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Rennet coagulation and calcium distribution of raw milk reverse osmosis retentate

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ABSTRACT

Reverse osmosis (RO) filtration at the farm is a method for reducing transport costs; this study investigated cheese making properties of RO concentrated milk. In this study, several combinations of concentration factor and rennet concentration were examined based on caseino-macropeptide (CMP) release and rheological attributes of the coagulum. Furthermore, the calcium distribution of retentate compared with raw milk was included in the study, as a possible explanation of observed differences. The results showed a clear influence of the ratio between rennet concentration and retentate dry matter on CMP release rate and coagulation onset. The calcium distribution shifted to a larger fraction of colloidal calcium in the retentate samples compared with raw milk.

1. Introduction

Reverse osmosis (RO) membrane filtration on the dairy farm is regarded as an option for saving transport costs and increase bulk tank capacity (de Boer & Nooy, 1980; Garcia & Medina, 1988), and one of the obvious utilisations of this retentate would be in cheese production. In modern cheese production, milk is concentrated and standardised according to protein content, to save rennet and cheese vat capacity. Ultra-filtration (UF) is often used to concentrate milk in this standardisation process. However, the UF process changes the milk composition, by allowing, e.g., lactose and ions to filter through the membrane as part of the permeate (Brans, Schroën, van der Sman, & Boom, 2004). Compared with using non-concentrated milk for cheese production, it could be assumed that cheeses produced from UF retentate have different properties, and further on, that cheese from RO versus UF retentate differs in cheese making properties due to altered milk component concentrations.

Casein is the group of milk proteins that makes the network of the basic cheese matrix. They exist as a micellar colloidal structure of α_{S1}-, α_{S2}-, β- and κ-caseins. The exact arrangements of the casein species are still being debated. However, the consensus is that α-caseins give the "framework", β-casein is dynamically drifting through the micellar structure and κ-casein is positioned primarily in the outer layer (Dalgleish & Corredig, 2012). The micelles are kept suspended in the water phase through a negative charged electrostatic repulsion and hydrophilic characteristics. This is especially due to the properties of the outer κ-casein (Fox & McSweeney, 1998; McSweeney & Fox, 2013).

Chymosin (EC 3.4.23.4) is an aspartic protease, commonly added to induce rennet coagulation of the milk during cheese production. The coagulation of milk is normally regarded as a two-phase process. The first phase is the enzymatic hydrolysis of κ-casein into CMP in the soluble fraction and para-κ-casein polypeptide being incorporated into the cheese matrix; the cleavage reduces the electrostatic repulsion between the casein micelles, and thus enables the casein to form a network structure. The second phase is the actual aggregation of the remaining insoluble part of the casein micelles (without CMP), and normally starts when approximately 70% of the κ-casein has been hydrolysed (Walstra, Wouters, & Geurts, 2006).

Calcium is overall of vast importance for the coagulation properties in milk. Bovine milk calcium exists as an equilibrium between colloidal calcium phosphate (CCP) and the serum calcium, where it is found as bound to various biomolecules, especially citrate and other organic acids, or as the free Ca^{2+}. The distribution is temperature dependent and reversible when re-heating after, e.g., cold storage (Malacarne et al., 2013). Lowering the pH furthermore results in higher Ca^{2+} activity due to higher solubility of the CCP complexes from the micellar phase (Koutina, Knudsen, Andersen, & Skibsted, 2014). Total Ca in milk is around 1200 mg L^{-1}, corresponding to approximately 30 mM, and of this approximately 20 mM

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is associated with the micelles. Of the around 10 mM in the serum phase, the native level of Ca^{++} constitutes around 2 mM, but also in some cases extra is added. Legally up to 20 g $CaCl_2$ per 100 L of cheese milk may be added (corresponding to approximately 2 mM increase in Ca) during the cheese making process to facilitate the aggregation (Lucey & Fox, 1993), but often less is added. Taken together, calcium in the ionic form is crucial for the aggregation process in the second phase of coagulation. In addition, micellar calcium is important for the integrity of the CN micelles and for their coagulation properties (Udabage, McKinnon, & Augustin, 2001).

