EFFECT OF pH ON WHEY PROTEIN SEPARATION USING HIGH PERFORMANCE TANGENTIAL FLOW FILTRATION

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JUDUL : EFFECT OF pH ON WHEY PROTEIN SEPARATION USING HIGH PERFORMANCE TANGENTIAL FLOW FILTRATION

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EFFECT OF pH ON WHEY PROTEIN SEPARATION USING HIGH PERFORMANCE TANGENTIAL FLOW FILTRATION

SITI HAZWANI BINTI TAHIR

A thesis submitted in fulfillment
of the requirements for the award of the Degree of
Bachelor of Chemical Engineering

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

DECEMBER 2010

"I hereby declare that I have read this thesis and in my opinion this thesis has fulfilled the qualities and requirements for the award of Degree of Bachelor of Chemical Engineering"

Signature :

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Date : 30 November 2010

"I declare that this thesis entitled "Effect of pH on Whey Protein Separation Using High Performance Tangential Flow Filtration" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

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DEDICATION

Special Dedication for my beloved family especially my father (Mr. Tahir bin Ramli) and my mother (Mrs. Salma Binti Musa). Unlimited appreciation to all my lecturers that give lots of knowledge, for my supervisor, Dr. Syed Mohd Saufi Bin Tuan Chik, for all my beloved friends and Mohd Hafifi Bin Sabri for the support and the cooperation given. I couldn't have done this without all of you. Thank you for the Care, Comitment and Encouraged on me and Support although indirectly or directly involves in this project.

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ABSTRACT

High-performance tangential flow filtration (HPTFF) is an emerging technology that enables the separation of proteins with similar size. Optimization the pH of whey solution has a significant impact on the sieving behavior of proteins in HPTFF systems. The purpose of this research was to separate protein component, especially for α-lactalbumin and β-lactoglobulin from whey using HPTFF. It was operate at different pH value which is from pH 2 to pH 6. HPTFF experiment was performed using a 30 kDa polyethersulfone membrane in KvickLab filtration system. In this research, the best whey separation was occurr at pH 5 because of higher optimization the yield of β Lactoglobulin (β-Lag) in permeate stream. pH was affect the charge and the size of the protein in the whey. The ability of HPTFF to separate and purify each single protein component from whey protein will added the value of specific protein compare to its original mixture.

ABSTRAK

Prestasi tinggi filtrasi aliran tangensial (HPTFF) adalah sebuah teknologi baru yang boleh memisahkan saiz protin yang hampir sama. Mengoptimumkan pH bagi larutan whey akan memberi kesan terhadap saiz protin dalam sistem HPTFF. Tujuan kajian ini adalah untuk memisahkan komponen protin, terutama bagi α-lactalbumin dan β-lactoglobulin daripada larutan whey dengan menggunakan HPTFF. Ia dijalankan pada pH yang berlainan bermula dari pH 2 hingga pH 6. Eksperimen ini telah dijalankan menggunakkan membran Polyethersulfone yang bersaiz 30kDa di dalam sistem penapisan KvickLab. Dalam kajian ini, pemisahan whey yang terbaik telah belaku di pH 5 kerana hasil yang tertinggi bagi mengoptimumkan β-lactoglobulin di aliran serapan. pH larutan whey menyebabkan cas dan saiz protein di dalam whey berubah. Keupayaan HPTFF untuk memisahkan dan memurnikan komponen di dalam whey kepada individu protin tertentu boleh menghasilkan sesuatu yang lebih bernilai berbanding dengan campuran asalnya.

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LIST OF ABBREVIATIONS

HPTFF	High Performance tangential Flow Filtration
CFF	Cross Flow Filtration
TMP	Trans Membrane Pressure
UF	Ultrafiltration
MF	Microfiltration
WPC	Whey Protein Concentrate
NF	Nanofiltration
rpm	Rotation per minute
NaCl	Sodium Chloride
RO	Reverse Osmosis
MWCO	Molecular Weight Cut Off
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
PES	Polyethersulfone
NaOH	Sodium Hydroxide
WF	Water Flux
NWP	Normalized Water Permeability
RPC	Reverse Phase Chromatography
TFA	Trifluoroacetic acid
BSA	Bovine Serum Albumin

LIST OF SYMBOL

%	Percent
°C	Degree Celcius
Psig	Pounds per Square Inch Gauge
mg	milligram
mL	milliLeter
psig	Pound per square inch gauge
mg/mL	milligram / millileter
nm	nanometer
µm	micrometer
g/L	gram / Liter
kDa	kilo Dalton
mS/cm	MilliSiemens / centimeter

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Membrane filtration is widely used for protein separation. There are two types of flow operation in membrane filtration which are dead end filtration or cross-flow filtration (CFF) as showed in Figure 1.1. In dead end filtration, the feed stream at the top side of the membrane is push through the pores of the membrane, which produce the permeate stream at the bottom side of the membrane. However, in dead end filtration, the cake layer will be develop and becomes increasingly thicker over the time. This cake layer formation will reduced the filtration rate and pressure need to push the feed through the membrane (Vogel and Todara, 1997).

In cross-flow filtration, the feed flow tangentially across the membrane, rather than perpendicularly into the filter. CFF is also known as a tangential flow filtration. The advantage CFF is the filter cake is substantially washed away during the filtration. This wills increase the filtration operation time because the clogging on the inner pore of the membrane can be minimized or prevented.CFF can be used to concentrate solids and semi-solids solution very effectively because it is designed to retain these solids on the top side of the membrane (retentate side) rather than penetrate through the membrane pore towards the permeate side.

However, conventional tangential flow filtration is limited to the separation of solutes that differ by ten-fold in size (e.g., cell– protein, virus–protein and protein–buffer). High-performance tangential flow filtration (HPTFF) has been developed to overcome the limitation of conventional tangential flow filtration. HPTFF is a two-dimensional purification method that exploited differences in both

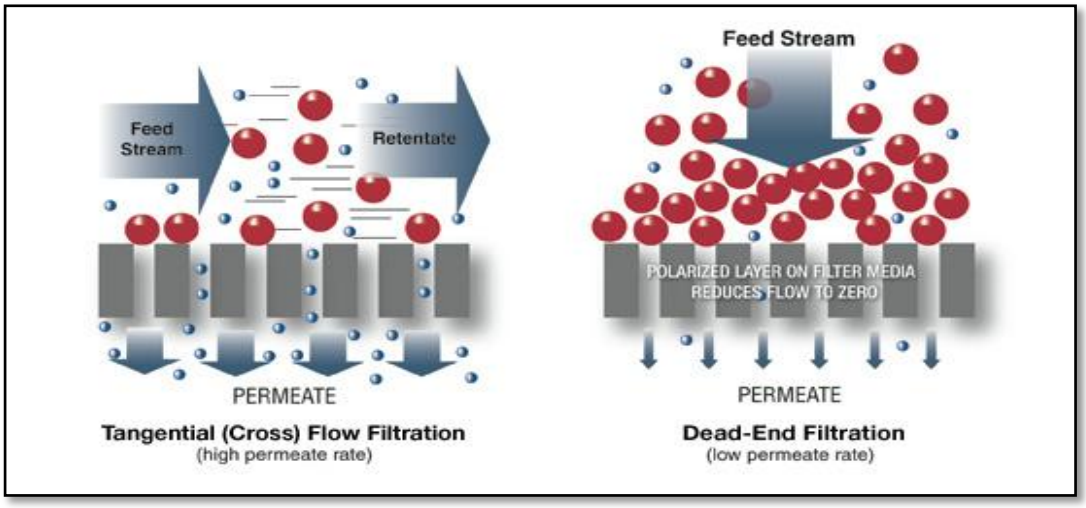


Figure 1.1: Tangential flow filtration and dead-end filtration operation in membrane separation process.

size and charge characteristics of biomolecules (van Reis and Zydney, 2001). Molecules that differ less than three-fold in size can be separated using highly selective charged membranes with careful optimization of buffer and fluid dynamics in HPTFF. HPTFF also provided high-resolution purification while maintaining the inherent high-throughput and high-yield characteristics of conventional UF (Saxena et al., 2008).

1.2 Problem Statement

Whey protein is a by-product or also known as waste in cheese production. Cheese is produced when casein is precipitated from milk, while the remaining liquid after precipitation formed is called as a whey protein. There is still a lack of awareness about the protein components present in the whey, which has its own value. These whey protein components could be purified into single pure protein to be used in specific application and had a higher market prices compare to it original protein mixture. Most of the protein components in whey differ less than 10 fold in size. In fact for the two major protein of α–lactalbumin (α–lac) and β–lactoglobulin (β–lag) only differ each other by less than 3 fold in size. So, it is impossible to separate these two components using normal CFF. With HPTFF concept, it seems to

be an ideal way to overcome this limitation as it can separate two or more molecules that differ even less than 3 fold in size.

1.3 Research Objective

The objective of this research is to separate protein component from whey using high performance tangential flow filtration at different pH operation. Besides that, to determine the pH that could optimize the yield of β–lag at permeate.

1.4 Research Scopes

The following scopes have been outlined in order to achieve the research objective:

i. Prepare and optimize the whey preparation method from fresh milk.
ii. Setup and operate HPTFF using 30 kDa polythersulfone membranes in Kvick Lab filtration system.
iii. Study the effect of HPTFF pH operation from pH 2 to 6 on the protein composition in retentate and permeate.

CHAPTER 2

LITERATURE REVIEW

2.1 Protein Bioseparation Methods

The most common techniques used for protein separation are precipitation and centrifugation, chromatography, electrophoresis and membrane separation (Ghosh, 2003). Sometime a combination of technique had been used to fulfil the require protein purity.

2.1.1 Precipitation and Centrifugation

Proteins can be partially purified using precipitation technique. This technique use salt (e.g. ammonium sulphate and sodium chloride), solvents (e.g. ethanol, methanol and acetone) or concentrated acids and alkali to partially precipitate the protein of interest from the feed mixture. Then, the precipitates are separated from the mixture using centrifugation by spinning the samples at a very high rotation speed.

2.1.2 Chromatography

Chromatography relies on the distribution of components to be separated between two phases: a stationary or binding phase and mobile phase, which carries these components through stationary phase. The mixture of the component enters the column along with the mobile phase, and each individual component is flushed through the system at a different rate depending on the interaction with the stationary phase. There are several types of column configuration is used in chromatographic

such as packed beds column, packed capillary columns, open tubular and monolith column. The most commonly used in biotechnology industries is packed beds column.

Chromatographic interaction can be based on four different sorption mechanisms, which are surface adsorption, partition, ion exchange and size exclusion. For the surface adsorption, separation mechanism depends upon differences in polarity between the different feed components. The more polar a molecule, the more strongly it will be adsorbed by a polar stationary phase. Similarly, the more non-polar a molecule, the more strongly it will be adsorbed by non-polar stationary phase. During a surface adsorption chromatography process, there is competition for stationary phase adsorption sites, between the materials to be separated and the mobile phase. Feed molecules of low polarity spend proportionally more time in the mobile phase than those molecules that are highly polar, which are retained longer. Therefore the components of a mixture are eluted in order of increasing polarity.

In partition chromatography, the stationary phase is coated onto a solid support such as silica gel, cellulose powder, or kieselguhr (hydrated silica). Assuming that there is no adsorption by the solid support, the feed components move through the system at rates determined by their relative solubilities in the stationary and mobile phases. In general, it is not necessary for the stationary and mobile phases to be totally immiscible. Hydrophilic stationary phase are generally used in conjunction with hydrophobic mobile phases (referred to as "normal-phase chromatography"), or vice versa (referred to as a "'reverse- phase chromatography"). Suitable hydrophilic mobile phases include water, aqueous buffers and alcohols. Hydrophobic mobile phases include hydrocarbons in combination with ethers, esters and chlorinated solvents (Groves, 2006).

In ion exchange process, the stationary phase consists of an insoluble porous resinous material containing fixed charge-carrying groups. Counter-ions of opposite charge are loosely complexed with these groups. Ion exchangers are either cation exchangers that exchange positively charged ions (cations) or anion exchangers that exchange negatively charged ions (anions). Passage of a liquid mobile phase,

containing ionized or partially ionized molecules of the same charge as the counter-ions through the system, results in the reversible exchange of these ions. The degree of affinity between the stationary phase and feed ions dictates the rate of migration and hence degree of separation between the different solute species. Resins with a low degree of cross-linking have large pores that allow the diffusion of large ions into the resin beads and facilitate rapid ion exchange. Highly cross- linked resins have pores of sizes similar to those of small ions. The choice of a particular resin will very much be dependent upon a given application. Cation (+) or anion (-) exchange properties can be introduced by chemical modification of the resin.

Size exclusion processes, also known as gel permeation chromatography, molecules of a feed material are identified according to their size or molecular weight. The stationary phase consists of a porous cross-linked polymeric gel. The pores of the gel vary in size and shape such that large molecules tend to be excluded by the smaller pores and move preferentially with the mobile phase. The smaller molecules are able to diffuse into and out of the smaller pores and will thus be retarded in the system. The very smallest molecules will permeate the gel pores to the greatest extent and will thus be most retarded by the system. The components of a mixture therefore elute in order of decreasing size or molecular weight.

2.1.3 Electrophoresis

Electrophoresis separates components by employing their electrophoretic mobility such as movement in an electric field. The mixture is added to a conductive medium then applies an electric field across it. The positively charged components will migrate to the negative electrode and the negatively charged component will move to positive electrode.

2.1.4 Membranes Separation

Although essentially all membrane processes are used for bioseparations, the greatest interest has been in the application of the pressure-driven processes of ultrafiltration (UF), microfiltration (MF), reverse osmosis (RO) and nanofiltration (NF). The size of the particles or components to be separated and the membrane

pores size are two important factors in membrane separation. The components that have smaller size than the membrane pores will pass through the membrane to the permeate side. While the larger components will be blocked from flow through and retain in the retentate side. Depending on the objective of the separation, either permeates or retentate can be used for collecting the product in membrane separation. Figure 2.1 show the membrane process based on the pore size and pressure drop used.

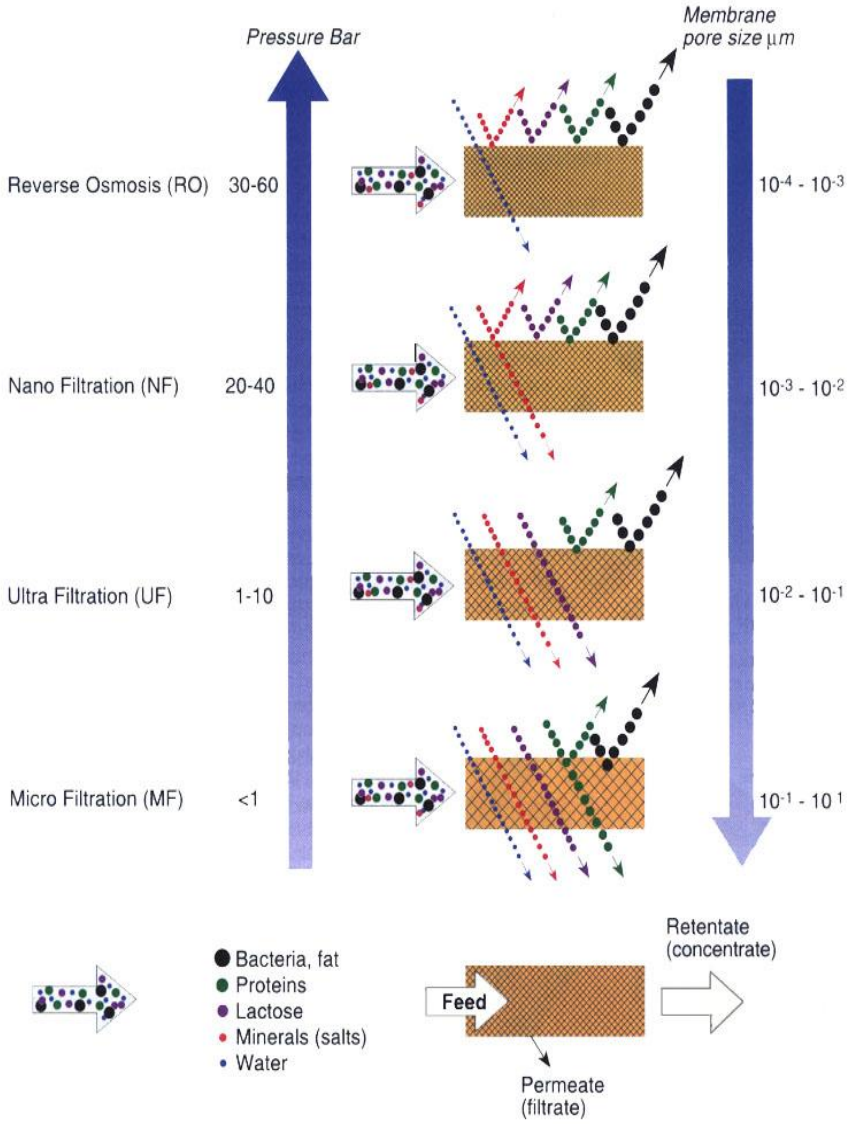


Figure 2.1: Principles of membrane filtration.

