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LINEARIZATION OF THE BRADFORD PROTEIN ASSAY TO APPLICATION IN COW MILK PROTEINS QUANTIFICATION BY UV-Vis SPECTROPHOTOMETRY METHOD

Linearização da metodologia de Bradford para aplicação na quantificação das proteínas totais do leite bovino por espectrofotometria no UV-Vis

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ABSTRACT

Reliable methods for determination and quantification of total protein in food are essential information to ensure quality and safety of food trade. The objective of this study was to evaluate the linearity of calibration curves obtained from different proteins (blood serum albumin-BSA, α-LA, β-LG, caseins (CN): α_s, β and κ-CAS) with the reagent of Bradford. Comercial UHT skimmed bovine milk was analyzed for the determination of total protein using the Bradford method by reading at 595 nm. The determination of the concentrations of total milk protein was achieved by linear regression. The Bradford method showed a high sensitivity for the determination of total proteins in bovine milk dilution 1:25 to values closer to those obtained by the Kjeldahl method. The results showed that the calibration curve of standard proteins β-CN and BSA obtained better linearity with less variation in the absorbance measurements for the determination of total protein of milk.

Keywords: bovine; skimmed milk; spectrophotometry; proteins quantification; β-casein; BSA.

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RESUMO

O desenvolvimento de métodos confiáveis para determinação e quantificação das proteínas totais nos alimentos é uma informação essencial para a garantia da qualidade e segurança dos alimentos comercializados. O objetivo deste trabalho foi avaliar a linearidade das diferentes curvas de calibração obtidas das proteínas (blood serum albumin-BSA, α-LA, β-LG, α_s-CAS, β-CAS e κ-CAS) com reagente de Bradford. Amostra de leite bovino desnatado UHT comercial foi analisada para a determinação das proteínas totais utilizando o método de Bradford com leitura em 595 nm. A determinação das concentrações das proteínas totais do leite foi alcançada pela equação de regressão linear. O método de Bradford mostrou alta sensibilidade para a determinação das proteínas totais do leite bovino na diluição 1:25 com valores mais próximo ao obtido pelo método Kjeldahl. Os resultados obtidos revelam que as curvas de calibração das proteínas padrão β-CN e da BSA mostraram melhor linearidade com menor variação nas medidas de absorbâncias para a determinação de proteínas totais do leite.

Palavras-chave: bovino; leite desnatado; espectrofotometria, quantificação de proteínas, beta-caseína ; BSA.

INTRODUCTION

The major bovine milk components concentration is estimated to contain proteins (3.25%), fats (3.9%), water (87%) and soluble solids (5.5%) which includes lactose, minerals and other substances in smaller amounts (WILLIAM, 2002). The milk proteins are distributed in caseins and whey proteins. Caseins comprise 80% of the total milk proteins and are essentially constituted by four individual molecules, the α_{s1}-, α_{s2}-, β-, and κ-casein. The whey proteins represent about 20% of the total milk protein distributed in β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA) and immunoglobulins (DALGLEISH; MORRIS, 1988).

The development of methods for determination and quantification of total protein in food is an important consideration in dairy research (DZIUBA et al, 2009). Kjeldahl is the official method for quantification of the total milk protein (HELRICH, 1990), however, this technique has been questioned because it is based on spectroscopic techniques to the quantification of samples total nitrogen which includes protein and non-protein nitrogen, besides being time-consuming and using corrosive reagents (DE KRUIF; HOLT, 2003). There are different ways to measure the total milk protein and methods based on spectrophotometry are commonly employed (LOWRY et al., 1951; GROVES et al., 1968). The ultraviolet spectrophotometry method has the advantage of not destroying the samples and to be very fast (PACE et al., 1995). One major limitation is that the results with complex samples may be imprecise when they are constituted by a mixture of substances (STOSCHECK, 1990).

