Iron bioavailability of fortified skim milk

An in-vitro study to investigate the influence of iron salt fortification on the phase distribution and oxidation state of iron in skim milk

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Abstract

Anaemia affects a third of women and almost 50% of infants and toddlers worldwide. Iron deficiency is the most common cause of anaemia, and the most common nutritional disorder in the world. To increase dietary iron intake, iron fortification of staple foods commonly consumed by the general public is an effective approach to prevent and control iron deficiency anaemia. It is important to maximise the iron bioavailability (absorption by the human body) in these fortified products, since iron overload is also known to cause negative health effects.

Since the diet of infants and toddlers revolves around milk, milk is a good food carrier to address iron deficiency anaemia in children. The aim of this project is to assess the influence of five factors on the bioavailability of iron fortified skim milk: (1) iron concentration, (2) molecular form of the added iron, (3) storage time after fortification, (4) the addition of an iron enhancer and (5) the addition of an iron inhibitor.

Skim milk was fortified with $FeCl_2$ and $FeCl_3$ to 5 and 20 mmol Fe L^{-1} and stored for 24 hours or one week. Part of the milk was further left untreated and to another part ascorbic acid (vitamin C) or tannic acid were added as respectively iron enhancer and iron inhibitor. The bioavailability of the iron in these milk samples was assessed on phase distribution and oxidation state, since it is believed that iron in the soluble phase of the milk and in the oxidation state Fe^{2+} is more bioavailable.

Analysis showed that the difference in iron concentration induced a pH decrease, which was greater for the higher concentrations of iron and the molecular form $FeCl_3$. The pH difference caused the percentage of iron present in the casein fraction to increase, in the protein fraction to decrease and did not influence the iron in the soluble phase. It was also found that the 20 mmol L^{-1} $FeCl_2$ showed more iron in the soluble phase and more Fe^{2+}, whereas no statistical difference was observed for the 5 mmol L^{-1}, which might also be due to the pH difference. The increase in storage time to one week was reflected by an increase in iron bound to the caseins. This shift was not reflected in a change of oxidation state. The addition of the iron enhancer (ascorbic acid) increased the iron present in the protein fraction and soluble phase, which was strengthened by the further decrease in pH. The chelation of iron by ascorbic acid did not necessarily reduce Fe^{3+} tot Fe^{2+}; the Fe^{2+} decreased for the 20 mmol L^{-1} $FeCl_2$, whereas it increased for the 20 mmol L^{-1} $FeCl_3$. Lastly the addition of the iron inhibitor resulted in a significant decrease of Fe^{2+}. Most iron was bound to the casein or precipitated as iron-tannic acid complex.

Overall, this research confirmed that the bioavailability of milk is not easily evaluated, since the behaviour of iron in milk depends on many different factors that can not be studied isolated. Only by evaluating many possible combinations of concentration, molecular forms, additives and storage conditions, the bigger puzzle can be solved. This project attributed to a part of the solution and is therefore a valuable contribution in the development of bioavailable milk products, that will reduce iron deficiency anaemia in children.

Acknowledgments

There are little people, that I know of, whose master thesis project is an easy ride. Also I encountered my share, and didn't escape the difficulties and setbacks; it is part of the journey to improve your academic skills, but they mainly contribute to your personal growth. This journey would not have been possible without the amazing support of the words and deeds of many around me.

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Contents

List of Abbreviations

ALARA	As low as reasonably achievable
CCD	Charge-coupled device
CPM	Counts Per Minute
CPS	Counts Per Second
DPM	Disintegrations Per Minute
EC	Electron Capture
Fe	Iron
ICP-OES	Inductively Coupled Plasma - Optical Emission Spectroscopy
I.S.	Isomer shift
MWCO	Molecular Weight Cut-Off
LSC	Liquid Scintillation Counting
QIP	Quench Indicating Parameter
Q.S.	Quadrupole splitting
RF	Radio Frequency
PMT	Photomultiplier Tube
LET	Linear Energy Transfer
UG	Ultima Gold XR

1

Introduction

Anaemia affects 30% of the women of reproductive age (570 million) and almost 50% of preschool-aged children (293 million) [1, 2]. The World Health Organization (WHO) endorsed a *Comprehensive implementation plan on maternal, infant and young child nutrition*, which specified six global nutrition targets for 2025 [2]. The second target of this plan is to decrease anaemia in women of reproductive age with 50% (to 230 million), compared to a baseline set in 1993-2005 [2].

Anaemia is a condition in which the haemoglobin concentration is below an established cut-off value (110 g/L and 120 g/L for resp. pregnant and non-pregnant women [3]). A decreased haemoglobin concentration compromises the capacity of blood to transport oxygen around the body, resulting in symptoms like fatigue, weakness and reduced concentration, among others [3–5]. For children, anaemia has also been linked to growth retardation, a worse motor and cognitive development, social inattention and decreased school performance [6]. Maternal anaemia has been associated with low birth weight, premature birth, and maternal and child mortality [1, 6]. That makes anaemia interlinked with three other global nutrition targets of the WHO: stunting, low birth weight and childhood overweight [1].

The most common cause of anaemia is iron deficiency, arising from prolonged negative iron balance [1]. This negative balance can be due to an inadequate intake or absorption of dietary iron; increased requirement for iron during menstruation, pregnancy or growth; or infections and intestinal worms. It is estimated that 50% of anaemia among women worldwide is caused by iron deficiency [1]. Other contributors to anaemia are diseases and infections like malaria; and genetic haemoglobin disorders [3].

In the *Global Nutrition Targets 2025 Anaemia Policy Brief*, the WHO recommends to prevent and control anaemia by improving the bioavailability and intake of micronutrients through food fortification or supplementation with iron, folic acid and other vitamins and minerals [1]. In their policy, they stress that diets containing adequate amounts of bioavailable iron should underpin all efforts, since iron deficiency anaemia is the most common cause and relatively easy to treat through dietary changes. Other causes of anaemia can be addressed by malaria control, deworming and delay cord clamping, among others [1].

The food fortification can be delivered through mass fortification, by the addition of iron to staple foods commonly consumed by the general public, such as wheat flour, maize flour and corn meals, rice and milk [1]. Since the diet of infants and toddlers revolves around milk, milk products as food vehicle for iron can play a crucial role in the control of anaemia in children. Many problems arise however in iron fortified milk products due to the chemical reactivity of iron, which makes it one of the hardest nutrients to fortify with [6, 7]. Due to the reactivity of iron, the milk product may change in colour, flavour and odour, which decreases the product quality [6]. Another issue related to iron fortification is that iron excess has proven to come with negative side-effects such as diarrhea; and has been related to several diseases, like as diabetes type 2 and colon cancer [6, 8]. Therefore it is of interest to maximise the bioavailability of the iron in the milk product, to reduce the total iron intake to prevent the negative health effects [8]; meanwhile finding a balance between sufficient bioavailability and product quality [6].

In order to develop food fortification strategies to tackle anaemia, it is crucial to assess the bioavailability of the iron fortified milk product. Frequently used methods to assess the bioavailability are centrifugation and dialysis [9], which give the solubility of a compound. However knowledge on the physiochemical environment of iron in milk (for example the oxidation state of iron and structure of the milk in interaction with iron), is of considerable interest to understand the bioavailability [10]. In 2000, Frédéric Gaucheron proposed in a review paper on the iron fortification in dairy industry, to use Mössbauer spectroscopy to determine the physiochemical environment of iron in milk. Although he proposed this over two decades ago, in the mean time only one paper has been published by Raouche *et al.* [11] that used Mössbauer spectroscopy on iron fortified milk.

To contribute in the fight against anaemia in children, the iron bioavailability of iron chloride fortified skim milk is assessed. *The aim of this project* is to study five factors that can influence the iron bioavailability in skim milk: the iron chloride concentration, the molecular form of iron chloride ($FeCl_2$ and $FeCl_3$), the storage time after fortification, the addition of an iron enhancer (ascorbic acid) and the addition of an iron inhibitor (tannic acid). As a measure for the iron bioavailability the phase distribution and oxidation state of iron in milk are used.

2

Theory

This chapter provides background information on the characteristics of iron, the absorption mechanism of iron by the human body, the concept of bioavailability and some complications that arise when fortifying food with iron (section 2.1-2.3). This project focuses on iron supplementation of milk, therefore the composition of milk is and methods to separate milk ingredients will be explained (section 2.4). Substantially, the bioavailability of iron can be assessed using these separation techniques (section 2.5). Lastly the analysis instruments are explained that can be used to assess these separated phases and the oxidation state of iron: ICP-OES, LSC and Mössbauer spectroscopy (section 2.6).

2.1 Characteristics of iron

2.1.1 Chemical characteristics of iron

Iron is a reactive element with atomic number 26 and average atomic weight 55.85 u. It has many oxidation states, where the most commonly encountered are Fe^{2+} (d^6) and Fe^{3+}(d^5). Where Fe^{2+} is extremely soluble in water, Fe^{3+} is rather insoluble. Iron plays an important role in the biological reactions that involve redox reactions, because of the variable redox potential of Fe^{2+}/Fe^{3+} [12]. For example, it is a component of hemoglobin, which is essential for the transport, storage and utilisation of oxygen [10]. The process of iron absorption by the human body is explained in section 2.2.1.

2.1.2 Isotopes of iron

Iron has four stable isotopes: ^{54}Fe (5.8%), ^{56}Fe (92%), ^{57}Fe (2.1%), ^{58}Fe (0.3%) [13]. The stable isotope ^{57}Fe is the most used isotope in Mössbauer spectroscopy, which is a non-destructive analytical technique to determine the chemical environment of ^{57}Fe in a sample [14]. The Mössbauer effect and Mössbauer spectroscopy will be described in section 2.6.3.

Besides the four stable isotopes iron has 26 radioactive isotopes [13]. ^{55}Fe ($t_{1/2}$ = 2.7 y) and ^{59}Fe ($t_{1/2}$ = 44.6 d) have a suitable half-life ($t_{1/2}$) for tracer studies. These studies track the behaviour of a radioactive isotope in for example a human body or chemical reaction, by measuring the decay product of the radioactive isotope. The other radioactive iron isotopes are either too short lived (~ms-min) or too long lived (~10^5 y) to perform a tracer study in which these decay products can be measured [13].

In this project ^{55}Fe is used, which decays to ^{55}Mn purely via electron capture (EC), whereafter Auger electrons or characteristic X-rays are emitted with a low energy of 5.8 keV (Figure 2.1) [13]. Because of the low energy of the decay radiation, Liquid Scintillation Counting (LSC) is suitable detection technique [15]. LSC will be described in section 2.6.2.

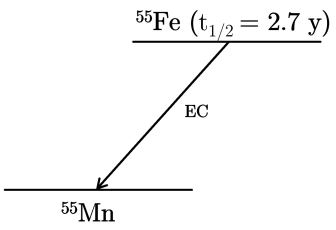


Figure 2.1: Decay scheme ^{55}Fe. ^{55}Fe decays purely via electron capture to ^{55}Mn. The half-life of ^{55}Fe is 2.7 y.

2.2 Absorption of iron by the human body

2.2.1 Iron absorption

Dietary iron is present in two forms: the relatively easily absorbed heme iron and less available non-heme iron [7]. Heme iron is primarily derived from animals, whereas non-heme iron is abundant in foods from animal and plant origin [16]. Both forms are mainly absorbed in the duodenum, but the absorption mechanisms are different, as is presented in Figure 2.2.

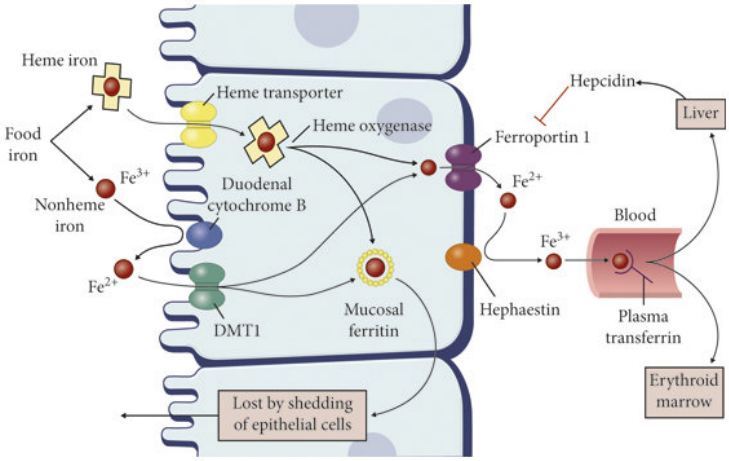


Figure 2.2: Schematic representation of the iron absorption mechanisms in the duodenal enterocytes. Heme iron is absorbed via a specific heme-carrier protein. Non-heme iron is mostly present in Fe^{2+} and Fe^{3+}. Fe^{3+} is first reduced to Fe^{2+} by the enzyme duodenal cytochrome B. Fe^{2+} traverses the membrane of the enterocyte via the divalent metal transporter (DMT-1). [17]

On the one hand, heme iron traverses the membrane of the enterocytes via a specific heme-carrier protein (HCP-1), which makes it almost unaffected by dietary factors. On the other hand non-heme iron traverses the membrane of the enterocytes via the divalent metal transporter-1 (DMT-1). This transporter also transports other divalent metals beside iron, such as calcium and zinc and therefore non-heme iron is more affected by dietary factors [6]. Non-heme iron mostly occurs in two oxidation states: Fe^{2+} and Fe^{3+}. Since DMT-1 requires divalent metals (oxidation state 2+), the Fe^{3+} needs to be reduced to Fe^{2+} by the enzyme duodenal cytochrome B before it can be absorbed by the enterocytes. Added molecular forms with the more soluble Fe^{2+} are generally assumed to be more bioavailable than forms with the less soluble Fe^{3+} [18]. However, since the iron is absorbed in the duodenum it is most important that the iron is in soluble form once it reaches the duodenum [6, 17, 19]. In case the reader is interested in the intracellular storage and iron export after absorption, further resources are for example Crichton [12], Shubham *et al.* [19] and Anderson *et al.* [16].

2.2.2 Iron bioavailability and dietary factors

The bioavailability of a food component, such as iron, is defined as the proportion of the ingested iron that is absorbed by the human body and used through normal metabolic pathways [20]. The bioavailability of iron can be influenced by dietary compounds and host factors [6]. Dietary compounds can either enhance or inhibit the absorption of iron in the enterocytes. Substances like ascorbic acid, which are present in fruit, have been proven to improve the absorption since they reduce Fe^{3+} to Fe^{2+} [7]. Other compounds like phytates and polyphenols, present in respectively whole grains and coffee, form insoluble complexes in the gut and impede its absorption [6, 16]. Lastly, divalent metals like calcium compete with iron for the DMT-1 transport, and therefore also affect the iron bioavailability [6]. Host factors that influence the absorption of iron are e.g. pregnancy, growth, physical activity and diseases [6]. One can imagine that improving the bioavailability of iron is an important factor in iron fortification of food, since the simple addition of iron to a food product does not equal improved absorption in the body necessarily.

2.3 The challenge of iron fortification

Besides the bioavailability of iron, some other factors have to be kept in mind during iron fortification because of the chemical reactivity of iron. The stability during processing and storage, and the compatibility with other components in the food [7], make it a hard fortification ingredient. Another issue is that iron excess of unbioavailable iron results in negative side-effects such as diarrhea [8]. Therefore iron fortification is a balance between food quality and and iron bioavailability.

Iron can be added to staple foods consumed by the general public, such as wheat flour, maize flour, corn meals, rice and milk [1]. In this project the focus is on bovine milk as a food vehicle for iron fortification. Although milk is a challenging product to fortify with iron (because iron in milk has a low bioavailability [21]), it is an important food vehicle for infants and toddlers, whose dietary revolves around milk.

Since fat in milk might interfere with the characterisation of the different phases [9], bovine skimmed milk is used in this project. In the following, 'milk' refers to bovine skimmed milk unless stated otherwise.

In section 2.5 it is explained how the bioavailability of iron in milk can be assessed, but before continuing on that subject, it is first of interest to understand the composition of milk and the binding of iron to milk compounds.

2.4 Composition of milk and binding of iron to milk components

Milk is a complex solution containing water, fat, proteins, lactose, vitamins and minerals. These compounds are divided in three phases or fractions. Lactose and most minerals can be found in aqueous solution, proteins are dispersed in this solution and the fat is present in an emulsified state [9, 22]. The protein phase can be fractionated in two groups: whey proteins (~20%) and casein proteins (~80%). The whey protein are present in the soluble phase. A small part of the casein proteins are present in the soluble phase, while the greater part is present in the casein micelles as colloidal particles [22]. Since the properties of milk are most affected by the proteins, and because this ingredient plays an important role in the distribution of iron in milk, the focus of this project is the milk proteins. When interested in the lactose, fat, minerals, vitamins and water in (whole) milk, the reader can consult Fox [9, 22] and Meurant [23].

2.4.1 Distribution of iron in milk

Unfortified whole milk contains only 0.2-0.5 mg/L of iron [7]. About 14% is associated with the fat, 24% is bound to casein micelles, 29% to whey proteins and the remaining 32% is bound to the low-molecular weight fraction [10], which may include salts, peptides and vitamins [24]. In skimmed milk, the fat is removed, and the natural occurring iron distribution is different. In skimmed milk, 50-60% is bound to the casein micelles, 18-33% to the whey proteins and 15-33% is in the low-molecular weight fraction [10].

The distribution of iron in iron-fortified milk has been reported in the literature as about 80-90% of the iron bound to the casein micelles, the remaining part is either bound to the whey protein or to small diffusible molecules [10, 11]. These studies have assessed the iron distribution by separating the different phases. Common methods to perform phase separation are by centrifugation or dialysis, which are described in the next section.

2.4.2 Phase separation

Phase separation or fractionation of milk is done to analyse whether a constituent is in the colloidal phase bound to the casein micelles, or present in the soluble phase as free ions or formed salts [25]. There are a great many techniques to perform phase separation, and although isolelectric precipitation is the most common technique to separate the casein from the non-casein fraction [9, 26, 27], it involves the acidification of the milk sample, which could influence the salt equilibria of fortified milk. Therefore two other often practiced techniques, dialysis and centrifugation, where the temperature and pH can be controlled, are used in this research en will be described hereafter. To separate the phases gel filtration [28], rennet coagulation [29], salting-out [30] and precipitation by ethanol [30] have also been used. Background information and comparisons studies can be found in Fox [9, 22], De la Fuente *et al.* [31], Franzoi *et al.* [32] and Lewis [33].

Dialysis

Equilibrium dialysis can be used to separate the soluble from the colloidal phase [22]. Dialysis is a process driven by diffusion or the concentration gradient (Figure 2.3). By placing a membrane of a pore size around

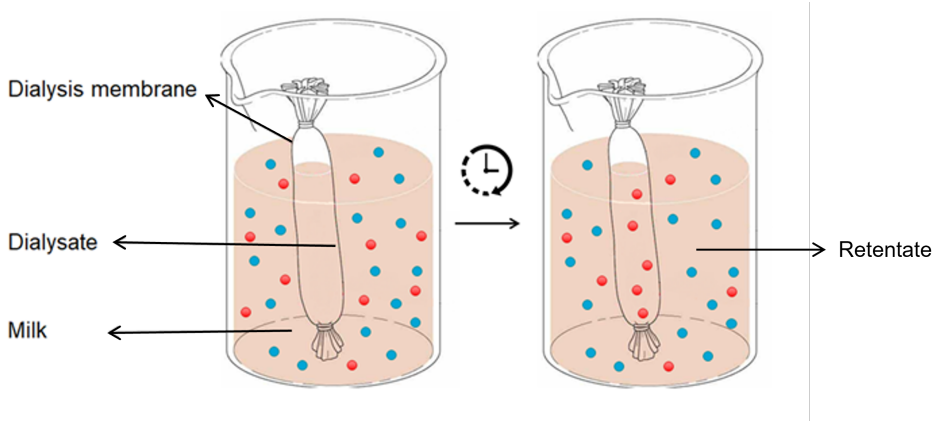


Figure 2.3: Schematic representation of the dialysis process. The red dots represent the soluble ions, which will equilibirate across the membrane over time. The colloidal phase is depicted in blue and is not able to penetrate through the membrane, due to its size. It is also possible to exchange places for the dialysate and the starting solution. Figure adapted from De Vos [34].

