Bioavailability of calcium in bovine skimmed milk

An *in-vitro* study to investigate the influence of calcium salt fortification and low pH conditions on the phase distribution of calcium in skimmed milk

By
Karishma Shanin Sewrattan

Bioavailability of calcium in bovine skimmed milk

An *in-vitro* study to investigate the influence of calcium salt fortification and low pH conditions on the phase distribution of calcium in skimmed milk

By

Karishma Shanin Sewrattan

4379195

in partial fulfilment of the requirements for the degree of

Master of Science

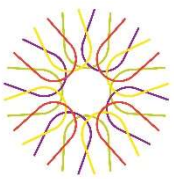
in Chemical Engineering

at the Delft University of Technology,

to be defended publicly on Wednesday August 17, 2022 at 13:00.

Thesis committee:	Dr. ir. R.M. de Kruijff,	TU Delft, Supervisor
	Dr. ir. A.G. Denkova,	TU Delft
	Dr. B. Bera	TU Delft

An electronic version of this thesis is available at http://repository.tudelft.nl/.



REACTOR INSTITUTE DELFT

TU Delft

Abstract

In the human body calcium is the most abundant mineral and is involved in a lot of vital processes. Calcium deficiency occurs when there is not enough calcium present in the bloodstream. In the Netherlands one in three women will face this problem in their lifetime. A major factor contributing to this problem is that the intake of calcium on average is below the recommended amount which is around 950 mg/day for adults in the Netherlands. Calcium deficiency when left unattended can lead to osteoporosis. Therefore it is important to consume enough calcium rich products or take calcium supplements. Milk is a good source of calcium and is considered a staple food. This makes bovine milk a good candidate for calcium fortification. Milk can be fortified by using calcium salts to increase the bioavailability the calcium present. In this thesis *in vitro* methods were used to study the effects of calcium salt fortification on the phase distribution of calcium in bovine skimmed milk under different pH conditions and by using the radioactive isotope ^{45}Ca to track the exchange between the phase casein micelles, serum proteins and the soluble phase when fortifying the milk.

For unfortified milk it was found that 36.0±0.9% was present in the soluble phase, 3.7±2.9% was present in the serum proteins phase and 60.3±3.0% in the casein micelles. Acidifying the milk to simulate the gastric environment resulted in an increase of the soluble phase from 55.1±2.7% to 97.0±3.3% which corresponds to a decrease in pH from pH 6 to pH 2.

For the fortification of the milk, five calcium salts were selected for synthesis. The salts were calcium carbonate, tri-calcium di-citrate, calcium gluconate, calcium lactate and tri-calcium phosphate. The fortification was performed with all of the synthesized salts except calcium lactate and tri-calcium phosphate. The starting material for the syntheses, calcium chloride, was also used for fortification. It was found that for the fortification of $CaCO_3$ under gastric conditions a bioavailability of 100% could be achieved. However the acidic environment also resulted in coagulation of the milk and therefore the bioavailability could not be accurately assessed. Calcium gluconate had the only increase in bioavailability under normal conditions.

In conclusion a better understanding of fortification with calcium salts was achieved and the influence of pH on the bioavailability of calcium in milk and the effects on the salt fortification.

Acknowledgements

First of all I would like to say thanks to my supervisors Robin de Kruijff and Baukje Terpstra. Robin, thank you for your guidance during this project and also thank you for steering me in the right direction when I was getting side tracked or thought too hard about a problem. Baukje, thank you for all your help in the lab and brainstorming sessions about experiments. I want to also give my thanks to the other members of my thesis committee Antonia Denkova and Bijoy Bera for being present during my defence.

I want to give my thanks to the ARI staff and students for all of the fun coffee and lunch breaks we had that sometimes were much longer that desired.

And finally I want to thank my friends and family for their support and in particular my boyfriend. Without you guys I would not be able to finish my thesis.

K.S. Sewrattan

Delft, July 2022

Table of Contents

List of abbreviations

Ca	Calcium
Ca_3N_2	Calcium Nitride
CaCi	Tri-calcium di-citrate
$CaCO_3$	Calcium carbonate
Cagluc	Calcium gluconate
CaLac	Calcium Lactate
CaLG	Calcium lactogluconate
CaO	Calcium Oxide
CPM	Counts per minute
CPS	Counts per second
DPM	Disintegrations per minute
ICP-OES	Inductively Coupled Plasma – Optical Emission Spectroscopy
LSC	Liquid Scintillation Counter
PMT	Photomultiplier tube
QIP	Quench Indicating Parameter
RF	Radio Frequency
Sc	Scandium
TCP	Tri-calcium phosphate
tSIE	transformed Spectral Index of External Standard
u	Unified atomic mass unit
XRD	X'ray Diffraction

1 Introduction

In the human body calcium is the most abundant mineral. It is not only needed for strong bones and teeth but it is also involved in a lot of vital processes in the body. In total the body contains around 2% of calcium. Of this 2%, about 99% can be found in the teeth and bones. The remaining calcium can be found in blood, muscle and other tissue [1]. The body receives the calcium needed by two methods: ingesting calcium supplements or by ingesting foods containing calcium, e.g. milk, yogurt, calcium fortified orange juice or almonds [2]. If not enough calcium is obtained by this dietary calcium, the body will draw calcium from the bones by using osteoclasts cells which can break down the calcium in the bones [3]. Up to the age of 30 the formation of bones exceeds the degradation of bones, after this age the balance turns around [4].

Calcium deficiency occurs when there is not enough calcium present in the bloodstream. Three major groups have a higher risk of being calcium deficient. Women, people who are lactose intolerant or have a milk allergy and the elderly or adolescents who have a reduced calcium uptake by their body [5]. Calcium deficiency can lead to osteoporosis. Osteoporosis makes the bones more fragile and this makes them more likely to break. Woman are at higher risk because after menopause the change in hormones affect calcium uptake from the body [3]. In 2020 in the Netherlands there were 507,200 registered cases of osteoporosis [6]. However this number might be much higher due to that a lot of people are undiagnosed. It is estimated that osteoporosis affects 1 in 3 women and 1 in 7 men in the Netherlands after the age of 60 [7].

A major factor contributing to this problem is that the intake of calcium on average is below the recommended amount which is around 950 mg/day for adults in the Netherlands [8]. The intake of calcium is decreasing around the world due to the negative health claims of the saturated fats present in milk [9]. In Asia the daily intake of calcium is less than 500 mg/day in South America and Africa the average intake ranges from 400 – 700 mg/day and only in Northern European countries the daily intake is more than 1000 mg/day [10]. This is due to the fact that on average more dairy products are consumed in those Northern European countries as it is a readily available staple food [11]. Milk is an important source of calcium and shown from studies has a relative high absorbability of calcium compared to other food sources like vegetables due to the matrix of milk [12]–[14]. Not all of the calcium present in food sources can be absorbed and used by the human body. This concept is called the bioavailability. To increase the bioavailability of a food source fortification can be applied. Therefore fortification of bovine milk is a smart way to increase calcium intake because of the nutritional value that milk has.

Generally there are two ways to assess the bioavailability, *in vivo* and *in vitro*. *In vivo* studies are mostly expensive and physiological effects of the subject have to be taken into account. *In vitro* assessment is a fast and inexpensive method and can give a good estimation of the bioavailability and the influence of the fortification with various calcium salts. Methods that can be used include ultrafiltration, ultracentrifuge combined with filtration, equilibrium dialysis, continuous dialysis and milk treatment with ion exchange resins [15]–[17].

There have been several studies that looked at the bioavailability of calcium under the influence of pH, temperature, time and added food components. The studies used either stable calcium to determine the bioavailability under these conditions or only determined to bioavailability at room temperature with a neutral pH for radioactive calcium [14], [16], [18]–[20].

The influence of different calcium salts using equilibrium dialysis and by stimulating the gastrointestinal tract was investigated by Sittikulwitit et al. [21]. Milk powder was used and the calcium salts that were used were calcium carbonate ($CaCO_3$), tri-calcium phosphate (TCP), calcium lactate (CaLac), tri-calcium di-citrate (CaCi), and calcium lactogluconate (CaLG). It was concluded that all of the added calcium salts caused an increase in the bioavailability, with the order being TCP > $CaCO_3$ > CaCi > CaLac > CaLG. López-Huertas et al. performed an *in vivo* study on humans with fortified milk using TCP [14]. They reported a significant increase of the bioavailability of calcium with the fortified milk in comparison to unfortified milk by using the radioisotope ^{42}Ca to perform the analysis. Singh et al. performed an

in vivo metabolic study on mice using the calcium salts calcium chloride ($CaCl_2$), CaLac and calcium gluconate (CaGluc) [19]. The study concluded that fortification with $CaCl_2$ decreased the bioavailability and fortification CaLac and CaGluc increased the bioavailability compared to unfortified milk. Based on these findings the calcium salts chosen to further investigate in this thesis are $CaCl_2$, CaCi, CaGluc, CaLac and TCP.

The aim of this project is to investigate the effects of the fortification with different calcium salts on the bioavailability of bovine skimmed milk and the effect of low pH conditions. To assess the bioavailability, phase separation was used by dialysis and ultracentrifugation and the fortified milk samples were analysed using the radioisotope ^{45}Ca.

2 Theory

2.1 Characteristics of calcium

Calcium is an alkaline earth metal. It is a reactive element that can form a mixture of calcium oxide (CaO) and calcium nitride (Ca_3N_2) when it comes in contact with the open air [22]. It has atomic number 20 and has an atomic weight of 40.078 u. The name comes from the Latin word '*calx*' which means lime. Calcium cannot be found freely in nature as the element. It is most commonly found in the form of carbonates and sulphates. It was first isolated in 1808 in England by Sir Humphrey Davy using electrolysis [1], [22]. The metal only became available on a large scale in the early 20th century.

2.1.1 Radioactive calcium isotopes

In total calcium has five stable isotopes, ^{40}Ca, ^{42}Ca,^{43}Ca, ^{44}Ca and ^{46}Ca, with ^{40}Ca being the most abundant at 96.4%. ^{48}Ca has a half-life of 6.4*10^{19} years, which is why it can also be considered stable. There are 19 other known isotopes of calcium which are all unstable [23]. Most of unstable isotopes have a very short half-life with a range from nanoseconds to a few days. ^{41}Ca and ^{45}Ca have a longer half-life of 1.3*10^{5} years and 162.61 d respectively.

For the purpose of this research, the radioactive nuclide ^{45}Ca will be used as a tracer to detect the distribution of the added calcium to milk upon fortification. The tracer is used to distinguish the calcium that was added to the milk from the calcium already present in milk. ^{45}Ca was chosen because of its half-life and its commercial availability.

In figure 1 the decay scheme of ^{45}Ca is shown. ^{45}Ca decays by beta-minus emission to its isobar ^{45}Sc with 0.0017% in the excited state of ^{45}Sc and 99.9983% in the ground state of ^{45}Sc [23], [24]. In beta-minus decay an unstable nucleus emits an electron (β^- particle) and an antineutrino. This is the result of a neutron changing into a proton. The added proton moves the element up by one in the periodic table. As a result of the change into a proton, the mass number does not change because this is equal to the number of protons + the number of neutrons. The resulting element is the isobar of the decayed element because they share the same mass number. The emission of the β^- particle is the radiation that can be detected and has an energy that varies between zero and $E_{\beta^-,max}$ [25]. ^{45}Ca decays to the ground state with a maximum beta energy of 256.9 keV. Due to ^{45}Ca being a 'weak' beta emitter, it can be counted very efficiently by using a Liquid Scintillation Counter (LSC).

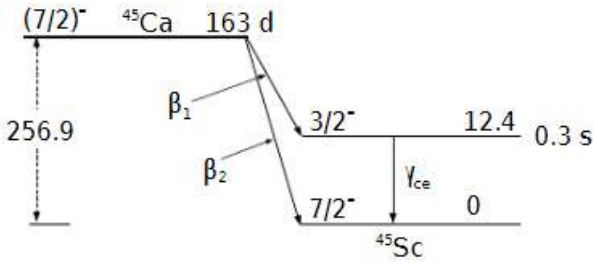


Figure 1. Decay scheme of ^{45}Ca to ^{45}Sc. Energies are in keV [24].

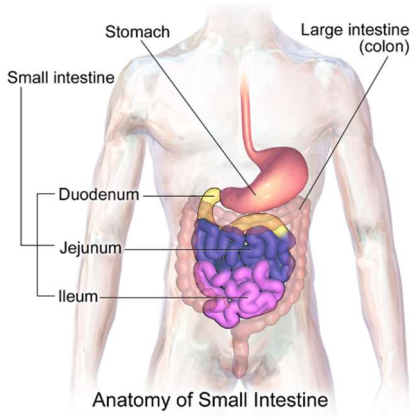


Figure 2. Anatomy of small intestine [26].

2.1.2 Uptake of calcium in the human body

The absorption of calcium can take place by two mechanisms, an active (saturable) transcellular process, primarily in de duodenum, and a passive (non-saturable) paracellular process that is present throughout the jejunum and ileum, see figure 2 [27]. First the pH of the dietary calcium is lowered by the stomach fluids to around a pH of 1-2. Under these conditions the dietary calcium becomes soluble as a salt and is present as the free ion Ca^{2+} [28]. After this the

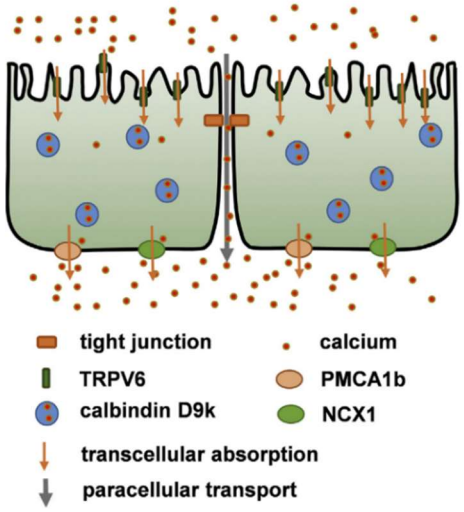


Figure 3. Calcium absorption in the small intestines using transcellular absorption and paracellular transport [5].

chyme moves further down the gastrointestinal tract to the duodenum. From the duodenum onwards the pH conditions become less acidic to neutral and the solubility of the calcium decreases [29]. Even though the calcium precipitates at higher pH, there are still some ions left in solution. The pathway for the uptake of calcium occurs as follows. If the intake of calcium is low, the transcellular absorption is more dominant than the paracellular transport. Transcellular absorption takes place through the cells and paracellular transport occurs between the intercellular space of the cells. A high calcium intake causes the transcellular absorption to be downregulated and the paracellular transport becomes the dominant absorption process [30].

The active transcellular absorption requires metabolic energy and is dependent on vitamin D. The transient receptor potential vanilloid type 6 (TRPV6) channel is responsible for initiating the absorption process across the brush border of the cell. There the calcium enters the cell through the TRPV6 channel and buffering proteins, calbindin D9k, bind to Ca. Subsequently the calcium is transported inside the cell by these proteins. Lastly the Ca is extruded out of the cell through the plasma membrane ATPase 1b (PMCA 1b) which is located at the basolateral membrane and by the sodium-calcium exchanger (NCX1) [28], [30].

Paracellular transport takes place at the tight junctions between the cells in the small intestine. The space between the cells are very narrow and allow the diffusion of small molecules and ions. Through this size- and ion selectivity can take place and acts like a permeable membrane. The diffusion of Ca^{2+} across these tight junctions is driven by the concentration and electric gradients throughout the epithelium. The permeability of the tight junction is regulated by many proteins [28]. From an experiment conducted by Marcus and Lengemann it was found that almost 90% of the time the chyme is present in the lower half of the small intestine in rats [31]. Most likely this is the same for humans. Resulting from the paracellular transport being exclusively present there, it is the major source of calcium uptake [30].

2.1.3 Bioavailability of Calcium

The dietary calcium is not necessarily the bioavailable calcium. Only a certain percentage of the dietary calcium can be absorbed by the human body. This percentage of calcium that is absorbed and used by the body is the bioavailable calcium. Calcium in the body can only be absorbed when it is ionized and in solution [32]. Different kinds of dietary calcium have different bioavailability. For example, high fibre content foods take longer to digest and this makes them a less efficient source for calcium [32]. Calcium absorbability can also depend on numerous physiological aspects. A few examples of these physiological aspects are age, pregnancy, menopause and diseases that limit Ca^{2+} absorption [33]. Just plainly adding more calcium to dietary calcium would not automatically increase the absorption of calcium.

2.2 Bovine milk composition

In general milk contains the nutrients that are required for the development of the infant. All female mammals can secrete milk and humans are the only mammalian species that drink milk directly from other species. Generally bovine milk is consumed and is a source of lipids, proteins, amino acids, vitamins and minerals. The concentration calcium in bovine milk is around 1.1 g/L [13]. Around 33% of the total calcium is found in serum in the free form Ca^{2+} [34]. The rest of the calcium in bovine milk is mainly bound to the casein micelles (insoluble) and a small percentage is bound to the serum (whey) proteins (soluble) [35], [36]. For the purpose of this thesis it was decided to use skim milk for all of the experiments so that we do not have to factor in the fats present in milk as they are 'considered to be one of the most complex of all natural fats' [37]. Milk contains around 400 different types of fatty acids and is therefore too complex to take into account for the purpose of this thesis [38]. In the next two subsections the casein micelles insoluble and the serum proteins will be further explained as these two are an important source of calcium in bovine milk.

2.2.1 Casein micelles

In bovine milk casein takes up to 80% of the total protein content [37]. Casein micelles are made out of a mixture of four different gene product components, the αs1-casein, αs2-casein, β-casein and κ-casein. Which make up around 93% of its dry mass. The other part consist out of inorganic material, referred as colloidal calcium phosphate (CCP) or micellar calcium phosphate (MCP) [39]. Milk has its white colour due to the strong light scattering of the casein micelles. The casein micelles are also responsible for the sensory and textural properties in milk. Many models of these almost spherical particles have been proposed [39]–[42]. They are important carriers of the many nutrients existing in milk due to their unique structure. Close to two third of the calcium, one third of the magnesium and half of the inorganic phosphate and a smaller part of citrate and some other small ions that are present in milk are contained by the micelles. Although these particles carry the name 'micelles', they cannot be compared with detergent micelles [43].

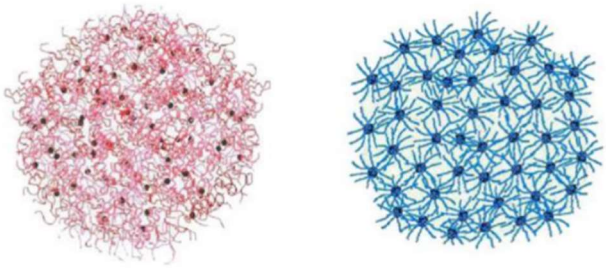


Figure 4. Two schematics of the structure of the hairy casein micelle as proposed by Holt linked by colloidal calcium phosphate (CCP) nanoclusters [39].

The bovine αs- and β-casein are heavy phosphorylated and causes the excess Ca^{2+} present in milk to precipitate due to the binding of these ions to the caseins. The κ-casein only have one phosphoserine group and consequently do not interact with the calcium ions. Because of this they play a major part in the stabilization of the micelles. Despite the huge debate around the structure of these micelles, there is a general consensus that the κ-casein should be located on the surface of the casein micelle and forms a hairy layer by the hydrophilic part. Inside the micelle a network is formed of nanoclusters with crosslinked CCP [39]. A schematic representation of these micelles can be seen in figure 4.

2.2.2 Serum proteins

The serum proteins make up 20% of the total protein content. Serum or whey protein consist out of β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin bovine lactoferrin) and lactoperoxidase and some other minor substances. Both β-lactoglobulin and α-lactalbumin have the property to bind calcium ions [44]. Serum proteins used to be regarded as a by-product of making cheese with having no nutritional value. Present day it is considered an important source of protein and plays an important role in the food industry [44]. For example it can be used to bind water in meat and sausage products or as flavour enhancers.

2.2.3 Separation methods

As mentioned in section 2.1.3, the calcium that is in its free ionized form is bioavailable. The bioavailability can be measured *in vivo* and *in vitro*. In vivo studies examine bioavailability through measuring absorbability or excretion. The results of the in vivo studies are heavily dependent on the physiological conditions of the human subjects or animal model and can lead to inconsistent results [9], [37]. In vitro studies are based on the gastrointestinal conditions. In general there are two methods that are commonly employed to measure the fraction of the calcium available for absorption. One is based on the solubility and the other one is based on the dialysability [20]. The two methods will be further explained as they are used for the determination of de bioavailability in this thesis.

Dialysis

Through dialyses the separation of molecules can take place because of their difference in size. In a dialysis sample a semipermeable membrane tube is placed in milk containing a buffer solution [45]. This mimics the absorption process

of the soluble calcium in the gastrointestinal tract. The buffer solution, called the dialysate, is where the soluble calcium can move to through the membrane, see figure 5. The membrane will only let through the free ionized calcium and will block the larger insoluble proteins (casein micelles and serum proteins) containing calcium. The soluble calcium is the calcium that is bioavailable. The difference in concentration of soluble calcium outside the membrane and inside the membrane is the driving force for this movement. The process will continue until an equilibrium is reached.

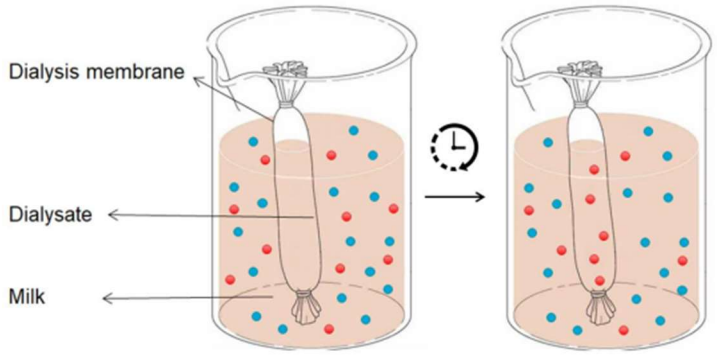


Figure 5. Schematics of the dialysis set-up adapted by de Vos [15]. The red dots represent the soluble calcium and the blue dots represent the protein bound insoluble calcium.

