Introduction

The buffalo’s milk, when compared to other species’ milk, possesses sensory features and better industrial efficiency, due to its higher total dry matter. Although it has a higher commercial value and its production is being increased, not much is made regarding the regulation of the authenticity and quality standards of the buffalo’s milk and its dairy products, making it difficult to control and inspect the products in the market (Sales et al., 2017).

The buffalo’s milk availability is seasonal, making it difficult to maintain a steady supply throughout the year (Czerwenka et al., 2010). During the off-season, the price of the buffalo’s milk elevates, reaching twice
the price of cow’s milk. With that, some breeders, suppliers and industries of dairy commit food fraud, which usually consist on adding cow’s milk to the buffalo’s milk and its dairy products for sale, without specifying it on the package, compromising the products nutritional values and causing losses to the consumer (Bonfatti et al., 2013).

The assessment of the authenticity of the buffalo’s milk delivered to the dairy producer is of great matter to the consumer and the food production authorities. Methods based on chromatography (Gonçalves et al., 2016), electrophoresis (Fuselli et al., 2015), immunochemistry (Dalmasso et al., 2011), mass spectrometry (Czerwenka et al., 2010) and biomolecular techniques (Agrimonti et al., 2015) were proposed to determine the authenticity of the buffalo’s milk and its dairy products. Although these methods provide results of high sensitivity and selectivity regarding the possible adulterations, they are expensive, they demand a lot of time, some demand the use of toxic chemical reagents, complex instrumentation and trained personnel, therefore they are not suited for routine analysis. The development of clean, efficient and fast analytic methods that guarantee the authenticity of the product is necessary, like the Spectroscopy in the Fourier’s Transform Infrared associated with Attenuated Total Reflectance (FTIR-ATR) (Lohumi et al., 2015; Arrieta-Almario et al., 2018).

The FTIR-ATR comprises the interaction between the electromagnetic radiation with the matter, able to generate spectrum that represent the “digital print” of a sample, to determine functional groups through the variation of the vibrational energy of atoms and molecules. It is considered a promising technology for the food industry, allowing fast and non destructive measurements. The advances in the data analysis with the application of chemometrics methods make this technology suited for a fast triage of large volumes of samples and ideal for monitoring food adulteration (Cassoli et al., 2011).

The chemometrics is a powerful tool that transforms complex analytic data in useful information, and techniques such as Principal Component Analysis (PCA) and Artificial Neural Network (ANN) have stood out as very important for the classification and detection of food authenticity (Souza et al., 2011).

The PCA is qualified tool that, reducing the dimensionality of the original data, aims to develop graphic models capable of separate samples of interest in different groups, according to similarities of differences (Kamal et al., 2015). The ANN consists in computer models, its processing units (artificial neurons) are capable of studying multiple dependent and independent variables simultaneously, with no necessity of previous information about the relation between them, also providing classification and prediction of the data (Silva et al., 2010; Goyal et al., 2012).

The aim of this study was to detect the addition of cow’s milk to the buffalo's milk through the electroscopic technique FTIR-ATR and the chemometrics tools PCA and ANN.

2 Materials and methods

2.1 Samples

Samples of raw whole milk of buffalo (Murrah) and cow (Dutch x Zebu) milk were obtained for 45 consecutive days and 9 combinations with rising levels of addition of cow’s milk to the buffalo’s (10% to 90%) with 10% interval and the reference samples of buffalo and cow (0% and 100% respectively) were prepared, 45 repetitions were performed, totaling 495 experimental units. Was realized the additions from 10% because it is considered that fraud in the industry does not occur in such low percentages and frauds involving milk mixtures of different species normally occur from 10% (Nicolaou et al., 2010). 1.0 mL aliquots were stored in eppendorfs and frozen at -20 ± 2 °C for 48 h and freeze-dried for 24 h in a FreeZone 4.5 L bench freeze-drier, at -48 ± 2°C and vacuum bomb of 86 L/min (Labconco, Kansas City, MO, USA), obtaining samples of around 0.4 g each. The composition analysis of the cow and buffalo milks were made according to the Association of Official Analytical Chemists - AOAC (1995).

