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Review

Heat-induced changes in milk salts: A review

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ABSTRACT

Although milk salts are classified as micronutrients, they strongly affect the behaviour of the proteins and the physical stability of milk and dairy products. This review focuses on heat-induced changes in milk salts, with particular emphasis on salt speciation, precipitation and association with proteins. In milk and other dairy liquids at neutral pH, heating results in precipitation of calcium phosphates. These precipitates may become stabilised by caseins and casein-derived (SerP-containing) peptides, with limited observable instability; in the absence of stabilising protein, precipitates can lead to notable instability. Increasing temperature reduces solubility of calcium phosphate, but, perhaps more importantly, also accelerates precipitate formation. In milk, heat-induced changes in salts are reversible, but only slowly, if heat-treatment is of moderate intensity. After treatment at temperatures >90 °C and/or for >20 min reversal may be incomplete. Heat-induced changes in milk salts are crucial for retaining functionality and stability during and after heating of milk.

Contents

1. Introduction

The salts in milk, and changes therein, play an important role in heat-induced changes in milk and other dairy products, such as the stability of milk to heat-induced coagulation. For instance, the influence of the addition of calcium salts and phosphate salts on the heat stability of milk is widely known; it is of high industrial relevance, with phosphate salts commonly added as stabilising salts in the manufacture of sterilised evaporated milk products (Huppertz, 2016). The salts affect several heat-induced changes in milk, including the heat-induced association of denatured whey proteins with casein micelles, the heat-induced dissociation of κ-casein from the casein micelles and the heat-induced changes in the pH of milk and dairy products (Anema, 2021; Huppertz, 2016). These reactions are crucial for the stability of milk during heat treatment, and also for the stability of heated milk products during storage; likewise, these heat-induced changes also strongly affect functional properties of the heated milk, e.g., acid gelation properties (Anema, 2021). Other examples of the relevance of temperature-induced changes in milk salts are their role in fouling of processing equipment, especially at UHT temperatures (Sadeghinezhad et al., 2013; Visser & Jeurnink, 1997), and the formation of

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precipitates in filtration permeate (Brulé, Real del Sol, Fauquant, & Fiaud, 1978). Mechanistic understanding of these key processes requires consideration of the salts in milk, their concentrations, their distribution and how this is affected by heat treatment.

In describing heat-induced changes in milk salts, we specifically use the term salt rather than the term mineral, because several of the components are not inorganic, and most of the inorganic components under discussion here do not occur in mines. We also avoid the word ‘ash’, which is frequently used to estimate the total salts content of dairy products and ingredients. Ash content gives relevant information only if batches of product are compared at the same protein content and composition, because P from casein SerP-residues and S from Met and Cys residues are included in the ash, and citrate, which is very relevant in ion-interactions, incinerates completely. Moreover, part of the Cl and SO_3 may evaporate, depending on the incineration temperature, so the method must be thoroughly standardised. All in all, measuring ash content is appropriate for monitoring purposes, but is of little use in research.

This paper reviews the state of the art with respect to the physical chemistry of the salts-casein system in bovine milk and changes therein upon heating. The components that we will focus on are the cations of Ca, Mg, Na K and H, and the anions of phosphate and citrate. Ions in such a mixture partly associate, and if activity product exceeds solubility product precipitation can occur, if given sufficient time. We will not discuss most ions that are present in concentrations of (much) less than 1 mmol kg^{-1}, such as Zn, Fe and Cu. Although these ions may strongly affect properties such as development of oxidative rancidity (Greenbank, 1948; O'Connor & O'Brien, 2006), or heat stability (Philippe, Le Graët, & Gaucheron, 2005), their behaviour mainly depends on the oxidation state, not on temperature or temperature history. Moreover, their natural concentration is too low to have any effect on protein stability and even if added concentrations up to the recommended allowance in a portion (max. 1 mmol kg^{-1}) the effects are still small.

Several models are available that quantify ion association and precipitation. The challenging and for industrial practice most relevant part of salts in dairy products is their interactions with the polyelectrolytes in milk, i.e., the proteins; for this, very few models are available and these are more empirical in nature. This review starts with the composition and distribution of salts in milk. Subsequent focus will be on temperature-induced changes in ultrafiltration permeates (Section 3), in whey (Section 4) and finally milk (Section 5), whereby the specific contributions of whey proteins and caseins can be distinguished. Throughout this review, focus will be on both changes resulting from altered solubility of salts, as well as the kinetics of these changes, and highlight that the latter is an important factor which has not featured strongly previously.

2. Composition and distribution of salts in milk

Average concentrations of the main salts in milk are shown in Table 1. For this, we use molal concentrations (mol kg^{-1}) because these can be more easily converted into activities, and because concentrations are usually reported in mg kg^{-1} rather than mg L^{-1}. Table 1 also shows the distribution of the salts between the milk serum and casein micelles. Salts in milk occur partly as free or complexed ions in solution, partly as ions associated to polyelectrolytes (counterions to proteins, peptides and phospholipids), and partly in undissolved form in the casein micelles; this applies especially to calcium and phosphate (Holt, 1997; Lucey & Horne, 2009). The non-dissolved salts are usually called colloidal or micellar salts and often collectively referred to as micellar calcium phosphate (MCP). It is important to note that counterions is not the same as the so-called Donnan-ions: upon (extensive) ultrafiltration or dialysis against water, proteins retain ions to the extent that net-charge-neutrality at both sides of the membrane is kept, which typically gives about 3–4 times higher concentrations for ‘bound’ ions, or Donnan ions, than for the counterions (Hiddink, de Boer, & Romijn, 1978). Donnan-ions explain why it is impossible to remove all salts from a product like whey protein isolate (WPI) despite very extensive diafiltration. The distribution of phosphorus is more complex than that of other elements, because part of P is inorganic, and part is organic and esterified to casein, carbohydrates, and phospholipids (Table 2). Sulphur in milk is largely in proteins as Cys and Met residues, and only about 10% of total sulphur in milk is the form of inorganic sulphate.

Table 3 gives the average number amino acids that can carry a positive or negative charge on the caseins and the main whey protein β-lactoglobulin (β-Lg), as well as their approximate pKa

Table 1
Concentrations of the major cations and anions milk and the proportion found in milk serum.[a]

Ion	Concentration in milk (mmol kg^{-1})	Fraction present in milk serum
Na	21	0.95
K	37	0.94
Ca	29	0.32
Mg	5	0.66
Cl	31	1
Inorganic PO_4	21	0.53
Citrate	9	0.92

[a] Data from Walstra, Walstra, Wouters, and Geurts (2005).