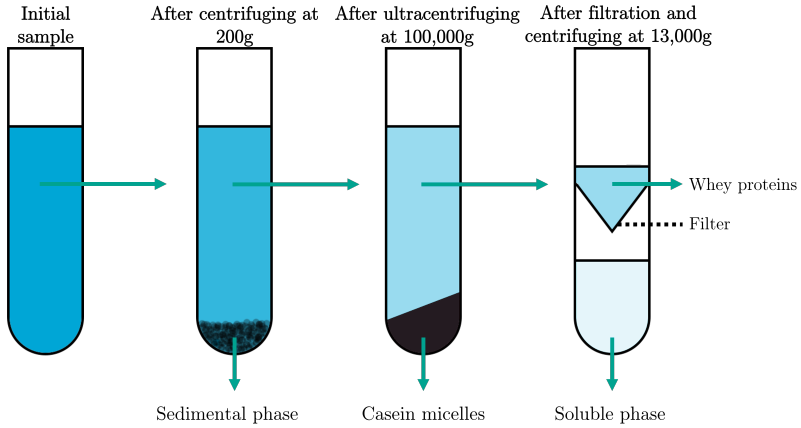


Figure 2.4: Schematic representation of the centrifugation process. The initial sample is centrifuged at 200g for 30 minutes to remove possible insoluble fortification salts. Next the non-sedimental phase is ultracentrifuged at 100,000g for 1 hour. The casein micelle fraction is the sedimented fraction. The supernatant is centrifuged with a filter at 13,000g for 40 minutes to separate the whey fraction from the soluble fraction. Figure adapted from De Vos [34].

10kDa, only the soluble fraction is able to penetrate through the membrane, whereas the colloidal phase is confined to the initial side. When equilibrium is reached, both sides can be analysed on the constituent of interest.

Centrifugation

Phase separation by centrifugation can be executed since casein micelles are large aggregates. These casein micelles can be sedimented by ultracentrifugation at 100,000g for 1 hour [22]. The supernatant contains the non-sedimentable whey proteins, which can be separated from the soluble phase over a 10kDa membrane [25, 35]. This process is shown in Figure 2.4, where the first centrifugation step is added to remove possible insoluble fortification salts [35].

Both dialysis and centrifugation can be used to assess the bioavailability of an iron fortified milk product as will be discussed in the next section.

2.5 Bioavailability assessment

In section 2.2.2 the bioavailability of a food component, such as iron, is defined as the proportion of the ingested component absorbed and used by the human body. There are several methods to estimate the bioavailability of iron in fortified foods. These methods can be divided in two groups: *in vivo* experiments with living beings (*in vivo*, meaning "within the living"); and simulated *in vitro* experiments performed in laboratory (*in vitro*, meaning "in glass"). *In vivo* experiments usually measure a response after consumption

of a food source, either by measuring blood plasma or examining faeces. However ethical restrictions when humans or animals are used limit this type of studies [36]. In contrast to *in vivo* studies, *in vitro* methods do not have these ethical restrictions. They are being extensively used since they are fast and safe, however they can only provide an indication of the bioavailability. In *in vitro* studies the digestion method is often simulated and the final concentration of a nutrient of a final extract is measured [36]. A measure for the bioavailability of this extract is the solubility of a nutrient [21]. In case of iron bioavailability specific, it is also possible to analyse the oxidation state [11, 19], since it is thought that Fe^{2+} is better absorbed than Fe^{3+} [18].

The phase separation methods that were discussed in 2.4.2 (dialysis and centrifugation) can be used to obtain the solubility of a nutrient by calculations showed below. The oxidation state of iron can be measured with Mössbauer spectroscopy, which will be explained in section 2.6.3.

2.5.1 Dialysis

As described in section 2.4.2, during dialysis the soluble fraction is able to equilibriate over the membrane. By measuring concentration of the constituent in the milk before dialysis and in the dialysate in equilibrium, the total soluble fraction in the initial sample can be calculated by:

$$Solubility\,[\%] = \frac{soluble\ constituent}{total\ constituent} * 100\% = \frac{[D_{equilibrium}] * V_{total}}{[M_{initial}] * V_{milk}} * 100\%, \tag{2.1}$$

where $[D_{equilibrium}]$ (g/mL) is the constituent concentration in the dialysate in equilibrium, V_{total} (mL) the volume of the milk and dialysate together, $[M_{initial}]$ (g/mL) the constituent concentration in the milk before dialysis and V_{milk} (mL) the volume of the milk [34].

The measurement can be verified by analysing whether the total amount of constituent after dialysis equals the amount at the start of the dialysis:

$$[D_{equilibrium}] * V_{dialysate} + [R_{equilibrium}] * V_{retentate} = [M_{initial}] * V_{milk}, \tag{2.2}$$

where $V_{dialysate}$ (mL) is the volume of the dialysate, $[R_{equilibrium}]$ (g/mL) is the constituent concentration in the retentate and $V_{retentate}$ (mL) is the volume of the retentate (which is equal to V_{milk} (mL) when the osmotic pressure is equal on both sides at the beginning of the dialysis).

2.5.2 Centrifugation

In Figure 2.4 it can be seen that in the centrifugation process, at the end of the the last filtration step the soluble phase is obtained. The solubility of the constituent is then given by:

$$Solubility\,[\%] = \frac{[F_{13,000g}]}{[M_{initial}]} * 100\%, \tag{2.3}$$

where $F_{13,000g}$ (g/mL) is the concentration of the filtrate. Besides the soluble phase, the centrifugation method can also provide information on the constituent fraction in the sedimental phase, casein micelles and serum proteins. These are calculated in the following way:

$$Sedimental\ phase\,[\%] = \frac{[M_{initial}] - [S_{200g}]}{[M_{initial}]} * 100\%, \tag{2.4}$$

$$Casein\ micelle\,[\%] = \frac{[S_{200g}] - [S_{100,000g}]}{[M_{initial}]} * 100\%, \tag{2.5}$$

$$Serum\ protein\,[\%] = \frac{[S_{100,000g}] - [F_{13,000g}]}{[M_{initial}]} * 100\%, \tag{2.6}$$

where $[S_{200g}]$ (g/mL) is the constituent concentration in the supernatant after centrifugation at $200g$ and $[S_{100,000g}]$ (g/mL) is the constituent concentration in the supernatant after centrifugation at $100{,}000g$.

2.6 Analysis instruments

In this project, the fortified milk and the products resulting from phase separation of fortified milk are analysed with three analysis instruments : the ICP-OES to determine the concentration of a chemical present in a sample (section 2.6.1), the LSC is used to perform relative measurements on a radioactive compound in the sample (section 2.6.2) and lastly Mössbauer spectroscopy is used to probe the chemical environment of iron in the sample (section 2.6.3). In this section a introduction to the working mechanism of these techniques are provided.

2.6.1 Inductively Coupled Plasma - Optical Emission Spectrometry

Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) is an analytical technique that is used to determine the concentration of chemical elements present in a sample. The ICP-OES instrument is a type of spectrometer that uses a plasma to produce excited atoms or ions. Upon deexcitation, these excited atoms or ions will emit light on element specific wavelengths. In Optical Emission Spectrometry the intensity of light at specific wavelengths is measured and can thereby be used to determine the concentration of a chemical element in the sample [37].

The ICP-OES instrument consists of two key parts: the plasma and the spectrometer. The mechanism of both parts is explained below and the explanation is mainly based on Boss and Redeen [37] and Nölte [38]. A schematic overview of the major components of the ICP-OES instrument is shown in Figure 2.5.

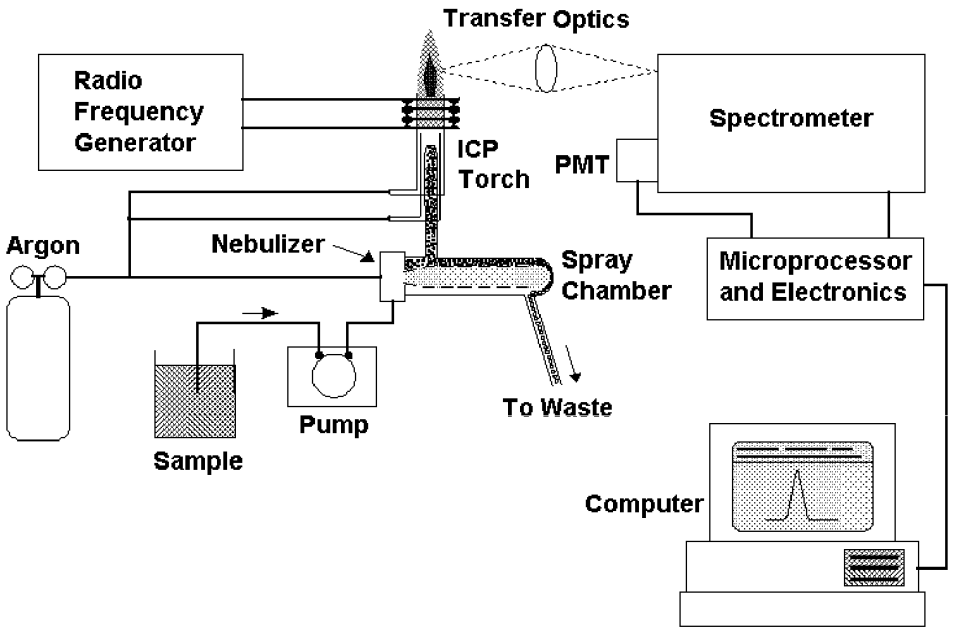


Figure 2.5: The major components of the ICP-OES instrument. Argon flows into the ICP-torch, which becomes a plasma due to the fluctuating magnetic field induced by the Radio Frequency generator. The sample is pumped into the nebulizer, where it is nebulized into aerosols. These aerosols are transported into the spray chamber with an argon flow. Small enough aerosols will be able to flow up into the ICP-torch, while larger aerosols have too much inertia to make the corner and go via the spray chamber to the waste. The small aerosols are introduced in the plasma, and will be excited by collisions with the electrons and ions in the plasma. Upon decay element specific light is emitted. With lenses this light is transferred to the spectrometer, which can separate the different wavelengths. With a photomultiplier tube and microprocessor, or in more modern devices a charge coupled device, the intensities of the emission lines are converted to a digital signal. On the computer the measured intensities can be compared to previous measured samples of known concentration. Thereby the concentration of the unknown sample can be determined. [37]

Plasma
An inert gas - often argon - is let into the torch of the ICP. At the end of the torch is a radio frequency (RF) generator, which creates an oscillating current in a coil surrounding the torch. This oscillating current induces an electric and magnetic field at the tip of the torch. The argon in the torch is ignited with a spark, which causes electrons to be stripped from the argon gas. These stripped electrons are accelerated by the magnetic field and will collide with other argon atoms, which in their turn will be stripped of their electrons. This cascade of electrons colliding with other argon atoms results in a plasma consisting of moving electrons, argon atoms and argon ions. This is known as the inductively coupled plasma.

Once the plasma is stable, the sample can be introduced to the system. In general (and also in this project), liquid samples are analysed with an ICP-OES instrument, so only this sample type will be described. The plasma should stay stable while the sample is introduced into the plasma, and therefore only small droplets can be injected into the plasma. These droplets are formed by the nebulizer, which converts the liquid sample into aerosols. These aerosols are transported to the plasma with a carrier gas, argon in this case. In the spray chamber, the gas flow changes direction (in Figure 2.5 the gas flows from the left and changes direction to the top). This direction changes ensures that only aerosols smaller than the cut-off diameter come into the plasma. The bigger aerosols that would destabilise the plasma cannot make the corner due to their inertia, and will travel through the spray chamber to the waste.

When the small droplets are injected into the plasma, first these aerosols are dried. The remaining solid particles are vaporised and then the molecules of this gas are dissociated into atoms. The free electrons and ions in the plasma can excite and ionise these analyte atoms and form positively charged ions. When these excited or ionised atoms fall back into a energetically lower state, the excess energy is emitted as light. The wavelength of this light is element specific, and upon measurement can be used to determine the elements in the sample.

Optical Emission Spectrometer
Lenses are used to focus the emitted light onto the spectrometer, which separates the different wavelengths by the use of a diffraction grating. With a photomultiplier tube (PMT) and microprocessor, or in more modern devices a charge coupled device (CCD), the intensities of the emission lines are converted to a digital signal, that can be analysed on the computer. At the beginning of a measurement a calibration line can be made of known concentrations versus measured intensities. Using interpolation the concentrations of unknown samples can be measured.

2.6.2 Liquid Scintillation Counting
Liquid Scintillation Counting (LSC) is a measurement technique that is used for the detection and quantitative measurement of radioactivity and is especially suitable for low energetic radiation. This radiation is hard to detect since it has a short range, and therefore gets absorbed in the sample itself, air or shielding material. To avoid (self-)absorption the sample is solved in a detection medium, a scintillation cocktail. This cocktail contains a solvent and a scintillator. The solvent absorbs the radiation energy emitted by the radionuclide in the sample. The activated solvent molecule then transfers this energy to a scintillator molecule, which is excited by this transferred energy. Upon deexcitation the molecule emits a photon that can be detected by a PMT. The PMT will convert the photo signal into an electrical signal. The intensity of the signal indicates the energy and type of decay, whereas the amount of light flashes is a measure for the activity of the sample. This liquid scintillation process is shown in Figure 2.6.

To reduce noise, two PMTs are used in an LSC; only electrical pulses that are detected simultaneously in both PMTs are considered as true counts. If a background event occurs in the PMT or the electronic circuit, it would result in a single count and therefore being rejected as background noise. The use of two PMTs reduces the background from 10,000 counts per minute (CPM) to about 10 CPM [15, 39]. Another reason to use two PMTs is that when the light is produced at the edge of the counting vial, the light is perceived brighter by the PMT close to that edge. However, by using two PMTs and summing the signal, the final intensity is not affected by the position of the decay [39].

Besides noise, the chemical composition of the sample also influences the efficiency of the measurement. The solvent can convert the radiation energy into heat instead of transferring it to a scintillator molecule. Or the solution can absorb the light emitted by the scintillator. This interference in the conversion of the radiation energy to photons detected by the PMT is called quenching. Four types of quenching are considered:

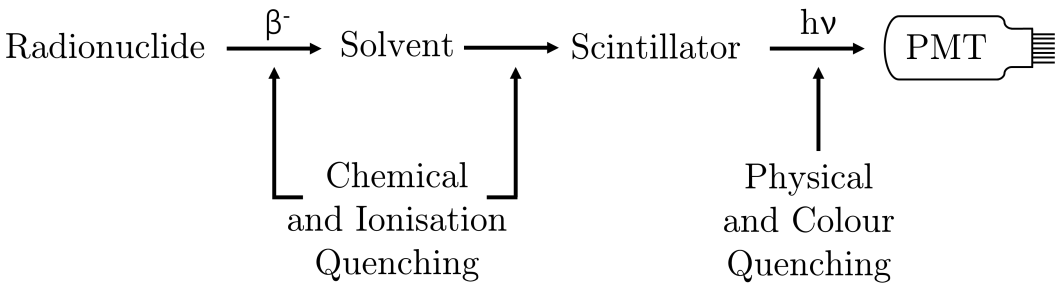


Figure 2.6: A schematic representation of the liquid scintillation process. The radiation energy emitted (e.g. β^--particle energy) by a decaying radionuclide is absorbed by the solvent. The activated solvent molecule transfers the energy to a scintillator molecule, which gets excited thereby. Upon deexcitation this molecule emits light, which is detected with a photomultiplier tube. This PMT converts the light into an electrical signal. Due to the sample composition and nature of the radiation energy, the energy of the decay may not reach the PMT. This phenomenon is called quenching. Chemical and ionisation quenching prevent (part of) the energy of the decay reaching the scintillator molecules, thereby reducing the number of counts and the intensity of the signal. Physical and colour quenching prevent the light emitted from the scintillator molecule from reaching the PMT, thereby reducing the number of counts. [39]

(1) physical quenching, (2) chemical quenching (3) ionisation quenching and (4) optical or colour quenching. Where these types of quenching occur in the liquid scintillation process is depicted in Figure 2.6.

Physical quenching
Physical quenching occurs when the radionuclide is physically separated from the scintillation cocktail. This effect can arise when the sample is not completely dissolved, and exist as solid particles. Proper homogenisation after adding the sample to the scintillation cocktail can prevent this type of physical quenching [40]. Another example is that fingerprints at the outside of the counting vial will block some of the scintillation light. This can be prevented by cleaning the vial before placing it into the LSC.

Chemical quenching
Chemical quenching can occur when compounds in the solvent or the sample absorb (a part of) the energy emitted by the radionuclide or the energy transferred by the solvent. Less energy will reach the scintillator molecules, and therefore the number and the intensity of counts is reduced. Chemical quenching cannot be prevented, however can be accounted for, which is explained in below in *Methods of quench correction.* [15, 39]

Ionisation quenching
Another type of quenching that reduces the amount of energy reaching the scintillator molecules is ionisation quenching. This occurs for radionuclides that decay by α-decay, low-energy β-decay and electron capture. The linear energy transfer (LET), or stopping power, of these radiation types is high. This means that per unit length, the energy transfer to the surrounding area is high. In case of LSC this results in a high concentration of excited solvent molecules, which increases the probability of interaction between two excited solvent molecules. In such an interaction one molecule deexcites by transferring its energy to the other excited molecule, which in its turn becomes super-excited. A super-excited molecule has a high change of becoming ionised. The eventual result is that both molecules lost (part of) their excitation energy and did not transfer this energy to a scintillation molecule, which results in less counts and a reduction of photo intensity [39, 41].

Colour quenching
The last quenching phenomenon is colour quenching. This occurs when colour in the sample absorbs the photons produced by the scintillator before being detected by the PMT, thereby reducing the amount of measured counts. Colour quenching can be prevented by either adding less sample or bleaching the sample before adding to the counting cocktail [15, 39].

Methods of quench correction
Two methods can be used to correct for quenching: the internal standard method, and the external standard quench indicating parameters method.

In the *internal standard method* a known amount of activity is added to an already counted sample. The counting efficiency for this particularly sample can than be obtained by:

$$\eta = \frac{CPM_{sample+standard} - CPM_{sample}}{DPM_{standard}} \tag{2.7}$$

where η is de efficiency, $CPM_{sample+standard}$ the counts per minute of the sample after the addition of the activity, CPM_{sample} the counts per minute of the sample alone, and $DPM_{standard}$ is the disintegrations per minute (DPM) of the added standard. The DPM of the sample can than be calculated by:

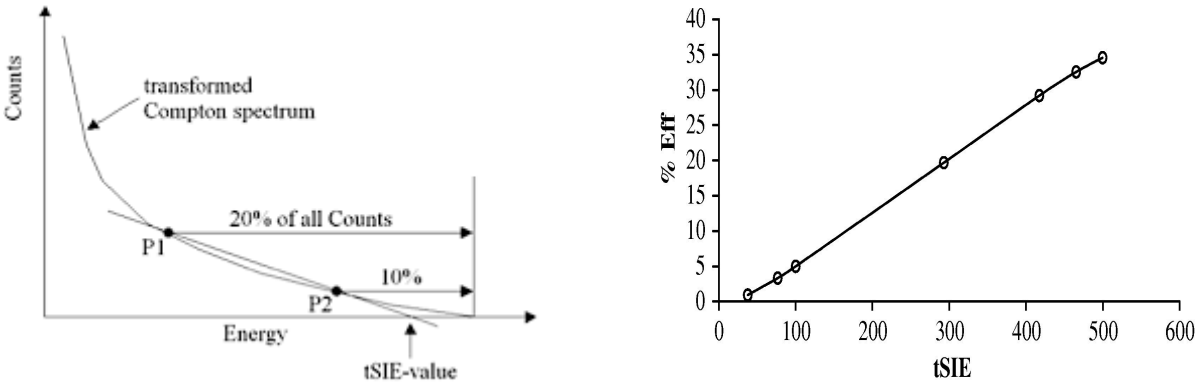
$$DPM_{sample} = \frac{CPM_{sample}}{\eta}. \tag{2.8}$$

When performed correctly, the internal standard method can provide extremely accurate results, but is however very labour intensive and therefore mainly used for a small number of samples [15, 39].

