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Comparison between official methods and ultrasound spectroscopy used to determine the physicochemical features of raw milk

Comparação entre métodos oficiais e espectroscopia por ultrassom na determinação das características físico-químicas do leite cru

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ABSTRACT: The aim of the current study is to compare official methodologies (OM) and ultrasound spectroscopy (US) used to determine the physicochemical features of raw milk. Twenty-five (25) milk samples deriving from a rural property in Bacabeira County, Maranhão State, were assessed. Fat and protein contents, density at 15°C, cryoscopic index, total dry extract (TDE) and defatted dry extract (DDE) were determined based on official analytical techniques and ultrasound spectroscopy, in laboratory environment. Mean protein densities and contents determined through US and OM did not differ from each other ($P > 0.05$). Mean fat contents and mean values recorded for cryoscopic index, TDE and DDE - determined through US and OM - differed from each other ($P = 0.026$, $P = 0.040$, $P < 0.001$ and $P = 0.014$, respectively); positive correlations were observed in the analysis of these parameters ($R = 0.470$, $R = 0.118$, $R = 0.087$ and $R = 0.315$, respectively); methods' accuracy reached 0.180, 0.058, 0.155 and 0.075, respectively. Ultrasound spectroscopy is a fast technique whose results correlate to those of official analyses. However, it is necessary adopting a specific profile by calibrating the equipment based on information deriving from official analyses. Results in the present study have suggested the need of conducting more comprehensive studies to validate its conclusions.

KEYWORDS: Dairy products; Laboratory analysis; Technological innovation; Alternative methods.

RESUMO: Objetivou-se com o estudo comparar as metodologias oficiais e a espectroscopia por ultrassom na determinação das características físico-químicas do leite cru. Para isso, foram avaliadas 25 amostras de leite oriundas de uma propriedade rural do município de Bacabeira, estado do Maranhão. Em ambiente laboratorial foram realizados a determinação dos teores de gordura e de proteína, densidade a 15º C, índice crioscópico, extrato seco total (EST) e extrato seco desengordurado (ESD), por meio de técnicas analíticas oficiais e espectroscopia de ultrassom. As densidades médias e teores médios de proteínas determinados pelo US e pelos MO não diferiram entre si ($P > 0,05$). Os teores médios de gordura e os valores médios do índice crioscópico, EST e ESD obtidos pelo US e pelos MO foram diferentes ($P = 0,026$, $P = 0,040$, $P < 0,001$, $P = 0,014$, respectivamente), as correlações obtidas na análise desses parâmetros foram positivas ($R = 0,470$, $R = 0,118$, $R = 0,087$ e $R = 0,315$, respectivamente) e as acurácias dos métodos foram de 0,180, 0,058, 0,155 e 0,075, respectivamente. A espectroscopia por ultrassom é uma técnica rápida e os resultados obtidos correlacionam-se àqueles das análises oficiais. Mas, é necessário o emprego de um perfil específico por meio da calibração do equipamento com base nas informações das análises oficiais. Os resultados deste trabalho sugerem a necessidade de se realizar estudos mais abrangentes a fim de validar as conclusões obtidas.

PALAVRAS-CHAVE: Laticínios; Análises laboratoriais; Inovação tecnológica; Métodos alternativos.

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INTRODUCTION

Raw milk and dairy production is an important Brazilian economy sector that has been active in Brazil for a long time. This scenario enabled the evolution of stages adopted to obtain and industrialize the raw material, the preparation of by-products, and the conduction of quantitative and qualitative analyses required to ensure high-quality products.

Normative Instruction (NI) n. 76, which was issued by the Ministry of Agriculture, Livestock and Supply – MAPA - Ministério da Agricultura, Pecuária e Abastecimento (Brasil, 2018) –, has approved the Technical Regulations establishing the identity and quality features to be presented by refrigerated raw, pasteurized and type A pasteurized milk types. According to Article (Art.) 2 of the aforementioned NI, refrigerated raw milk is the "milk produced in rural properties, refrigerated and sent to milk and dairy establishments under official inspection service".

