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Hydroxycarboxylate combinations for increasing solubility and robustness of supersaturated solutions of whey mineral residues

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ABSTRACT

Calcium phosphates present in whey mineral residue is a potential source of calcium for dietary purposes. Combinations of aqueous isocitrate and citrate were found more efficient than each of the isomers in dissolving dried insoluble whey processing mineral residues spontaneously forming supersaturated solutions. Hydrogen isocitrate was found around 30% less efficient in these non thermal dissolution processes compared to hydrogen citrate based on amount of dissolved calcium. In contrast, the lag phase of up to 4 h for precipitation of calcium citrate from the supersaturated solutions was significantly longer when calcium isocitrate was present. Highest degree of supersaturation with longest lag phase for precipitation was found for citrate/isocitrate combinations in a 1:1 ratio. Addition of calcium saccharate during dissolution further prolonged the lag phase simultaneously preserving the higher supersaturation degrees. Combinations of the three hydroxycarboxylates seem accordingly to provide a basis for increasing calcium availability from dried whey mineral fractions consisting mainly of calcium hydrogen phosphate and hydroxyapatite of low solubility with the perspective of transforming a side stream from cheese production into valuable functional foods.

1. Introduction

Calcium is an essential nutrient and the recommended daily intake is around one gram depending on age, gender, and living conditions (Bronner, 1987; Thorpe & Evans, 2011). Low bioavailability of calcium often causes malnutrition even for individuals with a high and constant dietary intake of calcium and osteoporosis is becoming epidemic in several population segments (Tondapu et al., 2009). The food industry is accordingly inspired to search for sources of calcium for development of functional foods with high bioavailability and a natural image.

Whey mineral residues consisting of mainly calcium hydrogen phosphate and hydroxyapatite hold the potential as a side stream from cheese production of becoming a valuable resource for the dairy industry for development of such functional foods with high calcium bioavailability and a natural and green image (Gaucheron, 2011). Whey mineral residues have previously been considered as a problematic waste and the use as a food ingredient been hampered by a low solubility.

The problem of low solubility of dried mineral residues from whey after whey proteins and lactose have been removed as valuable ingredients for use in food production was recently solved by the demonstration that the mineral residues readily dissolves in aqueous solutions of gluconolactone/gluconate combinations or of hydrogen citrate even without heating spontaneously forming strongly supersaturated solutions (Zawadzki & Skibsted, 2019, 2020). The degree of supersaturation has been found to be up to a factor of more than 20 for calcium hydrogen phosphate with a long lag phase for precipitation from the supersaturated solution depending on the nature and concentration of the hydroxycarboxylate used for solubilization (Vavrusova, Danielsen, Garcia, & Skibsted, 2018). Hydroxycarboxylic acids, like citric and gluconic acids are widely known and used in food and pharmaceutical industries, among other applications, due to theirs complexing capabilities (Kutus et al., 2020). That, allied with the fact that both minerals and these hydroxycarboxylic acids, as well as their salts, are naturally components of many foods, would facilitate the marketing of this type of functional foods.

Combinations of citrate and isocitrate have moreover recently been found to enhance the mutual solubility of their calcium salts in water and to lower the precipitation rate from such supersaturated aqueous solutions by some unknown mechanism (Cheng, Garcia, Tang,

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1. $H_3PO_4 \rightleftharpoons H_2PO_4^- + H_3O^+$

$K_{a1} = \frac{[H_3O^+]\gamma^+ \times [H_2PO_4^-]\gamma^-}{[H_3PO_4]}$ $K_c = \frac{[H_3O^+][H_2PO_4^-]}{[H_3PO_4]}$

$K_{a1} = K_c \gamma^+ \gamma^- = 7.537\ 10^{-3}$ pKa = 2.12 from Nims (1934).

2. $H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H_3O^+$

$K_{a2} = \frac{[H_3O^+]\gamma^+ \times [HPO_4^{2-}]\gamma^{2-}}{[H_2PO_4^-]\gamma^-}$ $K_c = \frac{[H_3O^+][HPO_4^{2-}]}{[H_2PO_4^-]}$

$K_{a2} = K_c \gamma^{2-} = 6.223\ 10^{-8}$ pKa = 7.20 from Nims (1933).

3. $H_3Citr + H_2O \rightleftharpoons H_2Citr^- + H_3O^+$

$K_{cit1} = \frac{[H_3O^+]\gamma^+ \times [H_2Citr^-]\gamma^-}{[H_3Citr]}$ $K_c = \frac{[H_3O^+][H_2Citr^-]}{[H_3Citr]}$

$K_{cit1} = K_{cc} \gamma^+ \gamma^- = 7.43 \times 10^{-4}$ pK_a = 3.13 from Bates & Pinching (1949)

4. $H_2Citr^- + H_2O \rightleftharpoons HCitr^{2-} + H_3O^+$

$K_{cit2} = \frac{[H_3O^+]\gamma^+ \times [HCitr^{2-}]\gamma^{2-}}{[H_2Citr^-]\gamma^-}$ $K_c = \frac{[H_3O^+][HCitr^{2-}]}{[H_2Citr^-]}$

$K_{cit2} = K_c \gamma^{2-} = 1.74 \times 10^{-5}$ pK_a = 4.76 from Bates & Pinching (1949)

5. $HCitr^{2-} + H_2O \rightleftharpoons Citr^{3-} + H_3O^+$

$K_{cit3} = \frac{[H_3O^+]\gamma^+ \times [Citr^{3-}]\gamma^{3-}}{[HCitr^{2-}]\gamma^{2-}}$ $K_c = \frac{[H_3O^+][Citr^{3-}]}{[HCitr^{2-}]}$

$K_{cit3} = \frac{K_c \gamma^+ \gamma^{3-}}{\gamma^{2-}} = 3.98 \times 10^{-7}$ pK_a = 6.4 from Bates & Pinching (1949)

6. $Ca^{2+} + HCitr^{2-} \rightleftharpoons CaHCitr$

$K_1 = \frac{[CaHCitr]}{[Ca^{2+}]\gamma^{2+} \times [HCitr^{2-}]\gamma^{2-}}$ $K_c = \frac{[CaHCitr]}{[Ca^{2+}] \times [HCitr^{2-}]}$

$K_1 = \frac{K_c}{\gamma^{2+}\gamma^{2-}} = 2.60 \times 10^3$ from Davies and Hoyle (1953)

7. $Ca^{2+} + Citr^{3-} \rightleftharpoons CaCitr^-$

$K_2 = \frac{[CaCitr^-]\gamma^-}{[Ca^{2+}]\gamma^{2+} \times [Citr^{3-}]\gamma^{3-}}$ $K_c = \frac{[CaCitr^-]}{[Ca^{2+}] \times [Citr^{3-}]}$

$K_2 = \frac{K_c \gamma^-}{\gamma^{2+}\gamma^{3-}} = 4.96 \times 10^4$ from Vavrusova & Skibsted (2016)

8. $Ca^{2+} + HPO_4^{2-} \rightleftharpoons CaHPO_4$

$K_3 = \frac{[CaHPO_4]}{[Ca^{2+}]\gamma^{2+} \times [HPO_4^{2-}]\gamma^{2-}}$ $K_c = \frac{[CaHPO_4]}{[Ca^{2+}][HPO_4^{2-}]}$

$K_3 = \frac{K_{c1}}{\gamma^{2+}\gamma^{2-}} = 500\ L\ mol^{-1}$ from Davies & Hoyle (1953)

Fig. 1. Equations for ion speciation.

Danielsen, & Skibsted, 2018). Notably, citrate may act not only as a solubilizer, but also at a later stage during digestion as a promoter of calcium absorption (Pak, Harvey, & Hsu, 1987). More recently, a positive correlation between high bone mass and circulating citrate seems to indicate that dietary combinations of calcium and citrate may increase calcium turnover and increase bone strength (Hartley et al., 2019). Accordingly, it seems timely to explore the effect of combining citrate with isocitrate, hydroxycarboxylates both naturally occurring in various fruits, for any synergistic effects in increasing solubility of calcium from whey mineral residues, also with the perspective of development of new functional foods with increased bioavailability. Calcium saccharate has been found to prevent precipitation from supersaturated solutions of other calcium hydroxycarboxylates despite a low solubility and was included in the investigation in order to optimize the formulation of strongly supersaturated calcium salts solutions with high robustness (Siegrist, 1949).

2. Materials and methods

2.1. Materials

Capolac® MM-0525 BG, a commercial dried milk mineral concentrate based on whey from bovine cheese production, was from Arla Food Ingredients (Viby J., Denmark). The same batch of Capolac® was used for all the experiments. According to manufacturer's information, Capolac® contains 24.0 – 29.0% calcium, 11.0 – 15.0% phosphorous, 6.0 – 10.0% lactose, less than 3.0% moisture, less than 3.0% protein and less than 3.0% fat. The informed content of citrates is 4.0%, which corresponds to less than 1.5% of the total citrate added in the dissolution experiments. As previously described (Zawadzki & Skibsted, 2019), the particular batch of Capolac® used in the present study was found to contain 26.5% of calcium and 13.3% of phosphorous corresponding to 30% $CaHPO_4{\cdot}2H_2O$ and 70% $Ca_5(OH)(PO_4)_3$, based on the

9. $Na^+ + HCitr^{2-} \rightleftharpoons NaHCitr^-$

$$K_4 = \frac{[NaHCitr^-]\gamma^-}{[Na^+]\gamma^+ \times [HCitr^{2-}]\gamma^{2-}} \quad K_c = \frac{[NaHCitr^-]}{[Na^+] \times [HCitr^{2-}]}$$

$$K_4 = \frac{K_c \gamma^-}{\gamma^+ \gamma^{2-}} = 8.7$$ from Daniele et al. (2008)

10. $Na^+ + Citr^{3-} \rightleftharpoons NaCitr^{2-}$

$$K_5 = \frac{[NaCitr^{2-}]\gamma^{2-}}{[Na^+]\gamma^+ \times [Citr^{3-}]\gamma^{3-}} \quad K_c = \frac{[NaCitr^{2-}]}{[Na^+] \times [Citr^{3-}]}$$

$$K_5 = \frac{K_c \gamma^{2-}}{\gamma^+ \gamma^{3-}} = 34.7$$ from Daniele et al. (2008)

