THE UNIVERSITY OF MANITOBA

Characterization of Biological Compounds by Mass Spectrometry

by

Mark Errol McComb

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A thesis submitted to the FACULTY OF GRADUATE STUDIES
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

Doctor of Philosophy

Dedicated to

my colleagues

my friends

my family

and my love

so long, and thanks for all the fish

Douglas Adams

All that we envision is possible.

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ABSTRACT

Mass spectrometry is a powerful method for the characterization of biological molecules. However, sample purification is often required due the presence of contaminants within the biological matrix which will affect the quality of spectra. In this thesis, two methods of "on-line" sample purification and separation were explored.

Non-porous ether type polyurethane (PU) membranes and porous PU thin films were used as sample supports for matrix assisted laser desorption time-of-flight (MALDI-TOF) MS. Protein and peptide samples were applied directly to the surface of the PU supports. Hydrophobic interactions between the proteins and peptides and the PU membrane allowed the incorporation of a washing step to remove salts and other matrix components present. This provided for an increase in resolution and mass accuracy and thus superior results compared with other supports. Peptide mass mapping was facilitated through on-membrane proteolytic digestion. A procedure for the sampling and off-site analysis of hemoglobin variants was developed by characterizing the Hb Shepherds Bush hemoglobin variant, b74 (E18)Gly->Asp. A novel application was explored by characterizing water insoluble wheat proteins which consist of gliadins, low molecular weight (LMW) and high molecular weight (HMW) glutenins. *In vitro* and *in vivo* qualitative and semi-quantitative analysis of plasma proteins demonstrated the first study of MALDI-TOFMS characterization of multiple proteins adsorbed onto the surface of a model biomaterial (PU).

On-line capillary electrophoresis electrospray ionization mass spectrometry (CE/ESI-MS) and tandem mass spectrometry (CE/ESI-MS/MS) experiments were conducted on a Micromass™ triple quadrupole mass spectrometer equipped with a Z-Spray™ source. On-line CE-time-of-flight mass spectrometry (CE/ESI-TOFMS) experiments were conducted using a reflecting TOF mass spectrometer built in-house. Gold coated sheathless interfaces were used to couple uncoated fused silica capillaries with the mass spectrometers. An electrical system and ESI interfaces were designed and characterized using standard peptides and proteins. For a standard peptide mixtures (10^{-4} to10^{-6} M) (pmol and sub-pmol injection), separation efficiency was typically characterized by $N > 10^4$ theoretical plates with S/N ca. 200 to > 400. Unit mass resolution (m/ΔmFWHM) was obtained on the QQQ and mass accuracy was better than 100 ppm. Reflecting TOFMS afforded mass resolution R > 6000 and mass accuracy of ca. 30 ppm. CE/ESI-MS/MS was used to sequence standard peptides. Selected ion electrophorograms of tryptic peptides of several proteins allowed for peptide mass mapping in spite of poorly resolved total ion electrophorograms. Citrate synthase, SB hemoglobin and equine myoglobin were characterized.

ABBREVIATIONS

AA	amino acid	MS	mass spectrometry
BGE	background electrolyte	MS/MS	tandem mass spectrometry
BI	bovine insulin	Mt	migration time
CE	capillary electrophoresis	N	theoretical plates
CE/MS	capillary electrophoresis mass spectrometry	NMR	nuclear magnetic resonance
CID	collision induced dissociation	PTMO	poly-tetramethylene oxide
CZE	capillary zone electro-phoresis	PU	polyurethane
DE	delayed extraction	PVDF	poly vinylidene difluoride
DTT	dithiothreitol	QQQ	triple quadrupole
EOF	electroosmotic flow	R	resolution
EP	electrophoresis	RF	radio frequency
ESI	electrospray ionization	Rt	retention time
FWHM	full width at half maximum	SB	Shepherds Bush
GC	gas chromatography	SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Hb	hemoglobin	SEAC	surface enhanced affinity capture
HMW	high molecular weight	SEM	scanning electron micros-copy
HPLC	high performance liquid chromatography	SIE	selected ion electrophoro-gram
IR	infrared	TDC	time-to-digital converter
LMW	low molecular weight	TEI	total ion electrophorogram
MALDI	matrix assisted laser desorption ionization	TFA	trifluoroacetic acid
MDI	methylene bis(p-phenyl diisocyanate)	Tn	tryptic peptide # n
Mr	molecular weight relative to ^{12}C	TOF	time-of-flight
		UV	ultraviolet

1 INTRODUCTION

1.1 MASS SPECTROMETRY

Mass spectrometry (MS) has been described as one of three key technologies available to probe cell proteins and to correlate the latter to their genes [1]. Information may be obtained on the molecular weight of molecular species with high mass accuracies i.e. greater than 10 ppm, far superior to traditional gel electrophoresis measurements. Structural information of a compound may obtained with the use of collision induced dissociation (CID). MS has been used to study a variety of applications in biochemistry from the sequencing of compounds to the study of macro-molecular structures. The roles of MS include investigation of non-covalent interactions, characterization of proteins, peptides and nucleotides, study of oligosaccharides and protein glycosylation, proteom characterization, peptide database mass mapping, 2-dimensional mapping of proteins from tissue slices, and intact virus identification.

This expanding ability of mass spectrometry to elucidate biomolecules has recently been made possible due to improvements in the design of mass spectrometers. New methods of gentle ionization have been developed and allow the introduction of non-volatile labile molecules into the vacuum system of a mass spectrometer. Improvements in sensitivity, resolution and accuracy of mass spectrometers have expanded applications to higher molecular weight proteins and other compounds. Decreasing instrument costs combined with improvements in ease of use, primarily though software controlled systems, have encouraged the production of bench-top systems. These may be run by moderately skilled technicians in a variety of laboratory settings and are not restricted to a dedicated mass spectrometry facility as they would have been only a few years ago. In the past decade mass spectrometry has thus emerged as a fundamental method for the characterization of biologically significant compounds [2-6].

1.2 SAMPLE INTRODUCTION

Traditionally MS was limited to the study of volatile analytes, i.e. small organic compounds, with electron impact sources [7]. Biologically derived samples, i.e. proteins,

peptides, glycans, nucleotides are for the most part non-volatile and are thermally labile. Therefore it is difficult to introduce these species into the vacuum system of a mass spectrometer. With the advent of new ionization methods, specifically matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), it has now become possible to characterize a variety of classes of biological compounds using mass spectrometry.

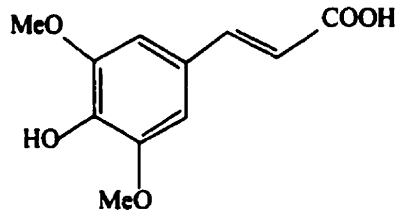
1.2.1 MALDI

MALDI time-of-flight mass spectrometry (MALDI-TOFMS) was introduced in 1988 by Michael Karas and Franz Hillenkamp and at the same time by Tanaka and colleagues [8,9]. Karas and Hillenkamp observed the mass spectrum of bovine serum albumin, molecular weight ca. 67,000 Da via laser desorption ionization using a UV absorbing matrix [8]. Their original work marked the first time that high molecular weight compounds could be analyzed with ease by laser desorption mass spectrometry. Now, some ten years later, MALDI-TOFMS is used for a tremendously large number of applications including the characterization of biological molecules [10-12]. Traditionally, MALDI-MS allowed one to determine the molecular weight of a compound to within ~ 0.1% accuracy. Now, with the advent of high resolution TOF mass spectrometers, the use of post source decay methods, and new methods of sample preparation, mass accuracy is superior and structural information may obtained.

1.2.2 The MALDI Experiment

MALDI is an example of solid state sample introduction. An analyte, i.e. protein or peptide, is mixed with excess matrix in usually a 1:100 to 1:1000 molar ratio in a suitable solvent on a MALDI probe. Matrices are chosen based on their ability to co-crystallize with the analyte, the wavelength of absorption and their ability to donate a gas phase proton (positive ion MALDI) [13,14]. A typical matrix is sinapinic acid (Figure 1-1) and consists of a chromophore for the absorption of an incident laser beam, and a free acidic proton. The matrix and analyte co-crystallize on the probe which is then placed within the mass spectrometer.

A. 3,5-dimethoxy-4-hydroxy-cinnamic acid



B. Matrix + protein co-crystallization

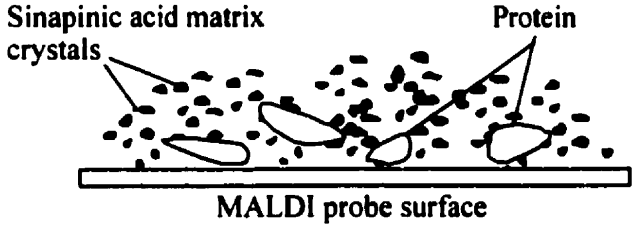


Figure 1-1. Matrix/ sample preparation for MALDI.
(a) sinapinic acid matrix, (b) sample preparation on a probe.

As MALDI is a pulsed ionization method the most common mass spectrometer used is a time-of-flight instrument operated in either the linear or reflecting mode. A laser (UV, IR) is used to ablate the surface of the probe with an irradiance of just enough intensity to produce ions seen in the mass spectrum (threshold intensity) (Figure 1-2). The matrix absorbs the incident radiation and literally explodes, producing a thermal plume of excited matrix and analyte molecules/ ions. In this plume, gas phase ion-molecule reactions take place transferring a proton (positive ion mode) from the matrix to the analyte [12,15]. The degree of ionization and the energetics of ionization are dependent on the relative proton affinities of the matrix/ analyte mixture, the type of matrix used and the laser intensity [6]. The analyte, which is now charged, is accelerated into the mass spectrometer via a potential applied between the target and the acceleration grids. In the case of a TOF analyzer, the time-of-flight of the analyte is measured relative to an internal or external calibrant and the corresponding mass calculated.

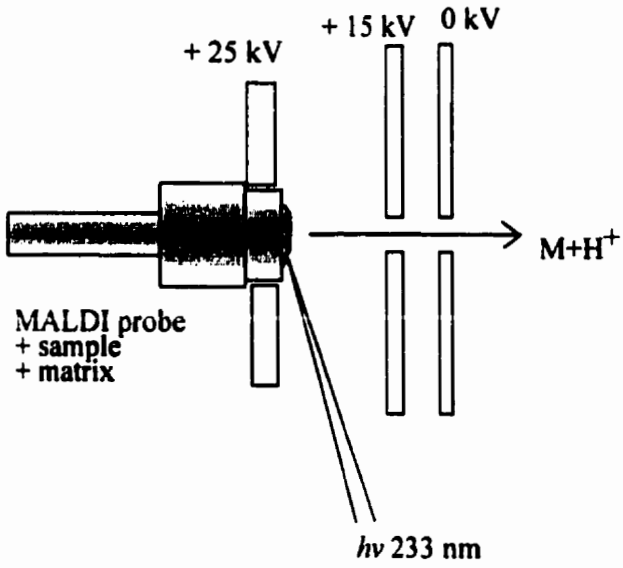


Figure 1-2. MALDI sample introduction.

1.2.3 Characteristics of MALDI

MALDI has several advantages for use as an ionization source. After simple sample preparation, mass spectra may be obtained within seconds with sub-pmol sensitivity. Compared with other ionization methods MALDI is more tolerant to the presence of interfering matrix components present within the sample [14]. The spectra produced by MALDI are relatively simple and typically consist of peaks of singly and doubly protonated species. This is advantageous in the case of mixture analysis, i.e. peptide digests where a large number of peaks may be present. MALDI is a soft ionization method and under appropriate conditions produces little or no fragmentation of the analyte. This allows the introduction of intact ions from a large number of biological molecules. Routine sensitivity is in the sub-pmol range and requires a relatively small sample size (1 μL). With modern TOF instruments, it is possible to measure an analyte's mass with better than 0.01% accuracy and greater than 5000 resolution (FWHM). MALDI is applicable to a wide range of analytes (peptides, proteins, DNA, RNA, glycans, synthetic polymers) with an extensive m/z range (800 Da > 100 kDa).

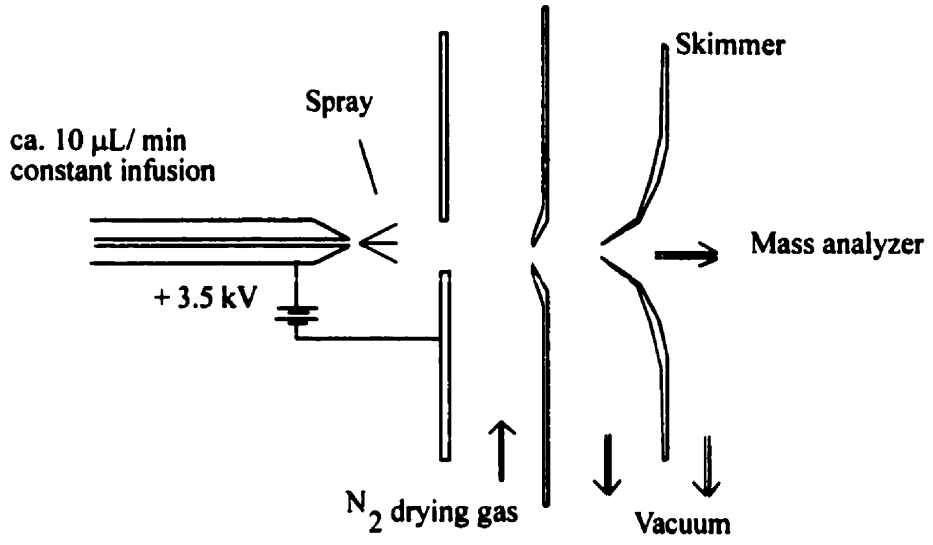
1.2.4 ESI

ESI was first introduced by Dole and co-workers who in 1968 produced evidence that intact molecular ions could be introduced into the gas phase without fragmentation [17]. By applying a potential to a narrow bore capillary they were able to produce an electrospray of free gas phase ions from analytes in a solution. The application to MS was only fully realized when Fenn and co-workers coupled an ESI source to a quadrupole mass analyzer and demonstrated ionization of liquid samples for MS at room temperature and pressure [18]. As did Dole, they observed multiple protonation of the analyte via the electrospray process allowing analysis of large M_r species within the limited mass range of the spectrometer. They were able to obtain mass spectra of bovine albumin dimer M_r ca. 133,000 Da [19]. Covey et al. also demonstrated that by using this "soft" method of ionization, intact macro-molecular ions could be characterized with relative ease [20].

1.2.5 The ESI Experiment

In ESI, an aqueous solution containing the analyte is passed through a narrow bore capillary at a relatively low flow rate. A potential gradient is applied to the capillary through space to the entrance of the mass spectrometer (2 - 5 kV). As the liquid emerges from the capillary it forms a meniscus which undergoes deformation and expansion due to the charge buildup at the surface of the liquid. After sufficient deformation the surface of the liquid breaks apart and begins to emit macroscopic droplets of different sizes each possessing an abundance of protons (positive ion mode) and hence positive charge. These droplets are drawn through space, via the potential gradient, towards the inlet of the mass spectrometer. The droplets evaporate which results in a buildup of charge (protons) near or on their surface. When the surface of a droplet can no longer support an increase in charge it undergoes a coulombic explosion which results in the formation of several, new, smaller droplets. Increasingly smaller droplets are formed until gas phase free ions are left either by evaporation [21,22] or until ions themselves are ejected from the droplets [23]. Although the exact mechanism has yet to be proven, the net result is a free or partially solvated multiply protonated analyte which may be introduced into the mass spectrometer. The ESI process is shown in Figure 1-3.

A. Interface showing spray



B. Electrospray process

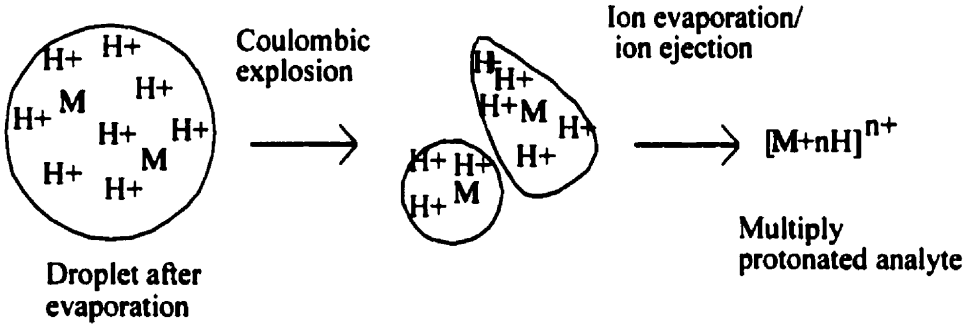


Figure 1-3. ESI fundamentals.
(a) typical interface (b) positive ion electrospray process

1.2.6 Characteristics of ESI

ESI has several key characteristics as an ion source which make it particularly attractive for characterizing biological molecules. The ESI mechanism is by far the softest form of ionization. Not only does this allow the study of intact tertiary structures of analytes, it allows quarternary structure analysis, i.e. the study of non-covalent complexes [24,25]. The other attractive feature is that in ESI, multiple protonation of the analyte occurs. This has a distinct advantage in mass spectrometry. As the m/z value of a species

is measured, by increasing z, it is possible to analyze increasingly high M_r compounds simply by increasing the charge, i.e. 66 kDa/ 45 charges = 1460 m/z [26,27]. A typical ESI mass spectrum contains a charge envelope of a multiply protonated analyte molecule, $[M+nH]^{n+}$ where n is the number of protons. The number of protons attached is dependent on the size and the degree of denaturation of the analyte. As a protein becomes denatured in solution (or the gas phase) it unfolds exposing an increasing number of basic sites which may be protonated [27,28]. A de-convolution algorithm may be used to produce the final mass spectrum in Da. Multiple charging also results in an increase in the sensitivity of analysis with typical solution amounts analyzed in the 10^{-6} M range and absolute amounts in the fmol and sub-fmol range.

The wide range of applications, introduction of the nanospray method [29] and the fact that ESI is easily coupled with on-line separation and purification methods such as HPLC and CE have made ESI one of the most popular sources available [21]

1.3 MASS ANALYZERS

Once the analyte is in the gas phase in an ionized form it enters the mass analyzer and is separated according to its mass to charge ratio (m/z). Separation and detection of ions may be performed in a scanning (filtered) mode as is the case with quadrupole and sector instruments or in a simultaneous mode as is the case with TOF instruments. The performance characteristics of the instruments are determined by the mass range (m/z max where z = 1), their resolution (m/Δm FWHM), ion transmission efficiency and the time required to complete one full m/z scan.

1.3.1 TOFMS

The first TOF mass analyzer was attributed to Cameron & Eggers in 194 [30] and the technique was later described by described by Wiley and McLaren in 1955 [31]. TOF was commercialized by Bendix Corp. who introduced the first commercial instrument [32]. TOF has been increasingly applied to the characterization of biological molecules based on several advantages it has over other analyzers [33,34]

In TOF ions are introduced into the analyzer and are transmitted to the detector as shown in Figure 1-4.

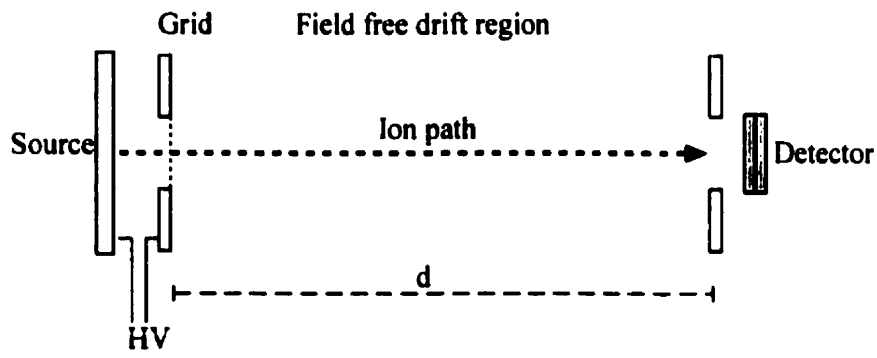


Figure 1-4. Schematic of axial injection linear TOF.

Mass analysis is based on relative flight times of the ions according to Newtonian mechanics:

$$K = \frac{m\upsilon^2}{2} = qV \qquad \textbf{Equation 1-1.}$$

where:

K is the kinetic energy of the ion
q is the charge where q = ze
υ is velocity
m is the mass of the ion
V is the applied potential

The time required for the ion to move a distance d within the field free region is:

$$t = \frac{d}{\upsilon} \qquad \textbf{Equation 1-2.}$$

Combining Equation 1-1 and Equation 1-2 yields the equation which relates the mass of the ion to its flight time:

$$t^2 = \frac{m}{z}\left(\frac{d^2}{2Ve}\right) \qquad \textbf{Equation 1-3.}$$

Thus m/z may be measured based on the time-of-flight of an ion. There is no scanning of fields to filter ions as required with sector and quadrupole instruments. Thus TOF

has a distinct sensitivity advantage over scanning instruments as all the ions introduced into the flight tube arrive at the detector. The elimination of a need for scanning also results in fast data acquisition rates which makes TOF ideal for use with separation methods such as GC-MS and CE-MS. Theoretically there is no upper mass limit in a time of flight analyzer. This has made the TOF instrument very attractive to biological mass spectrometry where analysis of high molecular weight compounds is common.

Early TOF mass spectrometers were not popular due to limits in resolution as a result of the considerable spacial and kinetic energy spreads of the ions when introduced into the flight tube. Resolution and ion transmission efficiency also suffered from axial injection of ions. Introduction of space focusing, ion mirrors and orthogonal ion extraction have improved resolution and facilitated coupling to ESI sources (Figure 1-5).

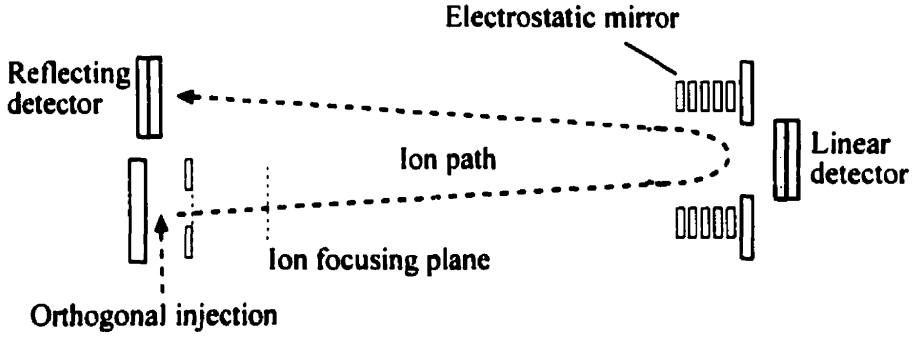


Figure 1-5. Reflecting TOF with ion mirror and orthogonal ion injection.

The first improvement for an ESI source was a result of the introduction of Wiley-McLaren space focusing, or time-lag focusing, correcting for the spacial displacement which ions may possess prior to acceleration into the flight tube [31]. In space focusing, ions are directed into a packet near the ion source which is then accelerated towards the detector. Time-lag focusing was later applied by Vestal for use with MALDI sources to correct for initial velocity distribution of ions within the MALDI plume [35].

The second improvement was the introduction of ion mirrors by Mamyrin, to correct for the kinetic energy distribution of ions entering the flight tube [36]. An ion mirror also has the advantage of effectively doubling the flight path of the ions, which increases

the flight time without increasing their time spread. Thus higher mass analytes may be characterized without a significant loss in resolution. Combined with MALDI, the use of ion mirrors and DE can yield high resolutions with R>15,000 [37]

The instrument design shown in Figure 1-4 is ideal for use with a MALDI source which introduces ions in a packet form, but is much less suited for use with a continuous beam source such as ESI. Orthogonal ion injection facilitates the coupling of ESI and other beam injection sources with TOF (Figure 1-5). The coupling of an ESI atmospheric pressure ion source with orthogonal injection TOF was first described by Dodonov in 1984 [38,39]. Ions are injected into an ion storage region located below the acceleration region. At a relatively fast rate (kHz) ions are pulse extracted into the acceleration region and then into the flight tube. Orthogonal introduction may be used in conjunction with space focusing and ion mirrors, yielding high resolution measurements with R>5000 [40]

1.3.2 Quadrupole MS and Triple Quadrupole MS/MS

Quadrupole mass analyzers are perhaps the most common in mass spectrometers owing to their ease of use, low cost and range of applications. Ferguson and colleagues first described the separation of ions with use of a quadrupolar field [41]. The quadrupole mass analyzer was developed extensively for mass spectrometry by Paul and Steinwegen in the 1950's [42]. Early commercialization was in the late 1950's by Finnigan, and the instrument became popular based on its scan speed, adequate resolution and ease of coupling to GC-MS [43,44].

Figure 1-6 shows a schematic of a typical quadrupole set up consisting of 4 cylindrical rods. Ions enter through a series of focusing lenses along the z-axis and normal to the direction of the quadrupoles.

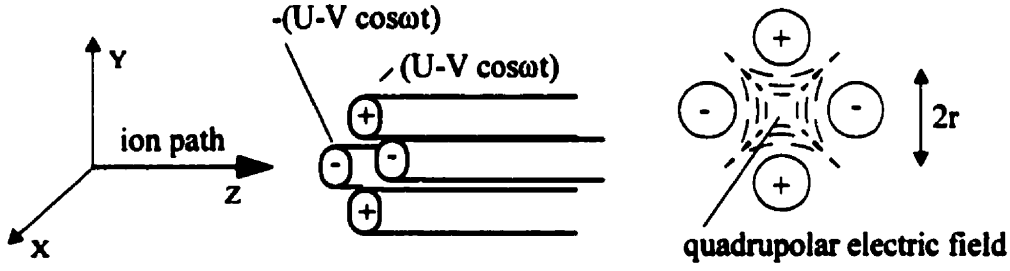


Figure 1-6. Quadrupole set up and applied potentials.

A combination of static DC and RF potentials are applied to the rods to generate an electric field:

$$\Phi = U - V\cos\omega t \qquad \textbf{Equation 1-4.}$$

where:

Φ is the total potential applied to the rods
U is the DC potential (500 to 2000 V)
V is the "zero to peak" amplitude of the RF voltage (0 to 3000 V)
ω is the angular frequency in rads/s = $2\pi f$ where f is the RF frequency

As the ions transverse the length of the quadrupole along the z-axis they experience the electric field potentials in the x and y planes (u = x or y plane) according to:

$$F_u = ma = m\frac{d^2u}{dt^2} = -ze\frac{\partial\Phi}{\partial u} \qquad \textbf{Equation 1-5.}$$

where:

F_u is the force on the ion
m is the mass
a is acceleration
z is the number of charges on the ion
e is electron charge
t is time

The stability of the ion within the applied field and its trajectory is described by the Mathieu equation [44]:

$$\varepsilon = \frac{\omega t}{2} \qquad \textbf{Equation 1-6.}$$

$$a_u = \frac{8zeU}{mr^2\omega^2} \qquad \textbf{Equation 1-7.}$$

$$q_u = \frac{4zeV}{mr^2\omega^2} \qquad \textbf{Equation 1-8.}$$

$$\frac{d^2u}{dt^2} + (a_u - 2q_u\cos 2\varepsilon)u = 0 \qquad \textbf{Equation 1-9.}$$

where:

r is the radius of the quadrupole field

If u (x and y) is less than the radius of the field then the ions will pass through the quadrupole to the detector. For a given quadrupole r and $\omega = 2\pi f$ remain constant while U and V may be varied (Figure 1-7).

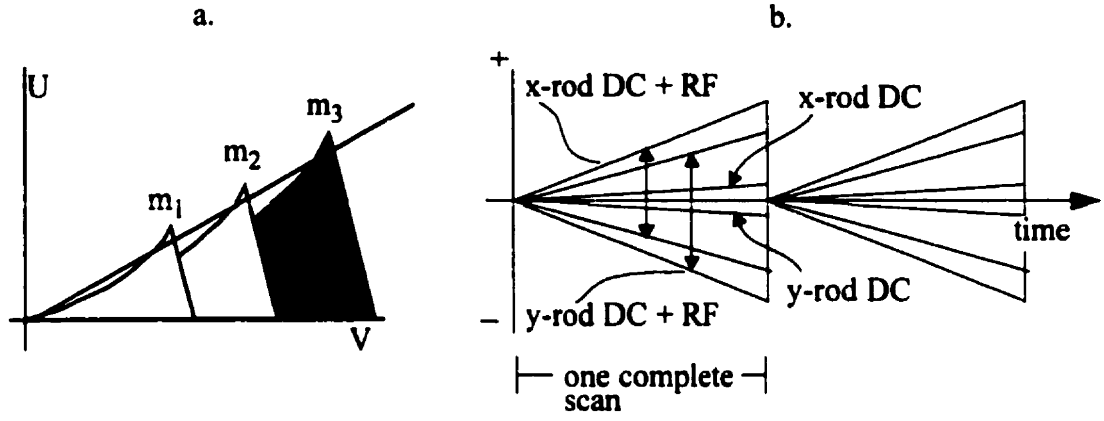


Figure 1-7. Scanning of ion stability diagram for quadrupolar field. (a) area under curves for ions m_1 to m_3 indicates combinations of U and V for ion stability, (b) RF and DC scanning of quadrupole potentials.

According to the relationships given in Equation 1-7 and Equation 1-8 ions of a given mass and charge will possess stable trajectories (indicated by the shaded areas in Figure 1-7.a. for individual masses). This allows ions of increasing m/z (m_1-m_3) to pass through the quadrupole (solid line in Figure 1-7.a.). By ramping U and V is with respect to time (Figure 1-7.b.) ions whose m/z correspond to a stable region within the stability diagram will pass through the quadrupole. Other ions will possess unstable trajectories and impinge upon one of the rods prior to reaching the detector.

1.3.3 Properties of a Triple Quadrupole Mass Analyzer

The instrument is considered to be of moderate resolution, typically unit mass resolution at m/z = 1000. Upper mass ranges are generally limited to 3000 to 4000 Da which is sufficient for most types of analyses when coupled with ESI. Ions are separated (filtered) in a scanning fashion with ca. 500 Da per second scan rate for unit resolution at m/z = 1000. This requires a wide time window for sample detection (i.e. peak widths of at least 8 sec-

onds) which makes for an ideal detector for use with on-line high performance liquid chromatography (HPLC) but less suitable for capillary electrophoresis (CE).

The most interesting application of quadrupole MS is realized when two or more quadrupoles are coupled together in series for use in tandem mass spectrometry (MS/MS) for biomolecule sequencing. McLafferty [45] and Jennings [46] described CID MS/MS. Peptide and oligonucleotide sequencing was developed extensively by Biemann in the late 1950's and 1960's [47]. MS/MS on a triple quadrupole mass spectrometer was introduced by Yost and Enke in 1978 [48]. They used a small RF only center quadrupole between two scanning quadrupoles. The RF quadrupole was used to re-focus ions after collision induced dissociation of ions selected within the first quadrupole. This led to improved ion yields and sensitivity.

1.3.4 MS/MS for Peptide Sequencing

In MS/MS, peptide fragmentation takes place by an ordered process dependent upon the nature of the peptide and the amino acid sequence [47,48]. MS/MS may be performed using a triple quadrupole mass spectrometer or other hybrid instrument, i.e. QqTOF (see Section 2.2.6) [50,51] or, for example, by using an ion trap.

In triple quadrupole MS/MS ions are introduced into the first quadrupole of the mass spectrometer. The ion of interest is isolated based on its m/z value (precursor ion) and allowed to pass through to the second, RF only, quadrupole. The second quadrupole acts as a collision cell and contains an inert gas, typically N_2, at a moderately high pressure relative to the rest of the vacuum system (i.e. $2x10^{-3}$ Torr). The ion undergoes fragmentation through CID with the collision gas to produce product ions. These product ions are then analyzed according to their m/z values in the third quadrupole. The overall process is shown in Figure 1-8.

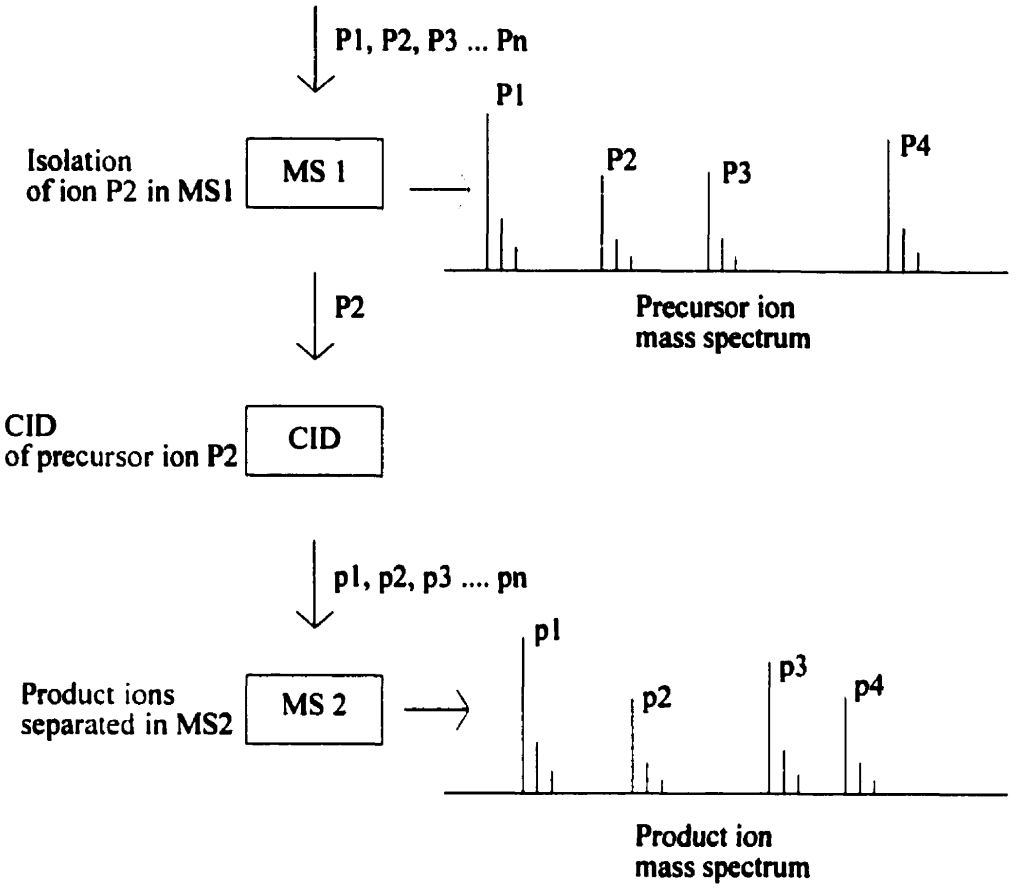
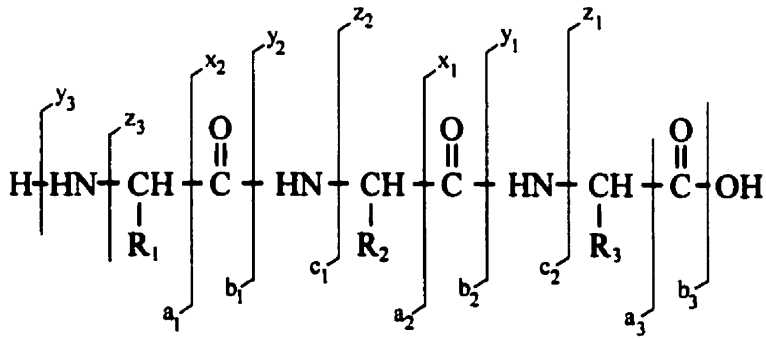
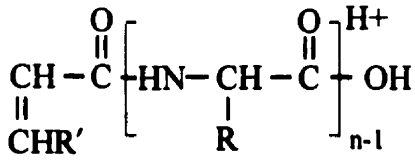


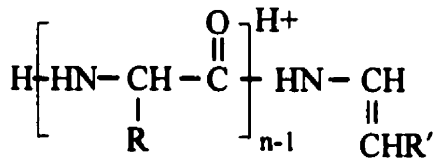
Figure 1-8. Overview of MS/MS with CID.

Nomenclature for peptide fragmentation have been developed based on empirical measurements and are outlined in Figure 1-9 (Roepstorff and Fohlman nomenclature) [47,49,52]. In low energy CID MS/MS, y and b ions tend to be prominent, as are certain immonium ions. As the fragmentation process is ordered it is possible to sequence the peptides based on their molecular weight. Sequencing is generally limited to low molecular weight peptides whose m/z are less than the upper mass range of the first quadrupole. By increasing the collision energy other ions may be formed, i.e. internal fragment ions, which may yield additional information on the peptide structure. Not all peptides are easily characterized. Isobaric amino acids i.e. Ile and Leu cannot be discerned without difficulty.

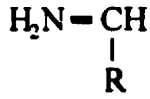




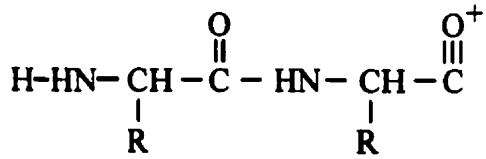
w_n



d_n



Immonium ion



Internal acyl fragment ion

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y1 = mass AA1 + 19	x1 = y1 + 26	z1 = y1 - 17
y2 = mass AA2 + y1	x2 = y2 + 26	z2 = y2 - 17
y3 = mass AA3 + y2	x(n-1) = y(n-1) + 26	zn = y3 - 17
yn = MH+		
b1 = AA1 + 1	a1 = b1 - 28	c1 = b1 + 17
b2 = AA2 + b1	a2 = b2 - 28	c2 = b2 + 17
b3 = AA3 + b3	an = bn - 28	c(n-1) = b(n-1) + 17
bn = MH+ -H_2O		

Figure 1-9. Peptide sequencing nomenclature [52].
Mass of AA = M_r amino acid - H_2O.

1.4 BIOLOGICAL MS SAMPLE PURIFICATION

MS by itself is not sufficient to achieve structural characterization of complex mixtures of biomolecules, and preliminary separation/purification steps are often required in order to obtain compounds that are reasonably pure and/or suitable in size and polarity for MS. Sample preparation is then perhaps the most important parameter influencing the quality of data produced in mass spectrometry with exception to instrument characteristics and instrument design. Biological samples tend to be incorporated within a complex matrix including mixtures of other analytes and buffer components such as salts. The presence of these components in the sample will interfere with both the ESI and MALDI ionization processes yielding poor quality mass spectra. Complete suppression of ionization, an overabundance of background ions, and the formation of analyte-interfering ion complexes called adducts may occur. A number of sample introduction, purification and separation methods exist for the analysis of biological compounds and several of these have been incorporated in on-line and off-line configurations interfaced with MS.

1.4.1 MALDI Sample Purification

Although MALDI is tolerant to relatively high concentrations of buffers, salts detergents, etc., samples must still be relatively clean. Sample preparation ultimately determines whether or not good results may be obtained. There are a large number of variables which will ultimately affect the sensitivity, resolution and accuracy of results [53]. Factors to consider are: type of analyte (i.e. peptide, protein, mixture of peptides), type of analyte contaminants (i.e. buffer, salts detergents), type of matrix, solution type for matrix preparation, addition of matrix modifiers and crystallization methods. For good quality MALDI spectra, one strong signal recorded at maximum resolution for each single component on the probe (with the exception of the matrix) is required. Interfering components such as salts, buffers, detergents may result in adduct formation which in turn will affect the sensitivity, resolution and accuracy of the results. In addition, the presence of other components in the sample may affect the crystallization to such an extent that no signal will be observed at all. As MALDI is an on-probe method sample purification is limited to an off-line approach.

1.4.2 ESI Sample Purification

In comparison with MALDI, ESI requires considerable sample purification prior to analysis. The method is overly sensitive to impurities. For example, salts present within the sample will compete with protons for free basic sites on the analyte. This results in selective ionization, overlapping charge envelopes, adduct formation and may lead to complete signal suppression.

Off-line purification and on-line sample purification may be used. On-line separation is preferable as it does not introduce any extra steps in the analysis scheme. In addition to an improved time frame of analysis the elimination of off-line sample manipulation reduces the possibility of sample degradation and loss. On-line separation is beneficial in the case of mixture analysis because it presents a single component of the mixture to the mass spectrometer at once. This is especially important when source CID is used for sequencing. Reversed phase HPLC and CE are easily coupled to mass spectrometers with ESI sources. HPLC-MS is routinely used while CE-MS is still a relatively new sample separation/ purification method. Both methods make it possible to characterize an analyte based on its retention time (Rt) or migration time (Mt) as well as the mass spectra. Selected ion chromatograms/ electrophorograms may be used to digitally enhance sensitivity and facilitate mixture analysis.

1.5 OVERVIEW OF THESIS

This thesis is based on these two very independent methods for the purification, separation, introduction and characterization of analytes by mass spectrometry.

The first section (Chapters 2-6) describes the use of polyurethane membrane as a sample support for MALDI-TOFMS. This work is based on previous experiments in our laboratory using nitrocellulose supports and work by others using porous PVDF membranes. The topic of sample supports is introduced in Chapter 2 for MALDI-TOFMS. The initial characterization of this approach is summarized in Chapter 3. Applications to the characterization of wheat proteins (Chapter 4), hemoglobin variants (Chapter 5) and biomaterials (Chapter 6) are presented.

The second section of this thesis describes the development of on-line capillary electrophoresis MS for sample separation, purification and introduction into the mass spectrometer. The subject is introduced in Chapter 7. CE-MS experiments are described in Chapter 8, using a triple quadrupole mass spectrometer for the characterization of peptides and MS/MS. Application to peptide mass mapping is shown. Chapter 9 outlines early results on interfacing CE with a reflecting time-of-flight mass spectrometer for peptide and protein characterization. Chapter 10 compares results obtained using the two instruments for the characterization of a medium molecular weight protein, citrate synthase.

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2 MALDI USING PU SAMPLE SUPPORTS

2.1 INTRODUCTION

MALDI is relatively tolerant of impurities, such as salts and buffers. Molecular ions of peptides and proteins can still be produced by MALDI, even with salts or buffers at concentrations which would hamper other ionization processes such as Electrospray (ES). However, in order to obtain the best results samples must be relatively pure prior to analysis. Numerous experimental parameters such as sample preparation may affect the sensitivity, resolution and accuracy of measurements. Several methods of sample preparation using different matrices have thus been developed for MALDI-TOFMS applications [1]. Purification is performed to remove components which may interfere with the signal during data acquisition. High salt concentrations strongly disrupt crystallization and quench MALDI signals. Smaller amounts result in adduct formation on the analyte molecular ions and thus produce broadened and poorly resolved peaks.

Techniques for sample purification prior to MALDI-TOFMS analysis have thus been developed. Many of these are based on traditional separation techniques including dialysis, filtration and chromatography. Although effective, these methods have limitations, including sample loss during processing and time-consuming sample preparation. Another approach is to purify the sample directly on the surface of the MALDI probe. By incorporating the sample purification step with the addition of matrix, the time frame of analysis may be reduced and only a minimal amount of sample is required.

Various approaches to on-probe sample purification based on a common theme have been explored. In all of these a substrate is first deposited onto the surface of the MALDI probe, either by mechanical adhesion to the surface (i.e. polymeric membranes and films), or by covalent attachment to the surface. The substrate possesses a chemical or physical property which allows it to selectively bind the analyte of interest. Once the analyte is bound, it is possible to remove other components from the surface by washing procedures. Addition of the matrix to the probe, in a suitable solvent, releases the bound analyte which then may co-crystallize with the matrix for analysis by MALDI. This overall

approach is easy and cost-effective. Moreover, it makes sample pre-concentration possible through application of successive sample aliquots to the probe. The process is outlined in Figure 2-1.

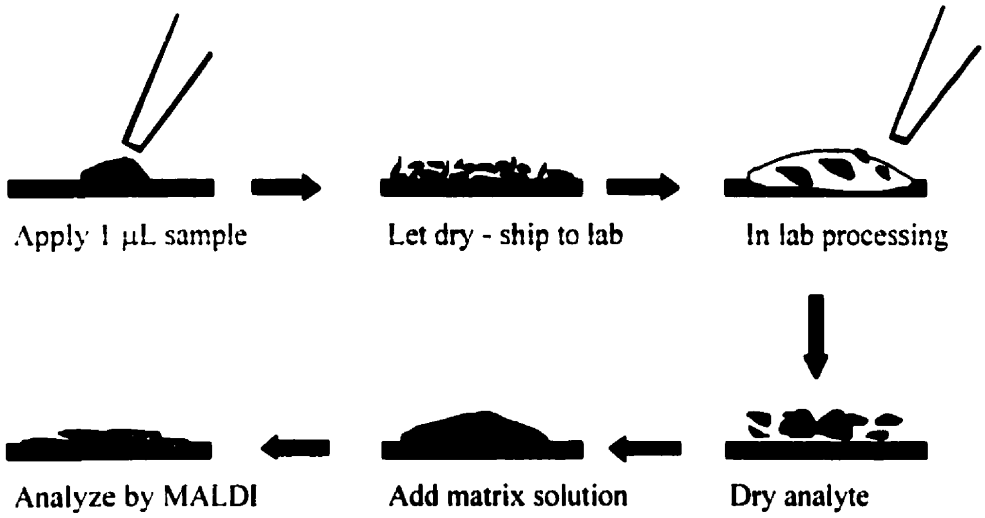

Figure 2-1. Sample preparation for on-probe purification.

Surface Enhanced Affinity Capture (SEAC), introduced by Hutchens and Yip [2], was an early example of sample pre-treatment directly on the MALDI probe. The SEAC probe surface was modified with a functional group (agarose) used to selectively bind the analyte of interest. Another similar example involved the use of a small amount of powdered chromatographic packing placed on the MALDI target [3]. Chemically-modified probes supporting active surfaces have also been developed. Examples of these have been reported by Brockman and Orlando and include a C18 reversed phase surface as well as an antibody/antigen surface [4,5]. MALDI probes with covalently-bound trypsin and other enzymes have been extensively developed by Nelson and co-workers [6] who recently demonstrated the on-probe digestion of bovine hemoglobin using a trypsin-active probe. According to their work [7], detergents were used to facilitate the denaturation of hemoglobin on the probe surface, which resulted in an increased extent of digestion compared to digests performed without the detergent.

However, modification of the probe surface as described above is time-consuming, and the probes have a limited lifetime. Also, there exists the possibility of sample carry-

over if the probe is re-used. In addition, samples must still be transported to the MALDI-TOFMS laboratory by conventional means, i.e. in solution and on ice.

An alternative technique involves modifying the probe surface by the addition of a polymeric membrane or film which binds to the sample protein and allows washing of buffer components. This approach, based on the use of membranes for automatic protein sequencing, is now used routinely for sample purification and introduction into the mass spectrometer [8-10]. Hydrophobic/ionic interactions between the sample and the film/ membrane allow the selective removal of interfering buffer components. Addition of matrix solution directly onto the surface promotes partitioning of the analyte and salts/ buffer components between the liquid phase and the surface of the film/membrane. Purification by on-probe washing and performance of on-probe enzymatic digestion result in minimal sample loss, since proteins and peptides are bound fairly strongly to the membranes by hydrophobic interactions. Derivatization and proteolytic digestion may be performed on the surface of the film/membrane. Membranes, which are readily available in large quantities and various sizes, are very convenient for transporting samples from the preparation site to the MALDI-TOFMS laboratory.

A number of different polymeric supports have been examined for use with MALDI. An early example by Mock et al. used an electrosprayed surface of nitrocellulose to create a thin film on a gold surface [11]. Since then, a number of other membranes and films have been used. These include cellulose [12], nitrocellulose [12-14], polyethylene [15,16], Nafion (perfluorosulfonated ion exchange membrane) [17], nylon [18], poly(vinylidene difluoride) (PVDF) [19], polypropylene [19], paraffin wax [20], and poly(tetrafluoroethylene) [21].

We introduced the use of non-porous PU membrane as a sample support for MALDI-TOFMS of peptides and proteins in 1997 (Appendix III). This research is summarized in Chapter 3. Several applications arose based on this work and results which were published or presented at conferences are described in Chapters 4-6.

2.2 EXPERIMENTAL

2.2.1 Common Reagents

All chemicals used in this study were of reagent grade unless otherwise stated. HPLC grade deionized water, prepared with a Milli-Q plus-TOC water purification system (Millipore, Bedford, MA), was employed in all solutions. Analytical grade acetic acid, HPLC-grade acetonitrile and electronic grade methanol were purchased from Mallinckrodt (Paris, KY). HPLC grade acetonitrile, dithiothreitol (DTT), and ethanol were from Fisher Scientific (Fair Lawn, NJ); trifluoroacetic acid (TFA) was from Sigma (St. Louis, MO). Sinapinic acid, used as the MALDI matrix (saturated in 70:30 or 50:50 water-acetonitrile (v/v), was obtained from Sigma.

Plasma protein standards (human, bovine and canine), obtained from Sigma Chemicals (St. Louis, MO), were made up in water and used without further purification. Protein standards were used as MALDI calibrants. Horse heart myoglobin, 16951 Da, bovine insulin, 5733 Da, bovine serum albumin, 66430 Da, carbonic anhydrase 29021 Da, human transferrin 79549 Da were purchased from Sigma, and bovine apotransferrin, 78030 Da, was obtained from Calbiochem (LaJolla, CA). Solutions of these standards were made up in water, and used without further purification.

2.2.2 Scanning Electron Microscopy

The PU membrane and film were characterized by scanning electron microscopy (SEM). Scanning electron micrographs were obtained on a Cambridge Instruments microscope. Samples prepared on PU membranes and on metallic surfaces were coated with gold and palladium by plasma deposition prior to analysis. Images were recorded with magnifications of 30 x and 300 x.

2.2.3 PU Sample Supports

The non-porous ether type polyurethane (PU) membrane, 25 μm and 50 μm in thickness (XPR625-FS), was supplied by Stevens Elastomerics (Northampton, MA). The PU membrane used consists of a co-polymer of polytetramethylene ether glycol and methylene bis(p-phenyl diisocyanate) (MDI) [22]. The membrane was washed with water and methanol prior to use in order to remove polar and non-polar surface contaminants. A cross

section of the membrane is shown in Figure 2-2. Properties of the membrane are discussed in Chapter 3.3.

