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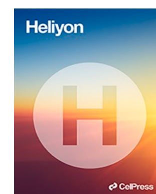

Development and validation of a versatile analytical method for absolute quantification of seven oligosaccharides in human, bovine, and goat milk

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ABSTRACT

Oligosaccharides are significant in mammalian milk, where they serve as prebiotics that promote the growth of beneficial gut bacteria in infants. Comprehensive research of milk oligosaccharides requires precise and validated analytical methods for compositional studies. To address this need, the focus of our study was to develop and validate an analytical method using UPLC-MS/MS to quantify seven specific oligosaccharides found in mammalian milk. The developed and optimized method has adequate linearity, accuracy, and precision parameters. The detection (LOD) and quantification (LOQ) limits for the seven compounds ranged from 0.0018 to 0.0030 μg/mL and 0.0054–0.0063 μg/mL, respectively. The sample preparation method yielded recovery rates above 90.5 %. Furthermore, no significant matrix effect was observed. The validated method was successfully applied to human, goat, and bovine milk samples, demonstrating its proficiency in identifying variances in the concentration of oligosaccharides across different mammals. This versatile method will allow future research about factors affecting oligosaccharide composition.

1. Introduction

Breastfeeding is the initial and most natural way newborns receive positive health benefits due to the rich composition of milk, which is abundant in bioactive compounds that promote the growth of infants [1]. In recent years, significant attention has been given to human milk oligosaccharides (HMOs), which represent the third most abundant solid component in mature milk (10–15 g/L), following lactose and lipids [2]. These complex sugars consist of two or more cyclic monomers linked together by glycosidic bonds and can form linear or branched chains. Approximately 150 different stereospecific structures have been identified in human milk (HM), including backbones of glucose (Glc), galactose (Gal), and N-ethylglucosamine (GlcNAc), which can be further decorated with fucose (Fuc), and sialic acid (Sia) residues [3]. The exact origin of these glycans remains unclear, but, three routes have been reported for the biosynthesis of HMOs [4]: (1) Lactose as the simplest direct acceptor substrate used in the formation of small oligosaccharides, such as 2′-Fucosyllactose (2′-FL), 3′-Sialyllactose (3′-SL), 6′-Sialyllactose (6′-SL), and 3-Fucosyllactose (3-FL). It is also an indirect acceptor substrate for larger oligosaccharides, such as Lacto-N-fucopentaose I (LNFPI), Lacto-N-neotetraose (LNnT) and Lacto-N-tetraose (LNT), and lacto-N-difucohexaose II (LNFDH II); (2) Key intracellular nucleotide sugar donors, such as UDP-GlcNAc and UDP-Gal, naturally

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produced in host cells as common metabolic intermediates, which can be originated from the initial intermediate glucose-6-phosphate. Another key donor, GDP-Fuc, has been reported to be produced by two typical pathways: *de novo* and salvage pathway; (3) The last and most important step for HMO synthesis depends on glycosyltransferases. Generally, β-N-acetylglucosaminyltransferases and β-galactosyltransferases are used to synthesize neutral-core HMOs, whereas α-fucosyltransferases and α-sialyltransferases are used to synthesize the fucosylated and sialylated HMOs, respectively.

Based on these biosynthetic routes, biotechnological production of HMOs has been chosen, which has accelerated preclinical and clinical research instead of the chemical synthesis of HMOs due to their limited availability, low yields, and high prices. Using bacteria as biotechnological machinery has allowed commercial applications, mainly the supplementation of infant formulas with up to 5 HMOs [5]. Undoubtedly, having a validated method for the quantification of HMOs is crucial to monitor and improve these biotechnological processes.

In humans, the three major oligosaccharides are: neutral fucosylated such as 2′-FL, 3-FL, and Lactodifucopentaose, representing 35 %–50 % of the total HMOs, and Neutral N-containing, like LNT and LNnT, with 42 %–55 %, and acid sialylated, including 3′-SL and 6′-SL with 12 %–14 % [6].

Although great discoveries about HMOs functions on infant health have been reported, some limitations on analytic methods are faced nowadays, including the availability of analytical standards, the number of steps for sample preparation, and the chromatographic resolution of isomeric species. According to a review by Ref. [7]; the three most common analytical methods used to quantify HMOs in 31 countries are high-performance liquid chromatography with fluorescence detection (HPLC-FLD) or ultraviolet (HPLC-UV), high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC), and liquid chromatography with mass spectrometry (LC-MS). The mean concentrations of HMOs in breast milk applying these techniques are 17.7 g/L in colostrum (0–5 days), 13.3 g/L in transitional milk (6–14 days), and 11.3 g/L in mature milk (14–90 days) [7].

The LC-MS/MS method offers several benefits in comparison to other analytical techniques.

LC-MS/MS enables accurate quantification of HMOs using a minimum volume required to the analysis (1–5 μL); the sample preparation process is rapid, involving 2–3 steps, and it does not necessitate derivatization, labeling, or fractionation of the HMOs extract (neutral and acidic) [8–10]. Furthermore, LC-MS/MS exhibits structural selectivity, as it relies on the chromatographic separation and ions filtered by the mass analyzer within complex mixture with isomeric species. Notably, instances have been reported where the successful quantification and identification of HMOs have been achieved [11–13]. While some advanced methods employing sophisticated allow the identification of isomeric oligosaccharides across diverse matrices, their utility often lies in the relative quantification of the compounds [14]. However, there are limited approaches that enable the absolute quantification of oligosaccharides using LC-MS/MS. Some of these methods concentrate on validating their effectiveness within a specific dairy matrix source [15].

