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Analytical Methods

Capillary zone electrophoresis for fatty acids with chemometrics for the determination of milk adulteration by whey addition

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ABSTRACT

Adulteration of milk with whey is difficult to detect because these two have similar physical and chemical characteristics. The traditional methodologies to monitor this fraud are based on the analysis of caseinomacropeptide. The present study proposes a new approach to detect and quantify this fraud using the fatty acid profiles of milk and whey. Fatty acids C14:0, C16:0, C18:0, C18:1, C18:2 and C18:3 were selected by gas chromatography associated with discriminant analysis to differentiate milk and whey, as they are present in quite different amounts. These six fatty acids were quantified within a short time by capillary zone electrophoresis in a set of adulterated milk samples. The correlation coefficient between the true values of whey addition and the experimental values obtained by this technique was 0.973. The technique is thus useful for the evaluation of milk adulteration with whey, contributing to the quality control of milk in the dairy industry.

1. Introduction

Brazil is the fifth largest producer of cow's milk, accounting for about 5% of global production from 2011 to 2013 (FAO, 2014). Much of this milk is used in the manufacturing of cheese. In 2013, global cheese production was estimated to be about 20,000 kT (kilotons), resulting in approximately 200,000 kT whey byproduct (FAO, 2014). Whey may be classified as sweet (pH ~ 6.5) or acidic (pH ~ 4.5). Sweet whey is formed by the cleavage of casein by adding rennet to milk, whereas acidic whey is formed through the conversion of lactose into lactic acid by the action of lactic acid bacteria, or by the addition of organic or mineral acids (FAO, 2013; Fischer & Kleinschmidt, 2015). In the dairy industry, the most common milk adulteration involves the addition of water, alkali agents, sodium chloride, sucrose and/or whey (Harding, 1995). The addition of whey is among the most common frauds committed in Brazil is, as this component is 90% cheaper than milk. The common fraud range is up to 20 or 25% of whey addition, because the consumer does not perceive differences in flavor, but fraud could reach up to 60% (Ferrão, Mello, Borin, Maretto, & Poppi, 2007). Brazilian law dictates that whey cannot be added to milk. However, due to the availability and low cost of whey, this fraud is economically attractive, thus harming consumers and law-abiding competitors (de Carvalho et al., 2015).

Commonly, fraud by whey addition is identified by detecting and quantifying caseinomacropeptide (CMP), present in sweet whey (Van Riel & Olieman, 1995). However, the activity of some bacterial proteases can generate false positive results due to the production of CMP (Recio, López-Fandiño, Olano, Olieman, & Ramos, 1996). Furthermore, the addition of acidic whey cannot be detected (Miralles, Bartolomé, Ramos, & Amigo, 2000).

There are several studies in the literature reporting various milk adulterants such as water to control the acidity, salt or sugar to correct the density, sweet or acid whey, hydrogen peroxide and many others (Sharma & Paradakar, 2010). Several techniques have been proposed for the detection of the adulteration of fluid milk, such as electrical admittance spectroscopy (Sadat, Mustajab, & Khan, 2006), enzyme-linked immunosorbent assay, liquid chromatography–tandem mass spectrometry (MacMahon, Begley, Diachenko, & Stromgren, 2012), mid-infrared microspectroscopy (Santos, Pereira-Filho, & Rodriguez-Saona, 2013) and nuclear magnetic resonance (Santos, Pereira-Filho, & Colnago, 2016). In addition, there are some studies that have identified milk adulteration by capillary electrophoresis (CE) (Cartoni, Coccioli,

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Jasionowska, & Masci, 1999; Herrero-Martınez, Simó-Alfonso, Ramis-Ramos, Gelfi, & Righetti, 2000; López-Tapia, Garcıa-Risco, Manso, & López-Fandiño, 1999; Miralles, Krause, Ramos, & Amigo, 2006; Pérez-Míguez, Marina, & Castro-Puyana, 2016; Recio, Amigo, & López-Fandiño, 1997; Recio, Garcıa-Risco, López-Fandiño, Olano, & Ramos, 2000). However, most of them are based on the analysis of proteins or amino acids.

