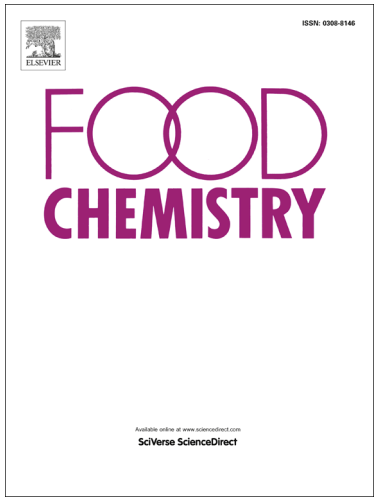

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PII: S0308-8146(16)31354-1
DOI: http://dx.doi.org/10.1016/j.foodchem.2016.08.114
Reference: FOCH 19760

To appear in: *Food Chemistry*

Received Date: 2 September 2015
Revised Date: 5 July 2016
Accepted Date: 27 August 2016

Please cite this article as: Du, J., Gay, M.C.L., Lai, C.T., Trengove, R.D., Hartmann, P.E., Geddes, D.T., Comparison of gravimetric, creamatocrit and esterified fatty acid methods for determination of total fat content in human milk, *Food Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.foodchem.2016.08.114

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

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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.

Keywords: Human milk, Gravimetric, Creamatocrit, Esterified fatty acids, Fat content, Lactation

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 Jr, 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 amongst mothers and as well as with stage of lactation (Czank, Simmer, & Hartmann, 2009; Kent, Mitoulas, Cregan, Ramsay, Doherty, & Hartmann, 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, Engstrom, Zuleger, Motykowski, Vasan, 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.

While 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 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 minutes and then homogenized with a mixer (ELMI Ltd., Riga, Latvia) for 15 seconds.

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 et al. (Folch, Lees, & 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, Gibbs, Lyster, & Baum, 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 x creamatocrit (%)) (Meier, Engstrom, Zuleger, Motykowski, Vasan, 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 (**Figure 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 the three different 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 creamatocrit method followed a similar pattern to the EFA method with a mean CV of 3.94 % and the highest CV observed in low fat milk (6.58 %) followed by medium (3.48 %) and high fat milk (1.75 %).

When comparing the three methods, the largest mean difference was observed between the gravimetric and the EFA methods in low, medium and high fat milk (**Table 1**). A smaller difference was observed between the gravimetric and the creamatocrit methods in low, medium and high fat milk (**Table 1**).

The box plots (**Figure 2**) show the percentage mean difference in low, medium and high fat milk in gravimetric-EFA methods (36.45 %, 19.13 % and 8.49 %,

185 respectively), gravimetric-creamatocrit methods (6.68 %, 4.58 % and 4.26 %
186 respectively) and creamatocrit-EFA methods (27.80 %, 11.01 % and 1.95 %
187 respectively).
188 The correlations between the methods were: gravimetric-EFA (r^2 = 0.994);
189 gravimetric-creamatocrit (r^2 = 0.995) and EFA-creamatocrit (r^2 = 0.988). The Bland-
190 Altman plots showed differences between these methods were within 2SD (**Figure 3**).

191 # 4. Discussion

192 In this study, we observed excellent correlations between the reference gravimetric
193 method for measuring milk fat content and both EFA and creamatocrit methods.
194 However, the linear mixed model analysis demonstrated a significant difference
195 between these three methods. Since the gravimetric method has been designated the
196 reference method for measuring fat in human milk, we have compared both the EFA
197 and the creamatocrit methods, which are simpler techniques for fat measurement, to
198 the gravimetric method.
199 Despite a strong correlation (**Figure 1A**) between the EFA and gravimetric methods,
200 which is consistent with previous literature (Atwood & Hartmann, 1992), we found
201 that the EFA method tended to underestimate the fat content by 7.62 to 10.73 g/L
202 (**Table 1**) with the percentage difference of 8.49 to 36.45 % compared to the
203 gravimetric method (**Figure 2A**). Underestimation of fat content in sow milk was also
204 observed by Atwood & Hartmann (Atwood & Hartmann, 1992). Underestimation of
205 fat content may be due to the fundamental principles underpinning the EFA method.
206 The EFA method disrupts the ester linkages of the triacylglycerol, which account for
207 98 % of the total fat in milk, whereas the gravimetric method partitions the fat and
208 measures its mass, essentially measuring total fat. Therefore, we should observe small
209 differences (0.59 to 1.79 g/L, based on the measured value using the gravimetric