Considering the crucial role of oligosaccharides, exploring alternative sources for developing formulas is essential for maternal-infant emergencies. Goat and cow milk are prominent sources of milk widely used in the food industry. However, the concentration and profile of oligosaccharides in humans significantly differ from other mammals [16]. For instance, concentrations of oligosaccharides in bovine milk (BMOs) are between 30 and 60 mg/L, where up to 50 varieties have been observed and 37 structures identified [17]; up to 1–2 g/L in colostrum [18]. On the other hand, goat milk is notable for having higher oligosaccharides concentration (up to 2.4 g/L in colostrum) compared to bovine and sheep [19,20].

Regarding the structural diversity and profiling, in goat milk, at least 77 structures have been identified, where only 40 have been characterized, of which 16 were neutral, 23 acidic, and one phosphorylated; the neutral and acidic represent 5 % and 95 % of the total GMOs, respectively (the acid GMOs 3′-SL and 6′-SL have been reported as the most abundant). On the other hand, 32 different structures have been characterized in bovine milk, of which 12 were neutral, 21 acidic, and two were phosphorylated; the neutral and acidic represent 9 % and 91 % of the total BMOs, respectively (the acid BMOs 3′-SL have been reported as the most abundant) [17].

Given the reported diversity and variability intra and interspecies, a comparative study of oligosaccharides across mammals can contribute to a better understanding, reveal new potential, and unearth unique functional properties applicable to various dairy products or complex formulations containing oligosaccharides. However, a reliable analytical method, compatible with diverse samples, is crucial for these efforts.

This research aimed to develop and validate a versatile UPLC-MS/MS analytical method for quantifying seven specific oligosaccharides, namely 2′-FL, 3′-SL, 6′-SL, 3-FL, LNFPI, LNnT, and LNT in human, goat, and bovine milk that represent about 80–90 % of the total oligosaccharides [17,21]. The development and validation of an analytical method for the absolute and simultaneous quantification of seven oligosaccharides allow its application for the study of mammalian milk that could contribute to knowledge about oligosaccharides, evaluation of complex formulations and factors associated with their production in humans or biotechnological processes for the benefit of maternal and infant health.

2. Material and methods

2.1. Ethical considerations

This study was conducted according to the ethical principles for medical research involving human subjects outlined in the Helsinki Declaration. Institutional review board approval (approval number: DEISC-PR-190122074; Hospital Regional Materno Infantil de Alta Especialidad) was obtained, and the donors provided written informed consent before participating in the study.

2.2. Samples

Ten women between the sixth and twelfth month of lactation were recruited, and an l milk sample was obtained from each and was analyzed separately. The volunteer was instructed to gently wash one breast with sterile distilled water; then, she performed a circular massage until drops of milk were produced. The first 2–3 drops were discarded, and the following volumes were collected in a 50 mL sterile polypropylene tube, obtained the same way from the other breast until reaching a final volume of 20–50 mL. The samples were immediately stored at −20 °C for no more than 48 h, then transported refrigerated, and finally frozen at −80 °C until use.

Eleven Goat and ten bovine milk samples were collected at a dairy goat farm in Allende, Nuevo León, Mexico, and at the Facultad de Agronomia of Universidad Autonoma de Nuevo Leon in Marin, Nuevo León, Mexico, respectively. All the animals were healthy and fed with conventional feeding materials. Mature milk was collected between the first and second month of lactation. Before performing manual milk collection, the udder surface of each dairy goat or bovine was washed with distilled water and disinfected with 70 % ethanol. After discarding the first three drops of milk, the samples were mixed and placed in sterile 50 mL plastic tubes and stored at −20 °C. Subsequently, they were transported on ice, then frozen at −80 °C.

2.3. Standards, reagents, and materials

The solvents used in the development of the methods were the following: acetonitrile LC-MS ≥99.9 % (34967-2.5L; Honeywell Riedel-de Haën), formic acid LC-MS ≥98 % (94318-250 ML; Sigma Aldrich, St Louis, MO), ammonium formate LC-MS ≥99.0 % (70221-25G-F; Sigma-Aldrich), water LC-MS ≥99.9 % (39253-4L; Honeywell Riedel-de Haën). The analytical standards used in the development of this method were the following: 2′-Fucosyllactose (SMB00933-10 M; Sigma Aldrich), 3′-Sialyllactose (SL302; Dextra, UK), 6′-Sialyllactose (SL306; Dextra, UK), 3-Fucosyllactose (L303; Dextra, UK), Lacto-N-Fucopentaose I (L502; Dextra, UK), Lacto-N-neotetraose (L404; Dextra, UK), Lacto-N-tetraose (L403; Dextra, UK). These analytical standards have been previously used and reported by other authors both for the identification and quantification of oligosaccharides [13,15,22]. All analytical standard stocks were prepared individually at 5000 μg/mL in water LC-MS ≥99.9 %, filtered at 0.22 μM. From the stocks, dilutions and appropriate working mixtures were made for the method validation.

2.4. Chromatographic and spectrometric conditions

The experiments were performed using an Acquity UPLC system (Waters, Milford, MA) equipped with Micromass Quattro Premier XE Mass Spectrometer (Waters, Milford, MA).

The initial LC-MS conditions were adapted from literature reported with modifications and optimized parameters in this study [15, 23].

An ACQUITY UPLC BEH Amide Column (Waters; 1.7 μm, 2.1 × 100 mm) coupled to an ACQUITY UPLC BEH Amide pre-column (Waters; 1.7 μm, 2.1 × 5 mm) was used for separation. Chromatographic conditions were optimized at 40 °C using a binary gradient.

