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Ultrafiltration isolation and characterization of caseinomacropeptide

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ABSTRACT

Biologically active peptides from milk whey, such as caseinomacropeptide (CMP), are of great interest for food technology and for the development of new functional foods. CMP is the soluble derivative of k-casein, generated by the cleavage of the enzyme chymosin enzyme in manufacture of cheese. The objective of this work was to isolate the CMP from whey by ultrafiltration, to determine the physical-chemical composition and to characterize it by high performance liquid chromatography (HPLC). The method applied was the ultrafiltration membrane 50, 30 and 5 kDa. The fraction of CMP isolated consisted of 73.7% protein content, 12% lactose and 2.5% sialic acid. The end fractions of the isolation presented chromatographic profiles compatible with CMP. Process parameters and characteristics of the raw material can change the efficiency of CMP isolation. Therefore, it is important to optimize all process step parameters for efficient isolation.

KEYWORDS: whey protein; caseinomacropeptide; ultrafiltration.

INTRODUCTION

Each year, the market for functional structural, and nutritional ingredients is growing, resulting in an increasing demand for separation of high-purity functional proteins and, especially, for the recovery of whey peptides. Mainly due to the large amount of whey produced during the cheese making process, it makes this by-product a valuable material to produce various ingredients for use in food products (Kilic-Akyilmaz *et al*., 2018). Caseinomacropeptide (CMP), the third most abundant protein/peptide in whey proteins produced from cheese, is a heterogeneous group of polypeptides formed by the cleavage of chymosin from kappa-casein (Delfour *et al.,* 1965). Approximately 50 % of bovine CMP is glycosylated, with sialic acid being the terminal residue in most carbohydrate chains (Saito & Itoh, 1992). The glycolyzed fraction (gCMP) has pI 3.15 and the non-glycolated half (aCMP) pI 4.15 (Kreuβ, Strixner, & Kulozik, 2009). Depending on the degree of glycosylation and phosphorylation, the isoelectric point ranges from 3.5 to 3.8 has also been reported (Lieske *et al.*, 2004). It is a randomly rolled peptide, without helix or sheet structure, with high conformational flexibility (Ono, Yada, Ytani, & Nakai, 1987; Thoma-Worringer *et al.*, 2006). The molar mass of CMP is reported to be 6-7 kDa for non-glycosylated and 9-11 kDa for glycosylated forms (Mollé & Léonil, 1995).

CMP has received considerable attention due to its health-promoting effects, such as body weight reducing activity, inhibition of bacterial and viral adhesion, regulation of blood circulation (Thoma-Worringer *et al*., 2006) and applications in diets for phenylketonuric patients due to the lack of phenylalanine in its sequence (Mollé & Léonil, 2005). Characteristics of heat stability, high solubility, with a soluble negative charge even at low pH values (Thoma *et al*., 2006; Abd El-Salam, 2009) and structural functions, such as emulsification, gelling, and foaming, has also been reported (Manso & Lopez-Fandino, 2004). For this reason, the CMP has been proposed to be used for the development of innovative food products with enhanced bioactivity and technological attributes, especially for special dietary (Abd El-Salam, 2009).

Several processes for the obtention of high-protein whey products have been described, including the recovery of CMP. Different technologies have been used for the whey protein fractionation, such as ion exchange, selective heat aggregation, chemical additives, pH adjustment, and membrane technology. However, many have low efficiency, in relation to purity and low protein recovery, as well as difficulties for implementation in an industrial plant or even a pilot plant (Lucena *et al*., 2007).

In this paper, the objective was to efficiently isolate CMP from whey by ultrafiltration, determine the physicochemical composition and characterize it by high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

MATERIALS

A sweet whey powder (WP), purchased from SOORO (Marechal Cândido Rondon, Paraná, Brazil) was used as starting material for the extraction of CMP. The whey protein isolate (WPI) was donated by Kraki & Kratschmer (Santo André,

São Paulo, Brazil). Standard sialic acid (Acros Organics, Geel, Belgium) and CMP were purchased from Sigma-Aldrich, Saint Louis, MO, U.S.A.).

