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Effect of freezing temperatures and time on mineral balance, particle size, rennet and acid coagulation of casein concentrates produced by microfiltration

Sara Mohamed Gaber [a, *], Anne-Grethe Johansen [a, b], Reidar Barfod Schüller [a], Tove Gulbrandsen Devold [a], Elling-Olav Rukke [a], Siv Borghild Skeie [a]

[a] Faculty of Chemistry, Biotechnology and Food Science (KBM), Norwegian University of Life Sciences (NMBU), 5003, N-1432 Ås, Norway
[b] TINE SA R&D, 7 Kalbakken, 0901, Oslo, Norway

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ABSTRACT

The effects of freezing temperature and storage time on mineral partition, particle size, and rennet and acid coagulation properties of microfiltered casein concentrates (CC) were investigated. The total inorganic phosphate content of the CC decreased ($P < 0.05$) in the frozen samples, while the calcium and inorganic phosphate concentrations in the serum fraction were dependent on the freezing temperature; serum levels of Ca and Pi decreased ($P < 0.05$) during 12 days of storage at −20 and −40 °C, but increased at −80 °C. The frozen samples formed aggregates of different mass median diameters, and the average size was dependent on the temperature and days of storage. Rennet coagulation properties were significantly altered by storing at −80 °C. The storage modulus of acid gels made from frozen CC had higher values compared with acid gels made from unfrozen CC. The process of freezing thus influenced the stability of the CC.

1. Introduction

Frozen storage of milk and milk concentrates started in the mid-1930s as a method for preservation or extension of shelf life (Muir, 1984). Since then, this technology has been used most often for cheese manufacturers who face milk seasonality (de Garnica, Santos, & Gonzalo, 2011; Fava, Serpa, Külkamp-Guerreiro, & Pinto, 2013; Kljajevic et al., 2016; Zhang, Mustafa, Ng-Kwai-Hang, & Zhao, 2006). Currently, however, economic and sustainability considerations are encouraging consumers to freeze milk as a potential method for the reduction of milk waste throughout the value chain (Fisher & Whittaker, 2018).

Previous studies have reported chemical and physical alteration of milk components during freezing due to its high water content (Addeo et al., 1992; Holt, 1985; Van Den Berg, 1961). Frozen storage of milk causes a degree of phase separation between the ice formed and the unfrozen solid content. Casein micelles tend to lose their stability during frozen storage; this change can result in the eventual formation of flocculates or aggregates, and their dispersion depends on the thawing method (Goff & Sahagian, 1996). De la Fuente (1998) reviewed this destabilisation of milk and milk concentrates during freezing and summarised it as changes related to the mineral partition, pH and micellar casein particle size. Lactose has also been reported as an influencing factor on casein stability during frozen storage due to its crystallisation. Only 40% lactose crystallisation was reported necessary to destabilise the casein micelle (Goff & Sahagian, 1996; Muir, 1984). Theoretically, dairy products from which the original water volume has been reduced should be more stable during freezing than those with a high water content (Goff & Sahagian, 1996). However, several authors also reported changes in the stability of milk concentrated by vacuum evaporation during frozen storage (Saito, Niki, & Hashimoto, 1963; Wells & Leeder, 1963): a decrease in acidity, increase in viscosity with casein precipitation and formation of aggregates, and loss of soluble calcium and phosphate were observed.

Interestingly, a few studies have examined milk concentrated by membrane filtration, which results in concentrates with a completely different composition compared with concentrates obtained by vacuum evaporation. Voutsinas, Katsiari, Pappas, and Mallatou (1995a,b) reported that frozen ultrafiltered (UF) milk with added 0.5% NaCl was destabilised after 2 months of storage

* Corresponding author. Tel.: +47 67232596.
E-mail address: Sara.mohamed@nmbu.no (S.M. Gaber).

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at −20 °C. The product was commercially unfeasible for cheese production because of defects in the structure and organoleptic properties of the produced cheeses. Koschak, Fennema, Amundson, and Lee (1981) studied the protein stability of frozen UF skimmed milk at −20 °C through viscosity and solubility index measurements and by visual appearance. The authors reported that removing 10–30% of the original milk volume by UF improved the stability of the frozen UF concentrate by partial removal of soluble calcium, phosphorous and lactose. They also noted that removing more than 30% of the milk's original volume reduced the protein stability of the frozen UF concentrates because of increased removal of soluble phosphorous and therefore increased solubilisation of colloidal calcium phosphate. Furthermore, regardless of the total solid content of milk and skimmed milk, better protein stability was obtained by slow freezing (−20 °C) than by very rapid freezing (−78 °C). This difference was attributed to the increase of ionic calcium concentration in solution during rapid freezing, preventing precipitation of tricalcium phosphate and causing an unfavourable environment for protein stability. A combination of low lactose content and low soluble calcium concentration is expected to increase the stability of milk proteins during frozen storage.

Today, a growing industry has emerged based on frozen milk concentrates shipped worldwide for their diverse applications and potential usage as a dairy ingredient due to their specific functionality. While recent studies of milk concentrates produced by membrane filtration focus on their functional properties and industrial applications (Jørgensen, Abrahamsen, Rukke, Johansen, & Skeie, 2017; Karlsson, Ipsen, Schrader, & Ardo, 2005; Lu, McMahon, Metzger, Kommineni, & Vollmer, 2015; Lu, McMahon, & Vollmer, 2017; Neocleous, Barbano, & Rudan, 2002; Schreier, Schafroth, & Thomet, 2010), there is a shortage of fundamental information in the scientific literature regarding the stability of these concentrates undergoing freezing and storage.

The objective of this study was to investigate the effect of freezing temperatures on microfiltration (MF) milk casein concentrates (CC) during the initial 15 days of storage. In the early days of frozen storage, gradual ice formation and solid concentration is still most probably occurring, thus affecting the casein micelle stability. The study focuses on changes in mineral balance, particle size, degree of destabilisation and coagulation properties.

2. Material and methods

2.1. Manufacture and preparation of casein concentrates

Three batches of raw bovine milk were obtained from the Animal Production Experimental Centre (SHF) at the Norwegian University of Life Sciences within 1.5 months (during January–February), to avoid seasonal variations. SHF has approximately 130 lactating cows of the Norwegian Red breed. The milk was separated (Westfalia Separator AG, MSD50-01-076, Oelde, Germany) at 55 °C into skimmed milk and cream. Pasteurisation of the skimmed milk was performed at 73 °C for 15 s using a plate heat exchanger (A3-HRB, Alfa Laval, Lund, Sweden) prior to MF. Pasteurised skimmed milk was fractioned into CC using a 0.14-μm ceramic membrane (INSIDE CéRAM™, TAMI Industries, Nyons, France) at uniform trans-membrane pressure (UTMP) 46.3 ± 5.1 kPa and average flux of 66 ± 4.5 L h^{-1} m^{-2} to a volume concentration factor of ~2.5 at 50 °C (Svanborg, Johansen, Abrahamsen, & Skeie, 2014).

