

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

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The separation and quantification of major milk proteins are 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 used 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 obtained when 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 of microfluidic chip technology using the SEP buffer solution were comparable to those obtained by SDS-PAGE for these proteins and to the data reported in the literature.

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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 thereby 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

spectrometers and mass spectrometers for quantification of protein fractions.^{3–5} The advantages and disadvantages of each of these techniques have been under discussion.^{6,7} Regarding the main advantages, automation and detection limit are the most cited. However, high consumption of toxic reagents that are subsequently discarded, long time required for sample preparation and high costs of most equipment, physical separation of the proteins and final integration and quantification of the individual protein components are considered as disadvantages of these techniques.

Recently, the microfluidic chip technique has been 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 per chip), the smaller amounts of reagents used, about 0.5 mL per chip, and the detection limit of the order of nanograms of the material in a microliter sample.^{7,10,11} Studies on milk proteins have been conducted to verify the potential application of this technique in evaluating the distribution of different protein fractions in milk. Thus, authors¹² 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

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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 analysis 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 the Embrapa Dairy Cattle National Research Center (Juiz de Fora, Minas Gerais, Brazil). Purified α -lactalbumin (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 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 mL^{-1} .

2.2 Microfluidic chip electrophoresis

Separation of individual milk proteins was performed using the microfluidic chip electrophoresis system (Agilent 2100 Bio-analyser) and the associated Protein 80 kit (Agilent Technologies, Germany). These kits contain the chips and proprietary reagents such as the gel matrix solution, proteins 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 0.1 mol L^{-1} tris chloride acid (Amresco, USA), pH 8.8, containing 2 mol L^{-1} urea (USB, Germany), 15% glycerol (Invitrogen, New Zealand) and 0.1 mol L^{-1} dithiothreitol (DTT) (Bioagency, Brazil). It was prepared according to the SOP (Standard Operating Procedure) available from the 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 L^{-1} urea (USB, Germany), 20 mmol L^{-1} trisodium citrate dehydrate (Synth, Brazil), 0.1 mol L^{-1} citric acid (Merck, Brazil) and 0.05% (w/w) hydroxypropylmethyl cellulose (Sigma-Aldrich, USA).¹⁵

Milk was diluted in a 1 : 4 ratio with TPS buffer, SEP buffer and pure water (Ultrapure Milli-Q; Millipore Corp., USA) to

compare and select the more efficient diluting agent. Samples were allowed for at least 2 h at $4 \text{ }^\circ\text{C}$ for protein solubilization before application in microfluidic chip electrophoresis which was performed using an Agilent 2100 Bioanalyser 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) $4 \text{ }\mu\text{L}$ of samples (milk; milk + TPS buffer; milk + SEP buffer; milk + pure water; and milk added with each individual protein + SEP buffer) were mixed with $2 \text{ }\mu\text{L}$ of 2-mercaptoethanol (Sigma-Aldrich, USA), heated ($95 \text{ }^\circ\text{C}$, 5 min), cooled in an ice bath, briefly spun in a centrifuge ($3000\times g$) and then $84 \text{ }\mu\text{L}$ of Milli-Q water was added to give a total volume of $90 \text{ }\mu\text{L}$.

Quantification was carried out considering the area under the 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 polyacrylamide gel electrophoresis (official method)

Raw milk samples were analysed in duplicate by SDS-PAGE. Samples were diluted in a 1 : 4 ratio with 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 $95 \text{ }^\circ\text{C}$ for 4 min. Samples ($40 \text{ }\mu\text{L}$) were loaded onto a 12% polyacrylamide Criterion XT Bis-Tris gel (Bio-Rad). A continuous buffer system was used consisting of 25 mL of $20\times$ 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) for 3 h at room temperature, according to the manufacturer's recommendations. Gels were destained with Milli-Q water for 6 h at room temperature. The Mark 12 unstained molecular mass standard (Bio-Rad) was used. Images were taken using 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 peak area or of the total whey protein peak area. The nitrogen content of bovine raw milk and its whey was measured using the Kjeldahl method. The results were converted to protein by multiplying N by 6.38.