2.1.4.1 Ultrafiltration

The pore size of UF membrane is normally from 0.001 to 0.02 μm. Common applications of UF are in the purification and concentration of enzyme, protein, cell, germs and polysaccharide, and in the clarification and decolorize of antibiotic fermentation. UF mainly has the advantages such as steady high permeated flux, easy operation, low energy and operation cost, less pollution discharging and compacted equipment.

2.1.4.2 Microfiltration

A typical MF membrane pore size range is 0.1 to 10 μm. MF membrane basically used for reduction of bacteria in skim milk, whey and brine, defatting whey intended for whey protein concentrate (WPC) and for protein fractionation. MF can remove effectively suspended particles, bacteria, colloid and solid protein. The common membrane modules for MF membrane include spiral-wound membrane, plate and frame membrane, tubular membrane and hollow fiber membrane.

2.1.4.3 Nanofiltration

The pore size of NF membrane is between RO membrane and UF membrane, which can remove NaCl under 90% rejections. NF membrane mainly removes the particle which diameter is near 1nm, MWCO 100~1000. In the drinking water area, NF mainly remove Ca^{2+} ,Mg^{2+}, peculiar smell, colour, pesticide, synthesized surfactants, dissoluble organic and the vaporized rudimental materials. The character of the NF is that it hold the charge itself, so under the low pressure, it also have a high desalted rate. The greatest field for the NF is to soften and desalt the brine water. NF has its own advantage included good chemical stability, long life and high rejection.

2.1.4.4 Reverse Osmosis

RO membrane is a liquid/liquid separation process that uses a dense semi-permeable membrane, highly permeable to water. A pressurized feed solution is

passed over one surface of the membrane. As long as the applied pressure is greater than the osmotic pressure of the feed solution, “pure” water will flow from the more concentrated solution to the more dilute through the membrane to desalt, purify, concentrate and separate the solution. RO membrane has molecular weight cut off (MWCO) under 100, which capturing pollutions, like inorganic salt, sugar, amino acid, biochemical oxygen demand (BOD), chemical oxygen demand (COD) and so on. RO membrane has been widely applied in the water treatment, such as desalting, pollution control, pure water treatment, wastewater treatment.

2.2 Membranes Configurations

Membrane configuration refers to the packing of the membrane in the module so that it can be installed in the system. Common configurations include plate and frame, tubular, spiral wound and hollow fiber. The following section will described the membrane configuration in detail.

2.2.1 Hollow Fiber

Narrow bore hollow fiber membranes for tangential flow microfiltration are made from a variety of polymers including polyethersulfone, polysulfone, polypropylene, polyvinylidien fluoride, and mixed cellulose esters. These fibers typically have inner diameters of 0.2–1.8 mm, providing laminar flow with moderate shear rates. Most hollow fibers have an asymmetric structure with the dense skin at the lumen side of the fiber. The fibers are self-supporting, so they can typically be cleaned by back-flushing from the filtrate-side. Pre-sterilized disposable hollow fiber modules have also been developed, eliminating the need for cleaning and regeneration (van Reis and Zydney, 2007). Figure 2.2 shows the picture of hollow fiber membrane that was glued together in a membrane module.

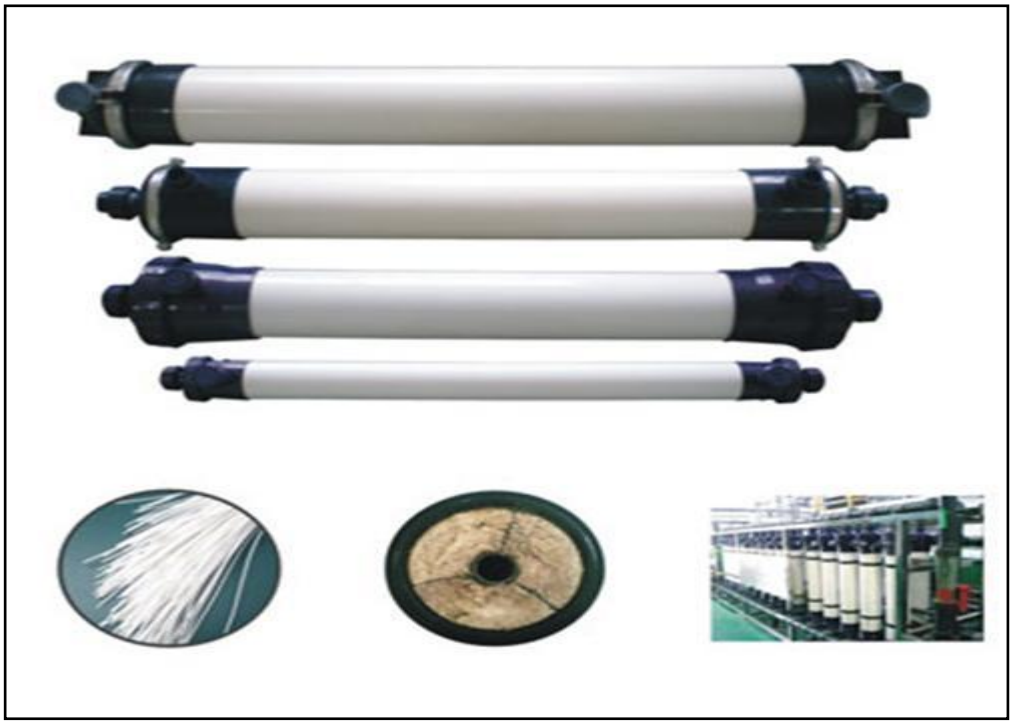


Figure 2.2: Hollow fiber membrane module.

2.2.2 Flat Sheet

Flat sheet membranes are typically cast on a non-woven substrate and can have either an isotropic or asymmetric structure. Uniform pore size is0.04m results in consistently high water permeability with minimal pore clogging. This asymmetric designed make membrane cartridge self-supporting and compact. The asymmetric membranes with the molecular-oriented skin layer were prepared by a simple dry/wet phase inversion technique with forced convection using a newly developed pneumatically-controlled casting system. A variety of polymers is available, including polysulfone, polyethersulfone, cellulose, and hydrophilized polyvinylidene fluoride. These materials are often surface modified to increase hydrophilicity and reduce fouling, and they can be cast as mixed polymers (e.g., with polyvinylpyrrolidone to increase wet ability). Membranes can be directly bonded or glued to plates or sealed using appropriate gaskets. Open channel systems are commonly employed for tangential flow microfiltration to minimize plugging by cell aggregates and debris (van Reis and Zydney, 2007).

2.2.3 Spiral Wound

Spirally wound modules are constructed from flat sheets of membrane glued back to back on three sides forming an envelope around a porous support material as showed in Figure 2.4. The open end of the membrane envelope is attached around a tube with holes which provide a route for permeate to flow out. The membrane is wound up around the centre tube to form a cylindrical element. Water that has passed through the membrane in service flows towards the centre tube through the porous support. The rolled up membrane leaves are separated by a mesh spacer, which also serves to promote turbulence in the feed channels. These membrane modules are designed for cross flow use, with the feed stream running mostly parallel to the membrane surface.

2.2.4 Tubular

Tubular membranes provide excellent capabilities for filtering and concentrating difficult process and waste streams because it have a wide centre

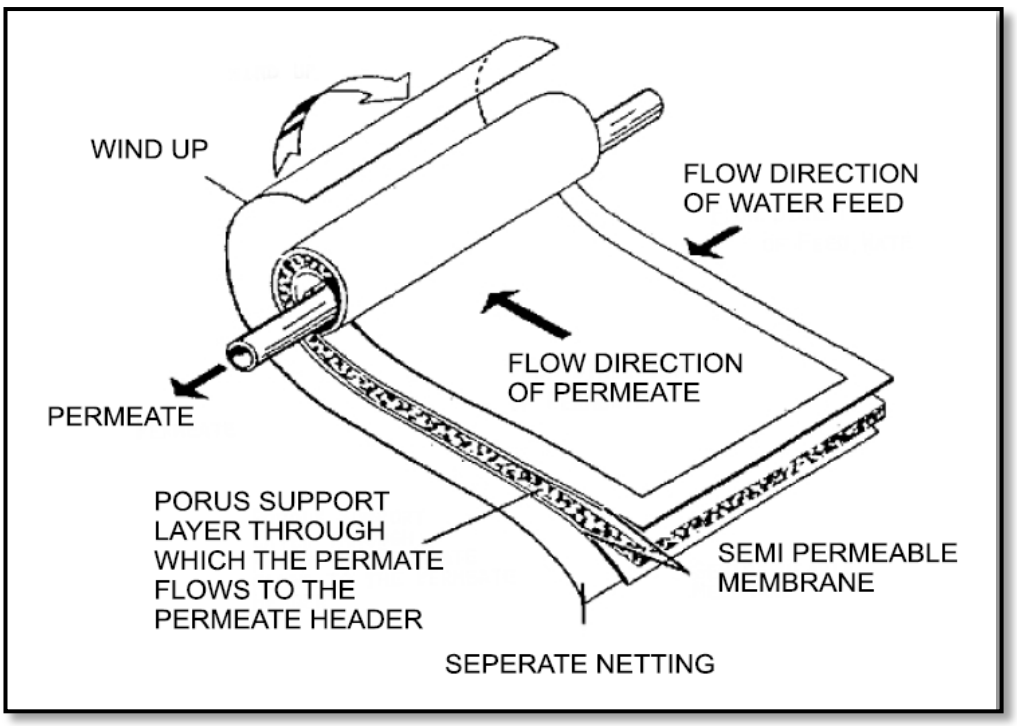


Figure 2.3: Spiral wound membrane module

channel which better handles feed streams with large solids and high levels of suspended soils without clogging. The ability to handle feed streams with widely varying compositions and characteristics makes these reliable, long-lasting tube membranes excellent replacements in nearly any existing in-plant system. Besides that, the tubular membranes feature excellent low-maintenance properties and prevent membrane fouling at high cross flow velocities especially in application with difficult process and waste streams. The tubular product range is from 6 to 12.5 mm diameter for liquids containing suspended solids and colloidal material. These tubular membranes from Figure 2.4 are designed to the most rigorous standards of performance, offering superior membrane composition with exacting tolerances.

2.2.5 Plate and Frame Module

This membrane is set up like a plate heat exchanger with the retentate on one side and the permeate on the other. The permeate is collected through a central collection tube. The plate and frame filter design is the standard in basic process depth filtration for clarification and pre-filtration in industries such as the pharmaceutical, chemical, cosmetic, food and beverage, and electric utility. Plate and frame as Figure 2.5 provide the lowest cost of filtration. Typically polymers that use as plate and frame membrane are polyethersulfone with polypropylene or polyolefin support. Range of plate and frame for UF is less than1 to 1000 kDa MWCO and for MF the range is 0.1 to 0.16 um diameter.

Figure 2.4: Tubular membrane module.

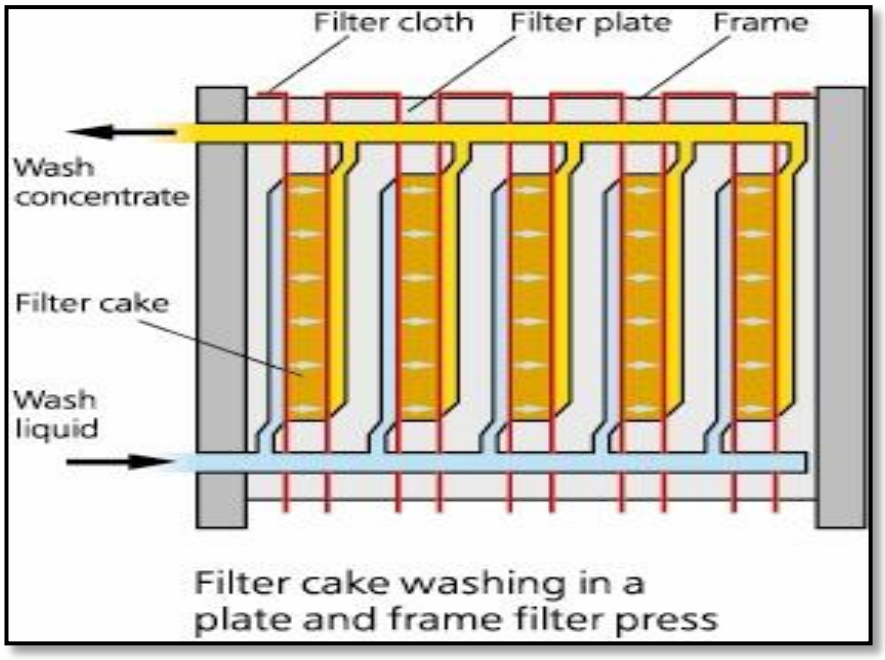


Figure 2.5: Plate and frame membrane module.

2.3 Whey Protein

Whey was discovered as a by-product of cheese production over 25 years ago. Cheese is made from milk and milk contains two major types of proteins which are casein and whey. Whey is the liquid that separates from the 'curd' or casein when cheese is produced. Whey contains a variety of proteins and large amounts of the milk sugar called lactose. Whey was traditionally thought to be worthless until some study had found that whey was loaded with a highly bioactive protein that is more similar to the protein found in human milk than any other known source. These proteins dissolved well in water, were highly digestible and contained an even better amino acid profile than the highly regarded egg white. The main problem with raw whey is it contains too much undesirable lactose, fat, and cholesterol. With the advance in separation technique, now it is able to extract the proteins from whey while preserving their integrity.

2.3.1 Whey Protein Components

The whey protein fraction contains a wide array of proteins with main components are summarized in the Table 2.1. Each individual whey protein components have their own unique nutritional, functional and biological characteristics that are largely unrealized in whey protein concentrates.

Whey proteins are commonly used in the food industry due to their wide range of chemical, physical and functional properties. The most important functional properties of whey proteins are solubility, viscosity, water holding capacity, gelation, and emulsification and foaming. In addition to their general properties, individual whey proteins have their own unique nutritional, functional and biological characteristics (Almecija,et al.,2006) as below:

i. **β-Lactoglobulin:** Is commonly used to stabilize food emulsions because of its surface-active properties. Besides that, β-lag so is a better foam stabilizer than the other whey protein components, and can be in the production of confection.

Table 2.1: Characteristics of major whey proteins (Andersson and Mattiasson, 2006).

Protein	Concentration[g/L]	Molecular weight[kDa]	IsoelectricPoint
β-Lactoglobulin	2 - 4	18	5.2
α-Lactalbumin	1.2 - 1.5	14	4.5–4.8
Immunoglobulin	0.65	150 –1,000	5.5–8.3
BSA	0.3–0.6	69	4.7–4.9
Lactoferrin	0.02–0.2	78–92	8–9.5
Lactoperoxidase	0.02–0.05	78–89	9.5
Glycomacropeptide	1–1.2	7	<3.8

ii. **α-Lactalbumin:** As a nutraceutical and a food additive in infant formula owing to its high content in tryptophan and as a protective against ethanol and stress-induced gastric mucosal injury (Almecija,et al.,2006). It also provides enhanced whip ability in meringue-like formulations. In addition, α-lac as strong affinity for glycosylated receptors on the surface of oocylates and spermatozoids and may thus have potential as a contraceptive agent.

iii. **Immunoglobulin :** Enhance the immunological properties of infant formula and they can be used therapeutically in the treatment of animal neonates and, in the form of special supplements, they can offer, in many situations, an important reduction of risk to acquire diarrhoea causing infections and other illnesses.

iv. **Bovine Serum Albumin:** Have gelation properties and it is of interest in a number of food and therapeutic applications, for instance, because of its antioxidant properties.

2.4 High Performance Tangential Flow Filtration

HPTFF is an emerging technology that enables concentration, purification, and buffer exchange in a single unit operation. HPTFF provides separation of solutes based on differences in both size and charge. Protein purification is possible due to enhanced selectivity and throughput. Significant improvement in performance has been achieved by operating in the pressure-dependent flux regime, generating similar flux throughout the membrane module, optimizing pH and conductivity, optimizing feed flow rate, bulk concentration and flux and using optimization diagrams to determine the best combination of selectivity and throughput for a specific process application.

In HPTFF of whey protein, the pH of whey protein will effect the composition of permeate and retentate side. Almecija et al. (2006) study the effect of whey pH on HPTFF operation using a 300 kDa tubular ceramic membrane in a continuous diafiltration mode. After 4 diavolumes, retentate yield for α-lac ranged

from 43% at pH 9 to 100% at pH 4, while for β-lag was from 67% at pH 3 to 100% at pH 4. In contrast, BSA, IgG and lactoferrin were mostly retained, with improvements up to 60% in purity at pH 9 with respect to the original whey.

It was, subsequently, recognized that significant improvements in performance could be obtained by controlling buffer pH and ionic strength to maximize differences in the effective hydrodynamic volume of the different proteins. For example, Saksena and Zydney (1994)showed that the selectivity (defined as the ratio of the protein sieving coefficients) for the filtration of BSA and IgG could be increased from a value of only two, at pH 7 and high salt concentrations, to more than 30 simply by adjusting the pH to 4.7 and lowering the solution ionic strength. The dramatic improvement in performance was due to the strong electrostatic exclusion of the positively charged IgG at pH 4.7, with the transmission of the (uncharged) BSA remaining fairly high. Similar improvements in performance by controlling pH and salt concentration have been reported for laboratory-scale filtration of BSA and hemoglobin (Eijndhoven van et al. 1995), BSA and lysozyme (Iritani et al. 1995), and myoglobin and cytochrome C (Yang et al. 1997 and van Reis etal. 1997) demonstrated that this approach can be used for protein separation processes (BSA monomer-dimer and BSA-IgG) by using a diafiltration mode to remove the more permeable species from the retained component.