11. $H_3isoCitr + H_2O \rightleftharpoons H_2Citr^- + H_3O^+$

$$K_{isocit1} = \frac{[H_3O^+]\gamma^+ \times [H_2isoCitr^-]\gamma^-}{[H_3isoCitr]} \quad K_c = \frac{[H_3O^+][H_2isoCitr^-]}{[H_3isoCitr]}$$

$$K_{isocit1} = K_{cc}\gamma^+\gamma^- = 5.13 \times 10^{-4}$$ $pK_a = 3.40$ from Brosset (1976)

12. $H_2isoCitr^- + H_2O \rightleftharpoons HisoCitr^{2-} + H_3O^+$

$$K_{isocit2} = \frac{[H_3O^+]\gamma^+ \times [HisoCitr^{2-}]\gamma^{2-}}{[H_2isoCitr^-]\gamma^-} \quad K_c = \frac{[H_3O^+][HisoCitr^{2-}]}{[H_2isoCitr^-]}$$

$$K_{isocit2} = K_c\gamma^{2-} = 1.95 \times 10^{-5}$$ $pK_a = 4.71$ from Brosset (1976)

13. $HisoCitr^{2-} + H_2O \rightleftharpoons isoCitr^{3-} + H_3O^+$

$$K_{isocit3} = \frac{[H_3O^+]\gamma^+ \times [isoCitr^{3-}]\gamma^{3-}}{[HisoCitr^{2-}]\gamma^{2-}} \quad K_c = \frac{[H_3O^+][isoCitr^{3-}]}{[HisoCitr^{2-}]}$$

$$K_{isocit3} = \frac{K_c\gamma^+\gamma^{3-}}{\gamma^{2-}} = 3.98 \times 10^{-7}$$ $pK_a = 6.40$ from Brosset (1976)

14. $Ca^{2+} + IsoCitr^{3-} \rightleftharpoons CaIsoCitr^-$

$$K_6 = \frac{[CaIsoCitr^-]\gamma^-}{[Ca^{2+}]\gamma^{2+} \times [IsoCitr^{3-}]\gamma^{3-}} \quad K_c = \frac{[CaIsoCitr^-]}{[Ca^{2+}] \times [IsoCitr^{3-}]}$$

$$K_6 = \frac{K_c\gamma^-}{\gamma^{2+}\gamma^{3-}} = 9.07 \times 10^3$$ from Cheng & Skibsted (2018)

15. $Ca^{2+} + Sac^{2-} \rightleftharpoons CaSac$

$$K_6 = \frac{[CaSac]}{[Ca^{2+}]\gamma^{2+} \times [Sac^{2-}]\gamma^{2-}} \quad K_c = \frac{[CaSac]}{[Ca^{2+}] \times [Sac^{2-}]}$$

$$K_6 = \frac{K_c}{\gamma^{2+}\gamma^{2-}} = 1.03 \times 10^3$$ from Garcia et al. (2016)

Mass balance equations:

16. $t_p = [H_3PO_4] + [H_2PO_4^-] + [HPO_4^{2-}] + [CaHPO_4]$
17. $t_{Citr} = [CaHCitr] + [\mathrm{CaCitr}^-] + [Citr^{3-}] + [HCitr^{2-}] + [H_2Citr^-] + [H_3Citr] + [NaCitr^{2-}] + [NaHCitr^-]$
18. $t_{Ca} = [CaHPO_4] + [CaHCitr] + [\mathrm{CaCitr}^-] + [CaisoCitr^-] + [Ca^{2+}] + [CaSac]$
19. $t_{Na} = [NaCitr^{2-}] + [\mathrm{NaHCitr}^-] + [Na^+]$
20. $t_{IsoCitr} = [H_3isoCitr] + [H_2isoCitr^-] + [HisoCitr^{2-}] + [isoCitr^{3-}] + [CaisoCitr^-]$
21. $t_{Sac} = [\mathrm{Sac}^-] + [CaSac]$

Fig. 1. (*continued*)

total calcium content.

Sodium citrate dihydrate ($Na_3Citr{\cdot}2H_2O$), sodium hydrogen citrate sesquihydrate ($Na_2HCitr{\cdot}1.5H_2O$), DL-isocitric acid trisodium salt hydrate ($Na_3isoCitr{\cdot}xH_2O$), calcium saccharate (CaSac), phosphorus standard for inductively coupled plasma optical emission spectroscopy, ICP-OES (9.992 ± 20 mg L^{-1}), calcium hydrogen phosphate dihydrate ($CaHPO_4{\cdot}2H_2O$), calcium standard for ICP-OES, (10.005 ± 20 mg L^{-1}) and, nitric acid 65% Suprapur®, were all from Sigma-Aldrich (Steinheim, Germany). Water purified by a Milli-Q Plus purification station (Millipore Corporation, Bedford, MA) was used throughout. Calcium chloride dihydrate was from Merck KGaA (Darmstadt, Germany). Q-Max RR syringe filters (filter diameter: 13 mm, membrane: 0.22 μm cellulose acetate) were from Frisenette ApS (Knebel, Denmark).

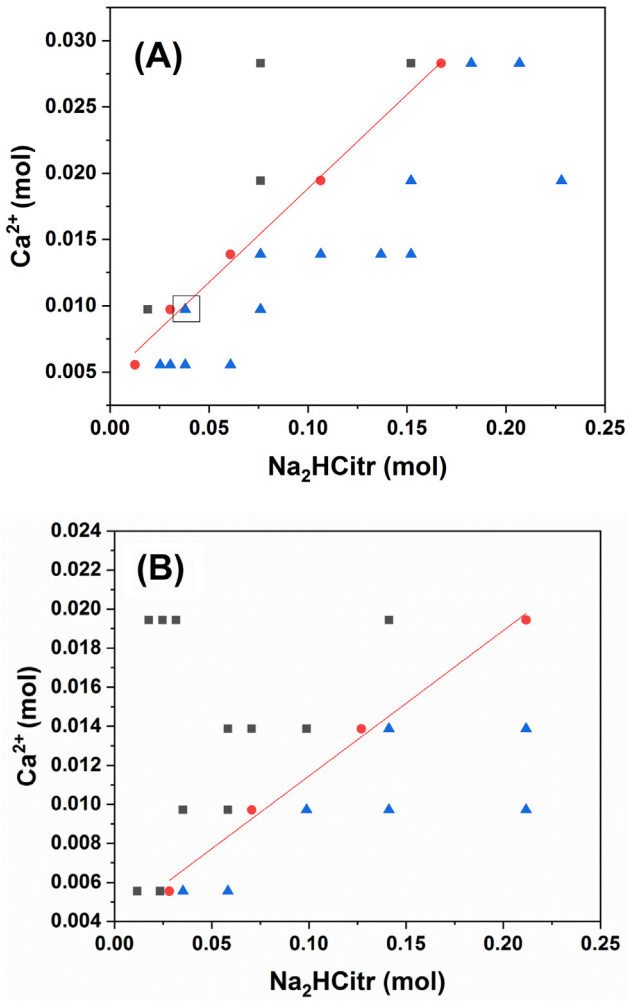

Fig. 2. Critical combination of (A) Capolac® or (B) $CaHPO_4{\cdot}2H_2O$ and Na_2HCitr /isoCitr for the formation of supersaturated solutions in water at 25 °C for a Na_2HCitr /isoCitr ratio of 1:1 as found by visual inspection. Two phase systems are represented by ■; critical calcium/citrate combinations for formation of homogeneous supersaturated solutions are indicated by ●; ▲ indicate the systems for which homogeneous supersaturated solutions were formed. Ion speciation of experiment marked with □ is shown in Table 4.

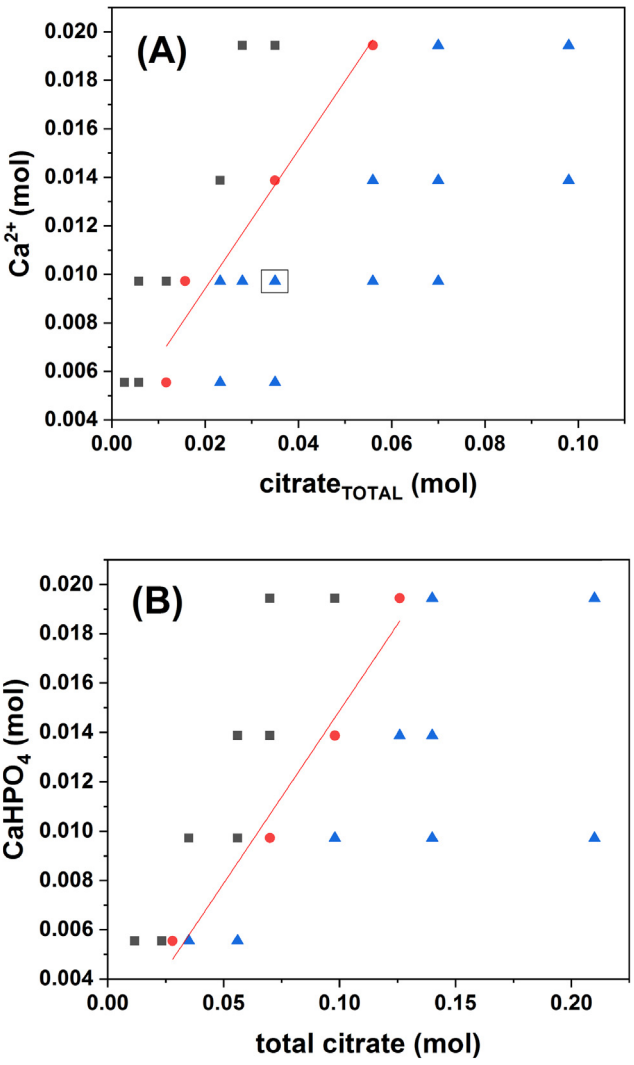

Fig. 3. Critical combination of (A) Capolac® or (B) $CaHPO_4{\cdot}2H_2O$ and Na_2HCitr /Na_3Citr for formation of supersaturated solutions in water at 25 °C for a Na_2HCitr / Na_3Citr ratio of 1:1 as found by visual inspection. Two phase systems are represented by ■; critical calcium/citrate combinations for formation of homogeneous supersaturated solutions are indicated by ●; ▲ indicate the systems for which homogeneous supersaturated solutions were formed. Ion speciation of experiment marked with □ is shown in Table 6.