Centrifugation

Another popular method for phase separation of milk is based on solubility. For this method centrifugation is used. First the milk sample is ultracentrifuged and the casein micelles form a pellet at the bottom of the ultracentrifuge tube. The supernatant is then transferred to a Eppendorf containing a 10 kDA filter on top. The solution is centrifuged in a microcentrifuge and because of the filter, the serum proteins are left behind. At the bottom the soluble phase can be found [34]. See figure 6 for a schematic representation of this separation process.

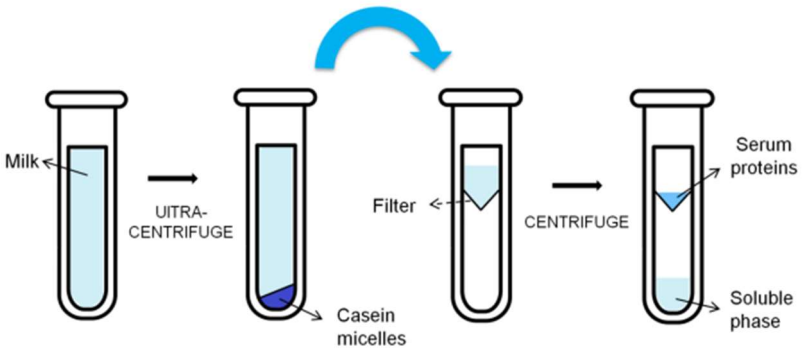


Figure 6. Schematics of the centrifuge set-up adapted by de Vos [15]. The casein micelles are first separated by ultracentrifugation. The supernatant is then centrifuged over a 10 kDa filter and finally the soluble phase is extracted.

2.3 Assessment of the bioavailability

In this section the calculation of the percentage soluble calcium is explained based on equilibrium dialysis and centrifugation. Both give us information about the solubility of the sample and centrifugation can give us also information on the casein micelles percentage and the serum proteins percentage in the milk sample.

2.3.1 Equilibrium dialysis

As explained in section 2.2.3, the soluble calcium is able to equilibrate over the semipermeable membrane during dialysis. By measuring the concentration of calcium in the milk before dialysis, $[M]_i$ (g/mL), and the concentration calcium of the dialysate in equilibrium, $[D]_{eq}$ (g/mL), the solubility of the initial sample can be calculated. The solubility

is the total percentage of soluble calcium present in the milk as is equal to the bioavailability. Correction for the volume is needed to give a more accurate value. This yields the formula that was used in this thesis (equation 2.1).

$$Solubility[\%] = \frac{[Ca\ Soluble\ phase]}{[Ca\ Total]} * 100\%$$
$$= \sum_{i=1}^{n} \frac{[D]_{eq,i} * V_t}{[M]_i * V_m} * 100\% \quad (2.1)$$

V_t (mL) is the total volume of the sample and is constructed out of the volume of milk V_m (mL) added and the volume of the dialysate combined. N is the total number of samples used to carry out one experiment (e.g., duplo n=2).

2.3.2 Centrifugation

With the centrifugation method calcium percentages of different phases in milk can be calculated. As seen in figure 6, the soluble phase can be extracted in the last filtration step. The concentration $[F]_i$ over the total concentration calcium in milk is the percentage of soluble milk (equation 2.2).

$$Solubility[\%] = \frac{[Ca\ soluble\ phase]}{[Ca\ total]} * 100\%$$
$$= \sum_{i=1}^{n} \frac{[F]_i}{[M]_i} * 100\% \quad (2.2)$$

Additional to the solubility, it is also possible to calculate the percentage casein micelles in the milk sample. This is done by measuring the concentration calcium in the supernatant after ultracentrifugation $[U]_i$. Subtracting from the total concentration yields concentration casein micelles (equation 2.3).

$$Casein\ micelles\ [\%] = \frac{[Ca\ total] - [Ca\ serum\ protein\ +\ Ca\ soluble\ phase]}{[Ca\ total]} * 100\%$$
$$= \sum_{i=1}^{n} \frac{[M]_i - [U]_i}{[M]_i} * 100\% \quad (2.3)$$

Similarly the percentage serum proteins can be calculated (equation (2.4).

$$Serum\ proteins[\%] = \frac{[Ca\ serum\ protein + Ca\ soluble\ phase] - [Ca\ solube\ phase]}{[Ca\ total]} * 100\%$$
$$= \sum_{i=1}^{n} \frac{([U]_i + [F]_i) - [F]_i}{[M]_i} * 100\% \quad (2.4)$$

2.4 Instruments used for analysis

In this thesis the milk and the fortified milk and their separated phases are analysed with two analysis techniques. The Inductively Coupled Plasma – Optical Emission Spectroscopy, which can determine the concentration of an element present in a sample and Liquid Scintillation Counting which is used to measure the activity present in the samples. The synthesized salts used for the fortification for salts are analysed by using X-Ray Diffraction, to see if the synthesis was successful. In this section the techniques that these instruments are based on are explained.

2.4.1 Inductively Coupled Plasma – Optical Emission Spectroscopy

Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) is an analytical technique used to determine the composition of samples by using the emission spectra of elements which are excited by a high temperature plasma. After excitation of the atoms, they deexcite and subsequently emit a light that is on an element specific wavelength. The intensity of this specific wavelength is measured and the concentration can be determined from this. In figure 7 an overview of the set-up is shown.

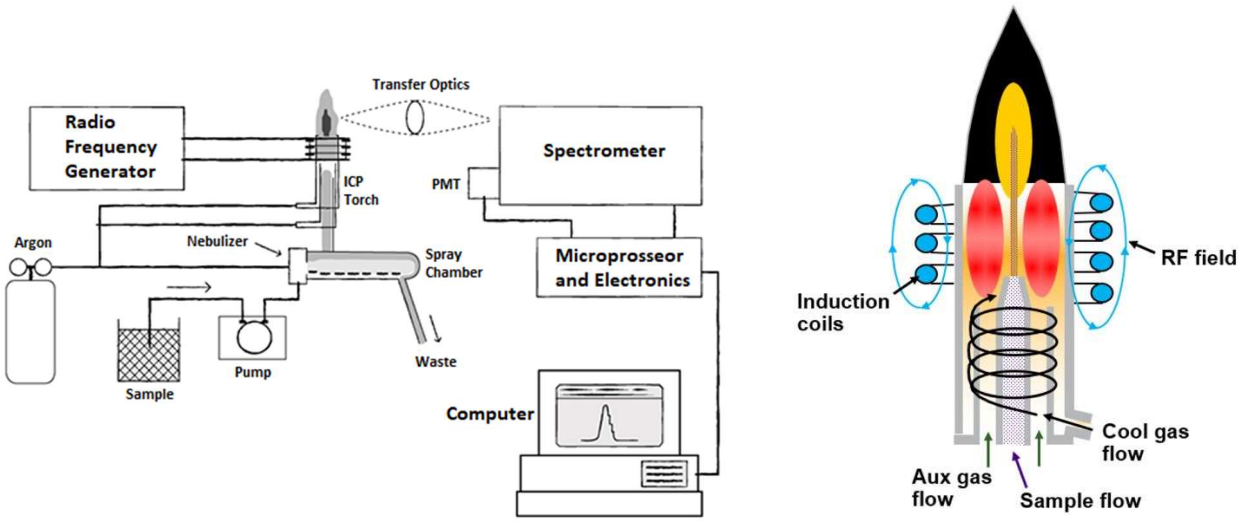


Figure 7. Left: Overview of ICP-OES set-up. Argon flows to the ICP torch and forms the plasma. The sample is introduced to the ICP through a pumping system. The sample is then aerosolized by the nebulizer so that the sample can enter the spray chamber together with the argon flow. Small particles of the sample will reach the plasma, while larger particles go to the waste. The small particles that reached the plasma will collide with the ions and electrons in the plasma and emit light at a specific wavelength due to the collisions. This is then transferred to the spectrometer by transfer optics that can separate the different wavelengths. With a photomultiplier tube and microprocessor the intensities of the emission lines are converted to a digital signal and are sent to the computer [46]. Right: The plasma generation in the torch due to the RF field. Argon flows in the outer tubes. The radio frequency generator at the end of the torch creates the spark needed for the plasma. The induction coils at the side accelerates the electrons that are stripped from the Argon flow and sustains the plasma. The sample flow is carried through the middle tube to the plasma [47].

There are four basic components in an ICP-OES, the sample introduction system, excitation source (plasma), spectrometer and the detector [47]. The liquid sample is introduced to the ICP through pumping. The sample is then moved to the nebulizer where the sample becomes an aerosol. The plasma in the ICP torch is formed by the inert gas Argon. There is a radio frequency generator at the end of the torch. This generator creates a RF field around the torch. The argon is swirled around in the torch and a spark is ignited. Some of the electrons of the argon are stripped by this and are accelerated by the magnetic field through the induction coils. Energy is added to these electrons and these electrons with high energy collide into the argon atoms and strip of more electrons. This chain reaction results in the argon gas being completely disintegrated in electrons, argon atoms and argon ions. The movement of all these things in the torch causes the temperature to rise to around 10 000 K and creates the inductively coupled plasma [48].

The aerosolized sample is introduced to the plasma by punching a hole in the plasma. There the sample is stripped down to its elements. The individual atoms or ions are excited and fall back to the ground state by emission of a photon. This photon has a characteristic wavelength and can be measured. See figure 8 for some examples of emission spectra

of a few metals. The light that is emitted is detected by a photosensitive detector such as a photo-multiplier tube (PMT), charge-injection device (CID) or a charge-coupled device (CCD). At the beginning of a measurement an calibration line is made of the element that is going to be measured. This is done by making four known concentrations and a blank sample. This is then coupled to the measured intensities and the concentration of the unknown samples can be measured [48], [49].

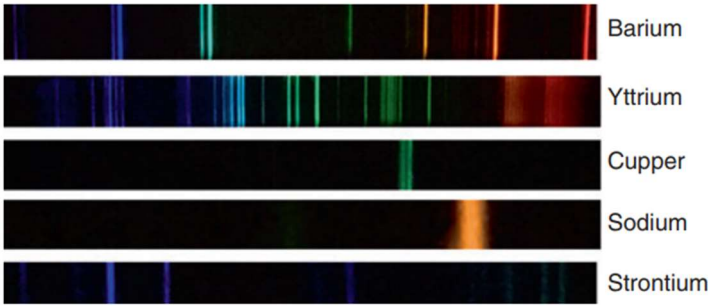


Figure 8. Emission spectra of Barium, Yttrium, Cupper, Sodium and Strontium. (Photograph taken by Michael B. Rutzke) [49].

2.4.2 Liquid Scintillation Counting

Liquid Scintillation Counting (LSC) is a good way to measure low energy β^- emitters and is therefore chosen as the method to measure the activity of ^{45}Ca. The LSC can measure the radioactivity of the unknown sample. To do this the sample containing the radioactivity is dissolved in a scintillation cocktail which contains an aromatic solvent and fluors (scintillators). The liquid scintillation cocktail is great for low energy emitters because they have a short energy range and prevent the self-absorption by the sample or the intermediate material [50]. The β particles that are emitted by the sample transfer energy to the solvent molecules and subsequently go to an excited state. The energy of these excited solvent molecules is transferred to the fluors in the cocktail. The fluors release photons (light) to get back to their stable ground state. The photons are then detected by the photon-multiplier tubes (PMT) present in the LSC, see figure 9. The photon signal is then converted to an electric signal. A single β particle can excite multiple solvent molecules in its path. This results into nearly simultaneously light pulses and is converted to one energy pulse. The number of light pulses per seconds can say something about the number of emissions and the intensity of the light pulse corresponds to the energy emitted [50], [51].

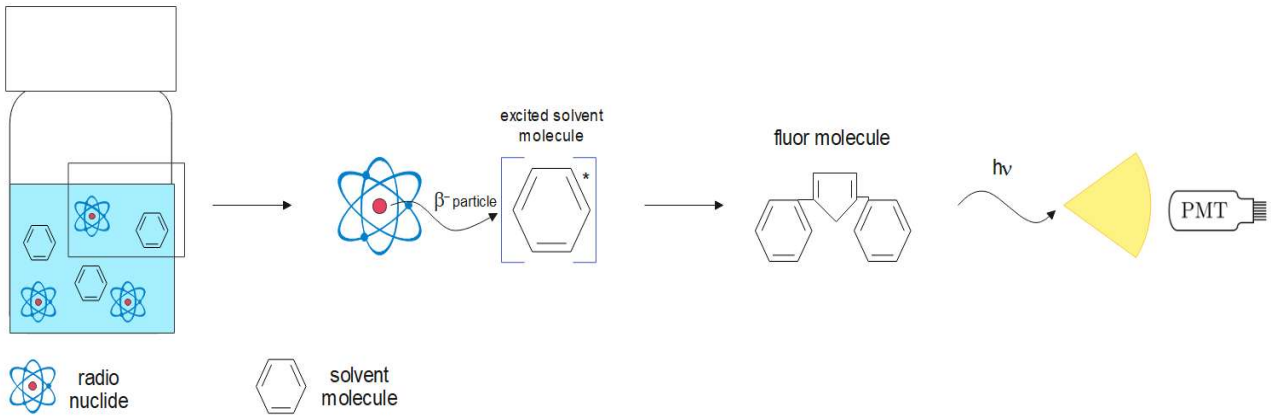


Figure 9. The radionuclide releases a beta particle and transfers its energy to the solvent molecules. The excited solvent molecules release photons to get back to the ground state. These photons are detected by the photon-multiplier tubes and can measure the radioactivity of the unknown sample by this.

Detection Efficiency

The counting efficiency of the detector is influenced by noise. This noise can be categorised as: non-quenchable noise and quenchable noise. Non-quenchable noise is caused by external factors (outside the sample). First of all background

(cosmic) radiation is always present. This can be corrected for by measuring a blank sample and subtracting this from the measured radiation. Secondly the PTMs can be shielded by lead to minimize the influence of this cosmic radiation and other radiation present inside the laboratory. There are two PMTs present in the LSC and only if they both measure a signal it is counted. This way background noise that only affects one of the two PMTs can be excluded.

Quenchable noise reduces the light coming from the sample and results into less counts measured by the LSC. Generally there are three types of quenchable noise: physical, chemical and colour quenching. Physical quenching occurs when the radionuclides in the sample are blocked from the scintillation molecules. Energy transfer cannot occur in that case or is partially blocked. This can happen if the sample is not properly dissolved is the scintillation cocktail and is present as solid particles. Therefore it is important to homogenise the sample before starting a measurement. Another physical quench can occur by the scintillation vial not being clean. The glass needs to be clean of dirt and fingerprints otherwise the fraction of light that can be measured is reduced.

Chemical quenching happens when the energy emitted by the radionuclide or the scintillation molecule is absorbed by another compound present in the solution. This can lead to the production of heat instead of light and subsequently less light is measured by the PMTs.

Colour quenching occurs when the photons that are emitted are absorbed by colour in the sample of the same wavelength. Less photons (light) reach the PMTs and therefore the measured counts are not the representative. This can happen for example in samples containing blood, urine and milk. It can be prevented by adding less of the sample to minimize this effect.

Quench correction

Quenching can be corrected for by using the internal standard method and the external standard method. With the internal standard method the sample is first measured normally in CPM on the LSC. Then a known activity in DPM is added to the sample and this is then measured again. The counting efficiency can then be obtained by:

$$\text{Counting efficiency} = \frac{\text{CPM}_{\text{sample+standar}} - \text{CPM}_{\text{sample}}}{\text{DPM}_{\text{standard}}} \tag{2.5}$$

$\text{CPM}_{\text{sample+stan}}$ are the counts per minute measured after the addition of the standard. $\text{CPM}_{\text{sample}}$ are the counts per minute of only the sample and $\text{DPM}_{\text{standard}}$ are the disintegrations per minute of the standard. With this information the disintegrations per minute of the sample can be obtained $\text{DPM}_{\text{sample}}$:

$$\text{DPM}_{\text{sample}} = \frac{\text{CPM}_{\text{sample}}}{\text{counting efficiency}} \tag{2.6}$$

This method is very accurate but also very labor intensive. To each measured sample a standard must be added and thus this method is only used for a small sample size [51], [52].

With the external standard method, the sample is first measured normally. After this a strong γ source is placed next to the sample. The sample is measured again and the Quench Indicating Parameter (QIP) can be determined. The γ emission causes a secondary emission of a Compton electron from the solvent molecule. This Compton electron behaves like a β^- particle and causes similar scintillation effects. The earlier measured counts from only the sample are subtracted from the measurement and only the counts originating from the Compton electrons remained. The counts are quenched the same way as the measurement of the sample and the measured spectrum of the Compton electrons are compared with the theoretical spectrum [51]. During this thesis a LSC device from PerkinElmer was used and the QIP is then expressed as tSIE (transformed Spectral Index of External Standard). To obtain the transformed spectrum of the external source a Reverse Transform Technique (RST) is deployed. Two points are chosen, P1 at 20% of the

counts and P2 at 10% of the counts. Between P1 and P2 a line intersecting with the x-axis is drawn. This intersection determines the tSIE value, figure 10. If a sample is unquenched tSIE is at 1000. A quench curve is usually stored in the device and with the tSIE value the counting efficiency can be determined. By using equation 2.6 the activity of the sample can be calculated.

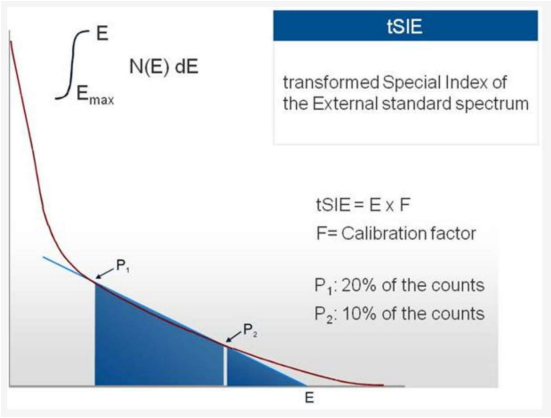


Figure 10. The tSIE value is determined by taking points P1 and P2 and drawing a line that intersects with the x-axis. The intersection is the tSIE value [52].

2.4.3 X-Ray Diffraction

For the fortification of the milk used in this project different salts were synthesized. X-ray diffraction (XRD) was used to determine if the synthesis were successful. The X-rays used by the XRD are generated in a x-ray tube where the cathode produces some electrons that are accelerated to the anode by applying voltage between the two. The anode is made out of copper (in most cases) and when these high energy electrons strike the anode, some of the electrons of the inner electron shell of the copper are knocked from their position. The outer electron then fill in the empty space and "fall down". To do this these electrons have to emit some energy in the form of X-ray photons. The X-ray that can leave the tube are angled towards the crystal (powder) that is being measured. The X-rays diffract at the surface of the powder in a pattern that is characteristic to the structure of the powder. The detector moves around the sample at different angels and a diffraction pattern can be generated with the intensity of the diffracted X-ray against the angle of the detector, 2Θ, see figure 11 [53].

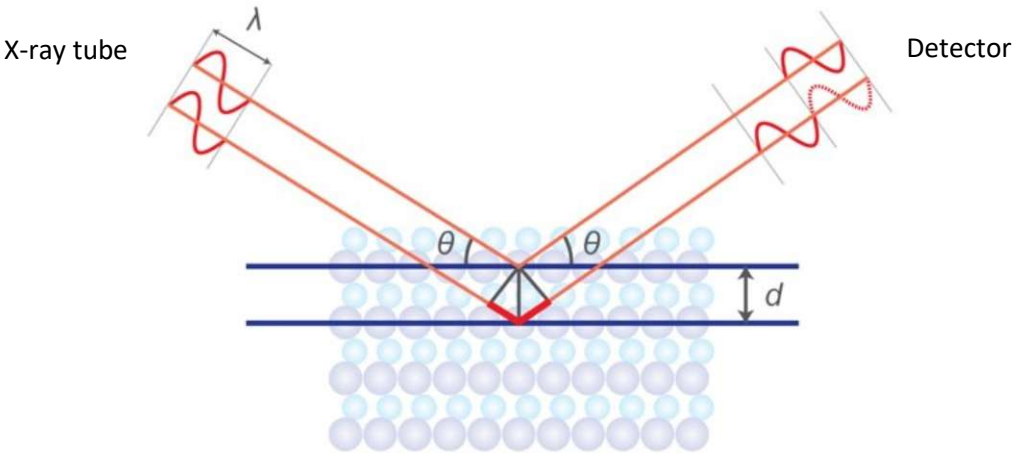


Figure 11. Two X-ray beams with identical wavelength and phase are angled towards the powder and are diffracted. Due to constructive interference satisfied by Bragg's law the powder can be analysed. Figure adapted from Rigaku [54].

$$n * \lambda = 2d * \sin(\theta) \quad (2.7)$$

The detection of these wavelengths is based on Bragg's law, equation 2.7, where because of constructive interference the wavelength can be measured. Here d is the spacing between the two diffracting planes in nm, Θ is the angle of the X-ray beams in degrees, n is an integer and λ the characteristic wavelength in nm. When Bragg's law is satisfied, the diffracted X-ray beams are in phase with each other and diffractions occurs in the direction defined by Θ. This gives each material an unique diffraction pattern and can be thought of as a chemical fingerprint. If the powder is crystalline then sharp clear peaks are detected [55].

3 Materials & Method

This chapter explains the materials used during the experiments and the methods used to conduct the experiments.

3.1 Materials

3.1.1 Chemicals

Table 1. List of chemicals used during the experiments of this thesis.

