Influence of calcium chelators on concentrated micellar casein solutions

From micellar structure to viscosity and heat stability

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Als je denkt: "Ik ben verslagen",
is de nederlaag een feit.
Als je denkt: "Ik zal niet versagen",
win je op den duur de strijd.
Als je denkt: "Ik kan het niet halen",
is de tegenslag op til,
want het overslaan der schalen
hangt voornamelijk af van wil.

Moedelozen gaan ten onder,
door hun twijfel, door hun vrees.
Vechters winnen, door een wonder
telkens weer een lange race.

Denk:"Ik kan het", en dan gaat het.
Iedereen vindt bij wilskracht baat
en in zaken wint de daad het
van het nutteloos gepraat.

Als je jammert: "Ik ben zwakker
dan mijn grote concurrent",
blijf je levenslang de stakker,
die je ongetwijfeld bent.

Niet de Goliaths en de rijken
tellen in de kamp voor zes,
maar de fermen, die niet wijken,
hebben vroeg of laat succes.

Voor Wil

Abstract

In practice it is challenging to prepare a concentrated medical product with high heat stability and low viscosity. Calcium chelators are often added to dairy products to improve heat stability, but this may increase viscosity through interactions with the casein proteins. The aim of this thesis was to obtain a better understanding of the influence of different calcium chelators on the physico-chemical properties of casein micelles and the resulting effect on viscosity and heat stability of concentrated micellar casein isolate (MCI) solutions. The calcium chelators disodium uridine monophosphate (Na_2UMP), disodium hydrogen phosphate (Na_2HPO_4), trisodium citrate (TSC), sodium phytate (SP), and sodium hexametaphosphate (SHMP) were studied.

Initially, the calcium-binding capacity of the phosphates was investigated and found to be directly related to the amount of charges. The resulting effects on physical changes of casein micelles were subsequently explored before and during heating. The viscosity of the MCI solutions increased upon addition of the calcium chelators, which was attributed to swelling of the caseins at decreasing calcium-ion activity. The calcium chelators induced different changes in turbidity of the MCI solutions, which could be related to the degree of dissociation of the casein micelles. Simulations of the ion equilibria indicated that the extent of casein micelle dissociation followed the calcium-binding capacity of the calcium chelators. Micelle dissociation occurred in the order of SHMP > SP > TSC > Na_2HPO_4 > Na_2UMP. The results on heat stability indicated that the calcium-ion activity and state of the micellar structure before and during heating determined the heat stability of the MCI solutions. Na_2UMP was the most effective heat stabilizer, as it bound sufficient free calcium ions to reduce protein aggregation without affecting the micellar structure. SHMP was the least effective heat stabilizer because of heat-induced changes occurring during heating. For polyphosphates, SHMP and SP, it was found that they decreased the isoint of casein by forming direct bindings with the caseins, for which calcium ions were not required.

In conclusion, this thesis has provided new insights in the relationships between calcium chelators and their influence on the casein micelle structure and on the physico-chemical properties of concentrated MCI solutions. Also, the practical relevance for the dairy industry was described, demonstrating how different calcium chelators can manipulate the viscosity and heat stability of dairy products.

Contents

Chapter 1

General introduction

1.1 Medical nutrition

Medical nutrition is specifically designed for the dietary treatment of a disease, because the human body often needs extra energy or protein during and after a period of illness. Medical nutrition is also frequently used by patients or elderly persons who suffer from swallowing disorders, reduced appetite, or loss of taste. This can reduce nutritional intake, leading to suboptimal nourishment, and, in the end, to malnutrition.
Medical nutrition can be, depending on the condition of the patient, consumed as oral or tube feed. A wide variety of medical products is available on the market in the form of liquids, texturized products, or powders. These products are not comparable to enriched food supplements, because they have to be tested in a clinical trial for their tolerance and functionality, and need to be officially registered and subscribed by a doctor or dietician. The nutritional composition has to follow official European legal guidelines for Food for Special Medical Purposes (FSMP). Medical products are often nutritionally complete, which means that they contain proteins, fats, carbohydrates, minerals, and vitamins. The required source and concentration of the constituents in a specific medical product depend on the target group of patients. Two typical compositions of medical products are shown in Table 1.1.

Table 1.1 illustrates that medical nutrition is highly concentrated in nutrients. One bottle of these medical products already delivers 20% of the advised daily intake for minerals, trace elements, and vitamins. To compare with retail milk: pasteurized, semi-skimmed cow's milk delivers 48 kcal per 100 ml and consists of 3.5 g protein, 5.0 g carbohydrates, and 1.5 g fat.

Table 1.1 Typical nutrient composition of a standard and a concentrated medical sip feed.

	Standard medical sip feed	Concentrated medical sip feed	
Energy	150	240	kcal
Protein	6	9.6	g
Fat	5.8	9.3	g
Carbohydrates	18.4	29.7	g
Minerals			
Na	90	96	mg
K	159	236	mg
Cl	87	91	mg
Ca	91	174	mg
P	78	174	mg
Mg	23	33	mg
Trace elements			
Fe	2.4	3.8	mg
Zn	1.8	2.9	mg
Cu	270	430	µg
Mn	0.5	0.8	mg
F	0.15	0.2	mg
Mo	15	24	µg
Se	8.6	14	µg
Cr	10	16	µg
I	20	32	µg
Vitamins			
Vitamin A	123	240	µg RE
Vitamin D	1.1	1.8	µg
Vitamin E	1.9	3	mg α-TE
Vitamin K	8	13	µg
Thiamine	0.23	0.4	mg
Riboflavin	0.24	0.4	mg
Niacin	2.7	4.3	mg NE
Pantothenic acid	0.8	1.3	mg
Vitamin B6	0.26	0.4	mg
Folic acid	40	64	µg
Vitamin B12	0.32	0.7	µg
Biotin	6	9.6	µg
Vitamin C	15	24	mg
Carotenoids	0.3	-	mg
Other			
Choline	55	88	mg

1.2 Challenges for the development of medical nutrition

It is challenging to develop medical nutrition that is both tasty and heat- and shelf-stable because of the many interactions between the ingredients. Stable means maintaining comparable physico-chemical properties during heat treatment and storage. Moreover, the microbiological quality of these products has to be high, because they are often consumed by a frail target group. To guarantee this quality, medical nutrition is normally sterilized at temperatures between 120°C and 130°C for several minutes. Because of this intensive heat treatment, the products will remain microbiologically stable at ambient temperature for approximately one year. Especially the proteins can be sensitive to intensive heat treatment, since they may exhibit conformational changes and react with the minerals present in the formulation. This may easily lead to physical instability of the products.

1.3 Concentrating medical nutrition

The dietary treatment of malnourished patients or frail elderly people is mainly focused on the intake of extra energy and proteins. Medical products often deliver a maximum of 150 kcal per 100 ml with a serving volume of 200 ml (standard sip feed, Table 1.1). Hence a large volume needs to be consumed to meet the daily intake of nutrients. Therefore, product development has focused on concentrating medical products to obtain a smaller volume of liquid that still provides the required daily intake of nutrients. Table 1.1 also shows a typical composition of such a concentrated medical sip feed. This concentrated medical sip feed delivers 240 kcal instead of 150 kcal per 100 ml. Upon increasing the concentration of dissolved solids in a medical product, the overall viscosity of the composition increases as well (Fig. 1.2).

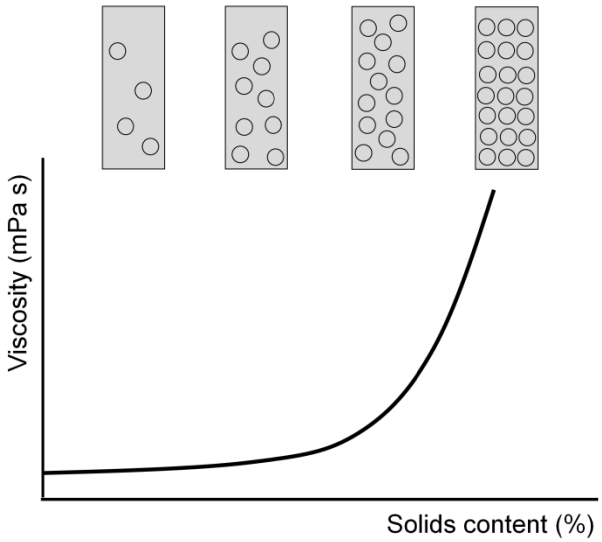

Figure 1.2 The viscosity of a medical product as a function of the solids content: adding more nutrients increases the product viscosity.

A higher viscosity makes the liquid nutritional composition often difficult to consume and may also change the taste of the product. This makes it challenging to develop a heat- and shelf-stable liquid nutritional composition with a high protein content (> 6 w/w% proteins) and an acceptable viscosity. The most frequently used protein source in medical nutrition is milk protein, which consists of casein and whey protein in a ratio of 80:20.[1] These proteins have a major impact on product stability, because physico-chemical conditions (e.g. mineral sources, ionic strength) and intensity of heat treatment affect the conformational state of casein and whey proteins in the product.

1.4 Characteristics of casein micelles

Casein appears in the form of intact casein micelles (e.g. present in milk protein concentrate/isolate, ultrafiltered milk, micellar casein isolate) or small casein aggregates (e.g. sodium, potassium, calcium, or magnesium caseinate). Intact casein micelles are naturally present in milk. Casein micelles are colloidal, polydisperse, and spherical with an average

diameter of 200 nm.[2] They are heterogeneous, hydrated, dynamic structures with a loose packing and a high porosity.[3] Casein micelles are composed of four different casein molecules, namely α_{s1}-, α_{s2}-, β-, and κ-casein, in a molar ratio of 4:1:4:1.[4-7] These molecules differ in hydrophobicity, net charge, phosphate concentration, and calcium sensitivity. They exhibit self-association, depending on the physico-chemical conditions.[2, 8, 9]

Casein micelles contain about 7 g minerals per 100 g dry casein, which is called colloidal calcium phosphate (CCP). CCP is more than just amorphous calcium phosphate, as it also contains sodium, potassium, magnesium, and citrate.[4, 10-12] The exact composition of CCP depends on the ionic environment, indicating that it has ion-exchange properties.[4, 12] The nanoclusters of CCP have an estimated diameter of about 2.5 nm.[1, 2, 4, 11, 13] CCP acts as "glue" in the micelles: the more CCP present in the micelles, the more rigid the micelles will be.[5] A typical casein micelle contains about 10^4 polypeptide chains of casein molecules associated with about $3 \cdot 10^3$ nanoclusters of CCP.[2, 4] About two-thirds of the casein is directly bound to the colloidal calcium phosphate through the negative charges of the phosphoserine residues, reducing the electrostatic repulsion in the casein micelles.[14] Hydrophobic interactions between the caseins are, furthermore, associating the caseins.[7] Casein micelles have in milk a voluminosity of about 3-4 ml water per g of dry casein [1, 15-17], giving them a spongelike colloidal structure, since they hold more water than dry matter. Relatively little of this water, around 0.5 g H_2O per g dry casein, is directly bound to casein.[17]

The nature and structure of the casein micelles have been extensively studied and different models have been proposed. However, the exact structure of the casein micelle is still not fully understood. Based on the physico-chemical properties of casein micelles three categories were defined: 1) coat-core model[7, 18]; 2) sub-micelle model[19-21]; and 3) internal structure model.[2, 8, 22] All proposed models agree that CCP plays an integral role in the structure of the casein micelle. In more recent studies[13, 15, 23, 24] dealing with the structure of the casein micelle the internal structure model is favored, because it was found that the smaller substructures detected by X-ray scattering were composed of CCP nanoclusters rather than sub-micelles. We also will use the internal structure model (Fig. 1.3) in this thesis to explain changes occurring in the casein micelle structure.

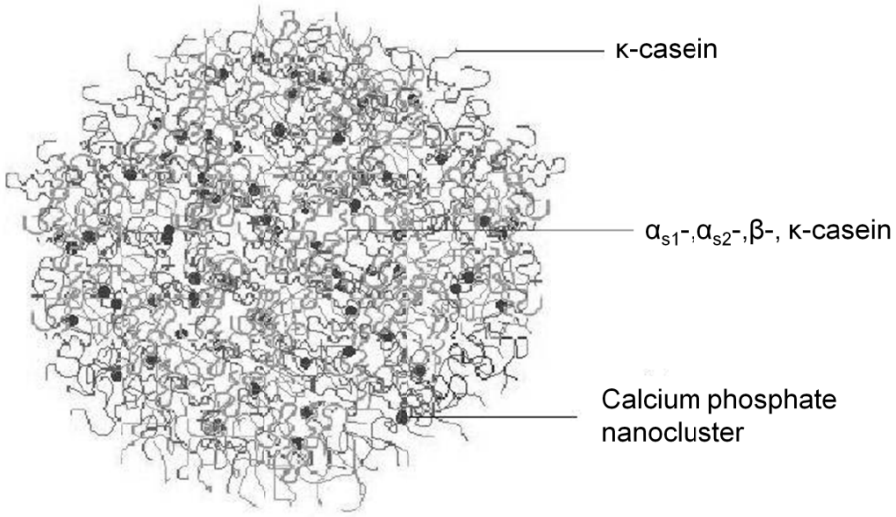

Figure 1.3 Internal structure model of a casein micelle as proposed by Holt and Horne.[22] The core of the casein micelle appears as a network and fairly open structure of polypeptide chains cross-linked by calcium phosphate nanoclusters (CCP, colloidal calcium phosphate). The external region has a low segment density and is known as the "hairy layer" of the casein micelle. The dark spots represent the calcium phosphate nanoclusters.

In this model the caseins and CCP nanoclusters are relatively homogeneously distributed in the casein micelle. The caseins are mainly bound together in the micelle by electrostatic and hydrophobic interactions.[15, 22] Also interactions, such as calcium-bridging, hydrogen bond formation and Van der Waals interactions occur and are important to maintain the casein micelle structure for instance upon cooling.[15] The form of the casein micelle is relatively spherical and the structure is open with groups of calcium phosphate partly cross-linked to the phosphoserine residues of the casein micelles. The interior of the micelle mainly consists of connected caseins (i.e. α_{s1}-, α_{s2}-, and β-casein) and calcium phosphate. The exterior of the micelle mainly consists of hydrophilic negatively charged κ-casein, which provides both electrostatic and steric stabilization to the casein micelle.[7, 22, 23] The negative charge is caused by dissociated carboxyl and ester phosphate groups, resulting in a zeta potential of about -20 mV for the casein micelle at neutral pH and ambient temperature. The κ-casein layer is also called the "hairy layer" of the micelle and has a hydrodynamic thickness of about 7 nm.[12, 22]

1.5 Effect of calcium chelators on the casein micelle structure

Calcium chelators, such as phosphate, citrate, hexametaphosphate, or ethylenediaminetetraacetic acid, are commonly used in the dairy industry to increase the heat stability or to retard age gelation in dairy products.[25-30] Calcium chelators shift the casein-mineral equilibria (Fig. 1.4), leading to a decrease in concentration of free calcium ions, dissolution of CCP from the micelle, and release of specific caseins from the micelle.[2, 4, 6, 31]

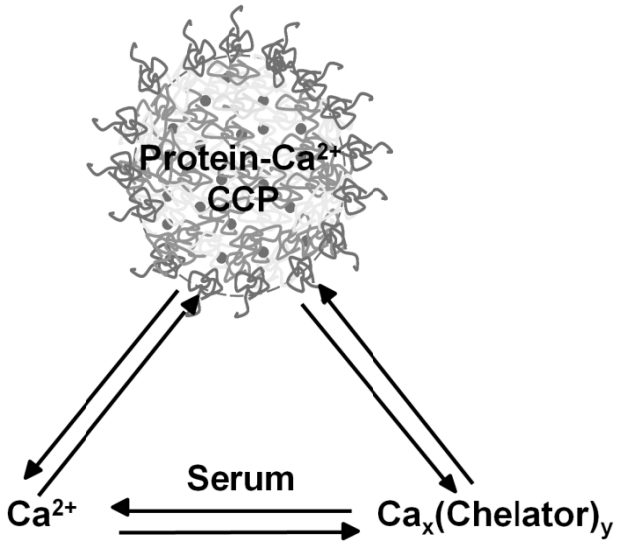

Figure 1.4 Equilibria between free calcium ions, calcium chelator complexes, and casein micelles in dairy solutions.

These shifts increase the repulsion between the negatively charged amino acids in the casein micelles, resulting in an increase in hydration and voluminosity of the micelles[32, 33] and a decrease in turbidity of milk solutions.[34-38] Casein micelles may eventually dissociate into smaller clusters upon addition of calcium chelators.[6, 13, 31, 36, 39-41] Hydrophobic interactions between the caseins also play an important role in the stability of the casein micelle structure.[1, 8, 15, 22, 42] For instance, urea will diminish hydrophobic interactions in the casein micelles, which also disrupts the micellar structure.[2, 43-45] The physical state of the casein micelle structure is strongly dependent on the physico-chemical conditions of the milk solution. Acidification,

cooling, or heating can strongly influence the viscosity, turbidity, and stability of the milk solution.[6, 15, 22, 31, 43, 46-48]

The impact of calcium chelators on the mineral equilibria and casein micelle structure might be different, as affinity for calcium ions (i.e. association and solubility constants) and interaction with the amino acids of caseins might be different.[4, 49-54] In this thesis the results of a study with sodium hexametaphosphate (SHMP), sodium phytate (SP), disodium hydrogen phosphate (Na_2HPO_4), disodium uridine monophosphate (Na_2UMP) and trisodium citrate (TSC) are described. SHMP and SP are strongly negatively charged and can bind, besides the calcium ions, to the positively charged amino acids of the casein residues in milk systems.[53-55] In various studies it is also reported that SHMP has the ability to cross-link caseins and cause gelling of milk solutions.[31, 54, 56] Addition of Na_2HPO_4 or TSC also affects the viscosity and turbidity of milk solutions.[37, 54, 56, 57] The calcium phosphate microcrystals formed upon addition of Na_2HPO_4 mainly precipitate in the casein micelles[58], whereas the calcium citrate complexes formed upon addition of TSC remain as stable, soluble complexes in the serum phase[53, 55, 56] or form insoluble calcium citrate crystals during storage. For Na_2UMP no information was found in literature about the interaction with casein.

The physico-chemical changes that occur in the casein micelle upon addition of calcium chelators may affect the heat stability of the milk solutions. Calcium chelators decrease the concentration of free calcium ions in the serum phase and this will reduce the calcium-induced protein aggregation, and, consequently, increase the heat stability of milk solutions.[25, 26, 28] Calcium chelators may also decrease the heat stability of milk solutions at a certain concentration, as they can chelate CCP from the casein micelle to a level at which the integrity of the micellar structure is lost.[25, 31]

1.6 Aim and outline of this thesis

Calcium chelators affect the physico-chemical properties of casein micelles and heat stability of milk solutions, but their specific interaction with the casein micelles may vary considerably. The aim of the research as described in this thesis was to obtain a better understanding of the influence of calcium chelators on the physico-chemical properties of casein micelles and the resulting effect on the viscosity and heat stability of concentrated micellar casein solutions.

Following the general introduction in chapter 1, Chapter 2 describes the differences in calcium-binding capacity of organic and inorganic ortho- and polyphosphates in a calcium chloride solution. This study was carried out to obtain a better understanding of the affinity of different calcium chelators for calcium ions, which gives useful information for understanding the interaction of calcium, phosphate, and casein micelles in dairy products. In Chapter 3 the influence of these phosphates and citrate on physical changes in the casein micelles in concentrated micellar casein solutions are described by measuring the calcium-ion activity, viscosity, turbidity, and sedimentation using ultracentrifugation. Citrate was also investigated in this study, because this compound is often used as heat stabilizer in medical products.[59] The study in Chapter 4 focuses on the effect of the calcium chelators on heat coagulation and heat-induced changes in concentrated micellar casein solutions. Chapter 5 describes to what extent casein micelles dissociate after addition of different types and concentrations of calcium chelators to a concentrated micellar casein solution to explain the differences in turbidity as discussed in Chapter 3. Dynamic light scattering was used to measure the particle size distributions in the casein solutions. In Chapter 6 the results are given of the binding of SHMP and SP to caseins by determining changes in the isoelectric point (IEP) in casein solutions through zeta potential measurements as a function of pH. SHMP and SP were added to sodium caseinate (calcium-poor) and micellar casein isolate (calcium-rich) solutions to elucidate if calcium ions were required for the binding to the caseins. Finally, Chapter 7 provides a general discussion on how the results of the previous chapters contribute to understanding the interaction of the different calcium chelators with the casein micelle in concentrated micellar casein solutions and recommendations for further research. Also, opportunities are described how knowledge obtained in this thesis may be applied in the dairy industry.

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Chapter 2

Calcium-binding capacity of organic and inorganic ortho- and polyphosphates

Based on: de Kort, E.J.P., M. Minor, T.H.M. Snoeren, A.C.M. van Hooijdonk, and E. van der Linden, *Dairy Science and Technology*, **2009**. *89*: p. 283–299.

Abstract

The aim of this research was to determine the calcium-binding capacity of inorganic and organic ortho- and polyphosphates. This calcium-binding capacity can be used to influence the stability of, for example, casein micelles in dairy systems. Four phosphates were selected: disodium uridine monophosphate (Na_2UMP, organic orthophosphate), disodium hydrogen phosphate (Na_2HPO_4, inorganic orthophosphate), sodium phytate (SP, organic polyphosphate), and sodium hexametaphosphate (SHMP, inorganic polyphosphate). Concentrations of up to 100 mmol L^{-1} phosphate were added to a 50 mmol L^{-1} $CaCl_2$ solution. The samples were prepared at pH 8.0 and were analyzed before and after sterilization for calcium-ion activity, conductivity, pH, sediment, and turbidity. Both SHMP and SP are strong chelators, as calcium ions bind to these phosphates in the ratio of 3:1 and 6:1, respectively. Calcium ions also strongly bind to Na_2HPO_4, but in a ratio of 3:2 with insoluble $Ca_3(PO_4)_2$ complexes as result. The equilibrium position of Na_2UMP is not strong towards the chelated complex, and significant levels of free calcium and free phosphate can exist. An equilibrium constant of 0.29 ± 0.08 L mol^{-1} was determined for calcium uridine monophosphate (CaUMP) complexes. Both calculation of the equilibrium constant and analysis on the CaUMP precipitate confirmed a reactivity of 1:1 between calcium and Na_2UMP. The CaUMP complexes are well soluble at ambient temperature, and insoluble complexes appear after sterilization, because the solubility of CaUMP decreases during heating. Finally, we concluded that the structure of phosphate molecules determines their calcium-binding capacity rather than organic or inorganic origin of phosphates.

2.1 Introduction

Calcium and phosphate are the most relevant minerals in dairy and medical nutrition products, and are part of the colloidal calcium phosphate (CCP) in casein micelles. CCP is important for the micelle structure and (heat) stability of dairy products[1]. Addition of calcium and phosphate causes interactions with casein micelles, CCP, and soluble calcium phosphate. As a result, insoluble or soluble calcium phosphate complexes will be formed.[2-7] Many calcium-binding agents are used in the dairy industry, for example, citrate, polyphosphates, or pyrophosphates, and they are named chelators.[3, 8] These chelators influence the activity of calcium in solution, the CCP concentration, and thus the (heat) stability of casein micelles in dairy systems.[3] Disodium hydrogen phosphate (Na_2HPO_4) and sodium hexametaphosphate (SHMP) are examples of chelating inorganic phosphates, which are commonly used in dairy products to influence and improve the quality of, for example, evaporated milk, processed cheese, or calcium-enriched milk.[9] Na_2HPO_4 and SHMP are ortho- and polyphosphates, respectively (Fig. 2.1).

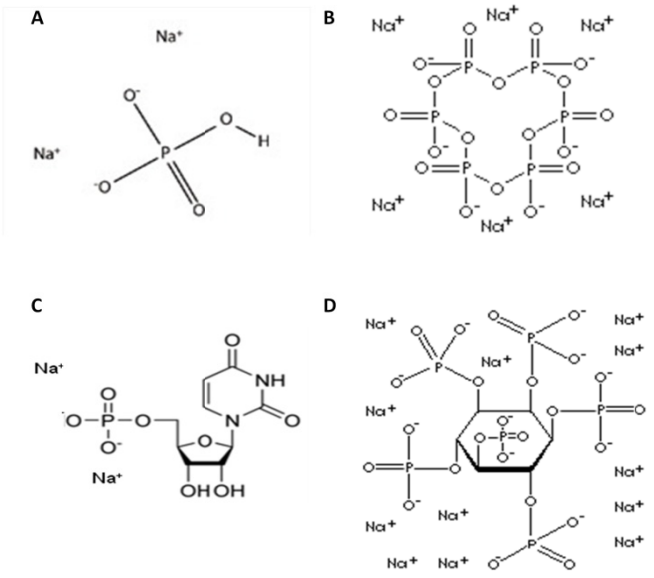

Figure 2.1 Phosphates used in this study: (A) Na_2HPO_4; (B) SHMP; (C) Na_2UMP; and (D) SP.

Na_2HPO_4 (Fig. 2.1a) influences casein micelles by interacting with casein or CCP.[3] Upreti *et al.*[10] and Pyne[11] summarized that CCP is composed of $CaHPO_4$, $Ca_3(PO_4)_2$, and even larger complexes including those containing crystal water. This indicates that calcium and Na_2HPO_4 react in a ratio of 1:1 or 3:2 around pH 6.7. Also, SHMP (Fig. 2.1b) influences the amount of CCP present in casein micelles by binding calcium ions.[4, 12-14] SHMP is a strong chelator and its binding to polyvalent cations is equal for calcium, magnesium, strontium, and barium.[15] Calcium hexametaphosphate can also bind with casein micelles. The extent of interaction of polyphosphates is, in general, dependent on the chain length of the polyphosphate.[15] Na_2HPO_4 binds less strongly with calcium than SHMP, even when extremely high Na_2HPO_4 concentrations are used.[4]

Nucleotides, such as disodium uridine monophosphate (Na_2UMP) (Fig. 2.1c), are organic orthophosphates, which naturally occur in human milk at low concentrations and are added to baby food.[16] To our knowledge, no information is available about the interaction of Na_2UMP with casein micelles. However, the interaction of multivalent cations with nucleotides has been studied in more detail.[17-22] These studies concluded that interaction with pyrimidine nucleotides (Na_2CMP and Na_2UMP) is solely determined by the alkalinity of the corresponding phosphate groups. This indicates that these pyrimidine molecules have a simple monophosphate group and that reactivity of Na_2UMP to cations should be similar to Na_2HPO_4.

Sodium phytate (SP) is an organic polyphosphate (Fig. 2.1d) and a strong chelator. It is naturally present in nuts, seeds, and grains, but is also added to foods as stabilizer, antioxidant, or preservative.[23, 24] It has 12 negative charges and binds all multivalent cations.[23] Below the pH of ~ 5.0, no significant binding of calcium with phytate occurs, but between pH 5.0 and 8.0 maximal calcium binding in the ratio of 6:1 occurs.[25] The solubility of these complexes show pH-dependent variations and are further determined by the type of multivalent cations and ionic strength.[23, 24] Mono- and dicalcium phytate are soluble complexes, but addition of a third calcium ion causes precipitation. If calcium is present in excess, insoluble pentacalcium phytate dominates the precipitate.[26] SP binds calcium ions irreversible and also interacts with proteins by binding to free lysine residues.[27] In this way it can affect the stability of dairy systems by binding with calcium and/or protein.

Both SHMP and SP are thus very strong chelators and are expected to have a strong influence on (heat) stability of dairy products. In contrast, Na_2HPO_4 and Na_2UMP bind less calcium ions at comparable concentrations and, consequently, are expected to have less influence on the (heat)

stability of dairy systems. Interactions between calcium and these phosphates must be known to understand the interactions between calcium, phosphate, and casein during the processing of dairy products. The aim of this research was to determine the calcium-binding capacity of organic and inorganic ortho- and polyphosphates before and after sterilization. Sterilization is included in this research, as long shelf life dairy products, and especially medical nutrition, will undergo this heat treatment.

2.2 Materials and Methods

Concentrations of up to 100 mmol L^{-1} phosphate were added to 50 mmol L^{-1} $CaCl_2$ solution. Samples were prepared at pH 8.0; maximal calcium-phosphate interactions were expected at this pH, resulting in soluble and insoluble calcium phosphate complexes. Samples were analyzed before and after sterilization for calcium-ion activity, conductivity, pH, sediment, and turbidity.

2.2.1 Sample preparation

Stock solutions of Na_2UMP (Yamasa Corporation, Chiba, Japan), Na_2HPO_4 (Merck & Co. Inc., Darmstadt, Germany), SHMP (VWR International Ltd., Poole, England), phytic acid dodecasodium salthydrate (Sigma-Aldrich GMBH, Steinheim, Germany), and calcium chloride (Kirsch Pharma GMHB, Salzgitter, Germany) were prepared using demineralized water. All stock solutions were adjusted to pH 8.0 with 1 mol L^{-1} sodium hydroxide (Sigma-Aldrich GMBH, Steinheim, Germany) or 1 mol L^{-1} hydrochloric acid (Merck & Co. Inc., Darmstadt, Germany). Subsequently, samples were prepared with 50 mmol L^{-1} $CaCl_2$, to which concentration ranges of 0–100 mmol L^{-1} Na_2UMP, 0–100 mmol L^{-1} Na_2HPO_4, 0–30 mmol L^{-1} SHMP, or 0–25 mmol L^{-1} SP were added. The pH of prepared samples, which represent 90% of the total volume at this moment, was measured and, if necessary, adjusted to pH 8.0 ± 0.1. After 1 h, the samples were measured a second time and brought to pH 8.0 ± 0.1. Subsequently, demineralised water was added to obtain the required concentrations, and the final pH was measured. No pH adjustments were made anymore in case the final pH deviated from 8.0. Also, a concentration range of 0–160 mmol L^{-1} was made for Na_2UMP in 20 mmol L^{-1} $CaCl_2$ solution for further study of the calcium-binding capacity of Na_2UMP. All samples were prepared and analyzed at least in duplicate, and the samples were analyzed with and without a sterilization

step. During the retort sterilization process, the samples were heated from 20 to 96°C for 271 s, followed by heating at 121°C for 960 s, and finally cooling to 40°C for 420 s (Stock, Grafton, USA). Samples were sterilized in 200 mL glass bottles with metal caps and were rotated during the process.

2.2.2 pH

Titrations and pH measurements were done with a 718 Stat Titrino (Metrohm, Herisau, Switzerland). The instrument was calibrated with stock solutions at pH 4.0–7.0. Calibration and measurements were done at ambient temperature. Samples were adjusted to pH 8.0 ± 0.1. No buffering agents were added; as a consequence, when a pH change was observed in the final samples, no pH corrections were made.

2.2.3 Conductivity

The ion conductivity was measured with an ion conductivity meter (CLM 381, serial number 50081031, Endress and Hauser, Weil am Rhein, Germany). Measurements were performed at ambient temperature. The cell constant of the meter was 0.475 cm^{-1}.

2.2.4 Calcium-ion activity

The calcium-ion activity was measured with a Mettler Toledo Seven Multi™ (with an Inlab® expert Pro pH-meter) calcium measuring device (Mettler Toledo, Greifensee, Switzerland) using an Orion 9300BH electrode and an Orion 900100 reference electrode. Calibration was performed at ambient temperature with standard solutions containing 20, 200, and 2000 mg kg^{-1} calcium (as $CaCl_2$) and 80 mmol L^{-1} KCl. Addition of this monovalent background electrolyte is beneficial, as it keeps the calcium-ion activity coefficient effectively constant in the calibration solutions. A calcium-ion activity coefficient ($\gamma_{Ca^{2+}}$) of 0.29 was calculated for the calibration solutions using the formula of Davies.[28, 29] The activity of calcium ions in each sample was determined by multiplying the experimental calcium-ion activities with the activity coefficient of 0.29. Electrodes remained in the 200 mg kg^{-1} stock solution for 30 min before calibration was started. Every solution was measured during 5 min until equilibrium was reached. The results are expressed in calcium-ion activity (mmol L^{-1}).

2.2.5 Turbidity

Turbidity was measured with a spectrophotometer (4053 Kinetics, LKB Biochrom, Midland, Canada). Plastic cuvettes of a length of 1 cm were used. Measurements were done at 700 nm and at ambient temperature.

2.2.6 Sediment

The amount of sediment was measured by filtering sample solutions through folded filters of Ø 185 mm type 595½ (Whatman, Schleider & Schuell, Dassel, Germany). The filters were dried at 37°C for 48 h and weighed at ambient temperature to determine the amount of sediment in each filter. The amount of sediment is expressed as gram per 100 g solution.

