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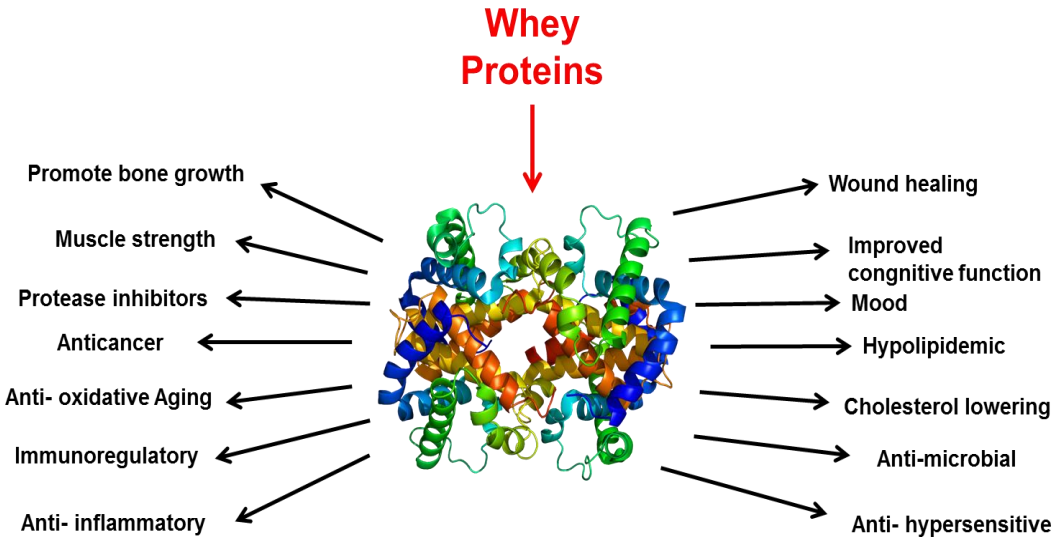
Determination of protein composition in whey fractions by molecular spectroscopy, gel electrophoresis and mass spectrometry



Zahra Salimi

Master thesis in Biological Chemistry
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University of Stavanger

TINE®

IRIS
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"If you want to live a healthy and active life, drink whey"

And

"If everyone was raised on whey, doctors would be bankrupt"

(Brink, 2005)

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Zahra Salimi

Abstract

Whey is becoming an important product for the dairy industry. In the last 15 years, the characterization of whey proteins (β-lactoglobulin (β-Lg), α-lactalbumin (α-La), Bovine serum albumin (BSA), Immunoglobulin (Ig), Lactoferin (LF), Glycomacropeptide (GMP) and Casein macropeptide (CMP)) has become an important issue for biochemists and dairy companies. Characterization of the protein in whey is necessary to obtain possible methods to achieve the best whey based products. This project is a comparison of three different methods for protein characterization in order to observe and compare the main proteins in whey and skimmed milk. Whey samples were generated through the microfiltration of skimmed milk where permeate is defined as native whey. During this whey sample preparation, a second type of whey was formed. This whey is the retentate generated from the ultrafiltration of permeate (native whey) and is called native whey protein concentrate from microfiltrated milk.

FT-IR spectroscopy, Gel electrophoresis and Mass spectrometry based proteomics were the methods that were used in this project to characterize the milk samples concentrate. Initially sample preparation methods were developed to obtain protein samples for analysis.

Fourier transform infrared spectroscopy (FT-IR) was used to evaluate differences between each sample according to the type of filtration or the concentration factor of each process. Principal component analysis (PCA) was utilized to FT-IR spectra in regions of 3043-898 cm^{-1} in order to identify the differences between skimmed milk, permeate and retentate proteins. Use of this method allowed analysis of the most important wavenumbers for each group of samples. PLS modelling was used to compare the data from FT-IR against other methods, by showing the correlation between samples.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to predict the presence of certain proteins in the samples based on their molecular weight. Gel scans show the distinct protein sizes in the whey and skimmed milk. The method does not allow identifying proteins but it may answer some biochemical questions.

Mass spectrometry based proteomics (using LC-MS/MS) is commonly used to identify proteins in biological samples. Protein characterization by this method is based on detection of peptides following trypsination of the samples, a so called bottom-up approach. Tandem mass spectra are collected from peptides and in order to identify the proteins, the results are searched against a database of proteins derived from genomic sequences.

B-lactoglobulin, α-lactalbumin and casein were the major proteins present in my samples. The existence of BSA, lactoferin, immunoglobulin and glycomacropeptide as the minor proteins were also detected. Most of the SDS-PAGE observations were in agreement with LC-MS/MS results. The results indicate that mass spectrometry provided the most information concerning the protein content of the samples. Although depending on the research needs SDS-PAGE or FT-IR analysis may be sufficient.

Aim of this Study

The main objective in this thesis was to evaluate and compare different sample characterization methods for skimmed milk and whey with focus on protein content. The methodology for generation of skimmed milk involved either microfiltration (MF) alone or both microfiltration followed by ultrafiltration (UF). The method used, has an influence on whey quality and functional properties of the whey product, thus influences are studied and evaluated for their significance for usability on whey proteins.

Microfiltration technology has been developed by membrane manufacturers. Samples from MF and UF were used for protein characterization with different methods as described below, (with the final results cross-correlated and FT-IR being calibrated against the other two methods):

1. FT-IR was used to obtain spectra significant to each sample. The differences between microfiltration and ultrafiltration technology were compared with this analysis.
2. Characterization of protein in skimmed milk and whey by SDS-PAGE. The protein in same profile of corresponding samples was compared and the marker used to group the protein more easily. The effect of microfiltration and ultrafiltration on protein profile was studied by this method.
3. More detailed determination of protein in skimmed milk and whey was done by mass spectrometry. Using the LC-MS/MS method. Comparison of the protein profiles in the samples was performed following different processes and sample preparation procedures.
4. The results of FT-IR, SDS PAGE and LC-MS/MS were cross correlated. The FT-IR method was calibrated with the other two methods.

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1. Introduction

Protein is an essential compound in human diets. Protein characterization is one of the most interesting and relevant topics for biochemists to consider. Determination and characterization of protein has been a challenging matter, and one that has not been completely solved yet (McKenzie, 2012).

1.1. Milk

Milk is an important nutrition source for people around the world. Recently, much consideration has been given to milk quality, especially milk protein (Shi, Yang, & ZUBAREV, 2010). Cow milk is comprised of approximately 3.3% protein, 4% fat, 87.1% water, 4.6% lactose, contains many essential vitamins (vitamin E and A), and is major source of calcium (Lindmark-Månsson & Åkesson, 2000; Dissanayake, 2011). Traditionally milk proteins have been divided in three crude groups, whey proteins, milk fat globular membrane (MFGM) proteins, and casein (Cunsolo, Muccilli, Saletti, & Foti, 2011). Currently, milk protein and its products are highly researched because of the significant impact on health and high biological value (Haug, Hostmark, & Harstad, 2007; M. Santos & Lies, 2015). Milk proteins play an important role for growth factors, enzymes, immune system function, hormones, and antibodies (Clare & Swaisgood, 2000; Korhonen, Marnila, & Gill, 2000). In addition, nutritional scientists found that using a higher amount of milk protein may build-up muscles, prevent bone breakdown, improve muscle movement, raise satiety, control blood sugar, and decrease the risk of some cancers (Melnik et al., 2013; Karen Giles-Smith, 2013). Milk proteins consist of 80% casein and 20% whey proteins. Casein is responsible for transporting calcium and phosphate and aiding efficient stomach digestion. Other major proteins in milk are α-lactalbumin, β-lactoglubulin, bovine serum albumin and immunoglobulins (Haug et al., 2007)

1.2. Whey proteins (WP)

Whey was considered a waste product before scientists and the dairy industry discovered its benefits. It was, then that whey became a major source of functional dairy components (M. J. Santos, Teixeira, & Rodrigues, 2012). Whey is the nutritional complement that is residual of milk coagulation. It is usually a secondary product in cheese or casein production (Wiley, 2014). It is full of proteins and peptides that are important to overall health and generally helpful for the body's functions, especially in children and as sports nutrition (Zimecki & Kruzel, 2007). Whey proteins are a specific group of proteins that have various biological, physiological and practical properties and are known as a rapid digested protein (Haug et al., 2007; P. Fox, 2001). Whey proteins have significant biological effect on the human immune and growth systems (Heino, 2010). Additionally, they have various effects in the human body such as being useful in human brain function and muscle protein synthesis (Markus et al., 2000; Heino, 2010). Whey proteins can be used

in dairy, beverages, and meat (Campbell, Adams, Drake, & Barbano, 2013). The advantages of drinking whey has been recognized for many years, with two ancient proverbs from the Italian city of Florence saying, "If you want to live a healthy and active life, drink whey" and, "If everyone was raised on whey, doctors would be bankrupt" (Brink, 2005).

Whey proteins contribute to 18-20 % of total milk proteins. The most important proteins in whey are bovine serum albumin (BSA) 10%, α-lactalbumin (α-LA) 20%, β-Lactoglobulin (β-LG) 50%, Casein, lactoferin (LF), immunoglobulins (Ig) 10% and glycomacropeptide (GMP) (P. F. Fox & McSweeney, 1998) (Jovanovic, Barac, Macej, Vucic, & Lacnjevac, 2007). The whey products are concentrates (WPC), hydrolysates (WPH) and isolates (WPI) (M. J. Santos et al., 2012). Environmental factor such as temperature, ionic strength and pH can change the functional properties of the protein in whey and the proteins themselves can affect the properties of the whey proteins (De Wit, Hontelez-Backx, & Adamse, 1988). For making new products, whey can be efficiently separated with different methods such as membrane techniques, precipitation, and chromatography, etc. (Dissanayake, 2011). Different types of WP are accessible in the form of WP powder, WP permeate and retentate (M. J. Santos et al., 2012). The reminder of milk proteins are caseins. Caseins have no secondary, tertiary or quaternary structures while whey proteins do exhibit this structural hierarchy. Caseins are also phosphorylated molecules, however whey proteins are not (P. Fox, 2001).

1.2.1. β-lactoglobulin

β-lactoglobulin (β-Lg) is the most important protein in whey with ~ 18 kDa molecular weight. It represents 50% of whey protein and also 12% of total protein in milk (Boland, Singh, & Thompson, 2014). Bovine β-Lg is a well-known protein that has been studied greatly (Dalgalarrondo, Dufour, Bertrand-Harb, Chobert, & Haertlé, 1992). β-Lg is able to bind to fatty acids and retinol (vitamin A) and because of this, it has great foaming and gelation properties (Heino, 2010).

1.2.2. α-lactalbumin

α-lactalbumin (α–La) is the second most important protein in whey and milk with a molecular weight of ~ 14 kDa. It comprises 20 % of total whey protein and also 3.5 % of total protein in milk. α–La has dependency on calcium (Ca^{2+}) ions and it is known as a metallo-protein (Thompson et al., 1988). α–La has a high tryptophan (Trp) content that is very useful for human brain function. It also has high level of the amino acids use in muscle protein synthesis (Markus et al., 2000).

1.2.3. Casein

Casein (CN) is the principle protein and represents 80% of the cow milk protein. Casein is known as micellar and there are 5 kinds of casein micelles in milk that are different in moleculear composition but are similar in structure (alpha S1 (αs1-CN), alpha S2 (αs2-CN), beta (β-CN), kappa (κ-CN) and (γ- CN) casein) (Heino, 2010). They typically have a molecular weight between 14 and 25 kDa.

1.2.4. Bovine serum albumin

Bovine serum albumin (BSA) with the molecular weight of 66 kDa is another cow milk proteins. BSA has significant biological effect on human health but its role in food and milk are not well known (Heino, 2010). BSA has only a slight effect on whey physiochemical properties due to its low concentration in milk (Boland et al., 2014). It is sometimes used as a protein standard in some experiments.

1.2.5. Lactoferin

Lactoferin (LF) has a molecular weight of about 76.5 kDa and is a multi-functional protein from the transferrin family (Yang et al., 2013). It exists in different liquids like milk, nasal, saliva and others. (Sanchez, Calvo, & Brock, 1992). LF has antibacterial activity in humans and interacts with nucleic acids (Pometto, Shetty, Paliyath, & Levin, 2014).

1.2.6. Glycomacropeptide

Glycomacropeptide (GMP) is the C-terminal portion of kappa casein and is sometimes called casein macropeptide (CMP). It has a molecular weight of 6-10 kDa. The amino acid composition of this protein is very unique. GMP has various chemical attributes like extensive emulsifying properties and is stable in a wide range of pH (Sharma, Rajput, & Mann, 2013). It can be said that GMP is a complex mixture of whey protein and casein micelles (Tovar Jiménez, Arana Cuenca, Téllez Jurado, Abreu Corona, & Muro Urista, 2012).

1.2.7. Immunoglobulin:

Immunoglobulin (Ig) is the immunological part of the milk (Hurley & Theil, 2011). Immunoglobulins are antibodies that can protect people against a wide range of bacteria and viruses (Korhonen et al., 2000). Human milk has the highest amount of Ig but cow's milk has low level of Ig (Carpenter Ea, 2010). Ig has a molecular weight of 150-1000 kDa. These proteins have immune-active peptides and therefore the presence of this protein is beneficial for a whey product (Tovar Jiménez et al., 2012).

Whey production is an important process. To release all non-protein compositions in whey, scientists decided to separate the large and small molecules. The size of the molecules and pressure in process, help to separate the molecules by pliable membrane (Flinn, 1970).

1.3. Membrane Filtration

Currently, membrane filtration is the chosen method in food and dairy industry to separate the protein, salt and minerals from the feed (Daufin et al., 2001). Membrane processing is used to remove particulates, bacteria, microorganism and minerals. Different filtration types are used for this technology in the dairy industry including: Reverse Osmosis (RO), Nanofiltration (NF), Ultrafiltration (UF) and Microfiltration (MF) (Bylund & Pak, 2003). These days, membrane processing of whey product is being developed. The use of NF and UF is a used for separating peptides and amino acids from milk (Timmer & Van der Horst, 1998) (Figures 3&4).

1.3.1. Concentration Factor

The concentration factor is a dimensionless factor that is used in filtration studies. The concentration factor is " the volume reduction achieved by concentration, i.e. the ratio of initial volume of feed to the final volume of concentrate" (Bylund & Pak, 2003). The content of the whey protein in skimmed milk will be effected by microfiltration, because the absorbency MF to the main whey protein components depends on the concentration factor (CF) (Outinen, Heino, & Uusi-Rauva, 2008). The concentration factor is directly proportional with the protein concentration.

Volume concentration factor was calculated:

$$CF = \frac{Flow\ retentate + Flow\ permeate}{Flow\ retentate} \qquad (1)$$

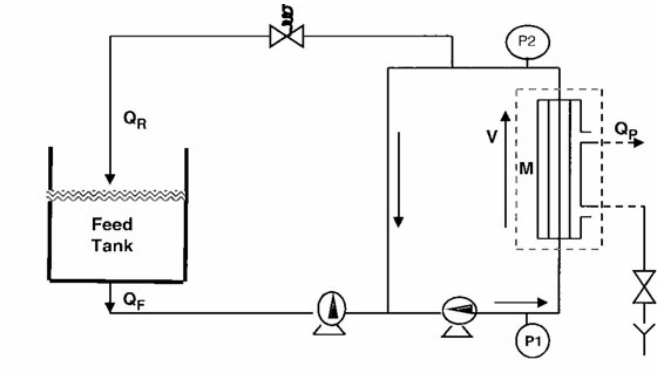


Figure 1. Schematic diagram of membrane pilot system. M:Membrane module; QF:Feed skimmed milk; QP: Permeate; QR:Retentate; V:Cross flow velocity; P1,P2: Pressure gauge (Mukhopadhyay et al., 2009)

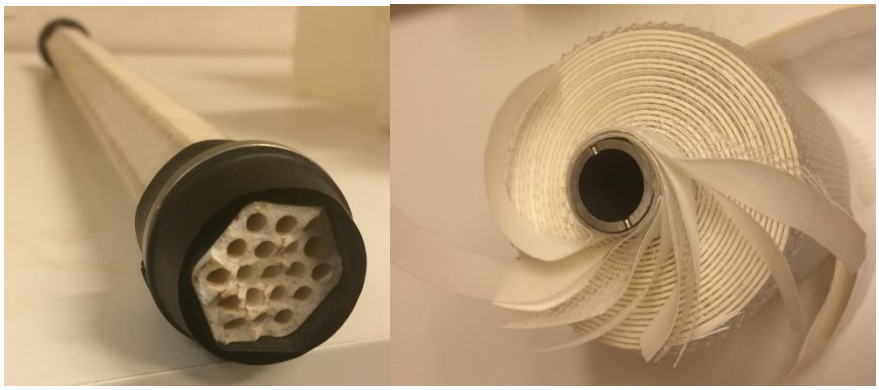


Figure 2. Membrane used for Micro and Ultra filtration

In this study microfiltration (0.05 and 0.15 µm) and ultrafiltration were both used; the differences between these methods were in the separation of whey proteins from skimmed milk. Ultrafiltration concentrated both casein micelles and whey proteins.

1.3.2. Microfiltration

Microfiltration (MF) is a low pressure cross flow membrane process used for separating particles in the range of 0.05-10 microns. Microfiltration is usually used for decreasing the bacteria in skimmed milk and whey. It also used to separate the fat from whey for protein fractionation of whey protein concentration (WPC) (GEA Filtration, 2015). MF is used for the protein extraction process to produce casein micelles and isolate the whey protein. Sweet whey is made when the skimmed milk go through the MF membrane with pore size of 0.1 or 0.2 µm. The whey from microfiltration can be attested as the bacteria and virus free (Daufin et al., 2001). Multichannel ceramic membrane and dynamic counter pressure are two different methods for removing bacteria by MF (Sandblom, 1978).

1.3.3. Ultrafiltration (UF)

Ultrafiltration is a separation technique used to separate solid components from a liquid mixture. UF membranes have a pore size less than 0.01 micron. Chemical interaction between the membrane and liquid component plus molecular the basis of UF separation. In this procedure, water molecules push through membrane by pressure to preserve the colloidal solids and salts (MMS, 2006). UF is a selective fractionation process that uses pressure around 10bar. It concentrates solutions of molecular weights higher than 1000 Daltons. Permeate from microfiltration process is usually used for ultrafiltration (GEA Filtration, 2015). Typically, a ceramic membrane with 300 kDa cut-off is used. This kind of membrane is used for removal of suspended solid in feed. UF normally generate a whey protein concentrate (WPC) with high solid ratios (77% to 90%) (Daufin et al., 2001).

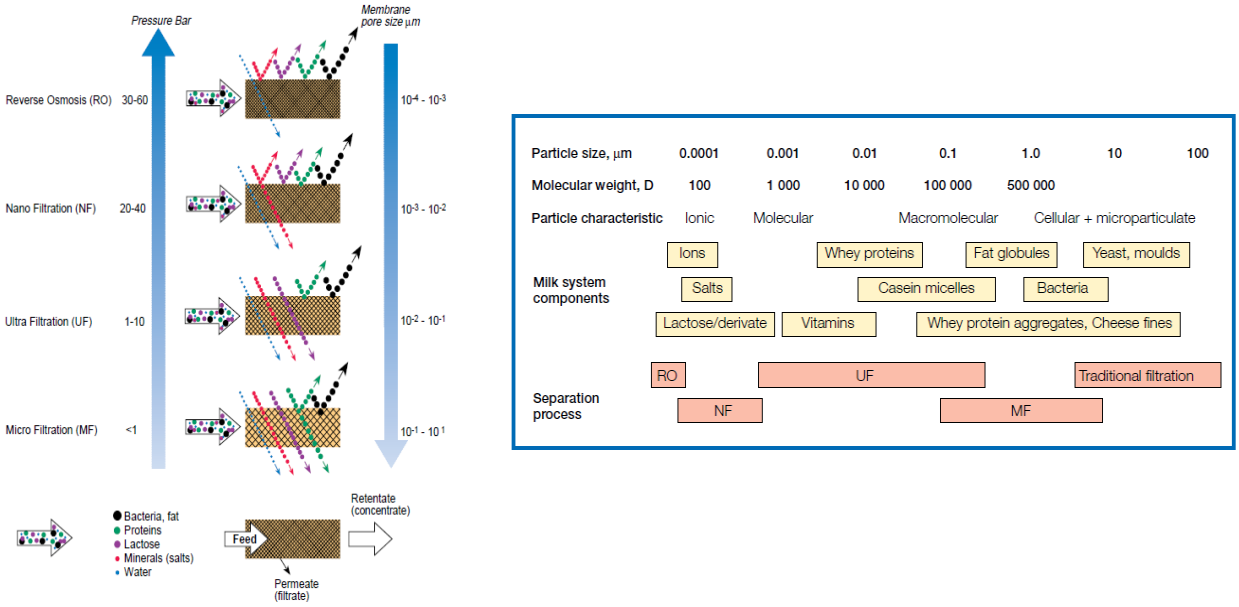


Figure 3.Principle of membrane processing in dairy industry (Bylund & Pak, 2003)

Microfiltration consists of a variety of membrane filtration systems with the largest pore size (0.1-3 microns). Ultrafiltration pore size range is 0.01 to 0.1 microns. MF fills the gap between ultrafiltration and traditional filtration according to the pore size. MF is usually used for decreasing the turbidity and raising the suspended solids. UF is typically used to eliminate color, some viruses, some colloidal organic substance, and smell. Both methods need a transmembrane pressure between 1 to 30 psi (Technical Service Center Water Treatment Group, 2010,).

In the dairy microfiltration industry, skimmed milk is fed to the microfiltration and the products are microfiltrated permeate and microfiltrated retentate, known as native whey and casein concentrates, respectively. Microfiltration permeate is the fed to ultrafiltration and the product is ultrafiltrated retentate, also called native whey protein concentrate(Jørgensen et al., 2015) (Figure 4).

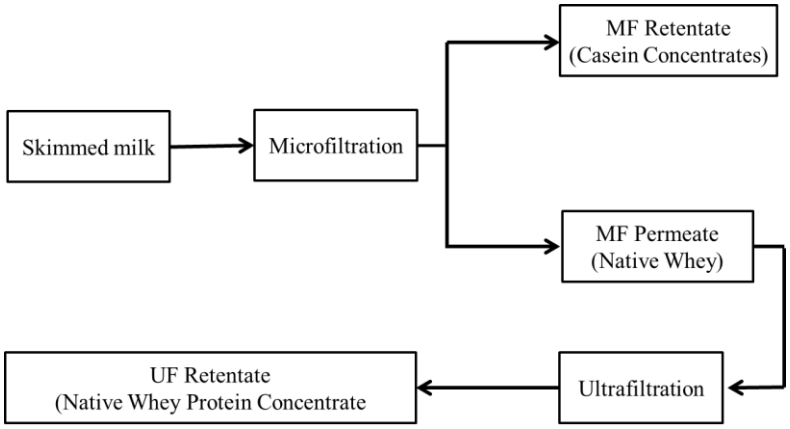


Figure 4. Filtration procedure flow chart

1.4. Gel Electrophoresis (SDS PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) is the standard method for protein sizing, although protein identification technology is a fast expanding field (Laemmli, 1970; Shapiro, Viñuela, & Maizel, 1967). SDS PAGE is an established method used to separate and identify proteins according to their movement by size of the molecules. The goals of this method are; characterizing proteins and enzymes, monitoring protein purity, studying environmental effects on protein composition studying, and classifying genetic variation (Cherry & Barford, 1988). SDS has a net negative charge as an anionic detergent which binds non-covalently to proteins. Moreover, SDS gives the polypeptide a total negative charge relative to its length. This negative charge in polyacrylamide gels aid in separating the proteins in an electrical field (Goetz et al., 2004). It binds to most soluble protein molecules over a wide pH range. Polyacrylamide gel is the normal medium for protein electrophoresis (Garfin, 2003). A polyacrylamide gel with a pre-determined polyacrylamide concentration prevents movement of larger protein molecules through the gel as fast as small protein molecules. Protein separation by SDS gel is usually used to characterize the molecular weight of the protein and shows the significant proteins present in the sample. The resolution of the size can be changed and modified by changing the gel composition. Proteins that are separated by SDS-PAGE can be used to characterize the approximate molecular weight of a protein and the abundance of major proteins in samples. The precision of sizing in SDS-PAGE is influenced different variables such as isoelectric point, structure, amino acid sequence, etc. The most important thing that can increase the accuracy of the results are controlling experimental conditions such as pH, temperature, staining times and gel composition (Goetz et al., 2004). One of the most important advantages of SDS PAGE is that, it is a reliable and simple method (Davey & Lord, 2003).

The electrophoresis in SDS gel is usually following with a staining step to visualize the bands. Comparing the test lane with a marker lane of known size helps in determining the molecular weight (Yazdanparast, Nezarati, Heshmati, & Hamzehlou, 2012).

Existence of proteins with similar molecular weight in a complex mixture is the reason that one dimensional gel electrophoresis is not as as two dimensional (Cherry & Barford, 1988).

1.5. FT-IR Spectroscopy

The combination of infrared spectroscopy with Fourier transform (FT-IR) is a fast, cheap and useful technique for characterizing the compositions of biological samples (Szymanska-Chargot & Zdunek, 2013). Fourier Transform is dependent on time and frequency. Generally FT-IR is a method to collect the infrared spectra, inelastic diffusion, imbibition and emission of compounds (Griffiths & De Haseth, 2007). FT-IR system measures the full spectrum of the sample in the same period of time (Van de Voort, Sedman, Emo, & Ismail, 1992). Infrared spectroscopy has been a common method for more than seventy years for materials studies like obtaining detailed information on chemical compound (Tauler, Kowalski, & Fleming, 1993). An infrared spectrum shows absorption peaks of samples, which correspond to the frequencies of vibrations between the bonds of the atoms. No two compounds have the same infrared spectrum since each material is a unique combination of atoms. Consequently, infrared spectroscopy is a positive identification (qualitative analysis) of some kinds of material. Furthermore, the amount of material present is shown by the size of the peaks in the spectrum. Infrared is a brilliant tool for quantitative analysis with new software algorithms (Nicolet, 2001). In a FT-IR spectrometer, radiation passes the sample through the measuring device to reach the detector, and there the signal is converted to digital signal by first amplified the analog to digital transformer. Finally, the converted signal is transferred to a computer that is connected to the instrument (UCDAVIS CHEMWIKI, 2015). FT-IR used together with PCA, allows better visualization of spectra (Szymanska-Chargot & Zdunek, 2013).

Principle Component Analysis (PCA) is a multivariate data analysis technique to find similarity and differences in samples (Van Der Ven, 2002; Brunn, 2006). The goal of this method is to analyze the data for modeling (K. H. Esbensen, Guyot, Westad, & Houmoller, 2002). To find a small set of principle component (PC) that describe the most mutability on these data set is another purpose of PCA (Szymanska-Chargot & Zdunek, 2013). It usually searches for linear combinations. The reason is that, in linear combination, less information is lost and data will be optimized. A multivariate data analysis technique is usually used to choose significant data when there is large data set to consider (Brunn, 2006). PCA method is helpful to explain the FT-IR spectra. FT-IR spectra are usually very complicated depending on the source of the samples (Szymanska-Chargot & Zdunek, 2013). Partial least squares (PLS) is another multivariate calibration. In PCA the principle component model of the correlation construction of X was made but PLS modeling depends on two matrices X and Y. The X matrix is the corresponding independent variable whereas Y contains dependent variables. PLS is a model to find the connection between X and Y. The starting point is always a set of known measurements collected for the data matrix X. The most accurate way for calibration in PLS modeling is that the range of X-area and Y-area should be as wide as possible to show the result clearly. Spectroscopy can concurrently measure many physical and chemical factors indirectly. It also can measure the data very quickly, reducing time spent in the laboratory (K. Esbensen, 2002).

1.6. Mass spectrometry (LC-MS/MS)

One of the main and most popular analytical methods for identification of the proteins is mass spectrometry (MS). MS is important because of its characteristic sensitivity and selectivity (Cooks, Chen, Wong, & Wollnik, 1997; Domon & Aebersold, 2006). Mass spectrometry has been extensively used for analyzing biological samples. The method is based on identification of ions according to mass over charge (m/z) ratios (Kang, 2012; Agilent Technologies, 2001). MS can be described as an ion visual instrument that uses the electric and magnetic fields to separate ions according to their m/z ratio (Carpenter Ea, 2010).

Electron spray ionization (ESI) charges the molecules in atmospheric pressure. ESI can be coupled with high performance Liquid chromatography (HPLC). This makes ESI a good ion source for complex mixtures in LC-MS like using Nano-LC with Nano-ESI tandem MS (MS/MS). The instrument and software are constantly improving velocity and precision making identification of proteins easier (Chen & Pramanik, 2008).

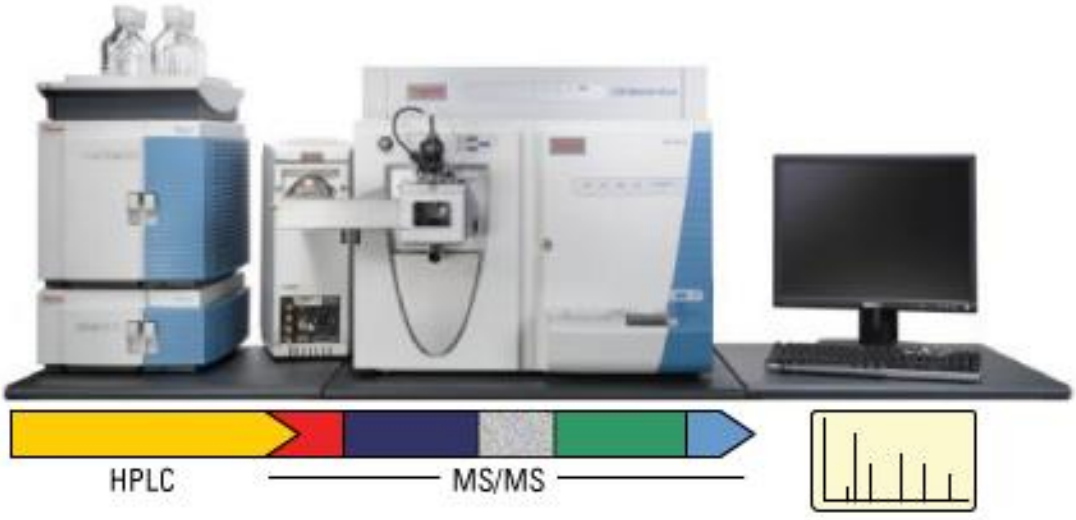


Figure 5. Schematic diagram of LC-MS/MS system

In chemistry, liquid chromatography is an essential separation technique especially for protein determination (Carpenter Ea, 2010). Normally C_{18} reverse phase is used as a cation exchange LC for the first step of separation. In online MS systems used for real time analysis the sample is directly electro-sprayed. All the peptides are retained on the cation exchange resin under acidic conditions. These bound peptides can then be eluted stepwise from the C_{18} resin. The C_{18}-bound peptides are separated using a conventional solvent gradient and electro-sprayed directly into the MS system for real-time analysis, in order to improve sequence coverage. In complex protein mixtures, peptides are very complicated so capillary LC-MS can be applied for high resolution (Chen & Pramanik, 2008).

To form molecular ions, gaseous molecules are ionized in the ion source and some of them will be fragmented. Ions with different m/z go through the mass analyzer separately to

reach the detector. When they impact the detector, ions can transform into an electrical signal that the detector translates to a digital response which can then be stored by a computer (Sparkman, 2000). In mass spectrometry, only ions are identified; continuous pumping preserves the vacuum which removes all the atoms that have no charge (Kang, 2012). Both positive and negative ions can be analyzed by MS, however the majority of analyzed ions are positive, since the positive ions are normally produced in larger number than the negative ions (Chiu & Muddiman, 2008).

The combination of a linear ion trap with Orbitrap analyzer is an increasingly common mass spectrometer step used to determine and specify proteins and chracterize proteomes (Michalski et al., 2012) .

2. Materials and Experimental Method

2.1. Materials

Three sample types Permeate (low protein), Retentate (high protein) and skimmed milk were sent from TINE SA in Ås. Permeate passes through the membrane and everything that is retained on the feed side of the membrane is defined as retentate (Figure 4 for shows the details on sample generation) (Hazer et al., 1996) . Skimmed milk was microfiltered with a ceramic membrane with 0.05, 0.10 and 0.15 μm pore sizes to a concentration factor 1.5, 2.5 and 2.8.

2.1.1. Preparation of whey samples

Skimmed milk was microfiltrated by ceramic membrane with 0.05 to 0.15 μm pore size. For Seri 1, the volume concentration factor (CF) was 1.5 when retentate (casein concentrates) was collected. After a while the concentration factor was set to 2.8, and new retentate was collected separately. Permeate (native whey) was collected from the start of microfiltration process. Retentate with CF around 1.5 means that the protein content of the feed is concentrated approximately 1.5 times. To concentrate the whey proteins, collected permeate was ultrafiltrated with spiral wound 25 kDa cut-off and concentrated native whey (permeate) called native whey concentrates (UF retentate). In series 2 and 3, skimmed milk was microfiltrated with 0.10 μm pore size. Permeate and retentate were collected with 1.5 concentration factor. Series 2 and 3 had the same conditions but different dates. Seri 4 was done with 0.15μm pore size and 2.5 CF but some conditions changed during the process to concentrate the permeate more (Table 1).

The samples were specified by MFP (microfiltration permeate = Native whey), MFR (microfiltration retentate = Casein concentrates), UFR (ultrafiltration retentate = Native whey concentrates) and Skimmed milk. Skimmed milk had been microfiltrated with ceramic membrane. Permeate, containing small molecules was passed through the membrane and retentate with large molecules was retained on the feed side. For ultrafiltration, permeate from microfiltration was used for further ultrafiltration. The differences between processes are flow rate, pore size (μm), length, type of membrane and the concentration factor.