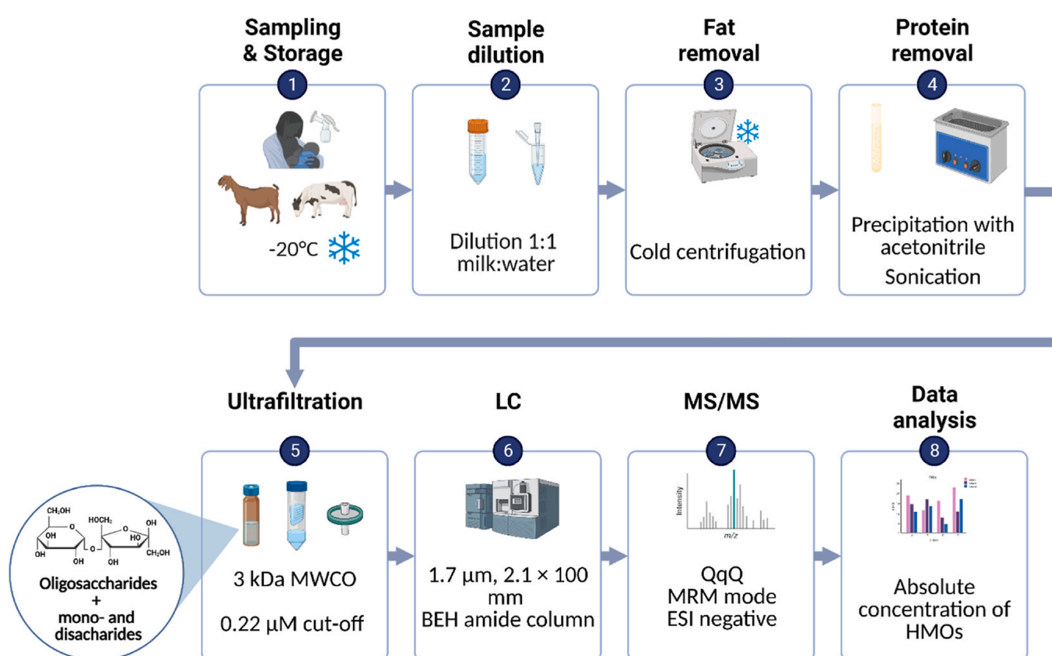

Fig. 1. LC-MS/MS workflow for absolute quantification of seven oligosaccharides.

The mobile phase A and B consisted of 10 mM ammonium formate in water and 99.9 % acetonitrile, respectively. The following linear UPLC gradient was used: 0−2 min from 98 % to 95 % B, 2−3 min to 65 % B, 3−8.5 min to 55 % A, 8.5−11 min to 55 % A, 11–11.2 min to 98 % A, 11.2–14 min to 98 % B. The flow rate was set to 0.4 mL/min.

Mass spectrometric analyses were performed with a triple quadrupole spectrometer equipped with an electrospray ionization source (ESI). ESI conditions were as follows: Capillary 4 kV, Cone 50 V, Extractor 3V, RF Lens 0 V, Source temperature 150 °C, desolvation temperature 300 °C, gas flow desolvation 720 L/h, gas flow cone 300 L/h. High-purity argon was used as collision gas. The mass spectrometer was operated in negative-ion, multiple reaction monitoring (MRM) mode. The analytical standards were directly infused to identify the precursor and product ions for 2′-FL, 3-FL, 3′-SL, 6′-SL, and LNFPI. In addition, we optimized the response of each of the compounds with the ideal cone and collision energies.

2.5. Sample preparation

Each milk sample was diluted at a 1:1 ratio with water and manually mixed through inversion until a homogeneous mixture was obtained. Next, the mixture was centrifuged at 12,000×*g* for 30 min at 4 °C, and the upper phase consisting of fat was removed. Before centrifugation, the sample was incubated at 4 °C for 15 min. The lower aqueous phase of carbohydrates plus proteins were transferred to 1.5 mL tubes and mixed with three volumes of acetonitrile LC-MS ≥99.9 % for protein removal. The mixture was sonicated for 10 min, incubated for 60 min at 4 °C, and centrifuged at 12,000×*g* for 30 min at 4 °C; in this step, the proteins were precipitated at the bottom of the tube, and the supernatant was recovered. For removal of residual protein, the supernatant was transferred to ultra-centrifuge tubes with a 3 kDa molecular weight cut-off membrane (Vivaspin 500, 3 kDa MWCO; GE28-9322-18; Sartorius, Stonehouse, UK) and centrifuged for 50 min at 7500×*g*, 4 °C [24]. A mixture mainly composed of lactose, mono-, di-, and oligosaccharides (carbohydrates fraction) was obtained in this step; the fraction could contain residual peptides <3 kDa. The carbohydrate fraction was filtered through a 0.22 μM nylon membrane to remove particles and stored at −20 °C until use; additional dilutions were necessary for adequate quantification. The workflow is shown in Fig. 1.

2.6. Method validation

The method validation was carried out, including calibration curves, accuracy, precision, recovery, the limit of detection (LOD), the limit of quantitation (LOQ), matrix effects, and stability in human and goat milk according to the validation of analytical procedures guideline of International Council for Harmonisation [25].

2.6.1. Calibration curves and linearity

The linearity of the method was assessed to establish the concentration ranges for quantification, and serial dilutions were prepared. Seven concentration points (1, 5, 10, 20, 40, 50, and 100 μg/mL) were used for 2′-FL, 3-FL, and LNFPI. Six concentration points (1, 5, 10, 20, 40, and 50 μg/mL) were employed for 6′-SL, 3′-SL, LNT, and LNnT; the mixture of standards was prepared in a solution containing 50 % acetonitrile in LC-MS grade water. The area obtained for each compound was placed on the Y-axis, and the theoretical concentration was on the X-axis in a linear regression plot. The coefficient of determination (R^2), the value of the y-intercept, and the linear equation slope were obtained.

