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Use of $^{31}$P NMR and FTIR to investigate key milk mineral equilibria and their interactions with micellar casein during heat treatment

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ABSTRACT

The thermal treatment of milk is one of the key processes routinely performed in the dairy industry. Several modifications occur in milk during heating, particularly with respect to its mineral equilibrium. As the temperature increases, the solubility of calcium and phosphate decreases leading to precipitation in the casein micelle as casein phosphate nanocluster. Recently, $^{31}$P NMR and Fourier Transform Infrared have been demonstrated to be capable of monitoring changes to its nanocluster. In this study, the effect of temperature on nanocluster during heating of milk to temperatures ranging from 25 °C to 80 °C followed by subsequent cooling were studied. It was also demonstrated that key ionic components of the mineral equilibria behaved differently with temperature, e.g., calcium influence was evident only at lower temperature, while the opposite was the case with phosphate. It was also shown that micellar casein concentration was influential at all temperatures, most notably at lower values.
1. Introduction

The complexity of milk makes its original composition easily susceptible to modification during different industrial processes. One of the more important industrial factors is temperature arising from thermal processing (Dalgleish & Corredig, 2012). The main reasons for heating milk are to (a) kill pathogens (pasteurisation), (b) increase its shelf life (Fox, Uniacke-Lowe, McSweeney, & O’Mahony, 2015), (c) increase the solids content by means of thermal evaporation before spray drying (Le Graet & Brule, 1982; Liu, Dunstan, & Martin, 2012) and (d) influence the heat classification of skim milk powder produced subsequent to the use of various preheat temperatures prior to evaporation.

An extensive range of temperatures is used during processing depending on application, e.g. from modest levels of heating (40–50 °C) during the advanced stages of milk evaporation (Liu et al., 2012) to values exceeding 130–140 °C in the case of ultra high temperature (UHT) processing (Fox et al., 2015). In addition, different heating systems feature different holding times. Previous studies showed how heating milk to certain temperatures influences the interaction of whey protein with casein micelles (Corredig & Dalgleish, 1996) and causes shifts in mineral equilibria (Gaucheron, 2005). Complex formation between whey protein and micellar casein during heat treatment relies extensively on the exposure of a thiol group during whey protein unfolding, denaturation and its resulting interaction with κ-casein (Donato & Guyomarc’h, 2009). However, in this study we focus on changes to the mineral equilibria in the course of heating milk between 25 °C and 80 °C. With increasing temperature, the solubility of calcium (Ca) and inorganic phosphate (P_i) in solution is reduced, leading to precipitation of phosphate and calcium (On-Nom, Grandison, & Lewis, 2010; Pouliot, Boulet, & Paquin, 1989a). At the same time, the pH of milk reduces within this temperature range (On-Nom et al., 2010). This change was found to be completely reversible if heating was conducted at 80 °C (Pouliot, Boulet, & Paquin, 1989b), but once it reaches > 90 °C an increase in soluble phase occurs (Wahlgren, Dejmek, & Drankenberg, 1990; Zhang & Aoki, 1996).
A limitation with studies to date on the influence of temperature on milk minerals is that they have been confined to milk as whole system. However, the mineral equilibria of milk are more complicated than a single equation (Holt, 2004). This study set out to show how key components of the mineral equilibria of milk respond differently when temperature increases. The experimental approach utilised $^{31}$P nuclear magnetic resonance ($^{31}$P NMR) and attenuated total refraction Fourier transform infrared (ATR FTIR), since both techniques are capable of directly probing the casein phosphate nanocluster structure as well as the repartitioning of phosphorus (Boiani, McLoughlin, Auty, FitzGerald, & Kelly, 2017; Boiani, FitzGerald, & Kelly, unpublished). A better understanding of the influence of heat treatment on the different components of milk is intended to provide dairy industry personnel with additional knowledge on how to better control physicochemical and functional properties during the manufacture of milk products.

