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A novel UHPLC-ESI-MS/MS method and automatic calculation software for regiospecific analysis of triacylglycerols in natural fats and oils

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ABSTRACT

Regioisomeric analysis of triacylglycerols (TAGs) in natural oils and fats is a highly challenging task in analytical chemistry. Here we present a software (*TAG Analyzer*) for automatic calculation of regioisomeric composition of TAGs based on the mass spectral data from recently reported ultra-high performance liquid chromatography electrospray ionization tandem mass spectrometry (UHPLC−ESI−MS/MS) method for analyzing TAG regioisomers. The software enables fast and accurate processing of complex product ion spectra containing structurally informative diacylglycerol $[M+NH_4-RCO_2H-NH_3]^+$ and fatty acid ketene $[RCO]^+$ fragment ions. Compared to manual processing, the developed software offers higher throughput with faster calculation as well as more accurate interpretation of chromatographically overlapping isobaric TAGs. The software determines results by constructing a synthetic spectrum to match the measured fragment ion spectrum, and by reporting the optimal concentrations of TAGs used to create the synthetic spectrum. This type of calculation is often extremely challenging for manual interpretation of the fragment ion spectra of isobaric TAGs with shared fragments, hence the need for automated data processing. The developed software was validated by analyzing a wide range of mixtures of regiopure TAG reference compounds of known composition and a commercial olive oil sample. Additionally, the method was also applied for regiospecific analysis of TAGs in human milk as an example of natural fats and oils with a highly complex TAG profile. The results indicate that the software is capable of resolving regioisomeric composition of natural TAGs even of the most complex composition. This novel calculation software combined with our existing UHPLC-ESI-MS/MS method form a highly efficient tool for regioisomeric analysis of TAGs in natural fats and oils.

1. Introduction

Triacylglycerols (TAGs) are the prime constituents of natural food lipids both of animal and plant origins. The molecular structures of TAGs consists of three fatty acids esterified to a glycerol backbone. The complex fatty acid composition and different combinations of fatty acids result in large numbers of different TAGs in natural fats and oils. It has been well documented that the regioisomerism in TAGs may strongly influence the bioavailability, nutritional properties, physiological effects and physical properties of fats and oils in the diet [1–4]. For example, the positional distribution of fatty acids in human milk TAGs plays an important role in nutrition, growth and development as well as the overall wellbeing of infants [5–7]. Especially the *sn*-positioning of palmitic acid in human milk has a major effect on optimal fat and mineral absorption, gut microbiota, bone strength, sleeping and crying behavior as well as bowel movements of infants [2,7–9]. While the effects of TAG regioisomers are increasingly being studied, more powerful and higher throughput methods for analysis are needed to support the development of next generation food ingredients and nutraceuticals with improved nutritional properties and health benefits.

Enzymatic hydrolysis and chemical deacylation methods used to determine the *sn*-positioning of FA distribution possess some limitations, as they are generally more time consuming and less accurate due to possible acyl migration [10,11]. Furthermore, the enzymatic methods only provide the overall composition of fatty acids at *sn*-1, *sn*-2 and *sn*-3 positions, without information on individual molecular species or regioisomers of TAGs. Studying the regioisomerism of TAGs is a challenging task due to structural variation and the sheer number of possible

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FA combinations. Additionally, TAGs are not easily volatilized and they are susceptible to acyl migration and thermal decomposition during long column runs at high temperatures, making gas chromatographic separation methodologies impractical and unreliable. Recent development of liquid chromatographic separation [12–14] and certain mass spectrometric methods [15–17] have facilitated the analysis of TAG regioisomers.

A comparative study of three different mass spectrometric methods [positive ion atmospheric pressure chemical ionization (APCI), positive ion ESI and negative ion chemical ionization (NICI) with ammonia as the reagent gas] using liquid chromatography for TAG analysis was reported by Leskinen et al. [18]. They found that out of the tested configurations, the UHPLC-ESI-MS/MS in positive ionization mode was best suitable for analysis of TAG regioisomers. Previously, our group reported the analysis of TAGs regioisomers in human milk as lithium adducts using UHPLC-ESI-MS/MS system [19]. However, over the long term, the use of lithium salt resulted in precipitation inside the chromatographic system and the ion source of the mass spectrometer, limiting the use of this method in routine applications. Our lipid team has previously also developed and applied a direct inlet negative ion chemical ionization MS/MS method and an automated calculation software for analyzing TAG regioisomers utilizing the relative abundances of both $[M-H-FA-100]^-$ and $[RCOO]^-$ fragment ions [20,21]. While the direct inlet method has its strengths, such as very fast analyses and easy setup, it is best suited for TAG samples containing mostly medium and long-chain FAs due to overlapping of some of the structurally informative $[M-H-FA-100]^-$ and $[RCOO]^-$ ions with ACN:DB (Acyl carbon number: double bonds) molecular species below 40.

