(2). The FA composition of the TAG depends on the species, the dietary FA composition, and the carbohydrate-to-lipid ratio of the diet(3). There is a high variation in TAG stereoisomers in oils and fats of different biological origin and sometimes even species specificity can be observed(4-6). It is generally accepted that the distribution of FAs between the different regio-specific positions of the TAG affect its nutritional (fat digestion, absorption), biochemical (biosynthesis), and physical (crystal structure, melting point) properties(6-8). Several clinical studies have shown that the type and position of the fatty acyl substituents of TAGs play an role in lipid digestion, absorption and metabolism (6-14).

Prominent implications of regiospecific TAG on physicochemical/texture properties of fats include the case of pork lard, where the presence of palmitic acid (P) in the sn-2 position contributes to desirable flakiness of pie crusts when lard is used as a baking shortening(6). In the case of cocoa butter, the unique positioning of P, O (oleic acid) and S (stearic acid) in two predominant TAG forms gives cocoa butter a sharp melting point just below body temperature(6). Furthermore, the food industry uses various inter-esterification processes to modify the distribution of FA and achieve their randomization among the sn-1/sn-3 or all three regiospecific positions(6). This way, the melting and crystallization behavior of fats can be improved. For instance, the hardening of low-viscosity oil by inter-esterifying it with a solid fat offers an alternative to the use of partial hydrogenation in the manufacture of margarines and spreads.
Indirect methods for regiospecific analysis of TAG were established by the partial hydrolysis of TAG with pancreatic lipase(15) or by a Grignard reagent followed by derivatization of the reaction products with \(n\)-butyl chloride(16) or naphthylethylurethane(17). Note, that the applicability of enzymatic methods is in general limited to oils with melting point below 45 °C. Liquid chromatography (LC) with ultra-violet/visible absorption (UV/VIS)(18) or evaporative light scattering detection (ELSD)(13,18-20) can be also used to characterize the intact TAG profiles, but these approaches lack sensitivity and specificity(21). Profiling of intact TAG classes without chemical derivatization by GC was reported by Destaillats(22) and Guyon(23).

The main analytical techniques applicable for the direct regiospecific analysis of intact TAG (no chemical derivatization) include nuclear magnetic resonance (NMR)(24-26) and liquid chromatography-mass spectrometry (LC-MS). NMR provides qualitative and quantitative information on the positional isomerism of fatty acids present in TAG based on characteristic chemical shifts. The applicability of this approach is limited in complex mixtures with abundant interferences and it does not provide information about the identity of individual TAG species. Several chromatographic methods were implemented for the separation of TAG(27), amongst which the three mainstream approaches are normal phase liquid chromatography (NP-LC)(28-30), silver-ion liquid chromatography (Ag-LC)(4,5,27,31-34) and non-aqueous reversed phase liquid chromatography (NARP-LC)(5,13,19,35-39).

NARP- and Ag-LC are orthogonal separation methods and were extensively compared by Holcapek et al (33). The retention in NARP mode is governed by the equivalent carbon number (ECN=carbon number-2*double bonds), while the retention in Ag-LC increases with the increasing number of DBs with a clear differentiation between \(cis\) and \(trans\)-FA(4,33,34). Unlike NARP-LC, Ag-LC can provide baseline resolution for regioisomer TAG with up to three double bonds(4,31). Resolution of Ag-LC is only partial for TAG regioisomers when the total number of double bonds higher than four (\(e.g.\) LLO and LOL) or for TAG pairs differing only by
one double bond (5,31,40). Note, that to date none of the existing LC methods is capable of resolving the entire complex TAG mixture of vegetable oils or animal fats.

The fingerprinting and tentative identification of TAG were described using numerous mass spectrometric methods including desorption electrospray ionization (41,42), direct analysis in real time (43,44) matrix-assisted laser desorption electrospray ionization (45), atmospheric solids analysis probe (46), extractive electrospray ionization (47), desorption atmospheric pressure photon ionization (48), and ambient sonic-spray ionization (49-51) and matrix assisted laser desorption ionization (MALDI) (25,52).

LC-MS enables the most efficient structural elucidation and quantitation of TAG, since using this approach the isobaric and isotopomer interferences can be separated and the interpretation of spectra becomes much more feasible. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are both commonly used for the analysis of TAG. APCI generates abundant in-source fragments and in some cases these can be used for structural elucidation of TAG (19,35,53,54). However, in the case of complex natural samples when several TAGs coelute and are present in wide dynamic range of abundances, this spontaneous and uncontrolled fragmentation rather complicates the assignment of parent-product relationship and hinders identification or data-dependent tandem experiments. In order to minimize the spontaneous, in-source fragmentation, ESI can be also used for the analysis of TAG by adding modifier buffers (e.g. ammonium formate, sodium-acetate) to the LC mobile phase. Such salts enhance the ionization process greatly and consequently allow lower detection limits to be reached by ESI than by APCI (19,21).

