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LIQUID CHROMATOGRAPHY CONTINUOUS-FLOW MATRIX-ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY FOR THE DETECTION OF BIOACTIVE PEPTI

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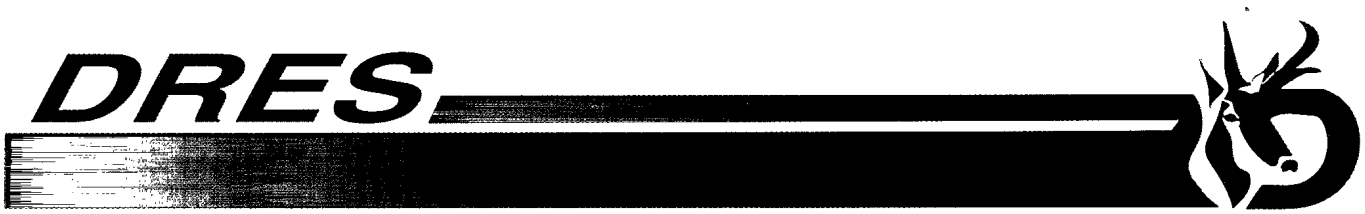

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Liquid Chromatography Continuous-Flow Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry for the Detection of Bioactive Peptides and Proteins

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Final Report

Title: Liquid Chromatography Continuous-Flow Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry for the Detection of Bioactive Peptides and Proteins

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Abstract

The general approach of combining liquid chromatography (LC) with matrix-assisted laser desorption ionization (MALDI) mass spectrometry for on-line detection of peptides and proteins based on the use of a continuous-flow (CF) probe is presented. Recent advances in time-of-flight mass spectrometric instrumentation for CF-MALDI are reported. Using 3-nitrobenzyl alcohol (3NBA) as the liquid matrix, stable flow can be readily achieved and the 3-port interface does not introduce chromatographic degradation for LC-MS. Separation and detection of low picomoles of peptides and proteins can be done with CF-MALDI LC-MS. Parallel ion extraction and time-lag focusing (TLF) are shown to provide enhanced performance with regard to mass resolution. However, mass resolution is generally poor for proteins with masses above ~6000 u using 3NBA as the matrix. Strong adduct ion formation with the use of 3-NBA as the liquid matrix is believed to be the main cause of this resolution reduction. It is argued that CF-MALDI is technically viable approach for LC-MS; but the overall performance and wide use of this method depend on the discovery of new liquid matrices that are suitable for continuous flow and provide much enhanced utility for MALDI over 3NBA, particularly for proteins. Finally, the results of time-lag focusing MALDI analysis of 15 peptides and proteins using solid matrices are presented. It is shown that the mass measurement error of TLF MALDI is generally less than 100 ppm. However, it is found that TLF MALDI does not offer any improvement in mass accuracy for bovine albumin (MW 66399.9) or chicken albumin (MW 67157.1). It is believed that TLF MALDI is best suited for the analysis of peptides and proteins with masses up to 30,000 u.

Introduction

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry is an important technique for chemical analysis [1]. MALDI has been widely used for the detection of biochemicals as well as industrial or synthetic polymers [2]. However, in contrast to electrospray ionization (ESI), the MALDI technique cannot be readily combined with solution based separation methods, such as HPLC, for on-line detection. An effective and reliable on-line LC-MALDI system is expected to complement LC-ESI in many analytical applications. For example, LC-MALDI can potentially become a two-dimensional separation technique analogous to 2D gel electrophoresis, where the LC separation can be based on mechanisms other than molecular weight. Individual LC peaks consisting of a mixture of several similar compounds can be ionized and separated by MALDI in a time-of-flight (TOF) mass analyzer.

Within the scope of MALDI, an on-line LC-MALDI system can provide several potential benefits for the complete analysis of complex samples. Direct analysis of mixtures by MALDI can suffer from signal suppression of one or more components. This ion suppression effect can be reduced or eliminated with the use of LC-MALDI where the individual components are either completely separated or partially separated into groups, each having similar MALDI properties. With an on-line detection system, sample throughput and MALDI analysis speed can be significantly increased. On-line LC-MALDI also improves sample handling efficiency and minimizes sample loss.

Despite the potential benefits that may be brought about by on-line LC-MALDI, the progress in developing such a system has been slow, mainly due to the technical and perhaps fundamental difficulties associated with interfacing LC to MALDI. One approach developed by Murray and Russell was to introduce the samples along with matrix solutions to a time-of-flight mass spectrometer as an aerosol beam [3,4]. MALDI is performed from the aerosol particle surfaces. Samples can be continuously introduced into the mass spectrometer at 1 mL/min and the technique has been used for LC detection [5]. The detection sensitivity was demonstrated to

be about 100 nmol with a mass resolution of 5-30 fwhm (full width at half maximum) on a linear TOFMS. Murray et al. [6] have recently improved the performance of this aerosol MALDI technique by incorporating a reflectron TOFMS. A mass resolution of 300–400 fwhm was shown for peptides.

We have been involved in developing a continuous-flow matrix-assisted laser desorption ionization (CF-MALDI) technique, for introducing solutions directly into a conventional MALDI MS system [7]. This technique uses a flow probe to continuously flow the sample and a liquid matrix, 3-nitrobenzyl alcohol (3-NBA), for MALDI analysis. In earlier work [7], a frit-type flow probe was used to deliver the sample and matrix at a flow rate in the range of 1 to 5 μL/min through a capillary tube and onto the probe surface, upon which laser desorption/ionization was carried out. 3-NBA liquid matrix was found to be important in achieving reproducible signals under the flow conditions used [7]. This method was further developed to combine it with microbore LC [8]. It was demonstrated that on-line LC-MALDI can be performed with a total sample injection in the low picomole region for protein analysis.

In this report, we first briefly review the general approach of LC-MS based on CF-MALDI. Some recent instrumental developments for CF-MALDI are then described along with the illustration of the improved performance brought about by these modifications. Experimental results relevant to the search for optimal liquid matrices are presented. The current limitations of this CF-MALDI approach are discussed. Finally, the accurate mass measurement of peptides and proteins by using time-lag focusing MALDI with solid matrices is presented.

CF-MALDI LC-MS

All initial studies on CF-MALDI were done using a linear TOF mass spectrometer where the sample probe was inserted orthogonal to the ion flight path [7-9]. Details of the design of the CF probe and the TOFMS instrument have been reported [7-9]. An on-line post-column matrix addition method was also developed to interface LC to CF-MALDI for protein detection [8]. Figure 1 shows the schematic diagram of the LC-MALDI interface, including the 3-port mixing

tee. The LC system consists of a Shimadzu LC-600 dual pump and a home-built solvent splitter. The latter is used to obtain a flow-rate compatible for the micro-column LC separation. The solvent splitter consists of a tee connected to a parallel combination of a coarse and fine metering valve, allowing a controllable flow-rate of 1-10 μL/min for the LC separation. The micro-column (LC Packings, Fusica C18, 5 cm × 320 μm I.D.) is connected directly to a Valco sample injector (60 nL injection volume) with finger tight fittings. For MALDI detection, the end of the capillary column is connected to the 3-port mixing tee through a short transfer tube. The second port of the tee is connected to a syringe pump (Harvard Apparatus, Model 11, Southnatick, MA, USA) that continuously feeds in the 3-NBA matrix solution (3-5 μL/min). This solution consists of 15% of 0.1% trifluoroacetic acid (TFA), 45% ethylene glycol, 25% 1-propanol, and 15% 3-NBA (all in volume). The resulting mixture flows out of the third port to the flow probe. A silica capillary extends from the mixing tee to the flow probe tip. The flow probe is inserted between the repeller and extraction plates of a linear TOFMS, where a 266-nm UV laser beam from a Nd:YAG laser operating at 10 Hz generates the MALDI ions. The resulting mass spectra are recorded and transferred in real time to a PC via GPIB from a digital oscilloscope using a data system developed in house [9].

