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CHEMICAL, STRUCTURAL AND PROTEOMIC PROFILE OF BUFFALO MILK POWDER PRODUCED IN MINI SPRAY DRYER

PERFIL QUÍMICO, ESTRUCTURAL Y PROTEÓMICO DE LECHE EN POLVO DEL BÚFALO PRODUCIDO EN MINI SECADOR POR ASPERSIÓN

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Abstract
The objective of this work was to obtain buffalo milk powder through the process of drying in mini spray dryer, and evaluate its physical, chemical, structural and proteomic features during storage. The buffalo milk was subjected to drying by atomization, air temperature of 140°C inlet. The samples were evaluated at times 0, 30, 60, 90, 120 and 150 days of storage. The moisture content, fat, protein, ash, density, acidity, solubility index, burning particles were analysed, along with structural analysis by Scanning Electron Microscopy (SEM) and proteomic study (SDS-PAGE). The production of milk powder from the set standards produced a buffalo milk powder with satisfactory results of physical and chemical compositions. In the SEM analysis was possible to observe an accumulation of milk powder particles with withered and wrinkled structures. Variations in the intensity of the bands of protein fractions were observed by electrophoresis, but no significant changes. The production secured buffalo milk powder with good quality characteristics, without any undesirable changes in the product during the storage time.
Keywords: microscopy, electrophoresis, composition, atomization, storage.

Resumen
El objetivo de este estudio fue obtener leche de búfala en polvo por el proceso de secado en un mini secador por aspersión y evaluar sus características físicas, químicas, estructurales y proteómicas durante el almacenamiento. La leche de búfala se sometió a secado por atomización, con la temperatura del aire de entrada de 140°C. Las muestras se evaluaron en los tiempos 0, 30, 60, 90, 120 y 150 días de almacenamiento. El contenido de humedad, grasa, proteína, cenizas, densidad, acidez, índice de solubilidad, partículas quemadas se analizaron, junto con el análisis estructural mediante Microscopía Electrónica de Barrido (MEB) y el estudio proteómico (SDS-PAGE). La producción de leche de búfala en polvo cumpliendo con los estándares establecidos aseguró una composición física y química satisfactoria. En el análisis de MEB fue posible observar una acumulación de partículas de leche en polvo con estructuras secas y arrugadas. Las variaciones en la intensidad de las bandas de las fracciones de proteína fueron observadas por electroforesis, pero sin cambios significativos. La producción de leche de búfala en polvo asegurado de las características de buena calidad, sin que ocurrieran cambios indeseados en el producto durante el tiempo de almacenamiento.
Palabras clave: microscopia, electroforesis, composición, atomización, almacenamiento.

1 Introduction

The buffalo dairy production has shown strong growth in recent decades, as evidenced by the increase in population of these animals in several properties and regions due to features like hardiness, disease resistance and adaptability to the climate (FAO, 2013). About 90 million tons of buffalo milk were produced in 2009, representing 13% of world milk production, behind only the production of cow's milk has about

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83.5% of production (IDF, 2010; Guimarães *et al*., 2013).

Despite being a species that expressively contributes to global production of milk, the reproductive seasonality (concentration of deliveries in a certain time of year) is a factor that interferes negatively in the production, supply and marketing of its dairy products, resulting in losses driven to the sector (Campanile *et al*., 2007). The solution to such problem has been sought by using techniques to change the reproductive seasonality, as well as processing techniques of fluid milk, curd freezing or even in the production of milk powder.

The buffalo milk has a high content of main components, having on average 7.73% fat, 4.23% protein and 4.90% lactose (Macedo *et al*., 2001; Rosati and Van Vleck, 2002; Tonhati *et al*., 2000). A higher value of total solids is a great advantage for the milk processing industry, for an increase of 0.5% in the solid content corresponds to a 11% increase in the yield of milk powder (CFIS, 2005).

