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**DEVELOPMENT OF METHODS FOR  
CAPILLARY ISOELECTRIC  
FOCUSING OF  
DAIRY PROTEINS**

**A THESIS PRESENTED IN FULFILMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE IN CHEMISTRY  
AT MASSEY UNIVERSITY,  
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# Abstract

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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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# Contents

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Abstract	i
Acknowledgements	ii
Contents	iii
List of Abbreviations	xiv
1 Overview	1
2 Literature Review	3
2.1 Composition of Bovine Milk	3
2.2 Introduction to Capillary Electrophoresis	5
2.3 Capillary Zone Electrophoresis	7
2.4 Micellar Electrokinetic Chromatography	7
2.5 Capillary Isotachopheresis	8
2.6 Capillary Electrochromatography	8
2.7 Capillary Isoelectric Focusing	8
2.8 Recent Reviews on CE of Large Biomolecules	9
2.9 CE of Dairy Proteins	9
2.9.1 Analysis of Casein	10
2.9.2 Whey Protein Separation	13
2.10 CE-MS	13
2.10.1 Coaxial Sheath-flow Interface	14
2.10.2 Sheathless Interface	15
2.10.3 Liquid-junction Interface	15
2.11 CE-MS Modes	16
2.11.1 CZE-MS	16
2.11.2 CIEF-MS	16
2.11.3 CITP-MS	17
2.11.4 MEKC-MS	17
2.11.5 CEC-MS	18
2.11.6 CGE-MS	18
3 Experimental Conditions	20
3.1 Chemicals	20
3.2 Sample and Buffer Preparations for CIEF Experiments	21
3.2.1 Whey Basic Protein Fraction	21
3.2.2 Whey Protein from Skim Milk	21
3.2.3 Casein Protein from Skim Milk	21
3.2.4 Standards	22

3.2.5	Buffers	22
3.3	CIEF-UV Experiments	22
3.4	CIEF-UV in a non-denatured system	23
3.5	CIEF-UV in a denatured system	24
3.6	CIEF-MS Experiments	25
3.7	Infusion MS experiments	27
3.8	CZE of Whey Proteins	27
3.9	CZE of Casein	27
3.9.1	Buffers	27
3.9.2	Sample Preparation	28
3.9.3	CZE Parameters	28
3.10	Flatbed IEF gel preparation	29
3.10.1	IEF Sample preparation	29
3.10.2	Skim Milk	29
3.10.3	Standards	29
3.10.4	Whey Basic Fraction	30
3.11	Flatbed IEF gel running conditions	30
3.12	Focusing	30
3.13	IEF gel staining	30
3.13.1	Coomassie Blue R-250 Stain	30
3.13.2	Coomassie De-Stain	31
3.13.3	Staining Procedure	31
3.14	2-Dimensional Gel Electrophoresis Experiments	31
3.14.1	Buffers	31
3.14.2	Sample Preparation	32
3.14.3	IEF Focusing	32
3.14.4	Second Dimension SDS-PAGE	32
4	Results	34
4.1	CIEF-UV Water Soluble Method	34
4.1.1	Method development protocol	34
4.1.2	Protein Concentration	34
4.1.3	Buffer Choice	37
4.1.4	Column Choice, Length & Internal Diameter	40
4.1.5	Detection Choice and Wavelength Selection	48
4.1.6	Ampholyte Choice	50
4.1.7	Focusing Times	56
4.1.8	Mobilisation Techniques	58
4.1.9	Changes in Voltage	60
4.1.10	Temperature Effects	64
4.1.11	Addition of Surfactants	67
4.1.12	Linearity of Standards	67
4.1.13	Method Repeatability	69
4.1.14	Applications of the CIEF-UV Method	72
4.2	Insoluble Dairy Proteins with UV detection	81
4.3	MS Infusion Experiments	82