2.2. Dissolution of Capolac® or $CaHPO_4{\cdot}2H_2O$ by sodium hydrogen citrate and isocitrate

Turbidity measurements was used to determine the critical mass of hydroxycarboxylates necessary to dissolve Capolac® or $CaHPO_4{\cdot}2H_2O$ Precipitates were assumed to be present in the samples with turbidity values of 100 NTU (Nephelometric Turbidity Units) or higher. A series of dissolution experiments with varying amounts of Capolac® or of $CaHPO_4.2H_2O$ and of $Na_2HCitr/Na_3isoCitr$ in a 1:1 or Na_2HCitr/Na_3Citr in a 1:1 ratio were performed. To the flasks containing Capolac® (1.40 g), 100 mL of Milli Q water were added, and the mixtures were equilibrated at 25 °C for 2 h followed by addition of $Na_2HCitr.1.5\ H_2O$ to yield concentrations of 0.38 mol L^{-1}. The solution was incubated under magnetic stirring at 25 °C, while calcium ion activity, turbidity, and pH were measured over 8 days. Aliquots were collected during every measurement period, to be further analyzed by ICP-OES. A similar experiment was performed with $Na_3isoCitr.xH_2O$ with pH adjusted to obtain a value of 5.6 as found for the $Na_2HCitr{\cdot}1.5H_2O$ experiment.

In order to investigate the effect of isocitrate together with citrate on the supersaturation of calcium salts, mixtures containing Capolac® and different combinations of Na_2HCitr and $Na_3isoCitr$ were prepared with $Na_2HCitr/Na_3isoCitr$ in a ratio of (A) 10:1 – $3.5\ 10^{-1}$ mol/$3.7\ 10^{-2}$ mol L^{-1}; (B) 4:1 – $3.0\ 10^{-1}$ mol/$7.7\ 10^{-2}$ mol L^{-1}; (C) 1:1 – $1.9\ 10^{-1}$ mol/$1.8\ 10^{-1}$ mol L^{-1}; (D) 7:5 – $2.7\ 10^{-1}$ mol/$1.9\ 10^{-1}$ mol L^{-1}. Samples were kept under magnetic stirring at 25 °C throughout the period of analysis.

2.3. Electrochemical determination of free calcium

Calcium chloride standard solutions of 1.00×10^{-4}, 1.00×10^{-3}, 1.00×10^{-2} mol·L^{-1} were used for the calibration of the electrode. Electrodes were calibrated before each measurement. Calcium ion activity was obtained using a calcium ion selective electrode ISE25Ca with a reference REF251 electrode from Radiometer (Copenhagen, Denmark). The determination of calcium ion activity $a_{Ca^{2+}}$ in the solution of equilibration was done using a linear standard curve based on Nernst equation of the potential (mV) against the corresponding $-\log(a_{Ca^{2+}})$, for conversion between activity and concentration:

$$a_{Ca^{2+}} = c_{Ca^{2+}} \times \gamma^{2+} \quad (1)$$

the activity coefficient γ^{z+} (z = 2 for calcium ions) was calculated

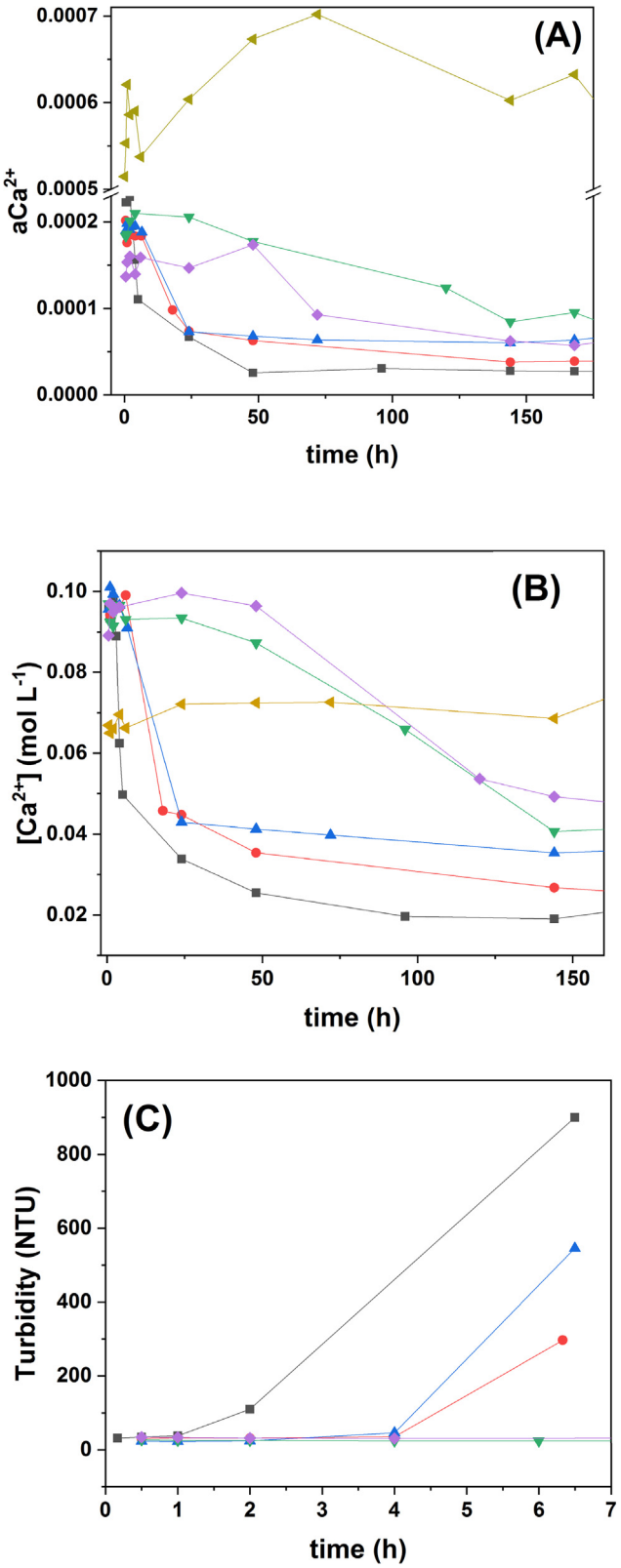

Fig. 4. (A) Calcium ion activity and (B) total calcium concentration and (C) turbidity (NTU) of homogeneous solutions containing 1.4 g of Capolac® in 100 mL of water dissolved in aqueous Na_2HCitr 3.8 × 10^{-1} mol L^{-1} (■), Na_2HCitr /isoCit 1:10 – 3.5 × 10^{-1} mol L^{-1} /3.7 × 10^{-2} mol L^{-1} (●), Na_2HCitr /isoCit 4:1 – 3.0 × 10^{-1} mol/7.7 × 10^{-2} mol L^{-1} (▲), Na_2HCitr /isoCit 1:1 – 1.9 × 10^{-1} mol/1.8 × 10^{-1} mol L^{-1} (▼); Na_2HCitr /isoCit 7:5 – 2.7 × 10^{-1} mol/1.9 × 10^{-1} mol L^{-1} (♦), isoCitr 3.8 × 10^{-1} mol L^{-1} (◀).

according to Davies' equation as previously described (Vavrusova et al., 2018).

2.4. Ion coupled plasma optical emission spectrometry (ICP-OES)

Samples collected during equilibration were filtered in syringe filters (pore size 0.22 μm), afterwards10 μL of the filtered sample were diluted to 10 mL in 5% HNO_3, and subsequently analyzed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) with an Agilent 5100 ICP-OES (Santa Clara, CA, USA), as previously described (Zawadzki & Skibsted, 2019).

2.5. pH measurement

A potentiometer (713 pH Meter, Metrohm, Herisau, Switzerland) with a glass electrode (602 Combined Metrosensor glass electrode, Metrohm, Herisau, Switzerland) was used for pH measurements, as previously described (Zawadzki & Skibsted, 2019).

2.6. Turbidimetry

Turbidity measurement were performed on a TurbiDirect AL450T-IR turbidity meter (Aqualytic, Dortmund, Germany), as previously described (Zawadzki & Skibsted, 2019) and turbidity expressed in Nephelometric Turbidity Unit (NTU).

2.7. Calculation of ion speciation

Ion speciation was determined considering fifteen equilibrium equations together with five mass balance equations that together describe the chemical equilibria of the supersaturated solutions (see Fig. 1). Total concentrations of calcium (t_{Ca}), sodium (t_{Na}) and phosphate (t_P) were obtained by ICP. Total citrate concentration (t_{citr}) was obtained as the sum of the total molar concentration of Na_2HCitr and Na_3Citr added to the solutions to obtain the homogeneous supersaturated solutions. The isocitrate concentration corresponded to the molar concentration of $Na_3isoCitr$ added to the solutions for obtaining the homogeneous supersaturated solutions ($t_{isoCitr}$). Constants shown in Fig. 1 together with their respective values reported in the literature are defined as: dissociation of phosphoric acid acid $K_{a1_{H_3PO_4}}$, $K_{a2_{H_3PO_4}}$ and $K_{a3_{H_3PO_4}}$ (Nims, 1933, 1934); dissociation of citric acid, K_{cit1}, K_{cit2} and K_{cit3} (Bates & Pinching, 1949); dissociation of isocitric acid, K_{isoc1}, K_{isoc2} and K_{isoc3} (Brosset, 1976); formation of the CaHCitr complex, K_1 (Davies & Hoyle, 1953); formation of the $CaCitr^-$ complex, K_2 (Vavrusova, Garcia, Danielsen, & Skibsted, 2017); formation of the $CaHPO_4$ complex, K_3 (Davies & Hoyle, 1953); formation of the $NaHCitr^-$ complex, K_4; formation of the $NaCitr^{2-}$ complex, K_5 (Daniele, Foti, Gianguzza, Prenesti, & Sammartano, 2008); formation of the $CaisoCitr^-$ complex, K_6 (Cheng et al., 2018). As the literature constants were reported for unity ionic strength, their values were corrected taking into account the respective activity coefficients at the actual ionic strength. The corrected constants were used to determine the numeric solutions for the ion speciation equations together with the measured pH and calcium ion activity. The total concentrations of citrate in solution were corrected for precipitated calcium citrate assuming that precipitated citrate is equal to 2/3 of the precipitated calcium ($[Ca^{2+}]_{MAX}$ - $[Ca^{2+}]_{ICP}$).

