An Investigation into the Large Scale Separation of Equine Blood Plasma

A thesis submitted in fulfilment of the requirements for the Degree of Master of Engineering in Chemical and Process Engineering at the University of Canterbury

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Summary

The large-scale separation of proteins from equine blood plasma was investigated, as an attempt to create a process that would separate multiple proteins. This contrasts to the current state, where methods target a single protein at a time. Ion exchange chromatography and ethanol precipitation were both used as techniques, due to their use in human blood plasma fractionation and their versatility as downstream separation methods. The bases of ethanol precipitation and the underlying percentages used were investigated, along with techniques specifically designed to remove albumin. Ion exchange fractions were based on those used in the separation of bovine plasma, with a specific focus on a 20-fold scale-up in column volume.

The results indicate that ion exchange chromatography is a feasible separation method. Separation was able to be carried out, with immunoglobulins in particular easily obtainable from this techniques. The overall profile broadly matched that of bovine blood plasma. However, there was considerable amounts of buffer required, along with significantly increased column pressure upon scale-up. These drawbacks can be overcome, but this may restrict the applicability of the process as an initial step due to the expense of buffers and columns that can withstand high pressures. Ethanol precipitations, on the other hand, proved effective in separation and would be easier to scale up for an industrial process, although requiring flame-proof equipment and facilities at large scale. While issues were found with the techniques used to identify the proteins, it was clear that in particular, albumin separation methods from the bovine plasma industry would easily be adaptable to equine blood.

From this, a complete process was obtained for use in both existing and new plants. With only three sub-categories of proteins commercialised from equine plasma, the scope for minimising waste protein was severely restricted. In addition, many of the methods of separation are quite expensive, further restricting the number of proteins that could be obtained. However, there is the ability to reduce waste through multiple protein production without major capital investment.

Acknowledgments

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List of Symbols and Abbreviations

BSA – bovine serum albumin

eCG – equine chorionic gonadotropin

ESA – equine serum albumin

$F(ab)_2$ – fragment antigen binding

HCV – Hepatitis C virus

HETP – Height equivalent of the theoretical plate

HIC – hydrophobic interactions chromatography

HSA – human serum albumin

IEC – ion exchange chromatography

Ig – immunoglobulin

IgG – immunoglobulin class G

pI – isoelectric point

Tris – Tris(hydroxymethyl)aminomethane

D_f – final column diameter (m)

D_i – initial column diameter (m)

d_p – particle diameter (m)

L – column length (m)

L_f – final column length (m)

L_i – initial column length (m)

V_f – final sample volume (m^3)

V_i – initial sample volume (m^3)

ΔP – pressure drop (Pa)

ε – voidage

u_0 – superficial velocity

μ - viscosity (unit)

Chapter 1: Introduction

Blood plasma, the mixture of dissolved proteins in blood excluding cells, has received increased scientific attention over the last century [1-3]. While this is partially due to the increased knowledge of the medical properties inherent to it, much is also owed to work aimed at separating, identifying and characterising the various proteins within it. This has resulted in new treatments for certain ailments, such as haemophilia, and created a large industry based on separating certain proteins [4, 5].

Knowledge of human blood plasma has been an understandable priority; meaning similar understanding of other mammalian systems has lagged behind, with far more basic studies undertaken. This is partially because of the decreased economic incentives of obtaining the various corresponding proteins. However, this has not stopped large scale production extending to the blood plasma of various animals [6].

That is not to say that these plasmas have not received any academic attention. Bovine plasma is the most explored, partially due to the quantities available and that proteins such as bovine serum albumin (BSA) have research applications in cell culture and as protein standards [7]. Equine plasma has been an additional target [8-10]; however this has been to a far lesser extent. This is despite the extraction of proteins from equine plasma such as immunoglobulin fragments, as many extraction processes are only designed to extract one type of protein [11, 12]. The ultimate result of this is a patchwork of different processes abounding, many of which cannot be used in combination. Meanwhile, there seems to be little focus on a comprehensive separation strategy.

An example of this is equine chorionic gonadotropin, which is generally produced using metaphosphoric acid precipitation [13]. This technique uses harsh conditions at around pH 3 which can result in protein deactivation in other proteins, lowering their commercial value. This indicates that in this, and other similar processes [10], there are no attempts being made to obtain as many products as possible, resulting in a considerable amount of waste.

For these reasons, this thesis focused on equine plasma and the proteins within it. Some identification of the plasma proteins was undertaken, but a greater priority was on the separation processes. The main objective was to determine which equine plasma proteins could be separated using normal industrial techniques. The additional constraint of ensuring as many proteins as possible would remain active was also applied.

1.1 Blood contents

While sequencing and structural studies have been carried out on many equine proteins [14-16], these have mostly focused on what proteins are present, rather than the respective quantities of these. Some studies have looked at quantities of albumin to globulins [17, 18] with the intent of determining the ratio between the two. Proteins that exist in smaller quantities have received far less attention, however.

Again, knowledge about human plasma is more advanced in this regard, out of medical necessity. Far more factors have been identified and quantified, as there are greater medical and academic interests in identifying these. Of particular interest have been clotting factors, which are used as haemophilia treatments [5]. Table 1.1 gives an outline of the typical composition of human blood.

Table 1.1: The composition of human blood. Produced with data from [19] and [20]

Blood Component	Sub-Component	Sub-amount (%)	Amount (%)
Cells			45
	Red Blood	44.9	
	White Blood and Platelets	0.10	
Plasma			55
	Water	49.5	
	Albumin	2.8	
	Immunoglobulins	1.0	
	Fibrinogen	0.2	
	Transferrin	0.3	
	Alpha-2-Macroglobulin	0.2	
	Alpha -1-Antitrypsin	0.2	
	Other	0.8	

While Table 1.1 relates to human blood plasma specifically, the results of the equine albumin/globulin ratios indicate these figures are accurate for horse plasma [17]. What is particularly noticeable is the extent to which these two protein families dominate the total protein mass. This means it can be difficult to determine proteins that exist in lower quantities. Exacerbating this is the natural variance between individuals, which can be of orders of magnitude in some proteins that exist in smaller concentrations [21]. Despite this, there have been some efforts to determine the quantities of similar proteins in bovine serum [22, 23].

1.2 Industrial separation methods

A number of separation methods exist within the blood plasma industries. There is a clear split in the objectives of these. For most mammalian systems, the focus tends to be on extracting a single protein. While similar systems exist for human plasma, there is a much greater emphasis on fractionating the entire proteome, due to the higher value of individual proteins, as can be seen in modern studies [4, 5].

There are three main stages to a separation; capture, intermediate processing and polishing. Capture involves ensuring the protein of interest is present, intermediate processing diminishes the presence of other proteins and polishing ensures the isolated protein is as pure as necessary. Figure 1.1 illustrates the way this may work in practice. Each of the following processes is designed to fit into at least one of these stages, with some having uses in all three.

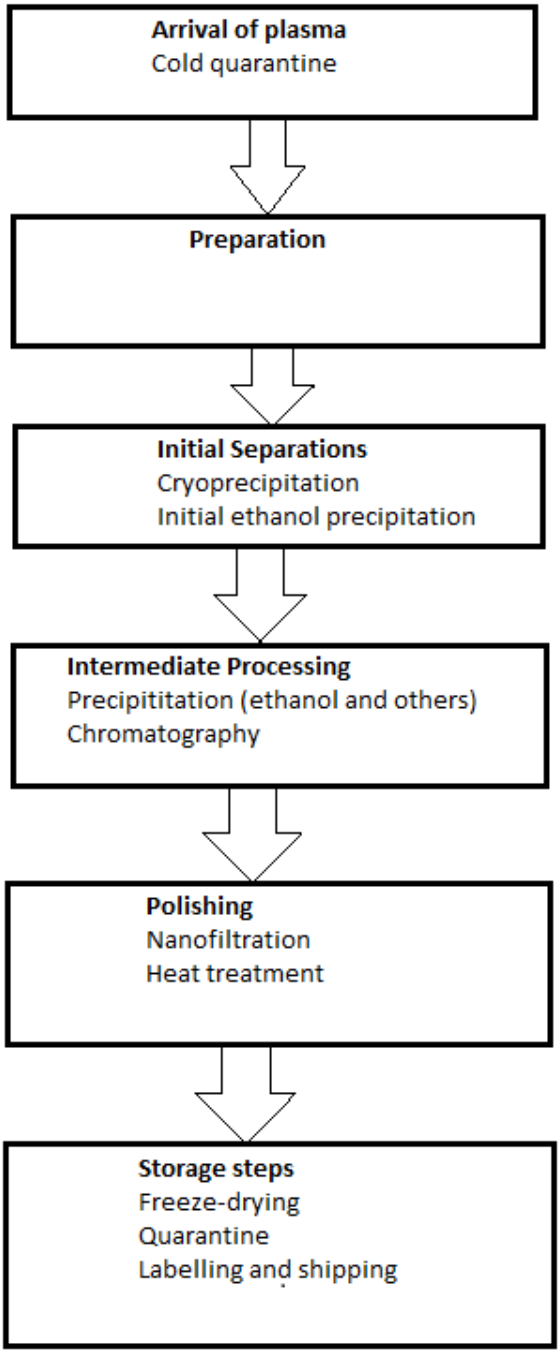

Figure 1.1: A typical broad separation scheme in practice for blood plasma. Based on data from [5]

Cryo-precipitation is the first step in many human plasma processing steps. The reason for this is that the cryo-precipitant is merely the precipitants from frozen plasma centrifuged out, and most plasma is stored and transported frozen to retain activity. This makes cryo-precipitation extremely easy to carry out initially. While investigations have been carried out on human plasma [24], the products obtained are mostly haemophilia factors, and are thus not considered practical in the equine industry.

Cohn fractionation was the first major method used within the industry, being developed during the Second World War as a method to separate albumin for medical use [25]. This method involves the addition of ethanol at varying concentrations. Precipitations are carried out at low temperatures, with the supernatant being exposed to the next ethanol step. The final supernatant contains almost pure albumin, although it is common for previous steps to undergo processing to increase the albumin yield.

Despite its age, this method is still used consistently in modern plasma fractionation. While it was originally a stand-alone method, it has now also been adapted to fit as a polishing stage for proteins such as immunoglobulins [26-28]. Other adaptions with different concentrations of ethanol and the addition of chromatography have been also used in an attempt to improve the process [29, 30].

Similar precipitation methods have been used as well, albeit far less extensively and generally in the extraction of particular proteins [31]. Occasionally, stand-alone ethanol precipitations and different pHs have been used. Likewise, different chemicals, such as ammonium sulphate and caprylic acid, are reasonably common precipitants.

During the 1970s and 1980s, however, liquid chromatography began to be used more commonly [32]. Chromatography is based on moving a mobile phase – in this case the blood plasma – through a stationary phase. The stationary phase is generally made up of a number of beads, which have some property designed to assist in separation. For instance, ion exchange chromatography has a number of ions bound to the beads, attracting proteins based on charge.

Initially, chromatography proved difficult to undertake, due to problems in ensuring a consistent bed, and thus good flow channels. However, there has been a development of more consistent beds, along with stationary phases becoming easier to produce [33]. This has eventually provided a powerful tool capable of processing large volumes, while also allowing different means of separation. Today, chromatography has become the backbone of plasma fractionation, with anion exchange being the most used form.

Less glamorous methods have also seen extensive use. Filtration is used both to separate cell matter and as a final polishing stage for certain proteins [34, 35]. Because of its antiquity and relatively simple separation principle, less attention has been focused on this method. However, it can cope with much larger volumes of plasma than many other methods, and plays a key role in product safety by removing viral elements that can infect blood.

Likewise, centrifugation is a key part of many separation studies, but is mostly used as a method of separation that is used to clarify another method, such as precipitation. For instance, ethanol separations often use centrifugation to separate out the precipitate. This is generally considered part

of a single separation step, for instance to separate a precipitated protein from the main plasma body. This means that little academic interest is generally devoted to it as an independent method [36].

1.3 The aims of this thesis

This thesis demonstrates a method to separate fractions from equine plasma. However, unlike previous research, there is an emphasis on ensuring that multiple fractions remain collectable. This means that harsh conditions, such as low pH, were avoided.

In addition, large-scale separation should be an achievable goal from this method. While many techniques exist that are proven on the small scale, these are often far too expensive to carry out at an industrial level, for instance electrophoresis. Thus, methods were only chosen if there was a realistic expectation that large volumes could be separated via this method.

New technologies provided an interesting dilemma. On one hand, many provide the means for separation that would fulfil most of the other requirements. This is weighted against the factors that many, by definition, are less proven at the large scale. Many are also currently unused in industry, meaning that introducing them would entail significant expense. For this reason, and that equine plasma products generally compete by being fairly cheap, established technologies were used as much as possible.

Furthermore, many techniques that cannot be used to separate multiple fractions, for instance affinity chromatography, were ignored. While indisputably helpful, these are better used as polishing techniques once the bulk of protein fractions have been removed. Techniques that are adaptable to a wide range of proteins were instead chosen. The intent of this was to allow any method designed to separate individual proteins to be added at the end to the appropriate fraction.

1.4 Scope and organisation of thesis

Chapter 2 of this thesis examines the literature based on the various methods of separation and blood plasmas in existence, trying to determine what methods are in existence already and could be used. Chapters 3 and 4 look at methodologies and results of two different types of separation technique; ethanol precipitations for Chapter 3 and ion exchange chromatography for Chapter 4. These two methods are the backbone of most blood plasma separations and provide the most promise for separation.

Chapter 5 discusses the implications of the results of the previous two chapters, aiming to put together a comprehensive separation process at an industrial scale. Chapter 6 looks at various conclusions from this and further recommendations for study.

Chapter 2: Literature review

Research of the current literature was undertaken in order to identify the information currently available about equine blood plasma and its separation. The aim of this was to determine techniques that could be used or adapted to ensure broad-scale separation. However, information on comprehensive separation is somewhat limited, due to certain gaps in the field. For this reason, other mammalian systems were examined when they were considered relevant.

In addition, a comprehensive study of separation techniques used was undertaken. Multiple fields of bioprocessing were considered for this, because techniques such as chromatography were demonstrated to work before being applied to blood plasma. However, as per the constraints of this thesis, they were not considered as methods for separation of blood plasma.

2.1 Plasma Types

Human blood plasma is usually to be obtained through volunteers [4, 5], a practice that is obviously impossible with animals. Slaughterhouses are common sources of bovine blood plasma, along with some less-used types of blood plasma [37-39]. Equine plasma, by contrast, tends to be obtained from animals specifically farmed for that purpose, due to the limited consumption of horsemeat in Western countries [6].

The result of this is that equine plasma proteins tends to be considerably less accessible than bovine counterparts. For this reason, research in them is considerably more limited than for human or bovine plasma. Most of this research is based on existing processes, rather than refining them further or discovering new opportunities. For many of the most recent trends in the industry, other forms of blood plasma are thus essential to consider.

Table 2.1 contains a number of the proteins present in human, bovine and equine blood plasmas, along with quantities where known. It is evident from this that human plasma is far better studied. Part of the reason for this is that many of the proteins present are noted primarily for their relationship with certain diseases or ailments, which are far less studied in cows and horses. For similar reasons, the existence of many of these proteins are presumed in bovine and equine plasma, but not confirmed.

Table 2.1: A list of the blood plasma proteins within human (h), bovine (b) and equine (e) plasmas, with known quantities of these proteins

Protein	Source	Quantity (g/L)	Reference
Albumin			
	h	35-50	[40]
	b	35	[41]
	e	30-39	[42]
Immunoglobulin G			
	h	8-16	[43]
	b	25	[44]
	e	10-25	[45]
Transferrin			
	h	2-4	[40]
	b	2-7	[46]
	e	4.3	[47]
Fibrinogen			
	h	3-4	[48]
	b	5.0-6.6	[49]
	e	0.9-2.3	[50]
Immunoglobulin A			
	h	0.5-4	[43]
	b	3-7	[44]
	e	2-3	[45]
Alpha-2-Macroglobulin			
	h	2	[51]
	b	-	
	e	-	
Immunoglobulin M			
	h	0.3-3	[43]
	b	0.1-0.6	[44]
	e	0.8-1.2	[45]
Alpha-1-Antitrypsin			
	h	1.4-2.3	[52]
	b	-	
	e	-	
C3 Complement			
	h	0.8-1.2	[53]
	e	-	
Haptoglobulin			
	h	0.6-2.5	[40]
	e	0.3	[47]
Apolipoprotein A-1			
	h	1.3-1.6	[54]

	b	-	
	e	-	
Apolipoprotein B			
	h	0.7	[40]
	b	-	
	e	-	
Alpha-1-acid glycoprotein			
	h	0.6-1.2	[55]
	b	-	
	e	0.012	[47]
Lipoprotein			
	h	-	
Factor H			
	h	0.1-0.6	[56]
Ceruloplasmin			
	h	0.2-0.4	[40]
	e	-	
C4 Complement			
	h	0.17-0.33	[53]
	e	-	
Complement Factor B			
	h	0.2	[57]
	e	-	
Prealbumin			
	h	0.16-0.35	[58]
C9 Complement			
	h	0.04	[57]
C1q Complement			
	h	0.066	[57]
C8 Complement			
	h	0.048	[57]
C5 Complement			
	h	0.05	[57]
Plasminogen			
	h	0.1	[40]
Immunoglobulin D			
	h	0.03	[59]
C1 Inhibitor			
	h	0.19	[57]
C6 Complement			
	h	0.06	[57]
C7 Complement			
	h	0.06	[57]
Complement Factor 1			

	h	0.04	[57]
Retinol Binding Globulin			
	h	0.04-0.05	[60]
iC3b			
	h	0.01	[61]
Thyroxin Binding Globulin			
	h	-	
C2 Complement Protein			
	h	0.03	[57]
Thrombus Precursor Protein			
	h	0.002-0.009	[62]
C-reactive Protein			
	h	0.003	[63]
Bb Fragment			
	h	0.001	[64]
C3a Complement Protein			
	h	0.0002	[65]
Anit-Testosterone Antibody			
	b	-	
Conglutinin			
	h	-	
	b	0.001-0.05	[66]
	e	-	
Gelsolin			
	h	0.24	[67]
	b	-	
	e	-	
Kininogen			
	h	0.07-0.12	[40]
	b	-	
	e	-	
Prothrombin			
	h	0.05-0.1	[68]
	b	-	

2.1.1 Human plasma

While human blood plasma is in many ways the worst comparison due to the widely differing requirements, it is also one on which large scale production is fully expanded. Bovine plasma tends

to instead be divided by vital component, as do other animal plasmas. This makes it difficult to assess what technologies are generally used, as major variance in the separation method used can occur between products. It also demonstrates a fundamentally different set of objectives towards non-human blood plasma.

It is also interesting to view developments in the human plasma industry, as these tend to anticipate similar applications in the animal plasma industry. Examples of this include Cohn fractionation, with a bovine variant developed some time later [25, 69], and, more recently, affinity chromatography [6, 70, 71]. One interesting development is the development of recombinant alternatives to blood proteins [31], such as $F(ab)_2$ fragments, that could compete with blood plasma products on a commercial level. However, it is unlikely that these will be cost-effective for equine plasma for some time.

Perhaps the definitive review of human blood plasma is that of Burnouf [5]. This covers a wide range of techniques used in the human plasma industry, complete with a description of extraction of many factors. While many of these factors are not relevant to equine plasma, what is interesting is the relatively low level of technology for many of the techniques used, with chromatography generally the most recent development. This demonstrates the conservative nature of the industry with regards to new processes.

Another demonstration of this is the relatively low proportion of blood plasma proteins that are used commercially. It has been noted that four proteins (albumin, IgG, Factor VIII and alpha-antitrypsin) make up around 70% of sales even in the human plasma industry [72]. This very low proportion highlights the difficulty in finding products from blood plasma.

This conservativeness is not without justification, as noted by Curling [73]. His discussion notes that because regulators tend to adopt the view that the process is the product, companies are often reluctant to introduce new methods into a process. This has a knock-on effect for mammalian blood plasmas, due to the fewer genuinely new innovations being produced and proven to work. In certain cases, innovations may even occur in the animal plasma industries before human plasma, thanks to fewer regulatory requirements.

Disease transfer has also been a historical risk. Past practices have been linked to HCV (Hepatitis C) outbreaks [74, 75]. These concerns have restricted development of existing practices for some time [76]. It also has served as a reminder of the dangers of poor separation and purification techniques.

Partially as a result of this, most of the recent focus has been on ensuring the safety of any future plasma products. Farcet [77] and Lovick [78] are both examples of this, each viewing risks involved with reducing hepatitis risk in blood donation. While these are crucial with regard to safe production,

they are generally less focused on new methods of separation. This indicates that from an industrial perspective, there has no dramatic progress in downstream technologies recently.

2.1.2 Bovine Plasma

A few studies have looked at total separation processes in bovine plasma. Talamo [22] was one of the first to comprehensively examine the proteins present in the blood plasma. The 2-D map obtained was predictably dominated by a few proteins, but nevertheless gave a demonstration of what could be expected from a typical mammalian system.