In the drying technique of spray drying, the powder produced meet high quality standards regarding the particle size of the product, final moisture content, uniformity, density and shape, and these features can be altered by modifications in process parameters (air temperature drying and processing time). Drying basically consists of three main steps: 1) The fluid is dispersed as droplets, producing a large surface area; 2) the droplets temperature increases due to contact with a steam of heathed air; 3) the solvent evaporates and the solid particles are formed (Oliveira and Petrovick, 2010). The mentioned process can be used for dehydration of various products such as juices and extracts (Guadarrama-Lezama *et al*., 2014; Gallo *et al*., 2015; Chavez-Rodríguez *et al*., 2016).

Electron microscopy is a tool that makes it possible to observe the milk powder surface ensuring the display of these changes that may occur and compromise the quality of milk. The type most commonly used in the literature is the Scanning Electron Microscope (SEM), one of the most versatile instruments available for the observation and analysis of microstructures of solid objects (Dedavid *et al*., 2007; Lapčík *et al*., 2015). The proteomic study is also among the other methods used to assess the characteristics of milk powder. Among these studies, electrophoresis is the most widely used to assess the protein fractions present in a sample (Silva, 2009).

Given the above, is aimed with this work to obtain whole milk powder from buffalo milk by drying in mini spray dryer and evaluate its physical, chemical, structural and proteomic during storage.

2 Materials and methods

2.1 Production of milk powder

The Murrah buffalo milk used for the production of milk powder was provided by a dairy during the months of October and November. Before drying, the milk was analyzed for acidity, density, fat, total solids (TS), nonfat dry extract (NDE), protein and cryoscopic index, as Brasil (2006). The slow pasteurization was performed (temperature of about 65°C for 30 min) and the milk was subjected to spray drying in a mini spray dryer MSD 3.0 (LABMAQ Brazil LTDA) co-current cycle, the air temperature at the inlet and outlet the dryer of 140°C and 80°C respectively, nozzle 1.0 mm thick with and air flow in the nozzle of 40 L/min, drying air flow of 3 m^3/min and peristaltic pump flow 1.07 L/h. The production of milk powder was carried out in three repetitions and 10 liters of buffalo milk were processed for each. The samples obtained were packed in a vacuum sealer model BS320 (Brazil R.BAIÃO LTDA), using laminated films for packaging (Incoplast) and stored for 30, 60, 90, 120 and 150 days at room temperature of about 27°C.

2.2 Yield of Milk powder

The yield of the process of obtaining milk powder was calculated from the following equation:

$$Yield\% = \frac{m_f}{m_i} \times 100 \quad (1)$$

In which: m_i = Mass of fluid milk (Kg); m_f = Mass of milk powder (Kg).

2.3 Analysis of the composition of milk powder

The samples were evaluated, in triplicate, after 0 (T0), 30 (T30), 60 (T60), 90 (T90), 120 (T120) and 150 (T150) days of storage, according to the methodology proposed by Brasil (2006). The moisture content was determined by infrared measurer model IV2000 (GEHAKA Brazil LTDA); the fat content determined using the Gerber method with Teichert butyrometer; the acidity was determined by titration with a sodium hydroxide N/9 standard solution,

using a 1% alcoholic phenolphthalein solution as an indicator; the ash content by means of the gravimetric method, using muffle LS20 model (FORLABO Brazil LTDA), 550ºC until constant weight; the protein analyses were performed using the micro Kjeldahl method; the solubility rate was determined by diluting and centrifuging the sample at 2012.4xg in an excelsa model 206 (FANEM and MERSE Brazil LTDA) centrifugal; the amount of burnt particles was determined by comparison with the ADPI graph: Burning Particles Standards for Dehydrated Milk.

2.4 Electrophoretic analysis SDS-PAGE

Eppendorf tubes were used to weight about 0.0020g of each powdered milk sample, which were then diluted in 800 μL Tris-HCl buffer solution (pH = 6.8) in the presence of 0.1% SDS and 5% of β-mercaptoethanol. Then, they were heated at 100oC for 3 minutes and added to 200 μL of the solution containing 10% glycerol and 0.01% bromophenol blue. Finally, they were frozen until the time of electrophoretic run (Egito *et al*., 2006).

