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A comparison of the heat stability of fresh milk protein concentrates obtained by microfiltration, ultrafiltration and diafiltration

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Research Article

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Abstract

The objective of this work was to evaluate the impact of changes during membrane filtration on the heat stability of milk protein concentrates. Dairy protein concentrates have been widely employed in high protein drinks formulations and their stability to heat treatment is critical to ensure quality of the final product. Pasteurized milk was concentrated three-fold by membrane filtration, and the ionic composition was modified by addition of water or permeate from filtration (diafiltration). Diafiltration with water did not affect the apparent diameter of the casein micelles, but had a positive effect on heat coagulation time (HCT), which was significantly longer (50 min), compared to the non diafiltered concentrates (about 30 min). UHT treatments increased the particle size of the casein micelles, as well as the turbidity of retentates. Differences between samples with and without diafiltration were confirmed throughout further analysis of the protein composition of the unsedimentable fraction, highlighting the importance of soluble protein composition on the processing functionality of milk concentrates.

Dairy ingredients, such as milk protein concentrates and isolates and micellar casein concentrates and isolates, are frequently used to achieve the desired nutritional and functional characteristics in high protein beverages, currently in high demand in the marketplace (de Kort *et al.*, 2011; Farkye and ur-Rehman, 2011; Agarwal *et al.*, 2015). To obtain these ingredients, different membrane filtration technologies may be used to concentrate milk before spray drying, and depending on processing conditions, the protein concentration and the composition of the soluble phase can be modulated. When ultrafiltration (UF) is used, all the major proteins are concentrated in the retentate, while, if larger pore sizes are used, with microfiltration (MF), it is possible to selectively concentrate caseins while transmitting whey proteins in the permeate. During MF, the amount of whey proteins present can be further decreased if diafiltration (DF) is used. This process consists of a dilution step with water to continue the selective removal of soluble molecules such as lactose, salts and whey proteins, in the case of MF. During extensive DF, in addition to the changes in the concentration ratio between colloidal and soluble fractions, the extensive removal of soluble calcium may affect the integrity of the casein micelles' supramolecular structure (Li and Corredig, 2014).

In milk protein beverages, the extended shelf-life is achieved by intense heat treatments such as ultra-high temperature (UHT) or retorting, which makes the structure of the caseins and their heat stability very important. Heat stability of milk protein concentrates is a challenge considering the inverse relationship between heat stability and protein concentration in milk systems (Singh, 2004). The interactions between whey proteins and caseins have been widely studied in skim milk. When heated, whey proteins are denatured and interact with themselves or associate with casein micelles, and form complexes mostly with κ-casein and α_{s2} casein. Protein-protein interactions are affected by time, temperature, rate of heating, pH, and protein concentration (Donato and Dalgleish, 2006; Anema, 2009; Donato and Guyomarc'h, 2009; Li *et al.*, 2015). Because of their sensitivity to heat, removal of whey proteins has been proposed as a way to obtain beverages with superior heat stability.

Micellar casein concentrates and isolates have been suggested as an ideal ingredient for the development of dairy beverages (Agarwal *et al.*, 2015). Studies have been conducted on the stability of micellar casein concentrates at different pH values, variable calcium concentration and distribution, and the presence of chelators (Beliciu *et al.*, 2012; de Kort *et al.*, 2012; Sauer and Moraru, 2012). These studies report poor heat stability of high protein solutions after simple redilution, and extra steps such as pH adjustment and addition of calcium chelators are necessary to achieve longer heat coagulation times and to avoid precipitation and coagulation during the commercial life of the product (Beliciu *et al.*, 2012; de Kort *et al.*, 2012; Sauer and Moraru, 2012). However, these results are based on studies on reconstituted powder

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dispersions, with a mineral balance substantially different than that of the original milk or the fresh retentates (Beliciu *et al.*, 2012; de Kort *et al.*, 2012; Sauer and Moraru, 2012).

Recent studies demonstrated that there are profound differences in heat stability between MF and UF concentrates, the latter containing a higher concentration of whey proteins (Renhe and Corredig, 2018). The removal of whey proteins increases the heat stability, when the concentrates are compared at the same concentration factor and under comparable serum composition (Renhe and Corredig, 2018). The processing history of the retentates is therefore critical to their stability. Processing changes such as the use of diafiltration, drying and reconstitution of the concentrates will lead to changes in mineral equilibrium, with consequences to the heat stability of the concentrates. To test this hypothesis, concentrates were obtained using UF, MF and DF, and compared at the same protein concentration. Concentrates were diafiltered using water or permeate from UF, to evaluate the effect of diafiltration with or without control of the composition of the serum phase. This work will improve the current understanding of the critical factors affecting the processing functionality of fresh protein concentrates.