Marco-Ramell [79] took this one step further, removing albumin and immunoglobulins from the plasma before running a 2-D electrophoresis gel. However, the focus of this study was on establishing the effectiveness of such methods as opposed to determining the proteins left behind. Their study thus only provides the maps created by the 2-D gels as useful components for the context of the aims of this thesis.

Alonso-Fauste [23] also examined bovine plasma using 2-D electrophoresis, albeit in a more limited context. Their results were more concerned with mastitis, and identifying differences between healthy bovines. This limited the usefulness of the 2-D maps of the gels obtained.

A common feature was that obtaining a bovine protein map was not a primary goal in any of these studies. This demonstrates the lack of perceived incentive in comprehensive separation strategies. This is partially due to the fact that there are so few proteins consistently separated from animal blood plasmas.

Another issue many of these papers have in common is that protein identification is generally not carried out. This applies particularly in the cases where albumin and immunoglobulins are removed. This seems unusual, as in these studies, identifying other proteins would be made considerably easier. Such identification needs to be a feature of further research.

2.2 Equine plasma

There have been few comprehensive studies on equine blood plasma. However, certain proteins such as immunoglobulins have been identified from the plasma. This allows for some examination of the components, if not of a comprehensive strategy for separation of these components. A white paper published some time ago by the USDA has noted that only a few products have been approved for

use, the majority of which are immunoglobulins [80]. However, this only refers to those designed for human consumption, as many extracted proteins are used for other applications. This means that many extracted proteins were not included in their count because they fell outside the parameters of the investigation.

One of the earliest attempts to determine the plasma contents was that of Keay [81], who examined the blood sera of cattle, sheep and horses. This gave the electrophoretic positions of albumin along with alpha, beta and gamma globulins relative to each other. This work was expanded on by Riond [42], who gave more precise quantities of these proteins. However, the lack of molecular weight testing meant that these could only be identified as electrophoretic fractions.

Other attempts have followed similar paths, using molecular mass as a point of difference instead of isoelectric point. Bokina [82] detailed protein bands of various masses and intensities, however the proteins found were not characterised. Similar work was carried out by Alberghina [17] and Quartuccio [18], with some identification of the proteins carried out. Neither paper contained more than passing mention of separation of the blood plasma.

On the other hand, there is a considerable amount of material covering the use of separation in the field of drug testing [83]. Two factors limit this applicability in industrial use for production. The first is scale, with all drug testing being practiced at much lower volumes than any industrial separation technique. The other is that, other than specific proteins being tested for, the other contents of the blood are generally not being tested for. This tends to result in antibody recognition and affinity systems that give poor results for overall separation.

This lack of information means that much of the information that can be used has to be put together through individual proteins rather than entire plasma information. A brief summary of some of the proteins and methods used for separation is given in Table 2.2. This demonstrates the paucity of proteins separated from equine plasma.

Table 2.2: A brief summary of the functional methods used to separate equine proteins

Protein	Method	Reference
Albumin + globulins	electrophoresis	[18]
eCG	magnetic ion-exchange chromatography	[10]
Albumin + globulins	electrophoresis	[17]
IgG	ammonium sulphate + caprylic acid precipitation	[8]
IgG F(ab)2	IEC/ affinity chromatography	[70]
IgG	caprylic acid precipitation	[84]
IgG	caprylic acid precipitation	[85]
IgG	caprylic acid/ pepsin	[86]
IgG F(ab)2	caprylic acid/ammonium sulphate precipitations	[87]
IgG	aqueous 2-phase system	[88]
IgG	Ethanol precipitation	[31]
eCG	Metaphosphoric acid precipitation	[13]

2.2.1 Immunoglobulins

There are several families of immunoglobulin protein, the most common of which being IgG. The common structure of IgG molecules includes two heavy chains is made of units each around 50 kDa in mass, and two light (and active) chains made up of units around 25 kDa each. These generally exist in pairs, as demonstrated in Figure 2.1. These also generally have one of the highest pI values of any blood protein, with values generally between 6 and 10 [89]. This increases the capability of separation by principles such as ion exchange.

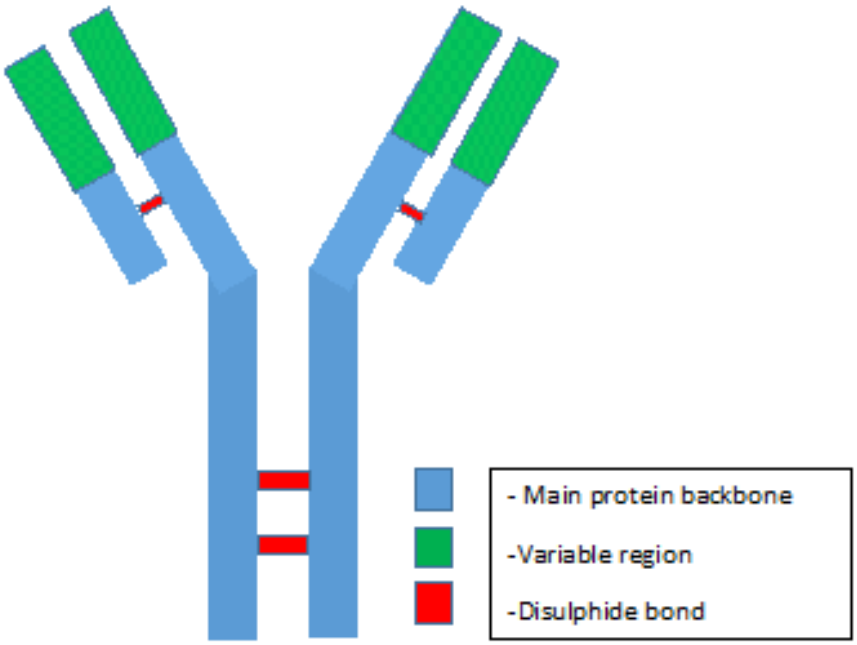

Figure 2.1: A simplified diagram of the structure of IgG. Created with information from [90]

Immunoglobulins (Ig) provide immune response to specific ailments. For this reason, they are excellent components of passive vaccines. Antibodies for the rabies virus are often purified from equine blood and used as a cheaper alternative to similar human antibodies in poorer nations [9, 12, 70, 91]. Meanwhile, snakebite immunoglobulins are also used in a similar manner [8, 11, 86, 88]. These traits make this class of protein probably the most studied of equine blood proteins. Furthermore, they are likely to have the highest commercial value of any equine blood waste product, providing that they are not already being produced. For this reason, they are probably the best way to improve the value of an industrial equine blood separation, making them a critical part of this investigation.

Consequently, more methods for separating immunoglobulins have been explored than for most other proteins. Ammonium sulphate precipitations have been historically common [6, 86, 92], although caprylic acid has emerged as an alternative [93-95]. Methods such as adapted Cohn fractionations have also been considered, with the use of ethanol [31].

Increasingly, chromatography has become the method of choice for separation. Affinity chromatography using Protein A and Protein G matrices are most common, because of the yields achieved [96]. Alternatives have also been used, with ion exchange [12, 70, 97, 98] and even metal affinity chromatography [98, 99] having been demonstrated to be effective.

2.2.2 ESA

Albumin is the largest protein component of blood plasma, including in equine plasma. Figure 2.2 illustrates its chemical structure. Its role is as a transport protein, with a large number of bonding sites able to convey crucial ions and proteins through the blood. With a molecular mass of around 70 kDa, it is relatively small, but has no easily defined subunits [100]. Its pI is considerably lower than that of immunoglobulins, at around 5.

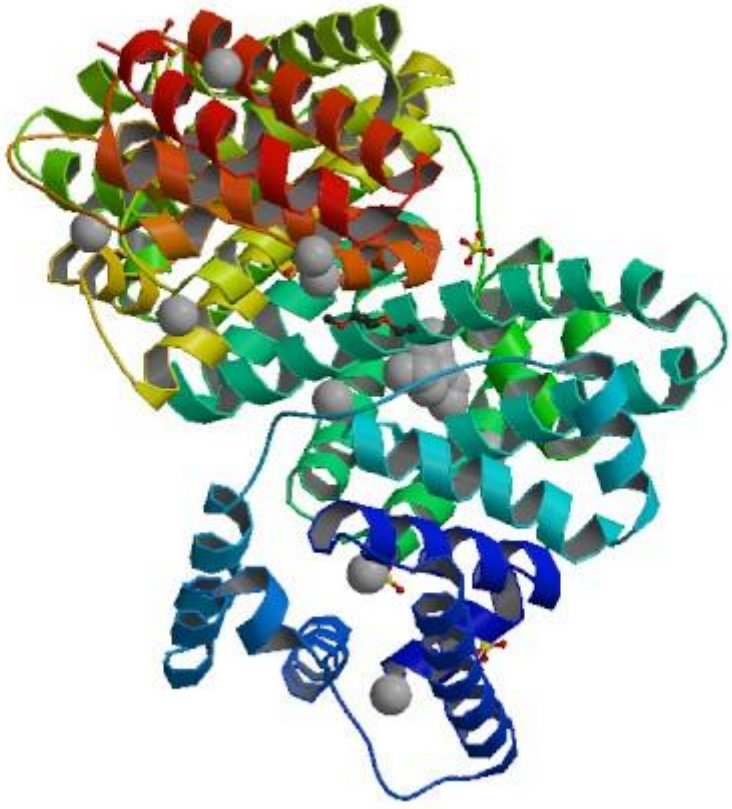

Figure 2.2: The full crystal structure of ESA. Image from the RCSB PDB (Protein I.D: 3V08). Protein structure determined by [101]

ESA is normally extracted using electrophoresis at an analytical level. This is not adequate for larger scale separation, as applying a charge gradient over large amounts of material is not practical.

At larger levels, Cohn fractionation adaptions are the most prominent methods for obtaining albumin [102, 103]. The original method has since been adapted for bovine plasma [104]. Despite some adaptions, the bulk of the process is still the same as that originally used by Cohn. Some chromatographic separations have also been noted, however [102, 103, 105].

For BSA, there has been a movement against using ethanol precipitations due to safety concerns of virus transmission [105]. Heat shock has instead been preferred because of the higher likelihood of virus removal. This has been reflected in the equine industry, where most ESA is produced similarly.

ESA has not been extensively explored as a veterinary tool [106], which has helped contribute to a lack of interest in it. Most applications are as a research substitute for BSA. However, it has some value in research roles [14], indicating that it may still be worthwhile separating this as a by-product of some other process. Belli [106] also noted its use as a tool in some animal health situations, however these are likely to be lower value than immunoglobulins.

2.2.3 Other proteins

eCG (equine chorionic gonadotropin) has applications as a supplement for a number of agricultural animals [107-110]. This protein exists in much smaller quantities in plasma and has a similar molecular mass to ESA at around 61 kDa, making fractionation a more difficult process. Processes to fractionate this have traditionally involved metaphosphoric acid precipitations [13], which have replaced chromatographic separations [111]. This presents problems, because the lowering of the pH potentially denatures other proteins. However, this is a key protein to be noted for potential production for any equine blood processes not already purifying it.

Fibrinogen has also been investigated, although this has partially been as a veterinary tool for analysis of equine health [112]. Scaled-up separation of fibrinogen has not been looked into, as it is unlikely to have commercial prospects in the foreseeable future. Porcine fibrinogen has been demonstrated as a meat additive [35], but this is unlikely to act as a good market for similar equine products.

A number of the more abundant proteins have also been examined, although primarily in terms of analytical systems for medical purposes. Haptoglobin and transferrin have both been used as medical markers of health [113]. Some medical applications have been found for human transferrin [114], but this is unlikely to extend to horses in practical applications because the diseases and applications are rare and complex respectively. Few other proteins are mentioned outside of comparisons between healthy and unhealthy horses, and none of these have had any real attention devoted to purification.

2.3 Existing Separation Methods

Blood plasma separation has been based on traditional methods used within the downstream bioprocessing industry. However, there are two significant distinguishing points; the volume of plasma processed, which is generally at the high end of purification processes, and that the upstream inputs

cannot easily be changed through modifications within the control of the purification group. These factors limit the application of certain techniques that would otherwise be usual.

Separation practices can vary widely, depending on the type of plasma being used and the desired product. As mentioned previously, human blood plasma has been the best analysed with a comprehensive strategy in mind, and a diagram of a practical separation scheme for this is shown in Figure 2.3.

One interesting aspect of Figure 2.3 is the uniformity of techniques. The vast majority of these proteins are separated by some combination of ethanol precipitation, chromatography and filtration. This contrasts with the equine industry, in which a large number of differing methods exist. There are distinct issues with using this system in an equine context, namely that the vast majority of proteins separated in this are not generally commercially produced. However, it provides a keen insight into the types of techniques that could be expected to be viable at an industrial level.

In this particular scheme, cryo-precipitation is the first technique used. However, its application is limited to the beginning of any given process, because blood plasma is generally frozen for storage. This means that most studies are based on exploiting this differently, rather than explicitly improving the technique. In addition, the only separated protein that may be valuable in equine systems is fibrinogen, dramatically reducing the usefulness of this process.

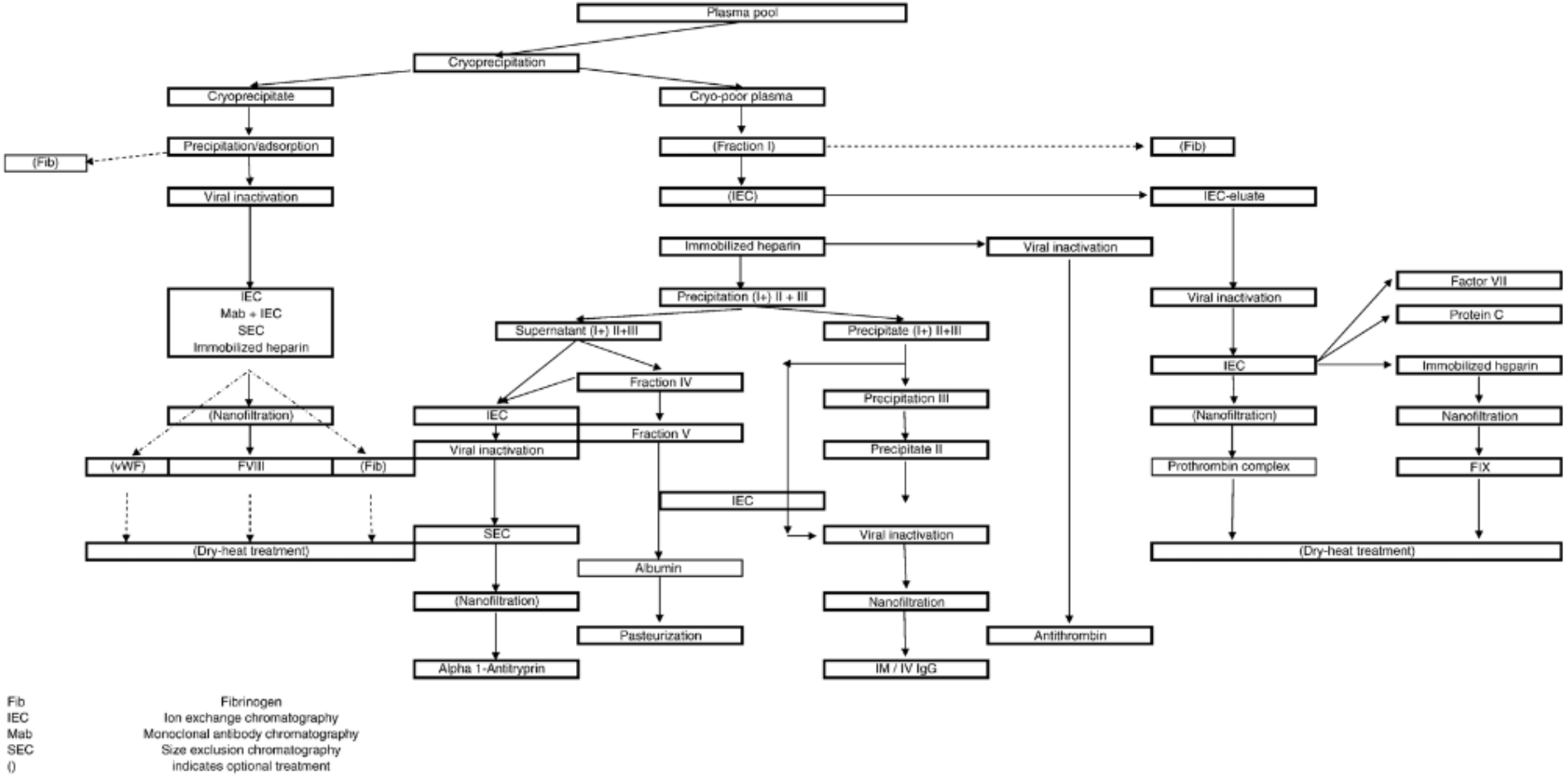

Figure 2.3: A scheme for the separation of human blood plasma. Reproduced with permission from [5]

2.3.1 Filtration

Various forms of filtration has a long history in blood plasma studies, primarily as a way to ensure the safety of blood products. Instances of nanofiltration use can be seen in a few studies [34, 77, 115, 116], along with virus removal [77]. This role has diminished as other separation methods have become available; however filtration still is used as a strategy. Ultrafiltration, on the other hand, tends to be used for clarification of precipitates [5, 11].

A variant method was tested on human plasma, involving ultrafiltration combined with centrifugation [117]. This proved effective at obtaining low molecular mass proteins. A few of these are currently useful from equine plasma, such as light IgG chains. In a similar vein, Pares [35] used a membrane filtration system as the final stage of their separation of porcine albumin.

Increasingly, filtration systems are expanding from their traditional purposes, with the advent of new membrane technologies. With membranes increasingly being developed to allow separation by means other than size exclusion, filtration technology may become more important to the industry. Although the principles of separation are quite different, aspects of filtration such as scale-up and anti-fouling will need to be further studied.

2.3.2 Centrifugation

As previously mentioned, centrifugation has been used reasonably extensively in the blood plasma industry [118, 119]. This has mainly come in the form of separating precipitates [25, 119], or in combination with filtration [118]. The flexibility and low expense mean that it is a popular technique in downstream processing.

The main disadvantage of centrifugation, as noted by Voisard [120], is that it is difficult to adapt a centrifugation technique to suit any situation. This has resulted in centrifugation not being seen as a stand-alone operation within downstream separation. In addition, centrifugation has had increasing competition from membrane technologies [121]. It is still likely that centrifugation will continue to be used in industrial processes, due to its use in existing processes in which it acts as a secondary part of the process.

2.3.3 Precipitations

Precipitation is well explored within the plasma industry, with a wide number of applications noted. These schemes avoid many of the problems associated with chromatography, such as difficult scale-up, complex operation and the additional flow-through of buffer. Various precipitation means have thus been developed both for isolating certain proteins and multi-protein fractionation schemes.

Cohn fractionation is the oldest of the major methods. It relies on multi-stage ethanol concentrations in low temperature blood plasma to separate albumin, generally giving good purity [25]. Yield is less outstanding, but can be improved by reprocessing early fractions. This process is still fundamental to the blood plasma industry, with most methods of obtaining albumin based on the principles this established [4]. In addition, many of the earlier precipitations are named and referred to in literature by the number of the stage, for instance Fraction I, Fractions II + III.

Despite the advantages of this separation, investigation into the precise steps have been limited [4]. Such precipitations rely on a number of factors separate to ethanol percentage being exact, such as temperature, pH and salt type and concentration. This makes them particularly difficult to study and critique, due to the number of factors that have to be optimised. While Cohn fractions have shown to be effective, they are not without purity and/or yield issues, implying that there may be ways to further optimise them.

Some processes have been designed with this intent, with variations in the ethanol percentages [29] and alternative procedures in place of certain steps [26]. Many of these methods have taken hold, with the methods used by Kistler [29] now forming the basis of separation used by companies such as Commonwealth Serum Laboratories, based out of Melbourne. This study has not retested the underlying basis of the ethanol levels used, however.

Other processes have had different uses of ethanol precipitations, generally relying on one or two steps instead of the full-scale instances of Cohn fractionation [27, 31]. These methods are often part of wider separation strategies however, as opposed to the stand-alone methods of Cohn and others. What is interesting about them is that they can be used to obtain proteins such as immunoglobulins and fibrinogen, which is part of the reason that Cohn fractionation is still used in modern industry.

A study into the effects of pH was undertaken by Yoshizawa [122]. This study altered pH levels during pH the precipitation of particular proteins, among them BSA. There was an appreciable difference in composition and quantity at differing pHs, but the total impact was limited. This indicates that pH is generally of low importance in the actual precipitation, with protein activity more critical. It is also worth noting that the pH range tested was only from 4 to 7; a small, albeit commonly used, area. This restricts the generalisations that can be made about this aspect.