Samples name

Table 1. Samples name

Seri	Type of samples	Industrial name of samples	Samples name	Pore size (µm)	Date of filtration	Concentration Factor (CF)
Seri 1	Microfiltration retentate	Casein concentrates	MFR 1.5 (S.1)			1.5
Seri 1	Microfiltration retentate	Casein concentrates	MFR 2.8 (S.1)	0.05-0.14	29.09.2014	2.8
Seri 1	Ultrafiltration retentate	Native whey concentrate	UFR (S.1)			-
Seri 2	Skimmed milk	Skimmed milk	Milk (S.2)			-
Seri 2	Microfiltration permeate	Native whey	MFP (S.2)	0.1	06.11.2014	-
Seri 2	Microfiltration retentate	Casein concentrates	MFR (S.2)			2.5
Seri 3	Skimmed milk	Skimmed milk	Milk (S.3)			-
Seri 3	Microfiltration permeate	Native whey	MFP (S.3)	0.1	26.11.2014	-
Seri 3	Microfiltration retentate	Casein concentrates	MFR (S.3)			2.5
Seri 4	Skimmed milk	Skimmed milk	Milk (S.4)			-
Seri 4	Microfiltration permeate	Native whey	MFP (S.4)	0.15	04.02.2015	-
Seri 4	Microfiltration retentate	Casein concentrates	MFR (S.4)			1.5

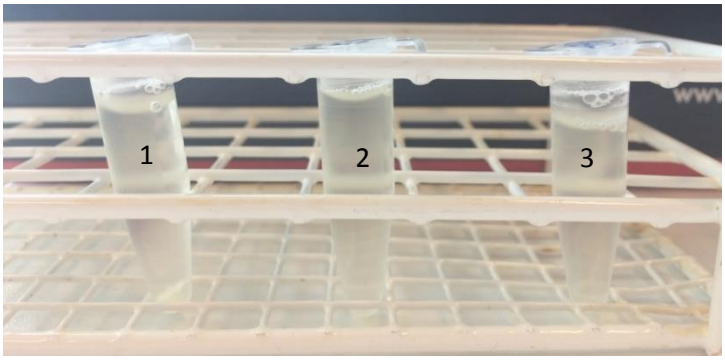


Figure 6. Permeate samples; 1: MFP (S.1); 2: MFP (S.2); 3: MFP (S.3)

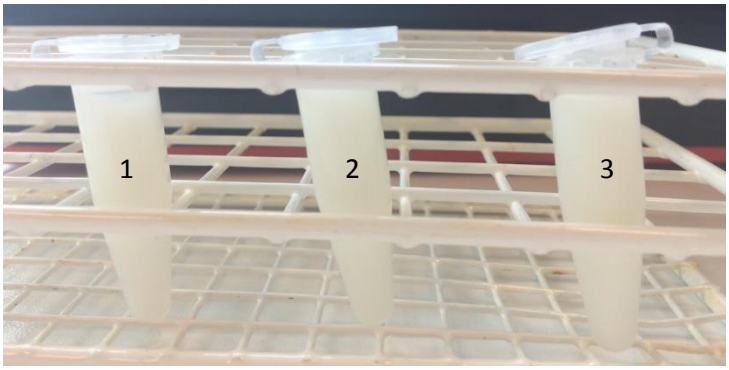


Figure 7. Milk samples; 1: Milk (S.1); 2: Milk (S.2); 3: Milk (S.3)

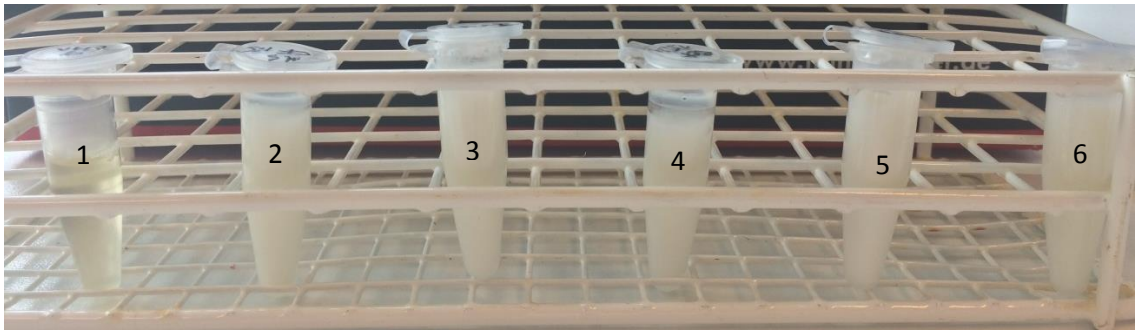


Figure 8. Retentate samples; 1: UFR (S.1); 2: MFR 1.5 (S.1); 3: MFR 2.8 (S.1); 4: MFR (S.2); 5: MFR (S.3); 6: MFR (S.4)

2.2. Method

2.2.1. Gel Electrophoresis (SDS-PAGE)

The gel casting and running was performed according to the Laemmli method. PAGE was performed in 12.5% of polyacrylamide separation gel containing 1% SDS and 4% of polyacrylamide stacking gel. Casting material included two spacers for each gel, a comb, one small and one large glass plate, casting block and the casting stand. All the materials were cleaned with ethanol before starting the experiment. The sandwich was assembled. The separation gel (for 5 mini gels) was prepared according to table below:

Table 2. Separation gel preparation material

ingredient	**Weight/Volume**
Urea	7.21 gr
Acrylamide (30%)	12.5 ml
8x Tris (pH 8.8)	3.75 ml
ddH2O	9 ml
Temed	15 µl
APS	50 µl

The gel was poured until 2 cm under the top and the rest was filled by water-saturated isobutanol and was incubated for one hour to polymerize. The water- isobutanol was drained completely and the stacking gel was poured. The solution for stacking gel is according to the table below:

Table 3. Stacking gel preparation material

ingredient	**Weight/Volume**
Acrylamide (30%)	0.8 ml
0.25M Tris (pH 6.8)	2.48 ml
ddH2O	1.6 ml
Temed	5 µl
APS	50 µl

The gel sandwich was attached to the electrode assembly. Running buffer was added as a cathode and anode buffer. Permeate and milk samples with lower protein were loaded in one gel and retentate samples with more protein was loaded in another gel. Retentate samples were diluted 1:15 plus 20 µl of detergent and permeate sample were inserted directly without dilution plus 15µl of detergent and milk samples were diluted 1.5:13.5 with 15 µl detergent. Samples were heated at 95ºC for 5 minutes and then centrifuged for 5 minutes afterwards. The marker used was Bench Marker pre-stained standard protocol (Life technology). Each gel was run with 15mA and 100 V for 2 hours. When the samples and marker reached the end of the gel, the electrode was disconnected. Gels were transferred to the plate carefully and then coomasie staining solution was added and stayed for one hour. The gel was washed three times with dH_2O and after was destained as much as needed.

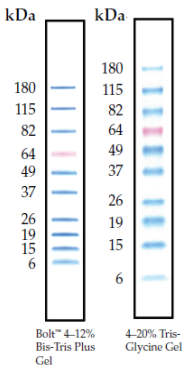


Figure 9. Bench Marker

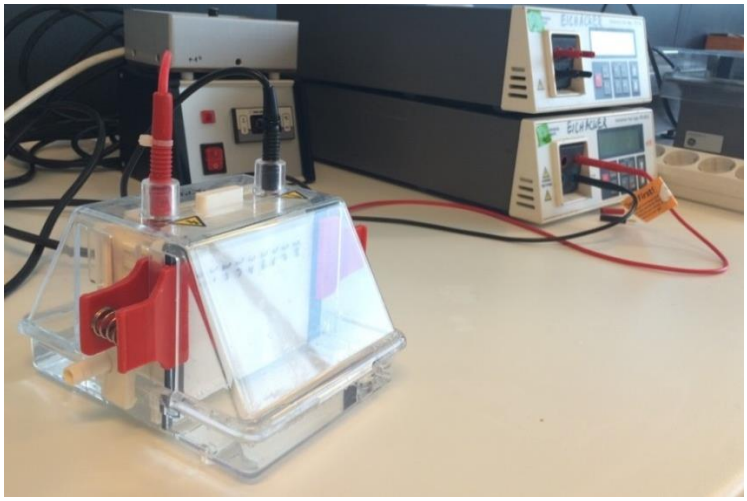


Figure 10. Gel Electrophoresis system

Image J software was used for quantitative analysis of the gels. Image J is software that can detect bands and calculate values according to the peaks strength. The results were transferred to the excel and are shown in the Appendix 8.2.

2.2.2. FT-IR Spectroscopy

FT-IR spectra were collected with a laboratory-scale Fourier transform infrared (FTIR) spectrometer (model Delta instrument, lactoscope FT-IR advanced, Type FTA 3.0). 70 ml of sample for five replicate of each sample under the same condition was examined. The measurement in the lactoscope was done automatically according to whey properties. The data was transferred to the computer to use the unscrambler X 10.3 software. The spectra were collected over the range 890 to 2900 cm^{-1}.

Figure 11. FT-IR Spectrometer

The averaged result of each sample was calculated to have an improved plot. Spectra were modified to remove peaks associated with water noises.

Finally, PCA and PLS modeling was done by using the unsrambler X software.

2.2.3. Mass spectrometry (LC-MS/MS)

2.2.3.1. LC-MS/MS sample preparation

Three sample preparations were evaluated:

1) Simple preparation without molecular weight cut-off filter
2) Preparation with molecular weight cut off filter step (10 kDa and 30 kDa)
3) Acetone precipitation result

The cut-off filter fractionation procedure was done as a first preparation but was then skipped due to low protein concentration in the generated samples.

- **Acetone precipitation**

Protein precipitation is usually used for fast sample clean-up and gets rid of non- protein components. 50 ml of acetone was cooled to -20º C for 1 hour. 500 µl of each sample plus 2 ml of cold acetone were added to Eppendorf tubes. The samples were vortexed completely and incubated at -20º C for 60 minutes. After that, samples were centrifuged at 14000 rpm at 4º C for 10 minutes. The supernatant was discarded and before evaporating the acetone, the Eppendorfs were placed in a fume hood with open lids. The pellet was dissolved in 500 µl ambic buffer (0.1 M). The pellet in this part is sticky; therefore, a warmer was used to dissolve it better. Then samples were then prepared for the Bradford assay.

- **Bradford (Protein concentration measurement)**

The precipitated protein quantification was done using the Bradford method (BRADFORD, 1976). For this purpose, a protein calibration curve was constructed using bovine serum albumin (BSA) as standard at concentrations from 0 to 0.4 mg/ml.

One part of Bio-Rad dye was taken with 4 part of dH_2O and filtered. A total of four BSA protein standards were prepared by using sequential dilutions from 0.1 to 0.4 mg/ml and were assayed at 595 nm. According to the plate layout, 10 µl of each standard solution and samples were added and after that 200 µl of diluted dye was added to each well. Measurement was taken at 595 nm, after incubation for 10 minutes. Data was transferred to calculate the protein concentration. Samples were diluted as far as needed to be within the range of standard curve.

Figure 12.Spectroscope, using Bradford method for finding the protein concentration

Calculation:

$Abs\ BG\ _{(BG=Background)} =$
$(Absorbance\ in\ 595\ nm\ for\ sample) - (Absorbance\ in\ 595\ nm\ for\ BSA\ standard\ curve)$ (2)

$Protein\ concentration\ \left(\frac{mg}{ml}\right) = (\frac{Abs\ BG}{Slope\ value\ of\ standard\ surve})(Diluton\ factor)$
(3)

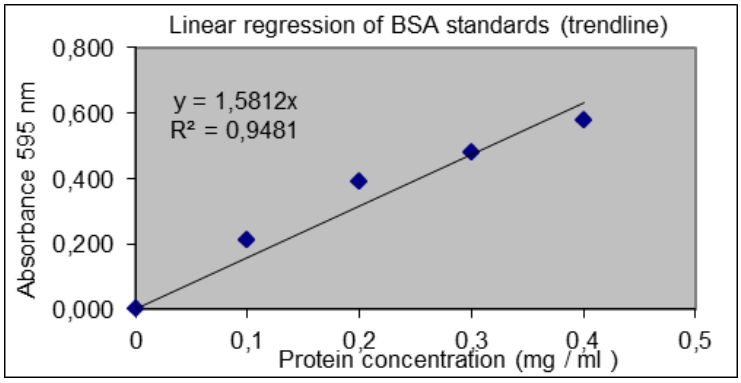


Figure 13. Example of BSA standard curve

- **Trypsination**

After the calculation of Bradford, the amount of ambic buffer for adding to samples was calculated to get 10µg protein at the end. For trypsination, Dithiothreitol (DTT) concentration should be 5mM, so 5 µl of DTT (0.1M) was added to the samples to reduce it and they were incubated for 30 min at 37 ºC. After that, 1.5 µl Iodoacetamide (IAA) was added to alkylate the samples, for a final concentration of 15 mM. They were incubated for 30 minutes in a dark shelf at room temperature. The last step was adding trypsin. The ratio of sample to trypsin should be 25:1, so 4 µl trypsin was added to each Eppendorf and the tubes were incubated overnight at 37 ºC.

With this tryptic digestion, the samples were prepared for LC-MS/MS analysis and protein identification. After trypsination, the samples were purified, washed and concentrated through pierce C_{18} spin columns.

- **Pierce C_{18} Spin columns**

Spin column is a perfect tool to remove the salts, solvents and protecting the materials that can affect the mass spectrometer or spectrometric analysis of the peptides. It is a simple device for sample cleaning and purifying components of the samples.

Figure 14. Pierce C_{18} spin column

- **Sample preparation**

As the ratio of sample and buffer should be 3:1, 90 µl of samples and 30 µl of buffer was added to the column. Column was fixed in receiver tube without any caps.

Column preparation:

200 µl of activation solution was added to make the resin wet. Columns were centrifuged at 1500 rpm for 1 minute and the flow through was discarded after that. This step was repeated once more. Equilibrium solution (200 µl) was added to the column, and then it was centrifuged and discarded as in the last step.

Sample binding:

Protein sample was added to column in this step with a new receiver tube. Tubes were centrifuged at 1500 rpm for 1 minute. The binding should be complete, therefore that step was done twice but this time the flow through was not discarded and was recovered.

Wash:

Column was placed in the new receiver tube. 200 µl of wash solution was added to the column and they were centrifuged at 1500 rpm for minute. The flow through was discarded. This step was repeated once more.

Elution:

Column again was moved to new receiver tube. Elution buffer was added around 20 µl at the top of the column. Centrifugation was done at 1500 rpm for one minute. Again 20 µl elution was added and they were centrifuged.

Last step was to put the tubes into the evaporator very carefully for 10 minutes. Then they were ready for adding to Orbitrap tubes.

2.2.3.2. LC-MS/MS Analysis

Samples were analyzed using the linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap hybrid mass spectrometer, Thermo Fisher Scientific, Walthman, MA, USA). The separation was carried out using a gradient from 2.5% to 64% acetonitrile in 0.1% formic acid.
The raw data files from the LC-MS/MS analyses were analyzed using proteome discoverer 1.4 (Thermo Fisher Scientific) with the Sequent algorithm with minimum precursor mass of 350 Da and 5000 Da. Trypsin, IAA and DTT were used in sample preparation and in the search criteria with trypsin set as the digestion enzyme. Oxidation (M) was set as dynamic modification and carbamidomethyl was set as a static modification. The software showed the PSMs, Amino acids, Coverage, peptides and molecular weights. The best scoring peptide-spectrum match (PSM) was considered as the peptide identification. For each protein the number of PSMs was exported to excel and grouped (Jensen, Provan, Larssen, Bron, & Obach, 2014).

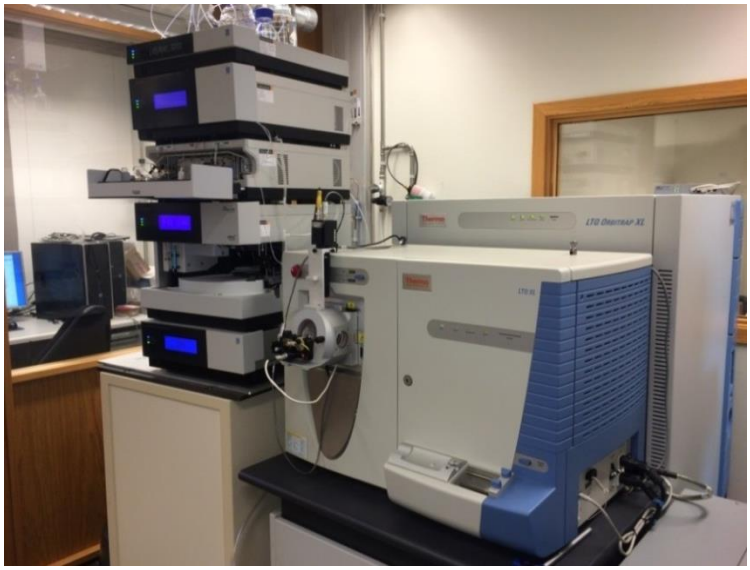


Figure 15. LC-MS/MS system

3. Results and Discussion

The results from this experiment are divided in three parts, FT-IR spectroscopy, Gel electrophoresis and Mass spectrometry. These three strategies were used to characterize the sample preparations of skimmed milk, permeate and retentate samples.

In this study MF and UF techniques were used to separate the whey protein from skimmed milk. Milk components were divided into the MF/UF retentate and permeate after filtration as shown in Table 1. Permeate from microfiltration has no microorganisms. Permeate from microfiltration is usually ultrafiltrated to remove excess casein and water (Froning, Wehling, Ball, & Hill, 1987).

3.1. Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is still considered an efficient method for studying denatured proteins (Goetz et al., 2004). The SDS PAGE electrophoresis method separates protein molecules according to their charge and molecular size.

3.1.1. Optimization of gel running conditions

The first experiments were performed to find the optimal concentration of each sample to run in the gel. The pores of the gel are an important variable in gel electrophoresis. Protein moves through the pores of a gel during electrophoresis. However it is difficult to measure in-situ the pore size of the gel accurately. It usually can be determined by the size limit of the protein that can go through the gel (Garfin, 2003). Figure 16 shows an overloaded gels with too wide-bands. It takes some time to find the best pore size.

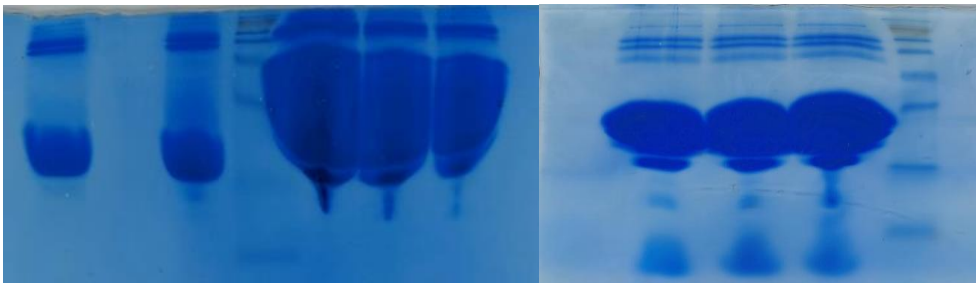


Figure 16. SDS-PAGE electrophoresis, Overloaded samples

In the gel electrophoresis part it also takes a lot of time to find the best conditions such as optimal cathode and anode buffers, best dilutions and running voltage.
Then one gel was run with dilution of 1:10 for each sample. The 1X running buffer was used as cathode and anode buffer. The electrode was fixed for 10 mA. The run took about three hours and the result was unsuccessful. The result is shown in Figure 22.

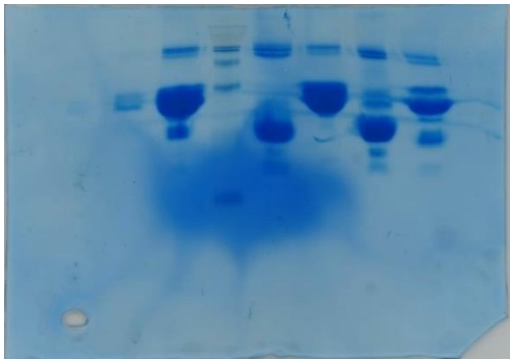


Figure 17. Unsuccessful gel using the wrong dilution

In the next experiment, the samples were run with 1:10 dilution. The electrode was fixed for 10 mA for each gel, but the 0.1% SDS buffer was used as the anode buffer and 1X buffer was used as the cathode buffer. The gel was again unsuccessful as is shown in Figure 18.

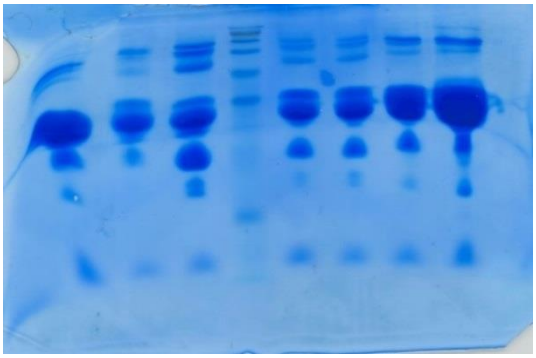


Figure 18. Unsuccessful gel using the wrong anode buffer

Until this experiment the SeeBlue Pre-stained Protein Standard (Life technology) was used. To see the smaller proteins in the gel a wider range molecular weight marker was needed. Bench Marker pre stained standard protocol (Life technology) was used in the future experiments. Again another gel was run with new dilution, retentate 1:10, UFR 0.5:9.5 and permeate was added without dilution. The 0.1% SDS buffer was used as anode and 1X buffer was used as cathode buffer. 15 mA was used for running. The gel is shown in Figure 19.

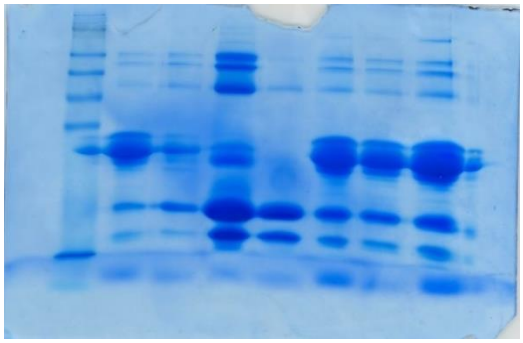


Figure 19. Unfinished gel, taking 3 hours for running

This was an acceptable dilution but it took about three hours to run, so a solution needed to be found. The buffer is the main factor for time of running; therefore it was decide to change the buffer in the next gel.
The next gel helped to do a better test afterwards. All the samples were diluted 1:10 except UFR by diluting 0.5:9.5. 20 µl of loading buffer were added to each sample. The electrode was fixed to 15 mA. The same cathode and anode buffers (1X buffer) were used and gel was run in 30 minutes. The time was unexpected but it was done. As shown in the picture, it was a clear gel.

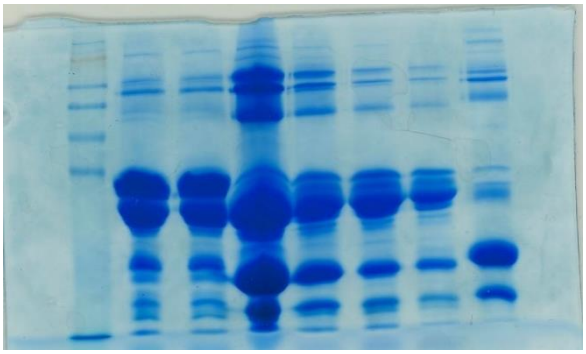


Figure 20. SDS gel, using the correct dilution and buffers

The samples were not all received at the same time; therefore one gel was run at a time. It was necessary to know the final gel conditions.
All the samples were available in the next experiment, so two gels that included all samples were run. Conditions were the same as previous experiment. The gels had the acceptable bands but there was a problem in casting so that the wells were not deep enough. The bands were likely connected to each other. The gels are shown in following figures:

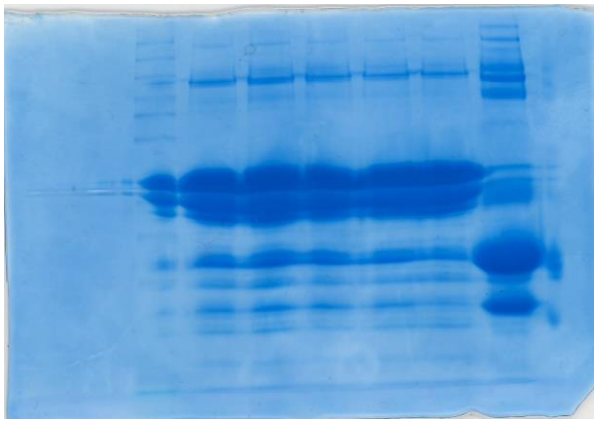


Figure 21. Unsuccessful gel because of imperfect casting

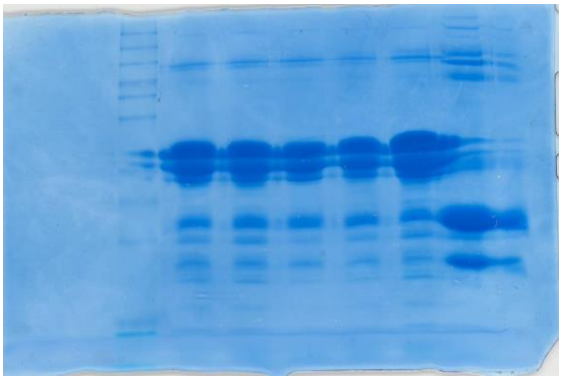


Figure 22. Unsuccessful gel because of imperfect casting

3.1.2. Characterization of protein content through SDS PAGE

SDS PAGE electrophoresis of different samples under different dilution was done. In 12.5% SDS-PAGE, 15 µl sample solution containing loading buffer as a detergent was loaded. Permeate and milk samples with lower protein amounts were loaded in one gel (Figure 23), and retentate samples with more protein were loaded in another gel (Figure 24). Retentate samples in Figure 24 were diluted 1:15 plus 20 µl of detergent, permeate sample were inserted directly without dilution plus 15µl of detergent and milk samples were diluted 1.5:13.5 with 15 µl detergent. A marker (Bench Marker pre stained protein standard protocol) with known molecular weights was also added to the gel. The SDS bands are clearly visible. The bands related to skimmed milk, permeate and retentate proteins (α-La, β-Lg, αs1-CN, αs2-CN, β-CN, κ-CN, BSA, Lactoferrin and immunoglobulin) were matched according to the marker on the SDS-PAGE gel and were marked on each gel. Major proteins including α-LA (14 kDa), β-Lg (18 kDa) and Casein (14-25 kDa) were predicted to be present in the whey samples. Lactoferin with molecular weight of 150-1000 kDa as a minor protein in whey was observed. In addition the presence of GMP with molecular weight of 6-10 kDa that could be a complex mixture of whey proteins and casein micelles was detected in the gel. Bovine serum albumin (66 kDa) and Immunoglobulin (Igs) were also predicted to be present in electrophoresis study part. The bands that are separated by SDS-PAGE are usually used to characterize the approximate molecular weight of a protein. Usually, the best way to have an understandable explanation of the gel bands is comparison of gel to gel or sample to sample (Goetz et al., 2004). Figures 23 and 24 show the normal scan of gels.

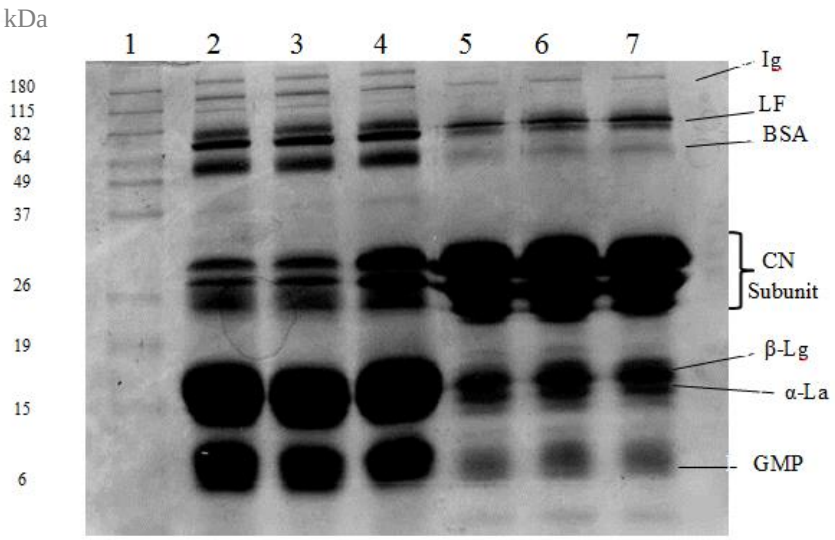


Figure 23. SDS-PAGE electrophoresis of whey proteins prepared; 1: Molecular weight marker; 2 Microfiltration permeate (S.2); 3: Microfiltration permeate (S.3); 4: Microfiltration permeate (S.4); 5: Skimmed milk (S.2); 6:Skimm milk (S.3); 7: skimmed milk (S.4);

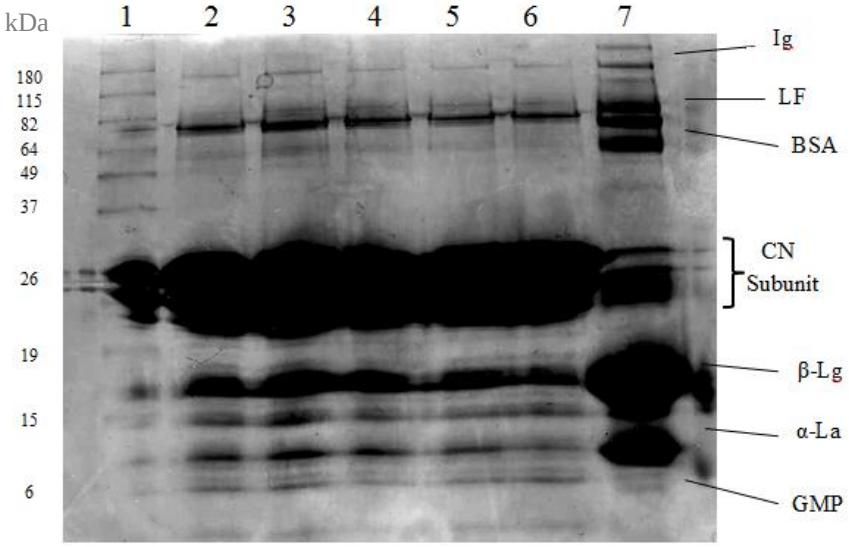


Figure 24. SDS-PAGE electrophoresis of whey proteins; 1: Molecular weight marker; 2 Microfiltration retentate (S.2); 3: Microfiltration retentate (S.3); 4: Microfiltration retentate (S.4); 5: Microfiltration retentate1.5 (S.1); 6: Microfiltration retentate2.8 (S.1); 7: Ultrafiltration retentate (S.1);

3.1.3. Comparison of samples

Gel electrophoresis of different WPCs and milk are shown in Figures 23&24. The protein bands were identified using broad range molecular weight marker (bench marker standards). As is shown in Figures 23&24 permeate samples have the lowest amount of protein and retentate samples have the higher amount of major protein based on strength of bands. The protein concentrations in this study are dependent on different factors such as type of filtration, concentration factor, pore size, etc.

The analytical method appears as the consistent as the same sample prepared on different dates show the same profiles. As shown in Figure 23, MFP (S.2) and MFP (S.3) were from the same process but different date, therefore lanes 2 and 3 have almost the same bands with all major and minor protein. As described, all samples from series 2 and 3 had the same bands as it expected. Skimmed milk in lanes 5, 6 and 7 are the feed for microfiltration, thus they have same protein bands. Skimmed milk has less β-Lg, α-La and BSA than permeate samples (lanes 2-4), β-Lg, α-La and BSA are the main proteins in whey (Figure 23).

Significant differences among the samples were found. Ultrafiltration offers the fundamental advantage of removing casein from native whey samples. This can be seen by the fact that there appears to be less Casein in the UFR than in any other sample type. Comparing skimmed milk, permeate and retentate, SDS-PAGE show that microfiltration did not affect the presence of proteins however proteins were distributed between permeate and retentate samples (Figures 23&24).

The protein bands are dependent on concentration factor so the samples with same concentration factor should have the same bands (MFR (S.2), (S.3), and MFR2.8 (S.1)) and (MFR (S.4), MFR1.5 (S.1)). In MFP (S.4) the membrane with CF=0.15 to get permeate with more protein was used in filtration system, so in lane 4, it is clear that the bands are darker showing higher concentration of the sample (Figure 23).

As clearly predicted in both gels, when casein was increased the levels of β-lactoglobulin and α-lactalbumin were decreased. Although the detergent was used for sample preparation for SDS gel, there was no way to avoid the overload of casein in retentate samples.

Figure 25 represents the comparison of gel bands by using the data from Image J software (the data is shown in Appendix 8.1). It clearly shows the ratio of proteins in each sample and helps to compare by demonstrating the direct and indirect proportions.

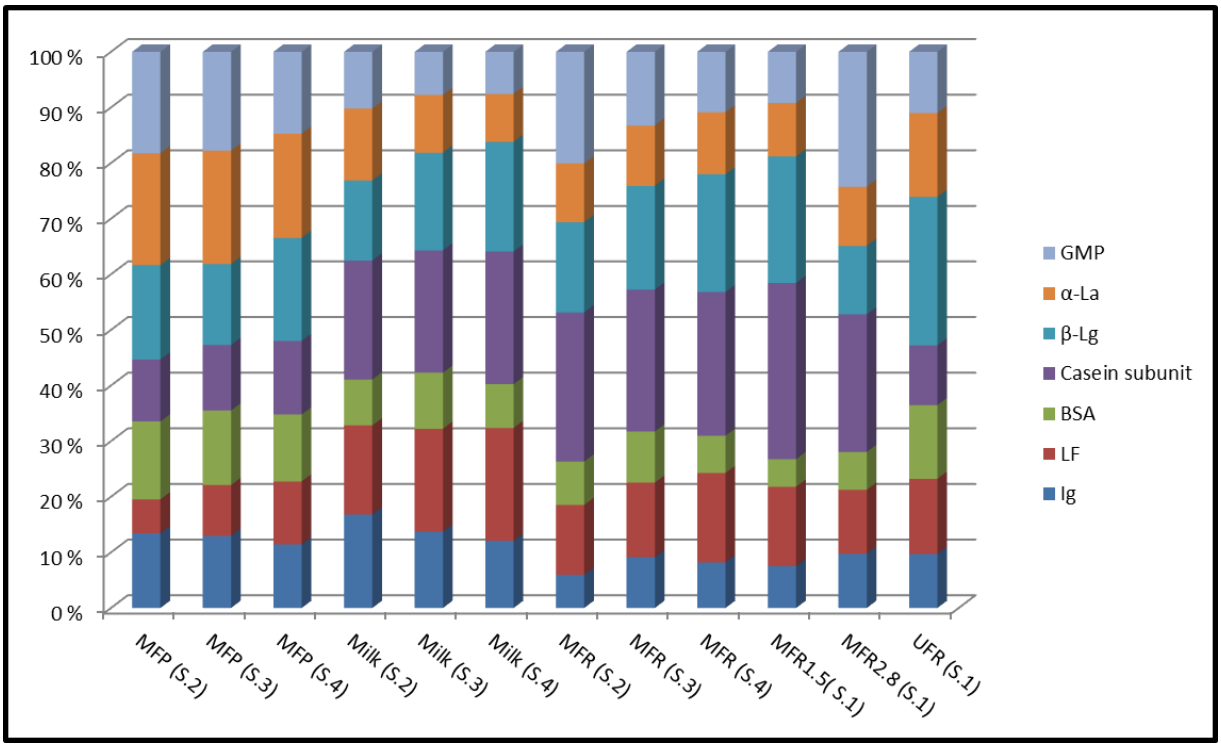


Figure 25. Stacked column 3D chart of comparing different protein in all samples by using Image J software

The method helps to visualize the different amount of the protein in each sample.

MF is used in an united protein extraction process to produce casein micelles and isolate the whey protein (Daufin et al., 2001). The ultrafiltration is used to remove the casein even more efficiently. In SDS gel electrophoresis results for ultrafiltration retentate, casein can be seen but in comparison to MFR is much less. Compared to other samples, UFR has more of other proteins such as β-Lg, α-La and BSA.

Skimmed milk is milk without fat that should have a combination of proteins, but according to the type of filtration and the size of the membrane, the amount of the proteins could vary. Cross flow filtration (0.05-0.15 µm) makes it possible to achieve the separation of skimmed milk micelle casein and soluble proteins.

FritzGerald (FitzGerald & Meisel, 2000) note that casein are the proteins that have high effect on blood pressure in human, therefore according to the results, microfiltration retentate samples have high level of casein so MFR potentially be medically used in addition to dairy industry. To compare the casein presence in samples can be shown like: MFR> Skimmed milk> MFP> UFR.

3.2. FT-IR spectroscopy

In FT-IR spectroscopy, uses infrared light in various wavelength to observe how the samples respond to each wavelength. Each compound has an individual set of absorption values in its infrared spectrum (Gallagher, 2009). FT-IR spectra can be related to functional properties of the samples, so an analysis of these properties by multivariate regression analysis was performed. PCA based on FT-IR data revealed differences between samples , as has been shown in previous work (Van Der Ven, 2002). In order to observe the differences between the samples, multivariate analysis of the spectra was performed (Jensen et al., 2014).

