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Icium Bioavailability and Exchangeability in Milk

An in vitro study to investigate the influence of calcium salt fortification using ^{45}Ca

M.E. de Vos

Calcium Bioavailability and Exchangeability in Milk

An *in vitro* study to investigate the influence of calcium salt fortification using ^{45}Ca

By

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Abstract

Calcium is an important nutrient for both young and elderly people which can, among others, stimulate healthy bone growth and prevent osteoporosis. Therefore, nutrition with sufficient calcium is of great importance. The highest calcium nutrient density for all foods can be found in bovine milk, which contains on average 1.2 g Ca / L. To increase the amount of bioavailable calcium, milk can be fortified by the addition of different calcium salts. In this research the bioavailability and exchangeability of calcium in unfortified and fortified skimmed milk was studied by *in vitro* methods using equilibrium dialysis and ultracentrifugation to separate different fractions in milk. Radioactive ^{45}Ca was used to follow the exchange behaviour of calcium between the three different fractions in milk: casein micelles, serum proteins, and soluble calcium.

For unfortified milk it was found that 34.9 ± 2.2% of the calcium was present in the soluble phase, 9.1 ± 0.6% was bound to serum proteins, and 56 ± 1.6% was present in the casein micelles, which confirmed the results in literature. In addition, with the investigation of the exchange behaviour of ^{45}Ca, it was confirmed that ~40% of calcium in casein micelles is hardly exchanged.

To investigate the influence of calcium fortification on the calcium equilibrium in milk, six different calcium salts were selected to be synthesized: calcium chloride, calcium carbonate, calcium gluconate, tri-calcium di-citrate, calcium lactate, and tri-calcium phosphate. Of these, calcium chloride, calcium carbonate, calcium gluconate, and tri-calcium di-citrate were successfully synthesized. Further experiments were performed with calcium chloride and calcium carbonate, intrinsically labelled with ^{45}Ca. From fractionation with ultracentrifugation, it was concluded that addition of calcium chloride led to an increase of calcium in the soluble phase, which might indicate an increase in bioavailability. Fortification of milk with ^{45}Ca labelled calcium carbonate did not lead to an exchange of ^{45}Ca with the calcium present in milk, indicating that calcium carbonate is unlikely to make calcium in milk more bioavailable. In conclusion, a better understanding of the solubility and exchangeability of Ca in milk and the influence of milk fortification with calcium chloride and calcium carbonate is obtained by using ^{45}Ca as a tracer.

Content

List of Abbreviations

ALARA	= As low as reasonably achievable
Ca	= Calcium
CaCi	= Tri-calcium di-citrate
$CaCl_2$	= Calcium chloride
$CaCO_3$	= Calcium carbonate
CaGluc	= Calcium gluconate
CaLac	= Calcium lactate
CaOH	= Calcium hydroxide
CaSac	= Calcium saccharate
CCP	= Colloidal calcium phosphate
CPM	= Counts per minute
CPS	= Counts per second
Cu	= Copper
FTIR	= Fourier-transform infrared
GIT	= Gastrointestinal tract
HTE	= Hard-to-exchange
ICDD	= International Centre for Diffraction Data
ICP-OES	= Inductively Coupled Plasma – Optical Emission Spectroscopy
IPA	= Instrument Performance Assessment
IR	= Infrared
KCl	= Potassium chloride
LSC	= Liquid scintillation counting
MQ	= MilliQ water
NaCl	= Sodium chloride
PMT	= Photo Multiplier Tube
QIP	= Quenching Indicating Parameter
SIS	= Spectral Index of the Sample
TCP	= Tri-calcium phosphate
tSIE	= Transformed Spectral Index of External Standard
WHO	= World Health Organization
XRD	= X-ray diffraction

1. Introduction

Calcium (Ca) is an important nutrient for both young and elderly people. Millions of people around the world suffer from osteoporosis, a skeleton disorder disease in which bones become more fragile and likely to fracture, resulting from bone density loss. The loss of bone density is related to a shortage of Ca mineral intake [1], [2]. Ca intake also reduces the risk of colon cancer, kidney stones, and hypertension [3]–[5]. In addition, Ca has a positive influence on the normal bone growth of infants and children. However, in some populations not even 50% of the reference Ca intake, which is 800-1300 mg/day, is reached [1], [2], [5]. To make sure both young and elderly people fulfil the recommended Ca intake, governments and health organizations encourage the Ca fortification of food products [5]. Milk is a good candidate for Ca fortification, since it is known that Ca in milk is more easily absorbed in the human body than Ca from fruits and vegetables, due to the specific food matrix of milk [5]. Besides, milk has a high nutritional value and is included in most of the dietary guidelines [6]. Therefore, bovine milk fortification with Ca salts is a good strategy.

However, the bioavailability of the Ca salts is not yet fully characterized [1]. The bioavailability of Ca is defined as the fraction of Ca that is absorbed and used by the body [7], [8]. Approximately 90% of the Ca absorption takes place in the small intestines and the Ca should be in the soluble ionised form (Ca^{2+}) or bound to organic molecules, like citrate or phosphate, to be absorbed through the intestinal wall [2]. Ca in milk exists in two phases: soluble and insoluble Ca. Another way to describe the Ca distribution in milk is according to three fractions: (1) soluble Ca, (2) Ca bound to proteins, and (3) Ca present in casein micelles. (1) Approximately one third of the Ca in milk is present as soluble Ca, which exists as Ca ions (Ca^{2+}) or is bound to organic molecules such as citrate or phosphate. The other two third is insoluble Ca, (2) of which ~10% is bound to proteins like casein and whey (serum proteins) and (3) ~90% is present in casein micelles, which are colloidal particles consisting of casein protein and calcium phosphate [9]. Of these phases/fractions the soluble phase represents the bioavailable phase of the milk, as this phase can penetrate the intestinal wall. However, the phase distribution of Ca in milk is influenced by many factors, such as pH and reactive precursors, and will change upon insertion to the gastrointestinal tract (GIT) [2]. According to a review study of Fardet et al. the Ca absorption of bovine milk in the human body is between 17 - 55%, with the majority of the *in vivo* studies having a Ca absorption of 20 – 30% [10].

Since *in vivo* studies are expensive and difficult to perform, often inexpensive, rapid and simple *in vitro* methods are used to investigate the solubility of Ca in milk. The results obtained from those studies do not reflect the actual bioavailability of Ca in the human body, but may give an indication of the bioavailability and the influence of several factors, such as Ca salt fortification [10]. Examples of *in vitro* methods are ultrafiltration, ultracentrifugation (UC), continuous and passive dialysis, and milk treatment with ion exchange resins [11], [12].

Several *in vitro* studies were performed to investigate the influences of factors such as pH, food components, and temperature on the bioavailability of Ca. Those studies were performed with either stable or radioactive isotopes of Ca [3], [7], [11], [13].

Yamauchi et al. used ^{45}Ca to investigate the exchangeability of ^{45}Ca in milk with dialysis. Dialysis is a method in which a semi-permeable membrane is used to separate the soluble and insoluble phase of Ca in milk. It was observed that approximately 40% of the Ca in the casein micelles was hardly exchanged with the ^{45}Ca added to the soluble phase of the milk. This Ca was defined as hard-to-exchange (HTE) Ca [14]. Pierre et al. and Zhang et al. observed similar results with resp. 35% and 45% HTE Ca [15], [16]. Additionally, the influence of time, temperature, and pH on the phase distribution

and exchangeability of ^{45}Ca in milk was studied [15]–[18]. However, in those studies the influence of milk fortification with Ca salt was not investigated.

Nonetheless, some other studies were performed to investigate the influence of Ca salt fortification on the bioavailability of Ca, using nonradioactive Ca. Sittikulwitit et al. studied the dialyzability of milk fortified with different Ca salts by simulating the GIT. The investigated Ca salts were calcium carbonate ($CaCO_3$), tri-calcium phosphate (TCP), calcium lactate (CaLac), tri-calcium di-citrate (CaCi), and calcium lactogluconate. All Ca salts caused an increase in Ca dialyzability, with TCP having the highest percentage dialyzable Ca followed by CaCi and $CaCO_3$ [4]. López-Huertas et al. performed an *in vivo* study in humans with milk fortified with TCP and an increase in Ca absorption was observed in comparison with unfortified milk [1]. Also Singh et al. investigated which calcium salts are suitable to fortify calcium in milk, using an *in vivo* study in mice. The metabolic study showed that fortification with Ca chloride ($CaCl_2$) led to a decrease in Ca absorption, while fortification with Ca lactate (CaLac) and Ca gluconate (CaGluc) led to an increase in Ca absorption in comparison with unfortified milk [5]. For further insights in the properties of different Ca salts and their interaction upon digestion, it is recommended to read the article of Vavrusova & Skibsted [2]. Based on previous research and the feasibility to synthesis the salts, the salts $CaCl_2$, $CaCO_3$, TCP, CaLac, CaCi, and CaGluc are further investigated in this research.

The aim of this thesis project is to obtain a better understanding of the solubility and exchangeability of Ca in skimmed bovine milk and the influence of milk fortification with different Ca salts, making use of ^{45}Ca to obtain additional insights on the exchange behaviour of Ca between the different fractions of milk. The used methods for milk phase separation are dialysis and ultracentrifugation.

This scientific question is addressed by answering the following sub research questions:

- How can ^{45}Ca in milk samples be measured?
- How can the different Ca salts ($CaCl_2$, $CaCO_3$, CaCi, CaGluc, TCP and CaLac) be synthesized starting from $CaCl_2$ and labelled intrinsically with ^{45}Ca?
- Are the same results obtained for phase separation with dialysis and ultracentrifugation?
- In which fraction of the milk is the Ca and ^{45}Ca present (soluble / protein bound / casein micelles)?
- What is the percentage of HTE Ca in the casein micelles?
- What is the influence of Ca salt fortification on the phase distribution?
- Which Ca salts are likely to make Ca in milk more bioavailable?

2. Theory

2.1 Calcium isotopes

Many isotopes of calcium (Ca) exist. The most abundant isotope is ^{40}Ca, which is a stable isotope. Other stable calcium isotopes are ^{42}Ca, ^{43}Ca, ^{44}Ca, and ^{46}Ca. All the other 19 Ca isotopes are unstable [19]. For this research radioactive Ca will be used as a tracer to detect the phase distribution of Ca. A radioactive isotope is preferred as this can be used to distinguish the added Ca from the Ca present in the milk.

2.1.1. Radioactive isotopes of calcium

Ca has two radioactive isotopes with suitable half-lives for the use as tracer, which are ^{45}Ca and ^{47}Ca with half-lives of 163 and 4.54 days, respectively. Most other radioactive Ca isotopes have a too short half-life, ranging from nanoseconds to minutes, except for ^{41}Ca and ^{48}Ca, which have very long half-lives of $1.3*10^{5}$ and $6.4*10^{19}$ years, respectively [19].

In this research, ^{45}Ca is chosen as tracer mostly because of its half-life but also based on commercial availability. Figure 1 shows the decay scheme of ^{45}Ca. ^{45}Ca is a β^- emitter which emits only low energy gammas (12 keV), therefore Liquid Scintillation Counting (LSC) is one of the most effective measurement techniques [20], [21].

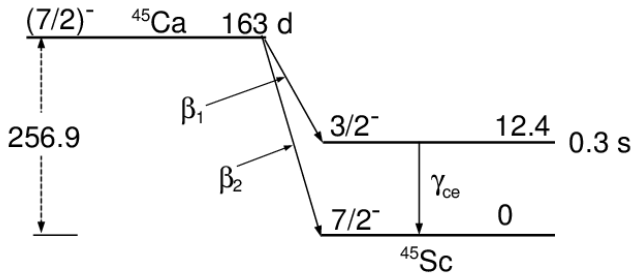

Figure 1: The decay scheme of ^{45}Ca. The energies are indicated in keV [22].

2.2 The dairy matrix

The addition of Ca to milk does not only influence the equilibrium of Ca in milk. All components of milk (e.g. carbohydrates, proteins, and minerals) are contained in a complex structure and together determine the physiological and nutritional properties of the milk [6], [10], [23]. This complex structure is called the dairy matrix.

For food in general, recent studies show that the nutritional properties are not only determined by the sum of the individual components and their properties, but by the food product as a whole, the so-called food matrix. In this complex structure constituents influence each other [23]. Although the food matrix for plant-based foods has already widely been investigated, still a lot of research has to be done concerning the food matrix of animal-based foods, such as milk [10].

2.3 Milk composition

From the macroscopic level, milk can be described as ''an oil-in-water emulsion formed by small milk fat globules dispersed in a serum phase'' [23]. From the microscopic level, milk is as a solution consisting of lactose, minerals, vitamins, soluble proteins, and other components in which casein micelles and non-soluble proteins form a suspension [23]. As proteins and casein micelles play an important role in the Ca distribution, these two constituents will be further discussed in the following two paragraphs [24].

As this research is about bovine milk, all compositions and properties of the milk are described for bovine milk. However, the composition and properties of different types of milk, such as human milk and infant formula, differ significantly. Differences being among others the ratio of serum proteins and casein micelles, the size of the casein micelles, and in which phase the Ca is mainly present. All these factors can influence the bioavailability of Ca [11]. The differences between various types of milk is widely investigated [7], [10], [11], [25]–[27].

2.3.1. Proteins in milk

Bovine milk contains two types of proteins; casein and whey. Approximately 20% of the proteins consist of whey protein, of which 70% β-lactoglobulin (which forms 50% of the soluble proteins), 20% α-lactalbumin, and 10% serum albumin [23]. The other 80% of proteins in milk consist of the phosphoprotein, casein, which is a mixture of; α_{S1}-, α_{S2}-, β-, and κ-caseins. These caseins differ in primary structure and therefore have different properties [28]. One of the differences is the amount of phosphorylated groups. The phosphorylated groups are responsible for the bindings of the casein protein with Ca. κ-casein has only one phosphorylated group and therefore is stable in the presence of Ca. α- and β-caseins are highly phosphorylated and therefore sensitive to the amount of Ca salts and will precipitate with an excess of Ca^{2+} [29]. A small part of the casein micelles is present in the soluble form, the other part of the casein proteins exist in a unique colloidal structure bound to phosphate; the casein micelles [2], [30].

2.3.2. Casein micelles

Casein micelles are colloidal particles which cause the white appearance of skim milk due to the strong light scattering [31]. Approximately 92% of the dry weight of the casein micelles consists of casein proteins (α_{S1}-, α_{S2}-, β- and κ-caseins), together with Ca, inorganic phosphate, magnesium, and citrate ions [30], [31]. Due to their unique structure casein micelles contain and transport many of the nutrients existing in milk. Approximately two third of the Ca, one third of the magnesium and half of the inorganic phosphate in milk, is contained in the casein micelles [23], [31]. Besides the casein micelles play an important role in the textural and sensory properties of the milk [28].

The particles are known as casein micelles, but should not be confused with classical micelles, like detergent micelles [31]. The structure of casein micelles is, despite years of research, still under discussion and many models have been proposed [28], [31], [32]. Most of the proposed models can be categorised in one of the following categories: the coat-core, sub-micelle, and the internal structure category. The original models in the specific categories are modified or discarded over the years [28], [29]. Further information on the categories, the models per category and adaptions over the years can be found in the review paper of Phadungath [29].

One of the more recent models is the nano-cluster model proposed by Holt in 1992 [28], [29]. In this model, a casein micelle is considered as ‘’a (homogeneous) protein matrix of caseins (a nanogel) in which the colloidal calcium phosphate (CCP) nanoclusters are dispersed as small ‘cherry stones’’’ (Figure 2)[28]. The phosphorylated casein proteins bind to the CCP nanoclusters and prohibit them from growing. The tails of the casein proteins stick out of the nanocluster network and form a homogenous network together, due to weak interactions leading to self-assembling. It is suggested that the protein κ-casein limits the self-assembly and therefore stabilizes the casein micelle [10], [28]. As discussed before, the difference between κ-caseins on the one side and α- and β-caseins on the other side, is the amount phosphorylated groups. κ-casein has only one phosphorylated group and therefore is stable in the presence of Ca ions. α- and β-caseins are highly phosphorylated [29], [33]. Besides, casein micelles are negatively charged due to the fact that κ-caseins are negatively charged. This negative charge stabilize the casein micelles, due to electrostatic repulsion and steric

hindrance [34], [35]. 90% of Ca in the casein micelles is present in CCP nanoclusters, 10% of the Ca is directly bound amino acids of the casein proteins [36]. Casein micelles can be modified by many factors, such as pH, temperature, ultrasounds, pressure, and the addition of chemicals [35], [34].

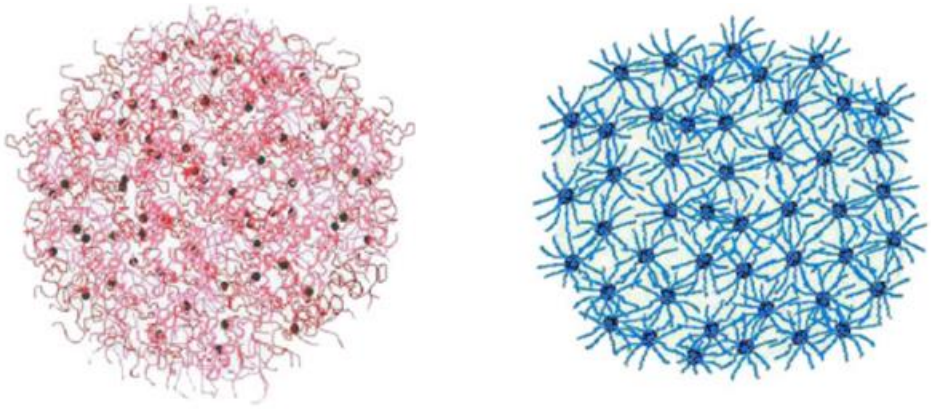

Figure 2: Two Impressions of a casein micelle according to the model proposed by Holt [28]*. The small dots represent the colloidal calcium phosphate nanoclusters surrounded by a hairy layer of casein proteins.*

2.4 Phase separation of milk

To determine the distribution of Ca in milk, the different phases and fractions of milk have to be separated. Frequently used methods to separate the phases are dialysis and ultracentrifugation and will be described in the following paragraphs.

2.4.1. Dialysis

Dialysis is a commonly used method to separate salts from a solution [37], [38]. This method can be used to separate insoluble Ca (present in serum proteins and casein micelles) from soluble Ca (present as Ca ions (Ca^{2+}) or bound to organic molecules, such as citrate or phosphate). Dialysis is a diffusion driven process in which molecules or ions can be separated over a semi-permeable membrane, based on their size (Figure 3). Smaller molecules (e.g. salts) can be separated from larger molecules (e.g. proteins) in this way. The molecules or atoms, which can penetrate through the membrane, will diffuse from a higher concentration to a lower concentration till equilibrium is reached (Figure 3). The solution where the ions diffuse to is called the dialysate. As the process is diffusion driven it is, according to Fick's law, depending on the concentration, diffusion path (distance), time, and temperature.