method in this study) between these two methods. However in reality, absolute reaction of the triacylglycerol in the EFA method is impossible leading to further underestimation (Casadio, Williams, Lai, Olsson, Hepworth, & Hartmann, 2010). On the other hand, the gravimetric method could potentially overestimate the fat as the partitioning step is selective toward all hydrophobic and hydrophilic compounds in milk and is not specific to only the lipid compounds. Cerbulis and Custer have reported that casein is also soluble in the extraction solvent (chloroform/methanol), thus further accentuating the difference between the gravimetric and the EFA methods (Cerbulis & Custer, 1967).

The creamatocrit and the gravimetric methods showed an excellent correlation (**Figure 1B**). However, the creamatocrit underestimated the fat content by 3.38 to 5.96 g/L (**Table 1**) with a small percentage difference of 4.24 to 6.68 % compared to the gravimetric method (**Figure 2B**). As the creamatocrit uses centrifugal force to separate the skim and cream layer, some fat is retained in the skim layer (Czank, Simmer, & Hartmann, 2009). Therefore, it is expected that the measured value by the creamatocrit would be lower compared to the gravimetric method. As with any sample handling, the milk fat globule can also undergo degradation into free fatty acids, which occupy less space than cream (Lucas, Gibbs, Lyster, & Baum, 1978), compounding the underestimation of fat by the creamatocrit method. Consistent with our finding, Ganguli et al. also observed that the creamatocrit method underestimated the fat content (by about 2 %) in sow and rat milk whilst providing good correlation when compared to the gravimetric method (Ganguli, Smith, & Hanson, 1969). A recent paper compared the creamatocrit method with mid infrared spectroscopy and concluded that the creamatocrit overestimated the fat content (O'Neill, Radmacher,

Sparks, & Adamkin, 2013). However, methodological concerns exist regarding centrifugation of the human milk samples. The samples were centrifuged for 15 minutes at 1315 *g* instead of the standard 15 minutes at 12000 *g* used in all other studies (Fleet & Linzell, 1964; Lucas, Gibbs, Lyster, & Baum, 1978; Meier, Engstrom, Murtaugh, Vasan, Meier, & Schanler, 2002). This would result in a lower compaction of the cream layer, and would account for the higher creamatocrit values observed and therefore higher fat content calculated as compared to the mid infrared spectroscopy.

When the creamatocrit method was compared with the EFA method, there was an excellent correlation (**Figure 1C**). However, compared with the creamatocrit method the EFA underestimated the fat content by 1.67 to 7.36 g/L (**Table 1**) with the percentage difference of 1.99 to 28.19 % (**Figure 2C**). The underestimation of the fat content by the EFA method could again be due to differences in the principles of the two methods. Our findings are similar to that observed by Meier et al. (Meier, Engstrom, Zuleger, Motykowski, Vasan, Meier, et al., 2006), where they also reported a mean difference of 6.80 g/L and good correlation (**Table 2**) between the creamatocrit and the EFA.

The relationships were further analysed by Bland-Altman plots, which showed no systematic error in the relationship between the fat content measured by gravimetric-EFA (**Figure 3A**), gravimetric-creamatocrit (**Figure 3B**) and creamatocrit-EFA methods (**Figure 3C**).

Each of the methods investigated here have inherent advantages and disadvantages. The gravimetric method requires the largest volume of milk (> 2 mL) among the

methods compared, and is also labor-, time- and solvent-intensive. Whilst this method is precise (CV = 1.7 %), due to its complicated procedures only one milk sample can be processed in an hour. The EFA method on the other hand only employs a small amount of milk (< 0.1 mL) and chemicals (< 0.1 mL). However, the EFA is also labor-intensive allowing only 10 samples to be processed per hour. Among the three methods investigated, the EFA has the lowest precision (CV = 5.7 %). Both the gravimetric and EFA methods require the use of laboratory equipment, and are not suitable for real-time analysis in a hospital setting. The creamatocrit method on the other hand is a reagent-free technique requiring only a small amount of milk (< 0.1 mL). In this study, the creamatocrit method has good precision (CV = 3.9 %) and has the highest throughput (60 samples per hour). Besides being an inexpensive analysis, it also does not require a skilled operator. Therefore, real-time analysis can be performed by clinicians in the hospital. Furthermore, this study has shown closer correlation of the creamatocrit measured fat content with the reference method (gravimetric) than the EFA method.