Figure 2 shows the LC-MS ion chromatogram of a mixture of cytochrome *c* (9 pmol) and lysozyme (8 pmol) on a micro-bore LC column (320 μm I.D.). The separation was carried out using isocratic conditions (60% solvent A containing 0.1% TFA and 40% solvent B consisting of acetonitrile-water (9:1) containing 0.1% TFA). Peak A in Figure 2 is from cytochrome *c* and peak B from lysozyme. The ion chromatogram was found to be similar to the UV chromatogram obtained with the same sample. Based on peak shapes and widths, it was determined that the interface shown in Figure 1 does not introduce a significant amount of peak broadening or distortion to the LC separation [8].

The above example demonstrates the possibility of doing on-line LC-MS detection based on CF-MALDI. However, the mass resolution obtained in this experimental configuration was poor, about 10-20 fwhm for both peptides and proteins. Thus the analytical utility of this system

was limited. Our subsequent research effort in instrumental development was focused on improving the resolution of the system.

Parallel Ion Extraction CF-MALDI

In TOFMS, ions can be extracted into the flight tube from a probe that is inserted orthogonal or parallel to the ion flight axis. In orthogonal extraction, the sample probe is conveniently placed between the repeller and extraction grids of the TOF instrument, thus allowing flow of the CF-MALDI liquid into the ionization region without electric breakdown. The MALDI ions expand perpendicular to the electric field in the acceleration region. However, ions are created in different regions of the electric field due to the finite width of the laser beam. Since this results in a spatial distribution of the ions, the resolution will correspondingly decrease. With the orthogonal configuration, the mass resolution observed in CF-MALDI as well as in static MALDI using 3-NBA liquid matrix is generally less than 20 fwhm [7,8]. To reduce the spatial distribution the sample molecules can be desorbed and ionized from an isoelectric surface, i.e., from a sample plane that experiences the same electric field at any point on the plane. This is achieved with parallel ion extraction. The ions formed in the source experience the same electric field as they expand in a direction parallel to the flight tube. The parallel ion extraction method requires the sample probe to be floated to the same high voltage as that applied to the repeller. Thus, the key in using parallel ion extraction for continuous flow experiments is to develop a flow probe that is able to handle the high voltage used.

Figure 3 shows a schematic diagram of the probe tip and the acceleration region of the linear TOF mass spectrometer that we developed for parallel ion extraction CF-MALDI. The acceleration plates are gridless and spaced by 4.7 mm. The CF probe is inserted into the center of the first acceleration plate. This probe can be floated up to ~15 kV, above which arcing can sometimes occur in the source region. The voltages applied to the acceleration plates were usually 12.0, 10.5, 8.0 kV, and ground, respectively. The tip of the flow probe is constructed of stainless steel and thus floated to the voltage of the first acceleration plate. An electrically

insulated probe heater (usually heated to 65 °C) prevents freezing of the matrix/analyte mixture on the probe tip. A video camera is used to monitor the liquid flow.

In this experiment, a matrix solution containing a known molecular weight standard is introduced through a syringe pump at 1.7 μL/min. The injected sample is introduced into the flow stream of a liquid chromatography pump (0.1 to 1.0 μL/min) and mixed with the matrix in a mixing tee as shown in Figure 1. The matrix solution contains: i) 70% 3-NBA, ii) 25% 1-propanol and iii) 5% water acidified to pH 2 with trifluoroacetic acid (TFA), with gramicidin S added as an internal standard. The LC effluent contains: i) 50% water (acidified with TFA) and ii) 50% acetonitrile. Under CF-MALDI conditions, the vacuum pressure is normally 1×10^{-3} Pa in the ionization region and 6×10^{-4} Pa in the flight tube.

With parallel extraction CF-MALDI, stable flow and reproducible analyte signals can be readily obtained. This is evident in Figure 4 where a flow profile from five repeat injections of 0.4 pmol myoglobin is shown. One benefit of parallel extraction is that it provides about 10-fold increase in detection sensitivity over orthogonal extraction. Mass resolution is significantly improved for small peptides, compared with the results we obtained earlier with the orthogonal ion extraction configuration. Figure 5 shows the CF-MALDI mass spectrum of bradykinin (9 pmol injection). The mass resolution is 213 fwhm in this linear time-of-flight instrument. As expected, improved mass resolution should result in the increase in mass accuracy. Table 1 lists the mass accuracy that was observed for a number of peptides using gramicidin S as an internal standard. They range from 0.02% to 0.13%.

Clearly, parallel ion extraction CF-MALDI improves the analytical performance in terms of mass resolution, mass accuracy, and sensitivity with respect to our earlier work using orthogonal extraction, and it also sets the stage for possible application of time-lag focusing to further improve mass resolution and accuracy. However, the performance of CF-MALDI is still inferior to static MALDI. MALDI performed on a solid insertion probe with a crystalline matrix in the same instrument without time-lag focusing yields spectra with a resolution of ~500 fwhm, for peptides and proteins up to ~10,000 u. When using 3-NBA as a matrix in either CF or static

mode of operation, a spectral resolution of 150 to 280 fwhm is typical for peptides up to 1500 u and resolution is generally less than 100 fwhm for higher mass peptides and proteins. Furthermore, the mass accuracy is typically 0.01% for internally calibrated peptides on the solid insertion probe with solid matrices.

One of the reasons lower resolution is observed when using 3NBA as the matrix is related to adduct ion formation. Figure 5 shows that alkali metal attachment to the peptide is a significant feature of the spectrum. As the peptide mass increases, alkali metal attachment becomes more extensive and it becomes more difficult to resolve the adduct peaks, limiting the utility of this liquid matrix for the analysis of high mass peptides and proteins. Consequently, our recent work in the area of CF-MALDI has been focused on searching for better matrices and matrix solution preparations. This is aided with the use of time-lag focusing MALDI TOFMS, capable of resolving adducts peaks at an extended mass range.

Liquid Matrices and Time-Lag Focusing MALDI

In CF-MALDI, the choice of matrix is very limited at present. There are only two known liquid matrices, 3NBA and 2-nitrophenyl octyl ether, that are suitable for peptides and proteins [10]. We found that the liquid 2-nitrophenyl octyl ether does not provide stable flow, partially due to its fast evaporation in a vacuum. In addition, it does not provide strong analyte signals for peptides and proteins. In contrast, stable flow can be readily obtained with 3NBA, which is not surprising because this matrix has been successfully used for continuous-flow fast atom bombardment (CF-FAB) MS [11]. Note that a thin film of 3NBA placed on the probe tip can remain as a liquid under the vacuum of 1×10^{-4} Pa for ~10 min. For a liquid to be effective as a CF-MALDI matrix it must remain a liquid under high vacuum conditions. This allows the analyte and matrix to continuously flow to the filter paper or other absorber wrapped along the probe adjacent to the tip surface. Otherwise, a sample memory effect would make chromatographic detection difficult.

To search for optimal matrix solutions that may be used for CF-MALDI, a great number of experiments were carried out. All these studies were done in a time-lag focusing MALDI TOF instrument that has been described previously [12].

Glycerol has been used extensively for FAB MS and provides stable flow in CF-FAB [13]. However, glycerol lacks a chromophore at the UV wavelengths of 266 nm from the Nd:YAG laser or 337 nm from the nitrogen laser. An additive is required to provide UV excitation [14]. Unfortunately, an investigation of a great number of glycerol mixtures including the use of laser dyes and solid matrices failed to yield any useful liquid matrix combination. For example, a mixture of coumarin 460 and glycerol can produce only very weak signals with poor resolution. In the case of mixtures of glycerol with solid matrices such as sinapinic acid (SA) or α-cyano-4-hydroxycinnamic acid (HCCA), the general observation was that analytically useful spectra can be obtained only after the glycerol had evaporated, leaving a dry preparation. This suggests that a mixture of glycerol and a common solid matrix such as SA or HCCA cannot be used for CF-MALDI.

