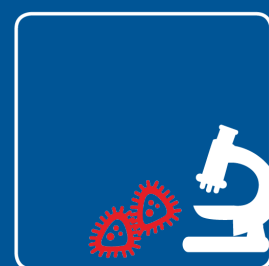


Leif Skibsted:

Super Calcium – Mineraler fra valle til målrettet ernæring

Super Calcium – Minerals from whey for
customized products



Final report

for collaborative projects funded via the Danish Dairy Research Foundation (DDRF)

1. Title of the project

UK: Super Calcium – Minerals from whey for customized products

DK: Super Calcium - Mineraler fra valle til målrettet ernæring

2. Project manager

Leif H. Skibsted, professor, Department of Food Science, University of Copenhagen, Rolighedsvej 26, DK-1958 Frederiksberg, +45 35 33 32 21, ls@food.ku.dk. From 1.1.2018 – 30.9.2019

Lilia Arhné, professor, Department of Food Science, University of Copenhagen, Rolighedsvej 26, DK-1958 Frederiksberg, +45 35 33 37 94, lilia@food.ku.dk. From 1.10.2019

3. Other project staff

Andressa de Zawadzki, post doc, Department of Food Science, University of Copenhagen, Rolighedsvej 26, DK-1958 Frederiksberg, andressa@food.ku.dk. From 1.2.2018 – 31.1.2020

4. Sources of funding

The project 'Novel Aging – Technologies and solutions to manufacture novel dairy products for healthy aging' funded by Innovation Fund Denmark as an international project together with Fundação de Amparo à Pesquisa do Estado de São Paulo /FAPESP Brazil (grants 7043-00006A and 2017/01189-0) served as cofinancing.

Mælkeafgiftsfonden

Arla Foods Ingredients

Københavns Universitet

5. Project period

Project period with DDRF funding:

Project start: 01/01/2018

Project end: 31/08/2020

6. Project summary

Dansk sammendrag

Formålet med Supercalcium-projektet var at udarbejde et grundlag for at kunne udnytte et restprodukt fra ostefremstilling og forarbejdning af valle til fremstilling af nye typer snacks og drikke med høj tilgængelighed af calcium. Produkterne vil, alene baseret på mælk og frugt, kunne markedsføres med et naturligt og grønt image. Calciumsaltene i den tørrede mineralfraktion fra valle blev for et kommercielt produkt med 26,5% calcium vist at bestå af 30% calciumhydrogenphosphat og 70% hydroxyapatit og have et Ca/P forhold på 1,5

i overensstemmelse med gældende anbefalinger for vores kost. Gluconolacton sammen med natriumgluconat kunne opløse denne ellers uopløselige mineralfraktion og danne opløsninger, der var stærkt overmættede med calciumsalte. Natriumhydrogencitrat havde samme evne, men de overmættede opløsninger udfældede calciumsaltene hurtigt. Isocitronsyre, som findes i brombær, gjorde de overmættede citratopløsninger mere robuste. Citronsyre kombineret med gluconat blev vist at forene effektiv opløsning og overmætning med høj beskyttelse mod udfældning.

English summary

The dried whey mineral fraction from bovine cheese production has been found to be a valuable source for dietary calcium. The commercial mineral fraction used for this investigation contained 26.5% calcium and 13.1% phosphorous, corresponding to a calcium/phosphate ratio of 1.5, which is close to the current recommendation for human nutrition. The calcium/phosphate ratio can be accounted for as a mixture of 30% calcium hydrogen phosphate and 70% hydroxyapatite out of the total calcium content as confirmed by X-ray diffractometry. The whey mineral mixtures have very low solubility in water and have often been considered a pollutant rather than a valuable side stream by the dairy industry. The mineral fraction was shown to dissolve in combinations of gluconolactone and sodium gluconate in water forming strongly supersaturated solutions with lag phase for precipitation of several weeks. Aqueous sodium hydrogen citrate had a comparable effect forming supersaturated solutions and for the combination of citrate and isocitrate the lag phase was prolonged to several weeks or even months following addition of small amount of calcium saccharate prior to dissolution of the whey mineral fraction. The side stream from cheese production and whey processing may accordingly find use for production of functional foods with high calcium availability. The combination of hydrogen citrate and gluconate seems most promising for practical application in drinks and snacks, since calcium citrate is known to have high bioavailability of calcium in human digestion while gluconate in the SuperCalcium project was found efficiently to increase the supersaturation robustness for whey minerals dissolved in citrate/gluconate combinations.