This raw material must be monitored by a group of laboratories belonging to the Brazilian Network of Laboratories for Milk Quality Control (RBQL - Rede Brasileira de Laboratórios de Controle da Qualidade do Leite), which are equipped to determine the quality features required by the legislation. It must be done in order to fulfill the primary purpose of monitoring and contributing to improve milk quality, based on goals of the National Milk Quality Improvement Program (PNQL - Programa Nacional de Melhoria da Qualidade do Leite). These laboratories are distributed in strategic-coverage geographic areas, countrywide. Milk processing establishments also carry out quality control procedures to assess suppliers' compliance with the composition and hygiene standards recommended by the legislation and, consequently, to assure quality products to their consumers, based on microbiological and physicochemical aspects.

Understanding the chemical composition of raw milk is essential for official policies focused on controlling its quality in programs that pay producers based on the quality of their products, in herd genetic enhancement programs, in determining the dairy cows' nutritional requirements and in monitoring animals' nutritional condition and clinical aspects. Dairy establishments can set different payment systems based on raw material-quality control actions, in order to encourage producers to produce high-quality milk. These systems are adopted in developed countries and their producers are paid based on the quality of the milk they produce.

Raw milk mandatory monitoring and the need of monitoring animals' nutritional status tend to increase the number of milk samples, as well as the frequency of milk analyses. There are several well-established methods focused on milk physicochemical analysis, such as the official ones enforced by the legislation, namely: Gerber (for fat) and Kjeldalh (for protein) methods (International Dairy Federation, 2001; International Dairy Federation, 2008). However, these methods require time, trained manpower and expensive reagents that, in some cases, can be dangerous to the ones handling them.

From this perspective, it is possible assuming that improving analysis methods is of paramount importance for the evolution of analysis methods and standards and, consequently, for the best quality of both raw materials and their derivatives (Ponsano *et al.*, 2007). Thus, automated analytical procedures, such as ultrasound spectroscopy, which are faster and more suitable for serial work (Buckin; O'Driscol; Smyth, 2003; Zhang *et al.*, 2011; Menegon *et al.*, 2020) were developed. According to Ponsano *et al.* (2007) and Venturoso *et al.* (2007), equipment developed for this purpose meet the new global demand, which is associated with the number of samples to be analyzed and with the frequency of analyses to be carried out.

The fundamental principle that matter can absorb sound by attenuating or changing its speed was the basis for the development of the ultrasound spectroscopy technique. Therefore, the range of this technology had its application expanded to feature physical and chemical mixes (O`Driscoll *et al.*, 2003; Ponsano *et al.*, 2007). Using spectroscopic techniques to analyze milk components is an important tool to provide useful information for milk producers, within a short period-of-time. These producers can use them to detect nutritional management issues and, consequently, to increase the production efficiency of their herds (Tsenkova *et al.*, 2000).

Using ultrasound spectroscopy is also of paramount importance for milk processing establishments, since it helps them to define the destination to be given to milk; to subsidize producers' differentiated payment, based on milk quality; and to make sure that their product is in compliance with milk composition standards required by the legislation (Ponsano *et al.*, 2007). In commercial establishments selling milk and dairy products, the main milk frauds are skimming, adding water, alkaline, preservatives, reconstituents, whey and mixing milk from different animal species (Abrantes; Campêlo; Silva, 2014).

There is legal support to replace the official analytical methods by the operational control ones, as long as their deviations from, and correlations to, their respective reference methods are well-known. Thus, the development and evaluation of alternative, faster, lower-cost and more accurate methods can benefit the production chain. However, the Brazilian literature lacks information about the correlation between methods officially used and recommended by official bodies to determine the physicochemical components of milk and ultrasound spectroscopy.

Wanderley *et al.* (2013) evaluated the behavior of official routine analytical methods (lipids, defatted dry extract, titratable acidity, relative density at 15 °C and cryoscopy) and fraud detection methods used for fluid milk. For the researchers, such analyzes were not effective in detecting adulterations, requiring additional analyzes of acidity neutralizing substances, preservatives and density restorers. Therefore, the aim of the current study was to compare official methodologies and ultrasound spectroscopy used to determine the physicochemical features of raw milk.

