Optimized Identification of Triacylglycerols in Milk by HPLC-HRMS

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Abstract
This work has developed an optimized workflow for the targeted analysis of triacylglycerols (TAGs) in milk by liquid chromatography coupled with a Q-Exactive Orbitrap mass spectrometer. First, the effects of resolution (17,500; 35,000; 70,000; 140,000) and automatic gain control target (AGC, from 2×10⁴, 2×10⁵, 1×10⁶, and 3×10⁶) have been optimized with the goal to minimize the injection time, maximize the number of scans, and minimize the mass error. Then, the flow rate of the liquid chromatography system was also optimized by maximizing the number of theoretical plates. The resulting optimized parameters consisted of a flow rate of 200 μL/min, mass resolution of 35,000, and AGC target of 2×10⁵. Such optimal conditions were applied for targeted TAG analysis of milk fat extracts. Up to 14 target triglycerides in milk fat were identified performing a data-dependent HPLC-HRMS-MS² experiment (t-SIM-ddMS²). The findings reported here can be helpful for MS-based lipidomic workflows and targeted milk lipid analysis.

Keywords LC-MS · Milk · Lipidomics · Triacylglycerols · Q-Exactive Orbitrap

Introduction
Triacylglycerols (TAGs) are the principal fats in milk. They are composed of three fatty acids (FA) esterified with glycerol. TAGs in cow’s milk are synthesized mainly from just 16 fatty acids, whose combination may generate up to 16³ = 4096 possible distinct triglycerides. Among these, 3454 molecular species of TAGs were recently detected (Liu et al. 2020). The great variability in the potential composition of triglycerides may constitute a new and unexplored chemical alphabet that may be able to detect and reconstruct the processing history of milk, such as the type of breeding of cows, their diet, the stage of lactation, and the season from which the milk is derived (Huppertz and Kelly 2009). Although it is widely recognized that the TAG profile determines the physicochemical properties of milk fat (Dimick et al. 1996; Narine and Marangoni, 1999; Smiddy et al. 2012; Tzompapa-Sosa et al. 2016), only very few studies on lipid composition of milk focused on TAG composition (Liu et al., 2017a, b).

One reason could be that the determination of TAGs in milk is still complicated nowadays (Indelicato et al. 2017). The analysis is generally based on chromatographic systems coupled with flame ionization (Gutiérrez et al. 2009), or mass spectrometry (MS) detectors (Fontecha et al. 2000). However, the analysis is complicated by the long sample preparation required to quantitatively extract TAGs, including transesterification for gas chromatography, the fast deterioration of the column performance (Lisa et al. 2011, Becacaria et al. 2014, Kadivar et al. 2013), and the low recovery of certain TAGs during pre-processing and analysis (Aparicio and Aparicio-Ruiz 2000). A few attempts have also investigated the possibility to detect TAGs by liquid chromatography (LC) coupled with detectors like ultraviolet (UV), evaporative light scattering (ELS), and refractive index detector (RID). However, such detectors have demonstrated low sensitivity toward TAGs (Carvalho et al. 2012).

Instead, a recent and promising approach for the analyses of TAGs in milk consists of the use of high-resolution mass spectrometry (HRMS) (Moulard et al. 2011), which has become the state-of-the-art technique for lipidomic studies (da Silva et al. 2021). HRMS can identify the fatty acid residues of each TAG and benefits from the recent advances in instrument sensitivity, mass resolution, and scanning speed (Kaufmann 2020). These advances lead to unprecedented high-accuracy mass measurements with exact mass resolving power (Makarov et al. 2006). Several attempts were made in recent years to improve the acquisition coverage.
of HPLC-HRMS–based metabolomics, such as data-set-dependent acquisition (Broeckling et al. 2018), nano-LC/nano electrospray MS (Danne-Rasche et al. 2018), and Data-driven Optimization of MS (Huffman et al. 2019) to name a few examples. Furthermore, HRMS detectors, like the Q-Exactive Orbitrap, offer numerous target or non-target workflows, which have found increased applications in milk lipidomics (Liu et al. 2017).

