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Advanced Tandem Mass Spectrometric Analysis of Complex Mixtures of Triacylglycerol Regioisomers: A Case Study of Bovine Milk Fat

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ABSTRACT: Comprehensive analysis of triacylglycerol (TAG) regioisomers is extremely challenging, with many variables that can influence the results. Previously, we reported a novel algorithmic method for resolving regioisomers of complex mixtures of TAGs. In the current study, the *TAG Analyzer* software and its mass spectrometric fragmentation model were further developed and validated for a much wider range of TAGs. To demonstrate the method, we performed for the first time a comprehensive analysis of TAG regioisomers of bovine milk fat, a very important and one of the most complex TAG mixtures in nature containing FAs ranging from short to long carbon chains. This analysis method forms a solid basis for further investigation of TAG regioisomer profiles in various natural fats and oils, potentially aiding in the development of new and healthier foods and nutraceuticals with targeted lipid structures.

KEYWORDS: *triacylglycerol, regioisomer, mass spectrometry, bovine milk*

INTRODUCTION

Fats and oils are essential components of the human diet, playing a crucial role in health and well-being at all ages. Triacylglycerols (TAGs) are a major component of natural fats and oils, consisting of a large variety of fatty acids (FAs) regio- and stereospecifically distributed in the glycerol backbone. The positional distribution of FAs in TAGs has a significant impact on the physical properties of fats and oils, but also nutritional properties, which has been well demonstrated especially in infant nutrition.[1−4] Effective methods for comprehensive analysis and in-depth understanding of TAG regioisomer compositions of natural fats and oils are essential for the development of food and nutraceuticals supporting a healthy diet with improved nutrition.

Analyzing the regioisomeric composition of TAGs in natural fats and oils is among the most challenging tasks in analytical chemistry due to the large number of TAG molecules resulting from different combinations of FAs. Various analytical methods have been used in numerous studies to investigate the FA distribution of TAGs in natural fats and oils. The methods used for such purposes can be categorized into enzymatic methods,[5−7] chemical methods,[7−9] chromatographic methods,[10−12] nuclear magnetic resonance (NMR) spectrometric methods,[13−16] and mass spectrometric methods.[17−20] Typically enzymatic and chemical methods are more time-consuming and produce less reliable results due to possible acyl migration.[21,22] More importantly, the enzymatic methods only provide information about the overall positional distribution of FAs instead of individual molecular species or regioisomers of TAGs. NMR, on the other hand, is a spectroscopic method based on characteristic chemical shifts, which provides both qualitative and quantitative information about positional distribution of FAs in TAGs. However, it does not provide information about the identity of individual TAG species and this method is not sufficient for analyzing complex TAG mixtures.[23] Existing chromatographic methods are typically focused on specific TAG regioisomers of interest and are not capable of resolving comprehensive regioisomer profiles of natural samples.[24]

Mass spectrometric (MS) methods for TAG regioisomer analysis are primarily based on the relative proportions of structurally informative fragment ions using collision-induced dissociation (CID). Studying the fragment ions is not straightforward, because the fragmentation efficiencies and the observed fragment ion ratios are influenced by the nature of the FAs in TAG molecules.[25−28] This creates challenges for quantitative analysis of TAG regioisomers, as the number of commercially available regiopure reference standards is low compared to the potential number of TAGs in natural samples. While some fragmentation techniques such as electron impact excitation of ions from organics (EIEIO)[29] or ozone-induced dissociation (OzID)[30] have been shown to produce *sn*-specific fragments, calibration curves with reference standards are still required to take into account the differences in the observed fragment ion abundances with different FA combinations in TAGs.

Another major obstacle in TAG regioisomer analyses is the interference of isobaric, structurally informative fragment ions

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from multiple TAG molecular species. Using calibration curves alone is not enough to quantify individual regioisomers when noticeable isobaric interference exists. One way of mitigating the effects of isobaric interference is adequate chromatographic separation of the isobaric molecular species. This is often challenging, and in many commonly used reversed-phase (RP) chromatographic analysis applications of natural fats and oils, multiple isobaric TAGs and their fragments are often chromatographically overlapping.

Previously, we have developed a UHPLC-MS/MS method for the analysis of TAG regioisomers based on the relative abundances of structurally informative diacylglycerol (DAG) fragments after fragmentation with CID.[25] For data processing and automatic calculation of complex samples, we recently developed *TAG Analyzer* software to address the challenge brought by chromatographically overlapping isobaric TAGs and their fragments.[19] *TAG Analyzer* uses a unique optimization algorithm to interpret the fragment ion data. Utilizing a fragmentation model calibrated with numerous reference standards, the algorithm finds the optimal TAG regioisomer composition that produces the best matching fragment spectrum, which can comprise regioisomers of different molecular species, including isobaric fragments from multiple sources. The fragmentation model was previously calibrated with TAG standards containing mainly long- and medium-chain FAs. In our current study, the first aim was to update the fragmentation model of *TAG Analyzer*[19] software by adding data of TAGs containing short-chain FAs to the existing calibration data set.

The second aim was to study the inter-instrumental applicability of the calibration data and robustness of the fragmentation model on a different mass spectrometer than we used previously. Finally, as an application example, the regioisomers of bovine milk fat TAGs were analyzed. Bovine milk fat is considered to be one of the most complex natural mixtures of TAGs, containing a wide range of fatty acids of short to long chains and varying number of double bonds.[31,32] Although molecular species of TAGs in bovine milk have been widely reported by previous studies,[24,33] the regioisomeric composition remains still largely unknown, mainly due to the lack of comprehensive analytical methods. It has been shown that cow breed can significantly influence the regioisomer ratios of certain TAGs in bovine milk.[34] While the amount of investigated TAG regioisomers in that study was limited to two pairs, it shows that there can potentially be substantial differences in the milk TAG regioisomer composition of different cow breeds. As bovine milk is a significant ingredient for food industry worldwide, deep characterization of the molecular structure will enhance our knowledge on its nutritional effects and show what factors potentially lead to differences in the regioisomer composition.