CHAPTER 3

METHODOLOGY

3.1 Chemicals and Buffer Preparation

Phosphate buffer was used in HPTFF experiment by mixing different ratio of 0.2M mono potassium phosphates, 0.2M di potassium phosphate and deionized water to achieve the desired pH as showed Table 3.1. For reverse phase chromatography (RPC) protein analysis, trifluoroacetic acid and acetonitrile was used as a buffer component. When necessary, the pH of any solution involved in this study was adjusted by using either hydrochloric (HCl) acid and sodium hydroxide (NaOH) solution. All the buffer prepared was filtered using at least 0.45 μm membrane filter.

Table 3.1: Recipe for buffer solution for cross flow filtration

Desired pH	Buffer Solution Recipes
2	○ 50 mL 0.2M KCl + 13 mL 0.2M HCl ○ Adjusted with distilled water to 200 mL
3	○ 100 mL 0.1M potassium hydrogen phthalate + 44.6 mL of 0.1M HCl. ○ Adjusted with distilled water to 200 mL
4	○ 41 mL 0.2M acetic acid + 9 mL 0.2M sodium acetate ○ Adjusted with distilled water to 100 mL
5	○ 14.8 mL 0.1M mono potassium phosphate + 35.2 mL 0.2M di-potassium phosphate ○ Adjusted with distilled water to 200 mL
6	○ 87.7 mL 0.1M mono potassium phosphate + 12.3 mL 0.2M di-potassium phosphate ○ Adjusted with distilled water to 200 mL

3.2 Whey Protein Preparation

Milk was centrifuged at 4 420 rpm at room temperature for 30 min for delipidation. The pH of the skimmed milk was adjusted to 4.7 by the slow addition of 5M HCl. After casein precipitation, the solution was stirred for a further 30 min to complete precipitation (Hahn et al., 1996). Casein was removed by centrifugation at 10 000 rpm and 25^{o}C for 30 min. The obtained whey was diluted with distilled water until a conductivity of 2.7 mS/cm was obtained. The pH of whey was adjusted to the desired pH from pH 2 to pH 6. Figure 3.1 show the step to involve in preparation of whey in this study.

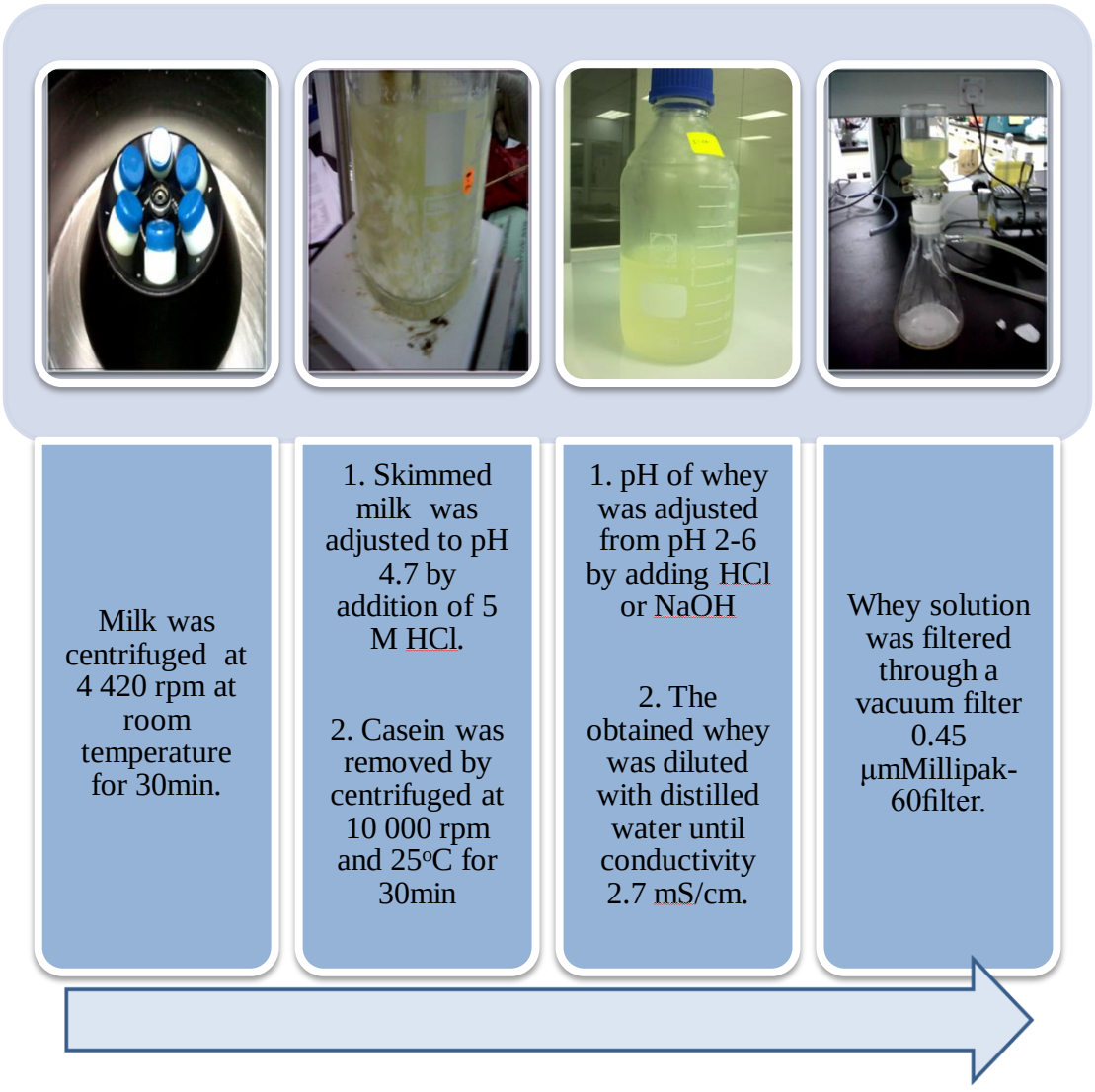


Figure 3.1: Whey protein preparation step.

3.3 Kvick Lab Cross Flow System

Cross-flow ultrafiltration experiments were performed using Kvick Lab cross flow system from GE Healthcare Technologies as showed schematically Figure 3.2 and Figure 3.3. The main component of the system include 2.5 L stainless steel jacketed reservoir, rotary lobe feed pump, Kvick Lab cassette holder, valves and in-line pressure gauge. The membrane use in HPTFF experiment was purchased from GE Healthcare which made from polyethersulfone with 30 kDa MWCO and 0.11 m^2 membrane areas.

3.4 Kvick Lab Running Protocol

Figure 3.4 show the running protocol in Kvick Lab cross-flow system. Each steps need to be followed in order to make sure the HPTFF experiment run smoothly and successfully.

Figure 3.2: Kvick Lab cross-flow system.

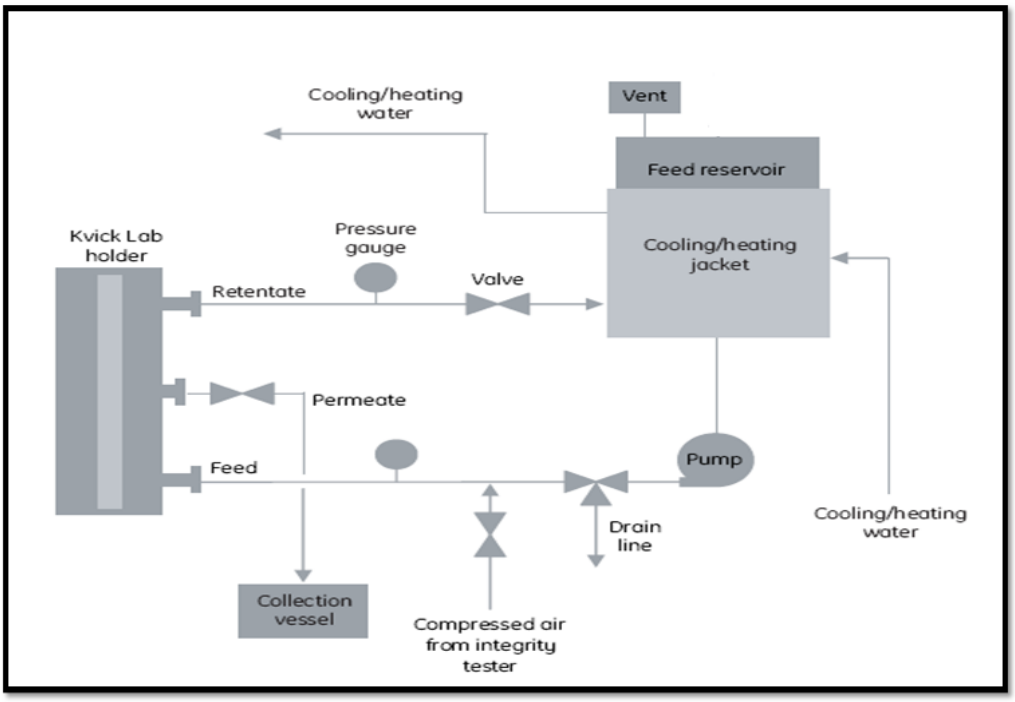


Figure 3.3: Kvick Lab cross-flow filtration system diagram.

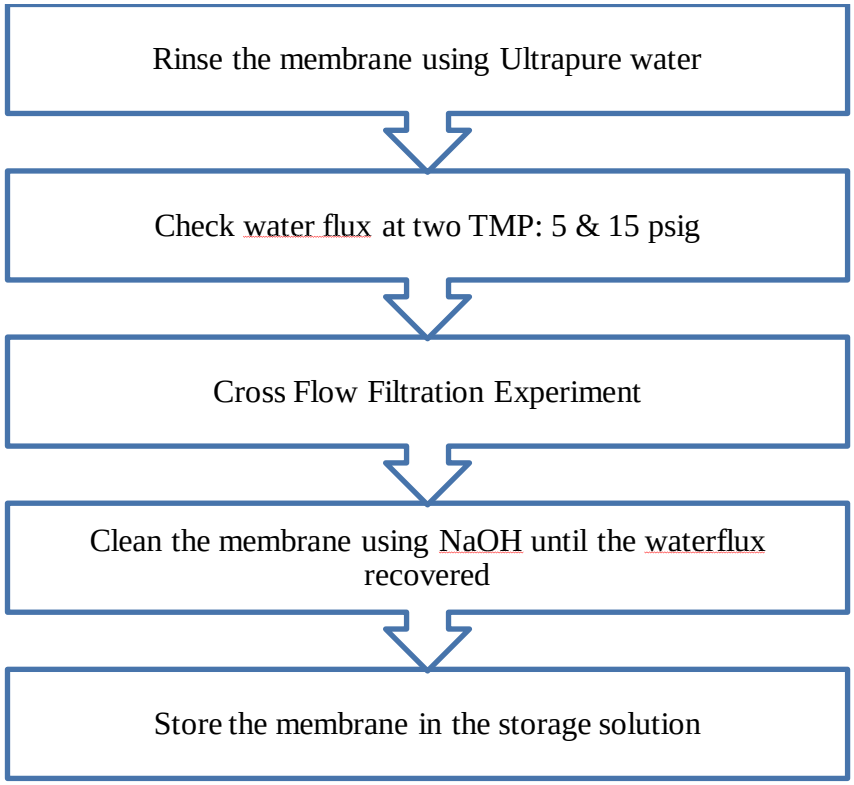


Figure 3.4: Running protocol in Kvick Lab cross-flow filtration system.

3.4.1 Membrane Rinsing

The membrane cassette was rinsed with water before using in cross-flow experiment to remove the storage solution inside the membrane. The membrane was placed in the membrane holder during the rinsing process. The reservoir was filled with 2 L of ultrapure water. Feed and retentate valves were opened and the permeate valve was closed. 10 percent of the water was pump through the retentate line to the waste. Next, the permeate valve was opened and the retentate valve was closed so the remaining water was pumped through the permeate line to waste. After rinsing, water flux of the membrane was measured.

3.4.2 Water Flux Testing

Clean water flux (WF) refers to the flux measurement made under standardized conditions on a new (and cleaned) membrane cartridge. The water flux obtain provide an indicator of the performance of the cassette. By tracking the water flux measurement, it can; (1) determined the effectiveness of cleaning cycles, and; (2) determined the cassette service life. Effectiveness of a cleaning protocol is usually examined by water flux recovery (%), comparing the water flux rate of a filter after cleaning against its initial water flux rate:

WF recovery (%) = (WF after cleaning / Initial WF) x 100

Water flux recovery may range widely, from 85% to 95%, after first use. Subsequent water flux recovery values should be near 90%, and low water flux recovery may indicate the need for cleaning method optimization. Because water flux is temperature sensitive, filter water flux should be normalized to 20°C (normalized water permeability, NWP). It is suggested to keep the water temperature constant when conducting filter water flux evaluation. Water flux measurement was made at transmembrane pressure (TMP) of 5 psig and 15 psig. Detailed calculation was showed in appendix A.

3.4.3 Cross Flow Filtration Experiment

The step in involve in HPTFF experiment was summarized in Figure 3.5. The HPTFF was run at the following condition: transmembrane pressure 5 psig, 200 rpm feed flow rate and temperature 30^{o}C. The membrane was firstly conditioned with 1 L running buffer for 15 min by circulating both retentate and permeate stream into the feed tank. Then 500 mL whey was fed to the system and run until the cumulative permeate volume achieved about 420 mL. Samples of initial feed, retentate and permeate were taken at each pH for quantification of individual proteins.

3.4.4 Cleaning Procedure

Membrane cleaning is necessary after several cycle of operation for the following reasons:

- To remove leftover product
- To prevent potential cross contamination
- To remove fouling materials
- To maintain and recovers filtration efficiency
- To prevent microorganism growth and remove their metabolites to keep a sanitary system

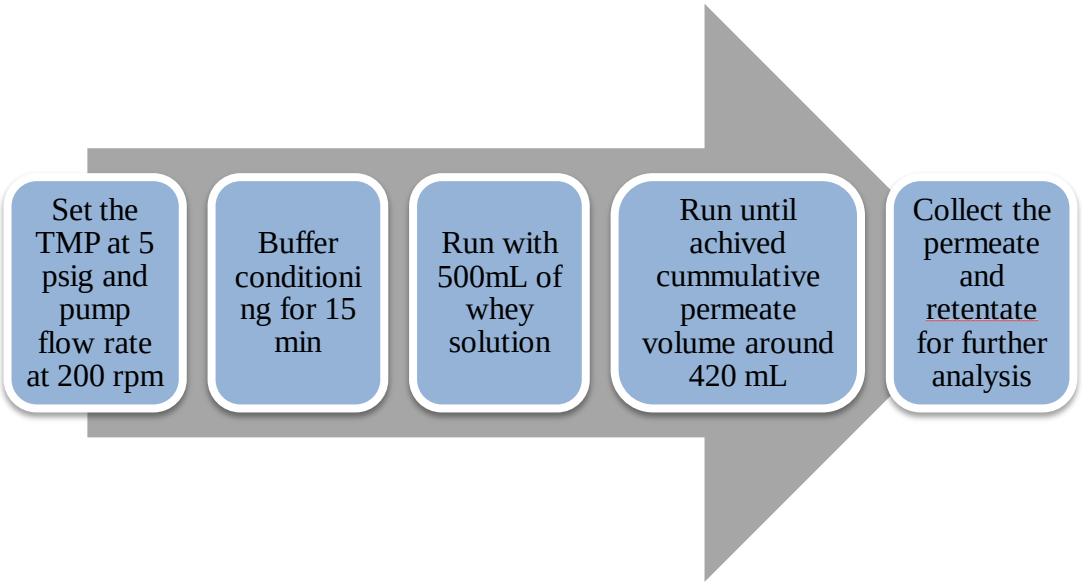


Figure 3.5: Cross-flow experiment protocol.

If the membrane is not cleaned effectively, its permeate flux will be reduced and the membrane life will be shortened. Different membranes may also require different cleaning strategies. In this study the following cleaning procedure was performed;

(1) Initial rinse with buffer solution for 10 min;
(2) Circulated with ultrapure water for 10 min;
(3) Circulated cleaning solution (0.5M NaOH) for 60 min;
(4) Flush the system using 2 L ultra pure water across the membrane for 2 hour;
(5) Lastly, change the water for every two hours until the water flux is recovered.

