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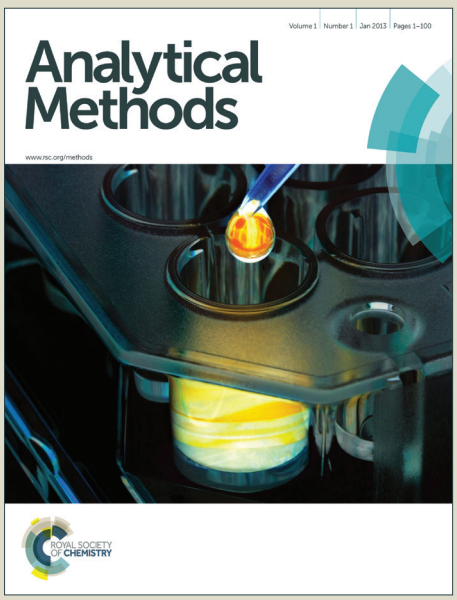

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Graphical and textual abstract

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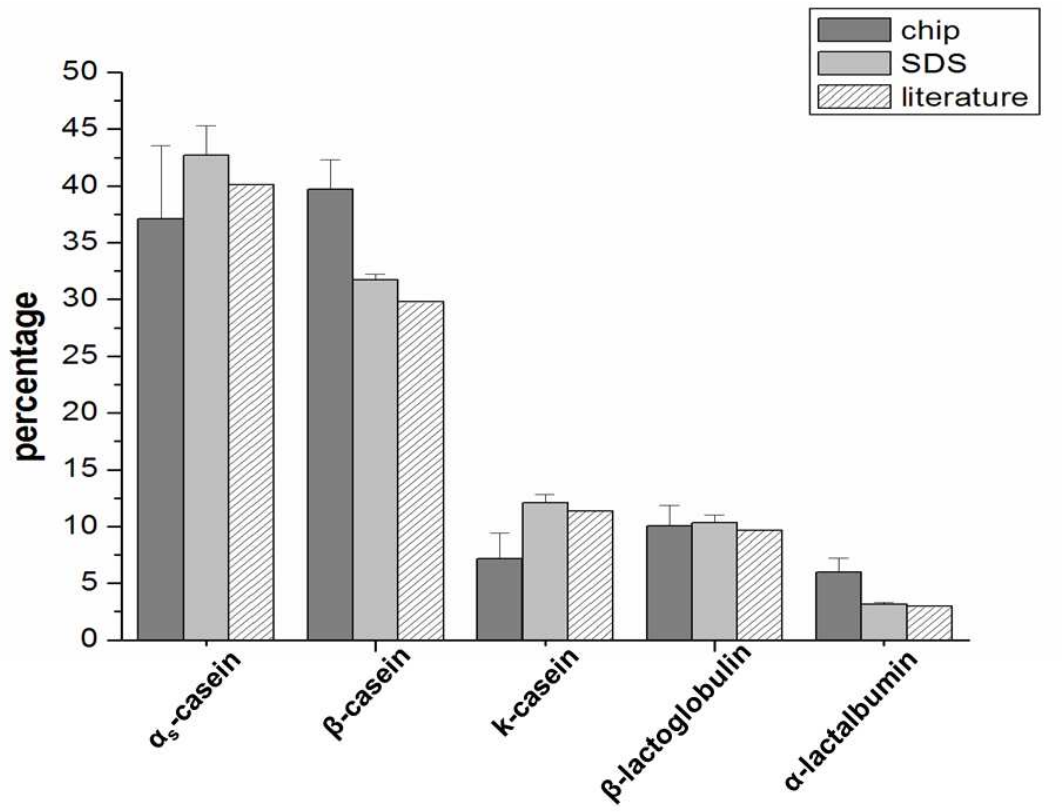

Microfluidic chip electrophoresis investigation of major milk proteins: study of buffers effects and quantitative approaching

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Abstract

The separation and quantification of major milk proteins is fundamental in dairy research. Therefore, accurate and rapid methods are profoundly important. The microfluidic chip technique is faster, and uses considerably fewer chemicals and materials than traditional techniques. The objective of this study was to improve experimental methods for separating and quantifying major milk proteins using the microfluidic chip technique. Deionized water, a total protein solubilization buffer (TPS buffer) and a separating milk protein buffer (SEP buffer) were added for the treatment of milk samples and their effects were evaluated. The results showed an excellent separation for whey proteins with α-lactalbumin migrating first, followed by β-lactoglobulin in the presence of both buffers. However, better results for major casein separation were achieved when the SEP buffer was added. The order of the migration time was: β-casein first, followed by α_s-casein and κ-casein. The quantitative analysis showed significant differences among the percentages of protein fractions from both buffers. The results using microfluidic chip technology using the SEP buffer solution were comparable to those obtained by SDS-PAGE for these proteins and with the data reported in the literature.

Key words: Microfluidic, electrophoresis, milk, proteins, SDS-PAGE

1. Introduction

The separation, identification and quantification of individual proteins that make up milk and dairy products provide important information about the physico-chemical properties of different dairy systems improving the technology of production of more stable products, which have better quality and longer shelf life. This information can be used to explain their influence on the biological activity, flavour, and functional properties of milk and dairy products and can also be used for product authenticity and history assessment[1]. Thus, for example, heat-induced denaturation and interactions of milk whey proteins have been studied in different milk protein systems under a variety of experimental systems[2].

