DEVELOPMENT OF METHODS FOR CAPILLARY ISOELECTRIC FOCUSING OF DAIRY PROTEINS

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Abstract

Capillary Isoelectric Focusing (CIEF) is a high-resolution technique which can be applied to the separation and characterisation of complex biological mixtures such as dairy proteins. Although dairy proteins are commonly analysed by traditional gel electrophoresis techniques including 2-Dimensional PAGE, CIEF offers the advantages of reduced analysis times, the ability to handle smaller sample volumes and increased sensitivity with improved separation efficiencies.

Several methods for capillary isoelectric focusing of dairy proteins have been developed herein. For the analysis of soluble whey proteins methods that can be used with either UV or mass spectrometry (MS) detection have been set up. For MS detection a coaxial sheath flow interface in conjunction with electrospray ionisation has been utilised. For analysis of the inherently insoluble casein proteins with UV detection denaturing and reducing agents have been introduced into the system. Results have shown very close similarities to those obtained by IEF gels.

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List of Abbreviations

2D	Two Dimensional
α-csn	α-Casein
α-Lac	α-Lactalbumin
Amy	Amyloglucosidase
β-csn	β-Casein
β-Lac	β-Lactoglobulin
β-Lac-A	β-Lactoglobulin-A
β-Lac-B	β-Lactoglobulin-B
BME	β-Mercaptoethanol
BSA	Bovine Serum Albumin
CA	Carbonic Anhydrase II
CCK	CCK Flanking Peptide
CE	Capillary Electrophoresis
CEC	Capillary Electrochromatography
CGE	Capillary Gel Electrophoresis
CIEF	Capillary Isoelectric Focusing
CITP	Capillary Isotachophoresis
CZE	Capillary Zone Electrophoresis
DNA	Deoxyribosenucleic Acid
DTT	$_{DL}$-Dithiothreitol
EDTA	Ethylenediaminetetra-Acetic Acid
EOF	Electroosmotic Flow
ESI	Electrospray Ionisation
GMP	Glycomacropeptide
HPLC	High Performance Liquid Chromatography

i.d.	Internal Diameter
IEF	Isoelectric Focusing
Ig	Immunoglobulin
IgG	Immunoglobulin G
κ-csn	κ-Casein
kV	Kilo Volt
Lf	Lactoferrin
Lp	Lactoperoxidase
mA	Milli Amps
Mb	Myoglobin
Mb-A	Myoglobin Acidic
Mb-B	Myoglobin Basic
MEKC	Micellar Electrokinetic Chromatography
MFGM	Milk Fat Globule Membrane
mg	Milli Gram
MHEC	Methyl 2-hydroxyethyl cellulose
mL	Milli Litre
MOPS	3-[N-Morpholino]propane-sulfonic acid
MS	Mass Spectrometery
MWCO	Molecular Weight Cut Off
NaOH	Sodium Hydroxide
nL	Nano Litre
PAGE	Polyacrylamide Gel Electrophoresis
PDA	Photo Diode Array
pI	Isoelectric Point (of a protein or peptide)
PP5	Proteose Peptone 5
PSI	Pounds per Square Inch

Rb	Ribonuclease
RNA	Ribosenucleic Acid
RP	Reversed Phase
SDS	Sodium Dodecylsulfate
TCA	Trichloroacetic Acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TI	Trypsin Inhibitor
TIC	Total Ion Count
Tris	Tris(hydroxymethyl)-aminomethane
Tryp	Trypsinogen
µg	Micro Gram
µL	Micro Litre
UV	Ultraviolet
V/cm	Volts per Centimetre (of column length)
v/v	Volume to Volume
v/w	Volume to Weight

1 Overview

Capillary Isoelectric Focusing (CIEF) is a technology that has developed in the last few years and is a technique whereby proteins and peptides are separated according to their isoelectric point (*pI*); such separations are generally as good as those obtained by flat bed isoelectric focusing (IEF) polyacrylamide gel electrophoresis (PAGE). Advancements in CIEF technology have been led by the requirements of proteomic research for high throughput analysis coupled with limited sample size. Routine methods for CIEF involve ultraviolet (UV) detection, but mass spectrometry (MS) detection is becoming more popular for many research groups. This is analogous to the time consuming method of 2-dimensional IEF/ PAGE in which spots on gels are excised, digested with enzyme, and the digests analyzed by high performance liquid chromatography-MS (HPLC-MS). CIEF-MS has the capability to reduce analysis times considerably and is used for a number of applications. Detection is of intact protein rather than hydrolyzed protein, which saves time on database searches. In recent years the CIEF-UV method that has traditionally only had applications to water soluble protein, has been modified for separation of proteins in denaturing systems. In this way proteins that are inherently insoluble can be separated by CIEF. Currently there is only one CIEF method within the literature that has a dairy application and this is based on the monitoring of glycosylation products of glycomacropeptide (GMP) (Tran et al. 2001).

Over the last few years dairy industries around the world have embarked on large-scale proteomic research, with a view to one or more of the following:

a.) The discovery of low abundance proteins and peptides that may have potential health benefit that could be explored in niche products of the future.

b.) Understanding expression and co-regulation of milk proteins.

c.) Acquisition of intellectual property for future strategic use.

The competitive edge of a dairy company is governed partly by the speed in which fundamental research can be translated into a commercial process or product. In this

respect it is mandatory to identify new technological areas and analytical techniques that may allow large time and cost savings in the commercialization pipeline. Capillary electrophoresis (CE) is one such analytical tool as it is rapid, has very good detection limits, can be interfaced to MS detection and requires very small sample size.

The aim of this research was to develop new methods in CE analysis that would be applicable to a wide variety of dairy-based samples, and could be used as rapid screening methods for proteomic applications. The CE mode of CIEF was investigated, as sample size in this format is generally 20 times larger than other modes of CE, thus enhancing detection sensitivity, and the method is able to separate proteins and peptides over a wide range of *pI* values. The method has the additional advantage that *pI* values can help in the identification of unknown protein. The technique is also very rapid and gives very good comparison to the IEF gel format, making this technology very much cheaper and less labour intensive to use.

Bovine dairy proteins are comprised of two main groups, the casein and the whey proteins. Caseins make up approximately 80 % of dairy protein and typically occur as micelles in milk, being inherently insoluble. Whey proteins on the other hand make up the remaining 20 % of protein and tend to be globular water-soluble proteins, while in addition there is another group of proteins collectively termed the milk fat globule membrane (MFGM) protein that makes up a very small amount (<1 %) of protein in milk. Taking these general properties into consideration the overall aim of this thesis was to develop methods of CIEF for the different types of dairy protein as follows:

- Develop methods using UV detection that are simple to run with minimum preparation and optimized for:
    - The major whey proteins
    - Casein proteins
    - Fractionated protein samples
- Compare these methods to IEF flat bed PAGE
- Develop methods of CIEF-MS for soluble proteins and if possible modify the method for insoluble proteins
- Compare CIEF-MS results to two dimensional PAGE (2D-PAGE) methods
- Compare CIEF methods to already developed CZE methods where applicable

2 Literature Review

2.1 Composition of Bovine Milk

Bovine milk is a complex mixture of proteins, lipids, carbohydrates, minerals, vitamins, and salts dissolved in water by way of being colloidly dispersed and emulsified. Some of the constituents of milk are transferred from the blood to the mammary gland, while other constituents are synthesized within the mammary gland. The amount of protein in bovine milk is typically 30-35 g protein/litre of milk, and can alter in amount and composition of protein due to time of lactation and breed of cow. There are two main types of milk proteins. Casein comprises the largest portion of these proteins at around 80 % and is represented by 4 gene products, α_{s1}-casein, α_{s2}-casein (α-csn), β-casein (β-csn), and κ-casein (κ-csn). There are, however some other constituents of casein within bovine milk, but they are derived from posttranslational modifications, such as phosphorylation and glycosylation, or proteolysis. Casein is inherently insoluble in water but can be stabilized by forming micelle structures in milk in which several caseins bind together and to calcium phosphate, forming spherical complexes. The other 20 % of milk protein comprises of whey proteins. These are usually classed as proteins which remain soluble after the pH of milk has been adjusted to 4.6 at 20°C. At this pH, the caseins precipitate out. The main components of whey include β-lactoglobulins (β-lac), α-lactalbumins (α-lac), immunoglobulins (Ig), and bovine serum albumin (BSA). For each of the major proteins there exist a number of different genetic variants. Table 1 describes the typical quantities of each of the major protein components and lists the genetic variants of each protein according to Swaisgood (1986).

Table 1 Major protein constituents of bovine milk including approximate concentration of each protein (depending on time of lactation) and genetic variants. From Swaisgood (1986).

Protein	Abbreviated Name	Approximate content in Bovine Milk	Known Genetic Variants	Notes
α-$_{s1}$ Casein	α-$_{s1}$-csn	12-15g/L	A,B,C,D	α-$_{s0}$ is derived from α-$_{s1}$-csn
α-$_{s2}$ Casein	α-$_{s2}$-csn	3-4g/L	A,B,C,D	α-$_{s3,4}$&$_{6}$ are derived from α-$_{s2}$-csn
β Casein	β-csn	9-11g/L	A^1,A^2,A^3,B,B_z,C,D,E	γ1,2&3 caseins of different genetic variants (1-2g/L) are derived from β-csn
κ Casein	κ-csn	3-4 g/L	A,B	
β Lactoglobulin	β-lac	2-4 g/L	A,A_{DR},B,B_{DR},C,D	
α Lactalbumin	α-Lac	1-1.5 g/L	A,B	
Bovine Serum Albumin	BSA	0.1-0.4 g/L		Many glycosylated forms present
Immunoglobulins	Ig	0.6-1g/L	G,G1,G2,A,M	
Proteose Peptone	PP	0.6-1.8	5,8	Proteolytic derivatives of β-csn

In addition to the major proteins listed above there are a number of minor proteins and enzymes found in milk that do not contribute more than 1 % of the total protein in bovine milk. These include MFGM proteins, lactoferrin (Lf), lactoperoxidase (Lp), angiogenins, transferrin, folate binding protein, and lysozyme to name a few. A number of these proteins and many others have health enhancing properties, and they are now becoming increasingly important to dairy industries around the world as new technologies have enabled isolation of such proteins for commercial products. The products are marketed as high value products and gain higher prices due to the perceived health benefit to the consumer. To isolate such products in the laboratory, and since these proteins are in such low abundance in milk compared to the casein and major whey proteins, researchers have to remove the high abundance proteins and effectively concentrate up their target minor component protein. This involves many techniques, in particular ion exchange chromatography. It is usually desirable to check the purity of different fractions produced in the laboratory and this is routinely done by PAGE or HPLC analysis. PAGE analysis takes time (often hindering progress of

the research) and HPLC, depending on the methodology used not only requires the use of solvents but may be misleading when 2 species of similar composition are separated, for example genetic variants. Typically however, HPLC does not resolve genetic variants very well. For example the reversed phase (RP) method of Elgar et al. (2000) for the separation of whey proteins shows poor resolution of β-lac-A&B variants, although the overall separation is good for quantitation of the major whey proteins. Capillary electrophoresis (CE) offers the researcher an advantage in that it is a rapid technique, requires very little sample and protein resolution in many of the CE modes is such that genetic variants can be separated.

Recently, results from rat feeding trials have suggested that certain fractions from whey protein may have potential benefit to bone health in humans (Kruger et al., 2005 a and b). However, it was not clearly understood which component or components of the fraction conferred the health benefit. To elucidate such bioactive components characterisation of protein fractions is required and rapid screening techniques such as CE would facilitate both component discovery and routine fingerprinting.

2.2 Introduction to Capillary Electrophoresis

Capillary electrophoresis (CE) has been an emerging technology in the last 15 years due to advances in technology and the manufacture of more reliable instruments. A typical schematic representation of a CE instrument is shown in Figure 1.

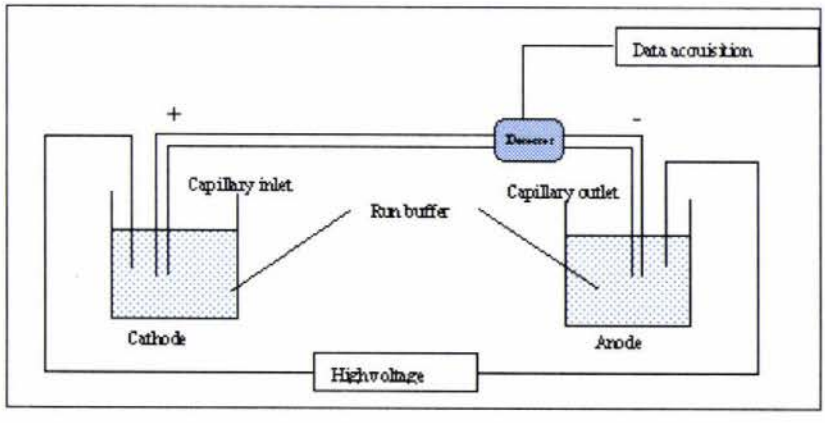

Figure 1 General Schematic overview of a CE instrument including cathode, anode, capillary, high voltage power supply, detector and data acquisition.

Typical modern automated instrumentation consists of a column in which multiple components in a sample are separated, internal pumping and vacuum generation

devices which pump electrode buffers through the column for equilibration and rinsing. These must be precise enough to give highly reproducible injection volumes. A high voltage power supply is incorporated to generate extremely high voltages to enable separation. In addition an integrated detection system sends output to a computer that has software that controls all aspects of the instrument to allow 24/7 automation. Columns and sample storage have temperature control, which ensure constant fluid viscosity hence allowing higher levels of reproducibility. The analyte can be detected a number of ways. Optical detection can include the use of UV detectors, photodiode array detectors, or fluorescence detectors. Other types of detectors range from amperometric detection to mass spectrometry detection. The data once obtained can then be processed rapidly through preset integration parameters for rapid quantification or qualification.

A large array of species separation can be performed by CE; from small inorganic ions through to organic acids, pharmaceutical, vitamins, peptides, large macromolecules such as deoxyribose nucleic acid (DNA), ribose nucleic acid (RNA), carbohydrates and proteins in many different sample matrices from biological to food stuffs. The most common modes of CE separation include capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary isotachophoresis (CITP), capillary gel electrophoresis (CGE), capillary electrochromatography (CEC) and CIEF. Most of these techniques have been developed as an analogous separation to PAGE or HPLC.

CE separations have many advantages to their analogous gel or HPLC techniques in that:

- They have an extremely high resolution of separation
- Detection is usually online so a result is more rapidly attainable
- Reproducible quantitation is achievable
- Very small amounts of sample and buffer are required so there are savings in cost of analysis.
- The chemicals used in CE are usually less toxic than some of those used in HPLC or PAGE, and if not, the volume of buffer required is many times lower, for example, RP-HPLC may require 1 litre of acetonitrile buffer to be made up in comparison to 4 millilitres of run buffer required for CE.

A survey of the modes of CE in relation to protein separation is discussed below.

2.3 Capillary Zone Electrophoresis

Protein separation by CZE technique is based on the hydrodynamic friction to charge ratio of the proteins in the sample solution. A sample aliquot is injected on to the column; both ends of the column are then placed in an identical run buffer solution. On addition of an electrical potential component separation occurs and the proteins flow to the detector due to electroosmotic flow (EOF). The pH of the run buffer is critical to the resolution of separation and efficient run time. The pH should give sufficient difference in mass to charge ratio for the proteins so that satisfactory resolution is achieved. Normally the pH of the buffer should be at least 1 pH unit greater or less than the *pI* of the proteins or the sample will be retained on the column for too long and resolution lost through peak tailing (Wehr et al. 1999). There is a vast array of literature on the use of CZE for protein separation with many techniques finding utility in food analysis and proteomics. Some recent key reviews include: Dolnik & Hutterer (2001), Recio et al. (1997a), Recio et al. (2001), Manabe (1999), Shen & Smith (2002), and Hu & Dovichi (2002).

2.4 Micellar Electrokinetic Chromatography

MEKC separates samples by differential portioning between two phases and is considered a chromatographic technique (Wehr et al. 1999). Separations are usually conducted in uncoated capillaries with basic conditions that allow a large EOF to occur. The run buffer contains a surfactant that is at a concentration above its critical micelle concentration and so surfactant monomers are in equilibrium with micelles. In the case of sodium dodecylsulfate (SDS) used as the surfactant, this compound is anionic so both surfactant monomer and micelle have electrophoretic mobility counter to EOF direction. The sample molecules are thus distributed between the bulk mobile phase and surfactant micelles depending on their hydrophobicity. Hydrophilic neutral species will remain in the aqueous phase and reach the detector in the time required for EOF to travel the length of the capillary. Hydrophobic neutral species will spend differing amounts of time in the micellar phase depending on their hydrophobicity, so their migration will be slowed due to the anodically moving micelles. Charged species have more complex interactions as they can migrate electrophoretically, or interact with the micelles electrostatically in addition to hydrophobic partitioning (Wehr et al. 1999). The MEKC technique is used mainly for small molecules such as drugs,

metabolites and peptides, and is also the basis of chiral separation with the addition of a chiral selector compound being added to the sample buffer system. Proteins are too large to partition into a surfactant micelle, and bind surfactant monomers to form surfactant-protein complexes. The review of Molina & Silva (2002) has outlined a number of current developments in the area of MEKC.

2.5 Capillary Isotachophoresis

The separation mechanism for CITP is that proteins are resolved as a contiguous zone that migrates in order of mobility. This can be achieved by injecting sample on to the capillary between a leading buffer of ion mobility greater than all the protein components, and a terminating buffer of ion mobility less than all the protein components. Zones migrate to the detector at equal speeds and are detected as steps with zone length proportional to concentration. CITP is not usually used as a separation method for proteins but is often used as a pre-concentration step for other CE techniques. (Gebauer & Boeck, 2002) give a good overview of recent developments in CITP.

2.6 Capillary Electrochromatography

CEC is a chromatography technique and uses capillaries that are packed with materials often used for RP-HPLC columns. This technique is rapidly developing into many sub-CEC methods utilising different types of capillaries. Generally the method requires the use of EOF to mobilise samples but separation is based not only on the sample's electrophoretic properties but on its chromatographic properties as well and thus this type of separation can achieve very high resolution of separations. Mostly this technique is involved with smaller molecules rather than proteins. Some good reviews on the different CEC techniques include those by Hilder et. al., (2002), Liu et. al., (2002), Rathore, (2002), and Li et al. (2004).

2.7 Capillary Isoelectric Focusing

Capillary isoelectric focusing was first pioneered by (Hjertén, 1985), who developed a capillary with an internal coating using acrylamide for the elimination of electroendosmosis and protein adsorption on the walls of the capillary. This method was further refined for the separation of human serum transferrin (Kilár & Hjertén, 1989).

The CIEF technique involves three main steps. Firstly a sample containing ampholytes is injected onto a capillary. This is followed by an addition of an electrical potential (where the ends of the capillary are at different pH's) in the second step that allows the sample and ampholytes to focus into narrow zones according to their isoelectric point (*pI*) within the capillary. The final step involves the detection of the sample and in most cases this is undertaken by mobilisation of the sample past or into a detector such as UV, fluorescence, or MS. It must be noted that there are a number of techniques used where whole column imaging detection is used. This topic is outside the scope of this literature review. However, a review by (Fang et. al., 1998) gives a good overview of the technique and detection systems used. A number of techniques for different types of sample separation by the CIEF method are outlined in Appendix 1 (Table 14).

2.8 Recent Reviews on CE of Large Biomolecules

There have recently been a number of key reviews focused on CE for the analysis of large biomolecules either for routine food analysis or for proteomic applications. The reviews give an overview of the use of different CE modes and their applications. (Hu & Dovichi, 2002) gave an extensive overview of the separation of biopolymers including protein, peptide, DNA, lipid and carbohydrate by different modes of CE with different detection systems. (Frazier, 2001) reviewed CE methods for food analysis investigating recent literature on protein analysis through to amino acid analysis, vitamins, toxins, and food additives for products such as cereals, milk and other foodstuffs. The author discussed the applicability of some CE modes over others for different types of analysis. (Bean & Lookhart, 2001) reviewed CE techniques used for meat, dairy and cereal protein applications. In the same year (Recio et. al., 2001) reviewed the analysis of food proteins of animal origin by CE very extensively. The review was mainly focused on dairy applications due to the author's extensive number of publications in dairy applications for CE, but also included egg and muscle protein separations.

2.9 CE of Dairy Proteins

In their review (Recio et. al., 2001) stated that in the eight years prior to the review there had been over 70 articles published in the field of milk protein separation using CE. Topics for papers have varied but include separation and quantification of the

different genetic variants of casein and whey proteins in milk, whey protein separation, analysis of adulteration of milk of different species, analysis of cheese proteolysis, and analysis of specific milk fractions such as those containing lactoferrin.

2.9.1 Analysis of Casein

Since CE has a high resolution of separation, the separation of very similar proteins that differ by only a few amino acid substitutions (such as genetic variants) is possible. Other techniques such as HPLC do not have the resolving power for such separations, while PAGE analysis is not particularly accurate for quantitative analysis.

The first application for the separation of milk proteins was developed by (Chen & Zang, 1992). Using CZE mode they trialled phosphate buffers from pH 6-9, with the addition of 4M urea to solubilize the proteins. Detection was at 200 nm. Separations were carried out on bare fused silica capillaries with no internal coating. The separations were not optimal for quantitation of all proteins in milk.

(de Jong et. al., 1993) first separated both whey and casein proteins by CZE using a hydrophilically coated capillary with a low pH (pH 2.5-3) citrate run buffer and samples dissolved in 6M urea and $_{DL}$-dithiothreitol (DTT). Both whey and casein protein could be separated in this system as casein micelles are disrupted by reducing with DTT and solubilizing in 6M urea. In this paper the researchers demonstrated the ability of the method to separate proteins of only 3 amino acids difference, specifically β-Casein-A1 and β-Casein-A2. They also showed differences between milk of different species (cow, goat and sheep milk) and investigated heat-damaged proteins. The method was compared with HPLC and illustrated the benefits of CE for separation of both whey proteins and casein and their genetic variants. This work was subsequently extended and the method optimised for determination of denatured whey proteins (BSA, α-Lac and β-Lac) in the casein fraction of heat treated milk, with the application of investigating whether milk powder has been added to pasteurised milk or investigating the whey protein to casein ratio in milk and milk products for tariffs regulation (Recio & Olieman, 1996). Further method refinement was undertaken to analyse genetic variants of the milk proteins from different species (Recio et. al., 1997) including cow, sheep and goat milk. The identification of a number of major

bovine proteins such as β-Caseins A1, A2, A3, B and C was determined and it was concluded this technique could be used for phenotyping individual cows due to the reproducibility of the method.

During rennet proteolysis of caseins and cheese making, the main κ-casein degradation product is para-κ-casein. In the general method defined by (de Jong et. al., 1993), para-κ-casein co-migrates with β-lac proteins so (Miralles et. al., 2001) further developed the method to allow quantification of both these components by changing the composition of the run buffer and lowering the voltage. As an addition to this work the same group examined other para-κ-casein type peptides with a view to using them as indicators of milk proteolysis. By identification of each peak the types of proteolysis reactions occurring in stored milk could be predicted (Miralles et al., 2003). Recently Xu (2003) claimed an improvement to the method of (de Jong et. al., 1993) in a short article outlining the method which used an untreated fused silica column to give better resolution of separation, and shorter run times for cheese proteins and peptides.

Several groups in Italy have furthered the method of (de Jong et. al., 1993), to give a more in-depth analysis of milk from different breeds of mares including Norico, Trotter, Haflinger, and Arabian and Ass (Civardi et. al., 2002). Prior to this the characterisation of ewe milk and analysis of cow, goat and ewe milk mixtures were performed (Cattaneo et. al., 1996), as this can be an issue for authenticity of different cheeses if goat milk or ewe milk is adulterated with cheaper cow milk. The method of analysis was based on the migration time differences of α_{s1}- casein for each species with a quantifiable amount as low as 8% cow milk in either goat or ewe milk.

Other applications where the original method of (de Jong et. al., 1993) has been used include the evaluation of authenticity of Serpa cheese made in Portugal (Roseiro et al. 2003). This cheese is made from ewe's milk and has a Protected Denomination of Origin (PDO) designation. The analysis of the proteolysis of authentic Serpa cheese versus similar cheese made by other means was investigated. The degradation of αs1- and β- casein was a good marker for the authenticity of traditional Serpa versus other similar cheeses. Another application where the de Jong method has been successfully used for identification of authentic cheeses is in the analysis of Ibérico cheese (Molina

et al. 2002). In this study the authors used 1 and 2 dimensional PAGE and compared the results with that of the de Jong CE method which had been altered slightly by using a different column coating and longer column to improve resolution of separation. Results from CE were comparable to the gel format. Due to the rapid automated nature of the CE method compared to gels, and that quantitation is possible with CE, the method is a complementary technique to PAGE.

Another study investigating mixtures of milks for authenticity in cheese making was conducted by Recio et al. (2004). In this study they investigated the detectability of goat's or cow's milk in Halloumi cheese that is traditionally made from ewe's milk in Cyprus. Monitoring of αs1- casein and para-κ-casein peaks was undertaken for cow's milk and goat's milk respectively, from a series of cheeses made from different ratios of milk species and matured for different amounts of time. Results showed that detection of less than 2 % addition of either cows' or goats' milk was possible. Studies on lactosylation of milk proteins due to the Maillard reaction during storage of skim milk powders was used to great effect with the de Jong method (Guyomarc'h et al. 2000). The method was modified slightly using phosphate in the buffer and a polyacrylamide internal column coating. Lactosylated protein appeared as extra peaks next to each main protein peak, but of a lesser height than the main protein peak depending on the degree of lactosylation. The amount of lactosylation could then be calculated as a ratio of the area of the lactosylated protein peak to the area of both the lactosylated and unmodified protein peaks. From results obtained the authors were able to show correlations between process conditions and lactosylation of skim milk powder and consequently investigated the best practice for storage of the powders to prevent lactosylation reactions occurring over time.

Recently the de Jong et al. (1993) method was applied to the analysis of β-casein variants A1, A2, and B and the ratios of these genetic variants present in the milk of Icelandic and other Nordic countries at different times of year (Iggman et al., 2003). The aim of the study was to investigate the composition of Nordic milks at different times of year, and then relate the composition to public health problems, in particular the relationship of the β-caseins variants to type-1 diabetes mellitus.

2.9.2 Whey Protein Separation

In addition to the de Jong et al. (1993) method where whey proteins can be separated along with the casein proteins, a number of methods have been developed to separate only the whey protein component using CZE. Due to the soluble nature of whey proteins, the methods generally do not use the intense solubilizing and reducing reagents that are required to disrupt inherently insoluble casein micelles.

Paterson et al. (1995a) separated the three major β-lac proteins in whey using a CZE method which was then used to identify phenotypes containing the β-lac-C variant (Paterson et al., 1995b). Comparison of this method with PAGE, HPLC methods and a newly developed SDS-CGE method (Kinghorn et al., 1995) showed that none of the methods were suitable at quantifying all of the constituents of different whey products. However, the CZE method did stand out as the best method, so further refinements to the method were made to quantitate β-lac-A&B, α-lac, BSA, and IgG (Kinghorn et al., 1996).

The method of Kinghorn et al. (1995) demonstrated the ability of CE to separate the β-lac-A, -B , &- C variants and its superior resolution has been applied in a recent study to determine the composition of Iceland and Nordic milk (Iggman et al., 2003).

2.10 CE-MS

CE–MS was first developed by Smith and co-workers (Olivares et. al., 1987); and (Smith et. al., 1998) in the late 1980s. CE–MS has now matured into a field of its own, as the coupling of the two instruments together has necessitated some differences in running conditions and applications. It has developed rapidly as a result of technology developments in MS and CE instrumentation.

Since the early work by Smith and co-workers on the development of the coaxial liquid sheath-flow interface for an electrospray ionisation (ESI) source, a number of key reviews outlining CE- MS have been published. These reviews have focused on a variety of topics, including applications, instrumentation, different modes of CE–MS analysis and different types of interfaces in use (Niessen et al., 1993; Smith et al., 1993; Cai & Henion, 1995; Tomer et al., 1995; Banks, 1997; Ding & Vouros, 1999; von Brocke et al., 2001, Monton & Terabe, 2005).

A device called an interface is required to connect a CE instrument to an MS detector. The interface acts to introduce the separated sample from the CE instrument into the MS detector for mass characterisation. As it acts as the cathode end of the CE and the anode end of the MS interface, it must be earthed to allow for charge dissipation. Because CE operates on very low flow rates, often too low for some MS instruments, a make-up flow has to be added to the flow from the capillary. Also, as coated capillary columns are used in some CE applications, the capillary must go all the way into the ESI source of the MS detector so that separation of the analytes is not compromised. A good interface will have all these features.

In the literature, there are reports of many types of interface that have been trialled by different groups. Most interfaces are made by individual researchers themselves and are modifications of three main types available.

2.10.1 Coaxial Sheath-flow Interface

The coaxial interface is the most widely used interface for CE–ESI–MS. The interface consists of a sheath that is connected to the cathode end of the CE capillary. A make-up flow is added to the sheath and mixing occurs with the use of a sheath gas. The sample is diluted but, at the same time, is in an environment more compatible with the MS instrument because the make-up flow usually consists of additives to increase mass sensitivity.

The interface is used mainly where the CE capillary wall is covalently coated with chemicals to form almost zero EOF. The coaxial sheath-flow interface was used throughout this work and a schematic of it is seen in Figure 2.

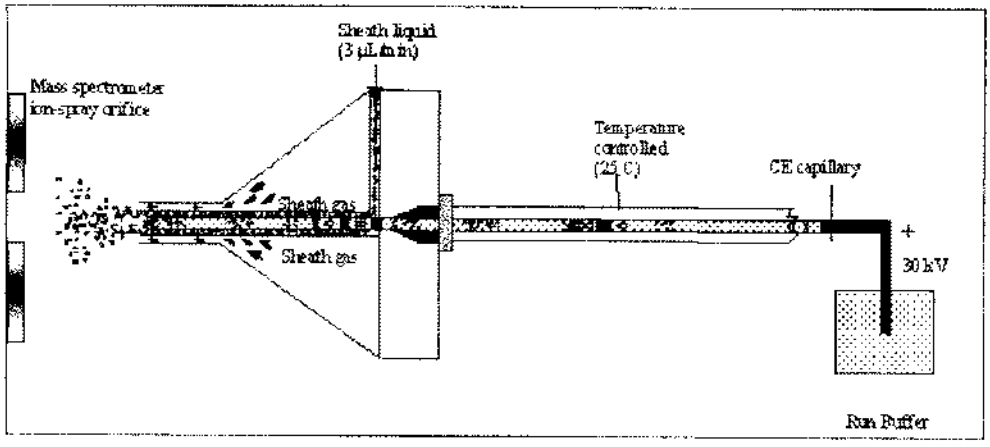

Figure 2 Schematic of the Finnigan coaxial sheath-flow CE–MS interface as used in this research.

2.10.2 Sheathless Interface

The sheathless interface was first proposed in 1988 by Olivares et al. Further development has been to sharpen the tip of the capillary to gain higher resolution. Electrical contact has been utilised by placing a piece of gold wire at the CE capillary tip to complete the electrical circuit for the CE and the ESI. The use of gold gives a good ESI spray stability from the solution entering the MS instrument. This interface is best used for addition to a nanospray ESI source because of the compatible flow rates. Samples are not diluted with the sheathless interface and no additional chemicals are added at the ionisation stage. Capillaries of internal diameter (i.d.) as low as 5–10 μm have been used (Wahl et al., 1994).

2.10.3 Liquid-junction Interface

Henion and co-workers developed the liquid-junction interface in 1989 (Lee et al., 1989). This type of interface uses a T-section that allows a piece of fused silica capillary to be inserted and electrically connected to the ESI emitter via the electrolytes introduced through the buffer reservoir. The gap between the emitter and the CE capillary is typically 10–20 μm. The advantage of the liquid-junction interface is that the CE capillary is disconnected from the ESI emitter. If any problems related to the emitter are encountered, then the emitter can easily be replaced without having to replace the entire capillary. However, this type of interface is not good if a coated capillary is being used, as the separation may be altered slightly because of EOF when

the sample goes through the bare fused silica. This EOF could affect the separation to give a slight loss of resolution.

2.11 CE-MS Modes

2.11.1 CZE–MS

There are an ever-increasing number of new methods for protein and peptide identification using CZE–MS. The selection of the background electrolyte buffer and make-up liquid is important in CZE–MS, to get a good resolution of separation and MS ionisation.

A survey of the literature indicates that many different groups have used any one of the three types of interface for CZE–MS. CZE often uses internally uncoated columns so that a liquid-junction column can be used. Buffers that are compatible with the MS detector are often used; since in this case no make-up flow is required, a sheathless interface can be used.

Unlike liquid chromatography, the background electrolyte for CE separation must contain a buffer system to avoid excessive changes in pH caused by electrolysis during the electrophoresis separation. It is also obligatory that these buffer components be volatile so as to improve MS detectability and to avoid problems with fouling the MS ion source with salt deposits. Furthermore, the conductivity of the buffer electrolytes should be low to minimise Joule heating.

The limited range of background electrolyte buffers that satisfy all these requirements has led to an overall lower dynamic range of protein measurement by CZE–MS compared with liquid chromatography–MS, although better sensitivities are achievable with CZE–MS (Shen & Smith, 2002).

2.11.2 CIEF–MS

This method is similar to the now commonly used proteomic technique of 2D PAGE coupled to MS. For 2D PAGE–MS, a sample is separated by IEF in the first dimension and then separated by molecular weight (SDS-PAGE) in the second dimension. Staining of the gel is then required, followed by excision of protein spots on the gel, destaining, hydrolysing the protein with trypsin or similar enzyme and then analysing the extracted peptide by HPLC–MS. Database searches must then be done

on the acquired peptide masses to find a protein match. The method of CIEF–MS replicates this procedure by a 1st dimension separation by *pI* followed by molecular mass identification (second dimension) of intact protein with MS detection. This type of procedure could potentially reduce analysis time by several days compared with the current 2D-PAGE-MS methods.

All CIEF–MS analyses reported in the literature have used a coaxial sheath-flow interface with ESI–MS. The sheath-flow liquid acts as an electrode to first establish the pH gradient within the capillary. Once the gradient is established (when focusing is complete), the sheath-flow liquid is changed (from a base to an acid) and this allows mobilisation of protein and ampholyte to occur.

Over time in a CIEF-MS separation the pH of the solution exiting the capillary into the MS changes, due to the pH gradient. However the overall pH of sample and sheath-flow is kept constant due to the dilution effect of the sheath-flow fluid. The constant pH creates an optimal ESI efficiency across the entire pH ampholyte range for optimal ionisation to occur, usually in the positive ion mode. The typical make-up liquid is a solution of 50 % v/v methanol and 1 % v/v acetic acid in water (Shen & Smith, 2002).

CIEF–MS has been successfully used for a number of applications particularly in proteomics, as outlined in Appendix 1 (Table 15). A recent review specifically on CIEF-MS outlined the history of the technique and modifications made to the method to optimise separation and sensitivity (Wehr, 2004).

2.11.3 CITP–MS

So far, very few methods have been reported in the literature for the CITP–MS technique. However, by using the pre-concentration technique of transient CITP (tCITP), a number of groups have separated proteins using tCITP–CZE–MS (Thompson et. al., 1993); (Naylor et. al., 1998) and tCITP–CIEF–MS (Mohan & Lee, 2002).

2.11.4 MEKC–MS

Coupling of MEKC separations to MS is not favourable as surfactants required for the separation create instability of the electrospray ionisation and contaminate the MS

detector. For this reason very few reports in the literature outline use of MEKC–MS applications (Molina & Silva, 2002).

2.11.5 CEC–MS

The CEC–MS technique has become more popular for small molecules in areas such as drug identification, steroid separation and the characterisation of oligosaccharides. So far, there has been no literature on protein separation; however, there have been two papers on peptide separation from one group (Gucek et al., 2000; Gaspari et al., 2001). Once the technology of CEC–MS matures, new opportunities for protein and peptide separation for dairy applications may emerge.

2.11.6 CGE–MS

Garcia & Henion (1992) developed CGE–MS for the analysis of organic anionic species utilising a liquid-junction interface. Since then, this method has generally stagnated. One of the major problems with this method is that the denaturing compounds required for the CGE separation are not compatible with the MS detector, and foul the detector.

The objective of this thesis was to develop new techniques using CE for dairy protein and/or peptide separation, with the aim that these would be utilised primarily for proteomic applications where samples of very different chemical composition could be analysed under similar operating conditions. For this reason, the preferred mode of detection was MS, although UV detection was also investigated. From the survey of literature for different CE techniques it was apparent that a number of techniques, although capable of separating proteins or peptides, could not readily be used with MS detection (e.g. CGE, MEKC, CITP, and CEC techniques). The two remaining CE techniques, CZE and CIEF, were, however, suitable for investigation as they could both be interfaced to MS. CZE separation as discussed earlier is based on mass to charge and is pH dependent whereas the CIEF method has the advantage that it can separate proteins and peptides of very different molecular weights and *pI* over a broad pH gradient. Furthermore, the method is able to give an approximate *pI* of the protein to aid sample identification.

With the above in mind, the intent of this thesis was to investigate methods for CIEF with potential application to dairy products, and compare results to currently used techniques as outlined in section 1.0.

3 Experimental Conditions

The following methods and operating parameters outlined in this section were used for the CE methods developed during this study. In further sections the discussion will focus on those parameters and conditions best suited to the different techniques.

3.1 Chemicals

Ammonium persulfate, DL-Dithiothreitol (DTT), urea, β-mercaptoethanol (BME), N,N,N',N'-tetramethylethylenediamine (TEMED), CHAPS, tris(hydroxymethyl)-aminomethane (Tris), anhydrous citric acid, Coomassie brilliant blue R-250, and acrylamide were all purchased from BioRad (Hercules, CA, USA) and were all electrophoresis grade. Ampholytes with different pH ranges were purchased from Beckman (pH 3-10) (Fullerton, CA, USA), Pharmacia (Pharmalyte pH 3-10) (Uppsala, Sweden), Fluka (pH 3-10, 7-9, and 4-6) (Buchs, Switzerland), Sigma (pH 3-7) (St. Louis, MO, USA), and Bio Rad (pH 3-10, 4-6). The following purified proteins were obtained from Sigma: β-lactoglobulin A, β-lactoglobulin B, α-lactalbumin, bovine serum albumin, proteose peptone-5 (PP5), glycomacropeptide (GMP), lactoferrin, lactoperoxidase, α-casein, β-casein, κ-casein (all from bovine milk), myoglobin (from horse heart), trypsin inhibitor (type I-S from soybean), trypsinogen (from bovine pancreas), carbonic anhydrase 1 (from human erythrocytes), amyloglucosidase (from *Aspergillus niger*). Also from Sigma were thiourea, 3-[N-morpholino]propane-sulfonic acid (MOPS), citric acid trisodium salt, and γ-methacryloxypropyltrimethoxysilane. Phosphoric acid, sodium hydroxide (NaOH), glacial acetic acid, isopropanol, ammonia, ammonium acetate, acetone, sodium tetraborate, Tween 20, ethylenediaminetetra-acetic acid (EDTA), glycerol, glycine, trichloroacetic acid (TCA), sodium dodecylsulfate (SDS), and sodium acetate were from BDH Laboratory Supplies (Poole, England). Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Methyl 2-hydroxyethyl cellulose (MHEC) was purchased from Aldrich (Milwaukee, WI, USA). Fluorescent *pI* markers with values of 3.0, 4.5, 5.1, 6.8, and 9.5 were purchased from Fluka (Buchs, Switzerland). A broad range (pH 3-10) isoelectric focusing calibration kit was

purchased from Amersham Biosciences (Buckinghamshire England). eCAP CIEF gel was purchased from Beckman. Deionized water was obtained from a Gradient Milli-Q system (Millipore, Billerica, MA, USA). Whey fraction samples and high purity lactoferrin were gifted from Dr Kate Palmano (Fonterra Research Centre, Palmerston North, New Zealand). Bacterial cell lysates were gifted from Dr Steven Flint (Fonterra Research Centre, Palmerston North, New Zealand).

3.2 Sample and Buffer Preparations for CIEF Experiments

3.2.1 Whey Basic Protein Fraction

Whey basic protein fraction samples were dissolved to an approximate concentration of 25 mg/ml (10 ml samples) in Milli-Q water and dialysed using 3500 molecular weight cut off (MWCO) membrane (Spectra /Por Houston, USA) to remove any salt. Dialysis was performed overnight at 4°C against several changes of water. The solutions were shell dried with a Just-A-Tilt shell freezer (FTS Systems, Stone Ridge, New York USA), and then freeze dried with a Freeze Mobile 12SL (The Virtis Company, Gardiner, New York USA). Powders were stored at 4°C in a desiccator until ready to be made up for CIEF or flatbed gel IEF analysis.

3.2.2 Whey Protein from Skim Milk

Fresh skim milk was diluted 1 to 1 with 0.2 M sodium acetate (pH 3.9) and then centrifuged in Eppendorf tubes at 14000 rpm for 6 minutes with a 5417C Eppendorf microfuge (Hamburg, Germany). The supernatant was removed and re-centrifuged at 14000 rpm for a further 6 minutes. 2.5 mL of the resulting supernatant was applied to a PD-10 de-salting column (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The fluid passed through the column was let go to waste, 3.5 ml of deionised water was applied to the column and the eluate collected. The column was re-equilibrated with addition of 25 ml-deionized water. Aliquots of the skim milk whey samples were frozen and used as required.

3.2.3 Casein Protein from Skim Milk

Fresh skim milk samples were centrifuged for 60 minutes at 14000 rpm (Eppendorf microfuge) and the pellet recovered and diluted with 8 M urea solution as required.

3.2.4 Standards

Casein protein standards were made up to between 6-9 mg/ml in Milli-Q water. All other protein standard solutions were made up in deionised water at concentrations of approximately 3-4 mg/ml.

3.2.5 Buffers

All buffers (anode, cathode and sample) were made up according to methods outlined in the following sections and filtered through 0.20 μm PVDF membranes (Titan Filtration Systems, Wilmington, NC, USA) prior to use.

3.3 CIEF-UV Experiments

All Capillary Electrophoresis experiments were performed on a P/ACE MDQ (Beckman Coulter, Fullerton, CA, USA) with a direct UV absorbance detector or photo diode array (PDA) detector both set at 4 Hz for data acquisition on 32 Karat software version 5.0.

A selection of coated capillary columns as follows were used in this work; eCAP Neutral Capillary (Beckman, Fullerton, CA, USA), ES20 weakly hydrophilic-coated fused-silica capillary column (SGE, Austin, Texas, USA), OV-1701-OH deactivated fused silica CE column (TSP-050375-P-10, BGB Analtyik AG, Switzerland), CE-100SA bonded phase open tubular CEC Zero flow and Low flow columns (MicroSolv Technology Corporation, Eatontown, New Jersey, USA), bare fused silica (Polymicro Technologies, Phoenix, Arizona, USA) and bare fused silica coated in-house with acrylamide as outlined by (Kilár & Hjertén, 1989). Briefly, this column was made as follows using the CE instrument; A 0.5 % (v/v) solution of γ-methacryloxypropyltrimethoxysilane in 50 % acetone was pumped into a 50 cm bare fused silica column at 25 psi for 10 minutes. After 1 hour the capillary was emptied by the pumping through of air (25 psi for 10 minutes). The capillary was then filled with a 4 % w/v acrylamide solution containing 0.4 μl TEMED and 0.5 mg ammonium persulfate per mL solution at 25 psi for 5 minutes and then left for 30 minutes, after which water was pumped through the column for 10 minutes (at 25 psi). The capillary was then pumped dry with air for 5 minutes at 25 psi. The ends of the capillary were cut freshly with a working column of 40 cm being used.

All capillaries had an internal diameter (i.d.) of 50 μm unless otherwise stated, and outer diameter (o.d.) of 360 μm. The length of the capillaries varied from 30-60 cm with a UV detector window being burnt into the column 10cm from the cathodic end to remove the polyimide coating. New columns were usually equilibrated before their first use by rinsing with deionised water for 10 minutes at 25 psi followed by rinsing with anode run buffer for 30 minutes at 25 psi. Before the start of each day the capillary was flushed for 20 minutes at 25 psi with anode run buffer.

3.4 CIEF-UV in a non-denatured system

Phosphoric acid at a concentration of between 10-91 mM was used as the anode solution and sodium hydroxide at a concentration of between 10-40 mM was used as the cathode solution throughout these experiments. In the case of chemical mobilisation, either a 20 mM phosphoric acid solution or a 1 % acetic acid solution was used as the cathode after focusing had taken place.

The general procedure for a non-denatured CIEF-UV run was as follows:

The capillary was first rinsed with anode buffer for 3-6 minutes (depending on capillary length) at 25 psi. This was followed by a solution of the sample plus ampholyte (at a concentration between 0.5-4 % v/v). These steps were performed such that at the cathode end a waste vial collected the solutions. To initiate focusing, the anode and cathode ends were placed in the phosphoric acid and sodium hydroxide solutions, respectively, and a voltage was applied at between 300-500 V/cm column length. The ramp time for this voltage was 0.17 minutes. Focusing took place for 3-10 minutes before the mobilisation step.

Mobilisation techniques included the following methods:

Pressure mobilisation was at 0.1-0.2 psi pressure unless a sample was diluted in eCAP CIEF gel, where 0.5 psi was used.

Chemical mobilisation was achieved by replacing the sodium hydroxide catholyte with either phosphoric acid or acetic acid after focusing was complete.

EOF mobilisation was trialled using the MicroSolv Low flow columns with a voltage applied to the capillary only.

Focusing and mobilisation were undertaken using different molarity anolytes and catholytes.

3.5 CIEF-UV in a denatured system

Four different urea sample buffers were used for these experiments and included the following:

- 8 M urea made up with 2 % w/v DTT
- 8 M urea made up with 5 % v/v BME
- 8 M urea made up in eCAP CIEF gel solution with 5 % v/v BME
- 7 M urea, 2 M thiourea, 1 % w/v DTT, and 2 % w/v CHAPS detergent.

In all cases DTT and BME were added freshly on the day of sample preparation.

Samples were incubated in sample buffer for 1 hour followed by centrifugation at 14000 rpm for 3 minutes in an Eppendorf centrifuge to remove bubbles.

Ampholyte solution with either a pH range of 3-10, or a mixture of pH range 3-10 and a narrow range ampholyte, was added to the sample solution to a final concentration of 2-4 % v/v.

In initial studies, TEMED was used at a concentration of 0.5-2 % v/v in the sample buffer to block the blind side of the UV detector when focusing proteins.

Firstly, a MicroSolv Zero flow capillary was rinsed with anodic buffer for 3-6 minutes (depending on column length) at 25 psi. Protein sample made up in one of the four sample buffers with ampholyte added was injected on to the column at 25 psi for 90 seconds to fill the entire length of column. Proteins were then focused to their isoelectric point by application of electric potential (300-500 V/cm) using phosphoric acid (anode) and sodium hydroxide (cathode) to form the pH gradient. Focusing took place for 3-10 minutes before the mobilisation step.

Mobilisation was undertaken by one of the following:

Pressure mobilisation was at 0.1-0.2 psi pressure unless a sample was diluted in eCAP CIEF gel, where 0.5 psi was used.

Mobilisation using a chemical gradient was achieved by using 20 mM phosphoric acid and 40 mM sodium hydroxide in those experiments where focusing and mobilisation occurred concurrently.

3.6 CIEF-MS Experiments

Capillary Electrophoresis–Mass Spectrometry experiments were performed on a Finnigan LCQ Ion Trap Mass Spectrometer (San Jose, CA, USA) equipped with an electrospray ionisation (ESI) source. Data acquisition was performed on Xcalibur software version 1.3. Deconvolution of proteins was performed using Bioworks 3.1. The previously described CE instrument was used for CE-MS experiments except the instrument was controlled via the Xcalibur Mass Spectrometer software. The CE and MS were interfaced by a co-axial sheath flow interface (Finnigan LCQ electrospray adaptor kit). The MS instrument was configured throughout to nano-spray mode using the Xcalibur software.

The procedure for CIEF-MS experiments was as follows:

A MicroSolv Zero flow capillary of approximately 90 cm in length was equilibrated with 1 % acetic acid for 6 minutes at 25 psi, then a 0.5-1 % ampholyte solution containing protein sample pumped through for 3 minutes at 25 psi. With the ESI source in an open position and the capillary pulled back 2 mm from the ESI tip, focusing of the protein ampholyte solution could occur. The end of the capillary in the CE instrument was used as the anode and was placed in 1 % acetic acid. The end of the capillary in the ESI source was used as the cathode with 1 % ammonia being pumped through the make up flow line to make a micro reservoir at the tip of the ESI source. Sheath gas was also applied to keep fresh ammonia on the tip but at gas pressures low enough to allow a droplet to form (liquid flow of 3 µl/min and gas flow setting of 7 in MS software) without being blown away. On addition of an electrical potential the current rapidly increased followed by a slow drop off during the focusing process. The current was monitored throughout and when a current value of

approximately 20 % of the initial starting current was attained, the proteins were sufficiently focused.

Mobilisation of the proteins was achieved by rapidly replacing the sheath liquid (make-up) with 50 % methanol, 1 % acetic acid. This was followed by aligning the capillary tip so that it was approximately 1 mm outside of the ESI source tip to give optimal spray into the MS detector. The ESI source was then closed up with pressure and voltage applied from the CE instrument. The MS was set to scan mode and a makeup flow of 3 µl/min used to ionise the proteins. Pressure and chemical mobilisation were used with this technique. A voltage of 30 kV was applied to the capillary for focusing and mobilisation. MS detection parameters are outlined in Table 2.

Table 2 LCQ Mass Spectrometry instrument settings for CIEF-MS experiments.

Parameter	Setting
Capillary spray voltage	5 kV
Sheath gas flow	10
Capillary Temperature	200 °C
Capillary Voltage	20 V
Tube lens offset	-25 V
Multipole 1 offset	0.75 V
Lens Voltage	-7 V
Multipole 2 offset	-4.0
Multipole RF Amplitude	960
Scan range	150-2000 m/z
Number of Microscans	10
Maximum inject time	50 ms
MS detection mode	Positive ion mode

MS parameters were optimised by performing infusion experiments using a standard of β-Lac-A at a concentration of 90 µl/ml in 50 % methanol/ 1 % acetic acid and setting the MS instrument to manual tune mode, tuning to a mass of 1531 m/z

3.7 Infusion MS experiments

Mass spectrometry infusion experiments were undertaken to measure the molecular mass of standard proteins. Samples were made up at an approximate concentration of 90 μg/ml in 50 % methanol/ 1 % acetic acid and infused at 3 μl/min. MS running conditions were identical to those outlined in section 3.6.

3.8 CZE of Whey Proteins

Whey proteins from skim milk were analysed by a CZE method modified from Kinghorn et al. (1996) as follows:

Whey protein was produced by acid precipitation of casein from skim milk at pH 4.6. The casein was removed by centrifugation at 14000 rpm for 6 minutes in an Eppendorf centrifuge. This also removed any fat that formed at the surface, with the resulting clear supernatant being a solution of whey protein. This was diluted 10 fold with Milli-Q water before being filtered through a 0.2 μm PVDF membrane (Titan Filtration Systems, SUN Sri, Wilmington, NC, USA) ready for CZE analysis. Sodium tetraborate (37.5 mM, pH 8.2) with 0.05 % Tween 20 additive was used as the run buffer. CZE was performed on the previously mentioned P/ACE MDQ CE system on a 60 cm x 50 μm i.d. (360 μm o.d.) bare fused silica column (Polymicro Technologies, Phoenix, AZ, USA). The column was equilibrated by rinsing with 0.1 M NaOH for 30 minutes followed by 10 minutes of flushing with Milli-Q water and a further 10 minutes flushing with run buffer. Each pressure rinse was performed at 25 psi. Samples were loaded at the cathode by pressure (0.5 psi for 5 seconds) and separated with 250 V/cm capillary length. Between runs the capillary was flushed with 0.1 M NaOH for 2 minutes, followed by 2 minutes with Milli-Q water and 3 minutes with run buffer, all at 25 psi pressure.

