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Separation and quantification of milk proteins with the addition of cheese whey by lab-on-a-chip

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Abstract – The objective of this work was to evaluate microfluidic chip electrophoresis, known as lab-on-a-chip technique, for the detection of milk adulteration using cheese whey in comparison with SDS-PAGE. Raw, pasteurized, processed at an ultra-high temperature (UHT), and powdered milk samples received increasing concentrations of cheese whey (0, 1, 2.5, 5, 10, 20, 30, 50, and 100% v/v), and were subjected to lab-on-a-chip electrophoresis and SDS-PAGE to detect their mixtures. The lab-on-a-chip methodology was able to separate and quantify milk proteins. In addition, the tested technique is easy, rapid, sensitive, and can detect the addition of cheese whey in milk from the lowest level tested (1%) for milk proteins α-casein and β-casein.

Index terms: electrophoresis, microfluidic electrophoresis, milk fraud, milk quality.

Separação e quantificação de proteínas do leite com adição de soro de queijo por *lab-on-a-chip*

Resumo – O objetivo deste trabalho foi avaliar a eletroforese com dispositivo de microfluidos, conhecida como técnica *lab-on-a-chip*, para detecção de adulteração de leite com soro de queijo, em comparação ao SDS-PAGE. Amostras de leite cru, pasteurizado, processado em temperatura ultra-alta (UHT) e em pó receberam adição de soro de queijo em concentrações crescentes (0, 1, 2,5, 5, 10, 20, 30, 50 e 100% v/v) e foram submetidas a eletroforese *lab-on-a-chip* e SDS-PAGE para detectar suas misturas. A metodologia *lab-on-a-chip* foi capaz de separar e quantificar as proteínas do leite. Além disso, a técnica *lab-on-a-chip* é fácil, rápida, sensível e pode detectar adição de soro de queijo no leite do nível mais baixo testado (1%) para as proteínas do leite α-caseína e β-caseína.

Termos para indexação: eletroforese, eletroforese microfluídica, fraude do leite, qualidade do leite.

Introduction

Milk proteins are elements of high importance from an economic point of view and to human health, due to their physico-chemical properties and nutritional value. There are two classes of proteins: caseins – alpha-casein (α-CN), beta-casein (β-CN) and κappa-casein (κ-CN) – and whey proteins represented by the albumin of the whey, lactoferrin, immunoglobulins, glycomacropeptides, beta-lactoglobulin and alpha-lactoalbumin (Beloti & Tamanini, 2015; Sharma et al., 2021).

According to the norms of sanitary inspection of products of animal origin, fraud in milk is considered when there is substitution of its characteristic components by other aggregates, such as the addition of substances of any nature to hide alterations or any deficiency in the quality of the raw material, or to cause an increase in the volume/weight ratio of the product (Brasil, 2017). The milk adulterated by the addition of whey has low commercial value and is rarely used in the manufacture of dairy products. Moreover, it is very common for milk that conforms to standards to be confused with adulterated milk, depending on the assessment technique used (Condé et al., 2020).

Brazilian legislation establishes the determination of the caseinomacropeptide marker (CMP) using the HPLC methodology as the official method for identifying milk fraud (Brasil, 2022a, 2022b). CMP marker is formed from the hydrolysis of κ-CN during cheese making and is not found in milk. However, CMP marker is not exclusively originated by enzymatic coagulation, once psychrotrophic microorganisms also produce enzymes that hydrolyze κ-CN (Lobato et al., 2020). Thus, the development of new methodologies to identify this type of fraud becomes important.

Thus, techniques based on the separation and quantification of milk proteins have been developed to assist in quality control, and to minimize and/or avoid illegal actions such as milk fraud. Conventional electrophoresis in polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) is considered an important study tool because it is already well established in analytical tests with milk (Anema, 2009; Sharma et al., 2021). Other methods have also been used to analyze milk proteins: liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), electrical impedance spectroscopy (EIS), isoelectric focusing (IEF), high-performance liquid chromatography (HPLC) by ion exchange, reverse phase and gel filtration modes, immunological methods and capillary electrophoresis (Raymundo et al., 2018; Masci et al., 2022; Oliveira et al., 2022). Although these methodologies are sensitive for fraud detection, they are expensive techniques.

