LAPPEENRANNAN-LAHDEN TEKNILLINEN YLIOPISTO LUT
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Improvement of whey fraction quality using chromatographic methods

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Abstract

Lappeenrannan-Lahden teknillinen yliopisto LUT
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Improvement of whey fraction quality using chromatographic methods

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The quality of whey can be improved by removing certain fractions from its chemical composition. These extracted components can also be utilized as a product of their own. The aim of this thesis is to study if two of these components could be removed with high selectivity from demineralized whey using ion-exchange chromatography. To simulate process scale scenarios, the whey used was left without pretreatment of any kind.

Four IE resins were used in the experimental part of this thesis, resins A and B were used for protein extraction, while C and D were applied for extraction of a vitamin component. Test started with flowrate experiments to study the effect of feed flow rate, followed by capacity experiments meant to map out the feed amount for which the resin bed retains it binding capacity. Samples were gathered and analyzed for their extracted components from the effluent streams using HPLC analysis equipment. Protein, amino acid, and various other analyses were conducted for select samples to gain a better picture of the phenomena's taking place.

Resins A and B showed potential in its target removal, with resin B showing remarkable binding capacity and selectivity, reaching up to 20 BV of feed with only minor reduction efficiency. Resins D and C showed no affinity towards the extraction of its target based on these studies, resulting near zero reduction rates.

Acknowledgements

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Jukka Nykyri
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ABBRIVIATIONS

AC - Activated Carbon

Ala – Alanine

Asn - Asparagine

Asp – Aspartic acid

α-la – Alpha lactalbumin

BCAA - Branched Chain Amino Acids

β-lg - Beta-lactoglobulin

BSA - Bovine serum albumin

BV – Bed volume

CMP -Caseinomacropeptide

Cys – Cysteine

DVB – Divinylbenzene

Gal - Galactose

Glu – Glutamic acid

Gln – Glutamine

GMP - glycomacropeptide

Gly – Glycine

HPLC – High performance liquid chromatography

IgG – immunoglobin G

Ile - Isoleucine

Leu - Leucine

Lys - Lysine

LF - lactoferrin

LP - lactoperoxidase

MAC - Membrane adsorption chromatography

Met – Methionine

Pro – Proline

Ser - Serine

Thr – Threonine

Trp - Tryptophan

Tyr - Tyrosine

TKN - total Kjeldahl nitrogen

Val - Valine

VB12 - Vitamin B12

WPI - Whey protein isolate

WPC - Whey protein concentrate

1. INTRODUCTION

The dairy industry produces vast amounts of whey as a side product of cheese production. During past decades utilization of whey for human nutrition has increased significantly as the demand of nutrient additives are on the rise. This has created demand for the study of new ways to utilize this protein-rich substance, once considered as unwanted side-product. Whey contains many nutrients beneficial to healthy human diet, including amino acids and vitamins. This makes whey a lucrative starting point for many refinement opportunities, one aspect of which is the separation of specific fractions for extraction or collection purposes. As some parts in whey play more nutritionally significant part, separation of minor proteins with lesser significance improves the quality of the whey can be done to improve the quality total amino acid composition of the whey.

Caseinomacropeptides, or CMP's for short, make up a significant part of whey's total protein composition, yet their structure lacks certain amino acids beneficial to human health. This peptide, along with its glycosylated variant glycomacropeptide or GMP, has previously been extracted from whey as a nutritional aid for people suffering from phenylketonuria and as a medicine for various bacterial toxins. Vitamin B12 is a health beneficial water-soluble vitamin found whey. In a balanced diet, daily recommendations of vitamin B12 are met without additional aids, with deficiencies being due to digestion and absorption problems rather than diet. Removal and potential extraction of this vitamin from whey may broaden the applicability of whey products for different purposes without possibly risking over consumption in risk groups, as well as open a new source for the production of the vitamin. Traditionally VB12 extraction has been done from bacterial sources in minor medical-scale streams using chromatographic methods.

Separation techniques applied in caseinomacropeptide and vitamin B12 removal from whey streams are typically applied in minor scale applications and capturing purposes with the focus revolving around efficiency rather than selectivity. In this study, the aim is to propose a chromatographic method applicable for large-scale industrial treatment of whey. In this scale it is paramount to gain high selectivity of removed components with as little as possible consumable chemicals, like pH adjusting agents. This causes the focus of the study to shift more onto the selection of proper resin, feed volume and flow selection. Chromatography ion-exchange and adsorption has previously been studied and applied in extraction of caseinomacropeptides in both analytical and separation scales. These methods have also been applied into small-scale separation and analytics for vitamin B12 from non-dairy sources. Selection of optimal ion-exchange or adsorption resin for whey streams without alteration of pH or salinity is a challenge, especially in the case of VB12 with its varying oxidation forms and its challenging properties.

2. LITERATURE REVIEW

In this literature review part of this thesis, the background of separation and analysis methods involved with whey proteins and vitamin B12 are inspected and reviewed. Gaining understanding of the main chemical composition and the properties of involved whey components is paramount when seeking an optimal method of separation for whey fractionations, as well as the general knowledge of the applied separation techniques.

2.1 Composition and origin of whey

Whey is produced as the side-stream of cheese production, when the protein casein coagulates and leaves behind a leftover liquid know as whey. Whey-to-cheese ratio in the production is about 8.5 whey liters produced per cheese kg, resulting in vast amounts of whey produced in European milk. (EWPA 2017) This results in a roughly 10 kilograms of whey and 1 kg of cheese produced for each 10kg of milk treated. Cheese production can be done with differing methods, the common examples being enzymatic isolation using renneting chemicals like chymosin and acid precipitation. The whey produced from acid precipitation at pH 4.6 is called acid whey, and the whey produced form renneting of cheese is commonly referred as sweet whey or cheese whey. (Krolczyk et al. 2016, De Wit 2011, Fox et al. 2015) The production method affects whey's properties. Liquid whey typically has the water content of roughly 93.5% with the remaining 6.5 percent consisting of solids. The solids of this whey mainly consist of high amounts of lactose and whey proteins. (De Wit 2011) The total protein consistency of sweet whey is made of whey proteins, as well as κ-casein derived caseinomacropeptides and leftover traces of renneting enzymes.

Whey can also be produced using microfiltration with filter pore size ranging from 0.1-0.8µm. In this method has the advantage of not using any additional coagulants, leaving no enzyme traces into either phases cheese or whey. The produced whey also contains no casein derived caseinomacropeptides, lactose, minerals and other milk derived water-soluble fractions. Some short-chained caseins are however permeated into whey. This whey, sometimes called ideal or native whey, is considered to have a more favorable proteins concentration compared to cheese whey, due to not containing any caseinomacropeptides. (Fox et al. 2015)

After renneting, the separated sweet whey still contains trace amounts of short caseins and fat globules. Before further treatments like demineralization, these traces are removed using methods such as using hydrocyclones, centrifugation, coarse filtration or sieving. The casein traces from this can be reused in cheese manufacturing or in other food products. (De Wit 2011, Kilara 2009) Removing these traces improves the effectivity of further refinement process steps and prevents possible clogging and blocking of process equipment due to larger particle sizes.

Traits associated with whey include its high protein content, which is beneficial for human muscle growth. Whey is also known for its gelling and foaming properties, which are commonly utilized in food texture modification and optimization. Traditionally whey has been considered as the more undesirable part of cheese production, usually being turned into animal feed or used in specialty whey cheese production or as an additive in food industry. In some historical cases, whey has also simply been neglected and ran off as a waste stream into nearby rivers. However, in Finland this was made illegal since 1961 water act, along with other waste waters, and has remained so to this day. (Water Act 1961) As whey is produced in high bulk amounts, finding a practical use for whey derivative products shows potential to be financially and ecologically beneficial solution.

2.1.1 Milk proteins

In order to efficiently understand the composition of whey, knowing the origin of its components is the key. Bovine milk contains the total protein content of roughly around 3.5%. These milk proteins are divided into two castes depending on their coagulability at acidic pH of 4.6. The part of the proteins which coagulate under these conditions are known as caseins, while the water-soluble proteins are known as whey proteins. In bovine dairy 80% of the contained proteins consist of caseins, while the rest are mainly whey proteins. In human milk the ratio amount of caseins is only 40%, while the rest 60% being whey proteins. Caseins differ from whey proteins, as they contain significant amounts of phosphorus, which gives them a high capability to bind calcium. Both groups are heterogenous, consisting of various proteins. (Fox et al. 2015) In addition to proteins milk and its derived products contain various peptides, short amino acids chains containing typically around 50 interconnected parts. Milk proteins contain all nine of the essential amino acids needed by adult humans; Threonine, Valine, Histidine, Methionine, Leucine, Isoleucine, Phenylalanine, Tryptophan, Lysine. (Outinen 2010) These essential amino acids function as the main ingredient in protein synthesis, that promotes muscle growth and welfare. Due to human body being unable to produce these amino acids by itself, consumption of them from diet is essential for healthy human activity and growth. The effect of caseins tends to be longer amino acid chains, their absorption from human diet tends to take longer time, compared to shorter whey proteins, effect of which is the absorption is more immediate.

2.1.2 Composition of whey

The chemical composition of whey highly varies, depending on the health, nutrition intake, area of origin and breed of the animal. As the production method of whey also affects the composition of whey, the whey studied in this thesis is considered as sweet whey produced via enzymatic renneting from edam and emmental type cheese production, also referred as cheese whey. Dry cheese whey solids contain 73 w-% of lactose, 8 w-% of mineral ash, 13 w-% proteins and minor water-soluble nutrients (De Wit 2011) Mineral ashes present in milk are almost completely made of varying salts. Whey is high in not only whey proteins, but also minor fractions of peptides originating from milk proteins. Figure 1 represents the total solid contents in milk and liquid whey in percentage of weight per volume. (Stanic et al. 2012)

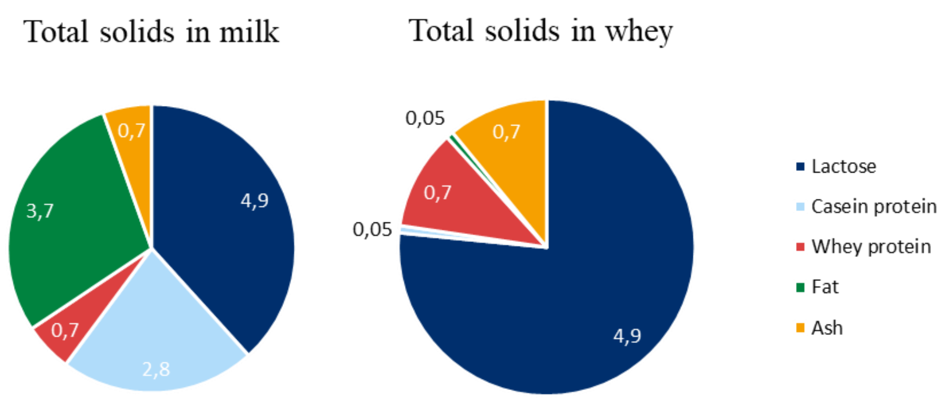

Figure 1 Composition of total solids in Milk (left) and Whey (right) based on percentage of weight per volume. (Based on Stanic et al. 2012)

The main proteins in whey are α-lactalbumin (14%), β-lactoglobulin (53%), serum albumins (12%), immunoglobulins (3%), caseinomacropeptides CMP (20%) and other minor protein traces, including minor proteins. Generalization of protein composition of cheese whey, manufactured from milk produced by Valio, is presented in Figure 2. (Outinen 2010) Of these, α-La and β-Lg hold the most prominent nutritional value for human consumption. Especially β-Lg contains high mounts of lysine and branched-chain amino acids (BCAA), which play a significant role in human protein synthesis. (Philips & Williams 2011) Bovine α-La closely resembles the α-La produced by humans and thus it is optimal and highly favorable for human consumption, factor which is especially important on infant nutrition. (Božanić et al 2014) Serum albumins and immunoglobins act as antibodies and are transported into milk and whey from bovine blood.

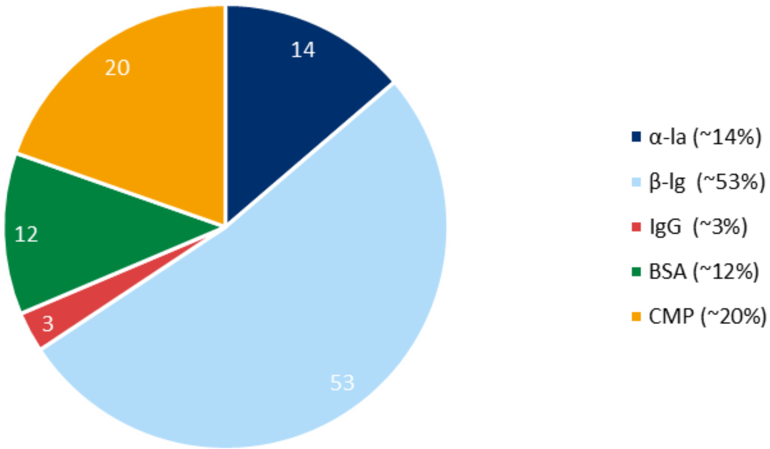

Figure 2 Generalized protein composition of whey from bovine cheese whey (Outinen 2010)

In this thesis, the properties and functions two non-whey protein components of vitamin B12 and caseinomacropeptides are studied. Both of these water-soluble organic compounds are transferred into whey from milk during the renneting phase. In order to improve the whey protein fractions to be more suitable for dietary purposes, removal of them is studied as the focus in this thesis.

2.1.3 Whey protein isolates and concentrates

Improvement of the protein quality of whey is most commonly done by removing the water and mineral content from the whey. Typical cheese whey contains mineral contents of 8-10g minerals per 100g of whey. (Božanić et al. 2014) Whey protein concentrates WPC that have decreased lactose content resulting in a varying protein content between 38 to 80 weight percentages. The lactose is typically reduced with crystallization, decantation and membrane filtration (De Wit 2011). Whey protein isolates or WPI consist high purities of whey proteins more than 90 w-%, with only minor traces of lactose and other non-protein material. (Prabhu 2006, Krolczyk 2011, De Wit 2011) WPI and WPC's are commercially sold as nutrient aids and other healthcare products, as well as used as additives or ingredients in other dairy products.

2.2 Demineralization of whey

One of the most common treatments done to whey is the process of demineralization. In demineralization process, the inorganic ash contentment is reduced as much as possible. This inorganic ash is composed mainly of salts and other mineral residues, and removing it is pursued to improve the taste, nutritional qualities and properties of the whey. As salinity and mineral contents of food products are carefully observed, demineralization has gained a lot of attraction in both markets and in fields of research.

Demineralization is usually done via nanofiltration NF, electrodialysis or ion-exchange methods. From these methods, ion-exchange is used for achieving high purity, while electrodialysis yields high selectivity in removal of certain minerals. (De Wit 2011) Electrodialysis technology has however taken leaps during recent developments and its production capacity is nearing that of traditional ion-exchange models. Demineralized whey retains its high lactose content. The degree of demineralized powder is typically 40, 50, 70 and 90 percent, which corresponds to 6, 4, 3 and 1% of the ash content, respectively. Demineralized whey typically still retains its high lactose content of around 78-83 percent of total solids depending on the degree of demineralization. The highest degree of demineralization can be achieved combining methods mentioned before.

Demineralization is an important step in removal of certain whey fractions, as it improves the selectivity and efficiency of separation techniques like ion-exchange by removing excess ions that compete with the aimed separated particles binding. The process also lowers the salinity of whey improving its quality as a food product. As is common in food industry, the taste and health benefits of the product must be taken into consideration when in the development phase, attributes both of which can be endangered with excessively high or low salt concentrations.

2.2.1 Ion-exchange demineralization

Ion-exchange demineralization uses electrochemical properties to bind minerals and charged particles from whey into a stationary media. The binding essentially happens as the exchange between two charged particles, one from the binding surface and one from the treated solution. These bound particles are then unbound into an effluent waste stream from the media using eluent of higher concentration and opposite charged ions. Depending on the charge and particle size of the resin used different molecules can be removed from the whey. (De Wit 2011)

In demineralization, the removed molecules are ionic salts present in the whey. As the aim is to remove major compositions of salts and other ash in a throughout manner, selectivity in demineralization is not as important as capacity. Systems of demineralization using IE typically consist of anion and cation exchangers in conjunction to achieve removal of ions of either charge. Systems used for ion-exchange consist of series of columns packed with ion-exchange resins, aimed to remove specific charged ions from the whey. Typically, anion exchange is followed by cation exchange and vice versa. More on the theory of ion exchange is reviewed in chapter 2.6.1. of this thesis.

2.2.2 Electrodialysis

Electrodialysis uses pairs of cation and anion exchange membrane cells located between two electrodes. Whey is conveyed into every-other channel between the cells, and the others are filled with brine or other ionic liquid. The ions in whey pass thought the membrane cells and end up in the brine. The selectivity of this system can be altered by modifying the conductive liquid concentration and electronegativity to match certain salts for specific channels. The typical range of demineralization achieved with electrodialysis is around 65-75%. (Andrés et al. 1995, De Wit 2011) Figure 3 by De Wit presents this system in an informative model. (De Wit 2011)

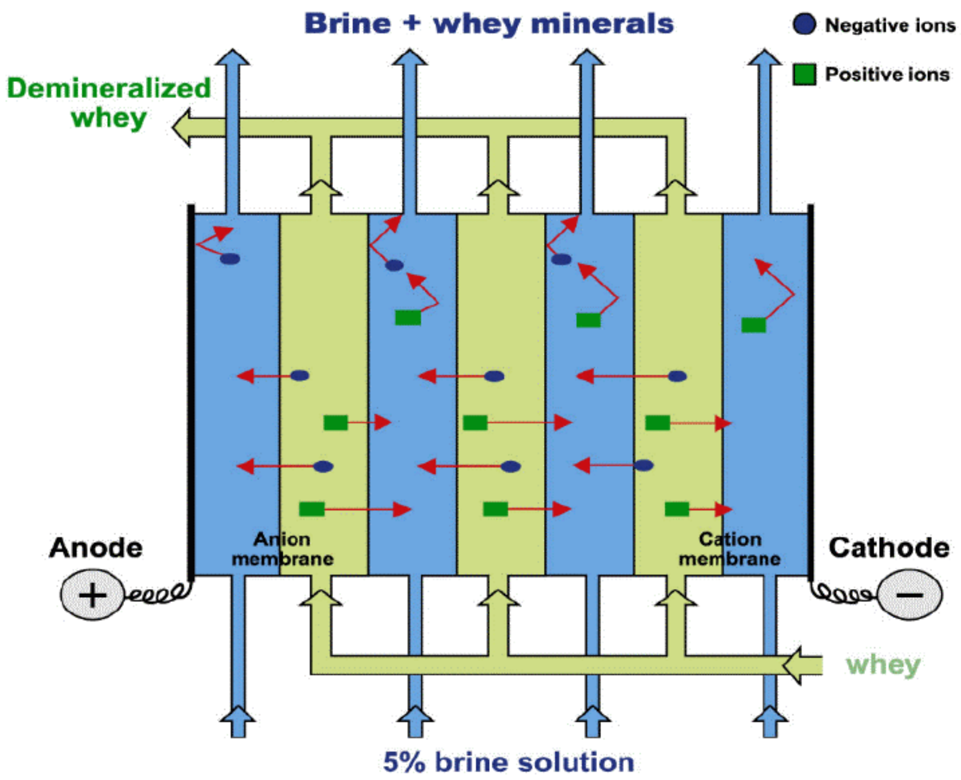

Figure 3 Principle of electrodialysis system according to De Wit (De Wit 2011)

2.2.3 Mebrane demineralization

Membrane filtration is used as an alternative to electrodialysis and ion-exchange in some cases. Using a membrane through which only the smallest components, with their particle size in the class of nanometers and their atomic mass in the field of hundreds or under, of whey could pass. As most of the particles fitting these descriptions in whey being salts and other ions, the protein composition of whey is left unaltered, while some of the minerals are partly removed. (Cuartas-Uribe Et al. 2007, De Wit 2011) Equipment for this purpose revolve around the use of nano or reverse osmosis membrane-based systems.

2.3 Vitamin B12

Water-soluble B12 vitamin is one of the nutrients that can be found in minor fractions present in cheese whey. B12 vitamin has four major forms, from which the cyanocobalamin is the commercially used. Other forms found in dietary, and thus in dairy, forms of VB12 are 5-deoxyadenosylcobalmin, methylcobalamin and hydroxocobalamin, and their different oxidation states. In milk, the predominant form is hydroxocobalamin, and are nearly completely bound to proteins. (Fox et al. 2015) The change in forms can also be caused difference in the charge of the central cobalt atom, or the degree of oxidation. Due to its water-soluble nature, vitamin B12 is conveyed into liquid whey solution. This vitamin contains a defining structure featuring rare element cobalt in its central corrin ring, connected into a ribose, nucleotide base and a phosphoric acid. According to measurements by Yariv et al. the particle size for standard B12 structure was about 847 (±127) nanometers. (Yariv et al. 2015)

The amount of B12 vitamin present in the bovine dairy differs on the minerals present in the feeding grounds of the bovine. In bovine milk, VB12 make up to 4µg per liter of milk. (Nohr & Biesalski 2009, Fox et al. 2015) Structure of cobalamin molecule, without vitamer specific groups, is shown in Figure 4. (Kumar & Kozlowski 2012) The corrin ring is noticeable around the central cobalt atom, consisting of four interconnected aromatic heterocyclic nitrogen containing pyrrole rings. The amount of VB12 in milk left unaltered by pasteurization, however ultra-high temperature processing or UHT treatment causes losses in VB12 concentration. (Fox et al. 2015)

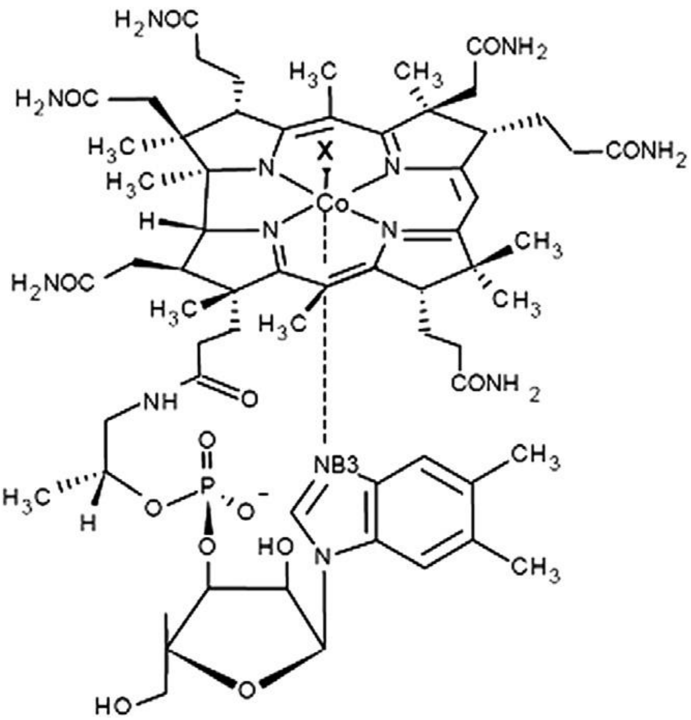

Figure 4. X-cobalamin molecule structure in its stabile form (X replaced with variant specific group i.e. cyano-group, CN) (Kumar & Kozlowski 2012)

Vital for humans and animals alike, it cannot be produced by human nor livestock itself, B12 is produced by bacteria commonly present in bovine rumen. B12 vitamin deficiency leads to neurological diseases and anemia. (Scott 1997, Agnoletti 2011) The optimal intake for an adult human is 3 µg/day according to Fox et al. and 4 µg/day according to European food safety association. For infants and children under the age of ten this is lower at 1.5 µg/day. (EFSA 2017, Fox et al. 2015). In developed countries, deficiencies in most cases are due to low absorption of B12, rather than low consumption from digested nutrients. (Fox et al. 2015) The elderly are suggested to consume slightly higher amounts of VB12 compared to adults, in order to make up for lowered absorption that comes with age. As a water-soluble vitamin, the excess of consumed VB12 is transferred into urine, hence the overconsumption of B12 does not possess any immediate dangers. However, as B12 is treated in liver and the excess in kidneys, people with liver and kidney problems should avoid overconsumption. B12 is highly sensitive to photolytic reactions, and thusly dissociates under high intensity light sources such as UV and sunlight. This, especially in the case of hydroxocobalamin, with the weak Co-OH bridge has shown attributes of photocatalytic activity in VB12 samples. (Kumar & Kozlowski 2012)

2.4 Caseinomacropeptides

Caseinomacropeptides are casein derived peptides which can be found in whey, making up around 15 to 25 % of total protein amount present in whey. (El-Salam et al. 1996) These proteins originate as the hydrophilic part of κ-casein, that break from the structure during the renneting of milk using enzyme chymosin. The main casein part of para-κ-casein is coagulated along other caseins into the curds further used in cheese production. Caseinomacropeptides consist of 64-part amino acid chain. This is described in Figure 5 by Fox et al. (Fox et al. 2015)

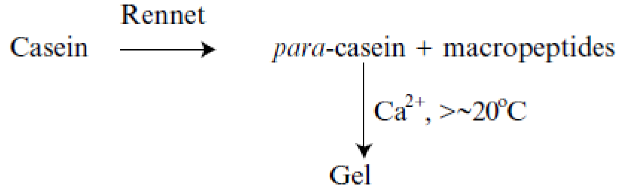

Figure 5 Breaking of casein into para-casein and macropeptide in renneting (Fox et al. 2015)

These caseinomacropeptides or CMP’s lack specific structures, typical for other proteins found in whey, and thus differ from whey proteins in their kind. As they originate from κ-casein, they are not whey proteins and share more properties with other caseins. Their composition is heterogenous and varies greatly, but they share similar properties due to sharing same functional groups. One of the main types of CMP is the glycosylated variant of caseinomacropeptide is known as glycomacropeptide or GMP. Non-glycosylated CMP-variants make up the majority of 50-60 percent of the total CMP content in most sweet whey sources. (Corinna et al. 2006)

Caseinomacropeptides, including Glycomacropeptides, differ from other proteins found in whey as they contain no aromatic amino acid structures, and thusly are cannot be detected under the typical protein identification wavelength of 280nm. (Brody 2000) Due to this, many of the spectrometric analysis done on CMP are conducted in wavelengths in the point of 214nm. It should be noted that in some studies the term GMP and CMP are used rather interchangeably, due to their functional similarities and irrelevance depending on the focus and scope of the study. In this thesis, the structural difference between GMP and CMP is acknowledged, with the GMP meaning the glycosylated variant of CMP exclusively.