2.4.1 Electrophoretic analysis SDS-PAGE milk powder

Electrophoretic characterization of samples from buffalo milk powder was performed according to the methodology recommended by Egito *et al*. (2006), by the method of polyacrylamide gel under denaturing conditions, in other words, with the addition of sodium dodecyl sulfate (SDS), in vertical electrophoresis apparatus from Apelex brand. SDS-PAGE technique was performed by separation gels of 15% polyacrylamide in 380 mmol/L Tris-HCl buffer, pH 8.8, containing 0.1% SDS gels and concentration/stacking 5% polyacrylamide in 125 mmol/L Tris-HCl buffer, pH 6.8.

The preparation of the separation gel and the concentration/stacking gel followed the methodology proposed by Egito *et al*. (2006).

Electrophoretic characterization of each sample was performed in triplicate. The migration of the protein lasted approximately 1 hour and 30 minutes. It was performed with a constant electric current of 250 V, amperage of 30 mA and controlled temperature between 4 and 8°C.

After the electrophoretic run the gel was removed from the glass plates and stained with silver nitrate according to protocol adapted Bloom *et al*. (1987).

All electrophoretic runs in the gels were registered by camera and its images stored for later identification of the bands.

2.5 Densitometry of protein fractions

For densitometric analysis, the software used was GelAnalyzer 2010a (G. A., 2015) for the quantitative evaluation of each protein band, separated by electrophoretic technique and to estimate the molecular mass for each band present in the gels of the samples.

2.6 Scanning electron microscopy

For the analysis of Scanning Electron Microscopy (SEM), samples of milk powder were mounted in the holder ("stub") with the use of double sided adhesive carbon guides and then coated with gold/palladium (metallization) to increase the conductivity of the sample surface. The metallization was performed on equipment SCD-050 model (Bal-Tec). After the metallization, readings of samples in the SEM model Quanta 250 (FEI Company) were made.

2.7 Experimental design

A completely randomized design-CRD to check the effect of the milk powder storage time on the composition parameters (fat, acidity, moisture, ash, solubility index, density and protein) performing analysis of variance (ANOVA) data and regression at significance level of 5% (α = 0.05), using the SAS software (Statistical Analysis System), version 9.0 (SAS).

3 Results and discussion

3.1 Analysis of buffalo milk in natura

The buffalo milk composition is essential to obtain a product with high quality characteristics. The buffalo milk used to carry out the work presented average expected results for the species' milk, 5.3% fat, 3.4% protein, 16ºD (degrees Dornic) acidity, 1.0315 g/mL density, 14.36% of total solids (TS), 9.03% nonfat dry extract (NDE) and -0.543ºH (Hortvet degrees) cryoscopic index. Close values were found by Araújo *et al*. (2011) when evaluating the influence of the seasons on the buffalo milk composition kept in cooling tank, and Costa *et al*. (2014) when assessing the seasonality and variation in the quality of buffalo

milk. It is important to note that there is no specific national legislation for buffalo milk that standardize its identity nor its quality. To date, only one state (São Paulo) has legislation for some identity and quality parameters of buffalo milk, by setting minimum values for fat 4.5%, acidity 14-23°D, nonfat dry extract (NDE) minimum of 8.57%, density at 15°C of 1.028 to 1.034 g/mL and cryoscopic index between -0.520 to -0.570ºH (São Paulo, 1994). So, considering that legislation, the buffalo milk showed adequate physicochemical characteristics, with results within established standards.

3.2 Obtaining the milk powder buffalo and its quality parameters

In the milk drying process, initially tests were conducted to determine the optimum parameters of the equipment, in order to obtain the highest yields of milk after the drying process. Thus, using these parameters, buffalo milk powder had a yield of 10% (volume of milk spent to obtain 1 kg of milk powdered), the maximum value in yield obtained in this work. Such value, when compared to the solids content in milk, could have been higher, but the equipment limitations such as the use of low volume of milk per hour and the concentration step which has not been performed, can be associated with losses in the drying process. According to Early (2000), to obtain a better quality product is necessary to remove from the milk as much water as possible before proceeding with spray drying.