There are reports concerning differences in the fatty acid (FA) profile of milk and whey from cheesemaking, especially those related to phospholipids (Bohdziewicz, 2006; Boyd, Drye, & Hansen, 1999; Jhanwar & Ward, 2014). To the best of our knowledge, there are no studies reporting on the use of the FA profile associated with CE to detect milk adulteration by whey. This separation technique has established itself as a good analytical tool for FA analysis in food samples in recent decades (de Oliveira et al., 2016; Oliveira et al., 2014).

In this context, this study presents an alternative approach to detect milk fraud by the addition of whey using the FA profile in milk and whey. The FA profile was analyzed by gas chromatography with flame ionization detection (GC-FID), a classic technique, in order to determine the key differences in the composition of FA in both samples. FA selected by GC-FID were then detected and quantified in milk samples intentionally adulterated with whey by a rapid method of capillary zone electrophoresis with ultraviolet detection (CZE-UV) (Barra et al., 2013) associated with chemometric tools.

2. Experimental

2.1. Chemicals and solutions

All reagents and standards used were of analytical grade. Water was purified by deionization using the Milli-Q system with a resistivity of 18.5 MΩ cm (Millipore, Bedford, MA, USA). Methanol (MeOH), acetonitrile (ACN), hexane, isopropanol, acetic acid, sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), anhydrous sodium sulfate (Na_2SO_4) and sodium hydroxide (NaOH) were purchased from Vetec (Rio de Janeiro, RJ, Brazil). Polyoxyethylene 23 lauryl ether (Brij 35) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A solution of sodium methoxide, sodium dodecylbenzenesulfonate (SDBS) and 1-octanol was purchased from Fluka (St. Louis, MO, USA).

FA standards for CE analysis such as tridecanoic acid (C13:0), myristic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:1c), elaidic acid (C18:1t), linoleic acid (C18:2) and linolenic acid (C18:3) were purchased from Sigma (St. Louis, MO, USA). Individual stock of FA solutions were prepared by dissolving appropriate masses at a concentration of 20.0 mmol L^{-1} in MeOH. All solutions were stored in the freezer until analysis. The FA standard mixtures were prepared daily from the appropriate dissolution of individual FA stock solutions in MeOH at a concentration of 0.5 mmol L^{-1}.

A commercial fatty acids methyl esters (FAME) standard mixture with 37 components Supelco (FAME 37) was purchased from Sigma (St. Louis, MO, USA) for analysis by GC-FID. This solution was stored in the freezer until analysis.

The extraction solution was prepared by adding three parts of hexane to two parts of isopropanol in a volumetric flask. A Brij 35 aqueous stock solution at a concentration of 50.0 mmol L^{-1} was prepared with the help of an ultrasonic bath for dissolution. This solution was kept at 4 °C to prevent mold formation and was used within 20 days of preparation. A mass corresponding to 0.5 mol L^{-1} of NaOH was dissolved in a volumetric flask with methanol. Na_2SO_4 solution (0.5 mol L^{-1}) and SDBS (100.0 mmol L^{-1}) were dissolved in water. Aqueous buffer stock solutions at concentration of 100.0 mmol L^{-1} were prepared from the weighed quantities of NaH_2PO_4 and Na_2HPO_4, corresponding to 50.0 mmol L^{-1} of each one in a 100.0 mL volumetric flask, resulting in final buffer concentration of 100 mmol L^{-1}. The background electrolyte (BGE) solution was prepared daily from the appropriate dilution of stock solution and solvent incorporation. The BGE solution was exposed to ultrasound for 10 min in order to remove air bubbles.

2.2. Samples

For this study, a set of seven samples of raw milk were acquired from different producers in the region close to the city of Juiz de Fora, MG, Brazil. Preliminary quality testing was performed. Based on the results of dornic acidity, pH, freezing point, fat and protein levels, all samples were within the standards required by local legislation. Thus, seven samples of raw milk were used for analysis by the traditional method of analysis of FA by GC-FID. Additionally, a mixture of equal parts of each of the seven initial samples was used for the construction of the analytical curve. These samples were analyzed by the CZE-UV method with indirect detection.