The unequivocal structural characterization of TAG requires tandem mass spectrometry and assessment of collision induced dissociation (CID) mass spectra. The most characteristic fragmentation steps of TAG are the loss of FA residues yielding diacylglycerol (DAG) fragments, but depending on experimental conditions the loss of fatty acyl-ketenes, various rearrangements, charge remote fragmentation involving β-cleavage with γ-hydrogen shift and
production of acylium ions was also described (19, 55). Numerous preliminary studies have reported that the neutral loss of FA from the outer sn-1/3 positions is energetically favored in comparison to cleavage from the middle sn-2 position (4, 13, 19, 34, 39, 54, 56-59). Based on this phenomenon, most studies have attempted to identify the prevailing FA in the sn-2 position by assuming that in a product ion spectrum it is represented by the lower relative abundance fragment (4, 60). This simple approach is very widely applied; however its substantial limitations and inherent errors became recently apparent. It has been demonstrated that the nature of fatty acids (mainly the double bond number and positions) (34, 35, 61, 62) and experimental conditions such as collision energy (58) or type of adduct (56) also affect the relative abundances of corresponding DAG fragment ions and this way the favored loss from the sn-1 and sn-3 positions (higher than from sn-2) might not be necessarily true for quite different FAs. Refined and more accurate approaches were described on quantitating the relative abundance of regioisomeric TAG based on the construction of calibration curves using identical standards of regioisomeric pairs mixed at different ratios (36, 39, 61, 63, 64). These studies revealed encouraging quantitation possibilities, but were restricted to TAG with commercially available standards. Holcapek et al. have utilized a range of standard regioisomeric mixtures synthesized using the micro scale randomization procedure (34). Their extensive data set measured in three different laboratories represents a good basis for the generalization of the fragmentation behavior in APCI mass spectra and the retention behavior in silver-ion chromatography.

In summary, the quantitation of unknown intact TAG regioisomers in complex oil/fat mixes still has not been accomplished principally, because all targeted LC-MS based quantitation approaches are restricted to TAG with commercially available standards, and these latter make up only approximately 1 % of the potentially occurring TAG.

The objective of this study is to enable the untargeted detection, structural elucidation and quantitation of the most common 28,000 TAG species in unknown oil mixtures. For this
purpose, a hybrid mass spectrometry based approach was developed and validated. The
detection of TAG was performed by NARP-LC coupled with high resolution ESI-MS, a
theoretical inclusion list of TAG and mass tagging criteria of their adduct pattern. The
identification of the TAG was performed based on the accurate mass and fragmentation
pattern obtained by data-dependent fragmentation. Quantitation of TAG was based on the
high resolution ion chromatograms, while relative proportion of $Sn-1(3)/Sn-2$ regioisomers was
calculated based on generalized fragmentation models and the relative intensities observed in
the product ion spectra.

EXPERIMENTAL

Chemicals & Samples

ULC grade ammonium-formate, methanol and isopropanol were obtained from Chemie
Brunschwig AG, Basel, Switzerland. LC grade sodium-formate, acetone and $n$-hexane was
purchased from Sigma-Aldrich, Buchs, Switzerland. Stable isotope labeled $^2$H$_5$-1,3-dipalmitoyl-
2-stearoyl-glycerol (catalogue number 110543) was obtained from Avanti Polar Lipids Inc.
(Alabaster, Alabama, US). TAG standards listed in Table 2, Table 3 were purchased from
Larodan/Chimie Brunschwig AG, Basel, Switzerland.

Standard solutions

Stock solutions of non-labeled TAG were prepared at 10 mg/mL in acetone. These were
further diluted to the required concentration with acetone:methanol 4:1. Solution of $^2$H$_5$-1,3-
dipalmitoyl-2-stearoyl-glycerol was prepared using acetone:methanol 4:1 at a concentration of
400 nanomoles/L.

Sample preparation
Oil/fat samples were melted at 65 °C. 10 µL sample was solubilized in 990 µL n-hexane. Next, 100 µL of this solution was added to 900 µL acetone:methanol 4:1. Finally, 50 µL aliquot of this latter solution along with 250 µL stable isotope labeled internal standard solution (400 picomoles/mL) was transferred into new glass vial and 700 µL acetone:methanol 4:1 was added. A 10 µL aliquot was injected for analysis, which corresponds 1 picomoles IS and 500 ng sample on-column injected absolute amount.

Liquid Chromatography

An Accela 1250 liquid chromatograph (ThermoFisher Scientific, Bremen, Germany) equipped with a Agilent Poroshell 120 EC-C18 (2.7 μm particle size, 2.1 x 250 mm) was used for separation of analytes. Solvent A was 1 mM ammonium-formate and 2 µM sodium-formate solubilized in methanol, whereas solvent B was isopropanol:n-hexane 1:1. The gradient was as following: 0-3 min isocratic 100 % A at 600 µL/min; 3-53 min gradient to 70 % A at 600 µL/min; 53-60 min gradient to 5 % A and to 400 µL/min; 60-70 min isocratic 5 % A at 400 µL/min; 70-73 min gradient to 100 % A and to 600 µL/min; 73-80 min equilibrate at 100 % A at 600 µL/min.