FT-IR spectra were collected by using a lactoscope FT-IR advanced (Delta instrument). Figure 26 present the spectra of all permeate retentate and milk samples in the range of 3043-898 cm^{-1} after lactoscope analysis. The result was transferred to the Unscrmbler X software to plot those (Figures 26-29). The FT-IR spectra showed that water can make noises (Appendix 8.5). These kinds of noises were in the range of 1700-1600 cm^{-1} and were removed. Variables with high loading weights that are responsible for main differences between FT-IR spectra of samples were mainly observed between 1600 -1200 cm^{-1}. Apparently, the FT-IR spectra were directly dependent on concentration, so that higher peaks in the graphs showed the higher concentrated sample. There is a sharp peak around (1500 cm^{-1}) region associated with aggregated proteins. In conjunction with this observation the presence of two peaks around (1000 and 2900 cm^{-1}) was observed and related to water and fat.

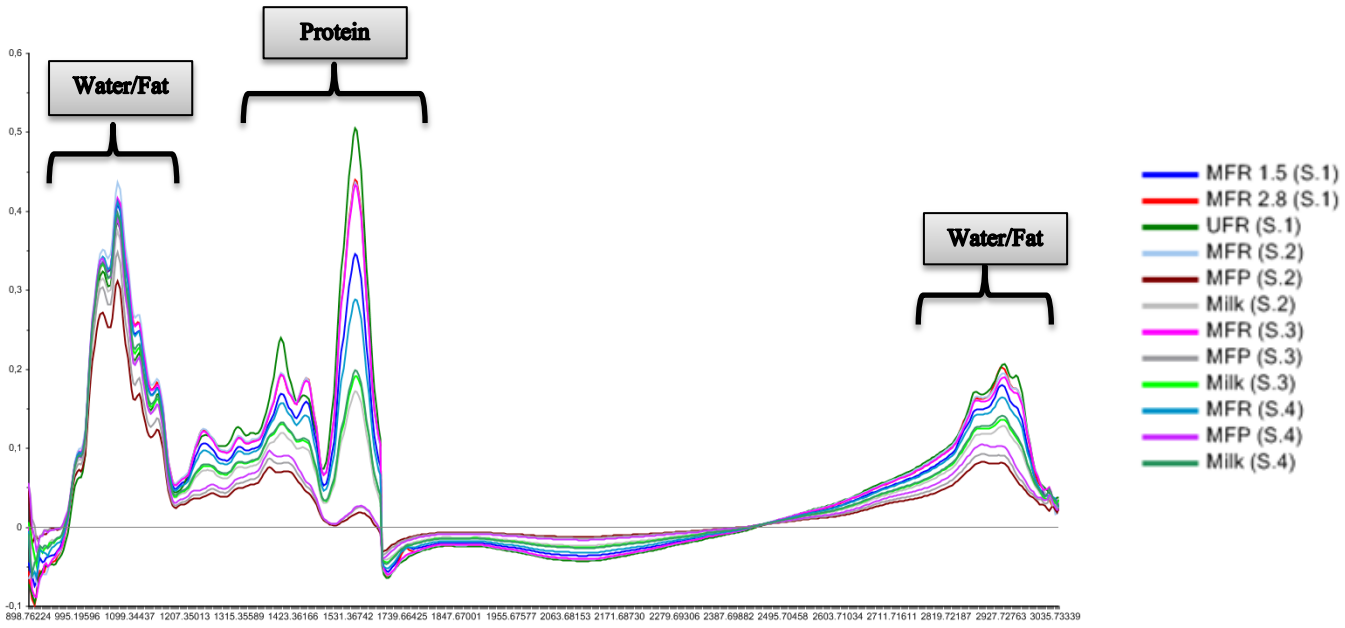


Figure 26. FT-IR spectra in 3043-898 cm-1 region of all samples

Figure 26 shows that samples with high concentration (Retentate) have the higher absorbance peaks than permeate and milk samples. The differences among them were clear and occurred in each region of spectra. The chemical properties of each sample cause the differences in the spectra.

Grouping the samples according to sample type, to obtain the spectra from each group was given useful information to discriminate the samples. Figure 27 represent the permeate samples that have less protein compare to other samples. Important FT-IR spectral values were in the range of 2400-750 cm^{-1}. As is shown, the peaks are lower because of low protein concentration in permeate samples. In microfiltration of sample Seri 4, the pore size was prepared according to get more protein in permeate and so, MFP (S.4) (green line) has the highest peak, as expected.

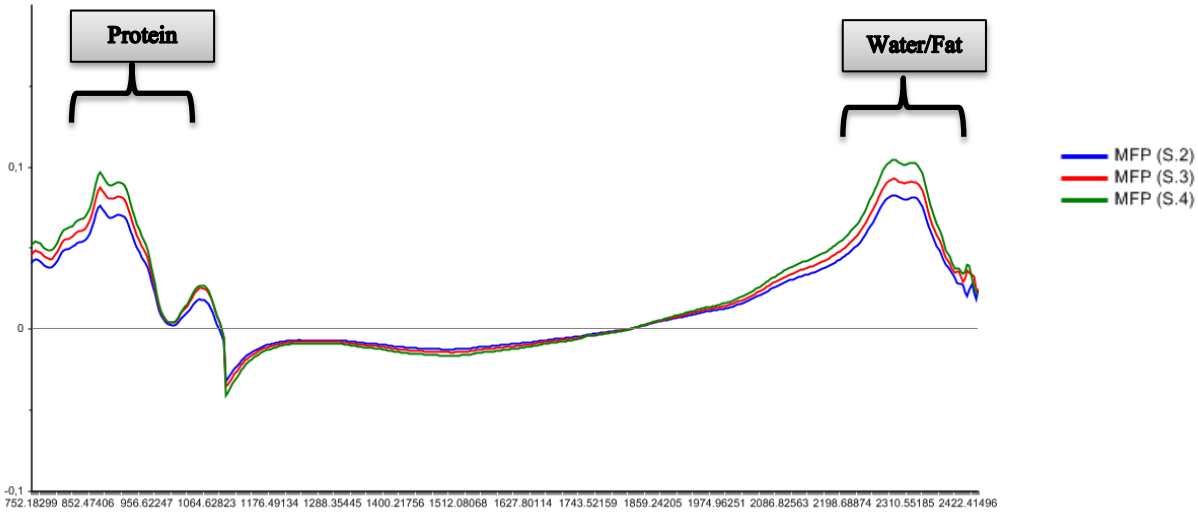


Figure 27. FT-IR spectra in 2400- 750 cm-1 region of Permeate samples

Figure 28 shows the retentate samples. FT-IR spectra were collected in the range of 2400 to 840 cm^{-1}. In this graph UFR has the highest peak and MFR (S.4) has the lowest. This can be related to the filtration process for Seri 4; when permeate has more protein, the protein in retentate was decreased. If the comparison is according to the concentration factor, the samples with similar CF have similar spectra.

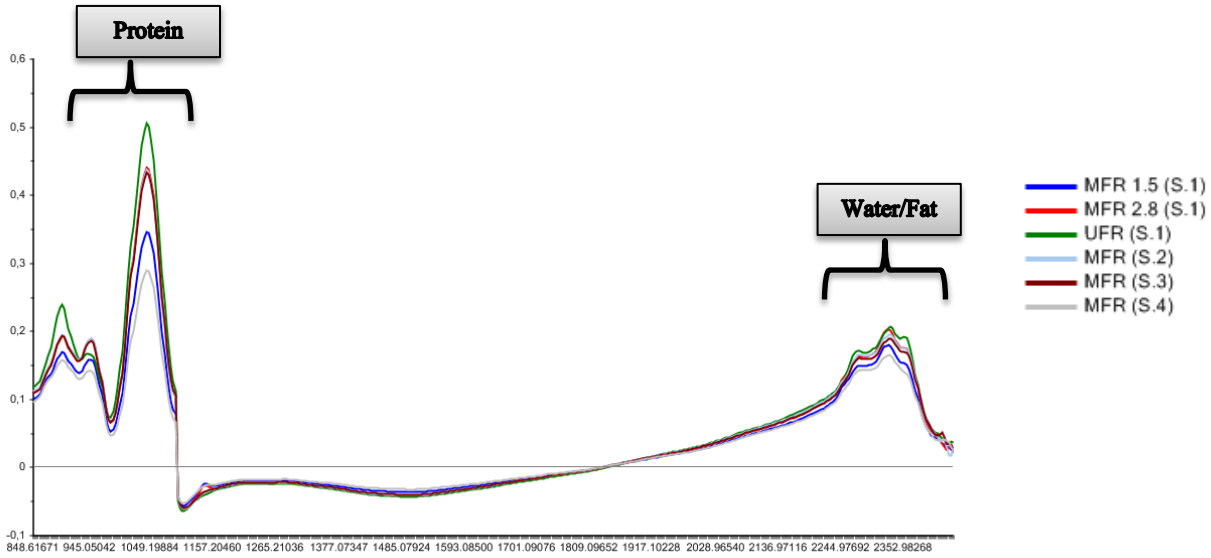


Figure 28. FT-IR spectra in 2400- 840 cm-1 region of Retentate samples

Figure 29 represents the milk samples. FT-IR spectra were collected in the range of 3000 to 800 cm^{-1}. The height of the peaks here are between permeate and retentate. Skimmed milk is the feed for microfiltration so it is clear that the spectra are similar.

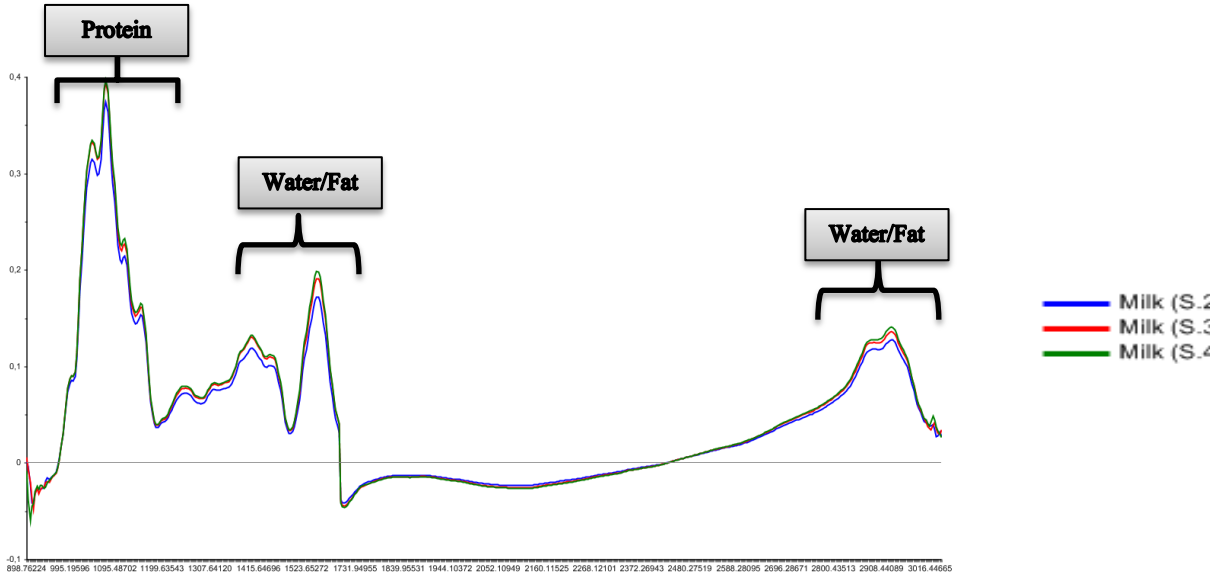


Figure 29. FT-IR spectra in 3000- 800 cm-1 region of Skimmed milk samples

The result of the principle component analysis of all samples data are shown by linear plot and score scatter plot (Figure 26 & 30), which represent the samples on the first two principle components (PC1 and PC2).

PC1 explain 97% of the variance between samples and PC2 explain 2% of the remaining variance. Samples are spread over the entire plot and are clustered in to groups. Three kinds of groups can be indicated as: Permeates, retentates and milk. The scatter plot represents information about the protein source. Arrangement of the samples illustrate that the three groups were indeed statistically different, but the only sample that is divergent from the others is MFP (S.4). This is because of the filtration process, as MFP (S.4) is supposed to have more protein than the other permeate samples. This sample contains more protein than the others because it was treated in microfiltration to have higher concentration than the other permeate. The UFR sample from retentate group is also far from the other retentate because it has less casein and due to going, it went through UF with smaller membrane.

The scores scatter plot of PC1 against PC2 shows differentiation between each group of samples. The scores scatter plot PC1 (explained above 97 % of variability), PC2 (explained around 2% of variability), were used to obtain separation of each group (Figure 30). As it expected, milk, permeate and retentate samples were grouped separately. Retentate samples were clustered in the negative direction of PC1, while milk and permeate samples were in the positive direction of PC1.

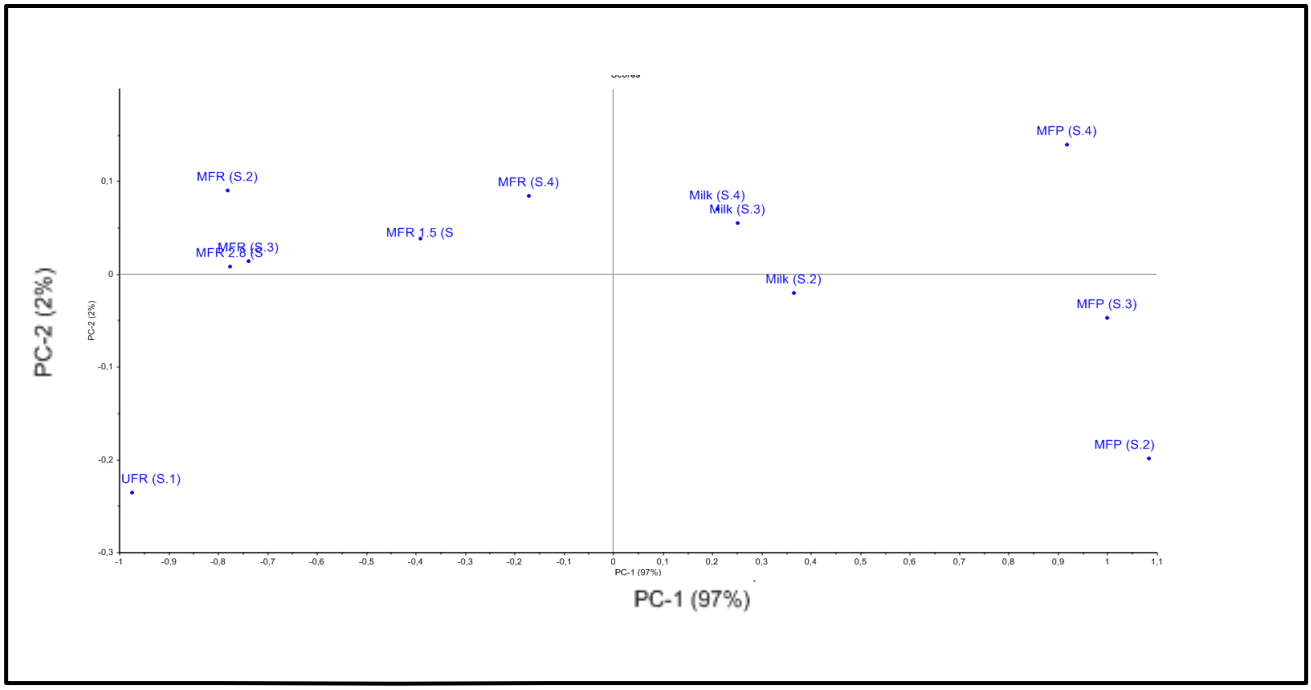


Figure 30. PCA scores scatter plots of all samples FT-IR spectra in the 3043-898 cm-1 region

As shown in Figure 30 the same samples are grouped in the same region. Combination of FT-IR and PCA analysis is a powerful method to obtain information and quick evaluation of the milk, permeate and retentate samples.

3.3. LC MS/MS

3.3.1. Optimization of sample preparation procedure

Mass spectrometry is a good technique to quantify and identify the protein in samples. Mass spectrometry with or without liquid chromatography, has been used for characterizing biological compounds. In this study, mass spectrometry had important use for monitoring the main proteins in whey, such as α-La, β-Lg and caseins (Cunsolo et al., 2011).
In the first set of experiments, molecular weight cut-off columns of 10 kDa and 30 kDa limits were used to separate the protein according to the molecular weight. The samples were added to the column after acetone precipitation and centrifugation (Appendix 8.4.2). The samples that had not been acetone precipitated were also tested. Afterwards proteins lower than 30 kDa were added to the 10 kDa columns and centrifuged again (Appendix 8.4.1). At the end, four different samples were generated (>30 kDa, >10, 30>sample>10 kDa and <10kDa). When the Bradford was done, the concentration of proteins less than 30 and 10 kDa was very low. Due to the low protein concentrations in the generated samples, analysis of samples generated through use of molecular weight cut-off filters was not done.

Initially in mass spectrometry analysis, the acetone precipitation was done to remove non-protein components from the samples. As described in FT-IR part, whey samples have a lot of fat and water. The protein concentration was measured by Bradford assay and was calculated according to each sample dilution. The BSA standard curve at 595 nm was found to be linear with a correlation coefficient of 0.995.

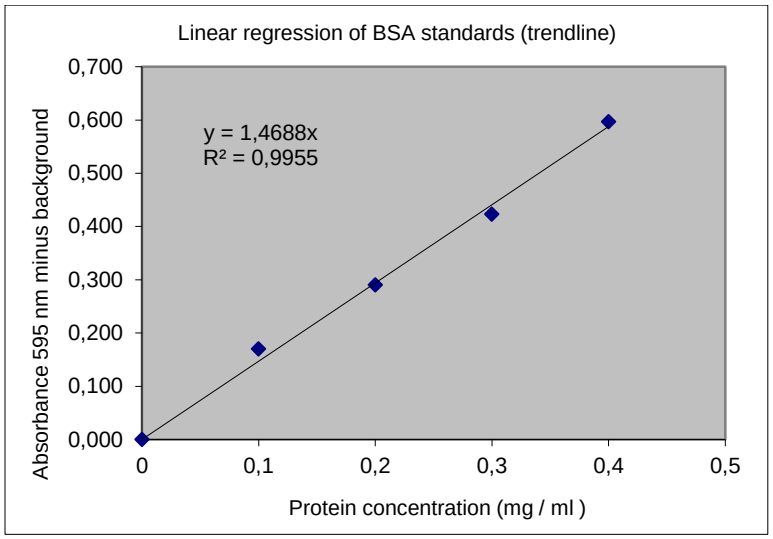


Figure 31. Standard curve

3.3.2. Characterization of prepared samples with LC-MS/MS

Protein concentration was measured with the Bradford method. Certain concentration methods significantly increased the true protein content. The typical protein concentration of each sample is shown in Table 4.

Table 4. Protein concentration

Sample Name	Protein Concentration (mg/ml protein)
MFR 1.5 (S.1)	38.34
MFR 2.8 (S.1)	37.81
UFR (S.1)	35.44
Milk (S.2)	21.88
MFP (S.2)	2.60
MFR (S.2)	31.94
Milk (S.3)	28.76
MFP (S.3)	3.10
MFR (S.3)	40.84
Milk (S.4)	23.47
MFP (S.4)	5.88
MFR (S.4)	35.01

Adding DTT for reducing and IAA for alkylation was done before trypsination. All samples were diluted to a volume corresponding to 10 µg protein. Trypsin digestions of approximately 1:25 dilution of each sample up to 15 hours was done and were carried out by cleaning the samples by spin C_{18} column for each digest sample. Then injecting 5 µl of each sample in to LTQ-Orbitrap (Thermo Fisher) to start analyzing the protein identification by LC-MS/MS. Peptide and mass spectrometry analysis was loaded in to proteome discover 1.4 software (Thermo scientific) to search against the Bos-Tourus database (Download from Uniprot 23.05.2014 containing 39125 sequences), trypsin was set as the digestion enzyme. The software settings were adjusted to account for the fact that the samples had been reduced and alkylated. The complete carbamidomethylation and dynamic oxidation of protein were confirmed by amino acid analysis. The main method used in the Bos-Tourus database search relies on reporting of the best scoring peptide-spectrum match (PSM) according to comparison of each observed spectrum to the theoretical spectra predicted from a genomic sequence.

Calibration of instrument was performed with following calibration solution (MSG15-IEA sigma).

Tables 5-7 show the PSMs, Amino acids, molecular weight and coverage of each sample according to mass spectrometry results. The rows specified by color are the important proteins in permeate, retentate and skimmed milk samples. It gives an overview to compare the samples.

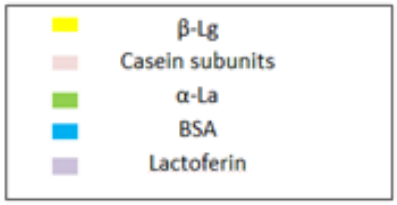


Table 5. Milk samples LC-MS/MS result

Number	Description	ΣCoverage	# AAs	MW [kDa]	Milk (S.2)	Milk (S.3)	Milk (S.4)
1	Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3	86,52	178	19,9	277	238	107
2	Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2	74,30	214	24,5	436	625	337
3	Major allergen beta-lactoglobulin OS=Bos taurus PE=2 SV=1	86,52	178	20,0	277	238	109
4	Alpha S1 casein OS=Bos taurus GN=CSN1S1 PE=2 SV=1	69,16	214	24,4	433	605	338
5	Alpha-lactalbumin OS=Bos taurus GN=LALBA PE=1 SV=2	83,10	142	16,2	27	24	33
6	Alpha lactalbumin (Fragment) OS=Bos taurus GN=alfaLA PE=3 SV=1	65,85	123	14,1	27	24	33
7	Alpha-S2-casein OS=Bos taurus GN=CSN1S2 PE=1 SV=2	54,05	222	26,0	166	190	51
8	Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2	21,43	224	25,1	178	209	88
9	Beta-lactoglobulin (Fragment) OS=Bos taurus GN=LGB PE=4 SV=1	86,11	36	4,2	91	58	14
10	Kappa-casein (Fragment) OS=Bos taurus x Bos indicus GN=CSN3 PE=4 SV=1	61,88	160	17,9	172	131	47
11	Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4	69,52	607	69,2	12		6
12	Kappa casein (Fragment) OS=Bos taurus GN=csn3 PE=4 SV=1	61,87	139	15,2	72	74	24
13	Glycosylation-dependent cell adhesion molecule 1 OS=Bos taurus GN=GLYCAM1 PE=1 SV=2	51,63	153	17,1	21	6	8
14	Enterotoxin-binding glycoprotein PP20K (Fragment) OS=Bos taurus PE=1 SV=1	100,00	20	2,3	52	26	
15	PIGR protein OS=Bos taurus GN=PIGR PE=2 SV=1	39,23	757	82,5	2		
16	Uncharacterized protein OS=Bos taurus GN=PIGR PE=4 SV=1	31,84	757	82,5	2		
17	Lactoperoxidase OS=Bos taurus GN=LPO PE=1 SV=1	43,82	712	80,6			
18	Complement C3 OS=Bos taurus GN=C3 PE=1 SV=2	21,25	1661	187,1			
19	Lactoferrin (Fragment) OS=Bos taurus PE=2 SV=1	27,25	690	76,2			

Table 6. Permeate samples LC-MS/MS result

Number	Description	ΣCoverage	# AAs	MW [kDa]	MFP (S.2)	MFP (S.3)	MFP (S.4)
1	Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3	86,52	178	19,9	434	311	250
2	Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2	74,30	214	24,5	35	29	18
3	Major allergen beta-lactoglobulin OS=Bos taurus PE=2 SV=1	86,52	178	20,0	411	296	250
4	Alpha S1 casein OS=Bos taurus GN=CSN1S1 PE=2 SV=1	69,16	214	24,4	35	29	18
5	Alpha-lactalbumin OS=Bos taurus GN=LALBA PE=1 SV=2	83,10	142	16,2	187	149	26
6	Alpha lactalbumin (Fragment) OS=Bos taurus GN=alfaLA PE=3 SV=1	65,85	123	14,1	179	148	26
7	Alpha-S2-casein OS=Bos taurus GN=CSN1S2 PE=1 SV=2	54,05	222	26,0	13	17	2
8	Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2	21,43	224	25,1	4	7	3
9	Beta-lactoglobulin (Fragment) OS=Bos taurus GN=LGB PE=4 SV=1	86,11	36	4,2	151	107	43
10	Kappa-casein (Fragment) OS=Bos taurus x Bos indicus GN=CSN3 PE=4 SV=1	61,88	160	17,9	6	13	8
11	Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4	69,52	607	69,2	87	47	14
12	Kappa casein (Fragment) OS=Bos taurus GN=csn3 PE=4 SV=1	61,87	139	15,2	4	6	5
13	Glycosylation-dependent cell adhesion molecule 1 OS=Bos taurus GN=GLYCAM1 PE=1 SV=2	51,63	153	17,1	44	25	7
14	Enterotoxin-binding glycoprotein PP20K (Fragment) OS=Bos taurus PE=1 SV=1	100,00	20	2,3	27	19	14
15	PIGR protein OS=Bos taurus GN=PIGR PE=2 SV=1	39,23	757	82,5	17	9	2
16	Uncharacterized protein OS=Bos taurus GN=PIGR PE=4 SV=1	31,84	757	82,5	12	6	2
17	Lactoperoxidase OS=Bos taurus GN=LPO PE=1 SV=1	43,82	712	80,6	23	12	
18	Complement C3 OS=Bos taurus GN=C3 PE=1 SV=2	21,25	1661	187,1	2	1	
19	Lactoferrin (Fragment) OS=Bos taurus PE=2 SV=1	27,25	690	76,2			

Table 7. Retentate samples LC-MS/MS result

Number	Description	ΣCoverage	# AAs	MW [kDa]	MFR 1.5 (S.1)	MFR (S.2)	MFR 2.8 (S.1)	MFR (S.4)	MFR (S.3)	UFR (S.1)
1	Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3	86,52	178	19,9	101	73	72	81	65	292
2	Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2	74,30	214	24,5	176	242	222	357	232	16
3	Major allergen beta-lactoglobulin OS=Bos taurus PE=2 SV=1	86,52	178	20,0	85	70	70	81	63	283
4	Alpha S1 casein OS=Bos taurus GN=CSN1S1 PE=2 SV=1	69,16	214	24,4	172	245	219	354	229	16
5	Alpha-lactalbumin OS=Bos taurus GN=LALBA PE=1 SV=2	83,10	142	16,2	47	30	40	15	57	102
6	Alpha lactalbumin (Fragment) OS=Bos taurus GN=alfaLA PE=3 SV=1	65,85	123	14,1	43	30	36	15	57	96
7	Alpha-S2-casein OS=Bos taurus GN=CSN1S2 PE=1 SV=2	54,05	222	26,0	60	47	91	83	116	10
8	Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2	21,43	224	25,1	43	56	48	68	49	11
9	Beta-lactoglobulin (Fragment) OS=Bos taurus GN=LGB PE=4 SV=1	86,11	36	4,2	21	6	9	24	17	68
10	Kappa-casein (Fragment) OS=Bos taurus x Bos indicus GN=CSN3 PE=4 SV=1	61,88	160	17,9	29	42	34	80	35	4
11	Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4	69,52	607	69,2	38	1	8	6	12	68
12	Kappa casein (Fragment) OS=Bos taurus GN=csn3 PE=4 SV=1	61,87	139	15,2	45	17	24	35	37	
13	Glycosylation-dependent cell adhesion molecule 1 OS=Bos taurus GN=GLYCAM1 PE=1 SV=2	51,63	153	17,1	13	6	12	8	16	25
14	Enterotoxin-binding glycoprotein PP20K (Fragment) OS=Bos taurus PE=1 SV=1	100,00	20	2,3					6	13
15	PIGR protein OS=Bos taurus GN=PIGR PE=2 SV=1	39,23	757	82,5	25		2	4	4	40
16	Uncharacterized protein OS=Bos taurus GN=PIGR PE=4 SV=1	31,84	757	82,5	16		2	4	4	27
17	Lactoperoxidase OS=Bos taurus GN=LPO PE=1 SV=1	43,82	712	80,6	25					40
18	Complement C3 OS=Bos taurus GN=C3 PE=1 SV=2	21,25	1661	187,1	14					67
19	Lactoferrin (Fragment) OS=Bos taurus PE=2 SV=1	27,25	690	76,2	12		4	1	1	20

The result from mass spectrometry represents the high sensitivity construction and analyses of complex matrices (whey) (Li et al., 2009)

Mass spectrometry gave the most information concerning protein identity among the methods that were tested in this project. Samples were run several times (Appendix 8.6) and Tables 5-7 shows the best and acceptable results from mass spectrometry data analysis. Samples were run a number of times, variability of results may be due to challenges with temperature of the instrument.

In mass spectrometry analysis, a lot of proteins were identified in each sample, however in these results only important proteins with high peptide coverage are shown (Appendix 8.6).

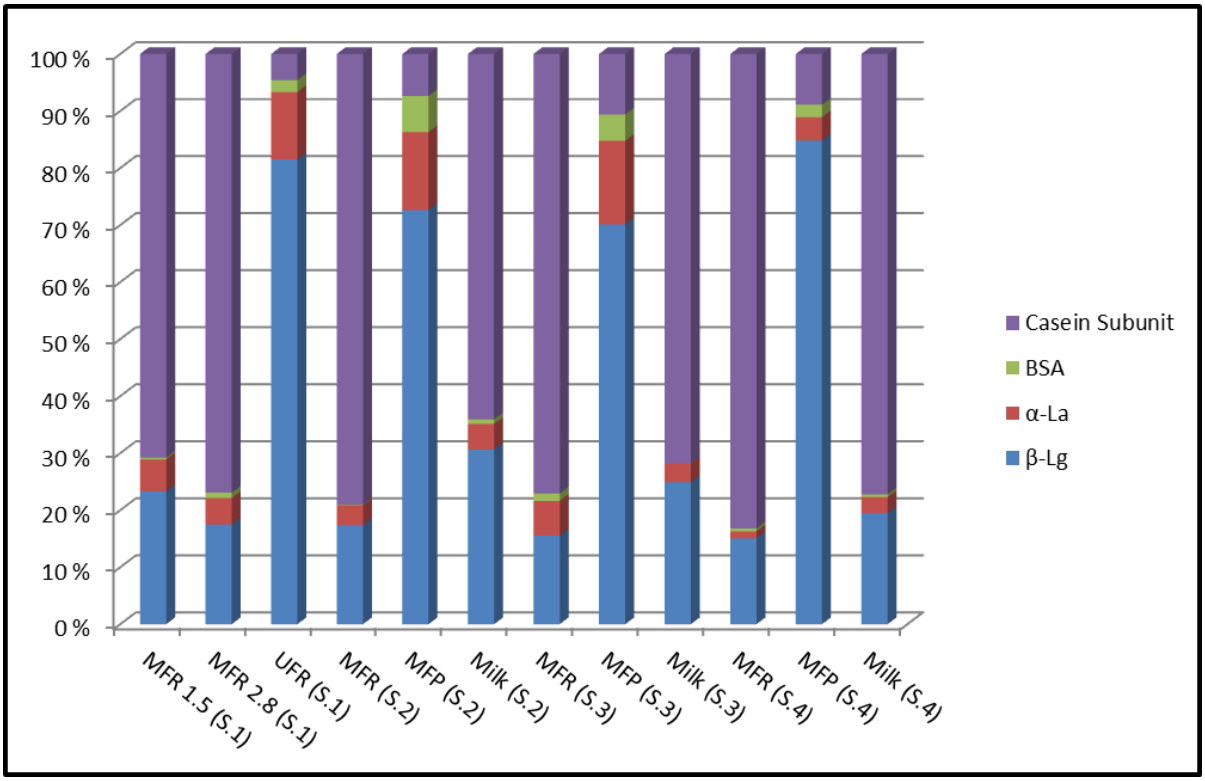


Figure 32. Stacked column 3D chart of comparing different protein in all samples by using LC-MS/MS results

Figure 32 shows the comparison between four different proteins according to the LC-MS/MS results. As mentioned previously, casein subunits exist high amounts in whey products so this graph shows the different amount of casein in each sample and aids in visualization.

LC-MS/MS method is used for detecting the peptides, so it can be concluded that casein subunit peptides had high peptide coverage and were detected more with Orbitrap compared to other proteins.

3.4. Cross comparison

FT-IR is a technique that can rapidly show a sample's molecular fingerprint. Recently it is becoming an interesting tool for biochemical research(Ami, Mereghetti, & Doglia, 2013). The data from FT-IR can be analyzed by different software. In this project, Unscrambler X software was used to evaluate the data.

The accurate measurement of qualitative and quantitative FT-IR Spectra in complex mixtures is very difficult. For this reason, a combination of multivariate analysis with FT-IR is the best way to analyze the FT-IR data (Ivanova, Tsalev, & Arnaudov, 2006).

The calibration method (PLS modeling) was done for comparison of FT-IR with the other two methods. PLS modeling was done by updating the program based on X and Y weights to $\frac{A}{SDev+B}$ $(A = 1), (B = 0)$. The classification model was validated by full cross- flow validation obtaining the statistical results shown in Figures 33-38. The graphs were chosen according to the explained variance to get the best factor and correlation for PLS modeling plots.

Figure 33 represents the calibration data between FT-IR spectra and mass spectrometry according to β-Lg. The X axis is the FT-IR sample wavelengths and the Y axis is the PSMs from summation of all β-Lg fragments for each sample (according to Tables 5-7). As shown in Figure 33 samples have a good correlation coefficient of 98% for a particular protein. Similar samples are in the same region and it is clear that MFP (S. 4) is near to milk samples when compared to permeate samples due to more proteins.

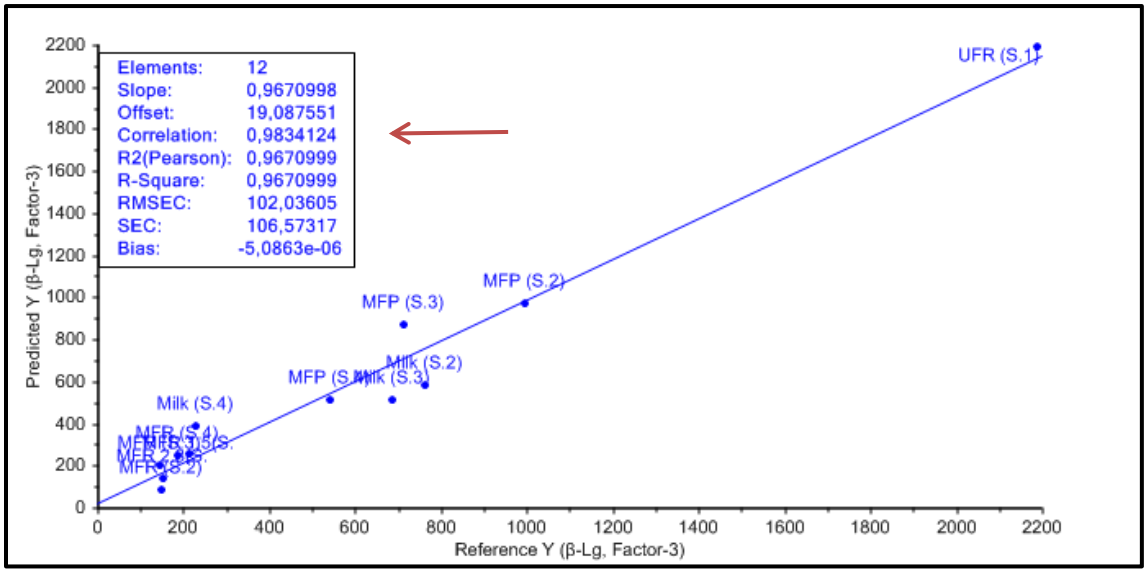


Figure 33. PLS modeling, FT-IR Vs Mass spectrometry data according to β-Lg

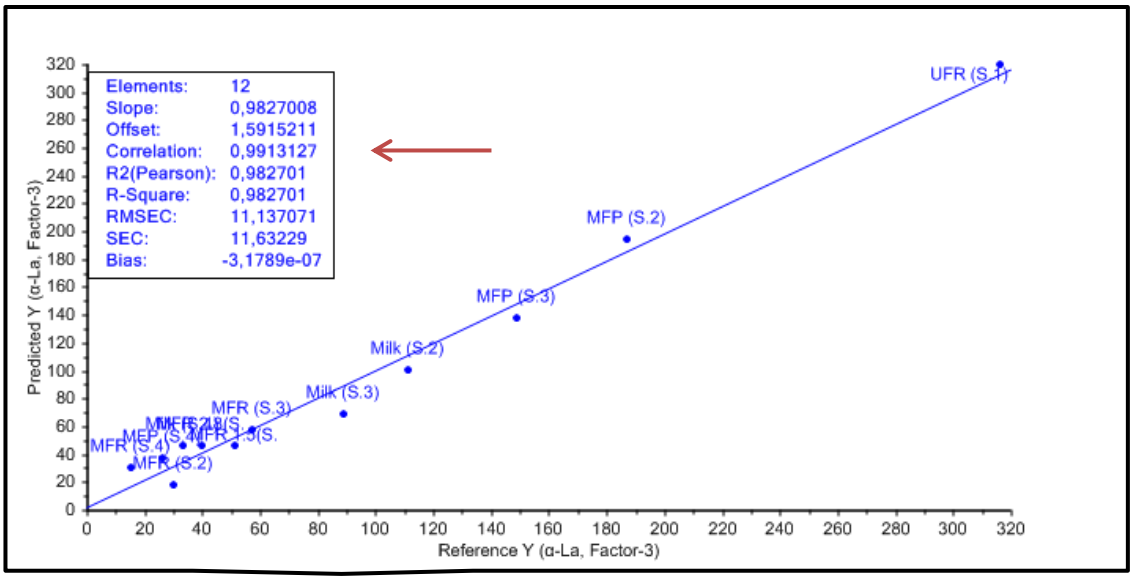


Figure 34. PLS modeling, FT-IR Vs Mass spectrometry data according to α-La

Figure 34 represents the calibration of FT-IR spectra with mass spectrometry PSMs according to α-La. Samples have good correlation to each other in this graph. Figure 35 has the same data but according to Casein subunits. In these graphs, the Y variable is the summation of all fragment of α-La and all casein structures. In Figure 35 the samples have a correlation coefficient of 88%, the same samples group in the same region.