Recently, we developed a new UHPLC-ESI-MS/MS method utilizing fragmentation with collision-induced dissociation (CID) for analyzing TAGs regioisomers [22]. In the case of CID, the loss of fatty acid from one of the outer *sn*-1 or *sn*-3 positions is energetically favored compared to that of the middle *sn*-2 position during the fragmentation process. This ultra-high performance liquid chromatography coupled with more accurate and robust ammonia adduct ESI-MS/MS method was used to analyze the reference compounds of 18 regiospecific pairs of AAB type TAGs and 5 regiospecific triplets of ABC type TAGs. Calibration curves for quantification of regioisomers of these TAGs were established based on intensity of ions representing DAG fragments $[M+NH_4-RCO_2H-NH_3]^+$ resulting from loss of a single fatty acid residue from the TAG precursor ions. It was also the first study, where calibration curves for ABC type TAGs was established using reference compounds.

Compared to individual reference compounds, analysis of TAG regioisomers of natural fats and oils is a far more complicated task due to the presence of multiple TAG species with identical molecular weights, which are often co-eluting in the chromatographic system. Additionally, many co-eluting isobaric TAG molecular species share structurally informative DAG fragments, making the determination of regioisomer ratios even more challenging. A large number of TAG molecular species are also often present even in samples with relatively simple fatty acid profiles. Separating isobaric TAG molecular species in complex samples would require much more sophisticated chromatographic equipment and, likely also require fractionation of the sample, increasing the total analysis time significantly, and thus being very impractical. It is critical to have automatic data processing and a calculation method that resolves the aforementioned challenges in order to make the analysis feasible for application in the analysis of natural fats and oils with a complex fatty acid composition such as human milk or bovine milk.

The aim of our current study was to further refine and streamline the data processing by developing an automated calculation software for our recent UHPLC-ESI-MS/MS method of TAG regioisomer analysis [22]. The new software uses mainly DAG fragments to calculate both the molecular species and regioisomers of each TAG based on the experimental data obtained with regiopure TAG reference standards. The software looks at the measured fragment ion spectra and then finds an optimal set of concentrations of TAGs to produce synthetic spectra to match the original. Functionality and accuracy of the developed calculation software were tested with analyses of various TAG reference standard mixtures of known regiospecific composition. The results were further validated with olive oil and human milk samples, comparing the results to our previous findings and data in other literature. The results show that the developed calculation software can be a powerful tool in interpreting complex product ion spectra for calculating TAG regioisomer ratios. This would be nearly impossible and extremely impractical for complex samples using manual calculations.

2. Experimental section

2.1. Abbreviation and nomenclature

Abbreviations for individual fatty acids are denoted as caproic acid (6:0) Co, caprylic acid (8:0) Cy, capric acid (10:0) Ca, lauric acid (12:0) La, myristic acid (14:0) M, palmitic acid (16:0) P, palmitoleic acid (16:1n-7) Po, stearic acid (18:0) S, oleic acid (18:1n-9) O, linoleic acid (18:2n-6) L, α-linolenic acid (18:3n-3) Ln, arachidic acid (20:0) A. Regioisomers of TAGs are denoted as AAB, ABA or ABC, where A, B and C are different FAs esterified in the *sn*-1, *sn*-2 and *sn*-3 positions of the glycerol backbone. No distinction between the *sn*-1 and *sn*-3 positions can be performed in this study. The structurally informative diacylglycerol $[M+NH_4-RCO_2H-NH_3]^+$ and fatty acid ketene $[RCO]^+$ fragment ions are denoted as DAG and RCO, respectively. ACN:DB denotes the acyl carbon number (ACN) and the number of double bonds (DB) in TAGs.

2.2. Reference compounds, reagents and samples

All the AAB, ABA and ABC type reference standards were bought from Larodan AB (Solna, Sweden) and were of at least 98% purity or higher. The complete list of reference standards is shown in Supplementary Tables S–1. The regiospecific standards (AAB/ABA and ABC/ACB/BAC) were used as pure as well as a mixture of different molar ratios for a total concentration of 0.1 mM dissolved in 2-propanol:hexane (4:1, v/v). All the solvents were purchased from Merck (Merck Oy, Espoo, Finland) and were of LC-MS grade, except for hexane, which was of HPLC-grade. Elga Purelab Ultra water purification system (Elga LabWater, Woodridge, IL) was used to purify the water. Ammonium acetate was of LC-MS grade and bought from Sigma-Aldrich Finland (Helsinki, Finland). The olive oil sample was purchased from a local grocery store in Turku, Finland. The human milk sample was pooled from milk aliquots of volunteer Finnish mothers (n = 7) breastfeeding infants younger than 6 months living in the Turku area. Ethics approval for collecting and studying the human milk samples was obtained from the Ethics Committee of Hospital District of Southwestern Finland. All mothers provided informed consent.

2.3. Sample preparation

The human milk TAGs were extracted using a modified Folch extraction and a solid phase extraction (SPE) as described in detail previously [21]. Briefly, 0.5 mL of human milk was first extracted with 1.5 mL MeOH and 2.5 mL $CHCl_3$. The chloroform phase was collected, followed by another extraction with 1.5 mL $CHCl_3$. For isolation of TAGs from other lipids, a Sep-Pak Vac silica 6 cc (500 mg) SPE column was used. TAG fraction was eluted from the column with a total of 11 mL diethyl ether. Solvent was evaporated and the sample was reconstituted in 1 mL of 2-propanol:hexane (4:1, v/v) for analysis.