3.5 Reverse Phase Chromatography

A 1 mL Resources reverse phase chromatography column (Amersham Biosciences, Uppasala, Sweden) chromatography was used to analyze the whey protein component according to method established by Elgar et al. (2000). The RPC column was attached to AKTA Explorer100 Liquid Chromatography System. In RPC, solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in Milli-Q water and solvent B was 0.09% (v/v) TFA, 90% (v/v) acetonitrile in Milli-Q water. The column was equilibrated in 80% solvent A. The gradient protocol used was: 0–1 min, 20% B; 1–6 min, 20–40% B; 6–16 min, 40–45% B; 16–19 min, 45–50% B; 19–20 min, 50% B; 20–23 min, 50–70% B; 23–24 min, 70–100% B; 24–25 min, 100% B; 25–27 min, 100–20% B; 27–30 min, 20% B. Detection was by absorbance at 214 nm. Prior to RPC analysis, all samples were filtered through 0.22μm nylon syringe filters and buffers were filtered through 0.45μm membrane filters and degassed.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Whey Protein Analysis

Figure 4.1 is show the example of RPC chromatogram for whey protein at pH 3. The major peaks, corresponding to the main whey proteins, α-lac, β-lag and BSA, are appeared at elution volumes of 15 mL, 20 mL and 22.5 mL, respectively. The concentration of β-lag in feed whey was 0.5 mg/mL as determined by developed standard curve forβ-lag as showed in Figure 4.2. The standard curve for β-lag was prepared by varied the concentration of single β-lag at 2 mg/mL, 1 mg/mL, 0.5 mg/mL 0.25 mg/mL, 0.125 mg/mL and 0.0625 mg/mL. The concentration of α-lac was not possible to calculate in this study due to the difficulty in getting a pure α-lac standard. However, the peak area of α-lac can be used as a guideline to calculate the percentages of α-lac in permeate and retentate side.

4.2 Effect of pH on α-lac and β –lag Separation.

Detailed results of the β –lag and α-lac separation from whey protein solution at initial feed, retentate and permeate by employing 30kDa polyethersulfone membrane are shown in Table 4.1, 4.2 and 4.3 under constant operating condition TMP 5 psig, 200 rpm. The percentage of protein in each side was calculated as the ratio between the mass of protein in the retentate or permeate respective to the mass of protein in the initial feed. The result was showed in Figure 4.3.

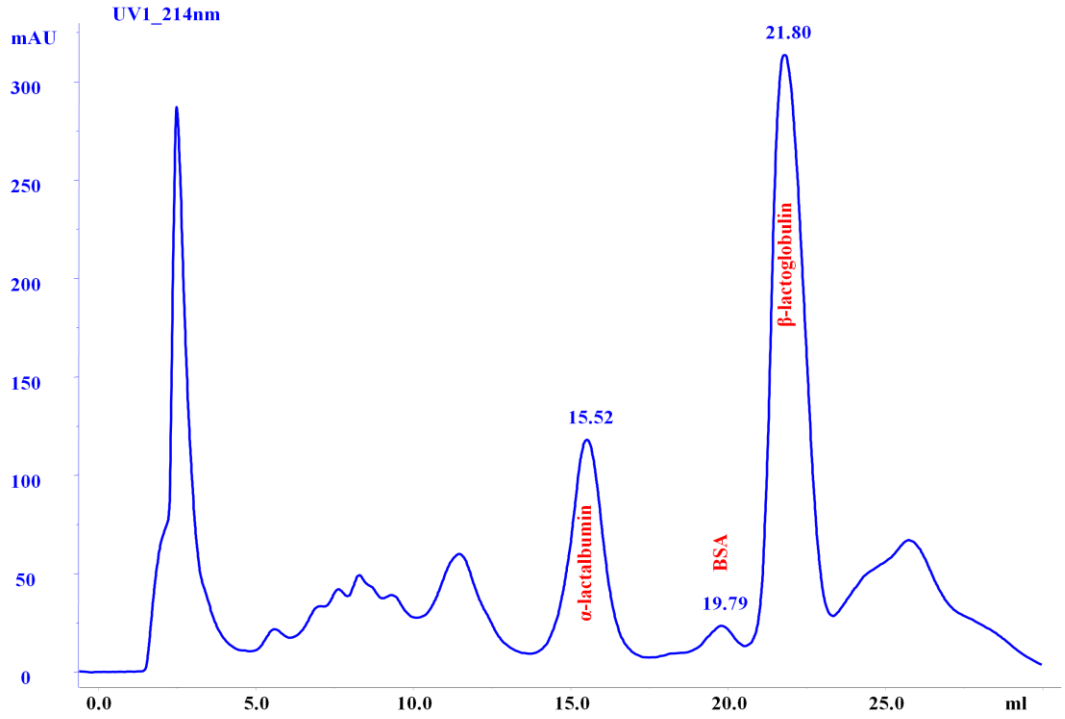


Figure 4.1: RPC chromatogram for feed whey

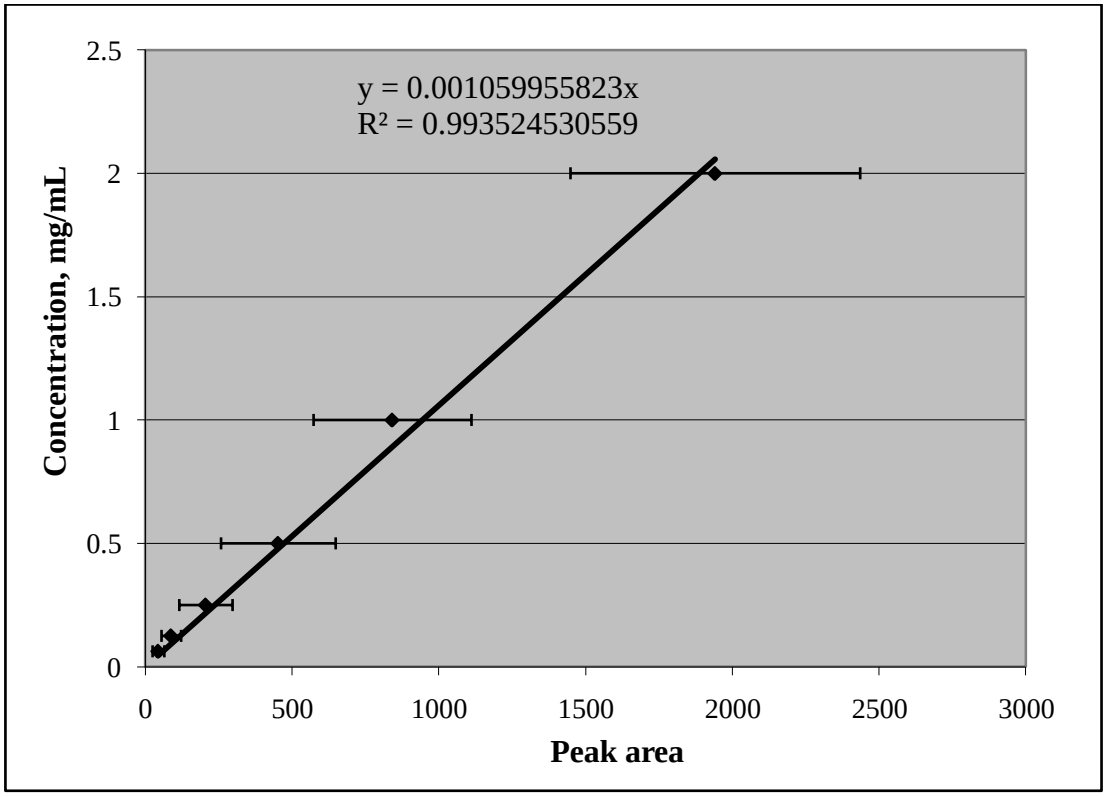


Figure 4.2: Standard curve for β-lag

The size of β -lag depending on the pH of the medium solution as summarized in Table 4.1. However, based on current result, direct correlation between the size of β –lag and the percentage of β -lag retained could not able to explain. At pH 2 and 3, most of the α-lac and β -lag were retained on the membrane. Less than 10 % were permeate. The α-lac permeate was slowly increase from pH 2 until achieved optimum value which are 80 % at pH 5. After pH 6, it reduced more than half, about 30 % of α-lac permeate. The similar pattern also was observed for β –lag in permeate side. In the retentate, pH 5 also retained less amount of α-lac. Based on the retentate percent, the best separation occurred at pH 5 which less than 2 % retained at retentate side and more that 80 % of α-lac permeate in the permeate side.

Table 4.1: Size of β–lactoglobulinon variable pH (Fee, et al. 2010).

pH	Structure	Size, kDa
< 3, > 8	Monomer	18.4
5.2 -7	Dimer	36.7
3.5 - 5.2	Octomer	140

The percentage of protein loss during the experiment was showed in Table 4.2. Two possible causes for the protein loss are : (1) protein adsorption to the membrane and clogged; (2) protein denaturation by shear stress caused by the circulation of the retentate stream at high velocities (Almecija, et al. 2006). The percentage of β–lag retaianed at pH 3 and 4 was high due to the formation of octomer structure of β–lag. The best pH for recover high percentage of β-lag on retetante side and α-lac on permeate side was determined at pH 5.

Table 4.2: Mass of α-lactalbumin and β-lactoglobulin for variable pH value at feed stream.

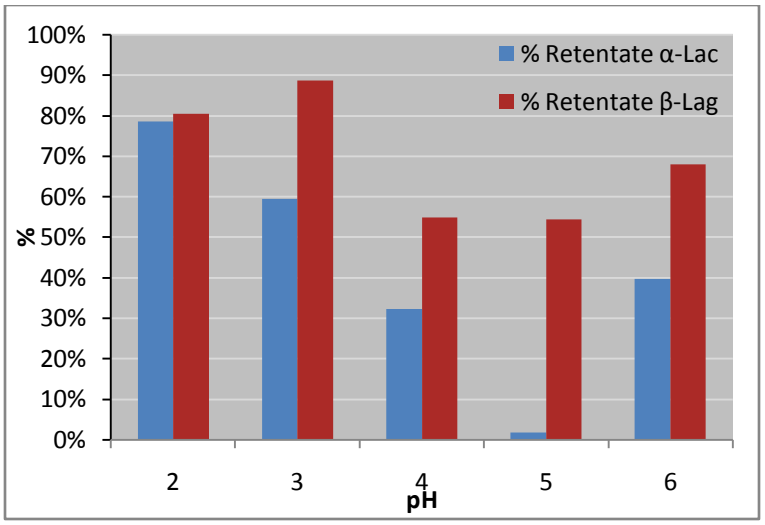
pH	Feed			
	Total mass, mg	Total area	%loss	
	β-Lag	α-Lac	β-Lag	α-Lac
2	373.59	118333.7	13%	18.91%
3	247.77	92957.9	5%	29.46%
4	384.32	126912.2	34%	32.87%
5	370.32	130884.0	7%	16.29%
6	371.60	127840.4	15%	27.43%

Table 4.3: Mass of α-lactalbumin and β-lactoglobulin for variable pH value at retentate stream.

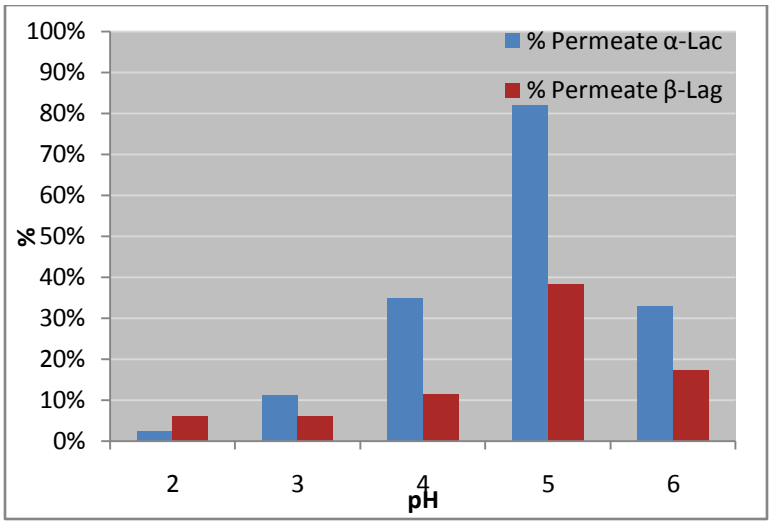
pH	Retentate			
	Mass, mg	Area	%retained	
	β-Lag	α-Lac	β-Lag	α-Lac
2	300.25	92888.66	80%	78.50%
3	219.66	55208.81	89%	59.39%
4	210.63	40863.35	55%	32.20%
5	201.36	2234.47	54%	1.71%
6	252.63	50662.56	68%	39.63%

Table 4.4: Mass of α-lactalbumin and β-lactoglobulin for variable pH value at permeate stream.

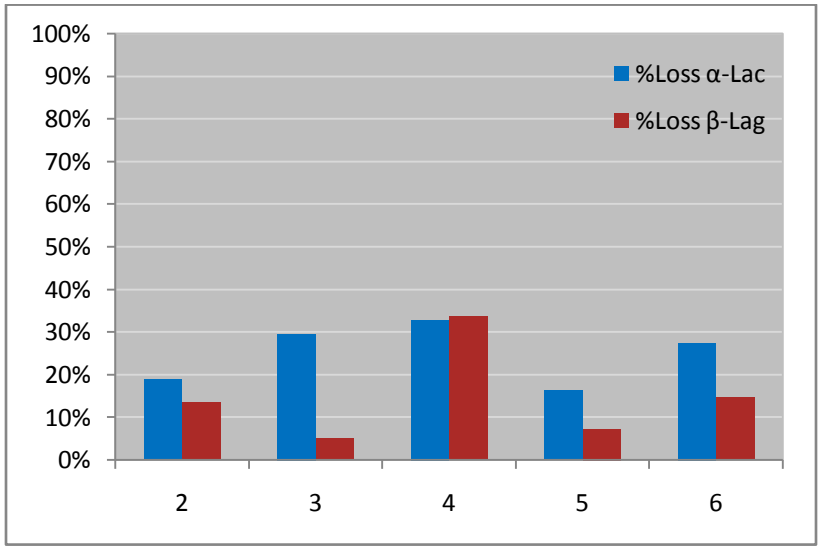
pH	Permeate			
	Mass, mg	Area	%permeate	
	β-Lag	α-Lac	β-Lag	α-Lac
2	23.00	3070.98	6%	2.60%
3	15.50	10360.71	6%	11.15%
4	44.10	44334.46	11%	34.93%
5	141.85	107325.84	38%	82.00%
6	64.70	42113.20	17%	32.94%



(a)



(b)



(c)

Figure 4.3: Percentage of α-lac and β–lag at all pH value; (a) Percent of retained; (b) Percent of permeate; (c) Percent of loss

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusions

HPTFF has a potential in separation of protein component that differ each other by less than 3 fold in size. In the current study, whey protein was fractionated by 30 kDa PES membrane at different pH. The best separation occurred at pH 5 which 80 % of α-lac permeate in the permeate side and less than 2 % α-lac retained at retentate side. However at this pH, there is still 38% β–lag was permeated. Enriched protein fraction from whey either at permeate or retentate side can be used in the specific application and had a higher value compare to its original mixture.

5.2 Recommendation

There a lot of parameter in HPTFF that can be study such as are ionic strength, pH, transmembrane pressure, feed flow rate and cross flow velocity. Each parameters should be carefully optimized in order to get higher protein fraction from HPTFF experiemnt.

In this study, 30 kDa PES membrane was used in the HPTFF, however another membrane with smaller MWCO also possible to be study especialy membrane with MWCO size near to the size of the α-lac and β–lag protein which is around 5 – 10 kDa. This will increse the selctivity between the protein to be separated. The arrangement of the membrane module in series or paralle will also have an effect on the membrane performance. This aspect should be investigated in the future on the fractionation of whey protein components.