The *external standard method* is performed with an external γ-radiation source within the LSC, to determine the Quench Indicating Parameter (QIP). The sample is first counted without the external source, and subsequently the LSC places the γ-source next to the counting vial, whereafter the Compton spectrum is measured. This Compton spectrum is quenched in the same way as the sample spectrum, and can therefore be used as a measure of the sample quenching. QIPs are determined in various manners and with various sources. The QIP used in this project is the transformed spectral index of the external standard (tSIE). For information about other QIPs the reader is directed to [39].

The tSIE-value is derived using the external standard Compton spectrum. To obtain the 'Reverse Spectrum Transform, a summation of counts in the individual measurements channels is done, starting at the end of the spectrum. This transformed spectrum can be seen in Figure 2.7a. Two points are chosen on this spectrum: P1, which is, starting at the back of the spectrum, at 20% of the counts and P2, which is at 10% of the counts. The line connecting P1 and P2 is intersecting the x-axis, this point is defined as the tSIE. An unquenched sample has a tSIE value of 1000. The tSIE-value is reset during the calibration of the LSC. [42]

A quench correction curve can be made by counting a series of samples, each containing a known amount of activity and different amounts of quenching material. Since the activity is known, and the count rate and tSIE-value are measured, the tSIE-value can be plotted against the efficiency per sample (Figure 2.7b. When subsequently an unknown sample is measured, the tSIE-value is determined and the counting efficiency can be read from the quench correction curve. By knowing the count rate and the efficiency the sample activity can be calculated via Equation (2.8) [15, 42].



(a) [42] **(b)** [43]

Figure 2.7: Determination of the tSIE-value and example of quench correction curve. In 2.7a the method to determine the tSIE-value is shown. In 2.7b a quench correction curve is shown for ^{55}Fe quenched with nitromethane.

2.6.3 Mössbauer Spectroscopy

Mössbauer spectroscopy is a non-destructive analytical technique, that provides information about the chemical, structural magnetic properties of a material. It is based on the recoilless γ-ray emission and absorption by nuclei, the so-called "Mössbauer effect". First the basic principle of the Mössbauer effect is explained, then it is described how this effect is applied in Mössbauer spectroscopy. Lastly the set-up of a Mössbauer spectrometer is presented.

Mössbauer effect

Consider a free excited nucleus with an energy of E_0 (eV), which on decay emits a γ-ray with energy E_γ (eV). Due to momentum conservation the free nucleus will receive a recoil momentum p_R (eV/c) to compensate for the momentum of the emitted γ-ray ($p_\gamma = E_\gamma/c$).

Under the assumption that $E_R << E_0$, the energy of the γ-ray is then given by:

$$E_\gamma = E_0 - E_R = E_0 - \frac{E_0^2}{2Mc^2}, \tag{2.9}$$

where E_R (eV) is the recoil energy of the free nucleus, M (eV/c^2) the mass of the free nucleus and c the speed of light in vacuum [44].

Consider now a second free identical nucleus. When this nucleus would get excited by absorbing the γ-ray emitted by the first nucleus, this is called nuclear resonance. Due to the aforementioned conservation law, the first nucleus emitted the γ-ray with an energy $E_0 - E_R$, whereas to overcome the recoil effect and be absorbed by the second nucleus, the emitted γ-ray energy should be $E_0 + E_R$. Nuclear resonance will generally not be observed for free nuclei, because this energy deficiency is $\approx 10^6$ times larger than the energy spread of the γ-ray, the natural line width Γ, which is shown in Figure 2.8.

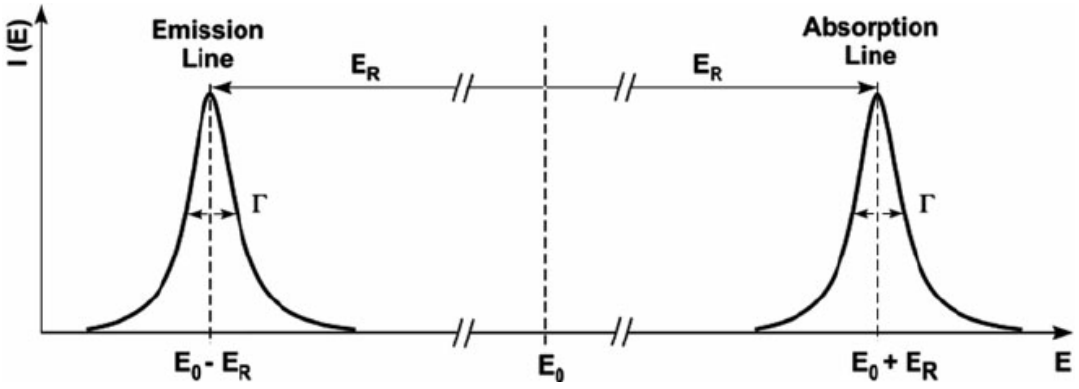


Figure 2.8: Energy separation due to recoil energy E_R between γ-ray emission and absorption for free nuclei. Since $2E_R \approx 10^6\Gamma$ nuclear resonance will generally not be observed. [44]

Instead of having two free nuclei, now imagine them being bound in a solid lattice. Upon decay of the first nucleus, the whole lattice recoils, taking up the energy as a lattice vibration, a phonon. The effective mass in Equation 2.9 is now the mass of the whole system. As a result E_R is negligible and γ-ray energy equals E_0. Phonons are quantised, and therefore when E_R is smaller than the smallest phonon energy E_{phonon}, there are two possibilities: (1) E_R is absorbed by the lattice or (2) no E_R is transferred to the lattice and the emitted γ-rays. In the latter case, the energy distributions of the emission and absorption line, shown in 2.8, will overlap, which makes nuclear resonance possible. However, both of these options violate momentum conservation. Due to quantum mechanics the solutions lay in the superposition of these two possibilities; the momentum is conserved by the probability of occurrence of both possibilities [45]. The probability of the zero-phonon process (possibility (2)) is known as the recoil-free fraction and given by the Lamb-Mössbauer (or Debye-Waller) factor [14]. The Lamb-Mössbauer factor increases with decreasing γ-photon energy, decreasing temperature and increasing Debye temperature (which is a measure for the strength of the lattice) [14].

Due to Heisenberg's uncertainty principle ($\Delta E \Delta t \geq \hbar$) the energy of the γ-ray cannot be determined exactly [14]. ΔE is the aforementioned natural linewidth Γ and depends on the lifetime Δt of the nucleus [44], $\hbar$ is

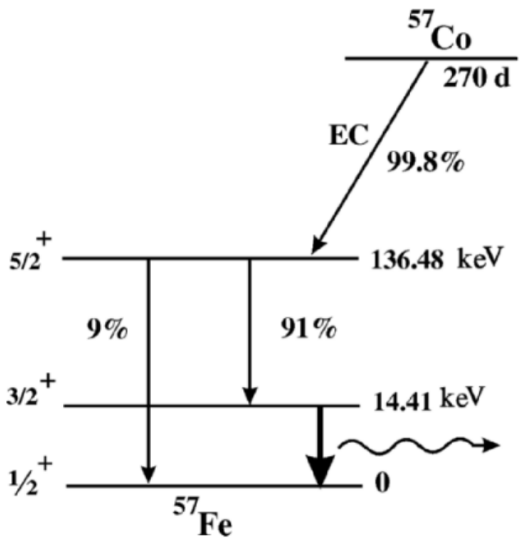


Figure 2.9: Decay scheme of ^{57}Co. During the decay of ^{57}Co to ^{57}Fe a γ-ray of 14.4keV is emitted, which is used in Mössbauer spectroscopy. [14]

reduced Planck constant. When $10^{-11}\ s < \Delta t < 10^{-6}\ s$, the natural linewidth can be used to probe hyperfine interactions in the nucleus, which are the interactions between a nucleus and its surroundings. This is the basis of Mössbauer spectroscopy, which will be explained in the next section. Longer lifetimes will produce absorption lines that are too small to measure, whereas shorter lifetimes have a too low resolution to be able to see the hyperfine interactions [14, 44]. Therefore not all isotopes can be measured with Mössbauer spectroscopy. Of all the used isotopes, ^{57}Fe is the most commonly used. It has a relatively long-lived excited state ($1.4{\cdot}10^{-7}\ s$) and a low lying excited state of 14.4 keV, which was important for the Lamb-Mössbauer factor. The radioactive source used for ^{57}Fe is ^{57}Co; its decay scheme is depicted in Figure 2.9.

Mössbauer spectroscopy
Mössbauer spectroscopy is a combination of the Mössbauer effect and the Doppler effect. In the previous section it was explained that nuclear resonance can occur when the energy of an emitted γ-ray equals the energy to absorb the γ-ray. However when the chemical environment of the nuclei differs slightly, the nuclear levels of the nuclei will be different, and nuclear resonance will not occur. By moving one of the nuclei in a controlled fashion with velocity v (m/s), the relativistic Doppler effect is used to tune the resonance energy:

$$E_\gamma(v) = E_0 \sqrt{\frac{1+v/c}{1-v/c}}. \tag{2.10}$$

In case of ^{57}Fe, when moving the source with 1 mm/s towards the sample, the energy of the photons is increased by ten natural linewidths. So when a known source sends out a γ-ray, through the sample, into the detector, the Mössbauer transmission spectrum can be obtained (Figure 2.10).

In case the source and absorber have an identical chemical environment, the Mössbauer spectrum would be a single absorption line at a doppler velocity of 0 mm/s. However the nuclear energy levels, and thereby the Mössbauer spectrum, can be changed by the following hyperfine interactions: (1) the isomer shift, (2) electric quadrupole splitting and (3) magnetic hyperfine splitting, which are depicted in Figure 2.11.

1. *The isomer shift* (I.S.) results from the Coulomb interactions between the positively charged nucleus and the negatively charged electron cloud. This interactions depend e.g. on the oxidation state of the nucleus. The isomer shift only shifts the nuclear levels without lifting the degeneracy [14, 45, 47].

2. *The electric quadrupole splitting* (Q.S.) results from the interaction with a non-spherical charge distribution around the nucleus, i.e. the deviation from a cubic symmetry of the charge distribution generated by the surroundings of the nucleus. When the nucleus experiences an electric field gradient, its

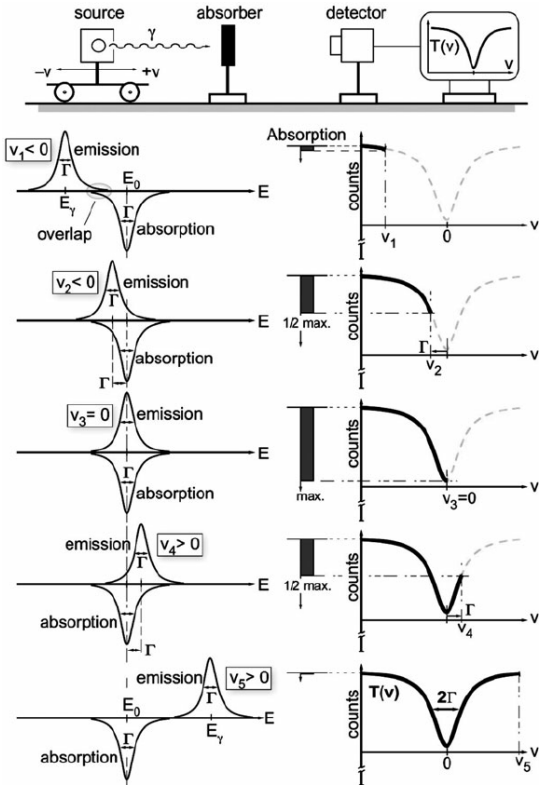


Figure 2.10: Schematic representation of collecting a Mössbauer spectrum. In this case of identical nuclei. [44]

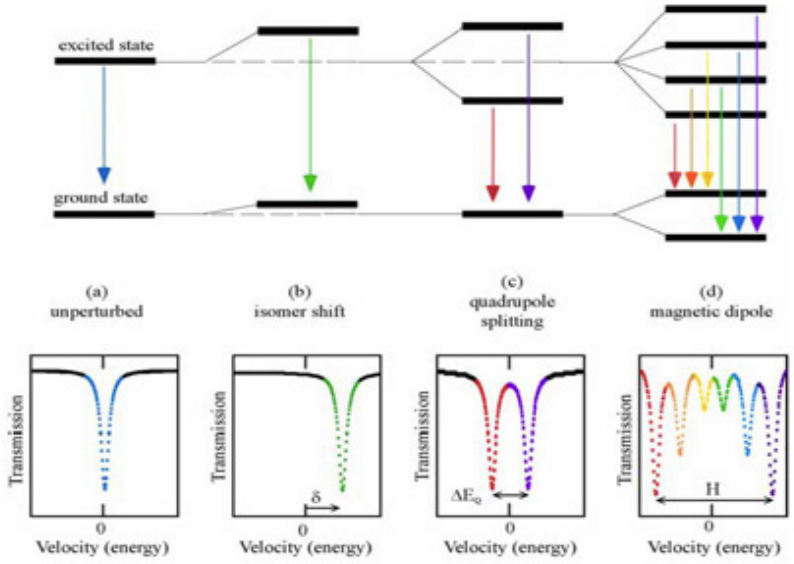


Figure 2.11: Overview of the hyperfine parameters and their influence on the nuclear energy levels and the resulting Mössbauer spectrum. [46]

excited state is splits in two states, as can be seen in Figure 2.11. In the spectrum this is observed as two absorption peaks [14, 45, 47].

3. *The magnetic hyperfine splitting* is caused by the interaction of the nuclear magnetic dipole moment with the magnetic field around the nucleus. This splitting is described by the Zeeman Effect. In case of ^{57}Fe the nuclear levels are splits into six levels, as shown in Figure 2.11. [14, 45, 47]

The effects described above can be observed together. For compounds consisting of multiple Mössbauer nuclei, the spectrum will consist of a combination of the single absorption peaks, not an average [45].

In case the reader is interested in mathematical and fundamental background on the Mössbauer effect and Mössbauer spectroscopy, the reader is directed to Verma [14], Yoshida and Langouche [48], Dickson [47], Gütlich *et al.* [44] and Zhang [49].

Mössbauer spectroscopy set-up
A Mössbauer spectrometer consists of several components (Figure 2.12). The source contains an isotope, which on decay becomes the Mössbauer isotope of interest. The source is mounted on a transducer, which lets the source move back and forth in a manner governed by the waveform generator. A typical velocity domain for ^{57}Fe is -10 mm/s to 10 mm/s. The γ-ray emitted by the source is altered by the velocity of the source, and its transmission spectrum is detected by the detector. The data is collected in the data acquisition system, where the measured Mössbauer spectrum is linked to the adequate channel number. Calibration of the system is done with an absorber with known peaks, so the channel number can be linked to a corresponding velocity [45, 47].

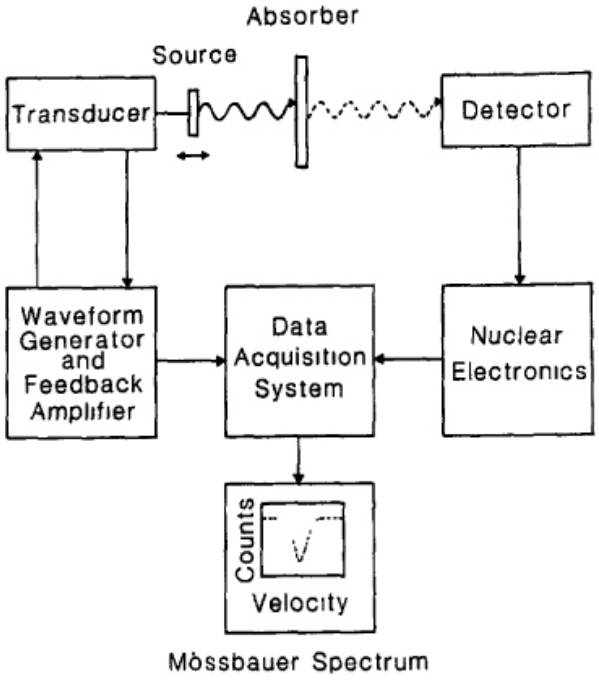


Figure 2.12: Schematic overview of the Mössbauer spectrometer. The source is moved by the transducer, in a manner governed by the waveform generator. The γ-ray emitted by the source travels through the absorber. The resulting transmission spectrum is detected by the detector. With the data acquisition system the spectrum is linked to the corresponding velocity of the source. [47]

Mössbauer sample preparation The measurement time of a sample depends on the amount of iron in the sample. To be able to measure lower iron concentrations with Mössbauer spectroscopy it is convenient to fortify the milk samples with enriched iron powder to reduce measurement time. The natural abundance of ^{57}Fe is 2.1%, therefore by using 95.40% ^{57}Fe (as in this project), the measurement time is reduced by a factor ~50.

3

Materials and Methods

3.1 Materials

3.1.1 Chemicals

The chemical compounds that were used in this research project are listed in Table 3.1.

Table 3.1: List of chemical compounds that were used to perform the experiments in this project.

Chemical	Formula	Supplier
HEPES, BioPerformance Certified, ≥ 99.5% (titration), suitable for cell culture	$C_8H_{18}N_2O_4S$	Sigma-Aldrich
Hydrochloric acid 30%, Technical	HCl	VWR Chemicals
Hydrogen peroxide 30%, (Perhydrol™) (stabilized for higher storage temp.) for analysis EMSURE ISO	H_2O_2	Supelco
Iron (powder) 98.4%, BAKER ANALYZED	Fe	J.T. Baker
Iron-55 Radionuclide, 74 MBq, Ferric Chloride in 0.5M HCl	$^{55}FeCl_3$	PerkinElmer
Iron-57 (metal powder), 95.40% Isotopic enrichment		
Iron(II) chloride tetrahydrate, for analysis EMSURE	$Fe(II)Cl_2{\cdot}4H_2O$	Supelco
Iron(III) chloride hexahydrate, for analysis EMSURE ACS, Reag. Ph Eur	$Fe(III)Cl_3{\cdot}6H_2O$	Supelco
Iron ICP Standard, traceable to SRM from NIST Fe(NO3)3 in HNO3 2-3% 1000 mg/L Fe Certipur		Merck
Lactorpure lactose, p.d. 20-7-2018, Non Food Grade		Wageningen University
L-Ascorbic acid, ACS reagent, ≥ 99%	$C_6H_8O_6$	Sigma-Aldrich
Nitric acid 65%, Suprapur	HNO_3	Supelco
Phosphoric acid solution, 85 wt. % in H_2O, FCC, FG	H_3PO_4	Sigma-Aldrich
Potassium Chloride, ACS Reagent, 99.7%	KCl	Sigma
Potassium Permangganate, 99.5%, BAKER ANALYZED	$KMnO_4$	J.T. Baker
Skimmed milk		Jumbo privat label
Sodium Chloride (crystals), BAKER ANALYZED	$NaCl$	J.T. Baker
Sodium Hydroxide, puriss. p.a., ACS reagent, reag. Ph. Eur., K ≤ 0.02%, ≥ 98%, pellets	$NaOH$	Sigma-Aldrich
Sulphuric acid, puriss. p.a., for determination of Hg, ACS reag., reag. ISO, reag. Ph. Eur., 95.0-97.0%	H_2SO_4	Sigma-Aldrich
Tannic acid, ACS reagent	$C_{76}H_{52}O_{46}$	Sigma-Aldrich
Ultima Gold™ XR		PerkinElmer

3.1.2 Products

In this project the following products were used:

- Polypropylene Bell-top Quick-Seal Centrifuge Tubes (11x32 mm), purchased from Beckman Coulter.
- VWR Centrifugal filters, Modified PES, 10 kDa MCWO, 500 μL, purchased from VWR International.
- Spectra/Por 4 Dialysis Membrane, Standard Grade Regenerated Cellulose tubing, 12-14 kDa MWCO 32 mm, purchased from Repligen.
- Dialysis tubing Membra-Cel MC24x100 Clr, 14 kDa MCWO, purchased from Viskase.
- 20 mL High Performance Glass Vial, for Liquid Scintillation Counting, purchased from PerkinElmer.
- 20 mL Gewindeflaschen, FIOLAX, purchased from Fischer Scientific.

