Delft University of Technology

The bioavailability of Calcium in Milk

Effect of Temperature

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1 Abstract

Calcium is an important mineral which the body uses to maintain strong bones and carry out many important bodily functions. According to the Dutch nutrition center people in the Netherlands consume 80-90% of their calcium through milk and other milk products. Often times people will further 'process' milk for other food products. Examples of these include boiling milk to make hot chocolate or freezing it for ice-cream. These temperature changes can have great influence on the bio-availability of the Calcium.

The goal of this thesis was to find the effect of temperature on the bio-availability of Calcium in milk. This was done through 2 experiments. Dialysing skim bovine milk at temperatures between 4-90°C and heating or cooling the bovine milk to temperatures between -18°C-95°C and then dialysing at room temperature. The dialysis separates the soluble and insoluble phase of milk allowing for the calculation of percentage soluble calcium which is a good indicator for bio-availability of calcium in milk.

It was found that there is a linear relationship between the temperature of the milk and the percentage soluble Calcium, the higher the temperature of the milk the lower the amount of bio-available calcium in the milk. Furthermore when looking at the heated milk it was found that heating to 60°C and above all showed a similar drop in percentage soluble calcium indicating some permanent loss of bio-available calcium. This permanent loss is however lower than what can be observed to be lost in the milk whilst hot indicating that a portion of the calcium that becomes insoluble upon heating returns to the soluble phase once returned to its original temperature.

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2 Abbreviations and acronyms

Abbreviation	
ICP-OES	inductively induced plasma optical emission spectrometry
IDF	International dairy federation
UHT	Ultra high temperature
LTLT	Low temperature long time
HTST	High temperature short time
OH	Over heated
β-LG	β-lactoglobulin
α-LA	α-lactalbumin
IG	immunoglobulins
BSA	bovine serum albumin
BLF	bovine lactoferrin
LP	lactorperoxidase
Ca	Calcium

3 Introduction

Calcium is an important mineral which the body uses to maintain strong bones and carry out many important bodily functions. It is needed for muscles to move, for nerves to carry messages, to help blood vessels move blood and help the releases of hormones and enzymes effecting almost every function in the human body [32].

According to the Dutch nutrition center people in the Netherlands consume 80-90 % of their calcium through milk and other milk products such as cheese, yoghurt and coffee milk for example [29]. Before a product such as milk lands on the shelf in a supermarket ready to be consumed it has undergone various temperature treatments to ensure it is safe to drink and lengthen shelf life, more on this in section 4.4 [19]. These treatments however do not just influence the microorganisms in the milk, they can also cause denaturing of proteins [47] [19], changes in the pH of milk [27] and overall just destabilize the milk [27]. All these different changes within the milk potentially influencing the bio-availability of the calcium in the milk. Since milk makes up such a large part of the calcium intake for the majority of people it is important to know the expanse of this influence.
Furthermore, milk is also often subjected to heat after it is bought by the consumer, such as boiling it to make hot chocolate or freezing it for ice-cream. This too can then have follow up effects as the milk is once more being subjected to a form of heat treatment.

There has been quite a bit of research done in the area of temperature and milk; Seiquer (2010) did a study comparing ultra high temperature (UHT) bovine milk and overheated (OH) bovine milk, the OH beeing 3 cycles of sterilization at 116°C for 16 min and the UHT being heating at 150°C for 6 seconds. A reduction in soluble calcium was observed in the OH milk compared to the UHT milk [47]. Sung-Ho Yoo (2013) did a study in Korea comparing raw milk with UHT, low temperature long time (LTLT) and high temperature short time (HTST) treated milk and found that the raw milk had the highest calcium bio-availability and LTLT, HTST and UHT following in decreasing order. The highest temperature therefore having the most effect on the calcium content [50]. On-Nom (2010) did a study where he measured the ionic calcium and the pH in milk at high temperature. Temperatures ranging from 20-80 °C and between 90 and 110°C were analysed and it was found that both the ionic calcium and the pH decreased at higher temperatures and that the relationship between ionic calcium and temperature was linear [33]. Burton (1984) also did a study into heat processes and claimed that a reduced soluble calcium content can be found in heated milk because the ionic calcium combined with the phosphates or the denatured proteins and turns into the colloidal calcium form while the calcium moves to the inside of casein micelles [5].
All of these papers point towards the same conclusion, that an increase in temperature means there is less soluble calcium and therefore the calcium is less bio-available. This is however not the case for all the research done in this area. Shandhu (1973) looked into the influence of different food processing on the mineral and vitamin contents of milk, he reported that the total calcium content in heat treated milk products does not change[63]. Furthermore Kitts (1991) too concluded that heat treatments do not adversely influence calcium bio-availability[22]. Lastly Weeks and King (1985) looked into The bio-availability of calcium in Heat-Processed Milk and compared the bio-availability of calcium in raw, HTST pasteurized, and stored UHT milks and found no significant effects were attributable to heat treatment[56]. From the two entirely different conclusions in these papers it is clear that temperature treatments applied to milk while already studied quite a bit are not yet fully understood, hence the following is to be researched:

What is the effect of temperature changes on the bio-availability of calcium in milk and to what extent is this effect permanent?

Having a better understanding of the effect of temperature on the bio-availability of Calcium in milk could pave the future for the way milk is processed. Changing this could then potentially lead to milk with a higher percentage of bio-available calcium without having to fortify the milk with anything. Furthermore having a better understanding of the effect of temperature could change the way people treat milk. If freezing for example has a negative effect it could stop people from storing it this way, or if boiling it causes a huge loss in calcium bio availability people would consider to be more careful with heating their milk. All in all gaining knowledge on the effect of temperature on the bio-availability of milk could increase the amount of Calcium people are able to gain from consuming milk.

3.1 Content Outline

In this thesis the effect of temperature will be investigated. This will be done through taking store bought skim milk and heating or cooling it to several temperatures. These temperatures lie in the range of -18°C up to 95°C as these are logical temperatures to which milk is brought in every day life. More details on these temperatures in section 4.5.
This report is structured as follows; section 4 the theoretical background is explained. The different experiments and materials used are explained in section 5. The results are presented and discussed in section 6 and finally the conclusion and recommendations are given in section 7.

4 Background Theory

4.1 Proteins in milk

Bovine milk has a protein content of 32 g/L[16]. There are two protein families which make up this content, the whey proteins and caseins, the caseins being the much more abundant proteins in bovine milk with around 80% being casein [16].

4.1.1 Whey proteins

Whey protein is the substance left over after the making of cheese. Whey proteins include β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulins (IG), bovine serum albumin (BSA), bovine lactoferrin (BLF), lactoperoxidase (LP) and various other minor components. [25].
All these proteins have their own function, such as β-LG is a transporter, able to bind retinol, palamitate, fatty acids, vitamin D and cholesterol[59] [40]. This binding of for example fatty acids facilitates the digestion of milk fat [34]and the binding of retinol enhances the uptake of vitamin A [46]. It is therefore a very important protein . β-LG is also the most abundant of the whey proteins in bovine milk [9].
α-LA, the second most abundant of the whey proteins, also has some important functions. It acts as a coenzyme for the synthesis of lactose [9]. Furthermore α-LA can contribute to reducing the risk of some cancers, as it can inhibit cell division[14]. α-LA also has the ability to bind to calcium and metal ions[55]. All the other less abundant whey proteins also have important functions, more information about these can be found in Madureira's paper on Bovine whey proteins [25].

4.1.2 Caseins

The caseins are made up of a mixture of 4 different phosphoproteins, α_{s1}-, α_{s2}-, β- and κ-casein's. These four phosphoproteins form colloidally dispersed particles with calcium phosphate called casein micelles [18].

α_{s1}-casein's are the main protein containing 8-10 seryl phosphate groups whilst β-casein contains about 5 phosphate groups [36]. Because these two casein's are highly phosphorylated they will precipitate with excess Ca^{2+} ions.[58] κ-caseins have only one phosphoserine group and are therefore stable in the presence of calcium ions, meaning they play an important role in stabilizing the casein micelle [53] [26].

There have been various models proposed for the structure of the casein micelle, these models fall into three categories; core-coat models, sub-micelle models and internal structure models[36]. There are two models which are most commonly accepted, within the sub-micelle category this is Walstra's model suggested in 1984 which is also considered the 'classic model'[45]. This model suggests casein micelles are built of spherical sub-micelles in the range of 12-15 nm, each cell containing 20-25 molecules. The sub-micelles are kept together by hydrophobic interactions between the proteins and by calcium phosphate linkages [53]. The sub-micelles are divided into two main types; one consisting of α_s-casein and β-caseins and the other type consisting of α_s-caseins and κ-caseins. The second type with the κ-caseins being the more hydrophilic due to the sugar residues on κ-caseins. As can be seen in figure 1 the κ-caseins are located on the outside of the micelle with their hydrophilic part protruding from the micelle surface forming a 'hairy' layer which will avoid further aggregation of the sub-micelles due to steric repulsions [36].