The microfluidic capillary electrophoresis, also known as lab-on-a-chip, is a more recent technique that is used and recommended in studies because it demonstrates satisfactory results in the detection of antibiotics and evaluation of milk protein profile (Santos et al., 2017). In addition, it is a faster technique and uses considerably less chemicals and materials, which makes it less expensive than traditional techniques (Costa et al., 2014; Bosma et al., 2020; Ragab & El-Kimary, 2021). Considering the previous results with raw milk analysis (Costa et al., 2014; Santos et al., 2017), this technique should be better evaluated as an alternative for milk fraud detection.

The objective of this work was to evaluate microfluidic chip electrophoresis, known as lab-on-a-chip technique, for the detection of milk adulteration using cheese whey in comparison with SDS-PAGE.

Materials and Methods

Samples of four types of milk (raw, pasteurized, UHT and powder) and cheese whey were analyzed. The raw milk samples were collected at the experimental field of José Henrique Bruschi of Embrapa Gado de Leite, in the municipality of Coronel Pacheco, in the state of Minas Gerais (MG), Brazil (21°33'23.7"S 43°16'09.2"W). The samples of pasteurized, UHT and powdered milk (all samples from Itambé – Itambé Alimentos S.A –, municipality of Belo Horizonte, Brazil) were purchased at a commercial establishment in the municipality of Juiz de Fora, MG. Powdered milk was reconstituted with ultrapure water according to the manufacturer's label instructions, maintaining a final concentration of 6.4 g/L (w/v).

Cheese whey used in the experiment was obtained by the manufacture in the Laboratório de Análises de Alimentos e Águas (a laboratory for food and water analyses) of Faculdade de Farmácia of the Federal University of Juiz de Fora, in Juiz de Fora, MG, Brazil. Samples that simulated different types of adulteration were prepared from mixtures of the four types of milk samples with cheese whey in increasing concentrations (0, 1, 2.5, 5, 10, 20, 30, 50, and 100% v/v).

For SDS-PAGE electrophoresis, samples were previously treated with cold acetone at a 1:3 ratio (v/v) and then stored at -20 °C for 2 hours. Subsequently, these samples were centrifuged at 20,000 *g* for 30 minutes. The supernatant was discarded and the resulting pellet was completely dried at room temperature and resuspended in 200 µL of buffer solution (0.02 mol L^{-1} bis-tris propane; 7 mol L^{-1} urea; pH 7.0) for hydration, homogenized and kept at 4°C for at least 15 hours. Finally, the samples were homogenized again and kept

at -20°C until use (GE Healthcare, 2004). Before the electrophoretic run on SDS-PAGE, these samples were thawed in an ice bath, homogenized and diluted 1:9 in ultrapure water.

Proteins were quantified according to the Bradford method using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, California, USA) following the manufacturer's instructions. The standard protein solution (BSA, Sigma, St. Louis, MO, USA) was prepared and serially diluted in ultrapure water (in the 200 μg mL^{-1} – 2,000 μg mL^{-1} range) to construct a standard curve. Milk samples added with whey cheese were previously diluted 1:9 in ultrapure water and the minimum volume of 4 μL of samples in 200 μL of diluted Coomassie Brilliant Blue R-250.

Absorbance was measured after a 5-minute incubation period at room temperature. Peaks were measured under UV light at 595 nm in the "Protein Bradford" module in a spectrometer NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

Microfluidic chip electrophoresis was performed in the Agilent 2011 Bioanalyzer with the 2100 Expert Software and the protein kit 80 (Agilent Technologies, Waldbronn, Germany). The denaturing solution used to reproduce the reducing condition was prepared by adding 3.5% (vol.) of 1 mol L^{-1} dithiothreitol to the sample buffer. Purified proteins α-lactoalbumin, β-lactoglobulin, α-casein, β-casein and κ-casein were obtained from Sigma-Aldrich (St. Louis, MO, USA). Solutions (10 mg mL^{-1}) were individually prepared with addition of ultrapure water (Ultrapure Milli-Q; Millipore Corp., USA). Mixed protein standards were prepared by combining each of the individual protein solutions (1 mL), obtaining a final volume of up to 10 mL until a concentration of 1 mg mL^{-1}.