Sweet whey from each individual sample and a mixture of raw milk were obtained by the enzymatic coagulation of milk samples using chymosin produced by *Aspergillus niger* var. *awamori* (coagulant HA-LA, Chr. Hansen, Valinhos, Brazil) with a coagulant strength of 1:3000/75 IMCU. One milliliter of liquid coagulant was added to 1.0 L of milk according to the label recommendations. The incubation time was 40 min at 36 °C. Then, the mass was removed and the whey was filtered using paper filter, with retention of particles 12–15 μm in size, and collected. Finally, whey was incubated at 80 °C for 5 min before use to stop the enzyme activity. In short, seven samples of raw milk and their respective whey fractions were analyzed by GC-FID.

The samples composed of a mixture of raw milk and the respective whey were used to prepare intentional adulterations, by adding mixed sweet whey into milk at different percentages (4.0, 8.0, 12.0, 16.0 and 20.0%), simulating five levels of adulteration. The samples were then analyzed in authentic duplicates by the CZE-UV method with indirect detection.

2.3. Extraction method

For extraction, 2.0 mL of milk, 7.5 mL of extraction solution and 5.0 mL of an Na_2SO_4 solution were pipetted into a glass tube with a screw cap and subjected to vortexing for 1 min at maximum speed. Then, it was allowed to stand in an ice bath to achieve phase separation. The organic (upper) phase was then transferred to a flat bottom flask. The solvent was evaporated under negative pressure at 40 °C in order to obtain the lipid residue in the flask. (Hara & Radin, 1978). This procedure was used prior to GC and CE analysis in duplicate for each technique.

2.4. GC sample preparation

The lipid extract was transesterified using base catalysis (Christie, 1993; Christie & Han, 2012). For this procedure, 2.0 mL of sodium methoxide in methanol was added to a flask containing the lipid extract and heated under reflux at 50 °C for 10 min. After cooling, 100 μL of cold glacial acetic acid was added to neutralize the solution, followed by the addition of 5.0 mL of deionized water and 3.0 mL of hexane. After phase separation using vortexing in an ice bath, the organic phase (upper) was transferred into a test tube. Then, 3.0 mL of hexane was added to the first mixture in order to increase extraction efficiency. After stirring and phase separation, the organic phase (upper) was transferred to the same test tube and 1.0 g of anhydrous Na_2SO_4 was added to dry the FAME solution. Then, the solution was transferred to a 5.0 mL volumetric

flask and the volume was made up with hexane. The solution was then transferred into a capped vial and stored in the freezer at −20 °C until analysis. Prior to injection into the GC-FID equipment, the solution was transferred to an auto-sampler vial and analyzed without dilution.

2.5. CE sample preparation

Lipid extracts were saponified by refluxing with 2.0 mL of a solution of NaOH in MeOH at 0.5 mol L^{-1} in a water bath (75–80 °C) for 20 min. After the saponification reaction, samples were transferred to a 5.0 mL volumetric flask and made up to the volume with MeOH. The solution was stored in a capped bottle in the freezer until analysis. Prior to injection into the CE device, samples were diluted with MeOH in a ratio of 1:2 (v/v) in 1.0 mL volumetric flask containing 0.5 mmol L^{-1} C13:0, used as an internal standard (IS).

2.6. GC instrumentation

The FAME analysis was performed on a Shimadzu gas chromatographic unit (GC-2010 Plus, Shimadzu, Kyoto, Japan) associated with flame ionization detection and split-splitless injection with an AOC-i-20 autoinjector. A capillary column of fused silica was used (CP-SIL 88 for FAME, 100 m × 0.25 mm × 0.2 μm, Agilent Technologies, Palo Alto, USA). The chromatographic conditions were: injection volume of 1.0 μL in split mode with a flow rate of 20 mL min^{-1} at 250 °C; the FID temperature was set at 270 °C; the column temperatures were initially programmed at 80 °C with an increase of 4 °C min^{-1} to 220 °C, held for 5 min, then the temperature increased 4 °C min^{-1} to 240 °C and maintained for 10 min. The carrier gas used was hydrogen at a flow rate of 1.0 mL min^{-1} and the pressure was 140.3 kPa. The compounds were identified by co-injection of standards and by the comparison of their retention times with that of the FAME 37 mixture. The FA were determined by area normalization and expressed in g per 100 g of fat (AOCS, Reapproved 1997 Revised, 1997 Revised 2001).