7. Project aim

The project explored the utilization of the phenomena of spontaneous supersaturation previously described for combinations of calcium hydroxycarboxylates, and also for calcium phosphates and hydroxycarboxylates for transformation of a whey processing waste into valuable functional foods with a green and sustainable image for prevention of osteoporosis. The role of milk proteins on calcium availability was further explored with the perspective for development of super-calcium drinks and calcium-enriched snacks based on dairy products.

8. Background for the project

Whey proteins and lactose are valuable side streams from cheese production. It is generally accepted that the mineral fraction from whey processing may be used for functional food with high calcium bioavailability. There seems to be good market possibilities for such mineral functional foods seen in the light of the epidemic development of osteoporosis and other age-related bone diseases. However, the low solubility has previously hampered development of such products based on the whey mineral residues. Previous studies have demonstrated that hydroxycarboxylates like gluconate and citrate dissolve calcium phosphates spontaneously forming strongly supersaturated calcium salts. Calcium saccharate was further demonstrated to increase the robustness of such supersaturated solutions, see Figure 1.

9. Sub-activities in the entire project period

	2018			2019				2020	
Effect of amino acids on supersaturation									
Characterization of whey minerals residues									
Development of calculation methods for ion speciation									
Dissolution of whey minerals in aqueous gluconate									
Dissolution of whey minerals in aqueous citrate									
Effect of whey proteins and caseins on calcium supersaturation									
Dissolution of whey minerals in combinations of aqueous hydroxycarboxylates, quantum mechanical calculations									

10. Project results

Valuable new information about the aqueous solution chemistry of calcium salts and especially mixtures of these salts as present in whey residues focusing on thermodynamics and kinetics of complex formation and precipitation in relation to supersaturation phenomena were obtained during the execution of this project.

