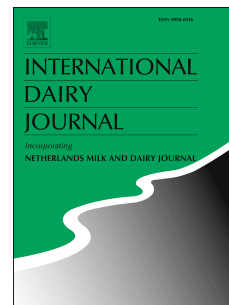


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Use of ^{31}P NMR and FTIR to investigate key milk mineral equilibria and their interactions with micellar casein during heat treatment

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

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26 ABSTRACT

27

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

39

40

41

42 1. Introduction

43

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

52 An extensive range of temperatures is used during processing depending on application, e.g.
53 from modest levels of heating (40–50 °C) during the advanced stages of milk evaporation (Liu et
54 al., 2012) to values exceeding 130–140 °C in the case of ultra high temperature (UHT) processing
55 (Fox et al., 2015). In addition, different heating systems feature different holding times. Previous
56 studies showed how heating milk to certain temperatures influences the interaction of whey protein
57 with casein micelles (Corredig & Dalglish, 1996) and causes shifts in mineral equilibria
58 (Gaucheron, 2005). Complex formation between whey protein and micellar casein during heat
59 treatment relies extensively on the exposure of a thiol group during whey protein unfolding,
60 denaturation and its resulting interaction with κ -casein (Donato & Guyomarc'h, 2009). However, in
61 this study we focus on changes to the mineral equilibria in the course of heating milk between 25
62 °C and 80 °C. With increasing temperature, the solubility of calcium (Ca) and inorganic phosphate
63 (P_i) in solution is reduced, leading to precipitation of phosphate and calcium (On-Nom, Grandison,
64 & Lewis, 2010; Pouliot, Boulet, & Paquin, 1989a). At the same time, the pH of milk reduces within
65 this temperature range (On-Nom et al., 2010). This change was found to be completely reversible if
66 heating was conducted at 80 °C (Pouliot, Boulet, & Paquin, 1989b), but once it reaches > 90 °C an
67 increase in soluble phase occurs (Wahlgren, Dejmek, & Drankenberg, 1990; Zhang & Aoki, 1996).

68 A limitation with studies to date on the influence of temperature on milk minerals is that
69 they have been confined to milk as whole system. However, the mineral equilibria of milk are more
70 complicated than a single equation (Holt, 2004). This study set out to show how key components of
71 the mineral equilibria of milk respond differently when temperature increases. The experimental
72 approach utilised ^{31}P nuclear magnetic resonance (^{31}P NMR) and attenuated total reflection Fourier
73 transform infrared (ATR FTIR), since both techniques are capable of directly probing the casein
74 phosphate nanocluster structure as well as the repartitioning of phosphorus (Boiani, McLoughlin,
75 Auty, FitzGerald, & Kelly, 2017; Boiani, FitzGerald, & Kelly, unpublished). A better understanding
76 of the influence of heat treatment on the different components of milk is intended to provide dairy
77 industry personnel with additional knowledge on how to better control physicochemical and
78 functional properties during the manufacture of milk products.

80 2. Materials and methods

82 2.1. Sample preparation

84 Pasteurised skimmed bovine milk obtained from the local market was used as reference
85 sample during investigation on temperature effects on milk salt equilibria. A further three samples
86 were prepared by increasing, respectively, the concentrations of micellar casein, P_i , and $\text{P}_i + \text{Ca}$.
87 The concentrated micellar casein (milk concentrate) sample was obtained by microfiltration (MF) at
88 $25\text{ }^\circ\text{C}$ and 1 bar using a NovaSetTM-LS membrane (TangenX, Shrewsbury, MA, USA), starting with
89 1 L of skimmed milk and collecting 0.5 L of permeate. The samples with higher mineral
90 concentrations were obtained by adding 10 mmol of sodium phosphate monobasic to 1 L of
91 skimmed milk or 10 mmol of sodium phosphate and 10 mmol calcium chloride. Samples were left
92 for 1 h to equilibrate at room temperature under gentle magnetic stirring following each mineral
93 addition. After 1 h initially, and every 30 min subsequently, the pH was corrected using 1 M NaOH

94 to the initial value of skimmed milk (pH = 6.8) until stabilised. Sodium azide as added (0.3 mg mL⁻¹)
95 ¹) to all samples to control microbial growth and stored overnight at 4 °C before analyses.

