



Surface assisted laser desorption ionization (SALDI) mass spectrometry
by Yu-Chie Chen

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Chemistry

Montana State University

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Abstract:

A new laser desorption ionization technique named "Surface Assisted Laser Desorption Ionization (SALDI) Mass Spectrometry" has been developed. SALDI avoids some drawbacks of the accepted laser desorption ionization method, Matrix Assisted Laser Desorption Ionization (MALDI). Instead of using a solid matrix, a suspension is used in SALDI that is composed of glycerol (SALDI solvent) with a graphite or activated carbon powder (SALDI solid). Sucrose is added to the SALDI suspension as an "adhesive" to immobilize the SALDI solid powder and limit contamination of the ion source.

The mass resolution of SALDI is almost identical with that of MALDI. Furthermore, the detection limit of SALDI is less than 10 fmole for analytes like peptides. However, the mass range is less than 20,000 Da. A very important advantage of SALDI is a low matrix background at low mass range.

Applications of SALDI to the analysis of amino acids, peptides, small proteins, organics, volatile organics, and polymers have been demonstrated. In particular, SALDI is very useful for peptide mixtures such as enzymatic digest products.

Activated carbon is the most useful SALDI solid. Activated carbon is also one of the most common adsorbents used in solid phase-extraction (SPE). The combination of SPE with SALDI has been developed. SPE/SALDI is a fast screening method and can be used for either qualitative or quantitative analysis. Furthermore, the development of TLC/SALDI is explored in this thesis.

SURFACE ASSISTED LASER DESORPTION IONIZATION

(SALDI) MASS SPECTROMETRY

by

Yu-Chie Chen

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APPROVAL

of a thesis submitted by

Yu-Chie Chen

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style and consistency, and is ready for submission to the College of Graduate Studies.

Jan Sunner

Jan Sunner
(Signature)

Jan. 16, 1997
Date

Approved for the Department of Chemistry

David M. Dooley

David M. Dooley
(Signature)

1/16/97
Date

Approved for the College of Graduate Studies

Robert L. Brown

RL Brown
(Signature)

2/3/97
Date

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ABSTRACT

A new laser desorption ionization technique named "Surface Assisted Laser Desorption Ionization (SALDI) Mass Spectrometry" has been developed. SALDI avoids some drawbacks of the accepted laser desorption ionization method, Matrix Assisted Laser Desorption Ionization (MALDI). Instead of using a solid matrix, a suspension is used in SALDI that is composed of glycerol (SALDI solvent) with a graphite or activated carbon powder (SALDI solid). Sucrose is added to the SALDI suspension as an "adhesive" to immobilize the SALDI solid powder and limit contamination of the ion source.

The mass resolution of SALDI is almost identical with that of MALDI. Furthermore, the detection limit of SALDI is less than 10 fmole for analytes like peptides. However, the mass range is less than 20,000 Da. A very important advantage of SALDI is a low matrix background at low mass range.

Applications of SALDI to the analysis of amino acids, peptides, small proteins, organics, volatile organics, and polymers have been demonstrated. In particular, SALDI is very useful for peptide mixtures such as enzymatic digest products.

Activated carbon is the most useful SALDI solid. Activated carbon is also one of the most common adsorbents used in solid phase-extraction (SPE). The combination of SPE with SALDI has been developed. SPE/SALDI is a fast screening method and can be used for either qualitative or quantitative analysis. Furthermore, the development of TLC/SALDI is explored in this thesis.

CHAPTER 1

INTRODUCTION

General

Extension of the mass range to include large molecules has long been an important problem in mass spectrometry. However, larger molecules tend to be polar, nonvolatile, and thermally labile. Traditional ionization methods in mass spectrometry, such as electron impact ionization (EI) and chemical ionization (CI), require high volatility of samples because molecules are ionized in the gas phase. If analytes have a low volatility, their mass and structure cannot be obtained using these traditional ionization methods. Therefore, extensive efforts have been made over many years to invent and develop new ionization methods for low volatility and thermally labile compounds. An ideal soft desorption ionization technique must have the advantages of high sensitivity, low ion background, easy operation, high mass range, etc. The methods that have been developed for this purpose can be divided into spray ionization and desorption ionization (DI) methods. The term "desorption ionization" refers to methods where analyte molecules are desorbed and ionized in a single step in vacuum. Here, we will be concerned solely with DI methods. In all these methods, energy is quickly deposited onto a liquid or solid surface. Ions ejected from the surface are detected by a mass spectrometer.

Several main types of desorption ionization methods have been developed: three types of particle-induced desorption methods and two types of laser-induced desorption methods. Particle-induced desorption methods include Plasma Desorption (PD) (Torgerson et al, 1974; Sundqvist & MacFarlane, 1984), Static Secondary Ion Mass Spectrometry (SIMS) (Benninghoven et al., 1970; Grade & Cooks, 1978), and Fast Atom Bombardment (FAB) (Barber et al., 1981). Each method has its unique advantages and disadvantages. For example, PD has low sensitivity and produces noisy spectra and thus is not used very often anymore. In SIMS, a very high energy ion beam bombards the sample. It is a "harder" desorption ionization method than the other DI methods. SIMS cannot be connected with chromatography in a simple way because solid samples are used. FAB has been interfaced with chromatography, FAB is noisy and has a low mass limit problem. The upper mass limit for all particle-induced DI methods is a few thousand Daltons. Thus, these methods cannot be used to analyze larger biomolecules, such as proteins.

In direct Laser Desorption (LD) (Honing et al., 1963), a pulsed laser is used to desorb analyte molecules and ions from a surface to vacuum. The upper mass range of LD is under 2,000 Da (Linder et al., 1985). This is not sufficient for large biomolecules, such as proteins. Two more recent techniques are ElectroSpray Ionization (ESI) and Matrix Assisted Laser Desorption Ionization (MALDI). Both are softer than the particle induced or the direct laser desorption techniques. In MALDI, a light absorbing material, called matrix, is mixed with the analyte in solution. Upon solvent evaporation, the matrix and analyte co-crystallize. During laser pulse irradiation in a vacuum, a desorption ionization process occurs. In this process, gas phase ions of both matrix and analyte are formed. High molecular weight

molecules, such as proteins, can be analyzed by MALDI or ESI. The maximum mass range in MALDI has been extended above 500 kDa. Partly for these reasons, MALDI and ESI have become revolutionary and practical techniques for routine biochemical structure determinations. Here, the history and mechanisms of LD and MALDI will be described first. Subsequently, the instrumentation will be discussed.

History of Laser Desorption Mass Spectrometry

It was demonstrated already in 1960's that when a laser beam hits a solid, the high energy laser pulse can desorb intact molecules from the surface, and that some of the molecules are ionized and can be analyzed by mass spectrometry. The first application of laser desorption ionization was published in 1963 (Honing et al., 1963). Honing et al. irradiated various solids, conductors, semiconductors, and insulators in a vacuum with a focused beam from a pulsed ruby laser. Intense pulses of electrons and positive ions were produced as a result of the laser irradiation. In the late 1960's, Vastola et al. used a 100 mJ ruby laser with a 500 ns pulse to desorb organic salts and were able to obtain mass spectra up to 200 - 300 Da (Vastola et al., 1968 & 1970).

In 1978, Posthumus and coworkers presented laser desorption mass spectra of small biomolecules such as oligosaccharides, glycosides, amino acids, peptides, steroids, antibodies, and chlorophyll (Posthumus et al., 1978). These researchers used a pulsed laser with a two orders of magnitude lower power density than used Vastola et al. (Vastola et al., 1968 & 1970). However, until 1985, the upper mass range in laser desorption experiments

remained at about 2,000 Da (Lindner et al., 1985). A breakthrough result was obtained by Tanaka et al. in 1988. In Tanaka's experiments, the maximum molecular weight was extended to over 30,000 Da (Tanaka et al., 1988). At about the same time, Hillenkamp developed a method that they named "Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI/MS)". With this method, spectra of compounds with molecular weights as high as 70,000 Da were demonstrated (Hillenkamp et al., 1988). A new era in biochemical mass spectrometry began with these discoveries. MALDI will be described in more detail in the following sections.

Matrix Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry

History

In Tanaka's laser desorption ionization experiment, an extremely fine cobalt powder of about 300 Å diameter was suspended in a glycerol liquid and the suspension mixed with sample solution. A nitrogen laser (337 nm) was used for desorption ionization. Spectra were obtained of several proteins including carboxypeptidase A with a molecular weight of 34,000 Da. However, the mass resolution was low, about 20, and the signal to noise ratio was poor. Tanaka et al. suggested that the desorption mechanism in their experiments involved a laser induced heating of the extremely small metal particles, followed by heat conduction to the surrounding glycerol solution and a thermal desorption process. Tanaka's method was, however, largely forgotten.

Hillenkamp and coworkers developed an LD method that they named "Matrix Assisted Laser Desorption Ionization (MALDI)." These researchers mixed an absorbing material called a matrix, with the analytes. In their initial study, they used nicotinic acid as the matrix. A quadrupled Nd-YAG laser (266 nm) was used for desorption ionization. Spectra of proteins such as bovine albumin, trypsin, β -lactoglobulin, and lysozyme were obtained by this method. Nicotinic acid has a very strong absorption at 266 nm. It seems that the nicotinic acid absorbs the UV laser energy and provides the energy to the intermolecular degrees of freedom in the crystal. Since 1988, many MALDI matrices have been found. The selection of matrix can strongly affect MALDI spectra.

Advantages of MALDI

MALDI has many important advantages. The sensitivity is very high. Only femtomoles of analytes are typically required for MALDI analysis in favorable case (Karas et al.; 1989b). Furthermore, the maximum mass range in MALDI is around 500,000 Da although mass resolution decreases at high mass (Overberg et al., 1990). With a careful selection of the matrix compound, MALDI can be used for a very wide variety of biomolecules. Because of ease of use and high sensitivity, MALDI has become a dominant technique for molecular weight determination and structural characterization of large biomolecules.

Matrices in MALDI

Matrices in MALDI play a very important role, and their selection critically affect the MALDI spectra. There are two major requirements for good matrices in MALDI. First, the matrix must solvate and isolate the biomolecules in the crystal. Second, the matrix must

absorb the laser pulse with a high absorptivity (500 to $15,000 \text{ Lmol}^{-1}\text{cm}^{-1}$) at the given laser wavelength (Levis et al., 1994). Good MALDI results depend on the selection of matrix, the tolerance toward impurities, and the optimum molar ratio of matrix to analyte, usually $100:1$ to $50,000:1$ (Hillenkamp et al., 1991). Experience has shown that different matrices have different sensitivities toward impurities such as buffer salts and detergents. Choosing the best matrix to match analyte solution requirements is a determinant factor for obtaining good MALDI results. Also, the optimum ratio of matrix to analyte depends on the matrix used and on the size of the analyte molecules. Usually, the larger the analyte molecules, the larger the ratio of matrix to analyte should be.

Generally speaking, there are three functions that the matrix needs to serve in MALDI (Karas et al., 1989). First, an excess of matrix prevents aggregation of the large biomolecules and reduces the intermolecular forces. Furthermore, the matrix has to co-crystallize with the analytes. Beavis et al. demonstrated that proteins that were not incorporated into the matrix lattice also were not detected in the gas phase (Beavis et al., 1993). Second, the matrix absorbs the laser energy via electronic excitations, transfers the energy into vibration of the solid lattice and induces the necessary strong perturbation and disintegration of a microvolume. Third, the matrix must give a high ionization yield of analytes by photochemical reactions, most probably via radical intermediates. In several studies, the ion to neutral yield for the MALDI laser-desorption process has been estimated to be $1:10^4$ to $1:10^5$ (Mowry et al., 1993; Ens et al., 1991; Spengler et al., 1991). In these studies, ionic and neutral products of MALDI were simultaneously detected in a reflectron time-of-flight mass spectrometer. The neutrals were photoionized with coherent vacuum

ultraviolet radiation. Based upon relative signal intensities of directly formed analyte ions and photoionized analyte neutrals, the ratio of neutrals to ions was obtained (Mowry et al., 1993).

Most matrices used in MALDI are acids that contain an aromatic ring and a carboxylic group. For example, nicotinic acid, benzoic acid derivatives, and cinnamic acid derivatives are common matrices for UV/MALDI. However, an aromatic ring with a carboxylic acid group is not strictly required. Fitzgerald and coworkers have reported several basic matrices such as benzene derivatives containing amino groups (Fitzgerald et al., 1993). With such matrices, also acid-sensitive compounds can be analyzed by MALDI. Table 1 shows a summary of common matrices used with either UV or IR lasers.

Table 1. Summary of common matrices in MALDI (Karas et al., 1987; Hillenkamp et al., 1991; Karas et al., 1991; Overberg, 1992)

Matrix	Form	Some usable wavelength
Nicotinic acid	solid	266 nm, 2.94 μ m, 10.6 μ m
2,5-Dihydroxybenzoic acid	solid	266 nm, 337 nm, 355 nm, 2.79 μ m, 2.94 μ m, 10.6 μ m
Sinapinic acid	solid	266 nm, 337 nm, 355 nm, 2.79 μ m, 2.94 μ m, 10.6 μ m
Succinic acid	solid	2.94 μ m, 10.6 μ m
Urea	solid	2.79 μ m, 2.94 μ m, 10.6 μ m
Thiourea	solid	266 nm, 2.79 μ m, 2.94 μ m, 10.6 μ m
Tris buffer (pH 7.3)	solid	2.79 μ m, 2.94 μ m, 10.6 μ m
Caffeic acid	solid	337 nm, 355 nm
Ferric acid	solid	337 nm, 355 nm
Vanillic acid	solid	266 nm
Ice (frozen aqueous solution)	solid	10.6 μ m
Glycerol	liquid	2.79 μ m, 2.94 μ m, 10.6 μ m
3-Nitrobenzyl alcohol	liquid	337 nm, 266 nm
2-Nitrophenyl octyl ether	liquid	266 nm
Triethanolamine	liquid	2.79 μ m, 2.94 μ m

Development of Liquid Matrices for Laser Desorption

As described above, Tanaka early used liquid glycerol with an extremely fine cobalt powder for laser desorption (Tanaka et al., 1988). However, the mass resolution was very poor. Partly for this reason, no other groups reported following up on Tanaka's work. MALDI almost exclusively uses solid matrices. Still, some groups have tried to find practical liquid matrices for MALDI because liquid matrices are expected to have important advantages. The advantages include: (1) allowing a method for continuous desorption from the same spot with excellent pulse-to-pulse stability (Lusting et al., 1991) (2) allowing for the analysis of a homogeneous solution of analyte(s) and preventing "sweet spots" (3) allowing for a direct interface with liquid separation system (Li et al., 1993).

Some UV-absorption liquids such as 3-nitrobenzyl alcohol (3-NBA) have been tried as matrices for UV-MALDI. However, problems with this matrix seem to be low mass resolution and high matrix background (Chan et al., 1994; Li et al., 1993; Yau et al., 1993). A second approach has been to dissolve a strong absorbing dye such as Rhodamine 6G in a non-UV absorbing liquid (Cornet et al., 1993). The mass resolution was again quite low, about 50. A third approach has been to use a fibrous material as a solid substrate to which a non-UV absorbing liquid was added. This method has been used to obtain high quality laser desorption mass spectra of porphyrins (Kim et al., 1994). A fourth approach has been to use IR-MALDI. Liquids like glycerol, triethanolamine, and water have absorption bands in IR range. Hillenkamp et al., have demonstrated a spectrum of lysozyme at 14,300 Da by using glycerol as the matrix in IR-MALDI (Overberg et al., 1990). They used a Q-switched Er-YAG laser which has a wavelength of 2.94 μm and a pulse duration of 200 ns.

Mechanisms of MALDI

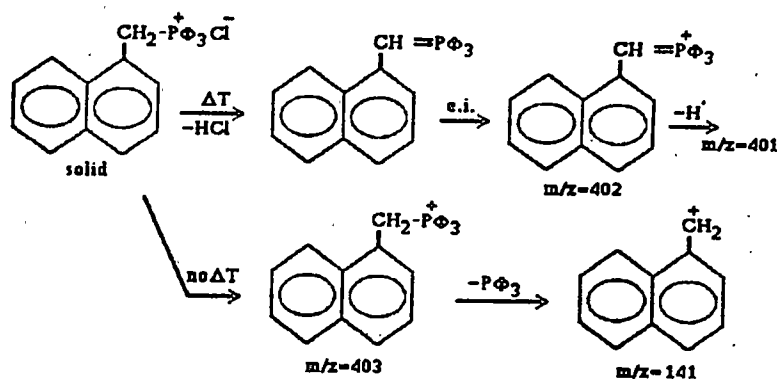
Since MALDI was introduced, many models and mechanisms have been proposed. To explain the MALDI process successfully, one question in particular must be answered. This is how the laser-induced process can transfer large molecules from the condensed phase to the gas phase without damaging the molecules (Vertes, 1991). The fundamental mechanisms of the MALDI process are still under active investigation. Some significant models will be described in the following.

Phase Explosion Model. Sunner et al. proposed a model called "phase explosion" for explaining the phenomenon of desorption ionization in liquid secondary ion mass spectrometry or FAB (Sunner et al., 1988b). This model can be equally well applied to the process in laser desorption since a high energy density is deposited in the material also in laser desorption.

The phase explosion model describes how the condensed phase transforms to a gas phase if the condensed phase is superheated, extremely rapidly to a high temperature. Above the temperature of "critical superheat", the matrix becomes mechanically unstable and undergoes a spontaneous "spinodal" decomposition. This phenomenon is called phase explosion or spinodal decomposition (Sunner et al., 1988b). The temperature for the "phase explosion" is $0.9 \times T_c$, where T_c is the critical temperature. It is explained that large molecules may survive intact despite the high energy density because of rapid cooling to a low temperature as the material transforms from the condensed phase to the gas phase. Furthermore, this model has been supported by a molecular dynamic simulation (Sunner et al., 1993). The

simulations showed that the spinodal decomposition mechanism can explain particle and laser desorption experiments

Cool Plume Model. This model was proposed by Vertes et al. (Vertes et al. 1988 & 1990a). It addressed the laser-solid interaction by describing the hydrodynamics of the laser-generated plume expansion. Subsequently, Balazes et al. refined this model (Balazes et al., 1991) to include also the phase transition, surface recession, and heat conduction processes in the solid, as well as electron-neutral inverse bremsstrahlung laser absorption, multiple ionization, and radiation cooling in the plume. The generated plume undergoes gas dynamic expansion and exhibits cooling. Two distinct time domains can be recognized in this model. The first time domain covers the time when the solid phase has not yet reached the phase transition temperature. This means that there is only a hot spot on the surface of the solid phase. The second time domain starts when the solid phase has been heated to the phase transition temperature. A dense plume is formed, and reactions like protonation, cationization, and adduct ion formation are proceeding in this plume. Subsequently, the temperature of the plume drops to a very low value due to expansion cooling. This cooling has a stabilizing effect on the entrained large molecules. An experiment using the fragmentation of a thermolabile compound, aryltriphenylphosphonium halide as a "thermometer" was claimed to support this model (Claereboudt et al., 1991). The fragmentation pathway is shown below (Claereboudt et al., 1991).



Ionization pathways of 1-naphtylmethyl-triphenylphosphonium chloride through thermal decomposition (ΔT) and in the absence of thermal decomposition (no ΔT).

This pathway has been studied and confirmed separately by electron impact, fast atom bombardment and laser desorption mass spectrometry. Without nicotinic acid present, thermal decomposition product ions, such as the ions at $m/z=401$ and 402 , appeared in the mass spectrum. With nicotinic acid matrix added, product ions, such as the ion at $m/z=403$, obtained from low internal energy aryltriphenylphosphonium ions were observed. No ions at $m/z=401$, 402 were found in the mass spectrum with nicotinic acid added. This showed the MALDI process including a strong cooling mechanism since none of laser-induced decomposition ions were formed.

Homogeneous Bottleneck Model. How can thermally labile molecules survive the very high energy density deposition used in MALDI? According to the homogeneous bottleneck model, there are two possibilities. Large molecules may survive due to an inefficient energy transfer process between matrix and analyte molecules (Vertes et al., 1990c). An additional cooling may result from two processes, phase transitions and heat conduction. Two effective phase transition processes are evaporation and sublimation. Cooling by heat conduction is

important only on a time scale much longer than the laser pulse. According to the authors, poor energy transfer between matrix and analyte molecules results from the mismatch of matrix-analyte interaction frequencies and matrix vibrational frequencies. If the frequencies are mismatched, energy will not be efficiently transferred to analyte molecules. A kinetic model of the energy transfer processes showed that, at an appropriately high sublimation rate, the analyte molecules will be desorbed internally cold (Vertes, 1990). The faster the energy transfer into the lattice, the faster is the cooling by sublimation. According to the computations by Vertes et al., by decreasing the energy transfer rate from the matrix to the analyte, the fragmentation of analyte ions can be decreased. This implies that in order to minimize fragmentation one should (1) keep the amount of analyte molecules as low as practically possible (energy transfer vs. detectability), (2) use a matrix host molecule with as poor a frequency overlap with the frequencies of the biomolecules as possible, (3) use a host matrix with a low sublimation temperature, and (4) use a laser pulse short enough to promote volatilization as opposed to degradation.

Shock Wave Model. Lindner and Seydel proposed a "shock wave model" for the laser desorption ionization process (Lindner & Seydel, 1985). The authors compared LD mass spectra obtained from a thick layer of sample (20 μm) under high intensity laser irradiation (10^{11} W/cm^2) with spectra obtained from a thin layer of sample (1 μm) under low intensity laser irradiation (10^8 W/cm^2). They concluded that under high intensity laser irradiation, little fragmentation and a high molecular ion yield resulted from a laser-driven shock wave. However, under low intensity irradiation conditions, extensive fragmentation and low

molecular ion yield resulted from thermal decomposition. That means that under high intensity irradiation, the heating rate is very high such that the matrix can reach the phase explosion limit without damage to the analyte molecules. Vertes et al. proposed another shock wave-induced mechanism for laser desorption of large molecules in MALDI (Vertes et al., 1988 & 1989). A one-component, one-dimensional hydrodynamics model was developed to describe the expansion of laser-generated plumes in the ion source. The model revealed two hydrodynamic phenomena: first, a compression wave propagating toward the interior of the target that is responsible for the splashing of molten material from a crater, and second, a shock wave in the plasma plume leaving the target that causes the appearance of energetic ions (Vertes et al., 1989).

Photoionization model. Ehring et al. have proposed a photoionization model to explain ionization in LD (Ehring et al., 1992). The authors stated that photoionization is the initial ionization step and that this is followed by ion-molecule reactions to give the final products. The gas-phase ionization energy for common organic compounds is about 7.5-9 eV. The laser photon energy in the UV range is about half of this ionization energy. (The photon energy at is 4.6 eV at 266 nm, and it is 3.7 eV at 337 nm.). In order for the molecules to become ionized, multiple photon absorption is required. This may occur through a two-step resonance absorption mechanism.