2.4.1 Chemical properties of CMP

The structure of caseinomacropeptide is considered as a random coil. Theoretical models of possible CMP structures have been proposed by Kreuß et al. featuring a model for possible 3D structure. (Kreuß et al. 2009) In Figure 6, a variant of caseinomacropeptide is described with two variants, A and B for two specific mutations. As can be seen from the structure, CMP is rich in amino acids proline, glutamic acid, serine, however lacking such amino acids such as tryptophan, tyrosine, phenylalanine and cysteine. From health beneficial branched chain amino acids present in CMP, isoleucine and valine are noticeably present in CMP, however with only one instance of leucine present in the chain. (Farrell et al. 2004, Neelima et al. 2013) From the various genetic variants of CMP, two major architypes, variants A and B, are found in typical bovine whey. Their structural difference is with the 31st amino acid threonine in variant A changing into isoleucine and 43rd asparagine being replaced with alanine, respectively.

1 10 20

H. Met – Ala – Ile – Pro – Pro – Lys – Lys – Asn – Gln – Asp – Lys – Thr – Glu – Ile – pro – Thr – Ile – Asn – Thr – Ile –

40

Ala – Ser – Gly – Glu – Pro – Thr – Ser – Thr – Pro – Thr – Thr* – Glu – Ala – Val – Glu – Ser – Thr – Val – Ala – Thr –

60

Leu – Glu – Asp** – Ser – Pro – Glu – Val – Ile – Glu – Ser – Pro – Pro – Glu – Ile – Asn – Thr – Val – Gln – Val – Thr –

64

Ser – Thr – Ala – Val. OH

Figure 6 Primary structure of variant A CMP, cut from kappa-casein with rennin. In mutation variant B; Thr* replaced with Ile and Asp** with Ala (Farrell et al. 2004, Neelima et al. 2013)

Two main groups of caseinomacropeptides have been discovered, glycosylated and phosphorylated GMP, and non-glycosylated and phosphorylated CMP. (Kreuß et al. 2009) Phosphorylation happens when a group of phosphoryl $P^{+}O_3^{2-}$ is attached into a protein structure.

2.4.2 Effect of glycosylation on CMP

Glycosylation is a phenomenon in which a carbohydrate is linked into a protein or a peptide via O- or N-bridge. This structural anomaly is common on caseinomacropeptides and takes place on Threonine and Serine amino acids parts which contain O-bridges suitable for glycan groups to attach into. (Neelima et al. 2013) These glycan-containing caseinomacropeptides are called glycosylated-CMPs or GMPs and depending on the attached carbohydrates their properties vary. Glycans attached to caseinomacropeptides consist of either monosaccharides or oligosaccharides. (Brody 2000) More specifically, four different oligosaccharides, and one monosaccharide (Saito & Itoh 1992), have been identified in GMP structures presented below: (El-Salam 1996)

1. Galβ (1 - 3) GalNAc-ol
2. NauNAcα (2-3) Galβ (1-3) GalNAc-ol
3. Galβ (1-3) [NeuNAcα (2-6)] GalNAc-ol
4. NeuNAcα (2-3) Galβ (1-3) [NeuNAcα (2-6)] GalNAc-ol
5. GalNAc-ol

in which Gal is galactose; NeuNAc is N-acetylneuraminic acid; GalNAc is N-acetylgalactosamine. (El-Salam 1996, Saito & Itoh 1992)

Caseinomacropeptides have the atom mass of around 7000 Da, and the GMPs mass is close to around 11000 Da. (Neelima et al. 2013, Kreuß et al. 2009) The weight difference is due to the addition of glycan group and is only an estimation as the mass varies based on the attached carbohydrate. The different genetic variations of caseinomacropeptides also cause a slight difference in atom mass.

CMP overall is considered as hydrophilic part of κ-casein; however, it shows hydrophobic properties it is structures under specific scenarios. In GMP, the glycan part gives certain properties as opposed to non-glycosylated CMP, for example protection from oxygenic degradation. Glycan part also functions as a negatively charged zone in the structure at pH values over the peptides iso-electric point. CMP coil structure itself is a negatively charged region. With the additional negatively charged glycan part, glycosylated CMP is slightly more negative zone. Figure 7 describes a crude model of CMP and GMP structure and its electrochemical domains and properties. (Kreuß et al. 2009)

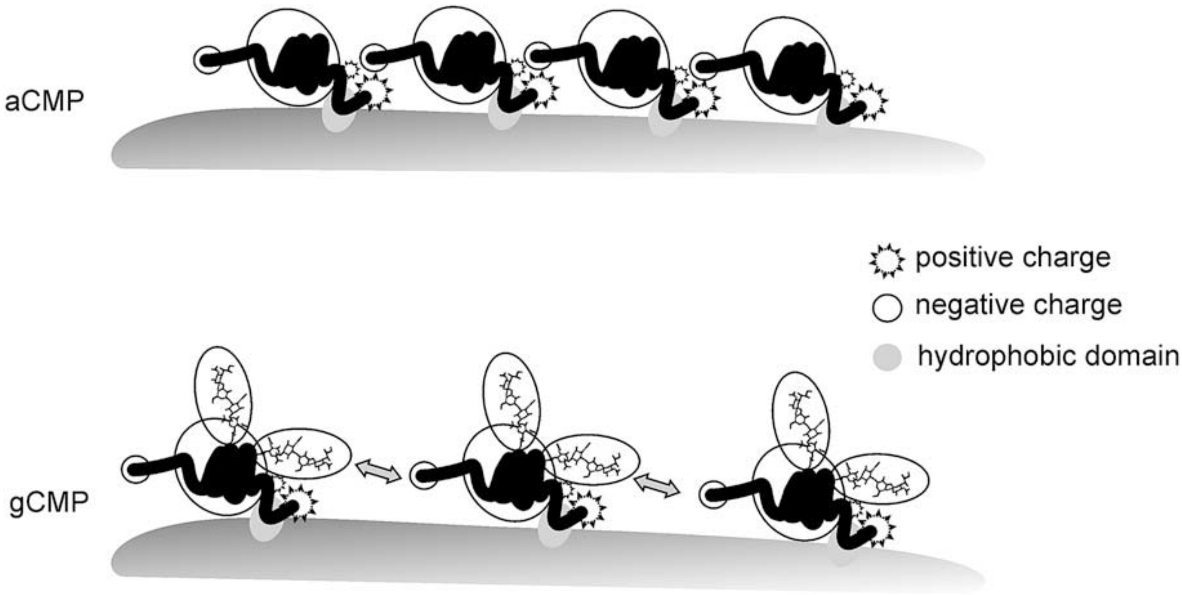

Figure 7 Schematic diagram of the different adsorption of CMP and GMP at a hydrophobic/hydrophilic interface at pH 7.0. (Kreuß et al. 2009)

Hydrophilic/phobic properties increase the particles adsorption in oil-water surface. This phenomenon can be exploited in the extraction of caseinomacropeptides from whey using adsorption. The difference between CMP and GMP hydrophilic properties can also be used to separate the two peptides from each other. These properties are however affected by pH changes, having its best adsorption into oil at its isoelectric point. Above this point, especially in the case of GMP, the electronegative zones decrease the effectivity of hydrophobic areas. This causes the GMP's adsorption into oil surface being challenging. (Kreuß et al. 2009) Separation techniques utilizing these properties will be studied more in chapter 2.6.2. According to Kreuß et al. the isoelectric point of CMP varies depending on the glycosylation of caseinomacropeptide, with the GMP having its isoelectric point at pH 3.15 and CMP at around 4.15. (Kreuß et al. 2009) This difference is caused by the glycan group attached to the peptide, which shifts the electrochemical properties of the molecule.

2.4.3 Biochemical properties of GMP

Glycomacropeptides show many biologically active features, from bacterial anti-toxin activity to protection from viral and bacterial adhesion. GMP has been found as an effective way to bind cholera and e. coli toxins. GMP also has shown to promote bifidobacterial growth in lower intestines, which protects the intestine from harmful pathogenic bacteria. (Brody 2000) GMP can be used as a protein source for patients suffering from phenylketonuria, a rare genetic disorder in which the patients must restrict the intake of phenylalanine from their diet. (Blau et al. 2010)

Due to these previously mentioned aspects, purification of GMP may show viable opportunities in medicine and nutritional industry. Commercial products based on GMP addition into the diet of phenylketonuria patients have been already manufactured by Arla Foods Ingredients with the product name of Lacprodan GCMP 10 and GCMP 20. First of which consisting of 75% GMP with 83% dry matter protein concentration, and the latter containing 95% GMP and 78% protein concentration. (Arla Foods Ingredients 2019) Other GMP product in the market has been launched by Agropur with their product BiPRO GMP 9000 (Agropur nutrition solutions 2020)

2.5 Analysis of whey fractions

In this chapter the methods, in which certain fractions present in whey, are identified and analyzed. Analysis for components in whey often utilize methods varying from high-performance chromatography to biological methods. Understanding of analysis methods used for these fractions may help to understand possible errors and anomalies present in the results, as well as shed light into properties of these materials. The focus in this thesis is around the identification and used analysis methods on vitamin B12 and caseinomacropeptides.

2.5.1 B12 vitamin analysis

Identification of vitamin B12 has been traditionally studied in food industry. As VB12 contains both organic structures as well as the inorganic cobalt center, various approaches for its identification can be taken. VB12 is near unique with its defining structure of corrin ring, and thus differs greatly from other vitamins and organic structures found in whey. Historically analysis of VB12 has been done using microbiological or chromatographic methods. Analytics can be done using atomic adsorption spectrometry AAS, using a cobalt hallow cathode lamp at 242.5nm, a standard method used for identifying cobalt. As the cobalt in whey is bound to VB12, the amount of cobalt correlates with the amount of VB12. Similar methods of cobalt identification can also be utilized in inductive-coupled plasma mass spectrometry ICP-MS. (Karmi et al. 2011) As the concentration of VB12 is a minor fraction in whey, these methods, especially AAS, have high amount of inaccuracy as the error margin can be quite significant compared to the concentration.

Microbiological analyze methods use bacteria such as *lactobacillus leishmania*, *bacterium coli* and *euglena gracilis* to determine the cobalamin content in sample liquids. These micro-organisms thrive in VB12 rich environment, and their growth can be used to conclude the amount of VB12 present in the solution. This method needs some pretreatment depending on the used micro-organism, for example *lactobacillus leishmania* works efficiently on cyanocobalamin, and thus introduction of cyano-groups into the solution is favorable. (Davis et al. 1973, Ford 1952) Microbiological methods lack the accuracy to tell difference between forms of VB12 and some forms may even be left unseen by some micro-organisms. While being highly mature and validated technology, microbiological assays function as effective analysis method in total VB12 content, however in deeper understanding of VB12 fractions is left unclear by this method. Microbiological assay method has also the disadvantage of taking rather long time as the growth of the organisms takes time.

Wyk & Britz studied a rapid analysis method for VB12 from milk and bacterial source of *Propionibacterium freudenreichii* cultures using HPLC-UV system with base-deactivated silica packing. Analysis wavelength used was selected at 361nm, resulting in a distinguishing VB12 peak at retention time of around 12min. Moreover, the results from the HPLC were comparable to ones achieved from more traditional microbiological assay, which have the disadvantage of being highly time consuming. (Wyk & Britz 2010) In more special and analytical cases, spectroelectrochemical VB12 analysis methods using mercury, platinum and gold electrodes were studied by Lexa et al. resulting in analytical results with the platinum and gold electrodes. The aim of this study was to define the electrochemical equilibrium between the vitamers of aquo/hydroxocobalamin and its oxidation states. (Lexa et al. 1977)

2.5.2 Analysis of caseinomacropeptides

Chromatographic methods are common in CMP analysis when combined with UV-detector, mass MS or nuclear magnetic resonance NMR spectrometer. These methods utilize the mass transfer properties between specific compounds to separate the composition of the feed solution into specific fractions based on the velocity of which the separated particles move through the system. Systems utilizing chromatography typically consist of a column packed with permeable material, through which the feed stock is fed. The material based on its properties affects the time it takes to pass though the column. This time is called the retention time. As the specific fractions leave the column at their specific retention times, analysis devices such as mass- or UV-spectrometry can be applied. Analysis-scale in high-performance liquid chromatography HPLC methods are commonly used to provide a reliable quantitative result in whey protein analysis. Various ways of analytical chromatography can be applied, for example reverse-phase LC and gel-filtration LC are common methods applied in detection of caseinomacropeptides.

RP-HPLC paired with a UV-detector can be used to analyze CMP and GMP form whey solutions, such as chromatographic elution streams in this case. Method for RP-HPLC detection of GMP/CMP from whey has been described by many authors described by Outinen et al, Thomä et al, and Elgar et al. These methods require the pretreatment of introducing trichloroacetic acid TCA to the whey solution and filtering it through a 0.45µm filter. If the solution contains clear phase differences due to casein or fat globules, removal of solids using i.e. centrifugation to avoid blockage in the column is necessary. The columns used are hydrophilic protein specific variants utilizing reverse phase techniques. The UV-spectrophotometry for this analysis uses the absorbance wavelength of 226 nm. As previously mentioned, the protein standard wavelength of 280nm is ineffective due to CMP being practically invisible under it. A standard solution of high purity caseinomacropeptide is needed for determination of reference points as a means of quantification for the results. (Thomä et al 2006, Outinen et al. 1995, Elgar et al. 2000) In Figure 8, an example of this analysis using HPLC-UV/vis from the study conducted by Thomä et al. is presented. (Thomä et al 2006) The glycosylated section can be identified as the low absorbance noise at around retention time (t_R) of 12-16 minutes. Variant A of CMP us drawn as a high absorbance spike at near 17-minute mark and B variant onto 18.5 minutes. The combined mark left by both variants of CMP is located between both peaks between 17-18 minutes of retention time.

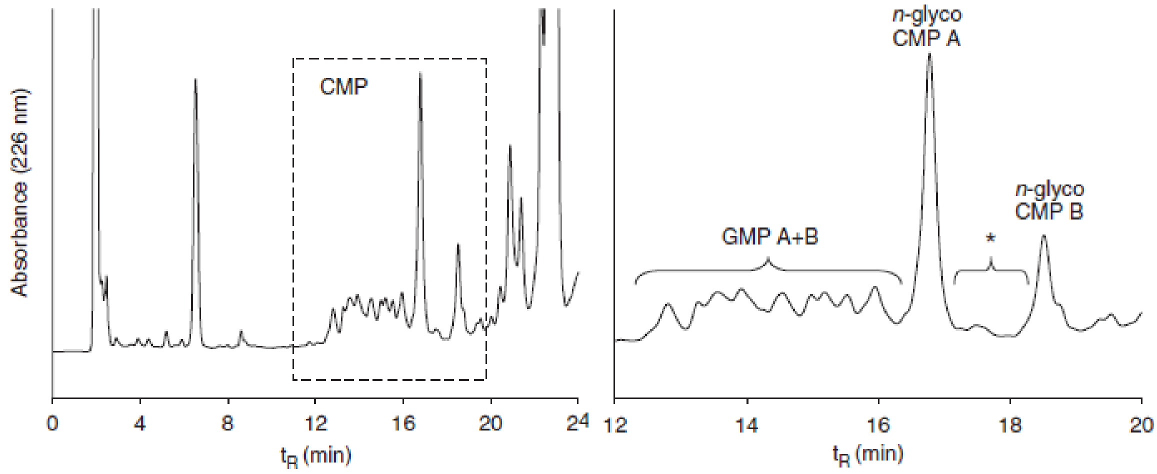

Figure 8 Spectrograms for CMP/GMP analysis under the wavelength 226nm for rennet whey (left) and zoomed in for CMP/GMP specific segment (right) *=CMP A+B (Thomä et al 2006)

The amount of glycosylation that takes place in caseinomacropeptides in whey is presented by a glycosylation degree. As previously mentioned, typically around half of the caseinomacropeptides are non-glycosylated variants. The main differences between these two similar particles are their atomic mass and the additional electronegative zones caused by the attached glycan groups. From HPLC analyses, Identification of GMP in spectrograms is easily distinguishable due to its low absorbance cluster caused by glycan groups at wavelength 226nm, example of this can be seen at figure 8. As there is a significant weight difference between GMP and CMP, the overall determination of them can be done using mass spectrometry (MS). (Thomä et al 2006, Dziuba & Minkiewicz 1996)

The effect of glycosylation on the CMP particles also were studied by Kreuß et al. in their study, revolving around the differences between CMP and GMP. When comparing zeta potentials of these peptides, it was found that the isoelectric point was slightly lower with the glycosylated variant. It was also found out that the creaming potential was higher in GMP compared to the one of CMP, due to the additional hydrophilic properties provided by the glycan. (Kreuß et al. 2009)

2.6 Separation of whey fractions

Various methods can be used for separation of different whey fractions. In this chapter overview of the most common proposed methods are presented for VB12 and CMP, opposed to the mineral and lactose removal that were described earlier in this thesis. In separation of whey fractions, a challenge of selectivity arises since many of the proteins share similar functional groups and physical properties. This can cause the unwanted removal of certain fractions, lowering the removal efficiency of the desired particles. In the case of VB12, some methods may leave some variants of cobalamin untouched. Many of these methods exploit similar techniques used in analysis, however in a larger scale with the sole aim in component separation and selectivity, as opposed to fractioning different parts the solution into clear detectable phases.

Separation methods in whey fractioning commonly utilize chromatographic and membrane methods, as well as various coagulation based novel techniques. Membranes are for example used in many cases where the separation can be done based on particle size of the components, like in the case of demineralization. In Figure 9, particle sizes and molecular weights are shown in a table describing the proper membrane suitable for their extraction. (De Wit 2011)

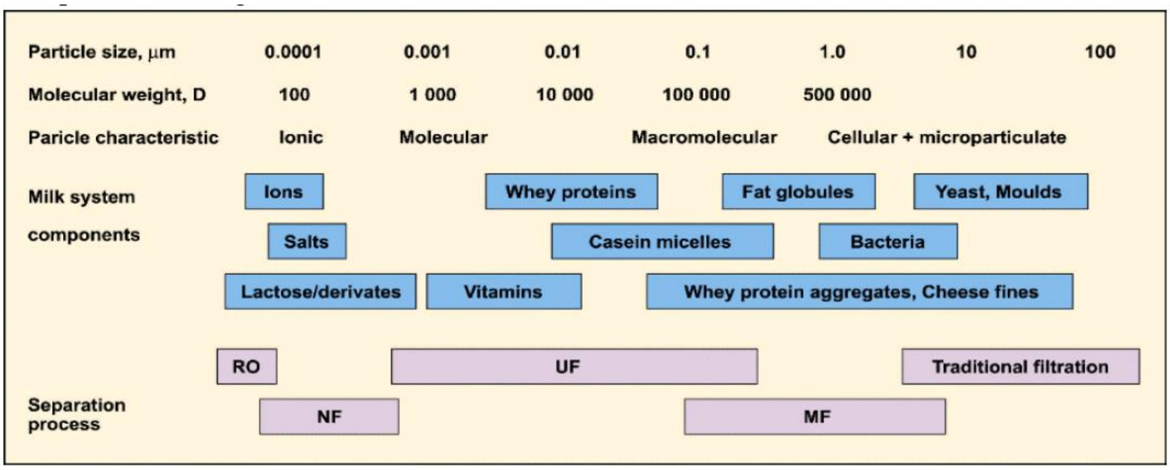

Figure 9 Suitable membranes for each component present in whey (De Wit 2011)

2.6.1 Removal Of Vitamin B12

Removal of vitamin B12 is commonly done in order to extract it from various sources, with the aim of capturing it with high purity. As with analysis of VB12, the cobalt and corrin formation is typically exploited as it is rather unique part of the cobalamin structure, possessing properties exploitable in extraction methods. From bovine milk VB12 extraction is usually done with using chromatographic methods using ion-exchange or adsorption. IEC and adsorption methods in VB12 reduction is investigated more in depth in chapter 2.6.3 of this thesis. The demand for vitamin B12 extraction is traditionally done in order to collect it for medicinal and nutritional aids. With the recent emergency in diets opting in for non-animal-based foods, such as veganism, the need for vitamin B12 supplements has also increased due to its absence in most plant-based foods. Due to its low concentrations in food sources, like whey, studies and applications revolving around the large-scale industrial removal and capture from whey are limited.

When the aim is to reduce the VB12 content of a solution, deconstructing it with photolytic methods may also prove as a valid opportunity, with the downside of inability to capture the vitamin. Photolysis starts with the degradation of cyanocobalamin into aquocobalamin and hydroxocobalamin, and further into smaller portions after cleaving of corrin ring via oxidation. This reaction can be intensified with pH adjustment with ascorbic acid. (Ahmad et al. 2017) The leftover chemicals, especially cobalt derived particles, may also cause unwanted effects, and their removal should be taken into consideration. The different properties brought by the group attached to the ligand site affect the effectivity of applied separation methods, as some of these groups have stronger bonds or different electrochemical properties, shifting their oxidation states and isoelectric points.

2.6.2 Removal of caseinomacropeptides

Removal and extraction of caseinomacropeptides has emerged as a rather new field of study in since 1990, due to the recent discovery of the peptide. Various methods have been developed for its extraction, mainly in the fields of ion-exchange, ultrafiltration and solvent partition.

Ion-exchange chromatography have been investigated for extraction of caseinomacropeptide, with the most revolving around the use of Ion-exchange. In large-scale production ion-exchange systems has typically been applied, due to the caseinomacropeptides isoelectric points vicinity of typical whey pH value. Ion-exchange chromatography in caseinomacropeptide removal and capture is investigated with greater detail in following chapter 2.7.

As opposed to packed bead chromatography, models using an ultrafiltration adsorption membrane have also been experimented on. Kreuß & Kulozik conducted a study using membrane adsorption chromatography or MAC to separate GMP from milk sweet whey, resulting in successful removal of GMP, however with diminishing efficiency after repeated batches due to membrane fouling. This however may be reduced using prefiltration. (Kreuß & Kulozik 2009)

One method for separation of CMP was studied by Bonnaillie et al. revolving around the use of supercritical carbon dioxide treatment on whey protein isolates, followed by filtration using UF-membranes, with the goal of increasing the β-Lg and α-La content in the isolate while also extracting high purity CMP. Supercritical carbon dioxide reactors cause precipitation of α-La and minor proteins, leaving behind a solution of β-Lg and CMP particles. Ultrafiltration was used to separate these two soluble particles from one another. (Bonnaillie et al. 2014, Yver et al. 2012)

2.7 Chromatography in whey fractioning

As the focus of this thesis revolves around the use of chromatographic methods, in this chapters this separation method is studied in greater detail.

Chromatographic methods have been applied for both separation and analytical purposes and they utilize various physicochemical properties. The simplified basics of these operations consist of a mobile phase that passes through a permeable stationary phase, leaving behind certain parts of it bound to the stationary phase. Depending on the properties of these particles they are separated into specific sections, or fractions, typically due to their varying velocity in the chromatographic system. The time in which a specific component takes to pass through a column is referred to as retention time. This phenomenon can be utilized in fractioning of certain feed stocks and comes especially useful in analytical chemistry, where the separation of different components into their own fractions is essential before further analysis. However, some systems are designed to selectively remove one specific type of substance with high ratio, acting as a method of extraction rather than fractioning.

The feed stream in this thesis is considered as highly demineralized whey powder, and thus the amount of competitive mineral ions is considered as next to none. As bulk industries, like whey treatment, the use of pH, conductivity and other properties altering consumable chemicals can become uneconomically high, it is paramount to design a system that uses them as few as possible or none if possible. This can be achieved with the proper selection of sorption materials and methods, complementing unaltered feeds chemical properties. Properties of the sorption materials can either be chemical between compounds functional groups or physical, for example the difference of porosity of the particles.

The basic fixed bed resin column chromatography uses a model where a feed solution is conveyed into ion-exchange or adsorption resin filled bed during the sorption, also referred as loading, phase. The charged particles are then bound into this medium, letting the effluent stream flow through the column. In ion-exchange chromatography the pre-existing ions exchanged from the resins are transferred into effluent stream. The bound particles are then released using elution solution, consisting high amounts of replacing ions, that releases the particles from the resins. After this the resins are typically regenerated, in order to restore them into their starting state, resetting the cycle (Nasef & Ujang 2012, Inglezakis & Zorpas 2012). This model of operation is described in Figure 10. (Nasef & Ujang 2012) In some cases especially in IEC, the resin is returned into its functional state already during elution, removing the need for a specific regeneration phase. This is typical for example in systems that lack pH alteration during elution. Regeneration is also used to reset the physical state of the resin bed as the state of the bed gets altered during normal process operations due to channeling of streams, formation of air bubbles and various other phenomena. This combined or replaced with, washing the column from impurities can be done in order to improve the longevity and effectivity of the resins. Backwashing with counterflow water to wash the resins is used to remove broken resin particles and impurities from the column, as well as reform the bed structure. The exact details of the methods presented greatly vary depending on the intended purpose, process parameters and applied techniques.

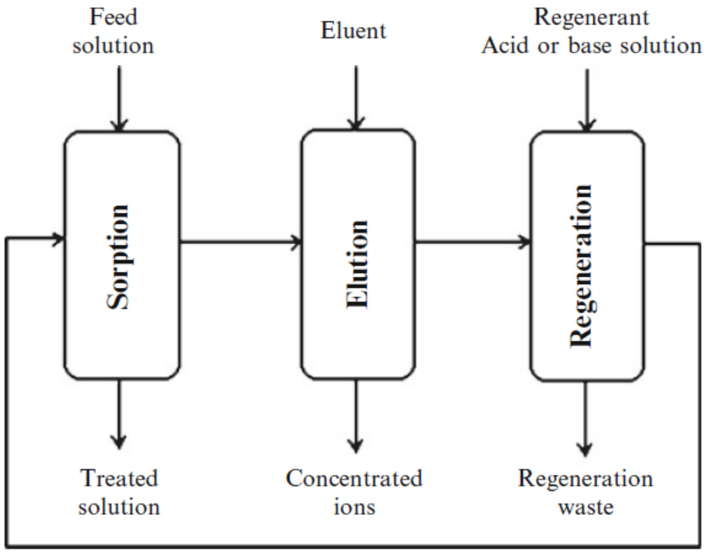

Figure 10 Schematic representation of ion exchange operation cycle (Nasef & Ujang 2012)

In the case of whey caseinomacropeptide removal using chromatography, the aim is to bind target compounds with high selectivity, thus leaving behind an effluent stream with high whey protein content. The selectivity of ion-exchange resin is also key, as the ions released into the effluent stream, should not endanger the food safety aspects and nutritional values of the purified whey. As production of resins may use chemicals unsafe for human consumption, or chemicals may emit from the resins, many of resins used for chromatography are deemed not usable in food industry.

2.7.1 Ion-exchange chromatography

In addition to demineralization of various solutions, ion-exchange chromatography can be also used in protein, amino acid and peptide removal from liquid sources such as whey. In this section, the overall functionality of ion-exchange chromatography, or IEC for short, is presented in moderate depth. Variations of this technique are used for large scale separation systems, as well as high in performance analysis methods. Following the similar principles as other chromatographic separation methods described previously, IEC uses electrochemical properties of particles to be bound into resin bed. After the binding, the particles are unbound by more reactive agent, typically basic or acidic solution or a highly saline water solution during elution.