Other precipitants are also used. Ammonium sulphate precipitations are particularly common in the context of purifying immunoglobulins [11, 35, 98, 123]. These are occasionally used in conjunction with pepsin in order to instead obtain the active $F(ab)_2$ fragments. These have the advantage of being simple, quick and easy to scale up, much like ethanol precipitations. The largest issue with these restricting them in a general separation strategy is the low pH and salting out conditions which are not generally conductive to other proteins' activity.

There have been questions about the necessity of pepsin in the $F(ab)_2$ process. Morais [86] noted that these digestions act best at pH levels below 3, which is not conducive to extracting other proteins. The reduction of yield caused by the digestion was also noted in the investigation. The other key disadvantage is that, like some ethanol precipitations, purity of product can be low at only 30-60%. This generally means additional processing steps are needed on top of the precipitations.

An alternative of caprylic acid precipitations has also been used for immunoglobulin precipitation [8, 87, 124, 125]. This is partially as a response to the low purity of the ammonium sulphate precipitations. These have the additional advantage of generally requiring fairly mild pH conditions outside of digestion, while maintaining yields at around the 65% mark [87].

One of the key limitations of caprylic acid precipitations is that of scale [95]. Although this study achieved very respectable yields of about 86% or above in equine plasma, the authors noted that constant agitation was required to achieve this, with the recommendation of improving centrifugation and filtration technology. Other studies have noted the difficulty of removing the precipitate [87]. While this may be the case, it is worth noting that mixing scale-up is typically easier to undertake than chromatography.

Eursakun [8] attempted to combine the two processes, using a combination of ammonium sulphate and caprylic acid to improve the separation. This attempt was unsuccessful, as the combined use did not provide any increased isolation. Interestingly, the comparative studies carried out between the two methods indicated that caprylic acid was more effective, however this could be reflective of the specific conditions chosen.

Morais [94] took a different approach, examining the precipitation of both BSA and immunoglobulins in bovine plasma. This proved moderately successful, although immunoglobulin purities and yields were lower than for single-protein methods. They also indirectly noted the ability for this to be used in equine plasma, with ESA replacing BSA as the product. This demonstrates the practical applications of this method, although no scale-up was attempted in this study.

Raweerith [126] originally demonstrated the potential effectiveness of caprylic acid precipitations when applied to equine plasma, using the precipitations directly on blood plasma combined with IEC

to extract immunoglobulins. A reasonable recovery of 66% was achieved, and the authors believed it compared favourably to other techniques at the time. It is worth noting that at the time, affinity mediums were more costly than at present. Since this was noted as the most effective method even then, this may affect the usability of this technique in a contemporary setting.

Some experimental procedures have tried to take advantage of principles within affinity chromatography by carrying out affinity precipitations [127]. These are designed to separate various materials without the difficulties and expenses of using chromatography. While this still requires the use of the specialist materials of affinity chromatography, it avoids the issues in flow rates and high pressures that impact chromatography. The disadvantage to these techniques is that, being affinity-based, they are poor for comprehensive separation.

Metaphosphoric acid precipitations, as mentioned previously, are often used for eCG separations. These precipitations reduce the number of steps required to a minimum, but the base method is dated [10]. Particularly notable is the large amount of ethanol used in order to undertake these precipitations.

2.3.4 Chromatography

Chromatography has been used extensively for some time now, and is a cornerstone in the blood plasma industry [5]. Since the 1980's, when materials became cheap enough to use in large enough quantities, this method's high purity, good flows and adaptability to scale-up have meant that few downstream processes are now carried out without it.

Ion exchange chromatography is of particular note, being the first to be properly developed, and still the most used in downstream processing. There have been claims that it can be used well in all three stages of processing [128], which highlights the versatility of the process.

In human plasma, IEC is used as part of a process to obtain many different haemophilia treatments and coagulation factors, as shown in Figure 2.2. Many of these are irrelevant when it comes to equine plasma, as haemophilia treatments are not generally required for equines. What IEC does provide is a reliable tool to examine and separate a protein mixture, regardless of the stage of separation. This assumes that affecting factors can be accounted for.

There has been some interest in examining pH as a factor of IEC, but one study that found key characteristics for equine plasma was Moure's [27]. This paper looked at separating bovine plasma with both a pH and salt gradient. Interestingly, similar results were achieved in each case. This may indicate that a higher salt level can substitute for pH in certain cases, which would have useful

consequences. It would enable a clearer connection between methods using differing pHs, whilst also avoiding potentially negative effects of pH extremes.

The other interesting feature of Moure's investigation was that it was one of the few to examine animal plasma separation without a goal of extracting a single protein or group of proteins. This gave it a global view many other papers do not have, with a larger focus on what could be obtained from a broader separation method. While key proteins were not extracted from the plasma, several were identified and salt gradients giving sharp peaks were also noted.

Other uses of ion exchange have been noted in production of immunoglobulins, albumin and eCG [13, 85]. Even though not all of the results were the most optimal obtained, the range of proteins it has been used for demonstrates that this technique is crucial to consider for a comprehensive separation process.

Affinity chromatography has increasingly become an alternative, with a large range of investigations into its use in blood plasma [71]. This method is not practical for a comprehensive separation strategy, but it is a viable option for obtaining certain proteins. There have been concerns about the conditions used being too extreme, but there is little difference between these conditions and the alternative means generally required for these proteins.

One of the most common uses of affinity chromatography is in immunoglobulin separations. Protein A and G separation techniques have been well established in a number of investigations [96, 129, 130]. These techniques are known for having high yields and selectivity, but also sometimes harsh conditions [131]. While there is strong competition from other methods, this is seen as the gold standard for purification of immunoglobulins.

Other blood plasma applications are considerably less common. A recent study demonstrates the use of affinity chromatography for human glycocalicin [132], but this is not a protein likely to be extracted from equine plasma, because it is used for an unusual human illness. A method separating Factor VIII has similar issues [133]. The high costs and low regeneration that these columns traditionally have [129] means that this is unlikely to be an effective method outside of clearly established uses.

Various other forms of chromatography also exist, although these are less used in blood plasma processing. Hydrophobic interactions chromatography (HIC) is an example of this, with well-developed uses in protein separation [134]. The biggest issue with HIC in practice is that many of the same functions can be performed by precipitations instead [94]. For instance, caprylic acid precipitations operate on similar principles without the difficulty and added expense of chromatography.

Size exclusion chromatography has had some use, but is restricted in general applications [135]. The reason for this is the extensive use of ultrafiltration, which at appropriate pore sizes can achieve exactly the same outcomes as SEC. However, filtration has fewer of the issues that limit scale-up, making it a much more valuable technique at the industrial level.

Metal affinity chromatography has been tested for separating immunoglobulins [99]. While this is somewhat effective, it is generally impractical in large scale production. This is because of the additional steps required of metal immobilisation [98], which is difficult to undertake given the volume of plasma and thus the volume of ions this requires. It is thus unlikely that such a technique will be effective at an industrial scale.

Despite its abundance in plasma separation, chromatography is not without issues. It is generally the most costly of the methods, particularly in terms of capital costs, and requires the most technical knowledge [136, 137]. Due to a number of factors, this information can be difficult to obtain. Many published methods, for instance, do not relate the reasons for the pH selected in IEC, unless these are being specifically tested. This could be because of a lack of desire to carry out the extensive and tedious work pH testing would require. This makes comparison difficult between methods as it cannot easily be determined what factor is most responsible for the results achieved in each study.

Furthermore, chromatography is not necessarily well-suited to industrial scale work. There have been debates about its effectiveness [73, 118, 136-138], primarily centred on the cost, time and complexity of chromatography. This is part of the reason ethanol precipitations are often more popular, as they avoid many of these problems. However, as Azevedo [137] notes, chromatography has carved out a niche of being able to resolve many intermediate purifications in a single step. This is something that no competing technique has demonstrated consistently.

Curling [73] noted that process improvements in the bioprocess industry have primarily been in the upstream sections. Because this is less relevant for the blood plasma industry, gains have been more modest. At the time of his review, improvements to downstream processes were beginning to be investigated. Since then, continuous chromatography has been investigated in more detail [139]. The largest obstacle to this proceeding is the requirement to convert all operations within a given process to continuous operation, a significant upfront expense for a company.

2.3.5 Membrane separation

With the debate and search for alternatives to chromatography, membrane separation has received increasing attention over the past decade. The technology is itself not new, with previous research noting its higher flowrates [136, 140]. Since then, the adaption of many traditional forms of

chromatography into membranes mean that this technology should be considered seriously as part of a separation strategy.

Size-exclusion was the first major principle to be incorporated into a membrane mechanism [140]. However, more recently ion exchange, hydrophobic and affinity membrane media have all been tested [141-143]. This demonstrates the clear intention to use the principles currently demonstrated through chromatography, while replacing the less desirable characteristics that packed beds have given.

There are some promising signs about the development of membrane technology. Lu [144] noted that membrane replacements for hydrophobic interactions chromatography took less time and consumed considerably less volume than standard columns. While hydrophobic interactions chromatography has limited applications in blood processing, this indicates the potential improvements membrane technology could bring.

Although there is certainly potential in this area of technology, the technology often has lower binding yields than that of chromatography [142]. In addition, there is little demonstrated application of these to separate blood proteins. A study by Wang [91] showed an application in separating bovine thrombin from blood; however this was carried out at very small volumes. For this technique to be considered successful difficulties such as fouling, electric stimulation and pressure would have to be solved, thus it is clear that this is not yet ready for application within the blood plasma industry. An older study by Bayramoglu [145] has similar flaws. These factors indicate that membrane separation in its most raw form is not yet suitable for application within the blood plasma industry.

2.3.6 Other techniques

While other technologies have been developed, many have significant issues to overcome, particularly scale. A method to separate eCG using adsorbents has been tested at the pilot scale [10]. This method has been tested at different scales, which is unusual in itself; however there is less evidence to demonstrate how it might perform at a higher scale-up. Many studies do not test scale-up to this extent, which may well be because the proteomics field is often the main innovator in new separation methods. This means that many such methods are designed for analytical investigations rather than industrial separations

The problem with this approach is that many techniques that work well at the small scale are either ineffective or far too expensive to carry out on a large scale. For this reason, new techniques should be tested at scale-up, as the Muller technique was. However, many are not, limiting their usefulness.

With chromatography generally seen as expensive, other options have been looked into. This applies especially in non-blood plasma industries, where there is more control over the inputs. Many of these are unlikely to be suitable for use within the blood plasma industry, as the inputs to the process are less able to be controlled. For instance, there is no record of using crystallisation to separate proteins from blood plasma.

Some studies have looked to improve chromatography rather than replacing it altogether. Przybycien [136] examined the possibility of fluidised bed chromatography to replace traditional packed beds. However, Curling [73] and Nfor [146] emphasised that this would be unlikely to replace traditional chromatography for some time.

The use of aqueous two-phase systems has been suggested as an alternative to chromatography [137, 147]. Nevertheless, this is as a general recommendation for the pharmaceutical industry rather than specifically for blood plasma fractionation. Vargas [88] managed to go one step further, separating immunoglobulins from equine plasma using this system. This achieved similar yields but lower purities than a comparison with caprylic acid. This indicates the technology is not yet ready for large-scale use.

2.4 Techniques investigated in this work

In spite of the efforts to find more cost effective or efficient means of separation, Azevedo's [137] statement that no other method resolves as many separation steps as chromatography still holds true. This explains the relatively low use of more recent technologies within large scale blood separation strategies and further supports the reason not to include new technologies in an expanded system.

Chromatography was used for a large number of applications within the blood plasma industry. For this reason, it was one the techniques examined for separation of equine plasma. Specifically, ion exchange chromatography was the main focus, due to its long history and excellent adaptability to a separation strategy. While ion exchange membranes are becoming increasingly studied, they are not yet in a position to be used for large-scale separation.

Due the amount of literature on them, ethanol precipitations are the other key area worth exploring for a broad separation strategy. These generally provide the gentlest forms of separation available, with a minimal impact on the other proteins. Because Cohn fractionation still has a large influence on blood plasma processes, the technique is also likely to be familiar to industry. This makes this method an excellent candidate for allowing industry to successfully separate a larger number of different proteins for sale.

Chapter 3: Separation of Equine Plasma by Ion Exchange Chromatography

As discussed, chromatography remains a dominant part of downstream separation methods despite the various flaws it possesses. For this reason, it was one of the processes examined more closely for advanced separation. Moure [27] demonstrated appropriate salt gradients for bovine plasma while using ion exchange chromatography. Because of the similarities between blood plasmas and the versatility of anion exchange chromatography, these were considered the starting point for investigation. However, scale up work was considered a larger priority. All work was carried out at the 6th floor School of Biological Sciences Laboratories at the University of Canterbury.

3.1 Materials and Equipment

Equine blood plasma was obtained from Bioniche (now Vetriquinol, Armidale, NSW, Australia) and was frozen at -18°C until required. Before use, this plasma was thawed overnight in a 4°C fridge while buffers were prepared on the day of chromatography. Analytical grade Tris (AppliChem, Darmstadt, Germany), NaCl (Merck KGaA, Darmstadt, Germany) and HCl (Merck KGaA) were all used in the ion exchange buffers. These were solubilised in deionised (MilliQ) water and had pH measured with a Denver International UltraBasic pH meter (Denver Instrument, Bohemia, NY, USA). For the phosphate buffer saline (PBS) NaCl, KCl, Na_2HPO_4 and KH_2PO_4 (all Merck KGaA) were used. Blood was spun down on a desktop centrifuge (Eppendorf Centrifuge 5415 R, Eppendorf, Hamburg, Germany).

Chromatography took place with 1 mL and 20 mL Sepharose Q Fast Flow columns purchased from GE Healthcare (Little Chalfont, UK). The 1 mL columns were used on an AKTA-Start liquid chromatography system (GE Healthcare), using UNICORN Start-1 software (GE Healthcare). No fraction collection took place at this step. Meanwhile, the 20 mL columns were run on an AKTA-10 machine while being controlled by UNICORN 5.31 software (both GE Healthcare). A GE Frac-950 fraction collector was used in this case (GE Healthcare).

For post-chromatographic investigation, a LABCONCO FreeZone 2.5 freeze-dryer (Labconco, Kansas City, MO, USA) was used, while concentrations were measured on a Nano-Drop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For gels, NuPAGE 4-12% Bis-Tris Midi gels were purchased from Novex (Thermo Fisher Scientific), along with NuPAGE LDS Sample Buffer, NuPAGE reducing agent, SDS running buffer and Novex Sharp pre-stained protein standard (all Thermo Fisher Scientific). These gels were stained with Coomasie Blue stain and images captured using a GE ImageQuart LAS 500 (GE Healthcare). The mass spectroscopy was carried out with ammonium hydrogen carbonate (Merck KGaA), acetonitrile (Thermo Fisher Scientific), dithiothreitol (Sigma Aldrich, St. Louis, MO, USA) and iodoacetimide (AppliChem) with trypsin (Sigma Aldrich) and formic acid (Merck KGaA) used in the protease and extraction buffers respectively.

3.1.1 Chromatographic preparation

A 200 mM Tris stock solution was made and had its pH adjusted to approximately 7.4 with concentrated HCl. This stock was used to make the start buffer of 20mM Tris and the running buffer of 20 mM Tris and 1000 mM NaCl. Both were adjusted to pH 7.4. These were filtered using a 40 µm filter and degassed for 10 minutes.

In preparation for the chromatographic run, the chromatography system was washed with filtered and degassed MilliQ water, followed by start buffer. The column was then washed and regenerated with one 5 CV step of start buffer, one 5 CV step of running buffer and finally with a second 5 CV start buffer step. Meanwhile, the defrosted equine blood plasma was centrifuged at 4°C and 8000 g for 5 minutes before being injected onto the column.

3.2 Running steps

The first chromatography was carried out on a 1mL column, aiming to use the salt gradients established by Moure [27]. These levels were read to be 0, 50, 100, 170 and 350 mM of NaCl. In addition, a 1000 mM NaCl step was added at the end of these steps. Each step was allowed to proceed for 5 CV, while 100 µL of blood plasma was used as a sample. The results of this are shown in Figure 3.1.

Having proof of concept, these steps were repeated on a 20 mL column. The sample size was increased to 2 mL to reflect this, while the linear velocity of the elution was altered from 250 cm/h to 240 cm/h to avoid exceeding the column pressure limits. All other factors were held the same. The results of this can be seen in Figure 3.2.

3.3 Post chromatography

The eluent was collected within 50 mL falcon tubes. The protein-containing fractions were snap-frozen to -80°C before being freeze-dried at -46°C and 0.018 kPa until all liquid was removed. The precipitate was then washed and dissolved in PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, and 1.8 mM KH_2PO_4), with the concentration of the redissolved precipitate being measured by UV absorbance. Samples from this were then taken, reduced and heated to be run on a sodium dodecyl sulphate polyacrylamide (SDS) gel. This was run at 200 V for 40 minutes after loading. This gel was then stained with Coomasie Blue stain. An image of the gel is shown with Figure 3.2.

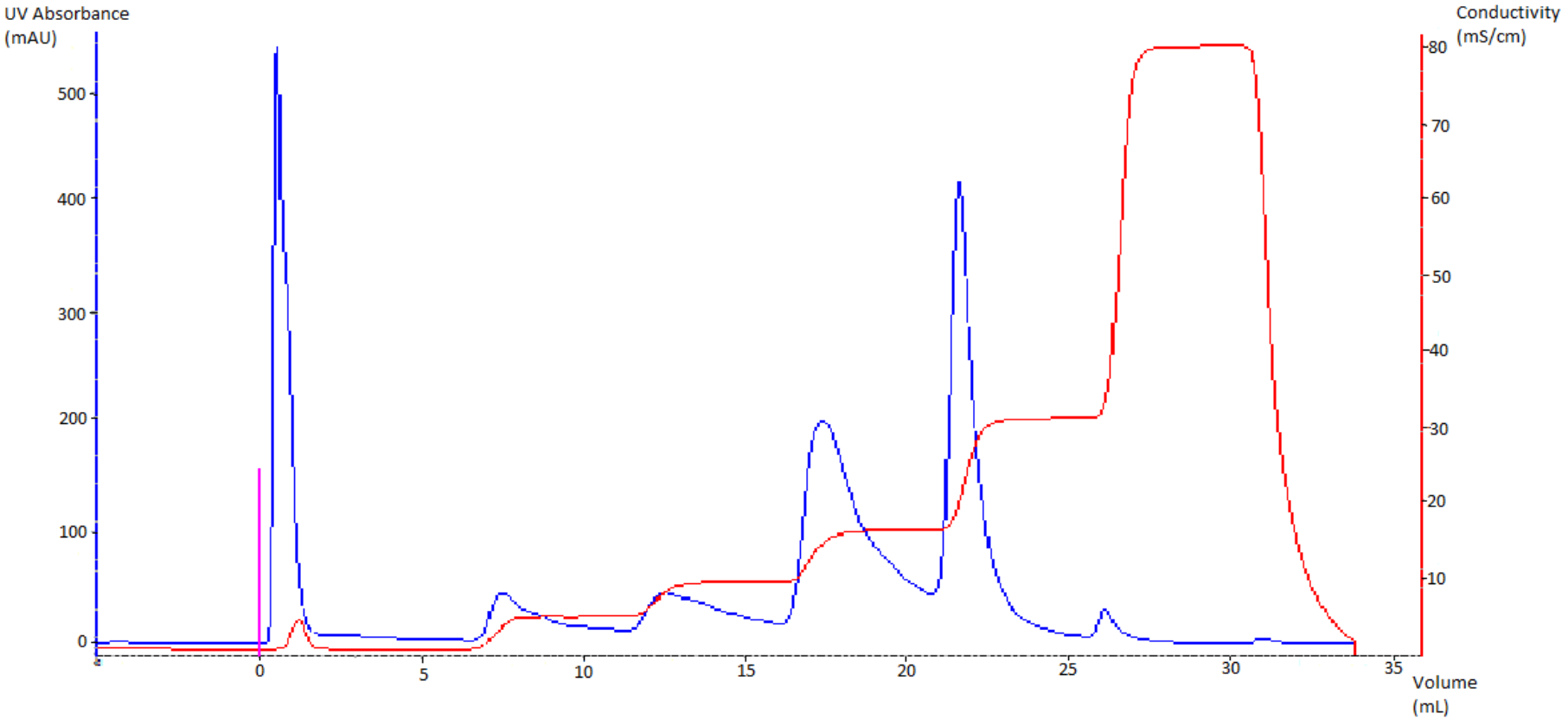

Figure 3.1: The separation of equine blood plasma on a 1 mL Q FF column using the salt concentrations established by Moure [27]