ULTRAFILTRATION (UF)

Two kilograms of sweet whey powder were rehydrated in one liter of deionized water (resistivity above 18 MΩ.cm-1) and mixed until full solubilization. The lactose was separated by filtration into a cheese desorption form with holes in the sides and bottom, diameter 14,5 cm and height 9,5 cm. The residue was washed with deionized water until a collection of three liters of the filtrate. The filtrate was heated at 80 °C for 30 min, and after that, this was cooled to 30 °C (at room temperature) and centrifuged (XPN-90, Beckman Coulter, Brea, CA) at 5,200 g at 4 °C for 20 min. The supernatant from the centrifugation was used for the ultrafiltration step. The used ultrafiltration system with the membrane cut-off 50 kDa, 30 kDa and 5 kDa with filtration on area of 0.5 m², polyethersulfone, polypropylene and polyurethane, respectively, as the filter material, skin and adhesive (Millipore Corporation, New York, USA). The pH of the resulting supernatant was adjusted to 7.0 and the supernatant was subjected to ultrafiltration (UF) through a 50 kDa membrane, which resulted in a permeate and a retentate, as shown in Figure 1. The generated permeate was subjected to a further ultrafiltration through a 30 kDa membrane, which resulted in a new set of permeate and retentate. These were in turn separately ultra-filtered through a 5 kDa membrane. The 5 kDa retentates were lyophilized (L101, Liobrás Ind. Com. & Serv. Ltda.) or spray dried (YC-015, Shanghai Politech Instrument & Equipment Co. Ltda.). Each step of UF was followed by diafiltration (DF) at apressure of 1 bar.

DETERMINATION OF THE PHYSICAL AND CHEMICAL COMPOSITION OF THE ISOLATED CMP

The moisture, ash, protein, and lipid contents were determined in triplicate according to the AOAC official method (2010). To convert nitrogen into protein, the conversion factor 6.38, adopted for milk protein, was used.

Determination of the sialic acid was carried out according to Fukuda (2004), with modifications. Briefly 0.5 mL of milk serum, retentate or permeate from the ultrafiltrations, were mixed with 0.5 mL of 20 % (v/v) trichloroacetic acid. After homogenization, samples were left standing for 30 min and then centrifuged at 2,000 g for 20 min at 4 °C. Aliquots from the supernatant were taken. After the volumes are completed to 1 ml, was added 1 mL of glacial acetic acid and 1 mL of acidic ninhydrin reagent. The mixture was homogenized and subjected to a water bath for 10 min. It was cooled in an ice bath and the UV/Vis spectrophotometric (600S, Femto, São Paulo, SP, Brazil) reading was taken at 470 nm. We measured the concentration of sialic acid compared to the standard curve.

The lactose content was determined according to Folin and Wu (1920).