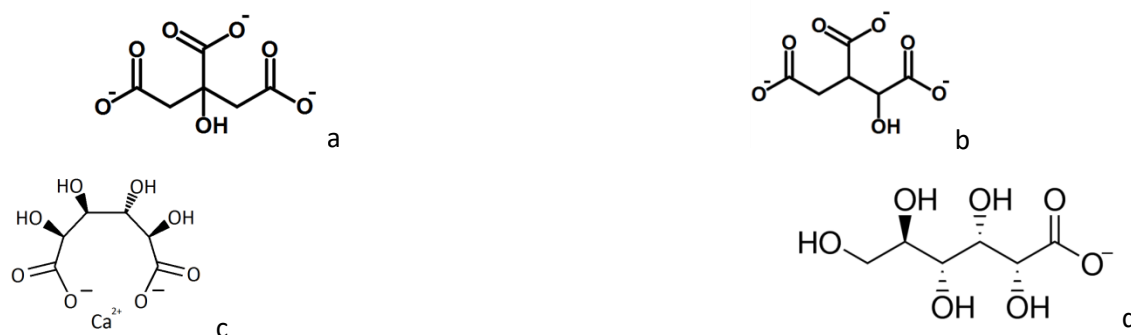
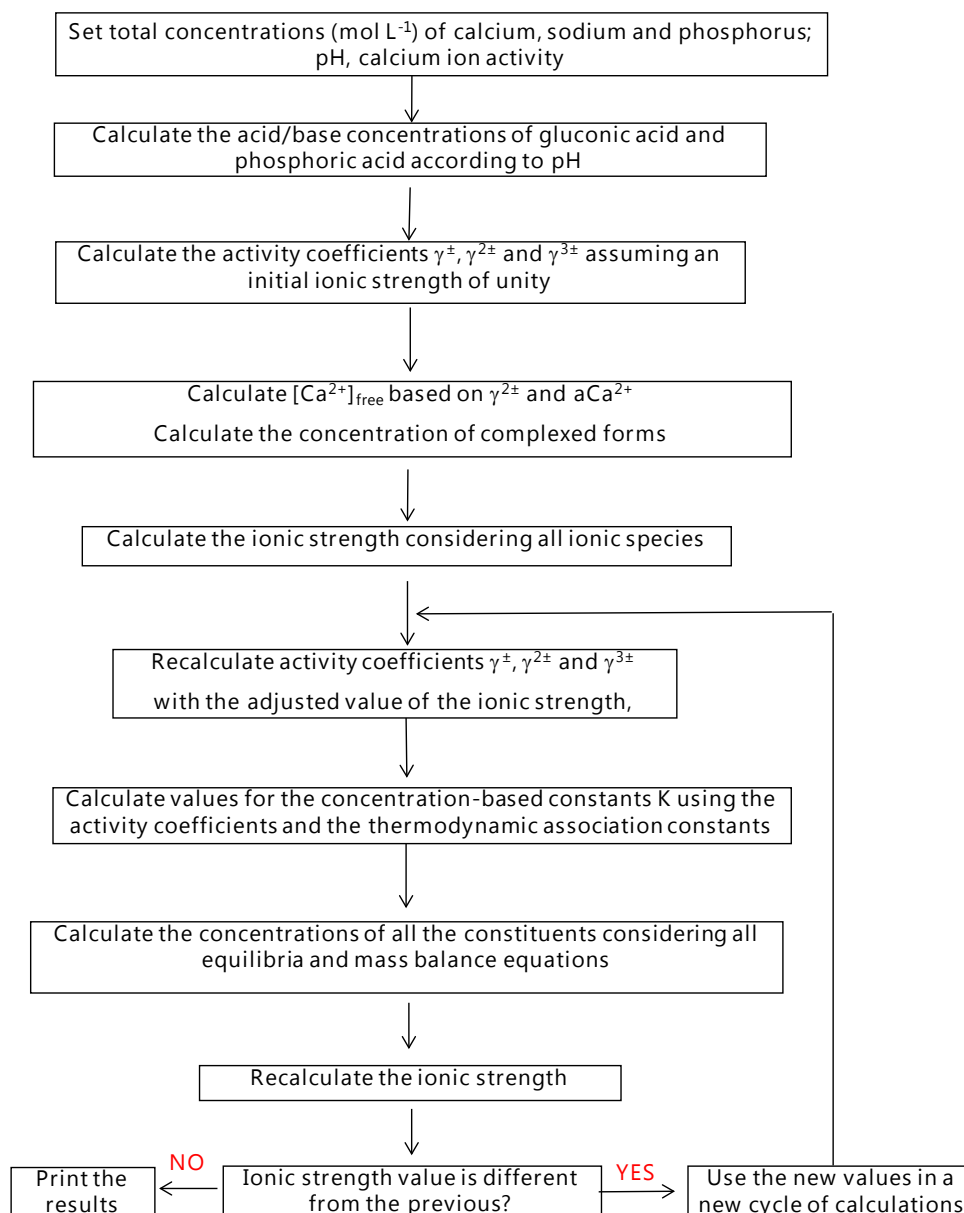


Figure 1. Structures of citrate ion (a), isocitrate ion (b), calcium saccharate (c), and gluconate ion (d). All these hydroxycarboxylates are naturally occurring like citrate in milk, gluconate as the first oxidation product of glucose, and lactate resulting from fermentations.

The mineral residues from processing of whey from cheese production have been characterized and the calcium salts in the commercially available residues used for the investigation was found to be a mixture of calcium hydrogen phosphate (30% of the calcium content) and hydroxyapatite (70% of the calcium content). The aqueous solubility of the whey residues was found very low. A mathematical framework for calculation of speciation in the supersaturated solutions has been developed using Matlab, see Scheme 1.

Scheme 1. Method for calculating ion speciation in supersaturated calcium hydroxycarboxylate solutions as shown for gluconate as dissolving hydroxycarboxylate using an iterative approach.



The spontaneous supersaturation is a unique phenomenon originally described for dissolution of calcium lactate in aqueous gluconate. Later calcium phosphates were found to dissolve in aqueous citrate also spontaneously forming solutions strongly supersaturated and from which calcium citrate only precipitated after a long lag phase. The most important finding of the SuperCalcium project is that the crude whey mineral

residues also form such spontaneously supersaturated solution with a surprising robustness and a high calcium availability as measured electrochemically. The spontaneous formation of the supersaturated solutions at a first glance apparently violating fundamental thermodynamics, seems to depend on a unique distribution of calcium complexes in which the ions important for the precipitation reactions are low despite the high total calcium concentration. For this reason, methods for calculations of ion speciation in solutions with high and varying ionic strength were developed.