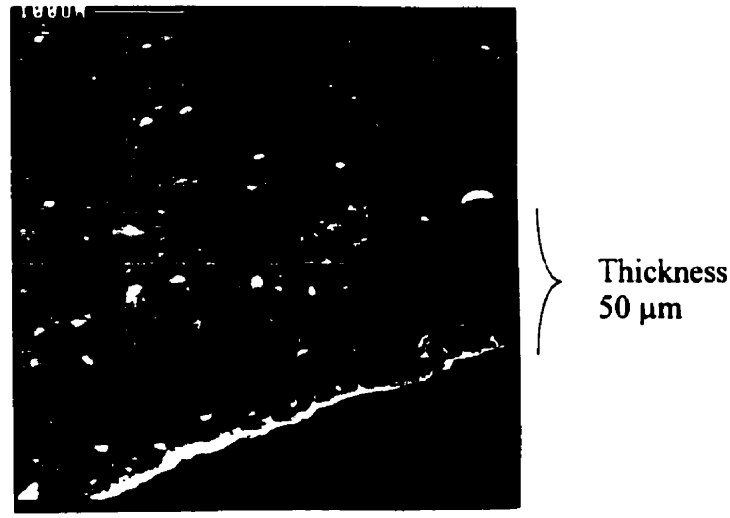

Figure 2-2. SEM of 50 μm PU membrane showing surface and edge.

Porous PU thin films were prepared by first dissolving the solid PU membrane in tetrahydrofuran and then depositing ca. 10 μL of the liquid onto the probe surface, spreading it around, and then allowing it to dry.

2.2.4 Sample Preparation Protocol

Sample preparation for MALDI was based on the dried-drop method [23]. The sample solution (2 μL) was placed on the membrane and the solvent allowed to evaporate slowly. Methanol (2 μL) was added and also allowed to evaporate. The matrix solution (2 μL) was then added and allowed to crystallize slowly. When incorporating a washing step, 20 μL aliquots of water were applied to the sample at intervals of one minute, and then removed prior to the addition of matrix. After the matrix had dried, the membrane was placed on a silver disk which had been coated with a thin layer of adhesive (Spraymount, 3M). Excess membrane was trimmed from the disk and the disk placed into the MALDI probe. This process is shown in Figure 2-1. The MALDI probe used is shown in Figure 2-3.

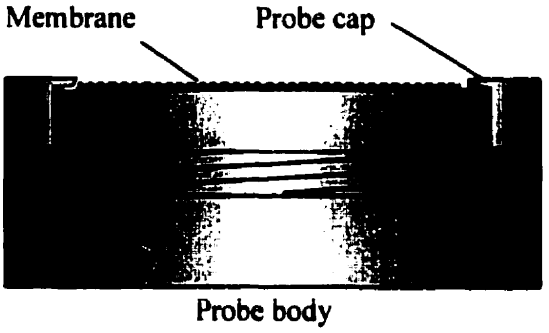

Figure 2-3. Two piece MALDI probe.

2.2.5 MALDI-TOFMS

Positive ion mass spectra were obtained in the linear modes on a MALDI-TOF mass spectrometer built in the Time-of Flight Laboratory, Department of Physics, University of Manitoba (Figure 2-4) [24]. A nitrogen laser (VSL 337 ND, Laser Science Inc., Cambridge, MA, USA) was used with intensity set just above threshold. For some of the measurements, a 2-grid delayed extraction system was employed [25], with a 25 kV DC accelerating potential on the probe and first grid, and a 3 kV pulse applied to the probe 1.2 μs after the laser pulse. The 3 kV pulse was supplied by a high voltage switch (Behlke, Frankfurt, Germany). For linear DC measurements extraction was employed with the target and first grid potentials of 30 kV and 18 kV or 25 kV and 15 kV, respectively. Following desorption and acceleration, ions drifted through a field-free region of 1.2 m in which the nominal pressure was 3×10^{-7} torr. The ions were detected with microchannel plates and the signal was recorded with a LeCroy 9350AM 500 MHz Digital Oscilloscope from which the spectra were summed by a UMAX Supermac C600 computer.

Each spectrum presented here results from the summation of 50 - 200 consecutive shots. External or internal calibration was performed. External calibrations for measurements using the PU membrane were performed with standards prepared on similar membrane targets.

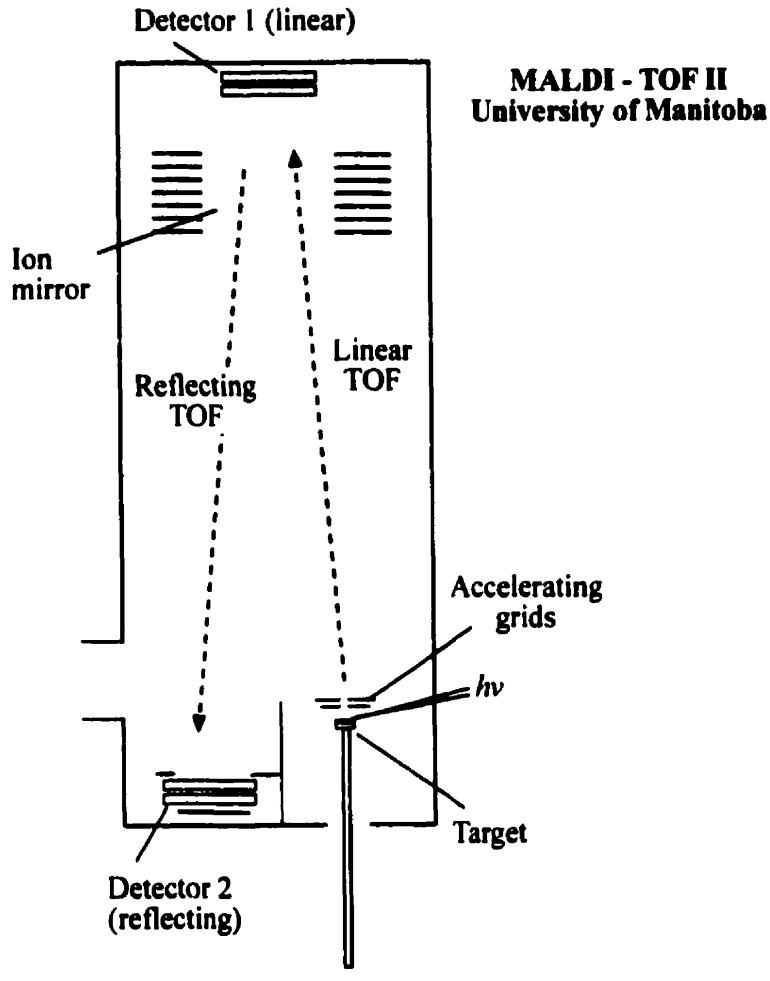

Figure 2-4. Schematic of Manitoba TOF II used for MALDI experiments.

2.2.6 MALDI-QqTOFMS

Positive ion mass spectra were obtained in the reflecting mode on a PE-SCIEX prototype MALDI-QqTOF mass spectrometer built the Time-of Flight Laboratory, Department of Physics, University of Manitoba (Figure 2-5). This instrument consists of a triple-quadrupole mass analyzer mated to a reflecting time-of-flight mass analyzer and has been used previously with an ESI source [26].

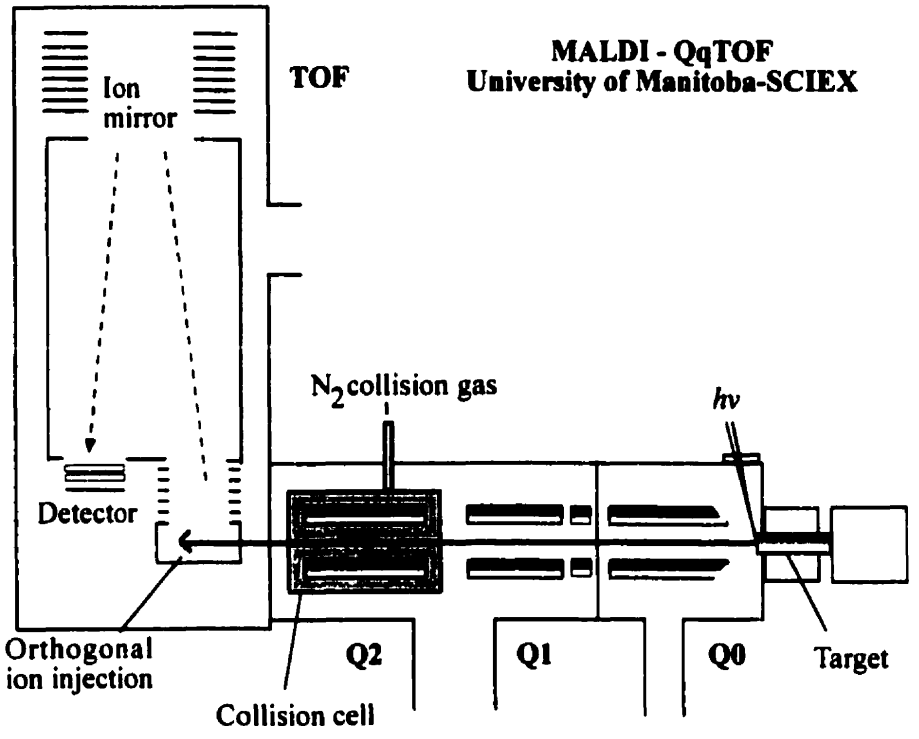

Figure 2-5. Schematic of the prototype QqTOF.

The second scanning quadrupole is replaced with a TOF analyzer which affords higher sensitivity and resolution. A MALDI ion source was constructed based on the ESI probe design. A nitrogen laser (VSL 337 ND, Laser Science Inc., Cambridge, MA, USA) was used to produce MALDI ions which enter the first quadrupole (Q0), normal to the ion path of the TOF analyzer. Here they undergo collisional cooling transforming the pulsed MALDI plume to a semi-continuous beam [27,28]. The second quadrupole (Q1) allows selection of the precursor ion which may be fragmented by collision induced dissociation in the second quadrupole (Q2) prior to analysis by TOF. Tandem MS was performed using N_2 as the collision gas. Collision energies and gas pressures were optimized on a sample to sample basis. This instrument is described in greater detail elsewhere [26,28].

Each spectrum presented here results from the summation of 50 - 200 consecutive shots. External calibrations were performed using standards applied to steel targets. The design of the instrument required calibration only once prior to acquiring spectra.

2.2.7 Safety Considerations

Standard precautions regarding the collection and handling of biological fluids were followed.

2.3 REFERENCES

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3 PU MEMBRANE AS A SAMPLE SUPPORT FOR MALDI-TOFMS OF PEPTIDES AND PROTEINS

3.1 INTRODUCTION

Investigation into using non-porous PU membrane as a sample support for MALDI-TOFMS is described here. The choice of PU is based on previous studies performed in our Chemistry Department on PU foam and PU membranes which demonstrate that uptake of a neutral analyte by PU is strongly favoured over uptake of a charged species [1]. PU membranes have been used previously for the separation and concentration of neutral metal complexes and organic dyes from aqueous solution [1,2]. They possess a unique two-phase structure consisting of hydrophobic soft domains and relatively hydrophilic hard domains. Proteins and lipids have been shown to adsorb through hydrophobic interaction with the soft domains of the polymer [3].

3.2 EXPERIMENTAL

3.2.1 Reagents and Materials

Immobilon-P membrane (PVDF), used as a standard MALDI-TOFMS sample support, was obtained from Millipore (Marlborough, MA). The PU and PVDF membranes were washed with water and methanol prior to use.

3.2.2 Proteolytic Digestion of Citrate Synthase

Wild type citrate synthase (E. coli. 47886 Da, 1.0 mg/mL in 20 mM Tris-HCl and 1 mM EDTA, pH = 7.8) was supplied by A. Ayed and H. Duckworth [4]. The molecular weight was derived from the Swiss-Prot sequence [5] (Appendix II) with the following modifications: loss of N-terminal M, post-translational modification (11:N-D) [6], conflict (289:F-V) [7]. Proteolytic digestion was performed on- and off-membrane with assistance from L. Donald, Chemistry Dept. University of Manitoba. On-membrane digestion was performed on 2 μL of protein solution, which was initially placed directly on the membrane and allowed to dry. Trypsin, (2-10 μL, 0.01 mg/mL in same buffer) was then placed onto the protein spots and the digestion was allowed to proceed for times ranging from 2 to 60

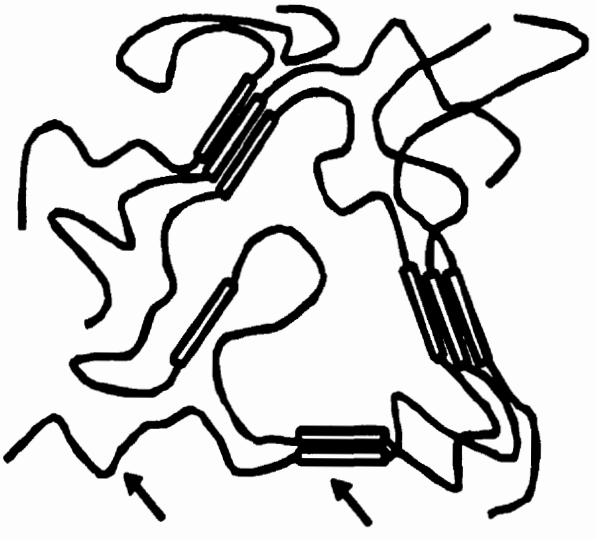
minutes. The digestion was stopped by adding 1 μL of a 1% solution of acetic acid. Digestion of citrate synthase was repeated in Eppendorf™ tubes under similar conditions, for time periods of 2 to 60 minutes and also for 3 hours, after which the digest was presumed to be complete.

3.3 RESULTS AND DISCUSSION

3.3.1 Properties of PU membranes

The ether-type PU membrane used possesses a unique two-phase structure consisting of hard (moderately polar) and soft (non polar) domains, as shown in Figure 3-1 [8,9]. The hard domains are microcrystalline regions on the surface of the polymer, where the isocyanate portions of the polymer chains are aggregated by hydrogen bonding between the carbamate groups on adjacent polymer chains. These hard domains are relatively polar in comparison to the soft segment domains. The soft domains consist of long chains which are relatively amorphous in character compared with the hard domains.

a.



b.

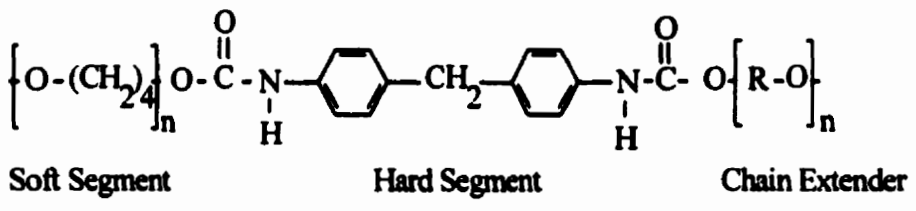


Figure 3-1. Structure of PU used in the membrane.
(a) macroscopic view [8] (b) chemical structure [9]

The two-phase structure of the polyurethane elastomer provides two different regions of possible membrane-protein interactions, differing in polarity and in ability to form hydrogen bonds. Hydrogen-bonding with the hard domains and hydrophobic interactions with the soft domains are believed to take place between the protein and the PU membrane, resulting in relatively strong analyte binding [2,3].

Initial work on PU membranes showed that the addition of methanol to samples deposited on the membrane caused swelling of the PU and enhanced protein sorption. Proteins prepared on PU without the addition of methanol were desorbed from the membrane more easily with washing. Addition of methanol possibly causes disruption of the intermolecular forces holding the polymer chains together, allowing an increase of the effective surface area available for protein sorption. Methanol also facilitates the partitioning of proteins and peptides from more polar components, such as salts.

Preparation of samples on PU membranes and introduction of the samples into the mass spectrometer were relatively straightforward. Both steps were facilitated by the probe design (Figure 2-3). Samples could be prepared on the PU membranes in our chemistry laboratory and affixed to the metallic disks allowing analysis at a later date in the MALDI-TOFMS lab. The dual-part probe design enabled the introduction of samples at a rate of one every few minutes. The longest delay was due to the evacuation of the mass spectrometer. It is noted that for larger probe surfaces several samples may be run at once by attaching the PU membrane to the surface of the probe with an adhesive (i.e. Spraymount, 3M).

The spectra of myoglobin obtained a) using a PU membrane and b) a metallic target are shown in Figure 3-2. The spectra were essentially identical. Equivalent resolution and mass accuracy were observed for several proteins of medium molecular weight, whether the samples were deposited on a PU membrane or on a metallic target, with irradiation at 337 nm. In general, spectral acquisition using the PU membranes was more facile and reproducible than with the metal targets using the dried-drop method of sample preparation. Results obtained for several myoglobin samples indicate an average mass resolution of 200 (linear DC mode) and an accuracy of ±0.026% with external calibration.

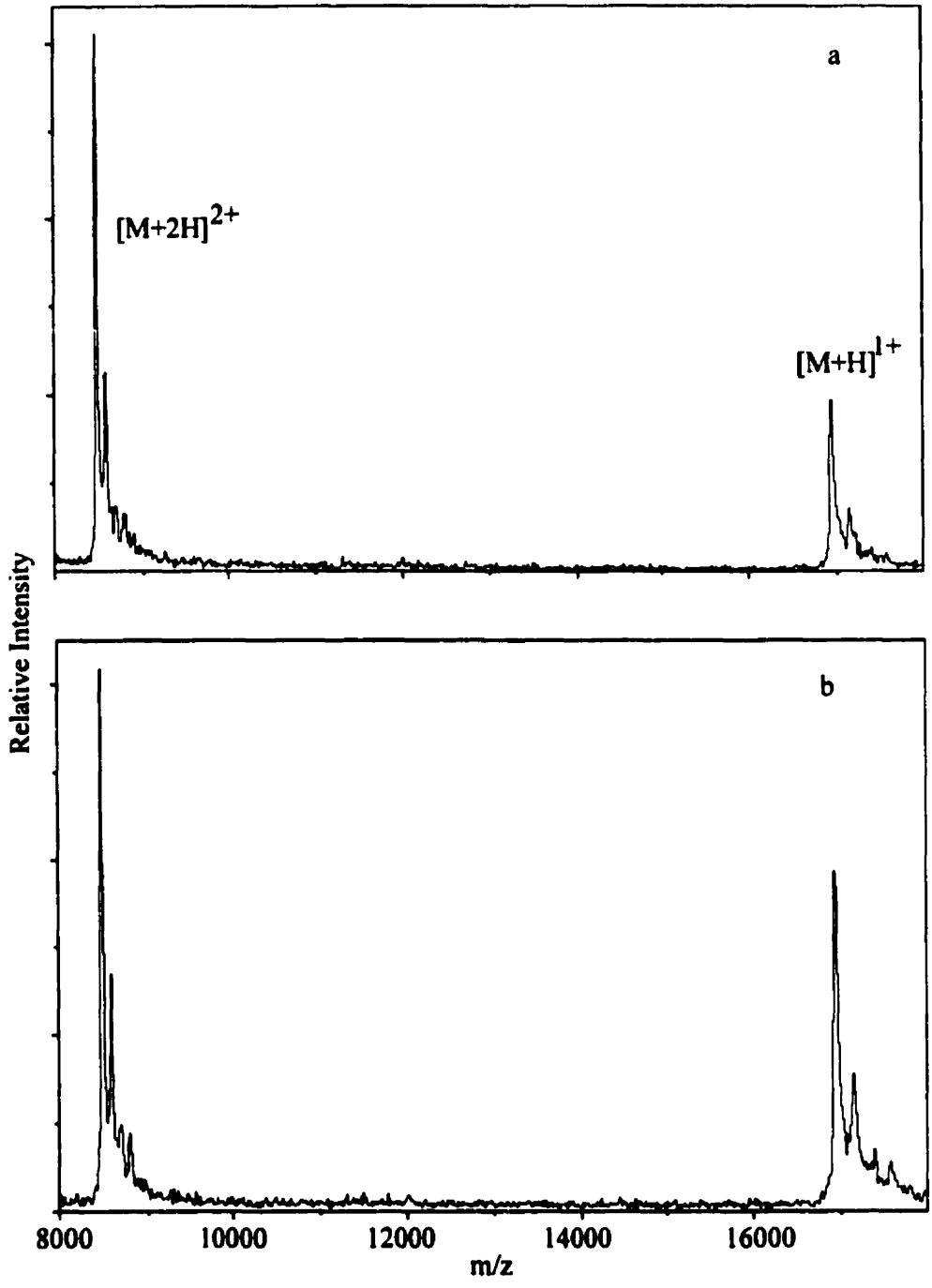


Figure 3-2. Myoglobin on PU vs. steel target.
200 pmol of myoglobin in sinapinic acid, obtained using (a) a PU membrane, and (b) a metallic target; accumulation of 50 shots.

Charging of the membrane, a phenomenon sometimes observed in MALDI [10], occurred at laser intensities well above threshold. The build-up of a static charge on the membrane affects the potential of the target surface and thus influences the flight time of the ions. This resulted in an increase in the time of flight of bovine insulin ions as a result of charging (Figure 3-3). Also, as the sampling frequency was increased from taking individual shots at less than 1 Hz to ~ 10 Hz, the charging phenomenon became more pronounced.

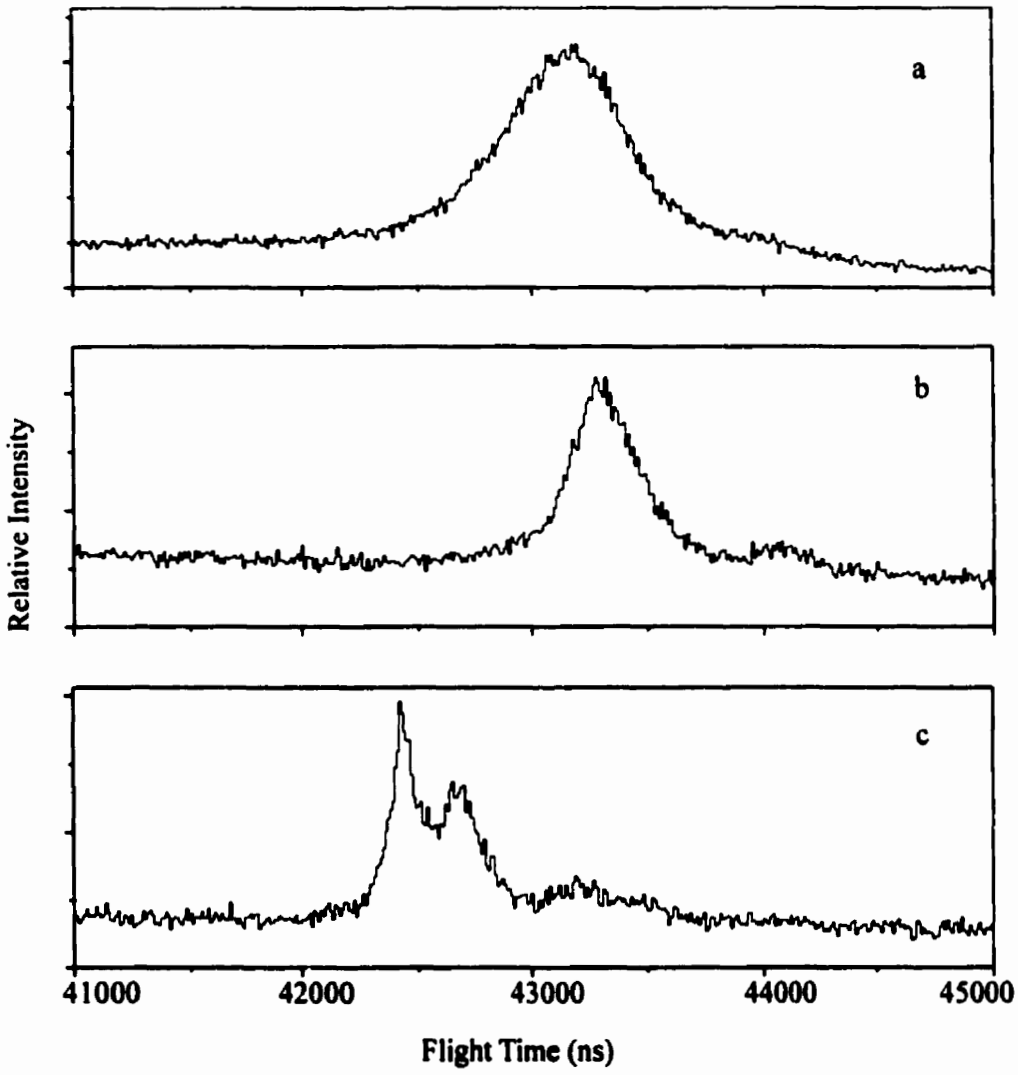


Figure 3-3. Charging effect on the spectra of bovine insulin.
(a) high laser fluence (b) high laser fluence and fast pulse rate,
(c) near optimum conditions.

This was attributed to shorter times being available for the dissipation of the static charge built up on the membrane. A decrease in resolution resulted, as well as longer flight times. However, the laser intensity used was substantially above the intensity required at threshold, and thus charging was not observed under normal operating conditions with PU.

3.3.2 Comparison Between PU, Metal and PVDF

A comparison between the time-of-flight of bovine insulin on a PU membrane and on a PVDF membrane is shown in Figure 3-4 for 5 - 250 pmol of sample. In general, flight times for proteins and peptides prepared on the PU surface were more precise.

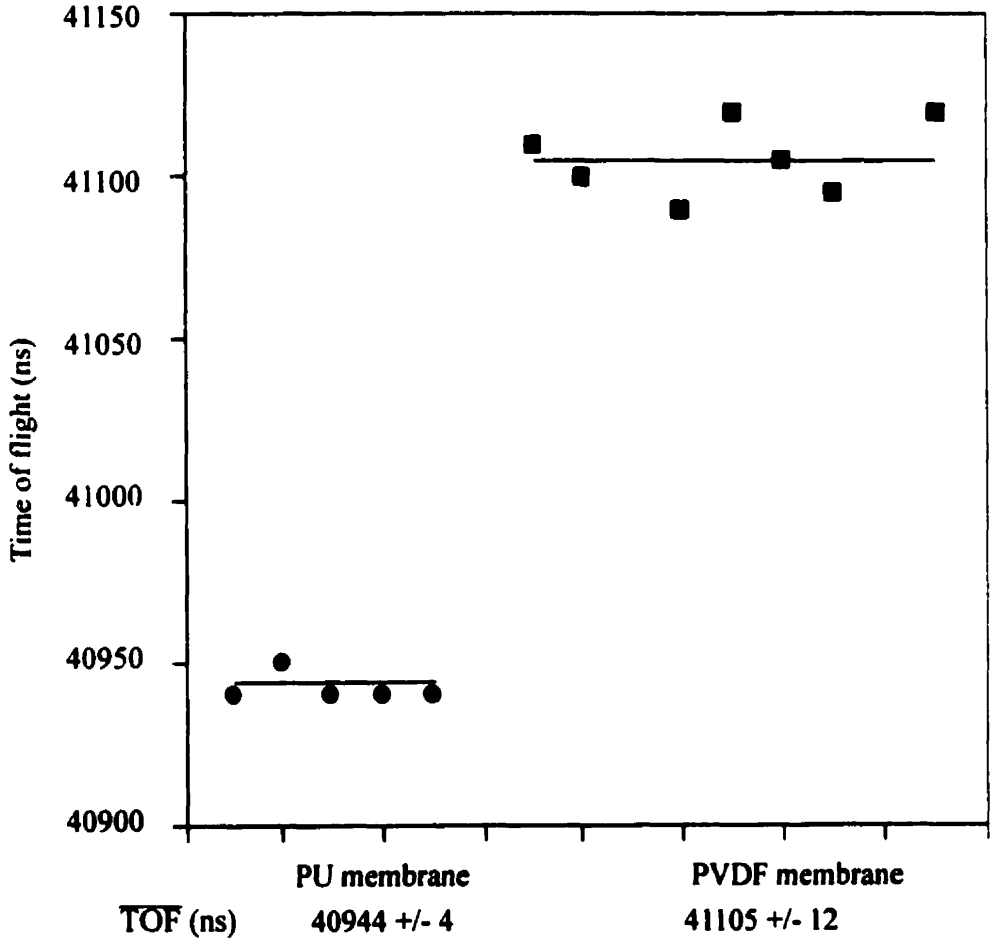


Figure 3-4. Comparison of the TOF of BI on PU and PVDF.
Flight times of bovine insulin $[M+H]^+$ ions generated by MALDI. (Bovine insulin: 5 - 250 pmol). Accumulation of 50 shots per measurement.

The distribution in the flight times for bovine insulin ions desorbed from PU membranes shows a similar variance to what is typically obtained on a metal target and a smaller variance than observed for ions desorbed from PVDF membranes. In general, peak shapes were better on PU membranes compared to metal targets, making centroid assignment more systematic. This was possibly due to partitioning of the bound protein molecules from interfering adducts (e.g. salts) which can affect the position of the peak centroid. PU-deposited samples were also tolerant of a large range of laser intensities, without observation of peak broadening due to charging or adduct formation. A larger variance in flight times was observed with PVDF due to the spatial distribution of sample within the pores and the larger laser intensity required to generate spectra. Relatively poor mass resolution was observed for samples prepared on PVDF membranes ($R = 211$, $n = 9$). In comparison, samples prepared on PU membranes ($R = 721$, $n = 4$) yielded similar values to those obtained on metal targets ($R = 764$, $n = 3$).

Comparisons among the spectra obtained for bovine insulin on a PU membrane, on a PVDF membrane and on a metallic target are shown in Figure 3-5 for 50 pmol of sample. The PU membrane (a) and metallic target (c) yielded comparable mass resolution and mass accuracy for 50 pmol amounts of bovine insulin. However, in our hands the mass accuracy observed with PVDF (b) was not as satisfactory with 50 pmol of sample.

A comparison was also made with 5 pmol amounts of bovine insulin (Figure 3-6). In this case, PU and the metallic target yielded comparable spectra to that observed with 50 pmol of sample while the PVDF membrane produced poor quality spectra. In the case of PVDF, the laser intensity required to obtain ionization threshold was higher than that required for PU and the metal target. This was attributed to the porosity of PVDF, which permits distribution of the analyte and matrix within the membrane [11]. The result is surface charging which causes an increase in the flight time, as shown in Figure 4b. In comparison, the non-porous nature of the PU membrane (or a metallic surface) favours crystal growth on the surface only. PU thus provides for enhanced spectral quality over membranes with porous structures such as PVDF.

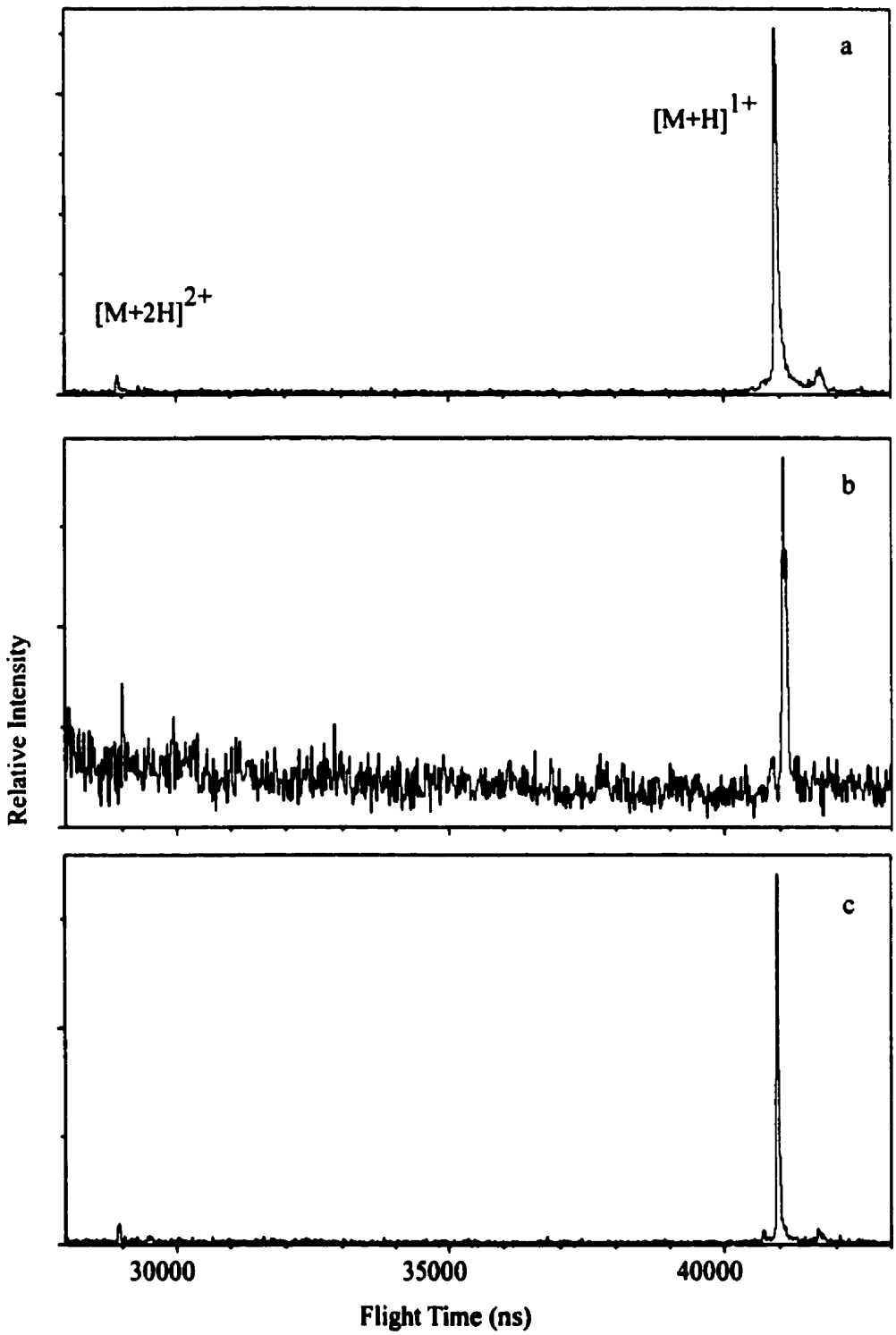


Figure 3-5. Comparison of PU, PVDF and steel targets for 50 pmol of BI. (a) PU membrane (b) PVDF membrane (c) metallic surface; accumulation of 50 shots.

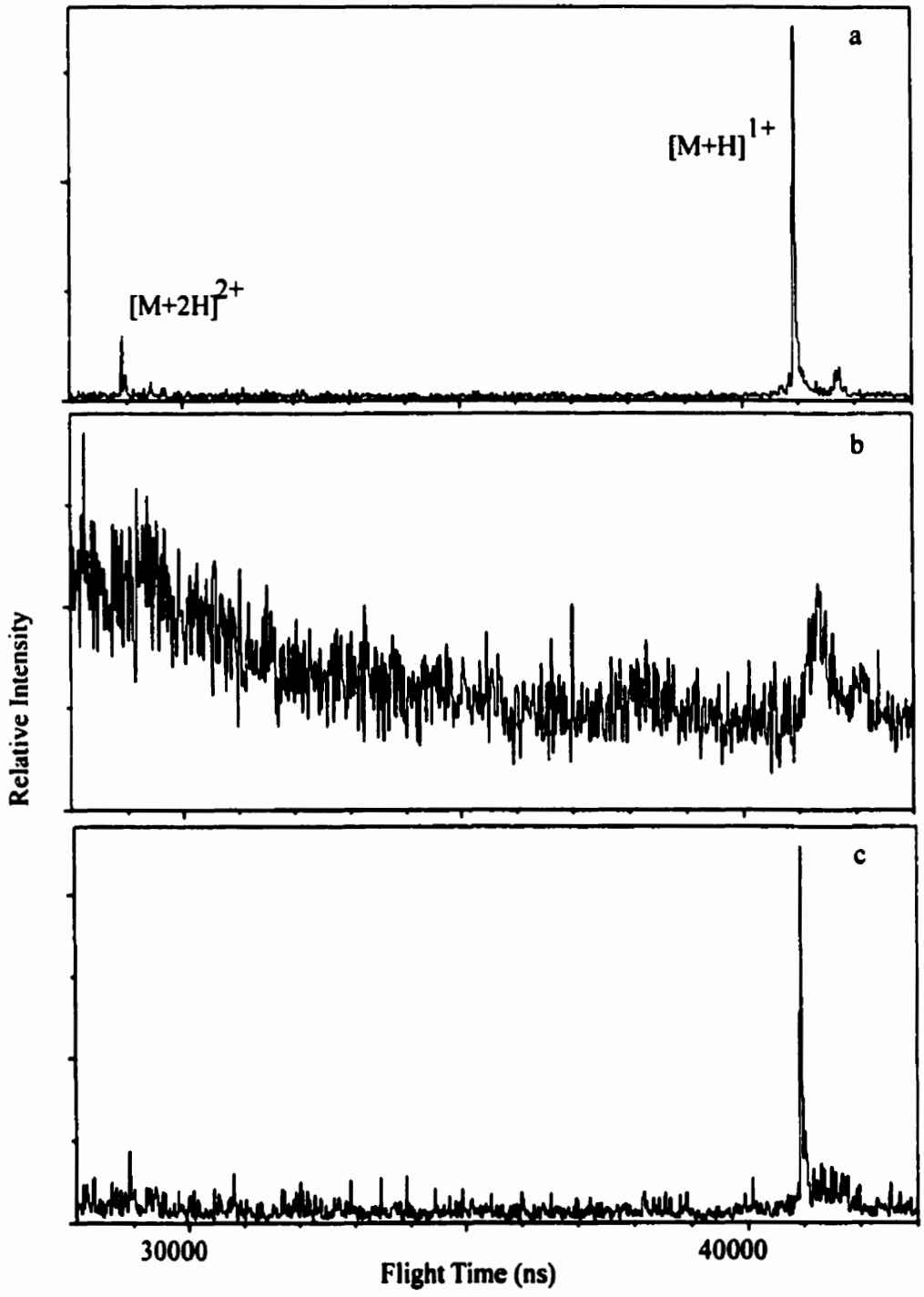


Figure 3-6. Comparison of PU, PVDF and steel targets for 5 pmol of BI. (a) PU membrane (b) PVDF membrane (c) metallic surface; accumulation of 50 shots.

When compared with metal targets for the analysis of NaCl-doped solutions, PU membranes brought a substantial improvement to the quality of the data, as indicated in Figure 3-7 (compare with Figure 3-2 for clean samples). Selective partitioning of the protein molecules, NaCl, and matrix components between the aqueous phase and the surface of the membrane likely occurs as areas of the target could be found which produced good quality spectra even in the presence of excess NaCl. This was not the case with the metal target. It has been shown that differences in the crystallization of the analyte with the matrix are known to affect the quality of the spectra [12-14].

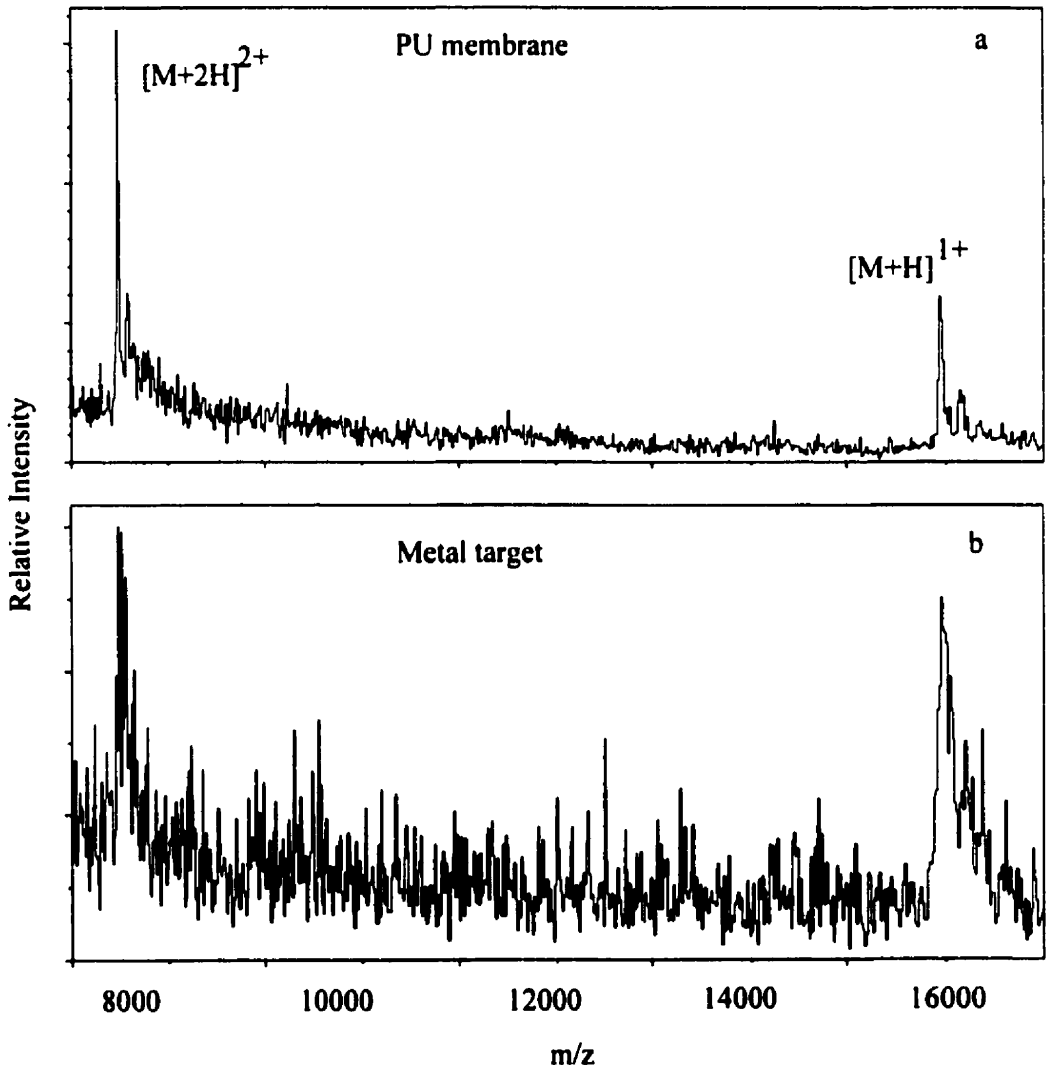


Figure 3-7. Effect of salt using a PU support.
200 pmol of myoglobin with 200 nmol of NaCl
(a) PU membrane (b) metallic target. Accumulation of 50 shots.

3.3.3 Development of a Washing Protocol

Application of NaCl-containing solutions to PU membranes resulted in a marked difference in the crystallization patterns of the protein and NaCl mixture before washing, after washing and with addition of matrix, as shown in Figure 3-8. Prior to washing (a), NaCl is visible on the surface as large crystals. After the addition of matrix (b), disrupted crystallization of sinapinic acid is observed due to presence of NaCl. Following one washing step (c) (Chapter 2.2.4), the "visible" amount of NaCl is removed and only a small amount of sample remains on the membrane. After two or more washing steps, protein and salt are not visible on the membrane surface. With the removal of NaCl, good quality sinapinic acid crystals are observed (d)

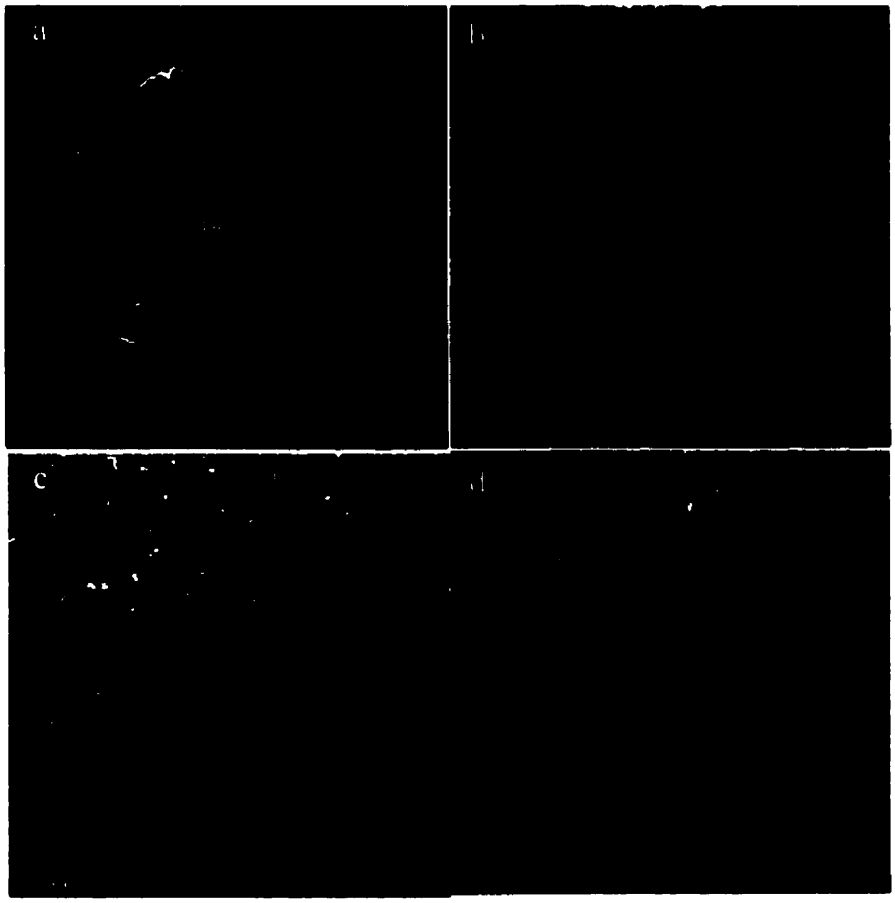


Figure 3-8. SEM of myoglobin (200 pmol) in 200 nmol of NaCl. Samples on a PU membrane (30x-magnification), (a) neat sample, (b) after addition of sinapinic acid, (c) after one wash and (d) after two washes and addition of matrix.

A sufficient amount of protein remains bound to the membrane as MALDI still produces strong signals. After washing, the addition of matrix (d), results in the formation of analyte-matrix crystals, typical of a clean sample which will produce a good MALDI spectrum.

The relatively strong interactions of the PU membrane with proteins and peptides enables the introduction of a washing step. The use of a washing step was examined for the analysis of samples with relatively high amounts of salts and buffer components. Samples of myoglobin were prepared in a 1000-fold excess of NaCl and applied to the membrane. In this case MALDI spectra were obtained using a wide laser beam to ensure sampling of the entire surface of the target including areas which contained NaCl, myoglobin and matrix. An overall improvement in peak shape and resolution were observed with increasing numbers of washes, as shown in Figure 3-9. Some peaks in the spectrum of the original sample (a) correspond to Na^+ adducts. These adducts cause peak broadening and make accurate mass assignment difficult. After successive washing steps (b - d), peaks become narrower as the abundance of Na^+ adducts decreases with the removal of NaCl. The resulting increase in resolution enables correct mass assignment.

3.3.4 On-Membrane Proteolytic Digestion of Citrate Synthase

Application of our membrane methodology to real samples was carried out by performing tryptic digests of citrate synthase directly on the membrane and comparing the results to those from samples digested in Eppendorf™ tubes. Digests were performed for periods of time varying from 2 minutes to 60 minutes on the membrane. Good quality MALDI spectra were observed following removal of the buffer components with the washing procedure an example of which is shown in Figure 3-10, for the 2 minute digest. Samples prepared on metallic targets did not produce spectra at all. Over the duration of the digest, the initially abundant high mass ions were replaced with lower mass ions. Also, the protein underwent significant digestion after only two minutes. This may indicate that the protein denatures upon sorption to the membrane, thus facilitating rapid digestion. Similar spectra were obtained for samples digested in Eppendorf™ tubes and on the PU membrane.

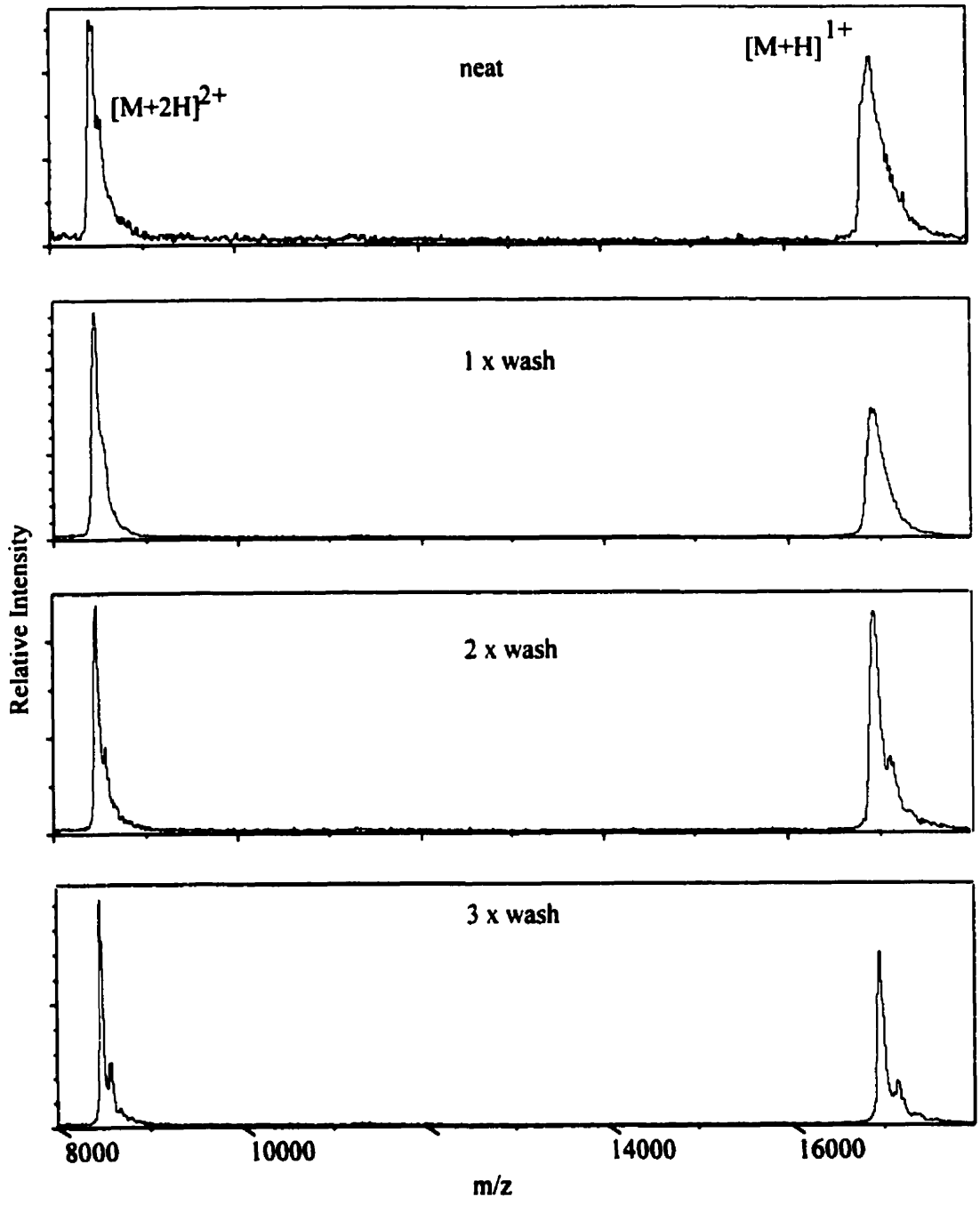


Figure 3-9. Effect of washing using a PU support.
400 pmol of myoglobin in the presence of 400 nmol of NaCl.
Accumulation of 50 shots.