3.9 CZE of Casein

Casein was analysed by a method similar to that of Recio et al. (1997) and is outlined in full in Fong et al. (2003).

3.9.1 Buffers

Electrophoresis Run Buffer

The electrophoresis run buffer consisted of 0.32 M citric acid, 20 mM trisodium citrate, 6 M urea, and 0.004 % MHEC. The buffer pH was typically 3.0 ± 0.1 pH units with no pH adjustment necessary if made correctly. Before use the electrophoresis buffer was filtered through a 0.2 µm filter PVDF membrane (Titan).

Sample Buffer

The sample buffer consisted of 167 mM Tris, 42 mM MOPS, 67 mM EDTA, 6 M urea and 0.004 % MHEC. Before use, DTT was added to 17 mM. The buffer was typically pH 8.6 ± 0.1 pH with no pH adjustment necessary if made correctly.

3.9.2 Sample Preparation

A liquid milk sample (300 µl) was diluted with 700 µl of sample buffer, and allowed to stand for 1 hour at room temperature. The sample was centrifuged for 6 minutes at 14000 rpm (Eppendorf microfuge) to remove fat and was then filtered through a 0.2 µm filter PVDF membrane (Titan) prior to CZE analysis.

3.9.3 CZE Parameters

CZE was carried out on the previously described P/ACE MDQ CE, with separations taking place on a 30 cm x 50 µm i.d. OV-1701-OH deactivated fused silica CE column (TSP-050375-P-10, BGB Analytik AG, Anwil Switzerland). The detector window on the capillary was made by burning a small section of polyimide coating 5 mm long and 10 cm from the capillary end. The separation was conducted at 333 V/cm capillary length (10 kV) with a voltage ramp to this potential over 3 minutes. Samples were injected at 0.5 psi for 5 seconds followed by a water dunk to clean the column and then a small run buffer injection (0.1 psi for 0.1 minutes). Run time was 45 minutes at 30 °C with detection at 214 nm using a PDA detector. The capillary was rinsed between samples with run buffer for 3 minutes (at 25 psi) in a reverse mode. New capillaries were first rinsed with 50 % methanol for 30 minutes, followed by a Milli-Q water rinse for 10 minutes and then a 30 minute rinse with run buffer (all at 25 psi).

3.10 Flatbed IEF gel preparation

A stock solution of acrylamide/BIS was made up as follows: acrylamide/BIS (37.5:1) (5 g), urea (48.05 g) and glycerol (15 g) were made up to 100 mL with Milli-Q water, the solution was filtered through a 0.45 μm membrane (Millipore, MA, USA) and stored in an amber Schott bottle at 4 °C to be used as required.

10 mL of acrylamide/BIS stock solution and 0.625 mL of carrier ampholytes were degassed for 10 minutes.

TEMED (9.4 uL) was added and mixed well, followed by 9.4 μL of 40 % ammonium persulfate (freshly prepared). Immediately after mixing the solution was poured onto a plastic IEF gel mould (258x124 mm) seated on a level glass plate. An electrophoresis film (Sigma) was placed onto the liquid acrylamide to form an acrylamide sandwich, ensuring no air bubbles were trapped. A roller was gently passed over the surface of the sandwich to extrude any air bubbles. A glass plate was placed on top for added weight and the gel allowed to set (1 hour). The glass plates were removed and the IEF gel stored at 4 °C in a plastic bag sealed under vacuum until use.

3.10.1 IEF Sample preparation

IEF sample buffer was made up freshly each day using urea (7 M), thiourea (2 M), DTT (65 mM), and CHAPS (2.5 % w/v).

3.10.2 Skim Milk

Milk (100 μL) was diluted into 900 μL of IEF sample buffer, mixed and incubated for 60 minutes. After centrifugation for 6 minutes at 14,000 rpm (Eppendorf microfuge), the fat layer was removed and the supernatant filtered through a 0.45 μm filter (Titan).

3.10.3 Standards

Pre-made whey and casein protein standards were made up as outlined in section 3.2.4 and were further diluted in IEF sample buffer to a concentration of 1 mg/ml before being loaded onto the IEF gel.

3.10.4 Whey Basic Fraction

Whey basic fraction samples were made up according to section 3.2.1. Samples were then made to a concentration of either 1.5 or 3 mg/ml in IEF sample buffer before being loaded on to the IEF gel.

3.11 Flatbed IEF gel running conditions

Approximately 3 mL of kerosene was placed on the bed of a Bio-Phoresis horizontal electrophoresis cell unit (Bio Rad). An IEF gel was then placed on top with the electrophoresis film side down. Rolling the gel to remove all bubbles from under the gel, the top mould was lifted to expose the acrylamide gel. The IEF electrode strips (Pharmacia) were wetted and then placed parallel along the width of the IEF gel with the cathode strip at the top and the anode strip at the bottom. The cathode strip was wet with 0.5 M NaOH and the anode strip wet with 0.5 M phosphoric acid.

3.12 Focusing

An electrophoresis power supply (Model 3000Xi, Bio Rad) was used to pre-focus the ampholytes by applying 2000 V, 15 mA, 4 W, for 30 minutes at 12 ^{0}C using a LTD6G water bath cooling system (Grant Instruments Ltd, Barrington, England). After pre-focussing, sample strips (Pharmacia) were placed on the anode side of the gel and 15 µL of sample added to the entire strip.

Sample pre-focussing was performed by application of 2000 V, 15 mA, 4 W, for 60 minutes at 12^{0}C. Final focusing of the sample was then achieved by application of 3000 V, 5 mA, 20 W, for 90 minutes at 12^{0}C.

3.13 IEF gel staining

3.13.1 Coomassie Blue R-250 Stain

Coomassie brilliant blue R-250 (2.5 g) was dissolved in isopropanol (1250 ml), glacial acetic acid (500 ml), and made up to a total volume 5 liters with Milli-Q water. This solution was stirred overnight and filtered under vacuum through a filter paper (Whatman No.1, Whatman International limited, Kent, England).

3.13.2 Coomassie Destain

Coomassie blue destain was made with a composition as follows:

Isopropanol (500 ml) and glacial acetic acid (500 ml) were diluted in Milli-Q water to a final volume of 5 litres.

3.13.3 Staining Procedure

The gel was fixed in 15 % TCA for 15 minutes followed by rinsing with water and addition of Coomassie blue stain for 1 hour. This was followed by overnight (approximately 20 hours) destaining in Coomassie destain. The resulting gel was scanned by laser densitometry using a Molecular Dynamics series 300 Personal Densitometer and analysed with ImageQuant software (Amersham Biosciences, Buckinghamshire England)

3.14 2-Dimensional Gel Electrophoresis Experiments

These were performed using pre-made IPG (immobilized pH gradient) strips and an Ettan IPGphor (Amersham Biosciences) for IEF in the first dimension with SDS-PAGE in the second dimension.

3.14.1 Buffers

Rehydration Buffer

This consisted of urea (12 g), CHAPS (0.5 g), IPTG buffer (125 ml), bromophenol blue (a few crystals), and Milli-Q water (16 ml). Rehydration buffer was kept in vials frozen at −20°C ready for use as required.

SDS equilibration Buffer

5 M Tris-HCl pH 8.8 (6.7 ml) was added to urea (72.07 g), glycerol (69 ml), SDS (4.0 g), bromophenol blue (a few crystals), with Milli-Q water to 200 ml.

Electrophoresis run buffer

Tris (15 g), glycine (72 g), and SDS (5 g) were mixed and made up to 1 litre in Milli Q water. The resulting buffer was stored at 4 °C and diluted 5 fold before use.

3.14.2 Sample Preparation

DTT was added to rehydration buffer at a concentration of 0.02 M prior to addition of freeze-dried sample to 2.5 mg/ml (sample volume 1ml). Sample was dissolved and allowed to stand for 1 hour before centrifugation (Eppendorf microfuge) at 14000 rpm for 3 minutes.

Sample solution supernatant (185 µl) was then dispersed onto an 11 cm IPG strip (Bio Rad 163-2014) of pH range 3-10. Dry Strip cover fluid (Mineral Oil) (Pharmacia Biotech, Uppsala, Sweden) was applied over the top of the strip to keep it from drying out and the strip allowed to passively rehydrate overnight at room temperature.

3.14.3 IEF Focusing

The rehydrated IPG strip was placed in a ceramic IEF casket (11 cm) (Amersham, Uppsala Sweden) with its gel side in contact with the electrodes. A piece of damp filter paper was placed over each electrode prior to strip placement to act as a salt sink. Another coating of Dry Strip cover fluid was applied and the casket placed appropriately on an Ettan IPGphorII system (Amersham Biosciences). The sample was then electrophoresed at a gradient of 1000 V/hr until 2000 V was achieved and then electrophoresed at this voltage for a further 22 hours (45000 V hours) at 20 °C. The IPG-strip was removed and prepared for the second dimension.

3.14.4 Second Dimension SDS-PAGE

DTT (10 mg/ml buffer) was added to SDS equilibration buffer and 5 ml of this solution placed in a test tube. The IPG strip was added to the test tube and equilibrated by gentle agitation for 15 minutes.

Vertical SDS PAGE was carried out using a midi (11 cm) 8-16 % Criterion™ precast Tris HCl gel system (BioRad, Hercules, CA, USA). This was prepared by rinsing the application well with water and then electrophoresis run buffer. The IPG strip was placed on the gel within the application well, ensuring complete contact with the surface of the gel. A layer of warmed agarose gel (0.5 % w/v in electrode buffer) was gently applied over the strip to seal it in place and then more electrophoresis run buffer was placed on top of the gel. The gel was placed into a Criterion electrophoresis tank (Bio Rad) and the reservoir filled with electrophoresis run buffer.

The gel was run at 210 volts, 70 milliamps and 12 watts for 2 hours using a Bio Rad model 1000/500 power supply.

The gel was stained overnight in Coomassie blue R-250 stain and destained for a further day in Coomassie blue destain (solutions are outlined in section 3.13.1 and 3.13.2, respectively). The resulting gel was imaged by densitometry as described in section 3.13.3.

4 Results

4.1 CIEF-UV Water Soluble Method

4.1.1 Method development protocol

Initial work on CIEF was performed with a Beckman CIEF kit, which included column, ampholytes, run buffers and sample buffer coupled with a PDA detector. Using this kit, good results were easily obtained for the standards provided. The work was further developed to produce optimized systems, which would be applicable to the analysis of soluble dairy whey proteins with either UV or MS detection. In addition, when the methods were optimized, the parameters were applied to a system designed to examine insoluble casein proteins, and membrane proteins. Initial developmental work was undertaken using a PDA detector, as there was several months delay in obtaining the UV detector required for CIEF analysis. Detector choice will be discussed later in the results section with relevant examples (Section 4.1.5).

General work on method development is outlined below.

4.1.2 Protein Concentration

In much of the developmental work, a series of protein *pI* markers of known *pI* and concentration were used. Generally the standard mixture consisted of trypsinogen, myoglobin, carbonic anhydrase I, trypsin inhibitor, and amyloglucosidase. However, in some later experiments ribonuclease-A and CCK flanking peptide were used instead of trypsinogen and amyloglucosidase, respectively, but where there was a change this will be noted in the text. The *pI* values for these proteins are listed in Table 3 along with typical working concentrations used. Additionally, a skim milk whey sample was made as in section 3.2.2 with the main protein constituents β-lac-B, β-lac-A, and α-Lac also being used as individual protein markers. The literature *pI* values of the major whey and all the other common dairy proteins are also listed in Table 3 as are the molecular weights of all the proteins and peptides used in this study.

Table 3 Literature values for isoelectric points and molecular weights of proteins used throughout this research. Typical CIEF working concentrations are also included.

Protein	Abbreviated Name	Literature *pI*	Literature Molecular Weight (Da)	Typical CE Concentration µg/ml
Ribonuclease-A	RBA	9.45	13700	30
Trypsinogen	Tryp	9.30	23700	30
Myoglobin Basic	Mb-B	7.35	16951	60
Myoglobin Acidic	Mb-A	6.85	16951	
Carbonic Anhydrase I	CA	6.55	29000	60
β-Lactoglobulin-B	β-Lac-B	5.34	18281	
β-Lactoglobulin-A	β-Lac-A	5.26	18367	
α-Lactalbumin	α-Lac	4.80	14172.5	
Trypsin Inhibitor	TI	4.50	20100	7.5
Amyloglucosidase	AM	3.50	70000- 89000	7.5
CCK Flanking Peptide	CCK	2.75	1074.1	7.5
Lactoferrin	Lf		83100	
Lactoperoxidase	Lp		77500	
Bovine Serum Albumin	BSA	5.13	66432	
Proteose Peptone 5	PP-5		12177- 12483	
Glycomacropeptide	GMP		6754-6780	
Immunoglobulin-G	IgG		150000	
α-Casein	α-CSN	4.96	23600	
β-Casein-A1	β-CSN-A1	5.27	24020	
β-Casein-A2	β-CSN-A2	5.19	23980	
κ-Casein	κ-CSN	5.43-5.64	19005-19037	

The general CIEF response under optimized conditions for a 30 cm column with UV detection can be seen in Figure 3. This is the first example of the major skim milk whey proteins being successfully separated by CIEF as opposed to one of the β-Lac genetic variants or α-Lac being used as a *pI* marker for the separation of other protein samples in CIEF. Each of the method development topics that follow investigates the reasoning for selection of these conditions, before coming to the final overall optimal separation.

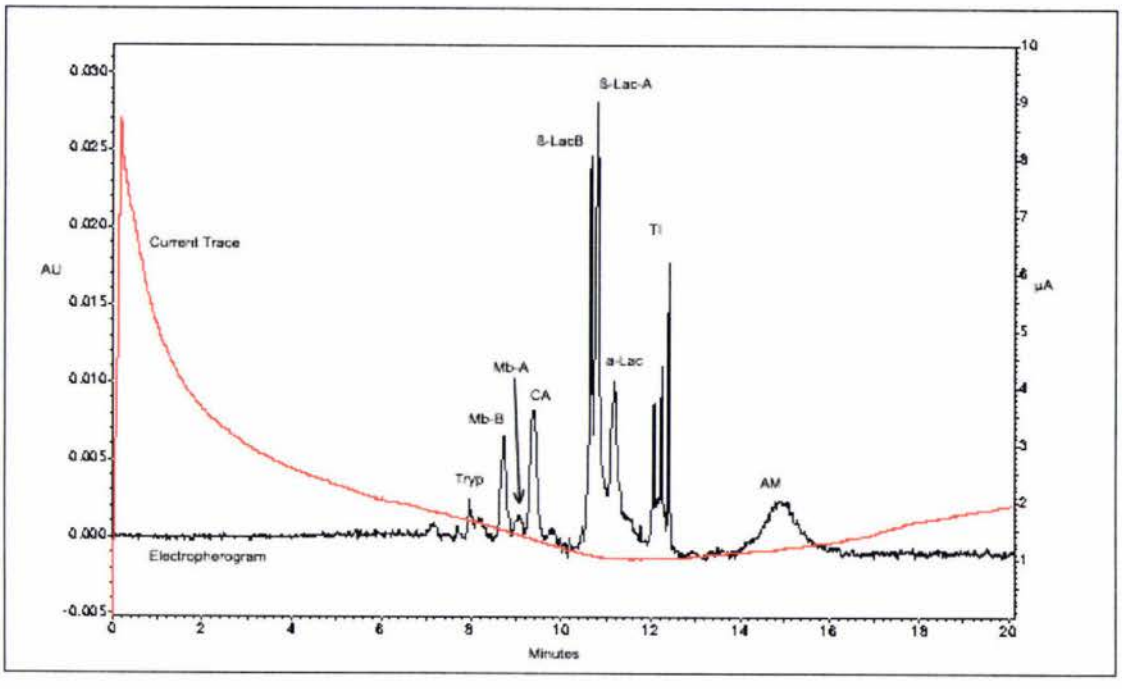

Figure 3 A typical electropherogram (Black) with current trace (Red) of whey protein from skim milk, with internal pI markers added. The sample was run on a 30 cm MicroSolv Zero flow column at 12 kV. Focusing was performed for 6 minutes followed by pressure mobilisation at 0.1 psi. Anode comprised 20 mM phosphoric acid and cathode buffer comprised 20 mM sodium hydroxide. Ampholytes used were Beckman 3-10 at 2 % (v/v) concentration. Tryp = trypsinogen, Mb-B = myoglobin basic, Mb-A = myoglobin acidic, CA = carbonic anhydrase I, β-lac-B = β-lactoglobulin-B, β-lac-A = β-lactoglobulin-A, α-Lac = α-lactalbumin, TI = trypsin inhibitor, AM = amyloglucosidase. Detection was UV at 280 nm.

The CIEF method of analysis effectively concentrates a protein into a very small region of the capillary at its isoelectric point. This concentrating effect can sometimes cause a protein to precipitate out of solution. The working concentrations of the standards thus varied due to precipitation problems with some standards. Trypsin inhibitor (TI) and amyloglucosidase (AM) were examples of proteins that readily precipitated out of solution during the CIEF process, resulting in a large spike observed in many electropherograms (for example TI in Figure 3) and so for this

reason concentrations were limited to approximately 7.5 μg/ml (Figure 3). The working concentrations of myoglobin (containing both the basic and acidic protein form) and carbonic anydrase II were 60 μg/ml, while 30 μg/ml was used for trypsinogen (and later the same for ribonuclease A). Standards were used to assess the effect of linearity for *pI* versus migration time on such parameters as: column coating, ampholyte brand, running buffer, sample buffer, mobilisation technique, focusing time, difference in voltage, temperature, method reproducibility and calculation of the resolution of separation. The red trace shown in Figure 3 is the current trace obtained from the CIEF process. The electrical potential was ramped up initially from 0 volts to 12 kV in 10 seconds, hence the sudden increase in current seen at the start of the trace. Once the current had peaked it started to drop off, initially at a rapid rate, but then at a progressively slower rate seen as a decreasing slope of the curve. This phenomenon can be explained as follows. The protein and ampholyte solution within the capillary are initially in a homogeneous mix. On addition of an electrical potential a concentration gradient is set up. Since the bulk of the ampholytes and proteins in solution are not at their isoelectric points and are charged species, a high current is formed. As the ampholytes and proteins move towards their respective isoelectric points along the pH gradient and a greater amount reach their *pI*, the current starts to decrease. This is because there is increasingly less protein and ampholyte with a nett charge. When all the ampholyte and proteins have been focused the current plateaus to a level a little greater than zero. The current does not drop to zero as protein and ampholytes constantly move in and out of equilibrium very slightly from their *pI* values. Salts in solution also add to this effect. In Figure 3 it can be seen that the current starts to rise again after 11 minutes. This is due to the mobilising effect of the CIEF procedure; as phosphoric acid is pushed into the column, heating occurs due to the voltage still being applied, and this results in a raised current. The current is therefore a very good indicator for monitoring whether a CIEF experiment is running well, and can be used to determine when the separation is complete in the case of unknown samples.

4.1.3 Buffer Choice

From prior literature, CIEF buffers generally consist of either phosphoric acid or acetic acid at different concentrations at the anode, and either sodium hydroxide or ammonia at the cathode. In general, phosphoric acid and sodium hydroxide are used

with UV detection (Rodriguez-Diaz et al., 1997) as a greater degree of resolution is achieved with these buffers than with acetic acid and ammonia run buffers that are compatible with mass spectrometry. This can be seen in the electropherogram in Figure 4, particularly in the area of the β-Lac-A and α-Lac peaks. With phosphoric acid and sodium hydroxide, separation of these peaks was observed. However, this was not the case with the acetic acid and ammonia buffers. A possible reason for the somewhat poorer separation with acetic acid and ammonia could be the slightly narrower pH range developed due to the weaker acid and base strength in comparison with phosphoric acid and sodium hydroxide. pH values for each buffer trialled are shown in Table 4. The pH value for phosphoric acid was somewhat lower than that of acetic acid or any of the other buffer combinations. In addition, the pH value for sodium hydroxide was somewhat higher than that for ammonia. The greater pH range for the phosphoric acid and sodium hydroxide buffers may allow the focusing process to give a more linear pH range resulting in a more even spread of ampholytes across the capillary.

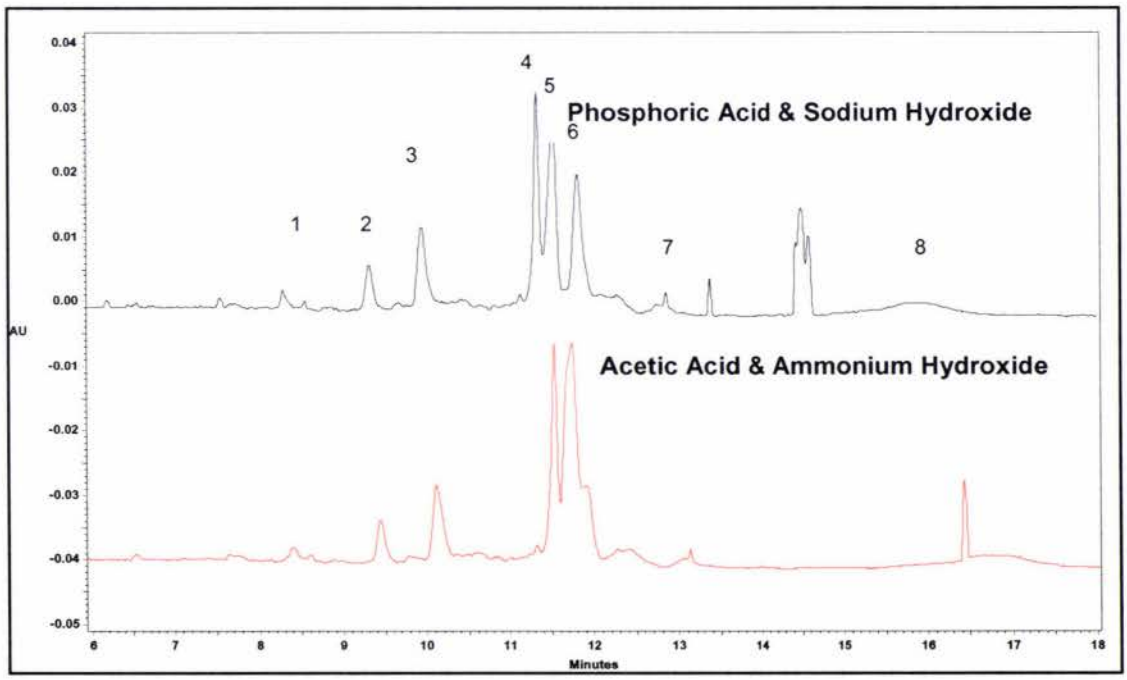

Figure 4 Comparison of buffer types. Electropherograms of skim milk whey protein with internal standards. Samples were ran in an identical manner to that in Figure 3 except bottom trace (Red) represents run with 1 % acetic acid at the anode and 1 % ammonia at the cathode. Peak 1 = trypsinogen, peak 2 = myoglobin, peak 3 = carbonic anhydrase, peak 4 = β-lactoglobulin-B, peak 5 = β-lactoglobulin-A, peak 6 = α-lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase.

Table 4 pH values for focusing buffers and mobilisation buffers in CIEF experiments.

Buffer Composition	pH	Buffer use
20 mM Sodium Hydroxide	12.9	Focusing
1 % Ammonia	12.6	Focusing
20 mM Phosphoric Acid	1.9	Focusing
1 % Acetic Acid	2.6	Focusing
1 % Acetic Acid & 50 % Methanol	3.0	Mobilisation
1 % Acetic Acid & 50 % Acetonitrile	2.9	Mobilisation
0.2 % Formic Acid & 50 % Methanol	2.8	Mobilisation
0.2 % Formic Acid & 50 % Acetonitrile	2.7	Mobilisation

In other work (not shown) there were many instances, particularly when using a 60 cm column, where peak resolution diminished and broader peaks were seen for acetic acid and ammonia buffers when compared to phosphoric acid and sodium hydroxide buffers. From Figure 4 it can be seen that the retention times for the proteins increased slightly from one buffer set to the other. With a longer column these retention times increased even further due to the increased focusing distance (See Figure 7 for a comparison of results using different length capillaries with phosphoric acid and sodium hydroxide).

For phosphoric acid and sodium hydroxide buffers there did not appear to be any advantage in using different buffer concentrations. Although buffer depletion can become an issue when large amounts of samples are run from the same buffer vials, it was decided that a 20 mM concentration of both was the best choice for general UV detection of water-soluble proteins. Use of sodium hydroxide at higher concentrations might have affected the column by making the silica of the column more brittle at the cathode end when constantly inserted in the alkali solution (Wehr et al., 1999, p 140).

Buffers were made up freshly each day from a stock solution of higher known concentration. This was particularly important for the sodium hydroxide as it can form carbonate compounds with atmospheric carbon dioxide gas (Wehr et al., 1999, p 140).

The same 2 ml buffer vials could be used for more than 40 de-salted samples run consecutively, with no changes in the separation taking place.

The use of a buffer system utilizing either 50 % methanol or 50 % acetonitrile with either formic acid (at 0.2 %) or acetic acid (at 1 %) was used as a means for chemical mobilisation to replicate conditions required for MS analysis. The results (not shown) showed that the use of formic acid on the capillary column affected the surface chemistry of the column causing it to malfunction. The capillary was irreversibly damaged, this occurring on 2 separate occasions. Further work showed that mobilisation with 50 % methanol and 1 % acetic acid (pH 3.0) gave similar results to mobilisation with 1 % acetic acid (pH 2.6) in Milli-Q water although there was a slight difference in pH of the buffers.

4.1.4 Column Choice, Length & Internal Diameter

4.1.4.1 Choice of Column

A number of different columns with different internal coatings were tried to not only optimise separation with regards to linearity of *pI* versus migration time but also test the robustness of the capillary. Figure 5 shows electropherograms of an identical sample of *pI* markers and dairy whey proteins isolated from skim milk run on different columns of the same length (30 cm) under the same conditions. All columns were equilibrated as per the manufacturer's instructions before use. By visually observing the electropherograms, elimination of several of the capillaries was possible, as the separations achieved were inferior to some others. This included the Beckman Neutral, MicroSolv Low Flow, and SGE polyethylene glycol columns. It must be noted that the Beckman column was purchased as a pair of pre-cut 30 cm columns which were particularly expensive. Good results were obtained with the first column although none of this work was performed with a UV detector or with the whey protein and *pI* markers used throughout this report. The second column was stored as per instructions for several months before being used (results in Figure 5). Unfortunately, it seemed that the column coating had deteriorated in this time period, as the separation resolution was extremely poor. The current traces obtained with this column indicated, however, that the IEF process was occurring as normal. In addition, the Beckman column was not suitable for CIEF-MS as it was only available as a 30 cm column, and a 90 cm column was required for this technique. The Beckman

column has an internal coating of acrylamide. An in-house acrylamide coated column was made using the method of (Kilár & Hjertén, 1989) but was found to give very irregular results and have a very short lifetime (results not shown).

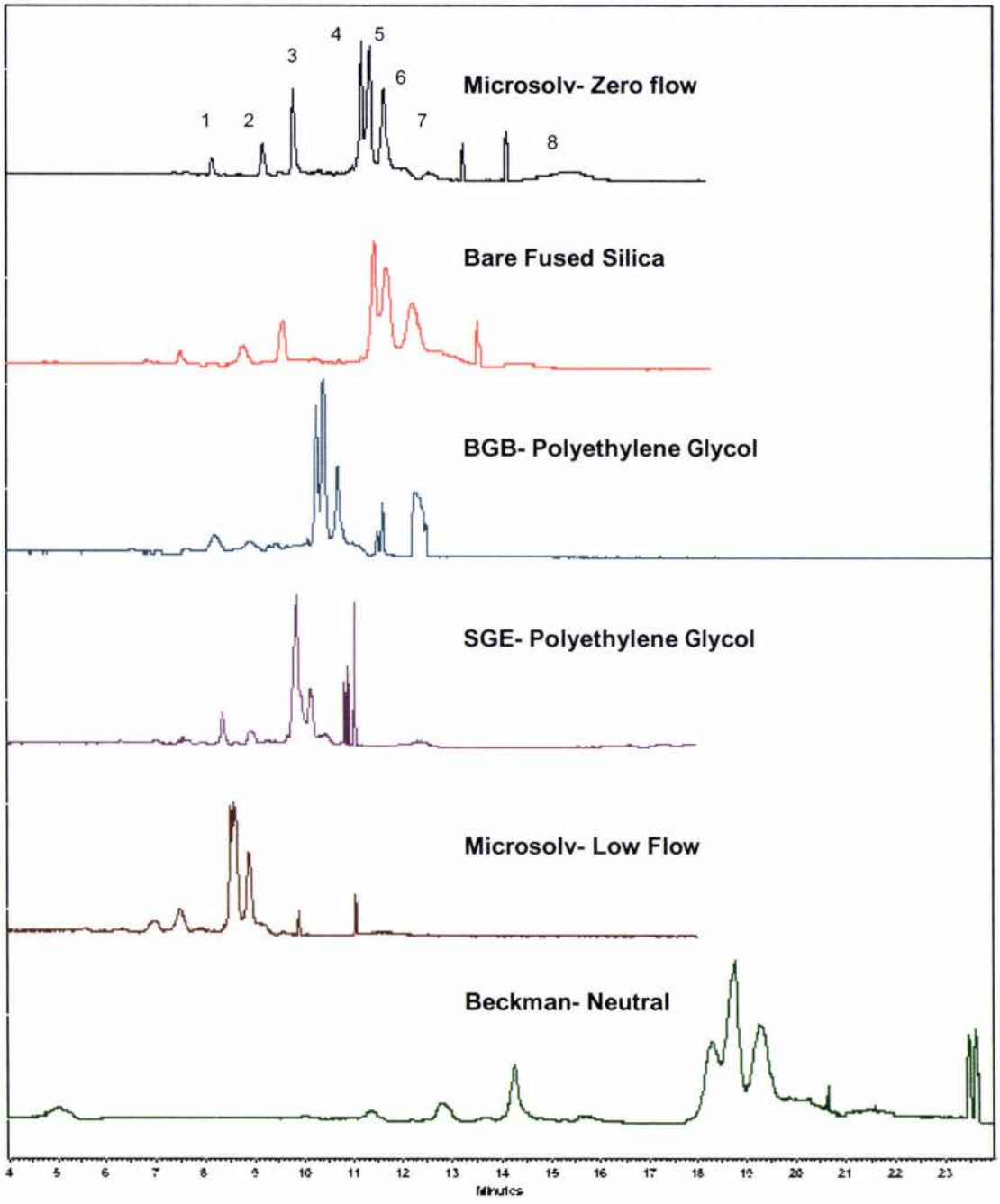

Figure 5 Comparison of column coatings. Electropherograms of whey proteins from skim milk and internal *pI* standards run in a manner identical to that in Figure 3 except different columns (30 cm) were used to generate each electropherogram. From the top trace: Black- MicroSolv Zero flow, Red- Bare fused silica, Blue- BGB, Purple- SGE, Maroon- MicroSolv Low flow, Green- Beckman neutral capillary. Peak 1 = trypsinogen, peak 2 = myoglobin, peak 3 = carbonic anhydrase, peak 4 = β-lactoglobulin-B, peak 5 = β-lactoglobulin-A, peak 6 = α-lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase.

The MicroSolv columns come in a range of different coatings with different levels of EOF control. In addition to the two columns tried here, there are medium and high flow columns available. Since in this work, the Low Flow column was unable to give baseline separation of β-Lac-A or β-Lac-B, and the first *pI* marker (trypsinogen) was detected just after focusing even when the column was new (having less EOF than an older column), the use of this column was not pursued; neither were the other higher flow columns. It is also interesting to note the peak shapes for myoglobin basic and carbonic anhydrase proteins were particularly poor using the Low Flow column, with diffuse humps rather than sharp peaks being observed. This may suggest some protein binding to the column.

The SGE column was also unable to separate β-Lac-A from β-Lac-B, and it was therefore considered that this type of polyethylene coating was not adequate for the study. Additionally, the peaks obtained for myoglobin or carbonic anhydrase were very low in intensity compared to those seen with the MicroSolv Zero Flow or Bare fused silica columns.

Although the BGB polyethylene glycol coated capillary gave excellent separation of β-Lac-A, β-Lac-B, and α-Lac, the use of this column was not pursued, as the peaks for many of the other proteins were poor including trypsinogen, myoglobin, carbonic anhydrase, and amyloglucosidase.

Bare fused silica gave very good results; because the capillary does not have a coating it is cheap and has an extremely long lifetime and in this sense it was a particularly desirable column to pursue. The use of bare fused silica columns has also been reported elsewhere with good results (Kilár et al., 1998) The linearity of the *pI* standards versus migration time was good for the 30 cm column compared to the MicroSolv Zero Flow column and BGB columns. However with numerous repeat runs, a lot of abnormal peak shapes and sizes occurred, particularly in the acidic region and it became very hard to positively identify some proteins in this region. Visual comparison of the overlaid electropherograms (Figure 5) clearly showed the MicroSolv Zero Flow column gave the best results in terms of peak shape and intensity and separation of proteins of similar *pI*. With the 30 cm column the linearity of *pI* versus migration time was generally not very good (Table 5) and gave low accuracy *pI* values for unknowns. However as will be discussed later, a longer column

gave very good linearity of standards and *pI* approximation for unknown proteins with good reproducibility.

Table 5 Comparison of the electropherograms obtained from using different 30 cm columns as shown in Figure 5.

Column Coating	*pI* vs Migration Time Equation	R^2 of Best Fit	% Error of unknowns	Separation of Proteins	Peak Shape
MicroSolv-Zero flow	y = -0.741x + 14.362	0.895	15.62	Very Good	Excellent
Bare Fused Silica	y = -0.7309x + 14.064	0.932	6.27	Very Good	Good
BGB	y = -1.0479x + 16.388	0.918	5.77	Very Good	Good
SGE	y = -1.1356x + 17.108	0.930	13.03	Poor	Average
MicroSolv Low Flow	y = -1.0238x + 14.892	0.902	17.26	Poor	Poor
Beckman Neutral	y = -0.2328x + 10.50	0.806	19.62	Poor	Very poor

From the electropherograms obtained in Figure 5 a plot of *pI* versus migration time was constructed for each column (Figure 6) with the results for linear fit being given in Table 5. The Table lists the separation and peak shape obtained with the columns in Figure 5 as good, average or poor, and includes the average error in calculating the *pI* of the whey proteins (from skim milk) from the standard curves outlined in Figure 6, using literature values as reference points. Table 5 also gives the R^2 values of the calibration curves for each of the columns in Figure 5. The results show that although R^2 is not always related to the percentage error (for example the SGE column), it is often a good indication of accuracy of results (for example Bare Fused Silica and BGB columns). From different results obtained at different times, there seemed to be some variation in column quality, as other 30 cm Zero flow capillaries gave (under the same conditions and sample concentrations) regressions up to 94.2 % linearity (Table 7).

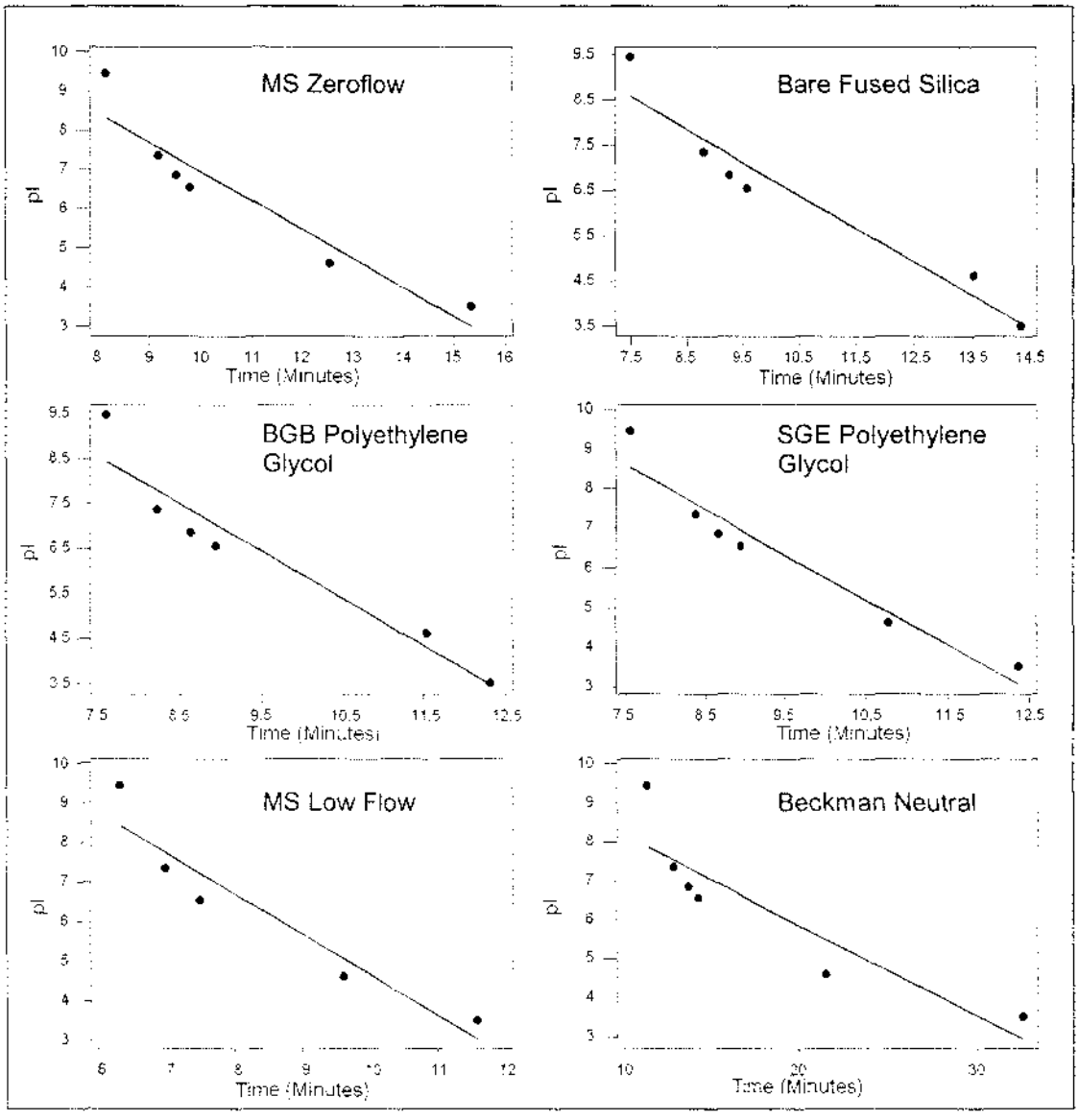

Figure 6 Calibration Curves of *pI* versus migration time for each column type compared in Figure 5. The equation and regression values for each column are expressed in Table 5.

4.1.4.2 Column Length

Column length affected linearity of *pI* versus migration time and hence the accuracy of determination of unknown proteins. In the comparison of column lengths (Figure 7), the samples were run at the same concentrations and under identical running conditions including the voltage applied per centimetre of column length. The results showed an increase in peak height and area for the 60 cm column as would be expected as the volume of sample injected is doubled with filling the entire length of the column (Table 6). Retention times were also increased as the focused protein bands had further distance to travel to the detector. An improvement in the linearity of standards was observed and is shown in Table 7. This was no doubt due to the longer

distance over which separation could take place along with the increased time for not only focusing but also mobilisation. There is often not much time between the focusing and mobilising step for a 30 cm column as proteins drift towards the detector due to EOF. In this case the basic proteins are closer to the detector irrespective of EOF, and so the pH gradient at this point is often not as stable. If the current traces of the 30 cm and 60 cm experiments are examined, then it is noticed that for the 30 cm column (as in Figure 3) the current curve is still moving down at a moderate gradient at the time the most basic protein is being detected whereas for the 60 cm column the gradient of the current at this time is very much less (data not shown). This would indicate that a more linear stable pH gradient exists within the capillary at the time of detection. Often it has been seen that the elimination of the most basic *pI* marker (and most acidic *pI* marker) will improve standard curve linearity and accuracy of *pI* determination for the 30 cm column.

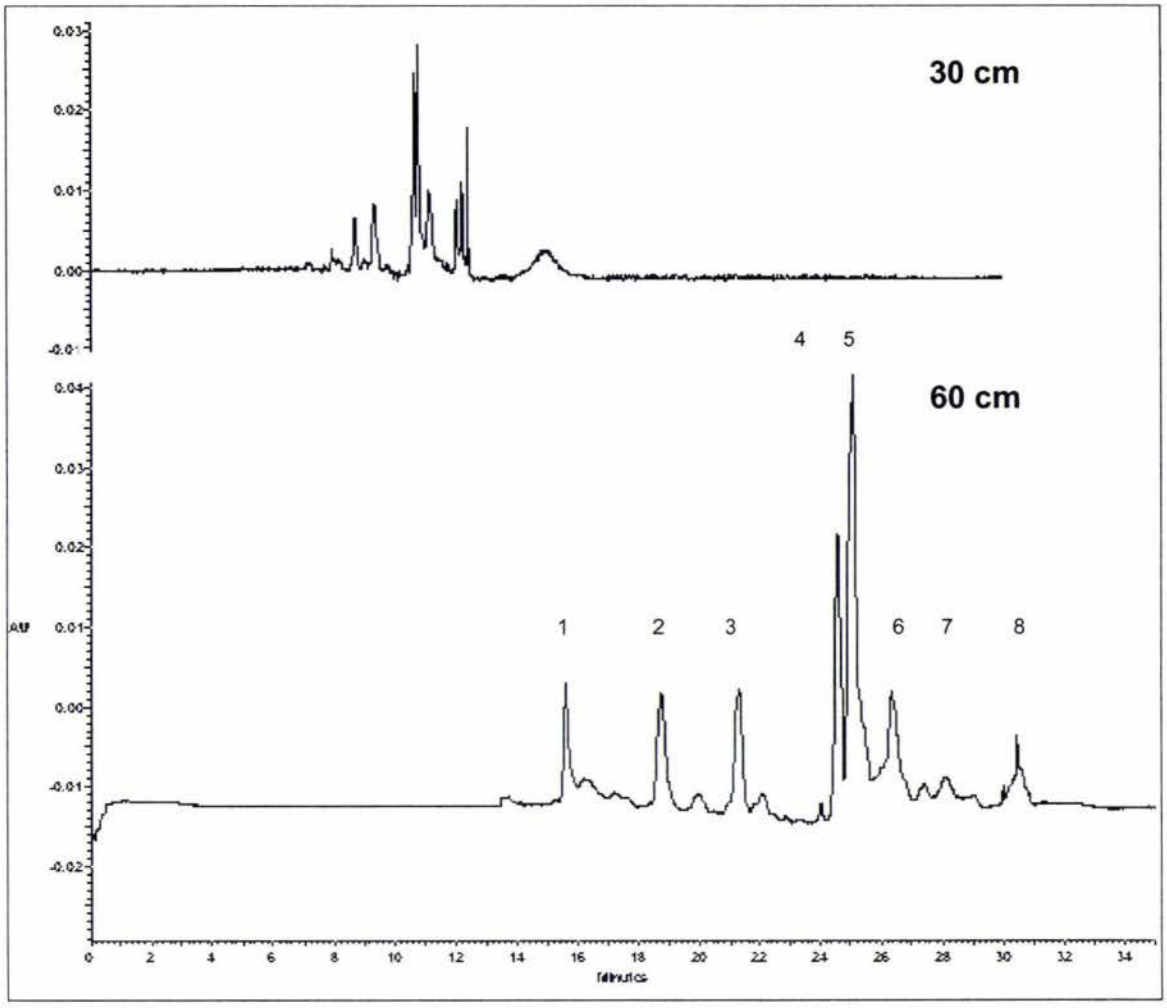

Figure 7 Comparison of column length. Electropherograms of skim milk whey proteins and internal *pI* standards. Both electropherograms run identically to Figure 3 except that the bottom electropherogram was run on a 60 cm column with a voltage of 24 kV to be consistent with the 30 cm column. Peak 1 = trypsinogen, peak 2 = myoglobin, peak 3 = carbonic anhydrase, peak 4 = β-lactoglobulin-B, peak 5 = β-lactoglobulin-A, peak 6 = α-lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase.

Table 6 Comparison of column volume (nl) when changing parameters such as length or internal diameter. Calculated from CExpert (Beckman Coulter).

Column Length (cm)	Column Internal Diameter (μm)	Column volume (nl)
30	50	589
30	75	1325
60	50	1178
60	75	2651

Table 7 Comparison of results from the electropherograms shown in Figure 7 for differences in column length on the MicroSolv Zero Flow capillary and between batches of capillary (For 30 cm results).

Column Length	Equation of pI vs Migration Time	Regression (R^2)	% Error of unknown proteins
30 cm	y = -0.7425x + 14.057	0.8872	16.05
30 cm	y = -1.1352x + 15.957	0.942	6.51
60 cm	y = -0.3568x + 14.316	0.9737	2.71

4.1.4.3 Column internal diameter.

A comparison of capillaries with different internal diameters and identical column coatings was undertaken to identify whether a larger diameter capillary would increase the sensitivity of the method. Figure 8 shows the separations achieved when the same sample was run using identical conditions on both 50 and 75 μm i.d. columns. Although the 75 μm i.d. column gave a faster separation, the resolution of separation was poorer as there was no separation of the whey proteins with similar *pI*'s. There also appeared to be no increase in sensitivity with the 75 μm i.d. capillary. This is somewhat surprising considering the increase in capillary volume is more than doubled (Table 6), allowing an increase in injection volume of two fold. Whether there was a problem with the 75 μm i.d. column is unknown; however from the literature the general trend for capillary internal diameter (Appendix 1 Table 14 and Table 15) was toward use of 50 μm i.d. and so for this study the use of 50 μm i.d. columns was continued.

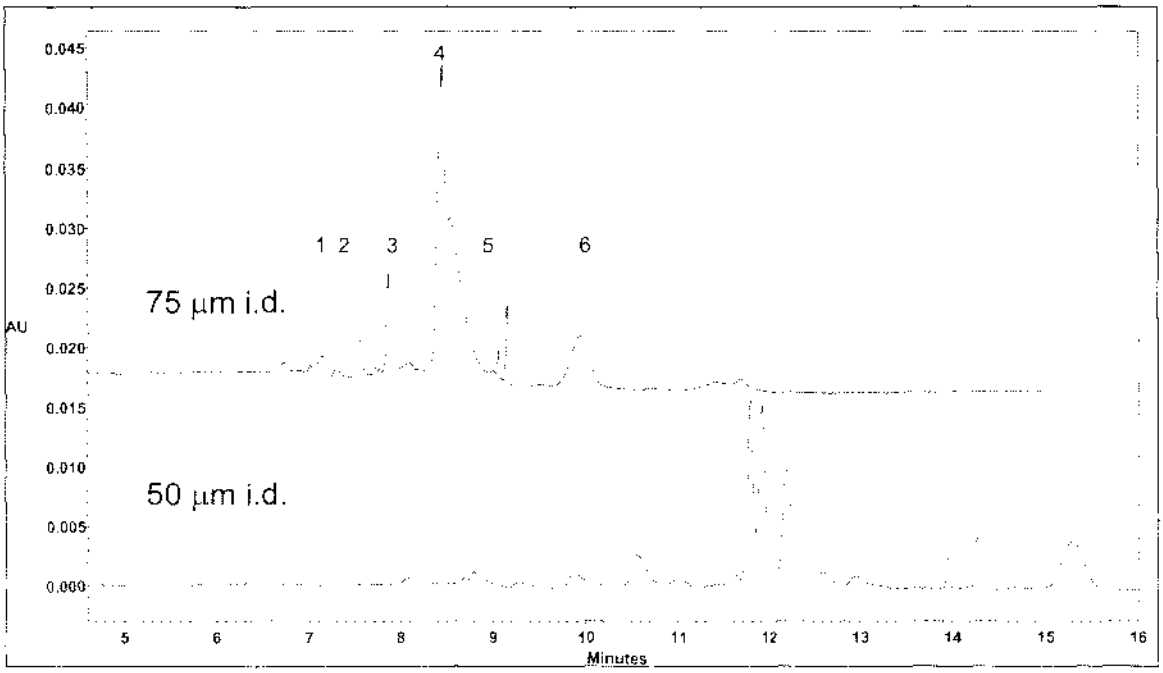

Figure 8 Electropherograms of whey protein with internal pI standards for capillaries of 75 µm i.d. (top) and 50 µm i.d. (bottom). Note standards are identical to those used in Figure 3 except trypsinogen is replaced with ribonuclease A and amyloglucosidase is replaced with CCK flanking peptide. Peak 1 = ribonuclease A, peak 2 = myoglobin, peak 3 = carbonic anhydrase, peak 4 = β-lactoglobulin-B, β-lactoglobulin-A, and α-lactalbumin peak 5 = trypsin inhibitor, and peak 6 = CCK flanking peptide.

4.1.5 Detection Choice and Wavelength Selection

The use of a filter UV detector rather than photo diode array (PDA) detector is preferred for CIEF as the filter detection system eliminates a lot of wavelengths of light from passing through the capillary. The use of a PDA means that all wavelengths of light pass through the detection window in the capillary. The extra wavelengths create noise and interference from the capillary's internal coating and so incomprehensible spectra are obtained. Figure 9 shows the effects of using a PDA detector at two different wavelengths (top two traces) as compared with a filter type UV detector (bottom two traces). In each run the current was monitored and shown to behave similarly to the current trace in Figure 3. From this it could be deduced that nothing was wrong with the separation itself but that the response was purely due to the detector.

Wavelength of detection is another problem with CIEF. For most dairy protein analysis it is desirable to detect proteins at 214 nm due to higher absorption coefficients at this wavelength. Unfortunately in CIEF it is not possible to use this

wavelength successfully as there is a lot of interference from the ampholytes at this wavelength. The use of 280 nm is better; although absorbance readings are lower compared to 214 nm, a relatively flat base line is achieved. Figure 9 shows these differences in signals obtained at 214 and 280 nm for whey proteins in skim milk. It is interesting to note that the scales of the top 3 electropherograms are identical, whereas the bottom trace (280 nm with UV filter detector) has a scale of approximately 10 % of the intensity of the 214 nm UV detector and PDA detector traces. Thus one of the problems for the CIEF technique compared to other CE methods is that the limits of detection are reduced 10 fold by using the 280 nm wavelength. On the other hand, in other CE techniques a sample injection of no more than 5 % of the column volume is used, whereas in CIEF the entire column is filled. Therefore the limits of detection of the CIEF method still remain higher due to higher sample load, but could be improved if ampholytes were produced that do not absorb at the lower wavelengths.

The use of mass spectrometry as a detector will be discussed later in section 4.4. , as it is a field of its own in which special conditions are used to make detection possible.

