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Short communication

Concordance analysis between estimation methods of milk fatty acid content

Mary Ana Petersen Rodriguez, Juliana Petrini, Evandro Maia Ferreira, Luciana Regina Mangeti Barreto Mourão, Mayara Salvian, Laerte Dagher Cassoli, Alexandre Vaz Pires, Paulo Fernando Machado, Gerson Barreto Mourão *

University of São Paulo, PO Box 9, CEP 13418-900 Piracicaba, SP, Brazil

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ABSTRACT

Considering the milk fatty acid influence on human health, the aim of this study was to compare gas chromatography (GC) and Fourier transform infrared (FTIR) spectroscopy for the determination of these compounds. Fatty acid content (g/100 g of fat) were obtained by both methods and compared through Pearson's correlation, linear Bayesian regression, and the Bland–Altman method. Despite the high correlations between the measurements ($r = 0.60–0.92$), the regression coefficient values indicated higher measures for palmitic acid, oleic acid, unsaturated and monounsaturated fatty acids and lower values for stearic acid, saturated and polyunsaturated fatty acids estimated by GC in comparison to FTIR results. This inequality was confirmed in the Bland–Altman test, with an average bias varying from −8.65 to 6.91 g/100 g of fat. However, the inclusion of 94% of the samples into the concordance limits suggested that the variability of the differences between the methods was constant throughout the range of measurement. Therefore, despite the inequality between the estimates, the methods displayed the same pattern of milk fat composition, allowing similar conclusions about the milk samples under evaluation.

1. Introduction

Nowadays, there is an increased concern about food composition, and search for healthier food has become very important. It is known that bovine milk is characterised by the predominance of saturated fatty acids (70%), which are associated with high levels of low density cholesterol (LDL) and, therefore, with cardiovascular diseases (Kromhout, Menotti, Kestleloot, & Sans, 2002). Among them, lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0) are the major fatty acids related to the increase of blood cholesterol (Bonanome & Grundy, 1988). However, milk also has many beneficial components, such as unsaturated fatty acids (Mensink, Zock, Kester, & Katan, 2003), especially oleic acid (C18:1*cis*-9) and conjugated linoleic acid (CLA). Thus, the knowledge about the composition of milk and, consequently, about the environmental and genetic factors that may influence or change the profile of fatty acids (FA), is very important to improve the nutritional quality of this product (Soyeurt, Dehareng, Mayeres, Bertozzi, & Gengler, 2008; Soyeurt et al., 2006; Soyeurt et al., 2007; Stoop, Van Arendonk, Heck, Van Valenberg, & Bovenhuis, 2008). Given this, the use of a fast, inexpensive and accurate way to quantify the levels of fatty acids in milk is a significant issue to be considered.

The determination of the fatty acid proportion in milk is performed by gas chromatography (GC) and Fourier transform infrared (FTIR) spectroscopy methods. Commonly used (Collomb & Buhler, 2000; Dorey, Brodin, Le Querler, & Kuzdzalsavoie, 1988; Soyeurt et al., 2006) due to its efficiency, GC allows the quantification of each FA. However, with the disadvantage of requiring the preparation of an esterified compound, this method is time consuming and requires specialized skills. In turn, the FTIR is an alternative method to GC, allowing the analysis of a higher number of samples, nearly 500 samples per hour (Foss, 2008; Soyeurt et al., 2006) compared to GC.

FTIR analyzes the vibrational motions of molecules and can be used for determination of FA in different ways. As there is no need for pre-preparation of the sample for analysis, this method becomes advantageous because of the low cost of reagents, time and specialized labour skills. Furthermore, FTIR is important for studies involving cellular responses, and it can be used as biochemical screening technique for explorative research, it requires

* Corresponding author. Address: University of São Paulo, Animal Science Department, PO Box 9, CEP 13418-900, Piracicaba, SP, Brazil. Tel.: +55 19 34294009; fax: +55 19 34294215.
E-mail address: gbmourao@usp.br (G.B. Mourão).