Chemical	Formula	Supplier
45-Calcium calcium chloride with an activity of 1 Ci (reference date 10th of sept 2020) in aqueous solution (0.1 M HCl)	$^{45}CaCl_2$	PerkinElmer (Groningen, the Netherlands)
Calcium chloride dihydrate, ≥99% ACS reagent	$CaCl_2 \cdot 2\ H_2O$	Sigma Aldrich (Zwijndrecht, the Netherlands)
Calcium ICP Standard, CertiPUR	Ca	Merck (Darmstadt, Germany)
Citric acid, anhydrous 99.5%-100.5% USP grade	$C_6H_8O_7$	Sigma Aldrich (Zwijndrecht, the Netherlands)
Ethanol, purum pur	C_2H_5OH	Honeywell (Delft, the Netherlands)
Gluconic acid (50% solution in water) for synthesis	$C_6H_{12}O_7$	Merck (Darmstadt, Germany)
Hydrochloric acid, suprapur 30% (w/w)	HCl	Merck (Darmstadt, Germany)
Hydrogen peroxide, 30% (w/w) ISO	H_2O_2	Merck (Darmstadt, Germany)
Lactic acid, 80-90% (w/w)	$C_3H_6O_3$	J.T. (Arnhem, the Netherlands)
Lactopure Lactose, p.d. 20-7-2018, Non Food Grade	$C_{12}H_{22}O_{11}$	Wageningen University
Nitric acid, 65% (w/w) supra pur	HNO_3	Merck (Darmstadt, Germany)
Potassium chloride, 99.7%, ACS reagent	KCl	Sigma Aldrich (Zwijndrecht, the Netherlands)
Potassium dihydrogen phosphate, crystallized GPR recta pur	KH_2PO_4	VWR Chemicals (Amsterdam, the Netherlands)
Skim milk	*NA*	Melkan (Superunie)
Sodium carbonate, anhydrous ACS reagent	Na_2CO_3	Sigma Aldrich (Zwijndrecht, the Netherlands)
Sodium hydroxide, ≥98% ACS	NaOH	Sigma Aldrich (Zwijndrecht, the Netherlands)
Tri-Sodium citrate dihydrate**,** purum pur	$C_6H_5Na_3O_7 \cdot 2H_2O$	Merck (Darmstadt, Germany)
Ultimate Gold XR	*NA*	PerkinElmer (Groningen, the Netherlands)

3.1.2 Equipment

The following equipment was used during the experiments:

- Polyallomer Bell-top Quick Seal Centrifuge tubes, (11x32 mm, 2 mL) purchased from Beckman Coulter (Woerden, the Netherlands).
- VWR Centrifugal filters, Modified PES, 10 kDa MCWO, 500 µL, purchased from VWR International.
- Dialysis tubing Membra-Cel MC24x100 Chr 14 kDa purchased from Viskase (Turnhout, Belgium).

- Spectra/Por 4 Dialysis Membrane, Standard Grade Regenerated Cellulose tubing, 12-14 kDa MWCO 32 mm, purchased from Repligen.
- 20 mL High Performance Glass Vial, for Liquid Scintillation Counting, purchased from PerkinElmer.

3.1.3 Instruments

- Inductively coupled plasma – optical emission spectrometry (ICP-OES), Optima 4300 DV, PerkinElmer.
  Protocol used: 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 used for the calcium measurements: 317.936, 396.852, 393.368, 422.658, 227.55, 315.892 nm. To calibrate the ICP a Calcium ICP standard was used. The calibration points used were: 4.041, 8.074, 11.928 and 15.816 mg/L. The calibration lines can be found in Appendix A1.
- Inductively coupled plasma - optical emission spectrometer (ICP-OES), Optima 8000, PerkinElmer.
  The used protocol was: Plasma flow rate 10 L/min, Auxiliary gas flow rate 0.2 L/min, Nebulizer gas flow rate 0.70 L/min, Power 1500 W , View distance 15.0, Plasma view axial and Sample flow rate 1.00 mL/min were used. HNO3 was used to wash between samples. The following wavelengths were used to measure for the calcium measurements: 317.933, 396.847, 393.366, 422.673, 227.546, 315.887 nm. For the calibration lines Iron ICP standard was used. To calibrate the ICP a Calcium ICP standard was used. The calibration points used were: 3.943, 7.933, 11.780, 15.685 mg/L. The calibration lines can be found in Appendix A2.
- X-ray diffractometer: PANalytical X'pert pro
  Used settings: 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 0.0160 °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).
- Liquid Scintillation Counter (tri-carb 2750TR/LL, Packard).
  The measurement time was 10 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.
  Two destruction methods were used: a temperature controlled program and a pressure controlled program. The temperature controlled program raised the temperature up to 180 °C in 40 minutes, then raised it to 200°C for 20 minutes, held at 200°C for 20 minutes and the cooled down to 7 °C in 20 minutes. For the pressure controlled program the power was increased in 60 minutes to 1300 W , then held at 1300 W for 20 minutes and then cooled to 70°C by delivering no power.
- Centrifuge, Jouan CR4i, Thermo.
  Used protocol: 200 x g , 30 minutes at 20°C for fortification
  700 x g, 10 min at 20°C washing synthesis
- Microcentrifuge, Micro star 17R, VWR.
  Used protocol: 13,000 x g , 40 minutes at 20°C.
- OptimaT M MAX Ultracentrifuge, Beckman Coulter.
  Used protocol: 100,000g , 60 minutes at 20°C.
- Freeze dryer, EZ-DRY EZ550Q, Kinetics thermal systems.
  Used protocol: temperature: -50°C, pressure: 25 mT (33 µbar, time: >4 days)

3.2 Methods

In this sections the methods used to synthesize the different calcium salts used, how the fortification takes place, the methods used for phase separation and the preparation for analysis are explained.

All the calcium salts used for fortification were first synthesized in the lab, except for the $CaCl_2$. After synthesis, the samples were characterized using a XRD to determine if the synthesis was successful. The calcium salts were then synthesized with the addition of a small amount of radioactive $^{45}CaCl_2$ that acts as a tracer during the phase separation

process. After the synthesis, samples are 'washed' by using a centrifuge to clear the sample of impurities. The obtained sample was divided over 50 mL centrifuge tubes and centrifuged for 7 min at 760 x g, 20°C. The supernatant was then carefully removed using a 5 mL pipet and 25 mL MilliQ was added to wash. This washing step was repeated two more times to ensure that no impurities remain. The sample was then dried in a vacuum oven at 60°C for 24h to do the XRD measurement. For the use of the fortification of milk, the sample was dried in the fume hood for 48h at room temperature. The described steps to wash the samples were used for all of the salts except for calcium lactate. The salts were analysed using a XRD.

3.2.1 Calcium carbonate – $CaCO_3$ synthesis

For the synthesis of $CaCO_3$ the method of Ohtani et al. was used [56]. In a 100 mL glass beaker with a stirrer, 52 mM of calcium chloride ($CaCl_2$) was dissolved in 30 mL MilliQ. Whilst stirring 52 mM of sodium carbonate ($NaCO_3$) was added to the solution. The suspension was stirred for 2h in the fume hood at room temperature. Afterwards the suspension was washed by using the aforementioned steps. The chemical reaction is:

$$CaCl_2\ (aq) + Na_2CO_3\ (aq) \rightarrow CaCO_3\ (s) + 2\ NaCl\ (aq)$$

To create [^{45}Ca]$CaCO_3$, 500 uL of 370kBq/10 mL of $CaCl_2$ was added in the first step of the synthesis, the solution was stirred for 2 min and then the $NaCO_3$ was added and the rest of the steps were carried out.

3.2.2 Tri-calcium-di-citrate – $Ca_3(C_6H_5O_7)_2$ synthesis

Method 1:

The first method to synthesize tri-calcium-di-citrate (CaCi) uses the synthesis as proposed by de Vos [15]. The first step is to synthesize calcium hydroxide. To do this first a 50 mL centrifuge tube was filled with 600 mM of sodium hydroxide ($NaCO_3$) in 15 mL MilliQ. Subsequently another 50 mL tube was filled 300 mM of $CaCl_2$ in 15 mL MilliQ. Both of the solutions were first homogenised and then the NaOH solution was added to the $CaCl_2$ solution. The obtained suspension was then stirred for 10 min at room temperature so that the reaction could be completed. Finally the suspension is washed and dried.

The calcium hydroxide that was synthesized was then used for the next synthesis step. The obtained calcium hydroxide from the previous step was dissolved in 30 mL MilliQ. Next 0.5763 g citric acid was added to the solution and the solution was stirred at room temperature for 1h. For the reaction to complete, the resulting suspension was left in the fume hood for at least 24h. The chemical reactions are:

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

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

Method 2:

A second method of synthesizing CaCi follows the method as described by Li et al. [57]. For this a 50 mL centrifuge tube was filled with 140 mM of $CaCl_2$ in 15 mL MilliQ. A second 50 mL tube was filled with 120 mM of tri-sodium-citrate in 15 mL MilliQ. Both of the tubes were then homogenised and the tri-sodium-citrate solution was then added to the calcium chloride solution. To this resulting solution was stirred and a mixture of 1:2 (v:v) ethanol:MilliQ was added. This mixture was then stirred for 1h at room temperature and resulted in a slurry of CaCi. The precipitate was then washed and dried. The chemical reaction is:

$$3\ CaCl_2\ (aq) + 2\ C_6H_5Na_3O_7\ (aq) \rightarrow Ca_3(C_6H_5O_7)_2\ (s) + 3\ NaCl\ (aq)$$

To create [^{45}Ca]CaCi for the fortification of milk, 500 uL of 370 kBq/10 mL of [^{45}Ca]$CaCl_2$ was added to the tube containing the $CaCl_2$ solution. The solution was stirred for 2 min and then the rest of the steps were carried out.

3.2.3 Calcium gluconate – $C_{12}H_{22}CaO_{14}$ synthesis

Method 1:

The first method is based on the synthesis proposed by de Vos [15]. For the first step in the synthesis of calcium gluconate (CaGluc) $CaCO_3$ is needed. This was synthesized using the method described in section 3.2.1. A 50 mL centrifuge tube was filled with 20 mL MilliQ and 12.8 mL gluconic acid 50% (w/w) was added and subsequently homogenised. To this solution 10 mmol of $CaCO_3$ was added and the effervescence was allowed to die away. This mixture was stirred for 2h at room temperature and after stirring was left in the fume hood for 48-72h for the reaction to complete. The precipitate was then washed and dried. The chemical reaction steps are:

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

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

To create [^{45}Ca]CaGluc,, 1 mL of 370 kBq/10 mL of $CaCl_2$ was added in the first step of the synthesis to the $CaCl_2$ solution, the solution was stirred for 2 min and afterwards the rest of the steps were carried out.

Method 2:

The second method is based on a patent by Vorage et al. [58]. Calcium hydroxide was first synthesized using step 1 from section 3.2.2 and washed and dried. To this quantity 20 mL MilliQ was added and this was them homogenised. Subsequently 6.4 mL of gluconic acid 50% (w/w) was added and the mixture was stirred for 2h at room temperature. Finally, the mixture was left in de fume hood for 48-72h for the reaction to complete. The precipitate was then washed and dried.

The chemical reactions are:

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

Step 2: $2\ C_6H_{12}O_7\ (aq) + Ca(OH)_2\ (aq) \rightarrow CaC_{12}H_{22}O_{14}\ (s) + 2\ H2O\ (l)$

3.2.4 Calcium lactate – $CaC_6H_{10}O_6$ synthesis

Method 1:

Ohtani et al. proposed a method for the synthesis of calcium lactate (CaLac) [56]. First, $CaCO_3$ was synthesized following the method described in section 3.2.1. A 50 mL centrifuge tube was filled with 40 mL MilliQ and 0.35 mL lactic acid 80-90% (w/w) was added to this. After homogenizing the solution, 10 mmol $CaCO_3$ was slowly added and the effervescence was allowed to die away. The mixture was stirred for 2h at room temperature. Subsequently the mixture was centrifuged for 7 min at 760 x g. The supernatant was carefully removed with a 5 mL pipet and transferred to a 50 mL centrifuge tube. Then 25 mL of MilliQ was added to the precipitate of the synthesis. This process was repeated two more times. The three centrifuge tubes obtained from washing the precipitate were first frozen in a freezer for at least 24h. The three tubes were then transferred to a freeze dryer and left there for at least 4 days. The chemical reaction steps are:

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

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

Method 2:

The second method is based on the findings of Park et al. [59]. A 150 mL glass Erlenmeyer filled with 100 mL MilliQ and 0.15 mL lactic acid 80-90% (w/w) was heated to 60°C by a water bath. The mixture was then stirred for 10 min. Next, 10 mmol of $CaCO_3$ was added slowly to the solution. The mixture was stirred for 2h at 60°C. The solution was then cooled to room temperature. The mixture was then centrifuged and the supernatant was removed as described in method 1 of this section. The obtained tubes were frozen first and then freeze dried. The chemical rection is the same as method 1.

To create [^{45}Ca]CaLac,, 1 mL of 370 kBq/10 mL of $CaCl_2$ was added in the first step of the synthesis to the $CaCl_2$ solution, the solution was stirred for 2 min and afterwards the rest of the steps were carried out.

3.2.5 *Tri-calcium phosphate – $Ca_3(PO_4)_2$ synthesis*

For the synthesis of Tri-calcium-phosphate (TCP) the method of Koo et al. was used [59]. First, the synthesis of $CaCO_3$ was carried out using the synthesis described in section 3.2.1. For the next step, calcium nitrate was synthesized by using a method proposed by Royal Society of Chemistry [60]. To do this, 20 mL MilliQ and 0.9 ml nitric acid 69% (w/w) was added to a 50 mL three-neck round bottom flask. The flask was heated by a water bath to 60°C. When the solution reached the desired 60°C, 7 mmol $CaCO_3$ was added in small quantities and the effervescence was allowed to die away between the additions. This mixture was then stirred for 10 min. Subsequently the temperature was raised to 80°C and 4.7 mmol of potassium dihydrogen phosphate and 9.3 mmol of sodium hydroxide were added. The mixture was stirred for 1h at 80°C at a pH of 9. Finally the solution was cooled down to room temperature and the suspension was transferred to a 50 mL centrifuge tube. The chemical reaction steps are:

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

Step 2: $CaCO_3\ (s) + HNO_3\ (l) \rightarrow Ca(NO_3)_2\ (aq) + H_2O\ (l) + CO2\ (g)$

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

3.2.6 *Milk sample preparation*

For the experiments skim milk was bought from the supermarket. The milk was used as is, a tracer was added, the pH was lowered or was fortified using the different synthesized Ca salts. The skim milk was fortified with 50 mg Ca / 100 mL milk as this was considered the optimal conditions for fortification taste and heat stability of the milk [19].

Lowering the pH

The pH of milk was lowered to pH 6, pH 4, pH 2 and pH 1. To achieve this milk was prepared by adding 30 mL milk to a 50 mL centrifuge tube. The tube was then placed in an ice bath to keep cool. Slowly the pH was adjusted using a pH meter and 1 M HCl while stirring the milk. After the desired pH was reached the milk samples were placed in a carousel in the refrigerator for 24h to equilibrate, see figure 12.

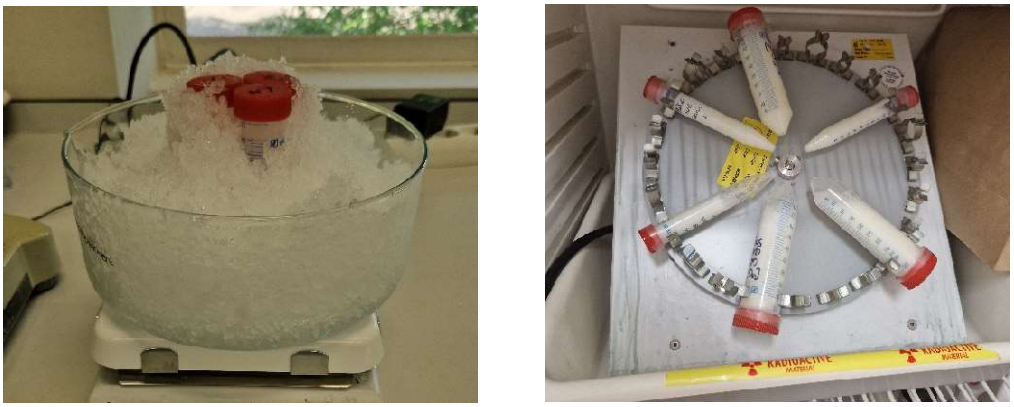


Figure 12. Left: Milk samples in ice bath to adjust pH. Right: Milk samples in the carousel in the refrigerator.

[^{45}Ca]$CaCl_2$ as a tracer

[^{45}Ca]$CaCl_2$ was used as a tracer to determine the relative Ca present in each phase. For this 10 mL of milk was added to a 15 mL centrifuge tube and 10 µL of 370 kBq/10 ml was added to this. The milk sample was then placed in the carousel in the refrigerator for 24h. The phase separation was carried out using the ultracentrifuge method. Analysis was done on the LSC.

Fortification with Ca salts

Fortification of milk was done with [^{45}Ca]$CaCl_2$, [^{45}Ca]$CaCO_3$, [^{45}Ca]CaCi, [^{45}Ca]CaGluc and [^{45}Ca]CaLac that were synthesized. For the fortification with [^{45}Ca]$CaCl_2$, , [^{45}Ca]$CaCO_3$, [^{45}Ca]CaGluc and [^{45}Ca]CaLac 1.25 mmol/ 100 mL milk of the synthesized Ca salt was added. For the fortification with [^{45}Ca]CaCi 41.3 µmol/ 100 mL milk was needed. After the addition of the Ca salt the milk sample was placed in the carousel in the refrigerator for 24h to equilibrate. To create [^{45}Ca]$CaCl_2$, first the $CaCl_2$ was added to the milk and subsequently 10 µL of 370 kBq/ 10 mL was added.

The fortification of [^{45}Ca]$CaCO_3$ was also performed at pH 4 and pH 2. The pH of the milk was first lowered and subsequently 1.25 mmol/ 100 mL milk of [^{45}Ca]$CaCO_3$ was added. The sample was placed in the carousel in the refrigerator for 24h to equilibrate.

3.2.7 Phase separation methods

The milk samples underwent phase separation by using two methods developed by de Vos: phase separation through dialysis and phase separation by (ultra)centrifugation and filtration [15]. The experiments were at least performed in *duplo* but were mostly carried out in *triplo*. The experiments were carried out at ambient lab temperature that varied between 18°C and 28°C unless stated otherwise.

Dialysis

To perform the dialysis a piece of 15 cm was cut from the membrane tubing. The membrane was soaked in MilliQ for approximately a hour. To open the membrane one side was carefully rubbed open and if this was too difficult a needle was used to open the membrane by carefully separating the membrane. Subsequently at the other side a knot was tied at the bottom that was tight enough to prevent the dialysate from leaking. The dialysate solution consist out of 87 mM NaCl, 350 mM KCl and 205 mM lactose dissolved in MilliQ. Using a pipette, 5 mL of the dialysate solution was added to the membrane. The membrane was then closed at the top with a clothespin. The membrane was the lowered into a measuring cylinder containing 100 mL of milk sample. The cylinder was then carefully sealed with parafilm at the top to prevent evaporation. The sample was then left stirring for 24h in the fume hood.

After the 24h the membrane was removed, the membrane was carefully removed from the cylinder. The top was cut open by using scissors a the dialysate was removed using a pipette. The milk outside the membrane was also removed to be analysed. The analysis was done by using the ICP-OES.

Ultracentrifuge

The ultracentrifuge tubes were carefully filled with 2 mL of the milk sample using a syringe. The top of the tubes were sealed by using several methods: a soldering iron, a tube topper or the metal seal former was held above a Bunsen burner an then transferred on top of the tube. After sealing a heat sink was placed on top of the metal sealer to cool down and was removed carefully using tweezers. The sealed tubes were then place in the ultracentrifuge at 100,000 x g for 1h at 20°C. After the ultracentrifugation the casein micelles precipitated and the supernatant was extracted. This was done by first inserting a small needle at the top of the tube to let air into the tube. Using a syringe and needle (0.9 x 40mm) 0.5 mL of the supernatant was transferred to an Eppendorf containing a 10 kDa filter, see figure 13. A small sample was also extracted for analysis on the ICP-OES for the 'cold' samples and LSC for the radioactive samples.

Figure 13. Removing process of the supernatant from the ultracentrifuge tube using needles and a syringe.

Subsequently the Eppendorfs containing the filter were placed in a microcentrifuge at 13,000 x g for 40 – 60 min at 20°C. A sample of the filtrate was taken for analysis with the ICP or LSC.

3.2.8 Sample analysis methods

XRD

An open sample holder was used for sample analysis with the XRD. The dried salts were grinded to a powder and were carefully placed on the sample holder using a spatula. It is important that the powder is distributed evenly across the holder and that the height of the sample does not exceed the border surrounding the sample. A small glass plate can help with this.

ICP-OES

The samples retrieved from the milk and the phase separation were first digested into a solution using a microwave due to the fact that the milk matrix cannot be directly introduced to the ICP-OES [61]. First 0.2 mL of the milk sample was added to the microwave vessels. Then a mixture of 4.5 mL nitric acid (HNO_3) 65% (w/w) and 1.5 mL hydrogen peroxide (H_2O_2) 30% (w/w) was added to the vessels to help with the digestion. The vessels were then placed in the microwave carousel and into the microwave. Two protocols were used: a temperature controlled program and a pressure controlled program. With the temperature controlled program the temperature is raised to 180°C in 40 minutes, then raised to 200°C for 20 minutes, held at 200°C for 20 minutes and the cooled down to 70°C in 20 minutes. For the pressure controlled program the power was increased in 60 minutes to 1300 W , then held at 1300 W for 20 minutes and then cooled to 70°C by delivering no power.

The digested samples were transferred to 50 mL volumetric flasks via a funnel and were diluted with MilliQ to 50 mL and homogenised. This is necessary to stay under the detection limit of the ICP. For the analysis on the ICP, 10 mL was transferred to 15 mL centrifuge tubes.