The working principle of the Q-Exactive Orbitrap is shown in Fig. 1. In details, analytes are firstly separated by LC, then ionized at the source of the Q Exactive Orbitrap. Ions are forced to pass through the S-lens, which filters non-charged compounds and impurities. Furthermore, specific ions of interest up to a wide range of ions are filtered by the quadrupole and, finally, transferred to the C-trap. Here, ions are collected and sent to the Orbitrap mass analyzer, where the mass-to-charge ratios (m/z) of the entering ions are accurately determined at high mass resolution (Zubarev and Makarov 2013).

Among the most important parameters that must be optimized, is the automatic gain control (AGC) target. This parameter sets the maximum number of ions that can be accumulated in the C-trap before they are transferred into the Orbitrap mass analyzer (Kalli et al., 2013). A further parameter is the maximum injection time (max IT). This sets the time limit after which the collected ions in the C-trap are transferred into the mass analyzer, even when the AGC target is not reached (Kalli et al., 2013). Finally, the third parameter to optimize is the ability to distinguish two peaks of slightly different mass-to-charge ratios in a mass spectrum (Zubarev and Makarov 2013). Overall, mass resolution, AGC target, and max IT greatly affect the resulting scan rate (i.e., number of spectra acquired per time unit) and resolving power of the instrument (Nie et al. 2016). Accordingly, their optimization is pivotal to achieve the best analytical performance. Only few studies attempted the systematic optimization of LC-MS acquisition parameters for lipidomics (Hutchins et al. 2019).

Accordingly, this study aims to optimize the ion detection of TAGs in milk samples. TAGs were chosen, as they represent the most abundant lipid species in milk. The proposed workflow aims to optimize ion acquisition and peak detection during MS analysis of milk lipids. The analytical standard trimargarin was used as model compound to optimize LC and MS parameters. Finally, the workflow was successfully applied for identifying 14 TAGs in milk fat samples.

**Materials and Methods**

**Chemicals and Reagents**

Trimargarin, LC-MS-grade formic acid and ammonium formate were purchased from Sigma Aldrich (Steinheim, Germany). LC-MS-grade methanol and acetonitrile were purchased from Honeywell (Selze, Germany),
and LC-MS-grade 2-propanol and methyl tert-butyl ether (MTBE) were purchased from Merck KGaA (Darmstadt, Germany) and not purified further. For HPLC methods, ultrasonicated Milli-Q water was employed.

### Standard Preparation

The trimargarin triglyceride TG 51:0 (TG(17:0/17:0/17:0)) was used for method optimization. First, 6.52 mg of standard were accurately weighed, transferred to a 20-mL volumetric flask, and brought to volume with 2-propanol to prepare the stock solution for method validation. The stock solution was diluted with methanol/2-propanol (50/50 \(v/v\)) to a final concentration of 0.48 \(\mu M\) to be used as working solution and filtered with a 0.45-\(\mu\)m syringe filter to prevent eventual clogging of the instrumentation. Dilutions in the range from 0.095 to 0.575 \(\mu M\) were prepared from the stock solution for the calibration curve and injected in triplicate.

### Samples

The samples consisted of whole raw milk provided by the Milk Federation of South Tyrol (Sennereiverband Südtirol). Fresh and unpasteurized bulk milk samples were collected from mountain dairy farms located in the north of Italy (South Tyrol, Italy). The samples were stored at \(-80^\circ C\) until analysis.

### Sample Preparation

The milk samples were thawed at 8°C overnight. The samples were pooled, carefully mixed, and six aliquots were taken. Fat extraction from the aliquots was carried out according to Breitkopf et al. (2017) based on the extraction method by Matyash et al. (2008) with modifications. In short, 200 \(\mu L\) of milk was mixed with 1.5 \(\mu L\) methanol and vortexed for 1 min. Then, 5 \(mL\) of MTBE was added and shaken at 200 rpm for 1 h at room temperature. Then, 1.2 \(mL\) of water was added and vortexed for 1 min. The mixture was centrifuged for 10 min at 1000\(\times\)g at room temperature. The upper phase was collected and the bottom phase re-extracted with 2 volume parts of MTBE/methanol/water (10:3:2.5, \(v/v/v\)). The combined upper phases were dried under nitrogen flow at room temperature (MultiVap 8; LabTech S.r.l., Milano, Italy). The dried extracts were dissolved in 5 \(mL\) methanol/2-propanol (50:50, \(v/v\)) and diluted 1:100 with the same solvent mix. Prior to injection, the sample was filtered with a 0.45-\(\mu\)m PTFE syringe filter. When needed, samples were also spiked with 0.48 \(\mu M\) of trimargarin to evaluate the MS performance during measurement.