Currently, polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) techniques are used for the separation of the main protein fractions of milk. These techniques may be coupled with separation equipment, such as ultraviolet spectroscopy and mass spectrometers for quantification of protein fractions[3,4,5]. The advantages and disadvantages of each of these techniques have been under discussion[7]. Regarding the main advantages, automation and detection limit are the most cited. However, the high consumption of toxic reagents that are subsequently discarded, the long time required for sample preparation and the high costs of most equipment, the physical separation of the proteins and the final integration and quantification of the individual protein components are considered as disadvantages of these techniques.

Recently, the microfluidic chip technique was developed for the separation and quantification of DNA, RNA and proteins in various fields such as proteomics, drug development, or medical diagnosis[8,9]. This technique has been recommended because of the good results it offers. The main advantages cited are the shorter time for sample preparation (~ 30 min/chip), the smaller amounts of reagents used, about 0.5 mL/chip, and the detection limit of the order of nanograms of material in a microliter sample[7,10,11]. Studies with milk proteins have been conducted to verify the potential application of this technique to evaluate the distribution of different protein fractions in milk. Thus, authors[12] have reported the ability of microchip electrophoresis (MCS) to rapidly separate and characterize whey proteins. However, the results in terms of optimization of the separation of individual proteins are still unsatisfactory when one follows the recommended manufacturer's methodology, due

to the overlaying of signals related to fractions of casein. The correct quantification of the percentages of protein fractions depends on the signals obtained. Data obtained with an unsatisfactory separation may underestimate or overestimate the amount of protein present, whereas a more efficient separation would provide more accurate results on the quantification of proteins[13].

This study determined the potential of the microfluidic chip technique as a rapid method of food control to separate and quantify the major milk proteins. The first aim was to evaluate the effects of adding two different buffers for the treatment of milk samples before the standard procedure recommended by the manufacturer of the electrophoresis equipment microfluidics in the separation and identification of the major milk proteins. Moreover, the quantitative achieved by the microfluidic chip technique, using the best buffer, was compared with the separation obtained using the traditional SDS-PAGE technique, and the literature.

2. Materials and Methods

2.1 Milk and Milk Proteins

Raw milk was supplied by Embrapa Dairy Cattle National Research Center (Juiz de Fora, Minas Gerais, Brazil). Purified α-lactoalbumin (α-La), β-lactoglobulin (β-Lg), α_s-casein (α_s-CN), β-casein (β-CN) and κ-casein (κ-CN) were obtained from Sigma-Aldrich (USA). Solutions (10 mg x mL^{-1}) of each individual protein were prepared by adding each individual protein to purified water (Ultrapure Milli-Q; Millipore Corp., USA) and stirring until dissolved. Mixed protein standards were prepared by combining each of the individual protein solutions (1 mL) and making the final volume up to 10 mL to give a mixed protein standard with an individual protein concentration of 1 mg x mL^{-1}.

2.2 *Microfluidic chip electrophoresis*

Separation of individual milk proteins was performed using the microfluidic chip electrophoresis system (Agilent 2100 Bioanalyser) and the associated Protein 80 kit (Agilent Technologies, Germany). These kits contain the chips and proprietary reagents such as the gel matrix solution, protein in a concentrated solution, a marker protein buffer solution and a protein molecular mass *ladder* solution to perform the electrophoresis.

The TPS buffer consisted of 0.1mol x L^{-1} tris chloride acid (Amresco, USA), pH 8.8, containing 2 mol x L^{-1} urea (USB, Germany), 15% glycerol (Invitrogency, New Zealand) and 0.1 mol x L^{-1} Dithiothreitol-DTT (Bioangency, Brazil). It was prepared according to SOP (Standard Operating Procedure) available from Food Standards Agency (FSA) of the United Kingdom[14].

The SEP buffer solution, pH 3.0, used to separate the proteins consisted of 6.0 mol x L^{-1} urea (USB, Germany), 20 mmol x L^{-1} trisodium citrate dehydrate (Synth, Brazil), 0.1 mol x L^{-1} citric acid (Merck, Brazil) and 0.05% (w/w) hydroxypropylmetyl cellulose (Sigma-Aldrich, USA)[15].

Milk was diluted 1:4 with the TPS buffer, the SEP buffer and pure water (Ultrapure Milli-Q; Millipore Corp., USA) to compare and select the more efficient diluting agent. Samples were allowed at least 2h at 4ºC for protein solubilization before application in the microfluidic chip electrophoresis which was performed using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Germany). The gel matrix, solutions and samples for electrophoresis were prepared according to the Bioanalyser protocols (Agilent Technologies, Germany). In eppendorf tubes (0.5 mL total volume) 4µL of samples (milk; milk + TPS buffer; milk + SEP buffer; milk + pure water; and milk added with each individual protein + SEP buffer) were mixed with 2 µL of 2-mercaptoethanol (Sigma-Aldrich, USA), heated (95 ºC, 5 min), cooled in an ice bath, briefly spun in a centrifuge (3000 x g) and then 84 µL Milli-Q water was added to give a total volume of 90 µL.

Quantification was carried out considering the area under electropherogram using the Agilent 2100 Expert software associated with the instrument. The results were expressed as percentages (%) according to all the proteins identified in the electropherograms.

2.3 *Sodium dodecyl sulphate poliacrylamide gel electrophoresis (official method)*

Raw milk sample were analysed in duplicate by SDS-PAGE. Samples were diluted 1:4 in Tris-Tricine sample buffer (Bio-Rad Laboratories, Hercules, CA) pH 6.8, containing 10% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.05% bromophenol blue and heated at 95°C for 4 min. Samples (40 µl) were loaded onto a 12% polyacrylamide Criterion XT Bis-Tris gel (Bio-Rad). A continuous buffer system was used consisting of 25 mL of 20 × XT SDS running buffer (Bio-Rad) with 475 mL of Milli-Q water in both tanks.