2. Materials and methods

2.1. Sample preparation

Pasteurised skimmed bovine milk obtained from the local market was used as reference sample during investigation on temperature effects on milk salt equilibria. A further three samples were prepared by increasing, respectively, the concentrations of micellar casein, $P_i$, and $P_i + Ca$. The concentrated micellar casein (milk concentrate) sample was obtained by microfiltration (MF) at 25 °C and 1 bar using a NovaSet™-LS membrane (TangenX, Shrewsbury, MA, USA), starting with 1 L of skimmed milk and collecting 0.5 L of permeate. The samples with higher mineral concentrations were obtained by adding 10 mmol of sodium phosphate monobasic to 1 L of skimmed milk or 10 mmol of sodium phosphate and 10 mmol calcium chloride. Samples were left for 1 h to equilibrate at room temperature under gentle magnetic stirring following each mineral addition. After 1 h initially, and every 30 min subsequently, the pH was corrected using 1 M NaOH.
to the initial value of skimmed milk (pH = 6.8) until stabilised. Sodium azide as added (0.3 mg mL$^{-1}$) to all samples to control microbial growth and stored overnight at 4 °C before analyses.

2.2. SDS PAGE analysis

The protein profiles of the samples and their distribution between retentate and serum fractions were assessed using reducing SDS PAGE. Gels of total protein and soluble fractions were performed using diluted samples with the Nu PAGE® SDS reducing buffer and obtained as already described in Boiani et al. (2017) using 12% bis-Tris precast gels (1.0 mm × 10 well; Novex® by Life Technologies™, Carlsbad, CA, USA).

2.3. NMR spectroscopy

$^{31}$P NMR spectra were collected using a 500 MHz Bruker spectrometer (Bruker UK Ltd, Coventry, UK) using an external phosphate solution as reference. Data acquisition was as previously described in Boiani et al. (2017). Three different spectra were collected for every sample: a first spectrum at 25 °C was collected as soon as the sample was loaded on the spectrometer. The sample was then warmed within the NMR spectrometer to collect the spectrum at higher temperatures. The skimmed milk, milk concentrate and milk plus P$_i$ and Ca samples were investigated at seven different temperatures: 25, 40, 60, 65, 70, 75 and 80 °C. The milk plus P$_i$ was investigated at 25, 35, 40, 45, 60, 65, 80 °C. Immediately following collection of the second spectrum, the NMR spectrometer and its entrapped sample, were cooled to 25 °C whereupon a third spectrum was collected to establish the effect of temperature reversibility. Each spectrum involved a 4 h collection time, thus, exposing the sample to a total time of 12 h in the spectrometer. Lock of the signal and topshim command was executed every time a new spectrum was gained. All spectra were analysed using Top Spin 3.2 software (Bruker UK Ltd). $^{31}$P NMR casein phosphate
nanocluster peak areas were identified using $P_i$ as reference and used to investigate the influence of the heating on the concentration of both phosphorus species.

2.4. **FTIR spectroscopy**

FTIR spectra were generated to investigate the recovery behaviour of samples after 80 °C for 4 h. The FTIR spectrometer used was a 27 Tensor FTIR (Bruker UK Ltd, Coventry, UK) equipped with an ATR BioATRCell II probe (Bruker UK Ltd, Coventry, UK). The spectra were collected as previously described (Boiani et al., unpublished), except that MF permeate was used instead of water as background sample. MF permeate was obtained from the microfiltration of milk. This microfiltration was similar to that used for preparation of the casein concentrate except that in this case the retentate was not recycled to the feed vessel during operation. After discarding an initial 250 mL of permeate, 3 mL of permeate was collected for use as background. All spectra manipulations were conducted using OPUS 5.5 (Bruker Optik GmbH, Ettlingen, Germany) software.

2.5. **pH measurement**

Sample pH was determined using a Mettler Toledo pH meter (Mettler-Toledo Ltd., Beaumont Leys, Leicester, UK). The pH meter was calibrated with standard pH solutions. pH measurements were taken at 25 °C.

2.6. **Statistical analysis**

Samples were analysed in duplicate for pH and NMR peaks area. Analysis of variance (ANOVA) was undertaken using Minitab version 17 (Minitab Inc.). The level of significance was
established at $P < 0.05$. Fisher’s multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

3. Results

3.1. Influence of temperature on milk minerals equilibria

The mineral equilibria of milk are influenced by temperature. As temperature increases, the shift towards the colloidal/solid phase (Pouliot et al., 1989a) may be observed in the spectrum generated by $^{31}$P NMR along with changes to the casein phosphate nanocluster (Fig. 1). $P_i$ and the casein phosphate nanocluster are visible at 1 ppm (sharp high intensity signal) and 2–3.5 ppm (multiplet), respectively (Boiani et al., 2017). $^{31}$P NMR analysis also shows that increasing the temperature of skimmed milk results in an initial reduction in the $P_i$ peak signal, however at temperatures > 70 °C a shift in the peak to higher ppm was visible (Fig. 2). At the same time, increasing temperature shifts the casein phosphate nanocluster peaks to lower ppm and expands their peak area (Fig. 3). However, this increase in casein phosphate nanocluster area was not linear and two different trends emerged (see Fig. 1 and Table 1). The initial increase in temperature had a minor effect on casein phosphate nanocluster change as reflected by the value (0.0011) of the initial slope of the linear regression; however, once the temperature exceeded 70 °C increased precipitation of $P_i$ to casein phosphate nanocluster raised the linear regression slope to 0.0085. The intercept of the two linear regression lines corresponds to a temperature of 68 °C (Fig. 1; Table 1).