2.2.7 UMP and uridine determination

UMP and uridine analyses were done with a reversed phase HPLC, using an Alltima C18 5 µ particles column 250 × 4.6 mm with precolumn 7.5 × 4.6 mm packed with the same material. Samples were prepared by addition of 800 µL 0.1 mol L^{-1} perchloric acid to 200 µL liquid sample. Nucleotides were extracted by vortexing the solution, followed by centrifugation, and 500 µL supernatant was neutralized with 20 µL 2.3 mol L^{-1} potassiumhydrogen carbonate. HPLC elution was done with solvent A, consisting of 0.15 mol L^{-1} $NH_4H_2PO_4$ solution containing 1% (v/v) methanol at pH 6.1 ± 0.05, and solvent B, consisting of 0.15 mol L^{-1} $NH_4H_2PO_4$ solution containing 40% (v/v) methanol at pH 6.1 ± 0.05. Gradient elution was done by 0–360 s 100% A, 360–540 s 2.5% B, 540–1140 s 20% B, 1140–1260 s linear gradient to 80% B, 1260–1500 s 80% B, 1500–1560 s linear gradient back to 100% A and 1560–2100 s re-equilibrate with 100% A. Flow rate was 0.013 mL s^{-1}. Quantification was done at UV absorbance of 210 and 254 nm and comparison was done with UMP and uridine standards.

2.2.8 Calcium content determination

The amount of calcium present in calcium uridine monophosphate (CaUMP) sediment was measured with an Inductively Coupled Plasma – Atomic Emission Spectrometer (iCAP 9300 series, Thermo Electron). Five to ten grams of samples were weighed into a 100 mL flask, to which 10 mL of 25% acetic acid was added. Samples were placed in a 100°C water bath for 90

min, followed by cooling and adjusting the volume to 100 mL using demineralised water. Finally, the sample was pumped into the ICP-AES and measured on its emission rate. Calibration was done with standard calcium chloride solutions of 1 and 10 g L^{-1}.

2.3. Results and Discussion

A solution of UMP molecules can hydrolyze into uridine and phosphate. This hydrolysis is temperature and pH dependent. The amount of hydrolysis was investigated between pH 2 and 8 in 50 mmol L^{-1} $CaCl_2$ with 50 mmol L^{-1} Na_2UMP solution (Fig. 2.2). These calcium and phosphate concentrations were selected, because the casein micelles in concentrated dairy products have comparable calcium concentrations.

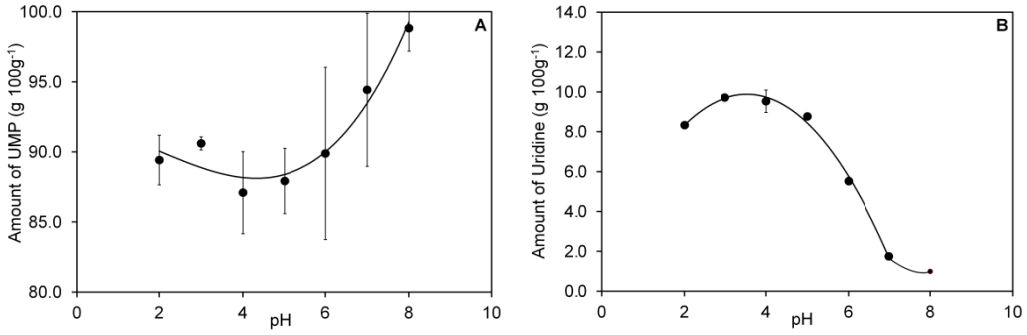

Figure 2.2 The pH-dependent hydrolysis of 50 mmol L^{-1} Na_2UMP into uridine and free phosphate. (A) Amount of UMP after sterilization. (B) Amount of uridine after sterilization.

Fig. 2.2a and 2.2b show that in acid conditions ~ 10% of Na_2UMP is hydrolyzed into uridine and phosphate. The pH-dependent hydrolysis of Na_2UMP is, however, negligible around pH 8.0 (~ 99% UMP and 1% uridine). Consequently, no free phosphate groups, able to interact with calcium ions, can be formed. Calcium-phosphate interaction is, in this way, solely determined by UMP molecules.

The extent of calcium-phosphate interaction is pH dependent: none of calcium and phosphate ions are in complexes below pH 5.2.[1] In Fig. 2.3 results are shown for titration of sterilized 50

mmol L^{-1} $CaCl_2$ with 50 mmol L^{-1} Na_2UMP solution at ambient temperature from pH 8.0 to 5.0, while measuring the calcium-ion activity and turbidity.

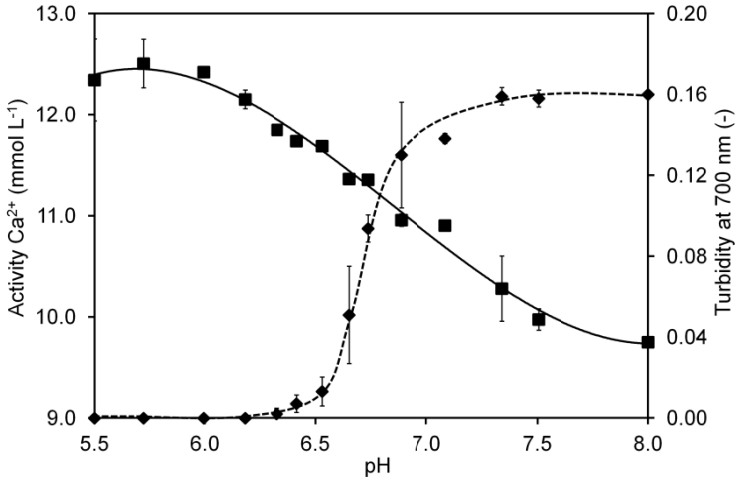

Figure 2.3 Calcium-ion activity and turbidity given as function of pH of heated 50 mmol L^{-1} $CaCl_2$ with 50 mmol L^{-1} Na_2UMP solution titrated from pH 8.0 to 5.0 at ambient temperature with 1 N HCl. (■) Calcium-ion activity and (- -♦- -) turbidity.

This titration indicated that maximum interaction between calcium and UMP is obtained at pH 8.0. Around pH 6.0 maximal calcium-ion activity and minimal turbidity were measured, indicating that calcium and UMP were not bound. For this reason, all samples were prepared at pH 8.0, assuming that other phosphates will give maximal binding with calcium at pH 8.0 as well.

Conductivity is often measured to analyze ion interactions. In our study, conductivities were determined as measure for the calcium-binding capacity. Theoretical conductivities were calculated using reported conductivities approaching infinite dilution, also named limiting conductivity.[30, 31] Calculations were done for sodium, phosphate, calcium, chloride, hydrogen, and hydroxide ions by taking into account calcium phosphate binding and pH. Conductivities were calculated at specific concentrations by using the expected reactivity of 3:2 with Na_2HPO_4, 3:1 with SHMP, and 6:1 with SP; Na_2UMP did not react with all available calcium ions and for

that reason conductivities were calculated using measured calcium-ion activities. No limiting conductivities were available for Na_2UMP, SHMP, and SP. Conductivities at phosphate concentration ranges of 0.5–40 mmol L^{-1} were therefore measured and plotted in conductivity-concentration curves. Extrapolation to infinite dilution resulted in limiting conductivities of 0.108 mS L $mmol^{-1}$ cm^{-1} for Na_2UMP, 0.282 mS L $mmol^{-1}$ cm^{-1} for SHMP, 0.660 mS L $mmol^{-1}$ cm^{-1} for SP, and 0.164 mS L $mmol^{-1}$ cm^{-1} for Na_2HPO_4. The latter value approximates the calculated conductivity of 0.166 mS L $mmol^{-1}$ cm^{-1} obtained from the limiting conductivity of reported individual ionic species.[30] Experimental and calculated conductivities are depicted in Fig. 2.4.

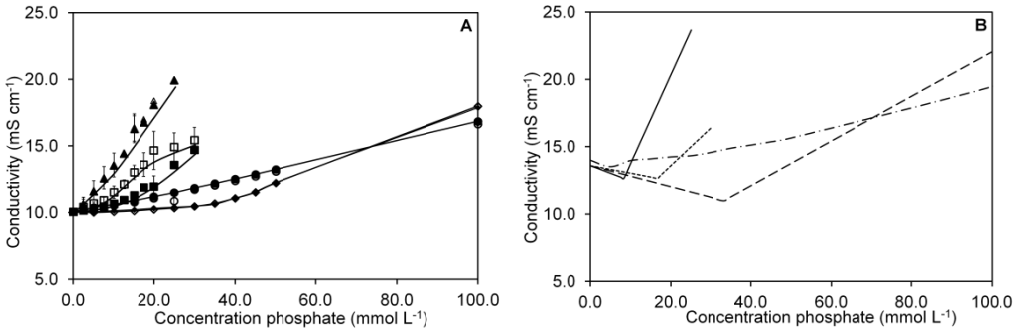

Figure 2.4 Experimental (A) and calculated (B) conductivity of phosphates in 50 mmol L^{-1} $CaCl_2$ solution. (●) Na_2UMP before heating; (○) Na_2UMP after heating; (♦) Na_2HPO_4 before heating; (◊) Na_2HPO_4 after heating; (■) SHMP before heating; (□) SHMP after heating; (▲) SP before heating; (Δ) SP after heating; (–·–) Na_2UMP; (— —) Na_2HPO_4; (- - - -) SHMP; (——) SP.

Conductivities were stable in Na_2UMP, Na_2HPO_4, and SP samples before and after heating, whereas increased conductivities were measured in SHMP samples after heating. This indicated calcium release from HMP during heating and negligible increase of calcium phosphate binding during sterilization. Slightly higher conductivities were calculated than measured, because conductivities calculated from limiting conductivities overestimate experimental conductivities.[30] The trends were comparable in the experimental and calculated conductivity curves. The minimum at 33.3 mmol L^{-1} in Na_2HPO_4 samples was also measured. The bends at 8.3 mmol L^{-1} in SHMP and 16.7 mmol L^{-1} in SP were not measured clearly. The bends in the curves illustrate the concentrations at which all available calcium had reacted with the specific

phosphate. Overall, the conductivity method was not sensitive and specific enough to determine the calcium-binding capacity of phosphates, as all species contribute to the conductivity. The calcium-ion activity method, however, specifically measures activity of calcium ions and can give useful information about the binding of calcium with these phosphates.

Experimental calcium-ion activities were compared with calculated curves of the phosphates (Fig. 2.5). We calculated the calcium-ion activities ($a_{Ca^{2+}}$) with the formula of Davies[28], that is based on the equation of Debye-Hückel, but is extended with a term that is proportional to the ionic strength of the solution and accounts for solvability and short-range interactions of ions. The Debye-Hückel equation is valid for ionic strengths up to 10 mmol L^{-1}, whereas the Davies equation can be applied for ionic strength of up to 500 mmol L^{-1}.[28, 29, 31] Fig. 2.5 shows that the calculated calcium-ion activity of 17 mmol L^{-1} (Fig. 2.5b) corresponds to the experimentally determined activity of $a_{Ca^{2+}} = 18 \pm 1$ mmol L^{-1} (Fig. 2.5a) for 50 mmol L^{-1} $CaCl_2$ solution with 0 mmol L^{-1} phosphate.

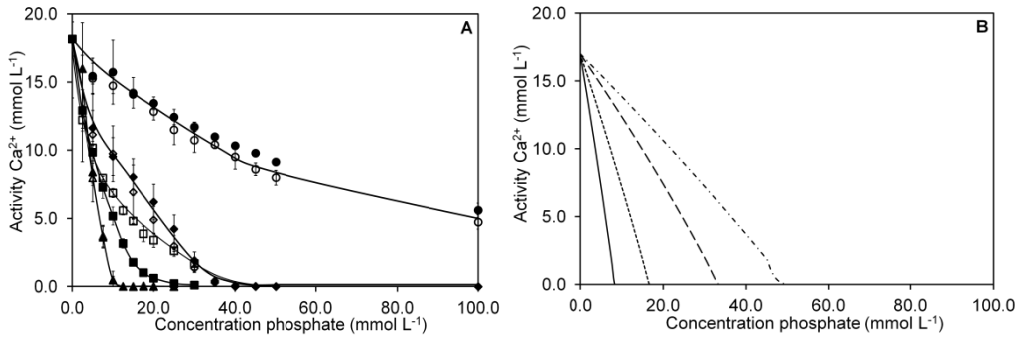

Figure 2.5 Experimental (A) and calculated (B) calcium-ion activity of phosphates in 50 mmol L^{-1} $CaCl_2$ solution. (●) Na_2UMP before heating; (○) Na_2UMP after heating; (♦) Na_2HPO_4 before heating; (◊) Na_2HPO_4 after heating; (■) SHMP before heating; (□) SHMP after heating; (▲) SP before heating; (Δ) SP after heating; (–·–) Na_2UMP; (— —) Na_2HPO_4; (- - - -) SHMP; (——) SP, resulting in CaUMP, $Ca_3(PO_4)_2$, $Ca_3(PO_3)_6$, and Ca_6phytate, respectively.

The calculated calcium-ion activities of Na_2HPO_4, SHMP, and SP were consistent with the experimental calcium-ion activities, showing calcium-binding capacity of 3:2 for Na_2HPO_4, 3:1 for SHMP, and 6:1 for SP before and after sterilization. Using the Davies equation we calculated

calcium-ion activities of zero for 33.3 mmol L^{-1} Na_2HPO_4, 16.7 mmol L^{-1} SHMP, and 8.3 mmol L^{-1} SP. The experimental calcium-ion activities approached zero values around these concentrations as well. Na_2HPO_4 can react with calcium in a ratio of 1:1 or 3:2 to form $CaHPO_4$ or $Ca_3(PO_4)_2$ complexes.[10, 11] Fig. 2.5a and 2.5b confirm that $Ca_3(PO_4)_2$ complexes are dominant in 50 mmol L^{-1} $CaCl_2$ solution at pH 8.0. The calculated Na_2UMP curve depicts binding of 1:1 between calcium and UMP. However, compared to Fig. 2.5a and 2.5b, Na_2UMP does not react with all available calcium ions: Na_2UMP has a lower equilibrium binding constant to calcium than the other phosphates. As a result, the calculated and experimental calcium-ion activity curves of Na_2UMP were different. Moreover, its calcium-binding capacity is not similar to the binding of calcium to Na_2HPO_4 for two reasons. First of all, the third proton in Na_2UMP is not easily released from the uracil ring, which is due to the mesomeric ring and pH. The proton will only be released above pH 10 (pK_{a3} is 9.5) and calcium will not be bound to uracil.[21, 22] Consequently, around neutral pH, a calcium-binding capacity of 1:1 is expected rather than 3:2 for UMP. Secondly, although the phosphate residue of the nucleotide largely determines the stability of cation-UMP complexes, the nucleobase is responsible for the selectivity or specificity of these complexes by hydrogen binding and cation coordination.[21, 22] As a consequence, UMP has a lower affinity for calcium ions than the other phosphates, and free calcium and free phosphate can exist simultaneously in solution. The equilibrium constant and the calcium-binding capacity of Na_2UMP are calculated in the last section of this study.

In SHMP samples, an increased calcium-ion activity was observed after sterilization, as a result of sterilization and pH decline. The pH decline was larger during sterilization, because of calcium binding and proton release from HMP at the same time. Final pH values in the samples before and after sterilization are depicted in Fig. 2.6. Furthermore, calcium binding could be reduced because of SHMP hydrolysis into sodium trimetaphosphate and sodium orthophosphate under acidic conditions.[32] For example, Gaucheron[3] and Walstra[1] stated that below pH 5.2, calcium phosphate bindings are less stable and below pH 3.5, no calcium phosphate binding was present anymore in aqueous solution. As SHMP samples remained above pH 5.2 before heating, all calcium ions should be bound to SHMP. However, after heating, the pH decreased to pH 4.2 and a part of the calcium ions, as shown in Fig. 2.5a, were released from SHMP.

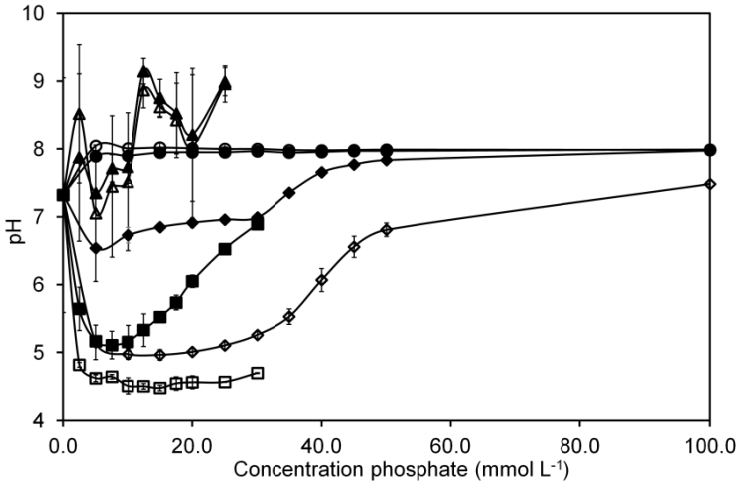

Figure 2.6 The pH of phosphates in 50 mmol L^{-1} $CaCl_2$ solution. (●) Na_2UMP before heating; (○) Na_2UMP after heating; (♦) Na_2HPO_4 before heating; (◊) Na_2HPO_4 after heating; (■) SHMP before heating; (□) SHMP after heating; (▲) SP before heating; (Δ) SP after heating.

Vujicic *et al.*[15] reported that addition of alkaline earth ions to polyphosphates like tetra- or hexametaphosphate causes release of sodium and protons bound to these polyphosphates as evidenced by the pH drop. The total binding between cations and phosphates depends on the amount of cations added and the amount of available binding sites on phosphates.[15] Consequently, the largest pH drop was expected with SHMP and SP followed by Na_2HPO_4. A pH drop was observed in SHMP samples, but not in SP samples. A larger pH drop was observed in Na_2HPO_4 samples in comparison with SP samples. Large fluctuations in SP samples were caused by a weak buffering capacity of SP around pH 8.0 (pK_{a7}, pK_{a8}, and pK_{a9} are 5.7, 6.9, and 7.6, respectively).[24] Calcium-ion activity results of the Na_2HPO_4 trial showed that $Ca_3(PO_4)_2$ was formed. Around pH 8.0, one or two protons had to be released to form $Ca_3(PO_4)_2$ (pK_{a2} is 7.2) and this resulted in a pH decrease. In case of Na_2UMP, a third proton could not be released from uracil and the pH remained at 8.0 in all Na_2UMP samples.

The amount of sediment and turbidity was measured before and after heating of all phosphates to confirm their calcium-binding capacity. Results are shown in Fig. 2.7 and 2.8.

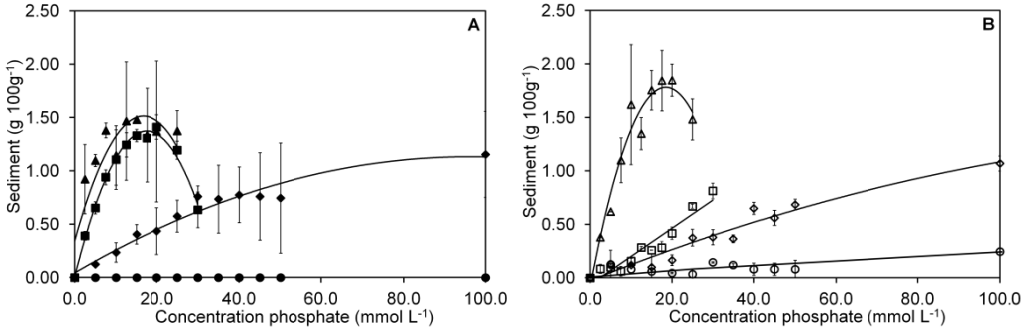

Figure 2.7 Experimental sediment of phosphates in 50 mmol L^{-1} $CaCl_2$ solution before (A) and after heating (B). (●) Na_2UMP before heating; (♦) Na_2HPO_4 before heating; (■) SHMP before heating; (▲) SP before heating; (○) Na_2UMP after heating; (◊) Na_2HPO_4 after heating; (□) SHMP after heating; (Δ) SP after heating.

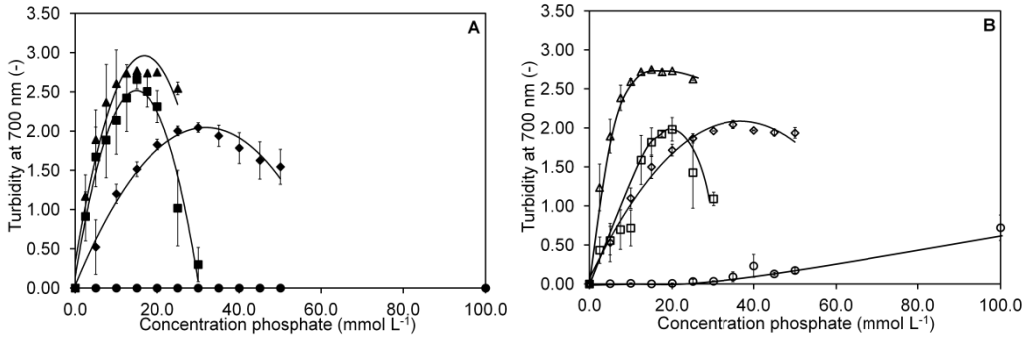

Figure 2.8 Turbidity at 700 nm of phosphates in 50 mmol L^{-1} $CaCl_2$ solution before (A) and after heating (B). (●) Na_2UMP before heating; (♦) Na_2HPO_4 before heating; (■) SHMP before heating; (▲) SP before heating; (○) Na_2UMP after heating; (◊) Na_2HPO_4 after heating; (□) SHMP after heating; (Δ) SP after heating.

Fig. 2.7 and 2.8 show that the same trends were obtained with measuring the amount of sediment and turbidity. Before heating no precipitation appeared in Na_2UMP samples, whereas after sterilization an increase in sediment and turbidity was measured. As similar calcium-ion activities were measured before and after sterilization, we concluded that CaUMP complexes are soluble, but precipitate during sterilization. Theoretical sediment amounts were calculated for $Ca_3(PO_4)_2$ from Na_2HPO_4, Ca_3HMP from SHMP, and Ca_6phytate from SP. For Na_2UMP, the

values could only be calculated by using equilibrium constant K_{CaUMP}, because Na_2UMP did not react with all available calcium ions. The calculated sediment was similar to the experimental sediment before heating. In the heated samples, however, less sediment was present due to the pH drop during sterilization, resulting in the release of calcium phosphate bindings. Although large standard deviations were found with sediment analyses, the results confirmed the reactivity with calcium ions of 3:2 for Na_2HPO_4, 3:1 for SHMP, and 6:1 for SP. The large standard deviations can be explained by low accuracy of sediment determination and by potential inclusion of crystal water during drying.

Above 16.7 mmol L^{-1} SHMP and 8.3 mmol L^{-1} SP, a decrease in both sedimentation and turbidity was observed. An excess of phosphates was present above these concentrations and an equilibrium exchange between already bound calcium and overdosed phosphates occurred which, consequently, decreased sediment and turbidity. Na_2HPO_4 is a weaker chelator than SHMP and SP and has a weaker effect on calcium release than SHMP and SP.

Na_2HPO_4, SHMP, and SP react, depending on their concentration and pH, with all available calcium ions and therefore have high equilibrium binding constants. Our results showed that Na_2UMP has lower affinity for calcium ions than Na_2HPO_4, SHMP, and SP, and as a consequence the calcium-binding capacity of UMP could not be determined directly. Free calcium, UMP, and CaUMP are in equilibrium in aqueous solution. The equilibrium constant K_{CaUMP} was calculated with results obtained from experimental calcium-ion activities. Concentration ranges of 0–160 and 0–400 mmol L^{-1} Na_2UMP in solutions of 20 and 50 mmol L^{-1} $CaCl_2$, respectively, were analyzed for their calcium-ion activity before and after heating. Results were plotted in a linear relation to calculate the calcium-binding capacity and equilibrium constant of CaUMP (Fig. 2.9). The deduction of this linear relation is described in the Appendix.

An equilibrium constant (K_{CaUMP}) was determined to be 0.26 ± 0.06 L mol^{-1} before heating and 0.32 ± 0.09 L mol^{-1} after heating. This resulted in an average equilibrium constant K_{CaUMP} of 0.29 ± 0.08 L mol^{-1}. Furthermore, linear fits were obtained with n = 1 and m = 1 indicating a calcium-binding ratio of 1:1 for UMP. No linear fits were obtained with other binding ratios between calcium and UMP. This best fitted binding ratio of 1:1 was in the line of expectation, because UMP has pK_a values of pK_{a1} 0.7–1.0, pK_{a2} 5.6–6.2, and pK_{a3} 9.5–10.0[18-20, 33, 34] and thus

is mainly in the divalent anionic form at pH 8.0. Moreover, analysis of the CaUMP sediment confirmed that equal amounts of calcium and UMP were present.

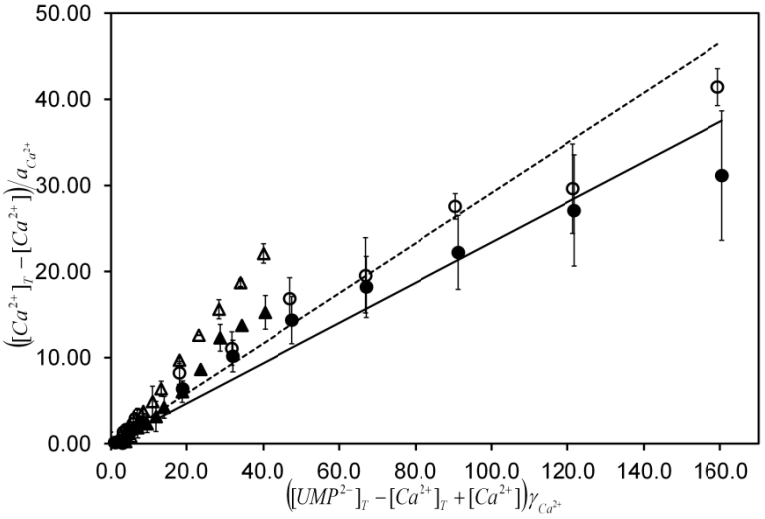

Figure 2.9 Model fits with n = 1 and m = 1 to determine K_{CaUMP} before (—) and after (---) heating. (Δ) 20 mmol L^{-1} $CaCl_2$ with 0–160 mmol L^{-1} Na_2UMP before heating; (▲) 20 mmol L^{-1} $CaCl_2$ with 0–160 mmol L^{-1} Na_2UMP after heating; (●) 50 mmol L^{-1} $CaCl_2$ with 0–400 mmol L^{-1} Na_2UMP before heating; (○) 50 mmol L^{-1} $CaCl_2$ with 0–400 mmol L^{-1} Na_2UMP after heating.

This study showed that Na_2HPO_4, SHMP, and SP have a strong calcium-binding capacity. They are useful additives in the dairy industry to reduce calcium aggregation during processing or shelf life of, for example, calcium-enriched milk, evaporated milk, or medical nutrition. Na_2UMP is a nutritional additive, which has less influence on the (heat) stability of dairy products.

2.4 Conclusions

The calcium-ion activity results showed that calcium reacts with Na_2HPO_4 in a ratio of 3:2 to $Ca_3(PO_4)_2$, with SHMP in a ratio of 3:1 to $Ca_3(PO_3)_6$, and with SP in a ratio of 6:1 to Ca_6phytate in a 50 mmol L^{-1} $CaCl_2$ solution at pH 8.0. Measuring calcium-ion activity was more sensitive

and specific for determining reaction ratios than measuring conductivity. Sediment and turbidity analyses elucidated that these three phosphates formed insoluble complexes with calcium. Na_2UMP reacted to a lesser extent with calcium ions than the other phosphates: CaUMP complexes were in equilibrium with free calcium and UMP at pH 8.0. These CaUMP complexes were soluble before sterilization and insoluble after sterilization. The hydrolysis of Na_2UMP into uridine and phosphate was negligible, whereas hydrolysis increased with decreasing pH. An average K_{CaUMP} of 0.29 ± 0.08 L mol^{-1} was determined with a calcium-binding capacity of 1:1 for UMP. Analysis of the CaUMP sediment confirmed a calcium-binding capacity of 1:1. The structure of phosphate molecules determined their calcium-binding capacity rather than organic or inorganic origin of phosphates. Polyphosphates were stronger chelators than orthophosphates. Overall, this study has elucidated the calcium-binding capacity of these phosphates, which is useful information to understand the interaction of calcium, phosphate, and casein micelles for the development of, for example, medical nutrition.

2.5 Appendix

Free calcium, UMP and CaUMP are in equilibrium in aqueous solution according to:

$$n\ Ca^{2+} + m\ UMP^{2-} \underset{\leftarrow}{\rightarrow} Ca_nUMP_m \tag{2.1}$$

The equilibrium constant K_{CaUMP} can be calculated indirectly from experimental calcium-ion activities and conservations of calcium and UMP, and is related to the activities of the different species according to:

$$K = \frac{a_{Ca_nUMP_m}}{\left(a_{Ca^{2+}}\right)^n \left(a_{UMP^{2-}}\right)^m}, \tag{2.2}$$

$$[Ca^{2+}]_T = [Ca^{2+}] + \frac{1}{n}[Ca_nUMP_m], \tag{2.3}$$

$$[UMP^{2-}]_T = [UMP^{2-}] + \frac{1}{m}[Ca_nUMP_m], \tag{2.4}$$

where $[Ca^{2+}]_T$ is the total calcium concentration (mmol L^{-1}), $[Ca^{2+}]$ is the experimental calcium ion concentration (mmol L^{-1}), $a_{Ca^{2+}}$ is the calcium-ion activity (mmol L^{-1}), $[UMP^{2-}]_T$ is the

added UMP concentration (mmol L^{-1}), and *n* and *m* are the binding ratio for calcium and UMP, respectively.

Combination of equations (2.2)-(2.4), and realizing that concentrations can be converted into activities by multiplying with the activity coefficient γ, results in:

$$K = \frac{\left(\left([Ca^{2+}]_T - [Ca^{2+}]\right)\gamma_{Ca_nUMP_m}\right) / \left(a_{Ca^{2+}}\right)^n}{\left(\left([UMP^{2-}]_T - \frac{n}{m}\left([Ca^{2+}]_T - [Ca^{2+}]\right)\right)\gamma_{Ca^{2+}}\right)^m} \tag{2.5}$$

If the numerator is plotted against the denominator, a straight line is obtained with slope K.

If n equals m, the activity coefficient γ_{CaUMP} of the complex is equal to 1 and the equation simplifies to:

$$K = \frac{\left([Ca^{2+}]_T - [Ca^{2+}]\right) / a_{Ca^{2+}}}{\left([UMP^{2-}]_T - [Ca^{2+}]_T + [Ca^{2+}]\right)\gamma_{Ca^{2+}}} \tag{2.6}$$

2.6 Acknowledgements

The authors would like to thank Gerrit Witte from Danone Research, Centre for Specialised Nutrition, in Wageningen for performing the HPLC analyses. The study was funded by Nutricia Advanced Medical Nutrition, Danone Research, Centre for Specialised Nutrition.

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Chapter 3

Effect of calcium chelators on physical changes in casein micelles in concentrated micellar casein solutions

Based on: de Kort, E.J.P., M. Minor, T.H.M. Snoeren, A.C.M. van Hooijdonk, and E. van der Linden, *International Dairy Journal*, **2011**. *21*: p. 907-913.

Abstract

The effect of calcium chelators on physical changes of casein micelles in concentrated micellar casein solutions was investigated by measuring calcium-ion activity, viscosity and turbidity, and performing ultracentrifugation. The highest viscosities were measured on addition of sodium hexametaphosphate (SHMP), because it cross-linked the caseins. For the weak calcium chelator disodium uridine monophosphate (Na_2UMP), physical changes in the solutions were negligible. Disodium hydrogen phosphate (Na_2HPO_4), trisodium citrate (TSC), and sodium phytate (SP) caused similar increases in viscosity, but had different effects on turbidity. The increase in viscosity was attributed to swelling of the casein micelles (i.e., increased voluminosity) at decreasing calcium-ion activity. The major decrease in turbidity was due to dissociation of the casein micelles. The extent of micellar dissociation was dependent on the type and concentration of calcium chelator. It seems that the micelles were dissociated in the order of SHMP ≥ SP > TSC > Na_2HPO_4 > Na_2UMP.