2.7. CE instrumentation

The FA experiments were performed in a CE system (CE 7100, Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector (DAD), with ultraviolet (UV) (224 nm) detection and a temperature control device (25 °C). Samples were hydrodynamically injected (12.0 mbar for 4 s) and the electrophoretic system was operated under normal polarity and constant voltage (+19 kV). For all the CE experiments, a fused silica capillary tube was used, which was 48.5 cm long (40 cm effective length), with a 75 μm internal diameter (ID) and 375 μm outside diameter (OD) and a fluoropolymer external coating (Polymicro Technologies, Phoenix, AZ, USA). When a new capillary was used, it was conditioned by a pressure flush using 1.0 mol L^{-1} NaOH solution (30 min), deionized water (15 min) and BGE (15 min). In between runs, the capillary was washed with 0.50 mol L^{-1} NaOH solution (2 min), deionized water (2 min) and fresh BGE (2 min, pressure flush).

2.8. Statistical data treatment

The files of fourteen GC-FID chromatograms, seven of milk and seven of whey, were exported and assembled into a single data table and used in the discriminant analysis by orthogonal partial least squares (OPLS-DA). For the CZE-UV method, the area of each FA was normalized by the area of the internal standard. A table containing these values was used to fit the multiple linear regression (MLR) model.

GC-FID chromatograms was obtained with GC Solution software (Shimadzu), CZE-UV electropherograms with HP ChemStation software (Agilent Technologies). OPLS-DA analysis was performed using SIMCA P+12 software (Umetrics) and the MLR model was calculated using MS Excel software (Microsoft).

3. Results and discussion

3.1. FA profile by GC-FID associated with OPLS-DA

The lipid composition of cow's milk contains approximately 150 different FA (Cruz-Hernandez et al., 2007). Fig. 1A for milk samples and Fig. 1B for whey samples show about 20 FA in the chromatograms. Whey has a lower magnitude for some FA because part of the lipid fraction is incorporated into the cheese mass, which is formed in the process of obtaining whey. Due to the complexity of the chromatograms of the milk and whey samples, it was difficult to conceive a reliable adulteration prediction model based on the integration of individual FA in each sample. However, the chemometric approach, taking into account multivariate statistical methods, may help in the elucidation of complex samples.

OPLS-DA was performed using a table containing fourteen chromatograms; the discriminant classes were milk and whey. This model was fitted with raw chromatograms, without transformation of the data using only centered scaling, presenting coefficients $R^2X = 0.972$ for the explained variance of the chromatograms and $R^2Y = 0.99$ for the explained variance of the discriminant classes with a capacity of prediction $Q^2 = 0.986$.

The score diagram, shown in Fig. 2A, have very good separation, with the group of milk samples on the left side of the diagram and the whey samples on the right side, with all samples inside the Hotelling T-square ellipse using a 95% confidence interval. The differences between individual samples were much less than the differences between milk and whey globally. This was expected because the FA concentration in whey is much lower than that in milk. However, it is important note that the OPLS-DA analysis was not used to show a predictable result, but rather to select the potential FA markers responsible for separating these two groups. At this point, Fig. 2B shows a graph of variable importance in the projection (VIP). This view assigns a weight to each input variable in the OPLS-DA model, giving more importance for the variables that contribute most to explaining the separation observed in the score diagram in Fig. 2A, i.e., among of all FA present in the sample, six (C14:0, C16:0, C18:0, C18:1t, C18:1c and C18:2) were the most important in separating milk and whey samples. The other FAs showed greater variation between samples within each group, or were close to the noise level, and were not considered to be good markers between the two groups.