5. Conclusions

This is the first study that has systematically compared three different methods of measuring fat content in human milk: gravimetric, EFA and creamatocrit. Both the EFA and creamatocrit methods showed excellent correlation with the gravimetric method. There were differences between methods in measured fat content, which can be explained by the different principles of measure and detected methods. The fat content measured by the creamatocrit method had values closer to that of the gravimetric method than the EFA method. Significant underestimation using the EFA method could be clinically relevant for low fat milk. The choice of method should

284 take into account whether the measurement is performed in the laboratory or clinical
285 setting and the requirements for accuracy and precision.

286 Acknowledgements

287 J. Du sincerely appreciates the International Postgraduate Research Scholarships
288 (IPRS), Australia Postgraduate Award (APA) and UWA Safety-Net Top-up
289 Scholarship for financial support. J. Du also wishes to thank G.Z. Zhao for his active
290 help and cooperation. This work was also supported by an unrestricted research grant
291 from Medela AG.

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357 **Table 1.** Fat content of the sample groups measured by gravimetric, EFA and creamatocrit methods.
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Sample group	Sample size	Mean fat content (g/L) (SD) Range			Mean difference [a]	Mean difference [b]	Mean difference [c]
		Gravimetric	EFA	Ceamatocrit			
Low fat milk	20	29.49 (0.42)	18.75 (2.11)	26.11 (1.76)	10.73 (1.89) [d]	3.38 (1.84) [d]	7.36 (2.92) [d]
		28.50 to 30.40	15.25 to 22.81	23.20 to 28.80			
Medium fat milk	20	53.80 (1.60)	43.47 (1.94)	48.94 (1.75)	10.32 (2.52) [d]	4.86 (1.87) [d]	5.47 (3.12) [d]
		51.10 to 52.70	40.65 to 47.62	45.60 to 52.40			
High fat milk	20	89.76 (0.87)	82.14 (1.55)	83.81 (1.50)	7.62 (1.71) [d]	5.96 (1.85) [d]	1.67 (2.42) [d]
		87.84 to 90.90	79.71 to 85.52	81.70 to 87.40			

359 [a] Mean difference of fat content measured by gravimetric and EFA presented as mean (SD).

360 [b] Mean difference of fat content measured by gravimetric and creamatocrit presented as mean (SD).

361 [c] Mean difference of fat content measured by creamatocrit and EFA presented as mean (SD).

362 [d] $P < 0.001$

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365 **Table 2.** Comparison of correlation coefficient by gravimetric, EFA and creamatocrit methods with previous studies.

Methods	Sample	Sample size	Correlation coefficient (r^2)	Reference
Gravimetric-EFA	**Human milk**	**60**	**0.99**	**Current study**
Gravimetric-EFA	Sow milk	33	0.99	(Atwood & Hartmann, 1992)
Gravimetric-creamatocrit	**Human milk**	**60**	**0.99**	**Current study**
Gravimetric-creamatocrit	Cow milk	16	0.99	(Ganguli, Smith, & Hanson, 1969)
Gravimetric-creamatocrit	Rat milk	4	0.99	(Ganguli, Smith, & Hanson, 1969)
Creamatocrit-EFA	**Human milk**	**60**	**0.99**	**Current study**
Creamatocrit-EFA	Human milk	37	0.95	(Meier, Engstrom, Zuleger, Motykowski, Vasan, Meier, et al., 2006)

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Figure captions:

Figure 1. Linear correlation between the fat content (g/L) measured by gravimetric and EFA methods (A), gravimetric and creamatocrit methods (B), and EFA and creamatocrit methods (C).

Figure 2. Differences of fat content (%) between methods for each sample group. gravimetric and EFA (A), gravimetric and creamatocrit (B) and EFA and creamatocrit (C).

