Original Article

Establishment of micromethods for macronutrient contents analysis in breast milk

Arum Choi, Gerhard Fusch, Niels Rochow, Natasha Sheikh and Christoph Fusch

Division of Neonatology, Department of Pediatrics, McMaster University, Hamilton, Ontario, Canada

Abstract

Commercially available milk analysers were originally developed for use in the dairy industry, but they are now used to analyse macronutrient content of breast milk in clinical studies and routine care of the premature or very low birthweight (VLBW) infants. Due to the different composition of cow and breast milk, these devices need to be validated against reference methods before they can be used in daily routine. However, current reference methods require a sample volume of 30–100 mL to analyse fat, protein and lactose. It is not feasible to obtain this volume of milk for research purposes, especially from VLBW infants as lactation may be delayed or impaired and the limited volume of breast milk must be provided to the infant. To support validation of milk analysers in both clinical and research settings, the aim of this study is to establish and validate micromethods for precise macronutrient analysis in small volume of breast milk and conduct a feasibility study of the micromethods as a post-validation. Methods include a modified Mojonnier ether extraction (fat), elemental analysis (protein) and ultra-performance liquid chromatography-tandem mass spectrometry (lactose). We were able to downsize volumes required for analysis of fat, protein and lactose to 1 mL, 260 μ L and 100 μ L; corresponding coefficients of variation are 1.7, 1.8 and 2.3%, respectively. The presented methods allow for reliable and precise analyses of macronutrients in ≤ 1.5 mL of breast milk and will be used to validate milk analysers.

Keywords: breast milk, macronutrients, preterm infants, neonatal nutrition, validation, quantitative methods.

Correspondence: Gerhard Fusch, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1. E-mail: gefusch@mcmaster.ca

Introduction

Breast milk is recommended as the primary source of nutrition and for promoting growth and development of preterm infants (O'Connor *et al.* 2003; Puntis 2006). Breast milk shows immunological benefits and reduces the risks of developing diseases such as necrotizing enterocolitis or sepsis (Schanler *et al.* 1999). Due to the higher nutritional uptake of

preterm infants compared to term infants, there is a need of additional macronutrients to compensate for inadequate protein and mineral contents in native breast milk. Therefore, fortifiers are routinely added to native breast milk in standard amounts. However, the macronutrient composition of breast milk (i.e. fat, protein, lactose) varies between mothers as well as between feeds. Due to the natural variation in macronutrient composition of breast milk, adding a

standardised fortifier may still sometimes fall short of recommended macronutrient levels for preterm infants (Polberger 2009). Alternatively, individualised fortification with adjustment of each macronutrient may be preferable (Polberger 2009; Arslanoglu *et al.* 2010). This concept of 'individualised fortification' for very low birthweight (VLBW) infants encompasses a system of nutritional management that precisely monitors the nutritional intake of each infant and promotes *in utero* growth rates recommended by the American Academy of Pediatrics (American Academy of Pediatrics, Committee on Nutrition 2004). In order to implement this system in clinical routine, a rapid analysis of fat, protein and lactose content of breast milk for an individual infant is needed, ideally as a point-of-care method.

The current gold standard methods for macronutrient analysis include Mojonnier ether extraction, Kjeldahl and high-performance liquid chromatography (HPLC) methods for fat, protein and lactose, respectively [International Committee for Animal Recording (ICAR) 2005]. They require sample volumes of 30–100 mL, which makes it nearly impossible to analyse milk samples from mothers of extremely preterm infants, especially in the early post-natal days. Each drop of breast milk is extremely valuable for early nutrition in these infants and therefore, it is not very likely that amounts of up to 100 mL will become available (Ellis & Picciano 1993; Meier *et al.* 2007). In light of the advantage that breast milk provides, obtaining such volumes would also pose ethical issues.

There are recent reports indicating that commercially available infrared (IR) spectroscopic milk analysers are now being used in neonatal intensive care units (NICUs) to provide the analytical basis to adjust macronutrient contents in small volume of breast milk (Corvaglia *et al.* 2010; Sauer & Kim 2011).

However, these devices have originally been validated for use in the dairy industry to measure cow milk. In consideration of the significant difference in the composition of cow and breast milk, there is a need for validation of these devices using breast milk samples before they can be reliably used for individualised fortification in a clinical routine or in research studies. As a general principle, validation must be performed using an appropriate number of samples in conditions that are identical to those intended for later use of the devices. Because the composition of breast milk changes with gestational and post-natal age, breast milk samples including those from mothers of extremely preterm infants as well as early lactation period need to be measured. Thus, precise micromethods will be needed to facilitate validation of milk analyser devices for clinical practice and research use in preterm nutrition.

Owing to the limited availability of preterm breast milk, the aim of this study is to establish and validate micromethods for the modified Mojonnier ether extraction, elemental analysis and ultra-performance liquid chromatography-tandem mass spectrometry that allow precise analysis of fat, protein and lactose content in less than 1.5 mL of breast milk. Furthermore, we demonstrate in a feasibility study that these methods have the potential to be successfully used in a clinical study.