It is not needed to digest the samples containing only soluble ions but it was still decided to this for all of the samples to ensure that all the samples were treated equally before analysis.

LSC

For the LSC measurements 20 mL glass vials were used. The samples were all prepared the same. First, the glass vials used were pre-rinsed with 0.5 mL of 100 mM $CaCL_2$ solution and afterwards rinsed with MilliQ. This is needed to prevent ^{45}Ca sticking to the glass wall when adding a sample [62]. To these vials 10 mL of the liquid scintillation cocktail was added. Then the samples obtained from the phase separation using the ultracentrifuge were added. Parafilm was wrapped around the cap of the vial and the vial was then vortexed for a few seconds to homogenise the sample. Every LSC measurement also had a blank measurement only containing the LSC cocktail. This was done to measure the background radiation and to subtract that from the measurements of the samples.

To determine how much activity was needed to be present in the samples to be able to measure them without interference from the background an activity curve was made. It is important to stay at least three times above the background count rate. As the working source that was available was almost a year old, different quantities ranging from 1 µL to 200 µL of the ~370 Bq source that was already diluted from the original source was added to 10 mL of the LSC cocktail.

was added to 10 mL of LSC cocktail determine how much CPS can be measured.

To ensure minimal quenching effects by adding milk to the LSC cocktail a quenching curve was made. Different vials containing 10 mL of the LSC cocktail and milk ranging from 0 to 3.5 mL were made. All of the vials contained the same amount of activity to compare the quenching.

4 Results & Discussion

In this chapter the results that have been obtained from the experiments will be reported and discussed. First the results of the synthesized calcium salts are discussed, followed by results from dialysis and ultracentrifuge. Finally the fortification of milk with the different calcium salts will be discussed.

4.1 Synthesis of calcium salts

To fortify the milk samples various calcium salts were synthesized. The results of the synthesis will be discussed in the next section. After the samples have been dried they were all measured on the XRD for an hour and those results were analysed with PANalytical X'Pert HighScore Software. This software compares the data to the ICDD database and match the diffractogram to the known diffractograms in the database. The match is represented in a score. As a rule of thumb a match score higher than 50 was considered successful. However there were a few exception which will be explained.

4.1.1 Calcium carbonate – $CaCO_3$ synthesis

The synthesis of $CaCO_3$ was successful using the method described in section 3.2.1. A XRD measurement was performed and the obtained diffractogram was analysed. As can been seen in figure 14, the peaks of the sample correspond to a diffractogram in the database with a score of 90. The synthesis method of $CaCO_3$ was used in the other methods that uses $CaCO_3$ as one of the reaction materials.

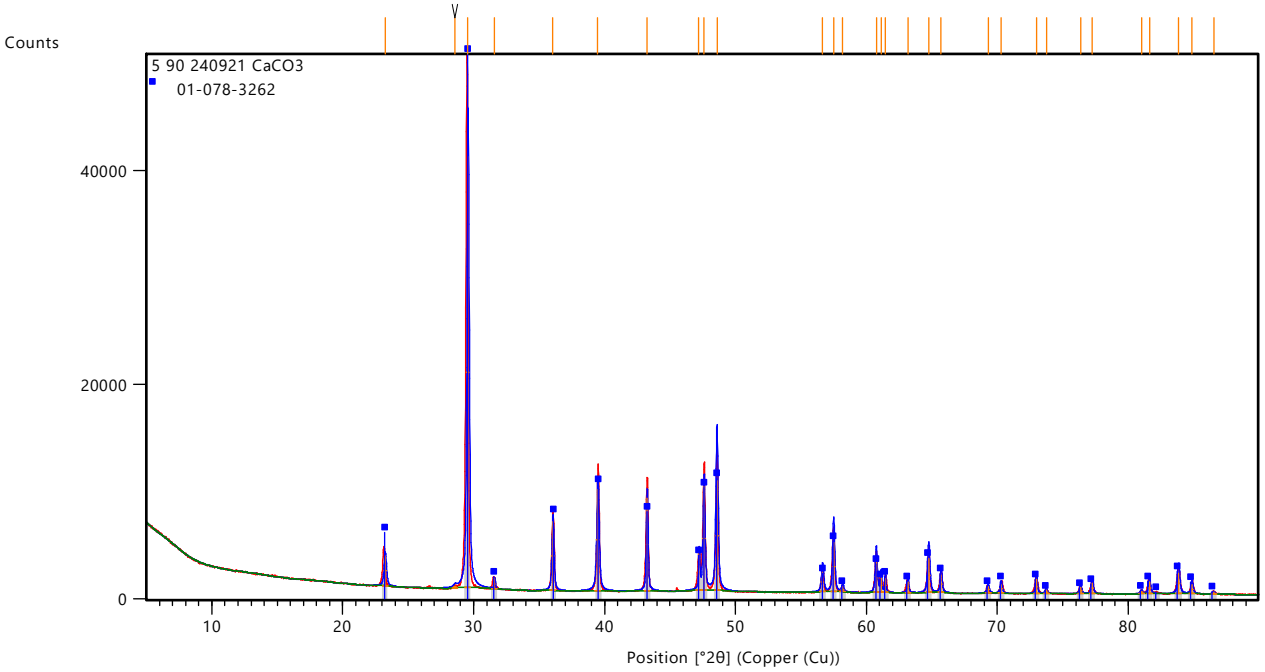


Figure 12. XRD diffractogram of the calcium carbonate sample. The red line is the diffractogram that was measured with the XRD and the blue lines are the peak that the software could match to the database with ref code 01-078-3262.

4.1.2 Tri-calcium-di-citrate – $Ca_3(C6H_5O_7)_2$ synthesis

Method 1:

The first method described in section 3.2.2 considered the synthesis of calcium di hydroxide ($Ca(OH)_2$) as an intermediate step. This synthesis described in this intermediate step was also analysed on the XRD. The diffractogram is shown in appendix B1. A match score of 83 was obtained and the reaction material could be used in the next step of the synthesis. For the CaCi that was subsequently synthesized a match score of 44 was obtained by using ref code 00-025-1568, which are the blue lines. There were still some peak unaccounted for and ref code 00-003-0038 was used

that gave a match of 38. The scores did not meet the requirement that was set. However it seems that both of the references complete the diffractogram that was measured. Therefore it can be said that the synthesis was still successful. To compare all of the peaks measured to a diffractogram in the database ref code 00-069-1274 that was used to compare the synthesis in the thesis of de Vos can be used [15]. Due to time constrains the results obtained in this thesis were not checked using this reference. Initially this reference does not show up in the program. It needs to be manually inserted in the program from the database.

In the measured diffractogram there are also many small peak present and some of them are broad. This indicates that the formed crystal is not entirely crystalline [56]. However, for the purpose of this thesis this was not a requirement. The peaks could be a result of drying the sample in the oven at 60°C. Oven drying removes some of the water content in the sample and could have an effect on the crystal structure. Li et al. used freeze drying to dry the CaCi samples which could be considered as an alternative drying method [57].

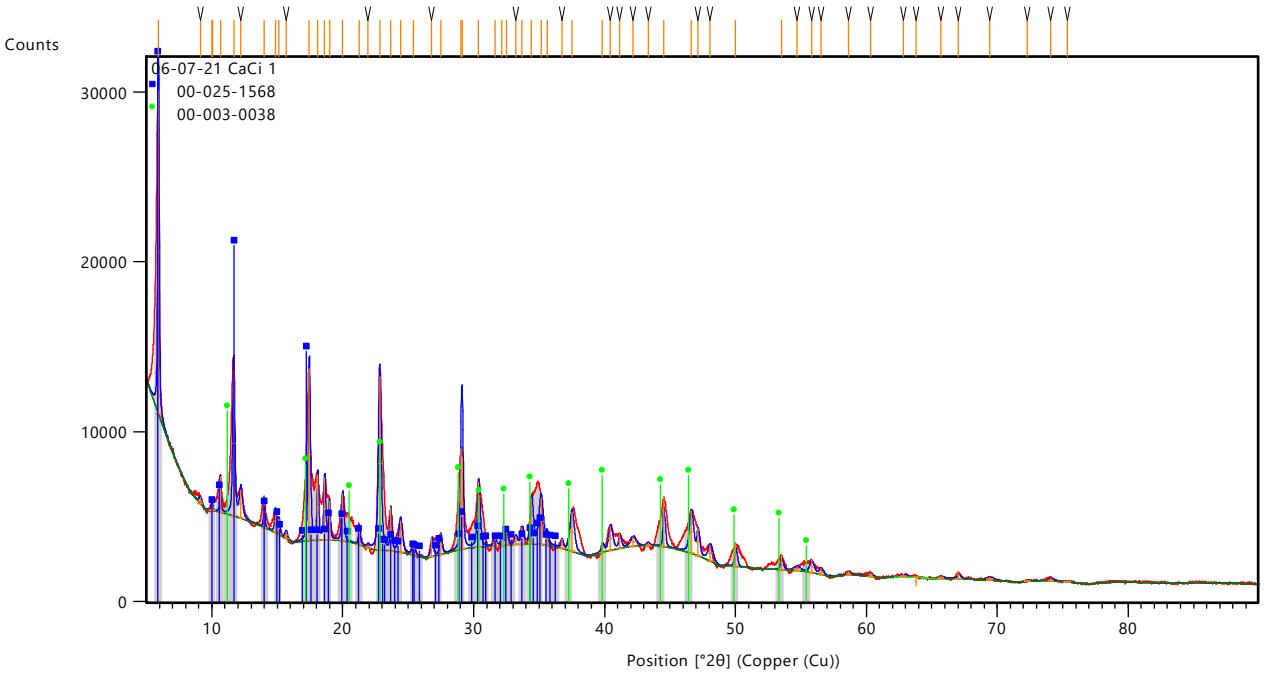


Figure 13. XRD diffractogram of the Tri-calcium di-citrate sample using method 1 of the synthesis. The red line is the diffractogram that was measured with the XRD and the blue and green lines are the peak that the software could match to the database with ref code 00-025-1568 and 00-003-0038 respectively.

Method 2:

The second method of synthesizing CaCi only uses one reaction step, making it faster and more efficient than the previous method. An XRD measurement was performed and showed a match of 49 and 43, see figure 16. Similarly to the previous analysis of the diffractogram, two references were used. In this diffractogram small and broad peaks are also present. Li et al. it is explained that with increasing the ratio ethanol:water to create the slurry of CaCi, the peaks broaden in the XRD results and causes a decrease in peak intensity [57]. In this experiment a ratio of 1:2 was used and should provide a better result. However the XRD results indicate that there are still some impurities left in the sample. The score of this synthesis was good enough to say that it was successful and the produced CaCi was used for the milk fortification experiments.

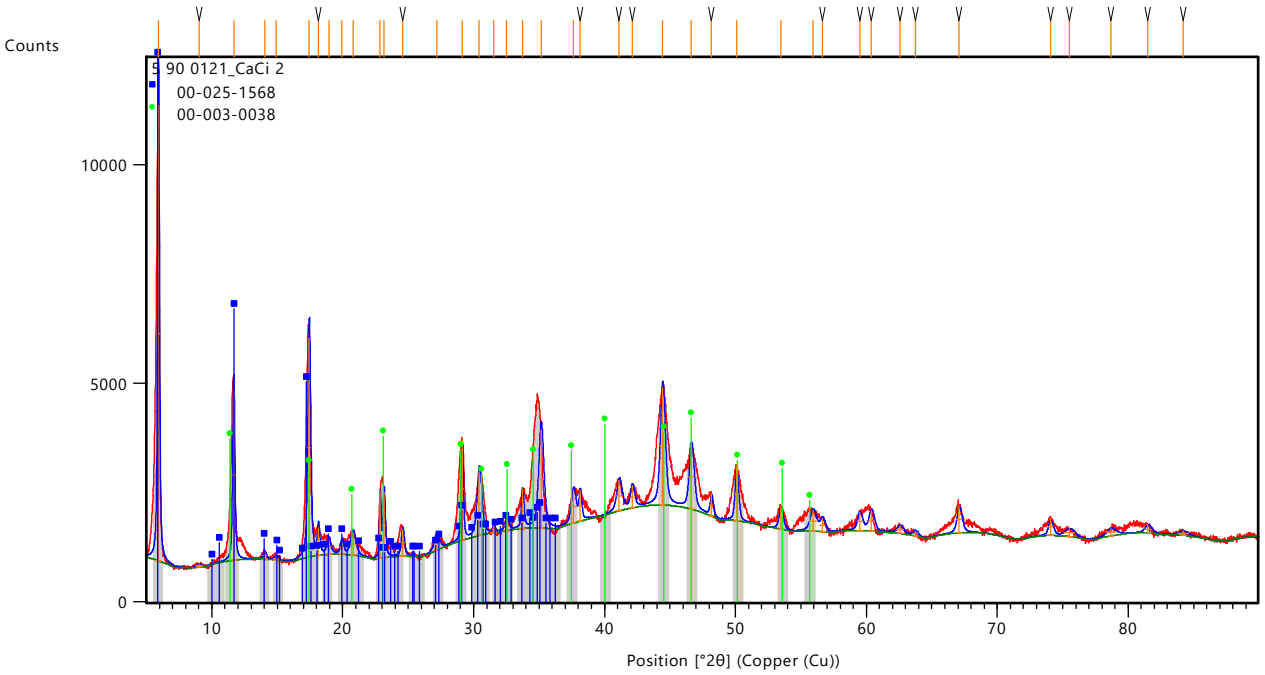


Figure 14. XRD diffractogram of the tri-calcium di-citrate sample using method 2. The red line is the diffractogram that was measured with the XRD and the blue and green lines are the peak that the software could match to the database with ref code 00-025-1568 and 00-003-0038.

4.1.3 Calcium gluconate – $C_{12}H_{22}CaO_{14}$ synthesis

Method 1:

Following the synthesis method 1 described in section 3.1.3 the synthesis was not successful. The formation of CaGluc was very slow over the course of several days and yielded not enough material to analyse it on the XRD. One of the reasons that the synthesis was not successful could be due to that during step 2 it is important to add an excess volume of gluconic acid to complete the reaction as described by de Vos [15]. In this experiment a molar ratio of 1:2 for the CaCO3:gluconinc acid was used. In future experiment different ratios could be tested to find the optimal ratio for the synthesis. Method 2 was successful and therefore no further experiments using method 1 were conducted.

Method 2:

The rection described in method 2 was also very slow and took 2-3 days to complete. Slowly spherical particles formed at the bottom of the liquid. A XRD measurement was performed and showed a match score of 41 using the ref code 00-005-0119. There are many small peaks present that could indicate that the sample is not crystalline. The reaction materials were also cross referenced but it was found that none of them were still present in the sample. That is why this method was chosen to continue with for the fortification. To achieve a better score other references in the database could be used to cross reference the measured diffractogram. It was noted that using the software not all of the diffractograms containing the measured material showed up. It can be useful to manually search the database to corresponding diffractograms. For example de Vos used a diffractogram using the ref code 00-069-1274 [15]. Again due to time constrains this was not tested for the obtained result.

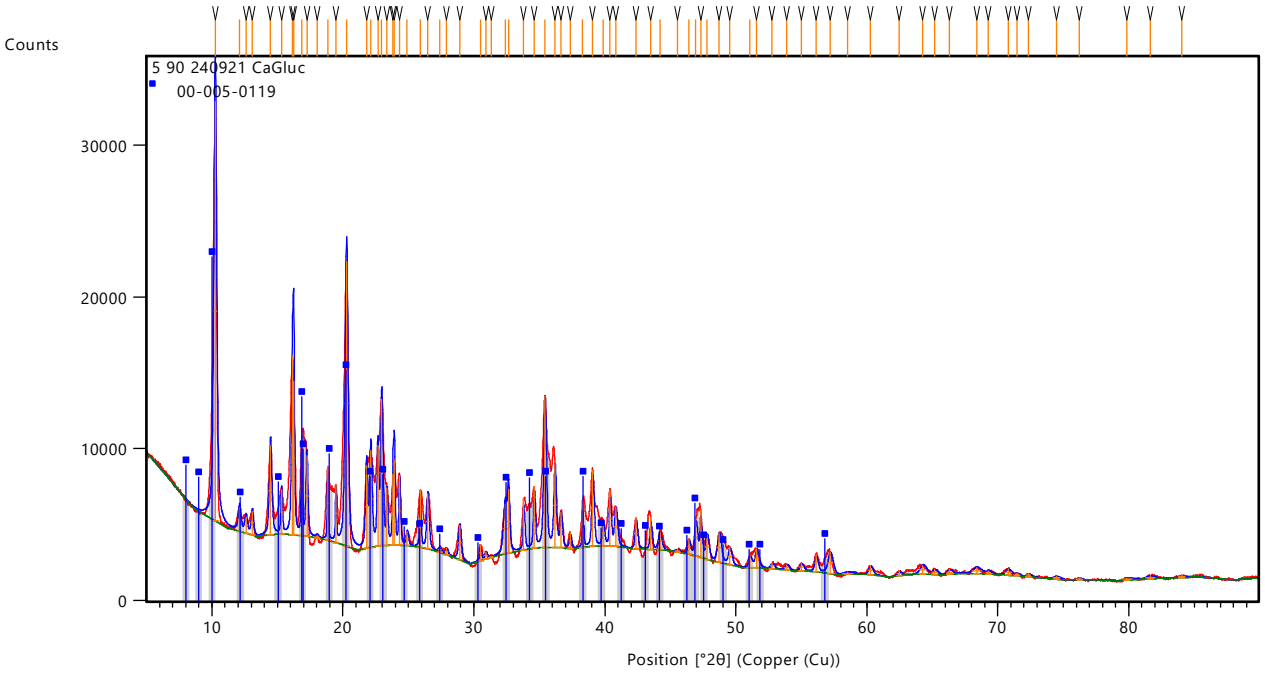


Figure 15.XRD diffractogram of the calcium gluconate sample using method 1 of the synthesis. The red line is the diffractogram that was measured with the XRD and the blue lines are the peak that the software could match to the database with ref code 00-005-0119.

4.1.4 Calcium lactate – $CaC_6H_{10}O_6$ synthesis

Method 1 was not successful for the synthesis of CaLac. At first the precipitate resulting from the synthesis of CaLac using method 1 and method 2 was used as the sample to measure on the XRD. However as can be seen from appendix B2 the material was a very crystalline $CaCO_3$ which was one of the starting materials. CaLac has a solubility of 58 g/L in water at 20°C which can be considered high. Therefore both of the synthesis were carried out again but this time the supernatant was preserved and freeze dried. Method 1 showed no material in the supernatant and method 2 had a residue of powder left in the liquid that was evaporated, see figure 18.

Figure 16. Samples of CaLac synthesis after freeze drying. The front rack shown is the synthesis resulting from method 2.

This sample was measured on the XRD and analysed. The result is shown in appendix B3. The XRD measurement shows a characteristic signal that can be assigned to an amorphous material. This is because freeze drying the material removed most of the water in the crystal structure. The water that normally is present in the material contributes to the crystal structure of the material [58]. Tansman et al. describes this phenomenon with the crystal structure of CaLac [59]. By oven drying their CaLac sample at 80°C al of the associated water was removed and resulted in a similar XRD diffraction pattern as described in appendix B3. The remaining powder that was not used for analysis on the XRD was

hydrated with a few drop of MilliQ and dried in the oven at 38°C for 12h as described in the paper of Tansman et al. [59]. This resulted in the XRD diffractogram in figure 19. The score obtained was 79 and the material had sharp peaks. According to Tansman et al. the material can lose a part of its X-ray diffraction capability due to the excessive drying under high vacuum [59]. Thus a more crystalline material can be obtained by just slowly drying the supernatant obtained from the synthesis under a relatively low temperature. This was not the aim for this thesis, therefore the synthesis method was used like this to perform fortification. As slowly drying the supernatant takes a long time and is not efficient. There are some peaks to right that could not be assigned to the CaLac. Upon further investigation it is shown in appendix B4 that those peaks are most likely from $CaCO_3$ still present.

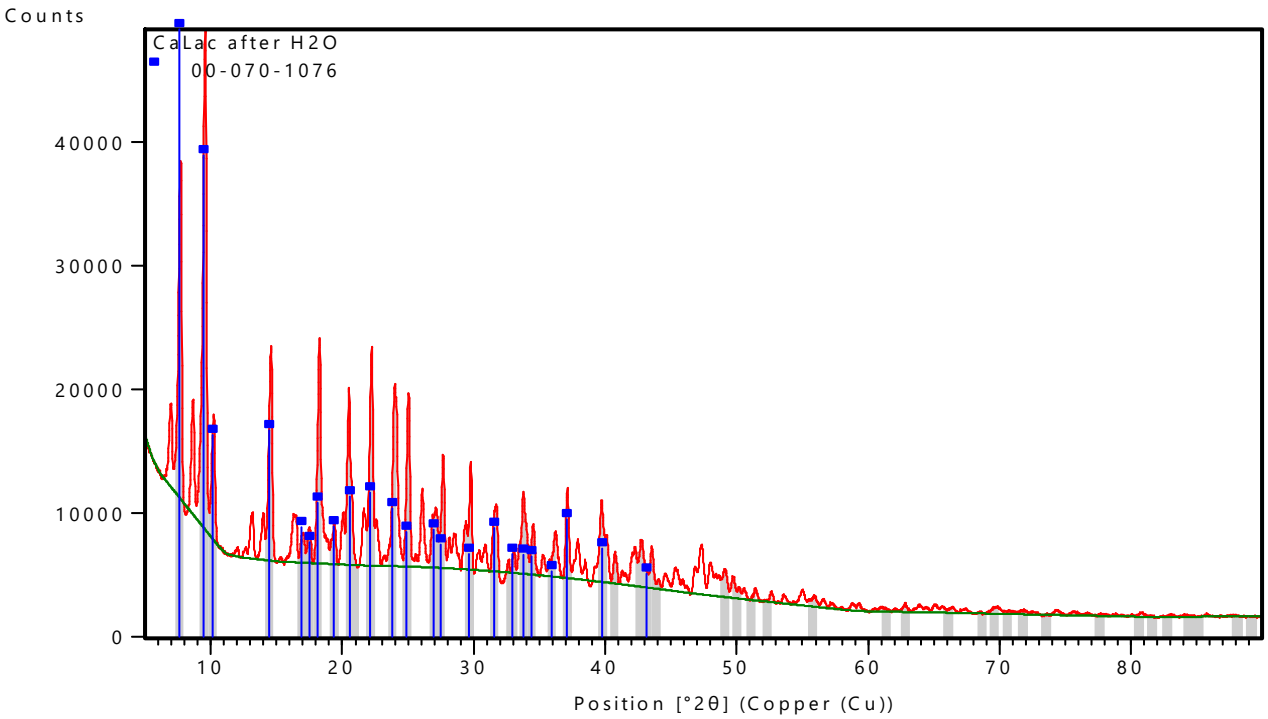


Figure 17. XRD diffractogram of calcium lactate. The red line is the diffractogram that was measured with the XRD and the blue lines are the peaks that the software could match to the database with ref code 00-070-1076 after hydrating the sample.