Solubility of whey minerals in aqueous gluconate

Almost insoluble milk mineral residues from whey processing dominated by hydroxyapatite and calcium hydrogen phosphate were found to dissolve isothermally in aqueous gluconate/ δ -gluconolactone spontaneously forming solutions supersaturated in both calcium hydrogen phosphate and calcium gluconate. The calcium concentration of the maximally supersaturated solutions was found proportional to the gluconate concentration indicating gluconate assisted dissolution, while gluconolactone increased calcium available for dissolution and supersaturation. Precipitation of calcium gluconate rather than of calcium hydrogen phosphate was found critical for supersaturation robustness. For ratios lower than 12 between the calcium gluconate ionic product and the solubility product of calcium gluconate, the supersaturated solutions was found to have a lag phase for precipitation of several weeks, which was increased to several months by addition of solid calcium saccharate prior to dissolution of the mineral residues. Such supersaturated solutions with up to 7 g calcium L⁻¹ corresponding to a factor of supersaturation of more than 100 times compared to equilibrium calcium hydrogen phosphate solubility, clearly should be exploited for increasing calcium availability from whey mineral based functional foods. The whey minerals were found to dissolve more easily than calcium hydrogen phosphate indicating the presence of some unknown components in the whey residue promoting dissolution in the presence of gluconate, see Figure 2 for the commercial whey mineral product Capolac® (Arla Foods Ingredients).

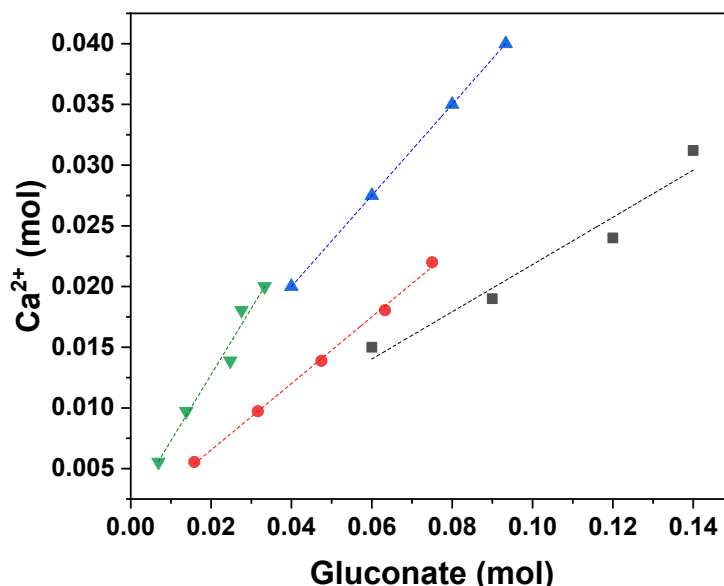


Figure 2. Critical combinations of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ or Capolac®, and gluconate for the formation of homogeneous supersaturated solutions in water at 25 °C for gluconate/gluconolactone with a ratio of 1:1 (■ for $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ and ● for Capolac® as a source of calcium) and 1:2 (▲ for $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ and ▼ for Capolac® as a source of calcium). The lines present the maximal supersaturation for 100 ml, below the lines the solution are also supersaturated, but may dissolve more calcium salt increasing the supersaturation. Any combinations above the lines are two-phase systems.

Solubility of whey minerals in aqueous citrate

Mineral residues from whey processing almost insoluble after drying and dominated by hydroxyapatite and calcium hydrogen phosphate were found to dissolve in aqueous sodium hydrogen citrate without heating spontaneously becoming supersaturated in both calcium hydrogen phosphate and calcium citrate. The maximal supersaturation was found to be proportional to total hydrogen citrate concentration and for 0.2 M hydrogen citrate, an initial calcium concentration of more than 0.1 M appeared corresponding to supersaturation of a factor of 10 more than for calcium hydrogen phosphate. Calcium citrate tetrahydrate, rather than calcium phosphates, precipitated from the supersaturated solutions. It was found that the time elapsing before precipitation began increased with increasing concentrations of excess of hydrogen citrate up to a certain hydrogen citrate concentration. For 0.2 M hydrogen citrate the lag phase changed from several hours to up to a day for moderately higher hydrogen citrate concentrations. For the higher concentrations of hydrogen citrate, the solutions became supersaturated only in calcium citrate but saturated in calcium hydrogen phosphate due to increasing binding of calcium by citrate. The observation that a maximal lag phase was found for moderately high supersaturation deserved further attention. Aqueous sodium hydrogen citrate may accordingly form the basis for formulation of functional foods with high calcium availability, but the relatively short lag phase for precipitation present challenges and inspired for investigation of hydroxycarboxylate combinations.