2.4 Statistical evaluation

A 3^3 Box-Behnken design containing three levels (-1 , 0 , and $+1$) and three factors (urea, hydroxypropylmethyl 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 the matrices in experimental design. The Tukey test was used to evaluate the differences among treatments. The statistical approaches (normality, homoscedasticity and independence) were performed using SPSS 8.0 for windows software. The lack of fit

Table 1 Box–Behnken (3^3) design for protein fraction separation^a

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

^a X₁-urea (mol L⁻¹): (-1) 5.0, (0) 6.0, (+1) 7.0; X₂-hydroxypropylmethyl cellulose (%): (-1) 0.04 (0) 0.05, (+1) 0.06; X₃-citrate/citric acid (mmol L⁻¹/mol L⁻¹): (-1) 10/0.5, (0) 20/0.1, (+1) 30/0.15.

analysis was performed using Microsoft Office® Excel 2007 software.

3. Results and discussion

3.1 Separation and identification of major milk proteins by microfluidic chip electrophoresis

As a starting 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 chip electrophoresis¹⁴ while the latter is commonly used for the separation of protein fractions of milk during the sample preparation for analysis by CE.¹⁵

Fig. 1 shows the electropherograms obtained from milk samples added to the SEP buffer (Fig. 1A), TPS buffer (Fig. 1B)

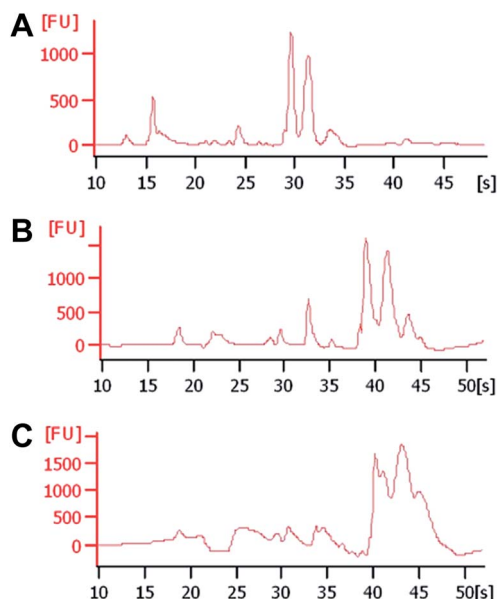


Fig. 1 (A) Electropherogram of milk proteins obtained by Agilent Bioanalysis 2100 of the milk sample added with SEP buffer. (B) Electropherogram of milk proteins obtained by Agilent Bioanalysis 2100 of the milk sample added with TPS buffer. (C) Electropherogram of milk proteins obtained by Agilent Bioanalysis 2100 of the milk sample added with deionized water for separation of milk proteins.

and deionized water (Fig. 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 in the electropherogram (Fig. 1C) while the addition of both the SEP and TPS buffers in the treatment of milk samples made it possible to separate different peaks corresponding to the major milk proteins with a good resolution. These results are explained because the milk caseins are dissociated by the addition of urea¹⁷ and both buffers contained urea, the TPS buffer had a concentration of 2 mol L⁻¹ and the SEP buffer had a concentration of 6 mol 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 electropherogram 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, authors¹⁸ 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 (Fig. 2). Thus, Fig. 2 shows 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 the 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 (Fig. 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, κ -CN 44

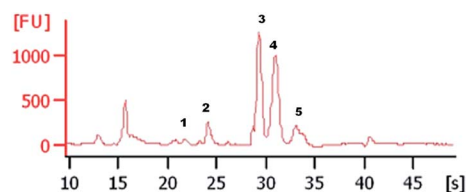


Fig. 2 Electropherogram obtained by Agilent Bioanalysis 2100 of samples of milk with SEP buffer solution for each protein identification – peaks: (1) α -lactalbumin; (2) β -lactoglobulin; (3) β -casein; (4) α_s -casein; and (5) κ -casein.

seconds, respectively. This different analysis time between both buffers could be due to a different pH ($\text{pH}_{\text{SEP}} = 3.0$ and $\text{pH}_{\text{TPS}} = 8.8$), ionic strength and, in particular, the viscosity.