It is also worth noting the difficulty in atomizing step because of the large presence of fat in buffalo milk, which caused the adhesion of the particles of milk on the walls of the spray dryer, and also it has been held in a continuous process of feeding. According to Schuck (2002), the physical and functional characteristics (powder density, hygroscopicity, thermal stability, solubility, dispersibility, degree of agglomeration, wettability and particle size) depend on both the parameters obtained during the drying process (equipment type, type of nozzle, flow of air and liquid, air pressure, drying air temperature) and the chracteristics of the concentrate to be dehydrated (composition, physical and chemical characteristics, viscosity, thermal stability and water availability). The control of these parameters will indicate whether the milk to be dehydrated presents high quality features, that ensure long lifespan to the final product (Whetstine and Drake, 2009).

The composition characteristics of buffalo milk powder right after the drying process and at storage times of 30, 60, 90, 120 and 150 days were observed. Among the equations of regression adjusted for the studied variables, it was posible to obtain a satisfactory linear regression ($p < 0.05$) only for humidity (y= 3.6382x + 0.0048), in which y = humidity and x = storage time, with the coeficient $R^2 = 0.52$, indicating that there was a significant increase in the moisture content of milk powder during storage. The other parameters analyzed were not significantly affected ($p > 0.05$) by the time of storage (Table 1).

The moisture content of milk powder showed results which ranged from 3.63% (time 0) to 4.2% (time for 150 days storage) at room temperature. According to Krey *et al.* (2009), the critical moisture for whole milk powder is 5.0%, from this percentage the product quickly loses its flavor and undesirable changes such as microbial deterioration and agglomeration begin, thus affecting their solubility and other physical properties.

Table 1. Avarege value of the buffalo milk powder parameters related to the time of storage.

Parameters	Storage Time (days)						Average
	T 0	T 30	T 60	T 90	T 120	T 150	values
Fat matter (%)	33.67	33.77	34.15	34.67	34.25	34.02	34.08
Moisture (%)	3.63	3.72	3.77	4.43	4.25	4.20	4.00
Solubility (mL)	0.21	0.20	0.20	0.20	0.20	0.20	0.20
Density (g/mL)	1.0420	1.0435	1.0423	1.0429	1.0420	1.0421	1.0424
Acidity (ºD)	11	10	11	11	11	11	10.83
Ashes (%)	4.84	4.73	4.77	4.79	4.80	4.88	4.80
Protein (%)	21.35	21.40	22.02	21.33	21.48	21.52	21.52
Burned Particles	Disc B	Disc B	Disc B	Disc B	Disc B	Disc B	Disc B

The acidity was expressed in Dornic degrees; ESD=fat free dry extract.

According to Brasil (1997), the optimal range for moisture in whole milk powder (specific legislation to cow's milk powder) is at most 3.5%, and partially skimmed and skimmed is a maximum of 4%. According to Medeiros *et al*. (2014), in much lower values for moisture, fat becomes more susceptible to oxidation reactions because the lipids lose the protective monomolecular layer of water, and in higher values the milk powder is susceptible to the reaction of lactose crystallization, the development of odors, dimming, protein insolubility, increased acidity, carbon dioxide production, among others.

According to Alves *et al*. (2008), during the storage period, the increase in the milk powder's moisture happens due to steam transfer through the package, thanks to permeation through the material or the air flow through flaws in the thermosealing. The same may have occurred with the buffalo milk powder produced, as moisture increased with the storage time, indicating possible problems related to the packaging of the product. Despite the indicated fact, the milk powder obtained has good quality characteristics considering the parameters.