96

97 2.2. SDS PAGE analysis

98

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

104

105 2.3. NMR spectroscopy

106

107 ³¹P NMR spectra were collected using a 500 MHz Bruker spectrometer (Bruker UK Ltd,
108 Coventry, UK) using an external phosphate solution as reference. Data acquisition was as
109 previously described in Boiani et al. (2017). Three different spectra were collected for every
110 sample: a first spectrum at 25 °C was collected as soon as the sample was loaded on the
111 spectrometer. The sample was then warmed within the NMR spectrometer to collect the spectrum at
112 higher temperatures. The skimmed milk, milk concentrate and milk plus P_i and Ca samples were
113 investigated at seven different temperatures: 25, 40, 60, 65, 70, 75 and 80 °C. The milk plus P_i was
114 investigated at 25, 35, 40, 45, 60, 65, 80 °C. Immediately following collection of the second
115 spectrum, the NMR spectrometer and its entrapped sample, were cooled to 25 °C whereupon a third
116 spectrum was collected to establish the effect of temperature reversibility. Each spectrum involved a
117 4 h collection time, thus, exposing the sample to a total time of 12 h in the spectrometer. Lock of
118 the signal and topshim command was executed every time a new spectrum was gained. All spectra
119 were analysed using Top Spin 3.2 software (Bruker UK Ltd). ³¹P NMR casein phosphate

120 nanocluster peak areas were identified using P_i as reference and used to investigate the influence of
121 the heating on the concentration of both phosphorus species.

122

123 2.4. FTIR spectroscopy

124

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

135

136 2.5. pH measurement

137

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

141

142 2.6. Statistical analysis

143

144 Samples were analysed in duplicate for pH and NMR peaks area. Analysis of variance
145 (ANOVA) was undertaken using Minitab version 17 (Minitab Inc.). The level of significance was

146 established at $P < 0.05$. Fisher's multiple-comparison test was used for paired comparison of
147 treatment means and the level of significance was determined at $P < 0.05$.

148

149 3. Results

150

151 3.1. Influence of temperature on milk minerals equilibria

152

153 The mineral equilibria of milk are influenced by temperature. As temperature increases, the
154 shift towards the colloidal/solid phase (Pouliot et al., 1989a) may be observed in the spectrum
155 generated by ^{31}P NMR along with changes to the casein phosphate nanocluster (Fig. 1). P_i and the
156 casein phosphate nanocluster are visible at 1 ppm (sharp high intensity signal) and 2–3.5 ppm
157 (multiplet), respectively (Boiani et al., 2017). ^{31}P NMR analysis also shows that increasing the
158 temperature of skimmed milk results in an initial reduction in the P_i peak signal, however at
159 temperatures $> 70\text{ }^\circ\text{C}$ a shift in the peak to higher ppm was visible (Fig. 2). At the same time,
160 increasing temperature shifts the casein phosphate nanocluster peaks to lower ppm and expands
161 their peak area (Fig. 3). However, this increase in casein phosphate nanocluster area was not linear
162 and two different trends emerged (see Fig. 1 and Table 1). The initial increase in temperature had a
163 minor effect on casein phosphate nanocluster change as reflected by the value (0.0011) of the initial
164 slope of the linear regression; however, once the temperature exceeded $70\text{ }^\circ\text{C}$ increased
165 precipitation of P_i to casein phosphate nanocluster raised the linear regression slope to 0.0085. The
166 intercept of the two linear regression lines corresponds to a temperature of $68\text{ }^\circ\text{C}$ (Fig. 1; Table 1).
167 By analysing the combined set of ^{31}P NMR and FTIR results, it was possible to explore the
168 influence of temperature on the recovery of the mineral equilibria (Fig. 4A,B). In Fig. 4A, no
169 statistical difference ($P > 0.05$) was observed within the area of casein phosphate nanocluster during
170 recovery at $25\text{ }^\circ\text{C}$; however, a comparison of the FTIR spectra of skimmed milk before and after 80
171 $^\circ\text{C}$ (Fig. 4B) clearly shows a reduction of the casein phosphate nanocluster signal at 1051 cm^{-1} .

172

173 3.2. *Casein micelle, phosphate and calcium influence*

174

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

182

183 3.2.1. *Micellar casein*

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

198

199 3.2.2. *Phosphate*

200 An increase in the concentration of phosphate has a dual influence, i.e., an effect on the
201 process of casein phosphate nanocluster formation and at the same time a role in calcium chelation
202 (Gaucher, Piot, Baucher, & Gaucheron, 2007). The amount of P_i added during experimentation was
203 set at 10 mM, to double the milk concentration (Gaucheron, 2005) of soluble phosphorus. As
204 expected, phosphate supplementation reduced the amount of casein phosphate nanocluster (Fig. 1)
205 based on the area of P_i as reference. Increasing P_i had a minor effect on the low temperature slope
206 (0.0008), while at high temperature the slope (0.002) was four times less than that of skimmed milk
207 (Table 1). The two regression lines intercepted at 56 °C compared with 69 °C for skimmed milk
208 (Table 1). Following temperature reversal to 25 °C, ^{31}P NMR analyses of the recovered samples
209 showed (Fig. 4E), with exception of the sample heated to 80 °C, that the casein phosphate
210 nanocluster content remained unchanged with reference to that of skimmed milk. However, when
211 the 80 °C heated sample was cooled to 25 °C an increase in the casein phosphate nanocluster signal
212 was detected by ^{31}P NMR, but not by FTIR (Fig. 4F). When the recovery effect was further studied
213 using SDS PAGE (Fig. 5), it was possible to observe an increase in soluble casein protein in the
214 supernatant of milk plus P_i sample after heating at 80 °C (Fig. 5C).