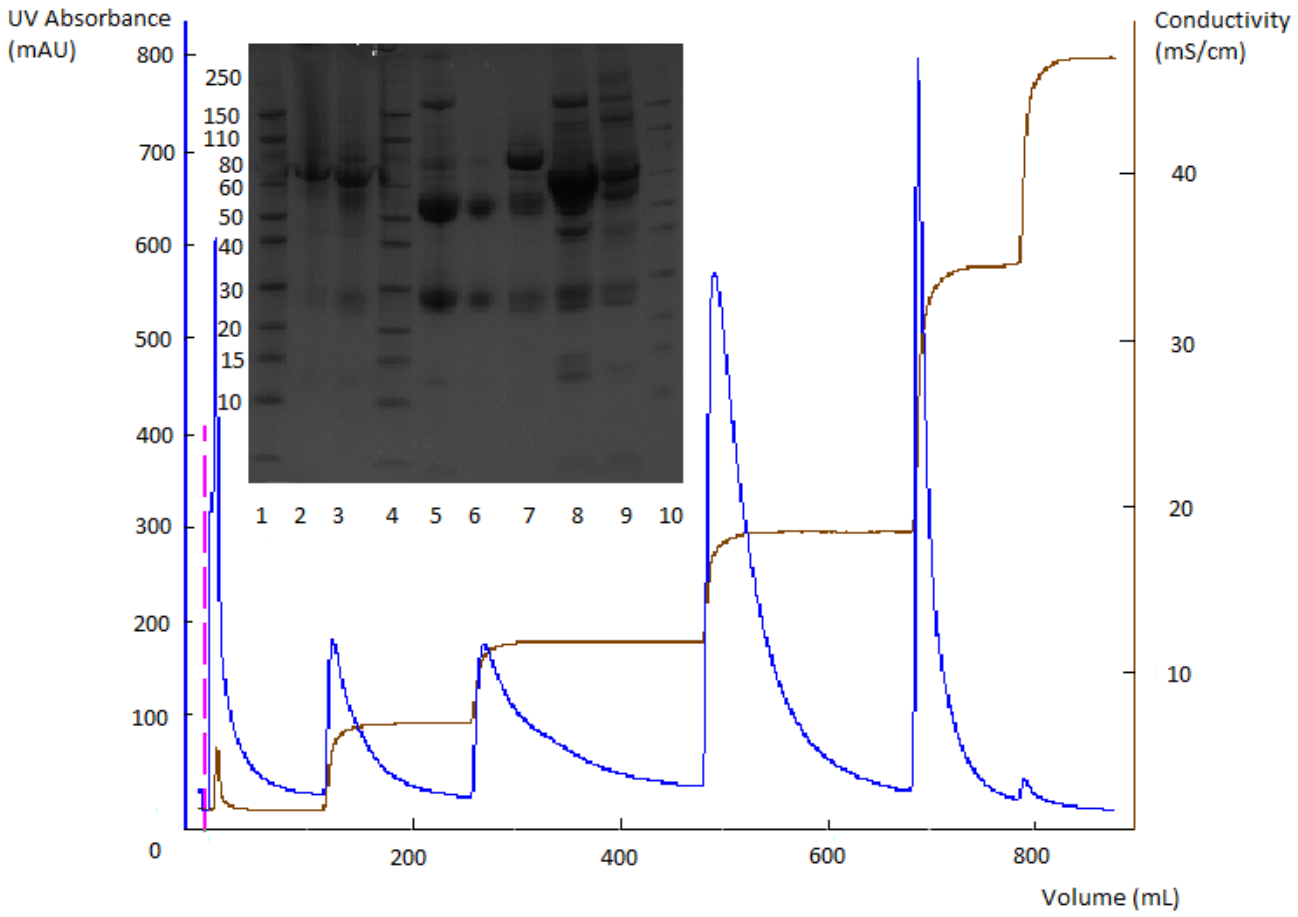

Figure 3.2: Results using the initial salt gradients from Moure [27] on a 20 mL column. Gel lanes are as follows (1) Protein standard; (2) Blood plasma; (3) 1:50 blood plasma dilution; (4) Protein standard; (5) Peak 1; (6) Peak 2; (7) Peak 3; (8) Peak 4; (9) Peak 5; (10) Protein standard. Numbers on the side of gels indicate molecular masses of protein standard (kDa)

3.4 Modifications

With these results obtained, separate variations were carried out. The first of these related solely to the length of elution for the various factors and involved increasing the time of elution until baseline UV absorbance was reached between peaks. This was once again tested on a 1 mL column and then scaled up to 20 mL columns. The elution volumes required to reach baseline UV absorbance are given in Table 3.1.

Table 3.1: Modified lengths of salt gradients

Salt Level (mM NaCl)	Length of Elution (CV)
0	5
50	7
100	11
170	10
350	5
500	5

In addition, the concentrations of salt were altered. This was undertaken by experimenting with separate salt gradients in 1 mL columns while adjusting the quantities required. A key focus was ensuring that the peaks gave as short as elution lengths as could be achieved. The salt levels that best provided that are given in Table 3.2.

Table 3.2: Alternative salt levels for use in anion exchange chromatography with associated lengths of elution

Salt Level (mM NaCl)	Length Required (CV)
0	5
30	10
80	20
130	25
170	23
350	8
500	5

It was noted during this process that a final salt concentration of 500 mM of NaCl and the associated conductivity achieved essentially identical results to the previous high point of 1000mM. Therefore, from this point the running buffer was made with 500 mM NaCl instead. This can be seen in Figure 5.1. The results from this chromatography can be seen in Figure 3.3.

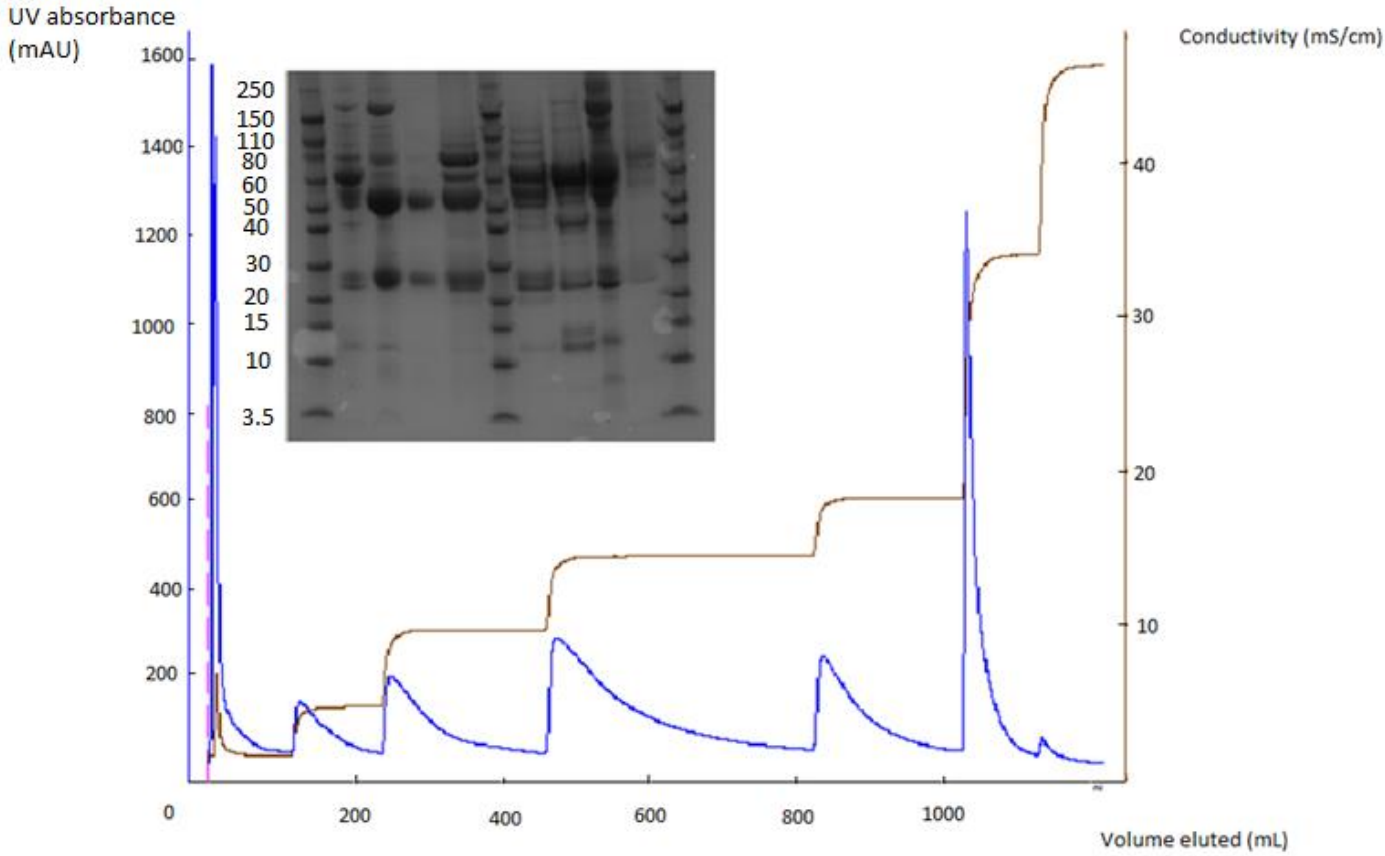

Figure 3.3: Chromatography carried out at the appropriate salt gradients determined in Table 1, with an associated gel. Lanes of the gel are (1) Protein standard (2) On sample at 1:50 dilution (3) Peak 1 (4) Peak 2 (5) Peak 3 (6) Protein standard (7) Peak 4 (8) Peak 5 (9) Peak 6 (10) Peak 7 (11) Protein standard. Numbers on the side indicate the molecular weights of the standards

3.4.1 Mass Spectroscopy

Some of the protein bands from the accompanying gel were removed using a knife and sliced into small cubes. The Coomasie Blue stain was then removed from the gel using an initial incubation at room temperature with 50% acetonitrile for 30 minutes and two further periods of incubation at the same temperature with pure acetonitrile for around 10 minutes. After each period, the acetonitrile was removed.

10 mM of dithiothreitol in 100 mM ammonium hydrogen carbonate was then added to the gel pieces and the mixture was incubated at 56°C for 30 minutes to remove any disulphide bonds within the proteins, after which the gel pieces was shrunk with more acetonitrile. The liquid contents were once again discarded.

55 mM iodoacetimide in 100 mM ammonium hydrogen carbonate was then added to the gel pieces to prevent the disulphide bonds reforming, and the samples were incubated in the dark at room temperature for 20 minutes. After another acetonitrile wash, a protease buffer consisting of 1 part 100 mM ammonium hydrogen carbonate: 1 part acetonitrile: 8 parts MilliQ with trypsin enzyme added at a concentration of 0.013 mg/mL was added to the gel pieces and the mixture was incubated overnight at 37°C. The following day, an extraction buffer consisting of 1.8 µL 90% formic acid, 670 µL acetonitrile and 328 µL MilliQ was added in 120 µL quantities to the gel pieces. This was then incubated at 37°C for 15 minutes. This liquid was then removed and freeze-dried, and the solid remains were dissolved in formic acid. This mixture was then sent to the Chemistry Department of the University of Canterbury for mass spectroscopy analysis.

3.4.2 Optimisation of chromatographic factors

Given the importance of ensuring that this process would operate correctly on a large scale, an investigation was undertaken to test how various factors affected the resolution and peak purity. Flow rate, length of elution and sample size were all identified as factors of relevance and each was tested.

To test elution speed, the chromatographic process was carried out at 60, 150 and 240 cm/h respectively on the 20 mL column. Higher superficial velocities were not attempted as these were not able to be carried out within the column pressure limits. The results of these comparisons are shown in Figure 3.4.

To test sample size, this factor was adjusted to 5 and 20 mL in the 20 mL column. The process was run at 240 cm/h, with all other factors being held constant. No fractions were collected for this process. The results for this comparison are given in Figure 3.5. In addition, the area underneath the curves was calculated using the UNICORN software. These results are given in Table 3.3.

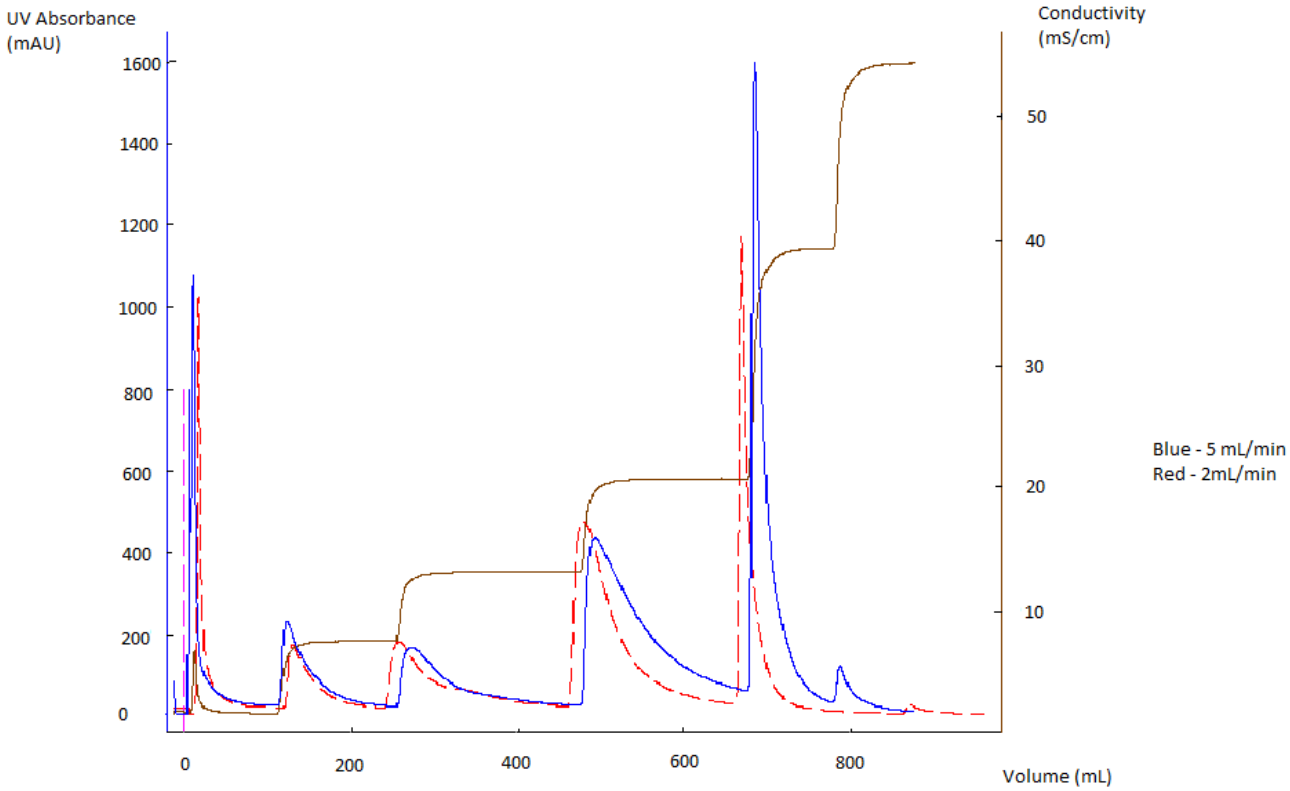

Figure 3.4: The effect of flow rate on the elution profile

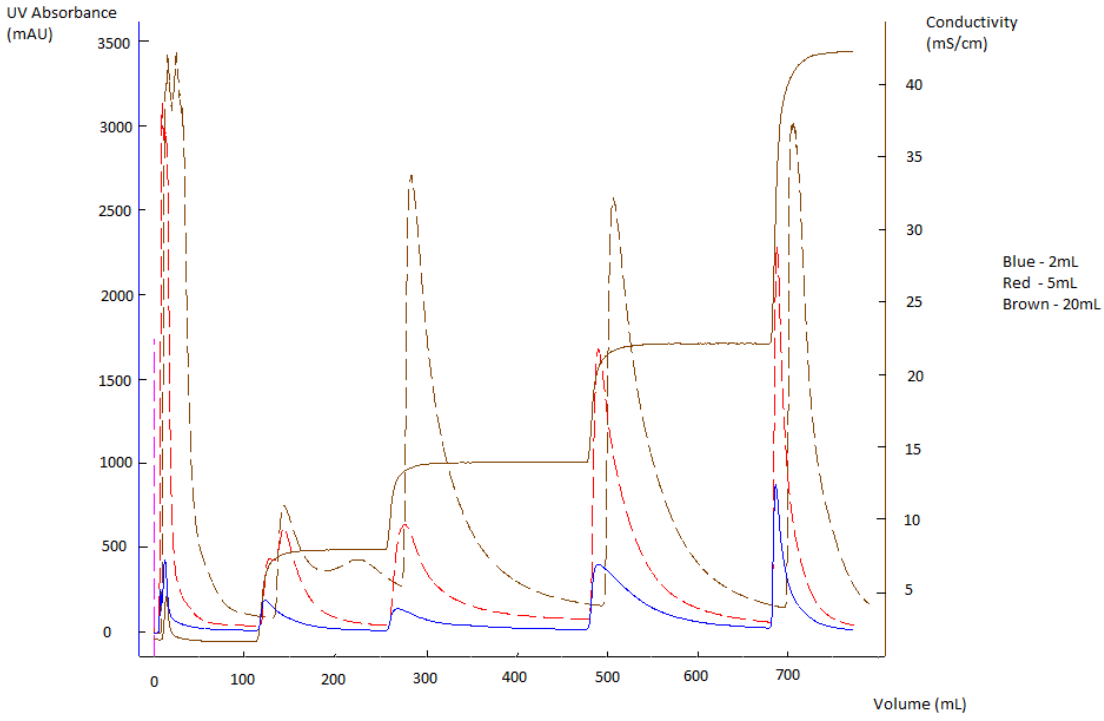

Figure 3.5: The effect of sample size on the elution profile

Table 3.3: The effect of increased sample size on the UV detection. Note that * denotes a peak that reached the maximum detection limit

Peak (mM KCl)	2 mL sample (mAU*mL)	5 mL sample (mAU*mL)	Proportion larger than 2 mL sample	20 mL sample (mAU*mL)	Proportion larger than 2 mL sample
0	13520	39920*	2.95*	108500*	8.03*
50	10660	24710	2.31	44270	4.15
100	14170	32210	2.27	110710	7.81
170	33500	65110	1.94	113680	3.39
340	18150	38360	2.11	82440	4.54

The effect of shortening the lengths of elution was done in a similar manner to the way in which the data in Table 3.3 was obtained. In this case, however, the data was obtained from a standard run at 240 cm/h and 2 mL sample size. Fractions of the overall length were calculated and the area under the curve was recalculated over these fractions. These areas are given in Table 3.4, with all measurements given as fractions of the overall area.

Table 3.4: The proportion of the area compared to length in shortened chromatography runs

mmol NaCl	Percentage of maximum area (%)			
% of peak	90	80	70	60
0	98.5	96.8	94.6	92.2
50	99.5	98.4	96.4	93.4
100	98.9	97.1	94.3	90.6
170	99.8	99.1	97.9	95.7
350	99.9	99.4	98.7	96.9

Chapter 4: Separation of Equine Plasma by Ethanol Precipitation

The second key technique examined was ethanol precipitation, because of its common use within the blood plasma industry. Because there has been a lack of examination of the basis of the ethanol levels, this was carried out. In addition, an examination of the Kistler-Nitschmann technique was carried out to test its compatibility with equine plasma.

4.1 Materials and equipment

The blood plasma was sourced in the same way as the samples in Chapter 3. In addition, analytical grade sodium acetate (Merck KGaA) was purchased in solid form and concentrated acetic acid (Merck KGaA) and ethanol (Sigma Aldrich) was purchased in liquid form.

The AKTA Explorer liquid chromatography systems from Chapter 3 were used in the chromatographic applications. Benchtop electronic balances were used to measure the masses of all Eppendorf tubes and contents.

4.1.1 Buffer Preparation

1500 mM NaCl and 200 mM sodium acetate stock solutions were prepared, with acetic acid used to adjust the pH to 5.2. These were filtered with 40 µm filters and degassed. This pH was selected because of its use within Cohn fractionation.

For chromatography, a 1:10 dilution of this stock solution was taken. The pH was altered to 5.2, and the buffer was filtered and degassed. This was used for running buffer in size-exclusion chromatography steps. For the ion exchange chromatography, Tris buffer was prepared as described in Chapter 3.

4.2 Precipitation test

The first stage was to test the blood plasma for ethanol precipitations to test the impact of initial precipitations. Because extremely large ethanol concentrations (up to 70%) had been used industrially, it was logical to test the necessity of such high levels.

Blood plasma was defrosted in the same manner as described in Chapter 3. This was pipetted into Eppendorf tubes with the stock buffer, deionised water and ethanol in the ratios given in Table 4.1.

These were mixed at room temperature for two hours before being refrigerated at 4°C overnight. The precipitate was removed through centrifugation and the supernatant was pipetted off. The masses of the precipitates were as given in Table 4.2.

Masses were measured by first weighing each individual Eppendorf tube before any material was placed within it. The masses were then measured complete with all material after being mixed. The precipitate was then removed from the suspension by centrifugation and the supernatant was removed from the Eppendorf tube by pipetting. The masses of the precipitates were thus calculated from these figures.