MATERIALS AND METHODS

Nomenclature and Abbreviations. The TAG regioisomer pairs and triplets are denoted as AAB/ABA and ABC/BAC/ACB types, respectively, where A, B, and C are different FAs esterified in three positions of the glycerol backbone. The middle letter denotes the *sn*-2 FA, while the first and third letters denote the *sn*-1/3 FAs interchangeably. No distinction is made between the *sn*-1 and *sn*-3 FAs. 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. The acyl carbon number (ACN) and the number of double bonds (DB) in FAs of the TAGs are denoted as ACN:DB. Abbreviations for individual fatty acids are as Bu for butyric acid (4:0), Co for caproic acid (6:0), Cy for caprylic acid (8:0), Ca for capric acid (10:0), La for lauric acid (12:0), M for myristic acid (14:0), Pd for pentadecylic acid (15:0), P for palmitic acid (16:0), Po for palmitoleic acid (16:1n-7), Ma for margaric acid (17:0), S for stearic acid (18:0), O for oleic acid (18:1n-9), L for linoleic acid (18:2n-6), Ln for α-linolenic acid (18:3n-3), A for arachidic acid (20:0), and G for gadoleic acid (20:1).

Reference Compounds, Reagents, and Samples. The complete list of reference standards used in this study is presented in Table S1. All regiopure reference standards were purchased from Larodan AB (Solna, Sweden). All solvents used in this study were of LC-MS grade, except for hexane, which was of HPLC grade; all of the solvents were purchased from Merck (Merck Oy, Espoo, Finland). Ammonium acetate of LC-MS grade was purchased from Sigma-Aldrich, Finland (Helsinki, Finland). Water was purified using an Elga Purelab Ultra water purification system (Elga LabWater, Woodridge, IL). The bovine milk sample was provided by Valio Ltd. (Helsinki, Finland).

Sample Preparation. The bovine milk TAGs were extracted using a modified Folch method and purified with solid-phase extraction (SPE) as described in detail previously.[18] Briefly, 0.5 mL of bovine milk was first extracted with 1.5 mL of MeOH and 2.5 mL of $CHCl_3$. The chloroform phase was collected, followed by another extraction of aqueous methanol phase with 1.5 mL of $CHCl_3$. The chloroform phases containing TAGs from the two extractions were combined. For the isolation of TAGs from polar lipids, a Sep-Pak Vac silica 6 cc (500 mg) SPE column was used. TAG fraction was eluted from the Sep-Pak column with 11 mL of diethyl ether. After evaporating the solvent, the sample was reconstituted in 1 mL of 2-propanol/hexane (4:1, v/v). Prior to analysis, a 1:100 dilution of the bovine milk TAG sample was prepared to prevent detector saturation. The TAG standards were diluted to 0.01 mM concentration with 2-propanol/hexane (4:1, v/v) and analyzed as such.

UHPLC-ESI-MS/MS Analysis Method. In our earlier studies, the Waters Quattro Premier triple quadrupole MS/MS instrument was used to analyze the regiopure reference TAGs for constructing the calibration curves and subsequently the first fragmentation model of the *TAG Analyzer* software.[19,25] In the current study, to establish the updated fragmentation model, we analyzed the reference compounds using a Shimadzu LC-MS 8045 triple quadrupole MS/MS instrument. In addition to the fragmentation model, individual calibration curves were also constructed for easier comparison of the calibration data from two different instruments for evaluating inter-instrument robustness of the two different instrument platforms (Figure S1).

A Waters Cortecs C18 column (150 × 2.1 mm^2, 1.6 μm particle size) with a Waters VanGuard C18 precolumn (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.[25]

Interface voltage was set at 4 kV. Interface temperature was set at 300 °C, desolvation temperature was set at 526 °C, and DL temperature was set at 200 °C. Nebulizing gas flow was at 2 L min^{-1}, heating gas flow was set at 15 L min^{-1}, and drying gas flow was set at 5 L min^{-1}. Initial MS scans in positive polarity with electrospray ionization (ESI) were carried out at m/z 200−1000 for preliminary TAG species identification. An estimation of the TAG species distribution was calculated based on the integrated peak areas of the TAG ammonium adduct ions. After the identification of major TAG species in the sample, product ion scans at m/z 50−700 of relevant precursor ions using CID at 30 eV were created to analyze the regioisomer composition. Argon was used as the collision gas for CID.