During the feeding phase the extractant particles, possessing opposing charge to the resin bed, are bound into the bed while exchanging places with pre-existing mobile ions in the resin bed. These mobile ions thus take the place of the extractant in the solution ending up into the effluent stream along with the rest of the feed solution. This phase is continued until the bed is saturated with extractant particles and the binding capacity of the bed is filled. Signs of this saturation can be seen as decrease in separation efficiency and increase in the amount of the particles desired to be extracted in the effluent stream.

In the elution phase, excess amounts of ions of opposing charge to the resin bed are used to replace the previously bound particles. The now released particles are released into the elution effluent stream as a separate fraction. As the binding capacity is reduced with the change of pH on some particles, elution with typically acidic solutions can be used to disengage bound particles from the binding media in weak ion exchangers. (Velickovic et al 2012, Park 2012) Examples of chemicals used as elution agents are salts like NaCl or KCl and acidic or basic solutions HCl and NaOH.

Both of the separated particles and the ion-exchange medium must possess a structure with electrical neutrality and consist of two structural parts of opposing electrical charges. The type of the IEC is determined depending on the electrical nature of fixed ion media used. If the fixed media is negatively charged and the exchanged mobile ion a positive cation, the method is called cation-exchange. In Figure 11, a basic structure of ion-exchangers model is shown, consisting of a firm backbone structure and mobile ions. (Park 2012) The materials used for the stationary phase are chosen depending on the properties of the molecule desired to be separated. The retainability of the bound molecule can be altered by changing the pH of the mobile phase. (Velickovic et al 2012) The resin materials are characterized into four groups depending on their functionality into strongly acidic, strongly basic, weakly acidic and weakly basic. (Park 2012)

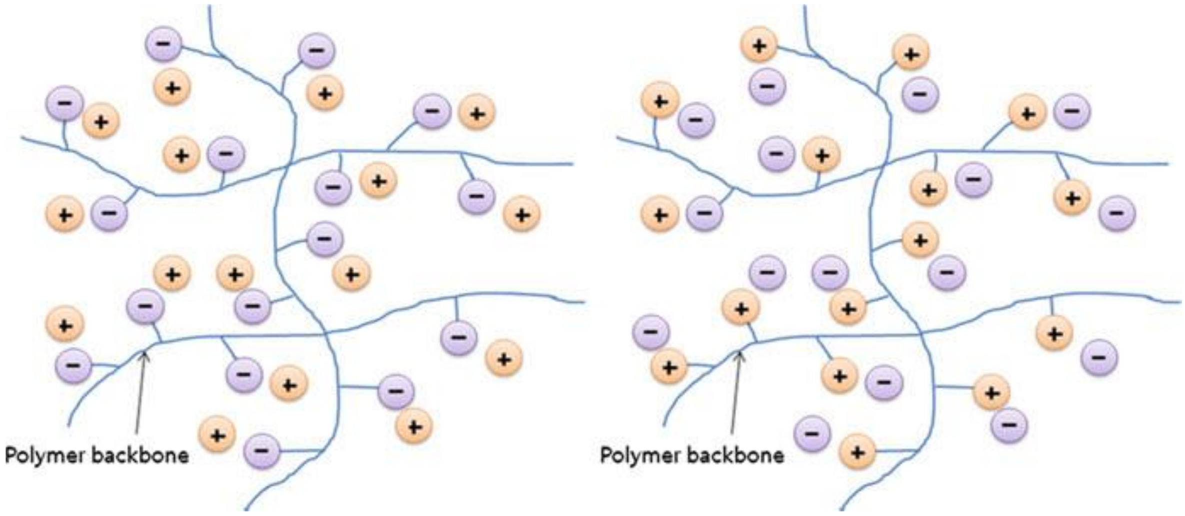

Figure 11 Structure of cation-exchanger (left) and anion-exchanger (right) (Park 2012)

For amphoteric substances, which act as either acid or base depending on the pH of the solution, the isoelectric point *pI*, the pH in which its net charge is zero, determines the used separation method. When the pH is higher than *pI* the amphoteric substance has negative charge, and while its lower the charge is positive. Proteins tend to contain amphoteric properties. The nearer the pH arrives to the isoelectric point, the lesser its ionic strength becomes, nearing neutrality at isoelectric point. The further it is the stronger the strength is. Overall, their properties depend on the specific amino acid content present in the protein. For example, amino acids arginine, lysine and histidine provide a positive charge, while aspartate and glutamate prove a negative charge when ionized. Peptide chains, while being shorter than most proteins, have more flexible effective pH-range, thus stronger ion-exchange systems can be applied. (Velickovic et al 2012) Anion exchange has shown potential to be used for extraction whey proteins β-Lg, α-LA and BSA, as they have specific isoelectric point *pI*-range varying from pH 4.7-5.4. (Stanic et al. 2012)

Traditionally, ion-exchange resins and membranes are polymeric hydrocarbon structures with alkali components as the exchanged ions. Typical exchanged ions present in IE-resins are alkali metals such as sodium and potassium. (Park 2012) The effectivity of the resins can be improved by increasing of its active reaction surface. This can be achieved with the increase of porosity or reducing the resin particle size and increasing their amount. However, the change in porosity and active surface area also affects the selectivity of the resin, as increase in particle size will accept larger particles with similar properties to bind themselves into the resin. Resins are typically separated into two structural types, macroporous and gel-type (microporous). Macroporous resins typically suffer less from volume changes due to their high degree and have a higher active surface area. Gel-type microporous resins in contrast offer typically higher sorption capacities. Sizes of these particles typically revolve around 0,5-20nm for gel-types and 20-200nm for macroporous. Resins typically consist of active functional groups and structural groups. Functional groups act as the part where the ion exchange itself happens. These groups are typically connected to each other by structural groups. Common structural group is divinyl benzene or DVB. Crosslinking between resins structures play important role in their functionality. Lower degree of crosslinking leads to higher absorbance of moisture, causing swelling and transformation of the resin's dimensions. This change in size can improve or decrease the properties of the resin, depending on its type. In gel type resins, the increase in size and moisture may improve its activity, however this may also increase the pressure of the water causing interference in the flow rate of the system. Macroporous resins tend to have higher degrees of crosslinking. (Nasef & Ujang 2012)

Capacity of the ion-exchange resin is determined based on its chemical and physical properties. This capacity can be expressed based in various ways depending on its state and purpose. Dry capacity, or weight capacity, is the ion-exchange capacity of the dry resin expressed based on equivalent number of univalent molecules in kilograms. Volume capacity is given as the amount of equivalent number of univalent molecules per volume, typically per liters. This value is typically determined using experimental data and provided by the manufacturer of the resin. When designing the amount of eluent to be used to meet the total capacity of an IE-systems, a safety factor is typically added towards this volume capacity in order to ensure saturation of the system. Selectivity of the ion-exchanger describes the affinity of the used resins towards certain components in the treated solution. The higher the selectivity is, the more likely it is that the resin binds the specific particle in the system. Compounds sharing lower rates of selectivity with the resin can be replaced by higher selectivity possessing compounds. This can cause specific fractions to form in the eluent, as materials with lower selectivity get replaced with higher selectivity ones, when the bed is saturated.

More analytical factors affecting the effectiveness of ion-exchange revolve around the principles of mass transfer and its subsequent thermodynamics. Selectivity coefficient between two components A and B with specific charges z for ion-exchanger systems can be calculated based on the following equation 1. (Lito et al 2012, Aniceto et al 2012).

$$K_{AB} = \frac{q_A^{|z_B|} c_B^{|z_A|}}{c_A^{|z_B|} q_B^{|z_A|}} \tag{1}$$

Where

q_i is the amount of substance *i* in solid phase, mol/l

c_i is the amount of substance *i* in liquid phase, mol/l

z_i is the charge of the substance involved

The protein concentrations can be acquired from the equation 2 presented below (Lira et al. 2009)

$$q = \frac{V(c_0 - c)}{M} \tag{2}$$

Where

V is the liquid phase volume, ml

M is the solid phase mass, g

c_0 is the liquid phase protein concentration at initial point, mg/ml^-1

c is the liquid phase protein concentration at equilibrium, mg/ml^-1

The values c_0 and c describe the concentrations from the initial influent flow and the beginning of breakthrough point, respectively. To determine the total nature of the ion-exchange phenomenon the saturation of the total bed is typically needed. This value for concentration *q* can be used to determine the selectivity between two components and is used to determine then isotherms involved with modeling the kinetic behavior of ion-exchanger units. These isotherms can be used to compare the effect that different factors such as temperature, pH and flowrate have on the ion-exchange units. Utilization of tests involving study of these factors, gives preliminary insight on the nature of ion-exchange and effects of which previously mentioned factors have on the possible separation.

In industrial-scale ion-exchangers fouling and loss of binding capacity causes problems, which are hard to detect in laboratory scale tests. (Rousseau 1987) The sorption phase should be continued until the binding capacity of the resins is filled. In some scenarios, it might be better to keep the total feed amount under the binding capacity, as beyond this point the removed material will flow into the effluent stream. When designing IE-columns, an evaluation on the value loss on impurity of product stream should be compared to the value loss on additional elution and regeneration steps. Simulations in laboratory for industrial scale applicable ion-exchange methods upscale can be done rathe well, with the major differences happening due to wall effect, an effect in which flow is disturbed by the column wall, due to the difference in diameter of the column and the relative wall area.

2.7.2 Chelating Ion-exchangers

Chelation, a phenomenon, in which two or more parts of an ionic organic compound or ligand are bound to a central metal ion, is utilized in novel forms in ion-exchange. Resins utilizing this method are traditionally used in treatment of waste waters rich in mineral and heavy metal content. In chelation, the ligand structure binds into a metal ion forming a claw-like structure around it. Exchange of this central atom or particle can be used similar to more traditional types of ion-exchange. Chelating ion-exchange resins, having a complicated crosslinked structure, tend to possess high selectivity towards specific compounds depending on their functional groups. The resin structure consists of two parts, the ligand part used for binding and a supportive polymer structure. The effect of solution pH is key when selecting chelating resin, as it affects the affinity of the ligands towards specific metal ions. (Nasef & Ujang 2012, Sud 2012)

2.7.3 Chromatography for B12 removal

Even though works on the removal of vitamin B12 from aqueous sources have been done in impressive quantities, previous works on VB12 removal from whey or milk seem are rather rare sight. A reason behind this is likely due to that most of commercially manufactured VB12 comes straight from a bacterial source, where the concentration is substantially higher. However, as where medical industry focuses on high effectivity and performance of VB12 manufacture, the focus in food industry revolves around selectivity due to high production amounts.

VB12 as a term is rather ambiguous, meaning the overall group of cobalamin-derived vitamers. As they all share the cobalamin structure as the basis, focusing on the removal methods applicable to this group could be utilized in removal of all VB12 variants. As pH and standard potential E° changes, cobalamin changes too, having an equilibrium at the pH 7.8 between hydroxocobalamin and aquocobalamin, lower values resulting in higher amounts of aquocobalamin and higher values into hydroxocobalamin. E°-pH-Diagram describing the change in forms of the three oxidation states of cobalamin is presented in Figure 12. (Lexa et al. 1977) As pH in whey is around 4-4.6, meaning that in this state the VB12 in the whey is present mostly aquocobalamin. The pH of the demineralized whey is usually slightly higher, around the value of pH 4.7. In this state, the charge of the cobalamin is neutral, and thusly its removal via IE-methods might pose a challenge, as altering the pH of the whey is non-ideal in industrial scales due to the amount of pH adjusting agents needed. The difficulty with this pH arrives, near the point of 4.7, cobalamin has neutral charge in its most oxidation states, thus finding a resin able to effectively bind this chemical is challenging.

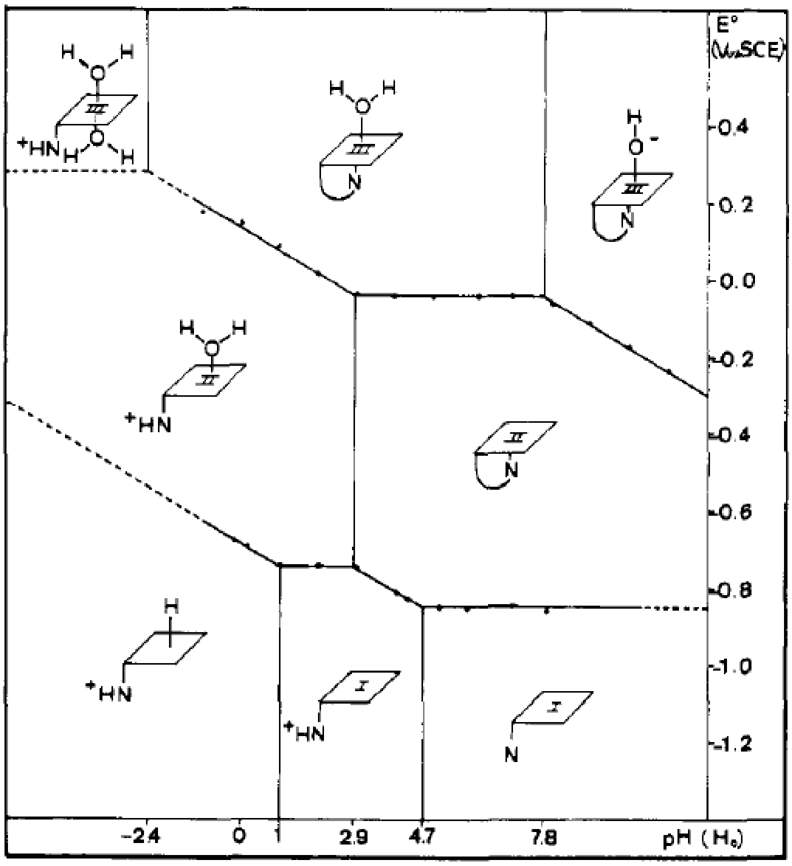

Figure 12 E°-pH-diagram describing the effect of pH and standard potential E° values onto cobalamin and its three-oxidation forms (Lexa et al. 1977)

Various adsorption materials and mechanisms have been studied in the general field of VB12 capture. In studies conducted by Guo et al. the potential of mesoporous carbon coated with poly-(methyl methacrylate) for VB12 extraction. Selection for mesoporous adsorbents was justified by the need for large active surface area, since the VB12 large particle size of 2.09nm. It was found that the implementing of higher pore diameter and surface properties increased the adsorption significantly. (Guo et al. 2005) As the study used VB12 mixed in a water solution, any side effects caused by the presence of other whey fractions might happen when using whey as a feed solution, causing decreased adsorption in similar studies.

Non-ionic adsorbents in VB12 extraction were studied by Ramos et al, using microporous polystyrene resins crosslinked with divinylbenzene in a fixed bed system functioning essentially as a chromatographic separation, and comparing it to a mixed tank system utilizing similar materials. The pH in this scenario was set to 5. The aim of the study was to determine adsorption parameters and take a deeper look into mechanisms present in adsorption of VB12 into non-ionic adsorbents. (Ramos et al. 2004) Studies in immunoaffinity chromatography, a method that utilizes the use of antibodies in a liquid chromatography system to bind biological agents (Moser & Hage 2010), have also been concluded in the field of VB12 extraction and detection, with promising rates. (Nakos et al. 2017, Campos-Giménez et al. 2012)

Other chromatographic methods utilizing activated carbon in VB12 removal, however in a water treatment scenario, have been concluded. For example Lorenc-Grabowska Rutkowski gained promising results using macroporous activated carbon (AC). In this study it was noted that the used sawdust polypropylene based AC had the pH point of zero charge at 9.19, while the feed solution having the pH of 8.1-8.4.(Lorenc-Grabowska & Rutkowski 2014) As in this point B12 is in the form of negatively charged hydroxocobalamin and the AC is under its point of zero charge, resulting in opposite charges, attraction is possible. This renders its effectivity in lower feed pH values drastically and thus the use in whey adsorption might be compromised.

Early models on ion-exchange using cation and anion beads for VB12 recovery were patented by Colovos in the year 1950. This method used cation beads either from sulfonic acid groups containing tannin- or formal-aldehyde materials followed by anion beads made out of aromatic amine structures with formaldehyde coating or melamine containing tertiary amino groups. The latter anion-exchange was done in order to purify the product retrieved from the cation-exchanger from other organic compound caught in the cationic beads. Regeneration for anion was done with alkali solutions, while for cation beads this was done with acidic solutions. (Colovos 1950) This method has not been applied to whey, and as suggested by the use of following anion purification of VB12 extract, the selectivity of these resins is considered to be low.

Chelating resins might show potential in ion-exchange of VB12. The use of chelating ion-exchange resins could be used to bind the central cobalt. However, as in pH 4.7 and in its third oxidation state, the cobalamin is in the form of aquocobalamin, a highly stabile and electroneutral form of cobalamin, the effect of chelation is reduced.

2.7.4 Chromatography for CMP removal

Removal of whey proteins and peptides is typically done using a packed-bed ion-exchange chromatography, as this method has been proven to be highly effective. This method can be applied into the removal of caseinomacropeptides, including the glycosylated variants.

As caseinomacropeptides and glycomacropeptides, like other protein and peptide components, are amphoteric substances their charge and thus eligibility to be used in ion-exchange is dependent on the pH of the working solution, the whey in this case.

$$\text{Protein's charge is positive} < \text{isoelectric point } pI < \text{Protein's charge is negative}$$

As this charge of the protein determines the choosing of the ion-exchange resin, between cation and anion, understanding this nature is important. Typical practice in protein fractionation is the selection of operation pH based on the target protein. According to calculations by Kreuß et al. the isoelectric point of CMP was found to be at 4.06, and at 4.15 in experimental values using zeta potential measuring. For glycosylated GMP pI was found at 3.15 using similar experiments. (Kreuß et al. 2009) Other amphoteric proteins found in whey near the *pI* value of CMP/GMP are the ones of β-Lg, α-LA and BSA, with β-Lg having its *pI* at near 5,2 and α-LA at 4,8. (Fox et al. 2015, Stanic et al. 2012) A change in pH over the isoelectric points of these proteins may cause competition of binding capacity of the resin, leading to lower selectivity, product purity and yield. This may cause problems in the pursuit high selectivity removal of the peptide.

Noteworthy, when treating food products, the used resins, eluents, and other materials should be food grade and the collected materials should not be contaminated by unsafe chemicals. The ion ending up in the purified whey streams should not cause any unwanted properties to the end product, such as increase in salinity or pH that might make the product unusable or unsafe. If these values rise too high, additional process operations might be needed to apply, however this could prove to be expensive and complicated.

Researches using anion exchange chromatography for CMP removal have been conducted by many authors. (Outinen et al. 1995, Doultani et al. 2003) In the production of whey protein isolates, the production of pure GMP and protein isolate in a same column has proven to be rather difficult, and thus the separation of caseinomacropeptides must be done in its own column. Demineralization of the whey improves the effectivity of ion exchange, as the mineral salts naturally present in the whey may compete with the CMP molecules for the ion-exchange medium. Doultani et al. studied the functionality of a Q-bead cation exchanger followed by a S-bead anion exchanger for CMP removal. The first cation exchanger was used to separate the whey proteins, and the followed anion exchanger to capture the glycosylated caseinomacropeptides. This system managed to recover 92% of whey proteins in the first column and achieve 96% of GMP captured in the second. In study by Outinen et al. CMP was extracted from microfiltered emmental cheese whey using Diaion HPA 75 strong basic anion exchange resin, a highly porous resin utilizing dimethyl ethanol ammonium functional group with styrene-DVB as structure. The resulting peptide stream contained CMP purity of 80%. (Outinen et al. 1995) Nakano & Ozimek studied the purification of GMP at pH range of 2-4,5 in contrast to the typical 4-5 suggested in many preceding studies. It was found that anion exchanger DEAE-Sephacel, functional group of dimethyl ethanol, could be used to effectively extract GMP from dialyzed whey under these conditions, and would have the pH optimal range between 2-4. (Nakano & Ozimek, 2000)

Lira et al. studied the thermodynamic properties of GMP removal using strong anion exchangers. It was suggested that increase in salinity of the feed lowered the adsorption capacity of sorbent material, while moderate increase in temperature from 25 Celsius degrees to 45 had a positive effect on GMP binding. The calorimetric studies indicated the adsorption in anion exchange of GMP to be endothermic reaction. (Lira et al. 2009)

Effects of various properties of the elution buffer used in anion-based IEC were studied by Tek et al. According to this, the buffer increases of pH 4 from pH 6 increased the amount of GMP captured, but also lowered the selectivity due to the high amount of captured β-Lg. Under the value of pH 4, GMP capture was highly diminished with high contents of it flowing through the column ending up in the effluent. With the salinity of 0.1, 0.2 and 0.3 M of NaCl in elution water, the GMP capture was favorable, with no distinguishing difference between them. However, with values 0 and 0.5 M the capture was significantly lowered. It was also found that the higher the conductivity of the buffer, the weaker the binding of GMP is. This was due to the anion exchangers losing their binding capacity in higher conductivity rates. (Tek et al 2005) As the salt concentration is needed, but its concentration difference has low impact on low dosages, the amount of its use should be kept at low values. Additional NaCl also transfers to the elution samples in minor fractions, increasing the salinity of the GMP concentrate produced.

The effective separation of GMP from non-glycosylated CMP using anion-exchange chromatography was studies by Kreuß et al. The study used membrane adsorption chromatography as opposed to a conventional fixed bed bead system. Beads used in this membrane were strong anion exchange beads from varying manufacturers. Elution was done with a mixture of NaCl, NaAc, NaCit and *N*-methylpiperazine. Study found out that at buffer pH of the range 4.0-4.1 achieved the best results in CMP and GMP fractionation. Under these conditions, the to be bound GMP was washed away with CMP into the effluent stream during elution. As with the separation of caseinomacropeptides, mentioned above, when the conductivity and ionic strength of the starting buffer was increased, the effectivity of GMP binding was also reduced, leading into unwanted results. It was also concluded that MAC systems in CMP fractionation tend to be more favorable in operation speed compared to the packed bead system. (Kreuß et al. 2008)

Recent studies in the field have also been published. Nakano & Betti studied the effectivity of food grade anion-exchange resins and chitin in separation of high purity caseinomacropeptides from whey. Elution in the study was done using NaCl solution of two molarities, 1M and 2M. Chitin was selected in this study as opposed to theoretically superior chitosan, due to the solubility of untreated chitosan in acidic whey solution. Study concluded that chitin could be used as an effective adsorbent and a competitive alternative to commercial ion-exchange resins in caseinomacropeptide recovery. (Nakano & Betti 2020)

3. EXPERIMENTAL WORK

As the aim of this thesis, is to find an optimal solution for separation of whey components, experimental work was conducted on the matter. This experimental work consisted of testing and comparing of four ion-exchange resins, two for CMP and GMP removal, and two for VB12, via sequence of tests studying their behavior at different flow rates and feed capacities. Due to the wishes by Valio Ltd, discretion is taken with the used materials, and thusly resins used in this thesis will be referred to as resins A, B, C and D.

Samples were taken from these tests and analyzed in order to better understand their separation effectivity and selectivity. All tests were done in a laboratory scale using non-pretreated feed solution. This was done to emulate the practices common to process environment as close to possible in laboratory scenario.

3.1 Materials and methods

In this chapter, the applied methods, as well as used materials, used in this thesis for the separation of whey fractions are described. These methods include both experimental methods involved with the chromatographic extraction and analytical methods following these experiments.

3.1.1 Materials

The feed solution for the experiments were provided by Valio from their manufacturing plant at Lapinlahti, Finland. The used solution was non-standardized 90% demineralized whey solution, gathered straight from the process stream after the demineralization process. Samples were kept in cold storage. The samples were introduced into room temperature and shaken by hand before sampling into feed tank, to improve homogeneity of the solution and solubility of precipitated particles present. Freezing of the samples was tried but was concluded to be detrimental to the experiment due to the increased formation of precipitated solids, suspected to being crystalized lactose or precipitated proteins. Also due to the sheer amount of feed solution needed for each experiment, thawing was concluded to being unacceptably time and energy consuming. As the feed stock was gathered from a process stream as an unfinished product, the composition and quality of the feed varied between the provided sample batches.

Two types of strong anion exchange resins were used for GMP and CMP removal, Resin A and Resin B. Both of these resins arrived in Cl^--form, and thusly were treated with NaCl during elution. Both resins were washed before operation to remove possible impurities and fractioned resins. Resins C and D arrived at Na^+-form and treated similarly to resins A and B with NaCl. Selection of these resins were done based on previous experiments conducted or ordered by Valio. Resin A had previous laboratory scale testing done in columns using pre-treated whey samples and acted as a reference point, while resin B only had been experimented in small scale decanter tests. Resin D had been studied in previously once by external sources, while resin C was the only resin not being previously tested on. The selected resins had to be food grade quality and certified for this purpose.

3.1.2 Applied methods

The experimental methods applied in this thesis revolved around ion-exchange in packed bed columns filled with resins selected for the respective extracted substance. Two types of columns were used in the experiments, both in a temperature-controlled setting. In nearly all of the experiments, the feed solution was left unaltered in order to yield accurate results, and thusly no filtering or pH adjusting was done. Two types of experiments were done in this study. First tests were done to determine the optimal feed flow rate. After selecting the optimal flow rate, a capacity experiment with the goal of testing the binding capacity of the resin bed as feed volumes increase was conducted. The aim was to repeat this testing cycle for each resin, however due to the limited time sampling and testing from resin C was limited only to capacity experiments using optimal flowrates based on previous experiments.

Samples were gathered from each experiment using a fraction collector in flowrate test and by hand in capacity tests. As some of the tests ran longer than the expected, due to slowing flowrate in present with last few bed volumes, the data points were correlated for the average flow rate of the system. The causes and proposed solutions for slowing flowrate are addressed in section 3.1.9 on column fouling.

All experiments followed basic batch process cycle, consisting of three parts: sorption (loading), elution and washing. The applied methods varied from the cycle presented in section 2.7 Figure 10, by including washing of the resin bed while replacing the regeneration phase, as with the resins used additional chemical treatment of the bed was not required. Additional chemical regeneration might improve the effectivity however, but study of this was left out of this thesis. Block chart describing the used process cycle is presented in Figure 13.

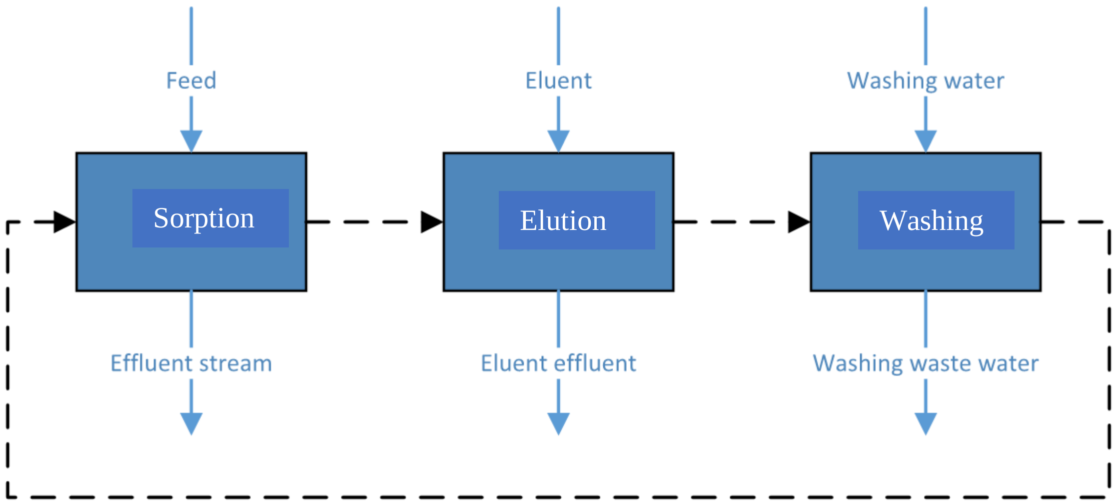

Figure 13 Process cycle steps presented in a simple block chart. Dashed lines describe the process flow and undashed material streams.