### Optimization of the MS Parameters in Flow Injection Mode

The system consisted of an Ultimate 3000 UHPLC instrument with UV–Vis detector coupled with a Q-Exactive hybrid quadrupole Orbitrap high-resolution mass spectrometer (HRMS) with heated electrospray ionization (HESI) ion source (Thermo Fisher Scientific, Waltham, MA, USA). The same Q-Exactive Orbitrap HRMS without UHPLC was used for the optimization of the MS parameters.

For flow injection analysis, the Ultimate 3000 UHPLC instrument was coupled to the Q-Exactive Orbitrap HRMS instrument via a Rheodyne switch valve with 5 \(\mu L\) sample loop and syringe injector. The UHPLC system delivered a continuous flow of 200 \(\mu L/\text{min}\) consisting of a mixture of solvent A (acetonitrile/water 60:40 \(v/v\) with 0.1% formic acid and 10 mM ammonium formate) and solvent B (2-propanol/ acetonitrile 90:10 \(v/v\) with 0.1% formic acid and 10 mM ammonium formate) at isocratic conditions with 83% solvent B. The HESI probe was set as follows: sheath gas flow at 40 (arbitrary units), auxiliary gas flow at 10 (arbitrary units), spray voltage in positive ionization mode at 4.00 kV, capillary temperature at 300°C, S-lens RF level at 50%, and aux gas temperature at 100°C. To determine the ideal MS parameters, the mass resolutions \(R = 17,500; 35,000; 70,000;\) and 140,000 and the automatic gain control (AGC) target values of \(2 \times 10^4, 2 \times 10^5, 1 \times 10^5,\) and \(3 \times 10^6\), with a fixed maximum injection time (IT) of 300 ms, were tested. Acquisition was performed in selected ion monitoring (SIM) mode.

### Milk Lipid Separation Using High-Performance Liquid Chromatography Coupled to High-Resolution Mass Spectrometry (HPLC-HRMS)

The chromatographic separation was performed following a modified method by Breitkopf et al. (2017) and optimized for TAG elution. In brief, the stationary phase consisted of a C18 column (Accucore RP-MS, 100 mm \(\times\) 2.1 mm i.d., 2.6 \(\mu m\) particle size; Thermo Fisher Scientific, Waltham, MA, USA) with a security guard cartridge system (Thermo Fisher Scientific). The mobile phase consisted of a combination of solvent A (acetonitrile/water, 60:40 \(v/v\) with 0.1% formic acid and 10 mM ammonium formate) and solvent B (2-propanol/acetonitrile, 90:10, \(v/v\) with 0.1% formic acid and 10 mM ammonium formate). The gradient at a flow rate of 200 \(\mu L/\text{min}\) was set as follows: 70% B \((v/v)\) for 2 min, then from 70% B to 83% B at 3 min, hold until 8 min then to 84% B at 13 min and hold until 14 min. Sample injection volume was 5 \(\mu L\) using an autosampler with a 20-\(\mu L\) injection loop. After each sample, a wash step with a blank (2-propanol) was introduced with the same chromatographic set-up as before but with a different gradient: from 84% B at 0 min to...
97% B at 2 min, hold 97% until 7 min, from 97% at 7 min to 70% B at 8 min followed by a re-equilibration step (70% B) from 8 to 10 min. Blank injection volume was 20 μL. During the wash and re-equilibration step, the flow from the HPLC was diverted to waste using a Rhodyne switch valve, while a flow of 3 μL/min 2-propanol was delivered to the MS using an infusion syringe pump (Thermo Fisher Scientific) to avoid clogging and minimize carry-over effects.