Table 4.1: Preparation of the blood/ethanol/water mixtures

Ethanol Concentration (%)	0	10	15	20	25	30	35	40	50	60	70
Water (mL)	800	700	650	600	550	500	450	400	300	200	100
Ethanol (mL)	0	100	150	200	250	300	350	400	500	600	700
Blood plasma (mL)	100	100	100	100	100	100	100	100	100	100	100
Buffer (mL)	100	100	100	100	100	100	100	100	100	100	100

Table 4.2: The masses of the precipitates of the blood plasma

Ethanol concentration (%)	0	10	15	20	25	30	40	50	60	70
Mass of precipitant (mg)	17.7	37.4	19.5	25.2	35.2	45.4	83.2	76.9	81.1	96.3
Mass of complete mixture (mg)	170.1	174.9	172.5	172.5	172.3	172	171.3	174.9	173.6	175.1
Proportion of mass to mixture	10%	21%	11%	15%	20%	26%	49%	44%	47%	55%

Preliminary chromatography in combination with the mass data displayed in Table 2 demonstrated that 50% and above levels resulted in little difference in the mass of the precipitates. For this reason, these were discontinued and smaller ethanol levels were tested instead. The supernatant was run on a Superdex 200 10/300 GL column (GE Healthcare). The results are as given in Figures 4.1, with the areas under the curves given by Table 4.3.

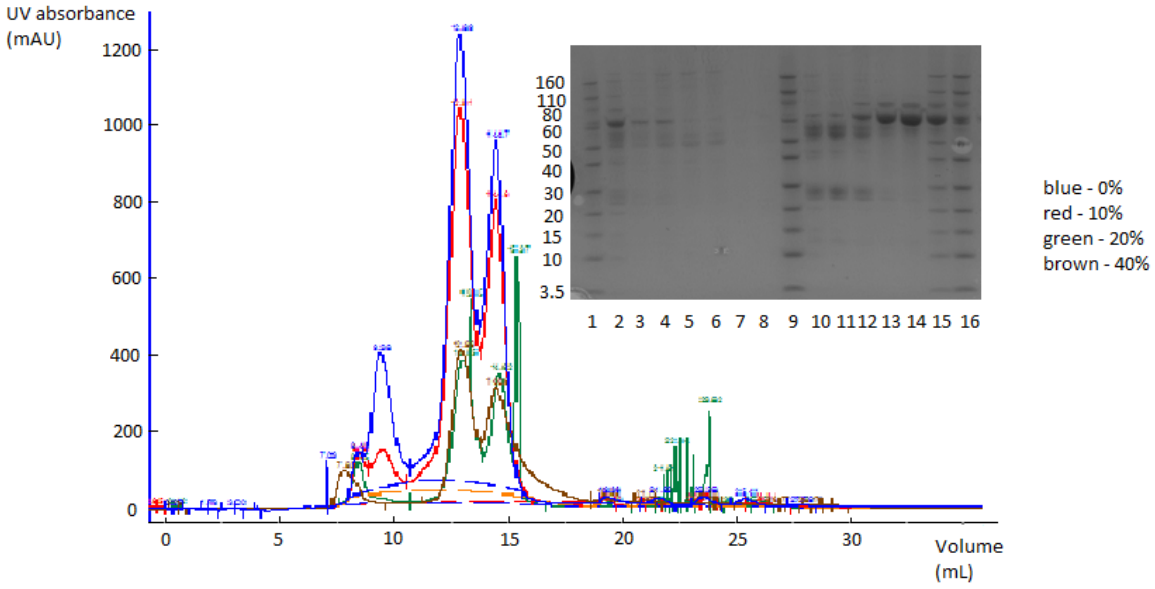

Figure 4.1: A demonstration of the effect of increasing ethanol levels with gel showing the 0% ethanol profile of the chromatograph. The lanes of the gels are as follows: (1) Protein standard; (2) through (8) initial peaks; (9) Protein standard; (10) and (11) Secondary peak; (12) Crossover between secondary and tertiary peak; (13) and (14) Tertiary peak; (15) and (16) Protein standard

Table 4.3: The areas underneath the curves of the runs with varying ethanol concentration of the sample

Ethanol Percentage	Initial peak	Second initial	Secondary peak	Third
0	54.14	367.99	1351.86	850.78
10	66.76	121.09	1194.95	759.43
20	82.1	0	424.9	431.6
40	83.64	0	435.42	478.29

In addition, blood plasma samples with the same ethanol concentrations, along with a 30% percent fraction, were run on a 1 mL Sepharose Q FF column, using the salt gradient steps already established. The results were given in Figure 4.2, with the areas under the curves shown by Table 4.

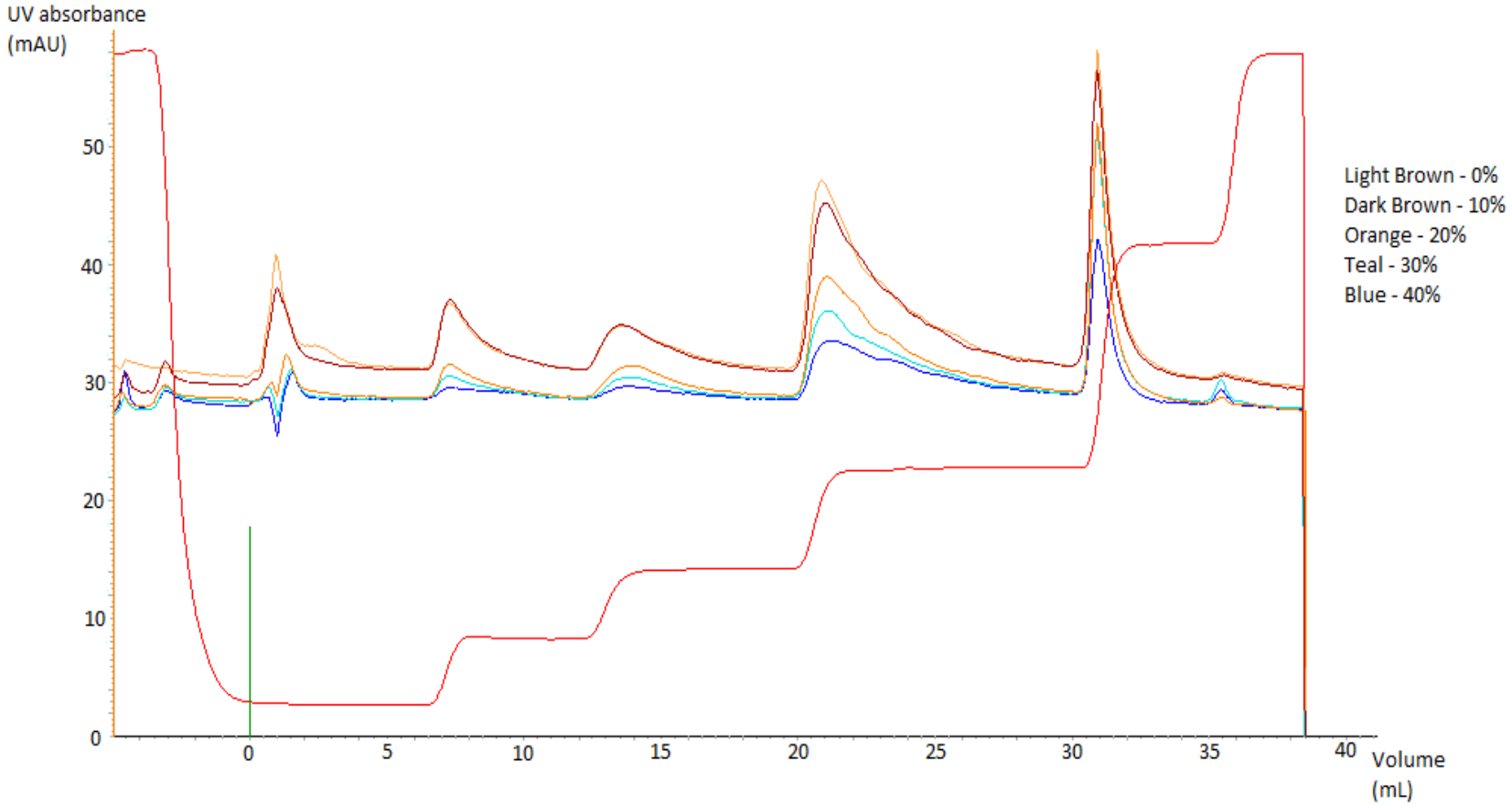

Figure 4.2: The effects of the level of ethanol on the supernatant

Table 4.4: Areas calculated underneath the curves at the given volumes

Peak (mL, approx.)	0% ethanol	10% ethanol	20% ethanol	30% ethanol	40% ethanol
7	9.7263	10.291	4.5102	2.6266	0
20	37.597	37.0608	17.5503	10.9953	7.36
30	22.0114	21.1355	17.0818	17.0056	10.6137

These experiments were performed again with the precipitate being freeze-dried after the supernatant was removed. The masses of these freeze-dried products was recorded and is shown in Table 4.5. Figure 4.3 gives a graphical representation of the data.

The other aspect that was checked was the protein concentrations of the supernatants of the resulting ethanol precipitations. These were examined using ion-exchange chromatography, with the salt levels established previously in Chapter 3. For this, Tris buffer was prepared as in Chapter 3. The results of these runs are given in Figures 4.4 through 4.7, with the areas under each curve given in Table 4.6.

Table 4.5: The effect of increased ethanol on the mass of the precipitant

Ethanol Concentration (%)	Precipitant Mass (g)	Error	Percentage of blood mass	Error
0	0.163	0.001	0.8	0.8
10	0.165	0.003	1.2	0
20	0.164	0.002	3	1
30	0.159	0.007	2.9	0.3
40	0.161	0.002	4.3	0.7

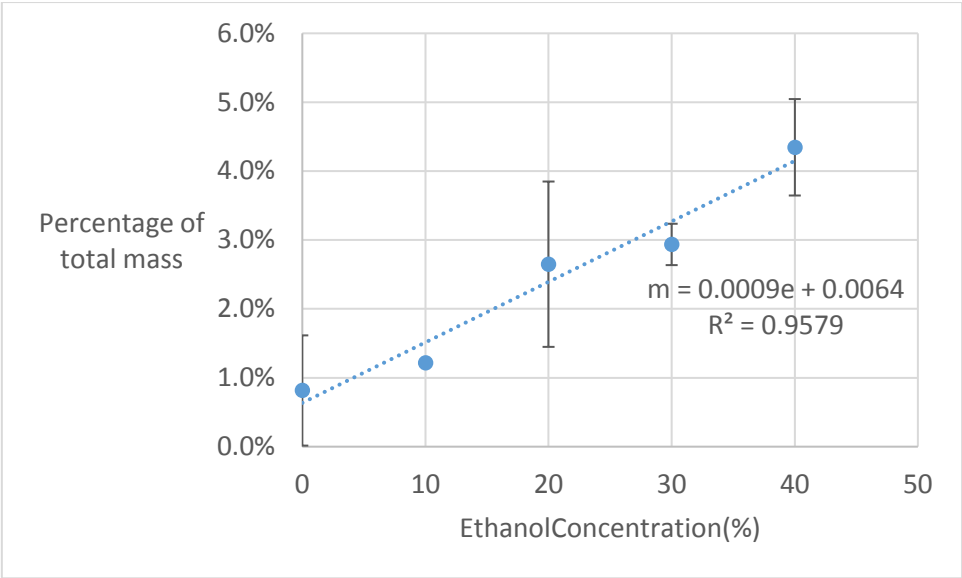

Figure 4.3: The relationship between the ethanol concentration and the mass of blood plasma removed.

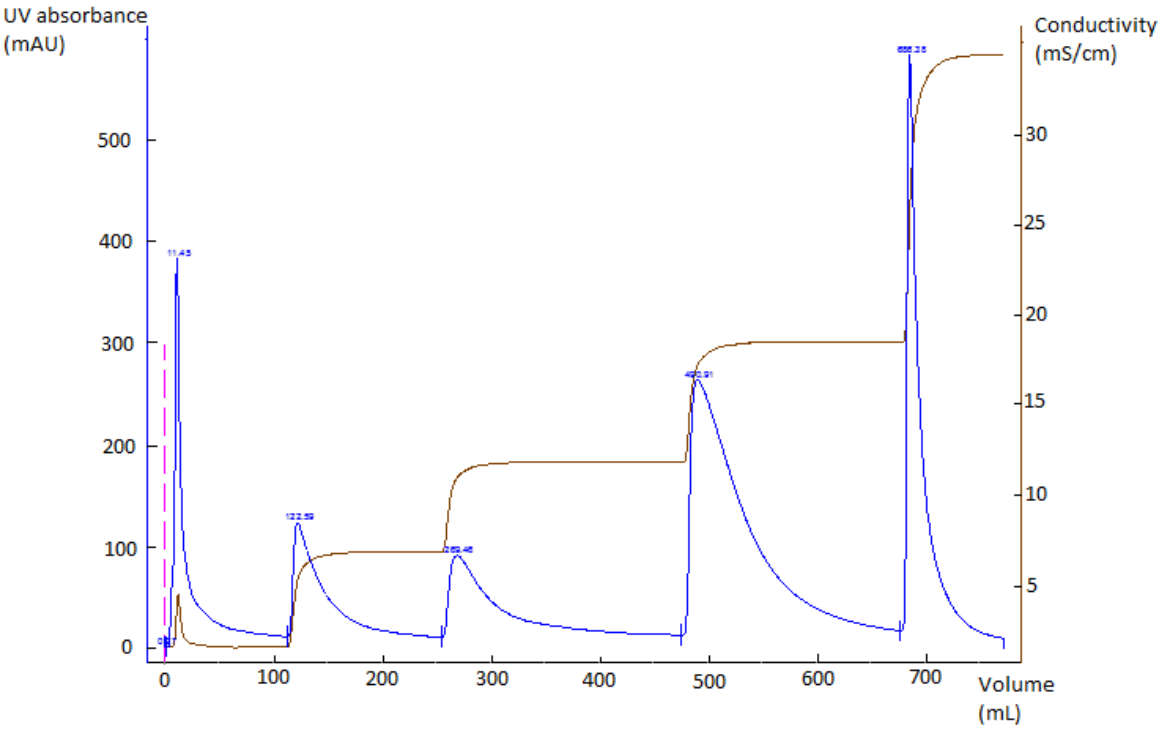

Figure 4.4: Ion exchange chromatography of the 0% ethanol reconstituted supernatant

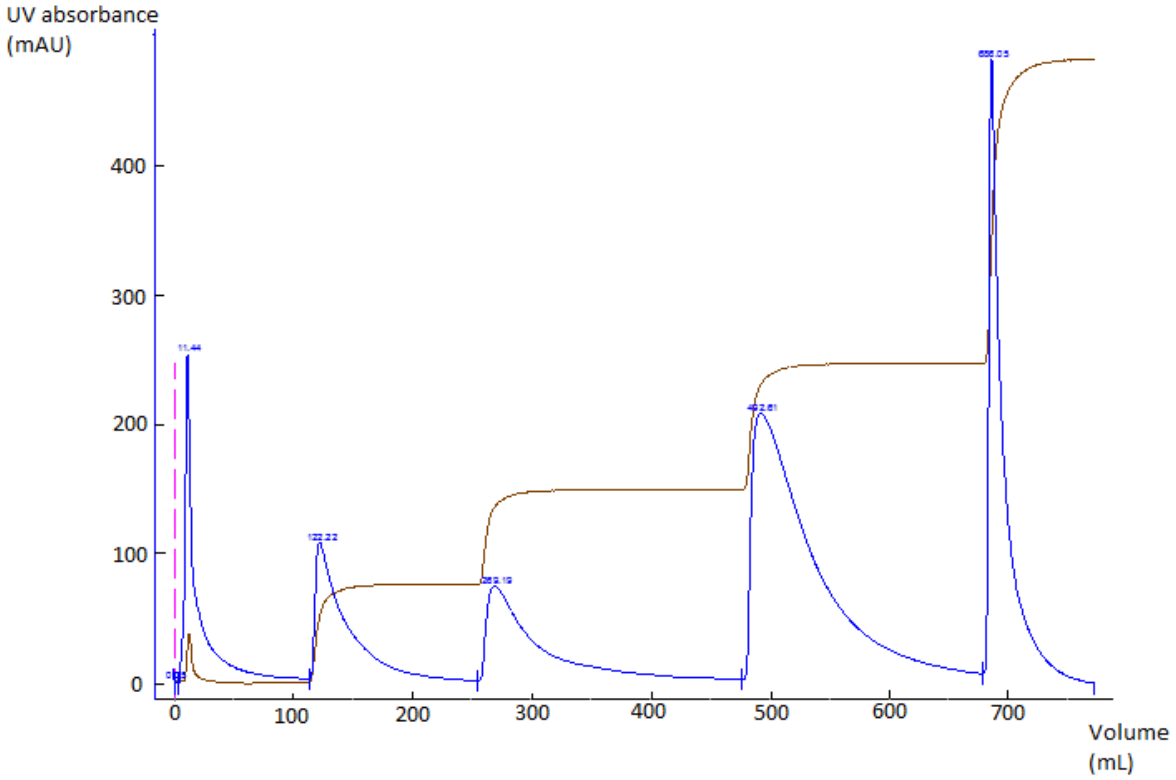

Figure 4.5: Ion exchange chromatography of the 20% ethanol reconstituted supernatant

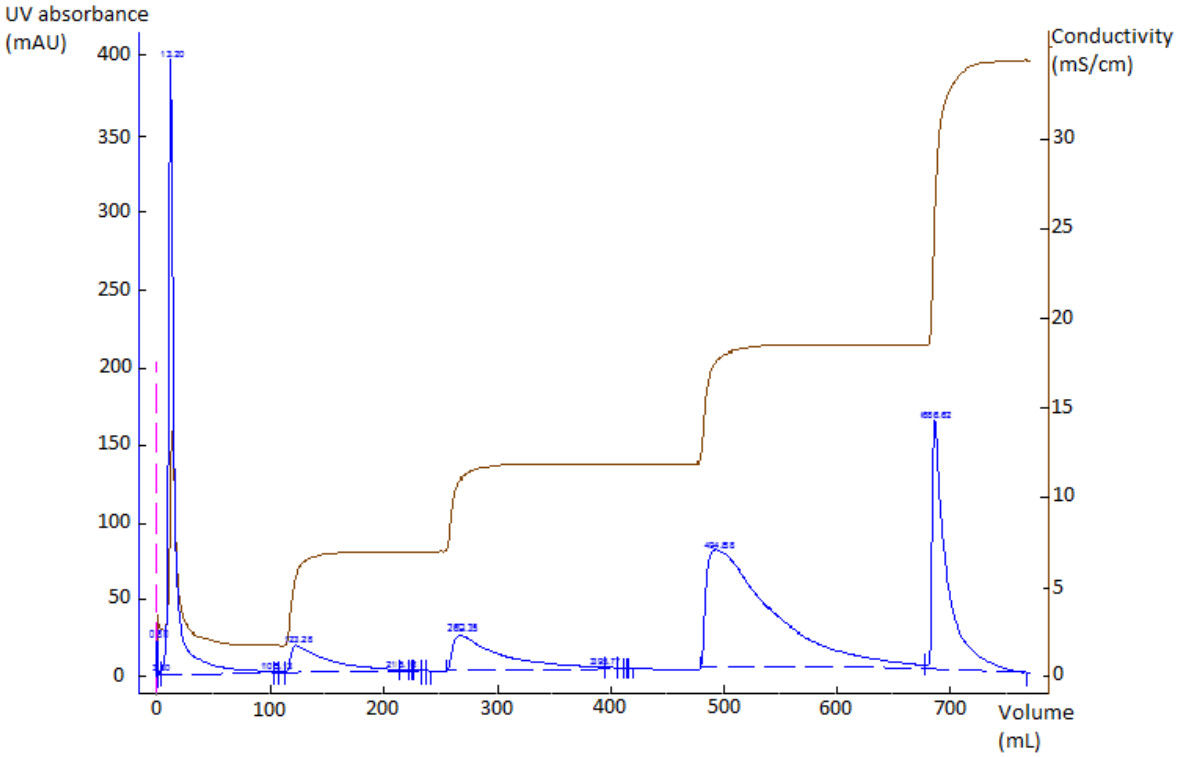

Figure 4.6: 30% ethanol supernatant run on ion exchange steps established in Chapter 3 (after freeze-drying and reconstitution)

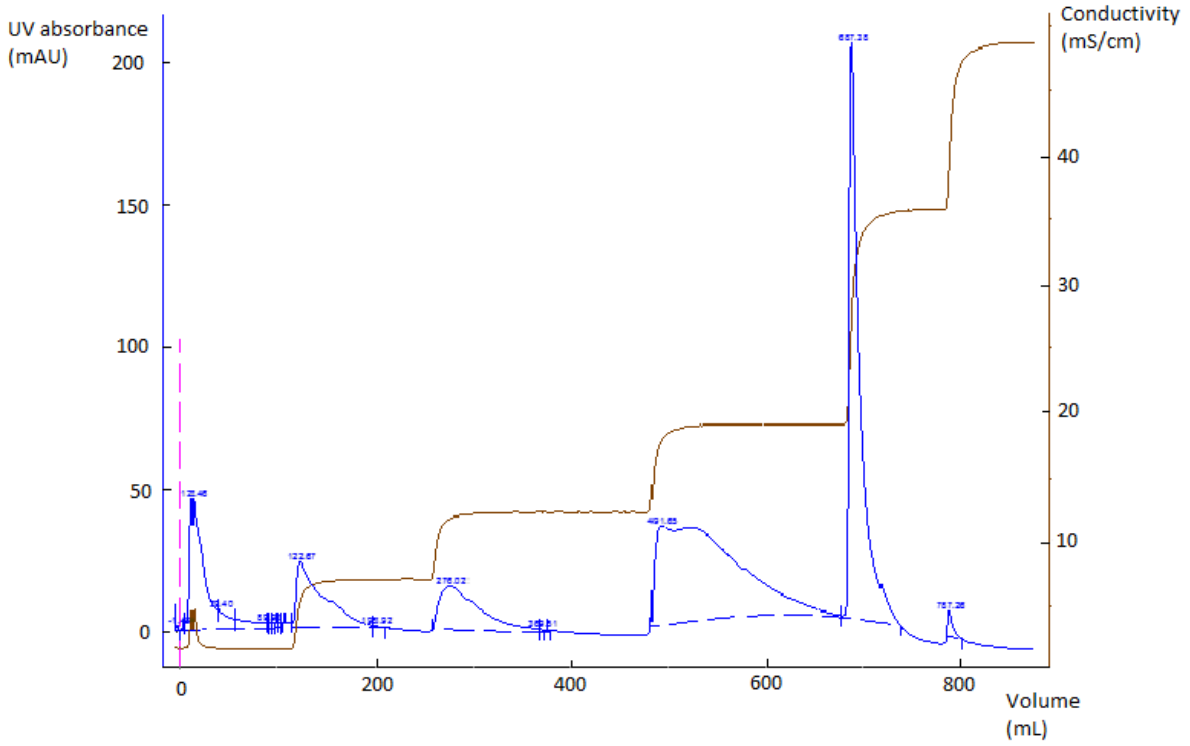

Figure 4.7: Ion exchange chromatography of the 40% ethanol reconstituted supernatant

Table 4.6: The areas under the curves of the freeze-dried supernatant

Salt concentration (mM)	0	10	%	20	30	%	40	%
0	4597.4	2715.8	59.1	2772.9	2795.9	60.8	633.51	13.8
50	4931.9	3233.8	65.6	679.09	1121.7	22.7	737.52	15.0
100	6379.4	3296.1	51.7	1006.03	2132.2	33.4	737.72	11.6
170	17369.1	12071.2	69.5	2109.09	6428.2	37.0	3303.14	19.0
350	9171.3	6595.4	71.9	1195.66	2826.5	30.8	2648.77	28.9

4.3 Albumin separation methods

Separations were trialled using the Kistler-Nitschmann method as a basis. The reason this step was selected was because of the high purity of albumin obtained. Even though the yield has been noted to be lower than other methods, any extraction would be beneficial.

