Original Article

Comparison of macronutrient contents in human milk measured using mid-infrared human milk analyser in a field study vs. chemical reference methods

Mei Zhu^{*}, Zhenyu Yang^{*}, Yiping Ren[†], Yifan Duan^{*}, Huiyu Gao^{*}, Biao Liu[‡], Wenhui Ye[‡], Jie Wang^{*} and Shian Yin^{*}

^{*}Key Laboratory of Trace Element Nutrition, Ministry of Health of China, National Institute for Nutrition and Health, Chinese Center for Disease Control and Prevention, Beijing, China, [†]Zhejiang Center for Disease Control and Prevention, Hangzhou city, Zhejiang Province, China, and [‡]Milk Powder Business Unit, Inner Mongolia Yili Industrial Group Co. Ltd, Hohhot, China

Abstract

Macronutrient contents in human milk are the common basis for estimating these nutrient requirements for both infants and lactating women. A mid-infrared human milk analyser (HMA, Miris, Sweden) was recently developed for determining macronutrient levels. The purpose of the study is to compare the accuracy and precision of HMA method with fresh milk samples in the field studies with chemical methods with frozen samples in the lab. Full breast milk was collected using electric pumps and fresh milk was analyzed in the field studies using HMA. All human milk samples were thawed and analyzed with chemical reference methods in the lab. The protein, fat and total solid levels were significantly correlated between the two methods and the correlation coefficient was 0.88, 0.93 and 0.78, respectively ($p < 0.001$). The mean protein content was significantly lower and the mean fat level was significantly greater when measured using HMA method ($1.0 \text{ g } 100 \text{ mL}^{-1}$ vs $1.2 \text{ g } 100 \text{ mL}^{-1}$ and $3.7 \text{ g } 100 \text{ mL}^{-1}$ vs $3.2 \text{ g } 100 \text{ mL}^{-1}$, respectively, $p < 0.001$). Thus, linear recalibration could be used to improve mean estimation for both protein and fat. There was no significant correlation for lactose between the two methods ($p > 0.05$). There was no statistically significant difference in the mean total solid concentration ($12.2 \text{ g } 100 \text{ mL}^{-1}$ vs $12.3 \text{ g } 100 \text{ mL}^{-1}$, $p > 0.05$). Overall, HMA might be used to analyze macronutrients in fresh human milk with acceptable accuracy and precision after recalibrating fat and protein levels of field samples.

Keywords: human milk analyser, Röese–Gottlieb method, micro-Kjeldahl method, HPAEC, macronutrients.

Correspondence: Zhenyu Yang and Shian Yin, National Institute for Nutrition and Health, Chinese Center for Disease Control and Prevention, No. 29 Nanwei Road, Xicheng District, Beijing 100050, China. Email: yang.zhenyuid@gmail.com; shianyin@126.com

Introduction

The World Health Organization recommends that infants should be exclusively breastfed for the first 6 months of life and be continuously breastfed with introducing appropriate complementary foods from 6 months through 2 years of age or beyond (World Health Organization, 2001; World Health Organization and UNICEF, 2003). The American Academy of Pediatrics also affirmed the recommendation of exclusively breastfeeding for about 6 months and continuous breastfeeding for 1 year or longer

(Section on Breastfeeding, 2012). Breastfeeding and human milk are the gold standards of infant feeding and nutrition (Section on Breastfeeding, 2012). Macronutrient (e.g. protein, fat and carbohydrate) contents in human milk are the common basis for estimating these nutrient requirements for infants and/or lactating women (Food and Nutrition Board Institute of Medicine, 2004). Therefore, it is critical to understand the distribution of human milk macronutrient contents for setting the recommended intakes of these macronutrients for infants and lactating women among various populations.