Gels were run for 90 min at 150 V and were stained with Coomassie Blue G-250 (Bio-Rad) during 3h at room temperature, according to the manufacturer's recommendations. Gels were destained in Milli-Q water during 6h at room temperature. The Mark 12 unstained molecular mass standard (Bio-Rad) was used. Images were taken with a versa Doc imaging system (Bio-Rad) and the software Quantity (Bio-Rad) was used for quantitative band analyses. Densitometric peak areas from different caseins and from different whey proteins were converted to percentages of the total casein peaks area or of the total whey protein peak area. The nitrogen content of bovine raw milk and its whey were measured using de Kjeldahl method. The results were converted to protein by multiplying N by 6.38.

2.4 Statiscal evaluation

A 3^3 Box-Behnken design containing three levels (-1, 0, +1) and three factors (urea, hydroxypropylmetyl cellulose and citrate/citric acid) was applied to the milk samples[16] Table 1 shows the contrast matrix for the 3^3 Box-Behnken designs. Microsoft Excel 2007 was used to calculate matrices in experimental design. Tukey test was used to evaluate differences among treatments. The statistical approaches (normality, homocedasticity and independence) were performed in SPSS 8.0 for windows software. The lack of fit analysis was performed in Microsoft Office® Excel 2007 software.

3. Results and discussion

3.1. Separation and identification of major milk proteins by microfluid chip electrophoresis

As a staring point, the analysis of the milk proteins of raw bovine milk was carried out using deionized water and two different buffers for the treatment of milk samples before the standard procedure recommended by the manufacturer of the electrophoresis equipment microfluidics. The two buffers compared were a total protein solubilization buffer (TPS buffer) and a separating milk protein buffer (SEP buffer). The first one is recommended for the preparation of milk samples before application in microfluidic electrophoresis[14] while the latter is commonly used for the separation of protein fractions of milk during the sample preparation for analysis by CE.[15]

Figure 1 shows the electropherograms obtained from milk samples added to the SEP buffer (Figure 1A), the TPS buffer (Figure 1B) and deionized water (Figure 1C). The addition of only deionized water to the milk sample resulted in an overlap of all signals, making it impossible to separate the individual major milk peak proteins from the base line on the electrophoregram (Figure 1C) while the addition of both, the SEP and the TPS buffers in the treatment of milk samples made it possible to separate different peaks corresponding to the major milk proteins with good resolution. These results are explained because the milk caseins are dissociated by the addition of urea[17] and both buffers contained urea, the TPS buffer had a concentration of 2 mol x L^{-1} and the SEP buffer 6 mol x L^{-1} of urea, respectively. On the other hand, the time of analysis was slightly shorter when the SEP buffer was employed (only 40 seconds of analysis). Moreover, a better resolution on the peaks to the base line of the electrophoregram was observed which affect positively the quantification showing that the treatment of milk samples with the SEP buffer should be preferred for the quantification of the major milk proteins by microfluidic chip electrophoresis.

The adaptation of techniques such as the addition of modified buffers is commonly used in studies involving analysis by HPLC, SDS-PAGE and CE[7]. Thus, authors[18] have employed the SEP buffer for the separation of casein in the supernatant of an ultracentrifugated milk sample before using CE. This protocol has been used in other studies to evaluate the protein profile of milk, employing CE[15,19].

In order to identify the peaks corresponding to each of the protein fractions, the addition of individual protein standards to the sample of milk was carried out. The identification was confirmed by the observation of an increased signal of each one of the individual proteins added (Figure 2). Thus, Figure 2 show the electropherogram of a milk sample with the addition of individual protein fractions of milk α-La, β-Lg, β-CN, α_s-CN and κ-CN when the SEP buffer was used in the treatment of the sample. According to these results, the order of separation of the individual proteins in milk, according to migration time in the samples, was α-La 21.65 seconds, followed by β-Lg 24.04 seconds, β-CN 29.63 seconds, α_s-CN 31.24 seconds and κ-CN 34.12 seconds (Table 3).

In the case of the utilization of the TPS buffer, as mentioned above, the analysis time was slightly longer but did not interfere with the separation of milk proteins (Figure 1B). In fact, a delay of 8 seconds in the migration time of each

protein was observed. Concretely, α-La had a migration time of 30 seconds, β-Lg 33 seconds, β-CN 39 seconds, α_s-CN 42 seconds, and lastly, the κ-CN 44 seconds, respectively. This different analysis time between both buffers could be due to a different pH (pH_{SEP} = 3.0 and pH_{TPS} = 8.8), ionic strength and, in particular, the viscosity.

The literature[13] showed that by following the conventional protocol of sample preparation under reducing conditions using microfluidic technology, it was possible to observe the separation between the main proteins in whey with α-La migrating first, followed by β-Lg. However, the caseins were not separated with good resolution and showed an overlap between the peaks corresponding to β-CN, which migrated first, followed by α_s-CN, second, and κ-CN which migrated last. This overlapping of signals observed with milk protein interferes with the quantification of individual fractions and may cause an incorrect estimation of protein quantification.

3.2 Effect of the concentration of chemical reagents used in the SEP buffer in the quantitative determination

In order to assess whether variations in the concentration of chemical reagents used in the SEP buffer could result in better separation and quantification of protein fractions, a 3^3 Box-Behnken design (Table 1) containing three levels and three factors: urea (5.0, 6.0 and 7.0 mol x L^{-1}), hydroxypropylmetyl cellulose (0.04, 0.05 and 0.06 % and citrate/citric acid with pH = 3.0 (10/0,5, 20/0,1 and 30/0,15) was applied.