Solubility of whey minerals in aqueous hydrocarboxylate combinations

Combinations of aqueous isocitrate and citrate were found more efficient than each of the two isomers in dissolving insoluble whey processing mineral residues consisting mainly of calcium hydrogen phosphate and hydroxyapatite spontaneously forming supersaturated solutions. Based on the amount of dissolved calcium, hydrogen isocitrate was found around 30% less efficient in these non-thermal dissolution processes compared to hydrogen citrate based on. In contrast, the lag phase of up to 4 hours for precipitation of calcium citrate tetrahydrate from the supersaturated solutions was significantly longer when calcium isocitrate also was present. Highest degree of supersaturation with longest lag phase for precipitation was found for citrate/isocitrate combinations. Addition of calcium saccharate 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 of low solubility, see Figure 3.

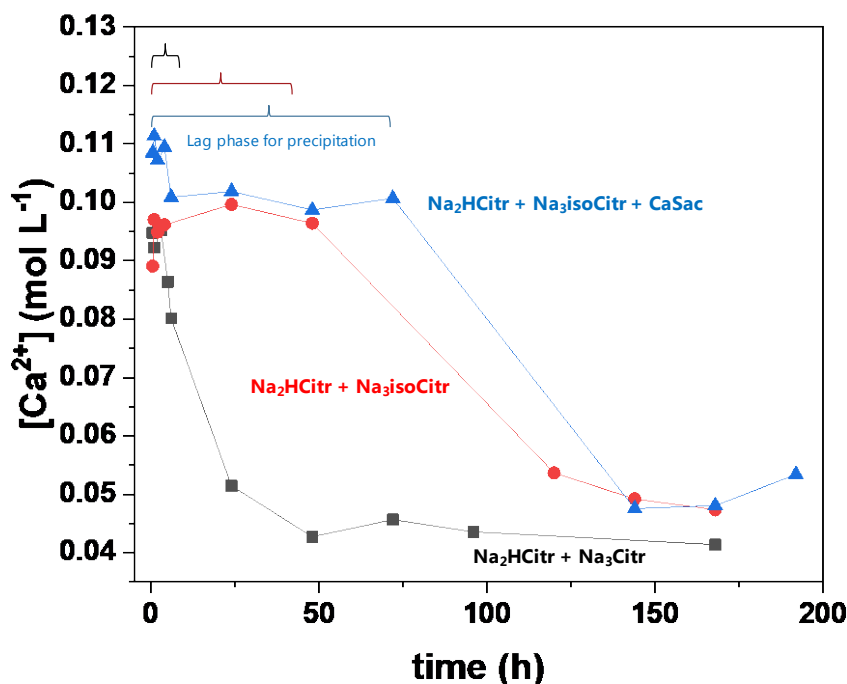


Figure 3. Example of effect on precipitation of calcium citrate from supersaturated solutions based on combinations of hydroxycarboxylates. Total calcium concentration of homogeneous aqueous solutions containing 1.4 g of Capolac® in 100 mL of water dissolved in aqueous Na₂HCitr/Na₃Citr 1:1 – $1.9 \times 10^{-1} \text{ mol L}^{-1}/1.8 \times 10^{-1} \text{ mol L}^{-1}$ (■), Na₂HCitr/Na₃isoCit 1:1 – $1.9 \times 10^{-1} \text{ mol L}^{-1}/1.8 \times 10^{-1} \text{ mol L}^{-1}$ (●), Na₂HCitr/Na₃isoCit 1:1 – $1.9 \times 10^{-1} \text{ mol L}^{-1}/1.8 \times 10^{-1} \text{ mol L}^{-1}$ plus $1 \times 10^{-2} \text{ mol L}^{-1}$ of calcium saccharate (▲). The calcium concentration was monitored by ICP-analyses. A similar pattern was found measuring calcium ion activity electrochemically.