The experiments were done in packed bed column systems utilizing a vertical glass or plastic column filled with resins respective to each experiment. The packing of the column was done by first inserting the dry resins into the column, and then introducing de-ionized water into the system. The water was flown through the column in with current and co-current to ensure the tight structure of the packing and prevent the formation of air pockets into the bed. All resin beds were eluted and regenerated before the experiments. Used resins were fresh from the supplier, and not used for any other experiment prior. In each column a freeboard space of about roughly 15-20% column volume was left due to possible bed expansion and to make backwashing easier.

Initial studies for ion-exchange behavior in CMP/GMP removal was conducted using Resin A strong base anion exchange resins in a 0,8l glass column provided by LUT-university. The column was equipped fine filters for both feed and effluent streams. While using a one-pump suction-based system, the use of filters however proved to be challenging, as the precipitated matter present in whey caused clogging of the preliminary filter. Due to this, the system was equipped with an additional feed-pump and the feed filter block was replaced with a silicone tube held by a funnel. The use of the second pump was mandatory, as without the feeding block the system was no longer airtight, and thusly siphoning could not be done. Both of these pumps were peristaltic pumps by Millipore. The cooling jacket of the system operated at around 7 Celsius degrees, measured from the outlet flow of the cooling water. Sampling was done using a fraction collector (Pharmacia Frac-100) installed into a pipeline split after the suction pump in the effluent line. Steady flow of the collected sample was ensured by the use of a smaller peristaltic pump (Amersham Bioscience Pump P-1).

The flowrate was determined by timing with a digital timer the effluent gathering from the suction end into a measuring glass. Initial setting of flow rate was repeated thrice to ensure accuracy, due to the cyclical pumping nature of peristaltic pumps. Several on process measurements and corrections on the flowrate were conducted to ensure the flowrate stayed as unchanged as possible. The feed pump was operated to keep the surface level of the feed and freeboard in check. Setup and a simplified flow chart of this system is shown Figure 14.

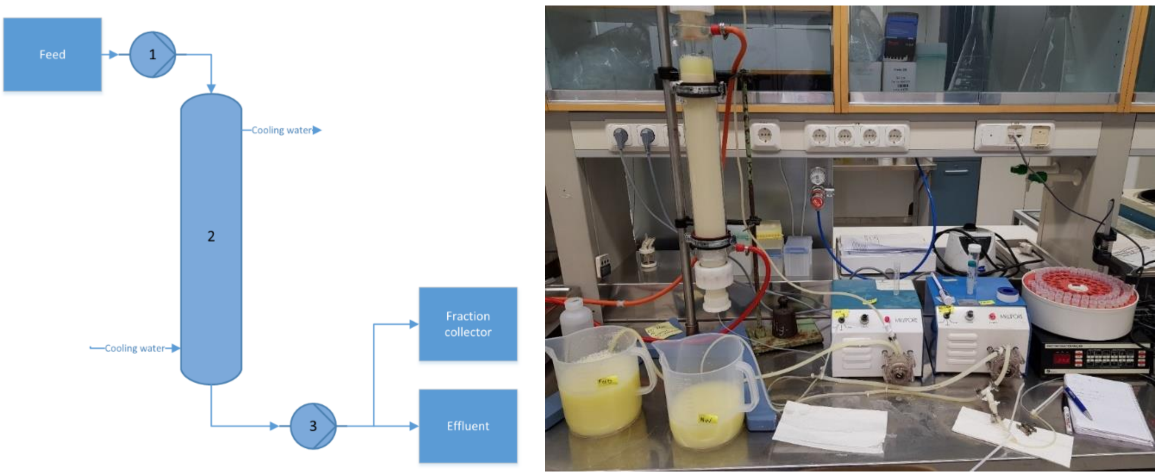

Figure 14 Process setup for extractions using Resins A, C and D, consisting of jacketed glass column (2), two peristaltic pumps (1, 3), fraction collector and feed & effluent tanks.

Flowrate tests were done for four different flow rates 1, 4, 7 and 10 BV/h in order to map the optimal operation parameter for flow rate. The tests were concluded at total of 20 bed volumes reached, with the exception of flow rates of 7 and 10 BV/h, which were abruptly stopped at near bed volume of 19, due to the clogging of the column. This clogging also caused the slowing of flowrate during the last bed volumes, rendering the accuracy of the last 2-4 bed volumes in tests for all flow rates except 4 BV/h. This was likely due to that the batch in which 4 BV/h tests for both resins A and B were experimented on was fresher and had visibly significantly less coagulated matter, resulting in only minor signs of increasing resistance due clogging.

Anion exchange resin B was used for CMP/GMP removal as a comparison. Study was conducted using a plastic column with height of 100 centimeters and diameter of 10 centimeters, located in a cold storage with ambient temperature around the column at 5 Celsius degrees. Watson-Marlow 630s peristaltic pump was used at the suction end of the column, causing the feed to be siphoned from the top of the column via a metal straw injected into the liquid freeboard space over the resin bed. Feed stock was added in small batches into the feed tank to minimize the setting of larger particles to the bottom of the feed tank in the absence of a mixer, ensuring the relative homogeneity of the feed. Sampling was done using a fraction collector, similar to the previously described experiments using resin A. Simplified flow chart and a picture of system setup is shown in Figure 15 below.

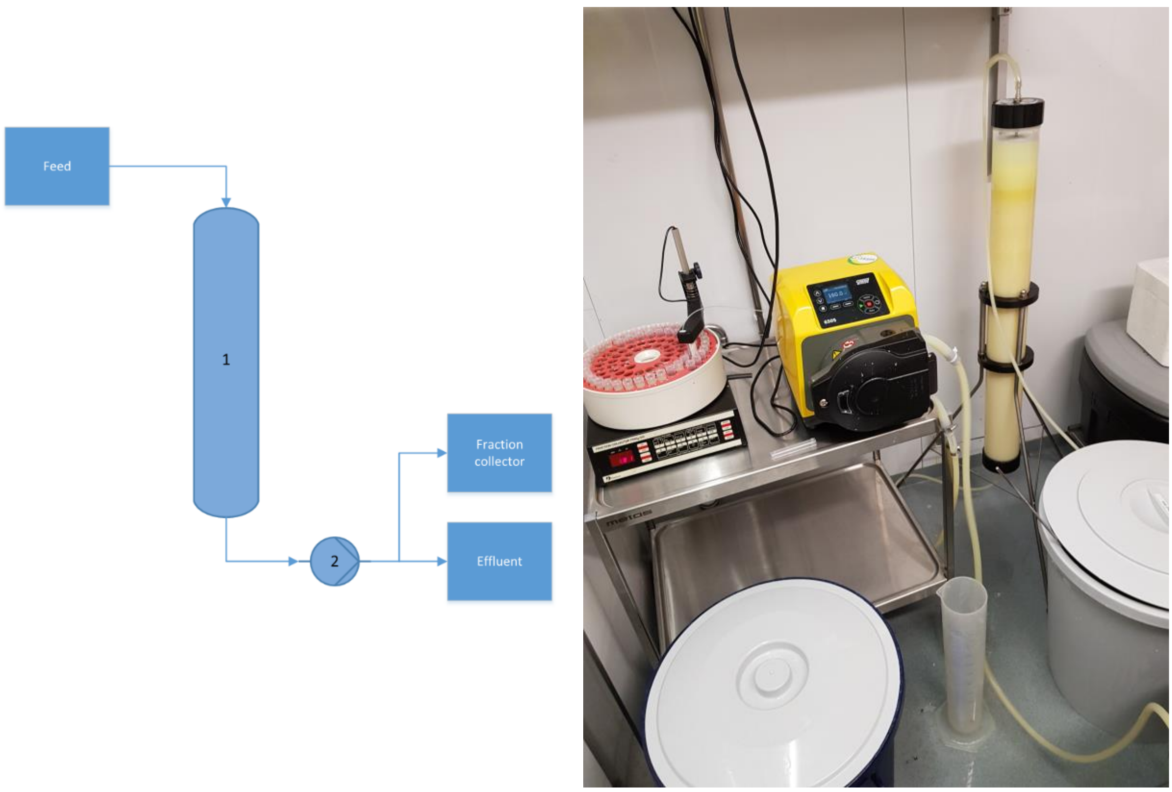

Figure 15 Process setup for extractions using Resin B, consisting of plastic column (1), peristaltic pump (2), fraction collector and feed & effluent tanks.

The flowrate experiments were conducted for flow rates 4, 7 and 10 BV/h. The 1 BV/h experiment left out due to non-promising results and technical difficulty present in the parallel Resin A experiment. Similar to the tests with Resin A, clogging was involved and thusly caused an early end to the test with 7 BV/h flow rate and caused similar slowing effect with 10 BV/h flow rate. For 10 BV/h test, it was noticed only after the tests and during sample analyzing, that half of the feed stock provided had significantly lower solid concentration than the rest. This was likely due half of the feed samples being taken after washing phase of the demineralization process equipment.

3.1.3 B12 extraction

The experiments for B12 extraction were conducted in a system similar to the one used with CMP/GMP extraction using resin A. Two resins were selected for these experiments, resin C and D. Resin C was a strongly acidic gel-type cation exchange resin, while resin D was weakly acidic chelating cation exchange resin.

In the following vitamin B12 experiments, the system setup was altered with the addition of light protective equipment. The fraction collector and column itself was covered with a cover made out of cardboard and the pipelines were fortified with aluminum foil. As B12 is degrades under certain wavelengths of visible light and UV, the protection was necessary in order to the results to be accurate. Analysis for these samples were done in a room with specific lighting for vitamin analysis. System setup is show in Figure 16.

Figure 16 Light protected process setup for extractions using Resin C and D. Effluent tank left unshielded while using fraction collector.

The experiment for resin C were conducted with the aim of reaching 30 BVs using the flowrate of 4 BV/h, which suggested to yield promising results with previous experiments using CMP/GMP. However, during the experiment the highest BV reached was only 9 BV before the resin bed was clogged. The experiment was retried with standardized pH of 5,35 to minimize the coagulate effect caused by denatured amino acids, however this only resulted in the reaching of 10 bed volumes. It can thusly be concluded that this raise of feed pH, plays nonsignificant part in the prevention of clogging. It should be noted that unlike with the resins A and B used for CMP extraction, the clogging mostly only occurred at the bottom of the resin bed at the point of outflow filter, and the clogging was significantly more sudden, happening with mere span of 1-2 bed volumes. This is likely due to the gelatinous nature of the resins letting the coagulated mass more easily pass onto the bottom levels of the column to be then stopped by the fouling filter. One suggestion to this problem maybe selection of a coarser filter, which would allow the passing higher particle size coagulated flocs pass through into the effluent.

Experiments for resin D, were conducted similar to A and B, with the goal of mapping out samples from various flowrates, before testing the binding capacity with the most promising flow rate. The selected flowrates were 1, 4 and 7 bed volumes per hour. From these, however the data gathered from 1 BV/h experiment was rendered short due to malfunction in the fraction collector at some point after 8.5 hours mark while the system was without supervision. At an unknown point after this the resin bed had also gotten clogged. Due to these challenges and the limited amount of time present, repeat test and thusly the possibility of capacity experiment using 1 BV/h was deemed too risky and challenging.

The binding capacity was thusly selected for 4 BV/h, as it resulted in more promising and comprehensive pH and conductivity results compared to 7 BV/h. However, this capacity experiment was cut short by coagulation of proteins and lactose in the system, just after 8 bed volumes. Repeat experiment was planned and prepared, however the samples provided for this experiment had abnormally low brix value for dissolved solids of 8-9%. As the time provided was running out, the experiment was put on hold, waiting for B12 extraction results from previous flow rate determination samples.

3.1.4 Washing of the system

After the sorption phase was concluded and the feed solution had passed thought the column, the system was washed with co-current feed of de-ionized water until the effluent was clear. This was followed by backwashing using counter-flow to rearrange the resin bed and remove air pockets and channels possibly generated by the previous experiment. Conductivity and Brix values for dissolved solids were monitored during washing to ensure the system returns as close as possible to its original setup. The de-ionized water was boiled in order to reduce presence of air within the water. In initial tests it was found that the air caused formation of air pockets and channeling. The amount of water needed for this washing depended on the used resins and column, but typically revolved around 5-6 bed volumes.

3.1.5 Elution and regeneration

After washing the system was eluted and thusly regenerated using 10% NaCl solution made from high purity food grade salt (ESCO Pure dried vacuum salt, 99.8% NaCl). The amount of solid ionic activator was calculated based on equation 3, presented below. From this value the total amount of the used solid activator, NaCl in this case, was calculated. The amount was diluted into 10% NaCl water solution with de-ionized water.

$$m_I = 2 \cdot M_I \cdot \ C_{eq} \cdot V_b \tag{3}$$

Where

m_I	is mass of the elution ion, g
M_I	is molar mass of the elution ion, g/mol
C_{eq}	is the resin specific volume capacity, eq/l
V_b	is the volume of the resin bed, l

The safety factor of 2 present in the calculation is used to ensure that the elution capacity is met, and the elution is not left incomplete. The specific volume capacities for each resin are presented below in Table I. These values were given by the suppliers of the respective resins.

Table I Resin specific volume capacities, C_{eq}

	RESIN A	RESIN B	RESIN C	RESIN D
C_{eq} *(eq/l)*	0.5	0.8	2.2	2.2

The saline eluent was fed introduced to the column in a feed velocity respective to the eluent to be spent in one hour. After the eluent was spent, de-ionized water was fed into the system until the conductivity of the outlet stream reached values similar to the starting stage. Upon reaching this, the feed current was changed, and the column was yet again backwashed.

From the capacity tests, samples of the elution were gathered and analyzed for CMP and GMP concentrations and other possible protein leftovers. This was done in order to gain a more in-depth perspective of the resin's selectivity and the strength of the elution, as well as gain preliminary intel on the purity of the separated GMP/CMP concentrate. The sampling in resin A elution was disturbed by technical difficulties, and the amount gathered was minor and less frequently select than with resin B. The tests done for these elution effluent samples were Kjeldahl total protein analysis, GMP and CMP detection via HPLC analysis and the contents of native whey proteins β-lg and α-la.

3.1.6 Effluent sample analysis

From each experiment, 40 to 60 ten milliliter samples were collected using a fraction collector (Pharmacia Frac-100 paired with Amersham Bioscience Pump P-1) between specific intervals. Two samples 50 milliliter from the feed stock were also collected from each experiment. After the experiments were concluded, the samples were frozen and later thawed for analysis using water bath set to 55 degrees Celsius. From these samples dissolved solids content, conductivity and pH were measured. Dissolved solids content was measured using a refractometer by Atago (Pocket refractometer), which utilized the Brix method. Conductivity of the samples was analyzed using electrical conductivity meter by Mettler Toledo (SevenCompact Conductivity). Finally, pH was measured with portable pH meter (Knick Portavo 904). All measurements were done in normal room temperature. Based on these, several samples were selected to be further analyzed using spectrophotometry, and later for HPLC analysis.

3.1.7 HPLC Analysis

HPLC analysis was conducted in a fashion described by Thomä et al. (Thomä et al. 2006) for determination of CMP and GMP concentration of the samples. The analysis itself was conducted by Valio R&D research analytics team, with the pretreatment done by the author. Selected 1ml samples were diluted using 0,1% trifluoroacetic acid (TFA) in one-to-one relation. The samples were then filtered through a 0,2 µm syringe filter, to remove any precipitated particles from the solution. Standards for analysis were obtained using Lacprodan GMP-based standard solution. The samples were loaded into Acquity ultraperformance LC by Waters Alliance HPLC system fitted with specific LC-column capable of analyzing hydrophilic proteins (Jupiter 5µ C18 300Å). UV-detector present was set to analyze between 226-280nm. As samples gathered from elution effluent had high concentrations of CMP/GMP, a higher degree of dilution of 1:10 or 1:100 was applied to these samples.

The analysis for vitamin B12 was conducted by external analysis company, Eurofins Scientific. The used method involved the use HPLC analysis methods, with details disclosed and not provided.

3.1.8 Additional analysis

In addition to previously described analyses, selected samples were also analyzed for their protein, lactose and chloride contents. These analyses were conducted by Valio R&D Research analytics team in Pitäjänmäki, Helsinki, and Lapinlahti.

The total protein content of the samples was analyzed using the Kjeldalh method using techniques based on ISO 8968-1:2014 standard. The native whey proteins β-lg and α-la were identified using gel high performance chromatography. In addition, an SDS-PAGE analysis was conducted by Valio research team to support the results for protein content.

Fat content analysis was done by using extraction with NH_4OH solution based on ISO 1211 standard for dairy fat content determination. Lactose content was determined using enzymatic means based on ISO 5765-1 standard, and Ash content based on ISO 8070 by incineration in a muffle oven at 550 Celsius degrees. Chloride content analyses were done by Valio R&D Research analytics in Lapinlahti, using titration utilizing the reaction and equilibrium between silver nitrate and chloride to form silver chloride.

In depth amino acid analysis was ordered from Eurofins scientific to determine the composition of whey samples in GMP/CMP capacity experiment. Methods used for this analysis were done along the lines of ISO 13903:2005 standard for most of the amino acids, with the exception of tryptophan for which method based on EU 152/2009 was applied. The specific details of this analysis are unknown.

Rapid analysis method for GMP/CMP content from samples was also tried as a means to identify potential samples before the use of HPLC analysis, as opposed to blindly picking promising samples. This method was done along the lines of method described by Fletouris et al. in 1993. This method utilized the UV-spectrum analysis of whey protein samples mixed with 6N guanidine-HCl reagent. As GMP/CMP lacks tryptophan and tyrosine, comparison from the sample’s fourth derivate of absorbance in wavelength 285.5 nm, where the amino acids create a typical trough, could be done to identify the relative concentrations of GMP/CMP present in the sample using a calibration curve. This curve was constructed using Lacprodan GCMP 20 GMP concentrate in increasing concentrations in the reagent solution.

However, while conducting the analysis the results received had highly varying values, with no seeming connection between them. This might have been due to the degradation of the reagent as the chemical was long past its suggested use before date. Since the schedule was rather tight, and samples were waiting to be analyzed, this method was scrapped.

One similar method using UV was also tried to verify the results from HPLC in later phases of the experiments. This method was used to construct a calibration curve from varying concentrations of GMP containing mixtures made of Lacprodan GCMP 20 concentrate from Arla and β-lg isolate from Davisco. The aim was to use similar 6N guanidine-HCl reagent mix, with fresher reagent, and analyze the UV-absorbance at the previously mentioned wavelength of 285.5 nm. From the acquired absorbance and the calibration, the concentration of the total GMP in the sample could be calculated based on the total protein content. However, yet again the method proved no coherent results, due to no clear peaks forming around the wavelength. Yet again, this method was scrapped due to timing and focusing to more pressing matters. With more time at hand, one could have found a way to gain usable results using UV-spectrophotometry as there exists previous studies in the matter.

3.1.9 Column fouling

As mentioned in previous chapters, the experiments were disturbed by clogging caused by a precipitate made of crystalized lactose and precipitated proteins that was present in nearly all provided feed solutions. This fouling was experienced as visible formation of white precipitated matter on top or the bottom of the resin bed, as well as formation of air pockets in the resin bed. As cause effect of this, the flow resistance increased, causing the need for increase in pumping strength in order to keep the flowrate at standard value. It was hypothesized that this precipitate formed over the transportation and storing that happened between sampling and experimenting. As the samples were transported over time period of 1-3 days ensuring their optimal storing was compromised, and it is likely that the samples had been exposed to unfavorable temperature conditions.

As the feed stocks pH was, in most samples, in the range of precipitation pH range of denaturized proteins, it is suspected that the coagulation of these proteins played a significant part in the clogging. Few samples were taken from the precipitated matter and analyzed for lactose and total protein contents. The analyses found both lactose and protein contents to be equally present in the samples. These samples however had high amounts of variance between each other and no valid verification on the composition of the coagulate could be gained from them. Examples of this precipitate is shown in Figure 17, taken from 1 BV/h flowrate experiment, where the formation of the precipitate was most prominently seen. The pictures were taken at 5- and 10-hour mark and after the experiment. Unseen in the figures, the precipitate barrier had concaved center caused by the flow of the feed stream.

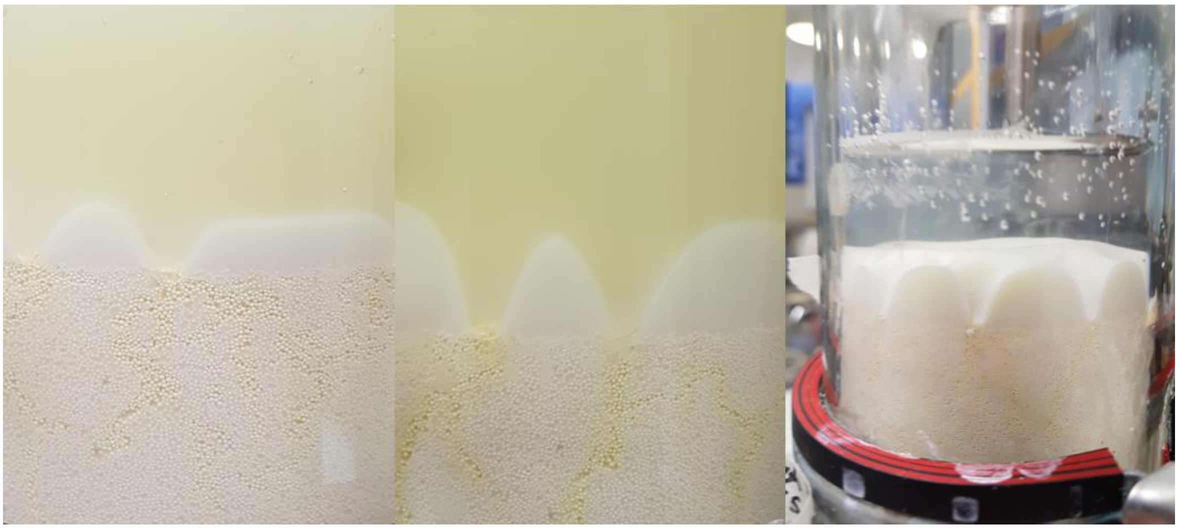

Figure 17 Examples of precipitate formation on the top of the resin bed in flowrate experiment for 1 BV/h. Pictures taken from at estimated 5-hour mark (left), 10-hour mark (Center) and during washing (right). Note that the center of the "structure" concaves into resin bed due to the flow.

In later experiments, more fresh samples, 1-2 days old, were acquired and experimented on. In these experiments it was noted that the feed stock was visibly clearer, and no clear precipitate was observed in the samples before operations. The experiments, these samples were involved in, no signs of clogging nor increase in flow resistance were experienced. As this was the case, and the existence of precedents of systems under similar pH and temperature conditions have been operated without similar difficulties, it was concluded that this is phenomenon is unlikely to cause problems in industrial environment. However, more study should be concluded on this by introducing a pilot scale model into the pre-existing de-mineralization systems and observe if similar problems occur.

3.2 Results and discussion

In this chapter results are presented and evaluated for both caseinomacropeptide and vitamin B12 extraction in their respective chapters. The results focus around the development of extraction efficiency and binding capacity of the resins, while comparing two resins for each extractant.

3.2.1 CMP/GMP: Optimal flow rate

Using the data gathered from the set of analysis described in section 3.1.6, a curve describing the development of solid contents in the system was constructed using the ratio dissolved solid content per each bed volume. From this curve the point in which the feed passes through the whole column can be determined. The results for both resins were quite similar resulting in solid contents passing through the column at points varying between 1-2 bed volumes, with the Resin B having taking slightly longer to gain full passthrough compared to Resin A. Example case of two curves is shown in Figure 18 describing the correlation between solid contents relative to feed for bed volumes for bed volumes consumed. The error bars signify the standard deviation between 5 bed volumes, with exclusion of initial loading phase for the first five. Data was only slightly processed by cleaning out significant outlier values.

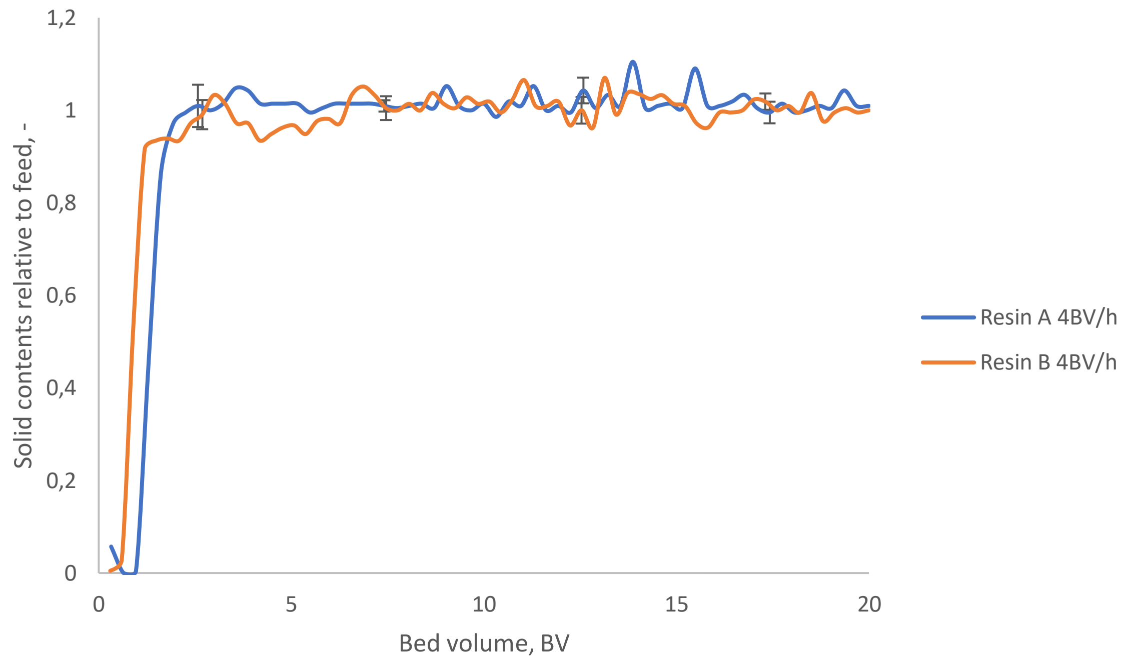

Figure 18 Solid contents relative to feed for bed volumes for 4 BV/h experiments for Resins A and B. Standard deviation selected for 5 bed volume intervals, excluding values before initial breaching point.