2.4. UHPLC-ESI-MS/MS method of analysis

The chromatographic and mass spectrometric analyses were carried out as described previously [22]. A Waters Acquity UHPLC coupled to a

Waters Quattro Premier tandem mass spectrometer with ESI source in positive ionization mode was used. A Waters Cortecs C18 column (150 mm × 2.1 mm, 1.6 μm particle size) with a Waters VanGuard C18 pre-column (1.6 μm particle size) was used for the separation of TAGs. The mobile phase consisted of solvent A, which was methanol:water (1000:1, v/v) with ammonium acetate (10 mM), and solvent B, which was 2-propanol:water (1000:1, v/v) with ammonium acetate (10 mM). The column oven was held at 60 °C. A solvent gradient program was used with an initial composition of 99% solvent A, which was changed linearly to 70% solvent A in 30 min, then linearly to 50% solvent A in 7 min, followed by isocratic 50% A for 1 min, and then changed linearly to 30% A in 2 min, and changed back to 99% A in 4 min, and finally isocratic until 50 min. Flow rate was set at 0.2 mL min^{-1} until 44 min, increased to 0.3 mL min^{-1} at 46 min and held at 0.3 mL min^{-1} for 4 min. The total analysis time was 50 min. Capillary voltage was set at 4.9 kV, the cone voltage at 22 V, the extractor voltage at 6 V, and the RF lens voltage at 0.1 V. The source was held at 120 °C and the probe heater at 350 °C. Desolvation gas flow was set at 750 L h^{-1} and cone gas flow at 200 L h^{-1}. MS/MS product-ion scans after CID at 35 eV were carried out with argon as the collision gas at a flow rate of 0.35 mL min^{-1}. Full scans of 120–950 *m/z*, and MS/MS product ion scans of 100–700 *m/z* were acquired.

2.5. The software (TAG Analyzer) for automated interpretation of MS/MS spectra for analysis of TAG regioisomers

The aim of the program is to find regioisomeric compositions of various TAGs, which have specific *m/z* values for the ammonium adducts of the molecular ions, and the approach is mainly based on analysis of the DAG fragment ion ratios. The program works in two phases. The program first finds the candidate TAGs consisting of specific fatty acid combinations. A TAG is a candidate if corresponding DAG fragments and fatty acid ketene RCO fragments are found in the spectra. The result of the first phase is a list of candidate TAGs that have fragments present in the spectra. In the second phase, the proportions of the regioisomers of the candidate TAGs found in the first phase are calculated. The concentrations are mainly determined based on the DAG fragments. The calculation is carried out based on recreating the DAG fragment peaks in the spectrum based on predetermined calibration curves. The RCO fragments can also be used. The same procedure to recreate the peaks is also used in that case. How the DAG and RCO fragments should be weighted to obtain the final result is configurable. The DAG fragmentation occurs in a more regular pattern and the calibration data is more accurate compared to data obtained with ketene fragments, hence by default DAG fragments are used exclusively.

2.5.1. Determining the candidate TAGs

Before the candidate TAGs are determined, preprocessing is done to enable matching the observed with the computed spectra. The spectra are first binned so that each peak is mapped to values representing the *m/z* ratios of the fragments. If peaks are spaced less than 1 *m/z* apart around the theoretical *m/z* value of the fragment, they are summed up. That means that the peaks are considered to belong to the same *m/z* value. After the binning of the spectra, the candidate TAGs can be found. The search space is formed by TAGs containing fatty acids with even number (4–28) of carbon atoms and in addition fatty acids of length 15 and 17. The number of double bonds considered is determined by the length of the fatty acid in order to consider all realistic fatty acids. In total, this gives 107648 unique TAGs to consider. The search is performed by creating a synthetic spectrum for the fragments for each TAG in the search space and checking if the fragmentation peaks in the synthetic spectra are found in the observed spectra. If they are found, the TAG is considered a candidate that is present in the spectra. A TAG is considered to be found if the DAG fragments and the RCO fragments have peaks above a user defined threshold. There are other MAG and FA fragments that could be utilized to filter the TAG candidates. However, the amount of these fragments varies significantly between samples, and they are hence not reliable for filtering TAGs that are present only in small amounts.

