Fractionation and Purification of Goat Milk Proteins
by Preparative High-Pressure Liquid Chromatography

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Preface

This project was conducted in corporation with the Proteins Team at Arla Strategic Innovation Centre (ASIC) in Brabrand, Århus. Therefore I would like to thank the entire team for letting me work with them, and to give me the opportunity to see how a job at Arla can be. I could not have gotten a better place to finish my education, and I feel very privileged. A special thanks to Mette Christensen, who has been my mentor at ASIC, and provided me with a lot of knowledge and excellent feedback and support over the last year. Furthermore I would like to thank Lars Haastrup Pedersen and the people at Aalborg University for letting me make use of their equipment as well, and to give me good feedback at our monthly presentation meetings.

The front page picture is from (Kübelbeck, 2010). Figure 7 and Figure 9 have been created with the guidance of Lene Buhelt Johansen, a technician in the Proteins Team at ASIC. If no source is cited at the illustration, it is created in the liquid chromatography software.
The enclosed USB flash drive contains the report as a pdf-file, chromatograms and Excel sheets.

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Date Ditte A. Madsen

List of Abbreviations

ACN	Acetonitrile
α_{s1}-CN	α_{s1}-casein
α_{s2}-CN	α_{s2}-casein
α -lac	α-lactalbumin
ASIC	Arla Strategic Innovation Centre
β-lg	β-lactoglobulin
BSA	Bovine serum albumin
β-CN	β-casein
°C	Degrees celsius
Da	Dalton
DTE	Dithioerythritol
e.g.	For example
ESI	Electrospray ionization
HPLC	High-pressure liquid chromatography
IS	Internal standard
κ-CN	κ -casein
L	Liter
LC	Liquid chromatography
M	Molar
mg	Milligram
MS	Mass-spectrometry
ml	Milliliter
min	Minute(s)
mm	Millimeter
mg	Milligram
N	Nitrogen
nm	Nanometer
No.	Number
NPN	Non-protein nitrogen
pI	Isoelectric point
Prep HPLC	Preparative high-pressure liquid chromatography
RPC	Reversed-phase chromatography
RT	Retention time
skgm	Skimmed goat milk
TOF LC/MS	Time-of-flight liquid chromatography-mass spectrometry
TFA	Tri-fluoro acetic acid
TP	Total protein
UV	Ultra-violet
µl	Microliter
µV	Microvolt
µg	Microgram
V	Volume

Abstract

The project was conducted in cooperation with the Proteins Team at Arla Strategic Innovation Centre in Brabrand, Århus.
It was attempted to produce pure fractions of the goat milk proteins α_{s1}-casein, α-lactalbumin and β-lactoglobulin for use as internal standard in liquid chromatography. These three goat milk proteins have masses of 18191.27 Dalton, 14194.12 Dalton and 23615.33 Dalton respectively (UniProt, 2014), and are not similar to α_{s1}-casein, α-lactalbumin and β-lactoglobulin from cow milk. Caseins are milk proteins which have an isoelectric point at 4.6 (McSweeney, 2003), and they do not have significant secondary and tertiary structures, which makes them very heat stable. This is due to their amino acid composition (Guelph, 2009). Whey proteins are the proteins left in the solution after precipitation (McSweeney, 2003).
The fractionation and purification was conducted with the use of preparative high-pressure liquid chromatography and freeze drying. The purity of the fractions was determined with time-of-flight liquid chromatography mass spectrometry.
All of the three previously mentioned goat milk proteins were attempted purified, but it was only proven possible to obtain 11 mg of ≥95% pure β-lactoglobulin from 38.12 ml skimmed goat milk, due to the time frame. This yield of 11 mg was only 10% of the maximum yield.
Furthermore a Bradford Protein Assay of the milk, and the purified protein, was made. This clarified that the Kjeldahl method and the Bradford Protein Assay does not provide the same values of protein concentrations for the same sample; the Kjeldahl method consequently gave higher concentrations of protein than the Bradford method did.

Contents

Introduction

Proteins, or polypeptides, are biomolecules which consist of chains of amino acids linked together by peptide bonds. These biomolecules, which are found in practically all living organisms, have different chemical and physical properties, depending on their unique amino acid compositions (Whitford, 2011). Proteins are also present in milk, which is produced by female mammalian species. This fluid is produced as a nutrition source for their infants, which is why milk is rich in lipids and minerals as well. Milk proteins can be divided into two groups; caseins and whey proteins. The caseins are the milk proteins which have their isoelectric point at 4.6. The proteins which are not precipitated at this pH are the whey proteins. The content and amino acid composition of the proteins in milk varies from species to species and amongst the individual animals as well. But, as mentioned above, all proteins have peptide bonds, and peptide bonds absorb UV light at 214 nm, which makes it suitable to analyze proteins with e.g. liquid chromatography (LC) (McSweeney, 2003). Furthermore the proteins can bind dye, which is a property that is used when proteins are analyzed with the Bradford Protein Assay method, which rely on absorbance at 600 nm and standard curves (Bio-Rad, 2014).
When analyzing samples with LC, it must be assured that the results are comparable over time. This can be done by using an internal standard. An internal standard assures that the results from analysis made on different days, can be compared without worrying about e.g. aging of the detector lamp. This method is very helpful when analyzing dairy products, since these samples are often desired analyzed during maturing over several weeks or months.
When choosing an internal standard, some criteria must be fulfilled; the standard must not elute from the column simultaneously as the compounds in the sample, it must be detectable at the given wavelength, and it must not interfere with the compounds in the sample (Dolan J. W., 2012).
A suggestion for an internal standard for analysis of cow dairy products, could be goat milk proteins, since goat milk contain the same types of proteins as cow milk, but their amino acid sequences are not entirely similar. But the standards of goat milk proteins available on the market are very expensive. So for this diploma project, it was decided to attempt to purify goat milk proteins for use as internal standard in the Proteins Team laboratory at Arla Strategic Innovation Centre.

Thesis statement

The purpose of the experimental work was to produce one or more protein standards from goat milk, with purities of >95% for use as an internal standard when analyzing cow dairy products.

The fractionation and purification of the proteins was done by preparative high-pressure liquid chromatography (Prep HPLC), and freeze drying. The purity of the fractions was determined by time-of-flight liquid chromatography mass-spectrometry (TOF LC/MS).
Furthermore the purified protein and the goat milk were analyzed utilizing the Bradford Protein Assay.

Theory

Proteins

Amino acids are the building blocks of peptides and proteins. Di-, tri-, and tetra peptides are short chains of respectively two, three and four amino acids linked together, while proteins, also called polypeptides, are longer chains of amino acids. To form peptides and proteins, the amino acids are linked together by peptide bonds. This linking of amino acids is called the primary structure of the protein (Whitford, 2011). Depending on the combination of amino acids, a protein can have a secondary, tertiary and quaternary structure as well, which is illustrated in Figure 1.

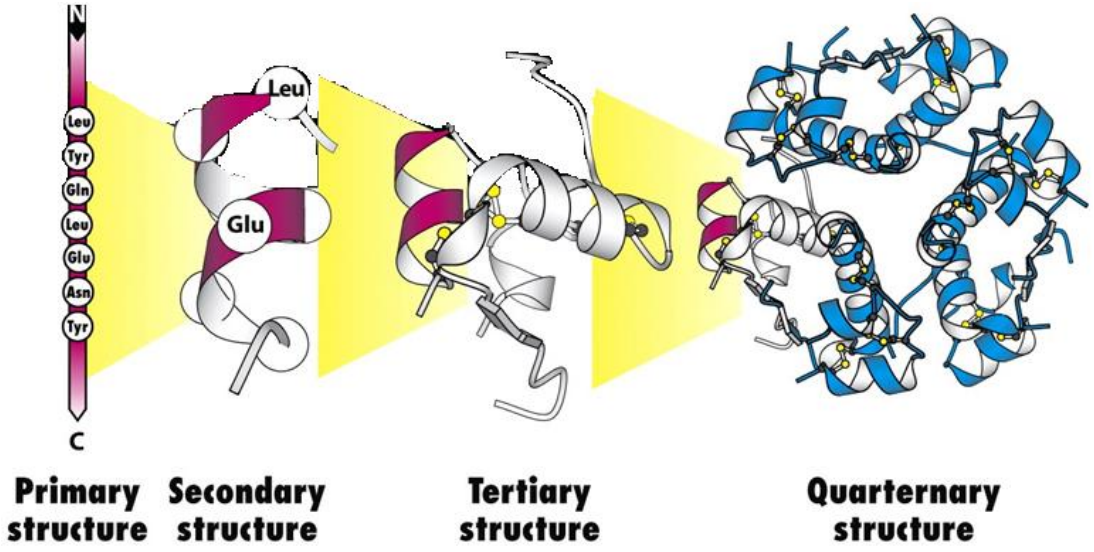

Figure 1. The four different types of protein structure, and their relation to each other. The combination of amino acids determines the structure of the protein (Jeremy M. Berg, 2007).

The peptide bonds which bind the amino acids together absorb light at 214 nm, which makes proteins and peptides detectable with a ultra-violet (UV) light detector. Furthermore the amino acids phenylalanine, tyrosine and tryptophan are able to absorb light at 260 nm and 280 nm as well, due to their aromatic ring, but not all proteins contain these amino acids, so to be able to detect all of the proteins in a mixture, it ought to be measured at 214 nm (University M. , 2014), (Whitford, 2011). In addition to the ability of absorbing UV light, proteins have other chemical properties as well. Depending on the amino acid composition, they can be polar or non-polar, or be charged or un-charged; the latter can be effected by the composition of the protein solvent (Whitford, 2011). These abilities can also be used in a range of analyses; charged proteins can bind dye, which can be used for spectrophotometric concentration analysis of protein mixtures and the polarity can be utilized in e.g. liquid chromatography (LC) for separation or purification of proteins. Proteins are present in an extremely wide range of food sources, but the proteins which may have been characterized the most are the milk proteins (McSweeney, 2003).