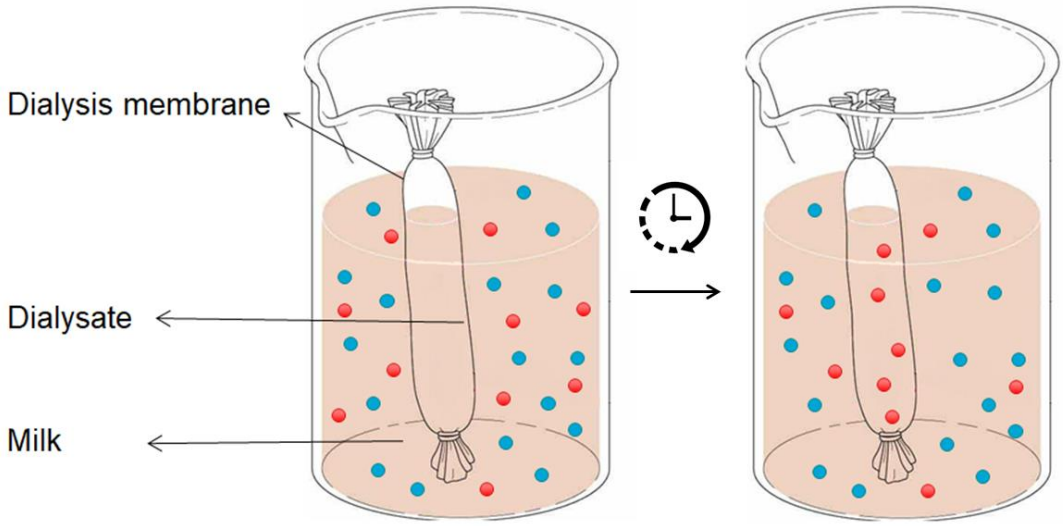

Figure 3: Sketch of a dialysis setup. The blue dots represent the insoluble calcium (bound to casein micelles and serum proteins) and the red dots represent the soluble calcium which will equilibriate over the membrane.

Additionally, osmotic pressure has an influence on the dialysis equilibrium. Milk has an osmotic pressure of around 7 bar [39]. The osmotic pressure in milk is mainly influenced by lactose, sodium, potassium and chloride [40].

2.4.2. Ultracentrifugation

A widely used method to perform phase separation is (ultra)centrifugation [40]. An advantage of this separation method is that not only the soluble and insoluble phase of milk, but also the casein micelles and serum proteins can be separated. The casein micelles are separated by precipitation due to ultracentrifugation [29], after which the serum proteins are separated with centrifugation over a 10 kDa filter [41] (Figure 4).

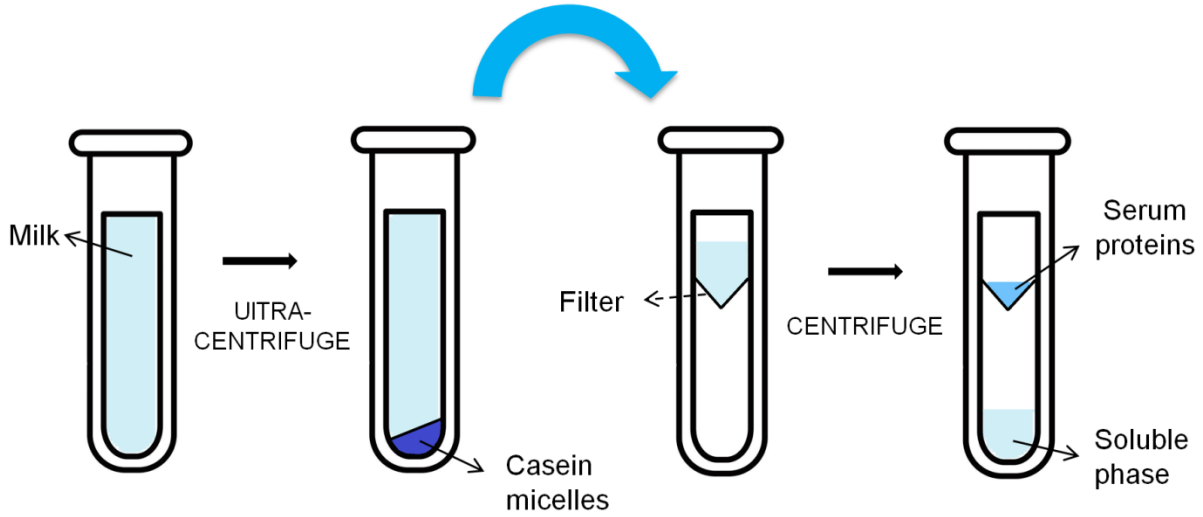

Figure 4: Schematic representation of the steps for ultracentrifugation. First casein micelles are separated from the milk in an ultracentrifuge. Then the supernatant is centrifuged with a filter, and the serum proteins are separated from the soluble phase (permeate).

2.5 Calcium bioavailability

By addition of Ca salts to milk, the bioavailability of Ca may be increased. Bioavailability is defined as the fraction of an ingest nutrient that is absorbed and used by the body for physiological processes or storage [7], [8]. As mentioned previously, *in vivo* and *in vitro* methods can be used to estimate the calcium bioavailability. However, *in vitro* methods may give an indication of the bioavailability but do not necessarily correspond to the bioavailability *in vivo*, as physiological effects can influence the bioavailability [42]. To measure the actual bioavailability animal models and human subjects are required [3]. *In vitro* methods often simulate the gastrointestinal digestion and can be used to measure the bio accessibility or the solubilisation of the calcium [10], [11], [42].

In general a distinction can be made between two ways to look at *in vitro* methods as an estimation of the bioavailability: (1) based on the dialyzability of the mineral and (2) based on the solubility of the mineral [11]. Both methods lead to different estimations of the bioavailability and this should be taken in consideration when comparing data. Therefore, the difference between dialyzability and solubility will be explained in the following paragraphs. In this research the solubility was used as estimate for the bioavailability.

2.5.1. Dialyzability

Equilibrium dialysis can be used to look at the dialyzability of minerals. By calculating the dialyzability, the dialyzable fraction is calculated. This is the fraction of mineral in the supernatant/dialysate after diffusion. This method is used to simulate the gastrointestinal digestion

of the food, by simulating the passive diffusion through the mucosa [11]. So in case the sample is outside of the dialysis tubing and the dialysate inside the dialysis tubing, the dialyzable fraction is the Ca inside the dialysis tubing (Figure 5). This method is frequently used to indicate the bioavailability [7], [11], [26], [42]. The dialyzable fraction is calculated according to Equation 1. Where D (mg) is the total amount of dialyzed Ca, [A] (mg/L) is the total concentration of Ca in milk, and V_s (L) the volume of the milk sample.

$$Dialyzability\ (\%) = \frac{D}{[A]*V_S} * 100\%$$

Equation 1

This method is assuming that only half of the soluble Ca will diffuse the mucosa and consequently ignores the fact that part of the soluble mineral will be present in the tube and the other part of the soluble element will be present outside the tube, due to equilibrium as explained earlier (2.4.1. Dialysis).

It should be mentioned that Roig et al. is performing the calculation of the dialyzability based on concentrations, instead of the amounts of mineral (Equation 2), taking both the concentration of dialyzed Ca [D] and the total concentration of Ca [A] in mg/100 mL [11]. This formula is only applicable if the amount of volume in the dialysis membrane and outside the dialysis membrane is exactly the same.

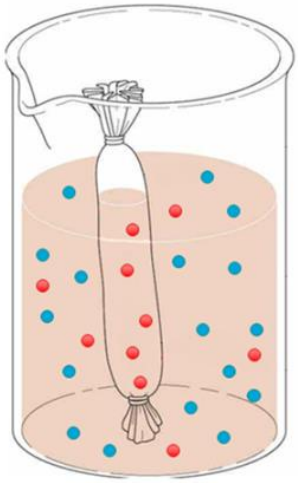

Figure 5: Sketch of a dialysis setup after equilibriating. The blue dots represent the insoluble Ca (bound to casein micelles and serum proteins) and the red dots represent the soluble Ca.

$$Dialyzability\ (\%) = \frac{[D]}{[A]} * 100\%$$

Equation 2

2.5.2. Solubility

To look at the solubility of the mineral, multiple methods can be used, among others: continuous dialysis [10], [11], equilibrium dialysis, and ultracentrifugation. For this research, equilibrium dialysis and ultracentrifugation were used and therefore are explained further.

2.5.2.1. Solubility - equilibrium dialysis

Equilibrium dialysis is the dialysis method as explained earlier (2.4.1. Dialysis). During equilibrium dialysis the soluble Ca penetrates the membrane until equilibrium is reached. In case of equilibrium, the concentration of soluble Ca is equal in- and outside the tube (Figure 5). The solubility is the total percentage of soluble Ca, so the amount of soluble Ca in the tube plus the amount of soluble Ca outside the tube. Kennefick & Cashman and Wolter et al. calculated the solubility according to Equation 3, which can only be used if the volumes in and outside the tubing are exactly the same, which is often not the case [3], [13]. In the equation D (mg) is the amount of dialyzed Ca, [A] (mg/L) the total concentration of Ca in milk, and V_S (L) the volume of the milk sample.

$$Solubility\ (\%) = \frac{2D}{[A] * V_S} * 100\ \%$$

Equation 3

A more general formula was used in this research project (Equation 4). Were [D] (mg/L) is the concentration of dialyzed Ca, V_D (L) the volume of the dialysate, V_s (L) the volume of the milk sample, and [A] (mg/L) the total concentration of Ca in milk.

$$Solubility\ (\%) = \frac{[D] * (V_D + V_S)}{[A] * V_S} * 100\%$$

Equation 4

2.5.2.2. Solubility – ultracentrifugation

Phase separation can also be performed with ultracentrifugation as explained earlier (2.4.2. Ultracentrifugation). The solubility can be calculated by taking the concentration of Ca in the sample, after removal of the casein micelles and serum proteins, divided by the total concentration of the milk sample (A) (Equation 5). It can be assumed that the total volume after removal of the casein micelles and the serum proteins stays approximately the same. Therefore, the concentrations can be divided.

$$Solubility\ (\%) = \frac{[Soluble\ phase]}{[A]} * 100\%$$

Equation 5

The percentage of Ca in the casein micelles and in the serum proteins can be calculated in a similar manner (Equation 6 and Equation 7).

$$Casein\ micelle\ (\%) = \frac{[A] - [serum\ protein + soluble\ phase]}{[A]} * 100\%$$

Equation 6

$$Serum\ protein\ (\%) = \frac{[serum\ protein + soluble\ phase] - [soluble\ phase]}{[A]} * 100\%$$

Equation 7

2.6 Calcium exchangeability

As discussed earlier Ca in milk exists in three fractions: in the casein micelles, bound to serum proteins, and as soluble Ca. A continuous dynamic equilibrium exists between the different fractions of milk. As discussed before casein micelles consist of casein proteins, Ca, inorganic phosphate, citrate, and magnesium. The ions and proteins in the casein micelles continuously exchange with the soluble phase of milk, forming a dynamic equilibrium [31], [34].

2.6.1. Hard to exchange calcium

Research has shown that part of the Ca in the casein micelles cannot freely exchange, this is the so called 'hard-to-exchange' (HTE) Ca [14], [17]. Yamauchi et al. has shown that the HTE Ca is approximately 40% of the Ca present in the casein micelles of skimmed milk. According to Yamauchi et al. it seems like the HTE Ca is present as hydroxyapatite, which is a calcium phosphate mineral present in the casein micelles [14].

2.6.2. Calculation exchangeability

The percentage of exchangeable Ca ($\%Ca_{exchange}$) in the insoluble phase can be calculated according Equation 8 [14]–[17], [43], [44]. In this equation $^{45}Ca_{insol}$, $^{45}Ca_{sol}$, Ca_{insol} and Ca_{sol} are the amounts of $^{(45)}Ca$ in the insoluble and soluble phase, expressed in weight units or percentages. The percentage of HTE Ca in the insoluble phase can be calculated according to Equation 9.

$$\%Ca_{exchange} = \frac{^{45}Ca_{insol}}{^{45}Ca_{sol}} * \frac{Ca_{sol}}{Ca_{insol}} * 100\%$$

Equation 8

$$\%Ca_{HTE} = 100\% - \%Ca_{exchange}$$

Equation 9

To understand how Equation 8 is derived, first $^{45}Ca_{sol}$ and $^{45}Ca_{insol}$ have to be defined. $^{45}Ca_{sol}$ can be defined by multiplying the total amount ^{45}Ca ($^{45}Ca_{tot}$) with the fraction of soluble Ca relative to the total amount of Ca that can be exchanged, this is the soluble Ca plus the exchangeable insoluble Ca (Ca_{sol} + ($\%Ca_{exch}$/100%)*Ca_{insol}) (Equation 10). $^{45}Ca_{insol}$ can be defined in the same way, however, in this equation the fraction of exchangeable insoluble Ca is used to multiply with the total amount of ^{45}Ca (Equation 11).

$$^{45}Ca_{sol} = {}^{45}Ca_{tot} * \frac{Ca_{sol}}{Ca_{sol} + \left(\frac{\%Ca_{exchange}}{100\%}\right) * Ca_{insol}}$$

Equation 10

$$^{45}Ca_{insol} = {}^{45}Ca * \frac{\left(\frac{\%Ca_{exchange}}{100}\right) * Ca_{insol}}{Ca_{sol} + \left(\frac{\%Ca_{exchange}}{100\%}\right) * Ca_{insol}}$$

Equation 11

Dividing Equation 11 by Equation 10 gives Equation 12. By multiplying both sides of Equation 12 with Ca_{sol}/Ca_{insol} Equation 8 is obtained.

$$\frac{^{45}Ca_{insol}}{^{45}Ca_{sol}} = \frac{\left(\frac{\%Ca_{exchange}}{100\%}\right) * Ca_{insol}}{Ca_{sol}}$$

Equation 12

2.7 Analysis

2.7.1. Inductively Coupled Plasma – Optical Emission Spectroscopy

To measure the concentrations of nonradioactive Ca in the milk, Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) is used. It is a widely used method to detect chemical elements and their concentrations. The technique is based on the formation of argon plasma by ionizing the argon gas with electrons. Atoms and ions of a sample are released in the argon plasma and will become ionised. When the excited electron in the atom falls back to the ground state, a photon with a specific wavelength is released; making it possible to distinguish what element is being

detected. In addition, the intensity of those wavelengths is proportional to the concentration of the element [45].

2.7.2. Liquid Scintillation Counting

As ^{45}Ca is a β^- emitter which emits only low energy gammas (12 keV), liquid scintillation counting (LSC) spectroscopy is one the most effective measurement techniques for ^{45}Ca [20], [21]. LSC is used to measure the radioactivity of a sample. For this technique the radioactive compound is solved in a detection medium (scintillation cocktail), which avoids self-absorption and absorption by air or shielding material. The detection medium consists of an aromatic organic solvent and scintillators (or fluor molecules). The aromatic solvent is excited by the energy released due to radioactive decay. This energy is transferred to the scintillator and can bring an electron, in the scintillator material, from the valence band to the conduction band. When the electron falls back to the valence band, a photon is emitted. This photon will be detected by the Photo Multiplier Tubes (PMTs) in the LSC (Figure 8).

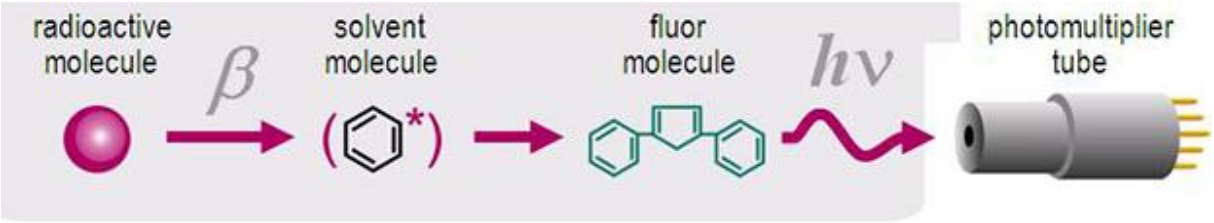

Figure 8: Detection of radioactivity by a photomultiplier tube in the liquid scintillation counter. The aromatic solvent molecule is excited by the energy of the β^- particle. The energy is transferred to the scintillator/fluor molecule and an electron is excited. Upon relaxation a photon is emitted which is detected by the photo multiplier tube (source: perkinelmer.com).

The photon will first reach the photo cathode in the PMT. At the photo cathode the photo electric effect takes place and, if the energy is high enough, a primary electron will be ejected from the photo cathode. The PMT consists of several dynodes, with a potential difference between each dynode. As soon as the primary electron reaches the first dynode, secondary electrons are emitted. Those electrons are again multiplied at the next dynode. Consequently, the amount of electrons is increased exponentially and a significant current pulse is created, which can be measured and is expressed in counts per minute (CPM) (Figure 7) [46]. These data are translated into a spectrum of CPM per energy level, where the maximum measured energy is the maximum emitted energy of the nuclide ($E_{\beta max}$) (Figure 6, curve 1).

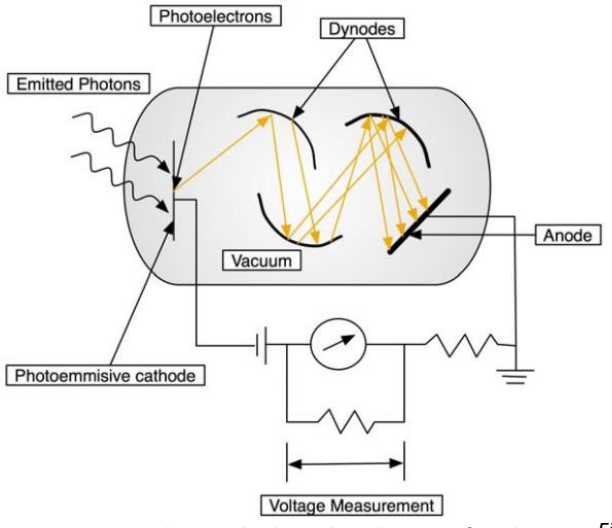

Figure 7: A photomultiplier tubes that transfers the energy of emitted photons to a current that can be measured (Source: Quinn 2015).

