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Comparison of gravimetric, creamatocrit and esterified fatty acid methods for determination of total fat content in human milk

Jian Du [a,*], Melvin C.L. Gay [a], Ching Tat Lai [a], Robert D. Trengove [b,c], Peter E. Hartmann [a], Donna T. Geddes [a,*]

[a] School of Chemistry and Biochemistry, The University of Western Australia, 35 Stirling Hwy, Crawley, Western Australia 6009, Australia
[b] Separation Science and Metabolomics Laboratory, 90 South Street, Murdoch University, Murdoch, Western Australia 6150, Australia
[c] Metabolomics Australia, Western Australia, Australia

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ABSTRACT

The gravimetric method is considered the gold standard for measuring the fat content of human milk. However, it is labor intensive and requires large volumes of human milk. Other methods, such as creamatocrit and esterified fatty acid assay (EFA), have also been used widely in fat analysis. However, these methods have not been compared concurrently with the gravimetric method. Comparison of the three methods was conducted with human milk of varying fat content. Correlations between these methods were high ($r^2 = 0.99$). Statistical differences ($P < 0.001$) were observed in the overall fat measurements and within each group (low, medium and high fat milk) using the three methods. Overall, stronger correlation with lower mean (4.73 g/L) and percentage differences (5.16%) was observed with the creamatocrit than the EFA method when compared to the gravimetric method. Furthermore, the ease of operation and real-time analysis make the creamatocrit method preferable.

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1. Introduction

Human milk contains a variety of nutrients and immunologically active components that are required for both optimal growth and the development of a newborn's immune system against an array of diseases and infections (LaKind, Amina Wilkins, & Berlin, 2004). Milk fat is the major source of energy for infants, contributing over half of the total energy of human milk (Hamosh, Bitman, Wood, Hamosh, & Mehta, 1985). However, fat is the most variable nutritional component in human milk, changing substantially within and between feeds, between breasts, and among mothers and as well as with stage of lactation (Czank, Simmer, & Hartmann, 2009; Kent et al., 2006). Despite the importance of milk fat for the rapidly growing human infant and the multiple methods of analysis of fat content available, no extensive comparative studies have been conducted on fat analysis of human milk.

It is standard in biological fluids, such as urine, urinary creatinine is normally used for comparison in the comparison of studies between different populations. In human milk, lipophilic compounds, such as persistent organic pollutants (POPs), bind to the central core of the milk fat globules and. Therefore, when making comparisons, values should be normalized to the fat content of human milk. For example, when estimating POPs dosage, precise measurement of fat will reflect more accurately the maternal-infant environment and associated risks. Unfortunately, the vast array of components in milk, such as proteins, hydrophilic components, micellar casein and fat globules, which are dispersed in the liquid colloid, make accurate measurement of fat challenging. Thus, total fat determination in milk requires a quantitative extraction of all lipid compound classes (Kumar, Lindley, & Mastana, 2014).

Several techniques have been employed to measure fat in milk. The gravimetric reference method is based on measurement of fat mass in a sample after liquid-liquid extraction (Bligh & Dyer, 1959). The esterified fatty acid (EFA) assay has been adapted from analysis of total fatty acids in blood and works on the principle of breaking ester linkages (–COO-R–) in lipid species, such as triacylglycerols, which constitute approximately 98% of the fat in milk, followed by spectrometry analysis (Jensen, 1995; Stern & Shapiro, 1953). Creamatocrit method has been developed as a rapid and feasible tool for use in the clinical setting (Lucas, Gibbs, Lyster, & Baum, 1978; Meier et al., 2006). Whole milk is centrifuged and measurements are made of the skim milk and cream layer to calculate the cream content of the milk.

Whilst differences in recorded fat content resulting from the detection methods employed are not unexpected, these differences have not been examined. Differences in measurements might lead

* Corresponding author at: School of Chemistry and Biochemistry, The University of Western Australia, 35 Stirling Hwy, Perth, Western Australia 6009, Australia.
E-mail addresses: 21192741@student.uwa.edu.au (J. Du), Donna.geddes@uwa.edu.au (D.T. Geddes).

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to errors in the calculation of the caloric content. This is important in situations where infant growth is paramount, such as in preterm infants. Similarly, estimation of fat-soluble contaminants is not possible without determination of fat content.

In this study, we compared three methods, specifically the gravimetric, EFA and creamatocrit methods for the analysis of fat content in human milk.

2. Material and methods

2.1. Sample

This study was approved by the Ethics Committee of The University of Western Australia. Term milk from the mother was thawed at 37 °C for one hour and was divided into four 100 mL aliquots. The first 100-mL aliquot was sub-divided into aliquots of 5 mL (medium fat content, n = 20). 50 mL from the second 100-mL aliquot was diluted 2-fold with 50 mL of double deionized (DDI) water. It was then divided into aliquots of 5 mL (low fat content, n = 20). The remaining two 100-mL aliquots were centrifuged at 750*g* for 5 min (Eppendorf 58410R, Hamburg, Germany) and 50 mL of skim milk was removed from each of the sample. The remaining content (containing fat and skim milk) in each tube were combined and divided into 5 mL aliquots (high fat content, n = 20).