To model the passing through rate of the column mean average of effluent sample brix was calculated. This value was then compared to the diluted solids brix value of the feed to gain a percentage describing the mean mass balance of the system. The results are described in Table II for resins A and B. For all the setups, the passing through rate was close to 100 %, indicating that the exchange between particles is near equivalent. Noticeable differences are present in the Resin A 7 BV/h experiment, where the lower balance is likely due to coagulated matter getting stuck in the resin bed. Seemingly high Resin B 10 BV/h balance is due to the previously mentioned variance in feed quality. With all these percentages it should be noted that error in feed sample reflects greatly on the balance, thusly these results are mainly directional in verifying the experiments accuracy and correctness. This data was used to map out the samples for further analysis, and to support the results from other analyses.

Table II Mean mass balance between mean diluted solids content of effluent samples and feed for resins A and B.

Set	Balance (± dev), %
Resin A 1BV/h	102,86 ± 3,83
Resin A 4BV/h	101,51 ± 2,18
Resin A 7BV/h	94,97 ± 5,20
Resin A 10BV/h	98,23 ± 4,42
Resin B 4BV/h	96,46 ± 6,03
Resin B 7BV/h	99,91 ± 4,14
Resin B 10BV/h	144,21 ± 21,97

In addition, curves for development of conductivity and pH were created, shown in Figure 19. From the development of conductivity, a notable rise in the conductivity is present after the point of the first feedstock passing through the column, followed by a decline back near to the original value. This is likely due to the initial release of Cl-ions caused by the binding of the feed components. As more of the Cl-ions get replaced, the amount released declines, also causing the decline in conductivity. This hints to the binding capacity of the resin used, as well as its effectiveness; a steep decline to the original value after the initial rise may suggest significant decline in binding capacity.

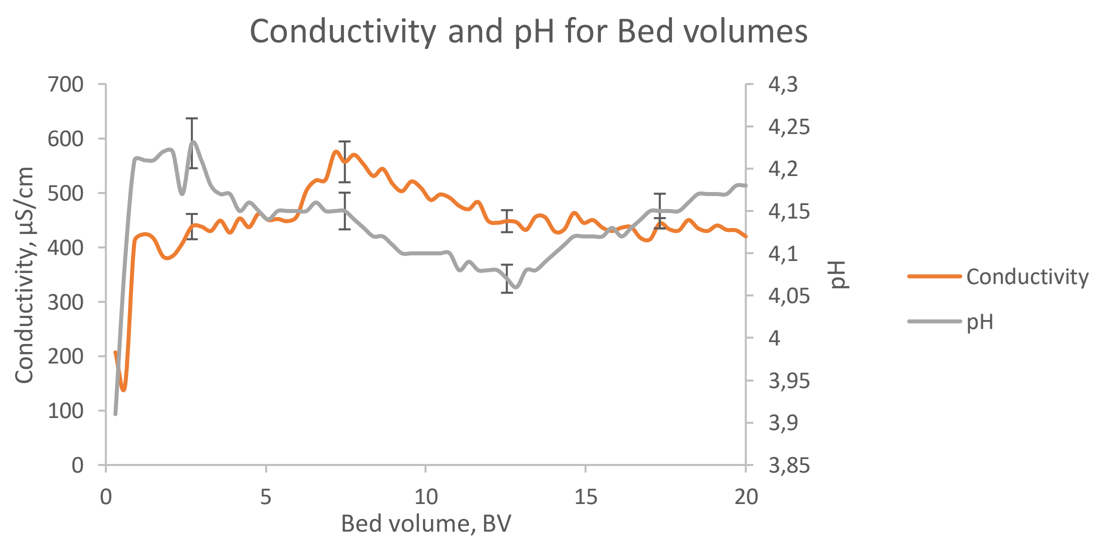

Figure 19 Conductivity (In orange) and pH (Gray) development based on bed volumes. Taken from experiment of 4 BV/h using Resin B. Standard deviation selected for 5 bed volume intervals, excluding values before initial breaching point.

As for pH, the values seem to take a minor decline during breakthrough, before settling into a value near the feed whey. This decline and seeming codependence of conductivity and pH can be explained with the releasing of bound chloride-ions which increase the conductivity of the solution while lowering the pH of the solution. The pH values revolve around the *pI* range of GMP at pH 3.15 and CMP at 4.15. This suggests high potential for binding, and thusly selectivity, as other major protein components isoelectric points are located higher in the pH scale, the nearest being at 4,7 for α-la.

From the HPLC analysis, the concentrations of CMP and GMP were acquired for a set of samples between intervals of 5 bed volumes, the feed solution and the point after the breakthrough. The results for reduction efficiency were calculated from these select points by dividing the result from the HPLC analysis with the corresponding result of the feed solution in a way described by equation 4. The reduction efficiencies were calculated for each effluent sample datapoint per volume fed.

$$x_i = 1 - \frac{c_i}{c_0} \tag{4}$$

Where

x	is reduction efficiency at point i, -
c_i	is CMP/GMP concentration of sample at point i, mg/ml
c_0	is CMP/GMP concentration of feed sample, mg/ml

These percentages were then constructed into a chart describing the development of CMP/GMP concentration per bed volume consumed. These charts are presented in Figure 20 describing the CMP and GMP reduction efficiencies for both resins over the time of bed volumes consumed. Detailed result tables are presented in addendum VI.

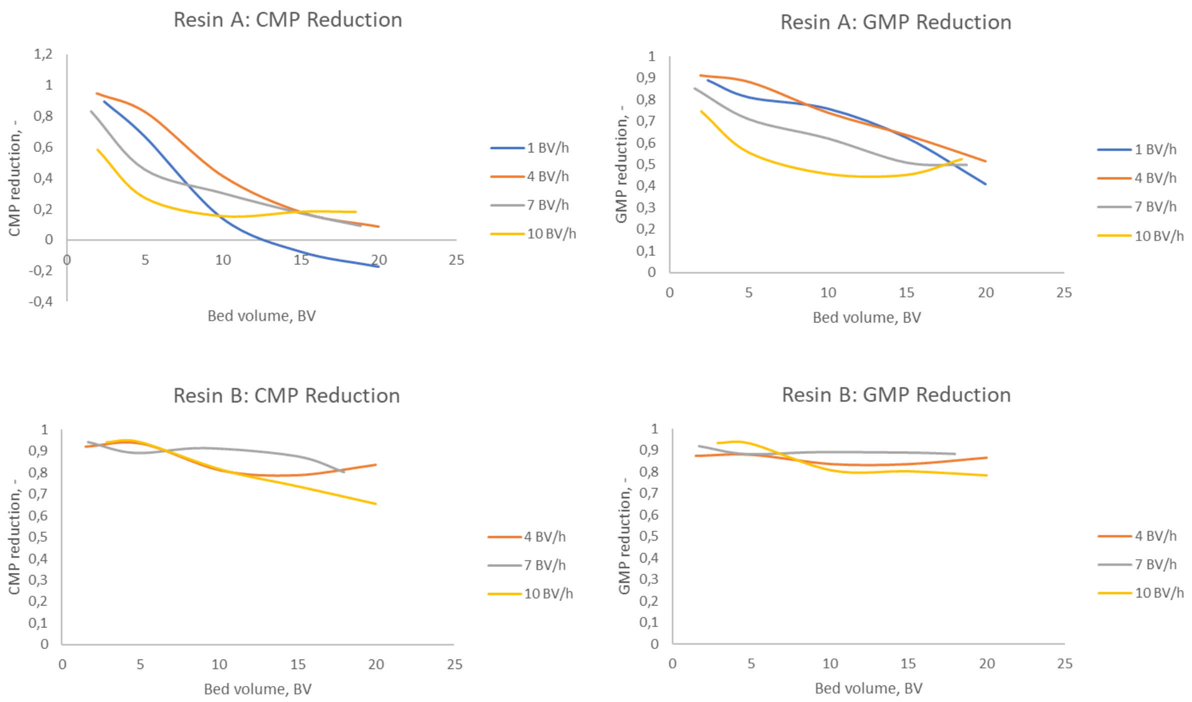

Figure 20 Development of CMP (left) and GMP (right) reduction for bed volumes for Resins A (Top) and B (Bottom) with differing flow rates: 1 BV/h (in blue), 4 BV/h (orange), 7 BV/h (gray) and 10 BV/h (yellow).

It can be observed that as the bed volumes are consumed, resin A starts to lose its initially high reduction efficiency, reducing it into half after 10 bed volumes even at its most promising flowrate. Resin B showed promising results throughout the whole 20 BV fed, with only reductions varying from 10-15% at it is more promising flowrates 4 and 7 BV/h. Even at the high flowrate of 10 BV/h, reduction efficiency remained higher than any of the comparative resin A's results. The results seem to mirror the volumetric capacities provided by the resin manufacturers, as resin B exceeds the capacity of resin A. The reduction of GMP was higher compared to the CMP, most likely due to the isoelectric point of CMP being rather close to the pH of the solution at 4.15, causing non-optimal conditions for anion exchange of CMP at points where pH drops below the *pI*. As with most experiments, it was observed that between 5-10 bed volumes, pH was commonly dropped below the point of 4.15, which correlates to a decline in reduction efficiencies at similar points. Though this could also be sign of the resin bed showing higher selectivity towards the binding of GMP rather than CMP, as the sooner decline in CMP concentration could be observed with nearly all experiments, independent on the effluent pH or conductivity.

As the flow velocity of 4 BV/h, resulted in the most optimal values in both resins, and had the least amount of clogging during tests, this flow rate was selected for the capacity tests. These results can be explained with the notion, that in higher flowrates the time required for the ion exchange phenomena to take place can be compromised due to lower exposure time to the resin bed. At lower speeds, the laminar flow is compromised in the resin bed due to the low velocity of certain particles, causing dispersion in particles flow patterns. In resin A 1 BV/h experiment the seeming increase compared to the feed amount is likely due to analytical error.

3.2.2 CMP/GMP: Capacity experiments

Determining the distance of which the Ion exchange system can be operated while retaining its binding capacity, was done as described in sections 3.1.3 and 3.1.4. The aim was to reach 30 bed volumes, however during the tests the furthest bed volume achieved was 22 using Resin B and 20 with Resin A. Both feed solutions were from the same batch, however with 2 days between experiments, which may have caused deterioration in the solution. The abrupt ending was due to the system clogging similar to which was experienced in previous flowrate tests. It was thusly concluded that using while using samples that are not freshly out of the process, the systems physical limits inhibit higher bed volumes to be experimented on in laboratory environment, without possible pretreatments.

From the gathered samples, brix, conductivity and pH were measured, as well as HPLC analysis was conducted to determine the GMP/CMP concentrations present. Seven samples were gathered from both experiments for further analysis. The mapping for these samples is presented below in Table III. Samples 1 and 2 BV were selected to represent the point after initial breaching of the system. Sampling was done between 5 BV intervals for the rest, however due to 5 BV sample in Resin A experiment having too little sample to analyze, point 6 BV was selected instead for both resins.

Table III Capacity experiment samples for analysis

BV: Resin A	0 BV (Feed)	2 BV	6 BV	10 BV	15 BV	20 BV	20,5 BV (Ending)
BV: Resin B	0 BV (Feed)	1 BV	6 BV	10 BV	15 BV	20 BV	22 BV (Ending)

Results for CMP and GMP reduction based on results from HPLC analysis are presented below in Figure 21. These results suggest the same as previously observed from the flowrate experiments, with both resins initially having high reduction capability, with Resin A having diminishing effectivity. The decline seems steeper in these experiments, however this may be due to the channeling caused by clogging, as air pockets were observed in the resin bed after these experiments with both resins A and B. These results can be observed in addendum VII, in a table format.

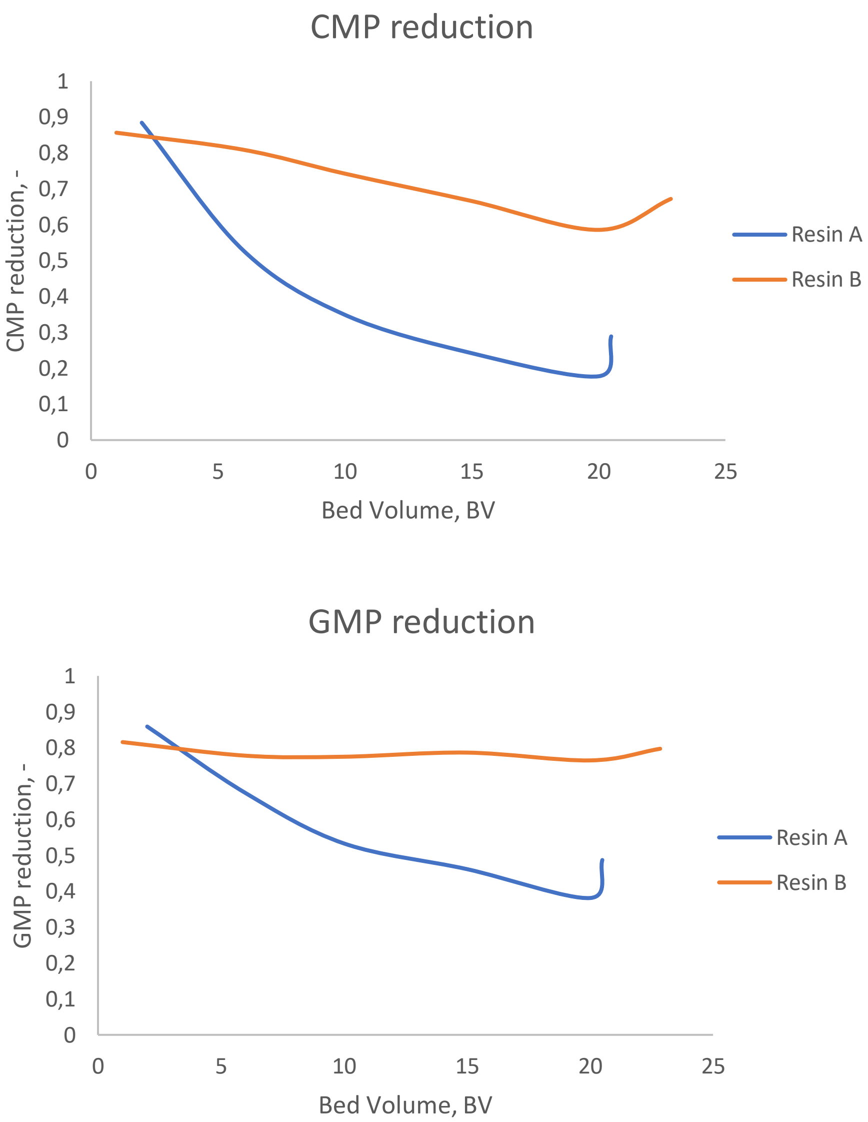

Figure 21 Development of CMP (top) and GMP (bottom) reductions for bed volumes for Resins A (Blue) and B (Orange). Last point is taken before concluding the experiment. Data from capacity experiment with 4 BV/h flowrate.

The results gathered from this capacity tests, mirrored the results from the previous test, however with lesser effectiveness for Resin B. This is likely due to the increased channeling caused by clogging of the column, which was more present during this test compared to the previous. The channeling limits the reactive surface area of the bed, as the flow runs through in them, leaving some resins outside these channels with lesser contact with the feed solution. Resin B still fared significantly better compared to resin A, which lost nearly half of its binding capacity before 10 bed volumes.

Plotting HPLC results for GMP/CMP reduction and conductivity of samples in the same curve in Figure 22 shows a seeming correlation between the two factors. This correlation could be explained with the conductivity caused by released chloride ions from the resin bed, which get replaced by the peptide particles. Based on these curves, it can be roughly hypothesized that as conductivity decreases at similar rate to the CMP/GMP in Resin A, the total binding capacity of the resin bed is achieved in the bed early, rather than the bed opting to bind other particles in the solution. If the resin would be selective towards other whey components, it would highly be likely to retain high conductivity after the decrease of CMP/GMP reduction rate. The curves seem to correlate especially well with the values of GMP, which might yet again hint a slightly higher selectivity towards GMP extraction.

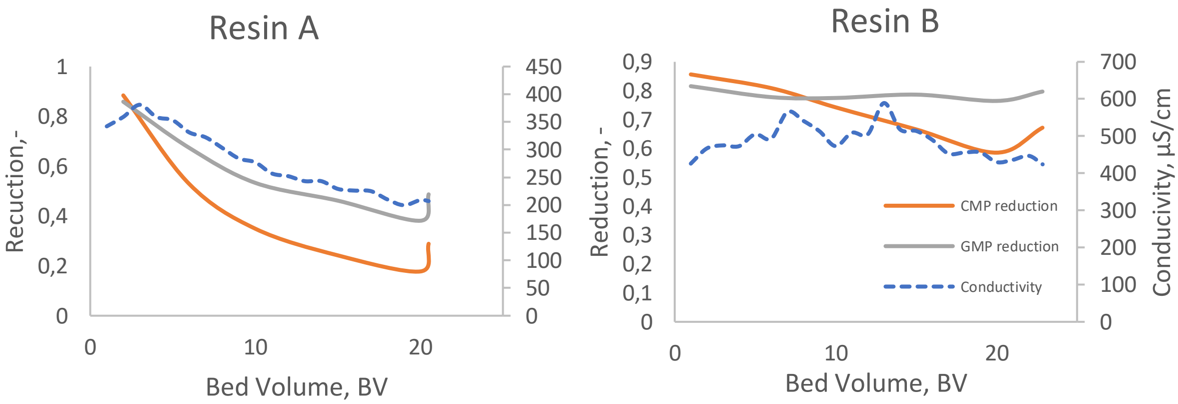

Figure 22 CMP and GMP reductions (undashed, CMP in orange, GMP in gray) and conductivity (dashed blue) for feed bed volume development.

3.2.3 GMP/CMP: Protein composition analyses results

Additional analyses were done to capacity experiment samples to gain a more in-depth detail of their composition. These analyses were done with methods described in section 3.1.9. and consisted of Kjeldahl protein analysis, lactose content mapping via enzymatic means, determination of fat content and determination of ash and total dry matter contents. Along these, the binding of native whey proteins into the resin were studied. From the samples of Resin B, it was found out that concentrations of both native whey proteins α-la and β-lg are left unaltered by the process. The concentrations of these main components are presented in Figure 23. No significant development was found for other observed parameters, signifying that the Resin B works with promising selectivity for CMP and GMP binding.

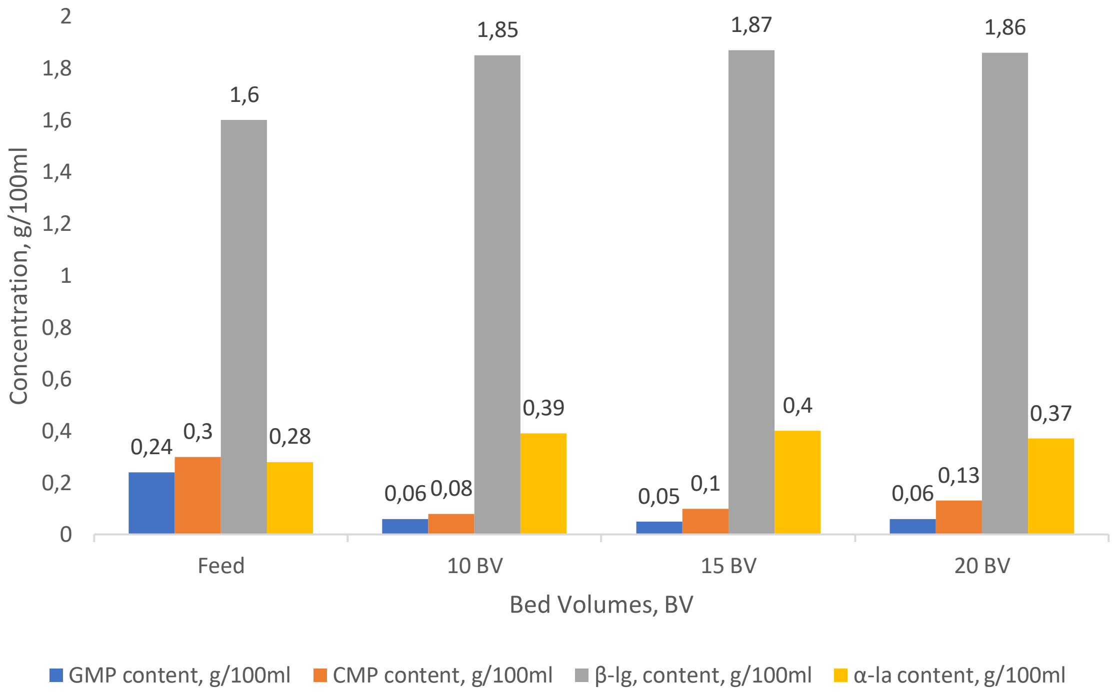

Figure 23 Protein composition of samples taken from feed, 10, 15 and 20 BV marks from Resin B capacity experiment. GMP (in blue), CMP (in orange) β-lg (in gray) and α-la (in yellow).

Results for resin A protein composition, shown in bar graphs in Figure 24, were rather similar to resin B's. However, a slight decrease in β-lg concentrations was observed as the fed bed volumes increased. The resulting reduction in β-lg is around 11.4% when comparing to the feed solution and 22.7% when compared to breakthrough point at 2 BV, which had the highest concentration of β-lg. However additional analysis of other components revealed a similar decrease in lactose content, which might indicate that the decrease is likely due to coagulation of lactose and proteins, rather than any significant binding into resin. The correlation between these values can be seen in Figure 25. For other analysis of other than proteins and lactose, it was again found out that there is no significant development in fat, ash and dry matter contents to be noticed as expected.

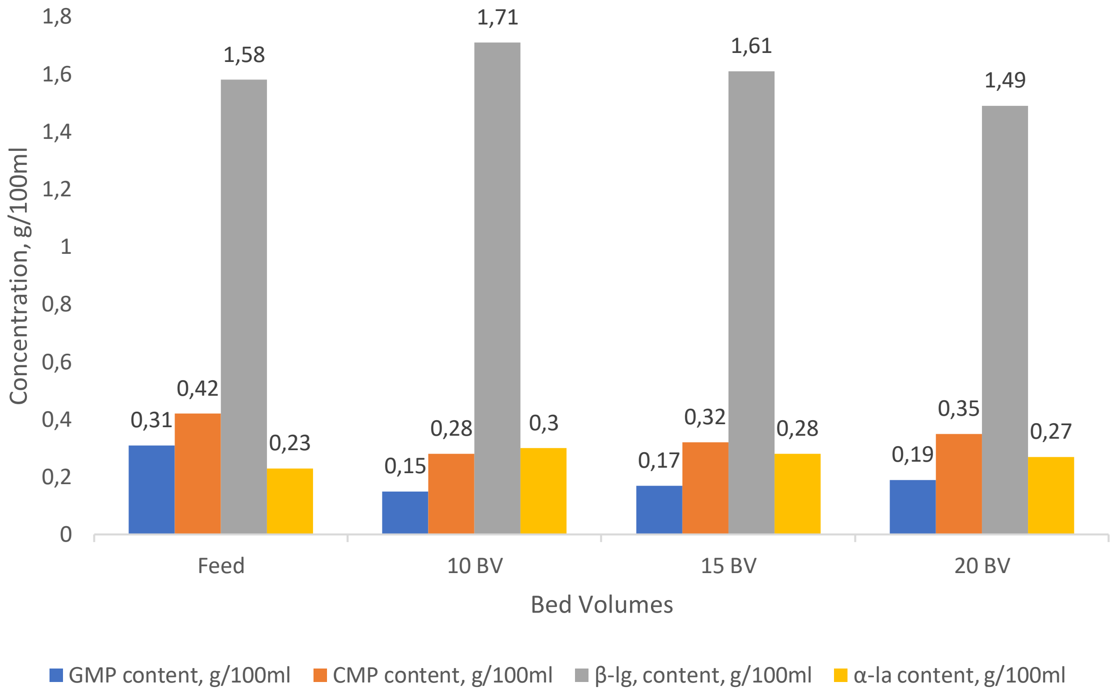

Figure 24 Protein composition of samples taken from feed, 10, 15 and 20 BV marks from Resin A capacity experiment. GMP (in blue), CMP (in orange) β-lg (in gray) and α-la (in yellow).

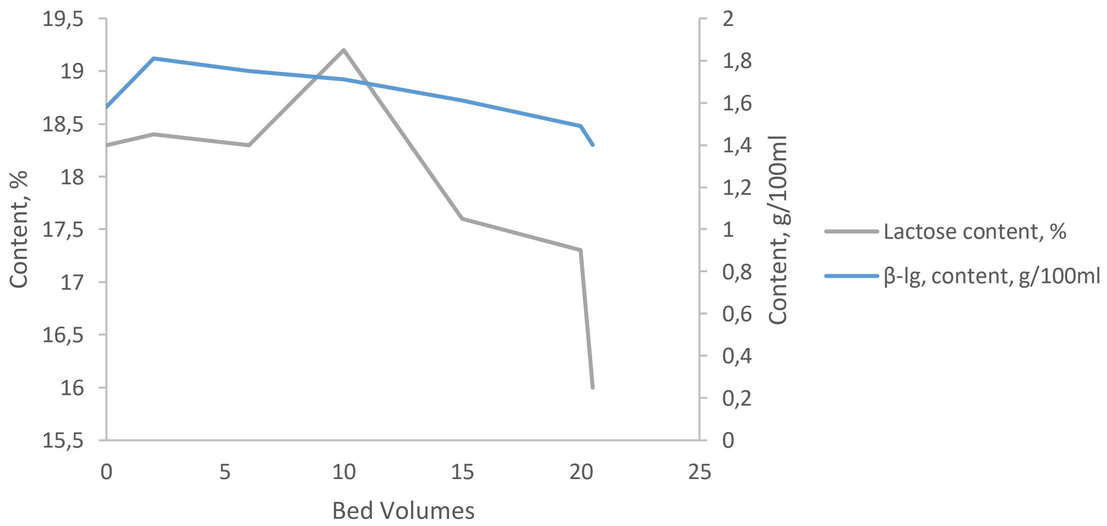

Figure 25 Content curves describing the correlation between β-lg content in g/100ml (Blue) and lactose content in % (grey).

From the Amino acid content analysis, the development between feed and 15 Bed volume samples were analyzed from resin B capacity experiment. Samples from the elution effluent near the peak value were also analyzed. Figure 26 describes the results in a bar graph. Figure 27 presents the results scaled with the respective protein contents of the samples. The noticeable decrease between feed and 15 BV results in threonine, glutamic acid, proline and serine, which are common amino acids found in caseinomacropeptide chains, seems to support the HPLC results in CMP/GMP reduction. Components lacking from caseinomacropeptides; tryptophan, tyrosine, phenylalanine and cysteine, show no major signs reduction during the 15 BV development. This would indicate the lack in reduction of native whey proteins, bovine serum albumin and immunoglobulins, in which these components are frequent. From the results from the eluent samples, similar conclusions can be made, as the previously mentioned CMP/GMP frequent amino acids are clearly visible, and while the missing ones are not present in the solution. These results, along with the protein content analysis, seem to suggest high selectivity of resin B for CMP/GMP removal. Amino acid content analysis was left out for resin A, due to its lackluster results in GMP/CMP analyses, leaving the nature of its selectivity relatively unknown. The seemingly exceeding high total amino acid content is due to analytical inaccuracy and the fact that the samples were taken from single datapoints.