The macro-composition of the retentate during the MF process was determined with a MilkoScan FT1 (CombiFoss 6500, Hillerød, Denmark) using Fourier-transform infrared analysis, as a fast method to monitor protein concentration.

When the protein concentration reached 8.0 ± 0.1%, aliquots of 10 L of CC were collected. If the protein concentration became too high during collection of the sample, dilution with permeate was made to adjust to the target concentration, and batch 2 and 3 required dilution with permeate. The casein concentrates were cooled to 4 °C prior to freezing. Before freezing, a sample of CC was taken for immediate analysis (0 day) and was further considered as a reference. The remaining CC were divided into 50-mL Falcon tubes and frozen at one of three different freezing temperatures: −20, −40 and −80 °C, representing slow, medium and rapid freezing speeds. The samples were analysed after 3, 6, 9, 12 and 15 days of frozen storage.

The effects of three different thawing methods were tested: room temperature, overnight at 4 °C and by warming in a water bath to 40 °C, on samples frozen at −40 °C and compared with unfrozen samples. The average particle size in nm was 161.8 ± 7.9, 159.6 ± 8.5, 159.1 ± 9.3 and 153 ± 1.6 for samples after room temperature, overnight at 4 °C, water bath to 40 °C and the reference, respectively. The ionic Ca level in mmol was 2.09 ± 0.00, 2.42 ± 0.02, 2.29 ± 0.01 and 2.48 ± 0.05 for room temperature, overnight at 4 °C, water bath to 40 °C and the reference, respectively. Given the non-significant change between the treatments of the frozen samples for particle size measurement and ionic Ca content for samples thawed overnight at 4 °C and the reference, 4 °C overnight was chosen as the standard thawing method prior to analysis. Methods for measurements are described below.

2.2. Analyses

The moisture content in the CC batches and frozen samples was determined according to IDF method 26A (IDF, 2010). The total contents of Ca, P, K, Na and Mg in the reference and frozen samples were quantified by inductively coupled plasma mass spectrometry (ICP-MS), as described by Jørgensen et al. (2015). To measure the mineral content in the serum phase, an ultrafiltration (UF) of the CC was performed for each freeze-thawed sample using a lab-scale UF system with 500-kDa membrane filters using a method adapted from Ketto, Abdelghani, Johansen, Øyaas, and Skeie (2019) with pre-heating to 30 °C and a filtration temperature of 30 °C. The collected UF-permeate was then used for serum (soluble) mineral analysis using ICP-MS. The UF permeate was also analysed to verify casein leakage from the casein micelles of the CC, using capillary electrophoresis (CE) as described by Jørgensen et al. (2016). The micellar mineral fraction was calculated as the difference between the total and the soluble mineral content.

Inorganic phosphate (Pi) levels in the reference, freeze-thawed CC samples and UF permeates were analysed as described by Izco, Tormo, Harris, Tong, and Jimenez-Flores (2003). The method was adapted to an Agilent (G1600AX) Capillary electrophoresis (CE), with Agilent ChemStation software (Agilent technologies, Waldbronn, Germany). Concentrations from 0 to 4.41 mmol of KH_2PO_4 were used for peak identification and the creation of a standard curve (R^2 = 0.9) for quantification. A solution of 0.735 mmol KH_2PO_4 was used as an external standard with every sample run. Samples were prepared by mixing 12 μL of thawed CC with 1-mL sample buffer, then filtered using a 25-mm syringe filter with 0.2-μm cellulose acetate membrane (VWR, Radnor, PA). Colloidal inorganic phosphate level was calculated by subtracting the total inorganic phosphate content from the serum inorganic phosphate.

Calcium ion activity was detected using an Orion 97–20 calcium ion selective electrode (Calcium ionplus^R Sure-Flow^R Plastic Membrane Combination ISE, Thermo Scientific, Chelmsford, USA) with a mV meter (PHM290, pH-STAT Controller, MeterLab™, Radiometer Analytica, Copenhagen, Denmark) as described by the

manufacturer's user guide. Serial dilutions of a calcium standard solution were measured before and after samples.

The particle size distributions of the CC samples were analysed by two different methods. The first of these was using a Zetasizer 3000HS (Malvern Instruments Ltd., Malvern, UK) for testing the thawing methods, the average casein micelle size of the thawed CC was determined by dynamic light scattering at 25 °C. Samples were prepared according to the method of Devold, Brovold, Langsrud, and Vegarud (2000). All samples were run in triplicate. The second methods was using a Malvern Mastersizer 3000 (Malvern Instruments Ltd) for testing of the frozen CC samples after thawing, the volume size distribution and the mass median diameter (D_{50}) were measured by laser diffraction (LD). The CC samples were pipetted drop-by-drop into a Hydro LV chamber with distilled water to achieve an obscuration of 6–8% at a 0.003 particle absorption index, 1.33 dispersant refractive index and 1.49 casein particle refractive index, as described by Logan et al. (2014). Continuous stirring at 1500 rpm took place in the chamber while adding the sample. The diluted sample was circulated through the wet cell, 10 readings were recorded, and all samples were run in triplicate. The particles showed a bimodal size distribution, and the results were thus split into two subsets: particle size <10 μm (subset a) and particle size >10 μm (subset b). The mass median diameter (D_{50}) was calculated for each subset. D_{50} is a numerical value that indicates that 50% of the particles are smaller and 50% are larger in diameter ($D_{50\text{-a}}$ for particles <10 μm and $D_{50\text{-b}}$ for particles >10 μm).

Acid coagulation properties were measured using a MCR 301 Rheometer with a bob (C-CC27/Ti diameter: 26,657, length: 40,003) and cup (C-CC27/T200/Ti diameter: 28,926 mm) measuring geometry (Anton Paar GmbH, Graz, Austria). Prior to measurement, the thawed CC sample (30 mL) was kept in a water-bath at 32 °C for 30 min. Further, 1.35 g of glucono delta lactone (GDL) was added, and the sample was stirred for 20 s. Then, 15 mL was transferred into the rheometer cup for immediate measurement, while in the remaining 15 mL, an immediate continuous pH measurement was started using a 742020 HACH sensION^{TM}+pH31 meter with 5011T probe (LANGE GMBH, Dusseldorf, Germany) connected to LabCom V2.1 software (Hach, lange GMBH, Germany). Small-amplitude oscillatory measurements were performed at 32 °C with a very low constant strain of 0.001 within the viscoelastic range, and a constant frequency of 1 Hz was applied to avoid any influence on the gel formation properties. Storage modulus (G′), loss modulus (G″), phase angle (δ) and the complex viscosity (η′) were recorded for 45 min. Each sample was analysed at least in duplicate; that is, more replicates were run when the replicates did not follow the same coagulation pattern. A modified Gompertz equation was used Tjørve and Tjørve (2017), as described by Gaber, Johansen, Skeie, Rukke, and Schuller (2019), to fit the G′ curves versus time data, allowing an estimation of the start time of coagulation (ACT) and the G′ value at 45 min. Samples with pH end values below ~4.6 or above ~5.1 were considered outliers and were later excluded from data analysis.