Instrumentation for Laser Desorption (LD) Time of Flight (TOF) Mass Spectrometry

Three components of the LD instrumentation will be described: (1) laser ion source, (2) time-of-flight mass spectrometer, (3) ion detectors, and (4) signal processing equipment. The instrumentation for MALDI and SALDI is the same as for LD.

Laser Ion Source

Laser interaction with solids can result in an ultrafast heating rate of the material to a high temperature. Rapid heating which can be produced by pulsed lasers (10^8 - 10^{12} K/s (Levis, 1994)) allows for thermally labile compounds to desorb intact from a condensed phase. The lasers used to deposit energy vary widely in terms of pulse length (CW to fs), irradiance (50 kW/cm² to 100 MW/cm²), and wavelength (151 nm to 10.6 μ m). The most commonly used lasers in LD are ultraviolet (UV) and infrared (IR) lasers. Commercial instruments are typically equipped with a nitrogen UV laser for 337 nm.

Time-of-Flight Mass Spectrometer

The selection of a mass analyzer is critical to obtain the best result in laser desorption mass spectrometry. Since laser interaction with solids may produce completely unexpected ionic and neutral species, the detection of all masses from every laser shot with a high transmission analyzer is preferred. Several types of mass analyzers have been used. When laser desorption was combined with a magnetic sector mass analyzer, the sensitivity was not good due to the low duty cycle (Eloy et al., 1971). Time of flight (TOF) analyzer is a much better alternative for pulsed laser desorption because of high ion transmission, and because the time taken for

the time taken for all the ions to pass through the flight tube is close to the time between laser pulses. In the 1970's, LD/TOF still had problems with broad ion kinetic energy distributions and slow signal processing. However, many improvements have been made since then. The major limitations of the quadrupole and magnetic sector instruments with respect to detecting all masses, scanning speed, and low ion transmission are avoided by the TOF principle itself (Vertes et al., 1993). The basic principle of TOF will now be discussed.

Basic Principles. TOF/MS was first proposed by Stephens in 1946 (Stephens, 1946). The basic linear TOF/MS instrumentation is shown in Figure 1. It consists of an ion source, a flight tube, and a detector. The distance (s) between the ion source plate and the accelerating grid is of the order of 0.5 cm. A fixed voltage V is applied to the ion source plate whereas the grid is grounded. The electric field is $E = V/s$. Ions that originate at the ion source plate are accelerated in the ion source region. They will all have essentially the same kinetic energy in the drift region of the flight tube.

$$\text{Kinetic energy} = mv^2/2 = zeV \quad (1)$$

$$v = (2zeV/m)^{1/2} \quad (2)$$

where m = the mass of ion, v = velocity of the ion, e = the charge of an electron, and z = number of ion charges. Ions of different m/z have different velocities in the vacuum flight tube. The ions travel a flight tube distance, l , to the detector, and the time-of-flight is given by:

$$t = l/v = l(m/2zeV)^{1/2} \quad (3)$$

Flight tube distances (l) ranging from 15 cm to 8 meters have been utilized. Typical

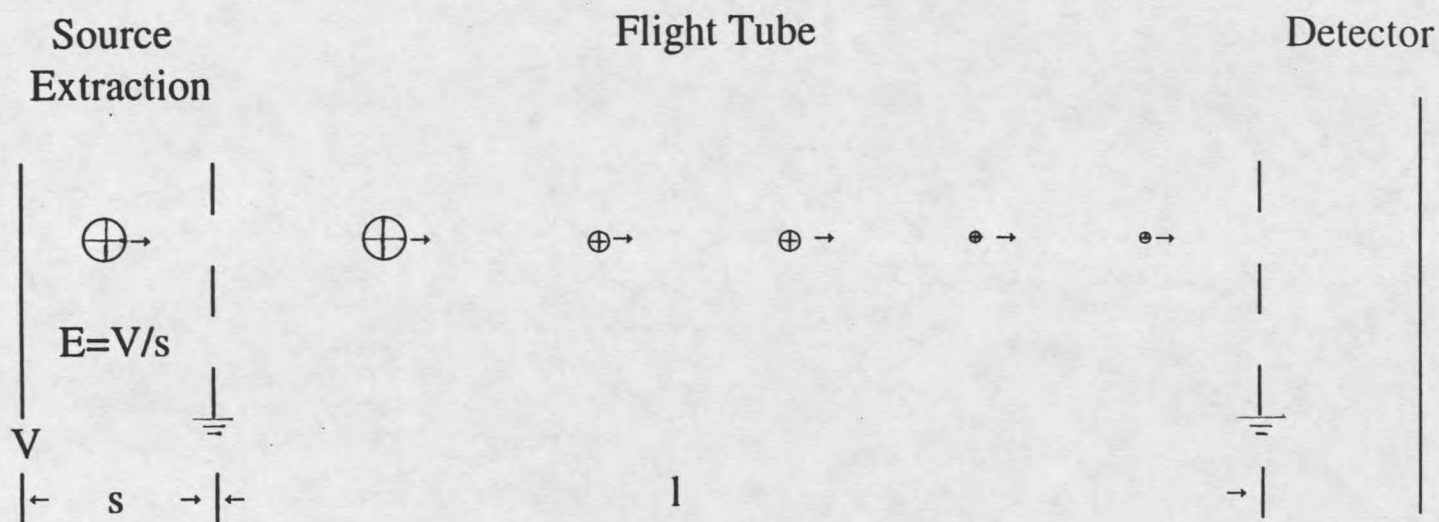


Figure 1. Linear time-of-flight mass spectrometer (from Cotter, 1994).

accelerating voltages (V) range from 3 kV to 30 kV, and typical flight times range from 5 to 100 microsecond (Cotter, 1994).

Advantages of TOF/MS. TOF/MS possesses many critical advantages (Price et al., 1994):

- (1) Ideal where ionization is pulsed.
- (2) Complete mass spectrum is obtained for each ionization event.
- (3) High ion transmission.
- (4) Fastest scanning mass spectrometer with repetition rates of up to 100 kHz.
- (5) High mass range (limited primarily by the detector).
- (6) Low cost.

Because TOF/MS has a very simple design and many advantages as described above, it has become the most popular mass analyzer for laser desorption.

Factors Affecting Mass Resolution of TOF. The mass resolution obtained in linear TOF/MS is usually fairly low. This is due to the time, space and kinetic energy distributions of ions as they fly from the ion source and through the flight tube (Cotter et al., 1992). Here, (1) the temporal distribution, (2) the spatial distribution, and (3) the kinetic energy distribution will be discussed.

(1) Temporal Distribution

Two ions with the same mass may be formed at different times during the ionization event but may still obtain the same kinetic energy in the flight tube. These ions will have different arrival times at the detector. This is referred to as the temporal distribution of the ions.

Because the mass resolution is determined by:

$$m/\Delta m = t/2\Delta t \quad (4)$$

when the time width of the arrival distance (Δt) is getting smaller, the mass resolution will be improved.

(2) Kinetic Energy Distribution

Another factor influencing the mass resolution in TOF/MS is the initial kinetic energy distribution. Ions of the same mass formed with different initial kinetic energies will have different arrival times at the detector, due to different velocities of ions through the flight tube.

(3) Spatial Distribution.

The mass resolution in TOF/MS will also be influenced by a spatial distribution. Ions of the same mass may be formed at the same time and with the same initial kinetic energy, but at different locations in the extraction field. These ions will experience a different acceleration voltage and will have different kinetic energies in the flight tube. They will arrive the detector at different times. This is referred to as the spatial distribution of ions.

Methods for Improving Mass Resolution in TOF/MS. Mass resolution in TOF/MS is affected by the temporal, spatial, and initial kinetic energy distribution as described above. By minimizing, or correcting, for these three distributions, mass resolution can be improved. Ionization by ultrashort pulsed lasers can minimize the uncertainties in the time of ion formation. The effects of the initial kinetic energy distribution can be minimized by using high extraction voltages or compensated for by a reflectron located in the mass analyzer

et al., 1994). The effects of the spatial distribution can be minimized by using low extraction voltages. Three techniques that have been used to improve the mass resolution in TOF/MS will be described: (1) two stage extraction (McLarne et al., 1955) (2) time lag focusing or delayed extraction (McLarne et al., 1955; Martin et al., 1995 & 1996), and (3) reflectron TOF/MS (Mamyrin et al., 1973).

(1) Two Stage Extraction

Because initial kinetic energy focusing demands a use of a high extraction voltages, one approach has been to use two-stage extraction. Two parallel grids define a low field extraction region above the sample plate and high field extraction region in front of the flight tube. For desorption techniques, in which ions are formed at a surface rather than in the gas phase, the spatial distribution is minimized. By accelerating ions in two source gaps with different electric fields, the mass resolution is improved (McLaren et al., 1955). However, the mass resolution still cannot be improved to more than about 1,000 Da with two stage extraction because the initial kinetic energy distribution cannot be compensated for by this method.

(2) Reflectron TOF/MS

Reflectron TOF/MS can solve the initial kinetic energy distribution problem. The principle was first proposed by Mamyrin in his Ph.D dissertation in 1966 (Mamyrin, 1966). The reflectron utilizes an electric field located at the end of the flight tube to reflect the ion trajectory around 180°, which is back along the initial flight path of the ions. A higher kinetic energy ion will penetrate the retarding field of the reflectron to a greater depth. Thus, high kinetic energy ions travel a longer distance, arriving at the detector at the same time as less

less energetic ions.

Two types of reflectron TOF/MS have been developed: (a) two-stage Mamyrin reflectron (Shmikk et al., 1972; Mamyrin et al., 1973) (b) coaxial reflectron (Della-Negra & Le Beyec, 1985).

(a) Two-Stage Reflectron

The two stage reflectron TOF/MS was suggested and designed by Mamyrin (Figure 2). The reflectron consists of three parallel grids. Ions enter the reflectron through the first grid, which potential is the same as that of the flight tube. The ions are retarded to about one third of their initial kinetic energy by the time they pass through the second grid, located at about 10% of the total reflectron depth. The ions are turned around in the second, longer stage which is terminated by a grid held a potential slightly above that of the ion source plate. Each ion exits with the reflectron with kinetic energy that is identical to its incoming energy, but with the velocity in the opposite direction. In the Mamyrin design, the reflectron is set at a small angle with respect to the flight axis such that the ion detector could be located off axis. At that time, the best resolution obtained with this design was $m/\Delta m = 3500$ for a $m/z = 1278$ ion (Mamyrin et al., 1973).

(b) Coaxial Reflectron

A coaxial reflectron TOF is shown in the bottom of Figure 2. Della-Negra and Le Beyec originally described this design in which exiting ions travel back along the same flight axis (Della-Negra, 1985). A doughnut-shaped detector is used in this reflectron design. The incoming ions pass through the center hole in the detector. Similar potentials are applied to the grids as in the Mamryin design.

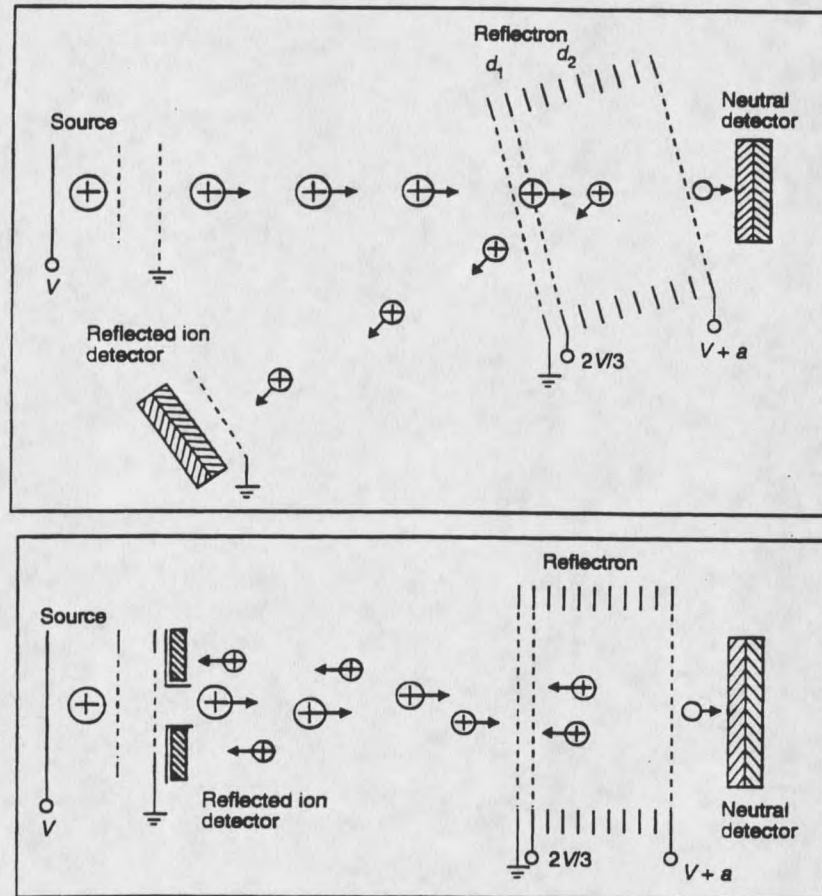


Figure 2. Two stage Mamyrin reflectron (top) and coaxial reflectron (bottom).
 (From Cotter, 1994).

(3) Delayed Extraction

Delayed extraction was first developed by Wiley and McLaren in 1955 (McLaren et al, 1955). They called it "time lag focusing". There is a time delay between the laser pulse and the application of a pulsed extraction field. This is different from the traditional way where the extraction field is applied continuously. Vestal et al. designed a new pulsed delayed extraction system for MALDI in 1995. In their system, the ions are produced in a weak electric field and subsequently extracted by the application of a high voltage pulse after a predetermined time delay (Vestal et al., 1995). By using this method, spatial, temporal, and energy distribution can be compensated. By combining the reflectron mode with delayed extraction, the mass resolution has been improved to about 7,500 Da for peptides (Martin et al., 1996).

Ion Detectors in TOF/MS.

The number of ions produced in LD, MALDI, or SALDI are too large for ion counting. Analog detection is therefore used. The most common ion detector are dual, microchannel plates in a Chevron geometry. However, combinations of one microchannel plate and a conventional dynode electron multiplier are also used.

Signal Processing.

In the 1970s, electronic techniques were developed that were fast enough to enable one to record full mass spectra in each time-of-flight cycle. Transient recorders (Cotter, 1994; Lys, 1994) are most commonly used for TOF/MS signal processing. The transient recorder is based on analog to digital conversion. It was introduced in the 1980's by several

companies. A transient recorder consists of sample and hold unit (S/H), analog to digital converter (ADC), and a memory (Figure 3). When packaged as a traditional oscilloscope, a transient recorder is referred to as a Digital Storage Oscilloscope, or DSO. When a trigger pulse is received by the unit, sampling begins at a rate determined by the clock, called the sample rate, and the digital representation of the voltage present at the input is stored in successive memory locations. Each start (trigger) pulse generates a complete representation of the entire signal. Each memory location corresponds to a small segment in time. The results of many repetition of an experiment can be averaged in the memory of the DSO, which can improve signal /noise ratio. Its value represents the voltage of the input signal during that time period after the trigger pulse. Each trigger can produce as many as 10^5 - 10^6 data values.

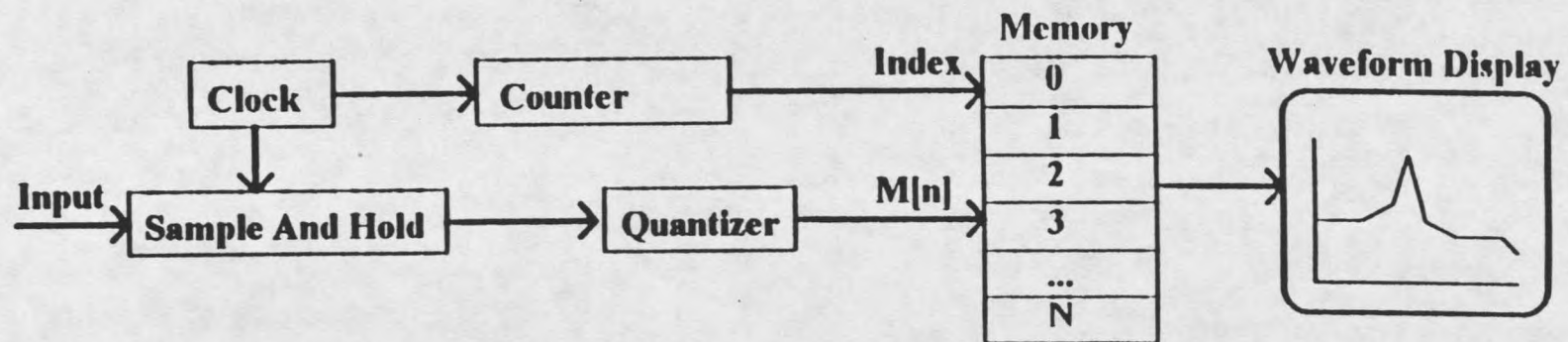


Figure 3. Block diagram of a transient recorder .

Objectives of the Thesis

Development of a New Laser Desorption Ionization Method

The major objective of this thesis is to develop a new laser desorption ionization method. The method was discovered at the beginning of this thesis work and has been named "Surface-Assisted Laser Desorption Ionization (SALDI) Mass Spectrometry." Established laser desorption ionization methods, particularly MALDI has disadvantages for some types of analysis as previously discussed briefly in Chapter 1. With the goals of overcoming some of these disadvantages, efforts will be made to characterize, develop, and improve SALDI in this study. The SALDI matrix system that we developed is composed of three major components, the SALDI solid, the SALDI solvent, and the SALDI "adhesive". The importance of each of these components to the analytical characteristics of SALDI will be studied.

SALDI Solids. In MALDI, organic matrices are used to absorb the UV laser light. The UV absorption results in a successful desorption ionization process if the matrix is well matched to the analyte. Because of a high molar ratio of matrix to analyte in MALDI, a high background of matrix ions cannot be avoided in the low mass range. This causes a problem in identifying analytes that have molecular weights below 200-400 Da in MALDI. In order to reduce this matrix background interference, a material which yields no or few ions at low mass should be used. This material should also be an efficient UV light absorber. Most UV-absorbing organics show extensive ionic fragmentation under intense laser irradiation. To avoid this problem, metal powders could be used since only a simple mass spectrum

consisting of M_n^+ ions (M = metal, $n=1,2, 3$, and so on) is obtained in laser desorption of the metals. Tanaka et al. showed that a cobalt metal powder can be used as the energy absorber in laser desorption (Tanaka et al., 1988). However, the mass resolution obtained by these researchers was quite low. We found that graphite or activated carbon can be used to couple UV-energy into the analyte solution and christened the basic method SALDI (Sunner et al., 1995). A low matrix ion interference was observed (Sunner et al., 1995). The characteristics of the SALDI solid will be studied.

SALDI Solvents. A decisive factor for obtaining good mass spectra in MALDI is the matrix/analyte co-crystallization process. Not every analyte has the ability to co-crystallize with available matrices. Poor matrix/analyte co-crystallization process results in poor MALDI mass spectra. A high matrix to analyte molar ratio (usually, 1000:1 to 50,000:1 (Overberg et al., 1990)) is required to obtain optimized MALDI mass spectra. Empirically, it is found that the co-crystals are not formed uniformly over the sample. There is always a need to move the UV laser focus over the sample to find the "sweet spots" where co-crystallization occurred. Furthermore, in a mixture, suppression of some analytes by the presence of other analytes is a common problem in MALDI. In addition, most MALDI matrices are acids, and some analytes are very sensitive to acids. For these reasons, analyte molecular ions are frequently not observed in MALDI mass spectra. For an unknown sample, trial and error must be used in order to obtain useful MALDI mass spectra. The use of a liquid matrix is a possible solution to the sweet spot problems. A liquid matrix does not require a co-crystallization process. Thus, the ratio of matrix to analyte is not expected to

be so critical. Furthermore, the analyte distribution should be much more homogeneous in the liquid matrix. It would then not be necessary to search around the sample surface for a "sweet spot". It may also be expected that the suppression effects should be reduced since there is no requirement for a crystallization process. Non-acid liquid solvents could be selected for the analysis of acid-sensitive compounds. Since the SALDI solid plays the role of absorbing the laser irradiation, liquid solvents do not need to be UV-absorbing.

SALDI "Adhesive". Two phases, the SALDI solid and the SALDI solvent, are used together in SALDI analysis. The solid used is a very fine powder. As the solvent evaporates in the vacuum chamber in the mass spectrometer, the powder will dry and may spread in the ion source. By introducing a non-volatile component with adhesive-like properties into the matrix system, the solid particles could possibly be made to remain in the sample well to reduce the contamination problem in the ion source of the mass spectrometer. Thus, a material that can make the SALDI solid particles "stick" together was searched for. This material will be referred to as the SALDI "adhesive".

SALDI Solvent Effects

The effects of the SALDI liquid solvent were studied in order to enhance the understanding of the SALDI process and to optimize the SALDI results. This includes a study of the effect on the mass spectra of the gas phase basicity of the SALDI solvent. The effects of adding acids or salts to the SALDI solvent were also studied.

Applications of SALDI

To demonstrate the potential of practical SALDI analysis, a variety of compounds will be analyzed by SALDI. The compounds will include: (1) Biochemicals; (2) Polymers; (3) Porphyrins; and (4) Volatile and involatile organics.

Solid Phase Extraction (SPE)/SALDI

The activated carbon used in SALDI has the ability to adsorb traces of organic compounds and biochemicals on its surface. Activated carbon is also used as the adsorbent in SPE. The potential for direct analysis of the organic compounds by SALDI on the SPE adsorbent used in SPE will be investigated. The combination of SPE and SALDI could possibly constitute a fast qualitative screening method. The possibility of quantitative analysis in SPE/SALDI will also be studied. Organic compounds such as drugs, herbicides, amino acids, peptides will be investigated by SPE/SALDI. The possibility to use solid phase microextraction (SPME) combined with SALDI was also explored.

Thin Layer Chromatography (TLC)/SALDI

The direct analysis of sample spots on the TLC plates by mass spectrometry has been explored since the 1960's (Kaiser et al., 1969). Different ionization methods such as EI, CI, FAB, LD, and MALDI have been tried. SALDI could seem to offer an attractive way to combine TLC with mass spectrometry. The new combination of TLC/SALDI was investigated. The potential to analyze biochemicals and organics by using this analysis method was explored.

