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Ion Mobility–Mass Spectrometry Approach for the Comparison of Sheep and Goat Milk Lipidomes

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Abstract: In this work, we report an analytical procedure to investigate the lipid compositions of sheep and goat milk. This approach is based on an ion mobility–high-resolution mass spectrometric method to facilitate the identification of complex lipid species and their regiochemistry. A common triacylglycerol profile was observed for sheep and goat milk samples, while a higher abundance of medium-chain fatty acids was observed at the sn-2 position for sheep milk. Furthermore, differences can be also observed in the levels of saturated and unsaturated fatty acids at the sn-2 position. In terms of lipid classes, goat milk showed higher levels of triacylglycerols, phosphatidylinositols and ether-linked phosphatidylethanolamines, while sheep milk showed higher levels of free fatty acids, lysophosphatidylethanolamines, lysophosphocholines and non-hydroxy fatty acid-dihydrosphingosine ceramides when compared with goat milk.

Keywords: lipidomics; triacylglycerols; lipid regiochemistry; IM-QTOF-MS analysis

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1. Introduction

Sheep milk is gaining worldwide interest for its nutritional and functional characteristics. The Italian sheep dairy industry accounts for 6.31 million head of ewes, mainly located in Sardinia (21.8%) and characterized by the breeding of razza sarda animals [1]. Sheep and goats are managed in semi-extensive systems, in which extensive use consists of artificial or natural pastures representing the first source of nutrients and constituting roughly 80% of the dry matter ingested annually by the animals. The latter factor influences the acid profile of the milk fat, which plays a fundamental role in defining the technological and nutritional characteristics of milk. In particular, sheep milk is characterized by a higher lipid content (6–7%) when compared to goat milk (4–5%) [2]. The lipid profile of a small ruminant's milk consists of triglycerides (TAGs), together with diglycerides (DAGs), phospholipids, ceramides, cholesterol, squalene, lanosterol and free fatty acids [3]. Milk fatty acids, representing 95% of the triglyceride structure, originate from the diet, both from rumen microbial activity, after which they are transferred to secretory cells via the blood and lymph, and from de novo synthesis in the endoplasmic reticulum of secretory cells. The milk lipid fraction is organized into fat globules with cores of neutral lipids (TAGs and cholesterol) and membranes with three or more layers. The milk fat globule membrane (MFGM) produced by secretory cells in the mammary gland consists of lipids, proteins and cholesterol. In addition to the major glycerophospholipids, i.e., phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs), MFGM contains sphingomyelins (SMs), glucosyl-(HexCer) lactosylceramides and gangliosides [4,5].

The complexity of the milk composition also makes its in-depth study extremely difficult. Furthermore, milk fat contains over 400 individual fatty acids and their isomers,

whose composition is influenced by various factors, such as the diet [6,7], lactation stage and animal conditions [8]. These reasons make the open profile study of the milk lipidome very difficult.

Similar to other -omics sciences, lipidomics is commonly used in profiling lipids present in cells, tissues and body fluids. Nowadays, this holistic technique represents a relatively new, stimulating and complex research area in the field of human nutrition, encompassing metabolic, nutritional, physiological, epidemiological and clinical aspects [9]. Lipidomics was recently proposed as a method for the profiling of goat milk [10,11], the digestibility evaluation of sheep and goat milk [12,13] and the identification of the geographical origin and lactation stage of goat milk [14]. Recently, Chen et al. reported a phospholipidomics approach for measuring phospholipids in sheep milk [15].

