VARIABILITY IN THE GASTRIC DIGESTION OF B-LACTOGLOBULIN ON IN-VITRO INFANT DIGESTION

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1 ABSTRACT

Cow's milk is one of the most common causes of food allergy in the first years of a humans life. β-Lactoglobulin (β-Lg), of which genetic variants A and B exist, is the main whey protein in cow's milk and one of the major allergen causing this allergy. Based on a previous thesis study of Wang (2017), which indicates that there might be a difference in digestion between β-Lg A and B, this study investigated the effect of genetic variants A and B of β-Lg on the in-vitro digestion in children below the age of two. The objective of this study was to investigate the differences in the in-vitro gastric digestion between β-Lg genetic variants A and B of raw bovine milk in 3-6M old infants, to see how reproducible the new 3-6M old in-vitro digestion model was and to investigate the differences between the composition of fresh milk and milk that has been stored in the freezer. A digestion model for 3-6M old infants was used to simulate the digestion, RP-HPLC was used to separate, identify and quantify the different proteins in bovine milk and SDS-PAGE was used to check the results of the RP-HPLC. Different results were obtained during this study compared to the previous study of Wang (2017). During this study the results showed that there was not a remarkable difference between the digestion of β-Lg A and B. There was also a lower breakdown of casein found and quite some variation was found between the measurements of the individual milk samples. The RP-HPLC measurements were checked by putting the samples on an SDS-PAGE gel. The results were similar and thereby excluded the fact that something was wrong with the RP-HPLC measurements. A pepsin assay has been conducted to check the activity of the enzyme that was used in the digestion model since the results were lower than expected. The assay showed that the pepsin activity was indeed lower than it should be and thereby explaining the lower breakdown of casein that was found.

This study suggests that there is not a significant difference between the digestion of β-Lg A and B and indicates that there is a lot of variation between the individual whey and casein proteins found in milk of individual cows. The 3-6M old infant digestion model is reproducible and storing raw bovine milk in the freezer for a long period of time does have an effect on the milk composition.

Keywords: β-Lactoglobulin, genetic variants A and B, bovine milk allergy, infant digestion, milk variation

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5 LIST OF ABBREVIATIONS

β-Lg = β-Lactoglobulin
α-La = α-Lactalbumin
kDa = kilo Dalton
BSA = Bovine Serum Albumin
IgE = Immunoglobulin E
DH = Degree of Hydrolysis
GIT = Gastrointestinal tract
SGF = Simulated Gastric Fluid
SIF = Simulated Intestinal Fluid
RSD = Relative Standard Deviation

6 INTRODUCTION

Breast milk is considered to be very important in the first few months of a humans life. However, it is not always possible to give a new-born human breast milk and in that case cow's milk, or cow milk-based formulas are usually provided. Cow's milk is one of the most common food allergies in the first years of a humans life. Even though most children outgrow the cow's milk allergy by the age of 4, still quite some proportion of the human population keeps the allergy throughout life. Cow's milk contains quite some proteins of which β-Lactoglobulin (β-Lg) and caseins are the major allergens in bovine milk and can be involved in the allergic sensitisation (Sélo et al., 1999)(Do et al., 2015).

β-Lg, of which it is known that genetic variants A and B exist, is absent in human milk. This protein and its two genetic variants A and B will be the main focus during this study. In previous studies it has been shown that β-Lg is resistant to pepsin digestion and acid denaturation. Due to the resistance of the breakdown of β-Lg during gastric digestion, this protein can stay intact and can be absorbed through the gut mucosa. When intact β-Lg comes in contact with the immune cells present at the start of the small intestine, these cells are able to trigger an immunological response.

From previous studies the resistance in digestion of β-Lg in young children is known, and due to this resistance β-Lg is labelled as a major allergen. However, there is a gap in knowledge about the two genetic variants and its digestion. A previous thesis study of Wang (2017) indicates that there could be a possible difference between the two genetic variants A and B, however this has not been further looked into due to its irrelevance during that particular study. Therefore, the aim of this thesis study is to expand the knowledge in the difference between the genetic variants A and B of β-Lg regarding digestion in young children, and to understand its mechanism. If there is in fact a difference between these variants with regard to their digestion in young children, this could be used to reduce the allergenicity of bovine milk in young children.

6.1 RESEARCH QUESTIONS AND HYPOTHESIS

6.1.1 Research Aim

The aim of this study is to investigate the variability in the breakdown of the genetic variants A and B of β-Lactoglobulin and other proteins after in-vitro gastric digestion of bovine milk in young children.

6.1.2 Research questions

- Is there a difference in the breakdown of genetic variants A and B of β-Lactoglobulin during gastric digestion?
- Is the new 3-6M old in-vitro digestion model reproducible?
- What kind of effect has the storage time in the freezer on the measured composition of the bovine milk?

6.1.3 Research hypothesis

A previous thesis study of Wang (2017) has shown that there is a difference in the breakdown of the genetic variants A and B of β-Lactoglobulin during digestion of unheated bovine milk in young children. Because this was out of the scope and irrelevant for that study this has not been further investigated. Through this study it is expected that there is a difference in the breakdown of genetic variant A and B.

Since the 3-6M old digestion model that is used is a newly made model it is important to see how reproducible the model is. There are quite some steps in the model that could have an effect on the

values such as pipetting, which could influence the distribution of the fluids and measuring the pH, which could have an effect on the casein micelles once they stick on the pH-meter. Since the new infant model is similar to the adult digestion model regarding pipetting steps and pH measurements, which is a model that has been used for a very long time, it is expected that the model will be reproducible. However, it is expected that there will be little variation, mainly in the caseins compared to the whey proteins since whey proteins are not part of the casein micelles.

Between measurements, the raw bovine milk is put back in the freezer at -20°C and when needed taken out again. The question is if storing raw bovine milk in the freezer for some time before using it again will have an effect on the composition of the milk, which in turn could have an effect on the data when using the milk for further measurements. It is expected that storing the milk in the freezer for a long period of time will have an effect on the composition of milk.

7 LITERATURE REVIEW

7.1 COW'S MILK

The general composition of bovine milk is shown in table 1 (Hettinga 2016). The milk composition is dynamic and can vary with stage of lactation, age, breed, nutrition, energy balance and health status of the udder (Haug et al., 2007). Lipids are in emulsified globules coated with a membrane, proteins are in colloidal dispersion as micelles and most minerals and all lactose are in true solution (Jensen et al., 1991). When investigating milk, which is the case during this thesis study, the variation mentioned above can have quite some influence on the results that are obtained during studies about milk.

Table 1 General composition of Bovine milk (Hettinga 2016*)*

Bovine milk		
Component	**Average content in milk (% w/w)**	**Average content in dry matter**
Water	86.6	
Solids-not-fat	9.0	
Lactose	4.6	34
Fat	4.4	33
Protein	3.4	25
Casein	2.7	20
Minerals	0.7	5.2
Organic acids	0.17	1.3

Bovine milk and other dairy products have been used in human nutrition for a long time. Bovine milk contains the nutrients that are needed for the development and growth of a calf and is therefore rich in various components such as lipids, proteins, amino acids, vitamins and minerals. In this thesis study proteins are the main interest. Proteins in bovine milk are in colloidal dispersions as micelles, consisting of proteins and salts, as shown in figure 1, which will be further discussed below. Due to the large variety of differences in for example cow breeds, age, nutrition and health status, the composition of bovine milk can vary (Haug et al., 2007).

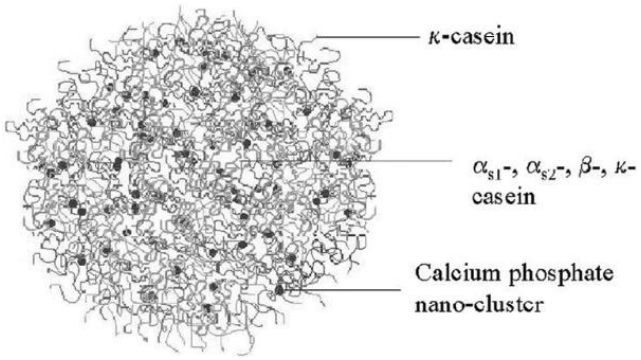


Figure 1 Schematic representation of a casein micelle

7.1.1 Bovine vs human milk

There are quite some differences between human breast milk and bovine milk proteins, as shown in figure 2 (Hambreus et al., 1977). Bovine milk contains far more proteins than human milk, especially caseins, and contains β-Lg which is absent in human milk. Even though β-Lg is naturally absent in human breast milk, it could be present if the mother has consumed bovine milk. Although this will be in very small quantities, it could still lead to allergic reactions. The nitrogen in milk is distributed among caseins, whey proteins and non-protein nitrogen. On the other hand, lactoferrin, which is an antimicrobial protein and relatively resistant to digestion in the gastrointestinal tract, is much more present in human milk compared to bovine milk (Haug et al., 2007). Lactoferrin and α-Lactalbumin play a key role in the immune system and are therefore important components in human milk whey protein.

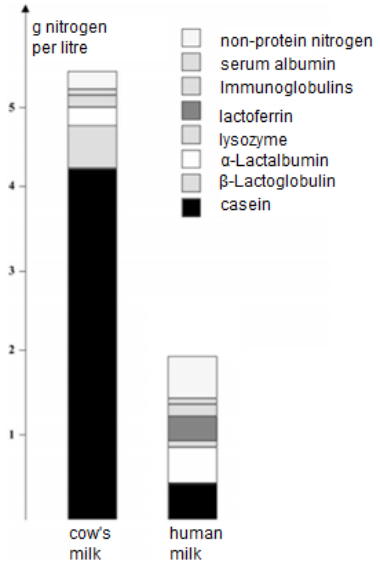


Figure 2 Protein composition of cow's milk and human milk. Nitrogen derived from various proteins and non-protein nitrogen are represented as grams of nitrogen per litre (Hambreus et al., 1977)

7.1.2 Proteins in bovine milk

Bovine milk contains about 32g protein/L. The nitrogen in milk is distributed among caseins, whey proteins and non-protein nitrogen of which whey and caseins are the main interest during this study.

7.1.2.1 Caseins

The casein content, which is about 78% of the total milk proteins, is a mixture of several components which are all characterized by the fact that they precipitate at low pH. There are four different caseins: α_{s1}-, α_{s2}, β-, and k-casein of which α_{s1} and β are present the most with 38 and 36 percent and α_{s2} and k are representing 10 and 12 percent of the total casein. Most of the casein carry calcium and phosphate and due to the low pH in the stomach they there form a clot, for efficient digestion. This clot is called a casein micelle, which is shown in figure 1. The micelles are stable structures that are able to handle high temperatures. Due to this, the milk can be heat-treated without the heat damaging the casein. The phosphorylated amino acids of the caseins are able to bind calcium ions in the form of colloidal calcium phosphate. This is very important in order to keep the micellar structure stable. Even though the micelles are very stable there are two ways in which aggregation can occur. These two ways are with enzymatic coagulation and acid coagulation. During enzymatic coagulation proteolytic enzymes can cause a splitting of k-casein and with acid coagulation the casein becomes insoluble due to the low pH, k-caseins are on the outside of the casein micelle, which leads to rejection between the micelles. Ones the pH value drops, the rejection decreases and the micelles will stick together and form protein flakes. (Hettinga 2016).

7.1.2.2 Whey proteins

The whey proteins are globular proteins that are more water soluble than caseins, and able to denature upon heating. During denaturation, the whey proteins unfold and aggregate with the casein micelles or with other whey proteins. The main whey proteins in bovine milk are: α-La and β-Lg, of which β-Lg will be the main interest during this thesis study due to its resistance to digestive enzymes. Due to this resistance, some of the intact β-Lg can be absorbed which can lead to a build-up of an allergic reaction, mainly in young children, which will be further discussed in the chapter about cow's milk allergy below.

7.2 β-LACTOGLOBULIN

As mentioned before, β-Lactoglobulin is one of the two most prevalent protein in whey and milk serum. It is 10% of the total milk protein, which is 58% of serum proteins. The percentage of β-Lg in infant formula is higher than 10%, sometimes even higher than 50%, since some caseins have been removed in order for the milk to become more similar to human breast milk. This removal leads to a higher percentage of whey-protein in the total amount of milk protein. The molecule has a size of 18kDa, has two disulphide bridges and one free cysteine (Wal, J-M., 2002). In contrast with caseins, milk whey proteins are fairly resistant to the action of plasmin. The amino-acid sequence and 3-dimensional structure of β-Lg shows that it is a lipocalin, a diverse protein family involved in the transport of small hydrophobic molecules, which can bind varies ligands. The allergen is capable of binding lipids including retinol, β-carotene, saturated and unsaturated fatty acids, and aliphatic hydrocarbons. β-Lg is manufactured specifically in the mammary gland for inclusion in milk. It is assumed that its importance in bovine milk is mainly to provide the offspring with sufficient amino acids. β-Lg occurs in various genetic variants of which in this study there will be only looked at the main variants A and B. During this study the main interest lays in the genetic variants A and B. (Kontopidis et al., 2004)(Walstra et al., 2005)(van Valenberg., Hettinga 2015).

7.2.1 Structure of β-Lactoglobulin

β-Lg is a calyx composed of an eight-stranded antiparallel β-sheet of which loops A-B, C-D, E-F and G-H surround the entrance of the cavity, while loops B-C, D-E and F-G close the opposite side of the β-barrel (Bello et al., 2007). Structural rearrangement occurs in the protein during the Tanford transition between pH 6-8. This is a reversible conformational change that occurs in loop E-F. At low pH, which is the case during gastric digestion, the loop adopts a closed conformation, blocking access to the calyx, whereas at high pH it folds back and opens to allow access of ligands to the calyx (Oliverira et al., 2001). This blocking access leads to the resistance to digestion which will be further discussed in section 'Bovine milk protein digestion and cow's milk allergy' below.

As shown in figure 3 (Botelho et al., 2000), variants A and B have a slightly different structure since they differ at two sites in the amino acid sequence: Asp64 in A is changed to Gly in B, and Val118 in A is changed to Ala in B. Due to this structural difference they differ in biophysical and biochemical properties. For example, their isoelectric points: pI= 5.26 for variant A and 5.34 for variant B.