4.1.5 Tri-calcium phosphate – $Ca_3(PO_4)_2$ synthesis

The synthesis of TCP was not successful according to the method described in section 3.1.5. After the synthesis the material was measured using the XRD. The XRD diffraction pattern could be matched to that of calcium phosphate hydroxide with a score of 59, see figure 20. TCP is a very specific synthesis where a lot of factors can influence the synthesis. Guo et al describes these factors influencing the reaction. The temperature and pH are very important factors in this synthesis and just a small shift can result in a different material. It can be tried to split up the synthesis steps rather than do all the steps in the round bottom flask. After the reaction of $CaCO_3$ with the nitric acid a sample should be measured on the XRD to see if the correct intermediate is synthesized. In the final step the pH should be measured and be controlled with the addition of the NaOH to keep the pH at 9.

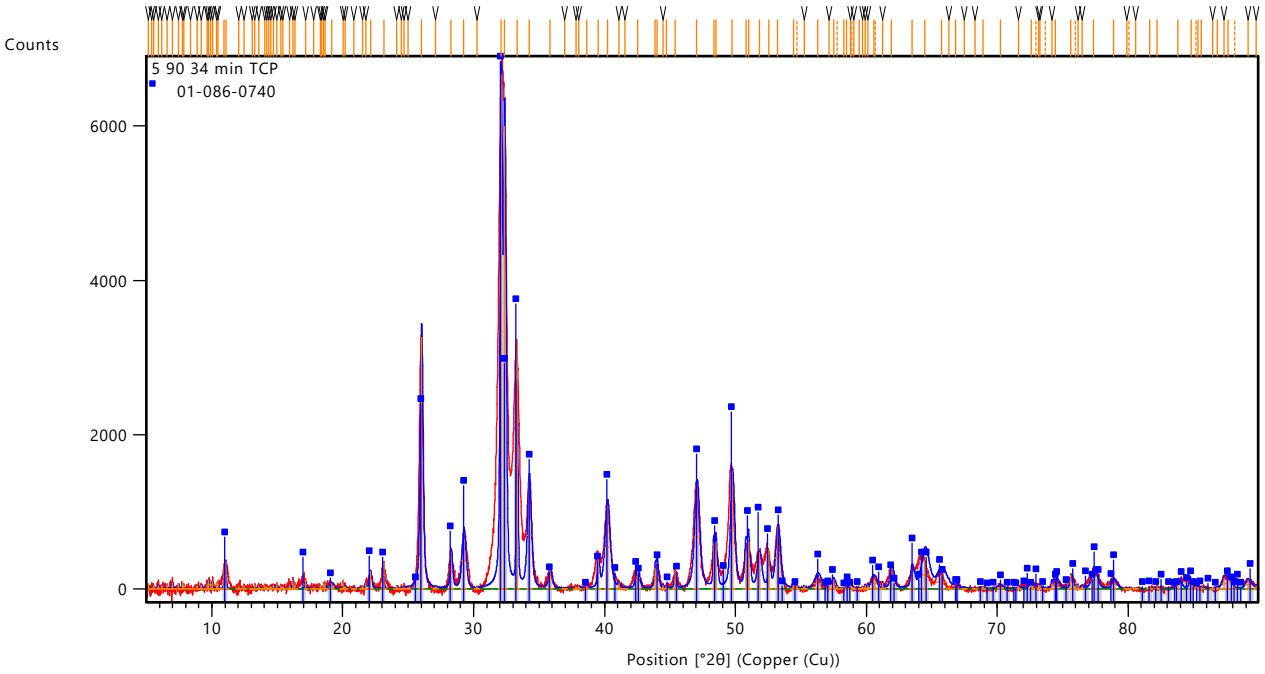


Figure 18. Figure 19. XRD diffractogram of calcium di nitrate. The red line is the diffractogram that was measured with the XRD and the blue lines are the peaks that the software could match to the database with ref code 00-86-0740.

4.2 Unfortified milk

To determine the distribution of the calcium present in bovine milk, two techniques can be used. These techniques were adapted from the protocol as described by de Vos [15].

4.2.1 Comparing dialysis with ultracentrifuge

Unfortified skimmed milk was separated with both dialysis and ultracentrifugation to obtain the percentage of calcium in the soluble phase. For dialysis 39.5±4.3% and for ultracentrifuge 36.0±0.9% of the total calcium was found in the soluble phase, see figure 22. These results were determined using the ICP. This is line with what was expected as a result. De Vos obtained a similar result with approximately 33% for both of the methods. Yamauchi et al. and Gaucheron et al. both reported a percentage of approximately 31.5% for the calcium present in the soluble phase in milk [18], [60]. Which are a bit lower compared to the results from this thesis. This could be due to the difference in brand of milk used or the external conditions, e.g. temperature in the lab.

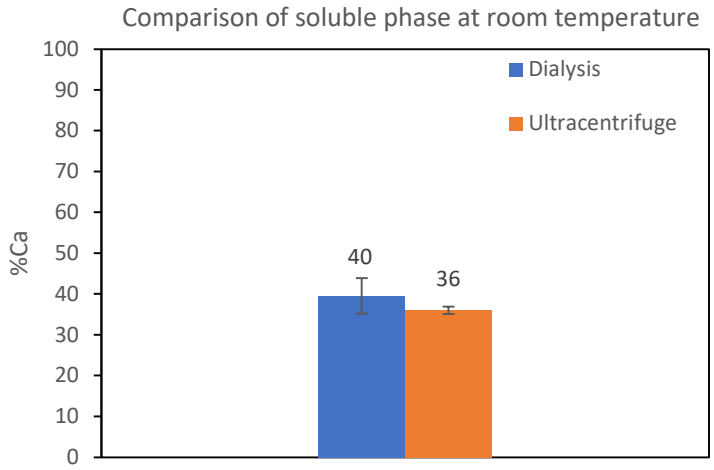


Figure 20. A comparison of the dialysis and ultracentrifuge method at room temperature used to determine calcium in the soluble phase of unfortified skimmed milk.

In figure 22 it is shown that both the dialysis and ultracentrifugation method yield similar results. This was verified by using a Welch's t-test where it was found that the two methods are not significantly different with a two tailed 95% confidence interval. This is also confirmed by literature [45]. Dialysis yields a slightly higher result compared to the ultrafiltration. However it also has a larger standard deviation. The experiments were performed in *triplo*, however one of the dialysis samples contained a very large deviation and was not included in the result. The deviation was due to coagulation of the sample because of high lab temperatures in the summer. The first sample measured a value of 739 for the concentration outside the membrane. The second and third sample measured 1316 and 1239 for the concentration. Although it is possible to do a standard deviation with only two samples, the error bar is not very meaningful. By using such a small sample size, the error bar is very wide. Other small deviations could be caused by incidental errors like pipetting errors. The results are also similar to the results obtained by de Vos and therefore it was concluded that dialysis and ultracentrifugation can be used to yield the same result [15]. For the rest of experiments the ultracentrifugation method was chosen as the preferred method for phase separation. This was due to the fact that with ultracentrifugation more information about the samples can be obtained and less sample is needed. Furthermore ultracentrifugation is faster than dialysis. Ultracentrifugation takes half a day while dialysis need a day and a half. The dialysis samples were stored in the fume hood with lab temperatures ranging from 18°C to 27°C in the summertime. This gave rise to different results when preforming the same experiment. With ultracentrifugation the %Ca can be determined in the micelle, protein and soluble phase. With dialysis only the %Ca in the soluble phase and protein + micelles can be determined. However the ultracentrifugation method created more waste and is more expensive.

The same experiment was also repeated at 37°C to simulate the temperature in the human body, see figure 23. For the dialysis method the samples were heated to 37°C for 24 hours so that an equilibrium could be reached. However heating the milk for such a long period of time resulted in coagulation of the milk. The results were 37.3±4.2% and are comparable to dialysis at room temperature. It could be that the sample already equilibrated before the coagulation started. For higher temperatures the equilibrium time is shortened. On-nom et al. found that for 40°C the equilibrium time was around 5 hours [61]. For the ultracentrifugation the milk was heated to 37°C and the ultracentrifuge and centrifuge machines were set at 37°C and resulted in a calcium content of 35.3±2.2%. There is almost no difference when comparing with the results at room temperature. For these reasons the rest of the experiments were carried out at room temperature.

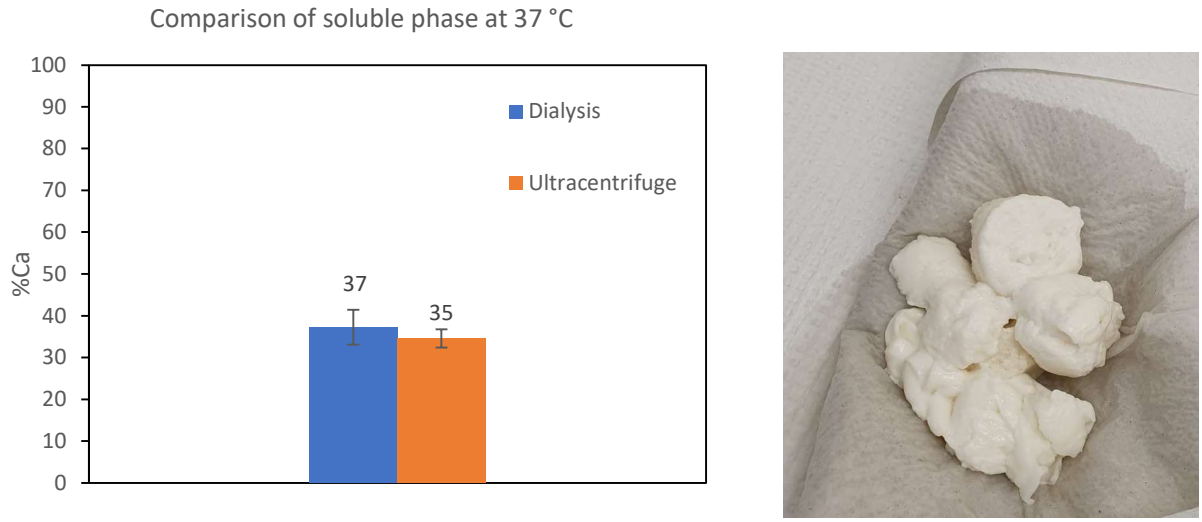


Figure 21. Left: A comparison of the dialysis and ultracentrifuge method at 37 °C used to determine calcium in the soluble phase of unfortified skimmed milk. Right: After heating the milk for 24 hours. The milk coagulated and became cheese like during dialysis.

4.2.2 Effect of pH on the calcium distribution

Skimmed milk normally has a pH of around 6.70. The pH of the milk was lowered by using 1 M HCl to a pH of 6, 4 and 2. Lowering the pH causes coagulation due to the isoelectric point of the casein micelles that lies around pH 4.6 [62]. That is why it was important to keep the temperature very low and stir the milk while adding the HCl. At room temperature the milk coagulated immediately upon adding HCl and the milk shifted, see figure 24.

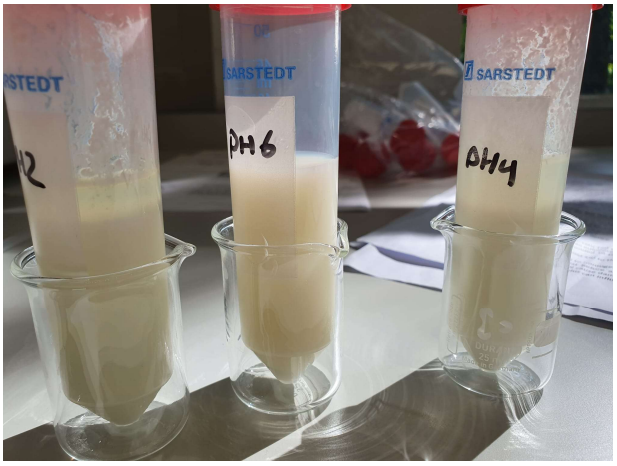


Figure 22. Shifted milk after adding HCl 30% at room temperature.

When decreasing the pH of the milk, more Ca becomes bioavailable as can been seen in figure 25. The Ca mostly comes from the casein micelles due to dissociation of the Ca from the micelles [63]. Acidifying the milk decreases the mol fraction bound to the micelles as reported by Holt et al. [64]. Under normal condition there is steric hinderance from the κ-casein layer together with electrostatic effects which prevent the casein micelles from coagulating. When the pH is decreased the net charge of the micelles also decreases. By lowering the pH protonation of the phosphates occurs and this causes the CCP to dissolve and more Ca^{2+} that came from the CCP to move to the soluble phase [65]. The Ca in the soluble phase increases from 55.1±2.7% to 97.0±3.3% in the range of pH 6 to pH 2 and a decrease in the Ca

content in the micelles can be seen from 38.3±4.1% to -0.2±1.3%. In the serum protein a slight decrease can be seen from 7.6±3.7% to 3.2±2.6%. However taking the error bars into account this difference is not significant.

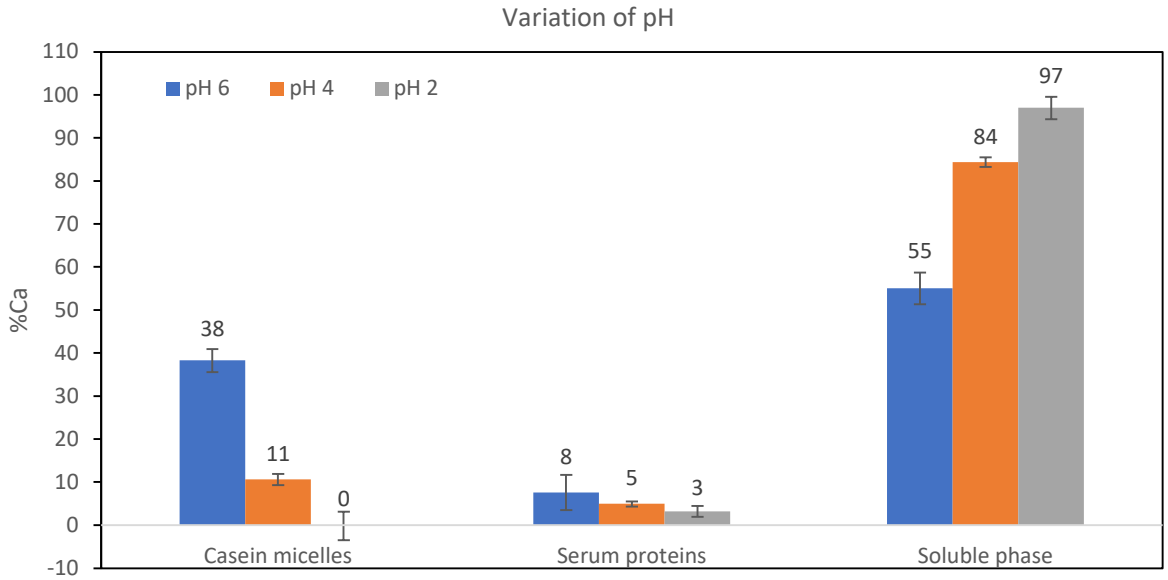


Figure 23. Phase separation upon decreasing the pH. The lower the pH of the milk, the more calcium becomes bioavailable due to dissociation of Ca from the casein micelles.

4.2.3 Tracer method

Liquid Scintillation Counting

The LSC was used in combination with the ultracentrifugation phase separation method to measure the ^{45}Ca present in each phase. During LSC measurements ^{45}Ca can be absorbed on the glass walls of the counting vials [66]. Due to the absorption of ^{45}Ca, less activity is detected. Hence the counting vials were rinsed with a non-radioactive $CaCl_2$ solution. The pre-rinsing causes the non-radioactive Ca to bind to the surface of the vials and after this the samples can be added without absorption of the ^{45}Ca. It was also necessary to rinse the vials with MilliQ after the $CaCl_2$, otherwise the samples would be very foamy and the top part of the vial would be 'dirty' and small little bubbles would be still present in the vial, see figure 26.This heavily influenced the LSC measurements by giving back results which were not expected when comparing it to the results from de Vos and had a lot of outliers [15].

Figure 24. The 'dirty' LSC vials without rinsing the excess non-radioactive $CaCl_2$ with MilliQ.

Before conducting the experiments with ^{45}Ca, the optimal count rate needed to perform the LSC measurements was determined, see figure 27. The count rates measured where then compared to the results of de Vos to estimate the activity added [15]. It was therefore determined to add 100 µL of the 370 Bq source to the milk, which corresponds to ~20 Bq.

Figure 27 also shows that a linear relation between the net count rate and the added volume of ^{45}Ca was obtained with a $R^2 = 0.999$ and thus resulting in no correction for the efficiency of measuring different activities had to be made.

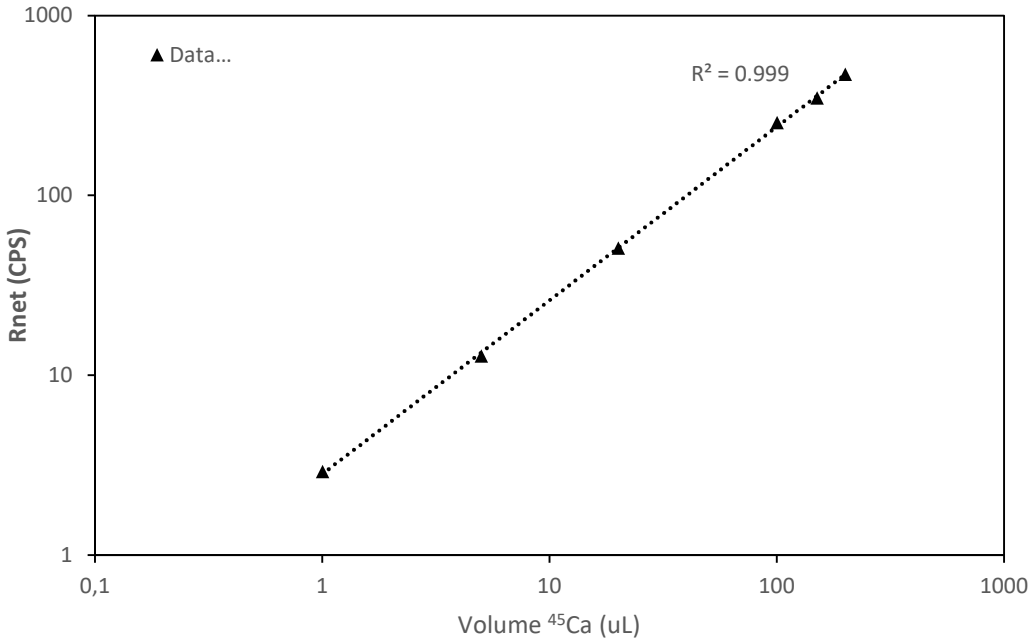
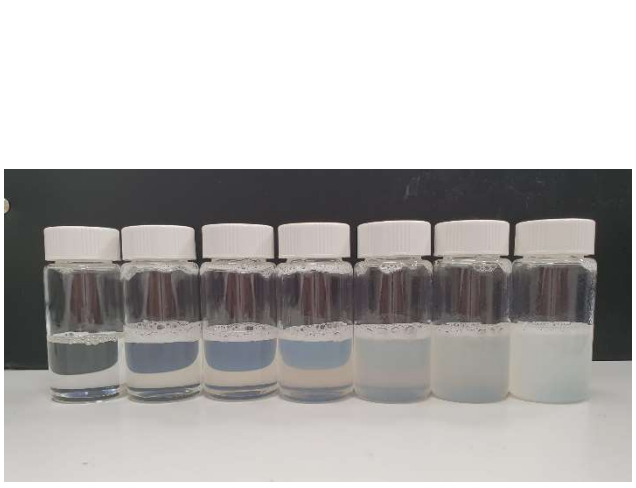


Figure 25.Volume of added ^{45}Ca (µL) plotted against the net count rate (cps) to determine the optimal count rate following the ALARA principle and still staying above three times the background rate.

Due to the fact that the sample contains milk, the effects of colour quenching were also determined. Because of colour quenching less activity can be measured by the LSC. The amount of added milk should be as minimal as possible. In figure 28 the visual effect of adding milk to the LSC cocktail can easily be seen. To make this quenching curve, different amounts of milk ranging from 0.05 mL to 3.5 mL was added to 10 mL of LSC cocktail containing a fixed amount of activity. Between 0 and 1 mL the net count rate is more or less the same. Only at 1 mL the net count rate was much higher and could be due to a pipetting error or an error in the measurement of the LSC device. Otherwise an exponential decrease can be observed. It was decided to add 0.2 mL of milk to all of the samples to ensure that enough sample from the filtration step could be added and if something went wrong during pipetting the sample not everything would be lost. From the filtration step usually around 0.4 mL of sample was obtained.