2.5.2. Fragmentation model

Calculation of the regioisomer composition of TAGs with specific FA combinations is based on the assumption that the fragmentation ratio of a TAG into different DAG (and RCO) fragments is known. The fragmentation ratios have been studied in a set of experiments with a wide range of regiopure TAG reference standards mixed at different ratios (Supplementary Figure S-1 and S-2 [22]). The set of TAGs containing two different fatty acids (AAB-type TAGs having AAB/ABA regioisomer pairs) have been processed separately from those that contain three different fatty acids, i.e. ABC-type TAGs having ABC/ACB/BAC regioisomer triplets. Conceptually they can be processed in the same way. However, since AAB/ABA TAGs give rise to two DAG ions instead of three as in the case of ABC-type TAGs, the precision of the analysis is increased when having a separate model for the two cases. For DAG fragments a fragmentation model has been developed that describes the probability that a fatty acid in a given position of TAG is detached. The fragmentation depends both on the relative lengths of the fatty acids and the number of double bonds in the *sn*-2 FA of the TAG as described previously [22]. For each AAB/ABA type TAG each calibration data point consists of the TAG pair, their concentrations and fragment ion ratios. For ABC/ACB/BAC type TAGs the calibration datasets consist of mixtures of the three TAGs with known concentrations combined with the associated fragment ion ratios. The structure of the fragmentation model is selected to be flexible enough to capture the observed fragmentation behavior, yet have few enough parameters to prevent over-fitting. The parameters of the fragmentation model are then tuned to fit the calibration data as well as possible.

2.5.3. Calculation of TAG ratios

The regioisomeric composition of a candidate TAG is determined by computing synthetic spectra of the candidate TAGs based on a fragmentation model and comparing it with the observed one. Only the DAG fragments are used by default to calculate the regioisomer ratios since the DAG fragments are produced in the most consistent and systematic manner. The synthetic spectra for each TAG are obtained based on fragmentation model calibrated on a set experiments with known concentrations of different TAGs. In the following equation, S_i denotes the spectra produced by the DAG fragments of candidate TAG $i \in 1 \ldots n$ where n is the number of candidate TAGs:

$$S(mz) = \Sigma_{i \in C} c_i S_{i,DAG}(mz)$$

To find the optimal set of TAG regioisomer ratios that make the synthetic spectra match the observed one, the following optimisation problem is solved to obtain the concentrations $c_1 \ldots c_n$ for TAGs $1 \ldots n$:

$$\min_{c1,\ldots,cn} \Sigma_{mz} (S_o(mz) - S(mz))^2$$

The sum of the square error of the difference in intensity value between the observed spectra S_0 and the calculated spectra S is minimized. This optimisation problem can be solved by numerical off-the-shelf optimisation algorithms. The Trust-Region Constrained Algorithm [23] from the open-source Python library SciPy [24] has been used.

The RCO fragments are also produced relatively consistently, but the fragmentation pattern is more complex, and it is unclear how the RCO fragments behave outside the TAGs used for calibrating the fragmentation model. The synthetic spectra for each TAG are obtained based on fragmentation model calibrated on a set of experiments with known regioisomers of different TAGs. The program finds the optimal set of regioisomer ratios that makes the synthetic spectra match the observed one. An example of the synthetic spectrum of ACN:DB 42:1 molecular species is displayed in Fig. 1. Four DAG fragments and four RCO fragments are observed in the spectrum. The *m/z* 155.14, 183.17, 211.21

and 263.24 ions are identified as the RCO fragments derived from Ca, La, M and L fatty acids, respectively. Matching DAG fragments consisting of *m/z* ions 439.38 (CaM/LaLa), 491.41 (CaL), 519.44 (LaL) and 547.47 (ML) are also found in the spectrum. The synthetic DAG fragment spectrum is displayed and overlaid on top of the actual, measured spectrum for a quick visual reference on the accuracy of the optimisation of the regioisomer ratios.

2.5.4. Limitations of the calculation software

The method can determine the candidate TAGs in a spectrum with high degree of confidence, limited only by the noise level. A high level of noise will potentially lead to false detections of TAGs, meaning that the detection threshold has to be set appropriately. There are systematic and stochastic errors that affect the accuracy of the TAG regioisomer ratio estimates. The systematic errors come from inaccuracies in the fragmentation model and the sampling of the spectra from the MS/MS scan. The stochastic errors come from the fact that there is random noise in the measurements. Only DAG peaks are used by default, which means that if the candidate TAGs do not share DAG fragments with the same *m/z* value, there are two DAG fragments for each AAB/ABA type TAG and three DAG fragments for each ABC/ACB/BAC type TAG. This is in general sufficient for accuracy comparable to the precision on the calibration data. In case the DAG fragments share *m/z* values, the number of DAG fragment peaks is fewer, and the precision somewhat decreases.

2.5.5. Applications of the automated calculation method

In order to validate the automatic calculation method by *TAG Analyzer*, regioisomeric analyses of known reference standard TAGs were performed both in sets AAB/ABA type regioisomer pairs and in ABC type regioisomer triplets of various concentrations. To further validate the results of regiospecific analysis using automatic software calculation, we have also analyzed the regiospecific composition of major ACN:DB species of a commercial olive oil sample of Italian origin. The same olive oil sample was previously analyzed with the current UHPLC-MS/MS method [22], but results were calculated manually. Additionally, a human milk sample with a more complex TAG composition was analyzed to demonstrate the functionality of the algorithm. The same human milk sample was previously analyzed in a different study using the direct inlet NICI-MS/MS method [21].