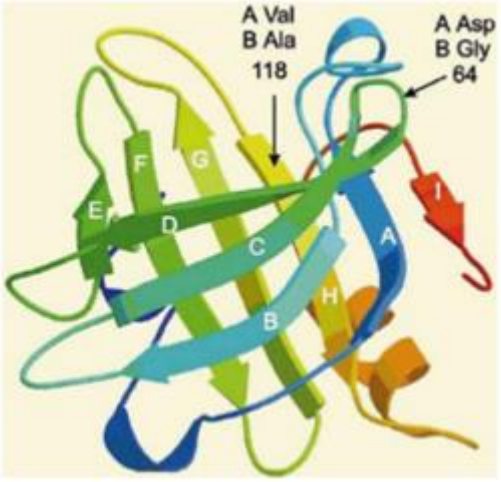


Figure 3 A general view of β-Lactoglobulin, indicating the positions of the substitutions of genetic variants A and B (Botelho et al., 2000)

It is assumed that proteins at pH above their isoelectric point are more susceptible to pepsin digestion (Zhang et al., 2014). Therefore at acidic conditions, which is below the isoelectric point and the case during gastric digestion, proteins are more resistant to digestion. As mentioned before, the size of β-Lg is 18 kDa. Since epitopes can still be found and bind at 2-3 kDa the protein should be cut into very small pieces in order for the allergen not to be reactive anymore. When knowing the DH of a protein, assumptions can be made about the allergenicity.

The differences in flexibility is also linked to the structural differences. The substitution Asp->Gly is just located before the highly flexible loop C-D. Therefore, when any conformational changes occurs before this loop, this could result in differences in flexibility (Oliverira et al., 2001). It could be assumed that flexible structures are more sensitive to proteolysis. The change in structure can also lead to a difference in net charge, which modifies the dimer-monomer dissociation equilibrium (Creamer et al., 2004). Variant A, containing more negative charges, could lead to more resistance due to more electrostatic interaction, which keeps it more stable.

7.3 BOVINE MILK PROTEIN DIGESTION AND COW'S MILK ALLERGY

Cow's milk allergy is one of the most common food allergies in young children and is usually developed in early childhood. Between two and three percent of children younger than three years old are allergic to milk. Even though most children outgrow the cow's milk allergy by the age of four, quite some proportion of the human population keeps the allergy throughout life. The allergy is mainly a type I, IgE mediated hypersensitivity reaction and around 20 different proteins in milk could be involved in the allergic sensitisation. However, only some of the proteins are labelled as being allergens of which β-Lactoglobulin and caseins are the most important allergens in bovine milk. Out of these two, β-Lactoglobulin is the major interest during this study. (Do et al., 2015)(Sélo et al., 1999).

β-Lg is quite resistant to the hydrolysis and proteases, this leads to some of the proteins remaining intact after gastric digestion. These intact β-Lg can be absorbed through the gut mucosa and build up an allergic reaction due to the immune cells present in the beginning of the small intestine (Wal, J-M., 2002).

7.3.1 Human digestion system

The human digestive tract can be divided into four individual processes that run after one another. These processes exist out of the oral, gastric, small intestine and large intestine processing of which the gastric processing will be the main focus during this study. Under normal circumstances there is no reverse flow within the four different processes. The gastric processing starts once the swallowed food arrives into the stomach. The stomach has a strong acidic environment with a pH around 2 due to the secretion of hydrochloric acid in the gastric wall. This is triggered by food entering the stomach. The enzyme pepsin, which is secreted into the stomach, catalyses the breakdown of the proteins present in the stomach which will be further discussed in the section 'digestion of milk proteins' below. The digested chyme leaves the stomach and arrives in the small intestine where the intestinal processing starts. Since this is irrelevant for this particular study this will not be further discussed. (Boland 2016)

7.3.1.1 Digestion of milk proteins

Protein digestion begins in the stomach, where an acidic environment favours protein denaturation. The primary proteolytic enzyme of the stomach is pepsin, a nonspecific protease that is maximally active at pH 2. Thus, pepsin can be active in the highly acidic environment of the stomach, even though other proteins undergo denaturation here (Berg et al., 2002). The proteins are further hydrolysed in the intestine, and then absorbed through the intestinal wall.

During digestion there is a difference in behaviour between different types of proteins. Caseins are relatively unstructured and more hydrophilic parts are exposed, this could lead to a more digestible protein. In contrast with caseins, milk whey proteins are fairly resistant to the action of plasmin. Whey proteins have a globular structure which results in less exposed hydrophilic bonds and therefore more interactions in order to keep the structure stable (Shandan et al., 2009). Due to the less exposed hydrophilic bonds there is less access for the proteases to break down the protein, meaning its harder to digest.

7.3.1.2 Digestion of milk proteins in infants

The younger the human, the less proteins are broken down during gastric digestion, which is shown in table 2. This is due to the lower level of proteases and higher pH in the stomach of young children compared to adults. Therefore the occurrence of cow's milk allergy is higher in children because of the less digested proteins, as mentioned before in the section about allergenicity.

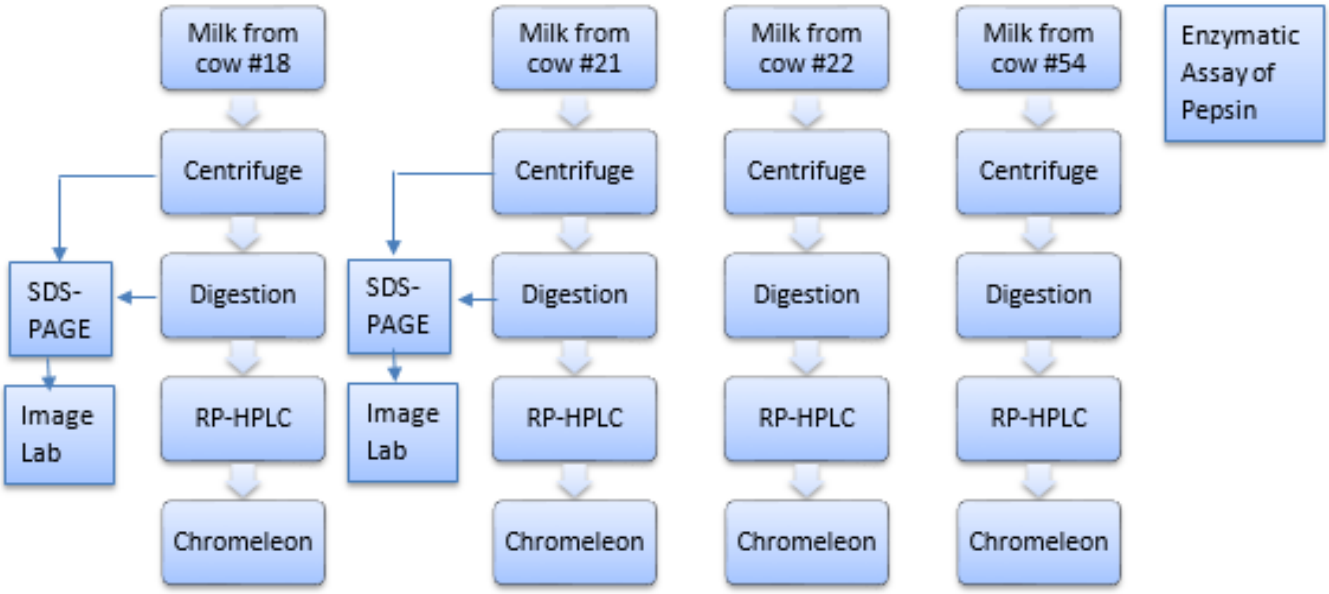
During a thesis study of Wang (2017) a difference in digestion between the two genetic variances of β-Lg was found. In table 2 the results of the in-vitro protein digestion in bovine milk are shown that were obtained during the study of Wang (2017). The percentages stand for the amount of protein that is broken down, where 100% means the protein is completely broken down and 0% means no protein has been broken down. It shows that the digestion of β-Lg in young children is far less than adults, which was already known. It also shows, which is the most relevant for this thesis study, that there is quite a big difference in the two genetic variants, which has not been seen before. Variant A is far less broken down compared to variant B, which will result in more intact β-Lg and therefore a higher chance of building up an allergic reaction to bovine milk with genetic variant A. Because further investigation on this difference was not executed, this difference first has to be confirmed by mimicking the in-vitro protein digestion. This will be done during this thesis study.

Table 2 The decrease of bovine milk proteins after gastric digestion in infant and adult (Wang 2017)

	κ-casein B	α_{s2}-casein	α_{s1}-casein	β-casein	α-LA	β-LG B	β-LG A
0-3M gastric	76.79%	75.20%	92.42%	96.96%	19.54%	17.89%	8.15%
3-6M gastric	100%	91.26%	96.58%	90.95%	92.86%	21.77%	8.41%
0-2Y gastric	100%	100%	100%	100%	94.87%	22.08%	9.06%
Adult gastric	100%	100%	100%	100%	99.89%	52.12%	53.82%

8 MATERIALS AND METHODS

Table 3 Overview of the measurements that have been performed during this thesis study



8.1 MILK SAMPLING

Milk samples were chosen focusing on β-Lg A and B since the difference between these genetic variants is of importance for this study. Accordingly, milk samples of β-Lg A and β-Lg B were used. Milk samples were taken from four cows: two β-Lg A and two β-Lg B.

These samples were picked out from an individual milk database provided by Hein van Valenberg from Wageningen University & Research Centre.

Since the amount of β-Lg present in a cow could have an effect on the degree of denaturation, it has been tried to pick samples with similar differences in amounts of β-Lg.

Table 4 bovine milk samples chosen for this thesis study

Farm van Ommeren								
		k-CN	**β-CN**		**β-Lg A**	**β-Lg B**		
Genotype	β-Lg A	AB	A2	**Amount of β-Lg**	7.27	0.79	**Cow number**	21
	β-Lg A	A	A1		7.58	0.61		22
	β-Lg B	A	A2		0.45	5.73		18
	β-Lg B	AB	A1,A2		0.22	5.92		54

Before any measurements were performed on the raw milk, the milk was centrifuged at 4 °C to be able to remove the fat to make sure this will not influence any of the measurements. The milk was centrifuged at 4 °C because at this temperature the fat forms a thicker structure and therefore the fat is easier to remove.

The raw bovine milk samples were used in the in-vitro infant static digestion model for 3-6 month old infants (Wang 2017). The protein composition and content was determined by RP-HPLC and the

resulting chromatograms were analysed through the Chromeleon 7.2 SR4 software. To see if the measurements of the RP-HPLC were done correctly the samples were put on an SDS-PAGE once. To check the pepsin activity an assay was performed on the pepsin that is used during the digestion.

8.2 METHOD

8.2.1 In-vitro protein gastric digestion model 3-6M

To mimic the results of Wang (2017) the same infant protein digestion model for 3-6M old infants was used because in this age category the largest difference between genetic variants A and B of β-Lg was obtained. To mimic the infant gastric digestion the following modifications were done to the standardised adult model of Minekus et al (2014): the pH of the gastric phase was changed from 3.0 to 3.5, the pepsin concentration in the gastric digestion mix was decreased by a factor of 4 (5000 U/mL), the trypsin concentration in the intestinal digestion mix was decreased by a factor of 10 (2000 U/mL), the bile salt concentration in the intestinal digestion mix was decreased by a factor of 4 (40mM) and the time of the gastric phase was changed from 2 hours to 1 hour.

8.2.1.1 Sample preparation

The whole milk samples were centrifuged at 4000g for 15 minutes at 4˚C (Beckman coulter Avanti J-26-XP centrifuge, rotor JA-25.15), this low temperature was used to make it easier to remove the top layer (fat). After the fat layer was removed the milk samples were ready to use.

Preparation of stock solutions of simulated digestion fluids. The volumes are calculated for a final volume of 500 mL for each simulated fluid. We recommend to make up the stock solution with distilled water to 400 mL instead, *i.e.* 1.25× concentrate, for storage at −20 °C. In the Experimental section, these 1.25× concentrates are referred to as Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) electrolyte stock solutions. The addition of enzymes, bile salts, Ca^{2+} solution etc. and water will result in the correct electrolyte concentration in the final digestion mixture. $CaCl_2(H_2O)_2$ is not added to the electrolyte stock solutions as precipitation may occur. Instead, it is added to the final mixture of simulated digestion fluid and food[a]

			SSF pH 7		SGF pH 5		SIF pH 7	
Constituent	Stock conc.		Vol. of stock	Conc. in SSF	Vol. of stock	Conc. in SGF	Vol. of stock	Conc. in SIF
	g L^{-1}	mol L^{-1}	mL	mmol L^{-1}	mL	mmol L^{-1}	mL	mmol L^{-1}
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	—	—	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	—	—
$CaCl_2(H_2O)_2$ is not added to the simulated digestion fluids, see details in legend								
	g L^{-1}	mol L^{-1}		mmol L^{-1}		mmol L^{-1}		mmol L^{-1}
$CaCl_2(H_2O)_2$	44.1	0.3		1.5 (0.75*)		0.15 (0.075*)		0.6 (0.3*)

[a] * in brackets is the corresponding Ca^{2+} concentration in the final digestion mixture.

Figure 4 Preparation of stock solutions of simulated digestion fluids (Minekus et al 2014)

Firstly, every solution was made with the right concentration (indicated in the red box) (e.g. KCl: make a solution with a concentration of 37.3 g/L). The volume was depended on the amount of samples needed. All the solutions were added in the right volume according to figure 4 (indicated in the blue boxes) (e.g. For SSF 15.1 ml of KCl with a concentration of 37.3g/L must be added). After addition of all the salts the total volume must be adjusted to 500 ml. This was done by adding Milli-Q water.

The solutions (SFF, SGF and SIF) were heated to 37 °C in a water bath for at least an hour before added to the digestion mixture.

8.2.1.2 The start for 3-6M infant digestion

Oral phase

5 mL of raw bovine milk (without fat) was mixed with 3.5 mL of SSF electrolyte stock solution. Then 25 µL of 0.3 M CaCl2 and 1.475 ml of Milli-Q water was added and then mixed.

Gastric phase

10 mL of the prepared mixture was mixed with 7.5 mL of SGF electrolyte stock solution. 5 µL of 0.3 M CaCl2 and 0.895 mL of Milli-Q water was added to the sample. The pH for infant digestion was adjusted to 3.5 by adding HCl with a pipette, while the pH was measured

After the pH was set to 3.5, 1.6 mL of enzyme solution was added. The enzyme solution was prepared by adding pepsin (pepsin from porcine gastric mucosa 3200–4500 U/mg protein, Sigma) to SGF electrolyte stock solution. To obtain an enzyme activity of 500 U/mL, 1,62 mg pepsin was added to 1 ml SGF. Depended on the amount of enzyme solution needed the amount of grams and ml was adjusted.