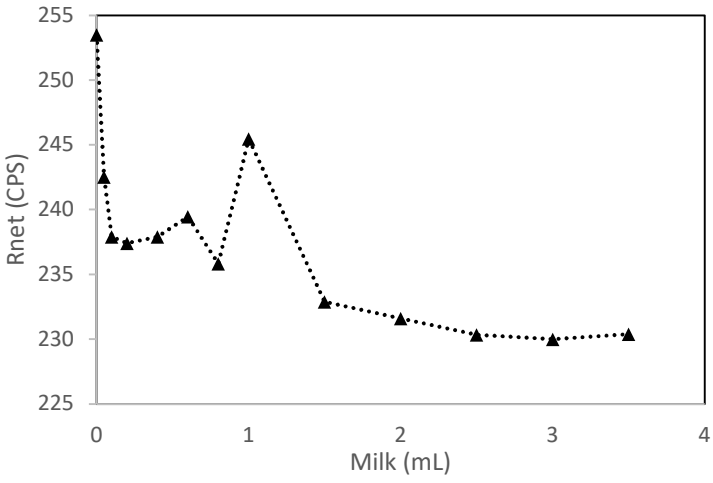


Figure 26. Left: The visual effect of quenching. Right: The quenching curve. Different amounts of milk with the same activity were measured on the LSC.

Phase separation using [^{45}Ca]$CaCl_2$ as a tracer

For the phase separation using [^{45}Ca]$CaCl_2$ as a tracer the activity was added to 30 mL milk and was left in the fridge to equilibrate for 24 hours. As seen in figure 29, the results of the non-radioactive Ca and the radioactive Ca are comparable. The radioactive Ca yields a slightly higher result of 41.10±0.45% because of hard to exchange (HTE) Ca being present in the casein micelles [45]. This can been seen as the result for the serum proteins is the same. So the HTE Ca must be present in the micelles. These HTE Ca can hardly exchange with the Ca and added ^{45}Ca in the soluble phase of the milk. Therefore the result of the soluble phase for the radioactive Ca is slightly higher. The result of the radioactive Ca is the base to which the fortified milk samples were compared to. When comparing the radioactive method with the non-radioactive method it is shown that the results are significantly different by using a Welch's t-test. Furthermore the casein micelles fraction is 60.3±3.0% and 53.1±1.2% for the non-radioactive Ca and radioactive Ca respectively. The serum proteins phase is 3.7±2.9% and 5.4±0.82%.

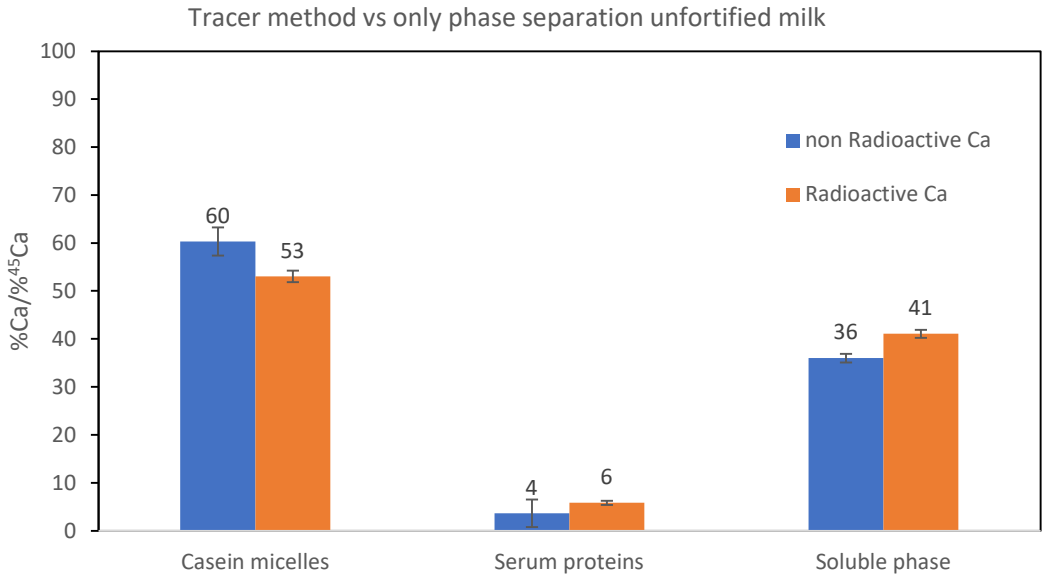


Figure 27. Phase separation using ^{45}Ca as tracer compared to the non radioactive method using the concentration measured by the ICP.

4.2 Fortified milk

Milk was fortified using the different Ca salts that were synthesized by adding 50 mg Ca/ 100 mL milk and using ultracentrifugation for phase separation. The LSC was used to determine the relative ^{45}Ca content in each phase.

4.2.1 Fortification with [^{45}Ca]$CaCl_2$

Milk fortification with [^{45}Ca]$CaCl_2$ resulted in a similar phase distribution as the unfortified milk when only a tracer was used. The soluble phase contains 42.8±0.6% of ^{45}Ca, see figure 30. The soluble phase increased slightly in its calcium content by fortification with [^{45}Ca]$CaCl_2$. This is different from what was reported by Singh et al. where a decrease in the Ca absorption was reported from 50.0% to 43.5% when fortifying with $CaCl_2$ [19]. The difference in results could be due to the fact that Singh et al. performed an *in vivo* study in mice which can have many physiological factors influencing the results [33]. It is important to note that the relative Ca content in each of the phases is determined. The overall calcium concentration in each of the phases is increased/ decreased. Most likely is that the concentration of the Ca in the casein micelles are increased because when fortifying with soluble Ca salts, casein mineralisation is increased [67]. This increase in casein micelles occurs due supersaturation of the milk serum that contains calcium phosphate, calcium citrate and Ca^{2+} ions by adding the $CaCl_2$. The calcium phosphate will bind to the casein proteins in the serum and micelles that causes the increase in the casein mineralisation.

It was reported that fortification with [^{45}Ca]$CaCl_2$ is not ideal because it gives the milk a slightly salty taste [19]. Nevertheless, it is a popular Ca salt used for fortification due to its high solubility.

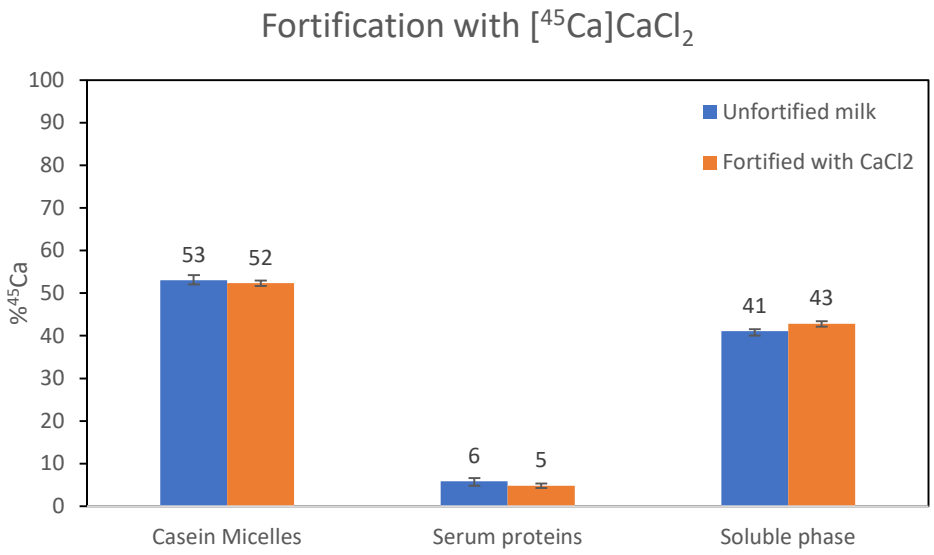


Figure 28. Milk fortification with [^{45}Ca]$CaCl_2$ and the relative calcium content in each phase compared to unfortified milk.

4.2.2 Fortification with [^{45}Ca]$CaCO_3$

The fortification with [^{45}Ca]$CaCO_3$ initially resulted in no exchange at all. After equilibrating in the fridge for 24 h, the samples were first centrifuged to remove sedimentation from the sample and subsequently no ^{45}Ca activity remained in the milk sample as measured by the LSC. This can be explained by the poor solubility in aqueous solutions of $CaCO_3$ see figure 31. Therefore the pH of the milk samples was lowered to pH 6, 4 and 2. This is similar to the gastric conditions described in chapter 1. The stomach has a pH of around 1-2 then when the chyme moves further down the digestive tract, the pH is slowly raised to an almost neutral pH. Therefore it is interesting to look at the lower pH conditions for $CaCO_3$. The salt was added to the milk and then the pH was then lowered to the desired pH level.

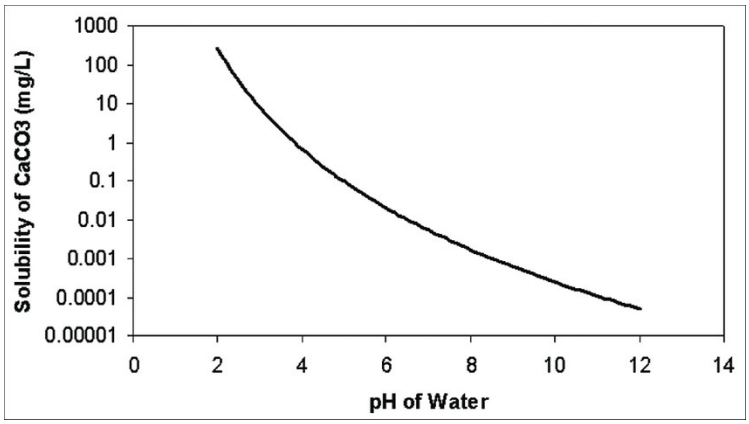


Figure 29. Solubility curve of $CaCO_3$ [68].

In figure 32 the results can be seen. The lower that the pH gets, the more Ca is present in the soluble phase with 2.1±0.2% at pH 6, 98.0±1.5% at pH 4 and 113.6±3.1% at pH 2. This is because the $CaCO_3$ will react with the protons present in an acidic environment and free Ca^{2+} becomes available. This free Ca^{2+} will then be present in the soluble phase and therefore increasing the bioavailability. The obtained results are higher than the results that were reported by Sittikulwitit et al. who simulated the gastrointestinal digestion conditions in their study by using pepsin-HCl to lower the pH to 2 [21]. For their experiments dialysis was used and a % Dialyzable Ca of 47.4±0.2 was reported. Due to acidifying the milk samples coagulation also occurred. After equilibrating in the fridge for 24h the sedimentation

remaining could not be centrifuged and this could result in a higher relative Ca in the other phases. This could be an explanation for the results obtained for pH 2. Due to not being able to centrifuge the samples the solid $CaCO_3$ might still be present or trapped in the coagulated milk. The ^{45}Ca could also not be evenly distributed throughout the sample. When measuring the ultracentrifugation phase a higher count rate was measured. This resulted in a soluble phase above the 100% and subsequently a casein micelles phase lower than 0% because it is relative to each other. The prevent this a stabilizing agent could be added so that the milk cannot coagulate.

Sittikulwitit et al. did not report for how long they dialysed the samples. This could explain the difference in results as the longer the $CaCO_3$ is in an acidic environment, the more it can dissociate.

Adding $CaCO_3$ for fortification to milk could impose some problems. For example as the salt is not soluble the milk cartons should have a label with shake before use otherwise all of the $CaCO_3$ but be at the bottom and the fortification would have no use. Furthermore adding $CaCO_3$ to milk leaves a chalky taste and is not favourable. It could be an option to add it to flavoured milk to combat the taste. $CaCO_3$ is also basic and this could raise the pH levels in the gastrointestinal tract and subsequently can lower the bioavailability or can cause the production of more acid in the stomach.

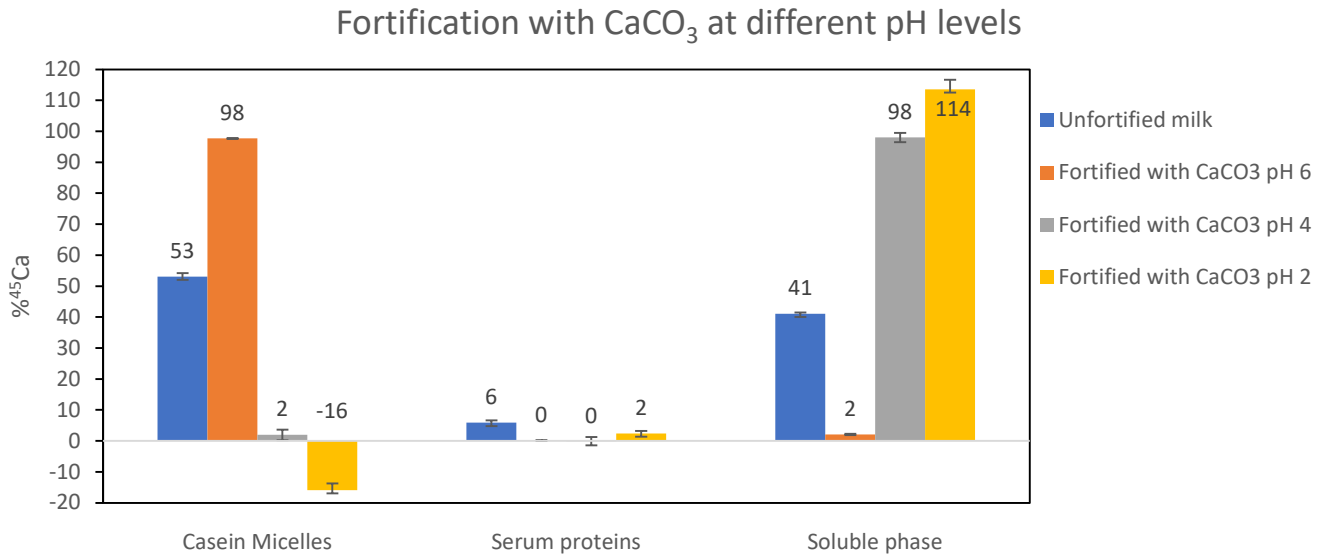


Figure 30. Milk fortification with $[^{45}Ca]CaCO_3$ at different pH levels and the relative calcium content in each phase compared to unfortified milk.

4.2.3 Fortification with $[^{45}Ca]Ca_3(C_6H_5O_7)_2$

Milk was also fortified by adding $[^{45}Ca]$CaCi to the milk samples. There was some exchange with a relative Ca content of 23.2±2.0% present in the soluble phase. Which is lower than the 47.6±1.1% which was reported by Sittikulwitit et al. and is a dialysability comparable to $CaCO_3$ in their study [21]. After centrifugation some of the CaCi was present as sedimentation at the bottom of the tube. The solubility of CaCi is relatively low in an aqueous environment. Lowering the pH will increase the solubility of the CaCi [69]. Sittikulwitit et al. performed the study under gastrointestinal conditions and therefore have a higher result for the fortification with CaCi. The same mechanism as for $CaCO_3$ when in an acidic environment is valid for CaCi. Due to the protons present in the acidic environment free Ca^{2+} becomes available and thus increases the relative Ca content in the soluble phase [70].

CaCi has a sour salty taste and that is also not an ideal taste to have in milk. With the sedimentation forming it has the same drawback as $CaCO_3$ when adding it to a milk carton. CaCi does not raise the pH of the stomach like $CaCO_3$ and could therefore be more favourable as a fortification method for people who do not cope well with increased acid production.

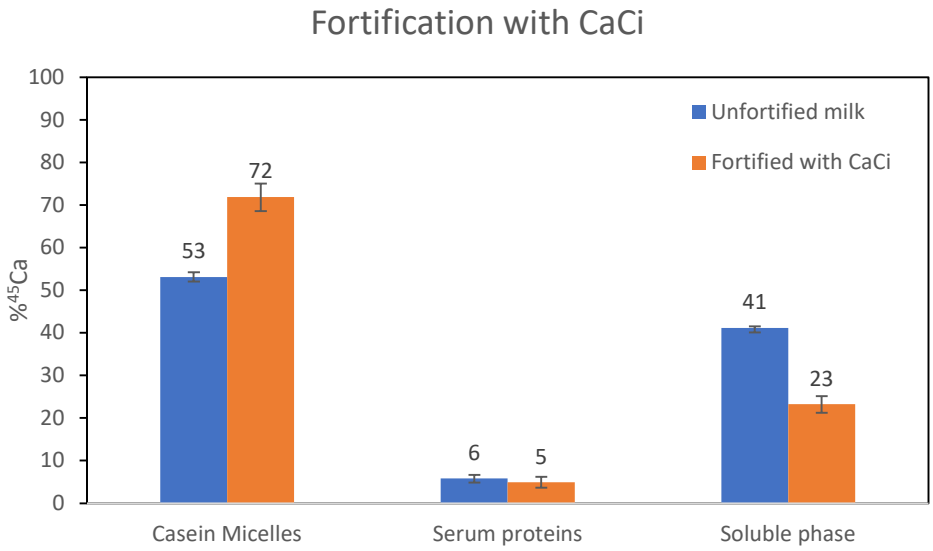


Figure 31. Milk fortification with [^{45}Ca]CaCi and the relative calcium content in each phase compared to unfortified milk.

4.2.3 Fortification with [^{45}Ca]$C_{12}H_{22}CaO_{14}$

Milk was also fortified using the soluble salt [^{45}Ca]CaGluc, see figure 34. The soluble phase contains 48.8±2.2% of the relative Ca. This is a slight increase from the unfortified milk samples. By itself CaGluc is an already highly soluble salt and this makes it an ideal candidate for milk fortification. Because of its high solubility, more free Ca^{2+} ions are present in the sample and those ions are needed so that the body can absorb the Ca [32]. The increased solubility is in accordance with the findings of Singh et al. who also reported an increase from 50.0% for unfortified milk to 57.3% [19]. It was also reported by Singh et al. that the addition of CaGluc causes a decrease in the pH of the sample and destabilizes the milk. The milk needs to be stabilized to ensure the heat stability by using chelating agents, e.g. disodium phosphate, or the pH needs to be corrected for. Otherwise the soluble Ca might react with the phosphates and proteins when heated during the production process and this will result in less bioavailable Ca [71].

CaGluc has a neutral taste and is therefore great to add to milk and because of its high solubility less sedimentation takes place. One of the drawbacks of CaGluc is the price to produce GaGluc [53]. It is higher than for example $CaCO_3$ which is quite inexpensive to produce. The Ca content of the organic salts that are added to milk are also lower and with the higher price tag to produce them might be unfavourable for companies who do not want to spend a lot of money to fortify their products.

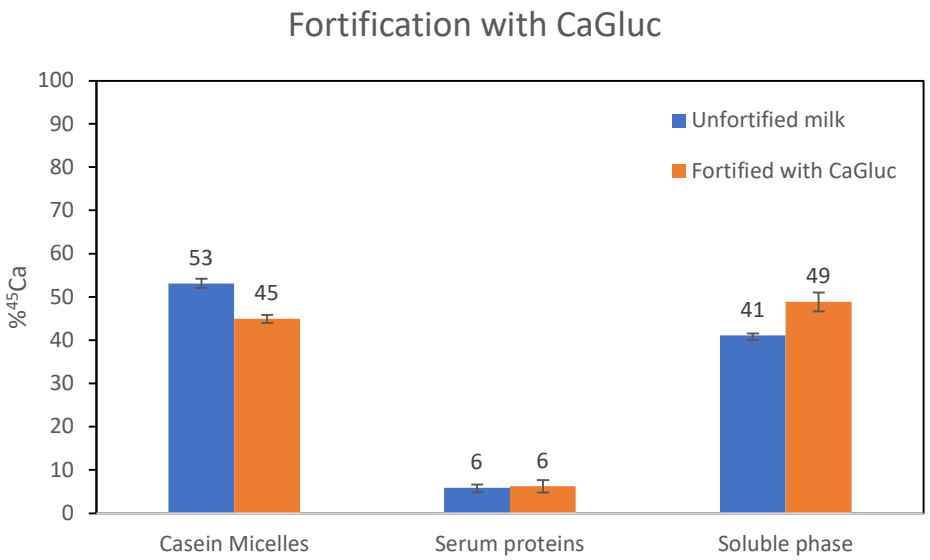


Figure 32. Milk fortification with [^{45}Ca]CaGluc and the relative calcium content in each phase compared to unfortified milk.

4.3 Comments on statistics

The samples were compared by determining the mean and the standard deviation of the results which is an appropriate method to calculated the uncertainty when having a low sample size N≤10 [72]. To compare if methods can be used interchangeably, a Welch's t-test was performed, see Appendix C for the calculations. A Welch's t-test can compare the mean of two independent groups. When using a Welch's t-test it is assumed that the population follows a normal distribution and that takes into account that the two populations have a different variance.

To reduce pipetting errors the pipettes used were first calibrated before each use. The experiments performed were false *triplo's.* For the unfortified milk and pH experiments one milk sample was created and from this three samples were taken for ultracentrifugation and the subsequent steps. To perform a valid *triplo* experiment, three initial milk samples should be created and from these samples the fractionation should be calculated and then compared to each other.

For the fortified milk samples the experiment was conducted using valid *triplo's* by having three initial milk samples fortified separately for each experiment.

5 Conclusion & Recommendation

5.1 Conclusions

During this thesis project the bioavailability of calcium in milk was assessed by using two methods of phase separation: dialysis and ultracentrifugation. The bioavailability was determined at 21°C and 37°C, for different pH levels, various calcium salts were synthesized and these salts were used to fortify bovine skimmed milk.

The methods to synthesize $CaCO_3$, CaCi, CaGluc and CaLac were found to be successful by using $CaCl_2$ as starting material. The synthesis of TCP was regrettably unsuccessful and requires further investigation.