Role of amino acids and milk proteins on calcium supersaturation

The effect of the presence of amino acids on the supersaturation of calcium was investigated in supersaturated solutions formed upon adding sodium hydrogen citrate to calcium hydrogen phosphate suspensions. Aspartate, glutamate, serine and o-phospho-L-serine were added as calcium binding amino acids to the supersaturated systems and their effect was evaluated through changes in calcium activity and calcium concentration prior to and during precipitation. The amino acids were surprisingly found to promote precipitation of calcium salts from the supersaturated solution by some still unknown mechanism. Due to the complexity of calcium binding to o-phospho-L-serine and the unexpected effect of this amino acid on calcium ion activity in the supersaturated solution, this study was transferred to two PhD projects. The amino acid o-phospho-L-serine is of particular interest as it is a major calcium coordinator of the caseins. The PhD student Yuan Jiang is currently investigating kinetics and thermodynamics of calcium binding to o-phospho-L-serine also including Density Functional Theory (DFT) calculations to investigate the quantum mechanical background for the role of amino acids in calcium salt supersaturation. The results of DFT calculations is expected to provide more detailed mechanistic information on the interaction between amino acids and calcium during supersaturation and in milk in general. The visiting Brazilian PhD student Marcella Oliva Paganelli has been investigating the effect of hydrolyzed whey proteins on precipitation from supersaturated whey mineral solutions. This study will be continued at University of São Paulo, São Carlos, Brazil, as part of the NovelAging project.

Supersaturation and calcium binding to milk protein hydrolysates

A commercial whey protein hydrolysate (Peptigen®) was compared to a commercial casein hydrolysate (Lacprodan®) for their effect on supersaturation of calcium salts from mineral residues from whey processing. For calcium hydrogen phosphate solubilized by sodium hydrogen citrate and spontaneously becoming supersaturated by a factor of up to 10 at 25°C, added whey protein hydrolysates or added casein hydrolysates initiated a rapid increase in free calcium ion concentration after a lag phase. This initial increase was followed by a slow decrease in both free and total dissolved calcium. From calcium hydrogen phosphate solubilized by hydrogen citrate, whey protein and casein hydrolysates may accordingly increase calcium bioavailability with a perspective for tailoring functional foods for calcium nutrition. This discovery needs clearly more investigations before firm conclusions can be reached, but the time profile discovered for calcium bioavailability in the presence of hydrolyzed milk proteins could probably be adjusted to match human digestion.

Future perspectives

The high supersaturations discovered in this project seem to be due to stronger calcium complex formation while the long robustness and long lag phase seems to be due to a simultaneous weaker binding by other components, which seems to lower the driving force of precipitation. The supersaturation phenomena of calcium salts might have an important role in calcium nutrition and also in bone biomineralisation. The spontaneous supersaturations, especially the unique role of citrate, might be an inspiration for the formulation of novel fortified foods and beverages with perspectives of improved calcium bioavailability. The combination of gluconate and citrate seems to be unique, since citrate increase the degree of supersaturation while gluconate prevent initiation of precipitation. This finding needs now to be tested in both liquid products like 'SuperCalcium' drinks and in dry products like snacks followed up by human intervention studies. High circulating citrate has lately been found to correlate with effective biomineralization in humans, and calcium citrates in animal studies found to increase rate of bone healing.

11. Deviations

11.1 Scientific

The content of one published paper and two submitted manuscripts correspond to the initial milestones together with a manuscript to be submitted soon. Two additional manuscripts are now under preparation.

11.2 Financial

Some of the more promising results will form the basis for new investigations in the framework of the Danish/Brazilian 'NovelAging' project. The PhD studies of Yuan Jiang and Marcella Oliva Paganelli are financed by Chinese and Brazilian funding agencies, respectively.

11.3 Timetable

The project has basically followed the original timetable. Additional activities have been added through the studies of the two PhD students.