3.1.3 Instruments

The following instruments were used in this project:

- Inductively coupled plasma - optical emission spectrometer (ICP-OES), Optima 4300 DV, PerkinElmer. Used protocol: Plasma flow rate 15 L/min, Auxiliary gas flow rate 0.2 L/min, Nebulizer gas flow rate 0.80 L/min, Power 1400 W, View distance 15.0, Plasma view axial and Sample flow rate 1.50 mL/min were used. HNO_3 was used to wash between samples. The following wavelengths were used to measure the iron content: 238.204, 239.562, 259.939, 234.349, 234.830, 238.863, 273.955 nm. For the calibration lines Iron ICP standard was used. The concentrations of the calibration points were: 5.014, 10.178, 15.112, 20.052 mg/L.
- 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. HNO_3 was used to wash between samples. The following wavelengths were used to measure the iron content: 238.205, 239.563, 259.941, 234.351, 234.830, 238.863, 273.955 nm. For the calibration lines Iron ICP standard was used. The concentrations of the calibration points were: 5.125, 10.954, 16.193, 21.381 mg/L.
- Liquid Scintillation Counter (LSC), tri-carb 2750TR/LL, Packard. The used protocol was: Count time between 10-60 minutes (depending on experiment), 1 cycle, data mode CPM, coincidence time 18 ns, no automatic background substract, Quench indicator tSie and no halflife correction were used. Three regions were measured: region A: 0.0 - 6.0 keV, region B: 2.0 - 6.0 keV, region C: 6.0-2000 keV. Region A was used for calculations.
- Microwave Reaction System, Multiwave PRO, Anton Paar. Two protocols were used: a temperature controlled program and a power controlled program. The temperature program went to 180°C in 40 minutes, then to 200°C in 20 minutes, held at 200°C for 20 minutes and lastly cooled down to 70 degrees in 20 minutes. The power controlled program was in 60 minutes increased to 1300 W, then held at 1300 W for 20 minutes and then cooled to 70°C by delivering no power.
- Transmission ^{57}Fe Mössbauer spectra were collected at room temperature with a conventional constant-acceleration spectrometer using a ^{57}Co(Rh) source. Velocity calibration was carried out using an α-Fe foil. The Mössbauer spectra were fitted using Mosswinn 4.0 program.
- Centrifuge, Jouan CR4i, Thermo. Used protocol: 200g, 30 minutes at 20°C.
- Optima^{TM} MAX Ultracentrifuge, Beckman Coulter. Used protocol: 100,000g, 60 minutes at 20°C.
- Microcentrifuge, Micro star 17R, VWR. Used protocol: 13,000g, 40 minutes at 20°C.
- Freeze drier, EZ-DRY EZ550Q, Kinetics thermal systems. Used protocol: temperature: -50°C, pressure: 25 mT (33 μbar, time: >24h).

3.2 Methods

This section first describes the synthesis of $FeCl_2$, subsequently the preparation of the milk samples and the following iron fortification (section 3.2.2) is explained. Afterwards it is described how the phase separation methods are performed (section 3.2.3). Then it is explained how the ICP-OES, LSC and Mössbauer spectrometer were used to analyse the milk samples (section 3.2.4). Lastly a short note on the statistical analysis of the experiments is given.

3.2.1 Synthesis of $FeCl_2$

To synthesise $FeCl_2$, the following reaction was used:

$$Fe + 2HCl \longrightarrow FeCl_2 + H_2.$$

Elementary iron in powder form was added to an Erlenmeyer flask. To obtain the preferred concentration (0.5 M or 1.7 M) first a certain amount of Milli-Q was added (depending on the amount of HCl needed for this concentration). Then an excess of 30% HCl was added to the solution to ensure that all the iron powder would dissolve ($Fe : HCl$ as 1:3, instead of the 1:2 needed for the reaction). This iron-solution was heated to 90°C for two hours. While heating, the Erlenmeyer flask was loosely closed with a stopper, so the H_2 could escape, whilst condensing water vapour. Meanwhile the solution was stirred without stirring bar. Iron powder is magnetic, so it stirs itself. A stirring bar could only be added after complete dissolution of the iron powder, since otherwise the powder would stick to the stirring bar instead of dissolving. The synthesis of the $FeCl_2$-solution was finished when the solution obtained a (pale) green colour and no iron powder was left in the solution, this was after approximately two hours. For smaller volumes the Erlenmeyer flask was exchanged for glass vials of 3 mL. The concentration of the obtained solution was checked by titration, see the section on titration below. Milk samples were fortified with the synthesised $FeCl_2$ and compared with milk fortified with off the shelf $FeCl_2{\cdot}4H_2O$. The $FeCl_2{\cdot}4H_2O$ was dissolved in the same conditions as the synthesised $FeCl_2$, to prevent pH differences from influencing the comparison.

Titration

Permanganate titration was used to check the obtained concentration of the synthesised $FeCl_2$ solution. The method was adapted from R.B. Fischer [50].

For the permanganate titration, a stirring bar was added to an Erlenmeyer flask. Afterwards 50 mL of Milli-Q water was added to ease the stirring, 6 mL of the Zimmermann-Reinhardt reagent was pipetted in the flask and 2.5 mL of the analyte (the $FeCl_2$-solution) was added. The Zimmermann-Reinhardt reagent was made by adding 4 g of $KMnO_4$ to an erlenmeyer, dissolving it in 60 mL of Milli-Q water, adding 10 mL of 95-97% H_2SO_4 and 10 mL of 85% H_3PO_4 [50]. The Erlenmeyer flask was placed on white paper on a stirrer. Then, depending on the expected concentration, a certain volume of 0.02 M permanganate solution was added to the Erlenmeyer flask until a faint pink colour persisted.

3.2.2 Milk sample preparation and iron fortification

Synthesised $FeCl_2$-behaviour in milk compared to off the shelf $FeCl_2{\cdot}4H_2O$

For the comparison of the iron behaviour in milk between synthesised $FeCl_2$ and milk samples fortified with off the shelf $FeCl_2{\cdot}4H_2O$, milk samples were fortified to 3 mmol/L and 10 mmol/L with the synthesised $FeCl_2$ and off the shelf $FeCl_2{\cdot}4H_2O$-solutions described in the previous section. To obtain these concentrations, the iron-solutions were pipetted into the milk and stored in the refrigerator for 24 hours, while stirring. The following phase separation procedure or Mössbauer spectroscopy preparation procedure are described below in section 3.2.3 and 3.2.4 respectively.

Other experiments

Milk was fortified with either $FeCl_2{\cdot}4H_2O$ or $FeCl_3{\cdot}6H_2O$ to achieve final concentrations of 5 and 20 mmol/L. This was obtained by dissolving the appropriate amount of the iron salt in Milli-Q water. To prevent the milk from being diluted by the iron solution, the volume of Milli-Q, used to dissolve the iron salt, was ~0.5% of the volume of the milk to be fortified. After the iron was dissolved, the milk was added to the iron solution and stirred for 5-10 minutes.

Following the stirring, the iron fortified milk was divided into four parts: a further untreated part, a further untreated part that would be stored for one week before analysis, a part to which ascorbic acid was added

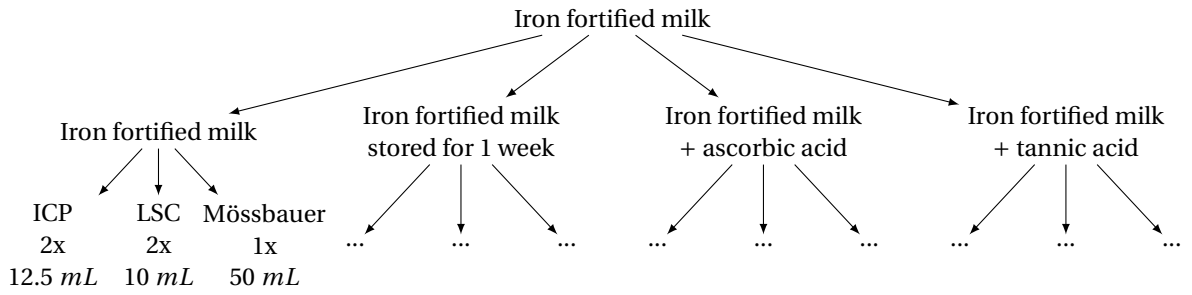


Figure 3.1: Schematic overview of the milk fortification process. Milk is fortified with either $FeCl_2{\cdot}4H_2O$ or $FeCl_3{\cdot}6H_2O$ to achieve final concentrations of 5 and 20 mmol L^{-1}. This iron fortified milk was divided into four parts: further untreated milk, further untreated milk that was stored for one week before analysis, milk to which also ascorbic acid was added and a part to which tannic acid was added. These samples were further analysed with the ICP, LSC and Mössbauer spectroscopy.

and a part to which was tannic acid was added (Figure 3.1). The ascorbic acid and tannic acid solutions were obtained in the same way as the iron solution as described above. After the ascorbic acid and tannic acid were solved, the iron fortified milk was added to these solutions. Of every sample type, ~10 mL was kept aside to measure the pH using a pH-meter.

The four types of samples were per type split into three categories: samples to be measured with the ICP-OES (2x 12.5 mL), LSC (2x 10mL) and Mössbauer spectroscopy (1x 50 mL). To the LSC-samples 17 μL of 0.1 GBq/L $^{55}FeCl_3$-solution was added as tracer. The samples were put in a carousel in the refrigerator for 24 hours or for one week.

3.2.3 Phase separation methods

Phase separation was performed using two experimental protocols: phase separation by dialysis and phase separation by (ultra)centrifugation and filtration. The experiments were performed in at least duplicate and a max quadruplicate. The experiments were performed at lab temperature which varied between 18°C and 28°C. The used protocols in this subsection are based on the protocols developed by De Vos [34].

Dialysis

The dialysis experiment was set-up as follows: First 15 cm dialysis membrane was soaked in Milli-Q water for approximately 30 or 60 minutes for respectively the Spectra/Por dialysis membrane and the Membra-Cel membrane. The now flexible membrane was opened at one side with a needle or when possible by rubbing the membrane. Then a half hitch (knot) was made at one side of the membrane, that was carefully tightened to prevent the dialysate from leaking out. The dialysate is a solution in Milli-Q water that contains 87 mM NaCl, 350 mM KCl and 205 mM lactose. Using a pipette, 5 mL of this dialysate was added to the membrane. The membrane was closed at the top by placing a clothespin transversely. The whole was hung in a graduated cylinder with 100 mL of milk sample and covered with Parafilm to prevent evaporation. The sample was then stirred for 24 hours in the fume hood.

After 24 hours the dialysis membrane was removed from the graduated cylinder. The membrane was cut open with scissors and the dialysate was removed with a pipette. Also the retentate milk sample was analysed. The analysis of dialysis samples was done using the ICP-OES instrument. The sample preparation for the ICP-OES is described in the section below on sample analysis (section 3.2.4). No dialysis experiments were performed using ^{55}Fe.

Centrifugation and filtration

After storage in the refrigerator, the milk samples were placed in the centrifuge at 200g for 30 minutes at 20°C. After centrifugation, 2 mL was extracted from the non-sedimental phase and transferred to an ultracentrifuge tube by using a syringe and needle. The ultracentrifuge tubes were sealed by placing a seal former on top. Several methods were used to heat the seal former: a tube topper, a soldering iron, or the seal former would be held above a burner and then transferred on top of the ultracentrifuge tube. After sealing a heat sink was placed on top immediately and afterwards the seal former was removed with tweezers.

The sealed ultracentrifuge tubes were placed in the ultracentrifuge at 100,000g for 60 minutes at 20°C. After the ultracentrifugation the supernatant needed to be transferred to an eppendorf with a 10 kDa filter. For

Figure 3.2: Needles in ultracentrifuge tube to remove supernatant with a syringe. The second needle is placed to prevent the occurrence of a vacuum.

the non-radioactive samples the top of the ultracentrifuge tubes was cut off with scissors, then a needle and syringe were used to transfer 0.5 mL of the supernatant to the eppendorf. In case of radioactive sample a small needle was inserted at the top of the ultracentrifuge tube to let air into the tube. A second needle (0.9x40 mm) and syringe was used to extract 0.5 mL from the tube and transfer the supernatant to the eppendorf. The needles were placed such that the precipitate was not punctured, while ensuring that the needles would not puncture the sides, see Figure 3.2. The eppendorfs with filter were placed in the microcentrifuge at 13,000g for 40 minutes at 20°C.

The initial sample, the non-sedimental phase, the supernatant and the filtered sample were analysed with the ICP-OES or the LSC.

3.2.4 Analytical methods for sample analysis

The samples were measured using three measurement techniques: the ICP-OES was used to measure the concentration of iron in the samples; the LSC was used to measure the radioactivity originating from ^{55}Fe; and Mössbauer spectroscopy was used to provide information on the chemical state of the iron in the samples. Below the used methods for these measurement techniques are described.

ICP-OES

Since the milk matrix cannot directly be introduced into the plasma of the ICP-OES, the milk samples were first digested into a solution [51]. This was done in three steps. First, 0.2-1 mL of the milk sample was added to microwave vessel liner. Then 4.5 mL of HNO_3 65% and 1.5 mL of H_2O_2 30% were also added to the microwave vessel liner. Next the liners were placed in the microwave vessel bodies in the microwave carousel and the whole was placed into the microwave digestion system.

During this project two protocols were used for the microwave digestion system: a temperature controlled program and a power controlled program. The transition from temperature to power controlled, was made since the temperature probe broke during the course of this project. The temperature controlled program started with heating to 180°C in 40 minutes, then further increased to 200°C in 20 minutes. It held at 200°C for 20 minutes and then cooled down to 70°C in 20 minutes. The power controlled program was in 60 minutes increased to 1300 W, then held at 1300 W for 20 minutes and afterwards cooled to 70°C by delivering no power. This temperature is sensed with an infrared sensor, that can not be used for a temperature controlled program.

To stay under the upper detection limit of the ICP-OES [51], the digested samples needed to be diluted. From the microwave vessel, the digested sample was transferred via a funnel into a volumetric flask of 25 mL or 50 mL, depending on the starting concentration of the sample. After transfer, the digested samples were diluted with Milli-Q water up to 25 mL or 50 mL and afterwards homogenised. Finally, the diluted samples were

poured into a 15 mL tube. The ICP-OES measurements were performed on the digested and diluted samples.

It is not necessary for samples that contain solely soluble ions, to be digested. However, to ensure that no differences would arise from different methods, an equal treatment was used for these type of samples.

LSC
To determine the percentage of radioactivity in each phase after the phase separation, the samples were analysed with the LSC. The samples were prepared in the following way: in 20 mL scintillation vials, 10 mL of liquid scintillation cocktail was added. Then for the initial and once centrifuged tannic acid samples, 10 μL of the sample was added, since the purple colour of tannic acid within iron fortified milk induced optical quenching. For the other samples 100 μL was added to the glass vial. To prevent the added sample from sticking to the bottom of the vial, after addition - and closure of the vial - the sample was shaken by hand immediately. After the samples were added to the vials, the vials were vortexed for approximately 20 seconds.

The measurement of the samples with the LSC was done as follows: before the start of the measurement, the LSC was calibrated using a ^{14}C, ^{3}H and a background standard. After calibration the samples were measured. Each sample batch started with a background sample, which contained 10 mL of liquid scintillation cocktail. Also two control samples were measured containing 10 and 100 μL of 0.17 MBq/L, which is the same activity as was added from the initial samples into the glass vials. All the samples were measured for 60 minutes, starting with the initial samples and the once centrifuged samples. It was done in this order because these two samples types unmix sooner, however still after measuring.

Before the first sample measurement with the LSC three control measurements were performed: (1) what amount of activity needed to be added to stay at least 10 times above the background, (2) what maximal volume of milk could be added while having minimal quenching and lastly (3) whether ascorbic acid and tannic acid induced chemical quenching. The samples for these checks were measured once and for 30 minutes per sample.

(1) The activity that needed to be added to the samples was determined by adding a range of activities of $^{55}FeCl_3$ to vials filled with 10 mL of liquid scintillation cocktail. The added activity was plotted against the count rate and compared to a background sample.

(2) The maximal volume of milk that could be added to the vials was determined by adding a fixed amount of activity and a varying amount of milk to the vials (10 μL - 5 mL). It was also checked whether time influenced the measurement, so by immediately measuring the samples, waiting for 1 hour or 18 hours before measuring the samples.

(3) Then lastly a measurement was performed to check whether ascorbic acid and tannic acid induced chemical quenching. This was done by adding 100 μL of 0.17 MBq/L to two vials, one for ascorbic acid and one for tannic acid. After each measurement 25 μL of ascorbic acid solution and tannic acid solution were added to these vials. In this way a range from 0 - 200 μL was made. The ascorbic acid and tannic acid solutions consisted of: 20 mM $FeCl_3 \cdot 6H_2O$ and 26 mM ascorbic acid or 2.7 mM tannic acid.

Mössbauer spectroscopy
The samples meant to be measured with Mössbauer spectroscopy were poured in a crystallising dish and covered with Parafilm, before being put in the freezer at -50°C for 24 hours. Afterwards the crystallising dishes were transported to the freeze dryer in crushed ice, to prevent the sample from melting. Then holes were made into the Parafilm, and the crystallising dish was placed into the freeze dryer for at least 30 hours. The remaining pellet was ground to fine powder with a mortar and pestle. Using the Lambert-Beer law it was determined that the samples, of the chosen concentrations, had to weigh 1.8 g. The samples were measured for at least 18 hours.

3.2.5 Statistics
The centrifugation and dialysis methods were run in at least duplicate. The experiments were not repeated with a new made iron solution. There were too few samples to perform a valuable Welch's test, therefore the results were normalised and compared at a confidence interval of two standard deviations (95%). Data was considered to be significantly different when the errorbars overlapped less than one arm [52].

4

Results and Discussion

This chapter reports first reports the results of the synthesis of $FeCl_2$. Subsequently, the influence of five factors on the bioavailability of iron in skim milk is presented. These factors are: the iron chloride concentration, the molecular form of iron chloride ($FeCl_2$ and $FeCl_3$), the storage time after fortification, the addition of an iron enhancer (ascorbic acid) and the addition of an iron inhibitor (tannic acid). As a measure for the iron bioavailability the phase distribution and oxidation state of iron in milk are used. Preliminary research such as determination of quench curves for LSC measurements, can be found in Appendix B.

4.1 Synthesis of $FeCl_2$

The measurement time with Mössbauer spectroscopy of the high iron concentrations of 5 and 20 mmol/L milk was ~1 day. To be able to measure lower concentrations of 0.07-0.2 mmol/L, which are often used in iron fortification for infant formula, it was desired to synthesise enriched $^{57}FeCl_2$ and $^{57}FeCl_3$ and use as fortificant. Since enriched iron is available to a lesser extent and more expensive than normal elementary iron, the synthesis of $FeCl_2$ was first performed with elementary iron, before trying to synthesise the salt with ^{57}Fe. Due to time constraints the synthesis of $FeCl_3$ was not performed.

4.1.1 Synthesis non-enriched $FeCl_2$

$FeCl_2$ was successfully synthesised according to the method described in section 3.2.1. The performed reaction was: $Fe + 2HCl \longrightarrow FeCl_2 + H_2$. Two concentrations were made (0.5 M, 1.7 M), to observe whether the concentration would impact the synthesis process, which was not found to be the case. These concentration were checked and verified by permanganate titration (results not shown). The beginning and end of the synthesis are shown in Figure 4.1a & b.