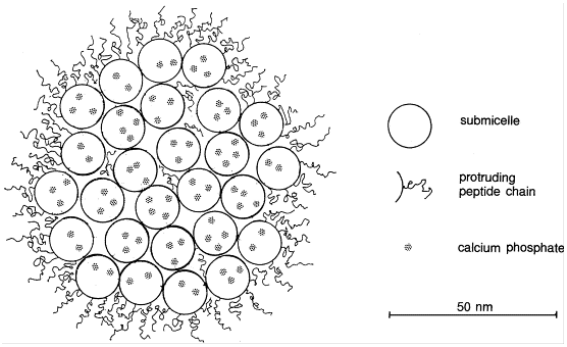

Figure 1: Schematic model of cross section of casein micelle as proposed by Waltra (1982). In this model the casein micelle is made up of submicelles containing calcium phosphate. The submicelles are surrounded by protruding peptide chains making up the 'hairy' layer of the micelle [54].

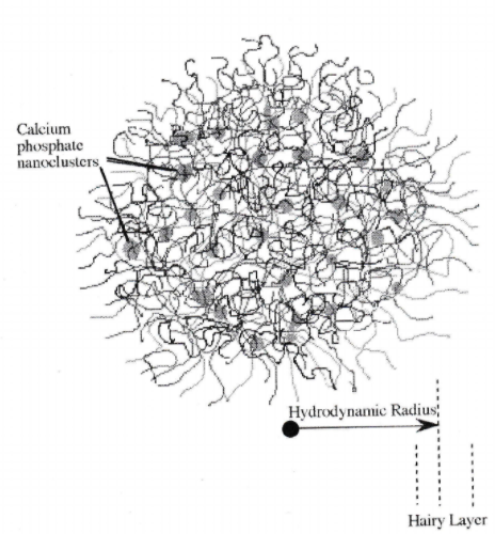

Figure 2: Schematic model of casein micelle as proposed by Holt (1992). In this model an open structure of polypeptide chains are cross linked with calcium phosphate to form the hairy layer [18].

The other commonly accepted model falls in the internal structure category and it is a model proposed by Holt in 1992. Holt and many others generally accepted the existence of the hairy layer he however did not agree with the notion of sub-micelles [36]. Holt stated the casein micelle is a tangled web of flexible casein networks as can be seen in figure 2. This network forms a gel like structure with granules of colloidal calcium phosphate throughout the caseins phosphate centers[13]. In this model, similar to Waltra's model the κ-caseins hydrophilic part form a hairy layer[17][18]. The two main features of this model are the colloidal calcium phosphate and the hairy layer of κ-caseins which stabilize the casein micelle [36].

4.2 Calcium in milk

Bovine milk contains on average 1.2 g/L of calcium [48] and it is present in three forms; free/ionized calcium, calcium complexed with inorganic anions and calcium bound to whey proteins [30]. The first of these forms belongs to the soluble phase, which only makes up around a third of the amount of calcium in bovine milk. The remaining two thirds are found in the insoluble phase [30]. The Calcium in milk is able to exchanges between the three forms. However, the calcium found bound to caseins is for the most part considered hard-to-exchange calcium [62]. In the caseins the calcium is bound to the α-caseins and β-caseins as these two are highly phosphorylated and thus much more sensitive to ca^{2+} ions [58]. In the caseins the calcium ions form calcium phosphate and because of this strong bond they mainly remain bound to the casein instead of exchanging with the rest of the calcium.
The insoluble phase is seen as not bio-available to humans as calcium is absorbed through the lining of the small intestine into the bloodstream, for this process it needs to be in the soluble phase [1].

4.3 Effect of Temperature changes

4.3.1 Effect on the Whey Proteins

β-lactoglobulin
As stated above the whey fraction is mainly made up of β-LG and α-LA [25] and thus it is important to see what the effect of temperature is on these specific proteins. Starting with β-LG, this protein can be seen in figure 3. The protein contains an exposed disulphide bridge (as can be seen in purple) and, shielded by an alpha helix, another disulphide bridge (as can be seen in orange) and a free cysteine (as can be seen in red). When in this natural form the alpha helix shield ensures these groups cannot interact [42]. However, between 60-70°C the total helicity of β-LG drops from 10% to 3% [41][44] thus allowing all the groups to be exposed. The free cysteine contains a thiol group which in turn is able to undergo thiol-disulphide exchange with the disulphide bridges and therefore allows the β-LG proteins to aggregate together [64].

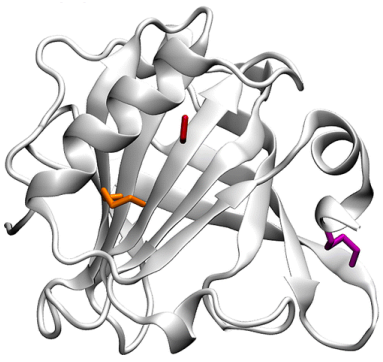

Figure 3: β-LG with the free cysteine shown in red, the buried bridge shown in orange and the exposed bridge shown in purple.[42]

α-lactalbumin
The α-LA protein can be seen in figure 4[21], it contains four disulphide bridges, all of which are exposed. These are the yellow spheres in the figure. Furthermore a purple sphere can be seen which is the bound calcium which increases the stability α-LA has against heat denaturation [35]. The temperature at which α-LA will denature lies in the rage of 78-94°C [57] which means that before α-LA starts to denature β-LG proteins will have already completely denatured and exposed its thiol group. This group will therefore interact with the disulphide bridges of the α-LA causing it to denature at a lower temperature. The α-LA thus forms aggregates with the denatured β-LG [52].

Figure 4: α-lactalbumin with the disulphide bridges seen as yellow spheres. There are four present, the fourth being hidden behind the turquoise helix. The purple sphere represents bound calcium [21].

For both proteins a fall in temperature does not have an effect until the cold denaturing temperature of β-LG is reached. This temperature turns out to lie between -15°C to -20°C [15] and at these temperatures the effect seems the same as at the higher temperatures, a loss of helicity causing the different groups to be exposed [15] and hence the aggregation of α-LA and β-LG.

4.3.2 Effect on the Casein micelles

The casein micelles have a higher stability and so don't denature until heated above 200 °C [39] which doesn't happen during any of the heat treatments (more on this is sections 4.4 and 4.5). However even though the casein micelles have a high heat stability [36] changes can still occur to the micelles at temperatures below this 200 °C.

It is the κ-casein that allows for the casein micelles to have such high heat stability. As seen in both models explained above this κ-casein forms brush like structures discouraging aggregation[36], even when the α- and β-casein's can already start to denature at around 100 °C [54] the κ-casein keep the micellar structure, meaning the α- and β-caseins are not able to denature and the micelles cannot aggregate. Temperatures at which the micelles do denatures and aggregate are not researched in this report, this is because there are no heat treatments or any other heating done after manufacturing reaching up to and beyond 200 °C (more information about this in sections 4.4 and 4.5). Hence the effect of the casein micelles or casein proteins denaturing are not of any influence as this does not occur at the to be researched temperatures[36].
However, casein micelles are effected by the denaturing of the β-LG. Once again the β-LG can undergo thiol-disulphide exchanges with its exposed thiol group, this time with the κ-casein on the edges of the casein micelles which disrupts its brush like form and now allows for the aggregation of the different micelles[20]. Thus upon heating the milk aggregates of whey protein and casein micelles are able to form.

4.3.3 Effect on Calcium

As stated above calcium is found in three forms, free/ionized calcium, calcium complexed with inorganic anions and calcium bound to the whey proteins [30]. To gain an understanding of what happens to calcium once the bovine milk is heated or cooled each of these phases needs to be looked at. Starting with the calcium complexed with inorganic anions, this is the hard-to-exchange calcium and makes up around 40% of the calcium in bovine milk [62]. When heated up Casein micelles are said to expand [10], this allows for more calcium to make its way into the micelles to form Calcium phosphate [36]. Furtermore, Calcium phosphate decreases in solubility at higher temperatures. This means at the higher temperatures it is even less likely to exchange with the rest of the calcium and thus more remains in the insoluble phase[38]. The calcium found bound to whey proteins can become trapped in the insoluble phase once whey protein aggregates and whey-casein micelles aggregates form. The calcium is now no longer able to exchange between forms and thus cannot balance the calcium between the phases, meaning the calcium traveling into the casein micelles is all coming from the soluble phase. Thus, the amount of Ca_{2+} found within the soluble phase drops once milk is heated meaning the bio-availability of milk falls with a rise in temperature [60].

The cooling of bovine milk also has an effect on the bio-availability of calcium. The solubility of calcium phosphate will be higher at the lower temperatures and thus the amount of soluble calcium should be higher as less will be present within the casein micelles [28]. One would expect this effect to be rather limited though seeing as the calcium phosphate in the caseins is seen as hard-to-exchange calcium [62]. At lower temperatures there is also possible added effect from cold denatured proteins which in turn would lower the bio-availability of calcium due to aggregation, it is however expected that this is less influential than the effect of the solubility of calcium phosphate as the cold denature temperature is so low [61].