3.1 Introduction

Foods for special medical purposes are concentrated in nutrients, in particular proteins and minerals, to meet the daily intake of nutrients in malnourished patients. High levels of protein raise the overall product viscosity, while patients appreciate low-viscosity products. To formulate products that fulfil these two opposing aspects, it is important to gain knowledge about the development of viscosity and related changes in the microstructure of concentrated systems.

Dairy proteins, in particular casein, are commonly applied in medical nutrition. Casein micelles in these concentrated systems (typically 6-10%, w/v, protein) may aggregate during ultra-high temperature sterilization, leading to undesirable instability. Interactions in and between casein micelles are not only strongly influenced by the concentration, size and internal structure of the micelles, but also by the environment. Parameters that determine the interactions include ionic strength, mineral composition, pH, and temperature.[1]

Calcium chelators (e.g., citrate, phosphate, EDTA) are often used to improve the heat stability of milk products [2], since they induce various physical changes in the casein micelle. Chelators shift protein-mineral equilibria leading to a decrease in the concentration of free calcium ions and depletion of colloidal calcium phosphate (CCP), and release of specific caseins from the micelle. These shifts are reported to increase the repulsion between the negatively charged amino acids in the casein micelles, resulting in an increase in hydration and voluminosity of the micelles[3, 4] and a decrease in turbidity of milk solutions.[5, 6] The micelles eventually dissociate into small clusters and dispersed proteins at higher chelator levels. The studies of Panouillé *et al.*[7] and Pitkowski *et al.*[8] have shown that intact and dissociated casein micelles can be present simultaneously in the milk solution after addition of chelators.

The effect of EDTA on the physical changes of the casein micelle in skim milk has been extensively studied.[9-15] Information is also available on the effects of phosphates and citrate on the voluminosity of the casein micelle and turbidity of the milk solution.[16-20]

The extent to which chelators affect the micellar structure depends on their calcium-binding capacity[21] and their interaction with the calcium ions and amino acids in the casein micelle.[22] Besides the generic property of calcium-binding, calcium chelators may generate specific effects. Disodium hydrogen phosphate (Na_2HPO_4) and trisodium citrate (TSC) both chelate

calcium ions from the casein micelle, but calcium phosphate precipitates in the casein micelle[23], whereas calcium citrate remains as stable, soluble complexes in the serum phase.[18, 20, 22] Strongly anionic polyphosphates, such as sodium hexametaphosphate (SHMP) and sodium phytate (SP), bind, as well as the calcium ions, to positively charged amino acids of the casein residues in milk systems.[24] This gives SHMP the ability to cross-link casein micelles and cause gelation of milk solutions.[18, 22, 25]

The effect of phosphates and citrate on physical changes of milk solutions has mainly been studied in skim milk systems, where about 20% of the protein is whey protein, with low concentration factors (maximally ~6.5%, w/v, protein), and relatively low chelator levels. Several of these studies focused on the formation of milk gels[22] or on age gelation after addition of chelators.[16, 25, 26]

The aim of this study was to investigate the effect of various phosphates and citrate on the physical changes of casein micelles in concentrated micellar casein solutions. Commercial micellar casein isolate (MCI) was selected, as it contains a negligible amount of whey protein. A 9% (w/v) protein solution was prepared from this MCI, to which phosphates and citrate were added at different concentrations. Physical changes in micellar structure were investigated through calcium-ion activity, viscosity, ultracentrifugation and turbidity measurements. The voluminosity of the casein micelle was derived from viscosity and ultracentrifugation measurements. The voluminosity of casein micelle in concentrated dairy systems has been calculated from viscosity via several equations.[27-30] The equation of Eilers was originally developed for hydrodynamically interacting particles[30, 31] and has been used to calculate the volume fraction of casein micelles in evaporated milk[3], ultrafiltrated milk[32] and skim milk concentrate[31]. Eilers' equation was also applied in this study.

3.2 Materials and methods

3.2.1 Sample preparation

MCI powder (Nutripro^{TM}) was supplied by DairyGold Food Ingredients (Cork, Ireland). This powder contains 85% (w/w) protein of which ≤5% (w/w) is whey protein. A MCI solution with 9% (w/v) protein was prepared for this study, which contains approximately 8.5 mmol L^{-1} sodium, 4.2 mmol L^{-1} potassium, 2.5 mmol L^{-1} chloride, 59.8 mmol L^{-1} calcium, 43.5 mmol L^{-1}

phosphorus, and 3.1 mmol L^{-1} magnesium. The MCI powder was dissolved in 80% of the total demineralized water at ambient temperature, while stirring at 600 rpm with a laboratory stirrer (RW 20.n, IKA Labortechnik, Staufen, Germany). The protein solution was homogenized with a two-stage high pressure laboratory homogenizer (NS2006L, GEA Niro Soavi S.p.A., Parma, Italy) at 35 MPa in the first stage and 5 MPa in the second stage. Individual casein micelles were obtained following homogenization with a diameter $D_{[4,3]}$ of 0.15 µm, as determined with a Mastersizer 2000 containing a hydro 2000G water bath (Malvern Instruments, Worcestershire, England). The temperature of the protein solution was 40°C after homogenization.

Subsequently, varying amounts of stock solution of disodium uridine monophosphate (Yamasa Corporation, Chiba, Japan), disodium hydrogen phosphate (Merck & Co. Inc, Darmstadt, Germany), sodium hexametaphosphate (VWR International Ltd, Poole, England), phytic acid dodecasodium salt hydrate (Sigma-Aldrich GmbH, Steinheim, Germany), or trisodium citrate (Gadot Biochemical Industries Ltd., Haifa Bay, Israel) were added to obtain final chelator concentrations of 0–105 mEq L^{-1} in the samples. These chelators contain a different amount of negative charges, which gives them different calcium-binding capacities.[21] Therefore, the concentration ranges of the calcium chelators were based on milliequivalents to add a similar amount of charges to the samples. Only sodium sources were used, because the type of counter-ion may also influence protein–mineral interactions.[33] The pH of the samples was adjusted, after stirring for 30 min, to 7.0 ± 0.05 with 1 mol L^{-1} sodium hydroxide (Sigma-Aldrich GmbH, Steinheim, Germany) or 1 mol L^{-1} hydrochloric acid (Merck & Co. Inc, Darmstadt, Germany). Finally, samples were brought to a final protein concentration of 9% (w/v) with demineralized water. Samples were stored overnight at 20°C for approximately 17 h to let the samples equilibrate. The pH of the samples was readjusted to 7.0 ± 0.05 the next morning, in case deviations had occurred during storage. Deviations in pH were always small and samples did not show any visible spoilage. Samples were analyzed at least in duplicate for their final pH, calcium-ion activity, turbidity, viscosity, and sedimentation after ultracentrifugation.

3.2.2 Viscosity

Samples were analyzed at 20°C with an MCR 300 rheometer (Anton Paar Physica, Graz, Austria) using a cup and bob geometry (CC27 cylinder). The viscosity was measured at shear rates of 1-1000 s^{-1}. The behavior of most of the samples was close to that of Newtonian liquids.

3.2.3 Calcium-ion activity

The calcium ion activity was measured with a Mettler Toledo Seven Multi™ (with an Inlab® Expert Pro pH-meter) calcium measuring device (Mettler Toledo, Greifensee, Switzerland) using an Orion 9300BH electrode and an Orion 900100 reference electrode. Calibration of the electrodes, sample measurements, and calculations of the calcium-ion activities were performed as described in De Kort *et al*..[21]

3.2.4 Turbidity

The turbidity was measured with a spectrophotometer (4053 Kinetics, LKB Biochrom, Midland, Canada). Plastic cuvettes with a pathway of 1 cm were used. Measurements were carried out at ambient temperature using a wavelength of 700 nm. Samples were diluted to 10% of their initial dry matter in demineralized water to be within the detection limits of the spectrophotometer.

3.2.5 Ultracentrifugation

Ultracentrifugation was done with a Centrikon T-1080A ultracentrifuge (Kontron Instruments Ltd., Milan, Italy) with a fixed-angle titanium rotor (type TFT 45.94). Samples were ultracentrifuged at 150,000 x *g* at 20°C for 60 min. A firm pellet and liquid supernatant were formed. The supernatant consisted of an opaque and translucent layer. The opaque and translucent layers were carefully removed, leaving a firm pellet. The opaque layer was included in the supernatant fraction. The pellet fractions were weighed and calculated for the amount of pellet per 100 g of total sample.

3.2.6 Protein content and casein composition

The protein content was determined in the ultracentrifuged pellets, supernatants, and total samples. The NA 2100 Nitrogen and Protein analyzer (CE Instruments, Milan, Italy) was used to determine the nitrogen content in the samples by the Dumas method. A conversion factor of 6.38 was used to convert nitrogen to protein content. Approximately 100 mg of sample was weighed in a tin cup. The sample was dried in an oven at 70˚C for 2.5 h. Then, 25 mg of absorbent (82009101, Interscience B.V., Breda, The Netherlands) was added and the cup was closed. The

cups were placed in the autosampler and analyzed for their nitrogen content. The amount of protein in the pellet was expressed as gram protein in the pellet per 100 g of total sample. The ultracentrifuged supernatants were also analyzed for their casein composition by capillary zone electrophoresis according to the method of Heck *et al.*[34].

3.2.7 Voluminosity

The voluminosity of the caseins was calculated in two ways: from viscosity and ultracentrifugation measurements. The viscosity measured at a shear rate of 50 s^{-1} was chosen for calculation, as this shear rate corresponds to the organoleptic shear during drinking. The viscosity values were inserted in Eilers' equation[30] to calculate the volume fraction (Φ) of the caseins in the solution. Volume fraction is a dimensionless number and defined as the fraction of the total volume occupied by the particles. Eilers' equation is as follows:

$$\eta = \eta_0 \left(1 + \frac{1.25\Phi}{1-\Phi/\Phi_{\max}}\right)^2 \tag{3.1}$$

where η_0 represents the viscosity of the continuous phase (taken in this paper as 1 mPa s), and where $\Phi_{\max}$ represents the maximum packing volume fraction. A value of 0.79 was used for $\Phi_{\max}$ in the calculations.[35] Eilers' equation is an extension of the Einstein relation, which describes the viscosity of dispersions in very dilute systems.[30] The voluminosity can be directly calculated by dividing the volume fraction by the protein concentration. Voluminosity is defined as the number of milliliters of solution occupied by a gram of protein material (mL g^{-1}).

The voluminosity of the casein micelle was calculated from the ultracentrifugation data by dividing the total pellet volume (mL g^{-1}) by the amount of protein in the pellet (g g^{-1}). The total pellet volume was calculated as described by van Hooijdonk *et al.*.[36] The volume the minerals occupy in the pellet is negligible, as the proteins dominate the pellet volume.

3.3 Results

3.3.1 Calcium-ion activity

The calcium-ion activity decreased upon addition of the phosphates and citrate (Fig. 3.1), because calcium ions are chelated from the serum phase and casein micelles. A comparable decrease in calcium-ion activity was measured for SHMP, SP, TSC, and Na_2HPO_4. The calcium-ion activity only slightly decreased upon addition of Na_2UMP, because Na_2UMP is a weak calcium binder. [21]

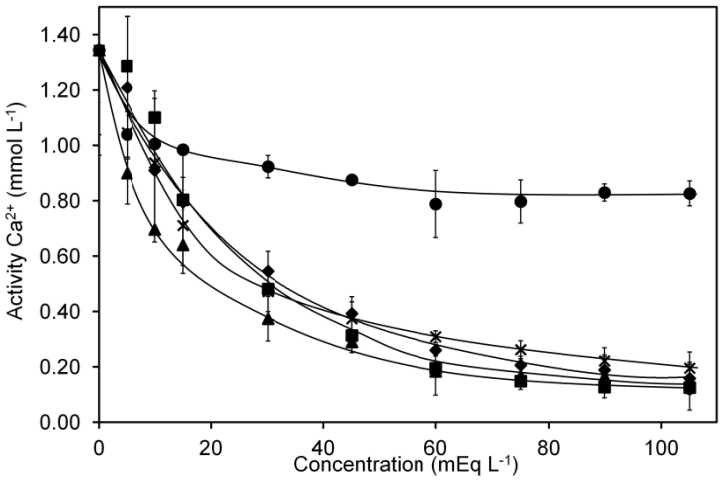

Figure 3.1 Calcium-ion activity of micellar casein isolate solutions as a function of concentration of phosphate and citrate salts: (●) Na_2UMP; (♦) Na_2HPO_4; (■) SHMP (▲) SP; (x) TSC. Results are the means for at least duplicates with standard deviations as error bars.

3.3.2 Viscosity

The viscosity increased to a comparable extent after addition of SP, TSC, and Na_2HPO_4 (Fig. 3.2). The largest increase in viscosity was measured for SHMP samples, which is due to the ability of SHMP to cross-link caseins.[18, 22, 25] This resulted in gel formation upon addition of more than 45 mEq L^{-1} SHMP. Na_2UMP only slightly shifted the mineral equilibria and, as a result, the viscosity of the Na_2UMP samples was negligibly affected.

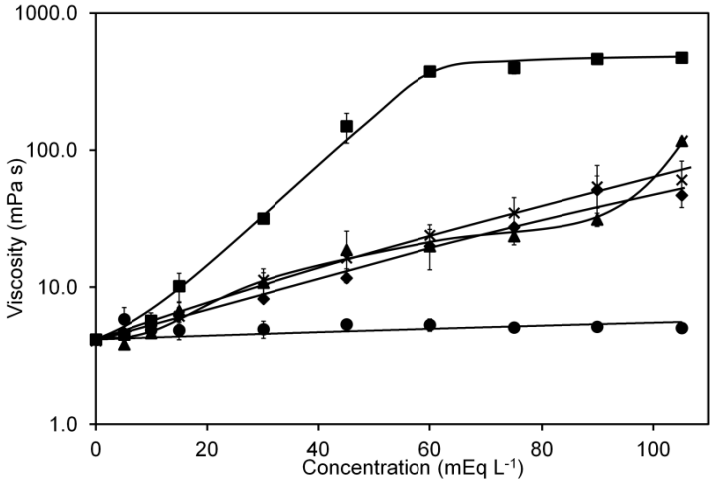

Figure 3.2 Viscosity at shear rate 50 s^{-1} of micellar casein isolate solutions as a function of concentration of phosphate and citrate salts: (●) Na_2UMP; (♦) Na_2HPO_4; (■) SHMP (▲) SP; (x) TSC. Results are the means for at least duplicates with standard deviations as error bars.

3.3.3 Voluminosity derived from viscosity

The viscosity values (at a shear rate of 50 s^{-1}) were inserted into Eilers' formula to calculate the voluminosity of the casein micelle. The solutions were shear-thinning at higher chelator concentrations (≥ 75 mEq L^{-1} for SP, TSC, and Na_2HPO_4 and ≥ 45 mEq L^{-1} for SHMP), when higher viscosities were measured. This seems to make interpretation via viscosities at 50 s^{-1} somewhat ambiguous. However, in this high-viscosity region, the sensitivity of the volume fraction calculated from Eilers' equation to the actual viscosity value is low: volume fractions are close to the value Φ_{max} for maximum packing. The results in Fig. 3.3 show that the casein micelle in the MCI solution has a voluminosity of 4.5 mL g^{-1}. Walstra *et al.*[1] measured a voluminosity of 4 mL g^{-1} for casein micelles in milk. Addition of Na_2UMP had a negligible effect on the voluminosity of the casein micelle. Higher volume fractions were calculated for SHMP samples than for the other phosphates and citrate because of gelling of the SHMP samples. For SHMP samples, accordingly, the voluminosity of the casein micelle cannot be deduced from the viscosity via Eilers' equation, because interactions between the micelles are no longer only of a hydrodynamic nature. The effect of SP, TSC, and Na_2HPO_4 on viscosity and thus the derived voluminosity were comparable. The voluminosity of the casein micelle

increased from 4.5 mL g^{-1} (Φ=0.41) to approximately 7.5 mL g^{-1} (Φ=0.69) upon addition of 105 mEq L^{-1} SP, TSC, or Na_2HPO_4. Addition of SP, TSC, and Na_2HPO_4 neither cross-linked casein micelles nor caused gelation in the studied concentration range, which is in agreement with the effect of TSC in milk.[17]

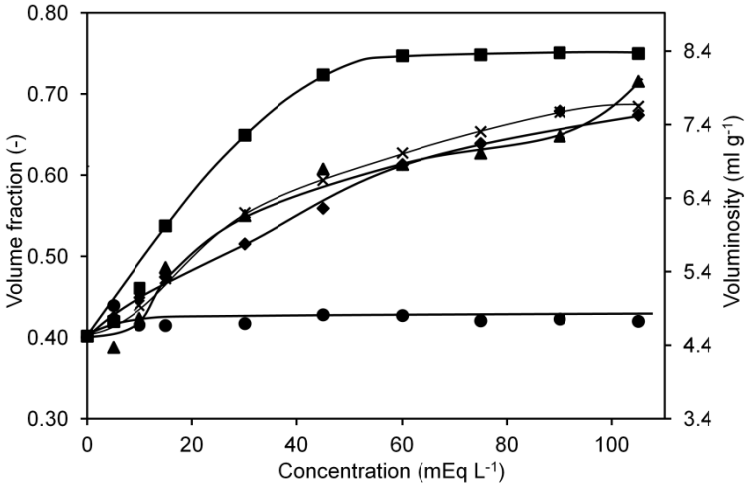

Figure 3.3 Voluminosity, calculated with Eilers' equation, at shear rate 50 s^{-1} of micellar casein isolate solutions as a function of concentration of phosphate and citrate salts: (●) Na_2UMP; (♦) Na_2HPO_4; (■) SHMP (▲) SP; (x) TSC.

3.3.4 Voluminosity derived from ultracentrifugation

The voluminosity of the casein micelle can also be deduced from ultracentrifuged fractions by dividing the total pellet volume by the amount of protein in the pellet. The amount of pellet and amount of protein in the pellet after ultracentrifugation for 1 h are shown in Fig. 3.5 and 3.6, respectively. The amount of ultracentrifuged pellet is influenced by the acceleration and time of centrifugation, the density and viscosity of the solutions, and the density and size of the casein micelles.[12]

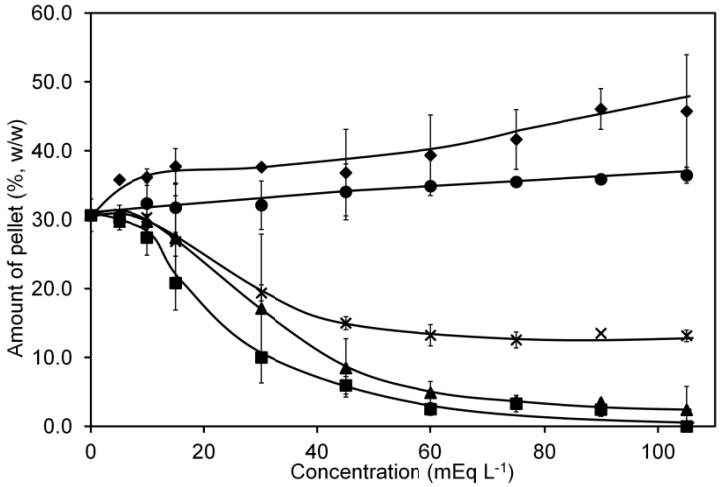

Figure 3.4 Amount of ultracentrifuged pellet of the micellar casein isolate solutions, expressed as gram pellet per 100 g of total sample (%, w/w), as a function of concentration of phosphate and citrate salts: (●) Na_2UMP; (♦) Na_2HPO_4; (■) SHMP (▲) SP; (x) TSC. Results are the means for at least duplicates with standard deviations as error bars.

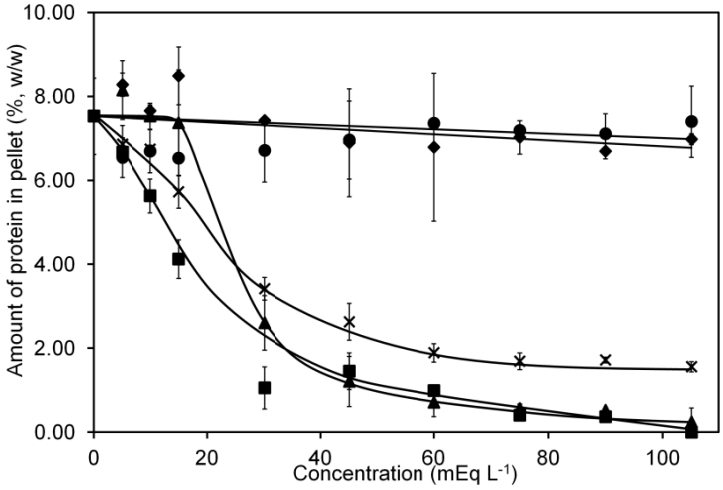

Figure 3.5 Amount of protein in ultracentrifuged pellet of the micellar casein isolate solutions, expressed as gram protein in the pellet per 100 g of total sample (%, w/w), as a function of concentration of phosphate and citrate salts: (●) Na_2UMP; (♦) Na_2HPO_4; (■) SHMP (▲) SP; (x) TSC. Results are the means for at least duplicates with standard deviations as error bars.

Approximately 30% (w/w) pellet was formed after ultracentrifugation for 1 h of the reference solution, and this pellet contained 7.5 g protein per 100 g of total sample. Prolonged ultracentrifugation (up to 8 h) caused a decrease in the amount of pellet in the reference sample. Calculation of the voluminosity also indicated a decrease in voluminosity. This was due to some squeezing of the casein micelles in the pellet. Dewan *et al.*[4] and Van Hooijdonk *et al.*[36] observed as well this phenomenon for ultracentrifugation of skim milk. For samples containing Na_2HPO_4 and Na_2UMP the amount of pellet increased and the amount of protein in the pellet slightly decreased at higher chelator concentration. This trend continued with prolonged ultracentrifugation and slightly decreased the voluminosity of the samples.

Ultracentrifugation for 1 h of the samples with TSC, SP, and SHMP showed that the amount of pellet and amount of protein in the pellet decreased with increasing chelator concentration. This decrease in amount of pellet was proportional to the decrease in amount of protein in the pellet and also with prolonged ultracentrifugation. Consequently, the voluminosity of the samples did not effectively change during longer ultracentrifugation times. Overall, an ultracentrifugation time of 1 h was chosen, because at this point sufficient protein was precipitated in the pellets of the chelator-containing samples to make calculation of voluminosities possible. Moreover, the unwanted effect of squeezing of the micelles stayed within an acceptable range.

Analyses of the casein composition of the supernatants ultracentrifuged for 1 h showed that the supernatants contained casein. The ratio of specific caseins in these supernatants was in agreement with the ratio as is naturally present in milk. The concentration and type of added chelator did not change the casein composition in the supernatants. This was also observed by Pitkowski *et al.*[8] and Griffin *et al.*[11] after addition of polyphosphate or EDTA, respectively.

Fig. 3.6 shows the correlation between voluminosities calculated from viscosity and ultracentrifugation measurements. In general, the voluminosities derived from ultracentrifugation were lower than the voluminosities derived from viscosity. For the reference sample, the voluminosity was approximately 15% lower, because the pellet was squeezed during ultracentrifugation. For the chelator-containing samples, the level of deviation was dependent on the concentration and type of chelator used.

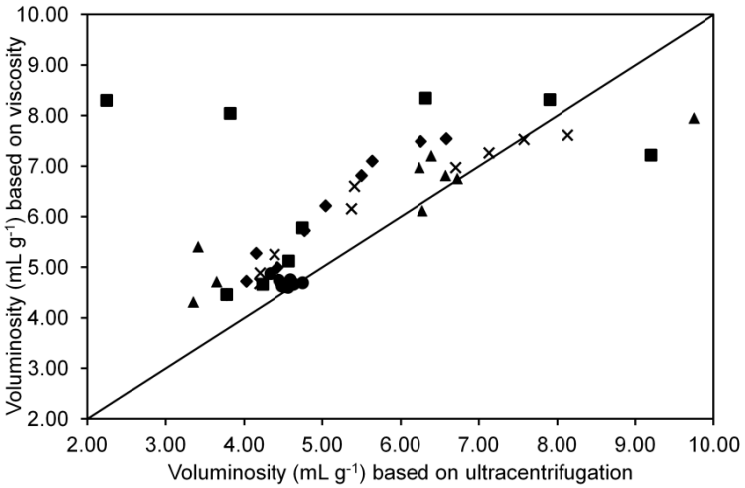

Figure 3.6 Correlation between voluminosities based on viscosity and ultracentrifugation measurements for: (●) Na_2UMP; (♦) Na_2HPO_4; (■) SHMP (▲) SP; (x) TSC; to guide the eye, the solid line indicates a correlation of 1.

The voluminosities of SHMP samples correlated up to 15 mEq L^{-1} SHMP, but the voluminosities derived from viscosity were overestimated at higher SHMP concentrations. This was due to the high viscosities measured in the SHMP samples. SHMP cross-linked the caseins[18, 22, 25], which interfered with calculation of the voluminosity of the caseins. Moreover, the amount of ultracentrifuged pellet was very low at higher viscosity, which caused large deviations in the voluminosities derived from ultracentrifugation. Therefore, the voluminosity of the casein micelle in SHMP samples cannot be calculated either via viscosity or via ultracentrifugation above a concentration of 15 mEq L^{-1} SHMP.

3.3.5 Turbidity

Calcium chelators also affect the turbidity of milk solutions.[6] The turbidity of the MCI solution decreased upon addition of the phosphates and citrate in the order SHMP ≥ SP > TSC > Na_2HPO_4 > Na_2UMP (Fig. 3.7). Mizuno *et al.*[17] also observed a decreasing order in turbidity for SHMP > TSC > Na_2HPO_4 in samples prepared from milk protein concentrate at pH 5.8.

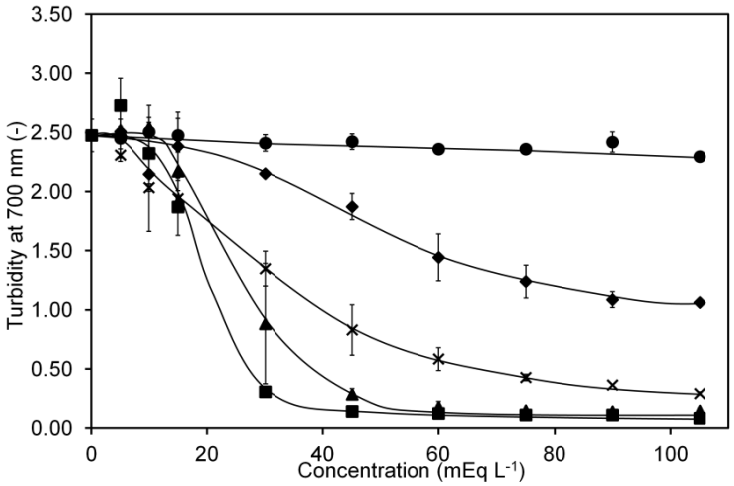

Figure 3.7 Turbidity of micellar casein isolate solutions diluted to 10% of their initial dry matter content in demineralized water as function of concentration of phosphate and citrate salts: (●) Na_2UMP; (♦) Na_2HPO_4; (■) SHMP (▲) SP; (x) TSC. Turbidity was measured as absorbance (dimensionless) at 700 nm. Results are the means for at least duplicates with standard deviations as error bars.

3.4 Discussion

3.4.1 Comparison of polyphosphates sodium phytate and sodium hexametaphosphate

It is remarkable that the turbidity decreased to a comparable extent after addition of ≤45 mEq L^{-1} SP or SHMP, whereas large differences in viscosity were measured. These calcium chelators probably affect the integrity of the micellar structure to the same extent, but SHMP has the ability to cross-link caseins, whereas SP has not. Our hypothesis is that this is due to the form and charge distribution around the molecules. SHMP has six homogeneously distributed negative charges around the molecule, whereas SP has twelve negative charges, clustered in pairs, around the molecule. This homogeneous charge distribution enables SHMP to interact with cations and the caseins at the same time. SP interacts with the caseins less easily than SHMP because of the charge distribution around the SP molecule and, in this way, cross-linking

is inhibited. SP also is a very strong calcium chelator and might immediately chelate free calcium ions to such an extent that no charges or calcium ions are any longer available for cross-linking the caseins. This was measured as a stronger decrease in calcium-ion activity for SP than SHMP (Fig. 3.1). These calcium chelators probably affect the mineral equilibria in the samples to a similar extent and, thus, have a similar impact on the integrity of the micellar structure. Mizuno *et al.*[22] investigated the cross-linking ability of tetrasodium pyrophosphate (TSPP) in milk protein concentrate solution. They suggested that TSPP acts with calcium as a cross-linking agent between the caseins. TSPP probably cross-links the caseins more easily than SHMP, as it has only four homogeneously distributed charges around its molecule. Nevertheless, further research is required to elucidate the exact mechanism of cross-linking caseins by different polyphosphates.

3.4.2 Effect of calcium chelators on physical changes in the casein micelle

It is remarkable that SP, TSC, and Na_2HPO_4 show a comparable increase in viscosity and voluminosity and decrease in calcium-ion activity, while they had a different impact on turbidity and ultracentrifuged (protein in) pellet. Calcium ions in the casein micelle are bound to the phosphoserine residues or are part of the CCP complexes. The added chelator competes with the phosphoserine residues and CCP in the casein micelle for the calcium ions. The chelators have a different affinity for calcium ions, which gives them the ability to release a different amount of CCP from the micelle.[21, 37-39] Moreover, solubilization of CCP from the micelle depends on the degree of saturation of the calcium complexes formed in the solution.[2] Therefore, calcium chelators can affect the integrity of the micellar structure to different extents.

3.4.2.1 Release of colloidal calcium phosphate and caseins from the casein micelle

In general, scattering of particles is determined by the concentration, particle size, and refractive index relative to that of the solution.[40] The caseins and CCP are mainly responsible for the light-scattering properties of the casein micelle.[14] Removal of CCP from the micelles reduces the refractive index of the casein micelles, which is measured as a decrease in turbidity of the milk solutions. The study of Smiddy *et al.*[41] on internally cross-linked casein micelles showed that after addition of 50 mmol L^{-1} citrate (150 mEq L^{-1}) to skim milk, a decrease in light scattering of approximately 50% was measured. These authors suggested that all CCP (7% of dry mass of the

casein micelle) was removed from the cross-linked micelles at this concentration, while the micelle remained intact. Fig. 3.7 shows that a decrease in turbidity of 97% for SHMP and SP, 87% for TSC, and 60% for Na_2HPO_4 upon addition of 105 mEq L^{-1} chelator to the MCI solution was measured. Hence, these decreases in turbidity cannot only be attributed to release of CCP from the micelle. Some caseins may also be released from the casein micelle upon addition of chelators to milk solutions, when the calcium-ion activity of the solution is kept effectively constant.[42] This decreases the turbidity of milk as well. However, no selective release of individual caseins could be detected in the ultracentrifuged supernatants of samples with Na_2HPO_4, TSC, SP, or SHMP, which is probably due to the strong decrease in calcium-ion activity upon addition of a low concentration of chelator.

3.4.2.2 Dissociation of the casein micelle

Rayleigh scattering indicates that the intensity of the scattered light varies as the sixth power of the particle size[40] and, accordingly, particle size makes the main contribution to the change in turbidity of the solution. The particle size of the casein micelles is affected, when the micelles swell or dissociate into smaller structures. Huppertz *et al.*[43] described that addition of 6 mol L^{-1} urea to internally cross-linked casein micelles induces swelling of the micelles, which is measured as a decrease in turbidity of 40%. The decrease in turbidity in our MCI samples is too large to be only attributed to the swelling of the casein micelles. A further explanation on the swelling of the casein micelles will be described in section 3.4.2.3.

The major decrease in turbidity is most likely due to the dissociation of the casein micelles into smaller structures. Dissociated micelles will precipitate less easily than intact casein micelles during ultracentrifugation, because the fragments of the dissociated micelles are smaller and lighter than the intact casein micelles. Based on these phenomena, the turbidity and ultracentrifugation results indicate that micellar dissociation occurred to the largest extent on addition of SHMP and SP, followed by TSC, and finally by Na_2HPO_4. Dynamic light scattering analyses has been performed to elucidate the concentration at which the various calcium chelators start to dissociate the casein micelle. Preliminary results indicate that micellar dissociation is responsible for the decrease in turbidity; a paper with these results is in preparation (Chapter 5). Micellar dissociation most probably did not occur in Na_2UMP samples, because only a slight increase in concentration of caseins in the supernatants was measured.