CHAPTER 2

EXPERIMENTAL

Instrumentation

The experiments were performed on a Perceptive LaserTec Benchtop II reflecting instrument with a 1.35 m vertical flight tube. A 337 nm nitrogen laser (CVI Laser, Newton, MA) was used for desorption. The accelerating voltage was 20 kV, the guide wire was set at 0.1%, and the guide voltage was set 70%, unless otherwise noted. The spectra were mass calibrated and plotted using Perceptive software and GRAMS (Galactic, Salem, NH). The laser power (LP) setting and the laser shot (i.e. scan average (SA)) will be described in the individual spectrum. The threshold laser power setting in this instrumentation for sinapinic acid is about 350 to 380. Unless otherwise noted, mass calibration was internal, and two known peaks in each spectrum were used as calibration points. The mass resolution ($m/\Delta m$) was based upon Δm being full width of the peak at half its maximum height.

Materials

Arginine, phenylalanine, isoleucine, adenosine, bradykinin, angiotensin I, angiotensin II, methionine enkephalin, melittin, polyalanine, carboxypeptidase Y (CPY), cytochrome C, and

myoglobin were obtained from Sigma. TPCCK-treated trypsin (88.3%) was purchased from Worthington. Urea and ammonium bicarbonate were obtained from Baker. All the organic solvents were obtained from Fisher. Diuretics were obtained from Aldrich or Sigma, and the triazine kit (Cat. no 4-9092) were obtained from Supelco. Sodium dodecyl sulfate, glucose, and fucose were obtained from Sigma. Glycerol, thioglycerol, 3-nitrobenzyl alcohol, mannose, diethanolamine (DEA), and triethanolamine (TEA), dithiothreitol and dithioerythritol were obtained from Aldrich. The poly(ethylene glycol) (PEG) standards were obtained from Aldrich. Magic bullet was made by dithiothreitol and dithioerythritol (3:5, w/w). Vacuum pump oil (100% paraffin oil) was obtained from Inland Vacuum Industry. Sinapinic acid (3,5-dimethoxy-4-hydroxy-cinnamic acid), α -cyano-4-hydroxy cinnamic acid, porphyrins, and Nafion (5%) were purchased from Aldrich. Sucrose (Western Family) was obtained in a grocery store. Adhesive was made by Itoya of America (Torrance, CA), and adhesive spray was obtained from Durco (Loctite Corporation, North American Group). Double side tape was Scotch. Acetic acid (99.7%) was purchased from EM. All the organic solvents including methanol, ethanol, chloroform, toluene, acetone, and acetonitrile were obtained from Fisher.

Several different types of graphites were used in this research. Graphite (10-150 μm), for lubrication and machining purposes, was used in the early work (Dixon No. 635; Lubricating Flake Graphite, Jersey City, NJ). Graphite (2 μm) powder, graphite flakes, and fluorinated graphite polymers $(\text{CF}_x)_n$, $n=0.8$; $(\text{CF}_x)_n$, $n=1$) were obtained from Aldrich. Different sizes of activated carbon were used. Activated carbon (Draco, 100 mesh), activated carbon (Draco, 12-20 mesh), and activated carbon (Draco, 4-12 mesh) were purchased from Aldrich.

Carbon black was obtained from Aldrich. Activated charcoal and iron powder were obtained from Matheson, Coleman & Bell. Carbo pack (120-400 mesh) was obtained from Supelco (cat. no, 5-7210) and C_{60} was obtained from TCI. Copper spray was obtained from Permatex (Loctite Corporation, Mexico City). Graphite aerosol spray was obtained from Ted Pella.

The vis-1 single processor (cat. no 5-7080) and filtration tubes (cat. no 5-7024) were purchased from Supelco. Polyethylene frits were included with filtration tubes. The tips were obtained from Fisher.

Sample Preparation for SALDI

In the following, SALDI sample preparation methods are described. Four methods were used throughout this study. In most cases, the SALDI sample preparation was based on the description given here. However, in the developments of SPE/SALDI, SPME/SALDI, and TLC/SALDI, the sample preparation was adjusted for specific requirements. Additional details are described in the individual chapters.

Method A and method B were used when SALDI was first developed. In both methods, the analytes were first dissolved in a volatile solvent like methanol or water. In method A, 0.50 μ L of glycerol was applied to the well or probe tip, and 1.0 μ L of the peptide or protein solution was added using a pipet. The graphite powder was then drop-placed on top of the liquid with a spatula in an amount sufficient to cover the liquid surface. For best results, the graphite particles should not be immersed in the liquid but should remain on the surface such that the surface had the appearance of a dry powder. However, method A is not recommended anymore since it causes serious contamination problem in the ion source.

In sample preparation method B, 50 vol% of glycerol was added to methanol. An approximately equal volume of graphite powder was mixed with the glycerol/methanol solution using a vortexer. One microliter of the slurry was pipetted into the probe well where the methanol evaporated after a few seconds. One microliter of a solution of the analyte in either methanol or water was subsequently added to the well. After 1-2 minutes in air at room temperature, the slurry had a dry appearance, and the probe was inserted into the ion source.

In addition to the methods A and B, used in the early SALDI work, the sample preparation was revised in an effort to improve the technique. Because of the contamination problems in the ion source caused by the SALDI solid, "adhesive" needed to be added to the SALDI suspension. The details are described under "SALDI Adhesive" in Chapter 3. Mainly, sucrose was used to immobilize the SALDI solid powder. The sample preparation is called "method C". In this method, the SALDI suspension was composed of three major components, the SALDI solid, the SALDI solvent, and the SALDI "adhesive". There are many choices for these three components. To simplify the description of the method C, glycerol is taken as representative of the SALDI solvent and graphite as representative of the SALDI solid in the following description. In method C, a solution was made by mixing sucrose/glycerol/methanol= 5/28/67 (w/w/v). First, a 15% sucrose in glycerol (w/w) solution was made first. Then, one volume of this 15% sucrose in glycerol solution was mixed with two volumes of methanol. An approximately equal volume of dry graphite powder was mixed with the sucrose/glycerol/methanol solution using a vortexer. One μL of this SALDI suspension was applied to a sample well, and 1.0 μL of analyte solution was added. This

sample was left in air at room temperature for at least 5 minutes to allow most of the volatile solvents to evaporate. The sample tray was then inserted into the ion source of the mass spectrometer. Method C worked well for polar SALDI solvent.

Sucrose will not dissolve in nonpolar solvents. An alternative way to add sucrose to the SALDI suspension will be referred to as method D. In method D, one volume of a nonpolar SALDI solvent such as NBA or PEGs was mixed with one volume of organic solvent such as methanol. A 5.0% sucrose in water (w/w) was made separately. An approximately equal volume of dry graphite powder was mixed with the nonpolar SALDI solvent matrix/methanol solution using a vortexer. One microliter of the SALDI suspension was pipetted to a sample well, and subsequently one microliter of a 5.0 (weight) % sucrose in water solution was added to this suspension. Finally, one microliter of an analyte solution was added to the same sample well. After 10 minutes in air at room temperature, the sample was inserted in the mass spectrometer for SALDI analysis.

CHAPTER 3

DEVELOPMENT OF SALDI METHOD

Introduction

In 1988, Tanaka reported a new laser desorption method, a solid inorganic material combined with a liquid matrix, fine cobalt powder with glycerol in laser desorption (Tanaka et al., 1988) as mentioned in the Introduction. A serious disadvantage of Tanaka's method is that the mass resolution is quite low (about 20). Alternative to metal powders, inorganic materials which have absorption in the UV range are possible assisting materials for UV laser desorption. According to a report by Ergun et al. (Ergun et al., 1961), graphite has an absorption band between 3.1 eV and 6.2 eV (200 nm and 400 nm) (Figure 4). Thus, graphite could possibly be used as the assisting material in laser desorption. Here we report a successful method to improve mass resolution and sensitivity in UV laser desorption using graphite powder with liquid solution. We have named this method Surface Assisted Laser Desorption Ionization (SALDI). In the following, results from graphite SALDI will first be presented. Additional, experiments on SALDI solid, SALDI solvent, and SALDI "adhesive" will also be described.

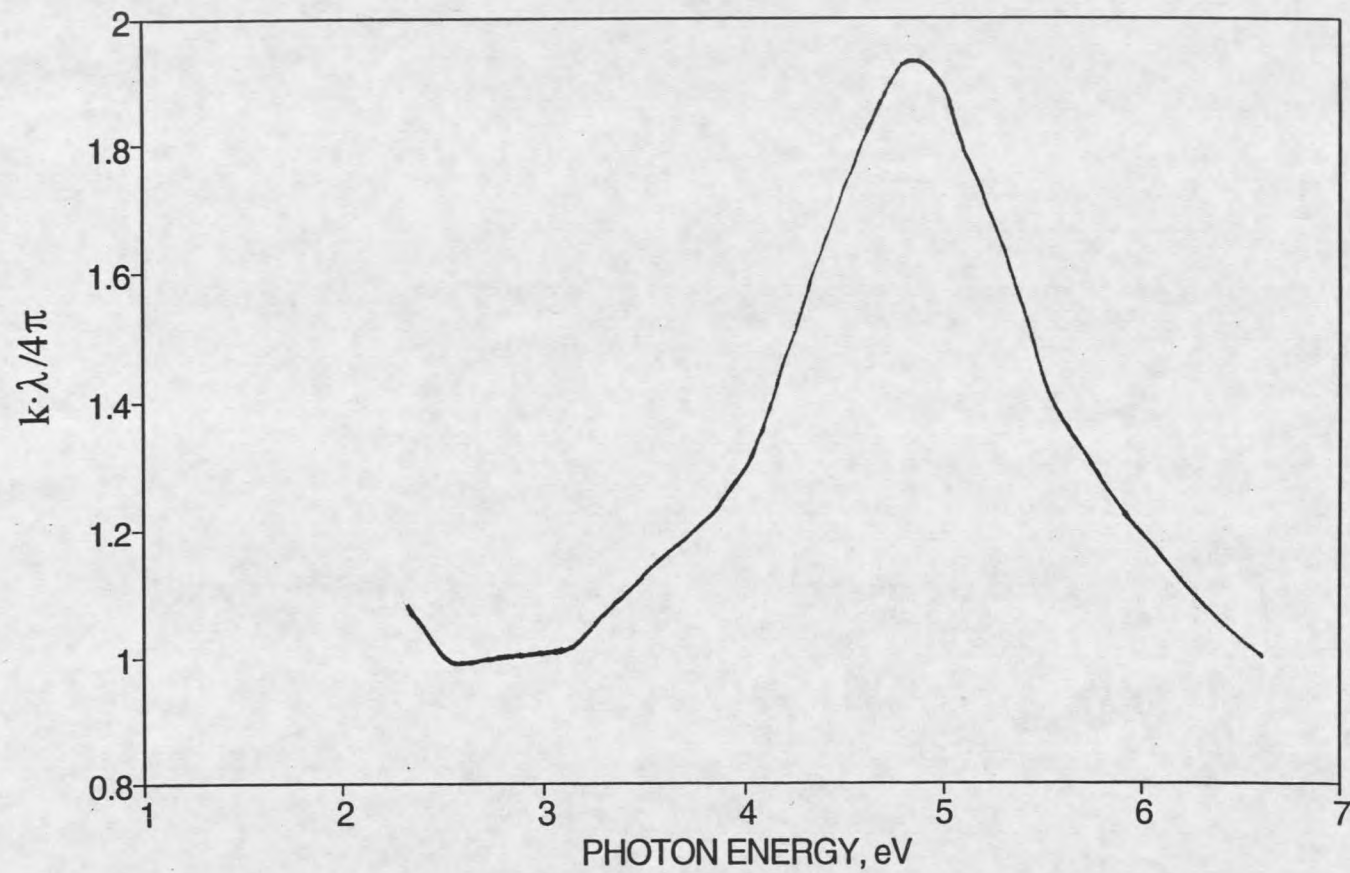


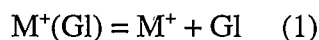
Figure 4. ($k \cdot \lambda / 4\pi$) of graphite as a function of the photon energy in the ultraviolet and wavelength range. k is the extinction coefficient, and λ is the wavelength (Ergun et al., 1961).

Early SALDI Results

Large graphite particles (10~150 μm) were used to couple UV laser-energy into the liquid solution. The large size of the graphite particles used is significant. Tanaka et al. used particles of near atomic dimensions, which diameter was smaller (300 $\text{\AA}$) than the wavelength of the laser light was used. When the cobalt powder is suspended in glycerol, a "bulk" desorption process is expected to result upon UV laser irradiation. In contrast, we are using particles that are many orders of magnitude larger than the wavelength, i.e., in the 10-150 μm size range. The graphite particles are as large as or larger than the laser focus. The results on neat glycerol (Gl) and diethanolamine (DEA) and on biochemicals dissolved in glycerol will be reported in the following (Sunner et al., 1995).

Two different sample preparation methods were used, and they will be referred to as methods A and B (see Chapter 2, Experimental). The low-mass region of a graphite surface-assisted laser desorption/ionization (SALDI) spectrum of Gl is shown in Figure 5. All major peaks are easy to identify. Thus, the ion at $m/z=93$ is protonated glycerol. This ion and the Gl fragments at $m/z=75$, 57, 45, 31, 29, and 19 are all prominent in the FAB spectra of Gl. The intense peaks at $m/z=23$, 39, and 133 are due to Na^+ , K^+ , Cs^+ , respectively. Ubiquitous alkali salt contaminants were observed in all SALDI mass spectra. However, the abundance of the different ions, in particular of Cs^+ , varied considerably between different experiments. The peaks at $m/z=115$, 131, and 225 are due to alkali ion/glycerol adducts, $\text{Na}^+(\text{Gl})$, $\text{K}^+(\text{Gl})$, and $\text{Cs}^+(\text{Gl})$, respectively. A series of low-intensity

peaks due to carbon clusters, $C_nH_m^+$, with $n = 1-25$ and beyond and with $m = 0$ to about 4, is also observed in some SALDI spectra. The spectrum in Figure 5 is, with the exception of the carbon clusters, very similar to FAB spectra of solutions of alkali halide salts in Gl (Sunner et al., 1993a). One major exception is that the Gl dimer at $m/z = 185$ was not observed in the graphite SALDI spectrum of Gl. Though a low dimer abundance may result from high analyte or salt concentrations (Sunner et al., 1988a), it is likely that the desorption temperature in SALDI is higher than in FAB and that the Gl ions are formed with a higher internal excitation energy. In this context, it is noteworthy that there is a pronounced asymmetry in the K^+ and Cs^+ ion peaks. This peak asymmetry can be explained by extensive metastable decomposition in the acceleration region of the TOF/MS. In this case, the reaction is declustering of cationized species,



Gl = glycerol

Figure 6 shows a graphite SALDI mass spectrum of a 0.25% DEA solution in Gl. Despite the relatively low analyte concentration, protonated DEA at $m/z = 106$ is seen to dominate over protonated Gl at $m/z = 93$. In FAB, proton transfer from Gl to a more basic analyte occurs during the desorption event (Sunner et al., 1988a & 1986). The gas-phase basicity (at 300K) of DEA of 220 kcal/mol is significantly higher than the gas-phase basicity of Gl at 196 kcal/mol (Sunner et al., 1986). Thus, the proton transfer reaction,



is exoergic. The same reaction should occur in SALDI. However, in the SALDI spectrum, the abundance of $DEAH^+$ relative to GIH^+ is about 10 times that observed in FAB for the

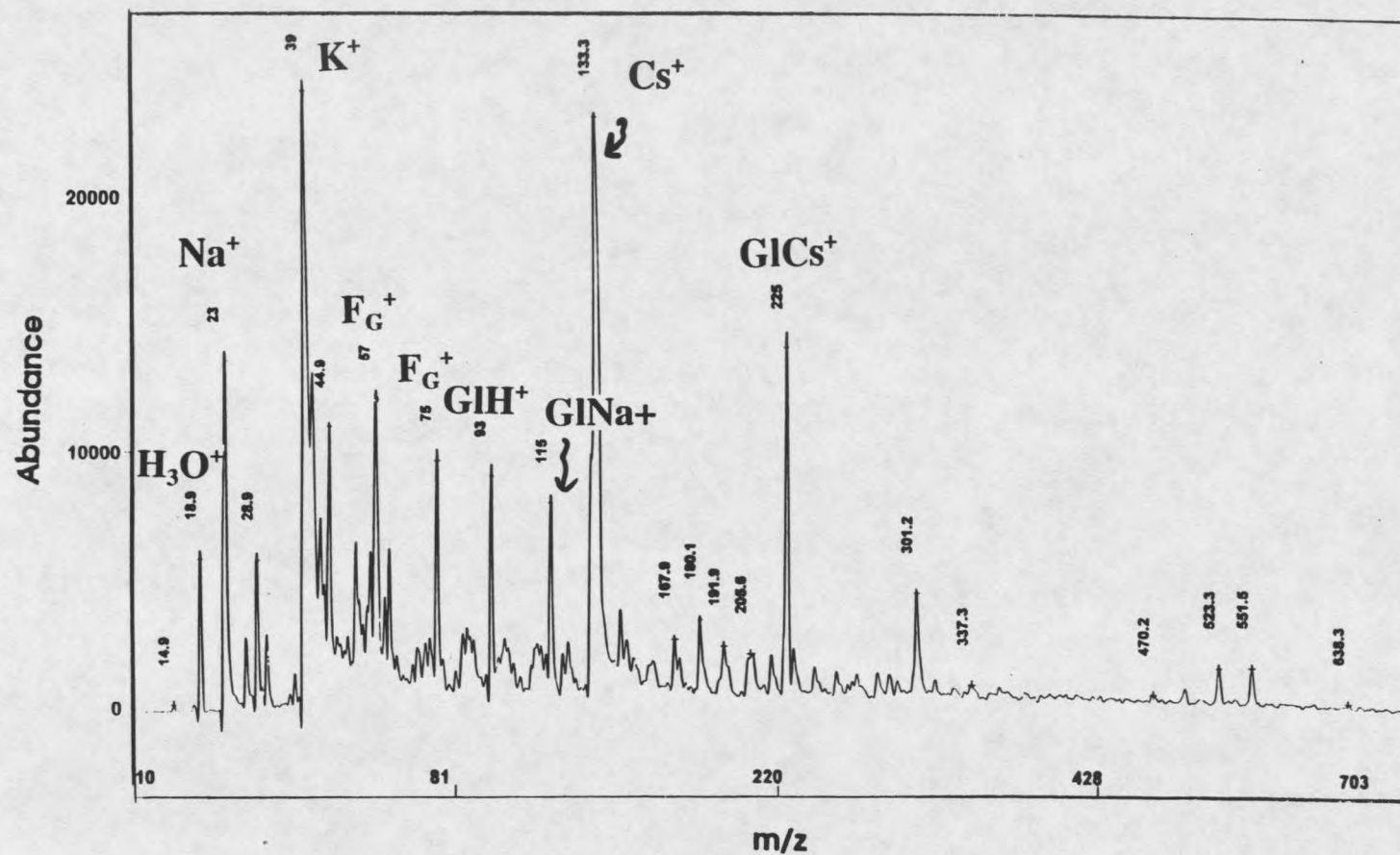


Figure 5. Graphite/SALDI (337 nm) mass spectrum of glycerol.

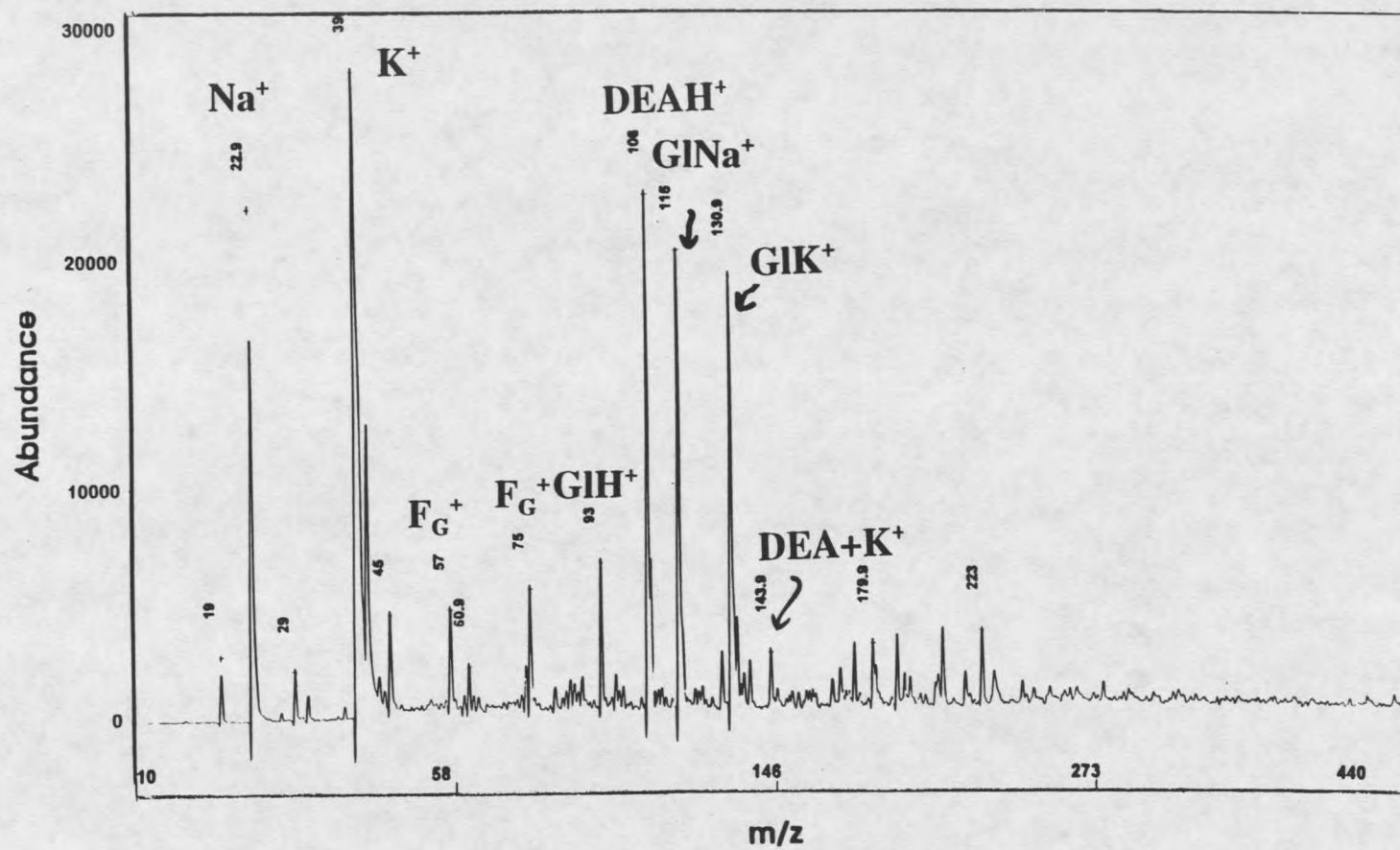


Figure 6. Graphite/SALDI (337 nm) mass spectrum of a solution of 0.25% (v/v) DEA in Gl.

same DEA concentration. This is not surprising, considering that the volume of the high-density selvedge (desorption plume), where reaction 2 occurs, is expected to be much larger in laser desorption than in FAB, resulting in a more complete reaction. In contrast, cationized GI ions [$\text{Na}^+(\text{GI})$ at $m/z=115$ and $\text{K}^+(\text{GI})$ at $m/z=131$] are much more abundant than cationized DEA ions [$\text{Na}^+(\text{DEA})$ at $m/z=128$ and $\text{K}^+(\text{DEA})$ at $m/z=144$] as expected in the absence of reactive coupling between these ionic species. One reason for this is likely that the Na^+ and K^+ ion affinities of GI and DEA are close.