2.6.2. Limit of detection (LOD) and quantitation (LOQ), and selectivity

LOD and LOQ of the chromatographic system were evaluated using ten consecutive blank measurements, which consisted of LC-MS grade water (n = 10). LOD and LOQ were calculated by referring to the standard deviation of the blank signal (SD) and the slope of the calibration curve (k) following the equations below: $LOD = (3.3 * SD)/k$; $LOQ = (10 * SD)/k$.

Selectivity was evaluated by analyzing different blank milk samples (Human, bovine, and goat) produced from the cleavage of the β-1,4 glycosidic bonds using a recombinant β-galactosidase to test for the specificity of filtering the masses of each compound in the detection system and possible interferences.

2.6.3. Accuracy and method precision (repeatability and intermediate precision)

The accuracy was measured by the ratio between the mean measured concentration and the theoretical concentration, described as a percentage of the theoretical concentration.

Repeatability was expressed as the relative standard deviation (RSD) of three concentrations of oligosaccharides standards (5, 25, and 50 μg/mL) in triplicate (n = 9). The intermediate precision was obtained from three determinations performed on three days (n = 9).

2.6.4. Extraction recovery, matrix effect, and robustness

For the evaluation of the recovery efficiency of the oligosaccharides extraction procedure (R_E), aliquots of human, goat, and bovine milk were spiked with a mixture of oligosaccharides standards at three levels of spike (5, 25, and 50 μg/mL) in triplicate for each concentration and subjected to sample preparation procedures along with aliquots of the unspiked milk. Then, the preparations were analyzed by LC-MS/MS, and the seven oligosaccharides were quantified. The percentage recovery of each oligosaccharide at each concentration was calculated as the difference between the concentration of each oligosaccharide in the spiked and non-spiked milk samples, then divided by the concentration of the standard mix multiplied by 100.

The matrix effect was evaluated for trueness in three dairy matrices: goat, bovine and human milk. Calibration curves for each

oligosaccharide were prepared in the mobile phase. Then, the matrix-matched calibration (MMC) curves for oligosaccharides were made using milk samples spiked with oligosaccharides standards at seven concentrations after extraction. The percentage of the ratio between the matrix-matched calibration slope and the solvent calibration slope to obtain the signal suppression/enhancement (SSE%); SSE >100 % indicates signal enhancement, and SSE <100 % shows signal suppression.

Regarding the method robustness, minor changes were made to evaluate the impact on the quantification of oligosaccharides. Parameters were column temperatures (40–50 °C), flow rate (0.4–0.3 mL/min), mobile phase A pH (7.5–8.0), and autosampler temperature (10–9 °C).

2.7. Statistical analysis

A Kolmogorov-Smirnov test was performed to assess the distribution of the data. The one-way ANOVA with posthoc Tukey HSD parametric tests was used for the data with a normal distribution. The Mann-Whitney U non-parametric test was performed for the data with a non-normal distribution. Statistical differences were considered when the P value was less than 0.05. It was carried out in triplicate to apply the method to the samples.

3. Results

3.1. Optimizing MS/MS conditions for oligosaccharides

First, upon testing the ESI interface in positive and negative modes, we observed that the best abundance values for the seven deprotonated compounds occurred in negative mode (data not shown). Under these ionization conditions, direct infusion into the MS detector allowed the identification of each compound's precursor and product ion. The precursor ion with the highest *m/z* ratio was LNFPI, with 852.3 *m/z*, followed by LNT, LNnT, 3′-SL, 6′-SL, 3-FL, and 2′-FL. The product ion with the highest *m/z* ratio was LNFPI with 325.0 *m/z*, followed by 6′-SL, 3′-SL, 2′-FL, 3-Fl, LNT, and LNnT (Figs. S1–S7). The *m/z* values were consistent with those reported in previous studies [13,23]. In addition, the optimized cone and collision energy values were obtained, ranging between 20-50 V and 30–32 V, respectively (Table 1).

3.2. Method validation

The method showed consistency in the detection of proportional signals according to each analyte concentration within the following ranges: from 1 to 100 μg/mL for 2′-FL, 3-FL and from 1 to 50 μg/L for 6′-SL, 3′-SL, LNT, and LNnT [25]. suggests a minimum of five concentration levels to evaluate a method's linearity. We obtained analytical curves of each oligosaccharide at seven concentration levels higher than those recommended by the revised guidelines. The R^2 value was maintained above 0.99 in the different compounds complying with the recommendation. In addition, the linear equation showed negative values at the Y-intercept in all analytes except for 2-FL and LNT (Table 2).

The LOD (minimum concentration required by the method for detecting oligosaccharides) ranged from 0.0018 to 0.0030 μg/mL; the lowest concentration was observed in 3-FL and 3′-SL and the highest in LNnT. On the other hand, the LOQ (minimum concentration required by the method for the quantification of oligosaccharides) ranged from 0.0054 to 0.0063 μg/mL; the lowest concentration was observed in 3′-SL and the highest in 2′-FL (Table 2). The LOD and LOQ were determined based on the standard deviation of the response and the slope in compliance with the ICH guidelines [25]. Regarding selectivity, no interference was detected in the retention time of each oligosaccharide and the standard (Table 1).

Accuracy was evaluated in nine determinations (three concentration levels by triplicate for each compound), and the results were in the accepted range between 70 and 120 %, as suggested by the ICH and AOAC [25,26]. The method accuracy varied between 97 % and 103 % at the low level, 97 % and 104 % at the medium level, and 96 % and 103 % at the high levels (Table 2).

[26] suggests that the coefficient of variation (CV%) should not be higher than 15 %. Regarding precision, the method showed excellent intra-day and inter-day repeatability and intermediate precision. The highest variability was observed in LFPI and LNT, with

Table 1
Optimized mass spectrometry conditions for the seven analytical standards through Multiple Reaction Monitoring (MRM) using an electrospray ionization (ESI) in the negative ion mode.