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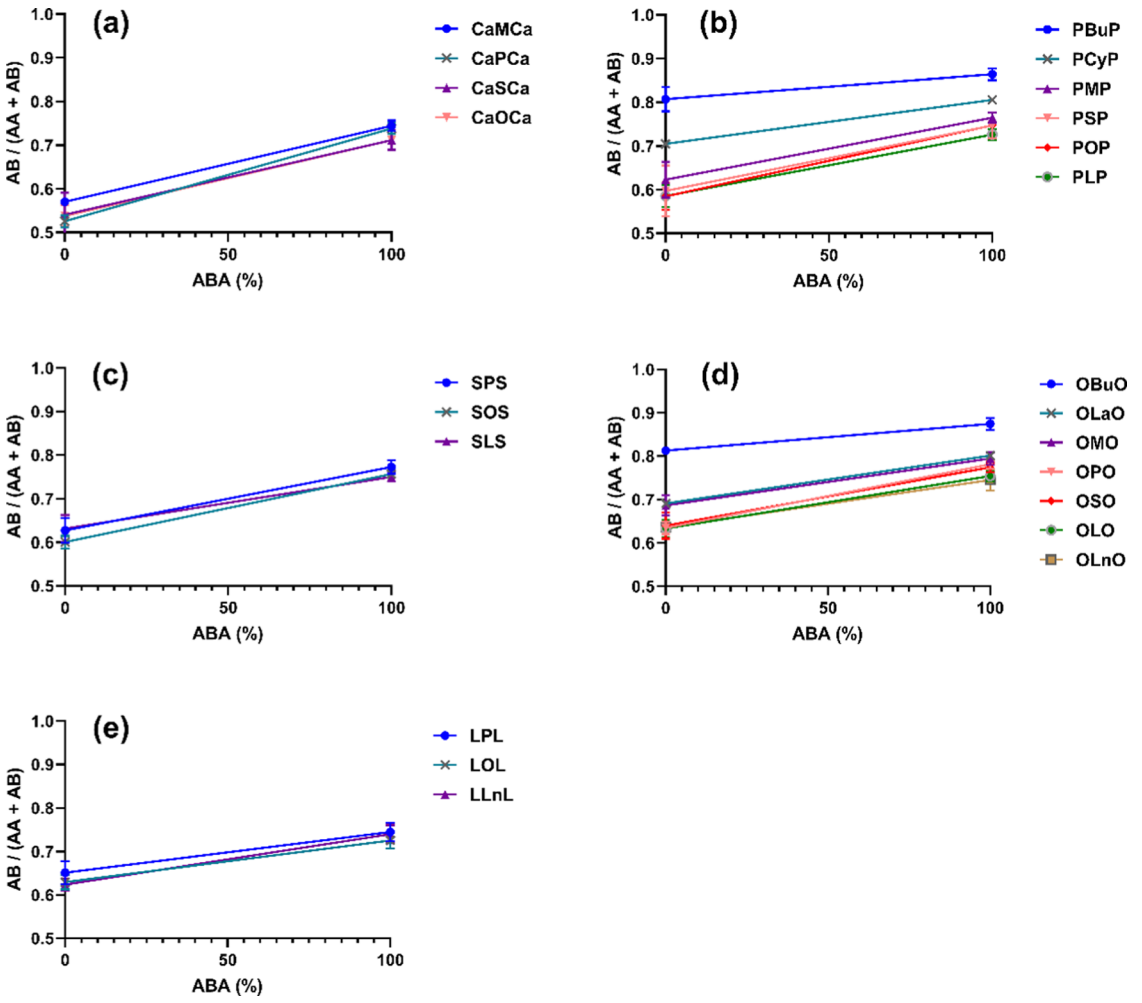

Figure 1. Two-point calibration plots of certain regiopure ABA/AAB-type TAG standards grouped together by the FAs; CaXCa series (a), PXP series (b), SXS series (c), OXO series (d), and LXL series (e), where X is a varying FA. *X*-axis indicates the percentage of ABA regioisomer, and *Y*-axis indicates the fragment ion ratios of AB/(AA + AB).

Standards and the bovine milk sample were analyzed in quadruplicate, and the injection volume was 1 μL.

***TAG Analyzer* Software.** Recently, we published the *TAG Analyzer* software for the analysis of TAG regioisomers in natural samples.[19] Briefly, the software has two important features for resolving complex TAG regioisomer mixtures using structurally informative fragment ions, mostly DAG fragments. First, a fragmentation model takes into account the influence of the FA composition of the TAGs in the fragmentation pattern. For example, as observed in our earlier study,[25] the chain length and number of double bonds of *sn*-2 FA have a strong influence on the abundance ratios of DAG fragment ions, while the *sn*-1/3 FAs play a minor role. The second vital feature of the *TAG Analyzer* is the optimization algorithm that mitigates the interference by isobaric DAG fragment ions dissociating from chromatographically overlapping isobaric TAG molecular species. Isobaric TAGs with the same equivalent carbon number (ECN) often elute simultaneously in reversed-phase liquid chromatography, creating isobaric structurally informative fragment ions that are difficult to resolve with the calibration curves of fragmentation models alone. The algorithm in *TAG Analyzer* creates a synthetic spectrum that matches the observed one utilizing the fragmentation model. Once the best fit is found, the software reports the regioisomer abundances that were used to create the matching synthetic spectrum. Generally, in TAG regioisomer analytics, the influence of overlapping isobaric TAGs and their isobaric fragments has been a major challenge, which the *TAG Analyzer* mitigates. Previously,[19] the fragmentation model of the *TAG Analyzer* software was calibrated with TAGs containing mainly long-chain FAs and some medium-chain FAs (18 AAB-type regioisomer pairs and 5 ABC-type regioisomer triplets). In the current work, the fragmentation model was updated with 8 additional AAB/ABA-type regioisomer pairs and 2 ABC/BAC/ACB-type regioisomer triplets containing short-chain and medium-chain FAs, allowing more accurate calculations for TAG regioisomers of a much wider range.