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APPENDIX A

Experimental Data for Water Flux Recovery

Table A1: Viscosity correction factor

Temperature(°C)	Correction factor
25	0.89
26	0.871
27	0.851
28	0.833
29	0.815
30	0.798
31	0.781
32	0.765
33	0.749

Formula:

a) Flux in LMH (L/ h.m^2) $= \frac{permeate \;\; flux \;\; in \;\; mL/min}{\text{Cassette surface meters}}$

b) TMP (psig) $= \frac{(pressure \;\; retentate \;\; + pressure \;\; permeate \;\;)}{2}$

c) Flow rate, Q $= \frac{Volume \;, mL}{time}$

i. Provided Data:

a) Cassette surface area, A = 0.11 m^2

2. Calculation (First experiment: pH 2)

i. Water flux before run the sample

a. TMP = (7 + 3) / 2

= 5 psig

b. Flow rate = 100mL / 30.6 s

= 3.2679

$$= 3.2679 \left| \frac{\text{mL}}{\text{s}} \right| \frac{\text{60 s}}{\text{1 min}} \left| \frac{\text{60 min}}{\text{1 h}} \right| \frac{\text{1 L}}{\text{1000 mL}}$$

= 11.7647 L/h

c. Flux = 11.7647 L/h ÷ 0.11 m^2

= 106. 952 L/ h.m^2

d. Viscosity correction factor

Normalized at 29.8 ^oC:

106. 952 L/ h.m^2 x 0.8014 = 85.7112 LMH

Normalized at 5 psig:

85.7112 LMH / 5 psig = 17.1422 LMH/psig

ii. Water flux after run the sample

a. TMP = (7 + 3) / 2

= 5 psig

b. Flow rate = 100 mL ÷ 30.767 s

= 3.25 mL/sec

$$= 3.25 \left| \frac{\text{mL}}{\text{s}} \right| \frac{\text{60 s}}{\text{1min}} \left| \frac{\text{60 min}}{\text{1hr}} \right| \frac{\text{1L}}{\text{1000mL}}$$

= 11.7 L/h

c. Flux = 11.7 L/h ÷ 0.11 m^2

= 106.3714 L/ h.m^2

= 106.3714 LMH

d. Viscosity correction factor

Normalize at 29.7 ^{o}C :

106.3714 LMH x 0.8031 = 85.4268 LMH

Normalize at 5 psig:

85.4268LMH /5 = 17.0854 LMH/psig

iii. Water flux recovery:

= 85.4268 LMH / 85.7112 LMH

= 99.67%

Summary for Water flux recovery:

Table A2: Water flux recovery during experiment at TMP 5psig

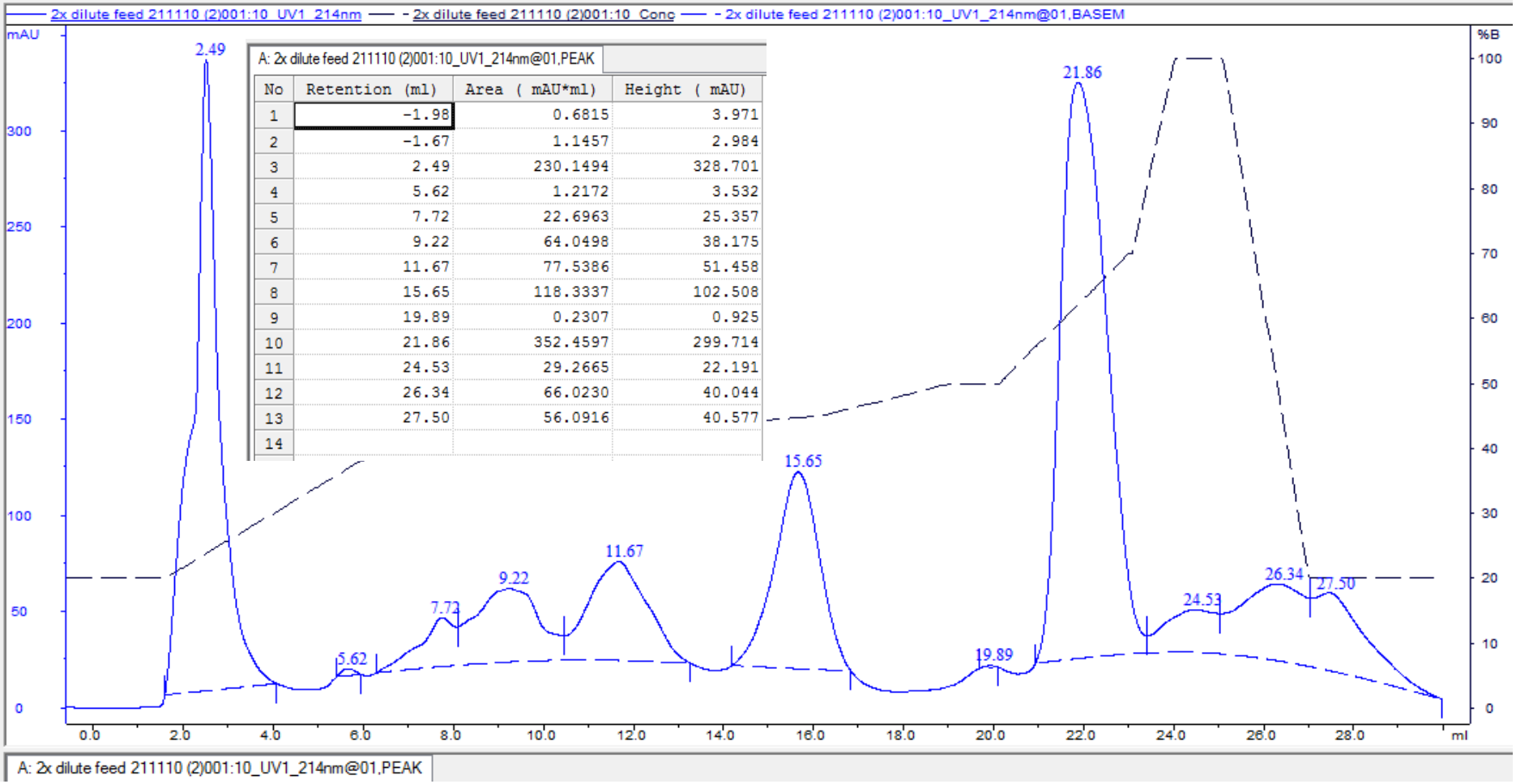
Experiment	**Water flux recovery**
Second (pH 3)	100%
Third (pH 4)	90%
Fourth (pH 5)	96%
fifth (pH 6)	97%

Table A3: Water flux recovery during experiment at TMP 15psig

Experiment	**Water flux recovery**
First (pH 2)	99%
Second (pH 3)	99%
Third (pH 4)	87%
Fourth (pH 5)	89%
fifth (pH 6)	90%

APPENDIX B

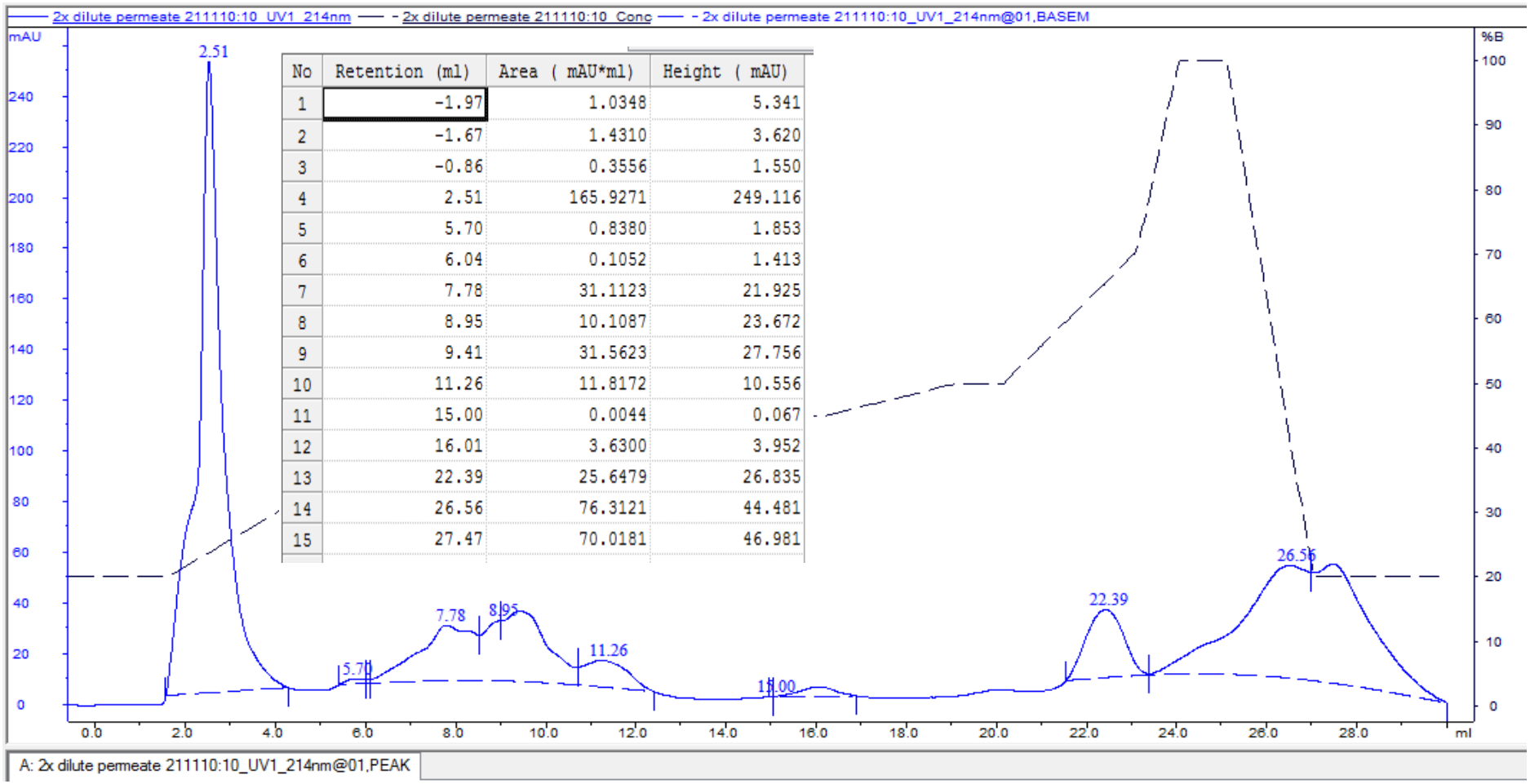
Results of Chromatogram RPC



A: 2x dilute feed 211110 (2)001:10_UV1_214nm@01,PEAK

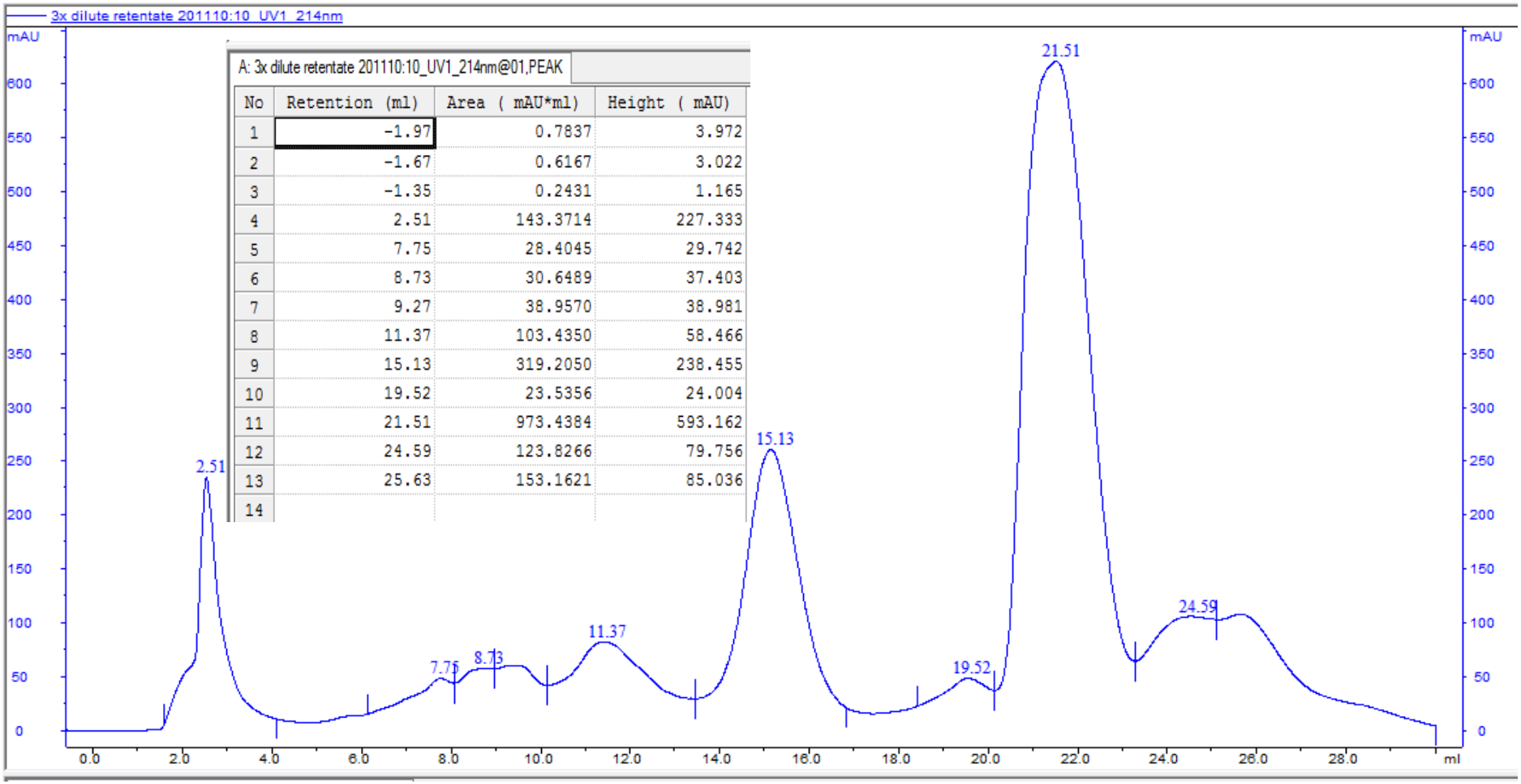
No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.98	0.6815	3.971
2	-1.67	1.1457	2.984
3	2.49	230.1494	328.701
4	5.62	1.2172	3.532
5	7.72	22.6963	25.357
6	9.22	64.0498	38.175
7	11.67	77.5386	51.458
8	15.65	118.3337	102.508
9	19.89	0.2307	0.925
10	21.86	352.4597	299.714
11	24.53	29.2665	22.191
12	26.34	66.0230	40.044
13	27.50	56.0916	40.577
14			

Figure B1: Chromatogram RPC at initial feed of pH 2



No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.97	1.0348	5.341
2	-1.67	1.4310	3.620
3	-0.86	0.3556	1.550
4	2.51	165.9271	249.116
5	5.70	0.8380	1.853
6	6.04	0.1052	1.413
7	7.78	31.1123	21.925
8	8.95	10.1087	23.672
9	9.41	31.5623	27.756
10	11.26	11.8172	10.556
11	15.00	0.0044	0.067
12	16.01	3.6300	3.952
13	22.39	25.6479	26.835
14	26.56	76.3121	44.481
15	27.47	70.0181	46.981

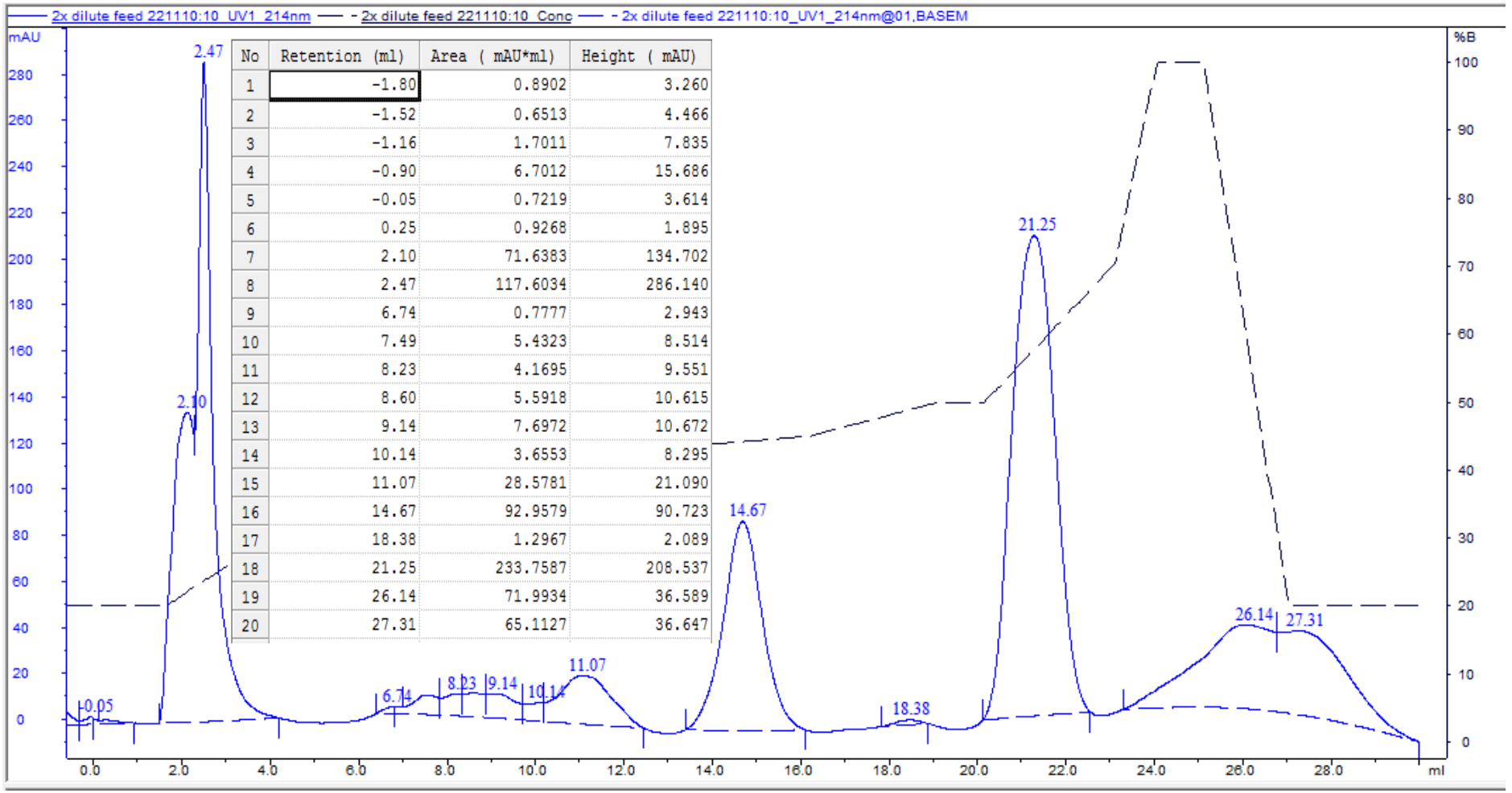
Figure B2: Chromatogram RPC at permeate line of pH 2