It is remarkable that urea present in the buffer is used in the dissociation of casein micelles into smaller fractions of polypeptide α_s-CN, β-CN e κ-CN, and its main function is to break the hydrogen bonds responsible for the interactions between these polypeptides[20,21,22]. High concentrations of urea (6.0 to 8.0 mol x L^{-1}) are necessary to maintain the state of denaturation of proteins after the disruptions of disulfide bonds by the addition of a thiol agent, which was used in the standard methodology for sample preparation prior to application in microchip analysis in the Bioanalyzer. The use of urea did not affect the charge of proteins assisting in the separation of polypeptides by their charge and molecular size.

Citrate/citric acid present in the SEP buffer helps to keep the pH constant (pH = 3.0) so as not to interfere with the burdens of keeping the polypeptides dissociated below the isoelectric point of caseins from milk (pH = 4.5). The use of

polysaccharides hydroxypropylmetyl cellulose assists in the molecular mobility of protein fractions of milk casein dissociated by the addition of urea[17]. The qualitative analysis of the protein separation profile in the electropherograms obtained from the 3^3 Box-Behnken designs (Table 1) showed no significant variation in the resolution of the signals between the treatments (data not shown). Therefore, the results showed that 6.0 mol x L^{-1} urea, 0.05% (w/w) hydroxypropylmetyl cellulose and 20 mmol x L^{-1} citrate trisodium citrate dehydrated / 0.1 mol x L^{-1} citric acid, pH = 3.0 (experiment 13), achieved the best separation and quantification condition.

3.3 Quantitative determination of major milk proteins by microfluid chip electrophoresis

Table 2 shows the results in percentages obtained by the distribution of protein fractions present in a sample of milk treated with deionized water, the TPS buffer and SEP buffer, as represented by the electropherograms in Figure 1. According to these results, a statistically significant difference ($P < 0.05$) was found among the percentages of protein fractions from all three treatments of the milk sample.

The results indicate an improvement in the separation of the peaks for each protein fraction in each milk sample diluted in the SEP buffer compared with those obtained from the TPS buffer and deionized water. Therefore, following the protocols recommended by the equipment manufacturer, we can infer that there was an improvement in the results for the percentage distribution of protein fractions, generating more accurate data as when a peak overlaps another peak during integration, there is an average estimate among the subsequent areas for each signal. When this separation occurs, better results are found, as they did not use any common approach to systems integration[23]. Variations with imprecise estimates yield results that can affect the understanding of the behavior of the system. The quantification of protein fractions in milk helps in understanding its physico-chemical properties.

After the optimization, a formal statistical procedure must to be applied in order to achieve the best information about the analytical system investigate is recommended. Within this context, the quantification of protein by microchip was achieved using regression models, which were applied through the linear ordinary least-square regression. In this case, the analytical curve of β-La demonstrated

heterocedasticity behavior, and the use of weighted least-square regression was required[23]. After regression employment, it was necessary to verify statistical assumptions through of statisticals test such as residues normality (Shapiro Wilk test), homoscedasticity (Levene – different replicates by level or Cochran - same replicates by level) and the lack of fit (linearity test) of the model through a priori test hypothesis using equation 1, according to recommended by IUPAC[24]. In the present case the assumptions were considered acceptable within 95% and 99% confidence interval, because the calculated values were lower than the critical values or p-value were higher than 0.05 or 0.01, respectively. The regression model diagnosis has been considered satisfactory with no lack of fit because the value of $F_{calculated}$ is lower than $F_{critical}$ for all milk proteins area within 95% or 99% confidence interval, indicating that the linearity test was considered acceptable in the concentration range considered and the mathematical approaches can be used for protein quantification. The values used for the regression model carried out are shown in Table 5.

$$F_{calc} = \frac{s^2_{\bar{y},x}}{s^2_{\bar{y}}} = \frac{\sum_{i=1}^{p} m_i(\bar{y}_i - \hat{y}_i)^2/(p-2)}{\sum_{i=1}^{p}\sum_{j=h}^{m_i}(y_{ij} - \bar{y}_i)^2/(m-p)} \quad (1)$$

The Table 6 shows the statistical results obtained: the lack of fit model, correlation (r) calculated and limit of detection (LOD) for each protein. The proteins in mixed milk protein standards, at a range of concentrations (concentration range of 0 - 1.0 mg x mL^{-1} for each protein) and a single milk sample were separated and quantified using the microfluidic chip and traditional SDS-PAGE techniques. The quantified proteins in the standards were used to generate standard curves for each of the individual milk proteins, and these curves were used to calculate the concentrations of the individual proteins in the milk sample. The LOD is expressed as the concentration that can be detected with reasonable certainty for a given analytical procedure. In case of linear calibration $y_i' = a(\pm S_a)x_i + b(\pm S_b)$, the slope is constant of concentration x_i (where subscript i in the expression denotes each different protein). According to ICH[25], LOD is defined as mathematical expression shown below:

$$LOD = 3.3\,\frac{s_b}{a} \quad (2)$$

Where s_b denotes intercept standard error and *a* is the slope of each protein curve calculated through the calibration method.