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minimal sample preparation and preserves the components in their natural environment (Najbjerg et al., 2011). Recently, a novel approach for FTIR characterization of the milk fatty acid composition based on dried film measurements has been presented and compared to a standard FTIR approach based on liquid milk measurements (Afseth et al., 2010; Najbjerg et al., 2011). However, despite of the potential for using this approach in routine measurements the dried film approach has not yet found industrial use (Najbjerg et al., 2011).

Thus, considering the particularities of each method, the aim of this study was to compare the measurements obtained by gas chromatography and Fourier transform infrared spectroscopy using validation methodologies, such as Pearson correlation, Bland–Altman and Bayesian linear regression, in order to verify the equivalence of both methods in fatty acids determination.

2. Materials and methods

2.1. Dataset

89 milk samples were collected from Holstein cows with lactations ranging between one and six. These samples, preserved with bronopol, were analysed by GC and FTIR to determine the concentration of FA, expressed as grams per 100 g of milk fat.

In GC analysis, 35 mL of bovine milk were centrifuged at 12,000 rpm (17,800×*g*) for 30 min at 4 °C to separate the fat from whey. Fat was transferred to a 1.5 mL eppendorf and centrifuged at 12,000 rpm (17,800×*g*) for 20 min at 20 °C (Feng, Lock, & Garnsworthy, 2004). After centrifugation, the fat had separated into three layers: the top layer of lipids; the middle layer of protein, fat and other water-insoluble solids; and the bottom layer of water. Then, an aliquot of the lipid extract was methylated in two steps with 2 mL of 0.5 M sodium methoxide (10 min at 50 °C), followed by addition of methanoic HCl (10 min at 80 °C), according to Kramer et al. (1997) and was stored at −20 °C in amber vials containing 1.5 mL of nitrogen to avoid possible oxidation.

After this step, a gas chromatography system (Agilent Technologies 7890A) was used equipped with a flame ionisation detector for the quantification and determination of FA. 10 μL of the sample were injected into the system with a 10 μL syringe. The identification of the FA in the samples was done by comparing the retention time of fatty acid methyl esters with a standard. The standard used was the Supelco® mix of 37 compounds (Sigma Aldrich) and individual patterns for the identification of C18:1 *trans*-11 (vaccenic acid), C18:2 *cis*-9 *trans*-11, C18:2 *trans*-10 *cis*-12 (Nu-CheckPrep) and C18:1-OH (Sigma Aldrich) were obtained. The dataset acquisition was performed using the software Chem Station (Agilent Technologies).

FTIR spectra were taken using a Delta Instruments Combi-Scope™ Filter equipment, Advanced Instruments, Inc., Norwood, USA. Based on these analyses, the samples concentrations of the FA palmitic (C16:0), stearic (C18:0), oleic (C18:1*cis*-9), groups of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated (MONO), polyunsaturated (POLY) were determined. Extreme values, identified as values larger or smaller than three standard deviations from the mean of each fatty acid, were considered to be outliers and not considered (Table 1).

2.2. Concordance analysis

The comparison of the results obtained by both methods was carried out using Pearson's correlation, Bland–Altman analysis and Bayesian linear regression.

The Pearson's correlation (r) quantifies the degree of linear relationship between two variables (x and y). Values of r near to −1 represents an inverse relationship between two variables, and values near to 1 indicates a positive relationship between them, while r equal to zero means that the variables are not correlated (Gasparini, Barbieri, & Mazzer, 2007; Polit & Hungler, 1995).

Table 1
Number of observations (N), mean, standard deviation, maximum and minimum (in g/100 g of fat) of fatty acids measures obtained by gas chromatography and Fourier transform infrared (FTIR) spectroscopy.