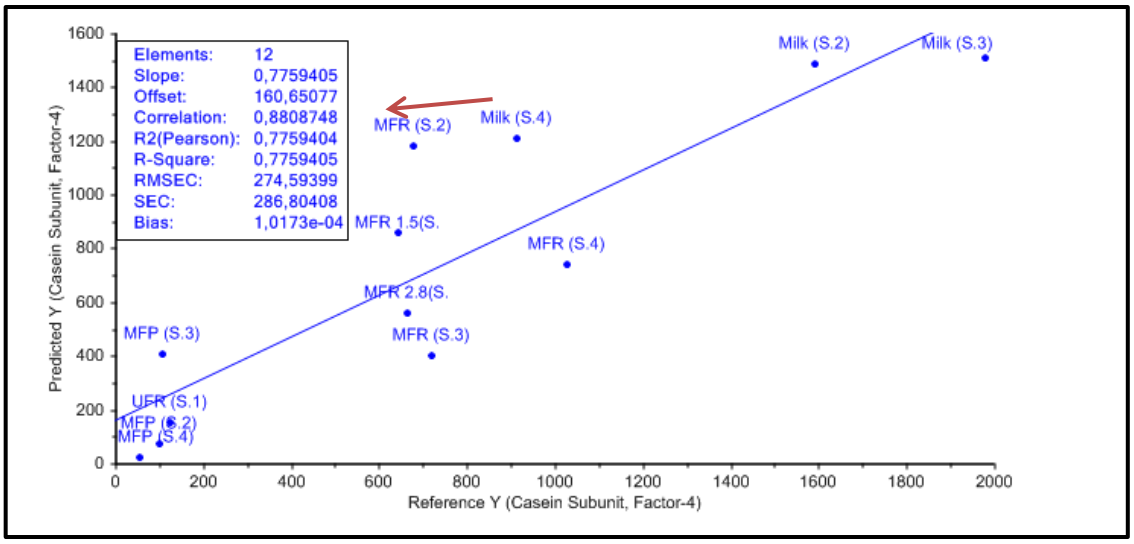


Figure 35. PLS modeling, FT-IR Vs Mass spectrometry data according to Casein subunits

Figure 36 shows the calibration data between FT-IR spectra and SDS-PAGE data according to β-Lg. The SDS-PAGE data was chosen from Image J software in the same way as in Figure 24. Samples have 91% correlation in this graph, except that UFR is far from the other samples. In figure 36, it shows that UFR has an effect on calibration graph. All the samples are near to each other except UFR. The reason can be the high level of β-Lg in UFR compare to other samples. If gel scan result considered, it is clear that UFR had much higher level of β-Lg even more than milk samples.

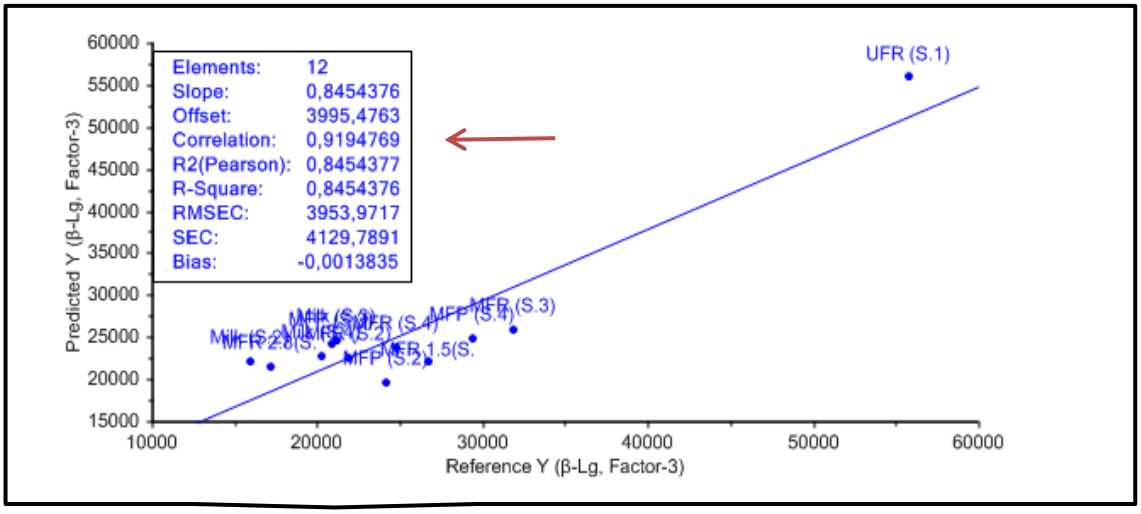


Figure 36.PLS modeling, FT-IR Vs SDS-PAGE data according to β-Lg

Figures 37 and 38 illustrate the FT-IR spectra versus SDS-PAGE according to α-La and casein subunits. Both plots have good correlation. All samples from same group are in same region.

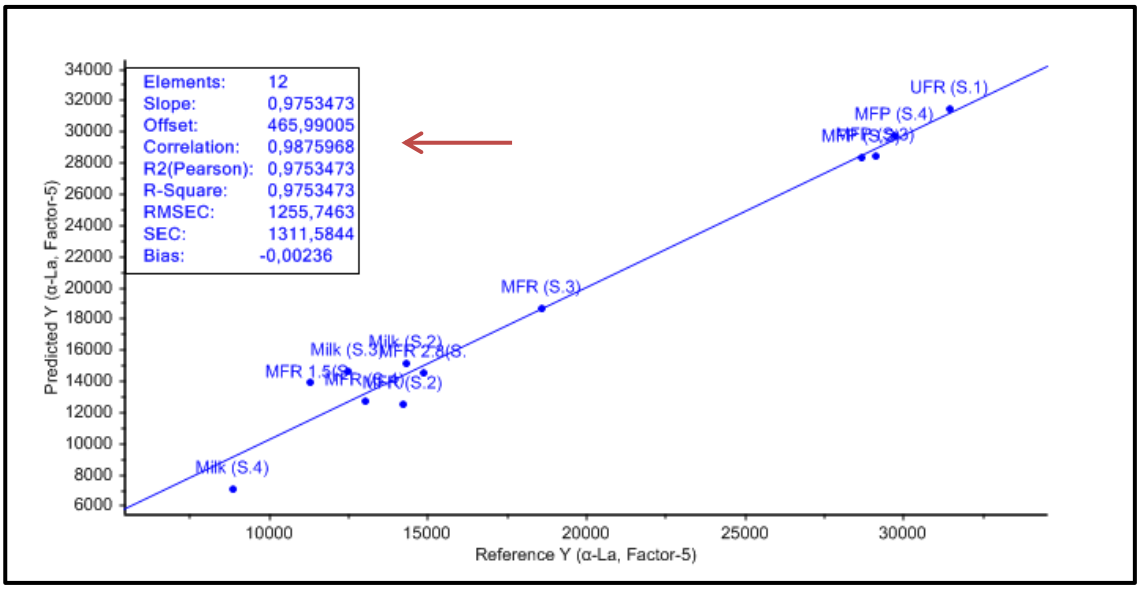


Figure 37. PLS mothod according to FT-IR and SDS-PAGE results according to α-La

As represented in Figure 38, MFP (S.4) is closer than the other permeate samples to UFR. This can be caused by the filtration system due to concentrating the MFP (S.4).

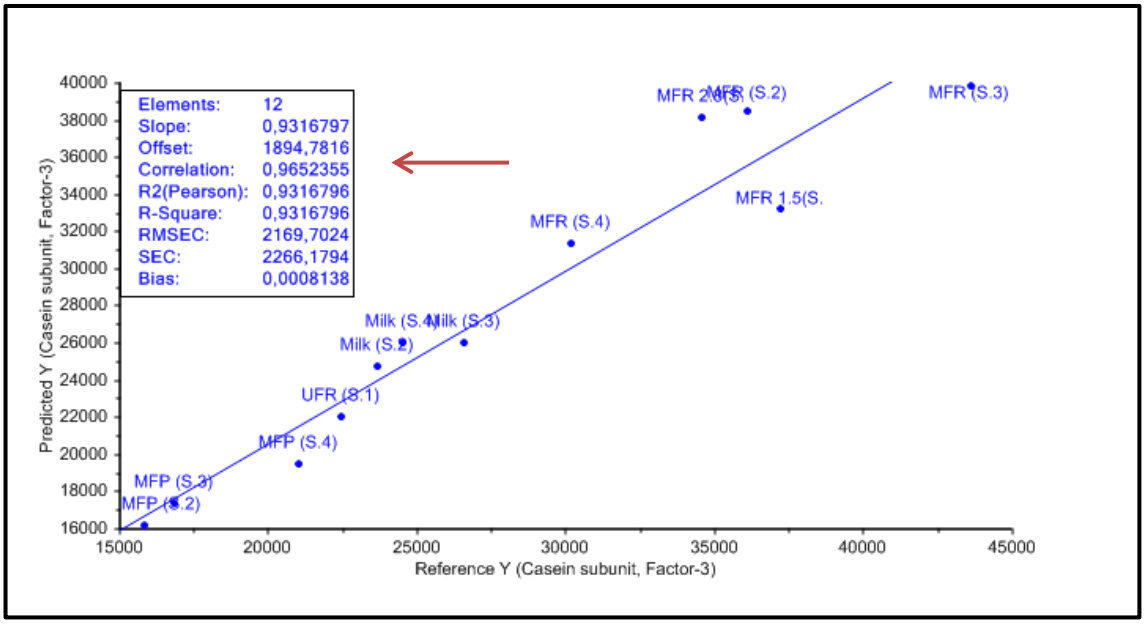


Figure 38. PLS mothod according to FT-IR and SDS-PAGE results according to Casein subunits

Figures 39-44 show the regression coefficient of data as it shown in previous figures. The peaks in these plots are the important part in the samples that contain proteins. The number of principle components were chosen from explained variance plot and PLS modeling by using Unscramler X software and Excel. The principle component percentages show which spectra explained better information about the samples.

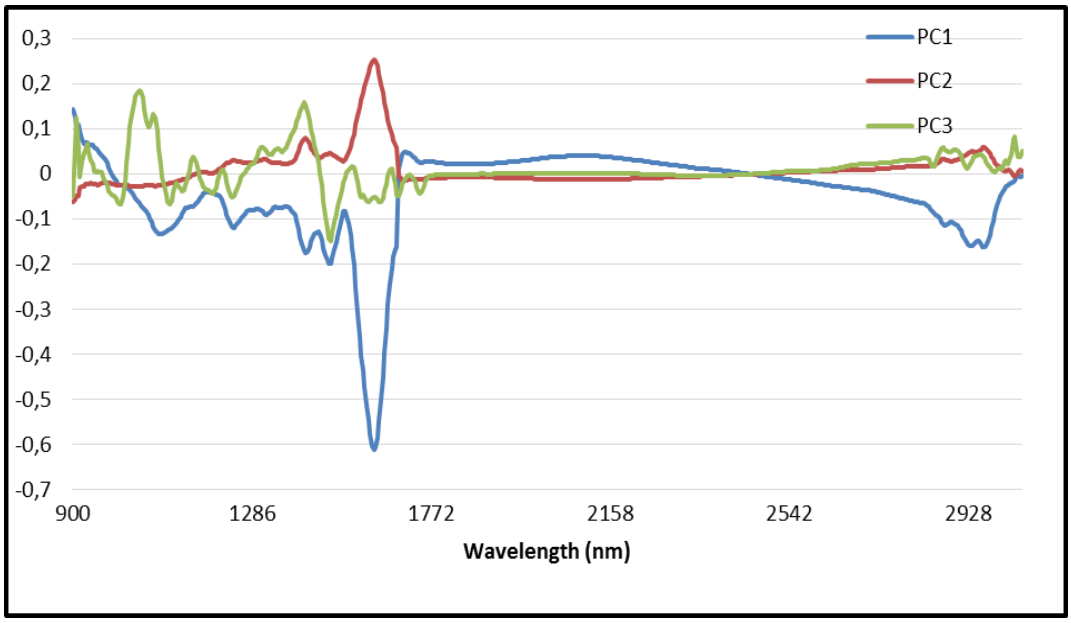


Figure 39. Regression coefficient plot according to the wavelength and Factors regarding the FT-IR and Mass spectrometry data based on β-Lg, variance explained: PC1= 15%, PC2=59% and PC3=23%

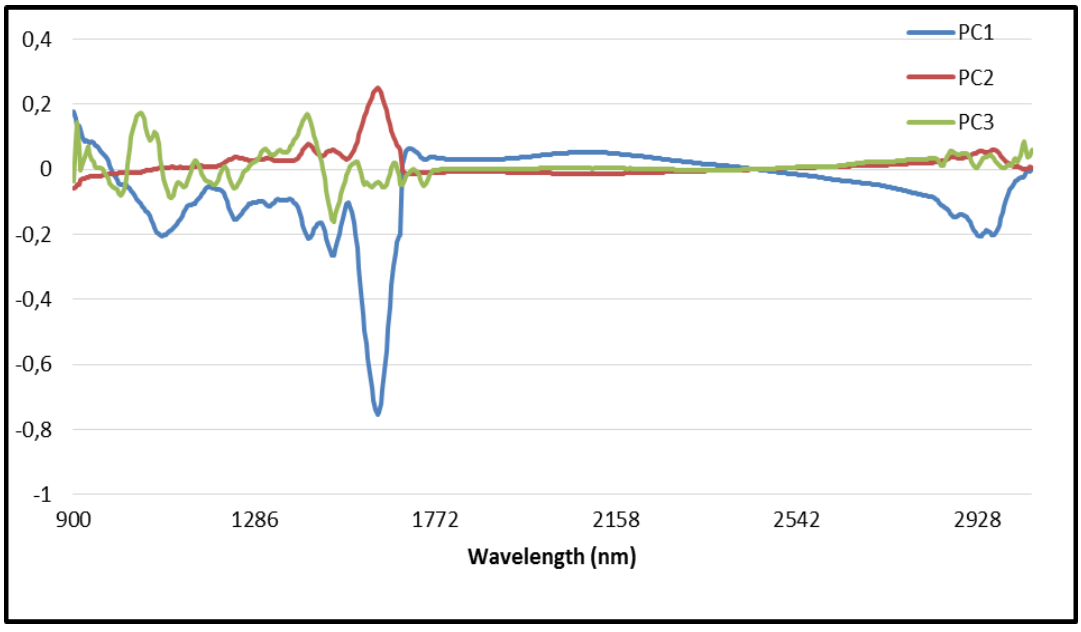


Figure 40. Regression coefficient plot according to the wavelength and Factors regarding the FT-IR and Mass spectrometry data based on α-La, variance explained: PC1= 39%, PC2=51% and PC3=9%

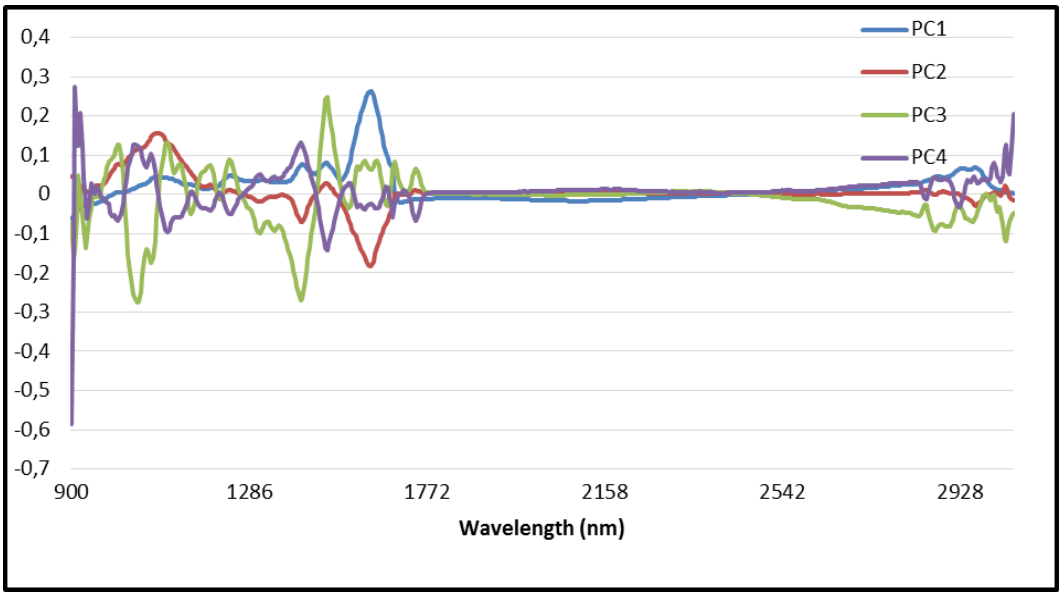


Figure 41. Regression coefficient plot according to the wavelength and Factors regarding the FT-IR and Mass spectrometry data based on Casein subunits, variance explained: PC1= 2%, PC2=15%, PC3=13% and PC4=48%

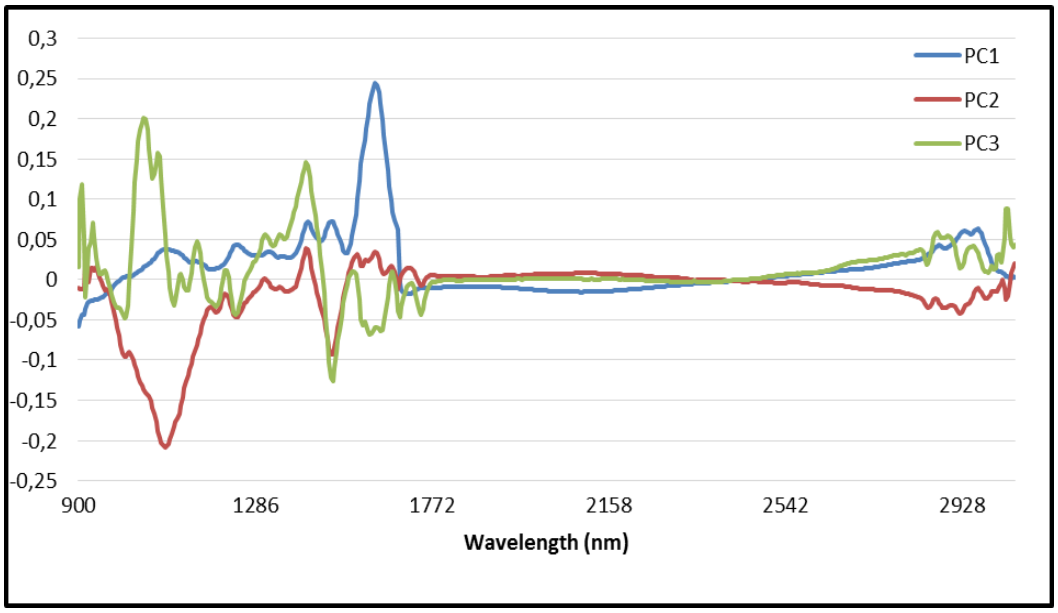


Figure 42. Regression coefficient plot according to the wavelength and Factors regarding the FT-IR and SDS-PAGE data based on β-Lg, variance explained: PC1= 17%, PC2=37% and PC3=31%

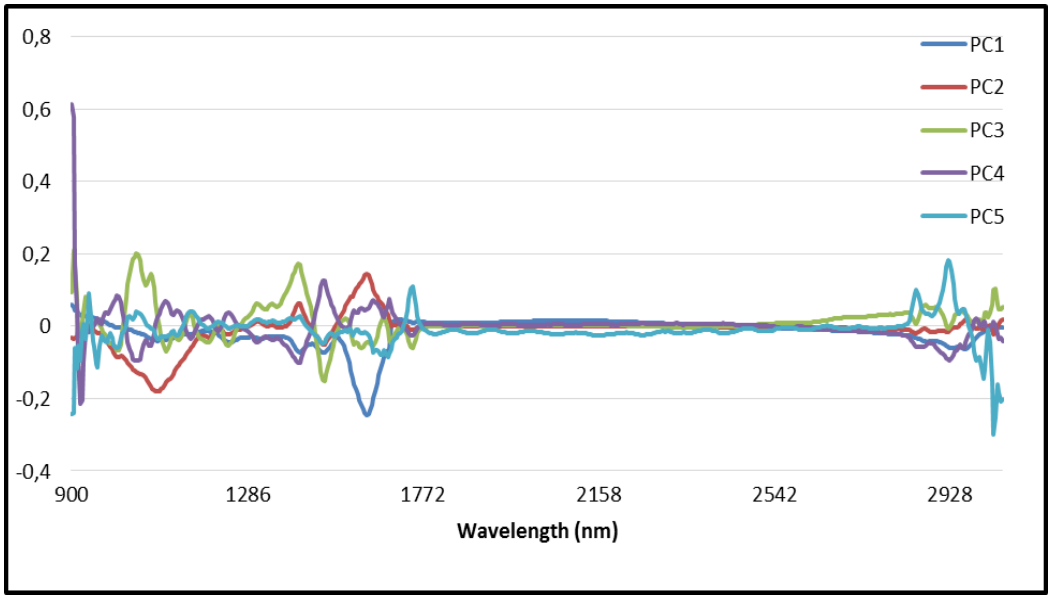


Figure 43. Regression coefficient plot according to the wavelength and Factors regarding the FT-IR and SDS-PAGE data based on α-La, variance explained: PC1= 14%, PC2=37% and PC3=19%, PC4=10%, and PC5=16%

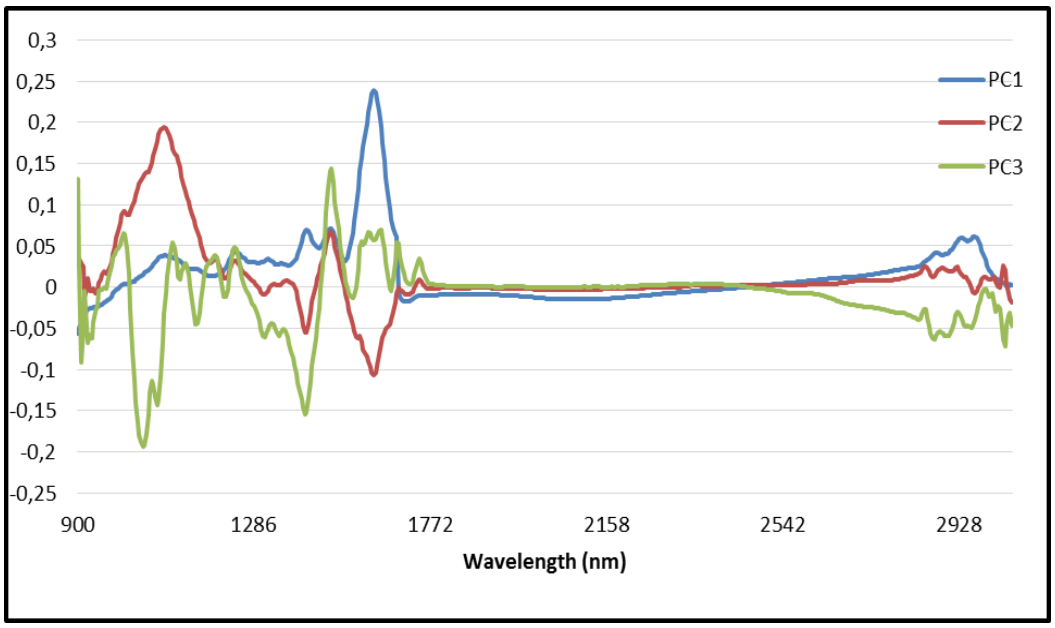


Figure 44. Regression coefficient plot according to the wavelength and Factors regarding the FT-IR and SDS-PAGE data based on Casein subunits variance explained: PC1= 60%, PC2=21% and PC3=12%

The PCA modelling shows the prediction of the samples and groups them but PLS modelling shows the correlation between each sample. Total multivariate analysis is useful for comparison between different methods.

3.5. Summary of results

Retention of casein in skimmed milk microfiltration (MF) was very high as expected. A higher ratio of α-LA/β-LG was observed in MF permeate compared to skimmed milk. The amount of α-LA/β-LG was very high in ultrafiltration (UF) compared to microfiltration (MF) retentate. It can be said that α-LA and β-LG are indirectly proportional to Casein subunits. Glycomacropeptides are the proteins with small molecular weight (6-10 kDa) which can be seen in the gel electrophoresis, but not identified in mass spectrometry. The LC-MS/MS analysis protocol involves many steps, therefore it cannot be excluded that some proteins are lost during sample preparation and analysis. Studies to investigate the quantitative recovery during sample preparation have not been conducted within this project. The mass spectrometry is a long method and there were different steps for protein precipitation and clean-up of the samples. The LTQ-Orbitrap in LC-MS/MS system is a very sensitive instrument, so sometimes in each run of samples there are different results. A lot of reasons can cause the differentiation in results. LC-MS/MS provides the most information about the samples, and although mass spectrometry with LTQ-Orbitrap is an expensive instrument, it has become a common instrument for protein separation in biological studies (Janini & Veenstra, 2002). Generally mass spectrometry has more sensitivity and a higher dynamic range (O'Donnell, Holland, Deeth, & Alewood, 2004).

For a number of the proteins there was a good regression between the results seen in SDS gel electrophoresis and for the LC-MS/MS analysis, for example the amount of casein subunits in UFR. Rattary (Rattray & Jelen, 1996) mentioned that UF retentate will be unchanged for casein ratio that in disagreement with my experiment where it is shown that the casein ratio is decreased in UFR. On the other hand, Rattray & Jelen (1996) conclude that liquid whey (such as permeate and retentate) will increase both total protein and the casein ratio in agreement with my results.

4. Conclusion

The main objective of this project was to study the results from three different methods in order to develop and compare methodology for determining and separating the protein in skimmed milk, permeate and retentate. The main techniques for determining the protein sizing, quantitation, molecular weight, were gel electrophoresis, spectroscopy and mass spectrometry and these were compared. FT-IR method was calibrated with the other two methods to compare by PLS modeling.

Skimmed milk, retentate and permeate samples were used in this project. More focus was on retentate and permeate samples, however skimmed milk was used for comparison. Skimmed milk, permeate and retentate protein properties were highly influenced by the type of filtration.

Obtaining the clear results in SDS-PAGE is dependent on appropriate sample preparation. The SDS-PAGE has clearly shown that MF increases the detected levels of major protein in permeate and retentate. This study suggested that the combination of traditional and modern methods for protein determination such as SDS-PAGE and LC-MS/MS makes the laboratory more efficient and productive.

Most of the SDS-PAGE observations were in agreement with LC-MS/MS. According to the (Tovar Jiménez et al., 2012) the proteins that were identified in their experiment (cheese whey) are similar to the skimmed milk whey in this project.

LC-MS/MS technique shows the high sensitivity, accuracy and characteristics of protein determination. Although the sample preparation and running the system involves a long procedure, this method helps to obtain more detailed analysis in complex mixtures such as whey. In this study, it was found that the most efficient protein precipitation was with acetone precipitation and the most efficient amount of trypsin was 1:25 (trypsin: protein).

This study showed the differences between permeate, retentate and skimmed milk proteins with different kinds of filtration and with FT-IR spectra in 1,800-1,200 cm^{-1} regions. Unscrambler X software helped to analyze the results with the PCA and PLS methods. The differences between samples were more apparent when the PCA was performed on the FT-IR region 1,800-1,200 cm^{-1}. Significant changes in peaks were observed for UF retentate. PLS modeling was used to calibrate the FT-IR method with the two other methods. FT-IR wavelength was an X variable in both calibration, and PSMs from mass spectrometry and SDS gel bands as Y variables were used to have a better comparison between samples.

In conclusion, all three methods have advantages and disadvantages. The FT-IR and principle component analysis show the existence of the proteins. The SDS-PAGE is a traditional method that is still useful and can be comparable to mass spectrometry. It clearly

shows the concentrations of different types of proteins. Mass spectrometry identifies proteins, peptides and amino acids and although it does require a long procedure to get the results, it has higher accuracy compared to the other two methods in this project. High cost is one of the disadvantages of mass spectrometry compare to FT-IR and gel electrophoresis.

Table 8. Compare the methods

Methodology	Application	Advantages	Disadvantages
SDS-PAGE	Separating protein by electrophoresis	1. Cheap method 2. Good predicting method for identify the protein 3. Result will be ready fast 4. Reliable and simple method	1. No confirmed identification 2. Takes time to get the good sample concentration 3. Polyacrylamide in SDS gel is hazardous 4. making small mistake in making buffers will destroy all experiment
FT-IR spectroscopy	Obtain an infrared spectrum of absorption	1.Fast procedure 2. Simple instrument maintenance 3. Result will be ready fast 4. possible to use the results in different software	1. Not identifying the specific proteins 2. Detect all minerals that makes noise in spectra 4. Not quantitative analysis
Mass spectrometry (LC-MS/MS)	Identify the amount and type of compounds present in a samples specially proteins	1.Accurate results 2. detailed information 3. High sensitive detector permits sensitive measurement 4. reliability and quantity results 5. requires small sample size 6. Versatile	1. Expensive instrument 2. Long procedure 3. Running the procedure and getting the result takes some days 4. Difficult instrument to operate and maintain

5. Future Work

Protein determination has an infinite world to investigate.

Suggestion of future works:

1. Bioactive protein monitoring by using LC-MS/MS to explore the proteins and peptides more. Identifying the bioactive peptide in whey to understand enzymatic digestion of whey proteins.
2. Mix the SDS-PAGE or 2D gel electrophoresis method with LC-MS/MS method to analyze the gel spot with Orbitrap to analyze each band in the gel and also analyze the complex proteins with low abundance or high molecular weight.
3. Whey protein analysis by using bioinformatics and proteomics methods that will help to advance the understanding of whey. Determine the impact of the peptide fraction on whey. Whey could have more products with more properties.
4. Ultrafiltration can be used to concentrate the whey protein for bio-peptide production.
5. Measuring the protein concentration before running the gel to get an accurate amount of proteins in the gel for more quantitative analysis.
6. Sample preparation methods for removing high abundant proteins to increase the number of proteins identified in LC-MS/MS analysis. Finding the way to remove the casein from high concentrated samples to see the other proteins more clearly

6. Symbols and Abbreviation

ACN	Acetonitrile
BSA	Bovine Serum Albumin
CF	Concentration Factor
CMP	Casein Macro Peptide
CN	Casein
DTT	Dithiothreitol
FT-IR	Fourier Transform Infrared spectroscopy
GMP	Glycomacropeptide
HPLC	High Performance Liquid Chromatography
IAA	Iodoacetamide
Ig	Immunoglobulin
kDa	KiloDalton
LC-MS	Liquid Chromatography Mass Spectrometry
LF	Lactoferin
MF	Microfiltration
MFP	Microfiltration Permeate
MFR	Microfiltration Retentate
PC	Principal Component
PCA	Principal Component Analysis

PSM	Peptide Spectrum Match
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TFA	Trifluoroacetic Acid
UF	Ultrafiltration
UFR	Ultrafiltration Retentate
WP	Whey Product
WPI	Whey Product Isolated
WPC	Whey Product Concentration
WPH	Whey Product hydrolysate
α-La	Alpha Lactalbumin

7. References:

1. Agilent Technologies. (2001). Basics of LC/MS.
2. Ami, D., Mereghetti, P., & Doglia, S. M. (2013). *Multivariate analysis for Fourier transform infrared spectra of complex biological systems and processes*: INTECH Open Access Publisher.
3. Boland, M., Singh, H., & Thompson, A. (2014). *Milk proteins: from expression to food*: Academic Press.
4. Brink, W. (2005). Fighting Cancer With Whey. from http://www.brinkzone.com/articles/fighting-cancer-with-whey/
5. Brunn, S. (2006). *Protein–Lipid–Carbohydrate Interactions and Importance to Food Quality. Spectroscopic Detection of Macromolecular Interactions. Biochemistry and Nutrition Group (BNG).* thesis. Technical University of Denmark.
6. Bylund, G., & Pak, T. (2003). *Dairy processing handbook*: Tetra Pak Processing Systems AB Lund.
7. Campbell, R. E., Adams, M. C., Drake, M., & Barbano, D. M. (2013). Effect of bleaching permeate from microfiltered skim milk on 80% serum protein concentrate. *Journal of dairy science, 96*(3), 1387-1400.
8. Carpenter Ea, H. D. R. H. S. M. M. C. A. (2010). *Natural variation in levels of Immunoglobulin A in bovine milk* (Vol. 70): New Zealand Society of Animal Production.
9. Chen, G., & Pramanik, B. N. (2008). LC-MS for protein characterization: current capabilities and future trends.
10. Cherry, J. P., & Barford, R. A. (1988). *Methods for protein analysis*: The American Oil Chemists Society.
11. Chiu, C., & Muddiman, D. (2008). What is Mass Spectrometry?, American Society for Mass Spectrometry.
12. Clare, D., & Swaisgood, H. (2000). Bioactive milk peptides: a prospectus. *Journal of dairy science, 83*(6), 1187-1195.
13. Cooks, R., Chen, G., Wong, P., & Wollnik, H. (1997). Mass spectrometers. *Encyclopedia of Applied Physics, 19*, 289-330.
14. Cunsolo, V., Muccilli, V., Saletti, R., & Foti, S. (2011). Applications of mass spectrometry techniques in the investigation of milk proteome. *Eur. J. Mass Spectrom, 17*, 305-320.
15. Dalgalarrondo, M., Dufour, E., Bertrand-Harb, C., Chobert, J., & Haertlé, T. (1992). Purification and characterization of two porcine β-lactoglobulin variants by NaCl salting-out and reversed phase-HPLC. *Le Lait, 72*(1), 35-42.
16. Daufin, G., Escudier, J.-P., Carrere, H., Berot, S., Fillaudeau, L., & Decloux, M. (2001). Recent and emerging applications of membrane processes in the food and dairy industry. *Food and Bioproducts Processing, 79*(2), 89-102.