Nevertheless, despite the use of high-throughput mass spectrometers, it is difficult to elucidate important structural features of lipids, such as constitutional isomers' identification, carbon–carbon double-bond positions and the presence of acyl-chain branching. However, ion mobility coupled with time-of-flight mass spectrometry may be used to separate ions in the gas phase based on their dimensions and shapes. Mixtures of ions can be separated based on changes in mobilities through a gas phase and then analyzed by a mass spectrometer measuring mass-to-charge (m/z) ratios. The great advantage offered by this analytical system is the acquisition of a further layer of separation compared to LC alone, as well as the use of an extra physiochemical property, i.e., collision cross-section (CCS, Å), which, in combination with the retention time and m/z value, can help in the lipid identification process [16]. Over the years, ion mobility spectrometry (IMS) has been increasingly central to the field of chemistry for the analysis of food composition, including nutrient and bioactive components, as well as for ensuring food safety, preventing food fraud and optimizing technological processes [17]. An instrumental setup such as ion mobility has already been used for the study of lipids present in complex matrices such as milk. By applying liquid chromatography–ion mobility–mass spectrometry, George et al., in 2020, characterized the triacyl glyceride profile of human milk and annotated 98 new TAGs [18].

The aim of this work was to develop a mass spectrometric workflow to study and compare the comprehensive lipidomes of goat and sheep milk samples. This workflow combines an iterative MS/MS method with a drift-tube ion mobility–LC-QTOF-MS/MS analytical platform to study complex milk lipids.

2. Materials and Methods

2.1. Chemicals

Analytical LC-grade methanol, tert-Butyl Methyl Ether (MTBE), acetonitrile, 2-propanol, ammonium acetate and ammonium formate were purchased from Sigma Aldrich (Milan, Italy). Bi-distilled water was obtained with a MilliQ purification system (Millipore, Milan, Italy). A standard deuterated lipid mixture (SPLASH® LIPIDOMIX®) was purchased from Sigma Aldrich (Milan, Italy).

2.2. Milk Samples

Morning milk samples from ten mature goats and sheep were obtained by pooling the raw milk of 120 pluriparous animals for each species. Bulk-tank milk samples were collected in June; both ewes and goats had access to the same pastures. The milk samples provided by the Sardinian Regional Animal Farmers Association (Associazione Regionale Allevatori, ARA, Sardegna) within the International Committee for Animal Recording (ICAR) program were collected in sterilized tubes; bronopol (2-bromo-2-nitro-1,3-propanediol) was added, and samples were then stored at −80 °C until analysis. Each bulk-tank milk sample was analyzed as 15 single samples.

2.3. Sample Preparation and UHPLC-QTOF/MS Analysis

After thawing at room temperature, milk samples were vortex-mixed. An aliquot of 150 µL of milk was placed in an Eppendorf tube, and 525 µL of methanol and 525 µL of MTBE were added and vortexed for 1 min. The centrifugation of samples was performed at 4000 rpm for 15 min and then at 12,000 rpm for 5 min. The supernatant was then filtered through a 0.22 µm MS nylon syringe filter and then placed into autosampler vials. Next, 10 mL of each bulk-tank milk sample was mixed, obtaining a new pool for quality control samples' preparation. An aliquot of 150 µL of this new pool was extracted with the procedure described above for the milk samples. All samples (15 samples for each bulk-tank milk) and 10 QC samples thus prepared were injected in IM-QTOF-MS and analyzed in positive and negative ionization modes.

2.4. IM-QTOF-MS Analysis

Milk samples were analyzed with a 6560 drift-tube ion mobility QTOF-MS connected to a 1290 UHPLC system (Agilent Technologies, Palo Alto, CA, USA). A volume of 1.0 µL was analyzed using a Kinetex 5 µm EVO C18 100 A, 150 mm × 2.1 µm column (Agilent Technologies, Palo Alto, CA, USA) heated at 50 °C and with a flow rate of 0.4 mL/min. For the positive ionization mode, the mobile phase consisted of (A) water containing 10 mM ammonium formate (60%) and acetonitrile 40% and (B) a 10 mM ammonium formate solution containing 90% isopropanol and 10% acetonitrile. For positive ionization mode, the separation gradient was 60% A, followed by a linear decrease to 50% A in 2 min, then at 1% in 5 min for 1.9 min and then equilibration for 1 min. In the negative mode, the mobile phase used varied only for the use of 10 mM ammonium acetate.