Materials and methods

Reference materials

Commercially available reference cow milk as well as custom-made reference breast milk were used for a quality assessment of macronutrient contents. An overview of reference materials and each analysis is demonstrated in Fig. 1. For cow milk, a set of refer-

Key messages

- Micromethods were established and validated to measure precise and accurate fat, protein and lactose concentrations using less than 1.5 mL breast or cow milk.
- With these new micromethods, valuable preterm milk can be analysed, which currently certified methods cannot measure due to its limited volume.
- These established micromethods can be used as a tool to validate milk analysers for bedside usage.

Fig. 1. Overview of externally analysed reference materials (cow and breast milk).

ence samples with known concentrations of fat ($n = 13$), protein ($n = 10$) and lactose ($n = 10$) contents were purchased from a certified laboratory (Central Milk Testing Laboratory, Edmonton, AB, Canada). Reference materials for breast milk are not commercially available. Therefore, we manufactured a set of reference breast milk samples ($n = 30$) from mothers' with preterm infants, which covered the variability of macronutrient concentrations in breast milk. These samples were analysed by two laboratories, which were accredited by the Standards Council of Canada. Due to the availability of laboratory capacity and sufficient volumes of breast milk samples, 18 samples were sent to Laboratory Services at University of Guelph (Guelph, ON, Canada) for analysis of total protein ($n = 18$) and true protein ($n = 9$). The remaining 12 samples were sent to Meyer Laboratory Services (Long-Sault, ON, Canada) to determine protein and fat. In six samples, only fat was analysed; in three samples, fat and total protein was determined; and in the remaining samples ($n = 3$), only protein was measured. Both laboratories used Mojonnier ether extraction and Kjeldahl method to analyse fat and protein, respectively. Additionally, to create a reliable internal standard for daily quality control, 500 mL of breast milk was pooled from a random selection of different breast milk samples, subsequently homogenised with heat and stir bar, and divided into 1 mL aliquots ($n = 500$).

Milk sample preparation

For each analysis of fat, protein and lactose content, stored cow and breast milk samples as well as the

daily quality control samples were thawed in a 37°C water bath and homogenised three times for 10 s using an ultrasonic vibrator (VCX 130; Chemical Instruments AB, Sollentuna, Sweden).

Fat analysis method: modified Mojonnier ether extraction

Mojonnier ether extraction (AOAC method 989.05), which is regarded as the gold standard method, was used for analysis of fat content in milk (AOAC International 2006). The sample volume was scaled down from originally 10 mL to 1 mL and was used for analysis of cow and breast milk samples. The principle of the method is to separate the fat fraction confined to the lipophilic ether phase from the rest of the milk sample. The dried ether phase is gravimetrically analysed to determine of total fat content.

Reagents

Ethanol (95%) was obtained from Commercial Alcohol (Brampton, ON, Canada). Anhydrous ethyl ether, petroleum ether, ammonium hydroxide (ACS grade, specific gravity 0.9 g mL⁻¹) and phenolphthalein were purchased from Caledon Laboratory Ltd (Georgetown, ON, Canada). The phenolphthalein was dissolved in 0.5% w/v ethanol and used to facilitate visualization as a pH indicator.

Procedures

The initial weights of approximately 1.0 mL of breast milk was measured in a clean and dry 15 mL Falcon

tube using an analytical balance (Mettler AT261, Greifensee, Switzerland) with a readability of 0.01 mg. 0.5 mL of ammonium hydroxide and two drops of phenolphthalein solution were added to the sample prior to the first round of extraction. About 1.0 mL of ethanol, 2.5 mL of ethyl ether and 2.5 mL of petroleum ether were subsequently added. The sample was vortexed for 30 s. To separate the two layers of the solution, the sample was centrifuged for 3 min at 4000 g. The ether phase was decanted into a pre-weighed dish and placed in a fume hood to allow for evaporation of the ether. Using the aqueous phase, the procedure was repeated twice with the addition of 1 mL of ethanol, 1.5 mL of ethyl ether and 1.5 mL of petroleum ether to the sample; however, the addition of ammonium hydroxide and phenolphthalein solution was omitted. After the ether had evaporated, the fat was dried in a gravity convection oven (Isotemp; Thermo Scientific, Waltham, MA, USA) at 100°C for 30 min to remove remaining volatile compounds. The sample was placed in a desiccator to cool for 10 min and was then weighed. A blank extraction was performed using 1 mL of water in place of milk sample. Calculations with obtained measurements were then performed to determine the fat content of the sample.

$$\text{Fat(g/kg)} = \frac{m_{\text{fat}} - m_{\text{empty}} - m_{\text{blank}}}{m_{\text{milk}}} \times 1000$$

with m_{fat} = weight of the dish with fat in grams, m_{empty} = weight of the empty dish in grams, m_{blank} = weight of the extracted blank water sample in grams and m_{milk} = weight of the milk sample in grams.