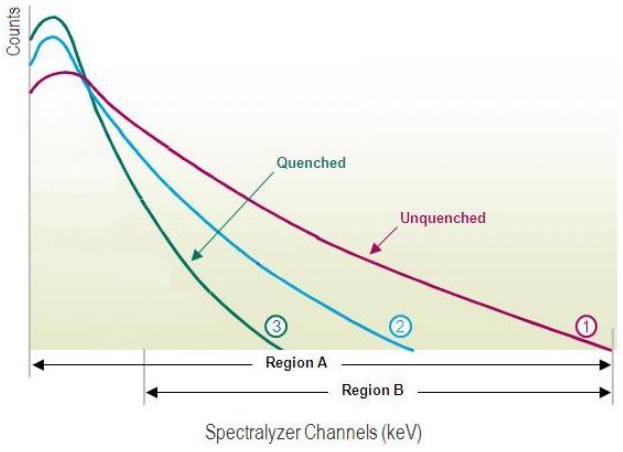

Figure 6: LSC spectrum of a quenched and unquenched sample. A spectrum of an unquenched radioisotope will be from E = 0 kEv till $E_{\beta max}$. Due to quenching relatively more counts with a lower energy and less counts with a higher energy will be measured (Source adapted from Perkinelmer.com).

Several factors can influence the effectiveness of LSC measurements. First of all, noise in the LSC device can disturb the measurements. Most LSC devices have two PMTs to keep the noise low. Only when an electrical pulse comes from both PMTs at the same time (within a range of nanoseconds) it is counted.

Secondly, quenching can have a significant influence on the LSC measurement. Quenching is a reduction of signal, resulting from the energy of the radioactive molecule that is not completely transferred to the PMT in the LSC. Therefore, due to quenching relatively more counts with a lower energy and less counts with a higher energy will be measured (Figure 6, curve 3).

Three types of quenching exist: (1) physical, (2) chemical and (3) colour (optical) quenching. All three types of quenching occur at a different stage of the energy transfer process (Figure 9). (1) Physcial quenching occurs when there is a physical blockage between the radioisotope and the scintillation cocktail. E.g. when the radioactive molecule is not completely dissolved in the scintillation cocktail and exist as solid particles. (2) Chemical quenching occurs when a part of the energy of the radioisotope is absorbed by a chemical compound (quencher) and transferred into heat instead of being transferred to the scintillation cocktail. (3) Colour quenching (or optical quenching) occurs when the photons produced by the scintillators, are (partly) absorbed by a dye in the sample (e.g. blood, milk, urine), instead of being transferred to the PMT.

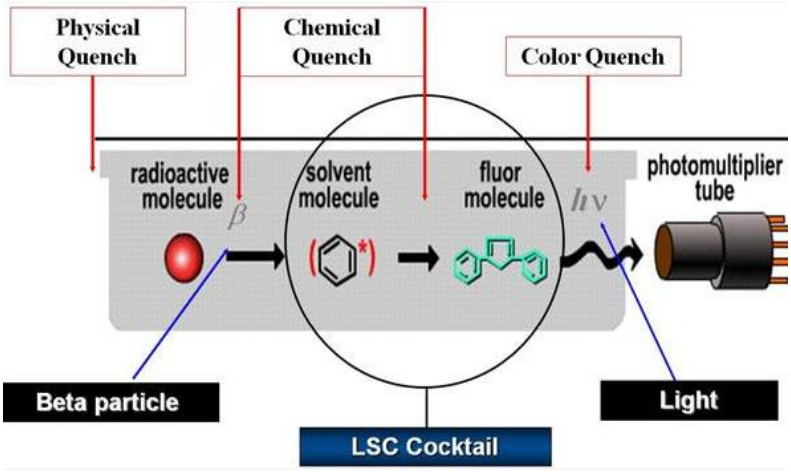

Figure 9: The different types of quenching occur at different stages in the process (source: perkinelmer.com).

During LSC meauserements with a radioisotope the amount of quenching can be meausured (1) by using an external closed γ-source and (2) by analysing the spectrum of the sample. (1) By using an external closed γ-source the Quenching Indicating Parameter (QIP) can be determined. For PerkinElmer LSC devices the QIP is expressed as tSIE (transformed Spectral Index of External Standard). A build-in γ-source is placed automatically next to the sample. The gamma radiation of the source will eject an electron from the atoms in the sample, a so called Compton electron. The Compton electron has similar properties to the β^- particles and can be measured due to scintillation, which gives a Compton electron spectrum. The shape of the Compton electron spectrum is influenced by quenching, in the same way the sample spectrum is. Hence, the LSC device can measure a QIP/tSIE value. The tSIE value decreases as quenching increases. (2) By analysing the spectrum of the sample a QIP can be determined as well, this is called the Spectral Index of the Sample (SIS) value and is determined by the endpoint of the spectrum. With an increase in quenching, the average kinetic energy of all β^- particles will decrease (Figure 6, curve 3), causing a lower endpoint of the spectrum and therefore a smaller SIS value.

When LSC meausurements are used for relative meausurements and all samples have the same composition, the QIP is relevant and can be used to check if all measurements are still correct. To obtain the same composition in a sample it is important to use the same amount of material that can cause quenching. The amount of scintillation cocktail is less important, as an excess will be added.

Lastly, luminescence can influence the efficiency of a LSC measurement. This can occur due to chemical- and photoluminescence. Chemical luminescence occurs if light is created within a chemical reaction in the sample, whereas photoluminescence can be caused by ultraviolet light from outside the sample. Both types of luminescence can activate the scintillator, which creates a photon that can be measured by the LSC device.

As both quenching and luminescence cause a difference in counts in energy regions, it can be chosen to measure two different energy regions in the LSC device (Figure 6). In this way the region in which most quenching and luminescence counts will occur, can be neglected for further calculations. Besides the optimization of the measurements itself, an Instrument Performance Assessment (IPA) of the LSC device should be done frequently. An IPA functions as a validity control for the experimental data. During an IPA, a background, ^{3}H and ^{14}C sample are measured for calibration, the obtained data are saved electronically and the device will give a warning if there is a change in system performance [47], [48].

2.7.3. X-ray diffraction

X-ray diffraction (XRD) is a method that can be used to determine the structure of a crystal and was used in this research to analyse the synthesized Ca salts. An X-ray diffractometer consist of a source of radiation, a sample, and a detector to measure the diffracted X-rays from the sample. The source of radiation is created by bombarding a metal, often copper (Cu), with an accelerated beam of electrons. The electrons will ionise some of the electrons in the Cu and by relaxation of the electrons characteristic X-rays are emitted. The X-ray beam can interact with matter (the sample) by scattering and absorption. The angles and intensities of the scattered beams are measured by a detector. Based on this information the electron density of a crystal can be determined, which gives information about the size and shape of the unit cell and the atomic number and position of atoms in the unit cell [49].

2.7.4. Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) was used to analyse the synthesized Ca salts. It is a technique that can be used to identify chemical compounds based on their functional groups. FTIR is a type of absorption spectroscopy in which infrared (IR) light is absorbed or transmitted by a sample. By detecting the transmittance of the IR spectrum through the sample, and therefore the absorption, functional groups of the sample can be identified.

3. Materials & Methods

3.1 Materials

3.1.1. Chemicals

The following chemicals were used to perform the experiments in this research project. Calcium chloride dihydrate ($CaCl_2$ *2 H_2O) ≥99% ACS reagent, citric acid ($C_6H_8O_7$) anhydrous 99.5%-100.5% USP grade, potassium chloride (KCl) 99.7%, ACS reagent, sodium carbonate (Na_2CO_3) anhydrous ACS reagent, sodium chloride (NaCl) 99.999% trace metal basis, sodium hydroxide (NaOH) ≥98% ACS reagent were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Calcium ICP Standard CertiPUR, hydrogen peroxide (H_2O_2) 30% (w/w) ISO, gluconic acid ($C_6H_{12}O_7$) (50% solution in water) for synthesis, nitric acid (HNO_3) 65% (w/w) Suprapur, hydrochloric acid (HCl) suprapur 30% (w/w), tri-sodium citrate dehydrate ($C_6H_5Na_3O_7$*$2H_2O$) p.a. and calcium carbonate ($CaCO_3$) suprapur were purchased from Merck (Darmstadt, Germany). Lactic acid ($C_3H_6O_3$) 80-90% (w/w) was purchased from J.T. (Arnhem, the Netherlands). Potassium dihydrogen phosphate (KH_2PO_4) crystallized GPR rectapur was purchased from VWR Chemicals (Amsterdam, the Netherlands). $^{45}CaCl_2$ with an activity of 1 Ci (reference date 10th of sept 2020) in aqueous solution (0.1 M HCl) and Ultimate Gold XR were purchased from PerkinElmer (Groningen, the Netherlands). Ethanol p.a. was purchased from Honeywell (Delft, the Netherlands). Lactopure Lactose, p.d. 20-7-2018, Non Food Grade was obtained via Wageningen University. Albert Heijn private label skimmed milk (0 g fat / 100 ml) was bought at the supermarket.

3.1.2. Equipment

The following equipment was used:

- Polyallomer Bell-top Quick Seal Centrifuge tubes, (11 x 32 mm, 2 mL) purchased from Beckman Coulter (Woerden, the Netherlands).
- Microcon-10 centrifugal filters, regenerated cellulose 10.000 NMWL purchased from Merck (Darmstadt, Germany).
- Dialysis Tubing-Visking 12-14,000 Da size No.2 (0.03 mm, 18/32in or 14.3mm,30m) purchased from Medicell Membranes (London, England).
- Dialysis tubing Membra-Cel MC24x100 Chr 14 kDa purchased from Viskase (Turnhout, Belgium).

3.1.3. Instruments

The following instruments and instrument settings were used:

- Inductively coupled plasma – optical emission spectrometry (ICP-OES), Optima 4300 DV, PerkinElmer. Used protocol: Plasma flow rate 15 L/min, auxiliary gas flow rate 0.2 L/min, nebulizer gas flow rate 0.8 L/min, power 1300 watts, view distance 15 and plasma view axial. The following wavelengths were selected for the quantification of calcium: 317.9, 315.9, 396.8, 422.7 and 227.5 nm. Calcium ICP standard was used to make the following concentrations stock solutions, which were used for the calibration lines: 2.05, 6.14, 10.19 and 14.28 mg/L. The calibration lines can be found in Appendix 1.
- Liquid Scintillation Counter (tri-carb 2750TR/LL, Packard). The measurement time was 1 min, data mode was CPM and no automatic background subtract was performed. The following three regions were selected: Region A: 0.0 - 5.0, region B: 5.0 - 256 and region C: 0 – 256. Region B was used for calculations.
- Microwave Reaction System, Multiwave PRO, Anton Paar.

- FTIR-8300, Shimadzu Scientific Instruments. Solid pellets with sample plus potassium bromide (KBr) were formed and measured. Background pellets with only KBr were made and subtracted from the sample data.
- X-ray diffractometer: PANalytical X'pert pro, Multi-Purpose Diffractometer (MPD) with copper X-ray tube. Intended wavelength type: K-Alpha. Generator settings: 40 mA, 45 kV. Angles: 5.0144 - 89.9804 °2θ degrees and step size 0.0170 °2θ degrees. For the analysis of the X-ray diffractograms the PANalytical X'Pert HighScore Software was used, which compares data with the reference data from International Centre for Diffraction Data (ICDD).

3.2 Methods

In this chapter the used methods will be described. First, the calcium salt synthesis methods will be described, subsequently the methods to prepare the different milk samples, then the methods to perform phase separation (dialysis and ultracentrifugation), and lastly the methods to analyse the samples (ICP-OES and LSC).

3.2.1. Calcium salt synthesis

All salts were first synthesized, as described in 3.2.1.1. Calcium carbonate - $CaCO_3$ until 3.2.1.5. Calcium lactate - $CaC_6H_{10}O_6$, without ^{45}Ca and the samples were analysed with XRD and FTIR. Subsequently, calcium carbonate was intrinsically labelled with radioactive ^{45}Ca as tracer. This was done by adding a small amount $^{45}CaCl_2$ to the calcium chloride solution before the start of the synthesis. After synthesis all samples were centrifuged for 7 min at 760 x g, 20 °C. The supernatant was removed with a 10 mL pipet and the sample was washed three times by adding 25 mL milliQ water (MQ) and centrifuging for 7 min at 760 x g. All samples were dried for at least 48 h at room temperature in the fume hood, by leaving the cap of the centrifuge tubes open. For the XRD and FTIR measurements the samples were dried in a vacuum oven at 60 °C.

The following methods were used for the synthesis of the calcium salts:

3.2.1.1. Calcium carbonate - $CaCO_3$

$CaCO_3$ was synthesized using the method of Ohtani et al. [50]. To synthesize 1.25 mmol $CaCO_3$, 52 mM of calcium chloride was dissolved in 30 mL MQ in a 50 mL centrifuge tube. An equimolar quantity of sodium carbonate was added. The resulting suspension was stirred at room temperature for 2 h.

To synthesize 1.25 mmol $CaCO_3$, labelled with ^{45}Ca, 3 kBq $[^{45}Ca]CaCl_2$ was added to the calcium chloride solution, before addition of sodium carbonate.

3.2.1.2. Tri-calcium di-citrate - $Ca_3(C_6H_5O_7)_2$

Method 1:

To synthesize CaCi, first calcium hydroxide (CaOH) was synthesized. First, a 50 mL centrifuge tube was filled with 15 mL MQ, then 600 mM sodium hydroxide was added and the solution was homogenised. Similarly, a 50 mL centrifuge tube was filled with 15 mL MQ and 300 mM calcium chloride was added, after which the solution was homogenised. The sodium hydroxide solution was added to the calcium chloride solution. The suspension was then stirred for 10 min at room temperature, to let the reaction complete. The suspension was centrifuged for 7 min at 760 x g, 20 °C. The supernatant was removed with a 10 mL pipet and the precipitate was washed three times with MQ by repeating the previous centrifugation step.

After washing the synthesized CaOH, the precipitate was dissolved by adding 30 mL MQ to the centrifuge tube and 100 mM citric acid was added. The resulting suspension was stirred at room

temperature for at least 1 h and then was left in the fume hood for at least 24 h for the reaction to complete.

Method 2:
CaCi was also synthesized using the method of Li et al. [51]. A 50 mL centrifuge tube was filled with 15 mL MQ, 120 mM tri-sodium citrate was added and the solution was homogenised. Another 50 mL centrifuge tube was filled with 15 mL MQ, 140 mM $CaCl_2$ was added and the solution was homogenised. The sodium citrate solution was added to the calcium chloride solution. The obtained mixture was stirred and a solution of 1:2 (v:v) ethanol:MQ of 30 mL was added to create a white slurry of CaCi. This solution was then stirred for 1 h at room temperature.

3.2.1.3. Calcium gluconate – $C_{12}H_{22}CaO_{14}$

Method 1:
To synthesize CaGluc, first $CaCO_3$ was synthesized according to method mentioned earlier (3.2.1.1. Calcium carbonate - CaCO3). Subsequently, a 50 mL centrifuge tube was filled with 20 mL MQ, 12.8 mL gluconic acid 50% (w/w) was added after which the solution was homogenised. Then, 100 mole of the earlier synthesized $CaCO_3$ was added and the solution was stirred for at least 2 h at room temperature and the effervescence was allowed to die away. Finally, the solution was left in the fume hood for at least 24 h for the reaction to complete.

Method 2:
To synthesize CaGluc, first calcium hydroxide was synthesized according to method 1 mentioned earlier in 3.2.1.2. Tri-calcium di-citrate - $Ca_3(C_6H_5O_7)_2$, using exactly the same quantities. After the washing steps of calcium hydroxide, 20 mL MQ and 6.4 mL gluconic acid 50% (w/w) was added, according to the method of Vorage et al. [52]. The solution was stirred for at least 2 h at room temperature. Finally, the solution was left in the fume hood for at least 24 h for the reaction to complete.

3.2.1.4. Tri-calcium phosphate – $Ca_3(PO_4)_2$

TCP was synthesized in three steps, using the method of Koo et al. [53]. First, $CaCO_3$ was synthesized according to the method mentioned earlier (3.2.1.1. Calcium carbonate - $CaCO_3$). Subsequently, calcium nitrate was synthesized using the method of RSC [54]. 20 mL MQ was added to a 50 mL three-necked round bottom flask with thermometer. 0.9 mL nitric acid 69% (w/w) was added. The solution was heated to 60 °C by placing the round bottom flask in a beaker glass with water on a heating plate (Figure 10). A stirrer was added to both the beaker glass and round bottom flask. For the third step, 7 mmol of the earlier synthesized $CaCO_3$ was added with a spatula in small portions, allowing the effervescence to die away between additions. The temperature was increased to 80 °C and 4.7 mmol potassium dihydrogen phosphate and 9.3 mmol sodium hydroxide were added. The solution was stirred for 1 h at 80 °C at a pH of 9. After cooling down, the suspension was transferred to a 50 mL centrifuge tube by rinsing with MQ.

This reaction was also performed at room temperature by adding 0.3 mL nitric acid 69% (w/w) instead of 0.9 mL.

Figure 10: Setup for tri-calcium phosphate synthesis

3.2.1.5. Calcium lactate - $CaC_6H_{10}O_6$

CaLac was synthesized using the method of Ohtani et al. [50]. Firstly, $CaCO_3$ was synthesized according to the method mentioned earlier (3.2.1.1. Calcium carbonate - $CaCO_3$). Secondly, a 50 mL centrifuge tube was filled with 40 mL MQ. Subsequently, 0.5 mL lactic acid 80-90% (w/w) and 0.55 mM of the synthesized $CaCO_3$ was added. The suspension was stirred at room temperature for 2 h. The effervescence was allowed to die away.

3.2.2. Milk sample preparation

Two types of milk sample were prepared before phase separation was performed: Skimmed milk samples with $[^{45}Ca]CaCl_2$ as tracer and skimmed milk samples fortified with $CaCl_2$, $[^{45}Ca]CaCl_2$ or $[^{45}Ca]CaCO_3$. For the fortification of skimmed milk 50 mg Ca / 100 mL milk was added [5]. In addition, the pH of the milk fortified with $CaCl_2$ and $CaCO_3$ was measured using a pH meter.

3.2.2.1. $[^{45}Ca]CaCl_2$ as tracer

For dialysis, samples of skimmed milk with $[^{45}Ca]CaCl_2$ as tracer were prepared by filling two 50 mL centrifuge tubes with 50 mL of milk and 1.9 kBq of $[^{45}Ca]CaCl_2$.
For ultracentrifugation the samples were prepared by adding 10 mL of milk to a 15 mL centrifuge tube and adding 375 Bq of $[^{45}Ca]CaCl_2$.
Both samples were homogenized, placed in a perspex box and left in the fridge at approximately 4 °C, for 24 - 48 h.

3.2.2.2. Fortification with $[^{45}Ca]CaCl_2$

The samples fortified with $CaCl_2$ were made by adding 1.25 mmol $CaCl_2$ / 100 mL milk.

The samples fortified with $[^{45}Ca]CaCl_2$ were made by adding 1.25 mmol $CaCl_2$ / 100 mL milk and 3.8 kBq $[^{45}Ca]CaCl_2$ / 100 mL milk. The samples were homogenized and left in the fridge at approximately 4 °C, for 24 - 48 h.