In the low-mass region of the SALDI spectra, close to unit mass resolution is obtained. In addition, SALDI spectra are considerably less congested in the low-mass region than MALDI spectra. Unit resolution, together with a relatively low chemical background, would seem to make SALDI suitable for the analysis of low molecular weight compounds, including smaller peptides in proteolytic digests.

Figure 7 shows a spectrum of the same DEA/GI solution as in Figure 6, except that the laser power used is significantly higher. The most notable feature of the spectrum is the high abundance of the C_nH_m^+ carbon cluster ions. In this spectrum, the cluster sequence can be followed up to $n=30$, and peaks that differ only in the number of hydrogens are resolved up to $n=13$. It is noteworthy that the larger carbon clusters have fewer hydrogen atoms and also that every fourth cluster (C_{11}^+ , C_{15}^+ , C_{19}^+ , etc.) is somewhat more abundant. A number of GI peaks are also seen in Figure 7, including GIH^+ at $m/z=93$ and the $\text{Cs}^+(\text{GI})$ peak at $m/z=225$, as well as the usual GI fragments. All these ions need not coexist. The "cold" glycerol ions may well originate from a lower laser pulse energy density region (peripheral) within the laser focus than the "hot" carbon cluster ions.

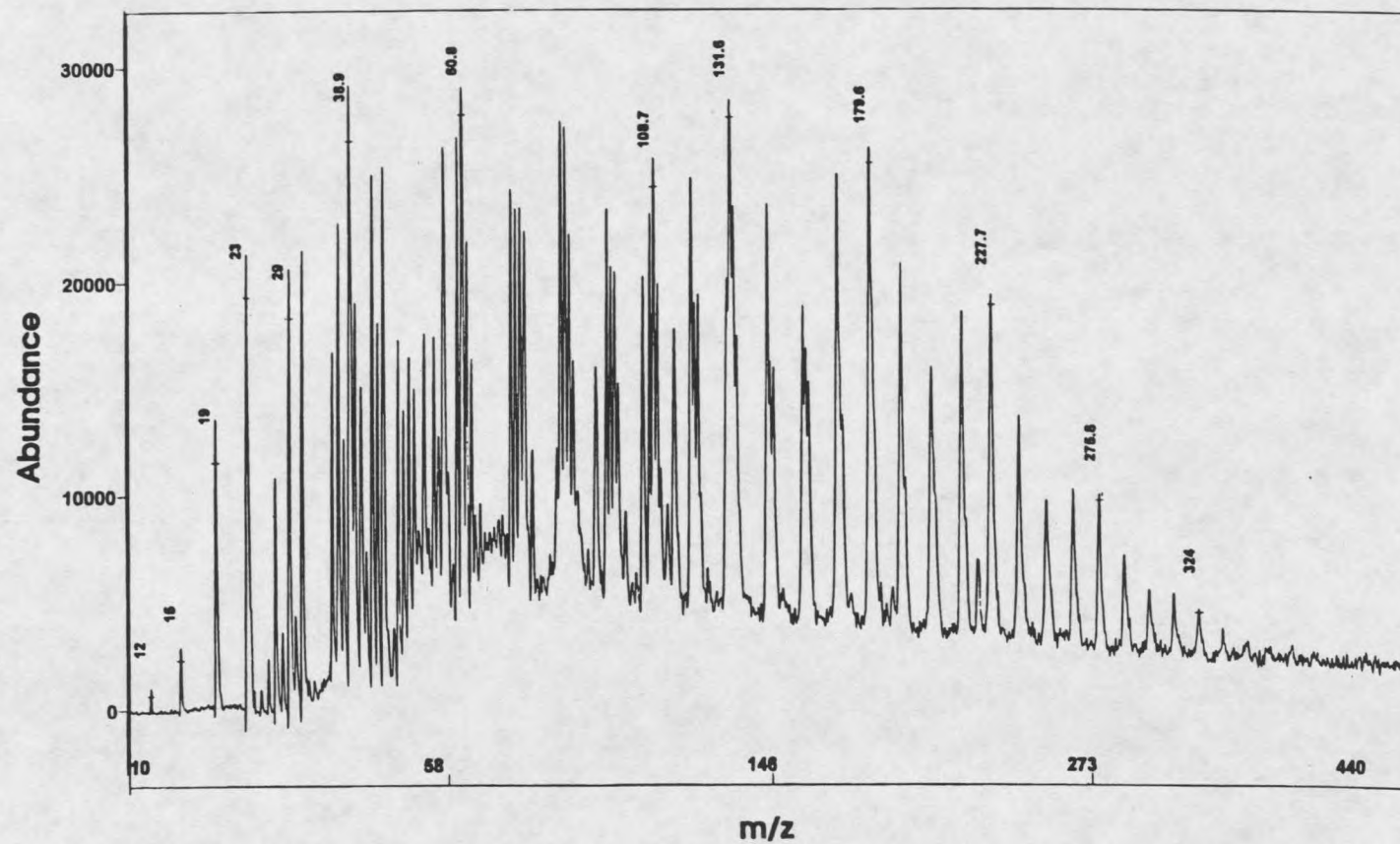


Figure 7. Graphite/SALDI mass spectrum (337 nm) of 0.25% DEA in Gl at an elevated laser power setting. The spectrum is dominated by carbon clusters.

Graphite SALDI can be used for the analysis of biomolecules. Figure 8 shows a spectrum of a 1.0×10^{-3} M solution of bradykinin in Gl (1 nmole sample). The inset shows the high-mass region in greater detail. The major peaks are due to protonated bradykinin at $m/z=1061$ and to adducts of the peptide with Na^+ , K^+ , and Cs^+ at $m/z=1083$, 1099, and 1193, respectively. The mass resolution for these peaks, measured at half-height, is about 300. In the low-mass region, the alkali and Gl ions are present. The alkali ions originate primarily from the surface of graphite. This was proven by the following experiment. Graphite powder was heated for 15 minutes on a heating plate before being used for SALDI. The SALDI mass spectrum of bradykinin (1 nmole) obtained from heated graphite powder is shown in Figure 9. The sodium adduct ions of bradykinin seem to dominate the spectrum. Furthermore, the potassium adduct ions of bradykinin are more abundant than protonated bradykinin molecular ions. The heating process likely released more sodium and potassium ions to the graphite surface. It may also be observed in Figure 9 that the mass resolution of protonated bradykinin ions is not as high as in Figure 8.

Graphite SALDI can be used to obtain also protein spectra. An example is shown in Figure 10 for a 800 μM cytochrome C solution in Gl. In the pseudo-molecular ion region, the Cs^+ -protein adduct gives a clearly separated peak. However, the mass peaks due to the protonated protein and to the Na^+ and K^+ protein adducts are not resolved, and the resulting composite mass peak is rather broad. The mass resolution, measured on the Cs^+ adduct peak, is again about 300. Though we have repeatedly obtained graphite SALDI spectra of cytochrome C and myoglobin, one usually has to search over the sample surface for a considerable time to obtain spectra such as that shown in Figure 10. In addition, such spectra

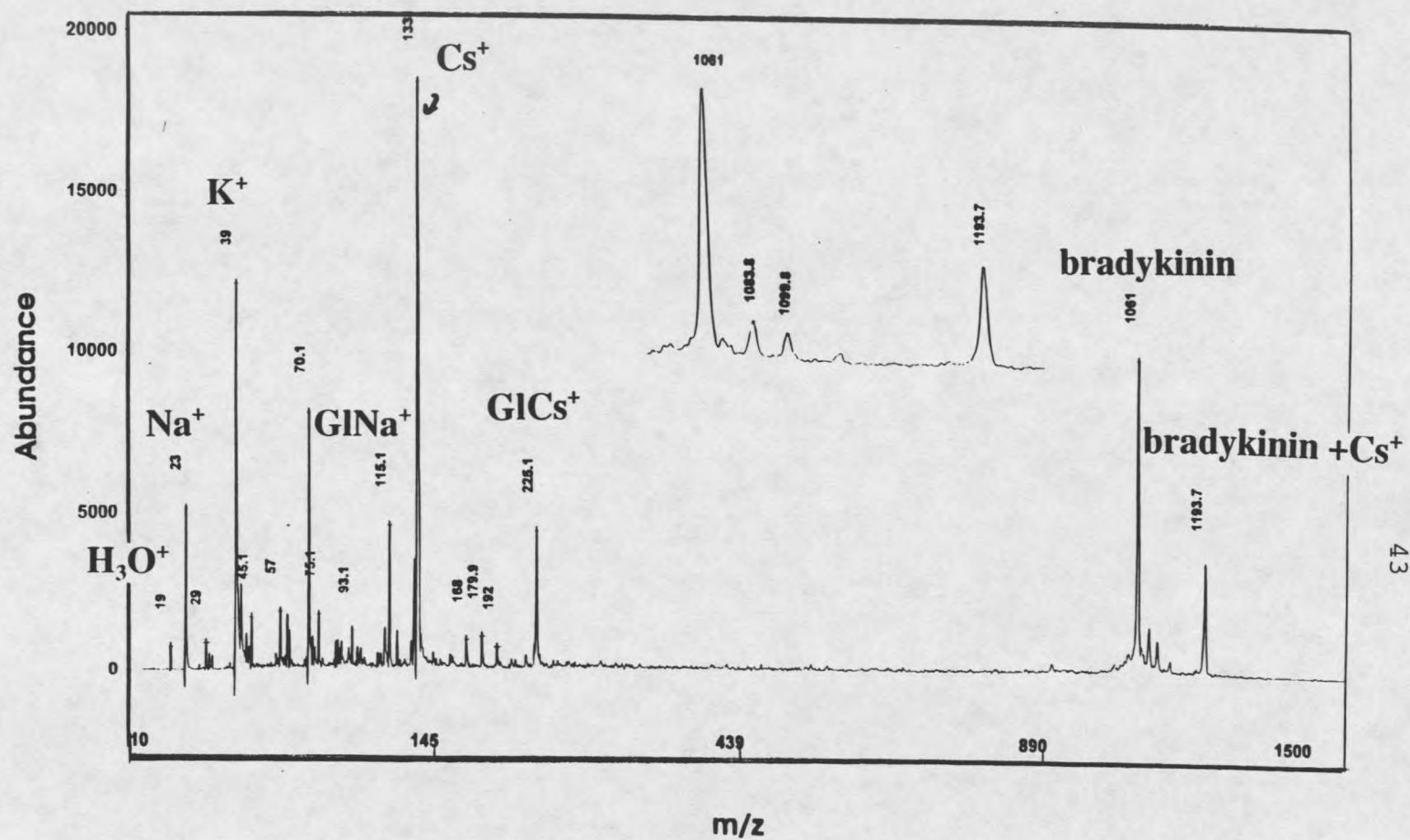


Figure 8. Graphite/SALDI mass spectrum of bradykinin ($\text{mw} = 1060.5$). Internal mass calibration using Cs^+ and $\text{Cs}^+(\text{bradykinin})$. Inset shows the bradykinin pseudo-molecular ion region on an expanded scale.

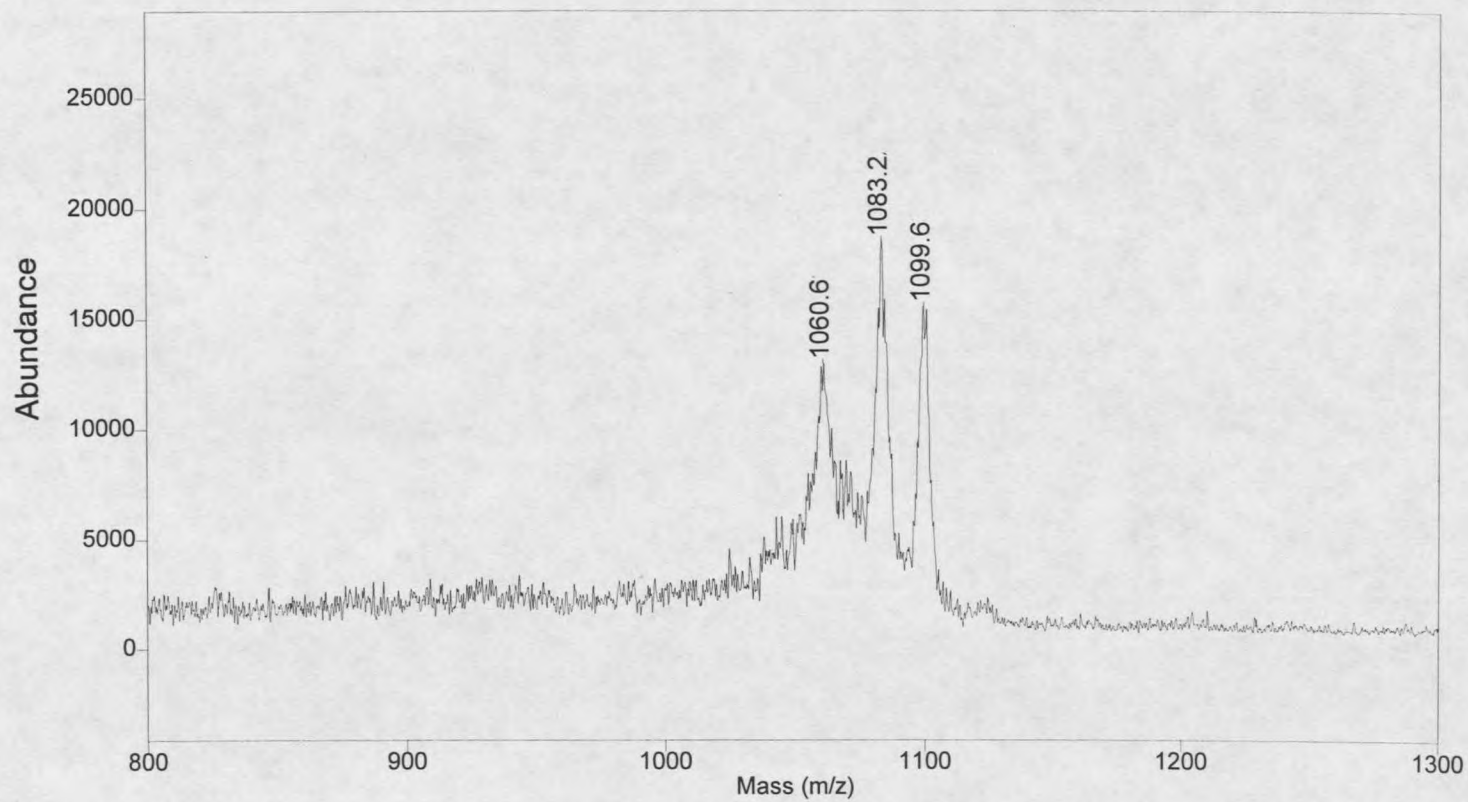


Figure 9. Graphite/SALDI mass spectrum of bradykinin (mw= 1,060.5). Graphite powder was heated on a heating plate for 15 minutes before it was used as the SALDI solid.

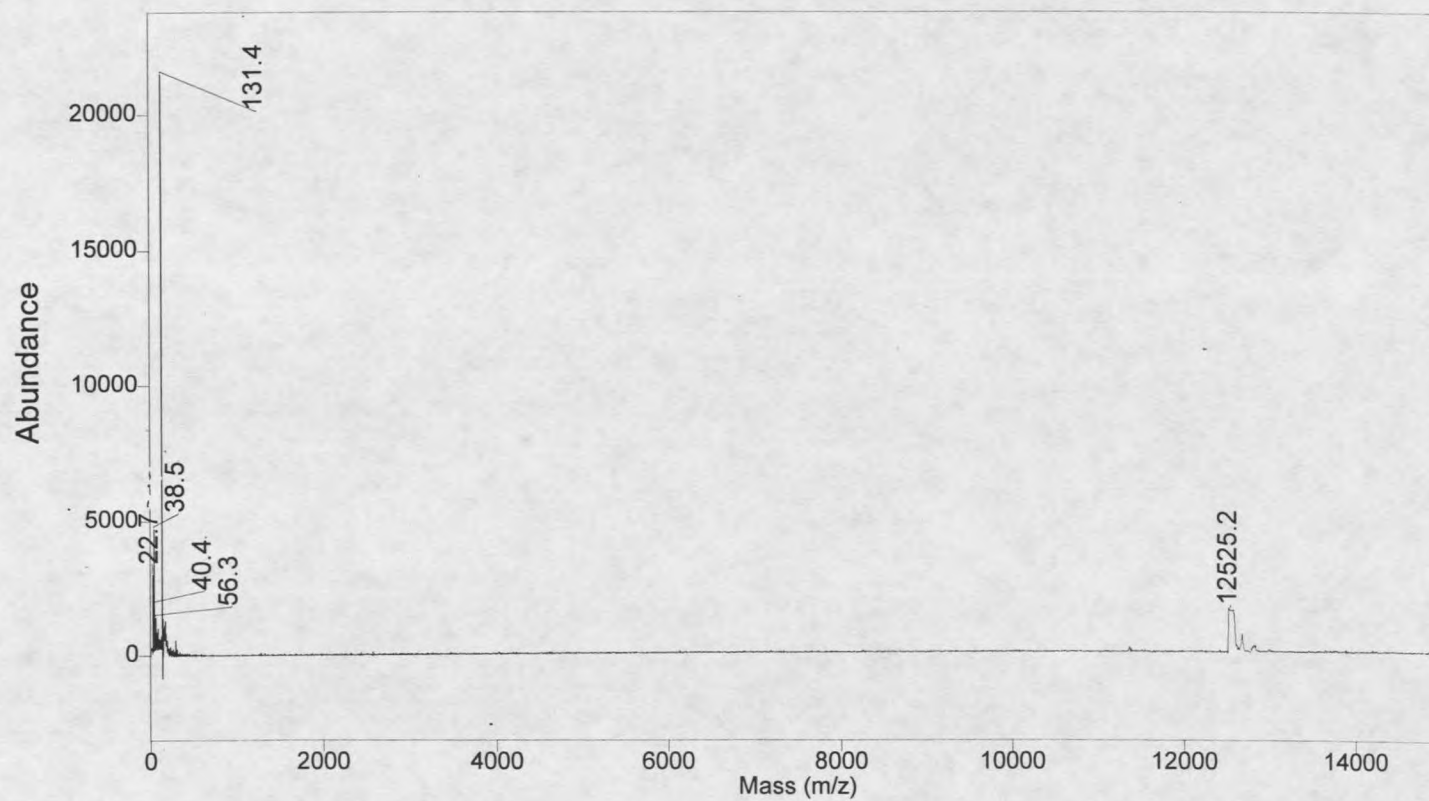


Figure 10. Graphite/SALDI mass spectrum of cytochrome C (mw= 12,360). Internal mass calibration using Cs^+ and $\text{Cs}^+(\text{cytochrome C})$.

are obtained for only about a second. Clearly, very special conditions on the sample surface must be fulfilled in order to obtain good protein spectra.

This first SALDI results were obtained with graphite. In an effort of finding new assisted materials, activated carbon was found as the other assisting material in SALDI. Furthermore, it has been proved that it is a good assisting material for easily obtaining the SALDI mass spectra of proteins such as cytochrome C and myoglobin. The details will be discussed in the next section of SALDI solid.

Possible materials for SALDI solids, SALDI solvents, and SALDI "adhesive" have been investigated. The results will be described in the following sections.

SALDI Solids

Tanaka discovered that an extremely fine cobalt powder (300 Å) can be used to induce a laser desorption ionization under laser irradiation (Tanaka et al., 1988). Although the resolution and sensitivity demonstrated were not very good, metal powders such as copper, iron, and bismuth have been studied for the use as a SALDI solid. The metal powder was mixed with glycerol plus analytes, and this mixture was analyzed by laser desorption. Metal ions were seen in the mass spectra. However, no analyte ions were observed in the mass spectra when the laser power was the same as that used in the graphite/SALDI analysis. However, when the laser power was increased very high, a very small peak of analyte molecular ions was sometimes observed in the mass spectrum. This shows that these metal powders are not useful as SALDI solids. A possible reason for these results is that these metal powders are not fine enough to be an efficient energy transfer medium. The surface

area in the larger particles is much smaller than that in the smaller particles for the same mass of particles. This results in inefficient energy transfer. Unfortunately, fine metal powder having the size around 300Å is hard to find.

Carbon materials such as graphite flakes, activated carbon, activated charcoal, carbon black, C₆₀, carbo pack and fluorinated graphite et al. have been tested as the SALDI solids. In our first SALDI results, graphite powder in the size range of 10-150 µm was used as the SALDI solid. Later, a finer graphite powder with sizes around 2 µm was used. However, no differences in the SALDI results were found. The 2 µm powder is the smallest size graphite that is commercially available. Large graphite particles such as graphite flakes (50 mm) can also be used as the SALDI solid. Figure 11 shows the graphite flake/SALDI mass spectrum of bradykinin (1.8 nmole). In addition to the pseudo-molecular ions (MH⁺) of bradykinin, dimer, trimer, tetramer, and quintamer ions of bradykinin are seen in the same mass spectrum. The sensitivity is lower, but the mass resolution is similar to the results with small size graphite powder. It was suspected that the energy transfer from flakes is less efficient than from the fine powder because the surface area of the flakes is much smaller than that of the powder. Thus, the desorption ionization process in graphite flakes/SALDI can be explained softer because the energy input on the analyte molecules is supposed to be lower. This shows that the analyte molecule ions are formed with a lower internal excitation energy; otherwise, the complex cluster ions cannot be formed (Sunner et al., 1988a). However, this mass spectrum was obtained only once. In most cases, the graphite flakes/SALDI mass spectrum of analytes has lower sensitivity, and only monomer molecular ions were seen. Apparently, some special sample condition must be met. What this is is not known.