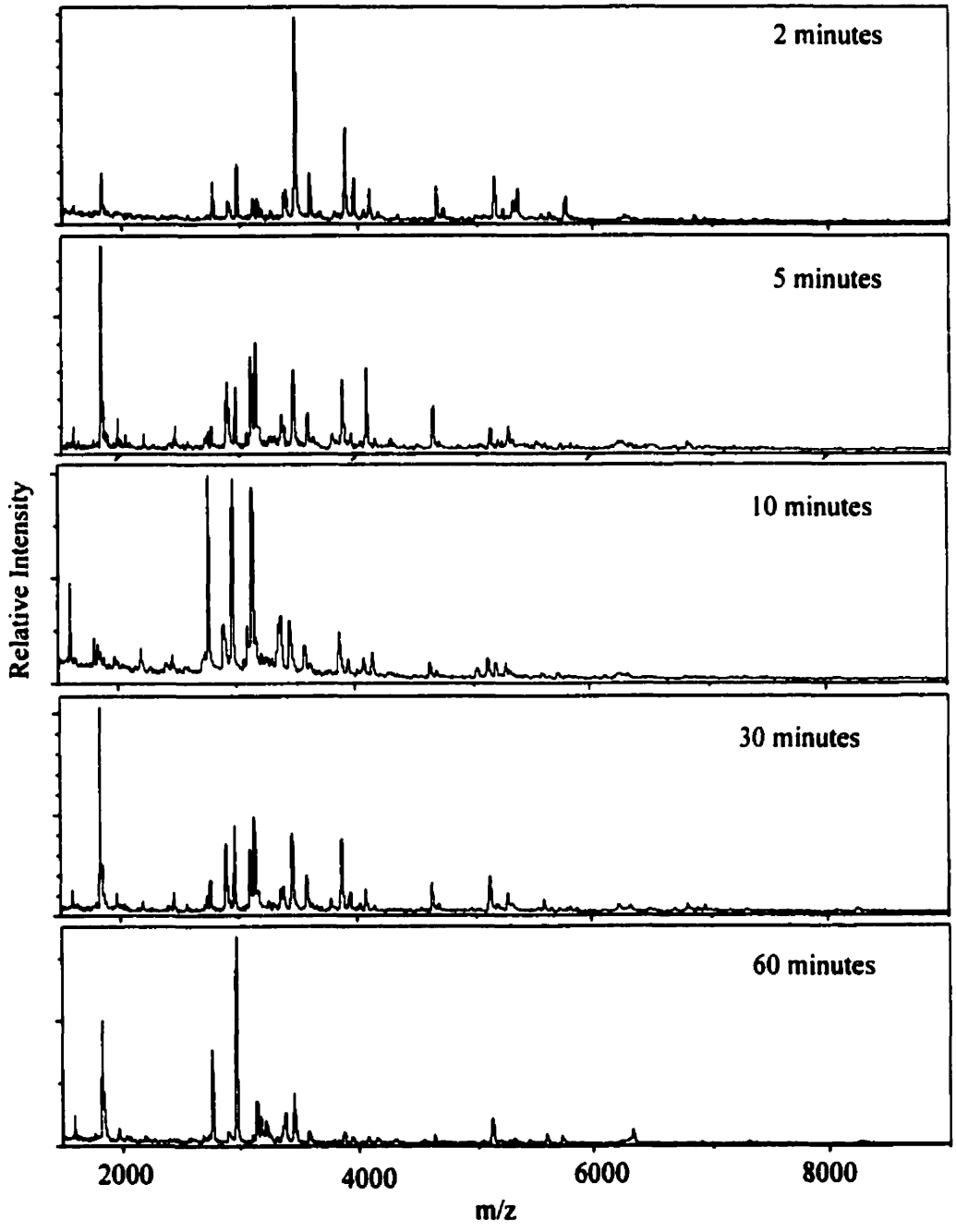


Figure 3-10. MALDI-TOFMS of the tryptic products of citrate synthase. Effect of the time of digestion on ion abundance. Accumulation of 50 shots.

Most segments of the protein were mapped against calculated fragments, as shown in Table 3-1. The entire on-membrane digestion process took less than 3 hours from start of the series of digests to the collection of data. Most of the time was devoted to acquisition and interpretation of spectra.

The 3-hr. proteolytic digestion was used to investigate the advantages of using delayed extraction with samples deposited on the PU membrane. The results presented in Figure 3-11 indicate a peak profile similar to the earlier digest profiles. The use of delayed extraction resulted in a substantial increase in resolution as shown in the inset where the oxidation product of the compound producing a peak at m/z 5759 may be observed. This enabled more accurate mass assignments as shown in Table 3-1.

Table 3-1. Tryptic fragments of citrate synthase on PU.

*Peak #	Sequence	TOF M/Z	Calc. $[M+H]^+$	Error	% Error
1	57-70	1599.74	1599.83	0.09	0.01
2	372-388	1831.25	1831.26	0.01	0.00
3	389-405	1972.55	1973.25	0.70	0.04
4	8-33	2771.59	2771.14	-0.45	-0.02
5	6-33	2970.44	2970.39	-0.05	-0.00
6	241-274	3140.23	3140.51	0.28	0.01
7	190-218	3366.15	3366.83	0.68	0.02
8	39-70	3462.76	3462.87	0.11	0.00
9	357-388	3581.05	3583.37	2.32	0.06
10	301-333	3881.80	3881.55	-0.25	-0.01
11	34-70	3949.45	3949.44	-0.01	-0.00
12	321-356	4164.60	4164.75	0.15	0.00
13	71-110	4642.48	4642.16	-0.32	-0.01
14	8-56	5121.08	5120.75	-0.33	-0.01
15	308-356	5759.30	5759.61	0.31	0.01
16	106-164	6819.18	6819.84	0.66	0.01
				Accuracy	0.0074

* Values correspond to peaks in Figure 3-11.

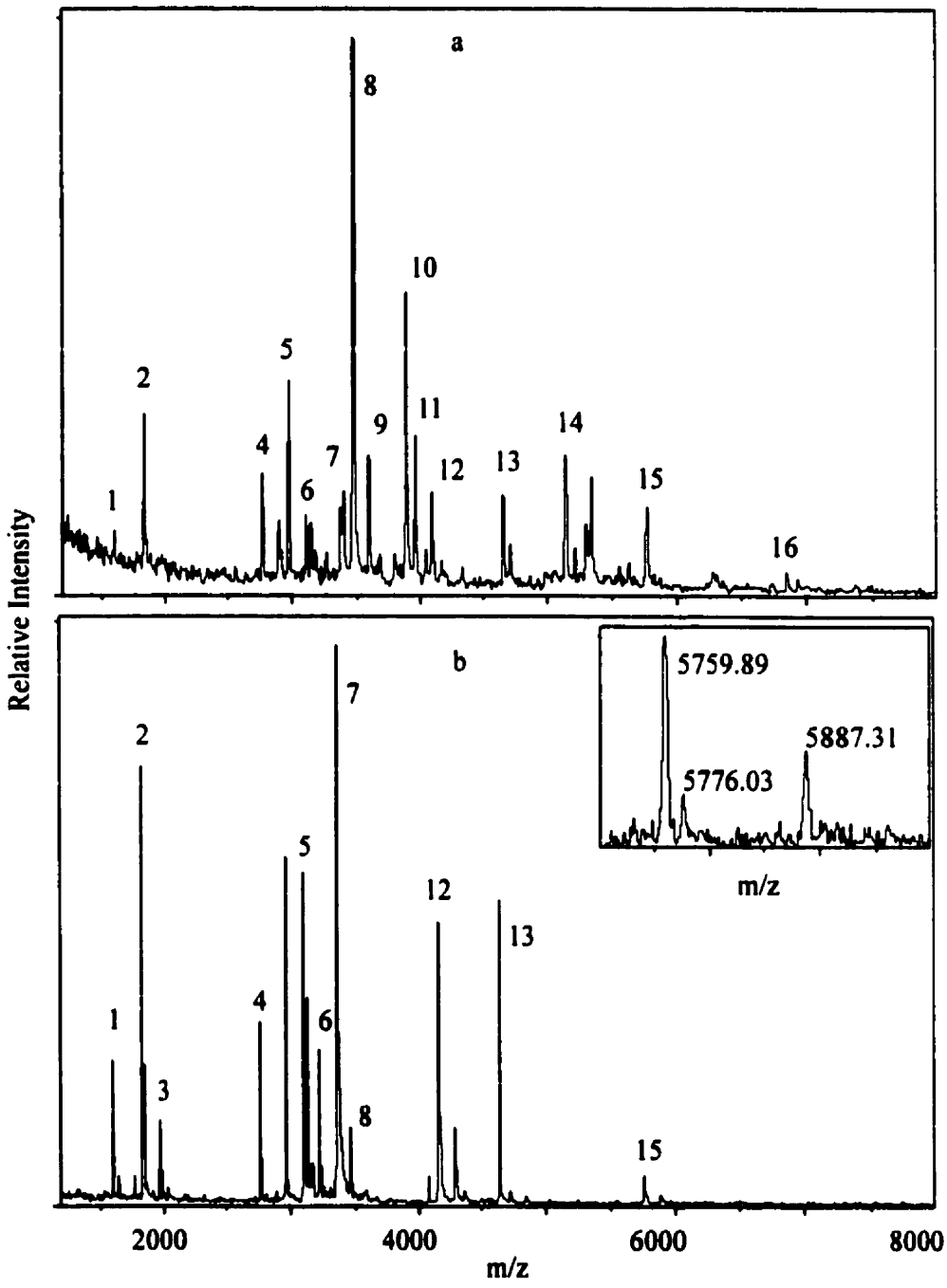


Figure 3-11. MALDI-TOFMS of the tryptic products of citrate synthase. Samples: (40 pmol) (a) 2-minute digest performed directly on-membrane (PU) (b) 3-hour digest with delayed extraction. Accumulation of 50 shots.

3.3.5 Application to High Mass Proteins

Application of our PU membrane technology to MALDI-TOF analysis of higher molecular weight proteins was briefly investigated. Spectra of bovine serum albumin and apotransferrin were comparable to those obtained using a metallic target (not shown, results obtained on higher molecular weight proteins derived from real samples are given in Chapters 4 and 6). A slight increase in mass was observed for the samples deposited on PU, likely due to charging and to use of external calibration. This phenomenon was observed only for higher m/z values and may be corrected with calibration under similar experimental conditions. Applications regarding high M_r proteins are discussed in Chapters 4 and 6.

3.4 CONCLUSIONS

The use of PU membranes as sample supports for MALDI-TOFMS analysis of proteins and peptides yields equivalent accuracy and resolution to values obtained with metal targets. The non-porous nature of the membrane facilitates crystal growth on the surface only and thus provides for enhanced spectral quality over porous membranes. The relatively strong interactions of the PU membranes with bound proteins and peptides enables the introduction of a washing step in order to remove salt and buffer components, which may interfere with MALDI analysis. Tryptic digestion of citrate synthase performed on the membrane surface yielded characteristic fragments, allowing for successful peptide mapping. As the method is simple and involves robust technology, it is now used in our laboratory on a routine basis (example applications are given in Chapters 4, 5 and 6).

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4 POROUS PU THIN FILMS AND NON-POROUS PU MEMBRANES FOR MALDI-TOFMS OF WHEAT PROTEINS

4.1 INTRODUCTION

In Chapter 3 the use of non-porous polyurethane (PU) membrane as a sample support for MALDI-TOFMS characterization of peptides and proteins derived from biological samples was introduced. The focus was on the analysis of low molecular weight proteins and peptides. The qualitative aspects of spectra, such as peak shape and peak width, depended on the laser fluence and repetition rate of data acquisition, consistent with electrostatic charging occurring near or on the membrane surface. This motivated the investigation on the use of thinner PU membranes (25 μm thickness) and thin PU films for use as sample supports. In this chapter, the use of non-porous PU membranes and porous PU thin films for MALDI-TOFMS, particularly in the analysis of wheat proteins is reported.

MALDI-MS has been used to accurately and quickly determine the masses of specific glutenin subunits of wheat [1,2], and our laboratory has recently characterized gliadins and glutenin subunits by MALDI-MS on metal targets as part of an ongoing collaborative project with the Canadian Grain Commission, Grain Research Laboratory [3]. All of these proteins, gliadins and glutenins, are insoluble in water. The wheat gliadins consist of a group of ca. 100 proteins, with a low molecular weight range of 30-40 kDa. The glutenins are large polymeric proteins composed of two groups of subunits: low molecular weight (LMW) (30-40 kDa) and high molecular weight (HMW) (65-90 kDa) subunits. These subunit proteins are heterogeneous and are thought to have some degree of post-translational modification, i.e. glycosylation. The reagents required for their isolation (i.e. 1% DTT, 2M urea, 0.082 M Tris-HCl) are not favorable for analysis by MALDI. We discuss the ease of sample preparation, time frame of analysis and show comparisons between spectra obtained using non-porous membranes, porous thin films and steel targets.

4.2 EXPERIMENTAL

4.2.1 Protein Extraction

Pure samples of the Biggar wheat variety from the Canada Prairie Spring commercial class were obtained from stocks maintained at the Grain Research Laboratory in Winnipeg, MB Canada. Wheat samples (25 g) were ground in a UDY cyclone sample mill equipped with a 1 mm sieve. Gliadins were extracted from ground grain (1.0 g) into 70% (v/v) ethanol (6 mL) at room temperature for 60 minutes. Glutenins were then extracted from the remaining grist with 1% dithiothreitol (DTT) in 100% n-propanol [3,4]. The LMW glutenin fraction was extracted by precipitating out the HMW glutenins with the addition of 1% DTT in 50% n-propanol to bring the final concentration to ca. 65% n-propanol. The HMW glutenin precipitate was washed with 1% DTT in 65% n-propanol and then re-solubilized in 1% DTT, 2 M urea in buffered (0.082 M Tris-HCL, pH=7.0) 50% n-propanol [4]. Extracts were then centrifuged at 20,000 g for 10 minutes. The extraction procedure is outlined in Figure 4-1.

4.2.2 Sample Preparation

The stock wheat protein solutions had concentrations varying from 30 to 600 mM. The matrix solution used in all analyses was a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in aqueous 0.1% TFA and acetonitrile (2:1 v/v). For samples on steel probes, the crushed matrix method was used [5]. A 2 μL aliquot of the matrix solution was first applied to the sample probe, allowed to dry, and then crushed. The stock solution was then mixed (1:10 v/v) with the matrix solution and a few microlitres of the mixture were applied to the crushed spot on the sample probe. When a visible film began to form after about 10 seconds, the spot was rinsed with cold deionized water during 10 seconds and then allowed to dry thoroughly prior to analysis. This process was repeated if necessary in order to deposit enough protein onto the surface of the probe to acquire MALDI mass spectra.

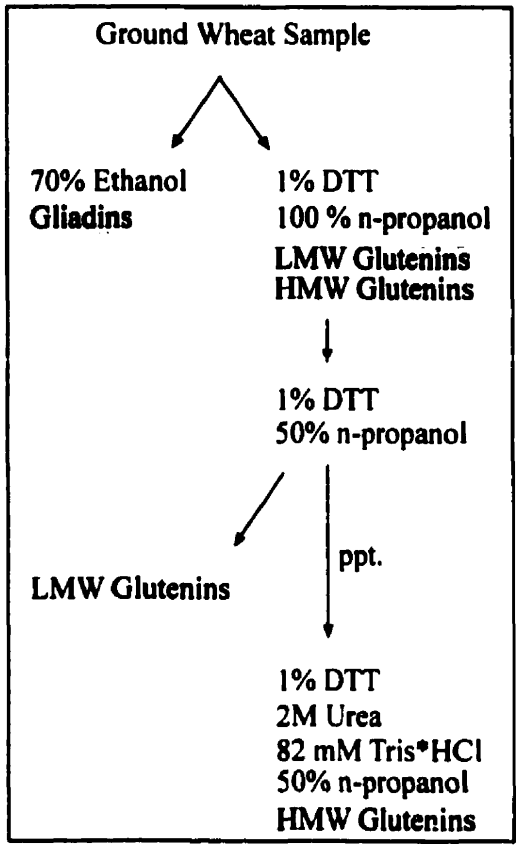

Figure 4-1. Sample preparation scheme for wheat proteins.

Samples were prepared on PU substrates by first depositing 2 μL of the protein solution onto the film or membrane surfaces. After the samples had dried, 2 μL of methanol were added to enhance protein sorption onto PU. For sample preparation without a washing step, 2 μL of the saturated matrix solution were added and allowed to dry prior to analysis. For preparation with a washing step, 25 μL of deionized water were deposited on the surface of each sample and allowed to stand for 1 minute to solubilize as much material as possible. This large droplet was then removed with a pipette. This step was repeated once more to ensure that the protein samples were as free from water soluble material as possible. The matrix was added just prior to analysis.

The acquisition of standard MALDI-TOF mass spectra obtained on steel targets was performed with the assistance of M. Znamirowski and R.G. Dworschak.

4.3 RESULTS AND DISCUSSION

4.3.1 Characterization of Polyurethane Films and Membranes

Membranes and films have different advantages when used as sample supports for MALDI analysis of proteins. While films may be directly deposited onto the surface of the MALDI target, membranes must be attached, usually with an adhesive, which introduces an additional step into the analytical scheme. Because films are thin, the possibility of surface charging is reduced or eliminated. One distinct advantage of membranes is that the sample may be applied prior to fixing the membrane onto the probe surface. This allows samples to be collected outside of the MALDI laboratory. In both cases a number of different polymer supports are available, thus a specific type may be chosen for a selected class of analytes.

For preparation of PU thin films, some PU membrane material is dissolved in THF, cast onto the surface of the MALDI probe and allowed to dry under ambient conditions. Figure 4-2 compares SEM images taken on the 25 μm thick PU membrane (a,b), and the PU film (c,d). The surface topology of both the membrane and film was flat, unlike the uneven rough surfaces typical of other membranes examined previously [6]. Unexpectedly, the surface of the PU film was porous as shown Figure 4-2 (c,d). Porosity is introduced when the film dries on the probe after casting. The extent to which it is observed varies with rate of drying and solvent volatility, among other parameters. The pore sizes ranged from ca. 1 μm to 8 μm in diameter, with most pores being in the 2-3 μm range. The film was less than 1 μm thick, i.e. thinner than ca. 1 μm as calculated from the amount of material deposited and the surface area of the probe.

MALDI spectra of bovine insulin were obtained using PU membranes and thin films and compared qualitatively with those obtained using steel targets (results not shown). Both PU surfaces produced spectra identical to steel probe spectra for samples applied to the surface in matrix solutions. The resolution and mass accuracy observed were typical of the instrument [7].

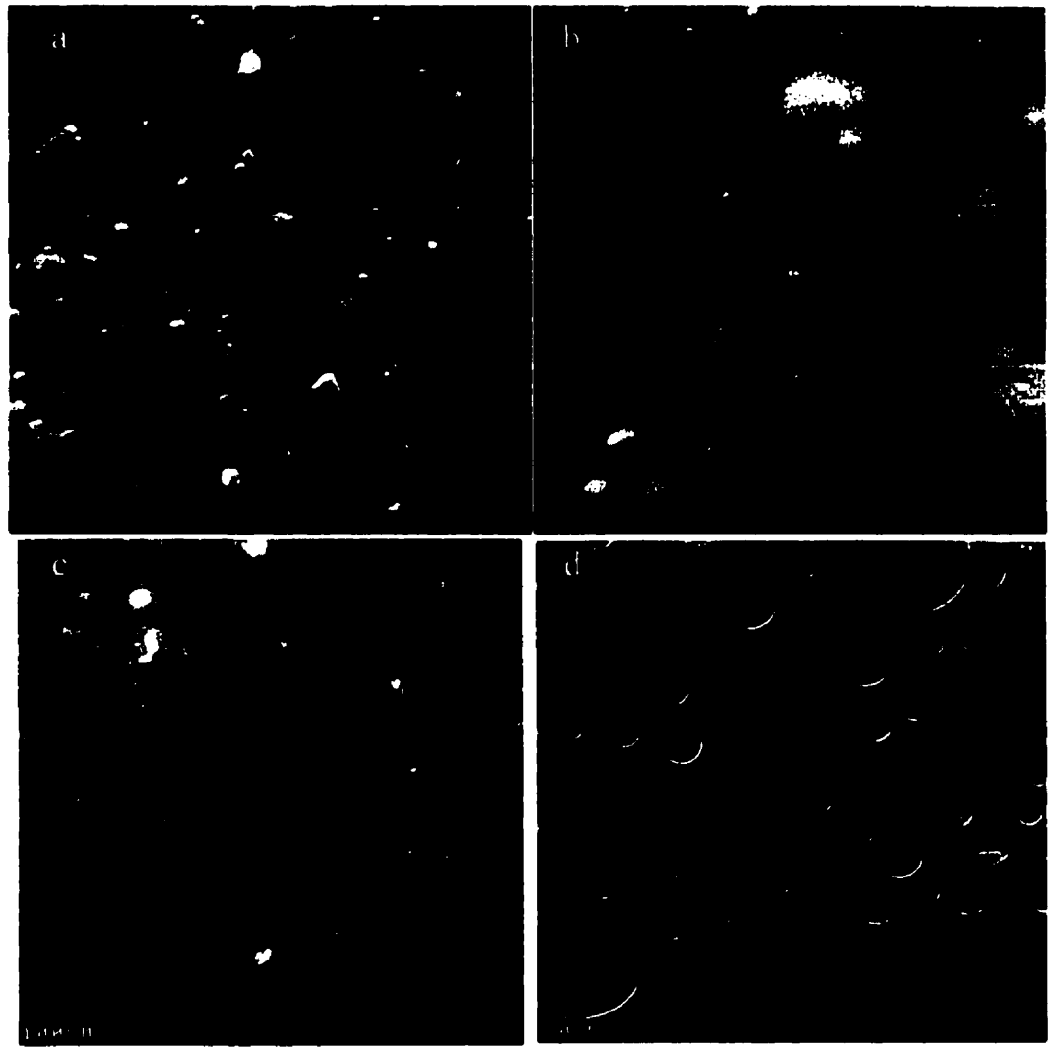

Figure 4-2. Scanning electron micrographs of PU membrane and film. (a) PU membrane 30x, (b) PU membrane 300x, (c) PU film 30x, (d) PU film 300x.

One important feature of using PU membrane or film was the lack of formation of PU adducts. This phenomenon was observed in our laboratory when using nitrocellulose films for MALDI sample supports and results in a decrease in resolution in the mass spectra of higher molecular weight components. The result of using PU is a cleaner mass spectrum without adducts.

4.3.2 Charging

Charging of the membrane, sometimes observed in MALDI [8], occurred with the 25 μm (Figure 4-3) and 50 μm thick PU membrane at laser intensities 2 to 3 times above threshold using a laser pulse rate of ca. 2 Hz on the laser. Charging was not observed with the PU thin films nor with the steel targets. We attributed this to the thinness and surface porosity of the film, which would be expected to facilitate dispersal of static charge. To overcome the charging observed with the membrane it was necessary to reduce the pulse rate of the laser to ca. 0.5 Hz to allow dispersal of the static charge.

Qualitative measurements of the charging observed are shown in Figure 4-3. The time-of-flight of bovine insulin $[M+H]^+$ ions was measured as a function of laser intensity on the three surfaces employed. With the 25 μm PU membrane, the time-of-flight increased by ca. 1.2% at higher laser intensity, relative to the value measured at threshold. Spectra obtained using the PU film and steel target were less influenced by the laser intensity and charging occurred approaching maximum intensity. It is noted that the maximum laser fluence used was about 6 x above threshold and would not normally be used in MALDI analysis.

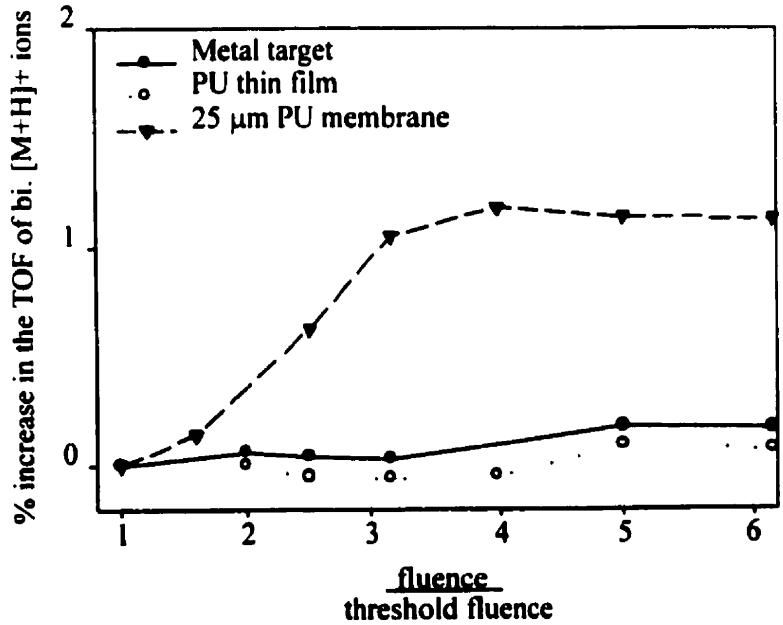

Figure 4-3. Influence of laser intensity on the TOF of bovine insulin. Comparison of sample supports.

4.3.3 Proteins from Wheat

Our laboratory has recently characterized some wheat gliadins and LMW and HMW glutenin subunits on metal targets by MALDI-MS [3]. The spectra obtained on wheat gliadins in the 70% ethanol fraction (see Experimental) were of good quality, informative and relatively easy to acquire. This was not the case for the spectra of LMW and HMW wheat glutenins. Extraction of the glutenin proteins requires buffers which are incompatible with the usual MALDI protocol. Successive applications of protein to the crushed matrix were necessary, followed by washing, in order to obtain informative spectra. Another consideration was the duration between sample extraction and MALDI analysis. We observed that the glutenins were not very stable in solution and necessitated analysis within ca. 1 day, once extracted. This is a limitation in a laboratory without a dedicated instrument. Dried samples were difficult to solubilize. In general, not all samples analyzed produced good mass spectra. Similar results were obtained in this work with spectra acquired on the steel targets using the crushed matrix method of sample preparation.

4.3.4 Gliadins

Figure 4-4 shows MALDI mass spectra of wheat gliadins obtained using the different sample supports. All 5 different sample preparation techniques employed here produced similar spectra. The main advantage of the PU supports for these proteins was the convenience of sample handling. Comparable spectra were obtained with the washed PU membrane and the washed steel target. Spectra of unwashed gliadin samples were also good, as the proteins were relatively clean after extraction with 70% ethanol. The pattern of relative intensities for the protein ions was different for PU and steel sample substrates. This may be due to a difference in protein affinity between the PU and the crushed matrix on the steel probe. Different extents of sorption would thus result in the release of variable amounts of protein during the washing and matrix application steps. These findings are similar to results obtained in our laboratory for comparison of PU and steel supports for peptide mixtures. Walker et al. have made similar observations with the use of polymeric supports for protein analysis. They suggested that some protein is removed during washing and not all the protein may be released from the polymer surface after applying the matrix solution [9].

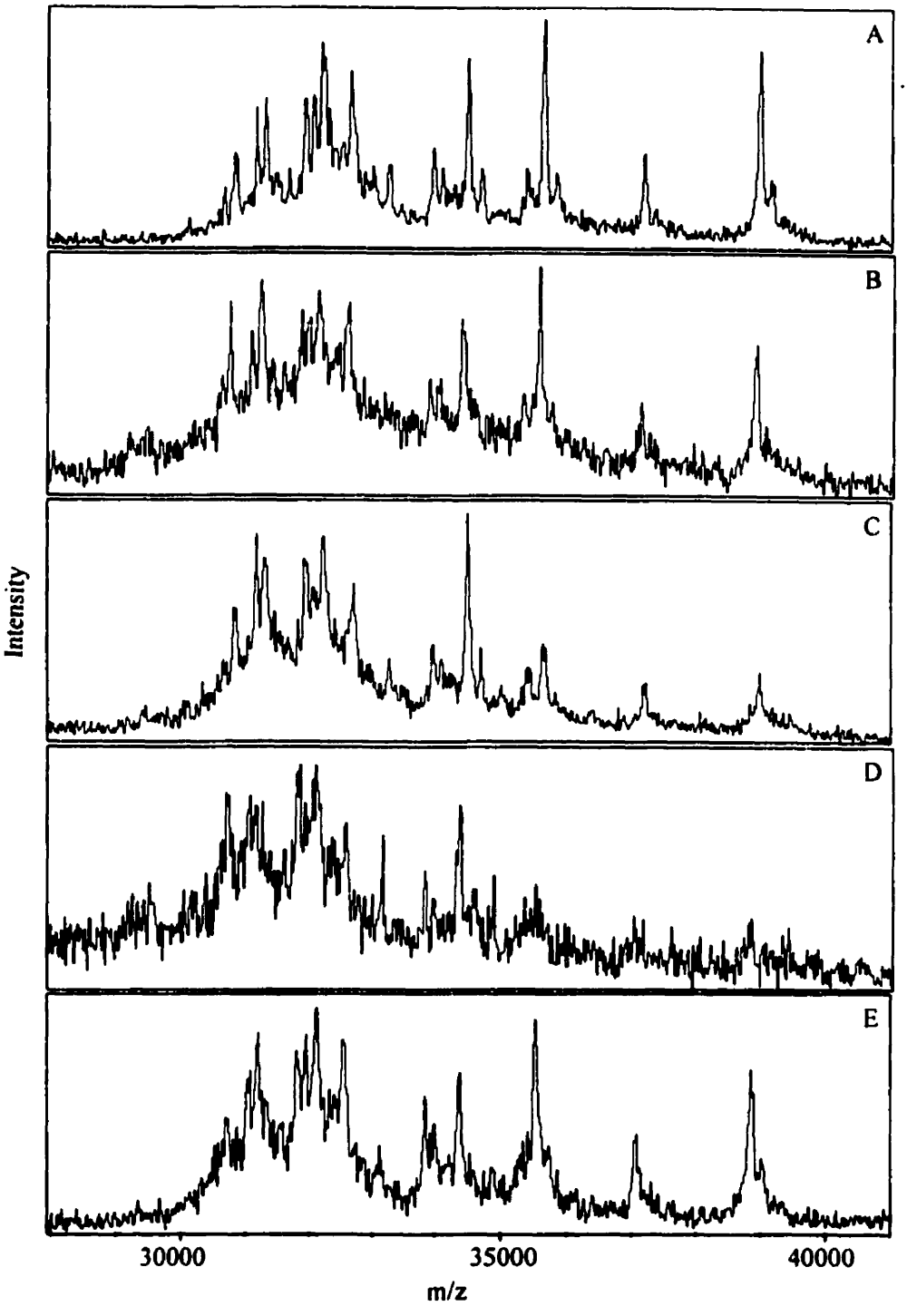

Figure 4-4. MALDI-TOF mass spectra of gliadins.
(a) steel stage with sample washing, (b) PU membrane without sample washing, (c) PU membrane with sample washing, (d) PU film without sample washing, (e) PU film with sample washing

4.3.5 Low Molecular Weight Glutenins

Considerable benefit of using PU substrates is apparent with the analysis of the LMW glutenins as shown in Figure 4-5. The washed sample on steel (a) produced a spectrum which is more difficult to interpret than those of the washed samples on PU (c and e). It was not possible to record spectra using the steel targets without washing, possibly due to the presence of 1% DTT and other components required for extraction of the LMW glutenins. Spectra were more easily obtained using the PU supports, which gave better signal uniformity and shot-to-shot reproducibility than metal targets. The washed samples on film and membrane (c and e) also yielded better signal-to-noise ratios in comparison with the unwashed samples (b and d). Spectra could be obtained from the entire matrix surface on samples prepared on the PU supports while samples prepared on the steel targets required rastering of the target in order to find useful sites to obtain results. Between 150 and 200 shots were required to obtain spectra on the steel targets while only ca. 50 shots were required using the PU supports.

Slightly different spectra were observed for washed samples on PU membrane and PU film. Spectra acquired with the membrane show more abundant ions in the 35-45 kDa range (Figure 4-5.c) while those acquired using the film show larger and better resolved peaks in the 30-35 kDa range (4e). Overall, the spectral differences were minor and did not influence the number of proteins detected nor their observed masses. Differences in the surface morphology of the film vs. membrane may contribute to the variations observed. Polyurethane surface composition is known to be dependent on the method of fabrication (e.g. casting, extrusion) and on the type of surface the polymer is in contact with while being cast [10]. The proportions of the hard (crystalline) and soft (amorphous) segments at the polymer surface will lead to different protein sorption characteristics [11]. In this application, casting the PU onto the MALDI probe may alter the surface composition of the polymer leading to different affinities for the different wheat proteins present.

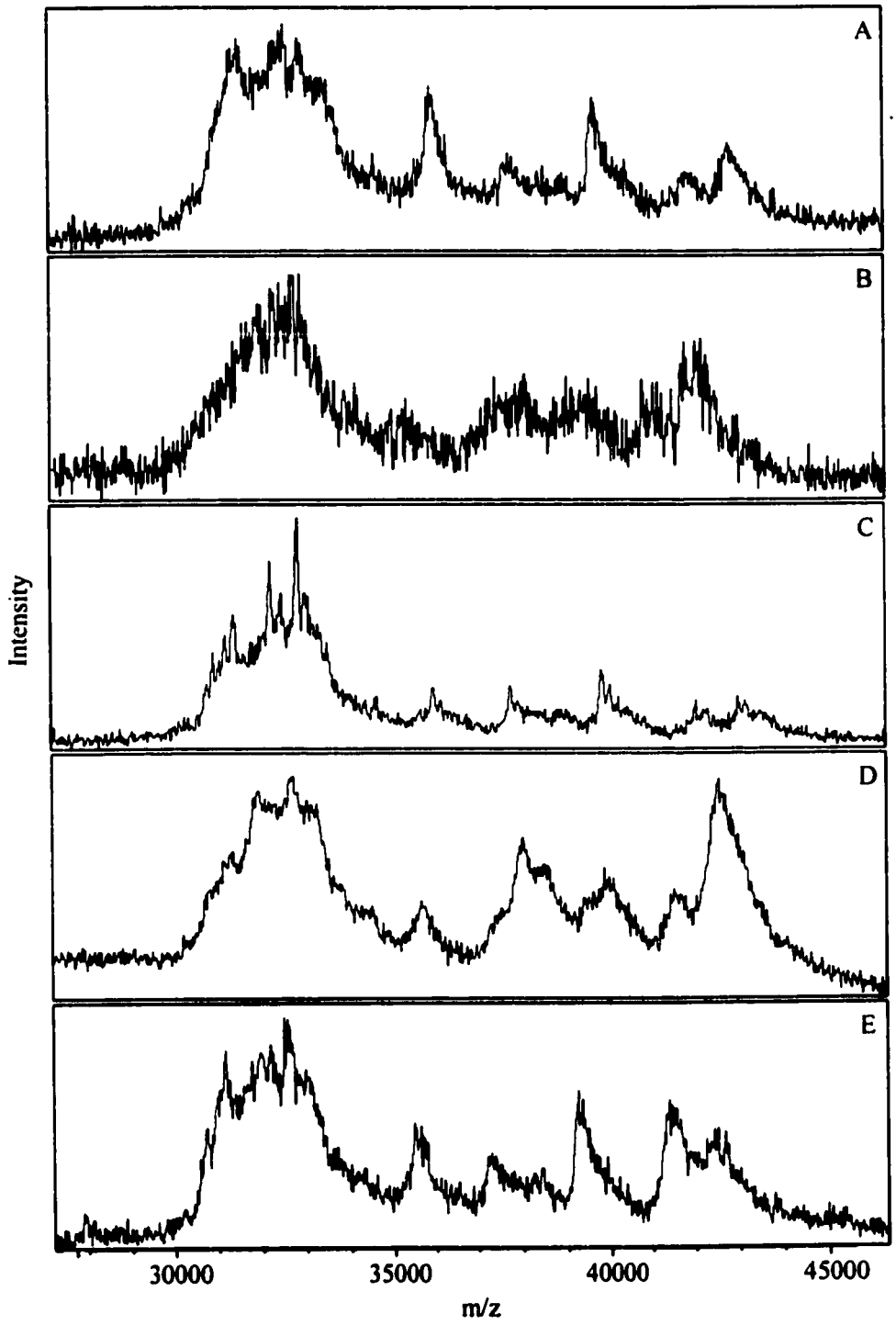

Figure 4-5. MALDI-TOF mass spectra of LMW glutenins.
(a) steel stage with sample washing, (b) PU membrane without sample washing, (c) PU membrane with sample washing, (d) PU film without sample washing, (e) PU film with sample washing.

4.3.6 High Molecular Weight Glutenins

Figure 4-6 shows results from the analysis of the HMW glutenin fraction. No signal was observed from any target without washing. More concentrated buffer components are present in the HMW fraction (1% DTT, 2M urea in buffered (0.082 M Tris-HCL, pH=7.0) Figure 4-1) than in other samples analyzed. The quality of the spectra was similar for the three washed substrates but as was the case of the LMW glutenins, PU targets yield better reproducibility and uniformity of signal. Different relative intensities were observed for the proteins on PU supports compared with steel, as was the case for the LMW glutenins. Again, this may be due to a difference in protein affinity between the two surfaces. In order to obtain similar quality spectra on the steel targets several attempts were required for each sample and a greater number of shots were required to obtain similar signal-to-noise ratios. The result was an overall increase in throughput using the PU supports. The samples, once applied to the PU supports, were stable for several days prior to analysis, giving an increase in confidence that the samples would generate good MALDI mass spectra.

4.4 CONCLUSIONS

Comparison of the two PU systems shows that the signals were slightly better with analysis on the membrane. This was attributed to an increase in surface area available for protein sorption on the membrane compared with the film and differences in surface morphology. More noticeable was the difference between the relative intensities of the protein ion signals measured using the steel targets and the PU supports. Nevertheless, each spectrum contained the same information regardless of the support used.

The washing procedures significantly improved the signal-to-noise ratios in the mass spectra, particularly for the LMW and HMW glutenin samples. Use of the PU supports allowed more vigorous sample washing due to the stronger interaction of the proteins with the surface of the PU compared with the matrix/steel target.

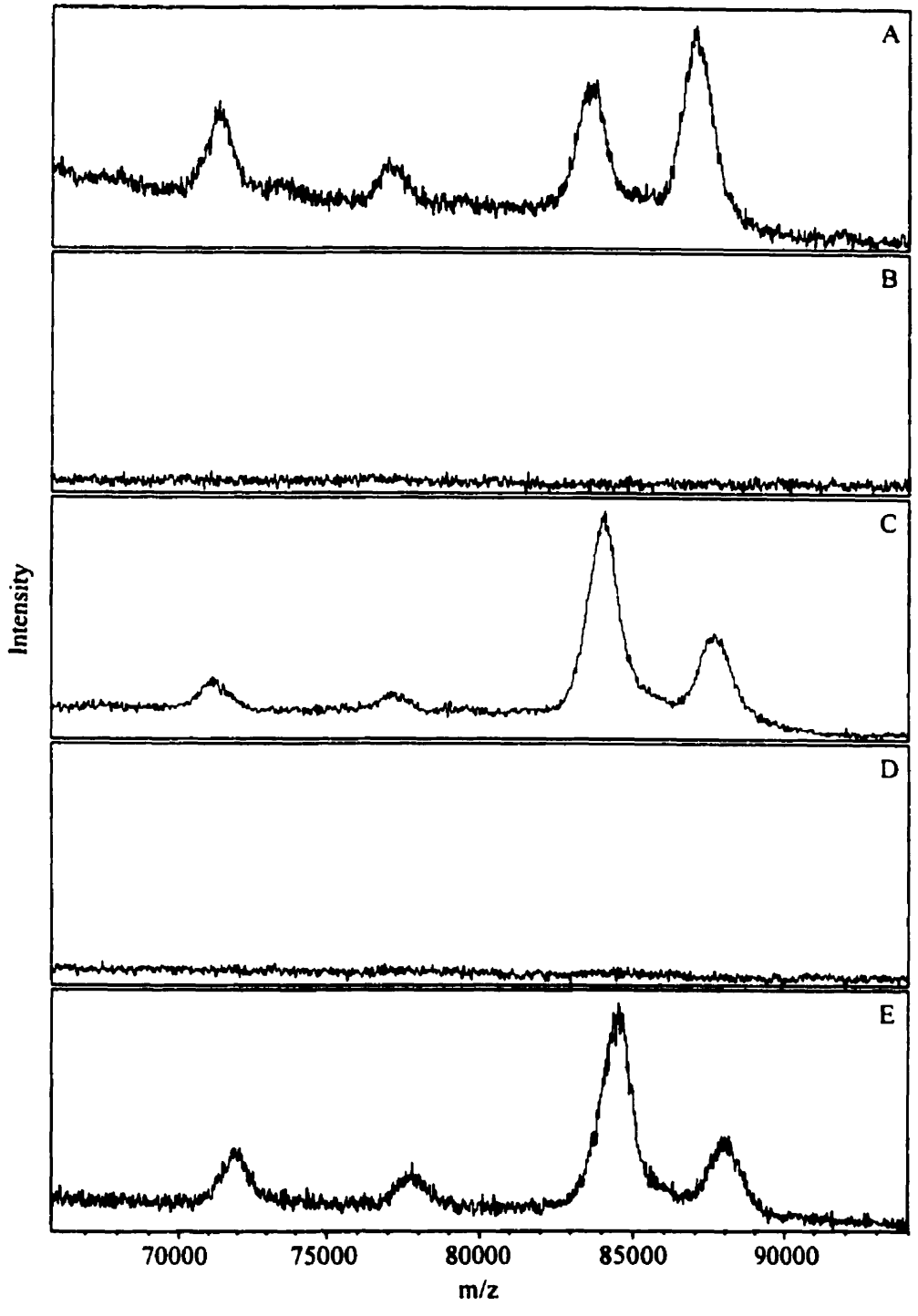

Figure 4-6. MALDI-TOF mass spectra of HMW glutenins.
(a) steel stage with sample washing, (b) PU membrane without sample washing, (c) PU membrane with sample washing, (d) PU film without sample washing, (e) PU film with sample washing.

The advantages of the PU supports which are not obvious from the spectra shown are the ease of sample manipulation and of obtaining and storing samples prior to analysis. Once the sample is placed onto the membrane or film, it is stable for several days to weeks, depending on the sample, prior to analysis. This was not the case with liquid samples of wheat proteins, which degraded rapidly during storage. Extraction of a dried sample into the matrix solution was facilitated using the PU supports as the extraction required only enough matrix to re-solubilize the analyte (ca. 2 μL). Similar dried-in-tube samples required a significantly larger amount of matrix or other extraction solvent to re-solubilize the sample. This resulted in dilution of the sample and an increase in the sample preparation time. In addition, use of the membrane bound samples allowed the removal of salts via washing thus increasing the quality of the mass spectra. The PU membranes were particularly useful as one large piece of membrane could be used to collect several samples outside of the laboratory and then store them prior to analysis. Spectra were obtained with greater ease and reproducibility on the PU supports, in particular the PU membrane, thus increasing the confidence that samples, once acquired, would yield results.

4.5 REFERENCES

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5 CHARACTERIZATION OF HEMOGLOBIN VARIANTS BY MALDI-TOFMS USING A PU MEMBRANE AS THE SAMPLE SUPPORT

5.1 INTRODUCTION

Hemoglobin disorders arise due to structural changes in the chains of hemoglobin and are responsible for a number of disease states. Essentially, four types of structural modifications may occur in abnormal hemoglobin: amino acid substitution, deletion of an amino acid, elongation of a chain, and fusion or hybridization of the DNA during meiosis. The most common modification is replacement of one amino acid with another. Several hundred variants have been described and approximately two thirds affect the beta chain [1]. The modifications may remain asymptomatic or may manifest themselves clinically. With the implementation of newborn screening programs, the health risks associated with the late detection of an abnormality could be minimized, but because of the large number of possible variants, the need exists for a rapid and cost-effective method of analysis.

The standard methods used for the characterization of hemoglobin variants are based upon traditional electrophoresis techniques and are time-consuming. This limits the number of samples that can be analyzed in given time period [1,2]. These methods involve isolation of hemolysate from red blood cells followed analysis by electrophoresis to establish normality. Further characterization may be performed by proteolytic digestion of the chains followed by electrophoretic or high performance liquid chromatographic (HPLC) separation of the fragments. The resulting peptide maps must be interpreted by a skilled technician in order to diagnose the existence of a variant. More complete characterization requires sequencing of the globin chains.

Application of mass spectrometry to the analysis of hemoglobin variants constitutes a new, rapid and accurate means for the detection and characterization of changes occurring in the globin chains. The role of mass spectrometry (MS) when applied to the identification of hemoglobin variants has recently been discussed in detail by Shackleton [3]. A number of approaches have been investigated, including analysis of the intact globin by ESI [9-12] and MALDI [4-8], and characterization of proteolytic fragments by liquid secondary ion

mass spectrometry (LSIMS) [13] and ESI [14]. The majority of these approaches involve the application of several sample pretreatment steps prior to mass spectrometric analysis, including sample purification to a varying extent. These steps are necessary in order to remove some of the components present in the blood matrix, such as plasma proteins and salts, which could cause interference during acquisition of mass spectra.

Characterization of hemoglobin specifically by MALDI has been the subject of a few reports, and some degree of sample purification was most often required [9-12]. For instance, MALDI was used to examine the extent of glycation of hemoglobin as a possible indicator for diabetes [10,11]. Recently, Houston and Reilly reported on the characterization of sickle cell hemoglobin [9]. In this example, good quality MALDI spectra of human hemoglobin were obtained by simply diluting whole blood prior to analysis.

It has been demonstrated that it is not necessary to use a liquid sample in order to apply mass spectrometry to the analysis of whole blood. Methods have been developed using samples of dried blood spots obtained from newborn screening cards [15]. D.E. Chace et al. developed a method for the diagnosis of phenylketonuria and maple syrup urine disease [16,17]. The procedure involved extracting the blood proteins from the screening card, followed by hydrolysis, chemical derivatization and analysis by tandem mass spectrometry (MS/MS). Wada et al. applied LSIMS to the characterization of gamma globins as part of a newborn screening program [13]. After reconstituting the dried blood from the screening cards, initial screening was performed using electrophoresis. The samples were then separated by HPLC to yield purified gamma globins. These were subjected to tryptic digestion and the fragments were characterized by LSIMS. These studies demonstrated that MS/MS and LSIMS can yield rapid, accurate and informative results when using dried blood as the analyte. However, both experiments required a relatively large sample size and considerable extent of workup prior to the analysis by MS.

In this chapter, we demonstrate the application of a PU membrane as a sample support for the mass spectrometric characterization of the Hb Shepherds Bush hemoglobin variant b74 (E18) Gly->Asp (Appendix II) [18-21]. Sampling is performed outside of the laboratory with a common lancet device, and less than 1 μL of whole blood is collected directly on the PU membrane. This method does not require a large volume of liquid sample

for analysis. Once dried, the sample may be sent to the mass spectrometry laboratory by regular mail, i.e. no particular precautions are required for transportation [15]. It is not necessary to transport the sample on ice, since the sample has been dried on the membrane. On-membrane washing protocols are used to process the sample with minimal extent of work-up prior to analysis by mass spectrometry. On-membrane proteolytic digestion allows for further characterization of the globin chains.

5.2 EXPERIMENTAL

5.2.1 Reagents and Materials

Regular hemoglobin was obtained from whole blood provided by M.E. McComb, R.D. Oleschuk and S. Shojania, Department of Chemistry, University of Manitoba. The Shepherds Bush variant hemoglobin was provided by M. Smith, Department of Pediatrics, University of California, Irvine.

5.2.2 Collection of Whole Blood on PU Membranes

The sampling protocol (Figure 5-1) is similar to that used for blood collection onto newborn screening cards [15-17]. Prior to sample collection, the PU membrane was cleaned in methanol and allowed to dry. Under aseptic conditions, using a common lancet device, approximately 0.5 to 1 μL of blood was drawn, which was sufficient for preparing 2 samples. The drop of blood was placed onto the PU membrane by lightly touching the membrane against the subject's finger. In order to avoid possible contamination, the contact time between the finger and the PU membrane was minimized. The samples were allowed to dry under ambient conditions. To some samples, 2 μL of methanol was added prior to drying in order to disrupt coagulation, enhance cell lysis and enhance protein binding onto the membrane. The Shepherds Bush variant samples were shipped to our MALDI-TOF laboratory via courier. No particular precautions were taken when transporting the samples, i.e. they were not shipped on ice. Sample preparation for mass spectrometry was performed according to the protocols previously developed and used with PU membranes (Chapter 2.2.4).

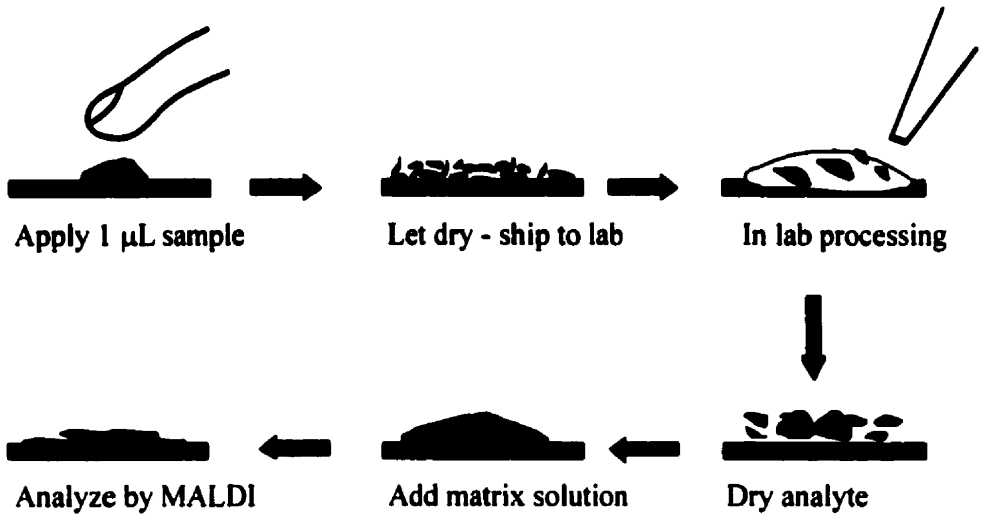

Figure 5-1. Sample protocol for blood analysis on PU.