Figure. 3 Bland-Altman plots showing the differences between gravimetric and EFA methods (A), gravimetric and creamatocrit methods (B) and EFA and creamatocrit methods (C) for all sample groups. The dotted line is the mean and the solid lines are ± 2SD of the mean.

379 **Figure 1.**

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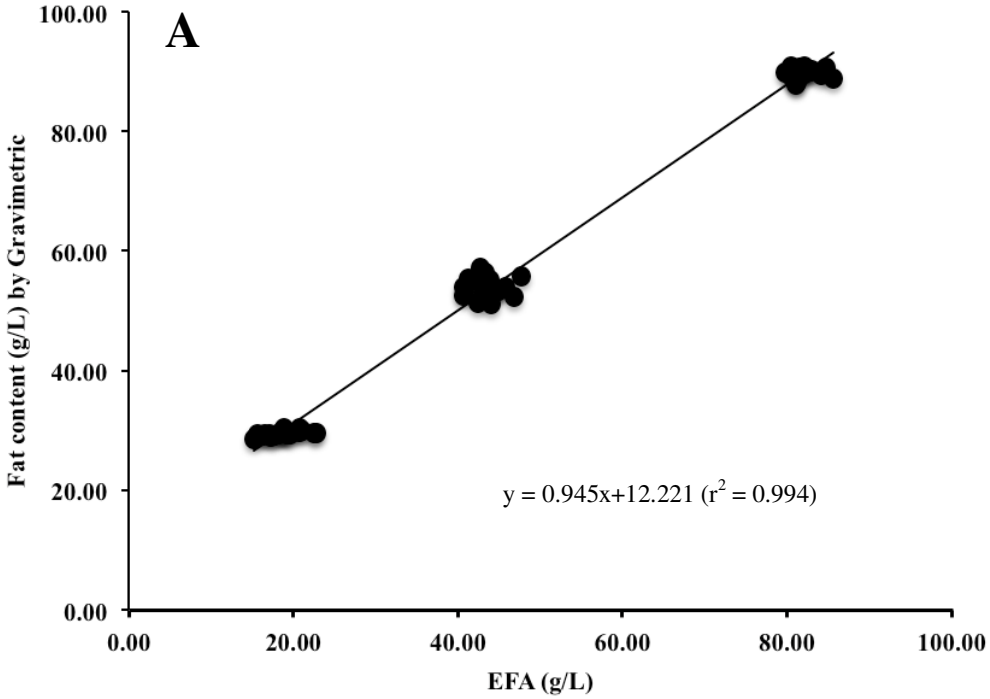

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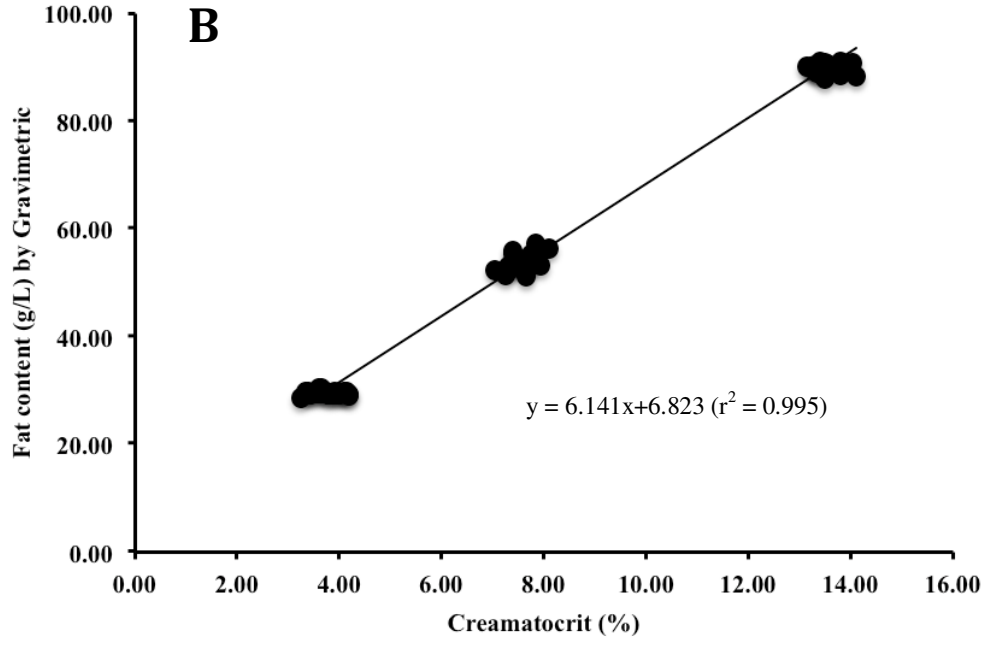