The gastric chyme was then incubated at 37 °C for 1 hour in a rotating device. After 30 minutes the samples were checked if the pH was still 3.5. When this was not the case, the pH was adjusted again. If the pH was 3.5 again the samples were put back and incubated for another 30 minutes.

The total incubation time was 1 hour.

Directly after the incubation of the gastric phase the pH was adjusted to 7 in order to stop the enzyme activity. This was done by adding NaOH with a pipette while the pH was measured with a pH meter.

From the total of 20 mL, 5 mL was taken out and put in the freezer to use for later measurements. The other 15 mL of gastric chyme was used for the next step.

Intestinal phase

15 mL of gastric chyme (with a pH of 7) was mixed with 8.25 mL of SIF electrolyte stock solution, 30 µL of 0.3 M CaCl2, 1.095 ml of water and 1.875 mL of bile salt (40mM) (0.3 g bile salt extract was added to 15 mL water to obtain around 15mL, depending on the amount of samples needed this was adjusted).

The pH was checked again, and if not 7, it was corrected.

The last step was to add 3.75 ml of enzyme solution. Enzyme solution was prepared by adding porcine pancreatin with an activity of 2000 U/mL (81,3 mg added to 1 ml of SIF gave this enzyme activity). Depended on the amount of enzyme solution needed this was adjusted.

After the addition of all the solutions, the duodenal chyme was incubated at 37˚C in a rotating incubator for 1 hour. After 1 hour the reaction was stopped by adding 0.007g of the protease inhibitor Pefabloc (1mM) (0.007 g).

If the samples were not used straight away, they were put into the freezer (-20˚C).

8.2.1.3 Calculations

The calculations that were done to obtain the results, which are shown in the results & discussion, are as following: in order to keep the dilution of the digested samples into account, the peak areas (mAU*min) of the digested samples were done times 2. The areas of the peaks of the digested samples were then divided by the peak area of the undigested samples. Example: 100-((digested peak area*2)/(undigested peak area))*100%.

8.2.2 RP-HPLC

The RP-HPLC was used to separate proteins according to their hydrophobic character. Quantification and identification was done via the Chromeleon 7.2 SR4 software to identify the peaks of the samples.

8.2.2.1 Sample preparation

The protein sample preparation followed the procedure stated by Bobe et al. (1999).

A solution comprised of 0.1 M Bis Tris buffer (pH adjusted to 6.8), 8 M Urea, 5.37mM sodium citrate and 19.5 mM DTT (pH 7) was added directly to the samples in a 3:1 ratio (v:v) at room temperature (for example: 0.6 mL solution 1 and 0.2 mL sample).

Solution 1 Volume	**Bis Tris 0.1M**	**Urea 8M**	**Sodium Citrate 5.37 mM**	**DTT 19.5 mM**	**Milli-Q water**
10 mL	0.20924 g	4.80 g	0.01579 g	0.03 g	

After thawing, samples were shaken for 10 seconds, incubated for 1h at room temperature, and centrifuged for 5 minutes at 16 000g in a micro-centrifuge.

The remaining solubilized sample was diluted 1:3 or 1:1 (for digestion chime) (v:v) with a solution (adjust the pH to 2) containing 6M Urea in solvent A, which consists of 0.1% TFA acid in water (v:v). 2mL Eppendorf tubes were used (for example: 0.4mL undigested milk sample and 1.2 mL solution 2 or 0.8 mL digestion chime and 0.8 mL solution 2).

Solution 2 Volume	**Urea 6M**	**0.1% TFA in Milli-Q water**
20 mL	7.21 g	

A blank was made to use as first two runs for the RP-HPLC sequence. To make this blank the same 1:3 dilution as mentioned before was used. Solution 1 and 2 were used for this blank (for example: 0.4mL solution 1 and 1.2mL solution 2).

Before injection into the RP-HPLC column, samples and blank were filtered through RC Ø15mm, 0.2 µm filters, by means of 2 mL syringe into HPLC vials.

The equipment used was HPLC, Thermo Scientific™ UltiMate 3000, with reversed-phase analytical column C18 (Aeris Widepore 3.6um XB-C18 RP, Phenomenex) with a silica-based packing (3.6µm, 300 Å, 250x4.6 I.D.).

For the analysis, the eluents that were used were labelled A and B: Eluent A was prepared as a solution of 0.1%(v/v) TFA acid in Milli-Q water: Eluent B was a solution of 0.1% (v/v)TFA in acid in acetonitrile. Every run lasted 42 min with the following gradient with ratio A: B; linear gradient from 65:35 in 5 minutes, 62: 37 in 4 minutes, 60:40 in 9 minutes, 59.5: 40.5 B in 2 minutes. 57: 43 in 0.5 minutes. Then in isocratic elutionat 57:43 during 4.5 minutes. Then returned to linear elution with ratio 56:44 in 3 minutes. 53:47 in 4.5 minutes. Afterwards isocratic elution at 53:47 for 5 minutes and returned linearly to the starting condition in 0.5 minute-equilibration under the starting conditions for 5 minutes. Temperature of samples was 4 °C, while that of the oven was 45°C. Injection volume was 5µL and the flow rate 0.4 ml/min.

The resulting chromatograms were analysed through the Chromeleon 7.2 SR4 software.

8.2.3 SDS-PAGE

The SDS-PAGE was performed once, in order to check if the protein quantity and composition measured with RP-HPLC was similar to what was found on an SDS-PAGE gel.

8.2.3.1 Sample preparation

2 µL sample was diluted in 5 µL of 4 times concentrated LDS sample buffer and 15 µL of Milli-Q water. The mixture was centrifuged at 2000 rpm for 1 min and then heated at 70 °C in a heating block for 10 minutes. Samples were loaded onto 12% Bis-Tris gels. Gels were run at 200 V and stained with Coomassie Blue R-250 0.1% (w/w), followed by distaining with washing buffer. The scanned gels were analysed by ImageLab software.

8.2.4 Enzymatic assay of pepsin

In order to check the pepsin activity of the pepsin, from porcine gastric mucosa 3200 – 4500 U/mg protein, an enzymatic assay of pepsin was performed ("Enzymatic Assay," n.d.). Since there was no polypropylene column with a coarse filter (90-130) µm, the Hemoglobin Stock Solution was not filtered.

8.2.4.1 Procedure

The substrate was pipetted into 6 glass tubes, of which 1 blank and 5 test tubes. The glass tubes were placed in a water bath of 37 °C for 10 minutes. In the 5 test tubes 1 mL of enzyme solution was added. In each test tube a different enzyme solution was present: 0.05 mg/ml, 0.04 mg/ml, 0.03 mg/ml 0.02 mg/ml and 0.01 mg/ml. All 6 tubes were mixed by swirling and incubated at 37 °C for exactly 10 minutes. After this, 10 mL of TCA solution was added to each of the 6 tubes to stop the reaction and 1 mL of enzyme solution was added to the blank.

All tubes were filtered through a 0.45 µm syringe filter and measured in a Cary 50 Bio UV-Visible Spectrophotometer.

9 RESULTS AND DISCUSSION

9.1 BOVINE MILK AFTER IN-VITRO PROTEIN DIGESTION

The in-vitro protein digestion model of Wang, Y. (2017) for 3-6M old infants was used on four raw, centrifuged bovine milk samples of which the fat was removed.

9.1.1 RP-HPLC

The digested and undigested bovine milk samples of four different cows were analysed with RP-HPLC and the different whey and casein proteins were identified. Chromatograms of the different digested and undigested bovine milk samples with the identified proteins are shown in the appendix, figure 6-8. Wang (2017) found that there was a difference in the digestion of the genetic variants A and B of β-Lg, but since this was not the main focus during that particular study this was not further investigated. To further investigate this difference this thesis study was conducted. However, during this thesis study different results were obtained which will be further discussed in the paragraphs below.

From the chromatograms of the undigested bovine milk samples (appendix, figure 6-8) it can be concluded that cow number 54 is not a β-Lg B cow, as was stated in the database, but contains both β-Lg B and β-Lg A in the milk. It can also be concluded that the milk that is used during this thesis study has a similar composition to what can be found in literature. In literature it is stated that the total casein content is about 78% and for the different caseins it is known that α_{s1} and β are present the most with around 38 and 36 percent and α_{s2} and k are representing around 10 and 12 percent of the total casein (Hettinga 2016). A study of de Vries (2017) also show similar values regarding the composition of bovine milk, which were also measured with HPLC. Therefore it is assumed that the milk that is used is similar to what can be found in literature. From table 5 it can be seen that the composition of the different undigested bovine milk samples are close to what can be found in literature and what has been found in previous studies. The small differences can be explained by the fact that the milk samples that were used came from individual cows, and therefore some variation can be found. Previous studies have already shown that milk protein variants can also have an effect on the protein composition. These studies have shown that variant A of β-Lg leads to a higher concentration of β-Lg and a lower concentration of casein, and that variant B of k-CN results in a higher concentration of k-CN in milk (Mayer et al., 1997)(Robitaille et al., 2002). Since during this particular study only four cows have been investigated and therefore only a few genetic variances, it is hard to conclude something out of these results regarding variation in milk composition. However, it can be seen that the numerical differences between β-Lg A and B that are found during this study, match to what is found in other studies (10% of A to 6% of B).

Table 5 The whey and casein protein ratio and percentages from the different raw bovine milk samples

Before digestion	% of whey	% of casein	Whey/casein ratio	k-CN A	k-CN B	αs_2-CN	αs_1-CN	β-CN	β-Lg A	β-Lg B
Cow #18	20.28%	79.72%	0.25	7.46%	-	12.41%	30.91%	28.93%	-	6.80%
Cow #21	19.33%	80.67%	0.24	3.08%	4.81%	14.76%	28.02%	29.99%	9.61%	-
Cow #22	15.49%	84.51%	0.18	6.34%	-	13.44%	29.70%	35.03%	9.79%	-
Cow #54	14.56%	85.44%	0.17	5.46%	-	18.12%	27.43%	33.44%	5.71%	3.34%

9.1.2 Bovine milk digestion after in-vitro gastric digestion for 3-6M old infants

After the gastric phase of the digestion, the digestion chyme was collected and analysed by RP-HPLC to check the protein composition. The obtained chromatograms were compared to the undigested bovine milk samples, of which the raw data can be found in the appendix, figure 6-8, to calculate the decrease of bovine milk proteins.

Table 6 The decrease of bovine milk proteins after 3-6M gastric digestion. The percentages mean the decrease of every protein which was calculated by the peak area of the RP-HPLC chromatograms

After 3-6M gastric	β-Lg A	β-Lg B	α-La	k-CN A	k-CN B	αs_2-CN	αs_1-CN	β-CN
Cow #18	-	34.21%	18.84%	63.20%	-	35.97%	46.06%	45.90%
Cow #21	28.94%	-	28.17%	66.32%	64.62%	40.14%	54.87%	58.17%
Cow #22	41.44%	-	42.43%	44.47%	-	39.05%	42.38%	43.75%
Cow #54	39.04%	37.63%	29.67%	62.34%	-	43.08%	47.34%	49.88%

Table 7 The decrease of bovine milk) proteins after 3-6M gastric digestion

	Cow #18	**Cow #21**	**Cow #22**	**Cow #54**
Casein	46.04%	54.42%	48.35%	46.04%
β-Lg	34.21%	28.94%	41.44%	38.52%
α-La	18.84%	28.17%	42.43%	29.67%

Table 6 and 7 show the results of the first measurement of the milk samples with the RP-HPLC. From these results it can be seen that there is not a big difference regarding the digestion of β-Lg A and β-Lg B as expected. Both β-Lg A and β-Lg B are broken down between 30-40%, and also when both genetic variants are present they are broken down around the same amount. The percentages of the other digested proteins such as k-CN, αs-CN and β-CN are also different than expected when comparing the results to the results that the previous thesis student Wang (2017) found, which are shown in table 8. The values for the digestion of casein found during this thesis study are much lower, around 45%, compared to Wang (2017) that found values around 90% and higher meaning the caseins were almost fully digested.

The overall digestion is also different since Wang (2017) found large differences between the breakdown of the different proteins, with low breakdown of β-Lg (20%) and high breakdown of casein (90%). During this study the β-Lg had a breakdown between 30 and 40% and the casein is broken down between 40 and 60%. This shows that less difference in the breakdown between the different proteins was found. When comparing the results from table 7 to a study of Inglingstad et al. (2010) it can be stated that during this thesis study around the same percentage breakdown of β-Lg was found. They found a β-Lg breakdown of 40%, which is similar to what was found during this study. However, they did not use an infant model meaning they had a lower pH during their digestion model. The bovine milk during that study was also not raw. It is known that the more a protein has been denatured, the more easy a protein is digested. It is therefore expected that during this study an even lower breakdown of β-Lg would have been found, due to its known resistance to pepsin and since the bovine milk was raw. It is not known why this was not found.

Table 8 Data from previous thesis student Wang (2017) showing the decrease of bovine milk proteins after 3-6M gastric digestion

	β-Lg A	β-Lg B	α-La	k-CN A	k-CN B	αs_2-CN	αs_1-CN	β-CN
3-6M gastric	8.41%	21.77%	92.86%	-	100%	91.26%	96.58%	90.95%

Data from Wang (2017) shows a very clear difference between the digestion of β-Lg A and β-Lg B, and very high breakdown of the other proteins. The reason that the results that are found are so different could be because of the fact that during the thesis study of Wang (2017) bulk milk was used whereas during this thesis study individual milk is used. It is known that there are large variations in individual milk, as mentioned before.

It could also be that when both genetic variants A and B of β-Lg are present in the milk, which is the case in bulk milk, the enzyme has a preference in breaking down genetic variant B. However, unintentionally cow #54 turned out to contain both genetic variant A as B which could give an indication on how the digestion would be when both genetic variants are presents. In this case no remarkable difference was found between the two genetic variants therefore indicating that there is no difference. During the study of Wang (2017) only one bulk milk sample was used and during this study four individual cow milk samples were investigated. These are low numbers of cows to be able to draw reliable conclusions but four is still more than one. Therefore it can be stated that there is a lot of variation in the composition and digestion of the individual cows since this is already found in only four cows.

9.1.3 SDS-PAGE

To exclude that the different data was obtained due to mistakes during the RP-HPLC measurement, some of the undigested and digested milk samples were also put on an SDS-PAGE gel as displayed in figure 5. The SDS-PAGE was used because this gives a more direct read out of what has been done. A down side of the SDS-PAGE however, is that it is harder to quantify. Since we are only looking at the differences between the digested and undigested samples it should give an indication if the results are similar to the results from the RP-HPLC.