3.2.2.3. Fortification with $[^{45}Ca]CaCO_3$

The samples fortified with $[^{45}Ca]CaCO_3$ were prepared in PLA bottles by adding 1.25 mmol $[^{45}Ca]CaCO_3$ / 100 mL milk of the earlier synthesized $[^{45}Ca]CaCO_3$ (3.2.1.1. Calcium carbonate - CaCO3). A stirrer was added to the solution and a stir plate was placed in the fridge. The solution was stirred for 24 h in the fridge (Figure 11).
For the sample preparation for ultracentrifugation the solution was transferred to centrifuge tubes, after stirring in the fridge. The solution was centrifuged for 30 min, 200 x g at 20 °C according to the method of Huppertz & Timmer [41].

Figure 11: Setup to equilibrate milk, fortified with calcium carbonate ($CaCO_3$), in the fridge.

3.2.3. Phase separation methods

The two following methods for phase separation were performed. All experiments were performed in triplicate.

3.2.3.1. Dialysis

To perform dialysis a piece of approximately 10 - 15 cm of membrane tubing was soaked in a beaker filled with MQ for approximately 1 h. Afterwards, the top of the membrane tubing was opened with a needle and the tubing was further opened by carefully rubbing the membrane. A knot was made at

the bottom of the tubing. A ratio of 1:20 dialysate:milk was used, to obtain a conveniently measurable concentration of Ca in both phases. So, 5 mL of dialysate solution, consisting of 87 mM NaCl, 350 mM KCl, and 205 mM lactose dissolved in MQ, was inserted at the top of the membrane tubing with a pipette. Subsequently, 100 mL of milk sample (pure skimmed milk, skimmed milk with [^{45}Ca]$CaCl_2$ as tracer or fortified with $CaCl_2$, [^{45}Ca]$CaCl_2$ or [^{45}Ca]$CaCO_3$) was added to a graduated cylinder and a stirrer was added. The membrane tubing was placed into the graduated cylinder with skimmed milk and was kept in place with a clip. The top of the graduated cylinder was covered with parafilm, to prevent evaporation. The solution was stirred for 24 h in the fume hood (Figure 12). To remove the solution from the membrane tubing, the membrane tubing was placed in a beaker and the liquid was removed by using a needle and syringe.

To make sure the osmotic pressure in the tube and outside the tube was the same, several measurements were performed with dialysates that differed in concentrations. The dialysates consisted of NaCl, KCl, and lactose dissolved in MQ. The concentrations differed between 10 - 174 mM NaCl, 40 - 700 mM KCl, and 146 - 366 mM lactose. After the preparation of the dialysate solutions, the solution were added to a membrane tube and weighted before dialysis. The dialysis was performed as described above for 24 h and weighted again.

The percentage Ca in the soluble phase was calculated by using Equation 4. Where [D] is the concentration of dialyzed Ca (mg/L), V_D the volume of the dialysate (L), V_s the volume of the milk sample (L), and [A] the total concentration of Ca in milk.

$$Solubility\ (\%) = \frac{[D]*(V_D+V_S)}{[A]*V_S}$$

Equation 4

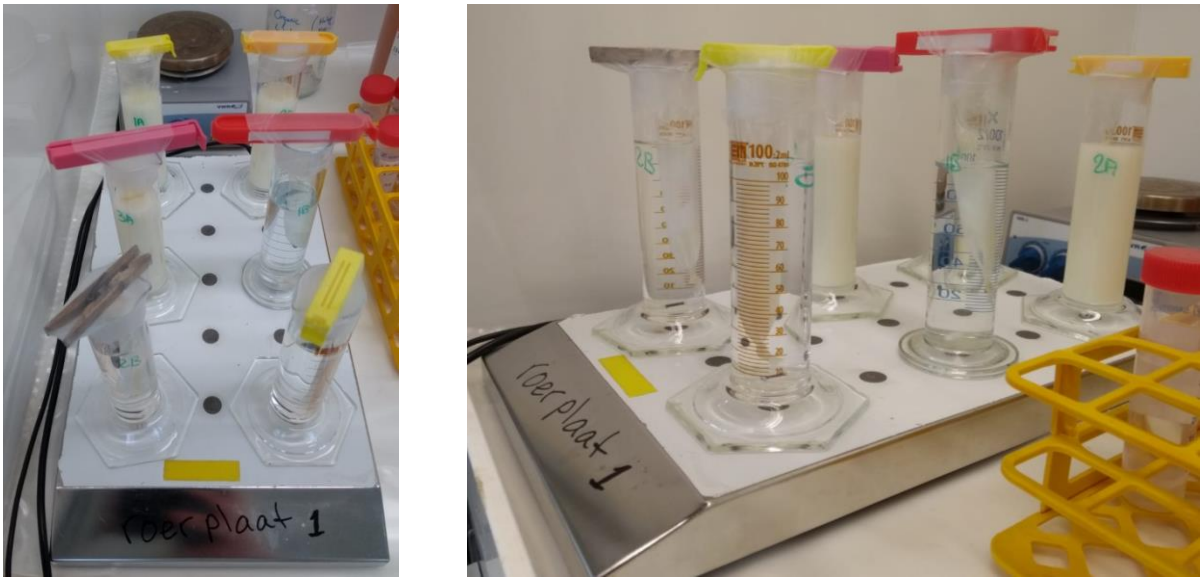

Figure 12: Setup for dialysis of milk. The graduated cylinders are placed on a stirring plate. The dialysis membrane is placed in the graduated cylinder. The clip makes sure that the dialysis membrane stays in place. The top of the graduated cylinder is covered with parafilm.

3.2.3.2. Ultracentrifugation

The method for ultracentrifugation was adapted from Huppertz & Timmer [41]. 2 mL of milk sample (pure skimmed milk, skimmed milk with [^{45}Ca]$CaCl_2$ as tracer or fortified with $CaCl_2$, [^{45}Ca]$CaCl_2$ or [^{45}Ca]$CaCO_3$) was inserted, with a needle and syringe, into an ultracentrifuge tube (Figure 13A). It was made sure that all ultracentrifuge tubes had the same weight, to prevent damage of the

ultracentrifuge. The top of the ultracentrifuge tubes were sealed by first placing a seal former and seal guide on top of the tube (Figure 13B). The tube topper was placed vertically on the ultracentrifuge tube and gentle pressure was applied while the seal former was hold in place. After the tube was sealed, the tube topper was removed and the heat sink was placed on the seal guide. The tubes were ultra-centrifuged for 60 min, 100.000 x g at 20 °C [55]. After ultracentrifugation the casein micelles were precipitated (Figure 13C). The supernatant was removed by first inserting one needle (0.9 x 40 mm) in the tube to prevent the occurrence of vacuum, after which a second needle (1.1 x 40 mm) with syringe was inserted to remove the supernatant (Figure 14). The needles were inserted away from the precipitate. 0.5 mL of the supernatant was inserted in an Eppendorf centrifuge vials with a 10 kDa filter. The vials were sealed with parafilm and centrifuged in the microcentrifuge for 40 min, 13.000 x g at 20 °C. By centrifugation the proteins were separated from the permeate. The whole sample, the supernatant (serum protein + soluble phase) and the permeate (soluble phase) were used for analysis.

The percentage of Ca in the different phases of milk is calculated by using the equations discussed in the theory: Equation 5, Equation 6, and Equation 7.

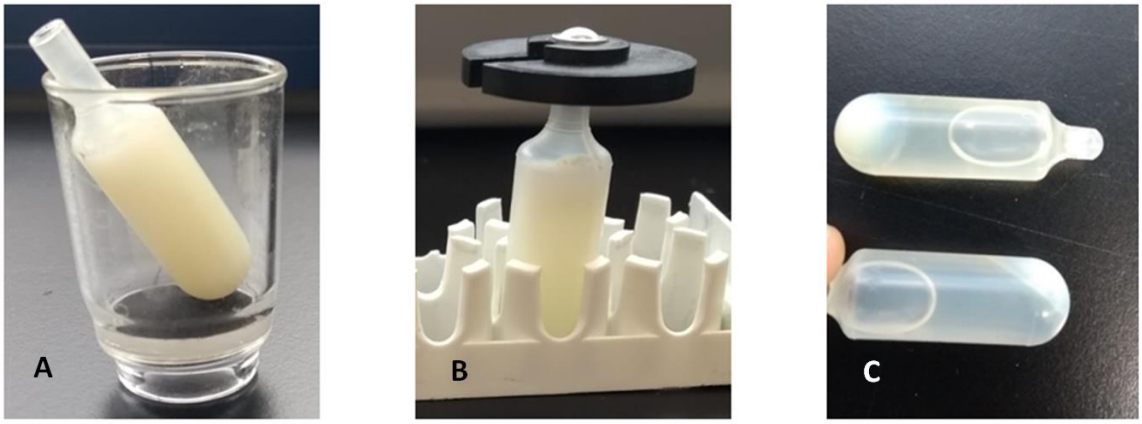

Figure 13: (A) Ultracentrifuge tube filled with sample, (B) Ultracentrifuge tube, filled with sample and seal guide and former on top of the tube, (C) Ultracentrifuge tubes after ultracentrifugation, the white layer at the bottom is the casein micelle layer.

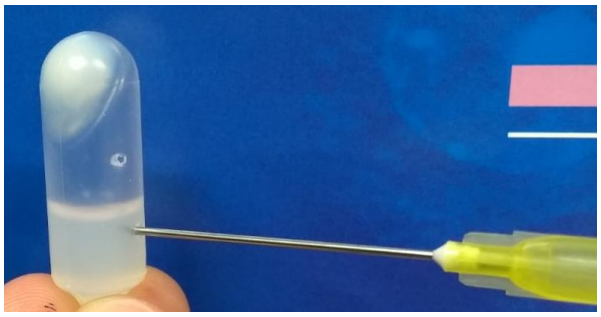

Figure 14: Ultracentrifuge tube being emptied with a needle. The hole above the needle prevents the occurrence of vacuum.

3.2.3.3. Calculation hard-to-exchange calcium

The percentage of HTE Ca (%Ca_{HTE}) is calculated by using Equation 13, which is a combination of Equation 8 and Equation 9. %$^{45}Ca_{sol}$ and %Ca_{sol} are the percentages of the ^{45}Ca and Ca in the soluble phase, respectively.

$$\%Ca_{HTE} = 100\% - \frac{(100\% - \%^{45}Ca_{sol})}{\%^{45}Ca_{sol}} * \frac{\%Ca_{sol}}{(100\% - \%Ca_{sol})}$$

Equation 13

3.2.4. Sample analysis

The concentration of Ca in the samples was measured using ICP-OES and the amount of radioactivity, due to ^{45}Ca, was measured using LSC.

3.2.4.1. ICP-OES measurements

The milk samples were digested in the microwave, before ICP-OES measurements were performed. For the microwave protocol the *Official Method* 21424:2018 and the method of Cruijsen et al. were slightly modified [56], [57]. 0.5 -1 mL of the sample, depending on the sample, together with 4.5 mL of nitric acid (HNO_3) 65% (w/w) and 1.5 mL of hydrogen peroxide (H_2O_2) 30% (w/w) were added to microwave vessels and placed in the microwave carrousel (Figure 15A&B). The following microwave protocol was performed: 30 min heating to 100 °C, 30 min heating to 180 °C, 20 min heating to 200 °C, 20 min at 200 °C, and 20 min cooling down. The digest in the microwave vessel was transferred to 50 mL volumetric flask using a funnel. The digest was diluted with MQ up to 50 mL and homogenised. 10 mL of the volume flasks was transferred to 15 mL centrifuge tubes (Figure 15C). For every digestion protocol a blank with 4.5 mL of HNO_3 65% (w/w) and 1.5 mL of H_2O_2 30% (w/w) was made and a reference sample with 1 mL milk, 4.5 mL of HNO_3 65% (w/w) and 1.5 mL of H_2O_2 30% (w/w).

After phase separation, the soluble phase is separated from the proteins and casein micelles and therefore contains solely soluble ions. Hence, physical interference during the ICP-OES measurement does not occur. However, it was still decided to digest those samples in the microwave, to perform an equal treatment method for all phases of the milk.

The digestion in the microwave was also performed with 6 mL *aqua regia*, a mixture of HCl and HNO_3 in a 3:1 molar ratio.

ICP-OES measurements were performed with the aforementioned samples digested in the microwave. During every measurement one of the stock solutions from the calibration line and a blank with 3 mL HNO_3 65% (w/w) and 7 mL MQ was measured.

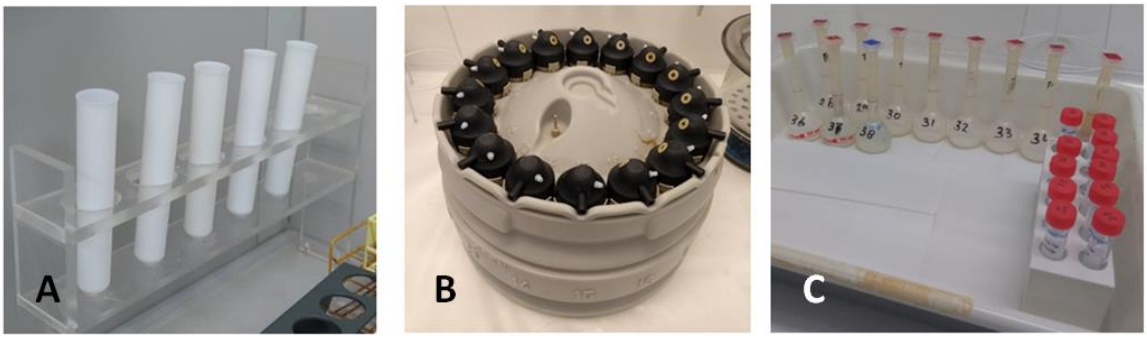

Figure 15: (A) Microwave vessels, (B) Microwave vessels placed in the microwave carrousel, (C) Volumetric flasks filled with microwave digest.

3.2.4.2. LSC measurements

For all measurements 20 mL glass LSC vials were used and all vials were pre-rinsed with 0.5 mL of 100 mM $CaCl_2$ solution. 10 mL scintillation cocktail was added to the LSC vials. The source, which had to be measured, was added to the scintillation cocktail and the vial was vortexed for a few seconds. For every LSC measurement a background measurement was performed by measuring a vial with solely

10 mL scintillation cocktail. The net count rate (R_{net}) was determined by subtracting the count rate of the background vial (R_{bkg}) from the count rate of the measured sample (R_{gross}) (Equation 14).

$$R_{net} = R_{gross} - R_{bkg}$$

Equation 14

To determine the maximal and minimal amount of activity that can be added to a counting vial, different activities of [^{45}Ca]$CaCl_2$ were added to the vials, corresponding to a range of 0.35 - 57 Bq. The added activity was then plotted versus the net count rate.

To investigate the quenching due to the addition of milk to the samples, a range of vials was made containing 0 -5 mL of milk with an increment of 1 mL, all samples containing 2.8 Bq of activity. The amount of milk was plotted versus the net count rate.

4. Results and Discussion

In this chapter the obtained results will be presented and discussed. First, the synthesized salts will be discussed, subsequently the methods to analyse the samples (LSC and ICP-OES), and lastly the performed dialysis and ultracentrifugation, including the results, will be discussed.

4.1 Calcium salt synthesis

Several calcium salts were synthesized, starting from $CaCl_2$. All calcium salts were analysed by XRD to check if the desired calcium salt was formed. Next to the XRD diffractograms presented in this chapter, the corresponding graphics of the peak lists can be found in Appendix 2. Additionally a FTIR measurement was performed for $CaCO_3$ and CaLac. However, as the XRD results were already sufficient to check if the desired Ca salts were formed, FTIR measurements were not performed for the other samples.

The XRD diffractograms were analysed with PANalytical X'Pert HighScore Software and compared with existing data from the ICDD. The software gives a score, when a score of 50 or higher is obtained it can be concluded that there is a match between the diffractogram of the sample and the ICDD data.

All the synthesized calcium salts will be discussed in this chapter.

4.1.1. Calcium carbonate - $CaCO_3$

$CaCO_3$ was successfully synthesized according to the method described in section 3.2.1.1. Calcium carbonate - $CaCO_3$. The performed reaction was: $CaCl_2$ (aq) + Na_2CO_3 (aq) → $CaCO_3$ (s) + 2 NaCl (aq).

A XRD measurement was performed and a match score of 91 was obtained, for the measured sample compared to the reference data. As can be seen in Figure 16, the diffractogram of the sample and of the reference data correspond to each other.

In addition A FTIR measurement of the synthesized $CaCO_3$ and purchased $CaCO_3$ was performed and it can be concluded the synthesis was successful, as the FTIR spectra are identical (Figure 17).

The synthesis method for $CaCO_3$ was also used as part of the synthesis of CaGluc, TCP, and CaLac.

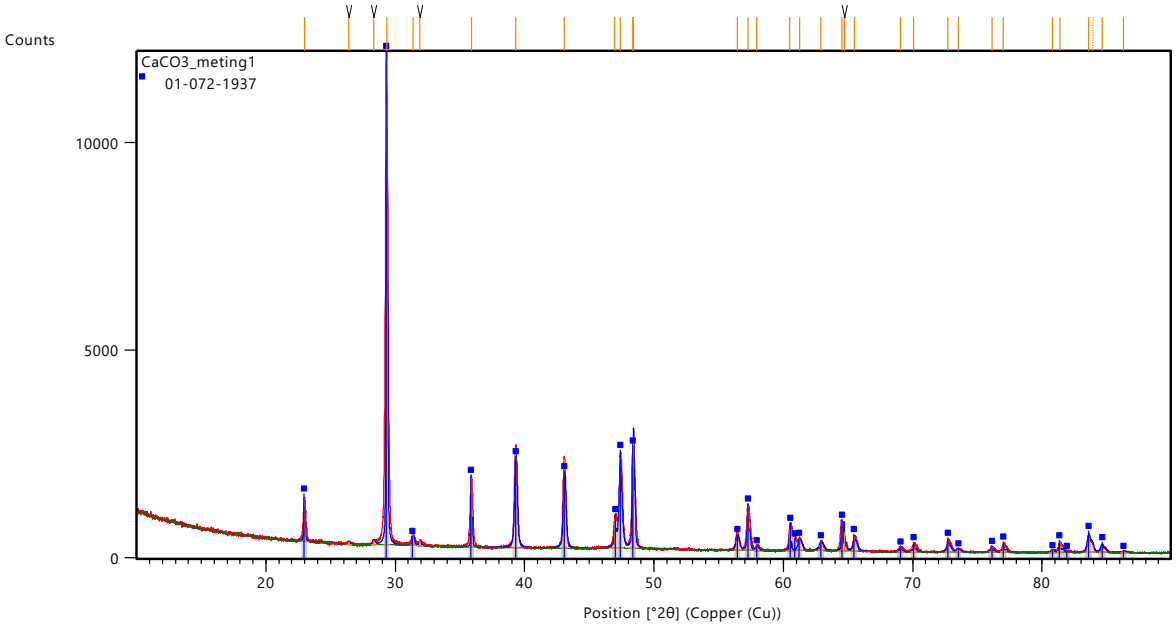

Figure 16: XRD diffractogram of the calcium carbonate sample. The red line is the measured diffractogram and the blue lines are the peaks of the matched calcium carbonate diffractogram from the database.