Recently, mid-infrared transmission spectroscopy has become available to determine the macronutrient contents in human milk, especially in clinical settings for preterm infant feeding (Menjo *et al.*, 2009; Casadio *et al.*, 2010; de Halleux and Rigo, 2013; Fusch *et al.*, 2014; Silvestre *et al.*, 2014). The method is applicable for analysing carbohydrates, fat and protein, which could be further used to calculate energy and total solids in human milk. This is a spectroscopic technique, which requires a very thin fluid film ($<100\ \mu\text{m}$) of the sample for exposure to infrared (IR) radiation. The transmitted IR wavelengths are detected at specific wavelengths within the IR spectrum (Menjo *et al.*, 2009). The absorbance for a substance in a specific wavelength is proportional to the concentration of the substance in the sample. The specific functional groups were selected for each major component in the milk. For example, carbonyl groups ($5.7\ \mu\text{m}$) and carbon hydrogen groups ($3.5\ \mu\text{m}$) were chosen for fat determination, amide groups ($6.5\ \mu\text{m}$) for protein determination and hydroxyl groups ($9.6\ \mu\text{m}$) for lactose determination (Sjaunja, 1984; Biggs *et al.*, 1987; Biggs and McKenna, 1989). The results of the analysis are available within approximately 1 min after a milk sample is injected (Chang *et al.*, 2012). These instruments were calibrated with known protein, fat and lactose concentration milk measured by chemical methods. Partial least squares-based model was commonly used to estimate the regression coefficients for macronutrient concentration calculation. Measurements can be performed rapidly, and only small amounts of milk are required (2–3 mL for each measurement). Carbohydrates, fat and protein are simultaneously determined. The analysis can be conducted in the field studies without sophisticated lab setting, which could avoid the need to freeze and thaw milk samples in the lab.

A few studies have been conducted to compare the results from the human milk analyser (HMA, developed by Miris (Uppsala, Sweden)) with those from various chemical methods (Menjo *et al.*, 2009; Casadio *et al.*, 2010; Fusch *et al.*, 2014; Silvestre *et al.*, 2014). However, the results were not fully consistent among these studies. Frozen milk samples were analysed using HMA in most of these studies. Various homogenization methods (e.g. vortex and ultrasonic homogenizing with different duration) were performed for these analyses in different labs, which might have affected the results (Fusch *et al.*, 2014). Fusch's study examined the effects of homogenization and freezing on macronutrient levels in human milk. Sample size varied significantly across these studies (e.g. more than 600 samples in Fusch's study and 32 samples in Menjo's study). Smaller sample size would have less robust parameter estimation between the two methods. In addition, no studies have been conducted to compare HMA method using fresh milk samples with chemical methods. Analysing fresh milk is a big advantage for HMA, which offers possibility for nutritionists and human biologists to study breast milk in the field studies (Miller *et al.*, 2013). The purpose of the study is to assess the accuracy and precision of the HMA method with fresh milk samples in the field studies with chemical reference methods with frozen samples in the lab.

Materials and methods

Subjects

Healthy volunteers (post-partum <11 months) were recruited from the Yunnan province, Guangdong province and Shanghai Municipality. The subjects were eligible for the study, if they were self-deemed healthy, with singleton delivery, no smoking and drinking. The

Key messages

- The protein, fat and total solid levels were correlated between human milk analyser (HMA) and chemical reference methods. The correlation coefficient was 0.88, 0.93 and 0.78, respectively.
- The mean protein content was lower, and the mean fat level was greater when measured using HMA method.
- There was no significant correlation for lactose between the two methods.
- Mean total solid level were not different between the two methods.

study was approved by the Institutional Review Board at National Institute of Nutrition and Food Safety, Chinese Center for Disease Control and Prevention. All subjects signed a consent form.

At least 40 samples are required for method comparison study (Westgard *et al.*, 2008). To meet the sample size needs for all four analysis (protein, lactose, fat and total solid), a total of 151 subjects were recruited from community health clinic for the current study. There were 99, 100 and 89 samples analysed for protein, lactose and fat to cover greater inter-subject variation, respectively. Because of limited breast milk volume, only 37 samples were analysed for protein, lactose, fat and total solids; 55 samples were analysed for both protein and lactose; 7 samples were analysed for protein and fat; 44 samples were analysed for fat only and 8 samples were analysed for lactose only.

Human milk collection and processing

One breast was emptied using an electric pump at 9–11 am, and approximately 30–50 mL of milk was collected into a bottle. After gently shaking the milk bottle up and down for approximately 10 times, a fresh milk sample (~3 mL) was taken using a disposable syringe (5 mL) for HMA analysis in the field studies.