Sodium acetate buffer was made up as described previously, with the pH kept at 5.2. This buffer was mixed with blood plasma and ethanol in the volumes shown in Table 4.7 to obtain an ethanol concentration of 19% and the pH was then adjusted to 5.85. This mixture was left overnight at 4°C.

The precipitate was then removed by centrifugation and additional ethanol was added to increase the ethanol concentration to 40%. The pH was once again adjusted to 5.85, and after a short period of mixing this mixture was left overnight at the same temperature. The precipitated was similarly removed and the pH was adjusted to 4.8 for another period of mixing and overnight incubation.

After this stage, these supernatants were run through ion-exchange chromatography in a similar manner to the blood plasma in Chapter 3. This was done in an attempt to determine the composition of the supernatant rather than for separation purposes. The results are given in Figure 4.8. Gels run for the various stages can be seen in Figure 4.9, while the area underneath the curves compared to a 0% ethanol concentration is given in Table 4.8. It is worth noting that the conductivity of this run was modified in an attempt to resolve the non-albumin separations in as timely a manner as could be achieved. For this reason, the steps below 120 mM NaCl were not carried out, aside from the original 0 mM step.

Table 4.7: The composition of the Kistler-Nitschmann separations.

Component	Quantity (mL)
Blood Plasma	3.55
Buffer	0.5
Ethanol (added at 1st step)	0.95
Ethanol (added after 1st step)	1.67

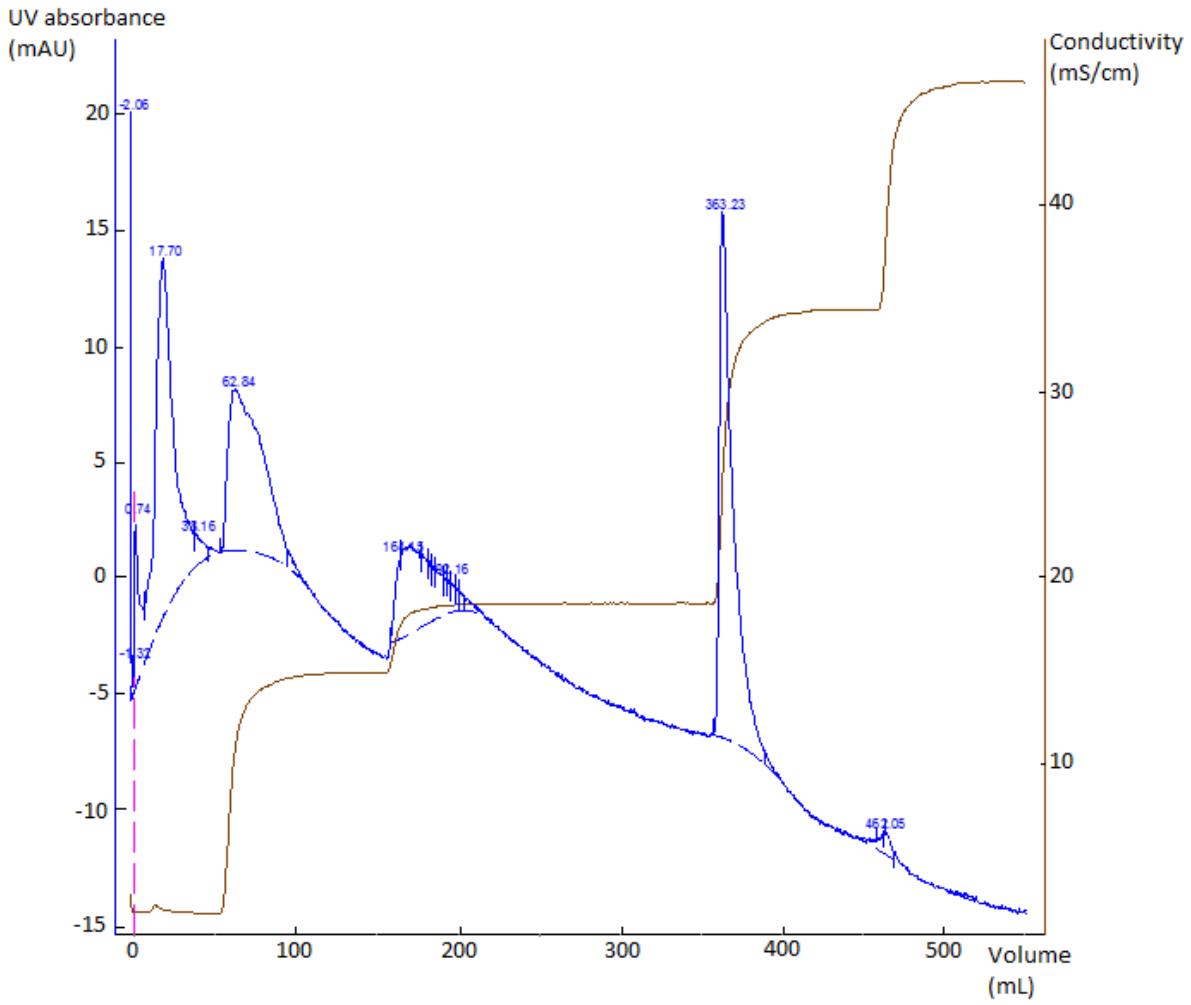

Figure 4.8: The effect of the Kistler-Nitschmann process on the profile of the blood plasma.

Table 4.8: The areas underneath each peak and their proportion of the total

Peaks	1	2	3	4
Area (mAU*mL)	210	171	104	226
Proportion	30%	24%	15%	32%
Proportions of 0% ethanol	11%	27%	41%	22%

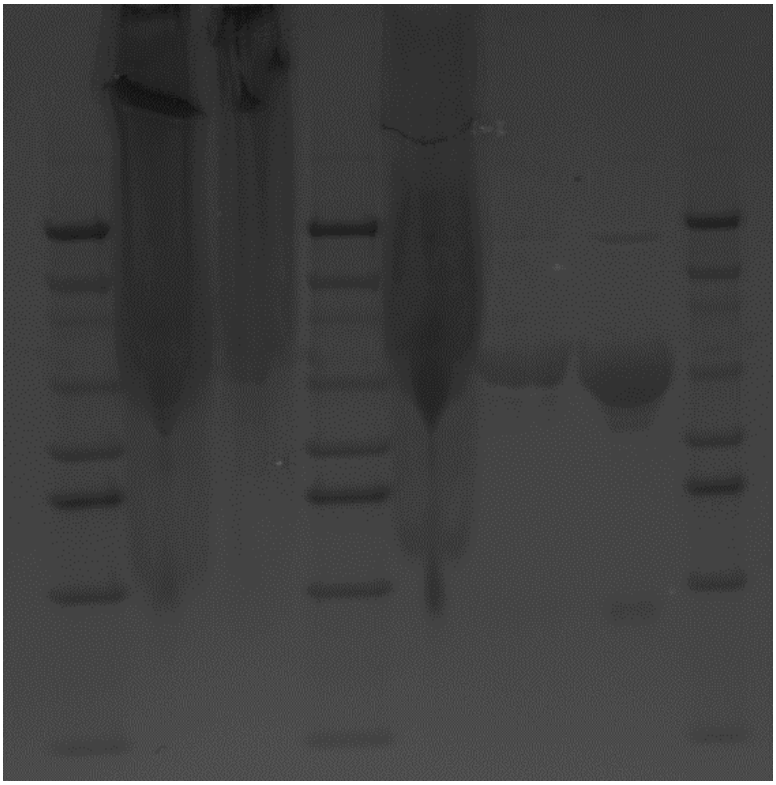

Figure 4.9: Gels of the various stages of Kistler-Nitschmann separations

Chapter 5.0 Discussion

5.1 Protein identification

While most of the results given from the mass spectrometry experiments did not give specific identification of proteins because of difficulties with gel bands having a too low protein concentration to identify, equine serum albumin was able to be identified successfully. Its corresponding band can be seen in Figure 5.1. Although its position was unsurprising, given the large amount of the protein within the gel and its molecular mass, it is still useful to have this as a base with which to compare other proteins.

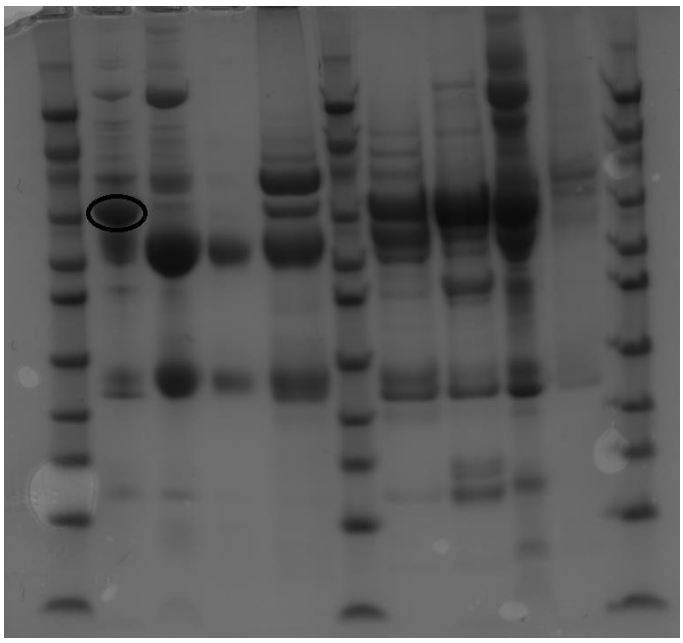

Figure 5.1: Equine Serum Albumin band circled in the diluted plasma after MS identification

This identification is important because a number of other proteins can be potentially identified from this band. For instance, immunoglobulin bands generally give a distinct pattern that can be seen in Figure 5.2. Other proteins can be determined thanks to some prior work in the field. For instance, the protein band at around 80 kDa is likely to be transferrin, based on the concentration and the location of the molecular weight band [114]. For similar reasons, the band at around 50 kDa that is not immunoglobulin appears to be antitrypsin [148] while the band at 40 kDa is possibly haptoglobin.

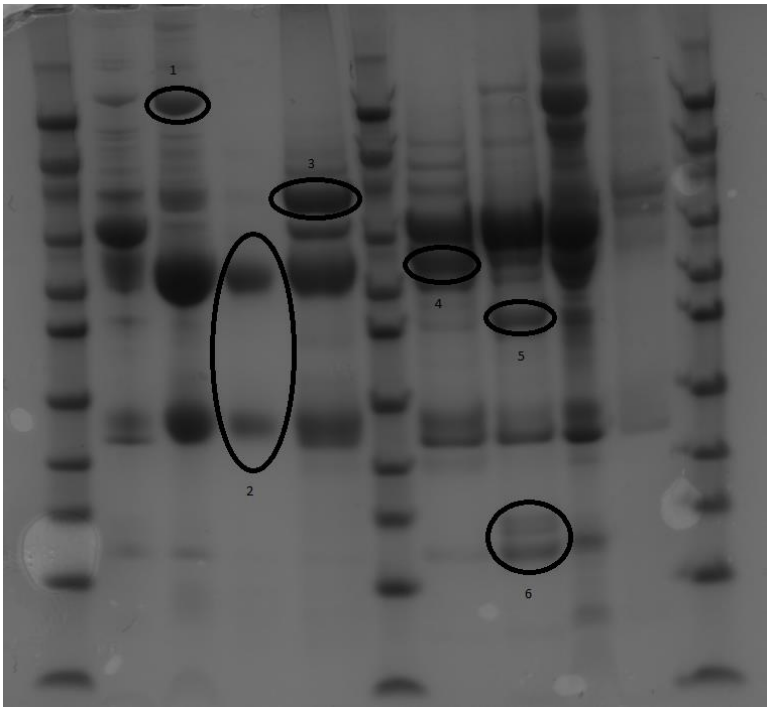

Figure 5.2: Long separation with protein estimations. The circled proteins match the following: (1) Immunoglobulin (2) Immunoglobulins; (3) Transferrin; (4) Antitrypsin; (5) Haptoglobin (6) Immunoglobulins

These protein identifications are only educated guesses and it is recommended that they be identified with more certainty further work. Given that immunoglobulins, for instance, can be identified this suggests a number of separation principles to be used. Immunoglobulins can be seen to elute earlier in the ion exchange gels, which means that ion exchange chromatography could be used to separate these immunoglobulins.

The key dilemma is that, unlike human plasma, there is not an existing market for large scale production of many of these proteins. Haemophilia is not a major veterinary priority for the equine industry, and many such factors extracted from human plasma are irrelevant for purification from equine plasma, primarily Factor VII. Some proteins may be able to act as substitutes for bovine proteins in similar fields, but the scope for these is generally limited to research purposes. This seems an extremely limited market for large-scale production. Likewise, the potential applications of lactoferrin, a transferrin protein, in nutraceuticals, medicine and food products [149] are limited by cultural biases against the consumption of horsemeat. Thus, determining practical uses for what are essentially waste products is rather difficult. While some may be used as purified research standards, it is difficult to imagine such a level of demand from this sector that justifies the industrial scale separation of these on a regular basis.

5.2 Comparison of different salt gradient steps

The salt gradients that have proven effective in bovine plasma were equally useful at separating equine plasma. The proteins obtained as shown in Figure 3.2 tended to be similar to their bovine counterparts, which is unsurprising. As in Moure [27], immunoglobulins were separated at low ethanol levels, while albumin was separated around the 170 mM level. This suggests a means of separation that could conceivably be used on a large scale.

The salt gradient steps shown in Figures 3.2 and 3.3 differ in the lower concentration salt steps. The key differences involved were in the intermediate proteins. Primarily, antitrypsin was better separated with increased salt gradients. However, this is generally not a critical protein for industrial separation, meaning that it affected the purities of other proteins more than any other factor. This could be rectified by further processing, indicating that this step is somewhat redundant. For this reason, the gradients obtained by Moure are most likely preferable, unless purifying antitrypsin is a priority.

The key proteins, as mentioned above, are the various globulin proteins, because these are likely to have the greatest commercial value. As their separation is not significantly improved by the increased number of steps, this indicates that the additional steps may not be necessary to the process. Likewise, the separation of albumin was not substantially improved with the additional steps, and in fact may be negatively impacted because of the increased volumes to separate downstream.

This redundancy is magnified by the effect of running an increased number of salt concentrations. The additional salt concentrations may mean less process difficulty in separating certain proteins downstream, but it is unlikely that there will be significantly fewer separation steps as an overall result. In addition, the extra time and buffer needed to run these steps all represent extra costs in an industrial process. Thus, unless some significant use for the proteins that the additional salt concentrations allow better separation for is found, it is probable that fewer salt steps are likely to be more efficient.

5.3 Proteins of interest

Immunoglobulins are clearly shown to elute at lower salt concentrations, with albumin eluting at much higher concentrations. This is consistent with data from Moure [27] and the respective pI values of the proteins. For a scheme aiming to utilise the use of antibodies or $F(ab)_2$, an ion-exchange processing step may be a valid first separation step, as little increase in conductivity is necessary for the elution of these proteins. Meanwhile, the higher salt concentrations needed to separate albumin mean that that is less suited to this process. eCG is difficult to determine due to its low total within the proteome.

Albumin clearly elutes at higher salt concentrations, primarily at 350 mM NaCl. However, the purity involved is considerably lower than for immunoglobulins. Many of the proteins that elute with albumin

are not of commercial value, so removing them through standard means such as heat shock is still feasible.

5.4 Process improvements

5.4.1 Buffer use and volume

Reducing the quantity of buffer used in the ion exchange step is a key consideration for adapting it to an industrial applications. With a minimum of around 750 mL of buffer used for a 20 mL column and 2 mL sample, the extremely large amounts of buffer required for separation would create considerable additional expense. A 400 L column and sample, for instance, may require 15 000 L of buffer if a proportional amount is used. The biggest reason for this use of buffer is the lack of sharp peaks obtained, requiring large amounts of tailing that requires a considerable amount of buffer to separate.

The height equivalent of the theoretical plate (HETP) values of the various peaks provides further evidence of the excessive use of buffer within the process. With the exception of the initial peak, all of the peaks' HETP values are very low, indicating the extensive tailing of the process. Additionally, when the measurement of the HETP is changed to only use the elution length at half of the maximum absorbance, the values are dramatically different. The difference in these values is due to the apparent changes in the number of theoretical plates, which is again indicative of the effects of peak tailing. Table 5.1 shows both chromatography runs (long referring to the set of salt concentrations with the longer elution period) with both the width of the full peak and that of the width over half the maximum absorbance (represented by W and $W_{1/2}$ respectively).

Table 5.1: The HETP values for the ion-exchange chromatography runs using full width and width at half the height of each peak

HETP values (m)	0 mM	30 mM	50 mM	80 mM	100 mM	130 mM	170 mM	350 mM
long W	0.98	0.013		0.0048		0.0031	0.00035	0.000024
long $W_{1/2}$	0.11	0.0023		0.0016		0.0014	0.000062	0.000015
Short W	0.98		0.011		0.0049		0.0011	0.00014
Short $W_{1/2}$	0.11		0.0025		0.0029		0.00021	0.000016

Attempts can be made to appropriately reducing the elution volume, as shown in Table 3.4. This demonstrates the extent of the peak lengths that can be reduced while still retaining much of the protein. Although the initial immunoglobulin peak has one of the highest depletion rates per volume reduced, it also requires the least volume of the various peaks, meaning that further reducing the elution volume is less critical than for other peaks. Furthermore, peaks could be combined to reduce this effect, for instance using only 30 mM and 170 mM NaCl steps. Such methods would require further

processing downstream of this chromatography operation. All of these efforts are ultimately partial solutions to the problem of large quantities of buffer, but would also significantly diminish the amount of raw materials used in the process. However, in order to reduce the amount of raw materials, chromatography should be preferred only when these options are able to be easily carried out or with smaller volumes of plasma.