When variations in coagulation properties are observed, it is meaningful to determine whether the change is due to first or second phase. This will allow more targeted actions. The distinction between first and second phase can be made by comparing the rate of CMP release on one hand, representing the first phase, with rennet coagulation time (RCT) and the curd firming rate (CFR) on the other hand, representing the second phase (Frederiksen et al., 2011a). Sandra, Ho, Alexander, and Corredig (2012) concluded that calcium activity plays a role only during the second phase of the coagulation process, and did not have direct influence on the first phase. It can be hypothesised that the calcium distribution and the general ion strength may change during RO filtration, and thus affect the second phase of coagulation.

During cheese production, not only the rennet coagulation time is of importance, but also the rate by which the coagulum is formed and the final firmness. Frederiksen et al. (2011b) and Logan et al. (2015) described in their studies that the size of both the casein micelles and fat globules have an influence on the development of curd firmness, and thus giving higher cheese yield. The combination of large fat globules and small casein micelles gave the highest curd firming rate, and the combination of small fat globules and large caseins the lowest. They ascribed this mechanism to smaller casein micelles being able to pack closer together, whereas the fat might only play a secondary role. These interactions could also be affected in a concentrated system, where the components are closer together and the water activity lower. The aim of this study was to examine the cheese making properties of RO retentate produced from raw milk at a farm. This includes the effects of the calcium distribution on the chymosin induced coagulation and further on the rheological behaviour.

2. Materials and methods

2.1. Raw milk samples and retentate production

The RO membrane filtration of the raw milk was conducted at Danish Cattle Research Centre (Aarhus University – Foulum, Tjele, Denmark), as an inline process with a 3.8″ pHt spiral wound membranes (Alfa Laval, Lund, Sweden), with a total surface area of 4.7 m^2. Pressure across the membranes was 30 bar, and the process temperature was kept at 4 °C. A closer description of the equipment and filtration process has been published by Sørensen, Jensen, Ottosen, Neve, and Wiking (2016). Samples of raw milk were acquired before the start of filtration. First, valves on the filtration equipment were set to 1.5 VCF and subsequently to 2 VCF. Retentate samples were acquired from both 1.5 VCF and 2 VCF after 30 min processing to ensure a stable process. The Danish Cattle Research Centre (Aarhus University – Foulum, Tjele, Denmark) supplied the raw milk (Danish Holstein breed) for the experiments. Three hundred litres of milk were collected from the bulk tank just after the morning milking by herringbone parlour milking system, and poured into the balance tank of the filtration plant. The experimental production was conducted as triplicates carried out at separate days.

2.2. Milk and retentate composition

The overall composition of the retentate, permeate and raw milk was measured by FT-IR (MilkoScan FT2, Foss, Hillerød, Denmark), giving values of dry matter content, fat, protein, lactose, and solids non-fat.

After 24 h of storage at 4 °C, samples of raw milk, 1.5 VCF retentate and 2 VCF retentate were skimmed by centrifugation (2000× *g* for 20 min at 4 °C), and removal of the fat phase. The milk serum phase was obtained by ultracentrifugation (Beckman–Coulter Optima L-80XP, Beckman Coulter Inc., Brea, CA) of skim milk at 100,000× *g* at 30 °C for 1 h in a T4-TI-70 rotor.

Calcium was measured as total, serum and ionic calcium, as published earlier (Poulsen et al., 2017). Before determination of serum calcium, the raw milk samples were prepared by ultracentrifugation and the serum phase was recovered. Briefly, total calcium of both skim milk, serum phase and retentate was measured by titration with ethylene-diamine-tetra-acetic acid (EDTA) after acidification to pH 4.3, centrifugation and collection of the supernatant. The titration was conducted by addition of 0.1 N borax buffer and measured with a calcium electrode (sclON 6.1241.070, Metrohm, Herisau, Switzerland) and a reference electrode (LL ISE Reference 6.0750.100, Metrohm) on an auto-titration system (862 Compact Titrosampler, Metrohm). Analytical replication was conducted as duplicates. The method for ionic calcium concentration measurements was based on work by Koutina Knudsen, Andersen, and Skibsted (2015) and Poulsen et al. (2017) using a Ca^{2+}-meter (LAQUA twin compact Ca^{2+} METER B-751, electrode model S050, Horiba, Kyoto, Japan) directly on the sample serum phase. The ionic strength obtained was converted to $[Ca^{2+}]$ through a standard curve of $CaCl_2$ solutions (Poulsen et al., 2017). The distribution of calcium fractions was calculated as follows:

$$[Ca_{\text{colloidial}}] = [Ca_{\text{total, skim}}] - [Ca_{\text{total, serum}}]$$

$$[Ca_{\text{serum bound}}] = [Ca_{\text{total, serum}}] - [Ca^{2+}]$$

2.3. Rheological measurements

2.3.1. ReoRox

The skimmed samples were pH adjusted to 6.5 with 10% (v/v) lactic acid and incubated at 33 °C for 30 min in a water bath. Chymosin (ChyMax, 200 IMCU mL^{-1}, Chr. Hansen, Hørsholm, Denmark) was diluted in Milli-Q ultrapure water (Millipore, Billerica, MA, USA) such that adding 20 μL to 10 mL milk would give 0.03, 0.04 and 0.05 international milk-clotting units (IMCU) as the final chymosin concentrations. Freshly diluted chymosin was added to the sample, mixed for 10 s and transferred to the ReoRox (ReoRox4, oscillatory rheometer, MediRox AB, Nyköping, SE) (1 mL in each of 3 channels). The ReoRox method has been fully described by Frederiksen et al. (2011a).

2.3.2. Oscillatory rheometry

Similar milk sample treatment as describe above was used for analysing the elastic modulus (G′) by a stress and strained controlled rheometer (AR G2TA Instruments, New Castle, DE, USA). The coagulating milk was measured for 40 min at 33 °C at an oscillatory strain of 6.89×10^{-5} rad at 1 Hz. RCT was chosen as the parameter from ReoRox to describe the second stage of coagulation. The G′ max value was not reproducible, and therefore not included, as the samples with firm gels would detach from the cup. The measurements were conducted as 3 technical replicates of 3 biological replicates, to a total of 9 measurements per combination of sample type and chymosin concentration.

2.4. CMP determination by liquid chromatography

The liquid chromatography (LC) method for CMP determination was adapted from Le et al. (2016). The samples of RO retentate of 1.5 and 2 VCF and raw milk were incubated at 33 °C for 30 min in a water bath prior to the start of the experiment, and pH was adjusted to 6.5 with 10% (v/v) lactic acid. Chymosin (ChyMax, Chr. Hansen, Hørsholm, Denmark) was diluted in milli Q water, such that adding 20 μL to 10 mL milk would give 0.03, 0.04 and 0.05 international milk-clotting units (IMCU) as the final chymosin concentration in each sample. One millilitre of milk sample was withdrawn at each time point, i.e., 0, 30 s, and 1, 2, 5, 10 and 20 min after addition of chymosin dilution, and mixed with 20 μL of pepstatin stock solution [1 mg of pepstatin A (Sigma–Aldrich, St. Louis, MO, USA) per millilitre of 10% (v/v) acetic acid in methanol], and placed on ice to inhibit the chymosin. The samples were carefully mixed with 100 μL of acetic acid (CH_3COOH; 10% for raw milk samples, 15% for 1.5 VCF retentate and 20% for 2 VCF retentate) to reach pH 4.6. After 2 min of incubation on ice, 100 μL of sodium acetate (CH_3COONa; 1 N for raw milk samples, 1.5 N for 1.5 VCF retentate and 2 N for 2 VCF retentate) was added as buffer, and the solution was mixed and centrifuged for 10 min at 10,000× *g* at 4 °C. The supernatants containing the CMP was recovered, and stored at −18 °C until further analysis of CMP. One hundred microlitres of supernatant was mixed with 300 μL 6 N guanidine hydrochloride (GdnHCl) and 6 μL 1 N dithioerythritol (DTE), and incubated in a shaker at 37 °C for 1 h, then centrifuged at 10,000× *g* at 7 °C for 10 min. The supernatant was filtered through filtervials (Mini-UniPrep syringeless filter device, 0.2 μm pore, PTFE filter media, Whatman, Maidstone, UK) and loaded into the LC. A commercial CMP (cGMP20, Arla Foods Ingredients, Nr. Vium, Denmark) was used as standard to confirm the retention times. The CMP standard was dissolved to a 5% (w/v) solution, and prepared with 6 M GdnHCl and 1 M DTE, according to the procedure described by Le et al. (2016). The samples were loaded (injection volume of 50 μL) into a LC-ESI/MS single Q-MS (Agilent LC 1100 series, Agilent Technologies, Palo Alto, CA, USA) using the procedure described by Le et al. (2016) and monitored at 214 nm. Data analysis was conducted through LC/MSD ChemStation (Agilent Technologies, Santa Clara, CA, USA). The CMP content was calculated as the curve area between 20 and 45 min retention time divided by the total curve area obtained between 20 and 75 min representing approximately total protein present in accordance with the elution times of detected compounds described by Le et al. (2016).