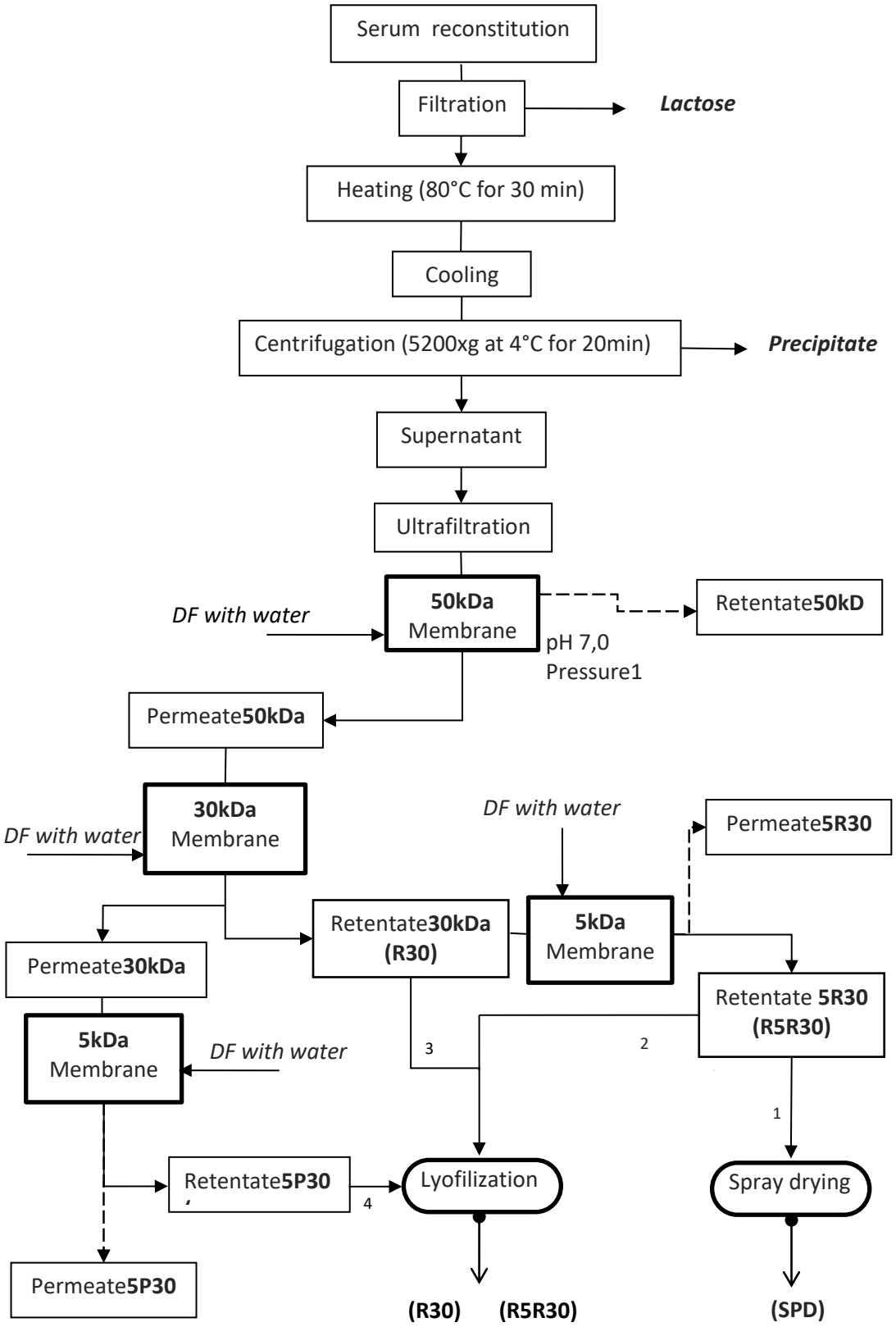

Figure 1 Flowchart of the CMP isolation by ultrafiltration: diafiltration (DF); permeate (P); retentate (R). Obtained process flow stage (1), (2), (3), (4), respectively, for the samples (SPD), (R5R30), (R30) and R5P30. Abbreviations in ( ) means CMP.

PROFILE ANALYSIS OF WHEY FRACTIONS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC)

The HPLC analysis was performed to characterize the CMP. The analysis of caseinomacropeptide chromatographic profiles was adapted from the method proposed by Thoma *et al.* (2006) and performed on a chromatograph Agilent 1200 series (Agilent Technologies Inc., NY, USA), consisting of a quaternary pump system, an automatic injection module and a photodiode assembly beam detector. The column used was a C18 OmniSpher, measuring 250 mm by 4.6 mm in diameter, with 5 micron particles. The flow used in the analysis was 1 mL min-1 at room temperature (25 °C). The elution of the samples was performed using a binary gradient. Solvent A consisted of 0.1 % trifluoroacetic acid in distilled water and solvent B contained 0.05 % trifluoroacetic acid in acetonitrile: water 80:20 (v/v). The gradient parameters were system stabilization from 0 to 2 min with 90 % A; from 2 to 14 min 51 % A; followed by 100 % B for 5 min; then isocratic for 2 min and finally return to initial equilibrium for 2 min. The wavelengths applied were 214 and 226 nm.