The chemical and equilibrium equations of Fig. 1 were transcribed into mathematical equations which were solved using the software Matlab ® R2019a (The MathWorks, Inc) implementing iterations. In the first cycle of calculations, the values of the resultant constants were considered at an initial ionic strength (I) of 1.0 mol L^{-1}. From the first set of solutions for the concentration of species in the supersatured solutions, new ionic strength values were calculated and used to obtain new activity coefficients (γ^{2+} or γ^{2-}) using Davies equation (Eq. (2)):

Table 1
Rate constants for precipitation of calcium from 1.4 g of Capolac® dissolved in 100 mL of 0.38 mol L^{-1} aqueous sodium hydrogen citrate or sodium hydrogen citrate/isocitrate combinations with total 0.38 mol L^{-1} hydroxycarboxylate concentration based on electrochemical determination of calcium activity and on total calcium concentration as determined by exponential fitting of the curves seen in Fig. 4A and B, respectively.

	Na_2HCitr	Na_2HCitr/isoCitr 10:1	Na_2HCitr/isoCitr 4:1	Na_2HCitr/isoCitr 7:5[a]	Na_2HCitr/isoCitr 1:1
aCa^{2+}	(13.3 ± 3.3) 10^{-2} h^{-1}	(5.0 ± 0.1) 10^{-2} h^{-1}	(6.0 ± 1.7) 10^{-2} h^{-1}	–	–
$[Ca^{2+}]$	(15.7 ± 0.5) 10^{-2} h^{-1}	(5.8 ± 1.7) 10^{-2} h^{-1}	(6.0 ± 1.5) 10^{-2} h^{-1}	(4.5 ± 0.3)[a,b] 10^{-4} mol L^{-1} h^{-1}	(4.3 ± 0.9)[b] 10^{-4} mol L^{-1} h^{-1}

[a] Total hydroxycarboxylate concentration 0.46 mol L^{-1}.
[b] Fit did not converge to an exponential curve and the zero-order rate constants[a] were determined by linear fitting.

$$\log\gamma^{z+} = -0.51z^2\left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.30I\right) \quad (2)$$

with the ionic strength:

$$I = \frac{1}{2}\sum_i c_i z_i^2 \quad (3)$$

where c_i is the concentration of the individual ions, and z_i is the charge.

The new activity coefficients were used to calculate new resulting constants for a second cycle of calculations. Calculations of ion speciation and iterations were repeated until obtaining a constant ionic strength for the actual equilibration time serving as a criterion for ending the iteration, as previously described (Garcia, Vavrusova, & Skibsted, 2018).

Association of Na^+ to isocitrate ions was not included in the calculations of ion speciation since no complex constants seem available. More importantly, association seems neglectable as concluded from a comparison of calcium association to citrate and isocitrate as determined both experimentally and by quantum mechanical calculations (Cheng et al., 2018). The acid/base distribution among the species of the triprotic acids citric acid, isocitric acid, and phosphoric acid was determined according to pH and was also taken into account in all calculations.

3. Results and discussion

3.1. Combinations of hydroxycarboxylates for calcium phosphate dissolution

Whey mineral residues dominated by hydroxyapatite and calcium hydrogen phosphate have been found to dissolve in aqueous sodium hydrogen citrate and sodium gluconate/gluconolactone to yield homogeneous solutions supersaturated in calcium hydrogen phosphate and calcium citrate or calcium gluconate with a lag phase for precipitation of hours and for gluconate even days or weeks (Zawadzki & Skibsted, 2019; 2020).

Combinations of isocitrate and citrate have been found to enhance the solubility of calcium salts compared to the individual isomers (Cheng et al., 2018). In order to explore this effect and to achieve a higher overshooting in the dissolution process of calcium phosphates from a commercial whey mineral product for practical use in functional foods, citrate and isocitrate were compared at varying pH together with their combinations for their capacity to form spontaneously supersaturated solutions upon dissolution of Capolac® and compared with $CaHPO_4{\cdot}2H_2O$

The formation of homogenous supersaturated solutions upon the addition of Na_2HCitr/Na_3Citr or $Na_2HCitr/Na_3isoCitr$ combinations to calcium hydrogen phosphate or to Capolac® was monitored by visual inspection. The critical concentrations of total hydroxycarboxylates (citrate plus isocitrate) necessary to dissolve Capolac® or calcium hydrogen phosphate were obtained from series of dissolution experiments varying both the amount of Capolac® or $CaHPO_4{\cdot}2H_2O$ and of hydroxycarboxylates.

The critical concentration of hydroxycarboxylates required for dissolving calcium from Capolac® or $CaHPO_4{\cdot}2H_2O$ forming supersaturated solutions with Na_2HCitr /$Na_3isoCit$ in a ratio 1:1 was found as seen in Fig. 2:

$$[Ca^{2+}] = (0.142 \pm 0.006)\,[citrate]_{total} + 0.002 \text{ for Capolac®} \quad (4)$$

$$[Ca^{2+}] = (0.075 \pm 0.005)\,[citrate]_{total} + 0.004 \text{ for } CaHPO_4{\cdot}2H_2O \quad (5)$$

Total citrate concentration added to calcium hydrogen phosphate or to Capolac® is the sum of the concentrations of citrate and isocitrate. Na_2HCitr /Na_3Cit in a ratio of 1:1 was compared to Na_2HCitr /$Na_3isoCit$ at the same ratio in order to determine the critical concentration of total hydroxycarboxylates required for dissolution of Capolac® or $CaHPO_4{\cdot}2H_2O$ of (Fig. 3). The critical amount of citrate to obtain supersaturated solutions with Na_2HCitr/Na_3Cit in a ratio of 1:1 was found to be:

$$[Ca^{2+}] = (0.285 \pm 0.043)\,[citrate] + 0.004 \text{ for Capolac®} \quad (6)$$

$$[Ca^{2+}] = (0.140 \pm 0.017)\,[citrate] + 0.001 \text{ for } CaHPO_4{\cdot}2H_2O \quad (7)$$

The total concentration of hydroxycarboxylates required for dissolving Capolac® was found to be two times less compared to $CaHPO_4{\cdot}2H_2O$ in both cases. The finding is very similar to what was found for solutions formed by dissolution of calcium from Capolac® and from $CaHPO_4.2H_2O$ by gluconate/gluconolactone (Zawadzki & Skibsted, 2019). Other compounds present in Capolac® may give an extra contribution to the solubility overshooting of calcium resulting in a synergistic effect when compared to the dissolution of $CaHPO_4{\cdot}2H_2O$.

However, when comparing Na_2HCitr /Na_3Cit to Na_2HCitr /$Na_3isoCit$ at the same ratio of 1:1, the concentration of hydroxycarboxylates required to obtain critical supersaturated solutions was found to be two times lower for Na_2HCitr /Na_3Cit. Experiments monitoring supersaturated solutions in relation to calcium ion activity, pH and total calcium and phosphorous concentration were performed in order to obtain a detailed picture of the speciation after dissolution and during precipitation in the search from an explanation of this difference between citrate and isocitrate.

3.2. Effect of combinations of isocitrate and citrate on calcium dissolution

Different ratios between $Na_2HCitr/Na_3isoCitr$ were used in order to identify the most suitable combination to optimize calcium dissolution from Capolac® forming supersaturated solutions. The presence of isoCitr in different ratios relative to citrate in the supersaturated solutions based on Capolac® were found to increase the lag phase for precipitation as well as to decrease the rate of precipitation, see Fig. 4 and Table 1. Rates of precipitation in Table 1 were obtained from an exponential fitting of the curves describing the decrease in the calcium ion activity, $a_{Ca^{2+}}$, or in the calcium ion concentration, $[Ca^{2+}]$, during precipitation assuming a (pseudo) first-order reaction.

The presence of 10% of isoCitr in a system containing Capolac® and 0.38 mol L^{-1} of total hydroxycarboxylates (Na_2HCitr/ $Na_3isoCitr$ in a

Table 2
Ion speciation (mol L^{-1}) in a filtered supersaturated solution made by the dissolution of 1.4 g Capolac® with 0.38 mol L^{-1} of Na_3isoCitr in water (100 mL) at pH 5.5.* No changes in concentration were seen for 200 h.

Time (h)	pH	$[Ca^{2+}]_{ICP}$	$[P]_{ICP}$	H_3PO_4	$H_2PO_4^-$	HPO_4^{2-}	Ca^{2+}	$CaHPO_4$	$H_3IsoCitr$	$H_2IsoCitr^-$	$HIsoCitr^{2-}$	$IsoCitr^{3-}$	$CaIsoCitr^-$	Na^+	I	Q	Q_i/K_i	Q_{CaHPO4}	Q_{CaP}/K_P
0.5	5.5	6.69×10^{-2}	4.04×10^{-2}	4.18×10^{-6}	2.95×10^{-2}	9.68×10^{-3}	1.66×10^{-3}	1.23×10^{-3}	6.92×10^{-5}	1.56×10^{-2}	0.21	9.50×10^{-1}	6.64×10^{-2}	0.50	1.18	4.12×10^{-11}	4.12×10^{2}	1.15×10^{-7}	1.39×10^{-1}

*Conditions for saturation were: 1.4 g of Capolac®; total isocitrate concentration of 0.39 mol L^{-1}; equilibration for 30 min at 25 °C. Iterative calculations are based on total calcium and phosphorous concentration as determined by ICP and shown in Fig. 4B and calcium ion activity as measured electrochemically and shown in Fig. 4A. Concentrations expressed as mol L^{-1}. $Q_{CaIsoCitr}$ and Q_{CaP} correspond to the ionic products defined, respectively, by $Q_i = [Ca^{2+}]^3 \times [IsoCitr^{3-}]^2$ and $Q_{CaP} = [Ca^{2+}] \times [HPO_4^{2-}]$. For ionic strength = 1.0, concentration based solubility product of calcium isocitrate is $K_{sp\text{-}Ca3IsoCitr2} = 1.0\,10^{-13}$ mol^5 L^{-5} (Cheng et al., 2018) and of calcium hydrogen phosphate is $K_{sp\text{-}CaHPO4} = 8.25 \times 10^{-7}$ mol^2 L^{-2} (McDowell et al., 1971), to be compared with the ionic products Q.

10:1 ratio) was found to decrease the rate of precipitation by a factor of 2.7 as well as to prolong the lag phase from 2 to 4 h when compared to supersaturated solutions only containing Na_2HCitr (see Fig. 4B and Table 1). This result was further confirmed by turbidity measurements (Fig. 4C).

