ELECTROPHORETIC ANALISYS TO DETECT AND QUANTIFY ADDITIONAL WHEY IN MILK AND DAIRY BEVERAGES[1]

Elizabeth Maria Talá DE SOUZA[2]; Sandra Fernandes ARRUDA[2]; Patrícia Oliveira BRANDÃO[2]; Egle Machado de Almeida SIQUEIRA[2,*]

SUMMARY

Polyacrylamide gel electrophoresis, SDS-PAGE system, was adjusted to detect the presence of additional whey in dairy beverages distributed in a Brazilian Government School Meals Program. Aqueous solutions of samples in 8 M urea were submitted to a polyacrylamide gel gradient (10% to 18%). Gel scans from electrophoresis patterns of previously adulterated milk samples showed that caseins peak areas decreased while peak areas of β -lactoglobulin plus α -lactalbumin increased as the percentage of raw milk powder replaced by whey powder increased. The relative densitometer areas of caseins or β -lactoglobulin plus α -lactalbumin plotted against the percentage of whey added to the raw milk showed a linear correlation coefficient square higher than 0.97. The caseins plot was used to determine the percentage of additional whey in 116 dairy beverages, chocolate or coffee flavor. Considering that the lowest relative caseins concentration found in commercial milk powder samples by the present method was 72%, the dairy beverages containing caseins percentages equal to or higher than this value were considered free of additional whey. Based on this criterion, about 49% of the coffee-flavor dairy beverages and 29% of the chocolate-flavor beverages, among all the samples analyzed were adulterated with whey protein to reach the total protein contents specified on their labels. The present method showed a sensitivity of 5% to additional whey.

RESUMO

ANÁLISE ELETROFORÉTICA PARA DETECTAR E QUANTIFICAR SORO DE LEITE ADICIONAL EM LEITE E BEBIDAS LÁCTEAS. A adulteração de bebidas lácteas, distribuídas no Programa Nacional da Merenda Escolar, pela adição de soro, foi investigada através da análise eletroforética em sistema de SDS-PAGE. Soluções aquosas de amostras em uréia 8M, foram submetidas a um gradiente de 10% a 18% de gel de poliacrilamida. Após a eletroforese, o gel corado com "Comassie Blue" foi submetido à densitometria para identificação e quantificação das proteínas lácteas. A partir de amostras de leite em pó, previamente adulteradas com diferentes proporções de soro em pó (0 a 100%) foi observada alta correlação linear entre a concentração relativa de soro adicionado ao leite e as concentrações de proteínas lácteas. Esta correlação foi inversamente proporcional ao percentual de caseínas (r^2 = 0,987) e diretamente proporcional à soma dos percentuais de β-lactoglobulina e α-lactalbumina (r^2 = 0,979) apresentados nos densitogramas. A partir da curva padrão de caseínas versus soro adicionado foi estimada a magnitude das adulterações encontradas nas bebidas lácteas sabor chocolate e café do programa da merenda escolar. Tendo em vista que, amostras de leite em pó comerciais analisadas através deste método apresentaram uma concentração mínima de 72% de caseínas, as bebidas lácteas, contendo percentual igual ou superior a este valor, foram consideradas livres de soro adicional. Baseado neste critério, cerca de 49 % das bebidas lácteas, sabor café, e 29 % sabor chocolate, de um total de 58 amostras analisadas por sabor, apresentaram adulteração. O método proposto apresenta uma sensibilidade de 5% de soro adicionado.

Palavras-Chave: Leite, proteínas lácteas, caseína, soro, adulteração.

1- INTRODUCTION

Dairy beverages are one of the products distributed by the National School Meals Program, a Brazilian government program, supplying 15% of the Recommended Dietary Allowances (RDA) of 30 million

children between 4 and 14 years old at public schools in Brazil. This program involves about 140 thousand tons of food and financial resources estimated at 200 million dollars a year (mean of six years [1]). Besides other components such as fat, coffee, chocolate and sugar, milk constitutes 13% of these dairy beverages. Due to the reduced price of whey in relation to raw milk, and the complexity of these mixtures, these drinks become very attractive targets for adulteration.