To utilize the 337 nm nitrogen laser, which is by far the most popular ionization laser used for MALDI, initial attempts were made at collecting spectra using 3NBA spiked with common solid MALDI matrix compounds such as SA and HCCA. Again, good spectra were obtained only after evaporation of 3NBA. Mixtures with solid matrix compounds were thus abandoned and we focused on using the 266 nm line (the absorption maximum of 3NBA is near 266 nm) from a frequency quadrupled Nd:YAG laser as the ionization source.

In the remaining experiments, for the study of 3NBA under time-lag focusing conditions, a peptide or protein solution was prepared in pure water or 0.06% TFA. A 0.5 μL aliquot of 3NBA was first applied to the probe tip followed by 0.5 μL of the peptide or protein solution. The tip was placed in a vacuum where the water evaporated. Partial removal of alkali salts from the 3NBA was done with water extraction. Equal volumes of 3NBA and pure water were mixed and the two solvents allowed to separate. The water was drawn off and another aliquot was added. This was repeated 3 or 4 times.

Figure 6 shows a spectrum of bradykinin using 3NBA as the matrix obtained by time-lag focusing MALDI. The resolution for the molecular ion peaks is about 2060 fwhm, which is sufficient to resolve the isotope peaks for this peptide. This is a significant improvement over the resolution of about 150 to 280 fwhm, typically obtained with static MALDI using 3NBA in the continuous extraction mode. Another example is shown in Figure 7 for insulin. Figure 7A was obtained using continuous extraction and Figure 7B was obtained using the pulsed ion extraction with time-lag focusing. Figure 7B shows a resolution of 835 fwhm which compares favorably with a resolution of ~1000 fwhm typically obtained with solid matrix preparations on this instrument for insulin. Note that the resolution obtained in the continuous extraction mode is about 70 fwhm for insulin using 3NBA. In the insert of Figure 7B, it is evident that strong alkali and matrix adduct peaks are formed. The alkali adduct peaks can be as intense as the protonated molecular peak for some samples. As an example, Figure 8 shows a spectrum of bovine insulin chain B (oxidized form). In Figure 8A potassium adduct peaks are prominent features. In a fashion consistent with that described for FAB MS [15], Figure 8B shows how the addition of 18-crown-6 ether to the 3NBA significantly reduced the adduct attachment to the peptide yielding a cleaner spectrum with stronger analyte peaks.

However, for proteins such as cytochrome *c*, we have not been able to observe any resolution enhancement by using the time-lag focusing technique with 3NBA as the matrix. In this case, to aid the dissolution of the proteins, a 50 mM solution of the nonionic surfactant n-octyl β-D-glucopyranoside in 3NBA was used in place of neat 3NBA [16]. This gave high sensitivity for proteins although the resolution is still poor (~10 fwhm). We speculate that the extensive overlap of multiple alkali adduct and matrix peaks prevent their resolution even by the time-lag focusing instrument. Despite a number of attempts to clean up the samples and matrices in the hopes of reducing adduct ion formation, we have not been able to observe signals with resolution better than 20 fwhm for proteins with masses above 8000 (i.e., ubiquitin). Clearly, 3NBA is a good viscous liquid useful for producing stable flow for CF-MALDI but it is a poor matrix for protein analysis.

Solid Matrices and Time-lag focusing MALDI

The use of TLF MALDI TOFMS operated at the normal conditions (i.e., use solid matrices) for the analysis of peptides and proteins was also evaluated. Peptide and protein stock solutions were prepared in 0.6 mL Eppendorf microcentrifuge tubes and stored at –20°C. All peptides and proteins were dissolved in water. Dilute solutions were prepared fresh daily in microcentrifuge tubes from the stock solutions using water. Small peptide samples were prepared for MALDI analysis using a modified fast evaporation method. A 1 μL aliquot of 50 mM HCCA dissolved in 99% acetone/water (v/v) was placed on the tip. After drying, 1 μL of 5 μM bradykinin solution, mixed 1:1 (v/v) with a saturated solution of matrix in 33% acetonitrile/0.1% trifluoroacetic acid (v/v), was added. Protein samples were prepared using the vacuum evaporation method. A 1.0 μL aliquot of a 50 mM solution of sinapinic acid in 90% methanol/water (v/v) mixed 1:1 with a 10 μM aqueous protein solution was applied to the probe tip and dried under a vacuum. The resulting crystal layer was lightly wiped away and a second 1.0 μL droplet was applied and again dried under a vacuum. The result was a homogeneous layer of small crystals.

Table 2 lists the results of mass measurement by TLF MALDI for a number of peptides and proteins. For peptides and small proteins, the error is generally less than 100 ppm. However, in the case of bovine albumin and chicken albumin, it was found that TLF does not provide any improvement in mass accuracy over that obtained by using continuous extraction MALDI. In our TLF instrument, the sensitivity of ion detection at $m/z > 60{,}000$ was found to be about 10-fold less than that of the Hewlett-Packard MALDI instrument with continuous extraction. Consequently, the HP instrument was used to analyze these two albumins. Due to the lack of an appropriate internal standard, the mass calibration was performed from the extrapolation of the molecular ion mass of bovine carbonic anhydrase II (MW 29024.7). For bovine albumin, the mass error is -0.08% and, for chicken albumin, the error is +0.14%. It should be noted that our TLF instrument has recently been demonstrated to provide accurate mass measurement of proteins with masses up to at least 30,000 u [17]. Due to the lack of

protein standards with homogeneous composition at masses above 30,000 u, the upper mass limit that the TLF technique can provide accurate mass measurement is currently unknown. This study of two albumins did indicate that the TLF method may not be effective at masses above 60,000 u.

Conclusions

On-line combination of LC and MALDI MS is feasible with the use of a continuous flow probe. Using 3NBA as the liquid matrix, stable flow can be readily achieved. The flow probe interface does not degrade the performance of the chromatographic separation. A limit of detection in the low picomole range for peptides and proteins can be routinely carried out.

The major limitation of this LC-MALDI system for analytical applications is the poor mass resolution observed with peptides and proteins. Parallel ion extraction where the flow probe is floated to a high voltage, up to 15 kV, can be done. It was demonstrated in this work that this mode of operation provides improved mass resolution over the orthogonal ion extraction mode of operation. However, for peptides with masses higher than about 1500 u, it did not offer any significant improvement in resolution. The use of time-lag focusing TOF was anticipated to significantly improve the resolution. Good resolution was obtained for peptides up to and including the protein insulin (MW 5700). But, with the use of 3NBA as the matrix, poor mass resolution was observed for proteins (above MW 8000) even with time-lag focusing TOFMS.

The resolution improvement brought about by the parallel ion extraction and the use of time-lag focusing provides some insights about the general performance of 3NBA as the MALDI matrix. This matrix requires the use of 266 nm laser beam for desorption, which limits its use in most commercial systems where a nitrogen laser emitting at 337 nm is commonly used. In addition, to accommodate the special need of the flow experiments, CF-MALDI LC-MS requires the modification of the ion source region and cannot be readily fitted to any commercial systems. A dedicated instrument would be needed. This work with parallel extraction as well as time-lag focusing clearly indicates that 3NBA is a poor matrix for MALDI of proteins. With 3NBA, a

broad peak is observed in the molecular ion region due to the formation of extensive adduct ions. Unfortunately, unlike the situation in static MALDI with solid matrices where a better matrix displaying less adduct ion formation can be selected among a pool of matrices, there is a very limited number of liquid matrices available. Choosing other matrices for CF-MALDI is not an option at present.