2.2 Spectroscopic Analysis (FTIR-ATR)

The Fourier Transform Infrared Spectroscopy associated with Attenuated Total Reflectance (FTIR-ATR) was used for obtaining the spectrum in the region between 4,000 cm⁻¹ and 600 cm⁻¹, with resolution of 4.0 cm⁻¹, 64 scans, with acquisition time of approximately 30 s (Agilent Cary 630, Danbury, CT, USA). A background spectrum reading was made before each collection, in standardized conditions. The samples were previously freeze-dried, for in this equipment the water bands overlap with the bands
of the functional groups of the milk, compromising the reading of the peaks. The freeze drying increases the concentration of the components of the milk, facilitating the interpretation of the results due to the lesser influence of humidity.

The peaks, the functional groups found and wavenumber range in which the samples were analyzed, were identified in the literature (Pappas et al., 2008; Nicolaou et al., 2010; Subramanian et al., 2011; Santos et al., 2013; Jaiswal et al., 2015).

2.3 Chemometrics analysis

The classification, quantification and prediction of the samples of buffalo’s milk mixed with cow’s milk were performed using techniques of pattern recognition: PCA and ANN, which used the peak data obtained by the FTIR-ATR analysis. The PC number was determined considering the criterion of interpretable factors and the Kaiser’s criterion, which select the first PC that represent more than 70% of the variance of the original data and presents eigenvalue bigger than one, respectively (Ferreira, 2011). Scatter charts were plotted and groups of similar samples were formed through visual examination. The object of PCA was to reduce the dimensionality of the data set, preserving its variance and transforming them in new non related variables, the principal components (PC), as well as find similarities between the samples and group them. The grouping of the reference samples (0% and 100%) and mixed samples were verified. Therefore, the data were standardized ($\mu = 0$, $\sigma = 1$) and the PC obtained using the statistical software Statistical Analysis System (SAS)® Studio.

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The ANN was used to separate and sort the groups of reference samples (0% and 100%), the samples of buffalo’s milk with low level of addition of cow’s milk (10% to 20%), medium level of addition (30% to 40%) and high level of addition (50% to 90%), as well as to predict the content of the samples of buffalo’s milk added with cow’s milk using the software Java Neural Network Simulator, JavaNNS, version 1.1 (Wilhelm-Schickard-Institute, WSI, Tübingen, Alemanha) (Fischer et al., 2001). The absorbance data of the spectrum were randomized and divided in two groups: training (80% of the data) and validation (20% of the data). The randomization and distribution of the data occurred on each group, consisting of 6 samples for training and 9 for validation, so that the number of data were the same for all groups, totalizing 225 samples for this analysis.

The ANN are configured in a mathematical algorithm that relates the inputs and outputs of the net and has the capability to perform the learning through its interactions. They were composed of artificial neurons interconnect with 15 input neurons (corresponding to 15 variables/15 peaks), 2 hidden layers, and 1 output formed by 5 neurons, corresponding to the groups of 0%, 100% and to those with levels of addition.

The signals received by the output neurons performed calculations for the generation of information, without previous knowledge of the relation between them and the bond strength, which was related to the weights applied during the learning stage. In the two hidden layers 0 to 50 neurons were tested, totalizing 10 different settings, 200 interactions being used until the best architecture be chosen.

The supervised methodology multilayer perceptrons (MLPs) with feed-forward connections with parameters from -1 a 1. The training algorithm was the Resilient Propagation, improved version of the Backpropagation, which makes the convergence process more efficient (Silva et al., 2010) and the activation function used was a hyperbolic tangent (tanh).

The choice of the best setting for the classification was based on the number of interactions, of hidden layers, in the maximization of the classification rate and minimization of the Root Mean Square Errors (RMSE) in the training and validation stages, expressed by the Eq. (1)

$$RMS E = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (x_d - x_p)^2}$$  

Where: n is the number of pairs of data and xd and xp are the experimental data (also called desired values) and predicted data, respectively. The data were classified according to the classification rate, the bigger the classification rate, better the capability of the network in differentiate and sort the groups (Rai et al., 2005).