By analysing the combined set of $^{31}$P NMR and FTIR results, it was possible to explore the influence of temperature on the recovery of the mineral equilibria (Fig. 4A,B). In Fig. 4A, no statistical difference ($P > 0.05$) was observed within the area of casein phosphate nanocluster during recovery at 25 °C; however, a comparison of the FTIR spectra of skimmed milk before and after 80 °C (Fig. 4B) clearly shows a reduction of the casein phosphate nanocluster signal at 1051 cm$^{-1}$. 
3.2. Casein micelle, phosphate and calcium influence

While the effect of heat treatment has been investigated extensively in the case of milk (Gaucheron, 2005), its influence on the behaviour of individual components of the mineral equilibria has yet to be studied in detail. It was, therefore, hypothesised that by increasing the concentration of micellar casein, $P_i$ and Ca, their respective influences would be accentuated at the different experimental temperatures, and hence, shed additional light on their individual roles. Thus, it was decided to focus on three components: micellar casein, $P_i$ and the Ca, all three of which are directly involved in the formation of casein phosphate nanocluster.

3.2.1. Micellar casein

A higher concentration of micellar casein increases casein phosphate nanocluster availability not only in the system but at the micellar core where $P_i$ and Ca most likely aggregate with increasing temperature. By means of MF, it was possible to increase the concentration of micellar casein while maintaining the concentration of all other components equal to that of skimmed milk. As expected, an increase in micellar casein concentration caused an increase in the casein phosphate nanocluster area signal in the $^{31}$P NMR spectrum (Fig. 1) such that the slope of the linear regression at low temperature (0.0032) became three times higher than that of skimmed milk, and contrasted with that of the upper temperature range where there was a $2\times$ increase in the value (0.0175) of the slope (Table 1). At the same time, the intercept of the two linear regression lines is at 69 °C, close to that found in skimmed milk (Table 1). When studying the reversibility of the temperature effects (Fig. 4C,D) following cooling to 25 °C, the reduction in casein phosphate nanocluster signal intensity observed after exposure to higher temperature was in agreement with the findings of previous workers (de la Fuente, 1998), while the FTIR spectrum (Fig. 5D) showed a similar reduction to that of skimmed milk (Fig 4B).
3.2.2. Phosphate

An increase in the concentration of phosphate has a dual influence, i.e., an effect on the process of casein phosphate nanocluster formation and at the same time a role in calcium chelation (Gaucher, Piot, Baucher, & Gaucheron, 2007). The amount of P$_i$ added during experimentation was set at 10 mM, to double the milk concentration (Gaucheron, 2005) of soluble phosphorus. As expected, phosphate supplementation reduced the amount of casein phosphate nanocluster (Fig. 1) based on the area of P$_i$ as reference. Increasing P$_i$ had a minor effect on the low temperature slope (0.0008), while at high temperature the slope (0.002) was four times less than that of skimmed milk (Table 1). The two regression lines intercepted at 56 °C compared with 69 °C for skimmed milk (Table 1). Following temperature reversal to 25 °C, $^{31}$P NMR analyses of the recovered samples showed (Fig. 4E), with exception of the sample heated to 80 °C, that the casein phosphate nanocluster content remained unchanged with reference to that of skimmed milk. However, when the 80 °C heated sample was cooled to 25 °C an increase in the casein phosphate nanocluster signal was detected by $^{31}$P NMR, but not by FTIR (Fig. 4F). When the recovery effect was further studied using SDS PAGE (Fig. 5), it was possible to observe an increase in soluble casein protein in the supernatant of milk plus P$_i$ sample after heating at 80 °C (Fig. 5C).