MIRIS HMA analyses

Fresh milk samples were warmed to 40°C in a water bath prior to analysis in the field studies. The unprocessed calibration procedure was used for sample analysis according to the manufacturer's recommendation (MIRIS AB, Sweden). A daily calibration check and cleaning steps every 10 analyses were performed using the calibration solution and cleaning solution, respectively. Both solutions were supplied by the manufacturer. One fresh milk sample (~2 mL) was injected into the analysis cell and measured for each subject. Crude protein rather than 'true' protein was measured in the HMA model used in the current study. The HMA measurements were conducted by different well-trained field workers across the three study sites.

Reference methods for lactose, fat and protein.

The remaining milk samples were aliquoted into 15 mL centrifuge tubes and were stored at –20°C in the field for further analysis in the lab. The frozen samples were shipped to the China CDC lab in frozen conditions and were stored at –80°C until analysis with the reference methods in the lab. To improve the accuracy and precision of lactose analysis in human milk and to reduce human milk sample volume used, the lactose was determined using high-pH anion exchange chromatography with pulsed amperometric detector (HPAEC model DX500) on a CarboPac PA-20 column (Dionex Corporation, Sunnyvale, CA, USA) by gradient elution using a flow of 0.45 mL min⁻¹: 0–13.0 min 250 mM NaOH (20%) and H₂O (80%), 13.1–15.1 min 250 mM NaOH (10%), 500 mM NaOAc per 100 mM (80%) and H₂O (10%), 15.2–17.2 min 250 mM NaOH (80%) and H₂O (20%), 17.3–25.0 min 250 mM NaOH (20%) and H₂O (80%). External standards were used for quantification. Approximately 0.5 g (to the nearest 0.0001 g) of human milk was used for lactose analysis. The detailed methods were previously published (Cai *et al.*, 2014).

Fat content was measured using the Roese–Gottlieb method (Jensen and Clark, 1984). Briefly, approximately 2.0 g (to the nearest 0.0001 g) of human milk was hydrolysed using ammonium hydroxide. Then fat was extracted with ethyl ether and petroleum ether. Fat content was weighed after evaporating the solvents.

Total nitrogen was determined by the micro-Kjeldahl method with approximately 1.0 g (to the nearest 0.0001 g) of human milk sample (Lonnerdal *et al.*, 1984). The protein content was calculated using a nitrogen-to-protein conversion factor of 6.25 (Lonnerdal *et al.*, 1984; Koletzko *et al.*, 2005).

The total solid content was measured by a direct drying method at 105°C with approximately 2.0 g (to the nearest 0.0001 g) of human milk sample (Ministry of Health China, 2010).

All samples were thawed and homogenized using MIRIS ultrasonic oscillatory mixing machine for 30 s. Then protein, lactose, fat and moisture were determined using the reference methods, respectively. All samples were run in duplicate. The mean CV for protein, fat and lactose were 0.7%, 2.4% and 2.5%, respectively.

Statistical analysis

To enable the two methods comparable, the unit of $\text{g } 100 \text{ g}^{-1}$ was converted to $\text{g } 100 \text{ mL}^{-1}$ based on the density of 1.02 g mL^{-1} of human milk in China (Jin *et al.*, 1988). Paired *t*-test was used to compare the mean difference between the reference methods and HMA method. Pearson correlation was analysed between the two methods. Passing–Bablok regression was used to assess agreement and possible bias between the two methods. Bland–Altman plots were drawn with reference method as the *x*-axis and the difference between reference method and HMA as the *y*-axis. All analyses were conducted using SAS software (version 9.2; SAS Institute Inc, Cary, NC, USA) and MedCalc® (MedCalc Software, Ostend, Belgium). Values are expressed by mean $\pm$ SD. *P*-value less than 0.05 was considered significant.

Results

As shown in Figs 1a– 4a, the protein, fat and total solid levels measured using the HMA were significantly correlated to those measured using reference methods ($P < 0.001$). The correlation coefficient was 0.88, 0.93 and 0.78, respectively. There was no significant correlation for lactose between the two methods ($r = 0.10$, $P = 0.30$, $n = 100$).