Automated data analysis was performed using the software Agilent 2100 Expert, which determined molecular weight and quantitative parameters, such as concentration (ng μL^{-1}), and total percentage (%) through the calculation of peak area from individual proteins in the sample.

The electrophoresis system used was of the horizontal type on the Amersham ECL Box support (GE Healthcare Life Sciences, Freiburg, Germany) with 12% acrylamide concentration gels produced by the manufacturer. As a molecular weight standard, Novex Protein, with an interval of between 3.5 and 260 kDa (Novex Sharp Pre-stained Protein Standard, Life Technologies, Carlsbad, CA, USA), and the mix of standard proteins were used. Solutions for protein separation and electrophoresis were prepared according to the manufacturer's instructions.

Gels were incubated for 15 hours in a staining solution (0.1% Coomassie Brilliant Blue R-250, 50% ethanol, 2% trichloroacetic acid) and then in destaining solution (25% ethanol, 8% acetic acid). Images of gels were scanned (Hewlett Packard Scanjet 2400, Palo Alto, CA, USA) and the molecular weight of each protein fraction was estimated using the software ImageQuant TL (GE Healthcare Life Sciences, Chicago, Illinois, USA).

Data were analyzed using a randomized block design where each of the chips represented a block and had all types of milk adulterated with cheese whey randomly distributed in each chip. Nine chips for each type of milk were used, with nine repetitions, in such a way that all treatments (increasing addition of cheese whey) were contained in the same chip. All data was analyzed with EZAnalyze program version 3.0 (Poynton, 2007). Least squares means were compared using Dunnett's test. Significance was considered at 5% probability.

Results and Discussion

The gel image obtained from the microfluidic electrophoresis technique, analyzing the raw milk samples and their respective fraud simulations, showed a protein profile with five protein bands in all samples of pure and unprocessed milk in the mixtures, while in the whey sample, it showed two bands of proteins (Figure 1). The migration and separation of milk proteins by the microfluidic electrophoresis technique was reached as in a previous work (Santos et al., 2017) using the lab-on-a-chip technique and the SDS-PAGE technique (Aquino et al., 2014). Whey proteins, alpha-lactalbumin (α-LA) and beta-lactoglobulin (β-LG) were the first to be separated and quantified, followed by β-CN, α-CN, and lastly κ-CN.

Regarding molecular masses, caseins were not particularly close to those described with the SDS-PAGE method, unlike whey proteins (Table 1). All caseins migrated with a higher molecular weight than that observed in the conventional SDS-PAGE

technique. The separation and quantification of milk proteins using the microfluidic electrophoresis technique were satisfactory, like the results found for bovine milk (Anema, 2009; Costa et al., 2014), buffalo milk (Buffoni et al., 2011), domestic donkey milk (Gubić et al., 2016) and goat milk with added cow's milk (Santos et al., 2017). The cited studies concluded that the method is efficient in comparison to the SDS-PAGE and that it can easily be used as an alternative method for the analysis of milk quality.

The lab-on-a-chip method generated quantitative data referring to height and peak area of the proteins (Table 2), the latter being the variable used as an indicator of the change in milk composition – fraud simulation – (Santos et al., 2017). From the comparative analysis of the information generated between the types