Protein analysis method: elemental analysis

Protein was analysed by measuring nitrogen content in cow and breast milk using an elemental analyser which is based on the Dumas combustion principle and well described elsewhere (ISO 14891 IDF 185 2002). The sample volume was scaled down from a recommended amount of 0.5 mL to 60 μL . To determine the total protein content in cow and breast milk, the total nitrogen (TN) content is multiplied

by a conversion factor of 6.38 (Loennerdal *et al.* 1984)

$$\text{Total protein} = \text{TN} \times 6.38$$

The true protein amount in breast milk is determined by measuring the difference between total nitrogen and non-protein nitrogen (NPN) and multiplying the result with 6.25 (Loennerdal *et al.* 1984). The total nitrogen and non-protein nitrogen were analysed in two different runs as described below.

$$\text{True protein} = (\text{TN} - \text{NPN}) \times 6.25$$

Reagents

Sulfanilamide and tryptophan were obtained from Elementar Americas Inc. (Mt. Laurel, NJ, USA) and Sigma (St. Louis, MO, USA), respectively.

Setting of elemental analysis

The elemental analyser (Vario PYRO cube, Elementar Americas Inc.) was configured in the sequential C and N analysis mode using the recommended standard reactor tube configuration. The oxidation and reduction oven was set to 950°C and 550°C, respectively. Sulfanilamide was measured to determine the daily correction factor and used to control for the stability of the system during each run. The calibration was performed for a mass range of 0.010 to 1.036 mg of nitrogen. Tryptophan was used as a daily quality control.

Procedures

For the measurement of total nitrogen content, 60 mg of breast milk was pipetted into a tin boat and weighed to the nearest 0.01 mg. The sample was dried under N_2 gas at 37°C and analysed. For the measurement of non-protein nitrogen, we modified volumes of the sample and reagents from the method of Barbano *et al.* (1997) to facilitate our small sample size. For protein precipitation, 200 μL of breast milk was treated with 20 μL of 50% trichloroacetic acid solution (TCA) and vortexed for 1 min. The sample

was left at 4°C for a minimum of 1 h to accelerate the process of protein precipitation. The sample was centrifuged at 16 000 g for 10 min. A 200-μL supernatant was weighed in the tin boat and then dried under N₂ gas at 37°C and subsequently analysed. The measured non-protein nitrogen content was corrected for both the dilution effect of the TCA and the volume loss of the fat and protein precipitation. The correction factor $R_p = 1.074$ was calculated using the following equation.

$$R_p = \frac{V_{\text{milk}} \times \delta_{\text{milk}} + V_{\text{TCA}} \times \delta_{\text{TCA}} - (V_{\text{milk}} \times \delta_{\text{milk}}) \times F}{V_{\text{milk}} \times \delta_{\text{milk}}}$$

with V_{milk} = volume of pipetted milk (200 μL), $\delta_{\text{milk}} = 1.03 \text{ g mL}^{-1}$, V_{TCA} = volume of pipetted TCA (20 μL), $\delta_{\text{TCA}} = 1.3 \text{ g mL}^{-1}$, $F = 0.052$, which is the multiplication factor, based on the assumption that breast milk fed to preterm infants contains a mass fraction of about 4.0% fat and 1.2% true protein (i.e. $0.040 + 0.012 = 0.052$) (Hibberd *et al.* 1982).

Lactose analysis method: LC-MS/MS

Lactose concentration in cow milk and breast milk was measured using a recently published method, applied liquid chromatography – tandem mass spectrometry (LC-MS/MS). Procedures and instrumental settings are described in detail in the literature (Fusch *et al.* 2011).

Reagents

[UL-¹³C₁₂]-lactose monohydrate was obtained from Omicron Biochemicals Inc (South Bend, IN, USA), lactose monohydrate was from Sigma-Aldrich (St. Louis, MO, USA). Water, acetonitrile and formic acid were obtained as Optima LC/MS grade from Fisher Scientific (Fair Lawn, NJ, USA).

Procedures

In order to have high sensitivity and specificity for LC-MS/MS detection, both cow and breast milk samples were diluted 150 000-fold with deionised

water. A 200-μL resultant solution was transferred into a last step vial. A solution of [UL-¹³C₁₂]-lactose monohydrate ($26.86 \mu\text{mol L}^{-1}$) as an internal standard was prepared and 100 μL was added to the sample. The final sample was vortexed for 1 min and injected into the LC-MS/MS.

Sample collection for feasibility study

In the study, 40 healthy mothers who delivered infants with a birthweight less than 1500 g and a gestational age less than 32 weeks (NICU, McMaster Children's hospital, Hamilton, ON, Canada) were included with informed consents. Those mothers were identified to produce exceeding amounts of milk for their own infant. With supervision of a lactation consultant, 5 mL sample of each fore, middle and hind milk were collected from a single lactation ($n = 120$) to manifest macronutrient variations in breast milk. Expressed breast milk was homogenised, divided into 1 mL aliquots and stored at −80°C until further analysis. The samples were tested as single analysis for each macronutrient. The study was approved by the Research Ethics Board at McMaster University.