Analyte	Molecular formula	Structure	Monoisotopic mass	Precursor Ion *(m/z)* [M − H]⁻	Product Ion *(m/z)*	Cone Energy (V)	Collision Energy (V)
2′-FL	$C_{18}H_{32}O_{15}$	Fucα1-2Galβ1-4Glc	488.2	487.3	205.0	30	20
3-FL	$C_{18}H_{32}O_{15}$	Galβ1-4(Fucα1-3)Glc	488.2	487.2	178.8	20	20
6′-SL	$C_{23}H_{39}NO_{19}$	Neu5Acα2-6Galβ1-4Glc	633.2	632.3	290.1	50	30
3′-SL	$C_{23}H_{39}NO_{19}$	Neu5Acα2-3Galβ1-4Glc	633.2	632.4	289.9	50	30
LNT	$C_{26}H_{45}NO_{21}$	Galβ1–3GlcNAcβ1–3Galβ1–4Glc	707.2	706.3	178.8	30	25
LNnT	$C_{26}H_{45}NO_{21}$	Galβ1–4GlcNAcβ1–3Galβ1–4Glc	707.2	706.2	142.9	30	32
LNFPI	$C_{32}H_{55}NO_{25}$	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	853.3	852.3	325.0	30	20

$[M - H]^-$: deprotonated ions; Glc: glucose; Gal: galactose; GlcNAc: N-acetylglucosamine; Fuc: fucose; Neu5Ac: N-acetylneuraminic acid.

Table 2
Linearity, LOD, LOQ, Accuracy, and precision of the developed method for the quantification of seven oligosaccharides.

Analyte	R^2	Linear equation	RT (min)	LOD (μg/mL)	LOQ (μg/mL)	Accuracy (%)			Precision (RSD, %)		
						5 μg/mL	25 μg/mL	50 μg/mL	Intra-day (n = 9)[a]	Inter-day (n = 9)[b]	Overall
2′-FL	0.999	y = 3.7x + 3.9	4.78	0.0021	0.0063	101	103	102	2.0	4.2	2.7
3-FL	1.000	y = 6.7x – 5.0	4.88	0.0018	0.0055	99	101	98	1.8	4.4	2.5
6′-SL	0.999	y = 19.7x – 4.3	4.7	0.0019	0.0057	102	100	97	1.9	4.6	2.8
3′-SL	0.999	y = 10.7x – 19.9	4.7	0.0018	0.0054	103	103	102	2.3	6.2	4.4
LNT	0.998	y = 1.3x + 0.6	5.09	0.0020	0.0060	99	104	103	4.7	9.3	6.8
LNnT	0.997	y = 14.8x – 31.3	5.10	0.0030	0.0055	97	97	96	1.0	4.3	2.5
LNFPI	0.999	y = 3.4x – 3.8	5.30	0.0023	0.0057	102	103	100	4.9	9.2	7.6

RSD: Relative standard deviation; LOD: Limit of detection; LOQ: Limit of quantification; R^2: Coefficient of determination; RT: retention time.
[a] The repeatability of three concentrations of HMOs standards (5, 25 and 50 mg/L) was assessed in triplicate on the same day.
[b] The intermediate precision was obtained of three determinations performed on 3 different days.

4.9 % and 4.7 intra-day and 9.3 % and 9.2 % inter-day, respectively. On the other hand, the HMOs that showed less variability were LNnT and 3-FL with 1 % and 1.8 % intra-day and 2′-FL and LNnT with 4.2 % and 4.3 %, as shown in Table 2.

The results of the recovery extraction of the seven oligosaccharides show no critical loss (<9.5 %) of the compounds after the preparation of the samples, including cleaning steps, such as removal of the fat and protein fraction, in the different evaluated dairy matrices. In human milk, recovery ranged from 90.5 % ± 7.1 %–99.7 % ± 4.1 % for LNFPI and 3-FL, respectively. In the results for goat milk, LNnT showed the highest and 3-SL the lowest recovery with 96.5 % ± 0.7 % and 99.7 % ± 0.7 %, respectively; 2-FL and LNFPI were not detected before spiking in goat milk samples. In addition, the recovery was between 96.9 % ± 2.0 % and 99.9 % ± 0.7 %; variability in recovery assays was ≤8.3 % (Table 3).

The percentage of SSE allowed us to conclude that the preparation of the samples used in this method is adequate to avoid a matrix effect that could affect the quantification of the seven oligosaccharides in the different milk matrices. When the SSE >100 %, there is an ion enhancement, and when SSE <100 %, there is an ion suppression, but a tolerance of ±10 % has been reported. The method showed SSE% values within that tolerance range. The SSE was from 94.3 % ± 0.8 %–97.2 % ± 1.3 % for human milk, 94.8 % ± 0.9 %–98.0 % ± 1.2 % for goat milk, and 96.6 % ± 1.9 %–100.8 % ± 1.2 % for bovine milk (Table 3).

The method showed robustness since minor changes in column temperature, flow, pH, and sample temperature did not significantly affect oligosaccharide quantification (Table S1).

3.3. Method application for quantifying oligosaccharides in human, goat, and bovine milk samples

Using the validated method, the seven oligosaccharides were quantified in ten samples of human, eleven goat, and ten bovine milks. Each sample was analyzed in triplicate, and the intra-sample CV% was calculated. In addition, the inter-individual variability of the different milk matrices was obtained.

In human milk, 2′-FL was the most abundant compound representing about 31.1 % of total HMOs, and the least abundant was 6′-SL, representing only about 3.3 %; the total HMOs were 4.550 ± 2.015 g/L. High variability was observed in the concentration of HMOs

Table 3
Recovery extraction and matrix effect evaluation of the method for the quantification of seven oligosaccharides in human, goat and bovine milk samples.