(a) **(b)** **(c)**

Figure 4.1: Three time steps in the synthesis of $FeCl_2$. (a) Iron solution at beginning of synthesis. HCl is added to elementary iron and heated to 90°C for two hours. (b) 0.5 M $FeCl_2$-solution at end of synthesis. (c) Undesired oxidised 0.5 M $FeCl_2$-solution after left standing for one week.

Within a week, the $FeCl_2$-solution turns from pale green to a yellow-green solution, as can be seen in Figure 4.1c. This can be explained by the following undesired oxidation reaction: $4FeCl_2 + O_2 + 4HCl \longrightarrow 4FeCl_3 + 2H_2O$. Titration was not executed to verify this explanation. To prevent potential influence this oxidation reaction, the $FeCl_2$-solution was always prepared right before use.

4.1.2 Synthesised $FeCl_2$-behaviour in milk compared to off the shelf $FeCl_2{\cdot}4H_2O$

A comparison on the behaviour in milk was made between off the shelf $FeCl_2{\cdot}4H_2O$ and the synthesised $FeCl_2$. Milk samples were fortified to a concentration of 3 and 10 mmol Fe/L milk. The phase separation was performed using centrifuging and dialysis; and Mössbauer spectroscopy was used to obtain information about the oxidation state. These results are first presented below and afterwards they will be discussed and compared.

Results $FeCl_2$-synthesis analysis by centrifugation

Figure 4.2 shows the phase distribution (obtained by centrifugation) of milk fortified to a concentration of 3 mmol Fe/L milk (Figure 4.2a) and 10 mmol Fe/L milk (Figure 4.2b). There is no statistical difference between the synthesised and off the shelf samples, except for the soluble phase of the 3 mmol/L samples (2.3±0.4% for synthesised and 2.9±0.4% for off the shelf). Since there is also no statistical difference between the 3 mmol/L and 10 mmol/L, the dialysis and Mössbauer spectroscopy are only performed on the 3 mmol/L to spend less iron and save time.

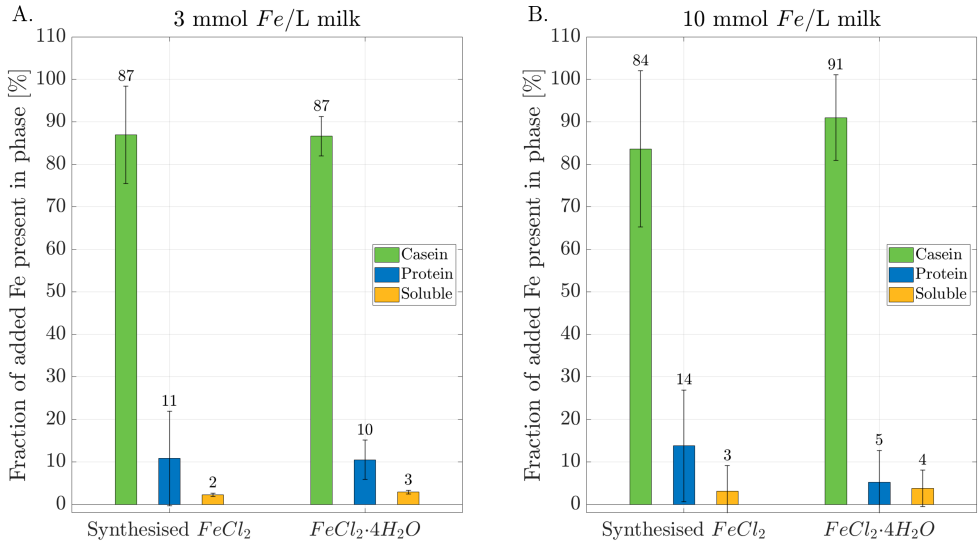


Figure 4.2: Phase separation by centrifugation. Comparison of in milk behaviour between synthesised $FeCl_2$ and off the shelf $FeCl_2{\cdot}4H_2O$ for 3 mmol Fe/L (A) and 10 mmol Fe/L milk (B). Both concentrations show no significant difference in iron percentage in the different phases. Only the soluble phase of the 3 mmol Fe/L milk shows a slight increase for the off the shelf $FeCl_2{\cdot}4H_2O$.

Results $FeCl_2$-synthesis analysis by dialysis

The results of the dialysis (Figure 4.3) also show that the percentage of iron in the soluble phase for the synthesised $FeCl_2$ is slightly higher than $FeCl_2{\cdot}4H_2O$ (3.6±0.1% versus 3.1±0.3%). The iron present in the retentate plus the iron present dialysate added up to the iron present at the start of the dialysis, therefore the retentate associated iron does not provide new information and is not shown in the figure.

Results $FeCl_2$-synthesis analysis by Mössbauer spectroscopy

In Table 4.1 the Mössbauer fitted parameters of milk fortified with the synthesised $FeCl_2$ are compared to the parameters of the milk fortified with off the shelf $FeCl_2{\cdot}4H_2O$. The isomer shift (I.S.), which is a measure for the oxidation state, is comparable for both samples. The spectral contribution of Fe^{2+} is slightly higher for the synthesised $FeCl_2$ compared to the $FeCl_2{\cdot}4H_2O$ (78±3% versus 71%). The quadrupole splitting (Q.S.), which is a measure for the crystal structure, differs significantly for the Fe^{3+} (2.39±0.01 mm/s versus 2.47±0.01 mm/s). This difference can be due to the difference in hydration state between the synthesised and off the shelf $FeCl_2$ [53]. This measurement was performed once and therefore incidental errors, as for example pipetting errors, cannot be identified.

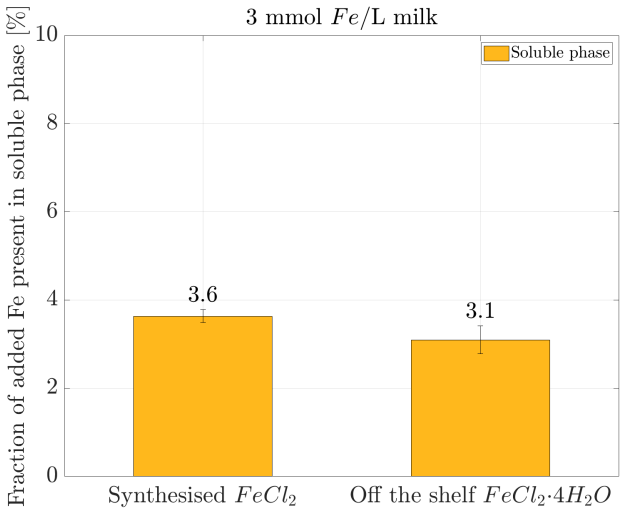


Figure 4.3: Phase separation by dialysis. Comparison of iron in milk behaviour between synthesised $FeCl_2$ and off the shelf $FeCl_2{\cdot}4H_2O$ for 3 mmol Fe/L. Note the axis is cut off at 10%. Together with the iron in the retentate, the iron content adds up to 100% of the iron in the sample at the start of the dialysis.

Table 4.1: Mössbauer fitted parameters of the milk fortified with synthesised $FeCl_2$ and off the shelf $FeCl_2{\cdot}4H_2O$. For both samples the concentration of iron in milk was 3 mmol/L. Experimental uncertainties: Isomer shift: I.S. ± 0.01 mm s^{-1}; Quadrupole splitting: Q.S. ± 0.01 mm s^{-1}; Line width: Γ ± 0.01 mm s^{-1}; Spectral contribution: ± 3%.

3 mmol Fe/L milk	IS [mm s^{-1}]	QS [mm s^{-1}]	Γ [mm s^{-1}]	Phase	Spectral contribution [%]
Synthesised $FeCl_2$	0.41	0.63	0.56	Fe^{2+}	78
	1.14	2.39	0.56	Fe^{3+}	22
Off the shelf $FeCl_2{\cdot}4H_2O$	0.41	0.63	0.55	Fe^{2+}	71
	1.17	2.47	0.55	Fe^{3+}	29

Comparing results of different analytical methods

The results for the three different methods showed similar behaviour of iron in milk for the synthesised $FeCl_2$ and off the shelf $FeCl_2{\cdot}4H_2O$. Both in centrifugation and dialysis the soluble phase was slightly higher for the off the shelf $FeCl_2{\cdot}4H_2O$. The Mössbauer results on the other hand show a higher spectral contribution of Fe^{2+} in the synthesised $FeCl_2$ samples, whereas it is believed that a higher amount of Fe^{2+} would result in a higher iron concentration in the soluble phase. Since the Mössbauer measurement was performed once and the centrifugation and dialysis experiments were fortified out of one stock solution of $FeCl_2$ or $FeCl_2{\cdot}4H_2O$, it is believed that these small differences probably arose due to incidental errors. It was concluded that the synthesis method performed good enough to synthesise $^{57}FeCl_2$.

4.1.3 Synthesis $^{57}FeCl_2$

During the synthesis of $^{57}FeCl_2$ some difficulties were encountered that were not present in the synthesis of the non-enriched $FeCl_2$. Where the normal elementary iron was dissolved in HCl in two hours at 90°C, the enriched iron did not dissolve in two days and after addition of extra HCl. Only after 300 minutes in an ultrasonic bath at 60°C, it was dissolved. Titration was not performed since the volume made was only 250μL. The small volume was not the cause of the insolubility, since the same volume was successfully prepared with $FeCl_2{\cdot}4H_2O$ beforehand. After synthesis, the enriched $^{57}FeCl_2$-solution was added to milk, however this milk samples coagulated due either the high temperature in the laboratorium (27°C) or due to the lower pH of the $^{57}FeCl_2$-solution. Due to time constraints this synthesis has not been performed again. Therefore it was chosen to fortify the milk to higher concentrations (5 mmol/L and 20 mmol/L) to be able to perform the measurements in one day, instead of using the more typical concentrations of iron fortified milk (~0.2 mmol/L [21]).

4.2 Phase distribution and oxidation state of iron chloride in milk

In this section results are presented of milk samples fortified with $FeCl_2$ and $FeCl_3$ to obtain final concentrations of 5 mmol/L and 20 mmol/L (section 4.2.1). The influence of storage time (section 4.2.2), the addition of an enhancer (section 4.2.3) and an inhibitor (section 4.2.4) were investigated. The phase distribution of the samples was analysed using centrifugation, and for the 20 mmol/L $FeCl_3$ also a tracer study was performed with $^{55}FeCl_3$. Information on the oxidation state of iron in the milk samples has been obtained by Mössbauer spectroscopy. In this section $FeCl_2{\cdot}4H_2O$ is addressed as $FeCl_2$ and $FeCl_3{\cdot}6H_2O$ as $FeCl_3$.

4.2.1 Influence of molecular form of iron chloride and concentration

Figure 4.4a presents the phase separation results of milk fortified with $FeCl_2$ to a concentration of 5 mmol/L and 20 mmol/L. It is observed that the percentage of iron associated to the casein fraction increased with higher concentration (73±8% versus 82±7%), whereas the protein associated fraction decreased (20±9% versus 9.6±0.8%). The percentage of iron in the soluble phase was not significantly different for both concentrations. The precipitation step has been executed to remove possible precipitation arising from the addition of the iron to the milk, that would otherwise wrongly be attributed to the casein fraction. For the $FeCl_2$-samples this is not an significant amount.

In Figure 4.4b the phase separation results of the milk fortified with $FeCl_3$ to a concentration of 5 mmol/L and 20 mmol/L are depicted. The experiment on the high concentration was also performed using the tracer ^{55}Fe. Since no significant difference is observed between the non-radioactive and radioactive sample, it is shown that ^{55}Fe can be used as tracer in the phase separation experiments and can be used to compare to the 5 mmol/L sample. The increase in percentage of iron present in the casein fraction is not observed for the $FeCl_3$-samples. The protein fraction significantly decreased (19±6% versus 2.6±0.7% (non-radioactive) or 3.3±0.4% (radioactive)). Comparing the precipitation fraction of the 5 mmol/L to the 20 mmol/L tracer sample, it appears that for higher concentration the iron precipitates.

Comparing the equal concentrations of the different salt shows that for 5 mmol/L the ferrous salt behaves similar to the ferric salt. The ferric 20 mmol/L has a significantly lower iron percentage associated to the protein and soluble fraction, and a higher percentage of iron precipitated.

Precipitation

The precipitation can be explained by considering the pH of the milk samples, since caseins coagulate around pH 4.6 and can then be removed by low-speed centrifugation [22]. The addition of $FeCl_2$ and $FeCl_3$ led to a decrease in pH (also observed by Gaucheron *et al.* [54]), which is stronger for $FeCl_3$ (pH 5.0 (20 mmol/L))

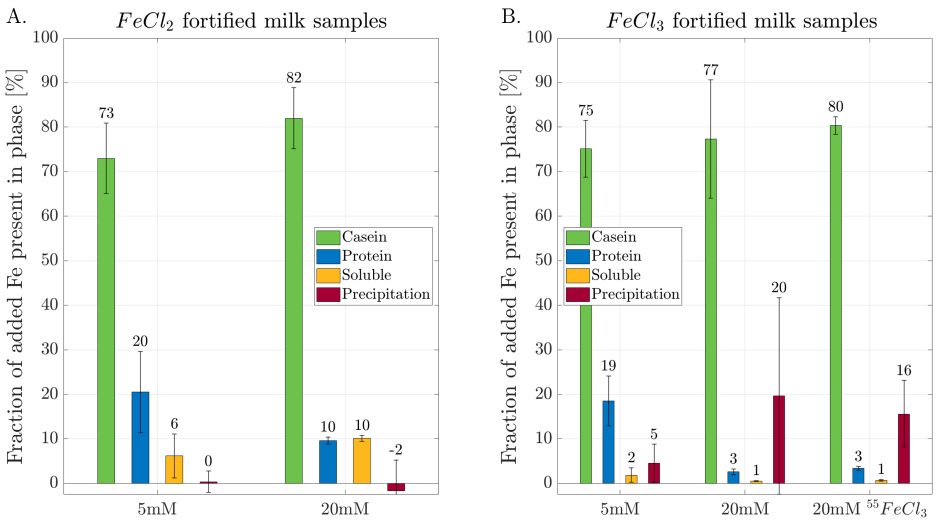


Figure 4.4: Milk fortified with $FeCl_2$ (A) and with $FeCl_3$ (B) to 5 mmol/L and 20 mmol/L. These results have been obtained using centrifugation. In green the iron percentage associated to the casein fraction, in blue the protein fraction, yellow the soluble phase and in red the iron the precipitated at low centrifugation speed. The radioactive sample was fortified to 20 mmol/L $FeCl_3$ and afterwards 17 kBq of $^{55}FeCl_3$ was added.

than for $FeCl_2$ (pH 5.8 (20 mmol/L)). Therefore (part of) the casein could have precipitated, leading to an underestimation of the iron present in the measured casein fraction. In this project, this effect is observed in all samples with a pH lower than 5.0. By chilling the milk in ice, and adding the iron solution dropwise while stirring, the coagulation can be reduced [11] and would therefore be good practice for further research.

Protein fraction
For both molecular forms of iron a decrease is visible in the iron associated to the protein fraction with increasing concentration. Also this effect can be attributed to the pH-difference (pH 6.5 to 5.8 for $FeCl_2$ and pH 6.1 to 5.0 $FeCl_3$), since the binding ability of the whey proteins decreases with lower pH, as observed by Hekmat and McMahon for $FeCl_3$ [55].

Casein fraction
Hekmat and McMahon [55] also observed an increase of iron present in the caseins for lower pH (83% for pH 6.7-5.3 versus 92% for pH ≤4.5), as also observed in this project for the $FeCl_2$. That this effect is not visible for the $FeCl_3$-samples can be attributed to the increase in precipitation. It is expected that the improved preparation procedure would show an increase in casein fraction for $FeCl_3$ too, by reducing the precipitation. The increase of iron present in the casein fraction is attributed to the pH difference instead of the concentration difference, since the increase is not observed for off the shelf samples as presented in section 4.2 (pH 6.6 for 3 mmol/L and pH 6.3 for 10 mmol/L), which were prepared using a different fortification procedure. Also Raouche *et al.* [11] fortified milk to 5 mmol/L and 20 mmol/L with $FeCl_2$ and $FeCl_3$, but did correct for the pH, and did not observe an increase in iron percentage bound to the casein micelles, endorsing the assumption that the increase in casein fraction for increasing concentration can be attributed to the pH difference.

Soluble phase and oxidation state
The soluble phase does not seem to be affected by the pH, although King *et al.* [27] observed an increase of 0.6% for pH 6.7-5.0. This accuracy was not reached in this project and therefore the effect may not be visible. However, the influence of the oxidation state of the added iron is visible when comparing the high concentrations (10.1±0.7% $FeCl_2$ versus 0.5±0.1% $FeCl_2$ or 0.7±0.2% $^{55}FeCl_2$). This is endorsed by the Mössbauer results (Table 4.2), where the difference in spectral contribution is significant for the 20 mmol/L samples (22±3% $FeCl_2$ versus 5±3% $FeCl_3$). Comparing the phase distribution with the spectral contribution of Fe^{2+} (22±3%), it shows that the ferrous iron is not only present in the soluble phase (10.1±0.7%). Raouche *et al.* [11] studied the Mössbauer spectra of micelles of milk fortified with $FeCl_2$ and $FeCl_3$ and found that 10% is associated to the micelles for milk fortified to 20 mmol/L. Making the crude assumption that these results are completely comparable, it means that the remaining 12% was distributed over the protein and soluble phase. Better assumptions can be made when both the caseins and the milk as a whole, originating from the same source, are measured with Mössbauer spectroscopy.

Table 4.2: Mössbauer fitted parameters for influence of iron salt and concentration on iron fortified milk samples. Milk samples were fortified with $FeCl_2$ and $FeCl_3$ to concentrations of 5 mmol/L and 20 mmol/L. Experimental uncertainties: Isomer shift: I.S. ± 0.01 mm s^{-1}; Spectral contribution: I ± 3%.

Sample		IS [mm s^{-1}]	Phase	I [%]
$FeCl_2$	5 mmol/L	0.40	Fe^{3+}	100
	20 mmol/L	0.41	Fe^{3+}	78
		1.15	Fe^{2+}	22
$FeCl_3$	5 mmol/L	0.41	Fe^{3+}	95
		1.13	Fe^{2+}	5
	20 mmol/L	0.41	Fe^{3+}	95
		1.15	Fe^{2+}	5

The iron in the $FeCl_2$ 5 mmol/L samples completely oxidised, due to the formation of iron-casein complexes [10]. The $FeCl_3$-samples surprisingly show 5±3% of Fe^{2+}. To the best of the author's knowledge, a reduction reaction has not been observed in milk without other additives beside iron. Therefore it would be interesting to measure the redox potential, to observe whether reducing conditions apply that account for this effect.

The results of the $FeCl_2$ and $FeCl_3$-samples will be used as reference in the following sections. For easier comparison they are showed again in the figures and tables as reference.

4.2.2 Influence of time

To investigate the influence of time between fortification and further analysis, on the phase separation and Mössbauer spectrum, the $FeCl_2$ and $FeCl_3$ fortified milk samples were stored for one week after fortification. The results are shown in Figure 4.5.

After one week, the iron was mainly associated to the casein fraction and increased in percentage (Figure 4.5 A,C) compared to the samples stored for 24 hours (Figure 4.5 B, D). Only the 20 mmol/L $FeCl_2$-sample did not show a significant difference. However, both the protein and soluble fraction decreased significantly and therefore it is plausible that either the casein fraction or precipitation fraction increased. The pH of the samples stored for one week was not measured, so it cannot be determined whether the increase in casein fraction originates from a decrease in pH as seen before. The increase in casein fraction originates from the decrease of the percentage of iron associated to the protein fraction and soluble phase.