For the MS parameters, the same HESI probe settings were used as described above. Three different MS experiments were used to acquire the standard and sample spectra using the optimized conditions. Full MS experiments were performed in a scan range from 150 to 1500 m/z with a resolution of 35,000 (at m/z 200), an AGC target of 2×10⁵, and a maximum IT of 200 ms. Targeted SIM (t-SIM) experiments were performed with a resolution of 35,000, AGC target of 2×10⁵, max IT of 125 ms, and an isolation window of 4 m/z. Finally, t-SIM–data dependent (dd) MS² experiments were performed with a resolution of 35,000, an AGC target of 2×10⁵, and a maximum IT of 125 ms for the MS1 acquisitions, and a resolution of 17,500, an AGC target of 1×10⁵, a maximum IT of 50 ms, a loop count of 5, an isolation window of 4.0 m/z, and a normalized stepped collision energy of 15, 30, and 60 eV, for the MS² acquisitions. For both the t-SIM and t-SIM-ddMS² experiments, an inclusion list containing the m/z of the targeted molecules to acquire and fragment was used.

Data Processing and Statistical Analysis

The analytical standard was injected in triplicate for each HPLC-HRMS condition and six times for the sample extracts. Data acquisition was performed with tune and Xcalibur software (Thermo Fisher Scientific). Correlation of chemical compounds relative abundances and integration of the area under each peak (HPLC-HRMS XIC integrations) was done using Xcalibur Quan Browser software (Thermo Fisher Scientific) and the LIPIDMAPS lipidomic database. The limit of detection and quantification (LOD and LOQ), as well as the precision and sensitivity, the latter being the slope of the regression line, were calculated for the trimargarin standard in solution from the regression line after calibration in agreement with Miller and Miller (2018). The obtained LOD and LOQ were theoretically calculated and not experimentally measured. Intraday repeatability was measured as relative standard deviation (RSD %) for the integrated areas of the major base peaks in the six replicates of the extracts. Mass error was calculated by dividing the difference of the actual mass and the theoretical mass by the theoretical mass and expressing in Δppm. The analysis of variance was conducted using XLSTAT annual version 2021.1.1 1092 (Addinsoft 2021, New York, NY, USA).

Results and Discussion

Optimization of HRMS Parameters by Flow Injection Analysis of Trimargarin

Standard solutions of trimargarin were analyzed by a flow injection system (200 μL/min) coupled with a Q-Exactive Orbitrap analyzer, working in positive selected ion monitoring (SIM) mode. A standard of trimargarin was selected as TAG standard for lipidomics. The ammonium adduct [M+NH₄]⁺ of trimargarin with a theoretical m/z of 866.8171 was determined. The injection of 0.48 μM of trimargarin (sample injection of 5 μL) resulted in a peak height of 2.24 ± 0.34 × 10⁶ counts and peak width of 7.76 ± 0.77 s.

Optimization of the MS parameters was next obtained by testing four different mass resolutions R (17,500; 35,000; 70,000; and 140,000 at m/z 200) and four automatic gain control (AGC) values (2×10⁴, 2×10⁵, 1×10⁶, and 3×10⁶). Accordingly, 16 independent experiments were designed, each tested in triplicate (n = 48). For each experimental condition, three parameters were determined: the injection time (IT) needed to acquire one spectrum, the number of scans per peak, and the mass accuracy (i.e., expressed as mass error, Δppm). In all experiments, a maximal injection time of 300 ms was set. This means that when the time to reach the desired AGC target value exceeds such threshold, a mass spectrum is still generated.

The results were examined by a two-way ANOVA. Both mass resolution and AGC target values show a significant effect on the resulting injection time (Fig. 2a), the number of scans per peak width (Fig. 2b), and the mass error (Fig. 2c) (Tukey HSD, p < 0.0001).

In details, Fig. 2a shows the effect of the mass resolution and AGC target values on the resulting number of acquired scans. Resolution was the most important factor (explaining 70% of the total variance), followed by AGC target (22%) and the combination of the two (7%). The interaction between resolution and AGC target was significant (F(1,2), = 170, p < 0.0001). At low resolutions (i.e., 17,500), the selection of the AGC target values was very important for assuring the highest number of scans, which was achieved at the lowest AGC values. Instead, at higher resolutions, the choice of the AGC target value was less important and negligible at 140,000. Similar conclusions were observed in proteomics (Michalski et al. 2011). This result is expected since the AGC target value controls the ion population which is accumulated in the C-trap before being injected into the Orbitrap mass analyzer. So, in general, a higher AGC target value is desired because it increases the ion population. However, at very high resolutions, the number of scans becomes small regardless of the AGC target value chosen.
Similarly, Fig. 2b shows the effect of the mass resolution and AGC target values on the injection time. Again, the main factors that affect the injection time are the mass resolution (explaining 57% of the total variance), followed by the AGC target value (43%) and the combination of the two (9%). The interaction between resolution and AGC target was significant ($F(1,2) = 2081, p < 0.0001$). At lower resolutions (i.e., 17,500), lower AGC target values led to fast injection times. Kalli et al. (2011) also observed higher injection times with the increase of AGC target for protein detection with the LQT-Orbitrap. Trap filling time was also described by da Silva et al. (2021) to be critical for sensitivity and saturation of lipidomic coverage. Instead, at higher resolutions, the effect of the AGC target was less significant. Ultimately, the AGC target value had no effect on the injection time at a resolution of 140,000. This result is expected as higher resolutions generally requires longer times of acquisition, which limit the number of data points that can be collected across a peak.