The effect of freezing on the pH of the CC solution was measured at 30 °C after thawing using the same sensION™ pH meter as for acid coagulation. The instrument was calibrated daily prior to analysis.

Rennet coagulation was measured using a Formagraph (Lattodinamografo; Foss Italia SpA, Padova, Italy) as described by Inglingstad et al. (2014). Firstly, 10 mL of a thawed CC sample was incubated at 32 °C for 30 min in the sample cuvette, followed by addition of 200 μL of rennet (CHY-MAX; Chr. Hansen A/S, Hørsholm, Denmark) previously diluted 1:50 in acetate buffer (pH 5.6), and the sample was immediately analysed for 45 min at 32 °C. Rennet clotting time (RCT; min), curd-firming time shown as the time from RCT until the width of the bifurcate was 20-mm (k20; min), and curd strength in mm distance of the bifurcate after 30 min coagulation (a30) were measured.

2.3. Statistical analysis

Statistical analyses were performed using packages and functions in R Studio (version 1.1.456). The data were analysed for significant differences between temperatures and storage days at 95% confidence level. Significant differences were declared at $P < 0.05$, using the Mixed Model ANOVA package. Post hoc means comparisons were made based on the P-value ($\alpha = 0.05$) using the Tukey-Kramer adjustment to obtain differences of means. Data were fitted using a linear mixed effect model, where batch was considered a random factor, and temperature (0, −20, −40 and −80 °C), storage days (0, 3, 6, 9, 12 and 15 days) and their interaction were used as the fixed variables.

A modified Gompertz model, as reviewed by Gaber et al (2019), was used to estimate the fit of G′ versus time data. The output was fitted using non-linear regression, and the differences were compared using a Tukey-Kramer test in MATLAB (MathWorks Natick, MA, USA). R-squared correlation coefficients of the factors versus the variables, as well as the variables versus the variables, were calculated using the regression equation function in Microsoft Excel 2016.

3. Results

3.1. Composition of casein concentrates

Table 1 shows the average of the total solids, protein, lactose and fat contents as measured by FTIR of the three batches of CC during MF. The moisture content of all CC batches was 85.5 ± 0.1% as measured by IDF 26A method, the calculated total solid content was 14.5 ± 0.1% and they all had a pH of 6.60 ± 0.01. Freezing temperature and initial frozen storage time (15 days) did not significantly influence the pH of the thawed samples. The CE analysis showed no leakage of casein from the micelle into the serum phase of CC (results not shown).

The mean total mineral contents of the three CC batches are shown in Table 1. The second CC batch had a significantly higher Ca and P content than batch 1 and 3, and the Ca content was strongly correlated ($R^2 = 0.9$) to the P content in all the samples. The total mineral content of the CC batches was not affected by freezing (as shown in Tables 2 and 4 for Ca and P, respectively). However, as shown in Tables 2 and 4, the distributions of micellar and serum Ca and P in the freeze-thawed samples were strongly affected by the

Table 1
Main composition as measured by FTIR and mineral composition as measured by ICP of the 3 batches of casein concentrate.[a]

Parameter	Casein concentrate
Composition by FTIR %	
Protein	7.97 ± 0.12
Fat	0.20 ± 0.00
Lactose	4.46 ± 0.01
Moisture content %	85.5 ± 0.10
Total minerals (mmol)	
Calcium, Ca	71.53 ± 1.45
Potassium, K	48.27 ± 0.12
Magnesium, Mg	7.53 ± 0.15
Sodium, Na	17.03 ± 0.12
Phosphate, P	65.47 ± 1.85

[a] Main composition was measured before freezing, while mineral composition is the mean (±standard deviation) of samples frozen at −20, −40, and −80 °C. Batches diluted with MF permeate to regain 8.0 ± 0.1% protein concentration.

Table 2
Calcium fractions (mmol) of freeze-thawed casein concentrates frozen at different temperatures for 3–15 days.[a]

T °C	Ref	−20 °C					−40 °C					−80 °C				
Day	D0	D3	D6	D9	D12	D15	D3	D6	D9	D12	D15	D3	D6	D9	D12	D15
Ca_T	71.5 ± 1.2	71.2 ± 1.2					72.0 ± 1.6					71.4 ± 0.9				
Ca_{Sol}	8.8 ± 0.5^{a}	6.5 ± 0.1^{c}		6.9 ± 0.2^{c}	5.3 ± 0.0^{d}		6.7 ± 0.2^{c}		6.0 ± 0.3^{d}	5.6 ± 0.0^{d}		6.9 ± 0.4^{c}		7.0 ± 0.1^{bc}	7.4 ± 0.0^{b}	
Ca_M	62.6 ± 0.9^{a}	64.6 ± 1.1^{ab}		64.3 ± 1.4^{ab}	65.9 ± 1.2^{b}		65.3 ± 1.4^{b}		66.0 ± 1.8^{b}	66.4 ± 1.6^{c}		64.5 ± 0.5^{ab}		64.3 ± 1.0^{ab}	63.9 ± 0.9^{abc}	
Ca^{2+}	2.3 ± 0.7	2.2 ± 0.0	2.0 ± 0.0	2.1 ± 0.1	2.1 ± 0.0	2.1 ± 0.1	2.0 ± 0.3	2.1 ± 0.0	2.1 ± 0.1	2.2 ± 0.2	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.0	2.1 ± 0.1	2.1 ± 0.0	2.2 ± 0.0

[a] Abbreviations are: Ca_T, total calcium as measured by inductive coupled plasma-mass spectrometry (ICP-MS); Ca_{sol}, soluble calcium as measured by ICP-MS; Ca_M, micellar calcium ($Ca_T - Ca_{sol}$). Values are the mean of 3 batch replicates ± SD; significant differences ($P < 0.05$) between means of the reference and each of the temperatures, between the days at each temperature, between the same day at different temperature, are shown with different superscript letters within a row.

freezing temperature and freezing time. Tables 3 and 5 show the significant effects of the Ca and P fractions, respectively, when using different explanatory variables in the statistical models. The content of serum Ca and Pi decreased significantly during storage at −20 °C and −40 °C while, at −80 °C, a significant increase in serum Ca was observed at day 12 of storage. The total inorganic phosphate (Pi) content in the CC samples was significantly lower ($P < 0.05$) in samples that had been frozen compared with the reference (CC sample prior to freezing), and Pi significantly decreased more in the thawed samples throughout storage at −20 and −40 °C than at −80 °C. The Ca^{2+} content of the CC samples was not significantly influenced by freezing (see Table 2).