A: 3x dilute retentate 201110:10_UV1_214nm@01,PEAK

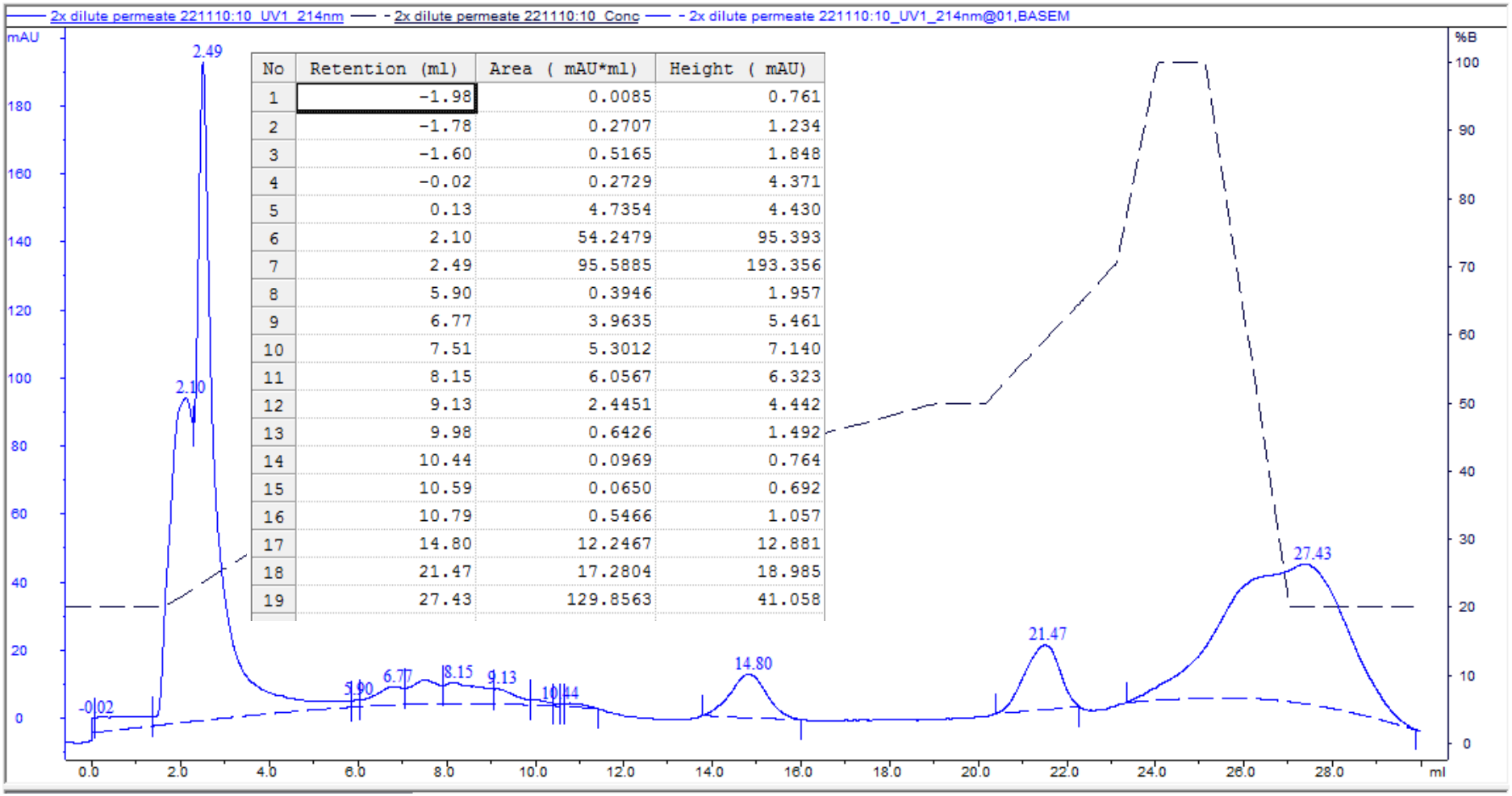
No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.97	0.7837	3.972
2	-1.67	0.6167	3.022
3	-1.35	0.2431	1.165
4	2.51	143.3714	227.333
5	7.75	28.4045	29.742
6	8.73	30.6489	37.403
7	9.27	38.9570	38.981
8	11.37	103.4350	58.466
9	15.13	319.2050	238.455
10	19.52	23.5356	24.004
11	21.51	973.4384	593.162
12	24.59	123.8266	79.756
13	25.63	153.1621	85.036
14			

Figure B3: Chromatogram RPC at retentate side of pH 2



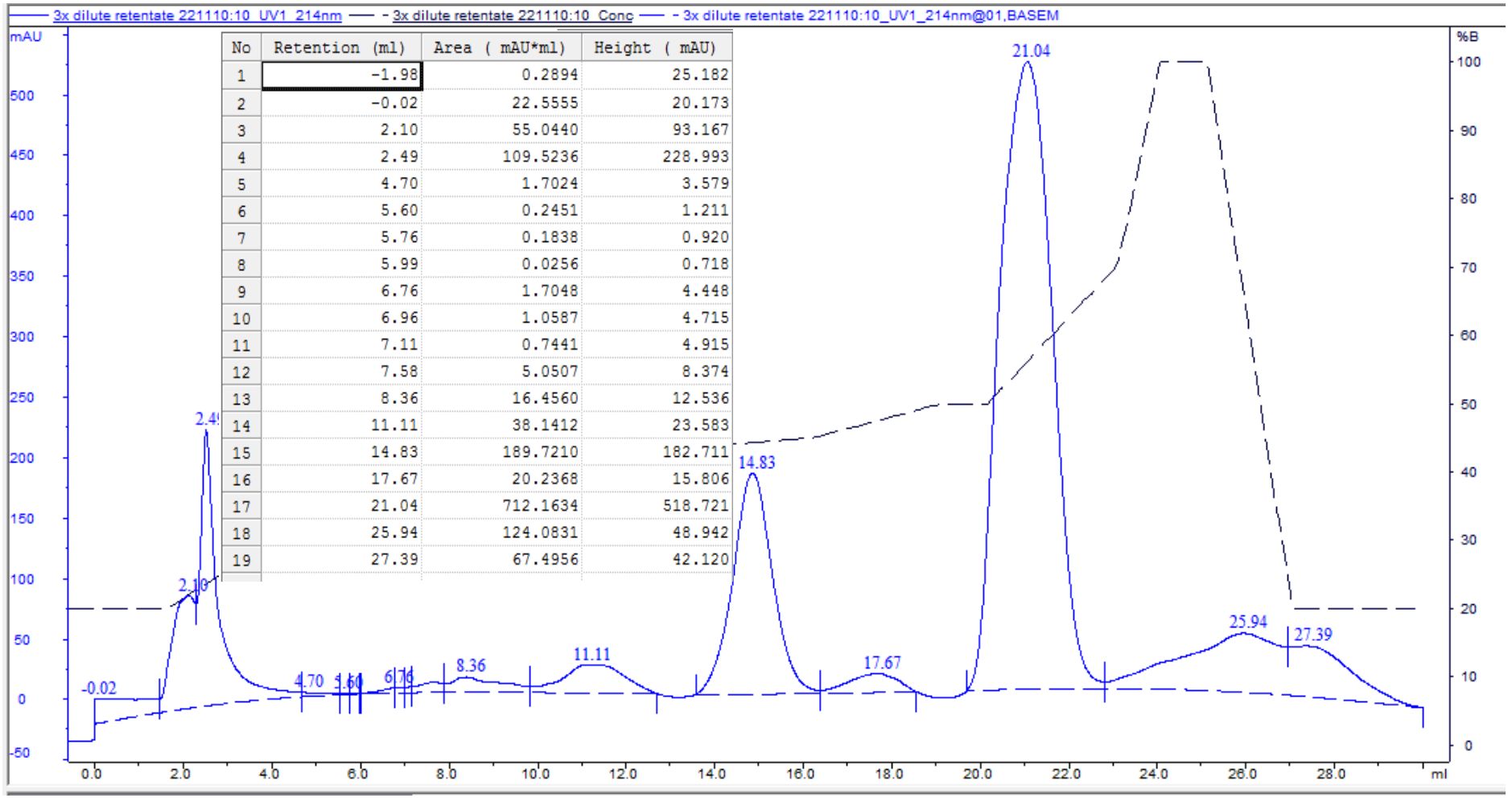
No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.80	0.8902	3.260
2	-1.52	0.6513	4.466
3	-1.16	1.7011	7.835
4	-0.90	6.7012	15.686
5	-0.05	0.7219	3.614
6	0.25	0.9268	1.895
7	2.10	71.6383	134.702
8	2.47	117.6034	286.140
9	6.74	0.7777	2.943
10	7.49	5.4323	8.514
11	8.23	4.1695	9.551
12	8.60	5.5918	10.615
13	9.14	7.6972	10.672
14	10.14	3.6553	8.295
15	11.07	28.5781	21.090
16	14.67	92.9579	90.723
17	18.38	1.2967	2.089
18	21.25	233.7587	208.537
19	26.14	71.9934	36.589
20	27.31	65.1127	36.647

Figure B4: Chromatogram RPC at initial feed of pH 3



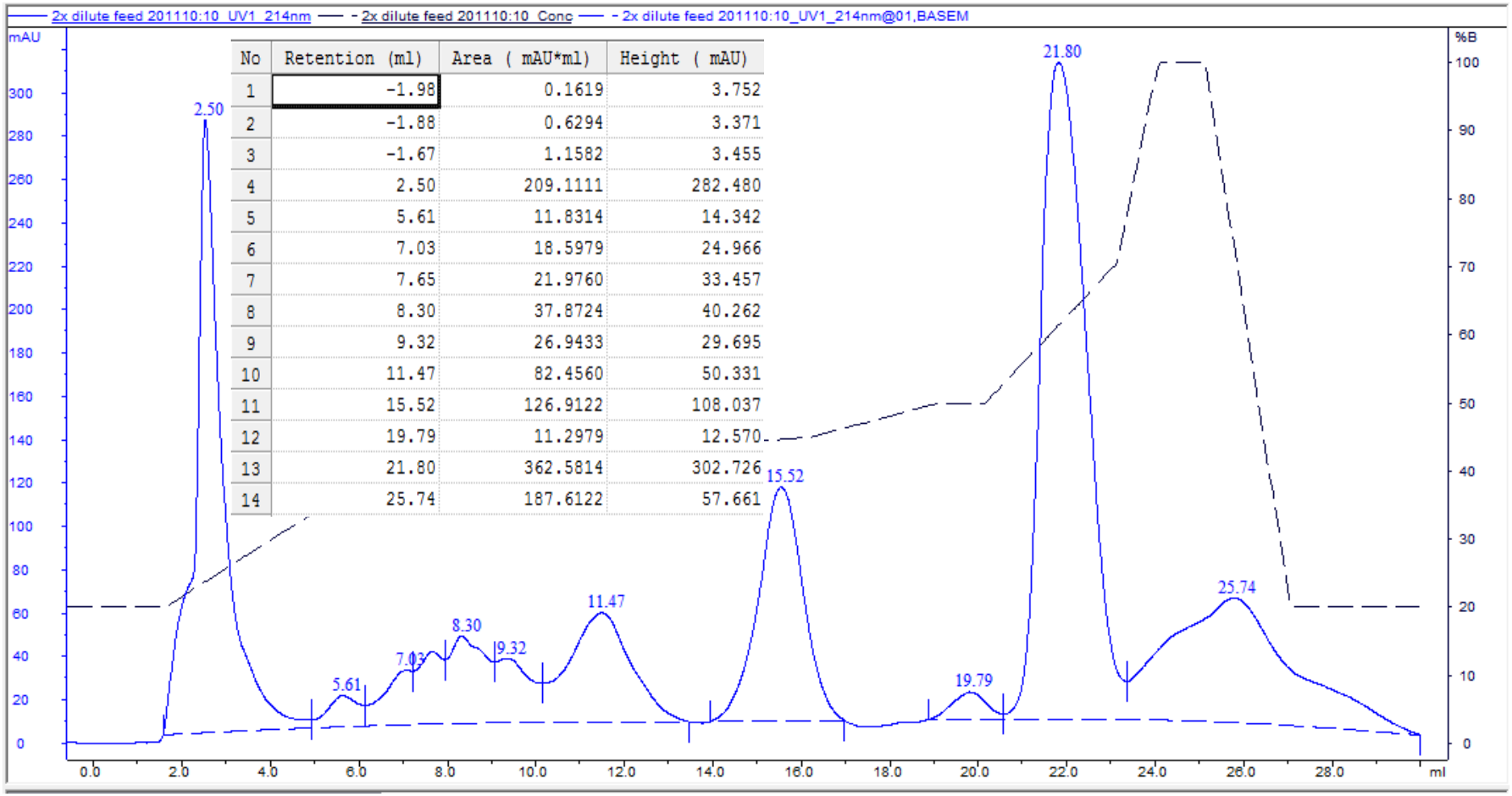
No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.98	0.0085	0.761
2	-1.78	0.2707	1.234
3	-1.60	0.5165	1.848
4	-0.02	0.2729	4.371
5	0.13	4.7354	4.430
6	2.10	54.2479	95.393
7	2.49	95.5885	193.356
8	5.90	0.3946	1.957
9	6.77	3.9635	5.461
10	7.51	5.3012	7.140
11	8.15	6.0567	6.323
12	9.13	2.4451	4.442
13	9.98	0.6426	1.492
14	10.44	0.0969	0.764
15	10.59	0.0650	0.692
16	10.79	0.5466	1.057
17	14.80	12.2467	12.881
18	21.47	17.2804	18.985
19	27.43	129.8563	41.058

Figure B5: Chromatogram RPC at permeate line of pH 3



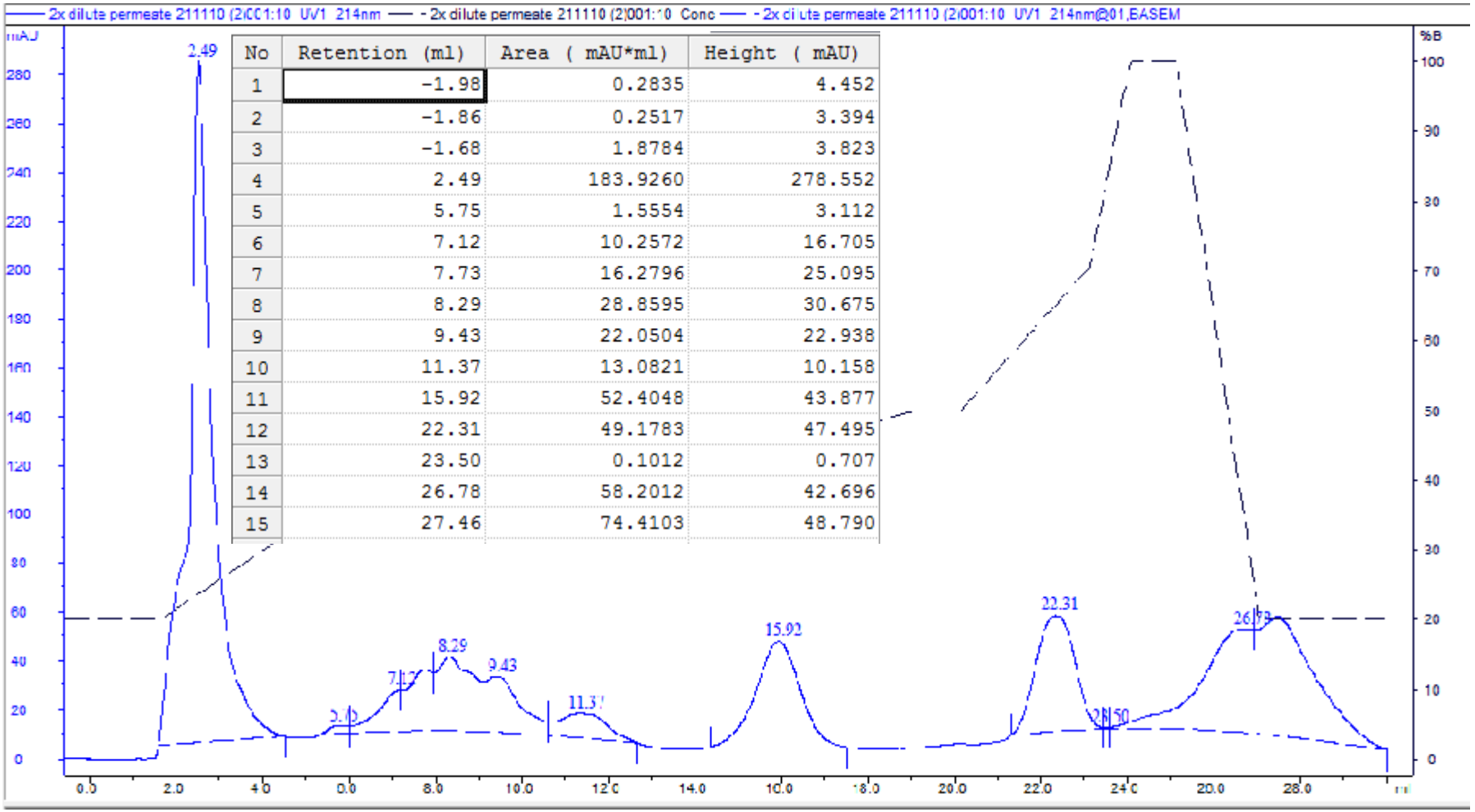
No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.98	0.2894	25.182
2	-0.02	22.5555	20.173
3	2.10	55.0440	93.167
4	2.49	109.5236	228.993
5	4.70	1.7024	3.579
6	5.60	0.2451	1.211
7	5.76	0.1838	0.920
8	5.99	0.0256	0.718
9	6.76	1.7048	4.448
10	6.96	1.0587	4.715
11	7.11	0.7441	4.915
12	7.58	5.0507	8.374
13	8.36	16.4560	12.536
14	11.11	38.1412	23.583
15	14.83	189.7210	182.711
16	17.67	20.2368	15.806
17	21.04	712.1634	518.721
18	25.94	124.0831	48.942
19	27.39	67.4956	42.120