The Coomassie Brilliant Blue (CBB), known as the Bradford method, is commonly used for protein determination in food (BRADFORD, 1976). This method is based on the electrostatic interaction between the dye, basic amino acids (arginine, lysine, histidine amino acid residues, and NH_2-terminal amino group) and aromatic amino acids residues. Van der Waals forces and hydrophobic interactions are also supposed

to be involved in the color formation. This technique is very reproducible, relatively fast, sensitive and specific (ZOR; SELINGER, 1996). When the protein binds to the red form of the reagent, it is converted to the blue form which is stable and non-protonated. Thus the method can provide a slight non-linearity in the Beer-Lambert law due to variation of the pH of the solution and the pattern of response due to overlapping of the spectra of two different types of reagent color (OWUSU-APENTEN, 2002; COMPTON; JONES, 1985; SPLITTGERBER; SOHL, 1989; TAL et al., 1985).

There are many studies for determination of protein comparing sensitivity, cost, robustness, and linearity of the different interference on the spectroscopic analysis. However it was not found studies comparing the linearity of the Bradford method standard curves with different proteins. Considering that milk is a complex solution and its composition has many different types of proteins with different conformations and structures, the aim of this study was to evaluate the linearity model using calibration curves obtained from different individuals proteins (α-, β- and κ-casein; α-LA, β-LG and BSA) to determine the concentration of total milk protein. The hypothesis was that these curves would present better reliability for quantification of total protein from bovine milk using the Bradford method with readings in the UV-Vis region of the spectra.

MATERIAL AND METHODS

Preparation of curves of individual proteins

The Bradford reagent solution (Bio-Rad Protein Assay, Hercules, California) was prepared according to manufacturer's instruction. The standard proteins: BSA, α-LA, β-LG, α-casein, β-casein and κ-casein (Sigma-Aldrich, St. Louis, MO, USA) were prepared in the following concentrations: 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 mg / mL using ultrapure water (Ultrapure Milli-Q water, Millipore Corp., Bedford, MA, USA). For standard procedure, 4 μL of each of them were added to 200 μL of Bradford reagent. After incubated for 5 minutes at room temperature, absorbance measurements were performed using "Protein Bradford" module at 595 nm in a spectrophotometer (ND-1000 NanoDrop Technologies, Wilmington, USA). The Bradford reagent was used as a blank. Standard curves were prepared for each standard protein and used to quantification of protein in milk samples.

Spectrophotometry determination of the total milk protein

Sample of commercial skimmed bovine milk was diluted (1:25, 1:50, 1:100) in ultrapure water (Ultrapure Milli-Q water, Millipore Corp., Bedford, MA, USA) and 4 μL of each dilution were added to 200 μL of Bradford reagent and subsequent vortex mixing. After 5 minutes at room temperature, absorbances were measured at 595 nm in a spectrophotometer ND-1000 NanoDrop as above.

Determination of Kjeldahl

The total protein content in milk was determined by the Kjeldahl method (HELRICH, 1990).

Statistical Analysis

The method of least squares (OLS) was used to calculate the linear regression curve for each protein concentration correlating to absorbance signal. The statistical significance of linear regressions obtained was evaluated by conducting an analysis of variance (ANOVA) for each regression (Microsoft Excel) and F test was applied. The samples were analyzed in triplicate.