With all the instrumental development, CF-MALDI will likely be best suited for the analysis of peptides (i.e., $m/z < 6000$) in a linear TOFMS with time-lag focusing. Note that other mass spectrometric analyzers have been used for CF-MALDI [17]. However, the potential advantages of these systems as a viable alternative to time-of-flight analyzers for LC-MS based on CF-MALDI remain to be seen. It is our view that the future advance of CF-MALDI with TOFMS is dependent on the discovery of new matrices that can be used for a flow system. Recent work using an infrared laser beam [18], instead of a UV beam, as the ionization source for MALDI may open new opportunity of discovery for liquid matrices useful for CF-MALDI.

On the other hand, time-lag focusing TOFMS operated at the conventional mode (i.e., use solid matrices) provides high mass accuracy over a broad mass range. Since the technique of MALDI can potentially analyze mixtures directly without extensive separation, future research should be focused on developing optimal sample and matrix preparation methods for direct analysis of peptides and proteins in a mixture.

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Table 1. Mass accuracy results for peptides determined by CF-MALDI with parallel ion extraction.

	Average Protonated Mass (u)		
Peptide	Calculated	Measured	Error (%)
Ac-KLEALEA-amide	814.96	815.9	0.12
Ac-TQDEQFIP-amide	1019.10	1019.3	0.02
Bradykinin	1061.23	1061.5	0.03
LYPVKLPVK	1220.54	1220.0	0.04
Ac-KLEALEAKLEALEA-amide	1569.84	1571.9	0.13

Table 2. Mass accuracy results for peptides and proteins determined by time-lag focusing MALDI TOFMS.

Compound	$(M+H)^+$ (u) Calculated	Experimental	Average and Std. Dev.	Error (ppm)
Substance P (7-11)	613.32	613.33 613.27 613.29 613.28 613.28	613.29 ± 0.02	-49
Substance P (6-11)	741.38	741.31 741.35 741.34 741.34 741.43	741.35 ± 0.05	-40
Substance P (4-11)	966.49	966.51 966.50 966.43 966.43 966.51	966.48 ± 0.04	-10
Substance P	1347.74	1347.82 1347.82 1347.80 1347.78 1347.61	1347.77 ± 0.09	+22
Bradykinin	1060.57	1060.45 1060.50 1060.44 1060.54 1060.44	1060.47 ± 0.04	-94
		1188.65 1188.64		

Lys-bradykinin	1188.66	1188.56 1188.67 1188.56	1188.62 ± 0.05	-34
Ile-Ser-bradykinin	1260.69	1260.67 1260.67 1260.67 1260.68 1260.68	1260.67 ± 0.01	-16
Met-lys-bradykinin	1319.70	1319.81 1319.73 1319.71 1319.71 1319.72	1319.74 ± 0.04	+30
Bombesin	1619.82	1619.74 1619.91 1619.74 1619.91 1619.73	1619.81 ± 0.09	-6
Neuropeptide Y (human)	4272.76	4272.94 4272.93 4272.92 4272.95 4272.94	4272.94 ± 0	+42
Neuropeptide Y (porcine)	4254.72	4254.59 4254.50 4254.79 4254.56 4254.59	4254.61 ± 0.11	-26
Neuropeptide Y (sheep)	4240.69	4240.94 4240.69 4240.64	4240.74 ± 0.13	+12

		4240.80		
		4240.64		
		14304.99		
		14305.80		
Lysozyme	14306.14	14305.80	14305.78 ± 0.52	-25
		14305.82		
		14306.47		
		66343.5		
		66353.1		
		66306.2		
Bovine Albumin**	66399.9*	66312.1	66346.3 ± 25.4	-0.08%
		66363.4		
		66371.0		
		66346.3		
		66374.4		
		67202.5		
		67250.3		
		67218.8		
Chicken Albumin**	67157.1	67283.6	67251.8 ± 81.0	+0.14%
		67415.8		
		67224.3		
		67167.0		

* Data were collected from the Model G2025A MALDI time-of-flight system (Hewlett Packard, CA), the instrument was operated in DC mode at +28 KV.

** Mass according to the gene sequence.

FIGURE CAPTIONS

Figure 1. Schematic of the micro-column LC-MALDI system using a continuous flow probe to interface with a time-of-flight mass spectrometer.

Figure 2. Ion chromatogram of LC separation of a mixture of horse heart cytochrome c and chicken egg white lysozyme by using a micro-column. The injected sample was a mixture of 9 pmol cytochrome c and 8 pmol lysozyme.

Figure 3. Schematic of the flow probe tip and the acceleration region of the linear time-of-flight mass spectrometer used for parallel ion extraction CF-MALDI.

Figure 4. Flow injection ion profile of 5 repeated injections of horse heart myoglobin.

Figure 5. Mass spectrum of bradykinin obtained by CF-MALDI with an injection of 9 pmol.

Figure 6. Mass spectrum of bradykinin by time-lag focusing MALDI with the use of 3NBA as the matrix.

Figure 7. MALDI spectra of insulin obtained by using 3NBA as the matrix with (A) continuous extraction and (B) time-lag focusing.

Figure 8. MALDI spectra of oxidized B chain of bovine insulin obtained by using 3NBA as the matrix (A) before the addition of 18-crown-6 ether and (B) after the addition. The total sample loaded is 10 pmol.