5.2.3 On-Membrane Tryptic Digestion

Tryptic digestions of blood samples were performed directly on the PU membrane. Prior to any washing steps, trypsin, (2-10 μL, 0.01 mg/mL in 20 mM Tris-HCl, pH = 7.5) was placed directly onto the protein spots on the PU membrane. Digestion was allowed to proceed for periods of time ranging from 2 to 150 minutes. Digestions were stopped by adding 1 μL of a 1% solution of acetic acid. The samples were then processed as outlined in Chapter 3 with the washing protocol.

5.3 RESULTS AND DISCUSSION

5.3.1 MALDI-TOFMS of Normal and Shepherds Bush Hemoglobin

Hb Shepherds Bush is a mutation in the beta chain of hemoglobin: Hb 74 (E18)Gly->Asp, which is presumed to result from a mutation in the DNA sequence at codon 74: GGC->GAC [18-20]. This variant results in an increased oxygen affinity and expresses itself as mild hemolytic anaemia in the patient. The variant hemoglobin makes up for ca. 24% of the total hemoglobin and exhibits thermal instability [18]. The Shepherds Bush mutation is relatively rare but serves as a good example to demonstrate the feasibility and advantages of our MALDI analysis protocol. The MALDI-TOFMS spectrum of the alpha and beta chains of normal hemoglobin is shown in Figure 5-2.

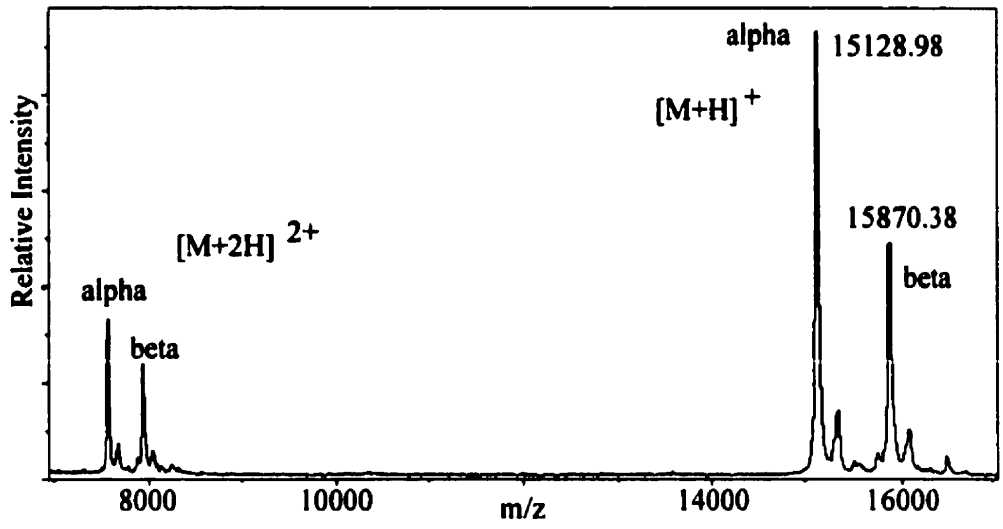

Figure 5-2. MALDI-TOFMS of normal hemoglobin.

The measured masses of the normal globin chains were: alpha $[M+H]^+$ = 15128.98 Da, beta $[M+H]^+$ = 15870.38 Da (n=3), in agreement with the calculated average masses derived from the sequence ($[M+H]^+$ = 15127.3 and 15868.2 Da respectively). Mass accuracy was ca. 0.01% using internal calibration and the resolution was ca. 800 (FWHM).

The mass spectrum of the Shepherds Bush variant is shown in Figure 5-3. The mass obtained for the variant was $[M+H]^+$ = 15928.83 Da, an increment of 58.45 Da (58.04 Da theoretical) relative to the normal beta chain, which was clearly observable in the mass spectrum. The peak ratio observed for the normal alpha and beta chains was similar to that reported by others with the alpha chain being predominant [9]. However, the peak area for the Shepherds Bush chain was observed to range from ca. 25% to 100% of the normal beta chain peak area. The upper value is larger than the reported value of 24% based on electrophoretic measurements [18]. This discrepancy may be attributed to non-uniform MALDI ionization phenomena [22,23].

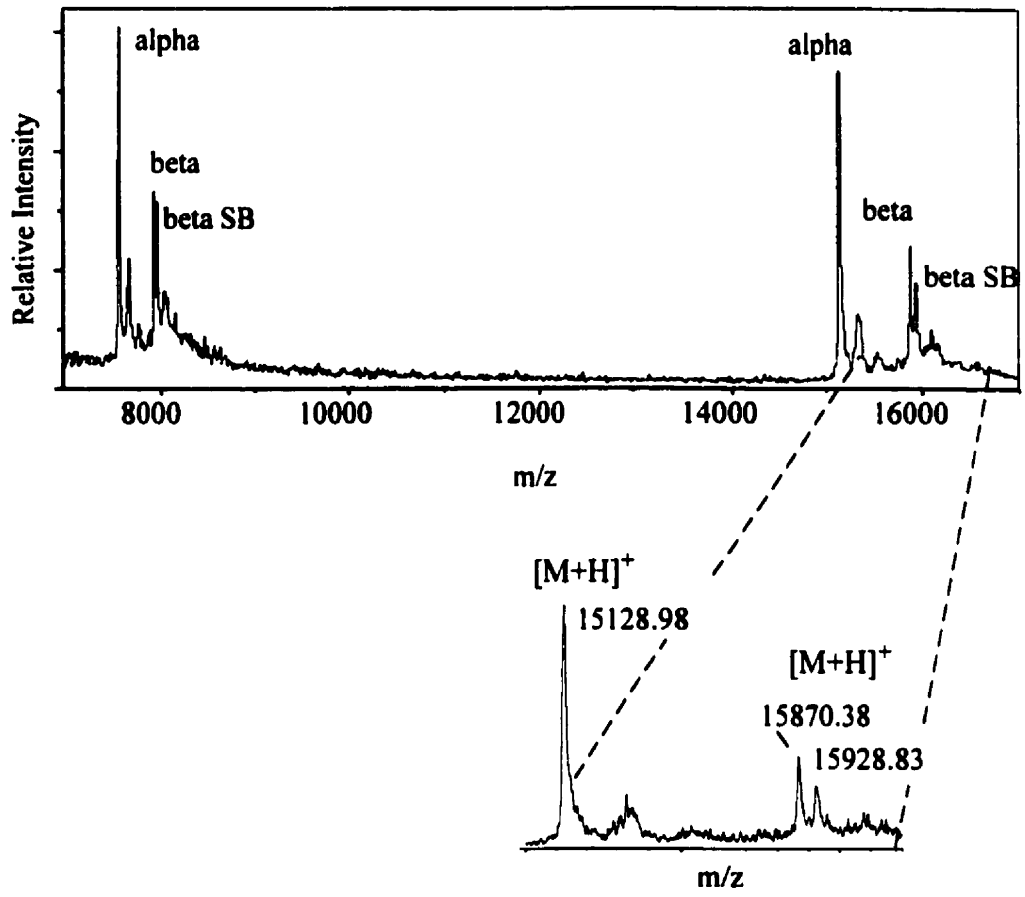

Figure 5-3. MALDI-TOFMS of the Shepherds Bush variant. The variant b74 (E18) Gly->Asp is indicated by a mass shift of 58 Da.

Preparation of samples on PU membranes and introduction of the samples into the mass spectrometer were relatively simple. Both steps were facilitated by the probe design. Samples could be prepared on the PU membranes and affixed to the metallic disks, allowing for analysis at a later date in the MALDI-TOFMS laboratory. The dual-part probe design enabled the introduction of samples at a rate of one every few minutes. The longest delay was due to the evacuation of the mass spectrometer.

Collection of samples of whole blood on the membrane with the lancet device constitutes a relatively non-invasive means of obtaining a sample, in comparison to the use of a syringe. The sampling protocol is easy to follow and may be performed by an untrained

individual. The presence of a health care practitioner is thus not necessary to draw a liquid sample. Samples were shipped to the laboratory by courier and no precautions were taken with regards to transportation. Samples were analyzed up to 7 days after acquisition with no observable change in the quality of the mass spectra. For prolonged storage, the sample-loaded membranes were placed in the freezer at ca. -20 °C.

When collecting the blood on PU membranes, smaller sample amounts provided for better results than larger amounts. More than 1-2 μL of whole blood resulted in excessive coagulation within the sample itself. This produced a scab which did not strongly bind to the membrane, and which in one case actually detached itself from the membrane during transportation. In addition, larger samples of whole blood proved to be more difficult to re-solubilize for co-crystallization with the matrix or for on-membrane digestion. In most cases, not all of the blood sample was re-solubilized. Excess material was removed in the washing procedure. However, this did not decrease the intensity of the MALDI-TOFMS signals, presumably due to the high sensitivity of the MALDI method. A 1-μL sample of whole blood contains approximately 23 picomoles of hemoglobin, significantly more than that required for MALDI-TOFMS.

The formation of scabs led us to investigate the use of modifiers which would disrupt coagulation of the sample on the membrane. Our initial work on PU membranes showed that the addition of methanol to samples deposited on the membrane caused swelling of the PU and enhanced protein sorption. Proteins prepared on PU without the addition of methanol were desorbed from the membrane more easily when washed with water. Methanol possibly disrupts the intermolecular forces holding the polymer chains together, thus allowing an increase of the effective surface area available for protein sorption. At the same time, it facilitates the partitioning of proteins and peptides from more polar components, such as salts. The effectiveness of methanol in disrupting the coagulation process was thus investigated. The addition of methanol to freshly collected liquid samples resulted in a decrease in the extent of coagulation compared with samples prepared without methanol. The MALDI-TOFMS spectra were slightly improved compared to those obtained for the samples prepared without the addition of methanol. This improvement was attributed to cell lysis rather than a decrease in coagulation.

As discussed previously by Houston and Reilly [9], a mass resolution of 1000 (FWHM) will allow the detection of a variant with a mass difference of ca. 16 Da at m/z = 15867 from that of the normal component. This is insufficient to resolve all possible variants occurring in the hemoglobin chains. One possible means of improving the identification of variants is to use ESI-MS in combination with a separation technique such as HPLC [3,5]. This improves the resolution to ca. 6 Da when deconvolution is used. Another method is to perform a proteolytic digestion of the alpha and beta chains of hemoglobin in order to produce smaller fragments. For example, with a mass resolution of 1000 (FWHM) and a molecular mass of a proteolytic fragment of 5000 Da, it would be possible resolve two peaks with a mass difference of 5 Da. This method would extend the number of variants which could be characterized using a MALDI-TOF instrument with moderate resolution.

Recently, Krutchinsky et al. introduced an orthogonal injection MALDI-TOF mass spectrometer with a resolution of ca. 5000 (FWHM) at 6000 Da, and a mass accuracy around 30 ppm [24]. With this resolution and accuracy it would be possible to identify mass differences of ca. 3 Da for the globin chains. Tryptic fragments may be characterized with isotopic resolution thus ensuring accurate peptide mass mapping.

5.3.2 On-Membrane Tryptic Digestion of Hemoglobin

Proteolytic digestion also allows for peptide mapping and may also be used to pinpoint the site of the mutation in the peptide chain. Peptide maps can be used to identify a protein, but in this case can also be used to generate patterns which are indicative of a specific variant [3]. Not only will there be a mass shift in one of the peptides as a result of the variant, but the extent of proteolysis and the cleavage sites may change due to the relative instabilities of variant chains compared with those of normal hemoglobin, which are generally more stable [18,19].

The membrane methodology was applied by performing tryptic digests of the normal and Shepherds Bush hemoglobin directly on the membrane. Digests were performed on the membrane for periods of time varying from 2 to 60 minutes. Good quality MALDI spectra were obtained once buffer components were removed using the washing procedure. Figure 5-4 shows the mass spectra obtained for the 2, 5, 10, and 60-minute digest of normal hemoglobin. During the digest, the initially abundant high mass ions were

gradually replaced with lower mass ions. Also, the protein underwent significant digestion after only two minutes. This may indicate that the protein becomes denatured upon sorption onto the membrane, thus facilitating rapid digestion. The digestion times were varied in order to optimize the procedure. Ideally, the best situation would be a relatively short digestion time which would allow formation of enough fragments to identify the variant. A 10-minute digestion was chosen as it produced an abundance of fragments within the mass range of 1000 - 9000 Da with good reproducibility.

Figure 5-5 shows the MALDI-TOF spectra obtained for 10-minute tryptic digests of (a) normal hemoglobin and (b) the Shepherds Bush variant. Most segments of the protein were mapped against calculated fragments by mass, as shown in Table 5-1. Digestion of the variant produced seven observable fragments which contained the mass shift corresponding to the Gly-Asp mutation. These are indicated with an asterisk (*) in Table 5-1.

Significant digestion was observed after 10 minutes, allowing for the characterization of the entire beta variant. Three ambiguous assignments appear in Table 5-1, at ca. 3267 Da, 5321 Da and 5593 Da. It was not possible to resolve these peptides using MALDI, due to the small mass difference. Further characterization would thus require digestion with a different enzyme or the use of sequencing methods. The 10-minute digestion of normal hemoglobin and of the variant produced a number of different fragments, as indicated in Table 5-1 and on the peptide map shown in Figure 5-5.

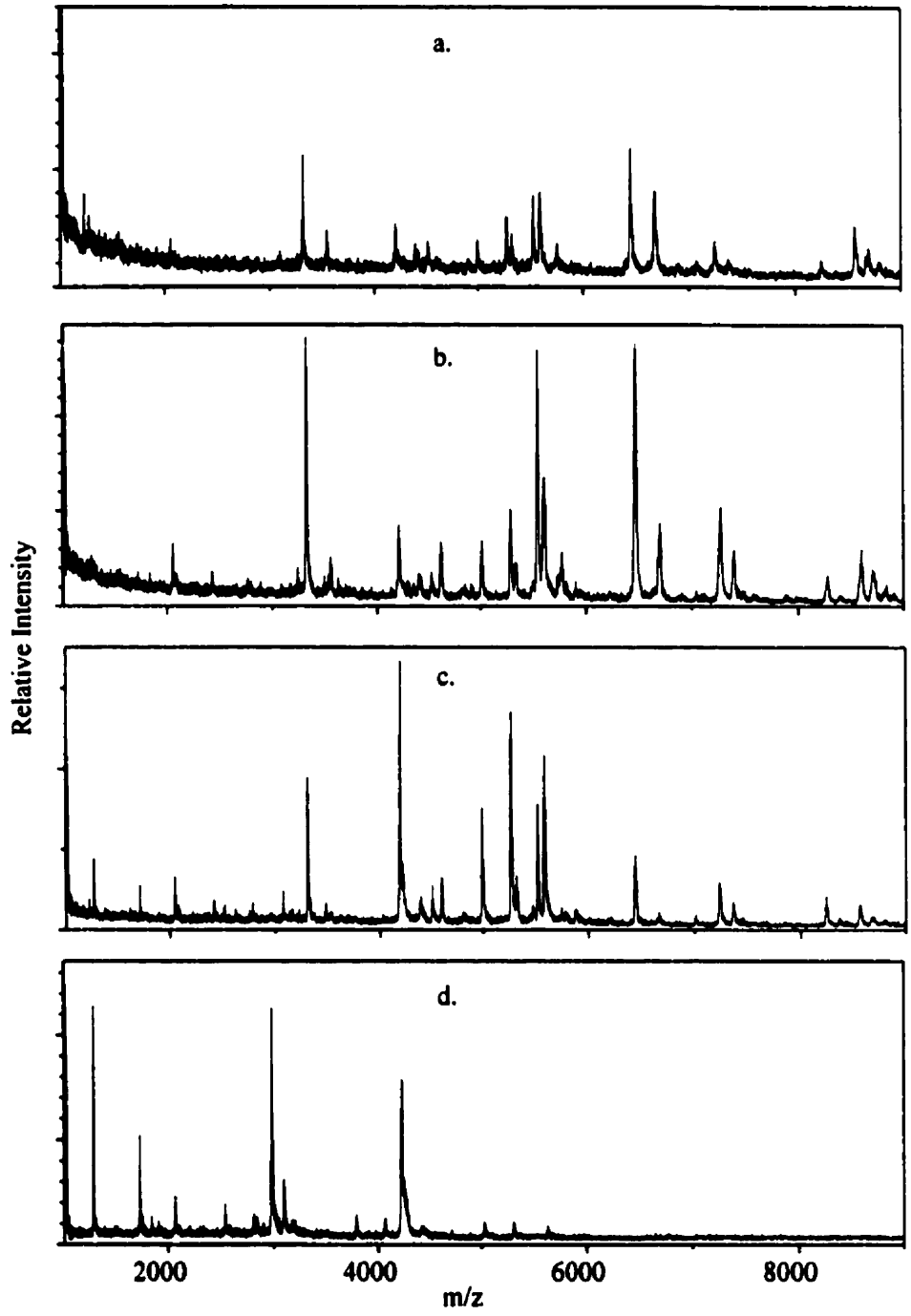

Figure 5-4. MALDI-TOFMS of tryptic fragments of normal hemoglobin. Digestion was performed directly on the membrane for (a) 2, (b) 5, (c) 10 and (d) 60 minutes. Each spectrum is the sum of 40-50 shots. Spectra were acquired in linear mode with internal calibration based on known peaks.

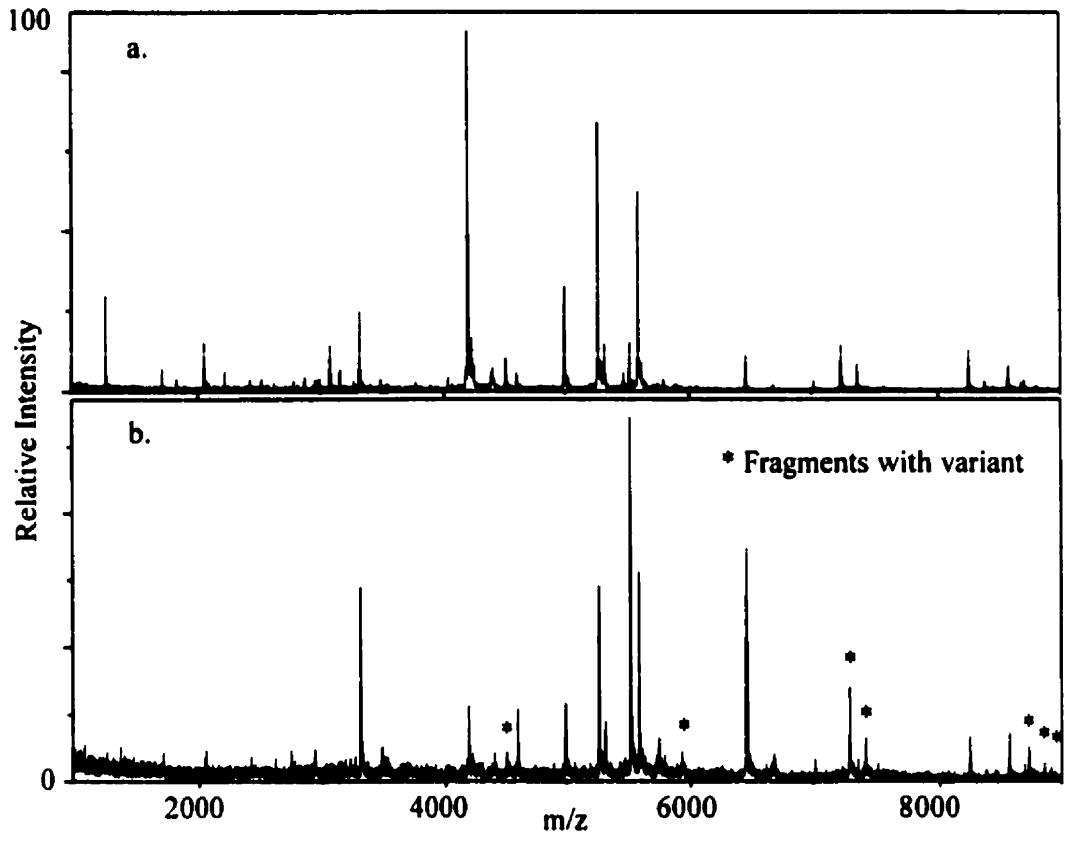

Figure 5-5. 10 minute digest of normal and Shepherds Bush hemoglobin. MALDI-TOF mass spectra of tryptic fragments of the alpha and beta chains of (a) normal hemoglobin and (b) hemoglobin containing the Shepherds Bush variant. Each spectrum is the sum of 40-50 shots. Delayed extraction was used with internal calibration based on known peaks. Each sample was digested directly on the membrane, with trypsin, for 10 minutes. Peak assignments are in Table 5-1. Asterisks peaks (*) indicate fragments derived form the variant b74(E18)Gly->Asp.

Table 5-1. Tryptic fragments of normal & Shepards Bush hemoglobins on PU.

Tryptic		Calculated	Normal		Shepherds Bush	
Fragment	Sequence	Mass $[M+H]^+$	Mass $[M+H]^+$	% error	Mass $[M+H]^+$	% error
b4	31 - 40	1275.55	1275.54	0.001		
b13	121 - 132	1379.56			1383.56	0.289
b12	105 - 120	1721.13	1720.52	0.035	1719.64	0.087
a6	41 - 56	1835.03	1834.27	0.041		
b5	41 - 59	2060.30	2059.99	0.015	2059.37	0.045
b2-3	9 - 30	2229.50	2229.69	0.009		
b10-11	83 - 104	2530.83	2531.07	0.009		
a5-6	32 - 56	2888.32	2888.64	0.011		
a12	100 - 127	2969.52	2968.77	0.025		
a9	62 - 90	2998.35	2997.17	0.039		
b12-13	105 - 132	3081.66	3082.02	0.012		
b1-3	1 - 30	3163.56	3162.45	0.035		
a9-10	62 - 92	3267.70	3267.39	0.009		
a5-7	32 - 60	3267.74	3267.39	0.011		
b4-5	31 - 59	3316.81	3316.63	0.005	3314.55	0.068
b2-4	9 - 40	3486.01	3483.72	0.066		
a7-9	57 - 90	3505.94			3500.99	0.141
a11-12	93 - 127	3769.45	3767.42	0.054		
a10-11	91 - 127	4038.80	4039.56	0.019		
a12-13	100 - 139	4203.98	4205.26	0.030	4202.02	0.047
b10-12	83 - 120	4232.93	4230.62	0.055		
a1-5	1 - 40	4250.87	4251.40	0.012		
a4-6	17 - 56	4399.94	4400.48	0.012		
b1-4	1 - 40	4420.07			4417.83	0.051
a12-14	100 - 141	4523.35	4524.37	0.023	4520.77	0.057
b5-9	*41 - 82	4519.14			4520.77	0.036
b3-5	18 - 59	4613.21	4613.72	0.011	4611.76	0.031
a11-13	93 - 139	5003.91	5003.91	0.000	5001.77	0.043
a10-13	91 - 139	5273.26	5273.60	0.006	5271.72	0.029
a6-9	41 - 90	5321.95	5323.34	0.026	5320.6	0.025
b11-14	96 - 144	5321.23	5323.34	0.040	5320.6	0.012
b2-5	9 - 59	5527.29	5527.58	0.005	5526.11	0.021

Table 5-1. Tryptic fragments of normal & Shepards Bush hemoglobins on PU.

Tryptic		Calculated	Normal		Shepherds Bush	
a6-10	41 - 92	5591.30	5593.17	0.033	5591.92	0.011
a10-14	91 - 141	5592.62	5593.17	0.010	5591.92	0.013
b10-13	83 - 132	5593.46	5593.17	0.005	5591.92	0.028
a2-7	8 - 60	5735.47			5733.28	0.038
b2-6	9 - 61	5754.59			5753.29	0.023
b9-12	*67 - 120	5942.85			5939.32	0.059
b1-5	1 - 59	6461.35	6461.53	0.003	6461.24	0.002
b1-6	1 - 61	6688.65	6687.57	0.016	6686.94	0.026
b10-15	83 - 146	7025.12	7023.64	0.021	7024.54	0.008
b9-13	67 - 132	7245.35	7245.85	0.007		
b9-13	*67 - 132	7303.38			7302.55	0.011
b8-13	66 - 132	7373.52	7374.04	0.007	7371.98	0.021
b8-13	*66 - 132	7431.55			7431.32	0.003
a9-13	62 - 139	8252.58	8251.99	0.007	8252.92	0.004
a8-13	61 - 139	8380.76	8380.84	0.001	8379.47	0.015
a9-14	62 - 141	8571.95	8571.95	0.000	8571.42	0.006
b9-15	67 - 146	8677.01	8677.13	0.001		
a8-14	61 - 141	8700.12	8699.75	0.004	8699.48	0.007
b9-15	*67 - 146	8735.04			8734.65	0.004
b8-15	66 - 146	8805.19	8803.01	0.025		
b8-15	*66 - 146	8863.21			8862.21	0.011
b1-9	*1 - 82	8920.19			8919.16	0.012

Peaks marked with an asterisk contain the variant.

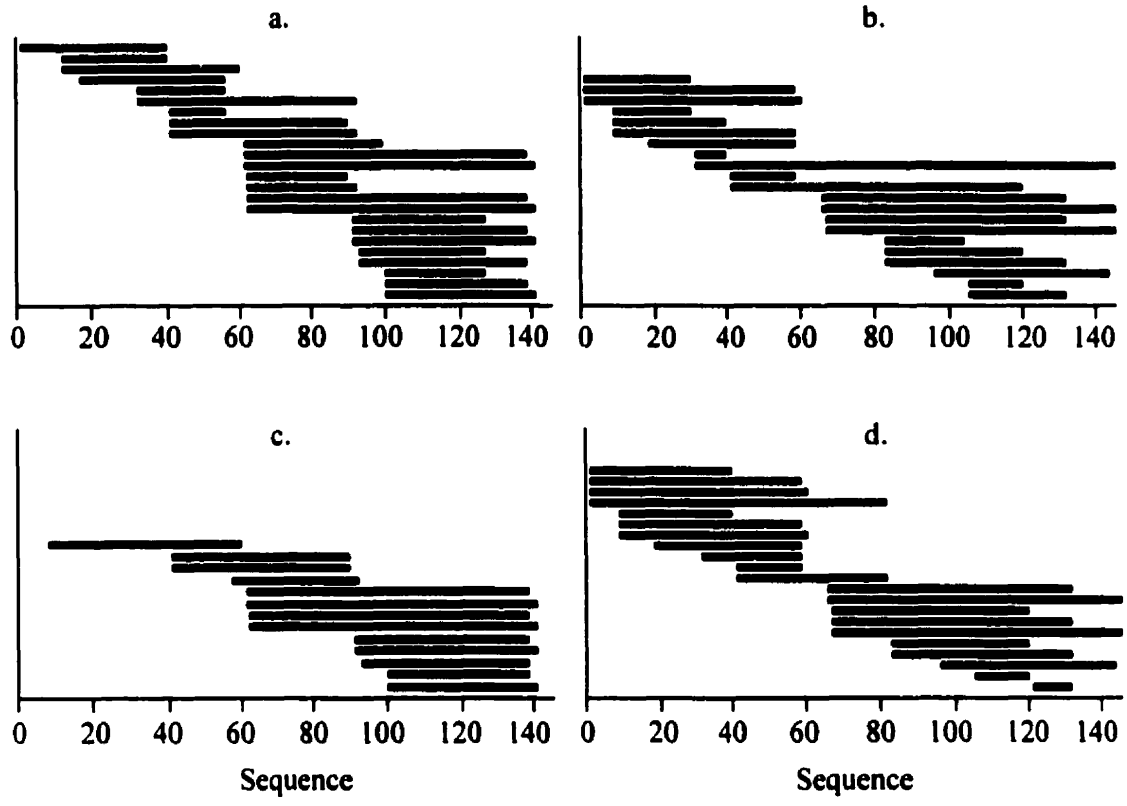

Figure 5-6. Peptide map of tryptic fragments of Hb A and Hb SB. (10 minute digest) of the (a) alpha and (b) beta chains of normal hemoglobin, and the (c) alpha and (d) beta chains of a sample containing Shepherds Bush variant hemoglobin

The peaks associated with beta chain fragments containing the variant were much more intense in comparison to analogous peaks from the normal beta chain appearing in the same mass spectrum. In some cases, the peaks corresponding to the normal fragments were not observed. For example, in the mass spectrum of Figure 5-5, there is no peak observed at 7245.85 Da (normal); only the variant peak at 7302.55 is observed. This is surprising, as the variant makes up for only 24% of the heterozygote. It is unlikely that the fragments containing the variant would produce a more intense signal based only on MALDI considerations, as the Gly-Asp shift should not enhance the proton affinity of the fragment. A possible but unlikely explanation is that the fragments containing the variant are retained more strongly on the PU membrane during the washing process. A more reasonable possi-

bility is that the variant hemoglobin is likely to undergo more extensive proteolytic digestion because of the decreased stability of the 3^{o} structure of the beta globin due to the amino acid substitution [18,19]. Replacement of Gly with Asp results in an increased charge within the heme pocket of the globin. This weakens the hydrophobic forces in the center and destabilizes the globin, allowing easier penetration of water. The digestion results suggest that the variant, due to its reduced stability relative to that of normal beta globin, undergoes slightly different denaturation and digestion.

Mapping of the proteolytic fragments may be used for positive identification of the variant in addition to simply measuring the mass differences of the globin chains. By performing the digest, it was possible to further characterize the Shepherds Bush variant. The mass difference observed in the beta chain, following the tryptic digest, could be assigned to the sequence 67-82. With a second digestion using a different enzyme, or with the use of post-source decay methods, it may be possible to sequence the chains and accurately pinpoint the substitution site.

This method allows for the rapid generation of a peptide map, which in turn allows for rapid identification with the help of computerized searches [24]. In combination with automated laboratory methods of analysis, this technology would open the possibility to routinely characterize samples from various locations and would favor accuracy and cost effectiveness.

5.3.3 Sequencing using a QqTOF Mass Spectrometer

In order to gain complete information on a variant it is necessary to sequence the peptide containing the site of mutation. Sequencing was performed on the prototype University of Manitoba-Sciex QqTOF mass spectrometer on samples of normal hemoglobin which had undergone digestion with trypsin. Spectral acquisition was performed with the assistance of M. Bromirski and A. Loboda. Figure 5-7 shows two example product ion mass spectra obtained on two tryptic fragments of hemoglobin.

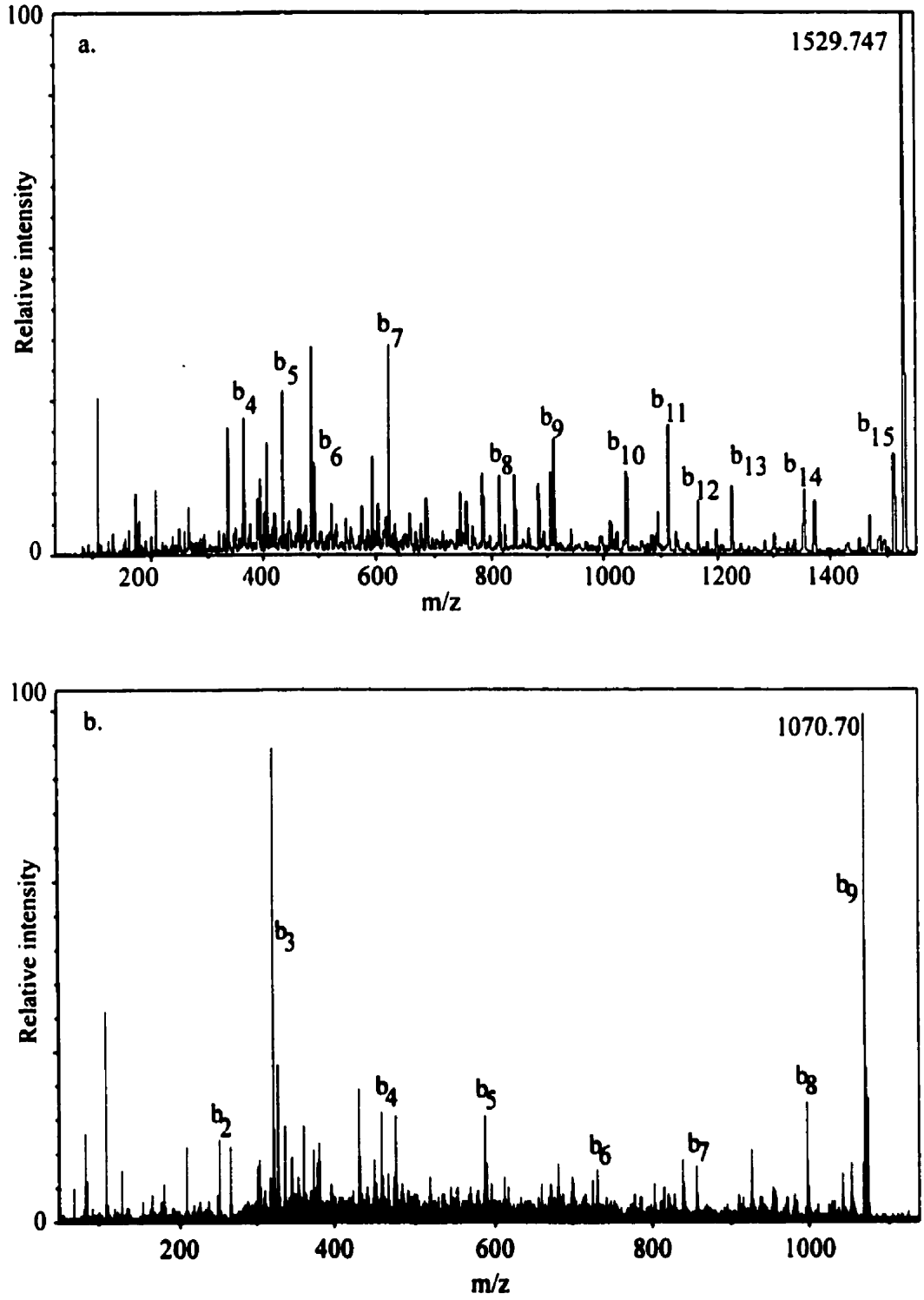

Figure 5-7. QqTOF sequencing of peptides from hemoglobin. Assignments as per nomenclature in Chapter 1.4.4.

Figure 5-7.a. shows sequence information from the peptide of mass 1529.747 obtained from a 1 hour digest of whole blood. The corresponding b-ion series was identified and the sequence identified as being from the alpha chain of hemoglobin; VGAHAGEYGAEALGR, theoretical mass 1529.630 Da, Ha(17-31), T4. Immonium ions and ions of other series were also identified (not shown).

This result, typical of the instrument, was obtained under ideal conditions with the laser fluence set slightly above threshold. With the instrument, ions of m/z < 3000 may be sequenced relatively easily given a sample amount of ca. 1 pmol. Mass accuracy (76 ppm) and resolution (R>5000) were typical of the day to day performance of the instrument and facilitated peak assignments. Analysis time was of the order of minutes and several precursor ions could be sequenced using a single sample.

Figure 5-7.b. shows results for a peptide from the alpha chain; LHAHKLRVD, theoretical mass 1070.700 Da, Ha(86-94), T?. In this case, the sequence and molecular weight did not correspond to a known tryptic fragment. Based on the b-ion series alone, the peptide was assigned to the alpha chain of hemoglobin, corresponding to the tryptic fragment; T9-11, VADALTNAVAHVDDMPNALSALSD*LHAHKLRVD*PVNFK, Ha(62-99). Here, the laser fluence used to obtain ions was significantly above threshold. This resulted in a two stage fragmentation process. Prompt fragmentation occurred near or at the target, or in the quadrupole ion guide used for collisional cooling. This produced a b-ion (b9) of mass = 1070.263 Da. The newly formed ion was then selected by the first scanning quadrupole and fragmented by CID in the collision cell prior to detection. The result is the tandem mass spectrum of a metastable fragment of a tryptic product. Such phenomena were observed periodically with the instrument and care was required to establish experimental parameters to minimize them. Use of lower laser fluences and less energetic matrices other than sinapinic acid resulted in less prompt fragmentation and was required when analyzing proteins of moderate M_r, m/z > 5000.

5.4 CONCLUSION

The preliminary results on the characterization/differentiation of normal and variant hemoglobin are favorable. MALDI mass spectra obtained on both the normal and Shepherds Bush hemoglobin samples are of good quality and informative. Sampling is easy to

effect and may be performed by an inexperienced user. Sample handling and shipment are facilitated by using the membrane, i.e. a liquid sample is not required. Analysis is rapid, and minimal time is required for hands-on preparation in the laboratory. Samples may be collected in any setting outside of the mass spectrometry laboratory, with a lancet device and a small piece of PU membrane (1 x 1 cm^2). Less than 1 μL of whole blood is sufficient to produce good quality MALDI-TOFMS spectra, and there is no need for a large volume of liquid sample. The analysis is sensitive because loss is minimized during digestion and during the washing steps as both processes are conducted on the membrane. The use of PU membranes with MALDI adds no significant cost to the analysis and thus may be applied on a routine basis. On-membrane digestion with data-base mapping should facilitate identification of abnormal amino acid sites in the sequence of the alpha and beta chains. MALDI-TOFMS spectra of the intact chains may give molecular weight information on the chain, which may constitute insufficient information for complete identification of a variant. Tandem MS of tryptic peptides was demonstrated and suggests the possibility of obtaining complete sequence information of a variant, on a single sample, in a minimal amount of time.

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6 CHARACTERIZATION OF PLASMA PROTEINS ADSORBED ONTO BIOMATERIALS BY MALDI-TOFMS

6.1 INTRODUCTION

Medical devices composed of polymeric materials have become ubiquitous for the treatment of certain disorders, however, implantation of polymeric devices within the human body can lead to a number of problems. Inflammation and thrombosis may cause the body to reject the implant, or the polymer material may be enzymatically degraded, leading to the deterioration or failure of the device. Protein sorption onto the surface of the biomaterial is a major factor contributing to the stability of materials in the body, and ultimately determines the in vivo performance of the polymer [1,2]. The adsorption of certain proteins is beneficial to inhibit further physiological response, while adsorption of others produces a detrimental effect, enhancing platelet adhesion and thrombus formation (shown schematically in Figure 6-1 and resulting in difficulties with the implanted device. Therefore, the characterization and measurement of surface adsorbed proteins is important for assessing the biocompatibility of a material.

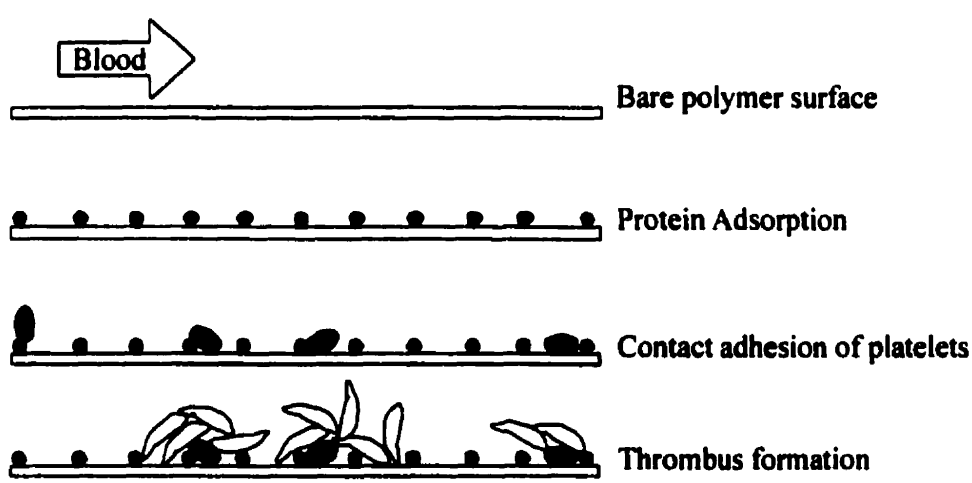

Figure 6-1. Protein sorption model.

Currently the characterization of adsorbed proteins is carried out using a number of techniques [3]. Traditional analyses utilizing radio-labeled proteins [4,5] have been performed, but are limited to monitoring the adsorption of one protein at a time. More elaborate protocols involving removal of adsorbed proteins from the biomaterial surface with a surfactant and subsequent analysis by SDS-PAGE [6,7] have also been used, but are extremely time-consuming. More recently, techniques such as Fourier transform infrared spectroscopy (FTIR) [8-10], Raman spectroscopy, and surface plasmon resonance (SPR) [11-13] have been utilized to quantify the amount of adsorbed protein on surfaces of materials, but are also unable to efficiently analyze several proteins simultaneously. Often, a physiological response to a biomaterial is caused by a complementary interaction [14,15] between two or more proteins. Techniques that can monitor the adsorption of only a single protein would not detect a synergistic interaction. A technique that can monitor and characterize the adsorption of several proteins simultaneously on the biomaterial would be ideal.

MALDI-TOFMS can simultaneously measure the molecular weights (M_r) of several proteins in a sample, allowing them to be identified by comparing experimentally determined M_r with literature values and has been used to analyze proteins prepared on different types of supports. Generally these supports have been polymeric membranes and have been used to remove polar components from protein samples. Membranes so far used for the purification of protein samples generally have a high binding affinity for a wide variety of proteins. In contrast, biomaterials usually adsorb certain proteins preferentially to give them enhanced in vivo stability. It has recently been suggested that MALDI-TOFMS would be a convenient method for the characterization of proteins adsorbed directly onto surfaces, such as biomaterials [16]. The biomaterial sample may be analyzed in an analogous manner to membrane MALDI-TOFMS samples and thus provide information about the type, and amount of protein bound to its surface following exposure to a biological fluid.

In this study, initiated by R.D. Oleschuk, PU was chosen as a model biomaterial based on its extensive use in the construction of biomedical implant devices [4] and previous characterization as a sample support for MALDI-TOFMS (Chapters 3 - 5). Protein

sorption onto PU in vitro and in vivo is well characterized within the literature, allowing MALDI-TOFMS to be compared with more traditional methods of analysis.

Polyurethane membrane material was exposed to plasma standards from three different hosts. Mass spectra of the plasma standards on the PU biomaterial showed the adsorption of several proteins ranging from 6.5 - 150 kDa. Proteins observed in the mass spectra directly correlate with those shown by other researchers to be adsorbed from plasma onto PU based materials, thus validating MALDI-TOFMS as a technique for biomaterial analysis. This type of application may also be used for screening among possible future biomaterials.

6.2 EXPERIMENTAL

6.2.1 Preparation of Plasma Standard Samples

Samples prepared on PU were based on protocols developed in our laboratory. Samples were prepared on the membrane by first depositing 2 μL of the plasma solution onto the surface of the membrane and allowing it to dry. A mark was placed on the underside of the membrane to allow the sample spot to be located after washing. The membrane was then washed with 50 μL of water for 1 minute to remove the non-adsorbed plasma components. The washing step was repeated. After drying, immediately before analysis, matrix (2 μL, saturated sinapinic acid in 1:1 H_2O/ acetonitrile) was placed on the membrane and allowed to crystallize. The membrane was then trimmed to size, affixed to the MALDI target using an adhesive spray (Spraymount, 3M) and subsequently analyzed by MALDI-TOFMS.

6.2.2 In Vitro Experiments

In vitro experiments were performed at the Institut des Biomatériaux du Québec, Université Laval by Y. Marois and M. King. Samples of PU membrane material were cut into 2.0 cm x 2.0 cm pieces and exposed to pooled samples of freshly collected canine plasma in neutral glass vials (Anchor Ltd, London, Ontario). Membrane samples were exposed (in triplicate) for periods of time from 0 to 4 hours at 37 °C. Following exposure, the samples were immediately washed with water to remove non-adsorbed plasma compo-

nents, dried under vacuum for 24 hours and later shipped to our laboratory at the University of Manitoba for MALDI-TOFMS analysis.

6.3 RESULTS AND DISCUSSION

6.3.1 Analysis of a Human Plasma Standard

The nature of protein sorption ultimately determines the in vivo stability of a material. PU possesses a two phase bulk structure consisting of hard (moderately polar) and soft (non polar) segments as shown previously. The chemical structure of each of these segments greatly influences the ability of the polymer to adsorb proteins [17]. Protein binding is believed to occur through a combination of hydrogen bonding and hydrophobic interactions between the protein and the surface of the polymer [18]. Several different types of PU have been used and tested for the construction of biomedical devices. For our study the PTMO (polytetramethylene oxide)/ MDI type PU was chosen because it can adsorb larger amounts of protein than can PU prepared with more hydrophilic soft segments [19].

Although MALDI-TOFMS is relatively tolerant of impurities such as salts and buffer components, the removal of these species yields greater resolution and mass accuracy. In addition, proteins with no affinity for the membrane material must be removed prior to analysis. To accomplish this a rinsing step may be performed. All of the components with a high affinity for the biomaterial remain adsorbed while polar components and non-adsorbed proteins are washed off the material. The washing process is analogous to membrane purification of proteins prior to MALDI-MS analysis [20]. This process is necessary considering the chemical nature of the sample. In addition to proteins, plasma contains a number of components in relatively large amounts (Table 6-1) which would inhibit the acquisition of MALDI spectra.

Aliquots of human plasma standard (2 μL) were placed onto the surface of the membrane and allowed to dry. Following rinsing and application of matrix, the samples were placed onto a MALDI probe and inserted in the mass spectrometer for analysis. Figure 6-2 shows MALDI-TOF mass spectra of human plasma proteins adsorbed onto the PU biomaterial. Tentative assignments for each of the proteins are shown in Table 6-2.

Table 6-1. Other solutes in blood.

Component	Concentration (mM) [22]
Bicarbonate	42 - 54
Calcium	4.2 - 5.2
Chloride	95 - 103
Cholesterol	0.038 - 0.063
Glucose	0.036 - 0.055
Iron	1.0 - 2.68 x 10-4
Magnesium	3.0 - 5.2
Phosphate	1.8 - 2.6
Potassium	8 - 9.6
Sodium	136 - 142
Sulfate	0.4 - 2.6
Urea	0.013 - 0.033

Table 6-2. Tentative assignments for human plasma proteins on PU.

Protein	Molecular Weight Literature (Da) [21,22]	Molecular Weight Experimental (Da)	Concentration in plasma (g/l) [22]
Immunoglobulin G	150,000	148,400	8-18
Immunoglobulin E	135,000	134,700	
Transferrin	79,754	78,510	2.0-4.0
Comp C3-	75,000	73,950	<1
Albumin	66,458	66,460	35-55
2-HS Glycoprotein	49,000	49,580	0.4-0.85
Apo A-4	43,400	44,180	<1
Apo E	34,236	33,110	<1
Apo A-1	28,000	27,500	<1
Apo D	22,500	22,170	<1
Apo H-III (HDL)	17,000	16,330	3
Transthyretin	13,761	13,560	<1
Apo C-II	8,914	8,950	<1
Apo C-III	8,764	8,780	<1
Apo C-I	6,630	6,630	<1

In order to obtain reasonable signals in the high mass range (>20 kDa) it was necessary to suppress the background from the matrix by turning on the electron converter/ microchannel plate detector, only after the matrix ions had time to reach the detector. This resulted in a decrease in background matrix ions. Good quality MALDI mass spectra were thus obtained and ions from at least 14 different proteins were visible.

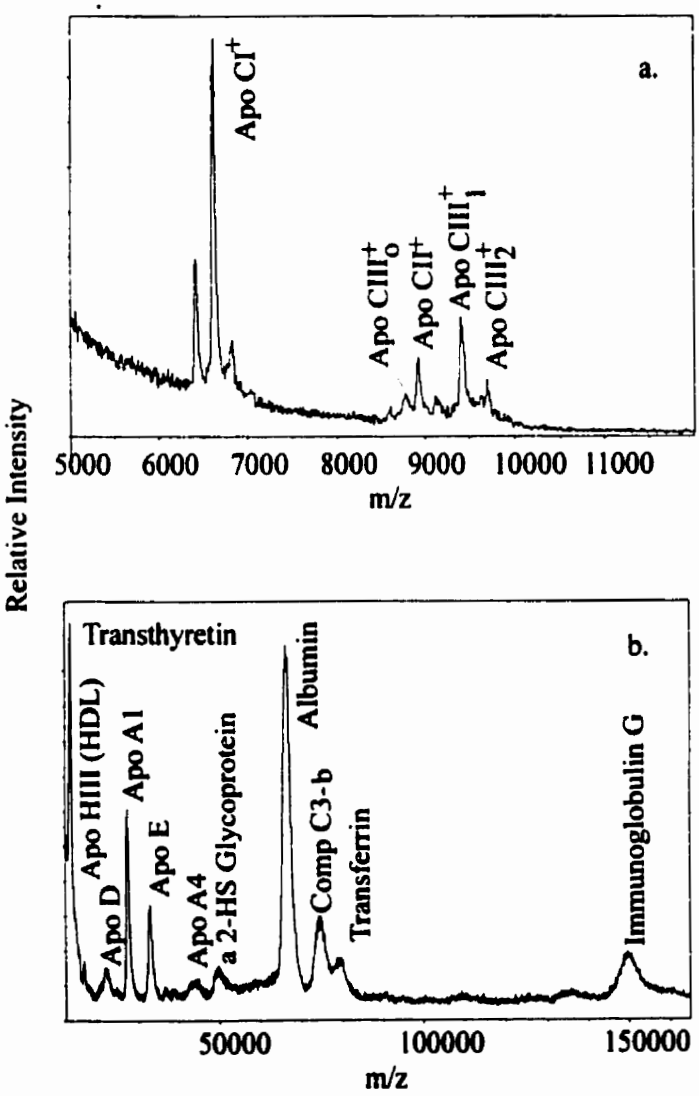

Figure 6-2. Human plasma proteins on PU.
(a) low M_r proteins (b) high M_r proteins.

The proteins ranged in M_r from ca. 6.5 kDa to 150 kDa. Mass resolution and accuracy were sufficient for identification upon comparison with M_r values from the literature [21,22].