Fatty acid	*N*	Mean	SD	CV	Maximum	Minimum
Gas chromatography						
C16:0	87	30.24	4.707	15.6	30.85	22.35
C18:0	86	11.25	2.795	24.8	20.07	4.82
C18:1*cis*-9	86	23.68	4.480	19.0	37.16	16.66
SFA	87	66.67	5.016	7.5	75.58	52.97
UFA	86	31.66	5.209	16.5	44.70	22.45
MONO	86	28.50	4.749	16.7	41.08	19.67
POLY	87	3.09	0.695	22.5	4.85	1.75
Fourier transform infrared spectroscopy						
C16:0	87	25.81	3.157	12.2	32.91	16.32
C18:0	86	18.17	3.587	19.7	32.56	11.49
C18:1*cis*-9	86	15.02	3.771	25.1	26.17	7.60
SFA	87	70.96	4.453	6.3	79.41	58.94
UFA	86	25.83	5.227	20.2	40.57	15.18
MONO	86	21.35	4.336	20.3	32.75	11.63
POLY	87	4.28	0.967	22.6	7.10	2.01

The CV values (the coefficients of variation) are expressed as (SD/Mean) × 100 (%). *Abbreviations:* C16:0, palmitic acid; C18:0, stearic acid; C18:1*cis*-9, oleic acid; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MONO, monounsaturated fatty acids; POLY, polyunsaturated fatty acids; SD, standard deviation.

The Bland–Altman analysis (Bland & Altman, 1999) is based on the construction of a scatter plot linking the average of results obtained by the two methods (abscissa axis) with the bias (ordinate axis), in order to evaluate the dimension of the differences between the methods, the dispersion of these differences around the mean, and possible outliers and trends. The average of the measurements was calculated by $(x_i + y_i)/2$, where x_i is the concentration of FA (in g/100 g fat) determined by the FTIR method for the ith milk sample and y_i is the concentration of the same FA measured by GC in the ith milk sample analyzed. Similarly, the bias was given by the difference between the measurements of each method on the same sample by the following equation: $(x_i - y_i)$. From the bias mean $(\overline{d})$ and its standard deviation (s_d), the limits of agreement (LA) were estimated using the equation $LA = \overline{d} \pm 1.96\, s_d$, which indicates the area where 95% of the differences in the studied cases can be found, considering a normal distribution of the data. The accuracy of the bias and the limits of agreement values were calculated using the standard error $(SE_{\overline{d}})$ and confidence interval (CI), the former being given by $SE_{\overline{d}} = s_d/\sqrt{n}$ where n is the sample size, and the latter estimated by $CI = \overline{d} \pm t \times EP_{\overline{d}}$, where t is the tabulated value of t distribution for $n - 1$ degrees of freedom.

The third method used was the simple linear regression with a Bayesian approach based in the model: $\boldsymbol{y} = \alpha + \beta\boldsymbol{x} + \boldsymbol{\varepsilon}$, where $\boldsymbol{y}$ is the vector of the observed values of fatty acid concentration estimated by GC, α is the regression intercept, β is the angular coefficient of regression, $\boldsymbol{x}$ is the vector of observed values of concentration of the same fatty acid found in the vector $\boldsymbol{y}$, but measured by FTIR, and $\boldsymbol{\varepsilon}$ is the residual vector ($\boldsymbol{\varepsilon} \sim N(\boldsymbol{0}, \boldsymbol{I}\sigma^2)$). For the vectors $\boldsymbol{y}$ and $\boldsymbol{x}$, the C16:0, C18:0, C18:1*cis*-9 FA as well as SFA, UFA, MONO and POLY FA groups were analyzed. For each linear regression, analyses were performed considering non-informative and informative *priors* for α (intercept) and β (inclination), and non-informative *priors* for τ (precision, $\tau = 1/\sigma^2$). For the α and β non-informative *priors*, normal distributions with mean equal to zero and precision equal to 10^6 were used, while for τ the *prior* the gamma distribution was used with shape and scale parameters equal to 10^{-3}. The informative *priors* for α and β were chosen assuming concordance between $\boldsymbol{y}$ and $\boldsymbol{x}$ in each regression, and therefore, $\alpha \sim N(0, 100)$, $\beta \sim N(1, 100)$. The range of 0 and 1 values by the