Effect of Fat on Protein Estimation in Milk and Its Correlation with Lactose in Different Milk Types: A Small-Scale Study

Sweekruthi A. Shetty¹, Melissa F. Young², Sunita Taneja³, Kannan Rangiah

ABSTRACT

Background: Estimation of macronutrients like protein and lactose is important to assess the quality of milk. To estimate these two macronutrients, ten raw milk samples obtained from each group of different animals (cow, goat, buffalo), ten pasteurized cow milk and ten human milk samples were analysed.

Methods: Bicinchoninic acid (BCA) method was used to estimate protein from different milk samples. Four different sample preparation protocols were compared to check the effect of fat on BCA based protein estimation: dilution (D), fat removal-protein precipitation (FR&PP), fat removal-dilution (FR&D) and dilution-fat removal (D&FR). For lactose quantification, ultrahigh-performance liquid chromatography-mass spectrometry-selected reaction monitoring (UHPLC-MS/SRM) method was developed and validated using $^{13}$C$_6$ lactose as internal standard (ISTD).

Result: Among these four different protocols, D&FR method showed consistent data for total protein content in animal milk (cow-3.16%, goat-3.21%, buffalo-3.81%, pasteurized-2.98%) and FR&PP showed consistent data in human milk samples (1.2%). Though BCA method is simple to use, proper sample preparation protocol has to be applied prior to protein estimation to avoid the interference due to fat or lactose. In case of lactose, inter-day validation showed the accuracy ranging from 97.13 to 100.54%, coefficient of variation varying between 0.1 to 1.53%, correlation $R^2$=0.999. Lactose is in the range of 4.1 to 4.8% in animal milk and 6.6% in human milk samples. The internal ratio of lactose/protein (1.28 to 1.55 in animal milk and 5.33 in human milk) will be useful to differentiate human milk from animal milk type and to assess the milk quality.

Key words: Milk, Protein, Lactose, Quantification, UHPLC-MS/SRM.

ABBREVIATIONS

D-Dilution; FR&PP-fat removal-protein precipitation; FR&D-fat removal-dilution; D&FR-dilution-fat removal; BCA- Bicinchoninic acid; UHPLC-MS/SRM-Ultrahigh Performance Liquid Chromatography-Mass Spectrometry-Selected Reaction Monitoring; CM-Cow Milk; BM-Buffalo Milk; GM-Goat Milk; PM-Pasteurized Milk; HM-Human Milk; LOQ-Limit of Quantification; LQC-Lower Quality Control; MQC-Middle Quality Control.

INTRODUCTION

Milk is a highly nutritious liquid formed in the mammary glands of mammals to sustain their new-borns during their initial months of life. The nutritional composition of milk is highly complex and it contains almost every single nutrient that human body needs. Milk contains essential micro and macronutrients; the nutritional wholeness of milk and its vital utility especially for children has been recognized by all cultures across the globe. Milk is known to be a major deciding factor in the early days of mammal’s life and WHO recommends exclusive breast-feeding for infants in the initial six months (WHO, 2011). Though animal milk is not recommended for initial year of life but still around 15% of infants receive animal milk along with breast milk in India (Mayuri et al., 2012). Majorly cow milk is the one used for human consumption (83%) in the overall milk production in the world. Next to cow milk, buffalo milk with 13%, goat milk with 2%, sheep milk with 1% and camel milk provide 0.3% be consumed around the globe (Food and Agriculture Organization, 1945). India is the largest producer of milk in the world (176 million tonnes during 2017-18) mainly from cow, buffalo and goat. Over all, around 92% of buffalo milk and 26% goat milk are produced in India (National Dairy Development Board, 1965). Milk is consumed not only in the liquid form but also in different forms like flavoured milk, ice cream, cheese, butter, yogurt, casein powder and many other dairy products. Due to the huge market value for milk and milk products, adulteration is quite rampant. Though the milk production is adequate, its nutritional quality is important to be maintained and checked routinely.

¹Food Safety and Analytical Quality Control Laboratory, CSIR-CFTRI, Mysore-570 020, Karnataka, India.
²The Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, USA.
³Centre for Health Research and Development, Society for Applied Studies, 45, Kalu Sarai, New Delhi-110 016, India.

Corresponding Author: Kannan Rangiah, Institute of Bioinformatics, Discoverer Building, International Tech Park, Whitefield, Bangalore-560 066, Karnataka, India.