Analyte	**R_E (%)**[a]						**SSE (%)**[b]					
	Human Milk		Goat Milk		Bovine Milk		Human Milk		Goat Milk		Bovine Milk	
	mean ± SD	CV	mean ± SD	CV	mean ± SD	CV	mean ± SD	CV	mean ± SD	CV	mean ± SD	CV
2-FL	95.6 ± 3.2	3.4	97.5 ± 2.0	2.0	97.3 ± 1.2	1.2	94.9 ± 0.6	0.6	95.5 ± 0.3	0.3	97.8 ± 0.5	0.5
3-FL	99.7 ± 4.1	4.1	98.2 ± 1.0	1.0	99.9 ± 0.7	0.7	94.3 ± 0.8	0.8	94.8 ± 0.9	1.0	98.5 ± 0.9	0.9
6-SL	98.8 ± 3.1	3.1	99.2 ± 1.9	1.9	96.0 ± 1.4	1.5	96.0 ± 0.8	0.8	96.7 ± 1.1	1.1	100.5 ± 1.6	1.6
3-SL	99.7 ± 2.4	2.4	99.7 ± 0.7	0.7	97.0 ± 1.6	1.7	97.2 ± 1.3	1.3	98.0 ± 1.2	1.2	99.9 ± 0.4	0.4
LNT	92.4 ± 6.2	6.7	99.1 ± 0.6	0.6	96.9 ± 2.0	2.0	95.3 ± 1.0	1.0	96.6 ± 1.4	1.5	100.8 ± 1.2	1.2
LNnT	90.8 ± 7.6	8.3	96.5 ± 0.7	0.8	98.0 ± 1.5	1.5	95.9 ± 0.5	0.5	96.5 ± 1.3	1.3	96.6 ± 1.9	2.0
LNFPI	90.5 ± 7.1	7.8	97.8 ± 3.0	3.1	97.2 ± 2.0	2.1	95.4 ± 0.7	0.7	95.8 ± 0.4	0.4	98.5 ± 3.1	3.2

R_E: recovery extraction; SSE: signal suppression/enhancement.
[a] Percentage of recovery extraction of each HMOs was calculated as the difference between the concentration of each HMOs in the spiked and non-spiked milk samples, then divided by the concentration of the standard mix multiplied by 100.
[b] The signal suppression/enhancement was calculated as the ratio between the matrix matched calibration slope and the solvent calibration slope. SSE >100 % indicates signal enhancement and SSE <100 % indicates signal suppression.

between individuals from 29 % to 78 %; 6′-SL and 3′-SL showed the highest variability, and LNFPI showed the lowest inter-sample variability (Table 4).

On the other hand, the most abundant oligosaccharides in goat milk were 3′-SL and 6′-SL, about 58.5 % and 34.2 %, respectively. The range of inter-individual variability was between 22 % and 37 %. Additionally, GMOs' total (the sum of the seven oligosaccharides) was 0.111 ± 0.035 g/L; 2′-FL and LNFPI were undetected (Table 4).

In bovine milk, we identified and quantified six oligosaccharides; 2′-FL was not detected. In the same way as goat milk, the principal oligosaccharides were 3′-SL and 6′-SL, with about 29.3 % and 24.0 % of the total BMOs, respectively. The inter-individual variability was between 50 % and 77 % (Table 4).

Finally, comparisons between the mammals revealed significant differences ($p < 0.05$) in 3-FL, 6′-SL, 3′-SL, LNT, LNnT, and the total analyzed in this study. In addition, significant differences ($p < 0.05$) in LNFPI were observed between human and bovine milk. Regarding inter-individual variability, similar results were observed between bovine and humans (Table 4).

4. Discussion

A versatile method of extraction and absolute quantification of seven oligosaccharides in different mammalian milk matrices was developed and validated. The method allowed the simultaneous analysis of seven oligosaccharides (three neutral fucosylated, two acidic non-fucosylated, and two neutral non-fucosylated). Negative mode ionization conditions during spectrometry allowed unequivocal identification based on m/z of the precursor and product ions of the seven compounds despite being three pairs of isomers (2′-FL/3-FL, 6′-SL/3′-SL and LNT/LNnT). The linearity derived from calibration curves allows the quantification of the different oligosaccharides in a wide quantification range (up to 100 μg/mL for 2′-FL, 3-FL, and LNFPI) without the need for additional sample dilutions or preparations; these limitations have been reported [13].

The present work shows similar validation results to those reported by other authors for oligosaccharide application in the human milk matrix [11]. developed a method to quantify 11 HMOs by HPLC-MS/MS system with a 3-μm Hypercarb column where they report linear ranges from 0.078 or 0.156–20 μg/mL ($R^2 > 0.998$) with a precision (CV) range from 1 % to 9 %, and accuracy was from 86 % to 104 % [11]. The lower LOQ was 0.039 μg/mL for LNDFH-I and 0.010 μg/ml for the remaining oligosaccharides. A study [13], developed a method for the quantification of 16 HMOs by HPLC-MS/MS system using a 3-μm PGC column with calibration curves ranging from 0.039 to 5 μg/mL with optimal linearity ($R^2 \geq 0.99$). LOQ was 0.156 μg/mL for LNFP I and 0.039 μg/mL for all the remaining HMOs. The precision range varied between 3 and 13 %, and accuracy ranged from 90 to 109 % [13]. The results of the present study were better in terms of precision and accuracy than those reported [11,13]. It is known that the UPLC system has a higher instrumental sensitivity than the HPLC system since using columns with a very low particle size is possible (1.7 μm). This may explain the lower LOQs in the present study.