Mass spectrometry

An LTQ-Orbitrap XL hybrid mass spectrometer (ThermoFisher Scientific, Bremen, Germany) was used. Electrospray ionization in positive ion mode was employed to form ions at 300 °C nebulizer temperature and 5 kV capillary voltage. Nebulizer and auxiliary gases were nitrogen at 40 and 20 units respectively. Tube lens was adjusted to 110 V, accumulation time was 100 ms. Other parameters were the typical values optimized during calibration. The Orbitrap was operated at 30,000 resolution in an m/z 100-2000 range. Data dependent events were triggered according to an inclusion list containing the accurate masses of ammoniated TAG, applying parent mass width criteria of ± 5 ppm. Inclusion list criterion for data-dependent
acquisition was established in MS Office Excel by calculating the elemental composition and corresponding accurate mass for TAG obtained by the combination of the 40 most common FA (see Table 1) on the glycerol backbone. The combination of these FA yields approximately 40,000 TAG: these species can be detected and identified using the herein described method, see also supplementary material. Note however, that due to the lack of commercially available standards and published data, only TAG constituting FA with maximum three DB (see Table 5) can be quantified representing approximately 28,000 TAG species.

Additional mass tagging of m/z 4.95540 (between ammoniated and sodiated adducts, see Figure 1) was applied in a parent intensity range of 0-100 %. Intensity threshold was 100,000 cps, preview mode for FT-MS master scans was enabled. Precursor ion isolation, fragmentation and detection was performed in the linear ion trap. Only the ammoniated adducts (low mass partner) were fragmented. Accumulation time was 50 ms, isolation width was 3 Da, normalized collision energy 30 %, activation Q value was 0.250, activation time was 30 ms. Note that the isolation width value 3 Da will result in mono-isotopic isolation of the parent ion of ammoniated TAG, and complete elimination of other isotopomers as experimentally confirmed by us. The reason for this unusual behavior of the linear ion trap is described by McClellan et al (65). The monoisotopic isolation of the parent ion is of crucial importance in order to eliminate unnecessary isotopomer interferences in the product ion spectra that would complicate the interpretation of the fragmentation pattern. Using a dynamic exclusion list and 30 sec exclusion time, ten data dependent events were triggered per two scan cycles (5 fragmentation events per one scan cycle). The dynamic exclusion parameters were: repeat count 1; repeat duration 0 s; exclusion list size 25; exclusion duration 2.5 s; exclusion mass width ± 5 ppm.

Data extraction
High resolution (5 decimals) Orbitrap and nominal resolution linear ion trap data (2 decimals) was extracted using the software Quanbrowser (ThermoFisher Scientific, Bremen, Germany). Chromatographic peak areas were obtained from ion chromatograms extracted in 10 ppm m/z windows. Interpretation of signals for TAG identification and quantitation is described below.

RESULTS & DISCUSSION

The global quantitation of TAG positional isomers is a long-standing and challenging problem in lipid analysis, despite the fact that TAG have strong impact on technological and nutritional properties of fats and oils (see Introduction). Because of these relevant aspects and most importantly due to the enormous diversity of TAG in nature, the development of a mapping method with quantitative capabilities is the objective of this study. For this purpose, we exploited 1) chromatographic retention time, 2) accurate mass, 3) adduct envelope, and 4) fragmentation pattern information. We have combined these data with a quantitative fragmentation model that we have obtained based on authentic TAG regioisomer standards and data reported in the literature.

Opposite to generic lipid profiling, the identification and distinct quantitation of TAG requires their chromatographic separation in order to obtain good spectral purity and correct assignment of accurate masses in unknown samples. For example, to separate the isotopomer envelope TAG pairs differing in one double bond (e.g. the 2x^{13}C substitution of PPO and PPS), the required full width at half maximum mass resolution would be 90,000 and a resolution allowing determination of accurate mass would certainly exceed 100,000. Accordingly without chromatography, the isotopomer envelope of the first TAG would overlap with that of the second TAG biasing the accurate mass of the second TAG.

*Chromatography*
In this study, the chromatographic resolution of TAG pairs differing in one double bond was accomplished by NARP. This approach is simple, robust and allowed the use of ESI and various salt additives that were helpful in identification of TAG. Several solvents were tested for the development of the final gradient including water, methanol, acetone, dichloromethane, isopropanol, n-hexane. The final gradient was optimized to allow appropriate retention of small TAG (abundant constituents of butter fat) but also to enable the elution of large TAG, as it is illustrated in Figure 2. The repeatability of this gradient was assessed by obtaining intra-batch/inter-batch precision data, see Table 3 and Table 4. The standard deviation of the relative retention time was well below 1% at both investigated concentrations. Furthermore, robustness of the method was also assessed by systematically varying the solvent composition A and B. The retention times were not affected significantly when changing the ammonium formate content from 1 mM to 0.8 mM or to 1.2 mM in solvent A. The retention times were slightly shifted when changing the n-hexane content in solvent B from 50 % to 45 % and to 55 % (see Figure 3, each data point stands for a specific TAG). The shift is in accordance with the classic hydrophobic interaction between the analytes and the stationary phase: the higher n-hexane content makes the solvent B stronger eluent translating into shorter elution times. The results in Figure 3 suggest that especially very long chain and saturated TAG might exhibit different retention times if uncontrolled fluctuations occur in the composition of eluents.