Care must be taken in the interpretation of the spectra. The MALDI signal intensity is not necessarily indicative of the amount of protein on the surface of the membrane, neither is the amount of a particular protein adsorbed onto the membrane indicative of its concentration in solution. The affinity of the protein for the membrane plays a more significant role than its concentration in solution. This is demonstrated by the absence of ions of several major proteins, and by the presence of several low concentration proteins ions (Figure 6-2, Table 6-2). Only proteins with an affinity for the membrane material are adsorbed and later detected, while those with low affinity are washed off [16]. Additionally, discrimination phenomena for protein and peptide mixtures has been discussed previously.

Plasma proteins which are known to adsorb onto PU materials in vivo include lipoproteins [23,24], albumin [25], immunoglobulin G (IgG) [27], C3 as well as others [4]. The spectra presented in Figure 6-2 suggest a direct correlation between the proteins detected in the mass spectrum of human plasma on PU and proteins known to adsorb onto PU in vivo, determined using other techniques.

The development of biomaterials has focused on the adsorption of three proteins in particular, as indicators of biocompatibility [3]. The adsorption levels of albumin, IgG and fibrinogen are used to assess the in vivo performance of a material. For biomaterials, albumin adsorption is beneficial owing to this protein's relative lack of glycosylation, which prevents platelet adhesion. On the other hand, adsorption of IgG and fibrinogen may cause a host defense reaction which increases platelet adhesion and promotes a physiological response. Since the adsorption of both albumin and IgG was observed in this study, MALDI-TOFMS was used to determine the level of adsorption of these proteins. It follows that MALDI-TOFMS could be used to assess biocompatibility of different materials by analyzing the amount of albumin or IgG adsorbed by a particular material. The third major protein, fibrinogen, was not observed because of its high M_r (ca. 700 kDa). In addition, fibrinogen is normally observed after longer periods of exposure than were used in this investigation.

6.3.2 Plasma Standards of Different Hosts

Studies suggest that there are slight differences in protein adsorption patterns between plasma samples from different hosts and that these are due to variations in hematological profiles [27]. Plasma samples from three hosts (i.e. human, canine and bovine) were used to determine the ability of MALDI-TOFMS to distinguish between protein adsorption patterns for different plasma standards. The spectra of three different plasma standards adsorbed on PU are shown in Figure 6-3.

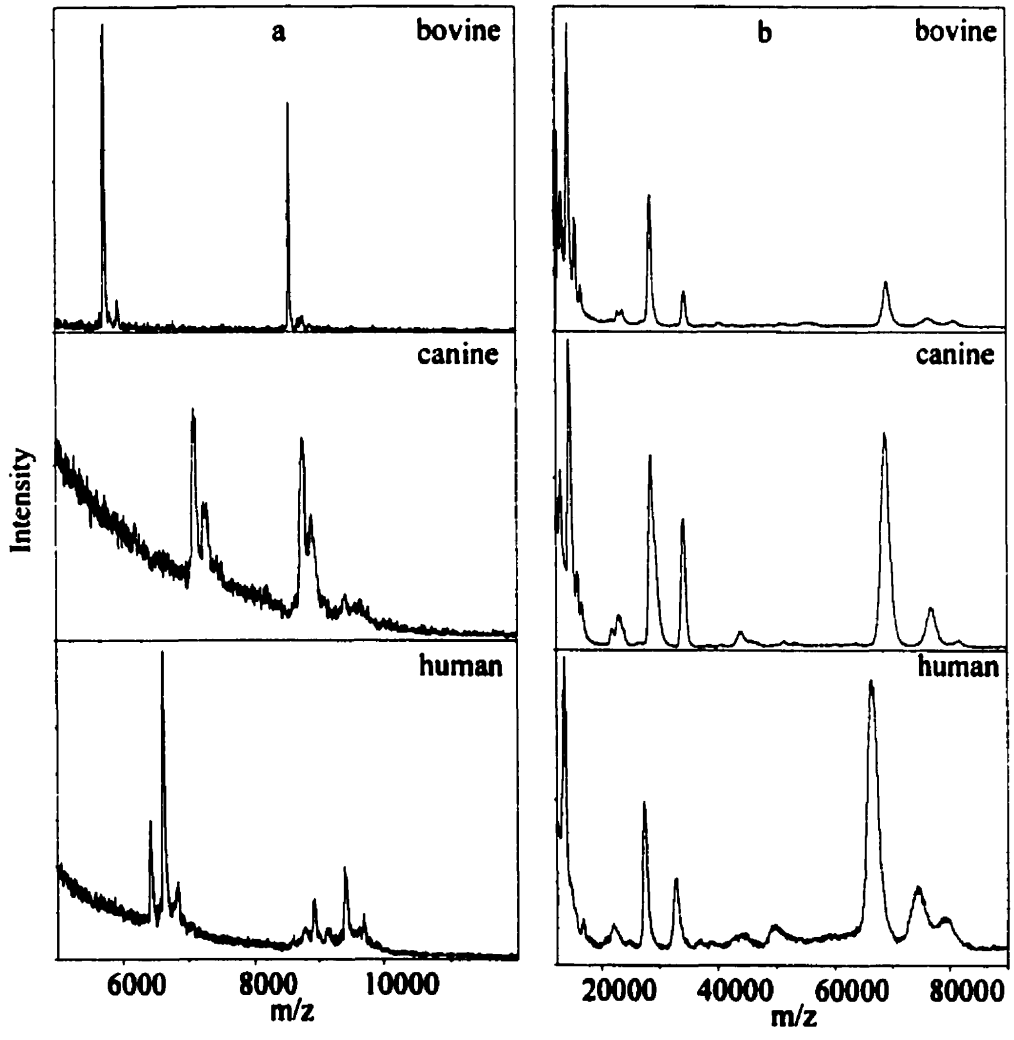

Figure 6-3. Plasma proteins from different hosts on PU.
(a) low molecular weight and (b) high molecular weight proteins.

These results indicate that proteins of similar high M_r were adsorbed (Figure 6-3.b) for each plasma standard. There were differences in the low M_r profiles (Figure 6-3.a). The human plasma standard resulted in the highest number of adsorbed proteins in the 5.5 - 10 kDa mass range, possibly lipoproteins. In comparison, the spectra obtained for bovine and canine plasma showed adsorption of fewer proteins in this region.

The adsorption of lipids onto PU surfaces has been associated with the biodegradation of PU implants [28]. It has been suggested that the interaction between lipoproteins and different domains of the PU facilitates lipid adsorption [4].

The variations between protein adsorption patterns could lead to differences in biocompatibility questioning the validity of testing biomaterials with other hosts [35]. MALDI-TOFMS, with its ability to distinguish between different protein adsorption patterns, can be seen as a valid method for biomaterial analysis.

6.3.3 Sample-to-Sample Reproducibility

The level of sample-to-sample reproducibility obtained with the preparation/ washing and subsequent MALDI-TOFMS measurements is exemplified by Figure 6-4, for 4 replicate samples of human plasma standard prepared under identical conditions. Each spectrum shown is the summation of 50 consecutive shots sampled over the entire surface of the target. Good shot-to-shot reproducibility was obtained when samples were subjected to the same sample preparation and MALDI conditions. Relative peak heights were consistent for each of the replicate samples when the surface of the target was rastered with the laser.

With good reproducibility, relative peak MALDI heights can be used as a semi-quantitative assessment of the concentration of different proteins adsorbed onto the biomaterial. In addition, small mass shifts in the protein adsorption profile of a material can be determined and allow better biomaterial characterization.

6.3.4 Influence of Sample Preparation Conditions

Different sample preparation methods were tested for their effect on ion signal intensities (Figure 6-5). In Figure 6-5.a, plasma was applied followed by subsequent wash-

ing and addition of matrix. In Figure 6-5.b, methanol was added following the application of plasma.

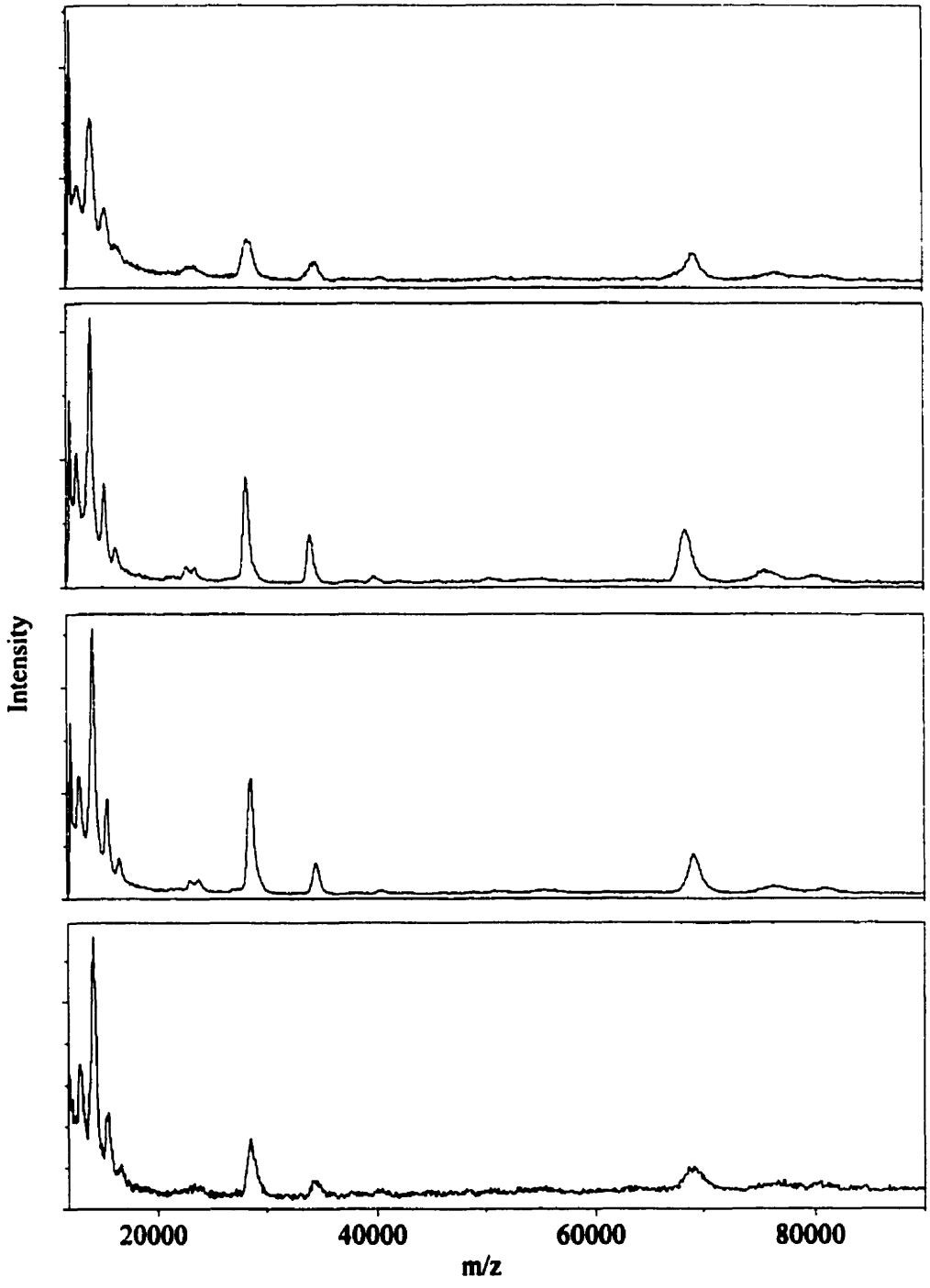

Figure 6-4. Reproducibility of sample preparation on MALDI spectra. Human plasma on PU, 4 replicate samples.

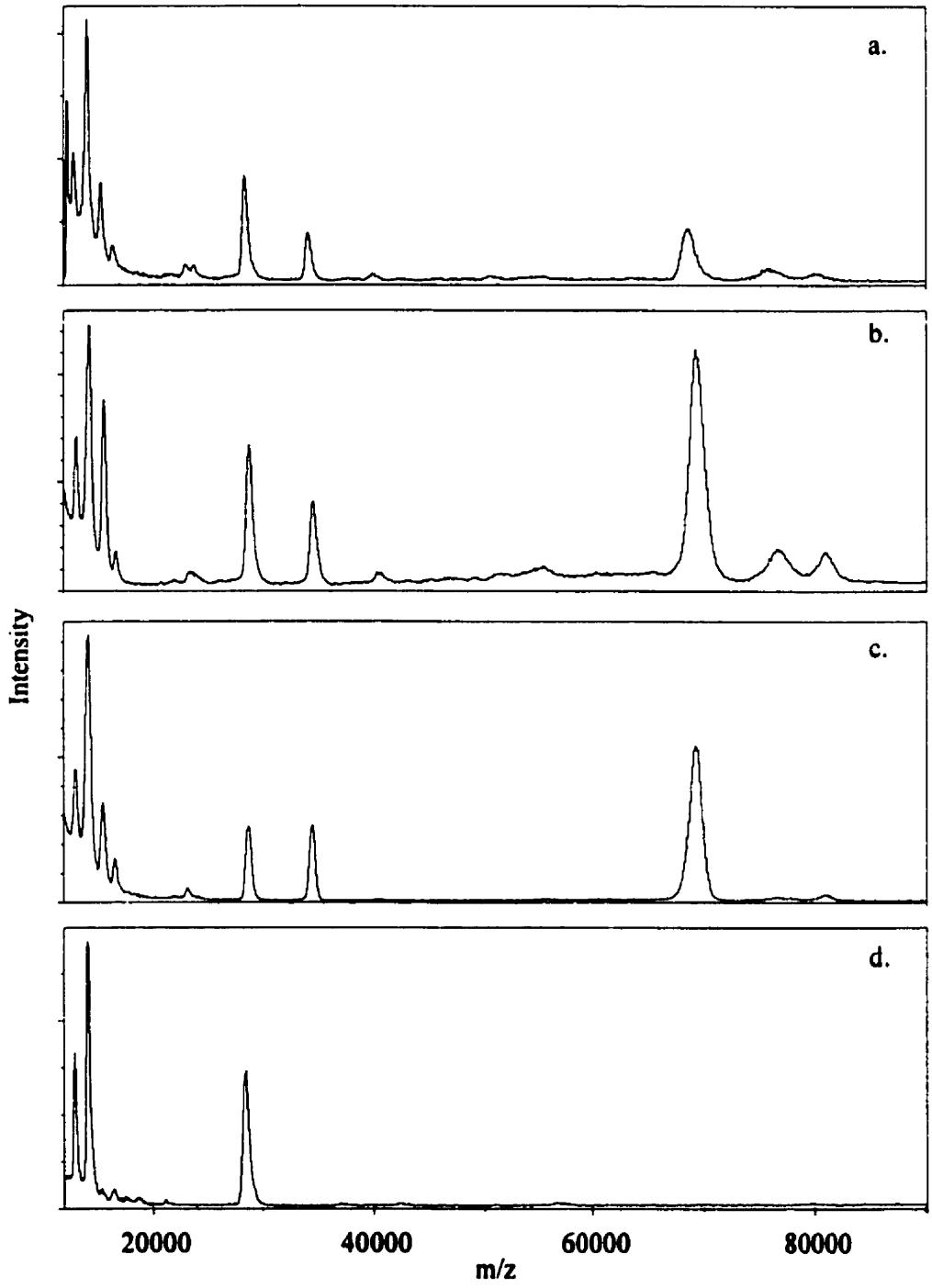

Figure 6-5. Effect of sample preparation on MALDI signal intensity. Human plasma on PU (a) plasma, dried, washed, dried, matrix. (b) plasma, dried, methanol, dried, washed, dried, matrix. (c) plasma + methanol, dried, washed, dried, matrix. (d) plasma + matrix solution, dried, washed, dried, matrix.

The addition of methanol resulted in an increase in the overall MALDI signal. A similar phenomenon was reported in Chapter 3. We associated this to enhanced protein binding on the membrane, an effect commonly exploited in SDS-PAGE electroblotting onto porous membranes. Methanol enhanced protein adsorption was also described for MALDI-TOFMS on porous membranes.

In Figure 6-5.c, methanol was added to the sample before the plasma sample had a chance to dry on the membrane. This resulted in the precipitation of the proteins from the plasma. Alcohol precipitation is often used to remove proteins from plasma/ serum in order to analyze other components. The result was an increase in signal intensity for some of the proteins, notably, albumin. Figure 6-5.d shows the effect of adding matrix solution to the sample before the plasma had a chance to dry on the membrane. In this case, the spectrum showed substantial differences relative to features observed in Figure 6-5.a-c. Even though the matrix material is dissolved in 50% (v/v) acetonitrile, it is acidic in nature. Protonation in solution will result in a net charge on the plasma proteins and will disfavor their sorption onto the predominantly hydrophobic membrane. The acidity of the matrix facilitates the removal of some more hydrophilic proteins from the surface so they may co-crystallize with the matrix for analysis by MALDI.

The different sample preparation protocols led to changes in the amount and nature of the proteins adsorbed and detected by MALDI. These discrepancies show that the same sample preparation method must be employed to directly compare protein adsorption patterns on different biomaterials. In one case signals were more intense when a single addition of methanol was made to the dried sample (Figure 6-5.b). However, this case does not reflect in vivo solution conditions. Such conditions were not used for the in vitro experiments.

6.3.5 In Vitro Analysis

The in vitro analysis of proteins adsorbed onto PU over different times of exposure to canine plasma was performed. Canine plasma was chosen as it is commonly used by biomaterials researchers and represents the worst case scenario for protein adsorption since it produces extensive platelet adhesion and thrombus formation. Canine plasma was placed in contacted with the PU material under specified conditions to facilitate protein sorption

for different lengths of time (0, 0.5, 1 and 4 hours) at 37°C. Representative mass spectra showing the effect of the time of exposure to canine plasma on the amount of proteins adsorbed are shown in Figure 6-6.

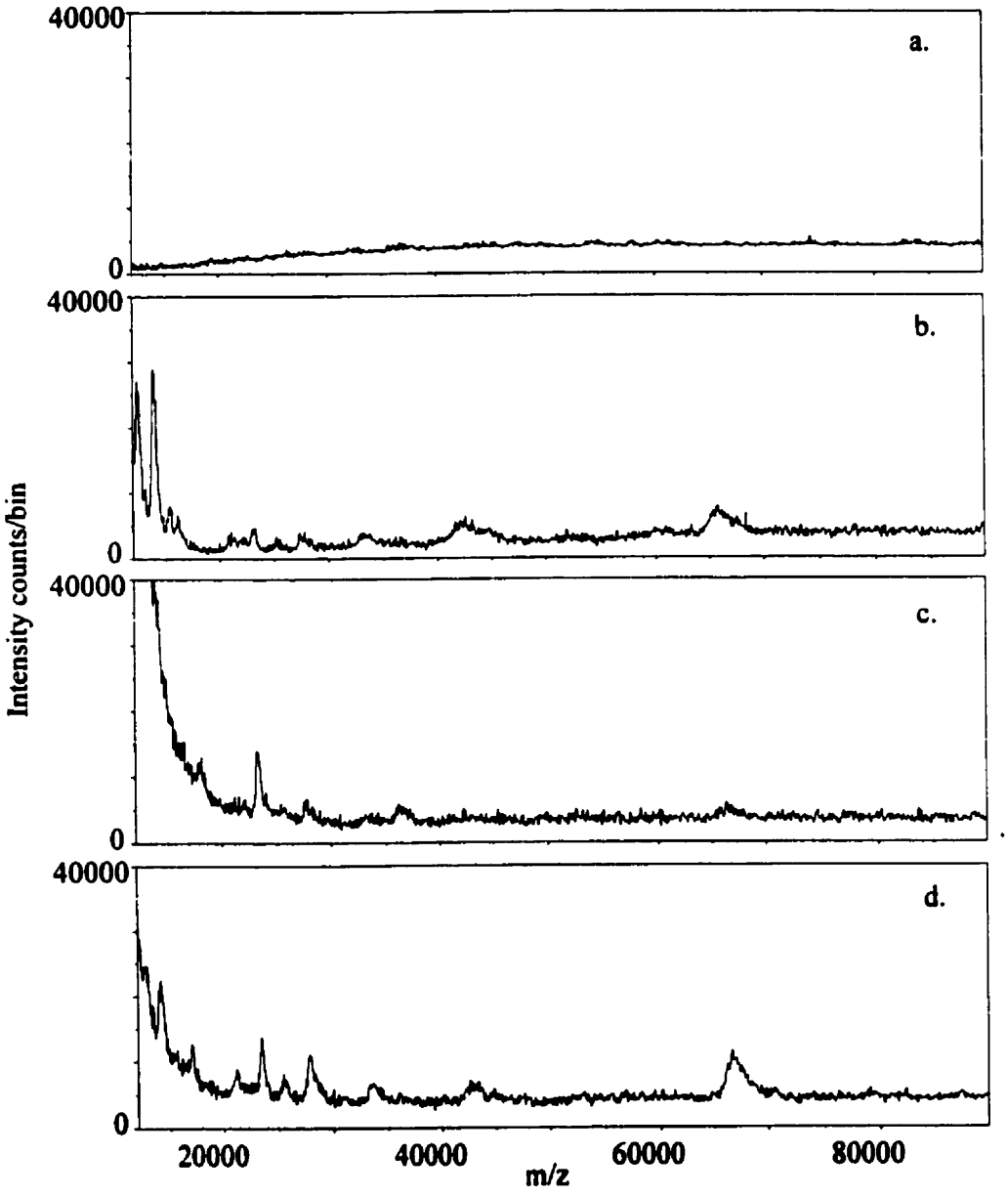

Figure 6-6. In vitro exposure of PU membrane to canine plasma.
Exposed to canine plasma for (a) 0 (b) 0.5 (c) 1 and (d) 4 hours.

In the control sample spectrum, (Figure 6-6.a) no peaks are observed. Analysis of the sample exposed to plasma for 0.5 hours shows several peaks due to adsorption of proteins onto the surface of the PU biomaterial. Continued exposure, up to 4 hours, yielded more intense protein signals. MALDI-TOFMS has been used for both the qualitative, semi-quantitative and quantitative analysis of protein and peptide mixtures [29,30]. The increase in the signal intensity may be interpreted as increasing amounts of protein being adsorbed on the surface of the polymer.

These data support the notion that protein adsorption occurs quite rapidly upon exposure of the polymeric material [1]. It has been suggested that for relatively short periods of exposure, protein adsorption fits a Langmuir isotherm [31]. The distribution of proteins adsorbed at the surface of a material is, however, time dependent. Proteins are constantly deposited, and displaced and thus the surface composition changes [32]. The rates of deposition and displacement can be determined by the relative concentrations of proteins within the plasma and their affinity for the material. Although proteins with a high plasma concentration may be adsorbed first, those with low concentrations but with a higher affinity for a particular surface will eventually displace them. The displacement of proteins occurs after the surface of the polymer has become saturated. This phenomenon was not explicitly observed in this study, even over the four hour exposure. Here, analysis of PU samples exposed to canine plasma in vitro yield spectra showing that, in general, the longer the exposure time, the larger the amount of protein present on the surface of the material.

The mass spectral profile observed, although weaker in absolute intensity, was similar to the profile shown for the canine standard (Figure 6-3.b). This resemblance indicates a strong similarity between the proteins observed from the in vitro experiments and those observed for the canine plasma standard. The use of plasma standards for biomaterials testing may therefore be sufficient as an initial screening process for the biomaterial. Here, the use of plasma standards is preferred because it is faster, simpler and less expensive than performing either in vivo or in vitro experiments.

6.4 CONCLUSIONS

Among the various kinds of interfacial phenomena occurring in the field of biomaterials, the adsorption of proteins at the interface between a liquid and a solid is one of the most significant areas that has been investigated. The analysis of proteins adsorbed onto biomaterials is a new application of MALDI-TOFMS. The technique is able to analyze several proteins simultaneously with sufficient resolution to allow the easy characterization of adsorbed proteins based on literature M_r. Experiments performed in this study showed direct correlations between the proteins known to adsorb on PU from plasma and those seen in the mass spectra of plasma proteins on PU.

The analysis of proteins adsorbed onto PU from plasma standards yielded good quality mass spectra, displaying a variety of proteins from 6.5 - 150 kDa. The MALDI spectra exhibited good shot-to-shot reproducibility with only minor shifts in peak intensity. These features may enable the semi-quantitative analysis of adsorbed proteins. Comparison of plasma standards from different hosts showed only small differences in the high M_r protein distribution, and more pronounced differences for low M_r proteins. Differences in sample manipulation were shown to have a profound effect on spectra, therefore care should be taken when developing experimental procedures for biomaterial characterization.

This study clearly demonstrates that MALDI-TOFMS may be used to monitor the adsorption of different proteins onto biomaterials. Therefore, this technique is helpful for determining the biocompatibility of the biomaterial in vivo. The method may be readily expanded to include testing implant devices removed from patients following implantation.

6.5 REFERENCES

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7 SHEATHLESS CE/ESI-MS

7.1 INTRODUCTION

7.1.1 Capillary Electrophoresis

Introduced by Jorgenson and Lukacs in 1981, high performance capillary electrophoresis (CE) constitutes one of the most powerful methods for the separation of biological molecules, including proteins and peptides [1]. CE is a high resolution separation method with efficiencies typically on the order of 10^5 to 10^6 theoretical plates. The method allows for the analysis of exceedingly small volumes of solution, i.e. on the order of few tens of nanoliters, which is advantageous considering that sample sizes may be limited when dealing with biological materials. The principles of CE are well understood, and explained in several review articles and textbooks, with details on various practical aspects [2-4].

The simplest form of CE is capillary zone electrophoresis (Figure 7-1). Separation takes place in a fused silica capillary with a small internal diameter, typically i.d. < 100 μm. The capillary spans two buffer reservoirs, one of which is held at the separation potential, 5 - 30 kV and the other held at ground. The capillary is filled with the background electrolyte (BGE) of low mM concentration. In solution the surface of the capillary will possess a net negative charge yielding the zeta potential (ζ) as a result of free silanol groups. In solution an electric double layer will be formed at the surface of the capillary wall (Figure 7-1.d).

When a potential is applied across the capillary the compact layer of ions is pulled towards the cathode (normal mode CZE) in a plug flow profile. This movement is termed electroosmotic flow (EOF) which may be defined as:

$$\mu_{EOF} = \frac{v_{EOF}}{E} = \frac{\varepsilon\zeta}{\eta} \qquad \textbf{Equation 7-1.}$$

where:

μ_{EOF} is the electrophoretic mobility of the EOF
v_{EOF} is the velocity of the EOF
E is the applied potential
ε is the dielectric constant of the BGE

ζ is the zeta potential on the capillary wall
η is the viscosity of the BGE

In addition to EOF the applied potential drives the separation process consisting of electrophoresis of the charged analyte, μ_{EP}:

$$\mu_{EP} = \frac{v_{EP}}{E} = \frac{q}{6\pi\eta r} \qquad \textbf{Equation 7-2.}$$

where:
μ_{EP} is the electrophoretic mobility of the analyte
v_{EP} is the velocity of the analyte
q is the charge of the analyte
η is the viscosity of the BGE
r is the ionic radius of the analyte

The total electrophoretic movement of the analyte, μ_{TOT}, is a combination of electrophoresis and the EOF:

$$\mu_T = \mu_{EOF} + \mu_{EP} \qquad \textbf{Equation 7-3.}$$

The plug flow profile (Figure 7-1.e) is responsible for the high resolution of CE compared with chromatographic methods of separation. There is essentially no friction induced pressure gradient at the walls of the capillary in CE while normal chromatographic flow is laminar or parabolic. Typical separation efficiency yields more than $N > 10^5$ theoretical plates and up to $N > 10^6$ to 10^7, where N is calculated as:

$$= 5.54\left(\frac{Mt}{PW}\right)^2 \qquad \textbf{Equation 7-4.}$$

where:
N is the number of theoretical plates
Mt is the migration time of the peak
PW is the peak width (*FWHM*)

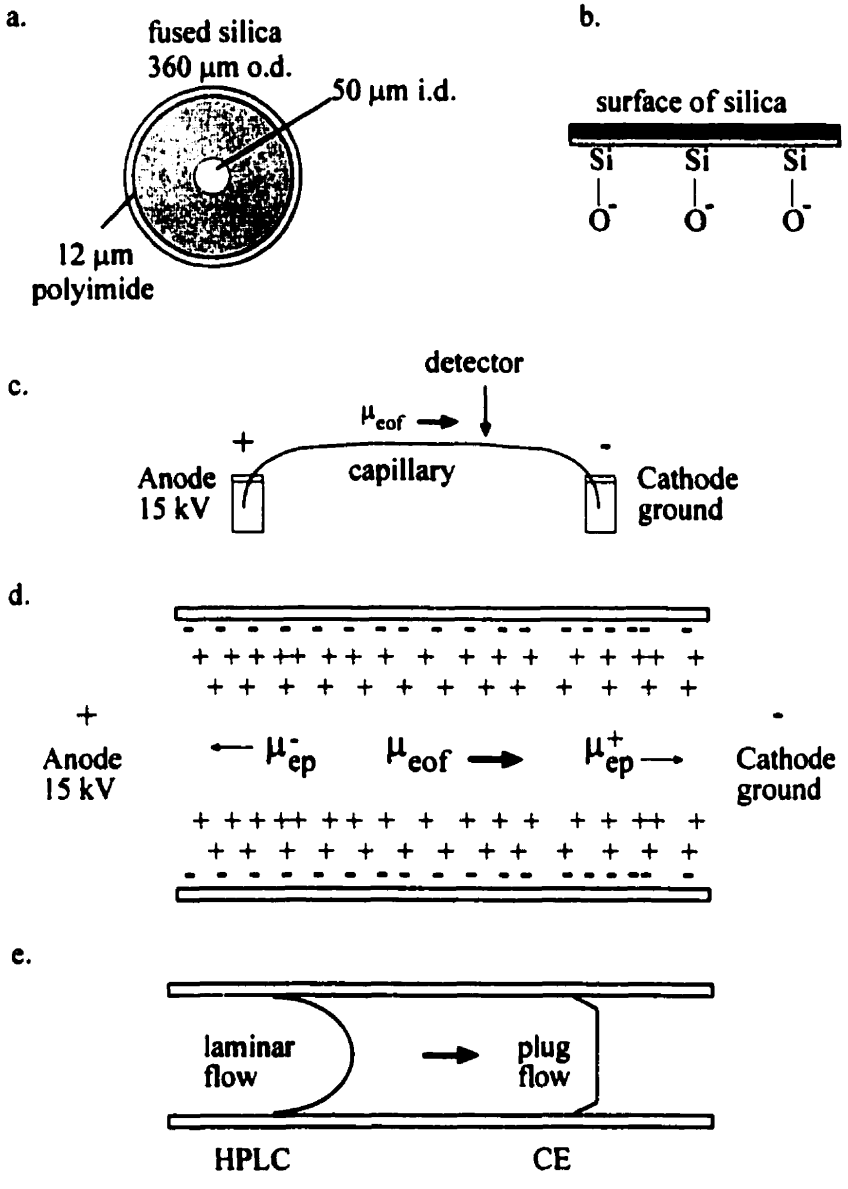

Figure 7-1. Capillary zone electrophoresis.
(a) cross section of capillary (b) free silanol groups
(c) potential driven separation (d) electroosmotic flow
(e) plug flow (CE) vs. laminar flow (chromatography)

7.1.2 CE-MS

Coupling mass spectrometry with CE yields a very powerful and sensitive means of detection of biologically significant components. CE-MS offers high resolution separation and mass spectral characterization of the biological molecule. Interfacing is best achieved with an ESI source as the low flow rates employed in CE are ideal for use with ESI. MS offers some of the best detection limits available to CE which is important as the sample sizes often employed in CE are of the order of nL, with pmol and sub-pmol amounts of material. Another consideration is the choice of the BGE which is typically limited to volatile buffered organic acids. Inorganic salts may be used with caution as they are non-volatile and not directly compatible with ESI/MS. Due to the high separation efficiencies often observed in CE, one problem encountered in MS is that narrow peak widths make it difficult to obtain full scan spectra without loss of information on the CE run. The use of a time-of-flight mass spectrometer is therefore beneficial compared to a quadrupole or other analyzers which operate with slower acquisition rates.

A number of different CE/MS approaches have been explored, and as a result, on- and off-line methods have been developed. Capillary electrophoresis has been coupled off-line with MALDI-TOFMS in order to take advantage of the sensitivity and robustness provided by MALDI. Various methods have been introduced for sample collection prior to MALDI-TOFMS. These methods differ in their complexity, ease of use and range of application [5-8]. Although CE/MALDI-TOFMS can be very helpful, sample handling is time-consuming and prevents the method from being used on a routine basis.

Several designs have been introduced for on-line coupling of CE to mass spectrometers. The most common approach involves electrospray ionization (ESI). The use of electrospray is favorable because the flow rates required by this ionization technique match those generated by CE, i.e. nanoliters per minute. Electrospray is also a soft ionization method which imparts low amounts of energy to the molecules and thus favours formation of intact molecular ions even for large, labile biomolecules, including non-covalent complexes [9-11]. Multiple protonation of molecules, typical with electrospray, allows the study of high mass molecules, by producing ions at m/z values which fit within the mass range of most commercial instruments.

Several types of CE/MS interface designs have recently been reviewed and compared [12-15]. Two of the most common CE/ESI interfaces are the sheathless and sheath-flow models. The sheath-flow design has been shown to be relatively easy to use and is most popular for commercial applications. Since acidic volatile buffers are used in the sheath liquid, suitable separation buffers for CE can be selected even if they are non-volatile. The major disadvantages of using a sheath liquid are reduction in sensitivity due to higher background, and dilution of the sample as it elutes from the capillary. Another problem is associated with a counterflow of anions from the sheath liquid, which may perturb separation efficiency [16].

A sheathless design has been used by several groups to avoid the above problems [17-23]. Electrical contact at the capillary terminus is provided through a conductive coating, typically gold. Enhanced sensitivity, as well as elimination of mixing problems associated with a sheath liquid, make the sheathless design favorable. Increased sensitivity may also be obtained with the microspray and nanospray designs, for which narrow bore capillaries, and pulled capillary tips, with i.d. of less than 10 μm, are used [19-21]. However, a gold-coated capillary tip as an electrospray needle requires careful manufacture and is somewhat more difficult and time-consuming to make and use compared with the sheath-flow design. The conductive coating used also has a limited lifetime which necessitates its frequent replacement. The sheathless interfaces require that the separation buffers be relatively dilute and volatile in order to maintain compatibility with ESI. This limitation restricts the variety of possible types of separations.

Other interfaces include several designs. The liquid junction interface made use of a T-junction to establish electrical contact with the capillary terminus [24]. Another method makes use of dialysis membrane at the end of the capillary to obtain electrical contact [25] while others have used an electrode inserted into the capillary terminus [26]. A sonic interface was recently introduced [27] and direct sheathless and electrodeless CE/ESI-MS has been also been demonstrated [28].

7.2 EXPERIMENTAL

7.2.1 Chemicals

Acetic acid (AR analytical reagent grade) was used to make up solutions of the running electrolyte and was obtained from Mallinkrodt (Paris, KY). Hydrofluoric acid (HF) used to prepare the capillaries was obtained from Fisher (Nepean, ON, Canada). Trypsin (TCPK-treated), used for the digestion, was obtained from Sigma Chemicals (St. Louis, MO).

Solutions of protein and peptide standards were prepared in water at concentrations of 10^{-6}-10^{-3} M. Standards: [Ile7]-angiotensin III, renin substrate (porcine), leucine enkephalin-Arg, luteinizing hormone releasing hormone (LH-RH), substance P, bovine insulin, myoglobin and cytochrome c were obtained from Sigma Chemicals (St. Louis, MO) and used without further purification.

7.2.2 Electrical Set-up

Two in-line resistor series were used to ensure stable CE and ESI potentials and current, and to reduce the possibility of arcing from the ESI capillary to the sampling cone of the mass spectrometer [29,30]. These were made from a series of 1 watt resistors with a summed resistance of 4.7 MΩ and 47 MΩ and were placed along the wires as shown in Figure 7-2.

7.2.3 Capillary Electrophoresis

Capillary electrophoresis was performed using a Spellman CZE 1000R 30-kV power supply (Plainview, NY) operated in the positive polarity mode. The ESI potential at the anodic end of the capillaries was generated using an EG&G Ortec No. 459 5-kV bias supply (Oakridge, TN) also operated in the positive polarity mode. Acetic acid (1 to 5%) in water was used as the running electrolyte.

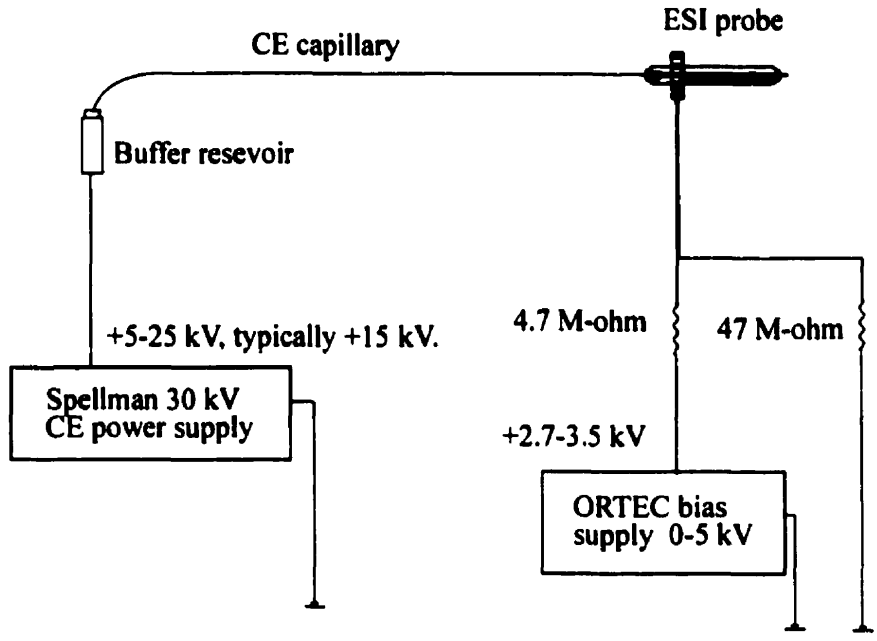

Figure 7-2. Electrical set-up for CE/ESI-MS.

7.2.4 Preparation of Capillaries

Fused silica capillaries of 350 μm o.d. and 50 or 25 μm i.d. were supplied by Polymicro Technologies (Pheonix, AZ). The lengths of the capillaries were 30 to 50 cm. The capillaries were prepared based on the sheathless CE-ESI approach [20,28]. Approximately 1 cm of capillary terminus was etched in 40% HF to form a conical end while infusing it with water. After etching, the capillary was cleaned with water and methanol, then dried at 110°C for ca. 1 hour. The etched conical end of the capillary was coated on one side with gold by vapor deposition at 10^{-5} torr at ca. a 45° angle for 3 minutes. The conical end of the capillary was then inverted and the coating was repeated on the other side. A 3-cm length of stainless steel tubing, 26 gauge, was slid over the capillary near the base of the tapered end to provide structural support and electrical contact with the ESI bias supply. Two-part silver epoxy (Circuit Works, Kennesaw, GA) was used to provide electrical connection between the sleeve and the gold coated tip.

7.2.5 Off-line Optimization of the CE/ESI-MS Interface

Optimization of the CE conditions were performed off-line using a test bench consisting of an ESI interface mounted opposite to a grounded 1.5 cm diameter steel plate which served as a counter electrode and simulated the entrance to the mass spectrometer (Figure 7-3). The sheathless ESI interface was mounted on a xyz stage to allow for mobility. A Wild M7A binocular microscope, equipped with a 35 mm camera mount (Wild Leitz Canada, Willowdale, ON), enabled visualization of the capillaries and of the effluent being electrosprayed. The separation potentials investigated ranged from 5 to 15 kV and the electrospray potential, from ca. 3.0 to 3.8 kV, with 1-10% solutions of acetic acid as the running electrolytes.

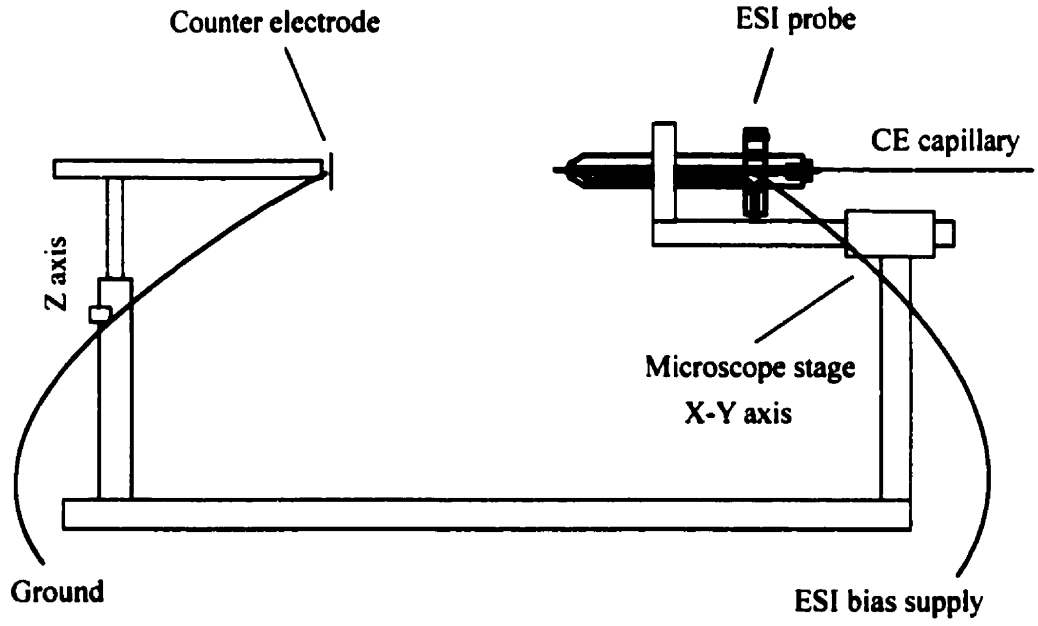

Figure 7-3. Capillary test bench.

7.2.6 Preparation and Testing of the Gold-Coated Capillaries

Optimization of CZE and ESI conditions was conducted using the test bench off-line. We chose to use single-piece, gold-coated, etched capillaries since they were simpler to manufacture than the dual-piece capillaries used by others [21]. The method for coating the capillaries was reliable and allowed for preparation of robust and reproducible gold-coating. Each capillary manufactured was inspected off-line using the test bench described

above. Approximately 65% of the capillaries tested were found to produce adequate electrospray without clogging. These were then rinsed with water, dried and stored for future use. These same capillaries, when used on-line, typically performed well for a minimum of 2 hours to a maximum of 8 hours (average ca. 3 hours). These conditions enabled us to study the effect of varying the CE and ESI potentials, which were optimized on a range of 5 - 15 kV (CZE) and 3.0 - 3.8 kV (ESI). Acetic acid solutions (1-5%, aqueous) yielded stable electrospray and provided suitable conditions for the separation of our standard mixtures of peptides and proteins. Better separation conditions were obtained with higher concentrations of acetic acid in the running electrolyte. At 10% acetic acid, separation of the three proteins was feasible. At lower acetic acid concentrations the separation was not possible due to the high concentrations of protein employed (10^{-3} M). Electrospray conditions favoured the reverse: lower concentrations of acetic acid yielded stable electrospray at lower potentials (1-5%). Higher concentrations of acetic acid (10%) required electrospray potentials greater than 4.0 kV, at which corona discharge occurred. The final conditions chosen were 9 kV separation potential with a 3.2 - 3.8 kV electrospray potential. A photograph of one of the capillaries showing the gold coating and stable electrospray is given in Figure 7-4.

Figure 7-4. Photograph of electrospray.
50 μm id. tapered capillary, ca. 3.2 kV ESI potential.

7.3 CE/ESI-MS AND CE/ESI-MS/MS

The instrument used was a Quattro-LC mass spectrometer (Micromass, Manchester, UK) equipped with a Z-Spray™ source configured for MegaFlow-Z™ ESI. Cone voltages of 20 - 60 eV were used. The sheath gas and nebulizer gas flows were disabled for the experiment. No curtain gas was used as the instrument at the time of these experiments was not equipped with a curtain gas cone. The instrument and source design are shown in Figure 7-5.

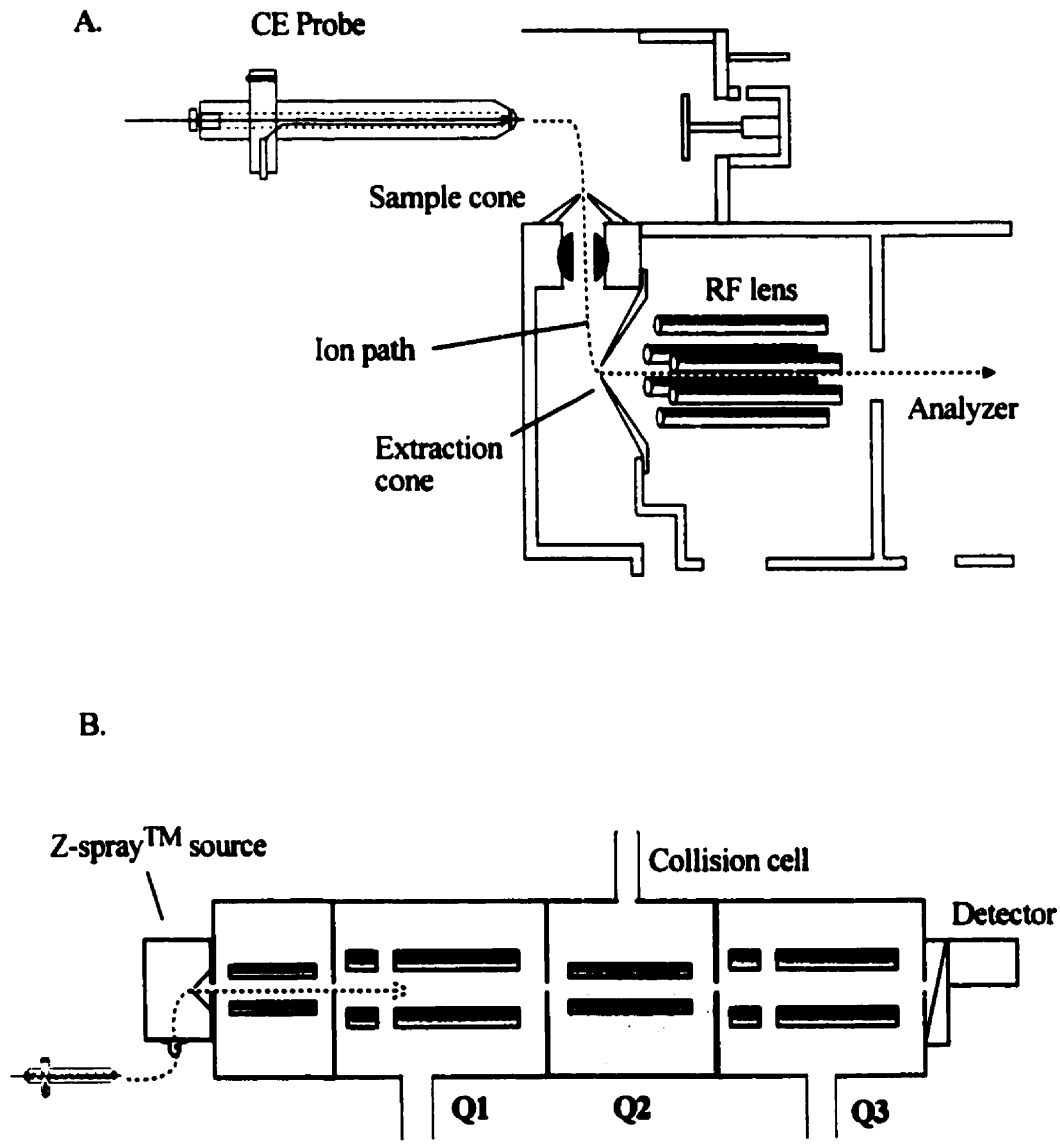

Figure 7-5. Micromass triple quadrupole instrument.
(a) Z-Spray™ source and (b) QQQ mass analyzer.

Mass spectra were acquired with Masslynx 3.0 software, using 8 points/Da and a scan rate of 500 Da/sec. This resulted in a complete spectrum ca. every two seconds and unit mass resolution within the mass range scanned (405-1000 Da). For MS/MS experiments, argon was used as the collision gas (1-2 x 10^{-3} torr) and the collision energy was of 20-40 eV (laboratory frame). Collision energies and gas pressures were optimized in a series of constant infusion experiments (standard ESI, Z-Spray^{TM} probe).

The fragmentation of the different precursor ions was monitored by pre-set MS/MS scan functions [31]. These product ion functions were effected in one of two ways. The first involved monitoring of all the MS/MS product ions for each of the 4 peptides consecutively and repetitively over the duration of the entire run. In the second, the MS/MS product ion functions were monitored sequentially over pre-set time windows specific for each peptide as it eluted from the capillary.

7.4 CE/ESI-REFLECTING TOFMS

On-line CE/ESI-TOFMS measurements were made on a reflecting TOF instrument built in the Time-of-Flight Laboratory, Department of Physics, University of Manitoba (Figure 7-6) [9,32]. The instrument was slightly modified to accept the sheathless CE/ESI interface. In this setup, the electrospray needle/ capillary interface is mounted on an xyz stage near the sampling orifice of the instrument and is held at the electrospray potential relative to ground. A gentle counter flow of N_2 heated to 70 °C is used as a curtain gas. The aerosol enters the mass spectrometer through a heated stainless steel capillary (~ 120 °C) held near ground. Ions are focused using an rf-only quadrupole ion guide which also provides collisional cooling of the ions before they enter the mass spectrometer. Ions are injected orthogonally into the flight tube of the mass spectrometer (2.8 m effective path length) by a 4 kV pulse with a repetition rate of 3400 Hz to give a duty cycle of about 20%. A single stage electrostatic mirror is used to correct for velocity distribution of the ions and increases the resolving power to R > 5000. Ions are detected using two 40 mm diameter microchannel plates in a chevron geometry. Mass spectra were recorded using single-ion counting with a multiple-stop time-to-digital converter (Orsay model CTN-M2), resulting in a dynamic range > 10^3 and a sensitivity in the low femtomole range. The resolution of the TDC is 0.5 ns but for these measurements, spectra were recorded in 2 ns channels. Total

ion electrophorograms (TIE) and selected ions electrophorograms (SIE) were recorded at 1, 2 and 5 Hz. Although higher rates are possible, no attempt was made to record the TIE or SIE at higher rates for these initial results. Data acquisition and analysis software (TOFMA) were developed in-house by V. Spicer and W. Ens. Initial instrument and source configuration was developed with the assistance of A. Krutchinsky.