Furthermore, as a general improvement to the validation of the fragmentation model, a leave-one-out cross-validation (LOOCV) was implemented to evaluate the accuracy of the models. When creating the model, LOOCV leaves out one reference standard from the calibration data and then calculates the result for that specific standard using the remaining data. This enables the estimation of the average error for a compound that is not included in the calibration data. This is important because there are potentially hundreds of regioisomers in complex natural samples, while the number of calibration standards is limited. To our knowledge, there are no existing software tools specifically dedicated to TAG regioisomer analysis. Some tools utilizing rule-based lipid identification such as Lipid Data Analyzer (LDA)[35] have the functionality of identifying the most abundant regioisomers within pairs/triplets based on simple fragment ion abundance ratios, neglecting the possibility of a mixture of different regioisomers. While this approach might be enough to identify the dominant regioisomers when there is only minor coelution of other regioisomers and isobaric molecular species, the accuracy dramatically decreases when the complexity of the coeluting isobaric and regioisomeric species increases. Based on our survey of other tools

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used in lipidomics identifications, only *TAG Analyzer* has the abundance optimization algorithm taking into account the effects of coeluting isobaric species, and the fragmentation model which considers the influence of different FA combinations of the molecular species on the fragmentation efficiencies. Both features are critical for accurate TAG regioisomer calculations in complex natural samples.

RESULTS AND DISCUSSION

Calibration Data for the Fragmentation Model. The calibration data was prepared by analyzing a total of 26 regiopure AAB/ABA-type TAG pairs and 7 ABC/BAC/ACB-type TAG regioisomer triplets, including the TAGs with short-chain FAs (see Table S1 for more details). As demonstrated earlier, two calibration points are sufficient to create linear calibration plots for TAG regioisomers.[25] Therefore, for each pair and triplet, only the pure compounds were analyzed as such without preparation of mixtures to create the calibration data for the fragmentation model. For visualization of the fragmentation behavior of AAB/ABA-type TAGs with different FA combinations, calibration curves were constructed by plotting the AB/(AA+AB) ratios of DAG fragments versus the fractional abundance of the TAG regioisomers, and the results were grouped by TAGs containing two same FAs and one varying FA (Figure 1). For example, Figure 1a shows the "CaXCa" series, where each calibration plot consists of TAGs with two capric acids and one varying FA.

The calibration curves of 18 AAB/ABA-type TAG regioisomeric pairs were compared with those established previously by using the same reference compounds (Figure S1). Overall, the calibration curves obtained in this study using a Shimadzu LC-MS 8045 triple quadrupole instrument were found to be close to those reported in a previous study using the Waters Quattro Premier.[25] Furthermore, the additional calibration curves for 10 regioisomer pairs (AAB/ABA-type TAGs) or triplets (ABC/BAC/ACB-type TAGs) containing the shorter-chain FAs (C4−C10) are shown in Figures S2 and S3.

As observed in our earlier study with a different instrument (Waters Quattro Premier triple quadrupole),[25] the slopes and intercepts of the calibration curves were mainly influenced by the identity of the *sn*-2 FA, as is evident especially in the OXO and PXP series containing the highest number of regiopure TAG standard pairs. With shorter-chain FAs in the *sn*-2 position, the proportion of the AB fragment increases and the slopes of the calibration curves decrease. If the curve goes completely flat, the method cannot distinguish the two regioisomers from one another, meaning that the fragment ion spectra are similar. For example, the PPBu/PBuP and OOBu/OBuO regioisomer pairs have lower positional sensitivity compared to other pairs. This makes quantifying these regioisomers more challenging because instrumental deviation in the fragment ion ratios may cause proportionally higher deviation in the calculated regioisomer results compared to other pairs.

Additionally, *sn*-1 and *sn*-3 FAs seem to have a minor impact on the fragmentation pattern, and the overall mechanisms that lead to the observed fragment ion ratio can be quite complicated. *TAG Analyzer* uses a machine learning-based fragmentation model that considers the chain length and number of double bonds in *sn*-2 and *sn*-1/3 FAs and deciphers how they influence the observed DAG fragment ion ratios. If a certain ACN:DB range of standards is not included in the calibration data, such as the short-chain FA-containing TAGs, the model might produce inaccurate results for that range. Correspondingly, the current model might not be ideal for TAGs containing very long polyunsaturated FAs such as FA 20:5 or FA 22:6, which are often found in lipids from marine sources. Additional calibration standards would still be required for the accurate quantification of TAG regioisomers containing these types of fatty acids.

Accuracy of the Fragmentation Model with Regiopure TAG Standards. Using the updated fragmentation model of the *TAG Analyzer* software, we calculated the results for all of the reference standard TAGs. The regioisomer results of 26 AAB/ABA-type regioisomer pairs are presented in Figure 2. Overall, the concentration of regioisomers in TAGs

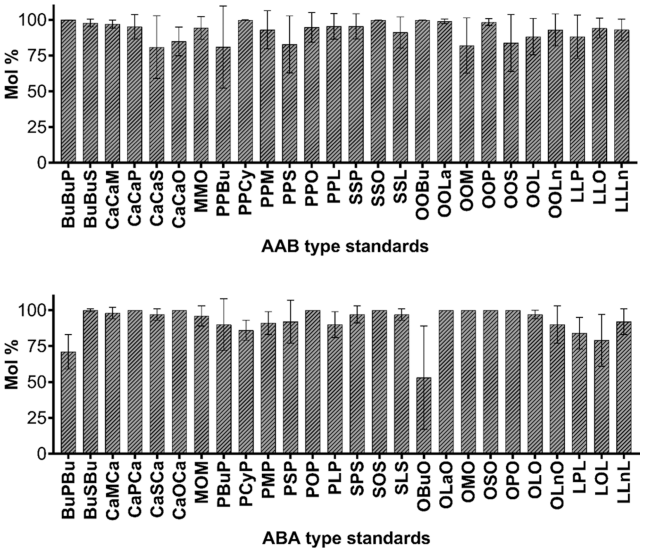

Figure 2. Molar ratios of AAB- and ABA-type TAG regioisomers in the reference standards calculated using the *TAG Analyzer software*.

standards calculated with the software was close to the actual amount present in the pure compounds. On average, the results for regiopure AAB and ABA standards were 100/0 ± 8.3 and 100/0 ± 7.8%, respectively. If a calculated result yields a molar ratio greater than 100%, then that result is reported as 100%. This can happen if, for example, the observed AB/(AA + AB) fragment ion ratio is higher than the end point of the established calibration curve or the fragmentation model.