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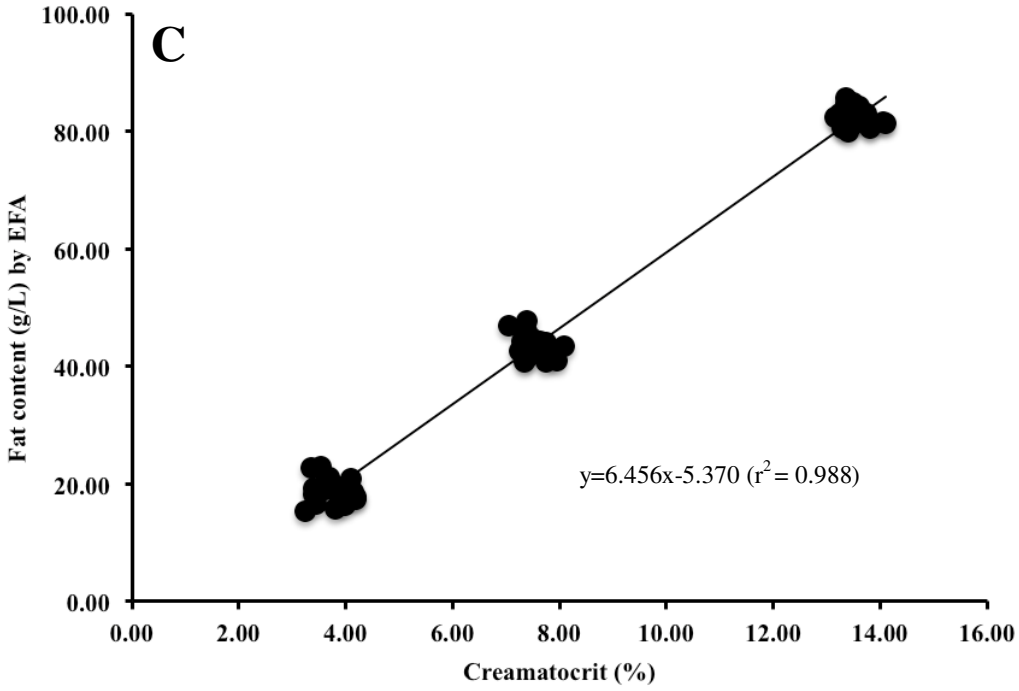

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388 **Figure 2.**

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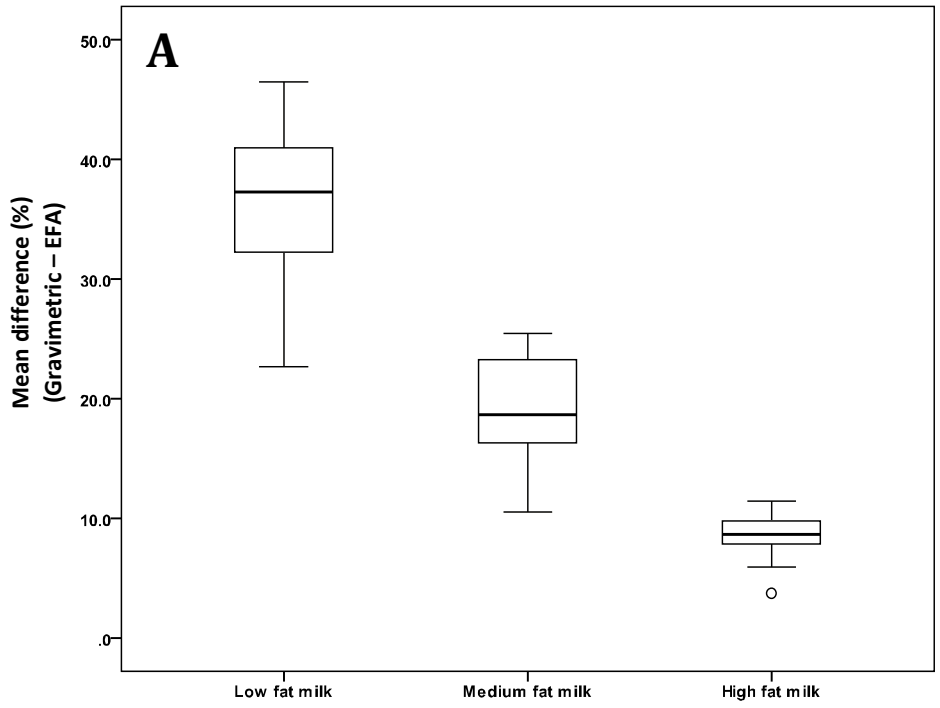