RESULTS AND DISCUSSION

ISOLATION OF CMP BY ULTRA-FILTRATION (UF)

Reconstituted dried whey used for the process of UF had 15.48 % protein, as shown in Table 1. A loss of 23.93 % of the protein was observed as a result of the dissolution of the whey powder in water and filtration for removal of lactose from the solution. This delactosed whey solution, which was the starting point for the heat treatment and UF, was equivalent to 30.263 L of serum, according to Richards (2002) wherein regular serum contains 0.9 % protein.

Table 1. Physicochemical composition and weight on a dry basis of CMP fractions obtained by ultrafiltration, WP and WPI

Composition	WP (g $100g^{-1}$)	WPI (g $100g^{-1}$)	R30kDa* (g $100g^{-1}$)	R5R30kDa* (g $100g^{-1}$)	SPD** (g $100g^{-1}$)
Moisture	2.55 ± 0.0	3.45 ± 0.1	--	--	--
Ash	4.92 ± 0.0	2.15 ± 0.0	5.44 ± 0.1^{e}	5.33 ± 0.1^{e}	7.83 ± 0.1^{a}
Protein	15.48 ± 0.3	92.31 ± 2.2	72.11 ± 0.2^{e}	71.94 ± 0.2^{e}	73.74 ± 0.8^{a}
Fat	1.86 ± 0.0	1.31 ± 0.1	--	--	--
Lactose	68.20 ± 0.3	0.92 ± 0.2	11.92 ± 0.0^{e}	11.85 ± 0.1^{e}	12.40 ± 0.0^{a}
Sialic acid	--	--	1.86 ± 0.1^{e}	2.15 ± 0.0^{i}	2.53 ± 0.6^{a}
Yield (g)	--	--	33.2^{e}	31.78^{a}	33.6^{e}

NOTE: Data represent mean of three replicates ± standard deviation. Values followed by different superscript letters in the same row are significantly different ($p < 0.05$); *Lyophilized, **spray-dried; (R30) sample originated from the retentate 30kDa, (R5R30) sample originated from the retentate 5kDa from the retentate to 30kDa, (SPD) sample originated from R5R30 with drying by spray dryer.

During heat treatment at 80 °C for 30 min, part of the protein was denatured and separated by centrifugation. The main change during heat treatment above 70 °C is the denaturation of whey proteins, followed by a series of reactions of aggregation and dissociation (Oldfield *et al.,* 1998). When elevating temperatures, β-lactoglobulin (β-lg) dissociates from a dimer to a monomer, exposing its thiol

group and interior hydrophobic residues, enabling thiol/disulfide exchange reactions. β-lg is the main protein responsible for heat-induced aggregation; however, α-lactalbumin (α-la) and bovine serum albumin present in WPI also participate in these hydrophobic, thiol-disulfide and electrostatic interactions (Kazmierski *et al.*, 2003).

Whey proteins can undergo unfolding, denaturation and aggregation during heating, and manufacturing processes, such as temperature, pH, ionic strength and drying, can affect the level of denatured proteins in dairy ingredientes (Barone *et al*., 2020).

The sialic acid content, protein and lactose of different fractions after UF are mentioned in Table 2. The protein concentration in delactosed serum has dramatically decreased as a result of heat treatment (see whey post-centrifugation). It is likely that such heat treatment caused loss of components of the CMP, as well as some structural change

Table 2. Concentration of sialic acid, protein and lactose in steps/fractions of ultra-filtration of whey