3.2. Particle size distribution

Results obtained by the Mastersizer indicated the presence of two distinct particle-size groups for most of the samples: a group with an average particle size <10 μm and another group with a particle size >10 μm. Fig. 1A shows two size classes (μm) of varying volume density (%) for the representative experimental samples and the reference. Two subsets were created to calculate the median mass diameter D_{50} for each size class: 1) $D_{50\text{-a}}$ for particles ranging from 0 to 10 μm and 2) $D_{50\text{-b}}$ for particles above 10 μm. Table 6 presents the average $D_{50\text{-a}}$ and their significances. Fig. 1B shows the effect of the freezing temperature and time on the larger particle size distribution ($D_{50\text{-b}}$) of the CC.

Freezing at −20 °C and below led to formation of aggregates, and the aggregates increased significantly in size in the thawed samples throughout the initial storage, mostly at freezing temperatures −20 and −40 °C. The $D_{50\text{-b}}$ of CC samples frozen at −20 and −40 °C increased significantly after the 9th day of storage. In contrast, the aggregates in the −80 °C samples were formed later and increased significantly after the 12th day of storage.

3.3. Acidification and rheological properties

Regardless of the freezing temperature and storage time, a linear relationship ($R^2 = 0.9$) was found between the start time of acid coagulation (ACT) and the gel firmness (G′): shorter ACT was correlated with higher storage modulus of the gel. Fig. 2 shows the average G′ curves during acid coagulation of thawed CC frozen after 3–15 days of storage at the three freezing temperatures. A larger variation in the replicability of acid coagulation between the freeze-thawed samples was observed for CC frozen at −20 and −40 °C than for CC frozen at −80 °C, resulting in higher standard deviations between the runs, supressing the possible significant differences. This is due to differences in water crystallisation and aggregate formation between the temperatures and days of storage, which are elaborated later in the discussion section.

Nevertheless, some trends could be observed. The thawed CC samples frozen at −20 °C had poor replicability when measured during the first days of freezing (<day 10), but consistency in replicability increased above 10 days of freezing. However, the gelation trend and average G′ for thawed CC samples measured after freezing at −20 °C for <10 days were similar to that of the CC reference, and the average G′ started to increase at prolonged storage at −20 °C. Freeze-thawed samples stored at −40° and −80 °C reached considerably higher G′ values than the reference, with the values increasing throughout the days of storage. Acid gels made from freeze-thawed CC samples frozen at −80 °C showed considerably higher average numerical G′ values, faster ACT and better replicability throughout the frozen storage period. Generally, frozen storage for longer periods (>10 day) improved replicability and storage modulus. During acid gelation, 87% of the

Table 3
Significant effects of the treatments on the calcium fractions using different explanatory variables in the statistical models.[a]

Factor	Item	Reference included		No reference included	
		P-value	Significant difference between treatment levels ($P < 0.05$)	*P*-value	Significant difference between treatment levels ($P < 0.05$)
1. T °C	Ca_{Sol}	<0.0001	0 > −20, −40, −80	<0.0001	−20, −40 < −80
	Ca_M	<0.0001	0 < −80, −40, −20 and −80 < −40	<0.0001	−20, −80 < −40
2. Days	Ca_{Sol}	<0.0001	0 > 3, 9,12	<0.0001	3 < 12 and 9 < 12
	Ca_M	<0.01	0 < 3,9,12	NS	
3. T °C × Day	Ca_{Sol}	<0.0001	Shown in Table 2 with different superscripts in a row	<0.0001	Shown in Table 2 with different superscripts in a row
	Ca_M	NS		NS	

[a] 1, using only temperature; 2, using only days of storage; 3, interaction effect of temperature and day. The two main columns show the effects when the reference was included or excluded in the model. T °C × Day interaction effect of temperature and day. NS, not significant (abbreviations for items as given in Table 2).

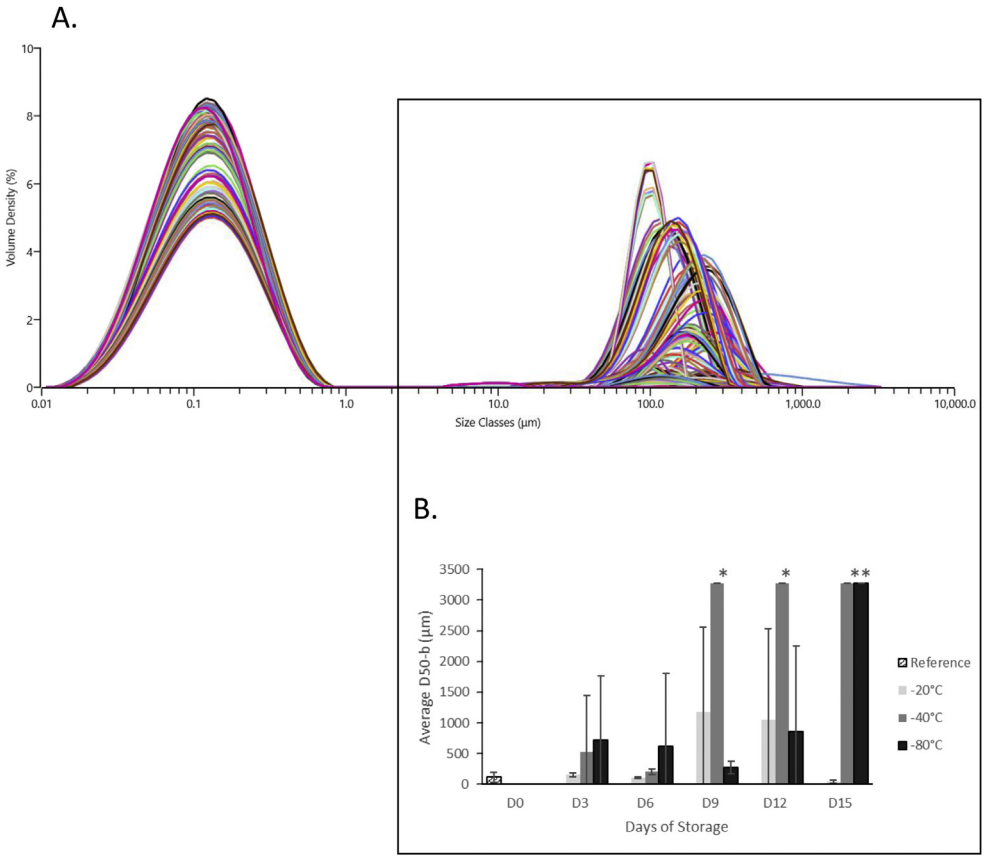

Fig. 1. Effect of freezing temperature and days on particle size distribution of casein concentrates as measured by Mastersizer. Panel A, the bimodal size distribution of all samples, showing two distinct peaks with particle size between 0.01 and 1.0 μm and one >10 μm. Panel B, the mass median diameter (D_{50}) of particle size > 10 μm of the reference (▨) and freeze-thawed samples −20 °C (□), −40 °C (■) and −80 °C (■) after 3–15 days of frozen storage. Mean-value of 3 batch replicates ± SD. An asterisk indicated occurred occasionally.

reference samples and the freeze-thawed CC samples frozen at −20, −40 and −80 °C (throughout the storage period) showed a similar acidification rate, with the pH end value ranging from ~4.6 to ~5.1 (Fig. 2).