To our knowledge, limited information is available to determine BMOs and GMOs. Previously, another study reported an analytical method for the quantification of six BMOs using a similar methodology with UPLC-MS/MS system with a selective reaction monitoring (SRM), shows LOD from 0.1 to 1.2 μg/mL and LOQ from 0.3 to 4.8 to 1.2 μg/mL [27]. In the present study, LOD and LOQ values were lower, and the reproducibility of the quantification of the seven oligosaccharides was significantly improved from a relative standard deviation (RSD) of 2.7 %–7.6 %, compared to the 17 % reported in literature [27].

Table 4
Method application for absolute quantification of seven oligosaccharides in human, goat, and bovine milk samples.

Analyte	Human milk (n = 10)			Goat milk (n = 11)			Bovine milk (n = 10)		
	Mean ± SD (g/L)	CV (%)		Mean ± SD (g/L)	CV (%)		Mean ± SD (g/L)	CV (%)	
		Inter-sample[a]	Intra-sample[b]		Inter-sample[a]	Intra-sample[b]		Inter-sample[a]	Intra-sample[b]
2′-FL	1.417 ± 0.703	50	1.2	ND	ND	0.7	ND	ND	0.8
3-FL	1.233 ± 0.480^a	39	1.0	0.023 ± 0.003^b	28	0.4	0.015 ± 0.009^c	60	0.6
6′-SL	0.151 ± 0.118^a	78	0.5	0.038 ± 0.014^b	37	0.6	0.018 ± 0.012^c	67	0.9
3′-SL	0.187 ± 0.145^a	78	1.1	0.065 ± 0.014^b	22	0.4	0.022 ± 0.017^c	77	1.0
LNT	0.158 ± 0.101^a	64	0.5	0.032 ± 0.003^b	24	1.2	0.004 ± 0.002^c	50	0.3
LNnT	0.156 ± 0.111^a	71	0.2	0.025 ± 0.001^b	27	0.5	0.007 ± 0.005^c	71	0.5
LNFPI	1.248 ± 0.357^a	29	0.4	ND	ND	1.2	0.009 ± 0.006^b	67	0.6
TOTAL[c]	4.550 ± 2.015^a	44	1.8	0.111 ± 0.035^b	32	1.4	0.075 ± 0.051^c	68	1.5

ND: Not detected; Different letters (superscripts) in the different milk samples indicate significant differences between values ($p < 0.05$).
[a] Coefficient of variation represents the observed inter-sample variability (10 human, 11 goat and 10 bovine milk samples).
[b] Coefficient of variation represents the observed intra-sample variability. Each sample was analyzed in triplicate.
[c] Total means the sum of the seven oligosaccharides.

Recently, a method was developed for quantifying nine BMOs using a UPLC system coupled to a fluorescence detector (UPLC-FLD) [10]. The study yielded satisfactory results in terms of linearity ($R^2 \geq 0.9992$), repeatability, and intermediate precision (RSD ≤4.46 %). The LOD for all investigated oligosaccharides ranged from 0.00197 to 0.00938 μg/mL, while the LOQ values ranged between 0.00684 and 0.03125 μg/mL [10]. In this study, the LOD and LOQ results showed a higher chance of detecting and quantifying BMOs in samples with a low abundance of compounds compared to those reported [10]. Although they used a 1.7-μM BEH column like the present work, the previous study used an FLD system with a lower instrumental sensitivity than the one used in this study, which may explain the variation in the LOD and LOQ values between the two studies.

To our knowledge, this is one of the first validated methods using UPLC-MS/MS for the absolute quantification of GMOs after Lu et al. [28]. In that study, the standard curve of each oligosaccharide showed an excellent linear relationship ($R^2 > 0.99$), and the RSD was less than 5.45 %. The LOD was between 0.00125 μg/mL and 0.00038 μg/mL. The LOQ was between 0.00415 μg/mL and 0.00127 μg/mL [28]. The LOD and LOQ were slightly lower than reported in this study, but they only quantified two (3′-SL and 6′-SL) of the seven oligosaccharides that were quantified in the present study.

An established method at the Bode Lab of the University of California San Diego allows the absolute quantification of 19 HMOs using a labeling system with 2-aminobenzamide by HPLC-FLD and offline mass spectrometric analysis on an LCQ Duo Ion trap mass spectrometer equipped with a Nano-ESI-source [2,9,29]. A hybrid configuration where they report lower detection limits (around 20 pmol) in comparison to those reported in this study.

Therefore, the advantage of the present method lies in its ability to be validated and applied to various milk matrices integrated into a single method, including those from human and non-human mammals of commercial importance, such as bovines and goats, for absolute quantification of oligosaccharides.

As well known, the dairy matrix presents a significant level of complexity and poses analytical challenges when studying its composition. In most of the developed and reported methods based on LC-MS/MS, minimal interference from the dairy matrix is described for the quantification of HMOs [11,13], and BMOs [27]. In the present study, the quantification of oligosaccharides was not affected by the milk matrix, suggesting that the carbohydrate fraction extraction from the complete milk matrix is sufficient (Table 3). However, even after sample preparation, the milk matrix has affected other detection systems, such as the refractive index or the phenol-sulfuric acid method [30,31].

Regarding the extraction of oligosaccharides, several authors have reported various strategies during sample preparation, which involve steps such as fat and protein removal, ultrafiltration, and elimination of lactose and free glucans [19,24,32]. Solid phase extraction (SPE) using porous graphited carbon (PGC) cartridges has been commonly employed to eliminate lactose and free glucans. The recovery percentages of oligosaccharides during extraction vary using PGC cartridges have been reported to range from 89 % to 110 % [11,13,33–35]. PGC chromatography offers good resolution and the ability to separate oligosaccharide isomers, including α- and β-anomeric configurations [11]. However, PGC chromatography typically involves additional steps of reducing and desalting the samples to achieve separation of HMOs, which can increase costs and be time-consuming for studying these compounds. The present extraction method does not involve using PGC cartridges and still reaches excellent recovery percentages comparable to those that use them.