Table 2
Approximate distribution of phosphorus in milk.[a]

Type of P	% of total P in milk
Esterified to casein	22
Esterified in phospholipids	1
Various (sugar) esters in serum	9
Inorganic, in MCP (‘colloidal’)	32
Inorganic, dissolved	36

[a] Data from Walstra et al. (2005).

Table 3
Number of amino acid residues that can carry positive or negative charges present in different bovine caseins.[a]

Parameter	Lys	Arg	His	Glu	Asp	SerP
α_{S1}-casein	14	6	5	25	7	8–9
α_{S2}-casein	24	6	3	24	4	10–13
β-casein	11	4	5	19	4	4–5
κ-casein	9	5	3	12	4	0–3
β-lactoglobulin	15	3	2	16	10	0
pKa	10.6	12.0	6.4	4.5	4.0	1.5, 6.5

[a] Data from Huppertz (2013) and Walstra et al. (2005).

Table 4
Regions on caseins with notable net-negative and net-positive charge.[a]

Casein	Negative charge	Positive charge
α_{S1}-casein	f (30–80)*, f (110–150)	f (1–20)
α_{S2}-casein	f (1–20)*, f (50–80)*	f (160–207)
β-casein	f (1–60)*	
κ-casein	f (120–169)	f (1–100)

[a] Regions indicated with * contain a phosphorylation cluster. Data from Huppertz (2013).

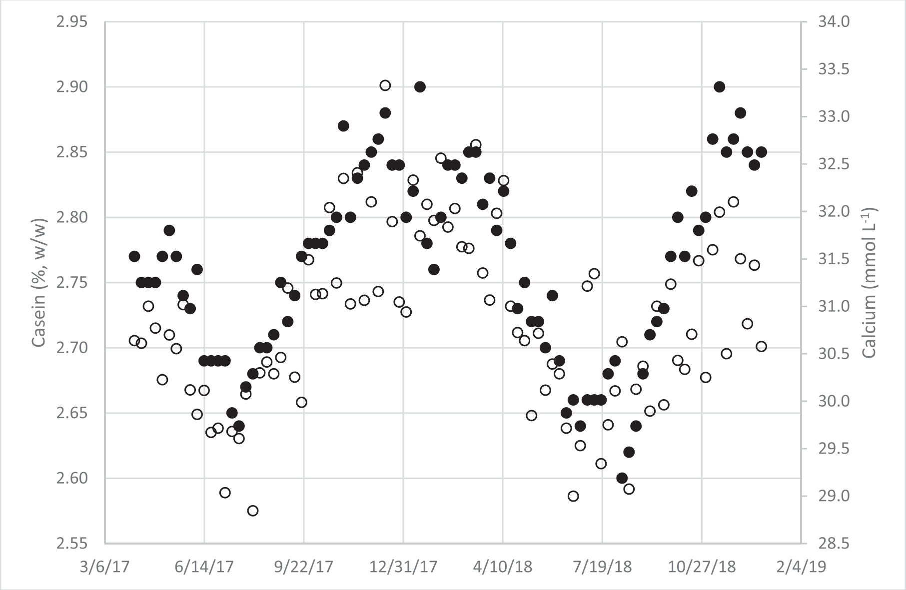

Fig. 1. Variation in casein (●) and calcium (○) content in bulk milk in the Netherlands between March 2017 and February 2019.

values of the amino acids that can carry a charge. Ion association to intrinsically disordered proteins, like caseins, at the moderate ionic strength of milk serum can be treated as the sum of the association to the individual residues, i.e., mainly the SerP residues and some pairs of Glu and/or Asp, except for nearest neighbour interactions (Bijl, Huppertz, van Valenberg, & Holt, 2019). Table 4 shows the regions on the caseins that carry a strong net-negative or net-positive charge. In principle, this also allows anions to be counterions at neutral pH, but, if any, these levels are often too low to be measured accurately, also because the strong binding anions, citrate and phosphate, are mainly present in the MCP (Pyne, 1962). Given the concentrations of positively charged amino acids in milk proteins (Tables 3 and 4), the low levels of measured anions acting as counterions are not expected; perhaps this could be taken as an indication of part of the positively charged amino acid residues being involved in intra- or intermolecular protein interactions, i.e., charged groups on proteins do not act completely independent. For native whey proteins, folding strongly affects ion binding; i.e., counterions cannot be calculated from individual residues. However, after heating the structure of whey proteins is (partially) unfolded and much less organised, which increases association with cations such as calcium (Phan–Xuan et al., 2014).

Several factors influence concentrations of salts and proteins in milk, one of which is season. Fig. 1 shows variation in calcium and casein content in pooled milk (one pooled sample prepared from bulk milk from several factories in the Netherlands) over a 2-year time period (data provided by QLIP, Zutphen, The Netherlands). Casein content varies from 2.6 to 2.9% (w/w) whereas calcium content varies between 29.0 and 33.5 mmol L^{-1}. Similar variation is observed, with maxima in winter and minima in summer. Variation is in line with previous reports from Bijl, van Valenberg, Huppertz, and van Hooijdonk (2013) and Karlsson, Langton, Innings, Wikström, and Lundh (2017). This may be related to year-round calving patterns in these countries. Small variations in total salts, however, may still be relevant for functional properties of dairy products, if only because the ion activities are the result of interactions between all ions. Concentrations of ions in milk vary a lot more between milk from individual cows (Huppertz, Heck, Bijl, Poulsen, & Larsen, 2021; White & Davies, 1958). While bulking of milk thus removes compositional variation, it is not known whether bulking also removes variability in functionality. See Table 5 for an illustration of variation in total and colloidal salts in individual cows.

Table 5
Variation in levels of total, soluble and colloidal calcium, phosphorus and magnesium in the milk of 48 Holstein-Friesian cows.[a]

Component		Total	Soluble	Colloidal
Ca	Average	31.4	9.6	22.7
	Min	26.1	6.3	18.7
	Max	37.3	12.0	29.5
P	Average	31.6	14.2	18.5
	Min	27.8	11.3	13.5
	Max	36.3	18.1	23.3
Mg	Average	4.6	3.1	1.7
	Min	3.5	2.4	0.5
	Max	5.4	4.0	2.8

[a] Data (in mmol L^{-1}) from Huppertz et al. (2021).