Compared with the reference CC, the average end pH of the acid-induced gels made from freeze-thawed CC decreased with increased storage at the three experimental freezing temperatures (Fig. 3). It is important to mention that the samples omitted because their pH was out of range (below 4.6 and above 5.1) mostly had a corresponding G′ value that did not show an odd behaviour. These samples followed the same rheological pattern as their same sample replicates which had a pH profile within the predefined range. This observation confirms the absence of a correlation between the final pH and the structural development of the gel.

3.4. Rennet coagulation

The average RCT of the CC reference sample was 15.2 ± 1.3 min. Rennet clotting time of the freeze-thawed samples was not significantly influenced by freezing temperature and time (results not shown), but an instability was observed with the replication of the freeze-thawed CC samples. The average value of RCT for the freeze-thawed CC samples, however, stayed within the range of the reference. Samples with a short freezing time (day 3) at −20 °C or the longest freezing times (day 9, 12 and 15) at −80 °C obtained an average RCT equivalent to that of the reference. The curd of all samples required from 2 to 3 min after RCT to firm (K20). Samples of thawed CC from the first 3–6 days of freezing at −20 and −40 °C took a significantly ($P < 0.05$) longer time to obtain K20 than samples frozen for a longer time or frozen

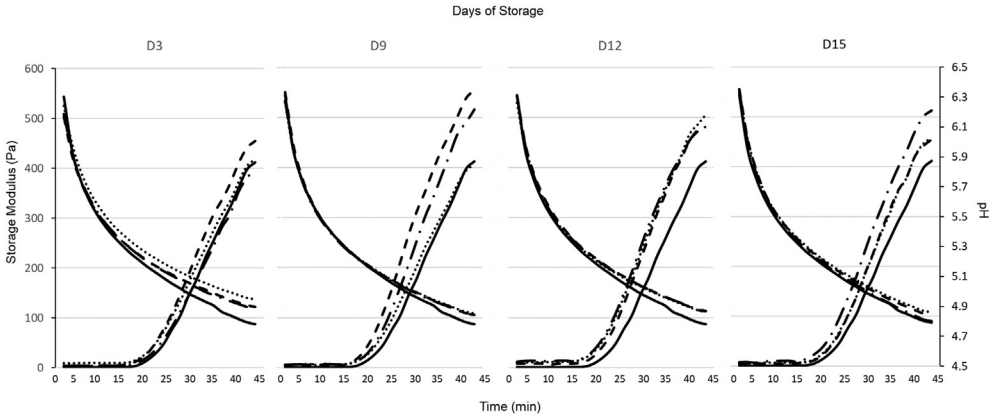

Fig. 2. Storage modulus [G'] of acid-induced gelation (left axis) of casein concentrates (CC); reference (——) and thawed samples of CC frozen at −20 °C (•••••), −40 °C (— · —), −80 °C (-----) for 3, 9, 12 and 15 days. pH curves during acid gelation shown on the right axis. Mean of 2–3 batch replicates (data for day 6 are not shown).

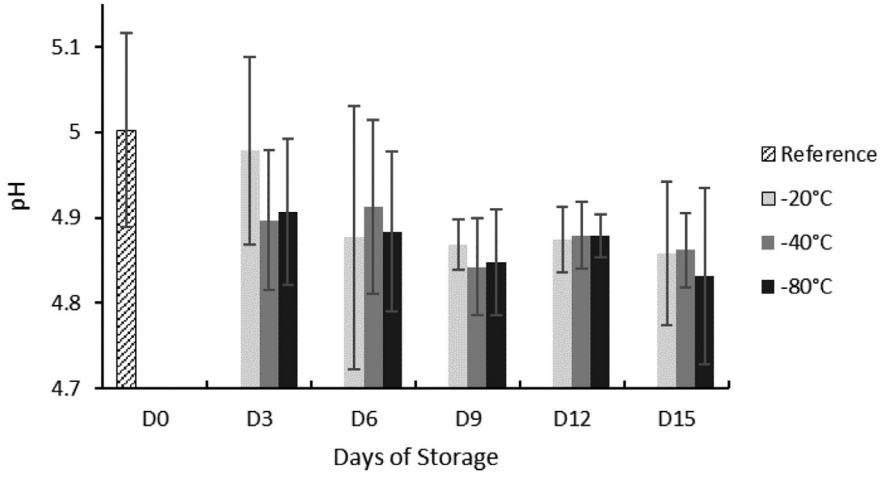

Fig. 3. pH end value of acid-induced gelation of milk casein concentrates; the reference (▨) and thawed samples of CC frozen at −20 °C (□), −40 °C (■) and −80 °C (■) for 3, 6, 9, 12 and 15 days. Mean of 2–3 batch replicates ± SD.

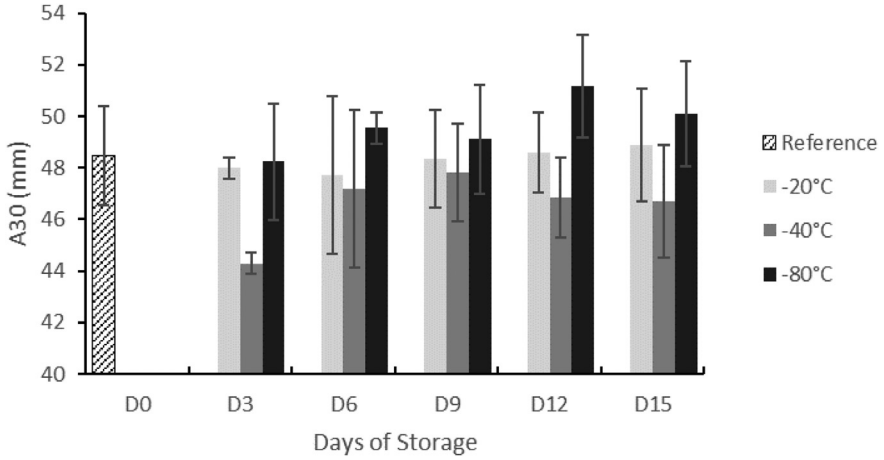

Fig. 4. Effect of freezing time and temperature on gel strength (A30) of rennet gels; the reference (▨) and thawed samples of CC frozen at −20 °C (□), −40 °C (■) and −80 °C (■) for 3, 6, 9, 12 and 15 days. Mean of 3 batch replicates (3 replicates per batch) ± SD.