Stages	Salic acid in total volume* (mg)	Protein in total volume* (g)	Sialic acid /Protein ratio (mg g^{-1})	Lactose (g)	Lactose (%)
Whey powder	---	357.56 ± 7.2	---	1575.4	68.2
Delactosed whey	2856.55± 0.1	272.37± 6.1	10.5	1155.5	24.3
Precipitate	---	115.05 ± 1.7	---	253.6	19.8
Whey post-centrifugation	3199.58± 0.0	164.23 ± 8.9	19.5	395.5	11.4
P50kDa	2411.51± 0.1	77.99 ± 1.4	30.9		
R50kDa	1412.25± 0.1	78.09 ± 0.7	18.1		
P30kDa	1444.70± 0.2	17.39 ± 1.2	83.1		
R30kDa	621.11± 0.1	26.35 ± 0,1	23.6		
R5P30kDa	675.31± 0.1	14.20 ± 0,1	47.6		
R5R30kDa	342.94± 0.0	23.86± 0.6	14.4		
P5P30kDa	---	3.53 ± 0,1	---		
P5R30kDa	---	2.50 ± 0,1	---		

NOTE: See also flowchart in Figure 1; * Average of two replicates ± standard deviation; Note: concentration is the ratio between mass of a substance and the volume of solvent in which the compound is dissolved. (P50) permeated 50kDa, (R50) retentate 50kDa, (P30) permeated 30kDa, (R30) sample originated from the retentate 30kDa, (R5P30) retentate 5kDa from the permeated 30kDa, (R5R30) sample originated from the retentate 5kDa from the retentate to 30kDa, (P5P30) permeated 5kDa from the permeated 30kDa, (P5R30) permeated 5 kDa from the retentate 30 kDa.

It is also seen in Table 2 that the lactose content was decreased from 68.2 % to 11.4 %. The greater amount of lactose was retained by filtration of an initial solution with high whey powder concentration. The reduction in the lactose content was 83 % throughout the process.

The concentration of sialic acid and protein and the relationship between them were also showed in Table 2. Thus, data suggest that CMP should be in the fractions P30 kDa and R5P30 kDa. However, this was not confirmed by subsequent HPLC analysis.

The quantification of sialic acid can be taken as a reference for CMP. Customary methods for determining the CMP are based on the determination of the soluble nitrogen in the trichloroacetic acid (TCA) solution. However, the validity of this approach is imprecise once the addition of 12 % TCA (w/v) to the set of CMP (glycosylated or unglycosylated) not precipitate carbohydrate-containing CMP but leads to precipitation of non-glycosylated CMP (Armstrong *et al.,* 1967; Creamer *et al.,* 1973). Thus, it was known that the different forms of glycosylated and unglycosylated macropeptide have different sensitivities to TCA precipitation (Vreeman *et al.,* 1986; Abd El-Salam *et al.,* 1996).

The results of the analysis of chemical composition of the CMP isolated, whey and WPI are shown in Table 1. Whey showed less protein content, but higher amounts of fat and lactose. The WPI showed a higher amount of protein and lower amount of lactose. The CMP isolated from whey during the process, increased the protein content and decreased the lactose content.

Two different processes were performed to obtain the CMP (Table 1): in one of them the material was dried by lyophilization (R30 and R5R30) and in the other by spray-drying (SPD), which is closer to industrial technology. After lyophilization, 33.16 g of the fraction R30 kDa and 31.78 g of the fraction R5R30 kDa were obtained, whereas spray-drying yielded 33.58 g of fraction R5R30 kDa. Thus, the yield showed a significant difference ($p<0.05$) between the same fraction R5R30 kDa from different drying. The sialic acid content was 1.86 g 100 g-1, 2.15 g 100 g-1 and 2.53 g 100 g-1, respectively, which is equivalent to 616.8 mg, 683.3 mg and 849.5 mg sialic acid per gram of powder isolate obtained. There were significant differences ($p<0.05$) between the R30 kDa, R5R30 kDa and SPD samples in terms of sialic acid content.

According to Martin-Diana *et al.* (2002), the percentage of sialic acid present in the CMP is 7-8 %. The CMP is present in cheese whey at concentrations ranging from 1.2 to 1.5 g L-1 which accounts for 10-25% of protein in whey (Oliva, 2002; Thoma-Worringer, Sorensen, & Lopez-Fandino, 2006). In this study, the CMP concentration in serum was 0.27 g L-1 for R30 kDa, 0.30 g L-1 for the R5R30 lyophilization and spray dryer was 0.37 g L-1, totaling 0.94 g L-1 in the process. The efficiency of ultrafiltration in accordance with a CMP content of 1.2-1.5 g per liter of serum was calculated to be between 63 - 78%.