Principles of the methodology to study the distribution of salts in milk have not changed much since the pioneering work of Van Slyke and Bosworth (1915) and that of White and Davies (1958): equilibrium dialysis or low-pressure ultrafiltration separates the solution of salts and lactose from proteins, whereas ultracentrifugation separates the casein micelles, including MCP, from peptides like proteose peptones, the serum proteins, and small protein complexes, mainly non-micellar caseins; in heated milk this non-sedimenting phase also contains aggregates of caseins and denatured serum proteins. Small differences between results from various studies are only to be expected, and not only when considering the use of different milk sources (Davies & White, 1960). Details in fractionation methods, e.g., molecular weight cut-off of dialysis or ultrafiltration membranes, as well as conditions of dialysis or ultrafiltration, affects permeation of peptides and proteins. Also, ultracentrifugation conditions strongly affect the sedimentation of small protein complexes and aggregates, which is especially relevant for heated milk that may contain a large fraction of non-micellar protein, i.e., not-sedimenting at the applied ultracentrifugation conditions. For pasteurised skim milk, Huppertz et al. (2017) studied sedimentation behaviour of the different caseins on centrifugation at 20,000–400,000×g for 1 h. With increasing centrifugal force up to 100,000×g, progressive reductions in non-sedimenting caseins were observed at natural milk pH, but little further effect was observed on further increasing centrifugal force to 200,000 or 400,000×g. However, when centrifugation time at 400,000×g was extended to 72 h, no residual caseins could be detected in the ultracentrifugal supernatant (Huppertz et al., 2017).

For studying heat-induced changes in milk salts, measurements can be done at the high temperature, or by applying a heat treatment and measuring effects after cooling. A very limited number of studies are available on in-situ measurements, e.g., pH, Ca-ion activity or NMR, at elevated temperature (Boiani, Fenelon, FitzGerald, & Kelly, 2018; Chandrapala, McKinnon, Augustin, & Udabage, 2010; Zhang & Aoki, 1996). Furthermore, some studies performed separation at elevated temperatures and measured composition of the fractions (Pouliot, Boulet, & Paquin, 1989a). Most studies, however, studied the effects of heat treatment by measuring differences in salt balances or indicators thereof after heat treatment of the milk. In the following three sections, we will discuss basics and heat-induced changes in systems of increasing complexity: permeates (Section 3), whey products (Section 4) and milk products (Section 5).

3. Heat-induced changes in the salts in ultrafiltration permeates

Ideally, ultrafiltration separates the solution of salts and lactose in water from the macromolecules and particles. Obviously, amino acids and other small molecules also pass through the membrane, but we will not consider these components in detail, because their concentrations are low in the permeates of good quality milk and we presume their effect to be insignificant. Behaviour of all permeates, e.g., of milk and those of whey from cheese-making or from acid casein manufacture using lactic or minerals acids, can be understood by applying the principles that are described below.

The key factor for understanding is that solutions such as milk permeate are not simply a mixture of Na^+, K^+, Cl^-, etc., according to the serum composition in Table 1, because ions in solution are also partly associated. The approximate concentration of the various species can be calculated from the composition and the intrinsic (i.e., calculated using activities, see below) association constants. Only part of the species can actually be measured; e.g., ‘free’ calcium can be measured using a Ca-selective electrode (Lewis, 2011). Furthermore, the Donnan membrane technique allows measurement of many ions simultaneously (Gao et al., 2009), but this technique is laborious and needs a long measurement time; therefore, it is seldomly used in the field of dairy science. In most cases, ion speciation models are applied, as further outlined in below.

Calculations must be based on activities (a, mol kg^{-1}), rather than on concentrations (m, mol kg^{-1}). Activity is the ‘effective’ concentration. Activity is the same as concentration for ideal solutions, i.e., a very dilute solution, having much lower concentrations than milk serum. The relation between a and m, for component x is:

$$a_x \equiv \gamma_x m_x \tag{1}$$

In which γ is the activity coefficient. Various models are in-use to calculate the activity coefficient; for use in dairy products the empirical Davies equation is useful because it covers the relevant range of ionic strength of 0.05–0.5 M:

$$\log \gamma = -0.5z^2\left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.3I\right) \tag{2}$$

Table 6
Calculated concentrations (in mmol kg^{-1}) of free anions and complexes with cations for phosphate, citrate and chloride in milk at pH 6.72.[a]

Ion	Free anion	Cation complex			
		Ca^{2+}	Mg^{2+}	Na^+	K^+
H_2Cit^-	<0.01	<0.01	<0.01	<0.01	<0.01
$HCit^{2-}$	0.05	0.01	<0.01	<0.01	0.01
Cit^{3-}	0.28	6.51	2.14	0.03	0.05
$H_2PO_4^-$	7.60	0.07	0.04	0.10	0.18
HPO_4^{2-}	2.84	0.57	0.37	0.41	0.56
PO_4^{3-}	<0.01	0.01	<0.01	<0.01	<0.01
Cl^-	33.72	0.26	0.08	0.42	0.74

[a] Based on data in Table 1; calculations were done with a salt speciation model slightly modified from that published by Holt et al. (1981).

In which z is the charge of the ion species, and I is the ionic strength, calculated as:

$$I = 0.5\sum_i m_i z_i^2 \tag{3}$$

The typical ionic strength of milk serum is about 0.07 M, resulting in activity coefficients for mono-, di- and trivalent species of 0.81, 0.42 and 0.14, respectively according to Eq. (2). Thus, activity in milk serum is significantly lower than concentration; e.g., if Ca^{2+} concentration in milk serum is 1.6 mM, then aCa^{2+} is 0.67 mM. Using activities, the various equilibria in solution can be calculated, e.g., the dissociation of citric and phosphoric acid, and the association between Ca^{2+} and $citrate^{3-}$. Calculations must be on all equilibria simultaneously. Holt, Dalgleish, and Jenness (1981) published a salt speciation model that became the standard for milk serum, and dairy salt solutions in general. A full discussion of speciation modelling in milk is outside the scope of this review; an excellent discussion of most aspects given by Noeparvar (2018). The calculated association of the main ions in milk serum, using a slightly modified version of Holt's model (Holt et al., 1981), is given in Table 6, for the average total concentrations in milk serum as shown in Table 1.