215

216 3.2.3. *Calcium*

217 As in the case of phosphorus, calcium ions have a double effect in milk – formation of
218 casein phosphate nanocluster and contribution to the supramolecular structure of micellar casein
219 and subsequent functionality in dairy product processing, e.g., coagulation (McMahon, Brown,
220 Richardson, & Ernstrom, 1984). For this reason, it was decided not to investigate calcium alone but
221 rather in combination with phosphate to avoid coagulation during analysis. P_i (10 mM) followed by
222 Ca (10 mM) were added to skimmed milk as outlined in the Materials and methods section. When
223 Ca was added to the P_i supplemented milk and subjected to the NMR heating, the linear regression

224 intercept was shifted to 66 °C from that of 56 °C in the case of milk plus P_i (Table 1). The casein
225 phosphate nanocluster content for milk plus P_i and Ca was also lower compared with skimmed milk
226 at 25 °C. As the heating temperature increased, the difference in casein phosphate nanocluster
227 contents between the milk and mineral supplemented samples was eliminated when they reached
228 similar values ($P < 0.05$) at 60 °C (Fig. 1). This trend was also evident from the slopes of the two
229 regression lines, i.e., at lower temperature (25–65 °C) milk plus P_i and Ca had the higher slope
230 (0.0028 for milk plus P_i and Ca versus 0.0011 for skimmed milk) while at higher temperatures (70–
231 80 °C) both slopes were virtually similar (0.0066 for milk plus P_i and Ca versus 0.0085 for
232 skimmed milk). No significant difference in the NMR signal was observed while recovering at 25
233 °C following heat treatment (Fig. 4G) while in the FTIR spectrum (Fig. 4H) it was possible to
234 observe an increase in the casein phosphate nanocluster signal.

235

236 4. Discussion

237

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

249

250 4.1. *Influence of temperature*

251

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

264 The non-linear nature of the NMR data obtained during heating (Fig. 1) revealed two
265 distinct phases relating to changes in phosphorus – a slow rate of precipitation during the first phase
266 as represented by the smaller slope of the linear regression line, and a more rapid rate of
267 precipitation during the second phase (Fig. 1). In this study, the change between the two phases
268 occurs close to $70\text{ }^\circ\text{C}$ (Fig. 1), while previous research shows that it occurred at $80\text{--}90\text{ }^\circ\text{C}$ (de la
269 Fuente, 1998). The lower intercept temperature observed in this study may be due to the protracted
270 period (4 h) that the samples were subjected to in the NMR instrument due to the long data
271 acquisition time compared with a maximum of 30 min used in other studies (Zhang & Aoki, 1996).
272 This protracted duration (4 h) may have exaggerated the influence of temperature. Follow-up
273 studies are, therefore, required with a view to adapting the above observations to the more
274 conventional milk heating regimes using the Pouliot approach. It is likely that the outcome of such
275 studies would result in a phase change similar to that detected in this study. Interestingly, it was also

276 found that addition of a calcium chelator such as the phosphorus to milk reduces the impact of
277 temperature change (Fig. 1). An increase in soluble casein in the case of milk plus P_i (Fig 5)
278 suggests that the first phase destabilisation of the micellar conformation takes place within a
279 narrower temperature range (25–56 °C). Addition of Ca countered this destabilisation effect thus
280 confirming that the calcium chelating effect of phosphate (i.e., in milk plus P_i) was the main reason
281 for the difference. It was also shown that the response to changes in the concentration of individual
282 components involved in the mineral equilibria depends on heating status i.e., the low and high
283 temperature phases.

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

299

300 4.2. *Reversibility and recovery study*

301

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

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

321

322 5. Conclusion

323

324 These results show how milk mineral equilibria are influenced in different ways by
325 manipulation of its components in conjunction with thermal treatment. It is possible to speculate
326 that during the first heating phase (25–60 °C) P_i and Ca precipitate within micellar casein to form
327 new casein phosphate nanocluster or as inorganic calcium phosphate salt in the case of low micellar

328 casein concentration. During the second heating phase (60–80 °C), it would appear that changes to
329 the micellar casein-casein phosphate nanocluster interaction predominates as a result of the
330 increased negative charge of P_i . The authors believe that this is the first time that the influence of
331 singular ionic constituents on milk mineral equilibria during heat treatment was made possible
332 using advanced analytical techniques such as ^{31}P NMR and FTIR. This paper added useful
333 knowledge regarding the interaction between the mineral-containing serum (P_i and Ca) phase of
334 milk and the colloidal (micellar casein and casein phosphate nanocluster) phase.

335

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337

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341

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343

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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 dodecylsulphate 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, α_S -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.

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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.