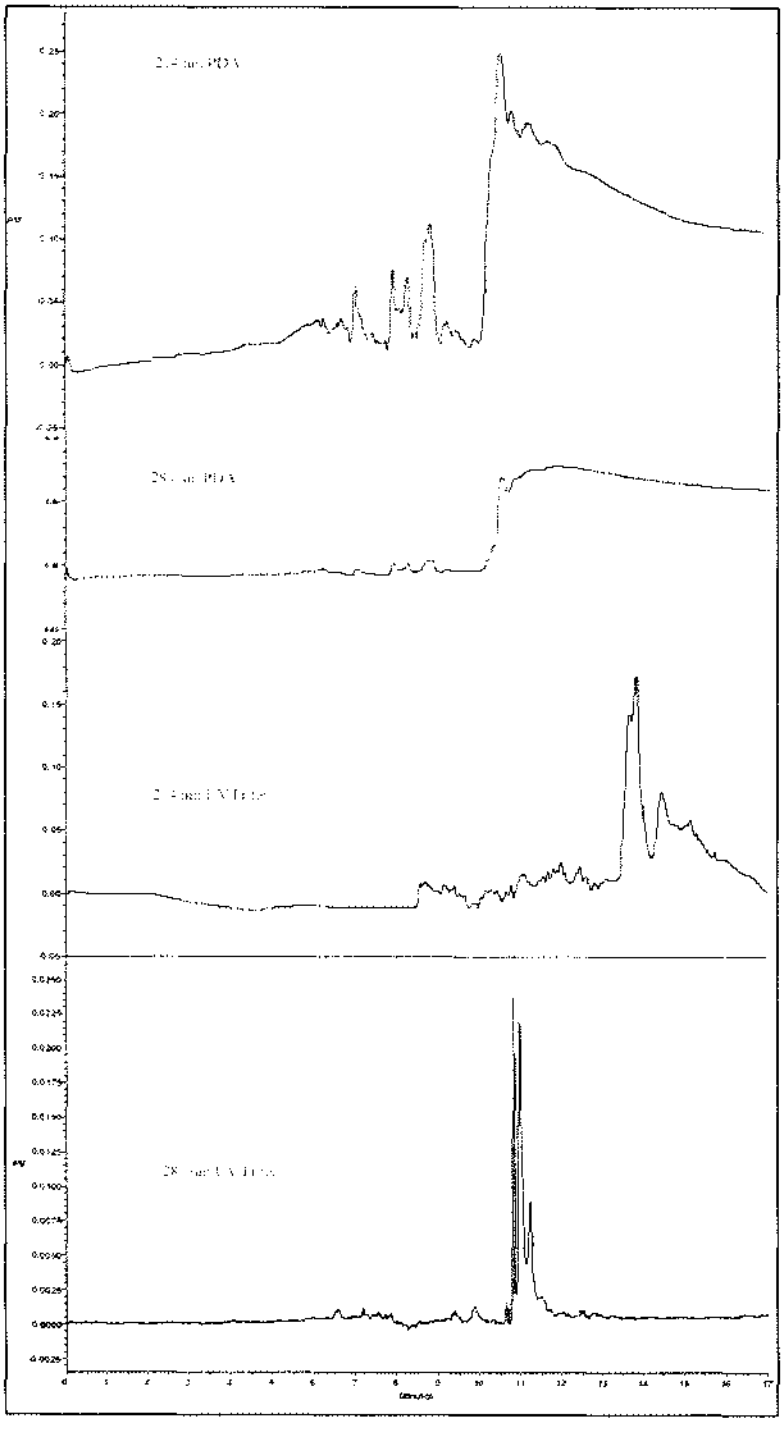

Figure 9 Comparison of detector type and wavelength. Samples are whey protein from skim milk run identically to Figure 3. From top to bottom: 214 nm PDA detector, 280 nm PDA detector, 214 nm UV detector, and 280 nm UV detector.

4.1.6 Ampholyte Choice

A survey of different ampholyte brands was performed to see which were best suited to CIEF since most commercially available ampholytes are designed for flat bed gel IEF. The experiments were all conducted on the same column (60 cm MicroSolv Zero

flow), using the same sample concentration of skim milk whey protein spiked with β-Lac-B (33 μg/ml sample) mixed with a 2 % (v/v) solution of different ampholytes. The majority of ampholytes used had a separation range of pH 3-10 and included the following brands: Beckman, Pharmalyte, Fluka and BioRad 3-10; Sigma ampholytes had a pH range 2.5-7. Figure 10 demonstrates the differences in ampholyte brands.

In the top electropherogram in Figure 10 Beckman ampholytes that are specifically designed for CIEF were used. This type of ampholyte clearly gave better results in terms of detector response than the other brands of ampholytes. The separation time was also fairly good but the peaks were not as well resolved as with other ampholyte types. This may be due to the quicker time for separation with some of the other ampholytes, particularly Fluka and Sigma, thus the protein peaks obtained from the Beckman ampholytes are broader due to the longer time the proteins remain in the column and the longer time they then require to pass the detector.

BioRad ampholytes gave poor results. Although these have been used for many other CIEF applications (Appendix 1 Table 14), and good results had been obtained with these ampholytes on IEF gels, results with the capillary column were not very good. Peaks were not resolved, and a replacement BioRad ampholyte was not purchased to test if the one used for this experiment had deteriorated.

Fluka ampholytes gave reasonable results except that sample suppression was an issue. Peak area and height were reduced compared to the other ampholyte brands. Peaks were detected very soon after focusing particularly when using a 30 cm column (data not shown).

Pharmacia ampholytes gave slightly different results to Beckman, Fluka or Sigma brands. Extra peaks were observed in the UV trace but when using these ampholytes with MS detection, no extra peaks were observed in the total ion count (TIC) (not shown). Although many research groups use these ampholytes particularly for MS detection and good results have been observed with flat bed IEF gels for the separation of milk proteins (Braun et al., 1990), in this work the Beckman ampholytes were considered a better alternative.

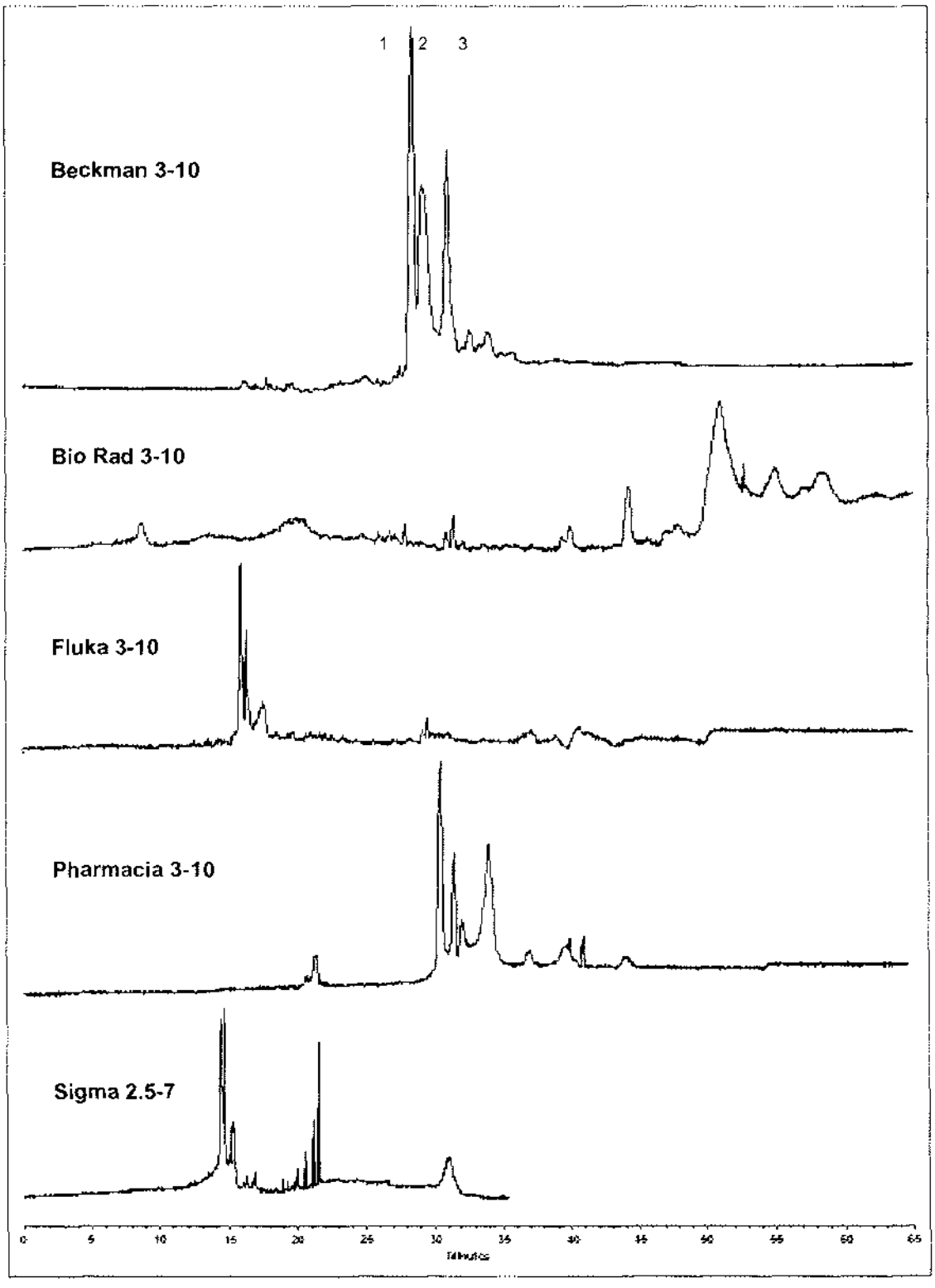

Figure 10 Comparison of different ampholyte brands. Each electropherogram represents whey protein from skim milk run on a 60 cm MicroSolv Zero flow column. All samples except that shown in the bottom electropherogram were spiked with β-lac-B. All other instrument settings were the same as those described in Figure 3. From the top: Beckman ampholyte 3-10, Bio-Rad 3-10, Fluka 3-10, Pharmacia 3-10, Sigma 2.5-7. Peak 1 = β-lactoglobulin-B, peak 2 = β-lactoglobulin-A, and peak 3 = α-lactalbumin.

Sigma ampholytes in a slightly narrower range of 2.5-7 pH units were tried, using whey protein from skim milk with no additional spiking with β-Lac-B (Figure 10). It was hoped the use of a narrower range ampholyte would give a better separation of proteins of similar *pI* than the 3-10 pH range, but this was not the case. Comparing the separation of whey proteins using the Sigma ampholytes to the others shows a separation no better than the best of the 3-10 range ampholytes. Use of a less basic cathode run buffer might have improved the separation. However, Sigma ampholytes were not routinely used as they produced a lot of spikes in the more acidic region of the electropherogram. This would make detection of low abundant proteins hard to achieve.

Optimum ampholyte concentration was investigated, as with MS detection, a lower concentration of ampholyte is required otherwise too much sample suppression is seen. From the literature (Refer to Appendix 1 Table 14 and Table 15), the usual concentration for ampholytes with UV detection is 2 % (v/v) and for MS detection 0.5 % (v/v). In developing the method for MS detection, it was desirable to monitor the separation with UV detection and compare it to that obtained with the usual 2 % ampholyte concentration. An example is shown in Figure 11. Good separation was still achieved with 0.5 % ampholytes, with a shorter run time. However, the 2 % ampholyte concentration was preferred for UV detection, as a slightly higher resolution of separation was achieved. It also ensured that the ampholyte to protein ratio was high enough to establish and maintain a linear pH gradient. This may not be as important a factor with MS detection for proteomic type analysis as a molecular mass would be derived for each protein enabling identification.

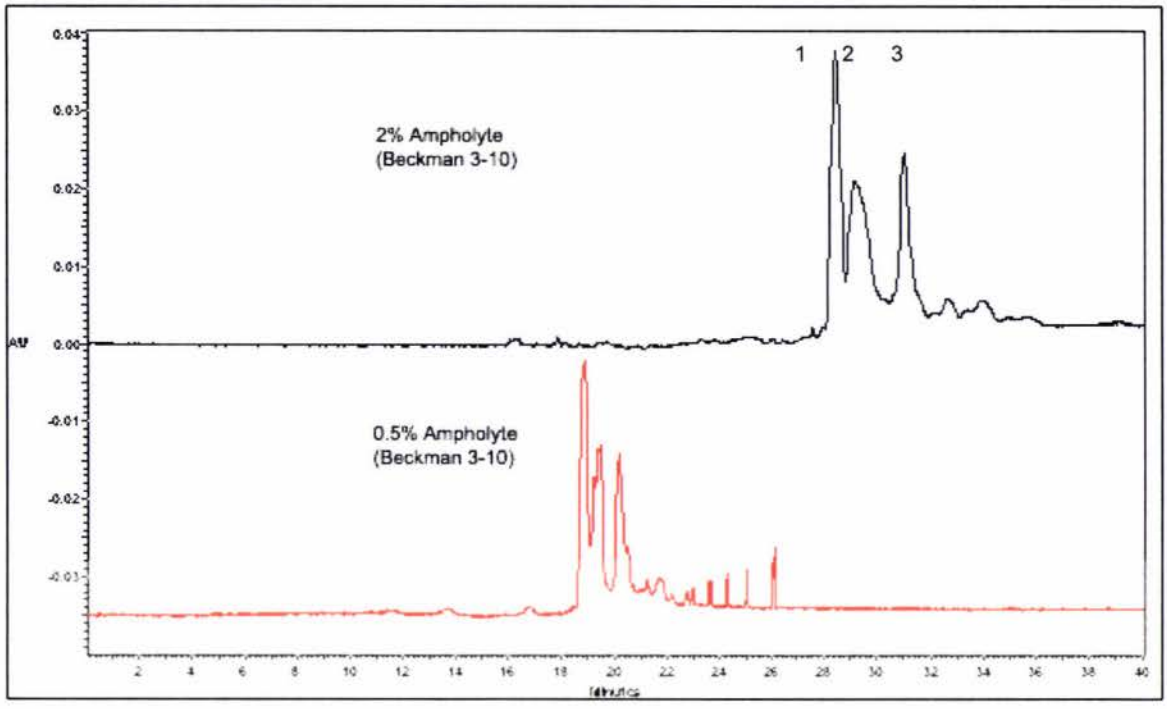

Figure 11 Comparison of ampholyte concentration. Electropherograms of whey protein from skim milk showing the effects of different concentrations of ampholytes added to the sample. Top: 2 % (v/v) ampholyte added, Bottom: 0.5 % (v/v) ampholyte added. All other parameters were the same as in Figure 3 except the separation was performed on a 60 cm column. Peak 1 = β-lactoglobulin-B, peak 2 = β-lactoglobulin-A, and peak 3 = α-lactalbumin.

The addition of narrow range ampholytes has been reported by several groups (Tran et al., (2000), Tran et al., (2001), and Lupi et al., (2000)) where they have managed to get a broader separation in a selected pH range for various samples containing proteins of very similar *pI* value. Most groups have reported using narrow range ampholytes in conjunction with a broad range ampholyte. Figure 12 shows the electropherograms for a whey basic protein fraction run with broad range 3-10 ampholyte only (Beckman) followed by electropherograms of sample run with the addition of Fluka and BioRad narrow range ampholytes. Spiking with narrow range ampholyte as in Figure 12 was done by adding 2 % narrow range ampholytes to 2 % broad range ampholytes (4% v/v total ampholyte concentration). These results simply show an increase in sample migration time and decrease in peak intensity with use of the narrow range ampholytes. The results shown were the best results obtained for this type of experiment. Other ratios of broad and narrow range ampholytes were also tried and in some cases the equivalent brand broad range ampholyte was used with its narrow range ampholyte (data not shown). However as seen in Figure 10, BioRad broad range ampholytes alone gave poor results and Fluka ampholytes were detected

early; similar problems were found to exist with the addition of narrow range ampholytes.

Some other experiments were conducted using narrow range ampholytes (e.g. pH range 7-9 for examining the whey basic fraction) alone at different concentrations but no useful results were obtained (data not shown). The addition of TEMED to block the blind side of the detector was also tried but again no recognizable separations were achieved (data not shown).

Throughout the experiments using narrow range ampholytes, the current was monitored and was seen to follow the pattern associated with normal CIEF (Figure 3 current trace), although for experiments with narrow range ampholytes alone, the maximum current intensity was significantly lower than for broad range ampholytes. With the addition of narrow range ampholytes to the broad range ampholytes the maximum current increased only slightly.

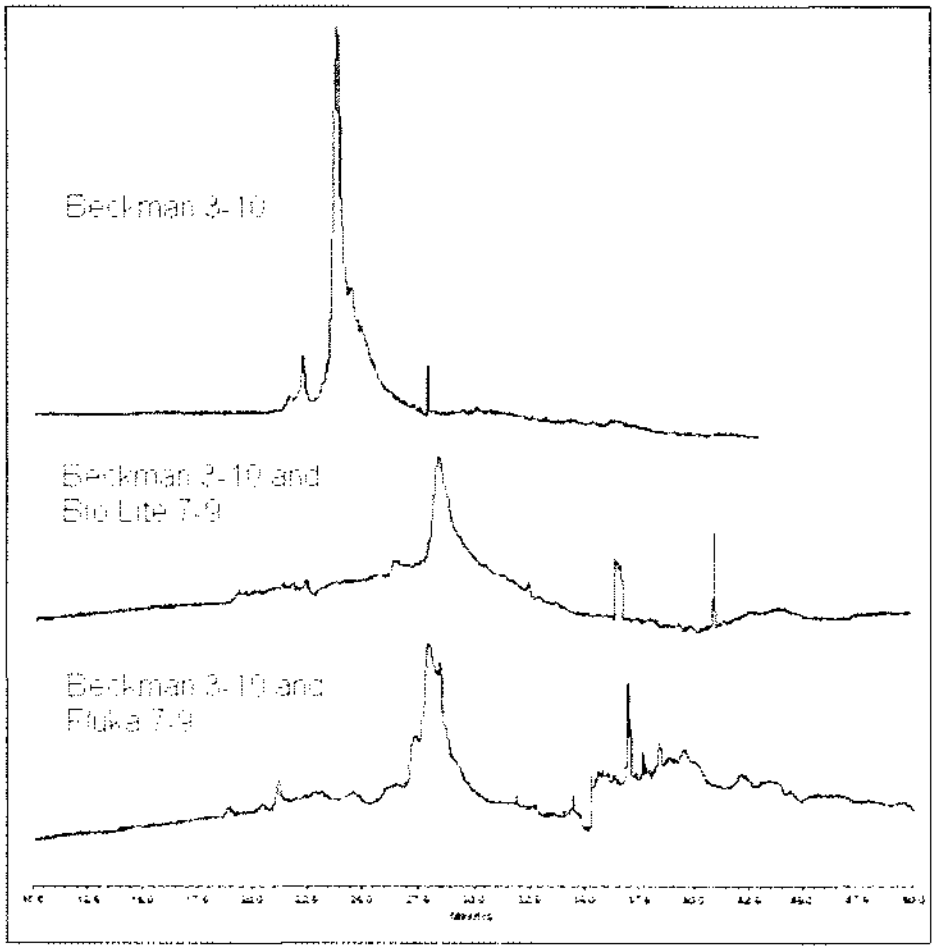

Figure 12 Effects of using narrow range ampholytes. Sample is whey basic protein fraction number 2 run identically to the sample in Figure 3 except for the addition of either 2 % (v/v) Bio Lite 7-9 or Fluka 7-9.

4.1.7 Focusing Times

A study on focusing times (Figure 13) was performed to see if this had any bearing on linearity of *pI* versus migration time. Focusing time was varied from 3 to 9 minutes and included continual focusing allowing EOF to mobilise proteins towards the UV detector. A new 30 cm MicroSolv Zero flow column was used for these experiments. The linearity of the standards for *pI* versus migration time was very similar for a 3 and 6 minute focus (Table 8) with similar regression (R^2) results also being obtained with the increased focusing time. However the percentage error for *pI* of the unknown proteins (β-lac-A, β-lac-B and α-lac) from whey greatly decreased on increasing focusing time. For the 3 minute focus an error of almost 10 % was seen, however this value decreased to 6.5 % for 6 minute focusing. With the 9 minute focus on the 30 cm column, the first *pI* markers were detected before mobilisation had started as a result of EOF. Even though the linearity and error of these results were extremely good (R^2

= 0.981 and error of the unknowns = 3.25 %) this focusing time could not be used as the integrity of the results would have been questionable from a linear standard curve in which a cross over of focusing to mobilisation had occurred during the separation. For a continuous focus (effectively EOF mobilisation) the results gave greatest error in the regression curve (R^2) but only by a small amount. The percentage error of the 'unknown' proteins relative to their literature *pI* values only slightly increased compared to the values obtained for the 6-minute focus. In conclusion, an optimum focusing time of 6 minutes was selected for the MicroSolv column, as with a shorter time, the linearity of the *pI* standards and hence calculations of unknown *pI*'s was inferior. In some instances when using an ageing 30 cm column, the first *pI* marker protein (trypsinogen) would be detected just before focusing had finished. This was a good indicator to replace the column as too much EOF was being generated in the column with the consequence that results started becoming increasingly irregular.

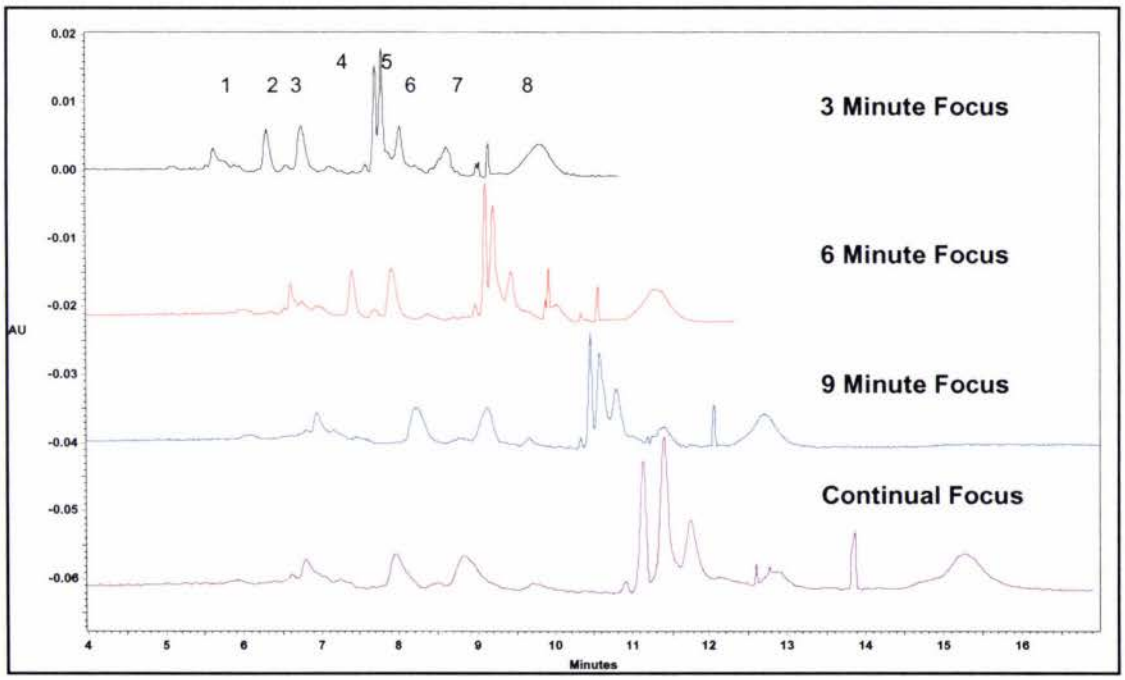

Figure 13 Electropherograms obtained using different focusing times on the same sample. All samples were run on the same 30 cm MicroSolv Zero flow column with operating parameters and sample identical to those in Figure 3 except for the focusing and mobilisation parameter changes. Peak 1 = trypsinogen, peak 2 = myoglobin, peak 3 = carbonic anhydrase, peak 4 = β-lactoglobulin-B, peak 5 = β-lactoglobulin-A, peak 6 = α-lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase.

Table 8 Comparisons of focusing times and mobilisation techniques. All samples were run on the same 30 cm MicroSolv Zero Flow column with instrument parameters identical to those in Figure 3 except for the focusing and mobilization parameter changes.

Focusing Parameters	Mobilisation Parameters	pI vs Migration Time Equation	Regression (R^2) of Best Fit	% Error of unknown proteins*
3 Minute Focus	0.1 psi	y = -1.2599x + 15.503	0.938	9.75
6 Minute Focus	0.1 psi	y = -1.1352x + 15.957	0.942	6.51
9 Minute Focus	0.1 psi	y = -0.9652x + 15.535	0.981	3.25
Continual Focus	-	y = -0.611x + 12.469	0.923	7.33
6 Minutes	Chemical Mobilisation	y = -0.7352x + 13.199	0.947	6.34

* = Percentage error of 'unknown' proteins from skim milk whey comprising β-lac-A, β-lac-B and α-lac and comparing the values obtained from the standard curve to known literature values as outlined in Table 3.

4.1.8 Mobilisation Techniques

For CIEF there are several ways of mobilising protein and peptides to be detected. These include hydrodynamic (pressure, gravity and vacuum), chemical (changing the cathode with anode solution or replacing the cathode solution with cathode solution with salt added to form a concentration gradient in the capillary after focusing), and electroosmotic flow (allowing the natural EOF of the column to take effect to mobilise the analytes).

All three of these techniques were tried. For hydrodynamic mobilisation only pressure was tried. The instrument could not be set up for gravity mobilisation with UV detection and from the literature, vacuum is rarely used. Chemical mobilisation comprised of replacing the basic cathode buffer with the anodic acidic buffer. This technique was used because it is the only way of performing CIEF with MS detection. EOF mobilisation was also tried to see how good the results could be from this technique and to ascertain the time required to elute all the proteins. From Table 8 the results with a 30 cm column and 6 minutes focusing show that both chemical and

pressure (0.1 psi) mobilisation gave very similar results for linear fit. Figure 14 shows the difference in separations seen for the 3 mobilisation techniques performed on the same 30 cm column. Pressure mobilisation at 0.1 psi gave the quickest run time but when mobilisation pressure was increased, there tended to be a merging of peaks, particularly the β-Lac peaks (not shown). Chemical mobilisation gave good results for *pI* versus migration time, and hence fairly accurate results in terms of percentage error for the unknown proteins. One problem with chemical mobilisation was that the peak shape was not particularly good for the more basic and acidic proteins (as also found with EOF mobilisation) when compared with pressure mobilisation. As a preference for CIEF with UV detection it was considered best to do analysis with pressure mobilisation; however when CIEF was coupled to MS detection, chemical mobilisation was mandatory.

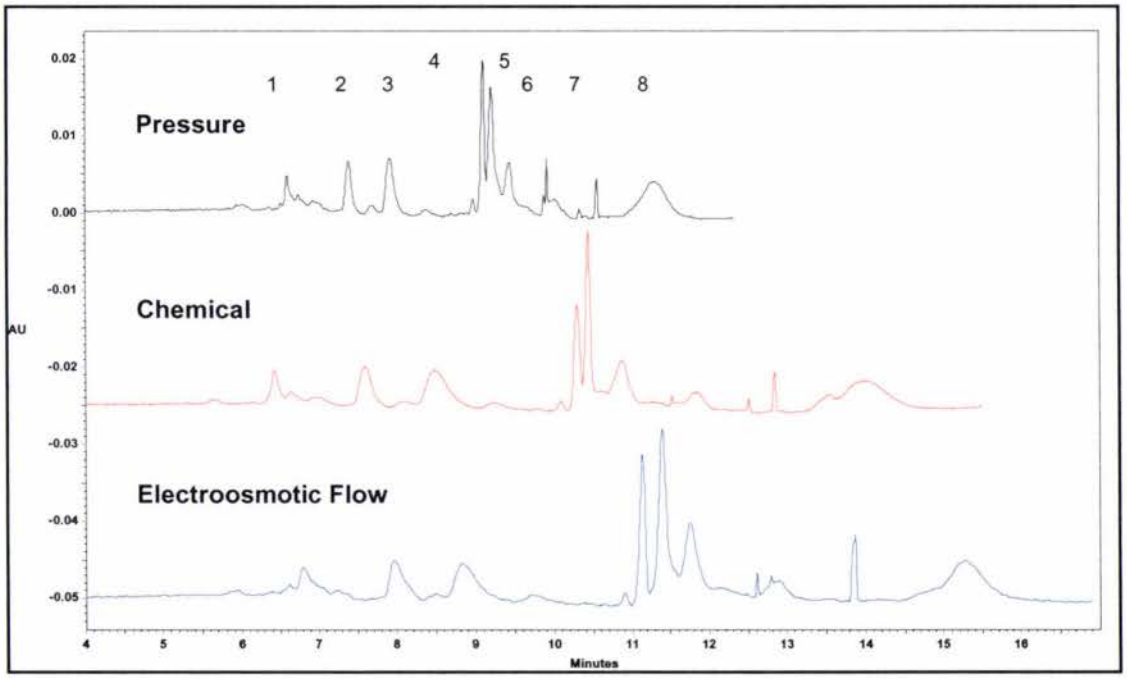

Figure 14 Mobilisation Techniques. Electropherograms of whey protein from skim milk with internal *pI* markers. Each sample was run identically to that in Figure 3 except different types of mobilisation was used. Top trace = pressure mobilisation at 0.1 psi, middle trace = chemical mobilisation, bottom trace = EOF mobilisation. Peak 1 = trypsinogen, peak 2 = myoglobin, peak 3 = carbonic anhydrase, peak 4 = β-lactoglobulin-B, peak 5 = β-lactoglobulin-A, peak 6 = α-lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase.

4.1.9 Changes in Voltage

Voltage choice is important in CIEF (as can be seen in Figure 15), as it can be a determinant of how fast the separation takes place, and hence the quality of the separation. Voltage also has an effect on peak shape, particularly at higher voltages where proteins can precipitate out due to too high a voltage due to them being focused into a very small region, or where peaks do not form at lower voltages. Voltage is described as an electric field strength or volts applied per centimetre of capillary length (V/cm). Figure 15 shows the effect of increasing the voltage across a 30 cm capillary on the separation of proteins in CIEF. At lower voltages (top traces 33 and 100 V/cm) the separation took longer and resolution of separation was poor. The time increase is due to less EOF being generated to mobilise proteins towards the detector (due to a low voltage). Separation is poor at the lower voltages as these voltages are not high enough to form a linear pH gradient within and throughout the entire capillary. As the voltage increases, the migration times of the proteins decrease as EOF has an increasing effect. The separation resolution increases as sharper peaks occur due to higher voltages promoting the generation of a more linear pH gradient. When the voltage becomes too high, resolution is lost on the extremes of the *pI* range. In Figure 15, at a voltage of 583 V/cm and higher, the more basic and acidic *pI* markers did not emerge as sharp peaks. For the basic and slightly acidic *pI* marker proteins, loss of resolution may be caused by the large amount of EOF generated that simply sweeps the proteins past the detector when they are still focusing. The β-lac whey proteins at these voltages formed very discrete bands of high resolution; at this point the highest level of focusing was observed. At very high voltages, the most acidic *pI* marker, amyloglucosidase was not detected. From 583 V/cm the peak broadened and at 833 V/cm was not detected.

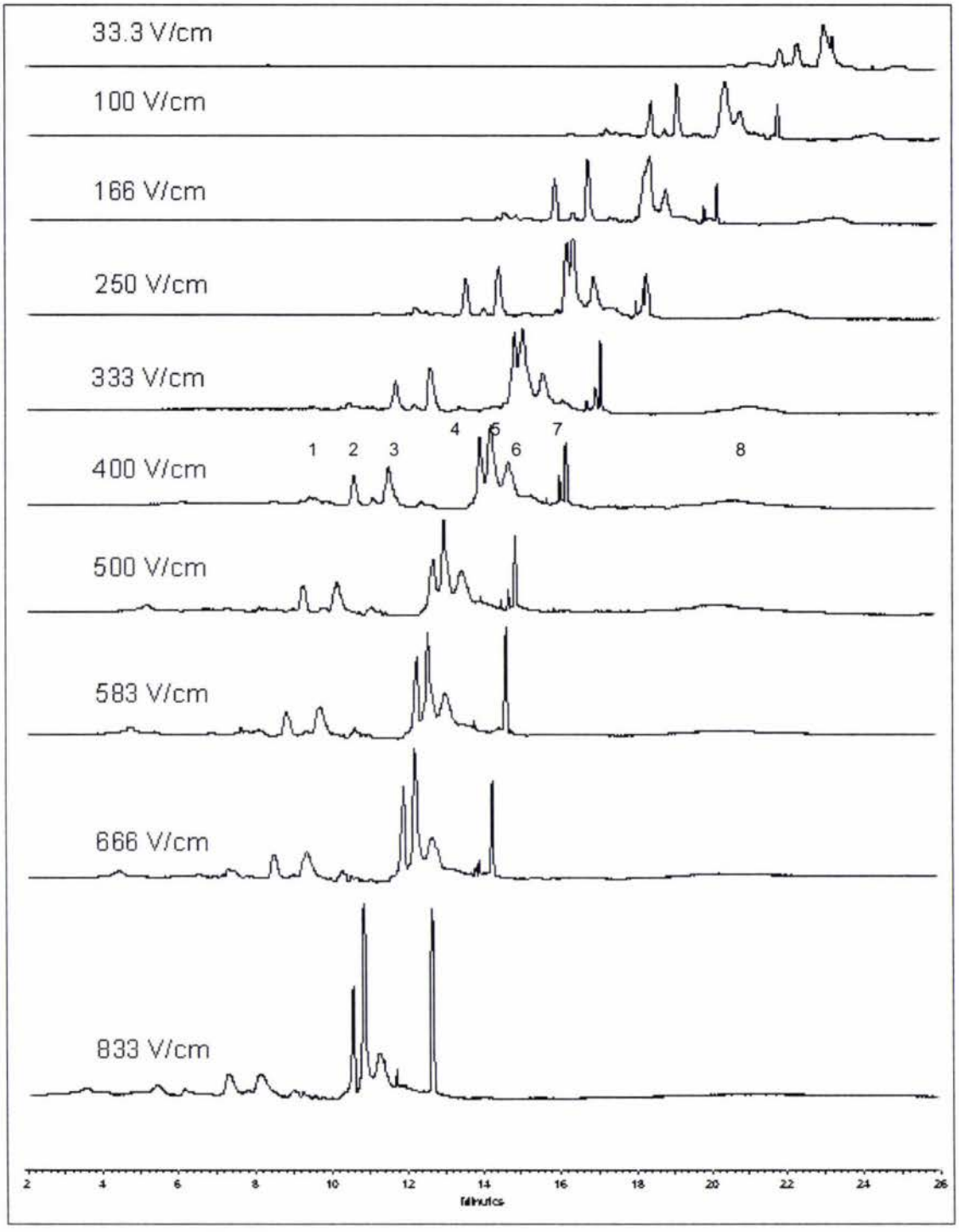

Figure 15 Effect of change in voltages across a capillary. Sample and experiment settings were identical to those outlined in Figure 3, except voltage was changed throughout. Peak 1 = trypsinogen, peak 2 = myoglobin, peak 3 = carbonic anhydrase, peak 4 = β-lactoglobulin-B, peak 5 = β-lactoglobulin-A, peak 6 = α-lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase.

At lower voltages linearity of all the standards was very good, however the voltage applied was not sufficient to separate proteins of similar *pI*. At the other extreme,

when a high voltage was applied, resolution of the separation of proteins of similar *pI* improved greatly.

High voltages increase the rate of EOF within the capillary. Since the CIEF columns are made of glass or silica, the surface of the capillary has a net negative charge due to silanol groups at the surface. The capillary is usually coated by firstly applying a spacer such as γ-methacryloxypropyltrimethoxysilane to the silanol groups of the capillary, which then allows the addition of a coating (eg acrylamide coating) to be applied to eliminate EOF, thereby facilitating the separation. Eventually through repeated use of the column, the coating will slowly degrade exposing the silanol surface. If the column is not properly coated, this would have an effect at the start of the column life with EOF affecting separation. It has been suggested (Wehr et al., 1999, p 140) that the columns will erode in the more basic regions first as high pH buffers such as sodium hydroxide or high pH ampholytes have a greater effect than phosphoric acid on the column coating (hence no rinsing was performed with sodium hydroxide between runs). The effect of EOF on the separation will be that a bulk flow will occur from the positive electrode to negative electrode, so basic compounds will travel faster as they get repelled from the negative silanol groups of the column coating, and move closer to the detector. On the other end of the pH gradient, the more acidic (positive) proteins will be slowed and fine bands dispersed due to the negative silanol groups as the positive proteins bind to the exposed silanol groups of the column.

It is important to note that although the linearity of *pI* versus migration time was not generally good over the tested voltages, particularly at the higher voltages (Table 9), when trypsinogen (*pI* 9.3) and amyloglucosidase (*pI* 3.5) were omitted from the standard curve the average error for the series decreased from 15.6 % to 5.3 % for estimation of *pI*'s of the major whey proteins relative to their literature values. In addition, the average R^2 value for the regression lines in the series increased from 0.882 to 0.988. The greatest amount of error was then observed with the lower voltages which gave typical average errors (between literature and observed *pI*'s) of approximately 7 %; at voltages between 400 and 833 V/cm, all values were below 4.5 %. From these observations it could be concluded that as long as the *pI* of the protein was not too low (< *pI* 4.5) or too high (> *pI* 7.4), then a reasonable determination of

pI could be made for a protein on the 30 cm column with the omission of the extreme *pI* markers trypsinogen and amyloglucosidase.

Table 9 Comparison of differences in separation for different voltages from data obtained in experiments in Figure 15.

Voltage per Centimeter (V/cm)	*pI* vs Migration Time Equation	Regression (R^2) of Best Fit	% Error of unknown proteins*	Separation	Peak Shape
33.3	y = -1.4445x + 39.106	0.948	12.08	Very Poor	Poor
100	y = -0.7711x + 21.723	0.929	14.58	Poor	Poor
166	y = -0.6195x + 17.386	0.915	15.70	Poor	Poor
250	y = -0.5486x + 14.974	0.905	15.36	Average	Average
333	y = -0.4899x + 13.285	0.892	14.00	Good	Good
400	y = -0.4576x + 12.398	0.877	14.23	Very Good	Very Good
500	y = -0.423x + 11.44	0.861	15.44	Very Good	Very Good
583	y = -0.4005x + 11.035	0.850	16.71	Very Good	Very Good
666	y = -0.393x + 10.825	0.847	17.13	Good	Good
833	y = -0.3307x + 9.8197	0.792	21.16	Good	Mixed good and poor

* = Percentage error of 'unknown' proteins from skim milk whey comprising β-lac-A, β-lac-B and α-lac and comparing the values obtained from the standard curve to known literature values as outlined in Table 3.

From Table 9, an optimal separation voltage for CIEF with UV detection was found to be ~400 V/cm. At this voltage the separation gave a good mix of peak shape, efficiency, resolution and migration time. With higher voltage a decrease in peak

efficiency for proteins of higher *pI* was seen, but at 400 V/cm the voltage was high enough to get a good resolution of separation for the β-Lac-A and -B proteins.

This series of experiments was performed four times due to the poor linearity of standard curves for the particular column used, with similar results being obtained each time. This, however, may be a reproducibility problem of the column manufacturer. As discussed earlier, other experiments (on other columns) gave better results.

4.1.10 Temperature Effects

The temperature at which the capillary cartridge is set can alter the separation behaviour markedly in CIEF. This may be due to protein adsorption to the capillary wall or the *pI* of a protein changing with temperature. Furthermore, at different temperatures the change in *pI* may be different from one protein to another.

Figure 16 shows a series of injections of the standard protein *pI* markers (except trypsinogen was replaced with ribonuclease) with skim milk whey proteins run on a 30 cm MicroSolv Zero Flow column at 12 kV (400 V/cm) with focusing for 6 minutes and mobilisation at 0.1 psi. The instrument in each experiment was set to allow the cartridge to equilibrate to temperature before each run. From the results in Figure 16 it can be seen that at a lower temperature less spiking occurred. This spiking is thought to be due to protein precipitation or salt effects and has only ever been seen at the acidic end of the *pI* range in this study. Another very noticeable change was that the acidic pI proteins began to disappear with increasing temperature. A visual example is the disappearance of α-Lac with increase in temperature.

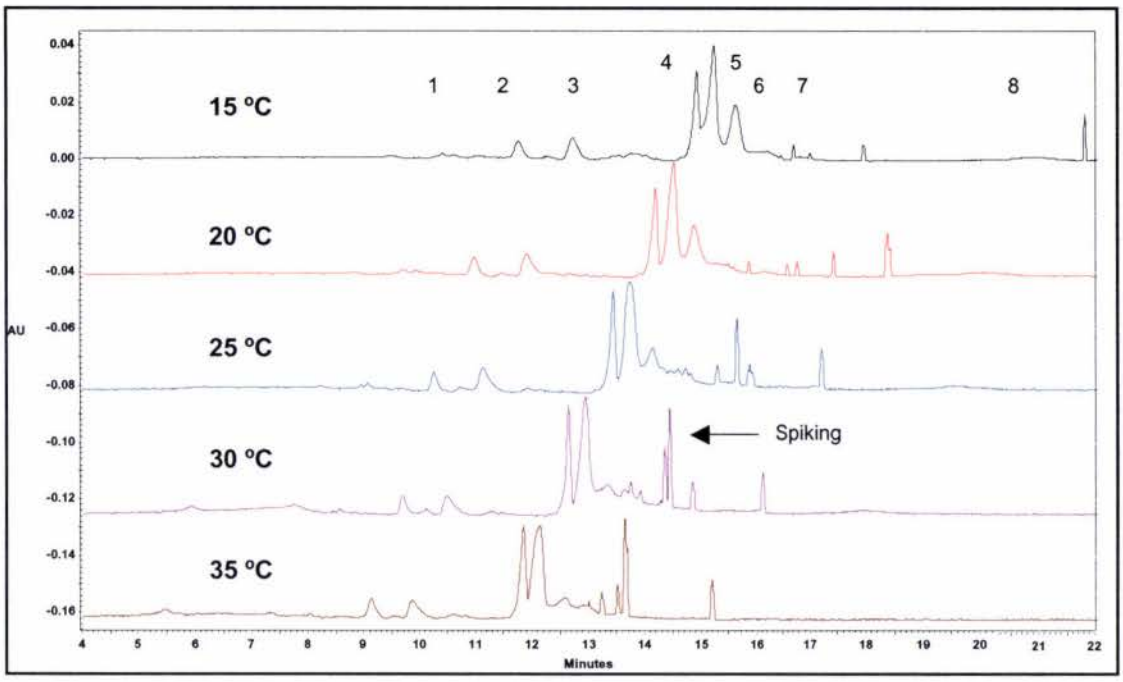

Figure 16 Change in temperature. Electropherograms of whey protein from skim milk with *pI* markers run identically to the sample in Figure 3 except that capillary temperature was altered and ribonuclease *pI* marker was substituted for trypsinogen. From top to bottom: 15, 20, 25, 30, and 35°C. Of particular interest is the disappearance of the α-Lac peak with increasing temperature and differences in the amount of spiking occurring in each electropherogram. Peak 1 = ribonuclease, peak 2 = myoglobin, peak 3 = carbonic anhydrase, peak 4 = β-lactoglobulin-B, peak 5 = β-lactoglobulin-A, peak 6 = α-lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase.

Figure 17 and Figure 18 examine the effect of different temperatures on the peak area of the major whey proteins and the percentage area of the 3 proteins, respectively. From Figure 17 it can be seen that the peak areas of α-Lac decreased with increasing temperature. The peak areas at 15°C was somewhat greater than the peak areas at 35°C with a general downward trend. There was no real difference in total area for the β-Lac proteins; however at 25°C the β-Lac-A peak increased before decreasing to its original area or a little lower. The β-Lac-B area remained similar throughout. When looking at the percentage peak area for the 3 whey proteins, β-Lac-A remained fairly constant, while β-Lac-B and α-Lac increased and decreased respectively. Examination of Figure 16 shows that the β-Lac-B peak increased with increased temperature while the α-Lac peak became smaller. The amyloglucosidase peak also disappeared with increase in temperature, while peak tailing started to occur for myoglobin and carbonic anhydrase. Changes in retention time were also noted; as temperature increased the proteins migrated faster through the column. It could be proposed that at higher temperature some proteins bind to the column hence peak tailing and peak

disappearance. At slightly raised temperatures some proteins may bind to one another, hence the disappearance of the α-Lac peak and the increase in size of the β-Lac-B peak. From the results obtained the optimal setting for the column temperature was seen to be 20°C. At this temperature there was baseline separation of the major whey proteins, the time required to perform the separation was not too long, there was a minimal amount of spiking and the spikes were not too large. This outcome for optimal column temperature ties in well with the standard nomenclature requirement to express the *pI* of a protein at 20°C.

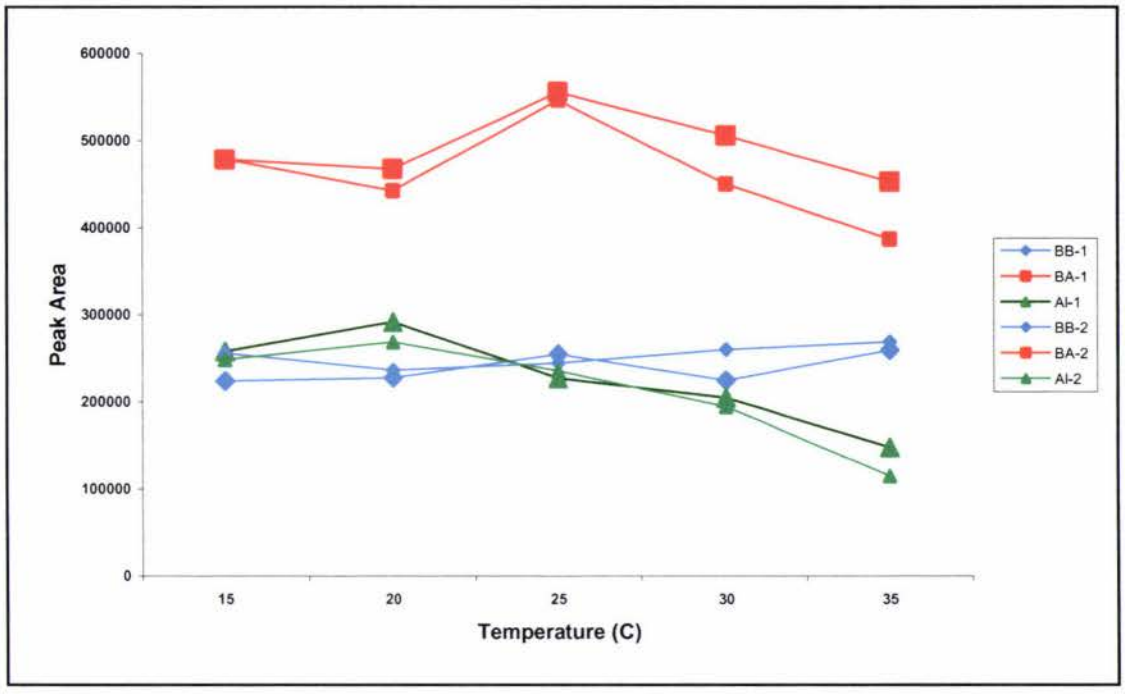

Figure 17 Differences in the peak areas of whey protein peaks from skim milk at different temperatures for 2 sets of data run identical to Figure 16. Al = α-lactalbumin, BA = β-lactoglobulin-A, and BB = β-lactoglobulin-B. 1 = sample set 1, 2 = sample set 2.

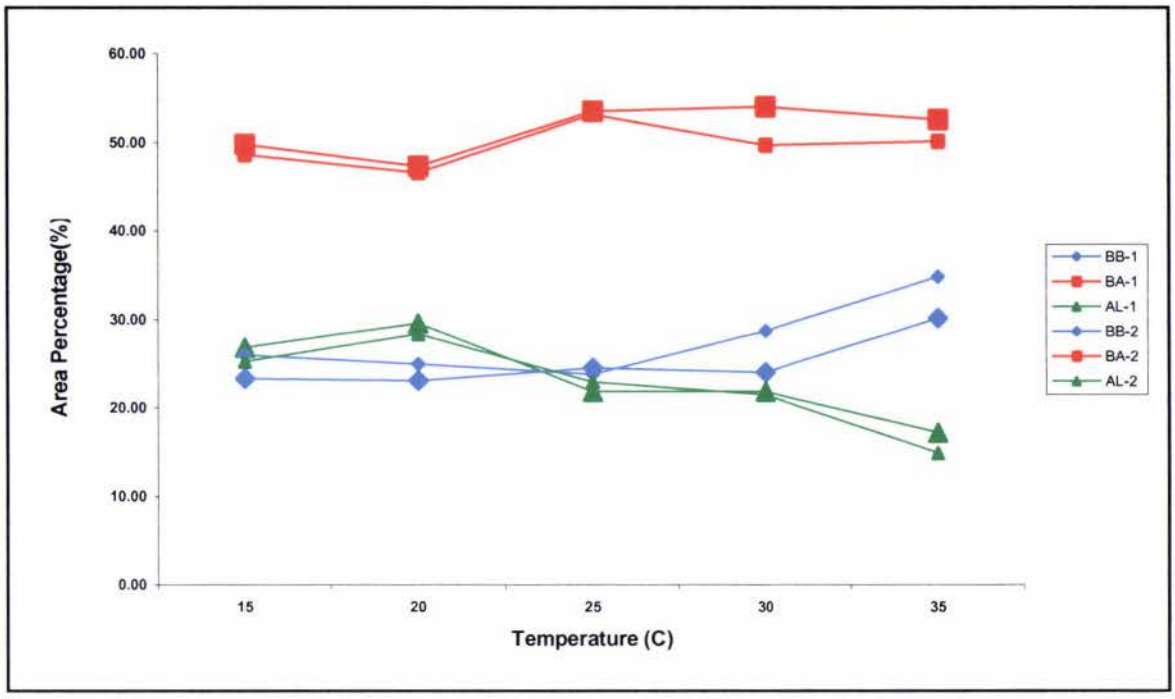

Figure 18 Differences in the percentage areas of the whey protein peaks identified in Figure 16. Percentages were calculated relative to the total area of the whey protein peaks. Samples were analysed identically to those outlined in Figure 16. AL = α-lactalbumin, BA = β-lactoglobulin-A, and BB = β-lactoglobulin-B. 1 = sample set 1, 2 = sample set 2.

4.1.11 Addition of Surfactants

The use of several surfactants such as Triton-X100, and Tween-20 was tried at different concentrations to minimise spiking in the acidic region of each separation. However, results obtained for these experiments were not ideal (results not shown) particularly when compared to the work of Zhu *et al.*, (1991) on γ-globulins, in which surfactants were used to suppress protein precipitation seen as spiking in their electropherograms.

4.1.12 Linearity of Standards

When considering a plot of *pI* against migration time for CIEF we expect a response that would be linear throughout the pH range of the ampholyte. However, this is not always the case. The linearity of *pI* against migration time for protein standards can vary considerably due to the following aspects:

- column internal coating
- column length

- true ampholyte range and distribution of ampholytes per pH unit
- condition of ampholytes
- focusing time
- column usage
- mobilisation technique
- buffer type and quality

There are many different column internal coatings available and throughout this work a number of different types of CE column coatings were tried with varying degrees of success. It was found that the best column in terms of lifetime, separation efficiency and *pI* linearity was the MicroSolv Zero flow column.

Column length is also a major factor for the linearity of *pI* markers versus migration time. This was illustrated in section 4.1.4 using equivalent voltage per centimetre, in general the longer the column the better the linearity as it takes longer for the proteins to pass the detector. However, it is usually very difficult to eliminate EOF from a column and so basic proteins will move at a different rate from more acidic proteins. The resulting effect is a skewed curve for *pI* versus migration time. It has been found that acidic proteins are retained on the column for a longer period of time with peaks sometimes being very broad. This is because a greater amount of diffusion occurs with acidic proteins the longer time they are in the capillary before detection when EOF is present.

The true pH range of ampholytes and the amount of ampholytes per pH unit are important factors (Righetti 2004) when wanting to calculate the *pI* of an unknown protein by CIEF. However, if calculation of *pI* is not required and a good separation occurs, then this is not a problem for CIEF. In the case of many commercial ampholytes often the ampholytes do not properly extend to the indicated full pH range. At the extremes of pH there often tend to be less ampholytes per pH unit and so a deviation from linearity is often observed (Righetti 2004). Another important factor as was discovered in this work was the condition of the ampholyte. In one such case

the BioRad brand ampholytes were found to be in bad condition resulting in unintelligible electropherograms (section 4.1.6).

Other important parameters of the CIEF process that have been optimised and discussed earlier include focusing time, column choice, mobilisation technique, voltage, capillary temperature and buffers used. Overall, the optimal conditions for CIEF for the analysis of dairy whey proteins in skim milk with internal *pI* markers are outlined in Table 10.