The standard curves for α-La, β-Lg, α_s-CN, β-CN and k-CN, generated from six separate chips and three separate gels, are shown in Figure 3. For both the microfluidic chip separation method and the traditional SDS-PAGE method, the standard curves for the individual proteins showed good linearity with $r^2 > 0.93$ while the data for all standard curves were combined and demonstrated higher correlations for a standard curve from a single chip or gel. The calculated concentrations in percentages of the milk proteins in the milk samples using microfluidic chip and SDS-PAGE are shown in Table 4. The concentrations of the individual caseins and whey proteins are in the range expected for fresh skim milk[26] and comparable concentrations were obtained by both the microfluidic chip and SDS-PAGE methods and compared with data from literature (Figure 4).

3.4. Comparison of the separation and quantitative determination using SDS-PAGE and the microfluidic chip technology

Figure 3 shows the SDS-PAGE analysis of raw bovine milk using the traditional SDS-PAGE technique (lane 3 and 5). The milk proteins shown in decreasing order of relative molecular weight bands for the whey proteins were: lactoferrin (Lf), bovine serum albumin (BSA); immunoglobulin G (IgG), after α_{s2}-CN, α_{s1}-CN, β-CN and κ-CN with a molecular weight between 35 and 24 kDa and lastly β-Lg and α-La with a molecular weight band of 18 KDa and 14,2 KDa respectively. A satisfactory separation of all milk proteins was achieved, in particular α_{s2}-CN, α_{s1}-CN, β-CN and κ-CN were clearly resolved. These results appear to agree completely with the observations on literature[13], as the peaks for BSA, Ig G and LF were considerably weaker for the microfluidic chip technique than for the traditional SDS-PAGE. However, the reason for this fact is unknown.

In order to make a comparison between the results obtained using the two different techniques, only the major whey proteins were considered. Table 4 shows the quantitative determination for the major milk proteins α_{s2}-CN, α_{s1}-CN, β-CN, κ-CN and β-Lg and α-La determined by SDS-PAGE as percentages of total protein. The results obtained are in accordance with the data in the literature. The proteins represent about 3.0% - 3.5% of the milk and caseins represent about 80% of total

proteins while whey proteins represent about 20% of total proteins[25,27]. The concentrations of the individual caseins and whey proteins are in the range expected for raw bovine milk[26] and comparable concentrations were obtained by both the microfluidic chip and SDS-PAGE methods (Table 4 and Figure 4).

4 Conclusions

The microfluidic chip electrophoresis represents a practical alternative for rapid analysis and quantification of major proteins: α-La, β-Lg, α_s-CN, β-CN and κ-CN of bovine milk. The addition of buffers in the treatment of the samples permitted more reliable results in the separation and quantification of protein fractions by electrophoresis chip in milk samples. The SEP buffer (6.0 mol x L^{-1} urea, 0.05% (w/w) hydroxypropylmetyl cellulose and 20 mmol x L^{-1} citrate trisodium citrate dehydrated 20/0.1 mol x L^{-1} citric acid, pH = 3.0) achieved the best quantification. The quantitative percentages of proteins fractions found were similar to those obtained by traditional SDS-PAGE technique and with the data reported in the literature.

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518 **Figure Captions**

519

520 Figure 1A. Electropherogram of milk proteins obtained by Agilent Bioanalysis 2100 of
521 milk sampled added with SEP buffer.

522

523 Figure 1B. Electropherogram of milk proteins obtained by Agilent Bioanalysis 2100 of
524 milk sampled added with TPS buffer.

525

526 Figure 1C. Electropherogram of milk proteins obtained by Agilent Bioanalysis 2100 of
527 milk sampled added deionizer water for separation of milk proteins.

528

529 Figure 2. Electropherogram obtained by Agilent Bioanalysis 2100 of samples of milk
530 with SEP buffer solution for each protein identification – Peaks: 1) α-lactalbumin; 2)
531 β-lactoglobulin; 3) β-casein; 4) α_s-casein; 5) κ-casein.

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533 Figure 3. SDS-PAGE electrophoretogram of a bovine milk sample. SDS-PAGE
534 analysis Lane 1: Kit of protein standards with different molecular weight. Lanes 2 and
535 4: Casein standard milk. Lanes 3 and 5: Raw bovine milk.

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537 Figure 4: Comparison graphical between official method, literature and microchip
538 analysis of major milk proteins.

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552 **FIGURE 1A**
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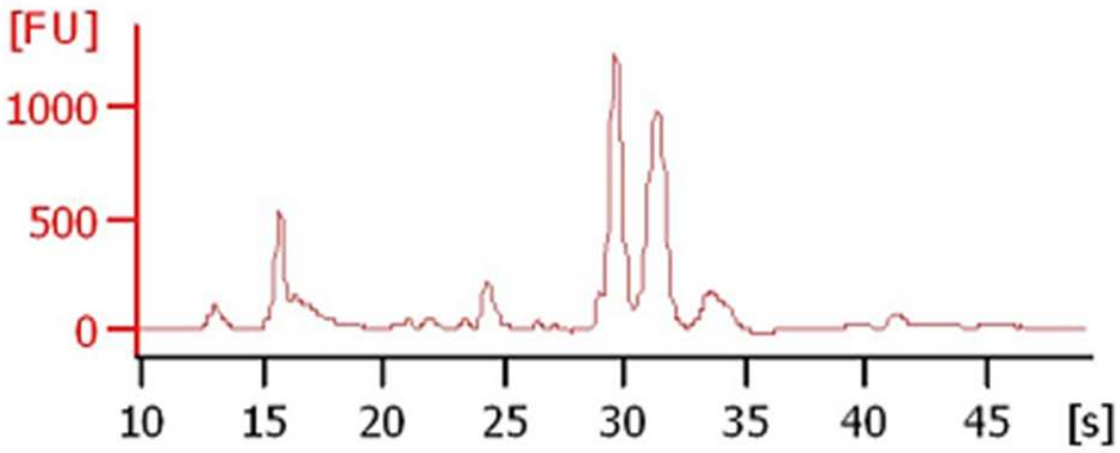