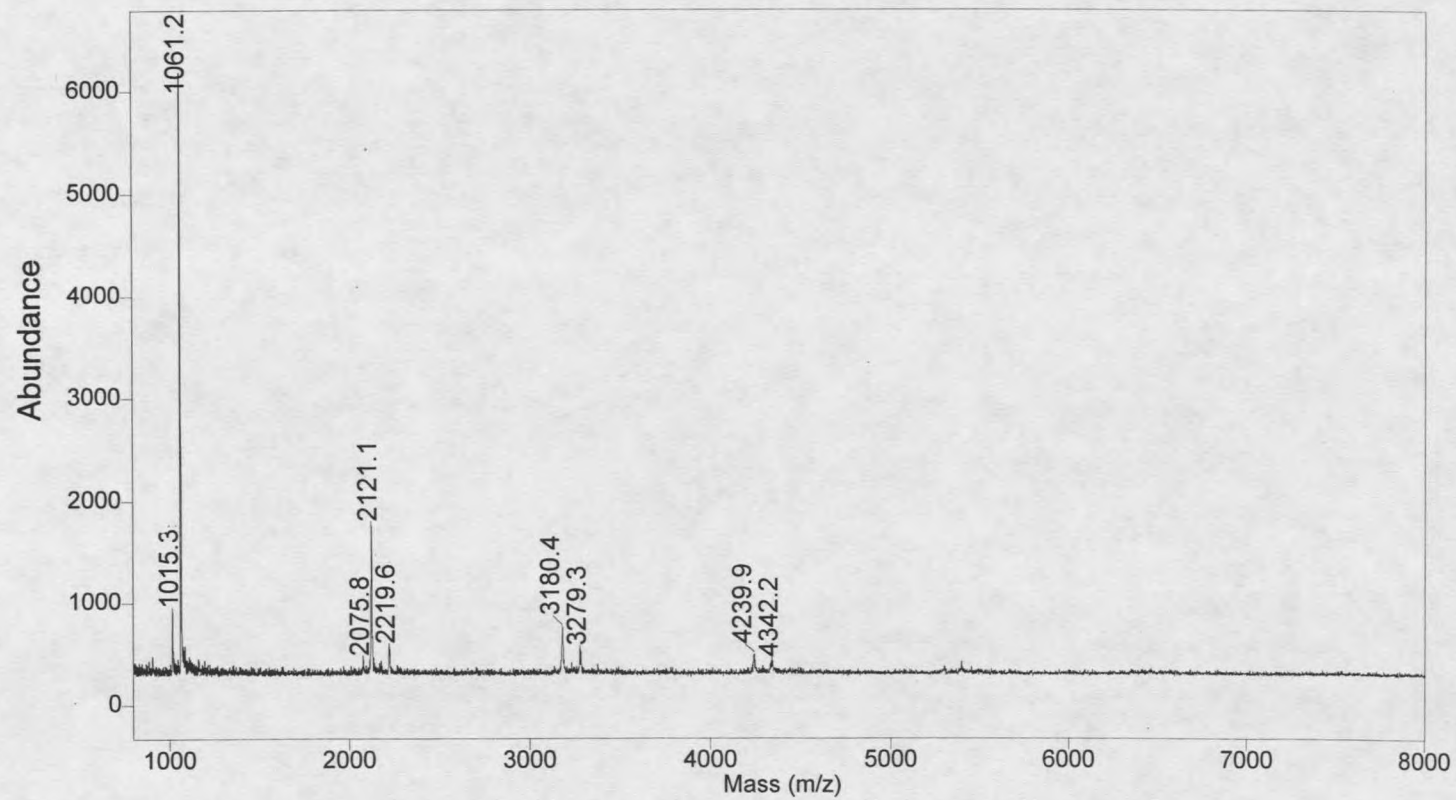


Figure 11. Graphite flakes/SALDI mass spectrum of bradykinin (mw= 1060.5).

When activated carbon, activated charcoal, and carbon black were used as SALDI solids, the same SALDI spectra as with graphite SALDI were obtained. The activated carbon, activated charcoal, and carbon black powders used were of similar size as the graphite powder (2-150 μm). Graphite, activated carbon, carbon black, and activated charcoal are produced from different materials and under different conditions. For example, the raw material for graphite is coke (Kelly et al., 1981), and the graphitization results from reactions with liquid reagents such as nitric acid or sulfuric acid under high temperature (above 600°C). Activated carbon is made from wood and is formed under solid phase reaction conditions at temperatures above 600-700°C (Mantell, 1968). When free carbon is liberated from its compounds below approximately 600-700°C by carbonization, it is either active or capable of activation. Primary carbons are activated by selective oxidation of the residual hydrocarbons on the surface. The oxidizing agent may be air, carbon dioxide, steam, or other substance. Figure 12 shows the scanning electronic microscope pictures of the surface of activated carbon and graphite. Comparing the surface appearances of graphite and activated carbon by observing the picture taken from scanning electronic microscope (SEM), the surface of activated carbon is extremely porous, and the surface of graphite is quite flat. The quality of graphite SALDI and activated carbon SALDI mass spectra is very similar. It is very easy to obtain spectra of small proteins with activated carbon SALDI, but very difficult with graphite SALDI. Two possible factors may result in this observation. First, the porous surface of activated carbon may help the desorption of protein from the surface. The reason would be that more liquid matrix may remain on the surface of activated carbon, and this

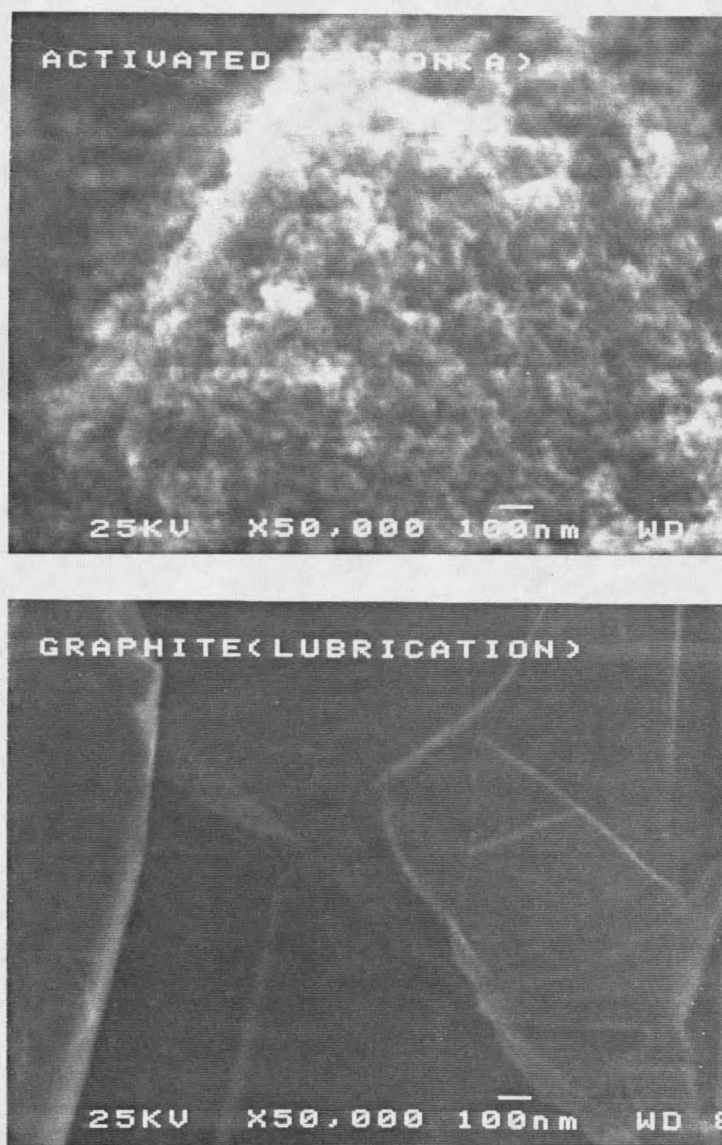


Figure 12. Scanning Electron Microscope (SEM) pictures of the surface of activated carbon (top) and graphite (bottom).

may result in a softer desorption process than for graphite SALDI. Large molecules such as proteins might then survive the desorption process without damaging the molecules. From experiments, the activated carbon/SALDI mass spectrum of glycerol has fewer fragments than that in the graphite/SALDI. Ions $m/z = 29, 45, 57, 75$ are not usually observed in the activated carbon spectra when activated carbon was used as the SALDI solid. However, these ions always show up consistently in the graphite/SALDI mass spectra. It might be another evidence that activated carbon/SALDI is softer. Second, the surface of activated carbon has been treated by special reagents for activation of the surface. The surface is modified through oxidation (Mantell, 1968). Functional groups such as $-COOH$ and $-OH$ have been produced on the surface of activated carbon. Under laser irradiation, these functional groups may be released into the gas phase. They may then participate in analyte ionization reactions which increase the ionization efficiency for analyte molecules. Activated carbon used in the experiments discussed above is also used for absorbing trace compounds from liquid phase. Another type of activated carbon is used for absorbing trace organics from the gas phase. This type of activated carbon usually has smaller cavities (the dimension of the cavity is less than $20 \text{ \AA}$) on the surface (Mantell, 1968). Figure 13 shows the SALDI mass spectrum of bradykinin by using this gas phase absorption activated carbon. An apparent difference between these two types of activated carbon powder is the intensity of alkali adduct ions of analytes in the SALDI mass spectra. Potassium adduct ions of bradykinin seem to be dominant in the mass spectrum. Furthermore, bradykinin with two attached potassium ions is observed. This is never observed in the SALDI mass spectra when liquid phase absorption activated carbon is used as the SALDI solid. No protonated

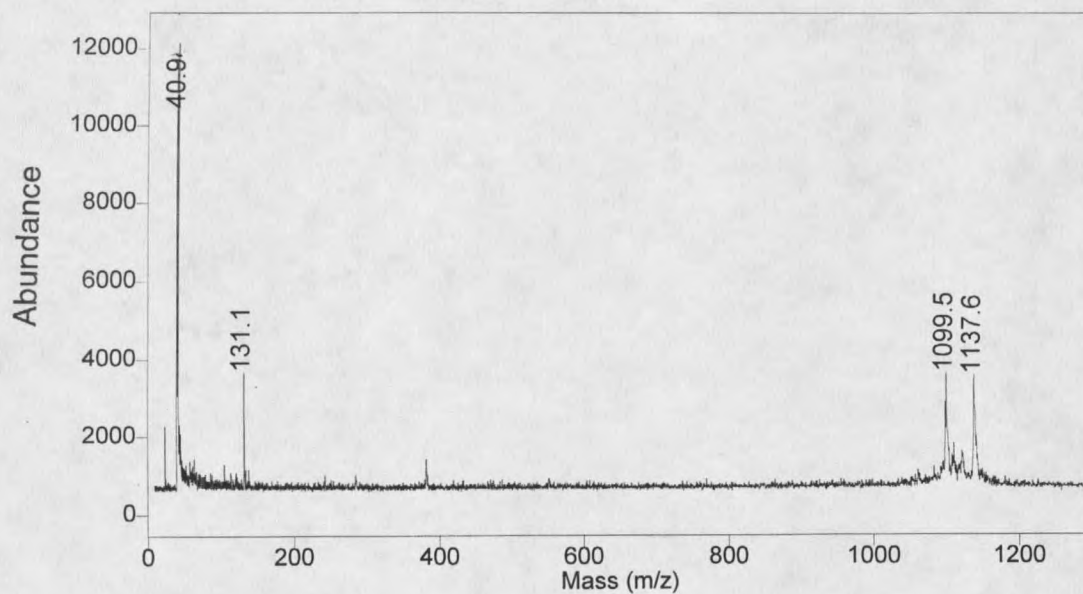


Figure 13. Activated carbon /SALDI mass spectrum of bradykinin (mw= 1,060.5). The activated carbon granule was ground to a fine powder before being used in the SALDI analysis. The activated carbon was of the gas phase absorption type. LP= 400. SA= 85.

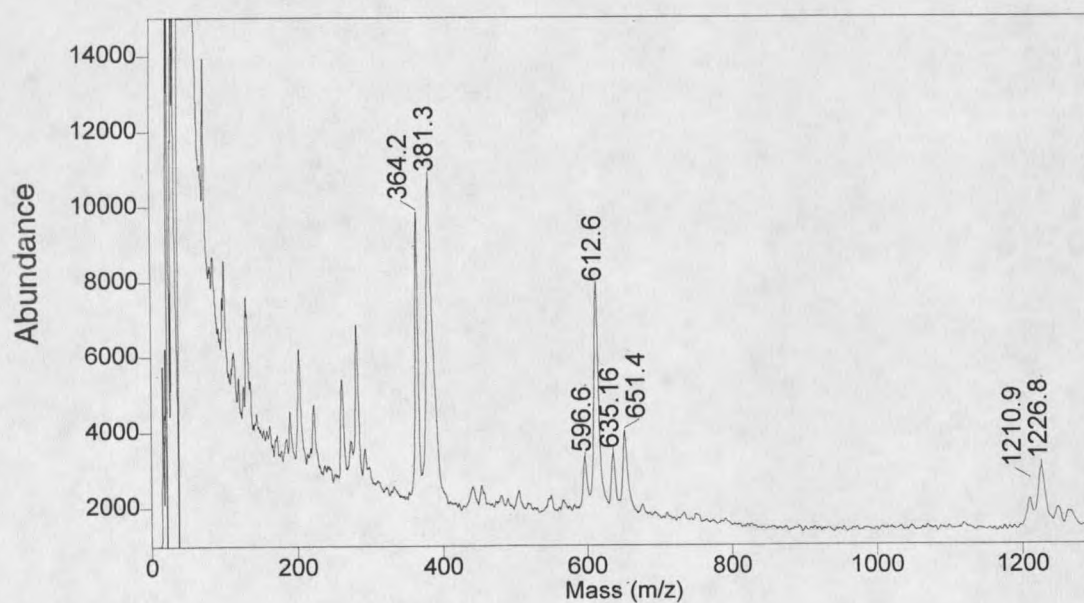


Figure 14. Carbopack/SALDI mass spectrum of methionine enkephalin (mw= 573.7). LP= 450. SA= 75.

bradykinin molecular ions are observed in the mass spectrum in Figure 13. In addition, potassium adduct ions of bradykinin peak is quite broad. It seems that gas phase absorption activated carbon contains much more potassium ions than the liquid phase absorption activated carbon. Also, the 20 Å (Mantell, 1968) pores help to improve neither the sensitivity nor resolution in SALDI mass spectra.

Carbo pack (120-400 mesh) is a common absorbent used for liquid extraction. Figure 14 shows the SALDI mass spectrum of methionine enkephalin with carbon pack as the SALDI solid. Potassium adduct ions of methionine enkephalin (MK^+) is the most intense peak. In addition, MNa^+ , $((M-H)^-Na^+)K^+$, and $((M-H)^-K^+)K^+$ are observed in the SALDI mass spectrum. Furthermore the alkali adduct ions of the dimer of methionine enkephalin molecules can be observed in the SALDI mass spectrum at $m/z=1211$ $((M-2H)Na_3)^+$. These results show the carbon pack contains many ions and potassium ions.

Michalak et al. reported a C_{60} -assisted laser desorption-ionization method (Michalak et al., 1994). C_{60} was selected for the laser desorption study at a laser wavelength of 337 nm because it has an absorption band at 328 nm (Hopwood et al., 1994). These authors placed a 0.50 μ l volume of a 1.0 mg cm^{-3} C_{60} solution in benzene on a flat stainless-steel sample holder. The sample was dried for 20 s at room temperature to remove the volatile compounds. The C_{60} matrix layer was then coated with a solution of the analytes. Spectra of proteins such as insulin, cytochrome C, and bovine albumin were reported. However, only smaller size of molecules like bradykinin could be repeated in our laboratory by this method, but the resolution and sensitivity are quite poor (results not shown). Also, intact C_{60} particles were used as the SALDI solid. The results were not positive, either. Bradykinin could still

be observed in C_{60} -SALDI, but the resolution and sensitivity were again poor. A possible explanation for this result is that the C_{60} particles were large. It looked more like granules than a powder.

Figure 15 shows the SALDI mass spectrum of angiotensin II with fluorinated graphite powder as the SALDI solid. The quality of this spectrum is not good. The ion intensity of angiotensin II, $m/z=1047.2$, is very low. The ion peak at $m/z=930$ is the fragment of angiotensin II obtained by losing an aspartic acid at the carbonyl terminal. The fluorinated modified graphite powder is not very useful for SALDI analysis. The powder size of the fluorinated graphite was as fine as unmodified graphite powder ($2\text{ }\mu\text{m}$). However, the absorption range for this fluorinated modified graphite might have shifted out of the UV range because of the modified surface.

The studies above have shown that the size of SALDI solid is an important factor in SALDI analysis. A particle size around $2\text{-}150\text{ }\mu\text{m}$ is acceptable for graphite, activated carbon, activated charcoal, carbon black, and carbo pack. In order to further understand the effects of particle size on SALDI spectra, different sizes of activated carbon powders were studied. A large activated carbon particle (4-12 mesh) was used as the SALDI solid. This single particle was attached on the sample tray by a double side tape. Then, $1.0\text{ }\mu\text{L}$ of 50% glycerol/methanol plus $1.0\text{ }\mu\text{L}$ of 5% sugar/water was added to this particle. One microliter of bradykinin solution (1 mM) was then added to the particle. Figure 16 shows the resulting SALDI mass spectrum of bradykinin. The protonated and alkali cationized analyte molecules and alkaline analyte ions are not resolved. However, when a large activated carbon particle was ground to fine powder and this powder used as the SALDI solid, the

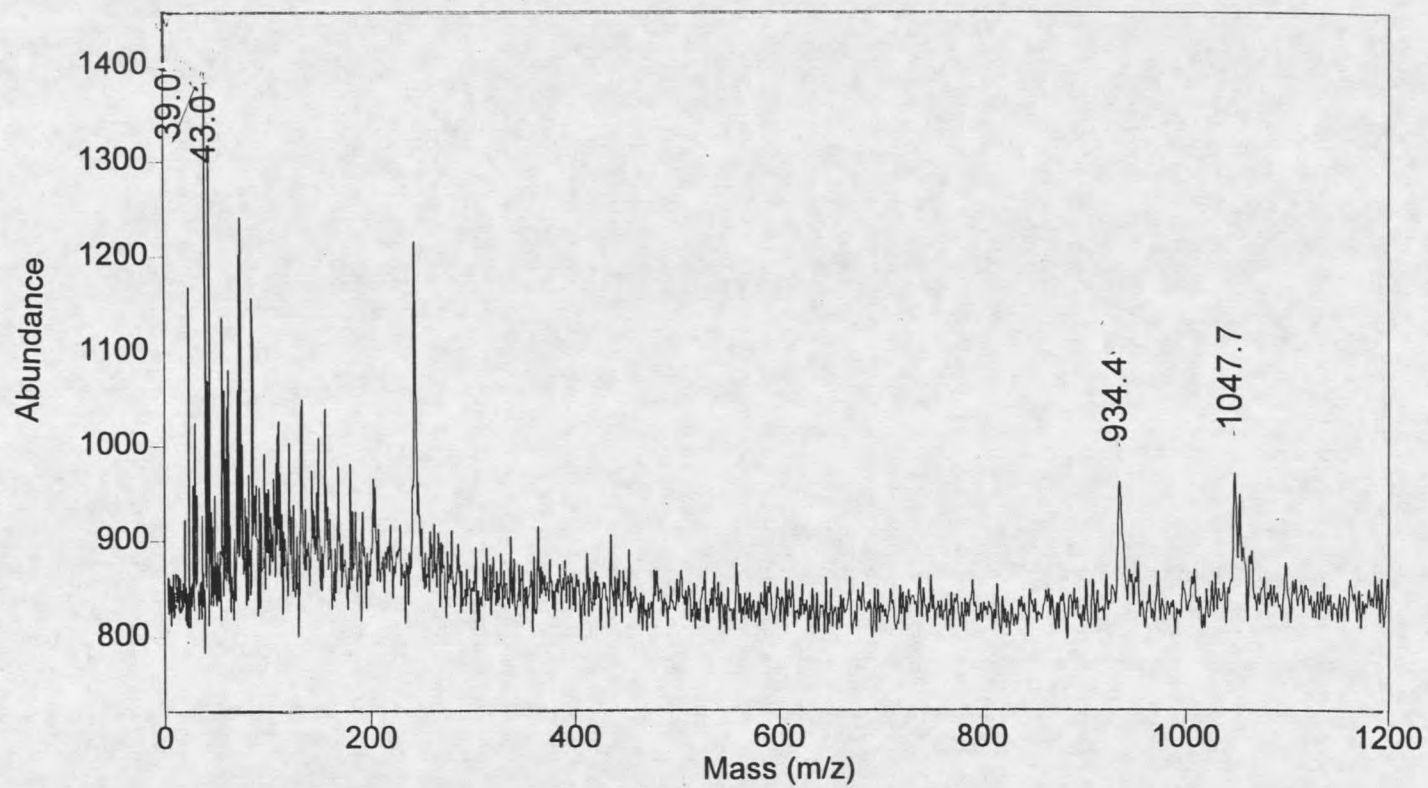


Figure 15. Fluorinated graphite/SALDI mass spectrum of angiotensin II (mw= 1,046.2). LP= 440. SA= 29.

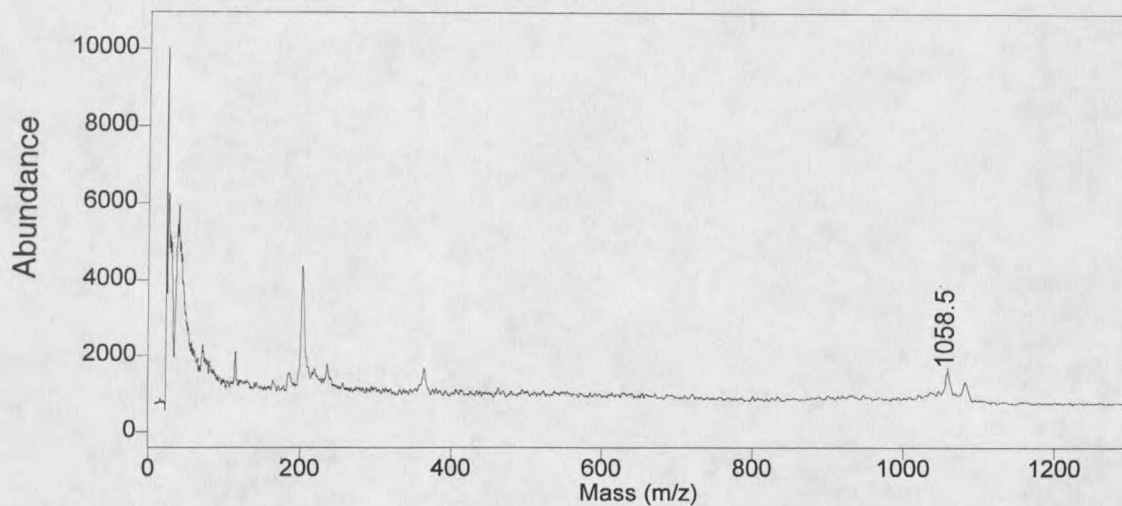


Figure 16. Activated carbon/SALDI mass spectrum of bradykinin (mw=1,060.5). A single granule (4-12 mesh) was used and attached to a sample plate.