Materials and methods

Sample preparation

Pasteurized skim milk (Crown Dairy Ltd., Guelph, Canada) was concentrated by microfiltration (MF) or ultrafiltration (UF), three times based on volume reduction. A custom made pilot scale spiral-wound system was used with a PVDF 800 kDa cutoff membrane for the microfiltration (Synder® Filtration, Vacaville, CA, USA) and a polyethersulfone (PES) with 10 kDa cutoff for the ultrafiltration (Koch, San Diego, CA, USA). The two other treatments evaluated were diafiltration carried out either with water or permeate from ultrafiltration. In this case, to the 3× MF retentates water or permeate was added back to the original volume, and then microfiltration was continued again up to 3× concentration.

In total 4 treatments were analyzed: (1) ultrafiltered retentate (UF); (2) microfiltered retentate (MF); (3) MF diafiltered with UF permeate (PF); and (4) MF diafiltered with water (DF). All treatments reached a 3× volume reduction (based on original volume) corresponding to a total protein volume of 10%.

Heat treatment

Thermal stability was evaluated by measuring the heat coagulation time (HCT), defined as the time during heating at 120 °C necessary for visible onset coagulation to occur. The system used was a silicone oil bath (Haake AC200 – ThermoFisher Scientific, Newington, NH) fitted with a custom made circulation device. Aliquots (3 ml) were transferred to a heat-resistant screw-cap test tube and immersed in the oil bath at 120 °C (Eshpari *et al.*, 2014). The samples were kept under agitation and the elapsed time between the immersion and the first visible precipitation was recorded as the HCT.

The same system was used to heat treat the samples at 120 °C for 10 min for further evaluation of the impact of heat treatment on these retentates. The samples were immediately cooled to room temperature by immersion in an ice bath. Samples were also submitted to a commercial heat treatment of ultra-high temperature (UHT) with pre-heating at 82 °C, final temperature of 136 °C and holding time of 6 s followed by homogenization at 3,450 kPa before cooling to 4 °C (MicroThermics, Raleigh, NC, USA).

Sample characterization

Total solids were measured by drying approximately 2 g of sample in an aluminum dish with pre-dried sand. The samples were dried at 105 °C in a gravity-flow convection oven (Fisher Scientific, Pittsburgh, PA) overnight. The pH of the retentates was measured at 25 °C, under agitation, using an Accumet pH meter (Fisher Scientific, Pittsburgh, PA), calibrated before use. Total and soluble protein concentration was measured by Dumas method Leco FP-528 (Leco Corp., St. Joseph, MI, USA) and a conversion factor of 6.38 was used to convert the nitrogen concentration into protein. The retentates were also separated in a precipitated and unsedimented fraction, obtained by ultracentrifugation at 100 000 × $\boldsymbol{g}$ for 1 h at 20 °C (Optima TM LE-80k, Beckman Coulter, Mississauga, ON, Canada). The whey proteins were measured by HPLC (Thermo Instruments Canada Inc., Mississauga, ON, Canada) as described in Li *et al.* (2015). Samples were filtered through a 0.2 µm membrane before analysis and eluted in a 1 ml/min flow gradient of 0.1% v/v trifluoroacetic (TFA) (solvent A) and acetonitrile, MilliQ water, and TFA in a ratio 900:100:1 (v/v/v) (solvent B). The gradient used started with 2% eluent B, increasing to 70% B in 40 min, achieving 100% B (in 41 min), and kept at 100% until 47 min.

Viscosity

Viscosity was measured using a controlled stress rheometer (Paar Physica MC 301, Anton Paar, Graz, Austria) using cone and plate geometry, with a set gap of 0.51 mm, and at 25 °C. The milk retentates samples were subjected to a shear sweep test from 10 to 300 s^{-1} and values at 100 s^{-1} were reported.