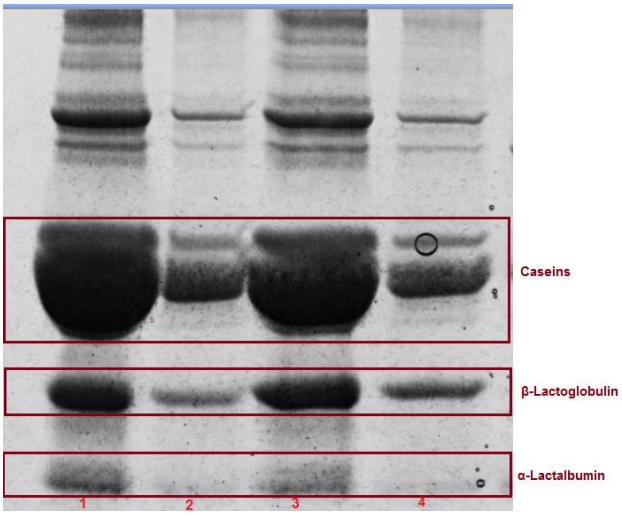


Figure 5 SDS-PAGE of bovine milk. Lane 1 displays the undigested milk of cow #18. Lane 2 shows the milk of cow #18 after 3-6M gastric digestion. Lane 3 displays the undigested milk of cow #18. Lane 4 shows the milk of cow #21 after 3-6M gastric digestion

ImageLab 5.2 was used to quantify the volumes of the bands to obtain a delta value and calculate the breakdown percentages of the protein to compare these results with the RP-HPLC. Since it is hard to see the different caseins in this gel the total of the caseins was used and compared. The raw data can be found in the appendix, table 16 and 17, and the results of the SDS-PAGE and the RP-HPLC of cow #18 and #21 are shown in table 9. Both HPLC and SDS-PAGE measurements each have their problems. With HPLC its problem is that peaks have more frequently issues with overlap which makes

identification of the proteins more difficult. On the other hand, the problem with SDS-PAGE is that its known for its inaccuracy. Whey protein tend to have a deviation of about 5% and the accuracy of casein is even less than that of whey protein. It is expected that SDS-PAGE would show a larger breakdown compared to the RP-HPLC measurements. The reason for this is that some breakdown products stay under the existing peaks in the chromatograms, which leads to underestimation of the actual digestion. Table 9 does show that the measurements of SDS-PAGE are in some cases higher compared to the measurements of the RP-HPLC. Since the values are somewhat similar, with an exception of α-La of cow #18, it can be assumed that the measurements of the RP-HPLC has been done correctly and the chromatograms should be trusted. Not much can be found in literature regarding percentages of casein after gastric digestion but a study of Dupont et al. (2010) indicates that casein is quite resistant to infant gastric digestion. Therefore, the high break down percentage (>90%) of casein after gastric digestion found by Wang (2017) seems to be invalid. A study of Inglingstad et al. (2010) found a breakdown of 31% for the casein after gastric digestion. Since during that study an adult digestion model was used, containing a lower pH than the infant model, it is expected that the breakdown of casein during this study would be even lower.

Table 9 Results of the RP-HPLC compared to the results of the SDS-PAGE of cow #18 and #21

Cow #21 after gastric digestion	**Measurements RP-HPLC**	**Measurements SDS-PAGE**
Caseins	54.42%	43.46%
β-Lg	28.94%	35.63%
α-La	28.17%	34.68%
Cow #18 after gastric digestion		
Caseins	46.04%	47.63%
β-Lg	34.21%	51.95%
α-La	18.84%	66.46%

As mentioned before there is a large variation found between the four cows, shown in table 4. Since the variation was not obtained due to errors during measurements with the RP-HPLC, something else could be the cause of the variation. This variation could be a result of the differences in digestion between the individual cows, or this variation could be due to a irreproducible model. Another reason that could explain the variation in results could be due to the fact that the pepsin that was used during the digestion is expired or has a lower activity than it should have.

To exclude the fact that the variation obtained in the results is due to a irreproducible model the reproducibility of the digestion model was investigated and the results of these measurements can be found in the paragraph ‘Reproducibility of the 3-6M old digestion model’ below. To exclude the fact that the pepsin is expired an enzymatic activity assay was performed and the results will be further discussed in the paragraph ‘Assay enzyme activity’ below.

9.1.4 Assay enzyme activity

As mentioned in the previous results it seems that the pepsin activity is lower than it should be. The overall decrease during gastric digestion in casein was about 50%, whereas the results of Wang (2017) were >90% for casein. Most chemicals have no problem when they are stored for a long period of time in the freezer but enzymes are different. Enzymes start losing their activity straight after the bottle is opened due to autolysis. Even when the enzyme is stored in the freezer this process slowly continues, leading to a reduction in activity. To check if the activity of the enzyme that was used is lower than it should be, an assay regarding the enzyme activity has been performed. The pepsin from porcine gastric mucosa should contain 3200 – 4500 U/mg, which is quite a wide range. The values that were obtained from the assay were around 2800 U/mg. If this is much lower than the activity that Wang (2017) had during his digestion is not known, since the enzyme activity was not measured during that study. If Wang (2017) used fresh pepsin, it could be that the activity was 4500 U/mg or maybe even higher. This would mean that the activity was nearly twice as high and could explain the lower values found for casein compared to the data of Wang (2017). The raw data of the performed assay can be found in the appendix, table 18. From this data it can be concluded that the pepsin used is indeed lower than it should have been.

9.2 Reproducibility of the 3-6M old digestion model

It is important that the model that was used is reproducible. The obtained data differs somewhat per measurement which could mean that the model that is used is not reproducible. Since the model has quite some pipette steps and measurements with a pH-meter this could result in differences in the rate of disappearing of the protein flakes that are formed. Protein flakes are a lot of casein micelles stuck together. Due to the many pipette steps it could be the case that the milk is sometimes more distributed than at other times. This could have an effect on the casein micelles. It is expected that there will be less variation in the whey proteins since these are not part of the casein micelles. During the digestion model there are some steps where the pH needs to be adjusted, during this measurement it could be that there are some protein flakes that stick onto the pH-meter and are washed off when cleaning the pH-meter for the next measurement. This could have an effect on the data.

To see how reproducible the 3-6M digestion model is, the digestion model has been performed again in fourfold with milk samples from cow #18 and #21. The raw data can be found in the appendix, table 19, 20 and 21. The relative standard deviation (RSD) from both cows was calculated and are shown in table 10. From the data it can be concluded that the digestion model of 3-6M is indeed reproducible since the RSDs are not too high. The RSD of k-CN A is quite high compared to the other individual proteins. It is expected that all the caseins would show a higher RSD due to the protein flakes that were washed off, however this is not the case. The reason that only k-CN A deviates from the others could be since the peaks of k-CN originate from breakdown products of other proteins which might overlap. This could lead to a less precise measurement of the k-CN peak and result in a higher RSD.

Table 10 Relative standard deviation of the RP-HPLC of the fourfold 3-6M old digestion of cow milk from cow #18 and #21

Cow #18	**β-Lg B**	**α-La**	**β-CN**	**k-CN A**	**k-CN B**	**αs_1-CN**	**αs_2-CN**
RSD%	2.86%	6.92%	3.14%	37.28%	-	1.58%	2.54%
Cow #21	**β-Lg A**	**α-La**	**β-CN**	**k-CN A**	**k-CN B**	**αs_1-CN**	**αs_2-CN**
RSD%	3.01%	2.40%	3.01%	18.14%	4.69%	3.83%	3.94%

9.3 EFFECT OF STORING MILK IN THE FREEZER

Since the milk samples have been stored in the freezer for some time this could lead to a difference in composition, therefore the same experiments have been performed on fresh milk. Raw data from these experiments can be found in the appendix, table 13-15 and figure 6-8.

The data of 1 day old fresh milk, is compared with the results from 15 day and 21 day old milk that has been stored in the freezer upon use, which can be found in table 11 and 12, and the raw data in the appendix. From these tables it can be concluded that in all the samples the whey goes a bit down and the casein goes a bit up after 15 days (unfortunately cow #54 was not delivered at the day of the measurements of the fresh milk and therefore is not taken into account). The casein and whey proteins do also differ individually. When comparing the fresh undigested milk to undigested milk that has been stored in the freezer for 21 days it can be concluded that the whey decreases with around 3% and the casein increases with around 3%. α-La and k-CN have definitely decreased, with around 3% whereas β-CN increases with about 5%. The other individual proteins stay somewhat the same. From a previous study (Nakanishi 2014) it was recognized that the β-CN fraction in comparison with whole casein increased after a long period of storage time in the freezer, this previous study suggest that this occurs as a result of flocculation. They suggest that the protein destabilization of milk results due to an increased salt concentration during frozen storage which is associated with lactose crystallization.

Table 11 Total whey and total caseins of the different undigested milk samples at different times stored in the freezer

	Cow #18		Cow #21		Cow #22	
	Total whey	**Total caseins**	**Total whey**	**Total caseins**	**Total whey**	**Total caseins**
Milk 1d old	21.2%	78.8%	18.7%	81.3%	17.9%	82.1%
Milk 15d old	20.3%	79.7%	19.3%	80.7%	15.5%	84.5%
Milk 21d old	17.8%	82.2%	15.7%	84.3%	14.1%	85.9%

Table 12 Individual proteins of the different raw milk samples at different storage times

	Cow #18					
	β-Lg B	**α-La**	**k-CN A**	**β-CN**	**αs_1-CN**	**αs_2-CN**
1d old	6.91%	14.3%	7.26%	27.4%	30.2%	13.9%
15d old	6.80%	13.5%	7.46%	28.9%	30.9%	12.4%
21d old	6.80%	11.0%	3.56%	32.3%	32.9%	13.4

	Cow #21						
	β-Lg A	**α-La**	**k-CN A**	**k-CN B**	**β-CN**	**αs_1-CN**	**αs_2-CN**
1d old	10.0%	8.66%	3.54%	5.26%	30.4%	26.8%	15.3%
15d old	9.61%	9.72%	3.08%	4.81%	30.0%	28.0%	14.8%
21d old	9.47%	6.27%	1.39%	3.33%	34.4%	29.3%	15.9%

	Cow #22					
	β-Lg A	**α-La**	**k-CN A**	**β-CN**	**αs_1-CN**	**αs_2-CN**
1d old	10.8%	7.09%	7.20%	32.2%	26.4%	16.3%
15d old	9.79%	5.70%	6.34%	35.0%	29.7%	13.4%
21d old	10.1%	4.06%	3.14%	40.0%	30.9%	11.8%

10 CONCLUSION

From the results obtained during this thesis study it can be concluded, unlike what was expected, that there is no remarkable difference in the breakdown of genetic variants A and B of β-Lactoglobulin during 3-6M old in-vitro gastric digestion. Since during this study individual milk was used in contrast to the previous study of Wang (2017) where bulk milk was used, it could be that there only occurs a difference between the genetic variants A and B when both are present in the milk, which is the case in bulk milk. However, unintentionally one of the four cows turned out to contain both genetic variants A and B. In this case there was also no remarkable difference found between the two genetic variants therefore indicating that there is in fact no difference. During the study of Wang (2017) only one bulk milk sample was used and during this study four individual cow milk samples were investigated. These are low numbers of cow milk samples to be able to draw reliable conclusions but four is still more than one and therefore it can be assumed that there is no large difference in the gastric infant digestion between β-Lg A and B. Comparing the overall digestion there was less difference found between the breakdown of the different protein compared to the results of Wang (2017). The percentages of the other digested proteins such as k-CN, αs-CN and β-CN were also different than expected when comparing the results to the results that the previous thesis student Wang (2017) found. The values for the digestion of casein found during this thesis study were much lower, around 45%, compared to Wang (2017) who found >90% meaning the caseins were almost fully digested.

During the experiments a lower breakdown of casein was found compared to the values of Wang (2017). This could indicate that something could have gone wrong during measurements. By putting some of the samples on a SDS-PAGE gel, which gave similar results to the data obtained from the RP-HPLC, it was excluded that something had gone wrong with the RP-HPLC. Since the 3-6M digestion model was a newly made model by a previous thesis student and only tested a few times on bovine milk, it could be that the model was not a reproducible model. To exclude the fact that this was the case the 3-6M digestion model was done in fourfold on two of the raw cow milk samples. The data obtained from these measurements indicated that the model that is used is in fact a reproducible model. Only very little differences were found, mainly in caseins, which was expected since some of the protein flakes might have got stuck onto the pH-meter and could have been washed off when cleaning the pH meter for the next measurement. Since the model was a reproducible model this could not have led to the lower breakdown values that were found after the digestion of the bovine milk proteins. By doing further research, such as an enzymatic assay on the pepsin that was used during the experiments, the source for the lower breakdown results was found. It turned out that the enzymatic activity of the pepsin that was used during the 3-6M old digestion model was lower than it should have been, most likely due to autolysis that occurred during storage of the pepsin in the freezer.

Between the measurements of this thesis study, the raw bovine milk was put back in the freezer at -20°C and defrosted again when it was needed for new experiments. Not much can be found in literature about the consequences of storing raw bovine milk in the freezer for a long period of time, therefore this has been looked into. As expected it turned out that when storing milk for 15 days it did not have a large effect on the whey/casein ratio of the raw bovine milk. But after 21 days the whey protein that was measured was less than before, compared to the casein. It was found that α-La and k-CN had definitely decreased, whereas β-CN somewhat increased. The other individual proteins stayed somewhat the same. From a previous study (Nakanishi 2014) it was recognized that the β-CN fraction in comparison with whole casein increased after a long period of storage time in the freezer, this study suggest that this occurs as a result of flocculation. They suggest that the protein

destabilization of milk results due to an increased salt concentration during frozen storage which is associated with lactose crystallization.

11 RECOMMENDATIONS

Since there was not enough time during this bachelor thesis to research all initial research questions it could still be interesting to research these initial questions that are stated in the proposal. My initial research plan was to also investigate the differences in breakdown of β-Lactoglobulin at different levels of denaturation. It is known that the more a protein has been denatured, the more easy the protein is digested. During denaturation the protein is randomly unfolded and will randomly aggregate, therefore the protein will not have a specific structure anymore. Because of this, it is expected that the structural differences between genetic variants A and B will be less relevant after denaturation than before and lead to no significant difference in behaviour. Even though there was not enough time during this particular thesis study, it is still relevant and interesting to do further research on this topic, and thereby taking the previous mentioned research questions into account.