The generalization capability of the network was verified to predict the cow’s milk percentage added to the buffalo’s milk. The predicted values by the
model and the experimentally obtained were used to achieve the correlation coefficient in the validation stage and observed its significance through the test F ($p < 0.0001$). The choice of the optimal number of hidden layers and its neurons was based on the net that displayed the smaller RMSE value and the biggest correlation coefficient.

3 Results and discussion

3.1 Spectroscopic Study (FTIR-ATR)

15 absorption peaks important in the spectral region between 4,000 cm$^{-1}$ to 600 cm$^{-1}$ were identified through FTIR-ATR, and its maximal absorbances correspond to the vibrations of the functional groups present in the milk (Figure 1) (Pappas et al., 2008; Santos et al., 2013; Botelho et al., 2015).

The spectrum of the buffalo’s reference samples (0%) and cow’s (100%) presented minor differences, evidenced in large part, by more intense absorptions in the peaks of the buffalo’s milk. According to Nicolaou et al. (2010), the degree of absorption of the peaks correlates to the quantity of the component present in each type of milk, fatty acids, proteins (amides I and II) and carbohydrates (lactose). For each sample, the replicates presented similar behavior in the spectra, for the milks of different species have the same components, the difference is the quantity of those, causing the absorbance intensity to be different. The similarity of the peaks justifies the use of chemometric analysis to separate and differentiate the formulations.

According to the data found in this work, for the composition the buffalo’s milk presented higher content of its main components (8.84% of fat, 4.27% of protein and 5.37% of lactose) when compared to the cow’s milk (3.95% of fat, 2.85% of protein and 3.89% of lactose) and these differences were observed on the absorption of its respective functional groups.

Most of the generated peaks refer to the fat bonds vibrations, such as the peaks of the wavenumber region 3,278 cm$^{-1}$, 2,919-2,922 cm$^{-1}$, 1,742-1,746 cm$^{-1}$ and 2,851-2,855 cm$^{-1}$ due to the presence of O-H, CH$_2$ and C-O, respectively, which were displayed more intensely on the buffalo’s samples, considering that the samples were previously freeze-dried and the peaks were not influenced by the presence of water (Ménard et al., 2010).

The peaks related to proteins and carbohydrates appear, in a smaller proportion than the other peaks, and this difference of intensity happened especially in the peak region of 1,147-1,149 cm$^{-1}$, related to the bonds C=O/C=C/C=O/CH of the carbohydrate (Pappas et al., 2008) that showed a larger energy absorption than the others (Figure 2). The peaks referring the vibration of the protein bonds in the region between 699-703 cm$^{-1}$ (N-H), 1,240-1,250 cm$^{-1}$ (C=O/N/H) were more intense for the buffalo’s samples (0%) (Jaiswal et al., 2015), also as the other peaks referring the carbohydrates (1,019-1,133 cm$^{-1}$ and 890-893 cm$^{-1}$) and the fat (768-780 cm$^{-1}$), confirming what the literature addresses regarding the relation between the degree of absorption and the
components content of each type of milk (Nicolaou et al., 2010).

The spectrum of the samples with addition of cow’s milk showed slight variations of absorbance, barely perceptible, both on the samples with smaller addition (10%) and with bigger addition (90%) (Figure 2). With the increase of the addition levels, the samples showed behavior similar to the cow’s samples (100%) and the samples with low levels of addition tended to the buffalo’s samples (0%) spectrum.

Nicolaou et al. (2010) verified differences present between the spectrum of the milks of goat, sheep and cow through the appearance of more intense peaks for the sheep’s milk in the region of 2,927 cm$^{-1}$ related to the fat (CH$_2$ e C-O), justifying the bigger absorption of the goat for its fat content compared to the other studied, which as similar to the result of this study.