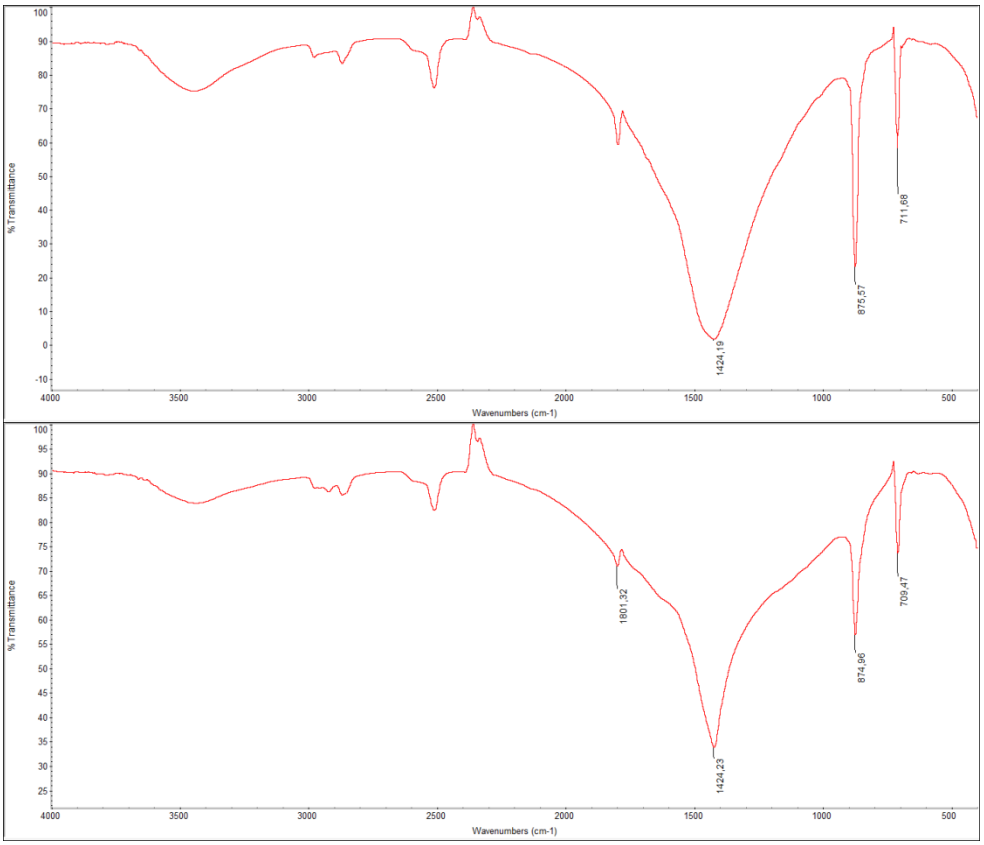

Figure 17: FTIR spectrum of $CaCO_3$. The top figure shows synthesized $CaCO_3$ and the bottom figure shows purchased $CaCO_3$.

4.1.2. Tri-calcium di-citrate - $Ca_3(C_6H_5O_7)_2$

CaCi was successfully synthesized according the two methods described in section 3.2.1.2. Tri-calcium di-citrate - $Ca_3(C_6H_5O_7)_2$.

Method 1

The first method consisted of the following two steps:

Step 1: $CaCl_2$ (aq) + 2 NaOH (aq) → 2 NaCl (aq) + $Ca(OH)_2$ (s)

Step 2: 3 $Ca(OH)_2$ (s) + 2 $C_6H_8O_7$ (aq) → $Ca_3(C_6H_5O_7)_2$ (s) + 6 H_2O (l)

A XRD measurement was performed and a match score of 67 was obtained for tri-calcium citrate. As can be seen in Figure 18, the diffractogram of the sample and the reference data correspond to each other. However, the many small and broad peaks in the diffractogram indicate that the formed crystal is not perfect. This could be due to the drying method, as for example Li et al. performed freeze drying instead of drying the sample in the oven [51]. A disadvantage of this synthesis method, in comparison with method 2, is that it contains two synthesis steps, which is inefficient. Besides, the reaction of CaCi was very slow and took several days to complete.

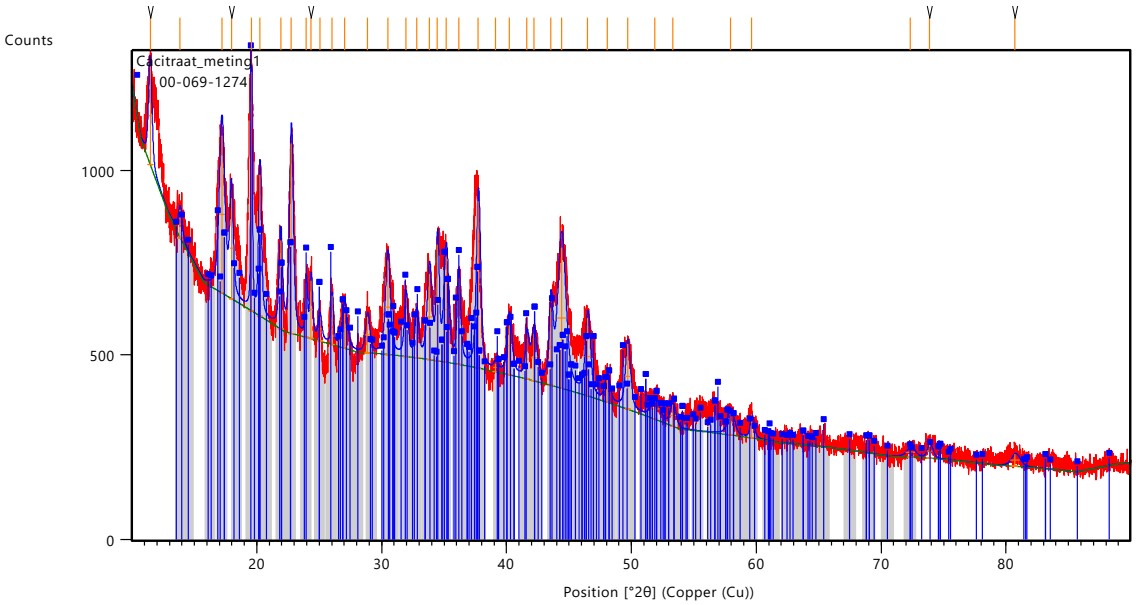

Figure 18: XRD diffractogram of the tri-calcium di-citrate sample synthesized according to method 1. The red line is the measured diffractogram and the blue lines are the peaks of the matched tri-calcium di-citrate diffractogram from the database.

Method 2

The second method consisted of the following reaction: 3 $CaCl_2$ (aq) + 2 $C_6H_5Na_3O_7$ (aq) → $Ca_3(C_6H_5O_7)_2$ (s) + 3 NaCl (aq).

A XRD measurement was performed and a match score of 63 was obtained for tri-calcium di-citrate hydrate ($Ca_3(C_6H_5O_7)_2$ * 4 H_2O). As can be seen in Figure 19 the diffractogram of the sample and the reference data correspond to each other. In addition, the peaks in the diffractogram correspond to the diffractogram measured by Li et al., with similar peaks at 5, 11, and 22 °2θ degrees. Further analysis of the diffractogram was not performed by Li et al. [51].

Also in this case the many small peaks in the diffractogram indicate that the formed crystal is not perfect. Again, this could be due to the drying method. However, it could also be due to the fact that nanocrystals might have formed [51].

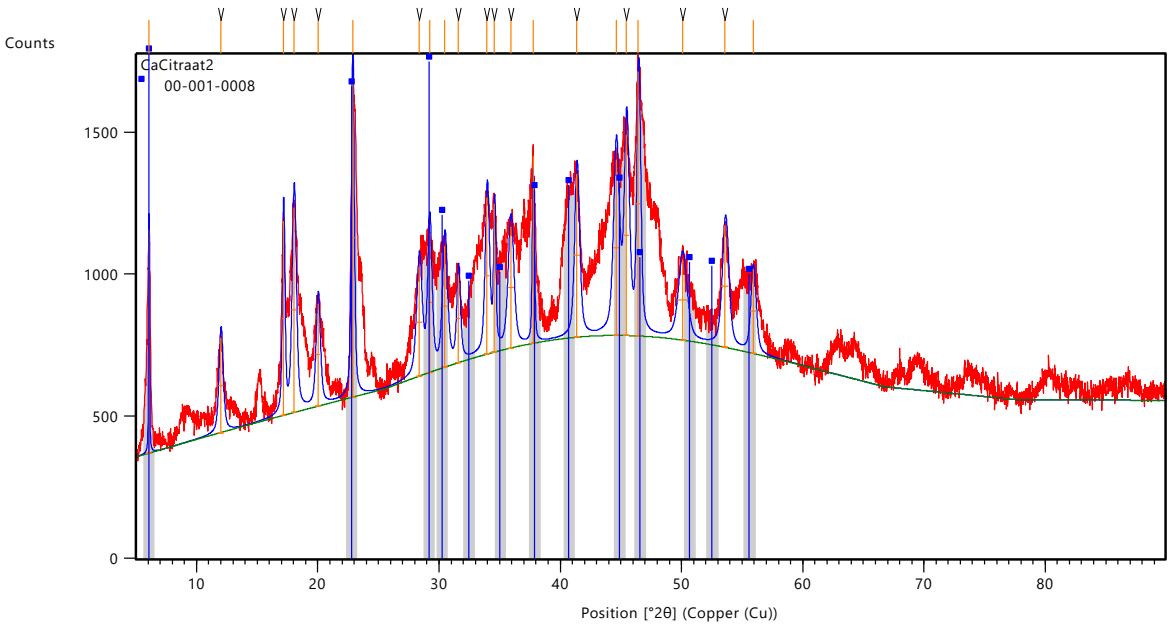

Figure 19: XRD diffractogram of the tri-calcium di-citrate sample synthesized according to method 2. The red line is the measured diffractogram and the blue lines are the peaks of the matched tri-calcium di-citrate hydrate diffractogram from the database.

4.1.3. Calcium gluconate – $CaC_{12}H_{22}O_{14}$

CaGluc was successfully synthesized according to two methods described in section 3.2.1.3. Calcium gluconate – $C_{12}H_{22}CaO_{14}$ For both methods, it was observed that the formation of CaGluc was very slow, in the range of days. The formed CaGluc precipitated in spherical particles.

Method 1

The first method consisted of the following two steps:

Step 1: $CaCl_2$ (aq) + Na_2CO_3 (aq) → $CaCO_3$ (s) + 2 NaCl (aq)

Step 2: $CaCO_3$ (s) + 2 $C_6H_{12}O_7$ (aq) → $CaC_{12}H_{22}O_{14}$ (s) + CO_2 (g) + H_2O (l)

For step 2, it was concluded that it is important to add an excess volume of gluconic acid in order to complete the reaction.

A XRD measurement was performed and a match score of 89 was obtained for CaGluc. As can be seen in Figure 20, the diffractogram of the sample and the reference data correspond to each other.

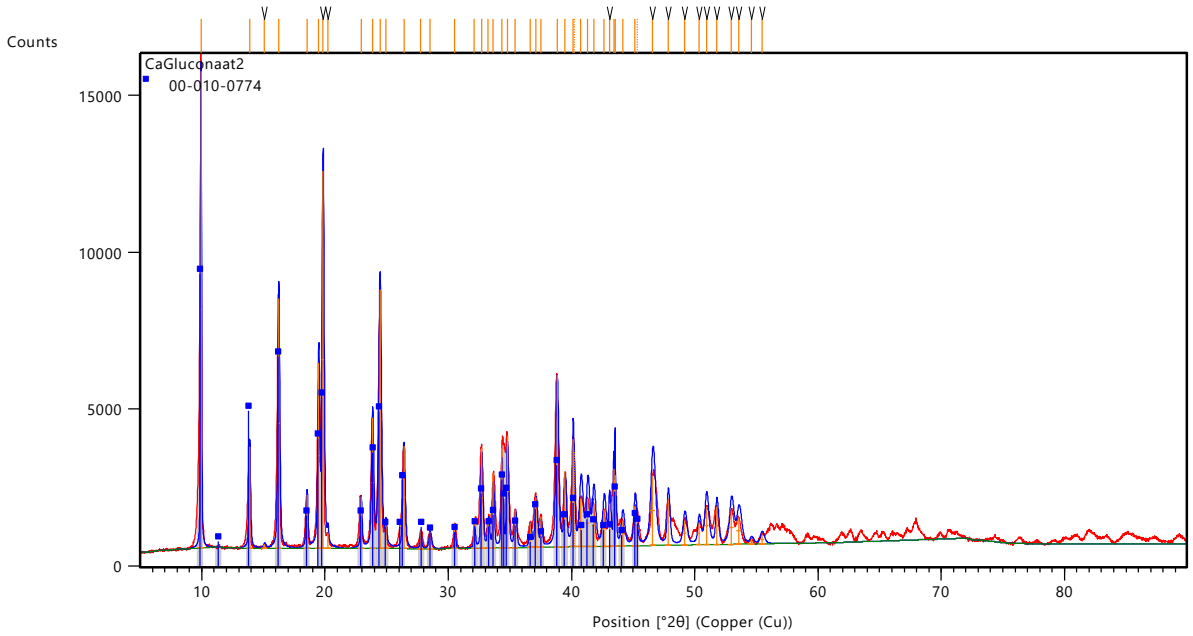

Figure 20: XRD diffractogram of the calcium gluconate sample synthesized according to method 1. The red line is the measured diffractogram and the blue lines are the peaks of the matched calcium gluconate diffractogram from the database.

Method 2

The second method consisted of the following two steps:

Step 1: $CaCl_2$ (aq) + 2 NaOH (aq) → 2 NaCl (aq) + $Ca(OH)_2$ (s)

Step 2: 2 $C_6H_{12}O_7$ (aq) + $Ca(OH)_2$ (aq) → $CaC_{12}H_{22}O_{14}$ (s) + 2 H_2O (l)

A XRD measurement was performed and a match score of 89 was obtained for CaGluc. As can be seen in Figure 21, the diffractogram of the sample and the reference data correspond to each other.

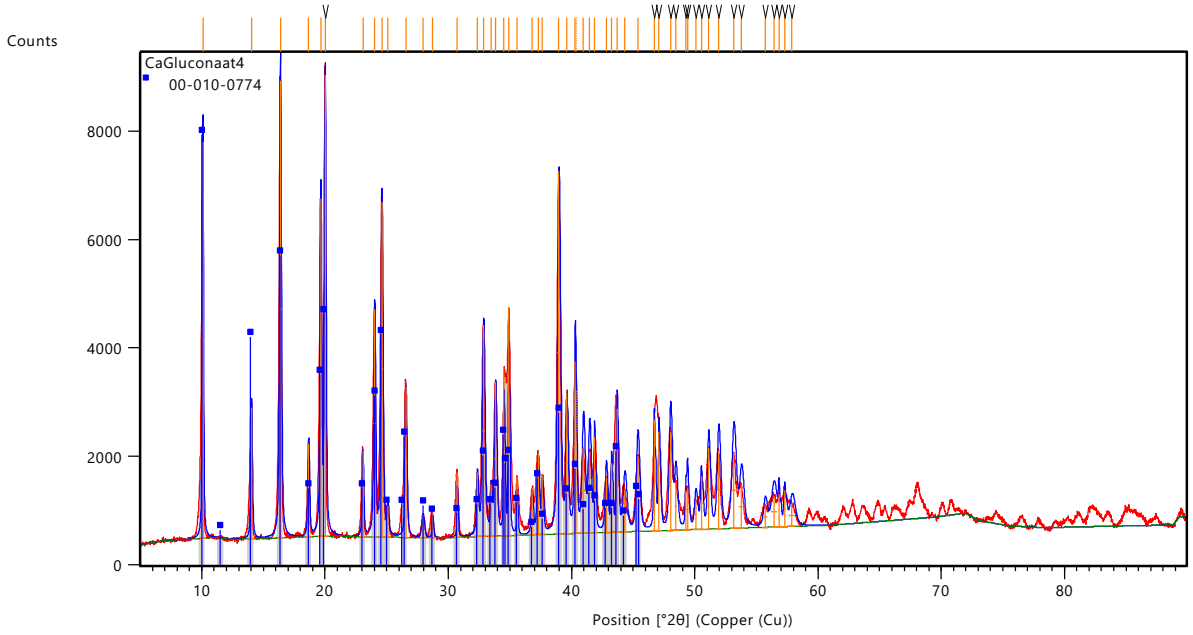

Figure 21: XRD diffractogram of the calcium gluconate sample synthesized according to method 2. The red line is the measured diffractogram and the blue lines are the peaks of the matched calcium gluconate diffractogram from the database.

4.1.4. Tri-calcium phosphate – $Ca_3(PO_4)_2$

TCP was not successfully synthesized according to the method described in section 3.2.1.4. Tri-calcium phosphate – Ca3(PO4)$_2$. The performed method was from Koo et al. and consisted the following three steps [53]:

Step 1: $CaCl_2$ (aq) + Na_2CO_3 (aq) → $CaCO_3$ (s) + 2 NaCl (aq)

Step 2: $CaCO_3$ (s) + HNO_3 (l) → $Ca(NO_3)_2$ (aq) +H_2O (l) + CO_2 (g)

Step 3: 3 $Ca(NO_3)_2$ (aq) + 2 KH_2PO_4 (aq) + 4 NaOH (aq) → $Ca_3(PO_4)_2$ (s) + 2 KNO_3 (aq) + 4 $NaNO_3$ (aq) + 4 H_2O (l)

The method was performed at room temperature and upon heating as described in 3.2.1.4. Tri-calcium phosphate – $Ca_3(PO_4)_2$. An XRD measurements were performed for both samples.

For the measurement at room temperature mainly $CaCO_3$ was present in the sample (with a match score of 67). However, a small amount of the not desired product calcium phosphate hydroxide ($Ca_5(PO_4)_3(OH)$), also known as hydroxyapatite, was formed with a match score of 37. According to Guo et al. hydroxyapatite and TCP often coexist, depending on the synthesis conditions [58]

As the desired product was not formed at room temperature, the experiment was repeated at a higher temperature, as indicated by Koo et al. 'in hot water'. However, no exact temperature was indicated by Koo et al. [53]. It was decided to perform the reaction at 80 °C, as a higher temperature would not be preferable when working with radioactive ^{45}Ca, due to steam formation.