Ionization and mass tagging

Using NARP chromatography, it was possible to use ESI instead of APCI. ESI results in cleaner mass spectra compared to APCI, since this latter generates abundant in-source fragments from TAG(53) (54) (35) (19) (35). In this study, we have taken advantage of the fact that ESI generates various adducts as pseudomolecular ions from TAG when used with buffers. The role of 1 mM ammonium formate and 2 µM sodium-formate was to allow
generation of predominant ammonium adducts and abundant sodium adducts from TAG. With the aid of these salts, both good ionization efficiency and characteristic adduct envelope can be achieved. The adduct envelope exhibiting the characteristic \( m/z \) difference between the ammoniated and sodiated adduct (\( m/z \) 4.955395, see Figure 1) is a useful descriptor that facilitates the deduction of charge carrier and determination of exact molecular weight. Note, that the relative proportion of ammoniated and sodiated forms of TAG varies depending on the types of esterified fatty acids. In this work only the peak areas of ammoniated ions are used for quantitation; the sodiated forms serve only the purpose of qualitative confirmation.

While this type of mass tagging helps reduce the number of false positives, it also slightly compromises the absolute sensitivity of the method due to the division of analytes into two ion populations (one ammoniated and one sodiated). Then again, the implications of this negative effect to this method are minimal since the selected data dependent triggering threshold (100,000 cps, see above) is still well above the absolute detection limits.

**Data dependent acquisitions based on accurate mass and mass tag**

Mapping of naturally occurring TAG commenced by on-the-fly screening of the high resolution mass spectra for the theoretical masses present in the inclusion list (see Experimental section). Once a peak possibly corresponding to an ammoniated TAG was picked, the corresponding sodiated analogue was searched using the \( \text{NH}_4^+ \Rightarrow \text{Na}^+ \) mass tag \( (m/z \ 4.955395) \). Overall, if the 1) inclusions list, 2) mass tag and the 3) intensity threshold criteria were met, the system performed a product ion scan on the respective ammoniated TAG, as shown in Figure 1. Using this approach, fragmentation pattern of TAG could be automatically obtained without the need of knowing and specifying which TAGs are in the sample.

The robustness of mass accuracy was evaluated by assessing the root mean square (RMS) mass errors in 0.2 µg/mL and 2 µg/mL standard solutions (equivalent to 0.4 and
4 weight % in an oil sample), see Table 3. The observed sub-ppm mass accuracy enables a very selective way of assigning the correct elemental composition for the TAG and reduces false positives. Despite of this state-of-the-art selectivity, specificity cannot be achieved and in rare cases triggering also occurs on substances which are not TAG. In these latter cases though, the fragmentation fingerprint can be used to confirm or exclude the presence of a TAG and perform its structural elucidation as described below.

**Structural elucidation of TAG**

Parent ion isolation, fragmentation and detection were performed at nominal mass resolution in the linear ion trap in parallel to the high resolution scan performed in the Orbitrap. This hybrid mode of operation allowed the simultaneous acquisition of fragmentation pattern and accurate mass at the chromatographic time scale. The peaks in the TAG product ion spectra allowed the determination of FA residues within the TAG by using an Excel template (see Supplementary material) that automatically calculates mass differences between the pseudo-molecular ion and the masses of the observed fragments. The calculated mass differences correspond to the masses of a fatty acid constituents and an ammonia unit, The Excel template automatically compares these mass differences to a pre-calculated list of fatty acids hereby automatically identifying which fatty acids are present in a given TAG. Then, one single built in macro constructs all possible combinations of the found fatty acids to generate TAG e.g. 18:0-18:0-18:3. The exact masses of these hypothetical TAG are calculated and compared automatically to the measured exact mass. The list of the generated TAG is then sorted according to their mass errors. All displayed TAG are considered found which have “mass error square” <10 ppm². Ultimately, the accurate mass and the fragmentation pattern clearly identify which FA are forming which TAG species in a given sample. For more detailed explanation on the calculations, see section “Example workflow of quantification routine” and Supplementary material.
Quantitation

The schematic of the quantitation process is visualized in Figure 4. In the first step, the sum concentration of TAG regioisomers was determined by integrating the corresponding peak areas in the high resolution ion chromatograms, normalizing them to the peak area of the internal standard ($^{2}$H$_{5}$-1,3-dipalmitoyl-2-stearoyl-glycerol) and substituting them into the experimentally determined generic calibration equation (described below). An Excel template can be used as a basis to calculate and express results as [weight %] and as [mM], see Supplementary material.

In the second step, the concentrations of the individual TAG regioisomers are calculated. The experimentally observed response was practically the same for the commercially available PPL (L-linoleic acid), LOO, LLS, POO, SOO, PSS TAG regioisomer pairs at 0.5 µg/mL and 5 µg/mL concentrations (data not shown) suggesting that there is no significant difference between the ESI responses of regioisomers of the same TAG. In addition, the experimentally observed fragmentation patterns of cis/trans isomers were identical (PPO and PPL cis/trans pairs, data not shown). Further, experimental data also showed that the fragmentation pattern of these TAG mixtures can be estimated by the combination of the individual fragmentation patterns of the TAG regioisomers (data not shown).