Unit of measurement

If necessary, units were converted from g kg^{-1} to g L^{-1} to compare measurements from the external laboratories thereby assuming that the average density of breast milk is 1.03 g mL^{-1} (Perez-Escamilla *et al.* 1995).

Statistical analysis

For the validation of the new methods, data acquisition was performed using varioPYROcube software v2.0.5 (Elementar Analysensysteme; Hanau, Germany) for protein and QuanLynx quantification software (Waters; Milford, MA, USA) for lactose. These raw data (protein and lactose) as well as the manually recorded weight data (fat) were entered into an Excel sheet and processed as described in the methods. Correlation analysis and Bland-Altman plots for fat and protein were performed using Microsoft Excel 2010 (Microsoft; Redmond, WA, USA).

The correlation plot shows data points, regression line and the 'line of identity', which indicates the ideal correlation between reference and micromethod. For the Bland-Altman plot, we decided to graph the reference method on the x -axis instead of using the mean value of both methods, which is more appropriate from a methodological point of view to demonstrate the agreement of the methods by comparing a new method against an established reference methods (Krouwer 2008).

The identity of the linear regression lines of the fat and protein subsets were tested using Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA), which employs an algorithm that is explained in detail by Zar (1999).

For the feasibility study, box plots were performed using Statistical Package for the Social Sciences v.18 software (IBM; Somers, NY, USA). Paired sample T -tests were run for fat, lactose and true protein to show significant changes of the macronutrients in between a single lactation period using a significance level of $P = 0.05$.

Results

Validation of fat analysis

The validation of the measurement of fat content in cow and breast milk samples is demonstrated in Fig. 2. A statistically highly significant correlation between reference method and our newly developed method is found for both subsets of substrates, cow milk ($y = 1.000x - 0.499$ with an $R^2 = 0.999$, $n = 13$) and breast milk ($y = 0.917x - 1.400$ with an $R^2 = 0.999$, $n = 9$). The correlation for all measurements (cow milk and breast milk, $n = 22$) revealed $y = 0.975x - 0.016$ with an $R^2 = 0.996$. Intra-day and inter-day variations ($n = 10$ each) were $43.3 \pm 0.6 \text{ g L}^{-1}$ [coefficient of variation (CV) = 1.4%] and $43.3 \pm 0.7 \text{ g L}^{-1}$ (CV = 1.7%), respectively.

Validation of protein analysis

The validation of total and true protein in cow and breast milk samples is shown in Fig. 3. Correlation for each subset of samples was highly significant (R^2 from

Fig. 2. Fat content validation: (A) Fat measurements from certified laboratories [Meyer Laboratory Services ($\diamond$) and Central Milk Testing Laboratory ($\bullet$)] vs. the modified Mojonnier ether extraction micromethod (dash line indicates line of identity); (B) Bland-Altman plot shows the differences between fat values obtained by reference laboratories and micromethods represented in the Y-axis. The results obtained using the reference values are represented in the X-axis.

0.91 to 0.99, slopes between 0.9 and 1.0 with negligible intercepts). The correlation of all available protein measurement in cow and breast milk samples confirmed the high accordance between both methods ($y = 1.005x + 0.240$ with an $R^2 = 0.995$). Intra-day and inter-day variations ($n = 10$ each) for total protein were $36.9 \pm 0.4 \text{ g L}^{-1}$ (CV = 1.1%) and $37.2 \pm 0.7 \text{ g L}^{-1}$ (CV = 1.8%), respectively.

Fig. 3. Protein content validation: (A) Total protein and true protein measurements from certified laboratories [University of Guelph (true protein ■, total protein △)], Meyer Laboratory Services (○) and Central Milk Testing Laboratory (○) vs. Elemental analysis micromethod (dash line indicates line of identity); (B) Bland-Altman plot shows the differences between fat values obtained by reference laboratories and micromethods represented in the Y-axis. The results obtained using the reference values are represented in the X-axis.

Validation of lactose analysis

The validation of the LC-MS/MS method to analyse lactose content of breast milk was reported previously (Fusch *et al.* 2011). Intra-day and inter-day variations ($n = 10$ each) were 69.2 ± 1.4 g L⁻¹ (CV = 2.0%) and 69.3 ± 1.6 g L⁻¹ (CV = 2.3%), respectively.