It is known that the processing conditions, such as heat or acid treatments, effect the stability of CMP (Siegert et al., 2012), and that, therefore, it is necessary to have a control parameter to obtain a good efficiency in the isolation of the CMP. Knowing the process of origin of the whey, which was the starting point for the isolation of the CMP, as well as establishing a parameter for the isolation process is necessary. The pH is obtained as an important parameter for isolation and functionality of proteins. Thus, the separation of whey proteins is based on pH adjustment. CMP is soluble and heat-stable even at low pH values, which allows its separation from other whey proteins with varying recovery of aCMP and gCMP depending on the process parameters (Lieske et al., 2004).

Of the CMP fractions, SPD had the highest ash, protein and lactose contents, these values were significantly different ($p< 0.05$).

In a technical specification of the sweet WP (SOORO, 2020), it mentions that the product was obtained from the manufacture of cheese, concentrated by nanofiltration membrane, skimmed, pasteurized, vacuum concentrated and dried

through a spray drier process. Depending on the process adopted to manufacture sweet WP ingredients (e.g., cheese-making parameters, the intensity of heat treatment, denaturation of proteins), the CMP concentration may vary (Croguennec et al., 2014).

CHROMATOGRAPHIC ANALYSIS (RP-HPLC)

The objective was to qualitatively assess the caseinomacropeptide profile of the UF fractions, to obtain the desired product. The chromatogram of the CMP standard at 10 mg mL-1 was not shown individually, but instead as multiple co-eluted peaks in the range between retention times 7 to 12 min, which prevented the quantitative analysis of the CMP under the conditions used. Such multiplicity of peaks (peaks 2) may be due to the heterogeneity of the composition as a consequence of genetic variability, as well as post-translational modifications that occur in the peptide chain, namely: glycosylation reactions of some amino acids with molecules of galactose, N-acetyl neuraminic acid and possibly also phosphorylation of the CMP. Another factor that may affect the process yield and chromatographic profiles of this macropeptide is the interference of the acidic pre-treatment performed, since pH reduction can induce partial losses due to solubility changes in different peptide fractions, mainly those unglycosylated (Thoma, 2006). Peaks were observed in the chromatographic profile; these peaks contained N-acetyl neuraminic acid (peak 1), CMP with glycosylation (peaks 2), unglycosylated CMP (peak 3).

Comparing the profile of peaks of the chromatogram in Figure 2 with the respective standard CMP, there was a sequence of co-eluted peaks in the region 7-10 min which are correspondent to CMP. At retention times between 10-12 min, there was the highest individualized peak. The presence of this peak was of little significance in the chromatogram of the standard CMP, but once this result was compared to the work of Thoma (2006), there was strong evidence that such prominent peak refers to an unglycosylated caseinomacropeptide (peak 3). Moreover, in comparison with a commercial CMP (LACPRODAN – CGMP-10 – ARLA), studied by Tullio (2007), the same profile of peaks is noticeable at equal retention times. Among the samples of CMP obtained by UF, the fraction that showed the highest peaks at the retention time 11 min was the fraction SPD (spray-dried 30 kDa membrane retentate). This major peak corresponds to the chromatographic profile demonstrated by Thoma (2006). The other two fractions R30 (30 kDa membrane retentate) and R5R30 (5 kDa membrane retentate from R30) also showed peak profiles similar to those of the CMP migration region, only with a visibly lower amount at the peak related to the retention time 11 min, coinciding with the profile shown in commercial CMP study Tullio (2007). Such profile similarities between both fractions obtained indicates that the ultra-filtration process was not critically selective (Fig 2).