Higher concentrations of Na_2HCitr relative to Na_3isoCitr resulted in shorter lag phases and increasing rates for precipitation. Such behavior can be explained by the stronger binding of calcium to citrate (complex formation described in Fig. 1 by eqn (7)) compared to isocitrate (Fig. 1, eqn 14) resulting in a higher concentration of $CaCitr^-$ which seems to be actively involved in the precipitation. The longest lag phase for precipitation was found for the combination Na_2HCitr/isoCitr ratio of 1:1 (Fig. 4A and B).

No significant changes were found in the calcium ion activity and total calcium concentration of mixtures containing Capolac® and Na_3isoCitr at pH 5.6 (Fig. 4A) during the period of equilibration investigated, providing evidence that the precipitation was not occurring for up to one week. Supersaturated solutions only containing isocitrate had longer lag phases for precipitation as well as higher calcium ion activities, however, the maximum total calcium concentration was found to be 30% less than the $[Ca^{2+}]_{MAX}$ achieved in the supersaturated solutions formed by dissolving Capolac® in solutions of Na_2HCitr or in Na_2HCitr /Na_3Cit and Na_2HCitr /Na_3isoCit combinations, see Fig. 4 and Table 2. During the dissolution of 1.4 g Capolac® in 100 mL of aqueous isocitrate at pH 5.6 corresponding to $HisoCitr^{2-}$, the solution never became totally homogeneous indicating that part of Capolac® was present as insoluble salts.

To assess the composition and the degree of supersaturation of the supersaturated solutions obtained upon the addition of Na_2HCitr, Na_3isoCit and/or Na_3Citr to Capolac®, ion speciation was established for solutions based on Capolac® in which the total hydroxycarboxylate concentration was 0.38 mol L^{-1} (Table 2, Table 3 and Table 4). Ion speciation for the other combinations was found to have a similar pattern for calcium ion activity and ion speciation were very similar to the corresponding solutions with 0.38 mol L^{-1} of total hydroxycarboxylates (results not shown).

The degree of supersaturation was obtained from the ratio between the ionic product (Q) and the solubility product (K_{sp}). Ionic products were compared with solubility products at unity ionic strength: $K_{sp\text{-}CaCitr} = 7.6 \times 10^{-17}$ mol^5 L^{-5} (Vavrusova & Skibsted, 2016); $K_{sp\text{-}CaIsoCitr} = 1.0 \times 10^{-13}$ mol^5 L^{-5} (Cheng et al., 2018); $K_{sp\text{-}CaHPO4} = 8.25 \times 10^{-7}$ mol^2 L^{-2} (McDowell, Sutter, & Brown, 1971) and $K_{sp\text{-}CaSac} = 6.17 \times 10^{-7}$ mol^2 L^{-2} (Garcia, Vavrusova, & Skibsted, 2016). For lower ionic strength, the solubility products were corrected by the activity coefficients and ion activities replaced ion concentrations, see footnotes to the tables. Ionic products for calcium citrate, calcium isocitrate and calcium hydrogen phosphate are, respectively, defined as:

$$Q_{CaCit} = [Ca^{2+}]^3 \times [Citr^{3-}]^2 \quad (8)$$

$$Q_{CaIsoCit} = [Ca^{2+}]^3 \times [IsoCitr^{3-}]^2 \quad (9)$$

$$Q_{CaHPO4} = [Ca^{2+}] \times [HPO_4^{2-}] \quad (10)$$

In the filtered solution obtained for Capolac® in isoCitr at pH 5.6, ion speciation was used to calculate the ionic products for $CaHPO_4$ and for $Ca_3(Isocitrate)_2$, see Table 2. The ratios Q/K_{sp} for calcium isocitrate and for calcium hydrogen phosphate in Capolac®/Na_3isoCitr at pH 5.6 were calculated to be 412 and 0.139, respectively, indicating that this solution is supersaturated in relation to $Ca_3(Isocitrate)_2$, but not in relation to $CaHPO_4$.

Notably, the solution containing 0.38 mol L^{-1} of Na_2HCitr was found to be slightly supersaturated in $CaHPO_4$ ($1 < Q_{CaP}/K_P$ less than 4), to have the highest rate of precipitation, the shortest lag phase for precipitation and the highest supersaturation degree for Ca_3Citr_2 ($Q_{Cit}/K_C > 10^4$) during the first 24 h prior to the precipitation process

Table 3
Ion speciation (mol L^{-1}) in supersaturated homogeneous solutions resulting from dissolution of 1.4 g of Capolac® in 100 mL of aqueous 0.38 mol L^{-1} Na_2HCitr.*

Time (h)	pH	%ppt	$[Ca^{2+}]_{ICP}$	$[P]_{ICP}$	H_3PO_4	$H_2PO_4^-$	HPO_4^{2-}	H_3Citr	H_2Citr^-	$HCitr^{2-}$	$Citr^{3-}$	Ca^{2+}	CaHCitr	$CaCitr^-$
0.5	4.9	–	$9.60\ 10^{-2}$	$5.98\ 10^{-2}$	$5.63\ 10^{-5}$	$5.81\ 10^{-2}$	$1.51\ 10^{-3}$	$2.76\ 10^{-4}$	$2.68\ 10^{-2}$	$1.12\ 10^{-1}$	$1.96\ 10^{-2}$	$1.79\ 10^{-3}$	$4.69\ 10^{-2}$	$4.72\ 10^{-2}$
3	4.8	10%	$8.90\ 10^{-2}$	$5.98\ 10^{-2}$	$5.84\ 10^{-5}$	$5.81\ 10^{-2}$	$1.46\ 10^{-3}$	$3.01\ 10^{-4}$	$2.84\ 10^{-2}$	$1.14\ 10^{-1}$	$1.94\ 10^{-2}$	$1.65\ 10^{-3}$	$4.42\ 10^{-2}$	$4.30\ 10^{-2}$
5	4.9	50%	$4.97\ 10^{-2}$	$5.95\ 10^{-2}$	$5.42\ 10^{-5}$	$5.78\ 10^{-2}$	$1.56\ 10^{-3}$	$2.82\ 10^{-4}$	$2.83\ 10^{-2}$	$1.22\ 10^{-1}$	$2.20\ 10^{-2}$	$8.37\ 10^{-4}$	$2.40\ 10^{-2}$	$2.49\ 10^{-2}$
168	5.0	78%	$2.15\ 10^{-2}$	$6.55\ 10^{-2}$	$4.06\ 10^{-5}$	$6.29\ 10^{-2}$	$2.50\ 10^{-3}$	$1.30\ 10^{-4}$	$1.86\ 10^{-2}$	$1.13\ 10^{-1}$	$2.87\ 10^{-2}$	$3.11\ 10^{-4}$	$8.52\ 10^{-3}$	$1.26\ 10^{-2}$

$CaHPO_4$	Na^+	$NaHCitr^-$	$NaCitr^{2-}$	I	Q_{CaCitr}	Q_{cit}/K_c	Q_{CaHPO4}	Q_{CaP}/K_P
$1.22\ 10^{-4}$	0.31	$9.11\ 10^{-2}$	$3.49\ 10^{-2}$	$6.03\ 10^{-1}$	$2.66\ 10^{-16}$	$2.91\ 10^{4}$	$2.44\ 10^{-7}$	3.28
$1.09\ 10^{-4}$	0.30	$9.07\ 10^{-2}$	$3.37\ 10^{-2}$	$6.01\ 10^{-1}$	$2.04\ 10^{-16}$	$2.25\ 10^{4}$	$2.17\ 10^{-7}$	2.92
$5.92\ 10^{-5}$	0.28	$9.08\ 10^{-2}$	$3.59\ 10^{-2}$	$6.08\ 10^{-1}$	$3.47\ 10^{-17}$	$3.73\ 10^{3}$	$1.18\ 10^{-7}$	1.58
$3.62\ 10^{-5}$	0.31	$9.43\ 10^{-2}$	$5.28\ 10^{-2}$	$6.39\ 10^{-1}$	$3.38\ 10^{-17}$	$3.25\ 10^{2}$	$7.24\ 10^{-7}$	0.94

*Conditions for saturation were: 1.4 g of Capolac®; total citrate concentration of 0.38 mol L^{-1}; equilibration for 168 h at 25 °C. Iterative calculations are based on total calcium and phosphorous concentration as determined by ICP and shown in Fig. 4B and calcium ion activity as measured electrochemically, see Fig. 4A. Concentrations expressed as mol L^{-1}. Q_{CaCitr} corresponds to the ion activity product for calcium citrate defined by $Q = [Ca^{2+}]^3 \times (\gamma^{2+})^3 \times [Citr^{3-}]^2 \times (\gamma^{3-})^2$. Q_{CaP} corresponds to the ion activity product for calcium hydrogen phosphate defined by $Q = [Ca^{2+}] \times (\gamma^{2+}) \times [HPO_4^{2-}] \times (\gamma^{2-})$. Activity based solubility product $K_{C\text{-}sp\text{-}activity} = K_{sp\text{-}\ Ca3Citr2} \times (\gamma^{2+})^3 \times (\gamma^{3-})^2$ was calculated to have the value $K_{C\text{-}sp\text{-}activity} = (1.11 \pm 0.19)\ 10^{-19}$ from the concentration based solubility product $K_{sp\text{-}\ Ca3Citr2} = 7.6 \times 10^{-17}$ $mol^5\ L^{-5}$ for ionic strength = 1.0 (Vavrusova et al., 2016). Activity based solubility product $K_{P\text{-}sp\text{-}activity} = K_{sp\text{-}CaHPO4} \times \gamma^{2+} \times \gamma^{2-}$ was calculated to have the value $K_{P\text{-}sp\text{-}activity} = 1.86 \times 10^{-7}$ from the concentration based solubility product $K_{sp\text{-}CaHPO4} = 8.25 \times 10^{-7}$ $mol^2\ L^{-2}$ for ionic strength = 1.0 (McDowell et al., 1971). $Q_{Cit/Kc}$ is the ratio between the ion activity product Q_{CaCitr} and the activity based solubility product for calcium citrate $K_{C\text{-}sp\text{-}activity}$. Q_{CaP}/K_P is the ratio between the ion activity product Q_{CaP} and the activity based solubility product for calcium hydrogen phosphate $K_{P\text{-}sp\text{-}activity}$. % ppt is the percentage of calcium precipitated as calculate from total calcium as determined by ICP.