Cows' milk has two main protein categories: caseins and whey proteins. Due to its high protein content and nutritional value [2,3], cows' milk is considered a complete food. Caseins represent about 80% of total milk proteins, while the whey proteins [lactalbumin, lactoglobulin, bovine serum albumin (BSA), lactoferrin and immunoglobulins] represent 20%[4,5]. Preliminary electrophoresis densitometric analysis of samples of dairy beverages showed a relatively high concentration of whey protein compared to caseins. This result suggests that additional whey has been used in these products to attain the total desirable protein concentration. Although whey proteins have a similar nutritional value to that of caseins, the substitution of raw milk by whey should be described in the dairy beverage specifications because whey is significantly cheaper than milk, so the product could be sold for a lower price. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [6] has been used to detect additional whey in milk and dairy beverages, but both low milk protein resolution and low sensitivity [7] have limited quantification by this method.

The present research shows an adjustment of the SDS-PAGE to improve the milk protein resolution and, consequently, the sensitivity of this system as a potential technique for quantifying milk and dairy beverage adulteration with whey.

2 - MATERIALS AND METHODS

2.1 - Milk and Whey samples

Samples: Commercial raw milk, commercial skim and raw milk powder and dairy beverages (from the Brazilian National School Meals Program) were used. Dairy beverage ingredients, informed by the manufacturer, were sugar, salt, milk powder, alkaline cocoa or soluble coffee powders with the following composition: 13.8% protein; 13.7% fat; 64.6% carbohydrates, 0.2% fiber and 47% moisture. Whey powder was obtained by precipitating raw milk with a commercial rennin; the supernatant was obtained by centrifugation (13.000rpm for 20 min at 25ºC), dialyzed against water (24 hours),

heated at 80ºC for 20 min to deactivate [8,9] the enzyme, and lyophilized.

2.2 - Preparation of raw milk powder samples with whey powder adulteration

Simulated adulterated milk samples were prepared replacing raw milk by whey in a mixture to obtain increased concentrations of whey (0 to 100%, w/w). The mixtures were diluted in distilled water (0.25g.ml^{-1}), and 100μl of this solution were placed in 900μl of urea 8M. Then 40μl of the solution were added to 10μl of sample buffer (20% 2-β-mercaptoethanol, 2MTris.HCl, 40% glycerol, 9.2% SDS, 0.04% bromophenol blue, pH 6.8). Samples were boiled for 2 min and 2μl were placed in gel electrophoresis.

2.3 - Standard Proteins

Caseins, β-lactoglobulin and α-lactalbumin were used as standards for milk proteins and BSA, egg albumin, pepsin, lysozyme and tripsinogen were used as molecular weight markers. All protein substances were purchased from Sigma.

2.4 - Preparation of gels

Samples were analyzed using a 10 to 18% acrylamide gradient concentration (SDS-PAGE), according to LAEMMLI [6]. For the 10% acrylamide gel concentration, a solution was prepared by mixing 3.3mL acrylamide aqueous solution (10% acrylamide, 0.27% bisacrylamide, 0.2% SDS, 0.02% ammonium persulfate and 2.7μL TEMED) and 0.2 ml gel buffer (3.1mol/L Tris.HCl, pH 8.8). For the 18% acrylamide gel, a solution was prepared by mixing 3.0mL acrylamide aqueous solution (18% acrylamide, 0.5% bisacrylamide, 0.2% SDS, 0.02% ammonium persulfate, 0.36ml glycerol and 2.7μL TEMED) and 0.2ml gel buffer. The gel gradient concentration was obtained by progressive addition of 10% acrylamide solution to 18% acrylamide solution, while stirring, as the glass plates (10 x 8cm) were filled with unpolymerized gel mixture. After 30 min an overlayer of stacking gel (aqueous solution 4.2% acrylamide, 0.1% bisacrylamide, 0.2% SDS, 0.05% ammonium persulfate and 2μl TEMED in 0.085 M Tris.HCl, pH 6.8) was added.The gel was run on Mini Protean II/Electrophoresis Cell system (BIO-RAD) using 0.25 M Tris.HCl, 0.18M glycine, pH8.9, containing 10% SDS as running buffer. The electrophoresis was carried out at room temperature with a current of 10mA for 30 min, then the current was increased to 20mA per gel, for 90 min. The gels were stained with Coomassie Blue R-250 and destained with 7% acetic acid solution.