Accordingly, the relative percentage of TAG isomers contributing to a complex fragmentation pattern can be estimated if the fragmentation patterns of the individual TAG isomers are known. In this work, we have relied on the experimentally observed fragmentation pattern of commercially available TAG standards and the fragmentation pattern published in the literature using linear ion trap instruments(34), see Table 5. In addition, we have evaluated the effect of fatty acid chain length on the fragmentation pattern. While the effect of fatty acid chain length is only minor on the fragmentation pattern, it can be estimated by the following equation:
Equation 1 \[ y = -0.1722 * x^2 + 5.0993 * x + 63.088 \]

where \( y \) is the calculated correction factor in % that takes into consideration the chain length, \( x \) is the carbon number of the fatty acids. These considerations altogether enabled to predict the fragmentation pattern of any TAG with the commonly occurring fatty acids up to three double bonds per fatty acid. Note that the prediction of TAG with more double bonds requires more detailed studies based on pure standards, which were not available for the current study.

In summary, using the theoretical fragmentation patterns of pure TAG, one can search for their combination which will match the experimentally obtained fragmentation pattern. In this study this was achieved by an Excel template and the Solver function of Excel (Add-in function under Data/Analysis), see Supplementary material. Briefly, the developed template automatically calculates the fragmentation pattern of pure regioisomers based on the type of fatty acids present in the TAG. This latter calculation is based on the reference patterns given in Table 5 and it also takes into account the length of the fatty acids, as described above in Equation 1. Note that based on the name of the fatty acids (e.g. 18:2) the template automatically recognizes the degree of unsaturation and which fragmentation case applies from Table 5. The calculated theoretical fragmentation patterns of the TAG isomers are automatically combined and the Solver function of Excel is used to optimize the relative proportions of isomers so that the resulting fragment intensities will match the experimental pattern. The absolute concentrations of the individual regioisomers are derived automatically from the sum concentration of TAG regioisomers simply by dividing it according to the relative proportions of the individual isomers; see the last step in Figure 4. A step by step explanation of this approach is given in the section “Example workflow of quantification routine” and Supplementary material.

Calibration
Response factors of commercially available TAG were determined by constructing calibration curves using standard solutions in the range of 0.1 µg/mL – 10 µg/mL (equivalent to 0.2-20 weight % in an oil sample). All calibration points were determined in triplicates. Most TAG exhibited bending calibrations which were best fitted by cubic functions in particular at concentrations below 2 µg/mL. Weighting factor of 1/x was used in all cases, in order to further improve accuracy at low concentrations. The precision of the replicate analyses was 4.1 % on average in the 0.1-1 µg/mL concentration range (Table 2). The overall accuracy of the individually fitted calibration curves was 0.1 % on average, see Table 2.

In order to extrapolate the semi-quantitative analysis of TAG species beyond these commercially available analytes, a generic calibration function was optimized that allows the semi-quantitation of TAG species with up to 3 double bonds per fatty acid chain, see Equation 2.

Equation 2 \[ y = A * x^3 + B * x^2 + C * x + D \]

Where \( y \) is the peak area ratio, \( x \) is the concentration, the constants \( A, B, C, D \) are:

\[ A = -0.00005042*(NCN)+-0.00484254*(DB); \]
\[ B = -0.00432127*(NCN)+0.10859674*(DB); \]
\[ C = 0.21785442*(NCN)+-0.77993196*(DB); \]
\[ D = -0.00132089*(NCN)+-0.11288858*(DB). \]

DB = number of double bonds within the TAG molecule, NCN is the normalized carbon number.

Equation 3 \[ NCN = ABS(carbon number-50)+50; \]

The detailed accuracy values obtained by using the generic calibration (Equation 2) averaged per analyte and averaged per concentration are shown in Table 2.

The sum quantity of identified triacylglycerols was varying between 65-96 % depending on the complexity of the sample, e.g. 96 % for pork lard and 67 % for butter fat. This is in accordance with the principle of the method, since more complex samples contain more low-abundance TAG which are below the triggering threshold of the method. More sensitive and
faster instrumentation will enable to capture higher percentage of TAG even in most complex samples such as butter fat.

Note, that the classical yield-recovery was not tested, since this method is based only on dilution and no extraction process is involved in the sample preparation. Further, it was not possible to perform matrix matched calibration due to the lack of an appropriate matrix with the absence of the respective TAG and the low available quantity of calibration standards.

*Example workflow of quantification routine*

In this section, we use the example of PSO TAG regioisomers in a beef tallow sample to demonstrate the workflow from data extraction, through substitution into calibration, and ultimate deconvolution of TAG regioisomers.