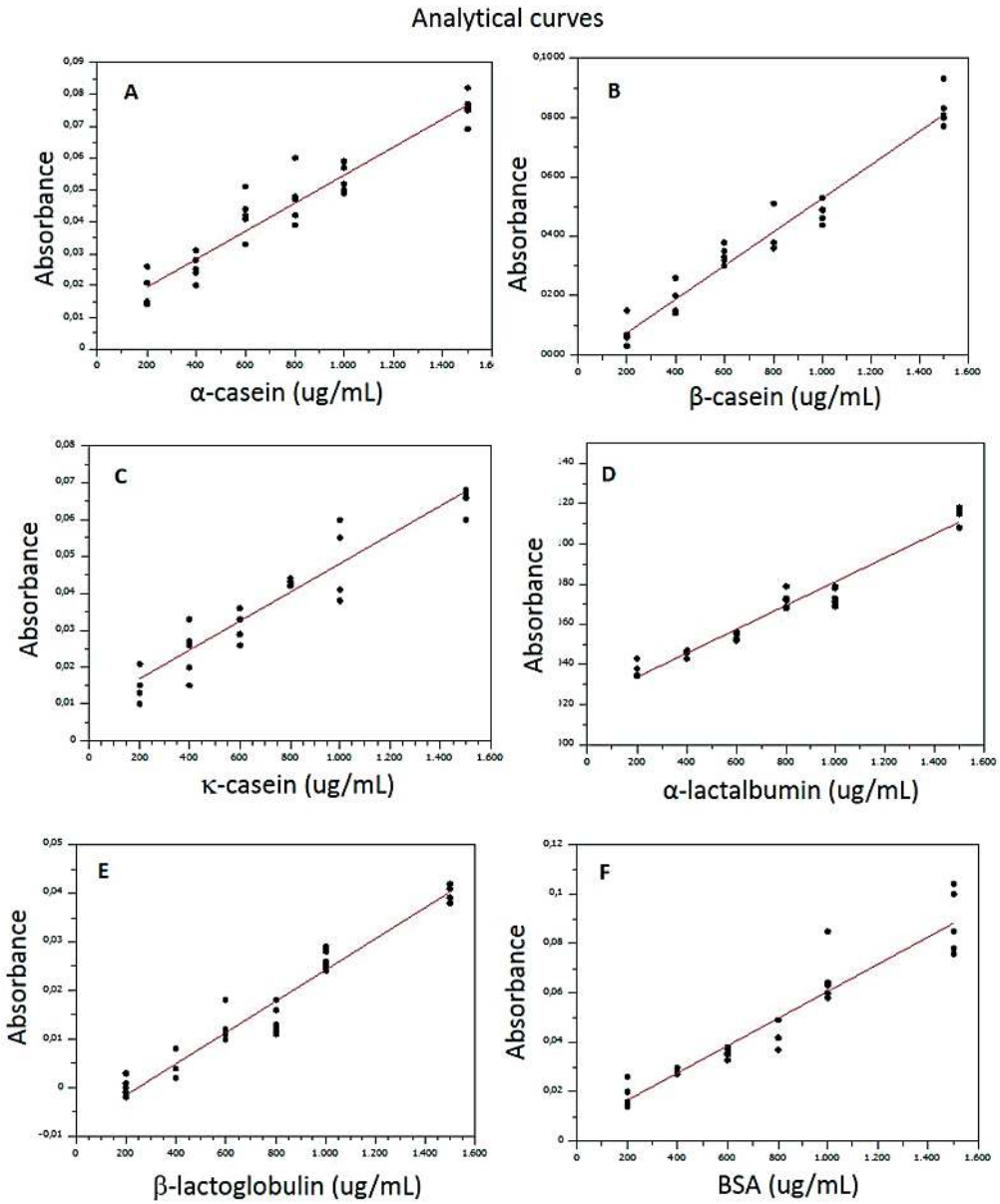


Figure 1 – Analytical curves for different protein fractions: A) α-casein; B) β-casein; C) κ-casein; D) α-LA; E) β-LG; F) BSA

RESULTS AND DISCUSSION

Figures 1A to 1F present the absorbance as a function of each protein concentrations and these graphs were used as standard curves. The determination of individual protein was done at 595 nm. The absorbance obtained for the proteins concentrations was correlated with the results of protein patterns as shown in standard curves of Figure 1 (A to F).

Results of variance analysis and the regression value were obtained for each parameter (Table 1). The significance of parameter F, given by the quotient of the mean square of the model and by the mean square residual indicates the existence of a significant linear relationship between the two variables. Some authors recommend that the significance of F value should be at least > 5 for the regression to have practical utility (AGTERDENBOS et al., 1981). The higher the F value the greater is the significance of the linear relationship between the two variables. Thus, the analysis of Table 1 provides the assertion that all the linear regression obtained in this study was significant, due to the high F values. The tabulated F ($F_{(0,05, 4, 24)}$) is 2.78, so the analysis of values of lack of fit of F contained in Table 1 leads to the conclusion that all of the standard curves, except for the α-LA and β-LG, were suitable for analysis because there was no evidence of lack of fit at 95% confidence.