MATERIAL AND METHODS

Ethical Aspects

The current study is part of a larger research project titled "*Rede Neural Artificial para Detecção de Fraude em Leite por Adição de Água*" [Artificial Neural Network for Fraud Detection in Milk Added with Water]. It is in compliance with the Ethical Principles in Animal Experimentation, and registered in the Ethics and Experimentation Commission (CEEUA - Comissão de Ética e Experimentação) of Maranhão State University (UEMA – Universidade Estadual do Maranhão), under Protocol n. 010/2020.

Study Site

Milk collections were carried out in a rural property located in Bacabeira County, Maranhão State. This property supplies milk to a small agro-industry whose state inspection service operates in the aforementioned county.

Bacabeira County's territory covers 542,962 km²; it was recently included in the Metropolitan Region of São Luís and its population comprises 17,446 inhabitants (Ibge, 2020). Its municipal human development index (MHDI) is 0.629 – which is classified as medium (Ibge, 2010). Agriculture, plant extractivism, permanent and temporary crops, government transfers, the business sector and informal jobs are the main income sources in this county (Correia Filho *et al.*, 2011).

Sampling

Twenty-five (25) crossbred Girolando cows were selected through systematic sampling from the total number of 60 producing females, in the aged group 36-48 months, between 60 to 120 lactation days, in semi-intensive breeding system. The models were subjected to physical examination in order to be included in the current study. Visual inspection and palpation of the mammary gland were carried out, and they were followed by tests focused on detecting mastitis, by serological test to detect brucellosis and by allergy test to detect tuberculosis.

The dark background mug test was used to investigate clinical mastitis, based on Santos; Fonseca (2007), whereas the California Mastitis Test (CMT) was adopted to investigate subclinical mastitis, based on Beloti; Tamanini; Nero (2015) - it was done based on using equal parts of milk and reagent/CMT (2 mL). After model assessment procedures were over, 25 samples (one liter of milk, each) were collected.

Laboratory Tests

Fat and protein contents, density at 15° C, cryoscopic index, total dry extract (TDE) and defatted dry extract (DDE) were determined in laboratory environment, based on official analytical techniques and on ultrasound spectroscopy.

Official techniques

Density at 15 °C was determined with the aid of Quennève thermo-lacto-densimeter, based on samples' temperature - ranging from 10°C to 20 °C (Laboratório Nacional de Referência Animal, 1981). Fat rate was determined based on the Gerber's butyrometric method (International Dairy Federation, 2008). Protein content was determined based on the Ekomilk-Milkana Stara technique - Zagora, Bulgaria micro-Kjeldahl (International Dairy Federation, 2001), by multiplying the nitrogen content found in the samples by conversion factor 6.38. DDE was calculated by subtracting the fat rate from the TDS value. TDS was indirectly determined with the aid of Ackermann disk, based on values recorded for fat rate and density at 15°C (Laboratório..., 1981). Cryoscopic index was determined by freezing volumes of the analyzed milk samples in cryoscope equipment. All analyses were performed in triplicate.

Ultrasound spectroscopy

The ultrasound spectroscopy device (master complete milk analyzer) was calibrated before the analysis, based on the method described by the manufacturer, in order to get more accurate results.

Fat content in non-skimmed (profile 1) and skimmed (profile 2) milk samples was assessed to standardize and better understand the physicochemical parameters of the analyzed milk, to enable using more accurate values in the calibration process and to measure each parameter per analyzed sample, in a more reliable manner.

After the calibration process was over, the analysis of all 25 samples was carried out by placing 5 mL of each sample in the equipment. Physicochemical results were displayed on the equipment's panel and printed, later on.

Data Analysis

Values recorded for all six physicochemical parameters in the adopted methods were compared through *t* test for paired samples, as well as through correlation and linear regression analyses, in the SAS software (1992), at 5% significance level. The accuracy of the investigated methods was estimated based on residual standard error (s(y,x)), as recommended by the International Dairy Federation (1999).

RESULTS AND DISCUSSION

The descriptive statistics applied to the physicochemical parameters of all 25 raw milk samples analyzed through official methods (OM) and ultrasound spectroscopy (US) is summarized in Table 1.