4.4 Heat Treatments

The international dairy federation states that any intentional heating above 50°C for such a time that a reduction in concentration of microorganisms is seen is considered a heat treatment [19]. This means there are infinite amounts of combinations of heat treatments possible with different temperatures and time combinations. The most commonly used can be seen in table 1 below. Heat treatment has been common practice since 1860 for preservation purposes destroying microorganisms, both pathogenic and spoilage for extending shelf life and ensuring milk is safe [31] and are essential to the dairy industry.

Heat Treatment	**Temperature-time conditions**	**Applications**
Thermisation	57-68 °C for 5sec-30min	Extending shelf life of raw milk before processing
Pasteurisation (LTLT)	65°C for 30 min	General drinking milk
Pasteurisation(HTST) with	72-80°C for 15-30 sec	General drinking milk
Pasteurisation (UHT)	135-140°C for 2-10 sec	Drinking milk with long shelf-life
In-container sterilisation	110-120°C for 10-20min or 125°C for 5 min	Drinking milk with long shelf-life at ambient temperature

Table 1: Most used heat treatments for bovine milk meant for drinking (not for further processing into other dairy products) [19][50].

In the Netherlands all fresh milk from the supermarket is pasteurized milk, so all the milk has gone through some form of this heat treatment. The 2 most commonly used pasteurization processes today are high temperature short time (HTST) and ultra high temperature (UHT)[19]. Thus depending on which type of pasteurization is done the effect on the different proteins can differ a lot. Seeing as the dutch milk companies do not release any further information about which treatment they exactly use for pasteurization or which other treatments are applied it is unclear what has already happened to the proteins in the bovine milk.

4.5 Milk Consumption Temperatures

Further heating after heat treatments can be seen as overheating the milk and can thus lead to further damage to the milk which in turn can mean even less calcium bio-availability[47]. A range of temperatures is listed in table 2 from -18°C up to 95°C, all temperatures indicating a reason for the milk to be at that temperature. Reason being that these are temperatures milk will be consumed at or brought to, and thus knowing the bio-availability at these temperatures is more useful.

Temperature	**Reason**
-18 °C	Ice cream
4 °C	Fridge milk
37 °C	Infant milk
60°C	Coffee milk
75 °C	Hot chocolate
90°C	Baking (Internal temperature of cake)
95°C	Boiling point of milk

Table 2: Temperatures to which milk is brought in everyday life. These are the temperatures which are to be researched [23][12][4]

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4.6 Separation method - Dialysis

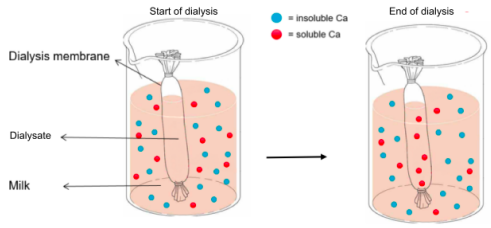

Figure 5: Dialysis set-up [8]

To determine the bio-availability of calcium in bovine milk, the soluble and insoluble phase need to be separated. It is then the calcium found in the soluble phase that is seen as bio-available calcium[].

Dialysis is the process of separating molecules in solution by the differences in their size through a semipermeable membrane, dialysis tubing[43].

In dialysis a sample, which in this case will be milk, and a buffer solution called the dialysate are separated by a semi-permeable membrane. The membrane allows for soluble calcium to pass through as these are small atoms, however the large protein particles which are large molecules(containing the insoluble calcium) cannot pass through[43]. This means that once osmosis has occurred and equilibrium is reached the soluble calcium will be spread throughout the dialysate and milk, with a homogeneous concentration and the insoluble milk will be left in the milk[49], as can be seen in figure 5.
Analyzing the amount of Ca in both phases will allow for the calculation of the percentage soluble calcium using the following formula:

$$\%SolubleCa = \frac{[Membrane](mg/L)*V_{\text{tube}}(L)+[Membrane](mg/L)*V_{\text{cylinder}}(L)}{[Total](mg/L)*V_{\text{cylinder}}(L)}$$

In this formula the concentration of calcium in the membrane is multiplied by the volume of the dialysate in the membrane. To this the concentration of calcium in the membrane multiplied by the volume of milk is added. This gives the total amount of calcium found in the soluble phase. This is then divided by the total amount of calcium which is calculated by multiplying the total concentration of calcium with the volume of milk in the cylinder. Multiplying this all by 100 gives the percentage soluble calcium which is an indicator for the bio-availability of calcium in the milk sample.

4.7 Analysis method - ICP-OES

For the analysis of the amount of calcium in the soluble phase and the insoluble phase the ICP-OES (inductively induced plasma optical emission spectrometry) is used. This is one of the most powerful and popular analytical tools used for inorganic analysis. It is able to detect the presence and amount of different elements in a sample [51].

The ICP-OES uses the fact that atoms and ions are able to absorb energy to move electrons from their ground state to an excited state. Those excited atoms then release a specific wavelength as they transition back to a lower energy level. The amount of light released at each unique wavelength is then proportional to the number of atoms/ions making the transition allowing for the calculation of the concentration of calcium in the samples [2].

In the ICP-OES an ICP torch provides the energy source for the analysis. The torch is made of three glass tubes, argon flows in the two outermost tubes. A spark from a Tesla coil in the two outermost tubes creates plasma and the energy from a radio frequency coil around the torch sustains the plasma. Through the middle of the torch argon caries up aerosols of the sample. An overview of what the torch looks like can be seen in figure 6. The heat of the plasma (around 10,000K [6]) then evaporates this sample, breaks the molecules down to ions and atoms and provides enough energy to excite the electrons [6].

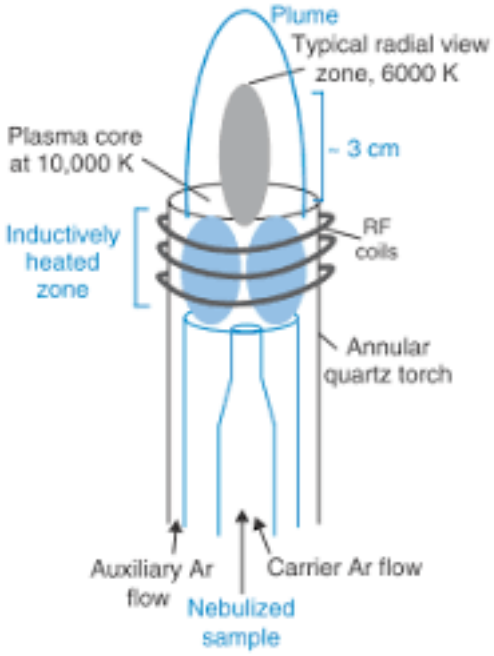

Figure 6: A schematic representation of the ICP-OES torch [3]

5 Materials and Methods

5.1 Materials

5.1.1 Chemicals

Table 3: Overview of the chemical used

Substance	**Chemical name**	**supplier**	**characteristics**
Sodium Chloride	NaCl	Sigma Aldrich	99.999% trace metal basis
Potasium Chloride	KCl	Sigma Aldrich	99.7% ACS reagent
Lactopure lactose	$C_{12}H_{22}O_{11}$	Obtained via Thom Hupperz	Non Food Grade
Nitric Acid	HNO_3	Suprapur	65% purity
Hydrogen Peroxide	H_2O_2	ISO	30% purity
Silicone oil	$C_6H_{18}OSi_2$	Alfa Aesar	usable range; -40°C up to 200°C
milliQ	H_2O	-	-
Bovine milk	-	Jumbo	skim milk
Calcium ICP standard	$Ca(NO_3)_2$ in HNO_3	CertiPUR	1000mg/l Ca

5.1.2 Instruments

Table 4: Overview of instruments used

Instrument	**Brand**
Heated stirrer	Arec.T
Scale	Mettler Toledo PB1502-S
Stirring plate	IKA RO10
Microwave	Anton Paar
ICP-OES	PerkinElmer – Optima 4300 DV

5.1.3 Materials

Table 5: Overview of other materials used

Material	**Details**
Dialysis tubing	Membra-Cel MC 24x100 Chr
Clips	-
Stirring bar	-
Measuring cylinder	100 ml
ICP tubes	15 ml
Volume flasks	50 ml
Crystallisation dish	190mm, 2000ml
Conical flasks	250 ml
Beaker glass	250 ml

5.2 Methods

Marije de Vos did a thesis on calcium bio-availability in fortified milk. The methods for dialysis, the microwave and ICP were adapted from this [8].

5.2.1 Heating

A Crystallisation dish with 600ml silicone oil was heated to 60°C using a heated stirrer to be an oil bath. A scale was used to measure out 100ml of milk, this was added to a conical flask and covered with aluminium foil. The conical flask was then added to the oil bath once it reached the desired temperature. It was left stirring in the oil bath for 30-45 minutes until the milk reached the same temperature as the oil (60°C). Once at temperature the milk was left to be heated for another hour. This was repeated for the following temperatures: 37°C, 75°C, 90°C and 95°C.