Light scattering

Particle size distribution was determined by dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK). The samples were diluted to the ratio 1 µl sample: 1 ml of filtered permeate from ultrafiltration (0.22 µm PVDF filters, Fisher Scientific) and analyzed at 25 °C in a backscatter measurement angle of 173°. Transmission diffusing wave spectroscopy (DWS) was used to measure the characteristics of casein micelles *in situ*, without dilution (Alexander *et al.*, 2006). DWS was used to measure the photon transport mean free path (l^*), which is defined as the length scale over which the scattered light has been totally randomized. l^* parameter can be related to turbidity ($1/l^*$) in highly turbid samples and depends on as particle size, particle concentration, and the dispersion medium (Alexander *et al.*, 2006).

Total, soluble and diffusible calcium and phosphate

The clear supernatant after precipitation with HCl and centrifugation for 15 min at 4,500 × $\boldsymbol{g}$ (Eppendorf centrifuge, 5415D, rotor R7018) was used for analysis of total calcium. Soluble fractions were analyzed from the supernatants after centrifugation at 100 000 × $\boldsymbol{g}$ for 1 h at 20 °C (Beckman Coulter). Diffusible phase was obtained after ultrafiltration of soluble phases in concentrators with molecular weight cutoff of 10 kDa (Corning® Spin-X®

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Table 1. Properties of the retentates obtained by ultrafiltration (UF), microfiltration (MF), microfiltration with permeate added (PF) and water added (DF)

	UF	MF	PF	DF
pH	6.7 ± 0[a]	6.7 ± 0.1[a]	6.7 ± 0[a]	6.9 ± 0[b]
Total solids (%w/w)	16.0 ± 0.5[a]	15.8 ± 0.4[a]	15.4 ± 0.3[a]	13 ± 0.1[b]
Total protein (%w/w)	10.5 ± 1.2[a]	9.9 ± 0.2[a]	10.0 ± 0.0[a]	10 ± 0.1[a]
Soluble protein (%w/w)	3.3 ± 0.6[a]	2.1 ± 0.5[b]	2.1 ± 0.2[b]	2.5 ± 0.1[ab]
α-lactalbumin (mg/ml)	18.5 ± 1.9[a]	14.1 ± 0.2[b]	12.3 ± 0.1[b]	13.9 ± 0.7[b]
% of WP retained	100[a]	82.8 ± 2.6[b]	77.5 ± 1.3[b]	86.6 ± 3.0[b]
Diameter (nm)	169 ± 5[a]	168 ± 5[a]	166 ± 3[a]	165 ± 2[a]
Viscosity (mPa s^{-1})	9.0 ± 2.0[a]	8.0 ± 0.9[a]	6.0 ± 1.4[a]	8.0 ± 0.6[a]

Values of pH; total solids; total protein; unsedimentable protein; concentration of α-lactalbumin; residual whey protein after concentration; apparent diameter of the casein micelles; viscosity. Values are means ± SD of at least 2 independent runs. Within a row, different superscripts indicate significant differences ($P < 0.05$).

UF), at 5000 × ***g*** for 30 min and used for both calcium and phosphate analysis. Ashes of original samples and soluble phase were solubilized with nitric acid and diluted in water for total and soluble phosphate analysis.

An Advanced Compact Ion chromatography (Ω Metrohm ion analysis, Metrohm Ltd., Herisau, Switzerland) was used to measure calcium and phosphate (Zhao and Corredig, 2015).

Characterization of soluble protein aggregates

Soluble protein aggregates were characterized by Size Exclusion Chromatography (SEC) using ÄKTA purifier 10 system (GE Healthcare, Uppsala, Sweden) as previously described (Li *et al.*, 2015). In brief, 10 ml fractions were collected from the elution peaks, for further analysis of the differences in the type and composition of the soluble aggregates through electrophoresis. The fractions were freeze-dried and then diluted in 1 ml sample buffer without β-mercaptoethanol. The electrophoresis analysis of selected peaks was conducted under reducing (with β-mercaptoethanol) and non-reducing conditions. SDS-PAGE was performed using a Bio-Rad electrophoresis unit (Bio-Rad Power Pac HC, Mississauga, ON, Canada) at 175 V for 50 min. The resolving gel contained 15% acrylamide and the stacking gel contained 4% acrylamide. Aliquots of 5 µl of 1% (w/w) standard solutions (sodium caseinate and a whey protein isolate) were loaded onto the gels. For the SEC peaks, 10 µl of samples were loaded on the gel.