Table 10 Optimised conditions for CIEF analysis of skim milk whey proteins and *pI* markers for a Beckman P/ACE CE. The optimised conditions were used on a number of other dairy applications for CIEF discussed in later sections.

Parameter	Optimized Conditions
Capillary type	MicroSolv Zero Flow
Capillary internal diameter	50 μm (or 75 μm for more senstivity)
Capillary length	60 cm (Often 30 cm is as good for rapid analysis)
Capillary temperature	20 °C
Voltage applied	400 V/cm
Focusing Time	6 minutes
Mobilisation Technique	Pressure mobilisation at 0.1 psi
Buffers	20 mM Phosphoric acid (anode) and 20 mM sodium hydroxide (cathode)
Ampholytes	Beckman 3-10 at a 2 % v/v concentration
Detector	UV filter detector set at 280 nm

4.1.13 Method Repeatability

A number of experiments were performed to test the repeatability of the optimized water-soluble method within day and between days on the same column and on different columns. The method consisted of injecting the same sample 10 consecutive times and analyzing the peak migration times as seen in Figure 19. The sample used for this analysis was again whey proteins from skim milk with added *pI* markers. In each case the migration time of each protein was monitored and the differences for each protein calculated as a percentage. Results are expressed in Table 11 and show that the migration times altered between days and columns considerably. As the column ages proteins are retained for less time, as a greater amount of EOF is generated. Sample set 1 and 2 were run on the same column that was near the end of

its useable lifetime with samples being tested on consecutive days with 30 other samples being run between the two sets. Set 3 was run on a new column with a few prior injections to check its efficiency. The differences between columns in protein migration times can be seen very clearly as can the difference between consecutive runs performed on the same column on consecutive days. The repeatability of the samples within a set of 10 identical injections (% difference) was between 0.45 % and 2.92 % for the differences in migration times. The results show that the later eluting acidic proteins (trypsin inhibitor (0.76-2.28 %) and amyloglucosidase (1.31-2.92 %)) have worse repeatability then basic proteins trypsinogen (0.45-0.75 %) and myoglobin (0.46-0.96 %).

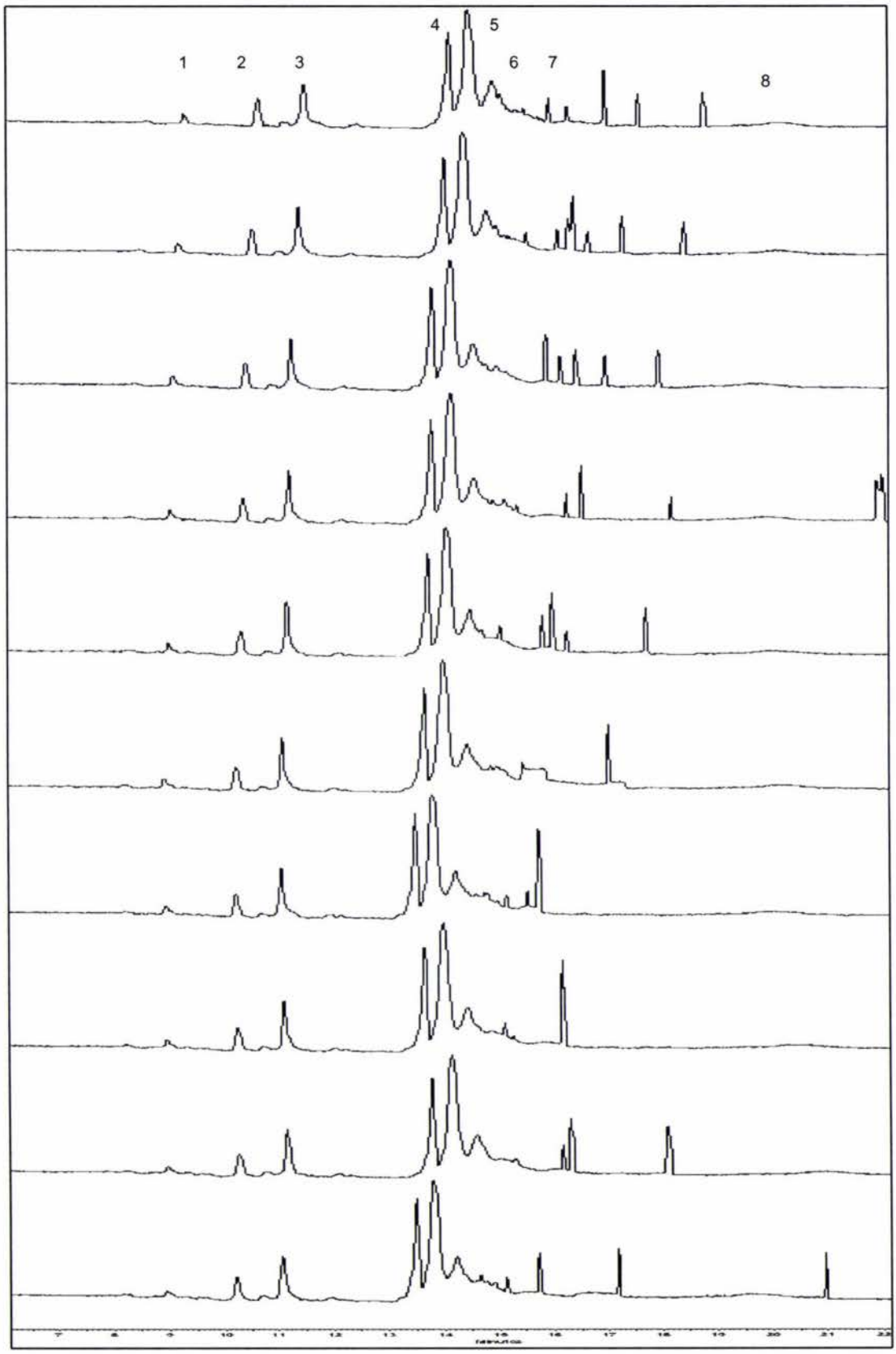

Figure 19 Method reproducibility as shown by 10 electropherograms of whey protein from skim milk with internal *pI* markers run consecutively. Samples were run under identical conditions to those used in Figure 3. Peak 1 = trypsinogen, peak 2 = myoglobin, peak 3 = carbonic anhydrase, peak 4 = β-lactoglobulin-B, peak 5 = β-lactoglobulin-A, peak 6 = α-lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase.

Table 11 Analysis of method reproducibility with the results of the average retention time, standard deviation and percentage difference for 3 sets of 10 samples run on different days. See text for details.

	Average Retention Time of each Data Set (Min)			Standard Deviation			Percentage Difference within each data set		
Protein	**Set-1**	**Set-2**	**Set-3**	**Set-1**	**Set-2**	**Set-3**	**Set-1**	**Set-2**	**Set-3**
Trypsinogen	8.02	7.63	9.16	0.04	0.04	0.07	0.45	0.53	0.75
Myoglobin-B	8.83	8.41	10.36	0.08	0.04	0.06	0.96	0.46	0.55
Myoglobin-A	9.16	8.73	10.85	0.09	0.04	0.07	1.02	0.47	0.62
Carbonic Anhydrase	9.51	9.02	11.25	0.10	0.06	0.07	1.02	0.62	0.66
β-Lac-B	11.00	10.36	13.87	0.24	0.06	0.15	2.14	0.55	1.11
β-Lac-A	11.15	10.49	14.13	0.24	0.06	0.17	2.15	0.60	1.20
α-Lac	11.56	10.86	14.63	0.26	0.07	0.18	2.25	0.62	1.26
Trypsin Inhibitor	12.61	11.83	16.07	0.29	0.09	0.27	2.28	0.76	1.66
Amyloglucosidase	15.58	14.88	20.66	0.45	0.19	0.55	2.92	1.31	2.64

4.1.14 Applications of the CIEF-UV Method

Analysis of whey protein fractions.

The analysis of fractionated protein components of milk is an ideal application for CIEF with UV detection. Although the analysis of some of these components is not possible with MS detection (as discussed in more detail in section 4.4), they can be monitored by UV detection. The isolation of minor protein components in bovine milk is desirable as it might lead the way to new value added consumer and commodity products with health benefits. Research in this area has increased as new technologies have become available to look at new components. Traditionally the purity of such samples would be assessed by PAGE, either separating by molecular weight or isoelectric point. Placing isoelectric focusing into a capillary format (CIEF) scales down the required sample size. This in itself can be essential as often with this type of research sample yield is minimal. CIEF also has the added benefit in that the

technique is rapid and automated so the results can be obtained relatively quickly. This is unlike gels which involve a lot of labour intensive steps.

CIEF offers an advantage to other CE techniques in that, in theory, the mechanism for separation will allow separation of proteins of a range of isoelectric points. For CZE separations, optimal conditions are obtained by selecting a sample buffer that has a pH of at least 1 pH unit either side of the *pI* of the proteins of interest, otherwise long analysis times are encountered (Wehr et. al., 1999, p 52). For CIEF, separation of proteins of *pI* ranging from 3-10 is achievable, and recently Mohan & Lee 2002 (b) were able to extend the pH range out to 12. The ability of CIEF to separate proteins over a wide pH range is a benefit as the same method can be used on fractions containing proteins of very different *pI* (for example in dairy applications, the whey basic fraction or acidic fraction) and results compared with relative ease. The analysis of samples of different whey basic protein fractions are shown in Figure 20 and Figure 21, while Figure 22 and Figure 23 shows different acidic whey protein fractions. Figure 20 shows the total whey basic protein fraction (fraction 1) (top electropherogram), followed by subfractions of fraction 1 obtained using ion exchange chromatography with sodium chloride salt step elutions; fraction 2 (middle) and fraction 3 (bottom) electropherograms. The top electropherogram shows the separation of the highly basic proteins lactoferrin, angiogenin and lactogenin which were known to be present in the sample. Peak identities were tentatively assigned by reference to 2D PAGE/MS results (Figures 33-35, section 4.6). The middle trace (1 M NaCl fraction) indicates the presence of the same proteins, while the bottom trace (0.4 M NaCl fraction) shows the presence of multiple protein peaks assigned as RNase, lactoperoxidase and Ig polymeric Ig receptor protein again on the basis of 2D PAGE/MS results.

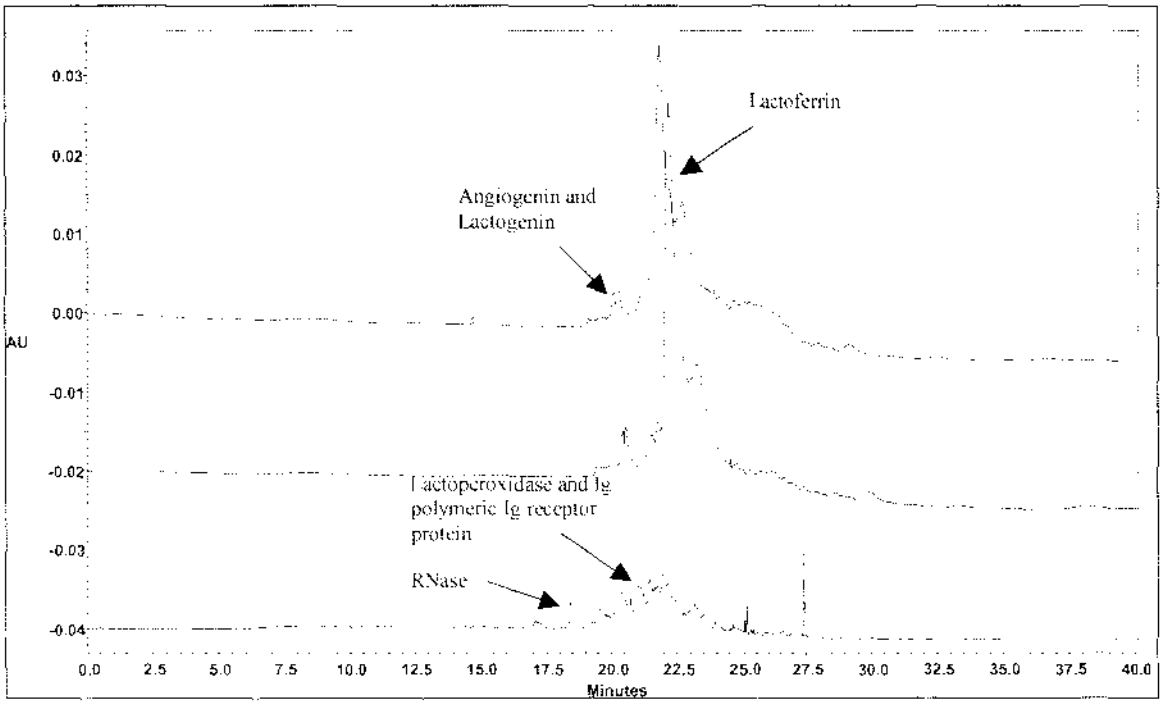

Figure 20 Separations achieved for several whey basic protein fraction samples. Top trace is the total whey basic protein fraction (fraction 1), middle trace is a subfraction of the top trace sample (fraction 2) as is the bottom trace (fraction 3). The main components of the sample are lactoferrin, lactoperoxidase and angiogenins. Each electropherogram was generated using the same parameters as used in Figure 3.

Other basic protein fractions that have been studied include the isolated angiogenin and lactogenin proteins (Figure 21). As can be seen by their electropherograms, these samples were of good purity. Further characterisation with MS infusion experiments (Appendix 3 Figure 78, Figure 79, Figure 80 and Figure 81) showed there were no other impurities of significance and masses obtained were similar to those expected for angiogenin or lactogenin.

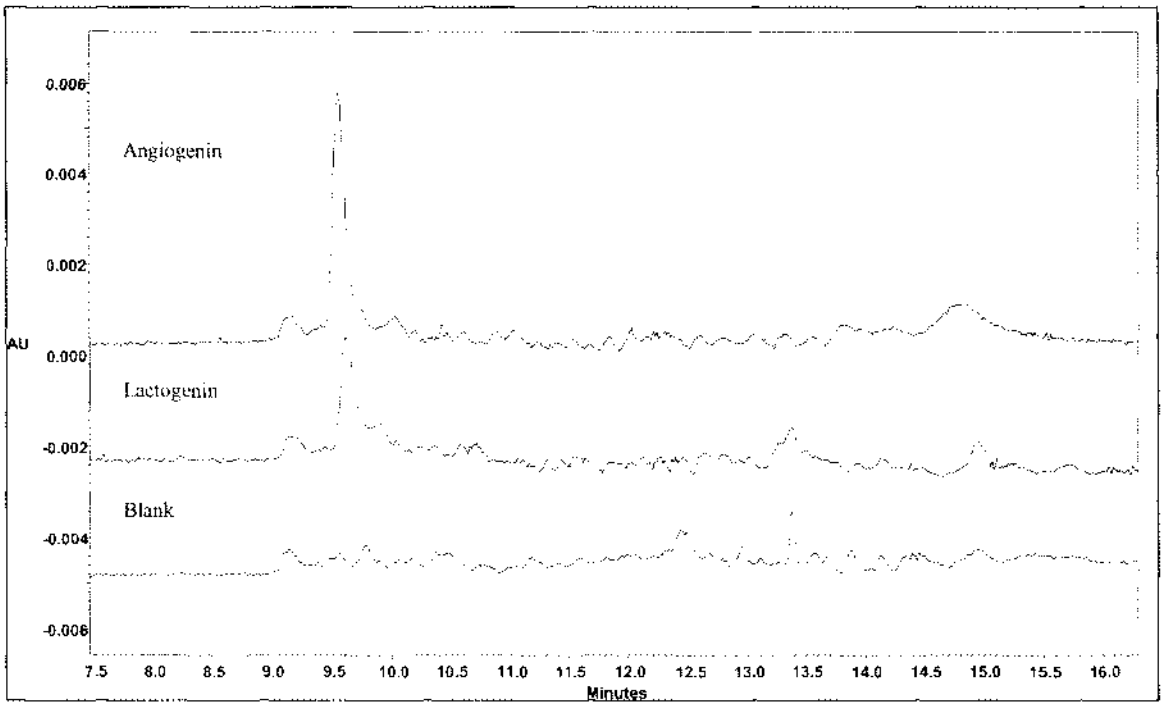

Figure 21 Electropherograms of angiogenin (top), lactogenin (middle), and a blank sample (bottom). The angiogenin and lactogenin samples are sub fraction samples of the total whey basic protein fraction and were found to have a *pI* > 9.1. Samples were run identically to those in Figure 3.

Minor components of whey protein with acidic *pI's* were also analysed using CIEF including a fraction rich in PP-5, a multiply phosphorylated β-casein fragment (Figure 22), and GMP, a heterogeneous κ-casein peptide (Figure 23).

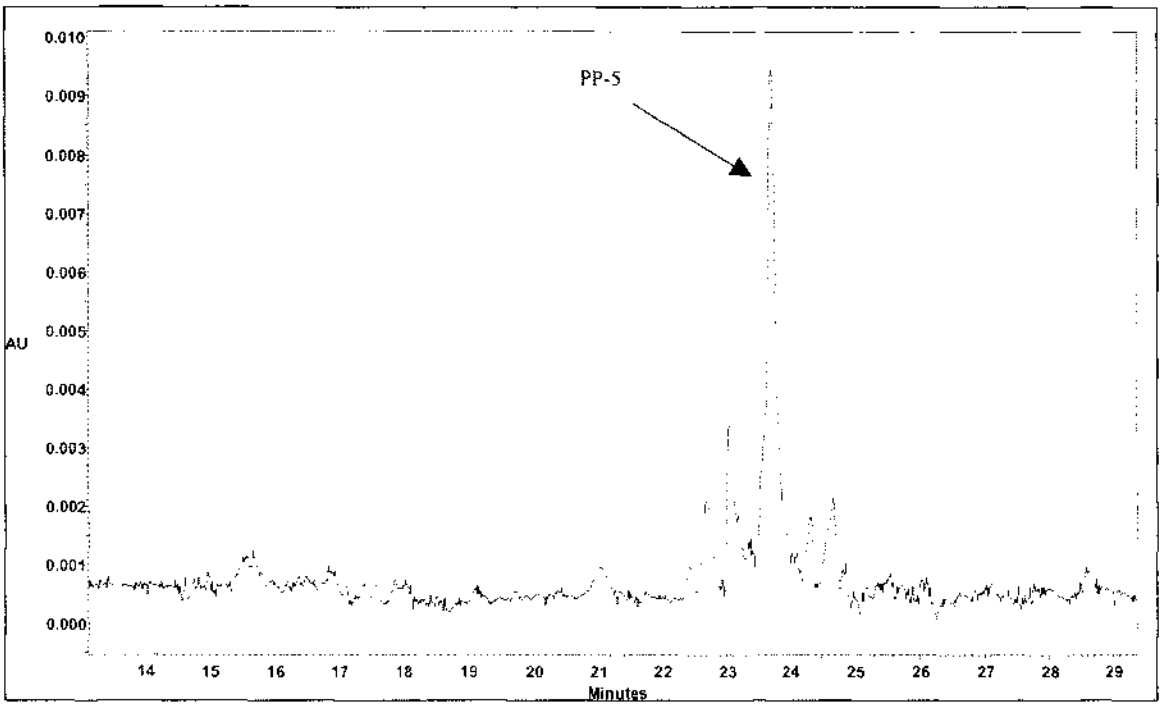

Figure 22 Electropherogram of a whey acidic protein fraction from mineral acid whey. Sample run identical to the sample in Figure 3.

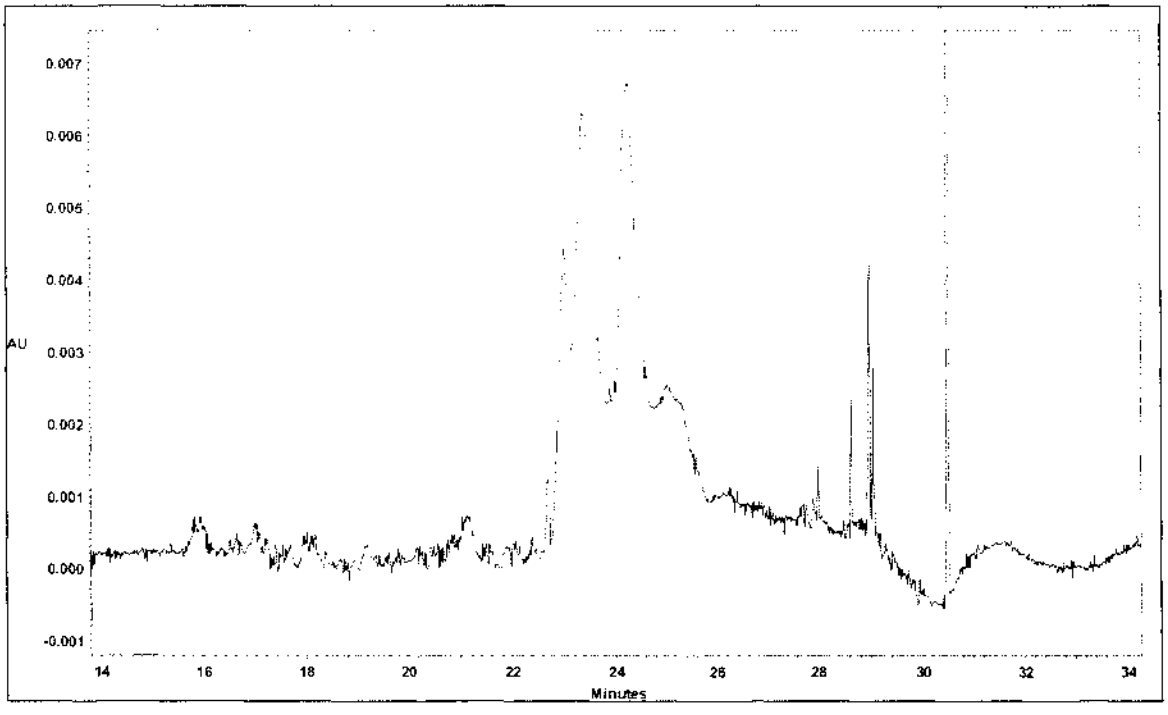

Figure 23 Electropherogram of a GMP fraction (cheese whey acidic protein fraction) isolated from a cheese whey retentate. Sample run identical to that in Figure 3.

In the industrial separation of lactoferrin from milk using ion exchange chromatography, a waste stream from the process is rich in an enzyme

lactoperoxidase. When lactoperoxidase is combined with thiocyanate and peroxide it forms the peroxidase system in milk which has antimicrobial qualities that can help preserve milk (Seifu et al. 2005). The monitoring of the first industrial scale trial separations of a fraction rich in lactoperoxidase was achieved using CIEF. Figure 24 shows a lactoperoxidase standard (top electropherogram) followed by the first 4 trials of the lactoperoxidase isolate. All samples and standard powders were made up to the same concentration (w/v) and run identically. As can be seen peak shapes were unusual and there appeared to be no resemblance to the standard. However analysis with PAGE (data not shown) reveals lactoperoxidase is present in each prototype sample, but with contaminants such as β-lactoglobulin and angiogenin (trials 1 and 3) or with β-casein (trials 2 and 4). The samples containing β-casein from PAGE results would probably be of limited solubility in the sample buffer used, could give some reason why the CIEF method for soluble proteins shows unusual peak shapes.

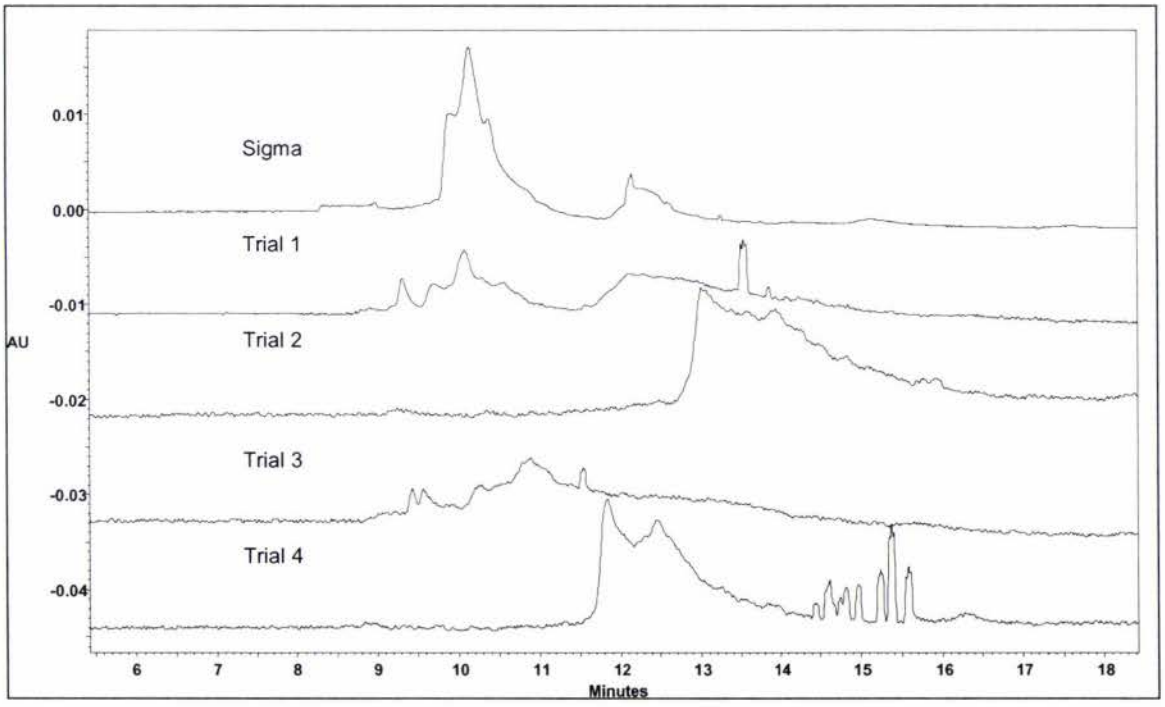

Figure 24 Electropherograms of industrial scale samples of lactoperoxidase protein. Top trace for reference purposes is a Sigma standard, the following four traces are four different prototype products.

Analysis of Hydrolysate Samples

The analysis of peptide samples using CIEF has been investigated previously (Shen et al., 2000a). Samples included the hydrolysis of bovine serum albumin standard and a

yeast cytosol with trypsin. DTT was used in the sample preparation to reduce any disulphide bonds and heat was used to denature the proteins before hydrolysis. Dairy companies, including Fonterra, produce a number of hydrolysate products for different applications. Fonterra produces hydrolysates from both whey and casein to use in products such as infant formula. One such whey based hydrolysate powder was analysed by CIEF (Figure 25) by simply dissolving the powdered product in water at a concentration of 3 mg/ml. Analysis of casein based hydrolysate products was performed using the same method. However, large spike peaks were seen in the electropherograms (data not shown), possibly due to insoluble casein material. These spikes might be eliminated by heating and reduction of the casein products with DTT prior to analysis. For future application work in the analysis of peptides, a lower voltage and longer column would be advisable as there are a large number of unresolved peaks seen in the electropherograms and these method alterations might help to improve resolution of peptides. The method shows some promise in profiling (fingerprinting) of industrial hydrolysates.

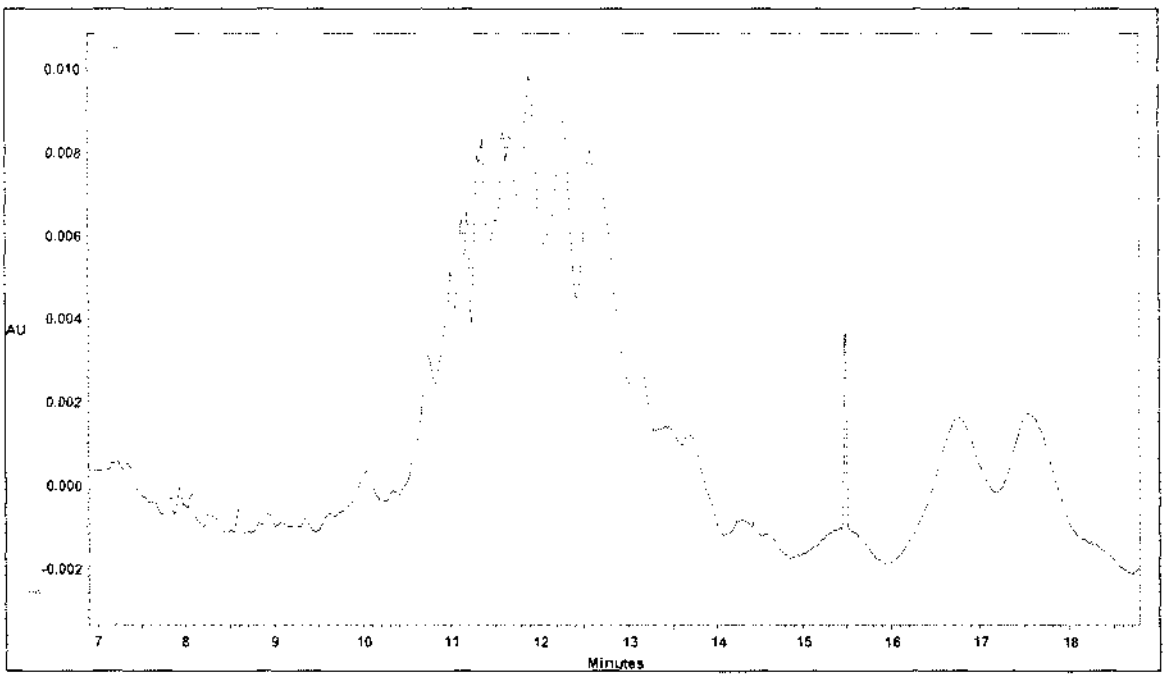

Figure 25 Analysis of a whey based industrial hydrolysate sample. Separation parameters were identical to those used in Figure 3. The sample was made at a concentration of 3 mg/ml (w/v) with 2 % Beckman 3-10 ampholytes added.

Analysis of Bacterial Cell Lysates

There have been several applications where cell lysates have been examined by CIEF. Shen et al., (1999) separated lysates of 2 different bacteria (*E. coli* and *D. radiodurans*) and yeast (*S. cerevisiae*), obtaining electropherograms with hundreds of peaks over a 50 minute separation. Within the dairy industry a number of different strains of bacteria are used for starter cultures in cheese and yoghurts. It is becoming more desirable to be able to characterise the different strains as, for example, different strains will give different flavour compounds in cheese. Several bacterial cell lysates with dairy application were gifted for investigation using CIEF. In Figure 26, the same bacterial cell lysate was analysed several times with a similar pattern emerging for each electropherogram. The electropherograms show numerous peaks throughout the electropherogram indicating the presence of numerous proteins. The traces indicate that insoluble proteins were present in solution seen as spiking in the electropherogram. Analysis of another bacterial cell lysate (Figure 27) showed that there were greater solubility issues with this sample. Figure 27 also shows the same sample injected several times in a row. The top electropherogram was the first electropherogram generated with an outcome of a great number of spiked peaks indicating protein insolubility. The following trace (middle) shows a lower level of peaks, suggesting a change in sample integrity, while the peaks were smaller still in the third trace (bottom). On inspection of the sample it was evident a pellet had formed in the vial and hence insoluble aggregated proteins had precipitated out over time. Although Shen et al. (1999) did not add any components to solubilise the proteins in the lysate, it appears further investigation of dairy lysate samples would need to be undertaken using a sample buffer with additives such as urea and BME or DTT to solubilise and denature proteins.

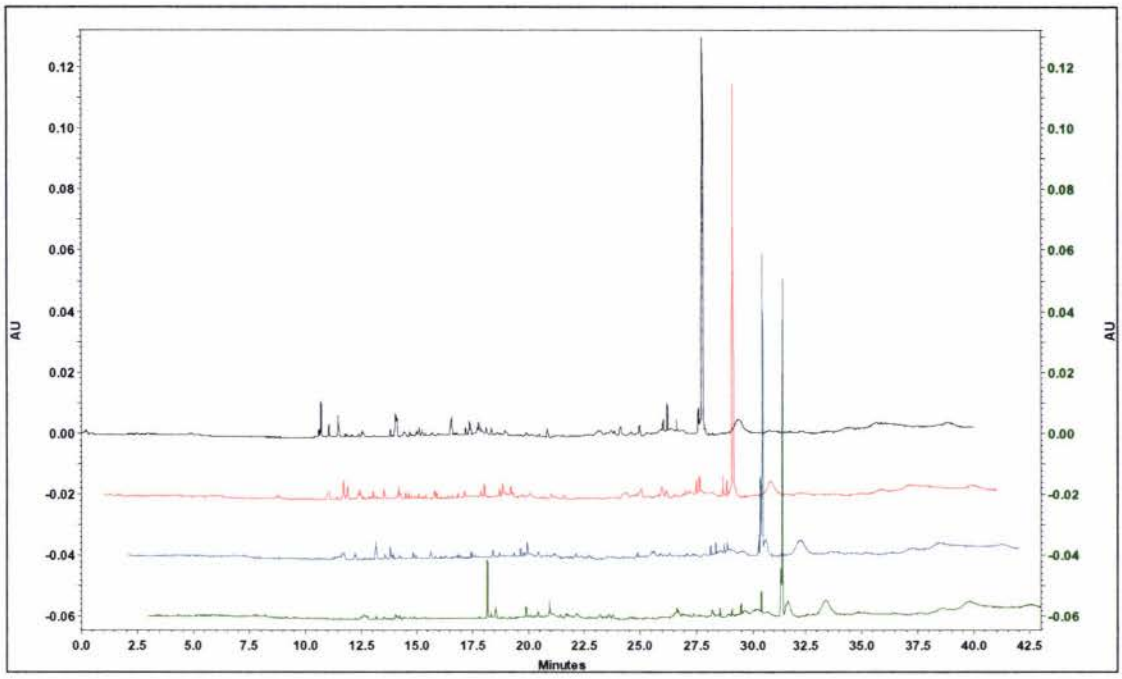

Figure 26 Electropherograms of bacterial cell lysate "B12" run 4 times (each electropherogram off set). Separation conditions were identical to that in Figure 3.

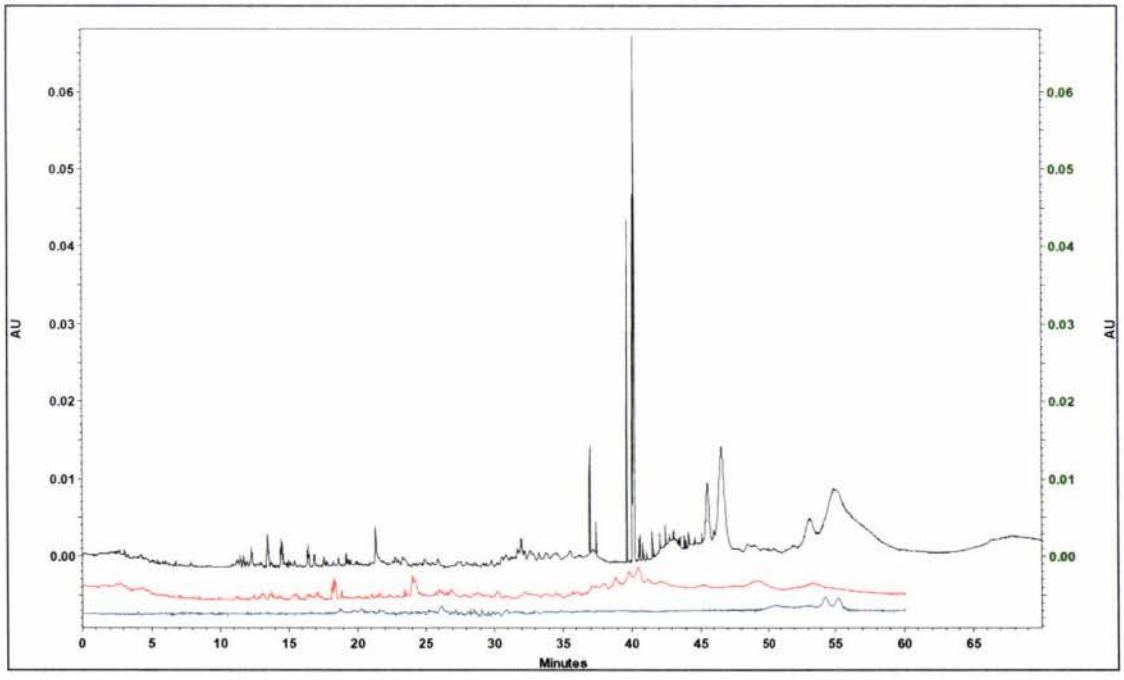

Figure 27 Electropherograms of bacterial cell lysate "X7" (Top and middle) run one after the other. After the second sample was run it was noticed that there was a pellet formed at the bottom of the sample vial. All samples run using conditions identical to that in Figure 3.

4.2 Insoluble Dairy Proteins with UV detection

The development of a CIEF method using sample buffers that were of a denaturing and reducing ability was first outlined by (Schwer, 1995). This method used reversed conditions to the normal procedure in that the separation took place in the shortest part from column end to detector window. Buffers were reversed and voltage applied in a negative mode. In the current study (refer to section 3.5 for methodology) a 30 cm capillary was initially used with the separation taking place over a distance of 10 cm under reversed conditions similar to Schwer (1995). To achieve this separation TEMED was used in the sample buffer to block the blind side of the detector. TEMED is a highly basic substance so that when a voltage is applied it focuses in an area beyond the ampholyte range and serves as a plug to block the blind side of the detector. Further development lead to use of the 30 cm column in the normal CIEF mode followed by extending the separation out to a 60 cm column. Initially the sample buffer consisted of 8 M urea and 2 % (w/v) DTT, and although this buffer gave good results, substitution of the DTT with 5 % (v/v) BME gave higher resolution of peaks (Figure 28). Several isoforms of κ- and β-casein were observed in these experiments; however there were no isoforms resolved for α-casein.

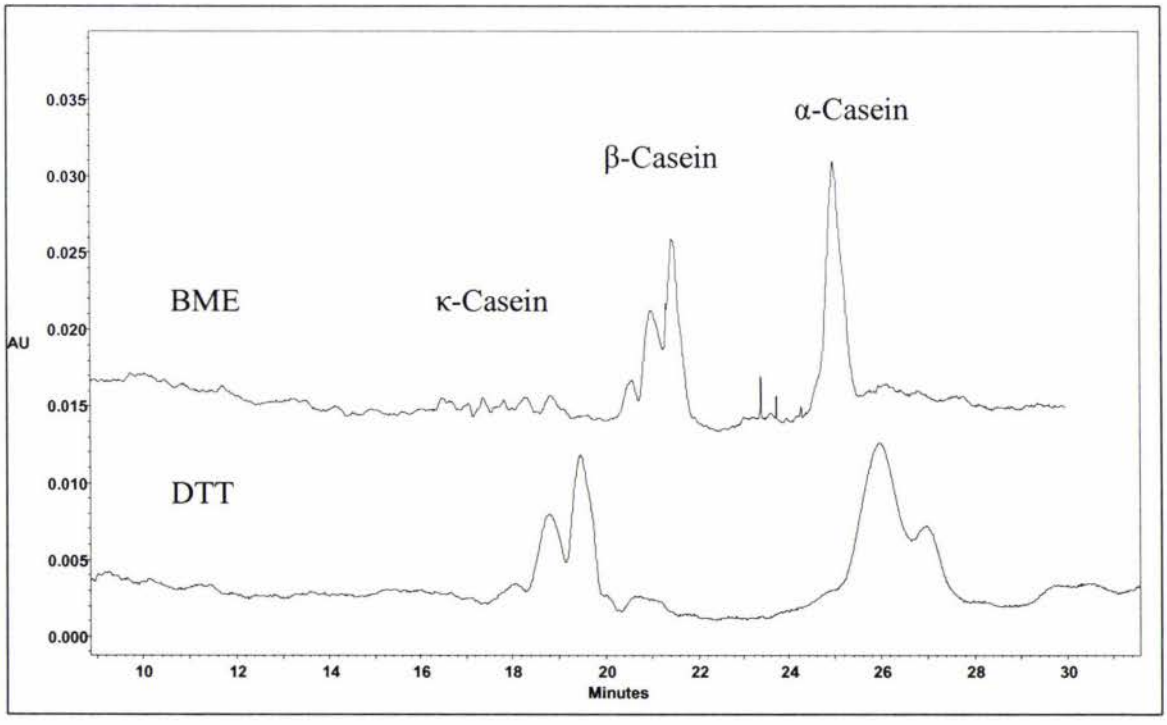

Figure 28 Electropherograms of skim milk run under identical conditions except the top trace utilised β-mercaptoethanol (BME), while the bottom trace utilised DTT in the sample buffer.

Good results were obtained following the method by Lopez-Soto-Yarritu et al., (2002) for the analysis of recombinant erythropoietin, where urea and BME were dissolved in Beckman eCap gel for the sample buffer. In addition, crystallization of the urea was greatly slowed using this method. It must be noted that the sodium hydroxide cathode buffer as used in these experiments should be replaced every 10 samples as the buffer deteriorates quickly over time due to sample with urea flowing in to this vial and a change in migration times was observed for repeat injections of the same sample. When re-injected into fresh cathode buffer sample, migration times returned to normal. Although this method has only been partially established in terms of what applications are possible with it, the method would be particularly good for analysis of casein products in that separation of genetic variants of β-casein has been observed (data not shown). Spiking experiments with whey protein showed that β-Lac-A and –B. and α-Lac co-elute with the β-casein species so there are some limitations to the method. Further work would have to be undertaken to find out if other β–casein genetic variants can be separated or if α-casein variants can be separated using this method.

A sample buffer consisting of 6 M guanidine in place of the urea was tried to see if this would work for a denatured CIEF method and perhaps give better results. However, even when low voltages were applied to the capillary a very high linear current was produced. No focusing occurred and no protein separation was detected.

4.3 MS Infusion Experiments

To aid in identification of proteins while developing the CIEF-MS method, infusion mass spectrometry experiments were carried out on standard proteins of whey and the *pI* marker proteins used throughout this work. A summary of results from these experiments are shown in Appendix 2 (Table 16). Mass values obtained from the literature are also included. Ionisation patterns with deconvolution results are shown in Appendix 2 (Figure 39 to Figure 70).

Results from the infusion experiments allowed a more rapid method development for CIEF-MS for two reasons.

Firstly, it allowed the researcher to determine if the method was running properly. With knowledge of the expected ionisation pattern for each protein in the test mix, the results could be determined online as the separation was taking place. This allowed the researcher to determine if there were problems and generate ideas for trouble shooting in the next experiment, or if all was going well, to refine the method in the next analysis.

Secondly, infusion experiments showed which proteins ionised well. The results in Appendix 2 show some proteins of fairly high molecular weight (>60 kDa) and smaller glyco proteins (such as GMP) that did not ionise well and others in the region of 14-30 kDa that did ionise very well. Although high MW proteins were made up to similar molar concentrations as lower MW proteins, the ionisation patterns were not as good for high MW proteins due to loss of resolution with having so many ionised species in the spectrum. Since the molecular weight is calculated from a mass to charge ratio, then more masses in a mass spectrum means less resolution is obtained compared to a protein of lower MW with less ionised species, where there is greater resolution. Since electrospray MS instruments do not have that great a resolution when there are many ionised species present, it is difficult to get accurate masses of proteins above 70 kD (Siuzdak 1996, p 86-93). This aspect has proven to be a difficult problem as several of the dairy proteins in the whey basic protein fraction (lactoferrin and lactoperoxidase) have MW's greater than 70 kD. These proteins are also glycosylated and if there are differences in glycosylation, then the mass spectra can become very messy and hard to interpret. In the preliminary infusion experiments it was possible, however, to get reproducible masses for these protein standards. Although a sample of deglycosylated lactoferrin was infused (Figure 59 of Appendix 2), results showed there was no advantage to clarity of the mass spectra for a deglycosylated large protein.

Samples of whey basic protein fraction (Figure 20) were also infused into the MS and it was possible to get some preliminary results on the contents of two of the 3 samples. From several repeat infusions of fractions 1 and 2, the following peaks outlined in Table 12 were identified. Although from Appendix 3 (Figure 74) there were several other peaks of greater intensity than that of molecular mass 14588, angiogenin is known to be present in this sample. According to Acharya et al., (1995)

the molecular mass of bovine angiogenin is 14595 and results with the MS infusion suggest a similar MW (Figures 78 and 79 Appendix 3). Many of the other peaks obtained from deconvolution of whey basic protein fractions 1 or 2, when examined in depth derive from the ionisation peaks associated with angiogenin and/or other peaks in the lactoferrin part of the mass spectrum.

Table 12 Results of MS infusion experiments of whey basic protein fraction samples

Sample	Deconvoluted Molecular masses	Protein Identification
Fraction 3	No peaks detected	None
Fraction 2	84866	Lactoferrin
	83322	Lactoferrin
	81475	Lactoferrin
	79571	Lactoferrin
	14588	Angiogenin
	13887	RNAse 4 (Lactogenin)
Fraction 1	87031	Possibly Lactoferrin
	85223	Lactoferrin
	83094	Lactoferrin
	81587	Lactoferrin
	14588	Angiogenin
	13887	RNAse 4 (Lactogenin)

4.4 CIEF-MS Detection

4.4.1 Method Development

To test that the buffer conditions required for CIEF with MS detection gave good separation of skim milk whey proteins and *pI* markers, samples and standards were first run with UV detection. The results are outlined in Figure 29 with a description of each buffer composition in Table 13. Each sample was run with different run buffers on a 30 cm column with all other conditions being kept the same. Voltage was altered

slightly from that of the usual UV detection method to give approximately the same voltage per centimetre that would occur with MS detection in a 90 cm column. Beckman 3-10 ampholytes were used at a concentration of 0.5 %, the same as that used for MS detection. As chemical mobilisation on its own takes a long time to complete, 0.1 psi pressure from the anode was also applied in these experiments. Other buffers such as formic acid were trialled in place of acetic acid as formic acid promotes better ionisation of proteins in the MS. However, errors occurred mid-run in these experiments and so results are not shown for these samples. The use of formic acid buffers had been trialled earlier in a similar experiment (section 4.1.3) and a similar problem occurred. It was thought that formic acid and acetonitrile buffers might irreversibly destroy the capillary column coating as the columns were unable to be regenerated after use with such buffers. However, acetonitrile used in conjunction with acetic acid gave satisfactory results (Figure 29) so it can be assumed formic acid was the detrimental agent.

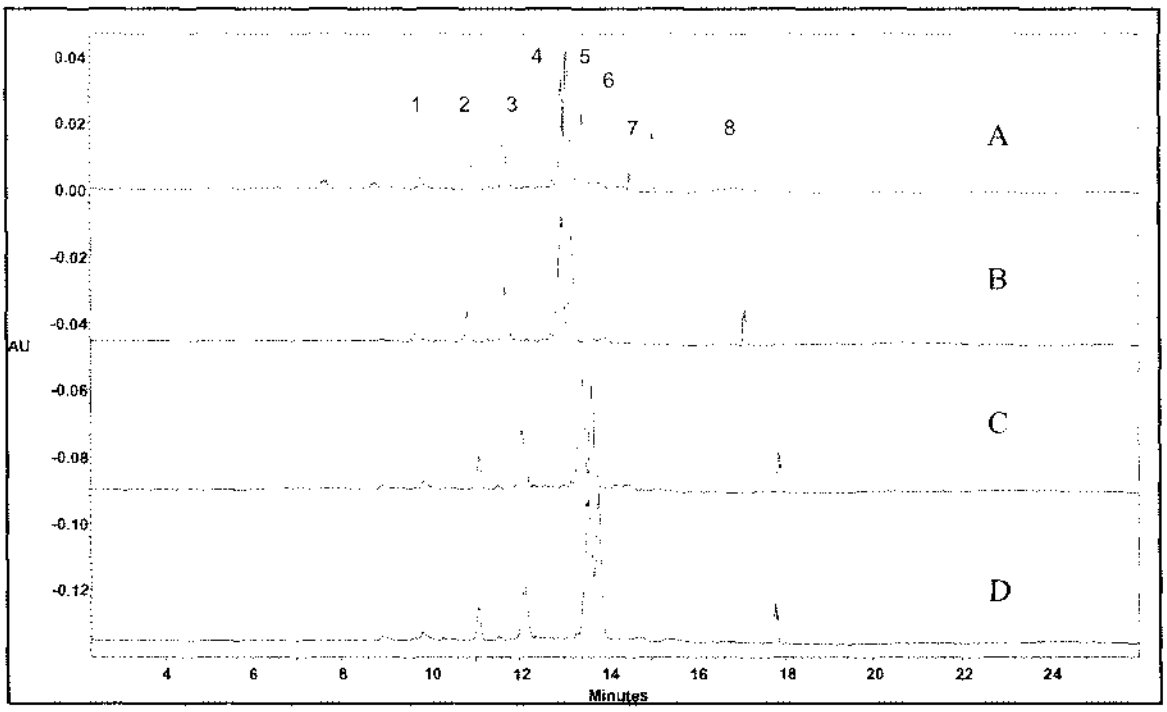

Figure 29 Comparison of different buffers under MS running conditions. Samples were whey protein from skim milk with standard *pI* markers. Samples were run identically to those in Figure 3, except that a voltage of 10 kV was applied to the 30 cm column. Buffers used are outlined in Table 13. Peak 1 = trypsinogen, peak 2 = myoglobin, peak 3 = carbonic anhydrase, peak 4 = β-lactoglobulin-B, peak 5 = β-lactoglobulin-A, peak 6 = α-lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase.

Table 13 Buffer compositions for the electropherograms shown in Figure 29. All buffer percentage compositions were in a v/v ratio.

Electropherogram	Focusing Buffers		Mobilisation Buffers	
	Anode	Cathode	Anode	Cathode
A	20 mM phosphoric acid	20 mM sodium hydroxide	20 mM phosphoric acid	20 mM phosphoric acid
B	1 % acetic acid	1 % ammonia	1% acetic acid	1 % acetic acid
C	1 % acetic acid	1 % ammonia	1% acetic acid	1 % acetic acid in 50 % methanol
D	1 % acetic acid	1 % ammonia	1% acetic acid	1 % acetic acid in 50 % acetonitrile

The results show that a better separation was obtained with phosphoric acid buffers, where β-Lac-A, β-Lac-B and α-Lac were separated from each other (electropherogram A). With acetic acid either on its own, or with methanol or acetonitrile, separation of all 3 of these proteins was not apparent. For MS detection the most likely buffer system that would be used for focusing would be acetic acid and ammonia with ammonia being replaced by 50 % methanol/ 1 % acetic acid for mobilisation (electropherogram C in Figure 29). Phosphoric acid and sodium hydroxide are not compatible with MS instrumentation whereas acetic acid and ammonia are. However, for good MS spectra, the addition of an organic buffer such as methanol will result in better protein ionisation than acetic acid on its own. Essentially this gives better sensitivity for the MS detection.

In the CIEF-MS system, the CE is connected to the MS electrospray ionisation source (ESI) via a coaxial sheath flow interface Figure 2. Because the flow rate of fluid from the outlet of a capillary is extremely low (approximately 13.5 nl/minute for a typical mobilisation at 0.1 psi, 20°C, and 50 μm i.d. capillary) a make-up flow (typically 2-3 μl/min) of 1 % ammonia buffer is added. This make-up flow can be utilised in the CIEF system as the cathode buffer. For focusing it was found that best results were obtained by having a low nitrogen gas flow rate that enabled a droplet to form at the

tip of the ESI source in an open position (Tao et al., 2002). On mobilisation the make-up flow buffer was changed to 50 % methanol, 1 % acetic acid. This buffer not only allowed chemical mobilisation to occur in the CE, but also allowed the proteins being eluted into the MS detector, to be ionised, and hence an ionisation pattern of mass to charge (m/z) for each protein was generated. Ampholytes did suppress the signal obtained for a protein, and in some cases where a standard mixture had been made up not all the proteins were detected possibly due to this in part (Figure 30). An ampholyte concentration of 0.5 % (v/v) is commonly used (Appendix 1 Table 15) and as seen in Figure 29 this is good enough to get good separation on a 30 cm column with MS buffers.