In the first stage, identification of TAG is performed based on its accurate mass and fragmentation fingerprint. The accurate mass of the ammoniated pseudomolecular ion is obtained from the raw data file, by displaying the mass spectrum, in our case at retention time 29.66 min. The corresponding mass spectrum in Figure 1 B shows the accurate mass of 878.81769 Da with the neighboring sodiated confirmatory ion (883.77275 Da). This mass is entered into an Excel template (TAGIDENTIFIER.xls), see Supplementary material. In the next step, the averaged fragmentation pattern is displayed for the 878 Da data-dependent channel; see Figure 1 D. The mass difference between each fragment and the parent ion represents a fatty acid loss with an ammonia unit, see labels on Figure 1 D. The spectrum list of the fragmentation pattern (signals above 5 %) is copied into the above mentioned Excel template. Once the accurate mass of the ammoniated pseudomolecular ion and the fragmentation list is copied into the template, the calculation of fatty acid losses corresponding to the fragments occurs automatically by launching a macro in the Excel template. This macro calculates the differences between all fragments and parent ion, and assigns the identified fatty acid loss by looking up these values on a pre-calculated list of fatty acid masses.
(integrated in the template). In the same process, the macro also constructs all possible combinations of the found fatty acids to generate TAG e.g. PPP, SSS, OOO, PPS, OOS, PSO and calculates their accurate masses, see the hit list for our example in Table 6. Finally, the exact masses of these hypothetical TAG are compared (automatically) to the experimentally measured exact mass (878.81769 Da). The hits of TAG are sorted according to their mass errors. All displayed TAG are considered possible hit which have “mass error square” <10 ppm². In our case, the only such TAG is PSO, all others have mass errors > 5 million ppm².

In the second stage, the sum concentration of TAG isomers (in our case PSO, SPO and POS) that have the same accurate mass and same retention time is calculated based on the peak area of the corresponding accurate mass ion chromatogram. In our case, the ion chromatogram of 878.81769 Da (Figure 1 C) yields a peak area of 697541884. This peak area is divided with the peak area of the internal standard (11538828) and is substituted into Equation 2 using the template “QUANTIFICATION_BY_CALIBRATION.xls”, see Supplementary material. Please note, that the carbon number (CN) and unsaturation (DB, number of double bonds) is required by the template in order to adjust the calibration parameters to the chemical nature of the respective TAG. Once these data are entered into the Excel template, the calibration parameters are calculated automatically as described in Equation 2. In our case, the CN is 52, the DB is 1. The calculated calibration parameters are A=-0.00726274; B=-0.09882424; C=9.67708010; D=-0.17629132. The resulting concentrations are derived using the Solver function of Excel (Add-in function under Data/Analysis). Briefly, the basic principle of Solver-based quantification is finding iteratively a concentration that returns a peak area ratio that is practically identical with the experimentally measured peak area ratio. This way, any type of calibration (e.g. cubic, quadratic, weighted by 1/x, 1/X²) can be applied without sophisticated calculations. In our case, we use Solver to minimize the difference between the calculated and experimental peak area ratio. Note that to enable this minimization, the squared difference between the calculated and experimental
peak area ratio is used. In our example, the solution was 7.03 μg/mL concentration in the vial, which corresponds to 163.36 millimoles/1000 g in the original sample.

In the third stage, the distinct quantification of TAG regioisomers occurs by deconvoluting the experimental fragmentation pattern (maximum 20 fragments) and dividing the above calculated sum concentration of TAG isomers accordingly. The deconvolution process is facilitated by the template “REGIOISOMER_CALCULATOR.xls”; for filling the template see Supplementary material. Briefly, first the theoretical fragmentation patterns of all identified TAG are predicted. In our example, this means the calculation of PSO, SPO and POS fragmentation patterns. The chain length and unsaturation of the fatty acid chains is used automatically by the template to locate the relevant fragmentation pattern from Table 5 (integrated in the template). In our case, this corresponds to the scenarios of 0:0:1 and 0:1:0. The look up process, the predicted individual intensities and the summation of all fragment intensities originating from various TAG isomers occurs also automatically in the Excel template. The deconvolution process is based on finding those proportions of the TAG regioisomers that will yield a sum fragmentation pattern that is practically identical to the experimentally measured fragmentation pattern. The deconvoluted proportions of TAG regioisomers are optimized using the Solver function of Excel (Add-in function under Data/Analysis). The target of Solver in our case is to minimize the differences between the calculated and experimental fragment intensities. Again, to enable the minimization process, the squared differences between the calculated and experimental fragment intensities are used. Once the relative proportions of TAG regioisomers are optimized by Solver, the sum absolute concentration of the TAG isomers (calculated above in the second stage) is divided automatically to yield the individual absolute TAG regioisomer concentrations. In our example the optimized proportions of PSO : SPO : POS were 7.68 : 27.03 : 65.29 %, which corresponds to final concentrations of 12.55 : 44.16 : 106.65 millimoles/1000 g.
Applications

A commercially available inter-esterified fat was analyzed using the present hybrid MS based method for its P content at the sn-2 position. Results showed that 57.3% of the overall P content was esterified at the sn-2 position. This number was practically identical with the result provided in the Certificate of Analysis of the product (56.9%).