Statistical analysis

The experiments were carried out in triplicate (i.e. three separate milk batches). Statistical significances were evaluated using analysis of variance (ANOVA) at $P < 0.05$ and *t*-test. The mean values were compared using Tukey test, with all data processed using Statistica 12 software.

Results and discussion

Sample characterization

The composition of the concentrates, viscosity and particle size distribution of the casein micelles are summarized in Table 1. UF and MF samples maintained the same pH as the original milk, as did the sample diafiltered with UF permeate (PF). On the other hand, DF concentrates showed a higher pH, as previously reported in the literature, due to removal of ions (i.e. inorganic phosphate) from the serum phase (Broyard and Gaucheron, 2015). The amount of total protein was comparable between all concentrates (approximately 10% w/w), a concentration that was about 3× that of the original milk. Total solids concentrations ranged between 15.4 and 16% w/w for UF, MF and PF concentrates, and, as expected, the total solids in the DF retentates were lower (13% w/w).

There was a difference in the amount of unsedimentable protein measured in the centrifugal supernatants, with UF milk showing the highest amount (3.3% w/w), and, as expected, a lower concentrations in the supernatants derived from MF and PF concentrates. The DF concentrates contained an unsedimentable protein concentration intermediate between the UF and the PF treatments. To better understand the extent of whey protein reduction in the MF, DF and PF retentates, the amount of α-lactalbumin residual was also quantified using HPLC, and, as expected, while UF retentates contained about 18.5 mg/ml of α-lactalbumin, all other treatments had approximately 80% of this protein reduced from the initial concentration.

All the MF treatments (with or without diafiltration) had lower levels of α-lactalbumin compared to UF concentrates. There was no significant difference between MF and diafiltration showing that, under these conditions, there was no further removal of whey proteins in the samples, either using diafiltration with water or permeate (Table 1). In addition, there was no difference in the apparent diameter of the casein micelles, as measured by light scattering (after re-dilution in ultrafiltered permeate) (Table 1). Furthermore, the viscosity of the concentrates was also comparable between samples (Table 1).

Figure 1 illustrates the electrophoretic pattern of the supernatant separated from the four retentates, analyzed under non-reducing and reducing conditions. The same volume of sample was loaded for all treatments and a qualitative comparison was made. All samples showed a predominant presence of whey proteins, with very little unsedimentable casein proteins. However, both UF and MF retentates showed a distinct β-casein band, and more caseins were present in the supernatant of UF concentrate. The lowest amount of unsedimentable caseins was shown in the DF retentates, even when compared to PF or MF concentrates (Fig. 1).

Calcium and phosphate play a major role in supramolecular structure changes of casein micelles and are critical to the heat

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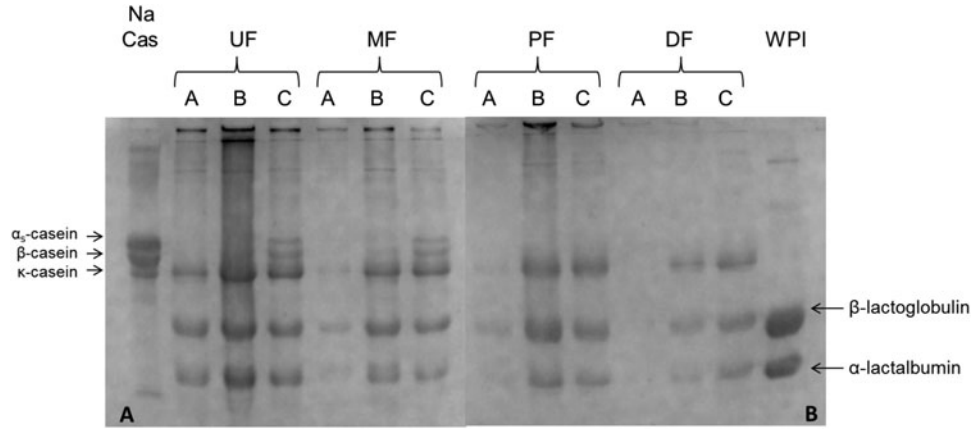

Fig. 1. SDS-PAGE patterns under non-reducing (a) and reducing conditions (b) of the centrifugal supernatants of fresh ultrafiltered (UF), microfiltered (MF) concentrates, as well as microfiltered concentrates diafiltered with permeate (PF) or water (DF).