12. The relevance of the results, including relevance for the dairy industry

The principle of spontaneous isothermal supersaturation is new. It was first discovered for dissolution of calcium lactate in aqueous sodium gluconate (Vavrusova, M.; Skibsted, L. H. *Food and & Function* (5), 2014, 85). This finding has now - in the SuperCalcium project - been transferred to commercial whey mineral products and the surprising effect fully confirmed, see Figure 4. This opens up for improving the calcium

bioavailability from the whey mineral fraction in functional foods for prevention of osteoporosis. Stabilization of strongly supersaturated calcium phosphate/citrate solutions by hydroxycarboxylates, like saccharate and isocitrate and especially combinations of citrate and gluconate, seems to hold the key for product formulation for commercial whey mineral residues from whey processing. It is now up to the dairy industry to develop commercial products. Andressa de Zawadzki and Xiaochen Liu will present the results to Arla Foods Ingredients in March 2020.

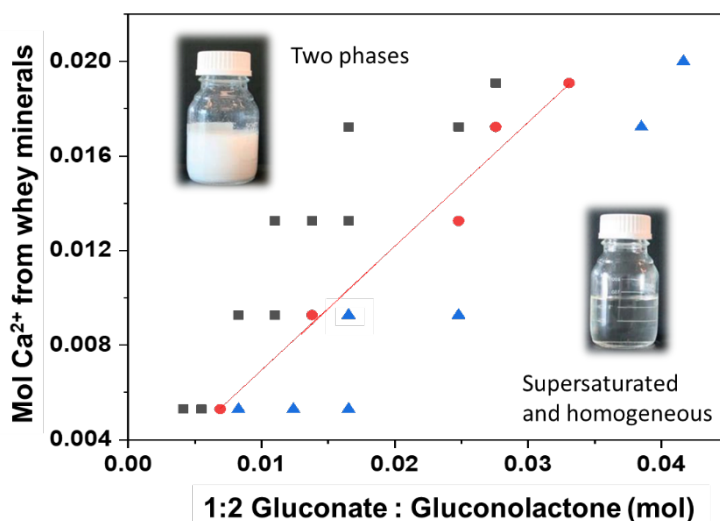


Figure 4. Red line shows the critical combination for spontaneous formation of supersaturated solutions of whey minerals in 100 ml of aqueous 1:2 gluconate:gluconolactone at 25°C.

13. Communication and knowledge sharing about the project

Papers in international journals:

de Zawadzki, A.; Skibsted, L.H.: Increasing calcium solubility from whey mineral residues by combining gluconate and delta-gluconate. *International Dairy Journal* (99) 2020, 104538.

Submitted papers:

de Zawadzki, A.; Skibsted, L.H.: Calcium availability from whey mineral residues increased by hydrogen citrate. *Food Research International*, submitted for publication.

de Zawadzki, A.; Paganelli, M.O; de Garcia, A.C.; Skibsted, L.H.: Increasing calcium availability from whey mineral residues by combining citrate and isocitrate. *Food Research International*, submitted for publication.

Other papers:

Skibsted, L.H.: Supercalcium – nyt koncept og nye produkter. *Mælkeritidende* (131) 2018, 8-9.

Skibsted, L.H.: Kostens tungtopløselige calcium: Ostwalds fase lov, spontan overmætning og biotilgængelighed. *Dansk Kemi* (99, 7) 2018, 14-16.

Skibsted, L.H.; Ahrné, L.; de Zawadzki, A.: Fra vallem mineral til supercalcium. *Mælkeritidende* 2020 (3), 12-13.

14. Contribution to master and PhD education

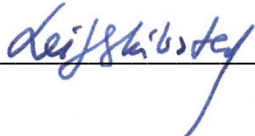
Marcella O. Paganelli enrolled as a PhD student at the University of São Paulo, São Carlos, has been on a secondment at FOOD, University of Copenhagen, working on the project.

15. New contacts/projects

The activities in SuperCalcium continues in a Danish/Brazilian project supported by Innovation Fund Denmark, "Novel ageing: Technologies and solutions to manufacture novel products for healthy ageing", with Professor Lilia Ahrné, University of Copenhagen, as the Danish project leader and Associate Professor Daniel R. Cardoso, from University of São Paulo, São Carlos, as the Brazilian project leader.

16. Signature and date

The project is formally finalised when the project manager and DDRF-representative (e.g. steering committee leader) have signed this final report.

Date: 25/3 2020 Signature, Project manager: 

Date: 6 April 2020 Signature, DDRF-representative: 