Panouille *et al.*[44] and Pitkowski *et al.*[8] both described how casein, which is fully dissociated after addition of a chelator, forms small micellar particles that contain 10-15 casein proteins, so called sub-micelles. The phenomenon of milk solutions containing intact and dissociated casein micelles upon addition of polyphosphate or EDTA was introduced previously by e.g. Lin *et al.*[12] and Griffin *et al.*[11]. However, the effect on viscosity was not taken into account in these studies. Our measurements show that, although the calcium chelators dissociate the micelles to different extents, an increase in viscosity of the same magnitude could be measured. It is most likely that intact and dissociated casein micelles contribute equally to the raise in viscosity, assuming that the viscosity of the continuous phase remains constant and equal to 1 mPa s.

3.4.2.3 Swelling of the casein micelle

Fig. 3.1 shows that the calcium-ion activity decreased to a comparable extent upon addition of SHMP, SP, TSC, and Na_2HPO_4. The electrostatic repulsion in the casein micelles increased because of the decrease in free calcium ions in the continuous phase. Consequently, the casein micelles became more hydrated and swollen, which is measured as an increase in viscosity of the MCI solutions (Fig. 3.2) and also an increase in voluminosity of the casein micelle (Fig. 3.3 and 3.6). The phenomenon of swelling of the casein micelles can be derived from the ultracentrifuged pellet in Na_2HPO_4 samples (Fig. 3.4). In these samples, the pellet volume increased at higher chelator concentrations, whereas in samples with SHMP, SP, or TSC the pellet volume decreased. Squeezing of the casein micelles in the pellets of samples with Na_2HPO_4 increased with prolonged ultracentrifugation, which indicates that the micelles were compressible. In the SHMP, SP, and TSC samples this squeezing effect was much smaller, probably because the dissociated sub-micelles have a higher resistance to compression.

Gaucher *et al.*[45] and Guo *et al.*[23] observed that orthophosphate precipitates with calcium in the casein micelles. We calculated that an amount of approximately 3 g $Ca_3(PO_4)_2$ can be formed upon addition of 60 mEq L^{-1} Na_2HPO_4, of which a large part of the calcium ions are already part of the casein micelles. The increase in molecular weight is negligible in comparison to the observed increase in amount of pellet. This increase is ascribed to the swelling of the caseins. Actually, it is not expected that an amount of 3 g $Ca_3(PO_4)_2$ could be formed, because, as earlier suggested by Gaucher *et al.*[45], the affinity of calcium is probably higher for micellar phosphate than for added orthophosphate. This suggestion was confirmed by the observation that most of the added orthophosphate remains in the diffusible phase.[38, 45]

Fig. 3.4 also shows that the casein micelle slightly swells upon addition of Na_2UMP, because the amount of pellet increased in these samples as well. These findings of swelling of the micelles were not observed by Lin *et al.*[12] and Udabage *et al.*[10] after addition of 1.2 mmol L^{-1} or 10 mmol L^{-1} EDTA to milk, respectively. Those authors described that a fraction of the micelles dissociated at these concentrations, but that the hydrodynamic radius of the residual casein micelles remained constant.[10, 12] However, Sood *et al.*[15] already questioned the observations of Lin *et al.*[12] that intact micelles will remain at a constant radius, because they measured an increase in voluminosity in the concentration range 0.2-2.0 mmol L^{-1} EDTA. Therefore, they concluded that the micelles should be able to swell or shrink when the calcium content in the casein micelles is changed.

Moreover, Huppertz *et al.*[43] showed by three light-scattering methods that even internally cross-linked casein micelles were able to swell upon addition of citrate or urea, which was measured as an increase in particle sizes and decrease in turbidity. These results are in line with our observations that calcium chelators induce swelling of the intact casein micelles and dissociation of a fraction of the micelles. This also suggests that loosely bound calcium, i.e., that bound to the negatively charged amino acids side chains and phosphate groups, is present in the casein micelle besides strongly bound calcium in the CCP complexes. The former type has a structural function and its release is related to swelling of the micelle, which is measured as an increase in viscosity. Release of the latter is related to the dissociation of the casein micelles, which is measured as a decrease in turbidity and amount of ultracentrifuged pellet.

The hypothesis that two types of calcium interactions are present in the casein micelle was proposed by Munyua *et al.*.[14] Overall, it seems that the calcium-ion activity is a good predictor for the observed viscosities and related swelling of the micelle, but a poor indicator when the casein micelle starts to dissociate.

3.5 Conclusions

The effect of different calcium chelators on physical changes in the casein micelles in concentrated micellar casein solutions can differ considerably. The increase in viscosity after addition of the calcium chelators is typically due to swelling of the caseins, except in the case of

SHMP because of its ability to cross-link caseins. The viscosity and related voluminosity can be linked directly to the calcium-ion activity until the casein micelles start to dissociate. The decrease in turbidity after addition of calcium chelators is due to dissociation of the casein micelles into smaller structures; the onset and extent of dissociation is dependent on the type and concentration of calcium chelator. These insights provide new opportunities for controlling the viscosity and turbidity of concentrated dairy systems by calcium chelators.

3.6 Acknowledgements

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Chapter 4

Effect of calcium chelators on heat coagulation and heat-induced changes of concentrated micellar casein solutions: the role of calcium-ion activity and micellar integrity

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Abstract

There is general consensus that calcium chelators enhance heat stability in milk. However, they can increase the heat stability to considerably different extents. For that reason, the effect of various calcium chelators on heat coagulation and heat-induced changes of concentrated micellar casein solutions was investigated by measuring the heat coagulation time (HCT) together with changes in calcium-ion activity, viscosity, turbidity, and zeta potential before and after heating. Surprisingly, the weakest chelator, disodium uridine monophosphate (Na_2UMP), gave the most pronounced increase in HCT. Stronger chelators, disodium hydrogen phosphate, trisodium citrate, and sodium phytate, gave a lower HCT compared to Na_2UMP. Sodium hexametaphosphate (SHMP) was the least effective heat stabilizer. Observed heat-induced changes for SHMP were the major cause for this reduced heat stability effect. Overall, differences in HCT caused by the addition of the various calcium chelators could be attributed to the calcium-ion activity and state of the micellar structure before and during heating.

4.1 Introduction

Foods for special medical purposes are mainly liquid concentrated drinks, which are sterilized at ultra-high temperature to remain stable for at least nine months at ambient temperature. Therefore, these products have to resist severe heat treatment. Most of these products contain a high concentration of casein. Casein micelles are remarkably stable against heat. Their stability is maintained by hydrophobic and electrostatic interactions, colloidal calcium phosphate (CCP), and steric effects of protruding chains of κ-casein.[1, 2] Nevertheless, physical and chemical changes occur in the casein micelles during heating of milk mainly due to shifts in the salt equilibria. These changes become partly irreversible after heating for several minutes above 120°C due to alterations in the structure and composition of the original micellar calcium phosphate into a more insoluble form.[3, 4] Other irreversible changes that occur during heating are hydrolysis of phosphoserine residues, degradation of lactose, and release of κ-casein from the micelle.[3, 5, 6]

Coagulation becomes visible when large aggregates have emerged or when a gel is formed. The resistance of milk against coagulation during heating is called heat stability. The time needed for coagulation is called the heat coagulation time, HCT.[6] The HCT of milk is highly dependent on protein concentration and pH. Concentrated milk (7.0-9.0% protein) has a much lower HCT than non-concentrated milk.[7] The pH is an important factor because it affects the protein charge (i.e. electrostatic repulsion between caseins), the concentration of free calcium ions in the serum phase, and the amount of CCP and casein in the micelle.[1, 6, 8] Moreover, the HCT of milk is influenced by the protein composition, explaining for instance the different effect of pH on the HCT of milk containing whey (Type A milk) and that of whey protein-free casein micelle dispersions (possible form of Type B milk).[9] The HCT-pH profile of Type A milk, which is the most common type of milk, shows a maximum and minimum in heat stability, whereas the HCT of Type B milk increases with increasing pH.[7, 9, 10]

The heat stability of dairy solutions can also be manipulated by addition of calcium chelators because of their effect on the concentration of free calcium ions and thereby on the integrity of the micellar structure.[1, 11-13] Phosphates and citrate, typically up to concentrations of 40 mmol per kg skim milk solids, are commonly used in the dairy industry as heat stabilizers.[11, 14] Addition of phosphate or citrate to milk solutions causes different HCT-pH profiles, which is

related to the precipitation of phosphate with calcium on the micelles and precipitation of citrate with calcium in the serum phase.[6, 15] Polyphosphates, such as sodium hexametaphosphate (SHMP) and sodium phytate (SP), increase the heat stability of milk by, not only binding calcium, but also by binding to positively charged amino acids of the casein micelle.[13, 16, 17] High concentrations of calcium chelators (e.g. 16 to 67 mmol EDTA per kg skim milk solids), might also decrease the heat stability of a milk system, as they can chelate a critical level of CCP from the casein micelle at which the integrity of the micellar structure is lost.[11, 18] In a previous study[19] it was suggested that a decrease in turbidity of casein micelle solutions is related to the dissociation of casein micelles upon addition of different types and concentrations of calcium chelators. These results suggest the micelle dissociation occurs in the order of SHMP ≥ SP > citrate > orthophosphate > disodium uridine monophosphate.

The heat stability of normal milk, concentrated milk, evaporated milk, and artificial casein micelle systems has been extensively studied.[1, 6, 20-22] However, to our knowledge, no studies have systematically evaluated the heat stability of commercial concentrated micellar casein isolate (MCI) solutions at sterilization conditions. An advantage of using MCI powder instead of concentrated milk is that it contains intact casein micelles and a negligible amount of whey protein. Although there is general consensus that phosphates and citrate enhance heat stability of milk systems, the effectiveness can differ considerably. Therefore, more knowledge is needed to understand the effect of the different calcium chelators on HCT. In a previous study it was shown that different calcium chelators had very different effects on the physical changes in the casein micelles in concentrated micellar casein solutions.[19] In this study the focus will be on the effect of the calcium chelators on heat coagulation and heat-induced changes of a concentrated MCI solution at different pH values.

4.2 Materials and Methods

4.2.1 Sample preparation

The protein powder micellar casein isolate, MCI (Nutripro^{TM}) was supplied by DairyGold Food Ingredients (Cork, Ireland). MCI powder contains 85 % (w/w) protein of which less than 5% is whey protein. A MCI solution with 9% (w/v) protein was dissolved and homogenized as described in De Kort *et al.*.[19] Homogenization of the MCI solution was required to split the

dispersed powder particles into individual casein micelles with a diameter $D_{[4,3]}$ of about 0.15 µm. The MCI solution contains approximately 8.5 mmol L^{-1} sodium, 4.2 mmol L^{-1} potassium, 2.5 mmol L^{-1} chloride, 59.8 mmol L^{-1} calcium, 43.5 mmol L^{-1} phosphorus, and 3.1 mmol L^{-1} magnesium. Stock solutions were prepared of disodium uridine monophosphate (Na_2UMP) (Yamasa Corporation, Chiba, Japan), disodium hydrogen phosphate (Na_2HPO_4) (Merck & Co. Inc, Darmstadt, Germany), sodium hexametaphosphate (SHMP) (VWR International Ltd, BDH Chemicals, Poole, England), phytic acid dodecasodium salt hydrate (SP) (Sigma–Aldrich GmbH, Steinheim, Germany), and trisodium citrate (TSC) (Gadot Biochemical Industries Ltd., Haifa Bay, Israel). Different amounts of the stock solutions were added to the MCI solutions in order to obtain final chelator concentrations of 0, 15, 30, 45, and 60 mEq L^{-1} in the samples. As these chelators carry different amounts of negative charges, this results in different calcium-binding capacities.[23] The concentration ranges of the calcium chelators were based on milliequivalents to obtain an equal amount of charges in the samples. An overview of the corresponding chelator concentrations in mmol L^{-1} and mEq L^{-1} for the different chelators is shown in Table 4.1. Only sodium sources were used, because the type of counter-ion may also influence protein–mineral interactions.[24]

Table 4.1 Overview of corresponding chelator concentrations in mmol L^{-1} and mEq L^{-1} for the different chelators

Type	Charge	15 mEq L^{-1}	30 mEq L^{-1}	45 mEq L^{-1}	60 mEq L^{-1}	
Na_2UMP	**-2**	7.50	15.00	22.50	30.00	mmol L^{-1}
Na_2HPO_4	**-3**	5.00	10.00	15.00	20.00	mmol L^{-1}
TSC	**-3**	5.00	10.00	15.00	20.00	mmol L^{-1}
SHMP	**-6**	2.50	5.00	7.50	10.00	mmol L^{-1}
SP	**-12**	1.25	2.50	3.75	5.00	mmol L^{-1}

The pH of the samples was adjusted, after stirring for 30 minutes, to 6.7 ± 0.05, 7.0 ± 0.05, and 7.3 ± 0.05 with 1 mol L^{-1} sodium hydroxide (Sigma-Aldrich GmbH, Steinheim, Germany) or 1 mol L^{-1} hydrochloric acid (Merck & Co. Inc, Darmstadt, Germany). The samples were brought to their final protein concentration of 9 % (w/v) with demineralized water and, subsequently, stored overnight at 20°C for approximately 17 hours to equilibrate. The pH of the samples was

readjusted the next morning to 6.7 ± 0.05, 7.0 ± 0.05, or 7.3 ± 0.05 in case deviations had occurred during storage. Deviations in pH were always small and samples did not show any visible spoilage. Samples with 0, 15, 30, 45, and 60 mEq L^{-1} calcium chelator were prepared (0 mEq L^{-1} is the reference sample) and analyzed in duplicate for their HCT in the Klaro-graph. In addition, samples with 0, 15, and 60 mEq L^{-1} calcium chelator were prepared in duplicate and heated for 0, 15, 35, and 55 minutes in a temperature-controlled oil bath. The pH, zeta potential, calcium-ion activity, turbidity, and viscosity of these samples were analyzed in duplicate before and after heating in the oil bath.

4.2.2 Analyses

The HCT of the samples was determined with a falling-ball viscometer using the Klaro-graph.[15, 25, 26] A sample of 13.5 mL was inserted in the inner part of a double-walled glass tube. The inner diameter of the tubes was 9.3 mm and the volume from the bottom to the expansion chamber was 20 mL. Two glass balls with a diameter of 9.0 mm were put in the tubes. The tubes were placed in the system and silicone oil was circulated around the tubes. The silicone oil was connected to a thermostatic oil bath of 126°C. The apparatus allows the use of eight tubes at the same time. The tubes were placed 10° from upright, so that the balls rolled along the wall of the tubes. The tubes were rotated 180° clockwise and anti-clockwise during the measurement as soon as the balls reach the bottom of the tubes, which was after approximately 20 s. When the samples became unstable, the balls were stopped by coagulated particles. The time needed to reach coagulation was recorded as the HCT. The heating-up period of about 4 min in the Klaro-graph tubes was not included in the reported heating times.

Heat-induced changes were determined by heating the samples for 15, 35, and 55 min in a temperature-controlled oil bath at 126 °C. The samples were inserted in heat-resistant glass tubes of 15 mL, which were closed with a lid (at least three tubes per sample). Samples with the same treatment were pooled after heating to obtain sufficient volume for analyses. The heating-up time of 6 min to reach a temperature of 126°C inside the heat-resistant glass tubes was not included in the final heating time of the samples. The analysis of the samples was carried out after cooling to ambient temperature in cold water for about 30 min.

The pH and calcium-ion activity of the samples were measured at ambient temperature with an Inlab® Expert Pro pH meter and a Seven Multi™ calcium measuring device (Mettler Toledo,

Griefensee, Switserland), respectively. The pH value and calcium-ion activity were read after gently stirring for 5 min. The pH meter was calibrated with standard buffer solutions of pH 4.0 and pH 7.0. The calcium-ion activities were obtained by the method as described by De Kort *et al.*.[23]

The zeta potential was measured with the Zetasizer Nano Z (Malvern Instruments, Worcestershire, UK) equipped with 4 mW He–Ne Laser. Measurements were based on the principles of Laser Doppler Electrophoresis. The electrophoretic mobility U_E was measured and the zeta potential was calculated by the Dispersion Technology Software provided by Malvern according to the Henry equation:

$$U_E = \left(\frac{2\varepsilon\zeta}{3\eta}\right) f(ka) \qquad (4.1)$$

where ε is the dielectric permittivity of the solvent, η the viscosity of the solution, and *f(ka)* Henry's function, respectively. A value of 1.5 was used for *f(ka)*, which is referred to as the Smoluchowski approximation, because the radius of the particles was much larger than the Debye Length of the electric double layer. Disposable folded capillary Zetasizer Nano cells of 1.5 mL (DTS1060, Malvern Instruments, Worcestershire, UK) were used for the measurements. Prior to analysis, samples were diluted 100-fold in demineralized water and subsequently filtered through disposable Nalgene® Syringe cellulose-acetate filters with a pore size of 0.8 µm (Nalgene, Nunc, Thermo Scientific, Rochester NY, USA). Analyses were performed at a cell temperature of 25°C and voltage of 100 V.

The turbidity of the samples was measured at ambient temperature with a spectrophotometer (4053 Kinetics, LKB Biochrom, Midland, Canada) using plastic cuvettes with a pathway of 1 cm. A wavelength of 700 nm was used for the measurements. Samples were diluted 10-fold in demineralized water to be within the detection limits of the spectrophotometer.

The viscosity of the samples was measured between shear rates of 1 to 1000 s^{-1} at a temperature of 20°C with an MCR 300 rheometer (Anton Paar Physica, Graz, Austria) using a cup and bob geometry (CC27 cylinder).

4.3 Results and Discussion

4.3.1 Heat coagulation time

The HCT of the MCI solution with and without calcium chelators was measured at pH 6.7, 7.0, and 7.3 with the Klaro-graph at 126°C for maximal 90 min. Calcium-ion activity, viscosity, and turbidity analyses were measured before heating to obtain information about changes in the concentration of free calcium ions and integrity of the micellar structure after addition of calcium chelators. Overviews of the results are shown for pH 6.7 in Fig. 4.2, pH 7.0 in Fig. 4.3, and pH 7.3 in Fig. 4.4. The HCT markedly increased upon addition of the calcium chelators, an effect which was most pronounced at pH 6.7. The differences in HCT were investigated in relation to the initial calcium-ion activity, viscosity, and turbidity of the samples. The results can be divided into four groups: 1) reference samples; 2) Na_2UMP; 3) Na_2HPO_4, TSC, and SP; 4) SHMP.

4.3.1.1 Reference samples

The HCT of the reference samples (no chelator addition) increased with increasing pH, from 2 min at pH 6.7 (Fig. 4.2), to 40 min at pH 7.0 (Fig. 4.3), and 55 min at pH 7.3 (Fig. 4.4). This strong increase in HCT is in line with the HCT as function of pH for whey-protein-free casein micelle dispersions heated at 140°C in sealed glass tubes in a temperature-controlled oil bath.[27] Van Mil & De Koning[25] found a strong correlation for HCT values determined with the Klaro-graph and with glass tubes placed in an oil bath. The increase in HCT as a function of pH is due to a lower calcium-ion activity, a lower hydrogen ion concentration, and a higher net negative charge on the casein micelles, inducing more electrostatic repulsion between the negatively charged caseins.[2] Both effects result in an improved heat stability.

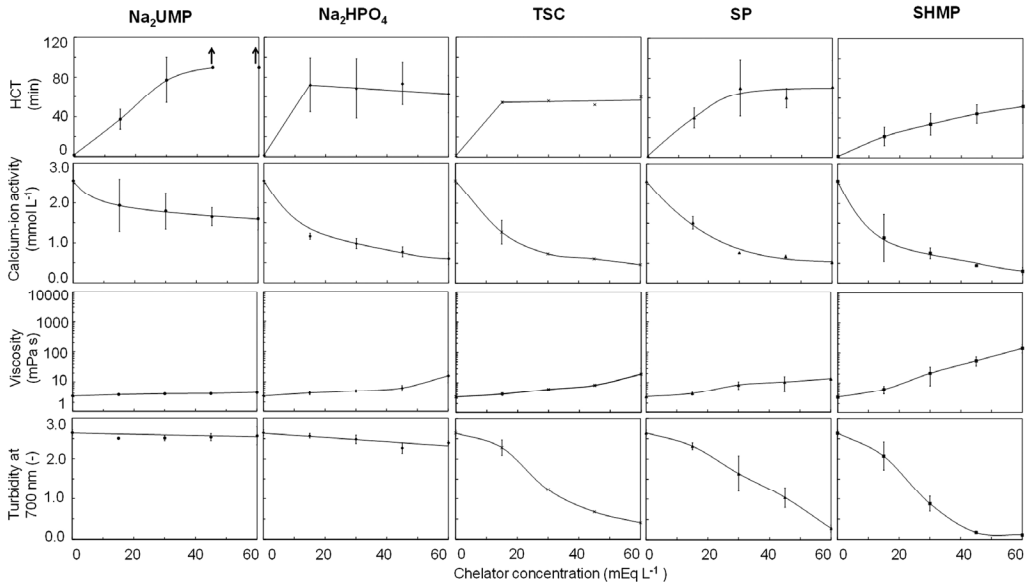

Figure 4.2 HCT, calcium-ion activity, viscosity, and turbidity of the MCI solution at a pH 6.7 as a function of calcium chelator concentration. Results are the means for at least duplicates; error bars represent standard deviations. (↑) indicates that no coagulation appeared after heating for 90 min.

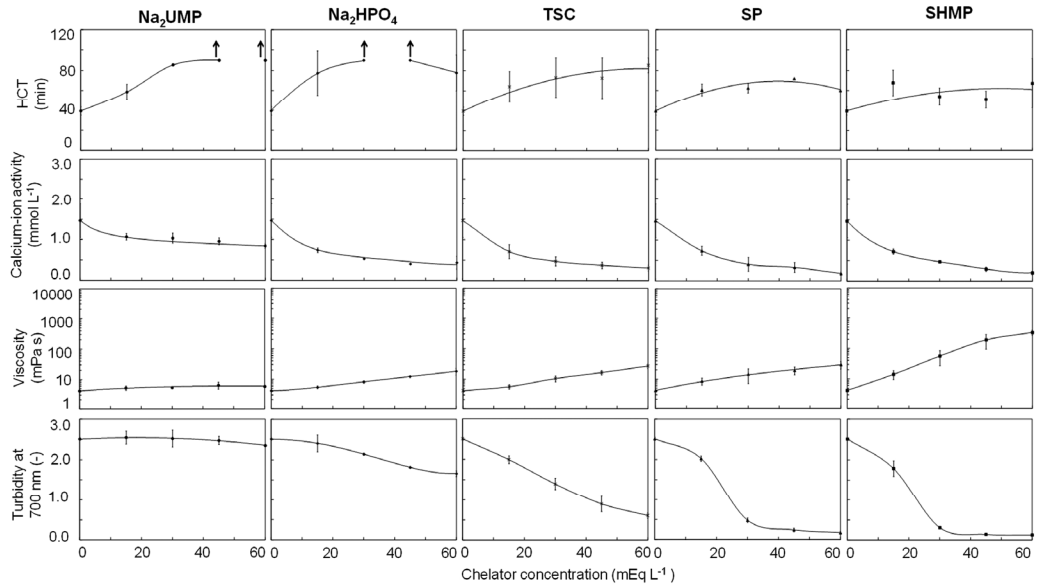

Figure 4.3 HCT, calcium-ion activity, viscosity, and turbidity of the MCI solution at a pH 7.0 as a function of calcium chelator concentration. Results are the means for at least duplicates; error bars represent standard deviations. (↑) indicates that no coagulation appeared after heating for 90 min.

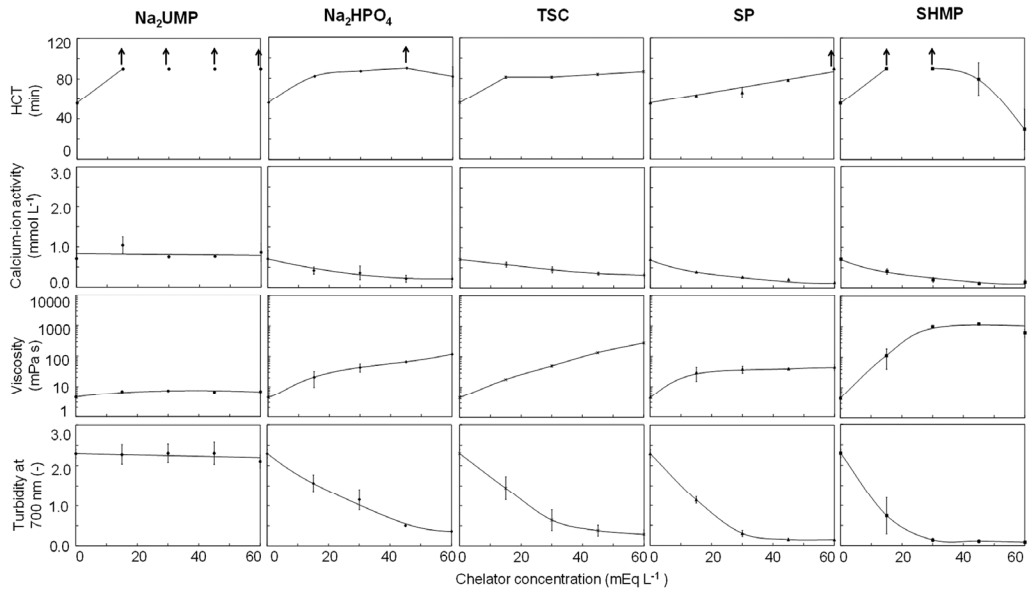

Figure 4.4 HCT, calcium-ion activity, viscosity, and turbidity of the MCI solution at a pH 7.3 as a function of calcium chelator concentration. Results are the means for at least duplicates; error bars represent standard deviations. (↑) indicates that no coagulation appeared after heating for 90 min.

4.3.1.2 Addition of Na_2UMP

Na_2UMP is very effective in increasing the heat stability of the MCI solution at all three pH values (Fig. 4.2–4.4). At higher pH a lower concentration of Na_2UMP was needed to give the MCI solution a HCT of more than 90 min. Addition of 45 mEq L^{-1} Na_2UMP reduced the concentration of free calcium ions with approximately 35% at pH 6.7 and 7.0. This suggests that a calcium-ion activity below ~2 mmol L^{-1} is sufficient to prevent protein aggregation. Consequently, a strong increase in HCT was measured for the Na_2UMP samples. Because Na_2UMP is a relative weak calcium-binder[23] the viscosity and turbidity of the solutions remained constant at all pH values, implicating that the micellar structure was not affected.[19] Therefore, it is most likely that the decrease in calcium-ion activity was the main driver for the increase in HCT in Na_2UMP samples.

4.3.1.3 Addition of Na_2HPO_4, TSC, and SP

Addition of Na_2HPO_4, TSC, and SP induced large increases in HCT at pH 6.7 and 7.0 (Fig. 4.2 and 4.3). The HCT at pH 7.3 (Fig. 4.4) was already high and additions of these chelators did not give strong pronounced changes in HCT. Slightly higher HCT values were measured for Na_2HPO_4 than for TSC and SP at pH 7.0 and 7.3 (e.g. no coagulation measured for 30 or 45 mEq L^{-1} Na_2HPO_4 at pH 7.0 and for 45 mEq L^{-1} Na_2HPO_4 at pH 7.3). The calcium-ion activity decreased and, in contrast with Na_2UMP, the viscosity increased to comparable levels after addition of Na_2HPO_4, TSC, or SP at each pH value. The decrease in calcium-ion activity became smaller with increasing pH, whereas the increase in viscosity became larger with increasing pH. This is related to the increase in calcium phosphate complex formation and increase in electrostatic repulsion between the caseins with increasing pH, respectively.[2] The strong decrease in calcium-ion activity at pH 6.7 with increasing chelator concentration is attributed to the calcium-binding capacity of Na_2HPO_4, TSC, and SP, which induced the strong increase in HCT. At pH 7.0 and 7.3 the calcium-ion activities stayed below 1.5 mmol L^{-1} and 1.0 mmol L^{-1}, respectively, which was sufficiently to give the samples a high HCT.

The slight differences in HCT that were measured for Na_2HPO_4, TSC, and SP might be related to their differences in turbidity before heating. In a previous study[19] it was concluded that the decrease in turbidity is most likely due to dissociation of casein micelles, which occurred in the order SP > TSC > Na_2HPO_4. It was further determined by capillary zone electrophoresis that the ratio of specific caseins in ultracentrifuged supernatants was similar to ratio as naturally present in milk, indicating that a mixture of intact and dissociated casein micelles was formed in the MCI solution upon addition of different types and concentrations of calcium chelators.[19] This conclusion was in agreement with the observations of Pitkowski *et al.*[28] and Griffin *et al.*.[18] Dynamic light scattering analyses confirmed that indeed smaller particles were formed upon addition of calcium chelators to MCI solutions. The onset and extent of micelle dissociation was determined by the calcium-binding capacity of the calcium chelators leading to dissolution of CCP from the micelle.[29] The turbidity results shown in Fig. 4.2 to 4.4 indicate that the concentration of dissociated casein micelles after addition of e.g. 30 mEq L^{-1} calcium chelator is highest for SP, followed by TSC, and Na_2HPO_4. Slightly higher HCT values were measured for Na_2HPO_4 than for TSC and SP at pH 7.0 and 7.3, suggesting that the heat stability decreases when the concentration of dissociated casein micelles increases in the solution. Dissociated casein micelles are apparently more sensitive for calcium-induced protein aggregation than

intact casein micelles, because the mainly inside the casein micelle located α_{s1}-, α_{s2}-, and β-caseins are more sensitive for aggregation with calcium ions than outside the micelle located κ-casein.[30] The exposure of α_{s1}-, α_{s2}-, and β-caseins to calcium ions strongly increases upon casein micelle dissociation, leading to increased calcium-induced protein aggregation. As a result, the heat stability decreased in the order of Na_2HPO_4 > TSC > SP. Augustin *et al.*[11] also observed that the decrease in heat stability of recombined concentrated milk containing Na_2HPO_4, TSC, or EDTA was dependent on loss of the micellar structure.

Panouillé *et al.*[31] observed that casein micelles dissociate into small micellar particles with a diameter of 24 nm after addition of polyphosphate to casein solutions. In another study of Panouillé *et al.*[32] it was mentioned that these small micellar particles have a similar size as the aggregates present in sodium caseinate solutions. The heat stability of sodium caseinate is relatively high[33], but can be markedly reduced in the presence of ionic calcium.[32] Fox *et al.*[33] also described that the heat stability of sodium caseinate and CCP-free milk shows a greater reduction in the presence of heat-precipitated calcium phosphate than milk containing unaltered casein micelles. This also confirms that lower heat stabilities can be expected in dissociated casein micelle solutions containing high concentrations of calcium ions.

The HCT of Na_2HPO_4 samples was considerably lower than for Na_2UMP samples upon addition of ≥ 45 mEq L^{-1} calcium chelator at pH 6.7. Addition of 15 mEq L^{-1} Na_2HPO_4 or Na_2UMP at pH 6.7 reduced the concentration of free calcium ions by approximately 55% and 25%, respectively, because Na_2HPO_4 has a stronger calcium-binding capacity than Na_2UMP. The decrease in free calcium ions at 15 mEq L^{-1} calcium chelator was sufficient to obtain a HCT of more than 70 min for Na_2HPO_4 and of just 40 min for Na_2UMP. Surprisingly, at higher chelator concentrations (at pH 6.7) the HCT increased more for Na_2UMP than for Na_2HPO_4. The calcium-ion activity in both samples was sufficiently low to increase the HCT. However, in Na_2HPO_4 samples calcium phosphate complexes are formed that precipitate on the casein micelle[34], whereas in Na_2UMP samples the micellar structure is negligibly affected. Precipitation of calcium phosphate complexes on the micelle reduced the protein charge in the casein micelles[33] and, subsequently, the heat stability of Na_2HPO_4 samples.

4.3.1.4 Addition of SHMP

The lowest HCT values were measured after addition of SHMP at pH 6.7 and 7.0 in comparison to the other calcium chelators. The SHMP samples became very viscous with increasing SHMP

concentration, making it difficult to determine coagulation accurately, because the glass balls could not freely move in the Klaro-graph tubes. The high viscosities are due to the cross-links formed between the caseins by SHMP.[19] Samples were gelled upon addition of more than 45 mEq L^{-1} SHMP at all three pH values. Addition of ≥ 45 mEq L^{-1} SHMP at pH 7.3 caused a sharp decrease in the HCT, which is probably due to the high initial viscosity. The net negative charge of the casein micelles and depletion of CCP from the casein micelles probably reached a critical value, releasing κ-casein from the micellar surface and inducing micelle dissociation during heating. The turbidity results indeed indicate that most of the casein micelles were dissociated at ≥ 45 mEq L^{-1} SHMP before heating. This may have caused the strong increase in coagulation for the SHMP samples, because, as explained in paragraph 4.3.1.3, dissociated casein micelles are more susceptible to calcium-induced protein aggregation.