3. Results and discussion

3.1. Analysis of regiopure reference standard TAGs

A total of 18 regiospecific pairs of AAB/ABA type TAGs were analyzed at five different molar ratios; 0/100, 25/75, 50/50, 75/25 and 100/0. Fig. 2 shows the ratios of AAB/ABA type TAGs analyzed from the mixture of regiospecific pairs of different composition. All manual calculations of the regiospecific TAG standards were performed using the calibration curves established in our previous study [22]. At all molar fractions, the ratios of most AAB/ABA type regioisomers calculated with the automated software were close to the values calculated manually.

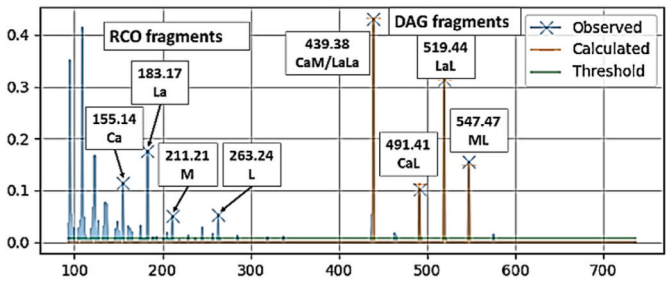

Fig. 1. The synthetic DAG spectrum of an ACN:DB 42:1 species overlaid on top of the measured fragment ion spectrum.

Some noticeable differences were also observed, most notably PPL/PLP, PPO/POP and LLLn/LLnL for the AAB/ABA type pairs.

In case of the ABC/ACB/BAC type TAGs, the analysis for each regioisomer triplet was performed at 13 different ratios: 100/0/0, 0/100/0, 0/0/100, 80/10/10, 10/80/10, 10/10/80, 70/15/15/, 15/70/15, 15/15/70, 50/25/25, 25/50/25, 25/25/50 and 33.3/33.3/33.3. Most automatically calculated results for ABC type TAGs were well in agreement with manual processing (Fig. 3) and close to the actual concentrations.

Out of all the investigated ABC type triplets the automatically calculated OPL/OLP/POL concentrations deviated slightly more compared to the other triplets. Some deviation is to be expected, as the software uses the general fragmentation model for all TAGs. The accuracy of the fragmentation model could still be further increased by analyzing additional regioisomer pairs and triplets with varying relative FA chain lengths and degree of saturation. Supplementary Figure S-1 and S-2 show the comparison between the calibration data and the corresponding fragmentation model generated for each reference standard pair or triplet. Overall, the results show that the regioisomer ratios obtained with automated calculation using *TAG Analyzer* were close to the actual ratios present in known mixtures and the results were similar to manual calculations. As the calibration curves and the fragmentation model are established using the Waters Quattro Premier tandem mass spectrometer, additional validation should be performed if the calculation software were to be used in conjunction with another instrument. There is likely variation in product ion ratios, causing potential errors in the final calculations if this is not considered. Collision energy might also need to be changed with another instrument to obtain optimal fragment ion abundances. The type of collision gas, for example argon or nitrogen, likely also influences the fragment ion behavior. In practice, validating the software again would mean reanalyzing the reference standard mixtures with the instrument in question and updating the fragmentation model.

3.2. Regiospecific composition of olive oil TAGs

The regiospecific composition of the olive oil sample was previously analyzed by manual calculation using the calibration curves established in the previous study [22]. The analyzed ACN:DB molecular species were 50:1, 52:1, 52:2, 52:3, 54:2, 54:3, 54:4, 54:5 and 56:2, totaling over 90% of all TAGs in the olive oil sample. In our current study, the regiospecific composition was analyzed with automatic calculation by *TAG Analyzer* and the results were compared with the manual calculations of the earlier study (Fig. 4).

In most cases the regioisomeric composition of the olive oil sample was very similar to our previous results (Fig. 4). However, there were also a few exceptions. The largest differences between the two calculation methods were observed in the isobaric OLnO/OOLn and LOL/LLO regioisomer pairs. Both OLnO/OOLn and LOL/LLO pairs are chromatographically partially overlapping and some of the DAG fragments share the same *m/z* ratio. In our previous study [22] using manual data processing, these pairs were resolved by taking the spectra from the rising front of the LOL/LLO peak and the descending tail of the OLnO/OOLn peak. This is not the ideal way as it is difficult to reliably know where the first pair ends and the second one starts. Additionally, there is some regioisomeric separation of the pairs even with this chromatographic method. Normally this is not noticeable, but the regioisomeric composition of the front or the tail of the peak might not be exactly the same as the average composition of the entire peak, causing possible inaccuracies in the manual calculations.