From the composition of the serum, using activities, the under- or supersaturation of milk serum with respect to various crystalline phases can be calculated. Ignoring the hydration water, the dissolution of, e.g., calcium citrate can be written as:

$$(Ca_3citrate_2)_{solid} \leftrightarrow 3\ Ca^{2+} + 2\ citrate^{3-}$$

For which the ion activity product (IAP) of the ions in solution is:

$$IAP = \left(a_{Ca^{2+}}\right)^3 \left(a_{citrate^{3-}}\right)^2 \tag{4}$$

And the solubility product constant K_{sp} is the IAP when the dissolved ions and the solid are at equilibrium. If IAP in a solution is smaller than K_{sp}, precipitation cannot happen, or the solid phase may (partially) dissolve, and if it is larger precipitation may occur. For predicting what may happen, the saturation ratio, Ω, is useful, it is defined as:

$$\Omega = \left(\frac{IAP}{K_{sp}}\right)^{1/\nu} \tag{5}$$

In which ν is the total number of participating ions the solid. If Ω is >1, precipitation may occur, if it is <1, dissolution. See Table 7 for the approximate intrinsic (i.e., calculated using activities) solubility products at 20–25 °C, and the Saturation Ratio of a typical milk serum or permeate. Milk serum is supersaturated with respect to

Table 7
Chemical formula, approximate intrinsic solubility products and saturation ratio in a typical milk salts solution for selected salts that may form a precipitate in dairy products.[a]

Compound	Intrinsic solubility product -$Log_{10}(IAP)$	Saturation ratio
$CaHPO_4.2H_2O$ (DCPD)	6.68	2.0
$Ca_5OH(PO_4)_3$ (HAP)	58.2	11
$Ca_4H(PO_4)_3.5H_2O$ (OCP)	46.9	1.8
$Ca_3Citrate_2.4H_2O$	17.6	0.8
$MgHPO_4.3H_2O$	5.82	0.5

[a] Data from van Kemenade & de Bruyn, (1987a,b) for the phosphates and from Walstra et al. (2005) for citrate.

almost all calcium phosphates, close to saturation for calcium citrate, but not saturated for magnesium phosphate. Thus, already at room temperature, a precipitate can form in milk permeate if given sufficient time. Typically, simulated milk ultrafiltrate (SMUF; Jenness & Koops, 1962) becomes unstable after a few hours to days at room temperature (Dumpler, Kieferle, Wohlschläger, & Kulozik, 2017), but milk permeate may be stable for (much) longer. Probably, this is due to the presence of a small amount of proteinaceous material in the UF permeate. Schmidt, Both, Visser, and van Rooijen (1987), reported a so-called ‘nucleation time extension factor’ of >50 for 5 mg L^{-1} of β-casein peptide f1–25, suggesting an important role of small amounts of proteinaceous material in increasing the stability of permeates against precipitation; this is further discussed in Section 4. In practice, permeate with higher nitrogen content tends to be more stable. Additional stability of small precipitate particles in permeate versus model salts solutions, as observed by Noeparvar and Morison (2018), may originate from the presence of lactose-phosphate in milk, that may co-precipitate with calcium phosphate and limit the growth of the precipitate particles. A full discussion on precipitation at room temperature is outside the scope of this paper; however, key factors relevant for heat-induced changes in milk salts are:

1. The SI for various crystals depends strongly on pH, because this determines the activity of HPO_4^{2-} and PO_4^{3-}, and also that of $Hcitrate^{2-}$ and $citrate^{3-}$, which is relevant because $citrate^{3-}$strongly associates with Ca^{2+}. Usually, if a crystalline phase forms, the initial type is dicalcium phosphate dihydrate (DCPD) at pH < 6.5 (e.g., pH as in cheese whey), whereas at higher pH (e.g., that of milk) this is octacalcium phosphate (OCP; Mekmene et al., 2009; van Kemenade & de Bruyn, 1987a,b). From the various studies on this topic, it is clear that small differences in experimental conditions, e.g., extent of initial supersaturation or buffering capacity of the system, may have a considerable effect on the type of precipitate that is formed.
2. If DCPD, or an amorphous precipitate having a similar composition, is formed from a solution at pH 6–7, pH decreases, due to re-equilibration of $H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+$. Thus, in most dairy systems, pH decreases upon precipitation of calcium phosphate (Gao et al., 2010).
3. Nucleation takes time, especially at moderate supersaturation; Schmidt and Both (1987) reported a nucleation time of about 1 h for a moderately supersaturated calcium phosphate solution at pH 6.5 at 50 °C. Nucleation times in (non-concentrated) milk permeate or whey are probably shorter, because the supersaturation of DCPD is about twice as high as in the solutions used by Schmidt and Both (1987). This is in-line with the observation that SMUF instantaneously becomes turbid upon warming to temperatures >45 °C (Rosmaninho, Rizzo, Müller–Steinhagen, & Melo, 2003).
4. Precipitation usually follows the Ostwald rule of stages; i.e., the phase that is thermodynamically least stable forms first. The first phase is often amorphous, but in some cases crystalline, and serves as a template for the formation of the thermodynamically more stable phase, e.g., at the typical pH of milk or whey (6.0–6.7), formation of hydroxyapatite (HAP) is always preceded by DCPD or OCP (van Kemenade & de Bruyn, 1987a,b).
5. Magnesium and citrate slow down precipitation of calcium phosphate and change the type of precipitate. Holt, Timmins, Errington, and Leaver (1998a) and Holt et al. (1988b) found that amorphous calcium phosphate developed into DCPD rather than HAP at pH 7.0 in the presence of Mg. For calcium phosphate solutions, Mekmene et al. (2012) reported formation of DCPD at low (drifting) pH, and of Ca-deficient apatite at a constant pH of 6.7, but in the presence of citrate, the precipitates developed very little crystallinity, and citrate was present in, or on, the precipitate.