Finally, Fig. 2c shows the effect of the mass resolution and AGC target values on the mass accuracy. Here, the main factor was the mass resolution (explaining 75% of the total variance), followed by the combination of AGC target and resolution (14%) and the AGC target (10%). As for the other variables, the interaction between resolution and AGC target was significant ($F(1,2) = 12, p < 0.0001$). As expected, the highest mass error was observed at the lowest resolution (i.e., 17,500). Conversely, increasing the resolutions, the mass accuracy greatly improved. However, it should be noted that the best mass accuracy could be achieved only with the lowest AGC target value. This result can be explained considering that high AGC target values promote longer injection times and the collection of large ion populations. This may lead to charge effects, which affect the ion distribution inside the Orbitrap and, ultimately, the mass accuracy (Makarov et al. 2006).

Optimal mass analyzer parameters were obtained by selecting those conditions that maximized the number of scans per peak width, minimized the mass error, and guaranteed best exploitation of the injection time (Huang et al., 2021). A desirability function was developed (Candioti et al. 2014) and the best conditions were a resolution $R$ of 35,000, with an AGC target value of $2 \times 10^5$. These conditions led to the highest possible number of scans per peak width ($4.58$ scans/s) with the smallest mass error (0.16 ppm) and in the shortest injection time. The second-best performance was achieved with a resolution $R = 70,000$ and with an AGC target value of $2 \times 10^4$, which led to 2.71 scans/s (number of scans per peak width) and a mass error of 0.162 ppm. Instead, the resolution of 140,000 led to the lowest number of scans, likely because of longer scan cycle times (Kalli and Hess 2012). Fewer data points across chromatographic peaks were also observed by Carlsson et al. (2022) when working with polarity switching which increases the cycle time of HRMS measurements. This underlines the importance to adjust scan cycle times depending on the resolution and application needed. In the case of targeted TAG acquisition, at 140,000 resolution, the time for collecting ions exceeded the maximum injection time allowed. This, in practice, resulted in insufficient scans per peak width for all the AGC target values. Conversely, the resolution at 17,500 led to a sufficient saturation of the scan cycle but with insufficient mass resolution.

Accordingly, all the next experiments were based on two optimal sets of experimental conditions, respectively, with $R = 35,000$ with AGC target $= 2 \times 10^5$ and $R = 70,000$ with AGC target $= 2 \times 10^4$.  

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**Fig. 2** Interaction plots of the mass resolution and AGC target (solid black circles = $2 \times 10^4$, empty black circles = $2 \times 10^5$, gray triangles = $1 \times 10^6$, gray squares = $3 \times 10^6$) and their influence on **a** scans per peak width, **b** injection time, and **c** mass error.
In-Column HPLC Flow Rate Optimization

The next step was the optimization of the chromatographic separation. The use of a column may limit the speed and depth of MS data acquisition (Huang et al. 2021), and may be critical to the MS efficiency (Shishkova et al. 2016). Therefore, two MS conditions were used, respectively, A: mass resolution \( R = 70,000 \) with AGC target value of \( 2 \times 10^4 \), and B: mass resolution \( R = 35,000 \) with an AGC target value of \( 2 \times 10^5 \). These two optimized conditions were used to study the effect of flow rate (150, 200, 250, and 300 μL/min) on the resulting peak height, peak width, number of theoretical plates, and number of scans for the analysis of trimargarin.