The literature¹³ showed that by following the conventional protocol of sample preparation under reducing conditions using microfluidic technology, it is 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 a 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 proteins 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 on 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 L⁻¹), hydroxypropylmethyl 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 polypeptides α_s -CN, β -CN and κ -CN, and its main function is to break the hydrogen bonds responsible for the interactions between these polypeptides.^{20–22} High concentrations of urea (6.0 to 8.0 mol L⁻¹) are necessary to maintain the state of denaturation of proteins after the disruption 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 to not interfere with the burden of keeping the polypeptides dissociated below the isoelectric point of caseins from milk (pH = 4.5). The use of polysaccharides hydroxypropylmethyl cellulose assists in the molecular mobility of the 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 L⁻¹ urea, 0.05% (w/w) hydroxypropylmethyl cellulose and 20 mmol L⁻¹ trisodium citrate dehydrate/0.1 mol L⁻¹ citric acid, pH = 3.0 (experiment 13), achieved the best separation and quantification conditions.

3.3 Quantitative determination of major milk proteins by microfluidic chip electrophoresis

Table 2 shows the results in percentages obtained by the distribution of protein fractions present in a sample of milk

Table 2 Percentage of protein fractions distribution in milk added with SEP buffer and TPS buffer

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

Table 3 Estimated migration time and percentage of protein fractions from the 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

treated with deionized water, TPS buffer and SEP buffer, as represented by the electropherograms in Fig. 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

Table 4 Main casein and whey protein fractions of raw bovine milk determined by SDS-PAGE

Proteins	SDS-PAGE percentage (w/w) of milk protein	Literature data ^a percent (w/w) of milk protein	Present work ^b 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	—

^a Source: literature.²⁶ ^b In the present work, standard deviation was calculated by taking into account the standard deviation of each protein by the ratio of the summation of all proteins.

Table 5 Values used to regression model with genuine replicates

Proteins	Concentration (mg mL ⁻¹)	Concentration		
		1 replicate	2 replicates	3 replicates
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

because when one peak overlaps with another peak during integration, there is an average estimation among the subsequent areas for each signal. When this separation occurs, better results are obtained, as they did not use any common approach to system 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. Within this context, the quantification of protein using a microchip was achieved using regression models, which were applied through the linear ordinary least-squares regression method. In this case, the analytical curve of β -La demonstrated heteroscedasticity behavior, and the use of weighted least-squares regression was required.²³ After regression employment, it was necessary to verify statistical assumptions through the use of statistical tests such as residue normality

(Shapiro–Wilk test), homoscedasticity (Levene – different replicates by level or Cochran – same replicates by level) and the lack of fit model (linearity test) through a *a priori* test hypothesis using eqn (1), according to regulations by IUPAC.²⁴ In the present case the assumptions were considered acceptable within 95% and 99% confidence intervals, because the calculated values were lower than the critical values or the *p*-values 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 $F_{\text{calculated}}$ is lower than F_{critical} for all milk proteins within a 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.

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

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 (in the concentration range of 0–1.0 mg 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 a reasonable certainty for a given analytical procedure. In the case of linear calibration $y_i = a(\pm S_a)x_i + b(\pm S_b)$, the slope is a constant of concentration x_i (where subscript *i* in the expression denotes each different protein). According to ICH,²⁵ LOD is defined as the mathematical expression shown below:

$$\text{LOD} = 3.3 \frac{S_b}{a} \quad (2)$$

where S_b denotes the 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 κ -CN, generated from six separate chips and three separate gels, are

Table 6 Statistical results: lack of fit model and *r* calculated for each protein^a

Proteins	F_{calc}	F_{tab}	Slope	Intercept	<i>r</i>	LOD (mg mL ⁻¹)
α_s -Casein	5.67	8.65 ^S	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