3.3 Analysis of scanning electron microscopy

The particles of buffalo milk powder displayed wilted and wrinkled appearance (Figure 1), usually formed due to the temperature used in the drying process milk which was 140°C and, according to Kim *et al*. (2009) and Hammes *et al*. (2015), milk drying performed at temperatures around it (between 140 and 145°C) promotes this type of structure. Kim *et al*. (2009) evaluated the effect of temperature (145 and 205°C) in the particle of milk powder by scanning electron microscopy, and found that the 205°C drying temperature accelerated the droplet drying rate, promoting rapid formation of a crust on powder surface, which caused the milk particle present a spherical and smooth appearance. However, when the drying temperature was lower (145°C), the particle remained moist and flexible for a longer time, emptying and wrinkling as it cooled, with wilted appearance.

Hammes *et al*. (2015) studied the influence of the addition of soy lecithin on the wettability of buffalo milk powder obtained by spray-drying at 140°C, and observed wilted appearance and furrowed in particles, expected features from powders produced in temperatures considered low and similar to those found in this work.

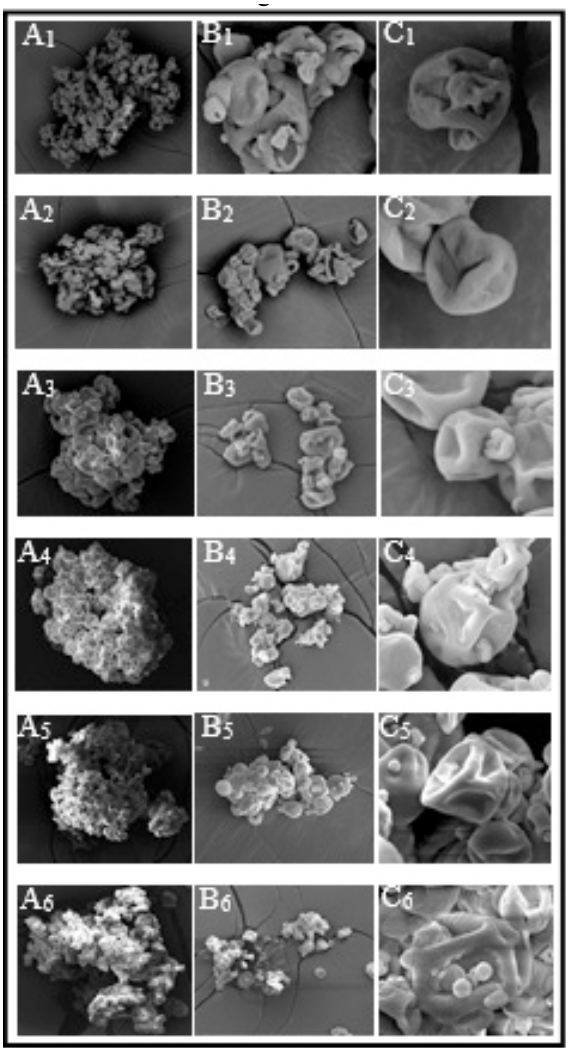

Fig. 1. Photomicrographs obtained through SEM of the buffalo milk powder samples. A = magnification 1000x; B = magnification 3000x; C = magnification 20000x. Storage time of the analysis 1= time 0; 2= time 30 days; 3= time 60 days; 4= time 90 days; 5= time 120 days e 6= time 150 days.

In the drying temperature, the droplet size produced by the atomizer nozzle is also responsible for the smooth or wrinkled appearance of powders, since smaller droplets at higher temperatures allow faster evaporation of water, due to their larger surface area, thus inducing the formation of smoother surfaces. On the other hand, at lower temperatures with larger droplets, the evporation is slower and there is a crust formation on the particle surface, a condition that allows the particle empty itself and wither (Nijdam and Langrish, 2006; Kim *et al*., 2009).