Table 2 shows the coefficients of the linear regression equations, developed from the six concentrations tested with five replicates each. Each protein was analysed by standard statistical parameters of the equation and the corresponding R^2 were obtained.

Table 3 shows the quantitative results using the Bradford method and the total protein concentration estimated from bovine milk in three dilutions (1:25, 1:50, 1:100) through each calibration standard curve prepared from the following proteins: BSA, α-LA, β-LG, α_s-casein, β-casein and κ-casein. The quantification of total milk protein using the standard curve of α-LA in the protein dilutions 1:100, 1:50 and 1:25 underestimate the concentration found by the Kjeldahl method. When compared with the measurements obtained from the curves in dilutions 1:100, 1:50 and 1:25 of β-LG. α_s-casein and κ-casein concentrations were higher than those found by the Kjeldahl method. Quantification of total protein in cow's milk 1:25 dilution obtained with the standard curves of the β-casein and BSA showed a result closer to that obtained from the Kjeldahl method.

Even though the Bradford method can provide different estimates of the concentration of total protein in milk for each standard curve, a better linearity of the curves was found. Kamizake et al. (2003) revealed that BSA and casein can be used as standard proteins and that the Bradford method for protein determination by spectrophotometry on total milk showed a higher sensitivity as compared with others standard curves. A possible explanation for these observations is the fact that the milk protein fractions have a differentiated structure presenting each physical and chemical property due to different amino acid composition and the same provision in the formation of the protein (DE KRUIF; HOLT, 2003). Although the Bradford method is rapid, sensitive and subject to a much smaller number of interference than other methods (BRADFORD, 1976), it presents the same drawbacks as the change in absorptivity to different specific proteins due to difference in hydrophobicity or molecular weight, and susceptibility to suffer interference in the presence of lipids and sodium and potassium chlorides (SHIBA et al., 1985; GOREN; LI, 1986; WIMSATT; LOTT, 1987). To circumvent the problems in specific absorptivity of the different proteins, some authors

Table 1 – Regression analyses of analytical curves for different protein fractions

Source	Sum of Squares	DF	Mean Square
		αs-casein	
Regression	1.0388E^{-02}	1	1.0388E^{-02}
Residual	9.6146E^{-04}	28	3.4338E^{-05}
Lack of Fit	1.8081E^{-04}	4	4.5202E^{-05}
Pure Error	7.6800E^{-04}	24	3.2000E^{-05}
F of significance		302.54	
F of lack of fit		1.41	
		β-casein	
Regression	1.6996E^{-02}	1	1.6996E^{-02}
Residual	7.6108E^{-04}	28	2.7181E^{-05}
Lack of Fit	1.9502E^{-04}	4	4.8756E^{-05}
Pure Error	5.9700E^{-04}	24	2.4875E^{-05}
F of significance		625.29	
F of lack of fit		1.96	
		κ-casein	
Regression	8.1890E^{-03}	1	8.1890E^{-03}
Residual	8.4364E^{-04}	28	3.0130E^{-05}
Lack of Fit	8.4925E^{-05}	4	2.1231E^{-05}
Pure Error	7.7200E^{-04}	24	3.2167E^{-05}
F of significance		271.79	
F of lack of fit		0.66	
		α-LA	
Regression	1.8956E^{-02}	1	1.8956E^{-02}
Residual	8.0064E^{-04}	28	2.8594E^{-05}
Lack of Fit	4.9440E^{-04}	4	1.2360E^{-04}
Pure Error	2.9400E^{-04}	24	1.2250E^{-05}
F of significance		662.93	
F of lack of fit		10.09	
		β-LG	
Regression	5.5040E^{-03}	1	5.5040E^{-03}
Residual	2.7347E^{-04}	28	9.7667E^{-06}
Lack of Fit	1.0453E^{-04}	4	2.6133E^{-05}
Pure Error	1.5400E^{-04}	24	6.4167E^{-06}
F of significance		563.55	
F of lack of fit		4.07	
		BSA	
Regression	1.6400E^{-02}	1	1.6400E^{-02}
Residual	1.8289E^{-03}	28	6.5317E^{-05}
Lack of Fit	4.1942E^{-04}	4	1.0486E^{-04}
Pure Error	1.3850E^{-03}	24	5.7708E^{-05}
F of significance		251.08	
F of lack of fit		1.82	

suggest increasing the concentration of dye (READ; NORTHCOTE, 1981), increasing the solubility of protein (FRIEDENAUER; BERLET, 1989) or heating the sample with urea or 2-mercaptoethanol (GOTHAN et al., 1988). However, the change of these variables depends on the molecular weight of each protein.