Figure 3 shows the chromatogram with the same technique used in the Other cromatograms, detection at 214 nm with a diluted sample of 10 mg mL-1 and injection of 30 µL for further comparison. According to Taylor & Woonton (2009), the retention time and total área count of the CMP peaks is from 4 to 8 minutes, from peptone proteose from 8 to 10 min, from a-lactalbumin from 10.5 to 11.5 min and b-lactoglobulin is 12.5 to 13.5 min. It is observed that the blue line that depicts the WPI and the green line that depicts the Whey, has the highest peak in

the retention time of 11 to 12 min. As is known, the most relevant protein contente of the composition of the WPI and Whey is β-lg with about 16 to 18 %. However, this protein is less stable to heat treatment and has a higher molecular weight than α-la and more distant from CMP.

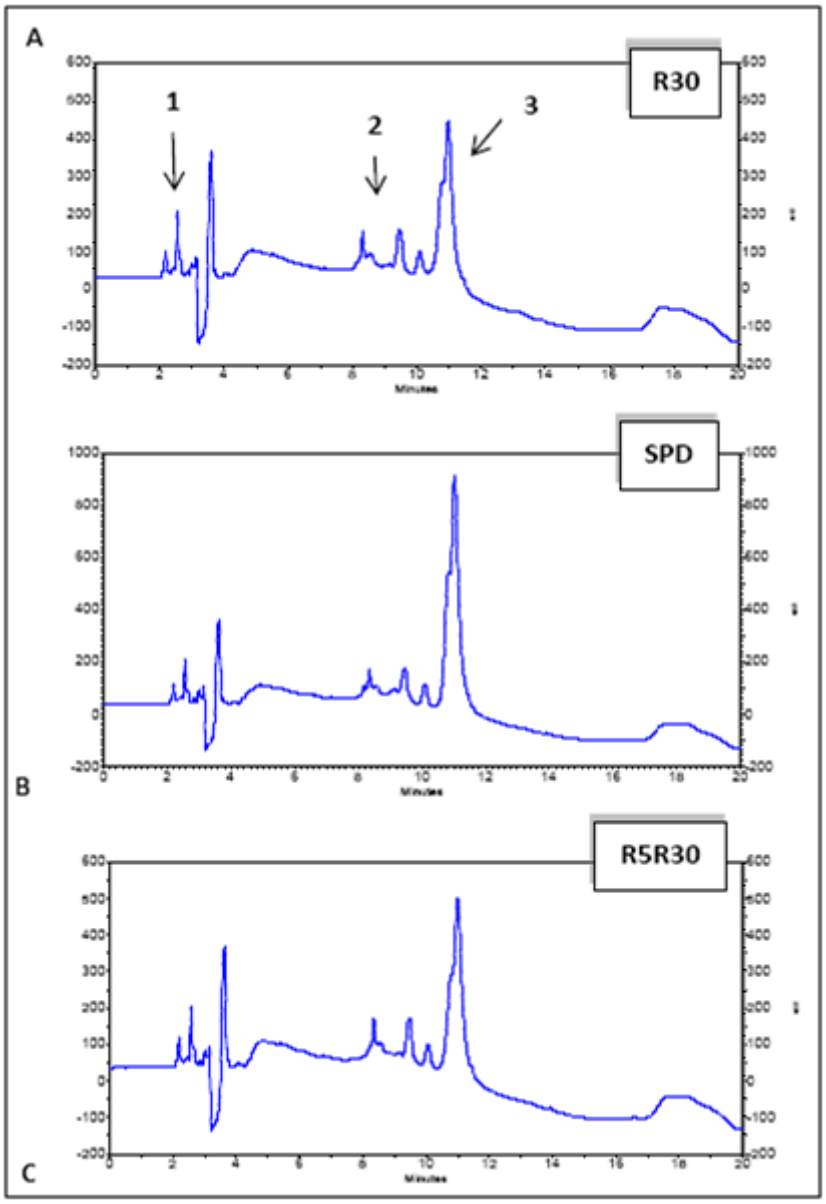

Figure 2. Chromatogram of fractions R30 (A), SPD (B) and R5R30 (C) obtained by the ultrafiltration at a concentration 10mg mL^{-1}, injection of 30µL using a OmniSpher C18 column with a flow rate of 1mL min^{-1}. (R30) sample originated from the retentate 30kDa, (R5R30) sample originated from the retentate 5kDa from the retentate to 30kDa, (SPD) sample originated from R5R30 with drying by spray dryer.