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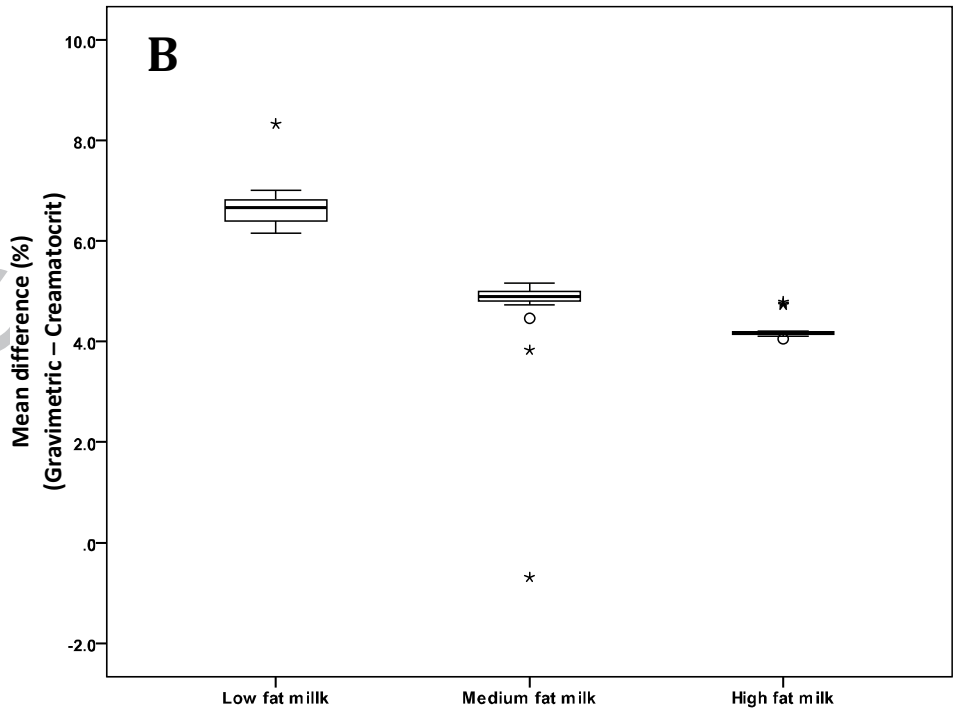

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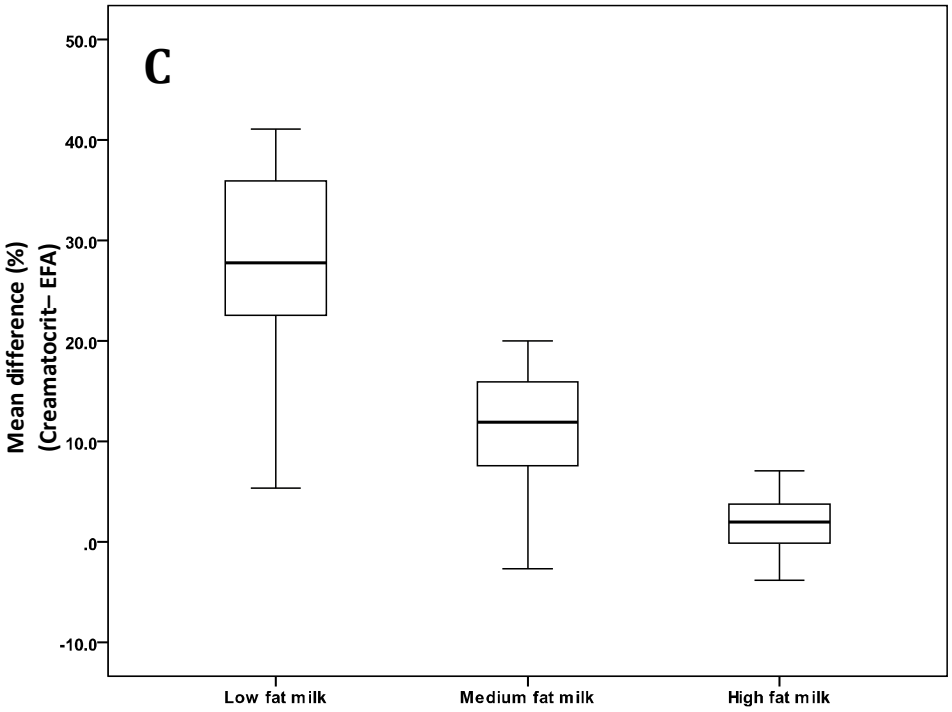