The mass spectrometry parameters were as follows: mass range, 50–1700 m/z; gas temperature, 200 °C; nitrogen 10 L/min; nebulizer gas nitrogen, 50 psig; sheath gas temperature, 300 °C; sheath gas flow, 12 L/min; capillary voltage, 3500 V for positive and 3000 V for negative; nozzle voltage, 0 V; fragmentor, 150 V; octapole, RF 7550 V; skimmer, 65 V; capillary voltage, 3.5 kV; collision energy, 20 eV in positive and 25 eV in negative mode; mass precursor per cycle = 3; threshold for MS/MS 5000 counts. Nitrogen worked as a drift gas with a trap fill time and a trap release time of 2000 and 500 µs, respectively. An Agilent reference mass mixture was used during the chromatographic run for mass calibration. Experimental data were obtained using the auto-MS/MS method consisting of an iterative mode with a mass error tolerance of 10 ppm and a retention exclusion tolerance of 0.2 min. In the iterative auto-MS/MS method, the sample was injected several times, while precursors previously selected for MS/MS fragmentation were rejected on a rolling basis [19].

2.5. Data Analysis

The MassHunter Lipid Annotator (Agilent Technologies, Palo Alto, CA, USA) was used for MS/MS data analysis. The lipid annotator uses a process that employs probability theory and least-squares fit to annotate lipids based on an in silico MS/MS database for Q-TOF fragmentation modeled from lipid standards for every lipid class. Lipid species were annotated from MS/MS fragmentation spectra. Lipid annotation is reported as the sum composition corresponding to the total carbon atom number and double bonds (carbon index and double bond index). Default method parameters were used, with identification based on MS/MS fragmentation data. The criteria were a Q-score > 30, a mass threshold < 10 ppm, a fragment score threshold > 30, and a total score > 60, and a dominant constituent level was reported if the relative abundance differential was >10%.

3. Results

3.1. Milk Lipidome Profiles

In this work, we investigated the lipid profiles of sheep and goat milk using an IM-QTOF-MS analytical platform. For the analysis, five different iterative injections of bulk milk samples were performed to evaluate the minimum number of runs with the maximum

number of lipid species detected. Four chromatographic runs were enough to cover the maximum number of milk lipid species (Figure S1). The percent lipid class compositions for sheep and goat milk samples acquired in the ESI+/− mode are reported in Table 1.

Table 1. Percent compositions based on relative abundance of lipid classes measured in sheep and goat milk samples.

Lipid Class	% Composition ± SD	
Positive Ionization	**Sheep**	**Goat**
TAG	65.25 ± 3.26	84.30 ± 4.21
PC	14.58 ± 0.73	8.27 ± 0.41
SM	11.14 ± 0.51	3.29 ± 0.16
PE	6.75 ± 0.73	3.10 ± 0.15
PS	1.71 ± 0.08	0.89 ± 0.04
LPC	0.30 ± 0.01	n.d.
HexCer_NS	0.15 ± 0.01	0.11 ± 0.01
ACar	0.03 ± 0.01	n.d.
DAG	0.03 ± 0.01	0.05 ± 0.01
Cer_NS	0.06 ± 0.01	n.d.
Negative Ionization	**Sheep**	**Goat**
PE	29.99 ± 1.50	32.76 ± 1.64
SM	19.28 ± 0.96	19.84 ±0.99
FA	18.32 ± 0.92	1.33 ± 0.07
PC	12.86 ± 0.64	20.61 ± 1.03
PS	8.85 ± 0.44	11.95 ± 0.60
PI	4.68 ± 0.23	6.41 ± 0.32
Cer_NS	2.72 ± 0.14	n.d.
Ether PE	2.32 ± 0.12	7.10 ± 0.35
LPE	0.96 ± 0.05	n.d.

n.d.: <0.01 mg/g fat.