2.4 - Gel scanning

Gel scans were obtained at 600nm using a CS-9301 PC SHIMADZU automatic computing densitometer. The relative concentration of the proteins (caseins, β -lactoglobulin and α -lactalbumin) was obtained by the densitometer integrator program, which measures the area under each densitometric peak.

3 - RESULTS AND DISCUSSION

Aqueous solutions of raw milk and whey were prepared with 8M urea to allow complete protein solubilization. Samples of these solutions and molecular weight markers were submitted to SDS-PAGE to identify the milk proteins. The caseins electrophoresis patterns exhibited molecular masses in the range of 25,000 to 31,000 (*Figure 1*), above 19,000 to 25,000 which is the range found for the major caseins [5,10]. BASCH et al. [11] found an even higher molecular mass range, above 30,000, for caseins by LAEMMLI gel [6] and they explain this abnormal behavior as a consequence of the competing equilibrium as the caseins either bind SDS or interact with other casein molecules. Our results showed that when the proteins are denatured by urea, which reduces non-specific molecular interactions, the caseins exhibit molecular masses closer than the values expected.
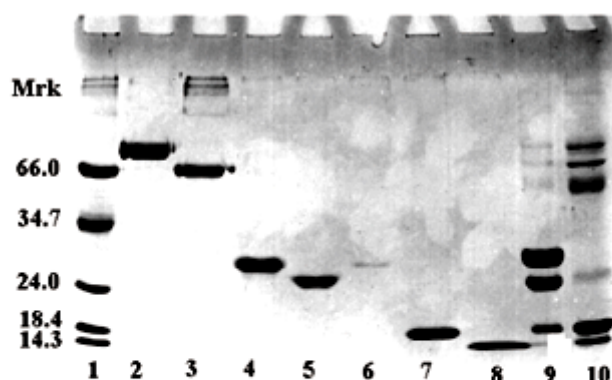

FIGURE 1. Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (gradient 10-18%). 1. molecular weight markers (from the bottom: lysozyme, β-lactoglobulin, trypsinogen, pepsin, albumin bovine); 2. lactoferrin; 3. BSA; 4. α-casein; 5. β-casein; 6. γ-casein; 7. β-lactoglobulin; α-lactalbumin; 9. raw milk; 10. whey powder.

Figure 2 shows typical densitometric scans from the raw milk (A) and whey (B) electrophoretic patterns and the respective densitometric responses are given in *Table 1.*The percentage of proteins obtained