^a $F_{\text{tab}}(\alpha = 0.05, \nu_1 = 3, \nu_2 = 10)$; $F_{\text{tab}}(\alpha = 0.01, \nu_1 = 3, \nu_2 = 10)$; $F_{\text{tab}}(\alpha = 0.01, \nu_1 = 2, \nu_2 = 8)$; $F_{\text{tab}}(\alpha = 0.01, \nu_1 = 3, \nu_2 = 5)$; ν_1 : numerator freedom degree; ν_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 ($C_{\text{critical}} = 0.684$): β -casein - $C_{\text{calc}} = 0.358$; β -lactoglobulin - $C_{\text{calc}} = 0.804$ (heteroscedasticity behavior); α -lactalbumin - $C_{\text{calc}} = 0.350$; Cochran test ($C_{\text{tab}} = 0.840$): κ -casein - $C_{\text{calc}} = 0.704$. Levene test (*p*-value): α_s -casein: 0.09.

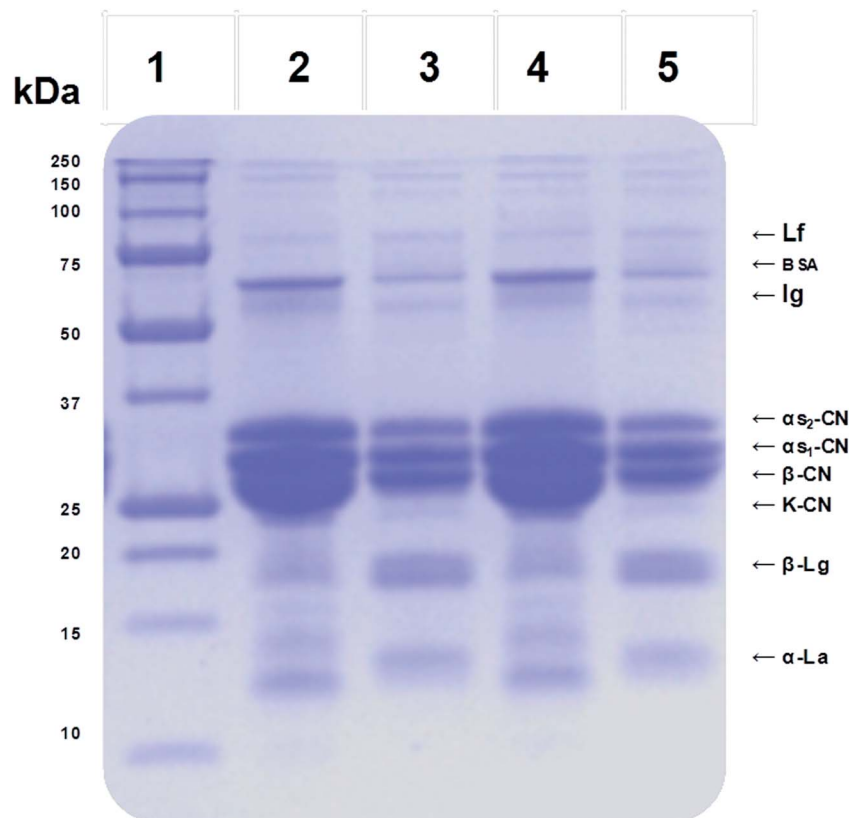


Fig. 3 SDS-PAGE electropherogram of a bovine milk sample. SDS-PAGE analysis lane 1: kit of protein standards with different molecular weights. Lanes 2 and 4: casein standard milk. Lanes 3 and 5: raw bovine milk.

shown in Fig. 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 methods are shown in Table 4. The concentrations of the individual caseins and whey proteins are in the range expected for fresh skim milk²⁶ and comparable concentrations were obtained by both the microfluidic chip and SDS-PAGE methods and compared with data from the literature (Fig. 4).