When compared to sheep milk, the percentage levels of TAGs (see Table 1) were higher in goat samples (84.30 ± 4.21% vs. 65.25 ± 3.26%). On the other hand, sheep milk samples showed higher levels of phosphatidylcholines (PCs) and sphingomyelins (SMs), when compared to goat milk. Notably, in the sheep milk samples, we found LysoPCs (0.30 ± 0.01%), non-hydroxy fatty acid-sphingosine ceramides (Cer_NS) (0.06 ± 0.01%) and acylcarnitines (ACar) (0.03 ± 0.01%). In the ESI mode, goat milk samples showed higher levels of phosphatidylethanolamines (PEs) (32.76 ± 1.64% vs. 29.99 ± 1.50%) (Table 1). While levels of SMs were comparable in the two groups, the milk levels of PCs, phosphatidylserines (PSs), ether-PEs and phosphatidylinositols (PIs) were found to be higher in the goat milk samples. Importantly, non-esterified fatty acid levels were more abundant in the sheep milk samples (18.32 ± 0.92%) when compared to goat milk samples (1.33 ± 0.07%). Among fatty acids annotated in sheep samples, we found palmitic acid and stearic acid to be predominant.

To enhance the confidence in the lipid annotations procedure, we decided to characterize the milk lipidomes by applying an ion mobility–mass spectrometry experiment to calculate CCS values, which, together with retention time and mass spectrometry characteristics, support the lipid annotation process. In Figure 1, we report an example of the usefulness of ion mobility spectrometry to discriminate TAG isomers.

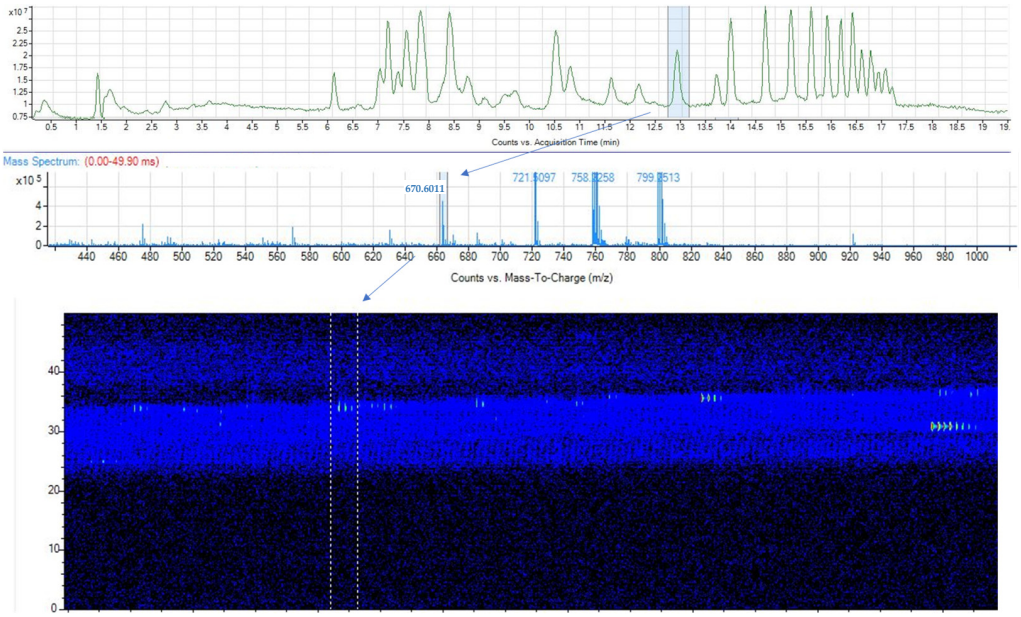

Figure 1. Ion mobility capability to discriminate isomers.

In Table S1, we report the mass spectrometry characteristics of each lipid, along with their sum composition and collision cross-section values (CCS, Å2) measured in the ESI+ mode. With this approach, we were able to annotate a total of 26 PCs, 14 PEs, 8 PSs and 17 SMs. In this table, TAGs are not reported but are discussed successively. In the ESI mode, we annotated the following lipids: free FA, PCs, PEs, PSs, PIs and SMs (Table S2). Interestingly, plasmalogens, membrane glycerophospholipids containing a fatty alcohol with a vinyl-ether bond at the sn-1 position, were also found in the form of ether-linked phosphatidylethanolamine (ether-PE). Low percentages of Cer-NS and LPE were also found only in sheep milk (Table 1).