4.3.2 Heat-induced changes

Samples with 0, 15, and 60 mEq L^{-1} phosphate or citrate were heated for 15, 35, and 55 min in the oil bath to determine heat-induced changes. A concentration of 15 mEq L^{-1} was selected, because the largest increase in HCT was measured between 0 and 15 mEq L^{-1}. The samples were analyzed for pH, calcium-ion activity, turbidity, viscosity, and zeta potential after heating. Based on the changes taking place during heating the results can be divided in three groups: 1) reference samples; 2) Na_2UMP, Na_2HPO_4, TSC, and SP; 3) SHMP.

4.3.2.1 Reference samples

The results of the reference samples containing no chelators are summarized in Table 4.5. The pH decreased by 0.29 to 0.63 units during heating and it decreased more in the samples with higher initial pH. The pH decrease is mainly attributed to calcium phosphate precipitation. The occurrence of maillard reactions seems to be less likely, because the MCI solution contains a negligible amount of lactose (≤ 0.05%, w/v). The pH decrease was less pronounced at pH 6.7 than at pH 7.0 or 7.3, which suggests that more calcium phosphate precipitated at higher pH. The samples coagulated within 15 min of heating at pH 6.7 as a result of the higher calcium-ion activity and lower negatively charged caseins. The increase in turbidity during heating indicated that protein aggregation occurred at all three pH values.

Table 4.5 Measured pH, calcium-ion activity, turbidity, viscosity, and zeta potential after heating the reference samples at a pH of 6.7, 7.0, and 7.3 for 0, 15, 35, and 55 min in a temperature controlled oil bath. Results are presented as means of at least duplicates with corresponding standard deviations.

pH	Time (min)	Measured pH (-)	Calcium-ion activity (mmol L^{-1})	Turbidity at 700 nm (-)	Viscosity (mPa s)	Zeta potential (mV)
6.7	0	6.70	2.57 ± 1.06	2.65 ± 0.20	3.31 ± 0.20	-22.83 ± 1.17
	15	6.48 ± 0.03	1.39 ± 0.43	nd*	coagulated	-27.85 ± 1.06
	35	6.46 ± 0.02	1.44 ± 0.32	nd*	coagulated	-28.00 ± 3.25
	55	6.41 ± 0.03	1.34 ± 0.25	nd*	coagulated	-26.60 ± 0.42
7.0	0	7.00	1.47 ± 0.41	2.51 ± 0.03	4.18 ± 0.37	-23.25 ± 3.77
	15	6.71 ± 0.02	0.91 ± 0.40	2.93 ± 0.01	3.04 ± 0.05	-22.30 ± 0.14
	35	6.71 ± 0.05	0.99 ± 0.51	2.95 ± 0.01	3.14 ± 0.07	-22.95 ± 3.33
	55	6.58 ± 0.04	0.97 ± 0.59	2.98 ± 0.02	3.72 ± 0.15	-21.73 ± 0.85
7.3	0	7.30	0.71 ± 0.21	2.30 ± 0.10	4.45 ± 0.39	-21.55 ± 0.06
	15	6.94 ± 0.16	0.80 ± 0.31	2.53 ± 0.02	3.12 ± 0.08	-19.56 ± 0.42
	35	6.85 ± 0.12	0.80 ± 0.20	2.50 ± 0.01	3.01 ± 0.01	-22.63 ± 0.97
	55	6.67 ± 0.13	0.84 ± 0.35	2.60 ± 0.07	3.10 ± 0.07	-24.04 ± 2.75

*nd= not detectable: turbidity exceeded the maximum detectable value of the spectrophotometer because of coagulation of the sample.

4.3.2.2 Addition of Na2UMP, Na2HPO4, TSC, or SP

The pH decrease after heating for 55 min in the oil bath for 15 and 60 mEq L^{-1} Na_2UMP, Na_2HPO_4, TSC, and SP samples at pH 6.7, 7.0, and 7.3 was comparable to the pH decrease that was measured for the reference samples (Table 4.5). None of these samples showed visible coagulation after heating for 55 min in the oil bath. The calcium-ion activities of these samples remained constant or slightly decreased. The calcium-ion activities before heating were already sufficiently low because of the calcium-binding capacity of the chelators and the stronger calcium phosphate binding in the micelles with increasing pH (Fig. 4.2 to 4.4). The changes in zeta potential were negligible in these samples. The turbidity increased and viscosity decreased in the samples during heating because of calcium phosphate precipitation and irreversible changes occurring in the caseins.[3-6] Panouillé *et al.*[32] also observed an increase in turbidity during heating (at 80°C) of casein solutions with polyphosphate, which was due to aggregation of the small micellar particles.

In Fig. 4.6 it is shown that the turbidity of the MCI solution with 60 mEq L^{-1} SP at pH 7.3 only slightly increased during heating. This sample behaved remarkably differently than the reference sample and samples with 60 mEq L^{-1} Na_2HPO_4 or TSC. The strongly negatively charged SP molecules have the ability to bind to the positively charged amino acid residues[16, 17], increasing the electrostatic repulsion between the caseins. This reduced protein aggregation and, as a result, a high HCT of more than 90 min was measured after addition of 60 mEq L^{-1} SP at pH 7.3 (Fig. 4.4). Only a slight decrease in viscosity and increase in zeta potential was measured for this SP sample during heating (Fig. 4.6), suggesting that the strong repulsion between the caseins and binding of SP to the caseins was maintained during heating.

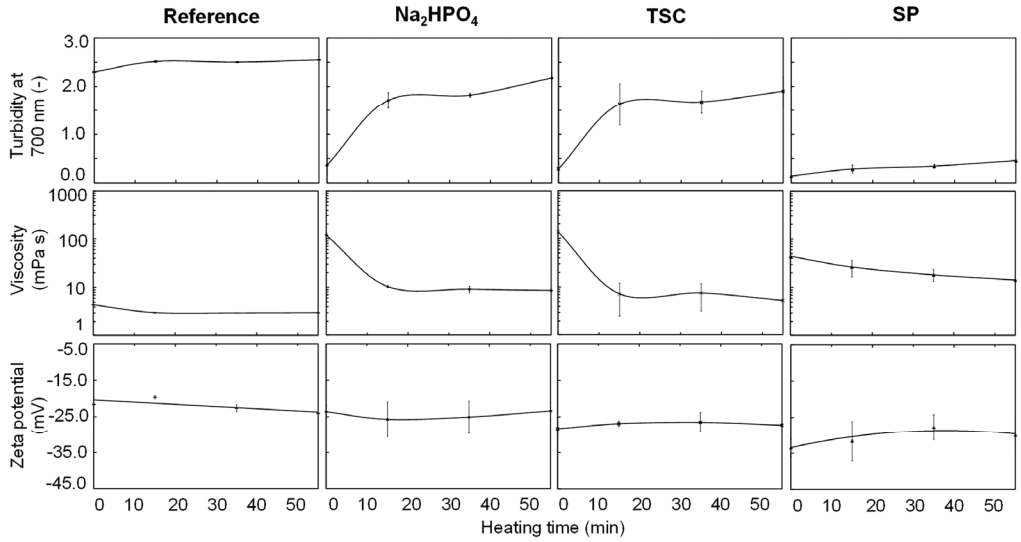

Figure 4.6 Turbidity, viscosity, and zeta potential of the MCI solution at a pH 7.3 of the reference samples and those with 60 mEq L^{-1} Na_2HPO_4, TSC, and SP as a function of the heating time in the oil bath. Results are the means of at least duplicates; error bars represent standard deviations.

Fig. 4.6 shows that the viscosity of the 60 mEq L^{-1} Na_2HPO_4 or TSC samples at pH 7.3 strongly decreased during heating to values that were slightly higher than the reference samples. The decrease in viscosity is related to the changes that occur in the micelles during heating. Fox[3] described for milk that during heating the viscosity decreases because of dissociation of the micelles (i.e. solubilization of casein and CCP and release of κ-casein). These heat-induced

changes make the dissociated casein micelles more susceptible to coagulation. Hence, with the onset of coagulation, the viscosity strongly increases. As the Na_2UMP, Na_2HPO_4, TSC, and SP samples did not coagulate in the oil bath, this strong increase in viscosity was not observed.

4.3.2.3 Addition of SHMP

SHMP gave a more pronounced decrease in pH during heating than the reference samples and the other calcium chelators at all three pH values: a pH decrease of 0.66-0.90 (Table 4.7) versus 0.29-0.63 (Table 4.5). This caused an increase in the concentration of free calcium ions, which made the samples more susceptible to calcium-induced protein aggregation. As a result, coagulation was measured after heating for 55 min in the oil bath upon addition of 15 or 60 mEq L^{-1} SHMP at pH 6.7. These low heat stabilities are in agreement with the low HCTs that were measured for these samples (Fig. 4.2). In a previous study a strong decrease in pH for SHMP in a calcium chloride solution upon heating was observed.[23] SHMP hydrolyzes into sodium trimetaphosphate and sodium orthophosphate in acidic conditions[35] and this hydrolysis probably occurred to some extent in the MCI solutions during heating as well. This induced, besides the pH decrease, the release of calcium ions, which can cause calcium-induced protein aggregation. The calcium-ion activity was lower at higher pH and upon addition of 60 mEq L^{-1} SHMP, as more calcium ions were part of the CCP complexes or bound to SHMP, respectively. Moreover, less SHMP will be hydrolyzed at higher pH.[35]

Table 4.7 Measured pH, calcium-ion activity, turbidity, viscosity, and zeta potential after heating samples with 15 mEq L^{-1} and 60 mEq L^{-1} SHMP at a pH of 6.7, 7.0, and 7.3 for 0, 15, 35, and 55 min in a temperature controlled oil bath. Results are presented as means of at least duplicates with corresponding standard deviations.

	pH	Time (min)	Measured pH (-)	Calcium-ion activity (mmol L^{-1})	Turbidity at 700 nm (-)	Viscosity (mPa s)	Zeta potential (mV)
15 mEq L^{-1} SHMP	6.7	0	6.70	1.13 ± 0.59	2.08 ± 0.35	6.04 ± 1.77	-25.85 ± 2.06
		15	6.21 ± 0.14	1.14 ± 0.19	2.91 ± 0.02	5.66 ± 2.17	-24.78 ± 1.30
		35	6.24 ± 0.17	1.37 ± 0.10	2.56 ± 0.01	coagulated	-24.13 ± 2.45
		55	6.04 ± 0.12	1.92 ± 0.11	2.63 ± 0.02	coagulated	-22.10 ± 0.57
	7.0	0	7.00	0.72 ± 0.07	1.79 ± 0.19	14.0 ± 4.48	-27.40 ± 0.78
		15	6.50 ± 0.09	0.61 ± 0.02	1.76 ± 0.08	5.04 ± 1.63	-27.57 ± 1.25
		35	6.36 ± 0.15	0.74 ± 0.16	2.13 ± 0.04	3.26 ± 0.24	-24.52 ± 3.02
		55	6.27 ± 0.02	0.42 ± 0.15	2.66 ± 0.11	3.77 ± 0.42	-25.13 ± 1.13
	7.3	0	7.30	0.41 ± 0.07	0.75 ± 0.45	115 ± 34.3	-31.33 ± 1.91
		15	6.76 ± 0.20	0.56 ± 0.03	2.62 ± 0.02	4.08 ± 0.08	-26.58 ± 2.81
		35	6.74 ± 0.13	0.30 ± 0.07	1.99 ± 0.01	3.89 ± 0.25	-27.40 ± 2.72
		55	6.60 ± 0.17	0.23 ± 0.13	2.36 ± 0.03	4.27 ± 0.45	-24.95 ± 2.36
60 mEq L^{-1} SHMP	6.7	0	6.70	0.30 ± 0.09	0.11 ± 0.01	144 ± 42.2	-33.10 ± 2.18
		15	6.06 ± 0.12	0.40 ± 0.05	2.79 ± 0.02	5.53 ± 2.43	-22.93 ± 0.90
		35	5.94 ± 0.19	0.49 ± 0.15	2.86 ± 0,02	6.53 ± 1.90	-24.40 ± 0.70
		55	5.85 ± 0.11	0.54 ± 0.07	2.52 ± 0.03	coagulated	-21.15 ± 0.21
	7.0	0	7.00	0.21 ± 0.05	0.13 ± 0.01	331 ± 62.9	-37.93 ± 3.39
		15	6.29 ± 0.01	0.30 ± 0.08	2.07 ± 0.02	5.54 ± 1.07	-27.87 ± 2.43
		35	6.17 ± 0.08	0.26 ± 0.02	2.58 ± 0.01	4.30 ± 0.11	-28.57 ± 4.09
		55	6.10 ± 0.08	0.31 ± 0.04	2.73 ± 0.07	coagulated	-23.25 ± 4.93
	7.3	0	7.30	0.14 ± 0.06	0.09 ± 0.03	593 ± 179	-43.00 ± 0.76
		15	6.78 ± 0.39	0.54 ± 0.10	1.86 ± 0.04	11.1 ± 5.92	-27.60 ± 5.16
		35	6.65 ± 0.37	0.28 ± 0.02	2.44 ± 0.03	10.8 ± 7.30	-26.53 ± 4.04
		55	6.57 ± 0.31	0.15 ± 0.02	2.49 ± 0.06	9.39 ± 5.67	-24.53 ± 3.99

The casein micelle becomes more negatively charged upon addition of SHMP, because the negatively charged SHMP molecule has the ability to bind to the positively charged amino acid residues of the casein micelle.[13] The strong decrease in viscosity and increase in zeta potential (e.g. from -33.10 to -21.15 mV at pH 6.7 for 60 mEq L^{-1}) in the SHMP samples at all three pH values, suggests that SHMP was released from the caseins during heating. As a consequence, SHMP cross-links between the caseins were released during heating as well. It is likely that also calcium ions are involved in the SHMP cross-links, because the calcium-ion activity increased

during heating. The increase in the concentration of free calcium ions during heating most probably initiated calcium-induced protein aggregation. The release of κ-casein from the casein micelles may also contribute to the strong increase in the zeta potential. κ-Casein depletion was found to be more pronounced at higher pH, which increased the sensitivity to calcium-induced protein-aggregation.[7, 36] As a result, a strong decrease in HCT was measured upon addition of ≥ 45 mEq L^{-1} SHMP at pH 7.3 (Fig. 4.4). The turbidity also strongly increased during heating in all SHMP samples, which is attributed to calcium-induced protein aggregation.[3] Overall, the MCI solutions with SHMP are more susceptible to heat coagulation than those with the other calcium chelators because of the strong decrease in pH and increase in calcium-ion activity during heating. Hence, contrary to the other calcium chelators, the heat-induced changes that occurred in the SHMP samples did play an important role in heat stability.

4.5 Conclusions

The heat stability of a MCI solution can be improved by increasing the pH or by addition of calcium chelators. Na_2UMP is the most effective heat stabilizer, as it binds sufficient free calcium ions to reduce protein aggregation without affecting the micellar structure. Na_2HPO_4, TSC, and SP induced a comparable increase in HCT in the MCI solutions, but the increase in HCT remained smaller compared to Na_2UMP. The slight differences in HCT for these samples were explained by the higher sensitivity of dissociated casein micelles for calcium-induced protein aggregation. SHMP was the least effective heat stabilizer. SHMP cross-linked the caseins, but these cross-links were apparently broken during heating, which decreased the pH and increased the calcium-ion activity during heating, resulting in reduced heat stability.

In conclusion, calcium chelators increase the heat stability of the MCI solution to different extents and these differences are attributed to the calcium-ion activity and state of the micelle structure before and during heating. This study showed that optimization of heat stability of dairy systems is complex and can be manipulated by careful selection of the type and concentration of calcium chelator.

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Chapter 5

Dissociation of casein micelles by calcium chelators

Based on: de Kort, E.J.P., V. Urbonaite, M. Minor, E. van der Linden, A.C.M. van Hooijdonk. *Submitted for publication*.

Abstract

Casein micelles in solution might dissociate upon addition of calcium chelators. Dissociation depends on the type and concentration of calcium chelator used. In this study dynamic light scattering (DLS) was used to determine to what extent different calcium chelators affect micelle dissociation. Small particles with a diameter of 30 to 50 nm were observed upon micelle dissociation. The calcium chelators induced micelle dissociation in the order of sodium hexametaphosphate > sodium phytate > trisodium citrate > disodium hydrogen phosphate. Simulations of the ion equilibria with an ion speciation model showed that the extent of casein micelle dissociation follows the calcium-binding capacity of the calcium chelators, which in turn leads to dissolution of colloidal calcium phosphate from the casein micelle.

5.1 Introduction

Casein micelles are present in milk as polydisperse spherical complexes with an average diameter of 200 nm.[1] Casein micelles are heterogeneous, hydrated, dynamic structures with a loose packing and a high porosity.[2-5] They consist of four types, namely α_{s1}-, α_{s2}-, β-, and κ-casein, and colloidal calcium phosphate (CCP). CCP is essential for maintaining the micellar structure: casein micelles dissociate when CCP is chelated or solubilized.[6, 7] Micelle dissociation can be induced by high-pressure treatment[8], pH decrease[9, 10], or calcium chelators.[11-13]

The effect of calcium chelators, such as polyphosphate, citrate, or EDTA, on micelle dissociation in milk has been investigated in several studies.[12-15] Panouillé *et al.*[13] determined that the small micellar particles formed upon micelle dissociation have a diameter of 24 nm. In another study[16] they mentioned that these small micellar particles appeared to be similar to the aggregates present in sodium caseinate solution.

Reported particle sizes of intact and dissociated casein micelles are not always consistent. Variability in reported particle sizes are due to using: 1) different particle size measurement techniques[12, 14, 15, 17-20]; 2) different dilution media[12, 14, 16, 18, 21]; 3) different temperatures for preparation and analysis of the samples[19, 21, 22]; 4) different milk types with varying casein concentration[12, 13, 15]; or 5) different concentrations or types of calcium chelator and mixtures.[12, 14, 15, 22, 23] These varying conditions and concentrations give different casein:chelator ratios in the milk solutions and hence different extents of micelle dissociation.

In a previous study[11] it was shown that various calcium chelators induce a different decrease in turbidity in a concentrated micellar casein solution. It was suggested that the differences could be explained by the degree dissociation of the casein micelles. In this study the aim was to investigate to what extent casein micelles dissociate after addition of different types and concentrations of calcium chelators to a concentrated micellar casein solution. Dynamic light scattering (DLS) was used to measure particle size distributions in the casein solutions and to determine the ratio between small particles and intact micelles. An ion speciation computer model[24] was used to investigate the effect of calcium-binding capacity of the calcium chelators on ion equilibria. With DLS and the computer model a relation could be established between the extent of casein micelle dissociation and the effect of calcium-binding capacity of the chelators on the dissolution of CCP from the casein micelle in a concentrated micellar casein solution.

5.2 Material and Methods

5.2.1 Sample preparation

Micellar casein isolate (MCI) powder (Nutripro^{TM}) was supplied by DairyGold Food Ingredients (Cork, Ireland). The powder contains 85% (w/w) protein, of which ≤5% is whey protein. A MCI solution with 9% (w/v) protein was prepared according to the procedure described by De Kort *et al.*.[11] Also, a sodium caseinate solution with 9% (w/v) protein was prepared. The preparation was done in the same way as for the MCI solution. Sodium caseinate was supplied by DMV (Veghel, The Netherlands).

Stock solutions were prepared of disodium hydrogen phosphate (Na_2HPO_4) (Merck & Co. Inc, Darmstadt, Germany), sodium hexametaphosphate (SHMP) (VWR International Ltd, Poole, England), phytic acid dodecasodium salt hydrate (SP) (Sigma-Aldrich GmbH, Steinheim, Germany), and trisodium citrate (TSC) (Gadot Biochemical Industries Ltd., Haifa Bay, Israel). Increasing amounts of these stock solutions were added to MCI solutions in order to obtain final chelator concentrations of 0-100 mEq L^{-1}. Addition of the chelators was based on milliequivalents so as to add a similar amount of negative charges to the samples. Only sodium sources were used, because the type of counter-ion might influence the ion equilibria.[25] Before the samples were brought to their final weight, the pH of the samples was set to pH 7.0 ± 0.05 with 1 mol L^{-1} sodium hydroxide (Sigma-Aldrich GmbH, Steinheim, Germany) or 1 mol L^{-1} hydrochloric acid (Merck & Co. Inc, Darmstadt, Germany). Samples were stored overnight at 20°C for approximately 17 h to let them equilibrate. The pH of the samples was readjusted to 7.0 ± 0.05 the next morning, in case changes had occurred during storage.

For DLS measurements, samples were diluted 100-fold in demineralized water, filtered through disposable Nalgene® syringe cellulose acetate filters with a pore size of 0.8 µm (Nalgene Nunc International Corporation, Rochester, NY, USA), and measured in disposable sizing cuvettes (MA DI67/754 PKG100 polystyrene, Sarstedt AG & Co., Nümbrecht, Germany). A dilution range indicated that the average particle diameter remained constant between 10- and 500-fold dilution in demineralized water. A gradual decrease of 10% in average particle diameter was measured (i.e. micelle dissociation) when the 100-fold diluted samples were kept at ambient temperature for 9 h. As all samples were measured within 30 min after dilution; the change in average particle diameter during the measurements was negligible. The decrease in particle size upon dilution in demineralized water was in line with the observations of Beliciu and Moraru[21].

It was concluded in that study that the particle diameter can be kept effectively constant upon dilution of the casein micelles in casein-depleted ultrafiltered permeate. However, depleted ultrafiltered permeate did not have a constant composition with increasing chelator concentration.

5.2.2 DLS measurements and size distribution analyses

Particle size distributions were determined by DLS using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, England) equipped with 4 mW He–Ne laser. All DLS measurements were performed at 25°C and at a scattering angle of 173° with backscattering. The particle size distribution in the samples was derived from a deconvolution of the measured intensity autocorrelation function of the samples. For this deconvolution a non-negative constrained least squares (NNLS) fitting algorithm was used. Examples of this algorithm are the general purpose and multiple narrow mode functions, which are both available in the Dispersion Technology Software of Malvern Instruments. Data were analyzed with the two algorithm functions. The multiple narrow mode algorithm function could be used, because the solutions showed a bimodal distribution. By analyzing the data with the multiple narrow mode function, which has a more aggressive alpha parameter (also called the regularizer) than the general purpose mode function, a baseline resolution between the two peaks was achieved.[26] Consequently, the volume peak areas (i.e. relative particle concentrations) of the two separated particle populations could be calculated by the Dispersion Technology Software. The volume peak area results were expressed as percentage of volume peak area.

In this paper DLS results are shown as Z-average diameter, which is defined as the intensity weighted mean size of all particles in the solution and is obtained with cumulants analysis as described in ISO13321. The size distribution curves are shown as volume distributions to emphasize the presence of a tail or second peak in the plots. The intensity particle size distribution curves were converted into volume size distribution curves by using the Mie theory.[27] For this conversion a refractive index of 1.57 was used for the caseins and smaller particles[12, 28] and 1.33 for the continuous phase (i.e. water). A refractive index of 1.57 for the casein particles is probably an overestimation, because it corresponds to the non-aqueous part of the casein micelle, while a casein micelle contains approximately 80% water. However, calculating the DLS results using refractive indices between 1.35 and 1.60 for the casein

micelles did not affect the particle size distribution. The viscosity of the 100-fold diluted samples was taken as the dispersant viscosity (water, 0.89 mPa s). Samples were equilibrated for 120 s before a measurement was started. Each measurement was performed in triplicate.

5.2.3 Equilibrium Ion Speciation model

The computer program AEsolve (Halotec Instruments, The Netherlands) was used to investigate the calcium-binding capacity of the calcium chelators. The Equilibrium Ion Speciation (EIS) model, which is based on intrinsic association constants and solubility products, was selected for the ion equilibria simulations. Details of the computer program AEsolve and the conditions of the EIS model are described by Gao.[24] Computer simulations were only performed for Na_2HPO_4, TSC, and SHMP. Casein and SP could not be simulated in the program, because the association constants are not available in the AEsolve program. The composition of Simulated Milk Ultrafiltrate (SMUF)[29] with increased $CaCl_2$ and KH_2PO_4 concentrations was used as starting point for the simulations. The concentrations of these salts were increased to a level as present in the MCI solution (60 mmol L^{-1} Ca and 44 mmol L^{-1} P). Calculations were performed with concentration ranges of 0 to 100 mEq L^{-1} Na_2HPO_4, TSC, and SHMP in the adapted SMUF solution in order to study the calcium binding competition in the mineral blend. The pH was set to 7.0 for all calculations.

5.3 Results and Discussion

5.3.1 Effect of calcium chelators on particle size distribution

DLS measurements were performed on the MCI solutions to which concentration ranges of 0-100 mEq L^{-1} Na_2HPO_4, TSC, SP, or SHMP were added. The measured particle size distributions are shown in Fig. 5.1. The particle size distribution of the reference sample (no chelator addition) is shown in every figure to guide the eye. As an approximation of the average diameter the top of the peak in the reference solution was used. The obtained value of 190 nm is in the range of reported diameters between 40 and 400 nm for casein micelles in milk.[1, 2, 30]

Dissociation of the casein micelles occurred when calcium chelators were added to the MCI solution. The DLS measurements in Fig. 5.1 indicate that both intact and dissociated casein micelles were present in the MCI solutions. Pitkowski *et al.*[14] also observed that a mixture of

intact and dissociated micelles was present in casein solutions after addition of polyphosphate or EDTA.

As is shown in Fig. 5.1, the calcium chelators induced micelle dissociation at different concentrations. The threshold concentration, at which smaller particles were detected, was at 25 mEq L^{-1} SHMP (Fig. 5.1A), 30 mEq L^{-1} SP (Fig. 5.1B), 35 mEq L^{-1} TSC (Fig. 5.1C), and 90 mEq L^{-1} Na_2HPO_4 (Fig. 5.1D). Micelle dissociation was most pronounced for SHMP samples, which is consistent with the strong decrease in turbidity found previously.[11] Micelle dissociation occurred less drastically with increasing concentration in the SP and TSC samples. The particle size distribution peak started to shift to particles with smaller diameters at 30 mEq L^{-1} SHMP, reaching values below 10 nm above 50 mEq L^{-1} SHMP. Micelle dissociation was less pronounced for SP than for SHMP: 45 mEq L^{-1} SP was required to dissociate the majority of the casein micelles. The shape of the particle size distribution peak slightly changed between 45 and 100 mEq L^{-1} SP, but the particle diameter remained larger than 10 nm. A possible explanation for the different behavior of SHMP and SP may be the different charge distributions around SHMP and SP molecules as earlier discussed by De Kort *et al.*.[11] For TSC, the onset of casein micelles dissociation starts at 35 mEq L^{-1} TSC but the larger part of the casein micelles are still intact upon addition of 100 mEq L^{-1} TSC. Fig. 5.1D shows that Na_2HPO_4 has only a weak ability to dissociate casein micelles into small particles. The results also show that addition of Na_2HPO_4 increases the diameter of the intact micelles from 190 to 220 nm. This suggests that the casein micelles swell upon addition of Na_2HPO_4, which is most likely due to the decrease in free calcium inducing increased repulsion between casein molecules.[11]

Overall, the results illustrate that the level of micelle dissociation is strongly dependent on the concentration and type of calcium chelator applied. Panouillé *et al.*[13] reported that polyphosphate, citrate, and pyrophosphate had the same dissociating effect in 1 g L^{-1} casein solutions, which might be a result of the relatively high chelator concentration used in their study.

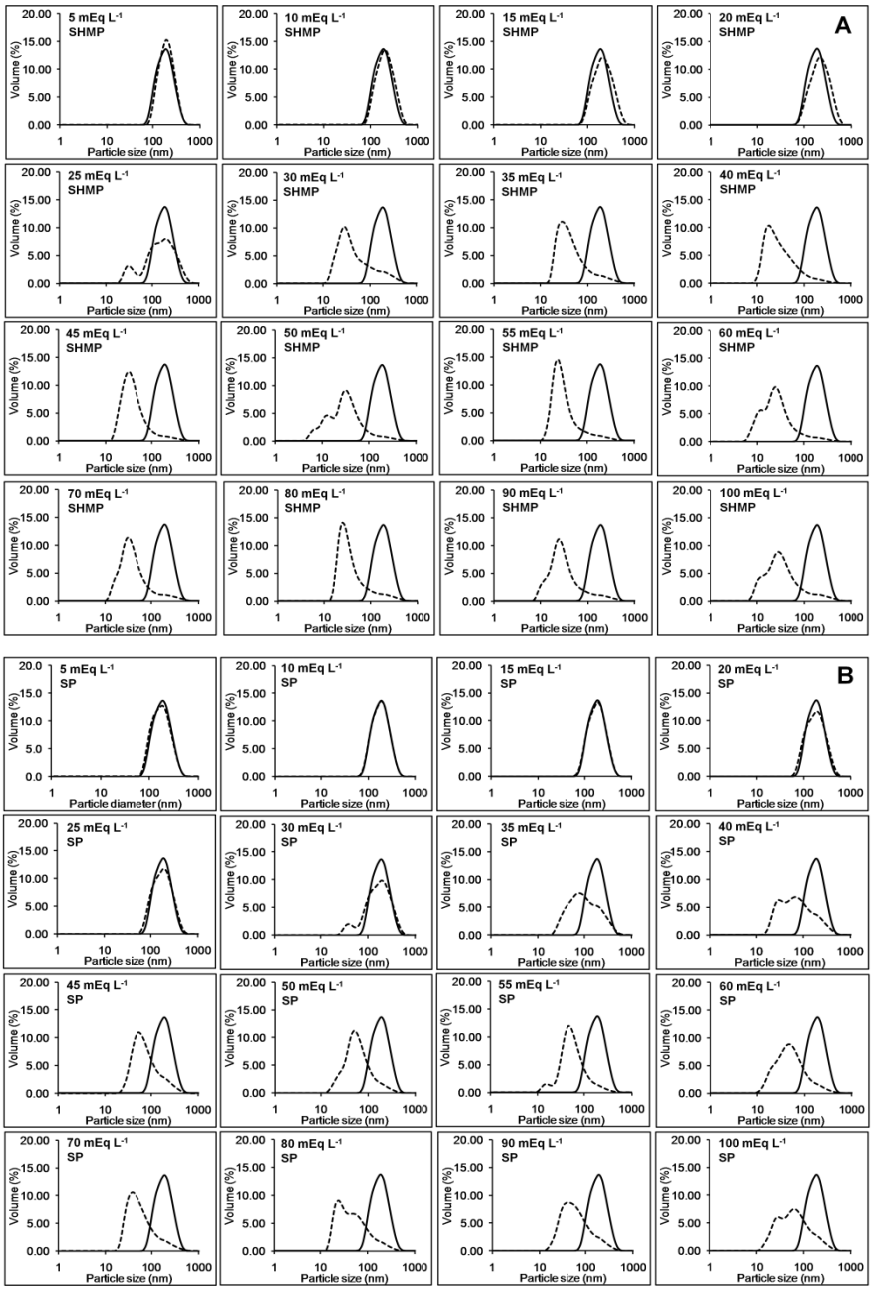

Figure 5.1 Particle diameter volume distributions in MCI solutions after addition of 0 – 100 mEq L^{-1} SHMP (A), SP (B), TSC (C), or Na_2HPO_4 (D) (---). The particle diameter volume distribution of the reference MCI solution (—) is shown in every figure to guide the eye. Results are the means of at least three measurements.