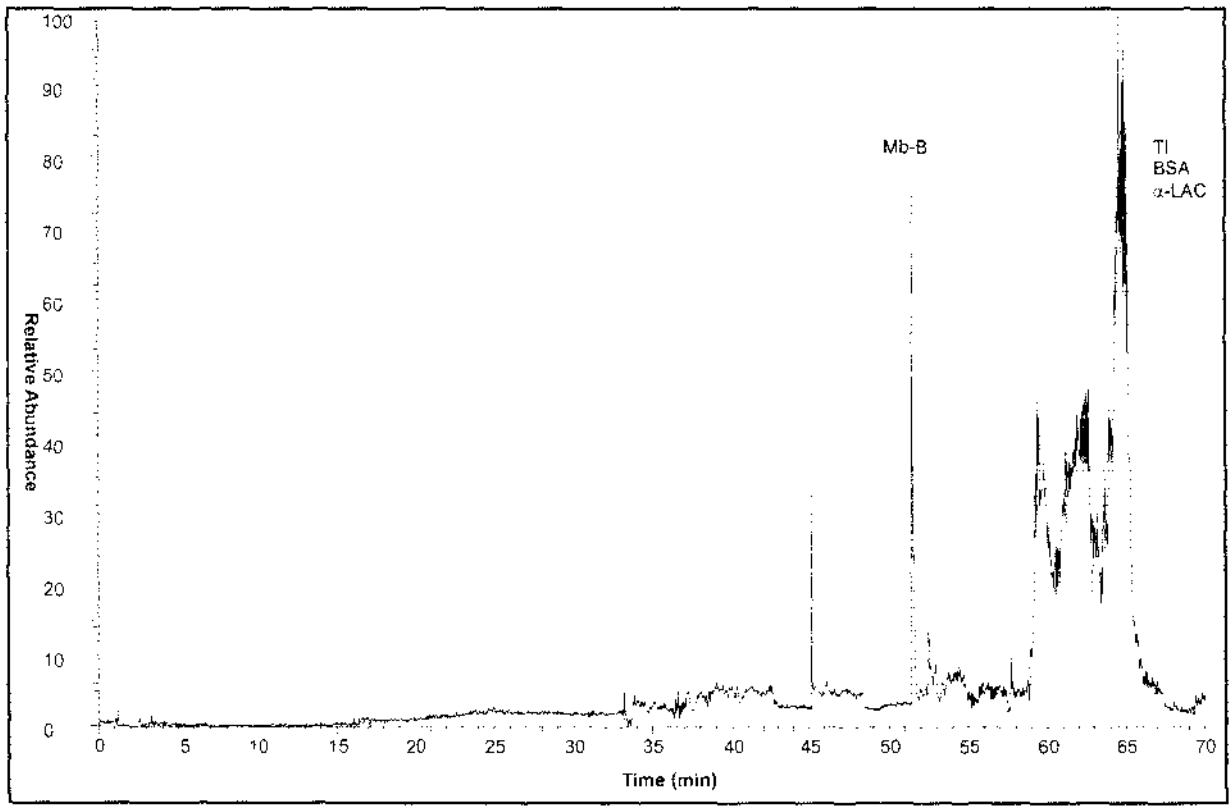

Figure 30 TIC of CIEF-MS of whey protein from skim milk spiked with minor whey proteins (BSA, GMP, and PP5) and *pI* markers.

Overall, this method unfortunately proved to be fairly difficult to develop in terms of reliable reproducible results. Problems which required trouble shooting were:

Make-up flow rate - This required setting at 3 μl/min for optimal results, otherwise an uneven baseline was obtained. Early experiments used 2 μl/min, but the data obtained was compromised compared to 3 μl/min.

Ampholyte condition - The literature showed that Pharmacia and BioRad ampholytes were most commonly used (Appendix 1 Table 14 and Table 15). However, these ampholytes were found to be routinely degraded; in particular the Pharmacia ampholytes proved to be troublesome, blocking the tip of the capillary repeatedly. Replacement with Beckman ampholytes overcame this problem giving good results with no suppression of signal noticed on the MS detector.

Adduct formation in MS results - Adducts of 50 mass units difference were repeatedly seen in the mass spectra from the early part of some electropherograms. This was reduced by lowering nitrogen gas flow slightly, and scanning at a higher mass range (900-2000 instead of 200-2000). Scanning at the higher range did not result in loss of any valuable data as proteins tend to ionise at greater than 900 m/z.

Height adjustment of CE - The CE system purchased came with a portable trolley that allowed the CE to be positioned in front of the MS detector. The trolley had height adjustment to precisely set the CE so that siphoning back into the anode did not occur as ocurred if the height was too low, or out of the cathode if too high. The trolley was found to need readjusting in height each day as it would slowly fall over time, which meant that on focusing, proteins would sometimes siphon back into the anode or retention time of analytes would be greatly increased.

However, when the system was established, it was possible to get reasonably reproducible results in terms of the repeatability of proteins being detected for the same sample run consecutively. A summary of optimized method parameters are outlined in section 3.6.

4.4.2 CIEF-MS Applications

Due to the limitations of the MS detector in that usual protein denaturing and solubilising reagents such as urea, SDS, and CHAPS cannot be used as they would damage the detector, CIEF-MS experiments are generally limited to soluble proteins. For dairy proteins, this poses a serious problem for the analysis of the sparingly soluble casein or milkfat globule membrane proteins. An alteration to the method was tried using UV detection where samples were made up in acetonitrile. However, the ampholytes proved to be insoluble in this medium. Although in some cases only low concentrations of solubilising/denaturing reagents would be required, and the make up

flow would further dilute the reagent by around 80 fold, this would still not be acceptable for the MS. A future extension of this method would be the investigation of a suitable sample buffer that could solubilise dairy proteins without damaging the MS detector.

CIEF-MS experiments were conducted with different samples of whey proteins from skim milk, some of which were spiked with minor components of whey in combination with *pI* markers. Although the technique was technically challenging, good results were obtained reproducibly. Figure 30 shows the TIC obtained from a CIEF-MS experiment where the sample comprised whey proteins from skim milk spiked with the minor whey proteins BSA, PP5 and GMP. Additionally, the standard proteins basic and acidic myoglobin, carbonic anhydrase-II, trypsin inhibitor, and trypsinogen were added. Figure 31 represents molecular weight versus time for the TIC shown in Figure 30, where every 10 microscans from the MS data were deconvoluted with Bioworks software. Unfortunately, with this sort of representation it is not possible to show the intensity of each protein as would be seen to some extent in a 2-D gel. Proteins were identified according to mass by comparison to infusion of standard protein solutions (Section 4.3).

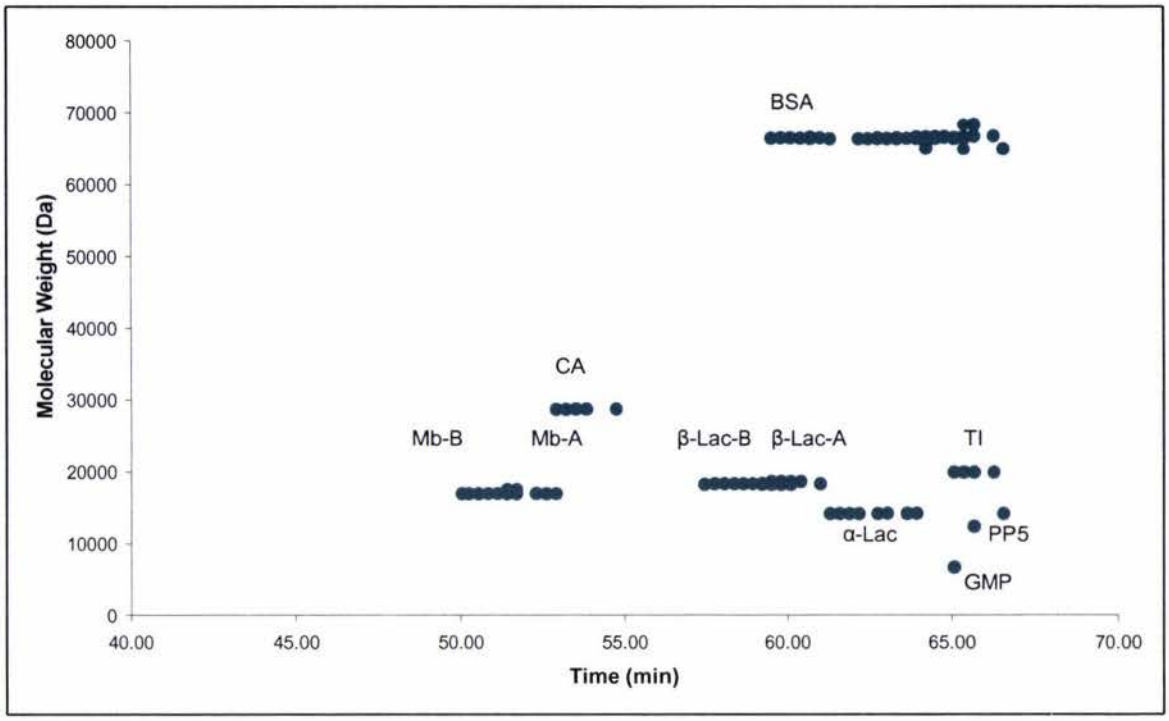

Figure 31 Representation of molecular weight versus retention time for the TIC in Figure 30. Every 10 microscans of the MS data were deconvoluted by Bioworks software. Proteins were then identified according to molecular mass with comparison to infused standards. Mb-B = myoglobin basic, Mb-A = myoglobin acidic, CA = carbonic anhydrase I, β-lac-B = β-lactoglobulin-B, β-lac-A = β-lactoglobulin-A, α-Lac = α-lactalbumin, TI = trypsin inhibitor, BSA = bovine serum albumin, PP5 = proteose peptone 5, GMP = glycomacropeptide.

Throughout the CIEF-MS experiments using the *pI* markers, the first marker that would normally be detected, trypsinogen (*pI* 9.3), was never detected. This was unusual as infusion experiments showed that it ionised very well (Appendix 2 Figure 69) compared to various other proteins. In other experiments (data not shown) focusing time was reduced to 3 minutes to ensure that trypsinogen had not eluted off the column before the focusing was complete and the mass spectrometer on line. The results in Figure 30 show that there was a very long lead time before the first proteins were detected and it is thought that trypsinogen should not have entirely eluted prior to completion of focusing. In some early method development experiments using whey protein only, it was noted that sometimes the β-Lac proteins were detected earlier than expected and that the amount of ionisation slowly increased to a sharp peak after which it dropped rapidly to zero. This is a typical outcome of a protein being only partially focused yet being mobilised at the same time, and could possibly occur due to the height of the CE being set too high and/or effects of EOF on the column. Since the column is filled with protein and ampholyte solution, protein that is towards the ends of the column would take longer to reach its *pI* value. In the CIEF-

MS system this would explain why β-Lac proteins were identified early on and how they reached a peak and rapidly dropped off. However, it does not tell us why the trypsinogen was not detected. The concentration of trypsinogen may have been the limiting factor in this system. One possible change to the method would be the use of a wider diameter capillary. Many other research groups performing CIEF-MS use a 75 μm rather than 50 μm internal diameter column. This would give a greater sample loading, and hence improve sensitivity as discussed in section 4.1.4.3.

Although both GMP and PP5 were spiked into the sample, they were not easily detected by MS. Infusion experiments (Appendix 2, Figure 51 and Figure 63 respectively) showed that the ionisation of these standards was poor as there seemed to be many products (glycoforms) for each standard. In analysis of CIEF-MS spectra an ionisation pattern for both GMP and PP5 was observed (Figure 31). Again, using this system with a 75 μm column could allow for greater sensitivity of proteins that do not ionise well and/ or are at a low concentration.

Many different ionisation species were observed for BSA although from analysis of infusion experiments (Appendix 2, Figure 47 and Figure 48) this was expected. Ionisation patterns and intensities showed that separation of β-Lac-A and β-Lac-B was still occurring with β-Lac-B eluting ahead of β-Lac-A. There was also a lactosylation product detected for both β-Lac-A and β-Lac-B.

From the literature it is apparent that the most successful CIEF-MS has involved the use of MS such as Fourier transform ion cyclotron MS instruments that have a much greater resolution and sensitivity than the older generation ion trap MS used in these studies. Enhanced MS capability could be one area of great improvement for this method, in particular for the ability to simulate 2-D gels for proteomic applications.

4.5 Flat Bed IEF Gels

Flat bed IEF gels were prepared and several samples including the whey basic protein fraction numbers 1, 2, and 3 were run. The gels were scanned by laser densitometry and gave mixed results. The whey basic protein fraction samples were high in salt concentration which had the effect of burning or smearing the gel (Figure 32). These samples required de-salting to obtain reasonable results. Samples such as skim milk

gave good results. Protein bands were easily identified when standards were run in an adjacent lane. The staining intensity of each band is determined by the ability of each protein to bind the stain. Whey proteins (although less abundant in skim milk) bind the stain better than caseins and so appear fairly intense in comparison to the casein proteins. The method of IEF PAGE has been utilised in dairy applications for determining protein concentrations (Braun et al. 1990) and this method has traditionally been the first step in creating a 2-D gel.

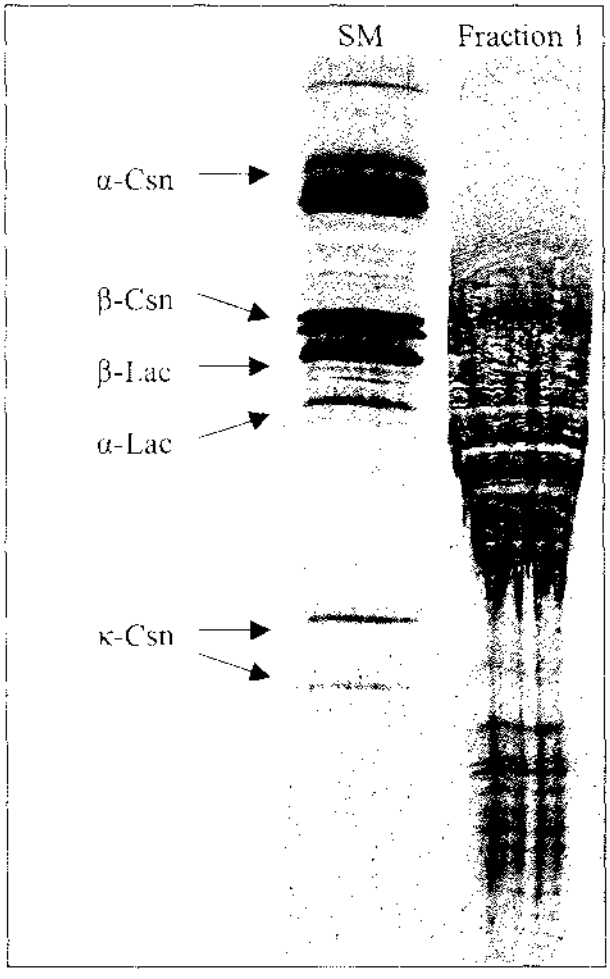

Figure 32 IEF flatbed gel of skim milk (SM, left lane) and whey basic protein fraction number 1 (right lane).

4.6 PAGE 2D Gels

Two-dimensional gels were generated for whey basic fraction protein samples 1, 2, and 3 (Figure 33, Figure 34, and Figure 35 respectively) and then analysed by MS by multidimensional protein identification technology (MudPIT) 2D HPLC (Fong et al. 2004). Through protein database searches of the MS data, the identities of the spots on

each gel were determined (Fong et al. 2005a & Fong et al. 2005b). Residual casein proteins were discovered to be present, as were small amounts of the major whey proteins β-Lac and α-Lac. Each sample contained other proteins such as lactoferrin, lactoperoxidase, angiogenins, RNAse, and IgG polymeric Ig receptor protein.

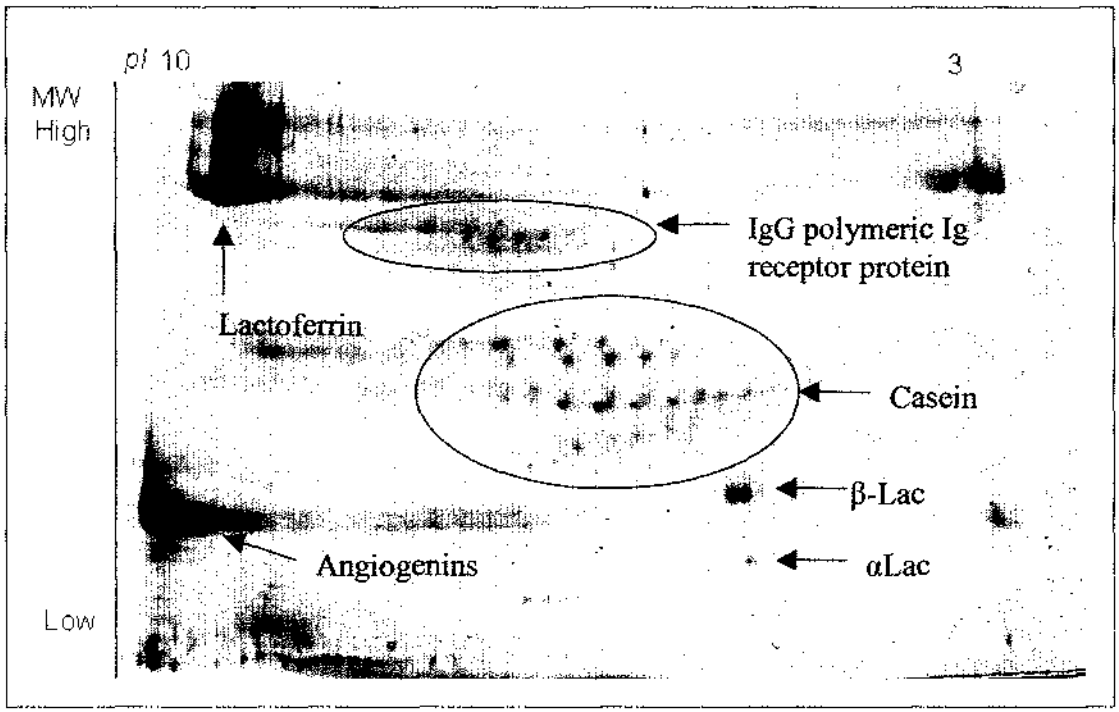

Figure 33 2D PAGE of whey basic protein fraction sample 1.

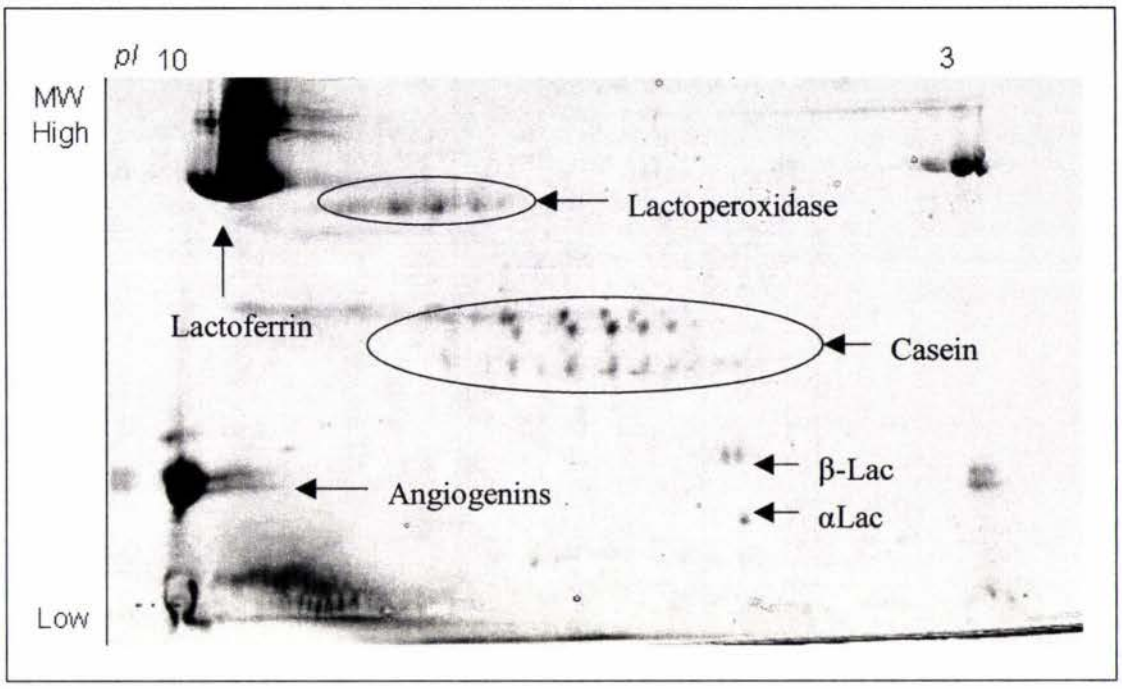

Figure 34 2D PAGE of whey basic protein fraction sample 2.

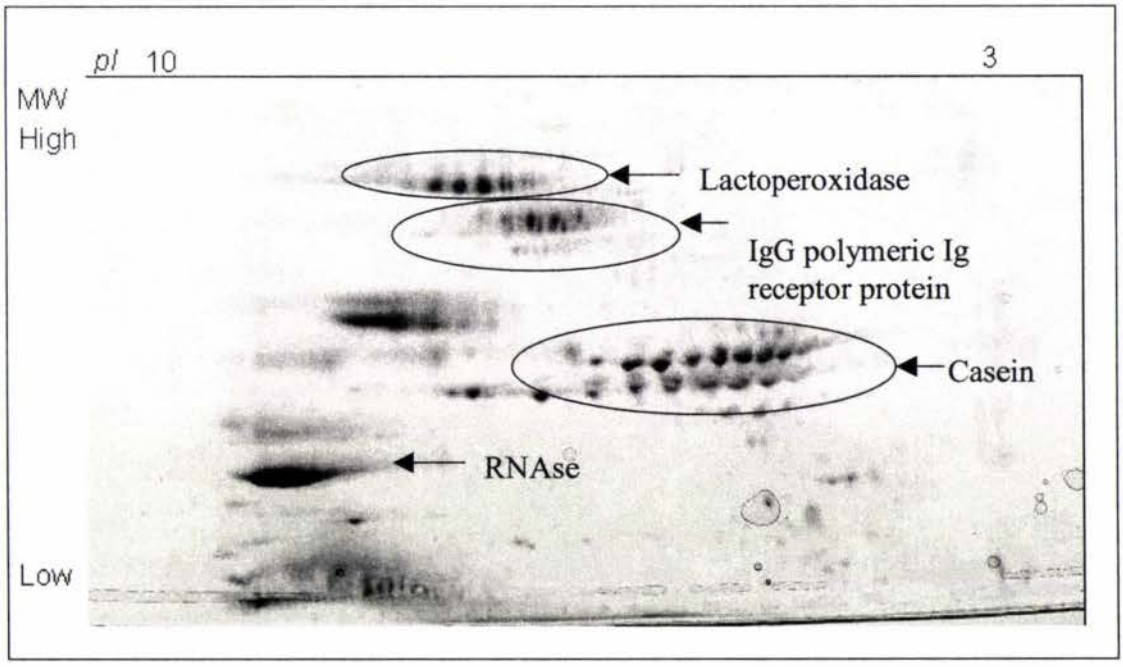

Figure 35 2D PAGE of whey basic protein fraction sample 3.

4.7 CZE of Dairy Proteins and Peptides

Key CZE methods for whey and casein protein separations were reproduced as these methods could be used as benchmarks for optimal separation of these proteins by CE with which to compare the CIEF methods developed in this study.

4.7.1 Whey Proteins

Separation of whey proteins from skim milk by a CZE method outlined by Kinghorn et al. (1996) was performed to compare the separation of whey using this method with a CIEF separation. The resulting electropherogram for whey protein is seen in Figure 36.

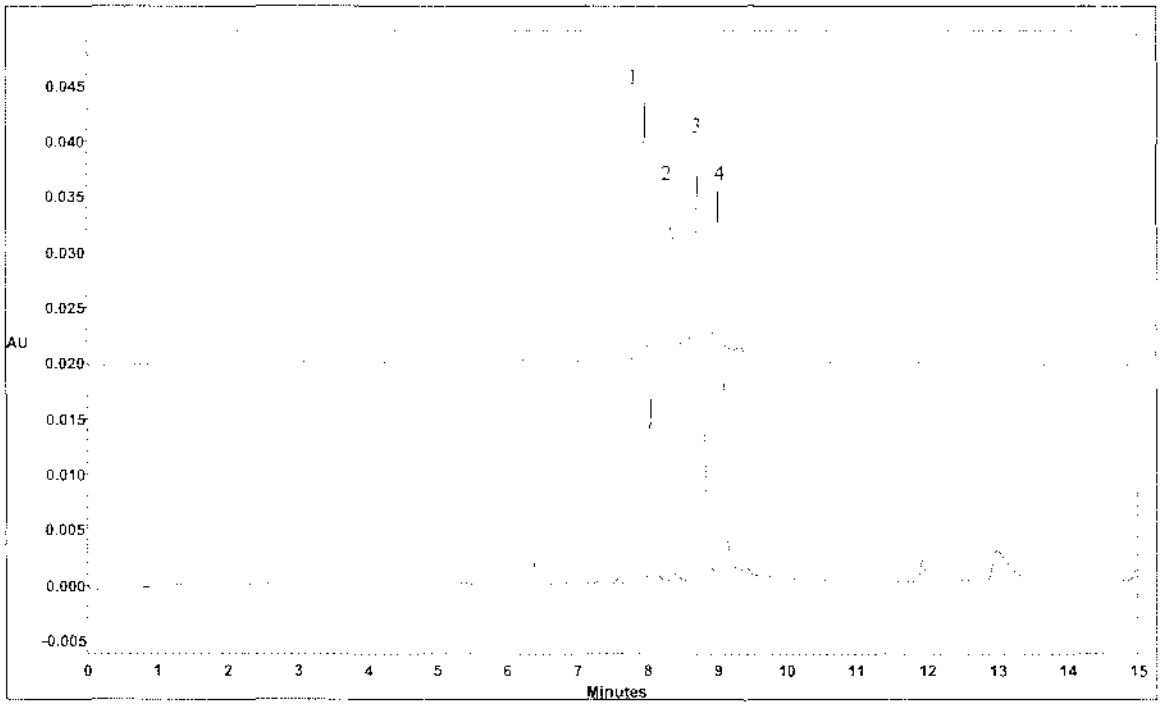

Figure 36 CZE separations of whey proteins from skim milk utilising the method of Kinghorn et al. (1996). The top trace represents protein standards of the major constituents of whey proteins, α-Lac (peak 1), β-Lac-A (peak 4), β-Lac-B (peak 3) and minor component β-Lac-C (peak 2) genetic variant. The bottom trace is the response for skim milk showing α-Lac, β-Lac-B, and β-Lac-A.

4.7.2 Casein

Figure 37 shows a typical CZE separation of milk proteins by the method of Recio *et al.* (1997). The separation of many of the genetic variants of casein was possible using this method, particularly the β-Casein variants A1, A2, B and C (latter not shown in figure). Separation of the α-S- casein variants was also possible (α-$_{S0}$, α-$_{S1}$ & α-$_{S2}$), but

this was not the case for the variants of α-s1-B & -C variants. The A and B variants of κ-Casein were also not able to be separated. This method was also limited for the separation of the major constituents of whey as β-lactoglubulin-A & -B co eluted. Overall, the general method of CZE originally developed by de Jong et al. (1993), has been used widely around the world for many different applications as outlined in section 2.9.1. It is likely to remain an important technique for dairy protein separations.

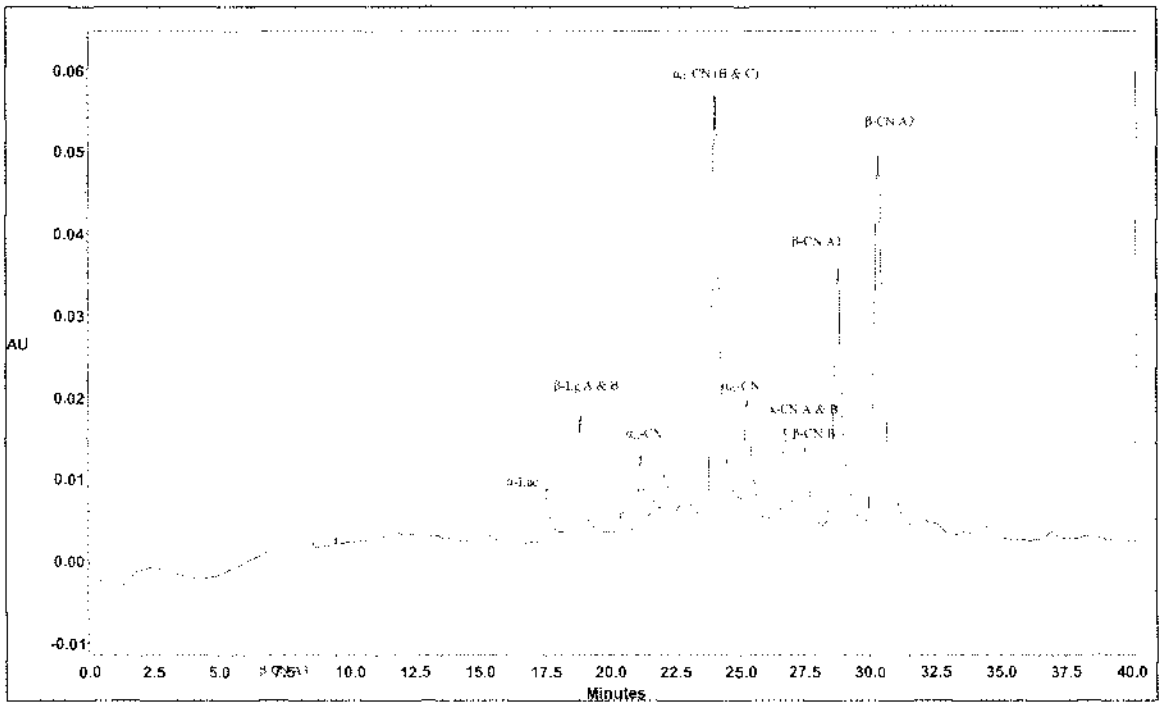

Figure 37 CZE separation of milk proteins from skim milk by the method outlined in section 3.9. The method was similar to that used by Recio et al., (1997).

4.8 Comparison of Methods

4.8.1 CIEF to CZE Methods

Water soluble proteins:

The main benefit of the CIEF method over the CZE method is its ability to separate soluble proteins over a large range of *pI* values. This is not typical for CZE as separations are usually designed for the separation of proteins of similar *pI* values. The time required to run samples is similar for both methods. However, the reproducibility of making identical run buffers while not a significant problem for CIEF, can sometimes be a problem for CZE. In the latter case, buffers must be made

carefully to give reproducible pH as differences in separation can occur between buffer batches.

In addition, CIEF method gives the added information of an approximate *pI* value for a protein, where this is not possible with CZE.

Insoluble Protein Method:

Overall the CZE method developed by de Jong et al. (1993) for the separation of the major dairy proteins is a far superior method to the CIEF method developed here for separation of casein proteins. The disadvantages of the CIEF method are that the major whey proteins and β-casein co-elute and there is a very small window of concentration limit such that proteins such as κ-casein, that are in low abundance and have a low absorption co-efficient at 280 nm can be difficult to detect. There is often separation of 2 α-casein species, but this is not always reproducible. The cathode run buffer also seems to deteriorate quickly due to sample buffer contamination as proteins run from anode to cathode, so regular changing of the cathode buffer is required. Alternatively a large reservoir buffer system could be used to dilute the effect of the sample buffer. In addition the samples seem to degrade, and/or dry out and crystallize rapidly. This may be due to using small sample sizes to minimise the use of expensive ampholytes. The CZE method seems to have a more stable run buffer system as it does not appear to degrade as quickly, and the sample size is usually larger hence the sample does not dry out and crystallize. Overall the CZE method has a greater resolution of separation compared to the CIEF method, with a large number of different types of genetic variants able to be separated in a single run. With the CIEF method for insoluble proteins it is also not possible to predict the sample *pI* from internal standards because the proteins do not elute according to their nominal *pI*. This is because the urea has an effect on protein *pI* value (Righetti, 2004). This effect is different for each individual protein because the unfolding of the protein by urea causes different surface amino acid groups to be presented with consequent changes in apparent *pI*.

Advantages of CIEF over CZE methods:

Typically in CIEF, sample is injected to completely fill the capillary before the sample is focused towards its *pI*. However, in CZE a typical maximum sample

volume is 5% of the capillary volume, hence CIEF would be better for investigation of dilute protein solutions.

CIEF also offers the benefit of being able to separate any protein, regardless of its *pI*, whereas CZE is optimised for a group of proteins of similar *pI* with a run buffer pH developed for that particular method. Therefore CIEF can be used as a screening tool in proteomics or other discovery research, with no method development required to optimise separation each time.

Disadvantages of CIEF over CZE:

Detection: Most dairy proteins have an absorbance maximum around 214 nm, and although another absorbance maximum is seen at 280 nm, this wavelength is usually 10 fold less sensitive then 214 nm. Unfortunately, as mentioned in section 4.1.5 the CIEF method is unable to detect at 214 nm, but also as mentioned in this section the difference in detection is only about four fold difference in sensitivity for an identical sample run at the two different wavelengths.

Some proteins with extreme *pI* values are either not detected (if highly basic) or are detected as broad peaks (acidic proteins). There is also a tendency for these high/low *pI* proteins to have their *pI* less accurately assigned. Low *pI* proteins also seem to have less reproducible migration times, possibly due to EOF effects and/or buffer degradation during a sample set.

4.8.2 CIEF to Gel Methods

Comparison of CIEF with UV detection to the IEF-PAGE format with laser densitometry scan (Figure 38), shows that the techniques are very complementary for skim milk proteins and allow a reproducible cross over for both methods. The advantage of CIEF is that it not only requires a much smaller sample size, but it is also a very rapid technique compared to the gel format with acquisition of results in less than an hour for one sample or overnight for a set of 20 samples. This compares to several days work required for an IEF gel. Thus CIEF is time and cost effective with the added advantage that it requires only small volumes of reagents. CIEF can be used for samples where sample size is an issue, or as a qualitative analytical tool when, for example, fractionating protein samples to rapidly identify the content or

purity. The method has proved to be very sensitive and therefore samples of fairly dilute concentration (7.5 μg/ml) can be used for analysis.

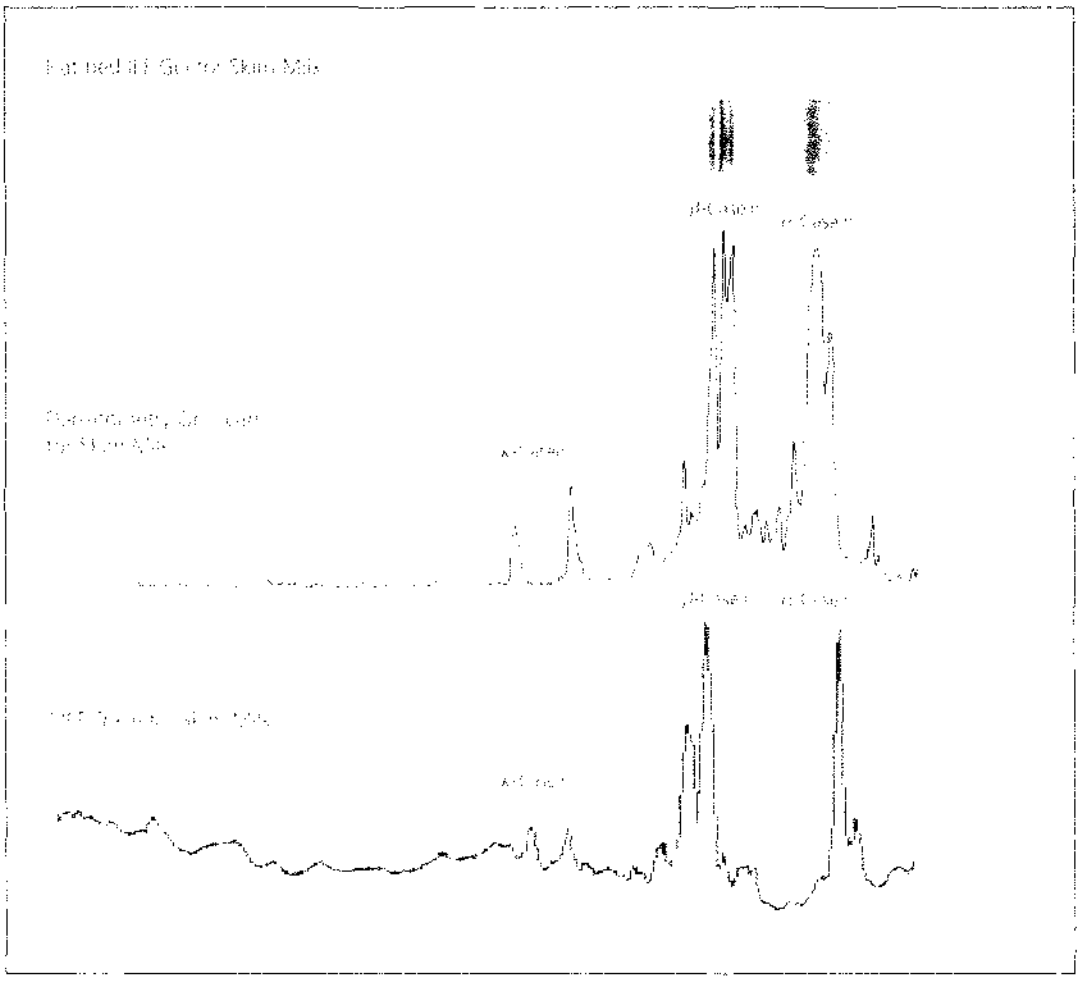

Figure 38 Comparison of flat bed IEF-PAGE with laser densitometry to CIEF-UV using the denaturing CIEF method (Section 3.5).

4.8.3 CIEF-MS to 2D-PAGE-MS

There have been certain limitations to the analysis of intact proteins by MS detection which means the CIEF-MS method is severely limited in sample types that can be analyzed for dairy applications, particularly for proteomics since many of the uncharacterised low abundant proteins are of high molecular weight. This shows then that the hydrolysis of proteins is a critical step in the analysis of protein fractions. Although a potential application for the CIEF-MS method could be for analysis of peptides from hydrolysis of 2D PAGE protein spots, the reality would be that due to the addition of ampholytes that suppress MS signal this would not be a viable option

and other techniques such as CZE-MS or nano-HPLC-MS would be preferable methods.

The *pI* of the protein has been shown to be a problem for CIEF analysis particularly in MS application, where the most basic proteins were never identified. Although there has been some interest in characterizing whey basic protein fractions, in this context the CIEF-MS method would be too problematic to pursue.

Finally, due to the insolubility of some dairy proteins the use of urea has been discussed in detail, i.e. with MS detection, it is not possible to use a run buffer containing urea. This further limits the uses of CIEF-MS and since for 2D-PAGE-HPLC-MS any residual buffer contaminants are removed in a desalting step on the HPLC. This gives the method of 2D-PAGE-MS a significant advantage. Some groups have solved the problem, in that they are now coupling the CIEF separation with a nano-HPLC system, which has a step to remove salts before separating intact proteins by reversed phase on line to the MS detector (Chen et al., 2002, Zhou & Johnston 2005). The use of powerful high resolution MS detectors then allows the analysis of large molecular weight proteins. Whether this type of separation would have application for the dairy industry would remain to be seen. Also, whether the slower analysis of removing protein spots from 2D-gels would outweigh the cost of such a high resolution MS would be a critical factor in purchasing such an instrument.

5 Conclusions

Proteomic research has been rapidly advancing in recent years due to advances in technology at the micro and nano level. The need for techniques that can deliver rapid results is required for research companies to get ahead of their competitors in the quest for intellectual property within the commercial sector. Traditional techniques such as 2D-PAGE, although very much tried and tested and able to deliver results for many applications, are however, very time consuming and more automated techniques are required. CIEF is a technique that can be used as an alternative method and has been used with success for many applications. The objective of this thesis was to take examples of CIEF techniques from the literature and modify them to optimise parameters for dairy proteomic applications. Techniques using CIEF with UV detection have been developed for both water soluble proteins (such as whey protein samples) and for insoluble protein samples (such as casein). The water soluble method with UV detection was optimised for different column lengths and several different sample types. In addition, the knowledge gained from the water soluble UV detection method was able to be used to optimise a CIEF separation with MS detection, before connecting the system to the MS detector.

Results from each method were compared to other techniques such as flat bed IEF PAGE, 2D-PAGE with HPLC-MS characterisation on a high resolution MS detector and to the currently used CZE separations of casein and whey proteins.

The CIEF-UV method for water-soluble proteins worked well, with a high accuracy of reproducibility and *pI* determination. Applications for this method included investigations of milk protein fractions such as the milk whey basic protein fraction, and acidic protein fraction. In addition peptides have been separated, and bacterial cell lysates with dairy applications have been investigated. Comparisons of this method with flat bed IEF PAGE support this technique as being able to replicate the gel format in a much quicker time frame (minutes/hours for CIEF versus days for PAGE).

Although the CIEF-UV method for insoluble proteins can give a good representation of what is seen by flat bed IEF PAGE, this technique does have a few limitations. Firstly, the concentration of dairy proteins used must be extremely low otherwise poor

resolution of separation is seen. With the low concentration, however, detection of some proteins becomes difficult. Secondly, the method was not able to be interfaced with MS detection; several alternative denaturing buffer systems were tried but failed. Thirdly, inclusion of the additives to the sample buffer system alters the *pI* value of the proteins. Although a reasonably good separation can be achieved, the determination of samples of unknown *pI* is not possible due to the denaturing buffer system. More research could be conducted on this technique to achieve better separation of proteins as there is potential a similar method could be used with MS detection by introducing another step of HPLC separation between the CIEF and MS.

The CIEF-MS method, although technically difficult, showed good similarities to that of 2D-PAGE in terms of 2D mapping. However, the use of a MS detector with greater resolution and sensitivity would dramatically improve results and make it a more worthwhile technique to pursue for other proteomic applications. It would also be interesting to see if modern high resolution MS detectors would be able to detect with great mass accuracy and reproducibility the molecular weight of large glycosylated dairy proteins such as lactoferrin and lactoperoxidase (as included in this study).

For the comparison of CZE to CIEF methods for separation of whey and casein proteins, the established CZE methods were the more preferred methods. For casein protein separation more genetic casein variants were detected with the CZE method. Additionally, the CZE method separated the whey proteins, whereas in the CIEF method the whey proteins co-eluted with β-casein. The CZE separation of whey proteins gives excellent separation of the β-lactoglobulin genetic variants and α-lactalbumin, although proteins such as BSA do not separate as discreet sharp peaks. The separation is also not optimised for other proteins such as lactoferrin or lactoperoxidase, whereas CIEF has been effectively optimised for all of these components as it was designed as a generic method of separation for proteins of very different *pI*.

Overall the CIEF methods developed here to date will not have as great an impact for the dairy industry as was originally envisaged. This is in comparison to established techniques that have proven the test of time as far as robustness and quality of results is concerned.

6 Future Work

Future work on CE for proteomic applications could include the following:

Develop a peptide CE-MS method for the analysis of 2-D PAGE spots that have been hydrolyzed and that would typically be analyzed by HPLC-MS. Reasoning; currently 3 identical gels must be run to gather enough protein for each spot to be analyzed. Since CE samples can be very much smaller than HPLC, then there may be an application whereby CE will outperform HPLC with MS detection.

Recently several papers have appeared where the use of CIEF-RP-HPLC-MS has been used for proteomic applications (Chen et al. 2002, Chen et al. 2003, Zhou & Johnston 2004 & 2005). The method utilises a standard CE instrument that has the cathode entering a HPLC switching valve. Once the proteins are focused in a capillary of zero EOF, zones of the focused proteins are then mobilised onto a reversed phase HPLC column. Buffer additives and ampholytes then pass through the column and are diverted to waste, before proteins retained on the column are further separated according to hydrophobicity and are eluted into a high resolution MS. The method uses a urea and DTT buffer system for the CIEF step, so samples could include insoluble proteins. This could have application to dairy proteomics and would be one future development for CIEF.

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Appendix 1 CIEF literature

Table 14 Summary of literature for CIEF with UV detection. Outlined are applications of samples separated, buffers used, running conditions and comments about each reference.

Samples	Buffer	CIEF conditions	Comments	Reference
Erythropoietin glycoform analysis	Anode: 91 mM phosphoric acid. Cathode: 20mM sodium hydroxide with different amount of phosphoric acid added to alter pH of buffer.	Beckman CIEF kit with several ampholyte brands including narrow *pI* range. Samples made in 7 M urea Beckman gel solution. 925V/cm ramped over 2 minutes was used.	Uses β-lac-A as internal standard. Range of pH is said to be 2-10 for the mixture used. Follow on work from Cifuentes, et al. (1999).	Lopez-Soto-Yarritu et al. (2002)
Erythropoietin glycoform analysis	Anode: 91 mM phosphoric acid. Cathode: 20 mM sodium hydroxide.	Beckman CIEF kit with several ampholyte brands including narrow *pI* range. Samples made in Tween 20 and other solutions. 925 V/cm ramped over 2 minutes was used.	Method compares well with IEF gels, and CZE.	Cifuentes et al. (1999)
Recombinant glycoproteins from human immunodeficiency virus	Anode: 100 mM phosphoric acid. Cathode: 20 mM sodium hydroxide	Beckman CIEF kit with polyvinyl coated capillary 47 cm x 75 μl i.d. and 425 V/cm. TEMED was used to block the blind side of the detector. 5 % ampholytes in a mixture of narrow and broad pH range.	One step CIEF in reverse polarity mode. Sample buffer also contained hydroxypropyl-methylcellulose, CAPS and saccharose. Comparisons of other sample buffers is shown using Triton X-100 and urea.	Tran et al. (2000)
O-glycosylated caseinomacropeptide (CGMP)	Anode: 91 mM phosphoric acid. Cathode: 20 mM sodium hydroxide.	Beckman CIEF kit with narrow range ampholines added. 2 % total ampholyte concentration 27 cm x 50 μm polyacrylamide coated column with 500 V/cm applied.	One and two step CIEF with reverse polarity. Samples were made in Beckman CIEF gel. Samples are very acidic so no need to use TEMED to block the blind side of the detector.	Tran et al. (2001)

Table 14 continued

Samples	Buffer	CIEF conditions	Comments	Reference
Mainly standards plus some monoclonal antibodies of IgG	Anode: 10-20 mM phosphoric acid. Cathode: 20-40 mM sodium hydroxide.	Alot of different techniques, column types, lengths, voltages, sample buffers, detectors were tried in this study.	Sample buffer includes the use of urea and β-mercaptoethanol	(Schwer, 1995)
α_1-Antitrypsin phenotypes to be used to determine if people will be susceptible to emphysema	Anode: 91mM phosphoric acid. Cathode: 20 mM sodium hydroxide both in 0.75 % polyethylene-oxide (PEO).	PEO coated column 27 cm x 50 μm 900 V/ cm, ampholytes *pI* range of 3.5-5.	Sample contains DTT. Comparison with flat bed gel methods is very good for desired result of phenotyping.	Lupi et al. (2000)
Bradykinin, cytochrome C, cytochrome C hydrolysate, and *pI* marker standards	Anode: 20 mM phosphoric acid. Cathode: 40 mM sodium hydroxide.	Hydroxypropyl-cellulose coated capillaries 37 cm x 50 μm i.d. 15 minute focusing at 500 V/cm, samples contained pharmalyte 3-10 and 9-11 from sigma. Gravity mobilisation.	In some experiments TEMED was used at a higher ratio to block blind side of detector and allow bradykinin (*pI* 12.0) to focus 9 cm before the detector. Mobilisation velocity ~1 cm/min.	Mohan & Lee (2002)
Haemoglobins	Anode: 10 mM phosphoric acid. Cathode: 20 mM sodium hydroxide	Polyacrylamide coated capillaries 14-20 cm x 25 μm i.d. 6kV applied for focusing and 8kV applied for chemical mobilisation. 2 % biolyte.	Early work on CIEF, some of the techniques used here have now changed slightly compared to more recent literature.	Zhu et al. (1991)
Drosophilia salivary gland protein digests	Anode: 0.1M acetic acid pH 2.5. Cathode: 0.5 % ammonium hydroxide pH 10.5.	Hydroxypropylcellulose coated capillaries 100 μm i. D. 30 cm long. Pharmalyte 3-10 at 2 % concentration. Voltage = 333 V/cm.	CIEF-RPLC-MS.	Chen et al. (2002)

Table 14 continued

Samples	Buffer	CIEF conditions	Comments	Reference
Peptides formed from hydrolysis of standard proteins	Anode: 0.1M acetic acid. Cathode at pH 2.5. Cathode: 0.5 % ammonium hydroxide pH 10.5.	Hydroxypropylcellulose coated capillary, 50 µm i.d. and 33 cm long, 500 V/cm in negative mode. 2 % pharmalyte. Another capillary is joined via a mirodialysis junction for CITP-CZE.	CIEF-CITP-CZE with UV detection at 214 nm.	Mohan & Lee (2002)
Protein standards such as β-lactoglobulin and myoglobin	Anode: 100 mM phosphoric acid. Cathode: 30 mM ammonium hydroxide.	Poly(vinylalcohol) coated capillary with 100 µm i.d. 30 cm in length and 500 V/cm applied. Samples made in 2 % ampholine.	CIEF as a micropreparative fraction collection method before MALDI-TOF MS detection.	Minarik et al. (2000)
Peptides formed from a tryptic digest of yeast cytosol and BSA.	Anode: 1 % acetic acid (pH 2.5). Cathode: 1 % ammonium hydroxide (pH 10.7).	Hydroxypropylcellulose coated capillary 50 µm i.d. and 65 cm long. Pharmalyte 3-10 at 1 % concentration was used. ~300 V/cm was applied and gravity mobilisation was used.	Detection was at 280 nm. Samples were reduced with DTT before use.	Shen et al. (2000a)
Yeast cells (Saccharomyces cerevisiae)	Anode: 1 % acetic acid (pH 2.5). Cathode: 1 % ammonium hydroxide (pH 10.7).	Hydroxypropylcellulose coated capillary columns 100 µm i.d. and 65 cm long. Pharmalyte 3-10 at 1 % concentration was used. ~300 V/cm was applied and gravity mobilisation was used.	Detection was at 280 nm. The authors monitored *pI* differences at different stages of cell growth.	Shen et al. (2000b)
Haemoglobin	Anode: 10mM phosphoric acid. Cathode: 20mM sodium hydroxide.	Bare fused silica capillaries 60 cm x 50 or 75 µm i.d. Separations at 333-500 V/cm. BioRad ampholytes at 2 % narrow and broad range used.	Detection at 280 or 415 nm. Samples ran 3-5 times.	Kilár et al. (1998)
Protein and peptide standards (Pharmalyte broad band *pI* kit)	Anode: 91 mM phosphoric acid. Cathode: 20 mM sodium hydroxide.	Beckamn eCAP n tral capillary 27 cm x 50 µm i.d. Pharmalyte or Servalyte at 1 % concentration. 500 V/cm. Pressure mobilisation.	TEMED and other reagents used. Aim was to determine *pI*'s by CIEF and compare to slab gel IEF.	Shimura et al. (2000)

Table 14 continued

Samples	Buffer	CIEF conditions	Comments	Reference
L-aspartate oxydase, thermylase, alcalase and a glycopeptide antibiotic of the teicoplanin family	Anode = 50 mM acetic acid (pH 3.5). Cathode = 50 mM lysine	Poly(AAP) coated column. 24 cm x 75 μm i.d. Ampholine of different narrow ranges (2.5% conc). 416 V/cm. Chemical mobilisation used.	Reagents such as CHAPS were used. Results compared to flat bed IEF gels.	Conti et al. (1997)
Proteins from lysates of microorganisms	Anode: 1 % acetic acid (pH 2.5). Cathode: 1 % ammonium hydroxide (pH 10.7).	Polyacrylamide, HPC and PVA coated capillaries of 65 cm x 50 μm i.d. Pharmalyte 3-10 at 1 % concentration used with ~300 V/cm applied. Mobilisation was by gravity.	Detection at 280 nm. High resolution of separation achieved with many peaks being obtained.	Shen et al. (1999)
Mixed standards	Anode: 10 mM phosphoric acid. Cathode: 20 mM sodium hydroxide.	Different coated capillaries from BioRad 34 cm x 50 μm i.d. ~220 V/cm applied. 2 % Pharmalyte 3-10. Continual focusing.	Detection at different wavelength with a PDA (214, 235, 254, and 280 nm). Aim of work was to look at reproducibility and protein adsorption on peaks in CIEF separations.	Graf & Wätzig et al. (2004)

Table 15 Summary of literature for CIEF with MS detection. Outlined are applications of different types of samples separated, buffers used, running conditions and comments about each reference.