3.2. TAG Regiochemistry and FA at sn-2 Position

Considering the nutritional importance of FA positions in the glycerol backbone of TAGs [20], we investigated and compared the regiochemistry of lipid species found in goat and sheep milk samples. With the aim to study the three isomeric forms of TAGs and the position of each FA in the glycerol backbone at sn-1, sn-2 and sn-3, we proceeded to perform an in-depth study of TAG mass spectra. An in-depth study of high-resolution MS/MS spectra of chromatographically resolved TAG molecules allows an easy understanding of the FA composition [11]. It is commonly assumed that for collision-induced dissociation-based MS/MS acquisition, compared to sn-1/sn-3 FA dissociation, the sn-2 position requires more energy [21]. Thus, DAG+ ions generated after the loss of the sn-2 FA are supposed to be of lower abundance [22]. This procedure was used to annotate the sn-2 FAs of TAGs in both milk samples in this study. The results of a structural analysis of TAGs with their mass characteristics and CCS values are reported in Table S3. For the same TAGs, we can notice the inversion of the FA position in sheep and goat milk samples, e.g., the TAG (36:1) at a retention time of 10.802 min and m/z 654.5699, where butyric acid (FA 4:0) is positioned at sn-2 in sheep milk but at sn-3/1 in goat milk samples. As theoretically expected, the different positions of butyric acid provided two different CCS values, 271.49 vs. 272.57 Å2. Furthermore, by analyzing the relative abundance of FAs at the TAG sn-2 position, we were able to report their distribution, along with their chain length (Figure 2) and saturation degree (Figure 3).

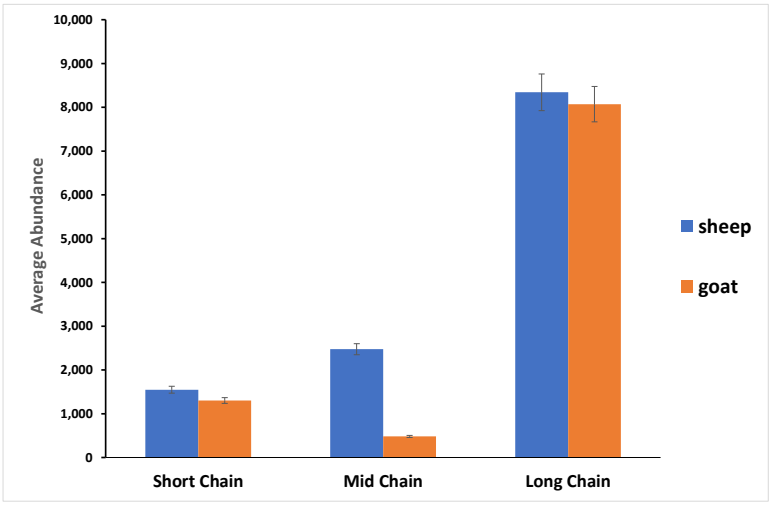

Figure 2. Average abundance values of the short, medium and long fatty acids at the sn-2 position.