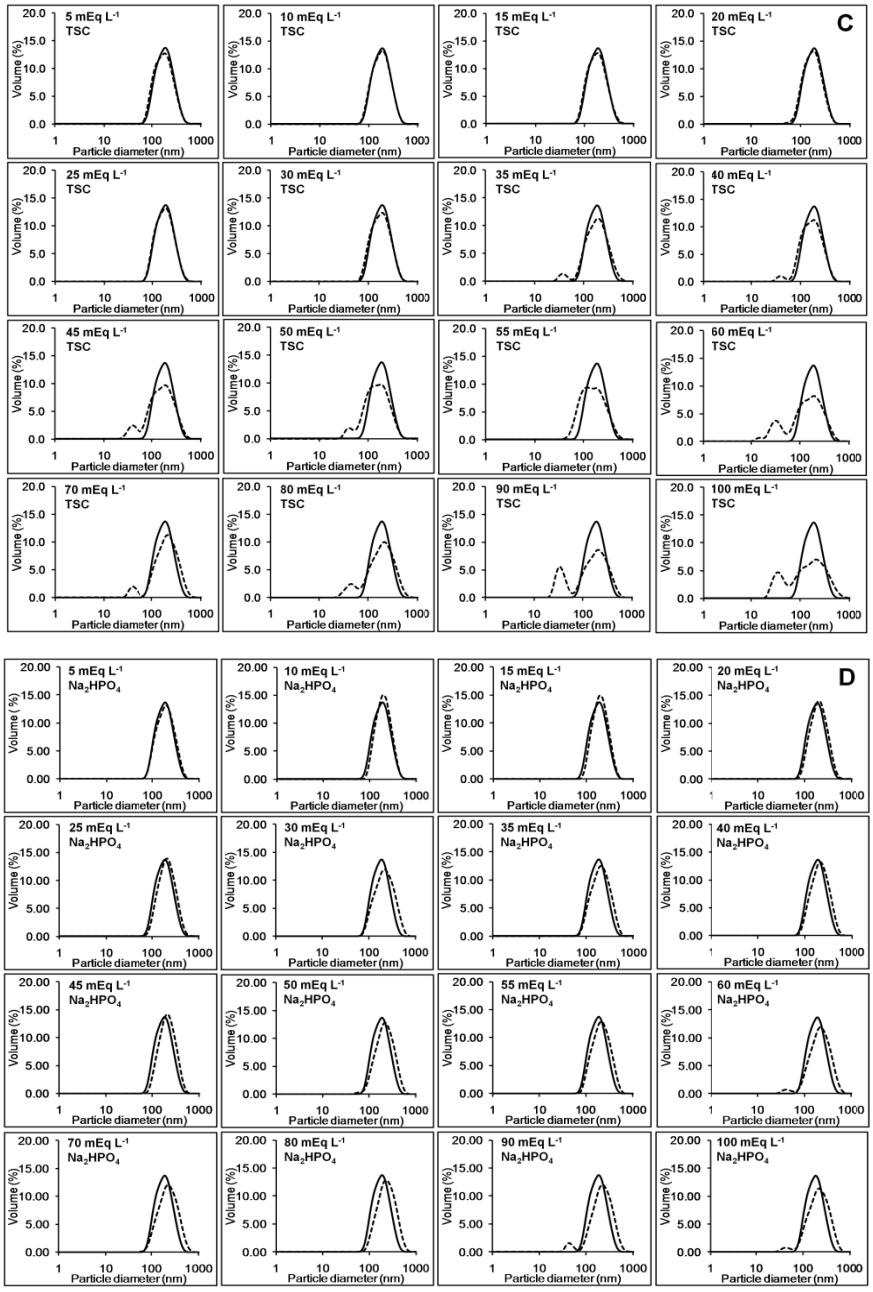

Figure 5.1 continued

The calcium chelators have in common that they all dissociate the casein micelles into small particles with a diameter between 30 and 50 nm. This observation is consistent with the results of Morr *et al.*[31], who determined that casein micelles disaggregate into particles with an average diameter of about 30 to 50 nm after removal of CCP. Casein-casein interactions must also play an important role in the formation of the micellar structure, because particles with diameters of 30 to 50 nm still exist after removal of all CCP from the casein micelles. In more recent studies[13, 14] it was observed that casein micelles dissociated into substructures with an average diameter of 20 to 24 nm after addition of 0.1 mol L^{-1} polyphosphate, citrate, or pyrophosphate to 1 g L^{-1} casein solution. In another study of Panouillé *et al.*[16], it was mentioned that the small micellar particles formed upon chelator addition appear to have a similar size as the aggregates present in sodium caseinate solution. HadjSadok *et al.*[32] determined an average diameter of 22 nm for the small aggregates present in a sodium caseinate solution with high ionic strength (>100 mmol L^{-1}). This is confirmed by our particle size distribution measurements on a sodium caseinate solution (Fig. 5.2), showing small particles with a peak appearing around 30 nm. This is a comparable particle diameter as was observed in the MCI solution after addition of the calcium chelators. The bimodal distribution of sodium caseinate was also observed by Chu *et al.*[33] and Farrell *et al.*.[34]

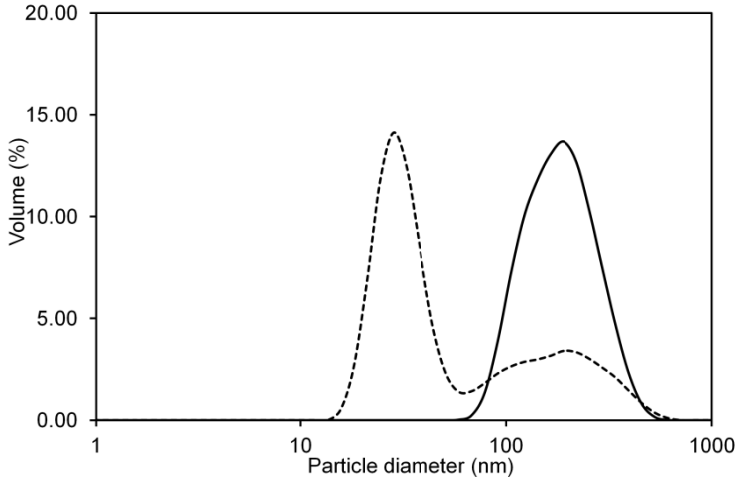

Figure 5.2 Particle diameter volume distribution in MCI solution (—) and sodium caseinate solution (---). Results are the means of at least three measurements.

5.3.2 Effect of calcium chelators on Z-average diameter

Fig. 5.3 shows the Z-average diameter of the particles in the samples against the added chelator concentration. A Z-average diameter of approximately 180 nm was measured for the reference MCI solution, which is in the same range as the top of the peak diameter as mentioned in section 5.3.1. This result is consistent with the average particle diameter of 200 nm for casein micelles in milk as determined by de Kruif [35] using DLS.

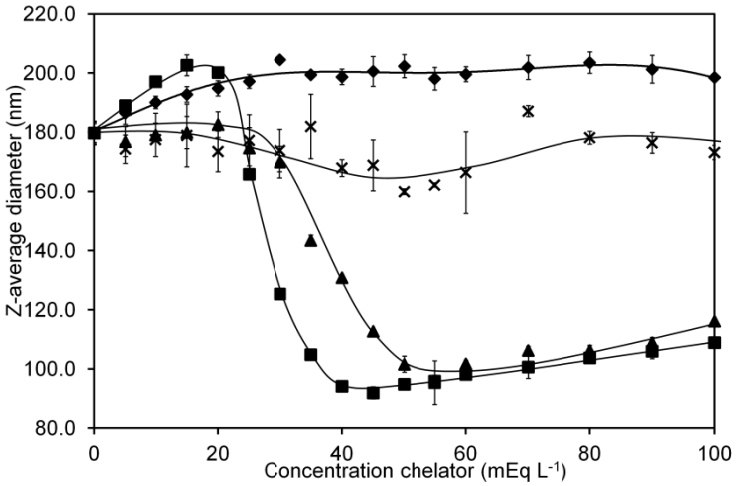

Figure 5.3 Z-average diameter of the MCI solution as a function of chelator concentrations. Symbols represent: (■) SHMP (▲) SP; (x) TSC; (♦) Na_2HPO_4. Results are the means of at least three measurements with standard deviations as error bars.

The Z-average diameter in the samples decreased in the sequence of SHMP, SP, TSC, and Na_2HPO_4, which is in line with the decrease in turbidity as found in a previous study.[11] The decrease of the Z-average diameter is concomitant with the decrease of the turbidity for SHMP and SP samples, as they both exhibit a sudden transition, indicating that turbidity reflects the dissociation of the casein micelles into smaller particles.

The strongest decrease in the Z-average diameter is observed in the SHMP and SP samples, which must be due to the highest concentration of small particles formed in the solution upon addition of the calcium chelators. The strong decrease starts with the onset of micelle dissociation (Fig. 5.1A and 5.1B). The Z-average diameter remains constant beyond a certain

concentration of dissociated casein micelles. The slight increase in the Z-average diameter at >40 mEq L^{-1} SHMP or SP might be due to aggregation of the particles with increasing ionic strength (i.e. chelator addition).

For TSC, only a slight decrease in Z-average diameter was measured, as the majority of the casein micelles remained intact up to 100 mEq L^{-1} TSC (Fig. 5.1C). For Na_2HPO_4 an increase in Z-average diameter of approximately 13% was measured with increasing Na_2HPO_4 concentration, indicating swelling of the casein micelles.[11] Also, for SHMP an increase in Z-average diameter of approximately 13% was observed up to 20 mEq L^{-1} SHMP, which probably is related to swelling of the casein micelles.

5.3.3 Calculation of relative concentration of small particles

Small particles are formed upon dissociation of the casein micelles (cf. Fig. 5.1). At low chelator concentrations, these smaller particles start to appear as a shoulder of the intact casein micelle peak. At higher chelator concentrations, this small-particle peak becomes more prominent. By using the multiple narrow mode algorithm function, the DLS results could be analyzed in terms of volume distributions of the two main sizes. SP was selected for these calculations, as this chelator showed the most gradual increase in peak area with increasing concentration.

In Fig. 5.4 the volume peak area of the small particles against the SP concentration is shown as well as the decrease in turbidity upon addition of SP (result taken from de Kort *et al.*[11]). The increase in concentration of small particles correlates well with the decrease in turbidity. The calculated volume peak areas indicate that small particles were already present at 25 mEq L^{-1} SP (Fig. 5.4) instead of at 30 mEq L^{-1} SP as deduced from Fig. 5.1. This was due to the higher resolution of the multiple narrow mode algorithm function in comparison to the general purpose algorithm function.[26] The volume peak area of the small particles upon initial detection was immediately 30%. This sudden appearance and strong increase in volume peak area are related to detection of small particles by DLS. As the intensity of scattered light varies as the sixth power of the particle diameter, large particles scatter more light than small particles.[27] Accordingly, light scattering is less sensitive for detecting small particles in a solution consisting of equal concentrations of large and small particles. The results indicate that a concentration of casein micelles (i.e. large particles) exists above which DLS cannot detect the smaller particles. Therefore, the volume peak area of the small particles was immediately 30% upon initial

detection. For SHMP and TSC samples an initial volume peak area of approximately 25% and 30%, respectively, was calculated. Approximately 90% of the casein micelles dissociated into small particles after addition of 55 mEq L^{-1} SP. This is the same SP concentration for which the turbidity reaches a minimum value (cf. Fig. 5.4).

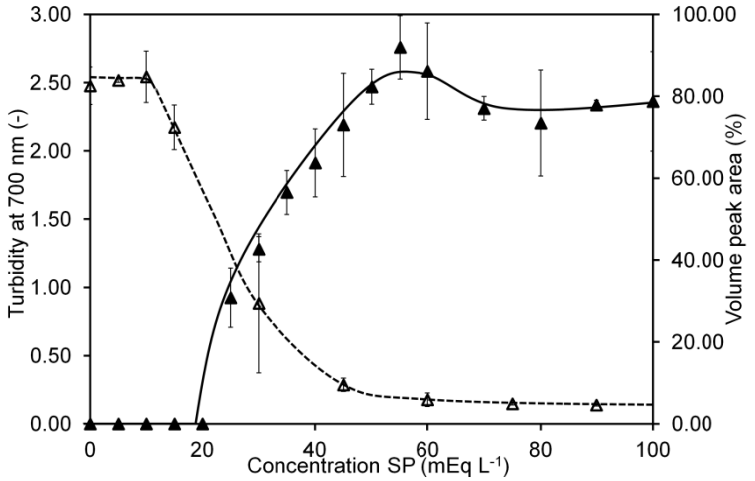

Figure 5.4 Turbidity of the MCI solution (measured as absorbance at 700 nm), diluted to 10% of the initial dry matter in demineralized water, as a function of the SP concentration (--Δ--). Volume peak area of small particles formed in the MCI solution as a function of the SP concentration (—▲—). Results are the means of at least three measurements with standard deviations as error bars.

5.3.4 Simulation of ion equilibria with the EIS model

Casein micelles dissociate when a threshold concentration of CCP is chelated from the micelle.[11-13] The DLS results in Fig. 5.1 show that different chelators start to dissociate casein micelles at different chelator concentrations and to different extents. To obtain an understanding of why these chelators show different behavior, it was investigated to what extent the chelators compete with CCP for calcium ions (i.e. calcium-binding capacity). Changes in the ion equilibria upon addition of chelators can be investigated with a so-called EIS model.[24] This model only applies to protein-free solutions. Gao[24] used the EIS model to investigate the ion equilibria in simulated milk ultrafiltrate (SMUF) solutions upon addition of e.g. $CaCl_2$ or Na_2HPO_4. Simulations with the EIS model indicated that a precipitate of octacalcium phosphate

(OCP, $Ca_4(HPO_4)_3{\cdot}5H_20$) was formed in the SMUF solution. In this SMUF solution OCP competes with the chelators for calcium ions. In our MCI solution CCP competes with the calcium chelators for calcium ions. Therefore, the OCP precipitate in the SMUF solution was used to simulate the CCP of the casein micelles in the MCI solution. As the concentration of calcium and phosphate (i.e. CCP in the casein micelles) is much higher in a MCI solution than in SMUF solution, it was decided for the simulations to adapt the calcium and phosphate concentrations in the SMUF solution to concentrations as present in the MCI solution. Accordingly, a higher concentration of OCP precipitate was calculated with the EIS model for the adapted SMUF solution. To simulate differences in calcium-binding capacity of the different chelators, concentration ranges of 0–100 mEq L^{-1} SHMP, TSC, and Na_2HPO_4 were added to the adapted SMUF solution. Unfortunately, it was not possible to include SP in the simulations, as no equilibrium constants for this chelator were available in the EIS model or literature.

An increase in OCP concentration of 7% was calculated for addition of 100 mEq L^{-1} Na_2HPO_4 to the adapted SMUF solution. This indicates that the added Na_2HPO_4 precipitated on the OCP complex with the free calcium ions available in the aqueous phase. Assuming OCP to act like CCP in the casein micelles implies that Na_2HPO_4 would only precipitate with free calcium ions on the casein micelles. This is in line with the measurements on phosphate enriched casein solutions performed by Guo *et al.*.[36] The ion equilibria simulations indicate that the majority of Na_2HPO_4 remains in the aqueous phase, since high concentrations of hydrogen phosphate, and sodium and potassium hydrogen phosphate, were calculated after adding more Na_2HPO_4 to the adapted SMUF solution. The high concentration of free phosphate was also observed in the ion equilibria simulations of Mekmene *et al.*[37] and in the measurements performed on phosphate enriched milk solutions of Gaucher *et al.*.[23] Apparently the calcium-binding capacity of the added Na_2HPO_4 is similar to that of CCP (and OCP). Consequently, Na_2HPO_4 will not chelate calcium ions from CCP and, therefore, will not induce casein micelles dissociation (cf. Fig. 5.1D).

After addition of 100 mEq L^{-1} TSC or SHMP, the OCP concentration in the adapted SMUF solution was found to gradually decrease, by 15% and 39%, respectively. Addition of TSC resulted in high concentrations of citrate and sodium, potassium, and calcium monocitrate, whereas addition of SHMP mainly resulted in high concentrations of dicalcium and monocalcium hexametaphosphate. This shows that SHMP has a much stronger affinity for calcium ions than TSC. The association constants of SHMP and TSC with calcium, as

summarized by Gao[24], suggest that SHMP should have a stronger calcium-binding capacity than TSC (e.g. pK_{ass} of Ca^{2+} with $citrate^{3-}$ is 5.22 and with $P_6O_{19}^{6-}$ is 10). If OCP is assumed to act like CCP, then the results imply that CCP is released from the micelles when SHMP or TSC are added. As the casein micelles dissociate when a threshold concentration of CCP is chelated from the micelle[11-13], it seems reasonable that the onset of micelle dissociation occurred faster for SHMP than for TSC. Hence, the calcium-binding capacity decreases in the order of SHMP > TSC > Na_2HPO_4, which is also the order found for micelle dissociation as determined by DLS (cf. Fig. 5.1) and for decrease in turbidity as measured by de Kort *et al.*.[11]

The size distribution analyses indicate that the onset of micelle dissociation was detected using the general purpose function at 25 mEq L^{-1} SHMP and at 35 mEq L^{-1} TSC (cf. Fig. 5.1A and 5.1C), simulating at these concentrations a decrease in OCP concentration of approximately 10.7% and 6.6%, respectively. As these decreases in OCP concentration are in the same range, this confirms that, assuming OCP to act like CCP, indeed a threshold concentration of CCP has to be chelated from the casein micelle to induce micelle dissociation. Hence, the ion equilibria simulations in an adapted SMUF solution can apparently predict the dissolution of CCP, responsible for the dissociation of casein micelles, in a concentrated micellar casein solution.

It may be tempting to deduce from the results on dissociation of the casein micelles into small particles that casein micelles consist of sub-micelles. However, the results do not prove that the small particles were already present as such in the native casein micelle. In fact, the small casein complexes that are found still allow for a sub-micelle model[38] as well as for an internal structure model.[2, 39, 40]

In Fig. 5.5 the peak area of the small particles formed after micelle dissociation as a function of the relative OCP concentration (simulating the CCP concentration in the micelles) is shown for SHMP and TSC. For both chelators it is shown that, upon dissolution of CCP, micelle dissociation only occurs above a threshold concentration of CCP. For TSC a linear relation was observed between 35 and 100 mEq L^{-1} TSC, because the concentration of small particles increased when the OCP concentration (i.e. CCP) decreased (cf. Fig. 5.1C). This implies that casein micelles dissociate upon dissolution of CCP from the casein micelles. For SHMP this linear relation was only observed between 20 and 40 mEq L^{-1} SHMP, because the concentration of small particles did not change above 40 mEq L^{-1} SHMP. This is due to the fact that the majority of the casein micelles was already dissociated above 40 mEq L^{-1} SHMP (cf. Fig. 5.1A), and, therefore, the concentration of small particles became independent of the change in OCP

concentration. Overall, the relation between volume peak area of dissociated micelles and simulations of the OCP concentration illustrates that the calcium-binding capacity of the calcium chelators is decisive for the extent to which casein micelles dissociate.

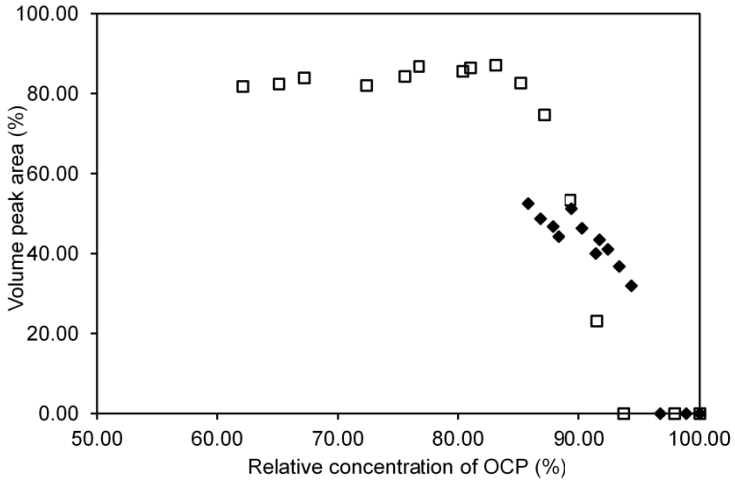

Figure 5.5 Relationship between volume peak area of small particles and relative concentration of OCP calculated with the EIS model after addition of 0-100 mEq L^{-1} SHMP or TSC to MCI solution and adapted SMUF solution, respectively. Symbols represent: (□) SHMP and (♦) TSC.

5.4 Conclusions

DLS is a useful method to investigate the effect of chelators on micelle dissociation. Our results show that micelle dissociation is strongly dependent on the type and concentration of the calcium chelator used. Small particles are formed with a diameter between 30 and 50 nm indicating dissociation of casein micelles. The degree of dissociation follows the order of the calcium-binding capacity of the chelators, i.e. SHMP > SP > TSC > Na_2HPO_4. This conclusion was further supported by simulating ion equilibria in an ion speciation model.

5.5 Acknowledgements

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Chapter 6

Investigating the binding of polyphosphates to caseins by determining changes in the isoelectric point

Abstract

The aim of this research was to investigate the binding of polyphosphates to caseins by determining changes in the isoelectric point (IEP) through zeta potential measurements as a function of pH. Sodium hexametaphosphate (SHMP) and sodium phytate (SP) were added to sodium caseinate (calcium-poor) and micellar casein isolate (calcium-rich) solutions to elucidate whether calcium ions were also required for the binding of the polyphosphates. SHMP and SP both shifted the IEP to more acidic pH. In the sodium caseinate solution a stronger decrease in IEP was measured for SHMP than for SP, indicating that more SHMP than SP was bound to the caseins. In the micellar casein isolate solution the shift in IEP was smaller than in the sodium caseinate solution and was comparable for SP and SHMP. The shift in IEP was smaller in the micellar casein isolate solution, because the polyphosphates also formed complexes with calcium. Overall, the results suggest that SHMP and SP both decrease the IEP of caseins by binding directly with the positively charged amino acids of caseins. Calcium ions are not essential for establishing this binding.

6.1 Introduction

Polyphosphates are commonly used in the dairy industry. They are added, for instance, to prevent and control age gelation in (concentrated) ultra-high temperature (UHT) sterilized milk[1-4] or to control the texture and meltability of processed cheese.[3, 5, 6] Typical concentrations of up to 3% of the final product weight are added to dairy products.[3, 5]

The effect of polyphosphates on the stability and texture of dairy products seems to be related to the interaction with proteins and calcium ions. It is reported that strongly negatively charged polyphosphates, such as pyrophosphate, metaphosphate, hexametaphosphate, or phytate, bind with protein via positively charged amino acid residues[3, 6-14] or via calcium ions.[1, 6, 10, 11, 13-15] This electrostatic interaction is pH dependent, as polyphosphates and amino acids have different pK_a values.[13, 16-18] Phytate, for instance, binds at acidic pH directly to the positively charged amino acids of soy proteins, whereas at alkaline pH calcium ions are involved in the binding of phytate to the negatively charged amino acids.[10, 11, 13, 14] The binding of polyphosphates to protein induces structural changes in the proteins, altering e.g. their hydration, solubility, and digestibility[3, 10, 14, 19], and this also changes the physico-chemical properties of the dairy product.

In previous chapters it was shown that sodium phytate (SP) and sodium hexametaphosphate (SHMP) affect the casein micelle structure and ion equilibria to different extents.[20-22] Especially the difference in viscosity upon addition of SP or SHMP was remarkable, which was explained by the cross-linking ability of SHMP.[20] Although SHMP is commonly used in the dairy industry, there is unclarity about the way SHMP interacts with casein.[2, 6, 8] The interaction of SP with soy protein and cations is described more extensively[10, 11, 13, 14, 18], because phytate is a common constituent in many plant tissues and related food products. To our knowledge, no literature is available about the interaction of SP with casein.

The aim of this research was to investigate the binding of SP and SHMP to caseins by determining changes in the isoelectric point (IEP) through zeta potential measurements as a function of pH. In this study the IEP is considered as the point where the zeta potential reaches a value of zero. Micellar casein isolate (calcium-rich) and sodium caseinate (calcium-poor) solutions were used for this study in order to investigate the role of calcium ions in the polyphosphate-casein binding. The IEP method was selected, because if the strongly negatively charged polyphosphates bind to the caseins, the charge on the caseins is altered, shifting the IEP

of the casein-polyphosphate complexes to more acidic pH. The approach of investigating the binding of phosphates to protein by measuring the IEP was, as far as we know, only used by Briggs[12] in 1940, who determined the binding of metaphosphoric acid to serum albumin by preparing titration curves and analyzing the change in IEP. Investigating the change in IEP upon addition of different polyphosphates to calcium-rich and calcium-poor casein solutions elucidates whether SP and SHMP have the ability to bind directly to caseins or whether calcium ions are involved in this binding.

6.2 Materials and Methods

6.2.1 Sample preparation

Micellar casein isolate (MCI) powder (Nutripro^{TM}) was supplied by DairyGold Food Ingredients (Cork, Ireland). The powder contains 85% (w/w) protein, of which ≤5% is whey protein. A MCI solution with 9% (w/v) protein was prepared according to the procedure described by De Kort *et al.*.[20] Homogenization of the MCI solution was required to split the dispersed powder particles into individual casein micelles with a diameter $D_{[4,3]}$ of about 0.15 μm. Also, a sodium caseinate (SC) solution with 9% (w/v) protein was prepared. The preparation was done in the same way as for the MCI solution. Sodium caseinate was supplied by DMV (Veghel, The Netherlands). Concentrations of 0, 60, and 120 mEq L^{-1} sodium chloride (NaCl) (Kirsch Pharma GmbH, Salzgitter, Germany), disodium hydrogen phosphate (Na_2HPO_4) (Merck & Co. Inc, Darmstadt, Germany), sodium hexametaphosphate (SHMP) (VWR International Ltd, BDH Chemicals, Poole, England), and phytic acid dodecasodium salt hydrate (SP) (Sigma–Aldrich GmbH, Steinheim, Germany) were added to the protein solutions and the pH was adjusted with 0.5 unit increments between 1.5 ± 0.05 and 7.0 ± 0.05. The concentrations of the minerals were based on milliequivalents (mEq) to obtain an equal amount of charges in the samples. For instance, SHMP carries six and SP twelve negative charges, so two times more SHMP than SP molecules were added to the solutions. After equilibrating the samples at 20°C for approximately 17 h, pH readjustments were made, followed by bringing the samples to a final protein concentration of 9% (w/v) with demineralized water. Finally, the samples were diluted 100-fold in demineralized water for the Zetasizer measurements. A flow diagram of the sample preparation is shown in Fig. 6.1.

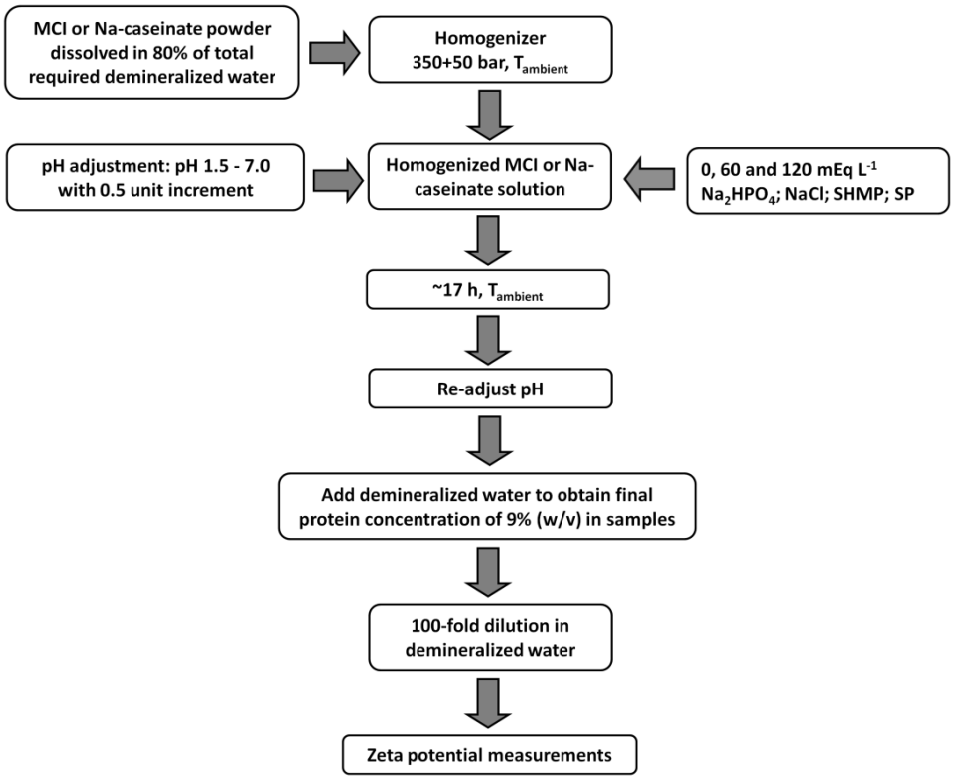

Figure 6.1 Flow diagram of sample preparation for zeta potential measurements

6.2.2 Zeta potential measurements

The zeta potential of the samples with pH values between 1.5 ± 0.05 and 7.0 ± 0.05 was measured with the Zetasizer Nano Z (Malvern Instruments, Worcestershire, UK) equipped with 4 mW He–Ne laser to determine the IEP. Measurements were based on the principles of Laser Doppler Electrophoresis. The electrophoretic mobility U_E was measured and the zeta potential (a value of 1.5 was used for the Smoluchowski approximation) was calculated by the Dispersion Technology Software provided by Malvern. Disposable folded capillary Zetasizer Nano cells of 1.5 mL (DTS1060, Malvern Instruments, Worcestershire, UK) were used for the measurements. Prior to analysis, samples were diluted 100-fold in demineralized water and subsequently filtered through disposable Nalgene® Syringe cellulose acetate filters with a pore size of 0.8 µm (Nalgene, Nunc, Thermo Scientific, Rochester NY, USA). A dilution range indicated that the

zeta potential deviated maximally ±5% between a 50- and 1000-fold dilution in demineralized water. Zeta potential measurements were performed after dilution within 30 min. The results collected between pH 1.5 and 7.0 were plotted in a graph to determine the IEP.

Zeta potential-pH titrations were performed on the samples adjusted to pH 7.0 ± 0.05. The samples were diluted 100-fold and a volume of 10 mL was inserted in the MPT-2 Autotitrator connected to Zetasizer Nano Z. The solutions were titrated from pH 7.0 ± 0.05 to pH 1.0 ± 0.05 with a 0.2 pH unit increment by using 1 mol L^{-1} hydrochloric acid (Merck & Co. Inc, Darmstadt, Germany). The IEP was automatically determined by the Dispersion Technology Software. The zeta potential measurements and zeta potential-pH titrations were both performed at a cell temperature of 25°C and voltage of 100 V. Samples were analyzed at least in duplicate.

6.3 Results and Discussion

6.3.1 Sodium caseinate: a solution poor in calcium ions

The IEP is the pH at which the zeta potential is 0 mV. The results in Fig. 6.2 illustrate that a decrease in IEP was obtained upon addition of SP and SHMP to SC solutions. The decrease in IEP was stronger with increasing chelator concentration. An IEP of 4.6 was measured for the reference sample (i.e. SC solution with 0 mEq L^{-1} mineral addition), which is in agreement with the IEP for casein.[17, 23] The IEP was negligibly changed upon addition of 60 or 120 mEq L^{-1} Na_2HPO_4 or NaCl, indicating that the IEP was negligibly affected by sodium and orthophosphate ions or by the increase in ionic strength of the solution.

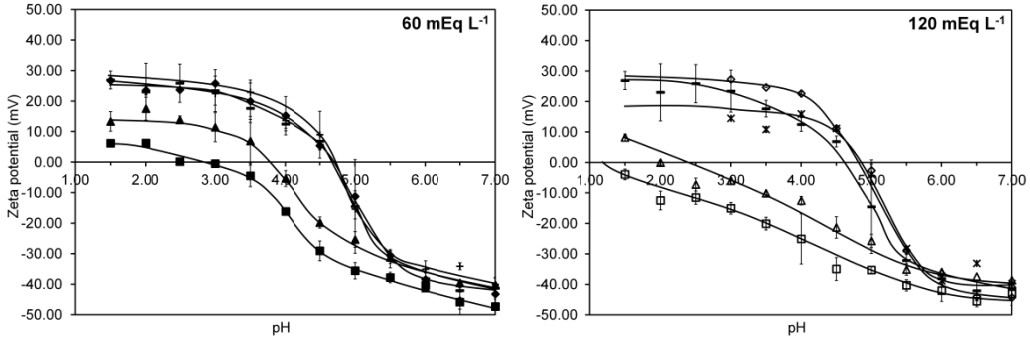

Figure 6.2 Zeta potential as a function of pH for sodium caseinate solutions containing 60 mEq L^{-1} and 120 mEq L^{-1} SHMP (■, □), SP (▲,Δ), Na_2HPO_4 (♦, ◊), or NaCl (+, x). Reference samples are presented as (–). The samples were diluted 100-fold in demineralized water prior to analyses. Results are the means of at least duplicates with standard deviations as error bars.

Also, zeta potential-pH titrations were performed on samples with an initial pH of 7.0 and the IEP was automatically calculated by the Dispersion Technology software. The IEP values obtained from the titrations and Fig. 6.2 are summarized in Table 6.3.