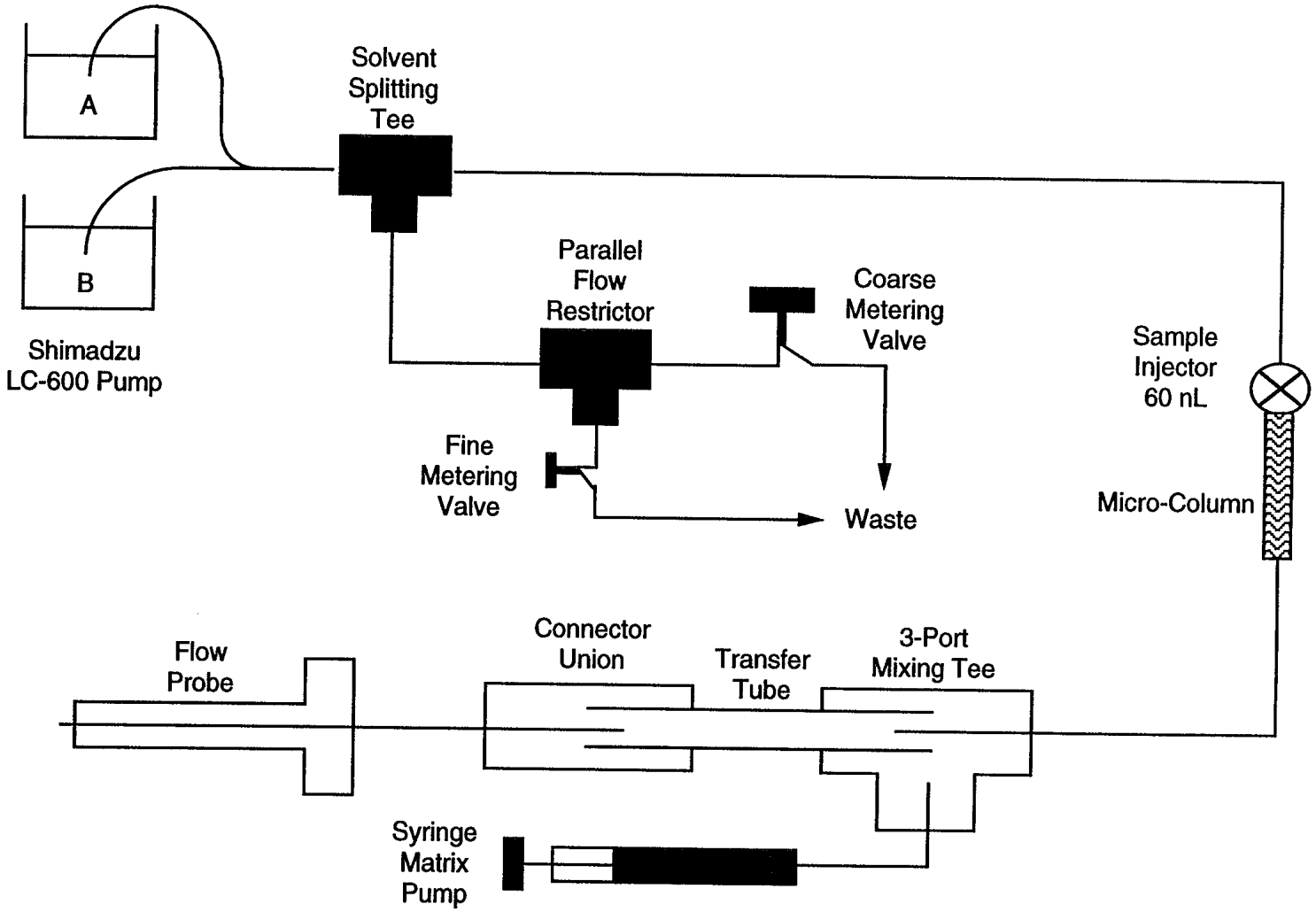

A
B
Shimadzu
LC-600 Pump
Solvent
Splitting
Tee
Parallel
Flow
Restrictor
Coarse
Metering
Valve
Fine
Metering
Valve
Waste
Sample
Injector
60 nL
Micro-Column
Flow
Probe
Connector
Union
Transfer
Tube
3-Port
Mixing Tee
Syringe
Matrix
Pump

Figure 1

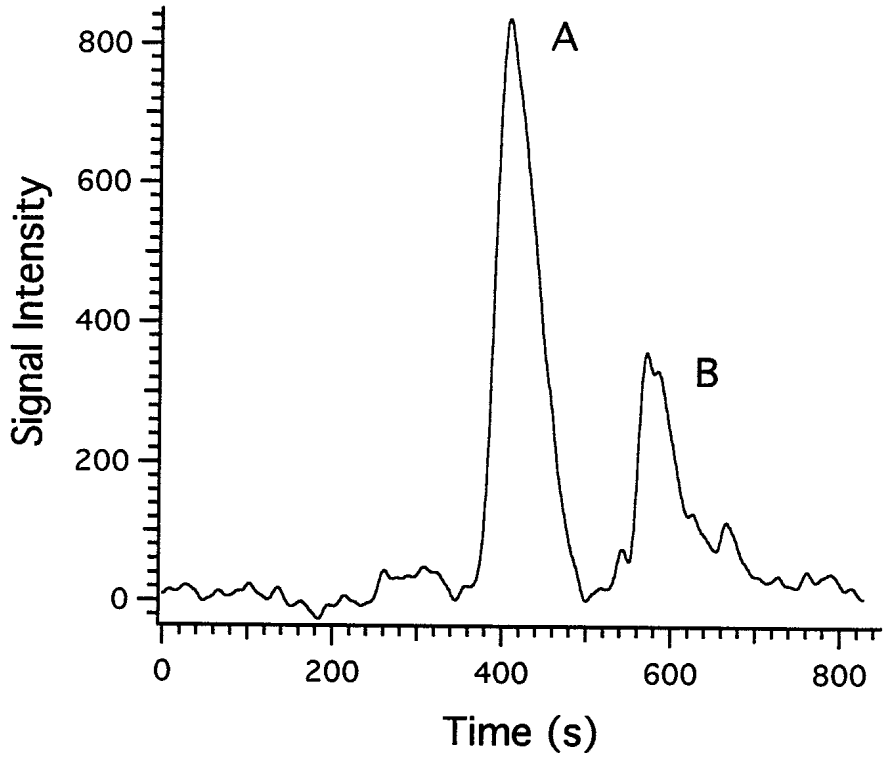


Figure 2

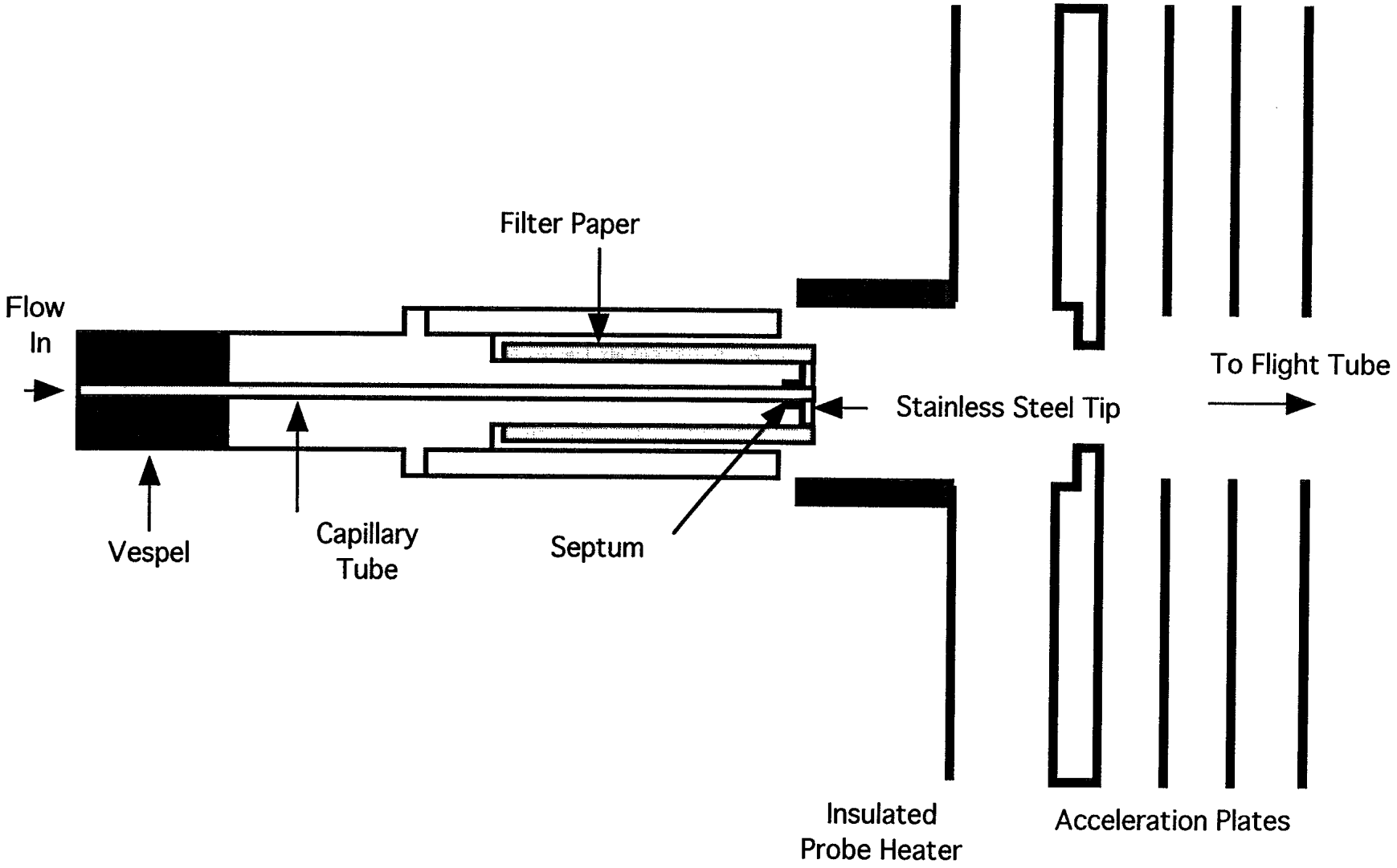

Filter Paper
Flow
In
To Flight Tube
Stainless Steel Tip
Vespel
Capillary
Tube
Septum
Insulated
Probe Heater
Acceleration Plates