for the whey sample confirmed that β-lactoglobulin and α-lactalbumin are the two major protein components of whey. The caseins percentage in the raw milk was about 78% (SD ± 2.3, n=5) and the two major whey proteins, β-lactoglobulin and α-lactalbumin, were present at concentration of 11% (SD ± 1.5, n=5) and 4% (SD ± 0.4, n=5), respectively. These results are consistent with the average composition of milk from different breeds of cattle previously determined by micro-Kjeldahl nitrogen [12].
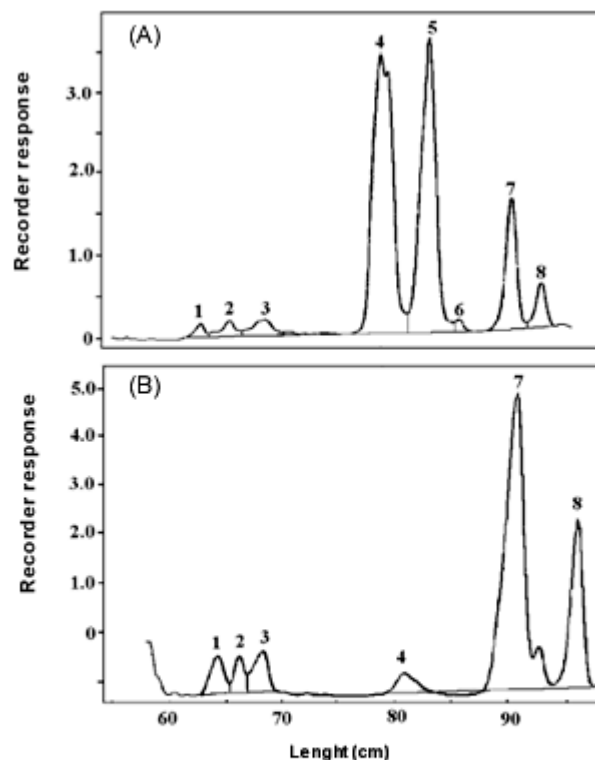

FIGURE 2. Typical Coomassie Blue gel scans from the sample of raw milk (A) and whey (B) electrophoresis pattern ($\lambda \neq 600$ nm). Gel length = 10,0 cm. Peak 1. lactoferrin; 2. BSA; 3. immunoglobulins; 4, 5 and 6. caseins; 7. β-lactoglobulin; and 8. α-lactalbumin.

TABLE 1 Densitometric response from the sample electrophoretic patterns.

Samples	Raw milk		Whey	
Proteins	Peak	(%)[a]	Peak	(%)[a]
Caseins	4, 5 and 6	78 ± 2.3	4	5 ± 0.7
β-lactoglobulin	7	11 ± 1.5	7	59 ± 4.2
α-lactalbumin	8	4 ± 0.4	8	19 ± 1.0

[a]Means of the three replicates of each sample run on separate gel.

In order to obtain standard curves that might correlate milk proteins with the relative concentration of added whey, mixtures of raw milk and whey powders in different proportions were prepared and submitted to the SDS-PAGE system (*Figure 3*). Densitometric scans from the gel electrophoretic patterns of the mixtures showed that the peak areas of caseins decreased while the peak areas of β -lactoglobulin and α -lactalbumin increased as the percentage of whey powder replacing raw milk powder increased.
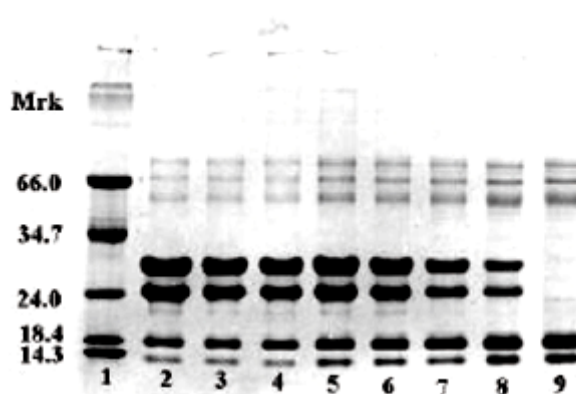

FIGURE 3. Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (gradient 10-18%) of raw milk powder and a mixture of raw milk powder containing different percentages of whey powder. 1) molecular weight markers (from the bottom: lysozyme, β -lactoglobulin, trypsinogen, pepsin, albumin bovine); 2) raw milk (14µg); 3-8. mixture of raw milk powder replaced by whey powder (14µg): 3) 5%; 4) 10%; 5) 20%; 6.) 30%; 7) 50%; 8) 75% and 9) whey powder (11µg).