3.2. FA quantification by CZE-UV with MLR

These FA selected by OPLS-DA analysis were examined by an alternative analysis method using CZE-UV with indirect detection, using a new set of samples. Fig. 3 shows the FA profile obtained by the CZE-UV method. In this CZE-UV method, it was not possible discriminate C18:1t and C18:1c because they had coeluting peaks; thus, the C18:1 shown in this figure represents the sum of C18:1c and C18:1t. With this method, it was also possible to separate out C18:3 in the same analysis.

Fig. 3 shows the FA used to compose a prediction model of the percentage of whey added to fluid milk samples. The CZE-UV method was able to analyze real samples, taking into account these six FA. The CZE-UV method offers some advantages over the GC-FID method, such as a reduction in the run time to less than 15 min, whereas the GC-FID method requires about 50 min time

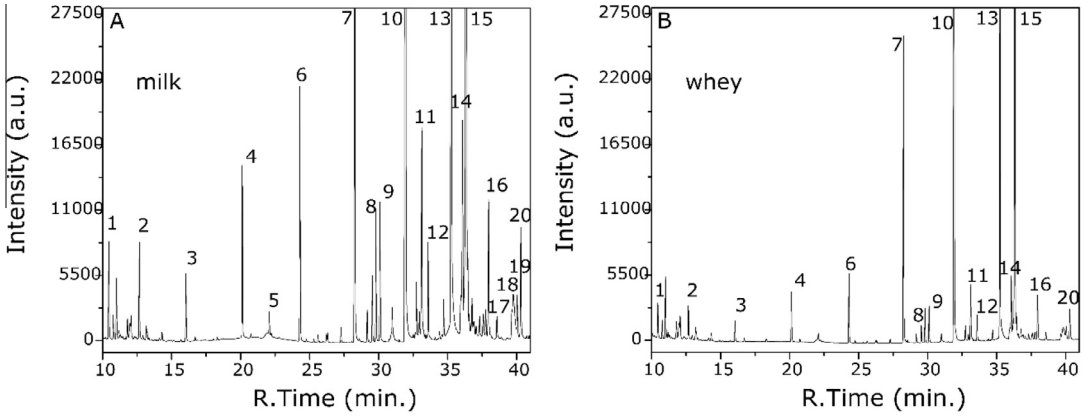

Fig. 1. GC-FID analysis to evaluate FA profile. (A) milk and (B) whey. Various FA peaks were identified in both milk and whey (Figure 1) by comparing with FAME 37 standard mixture: 1 – C4:0; 2 – C6:0; 3 – C8:0; 4 – C10:0; 5 – C11:0; 6 – C12:0; 7 – C14:0; 8 – C14:1; 9 – C15:0; 10 – C16:0; 11 – C16:1; 12 – C17:0; 13 – C18:0; 14 – C18:1t; 15 – C18:1c; 16 – C18:2; 17 – C20:0; 18 – C18:3γccc; 19 – C20:1; 20 – C18:3αccc.