Figure 7: Heating set up with oil bath.

5.2.2 Dialysis

For the dialysis 15cm of dialysis membrane was soaked in 100 ml of MilliQ for an hour. A buffer solution was made in MilliQ containing 87 mM Sodium Chloride, 350 mM Potassium Chloride and 205 mM Lactose.

A measuring cylinder was filled with 100 ml of skim bovine milk using a scale. Then a piece of membrane was carefully opened using a needle and a knot made at the base. It was then filled with 5 ml of buffer solution and clipped shut. Next it was balanced on the top of the measuring cylinder and covered with para-film to avoid evaporation. It was then stirred for 24 hours.

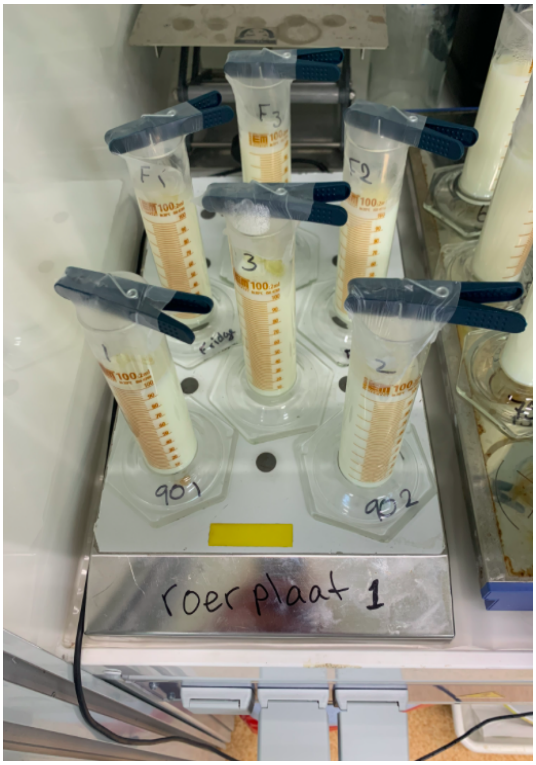

Figure 8: Dialysis set-up for room temperature dialysis

Figure 9: Figure A (top) shows how the membrane is prepared for heated dialysis and figure B (bottom) shows the dialysis set-up for heated dialysis.

5.2.3 Dialysis at temperature

Dialysis at higher temperatures was done in conical flasks or crystallisation dishes instead of measuring cylinders, it was a horizontal dialysis. The same set-up was used as described in the heating method (5.2.1) up to the milk being at the desired temperature. This was done for the following temperatures: 60°C, 75°C and 90°C. Next the same preparations for dialysis were done as stated in the dialysis method (5.2.2). However instead of clipping the membrane shut another knot was tied at the other end as can be seen in figure 9A. This membrane package was then dropped into the conical flasks or small crystallisation dishes with the milk and covered with aluminium foil to be dialysed. This dialysis was done for 2, 3 and 6 hours at 60°C and for 2 hours at 75°C and 90°C.

For cold temperatures the dialysis was done in the fridge at 4°C. This dialysis was done exactly according the the dialysis method (5.2.2) except the stirring plate was placed in the fridge. The cold dialysis was done for 24, 30 and 48 hours.

5.2.4 Microwave

A 0.5 ml sample of the buffer within the membrane or the milk was added to microwave tubes. Furthermore 1.5 ml hydrogen peroxide and 4.5 ml nitric acid was added to each tube. The tubes were then capped, inserted into ceramic microwave tubes and spread evenly around the microwave carousel as can be seen in figure 10.
The microwave was set to a program consisting of 40 minutes of heating to 180 °C, 20 minutes of heating to 200 °C, 20 minutes at 200 °C and finally 20 minutes of cooling down.
Once finished the microwave tubes were decanted into 50 ml volumetric flasks, each microwave tube was flushed 3 times with MilliQ and the contents were added to the volumetric flask each time as well to ensure all sample was transferred. Finally the volumetric flasks were filled up to 50 ml with MilliQ and homogenized. After which they were decanted into 15 ml ICP tubes.

Figure 10: Microwave carousel with 12 microwave tubes evenly spread around the carousel. An even spread of the tubes is necessary for the microwave to run.

5.2.5 ICP

For the measurements on the ICP-OES a calibration line was made. 5, 10, 15 & 20 mg/L samples were made using the chemicals and amounts indicated in table 6. A scale was used to weigh each step precisely so that exact concentration in each sample could be calculated.

Table 6: Volumes of all components in each calibration.

Volume stock (ml)	**Volume Nitric acid (ml)**	**Volume Hydrogen peroxide (ml)**	**Volume MilliQ (ml)**
0.25	4.5	1.5	43.75
0.5	4.5	1.5	43.50
0.75	4.5	1.5	43.25
1	4.5	1.5	43.00

The samples were analysed on the ICP-OES and the following detection wavelengths: 317.933, 315.887, 396.847, 422.673 and 227.546 nm were selected for the quantification of calcium.

6 Results and discussion

6.1 ICP Measurements

The ICP measured the intensity of calcium using 5 different wavelengths. These were 318, 316, 397, 423 and 228 nm. However only 3 wavelengths were used when calculating the final result for each sample. Reason for this is that at 423 nm the resulting concentration was always around 100 mg/L higher than measured at all other wavelengths. This means that most likely another substance also has an emission wavelength of around 423 nm which is interfering with the results. It is suspected that this substance is Argon which the ICP-OES uses to carry the sample. Argon has an emission line around 423 nm [24] and thus could be the cause of the higher concentration. Hence the measured intensity, resulting in the found concentration at this wavelength does not represent the amount of calcium in the sample. Furthermore the calibration curve at 397 nm only had a correlation coefficient of 0.998892 as can be seen in figure 11. This is too low for an accurate result as a minimum of 0.999900 is required for a good calibration. This correlation coefficient is probably lower than all the others because above a certain concentration of calcium the correlation between intensity and concentration is no longer linear. For the wavelength of 367 nm this linear region does not stretch up to 20 mg/L making the wavelength unsuitable for measuring the concentration of calcium within the samples.

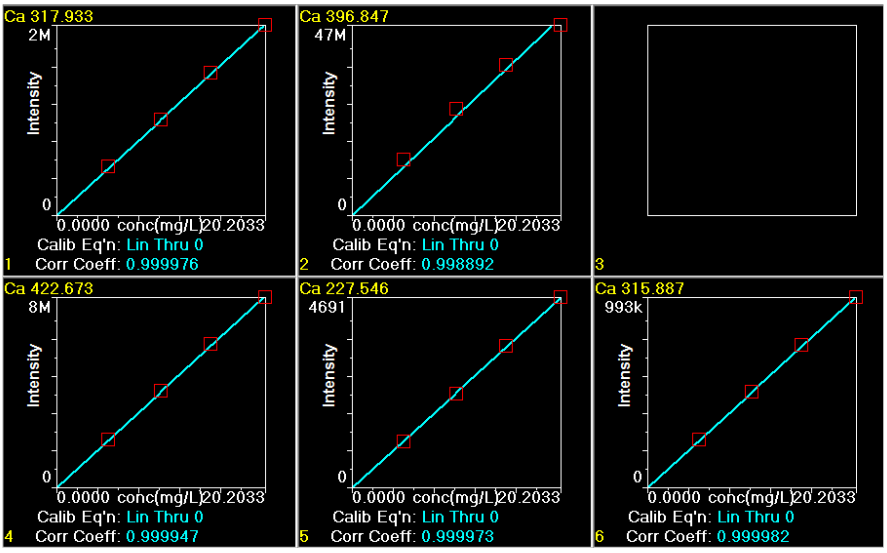

Figure 11: Calcium calibration curves used for ICP measurements. They showcase what intensities at different wavelengths fit with the given concentrations. The used wavelengths were 318, 316 and 228.

6.2 Percentage soluble calcium in bovine milk at different temperatures

6.2.1 Time period for dialysis at 60°C

As stated in the heated dialysis method the dialysis at 60°C was done for three different time frames, 2, 3 and 6 hours. This was done to to check whether equilibrium was truly reached after 2 hours as stated by On-Nom [33].

Figure 12 shows the amount of soluble calcium after having been dialyzed at 60 °C for different the time periods. The percentage of soluble calcium found is around the same value for each time frame, the values seem to fluctuate slightly however once the errors are taken into account this is not the case and they can be considered the same.

Seeing as On-Nom stated that at 60 °C the dialysis was slowest and for higher temperatures it only went faster[33] it can be assumed that the dialysis done at 75 and 90°C will also reach equilibrium in 2 hours.