Figure 3

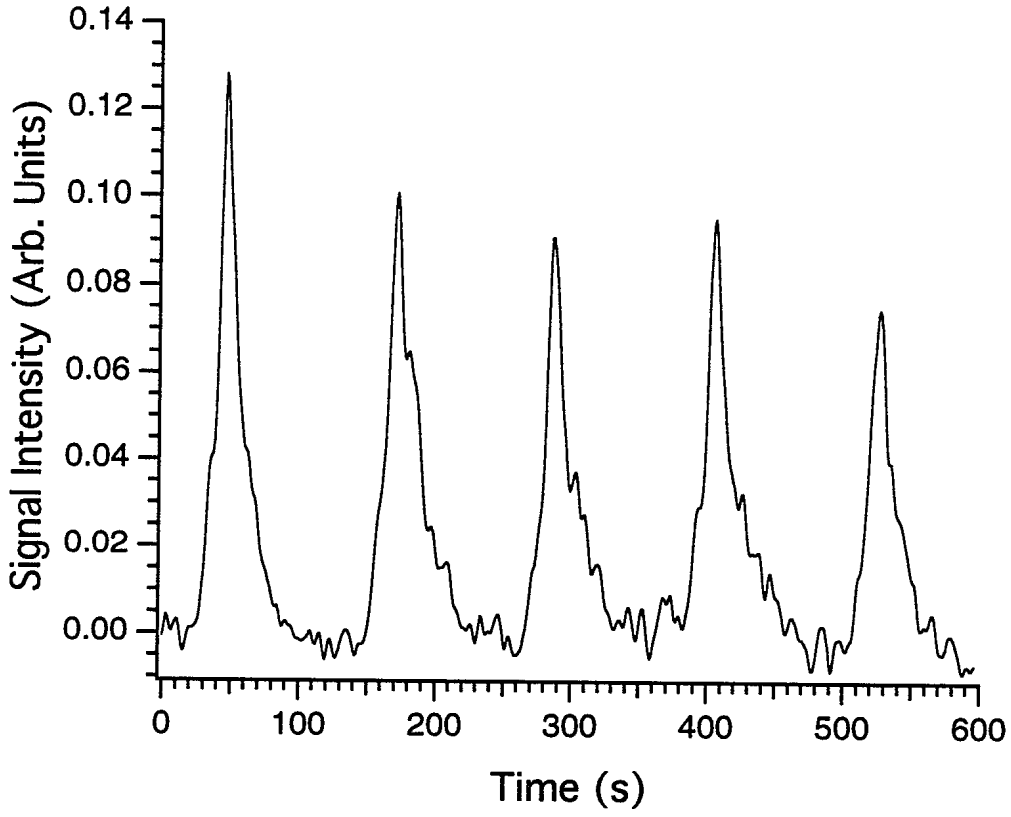

Signal Intensity (Arb. Units)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
100
200
300
400
500
600
Time (s)

Figure 4

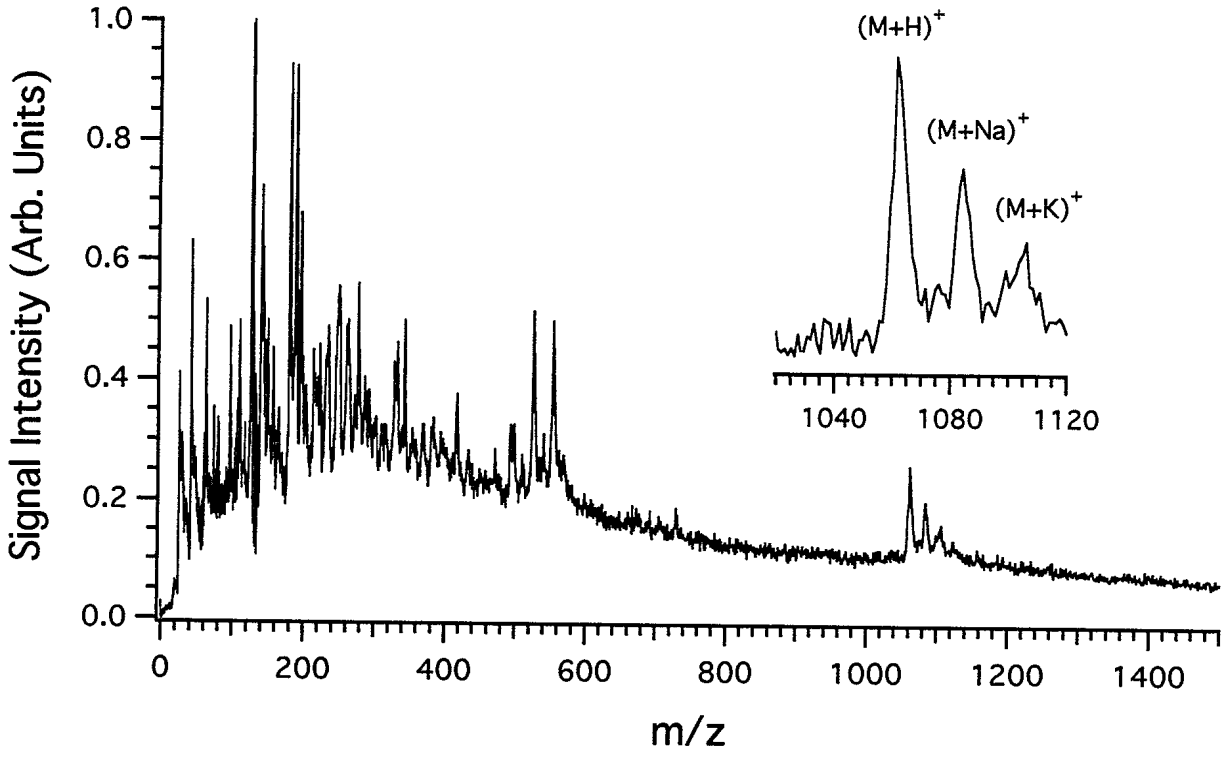

Signal Intensity (Arb. Units)
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
m/z
(M+H)+
(M+Na)+
(M+K)+
1040
1080
1120