3.2.3. Calcium

As in the case of phosphorus, calcium ions have a double effect in milk – formation of casein phosphate nanocluster and contribution to the supramolecular structure of micellar casein and subsequent functionality in dairy product processing, e.g., coagulation (McMahon, Brown, Richardson, & Ernstrom, 1984). For this reason, it was decided not to investigate calcium alone but rather in combination with phosphate to avoid coagulation during analysis. P$_i$ (10 mM) followed by Ca (10 mM) were added to skimmed milk as outlined in the Materials and methods section. When Ca was added to the P$_i$ supplemented milk and subjected to the NMR heating, the linear regression...
intercept was shifted to 66 °C from that of 56 °C in the case of milk plus P\(_i\) (Table 1). The casein phosphate nanocluster content for milk plus P\(_i\) and Ca was also lower compared with skimmed milk at 25 °C. As the heating temperature increased, the difference in casein phosphate nanocluster contents between the milk and mineral supplemented samples was eliminated when they reached similar values (\(P<0.05\)) at 60 °C (Fig. 1). This trend was also evident from the slopes of the two regression lines, i.e., at lower temperature (25–65 °C) milk plus P\(_i\) and Ca had the higher slope (0.0028 for milk plus P\(_i\) and Ca versus 0.0011 for skimmed milk) while at higher temperatures (70–80 °C) both slopes were virtually similar (0.0066 for milk plus P\(_i\) and Ca versus 0.0085 for skimmed milk). No significant difference in the NMR signal was observed while recovering at 25 °C following heat treatment (Fig. 4G) while in the FTIR spectrum (Fig. 4H) it was possible to observe an increase in the casein phosphate nanocluster signal.

4. Discussion

Previous studies have highlighted the effects of different heat treatments on milk minerals (de la Fuente, 1998). Most studies show that mineral equilibria shift towards the colloidal phase as temperature increases (Pouliot et al., 1989a) and pH of milk reduces (On-Nom et al., 2010). Furthermore, it has also been shown that once temperature reaches 90 °C alterations to mineral equilibria are no longer reversible (Pouliot et al., 1989b; Zhang & Aoki, 1996). This difference in reversibility behaviour points to a different structure of casein phosphate nanocluster as a result of its exposure to higher temperatures (Gaucheron, 2005). Using advanced analytical techniques now available (\(^{31}\)P NMR and FTIR) to investigate milk and dairy products (Boiani et al., 2017; Boiani et al., unpublished), the authors set out to confirm the findings of previous workers and to elucidate some new perspectives on the influence of temperature on casein phosphate nanocluster structure and mineral equilibria.
4.1. Influence of temperature

Using $^{31}$P NMR, it was possible to directly observe for the first time the influence of heating on the $^{31}$P NMR peaks depicting casein phosphate nanoclusters, $P_i$ distribution in milk and ionic charge. Figs. 2 and 3 clearly show how an increase in temperature not only influenced the repartitioning of minerals between colloidal and soluble phase (Gaucheron, 2005), but also had two additional effects. At temperatures $>70$ °C a shift of the $P_i$ signal was visible (Fig. 2); such a shift is usually associated with an increase in pH (Gonzalez-Jordan, Thomar, Nicolai, & Dittmer, 2015). However, in this case increasing the temperature caused a pH reduction (Fig. 1) in line with the observation of On-Nom et al. (2010). Hence, it is concluded that raising the temperature increases the negative charge of $P_i$, as already predicted by (de la Fuente 1998). On the other hand, the casein phosphate nanocluster signal shifts to lower ppm (Fig. 3), an effect that is associated with an increase in P:Ca. This indicates that increasing temperature leads to a change in casein phosphate nanocluster structure, and in particular, an increase in its phosphorus content (Boiani et al., 2017).

The non-linear nature of the NMR data obtained during heating (Fig. 1) revealed two distinct phases relating to changes in phosphorus – a slow rate of precipitation during the first phase as represented by the smaller slope of the linear regression line, and a more rapid rate of precipitation during the second phase (Fig. 1). In this study, the change between the two phases occurs close to 70 °C (Fig. 1), while previous research shows that it occurred at 80–90 °C (de la Fuente, 1998). The lower intercept temperature observed in this study may be due to the protracted period (4 h) that the samples were subjected to in the NMR instrument due to the long data acquisition time compared with a maximum of 30 min used in other studies (Zhang & Aoki, 1996). This protracted duration (4 h) may have exaggerated the influence of temperature. Follow-up studies are, therefore, required with a view to adapting the above observations to the more conventional milk heating regimes using the Pouliot approach. It is likely that the outcome of such studies would result in a phase change similar to that detected in this study. Interestingly, it was also
found that addition of a calcium chelator such as the phosphorus to milk reduces the impact of
temperature change (Fig. 1). An increase in soluble casein in the case of milk plus $P_i$ (Fig 5)
suggests that the first phase destabilisation of the micellar conformation takes place within a
narrower temperature range (25–56 °C). Addition of Ca countered this destabilisation effect thus
confirming that the calcium chelating effect of phosphate (i.e., in milk plus $P_i$) was the main reason
for the difference. It was also shown that the response to changes in the concentration of individual
components involved in the mineral equilibria depends on heating status i.e., the low and high
temperature phases.