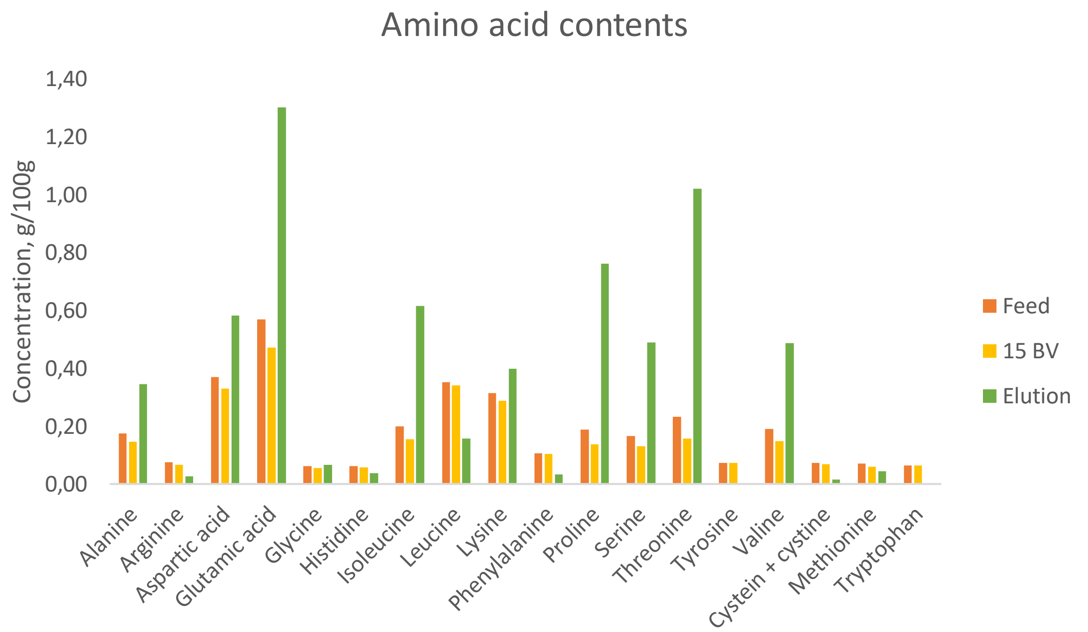

Figure 26 Amino acid composition of feed, at 15 bed volume fed, and elution effluent samples from Resin B capacity experiments.

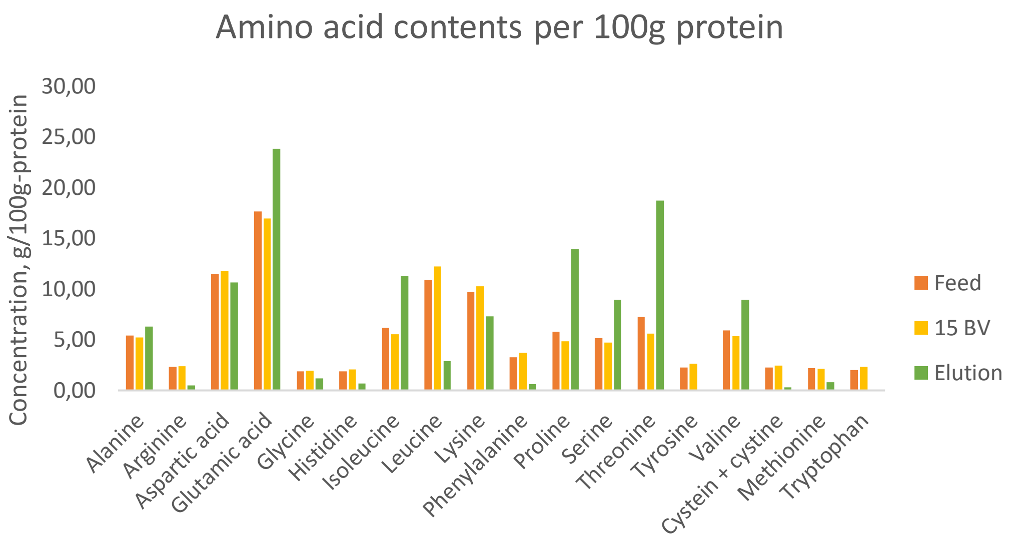

Figure 27 Amino acid composition per 100 grams of feed, at 15 bed volume fed, and elution effluent samples from Resin B capacity experiments.

According to these protein analyses it is most likely that at least Resin B is highly selective towards only CMP/GMP in this system, showing little to no signs of other organic whey components binding into the resin bed. To clarify the nature of the exchange the behavior of the bound ions is inspected in following chapter on chloride content analysis.

3.2.4 GMP/CMP: Chloride content analyses

Analysis for chloride content was carried out by Valio R&D research analyst team in Lapinlahti for 3 samples from both experiments. The results are presented below in Table IV. The results seem to support the previous results, as the amount of chloride present in the later bed volume Resin B samples compared to the ones from Resin A. This indicates that the Exchange of ions still happens throughout the experiment for Resin B, while the decline in chloride in Resin A samples indicates the decrease in ion exchange.

Table IV Chloride content of Resin A and B samples

	Resin A	Resin B
Feed	>12mg/100ml	>12 mg/100ml
6 BV	22 mg/100ml	32 mg/100ml
15 BV	12 mg/100ml	31 mg/100ml

These results also correlate with the measured conductivity values, where Resin A's conductivity started to decline around the same time due to the lack of free chloride ions in the solution. This correlation is shown in Figure 28 plotting both curves for chloride content and conductivity as feed volume develops. As Resin B held its binding capacity and thusly its ion exchange potential, the presence of chloride ions remained resulting in conductivity plateauing after initial rise, rather than declining.

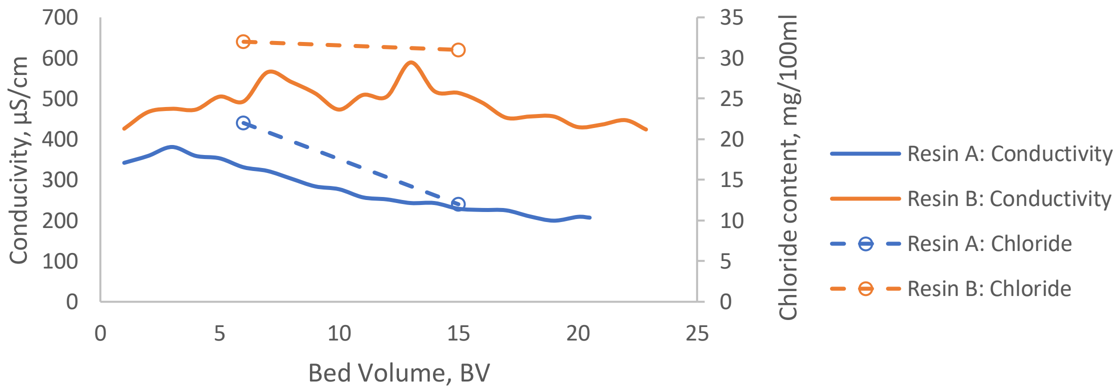

Figure 28 Chloride contents and conductivity for bed volume development. Resin A results in blue and Resin B in orange. Dotted line describes the chloride content in mg/100ml, and the fixed line conductivity.

Chloride contents correlate with the CMP/GMP reductions as predicted, confirming the hypothesis that the initial development of conductivity is caused by chloride ions freed from the resin bed by ion exchange. This is shown in Figure 29. This information that there is direct correlation between conductivity of the effluent and chloride ion contents and the binding capacity of the resin bed can become useful in rapid analysis predictions of binding capacity of the resin bed, as HPLC can be time extensive.

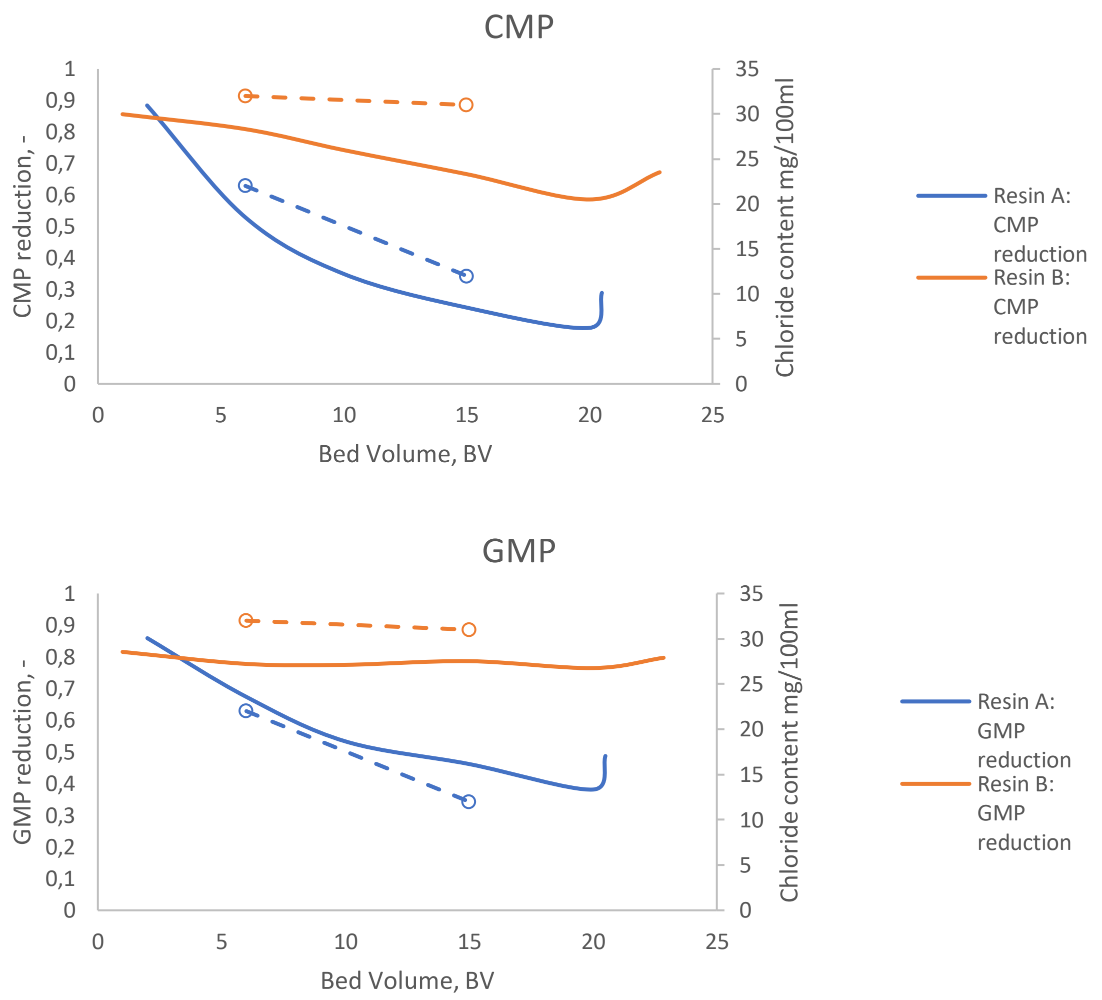

Figure 29 CMP (top) and GMP (bottom) reductions and chloride contents plotted for bed volume development.

3.2.5 GMP/CMP: Elution phase sample analysis

CMP/GMP was extracted in high concentrations from the resin beds, confirmed by HPLC analysis. Results for these CMP and GMP concentrations are presented in Table V. Initially the results acquired were in erroneously high scale, presumably due to humane mistakes in dilution, and results were presumed to be diluted in 1:10 rather than 1:100. This led the values to be in correct scale. However, not knowing the true cause of this and the lack of samples left, the results should be taken as directional and possibly inaccurate. After the total water presented in these results was expended, the conductivity was still high, indicating that the chlorine ions and excess NaCl was still emitting from the resin bed. The water feed was kept until the conductivity reached its starting value.

Table V GMP/CMP concentrations for effluent samples.

Resin	NaCl fed, BV	Water fed, BV	CMP, g/100ml	GMP, g/100ml
A	0,468	0	0,012	< 0,01
A	0,935	0	< 0,01	< 0,01
A	0,935	0,48	2,33	3,86
A	0,935	0,94	< 0,01	< 0,01
B	0,312	0	< 0,01	< 0,01
B	0,623	0	4,4	4,55
B	0,935	0	1,33	1,37
B	0,935	0,23	1,62	1,56
B	0,935	0,47	1,74	1,66
B	0,935	0,70	< 0,01	< 0,01

From these results the development of GMP and CMP was plotted and is presented in Figure 30, constructed from the results from resin B elution phase effluent samples. The axis of this figure is divided into two parts for 10% NaCl and de-ionized water feeds, respectively.

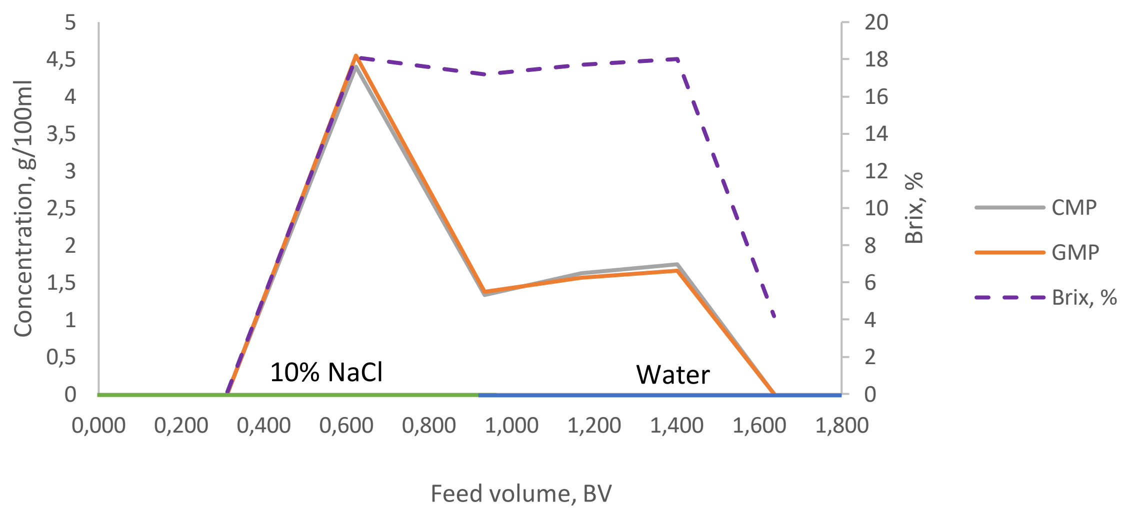

Figure 30 Development of CMP (Gray) and GMP (Orange) concentration and dissolved solids content Brix value (Dashed Purple) in effluent samples from elution phase. Taken from capacity experiments from resin B. In axis for feed volume, green indicates portion of feed using 10% NaCl solution, while blue feed with deionized water.

Scaling aside, it can be observed that the effluent of this phase has the highest concentration after the elution agent, NaCl in this case, has passed through the resin bed. The bed volume is saturated with the excess NaCl replacing the CMP/GMP particles in the resin surfaces. Protein analysis results from elution effluent showed no traces of β-lg nor α-la in neither resin. This results also clarifies that both resins are selective for GMP/CMP, with no affinity towards β-lg and α-la.

3.2.6 CMP/GMP: Mass balances

A simple mass balance was constructed for the systems based on the previously described results for resin B. This mass balance was constructed based on previous analytical results from 4 BV/h capacity experiments. The mass balance describes the quantity of major components present in a feed of 1000 kg of liquid whey feed into the system. The simple mass balances are described in Tables Table VI. GMP/CMP concentrate values are calculated based on calculated amounts left in the resin bed system after the sorption phase and supported by analytic results from effluent samples. Amount of NaCl in the concentrate is rough estimation based on the dry matter left after the other major components were calculated, and likely should be higher depending on the amount of eluent used and the extent of the elution.

Table VI Estimated mass balance for Resin B using 4 BV/h with the assumed feed of 1000kg of liquid feed solution.

Feed	% as is	kg
Dry matter, %	24,6	246
Total Protein Conter	3,23	32,3
GMP content	0,22	2,24
CMP content	0,28	2,80
β-lg, content	1,50	14,95
α-la content	0,26	2,62
Fat content	0,27	2,7
Lactose content	18,3	183
Other	2,80	28,00

Effluent 15 BV	% as is	kg
Dry matter, %	23,4	234
Total Protein Content	2,79	27,9
GMP content	0,05	0,47
CMP content	0,09	0,93
β-lg, content	1,50	14,95
α-la content	0,26	2,62
Fat content	0,27	2,70
Lactose content	17,90	179
Other	2,44	24,40

GMP/CMP concentrate	% as is	kg
Dry matter	17,75	12,00
Total Protein Content	6,51	4,40
GMP content	2,63	1,78
CMP content	2,76	1,87
β-lg, content	0	0,00
α-la content	0	0,00
Fat content	0	0,00
Lactose content	5,92	4,00
Other	5,325	3,6

Based on these results the purity of GMP and CMP contents could be calculated based on the respective total protein contents. These results are shown in Table VII.

Table VII GMP and CMP content purities in g/100g-protein based on mass balance results

	Feed	15BV Effluent	CMP/GMP concentrate
GMP content, g/100g-prot	7,43	1,79	40,36
CMP content, g/100g-prot	9,29	3,58	42,48

3.2.7 B12: Flowrate

Results between different flowrates resulted in breakthrough point at around 2 bed volumes of feed consumed. Example of solids development curve from these experiments is presented in Figure 31 taken from the results of 4 BV/h experiments with resin D.

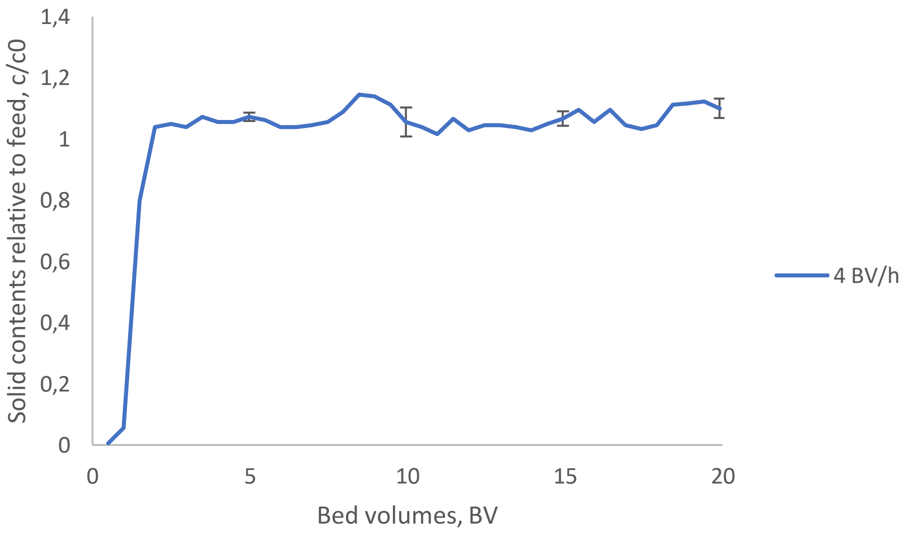

Figure 31 Solid contents per bed volumes fed for 4 BV/h experiments for Resins D. Standard deviation selected for 5 bed volume intervals.

The resin D showed little to no development in its pH and conductivity, excluding the eventual decline into the properties of the feed solution after the break point. It should be noted that in the experiment conducted with resin D using 7 BV/h conductivity and pH values rose initially as high as 1.6 mS/cm and pH 10.65. This was suspected to be due to the pristine quality of the resin, having only been treated with NaCl regeneration and washing with deionized water before the experiment, causing basic ions to emit from the resins. Figure 32 presents the correlation between pH and conductivity for 4 BV/h experiment, noting that the first two datapoints are influenced by the dead volume present in the systems pipelines. Curves for conductivity in all VB12 tests steadily declined towards the conductivity value of the original feed after the initial breakthrough spike. From what was observed from previous GMP/CMP experiments, this could show early signs of low amount or no free ions being released from the resin bed, as freed sodium ions are likely to cause noticeable rise in conductivity.

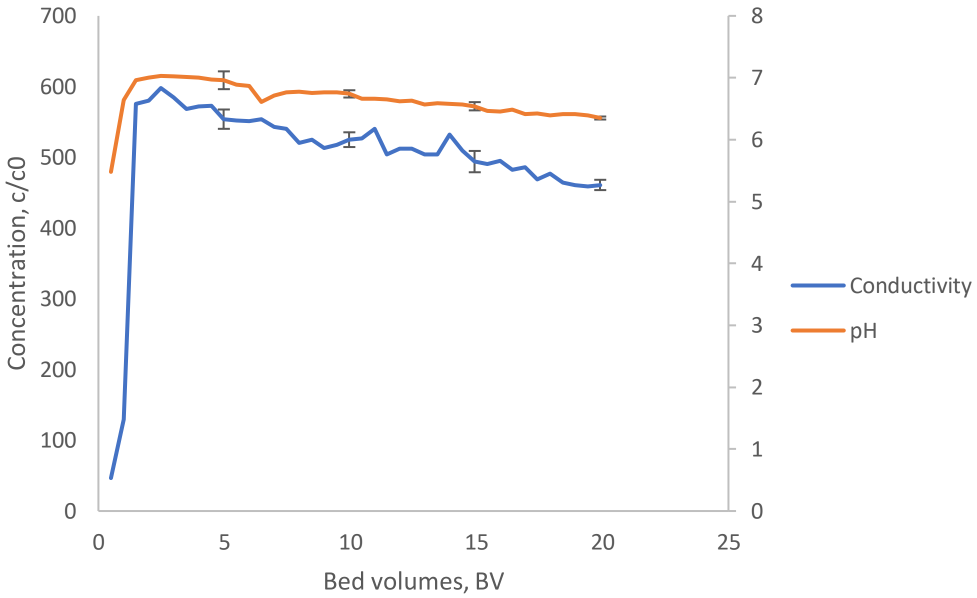

Figure 32 Conductivity (in blue) and pH (orange) development based on bed volumes. Taken from experiment of 4 BV/h using Resin D. Standard deviation selected for 5 bed volume intervals.

Mean mass balances were calculated for these experiments similar to the ones involving CMP/GMP. These balances with their respective standard deviations are shown in Table VIII. Variance from the total flow through rate of 100% is mostly present in resin D 1 BV/h, which is likely due to coagulation of matter in the column present in this experiment.

Table VIII Mean mass balance between mean diluted solids content of effluent samples and feed for resins A and B

Set	Balance (± dev)
Resin C: 4 BV/h	100,06 ± 1,85
Resin C: 4 BV/h pH adjst.	100,40 ± 1,50
Resin D: 1 BV/h	94.51 ± 4.31
Resin D: 4 BV/h	106.52 ± 3.06
Resin D: 7 BV/h	99,18 ± 2,64

Select samples were analyzed for their vitamin B12 concentrations using external analysis services provided by Eurofins Scientific. The most complete set of samples was gathered from test with Resin D using 4 BV/h flowrate, with the samples being taken with 5 bed volume intervals and one taken right after breakthrough point. Samples from Resin C and Resin D 1 BV/h experiments were taken from after breakthrough point and at the last feasible points. In addition, the breakthrough sample from pH adjusted Resin C experiment was also selected. From Resin D with flowrate of 7 BV/h experiment samples for 15 and 20 bed volumes were selected as earlier sample points in this set had abnormally high pH and conductivity. Feed samples from all experiments were analyzed. Results from the HPLC analysis for VB12 content are presented in Table IX.

Table IX Concentrations for vitamin B12 in µg/100g for feed and effluent samples from experiments with Resins C and D. *feed solution pH adjusted into 5.4.

Adsorbent	Flowrate	Sample	*c*(VB12)	Deviance ±	Sample	*c*(VB12)	Deviance ±
Resin C	4 BV/h	Feed	0,598	0,12	Feed*	0,712	0,142
		2 BV	0,773	0,155	2 BV*	0,664	0,133
		8 BV	0,766	0,153			
Resin D	4 BV/h	Feed	0,692	0,138			
		2,5 BV	0,62	0,124			
		5 BV	0,642	0,128			
		10 BV	0,664	0,133			
		15 BV	0,607	0,121			
		20 BV	0,621	0,124			
Resin D	1 BV/h	Feed	0,715	0,143			
		2,5 BV	0,695	0,139			
		7,75 BV	0,669	0,134			
Resin D	7 BV/h	Feed	0,675	0,135			
		15 BV	0,637	0,127			
		20 BV	0,656	0,131			

From this data, Figure 33 was constructed, describing the relation between the sample point VB12 concentrations and the corresponding feed sample values. It can be observed from the datapoints that no significant reduction in VB12 concentration happens during bed volume development with neither resin. No significant effect of the flowrate can be observed with this provided data. As the reduction of VB12 is at 12% at best, with the deviation on this point being nearly 20% of its value, the seeming reduction might also be due to analytical error rather than any significant sorption happening. Resin D was expected to function better compared to resin C, however according to these results, both results seem to suggest the same next to no extraction rate of resin C. The pH alteration done in resin C seems to have no effect on the extraction efficiency.

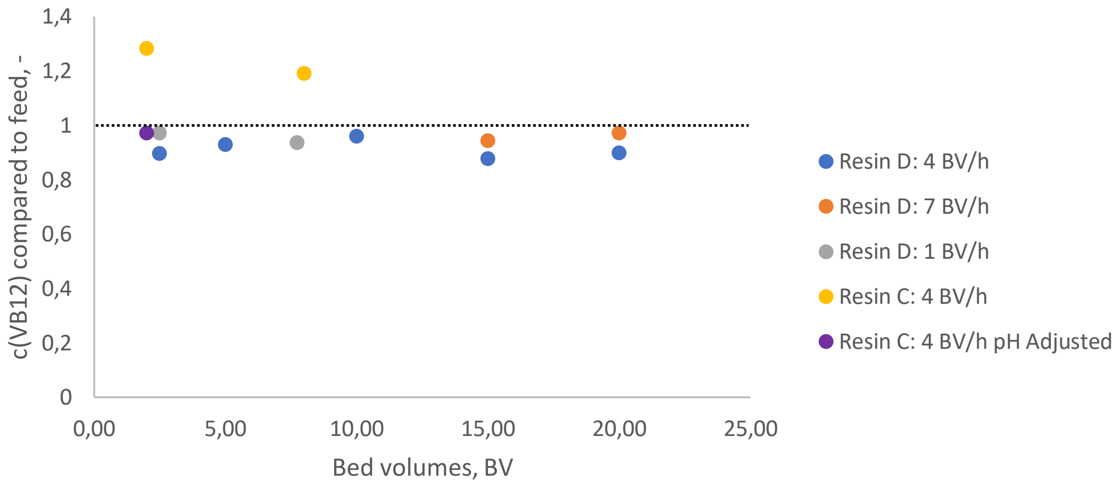

Figure 33 Effluent samples compared to feed VB12 concentrations plotted for bed volume development.