Before starting to work with digestion models make sure that the enzymes that are used are fresh. During this experiment the enzymes used were not new and it was unknown how long the enzymes had been stored in the freezer. Since autolysis of the enzymes occurs straight after the bottle is opened, even though it is stored in the freezer, this will have an effect on the enzyme activity and therefore will have an effect on the data. To exclude the enzyme activity being too low and to save time it is recommended to start with a fresh bottle of enzymes once research is performed with digestion models. Since pepsin from porcine gastric mucosa has quite a wide activity range it is suggested to regularly measure the pepsin activity.

It is recommended that when it is planned to perform experiments on bovine milk, fresh milk is used. Since storing milk in the fridge for a long period of time could in fact have an influence on the milk composition and therefore could have an effect on the data.

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13 APPENDIX

Table 13 Raw data from the peak area of 1d old bovine milk obtained from the Chromatograms measured with RP-HPLC. Unfortunately the farmer forgot to milk cow #54.

***BEFORE DIGESTION* (1d old milk)**	**#18 (β-B, βCN-A2)** ***mAU*min***		**#21 (β-A, βCN-A2)** **mAU*min**		**#22 (β-A, βCN-A1)** **mAU*min**	
β-Lg A	-		27.0708		31.6568	
β-Lg B	19.5235		-		-	
α-La	40.3653		23.4471		20.7776	
total whey	59.8888	**21.2%**	50.5179	**18.7%**	52.4344	**17.9%**
k-CN A	20.5234		9.5923		21.1027	
k-CN B	-		14.2461		-	
αs_2-CN	39.3536		41.3034		47.8761	
αs_1-CN	85.4118		72.6783		77.2817	
β-CN	77.4128		82.4228		94.5414	
total casein	222.7016	**78.8%**	220.2429	**81.3%**	240.8019	**82.1%**
Whey/Casein ratio		**0.27**		**0.23**		**0.22**

Table 14 Raw data from the peak area of 15d old bovine milk obtained from the Chromatograms measured with RP-HPLC

***BEFORE DIGESTION* (15d old milk)**	**#18 (β-B, βCN-A2)** ***mAU*min***		**#21 (β-A, βCN-A2)** **mAU*min**		**#22 (β-A, βCN-A1)** **mAU*min**		**#54 (β-AB, βCN-A1,A2)** **mAU*min**	
β-Lg A	-		26.0908		28.3465		22.2398	
β-Lg B	19.9815		-		-		12.9954	
α-La	39.6142		26.3812		16.5083		21.4505	
total whey	59.5957	**20.3%**	52.472	**19.3%**	44.85083	**15.5%**	56.6857	**14.6%**
k-CN A	21.9253		8.3577		18.3688		21.2524	
k-CN B	-		13.0583		-		-	
αs_2-CN	36.4795		40.0581		38.91665		70.5386	
αs_1-CN	90.824		76.0593		86.0068		110.6848	
β-CN	85.0204		81.4044		101.4454		130.2048	
total casein	234.6785	**79.7%**	218.9378	**80.7%**	244,7377	**84.5%**	332.7997	**85.4%**
Whey/Casein ratio		**0.25**		**0.24**		**0.18**		**0.17**

Table 15 Raw data from the peak area of 21d old bovine milk obtained from the Chromatograms measured with RP-HPLC

BEFORE DIGESTION **(21d old milk)**	**#18 (β-B, βCN-A2)** ***mAU*min***		**#21 (β-A, βCN-A2)** ***mAU*min***		**#22 (β-A, βCN-A1)** ***mAU*min***		**#54 (β-AB, βCN-A1,A2)** ***mAU*min***	
β-Lg A	-		24.5491		30.3084		20.3259	
β-Lg B	18.2402		-		-		13.0877	
α-La	29.5815		16.2663		12.2313		12.677	
total whey	47.8217	**17.8%**	40.8154	**15.7%**	42.5397	**14.1%**	46.0906	**13.6%**
k-CN A	9.5442		3.6133		9.4696		8.5485	
k-CN B	-		8.6305		-		-	
αs_2-CN	36.0198		41.2908		35.627		56.8661	
αs_1-CN	88.2018		75.8813		93.254		103.5647	
β-CN	86.4759		89.0944		120.6371		125.1839	
total casein	220.2417	**82.2%**	218.5103	**84.3%**	258.9877	**85.9%**	294.1632	**86.5%**
Whey/Casein ratio		**0.22**		**0.19**		**0.16**		**0.16**

Figure 6 RP-HPLC Chromatograms from fresh milk (1d old) before digestion and after gastric 3-6M digestion cow's #18, #21, #22

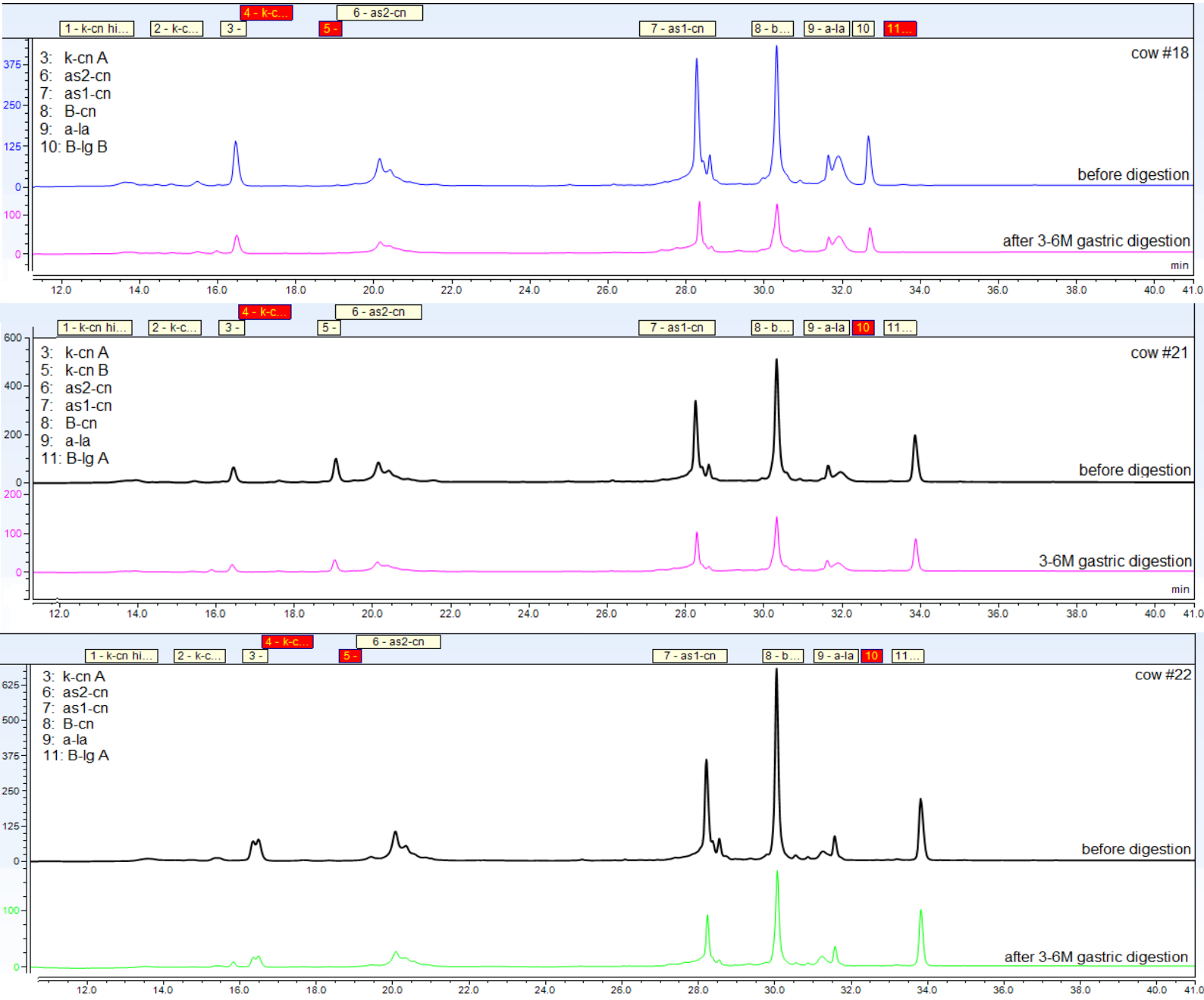

Figure 7 Chromatograms obtained from RP-HPLC, undigested milk samples of 15d old milk that has been stored in the freezer

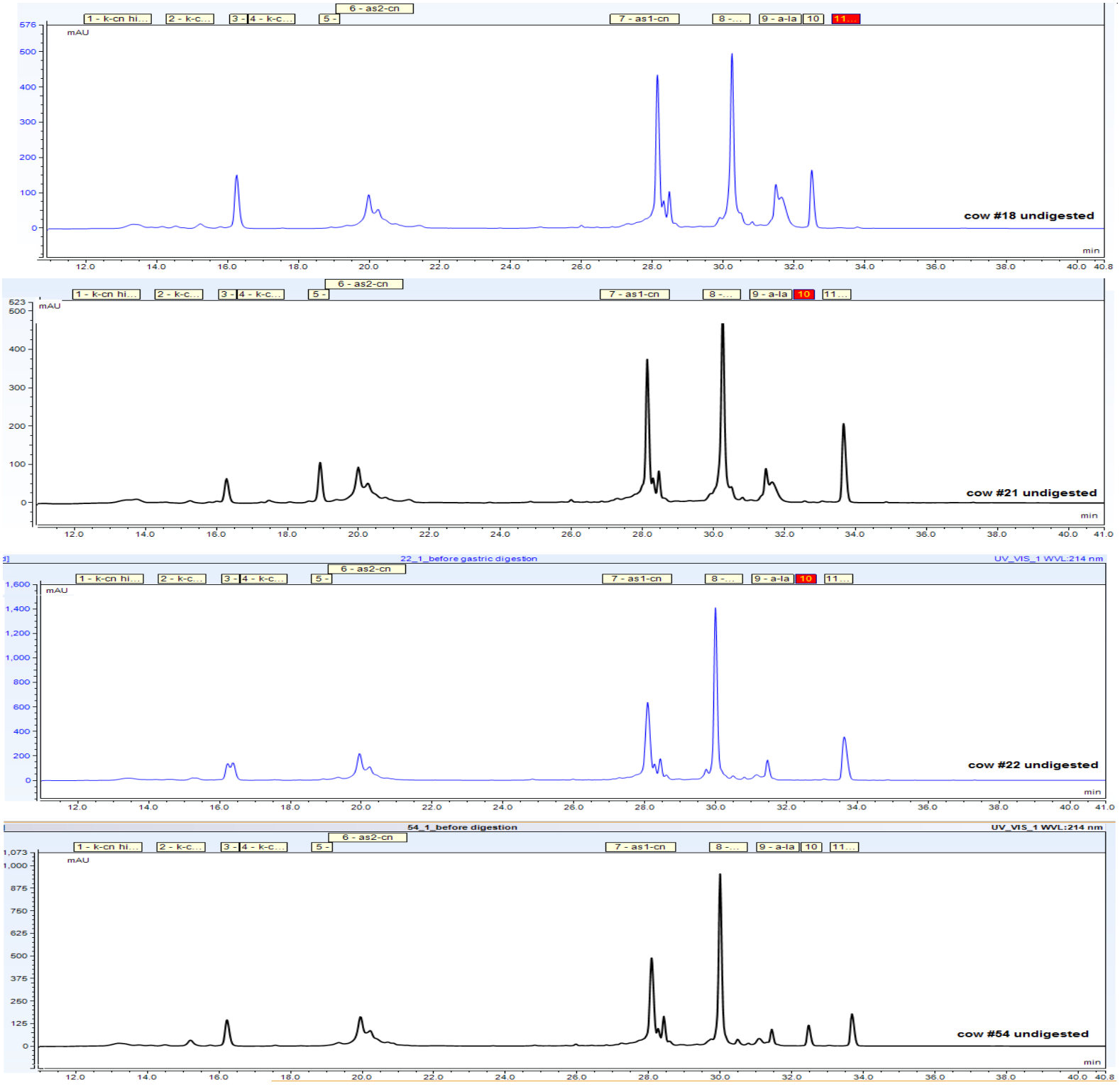

Figure 8 Chromatograms obtained from RP-HPLC, undigested milk samples of 21d old milk that has been stored in the freezer