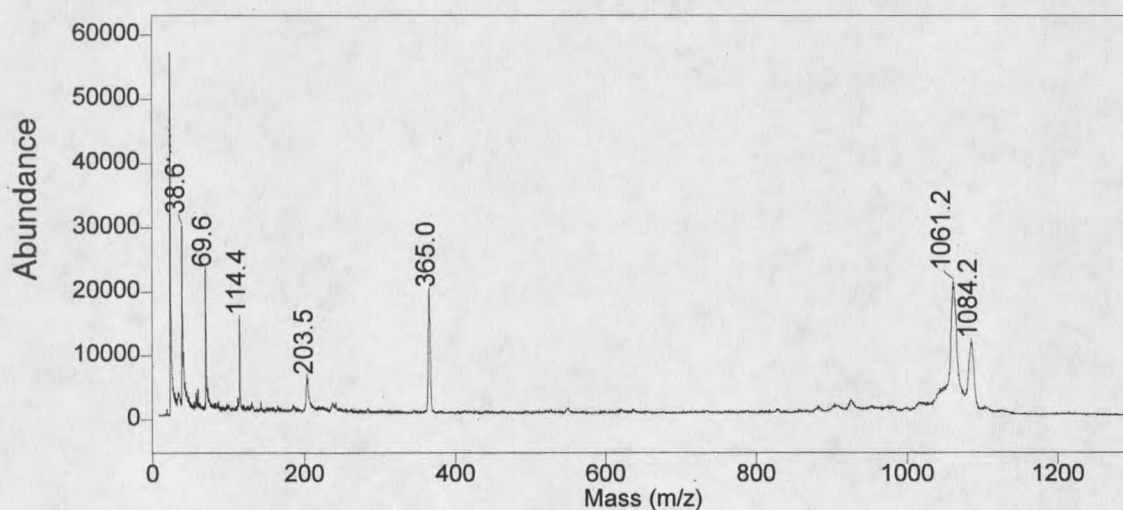


Figure 17. Activated carbon SALDI mass spectrum of bradykinin (mw= 1,060.5). The granule (4-12 mesh) was ground to a fine powder before being used in the SALDI analysis. LP= 375. SA= 59.

resolution becomes better. Figure 17 shows that protonated and sodium adduct ions of bradykinin are now resolved. This shows that when the size of SALDI solid becomes smaller, the resolution can be improved. Furthermore, the SALDI mass spectra of small proteins can be obtained by using the activated carbon powder ground from large activated carbon particles (4-12 mesh). Figure 18 shows the SALDI mass spectrum of cytochrome C. Only the molecular ions of cytochrome C is observed, and the mass resolution and sensitivity are quite poor.

A different type of SALDI solid material was tried by vapor deposition of carbon on a PVDF membrane. A very thin layer of graphite was coated on the PVDF membrane. The surface looks to the eye flat, but electron microscope shows structures the 10 μm size range. SALDI solvent (50% GI/MeOH) with analyte solution (angiotensin II, 1 nmole) was then deposited on the PVDF membrane. The SALDI mass spectrum is shown in Figure 19. The intensity of pseudo-molecular ion of angiotensin II is quite low, and the peak is broad. In addition, abundant alkali adduct ions of angiotensin II are observed in this mass spectrum. In particular, the potassium adduct ion of angiotensin II is the most intense peak. This is unusual because the sodium adduct ions of the analyte molecules are more intense than the potassium adduct ions in most of the SALDI mass spectra. It seems that the graphite used in carbon deposition contains more potassium ions. Another way of depositing a thin layer of graphite on a substrate such as paper or a membrane is to use a graphite aerosol spray. The graphite aerosol spray contains not only graphite, but also adhesive and volatile organic solvents. The graphite was first sprayed on a paper (white, 20-pound bond paper). The organic solvent was evaporated in 30 seconds. An analyte solution (1.0 μL) was applied to

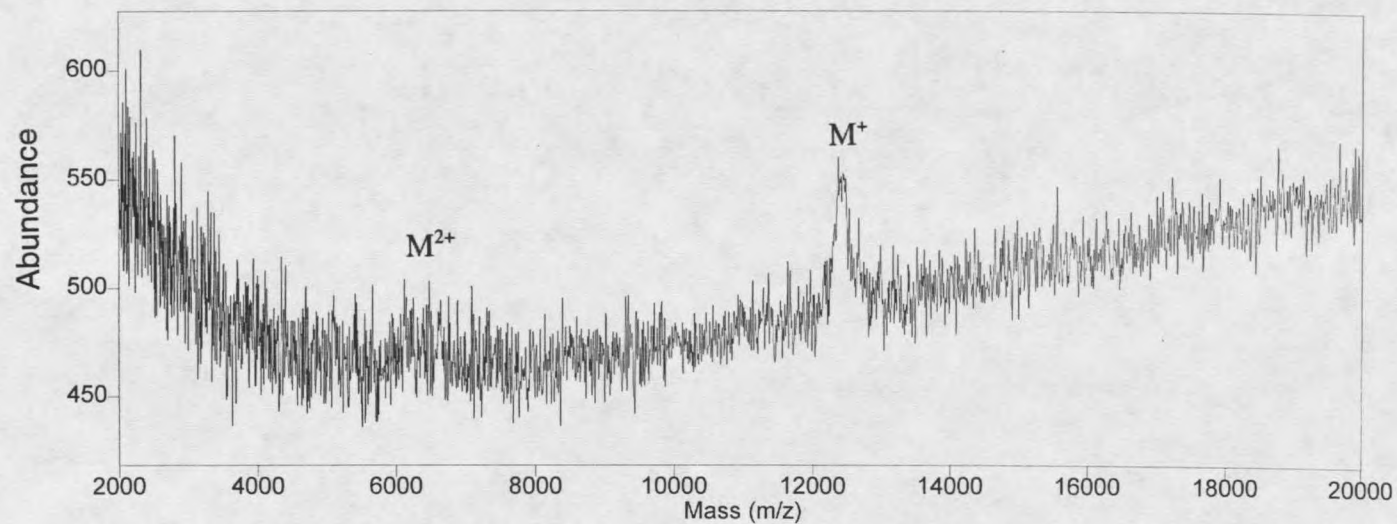


Figure 18. Activated carbon/SALDI mass spectrum of cytochrome C (mw= 12,360). The granule (4-12 mesh) was ground to a fine powder before being used as the SALDI solid. LP= 405. SA= 36.

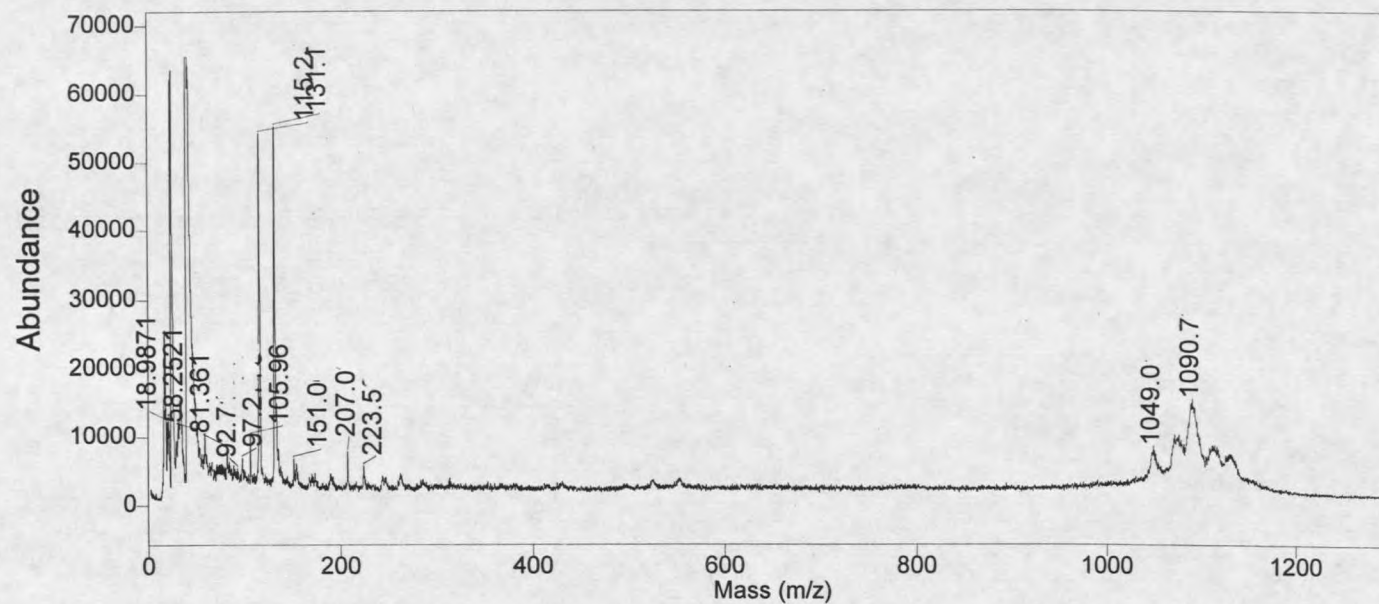


Figure 19. SALDI mass spectrum of angiotensin II (mw= 1,046.2). The SALDI solid was made by vapor deposition of a thin layer of carbon on a PVDF membrane. Analyte solution and SALDI solvent (50% GI/MeOH) were added to the coated membrane. LP= 406. SA= 37.

this paper with and without adding 1.0 μL of 50% glycerol/methanol. The SALDI mass

Table 2. SALDI solid materials

SALDI Solid	good	fair	poor
graphite (10-150 μm)	+		
graphite (2 μm)	+		
graphite flakes		+	
activated carbon (100 mesh)	+		
activated carbon (4-12 mesh)			+
activated carbon (12-20 mesh)			+
activated charcoal powder	+		
carbon black powder	+		
carbo pack (120-400 mesh)		+	
C ₆₀ particles			+
graphite coated PVDF			+
graphite aerosol spray			+
copper powder			+
iron powder			+
bismuth powder			+

spectra obtained by using this method were not promising, however (results not shown). The resolution and sensitivity were low. Protonated and alkali adduct ions were not resolved in the mass spectra. We tentatively conclude that a very thin layer of SALDI solid, deposited on a substrate does not work as well as a powder. It seems that a powder is needed to obtain good spectra. It seems likely that a rough surface SALDI will promote an intimate contact between the solvent and the surface, and that both phases are exposed to the laser irradiation. In conclusion, the requirements for good SALDI solid are (1) UV-range absorber (2) fine powder with sizes between 2 μm and 150 μm . The function of the solid material is to transfer the laser energy to the solvent. Graphite, activated carbon, activated charcoal, and carbon black powders all are useful as SALDI solid material. There are essentially no differences in the quality of the SALDI mass spectra with these materials. Table 2 shows a

summary of the result for all the SALDI solid as discussed above.

SALDI Solvents

In the early SALDI work, glycerol and DEA were used as SALDI solvent. Spectra can be obtained from graphite without adding a SALDI solvent. However, a poor mass resolution was obtained without adding liquid matrix into the SALDI matrix system. Figure 20 shows the SALDI mass spectrum of bradykinin without adding any SALDI liquid matrix. The intensity of protonated molecular ions ($m/z = 1061.2$) is low. Furthermore, abundant alkali adduct ions of analyte molecules are obtained (MNa^+ , $m/z = 1083$, MK^+ , $m/z = 1099$). The mass resolution is quite poor. These ions cannot even be resolved. Also, the signals of analyte ions could not last very long. In general, glycerol has been shown to a nearly universal SALDI liquid matrix that works well for both polar analytes and nonpolar analytes. It is even not necessary to dissolve nonpolar analytes to glycerol for SALDI analysis. Many other liquid compounds with low vapor pressure, high viscosity, and proper gas phase basicity can possibly also be used as the SALDI liquid matrix. FAB matrix compounds have these characteristics. Thus, a number of FAB matrices have been studied as SALDI solvents. Examples include 3-nitrobenzyl alcohol (NBA), polyethylene glycol (PEG), triethanolamine (TEA), magic bullet, thioglycerol, and crown ether.

Figure 21 shows the SALDI mass spectra of angiotensin II by using various SALDI solvents. NBA has an absorption band in the UV range. One of mass spectra in Figure 21 shows the SALDI mass spectrum of angiotensin II using NBA as the liquid matrix. The angiotensin II protonated molecules are seen to dominate the spectrum. NBA/angiotensin

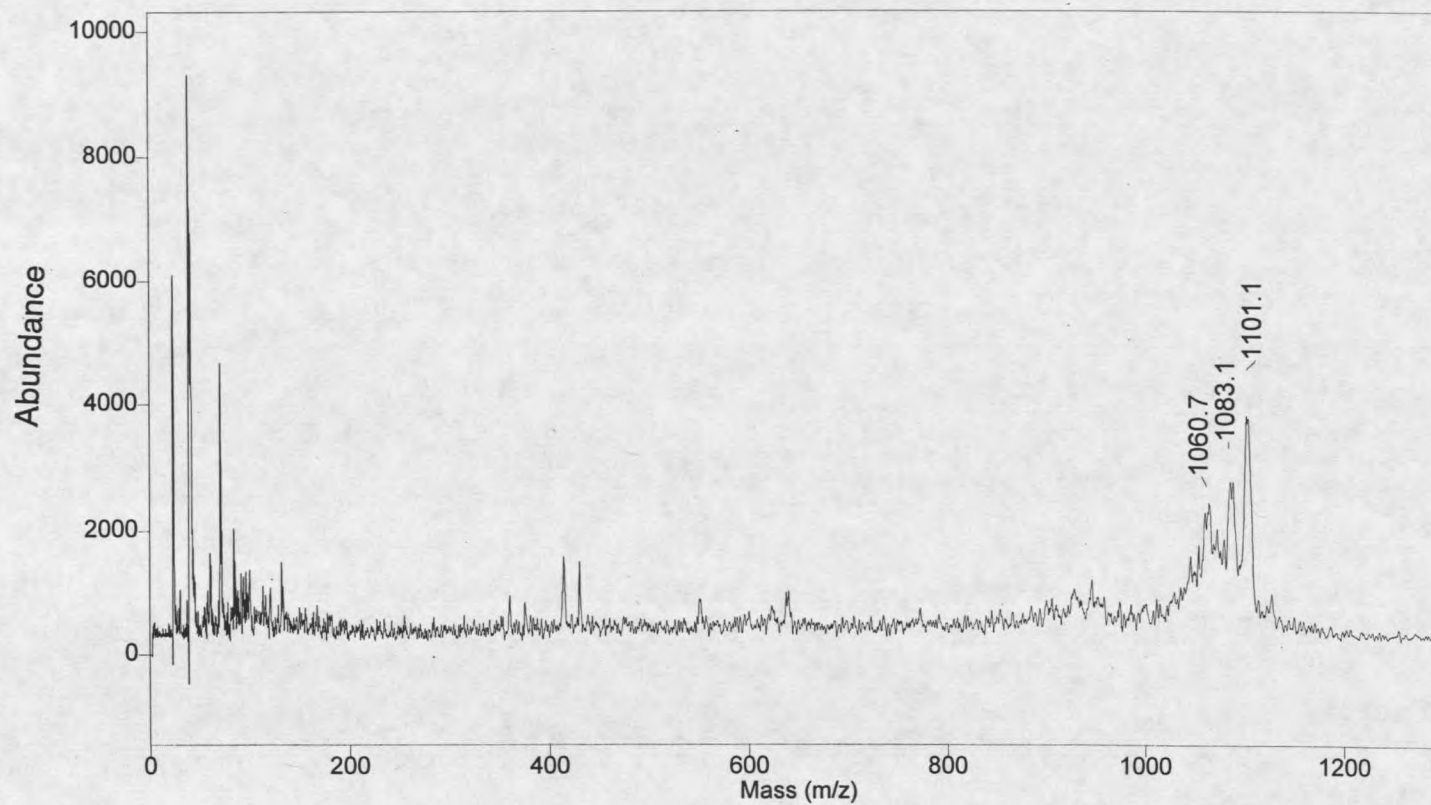


Figure 20. Activated carbon/SALDI mass spectrum of bradykinin (mw= 1,060.5) without adding any SALDI solvent.

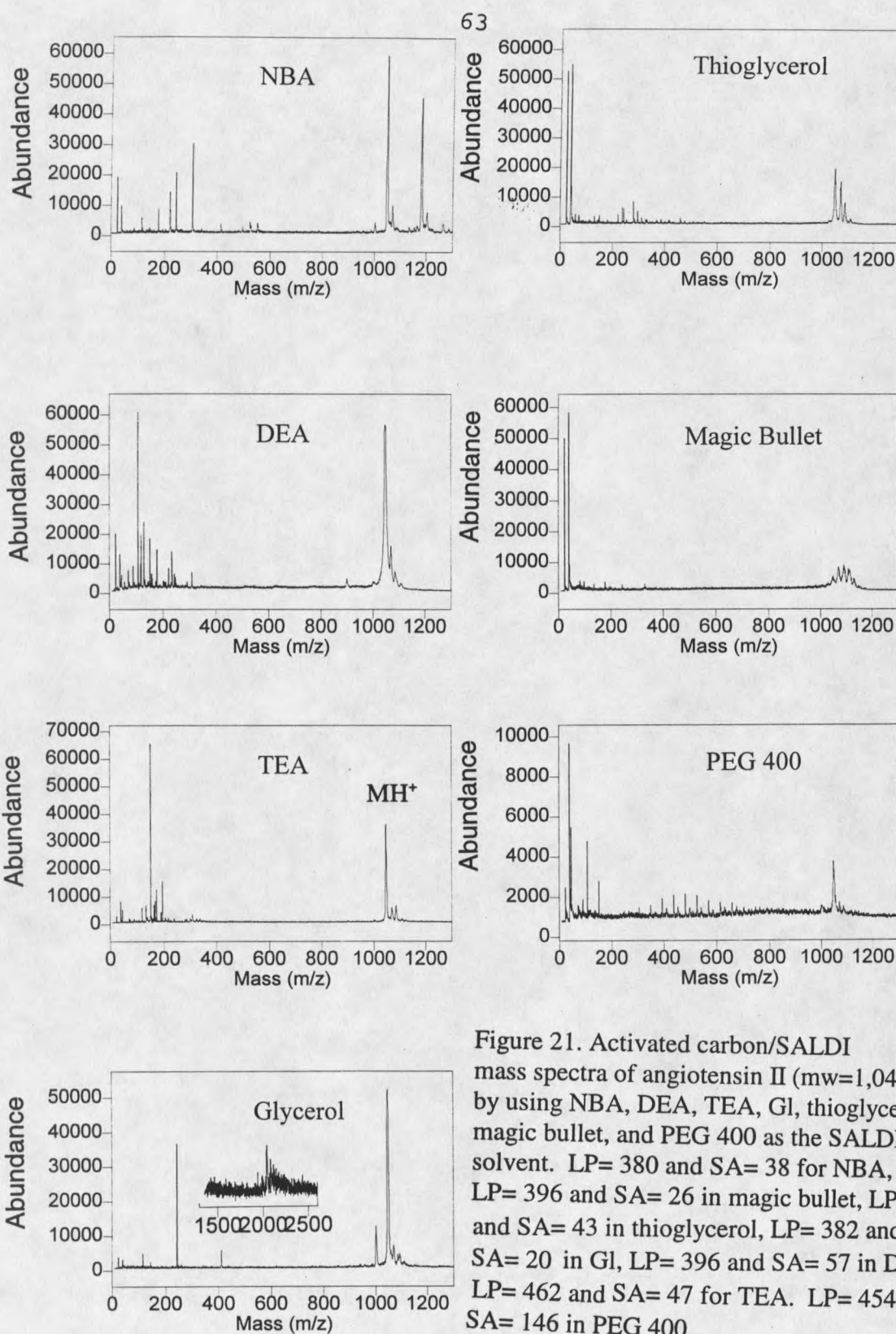


Figure 21. Activated carbon/SALDI mass spectra of angiotensin II ($mw=1,046.2$) by using NBA, DEA, TEA, Gl, thioglycerol, magic bullet, and PEG 400 as the SALDI solvent. LP= 380 and SA= 38 for NBA, LP= 396 and SA= 26 in magic bullet, LP= 356 and SA= 43 in thioglycerol, LP= 382 and SA= 20 in Gl, LP= 396 and SA= 57 in DEA, LP= 462 and SA= 47 for TEA. LP= 454 and SA= 146 in PEG 400.

II adduct ions are also abundant. For this reason, NBA has been used as the liquid matrix in continuous-flow matrix-assisted laser desorption ionization mass spectrometry (Li et al., 1993). However, the analyte pseudo-molecule ion peaks were quite broad. This is due to two factors. The broad peak was attributed to the formation of NBA/analyte adduct ions which cannot be resolved from the protonated and cationized molecular ions (Cornet et al., 1993; Dale et al., 1996). The second factor is that a dense plume was apparently formed above the sample surface (Cornet et al., 1993). It takes a longer time for some of the analyte molecular ions to escape from the plume, and this results in a worse mass resolution. When NBA was used as the SALDI solvent in protein analysis, a broad analyte peak was observed for high molecular weight analytes, as the mass spectrum shown in Figure 22. However, for smaller analytes such as peptides, the mass resolution was sufficient to resolve protonated molecular ions from NBA/analyte adduct ions. Because of the absorption ability of NBA toward laser irradiation, it is helpful for increasing the efficiency of desorption ionization in SALDI. That is, the total ion yield can be increased (Dale et al., 1996). DEA and TEA have weak absorption of UV light. As with NBA, the ion yields in SALDI mass spectra by using these two liquid matrices are somewhat higher than using non-UV absorbing liquid matrix like glycerol. This is seen by comparing the ion intensity of angiotensin II pseudo-molecular ions in the SALDI mass spectra with SALDI solvent NBA, DEA, TEA, and glycerol. NBA has the most abundant of ion intensity of angiotensin II pseudo-molecular ions, then DEA, TEA, and glycerol. The dimer of angiotensin II can be observed in the SALDI mass spectrum with glycerol as the SALDI solvent (see inset in G1 spectrum). It is possible that the plume temperature is significantly lower in glycerol than in the UV-absorbing matrices

due to the fact that glycerol does not absorb UV light.

One of mass spectra in Figures 21 is the SALDI mass spectra of angiotensin II with thioglycerol as the SALDI solvent. The alkali adduct ions of angiotensin II molecules are more abundant than for the other lower volatility SALDI solvents. A possible reason is that thioglycerol has a higher volatility and therefore less liquid remained on the surface of the SALDI solid during desorption (Dale et al., 1996). The quality of the SALDI mass spectrum with magic bullet as the SALDI solvent is very poor as the spectrum shown in Figure 21. The protonated angiotensin II ions have a lower intensity than the series of alkali adduct ions. Probably, magic bullet contains a relative high concentration alkali metal ions. Another spectrum shown in Figure 21 is the SALDI mass spectrum of angiotensin II with PEG 400 as the SALDI solvent. The protonated molecules of angiotensin II are still observed in the SALDI mass spectrum. However, the intensity is much less than with other SALDI solvents. The reasons may be that PEG 400 cannot provide enough protons to analyte molecules and that PEG does not absorb UV light.