2.5. Statistics

The effects of VCF was analysed by the model: $\gamma_i = T_{(i)} + e_i$; i = sample 1,…,9, where γ was the value of the dependant variable and T was the effect of type in sample 1 to 9, and e_i as the residual error, with variance test with a significance level of $P < 0.05$. Using statistical analysis in this study was processed through the statistical program R 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria). Further, Tukey's honest significant difference test was applied (R package agricolae, version 1.2–4) to test effect between groups (raw milk, 1.5 VCF retentate and 2 VCF retentate) and treatments (IMCU).

3. Results

3.1. Composition and calcium distribution in milk and retentate

The raw milk used for the experiments had an average protein content of 3.71% and a total dry matter content of 9.6% (Table 1). The retentate from 1.5 VCF concentration had an average protein content of 5.79% and dry matter content of 13.68%, and the 2 VCF retentate had 7.42% protein and 16.9% dry matter. Thus, the actual concentration factor of 2 VCF was at 1.8–2, and the 1.5 VCF was 1.4–1.5. The permeate had no measurable dry matter content by FT-IR (results not shown). Concentrating the milk by RO combined with cooling resulted in a significant decrease in pH, from 6.68 to 6.52 after 2 VCF, measured after 24 h storage at 4 °C.

Total calcium content increased from 1589 mg L^{-1} in raw skimmed milk to 3210 mg L^{-1} in the 2 VCF retentate, and from 497 mg L^{-1} in serum prepared from raw milk to 867 mg L^{-1} in serum from 2 VCF retentate (Table 1). Ionic calcium content did, however, not change between the raw milk and retentate samples. The permeate had no measurable calcium content, neither bound nor ionic (results not shown).

3.2. Curd formation

Chymosin was added in three different concentration to both the raw milk, and the two retentates, which result in different ratios between enzyme and protein content as shown in Table 2. Results obtained from oscillatory rheometry showed that raw milk had a shorter gelation time compared with retentate concentrated by a VCF of both 1.5 and 2 (Table 3), measured as the time needed for the elastic modulus to reach 1 Pa. The ratio between chymosin concentration and sample protein content had a significant impact on the gelation time ($P < 0.001$) (Table 3). Thus, gelation time was observed to decrease with increase in chymosin concentration, and increase with increasing retentate protein content. After 40 min of reaction time in the rheometer, both retentate samples (1.5 VCF and 2 VCF) reached a higher elastic modulus (G′) compared with the raw milk samples, where chymosin concentrations of 0.04 IMCU and 0.05 IMCU were used (Table 3). At 0.03 IMCU the coagulation of the VCF 2 retentate samples were delayed to such a degree, that there was very limited gel formation after 40 min. Likewise, G′ at 40 min was significantly influenced by both the chymosin concentration and the sample protein content as well as the ratio between them ($P < 0.05$). An example of the elasticity during the coagulation process for the three different sample types: Raw milk 1.5 VCF retentate and 2 VCF retentate, with a chymosin concentration of 0.05 IMCU is shown in Fig. 1. The RCT obtained from the ReoRox measurements were in line with the results from the rheometer (Fig. 2). Fig. 2 shows the relation between RCT and the chymosin/protein ratio. A clear relation was found, implying that a higher chymosin/protein ratio provides shorter rennet coagulation time in both raw milk and retentate.

3.3. CMP release

Fig. 3 shows representative results of LC measurements of whey from a retentate sample (2VCF), a raw milk sample and a CMP standard. The peak at 33 min represents CMP variant A, while the peak at 42 min represents the CMP variant B, while the areas from 20 to 30 and from 44 to 55 min are protein fragments (Le et al., 2016). The peaks between 60 and 75 min correspond to α-lactalbumin and β-lactoglobulin (variants A and B). The CMP eluted between 20 and 45 min, and the ratio between CMP peaks and the total peak area was used to represent relative content of CMP. The retentate samples generally had a larger peak area of CMP compared with samples from raw milk, but the peak area from the integrated whey proteins were of relatively higher intensity compared with the CMP peaks. Thus, the CMP content relative to whey proteins seemed lower in the retentate samples. CMP variant A appeared to be of slightly higher abundance than CMP variant B, which is in accordance to the results of Jensen et al. (2015) on milk samples from Danish Holstein cows.