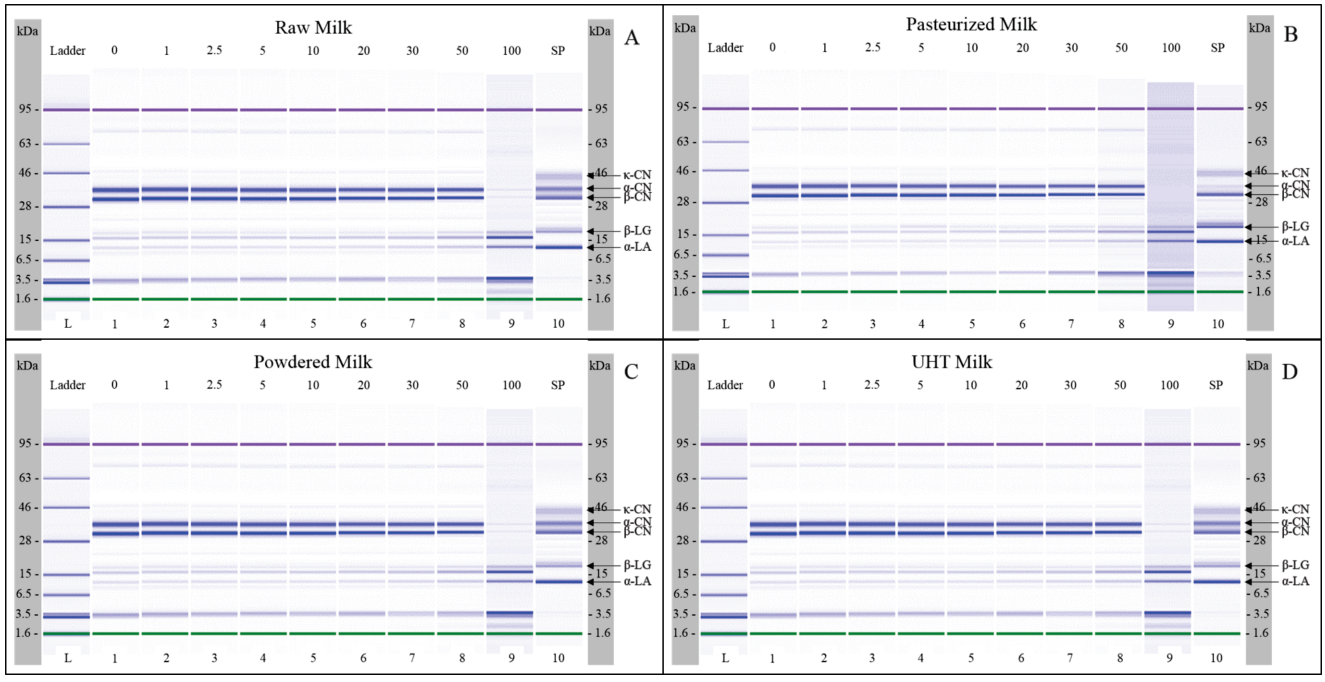

Figure 1. Gel-like images obtained in the microfluidic chip electrophoresis system with proteins extracted from raw (A), pasteurized (B), powdered (C), and UHT (D) milk samples. Cheese whey was added to the samples in increasing concentrations of 0, 1, 2.5, 5, 10, 20, 30, 50, and 100% (v/v). Ladder (molecular weight marker) and SP (standard proteins) were used. Proteins were separated and quantified according to molecular mass and retention time. α-LA, alpha-lactoglobulin; β-LG, beta-lactoglobulin; β-CN, beta-casein; α-CN, alpha-casein; κ-CN, kappa-casein.

Table 1. Estimated values of the molecular mass of milk caseins and whey proteins for samples of raw, pasteurized, UHT, and powdered milk, compared with the values of molecular mass described in the literature using the microfluidic technique.

Protein	Bovine milk		
	Estimated molecular mass by microfluidic chip (kDa)[(1)]	Reported molecular mass by microfluidic chip (kDa)[(2)]	Traditional SDS-PAGE (kDa)[(3)]
α-LA	12.5±0.2	12±1	11.03
β-LG	18.5±0.3	18±1	13.47
β-CN	32.5±0.8	29±2	25.90
α-CN	37.8±0.5	37±1	30.66
κ-CN	42.7±0.5	42±2	23.02

[(1)]Estimated molecular mass for samples from 9 repeated chips (all treatments per chip). [(2)]Reported molecular mass from Anema (2009) and Santos et al. (2017). [(3)]Estimated molecular mass for samples by traditional SDS-PAGE. α-LA, alpha-lactoglobulin; β-LG, beta-lactoglobulin; β-CN, beta-casein; α-CN, alpha-casein; κ-CN, kappa-casein.

Table 2. Mean values of the peak areas of the main protein fractions of samples of milk tupes (powdered, UHT, pasteurized, and raw) with addition of cheese whey in increasing levels obtained with the lab-on-a-chip technique.