Based on the results in Table 1, the flow rate greatly affected the retention time, the peak width, the number of theoretical plates, and the number of scans \( (p < 0.0001) \). Low flow rates led to lower peak heights, but very high number of scans \( (p < 0.0001) \). This can be easily explained considering that a lower flow rate allows a prolonged scan cycle time (Kalli and Hess 2012).

Similar conclusions could be derived at higher resolution (i.e., 70,000), although it should be noted that, when the flow rate is too high (300 μL/min) or too low (i.e., 150 μL/min), the number of ions collected is not sufficient to achieve the best number of scans. Instead, optimal performance was achieved at 200 μL/min, with a significant difference from the other flow rates (Tukey HSD, \( p < 0.0001 \)).

HPLC-HRMS Method Validation

Trimargarin standards were injected in the HPLC system at a concentration range from 0.191 to 0.575 μM. For each concentration, an extracted ion chromatogram (XIC) was generated (Fig. 3). The peak area versus concentration was plotted in Fig. 3 (inset). With a resolution of 35,000, the calibration curve gave a \( R^2 \) of 0.996 ± 0.006, limit of detection (LOD) of 0.06 ± 0.02 μM, sensitivity of 6.67 ± 0.31 × 10^7, and precision of 2% RSD (Table 2). Instead, with a resolution of 70,000, the quantitative analytical performance worsened, showing higher LOD (0.08 ± 0.03 μM), lower sensitivity (5.22 ± 0.17 × 10^7), and lower precision (4% RSD). This result can be explained considering that higher mass resolutions led to slower scan rates and the collection of less data points (Huang et al. 2021). Accordingly, the resolution of 35,000 was chosen for further experiments.

Table 1  The effect of the flow rate, resolution, and AGC on post-column detection of trimargarin (0.48 μM, injection volume = 5 μL) by HPLC-HRMS

| R/AGC target value | Flow rate (μL/min) | Retention time (min) | Peak width (s) | Peak height (×10^6 counts) | Number of theoretical plates (×10^3) | Number of scans per peak |
|-------------------|-----------------|-------------------|-------------|--------------------------|-----------------------------------|------------------------|
| 35,000/2×10^5     | 300             | 9.59 ± 0.01^a     | 18.0 ± 0.1^a | 2.84 ± 0.13^d            | 203^c                            | 125 ± 5^e               |
|                   | 250             | 10.75 ± 0.02^b    | 21.8 ± 0.7^e | 2.81 ± 0.10^d            | 236^d                            | 151 ± 5^d               |
|                   | 200             | 12.44 ± 0.01^c    | 23.6 ± 0.7^d | 2.61 ± 0.04^e,d          | 207^c,d                          | 163 ± 3^e               |
|                   | 150             | n.a.              | n.a.        | n.a.                     | n.a.                             | n.a.                   |
| 70,000/2×10^4     | 300             | 9.58 ± 0.01^a     | 17.2 ± 0.7^a | 2.01 ± 0.17^a            | 185^b                            | 66 ± 2^a                |
|                   | 250             | 10.74 ± 0.01^b    | 18.8 ± 0.4^b | 2.25 ± 0.16^a,b          | 176^b                            | 68 ± 4^e                |
|                   | 200             | 12.44 ± 0.01^c    | 24.2 ± 0.4^d | 2.45 ± 0.10^b,c          | 218^d,e                          | 91 ± 2^b                |
|                   | 150             | 15.39 ± 0.07^d    | 24.3 ± 1.3^d | 2.07 ± 0.04^a            | 143^a                            | 66 ± 3^a                |

For the parameters, a–e = significantly different between groups by Tukey (HSD). n.a. = not available.
Application of the Optimized Method for Analysis of Milk Fat Extracts

Next, the HPLC-HRMS method with optimized acquisition was applied for the analysis of milk fat extracts. Six aliquots of milk were extracted and analyzed by HPLC-HRMS. Preliminarily, a first MS acquisition was performed in full scan (full-MS) in the range from 150 to 1,500 m/z. This first mode was useful to determine the milk fat profile and choose the ions for SIM-mode acquisition. Figure 4 shows the resulting total ion current (TIC) chromatogram in positive ion mode. Fourteen cluster peaks were observed for the milk lipid profile with the m/z of the base peak of each cluster. The m/z of the most intense ion (base peak) was next used into an inclusion list for targeted SIM (t-SIM) acquisition.