Table 2. Concentration of calcium and phosphate ions present in the retentates, measured as total; soluble (present in the supernatant after centrifugation); and diffusible (permeating through an ultrafiltration membrane, and hence non associated to protein)

mM	UF	MF	PF	DF
Ca T	55 ± 11^{a}	47 ± 4^{a}	52 ± 5^{a}	32 ± 4^{b}
Ca Sol	12 ± 1^{a}	10 ± 3^{ab}	9 ± 3^{b}	7 ± 1^{b}
Ca Diff	5.9 ± 0.6^{a}	6.3 ± 0.7^{a}	5.3 ± 0.5^{a}	3 ± 0.3^{b}
P_i T	69.5 ± 4.0^{a}	67.5 ± 5.6^{ab}	67.2 ± 2.2^{ab}	59.5 ± 5.3^{b}
P_i Sol	18.4 ± 1.8^{a}	16.2 ± 2.1^{a}	14.8 ± 2.0^{a}	7.0 ± 0.3^{b}
P_i Diff	9.8 ± 1.1^{a}	11.4 ± 1.0^{a}	11.3 ± 1.1^{a}	3.9 ± 0.9^{b}

Values are the means of at least 2 independent runs. Within a row, different superscripts indicate significant differences ($P < 0.05$).

stability of milk (Singh, 2004; Lucey and Horne, 2009). Hence, the concentration of total, soluble and diffusible (non-associated with proteins) calcium and inorganic phosphate for the various fresh retentates was measured and summarized in Table 2. As expected, there were no significant differences in the amount of total calcium for UF, MF and PF samples. These retentates were concentrated without a change in the mineral balance of the serum phase. The comparable ionic composition of the serum phase is critical to better compare the heat stability properties of these retentates. On the other hand, the DF retentate showed significantly lower levels of total calcium. In the case of unsedimentable (soluble) calcium, which was defined as the amount of calcium present in the supernatant after centrifugation, the values were significantly lower for DF and PF samples, compared to UF. However, there were no statistical differences between diafiltration with water or permeate. It may be concluded that this lower level of calcium present in the soluble phase is due to the removal of soluble proteins associated with calcium. These proteins include α-lactalbumin, which accounts for 0.5 mmol/l of calcium (Lucey and Horne, 2009), but also some caseins that can leave the micelle during the processing. Table 2 also summarizes the changes in diffusible calcium. There was a similar concentration of calcium ions non-associated with the protein (and permeating through a UF membrane) for UF, MF and PF samples, while, as expected the DF retentates showed a significantly lower concentration. These results indicate that DF was more effective to remove ions of calcium but did not have the same efficiency for other forms.

It is known that while only one-third of total calcium is present in the soluble phase, half of phosphate ions are soluble (Gaucheron, 2005; Rahimi-Yazdi *et al.*, 2010). The amount of total phosphate ions, soluble and diffusible were also measured, and showed full agreement with the data reported for calcium ions. The total phosphate, as well as the value of soluble phosphate, and of diffusible phosphate were significantly lower for DF retentates, compared to UF, MF or PF, which did not differ from one another (Table 2).

Heat stability and heat-induced changes

Retentate samples were tested for their heat stability. It is important to note that all of the samples had comparable protein concentration, and only the DF retentates had a significant difference in the ionic serum composition. The results of the heat coagulation time (in minutes), as measured by heating the concentrates in an oil bath at 120 °C were: 28 ± 1 (UF), 33 ± 2 (MF), 31 ± 5 (PF), and 49 ± 1 (DF). UF, MF and PF retentates showed no statistical difference between them, whilst the fresh DF concentrate showed a significantly longer HCT. There was a significant difference in the HCT between PF and DF, indicating that the use of permeate for further filtration caused the proteins to maintain a processing functionality similar to that of the original milk. Furthermore, the extent of removal of whey proteins, under these conditions, was not sufficient to improve the HCT of the concentrates. The higher HCT of DF sample suggested that other factors play an important role in heat stability. Firstly, the pH of DF sample was 6.9 instead of 6.7, and it has been shown that the initial pH plays an important role in milk stability and calcium activity even with such a small change (Crowley *et al.*, 2014; Singh, 2004). It has been shown that an increase in pH and addition of phosphate can improve solubilization and heat stability of milk powders (de Kort *et al.*, 2012; Sauer and Moraru, 2012; Eshpari *et al.*, 2014).