Additional analyses for protein, β-lg, α-la, ash and dry matter contents were conducted for effluent samples to inquire the possibility off other components binding into the resin bed. These analyses however showed no change in any observed concentration for neither resin nor experiment. As with previous samples ash content was under the detection limit, so any changes in mineral contents were left unobserved. Based on the results from conductivity, HPLC and additional analyses, it is highly likely that no significant ion-exchange happens during the experiments, as opposed to resin bed being selective towards other components than B12.

The results can partly be explained with the stable structure of hydroxocobalamin which is likely the variant most common of cobalamin in feed solution. As the central cobalt is already tightly bonded to nitrogen and oxygen, as well as the typical corrin ring, breaking these bonds is energy intensive and unfavorable towards formation of chelation by the ligands around the central cobalt atom. Altering the conditions such as pH or oxidation level of the cobalamin complex, into more unstable form, might pose a possibility for chelation, however this should be investigated further in other works as this thesis was limited to unaltered feedstock.

3.3 Conclusions

From these results it can be observed that the initial CMP and GMP reduction after the breakthrough is high with both of the tested IE-resins, however as more bed volumes are consumed, resin A tends to lose its effectivity. Resin B seems to keep its binding capacity and effectivity at high, nearly over 80% with all flow rates, except the highest, throughout the whole 20 BV fed during the first set of samples. This correlates well with the volumetric capacities provided by the Resin supplier, with Resin B having higher value of 0,8 eq/l compared to the 0,5e q/l of resin A. And based on the results from protein composition and CMP/GMP extraction studies, it can be concluded that it was likely that resin A's binding capacity was filled towards CMP/GMP.

With both resins, GMP reduction was slightly higher compared to the total CMP reduction. Both resins were deemed to be highly selective towards CMP and GMP reduction in these process parameters, however Resin A had significantly lower total binding capacity compared to Resin B. Both resins also showed slightly higher selectivity towards GMP than CMP, this being evident especially with the Resin A. No major binding of other whey components was observed from the effluent samples for neither resin. This is likely partly due to the optimal binding settings present at the pH of the demineralized whey, being close to the *pI* of CMP/GMP, resulting in higher affinity towards their binding instead of other whey proteins with higher *pI* values.

As suggested by protein content analyses, Resin B performed in highly selective manner throughout the tested 20 bed volumes. As the signs of declining binding capacity were around 10% for CMP and had nearly no effect on GMP binding, the maximum binding capacity was not yet achieved with current feed amount and the feed could continue past 20 bed volumes, if the physical limitations caused by the coagulated proteins can be averted.

A suggestive correlation between conductivity, chloride content and binding capacity was observed, which seems to confirm the extent of ion-exchange between whey components and chloride-ions. As protein composition analyses suggested high selectivity towards CMP and GMP for resin B, development of conductivity and chloride content can be used as a rough estimation in CMP/GMP binding behavior in the tested scenario.

On the light of all previously mentioned results, it can be concluded that separation of CMP/GMP can be done in high selectivity for at least 20 Bed volumes feed using a packed bed system filled with strong anion exchange resin B. Comparatively, resin A results suggested similarly high selectivity, however with significantly lower binding capacity. Thus, based on these results the use of resin B is suggested for the separation of CMP/GMP.

As from the results gathered from vitamin B12 extraction experiments, no significant reduction was observed using resins C or D. The highest analytical reduction of 12% was achieved with resin D using 4 BV/h, however the role in this played by the resin is debatable. The amount vitamin B12 extracted based on the effluent samples can attributed to various other factors, such as analytical error, variations in feed solution homogeneity, light contamination, and possible degradation of samples during transportation and analysis. Thusly, based on the results provided by this study, resins C and D show no affinity for, nor prospect in, extraction of vitamin B12 from liquid demineralized whey solution. Results also suggest that the total ion-exchange happening with these resins is low or non-existent, due to no major changes being noticed between protein contents nor conductivity of the effluent.

As the experiments tended to end before the total saturation and meeting of the total binding capacity of a resin bed in most of the experiments, determination of optimal feed capacities and in depth analysis on mass transfer on the systems was left to only a fraction.

Ion-exchange methods show prospect in novel extraction of specific whey fractions. In the experiments done in this thesis it was found that extraction of CMP/GMP can be done in with high selectivity and binding capacity using anion exchange resins. Extraction on vitamin B12 was however left unsuccessful while using chelating cation ion-exchange resins. A simple summary of the results can be seen in Table X.

Table X Simplified compilation of results from all tests

	CMP/GMP		VB12	
	Resin A	Resin B	Resin C	Resin D
Selectitivity	High	High	None	None
Binding capacity	Low	High	None	None

4. PROPOSALS FOR FURTHER RESEARCH

Since the purpose of this thesis was limited to determine the effectivity of ion-exchange for whey fraction extraction in laboratory scales, there is room for additional research on the subject. In this chapter, few of these prospective research topics and ways to advance are introduced.

As the system used in this was meant to simulate industrial scale processes, a comparative pilot scale study should be conducted in order to determine the up scaling of the process. A pilot study with the feed integrated into the preceding demineralization process could also be done to clarify the behavior the coagulated proteins in the system. In this study it was briefly concluded that the precipitate was unlikely to cause problems in fresh and mixed feed stream, however before any major process investments a deeper study into this should be done in order to avoid fouling in industrial scale columns, which could prove to be highly expensive. Due to the previously mentioned precipitate many of the experiments in this study were left shorter than intended, for example capacity experiments reaching only 20 bed volumes, using the industrial stream could be done to determine the total binding capacity of the suggested resin B. Based on the results from this thesis, this resin could be used for longer than the twenty bed volumes.

Optimal flowrate for the system was only roughly estimated as only maximum of 4 flow rates were tested. Since the aim of this thesis was to study the plausibility and effectivity of extraction using various resins, the optimization phase was left to minimum detail. One of the next natural steps in refining this process could revolve around various process optimization aspects, such as, but not limited to, the previously mentioned flow rate determination. Changes in temperature, column size, pump type and various other process parameters or equipment should be considered in further research topics in the field of process development around this system.

Research on extracted CMP and GMP concentrate could also be concluded, as they also possess a niche market in healthcare and nutrition industries as mentioned in literary review part of this thesis. Further research could be done in demineralization and cleaning of this concentrate. In-depth analysis for the elution effluent concentrations should also be concluded as to map out the total contents of the solution.

As the resins C and D showed no sufficient affinity towards VB12 extraction, more studies using different resins or methods could be applied in this field of whey component extraction. If one were to find out method for extraction, a coexisting method for VB12 purification could also be investigated as this vitamin itself holds a significant value in pharmaceutical and nutritional markets. Since pH adjusted samples from resin C, showed slightly better results for first bed volumes additional analyses on the effect caused by increase in pH could also be studied.

More in depth analysis on the mass transfer phenomena and kinetics taking place in these systems might shed more light in the reasons behind the results provided by this thesis. Since the results varied between the flowrate determination and capacity experiments and no experiment with resin B reaching total capacity and no proper breakthrough curves could be drawn, calculation and modeling of the kinetics behind the reactions was left rather untouched.

The study in this thesis was limited only to process parameters similar to pre-existing process streams. Research on the effect of pH and conductivity altering of the feed solution could also be researched, as cobalamin comes in different forms depending on the conditions and the origin of the feed solution. In the end, more study on the subject of vitamin B12 sorptive extraction should be conducted before any functional or theoretical application to industrial scale processes.

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APPENDIX I: Flowrate experiment analysis results for Resin A

Table I. Resin A: 1 BV/h Flowrate analysis results

	t, min	BV	Brix, %	c/c0	Conductivity (µS/cm)	pH
Sample#						
1	15	0,22	0,00	0,00	134,10	3,56
2	30	0,43	0,00	0,00	119,90	3,60
3	45	0,65	0,00	0,00	106,70	3,70
4	60	0,87	0,60	0,03	133,90	3,96
5	75	1,09	10,60	0,52	389,00	4,22
6	90	1,30	16,40	0,80	501,00	4,39
7	105	1,52	18,70	0,91	544,00	4,45
8	120	1,74	18,50	0,90	580,00	4,45
9	135	1,96	20,60	1,00	573,00	4,45
10	150	2,17	19,50	0,95	585,00	4,37
11	165	2,39	21,30	1,04	602,00	4,28
12	180	2,61	21,20	1,03	622,00	4,20
13	195	2,83	20,30	0,99	628,00	4,13
14	210	3,04	21,30	1,04	634,00	4,04
15	225	3,26	21,50	1,05	658,00	3,94
16	240	3,48	21,30	1,04	672,00	3,90
17	255	3,70	20,30	0,99	672,00	3,85
18	270	3,91	20,80	1,01	677,00	3,84
19	285	4,13	21,20	1,03	663,00	3,81
20	300	4,35	21,80	1,06	654,00	3,81
21	315	4,57	21,60	1,05	649,00	3,81
22	330	4,78	21,10	1,03	627,00	3,82
23	345	5,00	21,80	1,06	603,00	3,83
24	360	5,22	21,00	1,02	610,00	3,83
25	375	5,43	21,70	1,06	598,00	3,84
26	390	5,65	20,40	1,00	608,00	3,85
27	405	5,87	21,40	1,04	606,00	3,88
28	420	6,09	21,50	1,05	593,00	3,89
29	435	6,30	20,60	1,00	582,00	3,91
30	450	6,52	20,70	1,01	576,00	3,92
31	465	6,74	21,10	1,03	571,00	3,90
32	480	6,96	21,50	1,05	567,00	3,94
33	495	7,17	21,60	1,05	559,00	3,95
34	510	7,39	21,20	1,03	551,50	3,99
35	525	7,61	21,20	1,03	544,00	4,02
36	540	7,83	21,20	1,03	536,00	3,99
37	555	8,04	20,00	0,98	536,00	3,99

38	570	8,26	21,90	1,07	520,00	4,00
39	585	8,48	23,40	1,14	525,00	4,01
40	600	8,70	21,60	1,05	526,00	4,02
41	615	8,91	23,10	1,13	516,00	4,02
42	630	9,13	22,50	1,10	524,00	4,05
43	645	9,35	24,50	1,20	530,00	4,06
44	660	9,57	21,80	1,06	524,00	4,07
45	675	9,78	21,60	1,05	497,00	4,07
46	690	10,00	21,40	1,04	488,00	4,09
47	705	10,22	21,40	1,04	488,00	4,09
48	720	10,43	20,70	1,01	487,00	4,11
49	735	10,65	20,30	0,99	489,00	4,11
50	750	10,87	21,40	1,04	474,00	4,12
51	765	11,09	21,50	1,05	461,00	4,13
52	780	11,30	20,80	1,01	461,00	4,14
53	795	11,52	21,30	1,04	451,00	4,15
54	810	11,74	20,00	0,98	438,00	4,15
55	825	11,96	20,20	0,99	439,00	4,16
56	840	12,17	20,40	1,00	435,00	4,15
57	855	12,39	20,70	1,01	429,00	4,18
58	870	12,61	20,50	1,00	424,00	4,20
59	885	12,83	20,50	1,00	434,00	4,21
60	900	13,04	20,60	1,00	419,00	4,21
61	915	13,26	20,90	1,02	410,00	4,22
62	930	13,48	20,80	1,01	400,00	4,24
63	945	13,70	19,50	0,95	396,00	4,26
64	960	13,91	20,20	0,99	402,00	4,25
65	975	14,13	21,40	1,04	396,00	4,27
66	990	14,35	19,70	0,96	402,00	4,26
67	1005	14,57	21,00	1,02	401,00	4,27
68	1020	14,78	21,40	1,04	386,00	4,28
69	1035	15,00	22,20	1,08	385,00	4,27
70	1050	15,22	20,50	1,00	383,00	4,28
71	1065	15,43	20,20	0,99	383,00	4,29
72	1080	15,65	20,90	1,02	379,00	4,29
73	1095	15,87	21,40	1,04	370,00	4,30
74	1110	16,09	20,60	1,00	372,00	4,29
75	1125	16,30	20,20	0,99	359,00	4,31
76	1140	16,52	21,00	1,02	358,00	4,32
77	1155	16,74	20,80	1,01	359,00	4,32
78	1170	16,96	21,10	1,03	353,00	4,33
79	1185	17,17	20,20	0,99	352,00	4,34
80	1200	17,39	21,00	1,02	348,00	4,34
81	1215	17,61	20,80	1,01	350,00	4,33

82	1230	17,83	20,50	1,00	330,00	4,36
83	1245	18,04	20,50	1,00	338,00	4,35
84	1260	18,26	21,00	1,02	338,00	4,35
85	1275	18,48	21,40	1,04	326,00	4,36
86	1290	18,70	21,30	1,04	324,00	4,36
87	1305	18,91	20,50	1,00	333,00	4,36
88	1320	19,13	21,00	1,02	327,00	4,36
89	1335	19,35	20,60	1,00	327,00	4,38
90	1350	19,57	22,40	1,09	319,00	4,39
91	1365	19,78	22,40	1,09	317,00	4,39
92	1380	20,00	20,30	0,99	310,00	4,39

Table II. Resin A: 4 BV/h Flowrate analysis results

Sample#:	t, min	BV	Brix, %	c/c0	Conductivity (µS/cm)	pH
1	5	0,32	1,20	0,06	N/A	N/A
2	10	0,65	0,00	0,00	N/A	N/A
3	15	0,97	0,10	0,00	700,00	4,93
4	20	1,29	9,40	0,45	248,00	4,71
5	25	1,61	18,10	0,86	317,00	4,61
6	30	1,94	20,40	0,97	345,00	4,46
7	35	2,26	20,90	1,00	364,00	4,42
8	40	2,58	21,20	1,01	378,00	4,36
9	45	2,90	21,00	1,00	391,00	4,32
10	50	3,23	21,30	1,01	400,00	4,24
11	55	3,55	22,00	1,05	403,00	4,21
12	60	3,87	21,90	1,04	397,00	4,18
13	65	4,19	21,30	1,01	409,00	4,22
14	70	4,52	21,30	1,01	423,00	4,21
15	75	4,84	21,30	1,01	426,00	4,23
16	80	5,16	21,30	1,01	425,00	4,22
17	85	5,48	20,90	1,00	415,00	4,21
18	90	5,81	21,10	1,00	407,00	4,23
19	95	6,13	21,30	1,01	402,00	4,24
20	100	6,45	21,30	1,01	395,00	4,24
21	105	6,77	21,30	1,01	389,00	4,25
22	110	7,10	21,30	1,01	391,00	4,27
23	115	7,42	21,20	1,01	378,00	4,30
24	120	7,74	21,10	1,00	370,00	4,31
25	125	8,06	21,20	1,01	370,00	4,32
26	130	8,39	21,30	1,01	370,00	4,34
27	135	8,71	21,10	1,00	368,00	4,35
28	140	9,03	22,10	1,05	349,00	4,37
29	145	9,35	21,20	1,01	341,00	4,39
30	150	9,68	21,00	1,00	341,00	4,39
31	155	10,00	21,30	1,01	333,00	4,42
32	160	10,32	20,70	0,99	325,00	4,43
33	165	10,65	21,40	1,02	321,00	4,45
34	170	10,97	21,20	1,01	314,00	4,45
35	175	11,29	22,10	1,05	308,00	4,47
36	180	11,61	21,00	1,00	304,00	4,48
37	185	11,94	21,20	1,01	307,00	4,47
38	190	12,26	20,90	1,00	296,00	4,51
39	195	12,58	21,90	1,04	299,00	4,51
40	200	12,90	21,10	1,00	290,00	4,52
41	205	13,23	21,70	1,03	284,00	4,53
42	210	13,55	21,20	1,01	283,00	4,53

43	215	13,87	23,20	1,10	267,00	4,57
44	220	14,19	21,10	1,00	270,00	4,57
45	225	14,52	21,20	1,01	264,00	4,57
46	230	14,84	21,30	1,01	260,00	4,60
47	235	15,16	21,10	1,00	259,00	4,61
48	240	15,48	22,90	1,09	253,00	4,63
49	245	15,81	21,20	1,01	255,00	4,64
50	250	16,13	21,20	1,01	254,00	4,64
51	255	16,45	21,40	1,02	244,00	4,67
52	260	16,77	21,70	1,03	245,00	4,65
53	265	17,10	21,10	1,00	239,00	4,65
54	270	17,42	20,90	1,00	235,00	4,66
55	275	17,74	21,30	1,01	234,00	4,66
56	280	18,06	20,90	1,00	229,00	4,66
57	285	18,39	21,00	1,00	224,00	4,69
58	290	18,71	21,20	1,01	224,00	4,71
59	295	19,03	21,10	1,00	223,00	4,70
60	300	19,35	21,90	1,04	223,00	4,71
61	305	19,68	21,20	1,01	220,00	4,72
62	310	20,00	21,20	1,01	216,00	4,72

Table III. Resin A: 7 BV/h Flowrate analysis results

Sample#:	t, min	BV	Brix, %	c/c0	Conductivity (µS/cm)	pH
1	4,28	0,39	0,00	0,00	88,40	4,08
2	8,56	0,78	0,30	0,01	99,00	4,10
3	12,84	1,18	12,30	0,51	403,00	4,22
4	17,12	1,57	22,20	0,91	528,00	4,20
5	21,4	1,96	24,50	1,01	546,00	4,08
6	25,68	2,35	24,80	1,02	560,00	4,04
7	29,96	2,75	24,00	0,99	583,00	4,01
8	34,24	3,14	24,70	1,02	563,00	3,99
9	38,52	3,53	24,90	1,02	546,00	3,99
10	42,8	3,92	24,90	1,02	534,00	3,97
11	47,08	4,32	24,80	1,02	512,00	4,00
12	51,36	4,71	25,80	1,06	502,00	4,00
13	55,64	5,10	24,10	0,99	494,00	4,00
14	59,92	5,49	23,90	0,98	487,00	4,00
15	64,2	5,89	23,20	0,95	498,00	4,03
16	68,48	6,28	23,20	0,95	489,00	4,03
17+18	72,76	6,87	21,10	0,87	509,00	4,06
19	77,04	7,06	23,60	0,97	455,00	4,12
20	81,32	7,46	24,20	1,00	445,00	4,13
21	85,6	7,85	23,10	0,95	460,00	4,13
22	89,88	8,24	23,00	0,95	455,00	4,16
23	94,16	8,63	22,40	0,92	455,00	4,13
24	98,44	9,03	22,90	0,94	445,00	4,15
25	102,72	9,42	24,40	1,00	391,00	4,16
26	107	9,81	22,80	0,94	418,00	4,18
27	111,28	10,20	22,90	0,94	420,00	4,18
28	115,56	10,60	22,00	0,91	406,00	4,22
29	119,84	10,99	22,90	0,94	397,00	4,21
30	124,12	11,38	22,60	0,93	411,00	4,21
31	128,4	11,77	22,50	0,93	395,00	4,21
32	132,68	12,17	23,20	0,95	381,00	4,20
33	136,96	12,56	23,00	0,95	383,00	4,23
34	141,24	12,95	23,00	0,95	382,00	4,24
35	145,52	13,34	22,30	0,92	382,00	4,20
36	149,8	13,74	21,80	0,90	389,00	4,23
37	154,08	14,13	22,10	0,91	378,00	4,24
38	158,36	14,52	22,30	0,92	373,00	4,25
39	162,64	14,91	22,00	0,91	373,00	4,24
40	166,92	15,31	21,50	0,88	364,00	4,25
41	171,2	15,70	22,70	0,93	351,00	4,25
42	175,48	16,09	22,00	0,91	355,00	4,27
43	179,76	16,48	24,40	1,00	319,00	4,25

44	184,04	16,88	21,90	0,90	339,00	4,26
45	188,32	17,27	24,80	1,02	315,00	4,26
46	192,6	17,66	22,30	0,92	345,00	4,26
47	196,88	18,05	21,30	0,88	367,00	4,25
48	201,16	18,45	20,80	0,86	370,00	4,28
49	205,44	18,84	21,70	0,89	362,00	4,26
Feed			24,3		163,1	4,57

Table IV. Resin A: 10 BV/h Flowrate analysis results

Sample#	t, min	BV	Brix, %	c/c0	Conductivity (µS/cm)	pH
1	3	0,39	0,00	0,00	14,11	6,15
2	6	0,79	0,30	0,01	396,00	4,78
3	9	1,18	7,60	0,34	448,00	4,28
4	12	1,58	21,70	0,97	546,00	4,09
5	15	1,97	22,90	1,03	536,00	4,00
6	18	2,37	20,80	0,93	555,00	3,97
7	21	2,76	19,40	0,87	564,00	3,97
8	24	3,16	23,60	1,06	533,00	3,94
9	27	3,55	23,90	1,07	512,00	3,96
10	30	3,95	22,80	1,02	492,00	3,95
11	33	4,34	22,40	1,00	510,00	3,99
12	36	4,74	24,00	1,08	450,00	3,96
13	39	5,13	22,20	1,00	476,00	4,00
14	42	5,53	22,30	1,00	474,00	4,04
15	45	5,92	22,40	1,00	453,00	4,07
16	48	6,31	22,60	1,01	438,00	4,08
17	51	6,71	22,20	1,00	440,00	4,07
18	54	7,10	21,40	0,96	429,00	4,09
19	57	7,50	22,30	1,00	411,00	4,10
20	60	7,89	22,80	1,02	405,00	4,10
21	63	8,29	22,40	1,00	416,00	4,11
22	66	8,68	21,90	0,98	400,00	4,12
23	69	9,08	21,30	0,96	384,00	4,14
24	72	9,47	21,60	0,97	398,00	4,16
25	75	9,87	21,10	0,95	388,00	4,15
26	78	10,26	21,30	0,96	403,00	4,16
27	81	10,66	22,60	1,01	374,00	4,17
28	84	11,05	21,90	0,98	372,00	4,16
29	87	11,45	21,40	0,96	387,00	4,17
30	90	11,84	23,50	1,05	354,00	4,18
31	93	12,23	21,30	0,96	352,00	4,20
32	96	12,63	21,80	0,98	367,00	4,19
33	99	13,02	22,70	1,02	348,00	4,19
34	102	13,42	21,40	0,96	340,00	4,23
35	105	13,81	21,80	0,98	340,00	4,25
36	108	14,21	20,30	0,91	368,00	4,24
37	111	14,60	21,00	0,94	363,00	4,24
38	114	15,00	20,50	0,92	358,00	4,24
39	117	15,39	21,50	0,96	358,00	4,24
40	120	15,79	21,00	0,94	379,00	4,24
41	123	16,18	20,80	0,93	368,00	4,23
42	126	16,58	22,70	1,02	343,00	4,23

43	129	16,97	21,80	0,98	340,00	4,24
44	132	17,37	23,00	1,03	330,00	4,24
45	135	17,76	21,00	0,94	356,00	4,24
46	138	18,15	21,40	0,96	348,00	4,24
47	141	18,55	21,20	0,95	358,00	4,26
Feed			22,30		176,00	4,55

APPENDIX II: Flowrate experiment analysis results for Resin B

Table I. Resin B: 4 BV/h Flowrate analysis results

Sample#:	t, min	BV	Brix, %	c/c0	Conductivity (µS/cm)	pH
1	5	0,29	0,1	0,005	207	3,91
2	10	0,59	0,6	0,028	145	4,09
3	15	0,89	11,3	0,528	411	4,21
4	20	1,19	19,6	0,916	424	4,21
5	25	1,49	20	0,935	416	4,21
6	30	1,79	20,1	0,939	383	4,22
7	35	2,09	20	0,935	384	4,22
8	40	2,39	20,8	0,972	407	4,17
9	45	2,69	21,2	0,991	438	4,23
10	50	2,99	22,1	1,033	438	4,21
11	55	3,28	21,7	1,014	430	4,18
12	60	3,58	20,8	0,972	449	4,17
13	65	3,90	20,8	0,972	427	4,17
14	70	4,18	20	0,935	453	4,15
15	75	4,48	20,3	0,949	437	4,16
16	80	4,78	20,6	0,963	462	4,15
17	85	5,07	20,7	0,967	450	4,14
18	90	5,37	20,3	0,949	452	4,15
19	95	5,67	20,9	0,977	448	4,15
20	100	5,97	21	0,981	457	4,15
21	105	6,26	20,8	0,972	504	4,15
22	110	6,56	22,1	1,033	523	4,16
23	115	6,86	22,5	1,051	524	4,15
24	120	7,16	22,1	1,033	575	4,15
25	125	7,46	21,5	1,005	557	4,15
26	130	7,76	21,4	1,000	570	4,14
27	135	8,05	21,7	1,014	552	4,13
28	140	8,35	21,4	1,000	531	4,12
29	145	8,65	22,2	1,037	544	4,12
30	150	8,95	21,7	1,014	517	4,11
31	155	9,25	21,5	1,005	503	4,1
32	160	9,55	22	1,028	521	4,1
33	165	9,85	21,7	1,014	509	4,1
34	170	10,14	21,8	1,019	487	4,1
35	175	10,44	21,3	0,995	497	4,1
36	180	10,74	21,9	1,023	491	4,1
37	185	11,04	22,8	1,065	476	4,08

38	190	11,34	21,6	1,009	470	4,09
39	195	11,64	21,6	1,009	483	4,08
40	200	11,94	21,8	1,019	449	4,08
41	205	12,23	20,7	0,967	445	4,08
42	210	12,53	21,4	1,000	448	4,07
43	215	12,83	20,6	0,963	446	4,06
44	220	13,13	22,9	1,070	432	4,08
45	225	13,43	21,2	0,991	456	4,08
46	230	13,73	22,2	1,037	455	4,09
47	235	14,02	22,15	1,035	429	4,1
48	240	14,32	21,925	1,025	433	4,11
49	245	14,62	22,1	1,033	463	4,12
50	250	14,92	21,7	1,014	445	4,12
51	255	15,22	21,6	1,009	450	4,12
52	260	15,52	20,8	0,972	436	4,12
53	265	15,82	20,6	0,963	430	4,13
54	270	16,11	21,3	0,995	436	4,12
55	275	16,41	21,3	0,995	437	4,13
56	280	16,71	21,4	1,000	417	4,14
57	285	17,01	21,9	1,023	415	4,15
58	290	17,31	21,8	1,019	444	4,15
59	295	17,61	21,4	1,000	433	4,15
60	300	17,91	21,6	1,009	431	4,15
61	305	18,21	21,3	0,995	450	4,16
62	310	18,51	22,2	1,037	435	4,17
63	315	18,81	20,9	0,977	430	4,17
64	320	19,10	21,3	0,995	440	4,17
65	325	19,40	21,5	1,005	432	4,17
66	330	19,70	21,3	0,995	431	4,18
67	335	20,00	21,4	1,000	420	4,18
Feed			22,4		98,6	5,12