Unlike the phase separation results, the Mössbauer spectrum does not indicate significant differences in the sample stored for one week before freeze drying compared to the sample stored for 24 hours; the ratio between Fe^{2+} and Fe^{3+} is similar for all samples (Table 4.3). The phase separation and oxidation state results are conflicting since the binding to the casein micelles induces the oxidation of iron from Fe^{2+} to Fe^{3+} [10], so an increase in Fe^{3+} should have been visible. It is not believed that the storage time between freeze drying and oxidation state analysis was of influence, since the difference ranged from measured 10 days earlier to 19 days later than reference sample, whereas the spectral contribution stayed similar. To the best of the author's knowledge, no other studies investigated the influence of storage time on a bioavailability parameter (longer than 24 hours). Therefore to track down the origin of the seemingly conflicting results, the casein fraction can be analysed with Mössbauer spectroscopy, to investigate whether the oxidation state of the iron in the casein micelles changes over time or stays similar. It can be that only Fe^{3+} binds to the casein over the course of the week. Also the quadrupole splitting can provide information on the binding of the iron to the milk. This parameter is measured and changes over time (see Appendix A, but evaluation was outside the scope of this project.

Table 4.3: Mössbauer fitted parameters for influence of time on iron fortified milk samples compared to the reference samples that were measured after 24 hours. Milk samples were fortified with $FeCl_2$ and $FeCl_3$ to concentrations of 5 mmol/L and 20 mmol/L and stored for one week after. Experimental uncertainties: Isomer shift: I.S. ± 0.01 mm s^{-1}; Spectral contribution: I ± 3%.

Sample		IS [mm s^{-1}]	Phase	I [%]
$FeCl_2$	5 mmol/L	0.40	Fe^{3+}	100
	20 mmol/L	0.41	Fe^{3+}	78
		1.15	Fe^{2+}	22
$FeCl_3$	5 mmol/L	0.41	Fe^{3+}	95
		1.13	Fe^{2+}	5
	20 mmol/L	0.41	Fe^{3+}	95
		1.15	Fe^{2+}	5
$FeCl_2$ stored for 1 week	5 mmol/L	0.40	Fe^{3+}	100
	20 mmol/L	0.42	Fe^{3+}	76
		1.18	Fe^{2+}	24
$FeCl_3$ stored for 1 week	5 mmol/L	0.41	Fe^{3+}	95
		1.25	Fe^{2+}	5
	20 mmol/L	0.41	Fe^{3+}	97
		1.12	Fe^{2+}	3

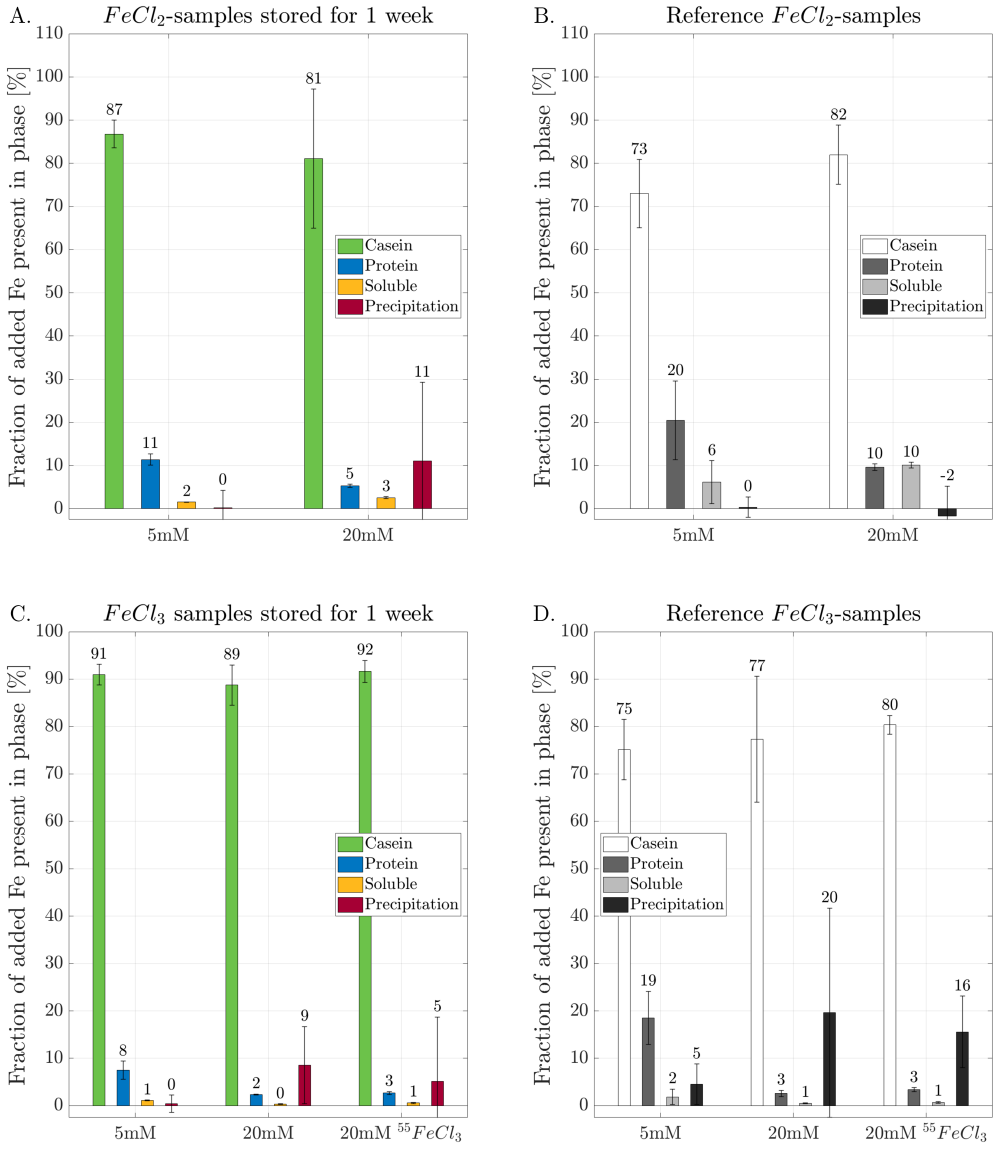


Figure 4.5: Milk fortified with $FeCl_2$ (A) and with $FeCl_3$ (C) stored for one week before freeze drying compared to the reference samples (B, D respectively) that were measured after 24 hours. In green (white) the iron percentage associated to the casein fraction, in blue (grey) the protein fraction, yellow (light grey) the soluble phase and in red (black) the iron the precipitated at low centrifugation speed. The radioactive sample was fortified to 20 mmol/L $FeCl_3$ and afterwards 17 kBq of $^{55}FeCl_3$ was added.

4.2.3 Influence of addition of enhancer

To maximise the bioavailability of an iron fortified product, iron enhancers are often added. Since the solubility is a measure of the bioavailability, it was expected that the addition of an iron enhancer, such as ascorbic acid, would increase the iron associated to the soluble phase [10, 56, 57]. Figure 4.6 shows that, except for the 5 mmol/L $FeCl_2$-sample, the iron associated to the soluble phase indeed increased significantly upon addition of ascorbic acid (vitamin C). The 5 mmol/L $FeCl_2$-sample did not change significantly.

Interestingly, the enhancing effect appears to be greater for the higher concentration samples, although the amount of ascorbic acid is equal for both samples. Therefore part of the effect can be attributed to the decrease in pH ($FeCl_2$ 5 mmol/L pH 6.5-5.5, 20 mmol/L pH 5.8-4.9; $FeCl_3$ 5 mmol/L pH 6.1-5.3, 20 mmol/L pH 5.0-4.5), as also observed by King *et al.* [27] who investigated the distribution of iron in milk at different pH (increase of 44% pH 6.8-4.5). As seen in section 4.2.1, the samples with a pH ≤5.0 show significant precipitation. The precipitation also explains the difference between iron in the soluble phase of the radioactive and non-radioactive 20 mmol/L sample, since the other fractions did not differ significantly and are measured with better accuracy. The increase in iron in the soluble fraction is balanced by the decrease of iron associated to the casein fraction; the ascorbic acid chelates the iron and forms a soluble complex [19].

Iron absoprtion is enhanced by ascorbic acid by reducing Fe^{3+} to Fe^{2+} and/or chelating the Fe^{3+} [58]. The Mössbauer parameters (Table 4.4) suggest that the addition of ascorbic acid does not necessarily reduce the Fe^{3+}. The 5 mmol/L sample still showed only Fe^{3+}, and the 20 mmol/L sample decreased from 22±3% to 16±3% of Fe^{2+} upon addition of ascorbic acid. Only the 20 mmol/L $FeCl_3$-sample shows that the addition of ascorbic acid results in significantly more Fe^{2+} (5±3% to 16±3%), even though the sample is measured two days later. It is not believed that the storage time between freeze drying and measurement was of influence on this measurement since the 20 mmol/L $FeCl_2$ was measured 7 days earlier than the reference sample and already showed a lower Fe^{2+} contribution. To observe whether it is rightfully assumed that time influence is absent, a sample can be measured multiple times: directly after freeze-drying and multiple times later over the course of a week or a month.

Table 4.4: Mössbauer fitted parameters of iron fortified milk with added ascorbic acid compared to the reference samples. Milk samples were fortified with $FeCl_2$ and $FeCl_3$ to concentrations of 5 mmol/L and 20 mmol/L. Subsequently 5 g ascorbic acid / L milk was added. Experimental uncertainties: Isomer shift: I.S. ± 0.01 mm s^{-1}; Spectral contribution: I ± 3%.

Sample		IS [mm s^{-1}]	Phase	I [%]
$FeCl_2$	5 mmol/L	0.40	Fe^{3+}	100
	20 mmol/L	0.41	Fe^{3+}	78
		1.15	Fe^{2+}	22
$FeCl_3$	5 mmol/L	0.41	Fe^{3+}	95
		1.13	Fe^{2+}	5
	20 mmol/L	0.41	Fe^{3+}	95
		1.15	Fe^{2+}	5
$FeCl_2$ + ascorbic acid	5 mmol/L	0.40	Fe^{3+}	100
	20 mmol/L	0.42	Fe^{3+}	84
		1.19	Fe^{2+}	16
$FeCl_3$ + ascorbic acid	5 mmol/L	0.41	Fe^{3+}	92
		1.15	Fe^{2+}	8
	20 mmol/L	0.42	Fe^{3+}	84
		1.19	Fe^{2+}	16

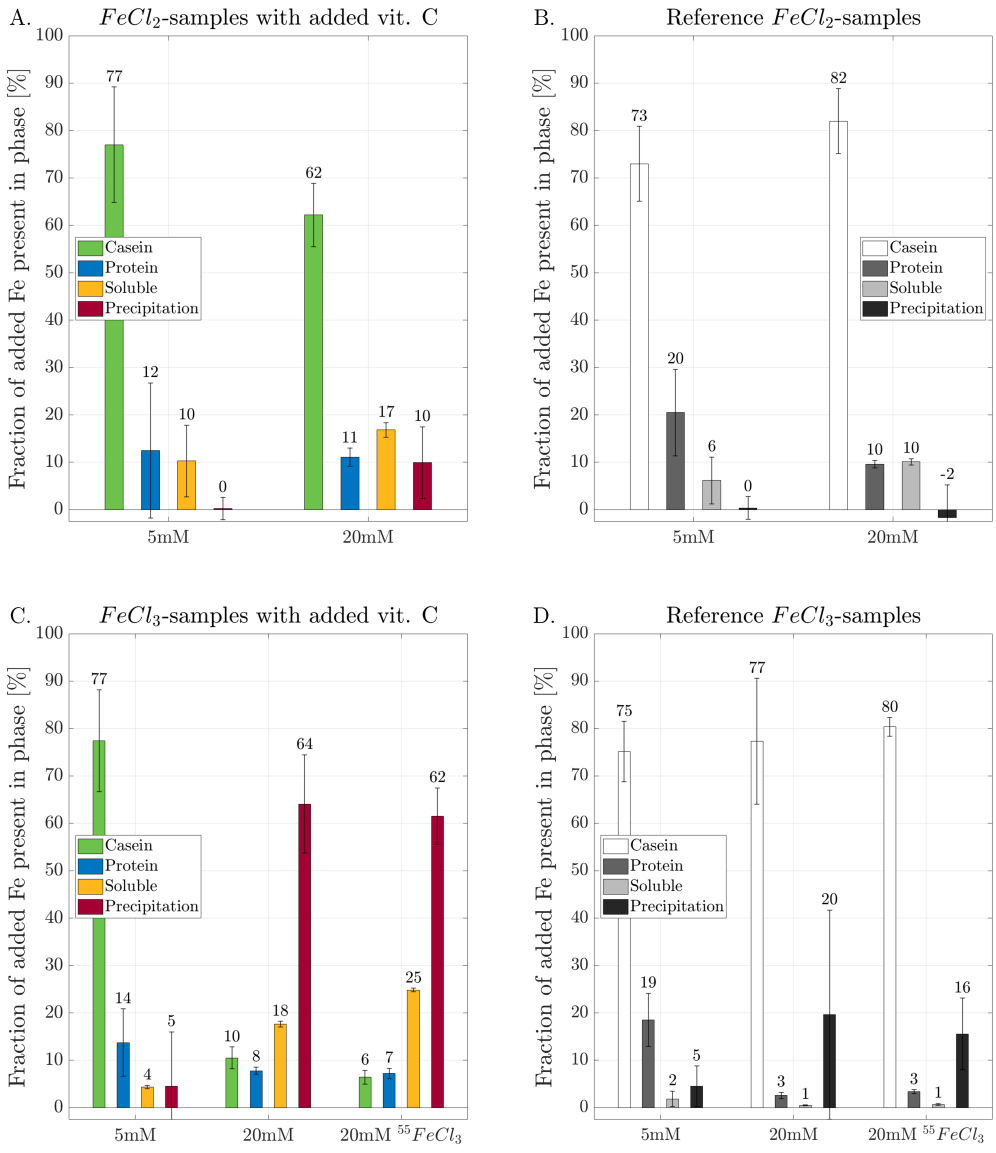


Figure 4.6: Milk fortified with $FeCl_2$ (A) and with $FeCl_3$ (B) + ascorbic acid (vitamin C) compared to the reference samples (B, D respectively) without ascorbic acid. Samples contained 5 g ascorbic acid /L milk. In green (white) the iron percentage associated to the casein fraction, in blue (grey) the protein fraction, yellow (light grey) the soluble phase and in red (black) the iron the precipitated at low centrifugation speed. The radioactive sample was fortified to 20 mmol/L $FeCl_3$ and afterwards 17 kBq of $^{55}FeCl_3$ was added.

4.2.4 Influence of addition of inhibitor

The influence on the iron distribution was also observed for the addition of an inhibitor, which is expected to lower the iron in the soluble state compared to the samples without the inhibitor. The results are shown in Figure 4.7.

The phase separation results clearly show a decrease in iron present in the protein fraction and the soluble phase, which was reflected in an increase in the casein fraction and precipitation associated iron. This corresponds with the results of Kalgaonkar and Lonnerdal [59] who also observed a significant reducing effect (9.9±1.1%) of tannic acid on the uptake of ferrous iron by Caco-2 cells.

Precipitation now also occurred for samples with pH > 5.0 (pH 5.7 for 20 mmol/L $FeCl_2$), meaning that the compound formed by iron bound the tannic acid precipitates. This is endorsed by a follow-up experiment, in which a comparison on solubility was made between tannic acid added to milk and tannic acid added to an iron solution. Tannic acid with milk or $FeCl_2$ resulted in a solution, whereas the tannic acid combined with $FeCl_3$ resulted in a suspension. A study performed by Hem [60] with 1:2 ppm - 1:50 ppm Fe:tannic acid did not show precipitation, meaning that the ratio between the tannic acid and iron should be more carefully chosen.

The Mössbauer parameters for these milk samples are presented in Table 4.5. Upon addition of tannic acid the Fe^{3+} contribution clearly increased, endorsing the reduction in soluble fraction observed in the phase separation experiment. It is not believed that the oxidation originates from the time difference between freeze-drying and analysis, since the 20 mmol/L $FeCl_2$ sample with tannic acid was measured after two days, whereas the reference sample was measured after 10 days. Even in this short time span, the sample contains less Fe^{2+} thereby suggesting that the tannic acid indeed reduced the Fe^{2+} present in the milk samples.

Table 4.5: Mössbauer fitted parameters of iron fortified milk with added tannic acid compared to the reference samples. Milk samples were fortified with $FeCl_2$ and $FeCl_3$ to concentrations of 5 mmol/L and 20 mmol/L. Subsequently 5 g tannic acid / L milk was addded. Experimental uncertainties: Isomer shift: I.S. ± 0.01 mm s^{-1}; Spectral contribution: I ± 3%.

Sample		IS [mm s^{-1}]	Phase	I [%]
$FeCl_2$	5 mmol/L	0.40	Fe^{3+}	100
	20 mmol/L	0.41	Fe^{3+}	78
		1.15	Fe^{2+}	22
$FeCl_3$	5 mmol/L	0.41	Fe^{3+}	95
		1.13	Fe^{2+}	5
	20 mmol/L	0.41	Fe^{3+}	95
		1.15	Fe^{2+}	5
$FeCl_2$ + tannic acid	5 mmol/L	0.41	Fe^{3+}	100
	20 mmol/L	0.42	Fe^{3+}	91
		1.17	Fe^{2+}	9
$FeCl_3$ + tannic acid	5 mmol/L	0.40	Fe^{3+}	100
	20 mmol/L	0.41	Fe^{3+}	100

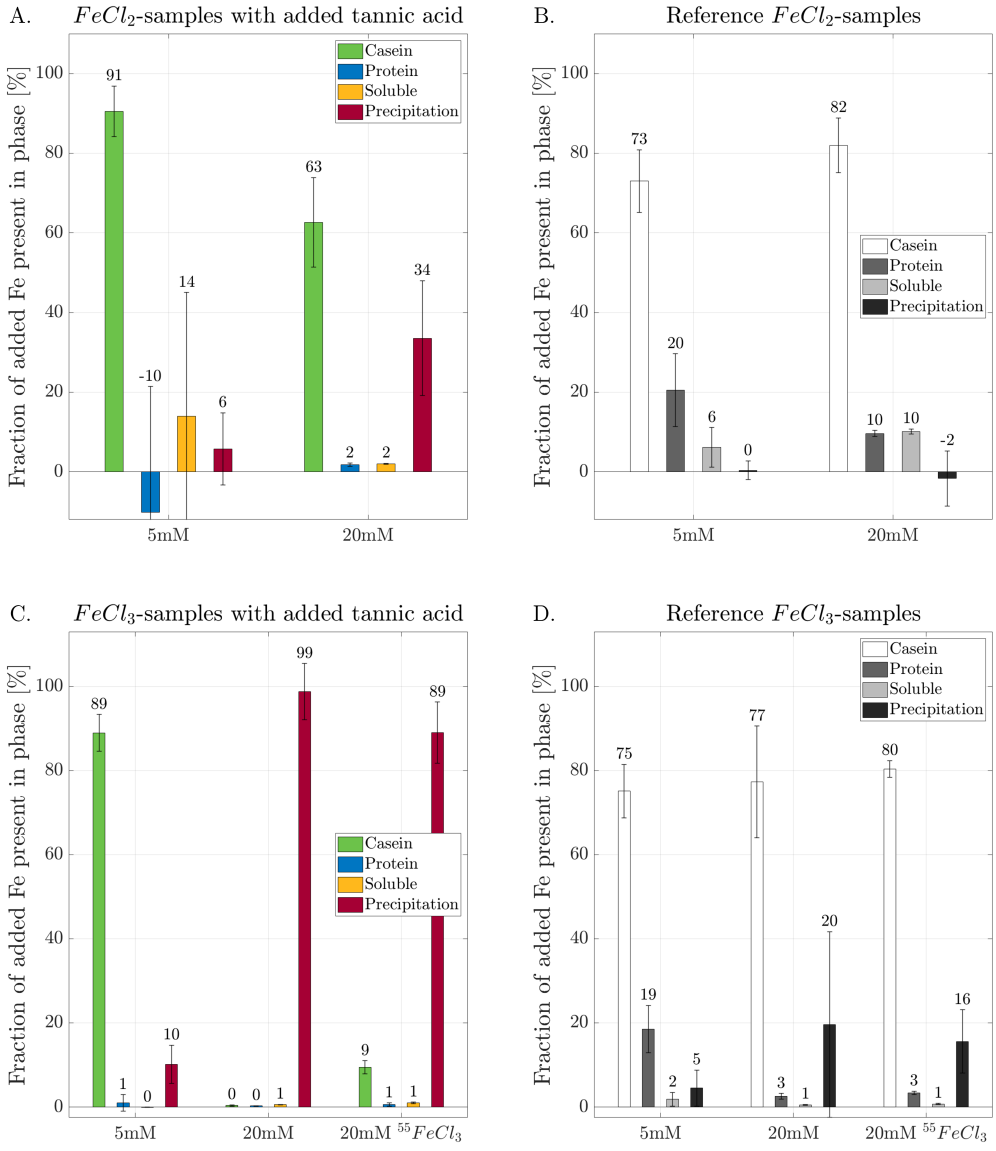


Figure 4.7: Milk fortified with $FeCl_2$ and with $FeCl_3$ + tannic acid compared to the reference samples (B, D respectively) without tannic acid. Samples contained 5 g tannic acid /L milk. In green (white) the iron percentage associated to the casein fraction, in blue (grey) the protein fraction, yellow (light grey) the soluble phase and in red (black) the iron the precipitated at low centrifugation speed. The radioactive sample was fortified to 20 mmol/L $FeCl_3$ and afterwards 17 kBq of $^{55}FeCl_3$ was added.