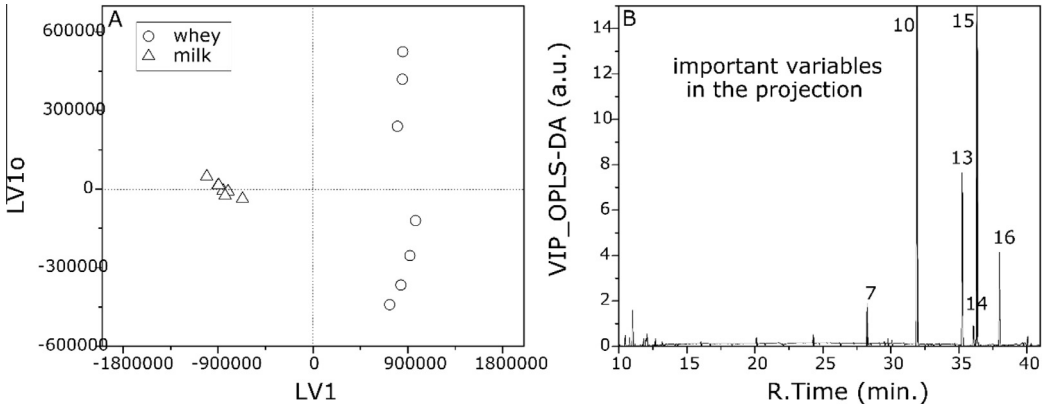

Fig. 2. Multivariate analysis associated to GC-FID results. In (A) is shown the score plot of OPLS-DA model and (B) is the most importance variables to explain the separation between milk and whey samples. Peaks identified by comparison with FAME 37 standards are: 7 – C14:0; 10 – C16:0; 13 – C18:0; 14 – C18:1t; 15 – C18:1c; 16 – C18:2.

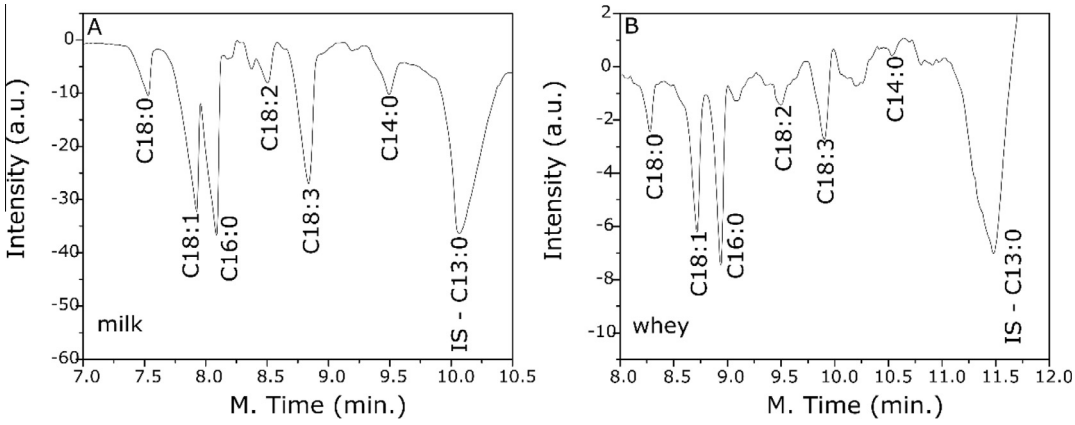

Fig. 3. FA profile analyzed by CZE-UV with indirect detection at 224 nm. Milk (A) and whey (B).

for each sample, although it does not require a derivatization reaction in the sample preparation step.

For the CZE-UV method, the area of each peak (C14:0, C16:0, C18:0, C18:1, C18:2, and C18:3) was normalized to C13:0, i.e. the IS area. Fig. 4 shows an example of duplicate analysis for samples of milk and whey. These bar diagrams provide a good overview of the distribution of the six monitored FA. Milk and whey have a very similar FA profile, and the concentration of each FA decreases in the following order: C16:0, C18:1, C18:3, C18:0, C18:2 and C14:0, in both samples. However, the magnitude of these concentrations was much higher in the milk samples, which can be used to adjust the regression equation to predict the mixture of these samples.

The samples with five levels of intentional adulteration by the addition of whey into milk, in authentic duplicates (ten analyses), and the content of each of the six monitored FA was used to fit am