4.4	CIEF-MS Detection	84
4.4.1	Method Development	84
4.4.2	CIEF-MS Applications	88
4.5	Flat Bed IEF Gels	91
4.6	PAGE 2D Gels	92
4.7	CZE of Dairy Proteins and Peptides	95
4.7.1	Whey Proteins	95
4.7.2	Casein	95
4.8	Comparison of Methods	96
4.8.1	CIEF to CZE Methods	96
4.8.2	CIEF to Gel Methods	98
4.8.3	CIEF-MS to 2D-PAGE-MS	99
5	Conclusions	101
6	Future Work	103
7	References	104
	Appendix 1 CIEF literature	116
	Appendix 2 Results of Infusion MS experiments	124
	Appendix 3 Results of MS infusion of basic protein fraction samples	144
	Appendix 4 Publications	150

## Table of Figures

- Figure 1 General Schematic overview of a CE instrument including cathode, anode, capillary, high voltage power supply, detector and data acquisition. 5
- Figure 2 Schematic of the Finnigan coaxial sheath-flow CE-MS interface as used in this research. 15
- 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,  $\beta$ -lac-B =  $\beta$ -lactoglobulin-B,  $\beta$ -lac-A =  $\beta$ -lactoglobulin-A,  $\alpha$ -Lac =  $\alpha$ -lactalbumin, TI = trypsin inhibitor, AM = amyloglucosidase. Detection was UV at 280 nm. 36
- 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 =  $\beta$ -lactoglobulin-B, peak 5 =  $\beta$ -lactoglobulin-A, peak 6 =  $\alpha$ -lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase. 38
- 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 =  $\beta$ -lactoglobulin-B, peak 5 =  $\beta$ -lactoglobulin-A, peak 6 =  $\alpha$ -lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase. 41
- 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. 44
- 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 =  $\beta$ -lactoglobulin-B, peak 5 =  $\beta$ -lactoglobulin-A, peak 6 =  $\alpha$ -lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase. 46

Figure 8 Electropherograms of whey protein with internal pl standards for capillaries of 75  $\mu$ m i.d. (top) and 50  $\mu$ m 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 =  $\beta$ -lactoglobulin-B,  $\beta$ -lactoglobulin-A, and  $\alpha$ -lactalbumin peak 5 = trypsin inhibitor, and peak 6 = CCK flanking peptide. 48

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. 50

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  $\beta$ -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 =  $\beta$ -lactoglobulin-B, peak 2 =  $\beta$ -lactoglobulin-A, and peak 3 =  $\alpha$ -lactalbumin. 52

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 =  $\beta$ -lactoglobulin-B, peak 2 =  $\beta$ -lactoglobulin-A, and peak 3 =  $\alpha$ -lactalbumin. 54

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. 56

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 =  $\beta$ -lactoglobulin-B, peak 5 =  $\beta$ -lactoglobulin-A, peak 6 =  $\alpha$ -

- lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase. 57
- 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 =  $\beta$ -lactoglobulin-B, peak 5 =  $\beta$ -lactoglobulin-A, peak 6 =  $\alpha$ -lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase. 59
- 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 =  $\beta$ -lactoglobulin-B, peak 5 =  $\beta$ -lactoglobulin-A, peak 6 =  $\alpha$ -lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase. 61
- 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  $\alpha$ -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 =  $\beta$ -lactoglobulin-B, peak 5 =  $\beta$ -lactoglobulin-A, peak 6 =  $\alpha$ -lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase. 65
- 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 =  $\alpha$ -lactalbumin, BA =  $\beta$ -lactoglobulin-A, and BB =  $\beta$ -lactoglobulin-B. 1 = sample set 1, 2 = sample set 2. 66
- 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 =  $\alpha$ -lactalbumin, BA =  $\beta$ -lactoglobulin-A, and BB =  $\beta$ -lactoglobulin-B. 1 = sample set 1, 2 = sample set 2. 67
- 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 =  $\beta$ -lactoglobulin-B, peak 5 =  $\beta$ -lactoglobulin-A, peak 6 =  $\alpha$ -lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase. 71
- 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. 74
- 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. 75
- Figure 22 Electropherogram of a whey acidic protein fraction from mineral acid whey. Sample run identical to the sample in Figure 3. 76
- 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. 76
- 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. 77
- 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. 78
- Figure 26 Electropherograms of bacterial cell lysate "B12" run 4 times (each electropherogram off set). Separation conditions were identical to that in Figure 3. 80
- 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. 80
- Figure 28 Electropherograms of skim milk run under identical conditions except the top trace utilised  $\beta$ -mercaptoethanol (BME), while the bottom trace utilised DTT in the sample buffer. 81
- 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 = $\beta$ -lactoglobulin-B, peak 5 = $\beta$ -lactoglobulin-A, peak 6 = $\alpha$ -lactalbumin, peak 7 = trypsin inhibitor, and peak 8 = amyloglucosidase.	85
Figure 30 TIC of CIEF-MS of whey protein from skim milk spiked with minor whey proteins (BSA, GMP, and PP5) and <i>pI</i> markers.	87
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, $\beta$ -lac-B = $\beta$ -lactoglobulin-B, $\beta$ -lac-A = $\beta$ -lactoglobulin-A, $\alpha$ -Lac = $\alpha$ -lactalbumin, TI = trypsin inhibitor, BSA = bovine serum albumin, PP5 = proteose peptone 5, GMP = glycomacropeptide.	90
Figure 32 IEF flatbed gel of skim milk (SM, left lane) and whey basic protein fraction number 1 (right lane).	92
Figure 33 2D PAGE of whey basic protein fraction sample 1.	93
Figure 34 2D PAGE of whey basic protein fraction sample 2.	94
Figure 35 2D PAGE of whey basic protein fraction sample 3.	94
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, $\alpha$ -Lac (peak 1), $\beta$ -Lac-A (peak 4), $\beta$ -Lac-B (peak 3) and minor component $\beta$ -Lac-C (peak 2) genetic variant. The bottom trace is the response for skim milk showing $\alpha$ -Lac, $\beta$ -Lac-B, and $\beta$ -Lac-A.	95
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).	96
Figure 38 Comparison of flat bed IEF-PAGE with laser densitometry to CIEF-UV using the denaturing CIEF method (Section 3.5).	99
Figure 39 Results of $\alpha$ -Lac standard infused into MS	127
Figure 40 Results of deconvolution of $\alpha$ -Lac	127
Figure 41 Results of amyloglucosidase standard infused into MS	128
Figure 42 Results of deconvolution of amyloglucosidase	128
Figure 43 Results of $\beta$ -Lac-A standard infused into MS	129
Figure 44 Results of deconvolution of $\beta$ -Lac-A	129
Figure 45 Results of $\beta$ -Lac-B standard infused into MS	130
Figure 46 Results of deconvolution of $\beta$ -Lac-B	130
Figure 47 Results of BSA standard infused into MS	131
Figure 48 Results of deconvolution of BSA	131
Figure 49 Results of carbonic anhydrase standard infused into MS	132
Figure 50 Results of deconvolution of carbonic anhydrase	132
Figure 51 Results of GMP standard infused into MS	133