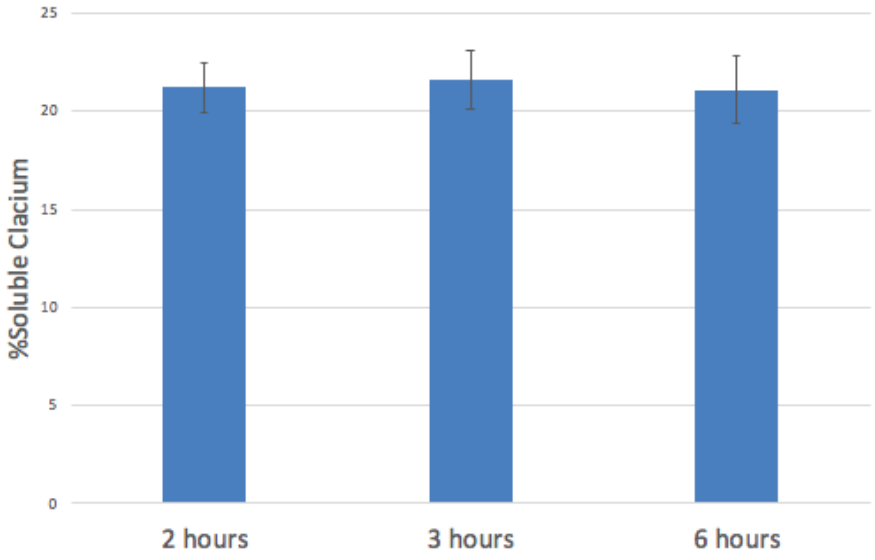

Figure 12: Dialysis of bovine milk at 60 degrees for different time frames (2 hours, 3 hours and 6 hours).

6.2.2 Time period for dialysis at 4°C

The solubility of calcium at lower temperatures was also researched seeing as this is mainly how people store milk. Dialysis was done in the fridge at a temperature of 4°C. As stated in the method this was done for 24, 30 and 48 hours in order to find out which time frame was suitable for cold dialysis. As can be seen in figure 13 the dialysis did not seem to be complete after 24 hours as the value of percentage soluble calcium is only 34.4%±1.1 whilst for 30 and 48 hours the value lies at 36.3%±1.6 and 36.1%±1.3 respectively. The values for 24 and 30 hours lie very close together when taking the errors into account, overlapping even and thus the difference may not seem significant. However, it does give an indication towards there not yet being equilibrium after 24 hours and thus a minimum time period of 30 hours was decided upon for the cold dialysis.
These values can sadly not be compared to literature values as nearly all experiments done with the influence of temperature on milk are done using ultra centrifuge or ultra filtration. Any research done which does use the dialysis method only does so at temperatures of 20 °C and above. This is possibly because dialysis at lower temperatures takes so much time. All that can be said for the results obtained as seen in figure 13 is that a longer time period does not seem to have a negative effect on the dialysis and thus, to be on the safe side and ensure equilibrium is reached, longer dialysing times are favorable hence the period of 30 hours.

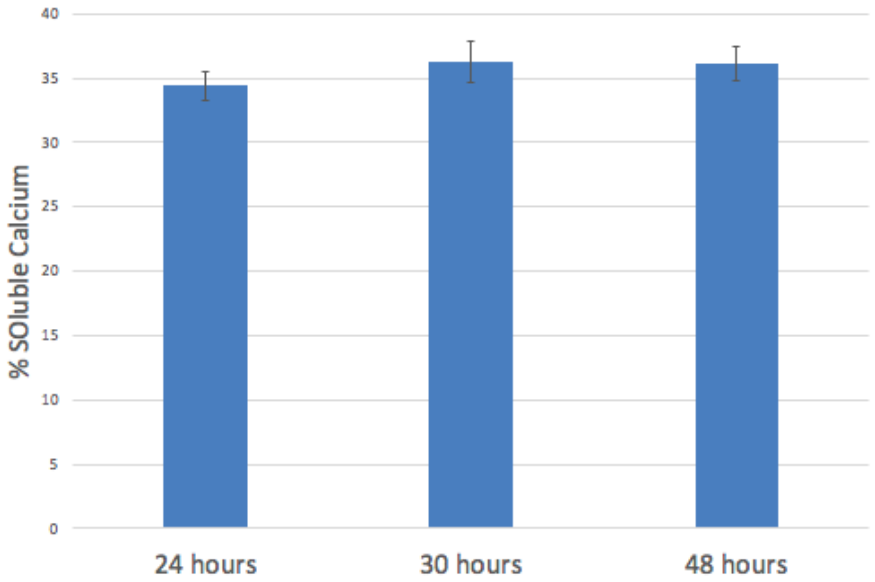

Figure 13: Dialysis of bovine milk at 4 degrees for different time frames (24, 30 and 48 hours).

6.2.3 Comparison of percentage soluble calcium in bovine milk at different temperatures

Finally dialysis was done a room temperature and 37°C for 24 hours (the later representing the temperature of the human body). This makes it in total 6 different temperatures at which dialysis was done: 4°C, 21°C, 37°C, 60°C, 75°C and 90°C. In figure 14 it can be seen that as the temperature is increased the percentage soluble calcium decreases in a linear fashion between 4 and 90°C with a gradient of -0.254. The highest percentage soluble calcium is seen at 4°C being 36.3%±1.6 and this linearly decreases until 90°C where the percentage soluble calcium is 16.6%±1.8. There were no measurements made above this temperature as the boiling point of milk is 95°C [7]. Hence warming the milk up to and beyond 95°C would result in it boiling, at this point it is no longer possible to do a dialysis using the method of these experiments.

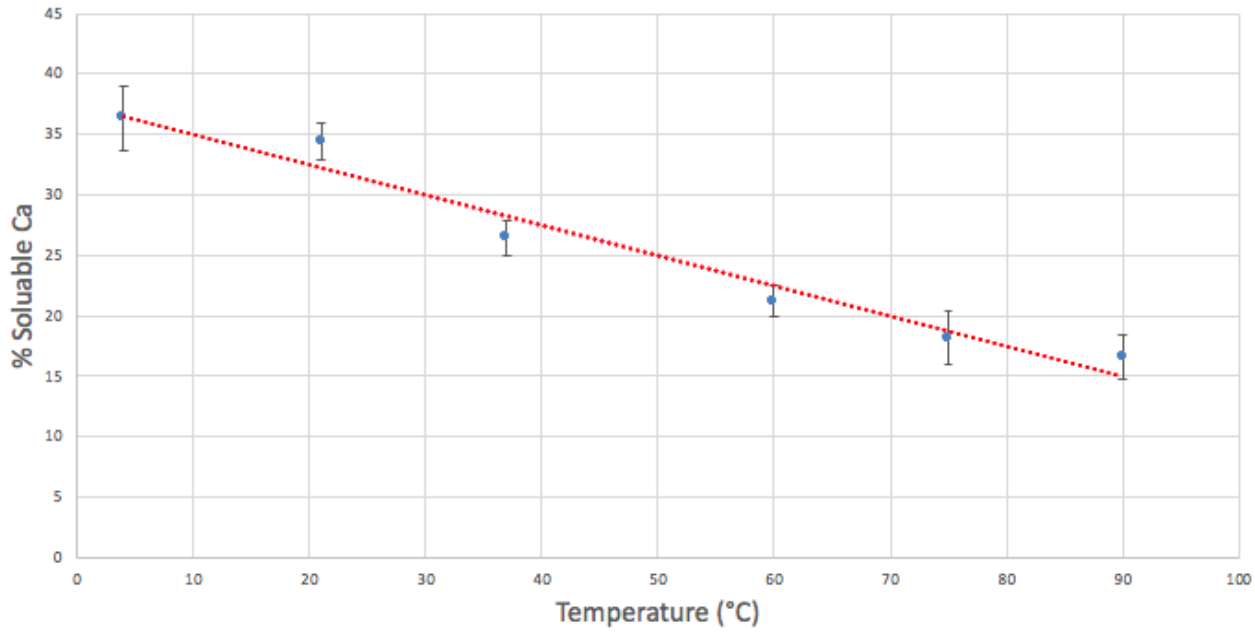

Figure 14: The Percentage of soluble calcium when milk is dialysed at different temperatures, a linear trend can be seen where the percentages of soluble calcium decreases with an increase in temperature.

The results found can be compared to results found by Pouilet et al. using an ultra centrifuge method to find the effect temperature on amount of soluble calcium. In table 7 these results can be seen compared with the results found. It should be noted that not all temperatures perfectly match with temperatures which Pouilet et al. used such as 40°C and 37°C, these differences are indicated in the table. A clear difference in results can be spotted, at all times the results found using dialysis are higher by a factor of 3-8%. That the results differ could be due to a different separation method being used by Pouilet et al. seeing as the values are always higher. It could also be due to Pouilet et al. starting of with raw milk which would contain less or no denatured whey protein to start with and thus could influence the results as well. What can also be noted about the comparison of the found results and Pouilet et al.'s results is that the difference is higher for 4 and 20°C milk, here the values found differ by 7-8% in comparison to the 3-5% difference on the rest of the results. This is rather peculiar because the result found for 21°C (34.4±1.6) is very close to the result found by Marije et al. who found the percentage soluble calcium to be 34.9% ± 2.2 at room temperature [8] indicating that this should be an accurate result.
However looking at figure 14 it can be seen that the value for 21°C does not lie on the trend line, not even the error bar extends to reach the trend line. This once again indicates that the value may not be completely accurate. The value for 4°C does lie on the trend line but this could be because Excel starts the trend line with the first point or it could be because the value is on trend, this is however not what Pouilet et al.'s results indicate as seen in table 7. Overall the accuracy of the results found for 4 and 21°C is debatable and further research is advisable.