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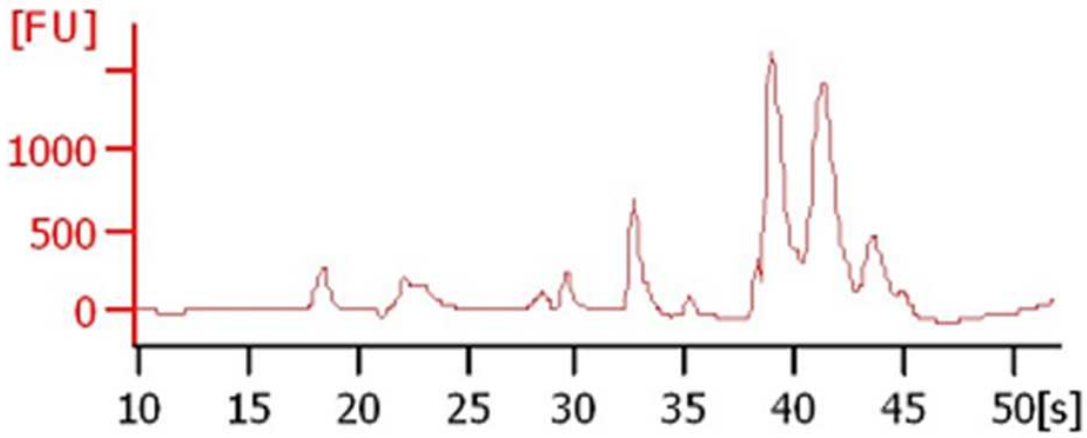
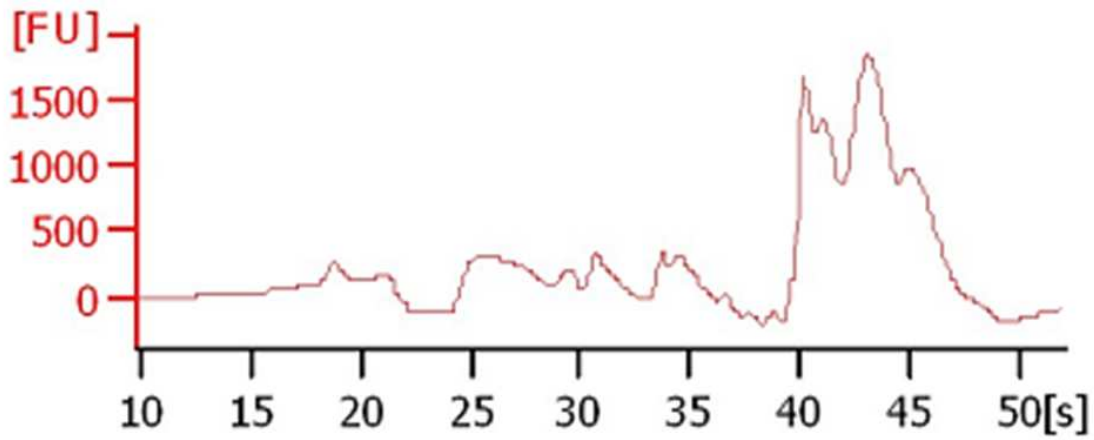
FIGURE 1B



588 **FIGURE 1C**



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606 **FIGURE 2**

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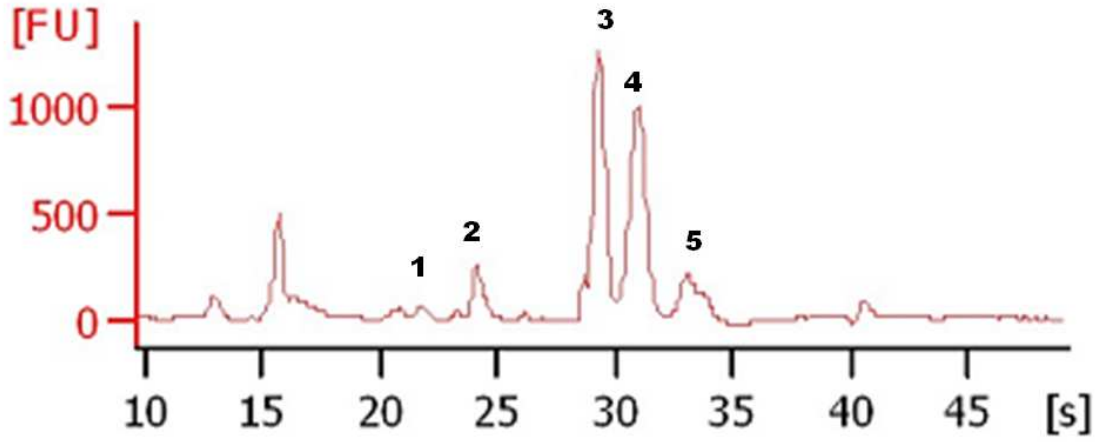