Pump oils have low volatility with high viscosity. They thus fulfill two of the requirements of a SALDI solvent. However, pump oils are hard to be ionized. That is, they cannot provide protons to the analytes so that no successful spectra were indeed obtained with pump oils as the SALDI solvent.

Cytochrome C (mw= 12,360) has been used as a large molecule analyte for comparing various SALDI solvents. Figure 22 shows the SALDI mass spectrum of cytochrome C by using the SALDI liquid matrix, NBA. The peaks are very broad presumably because NBA/analyte adduct ions cannot be resolved from pseudo-molecular analyte ions under this

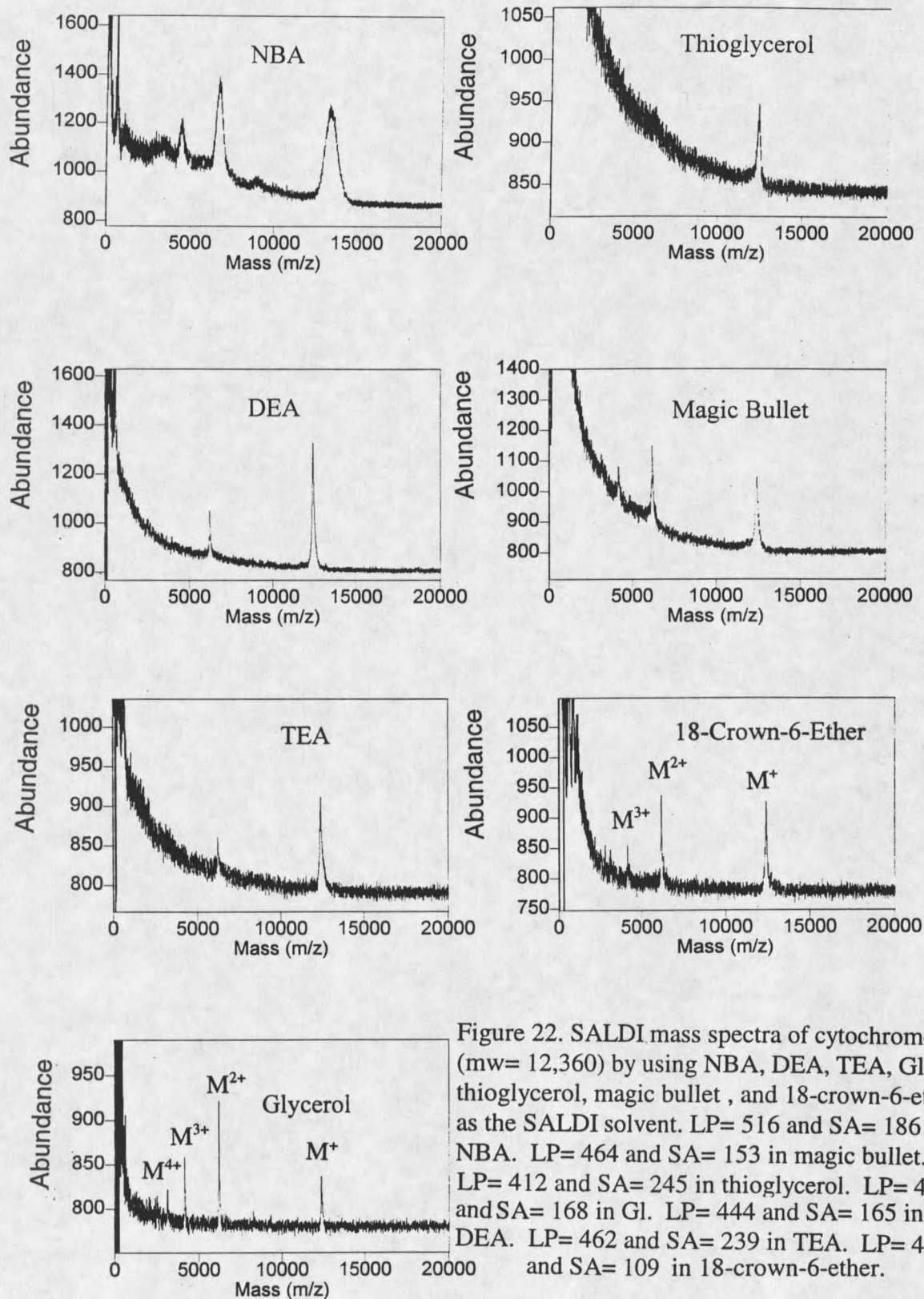


Figure 22. SALDI mass spectra of cytochrome C (mw= 12,360) by using NBA, DEA, TEA, Gl, thioglycerol, magic bullet , and 18-crown-6-ether as the SALDI solvent. LP= 516 and SA= 186 in NBA. LP= 464 and SA= 153 in magic bullet. LP= 412 and SA= 245 in thioglycerol. LP= 432 and SA= 168 in Gl. LP= 444 and SA= 165 in DEA. LP= 462 and SA= 239 in TEA. LP= 400 and SA= 109 in 18-crown-6-ether.

mass resolution. Furthermore, molecular ions (M^+), doubly charged (M^{2+}), and triply charged (M^{3+}) are observed with NBA. Even though NBA absorbs at 337 nm, no protein ions were observed without adding the SALDI solid. In Figure 22, the SALDI mass spectra of cytochrome C were shown with DEA and TEA as the SALDI solvent. The mass resolution is much better than with NBA. However, only M^+ and M^{2+} , but not M^{3+} were observed in either of these spectra. It might be due to the high gas phase basicity of DEA and TEA. This effect will be discussed in the section "Gas Phase Basicity of the SALDI solvent." Figure 22 also shows the SALDI mass spectrum of cytochrome C with glycerol as the SALDI solvent. The mass resolution is better than with DEA or TEA. In addition, multiply charged M^{4+} , M^{3+} , M^{2+} ions are observed in the glycerol solvent mass spectrum. Figure 22 also shows the SALDI mass spectrum of cytochrome C with thioglycerol as the SALDI solvent. The mass resolution is not as good as when glycerol was used. Only very weak M^{2+} peak is observed. With thioglycerol solvent, the SALDI analysis had to be performed immediately after the sample tray was injected into the ion source of the mass spectrometry. Otherwise, no cytochrome C molecular ions were observed. Thioglycerol has a higher volatility than the other SALDI solvents. This results in less liquid remaining on the surface of the SALDI solid. Although the SALDI suspension should have a nearly dry appearance for best results, too little liquid left in the sample will result in the failure of SALDI analysis. This is especially the case for large molecules like proteins. Another spectrum shown in Figure 22 is the SALDI mass spectrum of cytochrome C with magic bullet as the SALDI liquid matrix. In contrast to the peptide spectra, the mass spectrum of cytochrome C was almost as good as with glycerol. M^+ , M^{2+} , M^{3+} , and M^{4+} are observed in the mass spectrum. With PEG 400 as the

SALDI solvent, no molecular ions of cytochrome C were observed. A likely reason

Table 3. SALDI solvents

SALDI solvent	Peptides		Proteins	
	good	poor	good	poor
Glycerol	+		+	
NBA	+			+
DEA	+		+	
TEA	+		+	
Thioglycerol	+			+
Magic bullet		+	+	
PEG 400		+		+
Pump oils		+		+
Crown ether	+		+	

* If mass resolution is not a concern, NBA can be a good SALDI solvent for large analytes.

for this result is that PEG is difficult to ionize and is a poor proton donor. One of the spectra in Figure 22 shows the SALDI mass spectrum of cytochrome C with 18-crown-6-ether as the SALDI solvent. The spectrum quality is as good as when glycerol is used. Multiply charged ions are also observed in the spectrum.

To conclude, UV-absorbing compounds such as NBA, DEA, and TEA can be used as the SALDI solvents. According to the results obtained here, the mass resolution will be worse than with non-UV absorbing matrices such as glycerol. If mass resolution is not a concern, these NBA, DEA, and TEA can show higher sensitivity for the analyte in SALDI due to the higher total ion yield. The high abundance of alkali adduct ions with thioglycerol and magic bullet cause a lower resolution and a lower sensitivity for analytes. Being hard to be ionized, PEG 400 is not a good choice to be as a SALDI liquid matrix. Pump oils are not proton

donors which makes them fail as the SALDI solvents. To be a good SALDI solvent, a compound must have low volatility, high viscosity, and low ionization energy. Glycerol seems to fulfill all these requirements. Table 3 is the summary for the SALDI liquid matrices. The choice between "good" or "poor" was based on a combination of sensitivity and mass resolution.

SALDI "Adhesive"

In the early SALDI work, the sample preparation method used caused a problem of graphite contamination of the ion source of the mass spectrometer. After a few months of work, the ion source became noticeably dirty. It had to be cleaned by opening the ion source chamber. Sample preparation B (See Chapter 2) was developed to reduce the contamination problem; however, it was not very effective. In fact, all similar experiments, such as Tanaka's using an ultra-fine metal mixed with glycerol (Tanaka et al., 1988) and Zenobi's SALDI experiments (Dale et al., 1996), had the same contamination problem. However, no effort had apparently been made to solve the problem. There are primarily two concerns with contamination by SALDI solids. First, all the SALDI solids are electrical conductors. Thus, they might cause an electrical short-circuit problem in particular for the high voltage grid that is located about 2 mm above the sample tray. Second, carbon may possibly cause a problem with the bearings in the turbo pumps. Once contamination occurs, the powder will stay in the ion source because the materials are all non-volatile. The only way to remove the contaminant is to open the ion source and clean it. This is indeed the most important problem in SALDI to solve. It is necessary to immobilize the SALDI solid powder on the sample

plate. This can possibly be done in different ways.

Several additives have been experimented with to reduce the contamination problem. A major concern was that the additive should not result in a degradation of the SALDI spectra. The first approach tried was to mix a diluted commercial adhesive (Itaya glue, Canada) (50% adhesive/water) with the SALDI matrix. The analyte solution was then added to the SALDI suspension. However, the commercial adhesive was composed of polymers with molecular weights around 800 to 1,500 Da. The polymers gave intense mass peaks, and this caused interference in this mass range. Furthermore, analyte molecular ions were very weak in the mass spectra. Presumably, a thin layer of adhesive polymer was formed on the surface of the SALDI solid after most of the organic solvents had evaporated. This may have inhibited the energy transfer from the SALDI solid to the SALDI solvent and thus the desorption of the analyte molecules.

The second approach tried was to use Nafion to immobilize the SALDI solid. Nafion is a polymer that contains a fluorocarbon backbone with pendant chains terminated with sulfonated head groups. Nafion polymer membrane is used in electrochemistry (Inaba et al., 1993; Ramaraj et al., 1994). It provides a solid matrix with unique ion-exchange and swelling properties. However, when Nafion was used to immobilize the SALDI solid, no peptide analyte molecular ions could be found in the mass spectrum. The analyte signals were apparent totally suppressed by the Nafion polymer.

The third approach used to suppress ion source contamination was to use double side tape to immobilize the carbon powder. The SALDI solid such as activated carbon powder was sprinkled in a thin layer on one side of the double side tape. The SALDI liquid matrix and

analyte solution were then added to the top of the thin layer of powder. The SALDI mass spectra obtained using this method were not of good quality, and the intensities of analyte ions were weak.

The fourth approach tried to reduce ion source contamination was to make a pellet, under high pressure, from a mixture of the SALDI suspension with potassium bromide. This is similar to the method used for IR analysis of solid samples. The SALDI liquid matrix is necessary for the SALDI analysis. However, the liquid matrix made the pellets fragile and contamination still occurred. Also analyte peaks were of low intensity with extensive potassium adducts.

The final approach used saccharides as simple "adhesives". Adding saccharide to an aqueous solution increases the viscosity, and the saccharide works like an adhesive. Sucrose was chosen to immobilize the SALDI solid because it is inexpensive, is easily available, and is chemically similar to glycerol. Figure 23 shows SALDI mass spectra of angiotensin II (1 nmole) with decreasing amounts of sucrose added to the SALDI sample and with no SALDI solvent present. It is noteworthy that these spectra are clearly superior to bradykinin spectra obtained from activated carbon alone (Figure 20). It is seen that when the amount of sucrose is decreased, the mass resolution in the analyte molecular ion ($m/z(MH^+) = 1047.2$) region is improved. However, the mass resolution is improved much more when also glycerol is added to the SALDI matrix system. Figure 24 shows the SALDI mass spectra of angiotensin II without adding sucrose to the SALDI suspension (top) and with adding 5% sucrose/water solution to immobilize the SALDI solid (bottom). Comparing the protonated angiotensin II ion peak ($m/z = 1047.2$) in the two spectra, it is seen that the mass resolution is almost the

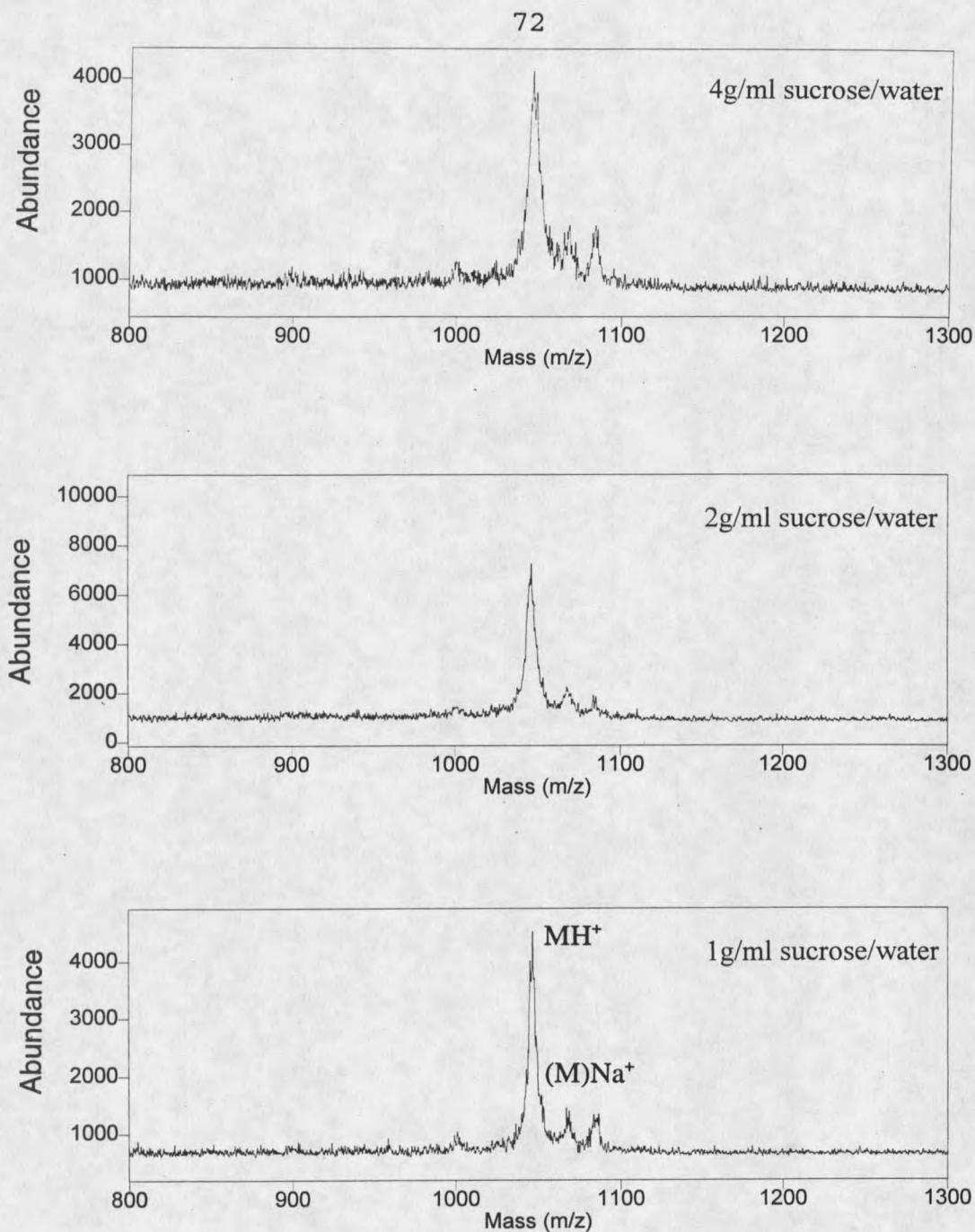


Figure 23. Activated carbon/SALDI mass spectra of angiotensin II (mw= 1,046.2). The SALDI solvents were 4.0 g/mL sucrose/water (LP= 330, SA= 35), 2.0 g/mL sucrose/water (LP= 330, SA= 29), and 1.0 g/mL sucrose/water (LP= 330, SA=30).

same. However, the peak intensity is lower with added sucrose. Three extra peaks, at $m/z=203$, $m/z=365$, and $m/z=381$, are observed in the mass spectra when sucrose is added. The remaining peaks are the same as shown in the top spectrum of Figure 24. The ion at $m/z=203$ is sodiated glucose ($m/z=180+23$), a fragment of sucrose. The ion at $m/z=365$ is the sucrose sodium adduct ion, and the ion at $m/z=381$ is the potassium adduct ion of sucrose. In the positive ion mode, protonated sucrose ions are not observed. However, in the negative ion mode, the deprotonated molecular ion of sucrose at $m/z=341$ is observed. In addition, the chlorine adduct ion of sucrose at $m/z=376$ is found in SALDI negative ion mass spectra. In the negative mass spectrum, a series of carbon clusters always appear in the low mass range.

SALDI mass spectra of larger analytes like proteins are still obtained with the sucrose additive. Figure 25 shows SALDI mass spectra of cytochrome C (1 nmole) with (bottom) and without (top) added sucrose. Whether sucrose is added or not, singly, doubly, triply, and quadruply charged proteins are observed in the SALDI mass spectra. It is demonstrated that sucrose does not appreciably affect the cytochrome C analysis by SALDI.

Monosaccharides can also be used to immobilize the SALDI solid powder. The effects of mannose and glucose are similar to what is observed for sucrose. Figure 26 shows SALDI mass spectra of angiotensin II (top) and cytochrome C (bottom) with a mannose additive (5% mannose/water). Alkali ion adducts of mannose ($m/z(MNa^+)=203$, $m/z((M-H)K^+Na^+)=242$) are observed in the low mass range in both spectra. A new ion is observed in the high mass range at $m/z=1209.9$ in the angiotensin II spectrum. This ion is identified as a mannose/angiotensin II adduct ion with the loss of a water molecule. In the

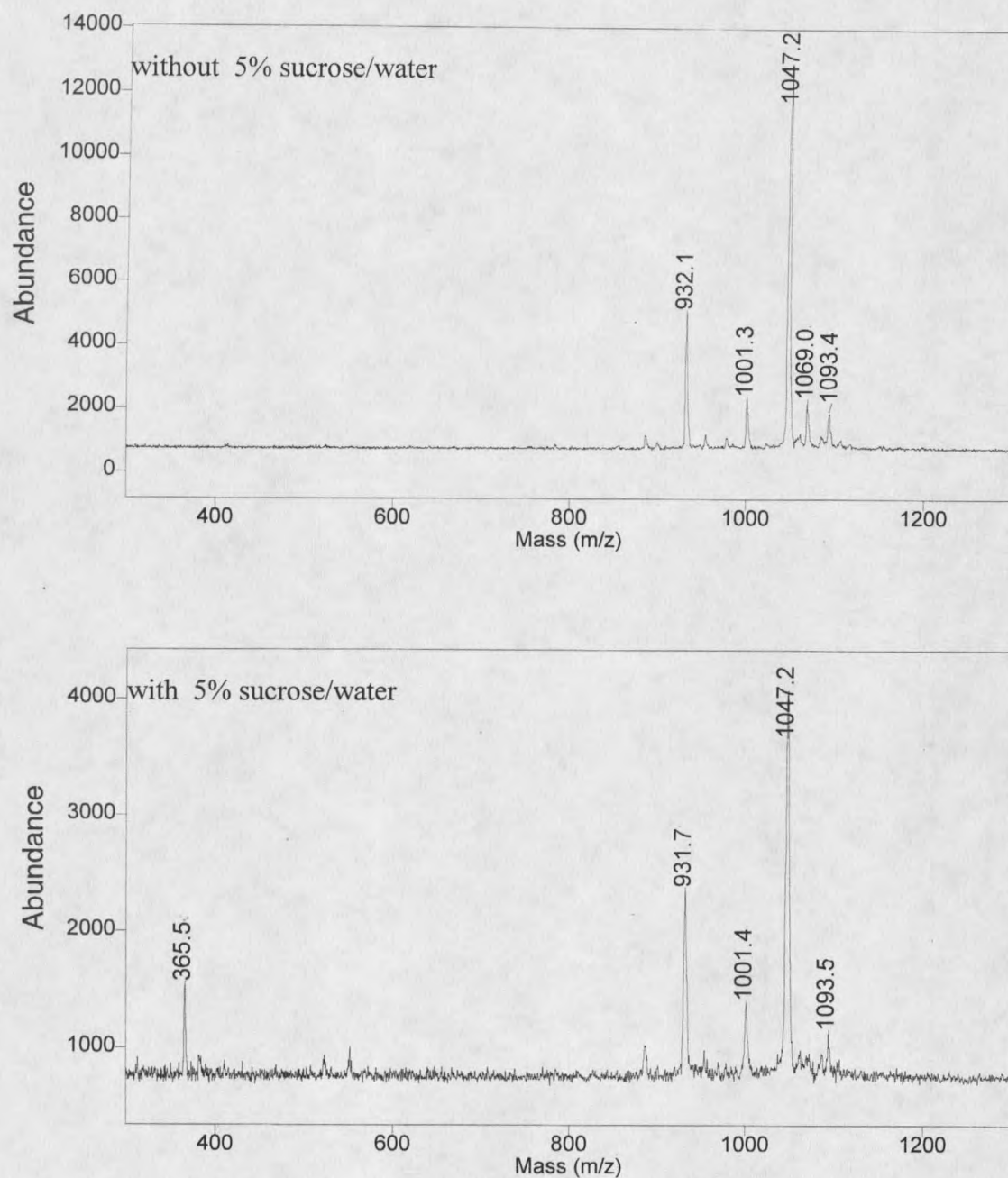


Figure 24. Activated carbon/SALDI mass spectra of angiotensin II ($m_w=1,046.2$) without added sucrose (top, LP= 400, SA= 50) and with adding 1.0 μL of 5% sucrose/water to the SALDI suspension (bottom, LP= 445, SA= 39).