Milk Proteins

Milk proteins can be divided into two groups; caseins and whey proteins, which have different chemical and physical properties. Caseins are primarily characterized by their ability to be acid-precipitated at pH below 4.6; milk has a natural pH value around 6.6 (McSweeney, 2003). Caseins do not have significant secondary and tertiary structures, due to their amino acid compositions, which make them very heat-stable (Guelph, 2009). Furthermore caseins are often phosphorylated, but the degree of phosphorylation can vary from casein to casein and from animal to animal; these variations of caseins are called natural variants (McSweeney, 2003). Phosphorylation occurs on amino acid level, and the amino acid which is most often phosphorylated is serine (Kahn, 2003). 95% of the caseins in milk exist in molecular spherical clusters, along with various minerals, called micelles. These micelles are responsible for the white appearance of milk. In bovine milk 76 to 86% of the total protein (TP) content is caseins, divided in four main types; α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN) and κ-casein (κ-CN). The composition and amount of caseins varies with species, feed and time since parturition. These factors also lead to the production of natural variants of the caseins.

The liquid remaining after precipitation of the caseins is called whey, hereby naming the proteins left in this solution whey proteins. The whey proteins β-lactoglobulin (β-lg) and α-lactalbumin (α -lac), are globular molecules, that constitute ~20% of the total protein in bovine milk. α-lac represents 20% of these whey proteins, while β-lg represents ~50%. β-lg does not occur in human milk, and is considered to be the most allergenic milk protein for human infants. (McSweeney, 2003). Below some physical properties of some caseins and whey proteins are listed.

Table 1. Characteristic features of some milk proteins (McSweeney, 2003), (Amrita, 2014), (UniProt, 2014).

Protein	Isoelectric point (pI)	Charge at pH 6.6	Mass (Da)
Bovine α-lactalbumin	4.80	Negative	16247
Bovine β-lactoglobulin	5.41 (theoretical 4.83)	Negative	19883
Bovine β-casein	4.6	Negative	25107
Goat α-lactalbumin	4.92	Negative	14192

Proteins in cow and goat milk are not similar

As well as natural variants of proteins exists amongst one single species, differences also occurs between the mammalian subdivisions; for instance between cows and goats. The proteins differ in size, sequence of amino acids and some physical properties which are likely a product of the different sequencing. For instance; people that are allergic to bovine α-lactalbumin, seems to be tolerant to α-lactalbumin found in goat milk (Rodden, 2006).

The UniProt Consortium has mapped the amino acid sequences of goat milk proteins, and has reported the natural variants listed in Table 2. The variants are, as mentioned before, an expression for the different phosphorylations of the caseins. The reason for the undetermined amino acid in κ-CN and α_{s1}-CN, is most likely due to the fact that different methods have been used in order to determine the sequences.

Table 3. Natural variants of caprine caseins (UniProt, 2014).

Protein type	Variants	Undetermined amino acid
α_{s1}-CN	A, B, C, D, E, F	Amino acid #24 is arginine or glutamine (174 or 146 Da)
α_{s2}-CN	A, B, C, CSN3-B, E, F	-
κ-CN	A, B, C, CSN3-B, E, F	Amino acid #134 is aspartate or aspargine (133 or 132 Da)

In order to determine the total protein content in the milk, the Kjeldahl method is often utilized. This is an analysis which determines the nitrogen content in organic material (Gyldendal, 2009), and then multiplies this number with a factor in order to obtain the protein content (Eurofins, 2014):

$$Protein\ content_K = N \cdot 6.38$$

But not all nitrogen in the milk is found in the proteins; this is called non-protein nitrogen (NPN). These NPNs must therefore be subtracted from the nitrogen content to obtain the value for the actual protein concentration. The NPNs are considered as the fraction of milk which contains nitrogen compounds that are soluble in 12% trichloroacetic acid (McSweeney, 2003). The main NPNs are listed below in Table 4.

Table 4. Main non-protein nitrogen compounds in cow milk, and their relative content pr. liter (McSweeney, 2003).

Component	N (mg/L)
Ammonia	6.7
Urea	83.8
Creatinine	4.9
Creatine	39.3
Uric acid	22.8
α-amino nitrogen	37.4

The content of NPNs in milk varies as well as the contents of proteins. The NPN content in goat milk is estimated to around 5-8% of the TP content (EFSA, 2012), (Colin G. Prosser, 2008). In cow milk this is slightly lower; around 5-7.5% of the TP content (Colin G. Prosser, 2008), (Ferguson, 1992). Therefore the result from the Kjeldahl method must be subtracted with 8% in order to obtain the total protein (TP) content in goat milk:

$$Total\ protein = (N \cdot 6.38) - 8\%$$

As mentioned earlier, proteins possess a lot of different properties, which can be utilized in analysis. For instance LC is a technique which can make use of the polarity of the proteins to e.g. divide them into different fractions. To understand the technique of LC, a review of LC, high-pressure LC (HPLC) and reversed phase chromatography (RPC) will follow.

Liquid Chromatography

To analyze and purify one or more components in a complex mixture, such as goat milk, liquid chromatography (LC) is an excellent analysis method. Liquid chromatography is a separation technique that consists of mainly three components: A mobile phase, a column containing a stationary phase and a detector (Ardrey, 2003). A simplified diagram of the high-pressure liquid chromatography (HPLC) principle can be seen in Figure 2 below.

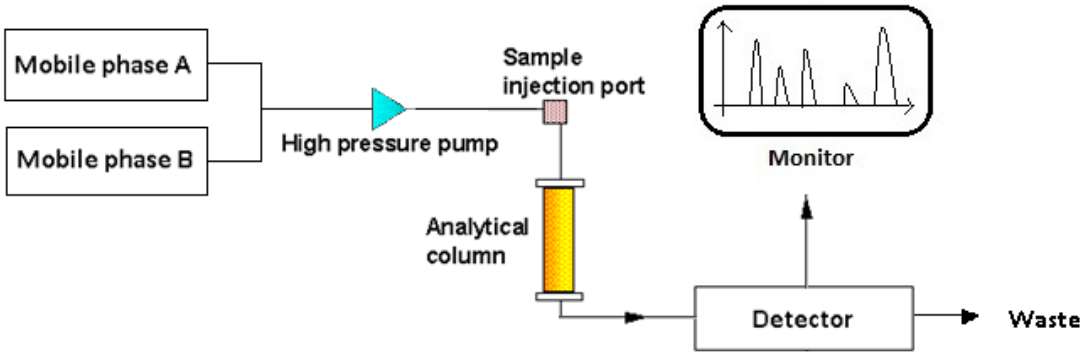

Figure 2. The HPLC principle. Two (or more) mobile phases are mixed in order to the required gradient, before being pumped onto the column. When the sample is injected, the sample compounds will elute from the column at different times and become detected, which is illustrated as a chromatogram on a monitor screen (University V. P., 2012).

In HPLC the pumps sustains a continuous flow of mobile phase in the system; from the containers the mobile phase flow past the sample injector, through the column, past the detector and into waste. The mobile phase often consists of a mixture of different solutions, e.g. solution A, which could be polar, and solution B, which could be non-polar. So when the sample is injected into the flow stream, the flow stream of mobile phase is a mixture of these two solutions in a proportion which make the compounds in the sample retain on the column in a desired degree. After the sample is loaded onto the column, the mixture proportions of the mobile phases, also called the gradient, can be changed in a way that makes the flow stream attract the compounds stronger than the stationary phase, hereby making them elute from the column (Ardrey, 2003). An example of elution of the compounds according to gradient is showed in Figure 3.

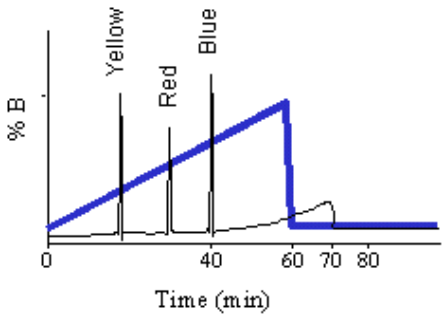

Figure 4. The blue line represents the gradient, which is percentage mobile phase B over time. A chromatogram is placed underneath, which shows the elution order of the compounds according to time and gradient (Guzzetta, 2001).

Note that the gradient is adjusted back to the starting amount of mobile phase A after the separation. If this was not done, the next sample would not be retained on the column, but elute immediately without getting separated.

Since peptide bonds absorbs light at 214 nm, the detector in the HPLC system should be a UV lamp, which is able to emit light at this particular wavelength; when the proteins elute from the column, they flow past the detector, and absorb some of the emitted light, hereby changing the intensity of the light. The system software is then able to convert these variations into a chromatogram; the more protein that elutes from the column pr. time unit, the more light will be absorbed, and hence the larger the peak will appear on the chromatogram (Ardrey, 2003).

In HPLC there are many types of stationary phases; ion exchangers, normal phase, reversed phase etc. (Waters, 2014). When analyzing milk samples reversed phase chromatography (RPC) is often used (McSweeney, 2003). RPC use non-polar stationary phases such as C4 or C18 packed columns (Waters, 2014). C18 is best suited for separation of small compounds such as peptides and nucleotides, while C4 is better suited for larger compounds such as proteins (Chem., 2011). Since the RPC column material is non-polar, the gradient must be increasingly organic when eluting the compounds from the column. Often acetonitrile or methanol is used as organic mobile phase (Waters, 2014). In addition the mobile phases must contain a small amount (0.1%) of acid like formic acid or acetic acid. This contributes to the accuracy of the chromatograms, since peak tailing is reduced (Dolan J. , 2014). Other ways to adjust the look of chromatogram, besides changing the gradients, are regulate the temperature of the column or adjust the flow rate in the system.

RPC is the LC technique which is most often used, since it is capable of processing a wide range of molecules, and the technique allows the user to easily control solvent-type, pH and temperature. Furthermore the reversed phase columns are efficient, stable and robust (GmbH, 2013).

The knowledge of the properties for mobile and stationary phases can help develop specific methods with focus on specific compounds in a given sample. Here it is important to have in mind that the strength of the stationary phase must not be too high, since this might make the compounds retain so strongly to the column that they cannot be eluted (Waters, 2014). Another type of HPLC is preparative HPLC.

Preparative HPLC

The major difference between analytical LC and preparative LC is the amount of sample that can be applied to the column (Huber, 2013). Standard LC systems normally inject 10 µl sample pr. injection, while a preparative column can be loaded with up to 900 µl sample pr. injection. Furthermore, due to the increased column volume, the flow of mobile phase is also higher than ordinary LC. This is illustrated in Figure 5 below.