After synthesis a XRD measurement was performed and it was concluded that a reaction took place, as there was no $CaCO_3$ present in the sample. However, calcium chloride phosphate hydroxide ($Ca_5(PO_4)_3Cl_{0.54}(OH)_{0.46}$) and calcium carbonate phosphate hydroxide ($Ca_{10}(PO_4)_{5.65}(CO_3)_{0.64}(OH)_{3.452}$) were measured, with a match score of 55 and 41 respectively. This result was surprising, since this synthesis method was also used by López-Huertas et al. and therefore expected to be repeatable [1]. However, Koo et al. only analysed the TCP with a particle size analyser, which does not confirm that TCP is formed. López-Huertas does not discuss an analysis method [1].

According to Guo et al. factors influencing the reaction mechanism are temperature, pH, and concentration of the reactants. Therefore, it can be tried to vary those factors. Besides, Vallet-Regí et al. gives an overview of possible synthesis methods for TCP with corresponding references [59].

4.1.5. Calcium lactate - $CaC_6H_{10}O_6$

CaLac was not successfully synthesized according to the method described in section 3.2.1.5. Calcium lactate - $CaC_6H_{10}O_6$. The method consisted of the following two steps:

Step 1: $CaCl_2$ (aq) + Na_2CO_3 (aq) → $CaCO_3$ (s) + 2 NaCl (aq)

Step 2: $CaCO_3$ (s) + 2 $C_3H_6O_3$ (aq) → $CaC_6H_{10}O_6$ (s) + CO_2 (g) + H_2O (l)

Both the XRD diffractogram and the FTIR spectrum showed that no CaLac was present in the sample and the sample consisted of only pure $CaCO_3$. However, during the reaction effervescence was formed, which indicates that CO_2 was formed and therefore it was expected that the reaction took partly place. As CaLac has a really high solubility in water (58 g/L @ 20 °C) it could be that the formed CaLac is removed during the washing steps. CaLac has a low solubility in ethanol, therefore an alternative could be to perform the reaction in an ethanol:water solution. In addition, it was concluded that the reaction is not complete, as there was still $CaCO_3$ present. Therefore, an excess of lactic acid should be added if the reaction is performed again.

4.2 Liquid scintillation counting

LSC was used to measure the relative amount of ^{45}Ca in each phase of the milk, after phase separation.

It is known that LSC measurements of [^{45}Ca]$CaCl_2$, can be influenced by the absorption of ^{45}Ca on the glass surface of the counting vials [21]. Due to the absorption of the ^{45}Ca on the glass wall, less activity can be detected. Therefore, all counting vials were pre-rinsed with a solution of nonradioactive $CaCl_2$. The nonradioactive Ca was absorbed on the surface of the glass, which prevents the binding of ^{45}Ca to the surface.

To determine the optimal amount of activity to perform a LSC measurement, vials with different amounts of activity were prepared. The different amounts of activity were plotted versus the net count rate (R_{net}) in counts per seconds (CPS) for every vial. As can be seen in Figure 22, a linear relation between the activity and the net count rate was obtained with a coefficient of determination of 0.999. It was concluded that an activity between 0.4 and 57 Bq can be used for the measurements. To comply with the ALARA principle (as low as reasonably achievable), it is preferred to use the lowest amount of activity possible. Therefore, the range with lower activities is of interest. It was concluded that an activity of 0.4 Bq can still be measured precisely with the LSC. However, it was decided to work with an activity of around 2.6 Bq to obtain a net count rate of at least 110 CPS, which is approximately three times the background count rate.

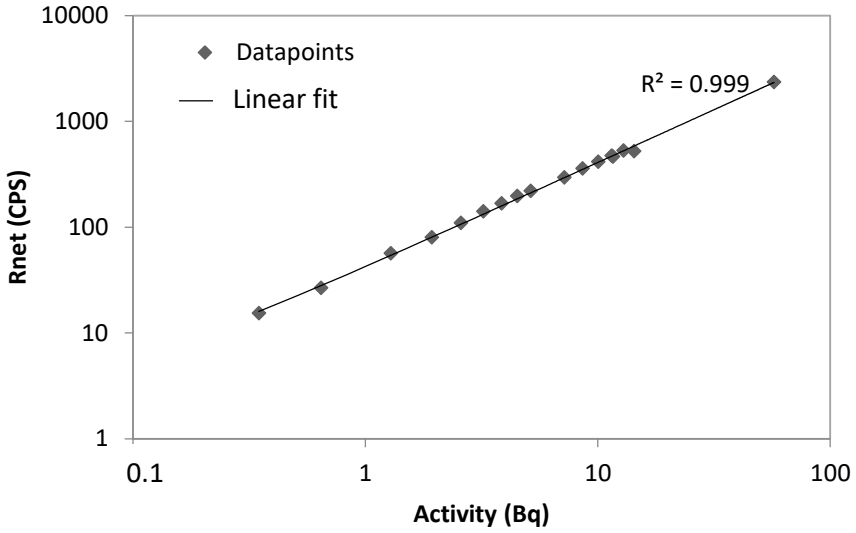

Figure 22: The activitity of different counting vials plotted versus the measured net count rate.

To measure the amount of activity in the different phases of milk, small amounts of milk sample, including ^{45}Ca with an activity of approximately 2.8 Bq, were added to the counting vials. The addition of milk to the sample might lead to quenching and the signal of the beta emission can be blocked [14]. To decrease the influence of quenching skimmed milk was used, which has a lower fat content and therefore will decrease quenching effects. To minimize the influence of quenching further, it was investigated how much milk can be added to the vials without a significant influence of quenching. Figure 23 shows the influence of the volume of milk in a sample on the measured net count rate (R_{net}), which is an indication for the quenching effect. The goal of the experiment was to determine a relation between the net count rate and the volume of milk, therefore the experiment was performed once. It can be seen in Figure 23 that the net count rate is lower from 1.5 mL onwards, relative to the samples with 0-1 mL milk. The net count rate for 0 and 1 mL milk was identical. The net count rate was 1.6% higher at 0.5 mL, in comparison to 0 and 1 mL. This error could

occur due to the fact that the experiment was performed only once and could be due to a deviation in the measurement of the LSC device or a pipetting error of the [^{45}Ca]$CaCl_2$. Alternatively, it could be that the net count rate at 0 mL deviates. However, this is not likely since according to Figure 22 for 2.8 Bq the net count rate is 115.5 CPS, which corresponds to the net count rate at 1 mL in Figure 23. So it is more likely that the count rate at 0.5 mL deviates.

Since the deviation at 0.5 mL is 1.6% and the net count rate at 0 and 1 mL are equal, it was concluded that a volume up to 1 mL of milk does not influence the quenching and therefore the measuring efficiency. Hence, volumes between 0.1 – 1 mL milk per counting vial were used in experiments, to measure the relative amount of ^{45}Ca.

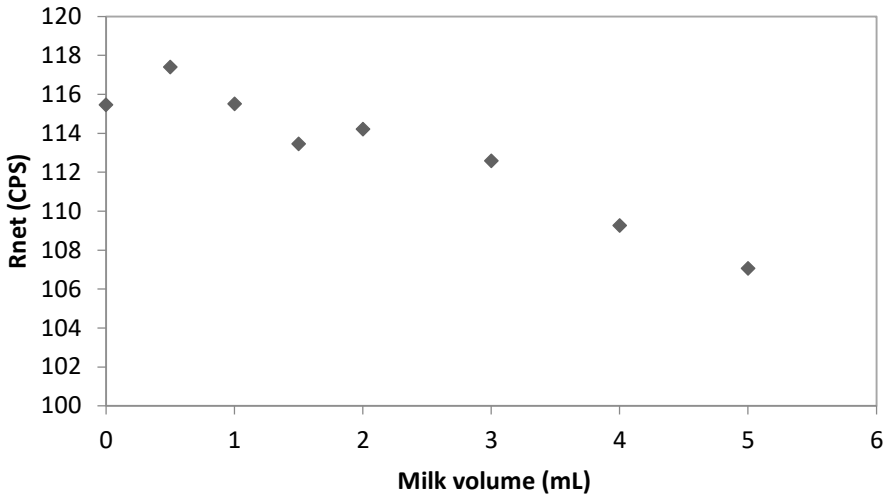

Figure 23: The amount of milk added to different counting vials with 2.8 Bq ^{45}Ca plotted versus the measured net count rate (R_{net}).

4.3 Microwave and ICP-OES

ICP-OES was used to measure the amount of nonradioactive Ca in each phase of the milk after phase separation. However, the milk matrix can cause physical interference in the ICP-OES, among others due to the proteins present. Therefore, prior to the ICP-OES measurement the milk was digested in the microwave.

Two different methods for milk digestion were used. Digestion of milk was performed by using *aqua regia*, as this is a widely used solution for sample digestion. Another digestion method was the addition of nitric acid and hydrogen peroxide to the milk samples [56], [57]. Hydrogen peroxide is added to reduce the carbon and nitrous oxide levels in the samples. Both methods showed complete digestion and accurate results. However it was chosen to use digestion with nitric acid and hydrogen peroxide for further analysis, as this is a commonly used method for milk digestion in literature [41], [56].

When complete digestion was performed the solution in the vessel had a clear yellow colour. Upon opening of the vessels a yellow/orange vapour was emitted, this was due to hydrolysis which takes place in the vessels [56].

4.4 Sample preparation & Phase separation

Different milk samples were prepared, after which phase separation and analysis was performed. Unfortified milk samples were prepared, with and without [^{45}Ca]$CaCl_2$ as tracer, and also milk samples fortified with [^{45}Ca]$CaCl_2$ and [^{45}Ca]$CaCO_3$ were prepared. The ^{45}Ca ions function solely as

tracer, since the amount of ^{45}Ca ions is so small (1 kBq ^{45}Ca ~ 10^{-14} mol Ca) it does not influence the salt equilibrium in milk [14].

It was chosen to fortify the milk with 50 mg Ca / 100 mL milk. In literature different concentrations of Ca for milk fortification were found. Both López-Huertas et al. and Kennefick and Cashman fortified milk samples with 40 mg Ca / 100 mL [1], [3]. Singh et al. prepared fortified milk samples with 50, 75, and 100 mg Ca / 100 mL and concluded that milk fortification with 50 mg Ca / 100 mL milk is optimal when taking sensory evaluation and heat stability into account [3]. Since Singh et al. concluded that 50 mg Ca / 100 mL of milk is optimal for Ca salt fortification, for this research it was chosen to fortify the milk with 50 mg Ca / 100 mL milk. However, it might be interesting to investigate how an increase or decrease of calcium fortification influences the phase distribution and the exchangeability of Ca in milk.

To check the influence of the milk fortification with different Ca salts on the pH of milk, the fortified milk samples were prepared with non-radioactive $CaCl_2$ and $CaCO_3$ and then pH measurements were performed. The measured pH of pure skimmed milk was 6.78, which is comparable with the pH of 6.89 ± 0.09 observed by Chen et al. for skimmed milk and the pH of 6.65 observed by Singh et al. [5], [60]. The pH remained constant upon addition of $CaCO_3$. This might be due to the low solubility of $CaCO_3$ in aqueous solutions. It was observed that the pH decreased slightly to a pH of 6.35 for milk fortified with $CaCl_2$. Disodium phosphate can be added to restore the pH to the original value [5]. However, this might influence the milk matrix and subsequently the results. In addition, according to Singh et al. a pH between 6.3 and 7 is a preferred range to investigate the bioavailability of milk. Therefore, the pH of $CaCl_2$ was not compensated [5].

After sample preparation, phase separation was performed. This was done by dialysis and ultracentrifugation.

4.4.1. Dialysis

With dialysis the soluble phase of the milk can be separated from the insoluble phase of the milk, including proteins and casein micelles.

It is important that the osmotic pressure inside and outside the dialysis tubing is equal. The osmotic pressure in milk is mainly influenced by lactose, sodium, potassium, and chloride [40]. Therefore, various dialysate solutions, containing NaCl, KCl, and lactose dissolved in MQ, were compared. Skimmed bovine milk contains approximately 20 mM Na^+, 40 mM K^+, 30 mM Cl^-, and 150 mM lactose [61]. Therefore, in the first place the experiment was performed with 10 mM NaCl, 40 mM KCl, and 146 mM lactose. However, it turned out that a dialysate solution consisting of 87 mM NaCl, 350 mM KCl, and 205 mM lactose was comparable to the osmotic pressure of milk. This composition of the dialysate solution was used in all experiments. It might be that this higher concentration of NaCl, KCl, and lactose in the dialysate, compared to the milk content, was required to compensate for other solute molecules present in the milk (e.g. citrate, phosphate, magnesium, and Ca). Nonetheless, it is known that addition of NaCl to milk can influence the properties of the casein micelles and can lead to an increase in soluble Ca. The positively charged sodium ions can shield the electrostatic repulsion between the negatively charged casein micelles, which can lead to formation of casein micelle aggregates [34]. However, aggregation of the casein micelles was not observed during the experiments and the fraction of Ca in the different phases of milk did not differ from literature, as will be discussed in 4.4.3.1. Unfortified milk with and without ^{45}Ca.

The addition of $CaCl_2$ and $CaCO_3$ for the fortification of milk could influence the osmotic pressure of milk. Therefore, the influence of the salts on the osmotic pressure was tested and it was concluded that the milk fortification does not influence the osmotic pressure.

4.4.2. Ultracentrifuge

Ultracentrifugation was successfully performed as an alternative method for phase separation. The advantage of ultracentrifugation is that in addition to the separation of the soluble and insoluble phase, also the casein micelles and proteins can be separated. Besides, ultracentrifugation is a fast, although expensive, method in comparison to dialysis. A disadvantage of ultracentrifugation is that an uncertainty is obtained, due to the fact that the concentration of Ca in the casein micelles and the proteins cannot be measured, but only calculated.

During ultracentrifugation it was observed that some of the samples formed not only a pellet of casein micelles at the bottom of the ultracentrifuge tube, but a white layer at the top of the tube as well (Figure 24). This might be due to low density proteins, like lipoproteins, which float to the top of the tube. Another options is the formation of for example carbon dioxide, due to the high pressure, which binds to the proteins and float to the top of the tube. As a non-homogenous layer is formed, this might influence the results for the phase separation of the soluble phase and the protein. However, no relation between the visibility of this layer and the used method was found. Also the samples with this layer did not have differentiating results from the samples without this layer, for the distribution of Ca between the various fractions.

Figure 24: Ultracentrifuge tube with the casein micelles on the bottom and a white layer in the top.

4.4.3. Calcium bioavailability

Phase separation can be used to determine the fraction of Ca in the soluble phase of the milk. The fraction of Ca in the soluble phase of milk is an indication of the bioavailability of Ca.

In the following paragraphs first of all the results of the Ca phase distribution for unfortified milk with and without ^{45}Ca will be discussed. In addition, the influence of the used method (dialysis and ultracentrifugation) and the influence of time, on the equilibrium of ^{45}Ca in the milk, will be discussed. Subsequently, the results of the Ca phase distribution of milk fortified with $[^{45}Ca]CaCl_2$ will be discussed. Lastly, the results of the Ca phase distribution of milk fortified with $[^{45}Ca]CaCO_3$ will be discussed.

4.4.3.1. Unfortified milk with and without ^{45}Ca

Both dialysis and ultracentrifugation were used to separate the calcium in the soluble phase of milk from the other phases. Dialysis and ultracentrifugation showed that approximately 33% of the calcium in milk is present in the soluble phase of milk (Figure 25). This is according to the expectation based on literature, which indicates that ~30% of the total Ca is found in the soluble phase [17], [18], [41].

Both dialysis and ultracentrifugation were also performed with ^{45}Ca as tracer, measuring the percentage of ^{45}Ca in the soluble phase with LSC (Figure 25). It was measured that the percentage of ^{45}Ca in the soluble phase is higher than the percentage of non-radioactive Ca in the soluble phase, approximately 42% and 33% respectively. So the solubility of ^{45}Ca in the soluble phase is higher than nonradioactive Ca in the soluble phase. As discussed by Yamauchi et al. this higher amount of ^{45}Ca in the soluble phase is due to the HTE Ca which is present in the casein micelles [14], [17]. This Ca in the

casein micelles hardly exchanges with Ca and ^{45}Ca present in the soluble phase. Therefore, the phase distribution of ^{45}Ca is not equal to the phase distribution of Ca that is already present in the milk and relative more ^{45}Ca is present in the soluble phase.

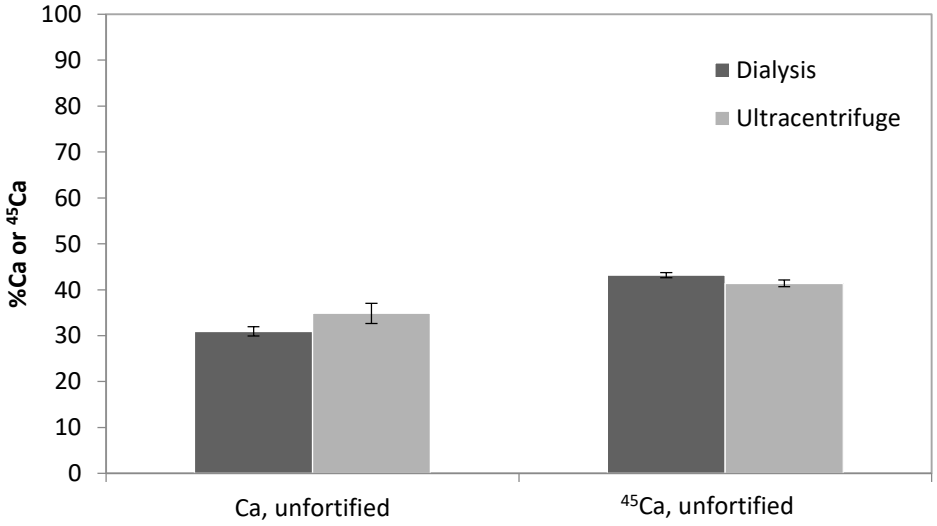

Figure 25: A comparison between phase separation performed with dialysis (dark grey) and ultracentrifugation (light grey) for an unfortified milk sample and a milk sample with ^{45}Ca as tracer.

Yamauchi et al. performed the phase separation with dialysis, Figure 25 confirms that both dialysis and ultracentrifugation obtain approximately the same results [14]. However, a small deviation between both methods was observed (Figure 25). A Welch T-test was performed, for both experiments with Ca and ^{45}Ca, to determine whether there was a statistically significant difference between the results of dialysis and ultracentrifugation. The used T-tests were for independent samples, unequal variances, and two-tailed significance [62]. It should be mentioned that the experiments were performed in triplicate. It is known that such a small sample size might induce an error in the T-test, even though T-tests are designed for sample sizes smaller than 30 samples [63]. To get a more reliable result of the T-test, more data are required.