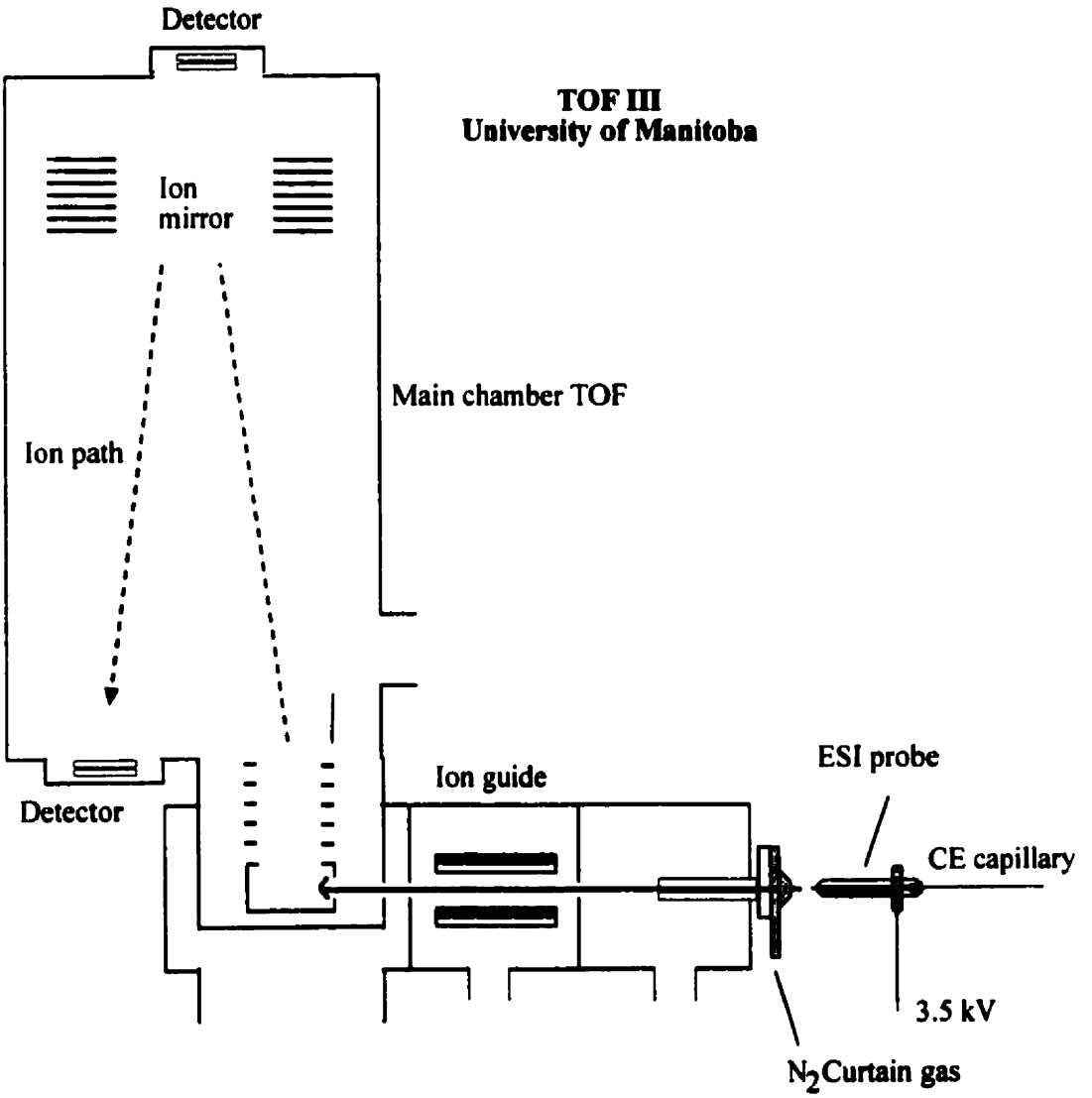

Figure 7-6. Manitoba TOF-III reflecting time-of-flight mass spectrometer.

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8 A SHEATHLESS CE/ESI-MS PROBE FOR OPERATION WITH A Z-SPRAY™ IONIZATION SOURCE

8.1 INTRODUCTION

The high cost of company-manufactured optional parts for mass spectrometers, such as CE interfaces, often restricts the variety of sample introduction techniques available to MS users. Along with the extensive use of on-line CE/ESI-MS to analyze biological molecules, there have been reports on in-house-built interfaces, as discussed in the previous chapter.

It is important to note that the majority of interfaces described previously have been operated axially relative to the ESI sampling cone. The novelty reported here lies in the design of a complete probe allowing the interfacing of CE with the Micromass Z-Spray™ source, more specifically configured for MegaFlow-Z™ electrospray. This probe allows easy and rapid replacement of CE capillaries and matches the orthogonal spraying requirements which characterize the Z-Spray™ mode. Although Major and Ashcroft [1] reported on the use of a coaxial sheathflow CE/ESI-MS interface to analyze a standard peptide mixture on a Micromass Quattro-II instrument, these results constitute the first detailed report on sheathless CE/ESI-MS involving a Quattro-LC instrument.

The performance of this interface has been tested with a standard mixture of peptides. Tandem MS is also demonstrated using two modes of switching between scan functions. Source CID experiments have also been performed. Application to peptide mass mapping is demonstrated on the digestion products of myoglobin.

8.2 EXPERIMENTAL

Equine myoglobin was from Sigma. A complete digestion was performed on a solution of myoglobin (10^{-4} M 20 mM Tris-HCl) using Trypsin.

Shepherds Bush hemoglobin was prepared and digested according to procedures mentioned in Chapter 5.

8.2.1 Probe Design and Electrical Set-up

The CE probe body was machined out of Lexan, based on the dimensions of the regular Quattro-LC MegaFlow-ZTM electrospray probe. A schematic of the probe is given in Figure 8-1. The end plate of the probe required a 1000-Ω resistor connection to by-pass the safety interlock on the Quattro-LCTM instrument. Electrical contact was established with the two pins on the safety interlock by pulling each connection end of the resistor through holes in the end plate. A non-corrodible nichrome wire was used to ensure connection between the stainless steel sleeve and the lead ending of the ESI bias supply. This wire ran from the side plate, through the probe shaft, and to the brass probe tip. To connect the Nichrome wire to the anodic end of the CE capillaries, a probe tip was machined out of brass, and soldered to the Nichrome wire. The external connection between the probe and the bias supply was made using silicone clad steel spark plug wire purchased at a local automotive store.

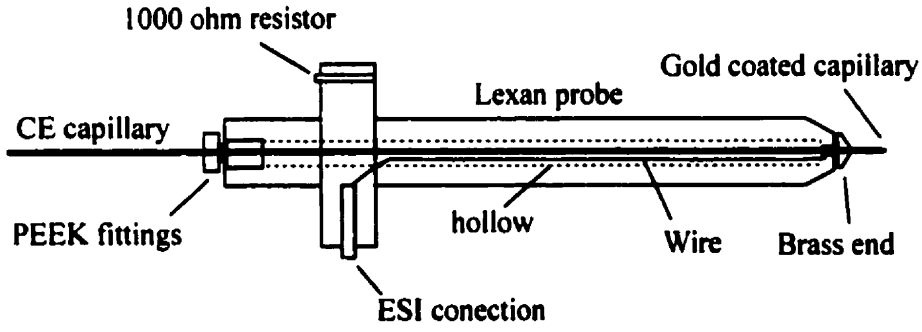

Figure 8-1. Probe design for CE/ESI-MS on the triple quadrupole.

8.2.2 CE/ESI-MS and CE/ESI-MS/MS

The instrument used was a Quattro-LC mass spectrometer (Micromass, Manchester, UK) equipped with a Z-Spray^{TM} source configured for MegaFlow-Z^{TM} ESI.

8.3 RESULTS AND DISCUSSION

8.3.1 Probe Design and Electrical Considerations

The CE/ESI-MS interface described here was designed to be easily adapted to the instrument within a minimal amount of time and with as little complication as possible.

Moreover, it was inexpensive and relatively easy to build. Changing from normal ESI or HPLC/ESI-MS modes of operation to CE/ESI-MS simply required removal of the commercial Micromass probe and insertion of the CE probe. The probe design did not allow the use of nebulizer gas which is often used to stabilize the electrospray. Instead, an attempt was made to use the sheath gas as configured on the Micromass instrument. However, it was not possible to obtain or maintain ESI when the gas was on, even during constant infusion experiments. This was possibly due to the development of corona discharge, which resulted in the loss of signal. As the Quattro-LC used in these experiments was not equipped with a curtain gas attachment, such gas was not used.

The electrical connections used as described in Chapter 7 resulted in the generation of stable CE and ESI potentials and current and hence a stable ESI. The 4.7 Mohm and 47 Mohm resistors were used to facilitate a constant current from the ESI bias supply and to ensure the ESI bias supply would not have to sink too much current from the CE power supply [2]. Without the resistors in place the ESI was not as stable. In some cases, the ESI bias supply would have to sink too much current, which resulted in its malfunction.

8.3.2 CE/ESI-MS

The background electrolyte (BGE), 1-5% acetic acid, was chosen to minimize interaction of the peptides with the negatively-charged silanol groups on the inner walls of the fused-silica capillary and to provide for a source of protons for ESI. The use of 1-5% acetic acid as a running buffer provided proper CE current values (ca. 3 to 10 μA) when a 50 cm x 50 μm i.d. capillary was used. Higher currents usually indicated the occurrence of corona discharge, which was often competing with electrospray if conditions were not carefully adjusted. Reduced capillary i.d. and/or tapered end i.d. seemed to reduce the extent of corona discharge vs. ESI. As each capillary was slightly different from the others, it was necessary to optimize spraying conditions for each of them for a few minutes using a constant infusion of a known peptide solution before a sample could be loaded. In general, a CE potential of 15 kV (i.e. 12 kV difference between the cathodic and anodic ends of the capillary, or 300 V/cm for a 40-cm capillary) gave rise to a stable electrospray when 5% acetic acid was used. The CE potential could be increased to ca. 20 - 25 kV; however, at values higher than 15 kV corona discharge or electrical breakdown within the capillary was

likely to occur, resulting in a loss of signal. ESI potentials of 2.8 to 3.5 kV were used depending on the i.d. of the capillary (25 or 50 μm i.d.) and the length of time the capillary was used. At higher ESI potentials oxidation of peptides was observed.

8.3.3 CE/ESI-MS of 4 Peptides

Figure 8-2 shows the initial CE/ESI-MS results for the separation of four peptides. A selected ion electrophorogram (SIE) was obtained for either the $[M+H]^+$ or $[M+2H]^{2+}$ ion of each peptide (the stronger ion signal was used, Table 8-1). The inset shows an example of on-line mass spectrum of [Ile7]-angiotensin III, of sequence RVYIHPI, Mr 896.51. A relatively concentrated solution (10^{-4} M of each peptide) was used, in order to match the small injection volume (ca. 45 nL) resulting in ca. 3.5 pmol of sample on the capillary (50 μm id capillary).

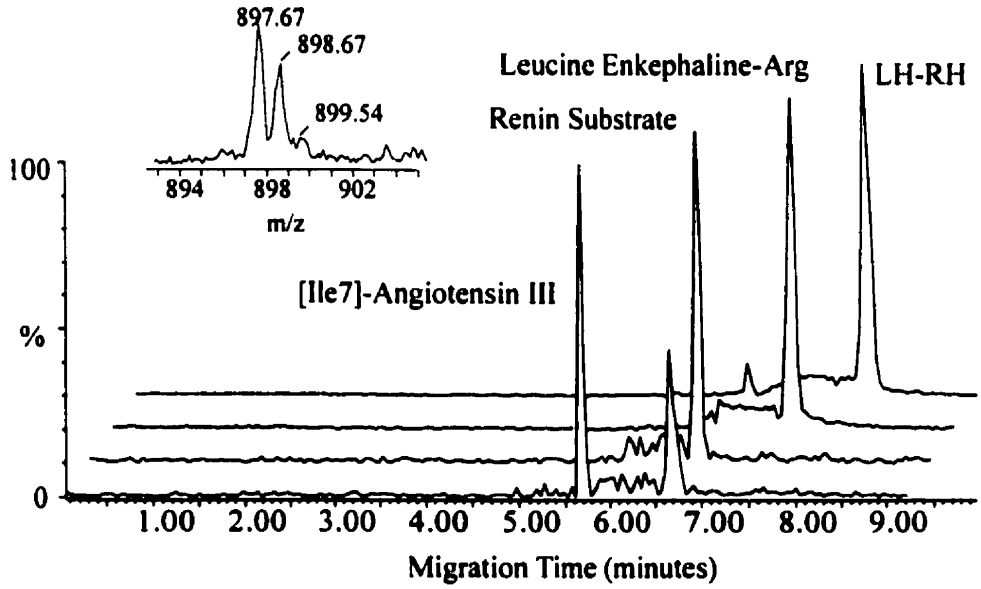

Figure 8-2. CE/ESI-MS of 4 peptides.

The separation efficiency was characterized by N = 24,000 to 78,000 theoretical plates, with an average S/N of ca. 190 over the total ion electrophorogram (TIE, m/z 405-1000 Da) and S/N of ca. 425 to 530 for SIE. Table 8-1 summarizes these CE/ESI-MS results, which were reproducible in terms of migration order. Migration times were matched within ca. 10% between runs due to manual sample injection and a delay time required to establish a stable ESI.

Table 8-1. CE/ESI-MS of four peptides.

peptide	sequence	Mt minutes	S/N	N	m/z calc.	m/z obs.
[Ile7]-Angiotensin III	RVYIHPI	6.06	528	29978	897.52^{1+}	897.7
Renin substrate	DRVYIHPFHLLVYS	7.06	471	24614	879.96^{2+}	880.6
Leucine Enkephalin-Arg	YAGFLR	7.84	425	50176	726.38^{1+}	726.5
LH-RH	pEHWSYGLRPG	8.40	424	78400	592.27^{2+}	591.8

Values correspond to peaks in (Figure 8-2).

Other reports on the use of sheathless interfaces give the following values: S/N = 4 on 4 fmol of myoglobin [3], S/N = 5 on 300 amol of leucine-enkephalin [4], and S/N > 20 on 30 fmol of digested cytochrome c [5]. It is expected that our interface will allow for similar sensitivity levels once fully optimized, i.e. with use of a curtain gas.

8.3.4 CE-In Source CID and CE/ESI-MS/MS

The use of the probe was successful with in-source collision induced dissociation (CID) and tandem MS. In-source CID experiments were performed with a cone voltage setting of 60 V, given that peptides were not co-migrating. This type of experiment may be performed if the peptides are completely separated by CE and if prior knowledge of the migration times of the peptides are already known. Under normal ESI-MS operating conditions, a cone voltage of 60 V is considered relatively low and does not induce extensive dissociation of peptides. The CE/ESI process and the Z-Spray™ source design thus seem to impart a significant amount of internal energy to the peptide molecules. Fragmentation patterns obtained in-source were similar to those observed by MS/MS discussed below.

Tandem MS experiments were performed using the collision cell and the product ion mode. Parameters such as collision energy, argon pressure, and cone voltage were optimized prior to separation using a constant infusion of the peptide mixture. Two different ways of combining product ion scan functions were used, the parameters of which are given in Table 8-2. The first combination was used when the migration times of the compounds were not known. In this case, fragmentation of each of the precursor ions was monitored by four different acquisition functions, which were cycled one after the other repetitively over

the entire run. The second combination of scan functions used required prior knowledge of the migration times of the peptides. Windows were identified, over which only the precursor ion of interest could be sequentially selected for fragmentation. This required a significant degree of reproducibility between CE runs, which was not always possible using CE with a home built set-up. However, the advantage was that the entire cycle time was spent on the selected precursor ion, resulting in an increase in S/N and in the quality of the MS/MS data.

Table 8-2. MS/MS scan function used.

MS/MS Parameter				
Peptide	[Ile7]-Angiotensin III	Renin substrate	Leucine Enkephalin-Arg	LH-RH
[1]Scan duration (sec.)	1.7	1.9	1.4	1.9
m/z range	50 - 900	50 - 1000	50 - 750	50 - 1000
Laboratory Frame Collision Energy (eV)	53	40	37	25
[2]Retention window (min.)	0 - 22 minutes (for entire CE-MS run).			
[3]Retention window (min.)	4.0 - 5.5	5.5 - 6.2	6.2 - 6.9	6.9 - 8.0

1. Scan rate of 500 Da per second with an inter-scan time of 0.1 seconds.
2. Repetitive scan function: each scan is cycled one after the other for the duration of the experiment. Total scan time = (1.7 + 1.9 + 1.4 + 1.9) sec. + 4 x 0.4 sec. = 7.3 sec.
3. Sequential scan function: each scan is performed exclusively for the time window indicated. Total scan time = value indicated (sec.).

SIE generated from CE/ESI-MS/MS of the peptide mixture using the first combination of the two MS/MS scan functions are shown in Figure 8-3. These SIE were obtained by monitoring characteristic fragment ions in the CID spectra of each precursor ion. Similar results were obtained for SIE obtained using the second combination of scan functions (not shown). In both cases the TIE obtained were of inferior quality possibly due to the high background observed when using acetic acid as a BGE. Use of a curtain gas would result in a reduction in the number of neutrals entering the vacuum inlet of the mass spectrometer and thus decrease the background signal. We are currently exploring this option.

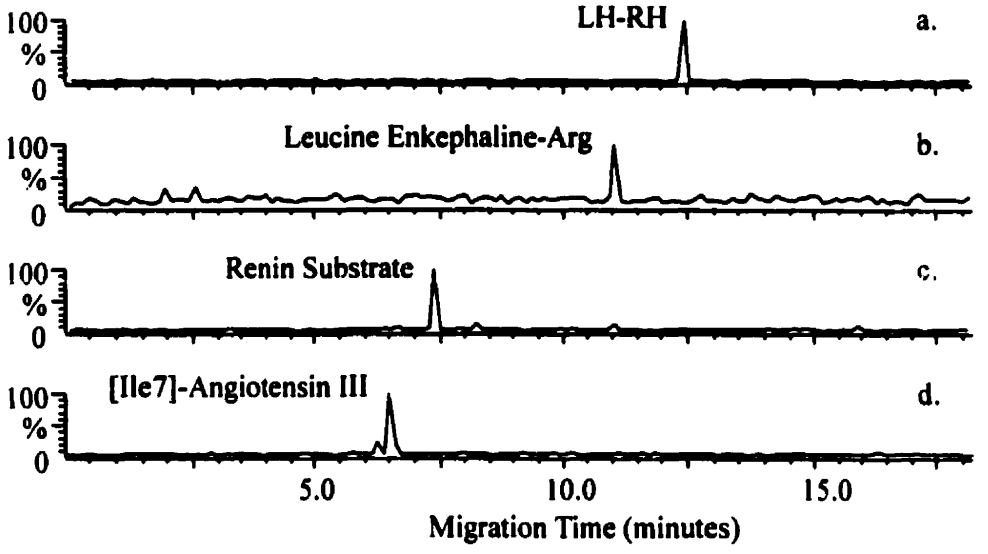

Figure 8-3. CE/ESI-MS/MS of 4 peptides.

The first combination of scan functions used (repetitive) resulted in a long cycle time between two subsequent scans for a particular precursor ion. For example, the peak at Mt = 12.44 minutes corresponding to LH-RH (Figure 8-3.a.) has a peak width of ca. 14.4 seconds at the base. Of the total time, 7.3 seconds, required to cycle through the 4 scan functions, only 1.9 seconds monitors the fragmentation of that particular peptide. Thus ca. 26% of the cycle is used to scan for the peptide which results in only ca. 2 scans being acquired. The remainder of the time is spent on other precursors. In all cases, the peptides eluted to some extent while the other precursor ions were being monitored. In some cases this resulted in poor S/N, and spectra, and necessitated repeating runs to ensure good data. This problem was not observed using the second combination of scan functions (sequential) where, within the defined time window, 100% of the cycle time is spent on each specific precursor ion.

In all cases considerable fragmentation was observed in the MS/MS spectra and the data obtained were comparable independent of the combination of scan functions used. Fragmentation patterns obtained by source CID were also similar to MS/MS patterns.

Two example CID spectra generated using the first combination of scan functions are shown in Figure 8-4. In Figure 8-4.a, product ions of $[M+2H]^{2+}$ ions of renin substrate (m/z 880.6) are observed. Some sequence-characteristic ions are found, creating a useful pattern considering that only 500 fmol of sample was analyzed over ca. 2 scans. As 23% of the scan time was used to monitor this precursor ion, only ca. 115 fmol of peptide is actually analyzed. The spectrum in Figure 8-4.b. contains numerous fragment ion peaks as well. Peak assignments were identified automatically using Masslynx 3.0 and confirmed manually.

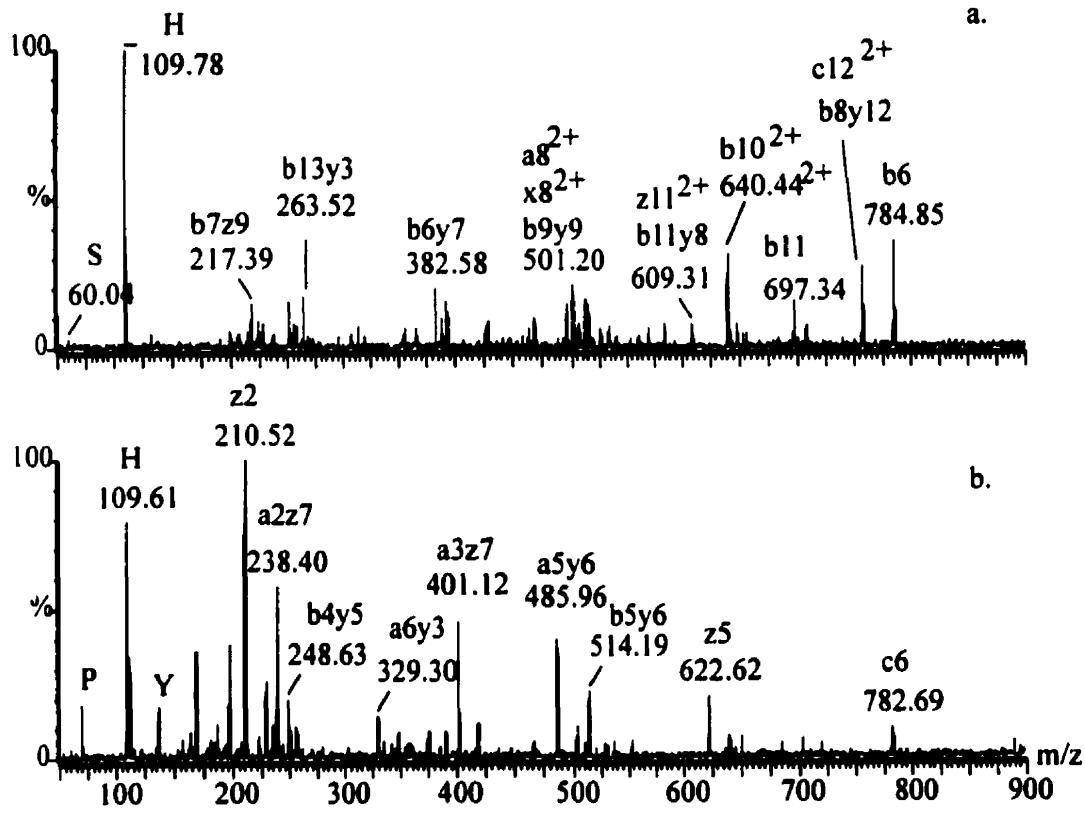

Figure 8-4. Example MS/MS spectra.
(a) renin substrate and (b) [Ile7]-angiotensin III.
Nomenclature from Chapter 1.4.4.

A comparison of MS/MS spectra obtained using the two combination modes of scan functions is shown in Figure 8-5, with LH-RH as an example peptide. Both modes produced very similar results with respect to the type and amount of fragmentation. However, there were differences is the peak intensities generated.

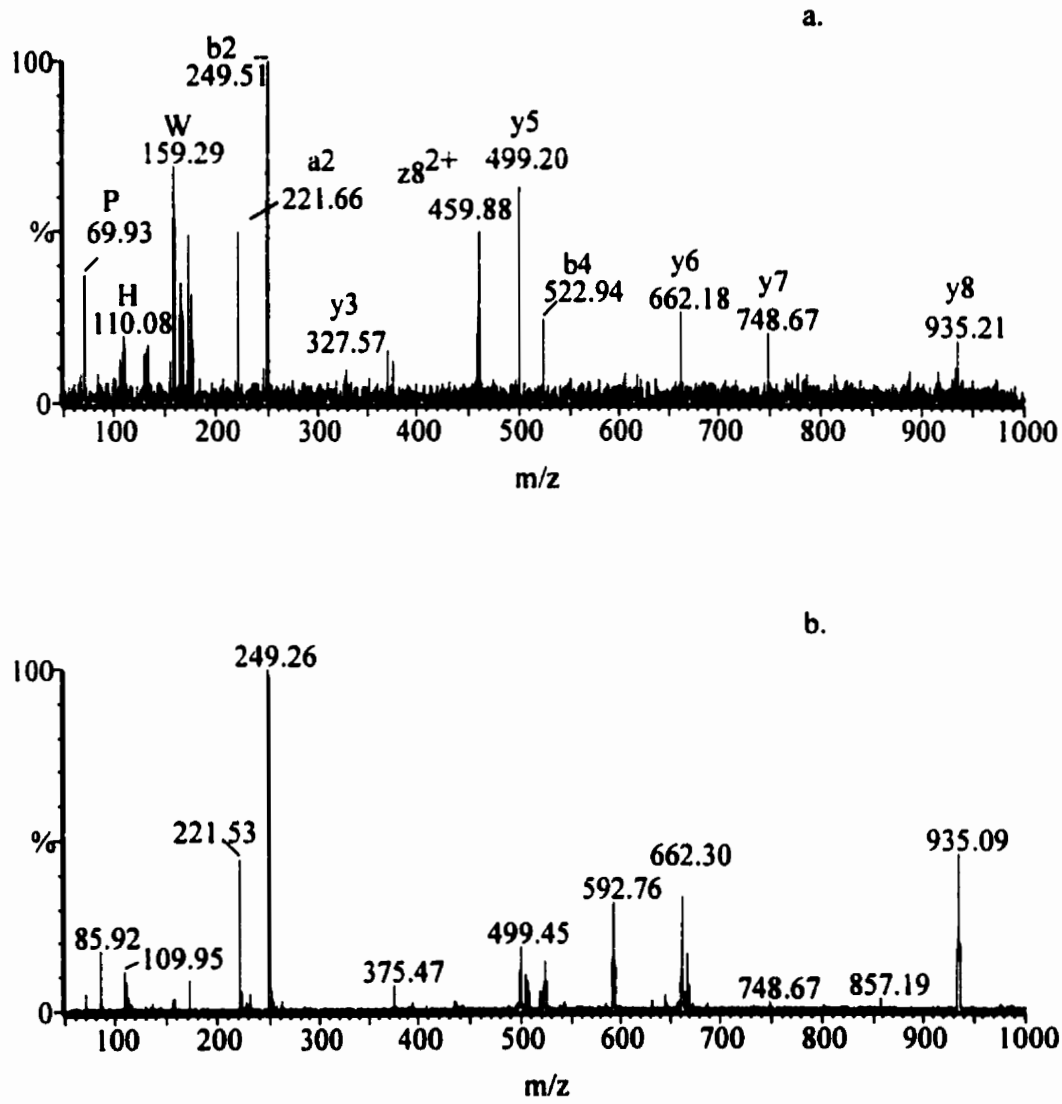

Figure 8-5. Comparison of CID-MS/MS scan functions.
LH-RH mass spectra (a) repetitive scan mode and (b) sequential scan mode. Nomenclature from Chapter 1.4.4

Overall quality and peak intensities obtained using the second combination mode (sequential) were superior to those obtained using the first (repetitive). For LH-RH, with a CE base peak width of 14.4 seconds and a scan cycle time of 2 seconds, 7 complete MS/MS scans were obtained using the second mode compared with only ca. 2 scans using the first. S/N values were thus higher using the sequential scan function due to longer time spent on precursor ions. Compared S/N values for the product ion peak at m/z 249 were 22 and 262 for the first and second combination modes respectively. The sequential mode yields a 10-fold sensitivity enhancement which facilitated peak assignments and spectral interpretation.

8.3.5 Myoglobin Protein Digest

The application described here aims at characterizing a tryptic digest of myoglobin. Myoglobin was digested with trypsin and the resulting peptides analyzed by CE/ESI-MS. For the analysis, the injection volume was ca. 3 nL (by gravity), and the protein concentration was ca. 10^{-4} M resulting in ca. 300 fmol of each peptide being injected.

The TIE obtained by analyzing the tryptic digest is shown in Figure 8-6.a. Because of the large number of peptides present, the background generated from the digest buffer and the BGE, very little information could be obtained from the TIE. The large number of peptides obtained from the digestion yielded a difficult separation considering that only a 30 cm capillary was used. Improved separation was obtained with the use of a longer capillary at the expense of an increase in the time of analysis. However, it was possible to gain useful information on the peptides through SIE as shown in Figure 8-6.b.

The SIE traces allow detection of single peptides among other components, thus facilitating their mass spectral identification. In total, over 35 SIE were identified which corresponded to known peptides (not all shown). These were assigned to tryptic and, in some cases, chymotryptic peptides. The most intense signals were obtained for singly and doubly charged molecular ions of peptides. The electrophoretic resolution was relatively high, with theoretical plate counts of $N > 10^4$. The SIE signal intensities were more than satisfactory, with S/N values ranging from ca. 45 to 440 on raw data. Table 8-3 lists tentative assignments for the peptides identified.

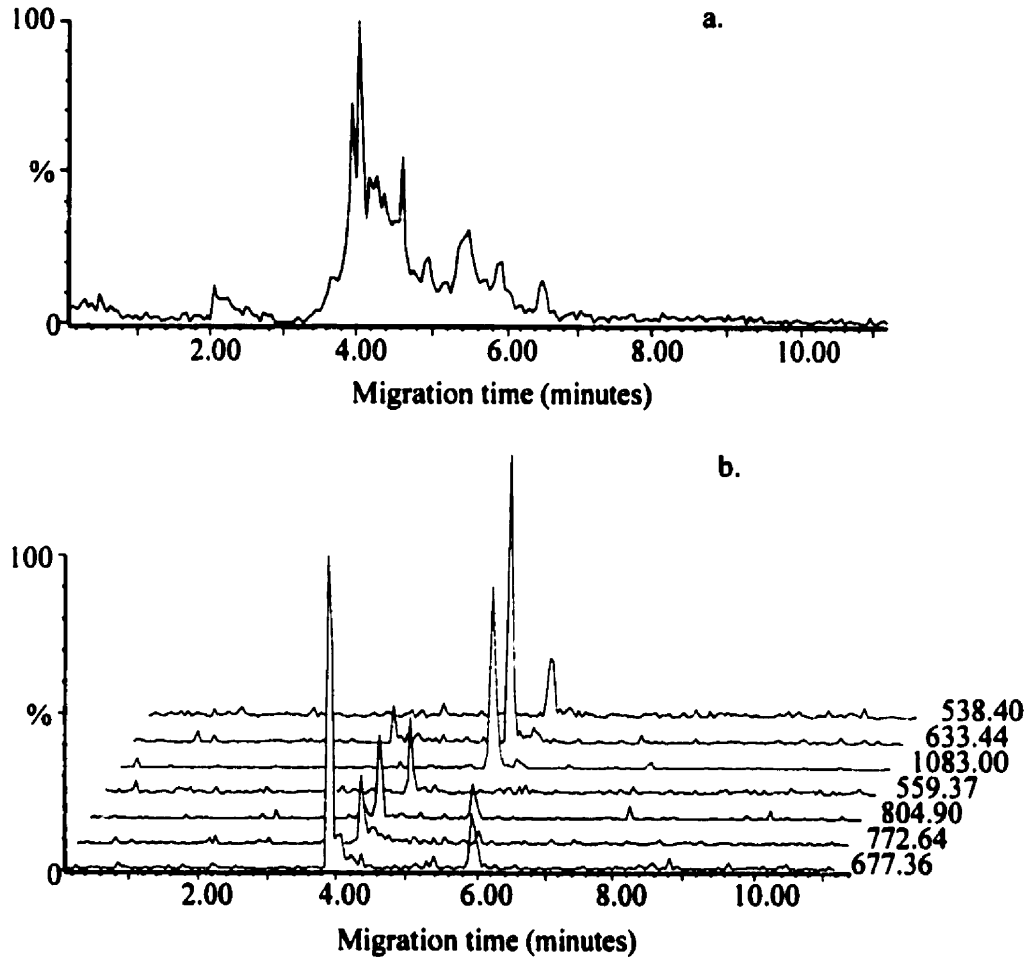

Figure 8-6. CE/ESI-MS of tryptic peptides of myoglobin. (a) TIE and (b) SIE.

Table 8-3. Digest fragments of myoglobin characterized by CE-MS.

Trypsin/ Chymotrypsin		Calculated		Charge state	Observed	Error
Fragment	Sequence	Mass	$[M+nH]^{n+}$	n	$[M+nH]^{n+}$	%
T21-22	99-103	632.39	633.40	1	633.44	0.01
T32-33	148-153	649.31	650.31	1	650.44	0.02
T12	51-56	707.32	708.32	1	708.50	0.03
T27-28	134-139	747.43	748.44	1	748.70	0.03
T12-13	51-62	1351.50	676.76	2	677.36	0.09
T15	64-77	1378.68	690.35	2	690.51	0.02
T5-7	32-43	1418.61	710.31	2	710.49	0.03
T4	17-31	1606.80	804.41	2	804.90	0.06
T5-8	32-45	1661.88	554.97	3	554.66	0.06
T19-21	88-102	1669.00	835.51	2	835.66	0.02
T6-10	34-47	1676.89	839.45	2	839.26	0.02
T28-33	139-153	1709.92	855.97	2	855.51	0.05
T11-13	48-62	1729.97	577.67	3	577.49	0.03
T1-3	1-16	1816.00	606.34	3	606.27	0.01
T19-22	88-103	1832.18	917.10	2	916.79	0.03
T9-13	46-62	2005.32	669.45	3	669.55	0.01
T25-27	119-138	2075.33	1038.67	2	1038.59	0.01
T13-15	57-77	2150.55	538.64	4	538.40	0.04
*T25-28	119-139	2231.52	558.89	4	559.37	0.09
T23-25	104-123	2274.61	759.21	3	759.00	0.03
T5-11	32-50	2315.7	772.91	3	772.64	0.03
T7-13	43-62	2395.76	1198.89	2	1198.59	0.03
T2-4	8-31	2660.03	887.68	3	888.26	0.07
T4-6	17-42	2860.22	716.06	4	715.82	0.03
T21-25	99-123	2889.39	723.36	4	723.51	0.02
*T5-12	32-56	3005.49	752.38	4	752.42	0.01
*T25-30	119-146	3007.38	752.85	4	752.42	0.06
*T4-7	17-43	3007.40	752.86	4	752.42	0.06
T25-31	119-147	3135.56	1046.19	3	1046.78	0.06
T26-33	124-153	3214.65	1072.56	3	1072.50	0.01
T11-15	48-77	3218.81	805.71	4	805.76	0.01
T4-8	17-45	3250.66	1084.56	3	1083.00	0.14

Table 8-3. Digest fragments of myoglobin characterized by CE-MS.

Trypsin/ Chymotrypsin		Calculated		Charge state	Observed	Error
*T24-28	107-139	3546.07	887.53	4	888.26	0.08
T2-6	8-42	3913.45	979.37	4	979.18	0.02
T15-22	64-103	4351.17	1088.80	4	1087.83	0.09
T21-27	99-138	4394.12	1099.54	4	1098.50	0.09
T23-29	104-145	4548.21	1138.06	4	1137.69	0.03
T21-29	99-145	5162.99	1291.76	4	1291.60	0.01

Peaks indicated with an asterisk may be assigned to more than one peptide.

A more detailed presentation of the peptides is shown in the contour map (Figure 8-7). The contour map provides a 3-D representation of the whole CE/ESI-MS data set and enables the detection of mixtures of compounds according to their migration time and mass spectra. This facilitated peak identification considering the degree of overlap of co-eluting peaks observed here.

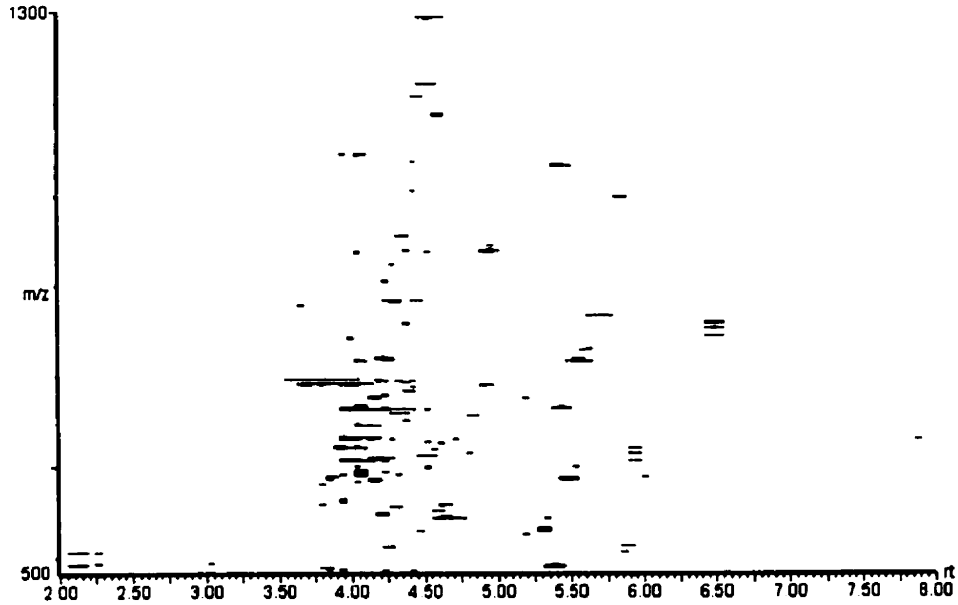

Figure 8-7. m/z contour map of tryptic peptides of myoglobin. Corresponds to the TIE in Figure 8-6.a.

Figure 8-8 shows the tryptic peptide map of myoglobin based on the results shown in Table 8-3. A complete peptide map was obtained based solely on the CE/ESI-MS results. The peptides identified compared well to those observed in constant infusion ESI and HPLC/ESI-MS results obtained on the same sample (not shown). The advantage of the CE analysis over constant infusion ESI was the degree of separation introduced by CE which resulted in cleaner mass spectra with increased S/N. Compared with HPLC/ESI-MS results on the same instrument, the CE analysis time was less than 7 minutes while typical HPLC runs were of the order of 50 minutes. The increased sensitivity, fast separation times and small sample requirements afforded quality results making CE/ESI-MS an important method for the analysis of peptides and proteins.

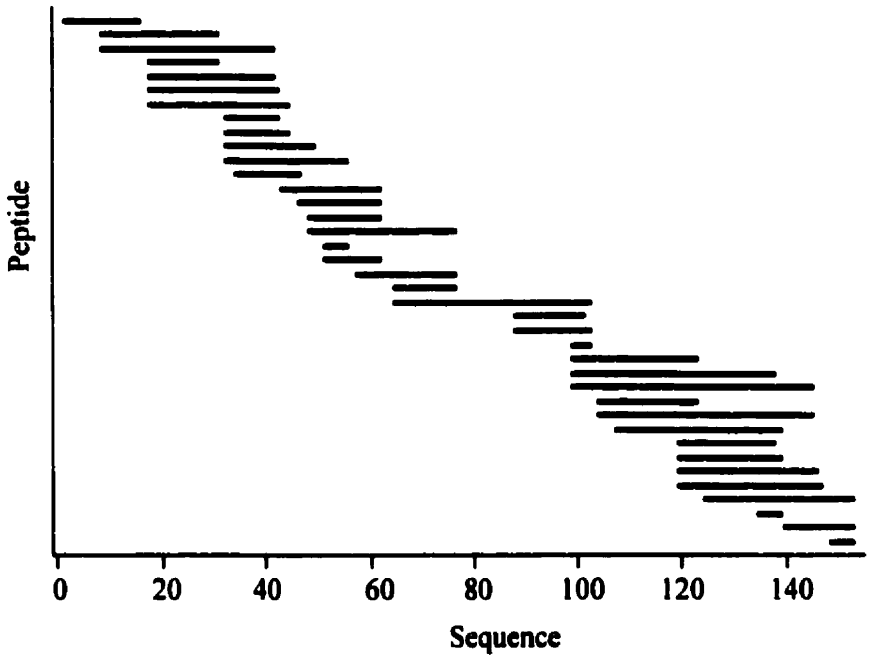

Figure 8-8. Peptide map for tryptic peptides of myoglobin.

8.3.6 CE/ESI-MS of Shepherds Bush Variant Hemoglobin

Application to a real sample is described here for the characterization of a partial tryptic digest of Shepherds Bush (SB) variant hemoglobin. This type of hemoglobin was characterized by MALDI-TOFMS in Chapter 5. The same sample was analyzed by CE/

ESI-MS here. Figure 8-9 shows the TIE and SIE from the digest. The TIE was similar to that obtained from the myoglobin digest and yielded little information. This was due to the limited digestion (10 minutes). The TIE consisted primarily of peaks from the undigested alpha and beta chains of SB hemoglobin along with some peaks from tryptic peptides.

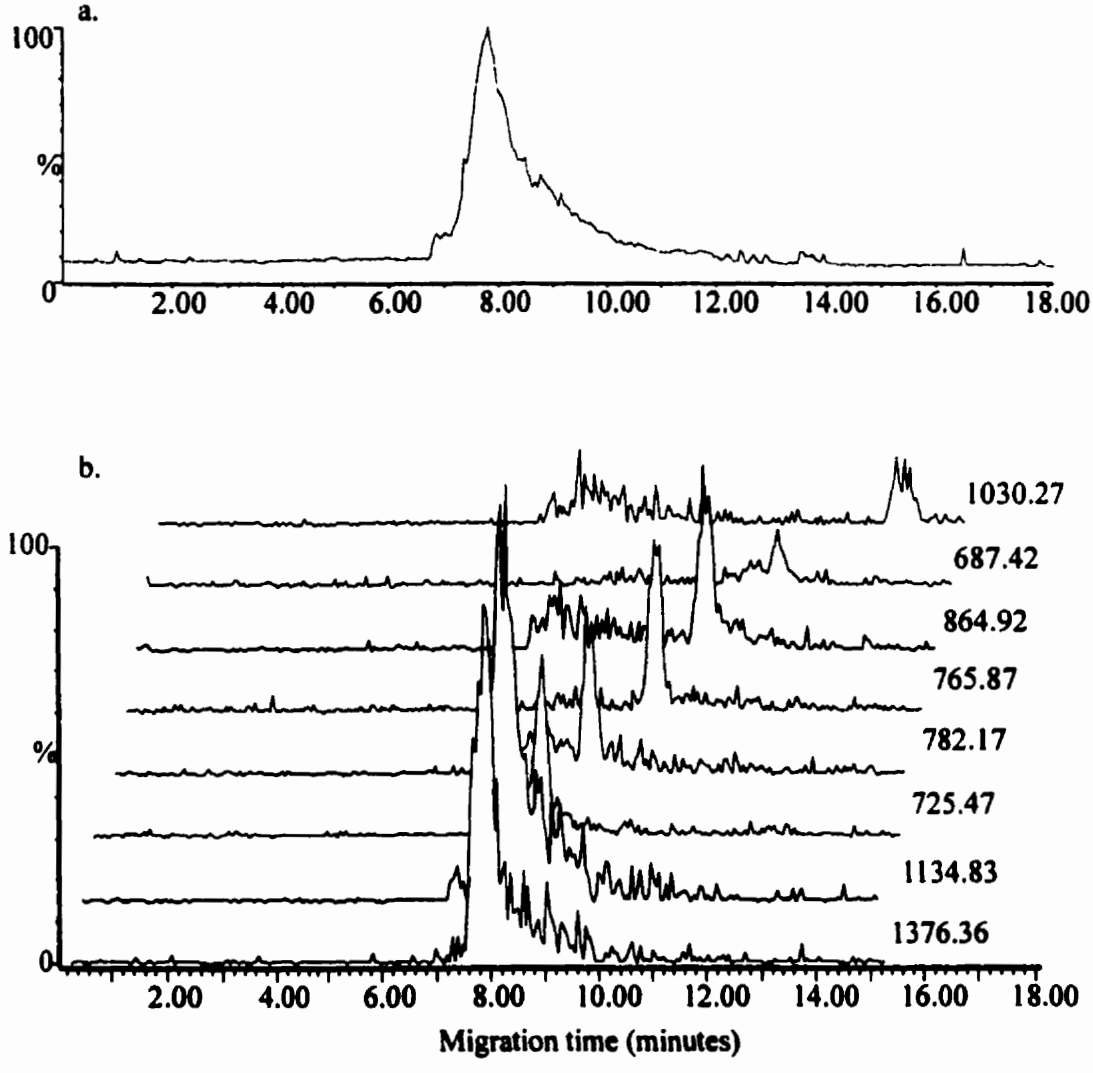

Figure 8-9. CE/ESI-MS of tryptic peptides of SB hemoglobin. (a) TIE and (b) SIE.

The overall separation was rather poor, mainly due to tailing of the undigested globin chains which tended to stick onto the capillary walls and thus contributed to worsened separation of tryptic peptides. This is better illustrated in the contour map shown in Figure 8-10. The separation efficiency was typically characterized by N > 5000 for peptides.

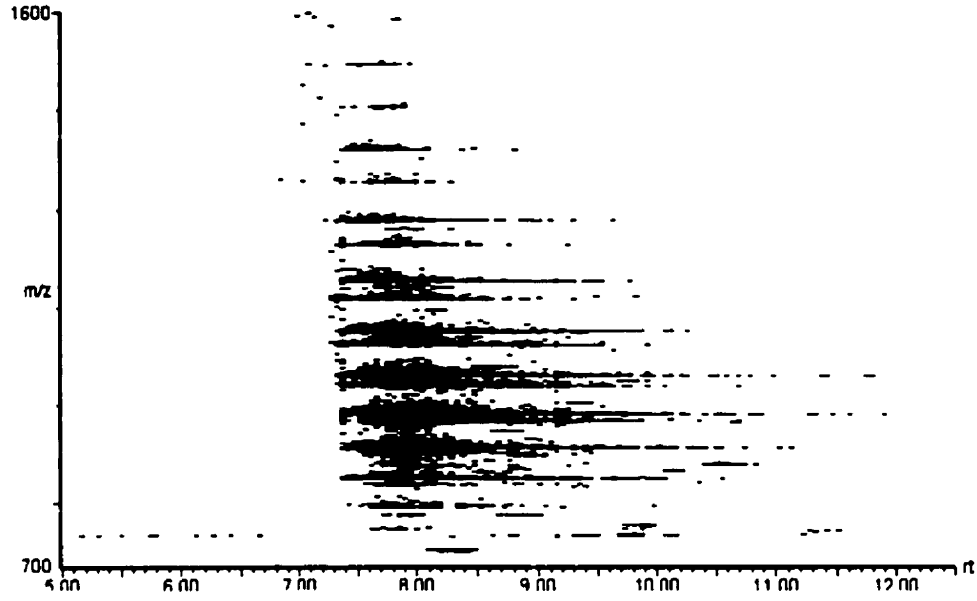

Figure 8-10. m/z contour map of tryptic peptides of SB hemoglobin. Corresponds to the TIE of Figure 8-9.a.

Figure 8-11 shows the mass spectrum of the charge envelope of the alpha and beta chains of the SB variant. Transformed data are shown as well. The variant was observed with a mass increment of 57.76 Da (58.04 calculated). Compared with the MALDI-TOFMS results of Chapter 5, the CE/ESI-MS results were less pronounced in the identification of the variant. This was due to the formation of Na and K adducts and oxidation of the analyte which occurs at the end of the capillary during the electrospray process. This resulted in a broader transformed parent mass for each of the alpha and beta chains. The MALDI-TOFMS results were not affected by the presence of salts and thus produced cleaner molecular ions.

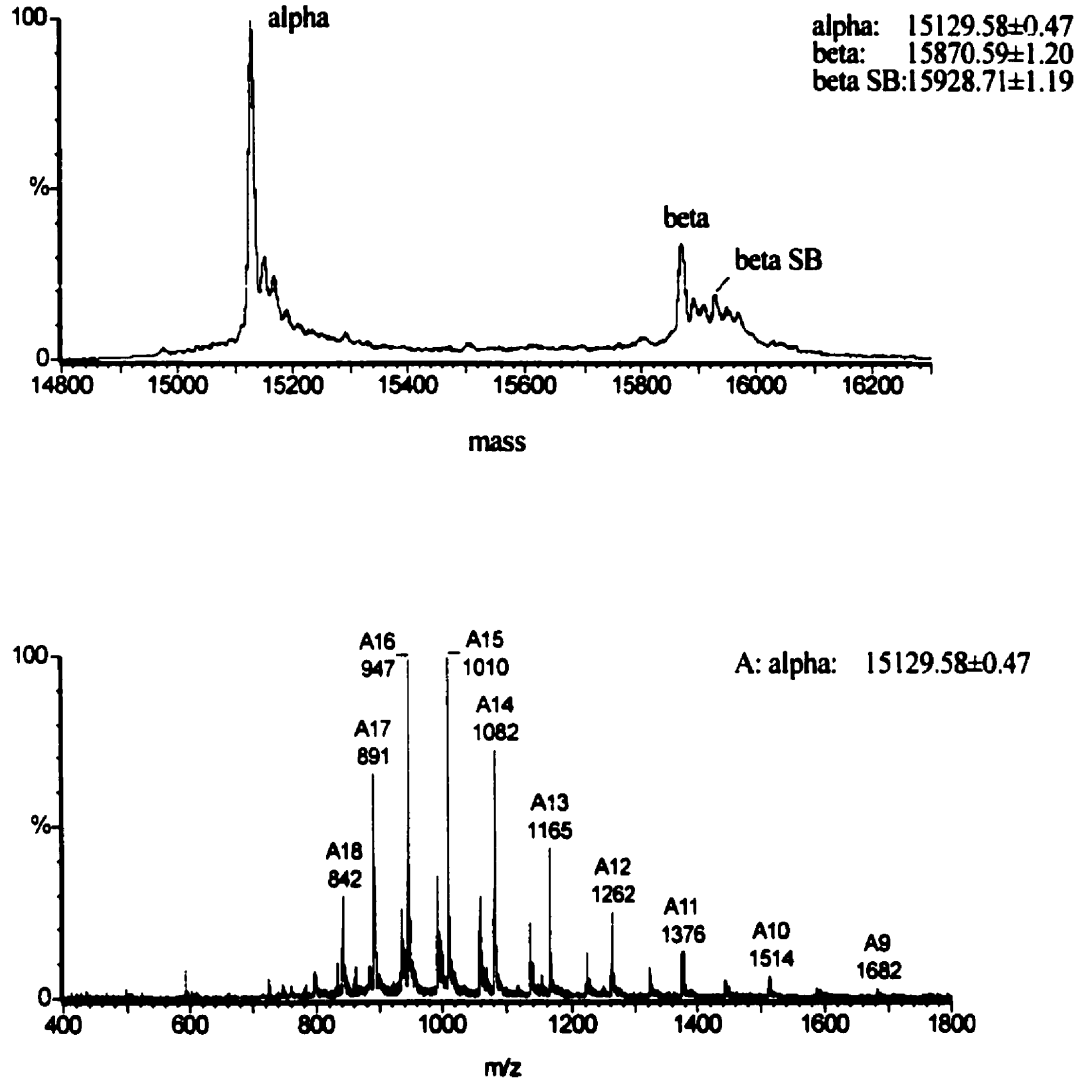

Figure 8-11. Alpha and beta chains of SB hemoglobin.
Variant indicated by a mass shift of 58 Da.
$[M+nH]^{n+}$ ion series shown for the alpha chain.