By performing the phase separation using dialysis and ultracentrifugation the results for the bioavailability of calcium was confirmed with the findings from literature [14], [19], [21], [60]. Using dialysis 39.5±4.3% of calcium is present in the soluble phase was present and for ultracentrifugation 36.0±0.9% was present. For the ultracentrifugation the calcium content in two other phases could be determined. 3.7±2.9% is present in the serum proteins phase and 60.3±3.0% is present in the casein micelles. Furthermore it was determined that dialysis and ultracentrifugation can be used interchangeably by comparing the results from this thesis to the results of de Vos [15]. The phase separation performed at 37°C raised some problems with coagulation during dialysis and the results are not reliable. The results from ultracentrifugation were comparable to ultracentrifugation at room temperature.

The effects of pH on the phase distribution of the calcium content was also studied. It was found that for a decrease in the pH of milk, more calcium was found in the soluble phase. The calcium content went up from 36.0±0.9% to 96.0±3.3% in the soluble phase. This indicated that more calcium might be bioavailable in the human body than was previously thought.

Fortification was also performed on milk. First a tracer was used to determine the phase separation. A higher calcium content was found in the soluble phase, 41.1±0.5%, due to not all of the ^{45}Ca exchanging with casein micelles and thus remaining as free ions in the soluble phase. Fortification with $[^{45}Ca]CaCl_2$ led to the bioavailability staying the same with 42.8±0.6% in the soluble phase. Fortification with $[^{45}Ca]CaCO_3$ initially did not yield any difference. However upon decreasing the pH to gastric conditions, the $CaCO_3$ dissociated at pH 4 and lower and the ^{45}Ca was present in its ionic form in the soluble phase and therefore increased the bioavailability. For fortification with CaCi most of the salt precipitated to the bottom and the measured bioavailability was 23.2±2.0%. CaCi was not investigated under gastric conditions which could influence the bioavailability like it did with the $CaCO_3$. Lastly, fortification with CaGluc resulted in an increase of the bioavailability with 48.9±2.2%.

In conclusion a lot of steps were made towards the fortification of bovine skimmed milk during this thesis with a lot of promising results. A better understanding of the influence of salt fortification is obtained, however there are still some pieces left to the puzzle and some recommendations are made to further help the investigation into salt fortification of milk with calcium. For this thesis it can be concluded that the best way to fortify milk is to use CaGluc. It is highly soluble, increases the bioavailability and does not leave a bad aftertaste.

5.2 Recommendations

- The TCP salt synthesis should be further investigated. It is a very difficult synthesis that is very sensitive to the pH and temperature. As it is a twostep synthesis, the intermediate step should be checked with the XRD if the $Ca(NO_3)_2$ was synthesized correctly. Other possible synthesis methods could be investigated, e.g. by Vellet-Reggi et al. [73].

- The phase distribution of calcium with fortification of CaLac should be investigated. Due to time constraints this was not performed during this thesis. If the synthesis of TPC does not work, then the fortification could be performed with readymade TPC. TCP is mentioned in literature as a good candidate for calcium salt fortification [21].

- All of the calcium salt fortifications should also be investigated under gastric conditions. The $CaCO_3$ fortifications proved to have a positive result by doing this and it makes for a more representative *in vitro* study. The pH in the digestive tract ranges from pH 2 to almost a neutral pH. So not only lowering the pH of the skimmed milk could be interesting but also increasing it again to investigate the effect on the salt equilibrium.

- To obtain an optimal experiment, the time that is needed for the Ca and added ^{45}Ca to reach an equilibrium should be investigated. Currently it is set at 24 hours, however a shorter equilibrium time might also be possible.

- After fortification of milk, the pH and therefore the heat stability of the milk is also changed [19]. It should be further investigated what the effect of adding calcium salts to milk is on the heat stability. This could be an important factor if the milk should be processed further before consumption.

Bibliography

[1] P. Pravina, D. Sayaji, and M. Avinash, "Calcium and its Role in Human Body," *Int. J. Res. Pharm. Biomed. Sci.*, vol. 4, no. 2, Accessed: Jun. 10, 2022. [Online]. Available: www.ijrpbsonline.com.

[2] Harvard, "Calcium | The Nutrition Source | Harvard T.H. Chan School of Public Health," *Harvard School of Public Health*, 2022. https://www.hsph.harvard.edu/nutritionsource/calcium/ (accessed Jul. 04, 2022).

[3] M. Almeida, "Aging mechanisms in bone," *Bonekey Rep.*, vol. 1, no. 7, 2012, doi: 10.1038/bonekey.2012.102.

[4] B. J. Campbell, "Healthy Bones at Every Age - OrthoInfo - AAOS," *American Academy of Orthopaedic Surgeons*, 2021. https://orthoinfo.aaos.org/en/staying-healthy/healthy-bones-at-every-age/ (accessed Jul. 04, 2022).

[5] J. A. Beto, "The Role of Calcium in Human Aging," *Clin. Nutr. Res.*, vol. 4, no. 1, pp. 1–8, Jan. 2015, doi: 10.7762/CNR.2015.4.1.1.

[6] Ministerie van volksgezondheid en zorg, "Osteoporose | Leeftijd en geslacht," 2020. https://www.vzinfo.nl/osteoporose/leeftijd-en-geslacht (accessed Jul. 04, 2022).

[7] "Feiten & Cijfers – Osteoporose." https://osteoporose.medischonline.nl/wat-is-osteoporose/feiten-cijfers/ (accessed Jul. 04, 2022).

[8] Voedingscentrum, "Calcium." https://www.voedingscentrum.nl/encyclopedie/calcium.aspx).

[9] U. N. Patwardhan, D. N. Pahuja, and A. M. Samuel, "Calcium bioavailability: An in vivo assessment," *Nutr. Res.*, vol. 21, no. 4, pp. 667–675, 2001, doi: 10.1016/S0271-5317(01)00278-0.

[10] E. M. Balk *et al.*, "Global dietary calcium intake among adults: a systematic review," doi: 10.1007/s00198-017-4230-x.

[11] R. Nicolau-Nos, J. Pujol-Andreu, and I. Hernández, "Milk, social acceptance of a new food in Europe: Catalonia, 19th-20th centuries," *Dynamis*, vol. 30, 2010, Accessed: Jul. 05, 2022. [Online]. Available: https://scielo.isciii.es/scielo.php?script=sci_arttext&pid=S0211-95362010000100005.

[12] R. R. Reeker, A. Bammi, J. Barger-Lux, and R. P. Heaney, "Calcium absorbability from milk products, an imitation milk, and calcium carbonate13," 1988. [Online]. Available: https://academic.oup.com/ajcn/article-abstract/47/1/93/4694854.

[13] A. Haug, A. T. Høstmark, and O. M. Harstad, "Bovine milk in human nutrition - A review," *Lipids in Health and Disease*, vol. 6. BioMed Central, p. 25, 2007, doi: 10.1186/1476-511X-6-25.

[14] E. López-Huertas *et al.*, "Absorption of calcium from milks enriched with fructo-oligosaccharides, caseinophosphopeptides, tricalcium phosphate, and milk solids 1-3," 2006, Accessed: Jul. 05, 2022. [Online]. Available: https://academic.oup.com/ajcn/article/83/2/310/4649990.

[15] M. E. De Vos, "Calcium Bioavailability and Exchangeability in Milk," *Master thesis*, 2021.

[16] Z. A. Abdel-Salam, I. I. Abdel-Mageed, M. A. Harith, and S. A. M. Abdel-Salam, "Evaluation of proteins in sheep colostrum via laser-induced breakdown spectroscopy and multivariate analysis," *J. Adv. Res.*, vol. 15, pp. 19–25, Jan. 2019, doi: 10.1016/J.JARE.2018.07.001.

[17] D. T. Davies and J. C. D. White, "The use of ultrafiltration and dialysis in isolating the aqueous phase of milk and in determining the partition of milk constituents between the aqueous and disperse phases," *J. Dairy Res*, vol. 27, p. 171, 2029, doi: 10.1017/S0022029900010256.

[18] K. Yamauchi and Y. Yoneda, "Effect of Some Treatments of Milk on the Exchangeability of Colloidal Calcium in Milk with Soluble Calcium•õ," 1977.

[19] G. Singh, S. Arora, G. S. Sharma, J. S. Sindhu, V. K. Kansal, and R. B. Sangwan, “Heat stability and calcium bioavailability of calcium-fortified milk,” *LWT - Food Sci. Technol.*, vol. 40, no. 4, pp. 625–631, May 2007, doi: 10.1016/J.LWT.2006.03.009.

[20] M. J. Roig, A. Alegría, R. Barberá, R. Farré, and M. J. Lagarda, “Calcium dialysability as an estimation of bioavailability in human milk, cow milk and infant formulas,” *Food Chem.*, vol. 64, no. 3, pp. 403–409, Feb. 1999, doi: 10.1016/S0308-8146(98)00151-4.

[21] S. Sittikulwitit, P. P. Sirichakwal, P. Puwastien, V. Chavasit, and P. Sungpuag, “In vitro bioavailability of calcium from chicken bone extract powder and its fortified products,” *J. Food Compos. Anal.*, vol. 17, no. 3–4, pp. 321–329, Jun. 2004, doi: 10.1016/J.JFCA.2004.03.023.

[22] “WebElements Periodic Table » Calcium » reactions of elements.” https://www.webelements.com/calcium/chemistry.html (accessed Jun. 10, 2022).

[23] “Livechart - Table of Nuclides - Nuclear structure and decay data.” https://www-nds.iaea.org/relnsd/vcharthtml/VChartHTML.html (accessed Jun. 10, 2022).

[24] C. R. Ponte Ponge-Ferreira, M. Fallone Koskinas, and M. Da Silva Dias, “Standardization of Ca-45 radioactive solution by tracing method,” *Brazilian J. Phys.*, vol. 34, no. 3 A, pp. 939–941, 2004, doi: 10.1590/S0103-97332004000500062.

[25] “Practical radiation protection.”

[26] Blauson.com staff, “Medical gallery of Blausen Medical,” *WikiJournal Med.*, vol. 1, no. 2, 2014, doi: 10.15347/wjm/2014.010.

[27] F. Bronner, “Issues and Opinions Calcium Absorption-A Paradigm for Mineral Absorption 1.” [Online]. Available: https://academic.oup.com/jn/article/128/5/917/4722394.

[28] F. Pu, N. Chen, and S. Xue, “Calcium intake, calcium homeostasis and health,” *Food Sci. Hum. Wellness*, vol. 5, no. 1, pp. 8–16, Mar. 2016, doi: 10.1016/J.FSHW.2016.01.001.

[29] A. de Zawadzki, X. C. Liu, L. M. Ahrné, and L. H. Skibsted, “Increasing calcium phosphate aqueous solubility and spontaneous supersaturation combining citrate and gluconate with perspectives for functional foods,” *Food Chem.*, vol. 374, Apr. 2022, doi: 10.1016/j.foodchem.2021.131701.

[30] F. Bronner and D. Pansu, “Recent Advances in Nutritional Science Nutritional Aspects of Calcium,” *J. Nutr.*, vol. 129, no. 1, pp. 9–12, 1999, [Online]. Available: https://academic.oup.com/jn/article/129/1/9/4723248.

[31] C. S. Marcus and F. W. Lengemann, “Absorption of Ca45 and Sr85 from solid and liquid food at various levels of the alimentary tract of the rat,” *J. Nutr.*, vol. 77, no. 2, pp. 155–160, Jun. 1962, doi: 10.1093/jn/77.2.155.

[32] F. Bronner and D. Pansu, “Nutritional Aspects of Calcium Absorption,” *J. Nutr.*, vol. 129, no. 1, pp. 9–12, Jan. 1999, doi: 10.1093/JN/129.1.9.

[33] A. V. Pérez, G. Picotto, A. R. Carpentieri, M. A. Rivoira, M. E. Peralta López, and N. G. Tolosa De Talamoni, “Minireview on Regulation of Intestinal Calcium Absorption,” *Digestion*, vol. 77, no. 1, pp. 22–34, 2008, doi: 10.1159/000116623.

[34] F. Masotti, S. Cattaneo, M. Stuknytė, V. Pica, and I. De Noni, “Analytical advances in the determination of calcium in bovine milk, dairy products and milk-based infant formulas,” *Trends Food Sci. Technol.*, vol. 103, pp. 348–360, Sep. 2020, doi: 10.1016/J.TIFS.2020.07.013.

[35] G.-B. Fransson and B. Lönnerdal, “Distribution of trace elements and minerals in human and cow’s milk,” 1983.

[36] A. R. Madureira, C. I. Pereira, A. M. P. Gomes, M. E. Pintado, and F. Xavier Malcata, “Bovine whey proteins - Overview on their main biological properties,” *Food Research International*, vol. 40, no. 10. pp. 1197–1211, Dec. 2007, doi: 10.1016/j.foodres.2007.07.005.

[37] S. L. Turgeon and G. Brisson, “Symposium review: The dairy matrix—Bioaccessibility and bioavailability of nutrients and physiological effects,” *Journal of Dairy Science*, vol. 103, no. 7. pp. 6727–6736, 2020, doi: 10.3168/jds.2019-17308.

[38] H. Lindmark Månsson, “Fatty acids in bovine milk fat,” *Food Nutr. Res.*, vol. 52, 2008, doi: 10.3402/fnr.v52i0.1821.

[39] C. G. De Kruif, T. Huppertz, V. S. Urban, and A. V. Petukhov, “Casein micelles and their internal structure,” *Advances in Colloid and Interface Science*, vol. 171–172. pp. 36–52, Mar. 2012, doi: 10.1016/j.cis.2012.01.002.

[40] D. G. Dalgleish, “On the structural models of bovine casein micelles - Review and possible improvements,” *Soft Matter*, vol. 7, no. 6. pp. 2265–2272, 2011, doi: 10.1039/c0sm00806k.

[41] D. S. Horne, “Casein interactions: Casting light on the black boxes, the structure in dairy products,” *Int. Dairy J.*, vol. 8, no. 3, pp. 171–177, Mar. 1998, doi: 10.1016/S0958-6946(98)00040-5.

[42] C. Holt, C. G. De Kruif, R. Tuinier, and P. A. Timmins, “Substructure of bovine casein micelles by small-angle X-ray and neutron scattering,” *Colloids Surfaces A Physicochem. Eng. Asp.*, vol. 213, no. 2–3, pp. 275–284, Feb. 2003, doi: 10.1016/S0927-7757(02)00520-4.

[43] E. Bijl, T. Huppertz, H. van Valenberg, and C. Holt, “A quantitative model of the bovine casein micelle: ion equilibria and calcium phosphate sequestration by individual caseins in bovine milk,” *Eur. Biophys. J.*, vol. 48, no. 1, pp. 45–59, 2019, doi: 10.1007/s00249-018-1330-2.

[44] R. L. Walzem, C. J. Dillard, and J. B. German, “Whey components: Millennia of evolution create functionalities for mammalian nutrition: What we know and what we may be overlooking,” *Crit. Rev. Food Sci. Nutr.*, vol. 42, no. 4, pp. 353–375, 2002, doi: 10.1080/10408690290825574.

[45] K. Yamauchi, Y. Yoneda, Y. Kola, and T. Tsugo, “Exchangeability of Colloidal Calcium in Milk with Soluble Calcium,” 1969.

[46] C. Grochowski *et al.*, “Analysis of trace elements in human brain: Its aim, methods, and concentration levels,” *Frontiers in Chemistry*, vol. 7, no. MAR. Frontiers Media S.A., 2019, doi: 10.3389/fchem.2019.00115.

[47] “ICP-MS Systems and Technologies _ Thermo Fisher Scientific - ZA,” Accessed: Jun. 20, 2022. [Online]. Available: https://www.thermofisher.com/nl/en/home/industrial/spectroscopy-elemental-isotope-analysis/spectroscopy-elemental-isotope-analysis-learning-center/trace-elemental-analysis-tea-information/icp-oes-information/icp-oes-system-technologies.html.

[48] C. B. Boss and K. Freeden, “Concepts,Instrumentation and Techniques in Inductively Coupled Plasma Optical Emission Spectroscopy,” *Emiss. Spectrosc.*, pp. 1–22, 2004.

[49] M. A. Rutzke, “Atomic absorption, inductively coupled plasma optical emission spectroscopy, and infrared spectroscopy,” in *Encyclopedia of Earth Sciences Series*, Springer, Cham, 2018, pp. 76–83.

[50] A. Chapon, G. Pigrée, V. Putmans, and G. Rogel, “Optimization of liquid scintillation measurements applied to smears and aqueous samples collected in industrial environments,” *Results Phys.*, vol. 6, pp. 50–58, Jan. 2016, doi: 10.1016/j.rinp.2016.01.018.

[51] national diagnostics laboratory staff, “Principles and Applications of Liquid Scintillation Counting A PRIMER FOR ORIENTATION –,” *Natl. Diagnostics USA*, no. Natl. Diagnostics USA, pp. 1–15, 2004.

[52] “Quench, Counting Efficiency, and Quench Correction | Application Support Knowledgebase | Lab Products & Services | PerkinElmer.” https://www.perkinelmer.com/nl/lab-products-and-services/application-support-knowledgebase/radiometric/quench.html#Quenchcountingefficiencyandquenchcorrection-InternalStandards (accessed Jun. 25, 2022).

[53] Zolotoyabko Emil, *Basic Concepts of X-Ray Diffraction*. Haifa: Wiley-VCH, 2014.

[54] “X-ray diffraction (XRD) | Rigaku Global Website.” https://www.rigaku.com/fr/node/713 (accessed Jun. 27, 2022).

[55] A. Chauhan, “Powder XRD Technique and its Applications in Science and Technology,” *J. Anal. Bioanal. Tech.*, vol. 5, no. 6, p. 5, 2014, doi: 10.4172/2155-9872.1000212.

[56] C. F. Holder and R. E. Schaak, “Tutorial on Powder X-ray Diffraction for Characterizing Nanoscale Materials,” *ACS Nano*, vol. 13, no. 7. pp. 7359–7365, 2019, doi: 10.1021/acsnano.9b05157.

[57] J. Li, Y. Liu, Y. Gao, L. Zhong, Q. Zou, and X. Lai, “Preparation and properties of calcium citrate nanosheets for bone graft substitute,” *Bioengineered*, vol. 7, no. 5, pp. 376–381, Sep. 2016, doi: 10.1080/21655979.2016.1226656.

[58] Y. Sakata, S. Shiraishi, and M. Otsuka, “Characterization of dehydration and hydration behavior of calcium lactate pentahydrate and its anhydrate,” *Colloids Surfaces B Biointerfaces*, vol. 46, no. 3, pp. 135–141, Dec. 2005, doi: 10.1016/J.COLSURFB.2005.10.004.

[59] G. F. Tansman, P. S. Kindstedt, and J. M. Hughes, “Powder X-ray diffraction can differentiate between enantiomeric variants of calcium lactate pentahydrate crystal in cheese,” *J. Dairy Sci.*, vol. 97, no. 12, pp. 7354–7362, Dec. 2014, doi: 10.3168/JDS.2014-8277.

[60] F. Gaucheron, “The minerals of milk,” *Reprod. Nutr. Dev*, vol. 45, pp. 473–483, 2005, doi: 10.1051/rnd:2005030.

[61] N. On-Nom, A. S. Grandison, and M. J. Lewis, “Measurement of ionic calcium, pH, and soluble divalent cations in milk at high temperature,” *J. Dairy Sci.*, vol. 93, pp. 515–523, doi: 10.3168/jds.2009-2634.

[62] M. Asaduzzaman, M. S. Mahomud, and M. E. Haque, “Heat-Induced Interaction of Milk Proteins: Impact on Yoghurt Structure,” 2021, doi: 10.1155/2021/5569917.

[63] D. G. Dalgleish and A. J. R. Law, “pH-Induced dissociation of bovine casein micelles II. Mineral solubilization and its relation to casein release,” *J. Dairy Res.*, vol. 56, pp. 727–735, 2029, doi: 10.1017/S0022029900029290.

[64] C. Holt, “An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk,” doi: 10.1007/s00249-003-0377-9.

[65] A. Gonzalez-Jordan, P. Thomar, T. Nicolai, and J. Dittmer, “The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution,” *Food Hydrocoll.*, vol. 51, pp. 88–94, Oct. 2015, doi: 10.1016/J.FOODHYD.2015.04.024.

[66] L. Rodriguez Barquero, J. M. Los Arcos, A. Grau Malonda, and E. Garcia-Toraño, “LSC standardization of 45Ca by the CIEMAT/NIST efficiency tracing method,” *Nucl. Inst. Methods Phys. Res. A*, vol. 339, no. 1–2, pp. 6–13, Jan. 1994, doi: 10.1016/0168-9002(94)91770-1.

[67] T. Huppertz and C. Timmer, “Salt equilibria in nutritional formulae for infants and young children,” *Int. Dairy J.*, vol. 110, Nov. 2020, doi: 10.1016/J.IDAIRYJ.2020.104805.

[68] P. W. Hart, G. W. Colson, and J. Burris, “Application of Carbon Dioxide to reduce water side lime scale in heat exchanges,” no. February 2012, 2015.

[69] M. Boulet and J. R. Marier, “Solubility of Tricalcium Citrate in Solutions of Variable Ionic Strength and in Milk Ultrafiltrates,” *J. Dairy Sci.*, vol. 43, no. 2, pp. 155–164, 1960, doi: 10.3168/JDS.S0022-0302(60)90138-7.

[70] W. J. Wiley, “XCIV. THE DISSOCIATION OF CALCIUM CITRATE,” May 1930.

[71] M. Münchbach and G. Gerstner, “Calcium Fortification in Dairy Products,” *Jungbunzlauer*, 2010.