Table 1
Composition of milk samples as dry matter (DM), total protein, pH and calcium content together with distribution between stages (bound to casein micelle, bound calcium in the serum phase and ionic calcium).[a]

Component	Raw	1.5VCF	2VCF	Significance level
DM (g 100 g^{-1})	9.60 ± 0.055[a]	13.68 ± 0.381[b]	16.90 ± 0.221[c]	$P < 0.001$
Protein (g 100 g^{-1})	3.71 ± 0.078[a]	5.79 ± 0.324[b]	7.42 ± 0.276[c]	$P < 0.001$
pH	6.68 ± 0.026[c]	6.59 ± 0.017[b]	6.52 ± 0.026[a]	$P = 0.01$
Total calcium skim (mg L^{-1})	1588.87 ± 32.094[a]	2466.13 ± 86.472[b]	3208.62 ± 49.06[c]	$P < 0.001$
Total calcium serum (mg L^{-1})	496.52 ± 24.639[a]	684.57 ± 14.442[b]	867.24 ± 24.733[c]	$P < 0.001$
Ionic calcium (mg L^{-1})	100.61 ± 3.550[a]	107.27 ± 4.613[a]	106.16 ± 1.463[a]	NS
Casein bound calcium %	68.7	72.2	73.0	
Serum bound calcium %	24.9	23.4	23.7	
Ionic calcium %	6.33	4.35	3.31	

[a] The standard error is calculated between sampling days; differences between sample types are indicated as different superscript letters with a significance level below 0.05% (NS, not significant).

Table 2
Overview of ratio between chymosin concentration and average protein content of the samples used throughout this study.

IMCU	Raw	1.5 VCF	2 VCF
0.03	0.008	0.005	0.004
0.04	0.011	0.007	0.005
0.05	0.013	0.009	0.007

The amount of CMP released relative to integrated whey protein content was calculated for raw milk, 1.5 and 2 VCF retentate samples during the first 20 min of reaction time. Fig. 4 shows the CMP release at 0.05 IMCU during the initial 20 min of renneting. The relative amount of CMP released did not have a consistent pattern during the first 2 min of reaction time, whereas the time interval from 2 to 10 min displayed some degree of linearity. The same CMP release pattern was observed for all combinations of chymosin concentrations and milk or retentate samples (results not displayed). The retentate samples released relatively less CMP compared with the raw milk sample at a constant chymosin concentration. Furthermore, the relative amount of CMP found after 10 min of reaction time was found to be positively correlated ($P < 0.001$) with VCF/chymosin ratio (Fig. 4). The level of CMP relative to integrated whey protein content after 10 min reaction time was negatively correlated to the total integrated protein content and positively correlated to chymosin concentration. The slope of the linear area between 2 and 10 min was negatively correlated to the protein content of the samples ($P < 0.05$), with no significant influence of chymosin concentration. After 20 min, the chymosin reaction was stopped by pepstatin addition for all samples, since excessive gelation made sample withdrawal inaccurate.

Table 3
Gelation time, as the time needed to reach a G′ of 1 Pa, of the different sample types and G′ measured 40 min after addition of rennet to the samples with increasing rennet concentration. Results obtained from oscillatory rheometry.[a]

IMCU	Gelation time (Pa = 1) min			G′ (Pa) at 40 min		
	Raw	1.5 VCF	2 VCF	Raw	1.5 VCF	2 VCF
0.03	24.7 ± 4.74[ab]	31.9 ± 3.52[b]	38.9 ± 2.60[b]	27.7 ± 0.25[b]	27.7 ± 0.30[b]	4.4 ± 0.09[a]
0.04	17.7 ± 2.12[ab]	22.2 ± 3.38[ab]	27.6 ± 3.34[b]	50.5 ± 0.22[c]	73.3 ± 0.73[e]	101.1 ± 0.92[f]
0.05	13.9 ± 1.65[a]	16.9 ± 2.15[ab]	21.8 ± 2.21[ab]	66.9 ± 0.24[d]	177.2 ± 0.66[g]	210.9 ± 0.91[h]

[a] The standard error is calculated between 3 separate sampling days. Differences between sample types are indicated as letters with a significance level below 0.05%.