The aforementioned studies indicate that in milk permeate at room temperature, calcium phosphate precipitate is usually amorphous and acidic, and that composition of the precipitate or crystals is only expected to vary considerably between different studies, due to small differences in composition and pH. Increasing temperature induces or accelerates precipitation, because solubility of the relevant calcium phosphates (amorphous or crystalline) is lower at higher temperature. However, detailed information, especially on the effects of temperatures above 40 °C, is scarce; most studies on calcium phosphate precipitation have been done at 25 °C. An important difference between crystallisation at 37 °C versus at 25 °C was the induction period was about a factor of 3 shorter and the crystal growth was faster (van Kemenade & de Bruyn, 1987a,b). Also, maturation into more stable forms occurred in shorter timescales, even at the significantly lower phosphate concentrations at 37 °C that were chosen to obtain similar supersaturations at different temperature. From these studies it also appears that solubility products of DCPD and HAP only slightly decrease with temperature, but that of OCP decreases much more (van Kemenade & de Bruyn, 1987a,b).

Brulé et al. (1978) studied effects of heating on milk permeate up to 90 °C for about 30 min; they observed a fine precipitate if temperature was increased above 30 °C, and precipitation of 40% of Ca and 25% of P after heating to 60 °C, and of 60% and 30%, respectively after heating to 90 °C. Precipitates were amorphous and the precipitate obtained at 60 °C had Ca/P ratio of ~1, whereas that obtained at 90 °C had a Ca/P ratio of ~1.5 (Brulé et al., 1978). This is not necessarily an effect of temperature (only), but may also be due to faster maturation into a thermodynamically more stable form at higher temperature, or simply due to the longer time needed to heat to a higher temperature.

Brulé et al. (1978) also tested the effects of pH and partial demineralisation on precipitation after heating at 80 °C; interestingly the Ca/P ratio of the material transferred to the precipitate varied between about 1.0 and 1.5 already for the reference samples in their various experiments; i.e., small details in composition affect the composition of the precipitate. It should be noted that the precipitate was separated after cooling, so partial reversal may also have affected results. Heat-treatment of permeate resulted in a pH decrease of about 0.3–0.6 units (Brulé et al., 1978), as expected from work on calcium phosphate solutions that are allowed to precipitate at room temperature (Gao et al., 2010; Mekmene et al., 2009).

Heating and concentrating at pilot conditions shows that the nucleation time for precipitation from permeate may be longer than the processing time: no precipitated material was observed in the concentrate after heating and concentrating milk-UF-permeate at 60 °C up to a concentration factor of slightly below 2 (Tanguy et al., 2016). However, from the published results it is not clear whether precipitation had already occurred in the evaporator; for concentration factor of 8.5, this was very considerable, about 1/3 of total Ca (calculated from concentration in feed and concentration factor of total solids). Full understanding requires detailed studies, like the one by Panova (2001) on precipitation from lactic and sulphuric whey permeate that was neutralised to pH 6.8, i.e., at high supersaturation, at 75 °C. This study showed subtle effects, such as the precipitation of Mg in the first few min of heating, presumably because it was incorporated into the amorphous calcium phosphate that was the first to precipitate, whereas Mg resolubilised upon longer heating, presumably because it was not part of the apatite-like or other crystal formed that formed during prolonged heating, in-line with the Ostwald rule of stages. Also, precipitation in neutralised sulphuric acid whey permeate started later and was less extensive than in neutralised lactic acid whey permeate, presumably because the latter contained much less citrate, because citrate was metabolised by the lactic acid bacteria (Panova, 2001).

From the above, it is clear that precipitation of calcium phosphates can readily occur in ultrafiltration permeates when the temperature is increased. Depending on composition and heat-treatment settings, the initial precipitate will be amorphous in nature due to the presence of magnesium and citrate, which hinder the formation of crystalline phases of calcium phosphate. Longer heating times and higher temperature promotes formation of thermodynamically more stable salts. In addition, polyelectrolytes, such as peptides, and small amounts of proteins may affect precipitation of calcium phosphates. Their concentration is low, but may be significant, in ultrafiltration permeates, but much higher in whey and microfiltration permeate, which are covered in Section 4, and milk, which is covered in Section 5.

4. Heat-induced changes in the salts in whey and microfiltration permeate

Whey represents the solution of milk salts, lactose and proteins. The salts and lactose content of cheese whey is comparable with that of milk ultrafiltration permeate, whereas acid whey from fermented products or caseinate production are about 3 × higher in Ca and 2 × higher in P, due to dissolution of the MCP during acidification (Wong, LaCroix, & McDonough, 1978). Most of the proteins in whey are globular serum proteins, such as β-lactoglobulin or α-lactalbumin (α-La). At neutral pH, these proteins weakly associate with cations; precise quantitative data seem to be lacking, but we estimate this to be about 1.5–2 mol of total cations per mol of protein. In addition, α-La tightly binds an additional 2 Ca ions per molecule (Brew, 2013). However, at the typical α-La content of milk, this represents only 0.10–0.15 mmol Ca kg^{-1} milk (i.e., <2% of non-colloidal Ca or <0.5% of total Ca in milk), and thus does not have a major effect on ion-equilibria in milk and whey, perhaps with the exception of whey protein isolates. Thus, ion activity in the solution is hardly affected by the presence of serum proteins in quantities as in whey; hence in cheese whey supersaturation of calcium phosphates is similar as in milk ultrafiltration permeate.

After heating at 80 °C for 30 min, Brulé et al. (1978) observed much less precipitation (measured as sedimenting after 30 min at 2000×g) of calcium phosphate from cheese whey than from milk permeate. In mixtures of cheese whey and milk permeate, a reduced amount of heat-induced precipitate was also observed compared with pure permeate, if the mixture contained more than 50% whey; i.e., at a whey protein content higher than ~0.5% (Brulé et al., 1978). This stabilising effect of whey components was investigated in detail by Schmidt et al. (1987), who measured the effect of various milk- and whey-derived peptides and proteins on the nucleation rate of calcium phosphate solutions at 25 and 50 °C. Inhibition was defined as the extension of the nucleation time, as measured by light scattering and the start of the pH-decrease, from a moderately supersaturated solution (having a nucleation time of ~1 h), which was obtained by slowly titrating $Ca(NO_3)_2$ and a mixture of KH_2PO_4 and K_2HPO_4 into a 0.07 M KNO_3 solution. They concluded that the β-casein peptides f1–105/107 and f1–28, i.e., proteose peptone 5 (PP5) and proteose peptone 8-fast (PP8-fast), were strong inhibitors of calcium phosphate precipitation, whereas all other proteins in whey, including the casein macropeptide (CMP), provided little or no inhibition of calcium phosphate precipitation (Schmidt et al., 1987). Inhibition resulted in slower formation of precipitates, or no precipitate at all, within the timeframe of the experiment, which was 100 h.