Email: rkannan007@gmail.com

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Protein, carbohydrate and fat are the three major macronutrients present in milk along with micronutrients like vitamins, amino acids etc. (Muehlhoff et al., 2013; Milk facts). Macronutrients present in animal/human milk are key essential components required to maintain the nutritional/energy balance in the early days of life. In case of milk proteins, it is present in two different types like casein (αS1, αS2, β, κ and γ) constitute around 80% and whey (α-lactalbumin, β-lactalbumin, bovine serum albumin, immunoglobulins, lactoferrin and lactoperoxidase) is around 20% (Layman et al., 2018). Milk proteins are considered as good protein source because of all nine essential amino acids are present in proportions needed as per human requirements. Most importantly, the branched-chain amino acid like isoleucine, leucine and valine contents are at higher levels in milk proteins than in many other food sources (Layman et al., 2018). Another macronutrient carbohydrate is chiefly present in the form of lactose (glucose-galactose) in milk with trace amounts of monosaccharides and oligosaccharides (Milk facts). There are many factors like different breeds of animals, stage of lactation, climate change around the globe, environmental pollution, feed contamination etc., which affects the milk quality. Thus, the estimation of total protein and lactose in animal/human milk becomes imperative to check nutritional quality consumed in the household level.

There are different methods been reviewed for macronutrient analysis in human milk by Miller et al., (2013). For protein estimation the methods like Kjeldahl analysis, Biuret assay, Lowry-Peterson assay, Bio-Rad assay, Bicinchoninic acid (BCA) assay are widely used (Keller et al., 1986; Bergqvist et al., 1989). The details of these methods were explained elsewhere, total nitrogen content using Kjeldahl analysis is considered as gold standard method for the protein estimation (Kjeldahl 1983; Lynch et al., 1999). The major constraints in this assay are higher sample volume (5 to 10 mL) and lower throughput. To check the protein content in milk, it has to be precipitated first from the nonprotein component and multiplied by a factor of 6.38 to express the data in terms of protein (Lynch et al., 1999). In the other calorimetric methods, the sensitivity is much higher but the specificity is lower due to the interference from other biological matrices. It has been shown that fat granules and lactose present in the milk are known to interfere with the colorimetric assay (Keller et al., 1986). It is necessary to remove these two components based on the milk type before doing protein estimation using colorimetric assay. Among the calorimetric methods, BCA method showed a good correlation with Kjeldahl analysis in the protein estimation from animal/human milk samples (Keller et al., 1986; Kamizake et al., 2003). It can also be made into high/medium through put using the micro-titre plate procedure, which can drastically reduce the analysis time compared to Kjeldahl analysis.

In case of lactose, there are official AOAC methods to quantify in milk, such as polarimetry, mid-infrared detection, gravimetry, differential pH, enzymatic methods detecting either the glucose or galactose moiety of lactose and HPLC based methods (Horowitz 2005 a,b,c; Lactose content 2007). Though these methods are simple to use, but they lack in sensitivity and specificity. Whereas LC-MS based methods are simple, quick and reliable with high precision and accuracy compared to values generated by subtraction of protein and fat contents in whole milk or enzymatic method (Fusch et al., 2011).

In the present work, in order to check the effect of fat on BCA based protein estimation, we have prepared samples in four different ways like dilution (D), fat removal-protein precipitation (FR&PP), fat removal-dilution (FR&D) and dilution-fat removal (D&FR) to estimate total protein from animal/human milk. For lactose quantification, we have diluted the milk samples and quantified using validated UHPLC-MS/SRM method. We have applied these established methods to estimate both protein and lactose in animal milk (n=10, in total 40 samples from cow, goat, buffalo and pasteurized cow milk) and in human milk (n=10) samples.

MATERIALS AND METHODS

Materials

Lactose was obtained from Sigma (Sigma-Aldrich, Bangalore, India) and Lactose-15C2 was obtained from Cambridge Isotope (Cambridge Isotope Laboratories, Inc., MA, USA). Bicinchoninic acid (BCA) kit was obtained from Pierce (Sigma-Aldrich, Bangalore, India). High quality solvents (Acetonitrile, Methanol and Ethanol) used for the sample preparation and LC-MS grade solvents (Acetonitrile, Water, Methanol) used in the mobile phase were obtained from Honeywell Research Chemicals (Honeywell Technology Solutions Lab Pvt. Ltd., Bangalore, India). Collection and storage of animal milk samples were as mentioned in the previous article (Shetty et al. 2020). The experiments were carried out between 2017 to 2019 at Food Safety and Analytical Quality Control Laboratory, Central Food Technological Research Institute, Mysore. Human milk samples were collected from the on-going study to look at the maternal nutrition and lactation in India, necessary approvals were obtained from the ethical committee (ERC: FWA00001757, IRB: 00007526) for the study.