Samples	Buffer	CIEF–MS conditions	Comments	Reference
Mixed standards	Cathode: 20 mM sodium hydroxide. Anode: 20 mM phosphoric acid. Sheath: 50 % methanol, 1 % acetic acid, pH 2.6.	20 cm x 50 µm i.d. x 192 µm o.d. linear polyacrylamide, 15 min focus, 500 V/cm with Pharmalyte Coaxial liquid sheath-flow Make-up 3 µL/min Finnigan MAT TSQ 700 MS.	First paper on CIEF–MS. Looks at different ampholyte concentrations.	(Tang et. al., 1995)
Haemoglobin variants	Catholyte: 0.5 % ammonium hydroxide in 50 % methanol. Anode: 0.5 % acetic acid. Sheath: 75 % methanol, 0.25 % acetic acid.	50 cm x 50 µm x 360 µm PVA-coated capillary, 1% Ampholytes, 500 V/cm Coaxial sheath-flow interface Make-up 1.5 µL/min Finnigan MAT TSQ 700 MS	Used tapered tip Three ampholytes used in a ratio of 1:1:1 (Ampholine/ Pharmalyte/ Servalyte all 3-10)	(Kirby et. al., 1996)
Transferrin glycoforms	Cathode: 20 mM sodium hydroxide. Anode: 20 mM phosphoric acid. Sheath: 50 % methanol, 1 % acetic acid, pH 2.6. Also used to immobilise proteins.	20 cm x 50 µm i.d. x 192 µm o.d. linear polyacrylamide, Pharmalyte 5-8 at 0.5 %, 500 V/cm. Coaxial liquid sheath-flow interface. Make-up 5 µL/min Finnigan MAT TSQ 700 MS.	Able to identify proteins around 78 kDa.	(Yang et. al., 1996)

Table 15 continued

Samples	Buffer	CIEF–MS conditions	Comments	Reference
Recombinant E. coli proteins	Cathode: 20 mM sodium hydroxide. Anode: 20 mM phosphoric acid. Sheath: 50 % methanol, 1 % acetic acid.	30 cm x 50 µm i.d. x 192 µm o.d. linear polyacrylamide, 500 V/cm, 0.5–2 % Pharmalyte. Coaxial liquid sheath-flow interface. Make-up 5 µL/min Finnigan MAT TSQ 700 MS instrument used.	Results compared very well with 2D PAGE. Used narrow range ampholytes for UV detection. Focusing was monitored and stopped when required.	(Tang et. al., 1997)
Model proteins including β-lactoglobulin A myoglobin carbonic anhydrase I	Cathode: 2 % acetic acid. Anode: 2 % acetic acid. Sheath liquid: 80 % methanol, 5 % acetic acid.	113 cm x 75 µm i.d. x 190 µm o.d. coated with PVA Pharmalyte pH 5–8 at 1 or 2.5 % at 265 V/cm focusing for 6 min. Coaxial sheath-flow interface Make-up 1 µL/min. Finnigan MAT SSQ 710 MS instrument used.	Use of a microdialysis junction to eliminate ampholytes. Anode and cathode both acetic acid.	(Lamoree et. al., 1997)
Ovalbumin phosphorylation using	Cathode: 20 mM sodium hydroxide. Anode: 20 mM phosphoric acid. Sheath: 50 % methanol, 1 % acetic acid, pH 2.6.	25 cm x 50 µm i.d. x 192 µm o.d. linear polyacrylamide, 600 V/cm. Coaxial liquid sheath-flow Make-up 3 µL/min.	Pharmalyte 4-6.5 used at 0.5 %.	(Wei et. al., 1998)
Protein standard mix containing β-lactoglobulin A	Cathode: 20 mM sodium hydroxide. Anode: 20 mM phosphoric acid. Sheath: 50 % methanol, 1 % acetic acid.	50 cm x 50 µm i.d. x 192 µm o.d. linear polyacrylamide, 260 V/cm, 15 min focus, 1 % 3-10 Pharmalyte. Coaxial liquid sheath-flow. Make-up 2 µL/min FTICR-MS.	Separation of dissociated subunits and intact protein complexes by two different ionisation methods.	(Martinovic et. al., 2000)

Table 15 continued

Samples	Buffer	CIEF–MS conditions	Comments	Reference
Recombinant E. coli proteins	Cathode: 50 mM ammonium acetate, pH 9.3. Anolyte: 0.2 M acetic acid. Sheath: 50 % methanol, 0.2 M acetic acid.	1 m x 50 µm i.d. x 180 µm bare fused silica, 0.5% Pharmalyte, 280 V/cm. Coaxial liquid sheath-flow interface. Make-up flow 2 µL/min. Either Finnigan LCQ ion trap or FTICR–MS used.	Stepwise mobilisation technique employed. Ampholytes also function to eliminate EOF (in addition to forming a pH gradient).	(Zhang et. al., 2000)
D. radiodurans cell proteins	Cathode: 20 mM sodium hydroxide. Anode: 20 mM phosphoric acid. Sheath: 50 % methanol, 1 % acetic acid, pH 2.6.	30–50 cm x 50 µm i.d. x 192 µm o.d. linear polyacrylamide, 0.5 % Pharmalyte, 260 V/cm for 10 min. Coaxial liquid sheath-flow interface. Make-up 2 µL/min FTICR–MS.	Isotope labelling for mass accuracy.	(Jensen et. al., 2000)
Six model proteins including β-lactoglobulin -A and –B, myoglobin, and carbonic anhydrase II.	Cathode: potassium acetate (pH 3 with formic acid). Anode: aspartic acid of same pH and concentration. Sheath: 50 % methanol, 20 mM formic acid.	90 cm x 75 µm i.d. x 375 µm o.d. linear polyacrylamide, 278 V/cm, Pharmalyte Make-up at 2 µL/min. Ampholytes of different concentrations Finnigan LCQ-DECA XP Ion Trap MS used.	Anolyte and catholyte are identical pH (pH 3.0). Separation depends on ampholyte concentration. Has characteristic of CITP separation. A plug of sample/ ampholyte was added.	(Chartogne et. al., 2002)

Table 15 continued

Samples	Buffer	CIEF–MS conditions	Comments	Reference
Cerebrospinal fluid, whole blood from diabetic and control patients, and mixed standards	Cathode: 50 % methanol, 1 % ammonium hydroxide. Anolyte: 50 % methanol, 1 % acetic acid. Sheath liquid: 50 % methanol, 1 % acetic acid.	37 cm x 50 μm i.d. x 360 μm o.d. PVA-coated capillary. 1 % Pharmalyte 540 V/cm focusing for 7 min. Coaxial sheath-flow interface. Make-up 3 μL/min Finnigan MAT 900 MS.	Able to use this technique successfully for haemoglobin α and β chains as well as cerebrospinal fluid.	Clarke & Naylor (2002)
Standard mixtures looking at isoforms	Cathode: 1 % ammonium hydroxide. Anode: 1 % acetic acid. Sheath liquid: 75 % methanol, 0.25 % acetic acid.	80 cm x 50 μm i.d. x 360 μm o.d. linear polyacrylamide coated capillary, 1 % Pharmalyte with 375 V/cm focusing for 40 min. Coaxial sheath-flow interface. Make-up flow of 2 μL/min. Finnigan MAT LCQ Ion Trap MS instrument.	Separated isoforms with long focusing times Used active capillary positioning Used standing drop technique while focusing.	Tao et al. (2002)
Haemoglobin variants A, C, S and F	Cathode: 20 mM sodium hydroxide. Anode: 20 mM phosphoric acid. Sheath liquid: 50 % methanol, 1 % acetic acid, pH 2.6.	20–30 cm x 50 μm i.d. x 192 μm o.d. linear polyacrylamide coated capillary with 500 V/cm with Pharmalyte 5-8 of varying concentration and focusing for 15 min. Coaxial sheath-flow interface. Make-up flow at 5 μL/min. Finnigan MAT TSQ 700 MS	Separation of proteins of *pI* 0.05 difference Used single ion monitoring in some MS experiments.	Tang et al. (1996)

Appendix 2 Results of Infusion MS experiments

Table 16 Results of infusion MS experiments. MS conditions used are outlined in section 3.7. Deconvolution of mass spectra was performed on Bioworks version 3.1. Literature masses were obtained from Mascot (www.matrixscience.com) web site. N/A = data not available due to lack of ionisation. Mass Spectra and deconvoluted data for each standard are presented in Figure 39 to Figure 70.

Protein Standard	Ionisation Pattern	Ionisation Charge Number	Deconvoluted mass	Literature Mass
α-Lac	1773.3	+ 8	14179	14172.5
	1576.5	+ 9		
	1419.0	+ 10		
	1290.0	+ 11		
Amyloglucosidase	N/A	N/A	N/A	78-80000
β-Lac-A	1837.4	+ 10	18364	18367
	1670.4	+ 11		
	1531.3	+ 12		
β-Lac-B	1828.4	+ 10	18275	18281
	1662.3	+ 11		
	1523.9	+ 12		
	1406.8	+ 13		
	1306.4	+ 14		
	1219.4	+ 15		
BSA	1480.3	+ 45	66434	66432
	1417.3	+ 47	66554	
	1387.6	+ 48	65403	
	1331.8	+ 50		
	1256.5	+ 53		
	1234.4	+ 54		
Carbonic Anhydrase II	1439.9	+ 20	28778	29000
	1371.4	+ 21		
	1309.1	+ 22		
	1252.1	+ 23		
	1200.0	+ 24		
	1152.2	+ 25		
	1107.8	+ 26		
	1066.8	+ 27		

Table 16 continued

Protein Standard	Ionisation Pattern	Ionisation Charge Number	Deconvoluted mass	Literature Mass
Glycomacropeptide (A and B variants) + phosphate content (P)	1697.6	+ 4	6754	6780.3 (A) + 1P
	1362.6	+ 5	6786	6867.4 (A) + 2P
	973.1	+ 6	6801	6754.4 (B) + 1P
	849.2	+8	6867	
ImmunoglobulinG	N/A	N/A	N/A	N/A
Lactoferrin	1845.4	+ 45	83015	83100
	1761.7	+ 47	84772	
	1695.0	+ 49	81420	
	1629.7	+ 51	79482	
	1537.6	+ 54		
Lactoferrin Deglycosylated	1769.0	+ 46	81352	74000
	1661.1	+ 49	83272	
	1596.2	+ 51	79709	
	1536.1	+ 53	77932	
	1480.2	+ 55		
Lactoperoxidase	1313.6	+ 60	78755	77500
	1292.1	+ 61		
	1211.7	+ 65		
	1159.2	+ 68		
	1094.6	+ 72		
Myoglobin	1695.9	+ 10	16950	16951
	1542.0	+ 11		
	1413.5	+ 12		
	1304.8	+ 13		
	1211.5	+ 14		
	1130.9	+ 15		
	1060.4	+ 16		
	998.0	+ 17		
Proteose Peptone 5	1746.3	+ 7	12216	12177.3
	1527.6	+ 8	12239	12442.6
	1358.3	+ 9	12174	12482.6
	1222.4	+ 10	12482	

Table 16 continued

Protein Standard	Ionisation Pattern	Ionisation Charge Number	Deconvoluted mass	Literature Mass
Ribonuclease	1955.3	+ 7	13683	13700
	1711.2	+ 8	13781	
	1521.3	+ 9		
	1369.3	+ 10		
Trypsin Inhibitor	1665.7	+ 12	19973	20100
	1537.4	+ 13	20082	
	1427.5	+ 14		
	1332.7	+ 15		
	1249.3	+ 16		
	1175.6	+ 17		
	1110.5	+ 18		
Trypsinogen	1845.6	+ 13	23979	23700
	1713.9	+ 14		
	1599.6	+ 15		
	1499.6	+ 16		