at −80 °C, which were similar to the CC reference. The mean numerical values of curd strength (A30) for the thawed CC samples frozen at −80 °C were relatively higher than those frozen at −20 and −40 °C and significantly ($P < 0.05$) higher at day 12 (Fig. 4). The thawed CC samples frozen at −40 °C had the lowest curd strength, while the thawed CC samples frozen at −20 °C tended to obtain a similar curd strength as the reference throughout the experimental period (Fig. 4).

Table 4
Phosphate fractions (mmol) of freeze-thawed casein concentrates frozen at different temperatures for 3–15 days.

T °C	Ref	−20 °C					−40 °C					−80 °C				
Day	D0	D3	D6	D9	D12	D15	D3	D6	D9	D12	D15	D3	D6	D9	D12	D15
P_T	65.4 ± 1.6	65.2 ± 1.6					65.9 ± 1.9					65.3 ± 1.3				
Pi_T	59.4 ± 3.9^{a}	48.8 ± 2.6^{ab}	44.3 ± 8.3^{b}	57.9 ± 7.7^{a}	38.4 ± 8.7^{bc}	42.4 ± 18.7^{b}	44.5 ± 5.0^{ac}	56.4 ± 0.4^{ab}	51.1 ± 4.1^{ac}	40.0 ± 1.2^{c}	42.5 ± 11.9^{bc}	50.9 ± 1.1^{ae}	46.7 ± 0.0^{abe}	41.6 ± 3.2^{e}	51.3 ± 6.8^{ace}	45.7 ± 0.8^{ae}
Pi_{Sol}		11.3 ± 0.6^{e}		12.1 ± 0.4^{d}	9.2 ± 0.0^{b}		11.5 ± 0.7^{de}		10.2 ± 0.2^{c}	09.6 ± 0.0^{b}		12.0 ± 0.8^{be}		12.5 ± 0.0^{bd}	12.6 ± 0.0^{a}	
Pi_{col}		37.4 ± 3.0^{ab}		47.3 ± 10.0^{a}	29.1 ± 8.2^{b}		33.0 ± 5.2^{a}		40.8 ± 4.0^{a}	30.4 ± 1.2^{ab}		38.8 ± 1.3^{ac}		29.0 ± 3.2^{c}	38.7 ± 6.8^{bc}	
P_M		53.8 ± 1.0^{ab}		53.1 ± 2.0^{b}	55.9 ± 1.6^{a}		54.4 ± 1.4^{a}		55.6 ± 2.1^{a}	56.3 ± 1.9^{a}		53.3 ± 0.6^{b}		52.8 ± 1.2^{b}	52.7 ± 1.3^{b}	

Abbreviations are: P_T, total phosphorous as measured by inductive coupled plasma-mass spectrometry (ICP-MS); Pi_T, total inorganic phosphate as measured by capillary electrophoresis (CE); Pi_{Sol}, soluble inorganic phosphate as measured by ICP-MS; Pi_{col}, colloidal inorganic phosphate ($Pi_T - Pi_{Sol}$); P_M, micellar phosphate ($P_T - Pi_{Sol}$). Values are the means of 3 batch replicates ± SD; significant differences ($P < 0.05$) between means of the reference and each of the temperatures, between the days at each temperature, between the same day at different temperature, is shown with different superscript letters within the row.

4. Discussion

4.1. Influence of freezing on salt partition and particle size

The change in salt partition between the serum and micellar phase of the thawed CCs after freezing was related to the freezing temperature and duration of frozen storage. The micellar Ca content of the freeze-thawed CC samples was higher than in the unfrozen reference CC, and the micellar Ca and P content of the thawed CC samples frozen at −20 °C and −40 °C increased significantly more during storage than that of the CC samples frozen at −80 °C. This shift in mineral content from the serum phase towards the micelle has been reported as an incorporation of P and Ca into the micellar phase over time where tricalcium phosphate $Ca_3(PO_4)_2$ is formed from $CaHPO_4$ and $Ca(HPO_4)_2$; this change happens as the amount of solvent water is reduced due to ice formation (Fox, 2010; Muir, 1984). Jonkman, Walstra, van Boekel, and Cebula (1999) also reported lower salt content in ice-cream plasma compared with milk and concluded that, at a low temperature such as −10 °C, association of calcium phosphate with the micelles is enhanced. This mechanism could be compared with the reported amorphous calcium phosphate formation observed when precipitates of milk-derived calcium phosphate from UF form during lyophilisation (Mekmene et al., 2012). The amount of frozen water increases as the freezing temperature is reduced, leaving a smaller amount of water as solvent for lactose and salts at lower temperature (Keeney & Kroger, 1974). At −20 °C, approximately 12.5–14% of the total water remained unfrozen in concentrated (CF 3x) milk (Keeney & Kroger, 1974; Muir, 1984). At −40 °C and −80 °C, the unfrozen water percentage is expected to be even lower. The relatively smaller changes in salt partitioning of CC samples frozen at −80 °C is most likely attributed to the faster ice-formation process at −80 °C and less available unfrozen water (Goff & Sahagian, 1996) for ion exchange during freezing. Wendorff (2001) also reported a decrease of stability in raw ovine milk when freezing was performed at temperatures above −20 °C (−15 °C) compared with freezing below −20 °C (−27 °C), which most likely is a consequence of the amount of available unfrozen water.

The development of large particles in the freeze-thawed CC samples in the present experiment shows that destabilisation of the casein micelles most likely occurred during freezing. Several authors observed similar formations and gave them different names, such as flocculates or aggregates (Fox & McSweeney, 1998; Keeney & Kroger, 1974; Muir, 1984; Wendorff, 2001). According to Walstra, Wouters, and Geurts (2006), the nomination is dependent on the reversibility; flocculation is weak and reversible, while aggregation occurs when storage is prolonged and is irreversible. Aggregation causes an increase in viscosity and may lead to gel formation. The re-dispersion properties of the freeze-thawed CC samples were not measured, but a visual increase in the CC viscosity was noticed after thawing. Therefore, the term “aggregates” is adapted to describe these large particles. However, this use of this term does not exclude the possible presence of formed reversible flocs within the system. It would be of interest, for future research, to identify the particle types formed, perhaps through an optical imaging technique.