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

Fig. 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 the 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 in the literature,¹³ as the peaks for BSA, IgG 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

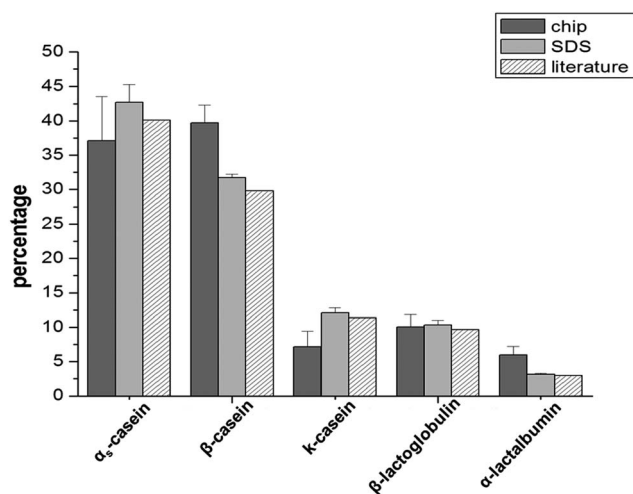


Fig. 4 Comparison graph between the official method, literature and microchip analysis of major milk proteins.

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 the 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 the total proteins while the whey proteins represent about 20% of the total proteins.^{25,27} The concentrations of the individual caseins and whey proteins are in the range expected for raw bovine milk²⁶ and comparable concentrations were obtained by both the microfluidic chip and SDS-PAGE methods (Table 4 and Fig. 4).

4. Conclusions

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 electrophoretic analysis of milk samples. The SEP buffer (6.0 mol L⁻¹ urea, 0.05% (w/w) hydroxypropylmethyl cellulose and 20 mmol L⁻¹ trisodium citrate dehydrate/0.1 mol L⁻¹ citric acid, pH = 3.0) achieved the best quantification. The quantitative percentages of protein fractions found were similar to those obtained with the traditional SDS-PAGE technique and to the data reported in the literature.