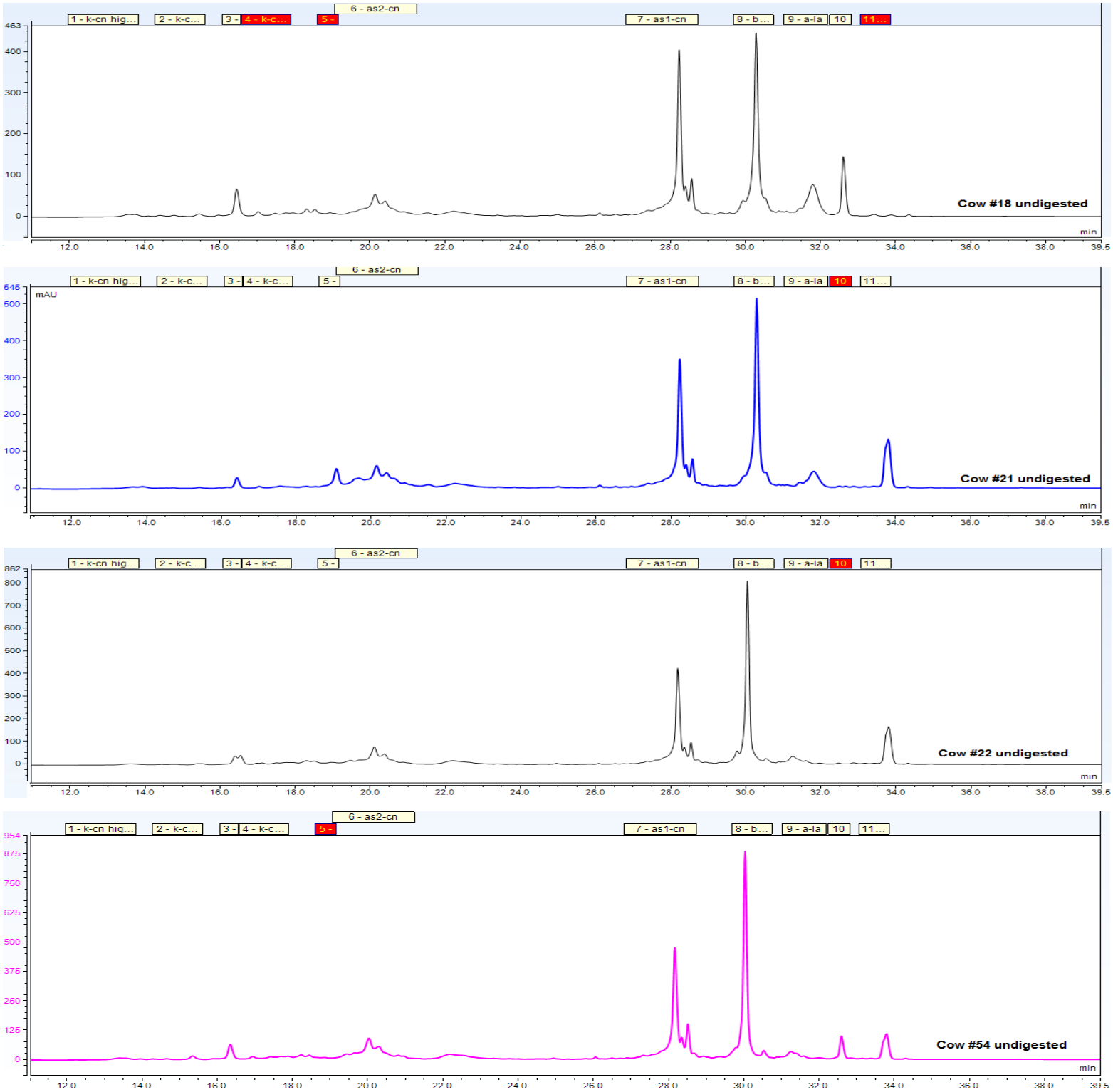

Table 16 Volume bands lane 1 and 2 and their protein breakdown in percentages

	Lane 1 (Volume (OD))	**Lane 2 (Volume (OD))**		
Band number	Cow #18 before digestion	Cow #18 after gastric digestion	*2	%
1	551.23	107.14	214.28	61.13
2	297.07	46.39	92.78	68.77
3	131.49	12.91	25.82	80.36
4	232.86	43.50	87.0	62.64
5	1736	275.47	550.94	68.26
6	605.09	88.37	176.74	70.79
7	1366.52	322.39	644.788	52.82
8	9841.42	2612.32	5224.64	46.91
9	2162.92	519.63	1039.26	51.95
10	688.03	115.37	230.74	66.46

Bands/proteins	**Lane 1 (Volume (OD))**	**Lane 2 (Volume (OD)) *2**	**% breakdown**
7+8 / Caseins	11207.94	5869.42	47.63%
9 / β-Lg	2162.92	1039.26	51.95%
10 / α-La	688.03	230.74	66.46%

Table 17 Volume bands lane 3 and 4 and their protein breakdown in percentages

	Lane 3 (Volume (OD))	**Lane 4 (Volume (OD))**		
Band number	Cow #21 before digestion	Cow #21 after gastric digestion	*2	%
1	506.60	112.82	225.64	55.46
2	265.46	67.07	134.14	49.47
3	170.51	40.94	81.88	51.98
4	285.79	37.29	74.58	73.90
5	1490.81	306.53	613.06	58.88
6	610.43	118.44	236.88	61.19
7	1471.05	537.09	1074.18	26.98
8	8817.27	2371.64	4743.28	46.20
9	2816.88	906.62	1813.24	35.63
10	515.85	168.48	336.96	34.68

Bands/proteins	**Lane 3 (Volume (OD))**	**Lane 4 (Volume (OD)) *2**	**% breakdown**
7+8 / Caseins	10288.32	5817.46	43.46
9 / β-Lg	2816.88	1813.24	35.63
10 / α-La	515.85	336.96	34.68

Table 18 enzyme activity assay of pepsin calculations

	A_{280}	A_{280} - $A_{280\ blank}$	Dilution factor (Df)	$\frac{A_{280} - A_{280}\ x\ Df}{10 \times 1 \times 0{,}001}$ (Units/mg enzyme)
Blank	0.3115			
0.05 mg/ml	1.7174	1.4059	20	2812
0.04 mg/ml	1.4620	1.1505	25	2876
0.03 mg/ml	1.2329	0.9214	33.33	3071
0.02 mg/ml	0.8406	0.5291	50	2646

Table 19 Results RP-HPLC of the fourfold 3-6M old digestion of cow milk from cow #18

	18 A **(mAU*min)**	18 B **(mAU*min)**	18 C **(mAU*min)**	18 D **(mAU*min)**
k-CN A	3.0568	1.6144	1.5382	1.6370
αs_2-CN	17.8292	18.1178	18.4493	18.9143
αs_1-CN	25.8569	26.4321	26.8667	26.4786
β-CN	37.2633	39.3191	39.8982	39.7669
α-La	3.1638	3.4563	3.2282	3.6775
β-Lg B	7.0468	7.5255	7.3753	7.2006

Table 20 Results RP-HPLC of the fourfold 3-6M old digestion of cow milk from cow #21

	21 A **(mAU*min)**	21 B **(mAU*min)**	21 C **(mAU*min)**	21 D **(mAU*min)**
k-CN A	2.0473	2.5570	2.2780	3.0979
k-CN B	3.9818	3.7186	4.1037	3.7619
αs_2-CN	12.2833	11.6275	12.2519	12.8080
αs_1-CN	21.4770	20.7942	22.1233	22.7295
β-CN	33.3985	32.9508	34.6404	35.1339
α-La	2.1453	2.1333	2.2469	2.1997
β-Lg A	9.7427	10.0106	10.4469	10.2511

Table 21 Relative standard deviation, average and standard deviation of the RP-HPLC of the fourfold 3-6M old digestion of cow milk from cow #18 and #21

Cow #18	**β-Lg B**	**α-La**	**β-CN**	**k-CN A**	**k-CN B**	**αs_1-CN**	**αs_2-CN**
STDV	2.08	2.34	12.24	7.31	-	4.16	4.66
AVG	72.871	33.815	390.619	19.616	-	264.086	183.277
RSD%	2.86%	6.92%	3.14%	37.28%	-	1.58%	2.54%
Cow #21	**β-Lg A**	**α-La**	**β-CN**	**k-CN A**	**k-CN B**	**αs_1-CN**	**αs_2-CN**
STDV	3.05	0.52	10.26	4.53	1.83	8.33	4.83
AVG	101.128	21.813	340.309	24.951	38.915	217.810	122.427
RSD%	3.01%	2.40%	3.01%	18.14%	4.69%	3.83%	3.94%

14 ETHIEK

Ad1 'Mijn alledaagse dagorde'

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Het verschil tussen de genetische varianten A en B van β-Lactoglobuline, met betrekking tot digestie in jonge kinderen.

Voor mijn thesis lab onderzoek heb ik melk monsters nodig van verschillende koeien met bepaalde eigenschappen. Deze monsters kies ik uit een database vol met individuele koeien van verschillende boerderijen waar ik verder niet van af weet. Het enige wat ik hoef te doen is het nummer van de koe met de gewenste eigenschappen door te geven aan mijn supervisor en een paar dagen later staat de melk klaar.
Ik weet verder niet of er op juiste wijze wordt omgegaan met deze koeien, en toch gebruik ik hun melk. Is het mijn plicht om te controleren of de melk monsters die ik gebruik voor mijn onderzoek op ethisch verantwoorde wijzen zijn verkregen, voordat ik ze gebruik, of mag ik er blind van uitgaan dat dit op de juiste manier gebeurt?

Hierbij beloof ik dat alles direct geciteerd of indirect aangeduid is met de namen van de desbetreffende auteurs. Indien ik andere informatie bronnen heb gebruikt heb ik dat aangegeven.

Ad2 Ethisch dilemma

Is het mijn plicht om te controleren of de melk monsters die ik gebruik voor mijn onderzoek op ethisch verantwoorde wijzen zijn verkregen, voordat ik ze gebruik, of mag ik er blind van uitgaan dat dit op de juiste manier gebeurt?

Ad3 Bijltjesmoment

Blind vertrouwen in de melkveehouderij?
Naar mijn idee leven wij in een land waar boeren goed met hun dieren omgaan en zou ik er blind vanuit moeten kunnen gaan dat deze koeien op de juiste manier behandeld worden. Helaas neemt deze gedachten het vervelende onderbuik gevoel niet weg en is de enige manier hoe ik er achter kan komen of de melk monsters daadwerkelijk op juiste manier verkregen worden en de koeien juist behandeld worden, door onderzoek te doen naar de desbetreffende boerderij. Ik kan vragen of mijn supervisor meer weet over de omgang van de boer met zijn koeien, aangezien hij al vrij lang contact heeft met deze boerderijen en al langer monsters hiervan gebruikt. Of is de enige manier om het echt zeker te weten door zelf op bezoek te gaan bij deze boerderijen en met mijn eigen ogen zien hoe deze boeren met hun koeien omgaan en of de melk op een juiste manier verkregen wordt? Maar is dit wel het juiste om te doen? Zou ik er niet blind vanuit moeten kunnen gaan dat de koeien op deze Nederlandse boerderij goed worden behandeld?

De oplossing van dit ethische dilemma, dat hierboven is beschreven, is naar mijn idee voornamelijk een deontologische oplossing. Bij deontologie draait de ethiek rond respect voor anderen als individuen, niet alleen mensen maar ook dieren horen juist en met respect behandeld te worden (Sward et al. 2006, 36). Niet alleen respect maar ook plicht speelt een rol en het feit dat je moet handelen volgens regels waarvan je wilt dat het een universele wet zou worden (Beyer 2013, 64) (van Erp 2000, 149-155). Wanneer ik vanuit deontologie beredeneer zou ik er dus vanuit mogen gaan dat er met respect en op de juiste manier gehandeld wordt met deze koeien op de boerderij. Als je kijkt naar de utilistische kant en er vanuit gaat dat de boeren niet op een juiste manier handelen dan zou

ik het met mijn eigen ogen moeten gaan bekijken en kijken hoeveel geluk het ons (de wetenschap) brengt, hoeveel geluk het de koeien brengt, hoeveel pijn het de koeien brengt en dat zou ik dan tegen elkaar af moeten wegen (Beyer 2013, 65) (van Erp 2000, 149-155) (Sward et al. 2006, 34). In dat opzicht brengt het ons, de wetenschap, een hoop geluk doordat we in dit geval onderzoek doen naar de vertering van een bepaald eiwit in koemelk om met die kennis misschien de allergeniciteit van koemelk bij jonge kinderen te kunnen verlagen. Hoeveel geluk het de koeien brengt is niet precies te zeggen, maar de melkkoeien moeten sowieso gemolken worden als ze melk produceren aangezien ze anders infecties krijgen, dus ik denk dat we ervan uit kunnen gaan dat het de koe geen verdriet doet (zolang het melken op de juiste manier gaat en het geen pijn geeft) en ook een soort van geluk geeft aangezien de melkkoeien veel melk produceren en daar vanaf moeten om infecties te voorkomen.

Maar wat is nou het juiste om te doen?
Als ik beredeneer vanuit de deontologie zou ik er van uit moeten kunnen gaan dat mens en dier met respect worden behandeld, en dus ook deze melkkoeien op de boerderij. Dan zou ik kunnen stellen dat wanneer iets een universele wet is, het volgens de deontologie het 'goede' is om te doen (Beyer 2013, 64) (van Erp 2000, 149-155). In dat geval ga ik er van uit dat ik er blind op kan vertrouwen dat het goed zit en zal ook mijn onderbuik gevoel weggaan. Omdat ik vind dat ik er vanuit kan gaan dat er in Nederland, anno 2017, de boeren op juiste wijze met hun dieren om gaan, zal ik hier ook naar handelen en dus ook niet bij de boerderijen langsgaan om dit na te gaan en kijken hoeveel geluk het ieder brengt. Het zit goed.

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Van Erp, Herman. 2000. *Moraal, geluk en verantwoordelijkheid: een filosofisch onderzoek naar de betekenis en het object van morele verplichting.* Assen: Van Gorcum & Comp.

Sward, Jac., Groothuis, Geny., Horbach, Jean., van der Valk, Jan. 2006. *Kan het ook anders?* Budel: DAMON.

15 BSC'S THESIS PROPOSAL

Thesis Title:	Differences in gastrointestinal digestion between genetic variances of β-Lactoglobulin in young children
Bachelor Student:	Roosje de Jong
Study Program:	BLT
Supervisors:	Kasper Hettinga
Department:	FQD: Dairy Science & Technology
Project Duration:	20.03.2017 – 07.07.2017

Abstract

Cow's milk is one of the most common causes of food allergy in the first years of a humans life. β-Lactoglobulin (β-Lg), of which genetic variances A and B exist, is the main whey protein in cow's milk and one of the major allergens causing this allergy. This study investigates the effect of genetic variants A and B of β-Lg on the digestion in children below the age of two. The objective of this study is to investigate the differences in the gastrointestinal tract (GIT) digestion between genetic variants A and B of raw bovine milk, to what extent these genetic variants are broken down at different levels of denaturation, to study the degree of hydrolysis (DH) and to find out the mechanism behind these differences. Knowing the mechanism could help reducing the allergenicity in cow's milk for children in their first years of life. RP-HPLC will be used to separate, identify and quantify β-Lg A and B in bovine milk and the OPA method will be used to measure the DH on purified β-Lg A and B from bovine milk.

Keywords: β-Lactoglobulin, genetic variant A and B, allergenicity, bovine milk allergy

Problem Definition

Cow's milk is one of the most common causes of food allergy in the first years of a humans life. β-Lactoglobulin, of which genetic variances A and B exist, is the main whey protein in cow's milk and one of the major allergens causing this allergy. During a thesis study of Wang (2017) a possible difference in the GIT digestion between the genetic variances has been found in young children below the age of two. In the past, several studies such as a study of Qin et al. (1998) already showed the structural differences between the genetic variants A and B of β-Lg and it is known that they differ at two sites in the amino acid sequence. However, no further research has been done regarding the digestion of these genetic variants. To fill in the gap of knowledge about this difference, further research will be done during this study. If there is in fact a difference to be found, the data from the experiments and the information obtained from literature about the known structural differences can be combined and could possibly provide knowledge in the mechanism. Knowing the mechanism could help reducing the allergenicity in cow's milk for children in their first years of life. Wang (2017) only looked at the amount of intact protein that remained. Because the degree of hydrolysis is also important, especially with regard to allergenicity, this will be further researched during this study. Since it is unlikely that young children will consume raw bovine milk, experiments should be performed at different levels of denaturation of β-Lg as well. This will give a more representative image for the current way that bovine milk in consumed. The level of denaturation influences the degree of digestion, therefore there will be looked at different levels.