In a few cases, more deviations were observed, most notably for BuPBu and OBuO regioisomers, where the calculated results highly deviated from the actual amount of pure compound. Especially for OBuO, the inaccuracy and the deviation are likely caused by the low positional sensitivity of the OOBu/OBuO regioisomers, resulting in similar fragment ion ratios and a quite flat calibration curve. The regioisomeric composition of 7 triplets of regiopure ABC/BAC/BCA-type TAGs was also analyzed (Figure 3), and the results were close to the actual concentration of the regioisomers with an average of 100/0 ± 4.9%. Among the ABC/BAC/BCA-type TAGs, a slightly higher deviation was observed for the CoPO/CoOP/OCoP triplet compared to others. Some inaccuracies in the calculations are to be expected since the software uses a general fragmentation model for all TAGs.

On average, the results calculated with the LOOCV models for AAB-, ABA-, and ABC/BAC/BCA-type TAG standards were 100/0 ± 9.9, 100/0 ± 9.4, and 100/0 ± 5.9%,

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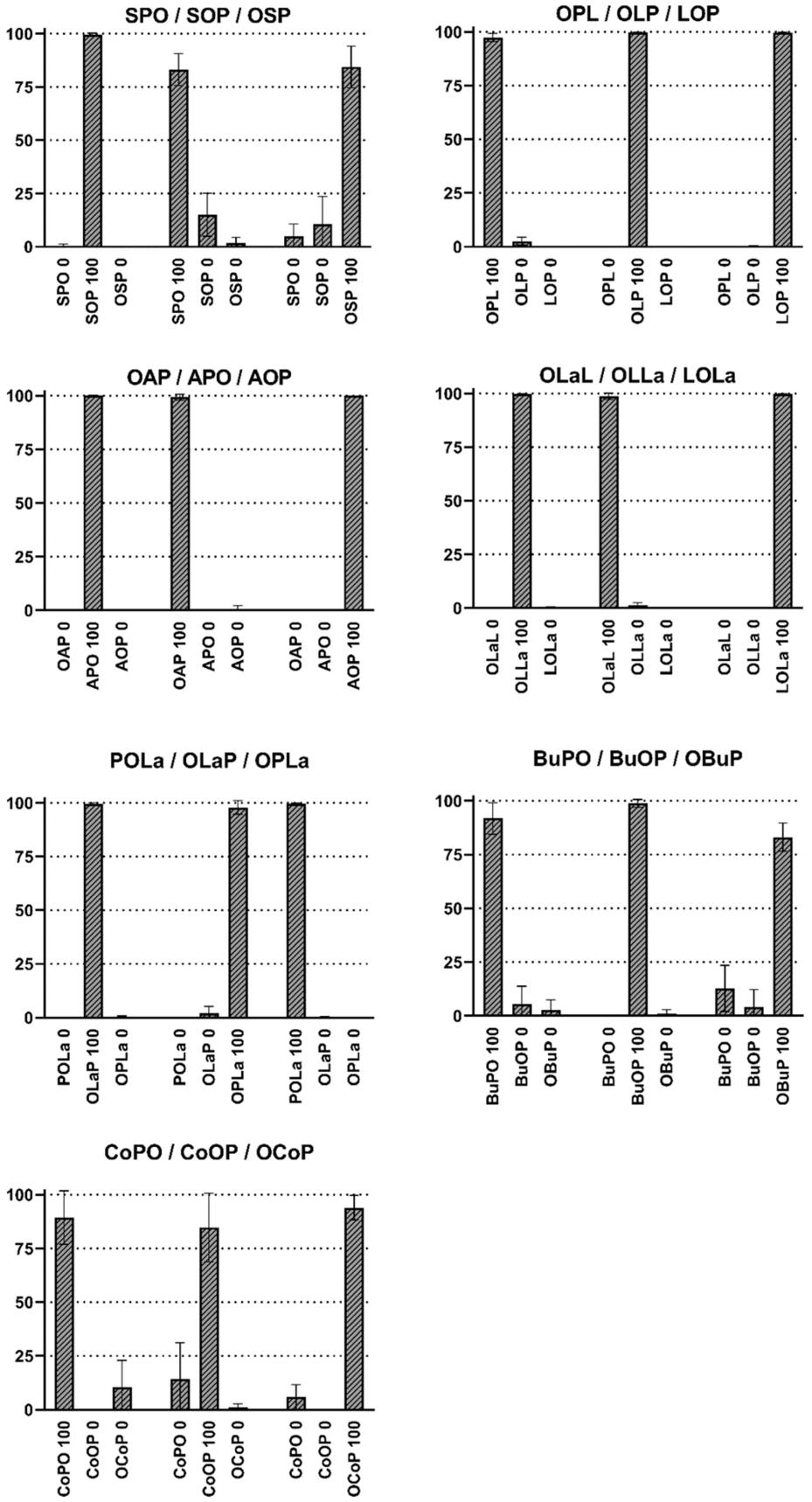

Figure 3. Molar ratios of ABC/BAC/BCA-type regioisomers in the reference standards calculated using *TAG Analyzer* software. The labels below the bars represent the actual regioisomer abundances of the standards.