Table 7: Comparison of results found by Pouliot et al using heated ultra centrifuge [37] and results from dialysis experiments

Temperature (°C)	% Soluble Ca Pouliot et al	% Soluble CA found	Difference between values
4	29.6	36.6	7.00%
20	26.5	34.4 (21°C)	7.90%
40	22.5	26.5 (37°C)	4.00%
60	18.6	21.2	2.60%
80	14.4	18.2 (75°C)	3.80%
90	11.8	16.6	4.80%

To compare the results from Pouilet et al. better with the results found using dialysis both can be plotted together and the gradients of the two linear lines can be compared as can be seen in figure 15. First looking at the red and green lines, red being the same line as in figure 14 above and green being the results found by Pouilet et al.. The gradient found using dialysis is -0.249 and the gradient found by Pouilet et al is -0.204. These two values lie relatively close together indicating similar findings, however when the fist two temperature of which the accuracy is unsure are taken out the new trend line is seen in blue in figure 15. The new gradient is then -0.190 which is even closer to Pouilet et al.'s findings. This was of course to be expected when looking at table 7. What this indicates is that the trend found for 60-90°C using the dialysis method is similar to literature values and more research must be done to be certain of the results found for 4 and 21°C as their accuracy is more questionable.

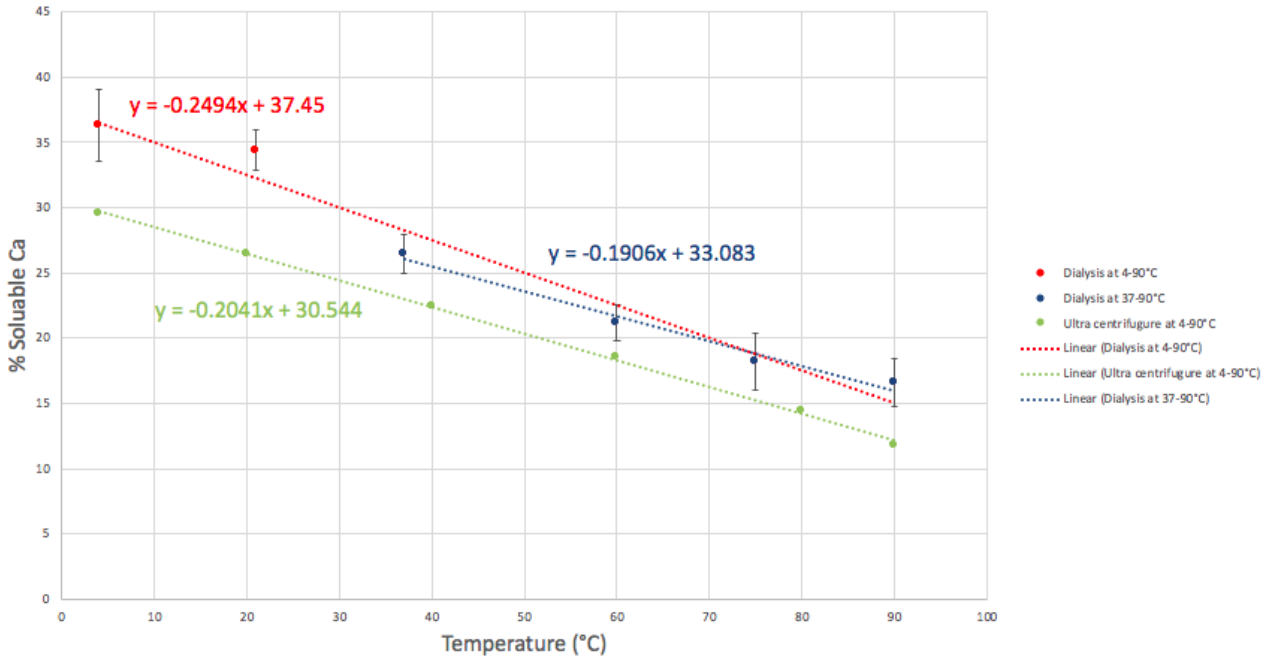

Figure 15: A comparison between the values found using dialysis and the values found by Pouilet et al. using ultra centrifuge[37]. Two trend lines can be seen for the dialysis results, once incorporating all results and one incorporating only temperature 37-90°C

The linear trend is something that can be found more often in literature, for example N. On-Nom et al. also found a linear trend using ultra-centrifuge [33]. These results cannot however be compared in the same way as Pouilet et al.'s because only a concentration of soluble calcium was calculated and no total amount of calcium was recorded in the report for a calculation of percentage soluble calcium. What can however be taken away from N. On-Nom et al.'s results is that a linear trend up to 90 °C seems to be an accurate finding.

Overall from figure 14 showing the percentage soluble calcium found when dialysing at different temperatures it can be said that temperature does indeed have an effect. An increase in temperature causes a decrease in percentage soluble calcium in a linear fashion. This is also in line with the theory in section 4. From 60°C up whey protein starts to denature and aggregate trapping calcium in the insoluble phase and no longer allowing it to exchange between the forms [41][60]. The casein micelles expand at higher temperatures allowing for more calcium ions to make their way into the micelles to form calcium phosphate[10]. The solubility of calcium phosphate decreases with increasing temperatures resulting in it exchanging even less with the rest of the calcium [37]. However, it is still unclear exactly which of these changes causes the loss in soluble calcium, or both, making it unclear how permanent the this change in percentage soluble calcium is. It could always still return to its original value once the milk is returned to its original temperature. Hence the next set of experiments done was heating or cooling milk and then allowing it to return to a set temperature for the dialysis to then compare how the percentage soluble calcium changes.

6.3 Percentage soluble calcium in bovine milk heated to different temperatures

6.3.1 Heating milk

Samples of milk were heated to various temperatures for 1 hour and dialysed for 24 hours at room temperature. The dialysis time of 24 hours was adapted from Marije et al.. It was assumed that dialysing for slightly longer would not have further effect on the dialysis as once equilibrium is reached nothing else happens. This was not entirely the case for the heated milk. When samples were accidentally left for longer than the 24 hour mark the milk would start to curdle, this looks like the start of the process of cheese making. This did not once occur for non-heated milk left out for slightly too long but if heated milk was left out for sometimes only an hour too much it would curdle as can be seen in figure 16. Thus is is very important to keep to exactly 24 hours otherwise one risks losing the sample. Furthermore it indicates something has clearly changed within the structure of the milk, it is now more eager to aggregate and phase separate.

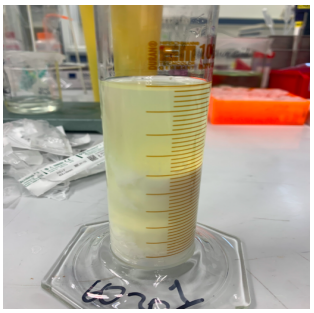

Figure 16: A dialysis left for 2 hours too long, the milk went bad and started to curdle, this sample was therefore unusable.

According to a study done by J.David et al. the yield of cottage cheese increases as a result of heating (74°C, 10 s), cooling (3°C), and storing (7 days) milk [11]. These time frames are of course very different to the 1 hour of heating and 24 hour dialysis done in the experiments however, the results found by J.David et al. do indicate that the curdling of milk occurring as explained above is not a one off scenario and that it has been observed before. J.David also found that the percentage casein was higher than the percentage whey in the untreated milk when doing a protein analysis [11]. Thus something similar may be occurring to the milk heated for the experiments here causing the aggregation and phase separation.

6.3.2 Time period of heating

To gain a good understanding of what time of heating was suitable an analysis of the effect of time of heating on the percentage solublility was done. The heating of milk at 60°C was done for 10 minutes, 30 minutes, 60 minutes and 120 minutes and in figure 17 the percentage of soluble calcium in milk heated for these different time frames can be seen.For 10 minutes of heating the percentage soluble calcium is 31.2%±2.6 whilst when the bovine milk was heated for 120 minutes the percentage soluble calcium drops very slightly to 30.1%±2.3. This difference between the time periods is so small, with errors much larger than the difference that it can be seen as negligible. The results therefore indicate that 10 minutes of heating and 1 hour of heating have the same effect.