Although all the concentrates presented similar total protein, the amount of total solids was lower in DF retentates, with a lower amount of soluble calcium and phosphate ions compared to the other treatments. All these factors play a role in determining the heat stability of the milk protein concentrates. Comparison with

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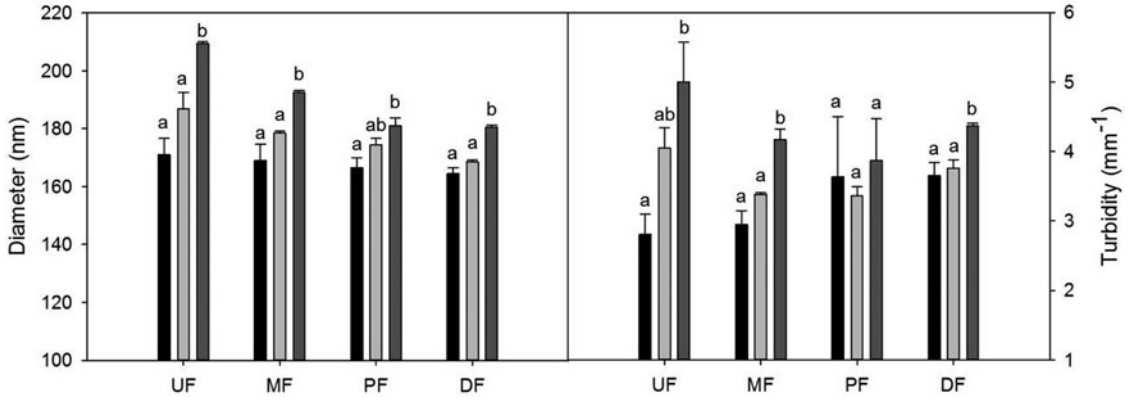

Fig. 2. Diameter (a) and turbidity (b) measured for UF and MF concentrates, as well as concentrates diafiltered with permeate or water (PF and DF, respectively). Before heat treatment (black bars); heated at 120 °C for 10 min in oil bath (light gray) or UHT 136 °C for 6 s (dark gray). Values are the average of two measurements. Bars represent standard deviation, and different letters show statistical significance at $P < 0.05$.

literature data was a challenge as previous research, for the main part, employed reconstituted concentrates and isolates, and not fresh retentates. To achieve higher protein concentrations, extensive filtration with diafiltration is applied which results in powder products with different calcium concentration and activity (Crowley *et al.*, 2014). Spray drying causes other major changes to milk proteins such as loss of solubility and stability (Beliciu *et al.*, 2012; Eshpari *et al.*, 2014) and simple solubilization in water may not be sufficient to fully recover the original organization and particle size of the casein micelles (Eshpari *et al.*, 2014).

To better compare the present results with current literature and to understand the changes during processing, concentrates were submitted to a batch heat treatment (120 °C for 10 min) and a UHT treatment (136 °C for 6 s). Samples were subsequently characterized for viscosity, particle size, and composition of the centrifugal supernatant. There were no significant differences in the retentates before or after heating experiments, for both heating at 120 °C for 10 min or UHT (data not shown). As shown in Fig. 2, the apparent diameter did not increase after treatment at 120 °C for 10 min, regardless of the treatment. On the other hand, there were significant statistical differences in the apparent diameter after UHT treatment. The apparent diameter was larger for all samples, and it was also significantly larger for UF retentate compared to the MF, PF and DF retentates. These results point, once again, to the importance of the whey proteins/casein ratio to the heat stability and functional properties of the retentates. More soluble proteins will lead to the formation of more aggregates (Donato *et al.*, 2007). Turbidity was not statistically significant for unheated retentates, and for the same retentates after batch heating, but the values were significantly higher for UHT retentates, apart from the case of the PF retentate. The extent of whey protein aggregation and the larger size of the casein micelles may be the main contributor to the increase in turbidity.

The unsedimentable fraction was analyzed by size exclusion chromatography, to better understand differences before and after each heat treatment, in size and composition of unsedimentable aggregates. Figure 3 illustrates the difference in the elution of the unsedimentable protein fraction, for each treatment: unheated; heated at 120 °C for 10 min; and UHT concentrates. The peaks were assigned as previously described (Donato and Dalgleish, 2006; Li *et al.*, 2015).