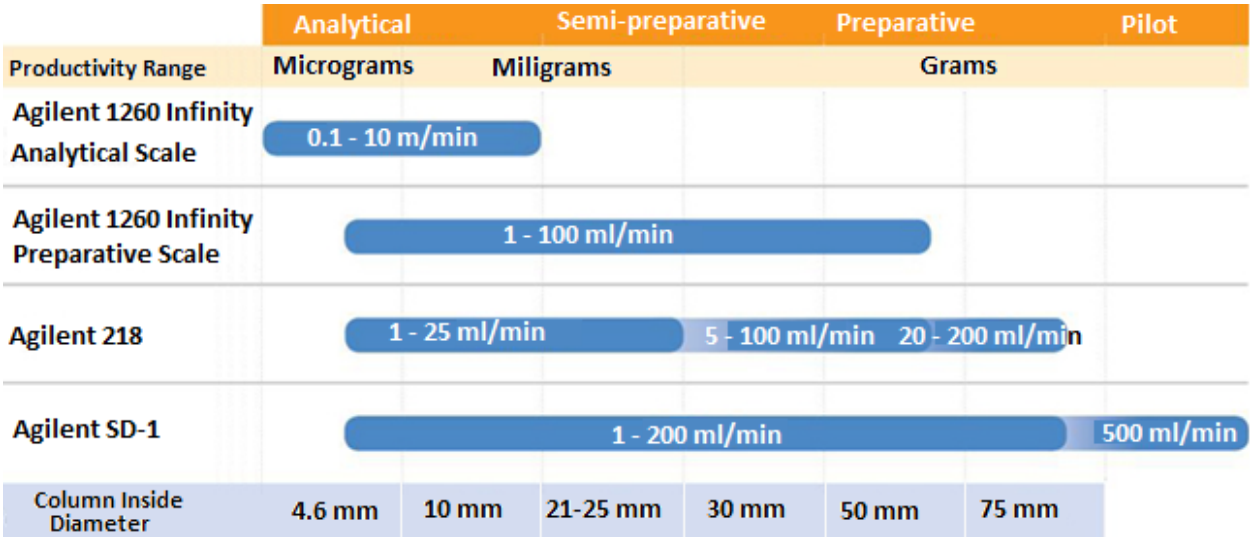

Figure 5. Different LC columns and their appurtenant flow capacities and inner column diameters (Agilent, 2014).

The fraction collection itself can be controlled by an automated fraction collector, which can be programmed to collect fractions at different retention times. To determine at which retention time the fractions should be collected, the chromatogram, made from the data collected by the UV detector, is used. An example of a chromatogram made on a C18 column can be seen below in Figure 6.

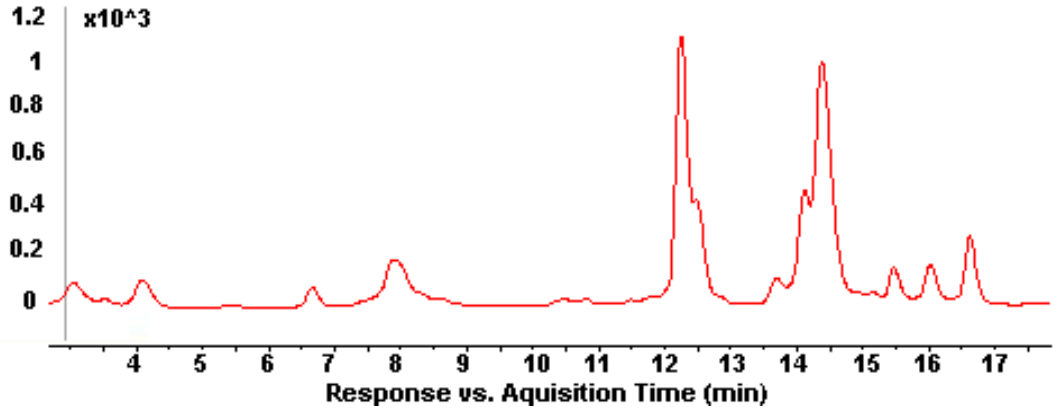

Figure 6. Proteins in skimmed cow milk, separated on a C18 column, and detected at 214 nm.

In order to be able to compare chromatographic results over time, the internal standard method has proved very useful.

Internal standard

An internal standard (IS) is a known amount of a component which is added to the sample matrix in order to estimate amounts of compounds in a matrix, or to be able to compare LC results over time. When an IS is used, it should be added to the sample as early as possible, so that any volumetric loss can be compensated for.

To identify which kind of IS should be used for a particular sample, the list below should be examined, since it outlines the criteria of a well suited IS:

- Should never be found in the original sample matrix
- Is able to be well-resolved in the given buffer
- Preferably eluting after the analyte of interest
- Stable
- Available in pure form
- Compatible with detector response
- Structure which is similar to analyte

So if all of these criteria are fulfilled, it is possible to use the IS as a benchmark between results from a brand new UV detector (UV_0) and a detector which has been used for 800 hours of analysis (UV_{800}), since the response of the detector declines over time. The IS peak at UV_0 will have a certain area, which can be connected to the given concentration of IS. When adding the same amount of IS to another sample measured at UV_{800} , the peak for this new IS can be compared to the area of the UV_0 IS peak hereby estimate which factor the peaks from the UV_{800} should be calculated with in order to find the true concentrations.

But if not being careful, adding an IS can be misleading. If the IS is badly chosen, and in some way disintegrated or precipitates during sample preparation, the remaining amount of IS in the sample will not be as expected. When comparing the areas of the peaks from time to time, the IS might be misleading since its lower response comes from degradation and not because the amounts in the samples are different. Furthermore the amount of added IS must be adjusted according to the concentration of compounds in the sample, since a too high amount of IS can be misleading by diminishing the responses from the compounds. Another disadvantage could be that the prospective loss of compound and IS is not similar. So if the IS is lost a little in some way, and the compound of interest is not, the comparison of peak areas might as well not show the expected ratio (Dolan J. W., 2012).

Beside LC combined with UV detection, there are other ways of analyzing proteins, which are both more precise, but also better for identification of unknown compounds in a sample mixture. This could be time-of-flight mass spectrometry.

Time-of-flight Mass-Spectrometry

In the late 1940's the time-of-flight mass spectrometry (TOF MS) technique was invented, but it was only until the 1990's that its utilization increased rapidly. Since then the TOF MS has also been successfully combined with LC (TOF LC/MS), hereby creating a very powerful and unique tool for accurate analysis and identification of compounds, which has become a core analysis technology today (Inc., 2014). The following section is related to the sources (Ashcroft, 2014), (Bhattacharjee, 2013), (MarywoodScience, 2011) and (Gates, 2004), unless otherwise stated.

The TOF LC/MS has three main components; a HPLC column, an ionization chamber and a TOF MS, see Figure 7. The HPLC system is similar to the one already described in the Liquid Chromatography section. The difference between HPLC and TOF LC/MS is what happens to the compounds after the UV detection.

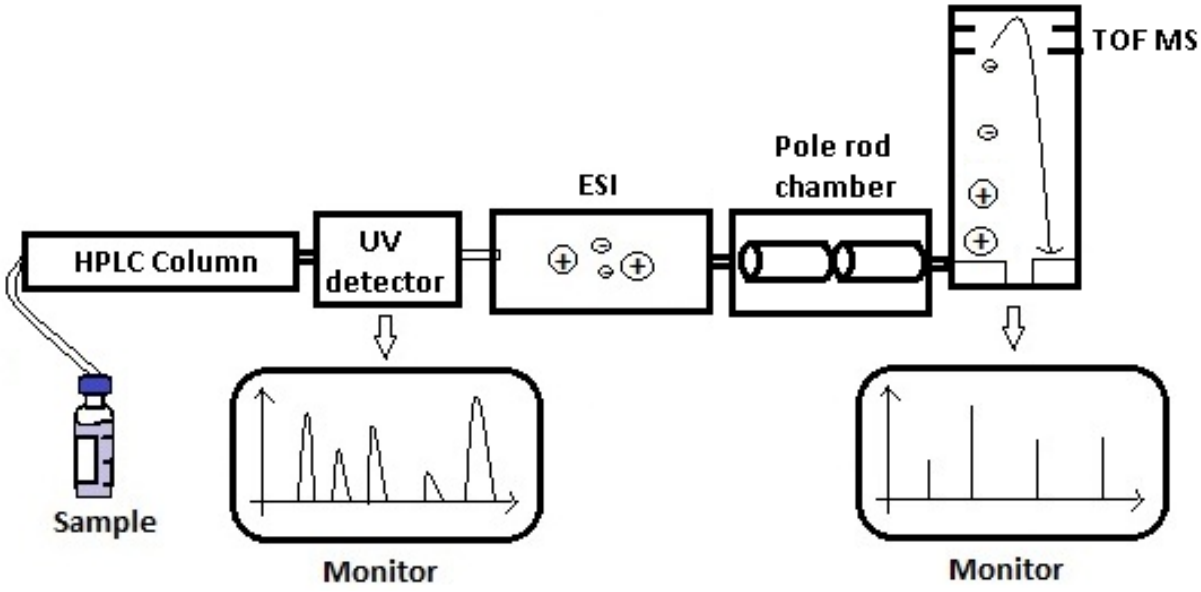

Figure 7. The entire TOF LC/MS procedure; HPLC, UV detection, electrospray ionisation, ion sorting pole rods and TOF MS.

When the compounds have passed the UV detector, they become ionized in order to be able to calculate their time-of-flight and mass-to-charge ratio. There are different types of ionization, and one of them is electrospray ionization (ESI). ESI is an ionization method which is well suited for larger molecules such as peptides and proteins.

After the detection the compounds, which are still dissolved in the solvent, arrives inside a charged needle tip (4000 V). From here they are sucked into a vacuum chamber and becomes sprayed into an aerosol of charged micro droplets. The vacuum chamber is 300-400 °C and contains N_2 which acts as a carrier gas when the solvent around the compounds evaporates. If the compounds are not within this solvent layer, they could be compromised or destroyed by the high voltage in the needle tip. Eventually all of the solvent is evaporated and the free ions are created. Figure 8 illustrates the ESI principle.