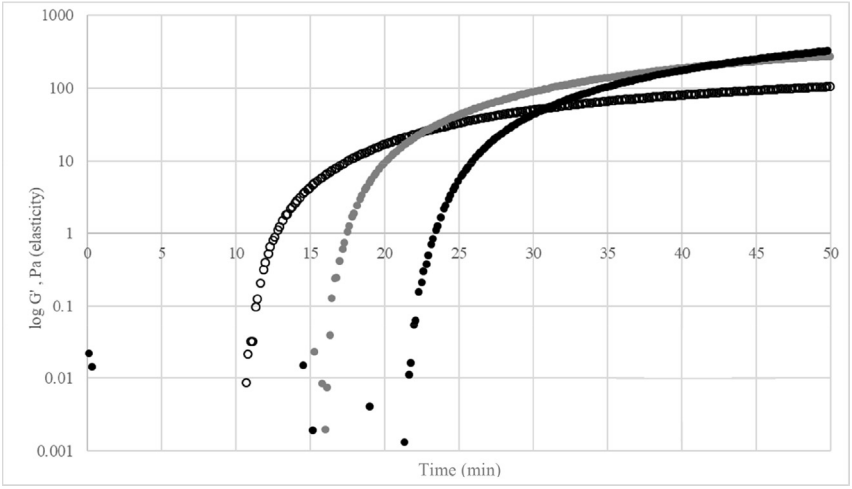

Fig. 1. Elasticity curve formed during the rennet coagulation process of raw milk (○), 1.5 VCF retentate (●) and 2 VCF retentate (●) with a rennet concentration of 0.05 IMCU. Data acquired through oscillatory rheometry.

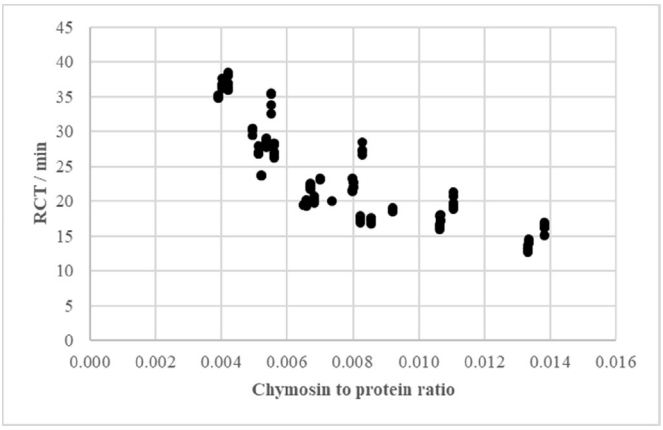

Fig. 2. Rennet coagulation time (RTC) as a function of chymosin to protein ratio in samples of raw milk and milk concentrated to 1.5 and 2 volume concentration factor, coagulated with chymosin solutions of 0.03, 0.04 and 0.05 IMCU. The results were obtained by ReoRox measurements.

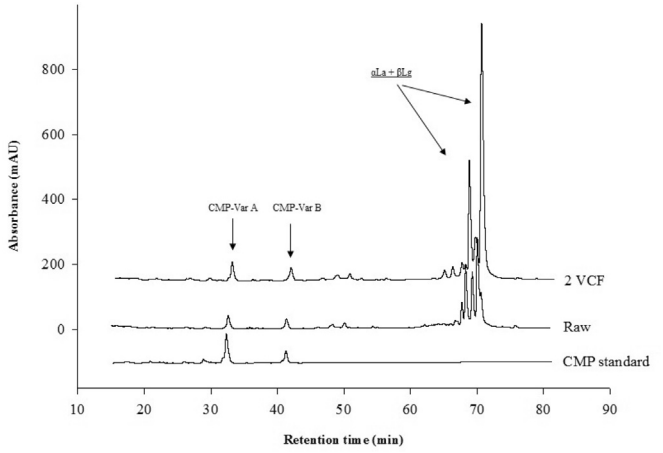

Fig. 3. LC absorbance spectrum at 214 nm of whey from a RO concentrated milk sample (2VCF) and a raw milk sample, reacted for 20 min with a 0.05 IMCU rennet concentration, compared to a commercial CMP standard.