3.3. Regiospecific composition of human milk TAGs

Human milk is a much more challenging and complex sample compared to the reference standards and olive oil. TAG regioisomers of 15 different ACN:DB species are presented in Figs. 5 and 6. These groups

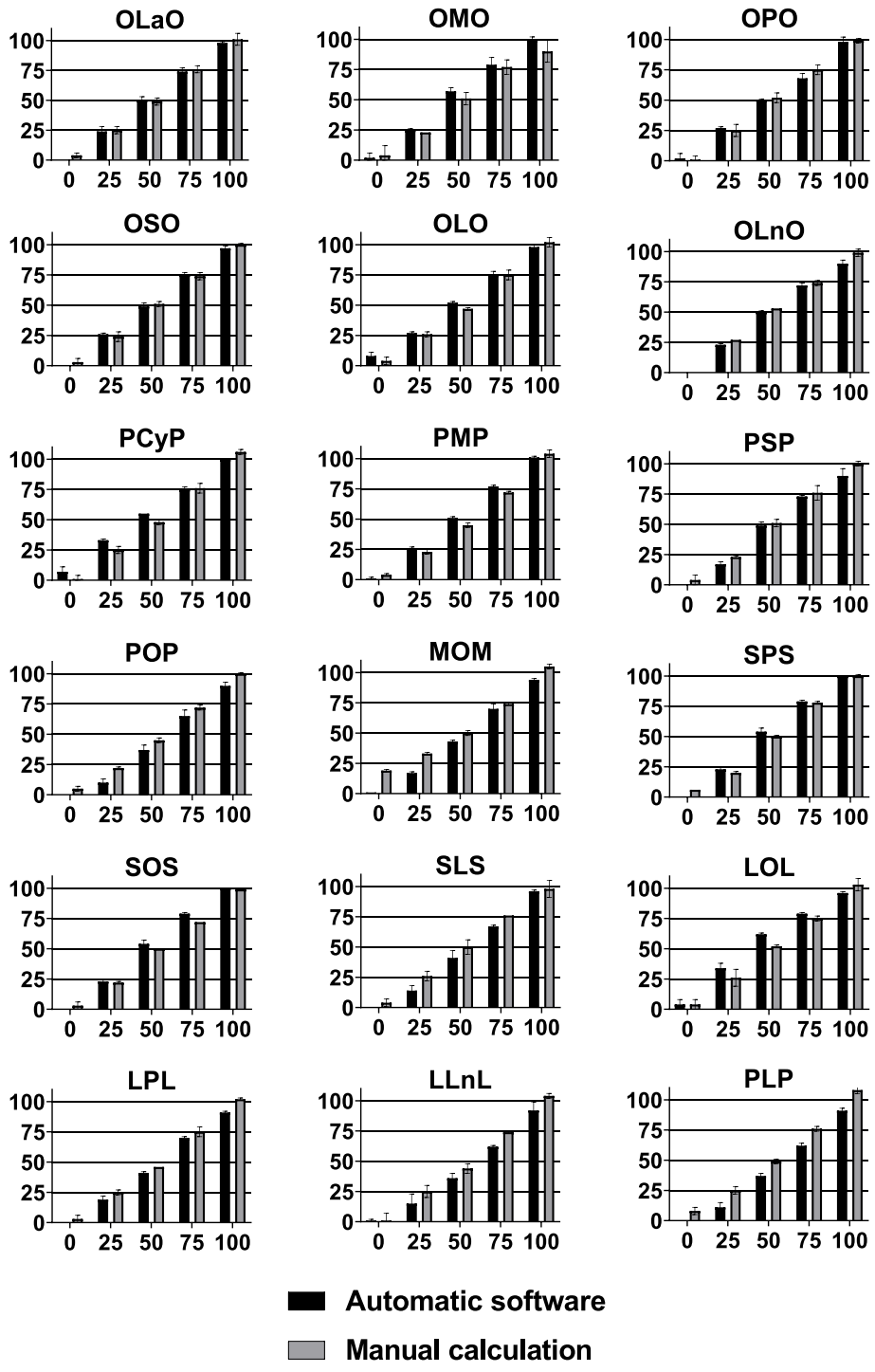

Fig. 2. Calculated concentrations of AAB/ABA type regioisomer pairs. The labels below the bars represent the actual ABA concentrations of the mixtures and Y axis represents the calculated proportions of ABA type regioisomers using automatic software and manual calculation.

of TAGs comprise of 46:0, 46:1, 48:1, 48:2, 48:3, 50:1, 50:2, 50:3, 52:1, 52:2, 52:3, 52:4, 54:2, 54:3 and 54:4 for a total of approximately 70% of all TAGs in the human milk sample. All regioisomer results, including the minor ACN:DB species are presented in the Supplementary Tables S–2. Most results for the abundant TAGs in the 15 selected ACN:DB groups were close to the previously reported values (Figs. 5 and 6) [21].