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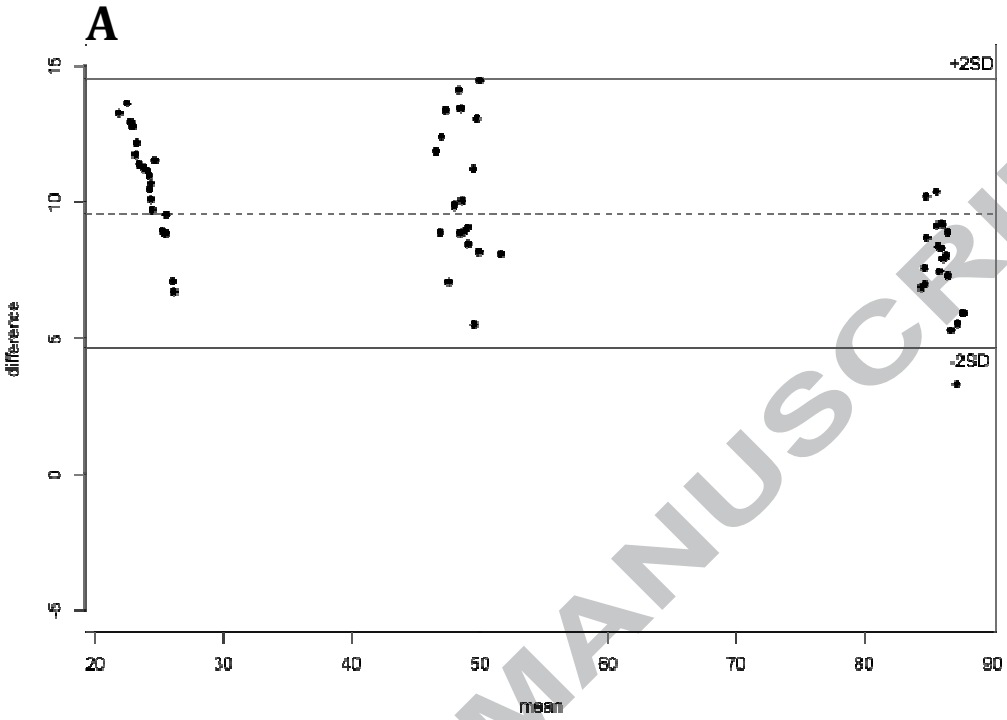
405 **Figure 3.**