Literature Review

Proteins in milk

There are quite some differences between human and bovine milk proteins, as shown in figure 1 below (Hambreus et al., 1977). Bovine milk contains far more proteins than human milk, especially caseins, and contains β-Lg which is absent in human milk. Even though β-Lg is naturally absent in human breast milk, it could be present if the mother has consumed bovine milk. Although this will be in very small quantities, it could still lead to allergic reactions. The nitrogen in milk is distributed among caseins, whey proteins and non-protein nitrogen. On the other hand, lactoferrin, which is an antimicrobial protein and relatively resistant to digestion in the gastrointestinal tract, is much more present in human milk compared to bovine milk (Haug et al., 2007). Lactoferrin and α-Lactalbumin play a key role in the immune system and are therefore important components in human milk whey protein.

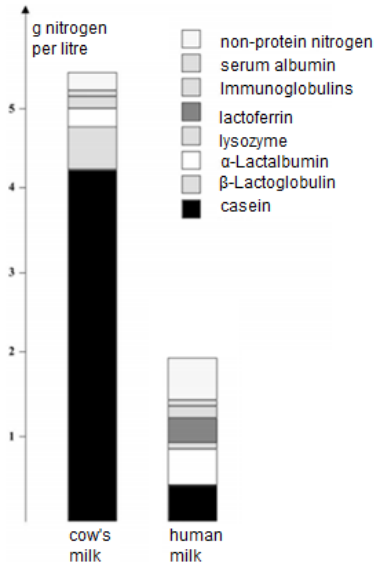

Figure 1 Protein composition of cow's milk and human milk. Nitrogen derived from various proteins and non-protein nitrogen are represented as grams of nitrogen per litre (Hambreus et al., 1977).

Some of the bovine milk proteins, such as β-Lg, are quite resistant to digestive enzymes (Haug et al., 2007). Due to this resistance, some of the intact β-Lg can be absorbed and lead to a build-up of an allergic reaction, mainly in young children, which will be further discussed in the section "allergenicity" below.

Allergenicity

β-Lg is quite resistant to the hydrolysis and proteases, this leads to some of the proteins remaining intact after gastric digestion. These intact β-Lg can be absorbed through the gut mucosa and build up an allergic reaction due to the immune cells present in the beginning of the small intestine (Wal, J-M., 2002). The known size of β-Lg is 18 kDa. Since epitopes can still be found and bind at 2-3 kDa the proteins should be cut into very small pieces in order for the allergen not to be reactive anymore.

During a thesis study of Wang (2017) a difference in digestion between the two genetic variances of β-Lg was found. In table 2 the results of the in-vitro protein digestion in bovine milk are shown that were obtained during the study of Wang (2017). The percentages stand for the amount of protein that is broken down, where 100% means the protein is completely broken down and 0% means no protein

has been broken down. It shows that the digestion of β-Lg in young children is far less than adults, which was already known. It also shows, which is the most relevant for this thesis study, that there is quite a big difference in the two genetic variants, which has not been seen before. Variant A is far less broken down compared to variant B, which will result in more intact β-Lg and therefore a higher chance of building up an allergic reaction to bovine milk with genetic variant A. Because further investigation on this difference was not executed, this difference first has to be studied by mimicking the in-vitro protein digestion. This will be done during this thesis study.

There has not been looked at the DH yet, which is of importance regarding the allergenicity. Therefore, during this thesis study there will also be looked at the DH.

Table 1 The decrease of bovine milk proteins after gastric digestion in infant and adult (Wang 2017)

	κ-casein B	α_{s2}-casein	α_{s1}-casein	β-casein	α-LA	β-LG B	β-LG A
0-3M gastric	76.79%	75.20%	92.42%	96.96%	19.54%	17.89%	8.15%
3-6M gastric	100%	91.26%	96.58%	90.95%	92.86%	21.77%	8.41%
0-2Y gastric	100%	100%	100%	100%	94.87%	22.08%	9.06%
Adult gastric	100%	100%	100%	100%	99.89%	52.12%	53.82%

β-Lactoglobulin

β-Lactoglobulin, with a size of 18kDa, is a prevalent protein in whey and milk serum and a major allergen causing food allergy. The molecule has two disulphide bridges and one free cysteine (Wal, J-M., 2002). It is 10% of the total milk protein, which is 58% of serum proteins. The percentage of β-Lg in infant formula is higher than 10%, sometimes even higher than 50%, since some caseins have been removed in order for the milk to become more similar to human breast milk. This removal leads to a higher percentage of whey-protein in the total amount of milk protein. The amino sequence and 3-dimensional structure of β-Lg shows that it is part of the lipocalin protein family that are involved in the transport of small hydrophobic molecules. In contrast with caseins, milk whey proteins are fairly resistant to the action of plasmin. β-Lg is manufactured specifically in the mammary gland for inclusion in milk and occurs in various genetic variants (Kontopidis et al., 2004)(Walstra et al., 2005)(van Valenberg., Hettinga 2015). During this study the main interest lays in the genetic variants A and B.

Structure

β-Lg is a calyx composed of an eight-stranded antiparallel β-sheet of which loops A-B, C-D, E-F and G-H surround the entrance of the cavity, while loops B-C, D-E and F-G close the opposite side of the β-barrel (Bello et al., 2007). Structural rearrangement occurs in the protein during the Tanford transition between pH 6-8. This is a reversible conformational change that occurs in loop E-F. At low pH, which is the case during gastric digestion, the loop adopts a closed conformation, blocking access to the calyx, whereas at high pH it folds back and opens to allow access of ligands to the calyx (Oliverira et al., 2001). This blocking access leads to the resistance to digestion which will be further discussed in section "Bovine milk protein digestion" below.

As shown in figure 2 (Botelho et al., 2000) below, variants A and B have a slightly different structure since they differ at two sites in the amino acid sequence: Asp64 in A is changed to Gly in B, and Val118 in A is changed to Ala in B. Due to this structural difference they differ in biophysical and biochemical properties. For example, their isoelectric points: pI= 5.26 for variant A and 5.34 for variant B.

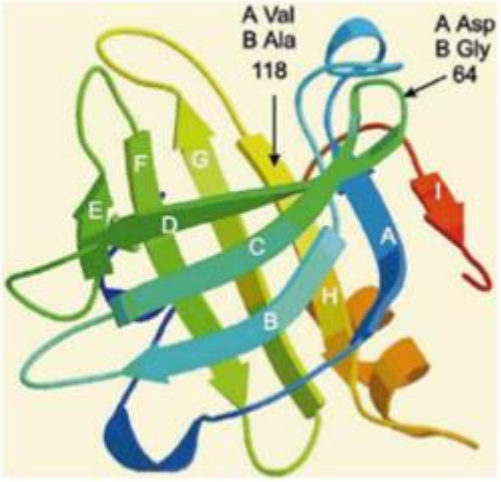

Figure 2 A general view of β-lactoglobulin, indicating the positions of the substitutions of variants A and B. (Botelho et al., 2000)

It is assumed that proteins at pH above their isoelectric point are more susceptible to pepsin digestion (Zhang et al., 2014). Therefore at acidic conditions, which is below the isoelectric point and the case during gastric digestion, proteins are more resistant to digestion.

The possible differences in denaturation between the genetic variants A and B could be linked to the structural differences within the proteins. The substitution Val->Ala results in the loss of two methyl groups, promoting formation of a cavity in the hydrophobic core of the protein. This could be responsible for the increased sensitivity of the B genetic variant to pressure and temperature (Oliverira et al., 2001)(Qin et al., 1998).

During denaturation, the protein is randomly unfolded and will randomly aggregate, therefore there will not be a specific structure anymore. Due to this, it is assumed that the structural differences between genetic variant A and B will be less relevant after denaturation than before.

As mentioned before the size of β-Lg is 18 kDa. Since epitopes can still be found and bind at 2-3 kDa the protein should be cut into very small pieces in order for the allergen not to be reactive anymore. When knowing the DH of a protein, assumptions can be made about the allergenicity.

The differences in flexibility is also linked to the structural differences. The substitution Asp->Gly is just located before the highly flexible loop C-D. Therefore, when any conformational changes occurs before this loop, this could result in differences in flexibility (Oliverira et al., 2001). It could be assumed that flexible structures are more sensitive to proteolysis. The change in structure can also lead to a difference in net charge, which modifies the dimer-monomer dissociation equilibrium (Creamer et al., 2004). Variant A, containing more negative charges, could lead to more resistance due to more electrostatic interaction, which keeps it more stable. This could lead to less degradation of the genetic variant A.

Variant A forms dimers and then octamers under increasingly acidic conditions, whereas variants B do not form octamers, but do form dimers, with dissociation constants in the micro-molar range at pH 7 and stability constants in the order B > A. Both A and B undergo pH-dependent conformational changes in the range pH 6.5 to pH 7.5 (Qin et al., 1998).

Bovine milk protein digestion

Protein digestion begins in the stomach, where an acidic environment favours protein denaturation. The primary proteolytic enzyme of the stomach is pepsin, a nonspecific protease that is maximally active at pH 2. Thus, pepsin can be active in the highly acidic environment of the stomach, even though other proteins undergo denaturation here (Berg et al., 2002). The proteins are further hydrolysed in the intestine, and then absorbed through the intestinal wall.

During digestion there is a difference in behaviour of proteins. Caseins are relatively unstructured and more hydrophilic parts are exposed, this could lead to a more digestible protein. In contrast with caseins, milk whey proteins are fairly resistant to the action of plasmin. Whey proteins have a globular structure which results in less exposed hydrophilic bonds and therefore more interactions in order to keep the structure stable (Shandan et al., 2009). Due to the less exposed hydrophilic bonds there is less access for the proteases to break down the protein, meaning its harder to digest.

The younger the human, the less proteins are broken down during gastric digestion. This is due to the lower level of proteases and higher pH in the stomach of young children compared to adults. Therefore the occurrence of cow's milk allergy is higher in children because of the less digested proteins, as mentioned before in the section about allergenicity.

Degree of denaturation

Native β-Lg is very resistant to pepsin digestion, while disruption of native structure and conformation by thermal treatment exposes susceptible peptide bonds and decreases the resistance to proteolytic digestion (Zhang et al., 2013). From literature it is known that the more a protein has been denatured, the more easy the protein is digested, since denatured proteins are more accessible as substrates for proteolysis than native proteins are (Berg et al., 2002). If a protein is more easily digested this could have a positive effect regarding allergenicity. Therefore, looking at the level of denaturation is important. During this thesis study there will be looked at three different levels of denaturation.

According to literature whey proteins are sensitive to unfolding at temperatures above 60˚C (O'Loughlin et al., 2002). Above this temperature the hydrophobic bonding is decreased. Therefore, as first level of denaturation 65˚C will be used, the second level will be 75˚C and the third 85˚C.

Serum proteins, such as β-Lg, are globular proteins which are easily denatured upon heating. Upon heating the proteins will precipitate on casein micelles. The casein micelle will be coated by whey proteins when milk is heated. After the milk proteins are denatured most conformational epitopes are destroyed. Enzymatic cleavage will mostly destroy linear epitopes but could also destroy conformational epitopes if the conformation is gone after cleavage (van Beresteijn et al., 1995). This could reduce the IgE binding and therefore the allergenicity. However, neo-epitopes should be kept in mind since they are formed during unfolding and could lead to new binding sites for the allergen to bind on.

Research Aim

The aim of this study is to investigate the differences in the breakdown of the genetic variants A and B of β-Lactoglobulin during digestion of unheated and heated bovine milk in young children.

Research questions

- Is there a difference in the breakdown of genetic variants A and B of β-Lactoglobulin during gastric and intestinal digestion?
- To what extent are the genetic variants A and B of β-Lactoglobulin broken down at different levels of denaturation?
- What is the degree and the rate of hydrolysis and do they differ between the genetic variants A and B of β-Lactoglobulin?

Research hypothesis

A previous thesis study of Wang (2017) has shown that there is a difference in the breakdown of the genetic variants A and B of β-Lactoglobulin during digestion of unheated bovine milk in young children. Because this was out of the scope and irrelevant for that study this has not been further investigated. Through this study it is expected that there is a difference in the breakdown of genetic variant A and B.

It is known that the more a protein has been denatured, the more easy the protein is digested. However, it is unknown if there is a difference between the genetic variants A and B regarding denaturation. During denaturation the protein is randomly unfolded and will randomly aggregate, therefore the protein will not have a specific structure anymore. Because of this, it is expected that the structural differences between genetic variants A and B will be less relevant after denaturation than before and lead to no significant difference in behaviour.

It is of importance to not only look at the amount of β-Lactoglobulin that is still intact after digestion, but also the degree of hydrolysis, to see how small the protein pieces are cut. In order for the protein to not be reactive anymore it should be cut into very small pieces, since epitopes can still be found around 2-3 kDa. The amount of time it takes before the protein is broken down is also important. If the proteins are broken down very slowly, they are longer intact and can cause problems for a longer time. Since the genetic variants A and B differ in structure it is expected that this could lead to a difference in the degree and speed of hydrolysis.

Research Plan

In order to do a thesis about the difference in digestion between the two genetic variances of β-Lactoglobulin in young children, this difference first has to be studied. Therefore, mimicking the laboratory work of a previous thesis study has to be done. This will be done with an in-vitro infant static digestion model. The protein composition and content will be determined by RP-HPLC and the resulting chromatograms will be analysed through the Chromeleon 7.2 SR4 software. If there is indeed a difference, which is assumed, then purified protein will be used to measure the degree of hydrolysis. This will be done according to the OPA method. The reason this hydrolysis is of importance for this study is because the DH can say something about the allergenicity. It will be performed with purified protein because only the DH of β-Lactoglobulin is of importance. Since raw bovine milk is hardly consumed there will also be looked at denatured protein in order to get a more representative image of how bovine milk is consumed. Since the level of denaturation affects the digestion there will be looked at three different levels of denaturation. The plan is to make an overview of what has to be done and perform this in the lab. While waiting to do this laboratory work, literature review on bovine milk proteins, in particular β-Lactoglobulin, will be performed to obtain more knowledge about the subject.

Milk sampling

Milk samples are chosen focusing on β-Lg A and B since the difference between these genetic variants is of importance for this study. Accordingly, milk samples of β-Lg A and β-Lg B will be used. Fresh milk samples will be taken from four cows: two β-Lg A and two β-Lg B.