There was also major deviation within some of the less abundant TAGs within the selected ACN:DB molecular species. For example, within ACN:DB 46:0, the results for PPM/PMP were consistent with the previous study [21], but especially MMS/MSM pair showed very different results with the two methods. This could be explained by the fact that PPM/PMP is the major and most abundant pair within the ACN:DB species. When the software makes adjustments to the synthetic spectra to find the optimal concentrations, the changes are accentuated in the less abundant TAGs because the intensity of their DAG fragments is low. This likely results in proportionally larger errors in those low-abundance TAGs within the ACN:DB. Similar behavior is observed in the ACN:DB group 52:3. The results for the OPL/OLP/POL triplet, which is one of the most abundant TAG species in human milk, are very

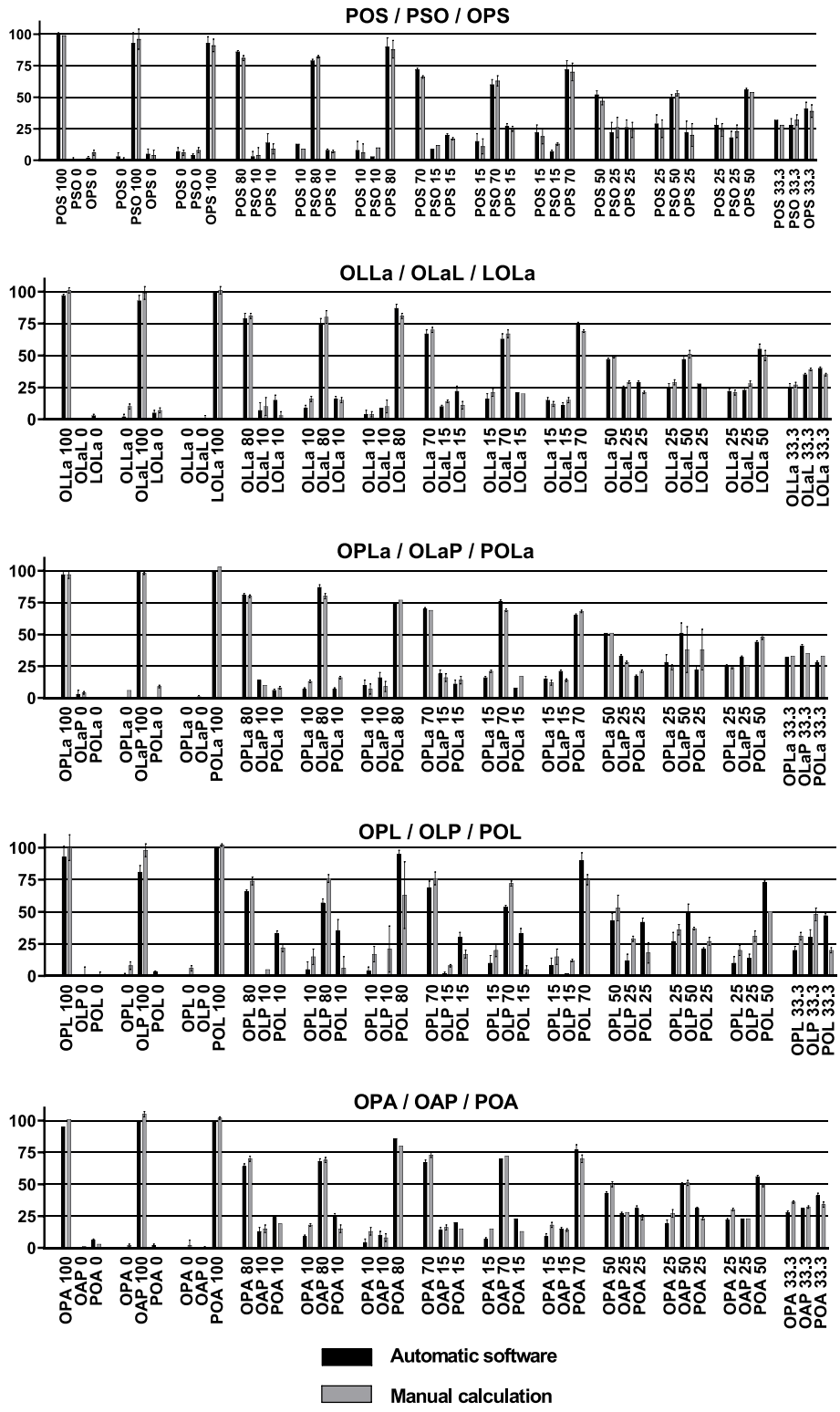

Fig. 3. Calculated concentrations of ABC/ACB/BAC type regioisomer triplets. The labels below the bars represent the actual concentrations of the mixtures and Y axis represents the calculated proportions of the regioisomers using automatic software and manual calculation.

consistent with previous literature [21,25]. The OPL/OLP/POL triplet is clearly dominating in the fragment ion spectra, and as a result, the low abundance OOPo/OPoO pair shows inconsistent behavior between replicates and different studies. Overall, the results show that the calculation software is accurate for the abundant TAGs within an ACN: DB species, but the accuracy decreases as the relative proportion of the TAG decreases.