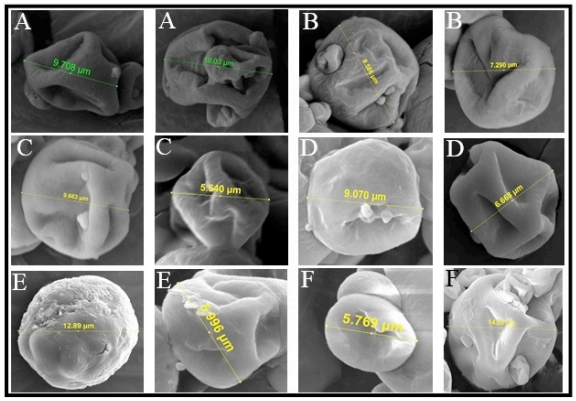

Fig. 2. Photomicrographs obtained through SEM containing the particle size of the buffalo milk powder. A, B, C, D, E and F correspond respectively to 0, 30, 60, 90, 120 e 150 days of storage.

That more withered and wrinkled structure observed in the sample obtained from buffalo milk powder suffered minor change during storage. By viewing the SEM images (Figure 1) obtained at time 0, one can realize the increased presence of shriveled particles and, with the increase of storage time, more spherical, smooth particles, and higher agglomeration thereof. The difference in the structure of the particles may be related to moisture absorption occurred with increase in storage time.

The buffalo milk powder produced displayed agglomerated particles from its obtention (Figure 1). According to Hardas *et al*. (2000) and Kim *et al*. (2002), the agglomeration can be related to wrinkled and withered structure, and a migration of fat to the surface, compromising the solubility properties (wettability, dispersibility and flowability), and it may promote an agglomeration of the particles, oxidation and the development of rancidity in the powder.

Foster *et al*. (2009) indicate the lactose as the main causes for agglomeration of powder milk, for the milk powder consists of spherical particles of amorphous lactose containing incorporated casein micelles and fat globules. The structure of the powder particles is changed when the amorphous lactose undergoes crystallization. When it happens, protein, mineral and fat are excluded from the ordered structure of the lactose crystals and expelled to the surface of the powder, thus causing the increase of the free fat.

On the other hand, agglomeration of the particles promotes advantages related to the solubility of milk powder. Some processes are performed in order to obtain agglomerated particles of milk powder, as the fluidizing and added lecithin, improving the properties

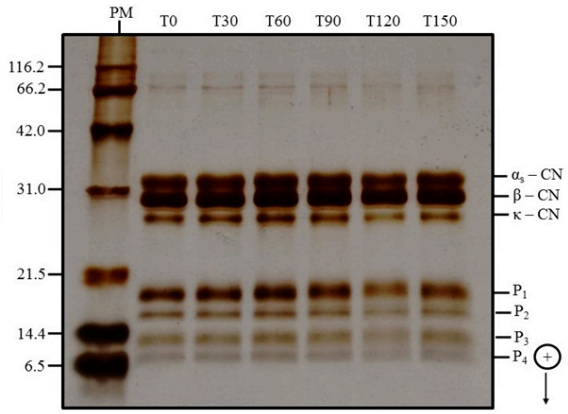

Fig. 3. Eletrophoretic profile (SDS-PAGE) of the buffalo milk powder samples. Uncovered gel with Coomassie Blue (a); gel stained with silver nitrate (b). 20 μg of protein were deposited in each pool. PM: Molar mass marker; T: samples in times of analysis (0, 30, 60, 90, 120 and 150 days of storage); αs-CN: αs-casein; β-CN: β-casein; k-CN: k-casein; P_1, P_2, P_3, P_4: peptides.

of wettability and flowability of milk powder. Due to agglomeration, buffalo milk powder showed good solubility, even if not used any of these methods. Thus, this agglomeration may have occured as a result of the factors mentioned above, such as temperature of the particles in the drying process, fat free preset in the surface of the milk powder and possible process of crystallization of lactose. The images obtained by SEM (Figure 2) show that the milk powder had particles of different sizes that varied mostly between 5 and 11 μm in diameter.