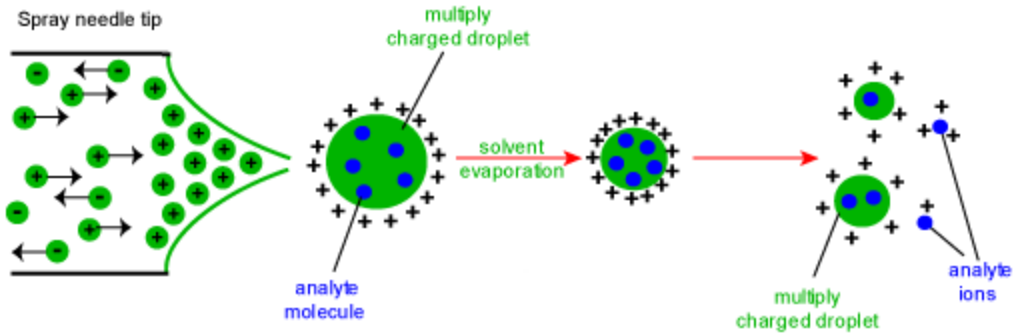

Figure 8. Electrospray ionization. The compounds are within the droplets of solvents which becomes charged, and afterwards the solvent evaporates leaving the compounds ionized and ready for the next step (Gates, 2004).

When the compounds are ionized and free from solvent, they now enter a chamber which contains two sets of pole rods; first the octupole, then the quadrupole. These are sets of charged rods, which change between negative and positive charges several thousand times per second. Ionized compounds which has charges of unwanted character, or moves too slow to travel past the rods, are separated from the other ions. The ions that travels past the octupole, emerges at the quadrupole, which is sorting the ions in the same way as the octupole, but here there is only four charged rods instead of eight. Figure 9 illustrates the rods and their functions.

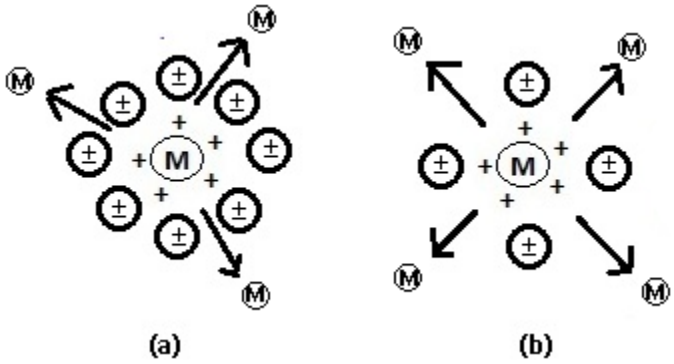

Figure 9. The poles seen from the front. The circles which have the ± symbol inside, are the charged rods, the circles with a M is the compounds. The octupole (a) makes the first sorting of the ions, then the quadrupole (b).

After being sorted by the quadrupole, the remaining ions are sent to the ion pulser. The ion pulser is a stack of plates with a hole in the middle. The ions enter this stack of plates from the side, just between the back plate and the first plate in the stack. Then a pulse of high voltage is applied to the back plate, hereby accelerating the ions through the holes in the entire stack of plates. The ions travel up into the flight tube, which has an ion mirror placed at the top. This mirror is able to reverse the direction of the ions and lead them back to the ion detector which is placed at the bottom, just beside the ion pulser, see Figure 10.

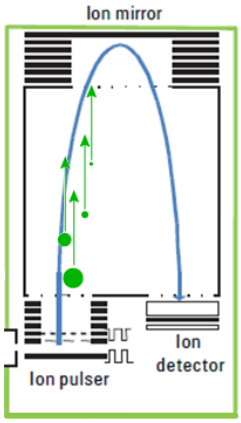

Figure 10. The TOF MS chamber, showing the path of the compounds and their separation by mass (Inc., 2014).

The compounds with the lowest mass will fly faster towards the ion detector than the high mass compounds. If two compounds have the same mass, then the compound with the highest charge will fly the fastest. After the time-of-flight detection, the mass of the compounds can now be calculated and connected to the retention times in the HPLC chromatogram. An example of the results from the TOF LC/MS is shown in Table 5 below.

Table 5. An example of results from a TOF LC/MS analysis at Arla Strategic Innovation Centre, connecting retention time on the LC chromatogram to a mass, from own analysis.

Retention time (min)	Mass (Da)
12.56	14193.99
13.92	23821.04
14.80	18191.27

When the masses haves been determined, the compound can be identified, using e.g. UniProt, which is a database of proteins. If the mass of 14193.99 Da shows to be consistent with e.g. the mass of α-lac, the peak at 12.56 minutes must represent this particular protein. The rest of the proteins are identified similarly.

Prior to the LC analysis, milk must be reduced so that the micelle structure of the caseins is disintegrated, and the structure of the individual protein becomes uniformed, hereby ensuring that a specific protein will occur at the same retention time each time the analysis is done. In order to do this the milk can be reduced in urea, sodium citrate and dithioerythritol (DTE) at e.g. 30 °C for one hour. The DTE will then break the disulfide bonds within and between the proteins, and the urea will reduce the hydrophobic regions inside the protein structure, hereby stretching out the protein and hence eliminate potential secondary, tertiary and quaternary structures (Lee, 2013).

All of the above mentioned chromatographic techniques are all well suited for analysis of proteins. Another method which is widely used is the Bradford protein assay. This is a spectrophotometric method, which makes use of a dye and standard curves to determine the protein concentration.

Bradford Protein Assay

The Bradford protein assay is a method which has been developed in order to determine the concentration of dissolved proteins in a solution by dying the proteins and then measure the relative absorbance. The dye used in the assay is Coomassie® Brilliant Blue G-250, which has an optimum absorbance maximum at 595-600 nm when bound to proteins. The dye preliminary binds to basic and aromatic amino acid residues, but interferences may occur caused by chemical-protein or chemical-dye interaction (Bio-Rad, 2014). Below in Figure 11 the chemical structure of the dye is illustrated.

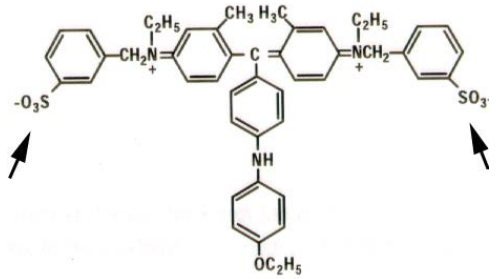

Figure 11. Coomassie® Brilliant Blue G-250 dye; the arrows indicate the active sites (McGill, 2014).

In this particular experiment an adaption of the original Bradford method is used, called the Bio-Rad Protein Assay. This assay uses 10 µl sample pr. well in a 96-well microplate, and has an incubation time of 5 to 60 minutes at room temperature. Triple determination is advised.

A standard curve should be made in order to connect absorbance and concentration. The best protein to use as a standard is a pure sample of the protein of interest. If this cannot be provided, another relative standard must be chosen, and if several assays are to be compared the same standard should be used in all of them. A common standard in these types of analysis is bovine serum albumin (BSA). In Figure 12 a standard curve from BSA is shown in a linear range of 2 mg/ml, which is the recommended concentration. When concentration and absorbance has been plotted, the linear equation for this relation can be used to calculate the protein concentration in the other samples, by using the absorbance of the given sample (Laboratories, 2014).

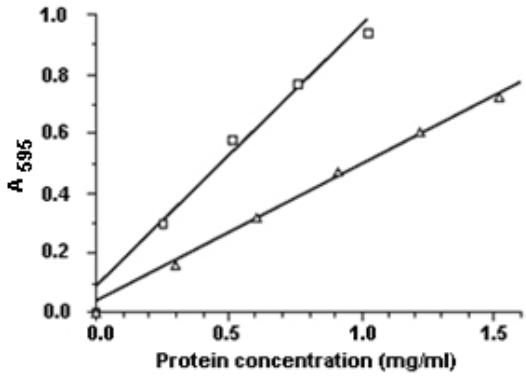

Figure 13. Typical standard curves for the Bio-Rad protein assay. BSA (□) and γ-globulin (Δ) (Bio-Rad, 2014).

Methodology

Below in Figure 14 is a flow diagram, showing all the steps of the fractionation and purification of the goat milk proteins.

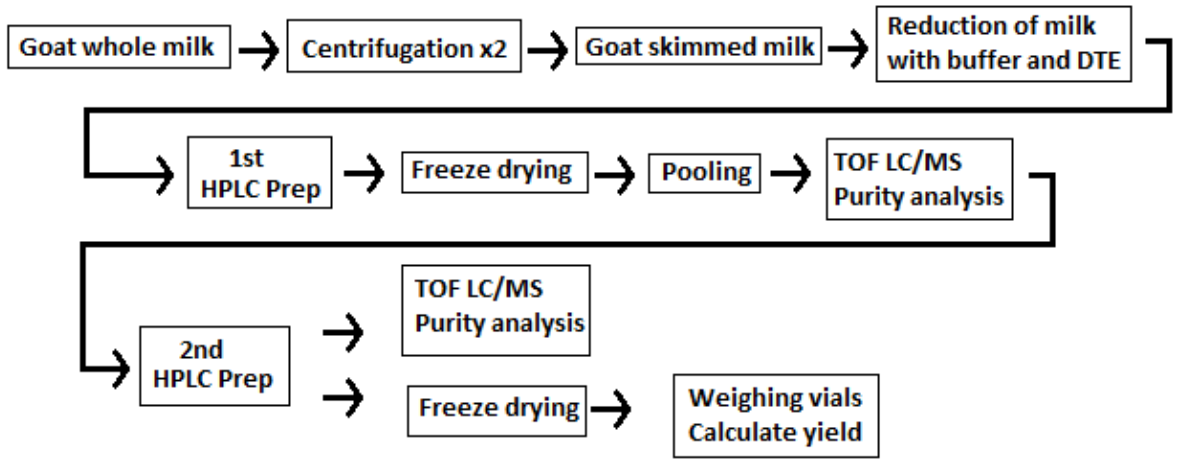

Figure 14. The fractionation and purification steps of the goat milk proteins.

Materials

Preparative High Pressure Liquid Chromatography System

- Agilent 1260 Infinity Binary LC
- Waters DeltaPak C4 Prep Column, 300Å, 15 µm, 7.8 mm X 300 mm
- Agilent 6 ml vials, Part. no. 8010-0022

Liquid Chromatography Mass Spectrometry System

- Agilent 1290 Infinity Binary LC System
- Waters BioSuite™ C18 reversed phase column
- Agilent 6230 Accurate-Mass Time-of-Flight (TOF) LC/MS System

Bradford protein assay

- EL808 microplate reader, Holm and Halby, Biotek

Solvents

Trisodium citrate (Merck, Lot: 1.06448.1000), acetonitrile (ACN, Rathburn, RH101), urea (Merck, 1.08487.1000), 1,4-Dithioerythritol, (DTE, Sigma Aldrich, D8161), trifluoroacetic acid (TFA, Merck, 1.08262.0100), Coomassie® Brilliant Blue G-250, BioRad, sodium phosphate buffer (Merck), bovine serum albumin standard (Sigma Aldrich).