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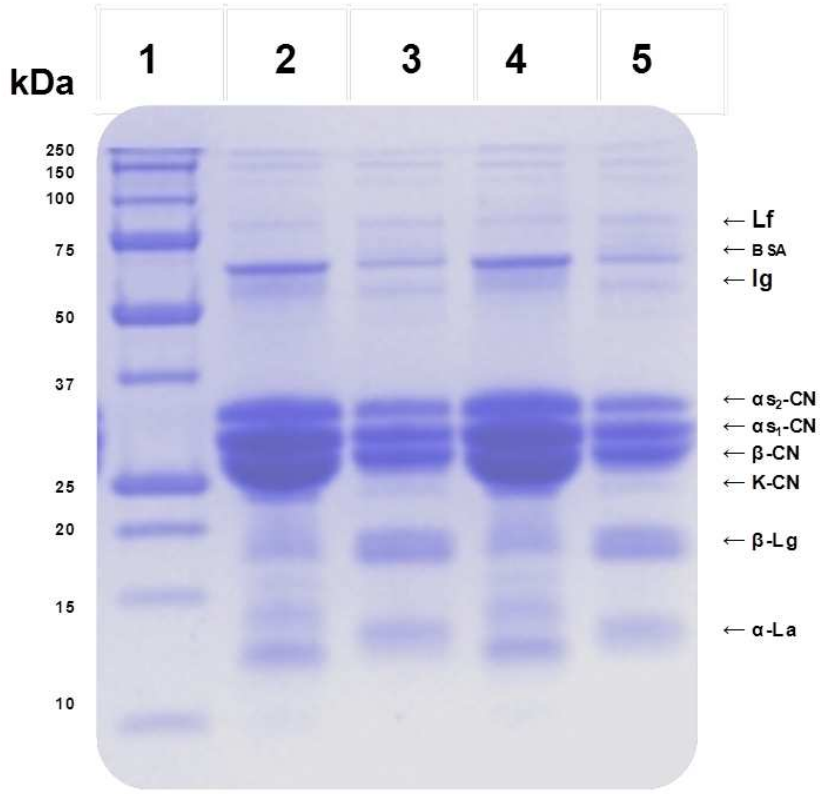
FIGURE 3



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643 **FIGURE 4**

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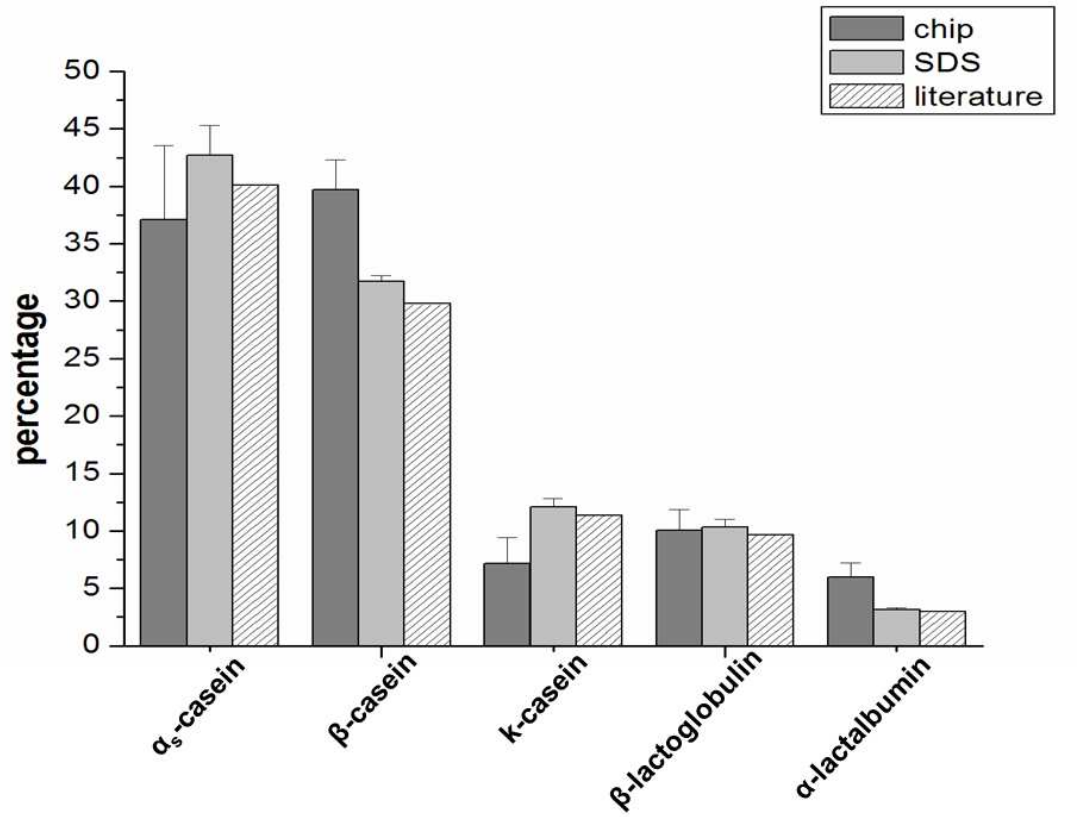

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Tables

Table 1: Box-Bhenken (3^3) design for protein fractions separation

Experiment	1	2	3	4	5	6	7	8	9	10	11	12	13
X_1	-1	+1	-1	+1	-1	+1	-1	+1	0	0	0	0	0
X_2	-1	-1	+1	+1	0	0	0	0	-1	+1	-1	+1	0
X_3	0	0	0	0	-1	-1	+1	+1	-1	-1	+1	+1	0

X_1-Urea (mol L^{-1}): (-1) 5.0, (0) 6.0, (+1) 7.0;

X_2-Hydroxypropylmethyl cellulose (%): (-1) 0.04 (0) 0.05, (+1) 0.06;

X_3-Citrate/citric acid (mmol L^{-1}/ mol L^{-1}): (-1) 10/0.5, (0) 20/0.1, (+1) 30/0.15

689 **Table 2:** Percentage of proteins fractions distribution in milk with SEP buffer and TPS
690 buffer added.