3.4 Electrophoretic analysis

From the electrophoresis, with the separation and identification of proteins present in buffalo milk powder, it was possible to observe the difference between the casein fractions during the storage time. The electrophoretic mobility of the casein fractions on SDS-PAGE gel from the buffalo milk powder (Figure 3) showed the migration of three upper bands with greatest intensity, corresponding to αs-casein, β-casein and κ-casein. The presence of other bands is also displayed, probably peptides formed through the process of degradation fractions.

It was observed an abscence of a sharp degradation of proteins during the storage time, even with moisture absorption occurring in the last analysis periods. It was possible to observe that the bands relating to α and κ-casein suffered a minor degradation denoted by

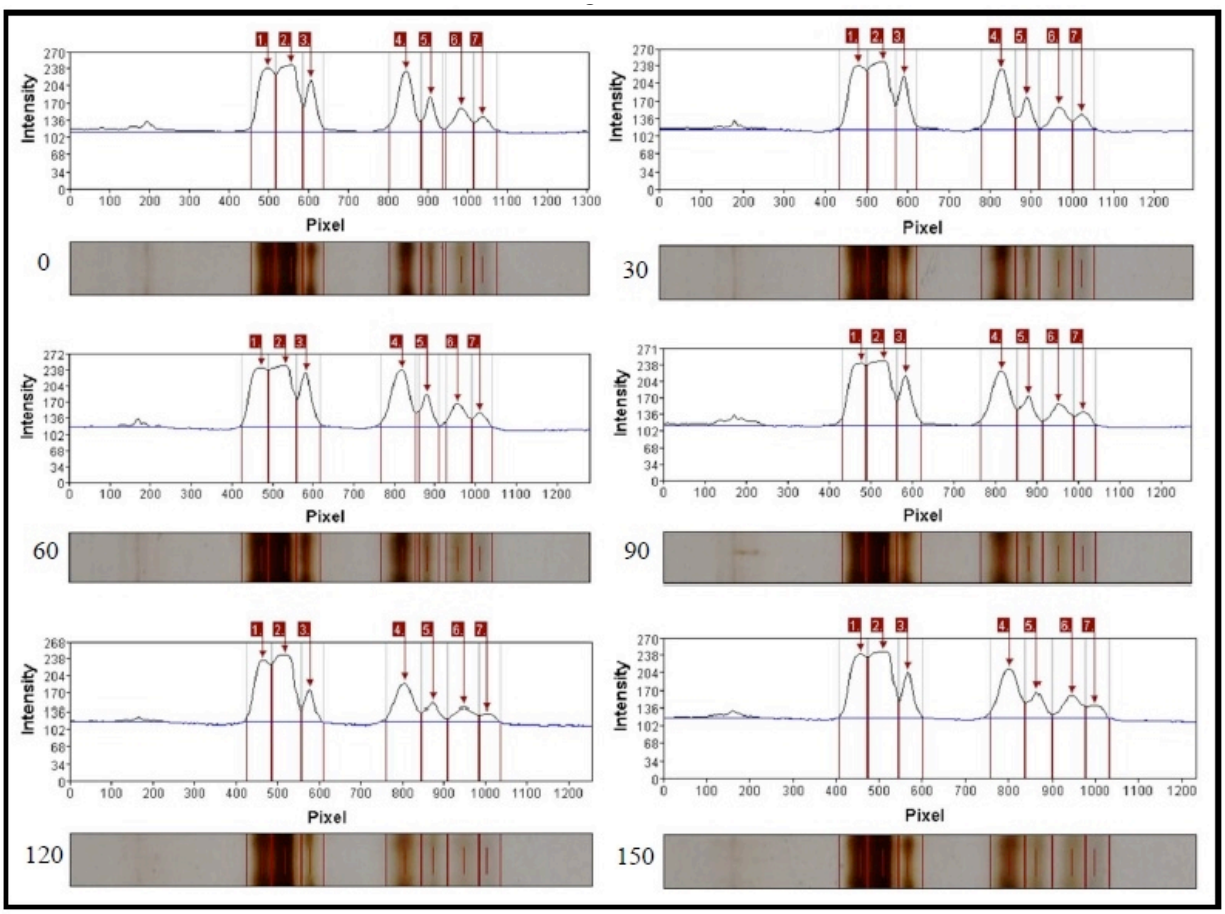

Fig. 4. Densitometry of the results obtained through electrophoresis SDS-PAGE, gel stained with silver nitrate. (1st peak) α-casein; (2nd peak) β-casein; (3rd peak) κ-casein; (4th, 5th, 6th e 7th peaks) peptides formed by the degradation of the protein fractions; storage times (T0, T30, T60, T90, T120 e T150).

a small reduction in color intensity of these bands from the time of 120 days of storage. The drying process, especially by spray drying, can induce changes in proteins (protein-protein interaction and degradation) that influence the hydrophobicity, solubility and denaturation of them (Kurozawa *et al*., 2009). The storage time and temperature lead to polymerization of the protein and a decrease in solubility, and the temperature and relative humidity are predominant factors involved in this process (Gaiani *et al*., 2009; Scheidegger *et al*., 2013).

With the results of electrophoresis, the quantification of bands from each fraction identified in silver nitrate stained gels was performed, by densitometric analysis (Figure 4), identifying the molecular weight of each protein fraction (α, β and κ-casein) and peptides formed.

In the first peak identified by densitometry, corresponding to α-casein, it is possible to observe a reduction in size in the time of 120 days of storage. The reduction indicates that in this period there was a degradation of the indicated protein fraction. In the second and third peaks corresponding to β-casein and κ-casein, it is also possible to notice the reduction in size of the peak formed during the storage time.