Table 4
Ion speciation (mol L^{-1}) in supersaturated homogeneous solutions resulting from dissolution of 1.4 g of Capolac® in 100 mL of aqueous 0.19 mol L^{-1} Na_2HCitr and 0.19 mol L^{-1} isoCitr *

Time (h)	pH	%ppt	$[Ca^{2+}]_{ICP}$	$[P]_{ICP}$	H_3PO_4	$H_2PO_4^-$	HPO_4^{2-}	H_3Citr	H_2Citr^-	$HCitr^{2-}$	$Citr^{3-}$	Ca^{2+}	CaHCitr	$CaCitr^-$	$CaHPO_4$	Na^+	$NaHCitr^-$	$NaCitr^{2-}$
0.5	5.4	–	$8.91\ 10^{-2}$	$6.01\ 10^{-2}$	$5.62\ 10^{-6}$	$4.12\ 10^{-2}$	$1.73\ 10^{-2}$	$7.94\ 10^{-6}$	$2.72\ 10^{-3}$	$3.53\ 10^{-2}$	$1.73\ 10^{-2}$	$1.24\ 10^{-3}$	$7.01\ 10^{-3}$	$5.72\ 10^{-2}$	$1.53\ 10^{-3}$	0.34	$3.93\ 10^{-2}$	$4.71\ 10^{-2}$
24	5.4	–	$9.96\ 10^{-2}$	$6.61\ 10^{-2}$	$6.14\ 10^{-6}$	$4.51\ 10^{-2}$	$1.90\ 10^{-2}$	$7.36\ 10^{-6}$	$2.52\ 10^{-3}$	$3.25\ 10^{-2}$	$1.57\ 10^{-2}$	$1.46\ 10^{-3}$	$7.69\ 10^{-3}$	$6.27\ 10^{-2}$	$2.00\ 10^{-3}$	0.38	$4.06\ 10^{-2}$	$4.83\ 10^{-2}$
48	5.4	5%	$9.64\ 10^{-2}$	$7.00\ 10^{-2}$	$6.37\ 10^{-6}$	$4.74\ 10^{-2}$	$2.05\ 10^{-2}$	$7.07\ 10^{-6}$	$2.44\ 10^{-3}$	$3.18\ 10^{-2}$	$1.54\ 10^{-2}$	$1.39\ 10^{-3}$	$7.31\ 10^{-3}$	$6.02\ 10^{-2}$	$2.10\ 10^{-3}$	0.40	$4.22\ 10^{-2}$	$5.06\ 10^{-2}$
120	5.4	46%	$5.37\ 10^{-2}$	$6.98\ 10^{-2}$	$5.19\ 10^{-6}$	$4.53\ 10^{-2}$	$2.33\ 10^{-2}$	$5.07\ 10^{-6}$	$2.04\ 10^{-3}$	$3.07\ 10^{-2}$	$1.71\ 10^{-2}$	$6.57\ 10^{-4}$	$3.48\ 10^{-3}$	$3.34\ 10^{-2}$	$1.17\ 10^{-3}$	0.37	$3.91\ 10^{-2}$	$5.41\ 10^{-2}$
168	5.4	52%	$4.74\ 10^{-2}$	$6.81\ 10^{-2}$	$4.68\ 10^{-6}$	$4.38\ 10^{-2}$	$2.29\ 10^{-2}$	$2.43\ 10^{-6}$	$1.07\ 10^{-3}$	$1.81\ 10^{-2}$	$1.17\ 10^{-2}$	$8.61\ 10^{-4}$	$2.38\ 10^{-3}$	$2.50\ 10^{-2}$	$1.34\ 10^{-3}$	0.39	$2.29\ 10^{-2}$	$3.58\ 10^{-2}$

$CaIsoCitr^-$	$IsoCitr^{3-}$	$H_3IsoCitr$	$H_2IsoCitr^-$	$HIsoCitr^{2-}$	I	Q_{CaCitr}	Q_{cit}/K_c	$Q_{CaIsoCitr}$	Q_i/K_i	Q_{CaHPO4}	Q_{CaP}/K_P
$2.21\ 10^{-2}$	$4.88\ 10^{-2}$	$2.92\ 10^{-5}$	$6.88\ 10^{-3}$	$1.00\ 10^{-1}$	0.95	$5.71\ 10^{-13}$	$7.51\ 10^{2}$	$4.54\ 10^{-12}$	45.4	$2.14\ 10^{-5}$	26.0
$2.58\ 10^{-2}$	$4.73\ 10^{-2}$	$2.88\ 10^{-5}$	$6.78\ 10^{-3}$	$9.84\ 10^{-2}$	0.96	$7.69\ 10^{-13}$	$1.01\ 10^{3}$	$6.93\ 10^{-12}$	69.3	$2.77\ 10^{-5}$	33.6
$2.54\ 10^{-2}$	$4.75\ 10^{-2}$	$2.83\ 10^{-5}$	$6.75\ 10^{-3}$	$9.86\ 10^{-2}$	0.98	$6.38\ 10^{-13}$	$8.39\ 10^{2}$	$6.04\ 10^{-12}$	60.4	$2.84\ 10^{-5}$	34.5
$1.50\ 10^{-2}$	$5.59\ 10^{-2}$	$2.16\ 10^{-5}$	$6.00\ 10^{-3}$	$1.01\ 10^{-1}$	1.00	$8.31\ 10^{-14}$	$1.09\ 10^{2}$	$8.87\ 10^{-13}$	8.87	$1.53\ 10^{-5}$	18.6
$1.78\ 10^{-2}$	$6.06\ 10^{-2}$	$1.64\ 10^{-5}$	$4.99\ 10^{-3}$	$9.48\ 10^{-2}$	0.93	$8.74\ 10^{-14}$	$1.15\ 10^{2}$	$2.35\ 10^{-12}$	23.5	$1.98\ 10^{-5}$	23.9

*Conditions for saturation were: 1.4 g of Capolac®; total citrate concentration of 0.38 mol L^{-1}; ratio between citrate and isocitrate of 1:1, equilibration for 168 h at 25 °C. Iterative calculations are based on total calcium and phosphorous concentration as determined by ICP and shown in Fig. 4B and calcium ion activity as measured electrochemically, see Fig. 4A. Concentrations expressed as mol L^{-1}. Q_{CaCitr}, $Q_{CaIsoCitr}$ and Q_{CaP} correspond to the ionic products defined, respectively, by $Q_{cit} = [Ca^{2+}]^3 \times [Citr^{3-}]^2$, $Q_i = [Ca^{2+}]^3 \times [IsoCitr^{3-}]^2$ and $Q_{CaP} = [Ca^{2+}] \times [HPO_4^{2-}]$. For ionic strength = 1.0, concentration based solubility product of calcium citrate is $K_{sp\text{-}\ Ca3Citr2} = 7.6 \times 10^{-17}$ $mol^5\ L^{-5}$ (Vavrusova & Skibsted, 2016), of calcium isocitrate is $K_{sp\text{-}\ Ca3IsoCitr2} = 1.0\ 10^{-13}$ $mol^5\ L^{-5}$ (Cheng et al., 2018) and of calcium hydrogen phosphate is $K_{sp\text{-}CaHPO4} = 8.25 \times 10^{-7}$ $mol^2\ L^{-2}$ (McDowell et al., 1971), to be compared with the ionic products Q. % ppt is the percentage of calcium precipitated as calculate from total calcium as determined by ICP.

(Table 3). A high supersaturation degree was also obtained for Ca_3Citr_2 in the combination $Na_2HCitr/Na_3isoCitr$ in a ratio of 1:1 ($Q_{Cit}/K_C > 10^2$), however, this system was found to be 100 times less supersaturated in Ca_3Citr_2 when compared to the supersaturated solutions formed with 0.38 mol L^{-1} of Na_2HCitr (compare Tables 3 and 4). Such finding may explain why the value for critical combinations between calcium salts and Na_2HCitr is two times higher compared to $Na_2HCitr/Na_3isoCitr$ combination in a ratio of 1:1. Na_2HCitr shows a higher efficiency in dissolving calcium to form supersaturated solutions compared to isocitrate, and a lower amount of hydroxycarboxylates is

Table 5
Ion speciation (mol L^{-1}) in supersaturated homogeneous solutions resulting from dissolution of 1.4 g of Capolac® in 100 mL of aqueous 0.21 mol L^{-1} Na_2HCitr and 0.18 mol L^{-1} isoCitr plus 1 × 10^{-2} mol L^{-1} of calcium saccharate.*

Time (h)	pH	%ppt	$[Ca]_{ICP}$	$[P]_{ICP}$	H_3PO_4	$H_2PO_4^-$	HPO_4^{2-}	H_3Citr	H_2Citr^-	$HCitr^{2-}$	$Citr^{3-}$	$HIsoCitr^{2-}$	$IsoCitr^{3-}$	Ca^{2+}	$CaHPO_4$	CaHCitr	$CaCitr^-$	$CaIsoCitr^-$
0.5	5.5	–	0.11	6.60 10^{-2}	6.44 10^{-6}	4.54 10^{-2}	1.84 10^{-2}	7.80 10^{-6}	2.56 10^{-3}	3.18 10^{-2}	1.49 10^{-2}	9.80 10^{-2}	4.54 10^{-2}	1.64 10^{-3}	2.18 10^{-3}	8.48 10^{-3}	6.64 10^{-2}	2.78 10^{-2}
1	5.6	–	0.11	6.85 10^{-2}	6.07 10^{-6}	4.59 10^{-2}	2.01 10^{-2}	6.53 10^{-6}	2.30 10^{-3}	3.03 10^{-2}	1.49 10^{-2}	9.57 10^{-2}	4.69 10^{-2}	1.62 10^{-3}	2.40 10^{-3}	8.13 10^{-3}	6.80 10^{-2}	2.92 10^{-2}
6	5.6	10%	0.10	6.23 10^{-2}	5.41 10^{-6}	4.18 10^{-2}	1.85 10^{-2}	6.33 10^{-6}	2.28 10^{-3}	3.12 10^{-2}	1.60 10^{-2}	9.65 10^{-2}	4.92 10^{-2}	1.44 10^{-3}	1.92 10^{-3}	7.25 10^{-3}	6.23 10^{-2}	2.62 10^{-2}
144	5.6	55%	0.05	7.02 10^{-2}	5.83 10^{-6}	4.72 10^{-2}	2.17 10^{-2}	4.34 10^{-6}	1.64 10^{-3}	2.36 10^{-2}	1.28 10^{-2}	1.02 10^{-2}	5.48 10^{-2}	7.77 10^{-4}	1.19 10^{-3}	2.91 10^{-3}	2.63 10^{-2}	1.54 10^{-2}
192	5.7	55%	0.05	8.29 10^{-2}	5.24 10^{-6}	5.20 10^{-2}	2.80 10^{-2}	7.29 10^{-7}	3.43 10^{-4}	6.29 10^{-3}	4.46 10^{-3}	8.48 10^{-2}	5.96 10^{-2}	1.58 10^{-3}	2.86 10^{-3}	1.44 10^{-3}	1.62 10^{-2}	2.96 10^{-2}