Proof of feasibility in 120 breast milk samples

Breast milk samples ($n = 120$) were measured by the validated micromethods. Average fat content was determined as 42.0 ± 20.6 g L⁻¹ [mean $\pm$ standard deviation (SD)], [37.9 g L⁻¹ (7.0; 109.3)] (mean $\pm$ SD), [median (min; max)]. True protein content was 13.8 ± 4.9 g L⁻¹, [12.4 g L⁻¹ (7.6; 33.9)]. Lactose content was 61.5 ± 11.4 g L⁻¹, [59.0 g L⁻¹ (39.7; 88.1)]. Figure 4 shows the data for fat, protein and lactose content in fore, middle and hind milk. The fat content was the highest in hind milk, being approximately twofold that of fore milk (mean: fore milk 26.3 g L⁻¹, mid milk 44.7 g L⁻¹, hind milk 54.8 g L⁻¹). The mean protein levels did not change during a single lactation (mean: fore milk 14.0 g L⁻¹, mid milk 13.8 g L⁻¹, hind milk 13.8 g L⁻¹). The lactose content demonstrated significant lower mean value for mid milk compared to fore and hind milk (mean: fore milk 63.8 g L⁻¹, mid milk 56.6 g L⁻¹, hind milk 63.9 g L⁻¹).

Additionally, the distribution of nitrogen for total protein, non-protein and protein as well as calculated values of total and true protein in breast milk is shown in Table 1. The non-protein nitrogen content was determined as approximately 18–19% of the total nitrogen content.

Discussion

In the present study, we were able to establish and validate three micromethods to measure fat, protein and lactose content in breast milk with corresponding CV's of 1.7, 1.8 and 2.3%, respectively. We managed to reduce the sample volume required for analysis of a full set of all three parameters from 30 mL to 100 mL down to 1.5 mL. We consider this reduction in sample size as a major step towards the analysis of breast milk samples. The small volume required will make it feasible to measure breast milk samples from mothers with limited milk production. The validated micromethods thus provide the basis to precisely evaluate commercially available milk analysers and will enable clinicians if indicated to monitor and improve nutritional intake of VLBW infants.

Fig. 4. Boxplot of macronutrient contents (lactose, true protein, fat) in breast milk ($n = 40$ for each fore, mid, hind milk) from a single lactation.

Fat measurement

The Mojonnier procedure has originally been accredited by the AOAC for fat extraction in cow milk. In the first step, we successfully validated our micromethod using small volumes of cow milk. As the next step, we extended the procedure to analyse small

volumes of breast milk. For this purpose, the 'true' fat content of our breast milk samples ($n = 9$, see Fig. 2) has been measured in a certified laboratory using the Mojonnier procedure. Although the Mojonnier method has been validated for cow milk, it seems to be reasonable to believe that the procedure can be also applied for breast milk. Breast milk and cow milk contain approximately the same amount of fat (4.2 vs. 3.9 g/100 mL, respectively). The lipophilic fraction in both cow and breast milk is of similar composition and contains primarily triglycerides which account for approximately 98% of total fat amount (Lammi-Keefe & Jensen 1984; Mansson 2008). The minor part of the lipophilic fraction (of approximately 2%) contains other milk lipids such as diglycerides, monoglycerides, phospholipids, cholesterol, glycolipids and free amino acids which is negligible (Lammi-Keefe & Jensen 1984; Mansson 2008). However, the distribution of triglyceride is composed differently in cow and breast milk. The majority of fatty acids in breast milk have a chain length of C10 to C18 with a small percentage of unsaturated long chain fatty acids, C20–C22. In contrast, cow milk contains a notable amount of short chain fatty acids, C4–C10, and medium chain fatty acids but an absence of long chain fatty acids. Although the pattern of the single fatty acids contained in cow and breast milk shows significant differences, the cumulative chain length of triglycerides expresses less variation (breast milk: C38–C60, cow milk: C26–C56) (Breckenridge & Kuksis 1967). Therefore, we do not expect that the Mojonnier ether extraction would influence the ability to accurately analyse fat content in breast milk. Although we managed to downsize the required sample volume already from 10 mL to 1.0 mL, the minimum sample volume could theoretically be reduced even further. However, this approach might become impractical as common scales have a readability of 0.1 mg in most laboratories. Lowering the sample volume further down would increase the error rate.

Protein measurement

In establishing a micromethod for protein analysis, elemental analysis is a simple, fast and convenient

Table 1. Sample description of the feasibility study and nutritional composition of the 120 breast milk samples

	Mean	Median	Standard deviation.	Minimum	Maximum
Postmenstrual age at sample collection (weeks)	31 ³ / ₇	29 ³ / ₇	5 ⁴ / ₇	23 ⁶ / ₇	40 ³ / ₇
Day of life (days)	38.3	26.0	36.9	4	180
Total nitrogen (g L ⁻¹)	2.75	2.47	0.87	1.44	5.81
Protein nitrogen (g L ⁻¹)	2.23	1.98	0.79	1.20	5.18
Non-protein nitrogen (g L ⁻¹)	0.52	0.51	0.17	0.06	1.22
Non-protein nitrogen (%)	18.0	19.0	5.0	4.2	21.0
Total protein* (g L ⁻¹)	17.6	15.8	5.6	9.6	37.1
True protein† (g L ⁻¹)	13.8	12.4	4.9	7.6	33.9
Fat (g L ⁻¹)	42.0	37.9	20.6	7.0	109.3
Lactose (g L ⁻¹)	61.5	59.0	11.4	39.7	88.1