Table 6.3 IEP of sodium caseinate solutions containing 60 mEq L^{-1} and 120 mEq L^{-1} Na_2HPO_4, NaCl, SHMP, or SP determined by performing zeta potential measurements on samples adjusted between pH 1.5 to 7.0 and by performing zeta potential-pH titrations on samples with an initial pH of 7.0. All samples were diluted 100-fold in demineralized water.

Mineral concentration	Concentration of 60 mEq L^{-1}		Concentration of 120 mEq L^{-1}	
Type of measurement	ZP measurements[1]	ZP-pH titrations[2]	ZP measurements[1]	ZP-pH titrations[2]
Reference	4.60	4.59 ± 0.14	4.60	4.59 ± 0.14
Na_2HPO_4	4.70	4.49 ± 0.05	4.80	4.67 ± 0.05
NaCl	4.75	4.41 ± 0.23	4.85	4.28 ± 0.20
SHMP	2.80	4.14 ± 0.06	1.10	2.96 ± 0.03
SP	3.80	4.13 ± 0.07	2.30	3.80 ± 0.09

[1] The IEP values determined with zeta potential measurements were obtained from Fig. 6.2.
[2] The IEP values determined with zeta potential-pH titrations were obtained from the Dispersion Technology Software. The IEP values of the titrations are presented as means of duplicate measurements with corresponding standard deviations.

The IEP values obtained from the titrations confirm that a shift in IEP is measured upon addition of SHMP and SP, and that the shift in IEP is stronger at higher chelator concentration. However, a smaller shift in IEP was determined with the titrations than with the zeta potential measurements; this is most probably related to the differences in sample preparation prior to analysis. For the zeta potential measurements, the undiluted samples were kept for approximately 17 h at 20°C at pH values between 1.5 and 7.0 to let the samples equilibrate. The samples were 100-fold diluted maximally 30 min before analysis. For the zeta potential-pH titrations, all samples were equilibrated at pH 7.0. After diluting 100-fold, the samples were titrated to pH 1.5 within approximately 4 h. Hence, the interaction between polyphosphates and caseins at a certain pH was more intensive in the case of zeta potential measurements, because the reaction time was longer and the solution was undiluted, which may explain the larger shift in IEP with this method.

SP and SHMP most probably induced a shift in IEP of the SC solution by binding to the positively charged casein residues. Several authors have reported[1, 6, 10, 11, 13-15] that strongly negatively charged polyphosphates, such as SP and SHMP, might also bind to the proteins via calcium ions. However, the SC solution contains a low concentration of calcium ions (i.e. 2.1 mmol L^{-1}), implying that binding via calcium ions is negligible in SC solution. Arginine, histidine, lysine, and α-NH_2 terminal group are the positively charged amino acids in a caseinate solution around neutral pH, since they have pK_a values of 12.0, 6.4, 10.6, and 7.6, respectively.[17] Casein (especially α_{s2}-casein) is rich in lysine[17] and, therefore, might be the major binding site for SP and SHMP molecules. For SP it was reported that binding with serum albumin occurred primarily via the α-NH_2 terminal group, followed by the ε-NH_2 group of lysine, then histidine, and finally via the guanidyl group of arginine.[13] For SHMP it was found that in a chitosan solution SHMP binds to the amine groups.[16] This information supports the assumption that SP and SHMP bind to the positively charged amino acids of caseins.

A shift in the IEP occurs when the strongly negatively charged polyphosphates bind to positively charged amino acids. The shift in IEP to more acidic pH was stronger with increasing SP and SHMP concentration, because probably more SP and SHMP molecules were bound to the caseins. The phenomenon of shifting the IEP by binding polyphosphate to protein was earlier described by Briggs[12], who determined that metaphosphate reacted with the ionizable basic groups of serum albumin protein, decreasing the IEP of the protein metaphosphate complex formed. The binding of SP to proteins was also described by Cheryan.[13] It was found that

phytate has the ability to shift the IEP of soy protein by binding directly to the protein, since the solubility profile of soy protein shifted to more acidic pH in the presence of phytic acid.

A stronger decrease in IEP was measured for SHMP than for SP in the SC solution (Fig. 6.2), suggesting that more SHMP than SP molecules bind to the caseins. There are several hypotheses that may explain the difference in binding of SHMP and SP to the caseins. 1) Polyphosphate concentration: two times more SHMP than SP molecules were present in the casein solutions that could bind to the caseins, because additions were done in milliequivalent concentrations (i.e. based on amount of charge per molecule). This hypothesis is supported by the data of Briggs.[12] 2) Charge distribution of the polyphosphates: SHMP contains fewer negative charges than SP, resulting in less repulsion between SHMP and the negatively charged amino acids than for SP around neutral pH. 3) pK_a values of the polyphosphates: the pK_a values of SP are divided into three groups, namely pK_{a1}-pK_{a6}: 1.1-2.1, pK_{a7}-pK_{a9}: 5.7-7.6, and pK_{a10}-pK_{a12}:10.0-12.0[13, 18]. Since SP has six pK_a values at low pH, the SP molecules remain strongly negatively charged at low pH. The pK_a values of SHMP were not found in the literature. Sanchez-Diaz[16] suggested that SHMP hydrolyses in acidic conditions into trimetaphosphate and orthophosphate, resulting in pK_a values of 2.64, 4.0, 6.1, 7.3, 10.0, and 11.7. Based on these pK_a values, the hydrolysed SHMP molecules are only weakly negatively charged at low pH. The repulsion between SHMP and the negatively charged amino acids therefore is much lower than for SP in acidic conditions. 4) Charge distribution of the caseins: the positively charged amino acid residues are inhomogeneously distributed on the different caseins (especially on α_{s1}- and α_{s2}-casein).[17] It is possible that the polyphosphates require different clusters of positively charged amino acids to enable binding with the caseins, resulting in different binding of the polyphosphates to the caseins. In this case several positively charged amino acids are involved in the binding of one negatively charged polyphosphate molecule. Calculations were performed to obtain a better understanding of the effect on the IEP by replacing positive charges by multiple negative charges. For instance, the IEP decreased by 0.45 when 2 positive charges of lysine groups were removed and 14 negative charges of phosphoserine residues were added to α_{s1}-casein. This indicates that the IEP of milk can already be shifted from 4.6 to 4.15 by binding two SP molecules (-7.5 charged around neutral pH) or six SHMP molecules (-3.3 charged around neutral pH) to α_{s1}-casein. Hence, binding small amounts of SHMP or SP to the caseins might cause a strong shift in the IEP.

Overall, it can be concluded from the results in SC solution that SHMP and SP can bind directly to the positively charged amino acids of the caseins. More research should be done to elucidate the difference in binding of SHMP and SP to the caseins.

6.3.2 Micellar casein isolate: a solution rich in calcium ions

The results in Fig. 6.4 show that in the MCI solution the IEP was negligibly changed upon addition of Na_2HPO_4 or NaCl, which is in agreement with the results in SC solution. SHMP and SP also have the ability to shift the IEP to more acidic pH in the MCI solution, indicating that in the presence and absence of calcium ions the IEP can be shifted by the polyphosphates. The decrease in IEP in the MCI solution was similar for SHMP and SP, whereas in the SC solution a stronger shift in IEP was measured for SHMP than for SP.

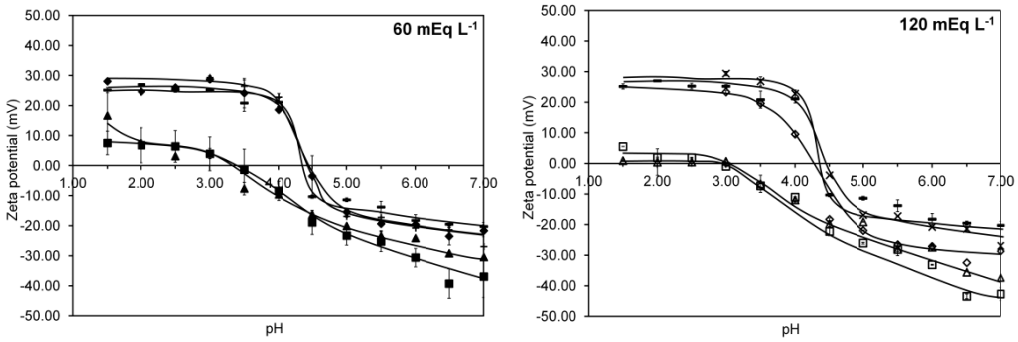

Figure 6.4 Zeta potential as a function of pH for MCI solutions containing 60 mEq L^{-1} and 120 mEq L^{-1} SHMP (■, □), SP (▲, Δ), Na_2HPO_4 (♦, ◊), or NaCl (+, x). Reference samples are presented as (–). The samples were diluted 100-fold in demineralized water prior to analyses. Results are the means of at least duplicates with standard deviations as error bars.

Zeta potential-pH titrations were performed on the MCI solutions with initial pH 7.0 as well. The IEP values obtained from Fig. 6.4 and titrations are summarized in Table 6.5. The results illustrate that the IEP shifted to a smaller extent with the titrations than with the zeta potential measurements. This smaller shift in IEP was also observed upon titrating the sodium caseinate solutions and was explained by the difference in sample preparation for the two methods (see paragraph 6.3.1).

Table 6.5 IEP of micellar casein isolate solutions containing 60 mEq L^{-1} and 120 mEq L^{-1} Na_2HPO_4, NaCl, SHMP, or SP determined by performing zeta potential measurements on samples adjusted between pH 1.5 to 7.0 and by performing zeta potential-pH titrations on samples with an initial pH of 7.0. All samples were diluted 100-fold in demineralized water.

Mineral concentration	Concentration of 60 mEq L^{-1}		Concentration of 120 mEq L^{-1}	
Type of measurement	**ZP measurements[1]**	**ZP-pH titrations[2]**	**ZP measurements[1]**	**ZP-pH titrations[2]**
Reference	4.30	4.56 ±0.04	4.35	4.56 ±0.04
Na_2HPO_4	4.45	4.37 ±0.27	4.30	4.54 ±0.00
NaCl	4.40	4.57 ±0.00	4.40	4.39 ±0.10
SHMP	3.30	4.04 ±0.09	2.95	3.94 ±0.23
SP	3.30	4.08 ±0.13	3.00	3.56 ±0.39

[1] The IEP values determined with zeta potential measurements were obtained from Fig. 6.4.
[2] The IEP values determined with zeta potential-pH titrations were obtained from the Dispersion Technology Software. The IEP values of the titrations are presented as means of duplicate measurements with corresponding standard deviations.

The shift in IEP upon addition of SHMP or SP was smaller in the MCI solution than in the SC solution (except for 60 mEq L^{-1} SP, which will be discussed later on), which might be explained by the high concentration of calcium ions present in the MCI solution (59.8 mmol L^{-1}). SHMP and SP are strong calcium chelators[9, 13, 15, 20, 24] and interact, as well as with the positively charged amino acids, with calcium present in the MCI solution. Approximately 5% of the total calcium in the MCI solution is present as free calcium ions in the solution.[20] The remainder of calcium is bound to phosphoserine residues and other negatively charged amino acids or is part of colloidal calcium phosphate (CCP) complexes in the casein micelles.[17, 20, 25, 26] In a previous study[20] it was shown that addition of 60 mEq L^{-1} SHMP or SP to MCI solution containing 9% (w/v) protein reduced the calcium-ion activity to a similar level. In another study[22] it was shown that the casein micelles were completely dissociated upon addition of 60 mEq L^{-1} SHMP or SP, because they chelated the calcium ions from the casein micelles. This implies that the strong calcium-binding capacity of SHMP and SP reduced the amount of SHMP and SP molecules available to bind to the positively charged amino acids in the MCI solution around neutral pH. Upon increasing the SHMP and SP concentration the decrease in IEP was smaller in the MCI solution than in the SC solution. A MCI solution contains around 120 mEq L^{-1} calcium,

indicating that the polyphosphates were still complexing calcium ions upon increasing the concentration from 60 to 120 mEq L^{-1} SHMP or SP. As a result, fewer SHMP and SP molecules were available to bind directly to the caseins, inducing a smaller shift in the IEP.

Several authors have reported[1, 6, 10, 13-15] that calcium ions are involved in the binding of polyphosphates to protein, suggesting that a stronger shift in IEP could have been measured in the MCI solution than in the SC solution. This effect was only observed upon addition of 60 mEq L^{-1} SP: IEP values of 3.80 and 3.30 were measured in the SC and MCI solutions, respectively. For SP it is described that at alkaline pH calcium ions are involved in the binding to soy protein, in which the imidazole group of histidine appears to be the major binding site for the formation of a protein-cation-phytate complex.[13, 14] At acidic pH, SP binds directly to the positively charged amino acids of soy protein.[10, 13, 14] The lower IEP value measured for the MCI solution than for the SC solution upon addition of 60 mEq L^{-1} SP suggests that SP was binding to the caseins via the positively charged amino acids as well as via the calcium ions. For the binding of SHMP to casein it was suggested by Mizuno *et al.*[6] that a caseinate-calcium-hexametaphosphate complex was formed at pH 5.8. Our results do not clarify whether this kind of complex was formed in the MCI solution between pH 1.5 and 7.0.

Fig. 6.2 and 6.3 both showed that Na_2HPO_4 did not shift the IEP of the casein solutions, indicating that Na_2HPO_4 did not bind to the caseins. However, in a previous study in this laboratory[22] and by other authors[27-29] it was found that Na_2HPO_4 precipitated with calcium ions on the casein micelle, which implies that binding of orthophosphate to the caseins occurs in casein solutions. Visser *et al.*[30, 31] determined that calcium and phosphate ions associated with the NH_3^+ groups of lysine and arginine of caseins in a cooperative manner, meaning that binding of phosphate to the caseins only takes place when calcium ions are present. It is possible that binding of phosphate to the caseins occurred in the MCI solution around neutral pH, but that this binding ceased with decreasing pH because of dissolution of calcium phosphate complexes at acidic pH.[17, 31] As a result, a similar IEP was measured for the reference SC and MCI solutions as well as for the casein solutions containing Na_2HPO_4.

6.4 Conclusions

The results have shown that the binding of polyphosphates to caseins can be determined by measuring the IEP of casein solutions. SHMP and SP both have the ability to bind directly to the positively charged amino acids of caseins, shifting the IEP to more acidic pH. Calcium ions were not required for the binding of SHMP or SP to caseins. The extent of shift in IEP was dependent on the concentration of polyphosphate and calcium present in the casein solution. The shift in IEP upon addition of polyphosphates to casein solutions creates new opportunities to formulate stable liquid dairy products between pH 3.5 and 6.0.

6.5 References

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Chapter 7

General discussion

7.1 Introduction

In practice it is challenging to prepare a concentrated medical product with high heat stability and low viscosity, since at higher total solids content the heat stability might decrease and the viscosity will increase (Chapter 1). As a more viscous product can be difficult to consume, it is important to develop concentrated medical products that have a low viscosity and a high heat stability at the same time. Calcium chelators, such as phosphates and citrate, are often added to dairy products to improve the heat stability.[1-4] The viscosity of a product is also affected by calcium chelator addition because of the interaction with colloidal calcium phosphate (CCP) in the casein micelles.[5-11] The specific interaction of the calcium chelators with the casein micelle can vary considerably, resulting in different effects on the physico-chemical properties of a product. The aim of this PhD research was to determine the influence of calcium chelators on the physico-chemical properties of casein micelles and the resulting effect on the viscosity and heat stability of concentrated micellar casein solutions. This chapter puts the results in a wider context by discussing relationships between the type of calcium chelator and the effect on the calcium balance, casein micelle structure and important properties such as, viscosity, turbidity, and heat stability of concentrated micellar casein solutions. Applications and recommendations for further research are provided as well to illustrate the practical relevance of this PhD research for the dairy industry.

7.2 Influence of calcium chelators on the casein micelle and concentrated micellar casein solutions

An overview of the results collected in Chapters 2 to 6 is given in Table 7.1. The type and concentration of calcium chelator are important for the physico-chemical properties of the casein micelle. This is expressed in calcium-ion activity, viscosity, turbidity, and heat stability of the concentrated micellar casein solutions.

Table 7.1 Overview of the effect of calcium chelators on the physico-chemical properties of casein micelles and concentrated micellar casein solutions.

Type of calcium chelator		Influence on casein micelle			Influence on concentrated micellar casein solution			
Chelator	Ca-binding capacity	Zeta potential decrease	Decrease in IEP	Casein micelle dissociation	Ca-ion activity decrease	Viscosity increase	Turbidity decrease	Heat stability increase
Na_2UMP	+	0	0	0	+	+	0	++++
Na_2HPO_4	++	0	0	0*	++	++	+	+++
TSC	++	0	0	+	++	++	++	++
SP	+++	++	++	++	++	++	+++	++
SHMP	+++	++	++	+++	++	+++	+++	+

+ = number of symbols indicates the extent of change; 0 = no change

* indicated as no change (0), because dissociation of the micelles was only detected at ≥ 90 mEq L^{-1} Na_2HPO_4

The effect of the different calcium chelators on the casein micelle and concentrated micellar casein solution will be discussed in paragraphs 7.2.1 to 7.2.7.

7.2.1 Calcium-binding capacity

According to Table 7.1, SP and SHMP were found to be stronger calcium chelators than TSC, Na_2HPO_4, and Na_2UMP (Chapter 2). Na_2UMP was found to be a weaker calcium chelator than the other chelators, since significant levels of free calcium and free phosphate remained in the solution. The calcium-binding capacity (i.e. amount of calcium ions bound per chelator molecule) was directly related to the amount of charges. Calcium ions were bound in a ratio of 1:1 for Na_2UMP, 3:2 for Na_2HPO_4, 3:1 for SHMP, and 6:1 for SP in a $CaCl_2$ solution. Based on these calcium-binding capacities, it was decided to add the calcium chelators in milliequivalent concentrations to the concentrated micellar casein solutions to introduce a similar amount of negative charges to the solutions.

7.2.2 Calcium-ion activity and viscosity

In Chapter 3 it was discussed that the viscosity of the concentrated micellar casein solutions was mainly determined by the calcium-ion activity (i.e. concentration of free calcium ions present in the continuous phase). A decrease in calcium-ion activity causes an increase in electrostatic repulsion between the negatively charged caseins because of reduced shielding of negative

charges on the caseins. The casein micelles subsequently become more hydrated and swollen, increasing the voluminosity of the caseins. For the calcium chelators Na_2HPO_4, TSC, and SP the increase in viscosity was comparable and directly related to the decrease in calcium-ion activity. Na_2UMP decreased the calcium-ion activity only slightly, causing a minor increase in the viscosity of the solutions. For SHMP the strong increase in viscosity was primarily related to cross-linking between the caseins by SHMP[12-14] and secondary to the decrease in calcium-ion activity.

7.2.3 Turbidity and casein micelle dissociation

Large differences in turbidity were measured in the samples containing the different calcium chelators (Chapter 3). The decrease in turbidity of the solutions was concluded to be mainly determined by the extent to which the micellar structure was dissociated (Chapter 5). Addition of calcium chelators to casein solutions induces changes in the casein-mineral equilibria (see Fig. 1.3, Chapter 1), leading to a decrease in concentration of free calcium ions, dissolution of CCP from the micelle, and release of specific caseins from the micelle.[6-9] The removal of CCP from the casein micelle by the calcium chelators may result in dissociation of the casein micelle structure.[7, 9, 15-19] The ion equilibria simulation data (Chapter 5) support the hypothesis that the onset and extent of micelle dissociation depends on the dissolution of colloidal calcium phosphate, which is determined by the concentration and type of calcium chelator. Differences between the calcium chelators are explained by the association and solubility constants with calcium ions and the amount and distribution of charges around the chelator molecule. Although the ion equilibria simulations in SMUF solution can only serve as a first approximation, because the ion equilibria in a concentrated micellar casein solution are more complex, useful information was obtained about the extent to which the calcium chelators dissolve CCP from the casein micelle and about the kind of calcium complexes that are formed in the concentrated micellar casein solutions. To further investigate the effect of calcium chelators on the ion equilibria in milk solutions, an ion equilibria model for milk solutions has to be developed.

The results in Chapter 5 have demonstrated that the casein micelles dissociate when a threshold concentration of CCP is chelated from the micelles, which is in agreement with literature.[7, 9] SHMP induced micelle dissociation at a slightly lower concentration than SP. This small difference might be related to differences in the association constant and the solubility product

of SHMP and SP with calcium ions. The effect of TSC on micelle dissociation was smaller than for SHMP and SP, which might be explained by the lower affinity of TSC for calcium ions: the pK_{ass} of Ca^{2+} with TSC is 5.22 and with SHMP is 10.[20] At a given concentration TSC apparently dissolutes less CCP from the casein micelle than SHMP or SP, resulting in a lower concentration of dissociated casein micelles in the concentrated micellar casein solutions. The ion equilibria simulations confirmed that TSC has a weaker calcium-binding capacity than SHMP (Chapter 5).

The minor effect of Na_2HPO_4 on micelle dissociation was also related to the competition for calcium ions between the casein micelle and phosphate and to the solubility product of calcium phosphate complexes. The ion equilibria simulations indicated that Na_2HPO_4 bound the free calcium ions available in the continuous phase, which decreased the calcium-ion activity of the solution. The newly formed calcium phosphate complexes subsequently precipitated in the continuous phase or on the casein micelle, because the continuous phase is saturated with calcium phosphate.[21] This was also reported by Gaucher *et al.*[22], Guo *et al.*[23], and Mekmene *et al.*.[24] Visser *et al.*[25, 26] derived that calcium and phosphate ions associated with the NH_3^+ groups of lysine and arginine of the caseins in a cooperative manner. Dissolution of CCP in the casein micelles did not occur in samples containing Na_2HPO_4 and, consequently, micelle dissociation was not detected in these samples.

Since micelle dissociation was negligible in Na_2HPO_4 samples, the decrease in turbidity could not be attributed to micelle dissociation. It is more likely related to swelling of the casein micelles. As explained in Chapter 3, calcium ions are bound to the casein micelle via the phosphoserine residues or other negatively charged amino acids or they are integrated in the CCP complexes. A decrease in binding of these calcium ions will induce swelling and/or dissociation of the casein micelles. As the association constant with calcium ions is smaller for phosphoserine residues than for phosphate ($2.2\ 10^4$ and $2.88\ 10^6\ M^{-1}$, respectively), it is likely that Na_2HPO_4 chelates calcium ions linked to phosphoserine, which induces swelling of the casein micelle. Accordingly, more continuous phase is present in the micellar structure, reducing the difference in refractive index of the casein micelle structure and continuous phase with a resulting decrease in turbidity of the solution.

Na_2UMP was found to be the weakest chelator and only induced small changes in the ion equilibria in the concentrated micellar casein solution. Consequently, the influence of Na_2UMP on the casein micelle structure was negligible. Dynamic light scattering measurements

confirmed that dissociation of the casein micelle was not detected upon addition of 100 mEq L^{-1} Na_2UMP (data not shown).

Overall, the results in Chapters 2 to 6 have shown that the type and concentration of calcium chelator added to the casein solutions determined their influence on the ion equilibria and, subsequently, on the onset and extent of swelling and dissociation of the casein micelles.

7.2.4 Viscosity and casein micelle dissociation

The viscosity of concentrated micellar casein solutions is mainly determined by the extent to which the casein micelles are aggregated and/or swollen. Aggregation between caseins occurred in samples containing SHMP because of the formation of cross-links between caseins, resulting in a strong increase in viscosity (Chapter 3). Swelling of casein micelles was clearly shown in samples containing Na_2HPO_4 (Chapter 3). Na_2HPO_4 induced swelling of the intact casein micelles, thereby increasing the viscosity. TSC and SP showed an increase in viscosity comparable to Na_2HPO_4, but had different ratios of intact and dissociated casein micelles (Chapter 5). This indicates that swelling occurred in both intact and dissociated casein micelles, because they are both subjected to increased electrostatic repulsion at decreased calcium-ion activity. More swelling is expected in the dissociated casein micelles than in the intact casein micelles, because a comparable increase in viscosity was measured in the samples. In conclusion, different mixtures of intact and dissociated casein micelles can contribute equally to the increase in viscosity. Therefore, viscosity is not always an indicator for the extent to which casein micelles are dissociated.

7.2.5 Casein micelle structure and dissociation

In the literature it is often described that the caseins are dispersed if a decrease in turbidity is measured in milk solutions containing calcium chelators[15, 27], but no information is provided about the effect of the calcium chelators on particle sizes. We determined that the casein micelles dissociated into particles with a diameter of 30-50 nm upon calcium chelator addition (Chapter 5). Based on this result, it may be tempting to conclude that casein micelles consist of sub-micelles. However, the results do not prove that the small particles formed upon micelle dissociation were already present as such in the native casein micelles. There are two models for casein micelles described in the literature that might explain this phenomenon: the sub-micelle

model and the internal structure model.[8, 28-32] In the sub-micelle model the micelle is built up of sub-micelles with diameters of about 14 nm[28]. This indicates that particles sizes of 30-50 nm can be formed if clusters of sub-micelles were formed upon micelle dissociation, which might be subjected to swelling as well. The internal structure model is nowadays most favored[5, 16, 33, 34]; in this model the average distance between CCP nanoclusters is about 18 nm.[5, 34] This does not give conclusive information about the particle sizes that might be formed upon micelle dissociation. In a sodium caseinate solution, which is a CCP-depleted casein solution, also particle sizes of 30-50 nm were detected (Chapter 6). As hydrophobic and electrostatic interactions both play an integral role in the integrity of the micellar structure[5, 31, 32, 35] and in the interactions between the caseins, neither of the two casein micelle models elucidates why particles sizes of 30-50 nm were formed upon micelle dissociation. Bouchoux *et al*.[36] recently introduced the spongelike casein micelle model, which contains hard regions with particle sizes of 10 to 40 nm that resist compression and contain the nanoclusters. The size range of these hard regions approach the size range we have measured for the dissociated casein micelles. Further research is required to investigate the composition and structure of the small particles formed upon micelle dissociation.

7.2.6 Zetapotential and isoelectric point

In Chapters 4 and 6 the effect of calcium chelators on zeta potential and isoelectric point (IEP) in casein solutions were described, respectively. Table 7.1 summarizes that only upon addition of SHMP and SP a decrease in zeta potential and IEP could be detected. SHMP and SP are strongly negatively charged molecules and bind directly with the positively charged amino acids of the caseins. Calcium ions were not essential for these bindings, because a shift in IEP to more acidic pH also occurred upon addition of SHMP or SP to sodium caseinate solutions (Chapter 6). The properties of the SHMP and SP molecules (e.g. amount and distribution of charges and pK_a values) seem to play an important role in their binding with caseins and calcium ions. More research needs to be done to elucidate the difference in binding of SHMP and SP to the caseins.
As described in Chapters 3 and 4, SHMP can form cross-links between the caseins. During heating these cross-links were released and most likely hydrolysis of SHMP occurred, which increased the calcium-ion activity in the solutions. This suggests that calcium ions were involved in the SHMP cross-links with the caseins. It was concluded from the results in Chapter 6 that

SHMP binds directly to the caseins, which suggests that casein–HMP–Ca–HMP–casein cross-links were formed in the micellar casein solutions.

Na_2UMP, Na_2HPO_4, and TSC are less negatively charged than the polyphosphates and, consequently, changes in zeta potential and IEP were not detected. Direct binding of these calcium chelators to the positively charged amino acids of caseins most probably was negligible or did not occur, because in the literature it is described that Na_2HPO_4 only binds to casein in the presence of calcium ions[25, 26] and TSC does not bind to caseins at all.[14, 27, 37]

7.2.7 Heat stability

An important aspect for the development of stable dairy products is heat stability. In general, calcium chelators increase the heat stability of dairy products by binding calcium ions present in the continuous phase, which decreases calcium-induced protein aggregation in the solutions.[1-4, 38] In Chapter 4 it is described that calcium chelators have a different influence on the heat stability, as they have a different influence on the casein micelle structure and physico-chemical properties of the concentrated micellar casein solutions (Table 7.1).

The results in Chapter 4 showed that the initial condition of the concentrated casein solutions gives an indication of the expected heat stability. A strong increase in heat stability is expected when the calcium-ion activity is below a critical value and when the turbidity and viscosity of the solution are negligibly affected. A slight decrease in calcium-ion activity can be established by increasing the pH of the solution. However, this increases the viscosity of the casein solution as well because of increased electrostatic repulsion between the caseins at higher pH (Chapter 4). Increasing the pH to increase the heat stability of the solution is therefore limited in practice because of viscosity requirements.

The calcium-ion activity can be reduced more effectively by adding a certain type and concentration of calcium chelator. Among the calcium chelators, Na_2UMP gave the strongest increase in heat stability, as Na_2UMP chelated a critical concentration of free calcium ions without affecting the micellar structure. The other calcium chelators affected the ion equilibria and herewith the casein micelle structure to a larger extent, which was measured as a strong decrease in calcium-ion activity and turbidity and increase in viscosity. These changes reduced the heat stability of the concentrated casein solutions, because dissociated casein micelles have a higher sensitivity to calcium-induced protein aggregation.[1, 7] The samples with Na_2HPO_4, TSC,

and SP contained different ratios of intact and dissociated casein micelles and this induced differences in heat stability for these samples. The heat stability for Na_2HPO_4 was higher than for TSC and SP, as no micelle dissociation occurred upon addition of Na_2HPO_4.

The effect of the calcium chelators on the heat stability is of course dependent on the added chelator concentration. For instance, a higher heat coagulation time was measured for Na_2HPO_4 than for Na_2UMP upon addition of 15 mEq L^{-1} chelator at pH 6.7, because at this chelator concentration Na_2HPO_4 caused a stronger decrease in calcium-ion activity than Na_2UMP without affecting the micellar structure. It is most likely that every calcium chelator has an optimal concentration, at which the calcium-ion activity is sufficiently reduced and the micellar structure is not affected yet, inducing a strong increase in heat coagulation time.

The heat stability of SHMP forms an exception to this general trend. The heat stability of SHMP samples could not be predicted by measuring the initial calcium-ion activity, turbidity, and viscosity of the solutions. It was found that the heat-induced changes played a major role in the heat stability of the solutions. SHMP cross-linked the caseins, but these cross-links were apparently broken during heating, because a strong decrease in viscosity was measured during heating. Indeed, due to hydrolysis of SHMP during heating, a strong decrease in pH was measured in these SHMP samples. The disappearance of the SHMP cross-links as well as the hydrolysis of SHMP during heating increased the calcium-ion activity in the samples and this reduced the heat stability of the concentrated casein solutions.

Overall, the heat stability measurements of the concentrated micellar casein solutions have shown that the heat stability can be increased by choosing the correct concentration and type of calcium chelator or pH. By selecting pH 7.0 or adding one of the calcium chelators, a high heat stable concentrated dairy product can be produced.

7.3 Summarizing overview

The discussion in previous sections has illustrated that the type and concentration of calcium chelator determine the physical changes in the casein micelle structure, and, consequently, the physico-chemical properties of the concentrated micellar casein solution. The relationship between the different calcium chelators, casein micelle structure, and functionality of the concentrated micellar casein solutions is summarized in Fig. 7.2.

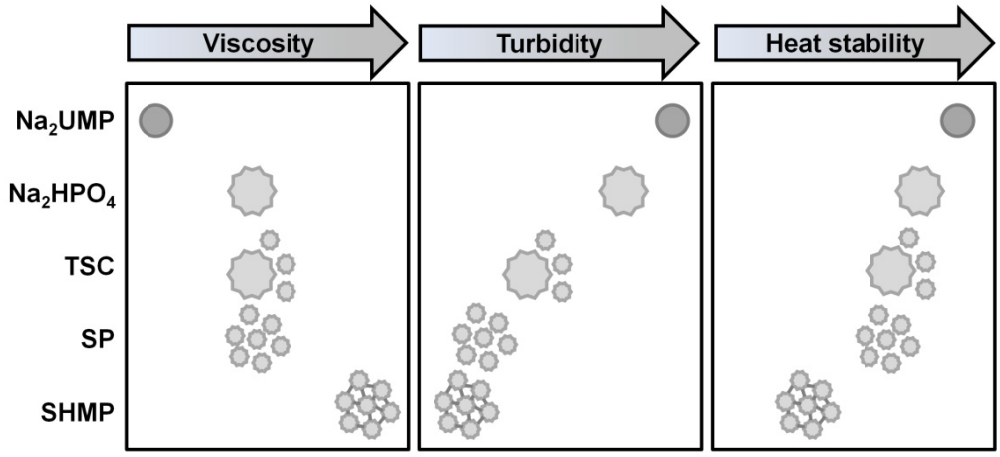

Figure 7.2 Relationship between the type of calcium chelator, effect on the micellar structure (swelling, (partial) dissociation, and cross-linking) and consequent effect on viscosity, turbidity, and heat stability of concentrated micellar casein solutions. The effect of the calcium chelators on the micellar structure is visualized as their ability to dissociate the casein micelle at a chelator concentration of about 60 mEq L^{-1}. The direction of the arrows denotes an increase of the indicated phenomenon.