Thereafter, a second acquisition was performed in SIM mode and Fig. 5 shows the resulting XICs. For each peak, 25–60 scans were recorded. For each peak, intraday repeatability of the area under the peak of six replicates of milk fat was always below 5%.

While characterization of isomer TAG species can be achieved with minimal chromatographic separation, if any (Xu et al. 2018; Wang et al. 2016), accurate detection of target lipids from milk can greatly benefit from improved acquisition parameters. Nevertheless, optimization of acquisition parameters for lipidomic studies has found only limited application, which concern especially untargeted analyses (da Silva et al. 2021). Most lipidomics studies on milk are reporting the profiling of the global milk lipidome or that of single lipid classes (Damário et al. 2015; Donato et al. 2011; Li et al. 2017; Liu et al. 2015; Ma et al. 2017; Tzompa-Sosa et al. 2018). Compared to these studies, this work describes the development of a method for optimization of MS parameters aimed to improve the detection and identification of single target lipids in milk.

Identification of Compounds via t‑SIM‑ddMS²

Identification of the 14 groups of target triglycerides in the milk fat extract was carried out performing a data-dependent HPLC-HRMS-MS² experiment (t-SIM-ddMS²). The resulting fragmentation spectra were used to characterize the target molecules. For each molecular ion, a chemical formula of the neutral mass was predicted (Table 3). TAG species were identified using the LIPIDMAPS lipidomics database, which classified TAGs based on the total number of carbons of the fatty acid residues (CN, TG x:–) and the total number of double bonds in the fatty acid residues (DB, TG –:y). For each group of classified TAG molecular species, several fatty acid residues were identified. The combination of those FA resulted in several possible TAGs within one cluster. The final tentative identification was obtained by comparing the resulting fragmentation spectra of each peak with the theoretical spectra generated in LIPIDMAPS (Table 3).

MS response of TAG molecules is knowingly very heterogeneous across species (Holcapek et al., 2005; Han and Gross, 2001). Choosing the correct MS parameters is therefore pivotal for efficient target acquisition. Most studies characterize the milk lipidome without considering the MS performance, which is not much explored. Accordingly, most studies perform lipid profiling rather than single lipid determination (Foroutan et al. 2019; Gresti et al. 1993; Liu

![Fig. 4](image-url) Total ion chromatogram acquired in full MS showing the lipid profile of a milk fat extract obtained by HPLC-HRMS in the ESI+ ionization mode. The m/z of the base peaks are displayed above each cluster peak
One of the possible reasons for this is a limited amount of TAG standards available on the market. There are not enough TAG standards available to cover all TAG groups, making the MS optimization laborious. Our study is showing how the use of even one analytical standard (trimargarin) allows to test the influence of MS parameters on the resulting acquisition of TAGs and develop a method to analyze target TAGs in real milk samples.