Total protein – Total nitrogen 6.38. †True Protein – PN * 6.25.

method that elicits a high degree of precision and accuracy. Elemental analysis precisely measures the total nitrogen content of a matrix which has to be converted to protein content. It is thereby assumed that all nitrogen detected is protein bound and that the average number of nitrogen atoms per amino acids is known. For purified protein, this factor has been established to be 6.25 (i.e. the total nitrogen content of protein is 16.0%). In other words, a nitrogen content of 1 g corresponds to 6.25 g of pure protein. However, in the dairy industry, it is common practice to use a factor of 6.38 to convert the total nitrogen value into a value for true protein. This slightly different factor corrects for the small fraction of non-protein nitrogen (e.g. urea, uric acid etc) present in cow milk (5%). However, in breast milk 15–25% of total nitrogen content is non-protein bound (Loenneker *et al.* 1984; Lynch & Barbano 1999). As a consequence, there is no accepted conversion factor to determine true protein content in breast milk without analytical pretreatment, like protein precipitation. Moreover, Loenneker *et al.* (1984) suggested following procedures to calculate true protein in breast milk; precipitate the protein, determine its nitrogen content and then apply the factor of 6.25. Like the reference laboratories, we used a conversion factor of 6.38 for the total protein (i.e. no analytical pretreatment) of cow and breast milk and 6.25 for the true protein of breast milk (after protein precipitation).

To obtain a 'true' (reference) protein content, our breast milk samples ($n = 18, 9$, and 6 ; see Fig. 3) have

been measured in a certified laboratory using the Kjeldahl method. The Kjeldahl method is based on the same principle i.e. to determine the nitrogen content of a matrix. Theoretically, there is a small difference of the number of N atoms per protein between cow and breast milk due to different composition of proteins. The major portion of protein in breast milk is lactalbumin, lactoferrin and casein. The ratio of these two protein classes changes during lactation from a casein: non-casein (whey protein) of about 10:90 in early lactation, 40:60 in mature milk and 50:50 in late lactation (Kunz & Loenneker 1992). In cow milk, a ratio of 80:20 is found. Taking available information about amino acid sequencing of standard lactalbumin, lactoferrin and casein molecules into account, the percentage of nitrogen can be calculated [15.4% for lactalbumin (Uniprot LALBA_HUMAN), 17.5% for lactoferrin (Uniprot TRFL_HUMAN) and 17.0% for casein (Uniprot CASK_HUMAN, E9PDQ1_HUMAN)] (UniProt Knowledgebase. <http://www.uniprot.org/>). As the ratios of these classes are different for cow and breast milk, the % N may vary by approximately 16.5–17.0%. However, being a systematic effect that affects both the reference method and our micromethod, this small variation in the conversion factor should not affect the correlation of both methods.

Other convenient and inexpensive protein determination methods include colorimetric assays, such as biuret assay, Lowry assay, Bio-Rad Coomassie Blue assay and Pierce bicinchoninic acid (BCA) assay. Keller *et al.* showed that these methods correlate

highly with Kjeldahl analysis (Keller & Neville 1986). Furthermore, they demonstrated that the assays show a varying sensitivity to the different breast milk proteins such as lactalbumin, casein and lactoferrin. A given standard may correlate with lactalbumin but shows larger deviation from the true value of lactoferrin. With the fact that the composition of milk proteins changes over the lactation period, this change in sensitivity leads to a problem of choosing the correct standard for the calibration curve and does not allow for precise determination of the true protein content. Bergqvist *et al.* (1989) reported that the BCA method overestimates the total protein in breast milk due to factors that interfere with accurate protein quantitation. Certain single amino acids such as cysteine, tyrosine or tryptophan in breast milk interfere with a protein-induced biuret reaction. Moreover, incubation time and temperature also interfere to produce colour in the BCA assay (Bergqvist *et al.* 1989).

Regardless of the chosen method, various assumptions have to be made to take account of the complexity of the matrix 'breast milk'. Determination of nitrogen content by elemental analysis or Kjeldahl method seems to be favourable over the colorimetric assays because they are more robust given the variability in the compositions of preterm breast milk.