Goat milk

Whole milk from dairy goats at Knuthenlund (Saanen, Toggenburger and Danish Landrace)

Experiments

Preparation of buffer and mobile phases

Reduction buffer: 6M urea in 100mM Na_3-citrate

72 g urea and 5.88 g Na_3-citrat was weighed in a measuring cylinder. 100 ml of Mili-Q water was added and the solution was mixed with a magnet stirrer until homogeneous and at room temperature. Note that this can take some time, since the mixture becomes cold when water is added. Then the cylinder was filled with water until reaching 200 ml. The buffer is stable for one week when stored in the fridge.

Mobilfase A and B: 0.1% TFA in water and 0.1% TFA in acetonitrile

4995 ml Mili-Q water or acetonitrile was measured and transferred to a bluecap bottle. Then 5 ml TFA was added, the cap is put on and the solution was mixed for five to ten minutes by a magnet stirrer.

Preparation of goat milk samples

Skimming the goat milk

The milk was centrifuged at 5 °C and 7500 rpm for 15 minutes to remove the fat. The supernatant was then transferred to a new container and centrifuged once more to be sure that as much fat was removed as possible, hereby having only the skimmed goat milk (skgm) left.

The skimmed milk was then transferred to 15 ml BD Falcon™ centrifuge tubes, 2 ml milk in each. The tubes were then stored in the freezer until further use.

Reducing of the milk prior to analysis and fractionation

Each tube containing 2 ml skgm was reduced with 10 ml reducing buffer and 200 µl 0.5 M DTE, then incubated at 30 °C for one hour. The samples were then transferred to eppendorf tubes and centrifuged at 4 °C and 13,200 rpm for 10 minutes. After centrifugation the supernatant was transferred to 1.5 ml vials, and is now ready for analysis and fractionation. The reduced milk samples can be kept for 48 hours when stored at maximum 5 °C. If it is necessary to make an analysis later than this, the samples have to be incubated once more, although this is not optimal conditions.

As a standard procedure a sample of whole milk and skimmed milk was sent to Eurofins for a total protein analysis. Eurofins utilizes the Kjelddahl method for protein analysis. The results are listed below in Table 6.

Table 7. Content of proteins in the milk samples.

Goat whole milk	Goat skimmed milk
4.41 g protein / 100 g milk (100 g milk = 97.54 ml) **45.21 g protein / L milk (- 8% NPN ≈ 41.59 g/L)**	1.93 g protein / 100 g milk (100 g milk = 99,42 ml) **19.4 g protein / L milk (- 8% NPN ≈ 17.85 g/L)**

Goat milk protein profiling

A sample of reduced skgm was analyzed with TOF LC/MS in order to make a profile of the goat milk proteins, and to precise at which retention times the different proteins would elute, hereby being able to determine at which retention times the fractions should be collected by the preparative HPLC.

The chromatogram showing the goat milk protein profile can be seen in Figure 15 in the Results section together with the identification of the proteins.

Preparative High-Pressure Liquid Chromatography

After the proteins had been identified and the retention times were been determined, the preparative HPLC method could be set up. It was decided to collect three different fractions. Below in Table 8 the method parameters are listed.

At the beginning of each day, prior to the fraction collection, the retention time of the proteins was checked, since this can vary from day to day (± 0.1 min), especially if the mobile phases have been renewed.

Table 8. Method parameters for the fractionation of skimmed goat milk.

Mobile phase A	0.1% TFA in H_2O
Mobile phase B	0.1% TFA in ACN
Injection volume	750 µl
Injection pr. vial	2
UV detection	214 nm
Flow rate	5 ml/min
Gradient	
Fraction volume	5 ml pr. vial (1 min pr. vial)
Autosampler temperature	5 °C
Column pressure at t_0	190-220 bar

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	68	32
2	68	32
15	55	45
16	0	100
17	0	100
18	68	32

After each fraction collection session (25-30 injections) the column was cleaned by injecting 2x 900 µl reduction buffer.

When the fractions had been collected, they were placed in a fume hood to evaporate for at least 12 hours. After evaporation they were frozen solid, and were now ready to be freeze dried. The freeze dryer utilizes vacuum for drying of the samples. After approximately 24 hours the fractions were dry.

After the freeze drying the related fractions were pooled, in order to gather one type of fraction in one container. This was done by adding 5 ml reducing buffer and 10 µl 0.5 M DTE to the first vial which was then Vortexed. Then this solution was transferred with a pipette to vial number 2, and Vortexed. This continues from vial to vial until the solvent volume is down to half of the original volume. Remember to use the same pipette for all of the transfers during the pooling, since residues of protein will remain on the pipette. Then 2.5 ml of reduction buffer and 5 µl 0.5 M DTE was added into the vial containing the already pooled fractions, and the rest of the vials were pooled as described above. The solution with the pooled fractions was stored in the fridge in a 15 ml BD Falcon™ centrifuge tube. The procedure of pooling the content of the vials was repeated two times more for the same vials, three times overall, hereby obtaining around 10 ml of solution with the protein fraction. The pooling method was repeated for the two other fractions as well.

After the pooling, 20 µl of each pooled fraction was analyzed by TOF LC/MS to clarify the purity. The chromatograms for this can be seen in the Results section.

2nd Preparative HPLC Process

When the purity had been determined, the rest of the pooled fractions were fractionized once more, to obtain even purer protein fractions. It was decided that the gradient should be changed in order to widen the peak to make the fractionation better; it was changed from 32% mobile phase B to 37% mobile phase B in the beginning. The rest of the gradient method was unchanged. In this fractionation the collection of fractions was done manually, in order to ensure that the vials were filled properly, and to avoid mix-ups if the retention times changed between injections. As can be seen in the Result section, the number of vials varied from four to six per injection. 50 µl of each new fraction was analyzed by TOF LC/MS to determine the purity. The rest of the content in the vials were evaporated and freeze dried, the same way as for the first preparative. These freeze dried fractions are the end product. The concerning chromatograms can be seen in the Result section, and additional chromatograms can be found on the USB flash drive.

Bradford Protein Assay

A pure caprine β-lactoglobulin fraction was chosen to be analyzed with the Bradford method as well as two goat milk samples, whole milk and skimmed milk respectively. For the milk samples bovine serum albumin (BSA) was used as a standard reference, since this protein standard is recommended for the Bio-Rad Bradford analysis. For the purified protein fraction, a bovine β-lactoglobulin standard was used.

Table 9. Standard dilutions of BSA and bovine β-lactoglobulin.

BSA standard (µg/ml)	Bovine β-lactoglobulin standard (µg/ml)
0	0
5	5
25	25
100	100
500	500
1000	1000
2000	2000

According to the results from Eurofins, the grade of dilution of the samples were chosen and calculated. This was done in order to have the protein concentrations in the samples to lye around the middle of the standard curves. The dilutions were done with a 0.5 M sodium phosphate buffer at pH 7, and below in Table 10 the dilutions of the samples are listed.

Table 10. Dilutions of milk samples and the caprine β-lactoglobulin fraction.

Goat whole milk			Goat skimmed milk (µg/ml)			Purified goat β-lactoglobulin	
Concentration (µg/ml)	Dilution grade		Concentration (µg/ml)	Dilution grade		Concentration (µg/ml)	Dilution grade
45	1000x		19	1000x		25	x60
301	150x		65	300x		100	x15
452	100x		194	100x		500	x3
1130	40x		970	20x		1000	x1.5
-	-		-	-		1500	Undiluted

For the Bradford analysis a 96 well microplate was used. In each well 10 µl of sample or standard was placed. Additionally 250 µl of Bradford Coomassie Blue was added to each well. The samples were then allowed to react for approximately 10 minutes, after which the plate is read on the ELISA plate reader at 600 nm and 25 °C. The standard curves and the result of the Bradford analysis can be seen in the Results section. Each sample or standard was triple determined.

Results

The UniProt values for goat milk protein masses are listed below in Table 11 together with the results from the TOF LC/MS analysis of the milk.

Table 12. Lookup masses and measured masses of goat milk proteins. To the left: Lookup values of the masses of the goat milk proteins from (UniProt, 2014)**. To the right: TOF LC/MS measured masses for three of the goat milk proteins.**

Goat milk proteins	UniProt masses (Da)
α-lactalbumin	14194.12
β-lactoglobulin	18191.27
κ-casein	19386.35
α_{s1}-casein	23615.33
β-casein	23820.96
α_{s2}-casein	25438.73

Measured masses (Da)	RT (min)	Protein
14193.9936	12.569	α-lac
14193.99	12.581	α-lac
14193.9526	12.61	α-lac
14193.9305	12.619	α-lac
23821.0241	13.888	β-cn
23820.9232	13.894	β-cn
23821.0363	13.92	β-cn
23820.8686	13.946	β-cn
18191.2979	14.717	β-lg
18191.2671	14.759	β-lg
18191.2334	14.804	β-lg

According to (Martin, 1992), the rest of the proteins in the goat milk were identified. The goat milk protein profile is showed below in Figure 15 together with the profile of cow milk proteins for comparison. From the chromatogram of the goat milk proteins, it was decided to collect and purify α_{s1}-casein, α-lactalbumin and β-lactoglobulin.