The obtained P-value for Ca was 0.075, which is higher than the standard significance level of 0.05. Therefore, although a really small sample size is used, the T-test indicated that there is no statistically significant difference between ultracentrifugation and dialysis. However, the P-value for the experiments with ^{45}Ca was 0.007 which is lower than the standard significance level of 0.05. This indicated a statistically significant difference between dialysis and ultracentrifugation for this experiment. This low P-value is mainly caused by the small standard deviations for both dialysis and ultracentrifugation with ^{45}Ca. This is related to the fact that the experiment is only performed three times. A larger sample size is required to indicate if the difference between ultracentrifugation and dialysis is significant.

It was also observed that there is no clear relation between the methods and the deviations, since none of the methods cause a lower/higher percentage for both Ca and ^{45}Ca. Therefore, the deviations are probably due to incidental errors, e.g. pipetting errors or loss of sample during the milk digestion. From these findings it was concluded that, despite the deviations, ultracentrifugation and dialysis can be used interchangeably.

In addition, the influence of time on the Ca equilibrium in milk was investigated (Figure 26 and Figure 27). Before phase separation, samples with ^{45}Ca as tracer and fortified samples were placed in the fridge to let the ^{45}Ca equilibrate. To make sure that equilibrium was reached, ultracentrifugation of

the samples was performed after 24 and 48 h of storage. Figure 26 and Figure 27 show that the amount of Ca in the different phases was equal after 24 and 48 h, for both unfortified and [^{45}Ca]$CaCl_2$ fortified milk, respectively. Therefore, it can be concluded that equilibrium is reached in at least 24 h. This confirms the results in literature [15], [17], [64]. To optimise the process it can be investigated whether equilibrium is reached in less than 24 h, as for example Recker et al. equilibrated the solution overnight [65].

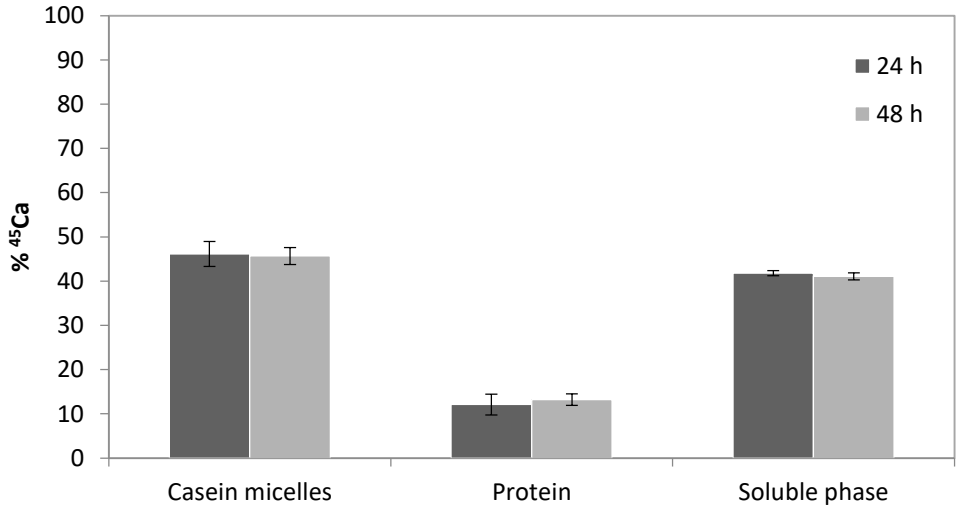

Figure 26: Phase separation, of unfortified milk with ^{45}Ca as tracer, by ultracentrifugation after equilibrating for 24 h (dark grey) and 48 h (light grey) in the fridge.

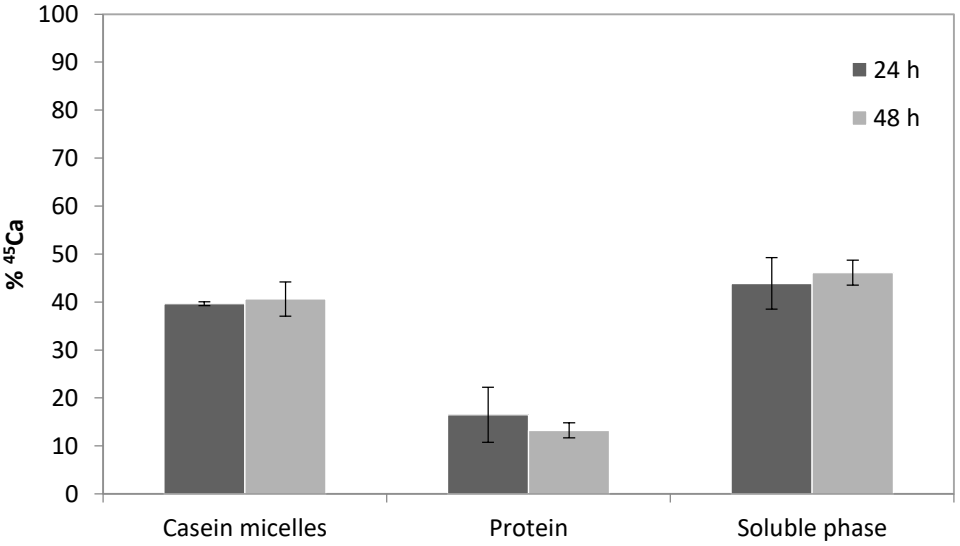

Figure 27: Phase separation, of fortified milk with [^{45}Ca]$CaCl_2$, by ultracentrifugation after equilibrating for 24 h (dark grey) and 48 h (light grey) in the fridge.

Phase separation with ultracentrifugation was performed for unfortified milk with and without tracer and for milk fortified with [^{45}Ca]$CaCl_2$ (Figure 28). Figure 28 shows that 56.0 ± 1.6% of Ca is present in the casein micelles, 9.2 ± 0.6% of the Ca is bound to the serum proteins, and 34.9 ± 2.2% of Ca is present in the soluble phase. This corresponds with the results of Huppertz that ~60%, ~10%, and ~30% of the Ca is present in the casein micelles, serum proteins, and soluble phase respectively [36]. However, slightly more Ca is present in the soluble phase than indicated by literature and observed after dialysis (Figure 25). As mentioned before, this might be due to errors in the procedure.

As discussed before, the amount of ^{45}Ca in the soluble phase is higher than the amount of non-radioactive Ca in the soluble phase, due to HTE Ca. Yamauchi et al. assumed that the HTE Ca was present in the casein micelles [17]. However, with dialysis this could not be confirmed since the serum proteins and casein micelles could not be separated. Figure 28 clearly shows that the HTE Ca is present in the casein micelles and not in the serum proteins and soluble phase, since the percentage of ^{45}Ca in the serum proteins and soluble phase is higher in comparison with the percentage of non-radioactive Ca in these phases.

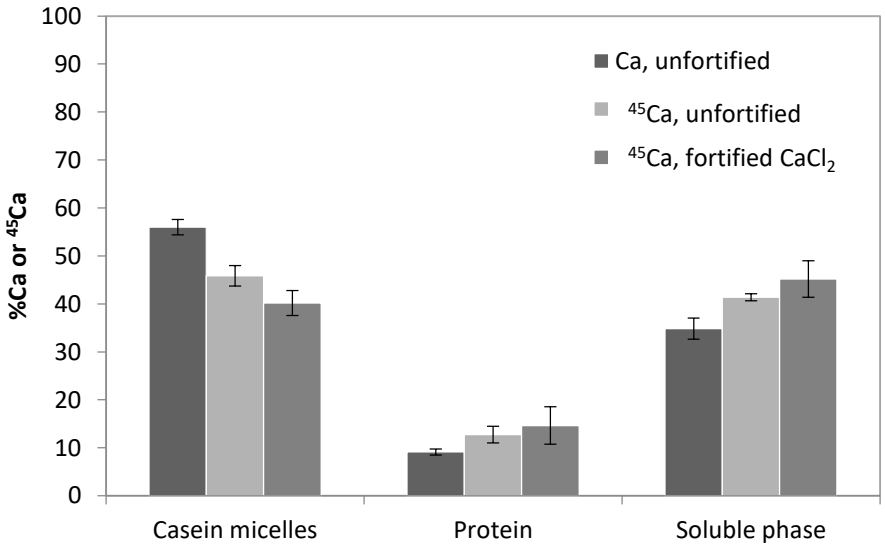

Figure 28: The phase distribution of Ca and ^{45}Ca in milk after ultracentrifugation of milk samples with unfortified Ca (dark grey) and ^{45}Ca (light grey), and fortified with [^{45}Ca]$CaCl_2$ (mid grey).

4.4.3.2. Milk fortification with [^{45}Ca]$CaCl_2$

Milk samples fortified with [^{45}Ca]$CaCl_2$ were also prepared and ultracentrifuged. The fortification of the milk with [^{45}Ca]$CaCl_2$ led to an increase of ^{45}Ca in both the soluble phase and the serum proteins, of ~4% and ~2% of ^{45}Ca respectively (Figure 28). The increase of ^{45}Ca in the soluble phase is an indication that fortification of the milk with $CaCl_2$ increases the bioavailability of Ca, however a part of the added $CaCl_2$ also binds to the serum proteins. Since these data are presented in percentages, it means that the relative amount of Ca in the soluble phase is increased and in the casein micelles is decreased. However, it is still likely that the actual concentration of Ca in the casein micelles is increased upon fortification, as discussed by Huppertz and Timmer: ''it is well-known that addition of soluble calcium salts (e.g. $CaCl_2$) increases casein mineralisation''. This is due to the fact that the milk serum, containing calcium phosphate, calcium citrate, and Ca^{2+} ions, gets supersaturated upon addition of $CaCl_2$. Due to the super saturation, the calcium phosphate in the solution will bind to the casein proteins in the serum and in the casein micelles, leading to an increase in casein mineralisation [41]. As a consequence, also inorganic phosphate and citrate ions are transferred from the soluble phase to the casein micelles [34].

The increase in Ca solubility, due to [^{45}Ca]$CaCl_2$ fortification is surprisingly, since Singh et al. observed a decrease in Ca absorption upon milk fortification with $CaCl_2$ during a metabolic study on mice [5]. This difference might be related to the many factors influencing Ca absorption *in vivo* [2].

Besides, a large standard deviation is observed upon fortification with $CaCl_2$ (Figure 27 and Figure 28). This might be due to the fact that $CaCl_2$ is hygroscopic, because of which a deviation per sample is obtained when weighing the $CaCl_2$.

4.4.3.3. Milk fortification with [^{45}Ca]CO_3

Milk was also fortified with [^{45}Ca]$CaCO_3$ and phase separated with ultracentrifugation. However, $CaCO_3$ is poorly soluble in aqueous solutions. To make sure the [^{45}Ca]$CaCO_3$ could exchange with the calcium present in milk, the samples were stirred in the fridge to equilibrate. As $CaCO_3$ directly precipitates when not stirred, before ultracentrifugation the $CaCO_3$ was first removed by precipitating the $CaCO_3$ with centrifugation, according to a method of Huppertz & Timmer [41]. After removal of the $CaCO_3$ from the milk solution, no ^{45}Ca was present in the milk. So no exchange between the additive [^{45}Ca]$CaCO_3$ in the milk and the Ca present in the milk took place. The lack of exchange of the additive $CaCO_3$ with the Ca present in milk shows that addition of $CaCO_3$ to milk does not increase the solubility of Ca in milk and therefore might also not increase the bioavailability of Ca in milk. This is contradictory to the results of Sittikulwitit et al. who studied the dialyzability of milk fortified with different Ca salts simulating the GIT by modifying the pH. The Ca dialyzability was increased with ~20% due to the fortification with $CaCO_3$ [4]. However, when milk is entering the GIT, the pH will decrease significantly, especially in the stomach where a pH of 2 will be achieved. $CaCO_3$ will react with the protons present in an acidic environment, according to the following reaction: $CaCO_3$ (s) + 2 H^+ → Ca^{2+} (aq) + CO_2 (g) + H_2O (l) [2]. In addition all CCP will be soluble at a pH 4.9, influencing the equilibrium significantly [40]. Therefore it will be interesting to investigate the influence of acidification on the calcium equilibria in milk, especially upon fortification with $CaCO_3$. Miller et al. has developed a method to perform dialysis under gastrointestinal conditions by changing the pH during dialysis [42].

Unfortunately, during this research project no time was left to investigate the influence of milk fortification with calcium citrate and calcium gluconate on the phase distribution in the milk.

4.4.4. Hard-to-exchange calcium

Besides the investigation of the phase distribution, the exchangeability of Ca between the soluble and insoluble phase was investigated. The percentage of HTE Ca in milk after dialysis was calculated to be 41.1 ± 2.0% (Figure 29). This is approximately the same result as obtained by Yamauchi et al. who stated that 40% of the Ca in the casein micelles is HTE Ca [14]. The result is also comparable to Pierre et al. and Zhang et al. who stated that 35% and 45% respectively, of the Ca in the casein micelles is HTE Ca [15], [16]. For ultracentrifugation the percentage of HTE Ca is calculated the same way as for dialysis, where the insoluble phase consists of the casein micelles and the serum proteins.

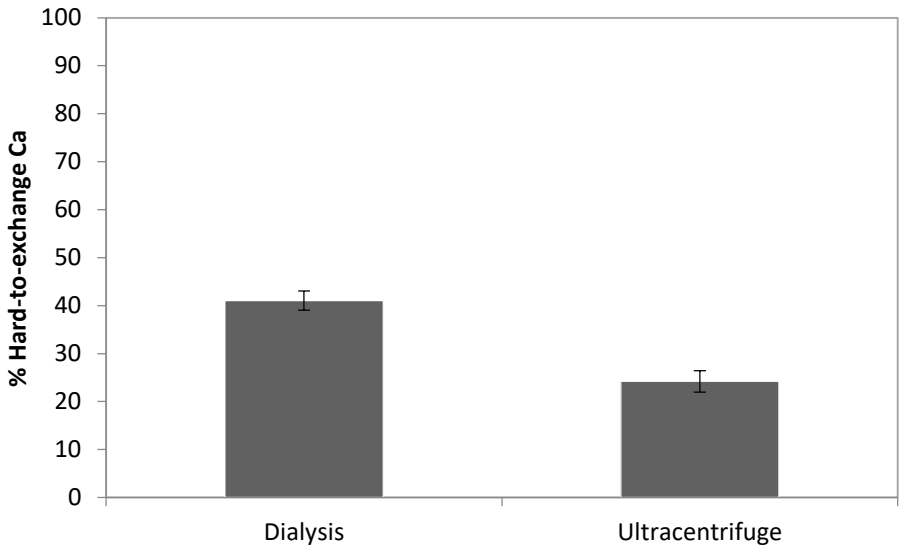

Figure 29: The percentage of hard-to-exchange Ca after dialysis and ultracentrifugation.

Remarkably the calculated percentage of HTE Ca after ultracentrifugation was much lower, namely 24.1 ± 2.3%. This decrease in the percentage of HTE Ca, is due to the fact that the percentage of soluble Ca and ^{45}Ca in the soluble phase for dialysis and ultracentrifugation slightly differ (Figure 25). For ultracentrifugation in comparison with dialysis, the percentage soluble Ca was higher and ^{45}Ca was lower (Figure 25). These deviations led to a decrease of the percentage HTE Ca, as can be seen from Equation 13.

$$\%Ca_{HTE} = 100\% - \frac{(100\% - \%^{45}Ca_{sol})}{\%^{45}Ca_{sol}} * \frac{\%Ca_{sol}}{(100\% - \%Ca_{sol})}$$

Equation 13

Since this calculation method is solely used for dialysis in the literature, the method assumes that the Ca is present in the insoluble phase, so both in the serum proteins and in the casein micelles [14]–[16]. However, as was concluded from Figure 28 the HTE Ca is present in the casein micelles. Therefore, it is more realistic to determine the %HTE Ca by ultracentrifugation and defining for the calculation the insoluble phase as solely casein micelles and the soluble phase as serum proteins plus soluble Ca. In this case the %HTE Ca will be 33.4 ± 3.0%, which is approximately 9% higher than the %HTE Ca calculated for ultracentrifugation in the original way. However, this result again has a deviation, since ultracentrifugation is used.

5. Conclusion & Recommendations

5.1 Conclusion

During this project a method to successfully measure ^{45}Ca in milk with LSC is developed. It is confirmed that very low activities can be measured with LSC, with an optimum around 2.6 Bq. In addition, it is found that up to 1 mL of milk can be added to a LSC vial with 10 mL counting cocktail, without significant effects of quenching.

Successful methods are developed to synthesize $CaCO_3$, CaCi, and CaGluc, starting from $CaCl_2$. Unfortunately, TCP and CaLac are not successfully synthesized and this should be further investigated.

By performing dialysis with unfortified milk, the results from literature were confirmed videlicet 1/3 of the Ca is present in the soluble phase and 2/3 of the Ca is present in the insoluble phase [17], [18], [41]. In addition, with the investigation of the exchange behaviour of ^{45}Ca, it was concluded that part of the Ca in the casein micelles is hardly exchanged. Which led to 43% of ^{45}Ca in the soluble phase, besides it was calculated that 41.1 ± 2.0% of the Ca in the insoluble phase was HTE Ca, both confirming the results of Yamauchi et al. [14].

Although deviations existed, results indicated that dialysis and ultracentrifugation can be used interchangeably. However, a larger sample size is required to confirm that there is no statistically significant difference between the two methods. In addition, it was confirmed that with ultracentrifugation the distribution of Ca between the casein micelles and proteins can be obtained.

Using ultracentrifugation, it was found that in unfortified milk 56.0 ± 1.6% of the Ca is present in the casein micelles, 9.1 ± 0.6% is bound to the serum proteins, and 34.9 ± 2.2% of the Ca is present in the soluble phase, confirming the results of Huppertz [36]. The fortification of milk with $[^{45}Ca]CaCl_2$, led to an increase of ^{45}Ca bound to the proteins and in the soluble phase, of ~2% and ~4% ^{45}Ca respectively, in comparison with unfortified ^{45}Ca. The latter might indicate an increase in bioavailability. Fortification of milk with $[^{45}Ca]CaCO_3$ did not led to an exchange of ^{45}Ca with the Ca present in milk. Indicating that $CaCO_3$ is not likely to make Ca in milk more bioavailable, however this could change in gastric conditions.

In conclusion, a better understanding of the solubility and exchangeability of Ca in milk and the influence of milk fortification with $CaCl_2$ and $CaCO_3$ is obtained by using ^{45}Ca as a tracer.