Fox and McSweeney (1998) suggested that aggregates are formed upon thawing; however, this experiment indicates that the freezing rate is also of importance for the formation of aggregates because the size of the aggregates, their volume density and frequency of formation depended strongly on the freezing rate and the duration of freezing.

Keeney and Kroger (1974) linked casein aggregation to lactose crystallisation. As more lactose crystallises, more colloidal calcium phosphate is formed due to the increase in the frozen water fraction

Table 5
Significant effects of the treatments on the phosphate fractions using different explanatory variables in the statistical models.[a]

Factor	Item	Reference included		No reference included	
		P-value	Significant difference between treatment levels ($P < 0.05$)	*P*-value	Significant difference between treatment levels ($P < 0.05$)
1. T °C	Pi_T	<0.05	0 > −20, −40, −80		
	Pi_{Sol}			<0.0001	−40 < −20 < −80
	P_M			<0.0001	−40 > −20 > −80
2. Days	Pi_T	<0.0001	0 > 3, 6, 12, 15		
	Pi_{Sol}			<0.0001	3 > 12 and 9 > 12
	P_M			<0.05	3 < 12 and 9 < 12
3. T °C × Day	Pi_T	<0.0001	Shown in Table 4 with different superscript in a row		
	Pi_{Sol}			<0.0001	Shown in Table 4 with different superscript in a row
	P_M			<0.05	

[a] 1, using only temperature; 2, using only days of storage; 3, interaction effect of temperature and day. The two main columns show the effects when the reference was included or excluded in the model. T °C × Day interaction effect of temperature and day. NS, not significant (abbreviations for items as given in Table 4).

Table 6
Mass median diameter of particles ranging from 0 to 10 μm (D_{50-a}) of casein concentrates, reference (D0) and frozen samples (−20, −40 and −80 °C) during frozen storage (D3–D15).[a]

T °C	D0	D3	D6	D9	D12	D15
Ref	114.6 ± 3.9[a]					
−20		118.0 ± 4.8[b]	116.4 ± 2.7[a]	116.7 ± 1.9[b]	116.3 ± 1.8[a]	117.2 ± 2.2[b]
−40		117.8 ± 3.8[b]	114.7 ± 2.7[a]	116.6 ± 1.7[b]	116.0 ± 2.7[a]	116.3 ± 2.4[a]
−80		116.3 ± 2.4[a]	114.0 ± 2.6[a]	118.4 ± 1.8[b]	113.7 ± 3.2[a]	116.6 ± 0.5[b]

[a] Values are the means of 3 batch replicates ± SD; significant differences ($P < 0.05$) between means of the reference and the interaction between days and temperatures is shown with different superscript letters.

and salt concentration. The precipitated calcium phosphate would then inter-link casein micelles and form aggregates of calcium caseinate-phosphate complexes. Jonkman et al. (1999) observed similar increase in micelle size of ice-cream plasma and explained them in terms of fusion of micelles due to decrease in freezing temperature. With greater micellar Ca and P contents, the average size of the aggregates increased in the thawed CC samples. The concentration of salts within the unfrozen water fraction starts prior to lactose crystallisation and develops greatly as lactose crystallise.

At a slow freezing rate, i.e., 20 °C, the ice formation is slow and therefore lactose crystals develop due to a higher amount of available unfrozen water compared with a faster freezing rate. An increased tendency of aggregate formation was observed for the thawed CC samples frozen at −20 and −40 °C, with a significant increase after the 9th day of frozen storage. At a faster freezing rate, −23 °C (Keeney & Kroger, 1974), lactose assumes an amorphous state and few or no crystals are formed due to lack of free water and high salt content. In contrast, in the thawed CC samples frozen at −80 °C, the size of the aggregates increased significantly at later days of frozen storage (after 12 days) compared with −20 °C and −40 °C. The aggregates could be observed visually, and their mechanism of aggregation seemed to be somewhat uncontrollable, presumably dependent on factors such as thawing, stirring or sample preparation. It is possible at this stage to conclude that formation of $Ca_3(PO_4)_2$ contribute to the formation of aggregates. Specific research on how freezing affect the micelle size itself is limited. Normal cooling conditions cause β-casein leakage (Creamer, Berry, & Mills, 1977; Downey & Murphy, 1970), which has been ascribed to cause an increase in micelle size (Walstra et al., 2006). During freezing, the speed of cooling is relatively fast, and no casein leakage was found in the serum phase (UF permeate, data not shown) of the thawed CC. It would be interesting to consider whether the leakage occurred during freezing or thawing at 4 °C and if pre-heating at 30 °C was sufficient to restore the β-casein into the micelle.

Jørgensen et al. (2016) showed that CC obtained using a 0.10-μm UTP membrane for MF of milk contained 0.86% whey protein (WP). This finding means that the CC in the present experiment may contain WP that may undergo changes upon freezing. Low temperatures, −20 °C and below, can cause globular proteins such as WP to denature and unfold by weakening of the hydrophobic interactions (Walstra et al., 2006). This phenomenon was also observed for WP concentrate samples in our lab, that showed change in reversed phase HPLC-diagrams after being frozen at −20 °C for several months (data not shown). These denatured whey proteins will most likely also take part in the formation of aggregates and contribute to the characteristics of the formed gels, as described below.

The observed reduction in the content of total Pi in the freeze-thawed CC compared with the reference suggests Pi precipitation. Hindmarsh and Watkinson (2017) reported immobile phosphorus bodies in ageing mozzarella cheese previously unclassified in the literature. These were not products bridging phosphoserine to CCP and were identified as unclassified bonding of calcium to phosphorus. As their study was on ageing mozzarella cheese, the applicability to similar immobile bodies requires further investigation.

In view of the discussed factors, it may be clear that the reduction of water content in MF concentrates does not necessarily improve the storage stability compared with milk, during frozen storage. High total solid content contributes to instability, despite the lower lactose: protein ratio in MF samples compared with milk. Lactose content in the CC is detrimental to the destabilisation of casein micelle, and a low lactose concentration or reduced lactose crystallisation is needed to ensure stable storage. Decreased lactose crystallisation may be achieved by a faster freezing rate at low temperatures, e.g., −80 °C.

4.2. Influence of freezing on acid-induced gel properties

Although the pH of the thawed samples was stable and similar for all samples before the addition of GDL, the duration of frozen storage influenced the pH value at the end of acidification, with lower pH end values after acidification with GDL for longer frozen storage periods. These results indicate that the buffer capacity of

the CC was altered by freezing. As colloidal Pi dissolves completely at pH = 5.2 (Gaucheron, 2005), thawed CC samples frozen for a longer time (>6 day) most likely obtained a completely dissolved colloidal Pi at an earlier stage (pH > 5.2) during acid coagulation than CC samples frozen < 6 days. This finding also suggests that the formation of $Ca_3(PO_4)_2$ and P precipitate altered the buffering capacity of the CC system, consequently affecting the rate of pH change (Lucey, 2016). The trend of an increased G′ with more days of frozen storage of the CC samples followed their decrease in pH end value. Anema (2008) showed a similar trend when demonstrating that the storage modulus (G′) of acid-skimmed milk gels was correlated to the acidification rate when temperature and GDL concentration were modified.