Unheated samples (Fig. 3a), showed a large peak at 110 min elution time. This peak contained mostly undenatured whey proteins (Donato *et al.*, 2007). As expected, supernatants of UF concentrates showed the highest peak, while the other supernatants showed a smaller peak because of the permeation of the whey proteins, during microfiltration. After heating either at 120 °C for 10 min (Fig. 3b) or by UHT (Fig. 3c), there was a significant decrease of this native whey protein peak. However, there were profound differences in the composition of the supernatant after UHT and batch heating. The two treatments showed a marked difference in the height as well as the distribution of the proteins between 45 and 90 min. Aggregates size at the most distinguish peak, 60–90 min, is on the range of 60–64 nm. The heat induced soluble aggregates elute in this region, and differences in elution time suggest differences in the size of the aggregates. It has already been shown that the unsedimentable aggregate peak increases with the extent of membrane concentration (Li *et al.*, 2015).

Figure 3b shows two distinct profiles after heating at 120 °C for 10 min. The aggregates present in the supernatant of UF and PF concentrates, after heating, eluted earlier than those of MF and DF concentrates. This would suggest the presence of larger aggregates in these samples. It is important to note that in these samples the residual protein present in the supernatant after centrifugation was lower than that present in UHT heated concentrates. After UHT treatment (Fig. 3c) the amount of aggregates increased, showing much larger peaks than those shown in Fig. 3b, with the exception of the elution chromatograms for supernatants from DF concentrates, which was very similar for both heat treatments. The elution of the aggregates occurred later, indicating smaller aggregates, and a larger amount of native protein remained, compared to the rest of the treatments. It is important to note that this concentrate was the only concentrate at pH 6.9, instead of pH 6.7.

In Fig. 3c, it is also clearly shown that UF retentates had the highest amount of unsedimentable aggregates, while MF and PF retentates showed smaller peaks. The difference in the elution pattern between the samples may suggest not only the presence of different amounts of soluble aggregates but also differences in composition and size. The aggregates eluted by chromatography were collected and analyzed by electrophoresis, as shown in Fig. 4.

The same amount of sample was loaded for all the concentrates because they are at the same concentration factor, allowing for a straight comparison between samples. As expected, UF presented more intense bands as result of more proteins in the

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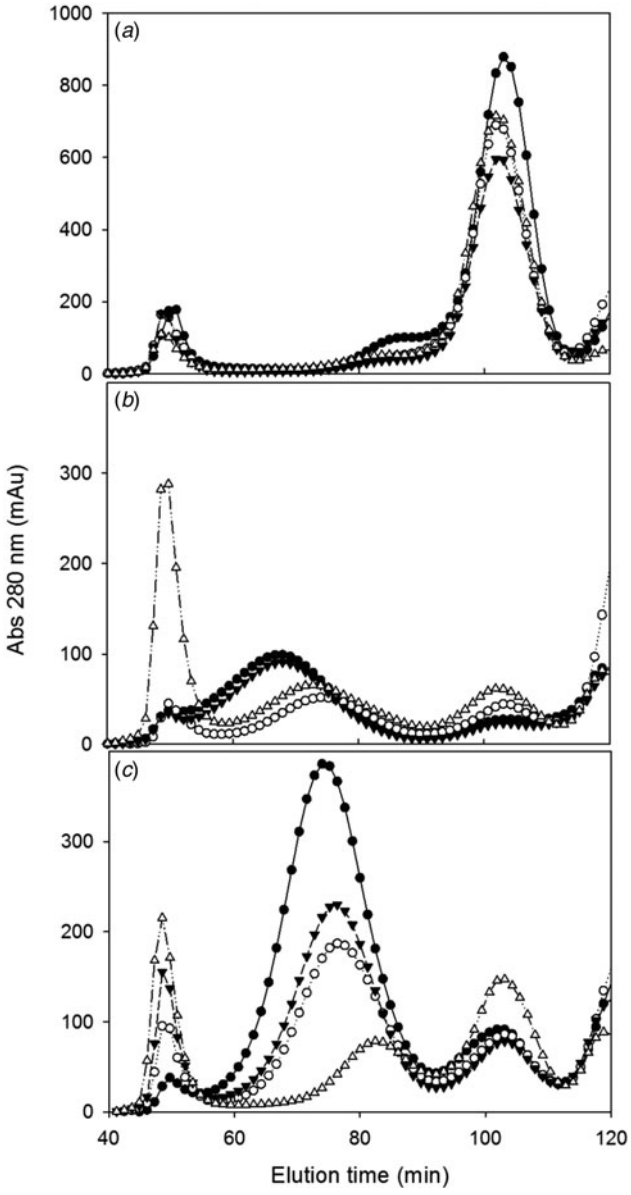