Similarly, the regiodistribution of P was compared between pork lard and beef tallow samples, see Figure 5. 83% of the overall P was found to be esterified to the sn-2 position in the investigated pork lard sample, while this value was only 17% in beef tallow sample. These results are in accordance with the literature since pork lard was reported to contain 80-90% P at the sn-2 position, while beef tallow only at 15% (4,6). Note that in addition to this information, the hybrid MS analysis also provides the regioisomeric balance of other FA (e.g. S, O), as illustrated in Figure 5. Further, the triacylglycerol data was interrogated to derive the quantity of FA present in the samples. This latter was then compared to the results obtained by the classical GC-FID analysis following transmethylation of the samples. Despite the fact that hybrid MS and GC-FID methods analyse different compounds (TAG versus FA), good agreement was found between the results (see Table 7) suggesting that majority of FA were captured during TAG analysis and FA profile can be also deducted from the TAG profile.

Finally, butter fat was analyzed using the described hybrid MS based method. The complexity of this sample and thus the key role of chromatographic separation is illustrated in Figure 2 B, depicting the ion chromatograms of the detected TAG extracted at 10 ppm extraction window. Altogether 565 TAG were identified and quantified, from which appr. 200 TAG were present at > 1 mM concentration. The regioisomeric balance of various FA is shown in Figure 6A. The results show that in butter fat the vast majority of the short-chain fatty acyl groups (C4-C6) are at the sn-1(3) position, which is in accordance with the literature (57,66). The most abundant odd carbon number FA were C17:0, C15:0 and C17:1, all of which were found enriched on the sn-2 position. Another advantage of the present hybrid MS
based method is that TAG are characterized in their intact form enabling to overview the size
distribution (e.g. sum carbon number of acyl chains) of TAG present in the sample. This is
illustrated in Figure 6 B, showing that the observed size distribution comprised a 24-54 range
in the investigated butter fat sample. The fact that the carbon number of acyl chains in the
smallest detected TAG was 24 suggests that butyric and caproic acids occur in butter fat
almost exclusively in combination with medium and long chain fatty acids, which is again in
accordance with the literature (67).

CONCLUSIONS

This work describes a new approach to detect, identify and semi-quantitate individual
regioisomers of TAG in fats and oils. The method is based on hybrid mass spectrometry which
enables the global detection and identification of TAG without the need of defining which TAG
are present in the sample. Since this method characterizes TAG in their intact form, the
overview of TAG size distribution is possible and the regioisomeric distribution of various FA
including that of P can be measured simultaneously. Further, since using this method there is
no need for enzymatic treatment, also fats/oils above melting point of 45 °C can be
characterized in contrast to conventional lipase-based methods. Limitation of the present
approach is that positional and geometrical isomers of FA (e.g. 18:3 n-3/18:3 n-6 or cis/trans
configuration) can not be distinguished, and currently only TAG containing FA with no more
than 3 double bonds (meaning 9 double bonds per TAG max) can be quantified due to the
limited availability of commercial standards.
OUTLOOK

The continuously expanding range of commercially available standards and the next generation of faster and more sensitive instrumentation will enable the characterization of more complex oils (e.g. fish oil) at even higher selectivity (e.g. MS$^3$). Further, the integration of ozone induced dissociation (68) to the present approach would bring information on FA isomerism. Finally, this work points out that radical future software developments are needed in order to facilitate the interpretation and exploit the identification/quantitation potential of hybrid datasets including various chromatographic, ion mobility, high resolution and multistage fragmentation information.
ACKNOWLEDGEMENT

The authors would like to thank Drs. Constantin Bertoli, Guillermo Napolitano, Brian David Craft and Pierre-Alain Golay for their useful comments and constructive criticism.
Reference List


FIGURE CAPTIONS

Figure 1

Typical results obtained from a beef tallow sample using the described hybrid MS approach. Insert A depicts the high resolution base peak ion chromatogram that is obtained in the Orbitrap. Insert B depicts the mass tag between ammoniated and sodiated ions that serves as an additional triggering criterion. Insert C depicts the high resolution ion chromatogram of ion 883.77275 Da that serves as a basis for quantification. Insert D depicts the averaged product ion mass spectrum of parent ion 878 Da obtained in the linear ion trap in parallel to the high resolution chromatogram. Note, that in window D only monoisotopic product ions are present representing the loss P, S, O and one ammonia unit.

Figure 2

The chromatogram of the TAG standard mixture is shown in the upper window (A) using the optimal conditions as described above. The numbering corresponds to the numbers in the tables. On the same scale, window B depicts the ion chromatograms of all TAG that were detected in butter fat. The letters stand for the following TAG: (a) - 16:0-16:0-4:0/4:0-14:0-18:0/4:0-18:0-14:0; (b) - 4:0-16:0-18:1/4:0-18:1-16:0; (c) - 4:0-12:0-18:0/4:0-14:0-16:0; (d) - 14:0-14:0-4:0/4:0-12:0-16:0/4:0-16:0-12:0; (e) - 16:0-16:0-18:1/16:0-18:1-16:0/18:0-14:0-18:1; (f) - 18:1-18:1-16:0/18:1-16:0-18:1; (g) - 16:0-16:0-16:0/16:0-14:0-18:0; (h) - 18:0-16:0-18:1/16:0-18:1-16:0/18:0-18:0-18:1; (i) - 14:0-16:0-18:1/16:0-14:0-18:1/18:0-12:0-18:1/16:0-16:1-16:0; (j) - 12:0-16:0-18:1/16:0-12:0-18:1/14:0-18:1-14:0/16:0-14:1-16:0/16:0-14:0-16:1/18:0-14:0-14:0/10:0-18:0-18:1; (k) - 16:0-16:0-12:0/16:0-12:0-16:0/14:0-16:0-14:0/14:0-12:0-18:0/12:0-18:0-14:0/10:0-18:0-16:0/16:0-10:0-18:0/10:0-16:0-18:0; (l) - 16:0-16:0-6:0/6:0-14:0-18:0/6:0-18:0-14:0; (m) - 4:0-16:0-18:0/4:0-18:0-16:0; (n) - 10:0-14:0-18:0/10:0-18:0-14:0/12:0-18:0-12:0/16:0-10:0-16:0/8:0-18:0-16:0; (o) - 16:0-14:0-16:0/16:0-12:0-
An extraction window of 10 ppm was used for all analytes.