It should be noted that some reports suggest that the use of organic solvents (such as ethanol and acetonitrile) may lead to recovery loss due to the co-crystallization of lactose and oligosaccharides [27,36]. To avoid the co-crystallization phenomenon, the extraction method involves the precipitation of proteins using acetonitrile followed by ultrafiltration to clean the samples. The present study results show a recovery extraction greater than 90 % in the oligosaccharides studied, demonstrating that our fast and straightforward sample preparation avoids potential co-crystallization effects when using organic solvents.

There are undoubtedly differences in the composition of mammalian milk (Table S2), we applied the current method to explore the differences in oligosaccharides concentrations from milk samples of human, goat and bovine. In human milk, carbohydrates are one of the most abundant components, with HMOs present in high concentrations from 5 to 20 g/L [6,7,37], consistent with the findings of the present study in 10 human samples with 4.5 ± 2.015 g/L.

A study quantified HMOs in the milk of 45 American women at different lactation stages, with concentrations ranging from 6.56 to 24.3 g/L. The concentrations decreased as lactation progressed; on four month after delivery, postpartum reported 8.64 ± 1.30 g/L [35]. In the present study, all samples were obtained from 10 Mexican women recruited between the sixth and twelfth month after delivery, which may explain the values obtained since the concentrations of HMOs decrease as lactation progresses; other study [7], report a concentration of 2-FL of about 2.28 g/L in mature milk.

Regarding HMOs variability [13], applied their method to quantify HMOs in 10 samples. They found that the most abundant HMOs were 2′-FL, 3-FL, and LNFPI, with concentrations of 3.233 ± 1.461 g/L, 0.687 ± 0.695 g/L, and 1.310 ± 1.250 g/L, respectively. They observed a high inter-individual variability ranging from 23 % to 142 %, consistent with the findings in this study from 29 % to 78 %. The inter-individual variability of HMOs concentrations in this study that previously reported factors could influence as socio-demographic conditions, lactation stage, health status, body mass index, and Nutrition [9,38,39].

BMOs concentrations were significantly lower (about 98.3 %) than HMOS in the present study. The concentration of the most abundant BMOs (3-SL, 6-SL, 6-sialyllactosamine (6-SLN) and disialyllactose (DSL)) range between 60 and 94 μg/mL for mature milk [27], concentrations similar to those found in this study. Regarding the BMOs profile, the dominant ones were sialylated oligosaccharides, as opposed to the fucosylated oligosaccharides, which are the dominant HMOs species [7]. This study showed 22 %–37 % variability, lower than those observed in humans and bovines. Quantitative analyses of GMOs have reported concentrations ranging from 60 to 350 mg/L in mature milk [40]. These levels are about 30-fold lower than those observed in human milk but approximately 10-fold higher than those found in domesticated dairy animals, such as bovine. These concentration values align with those found in this study. Undoubtedly, goats and humans had distinct differences in the oligosaccharide profiles. However, the concentrations of the

acidic structures (94–195 mg/L of 3′-SL and 13–129 mg/L 6′-SL) in goats are lower than those observed in humans. The main discrepancy lies in the abundance of acidic structures based on total GMOs (about 95 % of the total GMOs) [17].

Furthermore, neutral structures such as 2′-FL (2.2–31.6 mg/L) are present in lower concentrations or sometimes undetectable in goat milk, while they are more abundant in human milk [38,40,41].

In the present study, we observed higher concentrations of oligosaccharides in human milk compared to bovine and goat. Our findings are consistent with recent research [14], which employed relative quantification by UPLC-Q Exactive-HF Orbitrap LC-MS/MS system. They identified up to 70 oligosaccharide types in human milk, as opposed to 14 and 23 classes in bovine and goat milk, respectively. Other study, reported average concentrations of total GMOs, BMOs and HMOs of 0.084 g/L, 0.077 g/L and 1.5 g/L, respectively. These concentrations are lower than those found in the present study for goats and human but align with the results for bovine. Unlike our study, LNT was not detected in goat and 2′-FL was not detected in bovine [42].

Other researchers, developed a technique based on capillary electrophoresis to quantify three oligosaccharides (3′-SL, 6′-SL, and disialyl-lacto-N-tetraose (DSLNT)) in different mammals, including bovine, goat, and human. That study did not detect 6′-SL in the goat and cow samples, but 3′-SL was found in 12.4 (goat) and 30 times (bovine) less concentration than in humans [43].

Comparative studies on oligosaccharide concentrations in mammals, along with the identification or relative quantification of previously reported or unreported oligosaccharides are complementary to understanding the complexity and richness of these compounds in milk. [41]; confirmed that goat milk exhibits a greater diversity of oligosaccharides than bovine milk. However, the present study primarily focuses on the absolute quantification of the seven most representative oligosaccharides in mammalians.

5. Conclusion

An improved method based on UPLC-MS/MS was developed and validated for the absolute and simultaneous quantification of seven oligosaccharides in various mammalian milk matrices. The sample preparation procedure employed in this analytical method allows for efficient recovery of the oligosaccharides, eliminating the need for additional steps while avoiding matrix effects that could interfere with the compound's quantification. This developed method was effectively applied to establish differences in the profiles and concentrations of oligosaccharides among human, goat, and bovine milk.

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CRediT authorship contribution statement

Víctor H. Urrutia-Baca: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Cristina Chuck-Hernández:** Resources, Supervision, Writing – review & editing. **Janet Gutiérrez-Uribe:** Funding acquisition, Project administration, Writing – review & editing. **Perla A. Ramos-Parra:** Formal analysis, Supervision, Validation, Writing – review & editing. **Cuauhtemoc Licona-Cassani:** Supervision, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

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