Serum addition levels (%)	Mean peak areas of main milk proteins[(1)]				
	α-CN	β-CN	κ-CN	α-LA	β-LG
			Powdered milk		
0	773.90	1,141.46	22.78	12.59	16.08
1	727.87	1,075.37	17.38	16.89	11.88
2.5	651.78	989.76	14.27	12.66	12.50
5	592.64	901.33	14.54	16.99	11.68
10	536.19	847.49	14.42	18.98	9.86
20	421.97	673.11	13.24	19.37	9.68
30	355.21	580.01	10.27	18.18	8.55
50	260.64	424.82	7.68	28.22	13.01
100	-	-	-	61.88	25.83
			UHT milk		
0	686.60	772.07	47.38	40.87	23.56
1	642.80	733.09	42.81	43.59	23.70
2.5	536.90	614.70	25.17	35.08	23.52
5	481.80	571.32	25.93	32.71	24.37
10	431.90	514.28	19.68	32.01	24.43
20	358.20	432.49	10.50	30.59	26.06
30	305.70	369.76	2.64	30.37	23.84
50	239.30	284.93	2.31	40.49	31.56
100	-	-	-	52.31	38.71
			Pasteurized milk		
0	691.41	688.59	37.40	53.17	59.81
1	660.79	670.97	37.30	49.86	50.09
2.5	544.61	569.97	32.40	41.31	43.78
5	494.58	526.11	25.70	41.94	45.94
10	456.50	494.21	23.20	38.30	40.59
20	361.90	397.40	16.10	36.29	34.18
30	293.31	324.04	10.10	36.72	30.69
50	202.72	223.71	7.80	38.88	30.30
100	-	-	-	47.83	24.54
			Raw milk		
0	652.62	645.74	34.49	61.37	48.59
1	604.74	609.57	33.38	55.30	47.44
2.5	561.02	574.31	28.01	53.91	42.65
5	519.50	548.10	27.24	49.85	38.77
10	492.72	517.82	23.99	48.22	31.48
20	353.45	383.99	14.86	41.22	30.99
30	333.46	356.57	17.82	44.19	29.46
50	238.59	249.36	13.16	44.41	30.89
100	-	-	-	37.51	20.64

[(1)]α-LA, alpha-lactoglobulin; β-LG, beta-lactoglobulin; β-CN, beta-casein; α-CN, alpha-casein; κ-CN, kappa-casein.

of milk, it was evident that the area of α-CN showed similar behavior for all samples. It was observed that the peak of the area of α-CN reduced with the addition of cheese whey by 63.44% in raw milk, 70.68% in pasteurized milk, 65.14% in UHT milk, and 66.32% in powdered milk. These results proved to be statistically significant ($p<0.01$), supporting the purpose of identifying adulteration in milk from the lowest level of detection under the imposed laboratory conditions.

The mean protein peak area values of raw, pasteurized, UHT, and powdered milk were similar (Figure 2). The mean peak area values of the main protein fractions of raw milk samples with cheese whey added at increasing levels, obtained with the lab-on-a-chip technique, resulted in a significant difference for caseins (Table 3). For α ($p=0.0425$) and β ($p=0.0316$) caseins, the results were statistically significant ($p<0.05$) from 1% cheese whey addition, and this pattern was maintained until the 50% addition level ($p=0.0001$). There was a difference between the values of the protein peak areas. The means are not the same when comparing treatments with the negative control.