Table II. Resin B: 7 BV/h Flowrate analysis results

Sample#:	t, min	BV	Brix, %	c/c0	Conductivity (µS/cm)	pH
1	4,28	0,34	0,5	0,02	198,6	4,15
2	8,56	0,67	7,2	0,32	321	4,23
3	12,84	1,01	13,7	0,61	450	4,25
4	17,12	1,35	20,4	0,90	470	4,24
5	21,4	1,69	21	0,93	480	4,27
6	25,68	2,02	21	0,93	427	4,27
7	29,96	2,36	22,9	1,01	456	4,23
8	34,24	2,70	22,7	1,00	453	4,23
9	38,52	3,03	24,5	1,08	527	4,2
10	42,8	3,37	22,7	1,00	552	4,2
11	47,08	3,71	23	1,02	558	4,15
12	51,36	4,04	22,9	1,01	559	4,17
13	55,64	4,38	22,9	1,01	572	4,16
14	59,92	4,72	24,5	1,08	586	4,15
15	64,2	5,06	23	1,02	586	4,16
16	68,48	5,39	23	1,02	582	4,15
17	72,76	5,73	22,6	1,00	545	4,15
18	77,04	6,07	22,4	0,99	548	4,14
19	81,32	6,40	22,5	1,00	536	4,13
20	85,6	6,74	22,3	0,99	522	4,12
21	89,88	7,08	22,3	0,99	511	4,12
22	94,16	7,41	22,8	1,01	510	4,12
23	98,44	7,75	22,3	0,99	494	4,13
24	102,72	8,09	21,8	0,96	485	4,13
25	107	8,43	21,9	0,97	491	4,14
26	111,28	8,76	21,7	0,96	503	4,14
27	115,56	9,10	21,5	0,95	515	4,14
28	119,84	9,44	21,2	0,94	506	4,13
29	124,12	9,77	21,6	0,96	506	4,14
30	128,4	10,11	21,6	0,96	503	4,14
31	132,68	10,45	20,7	0,92	499	4,13
32	136,96	10,79	20,9	0,92	476	4,13
33	141,24	11,12	21,7	0,96	491	4,15
34	145,52	11,46	21,2	0,94	492	4,16
35	149,8	11,80	21,4	0,95	490	4,16
36	154,08	12,13	21,1	0,93	475	4,17
37	158,36	12,47	20,5	0,91	465	4,16
38	162,64	12,81	20,7	0,92	475	4,16
39	166,92	13,14	20,9	0,92	490	4,18
40	171,2	13,48	21,2	0,94	511	4,19
41	175,48	13,82	21,3	0,94	523	4,19
42	179,76	14,16	22,7	1,00	482	4,18

43	184,04	14,49	23,3	1,03	499	4,2
44	188,32	14,83	23,2	1,03	510	4,18
45	192,6	15,17	22,2	0,98	507	4,18
46	196,88	15,50	22,1	0,98	514	4,18
47	201,16	15,84	22,3	0,99	510	4,19
48	205,44	16,18	22	0,97	514	4,19
49	209,72	16,52	22,6	1,00	513	4,19
50	214	16,85	22,1	0,98	505	4,19
51	218,28	17,19	21,9	0,97	518	4,19
52	222,56	17,53	22,3	0,99	500	4,2
53	226,84	17,86	22,2	0,98	497	4,19
54	231,12	18,20	22,6		490	4,19
Feed			22,1		104,1	4,57

Table III. Resin B: 10 BV/h Flowrate analysis results

Sample#:	t, min	BV	Brix	c/c0	Conductivity (µS/cm)	pH
1	3	0,41	0	0,00	12,4	5,64
2	6	0,82	0	0,00	0	5,64
3	9	1,22	6,7	0,61	316	4,9
4	12	1,63	6,3	0,57	310	5,06
5	15	2,04	9,9	0,90	419	5,07
6	18	2,45	10,5	0,95	429	5,02
7	21	2,86	11,1	1,01	435	4,87
8	24	3,27	11,2	1,02	423	4,82
9	27	3,67	11	1,00	424	4,81
10	30	4,08	11,2	1,02	436	4,77
11	33	4,49	13,3	1,21	443	4,74
12	36	4,90	11,3	1,03	457	4,75
13	39	5,31	11,4	1,04	464	4,71
14	42	5,71	11,3	1,03	453	4,69
15	45	6,12	11,6	1,05	458	4,66
16	48	6,53	11	1,00	457	4,63
17	51	6,94	10,8	0,98	440	4,62
18	54	7,35	11,9	1,08	452	4,61
19	57	7,76	11,4	1,04	448	4,59
20	60	8,16	12,4	1,13	450	4,59
21	63	8,57	12	1,09	465	4,58
22	66	8,98	11,9	1,08	476	4,57
23	69	9,39	14,8	1,35	475	4,53
24	72	9,80	16,7	1,52	522	4,5
25	75	10,20	18,2	1,65	549	4,42
26	78	10,61	18,1	1,65	552	4,37
27	81	11,02	18,4	1,67	564	4,31
28	84	11,43	18,9	1,72	568	4,3
29	87	11,84	18,3	1,66	574	4,26
30	90	12,24	18,3	1,66	565	4,25
31	93	12,65	17,6	1,60	570	4,24
32	96	13,06	17,4	1,58	595	4,2
33	99	13,47	17,9	1,63	597	4,19
34	102	13,88	18,8	1,71	582	4,17
35	105	14,29	18,2	1,65	604	4,16
36	108	14,69	18,4	1,67	613	4,15
37	111	15,10	18,9	1,72	618	4,14
38	114	15,51	19,3	1,75	607	4,14
39	117	15,92	19	1,73	613	4,14
40	120	16,33	18,6	1,69	613	4,13
41	123	16,73	17,8	1,62	605	4,12
42	126	17,14	18,2	1,65	606	4,11

43	129	17,55	18,5	1,68	614	4,12
44	132	17,96	19,1	1,74	634	4,11
45	135	18,37	18,7	1,70	614	4,12
46	138	18,78	19,5	1,77	643	4,12
47	141	19,18	19,8	1,80	639	4,13
48	144	19,59	20,9	1,90	620	4,12
49	147	20,00	19	1,73	644	4,12
Feed			11		174,8	5,28

APPENDIX III: Experiment analysis results for Resin C

Table I. Resin C: 4 BV/h analysis results

BV	Brix, %	c/c0	Conductivity, (µS/cm)	pH
1	19	18,99	380	5,79
2	19,9	19,89	243	5,33
3	19,5	19,49	228	5,27
4	19,4	19,39	223	5,22
5	19,8	19,79	224	5,2
6	20	19,99	223	5,17
7	20,1	20,09	222	5,16
8	19,5	19,49	223	5,18
9	19,3	19,29	219	5,18

Table II. Resin C: 4 BV/h analysis results (pH altered experiment)

BV	Brix, %	c/c0	Conductivity, (µS/cm)	pH
1	17	16,99	282	5,29
2	19	18,99	362	5,52
3	19,1	19,09	365	5,59
4	19,3	19,29	360	5,63
5	19,2	19,19	363	5,67
6	19,8	19,79	354	5,67
7	19,6	19,59	356	5,66
8	19,3	19,29	353	5,65
9	19,7	19,69	356	5,6

APPENDIX IV: Flowrate experiment analysis results for Resin D

Table I. Resin D: 1 BV/h Flowrate analysis results

Sample#:	t, min	BV	Brix, %	c/c0	Conductivity (µS/cm)	pH
0,5	2,1425	0,25	0	0,00	1812	5,52
1	4,285	0,50	0,3	0,01	973	5,89
1,5	6,4275	0,75	0,1	0,00	577	6,15
2	8,57	1,00	8,2	0,34	468	6,05
2,5	10,7125	1,24	19,3	0,79	447	5,92
3	12,855	1,49	22,7	0,93	446	5,92
3,5	14,9975	1,74	22,6	0,93	437	5,94
4	17,14	1,99	23,7	0,98	440	5,94
5	21,425	2,49	23,7	0,98	435	5,92
5,5	23,5675	2,74	23,7	0,98	436	5,88
6	25,71	2,99	23,3	0,96	442	5,86
7	29,995	3,48	23,3	0,96	440	5,89
8	34,28	3,98	23,3	0,96	440	5,93
9	38,565	4,48	23,1	0,95	434	5,89
10	42,85	4,98	23,1	0,95	431	5,88
11	47,135	5,48	23,1	0,95	430	5,88
12	51,42	5,97	22,9	0,94	427	5,86
13	55,705	6,47	22,5	0,93	433	5,86
14	59,99	6,97	22,9	0,94	441	5,89
14,5	62,1325	7,22	24,3	1,00	419	5,91
15	64,275	7,47	22,9	0,94	434	5,88
16	68,56	7,97	23	0,95	429	5,9
Feed			24,3		151,7	5,09

Table II. Resin D: 4 BV/h Flowrate analysis results

Sample#:	t, min	BV	Brix	c/c0	Conductivity (µS/cm)	pH
1	4,285	0,50	0,1	0,01	46,6	5,48
2	8,57	1,00	1	0,06	129,5	6,64
3	12,855	1,49	14,3	0,80	576	6,96
4	17,14	1,99	18,6	1,04	580	7
5	21,425	2,49	18,8	1,05	598	7,03
6	25,71	2,99	18,6	1,04	585	7,02
7	29,995	3,48	19,2	1,07	568	7,01
8	34,28	3,98	18,9	1,06	572	7

9	38,565	4,48	18,9	1,06	573	6,97
10	42,85	4,98	19,2	1,07	554	6,96
11	47,135	5,48	19	1,06	552	6,89
12	51,42	5,97	18,6	1,04	551	6,87
13	55,705	6,47	18,6	1,04	554	6,61
14	59,99	6,97	18,7	1,04	543	6,71
15	64,275	7,47	18,9	1,06	540	6,76
16	68,56	7,97	19,5	1,09	520	6,77
17	72,845	8,46	20,5	1,15	525	6,75
18	77,13	8,96	20,4	1,14	513	6,76
19	81,415	9,46	19,9	1,11	518	6,76
20	85,7	9,96	18,9	1,06	525	6,74
21	89,985	10,45	18,6	1,04	527	6,66
22	94,27	10,95	18,2	1,02	540	6,66
23	98,555	11,45	19,1	1,07	504	6,65
24	102,84	11,95	18,4	1,03	512	6,62
25	107,125	12,45	18,7	1,04	512	6,63
26	111,41	12,94	18,7	1,04	504	6,57
27	115,695	13,44	18,6	1,04	504	6,59
28	119,98	13,94	18,4	1,03	532	6,58
29	124,265	14,44	18,8	1,05	510	6,57
30	128,55	14,93	19,1	1,07	494	6,54
31	132,835	15,43	19,6	1,09	491	6,46
32	137,12	15,93	18,9	1,06	495	6,45
33	141,405	16,43	19,6	1,09	482	6,49
34	145,69	16,93	18,7	1,04	486	6,41
35	149,975	17,42	18,5	1,03	469	6,42
36	154,26	17,92	18,7	1,04	477	6,39
37	158,545	18,42	19,9	1,11	464	6,41
38	162,83	18,92	20	1,12	461	6,41
39	167,115	19,42	20,1	1,12	459	6,39
40	171,4	19,91	19,7	1,10	461	6,35
Feed			17,9		95,1	4,95

Table III. Resin D: 7 BV/h Flowrate analysis results

Sample#:	t, min	BV	Brix, %	c/c0	Conductivity (µS/cm)	pH
1	4,285	0,50	1,9	0,10	21,8	9,75
2	8,57	1,00	1,5	0,08	815	10,01
3	12,855	1,49	14,6	0,79	1625	10,65
4	17,14	1,99	20,3	1,10	1437	10,49
5	21,425	2,49	18,5	1,00	1337	10,29
6	25,71	2,99	18,3	0,99	1274	10,11

7	29,995	3,48	18,3	0,99	1166	10
8	34,28	3,98	18	0,97	1121	9,89
9	38,565	4,48	17,9	0,97	1068	9,68
10	42,85	4,98	17,7	0,96	1004	9,51
11	47,135	5,48	18,2	0,98	978	9,34
12	51,42	5,97	17,6	0,95	931	9,21
13	55,705	6,47	17,6	0,95	890	8,98
14	59,99	6,97	17,6	0,95	882	8,91
15	64,275	7,47	17,6	0,95	800	8,86
16	68,56	7,97	18,1	0,98	807	8,73
17	72,845	8,46	18,2	0,98	719	8,66
18	77,13	8,96	18	0,97	722	8,52
19	81,415	9,46	17,9	0,97	753	8,42
20	85,7	9,96	18,5	1,00	699	8,32
21	89,985	10,45	18,3	0,99	698	8
22	94,27	10,95	18,3	0,99	685	7,85
23	98,555	11,45	18,4	0,99	687	7,82
24	102,84	11,95	18,5	1,00	671	7,77
25	107,125	12,45	18,4	0,99	696	7,65
26	111,41	12,94	18,4	0,99	695	7,68
27	115,695	13,44	18,2	0,98	658	7,59
28	119,98	13,94	18,4	0,99	670	7,54
29	124,265	14,44	18,5	1,00	650	7,5
30	128,55	14,93	18,9	1,02	650	7,433
31	132,835	15,43	18,6	1,01	640	7,33
32	137,12	15,93	18,8	1,02	659	7,27
33	141,405	16,43	18,5	1,00	665	7,23
34	145,69	16,93	18,5	1,00	645	7,32
35	149,975	17,42	18,5	1,00	635	7,28
36	154,26	17,92	18,7	1,01	614	7,26
37	158,545	18,42	18,7	1,01	628	7,21
38	162,83	18,92	18,4	0,99	617	7,18
39	167,115	19,42	18,7	1,01	613	7,05
40	171,4	19,91	18,9	1,02	610	7,05
Feed			18,5		98,8	4,71

APPENDIX V: Capacity experiment analysis results for Resin A and B

Table I. Capacity experiment results for Resin A

BV	Brix, %	c/c0	Conductivity (µS/cm)	pH
1	12,5	0,53	342	4,38
2	23,5	1,00	359	4,37
3	23,4	0,99	381	4,34
4	23,2	0,98	359	4,29
5	23,2	0,98	353	4,33
6	23,3	0,99	331	4,34
7	23,5	1,00	322	4,44
8	23,2	0,98	303	4,46
9	23,6	1,00	284	4,56
10	23,8	1,01	277	4,56
11	23,4	0,99	257	4,56
12	23,4	0,99	252	4,57
13	23,5	1,00	243	4,6
14	21	0,89	243	4,61
15	22,9	0,97	229	4,61
16	21,7	0,92	226	4,64
17	23,4	0,99	225	4,66
18	23,4	0,99	210	4,66
19	20,4	0,86	199,6	4,64
20	22,6	0,96	209	4,64
20,5	21,1	0,89	207	4,66
Feed	23,6		97,4	4,93

Table II. Capacity experiment results for Resin B

BV	Brix, %	c/c0	Conductivity (µS/cm)	pH
1	22,3	0,96	426	4,24
2	23,2	1,00	467	4,28
3	23,6	1,01	475	4,22
4	23,3	1,00	473	4,18
5	23,1	0,99	505	4,16
6	22,3	0,96	493	4,13
7	22,5	0,97	565	4,16
8	22,8	0,98	541	4,16
9	23	0,99	513	4,17
10	23,2	1,00	473	4,19
11	22,7	0,97	509	4,19
12	23	0,99	505	4,18
13	22,6	0,97	589	4,23

14	22,3	0,96	518	4,24
15	23,4	1,00	514	4,25
16	22,1	0,95	490	4,24
17	22,8	0,98	453	4,22
18	22,6	0,97	456	4,23
19	22,5	0,97	456	4,25
20	23,5	1,01	430	4,26
21	22,6	0,97	436	4,3
22	22,7	0,97	447	4,31
22,86	22,8	0,98	424	4,32
Feed	23,3		107,2	4,88

APPENDIX VI: GMP/CMP: HPLC analysis results Flowrate experiments for Resin A and B

Table I. CMP/GMP HPLC analysis results for Resin A Flowrate 1 BV/h experiment

BV	CMP sum	GMP sum	CMP Reduction	GMP Reduction
	g/100ml	g/100ml		
0 (Feed)	0,35	0,21		
2,39	0,04	0,04	0,89	0,89
5	0,12	0,07	0,67	0,81
10	0,30	0,09	0,14	0,76
15	0,38	0,13	-0,08	0,62

Table II. CMP/GMP HPLC analysis results for Resin A Flowrate 4 BV/h experiment

BV	CMP sum	GMP sum	CMP Reduction	GMP Reduction
	g/100ml	g/100ml		
0 (Feed)	0,36	0,25		
1,94	0,02	0,03	0,95	0,91
5,16	0,07	0,04	0,82	0,88
10,00	0,21	0,09	0,41	0,74
15,16	0,30	0,13	0,18	0,63

Table III. CMP/GMP HPLC analysis results for Resin A Flowrate 7 BV/h experiment

BV	CMP sum	GMP sum	CMP Reduction	GMP Reduction
	g/100ml	g/100ml		
0 (Feed)	0,42	0,33		
1,57	0,07	0,06	0,83	0,85
5,00	0,23	0,12	0,46	0,71
10,00	0,29	0,16	0,30	0,62
15,00	0,35	0,21	0,17	0,51
18,83	0,38	0,21	0,09	0,50

Table IV. CMP/GMP HPLC analysis results for Resin A Flowrate 10 BV/h experiment

BV	CMP sum	GMP sum	CMP Reduction	GMP Reduction
	g/100ml	g/100ml		
0 (Feed)	0,40	0,32		
1,97	0,17	0,10	0,58	0,75
5	0,29	0,18	0,27	0,56
10	0,34	0,22	0,15	0,46
15	0,33	0,22	0,18	0,45

Table V. CMP/GMP HPLC analysis results for Resin B Flowrate 4 BV/h experiment

BV	CMP sum	GMP sum	CMP Reduction	GMP Reduction
	g/100ml	g/100ml		
0 (Feed)	0,35	0,23		
1,49	0,03	0,04	0,92	0,88
5,07	0,02	0,04	0,93	0,88
10,14	0,07	0,06	0,81	0,84
14,92	0,07	0,06	0,79	0,84

Table VI. CMP/GMP HPLC analysis results for Resin B Flowrate 7 BV/h experiment

BV	CMP sum	GMP sum	CMP Reduction	GMP Reduction
	g/100ml	g/100ml		
0 (Feed)	0,32	0,21		
1,69	0,02	0,03	0,94	0,92
4,71	0,03	0,04	0,89	0,88
9,10	0,03	0,04	0,92	0,89
15,00	0,04	0,04	0,88	0,89

Table VI. CMP/GMP HPLC analysis results for Resin B Flowrate 10 BV/h experiment

BV	CMP sum	GMP sum	CMP Reduction	GMP Reduction
	g/100ml	g/100ml		
0 (Feed)	0,18	0,14		
2,86	0,01	0,01	0,94	0,94
4,90	0,01	0,01	0,94	0,93
10,20	0,03	0,04	0,81	0,81
15,10	0,05	0,04	0,73	0,80

APPENDIX VII: GMP/CMP: HPLC analysis results Capacity experiment

Table I. CMP/GMP HPLC analysis results for Resin A capacity experiment (4 BV/h)

BV	CMP sum	GMP sum	CMP Reduction	GMP Reduction
	g/100ml	g/100ml		
0 (Feed)	0,42	0,31	-	-
2,00	0,05	0,04	0,88	0,86
6,00	0,20	0,10	0,53	0,67
10,00	0,28	0,15	0,35	0,53
15,00	0,32	0,17	0,24	0,46
20,00	0,35	0,19	0,18	0,38

Table II. CMP/GMP HPLC analysis results for Resin B capacity experiment (4 BV/h)

BV	CMP sum	GMP sum	CMP Reduction	GMP Reduction
	g/100ml	g/100ml		
	0,30	0,24		
1,00	0,04	0,04	0,86	0,82
6,00	0,06	0,05	0,81	0,78
10,00	0,08	0,05	0,74	0,77
15,00	0,10	0,05	0,67	0,79
20,00	0,13	0,06	0,59	0,76

APPENDIX VIII: B12: HPLC analysis results experiment for resin C and D

Table I. B12 content HPLC analysis results for Resin C and D experiments

Sample:	BV	c(VB12) (µg/100g)	Deviation ±	Relative to feed	Reduction
Resin C	0,00	0,60	0,12		-
Resin C	2,00	0,77	0,16	1,28	-0,28
Resin C	8,00	0,77	0,15	1,19	-0,28
Resin C, pH alt	0,00	0,71	0,14		-
Resin C, pH alt	2,00	0,66	0,13	0,97	0,03
Resin D 4BV/h Feed	0,00	0,69	0,14		-
Resin D 4BV/h 1	2,50	0,62	0,12	0,90	0,10
Resin D 4BV/h 2	5,00	0,64	0,13	0,93	0,07
Resin D 4BV/h 3	10,00	0,66	0,13	0,96	0,04
Resin D 4BV/h 4	15,00	0,61	0,12	0,88	0,12
Resin D 4BV/h 5	20,00	0,62	0,12	0,90	0,10
Resin D 7BV/h Feed	0,00	0,68	0,14		-
Resin D 7BV/h 1	15,00	0,64	0,13	0,94	0,06
Resin D 7BV/h 2	20,00	0,66	0,13	0,97	0,03
Resin D 1BV/h Feed	0,00	0,72	0,14		-
Resin D 1BV/h 1	2,50	0,70	0,14	0,97	0,03
Resin D 1BV/h 2	7,75	0,67	0,13	0,94	0,06

APPENDIX IX: Additional content analysis for Resins A and B capacity experiments

Table I. Additional analyses from Resin A capacity experiment

Resin A	Feed	1	2	3	4	5	End
BV	0	2	6	10	15	20	20,5
Total Protein Content (Kjeldahl), %	3,18	2,76	2,81	2,91	2,95	2,79	2,56
GMP content, g/100ml	0,31	0,04	0,10	0,15	0,17	0,19	0,16
GMP content, g/100g	0,29	0,04	0,09	0,14	0,16	0,18	0,15
CMP content, g/100ml	0,42	0,05	0,20	0,28	0,32	0,35	0,30
CMP content, g/100g	0,39	0,05	0,19	0,26	0,30	0,33	0,28
β-lg, content, g/100ml	1,58	1,81	1,75	1,71	1,61	1,49	1,40
β-lg, content, g/100ml	1,48	1,69	1,64	1,60	1,50	1,39	1,31
α-la content, g/100ml	0,23	0,33	0,33	0,30	0,28	0,27	0,25
α-la content, g/100ml	0,21		0,31		0,31		0,28

Table II. Additional analyses from Resin B capacity experiment

Resin B	Feed	1	2	3	4	5	End
BV	0	1	6	10	15	20	22
	Feed	1 BV	6 BV	10 BV	15 BV	20 B	End at 22 BV
Total Protein Content (Kjeldahl), %	3,23	2,74	2,67	2,77	2,79	2,78	2,77
GMP content, g/100ml	0,24	0,05	0,05	0,06	0,05	0,06	0,05
GMP content, g/100g	0,22	0,05	0,05	0,06	0,05	0,06	0,05
CMP content, g/100ml	0,30	0,04	0,06	0,08	0,10	0,13	0,10
CMP content, g/100g	0,28	0,04	0,06	0,07	0,09	0,12	0,09
β-lg, content, g/100g	1,60	1,85	1,85	1,85	1,87	1,86	1,83
β-lg, content, g/100ml	1,50	1,73	1,73	1,73	1,75	1,74	1,71
α-la content, g/100g	0,28	0,39	0,39	0,39	0,40	0,37	0,36
α-la content, g/100ml	0,26	0,36	0,36	0,36	0,37	0,35	0,34

Table III. Amino acid content analyses from Resin B capacity experiment

	Feed			15 BV		
	c, g/100g	Deviance ±	g/100gprot	c, g/100g	Deviance ±	g/100gprot
Alanine	0,18	0,02	5,42	0,15	0,02	5,23
Arginine	0,08	0,01	2,33	0,07	0,01	2,39

Aspartic acid	0,37	0,05	11,46	0,33	0,05	11,79
Glutamic acid	0,57	0,08	17,62	0,47	0,07	16,92
Glycine	0,06	0,01	1,88	0,05	0,01	1,96
Histidine	0,06	0,01	1,89	0,06	0,01	2,09
Isoleucine	0,20	0,03	6,19	0,15	0,02	5,52
Leucine	0,35	0,05	10,87	0,34	0,05	12,22
Lysine	0,31	0,04	9,72	0,29	0,04	10,29
Phenylalanine	0,11	0,01	3,25	0,10	0,01	3,73
Proline	0,19	0,03	5,82	0,14	0,02	4,87
Serine	0,17	0,02	5,14	0,13	0,02	4,70
Threonine	0,23	0,03	7,21	0,16	0,02	5,63
Tyrosine	0,07	0,01	2,23	0,07	0,01	2,65
Valine	0,19	0,03	5,91	0,15	0,02	5,34
Cystein + cystine	0,07	0,01	2,23	0,07	0,01	2,47
Methionine	0,07	0,01	2,20	0,06	0,01	2,15
Tryptophan	0,06	0,01	2,00	0,06	0,01	2,31

Table IV. Amino acid content analyses from Resin B capacity experiment elution

	c, g/100g	Devance ±	g/100gprot
Alanine	0,34	0,05	6,31
Arginine	0,03	0,00	0,48
Aspartic acid	0,58	0,08	10,65
Glutamic acid	1,30	0,18	23,83
Glycine	0,07	0,01	1,21
Histidine	0,04	0,01	0,71
Isoleucine	0,62	0,09	11,29
Leucine	0,16	0,02	2,90
Lysine	0,40	0,06	7,28
Phenylalanine	0,03	0,00	0,60
Proline	0,76	0,11	13,93
Serine	0,49	0,07	8,95
Threonine	1,02	0,14	18,70
Tyrosine	<0,0023 (LOQ)	0,00	0,00
Valine	0,49	0,07	8,93
Cystein + cystine	0,02	0,00	0,27
Methionine	0,05	0,01	0,82

APPENDIX IX: Elution sample results

Resin	V_eluent, ml	V_water, ml	T ˚C	Brix,%	Conductivity, µS/cm	Protein, %	CMP, g/100ml	GMP, g/100ml
A	322,21	0,00	5,00	0,50	89,20	-	0,01	< 0,01
A	644,42	0,00	10,00	0,20	89,80	-	N/A	N/A
A	644,42	322,21	15,00	12,00	16980,00	11,30	2,33	3,86
A	644,42	644,42	20,00	0,10	332,00	-	< 0,01	< 0,01
B	2078,29	0,00	6,67	0,10	250,00	-	< 0,01	< 0,01
B	4156,58	0,00	13,33	18,10	8780,00	7,11	4,40	4,55
B	6234,88	0,00	20,00	17,20	75800,00	-	1,33	1,37
B	6234,88	1558,72	5,00	17,70	117400,00	3,80	1,62	1,56
B	6234,88	3117,44	10,00	18,00	41200,00	-	1,74	1,66
B	6234,88	4676,16	15,00	4,20	21800,00	1,31	< 0,01	< 0,01