The resolution of the peaks were all fairly similar to each other. However, none were especially high, with the highest being 0.31 for the 350 mM peak shown in Figure 3.2. This is far below what is generally considered acceptable in industrial levels. The most likely cause of this was that peak tailing was extremely evident within the various runs. To check a possible causes of this, a run of only BSA was made, under otherwise identical conditions to the equine plasma runs. BSA was selected because of its similarity to ESA, which as the dominant protein in equine blood plasma would give the best approximation of standard blood plasma.

In this run, tailing was almost entirely eliminated. This strongly indicates that tailing is a specific behaviour of the blood plasma as opposed to the column or process. The consequence of this is that tailing may be inevitable when processing blood plasma. Improving resolution between the various peaks is therefore a matter of improving the sample quality, which may not be possible for raw plasma. For this reason, ion exchange chromatography is unlikely to be considered suitable for initial separation. Processes further downstream may instead be a better position for this technique.

5.4.2 Scale-up

Scale-up operations were proven to work, with the same concentrations yielding essentially similar results as can be seen by the similarities between Figures 3.1 and 3.2. Of particular note was that the length of elution proved particularly long for many of the steps. This length was shortened to avoid excessive use of buffer, with negligible effects on the final results. For this reason, further shortening may be possible for further scale-up.

Biopharma [128] noted several parameters and their effects on scale-up. Key amongst these is to maintain constant linear velocity, length and sample concentration. With pure blood plasma being used, sample concentration is unlikely to change outside of individual fluctuations within the donor horses. While these can be minimized through means such as consistency of age of the horses, such measures were outside the scope of this investigation. Column length was more complex, given the comparatively short columns used. As it has been established that most columns have a maximum length of 0.2 m, it was decided that that would be sufficient for the total length in a scaled-up model.

5.4.3 Sample size

Figure 3.5 demonstrates a clear relationship between the size of the samples and the elution fractions. This is an indicator that the initial sample size of 10% of the column volume is not at the column's capacity. However, there is a loss of binding capacity evident with the increased sample size. Table 3.3 shows that the change in the amount of protein eluted at each stage is not directly proportional to the change in sample size.

The only fraction of which this may not be a direct representative is the initial peak. These fractions are also well below proportional levels, but the key difference is that in each case the UV detection limit was surpassed. This indicates that the "missing" protein may not be binding to the column. While it is also possible that the noted protein is remaining on the column for the entire process, post-operation cleaning indicates that that is unlikely. Losing blood proteins in the initial wash stage of chromatography is not necessarily a critical impediment to using larger sample sizes by proportion of the column volume, but the losses caused by this effect would have to be considered in any use of larger scale chromatography. The other factor to consider is that the relationship between the protein concentration and the UV absorbance is not linear at higher concentrations. This may contribute to the apparent gap in expected and actual concentrations.

Interestingly, the larger volume of plasma seems to indicate the presence of an additional protein group. Without the use of gels or some other identification method, it is difficult to determine what, if any, proteins are within this secondary peak. It is worth noting that the peak in question has a combination of immunoglobulin and albumin present. This means that said split may be simply the consequence of better separation between the two. However, the presence of transferrin complicates this, making overall conclusions difficult to make. Otherwise, there seem to be few differences in the chromatographic profile. This indicates that sample size can be increased easily as part of scale up without significant negative effect.

5.4.4 Speed of Elution

Speed of elution was shown to have a minimal impact on the separation. Figure 3.4 shows little change in the chromatographic profile despite the altered speed of elution. While there is some tightening of bands, there is less significant effect on the maximum absorbance and total protein eluted at various stages. This indicates that the chromatographic speed has a minimal impact on the overall separation with regard to peak appearance.

However, flow rate was also noted to have a key impact on the pressure of the column. There was a positive correlation between the speed of elution and the pressure drop over the column, which will be discussed in greater detail. Additionally, an increased speed of elution equates to a faster process overall; an obvious distinction, but one that is critical at an industrial level due to the extra cost in labour and utilities a longer elution requires. This creates a key dilemma, as an excessive pressure drop can have significant negative effects on the long-term viability of chromatography columns by

compressing the media and reducing the porosity and thus the overall binding area. This would result in a reduction in the separation capacity of the column.

5.4.5 Pressure

One of the key issues in ensuring scale-up is successful is ensuring that column pressure remains at acceptable levels. Over the scale-up that occurred in this thesis, the pressure drop increased greatly, with 350 kPa common when running at 240 cm/h, rather near the pressure limit of the column. Figure 5.3 shows the pressure while being run on 20 mL columns. This compares to only 10 kPa on the 1 mL column, at a speed of 250 cm/h. Decreasing the elution flow rate was noted to have reduced the pressure drop, but this is not an ideal method at an industrial scale. It was also noted that while linear velocity was one of the factors which should be kept constant according to Biopharma's recommendations, industrial columns in other industries are often run at much higher linear velocities.

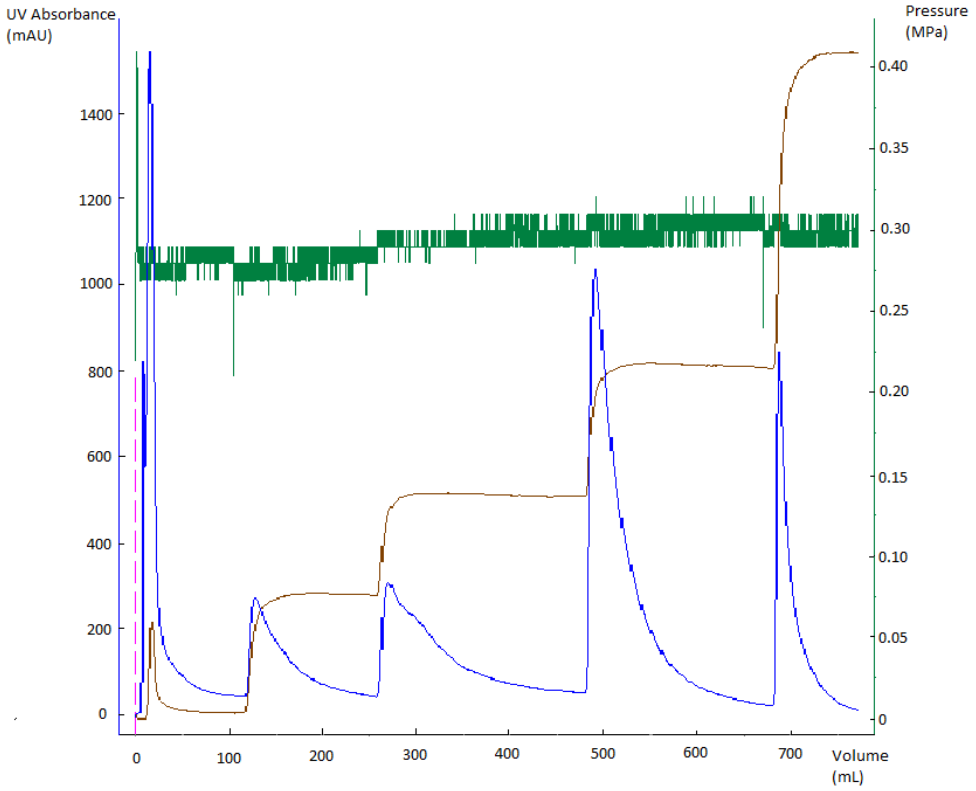

Figure 5.3: A demonstration of typical pressure levels at 20 mL chromatography

The Kozeny-Carman equation is given as Equation 5.1. This relation is considered a reliable indicator of scale-up pressure [150]. The key aspect of it is void volume, which is not given by GE Healthcare and is difficult to directly measure. To determine this, the pressure drop over the runs was substituted to obtain the voidage of the column. This was verified by checking the pressure drop over the alternate runs.

$$\Delta P = u_0 L \frac{150\mu}{{d_p}^2} \frac{(1-\varepsilon)^2}{\varepsilon^2} \quad (1)$$

The voidage obtained was around 0.052, which as an approximation was used for the rest of the scale-up. Applying this to a column that is 20 cm long (approximately the maximum length of industrial columns) and increasing the flow rate to 300 cm/h would give a pressure of around 1 MPa. This pressure is not exceptionally high for HPLC, though it is much higher than the levels which are recommended for laboratory-scale ion-exchange chromatography. The most logical method of reducing this pressure would be to increase either the voidage within the chromatographic media or the particle diameter. Given the availability of larger beads designed for large-scale chromatography, particle diameter is the easier parameter to change [151]. This would have the disadvantage of reducing resolution, but larger columns and samples tend to reduce the resolution. In addition, little change is needed to ensure a column run at the same flow rate would have an acceptable pressure, as a particle diameter of 150 µm or more would reduce the pressure drop to levels found in the 20 mL chromatography operation. Such sized beads are easily obtainable; GE Healthcare for instance advertises certain media with an average 200 µm diameter.

Curling [73] has noted that modern chromatography regularly handles elution speeds up to and including 700 cm/h. This is clearly considerably higher than what has been assumed here. Raising the speeds to these levels while maintaining the pressure drop established would require increasing the particle size to around 220 µm diameter, slightly beyond this average size. It is worth noting that the linear velocity is one of the factors advised to be held constant.

Flow rates compare somewhat differently for a larger column. The diameter of the column is the ideal aspect to increase, due the smaller effect it has on chromatographic separation. This can be seen in Equation 1, where increased column diameter has no effect on the pressure. Equation 2 demonstrates the way that the diameter can be adapted for the increased scale of the column

$$V_f = V_i \frac{{D_f}^2}{{D_i}^2} \frac{L_f}{L_i} \quad (2)$$

Using Equation 2, the diameter of a scaled-up column can be determined from the volume of the blood and the mentioned change in column length. With a standard process of 2000 L, which is a common raw human blood plasma batch [152], a column would have to be 11.3 m in diameter. Such a column would be extremely large by industrial standards, further suggesting that this chromatography operation is better used when the volume of the inlet is considerably reduced. Reducing the inlet volume to around 400 L decreases the required diameter under the same conditions to 5 m. This is still much larger than most industrial columns, which tend to have a maximum diameter

of 2 m. However, this would mean that only 6 to 7 parallel columns are required for the same column volume, instead of around 30.

5.5 Examination of ethanol concentration effects

One of the key problems found with analysing these precipitations was the ethanol interfering with the gel. Many of the high ethanol samples (above approximately 20%) would not run easily on the gel, causing the supernatants to appear far more dilute than what they actually were. This mostly affected relevant concentrations of the proteins present, particularly those that were less abundant before precipitation. However, there are still some indicators of key proteins from the gels, even if concentrations are difficult to determine. Chromatography was used partially to avoid the dilution, but this was instead affected by the reduction in the base line. In addition, it proved more difficult to determine exactly which proteins were precipitating at which stages with chromatography than with the gels.

The gel indicated that most proteins were removed from the blood plasma at an equal rate, although there were clear problems with the consistency of this with the masses of the various gels. While the less common proteins seemed to precipitate first, this may be simply a matter of resolution over any chemical factor. The proteins precipitating at no ethanol levels was most likely the cryo-precipitate, given that this process had not been carried out prior to the ethanol experiments.

There was a clear correlation between the ethanol levels (with the exception of one outlying point) and the amount of precipitant within mass measurements as given in Table 4.2, however. The only exception to this was at the upper extremes of ethanol concentration tested, where separation was independent to the level of ethanol. This indicated that after a certain point, there is little advantage in adding additional ethanol to the solution. Freeze-drying, as anticipated, gave a greater degree of accuracy in the trends noticed. Although the relationship was not a linear one, this can be explained by to the different proteins' responses to the ethanol.

The size exclusion chromatography carried out contained two main peaks as shown in Figure 4.1. The gels also in this figure indicate that these peaks are albumin and immunoglobulins, a conclusion supported by the literature [82]. The reduction of these peaks as the ethanol concentration was increased is also consistent with literature and with the rest of the results. More detailed interpretations are complicated by the presence of air bubbles within the 20% sample. Interestingly, the initial peak increased with increasing ethanol concentration, although the secondary peak vanished with this concentration increase. This may be due to aggregation of the protein due to precipitation.

Chromatographic investigation of the supernatant indicates that the albumin was the most soluble in ethanol, with other proteins removed with lower concentrations. This can be seen from the chromatographic results of Figures 4.4 through 4.7, which show a distinct decrease in all of the peaks, but particularly in the lower salt concentrations which have little to no albumin. This is best seen in the difference between Figures 4.5 and 4.6, which shows a dramatic reduction of the non-albumin peaks in comparison to the others.

However, analysis of the precipitates also indicated that considerable amounts of albumin were present in the precipitate. The reductions of the higher salt concentration peaks support this hypothesis. This correlates well with previous work that notes that albumin generally can be purified from lower ethanol concentration precipitates. This could be an option for increasing the process yield should ESA be a target for further purifications.

The supernatants of the blood plasma showed the same reduction in protein concentration. What is more interesting from a practical perspective is the lessened amount of albumin, given that this is generally the protein that is targeted to be retained. This retention appeared to drop at higher percentages of ethanol.

5.6 Kistler-Nitschmann separations

The results of the Kistler-Nitschmann separations were also affected by the side effects of the ethanol in the samples. This can be seen from the chromatography in Figure 4.8, where the calculation baseline reduced with increasing ethanol, and from the gel, where difficulties in loading samples resulted in the second stage appearing to have a lower protein concentration than the final stage. Given that these samples were both obtained from the supernatant, and a precipitate was observed within the sample in the final stage, additional protein in the last step that does not appear in the middle is clearly not possible. However, it is still possible to determine a number of details from these results.

The gel run indicates that the albumin has been retained in solution far more than any other proteins. While the obvious intensity change is partially due to precipitation and slight dilution, the immunoglobulin bands are much less intense by comparison to the albumin bands. This indicates that the albumin was better retained within the sample, as expected and supported by literature [29].

The chromatography gives slightly different results; however this is affected by the calculation software. The two peaks with the highest salt concentration in Figure 4.8, particularly the final one, have been shown to have most of the albumin in the ion-exchange experiments already detailed. The final peak contained a larger proportion of the total area, but the penultimate peak did not. This may be due to the precipitation of other proteins that would normally appear within this peak rather than

precipitated albumin. The low salt peak was dramatically reduced, but the initial peak increased. This could be because an increasing number of aggregation within the sample, causing a lack of binding.

One of the factors not tested for was recovery of albumin from the precipitate. Part of the reason for this is that ESA is not as commercially valuable as HSA, leaving less incentive to increase yields through albumin recovery. There are indications that not all albumin has been retained in the supernatant, most notably within the gels. Although this lack of value could be seen as an issue, the separations still have value as a method of separating other proteins from albumin, as opposed to the reverse. Should albumin need to be extracted, on the other hand, further processing most likely will be needed. This is due to the high purity (>95%) generally required for research, which is the biggest market for ESA. With heat shock being commonly used, this may be an option although there may be increased costs due to the higher volume of raw material.

5.6.1 Scale-up

Unlike chromatography, scale up was not directly tested, partially because of the considerable literature on similar technologies at an industrial level. The largest challenge with this is the fundamentally different methods of mixing. Because of the size of the samples, mixing could be carried out by rotating the entire container, while industrially impellers are preferred due to cost. However, testing was carried out at a number of different scales, ultimately giving some perspective, although the degree of difference was considerably lower (5x maximum) than the chromatography scale-up. In addition, some options can be determined from the literature.

The biggest impact that scale-up had was in the mixing stages, which took considerably longer with larger volumes. There did not appear to be any other negative effect on the results. Part of the reason for this is that the method did not have to be altered for any of these steps. With larger volumes, the method used may change and result in a considerably larger impact on separation.

While transferring to a larger tank would be a challenge, particularly in terms of ensuring that the impeller would not negatively affect the proteins, this could well be overcome. Thus, ethanol precipitations are more likely than ion-exchange chromatography to be a good initial step due to easier operation at higher volumes. Interestingly, few investigations centre on tank reactor scale up in the context of blood plasma. There is a wealth of literature on scale up of stirred tank bioreactors instead, which share many characteristics with precipitation tanks. However, there are several advantages in separating blood plasma over other common aspects of bioreactors. The most obvious is that oxygen transfer, traditionally a key concern, is unnecessary because no direct chemical reactions are taking place. The largest concern is potential damage to the proteins, which can be reduced with a large impeller at a low rotational velocity. The key advantage ethanol precipitation has over chromatography, however, is the fact that tank height is considerably less of a restriction than

for chromatography, allowing for a much lower cost in large scale use. This perhaps indicates the suitability of ethanol precipitations for an initial stage of separation.

For a 2000 L batch, a tank of around 1.15 m diameter and 2.88 m height would be sufficient to provide plenty of room and conform to an effective diameter to height ratio. This would require a 0.3 m impeller that causes a low shear force on the fluid. For instance, a marine impeller may be appropriate. However, this tank would be around 3000 L, considerably larger than required. The height, for instance, could realistically be reduced to 2.30 m without major effect on the mixing ratio, giving a volume of around 2070 L. This is perhaps a little small, giving little room for potential spillage. However, the foaming that is traditionally a concern for bioreactors is not an issue here, meaning less margin for this will be required.

5.7 A universal separation method for equine plasma

The critical element to this study was to establish a potential method for separation of whole horse plasma. Both ethanol precipitations and ion-exchange chromatography have been shown to do this. Technical issues, however, tend to favour precipitation, as it is easier and cheaper to scale up than chromatography. This can be seen by the volumes of buffer required for the chromatographic separation of blood. Though ethanol precipitations have been primarily used for albumin purification as seen in the literature, uses in separation for immunoglobulins have also been clearly demonstrated. As with any separation, scale up is an issue; however the challenges faced are far fewer than that of chromatography. For these reasons, chromatography should be used as a secondary step after any precipitations, in order to reduce the volumes and thus costs of operation. However, there were also issues found with the purity of products, particularly from one-stage separations.

As previously mentioned, Burnouf has noted the use of cryo-precipitation at the initial stage of human blood plasma separation. Unless fibrinogen is a target of separation, it is unlikely that this technique would be of any assistance for horse plasma. Few other proteins can be easily obtained from cryo-precipitation that are likely to have any commercial applications. An ethanol precipitation step is more cost-effective, as the proteins separated are far more valuable. Primarily, this precipitation step should aim to separate immunoglobulins as quickly as possible, due to their increased value and separation at lower levels of ethanol and chromatography.

5.7.1 Immunoglobulin waste recovery scheme

The key to an immunoglobulin production process is identifying the proteins available that will give the most alternative value. eCG stands as a potential option in such a scheme, while albumin may also

be able to be separated with some ease. Notably, the techniques normally used for immunoglobulin separation are not conductive to the activity of other proteins, meaning that any changes to techniques will likely have to be used as initial steps.

IEC would be an outstanding first step, should the scale of operation be around 500 L or less. An 80 mM NaCl concentration would allow for near total separation of Ig immunoglobulins from other blood products, meaning that all others could be processed as necessary while the immunoglobulins are either precipitated or run through affinity chromatography. However, this would be extremely expensive in terms of volume and quantities of buffer consumed if this were a first step with a much larger plasma volume.

A classic example of a process to adjust would be from Simisiriwong [87]. In this, the first step was a pepsin digestion at pH 7.4 using a Tris buffer. Ideally, similar conditions could be achieved from a chromatography step, due to the similarities in the buffers. Thus the first operation could be chromatography as described, should this prove economically viable. The immunoglobulins eluted could then be further purified using the techniques already established in the mentioned study. Meanwhile, other proteins such as ESA could be obtained by using greater concentrations of NaCl later in the elution.

Were these techniques to prove inadequate due to cost and scale restrictions of chromatography, other methods could be trialled. Ethanol precipitations, particularly at low concentrations similar to Cohn fractionation, are a prime candidate for separating large volumes of plasma, while retaining activity in all other proteins. There are two key disadvantages with this method however; a requirement to fundamentally change the immunoglobulin process already established and the difficulty in removing all solution from the precipitate. This effect can be seen from the results in Tables 4.2 and 4.5, which demonstrate a clear difference in masses after freeze-drying. Even though an equal interpretation of these results is that freeze-drying can successfully remove the excess precipitate, this operation would be expensive to repeatedly carry out on a large scale. For these reasons, chromatography should be preferred if it proves practical at the scale demanded.

Figure 5.4 provides an example of how to separate each aspect of the blood plasma. Part A demonstrates this with the use of ethanol precipitation, while Part B indicates how this would be undertaken with ion exchange chromatography. Key to this will be identifying where exactly eCG is eluted under these conditions, as this will affect the process steps after the ion exchange chromatography. This can be more easily determined by further study.