Mixed proteins from acid whey showed stronger inhibition than those from cheese whey (Schmidt et al., 1987), in-line with the finding by Van Boekel and Crijns (1994) that ~75% of proteose peptone is retained in cheese, presumably because it is associated to the MCP during cheesemaking, whereas all proteose peptone is expelled to acid whey. Phosphate-rich peptides from casein are even capable of bringing calcium phosphate precipitate in dispersion (Holt, 2000; Reeves & Latour, 1958). Essential for this capability is the presence of a so-called phosphate-centre, i.e., three or more phosphorylated residues in a short sequence (Lenton, Nylander, Teixeira, & Holt, 2014), which will be elaborated on further in Section 5.

Although the main stabilising effect in whey compared with permeate comes from the casein-derived peptides, small amounts of residual casein in cheese or acid whey may also contribute to the relatively high stability of the calcium-phosphate in whey; Schmidt et al. (1987) also showed that whole casein, and especially the Ba- and Ca-precipitating fraction of casein, was a strong inhibitor of calcium phosphate precipitation. In the calcium-phosphate solution used by Schmidt et al. (1987), the type of precipitate was not affected by inhibition: DCPD precipitated pH 6.1 or below, and OCP at pH 6.4 or higher, at conditions in which nucleation was slowed-down, but not fully inhibited for the time of the experiment. However, van Kemenade and de Bruin (1989) reported extensive formation of DCPD at the cost of OCP in the presence of caseins, for precipitation from calcium phosphate solutions in which in the absence of caseins a mixture of DCPD and OCP was formed at pH 6.1 and mainly OCP at pH 6.15. This again indicates that small differences in composition and conditions, like pH, can have a significant effect on the type of precipitate.

Studies on salts precipitation from whey other than that by Brulé et al. (1978) seem to be lacking, but (industrially) concentrated whey is known to contain a large amount of precipitated (i.e., sedimenting at 2000×g) salts, mostly calcium citrate in acid whey and a mixture of calcium citrate and calcium phosphate in cheese whey (Saulnier, Ferrero, Bottero, & Linden, 1995; Vavrusova, Johanson, Garcia, & Skibsted, 2017). Probably, the combination of the high viscosity of concentrated whey and the relatively small size for the precipitates, which (partly) originates in the stabilising effect of the proteose-peptones, results in slow sedimentation, which is not noticed at industrial conditions. Milk-microfiltration (MF) permeate was observed to have a very different stability compared with the UF permeate that was made from this MF permeate: up to concentration factor of 8.5, no calcium and phosphate was lost in the evaporator for the MF permeate, and the concentrate showed only a minor turbidity at low concentration factor (Tanguy et al., 2016). However, at high concentration factor considerable precipitation occurred (Tanguy et al., 2016). This is in-line with the stabilisation of calcium phosphate in nanoclusters by phosphoproteins or peptides (Lenton, Wang, Nylander, Teixeira, &

Holt, 2020), in MF permeate from milk, stabilisation is probably partly by caseins and partly by PP5 and PP8-fast.

Overall, if whey is heated and/or concentrated, activity of calcium and phosphate exceeds saturation, similar to ultrafiltration permeate as discussed in the previous section. However, due to the presence of PP5 and PP8-slow, and casein molecules in milk-MF-permeate, precipitation is inhibited to a certain extent, so these liquids can be heated to higher temperature and for longer time, and if precipitates form, they will usually be smaller, and predominantly acidic.

5. Heat-induced changes in the salts in milk

In milk, a large part of the Ca and phosphate, and some of the Mg and Citrate, are not in solution, but in the MCP in the casein micelles. MCP is formed in the lactating cell, in a crowded solution of casein in a salts solution that is high in calcium from the beginning and is slowly made more concentrated in especially inorganic phosphate (Farrell, Malin, Brown, & Qi, 2006). Principles in milk are not different from those discussed above for permeate (Section 3) and whey (Section 4): calcium phosphate precipitates, Mg and citrate prevent the formation of crystalline material, and phosphate-centres on the casein molecules stabilise the amorphous particles, also named calcium phosphate nanoclusters. Details of the in vivo formation of these nanoclusters are still not clear, but it is clear that formation of artificial micelles or casein-stabilised calcium phosphate nanoclusters requires careful and slow addition of salts and adjustment of pH (Aoki, Kawahara, Kako, & Imamura, 1987; Holt, Wahlgren, & Drakenberg, 1996), at a rate that is much slower than that of the usual heating processes. The empirical formula for MCP is $[Ca_{13.2}(P_i)_{6.5}Mg_{1.0}Cit_{1.3}Casein_{1.0}(SerP)_4]_{49}$ (De Kruif & Holt, 2003); i.e., the nanoclusters contain both the organic SerP residues and inorganic phosphate. Nanoclusters have an estimated radius of about 2.5 nm, and are stabilised, i.e., prevented from growing into large particles, by the casein-SerP which forms a shell around the calcium-phosphate core (De Kruif & Holt, 2003; Lenton et al., 2020). Single or double SerP residues are not capable of associating strongly with the nanocluster surface (Aoki, Umeda, & Kako, 1992; Schmidt & Poll, 1989), but α_S- and β-casein have one or more so-called phosphate-centres, with at least 3 SerP-residues adjacent in the primary structure (De Kruif & Holt, 2003). The cooperative effect of several SerP, and likely also the Asp and Glu residues that are adjacent to the SerP (Vavrusova & Skibsted, 2013), anchors the protein on the MCP surface, thus preventing the amorphous particles to grow into large crystals. Taking the proteins perspective, MCP is key for the structural integrity of the casein micelles: if they are removed, e.g., by adding calcium binders like citrate, EDTA or hexametaphosphate, the casein micelles dissociate into primary casein particles (PCP's), showing by a strong reduction in turbidity of skimmed milk (Huppertz et al., 2017).

An important factor for heat-induced changes in milk is that, usually, not all phosphate centres of the caseins in milk are sequestered on the surface of an MCP-nanocluster, i.e., most milks have spare-capacity for calcium-phosphate stabilisation, i.e., non-sequestered casein molecules, probably especially β-casein (Aoki, Yamada, & Kako, 1990a; Aoki, Umeda, & Kako, 1990b; Bijl et al., 2019; Huppertz et al., 2021). Similar to Bijl et al. (2019), we presume that nanoclusters are integers and that upon a change in conditions, e.g., temperature or pH, they dissolve per nanocluster; i.e., if a cluster starts to dissolve it goes completely, leaving others intact, and in supersaturated serum, nucleation happens at a phosphate centre, resulting in a new cluster of 2.5 nm radius, fully stabilised by casein.