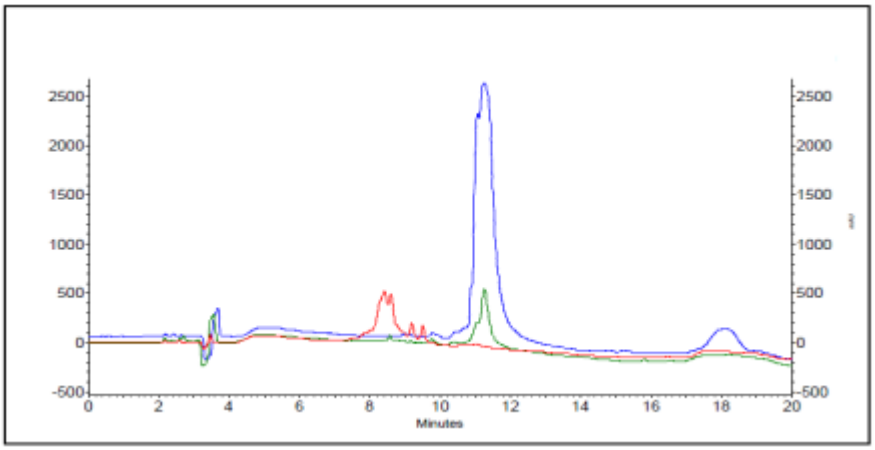

Figure 3. Chromatogram of WPI (blue line), CMP standard (red line) and Whey (green line) with 214 nm at a concentration 10 mg mL^{-1}, injection of 30µL.

Some authors report that ultrafiltration is not able to efficiently separate whey proteins, since α-la and β-lg have similar molar mass and isoelectric points (Alcântara, 2011).

Above all, this peak is more likely to be a-lactalbumin due to its retention time. The red line representes the CMP pattern, wich has several peaks in the retention time of 8 to 10 minutes. However, at 11 to 12 minutes there is no relevant peak.

CONCLUSIONS

Among the three different processes, the one that showed the highest efficiency was the R5R30 spray drier, afterwards the lyophilized R5R30, and in the end, with less efficiency was the R30. The SPD fraction showed higher yield, higher levels of protein, sialic acid, ash and lactose, as well as a significant difference ($p<0.05$) of these parameters in relation to the lyophilized fractions. All the processes were not very efficiency in the qualitative characterization by HPLC, due to the lack of knowledge of the pH and thermal parameters of the whey processing, initial raw material, and its influence on the CMP stability.

Isolamento por ultrafiltração e caracterização de caseinomacropeptídeo

RESUMO

Peptídeos biologicamente ativos do soro de leite, como o caseinomacropeptídeo (CMP), são de grande interesse para a tecnologia de alimentos e para o desenvolvimento de novos alimentos funcionais. O CMP é o derivado solúvel da k-caseína, gerado pela clivagem da enzima quimosina na fabricação do queijo. O objetivo deste trabalho foi isolar a CMP do soro de leite por ultrafiltração, determinar a composição físico-química e caracterizá-la por cromatografia líquida de alta eficiência (HPLC). O método aplicado foi a ultrafiltração com membrana de 50, 30 e 5 kDa. A fração de CMP isolada consistiu em 73,7% de teor de proteína, 12% de lactose e 2,5% de ácido siálico. As frações finais do isolamento apresentaram perfis cromatográficos compatíveis com CMP. Os parâmetros do processo e as características da matéria-prima podem alterar a eficiência do isolamento CMP. Portanto, é importante otimizar todos os parâmetros da etapa do processo para um isolamento eficiente.

PALAVRAS-CHAVE: proteína do soro; caseinomacropeptídeo; ultrafiltração.

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