The random formation of protein aggregates in the thawed CC samples most likely influenced the rheological properties of the acid gels and might explain the large deviations in gelation properties between the reference and the frozen CC samples. During acidification, the casein micelles are destabilised, their surface charge is reduced, micellar Ca and P is dissolved, and the internal interactions are modified. Hydrophobic interactions overcome the electrostatic repulsion, and aggregation occurs, forming a gel. More hydrophobic interactions result in stiffer gels (Anema, 2008; Lucey, 2016).

The size of the casein micelle has been reported to influence coagulation properties and specifically the gel firmness. Jørgensen et al. (2017) showed that smaller casein micelles gave higher G′ value for yogurts than larger micelles. The thawed CC samples frozen at −80 °C had significantly smaller $D_{50\text{-}a}$ compared with thawed CC samples frozen at −40 °C and −20 °C, and this difference may explain to some extent their higher G′. However, the lack of correlation between G′ and $D_{50\text{-}a}$ fits with the findings of Horne (2003), who found no effect of casein micelle size on the stiffness of GDL acid gels. The $D_{50\text{-}b}$ of the aggregates followed to some extent the change in the final G′ of the gels; after 6 days of frozen storage, the average size of the aggregates increased in CC samples frozen at −20 °C and −40 °C, and their G′ increased compared with the reference. This pattern is consistent with the findings of Wendorff (2001) of similar gel strength of thawed milk samples frozen at −27 °C compared with unfrozen samples, while thawed milk samples frozen at −12 °C had reduced gel strength. The $D_{50\text{-}b}$ of the thawed CC samples frozen at −80 °C remained relatively unaffected up to 12 days of storage, along with their consequent G′ value, which remained higher in the storage modulus compared with the reference. It seems that the thawed CC samples frozen at −20 °C and −40 °C needed a larger particle size formation to obtain a similar G′ value to the samples frozen at −80 °C.

It is highly relevant to reflect on how the aqueous phase contributes to the instability of these gels. If whey proteins present in the casein concentrates were assumed to undergo denaturation at −40 °C and −80 °C, then increased cross linking by denatured whey protein within the gels would increase the rigidity and G′ of the gels. Interestingly, the initial content of Ca and P in the micelles did not correlate with the G′ of the gels. It might be expected that a low amount of colloidal Ca and P would dissolve faster and lead to a stronger gel, but this process did not happen. It would be interesting to investigate the possible contribution of $Ca_3(PO_4)_2$ clusters to the gel strength during gel formation, as a G′ value increase followed the increase in micellar Ca and P content.

Understanding the mechanisms of how freezing influence the quality of acid coagulation of thawed CC can be challenging. This process is not a linear relationship between pH, particle size, CCP content and G′, but rather an unidentified random mechanism dependent on different factors. These factors might include the dissolution rate of aggregates, distribution of GDL within the sample, available bonding sites on micelles, etc.

4.3. Influence of freezing on rennet gels

Rennet coagulation properties are dependent on pH (Lu et al., 2017; Tsioulpas, Grandison, & Lewis, 2007), ionic calcium (Lewis, 2011), protein concentration and ionic strength (Daviau, Famelart, Pierre, Goudedranche, & Maubois, 2000; Guinee et al., 1997) and cold storage (Maciel et al., 2014). The results of this study show that RCT was not influenced by the freezing step followed by thawing and pre-heating at 30 °C for 30 min. This result is in line with the pH and ionic calcium measurements, which showed no significant change after freezing. Kljajevic et al. (2016) also reported no significant change in RCT for thawed caprine milk frozen at −27 °C for up to 60 days. In the firming stage of rennet coagulation, the aggregation rate and development of gel strength are to some extent dependent on the distance between particles and the amount of Ca^{2+} (Dalgleish & Corredig, 2012; Lewis, 2011; Walstra et al., 2006).

One possible explanation for the higher curd strength for thawed samples frozen at −80 °C for more than 12 days could be aggregate formation before renneting. In the primary stage of rennet coagulation, the hydrolysis of more than 85% of the κ-casein into para-κ-casein and caseinomacropeptide is the main reason for the observed reduction of electrostatic repulsion between micelles and initial aggregation (Dalgleish & Corredig, 2012). Thus, the number of κ-casein molecules on the micelle surface is of high relevance for gel formation. Several studies showed that small casein micelles have higher k-casein content on the surface (Dalgleish, Horne, & Law, 1989; O'Connell & Fox, 2000), and therefore, milk with small casein micelles forms stronger rennet gels (Gustavsson et al., 2014; Logan et al., 2015; Walsh et al., 1998) than milk with larger casein micelles. A reduction of electrostatic repulsion can also be caused when the salt content is increased in the milk (Montella, 2008).

The increased micellar Ca and inorganic phosphate content in the thawed CC samples frozen at −20 °C and −40 °C compared with the reference and −80 °C samples suggest their deposition on the casein micelle surface. This salt deposition will consequently shield the charges of the κ-CN hairy layer and reduce their availability for enzymatic cleavage, resulting in a smaller number of hydrophobic interactions and a comparably weaker rennet gel. It would also be interesting to further investigate whether aggregate formation influences the proportion of κ-CN on the aggregate surface compared with the micelles.

5. Conclusions

This study demonstrated that CC with 8% protein produced by microfiltration were destabilised during the initial frozen storage. The temperature and initial days of freezing influenced the rate of destabilisation after thawing. The mineral content was reduced in the serum phase of freeze-thawed CC, and temperatures ≤ −20 °C led to the formation of aggregates. The storage modulus of acid gels made from freeze-thawed CC samples had higher values compared with unfrozen CC samples. While the rennet clotting time was not influenced by the freezing conditions, curd firmness (A30) was influenced by storage of the CC at −80 °C prior to rennet coagulation. A combination of freezing temperature and time affected the rennet and acid coagulation properties of freeze-thawed CC. Freezing at −20 °C for 15 days, for example, gave similar acid gel properties of the thawed CC, as freezing at −80 °C for a shorter time. There is still room for investigation regarding the effects of

freeze-thawing methods, especially regarding the possible reversibility of destabilised systems and the nature of the aggregates formed. In conclusion, it is crucial to consider whether the changes occurring during frozen storage of CC may influence the product characteristics during further processing of the CC.

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