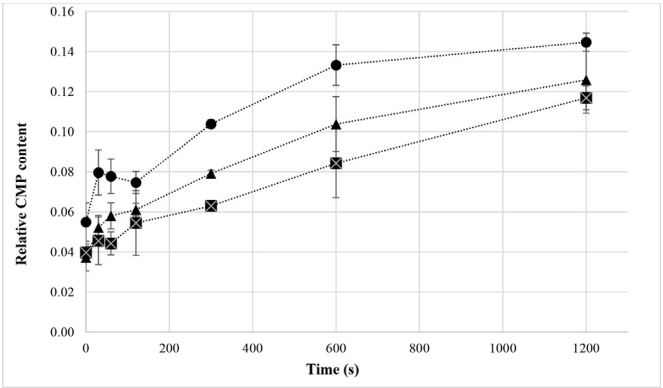

Fig. 4. CMP content, relative to integrated whey proteins, at certain time points during the renneting reaction on raw milk (●), 1.5 VCF retentate (▲) and 2 VCF retentate (⊠) with 0.05 IMCU rennet concentration. Standard error on results from the three trials is included as error bars.

4. Discussion

Several studies have dealt with coagulation and curd quality of UF retentate (Sandra, Cooper, Alexander, & Corredig, 2011; Sharma, Hill, & Mittal, 1993; Waungana, Singh, & Bennett, 1998), while studies with RO retentate are lacking. The primary distinction between UF and RO retentate is the ionic balance systems. Changes in ionic balance plays an important role during milk coagulation (Ferrer, Hill, & Corredig, 2008; Zhao & Corredig, 2016), and especially the calcium distribution and level of ionic calcium is known to greatly influence the coagulation properties (Klandar, Lagaude, & Chevalier-Lucia, 2007).

Release of calcium from the casein micelle into the serum is associated with a decrease in pH due to exchange between Ca^{2+} and H^{+}. This will typically, all other things equal, improve the coagulation properties as the optimum pH for chymosin is approached, as well as a neutralising effect of the casein micelle electrostatic repulsion due to the extra Ca^{2+}. In this study, the pH was standardised prior to the rheological tests, so the contribution of pH to rennet efficiency is not an explaining factor here. The observed decrease in pH during the RO process in the present study was likely a result of more Ca phosphate in the serum phase resulting precipitation of these, thus pushing the balance towards more H^{+} in the milk. Both protein content and pH of the raw milk used in this study are comparable with the levels found by Jensen et al. (2012) in milk with good coagulation properties from Danish Holstein-Friesian cows. As pH decreased due to the membrane filtration, it was not possible to make a complete distinction between concentration level and pH as the single factor influencing the calcium distribution. To do so, the pH should have been adjusted prior to determine the calcium content of the various phases.

The raw milk total calcium contents observed in this study, ≈ 1580 mg L^{-1} (Table 1), is in region of earlier reports (Jensen et al., 2012; Maciel et al., 2015), who reported total calcium concentrations in the range of 1360–1630 mg L^{-1}, depending on breed, for milk that exhibit good coagulation properties, and generally a high total calcium content has been correlated with higher curd firming rate and gel strength. Upon concentrating in the present study, the distributions of calcium fractions out of the total calcium content of the samples reveal that the ionic calcium decreased, while the casein bound calcium increased, and the serum bound remained constant. Since calcium has great impact on cheese making properties, it has been common to use it as a supplement to the cheese milk. The positive effect of ionic calcium is related to lowered surface charge of para-casein by improved interactions during aggregation (Dalgleish, 1983). Adding calcium to the system does not, however, result in the same calcium distribution pattern as observed in this study, though different conclusions have been drawn on the effect of calcium addition. Philippe, Gaucheron, Le Graet, Michel, and Garem (2003) observed an increase in serum and ionic calcium after supplementation rather than calcium binding to the casein micelles, whereas van Hooydonk, Hagedoorn, and Boerrigter (1986) found that addition of calcium to milk would result in calcium being absorbed by the casein micelles. The difference in observations to this present study might be ascribed not only to the calcium to protein ratio, which changes when calcium is added to the system rather than being constant as is seen for the retentate, but also to the time it takes before the calcium distribution reaches a new equilibrium after manipulations (18–24 h) (Koutina et al., 2014).