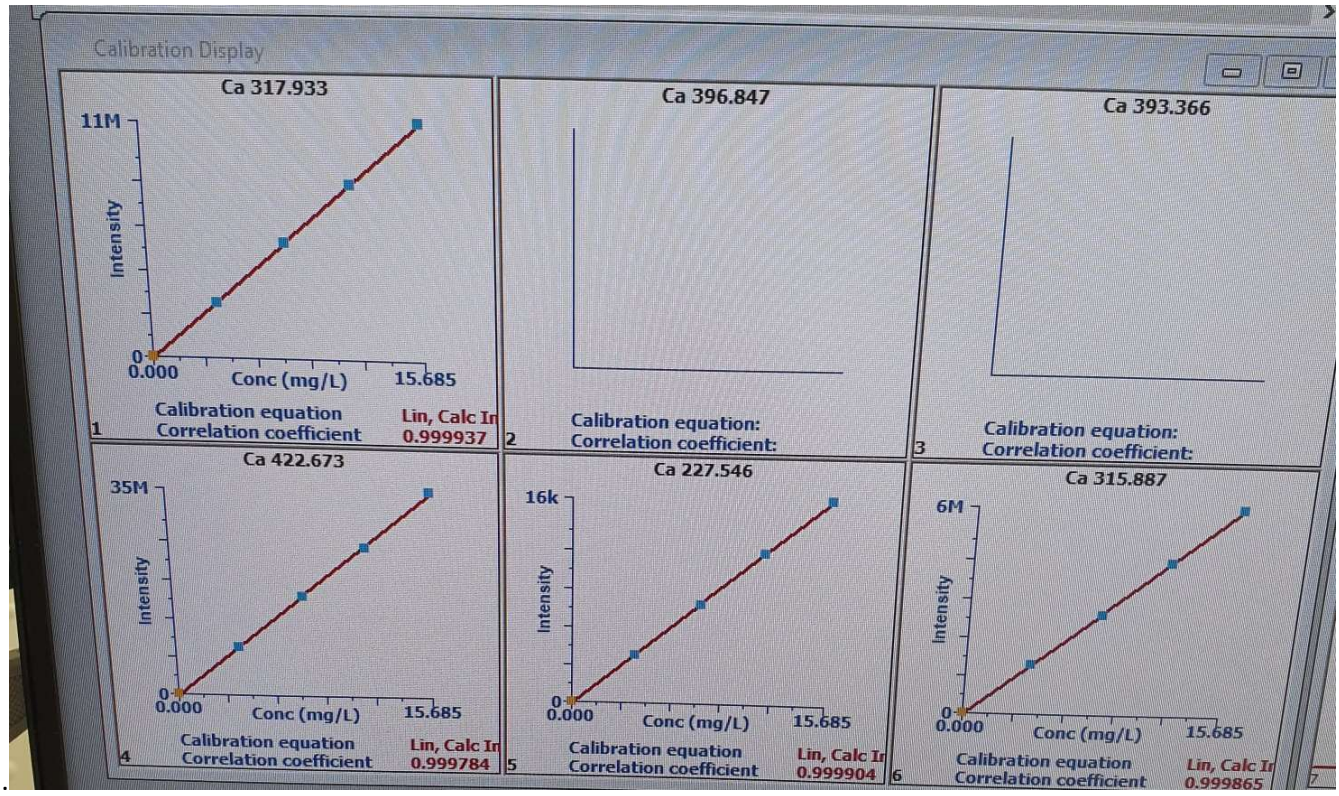
[72] I. G. Hughes and T. P. A. Hase, *Measurements and their uncertainties*. Oxford universtity press.

[73] M. Vallet-Regí and J. M. González-Calbet, “Calcium phosphates as substitution of bone tissues,” *Prog. Solid State Chem.*, vol. 32, no. 1–2, pp. 1–31, 2004, doi: 10.1016/J.PROGSOLIDSTCHEM.2004.07.001.

Appendix

A1. ICP Calibration Lines 4000

A2. ICP Calibration Lines 8000



B1. XRD $Ca(OH)_2$

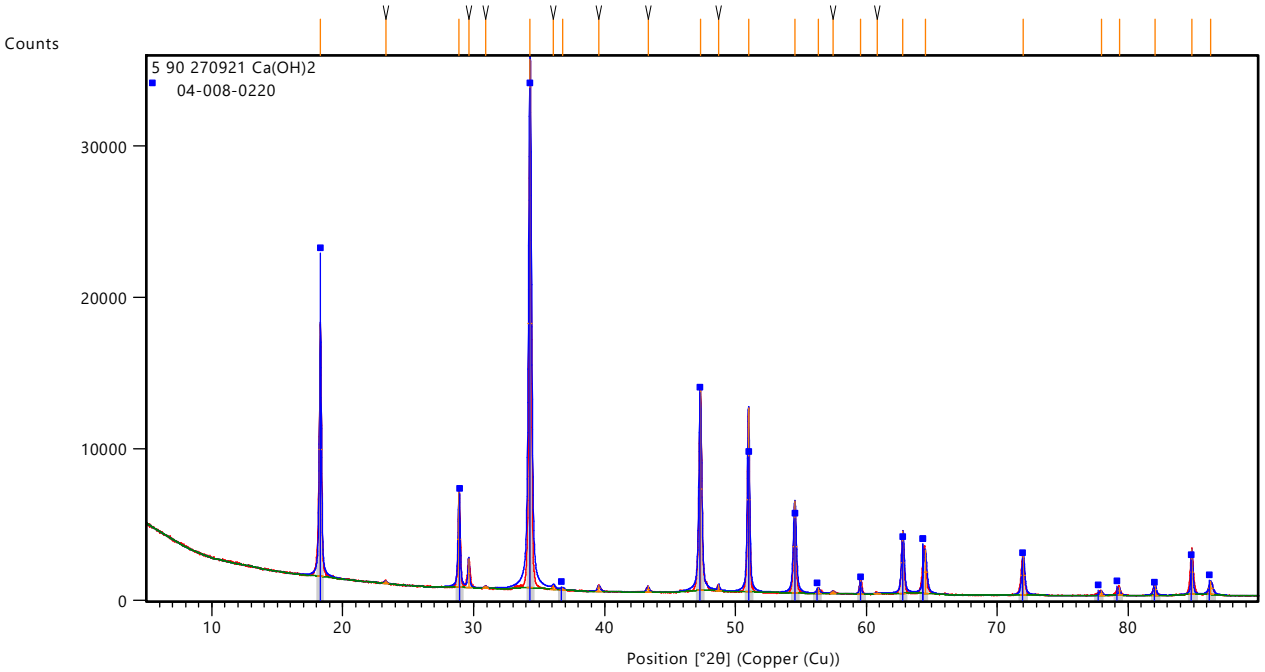


Figure Appendix 1. XRD diffractogram of the calcium di hydroxide. The red line is the diffractogram that was measured with the XRD and the blue lines are the peak that the software could match to the database with ref code 04-008-0220. A match score of 83 was obtained.

B2. XRD CaLac precipitate

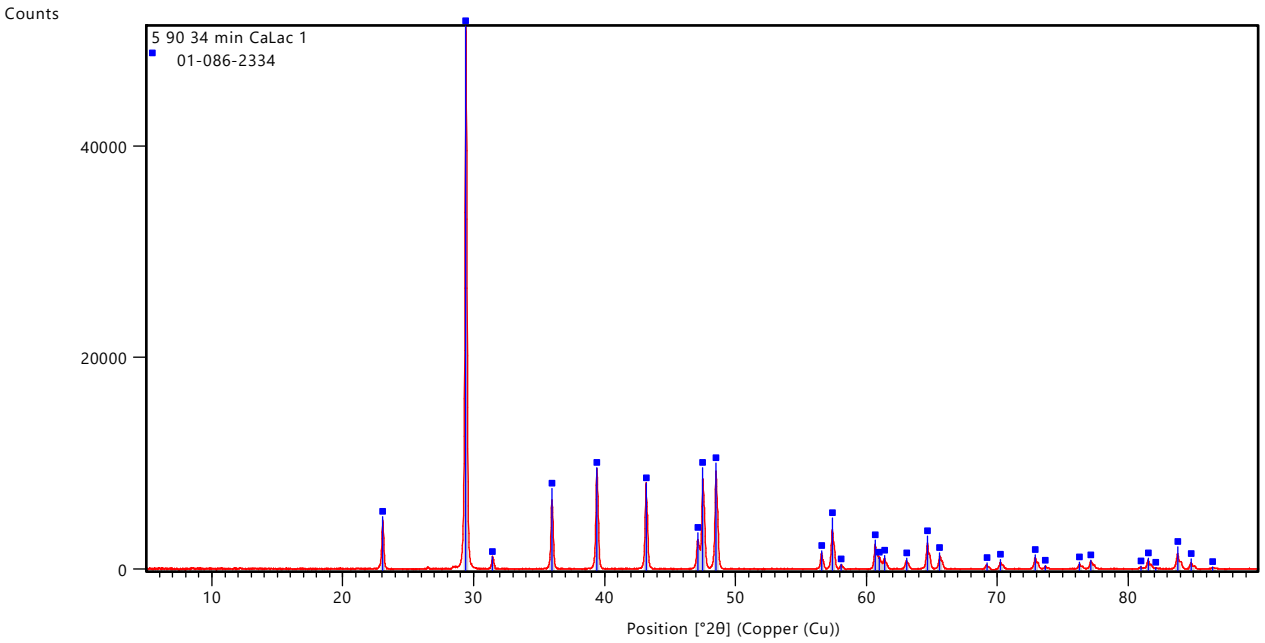


Figure Appendix 2. XRD diffractogram of the calcium lactate synthesis. The red line is the diffractogram that was measured with the XRD and the blue lines are the peak that the software could match to the database with ref code 01-086-2334. A match score of 99 was obtained for a comparison with $CaCO_3$.

B3. XRD CaLac after freeze drying

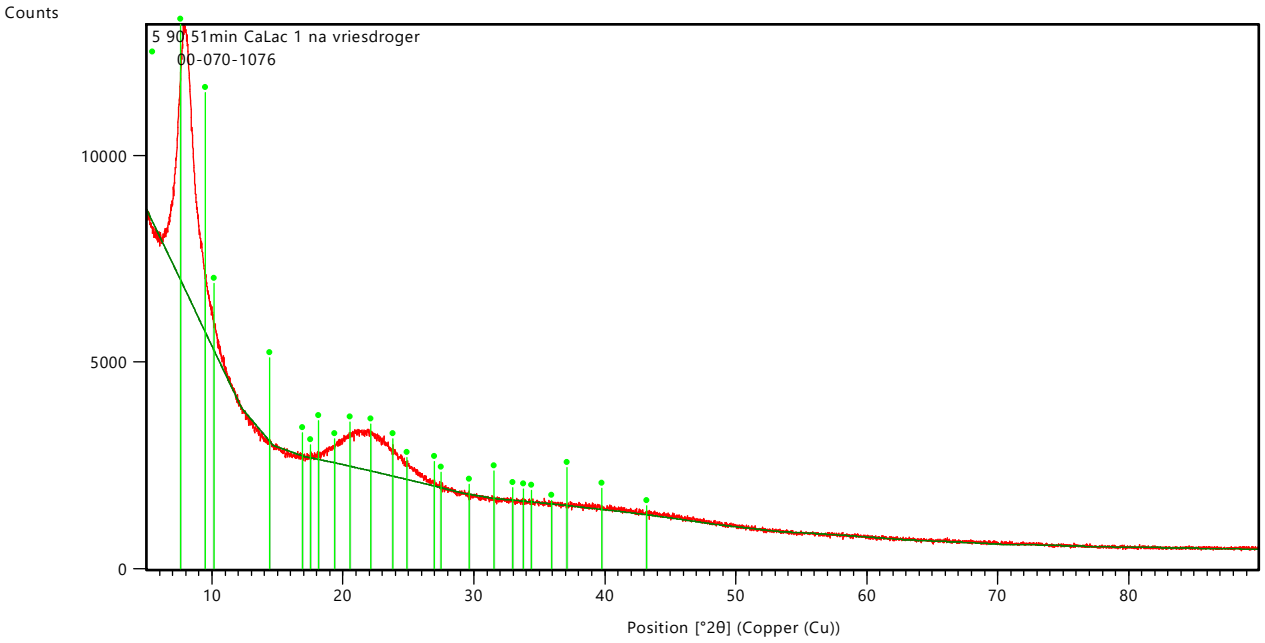


Figure Appendix 3. XRD diffractogram of calcium lactate. The red line is the diffractogram that was measured with the XRD and the green lines are the peaks that the software could match to the database with ref code 00-070-1076. This red line shown is characteristic for an amorphous sample and cannot be measured on the XRD.

B4. XRD CaLac after hydration remaining peaks

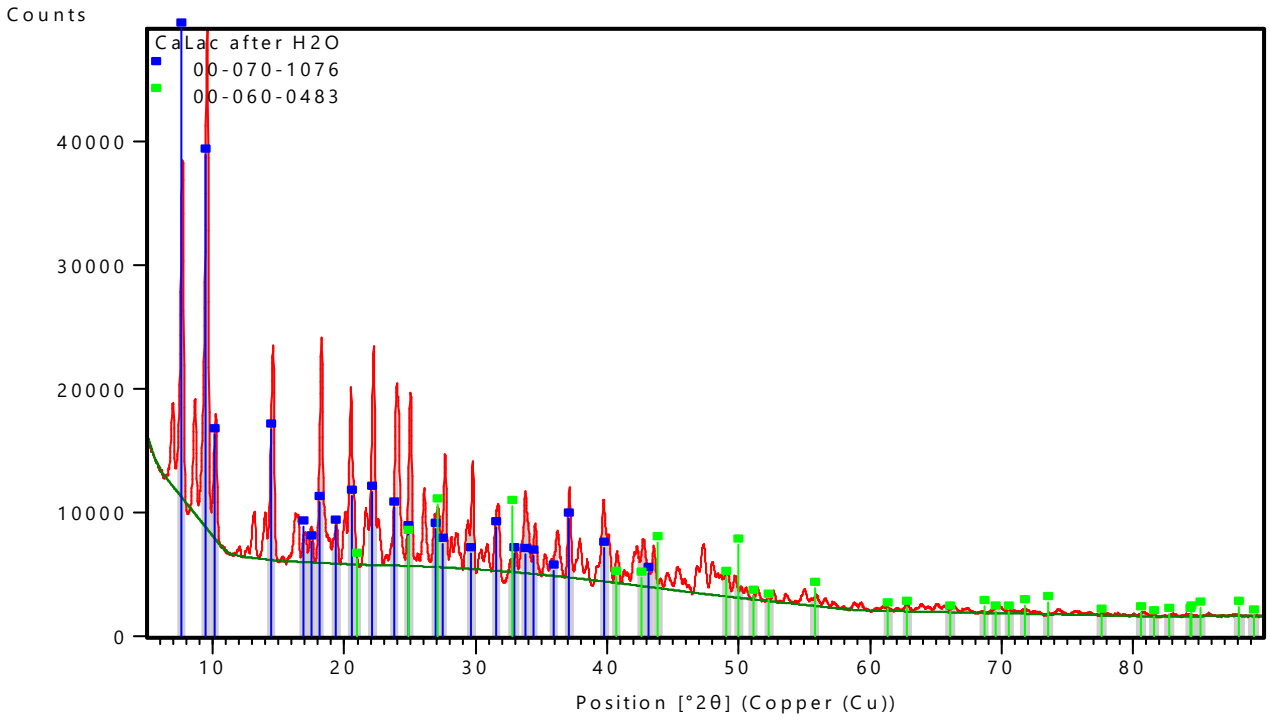


Figure Appendix 4. XRD diffractogram of calcium lactate. The red line is the diffractogram that was measured with the XRD and the blue lines are the peaks that the software could match to the database with ref code 00-070-1076 after hydrating the sample. There were still a lot of undefined peaks left and those can be assigned to calciumcarbonate. However the score was 79 so it was deemed successful.

C. Statistics

H0: ultracentrifugation (μ1) = dialysis (μ2)
H1: ultracentrifugation (μ1) ≠ dialysis (μ2)

μ1= 36.02
μ2= 39.54
s1= 0.89
s2= 4.32
N1 = 3
N2 = 3

$$t = \frac{\mu 1 - \mu 2}{\sqrt{\frac{s1^2}{N1} + \frac{s2^2}{N2}}} = 2.67$$

$$Degree\ of\ Freedom = \frac{(\frac{s1^2}{N1} + \frac{s2^2}{N2})^2}{\frac{(s1^2)^2}{N1-1} + \frac{(s2^2)^2}{N2-1}} = 2.167 => DOF = 2$$

From table tcrit = 4.303 for two tails 95% confidence interval

t < tcrit so H0 and H1 are not significant different from each other

H0: ultracentrifugation 21°C (μ1) = ultracentrifugation 37°C (μ2)
H1: ultracentrifugation 21°C (μ1) ≠ ultracentrifugation 37°C (μ2)

μ1= 36.02
μ2= 41.10
s1= 0.89
s2= 0.45
N1 = 3
N2 = 3

$$t = \frac{\mu 1 - \mu 2}{\sqrt{\frac{s1^2}{N1} + \frac{s2^2}{N2}}} = -13.26$$

$$Degree\ of\ Freedom = \frac{(\frac{s1^2}{N1} + \frac{s2^2}{N2})^2}{\frac{(s1^2)^2}{N1-1} + \frac{(s2^2)^2}{N2-1}} = 2.167 => DOF = 2$$

From table tcrit = 4.303 for two tails 95% confidence interval

t > tcrit so H0 and H1 are significant different from each other

cum. prob	$t_{.50}$	$t_{.75}$	$t_{.80}$	$t_{.85}$	$t_{.90}$	$t_{.95}$	$t_{.975}$	$t_{.99}$	$t_{.995}$	$t_{.999}$	$t_{.9995}$
one-tail	**0.50**	**0.25**	**0.20**	**0.15**	**0.10**	**0.05**	**0.025**	**0.01**	**0.005**	**0.001**	**0.0005**
two-tails	**1.00**	**0.50**	**0.40**	**0.30**	**0.20**	**0.10**	**0.05**	**0.02**	**0.01**	**0.002**	**0.001**
df											
1	0.000	1.000	1.376	1.963	3.078	6.314	12.71	31.82	63.66	318.31	636.62
2	0.000	0.816	1.061	1.386	1.886	2.920	4.303	6.965	9.925	22.327	31.599
3	0.000	0.765	0.978	1.250	1.638	2.353	3.182	4.541	5.841	10.215	12.924
4	0.000	0.741	0.941	1.190	1.533	2.132	2.776	3.747	4.604	7.173	8.610
5	0.000	0.727	0.920	1.156	1.476	2.015	2.571	3.365	4.032	5.893	6.869
6	0.000	0.718	0.906	1.134	1.440	1.943	2.447	3.143	3.707	5.208	5.959
7	0.000	0.711	0.896	1.119	1.415	1.895	2.365	2.998	3.499	4.785	5.408
8	0.000	0.706	0.889	1.108	1.397	1.860	2.306	2.896	3.355	4.501	5.041
9	0.000	0.703	0.883	1.100	1.383	1.833	2.262	2.821	3.250	4.297	4.781
10	0.000	0.700	0.879	1.093	1.372	1.812	2.228	2.764	3.169	4.144	4.587
11	0.000	0.697	0.876	1.088	1.363	1.796	2.201	2.718	3.106	4.025	4.437
12	0.000	0.695	0.873	1.083	1.356	1.782	2.179	2.681	3.055	3.930	4.318
13	0.000	0.694	0.870	1.079	1.350	1.771	2.160	2.650	3.012	3.852	4.221
14	0.000	0.692	0.868	1.076	1.345	1.761	2.145	2.624	2.977	3.787	4.140
15	0.000	0.691	0.866	1.074	1.341	1.753	2.131	2.602	2.947	3.733	4.073
16	0.000	0.690	0.865	1.071	1.337	1.746	2.120	2.583	2.921	3.686	4.015
17	0.000	0.689	0.863	1.069	1.333	1.740	2.110	2.567	2.898	3.646	3.965
18	0.000	0.688	0.862	1.067	1.330	1.734	2.101	2.552	2.878	3.610	3.922
19	0.000	0.688	0.861	1.066	1.328	1.729	2.093	2.539	2.861	3.579	3.883
20	0.000	0.687	0.860	1.064	1.325	1.725	2.086	2.528	2.845	3.552	3.850
21	0.000	0.686	0.859	1.063	1.323	1.721	2.080	2.518	2.831	3.527	3.819
22	0.000	0.686	0.858	1.061	1.321	1.717	2.074	2.508	2.819	3.505	3.792
23	0.000	0.685	0.858	1.060	1.319	1.714	2.069	2.500	2.807	3.485	3.768
24	0.000	0.685	0.857	1.059	1.318	1.711	2.064	2.492	2.797	3.467	3.745
25	0.000	0.684	0.856	1.058	1.316	1.708	2.060	2.485	2.787	3.450	3.725
26	0.000	0.684	0.856	1.058	1.315	1.706	2.056	2.479	2.779	3.435	3.707
27	0.000	0.684	0.855	1.057	1.314	1.703	2.052	2.473	2.771	3.421	3.690
28	0.000	0.683	0.855	1.056	1.313	1.701	2.048	2.467	2.763	3.408	3.674
29	0.000	0.683	0.854	1.055	1.311	1.699	2.045	2.462	2.756	3.396	3.659
30	0.000	0.683	0.854	1.055	1.310	1.697	2.042	2.457	2.750	3.385	3.646
40	0.000	0.681	0.851	1.050	1.303	1.684	2.021	2.423	2.704	3.307	3.551
60	0.000	0.679	0.848	1.045	1.296	1.671	2.000	2.390	2.660	3.232	3.460
80	0.000	0.678	0.846	1.043	1.292	1.664	1.990	2.374	2.639	3.195	3.416
100	0.000	0.677	0.845	1.042	1.290	1.660	1.984	2.364	2.626	3.174	3.390
1000	0.000	0.675	0.842	1.037	1.282	1.646	1.962	2.330	2.581	3.098	3.300
z	0.000	0.674	0.842	1.036	1.282	1.645	1.960	2.326	2.576	3.090	3.291
	0%	50%	60%	70%	80%	90%	95%	98%	99%	99.8%	99.9%
	Confidence Level										