A total of 60 samples were prepared and stored at −20 °C. Prior to analysis, each 5 mL aliquot was thawed at 37 °C for 30 min and then homogenized with a mixer (ELMI Ltd., Riga, Latvia) for 15 s.

2.2. Reagents and standards

Chloroform and methanol were obtained from Chem-Supply (Gillman, SA, Australia). Absolute ethanol was supplied by Merck (Darmstadt, Germany). Hydrochloric acid (32%, w/w) was obtained from Scharlau (Barcelona, Spain).

Hydroxylamine hydrochloride, sodium hydroxide, trichloroacetic acid, triolein standard stock solution, hydrochloric acid and ferric chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). DDI water used in the experiments was generated by Ibis Technology Ultrapure Water purification system (Perth, WA, Australia). All chemicals were of analytical grade and were used as received without further purification.

2.3. Determination of total fat content in human milk

2.3.1. Gravimetric method (FOL extraction)

The gravimetric method used is based on the modified method of Folch, Lees, and Sloane-Stanley (1957). Briefly, 2 mL human milk was mixed with 40 mL of chloroform/methanol (2:1, v/v). The mixture was homogenized thoroughly and centrifuged at 1509*g* for 10 min. The clear homogenate was transferred to a separating funnel. Subsequently, 7.8 mL of water was mixed with the homogenate and allowed to stand until phase separation was observed. The proportion of water to homogenate was 2:10 (v/v) to ensure that no interfacial fluff was formed in the biphasic system obtained. The lipid layer (lower layer) was collected. The aqueous layer (top layer) was rinsed with chloroform/methanol mixture (2:1, v/v) and was allowed to stand until phase separation. The ratio between the aqueous layer and the rinsing solvent was around 1:1 (v/v) to prevent interfacial fluff. The lipid layer was collected and combined with the previous collection. The combined lipid fraction was then evaporated to dryness in a rotary evaporator and dried to constant weight under vacuum and the lipid content determined gravimetrically.

2.3.2. Esterified fatty acids (EFA)

The EFA method used is modified based on the method of Stern and Shapiro (Atwood & Hartmann, 1992; Stern & Shapiro, 1953). Samples (2.5 μL) and standards (triolein, 0–200 mM, 2.5 μL) were pipetted in duplicate into a deep-well plate followed by addition of 400 μL of absolute ethanol and mixed well. Then, 100 μL of 2 M hydroxylamine hydrochloride and 100 μL of 3.5 M sodium hydroxide were added, mixed well and allowed to stand for 20 min at room temperature. The samples were acidified by addition of 100 μL of 4.08 M HCl. Color change from dark yellow to brown was observed after the addition of 100 μL of a ferric chloride/trichloroacetic acid solution (3.75 g TCA in 5 mL 0.37 M $FeCl_3$). Due to the hygroscopic nature of hydroxylamine hydrochloride and $FeCl_3$-TCA, they were freshly prepared. The mixture was thoroughly mixed and duplicate aliquots of 100 μL were pipetted into a flat bottom 96-well plate. The plate was then analyzed using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA) at 540 nm.

2.3.3. Creamatocrit

The creamatocrit method used is based on the modified method of Lucas et al. (Lucas et al., 1978). The milk sample was drawn into two 75 μL micro-hematocrit capillary tubes (Kimble, TN, USA) and one end of the capillary was sealed with critocaps (Kimble, TN, USA). The tubes were then centrifuged in a micro-hematocrit centrifuge (BHG Hermle, USA) at 12,000*g* for 10 min. The creamatocrit (%) was measured using Creamatocrit Plus™ (Medela AG, Switzerland), which was based on the ratio of cream layer and total milk volume. The creamatocrit (%) was converted to fat content (g/L) based on the following formula: fat content = 3.968 + (5.917 × creamatocrit (%)) (Meier et al., 2006).

2.4. Data analysis

Statistical analysis was carried out using R 3.2.0 using the package nlme for the linear mixed models (Pinheiro, Bates, DebRoy, & Sarkar, 2009) and the package Lattice for Bland-Altman plots (Sarkar, 2009). Linear mixed effects were used to determine the relationship between the fat content and the three different methods. The fixed effect factor was the method. The random effects were the group (low, medium and high fat) and individual aliquot. Differences were considered to be significant if $P < 0.05$. Results were expressed as mean and standard deviation (SD). Bland-Altman plots were used to investigate if there were systematic effects of the measured fat content on the difference between the measurement methods.

3. Results

Overall, the fat content measured was statistically different ($P < 0.001$) between the different analytical methods and also within each of the sample groups (low, medium and high fat). However, excellent correlations ($r^2 > 0.99$) were found between the methods (Fig. 1).

The fat content measured by the gravimetric method was significantly higher ($P < 0.001$) than that measured by both EFA and the creamatocrit methods in all three sample groups of low, medium and high fat milk (Table 1).

The intra-assay precision in each sample group (low, medium and high fat) within each method was also tested. The gravimetric method gave a mean coefficient of variation (CV) of 1.74%. The largest CV was observed in medium fat milk (2.89%) followed by low (1.40%) and high fat milk (0.94%). The EFA method gave a mean CV of 5.71% with the highest CV observed in low fat milk (10.95%) followed by medium (4.34%) and high fat milk (1.84%). The

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