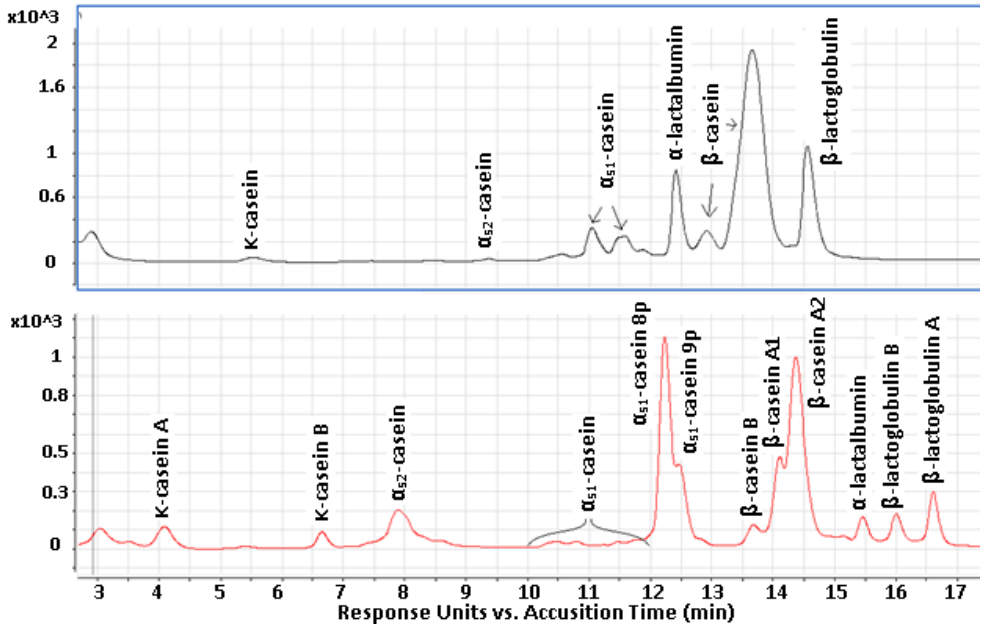

Figure 15. Protein profile of skimmed goat milk and skimmed cow milk made on a C18 column.

Fractionation of Proteins by Preparative HPLC

Below in Figure 16 a typical preparative HPLC chromatogram of the skimmed goat milk is showed. Each fraction was collected in its own vial. In total 38.12 ml milk was fractionated.

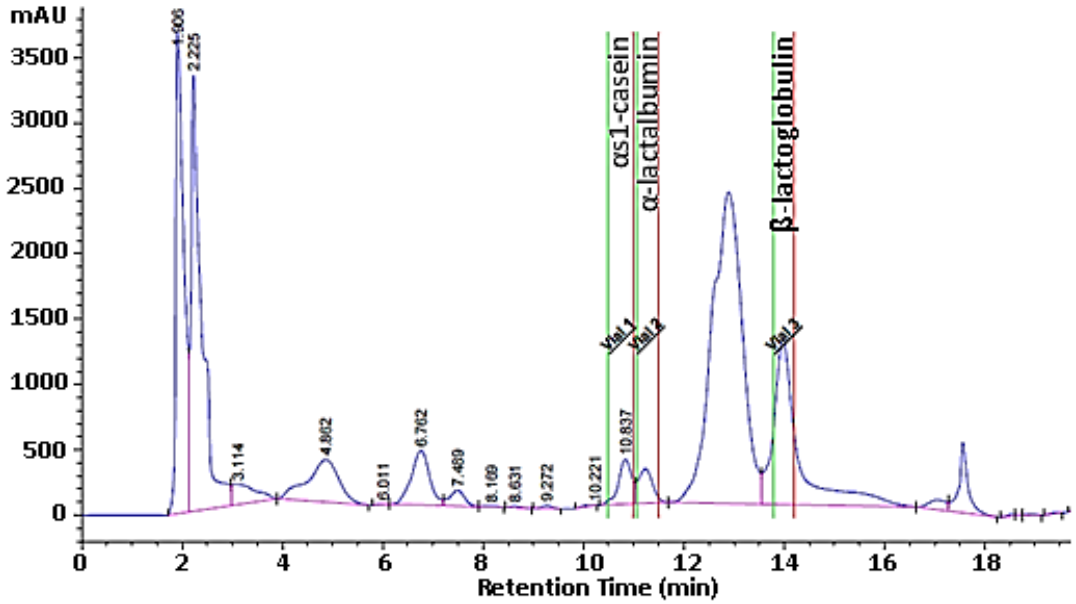

Figure 16. Goat milk proteins separated on the C4 preparative column.

After evaporation, freeze drying and pooling, a small sample of each pooled fraction was analyzed in the TOF LC/MS to determine the purity. The chromatogram can be seen below in Figure 17. The red graph represents the fraction with α_{s1}-casein, the black represents the α-lactalbumin fraction and the green represents the β-lactoglobulin fraction.

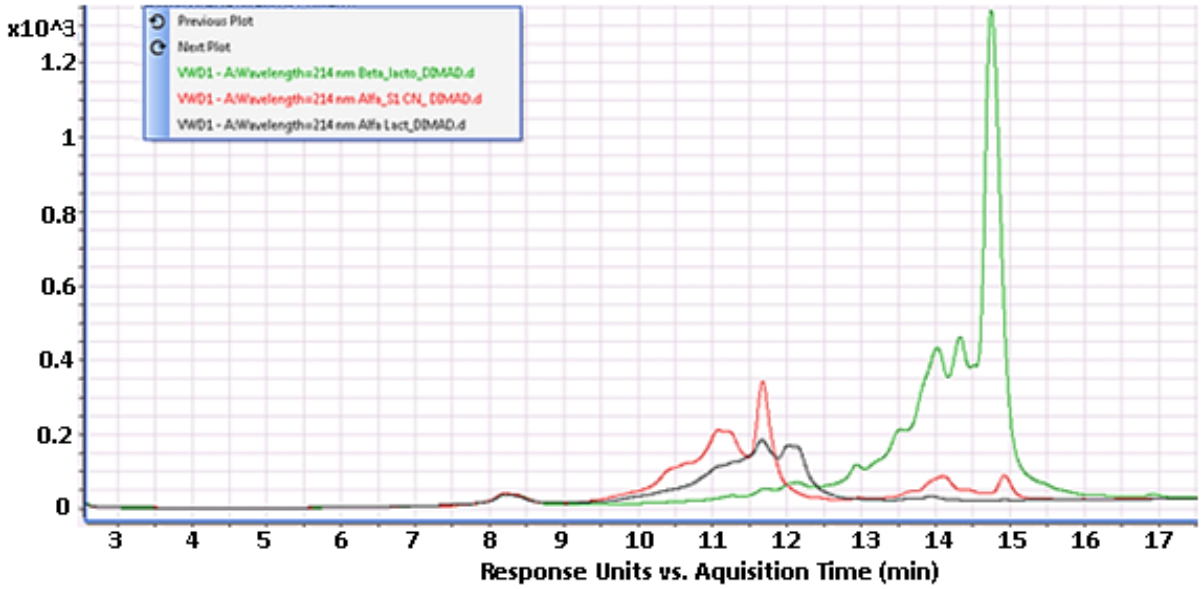

Figure 17. TOF LC/MS purity analysis results from the first fractionation with Prep HPLC.

It is clear that in both the first and second fraction α_{s1}-casein and α-lactalbumin were not separated at all. The β-lactoglobulin fraction was somewhat purer, although not as pure as intended. The required purity of the fraction in order to use it as an internal standard was decided to be >95%. In order to this it was decided to do a second preparative HPLC purification on this fraction, and store the others in the freezer for potential further purification.

2nd Preparative HPLC Purification

Ten injections in total were made from the pooled fractions, but in order to obtain purer fractions this time, the gradient on the Prep HPLC was increased with 5% of mobile phase B. This made the peaks much wider, and it was decided to divide these injections into fractions as well, in order to enhance the purity even more. A chromatogram from the second fractionation is showed below in Figure 18.

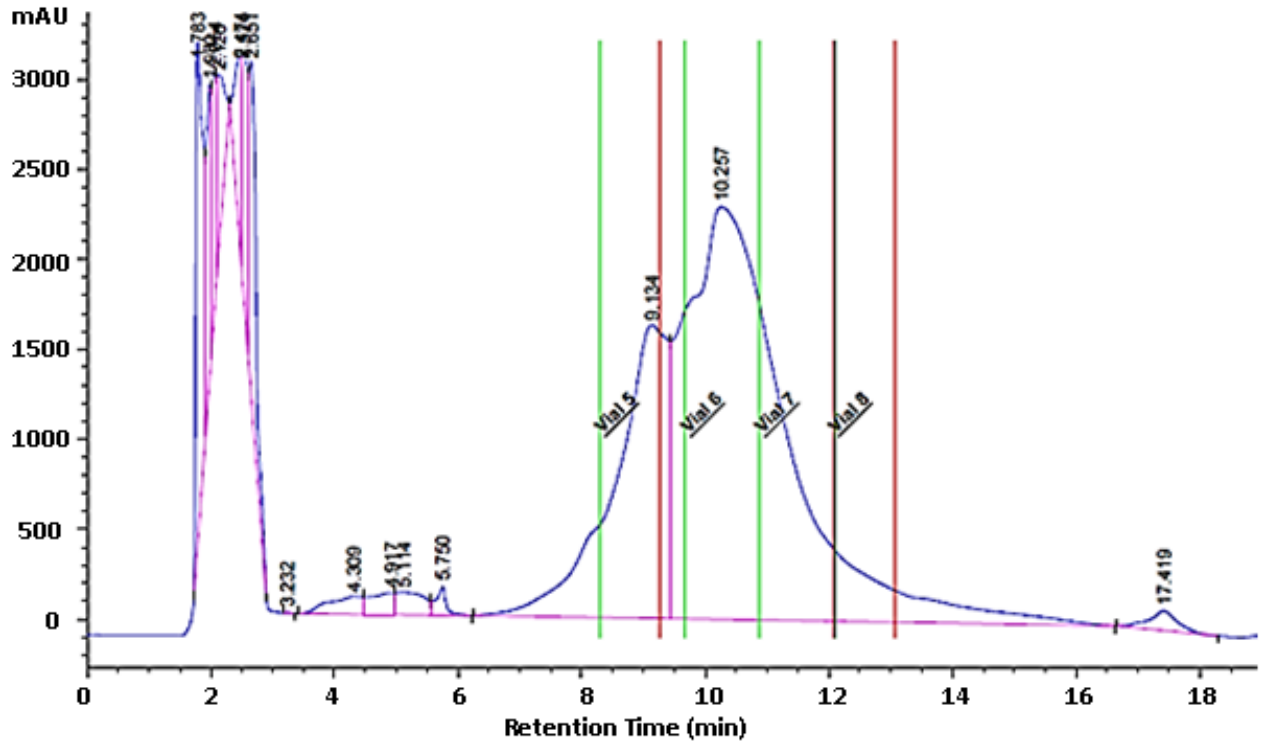

Figure 18. Pooled β-lactoglobulin fraction separated on the preparative HPLC column with a changed gradient.