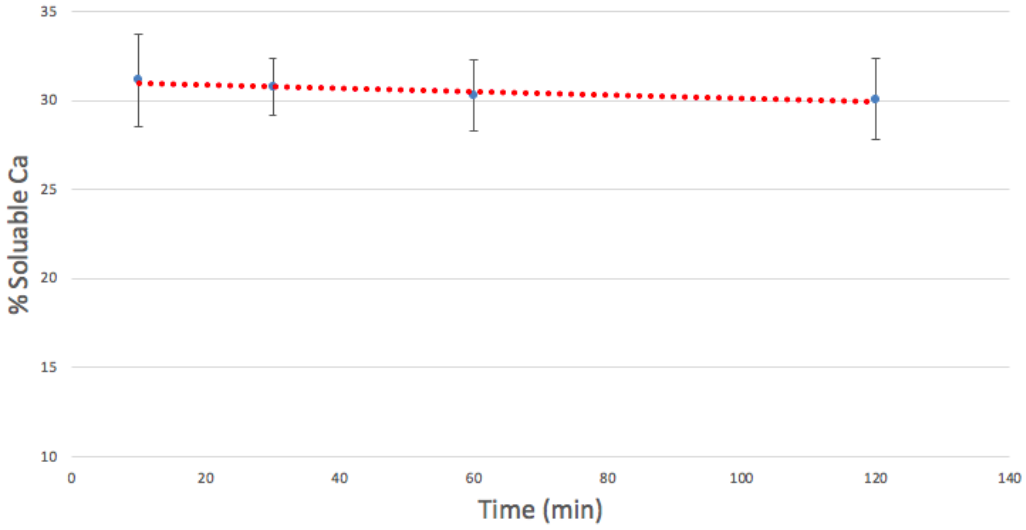

Figure 17: Percentage soluble calcium in milk heated for 10, 30. 60 and 120 minutes at 60°C. A downward linear trend can be seen where the longer the time the less soluble calcium is present in the sample.

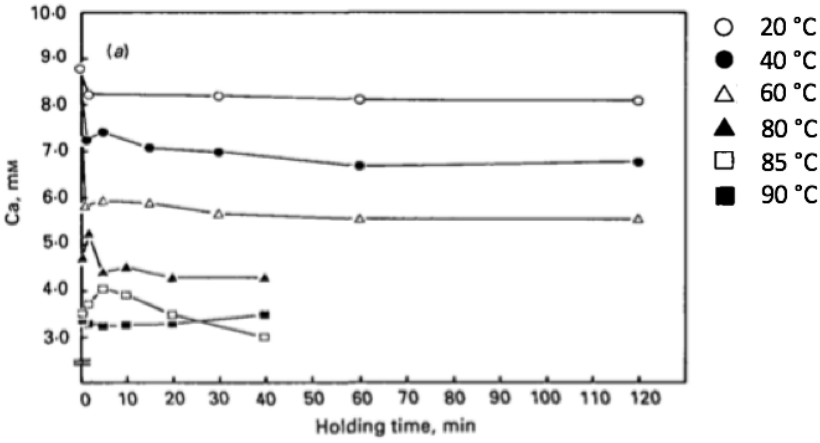

Figure 18: Pouiliot et al. did a study about the effect of heating time on the heat-induced salt balance changes in milk, the results found for the concentration of calcium in milk samples heated at 20-90°C for up to two hours can be seen. [38]

Pouiliot et al. did similar research except with the ultra-centrifuge method instead of the dialysis method. The result from this study can be seen in figure 18. It can be seen that for the temperatures of 20-60°C Pouiliot et al. has similar findings as in figure 17. The amount of soluble calcium is around the same value whether the bovine milk is heated for 10 minutes or 120. However, Pouiliot et al. went beyond the 60°C for this experiment and this is where the results also start to differ, they are no longer around the same value for all the heating times indicating that there is indeed a difference in amount of soluble calcium dependant on the time of heating.

These higher temperatures were not tested for different time periods in this thesis due to time constraints. However the results found by Pouiliot et al. are an indicator that at temperatures of 80°C and above a longer time period means more effect on the amount of soluble calcium can be seen.
Thus it was decided to do a longer time period of heating, as this had the biggest chance at showing significant differences between the different temperatures. This is of importance because with the chosen method the errors are around 1-3% and so a difference of only this much between temperatures would not give very trust worthy results. However, with two hours of heating many samples could not even be left for 24 hours to dialyse without the curdling issue described above. Only a limited few would make it past the dialysing step of the experiment which posed many issues with actually analysing samples dialysed for this time frame. This is most likely because the longer heating time caused the denaturing of more proteins in the milk as stated above and so the milk would be quicker to curdle. This is actually not a common problem when looking into influence of heating on bovine milk because nearly all research is done using ultra centrifuge. However, in the end with the 1 hour of heating and the precise 24 hour dialysis period the sample could be created without problems and thus this was the decided time frame.

6.3.3 Comparison of percentage soluble calcium in bovine milk heated to different temperatures

Milk from the fridge (4°C) was dialysed for 24 hours at room temperature, furthermore it was heated for 1 hour at 37, 60, 75, 90 and 95°C and once again dialysed for 24 hours at room temperature. The percentage soluble calcium found in these samples can be seen in figure 19. The unheated milk was found to have the highest percentage soluble calcium at 34.4%±1.0, this seems to gradually decrease as the milk is heated more. At 37°C the percentage soluble calcium does not drop, it is found to be 33.7%±1.7. At 60°C a drop can be seen, the value falling to 30.3%±1.9. Comparing this value to that found at 4°C and including both points error bars the value is clearly lower by at least 1.2% and at most 7%. This suggests that upon reaching 60°C something clearly changes within the milk affecting the percentage solubility. This all makes sense when comparing back to the literature. The whey protein starts to denature at 60°C[41], these are the proteins in milk which denature at the lowest temperature and thus the first to be influenced hence why not much happens when heating to 37°C. The denaturing of whey protein causes aggregation between the whey proteins themselves and between the whey and casein micelles[20]. The casein micelles having taken up more calcium when heated due to expanding would then no longer be able to exchange this calcium back to the soluble phase once cooled leaving permanent loss in percentage soluble calcium[10][36].
The value for percentage soluble calcium does not seem to decrease much further from 60 to 95°C. The difference between the results is rather small, especially in comparison to the error bars as seen on figure 19 and thus they can be concluded to not change after 60°C is reached. Once again going back to what was found in literature, 60°C is the denaturing temperature for the whey protein[41]. The next permanent change that could occur within the milk is the casein micelles denaturing, this however does not occur until 200°C is reached[39]. Thus that there is no further permanent change between 60 and 95°C is to be expected.

Looking at the overall trend line in figure 19 temperature and percentage soluble calcium seem to have a linear relation. The gradient this time however is only -0.074, much lower than the relation found for heated dialysis before which was -0.249. This indicates that while there is some permanent damage done to the amount of soluble calcium available it is not as much as what seems whilst the bovine milk is hot. A lot of the calcium is able to return to the soluble phase once cooled which suggest that whilst there is some further denaturing of whey proteins at heating above 60°C its effects are limited.

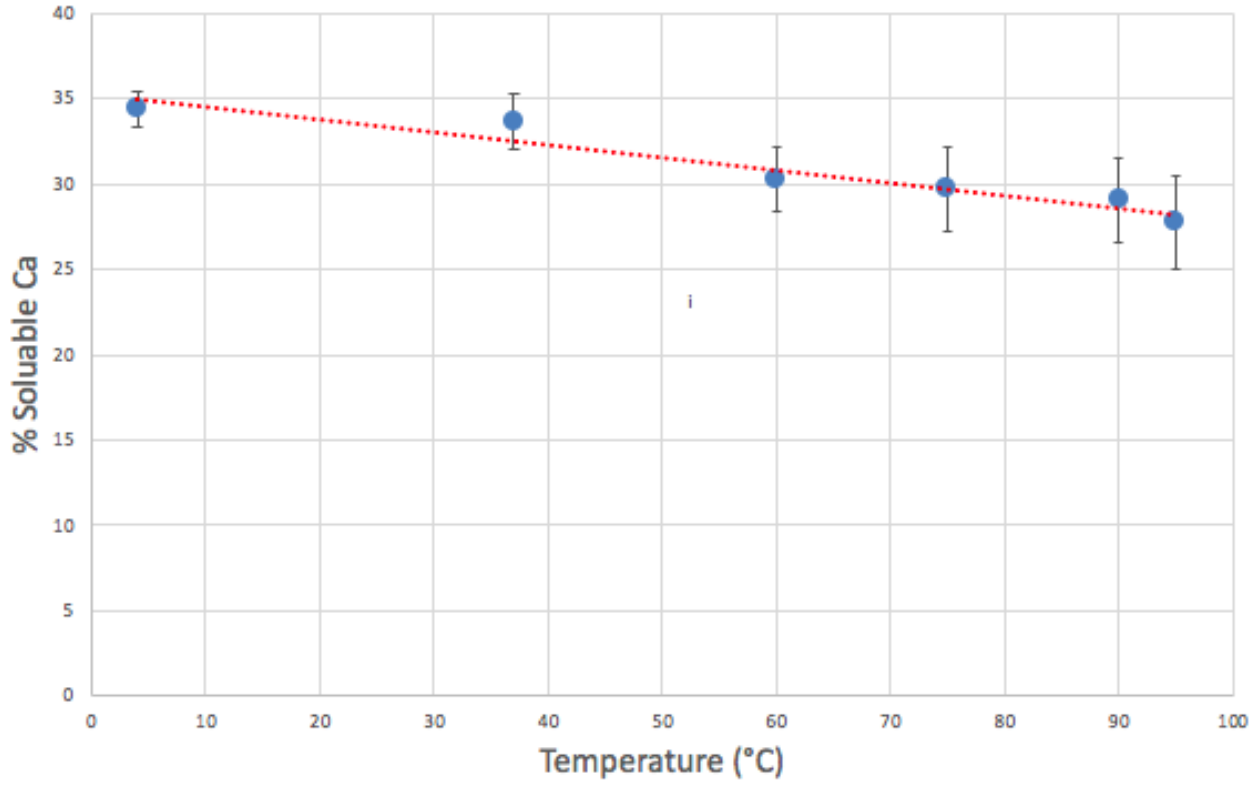

Figure 19: Percentage soluble calcium in milk heated for 1 hour at 37, 60, 75, 90 and 95°C and dialysed at room temperature. A linear trend can be seen where the percentage soluble calcium decreases with temperature.