4.3 Comments on the chosen method

This section explains choices on the used iron concentrations, enhancer and inhibitors.

Comments on the chosen iron concentrations
Whereas ~0.2 mmol/L milk is a typical concentration in iron fortified milk [21], in this research the significantly higher concentrations of 5 and 20 mmol/L are used. These high concentrations can be measured with Mössbauer spectroscopy. When lower concentrations are desired it is advised to synthesise the salts with ^{57}Fe, to reduce measurement time. An additional benefit is that the results could be compared to the research conducted by Raouche *et al.* [11], who investigated the Mössbauer spectra of casein micelles of milk fortified to 2, 5, 10 and 20 mmol Fe/L milk. To the authors knowledge, they are the only others to have studied the properties of iron fortified milk with Mössbauer spectroscopy.

Comments on the chosen enhancer and inhibitor
To observe whether the addition of an iron enhancer or inhibitor to iron would influence the iron bioavailability, ascorbic acid (vitamin C) was chosen as enhancer and tannic acid was chosen as inhibitor.

Ascorbic acid is a frequently mentioned enhancer of iron bioavailability [6, 7, 10, 19, 21]. A typical concentration of ascorbic acid in 0.2 mmol/L iron fortified milk products is 50 mg ascorbic acid/L milk [21]. Since the highest iron concentration used in this experiment is ~100x higher, the ascorbic acid is also increased by a factor ~100 to 5 g ascorbic acid/L milk. It was chosen to keep the ascorbic acid concentration the same for 5 mmol Fe/L samples.

There are many iron inhibitors naturally occurring in food [6, 7, 19], e.g. phytates and polyphenols. A type of polyphenol that was available is tannic acid. A common misconception is that tea contains tannic acid, instead of tannin [61]. However since tannic acid is a gallate, it can chelate iron [6], and therefore still acts as an iron inhibitor. To compare the influence of tannic acid to the enhancer ascorbic acid, it was chosen to add as much tannic acid as ascorbic acid (5 g/L milk). However it would have been better to add as much mol tannic acid as ascorbic acid. The molar masses differ about a factor 10 (tannic acid 1701 g/mol, ascorbic acid 176 g/mol).

Comments on multiplicity of samples
Of every fractionation step in the phase separation procedure, four samples were taken. However for the initial and once centrifuged samples, these were false quadruplicates: two tubes were filled with the milk, and two samples were taken from each of these tubes. From the ultracentrifugation onward the samples were divided in four. In order to analyse the validity of the false quadruplicates, real quadruplicates were made of the $FeCl_2$ and $FeCl_3$ at 5 mmol/L and 20 mmol/L. The standard deviation of these real quadruplicates was compared to the standard deviation of the false quadruplicates. In six out of eight samples the standard deviation was of the false quadruplicates was higher (1.3-4x), in two cases the standard deviation was lower (0.3 and 0.6x). Based on these eight numbers it was concluded that the results of the phase separation process could be used.

The calculations on the phase separation process were adjusted due to the use of the false quadruplicates. Normally of an initial sample, e.g. #1, the corresponding fractions are measured and calculated. Then these values are normalised to initial sample #1. This is also executed for sample #2-#4. Afterwards the values of sample #1-#4 are compared by calculating the mean and standard deviation of these samples. In contrast to this calculation procedure, in this case the mean and standard deviation over all four samples was taken per measurable fraction, and afterwards normalised to the mean of the four samples. The measurable fractions are the initial sample, supernatant of the once centrifuged sample, the supernatant of the ultracentrifuged sample and the filtered sample. The obtained normalised values were used to calculate the casein, protein and precipitation fraction.

In brief, normally first the sample fractions are calculated per sample and afterwards compared to the other measured samples. In this case, the measurable fractions are compared over all the measured samples, to construct an average sample. Of this average sample the non-measurable fractions were calculated.

In case of Mössbauer spectroscopy, only one sample was measured. This was to get an indication of the interesting samples. Due to time restrictions it was not possible to perform more experiments. This makes it impossible to assess the normal deviations when measuring two similar samples. In section B.3.3 some other questions on Mössbauer spectroscopy are described, that were not yet answered.

5

Conclusions and Recommendations

5.1 Conclusions

In this project five factors that can influence the iron bioavailability in skim milk were studied: the iron chloride concentration (5 mmol/L and 20 mmol/L), the molecular form of iron chloride ($FeCl_2$ and $FeCl_3$), the storage time after fortification, the addition of an iron enhancer (ascorbic acid) and the addition of an iron inhibitor (tannic acid).

Iron chloride concentration
It was found that the increase in iron concentration resulted in an increase of percentage of iron present in the casein fraction, a decrease in the iron present in the protein fraction and no significant effect on the soluble fraction. The effect on the casein fraction and the protein fraction is caused by the decrease in pH upon addition of the iron chloride. Therefore in the future the influence of $FeCl_2$ and $FeCl_3$ can be investigated for a range of pH-values. Also lowering the iron concentration in the milk can decrease impact on the pH-shift upon addition.

Molecular form of iron chloride
On comparing the differences in results of the ferrous iron and the ferric iron, the 20 mmol/L $FeCl_2$-sample has a higher percentage of iron associated to the soluble phase and more Fe^{2+}, indicating a higher bioavailability. This might not be visible for the 5 mmol/L $FeCl_2$-sample due to the lower pH of the $FeCl_3$-sample, which also influences the iron present in the soluble phase.

Storage time after fortification
The extension of storage time after fortification from 24 hours to one week, resulted in a higher percentage of iron associated to the caseins fraction and a lower percentage of iron associated to the protein fraction and soluble phase. This shift in iron distribution is not reflected in a change in oxidation state, which did not significantly change. In future work, comparing this experiment to a study that determines the oxidation state over time of the iron bound to the casein micelles, it can be determined whether the Fe^{2+} did not bind to the casein micelles over the week or that the different preparation procedures before analysis play a role.

Addition of iron enhancer - ascorbic acid
The addition of ascorbic acid to the milk samples resulted in a higher iron percentage associated to the protein fraction and soluble phase. Again this effect seems to be strengthened by the decrease in pH. The chelation of iron by ascorbic acid does not seem to reduce Fe^{3+} to Fe^{2+} necessarily: the Fe^{2+} fraction in 20 mmol/L $FeCl_2$ decreased, whereas it increased for 20 mmol/L $FeCl_3$.

Addition of iron inhibitor - tannic acid
The addition of tannic acid to the milk samples resulted in a significant decrease of Fe^{2+}. Almost all iron was bound to the casein or precipitated as iron-tannic acid complex.

As stated by Frédéric Gaucheron in his review paper on iron fortification in the dairy industry [10], results concerning the iron bioavailability are often conflicting, because the characteristics are not easily evaluated.

These characteristics also depend on many different factors, such as the chemical form of the added iron, the presence of other components in the milk product and storage conditions. Indeed this project showed that the phase distribution and oxidation state were not only influenced by the addition of the iron chloride, but also simultaneously by the decrease in pH induced by the addition. Also the storage time after freeze-drying might have influenced the oxidation state results. In such a complex puzzle, every piece is of value to the solution. Therefore combined with the recommended further work and the work of many giants before, this research is a valuable contribution in the assessment of iron bioavailability of fortified milk products. Thereby aiding in the development of bioavailable staple foods, that are main missiles in the fight against anaemia.

5.2 Recommendations

Several recommendations are made for improved and further research.

- Lower iron concentrations often occurring in iron fortification (4-12mg Fe/L milk) can be measured to study the phase separation and oxidation state of iron in milk. Mössbauer samples have to be fortified with ^{57}Fe to obtain workable measurement times.
- After addition of the iron chloride, pH can be adjusted to isolate the influence of pH difference on the comparison between $FeCl_2$ and $FeCl_3$. A pH-range can be made to study the behaviour of both salts under different acidic conditions, thereby also enabling the simulation of the gastrointestinal tract.
- Multiple experiments can be done to investigate the influence of sample processing on the phase distribution and oxidation state of iron:
  - Freeze-dried samples can be rehydrated and analysed on phase distribution to observe changes induced by freeze-drying.
  - Freeze-dried samples can be measured multiple times after freeze-drying to investigate the influence of storage time on the Mössbauer spectrum.
  - By measuring the Mössbauer spectrum at 77 K, it can be investigated whether the Lamb-Mössbauer factor of Fe^{2+} affected in the same manner as Fe^{3+} in the milk samples, since it can be that the Fe^{2+} fraction is underestimated.
  - Non-freeze-dried milk cannot be measured at room temperature or at 77 K, since the crystal is not rigid enough for the Mössbauer effect to occur. Cooling down to 4 K would be an option to study whether the milk is greatly influenced by the freeze-drying.
- Mössbauer spectroscopy can be applied on the casein micelles. For the protein fraction and soluble phase, too little material will remain after freeze-drying for analysis. However by analysing the whole sample and the casein fraction, information can be gained on these combined fractions too.
- Many more variations can be implemented by varying the added concentration, molecular forms of iron, using other inhibitors and enhancers or changing concentration. Besides, the study is not limited to skim milk, but can also be applied on whole milk, infant formula, and the other staple foods such as flour and rice.

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A

Appendix - Mössbauer

The next page shows the complete table of the measured Mössbauer fitted parameters.

Table A.1: Mössbauer fitted parameters for influence of iron salt and concentration on the iron bioavailability. Milk samples were fortified with $FeCl_2$ and $FeCl_3$ to concentrations of 5 mmol/L and 20 mmol/L. Experimental uncertainties: Isomer shift: I.S. ± 0.01 mm s^{-1}; Quadrupole splitting: Q.S. ± 0.01 mm s^{-1}; Line width: Γ ± 0.01 mm s^{-1}; Spectral contribution: I ± 3%.

Sample		IS [mm s^{-1}]	QS [mm s^{-1}]	Γ [mm s^{-1}]	Phase	I [%]
$FeCl_2$	5 mmol/L	0.40	0.59	0.51	Fe^{3+}	100
	20 mmol/L	0.41	0.63	0.45	Fe^{3+}	78
		1.15	2.54	0.45	Fe^{2+}	22
$FeCl_3$	5 mmol/L	0.41	0.60	0.48	Fe^{3+}	95
		1.13	2.50	0.48	Fe^{2+}	5
	20 mmol/L	0.41	0.57	0.44	Fe^{3+}	95
		1.15	2.54	0.44	Fe^{2+}	5
$FeCl_2$ stored for 1 week	5 mmol/L	0.40	0.62	0.52	Fe^{3+}	100
	20 mmol/L	0.42	0.60	0.45	Fe^{3+}	76
		1.18	2.53	0.45	Fe^{2+}	24
$FeCl_3$ stored for 1 week	5 mmol/L	0.41	0.61	0.50	Fe^{3+}	95
		1.13	2.67	0.50	Fe^{2+}	5
	20 mmol/L	0.41	0.57	0.44	Fe^{3+}	97
		1.25	2.45	0.44	Fe^{2+}	3
$FeCl_2$ + vit. C	5 mmol/L	0.40	0.56	0.51	Fe^{3+}	100
	20 mmol/L	0.42	0.55	0.43	Fe^{3+}	84
		1.19	2.54	0.43	Fe^{2+}	16
$FeCl_3$ + vit. C	5 mmol/L	0.41	0.55	0.48	Fe^{3+}	92
		1.15	2.43	0.48	Fe^{2+}	8
	20 mmol/L	0.42	0.54	0.42	Fe^{3+}	84
		1.18	2.55	0.42	Fe^{2+}	16
$FeCl_2$ + tannic acid	5 mmol/L	0.41	0.64	0.54	Fe^{3+}	100
	20 mmol/L	0.42	0.63	0.51	Fe^{3+}	91
		1.17	2.56	0.51	Fe^{2+}	9
$FeCl_3$ + tannic acid	5 mmol/L	0.40	0.62	0.57	Fe^{3+}	100
	20 mmol/L	0.41	0.62	0.51	Fe^{3+}	100

B

Appendix - Preliminary research

In this chapter the results of supporting research are presented and discussed. Supporting research entails the synthesis of $FeCl_2$, general findings on sample preparation, phase separation methods and the execution of the analytical methods (ICP-OES, LSC and Mössbauer spectroscopy).

B.1 Sample preparation

This section discusses the general notables that stood out during the sample preparation procedures. The explanation why certain concentrations were chosen can be found in the sections that specifically discusses the end results of sample measurements.

The fortification procedure of milk with iron salts improved over time. First it was found that direct addition of the solid form of the iron salt to the milk, resulted in little lumps of undissolved iron salt. This mainly occurred on addition of $FeCl_3{\cdot}6H_2O$, and to lesser extent for $FeCl_2{\cdot}4H_2O$. So it was decided to dissolve the iron salt in a small amount of Milli-Q water before adding to the milk. To prevent the milk from diluting by the addition of the volume of iron solution, it was chosen to dissolve the iron salt in ~0.5% of the total milk volume. The milk was added to this iron-solution, to prevent iron from remaining in the weighing boat when executed in reverse. This was not a suitable practice for the higher concentrations of $FeCl_3{\cdot}6H_2O$, since the milk would coagulate, which was probably due to the lower pH. Finally, to prevent the coagulation, the milk to be fortified was kept cool in crushed ice, when simultaneously the iron solution was added dropwise while stirring. This last method was also practiced by Raouche *et al.* [11] "ensuring fast and rapid mixing" and preventing coagulation. Due to time constraints the last method has not been applied apart from a trial experiment. The procedure where the milk was added to the iron solution was used instead.

After fortification, to some samples ascorbic acid or tannic acid was added. This was also done by dissolving the acid in Milli-Q in the same manner as the dissolution of the iron described above. Subsequently the milk was added to the acid solution. In the future it would also be advised to add these additives dropwise to the milk.

Of all these samples, a part was kept aside to measure the pH. This was documented solely to be able to interpret results, not as main research topic. It was found that the temperature of the milk significantly influenced the pH measurement, since the pH-meter did not correct automatically for temperature. Milk directly from the fridge (7°C) could have pH 6.8 whereas, the same milk on laboratory temperature had pH 6.4. It was chosen not to correct for the pH-change, due to the addition of different (amounts of) iron salts.

For the tracer studies, $^{55}FeCl_3$ was added to the fortified milk samples. This was only done for $FeCl_3{\cdot}6H_2O$ fortified milk samples. Tracer studies were not preformed on $FeCl_2{\cdot}4H_2O$, to prevent misinterpretation when the $^{55}Fe^{3+}$ behaved differently in the milk than the Fe^{2+}. The addition of $^{55}FeCl_3$ is negligible in molar contribution (1 MBq contains ~ 10^{-14} mol Fe), and therefore does not account for concentration differences compared to fortified milk without tracer.

After the sample preparation, the samples either went through the phase separation procedure, which is described in the next section; or to Mössbauer spectroscopy, which will be addressed later in section B.3.3.

B.2 Phase separation methods

In the experiments described in section 4.1.2, two phase separation methods were used: centrifugation and dialysis. Other phase separation experiments performed in this project are only conducted with centrifugation. This is for three reasons: (1) Centrifugation can provide information about the casein micelle, protein and soluble fraction, whereas dialysis is restricted to the soluble fraction (compare Figure 4.2 and Figure 4.3). (2) Centrifugation is a faster process, since the whole procedure can be executed in half a day, whereas dialysis has to equilibirate over at least 24h. (3) During this equilibriation, the dialysis samples are stored in the fume hood. The temperature could not be controlled and fluctuated between 18 and 27°C in the summer. In the refrigerator the temperature could be controlled, but then the equilibration might take longer. This time span has not been identified in this project. In contrast, the aperture used in the centrifugation process, was able to executed the method at fixed temperature.

Nevertheless, centrifugation has the disadvantage that it is more expensive and creates more waste. Also, for dialysis it is easier to compare the total amount of iron before and after dialysis, thereby having an extra verification of the results. This was not applied for the centrifugation method. It is however possible to dissolve the ultracentrifuge tube and the filter in the destruction microwave. Providing that this material does not contain iron, it can be used to also directly measure the casein and protein fraction, thereby providing an extra verification step.

B.3 Analytical methods

B.3.1 ICP-OES

The natural abundance of iron in milk could not be measured with the ICP-OES, due to the iron concentration being below the detection limit. Therefore no control samples consisting of non-treated milk were measured.

B.3.2 LSC

The LSC was used to perform a relative analysis of fortified milk samples with the added tracer ^{55}Fe. Before sample measurement first three parameters had to be determined: the activity of ^{55}Fe to add as tracer, the maximum volume to be added to prevent colour quenching and the quench correction curve to correct for chemical quenching.

Determination of activity to be added

To conduct a measurement with an acceptable signal to noise ratio, it is important to stay at least three times above the background count rate. However, it is good practice to keep the activity as low as reasonably achievable (ALARA). To determine this optimal count rate, a series of activities (1 Bq - 5 kBq) was prepared. In Figure B.1 the activity is plotted against the measured net count rate. The background was ~0.1 cps, and therefore the lowest activity that can be measured accurately is ~0.3 cps.

To be able to measure all the phases of a centrifugation experiment, it was desired that 2.5% of the added activity to the initial sample, would be measurable in the last centrifugation step (being 0.4 cps). Therefore an initial sample would preferably contain an amount of activity that gives rise to 13 cps (~17 Bq), when added to the counting vial. In the next section is determined how much sample could be added without inducing colour quenching. There is also explained how much activity is added to the initial sample, to obtain 17 Bq in the counting vial. The measurable percentage can easily be reduced by increasing the activity added to the initial sample.

From Figure B.1 it can also be concluded that the LSC measures the activity linearly over the measured range (R^2 being 1.00, for $y(x) = 0.7187 * x$ (fixed at origin)). This entails that no efficiency correction has to be made when measuring different activities.

Determination of maximum volume of milk

To prevent the impact of colour quenching on the measurement, the amount of milk sample added to the counting vial should be kept to a minimum. Also it was studied whether the time of addition of scintillation counting cocktail would influence the count rate. In order to do so, 100 Bq was added to a certain volume of milk and either put in the carousel for 1h, 18h or directly measured after the addition of 10 mL scintillation cocktail Ultima Gold XR (UG).

In Figure B.2a it can already visually been determined that milk induces colour quenching, when added in volumes starting from 0.1 mL. In Figure B.2b the net count rate is plotted against the added milk volume.