The number of fractions pr. injection would vary, since the fraction collection had to be done manually, in order to prevent ending up with too many vials, and vials containing too little amount of fraction. The fractions were collected in 4-6 vials pr. injection, which provided 51 new fractions. 20 µl of each new fraction was analyzed in the TOF LC/MS in order to determine the purity. None of the fractions were pooled this time, since the contents were varying. The determination of purities was done according to peak area comparison in the individual chromatograms.

Table 13 below visualizes the typical content of β-lg in each fraction from one injection, calculated from peak areas.

Table 14. Data of four of the collected fractions from the second preparative HPLC.

Fraction no.	Total yield (mg)	% β-lactoglobulin	Yield of β-lg (mg)
5	3	0	0
6	2.9	55.5	1.61
7	0.5	82.5	0.41
8	2.9	100	2.9

Figure 19 shows purity chromatograms from two of the fractions; the blue is vial 6, the purple is vial 8. Note that the concentrations of the two fractions are very different.

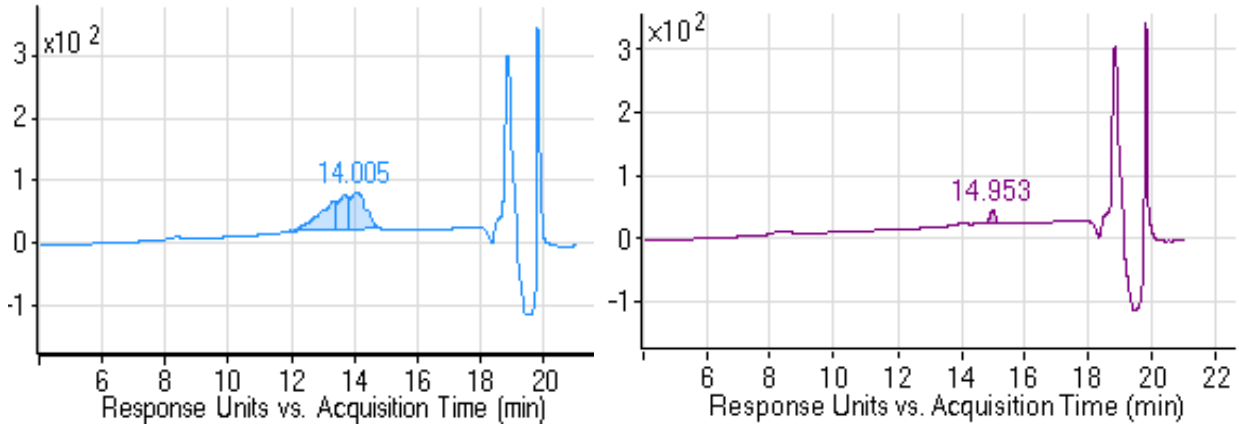

Figure 19. TOF LC/MS chromatograms showing the purity of the fractions from vial 6 to the left, and vial 8 to the right.

From the 51 new fractions, 12 were ~99% pure.

Mass Balance and Yield

In order to calculate the percentage wise yield and set up a mass balance, the peak area of the entire chromatogram in Figure 20 was calculated to a value of 101268.5 as reference.

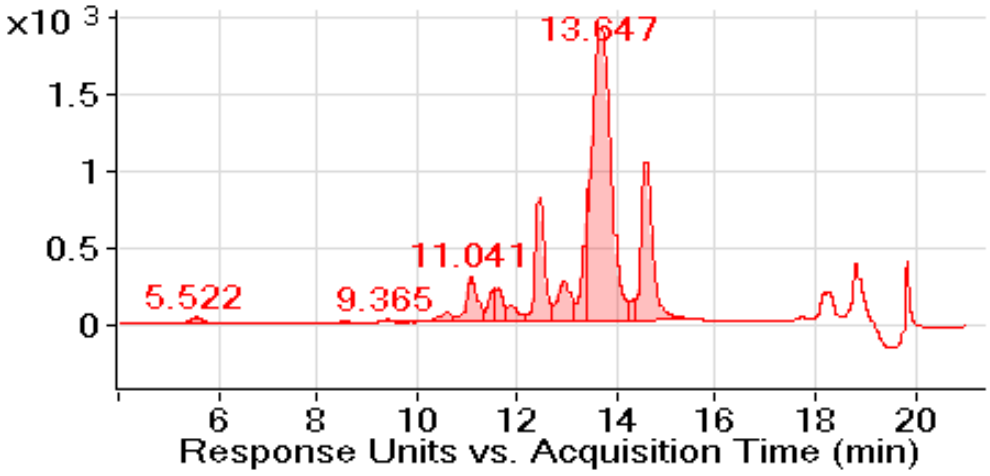

Figure 20. Goat milk protein profile used to calculate the mass balance.

Furthermore the areas for the different peaks and their percentage-wise share of the total peak area were calculated as can be seen in Table 15 below.

Table 16. Areas for the peaks seen in Figure 20, and their percentage-wise share of the total area.

Peak number	Retention time (min)	Area	Area %	Protein
1	5.522	895.98	0.885	κ-cn
2	7.443	96.58	0.095	Unknown
3	8.491	230.12	0.227	Unknown
4	9.365	250.73	0.248	α_{s2}-cn
5	10.556	1205.23	1.190	Unknown
6	11.041	4857.03	4.796	α_{s1_1}-cn
7	11.447	2151.35	2.124	α_{s1_2}-cn
8	11.576	2221.84	2.194	α_{s1_2}-cn
9	11.866	1570.28	1.551	Unknown
10	12.411	10326.27	10.197	α-lac
11	12.909	5005.93	4.943	β-cn
12	13.373	6098.97	6.0226	β-cn
13	13.647	48746.86	48.136	β-cn
14	14.309	1057.97	1.0447	Unknown
15	14.552	16553.36	**16.346**	**β-lg**

So from the total amount of proteins, 16.346% should be β-lg.

Calculation of the yield of β-lg

The protein content was determined to be 19.4 g/L, but the NPNs should be subtracted, which is ~8%.

Amount of total protein in skimmed goat milk: $19.4\frac{g}{L} - \frac{19.4\frac{g}{L}}{100\%} \cdot 8\% = 17.848\ g/L$

Amount of total protein in the fractionated milk: $17.848\frac{g}{L} \cdot 0{,}03812\ L\ milk = 0.66\ g$

Amount of β-lg in the fractionated milk: $\frac{0.66\ g}{100\%} \cdot 16.346\% = 0.11\ g$

The total weight of purified β-lg: 0.011 g

Percent-wise yield: $\frac{0.011 g}{0.0011} = 10\ \%$

In order to visualize the mass balance of the proteins in the milk, a flow chart of the process can be seen in Figure 21 below. The double arrows mean that some steps have been skipped, in order to simplify the illustration.

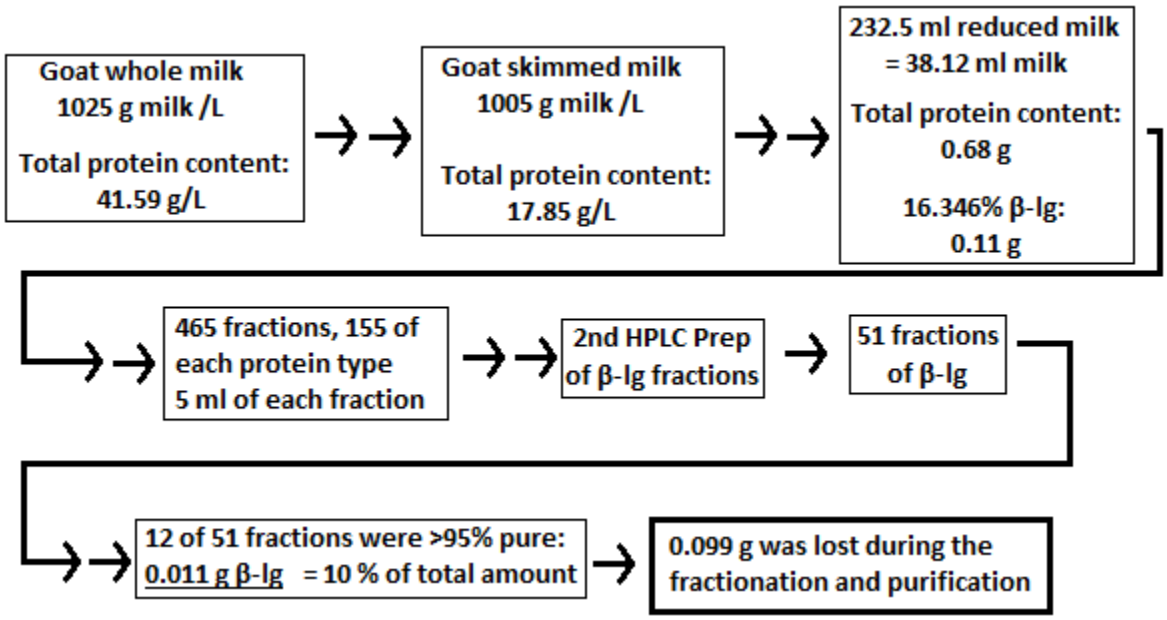

Figure 21. Flow chart of the milk protein mass balance. The double arrows mean that some steps have been skipped, in order to simplify the illustration.

Bradford Protein Assay

Curves for the two standards are showed below in Figure 22 including the regression lines for the linear part of each standard curve. The top equation represents the BSA standard, while the bottom equation represents the bovine β-lg standard.