5.2. Recommendations

- To validate that there is no statistically significant difference between dialysis and ultracentrifugation for milk samples with ^{45}Ca as tracer, the experiments should be repeated.
- The effect of milk fortification with CaCi and CaGluc on the phase distribution of Ca should be investigated using dialysis and/or ultracentrifugation.
- To investigate what the influence is of milk fortified with CaLac and TCP on the phase distribution of Ca, the salts should be synthesized successfully. For TCP the described method can be adapted or another method can be used. Vallet-Regí et al. gives an overview of possible synthesis methods for TCP [59]. For CaLac the method discussed in this report can be adapted by using an excess of lactic acid and/or performing the reaction in an ethanol:water solution, as discussed in 4.1.5. Calcium lactate - $CaC_6H_{10}O_6$. Additionally, the synthesis can be performed by first making CaOH, instead of $CaCO_3$ [2].
- To optimise the process it should be investigated whether equilibrium, between Ca in the milk and added ^{45}Ca, is reached in less than 24 hours.

- In this research it was investigated what the influence of Ca fortification with 50 mg Ca / 100 mL milk is, on the phase distribution and exchangeability of milk. However, it might be that an increase or decrease of the amount of fortification influences the equilibrium. Therefore, the Ca fortification in milk with e.g. 40, 75 and 100 mg Ca / 100 mL of milk should be investigated [1], [3], [5].
- As discussed in literature temperature and pH have an influence on the Ca exchangeability [15]–[18], [64]. Therefore, the influence of temperature and pH on the phase distribution and exchangeability of Ca should be investigated. Miller et al. has developed a widely used method to simulate the pH in the GIT and investigate the influence on phase separation using dialysis [42]. Also the influences of different pH's on the phase separation can be investigated using ultracentrifugation.
- In this research bioavailability and exchangeability of Ca in bovine skimmed milk is investigated. However, different types of milk, like human milk, bovine milk, and infant formula vary in their diary matrix and therefore in the Ca bioavailability and exchangeability [7], [11], [26]. Therefore, it should be investigated what the influence of fortification with different salts is on the bioavailability and exchangeability on the different types of milk.
- The influences of different food components on the bioavailability and exchangeability of Ca in specifically milk should be investigated as well.
- Another way to perform *in vitro* studies to investigate the bioavailability of Ca in milk is cell studies. As performed by Etcheverry et al. using radioactive Ca as tracer to investigate the influence of fortification of human milk [25]. It is interesting to investigate the influence of Ca fortification on the bioavailability of Ca in milk, using cell studies.
- Huppertz has developed a method to prepare casein micelles [9]. This method can be used to prepare casein micelles with ^{45}Ca included. Milk can be ultracentrifruged, removing the naturally present casein micelles from the milk. These casein micelles can be replaced by the synthesized casein micelles labelled with ^{45}Ca. Subsequently, the exchange behaviour of the ^{45}Ca from the casein micelles can be observed. Optionally the milk can be fortified with different calcium salts labelled with ^{47}Ca. In this way the exchange behaviour between calcium in the milk, ^{45}Ca in the casein micelles and fortified ^{47}Ca can be observed.

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Appendix 1

To perform ICP-OES measurements with Ca calibration lines were made, making use of stock solutions with various concentrations. Stock solution with the following concentrations were made and measured: 2.05, 6.14, 10.19, and 14.28 mg Ca/L. Figure 30 shows the concentrations of the various stock solutions, measured at different wavelengths, plotted versus the measured intensity. Figure 31 is an enlargement of Figure 30, showing only the wavelengths measured at lower intensities.

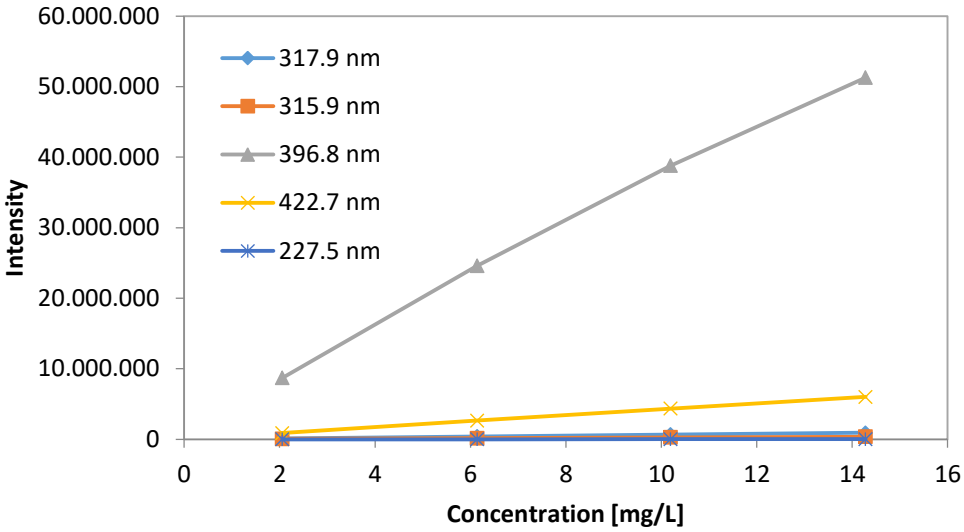

Figure 30: The concentration of different stock solutions, measured with the ICP-OES at different wavelengths, plotted versus the measured intensity.

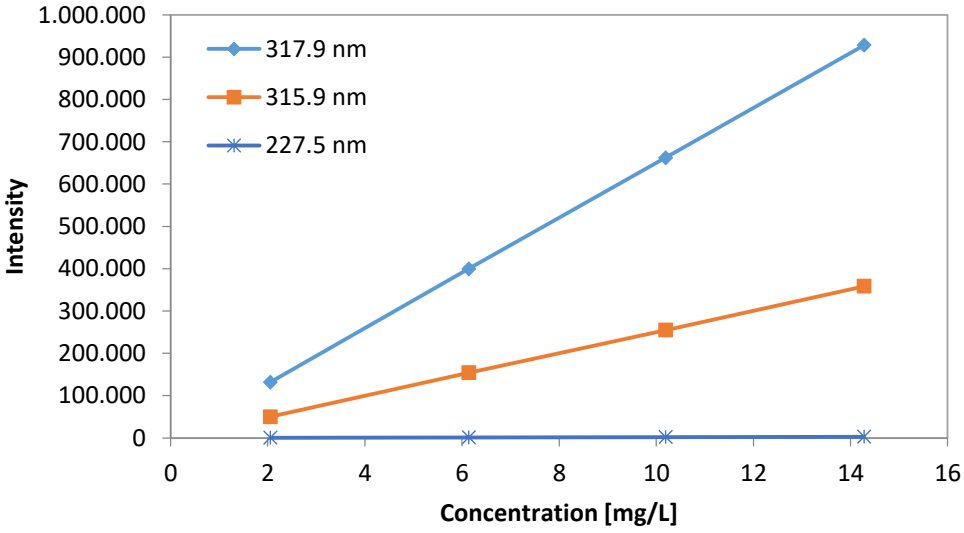

Figure 31: An enlargement of a part of Figure 30, showing the concentration of different stock solutions, measured with the ICP-OES at different wavelengths, plotted versus the measured intensity.

Appendix 2

Figure 32 - Figure 36 are the graphics of the peak lists of the XRD measurements of calcium carbonate, tri-calcium di-citrate, and calcium gluconate. The upper graphic presents the measured peak lines, while the lower graphic presents the peak list from the ICDD database.

Calcium carbonate

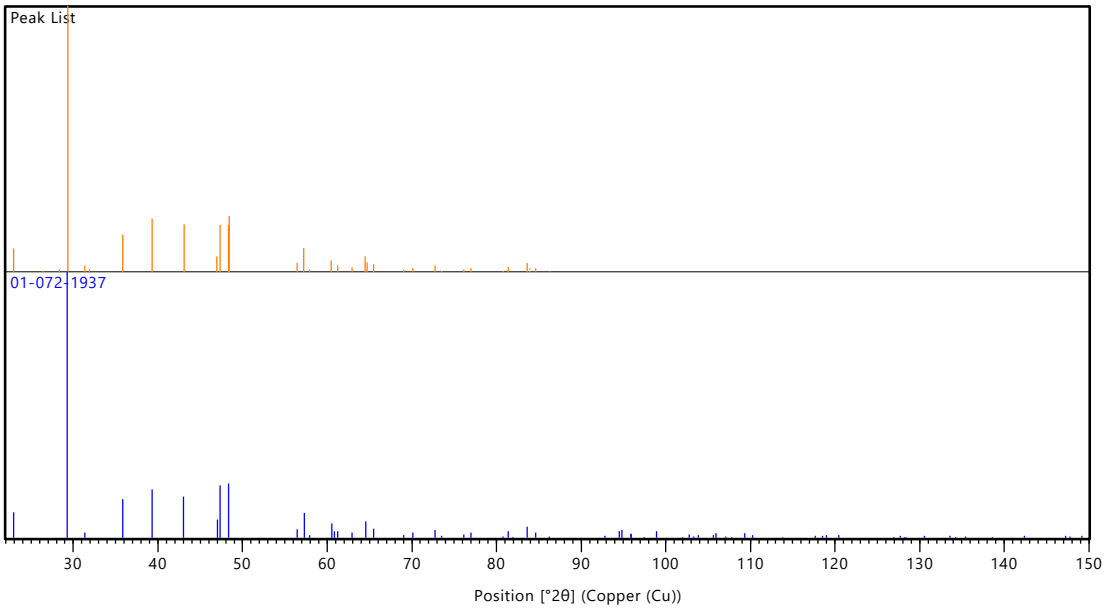

Figure 32: The upper graphic is the peak list of the calcium carbonate sample, measured with XRD. The peak lines present the relative intensity, measured in counts, of a peak at a certain angle. The lower graphic is the calcium carbonate peak list obtained from the ICDD database.

Tri-calcium di-citrate

Method 1

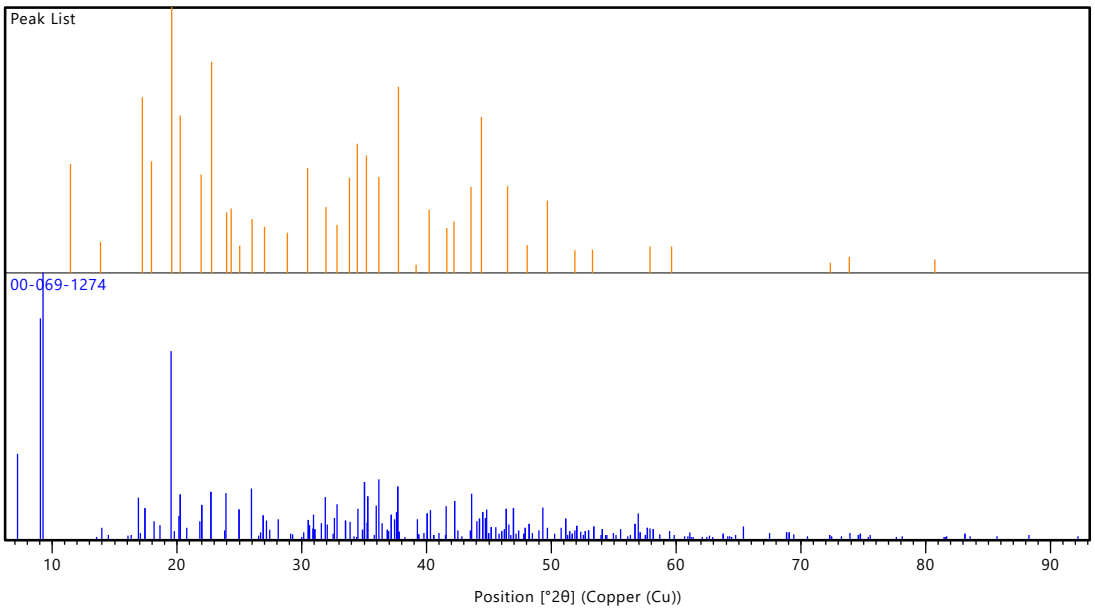

Figure 33: The upper graphic is the peak list of the tri-calcium di-citrate sample synthesized according to method 1, measured with XRD. The peak lines present the relative intensity, measured in counts, of a peak at a certain angle. The lower graphic is the tri-calcium di-citrate peak list obtained from the ICDD database.

Method 2

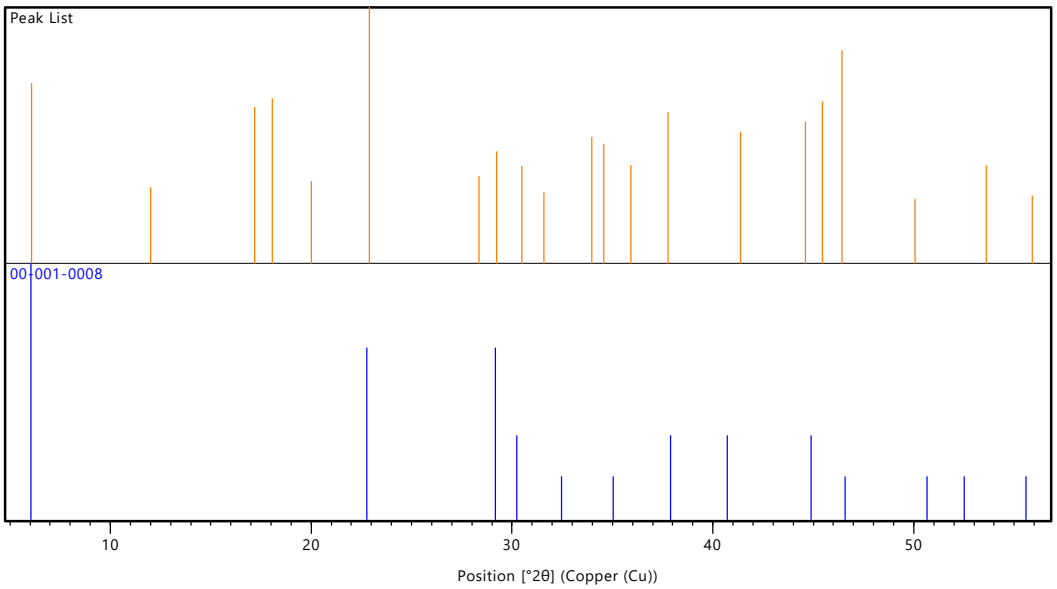

Figure 34: The upper graphic is the peak list of the tri-calcium di-citrate sample synthesized according to method 2, measured with XRD. The peak lines present the relative intensity, measured in counts, of a peak at a certain angle. The lower graphic is the tri-calcium di-citrate peak list obtained from the ICDD database.

Calcium gluconate

Method 1

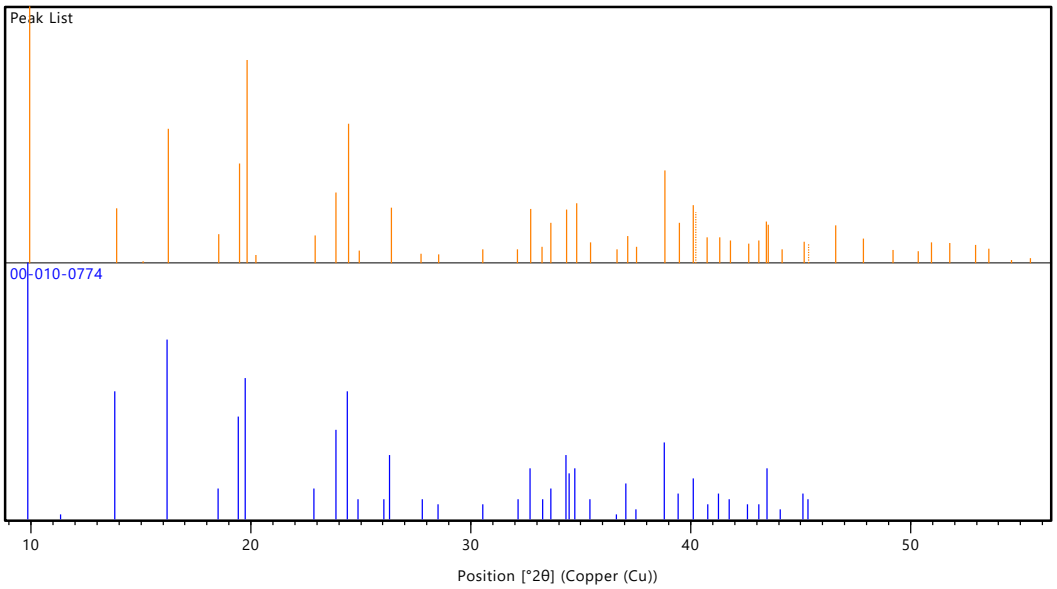

Figure 35: The upper graphic is the peak list of the calcium gluconate sample synthesized according to method 1, measured with XRD. The peak lines present the relative intensity, measured in counts, of a peak at a certain angle. The lower graphic is the calcium gluconate peak list obtained from the ICDD database.

Method 2

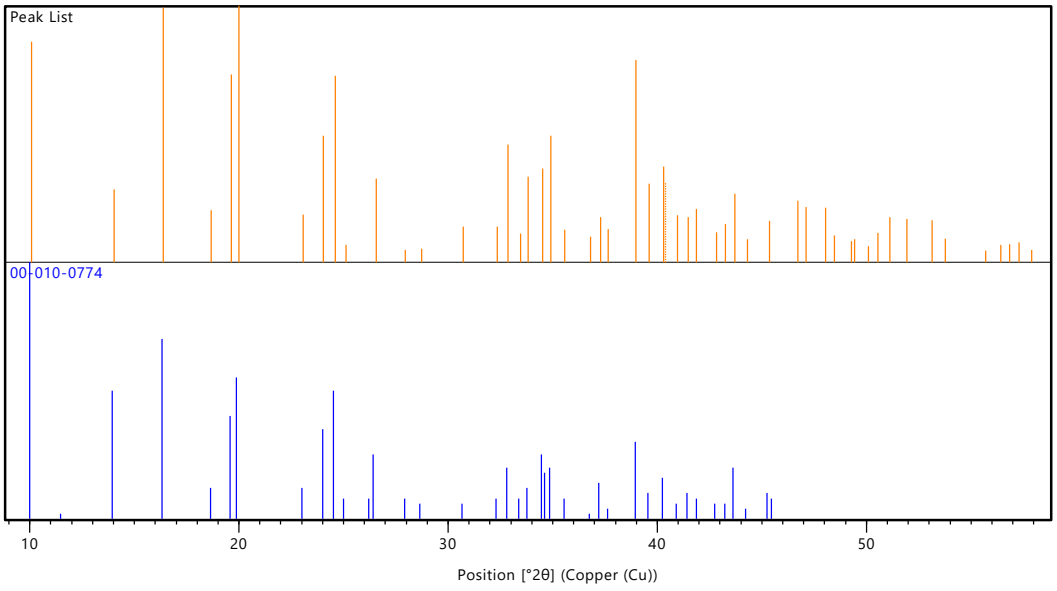

Figure 36: The upper graphic is the peak list of the calcium gluconate sample synthesized according to method 2, measured with XRD. The peak lines present the relative intensity, measured in counts, of a peak at a certain angle. The lower graphic is the calcium gluconate peak list obtained from the ICDD database.