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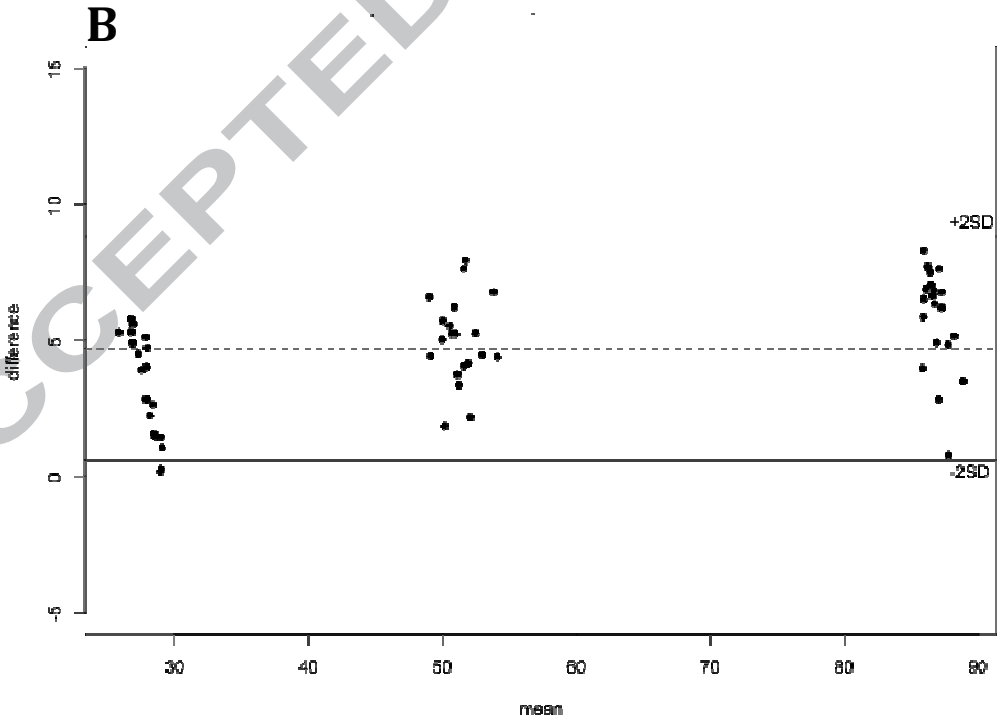

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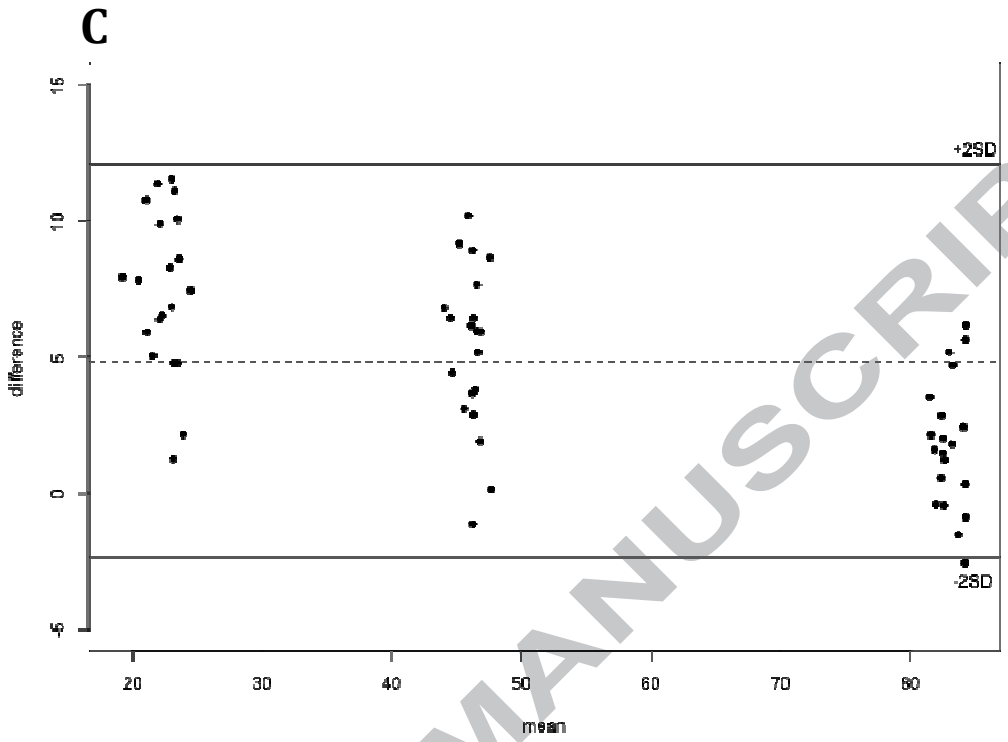

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Highlights

- First study to compare the gravimetric method with esterified fatty acid and creamatocrit methods to determine fat in human milk.
- The creamatocrit generated values were closer to those obtained by the standard gravimetric method.
- The creamatocrit method was more accurate and reproducible across different fat contents than the esterified fatty acid method.