The mean protein content measured using the reference method was significantly greater than using the HMA method ($1.2 \text{ g } 100 \text{ mL}^{-1}$ vs. $1.0 \text{ g } 100 \text{ mL}^{-1}$, $P < 0.001$), and the protein level was approximately 13% lower when analysed using HMA method (Table 1). Passing–Bablok regression showed that there existed constant and proportional difference in protein levels between the two methods (protein level by the HMA method = $-0.54 + 1.30 \times$ protein level by the micro-Kjeldahl method) (Fig. 1a). The adjusted protein levels would be $1.2 \pm 0.3 \text{ g } 100 \text{ mL}^{-1}$ based on the equation. Bland–Altman plot showed that the mean protein concentration difference was $0.16 \text{ g } 100 \text{ mL}^{-1}$ between the two methods (Fig. 1b). The flat line indicated that the mean protein concentration difference between the two methods was consistent across the range of 0.80 – $2.66 \text{ g } 100 \text{ mL}^{-1}$.

The mean fat content measured using the reference method was significantly lower than the one

Fig. 1. Comparison of the HMA method and the Kjeldahl method for determining protein concentration. A) Passing–Bablok regression (solid line: Passing–Bablok fit; dashed line: 95% CI; dotted line: identity); B) Bland–Altman plot (solid line: mean difference; dashed line: ± 1.96 SD; dotted line: identity).

measured using HMA method ($3.2 \text{ g } 100 \text{ mL}^{-1}$ vs. $3.7 \text{ g } 100 \text{ mL}^{-1}$, $P < 0.001$), and fat level was approximately 14% higher when HMA method was used (Table 1). Passing–Bablok regression showed that there existed constant and proportional difference in fat concentration between the two methods (fat level by the HMA method = $0.18 + 1.09 \times$ fat level by the Roese–Gottlieb method) (Fig. 2a). The adjusted fat levels would be $3.2 \pm 1.4 \text{ g } 100 \text{ mL}^{-1}$ based on the equation. Bland–Altman plot showed that the mean fat concentration difference was $-0.45 \text{ g } 100 \text{ mL}^{-1}$ between the two methods (Fig. 2b). The horizontal line indicated that the two methods agreed equally in determining human milk fat concentration across the range of 0.73 – $7.44 \text{ g } 100 \text{ mL}^{-1}$.

Fig. 2. Comparison of the HMA method and the Roese-Gottlieb method for determining fat concentration. A) Passing-Bablok regression (solid line: Passing-Bablok fit; (dashed line: 95% CI; dotted line: identity); B) Bland-Altman plot (solid line: mean difference; (dashed line: $7 \pm 96\text{SD}$; dotted line: identity).

Mean difference in lactose content between the two methods was $0.05 \text{ g } 100 \text{ mL}^{-1}$ ($6.7 \text{ g } 100 \text{ mL}^{-1}$ vs. $6.6 \text{ g } 100 \text{ mL}^{-1}$, $P > 0.05$), and the difference between the two methods was approximately 1% of the reference value. Passing-Bablok regression did not show systematic difference existed in lactose concentration between the two methods, but the two methods were not significantly correlated. The Bland-Altman plot showed that the difference was positively correlated to the actual lactose concentration (Fig. 3b). The correlated line indicated that the two methods did not agree equally in determining human milk lactose concentration across the range of $5.5\text{--}7.5 \text{ g } 100 \text{ mL}^{-1}$. At low lactose concentration levels, the HPAEC measurement was less than

Fig. 3. Comparison of the HMA method and the HPAEC method for determining lactose concentration. A) Passing-Bablok regression (solid line: Passing-Bablok fit; (dashed line: 95% CI; dotted line: identity); B) Bland-Altman plot (solid line: mean difference; (dashed line: $\pm 1.96\text{SD}$; dotted line: identity).

HMA measurement; at high lactose concentration levels, the HPAEC measurement was more than HMA measurement.

No mean difference was detected between the two methods in measuring total solid content in human milk ($12.3 \text{ g } 100 \text{ mL}^{-1}$ vs. $12.2 \text{ g } 100 \text{ mL}^{-1}$, $P > 0.05$), and the difference was less than 1%. Passing-Bablok regression showed no systematic difference existed in total solid concentration between the two methods. The difference between the two methods was not correlated to actual total solid concentration in the Bland-Altman plots. The horizontal line indicated that the two methods agreed equally in determining human milk total solid concentration across the range of $9.6\text{--}15.5 \text{ g } 100 \text{ mL}^{-1}$ (Fig. 4b).