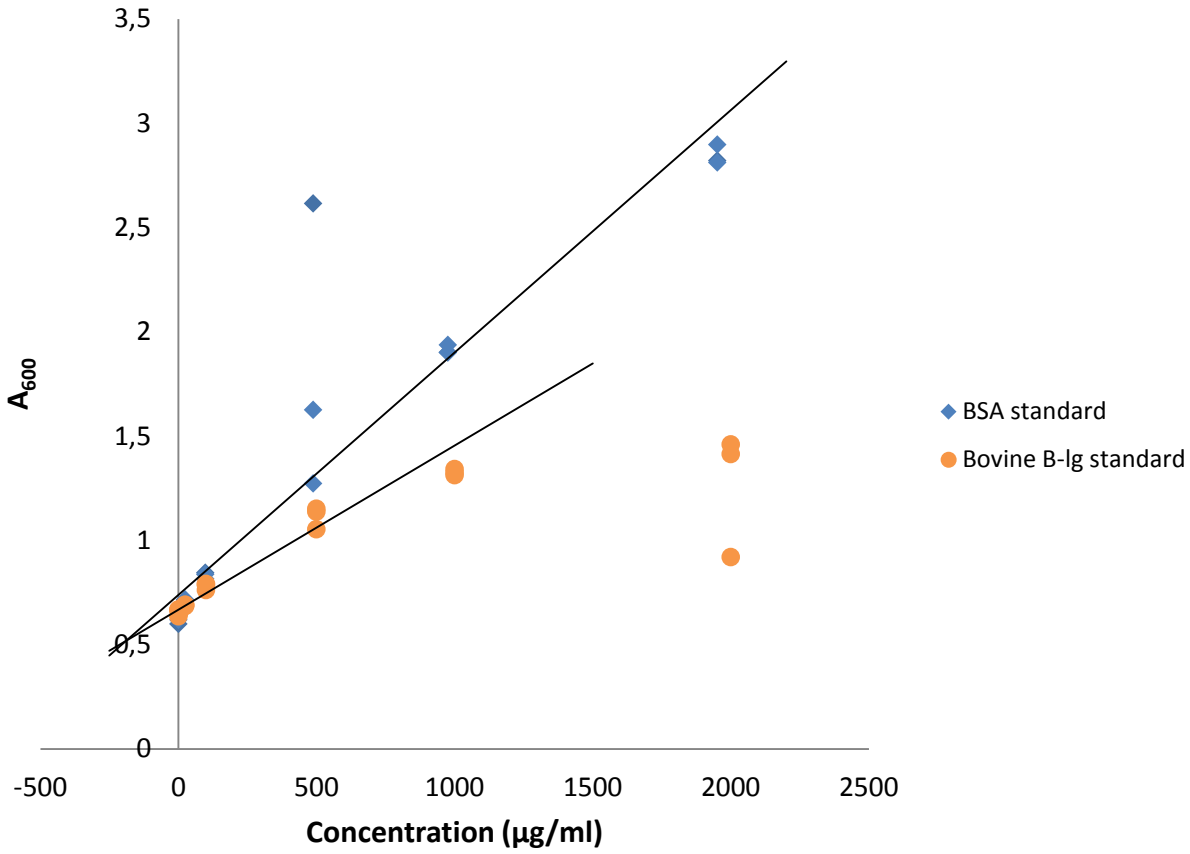

Figure 22. Standard curves of the two standards, including the regression lines for the linear part of each standard curve. The blue points represents the BSA standard, which have the linear regression values y = 0.0012x + 0.7376 and R^2 =0.9685, while the orange points represents the bovine β-lg, which have the values y = 0.0008x + 0.6673 and R^2 = 0.9758.

From the equations for regression lines of the standard curves, the absorbances of the different samples were used to calculate the concentrations of goat milk protein or caprine β-lg.

In Table 17 below, the results from the calculations are shown. The Excel sheet with the absorbances and calculations can be found on the attached USB flash drive.

Table 18. Expected and calculated protein concentrations made from the Bradford Protein Assay. ND*: Not detected. The measured absorbances for these samples were lower than the reference absorbances from the standard curves, which mean that these measurements cannot be included.

Whole milk: 41593.2 µg protein / ml milk		
Dilution	**Expected concentrations (µg/ml)**	**Bradford results (µg/ml)**
x40	1039.83	*373.111*
x100	415.932	*276.722*
x150	227.288	*143.666*
x1000	41.5932	*30.611*

Skimmed milk: 17848.0 µg protein / ml milk		
Dilution	**Expected concentrations (µg/ml)**	**Bradford results (µg/ml)**
x20	892.4	*332.278*
x100	178.48	*215.888*
x300	59.493	*27.278*
x1000	17.848	*ND**

Caprine β-lg		
Dilution	**Expected concentration (µg/ml)**	**Bradford results (µg/ml)**
Undiluted	1500	*27.125*
x1.5	1000	*15.875*
x3	500	*ND**
x15	100	*ND**
x60	25	*ND**

The results from the Kjeldahl method and the Bradford method can clearly not be compared directly. This make sense since the Kjeldahl analysis determines the protein content from the nitrogen content multiplied with a factor of 6.38, while in Bradford Coomassie® Brilliant Blue G-250 is used in the assay which binds to aromatic amino acids, and is therefore an expression for the amount of aromatic amino acids in the protein mixture. This will necessarily provide different results.

Discussion

Fractionation and Purification of Goat Milk Proteins

From the results of the purification experiments it is clear that the yield of β-lg can be significantly increased, since it was only accomplished to obtain 10% of the theoretically total amount of β-lg, which means that 0.099 g was lost during the purification of the 38.12 ml skimmed goat milk. To optimize the fractionation, the gradient, column temperature, and flow rate used in the method could be adjusted. If the gradient was changed, so that the amount of organic solvent was higher in the beginning, the fraction of interest would elute faster than with the current gradient, hereby making the fractionation time shorter for each injection. This change would also provide wider peaks, and combined with a lower flow rate, this could make it easier to collect purer fractions from the beginning.

If the column temperature is changed, it would affect various parameters. For one, the retention time is decreased ~2% for every degree the column temperature is raised, which is illustrated in Figure 23. So if the column is able to tolerate that the temperature is raised, this could contribute to the optimization of the fractionation method. With increasing temperature, the peak spacing is lowered as well, which can give problems since well separated peaks are desired. This can also be seen in Figure 23. Furthermore the viscosity of the mobile phase is lowered when the column temperature is increased, which lowers the column pressure and provide narrower peaks on the chromatogram. Narrowing of the peaks is desired since this increase peak intensity, and lowers the detection limits (John Dolan, 2014).

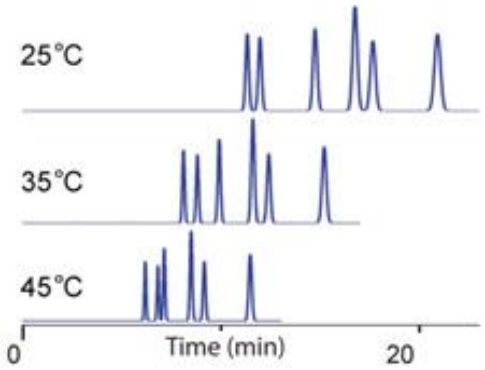

Figure 23. Retention time decreases with increasing column temperature (John Dolan, 2014).

Furthermore the number of purification steps could be reduced, since the amount of protein decreases at each step, which has been proved by the mass balance calculated in the Results sections. In addition, the protocol for pooling the fractions could be changed, so that fewer fractions were pooled together before second fractionation. This would result in less loss of protein, since during each transfer from vial to vial a little amount of protein is lost.

The required purity of the fractions could also be lowered; four of the 51 fractions from the second fractionation was ≥80%, which means that if the required purity has been lowered to this, the total yield of the process would have been bigger.

The reducing process of the skimmed milk could also be optimized, since the reducing method used in this project is optimized for cow milk, but due to a narrow time frame, this method was transferred directly to the reduction of the goat milk. To optimize the reducing process of the goat milk, the amount of added buffer solution could be adjusted, hereby possibly leading to a lower dilution of the milk, and hence be able to fractionate more milk pr. injection than before. This would mean a larger yield in the same time spent. But it must be assured that the reduction of the proteins still provides the largest amount of unfolded proteins, since this is the aim of the reduction. This could be tested by setting up an experiment with e.g. ten different dilutions of the skimmed milk, run them through the preparative HPLC system with the same gradient method, and compare the chromatograms. If no changes are detected, then other dilutions of the skimmed milk could be tested. The less the milk is diluted, while still reducing the proteins optimally, the better. In addition it would be an advantage to expand the fraction collector capacity in a way that more fractions could be collected overnight, to increase the amount of fractionated milk pr. day.

But if there is not much protein of interest present in the milk from the beginning, the purification is even harder. The first chromatogram in the Results section shows, that α_{s1}-casein would be preferable as internal standard, since this is the one that has a retention time which is most different from the proteins in cow milk. But it is also clear that this goat milk sample does not contain very much of this particular protein, compared to the amount of e.g. β-casein. So before attempting to adjust the fractionation parameters on the preparative HPLC, it could be a good idea to compare α_{s1}-casein content in the milk within the different goat breeds. The breeds from which the milk was collected is Saanen, Toggenburger and Danish Landrace at Knuthenlund Estate at Lolland, but the sources (Maga E. A., 2009) and (S. Clark, 2000) suggests that the breeds Alpine, LaMancha and Nubian should produce a higher amount of α_{s1}-casein in their milk. Furthermore it was stated that the overall amount of caseins in the milk was highest in the spring and summer, so it might also be an advantage to get milk from that time of the year, even though it is not clear from which time of the year this particular milk sample was collected.

Bradford Protein Assay

If a new Bradford protein assay should be made, different dilutions should be used, since dilutions which are in the middle section of the standard curve is desired for this type of analysis. This means, that for a second trial, the samples should not be diluted as much as they were in this assay. As a guideline for the new dilutions, the first dilutions of the samples could be used, meaning that the most diluted sample should only be x300 for the skimmed goat milk and x1.5 for the caprine β-lg.

Furthermore another standard than BSA could be tested, since this other standard might be more alike the goat milk proteins, hereby providing a more comparable standard curve.

Total Protein Content

In order to obtain another estimation of the total protein content in the milk, all of the proteins present in the milk could be collected by preparative HPLC, evaporated and freeze dried. Then the yield could be weighed, and the mass per volume could be estimated.

Conclusion

It was attempted to produce pure fractions of the goat milk proteins α_{s1}-casein, α-lactalbumin and β-lactoglobulin for use as internal standard in liquid chromatography, but it was only proven possible to obtain 11 mg of ≥95% pure β-lactoglobulin from 38.12 ml skimmed goat milk. This yield was 10% of the maximum yield. The other proteins were not purified.

The Bradford experiment showed that the Kjeldahl method and the Bradford method do not provide the same values of protein concentrations for the same sample; the Kjeldahl method consequently gave higher concentrations of protein than the Bradford method did. This means that the results from these two methods cannot be directly interpreted in order to determine protein concentration. But it was possible to create standard curves from both BSA and bovine β-lg standard and obtain equations for the linear part of the curves.

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