Another key factor is that part of Ca and inorganic phosphate (P_i) exchanges between serum and micelles at time scales of less than a second (Boiani et al., 2018), and that part of Ca and P_i in the MCP are exchangeable within the timescale of minutes to hours, as was shown by experiments using ^{45}Ca and $^{32}PO_4$ (Pierre, Brulé, & Fauquant, 1983; Yamauchi & Yoneda, 1977; Zhang & Aoki, 1996). Approximately 35% of P_i (i.e., ~4 mM), and 45% of Ca (i.e., ~9 mM), that was present in the colloidal phase of milk was estimated to be 'hard to exchange' (Zhang & Aoki, 1996); thus ~6 and 11 mM of Ca and P_i from MCP exchanged readily within the time scale of the experiments, i.e., typically >1 h. Heat-treatment hardly changed the proportion of 'hard to exchange' Ca if the ^{45}Ca was added after heating (Yamauchi & Yoneda, 1977), but if added before heating it significantly decreased; after 30 min at 120 °C no 'hard to exchange' Ca was measured (Pierre et al., 1983). More rapidly exchangeable calcium and phosphate may be considered counterintuitive when reasoning from the reduced solubility of calcium phosphates at different temperatures. However, when considering the influence of temperature on the rate of exchange, the increased levels of exchangeable calcium at elevated temperature (^{45}Ca added before heat-treatment) clearly indicate a mainly kinetic effect. Further studies are required to validate this.

Taking milk at 38 °C in the udder of the cow as the natural state, cooling results in dissolving of part of the nanoclusters. Warming back to 30–40 °C and heating to higher temperature results in formation of less ultrafiltration-permeable Ca and P, and possibly also Mg and citrate. Pioneering work on collecting ultrafiltration permeate at temperatures up to 110 °C was done by Rose and Tessier (1959), but because their UF-system suffered from strong fouling, we use later results by Pouliot, Boulet, and Paquin (1987, 1989a) for the discussion below, which were also later confirmed by Kaombe, Du, and Lewis (2012) and expanded to higher temperatures (up to 110 °C) by On-Nom, Grandison, and Lewis (2010). The results of Poulliot et al. (1987) show a strong decrease of Ca and P_i in the permeate collected at higher temperature, a small decrease of Mg and citrate, and no change in K and Na. However, it is important to realise that a large effect on serum concentration is a much smaller effect on colloidal content: taking 40 °C (2° above the udder temperature) as base value, cooling to 4 °C releases about 10% of colloidal Ca, and warming to 80 °C requires formation of 10% extra (Table 8); this range is far smaller than the variation in casein mineralisation observed between different cows (Huppertz et al., 2021).

The ratio $(Ca + Mg)/(P_i + citrate)$ for the heat-induced precipitate by Pouliot et al. (1989a) was similar to the 2.0 for the nanoclusters mentioned above: at bit higher at low temperature, very close to 2.0 at 40–80 °C, and about 1.8 at 90 °C. This is in-line with the observations, using ^{31}P NMR, of Boiani et al. (2018), who concluded that Ca/P_i is lower at high temperature, which was assumed to originate from the lower degree of protonation of phosphate at higher temperature (i.e., pKa is temperature-

Table 8
Serum concentrations in milk ultrafiltration permeate from milk held for prolonged periods at various temperatures, and calculated colloidal concentration.[a]

T (°C)	Ca (mM)		P_i (mM)		$(Ca + Mg)/(P_i + Cit)$, colloidal
	Permeate	Colloidal	Permeate	Colloidal	
4	8.78	22.4	11.46	9.8	2.28
20	7.86	23.3	11.32	10.0	2.24
40	6.67	24.4	9.89	11.4	2.09
60	5.52	25.5	8.54	12.7	1.99
80	4.27	26.7	7.88	13.4	1.97
90	3.51	27.4	7.45	13.8	1.79

[a] Periods held were 360 min at 20 °C, 30 min at 90 °C, intermediate time at intermediate temperatures. Total Ca and P_i were 30.8 and 21.4, respectively. Colloidal concentration was calculated as (total − permeate × 0.96), to adjust for the serum content of the milk. Data after Pouliot et al. (1987).

dependent). A large part of the transfer of salts to the colloidal phase occurred fast, within a couple of minutes (Pouliot et al., 1989a); however, changes on short time scales could not be investigated, if only because flushing the system took 1.5 min at 90 °C and up to 15 min at 4 °C. Anema (2009), who performed similar experiments to Pouliot et al. (1989a) for milk and milk concentrates up to 38% total solids, measured Ca, P_i and pH in permeate collected during 60 min of heating-recirculation. Results indicate that heat-induced changes of Ca and P_i in concentrated milk are similar to those in milk, despite the fact that pH of concentrates was lower than that of milk (at 20 °C 6.7 for milk and 6.3 for 38% solids concentrate, and at 80 °C 6.3 and 5.9. respectively. Almost all data in a plot of delta Ca versus delta P_i fell on a single line with a slope of 1.0 (Anema, 2009), which is lower, even when considering the inaccuracy that is inherent to such calculations, than the slope calculated from the data by Pouliot et al. (1987, 1989a): about 1.2 for delta Ca/delta P_i colloidal between 80 and 4 °C (calculated from the data in Table 8). Thus, the observations of Pouliot et al. (1987, 1989a) indicate that heat-induced precipitate is similar to the native MCP, and is possibly stabilised by the casein molecules that are not-sequestered at ambient temperature. The lower Ca:P_i ratio of the precipitate observed by Anema (2009) indicate precipitation of calcium phosphate with little or no additional association to SerP, i.e., growth of the inorganic part of the MCP only (resulting in slightly bigger nanoclusters), or the formation of a precipitate with little or no stabilisation by casein. An interesting distinction between the studies is that Pouliot et al. (1987, 1989a) started from fresh milk, whereas Anema (2009) used reconstituted skim milk powder.