Fig. 4. Comparison of the HMA method and the direct drying method for determining total solid concentration. A) Passing-Bablok regression (solid line: Passing-Bablok fit; dashed line: 95% CI; dotted line: identity); B) Bland-Altman plot (solid line: mean difference; dashed line: ± 1.96 SD; dotted line: identity).

Discussion

Our study showed that protein, fat and total solid concentration measured using HMA was highly correlated with using reference methods. The HMA method gave lower protein levels and higher fat levels than the

chemical methods, and the difference was 13% and 14% of the value obtained from the chemical methods for protein and fat, respectively. A correction could be made based on the linear relationship to improve mean estimation for protein and fat using the HMA method. Mean total solid concentration was similar between the two methods.

The major strengths of the current study are the following: (1) fresh human milk was used for HMA analysis; (2) the HMA analysis was conducted in the field studies; (3) nearly 100 subjects were included for fat, protein and lactose analysis; and (4) reference methods used were well accepted for dairy analysis (Jensen and Clark, 1984; Lonnerdal *et al.*, 1984). To our knowledge, four studies have compared the HMA method with chemical methods for protein, fat and lactose (Menjo *et al.*, 2009; Casadio *et al.*, 2010; Fusch *et al.*, 2014; Silvestre *et al.*, 2014). One study included more than 600 samples for both HMA and reference methods (Fusch *et al.*, 2014). The other studies included approximately 40 subjects for the two methods (Menjo *et al.*, 2009; Casadio *et al.*, 2010; Silvestre *et al.*, 2014). All these studies used frozen human milk samples in HMA analysis. Some studies vortexed thawed milk samples and others homogenized thawed samples with an ultrasonic vibrator for various durations (1.5 s per 1 mL or 30 s per 1.5 mL). Studies have shown that homogenization could have strong effects on infrared measurements and large fat globules could cause light scatter (van de Voort *et al.*, 1990; Fusch *et al.*, 2014). With longer duration of homogenization (i.e. 30 s per 1 mL sample), fat globule size could be more appropriate for HMA analysis (Fusch *et al.*, 2014). However, fresh samples remain the most ideal samples for HMA. If frozen samples are analysed, an ultrasound homogenizer is recommended to homogenize the samples by the HMA producer. The current study showed

Table 1. Comparison of macronutrient contents in human milk measured between reference methods and human milk analyser

Nutrient	<i>n</i>	Chemical method	HMA	Difference	Correlation coefficient	Difference (%)
Protein	99	1.2 $\pm$ 0.3	1.0 $\pm$ 0.4	0.16*	0.88*	13
Fat	89	3.2 $\pm$ 1.4	3.7 $\pm$ 1.5	-0.45*	0.93*	14
Lactose	100	6.7 $\pm$ 0.4	6.6 $\pm$ 0.4	0.05	0.10	1
Total solid	37	12.3 $\pm$ 1.4	12.2 $\pm$ 1.7	0.09	0.78*	1

HMA, human milk analyser. Values are expressed by mean $\pm$ SD; unit is g 100 mL⁻¹. **P* < 0.0001.

the effectiveness of HMA utilization in the field studies. The current study focused on mean macronutrient concentration estimation using HMA in a population level. Smaller variance of mean estimation was expected than individual variance. Therefore, HMA would have better precision in mean estimation when used in field studies.