Figure 5

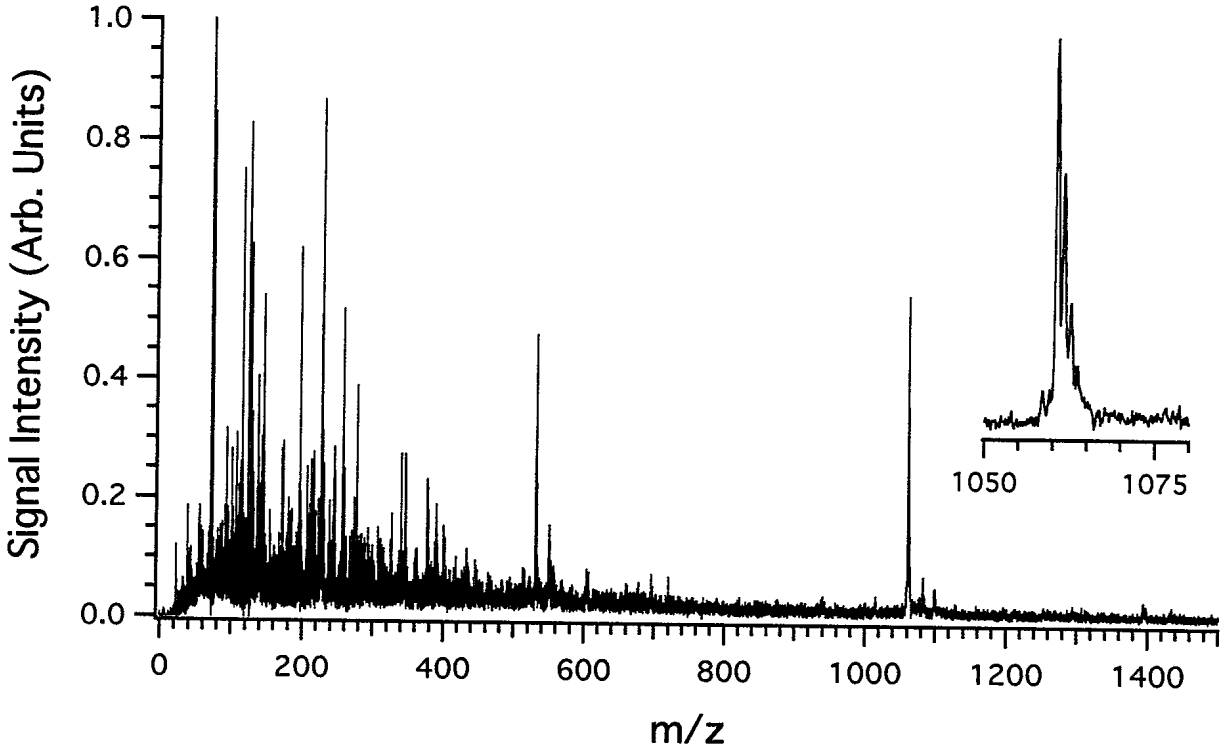
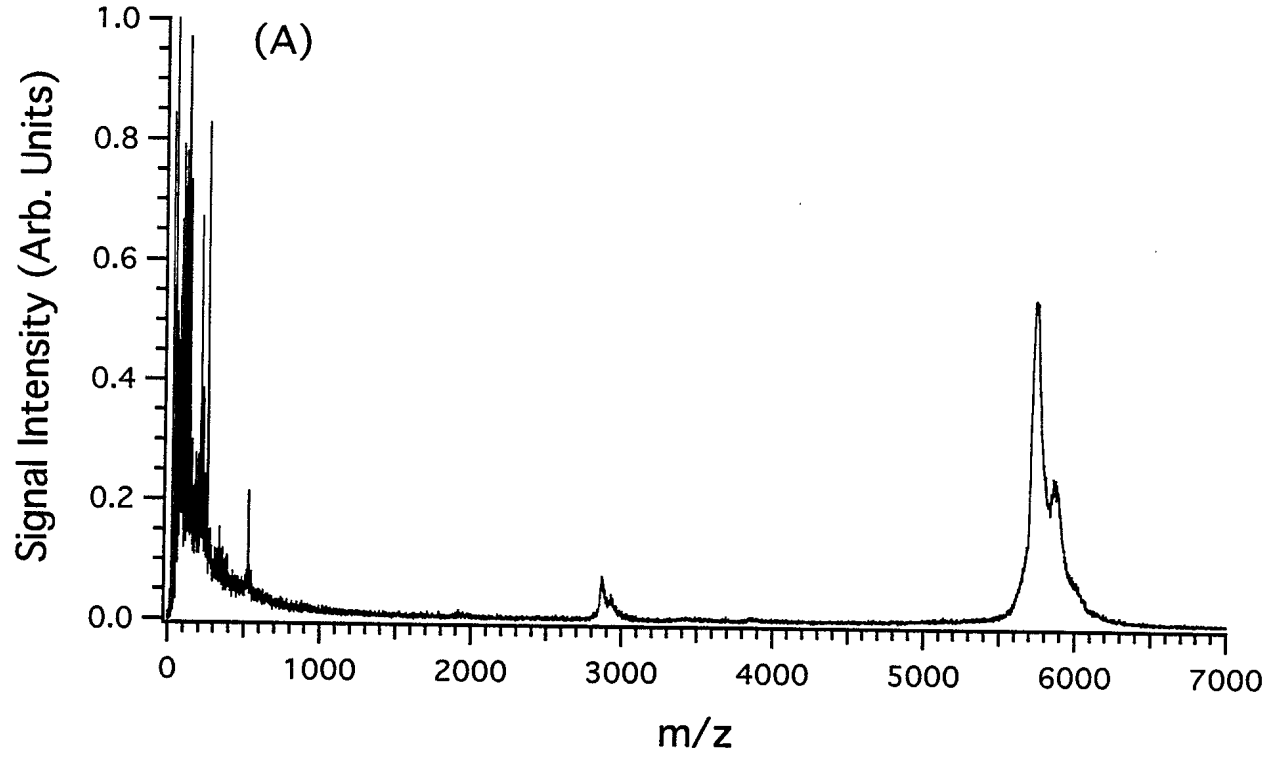

Signal Intensity (Arb. Units)
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
m/z
1050
1075

Figure 6


(A)
Signal Intensity (Arb. Units)
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
4000
5000
6000
7000
m/z