Figure B6: Chromatogram RPC at retentate side of pH 3



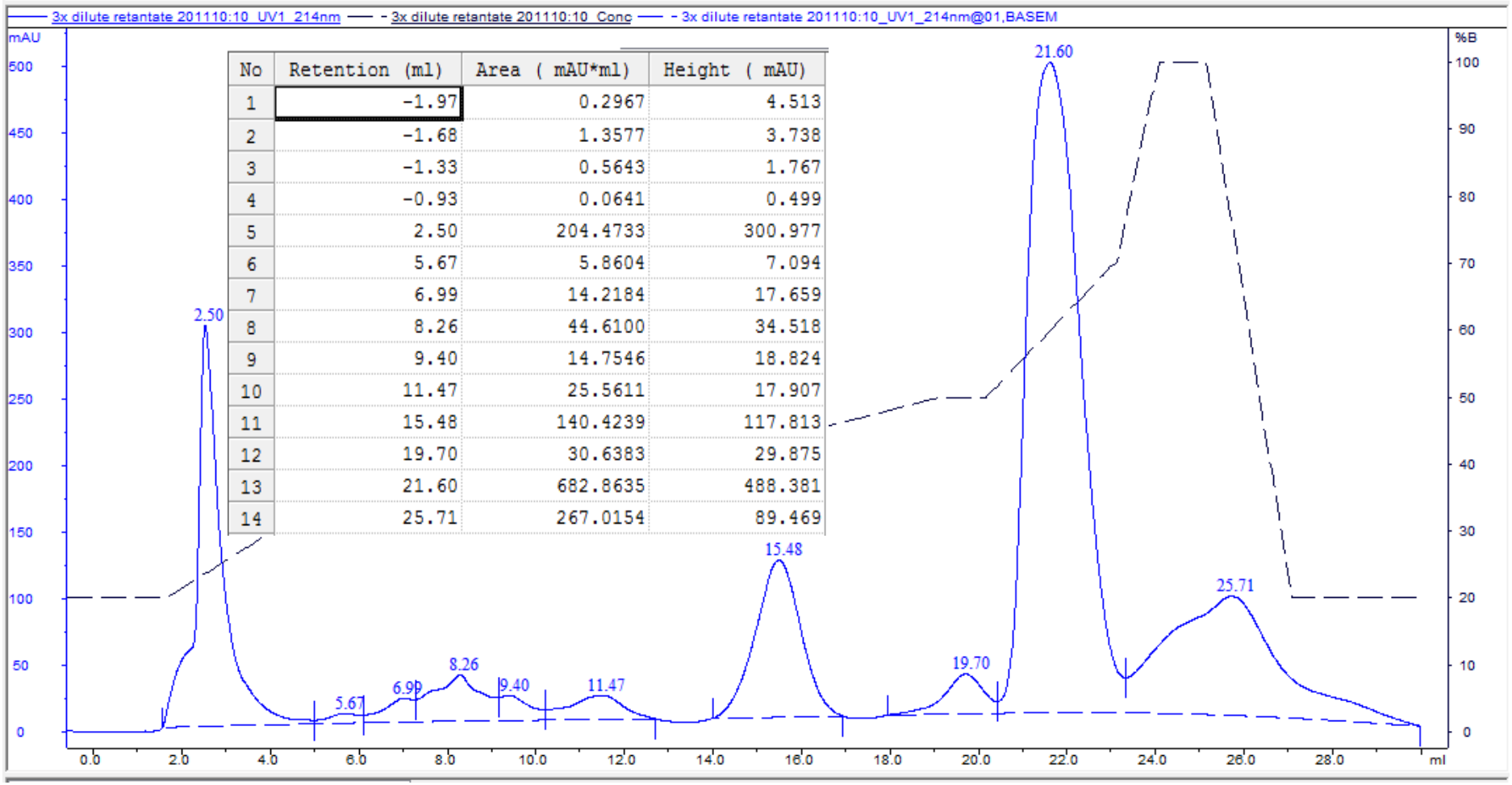
No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.98	0.1619	3.752
2	-1.88	0.6294	3.371
3	-1.67	1.1582	3.455
4	2.50	209.1111	282.480
5	5.61	11.8314	14.342
6	7.03	18.5979	24.966
7	7.65	21.9760	33.457
8	8.30	37.8724	40.262
9	9.32	26.9433	29.695
10	11.47	82.4560	50.331
11	15.52	126.9122	108.037
12	19.79	11.2979	12.570
13	21.80	362.5814	302.726
14	25.74	187.6122	57.661

Figure B7: Chromatogram RPC at initial feed of pH 4



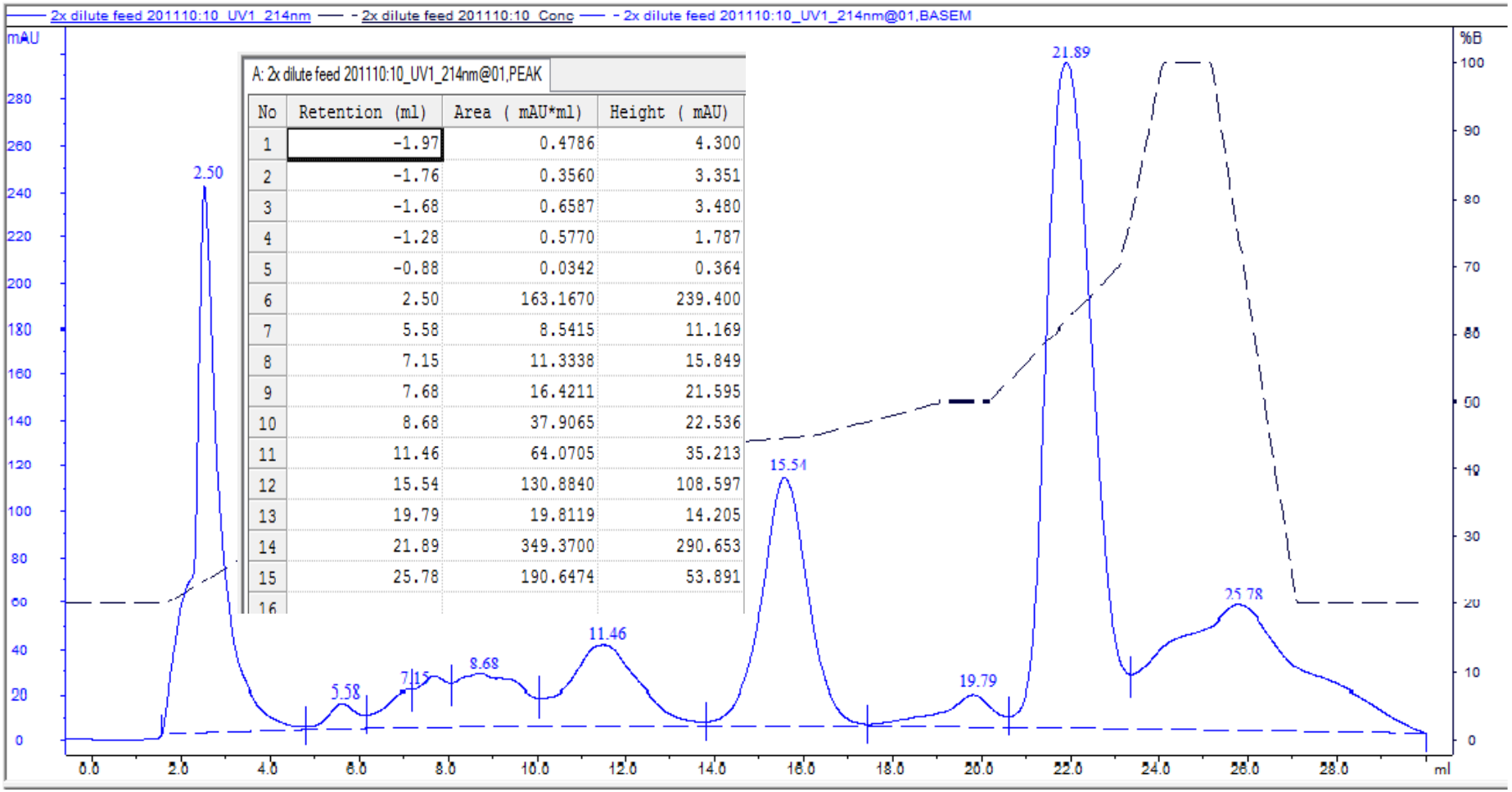
No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.98	0.2835	4.452
2	-1.86	0.2517	3.394
3	-1.68	1.8784	3.823
4	2.49	183.9260	278.552
5	5.75	1.5554	3.112
6	7.12	10.2572	16.705
7	7.73	16.2796	25.095
8	8.29	28.8595	30.675
9	9.43	22.0504	22.938
10	11.37	13.0821	10.158
11	15.92	52.4048	43.877
12	22.31	49.1783	47.495
13	23.50	0.1012	0.707
14	26.78	58.2012	42.696
15	27.46	74.4103	48.790

Figure B8: Chromatogram RPC at permeate line of pH 4



No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.97	0.2967	4.513
2	-1.68	1.3577	3.738
3	-1.33	0.5643	1.767
4	-0.93	0.0641	0.499
5	2.50	204.4733	300.977
6	5.67	5.8604	7.094
7	6.99	14.2184	17.659
8	8.26	44.6100	34.518
9	9.40	14.7546	18.824
10	11.47	25.5611	17.907
11	15.48	140.4239	117.813
12	19.70	30.6383	29.875
13	21.60	682.8635	488.381
14	25.71	267.0154	89.469

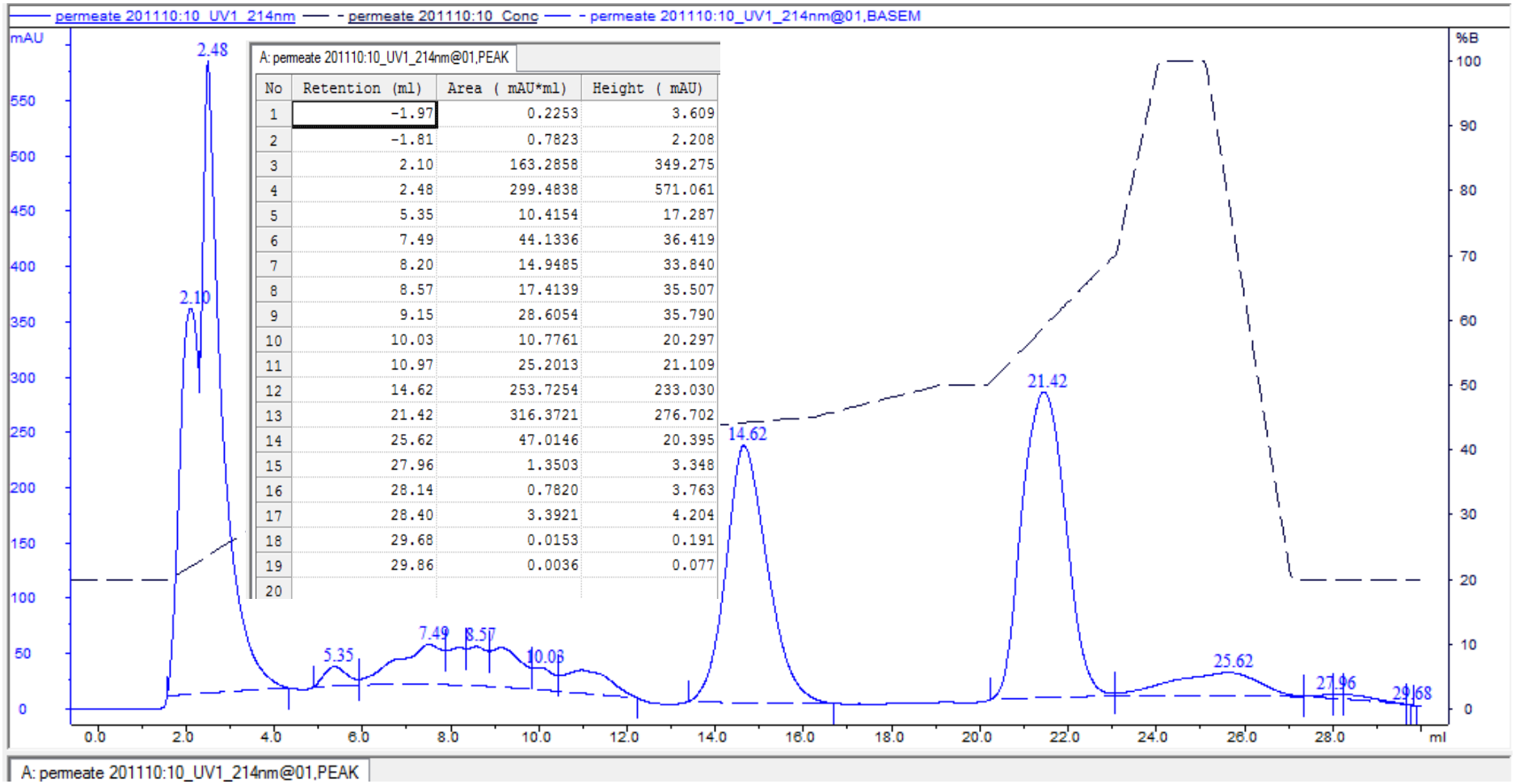
Figure B9: Chromatogram RPC at retentate side of pH 4



A: 2x dilute feed 201110:10_UV1_214nm@01,PEAK

No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.97	0.4786	4.300
2	-1.76	0.3560	3.351
3	-1.68	0.6587	3.480
4	-1.28	0.5770	1.787
5	-0.88	0.0342	0.364
6	2.50	163.1670	239.400
7	5.58	8.5415	11.169
8	7.15	11.3338	15.849
9	7.68	16.4211	21.595
10	8.68	37.9065	22.536
11	11.46	64.0705	35.213
12	15.54	130.8840	108.597
13	19.79	19.8119	14.205
14	21.89	349.3700	290.653
15	25.78	190.6474	53.891
16			