Protein estimation from milk

Total protein content was estimated in milk samples by BCA assay using BSA standard as described in the kit. Estimation of protein in milk was done after following four different ways of sample preparation.

Dilution (D)

Milk samples were diluted (50 fold- for cow, goat, pasteurized and human; 100 fold-for buffalo) in MS grade water and 20 μL of diluted sample was used to estimate total protein.

Fat removal and protein precipitation (FR&PP)

Milk samples were centrifuged (2000 rpm, 5 min, 10°C) to separate fat layer on top. From the bottom layer, 20 μL of...
sample was taken and mixed with 100 μL of methanol to precipitate proteins. It was then centrifuged (10000 rpm, 5 min, 10°C) to get the protein pellet and then pellet was further redissolved in 1 μL of MS grade water and 20 μL of sample was used for the protein estimation.

**Fat removal and dilution (FR&D)**

Milk samples were centrifuged (2000 rpm, 5 min, 10°C) to separate fat layer on top. After removing the fat layer from top, 20 μL of the bottom layer was taken and diluted to 1 μL in MS grade water and used for protein estimation.

**Dilution and Fat removal (D&FR)**

Milk samples (20 μL) were diluted directly to 1 μL ice-cold MS grade water (~5 to 10°C) first and centrifuged (2000 rpm, 5 min, 10°C) to separate residual fat layer on top. From the bottom layer, 20 μL of sample was used for the protein estimation.

The diluted milk (20 microliter) was mixed with 200 microliter reagent and incubated at 37°C for 10 min. The absorbance was checked at 562 nm using ELISA reader (Clario Star, BMG Lab Tech). All samples were taken in triplicate for protein estimation along with BSA standards in different amounts (1 to 20 μg).

**LACTOSE ANALYSIS**

**Standard preparation and LC-MS conditions**

Lactose standard (STD) and 13C5-lactose internal standard (ISTD) stock solutions of 1 μg/mL were prepared in ammonium acetate (10 mM, pH 5) and stored at -20°C. The highest STD 100 μg/mL was prepared in the same solvent and further diluted successive two fold dilutions to get 100 ng/10 μL to 1.56 ng/10 μL on column to construct the STD curve. Milk samples were analysed by using Agilent 1290 infinity II UHPLC (Agilent Technologies India Pvt. Ltd., Bangalore, India) connected with Sciex 6500 QTRAP (Sciex, Singapore) system. It is equipped with turbo V ion source for the effective ionisation. Scherzo SM-C18 column from Imtakt (C-18, 3 μm, 2.1x100 mm column), mobile phase A-Water (10 mM Ammonium acetate, 0.1% FA), B-Acetonitrile (0.1% FA), Gradient: 0 min- 2% B, 0 to 5 min -50% B, 5.1 to 7 min-100% B, 7.1 to 10 min-2% B and flow rate- 200 μL/min were used in the LC system. The MS conditions were: ion spray voltage-5500 V, Temperature-500°C, ion source gas 1-50 psi, ion source gas 2-30 psi and curtain gas-40 psi. In order to get the highest intense product ion, lactose STD and ISTD 10 μg/mL solutions were infused to MS through syringe pump and the other conditions like declustering potential, entrance potential, exist potential and collision energy were optimised through compound optimization mode.

**Sample preparation and method validation**

Since lactose content is higher (6-7%) in human milk compared to animal milk (4-5%), 5000-fold diluted human milk samples and 1000-fold diluted animal milk samples were used for the analysis. Briefly, 10 μL of milk (cow milk, goat milk, buffalo milk and pasteurized cow milk) was mixed with 10 μL of ammonium acetate (10 mM, pH:5) and vortexed, centrifuged (5 min, 5000 rpm). 10 μL of ISTD (from 100 μg/mL) was spiked to 90 μL diluted milk and from this 10 μL was injected to UHPLC-MS/SMR method for analysis. In case of human milk, it was further diluted to another 5-fold before injection. The method validation was done by constructing the STD curve (1.56 to 100 μg/mL) along with quality control samples (LOQ-3.12, LQC-6.25, MQC-25 and HQC-80 μg/mL) by spiking 5 replicates of each in ammonium acetate (10 mM, pH: 6). The same experiment was repeated thrice on different days to assess the inter-day validation. Method validation was done before quantifying lactose from animal and human milk samples.