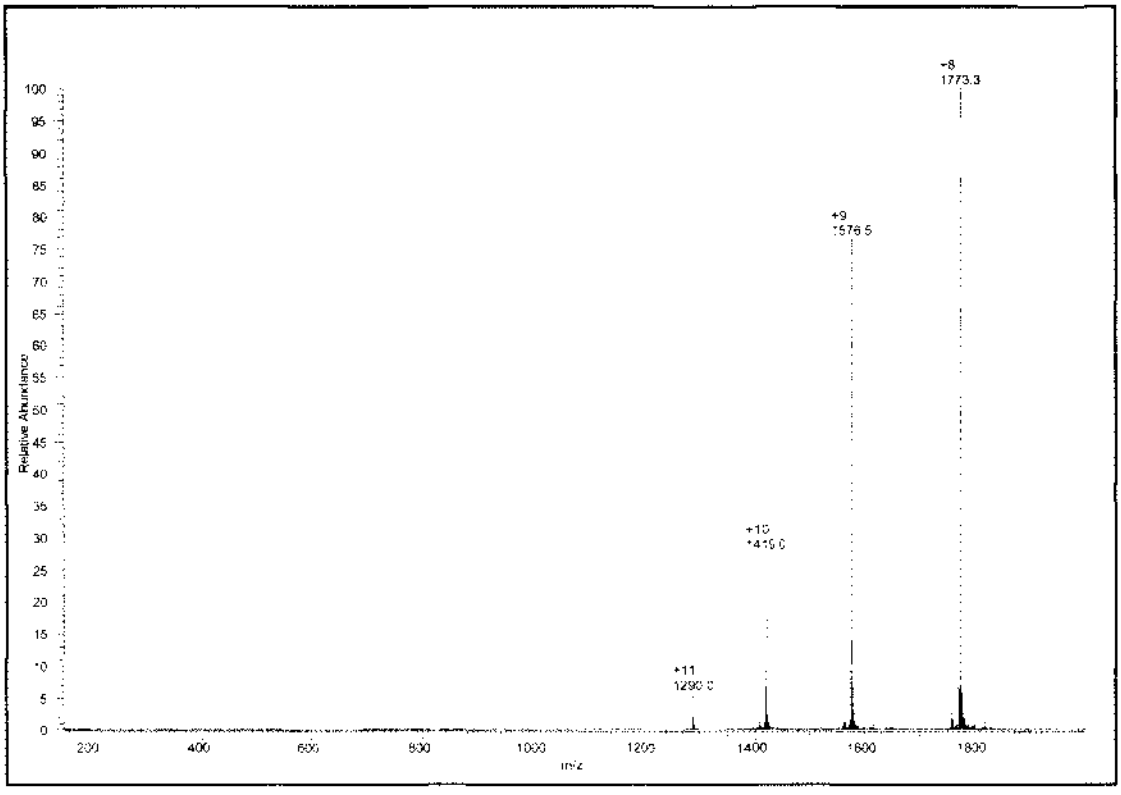

Figure 39 Results of α-Lac standard infused into MS

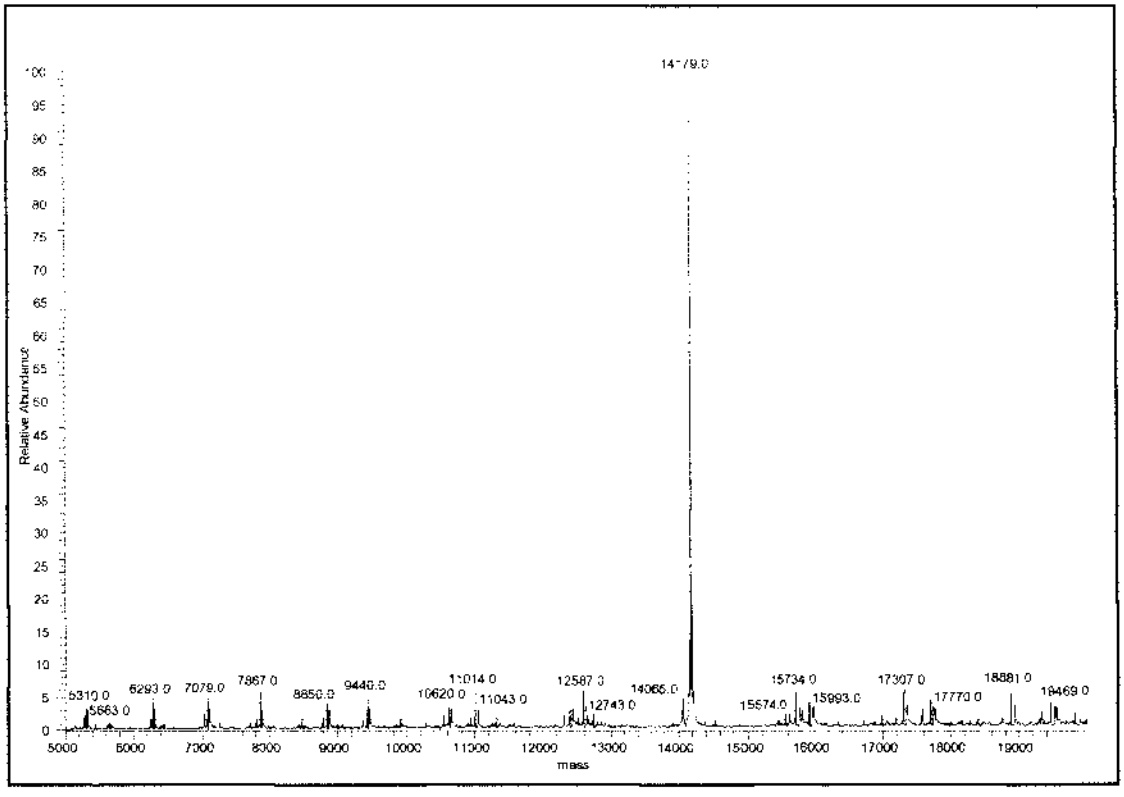

Figure 40 Results of deconvolution of α-Lac

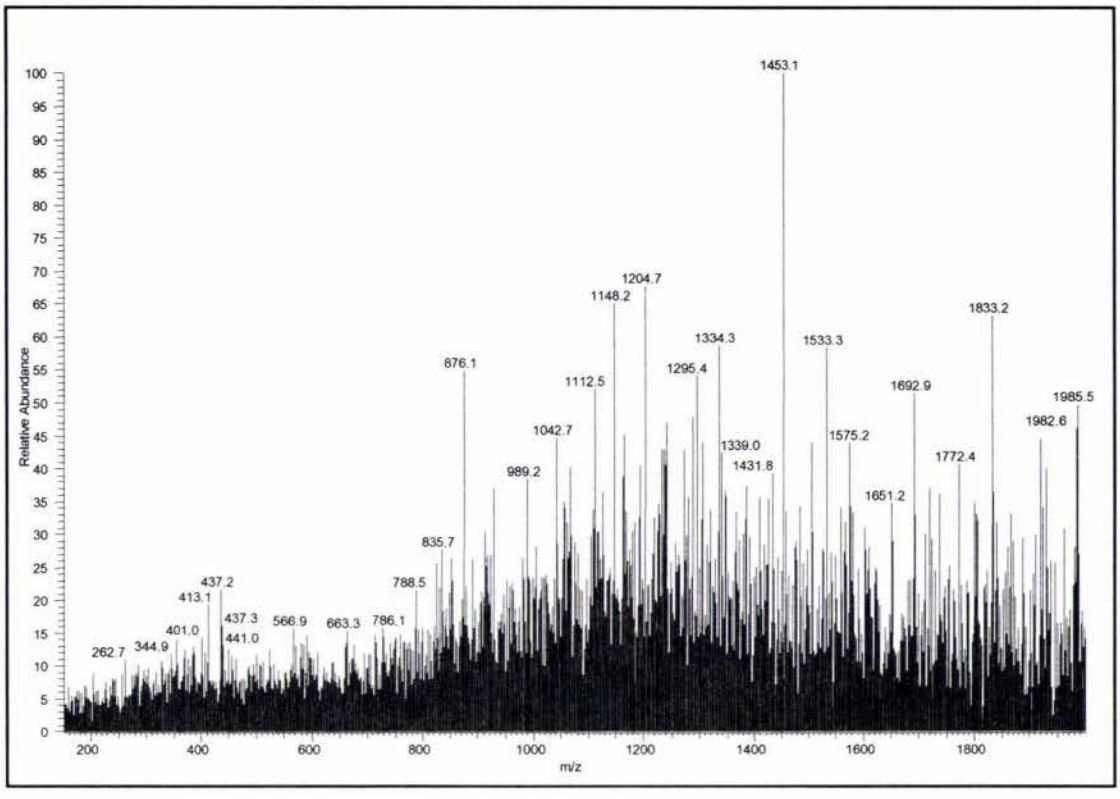

Figure 41 Results of amyloglucosidase standard infused into MS

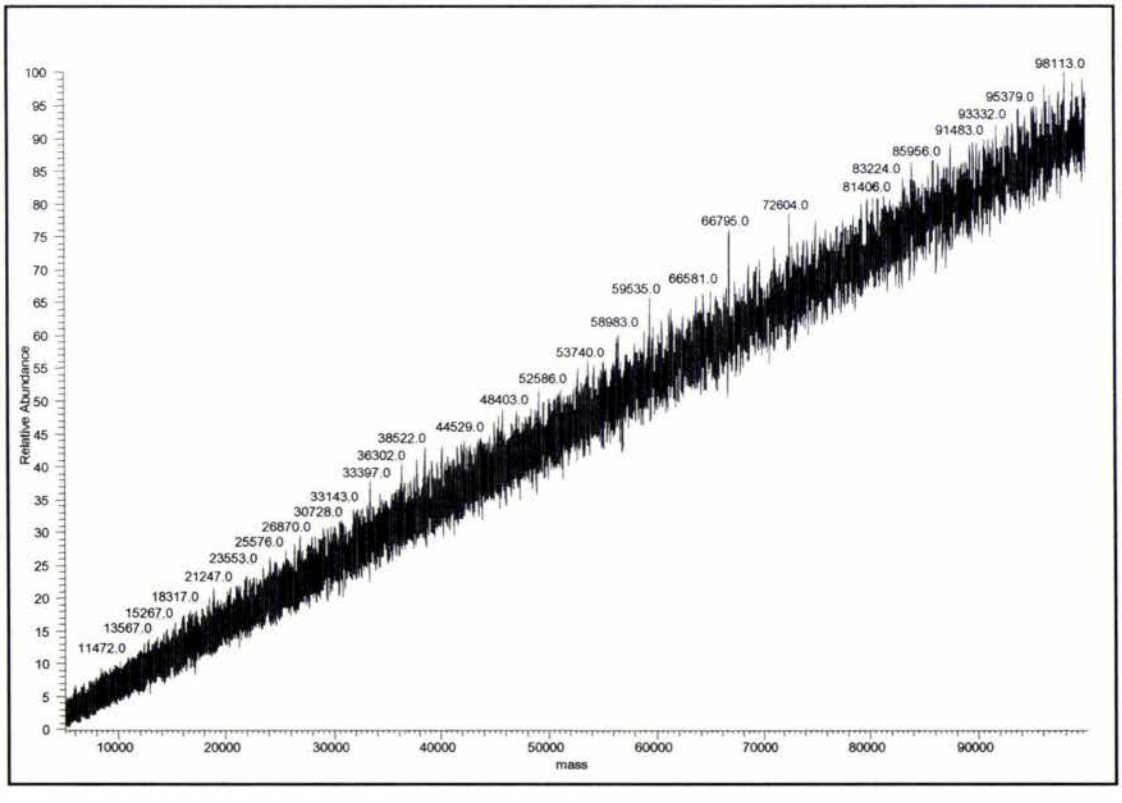

Figure 42 Results of deconvolution of amyloglucosidase

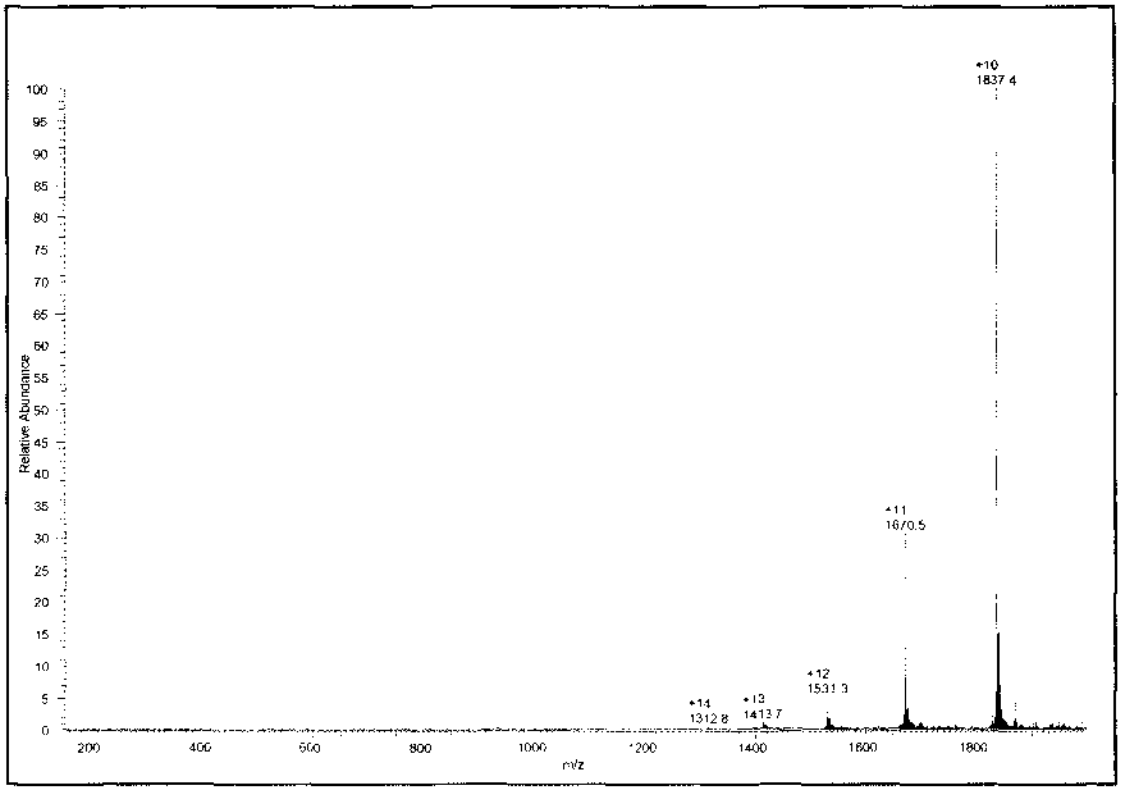

Figure 43 Results of β-Lac-A standard infused into MS

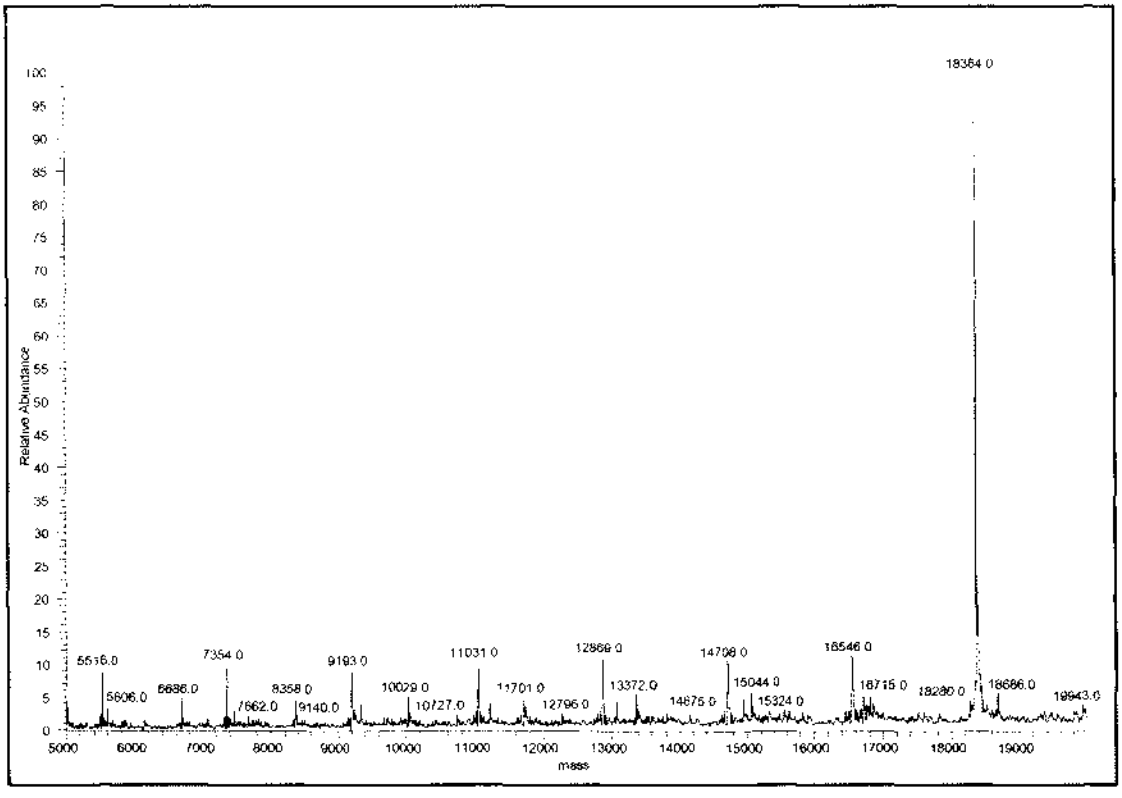

Figure 44 Results of deconvolution of β-Lac-A

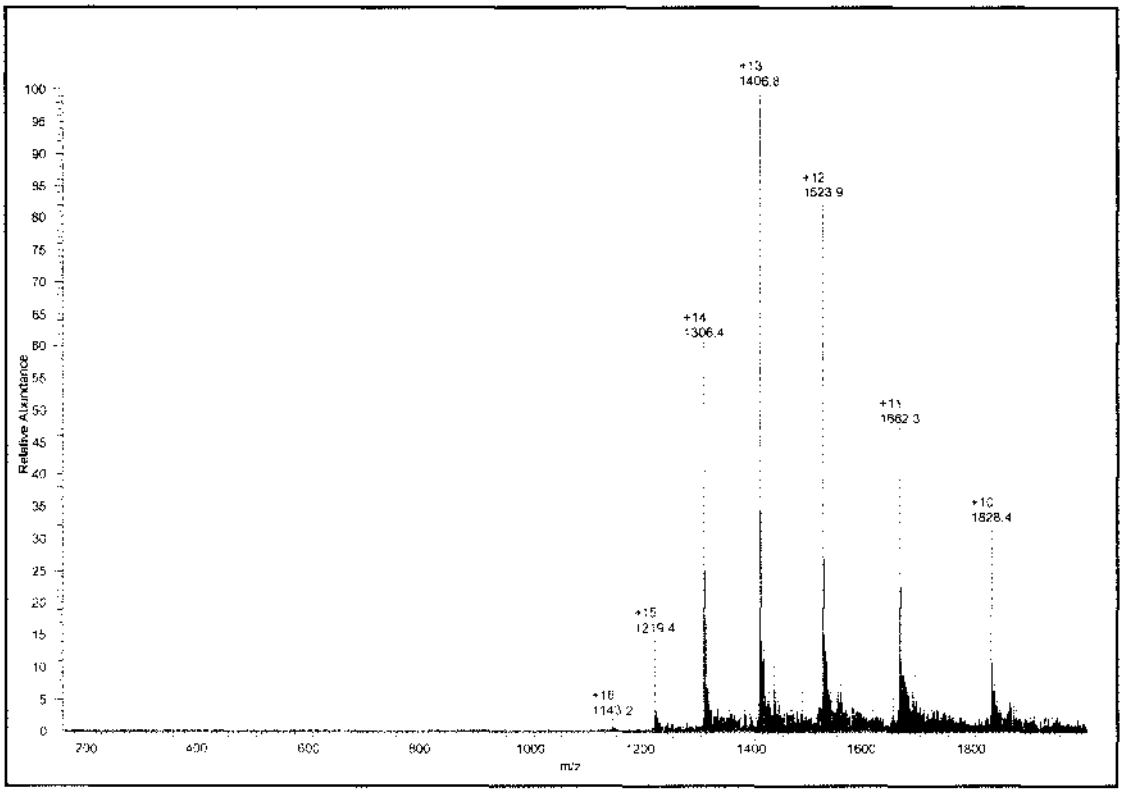

Figure 45 Results of β-Lac-B standard infused into MS

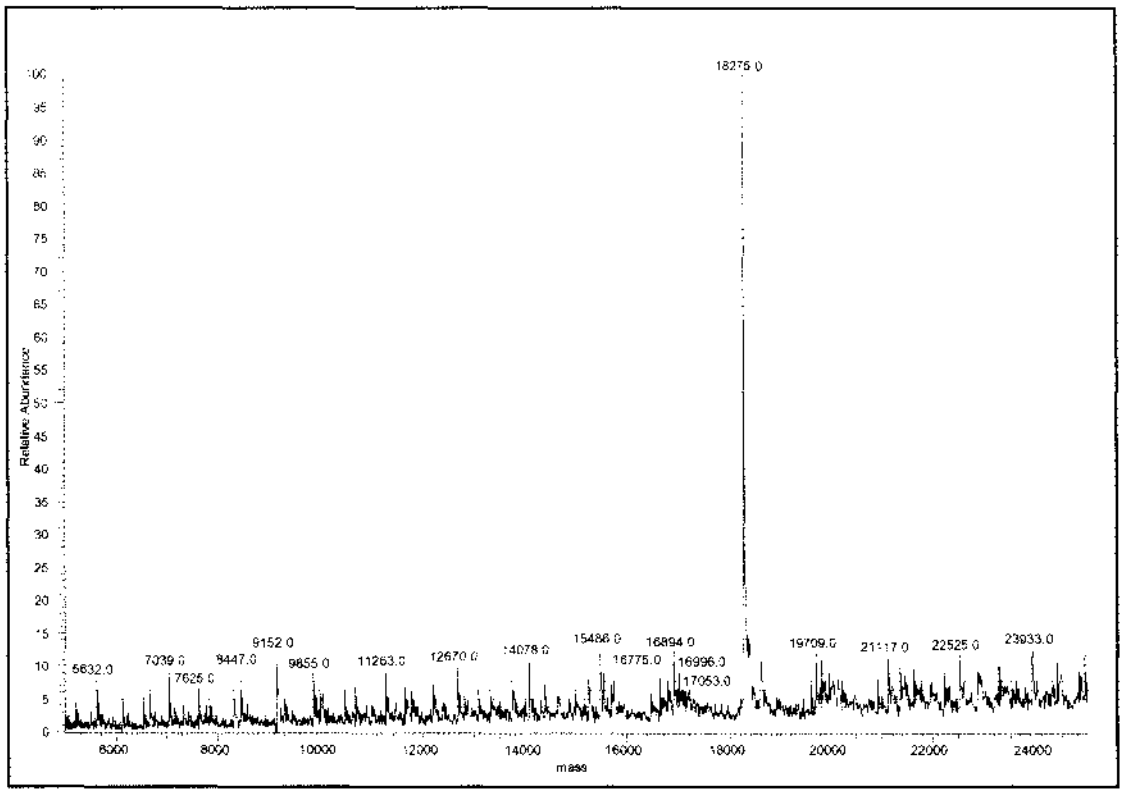

Figure 46 Results of deconvolution of β-Lac-B

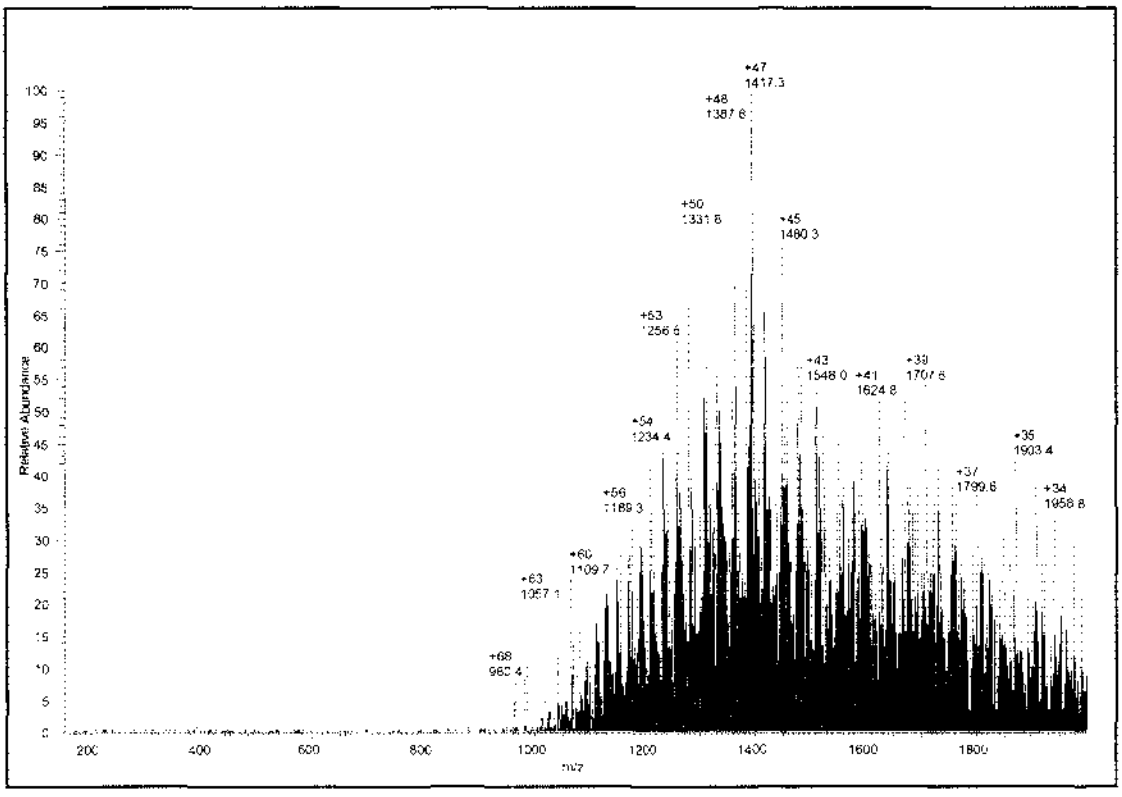

Figure 47 Results of BSA standard infused into MS

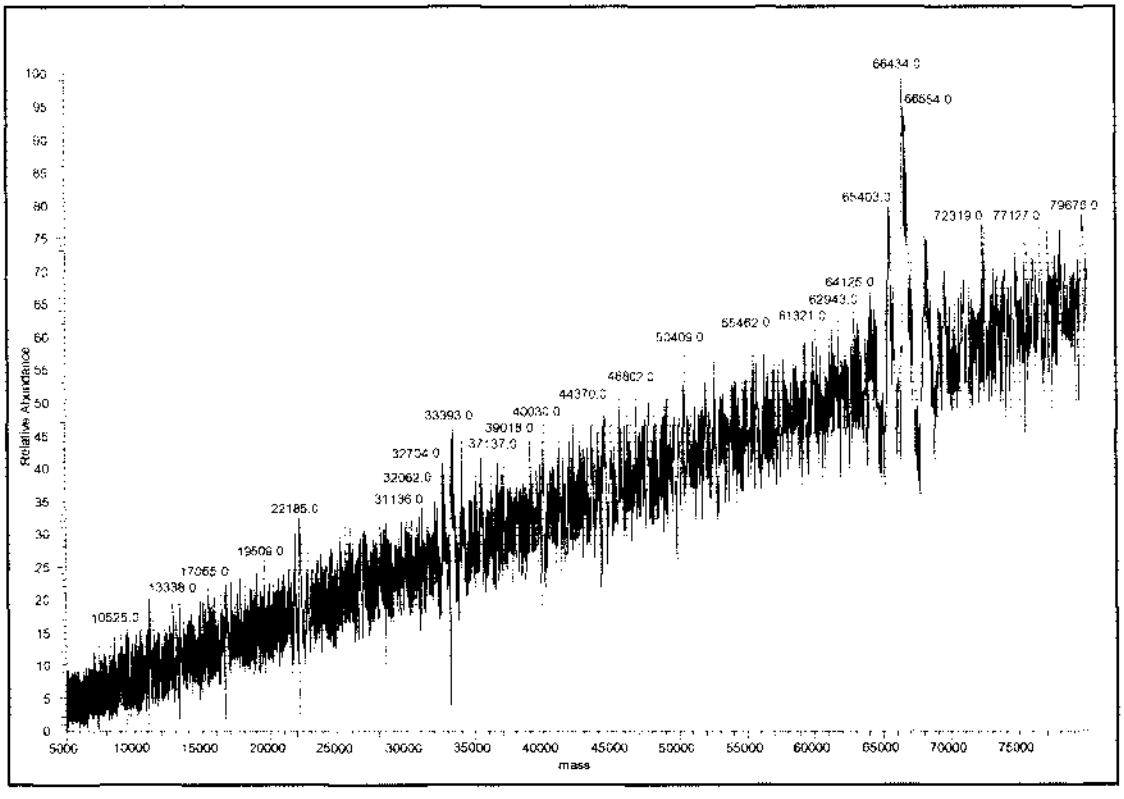

Figure 48 Results of deconvolution of BSA

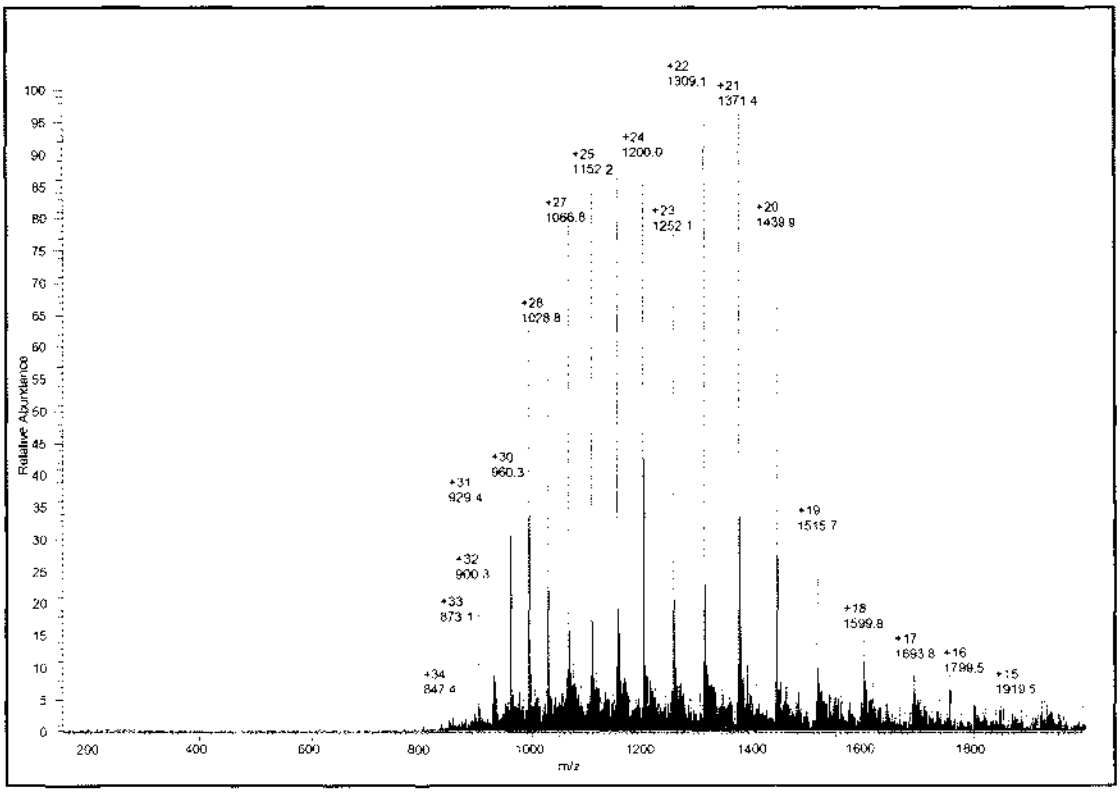

Figure 49 Results of carbonic anhydrase standard infused into MS

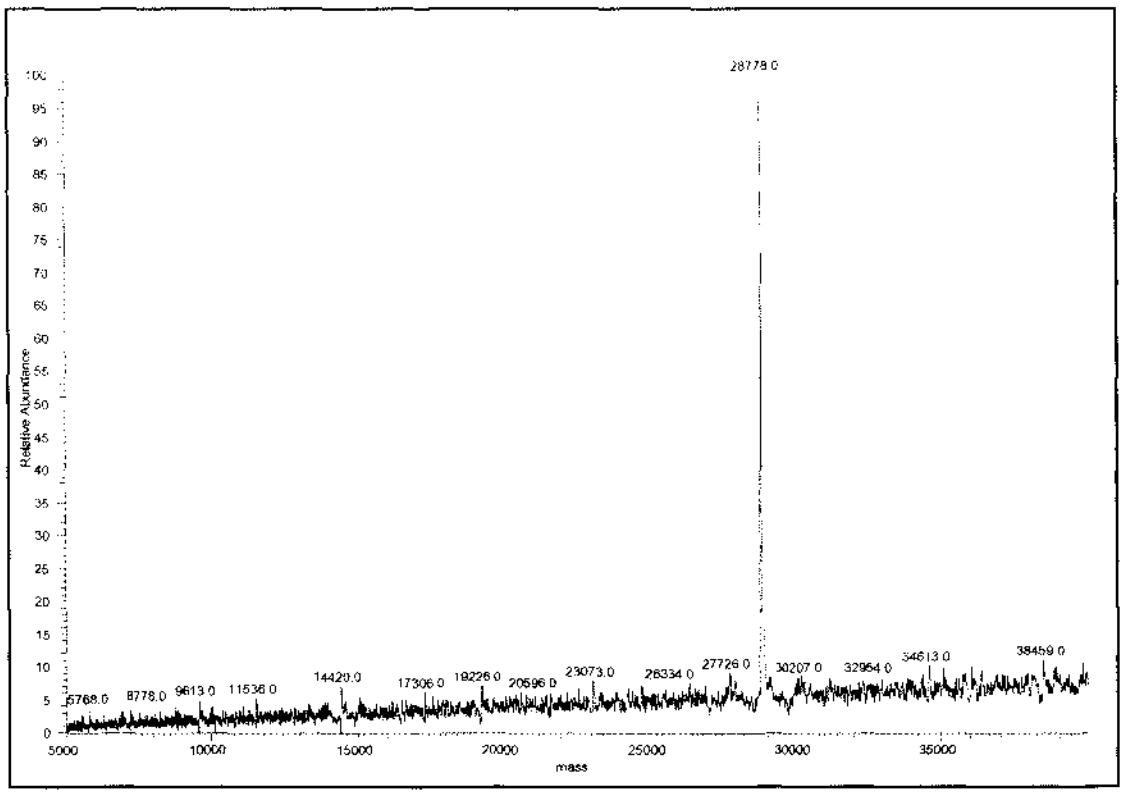

Figure 50 Results of deconvolution of carbonic anhydrase

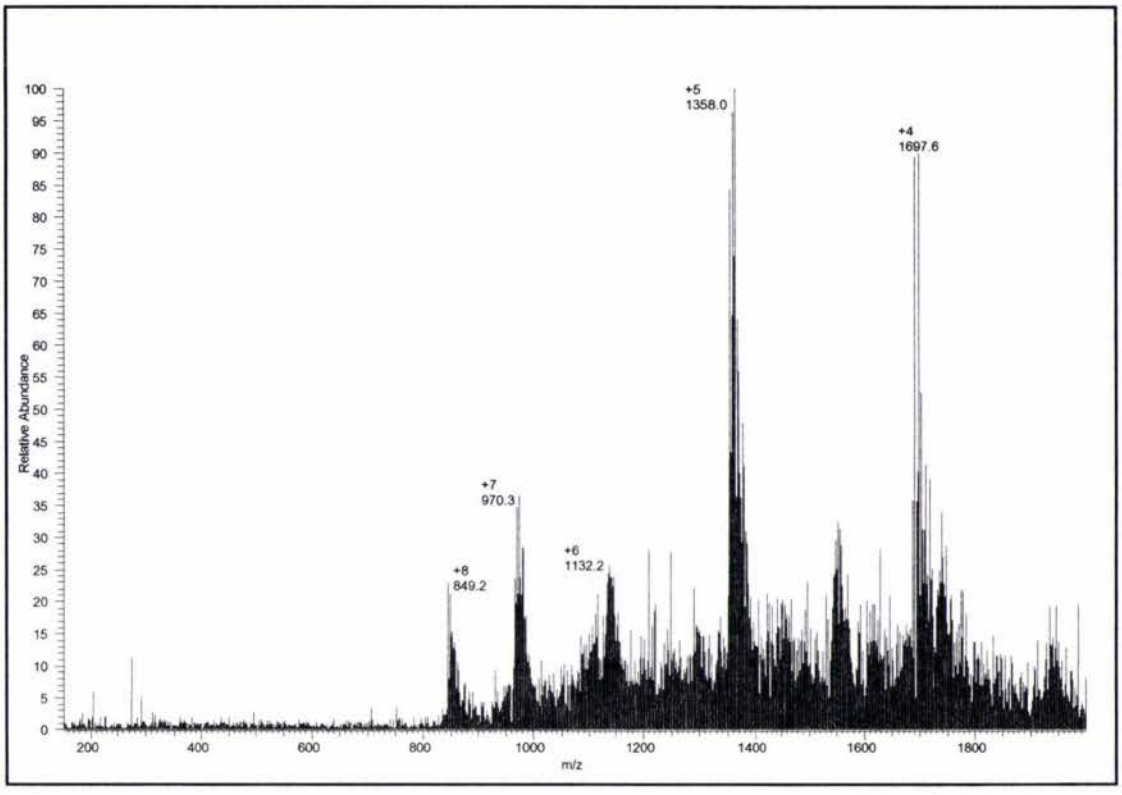

Figure 51 Results of GMP standard infused into MS

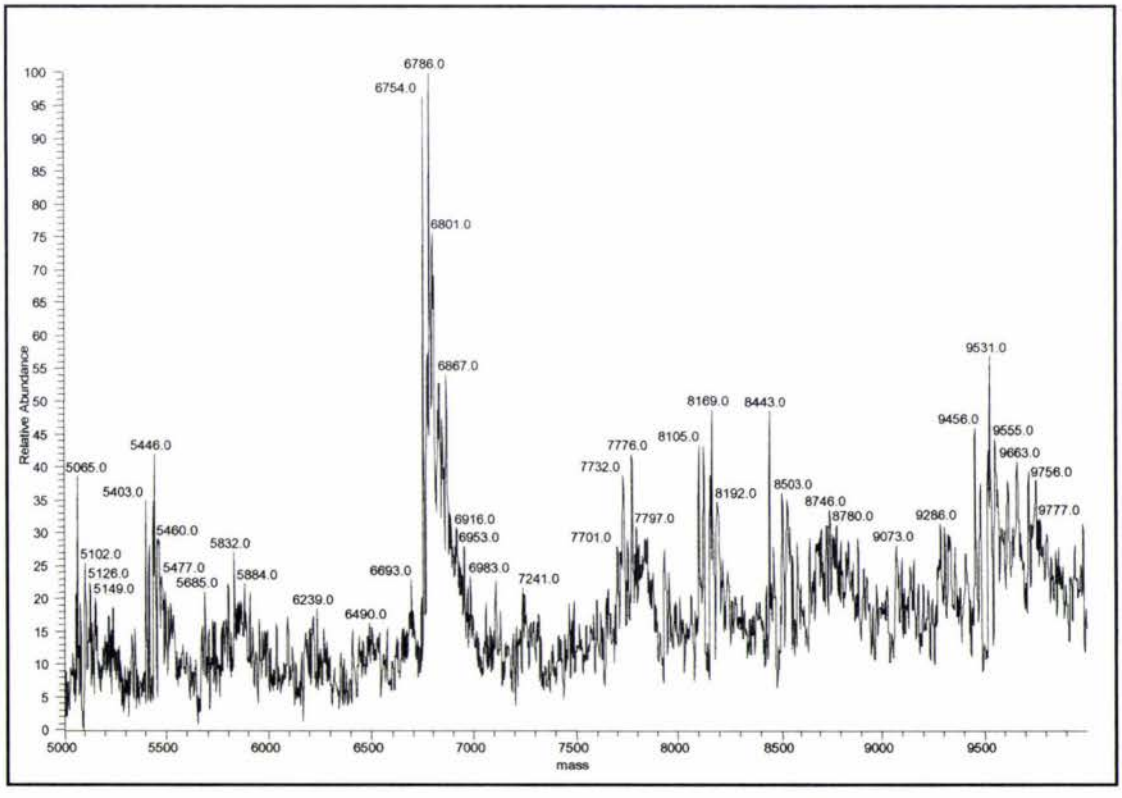

Figure 52 Results of deconvolution of GMP

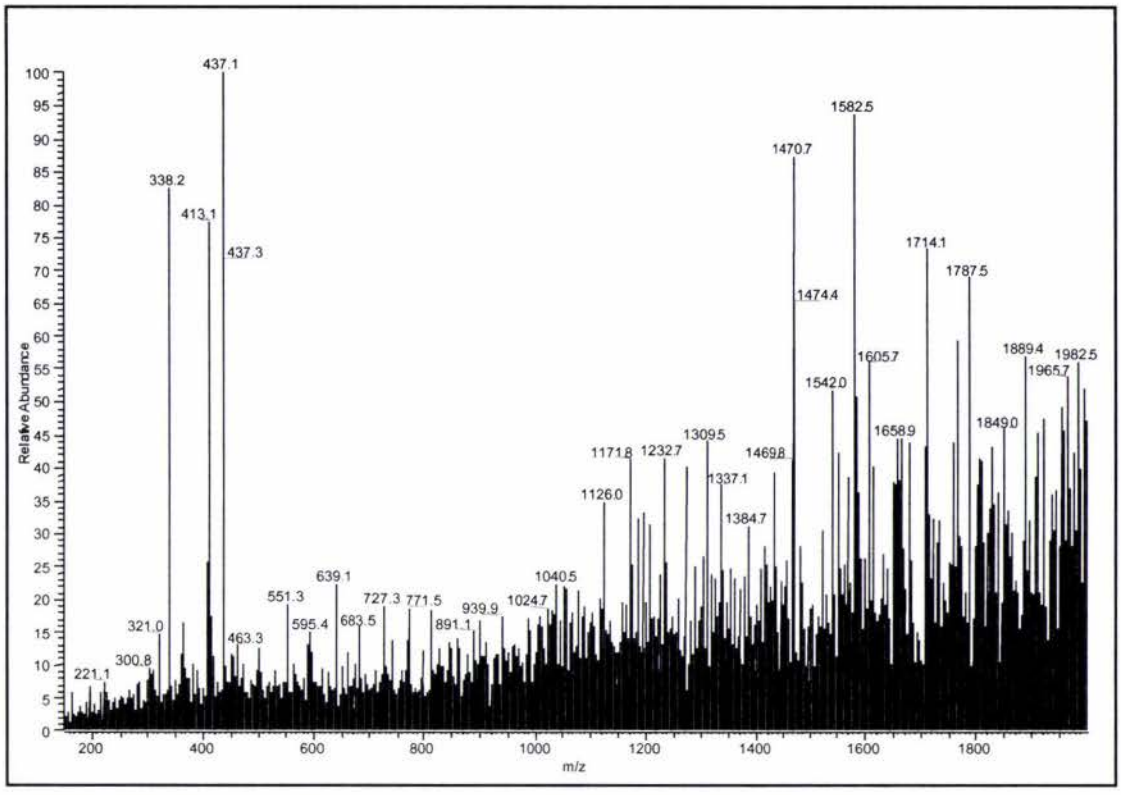

Figure 53 Results of IgG standard infused into MS

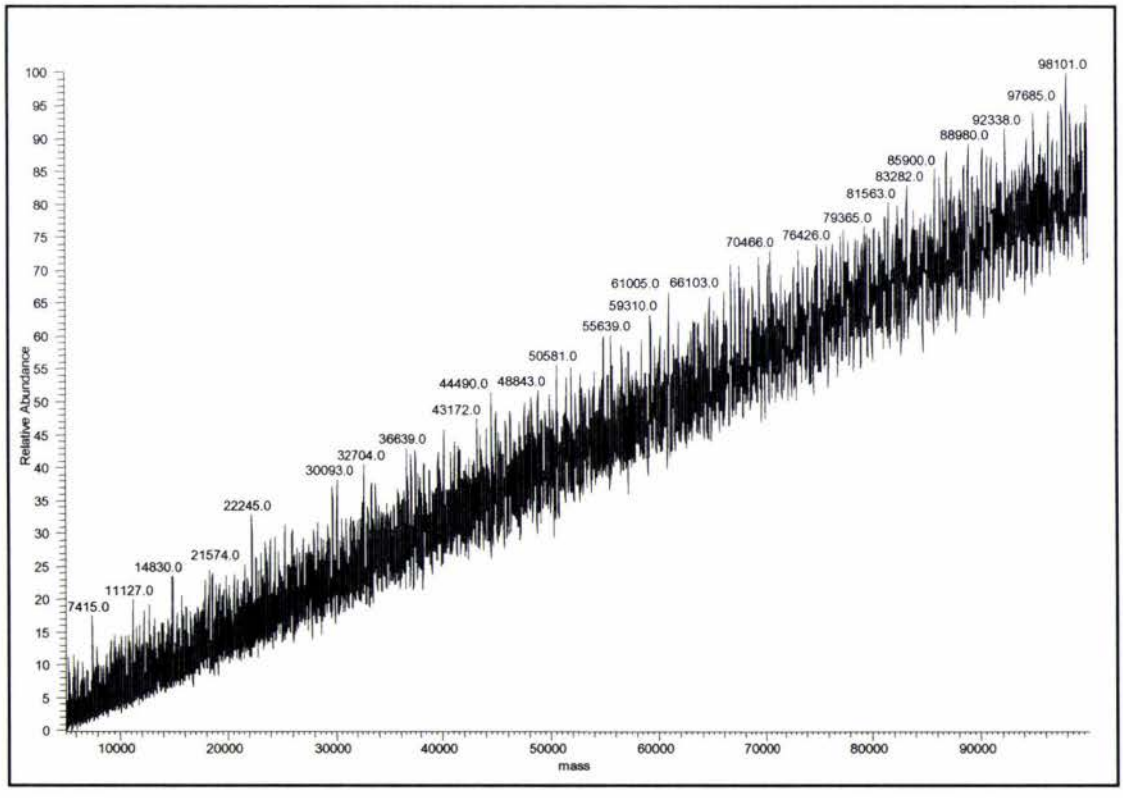

Figure 54 Results of deconvolution of IgG

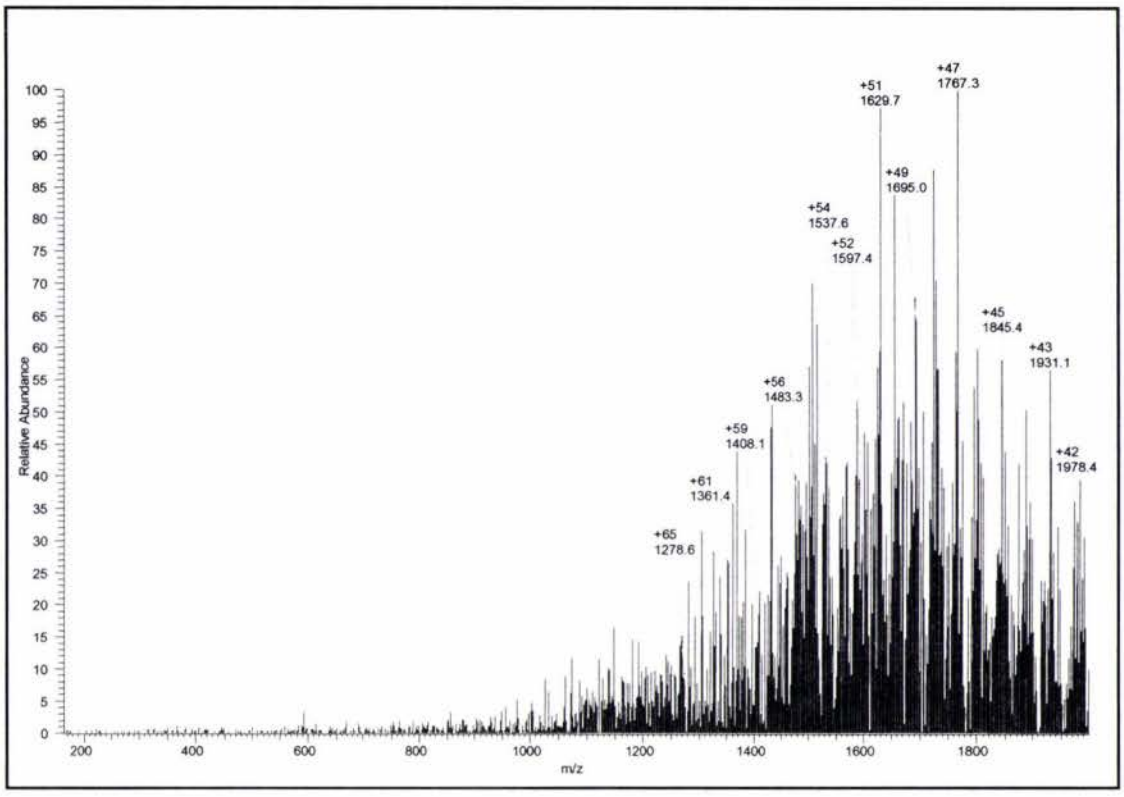

Figure 55 Results of lactoferrin standard infused into MS

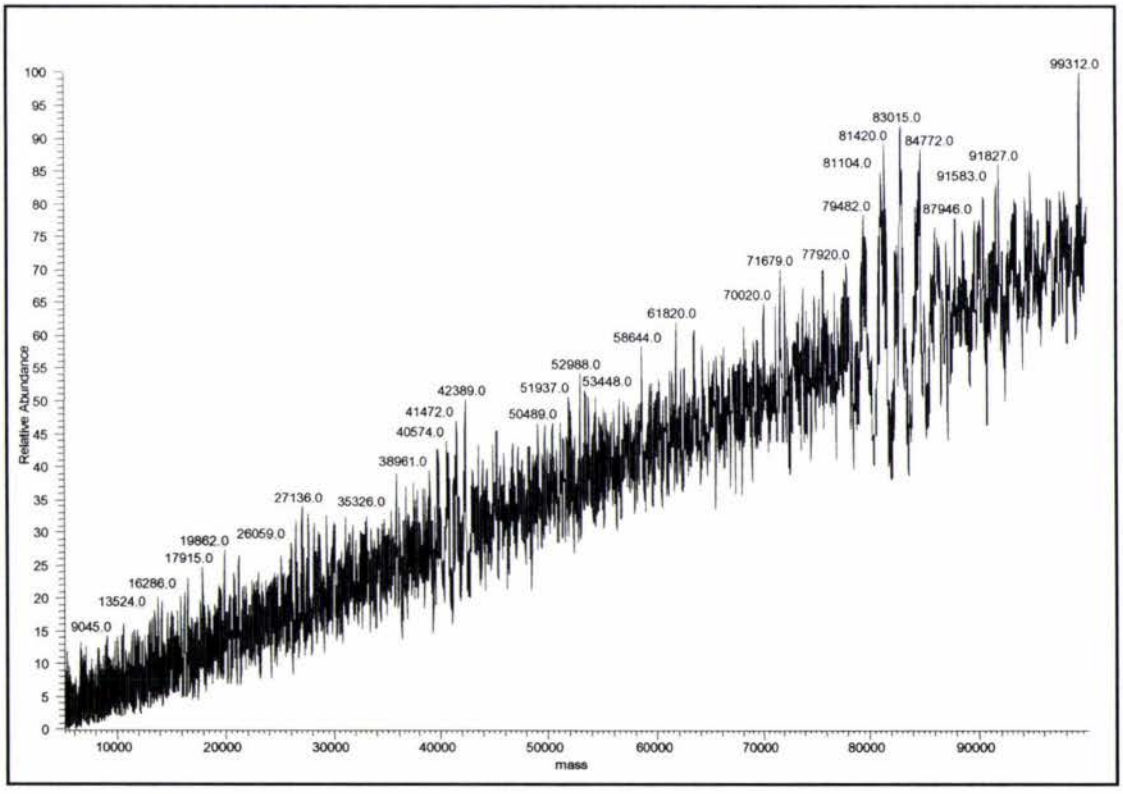

Figure 56 Results of deconvolution of lactoferrin

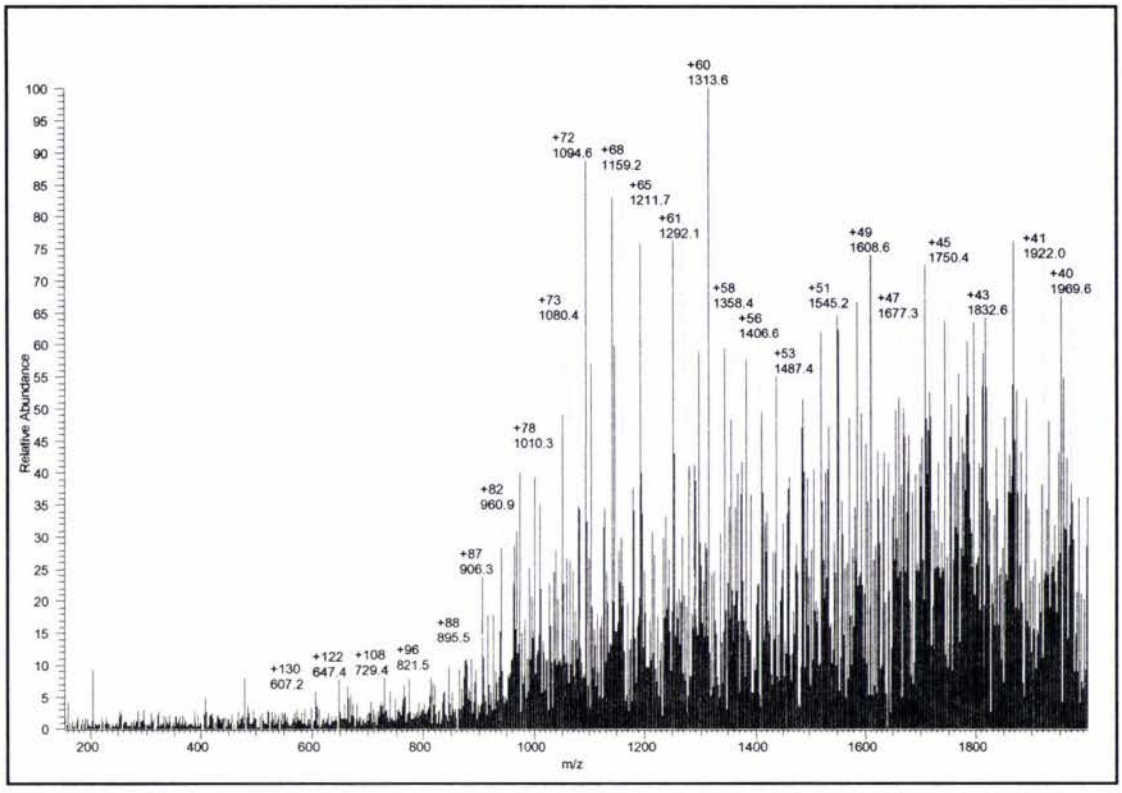

Figure 57 Results of lactoperoxidase standard infused into MS

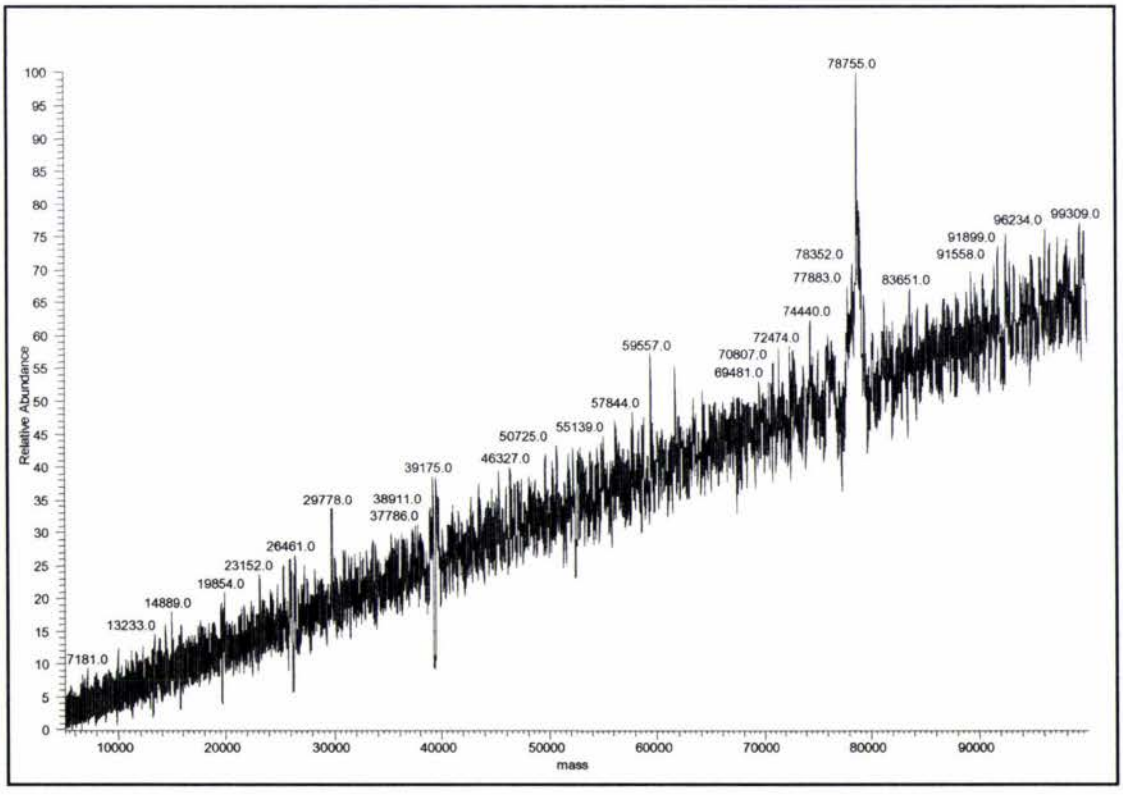

Figure 58 Results of deconvolution of lactoperoxidase

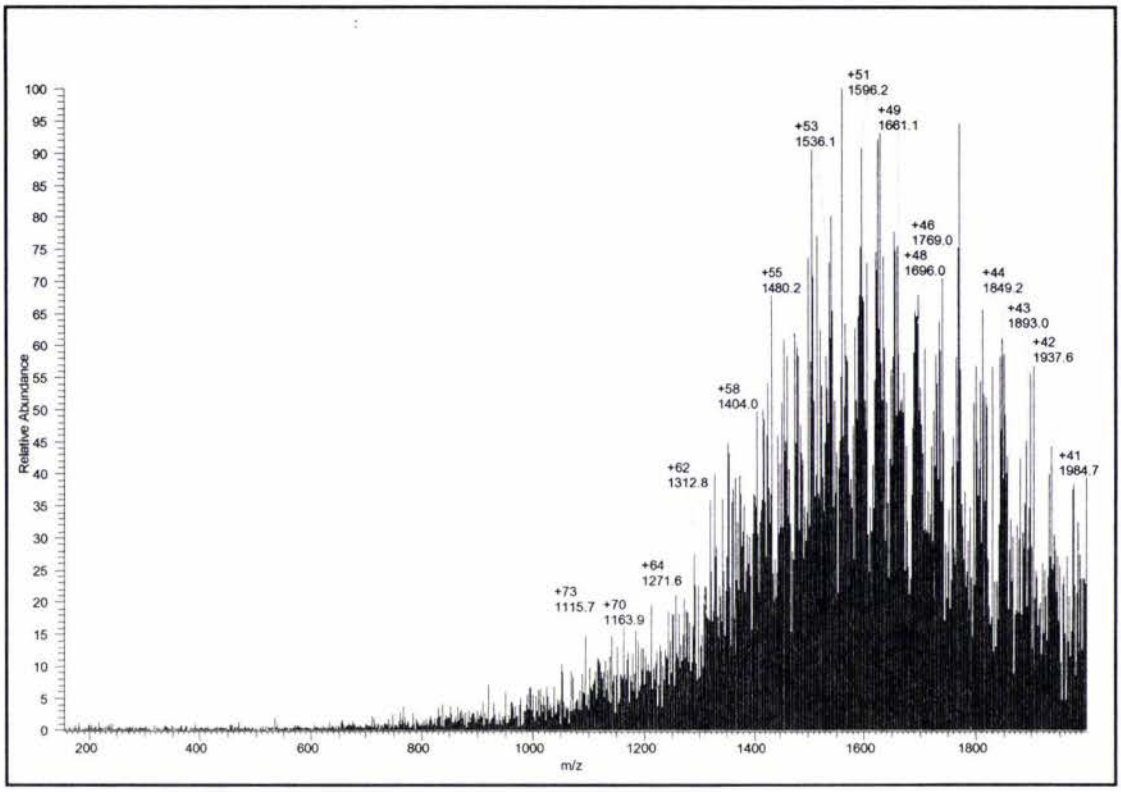

Figure 59 Results of lactoferrin deglycosylated infused into MS

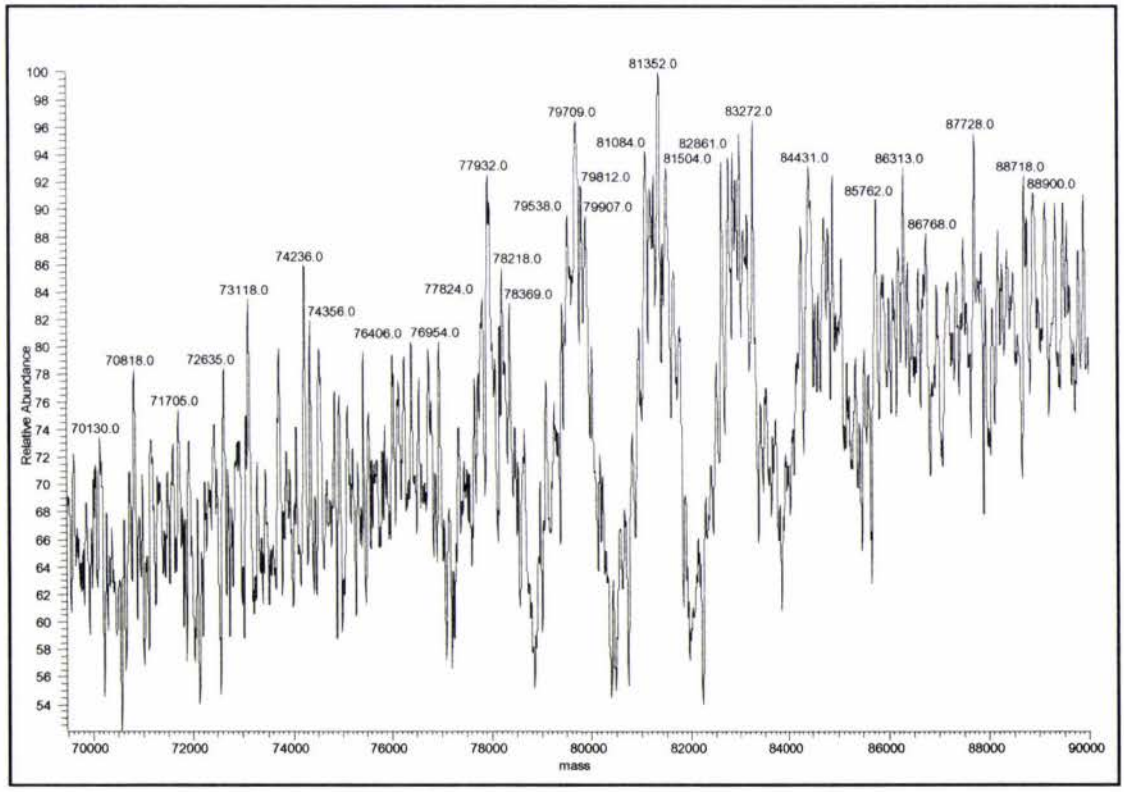

Figure 60 Results of deconvolution of deglycosylated lactoferrin

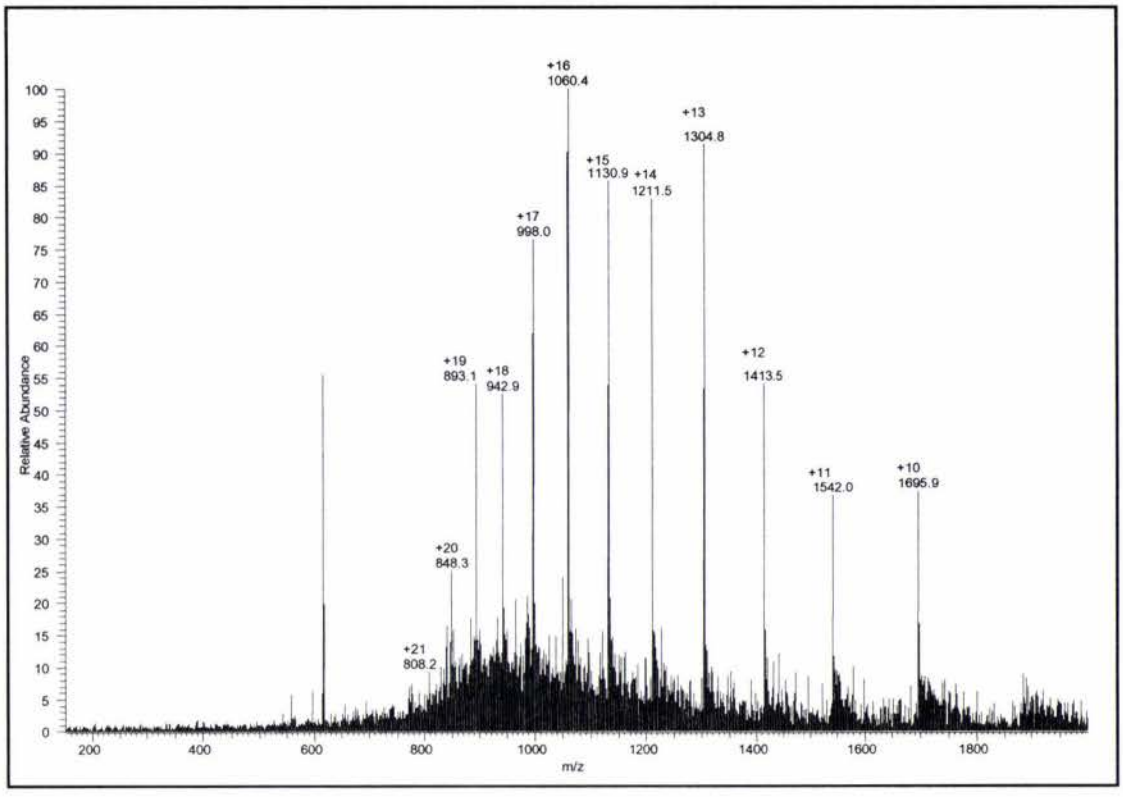

Figure 61 Results of myoglobin standard infused into MS

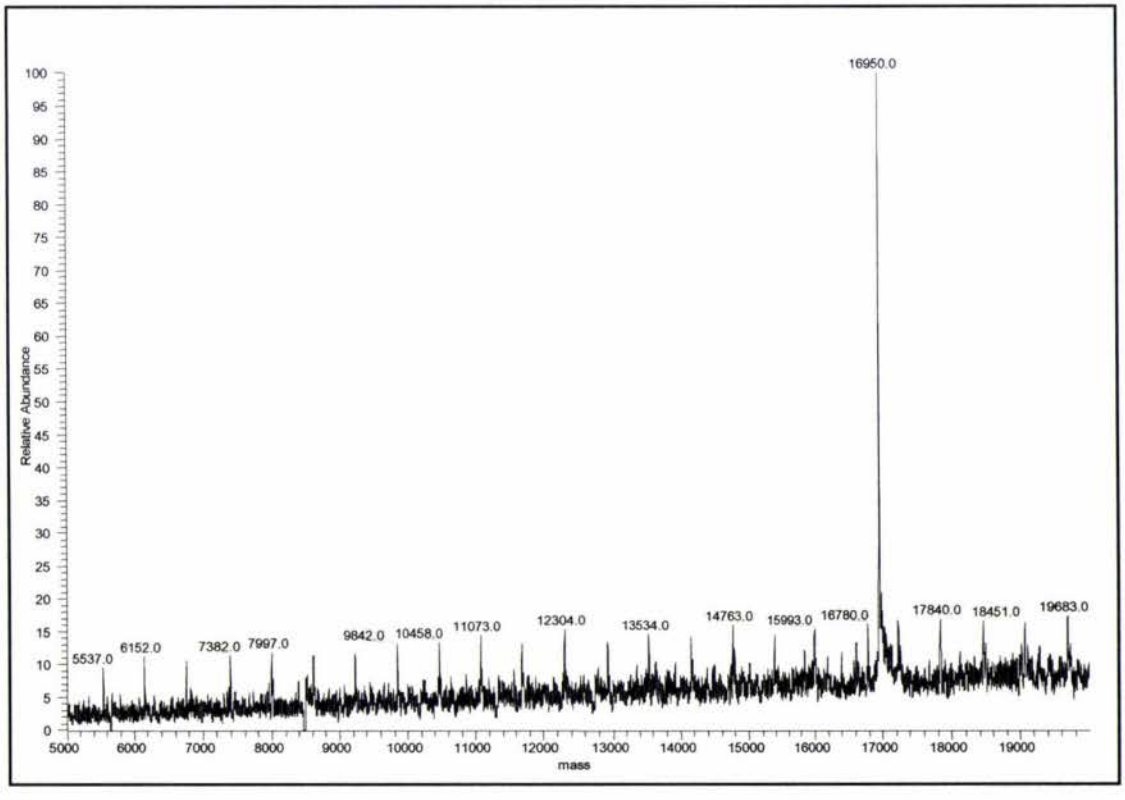

Figure 62 Results of deconvolution of myoglobin

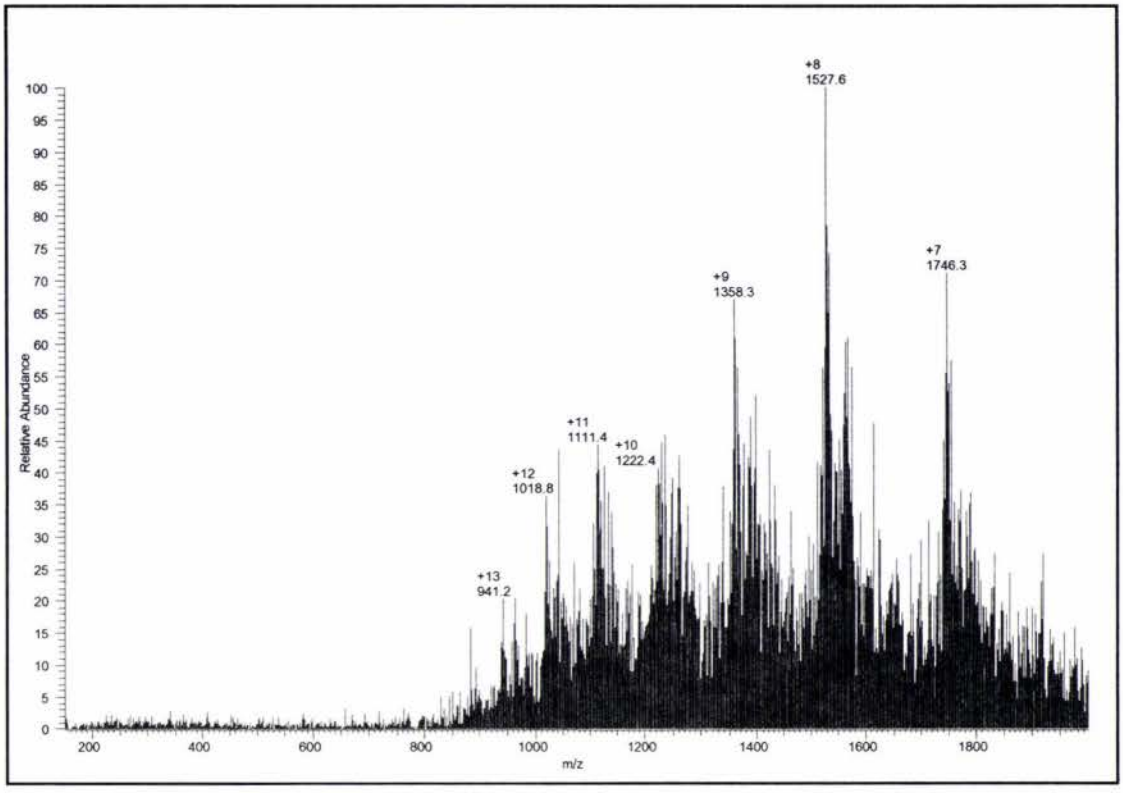

Figure 63 Results of PP5 standard infused into MS

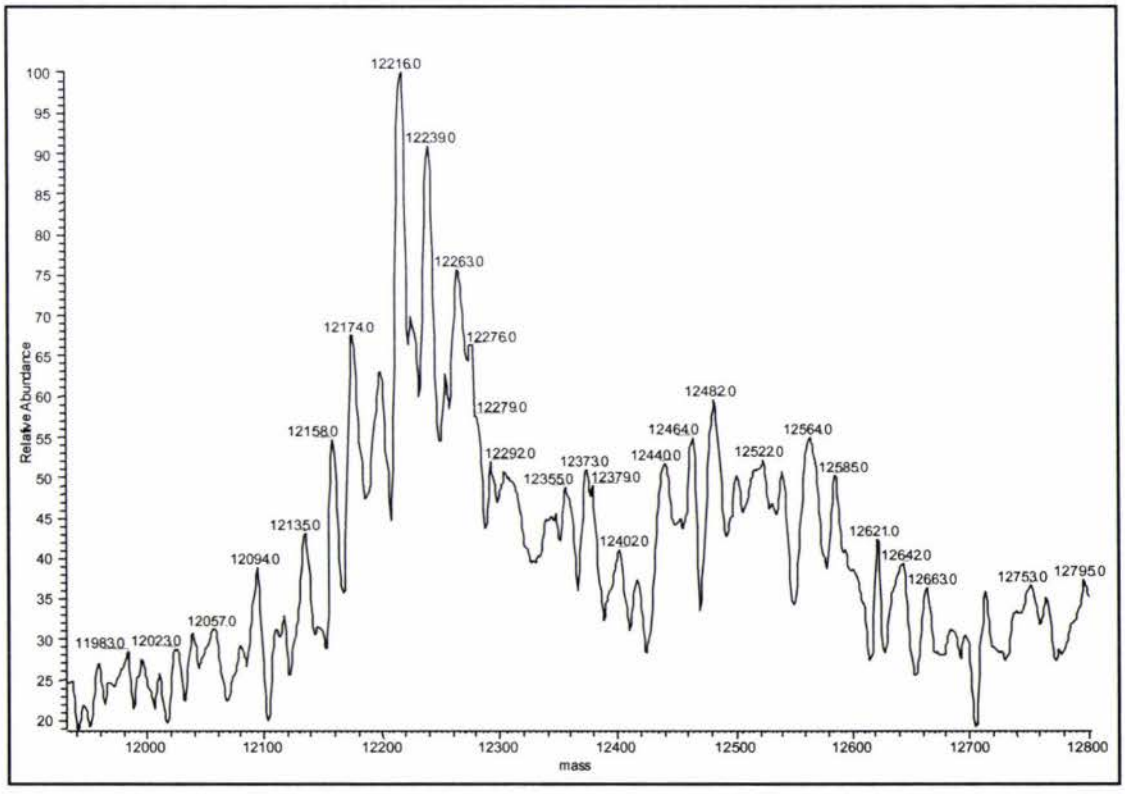

Figure 64 Results of deconvolution of PP5

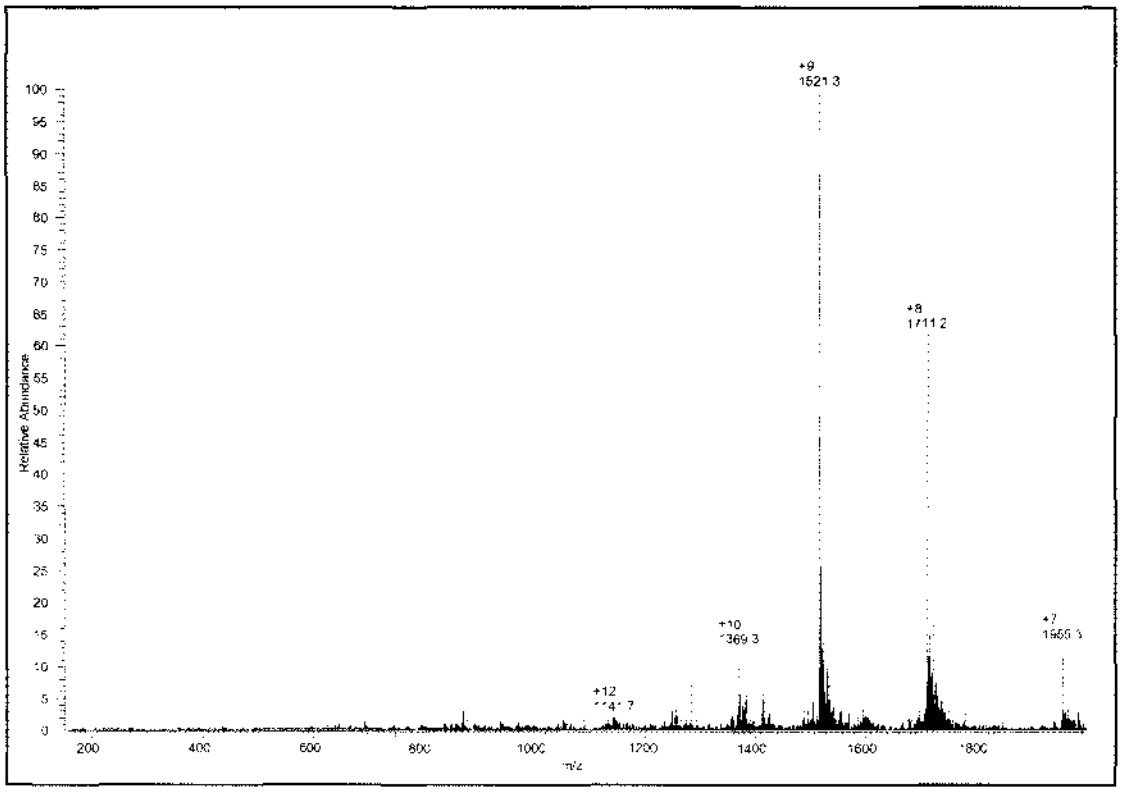

Figure 65 Results of ribonuclease standard infused into MS

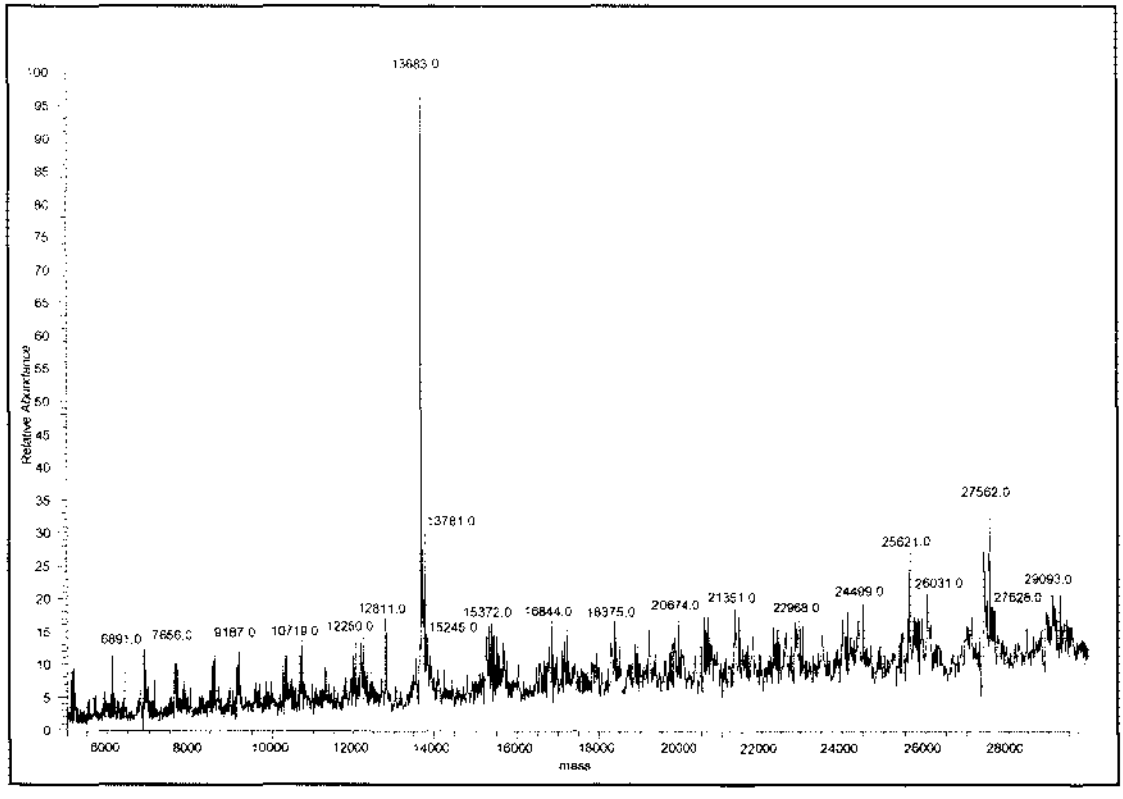

Figure 66 Results of deconvolution of Ribonuclease

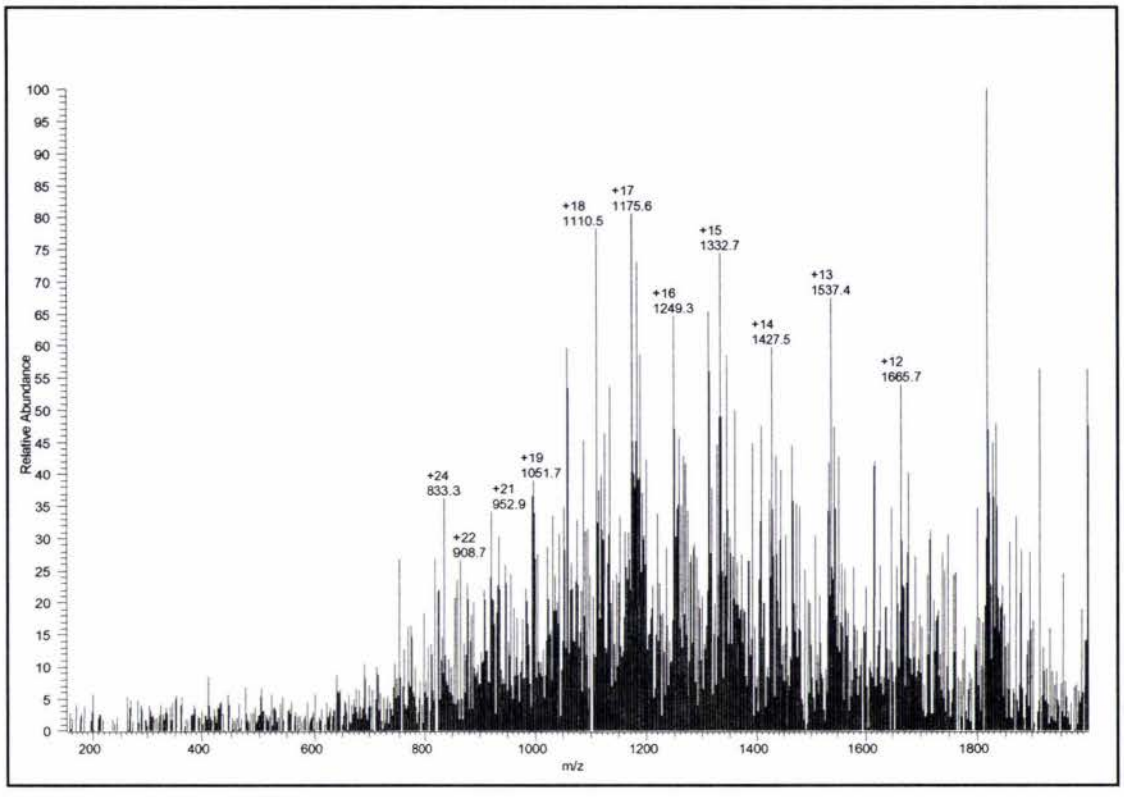

Figure 67 Results of trypsin inhibitor standard infused into MS

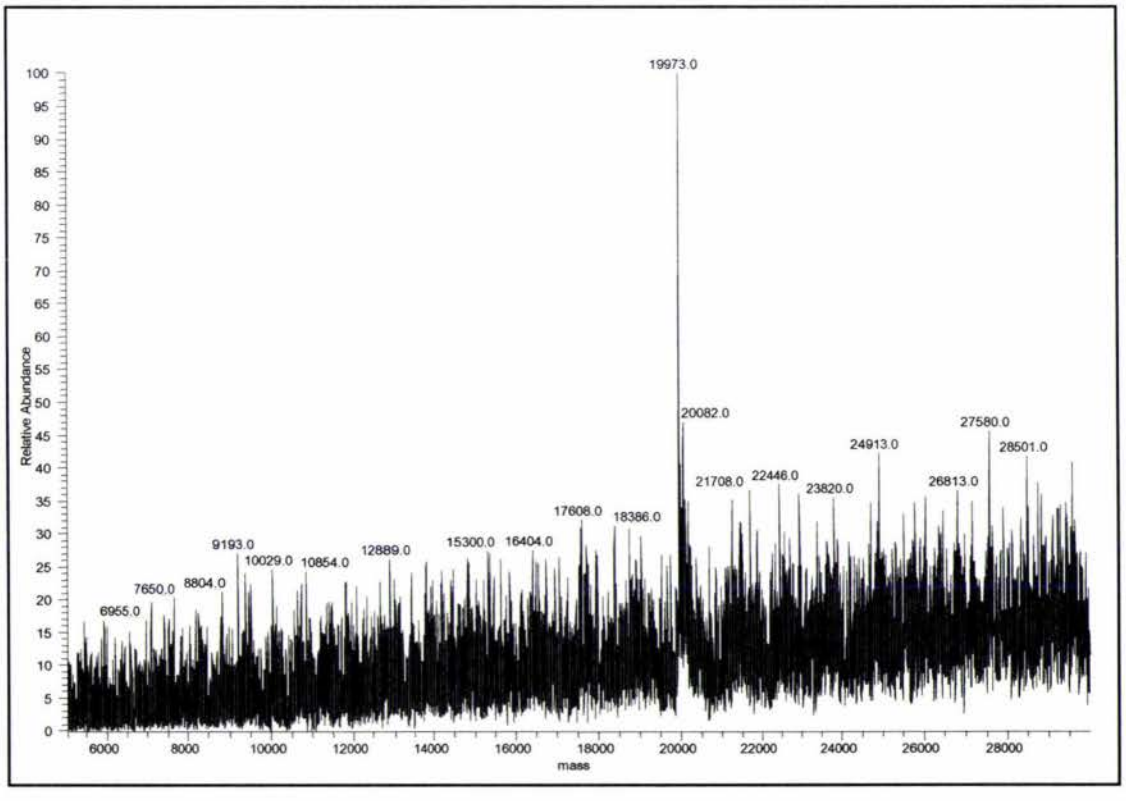

Figure 68 Results of deconvolution of trypsin inhibitor

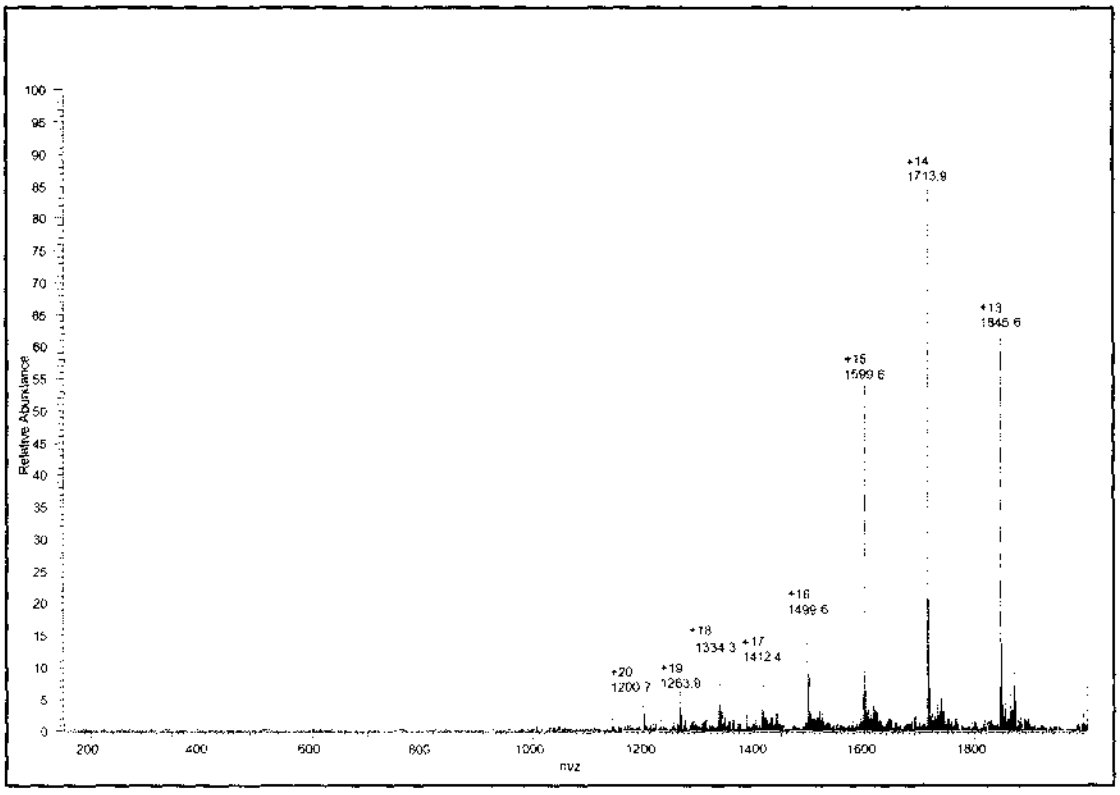

Figure 69 Results of trypsinogen standard infused into MS

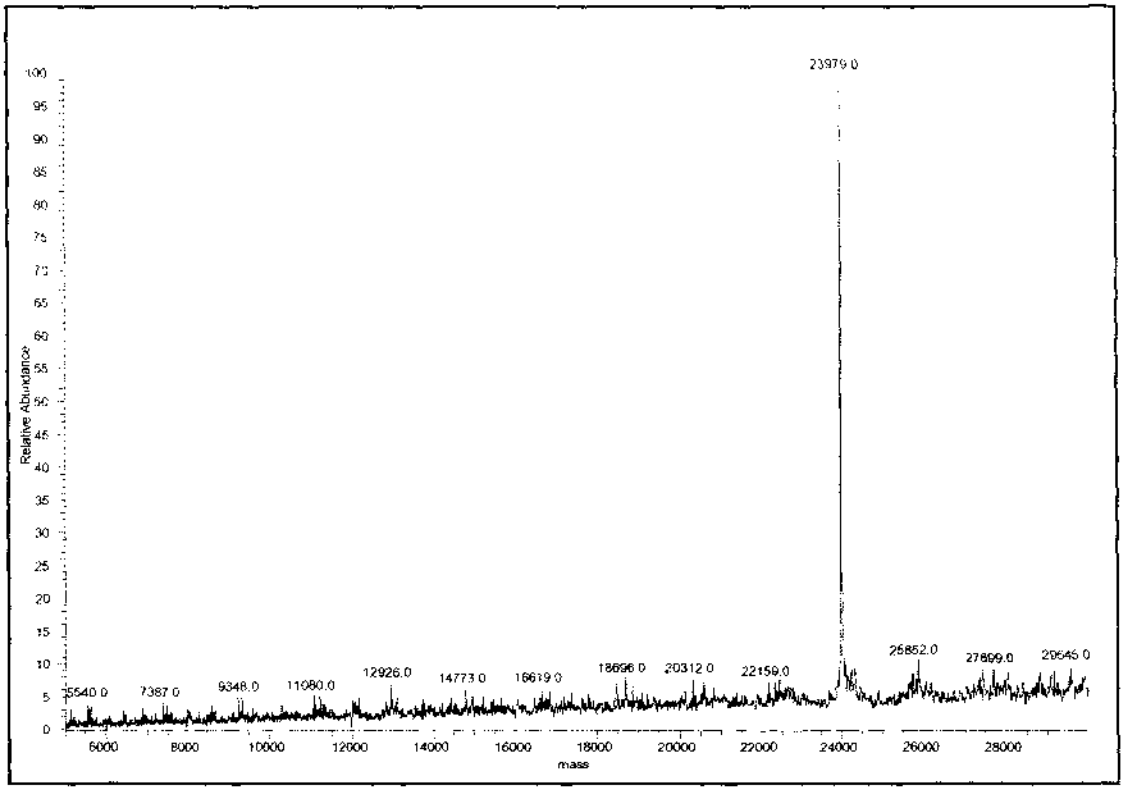

Figure 70 Results of deconvolution of trypsinsinogen

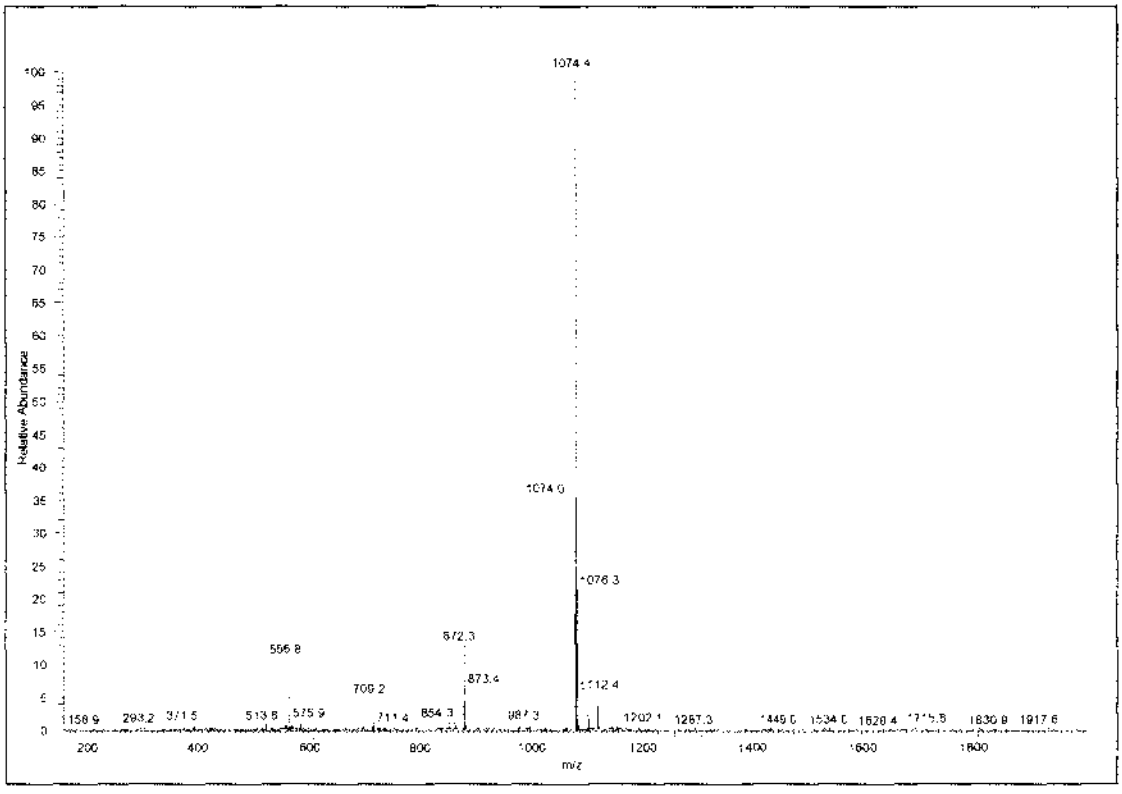

Figure 71 Results of CCK Peptide standard infused into MS

Appendix 3 Results of MS infusion of basic protein fraction samples

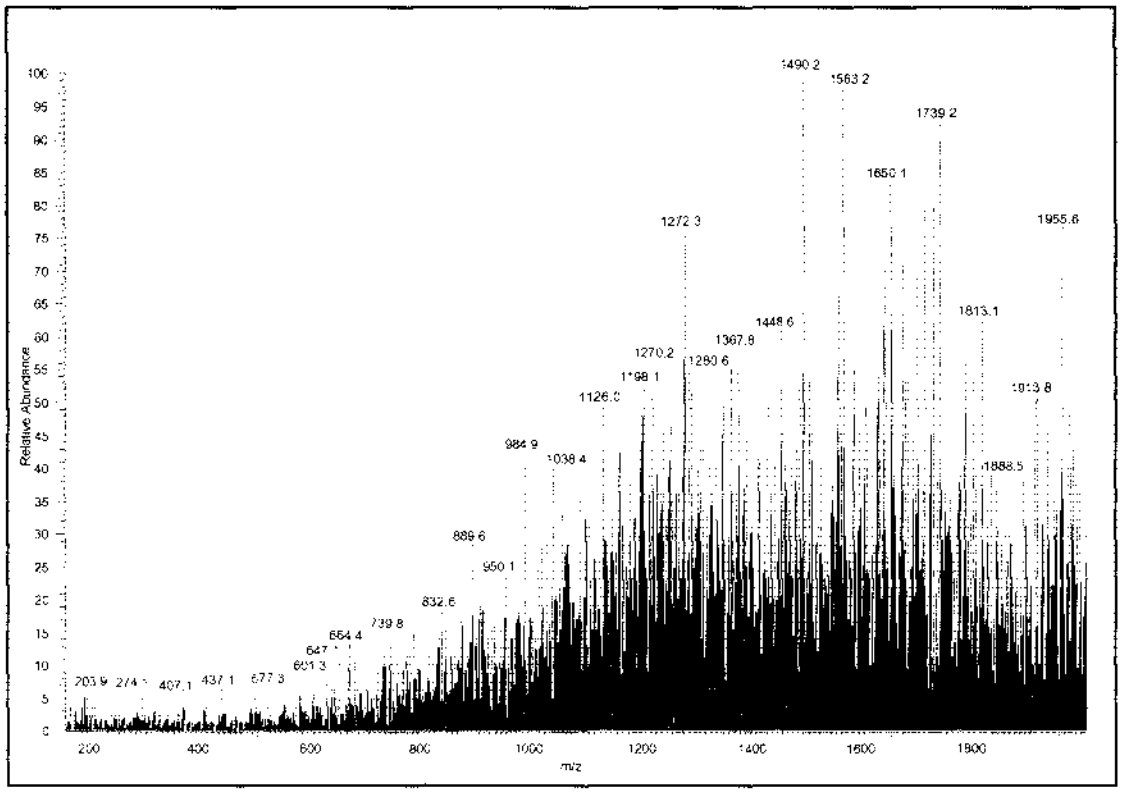

Figure 72 Results of whey basic protein fraction 3 sample infused into MS

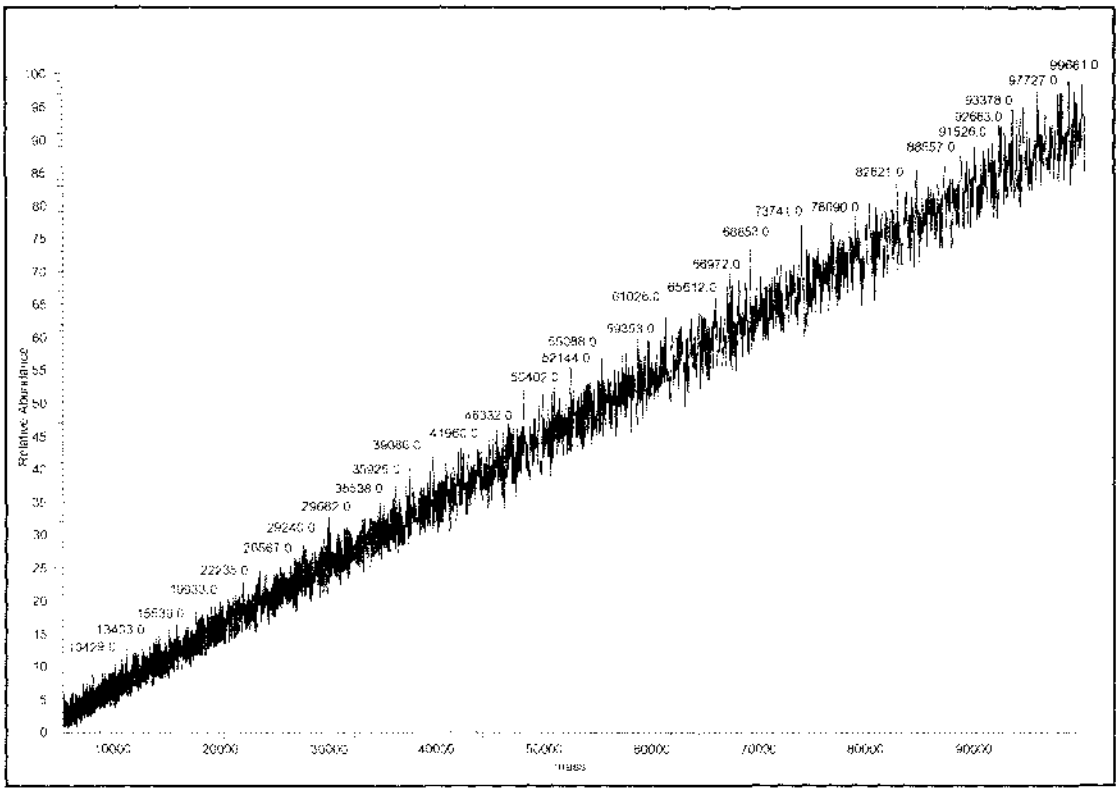

Figure 73 Results of deconvolution of whey basic protein fraction 3

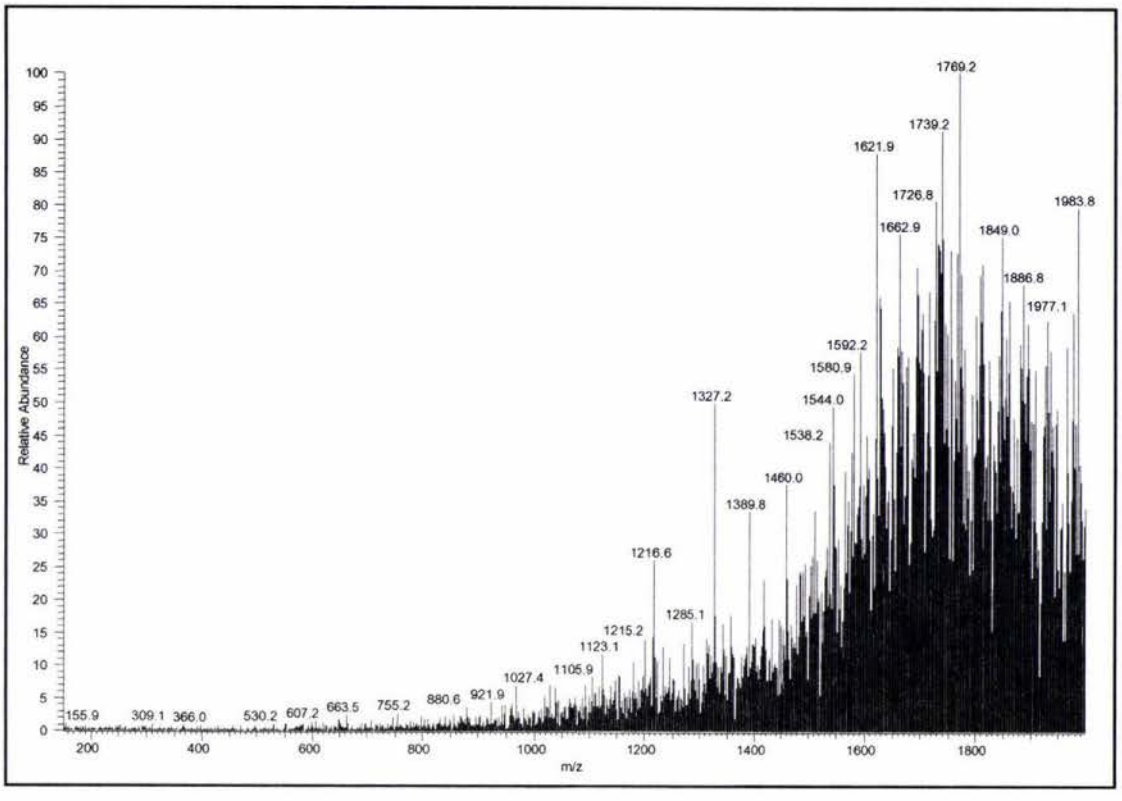

Figure 74 Results of whey basic protein fraction 2 sample infused into MS

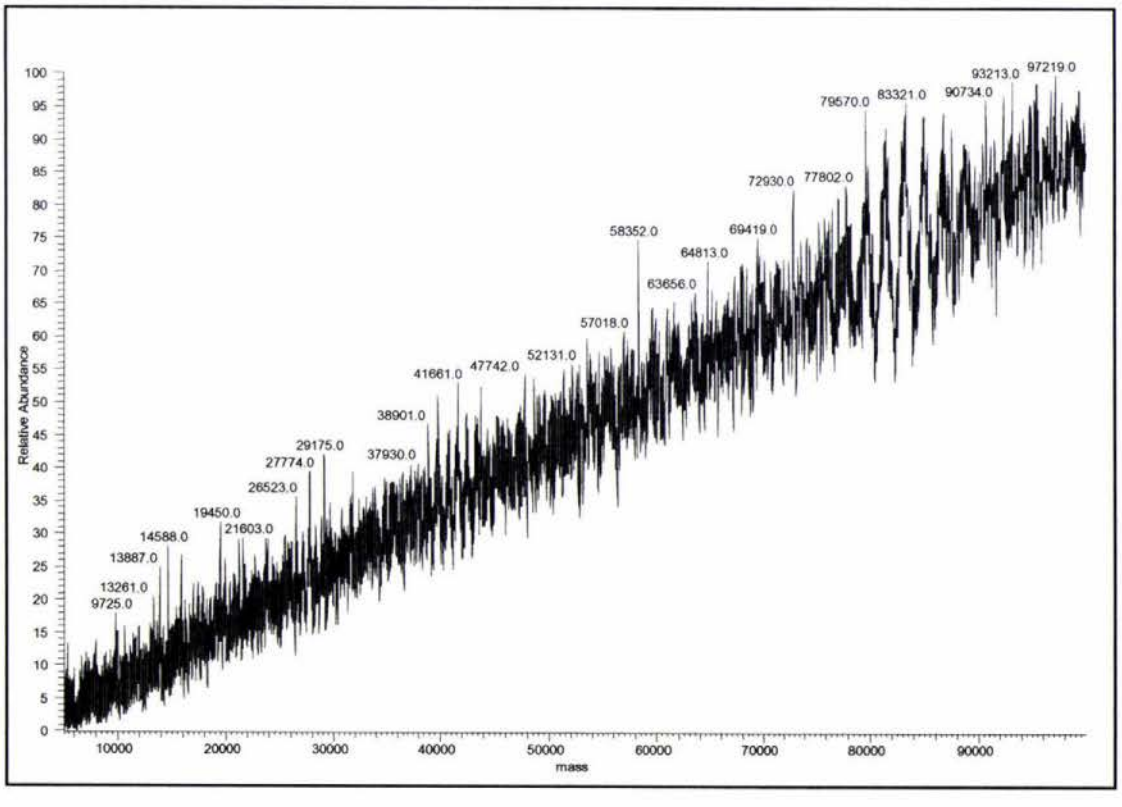

Figure 75 Results of deconvolution of whey basic protein fraction 2

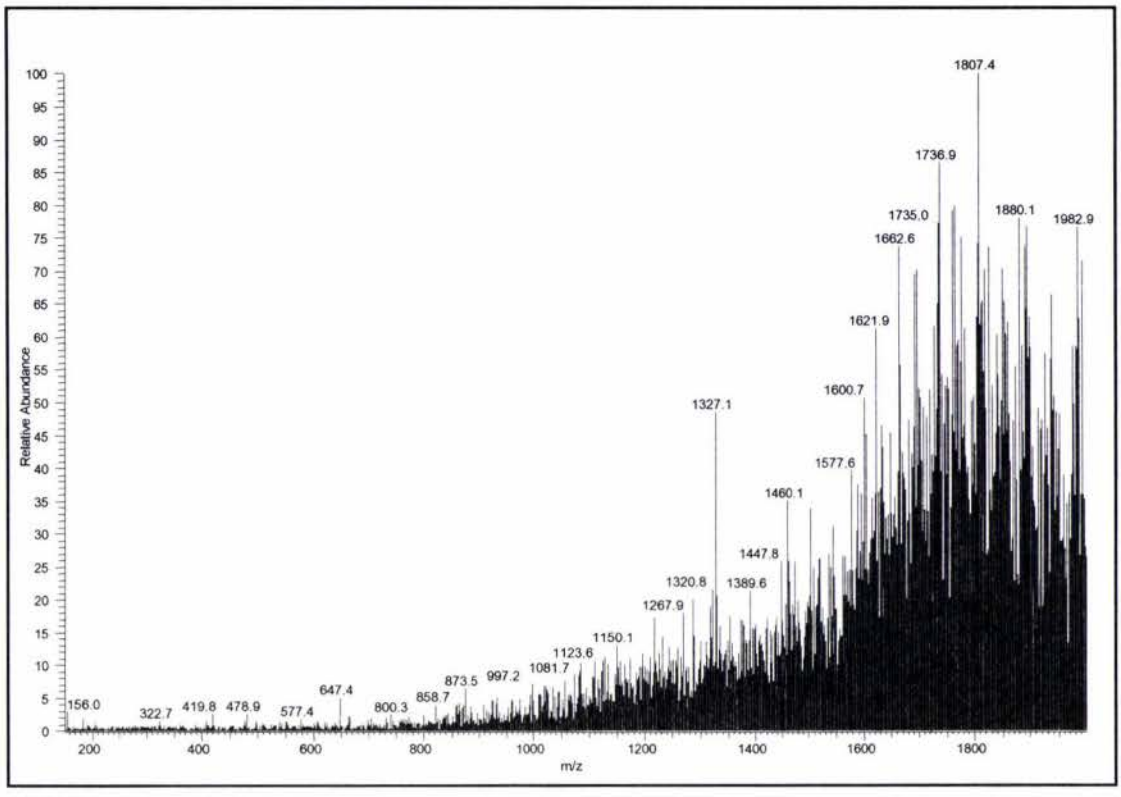

Figure 76 Results of whey basic protein fraction 1 sample infused into MS

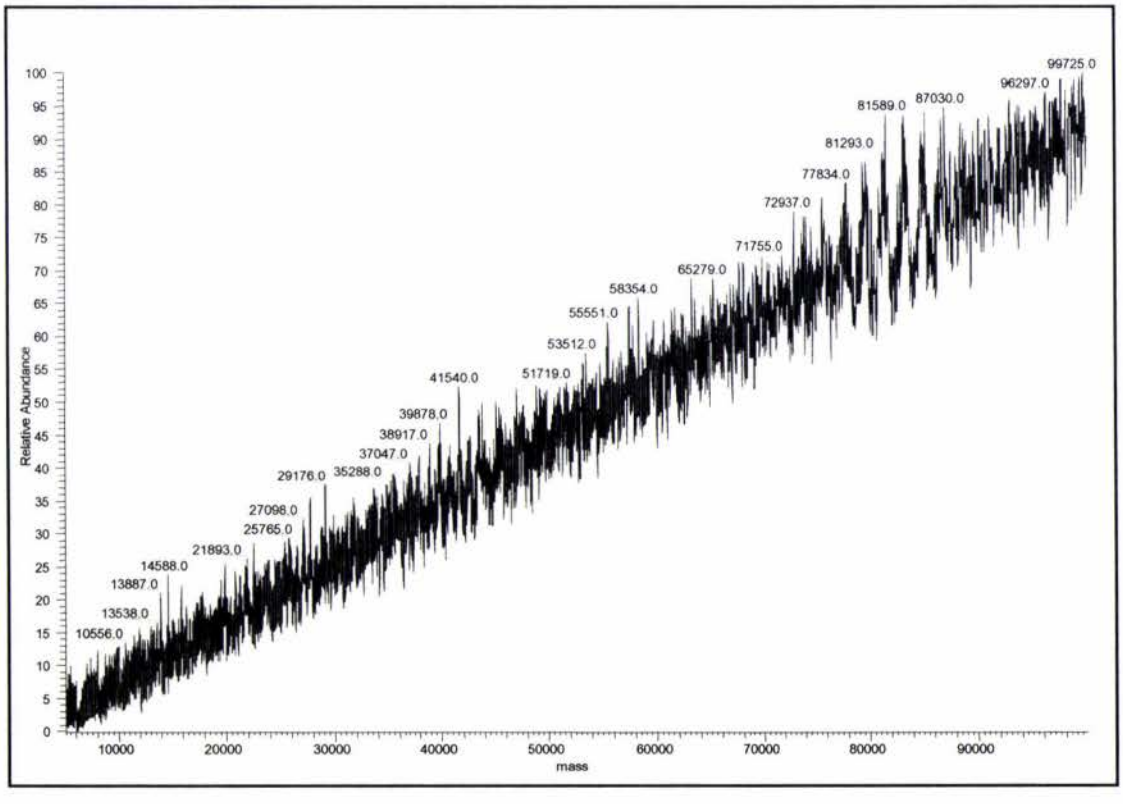

Figure 77 Results of deconvolution of whey basic protein fraction 1

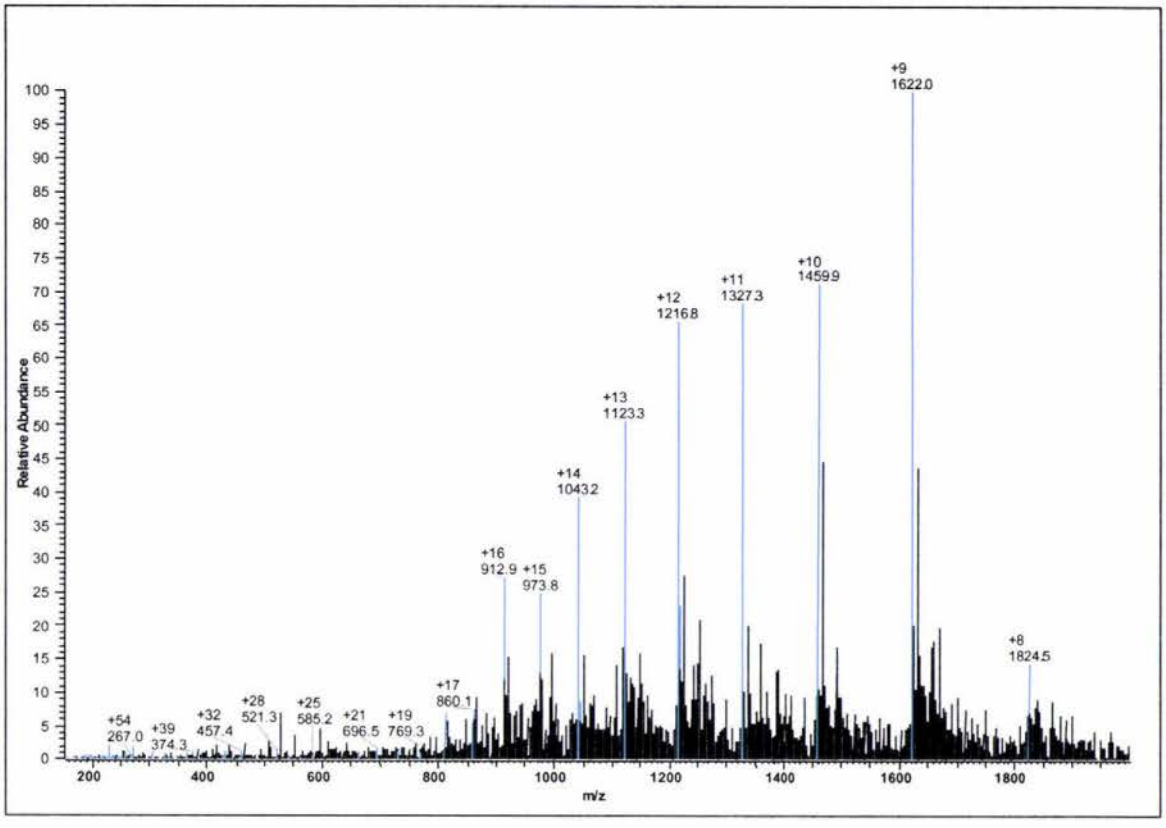

Figure 78 Results of angiogenin sample infused into MS

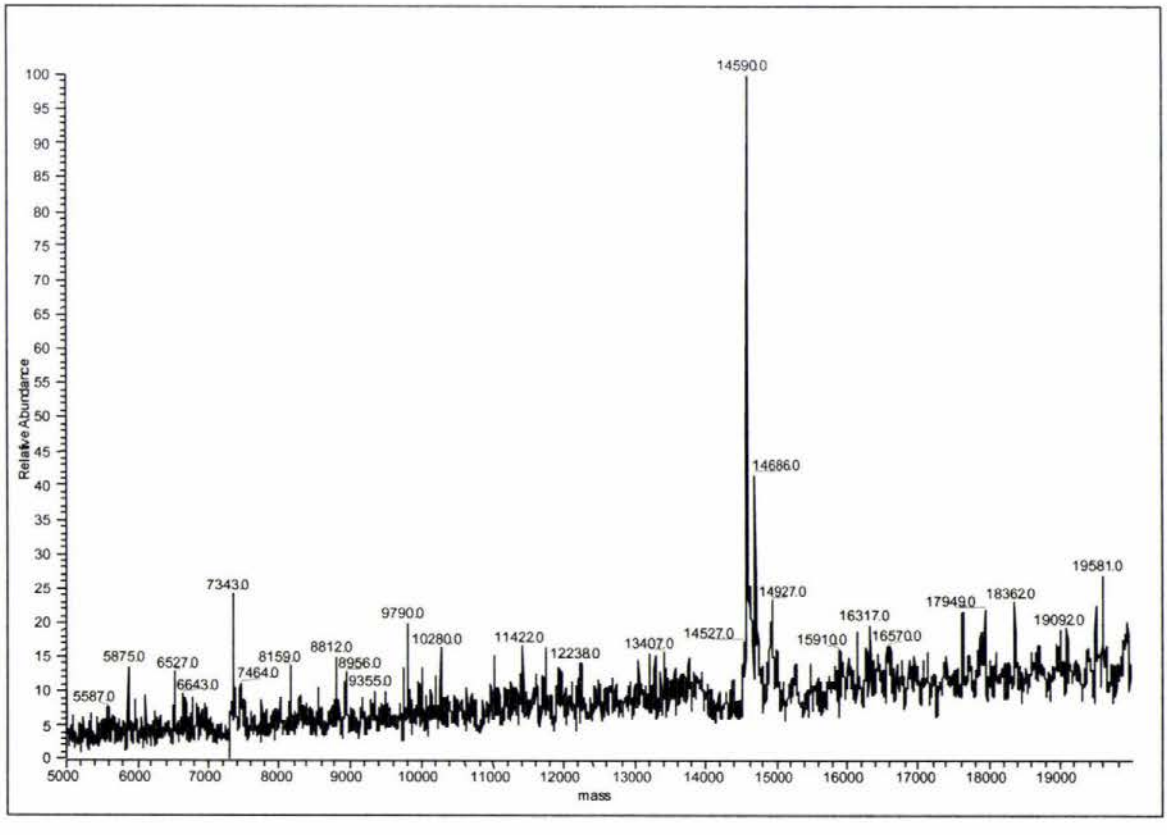

Figure 79 Results of deconvolution of angiogenin sample

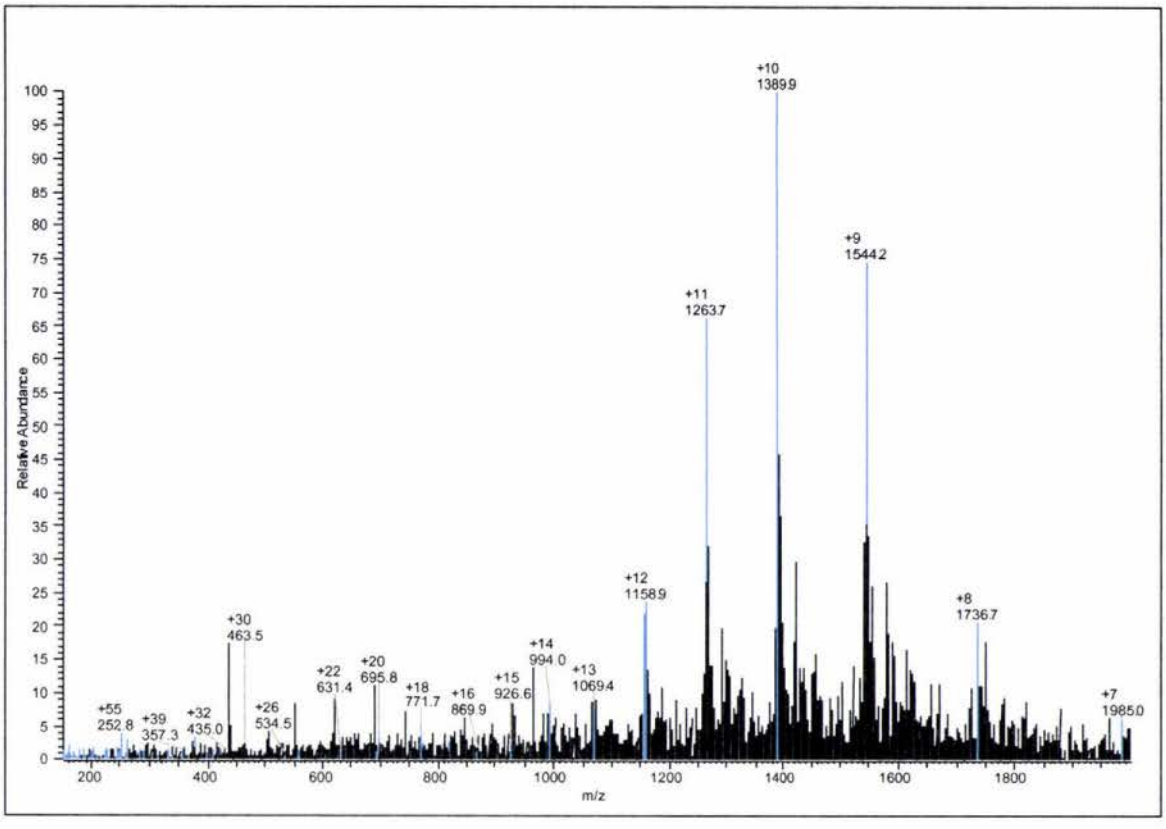

Figure 80 Results of lactogenin sample infused into MS

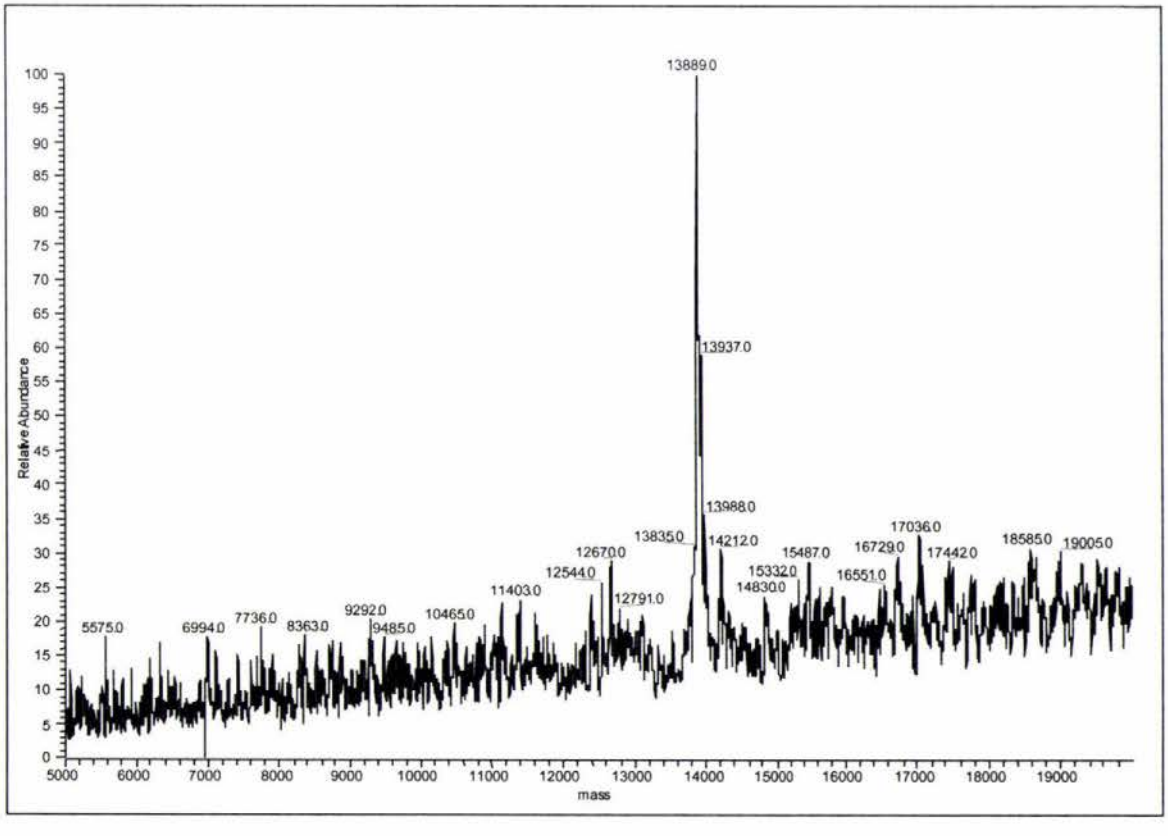

Figure 81 Results of deconvolution of lactogenin sample

Appendix 4 Publications

Capillary Isoelectric Focusing of Dairy Proteins, New Tools for Proteomic Research

Fonterra

Gapper, L.W.[1] Harding, D.R.[2] Palmano, K.P.
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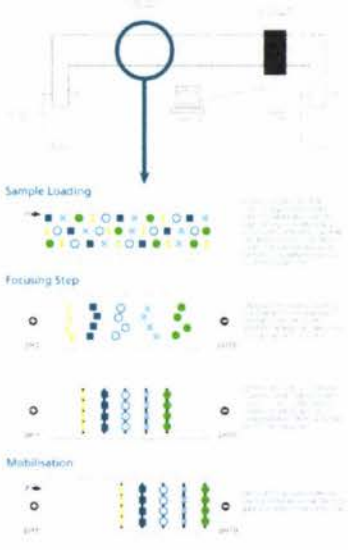
Introduction

- Capillary isoelectric focusing (CIEF) is a rapidly emerging tool for proteomic analysis (Shen & Smith, 2002).
- When coupled with mass spectrometry (MS) detection the technique is analogous to conventional 2D-IEF/SDS polyacrylamide gel electrophoresis (PAGE)-MS
- Excellent comparisons between CIEF-MS and 2D-PAGE have been demonstrated (Tang et al., 1997; Shen & Smith, 2002).
- At Fonterra-PN we are investigating the robustness of CIEF for proteomic applications within the dairy industry

Methods

CIEF with UV Detection



CIEF with MS Detection

Results

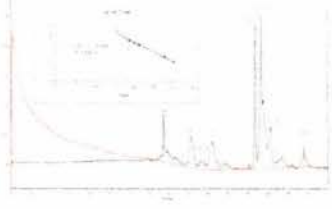


Figure 1. Electropherogram of skim milk whey with added pI dairy pI markers. UV detection at 280nm.

CIEF WITH UV DETECTION:
The major whey proteins β-lactoglobulin genetic variants A and B, and α-lactalbumin were identified in the electropherogram of skim milk whey (figure 1). The pI of these proteins were calculated from the standard curve, and were shown to be within 1.5-4.5% of the literature values.

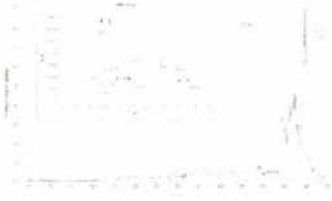


Figure 2. CIEF-MS. Total Ion Count (TIC) of a mixture of standard whey proteins and non-dairy pI markers.

CIEF WITH MS DETECTION:
Figure 2 shows a TIC trace of a CIEF-MS experiment where several major whey proteins have been included in a standard mixture of pI markers. Protein masses were assigned by deconvolution of the ionisation product data and plotted against pI to produce the 2D profile (shown figure 2 insert).

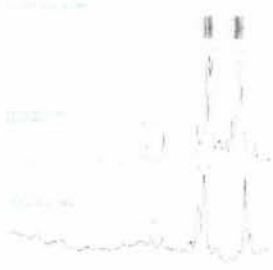


Figure 3. [illegible]

CIEF OF MILK COMPARE WITH UV DETECTION:
The milk proteins casein proteins are inherently insoluble. However, with the addition of 8M urea and pH neutralisation in a simple buffer, good resolution of the principal casein is achieved (figure 3). It can be seen that the results from CIEF sample based dairy and whey fare far better [illegible] using the method of Shen et al. (1997).

Discussion

These results illustrate the effective separation of both whey and casein proteins can be achieved using this technique.

Preliminary experiments suggest that CIEF-MS has wide potential in the analysis of:

- Minor protein component markers