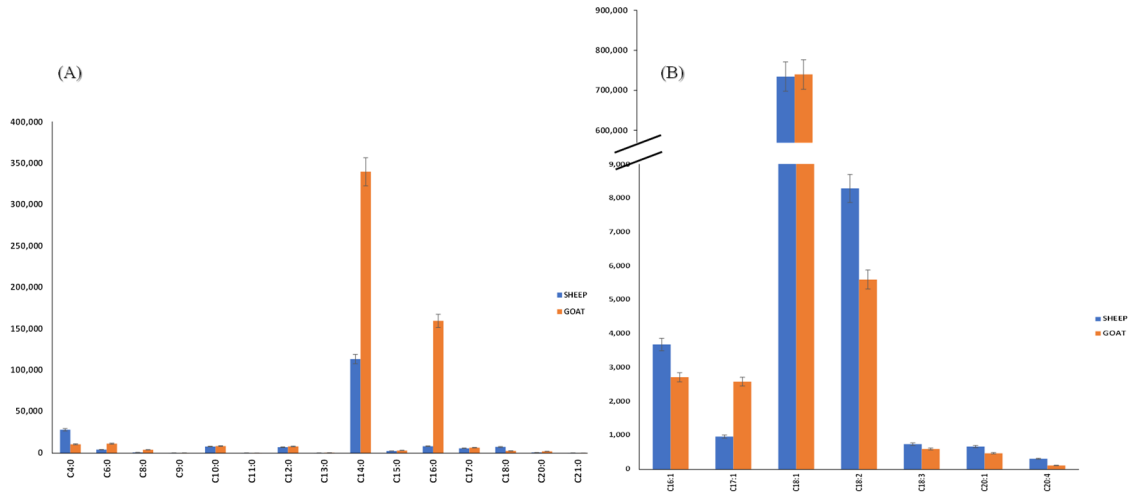

Figure 3. Triacylglycerols' relative abundance of saturated (**A**) and unsaturated (**B**) fatty acids at the sn-2 position.

Considering short-chain FAs with 4 to 8 carbon atoms, medium-chain FAs with 10 to 15 carbon atoms and long-chain FAs with 15 to 22 carbon atoms, as can be seen in Figure 2, TAGs in sheep milk are characterized by having more sn-2 FAs with a medium chain length. Overall, both sheep and goat milk samples showed higher levels of saturated FAs at sn-2 (Figure 2). However, we can highlight that goat milk is characterized by more saturated fatty acids, while sheep milk contains higher levels of butyric acid (C4:0) and stearic acid (C18:0) at the sn-2 position (Figure 3A). At the same time, looking at FAs at the sn-1/3 positions (Figure 4A,B), butyric acid (C4:0) was more abundant in sheep milk, while stearic acid (C18:0) was higher in goat milk.

Both milk samples were characterized by having predominant levels of oleic acid (C18:1) at the sn-2 position (Figure 3B). Sheep milk samples were characterized by higher levels of linoleic acid (C18:2), palmitoleic acid (C16:1) and gadoleic acid (C20:1) when compared to goat milk. Instead, higher levels of heptadecenoic acid (C17:1) and linolenic acid (C18:3) characterized goat milk.

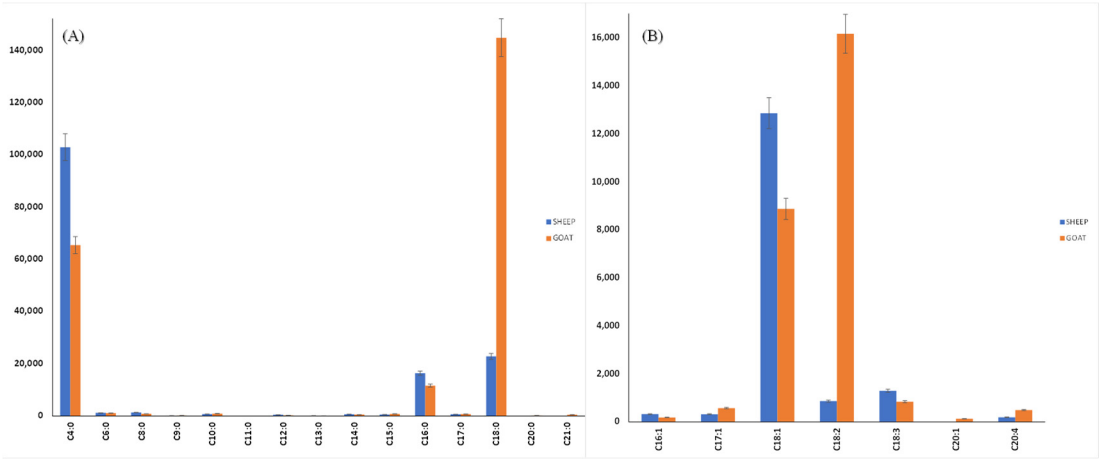

Figure 4. Triacylglycerols' relative abundance of saturated (**A**) and unsaturated (**B**) fatty acids at the sn-1/3 positions.