The relative densitometer areas of caseins and the sum of β -lactoglobulin and α -lactalbumin areas were plotted against the percentage of whey added to the raw milk. Casein and β -lactoglobulin + lactalbumin standard curve equations were y = 0.723x + 79,47 and y = 0.5931x + 15.79, respectively. Both curves showed a linear correlation coefficient square higher than 0.97.

Six samples of commercial raw and skim milk powder and powder for 116 dairy beverages, chocolate or coffee flavor, were analyzed in SDS-PAGE and the respective protein percentages were obtained by the densitometric method. The commercial milk powder samples showed 79 ± 7.0% (n=6) caseins. Considering that the caseins percentage average (79%) minus the SD (7%) obtained for those six commercial milk samples was 72%, the dairy beverages containing caseins percent equal to or higher than 72% could be considered free of additional whey. Based on this criterion, about 49% of the coffee

flavor dairy beverages and 29% of the chocolate flavor beverages analyzed contained additional whey to reach the total protein content specified on their labels (13%, w/w). Half of the twenty-eight adulterated coffee flavor samples contained 20 to 40% of additional whey, while only three of the sixteen adulterated chocolate flavor samples showed values between 20 to 24%. The averages of relative casein concentrations found in all dairy beverage samples analyzed considered free of adulteration, were no different from the casein percentages of the raw milk samples ($p < 0.05$). This result suggests that coffee and chocolate additives did not interfere in the present analytical method. The similar electrophoresis patterns found for commercial milk samples and both dairy beverage samples, which were prepared according to their commercial specifications, confirm the above statement.

Coomassie Blue gel scans from the electrophoresis patterns of the dairy beverages containing less than 72% caseins were quite different from those with higher caseins percentages (*Figure 4*). Special attention must be given to the quality control of products destined for governmental nutrition programs, such as The National School Meals Program, because they are produced and sold on a large scale. Therefore, replacing one ingredient by a cheaper one in the make-up of the product becomes a very attractive business. If it is possible to reduce prices without impairing quality, the profit from this substitution should revert to more food to cater to more children.
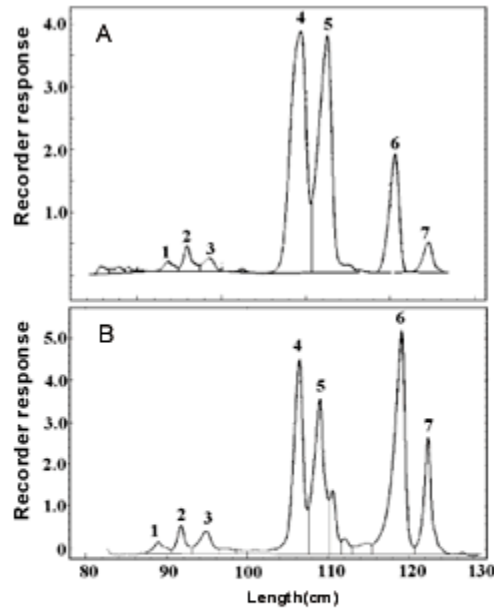
FIGURE 4 Typical Coomassie Blue gel scan from the electrophoresis patterns of two coffee-flavor dairy beverage samples containing: A) 76.9% caseins and B) 48.4% caseins ($\lambda \neq 600$ nm). Gel length = 10.0 cm. Peaks 1. lactoferrin; 2. bovine albumin serum; 3. immunoglobulins; 4 and 5. caseins; 6. β-lactoglobulin; 7. α-lactalbumin.

4 – CONCLUSION

The high linear correlation found between caseins as well as the β-lactoglobulin plus α-lactalbumin percentages and the percentage of whey added to the raw milk attests that the SDS-PAGE system developed in this research, followed by densitometric analysis, is suitable for identifying and quantifying adulteration by additional whey in complex dairy mixtures.

5 – REFERENCES

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[2] *Departamento de Biologia Celular. Universidade de Brasília, Brasília-DF.Cep 70910.900. Fax 55 61 273 86 74*

[*] *A quem a correspondência deve ser enviada.*