**RESULTS AND DISCUSSION**

**Protein estimation in milk samples**

As a macronutrient, the total protein content in milk is important to maintain the daily dose for child/human health. Protein estimation becomes vital to check the quality of human/animal milk samples that we use routinely. Among the different methods, Kjeldahl analysis is considered as a standard method for protein estimation (Kjeldahl 1983). However, this method requires higher sample volume and it is considered as low throughput due to multiple steps involved in the sample preparation before doing protein estimation. Indeed, there is a need for simple, accurate, sensitive, high/medium throughput method using lesser volume of milk for protein estimation to use routinely in the lab. There are always disputations in the literature while using other calorimetric methods for protein estimation in milk. Among the methods, BCA method showed comparable data with Kjeldahl analysis using defatted human milk samples (Keller et al., 1986; Bergqvist et al., 1989). It has been shown that milk fat globules interfere with calorimetric assay like BCA in human milk samples (Keller et al., 1986). In one of the recent studies, protein estimation in human milk showed 1.14% after dilution using BCA method without fat removal (Giuffrida et al., 2019). So there are controversies in literature, we wanted to check is there any real effect of fat on protein estimation using BCA method with BSA standard in micro-titre well plate procedure.

We compared four different sample preparation methods to estimate total protein concentration in animal milk (cow, goat, buffalo) and human milk. Pasteurized cow milk samples were also taken to check the total protein. Just 20 μL of diluted milk was mixed with BCA reagent in the micro-titre well plate procedure and the absorbance was checked at 562 nm. Seven different concentrations of BSA (0.312 μg to 20 μg) were used to construct linear standard curve and the mean of triplicate samples were used for estimation. The linear regression line equation (R=0.999) was used to calculate the concentration of total protein in the milk samples (Fig 1A). Reagent alone control and blanks were taken along with milk samples for each batch.
Before doing the estimation macronutrient analysis (protein and lactose) in milk samples, we checked in literature for the published values for these two in different milk types. We have compiled nine different studies for animal milk types and five different studies for human milk samples (Table 2) (Barłowska et al., 2011; Park et al., 2007; Linn 1988; Kanwal et al., 2004; Zhou et al., 2018; Kulkarni 2017; Kapadiya et al., 2016; Fangmeier et al., 2019; Wahid et al., 2011; Lubetzky et al., 2015; Moran-Lev et al., 2015; Hibberd et al., 1982; Beijers et al., 1992; Giuffrida et al., 2019). Keeping these values as reference, we checked four different sample preparation protocols as mentioned in method section for total protein estimation.

Direct dilution (D) of milk in MS grade water showed higher percentage of protein content in milk samples (buffalo-10.09%, cow-5.94%, goat-7.71%, pasteurized-6.25%, human-2.7%), these values are at least 2 fold higher compared to the published report (Table 2). This might be due to the interference of either fat/lactose or other nitrogen containing compounds like urea, amino acids present in the milk, which interfere in the assay, leads to overestimation of protein. To further check, fat content was removed from milk by centrifugation, which settles in the top. After removing the fat, using methanol, milk proteins were precipitated as shown in the method FR&PP. The protein pellet was redissolved in MS grade water and used for estimation. These samples showed lesser percentage of protein for animal milk (cow-2.29%, goat-1.47%, buffalo-2.09%, pasteurized-1.80%) compared to the published reports as shown in Table 2. In case of human milk samples, the estimated value (1.2%), which is very close to the published reports (Table 2). One of the reasons for the lower value for animal milk might be due to the lesser solubility of protein pellet back into water after precipitation. However, human milk has comparatively lesser protein concentration than animal milk, showed no issues in re-dissolving the protein pellet back into water. To avoid the issues in protein precipitation, the defatted milk samples were directly diluted in MS grade water and used for protein estimation as mentioned in FR&D. These samples showed slightly higher values (cow-3.93%, goat-3.89%, buffalo-5.7%, pasteurized-3.25%, human-1.6%) compared to the published reports as shown in Table 2. This might be due to the interference of residual fat/lactose contents still present in the sample preparation. Finally, instead of centrifuging the whole milk for fat removal, milk samples were diluted in ice-cold water (~5 to 10°C) first and centrifuged (2000 rpm, 5 min, 10°C) to separate the fat layer on the top as mentioned in D&FR. The bottom layer of the sample was used for the protein estimation, which showed comparable data for animal milk (cow-3.16%, goat-3.21%, buffalo-3.81%, pasteurized-2.98%) with published reports (Table 2). Whereas, human milk samples showed higher protein concentration in this protocol (2.1%). This might be due to interference of lactose, which is higher in human milk compared to animal milk samples.