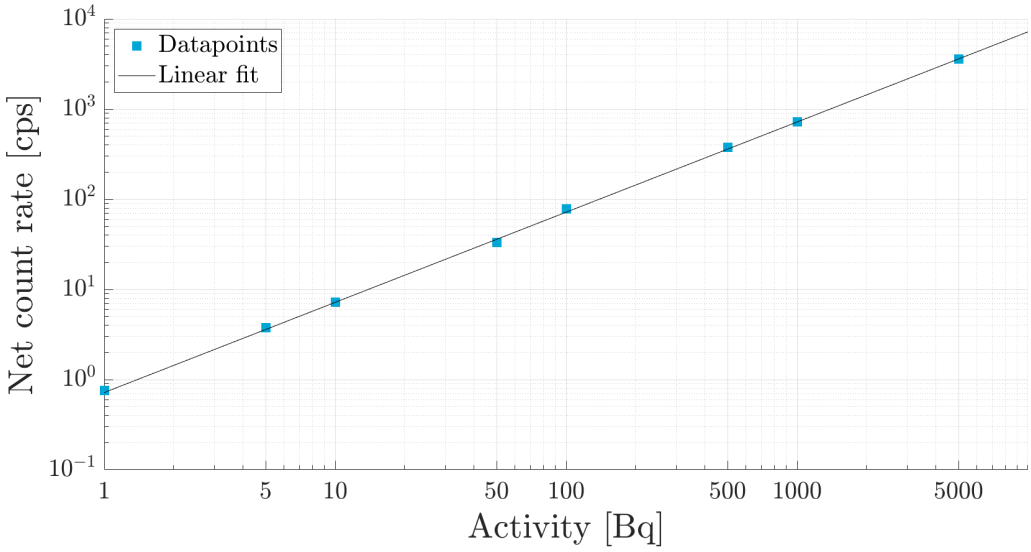


Figure B.1: Activity versus net count rate. The net count rate of vials with a known activity is measured to determine the activity needed to be added to a sample to stay above the background count rate (~0.1 cps). Besides it is shown that the LSC measures the activity range linearly, with a R^2 of 1.00, for $y(x) = 0.7187 * x$ (fixed at origin).

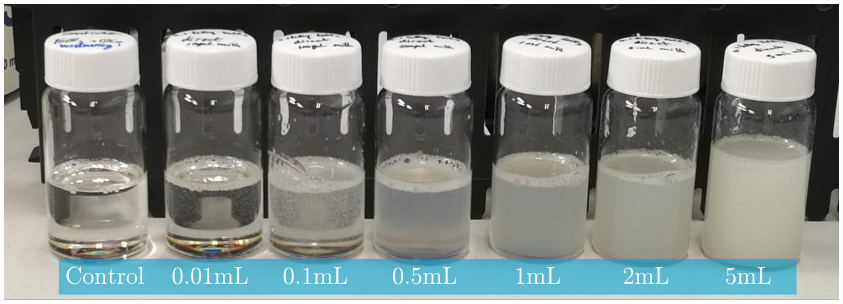
When the milk sample was directly added to the UG, vortexed and measured, it can be seen that the a volume up to 0.1 mL does not influence the measurement, despite the already slightly more opaque solution at 0.1 mL. Volumes higher than 0.1 mL steadily decrease the measured count rate.

The samples, of which the activity and milk were put in the carousel for 1 hour before addition of UG, show a slightly lower count rate than the directly added samples, but follow the same reduction pattern. The samples that were in the carousel for 18 hours, are not conclusively having lower or higher count rates than the directly added samples. The two data points that stand out: "UG added after 1h" for 0.01 mL milk and "UG added after 18h" for 0.1 mL milk, are probably outliers, since the "UG directly added" holds the same count rate as the sample with 100 Bq as shown in Figure B.1. The "UG added after 18h" for 2 mL milk was unfortunately lost and is therefore not shown in the figure. Although this experiment is not performed in multiplicet, and neglecting the outliers, it still gives a clear indication that the maximum of milk volume to be added to the UG is 0.1 mL.

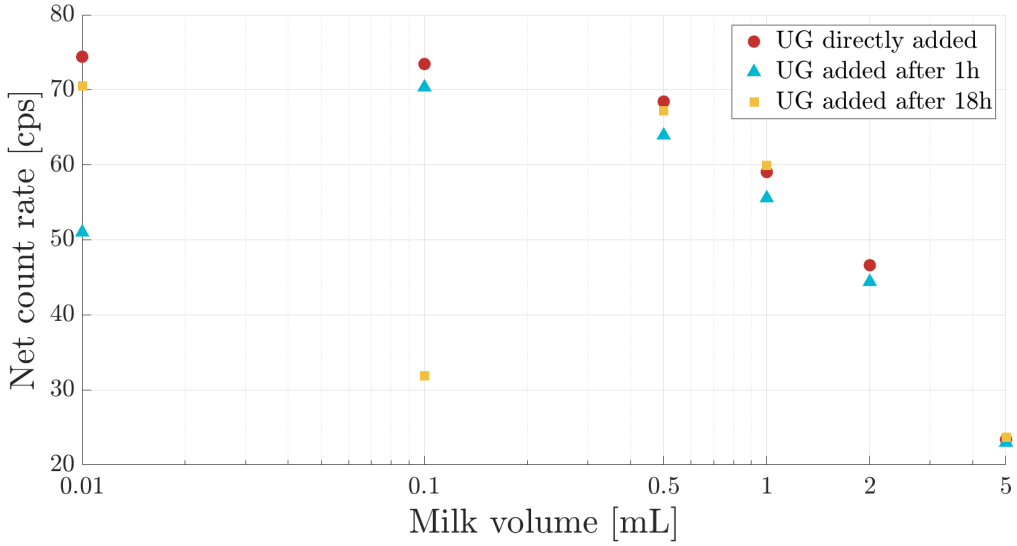
Next the result of the colour quenching (being able to add 100 μL of milk), and the necessity to add 20 Bq of activity to the counting vial to stay three times above the background, are combined to calculate that 20 kBq has to be added to a milk sample of 10 mL.

A remark about the colour quenching experiment is that the influence of colour quenching was only checked for normal milk. However to study the influence of an inhibitor and enhancer of iron, also tannic acid and ascorbic acid were added to milk. For the tannic acid it was very clear by only visual inspection, that 100 μL was probably quenching the solution, since it obtained a pink colour instead of the normally white transparent counting cocktail (see Figure B.3). It was verified that ~10% of the added activity was measurable. To minimise this colour quenching, it was decided to add 10 μL of the initial and once centrifuged sample, and 100 μL of the ultracentrifuged and filtered samples. The 10μL was still had a faint pink colour, but less sample was not easily pipetted. Therefore in the next section it is explained how a quench correction curve was made to correct for the colour quenching. In the section on the determination of activity to be added, it was concluded that the LSC measures the activity linearly, and therefore the difference in added volume was believed to not cause extra uncertainties in the calculations.

On visual inspection the ascorbic acid did not seem to induce colour quenching, however the tSIE-value, which is a quench indicating parameter, gave reason to believe that chemical quenching played a role. This is explained in the next section.



(a)



(b)

Figure B.2: Colour quenching due to addition of milk. A series of fixed activity (100 Bq) and varying milk volumes (0.01 - 5 mL) was made to observe the influence of colour quenching on the count rate. A control sample with 100 Bq of activity and directly added UG, had a count rate of ~74 cps. (a) shows the obtained colour of the various vials, (b) shows the milk volume versus the net count rate of these vials. The experiment was conducted thrice: once the counting cocktail Ultima Gold XR (UG) was added immediately after addition of ^{55}Fe to the milk. In the other two experiments the milk and activity were first stirred for respectively 1 hour and 18 hours before addition of UG. The separate series were performed once. The 2 mL sample of "UG added after 18h" was lost and therefore not shown. The values of 0.01 mL milk added after 1 hour and 0.1 mL milk added after 18 hours, are probably not trustworthy. It was concluded that a volume of 0.01-0.1 mL could be added to a counting cocktail without inducing colour quenching.

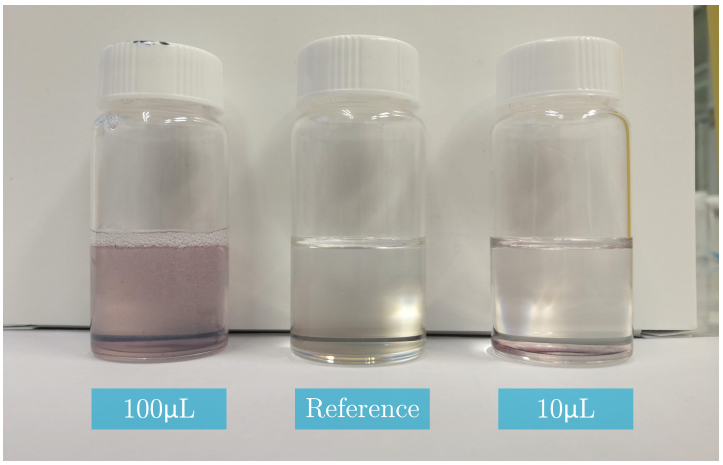


Figure B.3: Colour quenching due to addition of tannic acid. From left to right counting vials containing: 100 μL of milk with tannic acid, a blank reference sample to compare the colours, 10 μL of milk with tannic acid. In the 100 μL sample only 10% of the added activity could be measured. It was decided to add 10 μL, which reduced the quenching, although a faint pink colour persisted. Therefore also a quench correction curve was used.

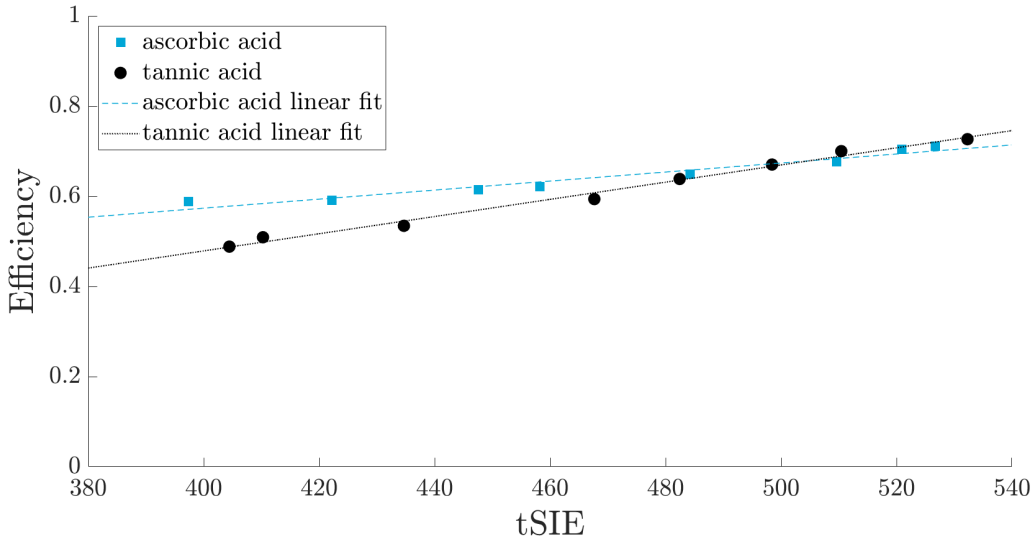


Figure B.4: Quench correction curve for ascorbic and tannic acid. From right to left on one line depicts every measurement point a increase of 25 μL, where the first point is a vial only containing activity and no quenching material. The quenching material was a solution of $FeCl_3{\cdot}6H_2O$ and ascorbic acid or tannic acid. A linear fit is drawn through the data points with an R^2 of 0.95 for ascorbic acid ($y(x) = 0.001906x - 0.2836$) and 0.99 for tannic acid ($y(x) = 0.001001x + 0.173$).

Quench correction curve

The tSIE-value is a measure for the amount of quenching. The higher the tSIE-value, the less quenching, where the maximum is 1000. In the case of milk, the tSIE-value does not alter for the different fractions in the phase separation, being ~520. Therefore it was concluded that: (1) the determination of the maximum amount of milk volume was performed correctly, because although the initial sample is whiter than the filtered sample, this did not alter the tSIE-value of the corresponding counting vial. And (2) the iron fortified milk did not induce chemical quenching, since the tSIE-value stayed constant. Another option is that the chemical in the milk that could be responsible for chemical quenching, was not removed during the phase separation process. This potential chemical would not influence the measurement, since this is a relative measurement, and the chemical would influence all samples in the same manner. Therefore a quench correction curve for the milk samples was not believed necessary.

For the ascorbic acid, on visual inspection no colour quenching could be deduced, however the tSIE-value deviated from the milk sample, being ~520 for the initial milk sample and ~430 for the initial ascorbic acid sample. However the tSIE-values increased further into the phase separation process (tSIE-value ranging from ~430-470), indicating that a chemical compound, that was decreased during the separation procedure, induced chemical quenching. Thus a quench correction curve was made for the ascorbic acid samples, and as explained above also for the tannic acid samples.

The quench correction curve was made as described in section 3.2.4; two counting vials containing 17 Bq each, were measured. After every measurement, 25 μL of $FeCl_3{\cdot}6H_2O$-ascorbic acid solution was added to one vial and $FeCl_3{\cdot}6H_2O$-tannic acid solution to the other vial. The concentration $FeCl_3{\cdot}6H_2O$, ascorbic acid and tannic acid in the added solution were equal to the concentration in the prepared milk samples. The resulting quench correction curve is shown in Figure B.4.

Although a quench correction curve is not necessarily linear, for these quenching chemical composition for ^{55}Fe in this domain of tSIE, it appears to be a good approximation (R^2 being 0.95 for ascorbic acid ($y(x) = 0.001906x - 0.2836$) and 0.99 for tannic acid($y(x) = 0.001001x + 0.173$)). The fit is used to calculate the efficiency for every sample containing ascorbic acid or tannic acid. With this efficiency the original count rate could be calculated.

A quench correction curve is usually made with the quenching compound. During this experiment it was assumed that the combination of $FeCl_3{\cdot}6H_2O$ and ascorbic acid or tannic acid was the quenching compound. It could be the case that a reaction between milk, $FeCl_3{\cdot}6H_2O$ and ascorbic acid or tannic acid would in-

Figure B.5: Not well mixed LSC sample. When the counting vials is not immediately swerved after addition of the sample to the counting cocktail, the sample sticks to the bottom of the counting vial. It is not possible to dissolve this later in time.

duce extra quenching. This effect could be present since for tannic acid normally 10μL of milk-iron-tannic acid-solution was added, and resulted in a tSIE-value of ~480, and for this quench correction curve 75μL was added. In case of ascorbic acid normally 100μL is added, which results in a tSIE-value of ~430. To obtain the same tSIE-value in the quench correction curve 125μL was added. Due to time constraints this quench correction was used, instead of producing a new curve with milk-iron-tannic acid or ascorbic acid solutions. Therefore the samples containing more quenching material may be underestimated compared to the samples that are future down the separation procedure.

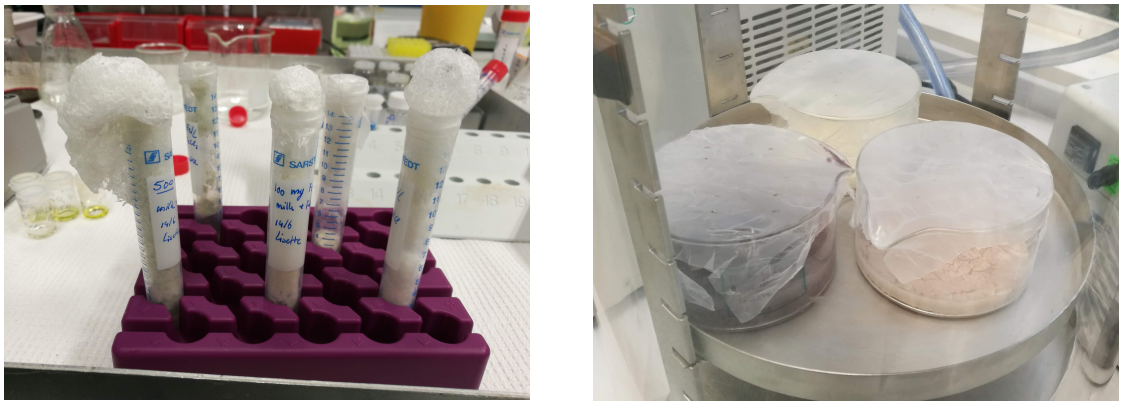
Although ^{55}Fe, which is used in this project, decays by electron capture, it is thought that ionisation quenching does not have to be corrected for in this project. This is because only relative measurements are done, and it is believed that the ionisation quenching reduces the counts in the same ratio for every sample. Even though different concentrations of ^{55}Fe are measured, the scintillation cocktail is present in such excess, that the probability of an interaction between two molecules - excited by decay of two different radionuclides - is negligible compared to the probability of an interaction between two molecules excited by the same decay. In case the reader is interested in ionisation quenching correction, Briks empirical model [62] is often used to correct for ionisation quenching [41, 63] and is recommended to look into.

Remarks on LSC preparation procedure
A remark on the preparation procedure of the LSC is that the counting vials have to be mixed immediately after addition of the milk solution. Otherwise the sample sticks to the bottom of the counting vial, as shown in Figure B.5. It is sufficient to swerve the counting vial for a couple of seconds to prevent the sticking. In this project this act was performed after a minute. The Dutch National Centre for Radiation Protection [15] adds 1 mL of Milli-Q water to enhance the solubility of the sample in the counting cocktail, since the counting cocktail is a bad solvent.

B.3.3 Mössbauer spectroscopy

Mössbauer spectroscopy was used to obtain information on the chemical environment of ^{57}Fe in the iron added to the milk samples. Since the preparation of enriched $^{57}FeCl_2$ was not successful, the measurements relied upon the natural abundance of ^{57}Fe. This only influences the measurement time. When lower concentrations are desirable it is convenient to use enriched iron to reduce measurement time.

To prepare the samples for Mössbauer spectroscopy the samples had to be freeze dried. Eventually this was done in crystallising dishes instead of small 15mL or 50mL tubes. This was practised since the use of small



(a) (b)

Figure B.6: Freeze dried samples - bad performed and well performed examples. (a) Samples that were slightly defrost before put in freeze dryer. In vacuum the fluid boils, resulting in an overboiled sample. Also the surface to volume ratio is not ideal, resulting in long freeze drying times. (b) Well frozen samples in a crystallising dish with a good surface to volume ratio.

tubes caused some problems: (1) the sample was not completely frozen when put in the freeze dryer, which resulted in an "overboiled" sample, because liquid will boil in vacuum; (2) the volume to surface ratio was to big, meaning that gas formed by sublimation cannot escape easily, resulting in a long freeze drying time (>4days); (3) the sample is taken too early out of the freeze dryer and therefore a part was still frozen, this mainly occurred together with (2) (combined with impatience). These problems were easily solved when wide crystallising dishes were used in stead of the small tubes.

The amount of milk that has to be freeze dried depends on the concentration or iron in the milk samples, because of the Lambert-Beer law. For the concentrations used in this project 1.8g of milk powder was sufficient. It was found that 50g of milk would be reduced to ~5g of milk powder after freeze-drying.

The preparation procedure of samples, including the freezing phase [64], can influence the chemical environment of iron. This can be observed in the Mössbauer spectrum. A couple of questions are not answered in this project, but necessary to judge the comparability of different projects. A non-exhaustive list of questions is presented in section 5.2.

The temperature during the measurement can also influence the Mössbauer spectrum, since the Lamb-Mössbauer factor is temperature dependent. This dependence can differ for Fe^{2+} from Fe^{3+}. By performing a measurement at 77K, the real ratio of Fe^{2+} to Fe^{3+} can be measured. Raouche *et al.* [11] performed Mössbauer spectroscopy on casein micelles found that the real proportion of Fe^{2+} was slightly underestimated. A measurement at casein micelles from milk fortified to 10 mmol Fe/kg, had a relative contribution for Fe^{2+} of 13% at room temperature and increased to 17% at 77K. Therefore it might be interesting to observe the temperature dependence for milk samples as well.