Lactose content

In establishing the micromethod for lactose analysis, we discovered that the highly sensitive and mass specific LC-MS/MS method (Fusch *et al.* 2011) is more precise and reliable than both the HPLC and the IR methods. This is because breast milk contains a significant amount of oligosaccharides, which constitute 10% of the total amount of carbohydrate content in contrast to an insignificant amount of oligosaccharides in cow milk. Oligosaccharides represent a class of molecules that are non-digestible for the human gut. All oligosaccharides have a lactose unit at the reducing end and the different oligosaccharides can be present in variable amounts in breast milk (Coppa *et al.* 1993). The lactose units bound in oligosaccharides can interfere with measurements of 'free lactose', which are not bound to oligosaccharides in

breast milk. The IR method analyses C-H, C-C and O-H bonds, which are found in both lactose and oligosaccharides making it difficult to accurately quantify lactose in breast milk samples without cross-contamination from oligosaccharides. The literature by Michaelsen *et al.* (1988) reported that the IR method measured oligosaccharides along with lactose, and a 10% difference of lactose content resulted when compared to results from an enzymatic lactose assay.

Some limitations of downsizing sample volumes refer also to how to reliably draw a representative milk sample. This is a particular consideration for breast milk; after thawing, fat has the tendency to cream and separate from the aqueous phase. To ensure an even distribution of components in milk and to enhance sample stability, homogenisation is a crucial step that needs to be meticulously followed in order to disperse fat globules uniformly in a small volume of milk. It also ensures reproducibility and reliability of measurements.

The principal strength of our study relies on precision, linearity, robustness and reproducibility of micromethods to determine macronutrient content in breast milk. In all 120 breast milk samples, it was feasible to obtain reliable results for all three parameters with a combined sample volume of 1.5 mL. The generated set of data seems quite reasonable with respect to the expected distribution of values. We could reconfirm the order magnitude for the variation of macronutrients among mothers as well as within a single lactation.

The data of our study also confirm that in breast milk about 20% of nitrogen molecules are derived from non-protein sources indicating that true protein provides a better basis for evaluating the amount of 'protein' from a nutrition standpoint.

From a methodological point of view, it is of interest to note when comparing the linear regression equations of fat (i.e. cow vs. human milk) and protein (i.e. total vs. true protein), the micromethods show small but statistically significant differences vs. the reference methods in these subsets of the samples. The fact that this systematical deviation becomes visible is mostly due to the high precision of both methods, reference and micromethods. Overall, these

observed differences are not considered to be of clinical significance.

It is unfortunate that there is no reference standard available for human milk and therefore neither our group nor other reference labs have the opportunity to test for the ultimate accuracy.

Considering the macronutrient variation in individual milk samples, consistent monitoring and subsequent addition of the appropriate amount of macronutrients to fortify the breast milk makes it possible to provide preterm infants with optimal nutrition. These micromethods will also be used to validate bedside milk analysers to provide an efficient means of determining macronutrient content of breast milk in the NICU. These validated milk analysers would improve individualised fortification of breast milk and would contribute to improving neonatal nutrition and health.

We conclude that the micromethods are feasible and reliable to incorporate in routine breast milk analysis for preterm infants, thereby promoting optimal nutritional outcomes.

Acknowledgement

We thank the research nurse, Lou-Ann Elliot for recruiting patients and sampling breast milk during this study. We would also like to thank the volunteers in our laboratory, Sunny Xia and Stephanie Fusch, for their valuable contribution of fat extraction and data acquisition.

Source of funding

None.

Conflict of interest

The authors declare that they have no conflicts of interest.

Contributions

All authors made substantial contributions. AC drafted and wrote the manuscript and processed samples. GF designed the laboratory methods, carried out the data analysis and drafted the manuscript. NR

contributed to the design of the study. NS performed laboratory tests. CF conceived the study, participated in interpretation of the results and editing the manuscript. All authors read and approved the final manuscript.

References

- American Academy of Pediatrics, Committee on Nutrition (2004) Nutritional needs of the preterm infant. In: *Pediatric Nutrition Handbook* (ed. R.E. Kleinman), 5th edn, pp 23–54. American Academy of Pediatrics: Chapel Hill, NC.
- AOAC International (2006) AOAC official method 989.05 fat in milk: modified Mojonnier ether extraction method. In: *AOAC international* (ed. W. Horwitz), p 33.2.26. AOAC International: Gaithersburg.
- Arslanoglu S., Moro G.E. & Ziegler E.E., The Wapm Working Group On Nutrition (2010) Optimization of human milk fortification for preterm infants: new concepts and recommendations. *Journal of Perinatal Medicine* **38**, 233–238.
- Barbano D.M., Lynch J.M. & Fleming J.R. (1997) Direct and indirect determination of true protein content of milk by Kjeldahl analysis: collaborative study. *Journal of the Association of Official Analytical Chemists* **74**, 281–288.
- Bergqvist Y., Karisson L. & Fohlin L. (1989) Total protein determined in human breast milk by use of coomassie brilliant blue and centrifugal analysis. *Clinical Chemistry* **35**, 2127–2129.
- Breckenridge W.C. & Kuksis A. (1967) Molecular weight distributions of milk fat triglycerides from seven species. *Journal of Lipid Research* **8**, 473–478.
- Coppa G.V., Gabrielli O., Pierani P., Catassi C., Carlucci A. & Giorgi P.L. (1993) Changes in carbohydrate composition in human milk over 4 months of lactation. *Pediatrics* **91**, 637–641.
- Corvaglia L., Aceti A., Paoletti V., Mariani E., Patrono D., Anocora G. *et al.* (2010) Standard fortification of preterm human milk fails to meet recommended protein intake: bedside evaluation by Near-Infrared-Reflectance-Analysis. *Early Human Development* **86**, 237–240.
- Ellis L. & Picciano M.F. (1993) Prolactin variants in term and preterm human milk: altered structural characteristics, biological activity and immunoreactivity. *Endocrine Regulations* **27**, 181–192.
- Fusch G., Choi A., Rochow N. & Fusch C. (2011) Quantification of lactose content in human and cow's milk using UPLC-tandem mass spectrometry. *Journal of Chromatography B Analytical Technologies in Biomedical and Life Sciences* **879**, 3759–3762.