Fat contents observed through both US and the official method (Gerber) were similar to each other; US and OM (R= 0.470) showed moderate correlation (P1 = 0.026) and accuracy of 0.180. Similar results were observed by Ponsano *et al.* (2007), whereas Menegon *et al.* (2020) reported that the same technique overestimated fat contents; Venturoso *et al.* (2007) mentioned that US

Table 1. Means, standard deviations and correlation coefficients of physicochemical parameters of all 25 raw milk samples analyzed through official methods and ultrasound spectroscopy, and descriptive levels of paired sample t tests and linear correlations.

Physicochemical Parameters	OM ($\bar{X} \pm s$)	US ($\bar{X} \pm s$)	P^1	R	P^2
Fat	4.0 ± 0.848	4.9 ± 0.947	0.026	0.470	0.021
Protein	2.93 ± 0.085	3.35 ± 0.904	2.971	-0.105	0.011
Density	1.026 ± 0.001	1.308± 0.335	5.437	0.066	0.004
Cryoscopic Index	-0.549 ± 0.005	-0.598 ± 0.023	0.040	0.118	0.012
TDE	17.39 ± 2.995	13.95 ± 1.862	<0.001	0.087	0.007
DDE	13.82 ± 2.226	9.05 ± 0.140	0.014	0.315	0.049

Wherein: X= means; s= standard deviations; R= correlation; OM= official methods; US= ultrasound spectroscopy; P^1 = paired sample t tests; P^2; linear correlations; TDE= total dry extract; DDE= defatted dry extract.

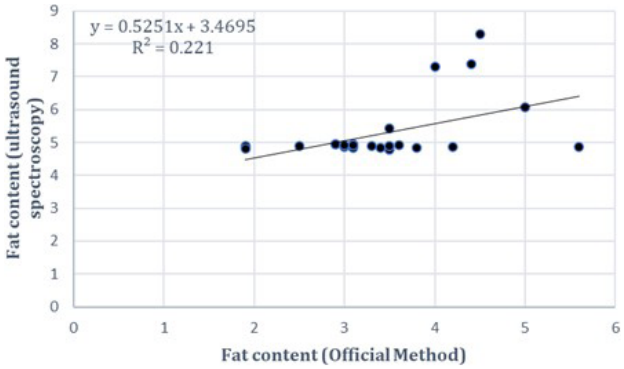
underestimated this parameter. Since US is based on the evaluation of the behavior presented by high-frequency sound waves when they propagate through a given sample, and given its subsequent calibration based on milk parameters obtained through official methods, it is possible stating that divergences concerning the calibration equations used in ultrasound devices can explain differences observed in the aforementioned studies.

Figure 1 shows the dispersion, the regression equation and the respective coefficient of determination recorded for fat content, based on OM and US. The coefficient of determination recorded for this variable has indicated that 22.10% of variability observed for fat content in the ultrasound spectroscopy is explained by the adopted official method.

Ultrasound spectroscopy (US) has overestimated the mean crude protein content in raw milk by up to 0.42 percentage points. Results recorded for this parameter have shown lack of statistically significant difference (P1 = 2.971) between mean protein levels, significant correlation between the Kjeldahl and US methods (P2 = 0.011) and correlation coefficient with significantly weak negative association (R = - 0.105). Similar to the reasons previously mentioned for milk fat - since ultrasound spectroscopy is based on the evaluation of the behavior presented by high-frequency sound waves when they propagate through a given sample - and given its subsequent calibration based on milk parameters obtained through official methods, it can be said that divergences concerning the calibration equations used in ultrasound devices can explain these differences.

Ultrasound spectroscopy is based on the evaluation of the behavior presented by certain parameters associated with high-frequency sound waves when they propagate through a given sample (Dukhin; Goetz; Travers, 2003). Sound speed is often the easiest ultrasound parameter to be measured; therefore, it is used in most ultrasound equipment (Nelligan, 2003). According to Buckin; O'Driscoll; Smyth (2003), milk density is directly linked to ultrasonic speed in US, since this parameter is extremely sensitive to molecular organization and to intermolecular interactions of the sample. Thus, it provides remarkably reliable information about components' concentration.