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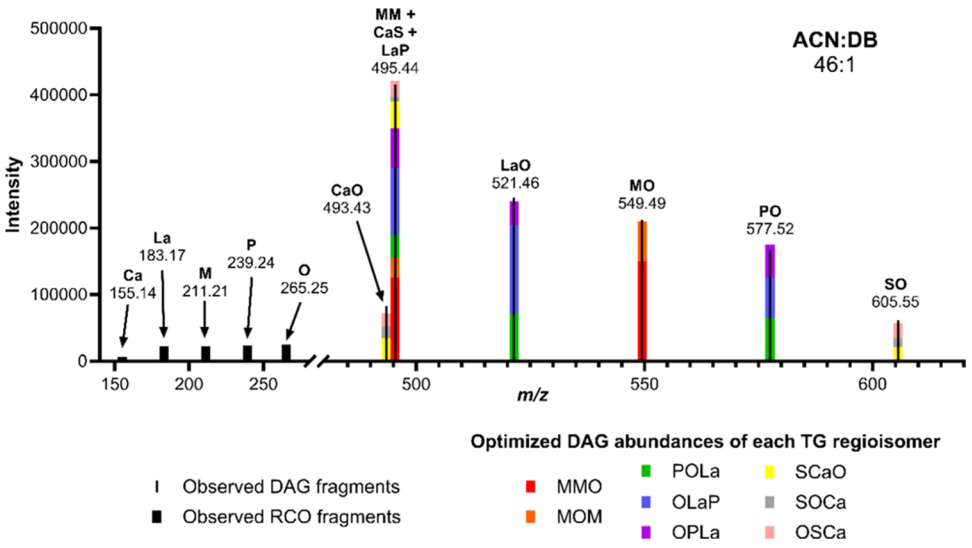

Figure 4. Visualization of the TAG regioisomer optimization process with the *TAG Analyzer* software in bovine milk TAG species 46:1. The colored bars represent the optimized, synthetic DAG fragment spectra overlaid on top of the observed DAG fragments.

respectively, which is only slightly less accurate than the final model. This shows that removing a standard from the calibration data somewhat decreases the accuracy for that specific TAG, but overall, it does not compromise the model. This demonstrates that the model is robust and reliable for the analysis of natural samples containing TAGs that are not included in the calibration data. While the results are mostly good, the accuracy of the fragmentation model and software could be further improved by incorporating the analysis data of additional reference TAGs with varying FA chain lengths and degrees of unsaturation.

Analysis of TAG Regioisomers in Bovine Milk. Due to the presence of a wide range of fatty acids, bovine milk TAGs represent one of the most unique and complex compositions among the natural fats and oils, of which the regioisomeric composition has remained mostly unknown. In one of our previous studies with a different negative ion chemical ionization (NICI) MS/MS method,[18] some regioisomers of bovine milk TAGs were analyzed. However, the NICI method and the accompanying *MSPECTRA*[36] software were not suitable for resolving TAGs of ACN below 44. As a result, regioisomers of less than 20% of TAG in bovine milk fat were resolved in that study. In the current study, we have comprehensively analyzed the TAG regioisomer composition of bovine milk for the first time using the current UHPLC-MS/MS method with the updated *TAG Analyzer* software. The relative abundances of all ACN:DB species identified in the initial survey MS scan are presented in Figure S4. The molecular weight distribution ranged from ACN 26 to 54. The most abundant ACN:DB species found in bovine milk was 36:0 (almost 8%), followed by 38:1 (6.97%), 38:0 (6.85%), 50:1 (6.20%), 34:0 (4.89%), 48:1 (4.89%), and 52:2 (4.20%).

An example of the TAG regioisomer abundance optimization and calculation process using *TAG Analyzer* is visualized in Figure 4. First, the software identifies the RCO fragments and their corresponding FAs of the selected precursor ion corresponding to a specific ACN:DB species. In this case, the ACN:DB species is 46:1. Based on the FA information obtained with the RCO fragments and the ACN:DB value, a list of candidate TAGs are determined. There are three different TAG molecular species and their regioisomers chromatographically overlapping in this example (MMO/MOM, POLa/OLaP/OPLa, and SCaO/SOCa/OSCa). Based on the identified TAG molecular species, the DAG fragments are identified next. The observed fragment m/z 495.44 is a net product of all three molecular species, making the optimization algorithm critical in the next phase. The software then uses the fragmentation model and the optimization algorithm together to find synthetic DAG fragment spectra that best match the original, observed one. Once the best fit is found, the software reports the TAG regioisomer abundances that produce matching synthetic spectra.

Fragmentation model or calibration curves alone would not be sufficient to accurately calculate the TAG regioisomers in this case, as there are isobaric DAG fragments from multiple TAG molecular species that would distort the results. The main benefit of the optimization algorithm is the simultaneous fitting of the fragmentation model to all identified molecular species, mitigating the distortion effects of the isobaric fragments.

The regioisomer composition of the most abundant ACN:DB species containing more than 2% of total TAGs in bovine milk is shown in Figure 5, and the regioisomer composition of all identified TAGs is presented in Table S2. In most cases, the results are reproducible with low deviations among the replicates, especially for the most abundant TAGs.