Figure 52 Results of deconvolution of GMP	133
Figure 53 Results of IgG standard infused into MS	134
Figure 54 Results of deconvolution of IgG	134
Figure 55 Results of lactoferrin standard infused into MS	135
Figure 56 Results of deconvolution of lactoferrin	135
Figure 57 Results of lactoperoxidase standard infused into MS	136
Figure 58 Results of deconvolution of lactoperoxidase	136
Figure 59 Results of lactoferrin deglycosylated infused into MS	137
Figure 60 Results of deconvolution of deglycosylated lactoferrin	137
Figure 61 Results of myoglobin standard infused into MS	138
Figure 62 Results of deconvolution of myoglobin	138
Figure 63 Results of PP5 standard infused into MS	139
Figure 64 Results of deconvolution of PP5	139
Figure 65 Results of ribonuclease standard infused into MS	140
Figure 66 Results of deconvolution of Ribonuclease	140
Figure 67 Results of trypsin inhibitor standard infused into MS	141
Figure 68 Results of deconvolution of trypsin inhibitor	141
Figure 69 Results of trypsinogen standard infused into MS	142
Figure 70 Results of deconvolution of trypsinsinogen	142
Figure 71 Results of CCK Peptide standard infused into MS	143
Figure 72 Results of whey basic protein fraction 3 sample infused into MS	144
Figure 73 Results of deconvolution of whey basic protein fraction 3	145
Figure 74 Results of whey basic protein fraction 2 sample infused into MS	146
Figure 75 Results of deconvolution of whey basic protein fraction 2	146
Figure 76 Results of whey basic protein fraction 1 sample infused into MS	147
Figure 77 Results of deconvolution of whey basic protein fraction 1	147
Figure 78 Results of angiogenin sample infused into MS	148
Figure 79 Results of deconvolution of angiogenin sample	148
Figure 80 Results of lactogenin sample infused into MS	149
Figure 81 Results of deconvolution of lactogenin sample	149

## Table of Tables

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).	4
Table 2 LCQ Mass Spectrometry instrument settings for CIEF-MS experiments.	26
Table 3 Literature values for isoelectric points and molecular weights of proteins used throughout this research. Typical CIEF working concentrations are also included.	35
Table 4 pH values for focusing buffers and mobilisation buffers in CIEF experiments.	39
Table 5 Comparison of the electropherograms obtained from using different 30 cm columns as shown in Figure 5.	43
Table 6 Comparison of column volume (nl) when changing parameters such as length or internal diameter. Calculated from CExpert (Beckman Coulter).	46
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).	47
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.	58
Table 9 Comparison of differences in separation for different voltages from data obtained in experiments in Figure 15.	63
Table 10 Optimised conditions for CIEF analysis of skim milk whey proteins and <i>pI</i> 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.	69
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.	72
Table 12 Results of MS infusion experiments of whey basic protein fraction samples	84
Table 13 Buffer compositions for the electropherograms shown in Figure 29. All buffer percentage compositions were in a v/v ratio.	86
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.	116
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. 120

Table 16 Results of infusion MS experiments. MS conditions used are outlined in section 3.7. Deconvolution of mass spectrums was performed on Bioworks version 3.1. Literature masses were obtained from Mascot ([www.matrixscience.com](http://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. 124

# List of Abbreviations

2D	Two Dimensional
$\alpha$ -csn	$\alpha$ -Casein
$\alpha$ -Lac	$\alpha$ -Lactalbumin
Amy	Amyloglucosidase
$\beta$ -csn	$\beta$ -Casein
$\beta$ -Lac	$\beta$ -Lactoglobulin
$\beta$ -Lac-A	$\beta$ -Lactoglobulin-A
$\beta$ -Lac-B	$\beta$ -Lactoglobulin-B
BME	$\beta$ -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 Isotachopheresis
CZE	Capillary Zone Electrophoresis
DNA	Deoxyribosenucleic Acid
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetra-Acetic Acid
EOF	Electroosmotic Flow
ESI	Electrospray Ionisation
GMP	Glycomacropptide
HPLC	High Performance Liquid Chromatography



i.d.	Internal Diameter
IEF	Isoelectric Focusing
Ig	Immunoglobulin
IgG	Immunoglobulin G
$\kappa$ -csn	$\kappa$ -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 Spectrometry
MWCO	Molecular Weight Cut Off
NaOH	Sodium Hydroxide
nL	Nano Litre
PAGE	Polyacrylamide Gel Electrophoresis
PDA	Photo Diode Array
<i>pI</i>	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

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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 glycomacropptide (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