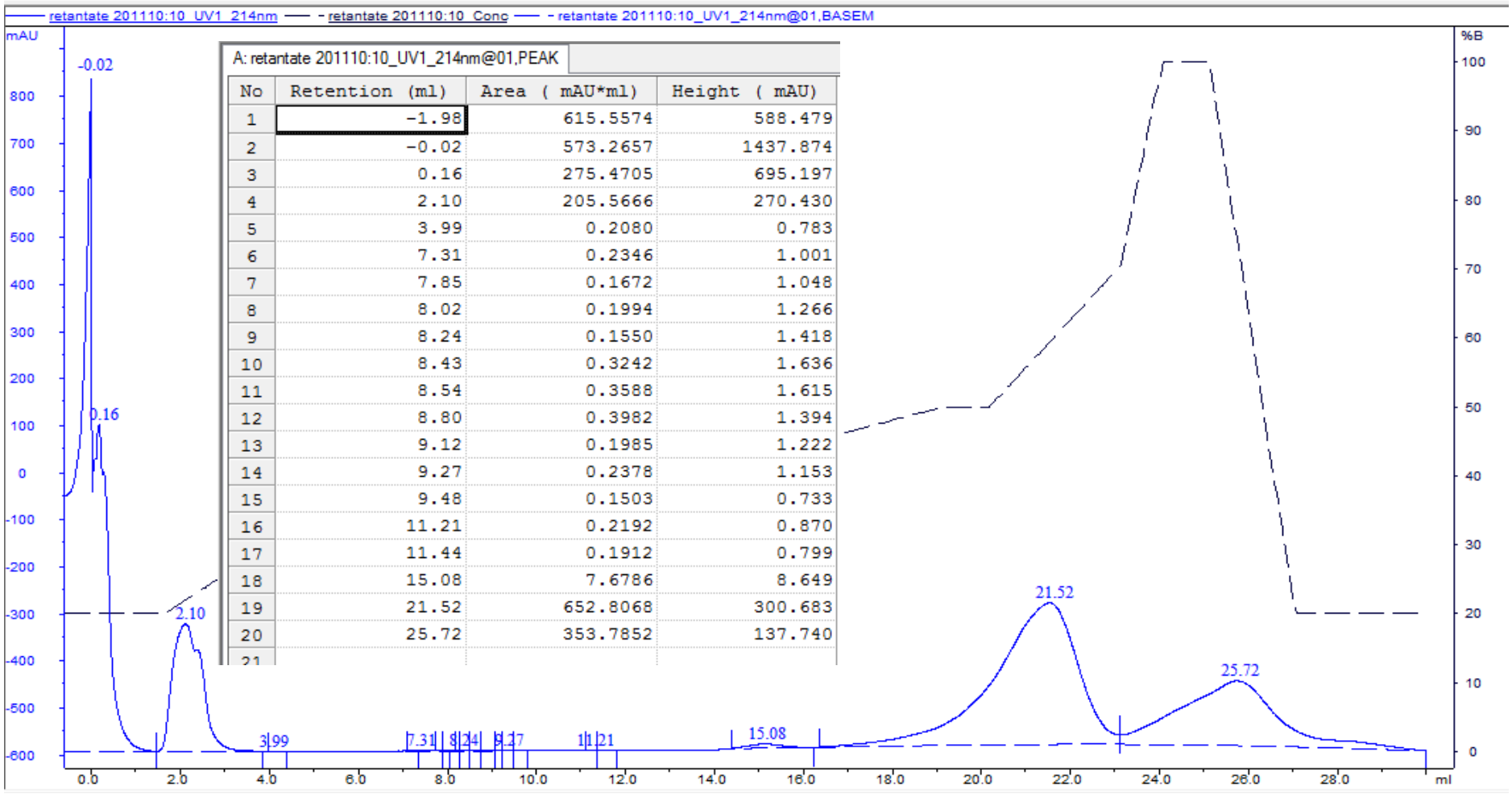
Figure B10: Chromatogram RPC at initial feed of pH 5



A: permeate 201110:10_UV1_214nm@01,PEAK

No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.97	0.2253	3.609
2	-1.81	0.7823	2.208
3	2.10	163.2858	349.275
4	2.48	299.4838	571.061
5	5.35	10.4154	17.287
6	7.49	44.1336	36.419
7	8.20	14.9485	33.840
8	8.57	17.4139	35.507
9	9.15	28.6054	35.790
10	10.03	10.7761	20.297
11	10.97	25.2013	21.109
12	14.62	253.7254	233.030
13	21.42	316.3721	276.702
14	25.62	47.0146	20.395
15	27.96	1.3503	3.348
16	28.14	0.7820	3.763
17	28.40	3.3921	4.204
18	29.68	0.0153	0.191
19	29.86	0.0036	0.077
20			

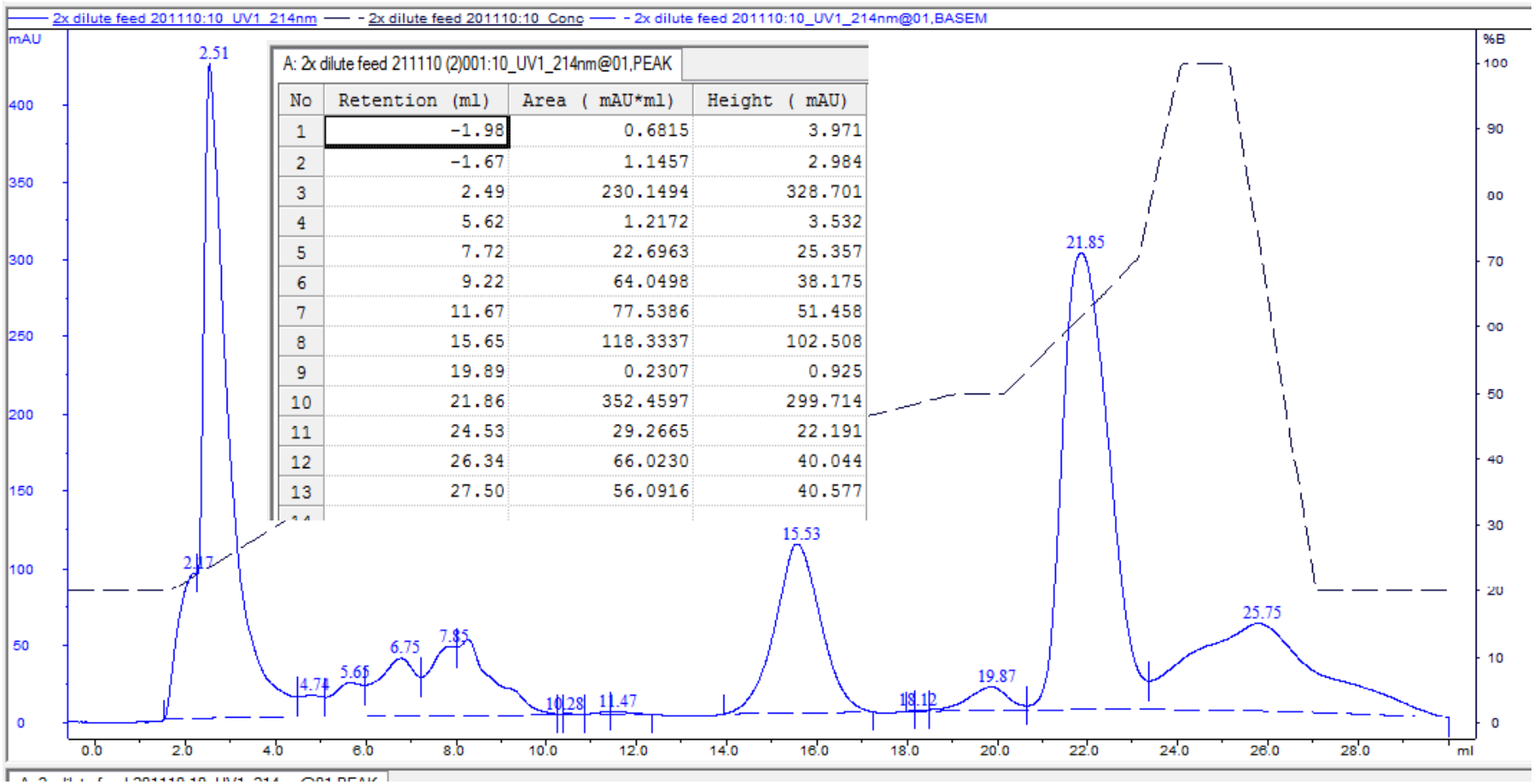
Figure B11: Chromatogram RPC at permeate line of pH 5



A: retantate 201110:10_UV1_214nm@01,PEAK

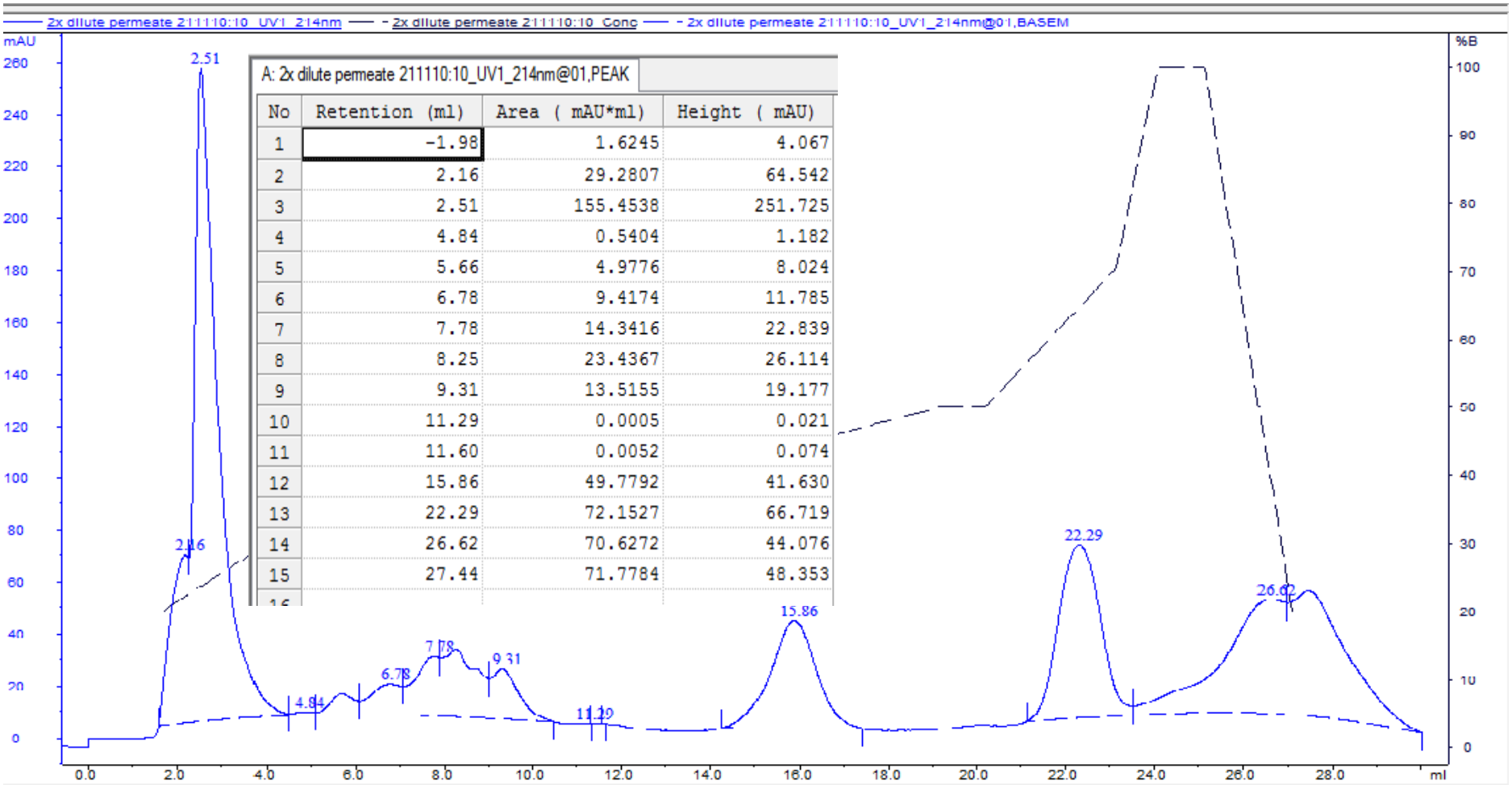
No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.98	615.5574	588.479
2	-0.02	573.2657	1437.874
3	0.16	275.4705	695.197
4	2.10	205.5666	270.430
5	3.99	0.2080	0.783
6	7.31	0.2346	1.001
7	7.85	0.1672	1.048
8	8.02	0.1994	1.266
9	8.24	0.1550	1.418
10	8.43	0.3242	1.636
11	8.54	0.3588	1.615
12	8.80	0.3982	1.394
13	9.12	0.1985	1.222
14	9.27	0.2378	1.153
15	9.48	0.1503	0.733
16	11.21	0.2192	0.870
17	11.44	0.1912	0.799
18	15.08	7.6786	8.649
19	21.52	652.8068	300.683
20	25.72	353.7852	137.740
21			

Figure B12: Chromatogram RPC at retentate side of pH 5



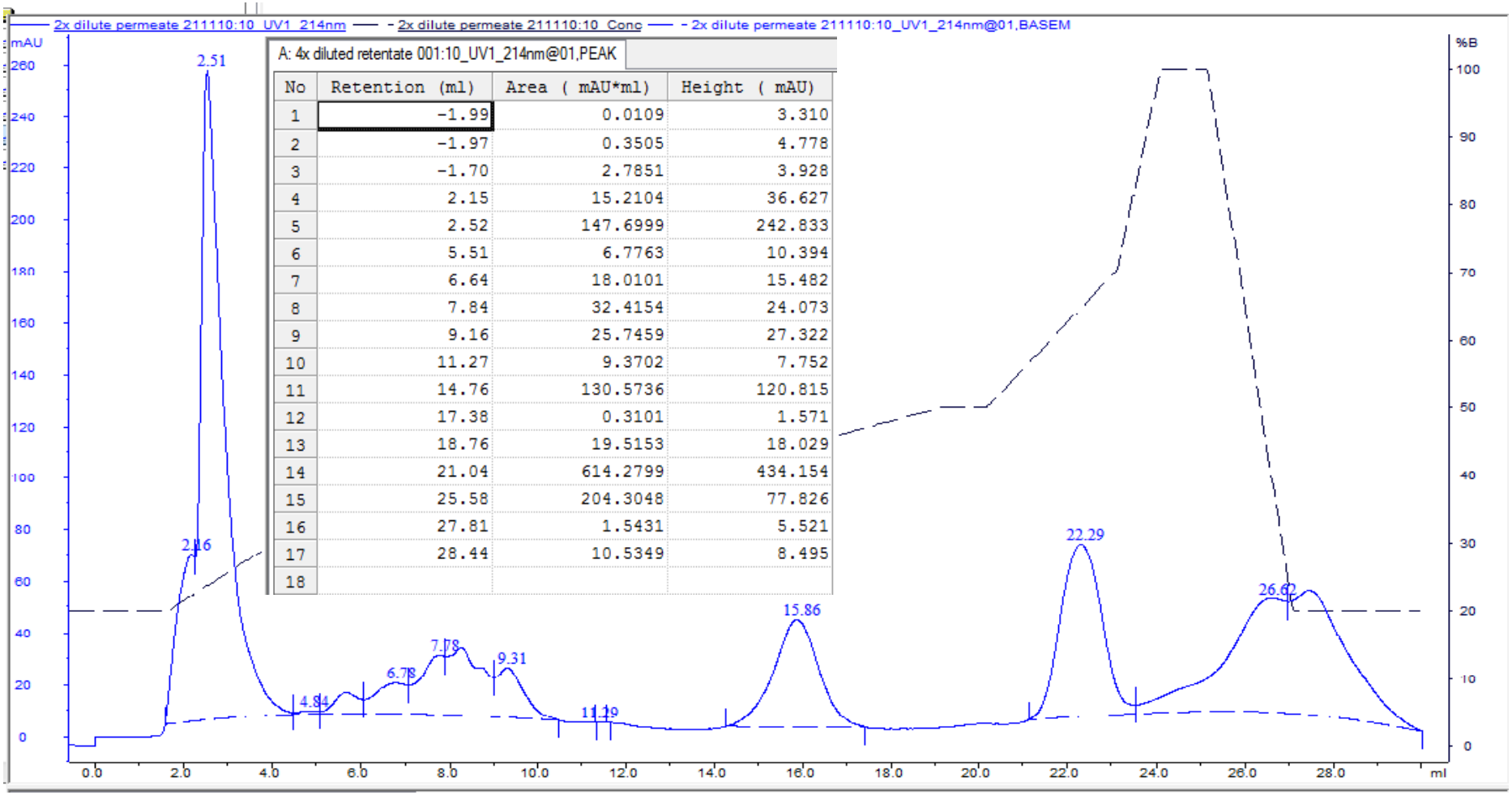
No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.98	0.6815	3.971
2	-1.67	1.1457	2.984
3	2.49	230.1494	328.701
4	5.62	1.2172	3.532
5	7.72	22.6963	25.357
6	9.22	64.0498	38.175
7	11.67	77.5386	51.458
8	15.65	118.3337	102.508
9	19.89	0.2307	0.925
10	21.86	352.4597	299.714
11	24.53	29.2665	22.191
12	26.34	66.0230	40.044
13	27.50	56.0916	40.577

Figure B13: Chromatogram RPC at initial feed of pH 6



No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.98	1.6245	4.067
2	2.16	29.2807	64.542
3	2.51	155.4538	251.725
4	4.84	0.5404	1.182
5	5.66	4.9776	8.024
6	6.78	9.4174	11.785
7	7.78	14.3416	22.839
8	8.25	23.4367	26.114
9	9.31	13.5155	19.177
10	11.29	0.0005	0.021
11	11.60	0.0052	0.074
12	15.86	49.7792	41.630
13	22.29	72.1527	66.719
14	26.62	70.6272	44.076
15	27.44	71.7784	48.353

Figure B14: Chromatogram RPC at permeate line of pH 6



A: 4x diluted retentate 001:10_UV1_214nm@01,PEAK

No	Retention (ml)	Area (mAU*ml)	Height (mAU)
1	-1.99	0.0109	3.310
2	-1.97	0.3505	4.778
3	-1.70	2.7851	3.928
4	2.15	15.2104	36.627
5	2.52	147.6999	242.833
6	5.51	6.7763	10.394
7	6.64	18.0101	15.482
8	7.84	32.4154	24.073
9	9.16	25.7459	27.322
10	11.27	9.3702	7.752
11	14.76	130.5736	120.815
12	17.38	0.3101	1.571
13	18.76	19.5153	18.029
14	21.04	614.2799	434.154
15	25.58	204.3048	77.826
16	27.81	1.5431	5.521
17	28.44	10.5349	8.495
18			

Figure B15: Chromatogram RPC at retentate side of pH 6