To our knowledge, no study has been reported on a comprehensive analysis of TAG regioisomers of bovine milk fat. For comparison of the bovine milk TAG regioisomer results, there are very limited studies that have taken into account the effects of isobaric fragment ion interference. Recently, Liu et al. demonstrated the regioisomeric analysis of specific bovine milk TAGs emphasizing chromatographic separation of interfering isobaric TAGs species with C30 stationary phase column.[34] The method was applied for analyzing the OPO/OOP and the OSO/OOS pairs using fragment ion intensity ratios. As a result, it provides structural information for targeted TAGs rather than comprehensive information on the regioisomer profile in bovine milk.[34] They concluded that the regioisomer ratios are affected by both cow breed and individual cows, but our OPO and OSO ratios are comparable with the average results in their study, having the

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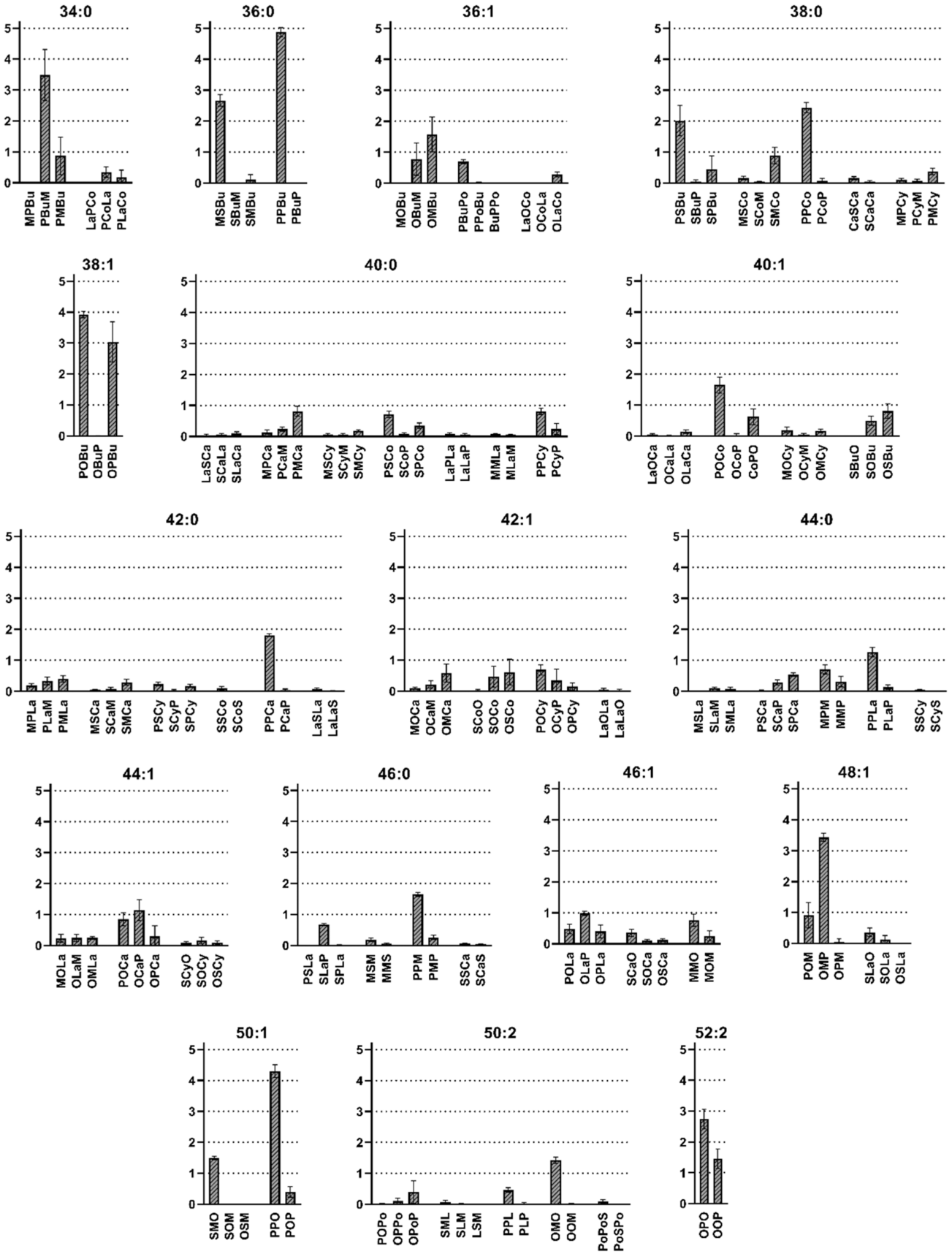

Figure 5. Distribution of TAGs regioisomers in the bovine milk fat sample grouped by the ACN:DB. TAG species containing more than 2% of all identified TAGs are shown. *Y*-axis represents the abundance of regioisomers as mol % of total TAGs of the bovine milk fat sample.

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closest matching profile of the two regioisomer pairs in the milk fat of the Jersey cow breed. As commercial milk provided by the largest dairy company (Valio Ltd., Helsinki, Finland), our milk sample was likely a mixture from several cow breeds. Nagai et al. used a C28 reversed-phase column for the separation of individual regioisomers, focusing on TAGs with a dipalmitoylglycerol backbone and one short- or medium-chain FA (PPX/PXP type TAGs).[24] In accordance with our results, they discovered that the PPX regioisomer (X = Bu, Co, Cy, Ca, La) was the dominant form, with the *sn*-2 position primarily occupied by palmitic acid. It has also been observed that the pregastric lipase of calf has a unique specificity for FAs 4:0−10:0 and preferentially hydrolyze the ester bond at *sn*-1/3 positions,[37,38] supporting our findings that these FAs are mainly located in *sn*-1/3 positions.