Using the peaks found in densitometry, areas identified for each fraction (Table 2) were obtained, and a regression analysis to verify these statistics difference between fractions of time during storage was performed. It was not possible to adjust any regression equation for the fractions, for there was no significant reduction to the period of 150 days of storage. By analyzing the electrophoresis gel is possible to observe a degradation due to the reduction in intensity of bands, but it was statistically not significant, therefore it would be necessary to analyze this milk for a longer period to detect significant changes in protein degradation.

Table 2. Areas of peaks values, found through densitometry and molecular weight of each protein fraction identified in electrophoresis SDS-PAGE.

Protein fractions	Areas of the fractions of the get stained with silver nitrate during the storage time (days)						MW (kDa)
	T0	T30	T60	T90	T120	T150	
alfa	5900	5780	5358	5505	4826	4845	33
beta	8324	8282	8999	9168	8200	7836	29
kappa	3614	3009	3619	3434	2045	2336	27
pep 1	5217	4351	5178	5341	3564	3418	15
pep 2	2089	1490	1652	1936	1219	1178	12
pep 3	2056	1473	1815	1883	1228	1327	9
pep 4	1101	786	1052	1047	642	637	7

MW= Molecular weight. kDa= kiloDalton.

It was possible to obtain, by densitometry, the molecular weight of protein, determined by the mobility of each fraction in the gel relative to standard molecular weight. Larger proteins such as α_{s1}-casein have greater difficulty with mobility in the gel due to the size of formed pores, with highest molecular weight, identified as the first band formed in gel band corresponding to 33 kDa, value close to the molecular weight standard 31 kDa. Since the peptide 4, the band identified at the bottom of the gel showed the lowest molecular weight of 7 kDa (Table 2).

Conclusions

It was possible to obtain whole milk powder buffalo by mini spray dryer drying, providing a product with good quality characteristics, but with a significant increase in moisture content during storage.

The particles of buffalo milk powder were agglomerated, presented with wilted and shriveled structures, with small changes in its structure during the storage time.

The proteomic study revealed that the protein fractions showed no difference throughout the storage period, no significant degradation occurring fractions.

Nomenclature

MW	molecular weight, KDa
NDE	nonfat dry extract, %
TS	total solids, %

Greek symbols

α	protein fraction, α-casein
β	protein fraction, β-casein
κ	protein fraction, κ-casein

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