At higher temperature, permeate Ca concentrations are still lower. On-Nom et al. (2010) observed a continuing decrease of total divalent cations in milk dialysate attained up to 120 °C, and pH and $a\mathrm{Ca}^{2+}$ of this permeate, after cooling to room temperature, was also lower with increasing temperature at which dialysis was performed, down to pH 5.9–6.0 and $a\mathrm{Ca}^{2+}$ of 0.2–0.3 mM after dialysis at 120 °C. In this work, ~5 mM Ca was dissolved and ~25 mM colloidal at 80 °C, changing to ~2.5 mM and ~27.5 mM, respectively, at 120–130 °C. Overall, the probability that all calcium and phosphate that becomes insoluble upon heating above 100 °C can be stabilised by casein is smaller, but this affects only a few mM of calcium and phosphate.

Reversibility after heating to up to 90 °C appears to be slow, as judged from results by Kannan and Jenness (1961): after cooling and dialysis against the unheated milk at 4 °C for 48 h, colloidal calcium and phosphate remained high, and total increased compared with raw milk, indicating that the heat-precipitated material dissolves so slowly that dialysis is able to restore serum composition before dissolution is complete (Kannan & Jenness, 1961). Presumably, the biggest part of the restoration of equilibrium by dialysis took less than one day, indicating that part of the heat-precipitated calcium phosphate is stable for at least a few hours after cooling. Slow reversibility or incomplete of heat-induced changes in the salts balance of milk has also been established by Law (1996), Muldoon and Liska (1972), Pouliot, Boulet, and Paquin (1989b), Van Dijk and Hersevoort (1992) and Wang and Ma (2020). Particularly heat treatments >90 °C can cause irreversible changes in the salt distribution.

A clear indication that not all calcium and phosphate that precipitates at high temperature is stabilised by casein, at least not at the salt:casein ratio as found in casein micelles, is that during heating of milk fouling of heating equipment can occur. Especially at the high temperature section of UHT plants, most of the material on surfaces is calcium-phosphate, usually with $\mathrm{Ca/P_i} = 1.5$ or even higher, especially at long running times where the precipitate could mature from amorphous to crystalline (Visser & Jeurnink, 1997). However, it must be realised that, for milk, the amount of Ca and P_i in a fouling layer is less than 0.5% of the total present in the milk that had passed the wall of the heater, so fouling may be a local phenomenon, in which the wall is a surface on which nucleation may happen from the supersaturated solution. Another indication that not all heat-precipitated calcium phosphate in milk is stabilised by casein is given by Nelson, Holt, and Hukings (1989), who showed the presence of poorly crystalline β-tricalcium phosphate in milk that was retort-heated for 5 min at 120 °C. But again, this was described as 'a small amount of poorly crystalline phase in a largely amorphous material'. If a large surface area is added to the milk, e.g. by adding yeast cells or cotton wool, heating induces precipitation of a large part of the calcium and phosphate on this surface, at a $\mathrm{Ca/P_i}$ of about 1.5, whereas no salts precipitated on the yeast cells at room temperature (Evenhuis & De Vries, 1955).

Overall, although it is clear that during heating to 80–140 °C most of the calcium and phosphate in (cows) milk serum becomes insoluble, details of product composition and heating rates may determine whether the additional precipitated material is stabilised by caseins. Starting from cold-stored milk, sufficient phosphate centres are surely available for warming up to 40 °C, and in most milks also for heating up to 80 °C, but probably not for heating to higher temperatures. Data to conclude whether the additional material, or all calcium phosphate in heated milk products, still resembles native nanoclusters, is lacking, although at moderate heating intensity is seems to be similar, not crystalline or having a very different $\mathrm{Ca/P_{i.}}$ Taking the proteins perspective, heat-treatment is known to result in a significant increase in not-UC-sedimentable casein, not only κ-, but also α_S- and β-caseins (Anema, 2021). Also, Aoki, Umeda, and Kako (1990) reported 'cleavage' of the linkage between casein and calcium phosphate after heating at 135–140 °C for 75 s and cooling, whereas no increase or decrease in casein linked to (micellar) calcium phosphate was measured after heating at 60–90 °C. Thus, during or after heat-treatment some casein dissociates from the micelles, and fewer caseins are linked to MCP, i.e., an irreversible effect, whereas during heat-treatment additional calcium phosphate precipitates, presumably in the micelles, which is reversible upon cooling. Thus, if MCP is defined as including the SerP-centres, it is fairly certain that moderately or high-heated milk, at least part of the MCP has changed. A relevant factor probably is the heating-rate: in the lactating cell, or in experiments on artificial micelles or nanoclusters (Farrell et al., 2006; Holt et al., 1996; Huppertz et al., 2017), supersaturation is very small due to slow addition of calcium and phosphate, or slow increase of pH, whereas heating is usually done within minutes, i.e., considerable supersaturation is attained quickly. Similar to in milk permeate, precipitated material in rapidly-heated milk may be amorphous, and similar to in whey, it is likely to be stabilised by casein molecules, which at high temperature may also be in the casein micelles, thus the precipitated salts will also be located in the micelles – nucleation will start at the phosphate-centre. Key is that the additional insoluble calcium-phosphate upon heating is stabilised by the casein, and it is only to be expected that composition and size of the insoluble material varies considerably between milk samples and also varies with details of processing conditions.

6. Conclusions and future perspectives

In this article, we reviewed the heat-induced changes in milk salts, in permeate, in whey and in milk. In all three systems, precipitates consisting of primarily calcium and phosphate are formed. Such precipitates are typically amorphous in nature, at least initially, and may mature into crystalline material, especially in permeate and whey. Heat-induced precipitation of calcium phosphates is related to the reduction in solubility with increasing temperature. However, a kinetic component should also not be overlooked, where precipitation reactions which occur over long time scales at low temperatures are accelerated to such an extent at higher temperatures that they occur within the time scale of heat treatments commonly applied in the dairy sector. In permeates, the presence of citrate and magnesium can hinder precipitate formation, whereas in whey, particularly the peptides PP5 and PP8 are potent inhibitors of calcium phosphate precipitate formation. In milk, caseins, in both micellar and non-micellar form provide additional stability against heat-induced precipitate formations. Mechanistic understanding of heat-induced changes in milk salts is still lacking in areas, which is at least partly related to the fact that in situ measurements are challenging. Advances in spectroscopic techniques, such as ^{31}P NMR at elevated temperature may facilitate further insights.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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