These samples will be picked out from an individual milk database provided by Hein van Valenberg from Wageningen University & Research Centre.

Since the amount of β-Lg present in a cow could have an effect on the degree of denaturation, it has been tried to pick samples with similar differences in amounts of β-Lg.

Farm van Ommeren							
		β-CN		**β-Lg A**	**β-Lg B**		
Genotype	β-Lg A	A2	**Amount of β-Lg**	7.27	0.79	**Cow number**	21
	β-Lg A	A1		7.58	0.61		22
	β-Lg B	A2		0.45	5.73		18
	β-Lg B	A1,A2		0.22	5.92		54

These raw bovine milk samples will be first centrifuged and the fat will be removed. Then the samples will be used in the in-vitro infant static digestion model for 3-6 month old infants (Wang 2017). The protein composition and content will be determined by RP-HPLC and the resulting chromatograms will be analysed through the Chromeleon 7.2 SR4 software.

These raw bovine milk samples will also be used to see if the difference remains after the milk is denatured upon heating. In this case these samples will be denatured upon heating in three different levels before they will be used in the in-vitro infant static digestion model, the RP-HPLC and the Chromeleon 7.2 SR4 as mentioned above.

Milk serum preparation

The whole milk samples were centrifuged at 1500g for 10 minutes at 10˚C (Beckman coulter Avanti J-26-XP centrifuge, rotor JA-25.15). The pellet (on top, hard material) was removed and the supernatant was transferred to the ultracentrifuge tubes followed by ultracentrifugation at 100.000g for 90 minutes at 4˚C (Beckman L-60, rotor 70 Ti). After ultracentrifugation, samples were separated into three layers. Of which the top layer was milk fat, the middle layer was milk serum and the bottom layer (pellet) was casein.

The top layer (fat) will be removed, that way the fat cannot have an influence on the breakdown of the proteins.

Degree of denaturation

According to literature whey proteins are sensitive to unfolding at temperatures above 60˚C. It is known that the more a protein has been denatured, the more easy the protein is digested, since denatured proteins are more accessible as substrates to proteolysis than native proteins are. If a protein is more easily digested this could have a positive effect regarding allergenicity.

During this thesis study there will be looked at three different levels of denaturation. The light level will be at 65˚C, the medium level will be at 75˚C and the hard level will be at 85˚C. They will be heated at the given temperature for 30 min so the milk has time to warm up.

These samples will be used in the in-vitro infant static digestion model, the RP-HPLC and the Chromeleon 7.2 SR4.which are further discussed below.

In-vitro protein gastric digestion model 3-6M

To mimic the results of Wang (2017) the same infant protein digestion model for 3-6M old infants will be used because in this age category the largest difference was obtained. To mimic the infant gastric digestion the following modifications were done to the standardised adult model of Minekus et al (2014): the pH of the gastric phase will be changed from 3.0 to 3.5, the pepsin concentration in the gastric digestion mix will be decreased by a factor of 4 (500 U/mL) and the time of the gastric phase will be changed from 2 hours to 1 hour.

In the GIT digestion, five parts of oral bolus, is mixed with 4 parts of simulated gastric fluid (SGF) stock electrolyte solution to obtain a final ratio of food to SGF of 50:50 (v/v) after addition of other recipients and water. Porcine pepsin (EC 3.4.23.1) is added to achieve 500 U/mL in the final digestion mixture, followed by CaCl2 to achieve 0.075 mM in the final digestion mixture. 1 M HCl is added to reduce the pH to 3.5. The mixture was then incubated at 37 °C for 1 h in a shaking incubator. The enzymatic hydrolysis was stopped by adjusting the pH to 7.

In the intestinal phase, five parts of gastric chyme is mixed with 4 parts of simulated intestinal fluid (SIF) electrolyte stock solution to obtain a final ratio of gastric chyme to SIF of 50:50 (v/v) after additions of other recipients and water. Porcine pancreatin (4 USP specifications) was added to reach an enzyme activity of 800 U/mL, followed by the addition of freshly prepared bile salts at a concentration of 160 mM. And then CaCl2is added to reach 0.3 mM in the final digestion mixture, the pH may need re-adjustment to 7. After incubation at 37 ° C for 2h in a shaking water bath, the enzyme was inactivated by the addition of the protease inhibitor Pefabloc SC to reach a concentration of 1 mM in the duodenal chyme. At last, the chyme was kept in freezer (-20℃) waiting for following experiment.

RP-HPLC

The RP-HPLC will be used to separate proteins according to their hydrophobic character. Quantification and identification will be done via the Chromeleon 7.2 SR4 software to identify the peaks in the samples.

Sample preparation

The protein samples preparation will follow the procedure stated by Bobe et al. (1998). A solution comprised of 0.1 M Bis Tris buffer (pH adjusted to 6.8), 8 M Urea, 5.37 mM sodium citrate and 19.5 mM DTT (pH 7) will be added directly to the samples in a 3:1 ratio (v:v) at room temperature. After thawing, samples will be shaken for 10 seconds, incubated for 1h at room temperature, and centrifuged for 5 minutes at 16000g in a micro-centrifuge. The remaining solubilized sample will be diluted 1:3 or 1:1(for digestion chime) (v:v) with a solution (adjust the pH to 2) containing 6M Urea in solvent A, which consists of 0.1% TFA acid in water (v:v).

Before injection into the RP-HPLC column, samples and solution will be filtered through 0.2 µm filters.

The equipment that will be used is HPLC, Thermo Scientific™ UltiMate 3000, with reversed-phase analytical column C18 (Aeris Widepore 3.6um XB-C18 RP, Phenomenex) with a silica-based packing (3.6µm, 300 Å, 250x4.6 I.D.).

Analysing chromatograms via Chromeleon 7.2. SR4 software

For the analysis the eluents that will be used will be labelled A and B; Eluent A will be prepared as a solution of 0.1%(v/v) TFA acid in Milli-Q water; and Eluent B will be a solution of 0.1% (v/v)TFA in acid in acetonitrile. Every run will last 42 min with the following gradient with ratio A: B; linear gradient from 65:35 in 5 minutes, 62: 37 in 4 minutes, 60:40 in 9 minutes, 59.5: 40.5 B in 2 minutes. 57: 43 in 0.5 minutes. Then in isocratic elutionat 57:43 during 4.5 minutes. Then return to linear elution with ratio 56:44 in 3 minutes. 53:47 in 4.5 minutes. Afterwards isocratic elution at 53:47 for 5 minutes and return linearly to the starting condition in 0.5 minute-equilibration under the starting conditions for 5 minutes. Temperature of samples will be 4 °C, while that of the oven will be 45°C. Injection volume will be 5µL and the flow rate will be 0.4 ml/min.

For bovine milk and human milk samples of 5ml and 1ml will be used.

Degree of hydrolysis

It is of importance to not only look at the amount of β-Lactoglobulin that is still intact after gastric digestion, but also the degree of hydrolysis, to see how small the protein pieces are cut. The amount of time it takes before the protein is broken down is also important. If the proteins are broken down very slowly, they are longer intact and can cause problems for a longer time. This measurements will be done via the OPA method with samples of purified protein, since it is necessary to only look at the β-Lg to be able to draw any conclusions. During the OPA method samples will also be checked at different times. When knowing the DH of a protein and the time it takes before the protein is broken down, assumptions can be made about the allergenicity. The OPA method is based on the specific reaction between ortho-phtaldialdehyde (OPA) and free primary amino groups in proteins in the presence of 2-(dimethylamino)ethanethiol hydrochloride (DMA), to give alkyl-iso-indole derivatives that show an absorbance at 340 nm. The determination is very specific for lysines and the protein N-terminal group over arginines. 4 mL cuvettes will be used for the measurements.

$$DH = \frac{number\ of\ hydrolysed\ peptide\ bonds\ per\ gram\ protein}{total\ number\ of\ peptide\ bonds\ per\ gram\ protein} * 100\%$$

To measure the degree of hydrolysis the protocol of the OPA method, obtained from the Food Chemistry chair group, will be performed according to protocol.

Protocol OPA method

Chemicals, Reagents and Apparatus

1. Spectrophotometer (340 nm)
2. 0.1 M di-sodium tertraborate buffer: Dissolve 19.07 gram di-sodium tetraborate-Decahydrat ($Na_2B_4O_7 \cdot 10H_2O$) in 500 mL milliQ water.
3. 10 % SDS:
   - Dissolve 10 gram Sodiumdodecylsulfate in 100 mL MilliQ water. (wear a fine dust mask and or work in the fume hood to avoid the inhalation of SDS).
4. OPA reagent:
   - The OPA-reagent is prepared ***daily*** by dissolving 40 mg OPA (Sigma, P-0657) in 1 mL methanol,
   - Add 25 mL 0.1 M di-sodium tertraboratebuffer and 200 mg DMA (Aldrich, D14.100-3)
   - add 5 mL 10% SDS.
   - Adjust the volume to 50 mL with MilliQ water.
5. 2 mM L-Leucine:
   - Dissolve 13.1 mg L-Leucine (Pierce, Mw. 131.18 g/mol) in 50 mL MilliQ water. Calculate the exact concentration.

Method

Sample preparation:

For protein hydrolysates or other samples in which aggregation can occur, samples should first be diluted in 2 % SDS to a protein concentration of 5 mg/mL and incubated for 1 hour at RT. Hydrolysates can be further diluted to 1 mg/mL in water before addition to the OPA reagent.

Calibration curve

1. Fill six quartz cuvettes with 3 mL of OPA reagent, weight and determine the absorbance (A_{blanc}) at 340 nm.
2. Add 10, 20, 40, 80, 120 and 150 µL of the 2 mM L-leucine stock solution (weight all) to 3 mL of OPA-reagent, yielding concentrations in the range from 0.0066 to 0.095 mM L-leucine.
3. Incubate 10 minutes at room temperature.
4. Determine the absorbance at 340 nm and calculate $\Delta A_{340} = A_{leucine} - A_{blank}$..
5. Fit data by linear regression. Calculate the molar extinction coefficient (e) of alkyl-iso-indole derivative (=slope of linear function, should be 7000 ± 500 $M^{-1}*cm^{-1}$).

Sample measurement

1. Fill quartz cuvettes with 3 mL of OPA reagent, weight and determine the absorbance (A_{blanc}) at 340 nm
2. Add 50 µL sample containing 5 mM NH_2-solution (approx. 10 mg/mL protein solution), weight and mix.
3. Incubated for 10 min at room temperature.
4. Determine the absorbance at 340 nm (A_{sample}).and calculate $\Delta A_{340} = A_{Sample} - A_{blank}$..
5. Calculate the molar concentration of NH_2: $\Delta A_{340}/e$
6. For further analysis of the data, it is important to know the total protein concentration in the samples you analyzed with the OPA method. This can be done by DUMAS, or by direct measurement of the absorbance at 280 nm. For both measurements, use the pure protein solution, without OPA reagent. .
7. Calculate the amount of free NH_2 groups on the protein by dividing the molar concentration of NH_2 by the molar protein concentration.

Remark

1. All measurements are performed at least in duplicate.
2. If OPA reagents turns into pink color instead of yellow before adding protein, most probably DMA is contaminated by secondary amines.

Chemicals, Reagents and Apparatus

1. Spectrophotometer (340 nm)
2. 0.1 M di-sodium tertraborate buffer: Dissolve 19.07 gram di-sodium tetraborate-Decahydrat ($Na_2B_4O_7 \cdot 10H_2O$) in 500 mL milliQ water.
3. 10 % SDS:
   - Dissolve 10 gram Sodiumdodecylsulfate in 100 mL MilliQ water. (wear a fine dust mask and or work in the fume hood to avoid the inhalation of SDS).
4. OPA reagent:
   - The OPA-reagent is prepared ***daily*** by dissolving 40 mg OPA (Sigma, P-0657) in 1 mL methanol,
   - Add 25 mL 0.1 M di-sodium tertraboratebuffer and 200 mg DMA (Aldrich, D14.100-3)
   - add 5 mL 10% SDS.
   - Adjust the volume to 50 mL with MilliQ water.
5. 2 mM L-Leucine:
   - Dissolve 13.1 mg L-Leucine (Pierce, Mw. 131.18 g/mol) in 50 mL MilliQ water. Calculate the exact concentration.
6. Preparation of the calibrant :
   - Dilute a stock solution of Leucine to the different concentrations: 0.4 mM ; 0.8 mM; 1.6 mM; 3.2 mM; 4.8 mM and 6 mM in 1 mL milliQ.

Method

Remark : for measurements with microtiter plates, the volume in each well should be the same.

Calibration curve

1. Fill six wells with 300 µL of OPA reagent and determine the absorbance (A_{blanc}) at 340 nm.
2. Add 5 µL of each concentration of leucine solution to 300 µL of OPA-reagent, yielding concentrations in the range from 0.0066 to 0.095 mM L-leucine. Calculate the exact concentrations.
3. Incubate 10 minutes at room temperature in the microplate shaker
4. Determine the absorbance at 340 nm ($A_{Leucine}$) and calculate $\Delta A_{340} = A_{leucine} - A_{blanc}$.
5. Fit data by linear regression. Calculate the molar extinction coefficient (e) of alkyl-iso-indole derivative (=slope of linear function, should be 5200 ± 200 $M^{-1}*cm^{-1}$).
6. Calculate the molar concentration of NH_2: $\Delta A_{340}/e$
7. For further analysis of the data, it is important to know the total protein concentration in the samples you analyzed with the OPA method. This can be done by DUMAS, or by direct measurement of the absorbance at 280 nm. For both measurements, use the pure protein solution, without OPA reagent. .

Sample measurement

1. Fill the wells with 300 µl of OPA reagent and determine the absorbance (A_{blanc}) at 340 nm.
2. Add 5 µL of a 5 mM NH_2-solution (approx. 10 mg/mL protein solution).
3. Incubate for 10 min at room temperature in the microplate shaker.
4. Determine the absorbance at 340 nm (A_{sample}).and calculate $\Delta A_{340} = A_{Sample} - A_{blank}$
5. Calculate the amount of free NH_2 groups on the protein by dividing the molar concentration of NH_2 by the molar protein concentration.

Remark

1. All measurements are performed at least in duplicate.
2. If OPA reagents turns into pink color instead of yellow before adding protein, most probably DMA is contaminated by secondary amines.

Thesis Timeline

	Number of weeks
Proposal writing and literature review	4
Laboratory work	8
Report writing	3
Preparations for presentation	1

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