17. Davey, J., & Lord, J. M. (2003). *Essential Cell Biology: Cell function* (Vol. 2): Oxford University Press.
18. De Wit, J., Hontelez-Backx, E., & Adamse, M. (1988). Evaluation of functional properties of whey protein concentrates and whey protein isolates, 3: functional properties in aqueous solution. *Netherlands Milk and Dairy Journal (Netherlands)*.
19. Dissanayake, M. (2011). *Modulation of functional properties of whey proteins by microparticulation.* Victoria University.
20. Domon, B., & Aebersold, R. (2006). Mass spectrometry and protein analysis. *science*, *312*(5771), 212-217.
21. Esbensen, K. (2002). Multivariate data analysis: an introduction to multivariate data analysis and experimental design: Alborg University, CAMO.
22. Esbensen, K. H., Guyot, D., Westad, F., & Houmoller, L. P. (2002). *Multivariate data analysis: in practice: an introduction to multivariate data analysis and experimental design*: Multivariate Data Analysis.
23. FitzGerald, R. J., & Meisel, H. (2000). Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *British Journal of Nutrition*, *84*(S1), 33-37.
24. Flinn, J. E. (1970). Membrane science and technology. Industrial, biological, and waste treatment processes. *Membrane science and technology. Industrial, biological, and waste treatment processes.*
25. Fox, P. (2001). Milk proteins as food ingredients. *International Journal of Dairy Technology*, *54*(2), 41-55.
26. Fox, P. F., & McSweeney, P. L. (1998). *Dairy chemistry and biochemistry*: Springer Science & Business Media.
27. Froning, G., Wehling, R., Ball, H., & Hill, R. (1987). Effect of ultrafiltration and reverse osmosis on the composition and functional properties of egg white. *Poultry Science*, *66*(7), 1168-1173.
28. Gallagher, W. (2009). FTIR analysis of protein structure. *Course manual Chem*, *455*.
29. Garfin, D. E. (2003). Gel electrophoresis of proteins. *Essential cell biology*, *1*, 197-268.
30. GEA Filtration. (2015). Reverse Osmosis, Nano, Ultra & Micro Filtration. from http://www.geafiltration.com/technology/membrane_filtration_process.asp
31. Goetz, H., Kuschel, M., Wulff, T., Sauber, C., Miller, C., Fisher, S., & Woodward, C. (2004). Comparison of selected analytical techniques for protein sizing, quantitation and molecular weight determination. *Journal of biochemical and biophysical methods*, *60*(3), 281-293.
32. Griffiths, P. R., & De Haseth, J. A. (2007). *Fourier transform infrared spectrometry* (Vol. 171): John Wiley & Sons.
33. Haug, A., Hostmark, A. T., & Harstad, O. M. (2007). Bovine milk in human nutrition–a review. *Lipids Health Dis*, *6*(25), 1-16.

34. Hazer, B., Boddeker, K., Fane, A., Koros, W., Lonsdale, H., Ma, Y. H., . . . Strathmann, H. (1996). Terminology for membranes and membrane processes.
35. Heino, A. (2010). Microfiltration in cheese and whey processing.
36. Hurley, W. L., & Theil, P. K. (2011). Perspectives on immunoglobulins in colostrum and milk. *Nutrients, 3*(4), 442-474.
37. Ivanova, B. B., Tsalev, D. L., & Arnaudov, M. G. (2006). Validation of reducing-difference procedure for the interpretation of non-polarized infrared spectra of n-component solid mixtures. *Talanta, 69*(4), 822-828.
38. Janini, G. M., & Veenstra, T. D. (2002). Methods for fractionation, separation and profiling of proteins and peptides. *Electrophoresis, 23*, 3048-3061.
39. Jensen, L., Provan, F., Larssen, E., Bron, J., & Obach, A. (2014). Reducing sea lice (Lepeophtheirus salmonis) infestation of farmed Atlantic salmon (Salmo salar L.) through functional feeds. *Aquaculture Nutrition*.
40. Jørgensen, C. E., Abrahamsen, R. K., Rukke, E.-O., Johansen, A.-G., Schüller, R. B., & Skeie, S. B. (2015). Improving the structure and rheology of high protein, low fat yoghurt with undenatured whey proteins. *International Dairy Journal, 47*, 6-18.
41. Jovanovic, S., Barac, M., Macej, O., Vucic, T., & Lacnjevac, C. (2007). SDS-PAGE analysis of soluble proteins in reconstituted milk exposed to different heat treatments. *Sensors, 7*(3), 371-383.
42. Kang, J.-S. (2012). Principles and Applications of LC-MS/MS for the Quantitative Bioanalysis of Analytes in Various Biological Samples. *Tandem Mass Spectrometry–Applications and Principles*, 441-492.
43. Karen Giles-Smith, M., RD,. (2013). Milk Proteins: Packing a Powerful Nutritional Punch Retrieved March, 2013, from http://www.todaysdietitian.com/newarchives/030413p26.shtml
44. Korhonen, H., Marnila, P., & Gill, H. (2000). Milk immunoglobulins and complement factors. *British Journal of Nutrition, 84*(S1), 75-80.
45. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature, 227*(5259), 680-685.
46. Li, M., Hu, B., Li, J., Chen, R., Zhang, X., & Chen, H. (2009). Extractive electrospray ionization mass spectrometry toward in situ analysis without sample pretreatment. *Analytical chemistry, 81*(18), 7724-7731.
47. Lindmark-Månsson, H., & Åkesson, B. (2000). Antioxidative factors in milk. *British Journal of Nutrition, 84*(S1), 103-110.
48. Markus, C. R., Olivier, B., Panhuysen, G. E., Van der Gugten, J., Alles, M. S., Tuiten, A., . . . de Haan, E. E. (2000). The bovine protein α-lactalbumin increases the plasma ratio of tryptophan to the other large neutral amino acids, and in vulnerable subjects raises brain serotonin activity, reduces cortisol concentration, and improves mood under stress. *The American journal of clinical nutrition, 71*(6), 1536-1544.

49. McKenzie, H. (2012). *Milk Proteins V1: Chemistry and molecular biology* (Vol. 1): Elsevier.
50. Melnik, B. C., Schmitz, G., John, S. M., Carrera-Bastos, P., Lindeberg, S., & Cordain, L. (2013). Metabolic effects of milk protein intake strongly depend on pre-existing metabolic and exercise status. *Nutrition & metabolism, 10*(1), 60.
51. Michalski, A., Damoc, E., Lange, O., Denisov, E., Nolting, D., Müller, M., . . . Belford, M. (2012). Ultra high resolution linear ion trap Orbitrap mass spectrometer (Orbitrap Elite) facilitates top down LC MS/MS and versatile peptide fragmentation modes. *Molecular & Cellular Proteomics, 11*(3), O111. 013698.
52. MMS. (2006). Membrane System Specialists, Inc,.
53. Nicolet, T. (2001). Introduction to fourier transform infrared spectrometry. *Information booklet*.
54. O'Donnell, R., Holland, J., Deeth, H., & Alewood, P. (2004). Milk proteomics. *International Dairy Journal, 14*(12), 1013-1023.
55. Outinen, M., Heino, A., & Uusi-Rauva, J. (2008). Microfiltration of milk II. Influence of the concentration factor on the composition of Emmental cheese milk and the k-casein macropeptide content of the whey. *Milchwissenschaft, 63*(3), 305-308.
56. Pometto, A., Shetty, K., Paliyath, G., & Levin, R. E. (2014). *Food biotechnology*: CRC Press.
57. Rattray, W., & Jelen, P. (1996). Protein standardization of milk and dairy products. *Trends in Food Science & Technology, 7*(7), 227-234.
58. Sanchez, L., Calvo, M., & Brock, J. H. (1992). Biological role of lactoferrin. *Archives of Disease in Childhood, 67*(5), 657.
59. Sandblom, R. M. (1978). Filtering process: Google Patents.
60. Santos, M., & Lies, M. (2015). Analysis of Casein and Whey Protein in Whole, 2%, and Skim Milk by Capillary Gel Electrophoresis. *Life sciences,*.
61. Santos, M. J., Teixeira, J. A., & Rodrigues, L. R. (2012). Fractionation of the major whey proteins and isolation of β-Lactoglobulin variants by anion exchange chromatography. *Separation and Purification Technology, 90*, 133-139.
62. Shapiro, A. L., Viñuela, E., & Maizel, J. V. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochemical and biophysical research communications, 28*(5), 815-820.
63. Sharma, R., Rajput, Y. S., & Mann, B. (2013). Chemical and functional properties of glycomacropeptide (GMP) and its role in the detection of cheese whey adulteration in milk: a review. *Dairy science & technology, 93*(1), 21-43.
64. Shi, L., Yang, T.-Z., & ZUBAREV, A. (2010). Rapid Purification and Identification of Osteopontin from Human Milk by HPLC Coupled to FT-ICR-MS. *Chemical Research in Chinese Universities, 26*(1), 17-22.
65. Sparkman, O. D. (2000). Mass spectrometry desk reference. *Journal of the American Society for Mass Spectrometry, 11*(12), 1144.

66. Szymanska-Chargot, M., & Zdunek, A. (2013). Use of FT-IR spectra and PCA to the bulk characterization of cell wall residues of fruits and vegetables along a fraction process. *Food biophysics, 8*(1), 29-42.
67. Tauler, R., Kowalski, B., & Fleming, S. (1993). Multivariate curve resolution applied to spectral data from multiple runs of an industrial process. *Analytical chemistry, 65*(15), 2040-2047.
68. Technical Service Center Water Treatment Group. (2010,). Water Treatment Primer for Communities in Need.
69. Thompson, M. P., Groves, M. L., Brower, D. P., Farrell, H. M., Jenness, R., & Kotts, C. E. (1988). The calcium-dependent electrophoretic shift of α-lactalbumin, the modifier protein of galactosyl transferase. *Biochemical and biophysical research communications, 157*(3), 944-948.
70. Timmer, J., & Van der Horst, H. (1998). Whey processing and separation technology: state-of-the-art and new developments.
71. Tovar Jiménez, X., Arana Cuenca, A., Téllez Jurado, A., Abreu Corona, A., & Muro Urista, C. R. (2012). Traditional methods for whey protein isolation and concentration: effects on nutritional properties and biological activity. *Journal of the Mexican Chemical Society, 56*(4), 369-377.
72. UCDAVIS CHEMWIKI. (2015). How an FTIR Spectrometer Operates,. Retrieved 17 February,, 2015
73. Van de Voort, F., Sedman, J., Emo, G., & Ismail, A. (1992). Assessment of Fourier transform infrared analysis of milk. *Journal of AOAC International (USA).*
74. Van Der Ven, C. (2002). *Biochemical and functional characterisation of casein and whey protein hydrolysates.*
75. Wiley, A. S. (2014). *Cultures of Milk*: Harvard University Press.
76. Yang, Y., Bu, D., Zhao, X., Sun, P., Wang, J., & Zhou, L. (2013). Proteomic analysis of cow, yak, buffalo, goat and camel milk whey proteins: quantitative differential expression patterns. *Journal of proteome research, 12*(4), 1660-1667.
77. Yazdanparast, S. A., Nezarati, S. S. M., Heshmati, F., & Hamzehlou, S. (2012). Comparison of cell wall proteins in putative Candida albicans & Candida dubliniensis by using modified staining method & SDS-PAGE. *Medical journal of the Islamic Republic of Iran, 26*(2), 45.
78. Zimecki, M., & Kruzel, M. L. (2007). Milk-derived proteins and peptides of potential therapeutic and nutritive value. *J Exp Ther Oncol, 6*(2), 89-106.

8. Appendix

8.1. Buffers

8X Tris-HCl: volume=100 ml

ingredient	Weight/Volume
Tris	36.35gr (adjust pH=8.8 by HCL)

2X Tris-HCl: volume=100 ml

ingredient	Weight/Volume
Tris	3.028gr (adjust pH=6.8 by HCL)

2X buffer (was used as a detergent): volume=20 ml

ingredient	Weight/Volume
2% SDS	4 ml
20 mM Tris-HCl, pH=6.8	4 ml
20% Glycerol	8 ml
160 mM DTT	3.2 ml
2mM EDTA	0.08 ml
Blue dye	(just until the solution become blue)

10X Running buffer: **volume=1000 ml**

ingredient	Weight/ Volume
Glycine	144.13 gr
Tris	30.3 gr
SDS	10 gr

Destain buffer: **volume=1000 ml**

ingredient	Weight/ Volume
Acetic acid	7.5 ml
Ethanol	100 ml

Coomassie staining solution: **volume=1000 ml**

ingredient	Weight/ Volume
Coomasie brilliant G250	0.5 gr
Ethanol (96%)	200 ml
Acetic acid	50 ml

Activation solution:

ingredient	Weight/ Volume
ACN	50%

Equilibration solution:

ingredient	Weight/ Volume
TFA	0.5%
ACN	5%

Sample Buffer:

ingredient	Weight/ Volume
TFA	2%
ACN	20%

Wash solution:

ingredient	weight
TFA	0.5%
ACN	5%

Elution Buffer:

ingredient	weight
ACN	70%

8.2. Gel bands value according to Image J software

Table 9. Gel bands values

Samples Name	Ig	LF	BSA	Casein subunit	β-Lg	α-La	GMP
MFP (S.2)	19183	8638,69	19954,93	15863,13	24174,47	28673,65	25955,74
MFP (S.3)	18552,52	13069,4	19178,88	16843,76	20833,15	29154,18	25437,26
MFP (S.4)	18170,42	17978,93	19097,93	21040,86	29435,03	29729,96	23390,59
Milk (S.2)	18620,83	17712,88	9150,154	23681,86	15933,03	14327,03	11275,59
Milk (S.3)	16615,93	22349,71	12169,71	26612,21	21230,76	12521,23	9398,589
Milk (S.4)	12435,42	20869,18	8103,296	24513,4	20305,4	8888,69	7763,347
UFR (S.1)	20297	28211,42	27643,25	22455,29	55747,44	31465,27	22995,08
MFR (S.2)	8022,811	16940,32	10532,83	36135,13	21888,78	14237,37	27030,97
MFR (S.3)	15583,1	22966,95	15717,61	43631,68	31909,29	18594,68	22642,66
MFR (S.4)	9603,539	18785,71	7836,125	30209,1	24747,56	13064,54	12708,37
MFR1.5(S.1)	8841,447	16792,54	5838,64	37235,1	26763,51	11296,9	10815,74
MFR2.8 (S.1)	13683,9	16047,05	9501,317	34583,72	17234,59	14874,13	33907,12

8.3. Protein concentration by Bradford

Table 10. Protein concentration (NO.1)

Sample Name	Protein Concentration (mg/ml protein)
MFR 1.5 (S.1)	27.99
MFR 2.8 (S.1)	37.79
UFR (S.1)	33.17
Milk (S.2)	22.24
MFP (S.2)	5.502
MFR (S.2)	38.75
Milk (S.3)	21.69
MFP (S.3)	4.02
MFR (S.3)	41.78
Milk (S.4)	22.88
MFP (S.4)	3.868
MFR (S.4)	30.70

Table 11.Protein concentration (NO.2)

Sample Name	Protein Concentration (mg/ml protein)
MFR 1.5 (S.1)	33.66
UFR (S.1)	48.54
MFP (S.2)	0.28
MFR (S.2)	30.02

Table 12. Protein concentration (NO.3)

Sample Name	Protein Concentration (mg/ml protein)
MFR 1.5 (S.1)	21.68
MFR 2.8 (S.1)	38.86
UFR (S.1)	29.85
Milk (S.2)	22.10
MFP (S.2)	0.68
MFR (S.2)	25.70

Table 13. Protein concentration (NO.4)

Sample Name	Protein Concentration (mg/ml protein)
MFR 1.5 (S.1)	22.80
MFR 2.8 (S.1)	18.10
UFR (S.1)	23.82
Milk (S.2)	16.98
MFP (S.2)	1.55
MFR (S.2)	24.19
Milk (S.3)	17.86
MFP (S.3)	1.60
MFR (S.3)	24.19
Milk (S.4)	26.38
MFP (S.4)	1.64
MFR (S.4)	20.52

8.4. Protein concentration using cut-off columns by Bradford

8.4.1. Before acetone precipitation

Table 14.Protein concentration before acetone precipitation (NO.1)

Sample Name	**Protein Concentration (mg/ml protein) <10 kDa from 30 kDa**
MFR 1.5 (S.1)	-0.007
UFR (S.1)	-0.006
MFP (S.2)	-0.005
Milk (S.2)	-0.009

Table 15.Protein concentration before acetone precipitation (NO.2)

Sample Name	**Protein Concentration (mg/ml protein) >30 kDa**
MFR 1.5 (S.1)	42.672
UFR (S.1)	91.103
MFP (S.2)	14.429
Milk (S.2)	45.521

Table 16. Protein concentration before acetone precipitation (NO.3)

Sample Name	**Protein Concentration (mg/ml protein) <10 kDa**
MFR 1.5 (S.1)	0.004
UFR (S.1)	0.223
MFP (S.2)	0.002
Milk (S.2)	0.015

Table 17.Protein concentration before acetone precipitation (NO.4)

Sample Name	**Protein Concentration (mg/ml protein) 10 kDa<sample<30 kDa**
MFR 1.5 (S.1)	0.033
UFR (S.1)	0.612
MFP (S.2)	0.039
Milk (S.2)	0.126

8.4.2. After protein precipitation

Table 18. Protein concentration after acetone precipitation (NO.1)

Sample Name	**Protein Concentration (mg/ml protein) <10 kDa from 30 kDa**
MFR 1.5 (S.1)	-0.003
UFR (S.1)	-0.012
MFR (S.2)	-0.008
MFP (S.2)	-0.003
Milk (S.2)	-0.006

Table 19. Protein concentration after acetone precipitation (NO.2)

Sample Name	**Protein Concentration (mg/ml protein) >30 kDa**
MFR 1.5 (S.1)	16.359
UFR (S.1)	22.534
MFR (S.2)	11.994
MFP (S.2)	1.703
Milk (S.2)	15.082

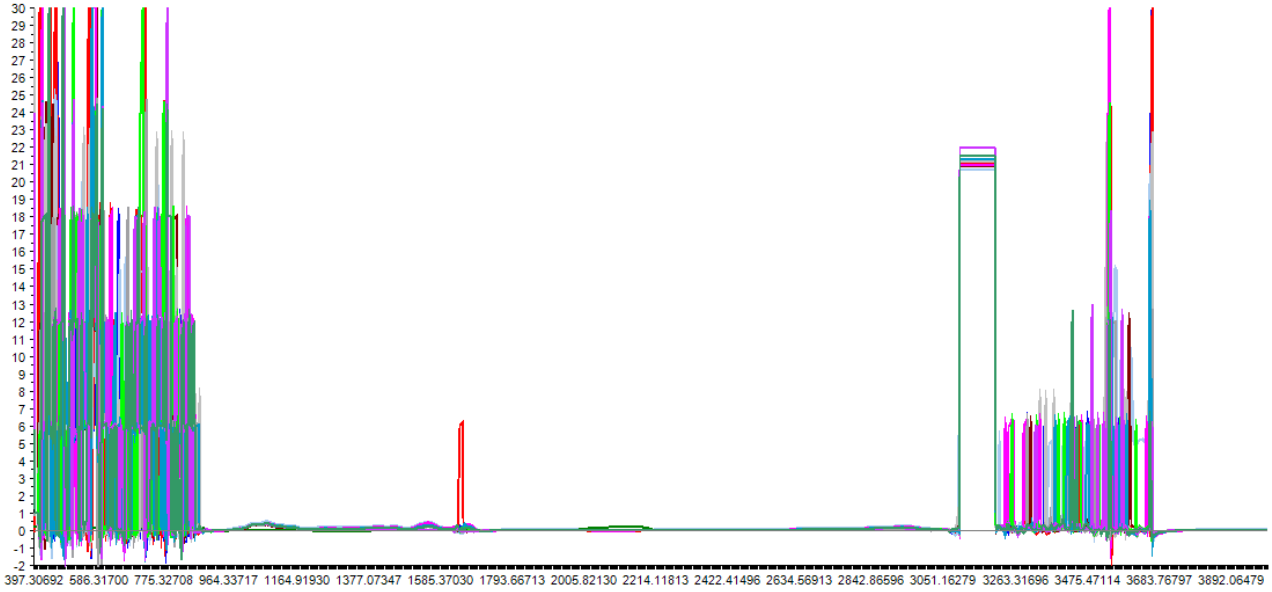
Table 20. Protein concentration after acetone precipitation (NO.3)

Sample Name	**Protein Concentration (mg/ml protein) <10 kDa**
MFR 1.5 (S.1)	0.009
UFR (S.1)	0.006
MFR (S.2)	0.021
MFP (S.2)	0.029
Milk (S.2)	0.027

Table 21.Protein concentration after acetone precipitation (NO.4)

Sample Name	**Protein Concentration (mg/ml protein) 10 kDa<sample<30 kDa**
MFR 1.5 (S.1)	0.011
UFR (S.1)	0.245
MFR (S.2)	0.019
MFP (S.2)	0.029
Milk (S.2)	0.420

8.5. Complete FT-IR figure



8.6. Complete LC-MS/MS results

Table 22. Milk samples (Complete LC-MS/MS results)

Description	ΣCoverage	AAs	MW [kDa]	Milk (S.2)	Milk (S.3)	Milk (S.2)	Milk (S.4)
Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3 - [LACB_BOVIN]	86,52	178	19,9	277	238	91	107
Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2 - [CASA1_BOVIN]	74,30	214	24,5	436	625	185	337
Major allergen beta-lactoglobulin OS=Bos taurus PE=2 SV=1 - [B5B0D4_BOVIN]	86,52	178	20,0	277	238	82	109
Alpha S1 casein OS=Bos taurus GN=CSN1S1 PE=2 SV=1 - [B5B3R8_BOVIN]	69,16	214	24,4	433	605	171	338
Alpha-lactalbumin OS=Bos taurus GN=LALBA PE=1 SV=2 - [LALBA_BOVIN]	83,10	142	16,2	27	24	58	33
Alpha lactalbumin (Fragment) OS=Bos taurus GN=alfaLA PE=3 SV=1 - [Q28049_BOVIN]	65,85	123	14,1	27	24	59	33
Alpha-S2-casein OS=Bos taurus GN=CSN1S2 PE=1 SV=2 - [CASA2_BOVIN]	54,05	222	26,0	166	190	85	51
Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2 - [CASB_BOVIN]	21,43	224	25,1	178	209	45	88
Beta-lactoglobulin (Fragment) OS=Bos taurus GN=LGB PE=4 SV=1 - [E7E1Q8_BOVIN]	86,11	36	4,2	91	58	7	14
Kappa-casein (Fragment) OS=Bos taurus x Bos indicus GN=CSN3 PE=4 SV=1 - [Q9N258_9CETA]	61,88	160	17,9	172	131	36	47
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4 - [ALBU_BOVIN]	69,52	607	69,2	12		63	6
Kappa casein (Fragment) OS=Bos taurus GN=csn3 PE=4 SV=1 - [Q5ZET1_BOVIN]	61,87	139	15,2	72	74	47	24
Glycosylation-dependent cell adhesion molecule 1 OS=Bos taurus GN=GLYCAM1 PE=1 SV=2 - [GLCM1_BOVIN]	51,63	153	17,1	21	6	23	8
Enterotoxin-binding glycoprotein PP20K (Fragment) OS=Bos taurus PE=1 SV=1 - [Q9TRB9_BOVIN]	100,00	20	2,3	52	26	11	
PIGR protein OS=Bos taurus GN=PIGR PE=2 SV=1 -	39,23	757	82,5	2		15	

[A6QNW3_BOVIN]							
Uncharacterized protein OS=Bos taurus GN=PIGR PE=4 SV=1 - [F1MR22_BOVIN]	31,84	757	82,5	2		15	
Lactoperoxidase OS=Bos taurus GN=LPO PE=1 SV=1 - [PERL_BOVIN]	43,82	712	80,6			5	
Lactoferrin (Fragment) OS=Bos taurus PE=2 SV=1 - [C7FE01_BOVIN]	27,25	690	76,2			3	
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [G5E5T5_BOVIN]	23,14	389	42,4	1		2	
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [G5E513_BOVIN]	19,26	457	49,9			2	
Uncharacterized protein OS=Bos taurus GN=A2ML1 PE=4 SV=2 - [F1MB32_BOVIN]	0,43	1396	155,5	25	11		
Putative uncharacterized protein OS=Bos taurus PE=2 SV=1 - [A6QM09_BOVIN]	14,66	232	24,7	3			3
Butyrophilin subfamily 1 member A1 OS=Bos taurus GN=BTN1A1 PE=1 SV=2 - [BT1A1_BOVIN]	27,19	526	59,2			4	
Isoform Short of Lactadherin OS=Bos taurus GN=MFGE8 - [MFGM_BOVIN]	17,38	374	41,5	7			
LOC532995 protein OS=Bos taurus GN=LOC532995 PE=2 SV=1 - [A6QP80_BOVIN]	6,84	395	45,4	1	29		
Osteopontin OS=Bos taurus GN=SPP1 PE=2 SV=1 - [Q58DM6_BOVIN]	10,45	201	22,2	3			
Myocyte-specific enhancer factor 2C OS=Bos taurus GN=MEF2C PE=2 SV=1 - [MEF2C_BOVIN]	9,52	441	47,8	10	4		1
Lipoprotein lipase OS=Bos taurus GN=LPL PE=1 SV=2 - [LIPL_BOVIN]	25,52	478	53,3	3		3	
Ribonuclease 4 OS=Bos taurus GN=RNASE4 PE=1 SV=4 - [RNAS4_BOVIN]	31,09	119	13,7			3	
Uncharacterized protein OS=Bos taurus GN=ZNF192 PE=4 SV=1 - [G3N3E2_BOVIN]	6,27	271	31,0	8	2	1	4
Secretoglobin family 1D member OS=Bos taurus GN=SCGB1D PE=3 SV=2 - [SG1D_BOVIN]	8,82	102	11,3			4	
Serotransferrin OS=Bos taurus GN=TF PE=4 SV=1 - [G3X6N3_BOVIN]	10,23	704	77,6			1	
Uncharacterized protein OS=Bos taurus GN=GOLGA4 PE=4 SV=2 - [E1B7E3_BOVIN]	0,63	2229	259,0		2	2	
Uncharacterized protein (Fragment) OS=Bos taurus GN=PTPN21 PE=4 SV=2 - [F1MRH9_BOVIN]	0,51	1180	132,3	5			
Uncharacterized protein OS=Bos taurus GN=SRGAP3 PE=4 SV=2 - [F1MJI3_BOVIN]	0,91	1099	124,4			3	
Alpha-2-adrenergic receptor (Fragment) OS=Bos taurus PE=2 SV=1 - [O97717_BOVIN]	48,98	49	5,6	6			
Uncharacterized protein OS=Bos taurus GN=PTBP2 PE=4 SV=1 - [F6Q0P6_BOVIN]	2,54	236	25,6	2	1		
Outer capsid protein (Fragment) OS=Bluetongue virus 10 GN=VP2 PE=4 SV=1 - [L7NSQ0_BTV1X]	8,64	162	18,8		2		
Uncharacterized protein OS=Bos taurus GN=PKHD1 PE=4 SV=2 - [E1BK86_BOVIN]	0,64	4071	443,9			1	
Sodium/hydrogen exchanger (Fragment) OS=Bos taurus GN=SLC9A7 PE=3 SV=1 - [G5E545_BOVIN]	1,67	660	73,5	2			
Uncharacterized protein OS=Bos taurus GN=TMEM126B PE=4 SV=1 - [E1BCS4_BOVIN]	8,70	230	25,3	7			
Hydroxyacid oxidase 2 OS=Bos taurus GN=HAO2 PE=2 SV=1 - [HAOX2_BOVIN]	2,27	353	39,2		7		
Fiber protein OS=Bovine adenovirus B serotype 3 GN=L5 PE=2 SV=2 - [SPIKE_ADEB3]	2,56	976	102,3	1	5		
UDP-glucose ceramide glucosyltransferase OS=Bos taurus GN=UGCG PE=2 SV=1 - [Q08DR4_BOVIN]	1,52	394	44,7	1	1		
Uncharacterized protein OS=Bos taurus GN=TXNDC16 PE=4 SV=1 - [E1BAG3_BOVIN]	2,67	824	92,9	3			
Rap guanine nucleotide exchange factor 2 OS=Bos taurus GN=RAPGEF2 PE=1 SV=2 - [RPGF2_BOVIN]	1,01	1486	165,0			1	
Uncharacterized protein OS=Bos taurus GN=PPP1R14A PE=4 SV=1 - [E1BE60_BOVIN]	16,33	147	16,6		1		
Uncharacterized protein OS=Bos taurus GN=ARHGEF12 PE=4 SV=1 - [F1MZA2_BOVIN]	0,39	1544	172,6	1	2		

Uncharacterized protein (Fragment) OS=Bos taurus GN=TBC1D5 PE=4 SV=2 - [F1N3C5_BOVIN]	0,87	804	89,5				4
Cation-independent mannose-6-phosphate receptor OS=Bos taurus GN=IGF2R PE=4 SV=2 - [F1MIE6_BOVIN]	4,80	2499	274,8			1	
Uncharacterized protein OS=Bos taurus GN=LRIG1 PE=4 SV=2 - [F1MZV0_BOVIN]	1,47	1090	118,3		2		
Uncharacterized protein OS=Bos taurus GN=CCM2 PE=4 SV=2 - [E1B8H2_BOVIN]	2,31	476	52,5		2		1
Uncharacterized protein OS=Bos taurus GN=DCLRE1B PE=4 SV=2 - [E1BFI5_BOVIN]	4,81	541	61,2	4			
Uncharacterized protein (Fragment) OS=Bos taurus GN=CACNA1F PE=3 SV=2 - [E1B9S9_BOVIN]	1,46	1708	190,9		2		2
26S protease regulatory subunit 10B OS=Bos taurus GN=PSMC6 PE=3 SV=2 - [F1MLV1_BOVIN]	8,74	389	44,1				
Putative malate dehydrogenase 1B OS=Bos taurus GN=MDH1B PE=2 SV=1 - [MDH1B_BOVIN]	3,59	473	53,1			1	
Olfactory receptor OS=Bos taurus GN=OR4K15 PE=3 SV=1 - [G5E586_BOVIN]	8,33	312	35,0	3			
F10 protein (Fragment) OS=Bos taurus GN=F10 PE=2 SV=1 - [Q3MHW2_BOVIN]	1,86	483	53,5	2			
Aldehyde oxidase 3L1 OS=Bos taurus PE=2 SV=1 - [M1ZMN6_BOVIN]	2,01	1342	148,1			1	
NOC3L protein OS=Bos taurus GN=NOC3L PE=2 SV=1 - [A5D7R2_BOVIN]	0,88	799	92,3	1			
Heat shock protein 90 beta (Fragment) OS=Bos taurus GN=hsp90 beta PE=2 SV=1 - [Q865A1_BOVIN]	8,12	234	27,8		3		
Centrosomal protein of 19 kDa OS=Bos taurus GN=CEP19 PE=2 SV=1 - [CEP19_BOVIN]	14,11	163	19,2		3		
60S ribosomal protein L13 OS=Bos taurus GN=RPL13 PE=2 SV=3 - [RL13_BOVIN]	4,74	211	24,3			1	
Outer capsid protein VP5 OS=Bluetongue virus 1 (isolate Australia) GN=S6 PE=3 SV=1 - [VP5_BTV1A]	3,04	526	59,2			1	
Fibroblast growth factor OS=Bos taurus GN=FGF6 PE=3 SV=1 - [E1BHC1_BOVIN]	14,42	208	22,6			1	
Olfactory receptor OS=Bos taurus GN=LOC506202 PE=3 SV=1 - [G3MWX1_BOVIN]	11,54	312	34,5			3	
Uncharacterized protein OS=Bos taurus GN=ARHGAP18 PE=4 SV=2 - [E1BIH5_BOVIN]	3,39	531	59,6			2	
Probable tRNA N6-adenosine threonylcarbamoyltransferase OS=Bos taurus GN=OSGEP PE=2 SV=1 - [OSGEP_BOVIN]	9,85	335	36,5				3
Isoform 2 of Collagen type IV alpha-3-binding protein OS=Bos taurus GN=COL4A3BP - [C43BP_BOVIN]	5,69	598	68,0	1			
CMRF35-like molecule 9 OS=Bos taurus GN=CD300LG PE=2 SV=2 - [CLM9_BOVIN]	9,02	255	28,0	2			
Protein odd-skipped-related 2 OS=Bos taurus GN=OSR2 PE=2 SV=1 - [OSR2_BOVIN]	11,59	276	30,5		2		
Genome polyprotein OS=Japanese encephalitis virus (strain SA(v)) PE=3 SV=1 - [POLG_JAEV5]	0,47	3432	379,9				2
Selenium-binding protein 1 OS=Bos taurus GN=SELENBP1 PE=1 SV=1 - [SBP1_BOVIN]	1,48	472	52,5	1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=LOC100848970 PE=4 SV=2 - [F1MYH7_BOVIN]	4,25	612	69,6		2		
Uncharacterized protein OS=Bos taurus GN=HNF1A PE=3 SV=1 - [F1MD26_BOVIN]	3,63	633	67,3	1			
Uncharacterized protein OS=Bos taurus GN=ACIN1 PE=4 SV=1 - [F1MQG6_BOVIN]	1,35	1336	150,7	1	1		
Malic enzyme OS=Bos taurus GN=ME1 PE=3 SV=2 - [F1N3V0_BOVIN]	4,92	569	63,7	2			
Glucose-6-phosphate 1-dehydrogenase OS=Bos taurus GN=G6PD PE=3 SV=1 - [F1MMK2_BOVIN]	3,11	515	59,3		1		
Uncharacterized protein (Fragment) OS=Bos taurus GN=HYDIN PE=4 SV=2 - [E1BED7_BOVIN]	1,07	5120	575,7				1
PSTK protein (Fragment) OS=Bos taurus GN=PSTK PE=2 SV=1 - [A7E3E4_BOVIN]	5,98	351	40,2	2			
Uncharacterized protein OS=Bos taurus GN=ASTE1 PE=4 SV=1 - [E1BIE0_BOVIN]	3,87	672	75,6		2		
Uncharacterized protein OS=Bos taurus PE=4 SV=2 - [F1N0Z8_BOVIN]	8,72	321	36,1	2			