**Figure 3**
Results of chromatographic robustness analysis are shown by varying the amount of \( n \)-hexane in solvent B. The shift of retention times are depicted as % compared to the retention time obtained using the optimal composition (50 % \( n \)-hexane). Each data point stands for a specific TAG eluted in the same order as indicated in Figure 2 A.

**Figure 4**
Schematics of TAG identification and quantitation is depicted.

**Figure 5**
Results on fatty acid regiodistribution are shown for beef tallow (A) and pork lard (B). The absolute quantity of fatty acids in the \( sn \)-1(3) and \( sn \)-2 position can be read on the vertical axis. 83 % of P is esterified at the \( sn \)-2 position in the case of pork lard sample, while on the contrary this number is only 17 % in the beef tallow sample.

**Figure 6**
Regiodistribution of fatty acids is shown in the case of butter fat sample (window A). Window B depicts the size distribution of 565 intact TAG detected in the same analysis and expressed as function of carbon number of acyl chains. Note that TAG with even acyl chain carbon number are more than ten times more abundant than TAG with the odd acyl chain carbon number.
<table>
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<tr>
<th>Trivial name</th>
<th>Abbreviation</th>
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<th>H</th>
<th>O</th>
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Table 1 List of FAs used for the generation of TAGs and inclusion list.
Table 2 Experimentally determined calibration coefficients and the corresponding precision and accuracy values expressed as relative percentage. Each point was determined in triplicates.
Table 3 Within- and inter-batch precision of mass accuracy, retention time and peak area ratio obtained for the commercially available TAG standards used for optimization and calibration. Results obtained at 0.2 μg/mL and 2 μg/mL are shown.
Table 4 Inter-batch precision of fragmentation pattern obtained for the commercially available TAG standards used for optimization and calibration. Results obtained at 0.2 μg/mL and 2 μg/mL are shown. (Note that the intra-batch precision is not shown, since it was practically the same as the inter-batch precision.)
### Table 5: Fragmentation models used to predict the fragment intensities in various product ion spectra.

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<th>Fragment1</th>
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<th>Fragment3</th>
<th>TAG</th>
<th>Fragment1</th>
<th>Fragment2</th>
<th>Fragment3</th>
<th>TAG</th>
<th>Fragment1</th>
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### Table 6: Accurate mass and fragmentation pattern of the parent ion 878 obtained from a beef tallow sample shown in Figure 1. The hit list of the possible TAG constructed from fatty acids P, S and O clearly shows that there is only one hit with acceptable mass error.

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<th>m/z of experimental ammoniated pseudomolecular ion</th>
<th>Reconstructed TAG</th>
<th>Mass error square in ppm²</th>
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</table>

**Fragments above 5% relative intensity**

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<th>m/z</th>
<th>Absolute Intensity</th>
<th>Relative Intensity</th>
<th>Neutral loss</th>
<th>Calculated fatty acid losses</th>
<th>Mass error square in ppm²</th>
<th>HIT!</th>
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<td>Pork lard</td>
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Table 7 Fatty acid content of an inter-esterified fat, beef tallow and pork lard sample calculated indirectly from the triacylglycerol data (hybrid MS method) and obtained by gas chromatography following transmethylation.
Figure 1

A. Relative abundance [%] vs. Retention time [min]

B. Mass spectrum showing [M+NH₃]⁺ at 878.81769 m/z with a mass tag at 883.77275 m/z.

C. Relative abundance [%] vs. Retention time [min]

D. Mass spectrum showing [FA1+NH₃]⁺ at 577.6 m/z, [FA2+NH₃]⁺ at 579.6 m/z, and [FA3+NH₃]⁺ at 605.6 m/z.

Figure 1
Figure 2
Figure 3
Mix with internal standards

Chromatography

Ionization with ammonium and sodium

Accurate mass inclusion criterion

Mass tag criterion

Product ion scan

Calculate fatty acid chains

Look up combinations of fatty acids matching the accurate mass

List the three isomeric forms of each identified TAG

Integrate high resolution peak area

Normalize to internal standard

Calculate concentration based on generic calibration

Calculate fragmentation pattern of all possible regioisomers

Optimize linear combination of regioisomers to match experimental data

Divide concentration according to proportions of regioisomers

Figure 4
Balance of fatty acids [weight % in fat]

Figure 5
Figure 6