- Hibberd C.M., Brooke O.G., Carter N.D., Haug M. & Harzer G. (1982) Variation in the composition of breast milk during the first 5 weeks of lactation: implications for the feeding of preterm infants. *Archives of Disease in Childhood* **57**, 658–662.
- International Committee for Animal Recording (ICAR) (2005) *Analytical methods used in milk recording* Available at: http://www.icar.org/documents/milk_laboratories_leray/list_analytical_methods_in_milk_recording.pdf (Accessed 4 December 2012).
- ISO 14891 IDF 185 (2002) Milk and milk products – Determination of nitrogen content – Routine method using combustion according to the Dumas principle.
- Keller R.P. & Neville M.C. (1986) Determination of total protein in human milk: comparison of methods. *Clinical Chemistry* **32** (1 pt 1), 120–123.
- Krouwer J. (2008) Why Bland-Altman plots should use X, not $(Y + X)/2$ when X is a reference method. *Statistics in Medicine* **27**, 778–780.
- Kunz C. & Loenneker B. (1992) Re-evaluation of the whey protein/casein ratio of human milk. *Acta Paediatrica* **81**, 107–112.
- Lammi-Keefe C.J. & Jensen R.G. (1984) Lipids in human milk: a review. 2: composition and fat-soluble vitamins. *Journal of Pediatric Gastroenterology and Nutrition* **3**, 172–198.
- Loenneker B., Smith C. & Keen C.L. (1984) Analysis of breast milk: current methodologies and future needs. *Journal of Pediatric Gastroenterology and Nutrition* **3**, 290–295.
- Lynch J.M. & Barbano D.M. (1999) Kjeldahl nitrogen analysis as a reference method for protein determination in dairy products. *Journal of AOAC International* **82**, 1389–1398.
- Mansson H.L. (2008) Fatty acids in bovine milk fat. *Journal of Food and Nutrition Research* **52**. doi: 10.3402/fnr.v52i0.1821
- Meier P.P., Furman L.M. & Degenhardt M. (2007) Increased lactation risk for late preterm infants and mothers: evidence and management strategies to protect breastfeeding. *Journal of Midwifery and Women's Health* **52**, 579–587.
- Michaelsen K.F., Pedersen S.B., Skafte L. & Peitersen B. (1988) Infrared analysis for determining macronutrient in human milk. *Journal of Pediatric Gastroenterology and Nutrition* **7**, 229–235.
- O'Connor D.L., Jacobs J., Hall R., Adamkin D., Auestad N., Castillo M. et al. (2003) Growth and development of premature infants fed predominantly human milk, predominantly premature infant formula, or a combination of human milk and premature formula. *Journal of Pediatric Gastroenterology and Nutrition* **37**, 437–446.
- Perez-Escamilla R., Cohen R.J., Brown K.H., Rivera L.L., Canahuati J. & Dewey K.G. (1995) Maternal anthropometric status and lactation performance in a low-income Honduran population: evidence for the role of infants. *American Journal of Clinical Nutrition* **61**, 528–534.
- Polberger S. (2009) New approaches to optimizing early diets. *Nestle Nutrition Workshop Series Pediatric Program* **63**, 195–204.
- Puntis J.W.L. (2006) Nutritional support in the premature newborn. *Postgraduate Medicine Journal* **82**, 192–198.
- Sauer C.W. & Kim J.H. (2011) Human milk macronutrient analysis using point-of-care near-infrared spectrophotometry. *Journal of Perinatology* **31**, 339–343.
- Schanler R.J., Shulman R.J. & Lau C. (1999) Feeding strategies for premature infants: beneficial outcomes of feeding fortified human milk versus preterm formula. *Pediatrics* **103** (6 pt 1), 1150–1157.
- UniProt Knowledgebase (2012) Protein Knowledgebase (UniProtKB). Available at: <http://www.uniprot.org/uniprot/> (Accessed 20 August 2012).
- Zar J.H. (1999) *Biostatistical Analysis*, 4th edn, Prentice-Hall: Upper Saddle River, NJ.