Uncharacterized protein OS=Bos taurus GN=LTN1 PE=4 SV=2 - [F1MTS8_BOVIN]	1,58	1898	213,6	2			
Phosphoglycerate kinase OS=Bos taurus GN=PGK2 PE=2 SV=2 - [Q32KN6_BOVIN]	4,08	417	44,7	2			
Uncharacterized protein OS=Bos taurus GN=CDK5RAP2 PE=4 SV=2 - [F1MGF7_BOVIN]	1,16	1902	212,6			1	
Uncharacterized protein OS=Bos taurus GN=C9orf172 PE=4 SV=2 - [E1B9Y2_BOVIN]	1,95	975	105,9				1
Chromosome 20 open reading frame 108 ortholog OS=Bos taurus GN=C13H20ORF108 PE=2 SV=1 - [A1A4K7_BOVIN]	8,33	192	20,4			1	
Uncharacterized protein OS=Bos taurus GN=SCUBE2 PE=4 SV=2 - [F1N7F6_BOVIN]	4,70	999	110,2				1
Uncharacterized protein OS=Bos taurus PE=4 SV=1 - [G3N0E3_BOVIN]	8,44	237	26,9			1	
Uncharacterized protein OS=Bos taurus GN=C2orf71 PE=4 SV=1 - [G3N2Z0_BOVIN]	3,20	1313	141,8			2	
Protein L (Fragment) OS=Adelaide River virus GN=L PE=4 SV=1 - [Q9E783_ARV]	5,33	450	52,2			1	
Sodium channel, voltage-gated, type II, beta-like OS=Bos taurus GN=SCN2B PE=4 SV=1 - [E1B757_BOVIN]	4,65	215	24,3			1	
Growth/differentiation factor 6 OS=Bos taurus GN=GDF6 PE=2 SV=2 - [GDF6_BOVIN]	3,19	470	52,1	1			
GRB2-related adapter protein OS=Bos taurus GN=GRAP PE=2 SV=1 - [GRAP_BOVIN]	9,68	217	25,4	1			
Putative helicase MOV-10 OS=Bos taurus GN=MOV10 PE=2 SV=1 - [MOV10_BOVIN]	0,80	1003	113,8			1	
Unconventional myosin-X OS=Bos taurus GN=MYO10 PE=1 SV=1 - [MYO10_BOVIN]	0,68	2052	235,7				1
Olfactomedin-like protein 3 OS=Bos taurus GN=OLFML3 PE=2 SV=1 - [OLFL3_BOVIN]	7,88	406	45,9	1			
Suppressor of tumorigenicity 7 protein-like OS=Bos taurus GN=ST7L PE=2 SV=1 - [ST7L_BOVIN]	6,31	555	62,6			1	
Core protein VP3 OS=Bluetongue virus 1 (isolate Australia) GN=S3 PE=3 SV=1 - [VP3_BTV1A]	1,44	901	103,1			1	
LOC510385 protein OS=Bos taurus GN=LOC510385 PE=2 SV=1 - [A7YWC9_BOVIN]	4,52	753	86,3		1		
Delta-like protein OS=Bos taurus GN=DLL4 PE=2 SV=1 - [Q0V7L8_BOVIN]	6,42	685	74,4		1		
Leukemia virus receptor BLVRcp130 OS=Bos taurus PE=4 SV=1 - [Q9GJU8_BOVIN]	3,88	644	73,6		1		
Acyl-CoA synthetase long-chain family member 6 OS=Bos taurus GN=ACSL6 PE=2 SV=1 - [Q2TA22_BOVIN]	5,02	697	78,0	1			
Cyclin B1 interacting protein 1 OS=Bos taurus GN=CCNB1IP1 PE=2 SV=1 - [Q2YDD5_BOVIN]	15,88	277	31,4		1		
Acetyl-CoA carboxylase 1 OS=Bos taurus GN=ACACA PE=3 SV=2 - [E1BGH6_BOVIN]	1,41	2346	265,2			1	
Uncharacterized protein OS=Bos taurus GN=CUBN PE=4 SV=2 - [F1MKV7_BOVIN]	1,22	3620	395,8		1		
Uncharacterized protein (Fragment) OS=Bos taurus GN=MYH7B PE=4 SV=2 - [E1BPX8_BOVIN]	0,31	1942	221,4		1		
Uncharacterized protein OS=Bos taurus GN=LOC100848786 PE=4 SV=2 - [F1MU59_BOVIN]	8,10	321	35,5	1			
Uncharacterized protein OS=Bos taurus GN=SMC6 PE=4 SV=1 - [E1BFH7_BOVIN]	3,18	1101	127,8				1
Uncharacterized protein (Fragment) OS=Bos taurus GN=ADAM1B PE=4 SV=2 - [F1MY02_BOVIN]	3,20	812	89,9	1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=GCN1L1 PE=4 SV=1 - [F1MZT7_BOVIN]	0,93	2676	292,9	1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=DNAH17 PE=4 SV=2 - [E1BLB4_BOVIN]	0,18	4392	501,0			1	
Uncharacterized protein OS=Bos taurus GN=WDR7 PE=4 SV=2 - [E1BEC1_BOVIN]	1,81	1490	163,6	1			
Uncharacterized protein OS=Bos taurus GN=CCDC136 PE=4 SV=2 - [F1N343_BOVIN]	2,00	1151	133,2			1	
Uncharacterized protein (Fragment) OS=Bos taurus GN=UBXN7 PE=4 SV=2 - [F1MUA8_BOVIN]	3,59	474	53,6	1			
HGD protein (Fragment) OS=Bos taurus GN=HGD PE=2 SV=1 - [Q2KIH3_BOVIN]	8,57	385	44,3	1			

Uncharacterized protein (Fragment) OS=Bos taurus GN=ZNF81 PE=4 SV=2 - [F1N1M0_BOVIN]	1,70	648	74,5			1	
Uncharacterized protein (Fragment) OS=Bos taurus GN=ZMYM4 PE=4 SV=1 - [F1MHR5_BOVIN]	1,71	1521	169,5		1		
Claudin OS=Bos taurus GN=CLDN14 PE=3 SV=2 - [E1BMT9_BOVIN]	11,39	237	25,0	1			
Uncharacterized protein OS=Bos taurus GN=FUT11 PE=3 SV=1 - [E1BIT9_BOVIN]	3,45	493	55,6	1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=CDCP1 PE=4 SV=1 - [G3MXT8_BOVIN]	3,46	693	77,8	1			
Uncharacterized protein OS=Bos taurus GN=RC3H2 PE=4 SV=1 - [E1BP22_BOVIN]	3,11	1191	131,6	1			
Uncharacterized protein OS=Bos taurus GN=AKAP4 PE=4 SV=1 - [F1MYH5_BOVIN]	1,89	848	93,9	1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=LOC100298119 PE=3 SV=1 - [G3N289_BOVIN]	11,07	307	34,8	1			
Olfactory receptor OS=Bos taurus GN=LOC614090 PE=3 SV=2 - [E1BMY3_BOVIN]	10,29	311	34,9	1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=CACNA1C PE=3 SV=2 - [F1N5T3_BOVIN]	2,13	2061	230,5				1
Uncharacterized protein OS=Bos taurus GN=EP400 PE=4 SV=2 - [F1MLB1_BOVIN]	1,43	3137	340,9	1			
Olfactory receptor (Fragment) OS=Bos taurus GN=LOC508785 PE=3 SV=1 - [G3MWH9_BOVIN]	9,30	344	39,6		1		
Uncharacterized protein OS=Bos taurus GN=SMPD2 PE=4 SV=1 - [F1N588_BOVIN]	6,15	423	47,6	1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=PIEZO2 PE=4 SV=2 - [F1N2A6_BOVIN]	1,61	2609	300,9	1			
Uncharacterized protein OS=Bos taurus GN=VAT1 PE=4 SV=2 - [F1MUP9_BOVIN]	2,74	402	42,8		1		
Uncharacterized protein OS=Bos taurus GN=LOC520023 PE=4 SV=2 - [F1N7H9_BOVIN]	3,72	457	52,3		1		
Phosphatidylinositol 3-kinase OS=Bos taurus GN=PIK3C3 PE=2 SV=1 - [A5PJQ9_BOVIN]	1,35	887	101,4		1		
Envelope glycoprotein (Fragment) OS=Bovine leukemia virus GN=env PE=4 SV=1 - [A9LXY8_BLV]	27,82	133	15,4	1			
LIN52 protein OS=Bos taurus GN=LIN52 PE=4 SV=1 - [A6QQR6_BOVIN]	7,76	116	13,0		1		
Zinc transporter ZIP4 OS=Bos taurus GN=SLC39A4 PE=1 SV=1 - [S39A4_BOVIN]	7,04	653	68,6		1		
Sugarbabe-like OS=Bos taurus GN=GLIS2 PE=4 SV=1 - [E1BKE4_BOVIN]	9,35	524	55,6				1
Uncharacterized protein (Fragment) OS=Bos taurus GN=CDC20B PE=4 SV=1 - [F1MF85_BOVIN]	9,55	440	48,5		1		
Uncharacterized protein OS=Bos taurus GN=ZMYM3 PE=4 SV=2 - [E1BKM9_BOVIN]	1,68	1372	152,5		1		
Uncharacterized protein OS=Bos taurus GN=CACNA1D PE=3 SV=2 - [F1MTK5_BOVIN]	1,10	2183	247,3				1
Classical MHC class I antigen (Fragment) OS=Bos taurus GN=BoLa PE=2 SV=1 - [Q3YFH4_BOVIN]	10,23	352	39,9		1		
Uncharacterized protein OS=Bos taurus GN=RBM27 PE=4 SV=2 - [E1BCT9_BOVIN]	2,08	1058	118,4		1		
Leptin (Fragment) OS=Bos taurus GN=obese PE=4 SV=1 - [Q6QLP8_BOVIN]	80,95	42	4,7		1		
NAT10 protein OS=Bos taurus GN=NAT10 PE=2 SV=1 - [A5D7R3_BOVIN]	3,41	1026	115,9		1		
Uncharacterized protein OS=Bos taurus GN=SIK1 PE=4 SV=2 - [E1BNS8_BOVIN]	5,56	773	84,1		1		
Similar to prolactin-like protein (Fragment) OS=Bos taurus PE=2 SV=1 - [Q862S7_BOVIN]	19,81	106	12,7		1		
Olfactory receptor OS=Bos taurus GN=LOC614592 PE=3 SV=1 - [G3X858_BOVIN]	9,20	326	36,8		1		
Uncharacterized protein OS=Bos taurus GN=PIGQ PE=4 SV=1 - [M5FMV9_BOVIN]	7,06	581	65,5		1		
Leucine zipper protein 5 (Fragment) OS=Bos taurus GN=LUZP5 PE=2 SV=1 - [Q0V8K4_BOVIN]	3,91	384	44,9		1		
Non-structural protein 3 OS=Rotavirus A (strain Cow/United Kingdom/UK/1975 G6-P7[5]-I2-R2-C2-M2-A3-N2-T7-E2-H3)	2,56	313	36,1			1	

PE=3 SV=1 - [NSP3_ROTBU]							
Outer capsid protein VP5 OS=Bluetongue virus 13 (isolate USA) GN=S6 PE=3 SV=1 - [VP5_BTV13]	5,13	526	59,2			1	
Uncharacterized protein OS=Bos taurus GN=ULK2 PE=4 SV=2 - [E1BH35_BOVIN]	2,51	1037	112,2			1	
Uncharacterized protein OS=Bos taurus GN=DHX57 PE=4 SV=1 - [F1N1A2_BOVIN]	2,03	1382	155,6				1
DNA-directed RNA polymerases I, II, and III subunit RPABC2 OS=Bos taurus GN=POLR2F PE=4 SV=1 - [F2Z4C9_BOVIN]	4,72	127	14,5			1	
T-complex protein 11-like protein 2 OS=Bos taurus GN=TCP11L2 PE=2 SV=1 - [T11L2_BOVIN]	3,85	519	58,1			1	
Uncharacterized protein OS=Bos taurus GN=DNMBP PE=4 SV=1 - [E1BIL8_BOVIN]	1,71	1579	178,0				1
Toll-like receptor 10 (Fragment) OS=Bos taurus GN=TLR10 PE=2 SV=1 - [Q56GY0_BOVIN]	27,94	68	7,9				1
Uncharacterized protein (Fragment) OS=Bos taurus GN=GFRAL PE=4 SV=1 - [F1MFI8_BOVIN]	6,17	324	36,2				1
Structural glycoprotein E2 (Fragment) OS=Bovine viral diarrhea virus GN=E2 PE=4 SV=1 - [Q6F5V5_BVDV]	9,29	140	15,7				1
Cytoskeleton-associated protein 2-like OS=Bos taurus GN=CKAP2L PE=2 SV=1 - [CKP2L_BOVIN]	3,49	744	82,6			1	
Fatty acid-binding protein, intestinal OS=Bos taurus GN=FABP2 PE=2 SV=3 - [FABPI_BOVIN]	23,48	132	15,0			1	
Histone-lysine N-methyltransferase SUV39H2 OS=Bos taurus GN=SUV39H2 PE=2 SV=1 - [SUV92_BOVIN]	7,07	410	46,5			1	
Replication factor C (Activator 1) 5, 36.5kDa OS=Bos taurus GN=RFC5 PE=2 SV=1 - [Q32PI3_BOVIN]	9,49	316	35,7			1	
Uncharacterized protein OS=Bos taurus GN=SUV420H2 PE=4 SV=2 - [E1B8M1_BOVIN]	5,03	457	51,7			1	
MTERFD2 protein (Fragment) OS=Bos taurus GN=MTERFD2 PE=2 SV=1 - [Q3MHX4_BOVIN]	7,16	335	38,6			1	
SYNCRIP protein (Fragment) OS=Bos taurus GN=SYNCRIP PE=2 SV=1 - [A7E355_BOVIN]	5,26	456	51,1			1	
Deoxyribonuclease (Fragment) OS=Bos taurus GN=DNASE1L1 PE=3 SV=2 - [F1MW13_BOVIN]	9,22	282	31,6			1	
Olfactory receptor (Fragment) OS=Bos taurus GN=LOC526713 PE=3 SV=1 - [G3MYS8_BOVIN]	10,26	312	35,1			1	
LOC100126054 protein OS=Bos taurus GN=LOC100126054 PE=2 SV=1 - [A5PJZ3_BOVIN]	5,81	258	29,2				1
Coagulation factor XIII, B polypeptide OS=Bos taurus GN=F13B PE=2 SV=1 - [Q2TBQ1_BOVIN]	3,18	661	75,1				1
Uncharacterized protein OS=Bos taurus GN=TP73 PE=3 SV=1 - [G3X6J7_BOVIN]	5,47	640	69,7				1
Uncharacterized protein C1orf198 homolog OS=Bos taurus PE=2 SV=1 - [CA198_BOVIN]	1,85	325	35,5				1
Uncharacterized protein OS=Bos taurus GN=NUDT19 PE=4 SV=1 - [E1BDS7_BOVIN]	4,99	381	42,6				1
Uncharacterized protein OS=Bos taurus GN=TBC1D21 PE=4 SV=1 - [F1MK40_BOVIN]	5,69	299	34,8				1
Truncated melanocortin 1 receptor (Fragment) OS=Bos taurus GN=MC1R PE=3 SV=1 - [H9BEA4_BOVIN]	34,71	121	13,0				1
MHC class II DQA2 (Fragment) OS=Bos taurus GN=BoLA-DQA2 PE=2 SV=1 - [P79462_BOVIN]	15,85	183	20,0				1

Table 23. Permeate samples (Complete LC-MS/MS results)

Description	ΣCoverage	AAs	MW [kDa]	MFP (S.2)	MFP (S.2)	MFP (S.3)	MFP (S.4)
Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3 - [LACB_BOVIN]	86,52	178	19,9	46	434	311	250
Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2 - [CASA1_BOVIN]	74,30	214	24,5	13	35	29	18
Major allergen beta-lactoglobulin OS=Bos taurus PE=2 SV=1 - [B5B0D4_BOVIN]	86,52	178	20,0	46	411	296	250
Alpha S1 casein OS=Bos taurus GN=CSN1S1 PE=2 SV=1 - [B5B3R8_BOVIN]	69,16	214	24,4	13	35	29	18
Alpha-lactalbumin OS=Bos taurus GN=LALBA PE=1 SV=2 - [LALBA_BOVIN]	83,10	142	16,2	31	187	149	26
Alpha lactalbumin (Fragment) OS=Bos taurus GN=alfaLA PE=3 SV=1 - [Q28049_BOVIN]	65,85	123	14,1	35	179	148	26
Alpha-S2-casein OS=Bos taurus GN=CSN1S2 PE=1 SV=2 - [CASA2_BOVIN]	54,05	222	26,0	3	13	17	2
Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2 - [CASB_BOVIN]	21,43	224	25,1		4	7	3
Beta-lactoglobulin (Fragment) OS=Bos taurus GN=LGB PE=4 SV=1 - [E7E1Q8_BOVIN]	86,11	36	4,2	3	151	107	43
Kappa-casein (Fragment) OS=Bos taurus x Bos indicus GN=CSN3 PE=4 SV=1 - [Q9N258_9CETA]	61,88	160	17,9	6	6	13	8
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4 - [ALBU_BOVIN]	69,52	607	69,2	10	87	47	14
Kappa casein (Fragment) OS=Bos taurus GN=csn3 PE=4 SV=1 - [Q5ZET1_BOVIN]	61,87	139	15,2	3	4	6	5
Glycosylation-dependent cell adhesion molecule 1 OS=Bos taurus GN=GLYCAM1 PE=1 SV=2 - [GLCM1_BOVIN]	51,63	153	17,1	13	44	25	7
Enterotoxin-binding glycoprotein PP20K (Fragment) OS=Bos taurus PE=1 SV=1 - [Q9TRB9_BOVIN]	100,00	20	2,3	12	27	19	14
PIGR protein OS=Bos taurus GN=PIGR PE=2 SV=1 - [A6QNW3_BOVIN]	39,23	757	82,5		17	9	2
Uncharacterized protein OS=Bos taurus GN=PIGR PE=4 SV=1 - [F1MR22_BOVIN]	31,84	757	82,5		12	6	2
Lactoperoxidase OS=Bos taurus GN=LPO PE=1 SV=1 - [PERL_BOVIN]	43,82	712	80,6		23	12	
Complement C3 OS=Bos taurus GN=C3 PE=1 SV=2 - [CO3_BOVIN]	21,25	1661	187,1		2	1	
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [G5E5T5_BOVIN]	23,14	389	42,4		1	3	

Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [G5E513_BOVIN]	19,26	457	49,9		5	3	
Xanthine dehydrogenase/oxidase OS=Bos taurus GN=XDH PE=4 SV=1 - [F1MUT3_BOVIN]	16,44	1332	146,7		2		
Putative uncharacterized protein OS=Bos taurus PE=2 SV=1 - [A6QM09_BOVIN]	14,66	232	24,7		5	7	3
Osteopontin OS=Bos taurus GN=SPP1 PE=2 SV=1 - [Q58DM6_BOVIN]	10,45	201	22,2		4	4	
Myocyte-specific enhancer factor 2C OS=Bos taurus GN=MEF2C PE=2 SV=1 - [MEF2C_BOVIN]	9,52	441	47,8		1		
Apolipoprotein A-I OS=Bos taurus GN=APOA1 PE=1 SV=3 - [APOA1_BOVIN]	13,96	265	30,3		4		
Epididymal secretory protein E1 OS=Bos taurus GN=NPC2 PE=1 SV=1 - [NPC2_BOVIN]	32,89	149	16,6		3		
Uncharacterized protein (Fragment) OS=Bos taurus GN=PTPN21 PE=4 SV=2 - [F1MRH9_BOVIN]	0,51	1180	132,3			2	1
Outer capsid protein (Fragment) OS=Bluetongue virus 10 GN=VP2 PE=4 SV=1 - [L7NSQ0_BTV1X]	8,64	162	18,8			1	
Vitamin D-binding protein OS=Bos taurus GN=GC PE=4 SV=2 - [F1N5M2_BOVIN]	6,96	474	53,3		1		
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [G3N0V0_BOVIN]	8,59	326	35,9		4		
Uncharacterized protein OS=Bos taurus GN=TXNDC16 PE=4 SV=1 - [E1BAG3_BOVIN]	2,67	824	92,9		1	1	
Olfactory receptor OS=Bos taurus GN=OR2T33 PE=3 SV=1 - [M0QVY4_BOVIN]	8,97	312	35,1		2	3	
Uncharacterized protein OS=Bos taurus PE=4 SV=1 - [G3N360_BOVIN]	10,76	158	17,9	1			
Rap guanine nucleotide exchange factor 2 OS=Bos taurus GN=RAPGEF2 PE=1 SV=2 - [RPGF2_BOVIN]	1,01	1486	165,0	1			
Uncharacterized protein OS=Bos taurus GN=PPP1R14A PE=4 SV=1 - [E1BE60_BOVIN]	16,33	147	16,6			2	1
Uncharacterized protein OS=Bos taurus GN=PTPRK PE=4 SV=2 - [F1MME1_BOVIN]	2,01	1445	162,9	1			
Uncharacterized protein OS=Bos taurus GN=WDR13 PE=2 SV=1 - [Q08D81_BOVIN]	1,65	485	53,7		2	1	
26S protease regulatory subunit 10B OS=Bos taurus GN=PSMC6 PE=3 SV=2 - [F1MLV1_BOVIN]	8,74	389	44,1		1		
Putative malate dehydrogenase 1B OS=Bos taurus GN=MDH1B PE=2 SV=1 - [MDH1B_BOVIN]	3,59	473	53,1	1			

Aldehyde oxidase 3L1 OS=Bos taurus PE=2 SV=1 - [M1ZMN6_BOVIN]	2,01	1342	148,1			1	
KTN1 protein OS=Bos taurus GN=KTN1 PE=2 SV=1 - [A7MB48_BOVIN]	0,69	1302	150,5		1		
Uncharacterized protein (Fragment) OS=Bos taurus GN=ANKRD29 PE=4 SV=1 - [G3X683_BOVIN]	4,42	294	31,7		1		
Uncharacterized protein OS=Bos taurus GN=ACKR2 PE=4 SV=2 - [F1MV88_BOVIN]	10,20	343	37,9			2	
Uncharacterized protein OS=Bos taurus GN=CRAMP1L PE=4 SV=1 - [E1BN92_BOVIN]	1,41	1274	135,0				2
Uncharacterized protein (Fragment) OS=Bos taurus GN=SYNE1 PE=4 SV=2 - [F1MGT1_BOVIN]	0,32	8760	1004,5				1
Uncharacterized protein OS=Bos taurus GN=CEP63 PE=4 SV=1 - [E1BDP9_BOVIN]	1,13	705	81,3	1			
Uncharacterized protein OS=Bos taurus GN=UFSP2 PE=4 SV=1 - [E1BK69_BOVIN]	4,26	469	53,1				1
Uncharacterized protein OS=Bos taurus GN=HECTD1 PE=4 SV=2 - [E1BLD1_BOVIN]	1,42	2610	289,2				1
Myosin-7 OS=Bos taurus GN=MYH7 PE=4 SV=2 - [F1N2G0_BOVIN]	1,39	1940	223,8		1		
Uncharacterized protein OS=Bos taurus GN=KDM5A PE=4 SV=2 - [F1MQ59_BOVIN]	0,53	1693	192,1				2
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1 (Fragment) OS=Bos taurus GN=PLCG1 PE=4 SV=2 - [F1MYF9_BOVIN]	2,13	1219	140,4		1		
Uncharacterized protein OS=Bos taurus PE=4 SV=1 - [G3N0E3_BOVIN]	8,44	237	26,9	1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=MED13L PE=4 SV=2 - [F1N4I1_BOVIN]	2,15	2189	240,4		1	1	
Nucleoprotein OS=Bovine coronavirus (strain F15) GN=N PE=3 SV=1 - [NCAP_CVBF]	5,36	448	49,4			1	
Uncharacterized protein OS=Bos taurus GN=LOC785144 PE=3 SV=2 - [E1BPG5_BOVIN]	11,15	314	35,0			2	
Probable glutathione peroxidase 8 OS=Bos taurus GN=GPX8 PE=2 SV=1 - [GPX8_BOVIN]	10,05	209	24,0			2	
UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 9 OS=Bos taurus GN=B3GNT9 PE=2 SV=1 - [B3GN9_BOVIN]	3,74	401	43,8				1
Cadherin-18 OS=Bos taurus GN=CDH18 PE=2 SV=1 - [CAD18_BOVIN]	4,18	790	87,7				1
Ras-related protein Rab-18 OS=Bos taurus GN=RAB18 PE=2	14,56	206	23,0				1

SV=1 - [RAB18_BOVIN]							
S100P-binding protein OS=Bos taurus GN=S100PBP PE=2 SV=1 - [S1PBP_BOVIN]	10,19	422	47,1		1		
Transmembrane protein 184B OS=Bos taurus GN=TMEM184B PE=2 SV=1 - [T184B_BOVIN]	7,37	407	45,5			1	
Core protein VP3 OS=Bluetongue virus 1 (isolate Australia) GN=S3 PE=3 SV=1 - [VP3_BTV1A]	1,44	901	103,1				
BMP and activin membrane-bound inhibitor homolog OS=Bos taurus GN=BAMBI PE=2 SV=1 - [Q1RMX1_BOVIN]	13,85	260	29,1		1		
TBC1 domain family, member 19 OS=Bos taurus GN=TBC1D19 PE=2 SV=1 - [Q08DJ1_BOVIN]	3,80	526	60,2				1
Uncharacterized protein (Fragment) OS=Bos taurus GN=ARHGAP21 PE=4 SV=2 - [F1MHG5_BOVIN]	0,93	1941	215,1				1
Uncharacterized protein OS=Bos taurus GN=LRRC9 PE=4 SV=2 - [F1MNH4_BOVIN]	0,66	1374	158,4		1		
Uncharacterized protein OS=Bos taurus GN=C11orf42 PE=4 SV=1 - [E1BJG7_BOVIN]	9,61	333	36,5			1	
Olfactory receptor OS=Bos taurus PE=3 SV=2 - [F1N486_BOVIN]	11,69	308	34,2		1		
Uncharacterized protein (Fragment) OS=Bos taurus GN=PTGFRN PE=4 SV=1 - [F1MT41_BOVIN]	1,02	882	98,8				1
Uncharacterized protein OS=Bos taurus GN=ARMCX3 PE=4 SV=2 - [E1BE36_BOVIN]	2,37	379	42,5		1		
Uncharacterized protein (Fragment) OS=Bos taurus GN=CAPRIN2 PE=4 SV=2 - [F1MCP8_BOVIN]	0,55	1086	121,4	1			
Uncharacterized protein OS=Bos taurus GN=PRRT3 PE=4 SV=1 - [F6QJQ2_BOVIN]	2,87	977	101,4			1	
Rab GTPase-binding effector protein 2 OS=Bos taurus GN=RABEP2 PE=2 SV=1 - [RABE2_BOVIN]	3,76	585	65,6		1		
Uncharacterized protein OS=Bos taurus GN=ALMS1 PE=4 SV=2 - [E1B958_BOVIN]	0,79	3287	367,0		1		
Uncharacterized protein OS=Bos taurus GN=CCDC88C PE=4 SV=2 - [E1BCQ3_BOVIN]	0,44	2028	227,8	1			
Alpha-(1,3)-fucosyltransferase 10 OS=Bos taurus GN=FUT10 PE=3 SV=2 - [F1N557_BOVIN]	8,60	477	55,6				1
Uncharacterized protein OS=Bos taurus GN=GRIP2 PE=4 SV=2 - [F1MFS5_BOVIN]	3,18	1099	118,1		1		
TTC21B protein OS=Bos taurus GN=TTC21B PE=2 SV=1 - [A7MB60_BOVIN]	1,14	1316	151,1			1	
Uncharacterized protein (Fragment) OS=Bos taurus	1,37	1679	183,2			1	

GN=PRDM2 PE=4 SV=2 - [F1N790_BOVIN]							
Uncharacterized protein OS=Bos taurus GN=WAPAL PE=4 SV=1 - [E1BGC3_BOVIN]	2,85	1193	133,2			1	
Uncharacterized protein (Fragment) OS=Bos taurus GN=ITGBL1 PE=4 SV=2 - [E1BK10_BOVIN]	8,16	392	42,8			1	
Potassium voltage-gated channel subfamily KQT member 3 OS=Bos taurus GN=KCNQ3 PE=2 SV=1 - [KCNQ3_BOVIN]	2,08	866	95,6	1			
Metastasis associated 1 family, member 2 OS=Bos taurus GN=MTA2 PE=2 SV=1 - [Q1RMW3_BOVIN]	2,85	666	74,8	1			
Putative uncharacterized protein MGC139448 OS=Bos taurus GN=MGC139448 PE=2 SV=1 - [A1A4H7_BOVIN]	3,04	461	51,8			1	
Uncharacterized protein OS=Bos taurus PE=3 SV=1 - [F6R9F1_BOVIN]	4,83	497	55,4	1			
Acyl-coenzyme A oxidase OS=Bos taurus GN=ACOXL PE=3 SV=2 - [F1N6R7_BOVIN]	2,24	580	65,4	1			
Uncharacterized protein OS=Bos taurus GN=SLC26A10 PE=4 SV=2 - [F1MWU6_BOVIN]	5,31	490	52,7		1		
ATP10D protein OS=Bos taurus GN=ATP10D PE=2 SV=1 - [A7Z029_BOVIN]	2,39	1422	159,4				
Uncharacterized protein OS=Bos taurus GN=CATSPERB PE=4 SV=2 - [E1B9V5_BOVIN]	1,91	1099	125,2			1	
RNA polymerase II-associated factor 1 homolog OS=Bos taurus GN=PAF1 PE=4 SV=2 - [G1K1Z2_BOVIN]	7,89	355	41,2		1		
Serine--pyruvate aminotransferase OS=Bos taurus GN=AGXT PE=2 SV=1 - [A7MBF1_BOVIN]	5,80	414	45,3	1			
CDKL1 protein OS=Bos taurus GN=CDKL1 PE=2 SV=1 - [A6QLF0_BOVIN]	3,41	352	40,7	1			
EVI5L protein OS=Bos taurus GN=EVI5L PE=2 SV=1 - [A5PK17_BOVIN]	1,73	807	92,7			1	
Uncharacterized protein (Fragment) OS=Bos taurus GN=SLC35F4 PE=4 SV=2 - [F1MGI4_BOVIN]	7,16	517	57,4				1
SH3 domain-containing YSC84-like protein 1 (Fragment) OS=Bos taurus GN=SH3YL1 PE=4 SV=1 - [H9GW32_BOVIN]	10,61	245	27,2			1	
RNA-dependent RNA polymerase (Fragment) OS=Schmallenberg virus GN=RdRp PE=4 SV=1 - [I1YZX9_SBV]	15,28	144	16,8	1			
ATP-dependent RNA helicase DDX19A OS=Bos taurus GN=DDX19L PE=2 SV=1 - [Q58DE5_BOVIN]	6,60	394	44,1			1	
Uncharacterized protein OS=Bos taurus GN=FLRT3 PE=4 SV=2 - [F1N0R7_BOVIN]	3,85	649	73,1			1	

Uncharacterized protein OS=Bos taurus GN=PIK3CB PE=4 SV=1 - [G3MWH3_BOVIN]	4,26	657	75,5		1		
Uncharacterized protein (Fragment) OS=Bos taurus GN=MLXIP PE=4 SV=2 - [E1BIZ7_BOVIN]	2,33	858	94,2			1	
Uncharacterized protein OS=Bos taurus PE=4 SV=1 - [E1BAL3_BOVIN]	21,71	129	14,4		1		
Uncharacterized protein OS=Bos taurus GN=ZHX2 PE=3 SV=2 - [F1MTC3_BOVIN]	4,77	838	92,1		1		
PNKP protein OS=Bos taurus GN=PNKP PE=2 SV=1 - [A4FV28_BOVIN]	7,73	194	20,9				1
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [G3MY02_BOVIN]	8,37	251	29,0		1		
DNA polymerase OS=Cowpox virus GN=CPXV_FRA2001_NANCY_070 PE=3 SV=1 - [G0XTY8_COWPX]	3,38	1005	116,7			1	
Glycoprotein GX OS=Bovine herpesvirus 1.2 (strain ST) PE=3 SV=1 - [VGLX_BHV1S]	5,18	444	46,7				1
TXNL-like protein (Fragment) OS=Bos taurus PE=4 SV=1 - [Q5D0G0_BOVIN]	37,50	64	7,3			1	
Uncharacterized protein OS=Bos taurus GN=ETV2 PE=3 SV=1 - [E1B7I2_BOVIN]	9,04	332	36,5			1	
Uncharacterized protein OS=Bos taurus GN=FAM76B PE=4 SV=1 - [E1BI86_BOVIN]	8,26	339	38,6		1		
Uncharacterized protein OS=Bos taurus GN=CEP76 PE=4 SV=1 - [E1B8D7_BOVIN]	5,75	661	74,5			1	
Interleukin 32 beta OS=Bos taurus PE=2 SV=1 - [I7FR42_BOVIN]	19,30	171	19,5		1		
Uncharacterized protein (Fragment) OS=Bos taurus GN=TRAC PE=4 SV=2 - [F1MBR7_BOVIN]	22,93	157	17,2				1

Table 24. Retenate samples part 1 (Complete LC-MS/MS results)

Description	ΣCoverage	AAs	MW [kDa]	MFR 1.5 (S.1)	MFR 1.5 (S.1)	MFR 1.5 (S.1)	MFR 2.8 (S.1)	MFR 2.8 (S.1)
Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3 - [LACB_BOVIN]	86,52	178	19,9	72	102	101	46	72
Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2 - [CASA1_BOVIN]	74,30	214	24,5	179	187	176	151	222
Major allergen beta-lactoglobulin OS=Bos taurus PE=2 SV=1 - [B5B0D4_BOVIN]	86,52	178	20,0	59	95	85	46	70
Alpha S1 casein OS=Bos taurus GN=CSN1S1 PE=2 SV=1 - [B5B3R8_BOVIN]	69,16	214	24,4	165	183	172	140	219
Alpha-lactalbumin OS=Bos taurus GN=LALBA PE=1 SV=2 - [LALBA_BOVIN]	83,10	142	16,2	30	51	47	15	40
Alpha lactalbumin (Fragment) OS=Bos taurus GN=alfaLA PE=3 SV=1 - [Q28049_BOVIN]	65,85	123	14,1	32	51	43	15	36
Alpha-S2-casein OS=Bos taurus GN=CSN1S2 PE=1 SV=2 - [CASA2_BOVIN]	54,05	222	26,0	87	68	60	60	91
Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2 - [CASB_BOVIN]	21,43	224	25,1	41	32	43	18	48
Beta-lactoglobulin (Fragment) OS=Bos taurus GN=LGB PE=4 SV=1 - [E7E1Q8_BOVIN]	86,11	36	4,2	4	15	21	3	9
Kappa-casein (Fragment) OS=Bos taurus x Bos indicus GN=CSN3 PE=4 SV=1 - [Q9N258_9CETA]	61,88	160	17,9	31	84	29	27	34
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4 - [ALBU_BOVIN]	69,52	607	69,2	36	3	38	10	8
Kappa casein (Fragment) OS=Bos taurus GN=csn3 PE=4 SV=1 - [Q5ZET1_BOVIN]	61,87	139	15,2	30	26	45	25	24
Glycosylation-dependent cell adhesion molecule 1 OS=Bos taurus GN=GLYCAM1 PE=1 SV=2 - [GLCM1_BOVIN]	51,63	153	17,1	18	6	13	14	12
Enterotoxin-binding glycoprotein PP20K (Fragment) OS=Bos taurus PE=1 SV=1 - [Q9TRB9_BOVIN]	100,00	20	2,3	11	4		9	
PIGR protein OS=Bos taurus GN=PIGR PE=2 SV=1 - [A6QNW3_BOVIN]	39,23	757	82,5	6	2	25		2
Uncharacterized protein OS=Bos taurus GN=PIGR PE=4 SV=1 - [F1MR22_BOVIN]	31,84	757	82,5	6	2	16		2
Lactoperoxidase OS=Bos taurus GN=LPO PE=1 SV=1 - [PERL_BOVIN]	43,82	712	80,6			25		
Complement C3 OS=Bos taurus GN=C3 PE=1 SV=2 - [CO3_BOVIN]	21,25	1661	187,1			14		
Lactoferrin (Fragment) OS=Bos taurus PE=2 SV=1 - [C7FE01_BOVIN]	27,25	690	76,2	9	2	12	5	4
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [G5E5T5_BOVIN]	23,14	389	42,4	1		8		
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [G5E513_BOVIN]	19,26	457	49,9	1	1	7		
Xanthine dehydrogenase/oxidase OS=Bos taurus GN=XDH PE=4 SV=1 - [F1MUT3_BOVIN]	16,44	1332	146,7			20		
Uncharacterized protein OS=Bos taurus GN=A2ML1 PE=4 SV=2 - [F1MB32_BOVIN]	0,43	1396	155,5		2	2		3
Putative uncharacterized protein OS=Bos taurus PE=2 SV=1 - [A6QM09_BOVIN]	14,66	232	24,7			3		
Serpin A3-1 OS=Bos taurus GN=SERPINA3-1 PE=1 SV=3 - [SPA31_BOVIN]	23,11	411	46,2			3		
Butyrophilin subfamily 1 member A1 OS=Bos taurus GN=BTN1A1 PE=1 SV=2 - [BT1A1_BOVIN]	27,19	526	59,2	5		7		
Serpin A3-2 OS=Bos taurus GN=SERPINA3-2 PE=3 SV=1 - [SPA32_BOVIN]	23,11	411	46,2			2		
Isoform Short of Lactadherin OS=Bos taurus GN=MFGE8 - [MFGM_BOVIN]	17,38	374	41,5	2		10		6
LOC532995 protein OS=Bos taurus GN=LOC532995 PE=2 SV=1 - [A6QP80_BOVIN]	6,84	395	45,4					
Osteopontin OS=Bos taurus GN=SPP1 PE=2 SV=1 - [Q58DM6_BOVIN]	10,45	201	22,2			6		
Myocyte-specific enhancer factor 2C OS=Bos taurus GN=MEF2C PE=2 SV=1 - [MEF2C_BOVIN]	9,52	441	47,8	1		1		
Lipoprotein lipase OS=Bos taurus GN=LPL PE=1 SV=2 -	25,52	478	53,3	1		16		1