3.2 Chemometrics analysis: PCA and ANN

The PCA was used to discriminate the buffalo’s and cow’s milk samples (0 and 100%) and verify the influence of the levels of addition of cow’s milk in the groups separation (Figure 3). Considering Kaiser’s criteria and interpretable factors, the three first principal components capable of explaining between 78-86.45% of the variability of the original data were chosen.

Based on Pearson’s correlation coefficients and on the correlation probability (0.65-0.7), the PC1 (44.59%) presented correlation with the variables corresponding to the fats (3,278 cm$^{-1}$, 1,458 cm$^{-1}$, 1,376 cm$^{-1}$, 768 cm$^{-1}$), to the proteins (1,647 cm$^{-1}$, 1,541 cm$^{-1}$, 1,241 cm$^{-1}$ e 700 cm$^{-1}$) and carbohydrates (1,023 cm$^{-1}$, 892 cm$^{-1}$). The PC2 (25.58%) showed itself highly correlated with most of the variables related to the fat (2,919 cm$^{-1}$, 2,081 cm$^{-1}$, 2,851 cm$^{-1}$, 1,742 cm$^{-1}$, 1,148 cm$^{-1}$, 1,541 cm$^{-1}$), while the PC3 (16.52%) presented significant correlation with some variables of fat (1,742 cm$^{-1}$, 2,919 cm$^{-1}$, 2,851 cm$^{-1}$). A pattern of grouping of the samples of buffalo’s milk (0%) and cow’s (100%) with the appearance of two homogeneous groups with little dispersion between them was noted (Figure 2).

Velioglu et al. (2017) discerned samples of cow’s and buffalo’s milk using the first two PC scores (99.91%) of the spectrum obtained through spectroscopy by graphic dispersion. Mabood et al. (2017) differentiated samples of goat’s, cow’s and camel’s milk through NIR spectroscopy analysis, also using the PCA, separating 54 studied samples.

As addition levels increased, samples of buffalo milk with addition of cow’s milk were grouped showing less dispersion between groups. Despite the difficulty in the separation of the smaller levels of addition, it was possible to observe the formation of a third group located between the reference samples (0% and 100%) from 40% of cow’s milk added to the buffalo’s milk (Figure 3).

With the increase of cow’s milk added to buffalo milk, the samples showed a similar behavior to that of cow’s milk, reason that no third group was formed between the samples containing 60% to 90% addition, since these showed similar behavior to cow’s milk. In contrast, with lower levels of cow’s milk addition, the samples resembled buffalo milk, making it more difficult to detect adulteration. Carvalho et al. (2015) also reported that in their study with samples of powdered milk added with whey, with smaller levels of serum addition (1% to 6%) presented the spectros with behavior similar to the samples with no addition, due to the small serum contend and the similarity of the composition between the studied samples.

It was possible to observe that the components PC2 and PC3, which presented correlation with most of the variables referring to fat, influenced in the separation of the samples depending on the levels of addition, as shown in Figure 4, the vertical separation between the samples can be verified, in which those with higher cow’s milk content (90% and 100%) separated from the others due to the influence of PC2, and the horizontal separation (0%, 10%, 20%), presented in different quadrants of the others (30%, 40%, 50%, 60%, 70%, 80%, 805) due to influence of PC3. Considering that the PCA is a triage analysis, it was possible to separate the samples in groups to perform the subsequent ANN analysis: buffalo’s (0%), cow’s (100%), group with low level of addition (10% to 20%), medium level of addition (30% to 40%) and high level of addition (50% to 90%).

For the ANN, the net with the best setting had fifteen neurons on the input layer, fifty neurons on the first hidden layer, twenty neurons on the second hidden layer and five neurons on the output layer with RMSE of 0.23, 95.55% of classification rate and 100% to the reference samples, with medium level of addition of cow’s milk to the buffalo’s milk (30% to 40%) and high level (50% to 90%) (Table 1).