In the milk concentrate samples, micellar casein had a dominant influence at all
temperatures (Fig. 1) with the result that the slopes of the two regression lines were different to
those of skimmed milk (Table 1). However, the micellar casein influence was not the same over the
two temperature phases. The greater impact occurred in the first phase where the slope of milk
concentrate line was three times higher than that of skimmed milk. In the second phase, a 2×
increase in the value of the slope was in direct response to the 2× increase in concentration of
micellar casein. The addition of $P_i$ to skimmed milk did not alter its first phase slope (Fig. 1; Table
1), even when the phase change occurred at an earlier temperature. However, during the second
phase precipitation was slowed by the presence of $P_i$, as deduced from the reduced slope of this
sample (Fig. 1; Table 1). When Ca was added to $P_i$, as in the milk plus $P_i$ and Ca sample, the first
phase showed a higher slope compared with skimmed milk. Thus, the casein phosphate nanocluster
concentration of milk plus $P_i$ and Ca was reduced relative to $P_i$ at 25 °C when compared with that of
skimmed milk. However, this difference reduced as the regression line converged at 60 °C with
skimmed milk, thus suggesting that the precipitation of $P_i$ and Ca occurred within the first phase,
but not during the second.

4.2. Reversibility and recovery study
Using both NMR and FTIR it was possible to investigate the influence of prolonged thermal exposure on mineral distribution. While NMR analysis did not demonstrate any difference within skimmed milk on recovery at 25 °C (Fig 4A), a reduction in casein phosphate nanocluster was found in the FTIR spectra of the same sample (Fig 4B). This is probably due to the limitations of NMR at the micellar casein concentration of milk. In fact, when micellar casein was increased in concentration, the NMR was able to distinguish a decreased casein phosphate nanocluster concentration at higher temperature (Fig 4C) in line with the result obtained using FTIR (Fig 4D), and as already established by previous work (de la Fuente, 1998).

The apparent contradictory results obtained with NMR and FTIR in milk plus P<sub>i</sub> may be explained by the increase in soluble casein found in this sample (Fig. 5C). In fact, NMR as a spectrophotometric technique is not intended to be used with colloidal systems such as milk (Belton & Lyster, 1991). Therefore, an increase in soluble casein would increase the signal of the casein on the spectra, without a real increase of the casein phosphate nanocluster concentration such that it is necessary to rely mainly on the result obtained from the FTIR in this instance (Fig. 4F). Hence, milk plus P<sub>i</sub> and Ca is the only sample that showed an increase in the FTIR signal after temperature reversal to 25 °C (Fig 4H). This increase, however, was not detectable by NMR (Fig 4G), and it suggests that part of the precipitate of P<sub>i</sub> and Ca is not generating new casein phosphate nanocluster, but forming inorganic calcium phosphate outside of the micellar casein that is only detectable by FTIR (Boiani et al., unpublished).

5. Conclusion

These results show how milk mineral equilibria are influenced in different ways by manipulation of its components in conjunction with thermal treatment. It is possible to speculate that during the first heating phase (25–60 °C) P<sub>i</sub> and Ca precipitate within micellar casein to form new casein phosphate nanocluster or as inorganic calcium phosphate salt in the case of low micellar
casein concentration. During the second heating phase (60–80 °C), it would appear that changes to
the micellar casein-casein phosphate nanocluster interaction predominates as a result of the
increased negative charge of $P_i$. The authors believe that this is the first time that the influence of
singular ionic constituents on milk mineral equilibria during heat treatment was made possible
using advanced analytical techniques such as $^{31}$P NMR and FTIR. This paper added useful
knowledge regarding the interaction between the mineral-containing serum ($P_i$ and Ca) phase of
milk and the colloidal (micellar casein and casein phosphate nanocluster) phase.