$H_3IsoCitr$	$H_2IsoCitr^-$	$HIsoCitr^{2-}$	Na^+	$NaHCitr^-$	$NaCitr^{2-}$	CaSac	Sac^{2-}	I	Q_{CaCitr}	Q_{cit}/K_c	$Q_{CaIsoCitr}$	Q_i/K_i	Q_{CaHPO4}	Q_{CaP}/K_P	Q_{CaSac}	Qs/Ks
6.44 10^{-6}	4.54 10^{-2}	1.84 10^{-2}	0.38	3.99 10^{-2}	4.57 10^{-2}	1.97 10^{-3}	8.04 10^{-3}	0.96	9.80 10^{-13}	1.29 10^{3}	9.11 10^{-12}	91.1	3.02 10^{-5}	0.33	1.31 10^{-5}	21.4
6.07 10^{-6}	4.59 10^{-2}	2.01 10^{-2}	0.39	3.97 10^{-2}	4.83 10^{-2}	1.98 10^{-3}	8.02 10^{-3}	0.98	9.51 10^{-13}	1.25 10^{3}	9.31 10^{-12}	93.1	3.26 10^{-5}	0.32	1.30 10^{-5}	21.0
5.41 10^{-6}	4.18 10^{-2}	1.85 10^{-2}	0.35	3.61 10^{-2}	4.55 10^{-2}	1.76 10^{-3}	8.24 10^{-3}	0.96	7.74 10^{-13}	1.02 10^{3}	7.28 10^{-12}	72.8	2.68 10^{-5}	0.26	1.19 10^{-5}	19.3
5.83 10^{-6}	4.72 10^{-2}	2.17 10^{-2}	0.38	2.97 10^{-2}	3.94 10^{-2}	1.02 10^{-3}	8.98 10^{-3}	0.95	7.75 10^{-14}	1.02 10^{2}	1.40 10^{-12}	14.1	1.69 10^{-5}	0.16	6.98 10^{-6}	11.3
5.24 10^{-6}	5.20 10^{-2}	2.80 10^{-2}	0.51	1.00 10^{-2}	1.69 10^{-2}	1.74 10^{-3}	8.26 10^{-3}	0.89	7.90 10^{-14}	1.04 10^{2}	1.41 10^{-11}	141.0	4.44 10^{-5}	0.29	1.30 10^{-5}	21.2

* Conditions for saturation were: 1.4 g of Capolac®; total citrate concentration of 0.21 mol L^{-1}; ratio between citrate and isocitrate of 1:1, equilibration for 192 h at 25 °C. Iterative calculations are based on total calcium and phosphorous concentration as determined by ICP and shown in Fig. 5B and calcium ion activity as measured electrochemically, see Fig. 5A. Concentrations expressed as mol L^{-1}. Q_{CaCitr}, $Q_{CaIsoCitr}$, Q_{CaHPO4} and Q_{CaSac} correspond to the ionic products defined, respectively, by $Qc = [Ca^{2+}]^3 \times [Citr^{3-}]^2$; $Qi = [Ca^{2+}]^3 \times [IsoCitr^{3-}]^2$; $Q_{CaP} = [Ca^{2+}] \times [HPO_4^{2-}]$ and $Q_{CaSac} = [Ca^{2+}] \times [Sac^{2-}]$, to be compared with the concentration based solubility products at ionic strength = 1.0: $K_{sp\text{-}CaCitr} = 7.6 \times 10^{-17}$ mol^5 L^{-5} (Vavrusova et al., 2016); $K_{sp\text{-}CaIsoCitr} = 1.0 \times 10^{-13}$ mol^5 L^{-5} (Cheng et al., 2018); $K_{sp\text{-}CaHPO4} = 8.25 \times 10^{-7}$ mol^2 L^{-2} (McDowell et al., 1971) and $K_{sp\text{-}CaSac} = 6.17 \times 10^{-7}$ mol^2 L^{-2} (Garcia et al., 2016). % ppt is the percentage of calcium precipitated as calculate from total calcium as determined by ICP.

Table 6
Ion speciation (mol L^{-1}) in supersaturated homogeneous solutions resulting from dissolution of 1.4 g of Capolac® in 100 mL of aqueous 0.19 mol L^{-1} Na_2HCitr and 0.18 mol L^{-1} Na_3Citr.*

Time	pH	%ppt	$[Ca^{2+}]_{ICP}$	$[P]_{ICP}$	H_3PO_4	$H_2PO_4^-$	HPO_4^{2-}	H_3Citr	H_2Citr^-	$HCitr^{2-}$	$Citr^{3-}$	Ca^{2+}	CaHCitr	$CaCitr^-$	$CaHPO_4$	Na^+	$NaCitr^{2-}$	$NaHCitr^-$
30 min	5.6	–	9.48 10^{-2}	6.54 10^{-2}	9.64 10^{-6}	5.58 10^{-2}	9.05 10^{-3}	6.55 10^{-6}	3.05 10^{-3}	5.58 10^{-2}	3.98 10^{-2}	8.94 10^{-4}	1.61 10^{-2}	7.73 10^{-2}	5.02 10^{-4}	0.34	5.87 10^{-2}	9.92 10^{-2}
3 h	5.6	–	9.52 10^{-2}	6.48 10^{-2}	9.56 10^{-6}	5.54 10^{-2}	8.97 10^{-3}	6.55 10^{-6}	3.06 10^{-3}	5.59 10^{-2}	3.99 10^{-2}	8.98 10^{-4}	1.62 10^{-2}	7.76 10^{-2}	4.99 10^{-4}	0.34	5.84 10^{-2}	9.89 10^{-2}
5 h	5.6	10%	8.64 10^{-2}	6.09 10^{-2}	9.54 10^{-6}	5.30 10^{-2}	7.62 10^{-3}	5.31 10^{-6}	2.69 10^{-2}	5.74 10^{-2}	5.10 10^{-2}	9.60 10^{-4}	1.36 10^{-2}	7.14 10^{-2}	3.49 10^{-4}	0.32	9.82 10^{-2}	4.99 10^{-2}
24 h	5.6	46%	5.15 10^{-2}	7.26 10^{-2}	1.05 10^{-5}	6.21 10^{-2}	1.03 10^{-2}	7.22 10^{-6}	3.45 10^{-2}	6.49 10^{-2}	4.77 10^{-2}	4.13 10^{-4}	8.58 10^{-3}	4.22 10^{-2}	2.61 10^{-4}	0.34	1.16 10^{-1}	6.69 10^{-2}
168 h	5.7	57%	4.14 10^{-2}	7.09 10^{-2}	7.91 10^{-6}	5.91 10^{-2}	1.16 10^{-2}	2.58 10^{-6}	1.73 10^{-2}	4.89 10^{-2}	5.70 10^{-2}	4.13 10^{-4}	5.12 10^{-3}	3.56 10^{-2}	2.34 10^{-4}	0.35	1.20 10^{-1}	4.60 10^{-2}

I	Q	Q_{cit}/K_c	Q_{CaHPO4}	Q_{CaP}/K_P
0.63	1.50 10^{-16}	1.49 10^{3}	1.83 10^{-6}	9.80
0.63	1.52 10^{-16}	1.52 10^{3}	1.82 10^{-6}	9.76
0.66	3.42 10^{-16}	3.03 10^{3}	6.98 10^{-7}	8.87
0.68	2.58 10^{-17}	2.11 10^{2}	4.13 10^{-7}	5.14
0.68	3.75 10^{-17}	3.01 10^{2}	4.67 10^{-7}	5.80

*Conditions for saturation were: 1.4 g of Capolac®; total citrate concentration of 0.37 mol L^{-1}; ratio between Na_2HCitr and Na_3Citr of 1:1, equilibration for 168 h at 25 °C. Iterative calculations are based on total calcium and phosphorous concentration as determined by ICP and shown in Fig. 5B and calcium ion activity as measured electrochemically, see Fig. 5A. Concentrations expressed as mol L^{-1}. Q_{CaCitr} corresponds to the ion activity product for calcium citrate defined by $Q = [Ca^{2+}]^3 \times (\gamma^{2+})^3 \times [Citr^{3-}]^2 \times (\gamma^{3-})^2$. Q_{CaP} corresponds to the ion activity product for calcium hydrogen phosphate defined by $Q = [Ca^{2+}] \times (\gamma^{2+}) \times [HPO_4^{2-}] \times (\gamma^{2-})$. Activity based solubility product $K_{C\text{-}sp\text{-}activity} = K_{sp\text{-}\ Ca3Citr2} \times (\gamma^{2+})^3 \times (\gamma^{3-})^2$ was calculated as $K_{C\text{-}sp\text{-}activity} = (1.20 \pm 0.05)\ 10^{-19}$ from the concentration based solubility product $K_{sp\text{-}\ Ca3Citr2} = 7.6 \times 10^{-17}$ $mol^5\ L^{-5}$ for ionic strength = 1.0 (Vavrusova et al., 2016). Activity based solubility product $K_{P\text{-}sp\text{-}activity} = K_{sp\text{-}CaHPO4} \times \gamma^{2+} \times \gamma^{2-}$ was calculated as $K_{P\text{-}sp\text{-}activity} = (7.98 \pm 0.08) \times 10^{-8}$ from the concentration based solubility product $K_{sp\text{-}CaHPO4} = 8.25 \times 10^{-7}$ $mol^2\ L^{-2}$ for ionic strength = 1.0 (McDowell et al., 1971). $Q_{Cit/Kc}$ is the ratio between the ion activity product Q_{CaCitr} and the activity based solubility product for calcium citrate $K_{C\text{-}sp\text{-}activity}$. Q_{CaP}/K_P is the ratio between the ion activity product Q_{CaP} and the activity based solubility product for calcium hydrogen phosphate $K_{P\text{-}sp\text{-}activity}$. % ppt is the percentage of calcium precipitated as calculate from total calcium as determined by ICP.

accordingly required to obtain supersaturation for citrate.