There was no significant difference (P^1 = 5.437) between mean density values obtained in thermo-lacto-densimeter and



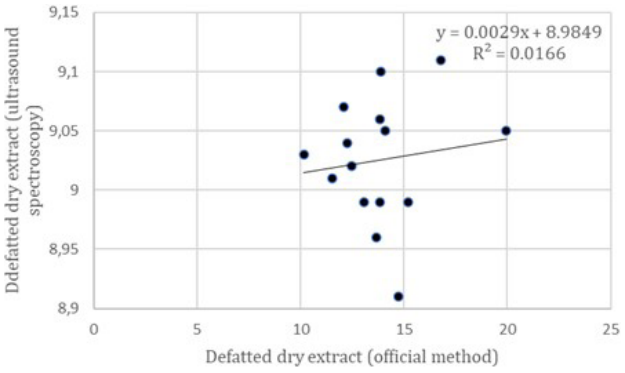
Source: Elaborated by the authors.

Figure 1. Scatter diagram of fat content between the official methodology and ultrasound spectroscopy representing the regression line and the coefficient of determination.

US (Table 1). There was significant correlation between these methods (P^2 = 0.004); their correlation coefficient reached 0.066 - this outcome has evidenced weak correlation between variables. It is worth emphasizing that density determination through the official method opens room for errors, since it depends on visual interpretation by laboratory technicians, on thermo-lacto-densimeter calibration and on the accuracy of values recorded at standard temperature (15° C).

Total dry extract (TDE) has evidenced remarkably weak correlation (P^1 < 0.01) between US and OM (R= 0.087), and accuracy of 0.058. On the other hand, mean DDE values were significantly different from each other (P^1 = 0.014); there was significant correlation between the evaluated methods (P2 = 0.049); correlation coefficient reached 0.315 (weak correlation) and the observed accuracy was 0.155. It is worth highlighting that, in order to determine DDE based on OM, it is necessary to previously determine TDE, which, in its turn, depends on density and fat content values. Therefore, any error at the time to determine these variables based on the official method will directly lead to errors in DDE values, a fact that can explain the statistical differences observed for the mean values recorded for the two methods.

Based on Figure 2, the coefficient of determination recorded for DDE, based on OM and US, indicated that



Source: Elaborated by the authors.

Figure 2. Scatter diagram of defatted dry extract between the official methodology and ultrasound spectroscopy representing the regression line and the coefficient of determination.

10.67% of its variability in ultrasound spectroscopy is explained by the adopted official method (subtracting fat rate from the TDE value).

Cryoscopic index values obtained through both US and the official method (cryoscopy) were close to each other. There was weak correlation (P1 = 0.040) between US and OM (R= 0.118); observed accuracy was 0.075.

Although the small number of studies comparing the performance of ultrasound spectroscopy to that of official methods used to determine the physicochemical features of raw milk and dairy products makes it hard to hold an in-depth discussion about this topic, there is consensus among researchers who have already investigated it (Miles; Shore; Langley, 1990; Buckin; O'driscoll; Smyth, 2003; Dukhin; Goetz; Travers, 2003; Nelligan, 2003; Ponsano *et al.*, 2007; Venturoso *et al.*, 2007; Menegon *et al.*, 2020) that ultrasonic analysis applied to milk samples is quite useful to provide information about their physicochemical composition, which is an essential factor for milk processing and quality control.

An important operational inference to be made in the current study lies on the fact that the physicochemical analysis of milk samples based on US has advantages over the one based on official methods, namely: (i) it does not require sample preparation; (ii) it uses minimal volumes of intact samples; (iii) it disregards the use of chemical reagents and specific glassware; and (iv) it simultaneously provides several results, based on two calibration profiles, within a few minutes. Results observed in this analysis provided important information for quick decision-making by milk and dairy products' processing establishments, as well as by regulatory and supervisory bodies, about the quality control method to be adopted.

Therefore, although ultrasound spectroscopy is a satisfactory method, analyses based on official methods should not, and cannot, be neglected. It is worth emphasizing that before placing one's total trust in equipment based on new technologies, it is necessary conducting studies to identify the limitations of the investigated device and, then, to calibrate it in the best way possible. Thus, it is recommended developing calibration equations based on a more comprehensive database in order to use this technique.

CONCLUSIONS

Results of raw milk physicochemical analysis based on the official methodology and on ultrasound spectroscopy presented positive correlation to each other (fat, cryscopic index, TDE and DDE). Results in the current study have suggested the need of conducting more comprehensive studies, based on a large number of samples, in order to validate the current conclusions.

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