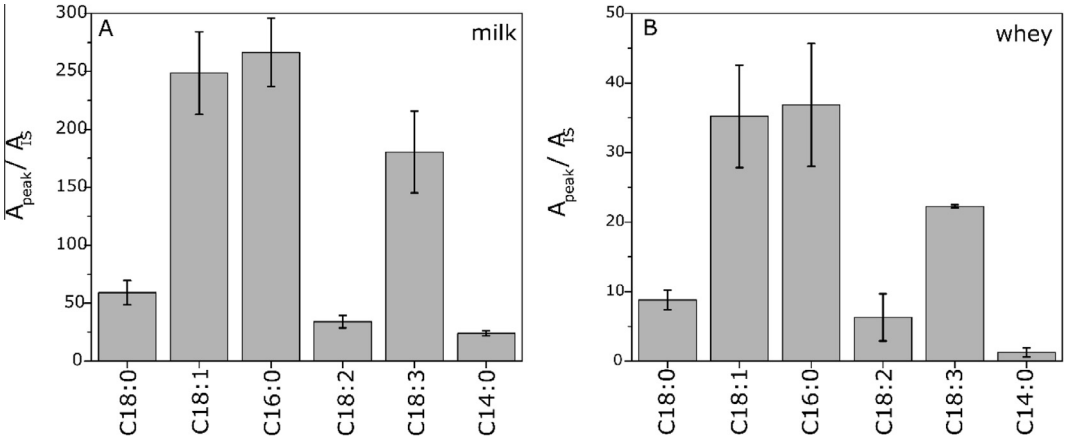

Fig. 4. Ratio between the area of the signal of each FA and the area of the IS peak to the CE analysis. Error bar represents the standard deviation of duplicate analysis.

MLR regression equation (Eq. 1), in which the matrix X represents the values of the peak area obtained by the electropherogram and matrix Y represents the adulteration levels prepared in the laboratory.

$$\beta = (X^T X)^{-1} X^T Y \quad (1)$$

Each β coefficient denotes the weight in the regression equation for each of the six FA as a result of the MLR, as show in Eq. (2). In this equation, A represents the area value of the peak indicated in the subscript. Fig. 5 shows, on the horizontal axis, the true values of the adulteration prepared in the laboratory and the vertical axis represents the values provided by the MLR model. The values obtained by the MLR model showed an excellent correlation with $R^2 = 0.973$, and the residual graph (Fig. 5B) without tendency indicates that the model was well-adjusted. The average values of the duplicates obtained by the CZE-UV method with the MLR approach were 3.9, 9.0, 11.1, 16.3 and 19.8% whey addition, with respective standard deviations of 0.6, 0.5, 0.9, 1.2 and 0.9%. For comparison, the true values are described in the sample description section.

$$\%_{\text{whey addition}} = 26.9 + \frac{1}{A_{IS}}(126.7A_{C18:0} + 18.5A_{C18:1} - 46.9A_{C16:0} - 178.6A_{C18:2} + 29.2A_{C18:3} - 54.2A_{C14:0}) \quad (2)$$

In order to complement the information of residual graph, the Durbin-Watson (Montgomery, Peck, & Vining, 2006) hypothesis test (p-value equal 0.896) was included after checking the presupposition of a normal distribution of the data by the Shapiro-Wilk test (Shapiro & Wilk, 1965) (p-value equal 0.808). The results show that there was no evidence at the 95% confidence level of a correlation of the residuals distribution between the values predicted by the MRL CZE method and the true value of whey addition, indicating that the model was well-adjusted.

The limits of detection (LOD) and quantification (LOQ) of individual FA analyzed by the CZE-UV were determined in one sample with 9% whey addition. The results are shown in Table 1. This procedure was performed for individual FA used in the MLR associated with the CZE-UV method to demonstrate the potential of the CE technique for this purpose.

The LOD and LOQ in Table 1 refer to the sensitivity of CZE-UV method to detect and quantify each individual FA. The LOD and LOQ associated with multivariate calibration of the MLR were determined from the graph of predicted values versus the true values, using the equations given below (Eq. 3) (Olivieri, 2015; Ortiz, Sarabia, & Sánchez, 2010). The LOD and LOQ values upon calculation were found to be 3.9% and 11.8% whey addition in fluid milk. Both LOD and LOQ for the individual FA and for the MLR prediction of fraud content present appropriate levels for the implementation of this approach, since the practiced fraud levels are commonly within the range of 20–25% whey addition, according to Ferrão et al. (Ferrão et al., 2007).

$$LOD = \frac{3.3S_{Res}}{a}\sqrt{1 + h_0 + \frac{1}{N}}, LOQ = \frac{10S_{Res}}{a}\sqrt{1 + h_0 + \frac{1}{N}} \quad (3)$$

where S_{Res} is the residual standard deviation, a is the slope of the linear fit between the predicted values versus the reference values, N is the total number of samples and h_0 is the leverage for the blank