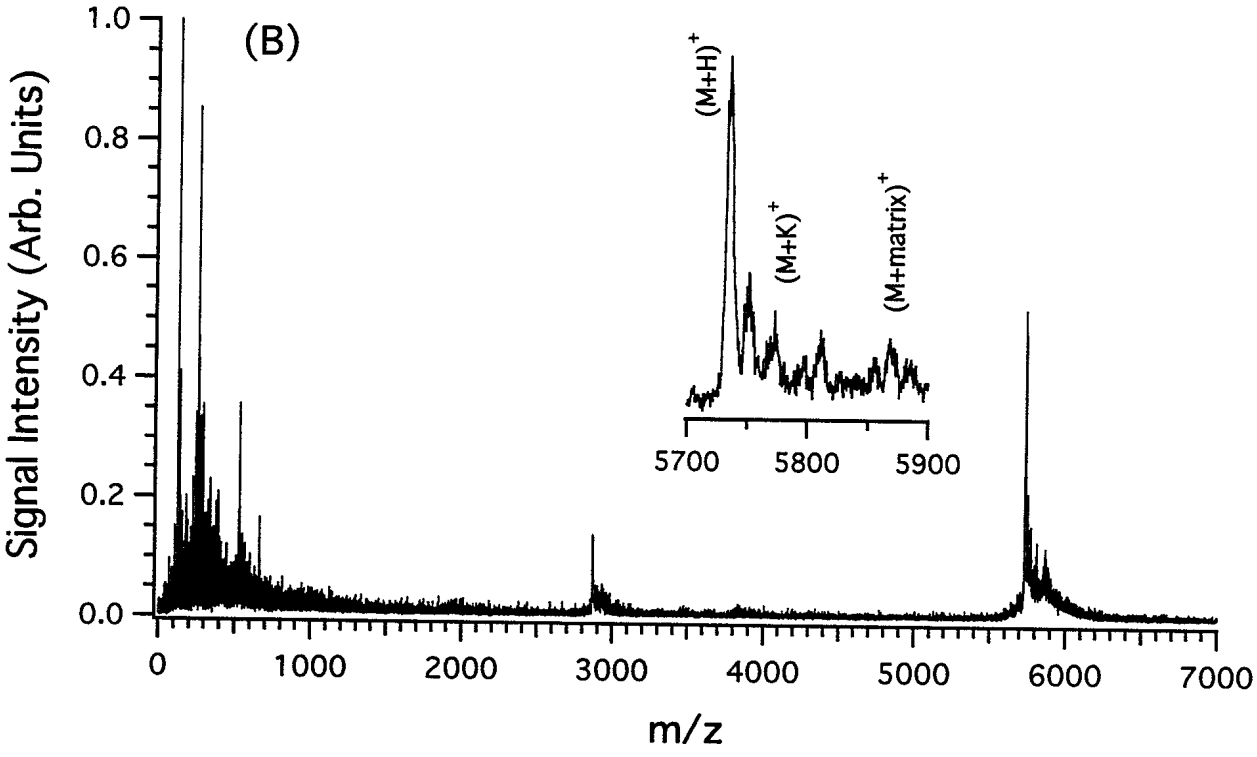
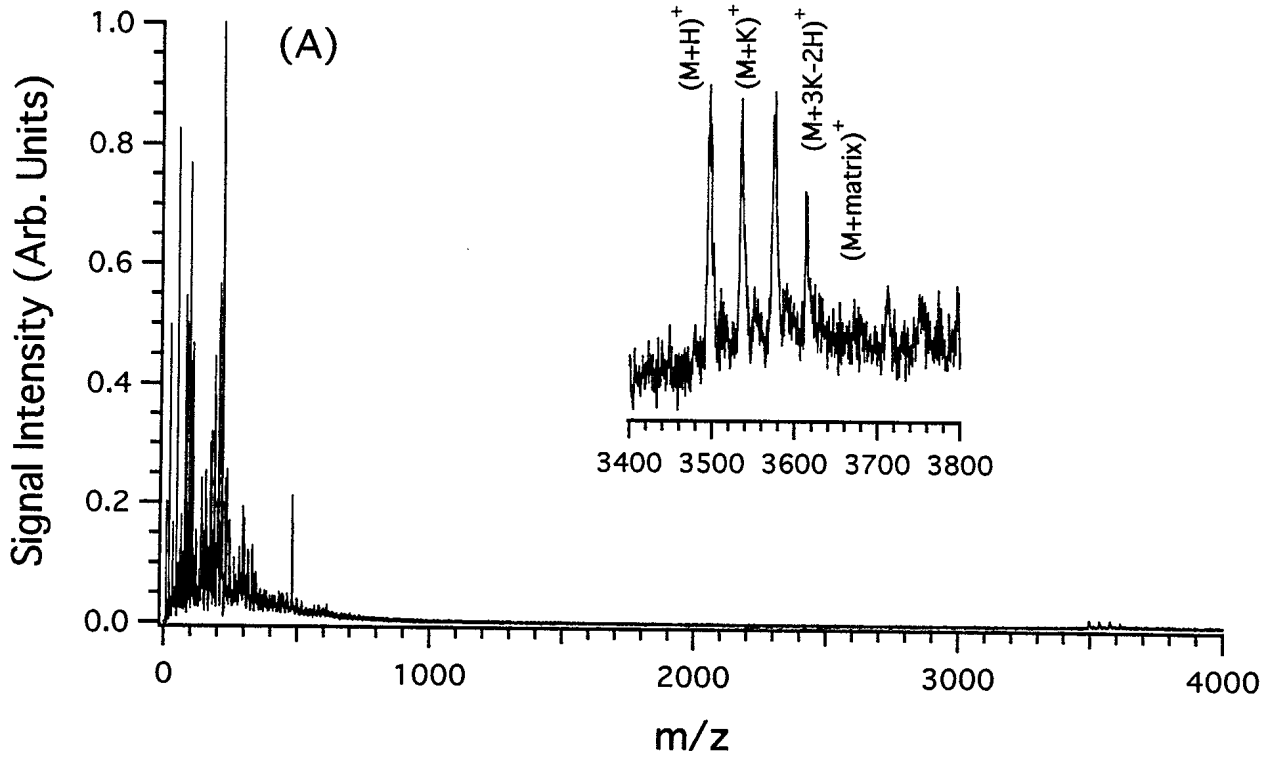

(B)
Signal Intensity (Arb. Units)
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
4000
5000
6000
7000
m/z
(M+H)+
(M+K)+
(M+matrix)+
5700
5800
5900

Figure 7


(A)
Signal Intensity (Arb. Units)
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
4000
m/z
(M+H)+
(M+K)+
(M+3K-2H)+
(M+matrix)+
3400 3500 3600 3700 3800

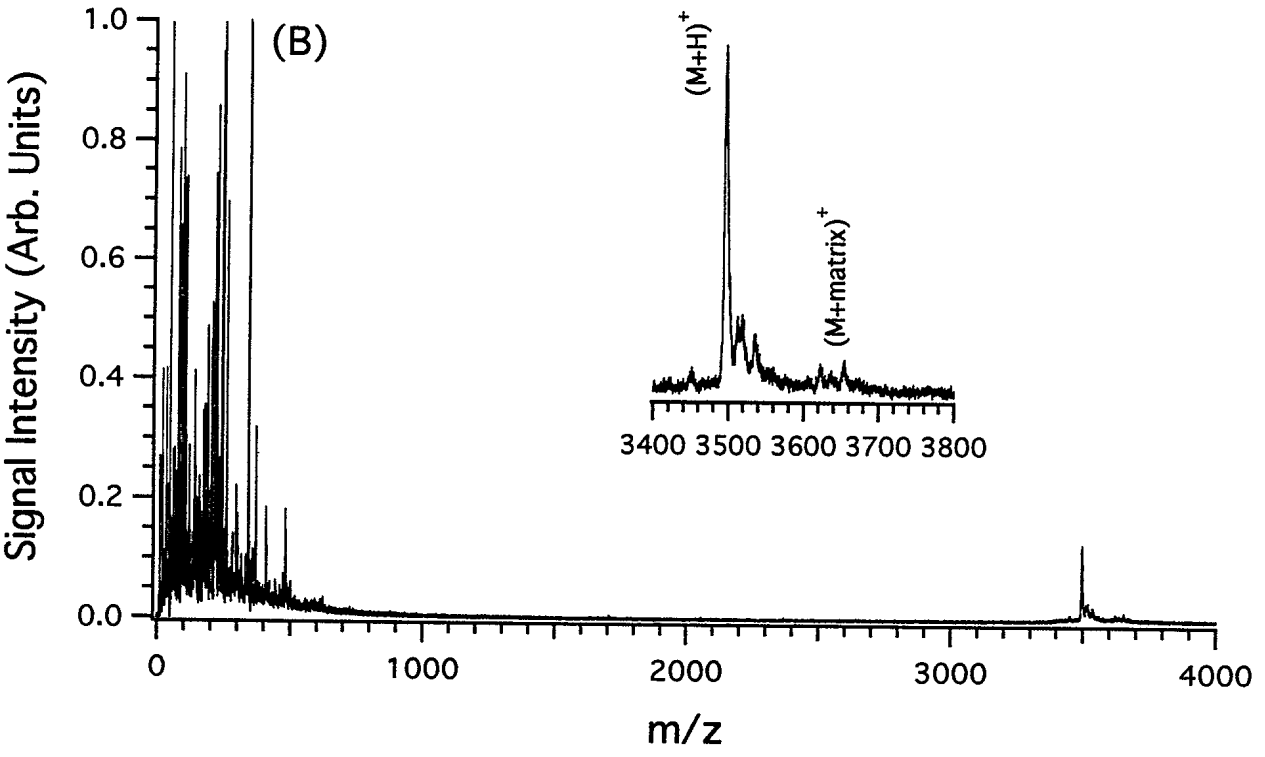

(B)
Signal Intensity (Arb. Units)
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
4000
m/z
(M+H)+
(M+matrix)+
3400 3500 3600 3700 3800

Figure 8

#503630