Considering the hydrophobicity of the proteins tested, BSA is a protein that has the lowest value followed by the α_s-casein = α-LA < β-LG = κ-casein < β-casein. With regard to molecular weight and number of amino acid residues, the α-LA is the smallest protein with less residues available for interaction followed by β-LG < κ-casein < α_s-casein < β-casein < BSA (WALSTRA; JENNESS, 1984). According to the hydrophobicity there was not a correlation since the best results for the quantification of total protein were obtained by BSA and β-casein. However there may be a correlation with the size of the protein and the amount of amino acid residues, since the less satisfactory results were obtained for measurement of the proteins of lower molecular weights (α-LA. β-LG and κ-casein) whereas, better results were obtained with higher molecular weight proteins (BSA and β-casein). These results may be explained due to the number of amino acid residues that interact with the colorant CBB. The higher the molecular weight observed in proteins tested the greater the amount of amino acid residues available for interaction with the Bradford reagent.

Table 2 – Coefficients analytical obtained through linear regression of the calibration curves of the standards protein: α_s-casein, β-casein, κ-casein, α-LA, β-LG e BSA

Regression	Proteins					
	αs-casein	β-casein	k-casein	α-LA	β-LG	BSA
A	$4.3963E^{-05}$	$5.6233E^{-05}$	$3.9033E^{-05}$	$5.9386E^{-05}$	$3.2000E^{-05}$	$5.5237E^{-05}$
SE_a	$2.5275E^{-06}$	$2.2488E^{-06}$	$2.3676E^{-06}$	$2.3065E^{-06}$	$1.3480E^{-06}$	$3.4860E^{-06}$
B	$1.0761E^{-02}$	$-3.6078E^{-03}$	$9.0589E^{-03}$	$2.1794E^{-02}$	$7.8667E^{-03}$	$5.3721E^{-03}$
SE_b	$2.1767E^{-03}$	$1.9366E^{-03}$	$2.0390E^{-03}$	$1.9863E^{-03}$	$1.1609E^{-03}$	$3.0021E^{-03}$
R^2	0.9153	0.9571	0.9066	0.9594	0.9527	0.8997

All curves consisted of six points with five replicates each. SE_A = Standard error of the coefficient of the straight and SE_b = standard error of the coefficient b of the line.

Table 3 – Concentration of total protein in a sample of bovine milk obtained from the calibration curve of standard protein by the Bradford method

Bovine Milk Diluted	Concentration (mg/mL)					
	α-LA	β-LG	α_s-casein	β-casein	k-casein	BSA
1:100	12.79 ± 2.83	147.09 ± 5.59	65.83 ± 12.22	58.74 ± 3.42	59.33 ± 16.82	46.71 ± 4.68
1:50	26.76 ± 1.08	94.17 ± 3.05	47.49 ± 7.58	47.52 ± 2.99	60.18 ± 4.10	42.70 ± 3.54
1:25	23.48 ± 2.29	63.65 ± 4.87	39.81 ± 0.65	30.88 ± 1.32	54.83 ± 1.90	27.95 ± 3.34

Total Protein = 30.3 ± 2.0 $mgmL^{-1}$ obtained by KJELDAHL method.

CONCLUSIONS

The calibration curves were assembled from standard proteins BSA and β-CN using the Bradford method. The correlation coefficients were greater than 0.9 and statistical evidence obtained by analysis of variance, exhibited a high degree of linearity. It may be used to estimate missing values accurately using curves that showed the best results for quantification of total protein in milk.

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