4. Discussion

In the negative ionization mode, PEs, SMs and PCs were the predominant lipid classes (Table 1). In particular, PE (18:1_18:1), PE (18:0_18:1), PE (16:0_18:1), SM d(34:1), SM d(41:1), SM d(42:1), PC (16:0_18:1) and PC (18:0_18:1) were the most abundant lipids in the two milk samples (Table 1). In this ionization mode, the percent levels of phospholipids were PE > SM > PS > PI. In particular, goat milk was characterized by higher percent levels of ether-phosphatidylethanolamine. The PC/PE ratio was 0.62 for goat milk, while for sheep milk, it was 0.42, thus reflecting the different sizes of fat globule membranes, which are smaller in goat milk (2.73 ± 0.15 µm and 3.63 ± 0.27 µm) [23]. Notably, sheep and goat milk were characterized by the unsaturated free fatty acid oleic acid (C18:1), while the saturated free fatty acid palmitic acid (C16:0) was found uniquely in sheep milk.

On the other hand, in the positive ionization mode for both sheep and goat milk samples, TAG was the most abundant lipid class, but considering that the regiochemistry of FAs in the glycerol backbone influences TAGs' nutritional importance, we decided to separately study this lipid class.

The positive ionization mode allowed us to annotate PC (16:0_18:1) and PC (18:0_18:1) as more abundant in sheep milk according to the literature (Calvano 2013). Furthermore, uniquely in sheep milk, we found several lysophosphatidylcholines, i.e., LPC (18:1), LPC (16:0) and LPC (18:0), which are hydrolysis products of PCs. Several studies have recently demonstrated the potential of dietary phosphatidylcholines as anti-inflammatory bioactive compounds in murine arthritis models [24].

Beneficial health effects are also provided by sphingomyelins (SMs) [25]. In our samples, SM levels were found to be higher in sheep milk (11.14 ± 0.51%) when compared to goat milk (3.29 ± 0.16%), and specifically, according to Calvano et al., SM d(34:1), SM d(36:1) and SM d(40:1) were the most abundant in sheep milk [26]. From the regiochemistry point of view, for SM, it is impossible to give the dominant lipid species due to the fact that isomers produce the same MS/MS spectra, particularly for SM d(34:1). A total of 18 potential lipids can be attributed to the same ion adducts and MS/MS fragments. Furthermore, it is known that dietary SMs may improve gut barrier function by preventing the translocation of inflammatory gut-bacteria-derived compounds and may protect against altered lipid metabolism, inflammation and gut dysbiosis [27].

Uniquely in sheep milk, we were able to annotate butyrylcarnitine C4:0 (ACar 4:0), and the percent levels found were 0.03 ± 0.01%. Acylcarnitines are a group of ester substances formed by the combination of free carnitine and acyl-coenzyme A formed by fatty acids. Mezhnin et al. (2020) recently reported that liver levels of butyryl carnitine were significantly increased when animals were under caloric restriction management, thus suggesting a favorable role in energy metabolism control while contributing to metabolic

flexibility under caloric restriction [28]. Furthermore, in the literature, carnitines were reported to be higher in sheep milk when compared with cow, horse, goat and human milk. Nevertheless, total carnitine did not show statistical differences between species, and the acid-soluble fraction of acylcarnitine was found to be higher in sheep milk [29].

Notably, in sheep and goat milk samples, we were able to annotate another bioactive lipid class corresponding to hexosyl non-hydroxy sphingosine ceramides, HexCer_NS d18:1/16:0 and HexCer_NS d18:1/23:0. The total percent levels of HexCer_NS found in sheep and goat milk were 0.15 ± 0.01% and 0.11 ± 0.01%, respectively. These ceramides serve as precursors of SMs, glycosphingolipid, sphingosine and FAs and are reported to reduce inflammation in a diet-induced obese mouse study [27].

Concerning the TAG regiodistribution analysis, the iterative auto-MS/MS and ion mobility experiments helped us to annotate TAGs with a chain length in the range of 36–54 carbon atoms for sheep milk and 36–59 for goat milk, with 6 as the maximum degree of unsaturation (Figure S2).

The most prevalent TAGs in goat milk samples were TAG (16:0_18:1_4:0) and TAG (18:1_14:0_4:0), while for sheep milk sample, they were TAG (16:0_18:1_4:0), TAG (16:0_6:0_18:1) and TAG (18:1_4:0_14:0) (Table S3).