4. Conclusions

Mass spectrometric analysis of TAG regioisomers produces large amounts of complex data. Chromatographically co-eluting TAGs may also have an identical *m/z* ratio. In addition, they often have DAG fragments with the same *m/z* ratio, making manual interpretation of the data not feasible. Especially the overlapping DAG fragments of different

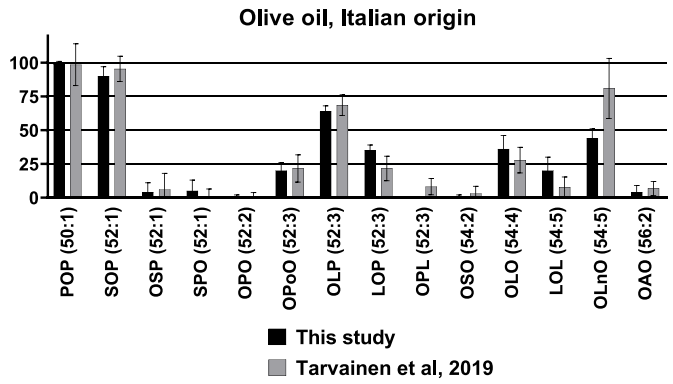

Fig. 4. Regioisomer composition of the TAGs in Italian olive oil sample.

TAG molecular species are a major challenge for accurate analysis of TAG regioisomers. The manual calculation method based on the calibration curves alone is sufficient for simple TAG mixtures that do not contain co-eluting isobaric TAGs with shared DAG fragments. Unfortunately, this is rarely the case in natural samples. To resolve these challenges chromatographically, significant advances in methodologies should be made for it to be practical. Currently, the reversed phase liquid chromatography applications are not sufficiently separating TAG molecular species with the same equivalent carbon number. The most practical approach to resolve TAG regioisomers in complex sample matrices is advanced data processing. In this study, we presented a unique solution (*TAG Analyzer*) based on an algorithmic recreation of the fragment ion spectra to find the optimal ratios of TAGs that have produced the observed fragment spectra. The method has some limitations, such as the inaccuracy of the low-abundance TAGs within one ACN:DB species. However, when comparing our results to previous literature, the precision of the software in most cases seems accurate enough even when analyzing a complex sample such as human milk.

CRediT authorship contribution statement

Md Abdullah Al Sazzad: Investigation, Formal analysis, Data curation, Writing – original draft. **Mikael Fabritius:** Investigation, Data curation, Writing – original draft, Visualization. **Pontus Boström:** Software, Validation, Writing – original draft. **Marko Tarvainen:** Methodology, Formal analysis, Resources. **Marika Kalpio:** Investigation, Writing – review & editing. **Kaisa M. Linderborg:** Resources, Writing – review & editing. **Heikki Kallio:** Conceptualization, Writing – review & editing. **Baoru Yang:** Conceptualization, Supervision, Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing, Funding acquisition.

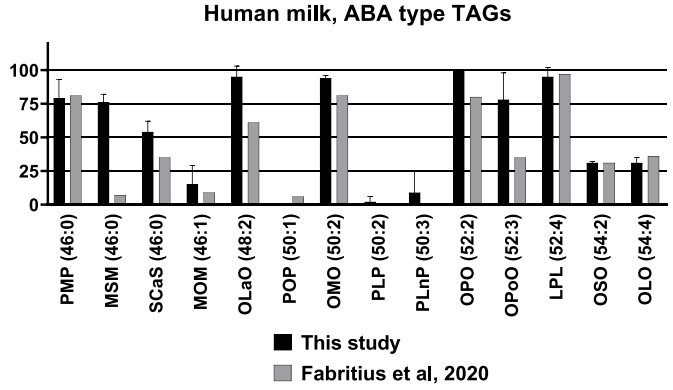

Fig. 5. Regioisomer composition of the most abundant AAB/ABA type TAGs in the human milk sample.

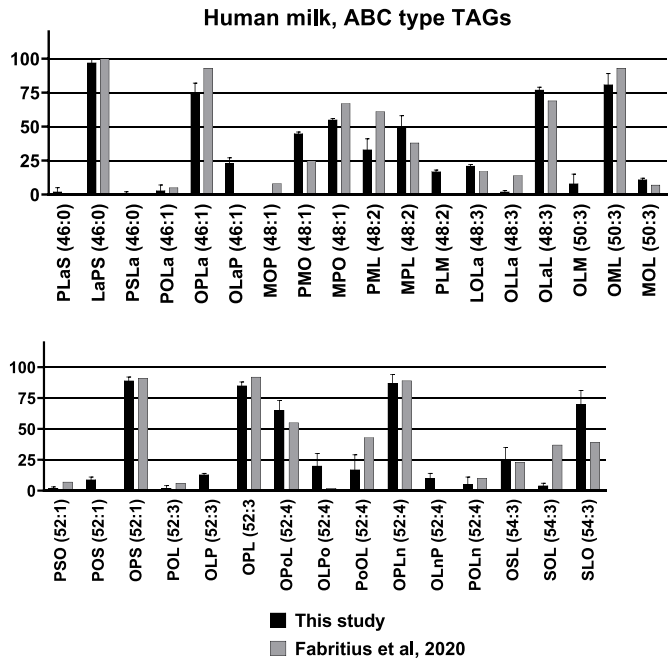

Fig. 6. Regioisomer composition of the most abundant ABA/ACB/BAC type TAGs in the human milk sample.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2022.339887.

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