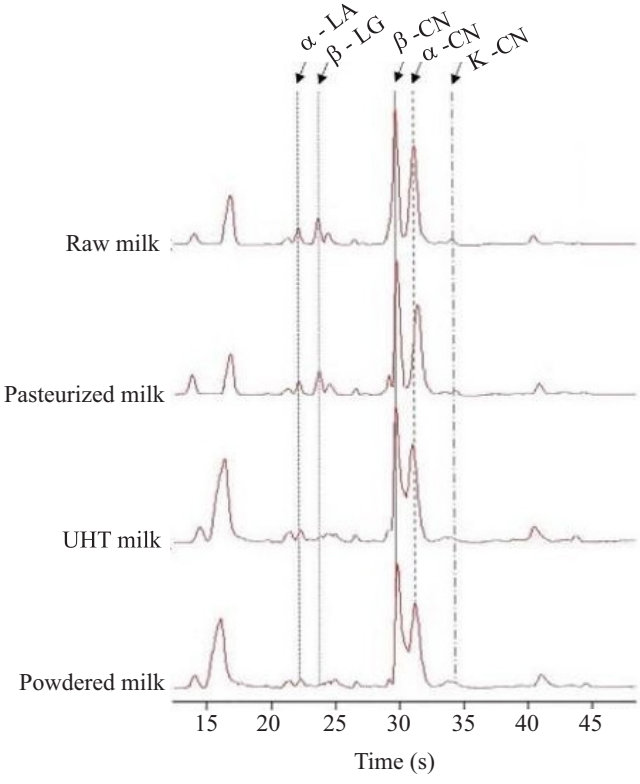

Figure 2. Electrophoretic profile of milk proteins obtained by microfluidic electrophoresis. α-LA, alpha-lactoglobulin; β-LG, beta-lactoglobulin; β-CN, beta-casein; α-CN, alpha-casein; κ-CN, kappa-casein.

Statistical analysis of the data for raw, pasteurized, UHT, and powdered milks showed that it was possible to detect fraud ($p<0.05$) with 1% addition of cheese whey for milk caseins, α-CN, and β-CN. As for the κ-CN protein, it was possible to detect fraud from 10%, 5%, and 2.5% of addition of cheese whey to the respective milks. In addition, for powdered milk, it was possible to detect fraud from 1% of addition of cheese whey. For whey proteins (β-LG and α-LA), it was not possible to identify adulteration at any level of cheese whey addition at the significance level of $p<0.05$.

The SDS-PAGE method was efficient regarding the separation of proteins and the estimates of their respective molecular masses (Figure 3). Qualitative changes in the intensity of the protein bands in the SDS gel were not representative. A slight reduction in the intensity of the κ-CN protein band was observed in the samples of raw milk from addition of 5% whey (Figure 3 A). In the types of pasteurized, UHT and powder milk analyzed, the intensity of the band started to be reduced after addition of 20% (Figure 3 B, C, D).

Farrell Jr et al. (2004), Anema (2009), Marques et al. (2011), and Santos et al. (2013) found molecular mass values for the main milk proteins like those obtained in this study (Table 4). It can be observed that there was little variation in the estimated molecular masses for milk caseins in the samples of raw, pasteurized, UHT, and powdered milk when compared to those described in the literature. Farrell Jr et al. (2004) reported that the main casein ranges are around 19 to 25 kDa; however, in this study, milk caseins migrated to a range close to 30 kDa, as presented by Marques et al. (2011) when analyzing powdered milk samples using the SDS-PAGE technique. This may have happened due to the binding, in different forms, of the proteins to the SDS or due to the interaction of the separated casein fractions (Marques et al., 2011).

Although the protein profile of the analyzed milks had little variation in relation to the molecular mass values of the main milk proteins, they are still very close to those obtained in the literature for the SDS-PAGE technique, which implies that this method was effective in separation of milk proteins.

Marques et al. (2011) showed that SDS-PAGE was efficient for the separation of proteins for milk samples and whey powder evaluated separately, but when

analyzing milk with added whey mixes to detect fraud, the SDS-PAGE technique was not satisfactory.

Similar analyses by the SDS-PAGE method were performed in the work of Aquino et al. (2014), which detected a qualitative change in the intensity of the α-CN, β-CN, and β-LG protein bands, and a quantitative change in the analysis of the values of their concentrations in samples of raw milk experimentally adulterated with cheese whey.

Table 3. Comparison of the significance levels of α-CN, β-CN, κ-CN, β-LG and α-LA proteins, between treatments applied to the four milk types (raw, pasteurized, UHT, and powdered), obtained by Dunnet's test.