Our data clearly indicates that simple dilution of milk is not enough to remove the interference due to fat or lactose content in the micro-titre plate based BCA assay. The effect of the other components like amino acids and urea in animal milk seems to be negligible when compared to fat or lactose, so D&FR seems to be the best for animal milk types. Since lactose content is higher (6-7%) in human milk compared to animal milk (4-5%), FR&PP seems to be the suitable method. The standard curve used for the protein estimation and estimated values using all four protocols for animal/ human milk samples were shown in the Fig 1.  

**Table 1: Lactose method validation table.**

| Validation | LOQ | LQC | MQC | HQC |
|------------|-----|-----|-----|-----|
| Concentration in (ng/mL) | 3.12 | 6.25 | 25.00 | 80.00 |
| Inter-day mean (n=3) | 3.09 | 6.07 | 24.95 | 80.43 |
| % CV | 1.53 | 0.58 | 0.88 | 0.10 |
| % Accuracy | 99.19 | 97.13 | 99.79 | 100.54 |

**Fig 1: Protein estimation from milk samples.**

(A) STD curve, (B) Protein estimation from animal milk samples, (C) Protein estimation from human milk samples using four different sample preparation protocols (D-Dilution, FR&PP-Fat removal and protein precipitation, FR&D- Fat removal and dilution, D&FR-Dilution and fat removal) (CM-cow milk, GM-goat milk, BM-buffalo milk, PM-pasteurized milk, HM-human milk). The median values are indicated for the human milk samples in all four protocols.
Lactose method validation and quantification from milk samples

To measure lactose, we have established UHPLC-MS/SRM method as mentioned in the method section. For initial standardisation, 10 µg/mL solution of lactose STD and lactose ISTD in ammonium acetate buffer (10 mM, pH ~5) were infused to mass spectrometer through the syringe pump at a flow rate of 10 µL/min. The mass spectrometry conditions were shown in the experimental section. Both lactose STD (343.13 m/z) and lactose ISTD (349.3 m/z) showed M+H ions in the positive polarity. However, in the previously published protocols negative ionisation mode was used for lactose quantification (Fusch et al., 2011). In this study, the dilution of lactose was done in ammonium acetate (10 mM, pH 5), which showed better signal in the positive ion mode for both lactose STD and lactose ISTD. The typical MS/MS spectra for both lactose STD and lactose ISTD were shown in the Fig 2A. One of the product ions is the water loss from parent mass (lactose STD-331.2 and lactose ISTD-325.2 m/z). The highest intense product ion is same for both lactose STD and lactose ISTD (163 m/z). Based on the MS/MS spectrum, the predicted the structures for the product ions were shown in the Fig 2B. The product ions (lactose: 343.2 → 163, lactose ISTD: 349.3 → 163 m/z) were considered for the estimation of lactose. The UHPLC-MS/ SRM chromatogram of lactose STD/ISTD is shown in Fig 3A. The separation of lactose from other disaccharides like sucrose and maltose is always challenging in the reverse phase chromatography. Since milk is known to contain disaccharide mainly in the form of lactose and further dilution of milk samples known to eliminate the effect of other disaccharides on lactose quantification.