Proteins	Distribution (%)	
	Milk diluted in SEP buffer	Milk diluted in TPS buffer
α-lactalbumin	1.03 ± 0.4	4.13 ± 1.3
β-lactoglobulin	7.74 ± 0.8	11.43 ± 2.8
α_s-casein	40.66 ± 2.2	36.09 ± 2.2
β-casein	41.12 ± 1.8	38.43 ± 3.1
κ-casein	9.45 ± 0.6	9.92 ± 1.9
Total	100	100

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Table 3: Estimated migration time and percentage of proteins fractions from microfluidic chip of milk submitted to the SEP buffer.

Proteins	Migration Time (s)	Percentage (%)
	Media ± SD	Media ± SD
α-lactalbumin	21.65 ± 0.06	1.03 ± 0.4
β-lactoglobulin	24.04 ± 0.14	7.74 ± 0.8
α_s-casein	29.63 ± 0.09	40.66 ± 2.2
β-casein	31.24 ± 0.16	41.12 ± 1.8
κ-casein	34.12 ± 0.05	9.45 ± 0.6

735
736 **Table 4:** Main casein and whey protein fractions of raw bovine milk determined by SDS-
737 PAGE.

Proteins	SDS-PAGE Percentage (w/w) of Milk Protein	Literature dates* Percent (w/w) of Milk Protein	Present work** Percentage (w/w) of Milk Protein
Total casein	81.25 ± 2.71	80.00	83.97 ± 11.29
α_s-casein	40.09 ± 2.59	39.0	37.12 ± 6.42
β-casein	29.79 ± 0.49	28.4	39.68 ± 2.59
κ-casein	11.37 ± 0.69	10.1	7.18 ± 2.27
Total whey protein	18.75 ± 1.38	19.30	------
β-lactoglobulin	9.68 ± 0.69	10.0	10.03 ± 1.81
α-lactalbumin	2.95 ± 0.15	3.1	5.99 ± 1.23
Others whey proteins	6.12 ± 0.70	5.6	-----

738 *Source: Literature[26]
739 ** In the present work, standard deviation was calculated taking into account
740 standard deviation of each protein by the ratio of the summation of all proteins.
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745 **Table 5:** Values used to regression model with genuine replicates.

Proteins	Concentration (mg/mL)	1 [a] Replicate	2 [a] Replicate	3 [a] Replicate
α_s-casein	0.500	521.20	585.90	554.40
	1.000	1024.80	882.20	1087.50
	3.000	2507.90	2242.60	2258.20
	5.000	3425.10	3109.30	3164.20
β-casein	0.125	134.00	121.80	117.20
	0.250	262.10	236.50	227.60
	0.500	525.80	465.90	444.40
	1.000	735.70	673.40	632.70
	2.000	1408.40	1369.90	1305.30
κ-casein	0.125	47.10	48.80	***
	0.250	64.00	59.90	***
	0.500	110.50	129.90	***
	1.000	193.40	167.60	***
	2.000	283.00	232.70	***
β-lactoglobulin	0.050	22.10	21.60	21.30
	0.100	55.20	44.20	46.00
	0.200	179.40	161.90	165.30
	0.300	135.50	251.80	255.40
	0.400	232.80	204.70	268.10
α-lactalbumin	0.025	9.40	9.60	11.10
	0.050	35.90	25.50	21.90
	0.100	48.40	37.90	34.50
	0.200	101.40	108.20	105.70
	0.300	168.30	153.20	166.00

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Table 6: Statistical results: Lack of fit model and r calculated for each protein.

Proteins	F_{calc}	F_{tab}	Slope	Intercept	r	LOD (mg/mL)
α_s-casein	5.67	$8.65^{\$}$	593.93 ± 28.24	369.69 ± 83.85	0.98	0.465
β-casein	5.93	6.55*	637.01 ± 21.56	83.69 ± 22.25	0.98	0.110
κ-casein	2.36	$5.41^{\&}$	111.71 ± 10.79	47.11 ± 11.15	0.93	0.329
β-lactoglobulin	5.08	6.55*	810.84 ± 59.25	-18.90 ± 0.70	0.97	0.003
α-lactalbumin	3.52	$3.71^{\#}$	553.28 ± 19.32	-5.56 ± 3.27	0.98	0.019

$^{\#}F_{tab}(\alpha=0.05, \upsilon_1=3, \upsilon_2=10)$; $^{*}F_{tab}(\alpha=0.01, \upsilon_1=3, \upsilon_2=10)$; $^{\$}F_{tab}(\alpha=0.01, \upsilon_1=2, \upsilon_2=8)$; $^{\&}F_{tab}(\alpha=0.01, \upsilon_1=3, \upsilon_2=5)$; υ_1:numerator freedom degree; υ_2: denominator freedom degree.

Shapiro-Wilk Test (p-value): α_s-casein: 0.039; β-casein: 0.013; κ-casein: 0.076; β-lactoglobulin: 0.049; α-lactalbumin: 0.587.

Cochran Test ($C_{critical}$ = 0.684): β-casein - C_{calc} = 0.358; β-lactoglobulin - C_{calc} = 0.804 (heterocedasticity behavior); α-lactalbumin - C_{calc} = 0.350; Cochran Test (C_{tab} = 0.840): κ-casein - C_{calc}= 0.704.

Levene Test (p-value: α_s)-casein: 0.09.