While the bovine milk TAG regioisomers have not been previously comprehensively characterized, there are several studies that have analyzed the positional distribution of FAs between *sn*-2 and *sn*-1/3 positions using enzymatic methodologies.[39−41] Although these studies only provide the composition and overall positional distribution of FAs in the TAGs, we can use this information to compare our findings by extracting the FA data from the TAG regioisomer results. The overall FA composition extracted from the TAG species identified and quantified in our current study is very similar to the FA composition reported in these three studies (Figure S5). Also, the general *sn*-2 and *sn*-1/3 FA profiles are largely similar (Figure S6) with the short- and medium-chain FAs mainly attached to the primary position in the TAGs of bovine milk in both studies, despite minor differences. Some of the differences may have been caused by factors such as cow breed,[34] influencing the regioisomer composition. Overall, the results are consistent with those of the previous studies. This shows that our methodology can produce results comparable to those of the enzymatic methods, while also providing regiospecific information on the structures of individual TAGs. Further, our comprehensive results on the bovine milk TAG regioisomer composition are in good agreement with the findings of previous studies targeted for specific TAG species. Therefore, the findings of the current study have demonstrated *TAG analyzer* to be a powerful tool for characterizing the TAG regioisomer profiles of complex natural samples such as bovine milk.

Future Improvements to the Methodologies. While our methodologies, including the *TAG Analyzer* software, are already quite effective in the accurate analysis of TAG regioisomers, there are still potential improvements and changes to be considered for achieving the maximum potential. The optimization algorithm in combination with the fragmentation model can mitigate the effects of interfering structurally informative DAG fragments, but the complexity within ACN:DB species often results in high relative deviation for especially the lower-abundance regioisomers. These effects could be further decreased by improving the chromatographic separation of isobaric TAG species. For example, a C30 reversed-phase column could be tested instead of the current C18 column to enhance the separation of TAG molecular species. While the software is designed to mitigate the influence of coeluting isobaric TAGs, the methodology would still benefit from improved chromatographic separation of the isobaric TAG species, especially when considering the low abundance TAGs. With our current methodology, we can quite confidently say whether certain regioisomers within ACN:DB species are of low abundance, but determining the ratios of those regioisomers within the pairs or triplets is sometimes challenging due to the high relative deviations as seen in the bovine milk results. The relative deviation of the low abundance TAGs would likely significantly improve if they would not be chromatographically overlapping with the more abundant isobaric TAGs, which is why we are still aiming to improve the separation. The optimization algorithm works not only better but also faster when there are less variables (isobaric DAG fragments) to consider. The calculation algorithm requires quite a lot of processing power, and any improvement would result in higher throughput. This optimization is important when we are eventually moving into untargeted lipidomics to make sure that the calculation process would not become a limiting bottleneck.

The second potential improvement is the use of different adduct ion types as precursors. Currently, we use ammonium acetate in the mobile phase as an additive, leading to the formation of ammoniated TAG ions $[M + NH_4]^+$. Ammonium ions are the most widely used complexing agent for TAG regioisomer analyses across different studies.[42] However, a recent study[43] suggested that $[M + NH_4]^+$ ions might not be the ideal adduct type for establishing a fragmentation model, as the fragment ion ratios of different ACN:DB combinations are not predictable, and sodiated $[M + Na]^+$ adducts might be more suitable for creating a general fragmentation model.[43] Contrary to the findings of Makarov et al., we have established an accurate fragmentation model using $[M + NH_4]^+$ adducts using two different triple quadrupole instruments (Shimadzu LC-MS 8045 and Waters Quattro Premier) used in this and a previous study.[19] We used argon as the collision gas for CID with both instruments, whereas helium was used in the study of Makarov et al. While it is difficult to evaluate the full effect without access to an instrument using different collision gases, the collision gas type could possibly explain at least some of the differences observed in the positional sensitivity (differences in the fragment ion ratios between the two regioisomers) of $[M + NH_4]^+$ adducts with different instruments. Testing $[M + Na]^+$ adducts as precursors of CID in ESI-MS/MS as suggested by Makarov et al. would also be worthwhile in future work of analyzing TAG regioisomers.

With the current fragmentation model and instrumental settings, a high deviation was observed for some specific TAG regioisomers containing short-chain fatty acids, indicating the need for further improvement in the accuracy of *TAG Analyzer* for quantifying regioisomers of low ACN:DB species. Additionally, TAGs containing very long-chain polyunsaturated FAs still need to be included in the fragmentation model. Finally, while we already have a considerable number of various regiopure TAG standards, additional calibration data for the model would increase the accuracy of the calculations. To address the financial strain of obtaining numerous regiopure TAG reference standards, an alternative solution for obtaining accurate calibration data should be considered. For example, a chromatographic separation of nonregiopure standards or natural samples could be attempted to establish fragment ion ratios of various TAG regioisomers. While such a chromatographic method would likely be time-consuming and impractical for routine analysis, it can be a useful and affordable method for obtaining calibration data for establishing an extensive fragmentation library.

Accurate analysis of TAG regioisomers in complex natural samples is one of the most challenging tasks in the field of

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analytical chemistry due to the possible presence of hundreds of regioisomers, several of which can be chromatographically overlapping isobaric TAG species that influence the structurally informative fragment ion spectra of each other. In this study, *TAG Analyzer* software was developed and validated using the calibration data sets derived from regiopure TAG reference compounds containing fatty acids of a wide range of chain lengths of C4−C20 and number of double bonds of 1−3. Further, the UHPLC-MS/MS method and the *TAG Analyzer* were applied for comprehensive analysis of TAG regioisomers of bovine milk, a highly complex sample with a wide range of FAs and many chromatographically overlapping isobaric TAG species. The results demonstrate that *TAG Analyzer* software is a powerful tool for analyzing TAG regioisomers in complex mixtures of natural fats and oils. This further opens possibilities for comprehensive characterization of TAG regioisomers in samples of various origins, as long as the calibration data of the fragmentation model is sufficient for the ACN:DB range of interest. Using methodologies such as the *TAG Analyzer*, the nutritional and biological significance of various TAG regioisomers can now be better understood.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c08536.

List of TAG reference standards, additional calibration curves, molecular species distribution of bovine milk TAGs, and comparisons of results with the literature (PDF)

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Notes

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