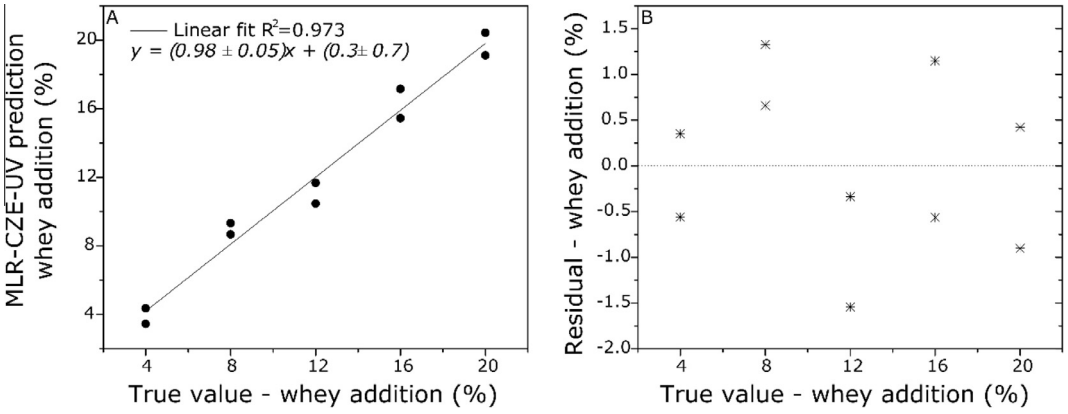

Fig. 5. Correlation graph for whey percentage prediction added to fluid raw milk between the values obtained by MLR and true values.

Table 1
LOD and LOQ of percentage of whey addition in milk for individual FA used in the MLR associated to CZE-UV analysis.

FA	LOD*	LOQ**
18:0	0.87	2.91
18:1	0.27	0.89
16:0	0.21	0.69
18:2	1.58	5.27
18:3	0.44	1.45
14:0	0.97	3.24

*LOD and **LOQ, both expressed in % of addition of whey.

sample; $h_0 = \frac{\bar{y}^2}{\sum_{i=1}^{N}(y_i-\bar{y})^2}$, in this notation, y_i represents the reference value and $\bar{y}$ is the average of the concentration of the prepared samples.

An error estimate of the prediction was calculated using the root mean square error of estimation (RMSEE). The value obtained for RMSEE was 0.88%. This value represents the global error of the predictions of the MLR model. Furthermore, the method most commonly used to detect the addition of whey in milk is the quantification of CMP, but this method applies only to whey obtained by enzymatic coagulation. This approach can provide false positive results due to the action of psychrotrophic bacteria in milk (Bremer, Kemmers-Voncken, Boers, Frankhuizen, & Haasnoot, 2008; de Carvalho et al., 2015; Martín-Hernández et al., 2009; Oancea, 2009). The important advantage of the method developed in this paper is the possibility of the quantification of fraud by whey addition from both enzymatic coagulation and acidification; this is a more comprehensive method than the most widely used method based on the quantification of CMP.

4. Conclusions

The adulteration of fluid milk by adding whey was investigated using FA profile analysis. The OPLS-DA analysis of the chromatogram sets of milk and whey samples revealed that the FA C14:0, C16:0, C18:0, C18:1c, C18:1t and C18:2cc were the most significant FA to be monitored upon suspicion of such adulteration. A multiple regression equation to monitor these FA (and C18:3) was successfully fit to quantify whey addition content in a range of 4–20% of adulteration, thus suggesting a new method that can be used for controlling the quality of milk by the industry and by government agencies.

It is important to note that the quantification of milk adulteration by CMP analysis detects only sweet whey. As the proposed method does not focus on proteins and amino acids, it allows for the detection and quantification of adulteration of milk by both acid and sweet whey. This observation supports the potential of this method to differentiate the type of whey used to adulterate milk, which makes it possible to trace the source of adulteration.

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