Serum addition levels (%)	p-value for main milk protein[1]				
	α-CN	β-CN	κ-CN	β-LG	α-LA
			Raw milk		
0	-	-	-	-	-
1	0.0425	0.0316	0.8685	0.8583	0.9998
2.5	< 0.0001	0.0002	0.0670	0.8374	1.0000
5	< 0.0001	0.0013	< 0.0001	0.9767	1.0000
10	< 0.0001	< 0.0001	< 0.0001	0.9576	1.0000
20	< 0.0001	< 0.0001	< 0.0001	0.9997	0.9999
30	< 0.0001	< 0.0001	< 0.0001	1.0000	0.7207
50	< 0.0001	< 0.0001	< 0.0001	1.0000	< 0.0001
			Pasteurized milk		
0	-	-	-	-	-
1	0.0410	0.2098	0.1316	0.9995	0.1316
2.5	< 0.0001	0.3140	0.0081	0.8950	0.0081
5	< 0.0001	0.2250	0.0108	0.9996	0.0108
10	< 0.0001	0.0130	0.0095	1.0000	0.0095
20	< 0.0001	0.0525	0.0026	1.0000	0.0026
30	< 0.0001	0.0177	< 0.0001	1.0000	< 0.0001
50	< 0.0001	0.5055	< 0.0001	1.0000	< 0.0001
			UHT milk		
0	-	-	-	-	-
1	0.0239	0.0347	0.1871	0.9943	0.9009
2.5	< 0.0001	< 0.0001	< 0.0001	0.0691	0.8860
5	< 0.0001	< 0.0001	< 0.0001	0.0067	0.9441
10	< 0.0001	< 0.0001	< 0.0001	0.0030	0.9474
20	< 0.0001	< 0.0001	< 0.0001	0.0005	0.9907
30	< 0.0001	< 0.0001	< 0.0001	0.0004	0.9119
50	< 0.0001	< 0.0001	< 0.0001	0.8503	1.0000
			Powdered milk		
0	-	-	-	-	-
1	0.0310	0.0210	0.0254	0.9995	0.9798
2.5	< 0.0001	< 0.0001	< 0.0001	0.8950	0.8678
5	< 0.0001	< 0.0001	< 0.0001	0.9996	0.9752
10	< 0.0001	< 0.0001	< 0.0001	1.0000	0.9973
20	< 0.0001	< 0.0001	< 0.0001	1.0000	0.9998
30	< 0.0001	< 0.0001	< 0.0001	1.0000	1.000
50	< 0.0001	< 0.0001	< 0.0001	1.0000	1.000

[1]α-LA, alpha-lactoglobulin; β-LG, beta-lactoglobulin; β-CN, beta-casein; α-CN, alpha-casein; κ-CN, kappa-casein.