Research done about permanent effects of heating tend to look into specific heat treatments. This makes for a more difficult comparison but can still indicate weather the conclusions found above point in the right direction. Sung-Ho Yoo et al. did a study comparing raw milk with UHT (135-140°C, 2-10 sec), LTLT (65°C, 30 min) and HTST (80°C, 15-30 sec) treatments [50]. In this study it was concluded that the higher temperature treatment caused the most loss of soluble calcium. This therefore seems to go against what has been concluded from figure 19 as Sung-Ho Yoo et al.'s results further heating above 60°C does have more impact. However, when comparing the values found at the the different temperatures it can be seen that the results are comparable and the values that have been found in the experiments with dialysis seem accurate.

Table 8: Comparison of results from Sung-Ho Yoo et al. using ultra centrifuge and results found from dialysis experiments [50]

% soluble Ca Sung-Ho Yoo et al.	% soluble Ca found
31,4 (65°C)	30,3±1,9 (60°C)
28,5 (80°C)	29,1±2,4 (90°C)
25,2 (135°C)	-

Sung-Ho Yoo et al. has indeed found bigger differences between results hence gaining a different conclusion. This could however be due to raw milk being the start point of the experiments in that study whilst already treated skim milk was used for the experiments here. Thus less whey protein is untouched and hence the effect of the temperature change was expected to be less significant. Furthermore Sung-Ho Yoo et al. also used a higher temperature of 135°C which was not tested in the experiments because with the current set up the milk could not be heated to such high temperatures.

Overall what this comparison does indicate is that the results found are of similar values to results found in literature. However, the differences between temperatures are more subtle when heating already heat treated skim milk in comparison to using raw milk. This is to be expected because as seen from Sung-Ho Yoo et al. results heat treatments already do permanent damage and so the amount of whey protein left to further denature is much more limited hence the effect of the percentage soluble calcium is less.

6.3.4 Effect of cold temperatures on the percentage solubility of calcium in bovine milk

To test the effect of cold temperatures frozen milk was defrosted and dialysed at room temperatures for 24 hours. Now unlike the heated milk this milk was not frozen for a set hour. It was frozen overnight simply because it would be very difficult to find the exact time the milk was completely frozen. This is also more realistic when wanting to represent real life, as milk is not frozen for just an hour. In figure 20 the comparison between the frozen milk (-18°C) and fridge milk (4°C) can be seen. For frozen milk the percentage soluble calcium was found to be 33.1%±2.3 and for the fridge milk it was found to be 34.4%±1.0 as stated above. This means there is over 1% difference between the two. This suggests that some form of cold denaturing has taken place in the time the milk was in the freezer. However, this cannot be said with certainty because the errors on the two results mean there is a large overlap and the percentage soluble calcium in the two samples could just be the same, it is still unclear from these results.

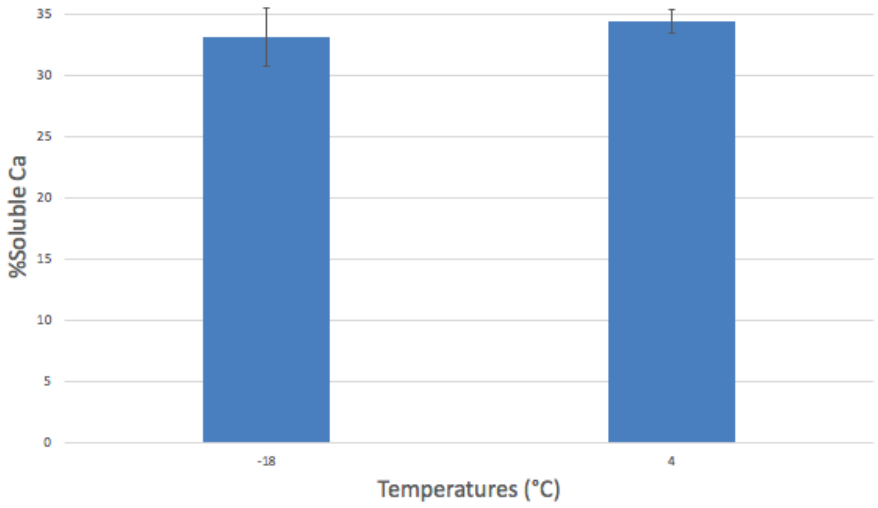

Figure 20: Percentage soluble calcium in frozen (-18°C) and fridge (4°C) milk dialysed at room temperature. An increase in percentage soluble calcium can be seen when moving from frozen to fridge milk of around 1%

Overall from figure 19 it can be said that temperature has a permanent effect on the percentage soluble calcium in bovine milk. However from the results it is simply heating to 60°C and above that causes further damages but it does not seem to matter to which temperature above 60°C the effect is all the same at least up to 95°C. For temperatures above this further research would be required before anything can be said.

7 Conclusion and Recommendations

7.1 Conclusion

The goal of this study was to find the effect of temperature changes on the bio-availability of calcium in milk, furthermore the extend of the permanence of this effect was also to be researched. To research this the percentage soluble Calcium has been used as an indicator for the bio-availability of calcium in bovine milk and the percentage soluble Calcium was found through separating the insoluble and soluble phases using dialysis and then analysing these phases using the ICP-OES.
It has been found that for dialysis at temperatures of 60°C and above a 2 hour dialysis period is sufficient and for dialysis in the fridge (at 4°C) a time period of 30 hours is required for equilibrium to be reached.
Furthermore when dialysis was done at temperatures of 4, 21, 37, 60, 75 and 90°C a linear relation with a gradient of -0.254 was found between temperature of the milk and percentage soluble Calcium. With the percentage soluble Calcium in bovine milk dropping 19,7% when comparing 4°C to 90°C.

For milk heated to 60°C dialysed at room temperature it was found that the time length of heating had no effect. Furthermore after heating milk to 37, 60, 75, 90 and 95°C it was found that heating to 60°C and above resulted in some loss of soluble calcium however it does not seem to matter which temperature it is up to 95°C.

Overall comparing the two sets of results the gradient of the heated milk dialysed at room temperature was lower at -0.074 whilst the gradient found for with the dialysis done at various temperatures resulted in a gradient of -0.254. This indicates that whilst there is some permanent damage and loss in soluble calcium it is clearly less than what would be suspected after doing dialysis of the heated milk. It seems a lot of the calcium is able to return to the soluble stage.

These results indicate that the heat treatment through which skim bovine milk in the Netherlands has to go does not seem to denature all whey protein and so further heating done at home to make coffee or hot chocolate will further denature the whey protein and cause permanent loss of calcium bio-availability. This loss occurs because at the warmer temperatures the un-aggregated casein micelles will expand and soluble calcium will move into the caseins[10], once the left over whey proteins denature they are able to aggregate to the casein micelles disallowing it from shrinking back as the milk is cooled down again and hence not allowing the calcium to exchange back to the soluble phase[20].

7.2 Recommendations

For further research looking into cold temperatures could be of interest. In this thesis only a fridge and freezer, and the temperatures to which these are set were used. For future research it would be interesting to look into exactly when cold denaturation takes place and the negative effects of cooling on the bio-availability of Calcium as only limited time was spent on this. Taking more temperatures in the negative range and different time conditions could give a better idea of the effects.
Also looking more into the higher temperature ranges, above the boiling point of milk has potential, this was also not done due to the limits of the experimental set-up in this thesis. This too could have effects on the calcium bio-availability outside of the expected linear relation or more permanent effect beyond what was seen in this thesis.
To gain a better understanding of the effect of temperature on the milk proteins and through that the bio-availability of Calcium in milk other tests can be done with the samples such as an SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis). It is a method which separates proteins based on their molecular masses, aggregates would therefore sink all the way to the bottom whilst the rest of the proteins would spread across the gel in accordance to their molecular weight. This would allow for a an analysis of what proteins are left and which have denatured and formed aggregates in the milk.
Lastly working with raw bovine milk would also be of interest in this research area, potentially finding heat treatment combinations which ensure higher Calcium bio-availability so that something like fortifying milk may not even be necessary as a higher Calcium bio-availability could potentially be gained in a much simpler way.

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