- Peptides
- Genetic variation
- Post translational and chemical modifications

The method can be modified for different applications by varying pH and cathode current, or using narrow range ampholytes to enable resolution of proteins with similar pI.

Conclusions

This technique shows potential for the analysis of dairy proteins as:

- It represents fast high throughput analysis compared to PAGE techniques
- Only very small amounts of expensive chemicals are required
- Very little sample is needed compared with PAGE analysis
- Results are highly reproducible from run to run and day to day
- The pI of dairy proteins can be calculated with accuracy.

Acknowledgements

[illegible]

References

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CAPILLARY ISOELECTRIC FOCUSING OF DAIRY PROTEINS, NEW TOOLS FOR PROTEOMIC RESEARCH

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Abstract

Capillary Isoelectric Focusing (CIEF) is a high-resolution technique, which can be applied to the separation and characterisation of complex biological mixtures such as dairy proteins. Although dairy proteins are commonly analysed by traditional gel electrophoresis techniques including 2-Dimensional PAGE, CIEF offers the advantages of reduced analysis times, the ability to handle smaller sample volumes and increased sensitivity with improved separation efficiencies.

We have developed several methods for capillary isoelectric focusing of dairy proteins. For the analysis of soluble whey proteins we have set up a method that can be used with either UV or mass spectrometry (MS) detection. For MS detection we have utilised a coaxial sheath flow interface in conjunction with electrospray ionisation. For analysis of the inherently insoluble casein proteins with UV detection we have introduced denaturing and reducing agents into the system. Our results have shown very close similarities to those obtained by IEF gels.

Introduction

- Capillary isoelectric focusing (CIEF) is a rapidly emerging tool for proteomic analysis (Shen & Smith, 2002).

- When coupled with mass spectrometry (MS) detection the technique is analogous to conventional 2D-IEF/SDS polyacrylamide gel electrophoresis (PAGE)-MS
- Excellent comparisons between CIEF-MS and 2D-PAGE have been demonstrated (Tang et al., 1997; Shen & Smith, 2002).
- At Fonterra- PN we are investigating the robustness of CIEF for proteomic applications within the dairy industry

Method

CIEF with UV Detection

Sample Loading

Ampholyte and protein (~50-100 μg/ml) solution is loaded under pressure (P) onto a 50 μm I.D. MicroSolv zero flow capillary column. The inside of the capillary is coated with sulfonic acid groups to eliminate electroosmotic flow (EOF).

Focusing Step

On addition of high voltage the ampholytes form a pH gradient and proteins migrate within the capillary to their respective pI's.

As the focusing occurs the current drops (Refer to red trace figure 1.) and when complete fine bands of concentrated proteins form inside the capillary.

Mobilisation

Pressure is applied from the anode to mobilise the protein bands towards the detector.

CIEF with MS Detection

CIEF with MS detection is achieved through the use of a coaxial sheath flow interface between CE and MS. With the ESI flange open the protein and ampholyte solution can be loaded onto the capillary. Focusing is achieved by using a basic (1% ammonia) makeup flow. Once focusing is finished the ESI flange is closed and the makeup flow is replaced with a methanol/acetic acid solution. MS detection is achieved in the positive ion mode.

Results

CIEF with UV Detection

The major whey proteins β-lactoglobulin genetic variants A and B, and α-lactalbumin were identified in the electropherogram of skim milk whey (figure 1). The pI's of these proteins were calculated from the standard curve, and were shown to be within 1.5- 4.5% of the literature values.

CIEF with MS Detection

Figure 2 shows a TIC trace of a CIEF-MS experiment where several major whey proteins have been included in a standard mixture of pI markers.
Protein masses were assigned by deconvolution of the ionisation product data and plotted against pI to produce the 2D profile shown figure 2 (insert).

CIEF with UV Detection of Insoluble Proteins

The milk micellar casein proteins are inherently insoluble. However, with the utilisation of 8M urea and B–mercaptoethanol as a sample buffer, good resolution of the principal caseins was achieved (figure 3). It can be seen that the results from CIEF compare favourably with those from flat bed gel IEF.

Discussion

The resolution of separation is very good particularly for using UV detection with phosphoric acid and sodium hydroxide. The technique has so far been applied to a number of different samples including bacterial cell lysates (dairy starter cultures), peptide samples, and different dairy powders and fractions. For example analysis of genetic variant proteins.
Addition of narrow range ampholytes gives better separation where a number of proteins have similar *pI*'s.

Conclusions

This technique shows potential for the analysis of dairy proteins as it is:

- Fast high throughput analysis compared to PAGE techniques
- Very small amounts of expensive chemicals required
- Very little sample needed compared with PAGE analysis
- Highly reproducible results from run to run and day to day

- Can calculate the pI of dairy proteins with reasonable accuracy

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Figures Text

Figure 1

Electropherogram of skim milk whey proteins with non-dairy pI markers at 280nm

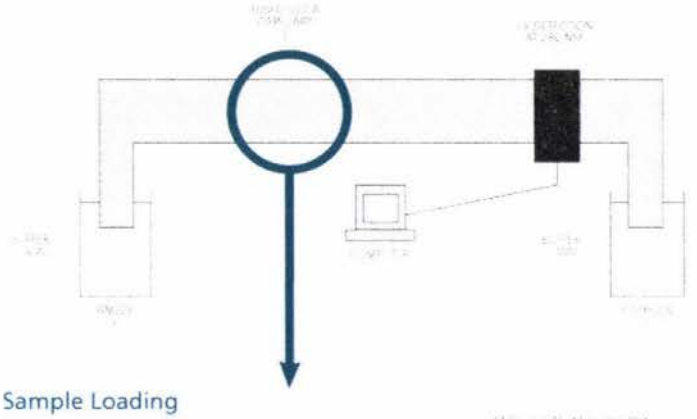
Figure 2

Total Ion Count (TIC) of a CIEF-MS experiment with a mixture of dairy protein standards with pI markers

Figure 3

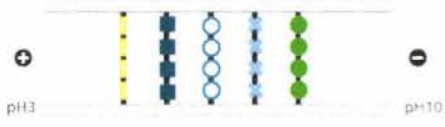
Comparison of flat bed gel IEF (top) with densitometry (middle) and CIEF (bottom) for skim milk proteins.

CIEF with UV Detection

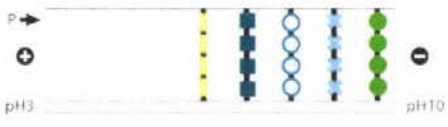


Sample Loading

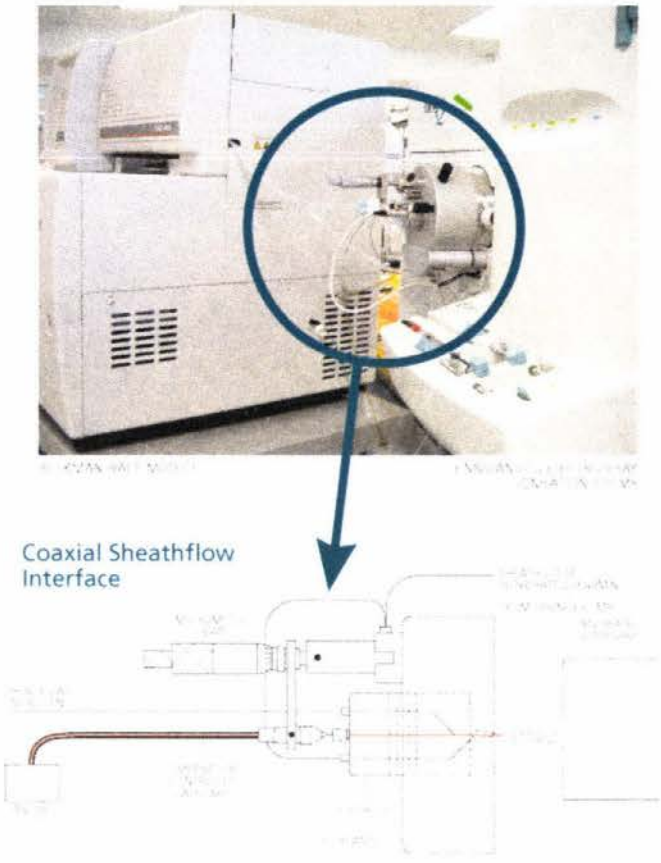
Focusing Step



Mobilisation



CIEF with MS Detection



CIEF with MS detection is achieved through the use of a coaxial sheath flow interface between CE and MS. With the ESI flange open the protein and ampholyte solution can be loaded onto the capillary. Focusing is achieved by using a basic (1% ammonia) makeup flow. Once focusing is finished the ESI flange is closed and the makeup flow is replaced with a methanol/acetic acid solution. MS detection is achieved in the positive ion mode.

Figure 1

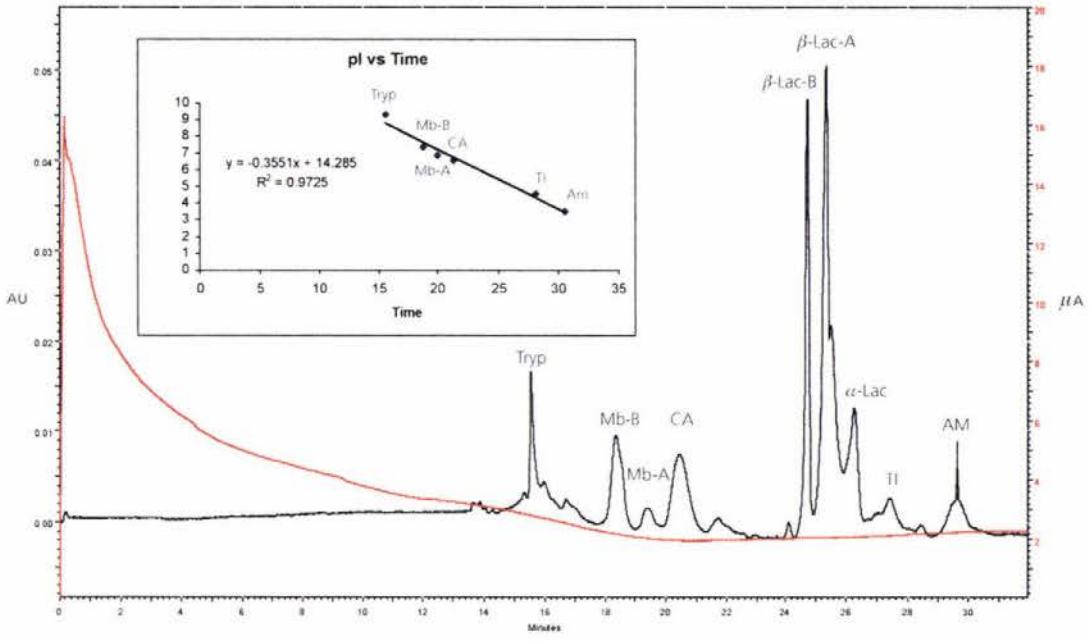

pI vs Time
y = -0.3551x + 14.285
R² = 0.9725
Tryp
Mb-B
CA
Mb-A
Ti
Am
Time
AU
μA
Minutes
β-Lac-A
β-Lac-B
α-Lac
AM
TI

Figure 2

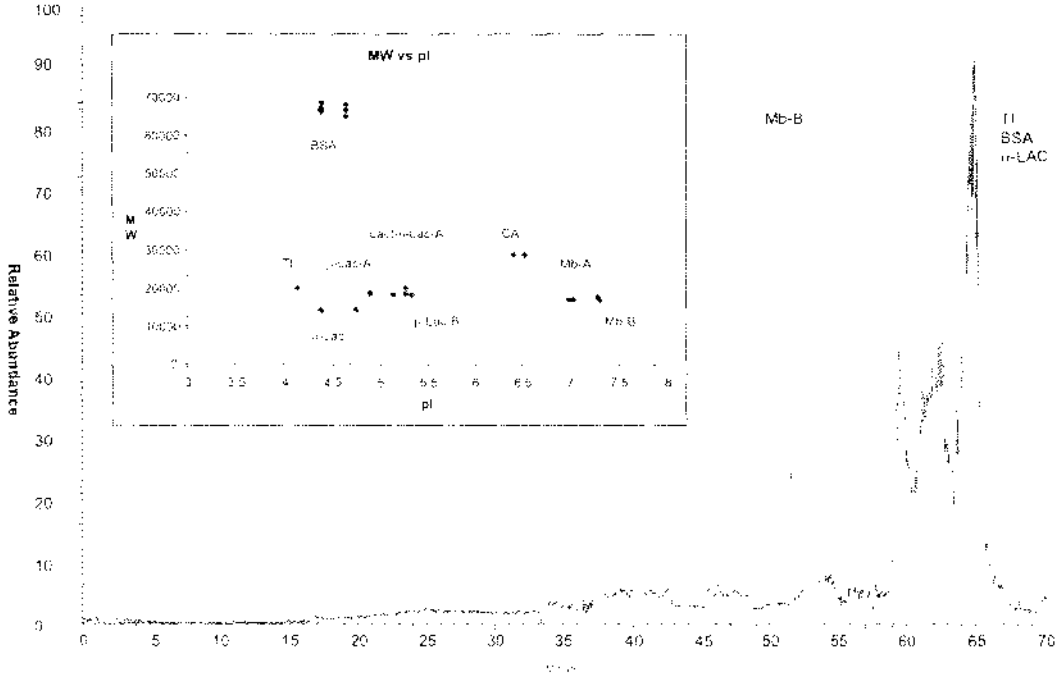


Figure 3