Table 2: Protein and lactose percentage in milk samples.

| No | Protein (%) | Lactose (%) | Protein (%) | Lactose (%) | Reference | Protein (%) | Lactose (%) | Reference |
|----|-------------|-------------|-------------|-------------|-----------|-------------|-------------|-----------|
| 1  | 3.42        | 4.82        | 3.26        | 4.51        | 4.38      | 4.79        | Barlowska et al., 2011 | 0.90      | 5.80      | Lubetzky et al., 2015 |
| 2  | 3.20        | 4.70        | 3.40        | 4.10        | 3.87      | 3.92        | Park et al., 2007      | 1.08      | 4.12      | Moran-Lev et al., 2015 |
| 3  | 3.50        | 4.60        |             |             | -         |             | Linn 1988              | 1.40      | 5.34      | Hlberd et al., 1982   |
| 4  | 4.03        | 4.66        | 3.87        | 3.92        |          |             | Kanwal et al., 2004    | 1.37      | -         | Beijers et al., 1992  |
| 5  | 4.50        | -           | 4.80        |             |          |             | Zhou et al., 2018      | 1.14      | 6.44      | Giuffrida et al., 2019 |
| 6  | -           | -           | -           | -           | 4.76      | 5.19        | Kulkarni 2017          | -         | -         |                    |
| 7  | 3.49        | 4.76        | 3.42        | 4.16        | 4.48      | 4.86        | Kapadiya et al., 2016  | -         | -         |                    |
| 8  | 3.39        | 3.83        | 3.24        | 4.46        | 4.30      | 5.55        | Fangmeier et al., 2019 | -         | -         |                    |
| 9  | 3.30        | 4.60        |             |             | 4.40      | 5.50        | Wahid et al., 2011     | -         | -         |                    |
| Mean| 3.38        | 4.48        | 3.33        | 4.38        | 4.43      | 4.97        |                        | 1.18      | 5.43      |                    |

Lactose /Protein

Fig 2: Analysis of lactose.

(A) MS/MS spectrum for lactose STD and ISTD, (B) predicted chemical structures for product ions.
Since the lactose is present in higher concentration in the milk samples, the STD curve was constructed in the range of 1.56 to 100 μg/mL against the ratio (lactose/lactose ISTD). It is linear over 64-fold concentration range, with regression of 0.9996 (Fig 3B). The validated methods showed excellent accuracy and precision. The inter-day accuracy (n=3) for LOQ (3.12 μg/mL) is 98.49%, LQC (6.25 μg/mL) is 98.75%, MQC (25 μg/mL) is 97.73% and for HQC (80 μg/mL) is 98.2%. The inter-day precisions (n=3) were in the range of 0.72 to 6.23 for LOQ and QCs (Table 1). Using this method, lactose concentration in different animal milk samples showed almost similar concentration, in cow milk-4.73%, goat milk-4.08%, buffalo milk-5.17% and pasteurized milk-4.95%. In case of human milk samples, it showed higher (6.6%) compared to animal milk. The lactose percentage value for five different types of milk is shown in the Fig 3C. In both animal and human milk samples, the estimated lactose value is similar to the published reports as shown in the Table 2. UHPLC-MS/SRM based methods using stable isotope labelled ISTD are considered as gold standard for quantification of biomolecules. These methods showed precise estimation compared to enzymatic assay kit and can detect lactose not only in the normal milk but also in the lactose free milk samples (Garballo-Rubio et al., 2018).

The lactose to protein ratio in cow milk-1.51, goat milk-1.35, buffalo milk-1.28, pasteurized milk-1.66 and human milk-5.33. It has close correlation with the published reports, which showed for cow milk-1.32, goat milk-1.31, buffalo milk-1.12 and human milk-4.61 (Table 2). The lactose/protein ratio will be useful to differentiate animal and human milk types.

CONCLUSION
Here we have established protocols to quantify the macronutrients (protein and lactose) from different animal milk and human milk samples. Dilution of milk in ice-cold water followed by separation of fat layer by centrifugation (D&FR) seems to be crucial for accurate estimation of protein from animal milk samples and fat removal and protein precipitation (FR&PP) is imperative to check total protein content in human milk samples using BCA method in micro-titre plate format. In case of lactose, simple dilution of milk in ammonium acetate buffer (10 mM, pH 5) seems to be important to look at the signal in positive ion mode in the mass spectrometry. We have applied these two methods to estimate protein and lactose concentration in milk samples (cow, goat, buffalo, pasteurized cow milk and human milk) and both of these methods showed comparable data with previously published values. The internal ratio lactose/protein (1.28 to 1.55 in animal milk and 5.33 in human milk) will be useful to differentiate human milk from other animal milk. Since these methods involve simple sample preparation and can be useful for the routine estimation of protein and lactose from milk samples.

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Conflict of Interest
The authors declare no conflict of interest.

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