Although the presence of isocitrate reflects a lower efficiency in the supersaturation of Ca_3Citr_2, a remarkable property of the combination of $Na_2HCitr/Na_3isoCitr$ in a ratio of 1:1 is that the supersaturation degree for $CaHPO_4$ is larger ($Q_{CaP}/K_P = 26$) than when only Na_2HCitr is present ($Q_{CaP}/K_P = 3.3$). The combination $Na_2HCitr/Na_3isoCitr$ in a ratio of 1:1 was also found to be supersaturated in $Ca_3IsoCitr_2$, but with a Q_i/K_i of 45 which is eight times lower than when only $Na_3isoCitr$ is present ($Q_i/K_i = 412$), as seen from a comparison of Tables 2 and 3. This difference may be assigned to the weaker binding of calcium to isocitrate compared to citrate.

Despite the higher efficiency of citrate to bind calcium as a hydroxycarboxylate complex, isocitrate seems kinetically more efficient in inhibiting precipitation of calcium hydroxycarboxylates from supersaturated solutions. Structural effects together with the dynamics of complex formation and salt precipitation might together determine the efficiency of hydroxycarboxylate dissolution overshooting resulting in spontaneous supersaturation. It is important to highlight that complex formation between calcium and citrate or isocitrate is far stronger in comparison with other food components binding calcium that represent antinutritional factors, such as polyphenols. Considering the antinutritional factors for preventing calcium supersaturation from citrate and isocitrate, the contribution of polyphenols may, accordingly, be neglected, since their association constant for binding calcium is around 7–8 mol L^{-1} and, thus, far weaker (Zhao, Vavrusova, & Skibsted, 2018).

Isocitrate is less efficient than citrate in reaching a high degree of supersaturation, but isocitrate prevents precipitation following solubility overshooting of calcium hydroxycarboxylates, and a long lag phase for precipitation from supersaturated solutions can accordingly be achieved using combinations of Na_2HCitr and $Na_3isoCit$.

3.3. Calcium saccharate

The nature of the hydroxycarboxylate was shown to be important for both the degree of spontaneous supersaturation, and for the length of the lag phase for precipitation. The use of additional hydroxycarboxylates like saccharate was considered as a strategy to increase the robustness of supersaturation. Calcium saccharate was included in the investigation of the lag phase for precipitation based on the results of previous studies in which calcium saccharate was found to prevent precipitation from supersaturated calcium hydroxycarboxylate solutions (Garcia et al., 2018; Siegrist, 1949; Zawadzki & Skibsted, 2019).

The combinations Na_2HCitr / $Na_3isoCit$ 1:1 and Na_2HCitr/Na_3Citr 1:1 were compared to the combination of Na_2HCitr / $Na_3isoCit$ of 1:1 in which 1×10^{-2} mol L^{-1} of calcium saccharate was added, all solutions having a comparable pH of approximately 5.6, see Table 5.

Notably, the addition of calcium saccharate to the solution containing Capolac® and $Na_2HCitr/Na_3isoCit$ in a ratio of 1:1 was found not only to prolong the lag phase for precipitation from the supersaturated solutions with isocitrate (Fig. 5), but also to achieve a higher degree of supersaturation, despite the addition of the far less soluble calcium saccharate. The ratio Q_{Cit}/K_c for the combination $Na_2HCitr/Na_3isoCit$ with a ratio 1:1 containing saccharate gave a higher value for supersaturation for citrate ($Q_{Cit}/K_C = 1.3\ 10^3$) and for isocitrate ($Q_i/K_i = 91$), but not for phosphate ($Q_{CaP}/K_P = 0.33$), when compared to $Na_2HCitr/Na_3isoCit$ combinations without calcium saccharate ($Q_{Cit}/K_C = 1.3\ 10^3$; $Q_i/K_i = 45$; $Q_{CaP}/K_P = 26$), as may be seen from a comparison of Tables 4 and 5. The supersaturation is comparable to the highly supersaturated solutions formed upon dissolving Capolac® in 0.38 mol L^{-1} Na_2HCitr ($Q_{Cit}/K_c = 2.91 \times 10^4$; $Q_{CaP}/K_P = 3.3$). The ionic product of calcium saccharate:

$$Q_{CaSac} = [Ca^{2+}] \times [Sac^{2-}] \quad (11)$$

was also calculated and, notably, supersaturated solutions of Capolac® dissolved in $Na_2HCitr/Na_3isoCit$ at the ratio 1:1 with calcium saccharate added were supersaturated in $Ca_3isoCitr_2$ ($Q_i/K_i = 91$) and in CaSac ($Q_s/K_s = 21$). The effect of saccharate on prolonging the lag phase for precipitation seems accordingly to be related to supersaturation of calcium hydrogen phosphate.

Combining the two isomers citrate and isocitrate makes it possible

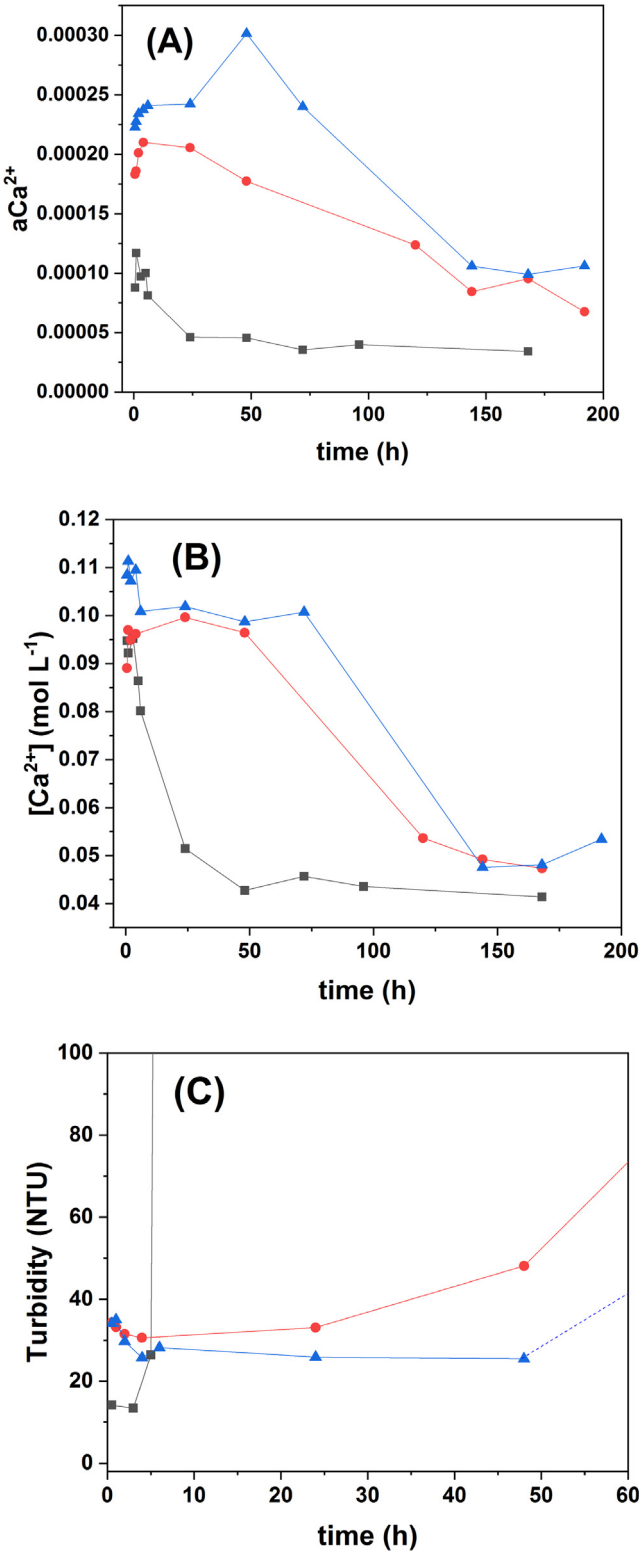

Fig. 5. (A) Calcium ion activity and (B) total calcium concentration and (C) turbidity (NTU) of homogeneous solutions containing 1.4 g of Capolac® in 100 mL of water dissolved in aqueous Na_2HCitr/Na_3Citr 1:1 – 1.9×10^{-1} mol L^{-1} /1.8×10^{-1} mol L^{-1} (■), Na_2HCitr /isoCit 1:1 – 1.9×10^{-1} mol L^{-1} /1.8×10^{-1} mol L^{-1} (●), Na_2HCitr /isoCit 1:1 – 1.9×10^{-1} mol L^{-1} /1.8×10^{-1} mol L^{-1} plus 1×10^{-2} mol L^{-1} of calcium saccharate (▲).

to obtain a synergistic effect for dissolving calcium and accordingly calcium availability for absorption. Together the two isomers also prolong the overshooting in calcium solubility, a time effect which can be further enhanced by the addition of calcium saccharate. Such synergistic effects need to be further investigated including other hydroxycarboxylates with the goal of providing combinations that can be used by the dairy industry in formulation of functional foods for prevention of osteoporosis. Our findings demonstrate the potential use of the supersaturation phenomenon for the design of functional foods with improved calcium bioavailability.

4. Conclusion

In conclusion, citrate binds calcium stronger than isocitrate and more easily forms supersaturated solutions by dissolution of calcium hydrogen phosphate and of dry mineral whey fractions from cheese processing. Isocitrate, however, binds calcium with less affinity but prolongs the lag phase for precipitation of calcium hydroxycarboxylates. Calcium saccharate further prolongs the lag phase for precipitation by some unknown mechanisms apparently related to calcium hydrogen phosphate solubility. These findings together may inspire for practical application but also for more detailed studies of the precipitation dynamics. For the more practical application, dried whey mineral fractions may be solubilized combining fruits rich in citrate and isocitrate to increase calcium bioavailability from beverages strongly supersaturated in calcium hydrogen phosphate. This conclusion should help the diary industry to solve a waste problem related to cheese production. Clearly the expected high bioavailability from dried whey minerals dissolved in aqueous solutions with combinations of citrate, isocitrate and saccharate still needs to be tested in human intervention studies.

CRediT authorship contribution statement

Andressa de Zawadzki: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Marcella Oliva Paganelli:** Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **André Castilho Garcia:** Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Leif H. Skibsted:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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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