[LIPL_BOVIN]								
Ribonuclease 4 OS=Bos taurus GN=RNASE4 PE=1 SV=4 - [RNAS4_BOVIN]	31,09	119	13,7	6		2	2	
Uncharacterized protein OS=Bos taurus GN=ZNF192 PE=4 SV=1 - [G3N3E2_BOVIN]	6,27	271	31,0			1		2
Secretoglobin family 1D member OS=Bos taurus GN=SCGB1D PE=3 SV=2 - [SG1D_BOVIN]	8,82	102	11,3	4		3		
Serotransferrin OS=Bos taurus GN=TF PE=4 SV=1 - [G3X6N3_BOVIN]	10,23	704	77,6	1				
Uncharacterized protein OS=Bos taurus GN=GOLGA4 PE=4 SV=2 - [E1B7E3_BOVIN]	0,63	2229	259,0	1	1	2		1
Uncharacterized protein OS=Bos taurus GN=SRGAP3 PE=4 SV=2 - [F1MJI3_BOVIN]	0,91	1099	124,4			3		
Nucleobindin-1 OS=Bos taurus GN=NUCB1 PE=2 SV=1 - [NUCB1_BOVIN]	5,70	474	54,9			1		
Fatty acid-binding protein, heart OS=Bos taurus GN=FABP3 PE=1 SV=2 - [FABPH_BOVIN]	36,09	133	14,8			6		
Keratin, type II cytoskeletal 68 kDa, component IA (Fragment) OS=Bos taurus PE=2 SV=1 - [K2CA_BOVIN]	6,04	182	18,1			4		
Uncharacterized protein OS=Bos taurus GN=PTBP2 PE=4 SV=1 - [F6Q0P6_BOVIN]	2,54	236	25,6		2			1
Outer capsid protein (Fragment) OS=Bluetongue virus 10 GN=VP2 PE=4 SV=1 - [L7NSQ0_BTV1X]	8,64	162	18,8	1	1	1		
Angiogenin-1 OS=Bos taurus GN=ANG1 PE=1 SV=4 - [ANG1_BOVIN]	14,86	148	17,0			2		2
Uncharacterized protein OS=Bos taurus GN=PKHD1 PE=4 SV=2 - [E1BK86_BOVIN]	0,64	4071	443,9	1				
Uncharacterized protein OS=Bos taurus GN=MAN2A2 PE=4 SV=1 - [E1BGJ4_BOVIN]	1,04	1150	130,1				1	
Platelet glycoprotein 4 OS=Bos taurus GN=CD36 PE=1 SV=5 - [CD36_BOVIN]	10,17	472	52,9			7		
Mucin-1 OS=Bos taurus GN=MUC1 PE=1 SV=1 - [MUC1_BOVIN]	3,45	580	58,1			7		
UDP-glucose ceramide glucosyltransferase OS=Bos taurus GN=UGCG PE=2 SV=1 - [Q08DR4_BOVIN]	1,52	394	44,7		1			
Uncharacterized protein OS=Bos taurus GN=TXNDC16 PE=4 SV=1 - [E1BAG3_BOVIN]	2,67	824	92,9					1
Rap guanine nucleotide exchange factor 2 OS=Bos taurus GN=RAPGEF2 PE=1 SV=2 - [RPGF2_BOVIN]	1,01	1486	165,0				3	
Uncharacterized protein OS=Bos taurus GN=PPP1R14A PE=4 SV=1 - [E1BE60_BOVIN]	16,33	147	16,6					1
Uncharacterized protein (Fragment) OS=Bos taurus GN=MROH2A PE=4 SV=2 - [F1MZK6_BOVIN]	0,83	1570	177,9	1				
Uncharacterized protein (Fragment) OS=Bos taurus GN=TSGA10 PE=4 SV=2 - [E1BE64_BOVIN]	1,68	891	103,6		2			
Putative malate dehydrogenase 1B OS=Bos taurus GN=MDH1B PE=2 SV=1 - [MDH1B_BOVIN]	3,59	473	53,1				1	
Neuroepithelial cell transforming gene 1 OS=Bos taurus GN=NET1 PE=2 SV=1 - [Q3ZC77_BOVIN]	2,61	536	60,7				2	
Uncharacterized protein OS=Bos taurus GN=NCAPD3 PE=4 SV=2 - [F1MPS0_BOVIN]	2,36	1482	166,4		1			
Similar to beta 2-microglobulin (Fragment) OS=Bos taurus PE=2 SV=1 - [Q862Q3_BOVIN]	53,76	93	11,0			2		
Aldehyde oxidase 3L1 OS=Bos taurus PE=2 SV=1 - [M1ZMN6_BOVIN]	2,01	1342	148,1					1
KTN1 protein OS=Bos taurus GN=KTN1 PE=2 SV=1 - [A7MB48_BOVIN]	0,69	1302	150,5	1				
Prostamide/prostaglandin F synthase OS=Bos taurus GN=FAM213B PE=2 SV=1 - [PGFS_BOVIN]	32,34	201	21,5	1				
Uncharacterized protein (Fragment) OS=Bos taurus GN=BNC2 PE=4 SV=1 - [F1N447_BOVIN]	5,26	837	92,3					1
Isoform Short of Beta-1,4-galactosyltransferase 1 OS=Bos taurus GN=B4GALT1 - [B4GT1_BOVIN]	3,08	389	43,5			3		
Pyrroline-5-carboxylate reductase 1, mitochondrial OS=Bos taurus GN=PYCR1 PE=2 SV=1 - [P5CR1_BOVIN]	8,75	320	33,4		1			1
Uncharacterized protein OS=Bos taurus GN=GP2 PE=4	4,87	534	59,2			1		

SV=1 - [F1N726_BOVIN]								
Peroxisomal membrane protein PEX13 OS=Bos taurus GN=PEX13 PE=2 SV=1 - [PEX13_BOVIN]	12,90	403	44,3					1
Uncharacterized protein OS=Bos taurus GN=HNF1A PE=3 SV=1 - [F1MD26_BOVIN]	3,63	633	67,3					1
Glucose-6-phosphate 1-dehydrogenase OS=Bos taurus GN=G6PD PE=3 SV=1 - [F1MMK2_BOVIN]	3,11	515	59,3					1
Uncharacterized protein OS=Bos taurus GN=CEP63 PE=4 SV=1 - [E1BDP9_BOVIN]	1,13	705	81,3	1				
Uncharacterized protein (Fragment) OS=Bos taurus GN=UMODL1 PE=4 SV=2 - [F1MN54_BOVIN]	2,25	1333	144,3				1	
Uncharacterized protein OS=Bos taurus GN=NEB PE=4 SV=2 - [F1MT60_BOVIN]	0,29	4888	567,4	1				
Uncharacterized protein OS=Bos taurus GN=C9orf172 PE=4 SV=2 - [E1B9Y2_BOVIN]	1,95	975	105,9			1		
G protein-coupled receptor 43 OS=Bos taurus GN=GPR43 PE=2 SV=1 - [B9VJV9_BOVIN]	1,82	329	36,8	1				
Uncharacterized protein OS=Bos taurus GN=APBB2 PE=4 SV=1 - [F1MDE6_BOVIN]	2,51	758	83,1			2		
Uncharacterized protein OS=Bos taurus GN=SCUBE2 PE=4 SV=2 - [F1N7F6_BOVIN]	4,70	999	110,2		1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=ATP12A PE=3 SV=2 - [F1N1K4_BOVIN]	2,46	1099	121,5					1
Coiled-coil domain-containing protein 126 OS=Bos taurus GN=CCDC126 PE=2 SV=1 - [CC126_BOVIN]	13,57	140	15,6				1	
Uncharacterized protein OS=Bos taurus GN=HHIPL2 PE=4 SV=1 - [E1BGX8_BOVIN]	2,62	724	81,1			2		
tRNA (adenine(58)-N(1))-methyltransferase non-catalytic subunit TRM6 OS=Bos taurus GN=TRMT6 PE=2 SV=1 - [TRM6_BOVIN]	5,23	497	55,9	1				
SEC22 vesicle trafficking protein homolog B (S. cerevisiae) OS=Bos taurus GN=SEC22B PE=2 SV=1 - [Q3T0L9_BOVIN]	7,17	251	28,7		1			
Cytosolic purine 5'-nucleotidase OS=Bos taurus GN=NT5C2 PE=1 SV=1 - [5NTC_BOVIN]	3,04	560	64,8				1	
Ankyrin repeat and zinc finger domain-containing protein 1 OS=Bos taurus GN=ANKZF1 PE=2 SV=2 - [ANKZ1_BOVIN]	2,20	728	81,1				1	
DnaJ homolog subfamily A member 2 OS=Bos taurus GN=DNAJA2 PE=2 SV=1 - [DNJA2_BOVIN]	6,07	412	45,7			1		
Protein kintoun OS=Bos taurus GN=DNAAF2 PE=2 SV=2 - [KTU_BOVIN]	3,74	829	90,9					1
Hormone-sensitive lipase OS=Bos taurus GN=LIPE PE=1 SV=2 - [LIPS_BOVIN]	4,63	756	82,6			1		
Nucleolar protein 16 OS=Bos taurus GN=NOP16 PE=2 SV=1 - [NOP16_BOVIN]	7,30	178	21,1			1		
Radial spoke head 10 homolog B OS=Bos taurus GN=RSPH10B PE=2 SV=2 - [RS10B_BOVIN]	0,95	840	95,8				1	
PPIL5 protein OS=Bos taurus GN=PPIL5 PE=2 SV=1 - [A6QPV4_BOVIN]	1,42	424	47,8		1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=MYO9A PE=4 SV=1 - [G3MXE8_BOVIN]	0,82	1951	225,3	1				
Uncharacterized protein OS=Bos taurus GN=XRN2 PE=4 SV=1 - [F1MKX7_BOVIN]	0,84	951	108,4		1			
Uncharacterized protein OS=Bos taurus GN=HMCN2 PE=4 SV=2 - [E1B9K4_BOVIN]	0,79	5073	545,0	1				
Uncharacterized protein OS=Bos taurus GN=ARAP2 PE=4 SV=2 - [F1MTN6_BOVIN]	0,94	1711	194,7		1			
Uncharacterized protein OS=Bos taurus GN=TRIM69 PE=4 SV=1 - [E1BID7_BOVIN]	3,39	502	57,5		1			
Uncharacterized protein OS=Bos taurus GN=OTUD4 PE=4 SV=2 - [E1BN58_BOVIN]	2,52	1109	122,3		1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=DST PE=4 SV=2 - [F1MPT5_BOVIN]	0,49	7493	843,7			1		
Uncharacterized protein OS=Bos taurus GN=ZNF608 PE=4 SV=2 - [F1N312_BOVIN]	1,26	1512	161,9					1
Tyrosine-protein kinase OS=Bos taurus GN=TYK2 PE=3 SV=2 - [F1MCX4_BOVIN]	2,53	1185	132,6			1		

Uncharacterized protein OS=Bos taurus GN=SCN1A PE=3 SV=1 - [M0QW12_BOVIN]	0,80	1998	227,6				1	
Uncharacterized protein OS=Bos taurus GN=WHSC2 PE=4 SV=2 - [F1MW50_BOVIN]	6,71	432	45,9	1				
Tyrosine-protein kinase OS=Bos taurus GN=FER PE=3 SV=1 - [E1BNE0_BOVIN]	5,11	822	94,7			1		
Uncharacterized protein (Fragment) OS=Bos taurus GN=TRPC4AP PE=4 SV=2 - [F1N232_BOVIN]	1,96	816	92,7				1	
Uncharacterized protein OS=Bos taurus GN=PRSS53 PE=3 SV=2 - [F1N116_BOVIN]	3,62	553	59,2					1
Uncharacterized protein OS=Bos taurus GN=ARHGAP23 PE=4 SV=2 - [F1MMP0_BOVIN]	0,56	1248	135,6					1
E3 ubiquitin-protein ligase BRE1A OS=Bos taurus GN=RNF20 PE=2 SV=1 - [BRE1A_BOVIN]	3,18	975	113,6					1
Uncharacterized protein OS=Bos taurus GN=B4GALT7 PE=2 SV=1 - [Q17QI0_BOVIN]	4,25	259	29,5				1	
Uncharacterized protein (Fragment) OS=Bos taurus GN=LARP4 PE=4 SV=2 - [F1MHD9_BOVIN]	4,19	621	68,3					1
Uncharacterized protein (Fragment) OS=Bos taurus GN=PARD3 PE=4 SV=2 - [F1MZ23_BOVIN]	1,26	1267	141,2					1
IIIA OS=Bovine adenovirus B serotype 3 PE=4 SV=1 - [O71095_ADEB3]	3,52	568	63,2	1				
Cytochrome b-245 heavy chain OS=Bos taurus GN=CYBB PE=2 SV=1 - [CY24B_BOVIN]	6,49	570	65,6		1			
Envelope glycoprotein B OS=Bovine herpesvirus 2 (strain BMV) PE=3 SV=2 - [GB_BHV2B]	2,07	917	101,8				1	
Uncharacterized protein OS=Bos taurus GN=LPIN3 PE=4 SV=2 - [E1BLQ9_BOVIN]	4,59	850	93,1					1
Uncharacterized protein OS=Bos taurus GN=GGA3 PE=4 SV=1 - [G5E690_BOVIN]	2,93	581	62,2				1	
Uncharacterized protein OS=Bos taurus GN=LEMD3 PE=4 SV=2 - [E1BIJ4_BOVIN]	1,31	913	100,0			1		
Uncharacterized protein OS=Bos taurus GN=ABCA7 PE=3 SV=2 - [E1BCG1_BOVIN]	1,06	2169	236,0			1		
E2 glycoprotein (Fragment) OS=Bovine viral diarrhea virus PE=4 SV=1 - [L7RZ74_BVDV]	11,01	218	24,3			1		
Histatherin OS=Bos taurus PE=4 SV=1 - [C6KGD7_BOVIN]	20,69	58	7,2			1		
Kynurenine 3-monooxygenase OS=Bos taurus GN=KMO PE=3 SV=2 - [E1BN59_BOVIN]	7,71	480	55,0				1	
Uncharacterized protein OS=Bos taurus GN=LRRN3 PE=4 SV=2 - [F1MSM1_BOVIN]	3,53	708	79,0					1
Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial OS=Bos taurus GN=SUCLA2 PE=3 SV=2 - [F1MGC0_BOVIN]	7,99	463	50,1	1				
DDX31 protein OS=Bos taurus GN=DDX31 PE=2 SV=1 - [A6QP73_BOVIN]	2,45	734	80,8	1				
Uncharacterized protein OS=Bos taurus GN=ABCG1 PE=3 SV=1 - [E1BDU6_BOVIN]	3,61	665	74,1				1	
Uncharacterized protein OS=Bos taurus GN=FAM205A PE=4 SV=2 - [E1BHB6_BOVIN]	2,92	1334	148,2					1
SHD protein OS=Bos taurus GN=SHD PE=2 SV=1 - [A6QLW7_BOVIN]	8,82	136	15,4			1		
Uncharacterized protein OS=Bos taurus GN=DIRAS1 PE=4 SV=1 - [G5E6L2_BOVIN]	6,57	198	22,4				1	
Uncharacterized protein OS=Bos taurus GN=EHBP1L1 PE=4 SV=2 - [E1BL45_BOVIN]	1,24	1451	153,7	1				
GPR128 protein OS=Bos taurus GN=GPR128 PE=2 SV=1 - [A4IFD4_BOVIN]	2,64	795	88,3				1	
Adaptor-related protein complex 1, beta 1 subunit OS=Bos taurus GN=AP1B1 PE=2 SV=1 - [Q2KJB2_BOVIN]	2,54	828	90,4		1			
P24 (Fragment) OS=Borna disease virus PE=4 SV=1 - [O10404_BDV]	14,93	201	22,4	1				
Protein phosphatase 2, regulatory subunit B', alpha isoform OS=Bos taurus GN=PPP2R5A PE=2 SV=1 - [A2VDZ0_BOVIN]	5,32	489	56,5					1
Uncharacterized protein OS=Bos taurus GN=SAAL1	8,88	473	53,1			1		

PE=4 SV=1 - [E1BD67_BOVIN]								
Uncharacterized protein OS=Bos taurus GN=CCDC134 PE=4 SV=1 - [E1BFW1_BOVIN]	7,42	229	26,4				1	
Uncharacterized protein OS=Bos taurus GN=S1PR4 PE=4 SV=2 - [F1ME98_BOVIN]	6,23	385	41,8		1			
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=2 - [F1N3N1_BOVIN]	4,87	472	53,9	1				
Uncharacterized protein OS=Bos taurus GN=FLAD1 PE=4 SV=1 - [F6R6Q1_BOVIN]	3,67	490	54,2			1		
Perilipin OS=Bos taurus GN=PLIN2 PE=3 SV=1 - [F1MQB0_BOVIN]	2,19	411	45,1			1		
Mitochondrial glutamate carrier 1 OS=Bos taurus GN=SLC25A22 PE=2 SV=1 - [GHC1_BOVIN]	9,01	322	34,5	1				
Uncharacterized protein OS=Bos taurus GN=CRISP3 PE=4 SV=1 - [F6R3I5_BOVIN]	6,61	242	27,1			1		
Fibroblast growth factor OS=Bos taurus GN=FGF21 PE=3 SV=1 - [E1BDA6_BOVIN]	21,05	209	22,6	1				
CPXV074 protein OS=Cowpox virus GN=CPXV074 PE=4 SV=1 - [G0XUK2_COWPX]	7,33	273	31,9			1		
ATP-sensitive inward rectifier potassium channel 12 OS=Bos taurus GN=KCNJ12 PE=2 SV=1 - [A2VDS5_BOVIN]	9,13	427	48,4					1
Uncharacterized protein OS=Bos taurus GN=ELK1 PE=3 SV=2 - [F1MT98_BOVIN]	10,68	440	46,1			1		
60S ribosomal protein L6 OS=Bos taurus GN=RPL6 PE=2 SV=1 - [A8NJ40_BOVIN]	10,23	215	24,0		1			

Table 25. Retenate samples part 2 (Complete LC-MS/MS results)

Description	**ΣCoverage**	**AAs**	**MW [kDa]**	**UFR (S.1)**	**UFR (S.1)**	**UFR (S.1)**	**MFR (S.2)**	**MFR (S.2)**	**MFR (S.3)**	**MFR (S.4)**
Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3 - [LACB_BOVIN]	86,52	178	19,9	166	983	292	69	73	65	81
Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2 - [CASA1_BOVIN]	74,30	214	24,5	35	50	16	183	242	232	357
Major allergen beta-lactoglobulin OS=Bos taurus PE=2 SV=1 - [B5B0D4_BOVIN]	86,52	178	20,0	156	951	283	61	70	63	81
Alpha S1 casein OS=Bos taurus GN=CSN1S1 PE=2 SV=1 - [B5B3R8_BOVIN]	69,16	214	24,4	31	50	16	173	245	229	354
Alpha-lactalbumin OS=Bos taurus GN=LALBA PE=1 SV=2 - [LALBA_BOVIN]	83,10	142	16,2	82	316	102	24	30	57	15
Alpha lactalbumin (Fragment) OS=Bos taurus GN=alfaLA PE=3 SV=1 - [Q28049_BOVIN]	65,85	123	14,1	87	304	96	27	30	57	15
Alpha-S2-casein OS=Bos taurus GN=CSN1S2 PE=1 SV=2 - [CASA2_BOVIN]	54,05	222	26,0	13	9	10	81	47	116	83
Beta-casein OS=Bos taurus GN=CSN2 PE=1 SV=2 - [CASB_BOVIN]	21,43	224	25,1			11	30	56	49	68
Beta-lactoglobulin (Fragment) OS=Bos taurus GN=LGB PE=4 SV=1 - [E7E1Q8_BOVIN]	86,11	36	4,2	10	255	68	4	6	17	24
Kappa-casein (Fragment) OS=Bos taurus x Bos indicus GN=CSN3 PE=4 SV=1 - [Q9N258_9CETA]	61,88	160	17,9	11	6	4	33	42	35	80
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4 - [ALBU_BOVIN]	69,52	607	69,2	90	56	68	44	1	12	6
Kappa casein (Fragment) OS=Bos taurus GN=csn3 PE=4 SV=1 - [Q5ZET1_BOVIN]	61,87	139	15,2	5	5		43	17	37	35

Glycosylation-dependent cell adhesion molecule 1 OS=Bos taurus GN=GLYCAM1 PE=1 SV=2 - [GLCM1_BOVIN]	51,63	153	17,1	18	49	25	19	6	16	8
Enterotoxin-binding glycoprotein PP20K (Fragment) OS=Bos taurus PE=1 SV=1 - [Q9TRB9_BOVIN]	100,00	20	2,3	27	87	13	9		6	
PIGR protein OS=Bos taurus GN=PIGR PE=2 SV=1 - [A6QNW3_BOVIN]	39,23	757	82,5	36	31	40	8		4	4
Uncharacterized protein OS=Bos taurus GN=PIGR PE=4 SV=1 - [F1MR22_BOVIN]	31,84	757	82,5	24	21	27	8		4	4
Lactoperoxidase OS=Bos taurus GN=LPO PE=1 SV=1 - [PERL_BOVIN]	43,82	712	80,6	23	21	40				
Complement C3 OS=Bos taurus GN=C3 PE=1 SV=2 - [CO3_BOVIN]	21,25	1661	187,1		9	67				
Lactoferrin (Fragment) OS=Bos taurus PE=2 SV=1 - [C7FE01_BOVIN]	27,25	690	76,2	21	6	20	3		1	1
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [G5E5T5_BOVIN]	23,14	389	42,4	1	9	18			4	1
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [G5E513_BOVIN]	19,26	457	49,9	5	6	16			3	1
Xanthine dehydrogenase/oxidase OS=Bos taurus GN=XDH PE=4 SV=1 - [F1MUT3_BOVIN]	16,44	1332	146,7		6	23				
Uncharacterized protein OS=Bos taurus GN=A2ML1 PE=4 SV=2 - [F1MB32_BOVIN]	0,43	1396	155,5					4	5	5
Putative uncharacterized protein OS=Bos taurus PE=2 SV=1 - [A6QM09_BOVIN]	14,66	232	24,7	2	5	6			3	3
Serpin A3-1 OS=Bos taurus GN=SERPINA3-1 PE=1 SV=3 - [SPA31_BOVIN]	23,11	411	46,2			20				
Butyrophilin subfamily 1 member A1 OS=Bos taurus GN=BTN1A1 PE=1 SV=2 - [BT1A1_BOVIN]	27,19	526	59,2	1		7	3		2	
Serpin A3-2 OS=Bos taurus GN=SERPINA3-2 PE=3 SV=1 - [SPA32_BOVIN]	23,11	411	46,2			20				
Isoform Short of Lactadherin OS=Bos taurus GN=MFGE8 - [MFGM_BOVIN]	17,38	374	41,5						10	3
LOC532995 protein OS=Bos taurus GN=LOC532995 PE=2 SV=1 - [A6QP80_BOVIN]	6,84	395	45,4		1					
Osteopontin OS=Bos taurus GN=SPP1 PE=2 SV=1 - [Q58DM6_BOVIN]	10,45	201	22,2		2	5		1	1	
Myocyte-specific enhancer factor 2C OS=Bos taurus GN=MEF2C PE=2 SV=1 - [MEF2C_BOVIN]	9,52	441	47,8			1	1	4	4	
Lipoprotein lipase OS=Bos taurus GN=LPL PE=1 SV=2 - [LIPL_BOVIN]	25,52	478	53,3						2	
Apolipoprotein A-I OS=Bos taurus GN=APOA1 PE=1 SV=3 - [APOA1_BOVIN]	13,96	265	30,3	2		10				
Ribonuclease 4 OS=Bos taurus GN=RNASE4 PE=1 SV=4 - [RNAS4_BOVIN]	31,09	119	13,7				1			
Epididymal secretory protein E1 OS=Bos taurus GN=NPC2 PE=1	32,89	149	16,6	6	2	6				

SV=1 - [NPC2_BOVIN]										
Uncharacterized protein OS=Bos taurus GN=ZNF192 PE=4 SV=1 - [G3N3E2_BOVIN]	6,27	271	31,0					1	1	1
Secretoglobin family 1D member OS=Bos taurus GN=SCGB1D PE=3 SV=2 - [SG1D_BOVIN]	8,82	102	11,3				4			
Serotransferrin OS=Bos taurus GN=TF PE=4 SV=1 - [G3X6N3_BOVIN]	10,23	704	77,6	2		2				
Uncharacterized protein OS=Bos taurus GN=GOLGA4 PE=4 SV=2 - [E1B7E3_BOVIN]	0,63	2229	259,0				1			4
Uncharacterized protein (Fragment) OS=Bos taurus GN=PTPN21 PE=4 SV=2 - [F1MRH9_BOVIN]	0,51	1180	132,3		4	2				
Monocyte differentiation antigen CD14 OS=Bos taurus GN=CD14 PE=3 SV=1 - [A8DBT6_BOVIN]	9,12	373	39,6	3		4				
Uncharacterized protein OS=Bos taurus GN=SRGAP3 PE=4 SV=2 - [F1MJI3_BOVIN]	0,91	1099	124,4				1		1	
Uncharacterized protein OS=Bos taurus GN=SERPING1 PE=3 SV=2 - [E1BMJ0_BOVIN]	9,40	468	51,7			7				
Nucleobindin-1 OS=Bos taurus GN=NUCB1 PE=2 SV=1 - [NUCB1_BOVIN]	5,70	474	54,9		2	5				
Fatty acid-binding protein, heart OS=Bos taurus GN=FABP3 PE=1 SV=2 - [FABPH_BOVIN]	36,09	133	14,8	1		3	1			
Uncharacterized protein OS=Bos taurus GN=SERPINA3 PE=3 SV=1 - [G8JKW7_BOVIN]	5,83	412	46,3			5				
Keratin, type II cytoskeletal 68 kDa, component IA (Fragment) OS=Bos taurus PE=2 SV=1 - [K2CA_BOVIN]	6,04	182	18,1			3				
Uncharacterized protein OS=Bos taurus GN=ATMIN PE=4 SV=2 - [F1MEP8_BOVIN]	0,97	822	88,4		3	4				
Alpha-2-adrenergic receptor (Fragment) OS=Bos taurus PE=2 SV=1 - [O97717_BOVIN]	48,98	49	5,6							3
Uncharacterized protein OS=Bos taurus GN=PTBP2 PE=4 SV=1 - [F6Q0P6_BOVIN]	2,54	236	25,6						1	2
Outer capsid protein (Fragment) OS=Bluetongue virus 10 GN=VP2 PE=4 SV=1 - [L7NSQ0_BTV1X]	8,64	162	18,8				1			2
Angiogenin-1 OS=Bos taurus GN=ANG1 PE=1 SV=4 - [ANG1_BOVIN]	14,86	148	17,0						5	
Hemopexin OS=Bos taurus GN=HPX PE=2 SV=1 - [HEMO_BOVIN]	4,36	459	52,2			4				
Structural glycoprotein E2 (Fragment) OS=Bovine viral diarrhea virus PE=4 SV=1 - [K7XEH4_BVDV]	3,15	286	31,7			4				
Sodium/hydrogen exchanger (Fragment) OS=Bos taurus GN=SLC9A7 PE=3 SV=1 - [G5E545_BOVIN]	1,67	660	73,5				3			1
Vitamin D-binding protein OS=Bos taurus GN=GC PE=4 SV=2 - [F1N5M2_BOVIN]	6,96	474	53,3			3				
Uncharacterized protein (Fragment)	8,59	326	35,9		3					

OS=Bos taurus PE=4 SV=1 - [G3N0V0_BOVIN]										
UDP-glucose ceramide glucosyltransferase OS=Bos taurus GN=UGCG PE=2 SV=1 - [Q08DR4_BOVIN]	1,52	394	44,7						2	1
Olfactory receptor OS=Bos taurus GN=OR2T33 PE=3 SV=1 - [M0QVY4_BOVIN]	8,97	312	35,1				1			
Uncharacterized protein OS=Bos taurus PE=4 SV=1 - [G3N360_BOVIN]	10,76	158	17,9	2			1			
Alpha-1-antiproteinase OS=Bos taurus GN=SERPINA1 PE=1 SV=1 - [A1AT_BOVIN]	3,61	416	46,1			3				
Apolipoprotein A-IV OS=Bos taurus GN=APOA4 PE=3 SV=1 - [F1N3Q7_BOVIN]	4,47	380	43,0			3				
Uncharacterized protein OS=Bos taurus GN=ARHGEF12 PE=4 SV=1 - [F1MZA2_BOVIN]	0,39	1544	172,6		2					
Uncharacterized protein (Fragment) OS=Bos taurus GN=MROH2A PE=4 SV=2 - [F1MZK6_BOVIN]	0,83	1570	177,9	1						
Uncharacterized protein (Fragment) OS=Bos taurus GN=TSGA10 PE=4 SV=2 - [E1BE64_BOVIN]	1,68	891	103,6		1			1	1	
Uncharacterized protein OS=Bos taurus GN=PTPRK PE=4 SV=2 - [F1MME1_BOVIN]	2,01	1445	162,9				3			
GTP-binding protein 1 OS=Bos taurus GN=GTPBP1 PE=4 SV=1 - [G3MX26_BOVIN]	2,32	603	65,9	3						
Protein HP-25 homolog 2 OS=Bos taurus PE=2 SV=1 - [HP252_BOVIN]	13,02	215	22,9			3				
Cation-independent mannose-6-phosphate receptor OS=Bos taurus GN=IGF2R PE=4 SV=2 - [F1MIE6_BOVIN]	4,80	2499	274,8	2						
Uncharacterized protein OS=Bos taurus GN=LRIG1 PE=4 SV=2 - [F1MZV0_BOVIN]	1,47	1090	118,3				1	1		
Uncharacterized protein OS=Bos taurus GN=CCM2 PE=4 SV=2 - [E1B8H2_BOVIN]	2,31	476	52,5							1
Inter-alpha-trypsin inhibitor heavy chain H4 OS=Bos taurus GN=ITIH4 PE=4 SV=2 - [F1MMD7_BOVIN]	1,42	916	101,5			2				
26S protease regulatory subunit 10B OS=Bos taurus GN=PSMC6 PE=3 SV=2 - [F1MLV1_BOVIN]	8,74	389	44,1		1	1				
CutA divalent cation tolerance homolog (E. coli) OS=Bos taurus GN=CUTA PE=2 SV=1 - [Q1RMP3_BOVIN]	3,92	153	16,3				1			
Neuroepithelial cell transforming gene 1 OS=Bos taurus GN=NET1 PE=2 SV=1 - [Q3ZC77_BOVIN]	2,61	536	60,7				1			
45 kDa calcium-binding protein (Fragment) OS=Bos taurus GN=SDF4 PE=4 SV=2 - [F1MKI5_BOVIN]	7,48	254	29,8			2				
Uncharacterized protein OS=Bos taurus GN=NCAPD3 PE=4 SV=2 - [F1MPS0_BOVIN]	2,36	1482	166,4	2						
Uncharacterized protein OS=Bos taurus GN=CREG1 PE=4 SV=1 - [F1MX50_BOVIN]	9,50	221	23,9			4				

Similar to beta 2-microglobulin (Fragment) OS=Bos taurus PE=2 SV=1 - [Q862Q3_BOVIN]	53,76	93	11,0		1					
F10 protein (Fragment) OS=Bos taurus GN=F10 PE=2 SV=1 - [Q3MHW2_BOVIN]	1,86	483	53,5					1		
KTN1 protein OS=Bos taurus GN=KTN1 PE=2 SV=1 - [A7MB48_BOVIN]	0,69	1302	150,5		1					
NOC3L protein OS=Bos taurus GN=NOC3L PE=2 SV=1 - [A5D7R2_BOVIN]	0,88	799	92,3						2	
Prostamide/prostaglandin F synthase OS=Bos taurus GN=FAM213B PE=2 SV=1 - [PGFS_BOVIN]	32,34	201	21,5					1		
Uncharacterized protein OS=Bos taurus GN=DOCK11 PE=4 SV=1 - [G5E580_BOVIN]	1,01	2074	237,8		3					
Uncharacterized protein OS=Bos taurus GN=ARHGAP18 PE=4 SV=2 - [E1BIH5_BOVIN]	3,39	531	59,6				1			
STAU1 protein (Fragment) OS=Bos taurus GN=STAU1 PE=2 SV=1 - [A6QNY4_BOVIN]	4,71	531	58,2		1			1		1
Uncharacterized protein (Fragment) OS=Bos taurus GN=BNC2 PE=4 SV=1 - [F1N447_BOVIN]	5,26	837	92,3			1				
Pyrroline-5-carboxylate reductase 1, mitochondrial OS=Bos taurus GN=PYCR1 PE=2 SV=1 - [P5CR1_BOVIN]	8,75	320	33,4							1
Uncharacterized protein OS=Bos taurus GN=GP2 PE=4 SV=1 - [F1N726_BOVIN]	4,87	534	59,2	1		1				
Uncharacterized protein OS=Bos taurus GN=ACKR2 PE=4 SV=2 - [F1MV88_BOVIN]	10,20	343	37,9		1					
Alpha-2-HS-glycoprotein OS=Bos taurus GN=AHSG PE=1 SV=2 - [FETUA_BOVIN]	4,74	359	38,4	2						
Isoform 2 of Collagen type IV alpha-3-binding protein OS=Bos taurus GN=COL4A3BP - [C43BP_BOVIN]	5,69	598	68,0			1				
Peroxisomal membrane protein PEX13 OS=Bos taurus GN=PEX13 PE=2 SV=1 - [PEX13_BOVIN]	12,90	403	44,3							1
Selenium-binding protein 1 OS=Bos taurus GN=SELENBP1 PE=1 SV=1 - [SBP1_BOVIN]	1,48	472	52,5							1
Uncharacterized protein (Fragment) OS=Bos taurus GN=SPTB PE=4 SV=2 - [F1MKE9_BOVIN]	0,64	2335	268,7				1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=DNAH6 PE=4 SV=2 - [F1MZU0_BOVIN]	0,39	1794	207,4					2		
Uncharacterized protein OS=Bos taurus GN=ALS2CR11 PE=4 SV=2 - [E1BEA6_BOVIN]	1,95	1847	211,5		1		1			
Uncharacterized protein OS=Bos taurus GN=UFSP2 PE=4 SV=1 - [E1BK69_BOVIN]	4,26	469	53,1					1		
Uncharacterized protein (Fragment) OS=Bos taurus GN=CACNA1H PE=4 SV=2 - [F1MQV2_BOVIN]	0,44	2258	248,6							2
Uncharacterized protein (Fragment) OS=Bos taurus GN=IGFN1 PE=4 SV=1 - [G3MZU6_BOVIN]	0,53	2629	266,9			1				
Uncharacterized protein (Fragment) OS=Bos taurus GN=SYF2 PE=4	5,11	235	28,0			1				