**Conclusions**

In this study, targeted milk TAG analysis was performed with an optimized HPLC-HRMS method. Flow rate, mass resolution, and AGC target were optimized on a Q-Exactive Orbitrap HRMS to exploit its full functionalities. Although the current study is not proposing a thorough characterization of all TAG species present in bovine milk, it provided a solution to optimize the ion acquisition and target identification of TAG groups. Accordingly, a flow rate of 200 μL/min, mass resolution of 35,000, and an AGC target value of $2 \times 10^5$ yielded the best data point acquisition, peak generation, and peak area reproducibility for a trimargarin standard and was applied to a real milk fat extract. With the optimized method, a robust analytical performance was achieved. The number of scans for each peak could be maximized for best collection of data points and good peak area reproducibility. The here presented workflow constitutes a valuable addition for lipidomic research. For future studies, this work provides a tool for the optimized acquisition of biomarkers from milk lipids using HRMS and can find application for further food matrices and research fields.
| m/z [M+NH₄]+  | Mass error Δppm | Predicted chemical formula | Classification (CN:DB*) | Fragments | Tentatively identified fatty acid moieties |
|---------------|-----------------|-----------------------------|--------------------------|-----------|------------------------------------------|
| 516.4268      | 1.8             | C29H54O6 TG 26:0            |                          | 579.5222; 411.3401; 383.3616; 341.0173; 221.0840; 145.0856; 71.0856 | Butyric (4:0); caproic (6:0); caprylic (8:0); capric (10:0); lauric (12:0); palmitic (16:0) |
| 544.4575      | 0.6             | C31H58O6 TG 28:0            |                          | 439.3798; 411.3487; 383.3177; 355.2858; 327.2544; 299.2299; 271.1913; 211.2063; 183.1747; 155.1434; 137.1333; 127.1123; 109.1016; 99.0808; 81.0701; 71.0494 | Butyric (4:0); caproic (6:0); caprylic (8:0); capric (10:0); lauric (12:0); myristic (14:0) |
| 572.4886      | 0.2             | C33H62O6 TG 30:0            |                          | 467.4111; 439.3808; 411.3471; 383.3164; 355.2852; 327.2541; 299.2226; 271.1901; 239.2386; 211.2061; 183.1749; 155.1437; 137.1331; 127.1119; 109.1016; 99.0806; 81.0702; 71.0494 | Butyric (4:0); caproic (6:0); caprylic (8:0); capric (10:0); lauric (12:0); myristic (14:0); palmitic (16:0); stearic (18:0) |
| 600.5193      | 0.8             | C35H66O6 TG 32:0            |                          | 495.4422; 467.4152; 411.3465; 383.317; 355.2852; 327.2538; 299.2226 | Butyric (4:0); caproic (6:0); caprylic (8:0); capric (10:0); lauric (12:0); myristic (14:0); palmitic (16:0); stearic (18:0) |
| 628.5504      | 1.0             | C37H70O6 TG 34:0            |                          | 355.2854; 383.3171; 523.4732; 495.4388; 411.3452; 383.3171 | Butyric (4:0); caproic (6:0); capric (10:0); lauric (12:0); palmitic (16:0); stearic (18:0) |
| 656.5818      | 0.8             | C39H74O6 TG 36:0            |                          | 551.5046; 523.472; 411.348; 383.3169; 355.2857; 239.2375 | Butyric (4:0); caproic (6:0); capric (10:0); lauric (12:0); palmitic (16:0); stearic (18:0) |
| 684.6130      | 1.0             | C41H78O6 TG 38:0            |                          | 579.5359; 411.3483; 383.3171; 239.2373; 71.0858 | Butyric (4:0); caproic (6:0); myristic (14:0); palmitic (16:0); stearic (18:0) |
| 712.6447      | 0.4             | C43H82O6 TG 40:0            |                          | 607.5632; 597.5341; 439.3798; 411.3482; 267.2628; 239.2376; 155.1431; 137.1329; 127.1123; 99.0805; 81.0701; 71.0493; 53.039 | Butyric (4:0); caproic (6:0); myristic (14:0); palmitic (16:0); stearic (18:0) |
| 890.7291      | 0.9             | C57H92O6 TG 53:2            |                          | 603.5335; 591.5377; 339.2892; 265.2524; 247.2418 | Margaric (17:0); oleic (18:1) |
| 794.7225      | 0.9             | C49H92O6 TG 46:1            |                          | 605.551453; 577.5211; 551.503052; 549.4897; 523.472656; 521.535339; 495.4422; 493.432587; 265.25625; 239.237991; 211.206589; 183.17363; 165.166016; 155.143402; 137.132767 | Capric (10:0); lauric (12:0); myristic (14:0); palmitoleic (16:1); stearic (18:0); oleic (18:1) |
| 896.7690      | 1.3             | C57H98O6 TG 54:6            |                          | 599.5044; 263.2371 | Linoleic (18:2) |
| 898.7850      | 0.9             | C57H100O6 TG 54:5           |                          | 601.5203; 599.5050; 339.2891; 265.2524; 263.2378; 247.2417; 245.22653 | Oleic (18:1); linoleic (18:2) |
| 900.8007      | 0.9             | C57H102O6 TG 54:4           |                          | 603.535461; 601.520203; 339.290131; 337.275146; 265.252689; 263.237549; 247.24188; 245.22653 | Oleic (18:1); linoleic (18:2) |
| 902.8167      | 0.4             | C57H104O6 TG 54:3           |                          | 603.5361; 339.2901; 265.2536 | Oleic (18:1) |

*CN:DB = carbon number-total double bond number of the 3 FA
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Declarations

Ethical Approval This article does not contain any studies with human or animal subjects.

Conflict of Interest The authors declare no competing interests.

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