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References

- H. K. Mayer, J. Bürger and N. Kaar, Quantification of cow's milk percentage in dairy products—a myth?, *Anal. Bioanal. Chem.*, 2012, **403**, 3031–3040.
- C. Pinho, Z. E. Martins, C. Petisca, A. M. Figurska, O. Pinho and I. M. P. L. V. O. Ferreira, Size exclusion and reversed-phase high-performance liquid chromatography/UV for routine control of thermal processing of cows and donkey milk major proteins, *J. Dairy Res.*, 2012, **79**, 224–231.
- J. Dziuba, D. Nalecz and P. Minkiewicz, Reversed-phase high-performance liquid chromatography on-line with the second and fourth derivative ultraviolet spectroscopy as a tool for identification of milk proteins, *Anal. Chim. Acta*, 2001, **449**, 243–252.
- B. Miralles, V. Rothbauer, M. A. Manso, L. Amigo, I. Krause and M. Ramos, Improved method for the simultaneous determination of whey proteins, caseins and para-kappa-casein in milk and dairy products by capillary electrophoresis, *J. Chromatogr. A*, 2001, **915**, 225–230.
- L. Donato and D. G. Dalgleish, Effect of the pH of heating on the qualitative and quantitative compositions of the sera of reconstituted skim milks and on the mechanisms of formation of soluble aggregates, *J. Agric. Food Chem.*, 2006, **54**, 7804–7811.
- J. Wang, Q. H. Zhang, Z. H. Wang and H. M. Li, Determination of major bovine milk proteins by reversed phase high performance liquid chromatography, *Chin. J. Anal. Chem.*, 2009, **37**(11), 1667–1670.
- H. Goetz, M. Kuschel, T. Wulff, C. Sauber, C. Miller, S. Fisher and C. Woodward, Comparison of selected analytical techniques for protein sizing, quantification and molecular weight determination, *J. Biochem. Biophys. Methods*, 2004, **60**, 281–293.
- M. Poitevin, Y. Shakalisava, S. Miserere, G. Peltre, J. L. Viovy and S. Descroix, Evaluation of microchip material and surface treatment options for IEF of allergenic milk proteins on microchips, *Electrophoresis*, 2009, **30**, 4256–4263.
- H.-Y. Li, V. Dauriac, V. Thibert, H. Senechal, G. Peltre, X.-X. Zhang and S. Descroix, Micropillar array chips toward new immunodiagnosis, *Lab Chip*, 2010, **10**, 2597–2604.
- U. Bütikofer, J. Meyer and B. Rehberger, Determination of the percentage of α -lactalbumin and β -lactoglobulin of total milk protein in raw and heat treated skim milk, *Milchwissenschaft*, 2006, **61**(3), 263–266.
- D. Wu, J. Qin and B. Lin, Electrophoresis separations on microfluidic chips, *J. Chromatogr. A*, 2008, **1184**, 542–559.
- J. N. Buffoni, I. Bonizzi, A. Pauciuolo, L. Ramunno and M. Feligini, Characterization of the major whey proteins from milk of Mediterranean water buffalo (*Bubalus bubalis*), *Food Chem.*, 2011, **127**, 1515–1520.
- S. G. Anema, The use of lab-on-a-chip microfluid SDS electrophoresis technology for the separation and quantification of milk proteins, *Int. Dairy J.*, 2009, **19**, 198–204.
- J. Dooley, H. Brown, S. Wellu, B. Burch and P. Jasionowicz, *Determining the milk content of milk-based food products*, Food Standards Agency., (FSA Final Report Q01117), 2010.
- A. M. Gouldsworthy, J. M. Banks, A. J. R. Law and J. Leaver, Casein degradation in Cheddar cheese monitored by capillary electrophoresis, *Milchwissenschaft*, 1990, **54**, 620–623.
- S. L. C. Ferreira, R. E. Bruns, H. S. Ferreira, G. D. Matos, J. M. David, G. C. Brandão, E. G. P. da Silva, L. A. Portugal, P. S. dos Reis, A. S. Souza and W. N. L. dos Santos, Box–Behnken design: an alternative for the optimization of analytical methods, *Anal. Chim. Acta*, 2007, **597**, 179–186.
- B. D. Hames and D. Rickwood, in *Gel Electrophoresis of Proteins: A Practical Approach*, Oxford University Press, 4th edn, 1998, pp. 98–145.
- F. F. Costa, J. V. Resende, L. R. Abreu and H. D. Goff, Effect of calcium chloride addition on ice cream structure and quality, *J. Dairy Sci.*, 2008, **91**(6), 2165–2174.
- Z. Zhang and H. D. Goff, Protein distribution at air interfaces in dairy foams and ice cream as affected by casein dissociation and emulsifiers, *Int. Dairy J.*, 2004, **14**(7), 647–657.

- 20 P. F. Fox and P. L. H. McSweeney, in *Dairy Chemistry and Biochemistry*, Blackie Academic & Professional., London, 1998, pp. 220–298.
- 21 C. Holt, An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk, *Eur. Biophys. J.*, 2004, **33**, 421–434.
- 22 E. Smyth, R. A. Clegg and C. Holt, A biological perspective on the structure and function of caseins and casein micelles, *Int. J. Dairy Technol.*, 2004, **57**, 121–126.
- 23 D. L. Massart, B. G. M. Vandeginste, L. M. C. Buydens, S. De Jong, P. J. Lewi, and J. S. Verbeke, in *Handbook of chemometrics and qualimetrics: Part A*, Elsevier, New York, 1997, pp. 32–56.
- 24 K. Danzer and L. A. Currie, Guidelines for Calibration in Analytical Chemistry Part 1: Fundamentals and Single Component Calibration, *Pure Appl. Chem.*, 1998, **70**(4), 993–1014.
- 25 ICH Harmonized Tripartite Guideline, Validation of Analytical Procedures Methodology, 14 November, 1996.
- 26 P. Jelen and S. Lutz, Functional Milk and Dairy Products, in *Functional Foods. Biochemical & Processing Aspects*, ed. G. Mazza, Technomic Publishing Company. Inc., Lancaster, USA, 1998, pp. 265–365.
- 27 P. F. Fox, The major constituents of milk, in *Dairy processing – Improving quality*, G. Smit, Woodhead Publishing Limited, Cambridge, England, 2003, ch. 2, pp. 5–41.