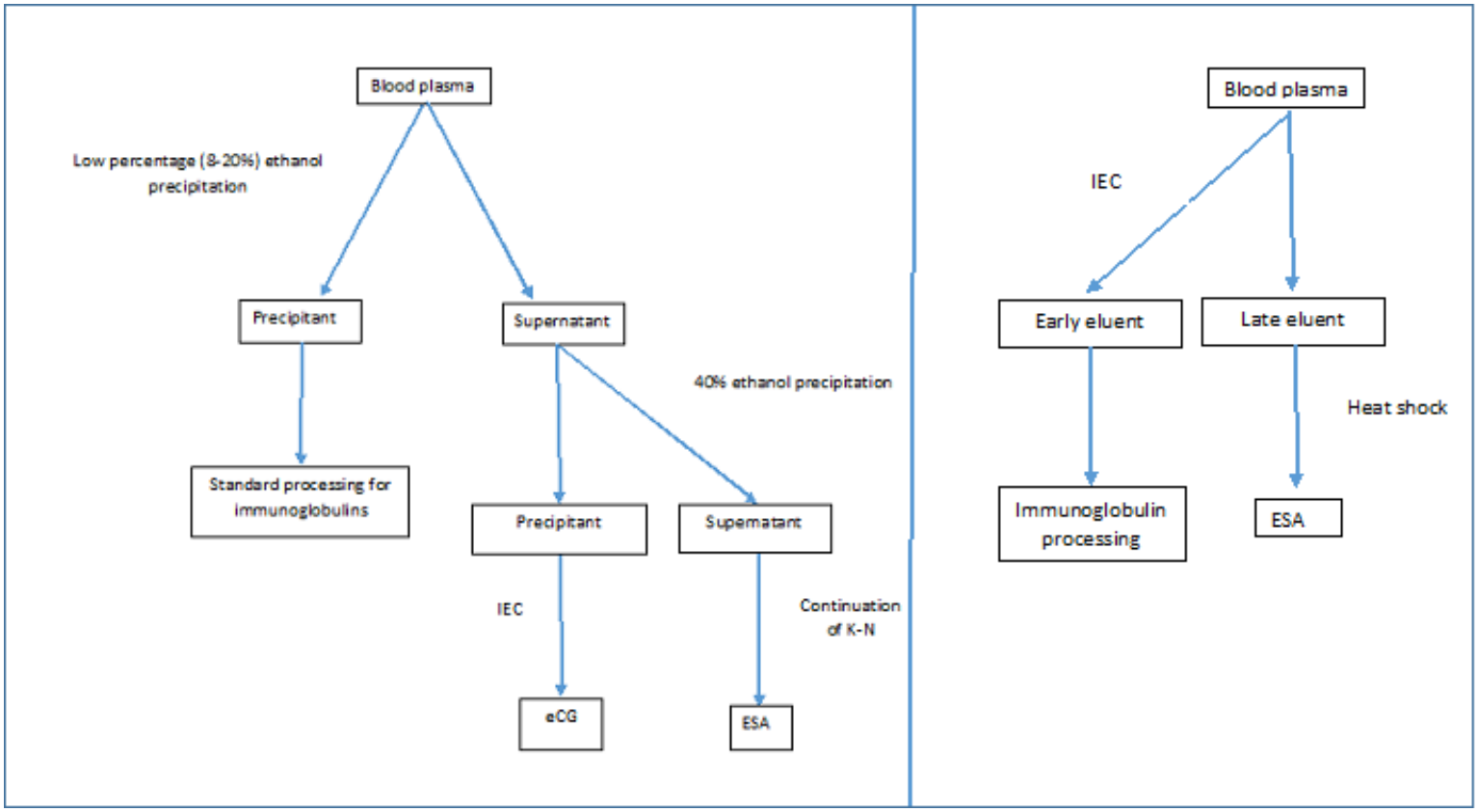

Figure 5.4: A schematic of potential modifications to immunoglobulin purification using a) ethanol precipitation and b) ion exchange chromatography

5.7.2 eCG waste recovery scheme

As previously noted, the first step in this process is generally an ethanol precipitation, somewhat simplifying the issue of waste protein recovery. Because a 20% ethanol cut would allow most of the immunoglobulins to be easily obtained from the precipitate, little change needs to be made to the initial operation. To recover the immunoglobulins from this, the precipitate could be resolubilised and processed by means such as ammonium sulphate precipitations. However, an IEC step could be added after this stage to further purify the immunoglobulins for preparation if necessary. Avoiding excessive buffer solution in the precipitate is once again a key concern.

Albumin is a more challenging prospect, due to its retention in both stages of the initial precipitation. However, the later operation of an extremely high ethanol concentration could be undertaken at a lower ethanol concentration, which would allow for both the retention of albumin, while reducing the raw material needs of the operation. However, this is a much lower priority than retaining immunoglobulins, and could be ignored if necessary.

This would give a scheme as shown by Figure 5.4 in Part A. The scheme listed is in many ways the most natural of the various combination methods, as there is no requirement of a step prior to current operating practices. In each case, additional proteins can simply be purified from the already existing waste. The biggest disadvantage, aside from the difficulty in obtaining ESA, is the harsh conditions that are generally used for eCG purification. This may result in the loss of activity of some proteins; however given that these are currently simply disposed of it will likely take major losses of activity to render the methods completely impractical.

5.7.3 Albumin waste recovery scheme

Most ESA commercially available is currently produced by heat shock. In the absence of a large scale shift in the production method of albumin, it is difficult to see how to obtain alternative proteins from this waste in any reliable fashion. This is because the heat shock method relies specifically on the deactivation of all other proteins, relying on a stabilising agent to avoid this occurring to albumin. Although this step could be delayed until after earlier separation methods, doing so would render the advantage of the lack of unnecessary steps entirely moot.

A shift to existing ethanol methods may help rectify this. Even though this is a dramatic change from the previous method, the ubiquity of ethanol precipitation in human plasma indicates that this may be a more cost-effective strategy. In this instance, a Kistler-Nitschmann method adaption may prove the best strategy. To retain as many proteins as possible, the 19% precipitate could be then be resolubilised and have immunoglobulins purified from the resulting solutions. Similar methods could be applied to the later precipitations to retain eCG. While this is a highly speculative method, the harshness of current methods make an almost entirely new strategy necessary. The supernatant could then be treated as normal for an albumin separation.

5.7.4 Initial process

Although these techniques could be used in an attempt to improve existing processes, this is of less assistance to those attempting simply to separate raw plasma as a first step. Therefore, it makes sense to examine the entire process with no particular protein in mind, to examine the best method for a hypothetical start-up.

Once again, precipitation methods are generally the best to begin with, aside from cryo-precipitation. For this reason, a Kistler-Nitschmann initial separation is the best to begin with. The 19% supernatant could then be used for albumin and eCG purification, while the precipitate could then be used for immunoglobulin production. This supernatant could be best resolved by additional steps of the Kistler-Nitschmann process, allowing for the separation of the eCG from albumin. These could then be purified by cation exchange chromatography and heat shock respectively. Alternatively, anion

exchange chromatography could be used to separate the albumin, should heating the volume of material prove too expensive for this separation. Meanwhile, the 19% precipitate could be solubilised again and separated by the method of choice in order to obtain the relevant immunoglobulins.

On the other hand, there is no large scale demand for ESA at this point in time. Although there are markets for it in research areas, this is unlikely to sustain the influx of product that large-scale production would provide. This implies that the production of this in large volumes is unnecessary. However, this could instead be replaced by some being produced to sell for the research industry.

5.8 Future directions

One of the key advantages blood plasma processing has had over other downstream processes is the absence of upstream processes involved. This has both ensured consistency in the input material and allowed a focus on the downstream steps that has been absent in other processes until recently. The result of this is a large number of precipitation methods being present for use. However, this advantage has been coupled with the lack of development in new processes.

The problems with ion exchange chromatography are evidence of this lack of new processes. One of the themes of the combined methods is that ion exchange chromatography requires considerable attention to factors such as pressure drop, uses large amounts of buffer and is difficult to optimise. These issues all make this form of separation less feasible, despite the fact that it has been shown to give clear separations. If membrane chromatography, for instance, were to be successfully developed, this may remove a large number of these problems. This would significantly alter the techniques that could be considered optimal.

The other point to consider is the applications of the proteins available. A key example of this is albumin, which is currently not extensively used from equine plasma, but is from human plasma. This may become more able to be used as the costs of downstream processing decrease. For this reason, it is critical to consider the existing applications of parallel proteins. Most of these proteins are clotting factors, which are less likely to see extensive use.

ESA has some potential to be used more extensively in the future. Unlike human albumin, it is not used for medical purposes because of a lack of economic incentive. However, as downstream processing of blood plasma becomes less expensive, this may change. Using the techniques already established may therefore be useful for production. Fibrinogen may also see use as a coagulation factor with veterinary applications, however this is currently inhibited by a lack of research into its application.

Much of this relates to the expense of downstream processing. Innovation in the use of alternative proteins is unlikely to become more common until the methods of separation become cheaper. Likewise, companies will not reduce their waste outflows if processing them for further proteins is not seen as profitable. Consequentially, there needs to be considerable movement towards more modern and cost-effective methods of separation, given the antiquated nature of much of the current separation technologies.

Chapter 6: Conclusions and future directions

The understudied nature of equine plasma has resulted in considerable amounts of waste being generated through the process because of the small number of proteins available. However, no efforts have been made to separate the blood plasma comprehensively, which was what this thesis attempted to show.

Ion exchange chromatography was demonstrated to separate blood with repeatable results and a degree of purity, particularly with regard to immunoglobulins. Because these are the most valuable proteins generally available, this gives a clear direction of separation. Even though a direction for scale up has been obtained, there are still a number of issues surrounding the use of chromatography on a large scale. In particular, the large use of buffer restricts the usability due to the potential costs. If these can be reduced through means such as smaller samples, cheaper techniques relying on the same principles or some other development, then this would be an ideal method. However, some work is still required in determining effective salt levels for better separation of albumin.

Ethanol precipitations, on the other hand, provide a far cheaper method for separation, although less effective than ion-exchange for immunoglobulins. These also have the advantage of utilising equipment most likely present in any industrial processing plant, as opposed to the more specialised requirements of chromatography. The biggest disadvantage is that from the method tested, albumin is the best separated, which is one of the least valuable proteins normally commercially separated.

One of the key limitations of this process is the lack of proteins separated from equine plasma. Increased research in downstream techniques and their adoption in industry may result in better use of this resource, but it is of note that the blood plasma industry has already taken a large number of steps to improve this. Precipitation steps are far more common and better explored within this than in other industries [153, 154]. Although membrane technologies are being heavily explored, there is still no guarantee that these will prove adaptable to the blood plasma industry. This places limitations on the strategies that can be obtained for the production of many of these proteins.

However, the advantages of the separation system determined is its conformation with current technologies while retaining the ability to be adapted as new technology arrives. New proteins determined may result in alterations in this method. Given that this may well come from low concentration proteins, it is difficult to predict precisely which processes will be optimal to separate these. Fortunately, the current system is fairly robust, with little damage being done to other proteins and thus any new proteins will likely be able to be separated from the stages already established.

Although the lack of waste processing was originally seen as a critical issue to be overcome, evidence suggests that this is at least partially because there are so few equine blood proteins with applications

when purified. However, as the production of downstream processing units becomes cheaper, many more proteins will become more likely to be produced due to the reduced economic disincentive. A key advantage of the methods trialled in this thesis is that they are often used industrially, which would reduce the costs further if existing equipment is being used.

The key areas of research in the future must therefore be to examine other proteins within the proteome to determine their effectiveness in veterinary or other applications. Developments in downstream technologies, particularly in chromatographic effectiveness, are also necessary. While the increasing studies of membrane separation may yield improved results, other means may also prove effective.

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Appendices

Appendix A: The data for the chromatography run shown in Figure 4.1

Comparison of ethanol precipitations. Data for each below

Table A1: The raw data of the 0% ethanol concentration curve in Figure 4.1

Volume point (mL)	Area (mAU*mL)	Maximum peak height (mAU)
-4.01	1.4522	288.356
-3.77	1.2020	7.394
-3.61	4.3870	6.351
0.27	1.0340	3.324
0.97	1.0246	3.765
1.78	1.4082	4.327
2.07	1.8414	4.432
3.00	2.1137	5.528
3.14	1.7265	5.753
4.00	1.0322	8.458
7.03	4.0663	106.258
8.45	54.1435	110.693
9.38	367.9850	351.409
12.89	1351.8614	1169.629
14.47	850.7763	904.813
19.36	12.9426	16.045
21.63	12.0456	17.949
23.56	14.6414	22.098
25.41	14.1278	18.426
27.79	2.1176	3.173

Total number of detected peaks 73

Total area (mAU*ml) 2708.5530

Area in evaluated peaks (mAU*ml) 2701.9292

Ratio peak area / total area 0.997554

Total peak width (ml) 19.17

Column height (cm) 30.00

Column V0 (ml) 8.00

Calculated from S200 BYPASS 291015001 0PC001:10_UV1_280nm

Baseline S200 BYPASS 291015001 0PC001:10_UV1_280nm@01,BASEM

Peak rejection on

Maximum number of peaks () 20

Table A2: The raw data of the 10% ethanol concentration curve in Figure 4.1

Volume point (mL)	Area (mAU*mL)	Maximum peak height (mAU)
-4.01	0.0112	2.251
-3.97	0.0675	2.649
-3.82	8.7661	12.041
-1.83	0.8836	3.343
-1.64	0.8868	3.325
-1.33	1.2324	3.303
-0.92	0.9979	3.313
-0.65	0.9907	3.326
-0.26	1.3483	3.361
8.48	66.7633	110.536
9.48	121.0857	112.557
12.91	1194.9544	1001.413
14.46	759.4330	766.014
19.40	0.5247	1.625
20.97	0.0059	0.093
21.67	0.1882	1.092
23.27	8.3455	7.210
24.07	5.3404	8.698
24.42	9.8066	9.368
26.24	11.2169	8.979

Total number of detected peaks 24

Total area (mAU*ml) 2192.8653

Area in evaluated peaks (mAU*ml) 2192.8490

Ratio peak area / total area 0.999993

Total peak width (ml) 18.69

Column height (cm) 30.00

Column V0 (ml) 8.00

Calculated from S200 BYPASS 291015002 10PC001:10_UV1_280nm

Baseline S200 BYPASS 291015002 10PC001:10_UV1_280nm@11,BASEM

Peak rejection on

Maximum number of peaks () 20

Table A3: The raw data for the 20% ethanol concentration curve in Figure 4.1

Volume point (mL)	Area (mAU*mL)	Maximum peak height (mAU)
-3.81	2.6534	7.298
0.42	1.6304	3.493
8.45	82.1012	109.446
13.05	289.8503	373.702
13.35	135.7308	534.204
14.62	283.9453	340.186
15.37	150.7489	650.799
19.48	1.0769	1.878
21.17	0.9302	2.290
21.81	4.1095	83.346
21.98	5.8883	94.548
22.16	4.8974	106.257
22.34	9.5270	155.730
22.54	7.5928	179.055
22.86	7.9120	180.052
23.09	10.2126	138.940
23.82	37.6859	260.904
24.30	0.9115	3.802
24.93	3.1564	4.508
25.85	2.4260	3.102

Total number of detected peaks 109

Total area (mAU*ml) 1051.8460

Area in evaluated peaks (mAU*ml) 1042.9869

Ratio peak area / total area 0.991578

Total peak width (ml) 16.33

Column height (cm) 30.00

Column V0 (ml) 8.00

Calculated from S200 BYPASS 291015003 20PC001:10_UV1_280nm

Baseline S200 BYPASS 291015003 20PC001:10_UV1_280nm@13,BASEM

Peak rejection on

Maximum number of peaks () 20

Table A4: The raw data for the 40% ethanol concentration curve in Figure 4.1

Volume point (mL)	Area (mAU*mL)	Maximum peak height (mAU)
-3.77	1.2295	2.862
0.25	0.6860	1.767
0.62	0.3361	1.346
7.85	83.6428	86.586
12.96	435.4159	397.594
14.46	478.2894	300.845
19.32	7.4597	8.637
21.02	3.7063	6.651
21.52	0.3715	2.251
23.63	0.6617	2.258
23.94	0.3838	1.828
24.28	0.5508	2.481
24.41	1.1075	2.717
26.77	0.7038	1.895
26.94	0.3866	1.585
27.37	0.6512	1.525
28.17	0.4282	1.460
28.80	0.3204	2.413
29.22	1.2821	3.660
29.30	1.3170	3.767

Total number of detected peaks 50

Total area (mAU*ml) 1021.7789

Area in evaluated peaks (mAU*ml) 1018.9304

Ratio peak area / total area 0.997212

Total peak width (ml) 18.69

Column height (cm) 30.00

Column V0 (ml) 8.00

Calculated from S200 BYPASS 301015005 40PC001:10_UV1_280nm

Baseline S200 BYPASS 301015005 40PC001:10_UV1_280nm@14,BASEM

Peak rejection on

Maximum number of peaks ()	20

Appendix B: Kistler-Nitschmann separation data

Table B1: The chromatographic profile of the Kistler-Nitschmann separation with tabulated peaks and area underneath the curve

Volume point (mL)	Area (mAU*mL)	Maximum peak height (mAU)
-2.06	6.6974	25.334
-1.32	1.6206	1.561
0.74	26.3628	7.394
17.70	202.9837	15.486
38.16	7.2652	1.410
62.84	171.0630	6.960
164.15	12.8873	3.616
170.22	43.8064	3.847
177.41	12.8727	3.191
181.59	7.7546	2.769
184.81	4.2094	2.340
186.61	9.9157	2.148
192.16	3.0498	1.595
193.90	2.6197	1.455
195.86	3.1754	1.231
198.76	2.2209	1.001
201.56	1.6860	0.833
363.23	226.0455	22.667
462.05	2.6811	0.904
464.64	3.5122	0.959

Total number of detected peaks 140

Total area (mAU*ml) 771.1657

Area in evaluated peaks (mAU*ml) 752.4294

Ratio peak area / total area 0.975704

Total peak width (ml) 175.09

Column height (cm) 10.00

Column V (ml) 18.60

Calculated from alb checker kn method001:10_UV1_280nm

Baseline alb checker kn method001:10_UV1_280nm@01,BASEM

Peak rejection on

Maximum number of peaks () 20

Appendix C: Mass measurements of the Eppendorf tubes and precipitates

All measurements in grams unless otherwise noted

Table C.1: The masses of the Eppendorf tubes without any material

Ethanol concentration (%)	1	2	3
0	1.014	1.029	1.017
10	1.04	1.034	1.013
20	1.007	0.991	1.014
30	1.014	1.012	1.014
40	1.039	1.014	1.011

Table C.2: The masses of the Eppendorf tubes and blood plasma

Ethanol concentration (%)	1	2	3
0	1.177	1.193	1.179
10	1.208	1.197	1.176
20	1.169	1.156	1.178
30	1.166	1.171	1.179
40	1.202	1.173	1.172

Table C.3: The masses of the Eppendorf tubes and the precipitates after the supernatants was removed

Ethanol concentration (%)	1	2	3
0	1.063	1.085	1.067
10	1.096	1.074	1.053
20	1.047	1.043	1.056
30	1.074	1.065	1.063
40	1.108	1.082	1.088

Table C4: The masses of the Eppendorf tubes and the freeze-dried precipitates

Ethanol concentration (%)	1	2	3
0	1.016	1.031	1.017
10	1.042	1.036	1.015
20	1.012	0.996	1.017
30	1.018	1.017	1.019
40	1.046	1.02	1.019

Table C5: The masses of the blood plasma in each Eppendorf tube

Ethanol concentration (%)	1	2	3	Average mass	Uncertainty
0	0.163	0.164	0.162	0.163	0.001
10	0.168	0.163	0.163	0.165	0.003
20	0.162	0.165	0.164	0.164	0.002
30	0.152	0.159	0.165	0.159	0.007
40	0.163	0.159	0.161	0.161	0.002

Table C6: The masses of the precipitates in each Eppendorf tube

Ethanol concentration (%)	1	2	3	Average mass	Uncertainty
0	0.049	0.056	0.05	0.052	0.004
10	0.056	0.04	0.04	0.05	0.01
20	0.04	0.052	0.042	0.045	0.007
30	0.06	0.053	0.049	0.054	0.006
40	0.069	0.068	0.077	0.071	0.006

Table C7: The masses of the freeze-dried precipitates in each Eppendorf tube

Ethanol Concentration (%)	1	2	3	Average mass	Uncertainty
0	0.002	0.002	0	0.001	0.001
10	0.002	0.002	0.002	0.002	0
20	0.005	0.005	0.003	0.004	0.001
30	0.004	0.005	0.005	0.005	0.001
40	0.007	0.006	0.008	0.007	0.001

Table C8: The mass of the freeze-dried precipitate as a proportion of the total blood plasma mass

Ethanol concentration (%)	1	2	3	Average proportion	Uncertainty
0	1.2%	1.2%	0.0%	0.8%	0.8%
10	1.2%	1.2%	1.2%	1.2%	0.0%
20	3.1%	3.0%	1.8%	3%	1%
30	2.6%	3.1%	3.0%	2.9%	0.3%
40	4.3%	3.8%	5.0%	4.3%	0.7%

Table C9: The mass of the freeze-dried precipitate as a proportion of the total precipitate mass

Ethanol concentration (%)	1	2	3	Average proportion	Uncertainty
0	4%	4%	0%	3%	3%
10	4%	5%	5%	5%	1%
20	13%	10%	7%	10%	3%
30	7%	9%	10%	9%	2%
40	10%	9%	10%	10%	1%