One way to evaluate coagulation properties is to measure elastic modules during formation of the coagulum. This provides comparable data of both the time it takes to form the coagulum (either the time it takes to change from mainly viscous to mainly elastic

properties, or the time to reach maximum coagulum strength) and the strength of the coagulum. These properties are important, and need to be considered in cheese production, to control the production process (Frederiksen et al., 2011a). In the present study it was observed that a higher dry matter content, and, thereby higher protein content as found in the RO retentates delayed the rennet coagulation, but increased CFR. However, the increase in RCT can be linked to the enzyme to substrate ratio, being lower in the retentate samples compared with the raw milk samples (Table 3). Similar effects on delayed coagulation onset and increased CFR have been observed in earlier studies of UF retentates. The higher CFR found for retentate can be explained by a stronger interaction between casein micelles with the initial distance between them being shorter (Karlsson, Ipsen, & Ardö, 2007). The results of CMP release indicates that κ-casein is hydrolysed at a lower rate in concentrated milk, presumably due to the decreased enzyme to substrate ratio. Karlsson et al. (2007) and Caron, St-Gelais, and Pouliot (1997) found increased gel firmness in rennet coagulated milk with elevated protein content by addition of milk powder. The results of these studies are very much in line with the observations made in this current study, even though the setup differs. The electrostatic interactions between casein micelles are likewise affected by the ionic balance of both Ca^{2+}, Na^{+} and pH (Karlsson, Ipsen, Schrader, & Ardö, 2005), all of which are influenced by the RO process.

To distinguish between effects of RO concentration on coagulation process to be related to changes in first or second phase of coagulation, the study on the hydrolysis rate of κ-casein were used to describe the first phase. Rennet induced aggregation of casein micelles normally happen when approximately 70% of the κ-casein has been hydrolysed as a pseudo first order reaction rate (Mellema, Leermakers, & De Kruif, 1999). Increasing the rennet concentration does, however, change the reaction rate with increased slope in the linear phase (Lomholt & Qvist, 1999; Sandra, Alexander, & Dalgleish, 2007). When comparing the coagulation time from Table 3 for samples added 0.05 IMCU with the released concentration of CMP in Fig. 4, it is clear that raw milk reaches a gel faster and also have a much greater CMP formation. 1.5 VCF and 2 VCF differ in gelation time, but at gelation point, approximately the same amount of relative CMP is found in the serum. The enzyme to substrate ratio is vastly higher for raw milk compared with the difference between 1.5 VCF and 2 VCF. Generally, when comparing the enzyme-to-substrate ratio of the samples (Table 2) used in the gelation time study (Table 3), it is clear that a direct correlation is found, making enzyme-to-substrate ratio the dominant factor influencing gelation time and κ-casein hydrolysis rate. Sandra et al. (2011) found a significant relation between protein content in UF retentate and gel strength, but not changes in the amount of κ-casein that needed to be hydrolysed for the samples to start to coagulate. Thus, the UF treatment did not seem to affect the first phase of rennet coagulation. In accordance with the present study, Lomholt and Qvist (1997) found that increased rennet concentration contributed to a higher curd-firming rate, even after all κ-casein had been hydrolysed, and thus speculated rennet to contribute to the final gel strength beside the hydrolytic process per se. Since the enzyme to substrate ratio was lower in RO retentate compared with raw milk, this effect has likely not been a contributing factor in the present study (Table 3). This confirms the theory that the changes in rennet coagulation properties of RO retentate was affected by the first phase trough the change in ratio between enzyme and substrate.

5. Conclusion

Concentrating raw milk by RO caused a shift in calcium distribution towards increase in colloidal calcium relative to ionic calcium, despite a slightly decreased pH. A difference in coagulation properties between RO retentate and raw milk was observed, leading to longer RCT, but higher CFR. By lowering the amount of chymosin added, the retentate samples showed decreased CFR and longer RCT. These properties were highly ascribed to the ratio between added rennet and the protein concentration in milk or retentate. Furthermore, the chymosin reaction rate was highly dependent on the sample protein content and the rennet concentration. Thus, it appears that the mechanical process of RO does not contribute to changes in coagulation properties per se, as it is primarily an effect of enzyme to substrate ratio, which could explain the delay in RCT. The increased CFR could be related to the calcium distribution and ionic balances, and this would require further studies to confirm.

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