Examination of the SIE identified several peptides with masses other than those belonging to the globin envelope. Table 8-4 lists the assignments for the tryptic peptides whose SIE are shown in Figure 8-9. These masses were confirmed by HPLC-MS (results not shown). Over 25 peptides were tentatively identified including several which contained the variant b74 (E18) Gly->Asp.

Table 8-4. Digest fragments of SB hemoglobin characterized by CE-MS.

Tryptic fragment	Sequence	Calculated Mass $[M+nH]^{n+}$	Observed Mass $[M+nH]^{n+}$	Charge (n)	% Error
b4	31-40	427.13	425.85	3	0.30
b1-2	1-17	467.23	467.54	4	0.07
b1-2	1-8	476.94	476.76	2	0.04
a4	7-31	509.13	510.88	3	0.34
b5	41-59	515.00	515.83	4	0.16
a3	12-16	532.24	532.29	1	0.01
a10-11	91-99	544.25	544.65	2	0.07
a1-2	1-11	586.53	586.69	2	0.03
a6	41-56	613.86	612.35	3	0.25
m8-9	66-82	619.35	619.71	4	0.06
b3	18-30	657.02	658.22	2	0.18
b5	41-59	687.42	687.44	3	0.00
b14-15	133-146	725.47	725.85	2	0.05
a9	62-90	750.50	750.34	4	0.02
m7-9	62-82	751.01	750.86	3	0.02
a4	17-31	765.87	765.82	2	0.01
b12-13	105-132	771.96	771.17	4	0.10
a6-8	41-61	781.41	781.55	3	0.02
a8-9	61-90	782.17	782.39	4	0.03
a2-4	8-31	828.02	829.59	3	0.19
m9	67-82	864.92	864.98	2	0.01
a6	41-56	918.17	918.02	2	0.02
b6-10	60-95	957.24	956.61	4	0.07
a5-6	32-56	964.77	963.45	3	0.14
a3-4	12-31	1022.61	1022.62	2	0.00
b5	41-59	1030.27	1030.65	2	0.04
a8-9	61-90	1041.51	1042.85	3	0.13
b11-13	96-132	1048.78	1048.23	4	0.05
a8-11	61-99	1049.81	1049.70	4	0.01
b1-3	1-30	1055.17	1055.19	3	0.00
b6-9	60-82	1211.43	1210.42	2	0.08
b3-6	18-61	1211.43	1210.88	4	0.05

Figure 8-12 shows the tryptic peptide map of SB hemoglobin based on the results shown in Table 8-4. A partial peptide map of the alpha chain and a complete peptide map of the beta chain were obtained. Improved separation and a more thorough digestion would have yielded better results as several peptides possessed theoretical masses which corresponded to masses from the charge envelopes of the globin chains.

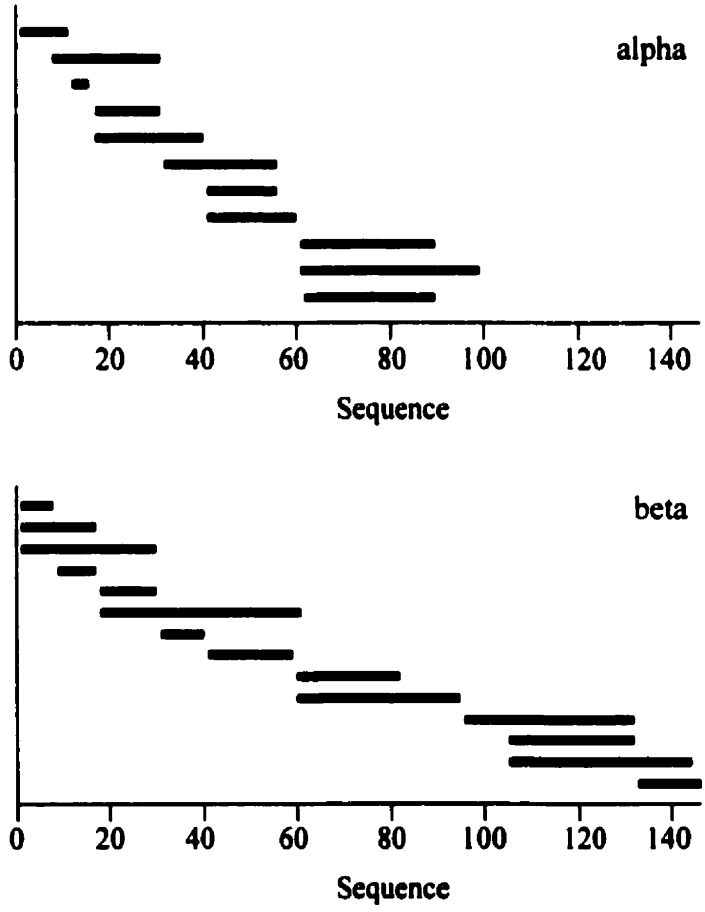

Figure 8-12. Tryptic peptide map for SB hemoglobin.

8.4 CONCLUSIONS

The CE/ESI-MS interface described in this article constitutes a practical and simple means of connecting a CE system to the Z-Spray™ source of a Quattro-LC mass spectrometer configured for the MegaFlow-Z™ electrospray mode. Construction of the probe was inexpensive and simple. The probe allows one to switch from normal ESI or HPLC/ESI-

MS to CE/ESI-MS in a matter of minutes without removing the ESI source as is the case with the commercial sheathflow CE/ESI-MS probe. The gold-coated CE capillary tips used for sheathless interfacing produced a stable electrospray under restricted CE voltage conditions which were still satisfactory for efficient separations. Good quality CE separation and MS detection of a mixture of standard peptides were obtained. It was also possible to perform on-line CE/ESI-MS/MS experiments on the same peptide mixture and obtain sequence information. A myoglobin tryptic digest was subjected to CE/ESI-MS analysis under the same conditions as used for the standard peptides. Separation was limited by overlapping of the tryptic peptides. Nevertheless, use of SIE allowed identification of several tryptic peptides and a complete peptide map was obtained. We are currently refining the probe design and interface set-up in order to increase the sensitivity of analyses.

8.5 REFERENCES

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9 CE/ESI-TOFMS

9.1 INTRODUCTION

In order to exploit the full potential of mass spectrometry as a detector for CE, a complete mass spectrum should be recorded at suitable intervals. To accurately represent the analyte peaks, which may be on the order of one second in width, several mass spectra should be recorded per second. This presents a problem for most of the types of mass spectrometers which have been used as detectors for CE/ESI, such as quadrupoles, ion traps, and ion cyclotron resonance cells [1-5]. In these instruments, particularly in scanning instruments such as quadrupole mass filters and ion traps, rapid data acquisition can only be accomplished at the expense of mass resolution (m/ΔmFWHM) or sensitivity, or both. The performance limits of conventional scanning mass spectrometers generally limit the spectral range to a few hundred daltons when the scan rate exceeds one spectrum/ sec. These instruments are therefore often used to acquire the total ion electrophorogram (TIE) and selected ion electrophorograms (SIE) for one or a few ions at a time.

Time-of-flight mass spectrometry has a distinct advantage as a detector for CE because it uses parallel detection and is capable of high repetition rates (up to several kilohertz). The rate at which useful spectra can be recorded is determined by the rate at which ions can be injected into the mass spectrometer, i.e. by the available sample and the instrument sensitivity, and not by characteristics of the measurement itself. Therefore, recording the complete mass spectrum at each selected interval rather than the intensities of only a few ions does not compromise the performance of the instrument. Total and selected ion electrophorograms can then be extracted from the single recorded data set. Reflecting ESI-TOFMS for peptides and proteins up to molecular mass of 10,000 Da, with mass resolution higher than 5000, mass accuracy in the range of 10 ppm, and femtomole sensitivity, is well suited to CE [6]. Moreover, TOF instruments have an unlimited m/z range such that high mass ions with lower charge-states (such as non-covalent complexes) can be analyzed.

The use of orthogonal injection ESI/TOF as a detector for CE has recently been reported by a limited number of groups [7-11]. Both sheath-flow and sheathless designs

have been adopted with orthogonal injection. The mass analyzer modes used in these studies included linear TOF [9,10], inverted perfectron TOF [8], and ion-trap reflectron TOF [7,11]. Early work by L. Fang et al. employed a sheathless interface which used a 25 μm gold wire for electrical contact placed inside the terminus of a fused silica capillary [9]. Spectra from a separation of peptides were acquired at 10 kHz on a linear TOF spectrometer with a resolution of ca. 100. A detection limit of ca. 40 - 80 fmol was reported. J.F. Banks et al. demonstrated fast CE separations of peptides and proteins with coated capillaries using a co-axial sheathflow ESI interface coupled with a linear TOF mass spectrometer [10]. TIE were integrated at rates up to 8 Hz with a scan rate of 8192 Hz. A resolution of 1000 was obtained and a sensitivity limit of 8 fmol shown for leucine enkephalin. An inverted perfectron TOF mass spectrometer was employed by D.C. Muddiman et al. for the separation of three peptides [8]. Coated capillaries were used and coupled via a gold-coated sheathless interface. Mass spectra were generated at a rate of 3500 Hz but due to limited signal averaging capabilities only 1 out of 70 spectra acquired were recorded with ca. 2 Da. bin width. TIE were obtained with a S/N of 6 on raw data. An ion-trap storage/reflectron TOF mass spectrometer has recently been interfaced as a detector for CE [7,11]. Separations of peptides from proteolytic digests of proteins has been performed using coated capillaries coupled via a sheathless interface. TIE were generated with an integration rate of 0.5 - 4 Hz on fmol amounts of material, resulting in a resolution of ca. 1500 (10 ns bin width) and a S/N of 17.2 on raw data. Detection limits of 2 - 5 fmol were reported.

In this chapter, the feasibility of on-line CE/ESI-TOFMS coupled using a sheathless interface on the Manitoba reflecting ESI-TOF instrument is demonstrated. In contrast to previous work, this instrument uses an ion mirror and single ion counting with a time-to-digital converter (TDC). The instrument performance is better than the TOF instruments mentioned above and the goal was to maintain this high degree of performance when coupled with an on-line separation method such as CE. The use of reflecting ion mirror and single ion counting afforded high mass resolution > 6000 and mass accuracy at the 10 ppm level. With the high repetition rate of the instrument, exceptional S/N was observed on raw data, demonstrating that fmol sensitivity is easily achieved. Such S/N values have not been reported before on raw data and are a direct result of the high quality performance of the Manitoba ESI-TOF mass spectrometer [6,12]. In these measurements a complete mass

spectrum was acquired but not recorded in full at each time interval because storage and software facilities were not yet implemented to record the large data sets associated with CE/MS [6,11]. To demonstrate the potential of CE/ESI-TOFMS, TIE and SIE were recorded on separate runs. The necessary technology to realize the full potential was developed later during this research and is discussed in Chapter 10.

9.2 EXPERIMENTAL

CE/ESI-TOFMS experiments were performed on a reflecting TOF mass spectrometer, an instrument built in-house in the Time-of-Flight Lab, Physics Department, University of Manitoba. Spectral analysis was carried out using TOFMA software written in-house. Spectra were recorded in 2 ns channels.

9.3 RESULTS AND DISCUSSION

9.3.1 Constant Infusion of Substance P

Initial on-line testing of the capillaries was performed using a constant infusion of a solution of substance P (3×10^{-5} M in 1% aqueous acetic acid). A 9-kV separation potential was applied and flow rates of 100 - 300 nL/ minute were generated, making the CE system compatible with the ESI-TOF instrument. The spectrum shown in Figure 9-1 was obtained during this constant infusion experiment. Performance was typical of the instrument [6,12], with mass resolution > 6000, enabling baseline isotopic resolution as shown inset, and mass accuracy < 10 ppm.

The amount of sample consumed was ca. 50 picomoles over 4 minutes and the electrospray produced by constant infusion was stable for several hours. Under these conditions, an average of 1.25 ions were detected for each TOF ion injection pulse which for a pulse repetition-rate of 3400 Hz corresponds to about 4000 counts/ sec. In contrast, the background signal for constant infusion of a 1% solution of acetic acid produced less than 175 counts/ sec. with a variance of $(175)^{1/2} = 13$. These values give an indication of the performance that can be expected of CZE/MS. If peaks are of the order of 1 sec. in width, then the S/N in the TIE should be about 300.

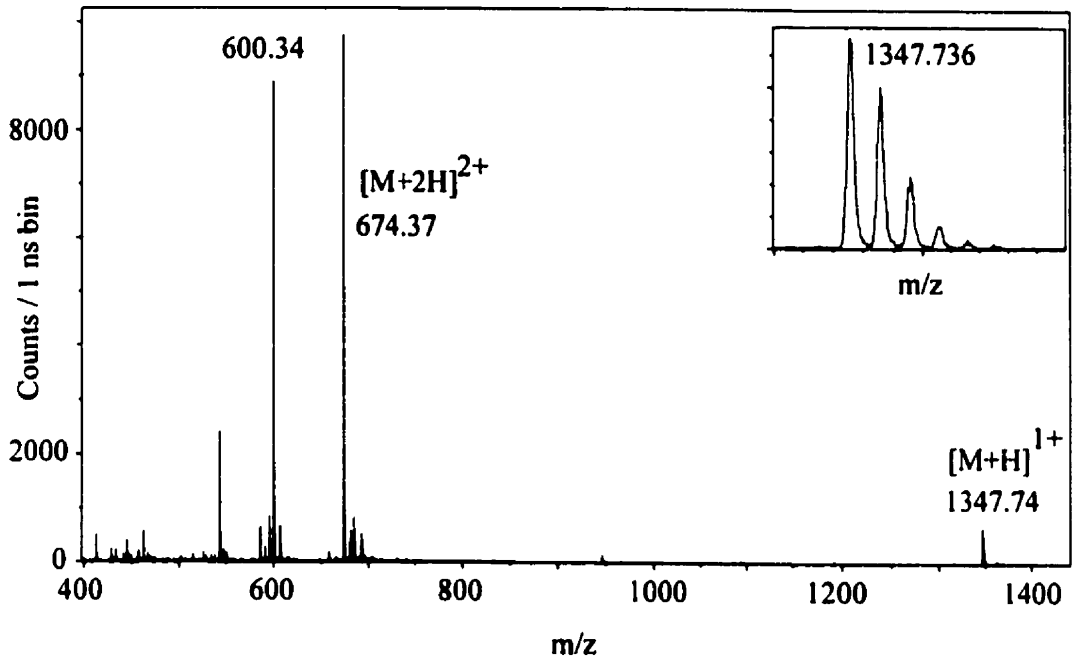

Figure 9-1. CE/ESI-TOFMS constant infusion of substance P. [substance P] = 30 mM. Electrolyte 1% acetic acid, 9 kV separation potential, 3.2 kV ESI potential. Amount consumed ca. 50 pmol over 4 minutes.

Even if spectra are recorded and integrated at 10 per second, the ratio of peak height to background-variance will be about 100. When spectra are recorded for a shorter time during CE/MS, no sacrifice in instrument performance is necessary. Only the number of counts/ sec. is reduced which limits the dynamic range of individual spectra.

9.3.2 Separation of 5 Peptides

The total ion electrophorogram (TIE) obtained for on-line CZE/MS separation of a mixture of five peptides is shown in Figure 9-2. The concentration of the peptides in 1% acetic acid was 10^{-5} M, and approximately 230 fmol of sample were loaded onto the capillary. Separation proceeded with 5% acetic acid as the running electrolyte. In order to generate the TIE trace, a mass spectrum was acquired once every second and the total counts in a mass window (m/z 400-2500) were integrated and recorded.

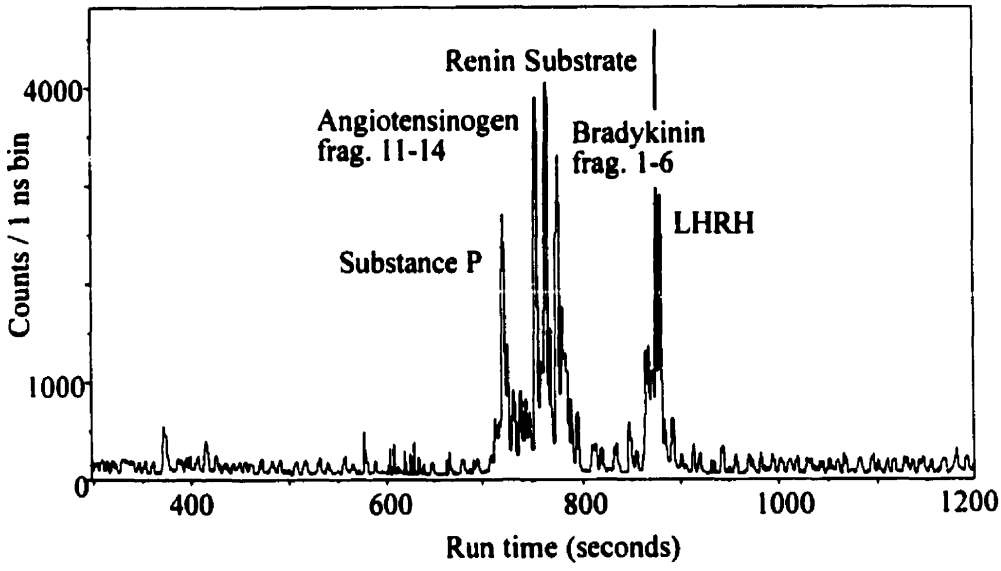

Figure 9-2. CE/ESI-TOFMS of a mixture of five peptides.
230 fmol injected, electrolyte 5% acetic acid, 9 kV separation potential, 3.6 kV ESI potential.

During the elution of the analytes, approximately 5000 counts/ sec. were measured, corresponding to 5000 ions summed over the one second recording interval. It was not necessary to integrate more than one spectrum per second in this case to represent the peaks, which are typically 3 - 5 seconds in width with a S/N = 270 - 450 (Table 9-1).

Table 9-1. CE/ESI-TOFMS of 5 peptides.

peptide	sequence	Mt seconds	S/N	N	Mass	observed
Substance P	RPLPQQFFGLM-NH2	721	270	180000	1347.701	1347.727
Angiotensin 11-14	VIHN	753	390	350000	482.265	482.285
Renin substrate	DRVYIHPFHLVYS	763	400	360000	1758.925	1758.910
Bradykinin 1-6	RPPGES	775	330	208000	660.339	660.354
LH-RH	pEHWSYGLRPG	878	450	87000	1182.557	1182.581

The high ion current during elution (given a S/N > 300) indicates that considerably high integration rates can be used and that analytes in lower concentrations may be detected using this on-line system. Trace amounts of salts present in the original peptide sample eluted at ca. 380 and 410 sec. The electrophoretic resolution was between 3.3 and 18.7, with theoretical plate counts of 87,000 to 360,000. The broad base of the cluster of peaks at around 700 sec. is due to intermittent perturbations in the spray as components eluted from the capillary.

Figure 9-3 shows the cumulative mass spectrum obtained once the CZE/ESI-TOFMS experiment was completed. This spectrum contains ions produced by the five peptides, some of which are doubly or triply charged in the case of larger peptides. Very low background signal appears in this spectrum. Resolution > 6000 and ppm accuracy facilitated identification of multiple charge states for each of the peptides.

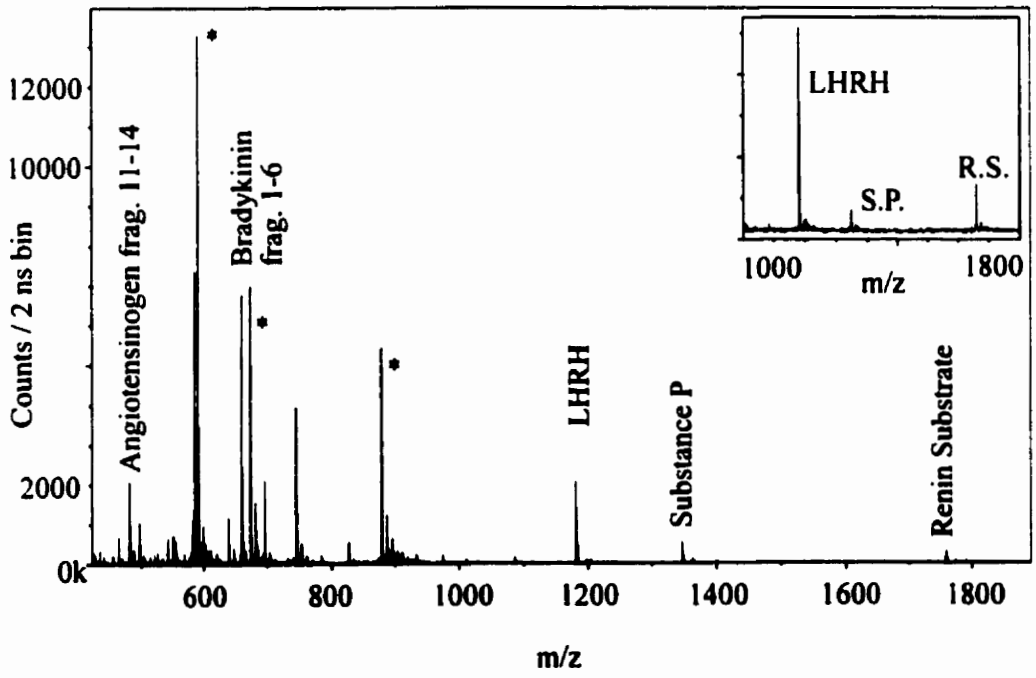

Figure 9-3. CE/ESI-TOFMS mass spectrum of five peptides.
* indicates multiple charged species.

Figure 9-4 shows SIE derived from a selected ion monitoring (SIM) experiment performed on the peptide mixture, using sampling windows of ca. 5 Da for each peptide and integrated at a rate of 2 Hz.

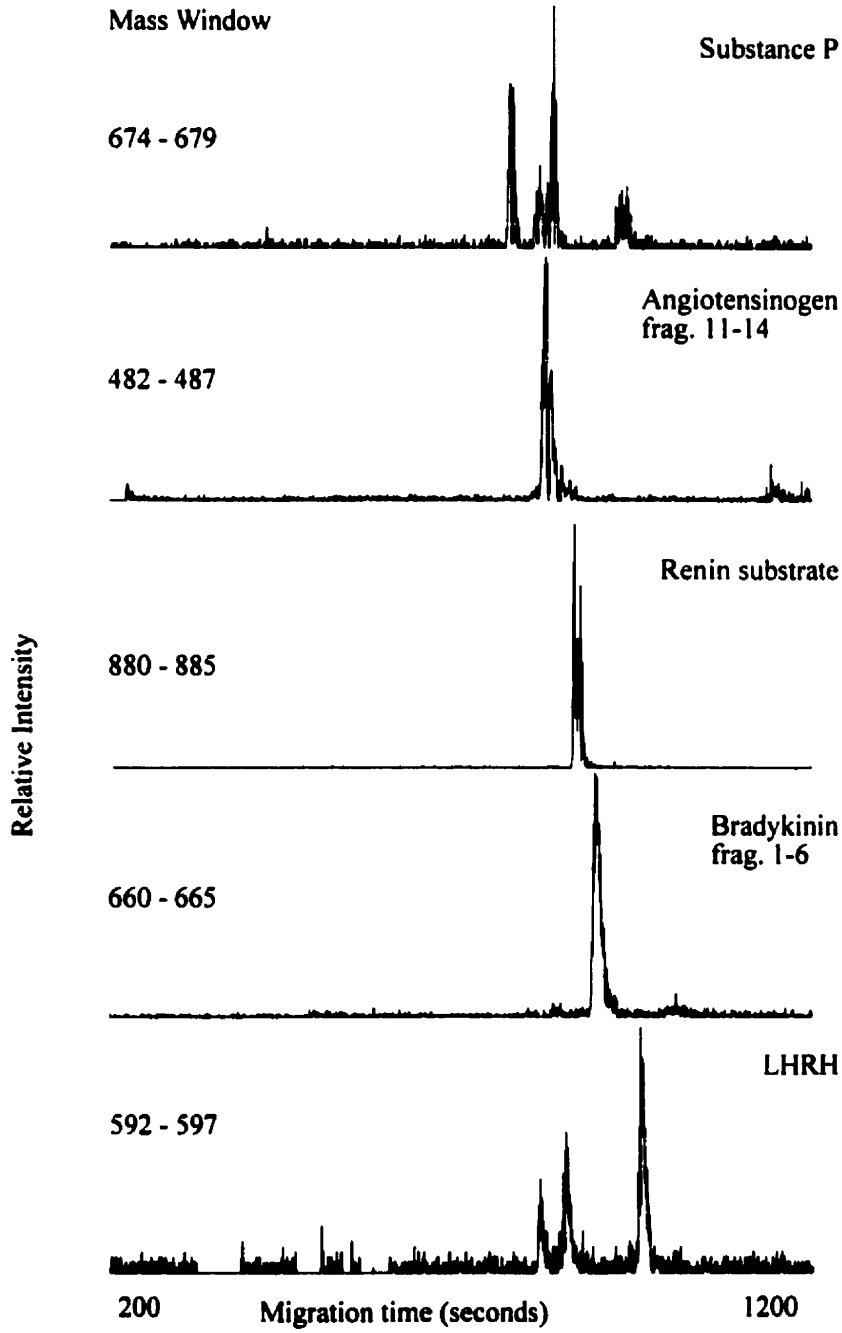

Figure 9-4. CE/ESI-TOFMS SIE of five peptides.
350 fmol injected, electrolyte 5% acetic acid, 9 kV separation potential, 3.6 kV ESI potential.

As mentioned in the introduction, the data for all SIE are available simultaneously but because of limitations in our present data storage and handling, separate runs were necessary to record and store them. The peaks were between 4 and 6-seconds wide and signal intensity was in excess of 8000 counts/ sec. for each peptide eluted. This corresponds to > 4000 counts integrated at each half-second interval. Some peptides produced peaks which coincided with the selected window of other peptides, due to multiple charging or fragmentation, and this caused some electrophoretic traces in Figure 9-4 to contain more than one peak. For example, the m/z windows monitored for substance P and LHRH contained peaks due to other mixture components which produced ions of m/z which fell within the recorded window. Due to higher loading, peptide peaks could not be resolved in the TIE (not shown) but are resolved in the series of SIE's shown here. Another significant advantage of recording SIE's is the increase in S/N observed over a small mass window, due to lower background noise present in different regions of the mass spectrum. As an example, a S/N value of ca. 760 was observed for the renin substrate peak, whereas the LHRH peak was recorded with slightly higher background noise and thus a S/N value of only ca. 30.

An example of what is observed during the elution of a compound is shown in Figure 9-5 for the $[M+H]^+$ peak of LHRH. Before and after elution of the peptide, very low background noise, with count rates less than 200 counts/ second, is recorded in the mass window shown here (a). As the component eluted, the mass spectrum was acquired (b). The mass spectrum of LHRH was recorded during 60 seconds with the peak width of LHRH of ca. 5 seconds. The spectrum was produced by ca. 350 fmol of peptide. Resolution was about 5000 allowing baseline resolution of the isotopic pattern. The total number of ions contributing to the $[M+H]^+$ peak is about 10,000 indicating that if the spectra were acquired at 10 per second there would still be > 200 ions in the molecular ion peak. As mentioned above, this higher spectrum rate does not sacrifice performance of the instrument; only the dynamic range is reduced in shorter acquisition times. The mass spectrum in Figure 9-5 which represents about 5 seconds of elution has an excellent S/N ca. 500 and dynamic range indicating good sensitivity at 350 fmol. Spectra may thus be obtained with much lower amounts of material [6,12].

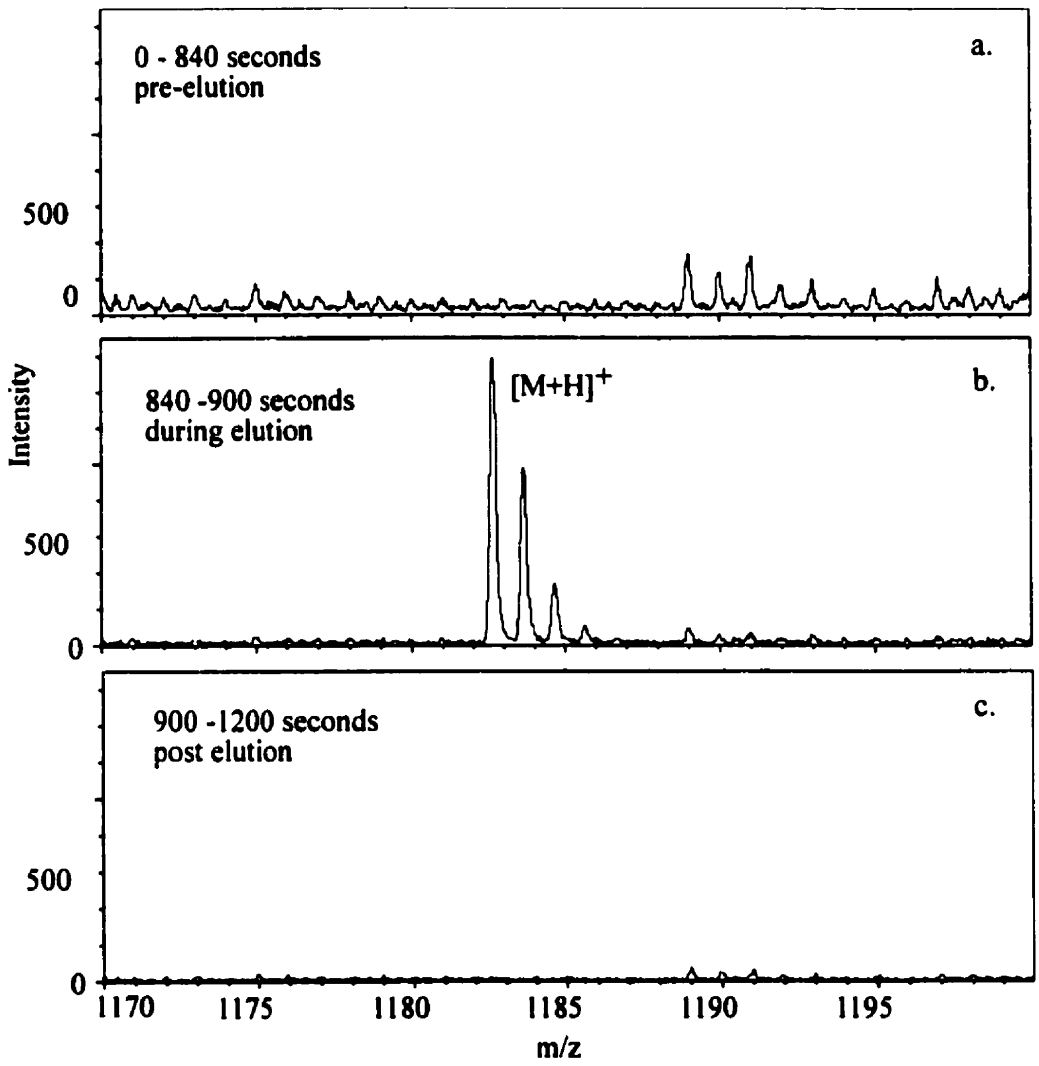

Figure 9-5. Time window monitoring of MS data.
Mass spectra recorded (a) before (b) during and (c) after elution of LHRH. Electrolyte 5% acetic acid, 9 kV separation potential, 3.6 kV ESI potential.

9.3.3 Separation of 3 Proteins

The TIE obtained from the separation of a mixture of three proteins, myoglobin, bovine insulin and cytochrome c, is shown in Figure 9-6. The amount injected was ca. 3.5 pmol of each protein, i.e. larger than in the case of the peptide mixture. A sampling rate of 5 Hz was used. The peaks obtained were broad and not well resolved between the first two eluted components. Resolution values for the CZE separation ranged from 0.8 to 6.5, and

the theoretical plate counts were from 2800 to 5500 due presumably to the large sample size and interaction of the sample with the capillary walls. Perturbations in the baseline at ca. 300 and 400 sec. were caused by salts present in the sample. The ion count rate was between 2200 and 4000 counts/ sec. during elution of the proteins with low background signal of ca. 200 counts/ sec. The S/N value was between 110 - 225 on raw data recorded at 5 Hz. The large peak widths (> 10 sec.) enabled acquisition of a good quality mass spectrum for each protein.

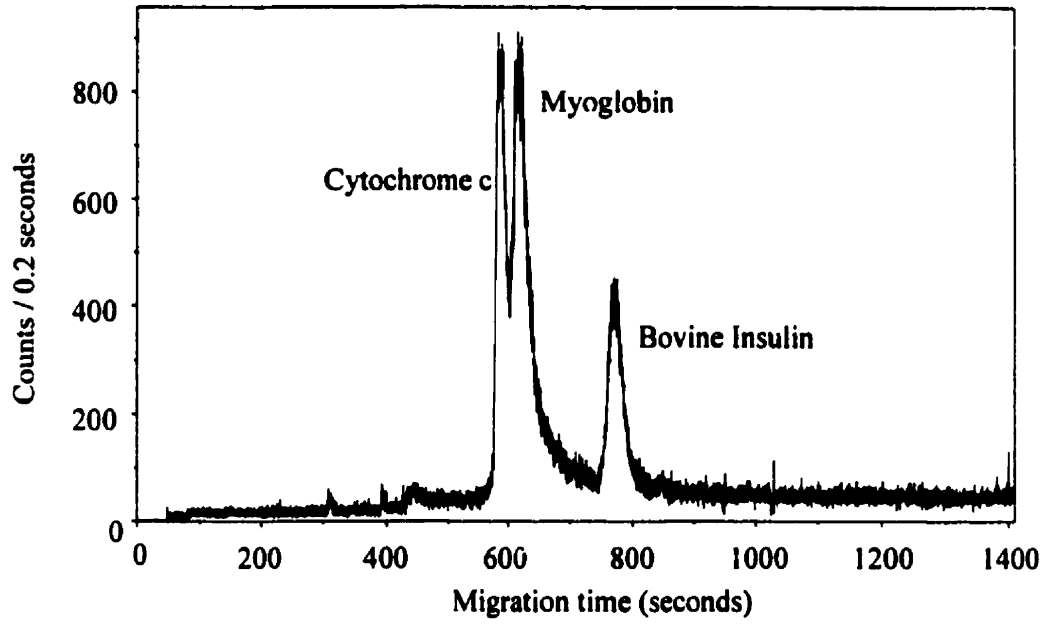

Figure 9-6. CE/ESI-TOFMS TIE of three proteins.
3.5 pmol injected, electrolyte 5% acetic acid, 9 kV separation potential, 3.6 kV ESI potential.

The spectra, recorded for 4-5 seconds for each component, are shown in Figure 9-7. Multiply charged ions were observed and the deconvolved spectra are presented in the insets. The high resolving power of the TOF mass analyzer made it possible to resolve peaks of oxidation products of bovine insulin at m/z intervals of 16 (c). In the deconvolved spectrum of myoglobin (b) it is possible to observe molecular ions of the protein with and without adduction of acetate moieties from the electrolyte. In all cases, the mass spectra obtained were essentially identical to those observed during the off-line experiments performed earlier.

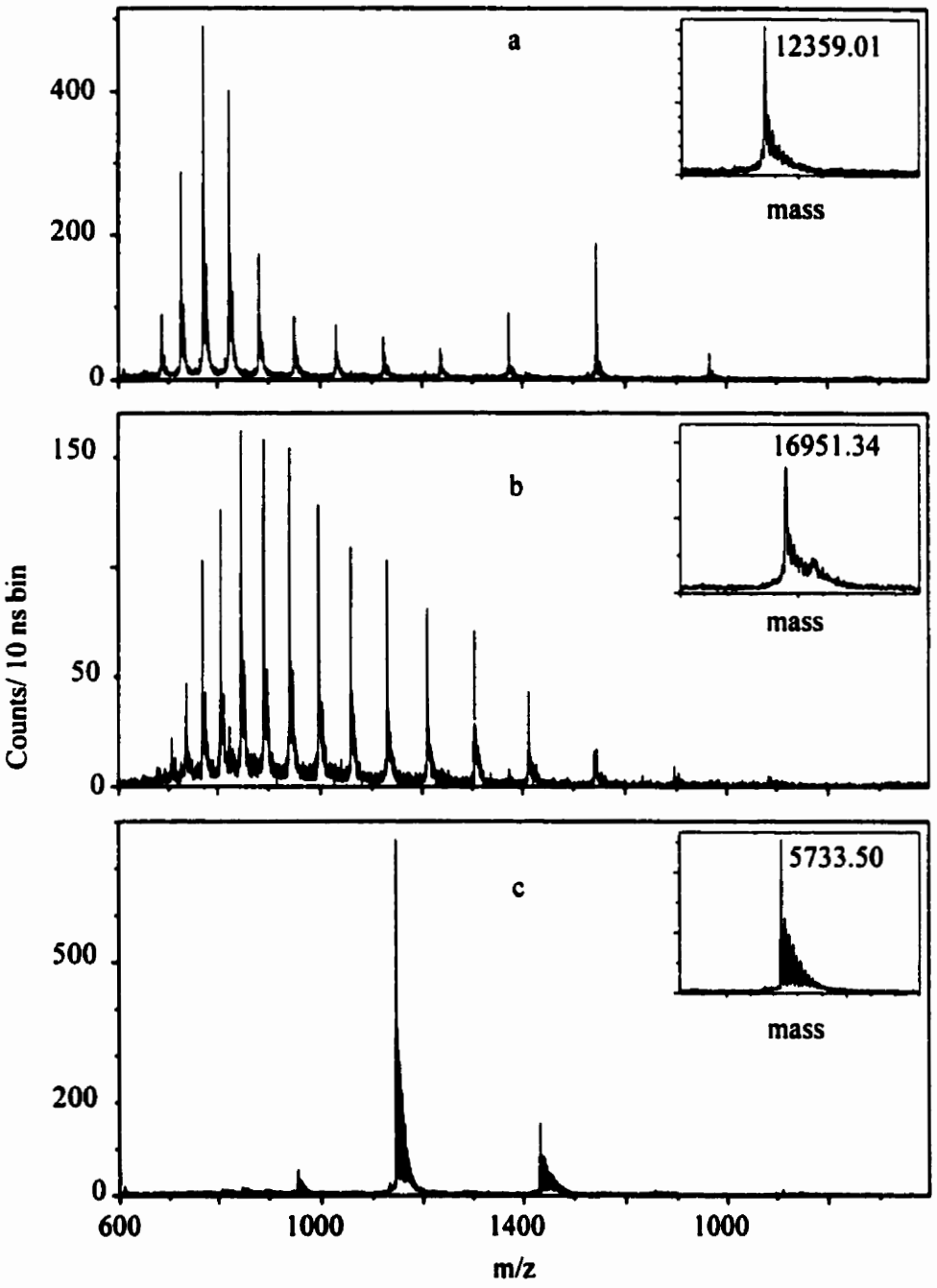

Figure 9-7. CE/ESI-TOFMS mass spectra three proteins. (a) cytochrome c (b) myoglobin (c) bovine insulin. Deconvolved spectra are shown inset.

9.4 CONCLUSIONS

Off-line and on-line CZE/ESI-TOFMS were investigated and found to be useful and produce good results for the separation and analysis of peptides and proteins. The use of a test bench enabled optimization of ESI and CZE conditions prior to performing on-line experiments. The gold-coated capillaries used as the sheathless ESI interface produced a stable electrospray for extended periods of time. On-line CE/ESI-TOFMS produced typical separations with 35,000 to 87,000 theoretical plates and fmol-level sensitivity. Electrophoretic separations were not compromised on coupling to the mass spectrometer. The mass spectra obtained were not adversely affected by the electrophoretic process. Reflecting TOF mass spectra were recorded with mass resolution and accuracy typical for the instrument used ($R > 6000$, 10 ppm). Single ion counting resulted in good S/N values obtained for TIE and SIE indicating fmol sensitivity.

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10 CE/ESI-MS AND CE/ESI-TOFMS CHARACTERIZATION OF CITRATE SYNTHASE

10.1 INTRODUCTION

This chapter describes the validation and performance characterization the QQQ and TOF mass spectrometers for use as detectors for CE/ESI-MS. The substance of choice for validation was citrate synthase, a protein of medium molecular weight (M_r = 47885 Da, wild type). This protein was chosen as a model based on prior characterization by ESI-TOFMS [1] and MALDI-TOFMS (Chapter 3). Citrate synthase readily undergoes near complete digestion and with 44 tryptic cleavage sites produces an abundance of peptides suitable for analysis by MS.

Complete mass spectral characterization was performed on the tryptic peptides by on-line CE/ESI-MS and CE/ESI-TOFMS. For these on-line experiments, a Quattro-LC triple quadrupole mass spectrometer (Micromass) and a home built reflecting time-of-flight mass spectrometer were used. Data logging software for CE/ESI-TOFMS was developed by W. Ens and V. Spicer and evaluated.

10.2 EXPERIMENTAL

10.2.1 Tryptic Digest of Wild-Type Citrate Synthase

The protein was prepared from E. coli as described [2] and the wild type made available by A. Ayed and H. W. Duckworth [3] and is described in more detail in Chapter 3.

The protein (1.0 mg/mL in 20 mM Tris-HCl and 1 mM EDTA, pH = 7.8) was incubated with 0.01 mg/mL TPCK-treated trypsin for 3 h at 37°C. It should be noted that dialysis [3] was omitted for citrate synthase samples subjected to enzymatic digestion.

10.2.2 Capillary Zone Electrophoresis

CE/ESI-MS experiments were performed on a Quattro LC (Micromass, UK) mass spectrometer equipped with a Z-Spray™ interface. CE/ESI-TOFMS experiments were performed on a reflecting TOF mass spectrometer, an instrument built in-house in the

Time-of-Flight Lab, Physics Department, University of Manitoba. The instruments are described in detail in Chapters 7 through 9.

CE/ESI-TOFMS spectral analysis was carried out using newly written TOFMA software. The software is based on the principle of data logging. Instead of storing a series of individual spectra during a CE experiment, the entire TDC output is spooled into computer memory along with real time markers. Time markers were stored at 60 Hz (16 ms) for convenience and may be stored as fast as 5 kHz, the start pulse rate of the TOF instrument. By storing the entire data set all instrumental parameters such as resolution (625 picoseconds) are retained. This allows the generation of SIE and subsequent individual mass spectra without loss of information. In order to process the data set a significant amount of computer memory (ca. 300 MB) was required. Count rates range from 300 ions per second to 100,000 ions per second for TOF analysis depending on the amount of analyte present. This can result in up to ca. 0.4 MB of data spooled per second into memory. Typical CE/ESI-TOFMS experiments on sub-pmol amounts of proteolytic digests resulted in data files in excess of 200 MB per run. However, these data sets may be reduced by using compression algorithms which can eliminate 20% - 70% of the file by eliminating regions which contain little or no information. A more complete description of the software may be found elsewhere [4].

10.3 RESULTS AND DISCUSSION

10.3.1 CE/ESI-MS and CE/ESI-TOFMS

Simple M_r measurements on proteins such as citrate synthase can be effected very efficiently by sample infusion ESI-MS (not shown). The M_r measurements yielded 47887.9 Da for wild-type citrate synthase. The corresponding calculated value (average mass) was 47884.95. The error on this measurements is thus of the order of 60 ppm.

In order to further characterize wild-type citrate synthase and test the performance of the instruments, a 3-h tryptic digest was performed and the resulting peptides analyzed by on-line CE/ESI-MS and CE/ESI-TOFMS. For both analyses, the injection volume was ca. 40 nL (by gravity), and the protein concentration was ca. 2×10^{-5} M resulting in ca. 800 fmol of each peptide being injected. The background electrolyte, 5% acetic acid was chosen

to minimize interaction of the peptides with the negatively-charged silanol groups on the inner walls of the fused-silica capillary and to favour protonation of the analytes.

The TIE obtained by analyzing the tryptic digest on the Micromass triple quadrupole mass spectrometer is shown in Figure 10-1. Because of the large number of peptides present and of the abundant chemical background associated with the separation buffer used, very little information could be obtained from the TIE, which exhibits a S/N value of ca. 5 for the peak at 12 minutes. Moreover, the S/N of this TIE trace was compromised by the presence of contaminants in the CE electrolyte. A reconstructed TIE excluding signals from impurities would feature a considerably higher S/N value.

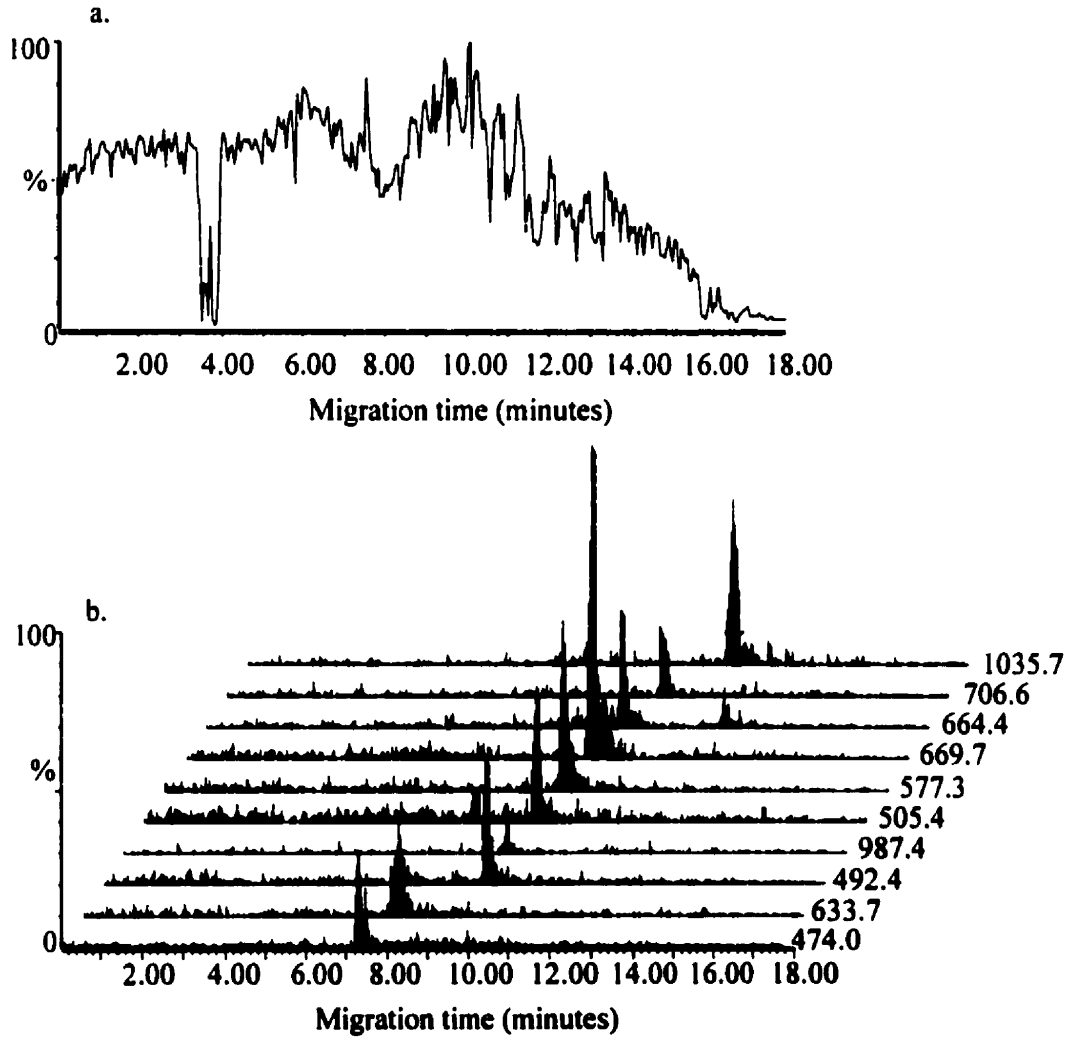

Figure 10-1. CE/ESI-MS of tryptic peptides of citrate synthase. (a) TIE and (b) typical SIE of selected peptides.

The TIE obtained by CE-ESI-TOFMS of a similar citrate synthase digest is shown in Figure 10-2. The performance of the CE-ESI-TOFMS setup has been characterized, Chapter 8, as yielding S/N values ca. 300 in the TIE on 230 fmol of standard peptides. The results here were typical of the instrument with high S/N values, resolution and mass accuraccy. Although the TIE was of better quality, little information may be gained due to overlapping peaks.