From the study of the TAG regiochemistry, it appears that there are profound differences in terms of the number of carbons of the FAs that occupy the sn-2 positions in sheep and goat milk (for further details, see Table S4). As can be seen in Figure 2, the most prevalent FAs in sn-2 positions are long-chain ones (C13–C25) in both sheep and goat milk. However, these FAs are more abundant at the TAG sn-2 positions in sheep milk. The most significant difference can be found by observing medium-chain FAs, which were more abundant in sheep milk. Surprisingly, even when looking at short-chain FA levels, the latter appear to be more abundant in sheep milk than in goat milk. Considering these results, we decided to analyze MS/MS spectra one by one and look at TAGs bearing a short-chain fatty acid at the sn-2 position. From this analysis, we were able to observe that goat milk is characterized by a higher number of TAGs with short-chain FAs at sn-2, while in sheep milk, they are higher in terms of concentration.

During digestion, sn-1,3-specific pancreatic lipase hydrolyzes TAGs, forming sn-2 monoacylglycerols (MAGs) and free fatty acids (FFAs) [30]. Enterocytes present in the intestinal mucosa are able to efficiently absorb sn-2 MAGs, which are used for de novo lipid synthesis (TAGs and phospholipids) [31]. Certainly, in the human intestine, the absorption efficiency of FAs derived from TAGs is related to the FA *sn* position on the glycerol backbone. This is particularly true for infants. Palmitic acid (C16:0) is known to be better absorbed when in the sn-2 position as compared to the sn-1/sn-3 positions [22]. Indeed, TAGs possessing saturated FAs at sn-1 and sn-3 positions may have an adverse effect on digestion. Consequently, the determination of the saturated or unsaturated FA position is of paramount importance for understanding differences in the digestibility of milk containing several TAG isomers.

Our results show that goat milk is characterized by higher levels of esterified saturated FAs at the sn-1 and sn-3 positions. In more detail, in goat milk, the predominant FA esterified at the sn-1/3 positions was FA 18:0, suggesting lower adsorption.

Furthermore, considering that short-chain fatty acids are easily adsorbed at the gastric and duodenal tracts, sheep milk showed higher levels of butyric acid (C4:0) at all sn-positions when compared to goat milk (see Figures 3A and 4A).

5. Conclusions

In conclusion, iterative high-resolution MS/MS and ion mobility methods used for the profiling of milk lipids allowed the annotation of common and uncommon features while allowing the fast exploration of lipids with biological significance, such as SMs and Hex-Cer, and potential health benefits. The development of this analytical method lays the foundation for expanding and better understanding the characterization of ruminant milk used to produce high-nutritional-value dairy products.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app13063535/s1. Figure S1: Number of annotated lipid features plotted as number of features annotated versus the number of iterative injections of sheep milk (**a**) and goat milk (**b**) acquired in the negative (blue) and positive (orange) ionization mode; Figure S2: TAG distribution with the relative carbon number in sheep (**A**) and goat (**B**) milk samples; Table S1: Mass spectrometry and collision cross section characteristics of lipids detected in sheep (S) and goat (G) milk samples and acquired in the positive ionization mode; Table S2: Mass spectrometry and collision cross section characteristics of lipids in the negative ionization mode (G = goat and S = sheep); Table S3: TAG concentration in sheep and goat milk (S = sheep and G = goat); Table S4: List of TAG annotated by MS spectra analysis and comparison of reference mass spectra.

Author Contributions: Conceptualization and experimental design, P.C., P.S., M.A. and C.M.; methodology, P.C., P.S., V.G. and C.M.; software, P.S. and C.M.; validation, P.C., C.M. and P.S.; formal analysis, C.M.; investigation, P.C. and P.S.; data curation, P.C., P.S., V.G. and C.M.; resources, I.I.; writing—original draft preparation, P.C., P.S., V.G., M.A., I.I. and C.M.; writing—review and editing, P.C., P.S., M.A., I.I. and C.M. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: None of the data were deposited in an official repository. The data that support the study findings are available from the authors upon request.

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