Our study found that HMA underestimated the protein level by $0.16 \text{ g } 100 \text{ mL}^{-1}$, which was slightly less than a previous study ($0.2 \text{ g } 100 \text{ mL}^{-1}$) (Fusch *et al.*, 2014) but was much less than another study ($0.6 \text{ g } 100 \text{ mL}^{-1}$) (Silvestre *et al.*, 2014). The third study found that HMA overestimated the protein level by $0.2 \text{ g } 100 \text{ mL}^{-1}$ (Menjo *et al.*, 2009). The difference among these studies might be due to different methods used for quantifying protein (e.g. micro-Kjeldahl, Bradford method and elemental analysis) (Menjo *et al.*, 2009; Fusch *et al.*, 2014; Silvestre *et al.*, 2014). Bovine sero-albumin was used as the standards for Bradford method in Silvestre's study, which could have different amino acid composition from human milk protein (e.g. percentage of arginine and lysine) and might overestimate total protein levels in human milk. Elemental analysis was developed to measure protein level in human milk with small sample volume. The current study used micro-Kjeldahl method, which was used for calibrating the HMA by the manufacturer. In addition, about 15–24% of total nitrogen was non-protein nitrogen in human milk (Dupont, 2003; Choi *et al.*, 2013). High concentrations of non-protein nitrogen may explain some of the difference. Further, the slightly better performance of HMA in our study might be due to fresh human milk samples used. These results suggested that recalibration of HMA for protein levels and conducting linear adjustment might be required due to the high correlation between the HMA method and the chemical method that was revealed without adjusting the analytical setup of HMA. Similar suggestions were proposed by other studies (Fusch *et al.*, 2014).

Fat concentration was overestimated by $0.45 \text{ g } 100 \text{ mL}^{-1}$ by HMA in the current study, which was similar to a previous study ($0.4 \text{ g } 100 \text{ mL}^{-1}$) (Casadio *et al.*, 2010) and was much less than the Silvestre *et al.* study ($0.75 \text{ g } 100 \text{ mL}^{-1}$) (Silvestre *et al.*, 2014). No difference between HMA and a chemical method was seen in the

third study (Fusch *et al.*, 2014). A difference in methods (e.g. a spectroscopic esterified fatty acid method, Gerber method and ether Mojonnier extraction method) could partially contribute to the different results (Casadio *et al.*, 2010; Fusch *et al.*, 2014; Silvestre *et al.*, 2014). Different pretreatment might also explain some of the discrepancy. Overall, these results support that a recalibration of fat for HMA might be required. In addition, a linear correction could be conducted based on the high correlation between the two methods.

Mean lactose concentration was comparable between HMA and high-performance anion exchange chromatography method in our study. However, there was no correlation between the two methods, which was confirmed by the previous studies (Fusch *et al.*, 2014; Silvestre *et al.*, 2014). High concentration of oligosaccharides in human milk may explain the uncorrelated results, which always contains a lactose terminal. HMA might not differentiate free lactose from the lactose moiety in the oligosaccharides because of the low specificity of HMA (Fusch *et al.*, 2014). The sum of the two components tends to be constant to some degree. Although, it might not be necessary to calibrate for mean lactose estimation; however, further development of HMA would be needed to increase the accuracy of lactose measurement for individual use. An external standard was used for lactose quantification in the current study, which might introduce some errors, as internal standards are recommended for quantifying lactose, and detection response could vary across different runs (Thurl *et al.*, 1996; Thurl *et al.*, 2010). Mean total solid concentration was similar, and there was a significant correlation between the two methods.

The limitations of the current study are as follows. Firstly, frozen human milk samples were used for the reference method analysis rather than fresh samples. Frozen samples were homogenized using MIRIS ultrasonic oscillatory mixing machine for 30 s, which might minimize the effects of freezing on results. Secondly, HMA analysis was conducted by different field workers with different HMA, which might introduce inter-person and inter-device variation. However, the results could be more applicable for different fields and field workers. Thirdly, external standards rather than internal standards were used for lactose quantification, which might introduce some errors across different

runs. However, the recovery rates of the method were 99.53–102.40%, and the relative standard deviations were 0.72–1.53% in the method development experiment (Cai *et al.*, 2014).

In conclusion, HMA might be used to analyse macronutrients (fat, protein and total solid) in fresh human milk in a population-based field setting. A conversion factor would be needed to improve accuracy of measuring fat and protein using HMA. Mean difference between HMA and chemical methods for these nutrients is approximately 1% after recalibrating fat and protein levels in HMA for population-based studies. Therefore, HMA might be a useful tool for large-scale population based-studies.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Contribution

ZY and SY designed the study; MZ, YR, YD, HG, BL, WY and JW implemented the study; MZ and ZY drafted the manuscript and all authors review the final version.

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Supporting information

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