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Figure legends

**Fig. 1.** Changes of casein phosphate nanocluster $^{31}$P NMR integral with changing temperature: ⚫, skimmed milk samples; ◆, skimmed milk samples concentrated 2× by microfiltration; ▲, skimmed milk samples with 10 mM orthophosphate; ◇, skimmed milk samples plus 10 mM orthophosphate and 10 mM calcium. The dotted lines represent the linear regression for the different phases; values of slope, $R^2$, the intercept of the regression line and the pH value of the sample after heating at 80 °C for 4 h are given in Table 1.

**Fig. 2.** Soluble phosphate $^{31}$P NMR spectra obtained from skimmed milk at (from bottom to top) 25, 40, 60, 65, 70, 75 and 80 °C.

**Fig. 3.** Casein phosphate nanocluster $^{31}$P NMR spectra obtained from skimmed milk at (bottom to top) 25, 60, 65, and 80 °C.

**Fig. 4.** Reversibility and recovery study using casein phosphate nanocluster $^{31}$P NMR integral (A, C, E, G) and Fourier transform infrared spectra (B, D, F, H); the black lines show the results obtained at the different temperatures, the grey line shows the reversibility signal at 25 °C. Panels A and B, skimmed milk samples; panels C and D, skimmed milk samples that were 2× concentrated using microfiltration; panels E and F, skimmed milk samples plus 10 mM orthophosphate; panels G and H, skimmed milk samples with 10 mM orthophosphate and 10 mM calcium.

**Fig. 5.** Reducing sodium docecylsulphate polyacrylamide gel electrophoresis of: A, skimmed milk; B, skimmed milk 2× concentrated using microfiltration; C, skimmed milk plus 10 mM
orthophosphate; D, skimmed milk plus 10 mM orthophosphate and 10 mM calcium. For each gel: lane 1, protein markers; lane 2, αs-casein; lane 3, β casein; lane 4, skimmed milk; lane 5, sample; lane 6, supernatant of the sample obtained by centrifugation; lane 7, sample after heating at 80 °C for 4 h; lane 8, supernatant after heating at 80 °C for 4 h.
Table 1

Changes of casein phosphate nanocluster $^{31}$P NMR integral with changing temperature: values for slope, $R^2$, the intercept of the regression line and the pH value of the sample after heating at 80 °C for 4 h. a

| Parameter | M      | M×2    | M+P    | M+P+Ca |
|-----------|--------|--------|--------|--------|
| Low T slope (CPN area °C$^{-1}$) | 0.0011 | 0.0032 | 0.0008 | 0.0028 |
| Low T regression line $R^2$ | 0.9723 | 0.9973 | 0.9859 | 0.9997 |
| High T slope (CPN area °C$^{-1}$) | 0.0085 | 0.0175 | 0.002  | 0.0066 |
| High T regression line $R^2$ | 0.9986 | 0.9999 | 0.9998 | 0.9935 |
| Intercept (°C) | 68     | 69     | 56     | 66     |
| pH | 6.42 ± 0.03 | 6.47 ± 0.03 | 6.42 ± 0.01 | 6.34 ± 0.04 |

a Values were calculated from the data presented in Fig. 1. Abbreviations are: M, skimmed milk samples; M×2, skimmed milk samples concentrated 2× by microfiltration; M+P, skimmed milk samples with 10 mM orthophosphate; M+P+Ca, skimmed milk samples plus 10 mM orthophosphate and 10 mM calcium.
80 °C

65 °C

60 °C

25 °C

CPN
**A**

| α-casein | β-casein | Skim milk | Sample | Sample supernatant | Sample supernatant after heating |
|----------|----------|-----------|--------|--------------------|----------------------------------|
| 35 kDa   |          |           |        |                    |                                  |
| 25 kDa   |          |           |        |                    |                                  |
| 15 kDa   |          |           |        |                    |                                  |

**B**

| 35 kDa   |          |           |        |                    |                                  |
| 25 kDa   |          |           |        |                    |                                  |
| 15 kDa   |          |           |        |                    |                                  |

**C**

| α-casein | β-casein | Skim milk | Sample | Sample supernatant | Sample supernatant after heating |
|----------|----------|-----------|--------|--------------------|----------------------------------|
| 35 kDa   |          |           |        |                    |                                  |
| 25 kDa   |          |           |        |                    |                                  |
| 15 kDa   |          |           |        |                    |                                  |

**D**

| 35 kDa   |          |           |        |                    |                                  |
| 25 kDa   |          |           |        |                    |                                  |
| 15 kDa   |          |           |        |                    |                                  |