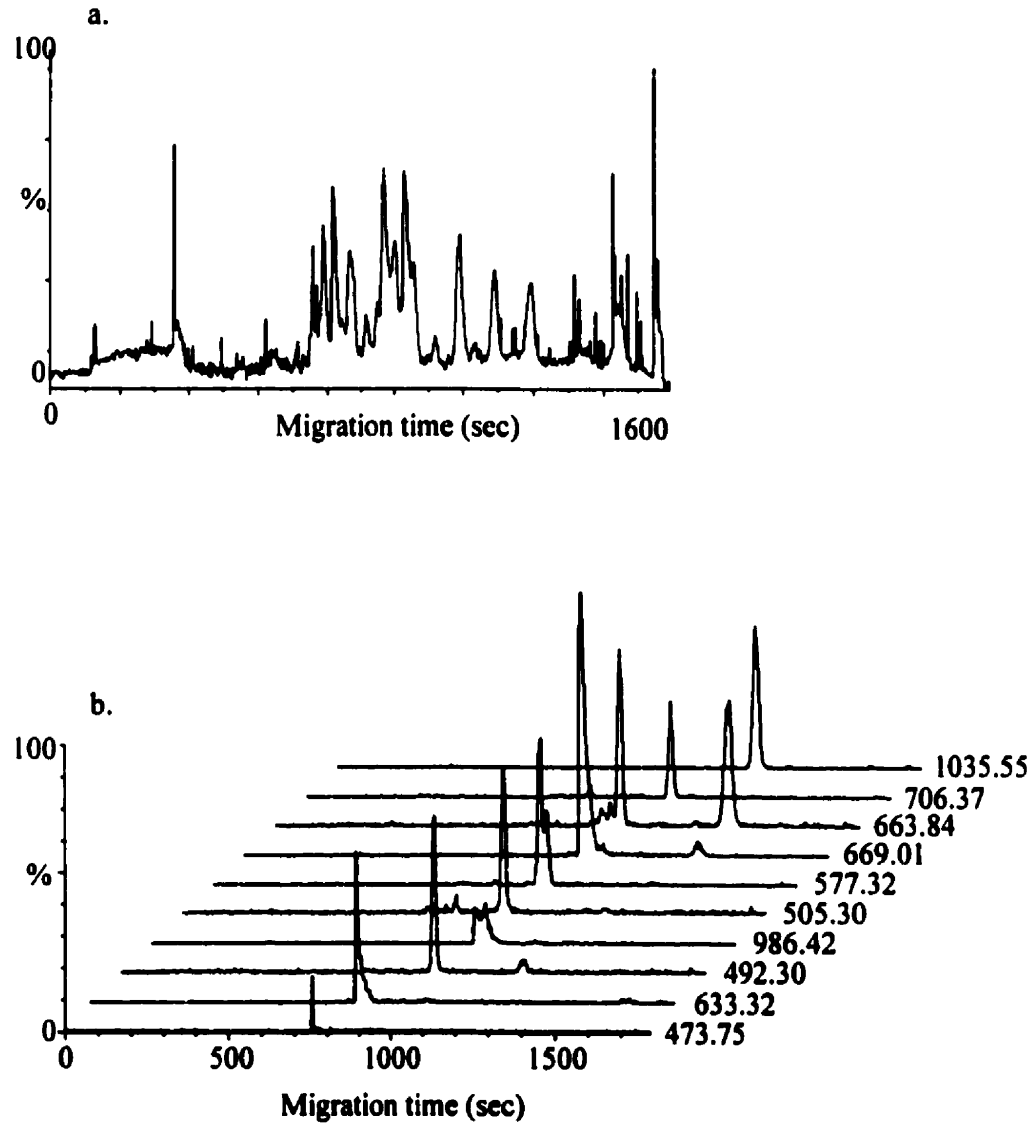

Figure 10-2. CE/ESI-TOFMS of tryptic peptides of citrate synthase. (a) TIE and (b) typical SIE of selected peptides.

Citrate synthase has a total of 44 possible tryptic cleavage sites. A large number of peptides may thus be obtained from a single digestion which in turn provides for a difficult separation. However it is possible to gain useful information on the peptide through selected reconstructed ion electrophorograms (SIE) as shown in Figure 10-1.b and Figure 10-2.b. The SIE traces thus obtained allow detection of single peptides among other components, thus facilitating their mass spectral identification. In total, 41 SIE were obtained with S/N varying from ca. 15 to 120 on raw data (not all shown) for the QQQ results. Additional peptides were identified from the digest, but due to poor signal intensity, they were not included here. The most intense signals were obtained for $[M+H]^+$ and $[M+2H]^{2+}$ ions of peptides. Over 70 peptides were identified by their SIE in the TOF results. S/N values were higher and ranged from ca.100 to 500.

Table 10-1 shows S/N and theoretical plate count values derived from three SIE traces shown in Figure 10-1 and Figure 10-2 (m/z 577.3, 633.4 and 1035.7). The mass spectra of the three peptides corresponding to these traces are shown in Figure 10-3 (b,d,f) for QQQ results and in Figure 10-3 (a,c,e) for TOF results. These values were typical of the peptides observed on the Quattro-LC and the TOF.

Table 10-1. Comparison of QQQ with TOF for CE-MS.

		QQQ		S/N	TOF		S/N
Tryptic Peptide	m/z	Mt (minutes)	N	(SIE)	Mt (minutes)	N	(SIE)
9	577.3	14.84	37655	53.1	16.71	25817	422
10	633.4	12.81	25182	54.91	13.60	83420	503
23	1035.7	17.01	33118	37.88	21.48	28979	266

For both separations the electrophoretic efficiency was relatively high, with theoretical plate counts (N) from ca. 8,000 (m/z 633.4) to ca. 88,000 (m/z 540.9). Typical N values of less than 50,000 were attributed to peptide adsorption on bare fused silica, and to the use of an acidic separation buffer (5% acetic acid). Moreover, the TRIS buffer used to perform the digestion has a higher conductivity than 5% acetic acid, and thus sample stacking (isotachophoresis) would not have occurred. In general, the electrophorograms were reproducible in terms of migration order of the tryptic peptides, and average N values on CE peaks. The results presented here are representative of the CE/ESI-MS and CE/ESI-TOFMS system used.

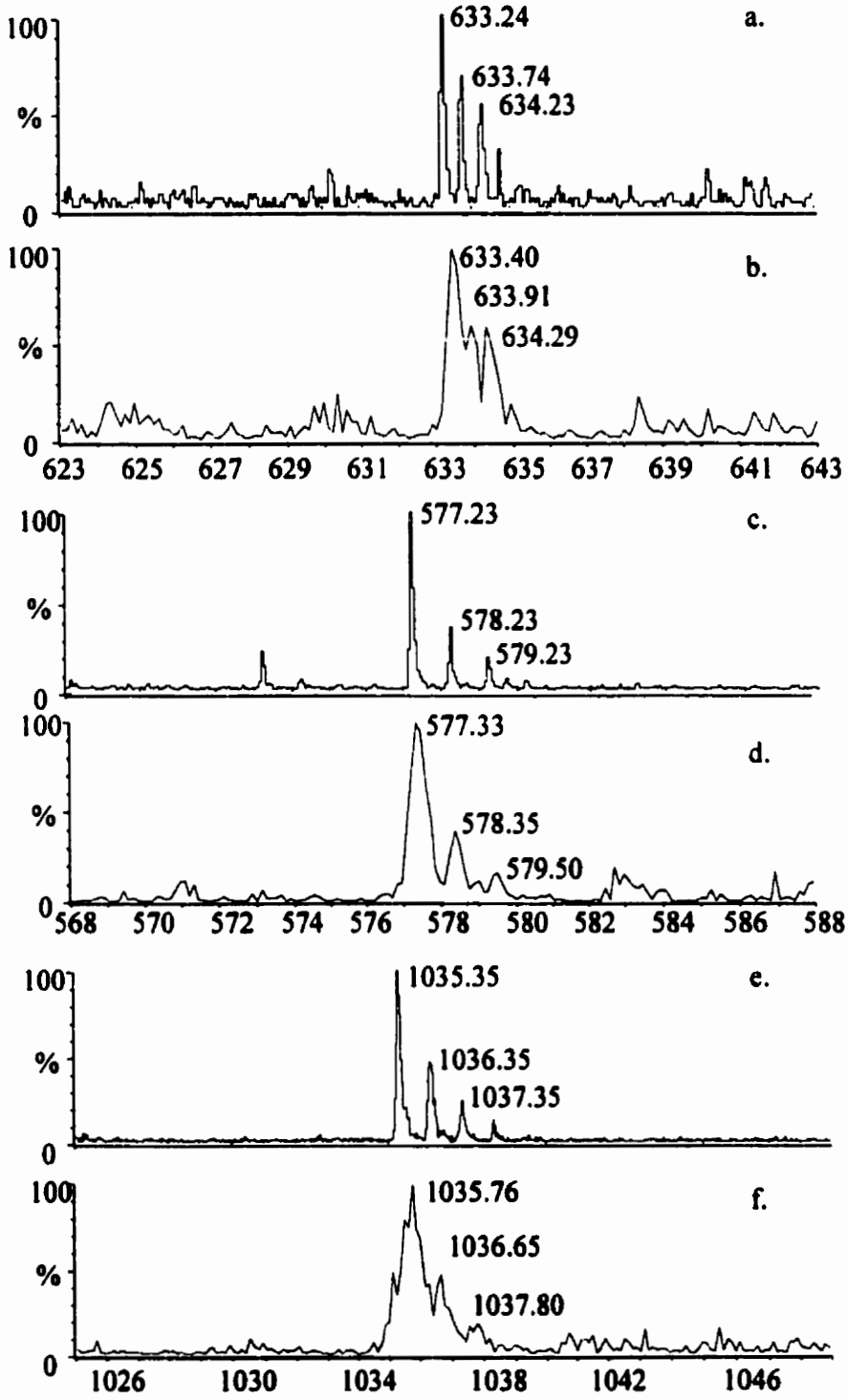

Figure 10-3. Comparison of mass spectra of citrate synthase tryptic peptides.
Instrument (a,c,e) time-of-flight (b,d,f) triple quadrupole.

Of note were the higher S/N values obtained using the TOF instrument compared with those obtained using the QQQ instrument (Table 10-1). In general, TOF was an order of magnitude more sensitive. There are two reasons for this. The first is that a curtain gas was used for the TOF instrument which resulted in the reduction of background from the electrolyte. More important was the higher scan rate of the TOF instrument. Under normal operating conditions the TOF scan rate (pulse rate) is 5 kHz. In comparison mass spectra were obtained at 500 Da per second for the QQQ measurements, which corresponds to a scan rate of ca. 0.5 Hz over the mass range observed. Thus, in theory, ca. 10^3 more ions are sampled by the TOF instrument. This is reflected in the S/N values shown.

Some comparisons between the mass spectra (raw data) obtained on the two mass spectrometers are presented in Figure 10-3. The mass resolution was superior with the TOF mass spectrometer compared with the Quattro-LC instrument, and high resolution facilitated the assignment of multiply-charged fragment ions. This performance was typical of the ESI-TOF instrument with mass resolution R > 6000, enabling baseline isotopic resolution. In comparison, the triple quadrupole analyzer was tuned for unit mass resolution. Although lower, the extent of resolution still allowed the identification of the peptides over the mass range investigated, albeit with more difficulty in the case of higher charge states. In general, sensitivity was higher on the TOF instrument than on the triple quadrupole instrument, and comparable CE efficiency was obtained on the two mass spectrometers.

A mass spectrum was obtained from each SIE, resulting in the identification of 44 tryptic fragments based on the QQQ results. Substantially more peptides were identified with the TOF results due to the higher sensitivity of the instrument and the higher resolution which facilitated peak identification. The peptides were tentatively identified and matched with literature values as shown in Table 10-2. It is noted that a few peptides were observed from their SIE, with S/N values less than 5, and were therefore not included in Table 10-2. In some cases, molecular ions corresponding to two charge states were observed for a single peptide, and these results were confirmed by similar SIE for each charge state. The charge states of molecular ions observed on the two instruments varied. This was due, presumably, to different ESI and declustering potentials and different interface designs.

Table 10-2. Tryptic fragments of citrate synthase by QQQ and TOF.

Sequence	Calculated $[M+nH]^{n+}$	QQQ $[M+nH]^{n+}$	n	% Error	TOF $[M+nH]^{n+}$	n	% Error
156-157	312.18				312.176	1	0.00
290-292	375.25				375.217	1	0.01
120-125	395.72				395.728	2	0.00
419-421	409.21				409.257	1	0.01
307-309	409.24				409.257	1	0.00
1-4	434.22				434.225	1	0.00
293-299	441.21				441.205	2	0.00
283-289	449.245				449.252	2	0.00
164-167	461.27				461.250	1	0.00
291-294	462.26				462.260	1	0.00
120-126	473.27	474.0	2	0.15	473.271	2	0.00
320-327	479.74				479.737	2	0.00
218-221	492.22	492.4	1	0.04			
156-163	499.28				499.087	1	0.04
418-425	504.28				504.128	2	0.03
419-426	505.08	504.9	2	0.04			
33-37	505.30	505.4	1	0.02	505.289	1	0.00
290-306	509.84	509.0	4	0.16			
307-314	527.27	527.3	2	0.01	527.297	2	0.01
291-299	540.77	540.9	2	0.02	540.772	2	0.00
328-332	547.31				547.310	1	0.00
418-421	565.31				565.272	1	0.01
168-177	573.24				573.281	2	0.01
105-109	577.33	577.3	1	0.01	577.307	1	0.00
315-319	577.31	577.3	1	0.00	577.307	1	0.00
410-418	579.30				579.294	2	0.00
22-32	593.82				593.805	2	0.00
405-409	612.39	613.9	1	0.25	612.389	1	0.00
422-426	618.36				618.355	1	0.00
1-6	633.36	633.4	2	0.01	633.319	2	0.01
295-299	638.29				638.297	1	0.00
388-404	657.96	658.3	3	0.05	657.945	3	0.00

Table 10-2. Tryptic fragments of citrate synthase by QQQ and TOF.

Sequence	Calculated $[M+nH]^{n+}$	QQQ $[M+nH]^{n+}$	n	% Error	TOF $[M+nH]^{n+}$	n	% Error
178-188	663.34				663.288	2	0.01
310-314	664.31	664.4	1	0.01	664.291	1	0.00
315-332	682.81	682.2	3	0.09			
158-163	706.39	706.4	1	0.00	706.361	1	0.00
120-125	790.44	791.5	1	0.13	790.432	1	0.00
33-35	790.21	791.4	3	0.15			
164-177	795.04	795.2	2	0.02			
405-417	799.43	800.2	2	0.09	799.790	2	0.05
300-319	804.96	803.6	1	0.17			
290-309	809.61	811.4	3	0.22			
300-306	817.99	817.6	1	0.05	817.270	1	0.09
357-370	821.45	822.2	2	0.09	821.427	2	0.00
293-306	840.95	841.0	2	0.01			
293-299	881.41				881.395	1	0.00
356-370	886.06	885.8	2	0.03			
38-69	886.47	886.5	4	0.00			
126-157	889.26	890.2	4	0.11			
283-289	897.49	897.6	1	0.01	897.488	1	0.00
5-21	901.00				900.968	2	0.00
371-387	916.14	916.5	2	0.04			
7-32	923.83				923.462	3	0.04
291-306	940.57	941.6	2	0.11	940.730	2	0.02
38-55	941.53	941.2	2	0.04			
120-126	946.54				946.532	1	0.00
320-327	958.47				958.414	1	0.01
274-306	983.90	982.0	4	0.19	984.300	4	0.04
388-404	986.44	987.4	2	0.10	986.410	2	0.00
5-32	990.80	990.7	3	0.01			
156-163	999.55				999.515	1	0.00
222-239	1003.03	1003.9	2	0.09	1002.990	2	0.00
418-425	1008.55				1008.440	1	0.01

Table 10-2. Tryptic fragments of citrate synthase by QQQ and TOF.

Sequence	Calculated $[M+nH]^{n+}$	QQQ $[M+nH]^{n+}$	n	% Error	TOF $[M+nH]^{n+}$	n	% Error
274-282	1035.54	1035.7	1	0.02	1035.492	1	0.00
240-273	1047.51	1047.5	3	0.00			
283-290	1053.60				1053.577	1	0.00
320-356	1073.99	1073.7	4	0.03			
291-299	1080.54				1080.546	1	0.00
310-327	1082.25				1081.850	2	0.04
328-356	1117.57				1117.506	3	0.01
357-387	1152.40	1152.9	3	0.04			
5-37	1152.99	1152.9	3	0.01			
410-418	1157.60				1157.590	1	0.00
70-109	1161.29	1161.3	4	0.00			
22-32	1186.64				1186.573	2	0.01
300-309	1207.64				1207.452	1	0.02
283-292	1252.73				1252.948	1	0.02
110-119	1265.64				1265.656	1	0.00
178-188	1326.68				1326.626	1	0.00
240-282	1385.69				1385.195	3	0.04
156-167	1440.84				1440.524	1	0.02
5-32	1484.81				1484.799	2	0.00
56-69	1598.85				1598.781	1	0.00
7-21	1601.86				1601.811	1	0.00
357-370	1641.89				1641.810	1	0.00
22-37	1672.92				1673.170	1	0.01
5-21	1801.00				1800.885	1	0.01
291.306	1879.94				1879.738	1	0.01
388-404	1971.88				1971.848	1	0.00
222-239	2005.05				2005.025	1	0.00
			Mean% error	0.064		Mean% error	0.009

More peptides were readily observed and identified in the TOF mass spectra. A slight difference may have resulted as the samples run by CE/ESI-MS and CE/ESI-TOFMS were digested separately and at different times, using enzymes from different batches. In some cases, the assignments were ambiguous and could not be confirmed due to peptides which had similar m/z values, i.e. m/z 577.33 and 577.31. It was not possible to differentiate these peptides based on mass alone. Further characterization would require digestion with a different enzyme or the use of sequencing methods, which we are currently investigating. The use of tandem MS on-line with CE is also under investigation.

The m/z values in Table 10-2 indicate improved mass accuracy for CE/ESI-TOFMS measurements compared to the CE/ESI-MS experiments. Under ideal conditions data produced using the TOF instrument had accuracies of 10-30 ppm. Here the results suffer somewhat due mainly to calibration drift of the TOF instrument which occurs over the duration of the run and centroid errors which become more pronounced with peaks of low S/N.

10.3.2 Peptide mass mapping and database search

Mapping of the proteolytic fragments is useful for positive identification of the protein by database searching. Figure 10-4 shows the tryptic peptide map of citrate synthase based on the QQQ results shown in Figure 10-2. A complete peptide map was obtained based solely on the CE/ESI-MS results obtained on the QQQ instrument with the exception of the tryptic fragment 178-188, which was observed only with the ESI-TOF mass spectrometer.

The m/z values of the most intense singly charged peaks were used to perform an on-line database search, in order to investigate the possibility of identifying the protein using the peptide information alone. The search parameters were as follows: Protein name: unknown; Mr range 40 kDa +/- 20%; Species: Escherichia coli; peptide masses: 492.4, 505.4, 577.3, 613.9, 664.4, 791.5, 795.2, 822.2, 897.6, 1035.7; Minimum number of peptides to match: 4; Enzyme: trypsin; Missed cleavage sites: 2; Cysteine: reduced form. The search was performed on the 08-Feb-1999, SWISS-PROT Release 37 and updates up to 05-Feb-1999: 78197 entries [3]. The search produced one protein match: Score: 0.5; # peptide matches: 6; Accession #: P00891; ID: CISY-ECOLI; Description: CITRATE SYNTHASE

(EC 4.1.3.7).The search was performed in less than one minute on-line and resulted in a correct match for the protein studied. We note that a combination of limited search parameters must be used in order to minimize the number of results from the search. Eliminating the species name and Mr range of the intact protein resulted in over 100 search results which would make the correct identification of the protein much more difficult.

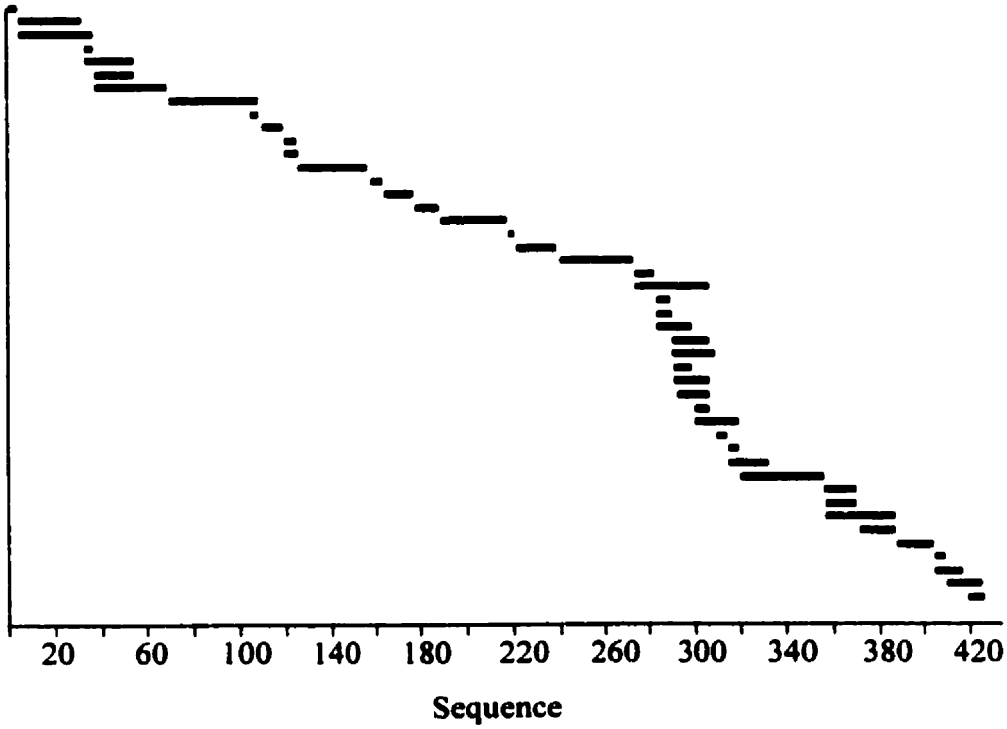

Figure 10-4. Tryptic peptide map of citrate synthase.
Peptides identified based on QQQ results.

10.4 CONCLUSIONS

On-line CE/ESI-MS and CE/ESI-TOFMS produced significant results for the separation and analysis of tryptic peptides of wild-type citrate synthase. It should be emphasized that CE/MS analyses were performed on non-purified protein samples, which traditionally require extensive buffer exchange via dialysis prior to further characterization. We have confirmed the usefulness of on-line CE-MS, as performed on two different mass spectrometers, in enabling the complete sequence elucidation of wild-type citrate synthase.

10.5 REFERENCES

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11 SUMMARY

11.1 PU SAMPLE SUPPORTS FOR MALDI-TOFMS

Non-porous PU membranes and porous PU thin films were used as sample supports for MALDI-TOFMS analysis of proteins and peptides. Mass accuracy and resolution were equivalent to values obtained with metal targets and superior to results obtained using porous PVDF membranes. The non-porous nature of the PU membranes facilitated crystal growth on the surface only and thus provided for enhanced spectral quality over porous membranes. Relatively strong interactions between the PU membranes/ PU films and bound proteins and peptides facilitated the removal of salts and buffer components through washing protocols. Signals obtained on PU membranes were slightly better than those obtained on porous films. This was attributed to an increase in surface area available for protein sorption on the membrane compared with the film and differences in surface morphology. Differences between the relative intensities of protein ion signals measured using steel targets and the PU supports were observed in some cases presumably due to selective protein binding to the PU surface during the sampling and washing protocols.

On-membrane digestion protocols were developed. Trypsin digestion of proteins were performed on the membrane surface and yielded characteristic fragments, allowing for successful peptide mapping. The analysis was sensitive because loss was minimized during digestion and during the washing steps as both processes were conducted on the membrane. Tandem MS of tryptic peptides was demonstrated and suggests the possibility of obtaining complete sequence information on a single sample, in a minimal amount of time.

The primary advantages of using the PU supports were the ease of sample manipulation and of obtaining and storing samples prior to analysis. Samples, once placed onto the membrane or film, were stable for several days to weeks, prior to analysis. PU membranes were particularly useful as one large piece of membrane could be used to collect several samples outside of the laboratory and then store them prior to analysis. Off-site sample collection was demonstrated for wheat proteins and hemoglobin variant analysis. Use of the

on-membrane washing and digestion protocols enhanced the MS characterization of these samples.

A novel application of PU membranes was explored by characterizing plasma proteins adsorption onto the membrane surface by MALDI-TOFMS. In this case the PU membrane acted as a model biomaterial. Several proteins were analyzed simultaneously with sufficient resolution to allow characterization based on literature molecular weights. Direct correlation between the proteins known to adsorb onto PU from plasma and those seen in the mass spectra were observed.

11.2 CE/ESI-MS, CE/ESI-MS/MS AND CE/ESI-TOFMS

A CE/ESI-MS interface was constructed for use with the Z-Spray^{TM} source of a Quattro-LC mass spectrometer configured for the MegaFlow-Z^{TM} electrospray mode. The probe design was inexpensive and easy to manufacture. The interface was designed to allow one to switch from normal ESI or HPLC/ESI-MS to CE/ESI-MS in a matter of minutes without removing the ESI source as is the case with the commercial sheathflow CE/ESI-MS probe.

Gold-coated CE capillary tips used for sheathless interfacing produced a stable electrospray under restricted CE voltage conditions which were still satisfactory for efficient separations. Good quality CE separations ($N>10^4$ theoretical plates) and MS detection (pmol to fmol-level sensitivity) of mixtures of standard peptides and proteolytic fragments of proteins were obtained. Electrophoretic separations were not compromised due to coupling with the mass spectrometer nor were the mass spectra affected by the CE coupling. On-line CE/ESI-MS/MS experiments were demonstrated on a peptide mixture and sequence information obtained using two MS/MS scan modes.

Tryptic digests of proteins were subjected to CE/ESI-MS and CE/ESI-TOFMS analysis under the same conditions as used for standard peptides. Although in some cases separation was limited by overlapping tryptic peptides, the use of SIE allowed identification of several tryptic peptides and complete peptide maps were obtained.

A direct comparison was made between on-line CE/ESI-MS using the Quattro-LC QQQ mass spectrometer and CE/ESI-TOFMS using Manitoba TOF-III for the analysis of

tryptic peptides of wild-type citrate synthase. Both instruments yielded results typical of their normal modes of operation in terms of resolution and mass accuracy. While the sensitivity and resolution of the TOF mass spectrometer were superior to that of the QQQ mass spectrometer the later did enable the generation of peptide maps and enabled data-base mass mapping of tryptic fragments of a medium M_r protein.

12 CONCLUSIONS

12.1 CONCLUSIONS

Mass spectrometry is a rapidly evolving field and may be likened to NMR research a few decades ago. To state the direction which MS may take in the future would only result in an oversimplification of new possible technologies and applications. Mass spectrometry in its present form complements almost every branch of science in some aspect. The general direction taken by MS will follow the same pattern as other new technologies have followed in the past. New instrument designs have made instrumentation user friendly to the point of allowing open area analysis to take place. As the cost of MS instrumentation continues to decline more laboratories are now incorporating MS as a routine means of analysis. New applications in all areas of MS are being investigated and range from traditional small molecule characterization and peptide sequencing to mega dalton DNA characterization, proteomics, high resolution mass measurements, post-translational modification, zeptomol sample introduction and others.

This research has been accepted well by the scientific community and has been complimented and referenced a number of times by other groups in similar areas. The work presented here does not represent the full extent of the author's research over the last four years and in no way is it complete. The research presented in this thesis, was new at the time of presentation at conferences or in publication [Appendix 3] but is in fact, now, somewhat dated. Research in the area of using membranes for MALDI sample supports is now explored by several groups with new substrates and applications continually being presented. The same holds true for CE-MS fundamentals and applications. The field has become very aggressive from a research point of view. Any new idea or application worth investigation is duplicated and improved upon within the year after presentation, and in some cases, earlier.

12.1.1 MALDI-TOFMS on PU Supports

This research demonstrates the first use of non-porous PU membranes and porous PU thin films for MALDI-TOFMS. Prior to this work the majority of sample supports used

for MALDI sample preparation were porous which necessitated appropriate experimental conditions to compensate for their porosity. Film support studies tended to revolve around the use of nitrocellulose, which, because of its chemical nature resulted in adduct formation and a loss in resolution of mass spectra. Systematic characterization of the non-porous PU membranes demonstrated their unique features in protein and peptide binding and improvements in the quality of mass spectra obtained on them compared with standard methodology. Results obtained using the PU membranes led to the investigation of the physical and chemical properties of porous PU thin films and, in general, a more complete understanding of the phenomena involved with using membranes and films as samples supports.

Applications of the new methodology developed early in this research were applied to real samples and not limited to simply investigating off-the-shelf standards. An improved sample preparation method was developed for the MS characterization of wheat proteins. This new application resulted in an improvement in the ease of obtaining mass spectra on this class of compounds. The application itself is novel in the fact that it lies outside the normal realm of MS research which is primarily focussed on biological molecules and pharmaceuticals. The sample preparation protocols developed for these samples may be applied to a larger number of samples containing components which are not suitable for traditional MALDI-TOFMS characterization.

A new method for the sample collection, manipulation and characterization of hemoglobin variants was investigated. The approach investigated here is non-invasive, more cost effective, results in easier sample manipulation and with the possibility of automation may provide for a faster analysis of the variant compared with other methods. These factors are significant as the implementation of new-born blood screening programs will result in a tremendous amount of samples for characterization. Additionally, underdeveloped countries may lack adequate facilities for the characterization of a new or non-standard variant and thus would require off-site sample collection followed by characterization at a central laboratory. The first detailed MS characterization of the Sheperds Bush variant hemoglobin was presented which confirmed earlier results in the literature derived via more traditional measurements.

This work also demonstrated the first example of the characterization of PU membrane for use in biomaterials evaluation. This was demonstrated in-vitro using plasma standards and in-vivo for canine plasma. Compared with traditional methods, MS offers the opportunity for the rapid and accurate characterization of protein sorption onto the surface of a biomaterial. This would allow suitable biomaterials to be critically evaluated in-vitro for biocompatibility prior to use within a host. This approach is inexpensive, easy to implement and rapid enough to enable the testing of a multitude of several biomaterial candidates at once.

12.1.2 CE/ESI-MS

A new CE/ESI-MS interface was constructed for use with the Z-Spray^{TM} source of a Quattro-LC mass spectrometer configured for the MegaFlow-Z^{TM} electrospray mode. The probe and electrical set-up were designed to be inexpensive and simple to use. Experimental details are given in such detail that they may be easily duplicated by other researchers who have similar instrumentation and wish to explore the option of using a CE/ESI interface. This work thus may easily be duplicated and provides a suitable base for new laboratories which consider using CE with MS.

Initial results of this research on CE/ESI-TOFMS represented the 5th published report on TOF detection coupled with CE, results of which were equivalent to or superior to others at the time. As a direct consequence of this research, improved MS software capable of handling the large amount of data obtained during a CE/ESI-TOFMS experiment was developed. The initial results, using the improved software, the MS performance characteristics of the TOF instrument and the high resolution separations possible with CE demonstrate the exceptional capabilities of this method of sample characterization by MS.

This work demonstrated the first direct comparison of QQQ with TOF for MS detection of tryptic peptides under controlled and near identical experimental conditions. The TOF results were superior from a brute force point of view. In comparison the QQQ results showed that very good data were obtainable on a commercial QQQ mass spectrometer.

12.1.3 Prospective

The use of PU membranes and films in addition to other membrane materials is still under investigation in this laboratory. The methodology developed is simple and involves robust technology and is now used on a routine basis for protein and peptide characterization by MALDI-TOFMS. The protocols developed are currently being used for the characterization of wheat proteins and new applications are being sought. Application to hemoglobin variant analysis is on-going and improvements in sampling and digestion protocols are being investigated. The use of PU as a model biomaterial is still being explored and experiments are in progress to characterize different plasma proteins sorbed onto the surface of PU both qualitatively and semi-quantitatively. Methodology for using of the QqTOF instrument for peptide sequencing is being developed.

CE-MS experiments using TOF and QQQ detection continue. The data logging software developed for TOFMS has been revised and new features added to improve analysis speed and data and file manipulation. CE-MS and CE-TOFMS have been applied to other proteins and peptides in order to characterize them by MS. A new interface is in the process of being developed for use with a newly acquired commercial CE instrument.

Several avenues of the PU membrane and CE-MS research have been adopted by other members of the laboratory, based on the methods developed here, and are being applied to the characterization of an increasing number of real, significant, samples.

13 APPENDIX I

13.1 PROPERTIES OF AMINO ACIDS

Table 13-1. Molecular Weights and Composition of Common Amino Acids.

Name	Symbol	Average Mass	Monoisotopic Mass	Side Chain
Alanine	A, Ala	71.079	71.0788	CH3-
Arginine	R, Arg	156.188	156.1876	HN=C(NH2)-NH-(CH2)3-
Asparagine	N, Asn	114.104	114.1039	H2N-CO-CH2-
Aspartic acid	D, Asp	115.089	115.0886	HOOC-CH2-
Cysteine	C, Cys	103.145	103.1448	HS-CH2-
Glutamine	Q, Gln	128.131	128.1308	H2N-CO-(CH2)2-
Glutamic acid	E, Glu	129.116	129.1155	HOOC-(CH2)2-
Glycine	G, Gly	57.052	57.0520	H-
Histidine	H, His	137.141	137.1412	N=CH-NH-CH=C-CH2-
Isoleucine	I, Ile	113.160	113.1595	CH3-CH2-CH(CH3)-
Leucine	L, Leu	113.160	113.1595	(CH3)2-CH-CH2-
Lysine	K, Lys	128.17	128.1742	H2N-(CH2)4-
Methionine	M, Met	131.199	131.1986	CH3-S-(CH2)2-
Phenylalanine	F, Phe	147.177	147.1766	Phenyl-CH2-
Proline	P, Pro	97.117	97.1167	-N-(CH2)3-CH-
Serine	S, Ser	87.078	87.0782	HO-CH2-
Threonine	T, Thr	101.105	101.1051	CH3-CH(OH)-
Tryptophan	W, Trp	186.213	186.2133	Phenyl-NH-CH=C-CH2-
Tyrosine	Y, Tyr	163.176	163.1760	4-OH-Phenyl-CH2-
Valine	V, Val	99.133	99.1326	CH3-CH(CH2)-

14 APPENDIX II

14.1 PROTEIN SEQUENCES

Sequences adopted from the Swiss-Protein databank.

14.1.1 E. Coli Citrate Synthase

SEQUENCE 427 AA; 48087 MW; 7AC57D4A CRC32;

MADTKAKLTL NGDTAVELDV LKGTLGQDVI DIRTLGSKGV

FTFDPGFTST ASCESKITFI DGDEGILLHR GFPIDQLATD

SNYLEVCYIL LNGEKPTQEQ YDEFKTTVTR HTMIHEQITR

LFHAFRRDSH PMAVMCGITG ALAAFYHDSL DVNNPRHREI

AAFRLLSKMP IMAAMCYKYS IGQPFVYPRN DLSYAGNFLN

MMFSTPCEPY EVNPILERAM DRILILHADH EQNASTSTVR

TAGSSGANPF ACIAAGIASL WGPAHGGANE AALKMLEEIS

SVKHIPEFFR RAKDKNDSFR LMGFGHRVYK NYDPRATVMR

ETCHEVLKEL GTKDDLLEVA MELENIALND PYFIEKKLYP

NVDFYSGIIL KAMGIPSSMF TVIFAMARTV GWIAHWSEMH

SDGMKIARPR QLYIGYEKRD FKSDIKR

14.1.2 Human Hemoglobin Alpha Chain

SEQUENCE 141 AA; 15126 MW; 5EC7DB1E CRC32;

VLSPADKTNV KAAWGKVGAH AGEYGAEALE RMFLSFPTTK

TYFPHFDLSH GSAQVKGHGK KVADALTNAV AHVDDMPNAL

SALSDLHAHK LRVDPVNFKL LSHCLLVTLA AHLPAEFTPA

VHASLDKFLA SVSTVLTSKY R

14.1.3 Human Hemoglobin Beta Chain

SEQUENCE 146 AA; 15867 MW; EC9744C9 CRC32;

VHLTPEEKSA VTALWGKVNV DEVGGEALGR LLVVYPWTQR

FFESFGDLST PDAVMGNPKV KAHGKKVLGA FSDGLAHLDN

LKGTFATLSE LHCDKLHVDP ENFRLLGNVL VCVLAHHFGK

EFTPPVQAAY QKVVAGVANA LAHKYH

14.1.4 Equine Myoglobin

SEQUENCE 153 AA; 16950 MW; 7ADA30F4 CRC32;

GLSDGEWQQV LNVWGKVEAD IAGHGQEVLI RLFTGHPETL

EKFDKFKHLK TEAEMKASED LKKHGTVVLT ALGGILKKKG

HHEAELKPLA QSHATKHKIP IKYLEFISDA IIHVLHSKHP

GNFGADAQGA MTKALELFRN DIAAKYKELG FQG

15 APPENDIX III

15.1 PUBLICATIONS/ PRESENTATIONS RELATED TO THESIS WORK

15.1.1 Publications:

1. Oleschuk, R.D., McComb, M.E., Marois, Y., King, M.W., Chow, A., Ens, W., Standing, K.G., and Perreault, H., 1999, Characterization of Protein Adsorption onto Biomaterials by MALDI-TOFMS, *Biomaterials*, in submission.
2. McComb, M.E., Dworschak, R.G., Oleschuk, R.D., Chow, A., Preston, K., Ens, W., Standing, K.G., and Perreault, H., 1999, Application of Polyurethane Thin Films and Membranes for MALDI-MS of Wheat Proteins, *J. Am. Soc. Mass Spectrom.* in submission.
3. McComb, M.E., and Perreault, H., 1999, Design of a Capillary Electrophoresis-Mass Spectrometry Probe for Operation with a Z-Spray^{TM} Ionization Source, *Electrophoresis*, in press.
4. McComb, M.E., and Perreault, H., 1999, Electrospray Ionization Mass Spectrometry and on-line Capillary Electrophoresis Mass Spectrometry for the Characterization of Citrate Synthase, *Can. J. Chem.*, 77, 1752-1760.
5. McComb, M.E., Oleschuk, R.D., Chow, A., Ens, W., Standing, K.G., Smith, M., and Perreault, H., 1998, Matrix Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry Analysis of Hemoglobin Variants using Non-porous Polyurethane Membrane as a Sample Support, *Anal. Chem.*, 70: 5142-5149.
6. McComb, M.E., Krutchinsky, A., Ens, W., Standing, K.G., and Perreault, H., 1998, Sensitive High Resolution Analysis of Biological Molecules with Capillary Zone Electrophoresis Time-of-Flight Mass Spectrometry, *J. Chromatogr. A.*, 800: 1-11.
7. McComb, M.E., Oleschuk, R.D., Manley, D.M., Donald, L., Chow, A., O'Neil, J.D.J., Ens, W., Standing, K.G., and Perreault, H., 1998, Use of a Non-porous Polyurethane Membrane as a Sample Support for Matrix Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry of Peptides and Proteins, *Rapid. Commun. Mass Spectrom.*, 11: 1716-1722.

15.1.2 Conference Proceedings, Non-Refereed:

8. McComb, Mark E.; Krutchinsky, Andrew; Ens, Werner; Standing, Kenneth G.; Perreault, H., 1999, CE/ESI-TOFMS and CE/ESI-MS/MS Analysis of Protein Digests, Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, Dallas, TX, June 13-18. # 1090
9. C.H. Lee, M.E. McComb, W. Ens, K.G. Standing, H. Perreault, 1999, Method Development for the Characterization of Phosphorylated Proteins by Mass Spectrometry, Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, Dallas, TX, June 13-18. # 1413

10. M. E. McComb, R. D. Oleschuk, Y. Marois, A. Chow, M. King, W. Ens, K. G. Standing, H. Perreault, 1999, Characterization of Plasma Protein Sorption onto Biomaterials (PART-2) by MALDI-TOFMS, Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, Dallas, TX, June 13-18. # 1079.

11. M.E. McComb, R.D. Oleschuk, M.P. Bromirski, A. Chow, W. Ens, K.G. Standing, M. Smith, H. Perreault, 1999, Hemoglobin Variant Analysis by MALDI-TOFMS (PART-2) Using Polyurethane Membrane Sample Supports, Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, Dallas, TX, June 13-18. # 1087.

12. W. Ens, A. Krutchinsky, A. Loboda, C. Lock, M.E. McComb, K.G. Standing, A Method to Record LC/MS and CE/MS Data by Data-Logging, Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, Dallas, TX, June 13-18. # 123.

13. McComb, M.E., Krutchinsky, A., Ens, W., Standing, K.G., and Perreault, H., 1998, Biomolecule Characterization by CE-ESI/TOFMS and CE-ESI/MS/MS, Proceedings of the 46th ASMS Conference Mass Spectrometry and Allied Topics, Orlando, FL, May 31-June 4. # 1269.

14. McComb, M.E., Oleschuk, R.D., Marois, Y., King, M.W., Chow, A., Ens, W., Standing, K.G., and Perreault, H., 1998, Characterization of Protein Adsorption onto Biomaterials by MALDI-TOFMS, Proceedings of the 46th ASMS Conference Mass Spectrometry and Allied Topics, Orlando, FL, May 31-June 4. # 175.

15. McComb, M.E., Dworschak, R.G., Oleschuk, R.D., Chow, A., Preston, K., Ens, W., Standing, K.G., and Perreault, H., 1998, Application of Polyurethane Thin Films and Membranes for MALDI-MS of Proteins and Glycoproteins, Proceedings of the 46th ASMS Conference Mass Spectrometry and Allied Topics, Orlando, FL, May 31-June 4. # 174.

16. McComb, M.E., Oleschuk, R.D., Manley, D.M., Chow, A., Ens, W., Standing, K.G., and Perreault, H., 1997, Polyurethane Membrane as a Sample Support for MALDI-MS of Biological Molecules, Proc. 45th ASMS Conf. Mass Spectrom. Allied Topics, Palm Springs, CA., June 1-5, #309.

17. McComb, M.E., Krutchinsky, A., Ens, W., Standing, K.G., and Perreault, H., 1997, Capillary Electrophoresis Time-of-Flight Mass Spectrometry for the Analysis of Biological Molecules, Proc. 45th ASMS Conf. Mass Spectrom. Allied Topics, Palm Springs, CA., June 1-5, #500.

18. McComb, M.E., and Perreault, H., 1996, Analysis of Glycoprotein Components Using CE/ UV-Vis and Off-line CE/ MS, Proc. 44th ASMS Conf. Mass Spectrom. Allied Topics, Portland, OR., May 12-16, #1070.

15.1.3 Conference Presentations, Non-Refereed:

19. M.E. McComb and H. Perreault, 1999, Applications of PU membrane as a sample support for MALDI-MS of peptides and proteins, 82cnd Canadian Society for Chemistry Conference and Exhibition, Toronto, ON.

20. M.E. McComb and H. Perreault, 1999, Application of CE/ESI-MS to the characterization of Citrate Synthase, 82cnd Canadian Society for Chemistry Conference and Exhibition, Toronto, ON.

21. C.H. Lee, M.E. McComb, H. Perreault, 1999, Optimization of Conditions for On-membrane Digestion of Phosphorylated Proteins for their Characterization by MALDI-TOFMS. 82nd Canadian Society for Chemistry Conference and Exhibition, Toronto, ON, May 30-June 2, #627.

22. McComb, M.E., Oleschuk, R.D., Chow, A., Ens, W., Standing, K.G, and Perreault, H., 1998, Application of PU membrane for MALDI-TOFMS Characterization of Hemoglobin Mutants, 216th ACS National Meeting, Boston, MA., August 23-27, ANYL-100.

23. Manley, D.M., Witzke, K., McComb, M.E., Oleschuk, R.D., Donald, L., Duckworth, H., Chow, A., Perreault, H., and O'Neil, J.D.J., 1998, Over-expression, Purification, and Characterization of Escherichia Coli Integral Membrane Protein Glycerol Facilitator, 41st Canadian Federation of Biological Societies, Edmonton, AB., June 18-20.

24. Oleschuk, R.D., McComb, M.E., Ens, W., Standing, K.G., Perreault, H., and Chow, A., 1998, Direct Determination of Proteins Adsorbed onto Polyurethane Biomaterials by Mass Spectrometry, 81st Canadian Society for Chemistry Conference and Exhibition, Whistler, BC., May 31-June 4, #392.

25. R.D. Oleschuk, McComb, M.E., Manley, D.M., Donald, L., Chow, A., O'Neil, J.D.J., Ens, W., Standing, K.G., and Perreault, H., 1997, Use of a Non-porous Polyurethane membrane as a Sample Support for MALDI Mass Spectrometry of Proteins, 80th CSC Conference and Exhibition, Windsor, ON., May 28-June 2, #225.

26. Standing, K.G., McComb, M.E., Krutchinsky, A., Chenushevich, I.V., and Ens, W., 1997, Characterization of Protein Components by Off- and On-line Capillary Electrophoresis/ Time-of-Flight Mass Spectrometry, The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlanta, GA., March 16-21, #1041.

27. McComb, M.E., Perreault, H., 1996, Analysis of Glycoprotein Components Using CE/ UV-Vis and Off-line CE/ MS, 79th CSC Conference and Exhibition, St. Johns, ND., June 23-26, #501.

15.1.4 Patents:

28. McComb, M.E., Oleschuk, R.D., Manley, D.M., Donald, L., O'Neil, J.D.J., Chow, A., Ens, W., Standing, K.G., Perreault, H., 1998, Use of Polyurethane Membrane for MALDI-TOFMS Analysis of Whole Blood, Canadian Patent Application, January 30, # 2,228,413. US Provisional Patent, February 2, #073,364.

15.1.5 Invited Speaker:

29. McComb, M.E., Oleschuk, R.D., Chow, A., Ens, W., Standing, K.G., Perreault, H, 1998, Matrix Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry Analysis of Hemoglobin Variants using Non-porous Polyurethane Membrane as a Sample Support. Mid-Canada AOAC Conference and Exhibition, Winnipeg, MB., June 18.

30. Oleschuk, R.D., McComb, M.E., Manley, D.M., Donald, L., Chow, A., O'Neil, J.D.J., Ens, W., Standing, K.G., and Perreault, H., 1997, Polyurethane Membrane as a Sample Support for MALDI-MS of Biological Molecules, Mid-Canada AOAC Conference and Exhibition, Winnipeg, MB., June 16.

31. McComb, M.E., Krutchinsky, A., Ens, W., Standing, K.G., and Perreault, H., 1997, Capillary Electrophoresis Time-of-Flight Mass Spectrometry for the Analysis of Biological Molecules. Mid-Canada AOAC Conference and Exhibition, Winnipeg, MB., June 16.

16 APPENDIX IV: OTHER PUBLICATIONS

16.1 RESEARCH PERFORMED AT THE UNIVERSITY OF MANITOBA

16.1.1 Publications

32. Shojania, S., McComb, M.E., Oleschuk, R.D., Chow, A., Gesser, H.D., Fingerprint Analysis of Volatile Organic Compounds Using the INCAT Device, *Can. J. Chem.*, 1999, 77, 1716-1727.

33. McComb, M.E., Gesser, H.D., Analysis of Trace Metals in Water by In-Situ Sample Pre-concentration Combined with WDXRF and ICP-OES, *Talanta*, 1999, 49, 869-879.

34. Shojania, S., McComb, M.E., Oleschuk, R.D., Chow, A., Gesser, H.D., The Active and Passive Sampling of BTEX Compounds Using the INCAT Device, *Talanta*, 1999, 50, 193-205.

35. McComb, M.E., Oleschuk, R.D., Giller, E., Gesser, H.D., Microextraction of Volatile Organic Compounds Using the Inside Needle Capillary Adsorption Trap (INCAT) Device, *Talanta*, 1997, 44, 2137-2143.

36. McComb, M.E., Gesser, H.D., Preparation of Poly(acrylamidoxime) Chelating Cloth for the Extraction of Heavy Metals from Water, *J. Appl. Polym. Sci.*, 1997, 65, 1175-1190.

37. McComb, M.E., Gesser, H.D., Passive Monitoring of Trace Metals in Water by In-situ Sample Preconcentration Via Chelation on a Textile Based Solid Sorbent, *Anal. Chem. Acta.*, 1997, 341, 229-233.

16.1.2 Conference Presentations, Non-Refereed:

38. S. Shojania, M.E. McComb, H.D. Gesser, H. Perreault, A. Chow, 1999, Application of the INCAT Device to the Analysis of Complex Mixtures of VOC's, 82nd Canadian Society for Chemistry Conference and Exhibition, Toronto, ON, May 30-June 2, #700.

39. Shojania, S., McComb, M.E., Oleschuk, R.D., Chow, A., and Gesser, H.D., 1998, Analysis of BTEX Compounds Using the INCAT Device, 216th ACS National Meeting, Boston, MA., August 23-27, ANYL-100.

40. Shojania, S., McComb, M.E., Oleschuk, R.D., Chow, A., and Gesser, H.D., 1998, The Active and Passive Analysis of BTEX Compounds Using the Inside Needle Capillary Adsorption Trap (INCAT) Device, 81st CSC Conference and Exhibition, Whistler, BC., May 31-June 4, #391.

41. Shojania, S., McComb, M.E., Oleschuk, R.D., Chow, A., and Gesser, H.D., 1997, Analysis of Wine Using the Inside Needle Capillary Adsorption Trap (INCAT) Device, 11th CSC Western Canada Undergraduate Chemistry Conference, Winnipeg, MB, May 1-3.

42. McComb, M.E., and Gesser, H.D., 1996, Passive Monitoring of Trace Metals in Water, 79th CSC Conference and Exhibition, St. Johns, ND., June 23-June 26, #361.

43. McComb, M.E., Oleschuk, R.D., Giller, E., and Gesser, H.D., 1996, Microextraction of Volatile Organic Compounds Using the Inside Needle Capillary Adsorption Trap (INCAT) Device, 79th CSC Conference and Exhibition, St. Johns, ND., June 23-June 26, #379.

44. Gesser, H.D., and McComb, M.E., 1995, The Development of a Passive Monitor for Trace Metals in Water, International Chemical Congress of Pacific Basin Societies, Koloa, Kauai, Hawaii.

45. McComb, M.E., and Gesser, H.D., 1995, A Passive Monitor for Trace Metals in Water, 78th CSC Conference and Exhibition, Guelph, ON., May 28-June 1, #1037.

46. Giller, E., McComb, M.E., and Gesser, H.D., 1994, Passive Monitors for Organic Pollutants in Air and Water and for Traces of Heavy Metals in Water, 77th CSC Conference and Exhibition, Winnipeg, MB., May 29-June 2, #084.

16.1.3 Thesis:

47. McComb, M.E., 1995, A Passive Monitor for Trace Metals in Water, University of Manitoba, M.Sc. Thesis.