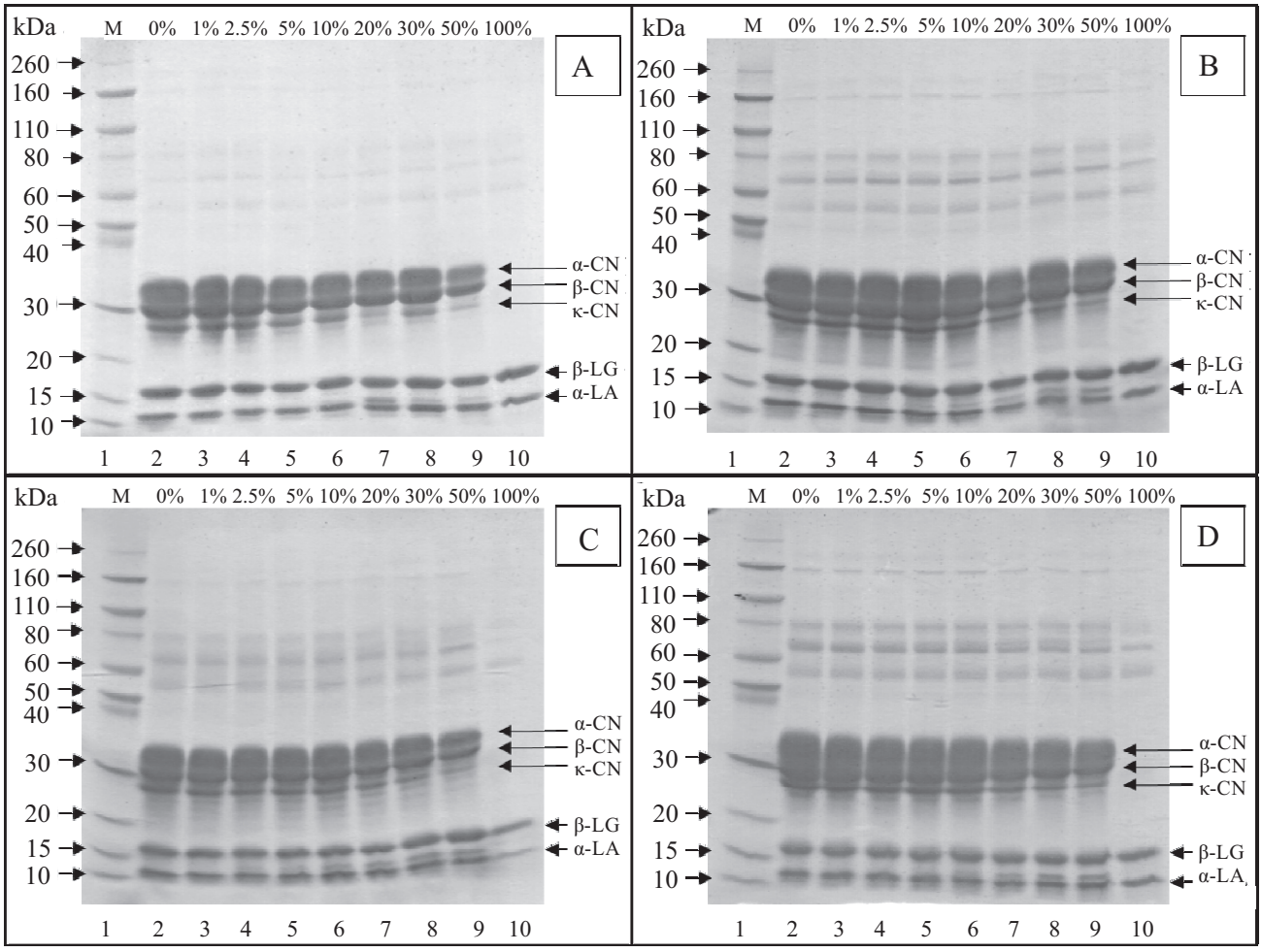

Figure 3. SDS-PAGE electrophoretic profile of milk proteins. A, B, C, and D – samples of raw, pasteurized, UHT, and powdered milk adulterated with whey, respectively. Well 1 – NovexProtein molecular marker. The values from 10 to 260 correspond to the values of the molecular marker in kDa. Wells 2 to 10 – % whey addition. Arrows indicate the main milk proteins. α-LA, alpha-lactoglobulin; β-LG, beta-lactoglobulin; β-CN, beta-casein; α-CN, alpha-casein; κ-CN, kappa-casein.

Table 4. Estimated values of the molecular mass of milk caseins and whey proteins for samples of raw, pasteurized, UHT, and powdered milk, compared with the values of molecular mass described in the literature using the SDS-PAGE technique.

Milk proteins[1]	Molecular mass described in the literature (kDa)			Molecular mass of milk (kDa)			
	Bovine milk			Raw	Pasteurized	UHT	Powdered
α-CN	30±3[2]	23.6[3]	29.94[4]	30.66	32.09	31.58	32.41
β-CN	27±4	25.2	28.03	25.90	26.33	26.64	26.29
κ-CN	24±4	19.0	25.72	23.02	23.06	23.45	23.24
β-LG	19±2	18.3	16.63	13.47	13.35	13.59	13.57
α-LA	13±3	14.1	12.56	11.03	10.85	11.04	10.99

[1]α-LA, alpha-lactoglobulin; β-LG, beta-lactoglobulin; β-CN, beta-casein; α-CN, alpha-casein; κ-CN, kappa-casein. [2]Molecular masses estimated by Anema (2009). [3]Molecular masses estimated by Marques et al. (2011). [4]Molecular masses estimated by Santos et al. (2017).

Conclusions

1. The lab-on-a-chip method detect fraud from 1% addition of cheese whey to all types of milk tested.

2. The lab-on-a-chip electrophoretic method is able to separate milk protein band with the same electrophoretic pattern in all the types of milk analyzed.

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