SV=2 - [F1N607_BOVIN]										
Myosin-7 OS=Bos taurus GN=MYH7 PE=4 SV=2 - [F1N2G0_BOVIN]	1,39	1940	223,8							1
Uncharacterized protein OS=Bos taurus GN=NEB PE=4 SV=2 - [F1MT60_BOVIN]	0,29	4888	567,4				1			
Protein OS-9 OS=Bos taurus GN=OS9 PE=4 SV=1 - [F1MX65_BOVIN]	2,40	667	75,8			1				
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1 (Fragment) OS=Bos taurus GN=PLCG1 PE=4 SV=2 - [F1MYF9_BOVIN]	2,13	1219	140,4		1					
G-protein coupled receptor 39 OS=Bos taurus GN=GPR39 PE=2 SV=1 - [GPR39_BOVIN]	5,95	454	51,4							2
Uncharacterized protein OS=Bos taurus GN=CDH23 PE=4 SV=2 - [F1MXP9_BOVIN]	0,42	3354	369,0							2
Cyclin-dependent kinase 1 OS=Bos taurus GN=CDK1 PE=2 SV=2 - [CDK1_BOVIN]	3,03	297	34,0				1			
G protein-coupled receptor 43 OS=Bos taurus GN=GPR43 PE=2 SV=1 - [B9VJV9_BOVIN]	1,82	329	36,8	1						
Uncharacterized protein OS=Bos taurus GN=CCAR2 PE=4 SV=1 - [E1B9H3_BOVIN]	2,51	916	101,9		2					
Uncharacterized protein OS=Bos taurus GN=DOCK4 PE=4 SV=2 - [E1BMG8_BOVIN]	1,12	1973	225,4	2						
Uncharacterized protein OS=Bos taurus GN=N4BP3 PE=4 SV=1 - [E1BLB8_BOVIN]	5,34	543	60,3		1					
Uncharacterized protein OS=Bos taurus GN=POU4F2 PE=3 SV=2 - [E1BLU8_BOVIN]	6,13	408	43,0	2						
Uncharacterized protein (Fragment) OS=Bos taurus GN=SYTL5 PE=4 SV=2 - [F1MMM4_BOVIN]	5,45	752	83,7		2					
WC1-12 OS=Bos taurus PE=2 SV=1 - [G1FM81_BOVIN]	3,70	1377	147,4			1	1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=ATP12A PE=3 SV=2 - [F1N1K4_BOVIN]	2,46	1099	121,5							1
Coiled-coil domain-containing protein 126 OS=Bos taurus GN=CCDC126 PE=2 SV=1 - [CC126_BOVIN]	13,57	140	15,6	1						
Nucleoprotein OS=Bovine coronavirus (strain F15) GN=N PE=3 SV=1 - [NCAP_CVBF]	5,36	448	49,4			1				
Sodium channel, voltage-gated, type II, beta-like OS=Bos taurus GN=SCN2B PE=4 SV=1 - [E1B757_BOVIN]	4,65	215	24,3				1			
Uncharacterized protein OS=Bos taurus GN=DPP8 PE=4 SV=2 - [F1N1L4_BOVIN]	4,23	898	103,3		2					
Uncharacterized protein (Fragment) OS=Bos taurus GN=AMPD1 PE=4 SV=2 - [F1MLX6_BOVIN]	6,61	620	72,3						2	
Uncharacterized protein OS=Bos taurus GN=LOC533821 PE=4 SV=2 - [E1BCY7_BOVIN]	2,98	1408	150,1			1				

tRNA (adenine(58)-N(1))-methyltransferase non-catalytic subunit TRM6 OS=Bos taurus GN=TRMT6 PE=2 SV=1 - [TRM6_BOVIN]	5,23	497	55,9		1					
Uncharacterized protein OS=Bos taurus GN=SLC38A10 PE=4 SV=2 - [E1BF27_BOVIN]	1,75	1084	115,6			1				
Mannose-P-dolichol utilization defect 1 OS=Bos taurus GN=MPDU1 PE=2 SV=1 - [Q148D6_BOVIN]	2,44	246	26,6		2					
NADH-ubiquinone oxidoreductase chain 5 OS=Bos taurus GN=ND5 PE=3 SV=1 - [I3PEU1_BOVIN]	5,45	606	68,3			2				
SEC22 vesicle trafficking protein homolog B (S. cerevisiae) OS=Bos taurus GN=SEC22B PE=2 SV=1 - [Q3T0L9_BOVIN]	7,17	251	28,7						1	
HFE OS=Bos taurus GN=HFE PE=2 SV=1 - [Q5EEZ1_BOVIN]	7,02	356	40,6			2				
Nucleolar protein NOP52 OS=Bos taurus GN=D21S2056E PE=2 SV=1 - [A1L546_BOVIN]	4,87	411	46,5							2
Coronin-2A OS=Bos taurus GN=CORO2A PE=2 SV=1 - [COR2A_BOVIN]	6,67	525	59,8			1				
Cleavage and polyadenylation specificity factor subunit 3 OS=Bos taurus GN=CPSF3 PE=1 SV=1 - [CPSF3_BOVIN]	4,24	684	77,4				1			
Dematin OS=Bos taurus GN=DMTN PE=2 SV=1 - [DEMA_BOVIN]	6,40	406	45,5							1
Inter-alpha-trypsin inhibitor heavy chain H5 OS=Bos taurus GN=ITIH5 PE=2 SV=1 - [ITIH5_BOVIN]	2,87	940	104,3		1					
Gag-Pol polyprotein OS=Bovine immunodeficiency virus (strain R29) GN=gag-pol PE=1 SV=2 - [POL_BIV29]	0,41	1475	168,0							1
Trifunctional purine biosynthetic protein adenosine-3 OS=Bos taurus GN=GART PE=2 SV=1 - [PUR2_BOVIN]	1,29	1010	107,8				1			
DNA-directed RNA polymerase OS=Bos taurus GN=POLR1B PE=2 SV=1 - [A6QLS0_BOVIN]	3,52	1135	128,3							1
MGC166151 protein OS=Bos taurus GN=MGC166151 PE=2 SV=1 - [A7MB79_BOVIN]	11,33	203	23,3						1	
INTS10 protein OS=Bos taurus GN=INTS10 PE=2 SV=1 - [A5PJT2_BOVIN]	3,09	711	82,1	1						
Uncharacterized protein OS=Bos taurus GN=VPS13D PE=4 SV=2 - [E1BIF6_BOVIN]	0,30	4332	484,4		1					
Uncharacterized protein OS=Bos taurus GN=SEC22A PE=4 SV=2 - [F1N2T5_BOVIN]	9,48	306	35,0			1				
Uncharacterized protein (Fragment) OS=Bos taurus GN=ANK3 PE=4 SV=2 - [E1BNC9_BOVIN]	0,81	3342	366,7						1	
Uncharacterized protein OS=Bos taurus GN=TCF20 PE=4 SV=2 - [E1B8T3_BOVIN]	1,32	1968	211,9				1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=IL1RAP PE=4 SV=2 - [E1BFL8_BOVIN]	6,11	687	78,5	1						
Uncharacterized protein OS=Bos taurus GN=FANCI PE=4 SV=2 -	2,41	1327	149,3	1						

[E1BDI4_BOVIN]										
Uncharacterized protein OS=Bos taurus PE=4 SV=1 - [G3MZY7_BOVIN]	46,07	89	10,0			1				
Uncharacterized protein (Fragment) OS=Bos taurus GN=FLII PE=4 SV=1 - [F1N365_BOVIN]	3,39	1268	144,1			1				
Uncharacterized protein (Fragment) OS=Bos taurus GN=LOC101909859 PE=4 SV=2 - [F1MF62_BOVIN]	1,33	1882	213,2		1					
Iodothyronine deiodinase OS=Bos taurus GN=DIO1 PE=3 SV=2 - [F1MDU1_BOVIN]	19,20	125	14,4		1					
Uncharacterized protein OS=Bos taurus GN=RNF213 PE=4 SV=2 - [E1BHN4_BOVIN]	0,64	3275	374,0	1						
Uncharacterized protein (Fragment) OS=Bos taurus GN=SPECC1 PE=4 SV=2 - [F1MEN2_BOVIN]	2,67	974	107,7							1
Uncharacterized protein OS=Bos taurus GN=CNBD2 PE=4 SV=1 - [E1BIR4_BOVIN]	1,37	583	68,4							1
Uncharacterized protein OS=Bos taurus GN=PRR12 PE=4 SV=2 - [F1MSK1_BOVIN]	1,13	2038	210,7					1		
Uncharacterized protein (Fragment) OS=Bos taurus GN=SHROOM3 PE=4 SV=2 - [F1N643_BOVIN]	1,01	1881	203,3							1
Putative uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1 - [A0A2V0_BOVIN]	16,37	171	16,6					1		
Uncharacterized protein OS=Bos taurus GN=KBTBD8 PE=4 SV=2 - [F1N465_BOVIN]	1,16	601	68,8						1	
Uncharacterized protein OS=Bos taurus GN=IL17RE PE=4 SV=1 - [E1B8L3_BOVIN]	4,94	668	74,0	1						
Uveal autoantigen with coiled-coil domains and ankyrin repeats protein OS=Bos taurus GN=UACA PE=4 SV=2 - [F1MKQ9_BOVIN]	0,64	1401	161,2	1						
Uncharacterized protein OS=Bos taurus GN=DOK6 PE=4 SV=1 - [G3N3X9_BOVIN]	4,23	331	38,2					1		
Mucin-5B OS=Bos taurus GN=MUC5B PE=4 SV=1 - [F2FB42_BOVIN]	1,71	6724	689,4		1					
TEK tyrosine kinase, endothelial OS=Bos taurus GN=TEK PE=2 SV=1 - [Q0IIL9_BOVIN]	2,22	1124	125,7			1				
Uncharacterized protein OS=Bos taurus GN=COG2 PE=4 SV=1 - [F1MQ89_BOVIN]	1,76	739	83,4	1						
Ankyrin repeat protein OS=Lumpy skin disease virus GN=LW152 PE=4 SV=1 - [Q8JTM7_LSDV]	3,48	489	57,4							1
Polycystin-2 OS=Bos taurus GN=PKD2 PE=2 SV=1 - [PKD2_BOVIN]	1,13	970	109,7		1					
Serine/threonine-protein kinase haspin OS=Bos taurus GN=GSG2 PE=4 SV=1 - [F1MG77_BOVIN]	2,05	781	86,3							1
Uncharacterized protein (Fragment) OS=Bos taurus GN=LOC505468 PE=3 SV=2 - [F1N6N4_BOVIN]	4,67	493	55,5				1			
Uncharacterized protein OS=Bos taurus GN=KIAA1549L PE=4 SV=2 - [F1MD25_BOVIN]	1,62	1847	198,3					1		

Uncharacterized protein OS=Bos taurus GN=ATG2B PE=4 SV=2 - [E1BH30_BOVIN]	1,06	2082	232,4		1					
Uncharacterized protein (Fragment) OS=Bos taurus GN=COL25A1 PE=4 SV=1 - [G3MYG7_BOVIN]	4,06	493	47,6			1				
CCDC80 protein OS=Bos taurus GN=CCDC80 PE=2 SV=1 - [A5PKA3_BOVIN]	2,73	954	108,2			1				
DnaJ (Hsp40) related, subfamily B, member 13 OS=Bos taurus GN=DNAJB13 PE=2 SV=1 - [Q3SZW9_BOVIN]	4,43	316	36,1		1					
BTB (POZ) domain containing 12 OS=Bos taurus GN=SLX4 PE=4 SV=2 - [F1N7Q0_BOVIN]	0,71	1828	197,5							1
Centromere protein Q OS=Bos taurus GN=CENPQ PE=2 SV=1 - [Q08DW5_BOVIN]	6,69	269	31,0			1				
LOC522691 protein OS=Bos taurus GN=LOC522691 PE=2 SV=1 - [A5D7C7_BOVIN]	4,04	569	63,3				1			
Uncharacterized protein OS=Bos taurus GN=KPNB1 PE=4 SV=2 - [E1BFV0_BOVIN]	2,63	876	97,2			1				
Uncharacterized protein OS=Bos taurus GN=SGMS2 PE=4 SV=1 - [E1BNX6_BOVIN]	6,85	365	42,4							1
Transferrin receptor 2 OS=Bos taurus GN=TFR2 PE=2 SV=1 - [D5KB40_BOVIN]	2,86	803	88,3				1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=MIPEP PE=3 SV=1 - [F1MX73_BOVIN]	1,26	712	81,0				1			
Dimethylaniline monooxygenase [N-oxide-forming] OS=Bos taurus PE=3 SV=2 - [F1N007_BOVIN]	5,62	534	61,2		1					
Uncharacterized protein OS=Bos taurus GN=FLNC PE=4 SV=1 - [E1BE25_BOVIN]	0,40	2723	290,6						1	
Uncharacterized protein OS=Bos taurus GN=MUSK PE=4 SV=2 - [E1BKP7_BOVIN]	0,92	868	96,8		1					
Uncharacterized protein OS=Bos taurus GN=IFT172 PE=4 SV=1 - [E1BJT7_BOVIN]	1,14	1749	197,1			1				
Uncharacterized protein OS=Bos taurus GN=L3MBTL3 PE=4 SV=2 - [F1MZS7_BOVIN]	0,79	755	85,7				1			
Melanoma antigen family B-like OS=Bos taurus GN=MGC133764 PE=2 SV=1 - [Q2T9P7_BOVIN]	6,65	361	39,4				1			
Uncharacterized protein OS=Bos taurus GN=LUC7L2 PE=4 SV=2 - [F1MYQ8_BOVIN]	6,38	392	46,5			1				
Uncharacterized protein OS=Bos taurus GN=EFCAB5 PE=4 SV=2 - [E1B7P6_BOVIN]	2,45	1385	158,1	1						
Uncharacterized protein OS=Bos taurus GN=MME PE=4 SV=1 - [E1BPL8_BOVIN]	1,33	750	85,8	1						
Nuclear factor NF-kappa-B p105 subunit-like protein (Fragment) OS=Bos taurus PE=4 SV=1 - [Q5MAD5_BOVIN]	8,75	80	9,0	1						
Polyprotein (Fragment) OS=Bovine viral diarrhea virus PE=4 SV=1 - [R9RZB4_BVDV]	2,69	1078	121,5			1				

Putative uncharacterized protein OS=Cowpox virus GN=CPXV_FIN2000_MAN_034 PE=4 SV=1 - [G0XT89_COWPX]	10,48	229	27,0	1						
SNTB2 protein OS=Bos taurus GN=SNTB2 PE=2 SV=1 - [A7YWQ4_BOVIN]	2,96	540	57,9			1				
Complement C5a anaphylatoxin OS=Bos taurus GN=C5 PE=4 SV=2 - [F1MY85_BOVIN]	2,44	1677	188,9		1					
L-serine dehydratase/L-threonine deaminase OS=Bos taurus GN=SDS PE=4 SV=1 - [F1N0R8_BOVIN]	3,36	327	34,4		1					
Uncharacterized protein (Fragment) OS=Bos taurus GN=SOX7 PE=4 SV=1 - [G3N2M6_BOVIN]	3,69	406	43,9							1
Uncharacterized protein OS=Bos taurus GN=ABCA13 PE=4 SV=1 - [E1BM08_BOVIN]	3,43	787	88,5					1		
Actin-related protein T2 OS=Bos taurus GN=ACTRT2 PE=2 SV=1 - [ACTT2_BOVIN]	7,43	377	41,9		1					
ATP synthase subunit a OS=Bos indicus GN=MT-ATP6 PE=3 SV=1 - [ATP6_BOSIN]	16,37	226	24,7		1					
Periodic tryptophan protein 1 homolog OS=Bos taurus GN=PWP1 PE=2 SV=1 - [PWP1_BOVIN]	4,60	500	55,8			1				
DDX31 protein OS=Bos taurus GN=DDX31 PE=2 SV=1 - [A6QP73_BOVIN]	2,45	734	80,8							
Uncharacterized protein (Fragment) OS=Bos taurus GN=CASKIN1 PE=4 SV=2 - [F1MB71_BOVIN]	1,77	1413	147,3			1				
Uncharacterized protein OS=Bos taurus GN=PPP2R1B PE=4 SV=2 - [F1MQN0_BOVIN]	3,31	695	76,7	1						
Vacuolar protein sorting-associated protein 51 homolog OS=Bos taurus GN=VPS51 PE=4 SV=2 - [F1MJ84_BOVIN]	1,70	706	78,0						1	
Uncharacterized protein (Fragment) OS=Bos taurus GN=NHS PE=4 SV=1 - [G3X6S6_BOVIN]	1,83	1363	147,7				1			
Uncharacterized protein OS=Bos taurus GN=EPHX2 PE=4 SV=1 - [F6QS88_BOVIN]	5,23	555	62,7		1					
Chromodomain-helicase-DNA-binding protein 1-like OS=Bos taurus GN=CHD1L PE=4 SV=2 - [F1MFS2_BOVIN]	4,35	896	101,2		1					
Uncharacterized protein OS=Bos taurus GN=KIAA1430 PE=4 SV=1 - [F1MSC2_BOVIN]	3,42	527	58,7			1				
Uncharacterized protein (Fragment) OS=Bos taurus GN=AGRN PE=4 SV=1 - [F1MSI2_BOVIN]	0,79	2032	215,1		1					
Uncharacterized protein OS=Bos taurus GN=INHBE PE=3 SV=1 - [E1BFT5_BOVIN]	4,89	348	38,3				1			
T cell receptor, alpha OS=Bos taurus GN=TRA@ PE=2 SV=1 - [Q3SZN6_BOVIN]	9,33	268	29,5				1			
Uncharacterized protein OS=Bos taurus GN=PTCHD1 PE=4 SV=1 - [G5E5Q5_BOVIN]	2,70	888	101,2				1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=FAM102B PE=4 SV=2 - [F1N4Z9_BOVIN]	7,78	334	36,7			1				

CRY1 protein OS=Bos taurus GN=CRY1 PE=2 SV=1 - [A7YWC2_BOVIN]	3,58	587	66,4						1	
Uncharacterized protein OS=Bos taurus GN=EFTUD1 PE=4 SV=2 - [E1BH79_BOVIN]	2,21	1129	126,1		1					
Uncharacterized protein (Fragment) OS=Bos taurus GN=LOC100139826 PE=4 SV=1 - [G3N2C2_BOVIN]	33,63	113	12,9				1			
NADPH oxidase 4 (Fragment) OS=Bos taurus PE=2 SV=1 - [Q6V1P7_BOVIN]	5,02	518	59,9							1
Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=2 - [F1MT34_BOVIN]	11,22	303	34,2		1					
Uncharacterized protein (Fragment) OS=Bos taurus GN=C1orf168 PE=4 SV=1 - [G3MWP7_BOVIN]	3,01	697	78,5	1						
Uncharacterized protein OS=Bos taurus GN=GRK6 PE=3 SV=2 - [E1BP29_BOVIN]	3,69	597	67,9				1			
RBM39 protein (Fragment) OS=Bos taurus GN=RBM39 PE=2 SV=1 - [Q2HJD8_BOVIN]	6,63	528	59,1		1					
Protein phosphatase inhibitor 2 OS=Bos taurus GN=PPP1R2 PE=4 SV=1 - [F1MTZ0_BOVIN]	20,29	207	23,0	1						
ATP-binding cassette sub-family G member 2 OS=Bos taurus GN=ABCG2 PE=3 SV=2 - [ABCG2_BOVIN]	3,05	655	72,7						1	
Uncharacterized protein OS=Bos taurus GN=C11orf49 PE=4 SV=1 - [F1N766_BOVIN]	3,68	326	37,4		1					
Vimentin-type intermediate filament-associated coiled-coil protein OS=Bos taurus GN=VMAC PE=2 SV=1 - [VMAC_BOVIN]	24,10	166	17,8							1
Matrix metallopeptidase 19 OS=Bos taurus GN=MMP19 PE=2 SV=1 - [Q08DI9_BOVIN]	7,62	499	56,4	1						
Uncharacterized protein OS=Bos taurus GN=ZNF784 PE=2 SV=1 - [A5PJI8_BOVIN]	10,83	314	33,6				1			
Uncharacterized protein (Fragment) OS=Bos taurus GN=IGLL1 PE=4 SV=1 - [G3N2D7_BOVIN]	12,93	116	12,1	1						
Adenylate cyclase type 1 (Fragment) OS=Bos taurus GN=ADCY1 PE=3 SV=2 - [F1MBR9_BOVIN]	3,44	1133	123,8		1					
10 kDa interferon-gamma induced protein (Fragment) OS=Bos taurus GN=CXCL10 PE=2 SV=1 - [Q3MQ28_BOVIN]	8,99	89	9,8				1			
Uncharacterized protein OS=Bos taurus GN=C19orf38 PE=4 SV=1 - [E1BKF5_BOVIN]	13,33	225	24,4							1
Uncharacterized protein OS=Bos taurus GN=AARD PE=4 SV=1 - [E1BHW0_BOVIN]	12,42	153	17,1					1		
Envelope glycoprotein gp51 (Fragment) OS=Bovine leukemia virus PE=4 SV=1 - [Q6S017_BLV]	32,74	113	13,1		1					
39S ribosomal protein L13, mitochondrial OS=Bos taurus GN=MRPL13 PE=1 SV=1 - [RM13_BOVIN]	5,06	178	20,6			1				
G protein-coupled receptor, family C, group 5, member B OS=Bos taurus	9,82	387	43,0		1					

GN=GPRC5B PE=2 SV=1 - [Q1JPD9_BOVIN]										
Uncharacterized protein (Fragment) OS=Bos taurus GN=LOC614159 PE=4 SV=1 - [G3N2N0_BOVIN]	10,74	149	16,7		1					
CPXV034 protein OS=Cowpox virus GN=CPXV034 PE=4 SV=1 - [U5TNH1_COWPX]	14,56	261	28,4		1					

8.7. LC-MS/MS instrument (LTQ-Orbitrap XL)

Complex protein mixture analysis is an interesting issue in proteomics studies. The clue is MS/MS experiments to see accurate and sensitive results. A requirement for getting the best result from MS/MS is wide dynamic range, outstanding mass accuracy, fast cycle times and high accuracy.

Figure 45. LTQ Orbitrap XL

Orbitrap mass analyzer and linear ion trap MS combines for a hybrid FT mass spectromter. LTQ Orbitrap Xl is useful for the analysis of polar compounds such as proteins, peptides and nucleotides.

Ions made by API are collected in the LTQ XL followed by axial ejection to the C-shaped storage trap that is used to store the cool ions before injection into orbital trap.

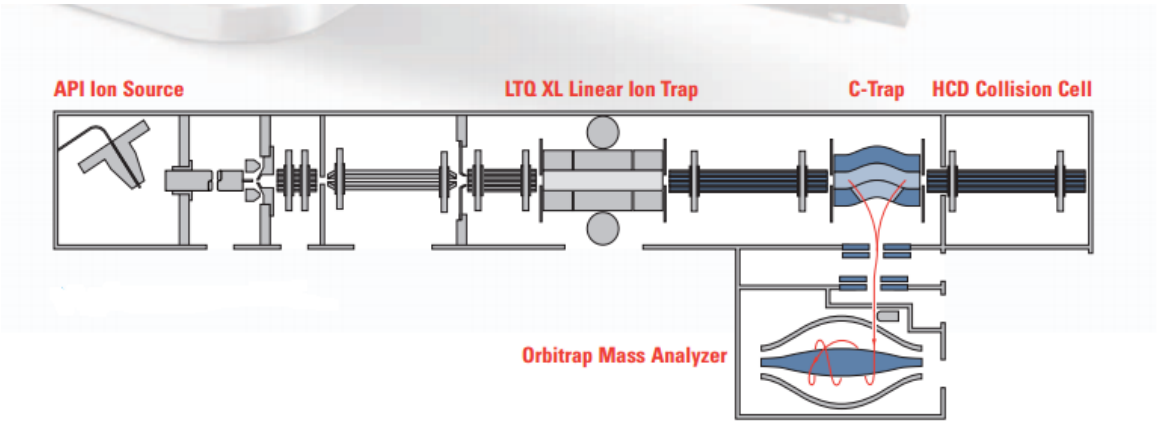


Figure 46. Schematic of LTQ Orbitrap XL

Then the ions are transferred from C-Trap by increasing the electrical filed rapidly. Signals from each of the orbital trap outer electrodes are amplified and converted in to a frequency spectrum by fast Fourier transformation that is finally transformed into a mass spectrum.

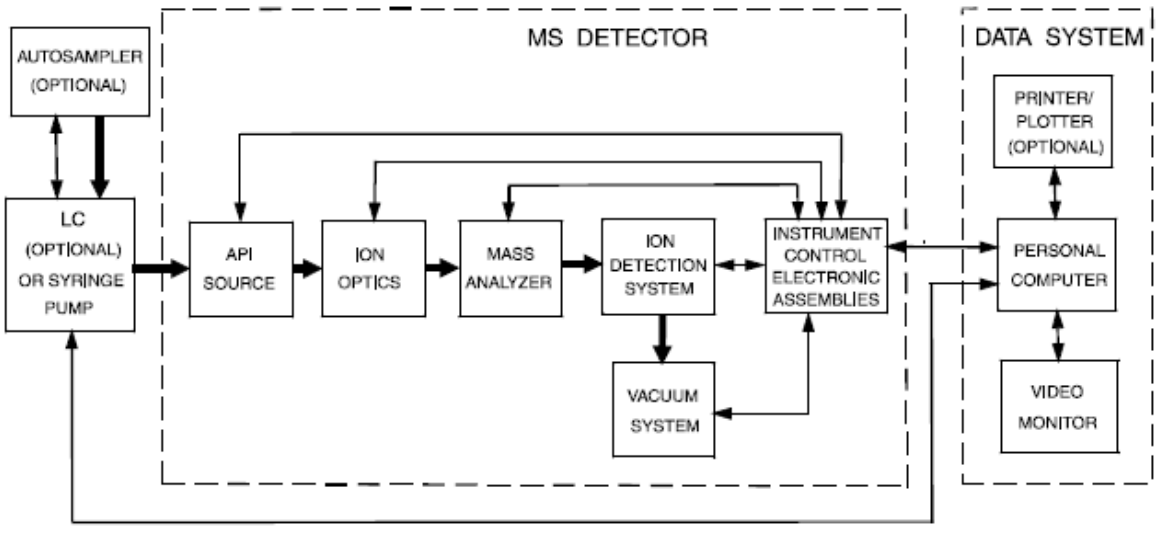


Figure 47. Mass spectrometry analysis flowchart

Ions can be selected in the linear ion trap and fragmented either in the ion trap (CID) or in the new collision cell (HCD).

Figure 48. sample preparation for LTQ Orbitrap XL

Electrospray ionization can be described as follow:

1. The sample solution enters the ESI needle with high voltage.
2. The ESI needle sprays the sample solution into droplets that are electrically charged at their surface.

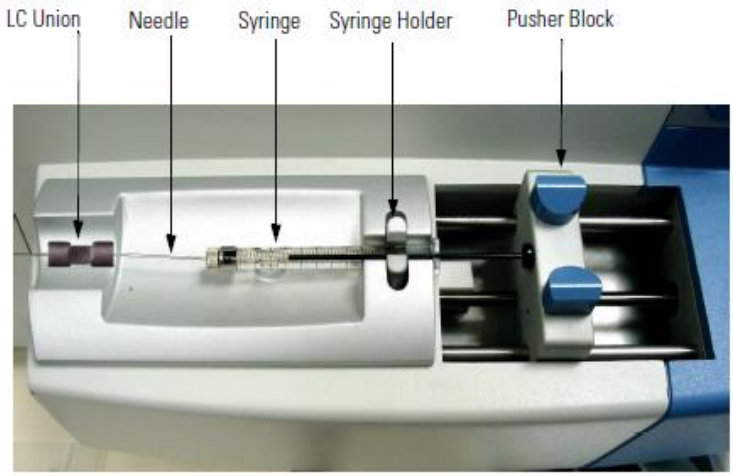


Figure 49. Syringe pump

3. As solvent evaporates from the droplets, the electrical charge density at the surface of the droplets increases.
4. The electrical charge density at the surface of the droplets increases to a critical point, recognized as the Rayleigh stability limit. At this critical point, the droplets split into smaller droplets because the electrostatic repulsion is greater than the surface tension. To form very small droplets the process is repeated many times.
5. By electrostatic repulsion, sample ions are expelled into the gas phase from the very small, highly-charged droplets.
6. The sample ions pass through an ion transfer capillary, enter the MS detector and are analyzed.

An important goal of proteomic analysis is not only the identification of all proteins in complex biological samples but also the accurate determination of their relative concentrations. The method is based on the measurement of particular reporter ions in the low m/z ratio of MS/MS spectra of target peptides.

The results of mass spectrum are typically a series of peaks consistent to scattering of multiply charged analyzed ions.

Figure 50. PC connected to LTQ Orbitrap to transfer the data

For MS/MS full scan applications the ion isolation persuades that dissociation steps are performed once. At the end sample ions are scanned out. Most of the ions are focused toward the ion detection system where they are detected.

8.8. FT-IR instrument (Lactoscope)

The Lactoscope FT-IR Advanced is called Fourier transform interferometer. It relies on the interference of infrared energy, passing through beam splitter and modifying of reflected and time shift signals.

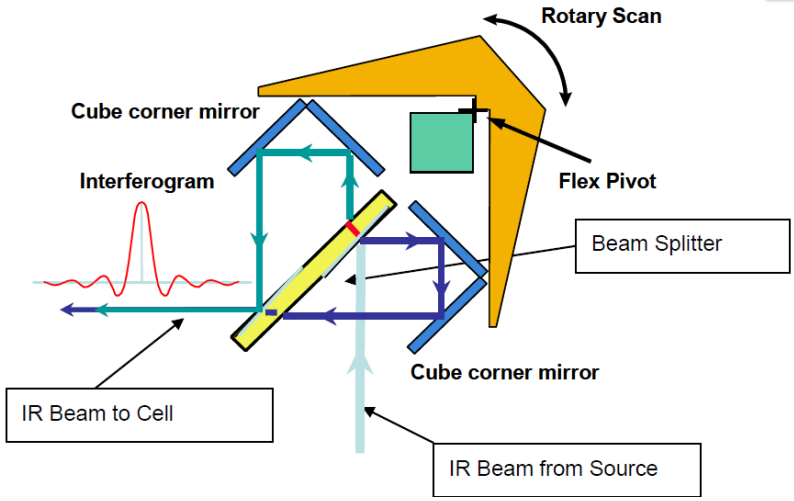


Figure 51. Schematic of FT-IR lactoscope

The infrared energy comes from an IR source is divided into a two beam splitter. The beam splitter performs as a mirror for 50% by reflecting one part to the first cube corner mirror. The other 50% is passing the beam splitter and hits the other cube corner mirror. Then both beams are reflected by the cube corner mirrors and will hit the beam splitter one more time.

Figure 52. Lactoscope FT-IR advanced

The sample is taken by suction, made by a peristaltic pump and heated by an inline heater to 40°C. The pipette system is prepared with a milk sensor to identify if liquid is available at the pipette. If not, the pump will not start and a message will be give in the screen. Then the sample will pass the inlet valve to be pumped with high pressure to homogenize the fat globules.

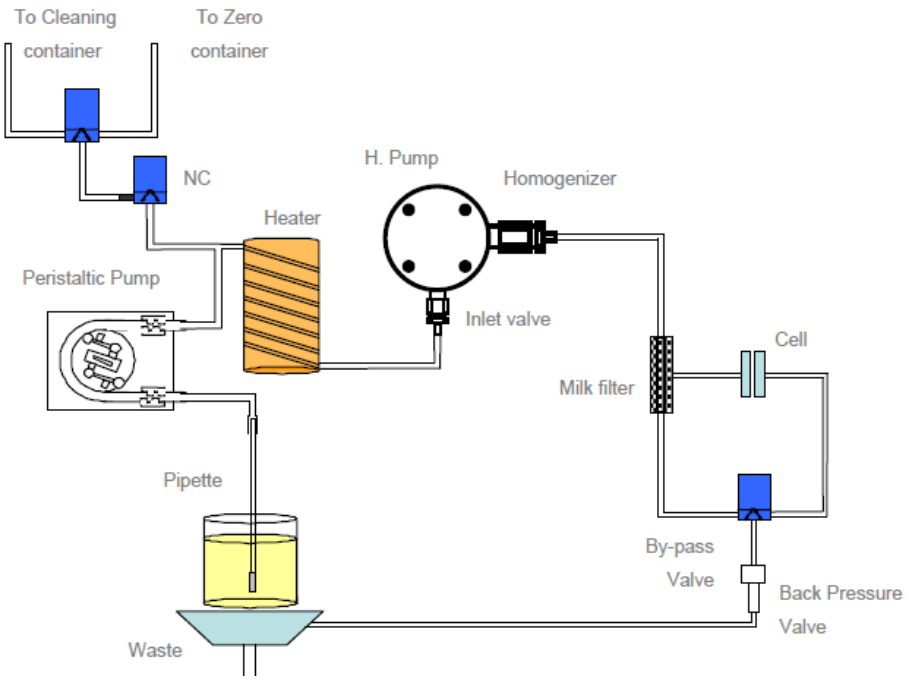


Figure 53. LactoScope FTIR Advanced Flow System

The infrared energy is lead through a measuring cell containing the sample. More or less infrared energy will be absorbed by specific molecular bonds on various wavelengths because of molecular vibration. The interferometer produces an interferogram rely on the absorbed energy of the sample. A computer will utilize to calculate the infrared spectrum from the interfrogram. The mathematical model will calculate the predictions for the components like fat, protein and lactose.

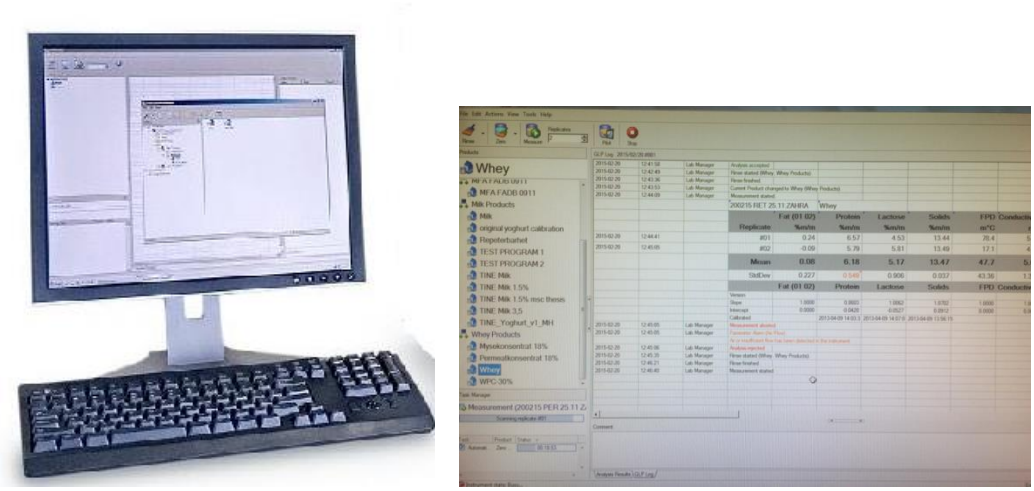


Figure 54. PC connecting to lactoscope for utilizing the data