Fig. 7.2 illustrates schematically how the different calcium chelators affected the micellar structure at a chelator concentration of about 60 mEq L^{-1}: Na_2UMP had negligible effects on the casein micelle structure; Na_2HPO_4 induced swelling of the intact casein micelles; TSC formed a mixture of swollen intact and dissociated casein micelles; SP dissociated all casein micelles into small, swollen particles; SHMP dissociated all casein micelles into small, swollen and cross-linked particles.

The different effects on the micellar structure affected the viscosity, turbidity, and heat stability of the concentrated micellar casein solutions. Fig. 7.2 shows that viscosity is determined by the extent of swelling and aggregation of the caseins (and not by the extent to which the casein micelles are dissociated). The turbidity is strongly related to the extent to which the casein micelles are dissociated. Cross-linking (by SHMP), however, did not increase the turbidity. Heat stability is dependent on the extent to which the casein micelles are dissociated or aggregated: dissociation and aggregation reduced heat stability.

In conclusion, the calcium chelators have a different influence on the micellar structure and the physico-chemical properties of the concentrated micellar casein solution. Which calcium chelator is most appropriate depends on the required functionality of the dairy application.

7.4 Practical relevance for the dairy industry

As described in Chapter 1 a low-viscosity product is often desired for medical nutrition, but it is challenging to find an optimal balance between high heat stability and low viscosity for a concentrated product. One approach to this challenge is to choose the right type and concentration of calcium chelator. This should be done in accordance with other minerals to obtain the required nutritional complete medical product (according Foods for Special Medical Purposes, FSMP).

An important finding in this thesis is that Na_2UMP is a very good heat stabilizer due to its chelating properties. Na_2UMP is commonly added to baby and medical products for its nutritional value as a nucleotide, but its behavior as calcium chelator has been previously unexplored. Upon addition of a certain amount of Na_2UMP, free calcium ions can sufficiently be bound by Na_2UMP to reduce calcium-induced protein aggregation without affecting the micellar structure. In this way, a high heat stability and low viscosity is obtained (PCT/NL2011/050521).

Another possible chelator is citrate. The heat stability of a product is improved by addition of citrate, but this increases the viscosity. If the citrate levels are reduced, this may lead to insufficient levels of sodium or potassium in the product from a nutritional point of view, since the most commonly used citrate sources in medical products are sodium and potassium citrate. Sodium and potassium can also be added as chloride or phosphate salts, but this may result in too high levels of chloride or phosphate on the product label. Lactate does not interact with CCP in the casein micelle, does not contribute to the product label, and is allowed in medical products. Consequently, by replacing sodium and potassium citrate by sodium and potassium lactate, the required cation levels can be reached and the viscosity can be kept low in the product (PCT/NL2012/050121).

The knowledge obtained about the effect of calcium chelators on the viscosity can also be used for the processing of dairy powders. This process generally starts with dissolving all ingredients in a liquid phase followed by spray-drying the resulting solution into a powder. The

concentration factor of the liquid phase is important for the process, because the higher the total solids content of the liquid phase the less energy is required to turn the liquid phase into powder. However, the viscosity of the liquid phase strongly increases with increasing solids content (see Fig. 1.2, Chapter 1), making it more difficult to process. Accordingly, the viscosity of the liquid phase restricts the possible total solids content in the liquid phase. The majority of the minerals are dissolved together with the proteins in the liquid phase, including citrate and phosphate sources, but this may easily increase the viscosity of the concentrated liquid phase. The extent these mineral sources affect the viscosity is dependent on the total solids content and the calcium chelator type and concentration added to the liquid phase. By eliminating the calcium chelators from the liquid phase, a higher total solids content with a lower viscosity can be obtained in the liquid phase. This reduces the process energy, and hence costs, in the rather expensive spray-drying process.

Calcium chelators can also be used to increase the viscosity in a product (WO2011/112087). Texturized medical products are often used by patients with swallowing disorders (i.e. dysphagia). For these patients it is important that the product remains viscous in the mouth. Starch is often used to obtain the required texture in the product, but starch is decomposed by amylase, resulting in loss of viscosity already in the mouth. By using calcium chelators amylase-resistant viscous products can be formulated for dysphagia patients. The required viscosity of the product can be obtained by selecting a suitable type and concentration of calcium chelators. SHMP seems to be the most suitable phosphate to formulate gel-like products.

Calcium chelators are also useful additives to create certain translucency in a product (WO2011/112087). For instance, (semi-)translucent fat-free high protein drinks can be formulated by adding a certain concentration of TSC, SP, or SHMP.

The knowledge obtained on the binding of polyphosphates to caseins may give interesting opportunities for the development of dairy products as well, since the polyphosphates affect the charge on the caseins. Liquid dairy products are usually formulated between pH 6.0 and 7.0 or below a pH of about 3.5, because milk proteins induce product instability around their IEP (i.e. around pH 4.5-5.0[39]). In Chapter 6 it was reported that the polyphosphates shift the IEP of the caseins to more acidic pH. Hence, by adding polyphosphates to the dairy product more robustness can be created to changes in pH, giving liquid and stable dairy products between pH 3.5 and 6.0.

7.5 Concluding remarks

The aim of this thesis was to determine relationships between calcium chelators and their influence on the casein micelle structure and on the physico-chemical properties of concentrated micellar casein solutions. The results in this thesis showed that calcium chelators have different influences on the micellar structure and physico-chemical properties of concentrated micellar casein solutions. The calcium chelators induced swelling, dissociation, and/or aggregation of the casein micelles. The viscosity, turbidity, and heat stability of concentrated casein-based products could, therefore, be manipulated by choosing the most appropriate type and concentration of calcium chelator (Fig. 7.2).

By further studying changes in the ion equilibria upon addition of calcium chelator, new insights into the interaction of calcium chelators with the casein micelle and calcium ions can be obtained. The simulations of the ion equilibria with the EIS model in the adapted SMUF solution indicated that the calcium chelators have different calcium-binding capacities and, consequently, affect the ion equilibria to different extents. These simulations were useful to understand the changes in the ion equilibria and micellar structure in concentrated micellar casein solutions upon calcium chelator addition. For this simulation the assumption had to be made that CCP in the casein micelles acts in a similar way to OCP in the adapted SMUF solution. To get a better understanding of the competition of calcium chelators and CCP in the casein micelles for calcium ions, it is recommended to develop an ion equilibria model for milk systems as well. Efforts have been made by Gao[20] and Mekmene *et al.*[24] to develop a model simulating the ion equilibria in milk. In both models assumptions had to be made for the composition of CCP, which did not completely correspond to the nature of CCP in the casein micelles.[20, 24] Therefore, efforts should be made to adapt these models to enable simulations of the ion equilibra in milk systems upon calcium chelator addition. It would also be interesting to determine and include the association constants and solubility products of SP in the model to make simulations for this calcium chelator possible as well.

Another interesting line of research would be to determine the concentration of calcium ions in the serum phase, casein micelles, and calcium complexes in the serum phase (see Fig. 1.4, Chapter 1) to learn more about the effect of the calcium chelators on the ion equilibria. In this thesis only the concentration of calcium ions in the serum phase (i.e. calcium-ion activity) was measured. Unfortunately, this did not provide any information about the distribution of calcium

ions in the casein micelles and in the calcium complexes in the serum phase (e.g. similar calcium-ion activity decrease for Na_2HPO_4, TSC, SP, and SHMP). Separation of the calcium complexes formed in the serum phase from the casein micelles may give useful information about the different calcium complexes that will be formed upon addition of the various chelators. Efforts have been made to obtain this separation by centrifugation or microfiltration (results not shown). However, casein micelles are strongly influenced by their environment, making it difficult to obtain different fractions without changing the ion equilibria. More research should be done to make quantification of calcium in the different fractions possible, so as to learn more about the competition of the caseins and chelators for calcium ions.

Finally, it was observed that SHMP affected the viscosity and heat stability of the concentrated micellar casein solution to a different extent than the other calcium chelators. In the literature SHMP is described as an effective additive to control age gelation or coagulation in ultra-high temperature milk.[12, 40, 41] In our case, SHMP caused gelling in unheated samples, which strongly reduced the heat stability. Kocak *et al.*[41] mentioned that SHMP samples supplied by different manufacturers differed in their ability to control age gelation. Gao[20] mentioned that SHMP can be considered as a cyclic or a linear molecule. It is recommended to investigate the SHMP molecule in more detail to obtain a better understanding of the influence of the different types of SHMP in milk systems. This will give the possibility to select a specific SHMP molecule to establish a required effect in a dairy product.

In conclusion, this thesis has provided new insights in the relationships between calcium chelators and their influence on the casein micelle structure and on the physico-chemical properties of concentrated micellar casein solutions. It was found that the viscosity and heat stability of a dairy product can be manipulated with the concentration and type of calcium chelator. Further studies on the influence of calcium chelators on ion equilibria in milk systems may open the way for new, innovative solutions in the development of dairy products.

7.6 References

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Summary

Medical products are often highly concentrated in nutrients, as they are designed for frail elderly and malnourished patients. Interaction between the nutrients (especially between casein proteins and minerals) increases viscosity and this is undesirable with respect to consumption. Medical products require an intensive heat treatment to guarantee a high microbiological quality, but this affects the physical stability (including viscosity) of the product. Calcium chelators are commonly added to dairy products to improve the heat stability, but these additives can easily increase the viscosity of a product. The challenge is, therefore, to formulate medical products with high heat stability and low viscosity. The aim of this thesis was to obtain a better understanding of the influence of different calcium chelators on the physico-chemical properties of casein micelles and the resulting effect on the viscosity and heat stability of concentrated micellar casein solutions.

In Chapter 1 a general introduction is given about medical nutrition and the challenges that are faced to formulate this type of products. The casein micelle and effect of calcium chelators on the mineral equilibria are discussed, as interactions between these constituents have a large impact on the viscosity and heat stability of a medical product.

In Chapter 2 the differences in calcium-binding capacity of disodium uridine monophosphate (Na_2UMP), disodium hydrogen phosphate (Na_2HPO_4), sodium phytate (SP), and sodium hexametaphosphate (SHMP) in a calcium chloride ($CaCl_2$) solution were investigated. This study was carried out to obtain a better understanding of the affinity of different calcium chelators for calcium ions, which gave useful information for understanding the interaction of calcium, phosphate, and casein micelles in dairy products. It was demonstrated that the calcium-binding capacity of the phosphates was directly related to their amount of charges: i.e. calcium ions were maximally bound in a ratio of 3:2 for Na_2HPO_4, 3:1 for SHMP, and 6:1 for SP in the $CaCl_2$ solution. Na_2UMP was found to be a weaker calcium chelator than the other phosphates, as significant levels of free calcium and free phosphate remained in the solution. An equilibrium constant of 0.29 ± 0.08 L mol^{-1} was determined for the formed calcium uridine monophosphate (CaUMP) complexes. Calculation of the equilibrium constant and analysis on the CaUMP precipitate confirmed a reactivity of 1:1 between calcium and Na_2UMP.

In Chapter 3 the effect of the calcium chelators on physical changes of casein micelles in concentrated micellar casein isolate (MCI) solutions was described. Trisodium citrate (TSC) was also investigated, because this calcium chelator is often used as heat stabilizer in medical products. Concentration ranges of Na_2UMP, Na_2HPO_4, TSC, SHMP, and SP were added to the concentrated MCI solution and the samples were analyzed for their calcium-ion activity, viscosity and turbidity. The samples were also ultracentrifuged in order to calculate the voluminosity of the caseins via ultracentrifugation and viscosity measurements. A strong correlation was found between the two methods for the voluminosity of the caseins. The effect of the calcium chelators on physical changes in the casein micelles in concentrated MCI solutions differed considerably. The highest viscosities were measured upon addition of SHMP, because it cross-linked the caseins. Na_2UMP showed negligible physical changes in the solutions, as its effect on the mineral equilibria and, herewith, the casein micelle was negligible. Samples with Na_2HPO_4, TSC, or SP showed similar increases in viscosity, but the turbidity of the samples decreased in the order of SP > TSC > Na_2HPO_4. The increase in viscosity was attributed to swelling of the caseins (i.e. increase in voluminosity) at decreasing calcium-ion activity. The major decrease in turbidity seemed to be due to dissociation of the casein micelles.

In Chapter 4 the heat stability of the concentrated MCI solutions was studied containing several calcium chelators at different concentrations. The effect of calcium chelators on heat coagulation and heat-induced changes of concentrated MCI solutions was investigated by measuring the heat coagulation time (HCT) together with changes in calcium-ion activity, viscosity, turbidity, and zeta potential before and after heating. It was found that the heat stability of the MCI solution improved by increasing the pH or by addition of calcium chelators. Na_2UMP was the most effective heat stabilizer, as it bound sufficient free calcium ions to reduce protein aggregation without affecting the micellar structure. The HCT of the MCI solutions after addition of Na_2HPO_4, TSC, and SP increased to comparable levels, but remained smaller compared to Na_2UMP. The slight differences in HCT for these samples were explained by the higher sensitivity of dissociated casein micelles for calcium-induced protein aggregation. SHMP was the least effective heat stabilizer. SHMP cross-linked the caseins, but these cross-links were apparently broken during heating, which decreased the pH and viscosity and increased the calcium-ion activity during heating. These observed heat-induced changes for SHMP were the major cause for the reduced heat stability. Overall, it was concluded that the differences in HCT

caused by the addition of the various calcium chelators could be attributed to the calcium-ion activity and state of the micellar structure before and during heating.

The results in Chapter 3 and 4 both showed that the calcium chelators induced different decreases in turbidity in the concentrated MCI solution. It was suggested that the differences could be explained by the degree of dissociation of the casein micelles. The aim of the study in Chapter 5 was to determine to what extent different calcium chelators affect micelle dissociation. The approach chosen was to measure particle size distributions in the casein solutions by using dynamic light scattering. Small particles with a diameter of 30 to 50 nm were observed upon micelle dissociation. The calcium chelators induced micelle dissociation in the order of SHMP > SP > TSC > Na_2HPO_4 > Na_2UMP, which was in agreement with the order of decrease in turbidity in the MCI solutions. Simulations of the ion equilibria with an ion speciation model showed that the extent of casein micelle dissociation followed the calcium-binding capacity of the calcium chelators, which in turn led to dissolution of colloidal calcium phosphate from the casein micelle. The results in Chapter 5 confirmed that the extent of micelle dissociation was found to be dependent on the type and concentration of calcium chelator.

Chapter 6 focused on investigating the binding of SHMP and SP to caseins. This was done by determining changes in the isoelectric point (IEP) through zeta potential measurements as a function of pH. SHMP and SP were added to sodium caseinate (calcium-poor) and MCI (calcium-rich) solutions to elucidate if calcium ions were required for the binding. The results indicated that the chelators bind to caseins, as they both shifted the IEP to more acidic pH. In the sodium caseinate solution a stronger decrease in IEP was measured for SHMP than for SP, indicating that more SHMP than SP was bound to the caseins. In the MCI solution the shift in IEP was smaller than in the sodium caseinate solution and was comparable for the two chelators. The shift in IEP was smaller in the MCI solution, because the chelators also formed complexes with calcium. SHMP and SP both decreased the IEP of casein solutions by forming direct bindings with the positively charged amino acids of caseins, for which calcium ions were not required.

Chapter 7 comprises a general discussion on the main results obtained in this study. The influence of the calcium chelators on the casein micelle structure and concentrated MCI solution is discussed and recommendations for further research are suggested. Also, the practical relevance for the dairy industry is described, demonstrating how different calcium chelators can manipulate the viscosity and heat stability of dairy products.

Samenvatting

Medische producten zijn vaak erg geconcentreerd in nutriënten, aangezien ze ontworpen zijn voor kwetsbare ouderen en ondervoede patiënten. Interacties tussen de nutriënten (met name tussen caseïne-eiwitten en mineralen) verhoogt de viscositeit en dit is ongewenst met betrekking tot de consumptie. Medische producten vereisen een intensieve hittebehandeling om een hoge microbiologische kwaliteit te garanderen, maar dit beïnvloedt de fysische stabiliteit (waaronder de viscositeit) van het product. Calciumbinders worden vaak toegevoegd aan zuivelproducten om de hittestabiliteit te verbeteren, maar deze additieven kunnen ook de viscositeit van het product verhogen. De uitdaging is dan ook om medische producten te ontwikkelen met een hoge hittestabiliteit en een lage viscositeit. Het doel van dit proefschrift was om een beter begrip te verkrijgen van de invloed van verschillende calciumbinders op de fysisch-chemische eigenschappen van caseïnemicellen en de daaruit voortvloeiende effecten op de viscositeit en de hittestabiliteit van geconcentreerde oplossingen van micellair caseïne.

In hoofdstuk 1 wordt een algemene inleiding gegeven over medische voeding en de uitdagingen die er zijn om dit soort producten te ontwikkelen. Het caseïnemicel en het effect van calciumbinders op de mineraalevenwichten worden besproken, aangezien interacties tussen deze componenten een grote invloed hebben op de viscositeit en hittestabiliteit van een medisch product.

In hoofdstuk 2 zijn de verschillen in calciumbindend vermogen van dinatriumuridinemonofosfaat (Na_2UMP), dinatriumwaterstoffosfaat (Na_2HPO_4), natriumfytaat (SP), en natriumhexametafosfaat (SHMP) in een calciumchlorideoplossing ($CaCl_2$) onderzocht. Deze studie werd uitgevoerd om een beter begrip te verkrijgen van de affiniteit van verschillende calciumbinders voor calciumionen, die nuttige informatie gaf voor het begrijpen van de interacties van calcium, fosfaat, en caseïnemicellen in zuivelproducten. Er werd aangetoond dat het calciumbindende vermogen van de fosfaten direct gerelateerd was aan het aantal negatieve ladingen. Dat wil zeggen: calciumionen werden maximaal gebonden in een verhouding van 3:2 met Na_2HPO_4, 3:1 met SHMP en 6:1 met SP in de $CaCl_2$-oplossing. Na_2UMP bleek een zwakkere calciumbinder te zijn dan de andere fosfaten, omdat er aanzienlijke niveaus vrij calcium en fosfaat in de oplossing aanwezig bleven. Een evenwichtsconstante van 0,29 ± 0,08 L mol^{-1} werd bepaald voor de gevormde calciumuridinemonofosfaat (CaUMP) complexen. Berekening van de evenwichts-

constante en de analyse van het CaUMP-neerslag bevestigde een reactiviteit van 1:1 tussen calcium en Na_2UMP.

In hoofdstuk 3 is het effect van de calciumbinders op de fysieke veranderingen van caseïnemicellen in geconcentreerde micellaire caseïne-isolaatoplossingen (MCI) beschreven. Trinatriumcitraat (TSC) is ook onderzocht in deze studie, omdat deze calciumbinder vaak wordt gebruikt als hittestabilisator in medische producten. Concentratiereeksen van Na_2UMP, Na_2HPO_4, TSC, SHMP en SP werden toegevoegd aan de geconcentreerde MCI-oplossing en de monsters werden geanalyseerd op hun calciumionactiviteit, viscositeit en troebelheid. De monsters werden ook geültracentrifugeerd om de voluminositeit van de caseïnes via ultracentrifugeren en viscositeitsmetingen te berekenen. Een sterke correlatie werd gevonden tussen de twee methoden voor bepaling van de voluminositeit van de caseïnes. Het effect van de calciumbinders op fysieke veranderingen in de caseïnemicellen in geconcentreerde MCI oplossingen verschilden aanzienlijk. De hoogste viscositeiten werden gemeten na toevoeging van SHMP, omdat het bindingen vormde tussen de caseïnes. Na_2UMP toonde verwaarloosbare fysieke veranderingen in de oplossingen, aangezien het effect op de mineraalevenwichten, en hiermee op het caseïnemicel, te verwaarlozen was. Monsters met Na_2HPO_4, TSC of SP toonden een vergelijkbare toename in viscositeit, terwijl de troebelheid van de monsters afnam in de volgorde van SP > TSC > Na_2HPO_4. De toename in viscositeit werd toegeschreven aan zwelling van de caseïnes (i.e. toename in voluminositeit) door het verlagen van de calciumionactiviteit. De belangrijkste daling in troebelheid van de MCI oplossingen leek te worden veroorzaakt door dissociatie van de caseïnemicellen.

In Hoofdstuk 4 is de hittestabiliteit van de geconcentreerde MCI-oplossingen onderzocht door het toevoegen van calciumbinders in verschillende concentraties. Het effect van calciumbinders op hittecoagulatie en hittegeïnduceerde veranderingen in geconcentreerde MCI-oplossingen is onderzocht door het meten van de hittecoagulatietijd (HCT) en de veranderingen in calciumionactiviteit, viscositeit, troebelheid, en zetapotentiaal voor en na verhitting. De hittestabiliteit van de MCI-oplossing bleek te verbeteren door het verhogen van de pH of door toevoegen van calciumbinders. Na_2UMP was de meest effectieve hittestabilisator, omdat het voldoende vrije calciumionen bindt om eiwitaggregatie te verlagen en een verwaarloosbaar effect heeft op de micellaire structuur. De HCT van de MCI-oplossingen met Na_2HPO_4, TSC en SP nam tot een vergelijkbaar niveau toe, maar de toename bleef kleiner dan met Na_2UMP. De kleine verschillen in HCT voor deze monsters werden verklaard door de hogere gevoeligheid

van de gedissocieerde caseïnemicellen voor calciumgeïnduceerde eiwitaggregatie. SHMP was de minst effectieve hittestabilisator. SHMP vormt bruggen tussen de caseïnes, maar deze bruggen worden waarschijnlijk verbroken tijdens verhitting. Dit zorgde tijdens verhitting voor een verlaging van de pH en de viscositeit en voor een verhoging van de calciumionactiviteit. Deze waargenomen hittegeïnduceerde veranderingen met SHMP waren de belangrijkste oorzaak voor de verminderde hittestabiliteit. Over het algemeen kon worden geconcludeerd dat de verschillen in HCT, veroorzaakt door de toevoeging van de verschillende calciumchelatoren, kunnen worden toegeschreven aan de calciumionactiviteit en de toestand van de micellaire structuur voor en tijdens verhitting.

De resultaten in hoofdstuk 3 en 4 hebben allebei laten zien dat calciumbinders verschillende afnames in troebelheid veroorzaken in de geconcentreerde MCI-oplossingen. Deze verschillen kunnen waarschijnlijk worden verklaard door de mate van dissociatie van de caseïnemicellen. Het doel van de studie in Hoofdstuk 5 was om te bepalen in welke mate de verschillende calciumbinders invloed hebben op de dissociatie van de caseïnemicellen. Hiervoor werden de deeltjesgrootteverdelingen bepaald in de caseïneoplossingen met behulp van dynamische lichtverstrooiing. Kleine deeltjes met een diameter van 30 tot 50 nm werden waargenomen als de caseïnemicellen dissocieerden. De calciumbinders induceerden dissociatie van de caseïnemicellen in de volgorde: SHMP > SP > TSC > Na_2HPO_4 > Na_2UMP dat in overeenstemming was met de volgorde van afname in troebelheid in de MCI-oplossingen. Simulaties van de ionevenwichten met een ionspeciatiemodel toonden aan dat de mate van dissociatie van de caseïnemicellen afhankelijk was van het calciumbindend vermogen van de calciumbinders, wat op zijn beurt leidde tot ontbinding van colloïdaal calciumfosfaat uit de caseïnemicellen. De resultaten in hoofdstuk 5 bevestigden dat de mate van dissociatie van de caseïnemicellen afhankelijk bleek te zijn van type en concentratie van calciumbinders.

Hoofdstuk 6 richt zich op onderzoek naar de binding van SHMP en SP aan de caseïnes. Dit werd gedaan door het bepalen van veranderingen in het iso-elektrische punt (IEP) door middel van zetapotentiaalmetingen als functie van de pH. SHMP en SP werden toegevoegd aan natriumcaseïnaat (calciumarm) en MCI-oplossingen (calciumrijk) om op te helderen of calciumionen nodig waren voor binding aan de caseïnes. De resultaten lieten zien dat de chelatoren binden aan caseïne, omdat ze allebei een verschuiving in het IEP naar meer zure pH gaven. In de natriumcaseïnaatoplossing werd een sterkere daling van het IEP gemeten voor SHMP dan voor SP, wat aangeeft dat meer SHMP dan SP gebonden werd aan de caseïnes. In de

MCI-oplossing was de verschuiving in IEP kleiner dan in de natriumcaseïnaatoplossing en was vergelijkbaar voor de twee calciumbinders. De verschuiving in IEP was kleiner in de MCI-oplossing, omdat de calciumbinders ook complexen vormden met calcium. SHMP en SP zorgden allebei voor een verlaging van het IEP van caseïneoplossingen door directe bindingen te vormen met de positief geladen aminozuren van de caseïnes, waarvoor calciumionen niet vereist waren.

Hoofdstuk 7 bevat een algemene discussie over de belangrijkste resultaten in deze studie. De invloed van calciumbinders op de caseïnemicelstructuur en geconcentreerde MCI-oplossing werd besproken en aanbevelingen voor verder onderzoek werden voorgesteld. Ook is de praktische relevantie voor de zuivelindustrie beschreven, waarbij wordt aangegeven hoe calciumbinders de viscositeit en hittestabiliteit van zuivelproducten beïnvloeden.

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Esther

List of publications

Peer-reviewed journals

De Kort, E.J.P., M. Minor, T.H.M. Snoeren, A.C.M. van Hooijdonk, and E. van der Linden, Calcium binding capacity of organic and inorganic ortho- and polyphosphates. *Dairy Science and Technology*, **2009**. *89*: p. 283–299.

De Kort, E.J.P., M. Minor, T.H.M. Snoeren, A.C.M. van Hooijdonk, and E. van der Linden, Effect of calcium chelators on the physical changes of casein micelles in concentrated micellar casein solutions. *International Dairy Journal*, **2011**. *21*: p. 907-913.

De Kort, E.J.P., M. Minor, T.H.M. Snoeren, A.C.M. van Hooijdonk, and E. van der Linden, Effect of calcium chelators on heat coagulation and heat-induced changes of concentrated micellar casein solutions: the role of calcium-ion activity and micellar integrity. *Accepted for publication in International Dairy Journal*, **2012**.

De Kort, E.J.P., V. Urbonaite, M. Minor, E. van der Linden, and A.C.M. van Hooijdonk, Dissociation of casein micelles by calcium chelators. *Submitted for publication.*

Patents

De Kort, E.J.P., R.J.J. Hageman, M. Groenendijk, P.J.G. Kamphuis, Liquid nucleotides/nucleosides-containing product. Publication number: WO2009082203, **2007**.

Minor, M., E.L. Sliwinski, N.E. Hotrum, W.H.A. Kiers, S.M. van Steenis, A. Waterink, E.J.P. de Kort, Protein-dense micellar casein-based liquid enteral nutritional composition. Publication number: EP2249666, **2008**.

De Kort, E.J.P., M. Groenendijk, P.J.G. Kamphuis, A palatable nutritional composition comprising a nucleotide and/or a nucleoside and a taste masking agent. Publication number: WO2009082227; EP2244591, **2008**.

De Kort, E.J.P., M. Minor, Controlling the texture of high-protein nutritional compositions comprising micellar casein. Publication number: WO2011/112087, **2010**.

Sliwinski, E.L., E.J.P. de Kort, C.T van Ravesteijn, A. Le Fur, Pre-thickened compact liquid nutritional composition for dysphagia patients. Filing number: PCT/NL2010/050342; PCT/NL2011/050390, **2010**.

De Kort, E.J.P., M. Minor, Use of a nucleotide for improving the heat stability of an aqueous micellar casein composition. Filing number: PCT/NL2011/050521, **2011**.

De Kort, E.J.P., Energy-rich liquid nutritional composition having improved organoleptic properties. Filing number: PCT/NL2012/050121, **2012**.

Conference abstracts

De Kort, E.J.P., M. Minor, T.H.M. Snoeren, A.C.M. van Hooijdonk, and E. van der Linden, Calcium binding capacity of organic and inorganic ortho- and polyphosphates. *Proceedings of the IDF/INRA 1st international symposium on minerals & dairy products,* **2008**. Saint Malo, France.

De Kort, E.J.P., M. Minor, T.H.M. Snoeren, A.C.M. van Hooijdonk, and E. van der Linden, Voluminosity of casein micelles in concentrated systems enriched with orthophosphates, polyphosphates, or citrate. Proceedings of the 13th Food Colloids 2010 – On the road … from interfaces to consumers. **2010**. Granada, Spain.

Curriculum vitae

Esther Jacqueline Petra de Kort was born in Dordrecht on 24 May 1979. In 1991, she started her secondary school at Emmaus College (Rotterdam, The Netherlands) and in 1998 she passed her secondary school exam at Sint Oelbert Gymnasium (Oosterhout, The Netherlands). In the same year she started the study Food Technology at Wageningen University (Wageningen, The Netherlands). As part of her MSc study, she performed a major thesis in the Laboratory of Food Chemistry and a minor thesis in the Department of Toxicology. She also performed two internships during her studies. She worked as a trainee at Nestlé Research Centre (Lausanne, Switzerland) from February till July 2003 and at Nutricia (Zoetermeer, The Netherlands) from March till June 2004. In June 2004, she graduated from Wageningen University with an MSc degree. In November 2004 she started as a Junior Scientist in the group of Product and Process Development at Numico Research (Wageningen, The Netherlands), which was renamed to Danone Research, Centre for Specialised Nutrition, in April 2008. In January 2007, a PhD project was started in collaboration with the Dairy Science & Technology group and Physics & Physical Chemistry of Foods group of Wageningen University. The results collected for the PhD project are described in this thesis. Currently, Esther works as a Scientist in the group of Product Development Medical Nutrition at Danone Research, Centre for Specialised Nutrition.

Overview of completed training activities

Discipline specific activities

Courses

Gels, thickeners and stabilizing agents, Leatherhead Food International, Leatherhead, UK (2005)

Basics of rheology for food applications, Anton Paar Benelux, Wageningen, The Netherlands (2006)

Mastersizer & Zetasizer training, Sysmex, Etten-Leur, The Netherlands (2007)

Malvern Zetasizer Nano training, Sysmex, Etten-Leur, The Netherlands (2011)

Congresses, symposia

International Technology symposium, Numico Research, Wageningen, The Netherlands (2005, 2006, 2007, 2008)

Global Technology symposium, Danone Research, Doorwerth, The Netherlands (2009, 2010, 2011, 2012)

Soya drinks & desserts conference, Prosoy research & strategy, Cologne, Germany (2005)

8th International Hydrocolloids conference, NTNU, Trondheim, Norway (2006)

1e Voedings- & gezondheidscongres, Amsterdam, The Netherlands (2006)

World Dairy Summit, Dairying – can it manage change? IDF, Dublin, Ireland (2007)

Food Colloids 2008 – Creating structure, delivering functionality, Université du Maine, Le Mans, France (2008)

1st International symposium on minerals & dairy products, IDF/INRA, Saint Malo, France (2008)

Delivery of functionality in complex food systems – physically-inspired approaches from nanoscale to microscale, Wageningen University, Wageningen, The Netherlands (2009)

NIZO Dairy conference, Dairy ingredients: innovations in functionality, Papendal, The Netherlands (2009)

Food Colloids 2010 – On the road... from interfaces to consumers, University of Granada, Granada, Spain (2010)

General courses

Numico Excellence Training (NEXT) General, The Netherlands (2006-2007)

International postgraduate course – Design of Experiments, Wageningen Business School, Wageningen, The Netherlands (2007)

Techniques for writing and presenting a scientific paper, Wageningen Graduate Schools, Wageningen, The Netherlands (2008)

Teaching is not presenting, Pentacle-learning to transform, Amsterdam, The Netherlands (2010)

Danone Odyssee Explorer, Insights Benelux BV, Doorwerth, The Netherlands (2010)

Finance for non-financials, Danone Research, Wageningen, The Netherlands (2010)

Project management soft skills, Management Centre Europe, Doorwerth, The Netherlands (2011)

Convince in 15 minutes, Agent Majeur, Doorwerth, The Netherlands (2011)

Getting things Done, Tijdwinst.com, Wageningen, The Netherlands (2011)

CODE Developer, Impact NV, Doorwerth, The Netherlands (2011)

Optionals

Preparation PhD research proposal

Course Colloid Science, Wageningen University, Wageningen, The Netherlands (2008)

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