Fig. 3. Size Exclusion Chromatography of the soluble phase of original concentrates (a); after heating at 120 °C for 10 min (b); and after UHT at 136 °C for 6 s (c). ● UF; ○ MF; ▼ PF; Δ DF. Note the difference in scale in (a).

soluble phase. MF and PF had similar intensity while DF presented faint bands indicating lower protein content, in agreement with the lower peak of aggregates in Fig. 3c. All treatments showed similar composition of the aggregates, with whey proteins (α-lactalbumin and β-lactoglobulin) and κ-casein being the most prominent components. UF, MF and PF presented similar profiles, with the most intense bands present in the peak eluting between 70–80 min. Consistent with the peak elution profile showed in Fig. 3c, DF fractions presented a delayed elution profile and much lower intensity bands compared to the other treatments.

It is also important to note that α- and β-casein were only present on the last band of UF and MF samples (80–90 min), indicating their presence in the smaller, soluble aggregates eluting close to the native whey proteins. The results shown in Fig. 4 for fractions eluted from the supernatant of UF concentrates are in agreement with previous reports on UF aggregates after heat treatment (Li *et al.*, 2015). The characteristics and size of formed aggregates reinforce the importance of processing history on serum composition with consequences on concentrates properties and the higher heat stability of diafiltered sample.

In conclusion, all fresh milk protein concentrates, regardless of the membrane treatment applied, showed good heat stability, with minimum HCT of 30 min when heated at 120 °C. The retentates obtained using DF showed a significantly higher HCT than the concentrates obtained only using UF or MF or those where permeate was used as diafiltration medium. This is due to the removal of calcium and phosphate from the soluble phase, together with the higher pH and lower amount of lactose. These results showed that fresh concentrates do not show the same heat stability behavior than those reported widely in the literature, mostly reconstituted from powders. Analysis of the heat-induced aggregates present in the unsedimentable phase of these retentates showed important differences between different heating treatments (batch 120 °C for 10 min *vs.* UHT). There were significantly higher levels of soluble complexes present in UHT treated retentates, and it was also concluded that the composition and size of the heat induced whey protein aggregates was also different depending on the membrane processing history of the retentates, which impacts the final stability of the samples with possible impacts on storage. This study brings novel understanding of the heat stability of fresh concentrates, and it was concluded that by modulating the conditions during membrane filtration and the ionic composition of the serum phase it is

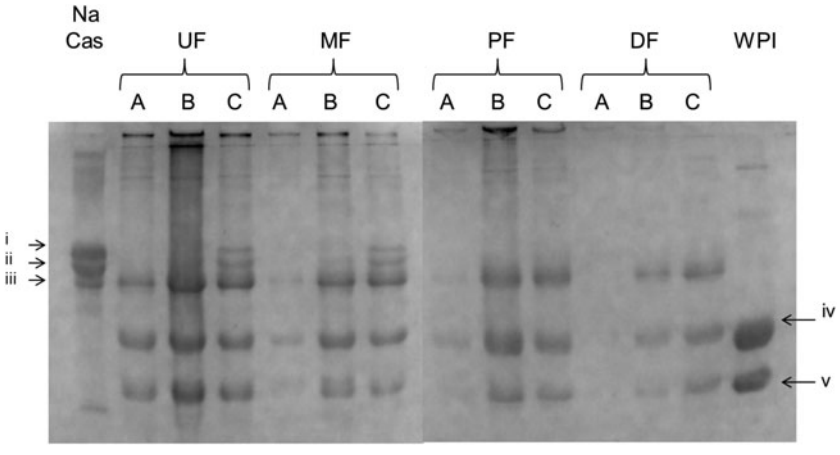

Fig. 4. SDS-PAGE patterns under reducing conditions of the serum in UHT treated samples collected from size exclusion chromatography at elution time ranging from 60–70 min (a), 70–80 min (b) and 80–90 min (c). The bands in the gels are identified as (i) αs-casein; (ii) β-casein; (iii) κ-casein; (iv) β-lactoglobulin; and (v) α-lactalbumin.

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possible to improve the heat processing properties of milk concentrates. More work is needed to better understand how the aggregates present in the unsedimentable fraction may affect commercial shelf life, or other important technological functionalities.

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