The group containing samples with low level of addition (10% to 20%) presented smaller classification rates for most of the tested architectures, when compared to the other groups, making it possible to predict the complexity of the separation of the
Fig. 3. Graphic of scatter of the samples of buffalo’s milk (0%) and cow’s milk (100%) (a) and buffalo’s milk added with different levels of cow’s milk 10% (b), 20% (c), 30% (d), 40% (e), 50% (f), 60% (g), 70% (h), 80% (i), 90% (j).
Table 1. Setting of the tested Artificial Neural Network, Root Mean Square Error (RMSE), and classification rates (%) of the buffalo’s milk (0%), cow’s milk (100%), of the group of samples with low level of addition of cow’s milk to the buffalo’s milk (10% a 20%), with medium level of addition of cow’s milk to the buffalo’s milk (30% a 40%), with high level of addition of cow’s milk to the buffalo’s milk (50 a 90%) and total classification rate (T).

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NIL = Neurons of the input layer / HL = Hidden layer / NOL = Neurons of the output layer / RMSE = Root Mean Square Errors.

Table 2. Settings of the neural networks tested for the prediction and the predictive coefficients.

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NIL = Neurons of the input layer / HL = Hidden layer / NOL = Neurons of the output layer / RMSE = Root Mean Square Errors.

adulterated buffalo’s samples, especially with low content of cow’s milk.

The detection of mixtures of buffalo’s and cow’s milk, especially with low percentages of cow’s milk, turned out to be more complex than the differentiation of the reference samples (0% and 100%), for these are similar compositions in their qualitative aspects, as it happened with the differentiation of the samples of cow, goat and sheep in the study of Nicolaou et al. (2010). When it is not detected, the fraudulent addition of cow’s milk to the buffalo’s milk harms the final quality of the product and its dairy products, generating a nutritional and economic loss for the consumer (Gonçalves et al., 2016).

The ANN have been implemented on studies of milk adulteration with replacers and with addition of other species (Balestrieri et al., 2001; Valente et al., 2014). However, one of the obstacles found for this and others techniques is the similarity between the milk samples of certain species, which make their classification in lower levels of adulteration difficult. This technique is set in a non linear system, capable of separating data that would not be separable linearly and has the advantage of being capable of learning and improving the performance of new data, which allows the generation of a model with great importance in the detection of mixtures between cow’s and buffalo’s milks.
Fig. 4. Graphic of mean values of the scores of the samples of buffalo’s milk (0%) and cow’s milk (100%) and buffalo’s milk added with different levels of cow’s milk (10% a 90%).

Fig. 5. Experimental values versus predicted values by the net of the samples of the buffalo’s milk (0%), cow’s milk (100%), and buffalo’s milk added with different levels of cow’s milk (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%).

To predict the addition of the cow’s milk content to the buffalo’s milk, the ANN that presented the best setting included two hidden layers with 50 and 20 neurons, respectively, which displayed satisfactory RMSE and correlation coefficient, of 0.23 and 0.971 (Table 2).

The net that obtained the smallest RMSE and biggest correlation presented good capability of prediction and/or generalization, whose scatter chart displayed the data in ascending order and concentrated around the tendency line with the experimental and/or real values and the values predicted by the net highly correlated ($R^2 = 0.971$), with significant correlation ($p < 0.001$) according to the F test (Figure 4).

The results of this study were shown to be important in the differentiation between the samples of milk of the bovine and buffalo species and in the prediction of adulterated samples to aid in the detection of the buffalo’s milk adulteration, indicating the PCA and ANN, associated with FTIR-ATR, as an important non destructive tool for triage the authenticity of the buffalo’s milk.

**Conclusions**

The spectroscopic analysis FTIR-ATR has been shown to be efficient in generating important data for the classification and prediction of the samples of buffalo’s milk added with cow’s milk demonstrating great potential for adulteration detection. The FTIR-ATR associated to the chemometry is a qualitative method, especially when combined with the PCA and ANN chemical analysis, being able to identify the presence of adulteration as well as the levels of addition.

The PCA allowed the summarization of the data and the detection of the addition of cow’s milk to the buffalo’s milk, highlighting the importance of the variables related to the fat for the classification and differentiation of the samples. It was observed that the ANN can be used as an identification and prediction tool of the studied samples, with good classification rates, satisfactory errors and high capability of generalization.

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