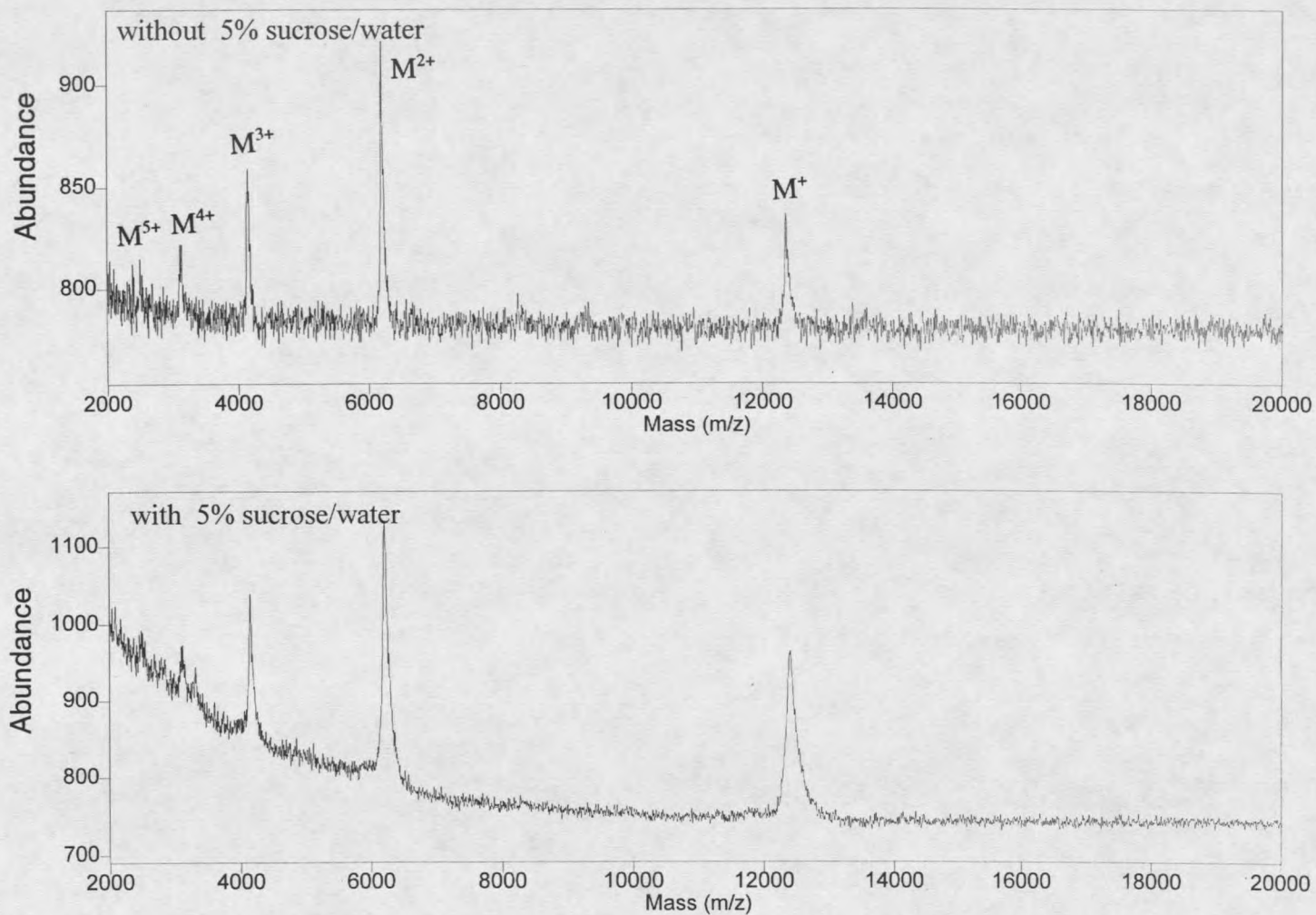


Figure 25. Activated carbon/SALDI mass spectra of cytochrome C (mw= 12,360), without adding sucrose (top), and with adding 1.0 μ L of 5% sucrose/water (bottom, LP= 555, SA= 256).

SALDI mass spectrum of cytochrome C with the mannose additive, the singly and doubly charged cytochrome C are seen in the mass spectrum.

By using saccharides to immobilize the SALDI solid powder, the contamination of the source is greatly reduced. Without adding saccharides to the SALDI suspension, the grid and the ion source chamber appeared quite black when opened after running SALDI experiments for three months. Carbon powder was found everywhere in the ion source. However, with saccharides added to the SALDI suspension, the ion source had only a very thin black coating after being used for three months. Thus, it is now only necessary to clean the ion source every six months. For the future, a better method that completely eliminates the contamination is needed.

In conclusion, saccharide additives can be used to immobilize the SALDI solid and reduce most of the contamination of the ion source. It has also been demonstrated that the SALDI mass spectra are not appreciably degraded by the added saccharide.

SALDI Analyte Solvents

Both polar and non-polar organic solvents can be used to dissolve analytes for SALDI analysis. It does not cause any apparent problems when a non-polar analyte solution is mixed with a SALDI polar solvent. Presumably, when the surface of the sample has a dry appearance, most of the organic solvents has evaporated and the analyte is absorbed on the surface of the SALDI solid.

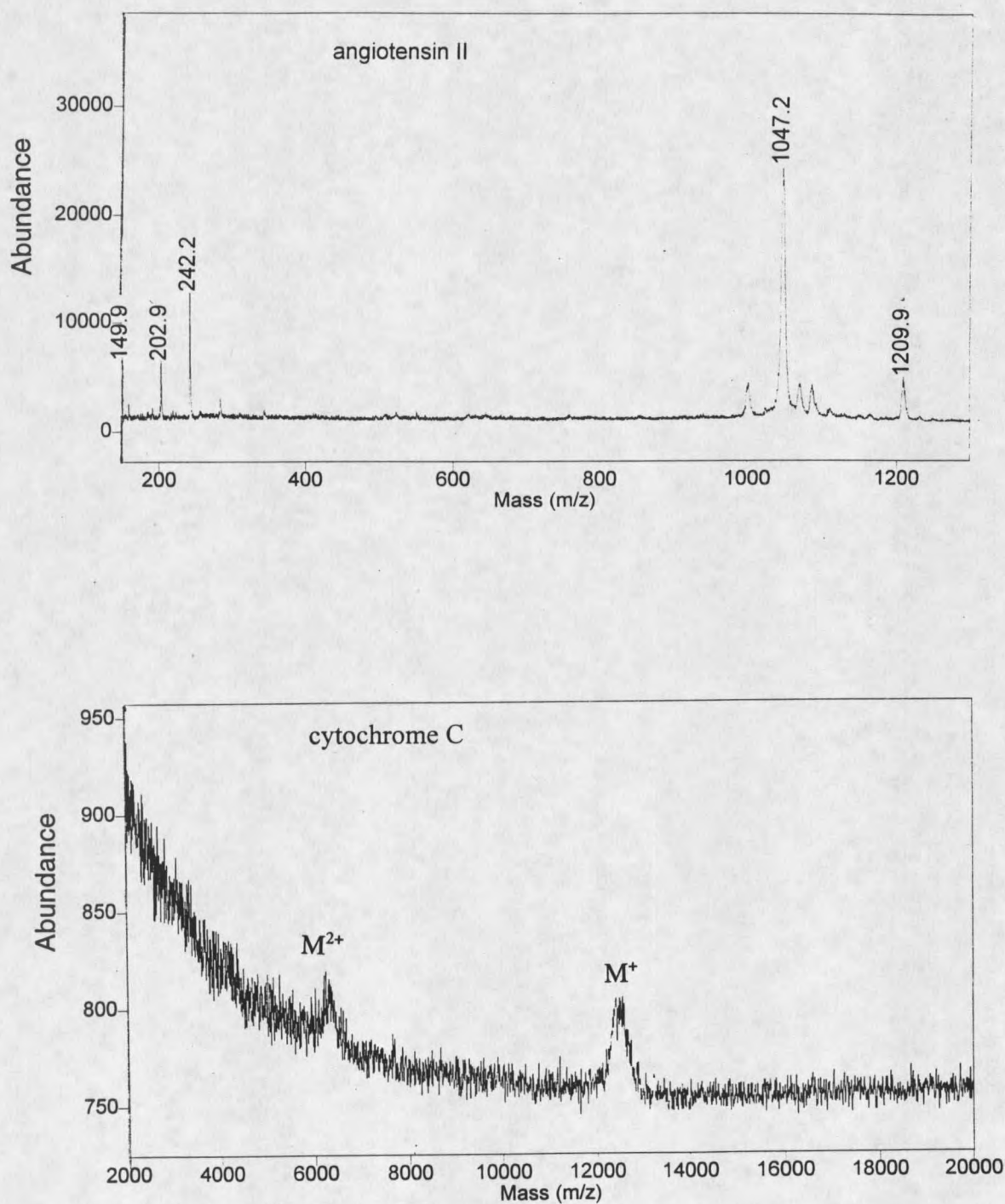


Figure 26. Activated carbon/SALDI mass spectra of angiotensin II (mw= 1,046.2) (top, LP= 414, SA= 63) and cytochrome C (mw= 12,360) (bottom, LP= 522, SA= 185) with mannose used to immobilize the SALDI solid.

Internal Energy of SALDI Ions

In order to probe the internal energy of SALDI generated ions, adenosine ($m_w = 267$) was used as a "thermometer". Protonated adenosine is easily fragmented, and a fragment ion at $m/z = 136$ is observed in the mass spectrum, in addition to the protonated molecule at $m/z = 268$. The more energy deposited in the protonated molecule, the more extensive is the fragmentation. Thus, the 136/268 ratio is a measure of the internal energy of the protonated adenosine ions.

How fragmentation is detected in TOF/MS depends on where the fragment ions are formed. Fragmentation may occur immediately after ion formation. This "prompt" fragmentation is simply detected in the linear mode. If the fragment ions are formed in the time-of-flight tube, the fragmentation can only be detected by post source decomposition (PSD) in the reflectron mode. Figure 27 shows the MALDI-PSD (top) and SALDI-PSD (bottom) mass spectra of adenosine. Only two peaks are observed in either mass spectra; protonated adenosine at $m/z = 268$ and the fragment ion at $m/z = 136$. In comparison with the MALDI-PSD mass spectrum of adenosine, the relative intensity of ions 136 to 268 in the SALDI-PSD mass spectrum is much higher. More metastable fragmentation occurs in SALDI. This shows that SALDI is a "harder" ionization method than MALDI. That is, the internal energy of analyte ions in SALDI is higher than in MALDI.

In SALDI, several different solid materials can be used. The internal energy of ions can be expected to be different for each material. Again, adenosine was used as the "thermometer" probe. Linear mode was used for this experiment. The results are shown in

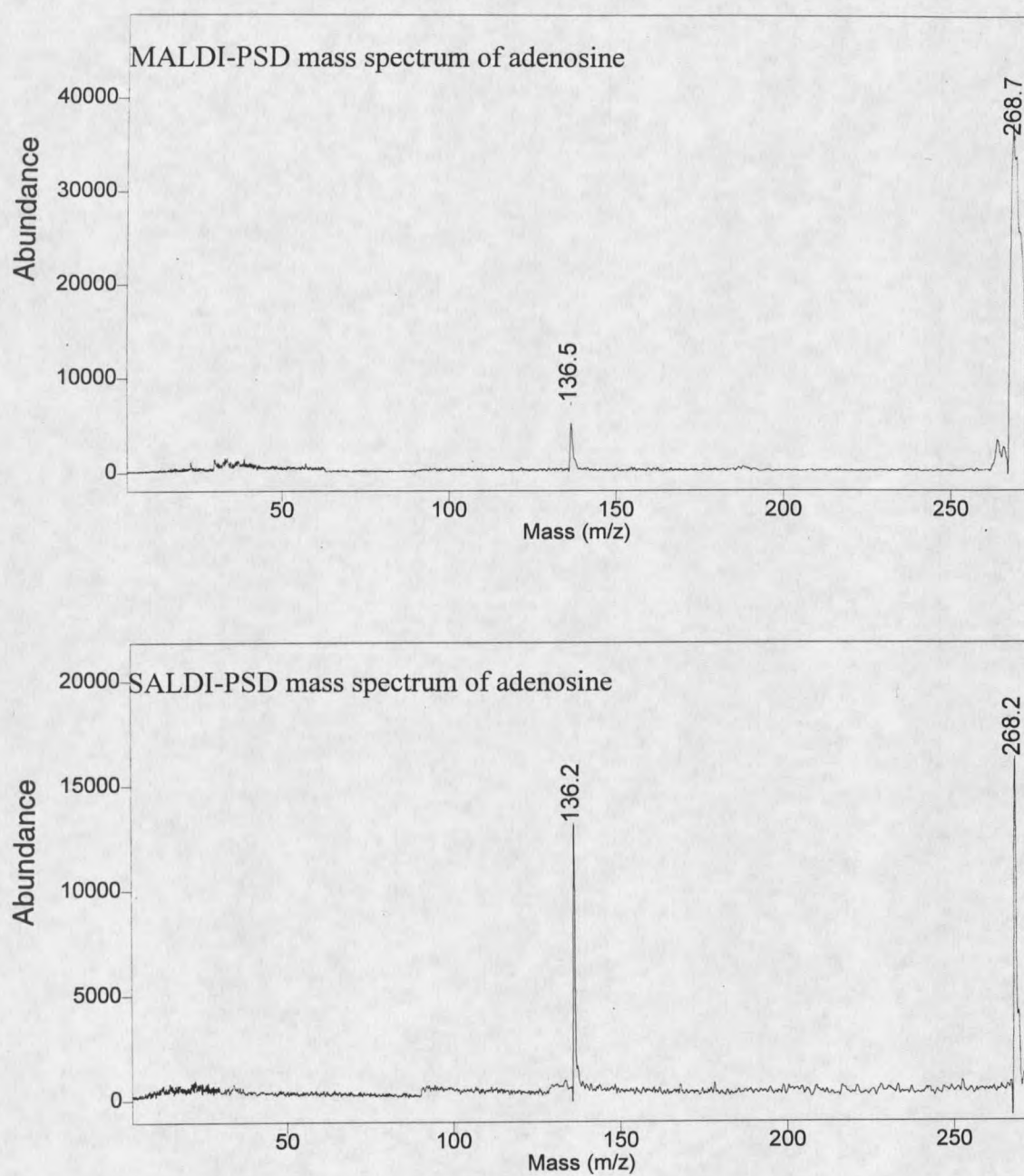


Figure 27. Activated carbon/SALDI-PSD mass spectrum of adenosine (mw=267.2) (200 nmole) (bottom) MALDI-PSD mass spectrum of adenosine (200 nmole) (top).

Table 4. It is seen that the 136/268 ratio for activated carbon and activated charcoal/SALDI is higher than five. However, the ratio for graphite is lower, about 2.3. Activated carbon causes more extensive fragmentation than graphite in SALDI. It is of interest to consider why this may be so. One interpretation is that activated carbon can transfer more energy to the analyte molecules such that fragmentation is more extensive as compared to graphite. The larger surface area of activated carbon would result in higher internal energy of analyte molecular ions in activated carbon/SALDI. It is also seen in Table 4 that the 136/268 ratio is higher for graphite powder than for graphite flakes. Again, the larger surface area of the graphite powder corresponds to more extensive fragmentation.

With fluorinated graphite and iron powder as the SALDI solid, the 136/268 ratios are lower than for graphite. However, the total ion yields were also quite low with these materials. It should be noted that when iron powder was used as the SALDI solid, the laser power needed was higher than normally used. The 136/268 ratio in LD (i.e. with stainless steel as the energy transfer surface) is 1.2 which is about the same value as that for iron. Presumably, this is due to less energy provided to the analyte molecules by iron and stainless steel under laser irradiation. The internal molecular energy of analytes was low, and not much fragmentation occurred. It was also observed that the total ion yield was lower with iron, fluorinated graphite, and stainless steel. It is likely that the energy provided by these three materials was not enough to desorb many molecules into the gas phase as graphite or activated carbon do.

SALDI seems to be a significantly harder ionization technique than MALDI since the 136/268 ratio in MALDI is only 0.26 compared with 5.0 for activated carbon SALDI. The

internal energy of analytes is higher in SALDI than that in MALDI. The total ion yields in SALDI and MALDI are almost identical. That is, the desorption ionization efficiency is similar in the two methods. The upper mass limit in SALDI is about 20,000 Da, whereas it is much higher in MALDI. A possible reason is that too high an internal molecular energy of analyte molecular ions in SALDI causes fragmentation of all large analyte molecules.

Table 4. The abundance ratio of 136/268 in linear TOF mode with different SALDI solids. Sample preparation C was used in all experiments.

SALDI Solid	Ratio of 136/268
Graphite powder (2 μ m)	2.3
Graphite flakes	1.6
Fluorinated graphite powder	1.1
Activated carbon powder	5.0
Activated charcoal powder	4.8
Iron powder*	1.2
MALDI (sinapinic acid)	0.26
LD (stainless steel)	1.2

* Higher laser power was used to obtain the SALDI mass spectra from the iron powder

Combining SALDI with MALDI Matrix

SALDI is a "harder" desorption ionization method than MALDI as shown by these studies. It was considered that intermediate internal energies might be obtained by mixing SALDI and MALDI matrices together in the same sample. Figure 28 shows a mass spectrum of bradykinin (0.10 nmole) using a mixture of a 0.50 μ L of SALDI suspension with 0.50 μ L of MALDI matrix. The SALDI suspension was made by mixing approximately equal volumes of dry activated carbon powder and a sucrose/water (1.0 g/mL) solution. The MALDI matrix was a saturated sinapinic acid solution in acetone/water (1/1 (v/v)) solution. A SALDI solvent was not added to this mixture because sinapinic acid was expected to form crystals

as the water and acetone evaporated. Figure 28 shows that the mass resolution of the pseudo-molecular ions of bradykinin is low. Neither sinapinic acid protonated molecule nor its fragment ions are seen in the mass spectrum. It seems likely that sinapinic acid did not form crystals in this sample so that it could not play the same role as it does in MALDI. The sodiated adduct ions of sucrose at $m/z = 365$ and the sodiated adduct ions of glucose at $m/z = 203$ were observed, however.

Figure 29 shows the mass spectrum of bradykinin using 0.50 μL of MALDI matrix with 0.50 μL of SALDI solvent. The MALDI matrix was saturated α -cyano-4-hydroxy cinnamic acid dissolved in acetone/water (1/1 (v/v)). The SALDI solvent was 50% glycerol in methanol. In Figure 29, the bradykinin pseudo-molecular ion is sharp at the top of the peak but has a triangular shape at the bottom. This is similar to what was observed for the UV-absorbing liquid matrix. It would seem that α -cyano does not to form crystals in the presence of glycerol. As has been discussed previously, the poor mass resolution is likely due to a dense plume formed above the sample well and to matrix/analyte adduct ion formation. Finally, the dimer ions of bradykinin are observed in the mass spectrum.

Optimum SALDI Sample Preparation Conditions

Optimum Proportions of SALDI Suspension Components

The SALDI solid, the SALDI solvent, and the SALDI "adhesive" are the major components in the SALDI matrix system. It was concluded above that graphite powder and activated carbon (or charcoal) powder are the best SALDI solids found to date. Glycerol is an universal SALDI liquid matrix for polar and nonpolar analytes. Sucrose was introduced

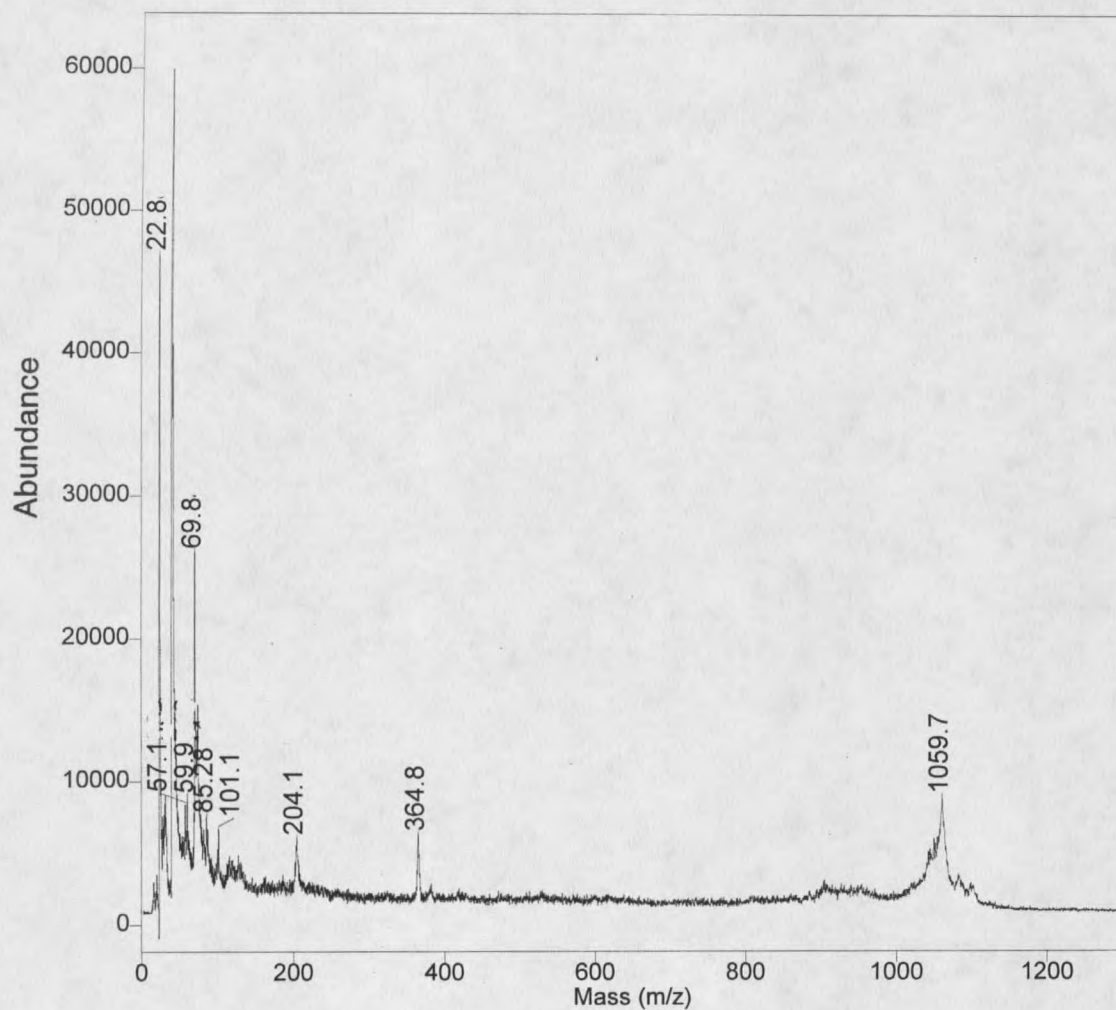


Figure 28. SALDI-MALDI combination mass spectrum of bradykinin (mw= 1,060.5).

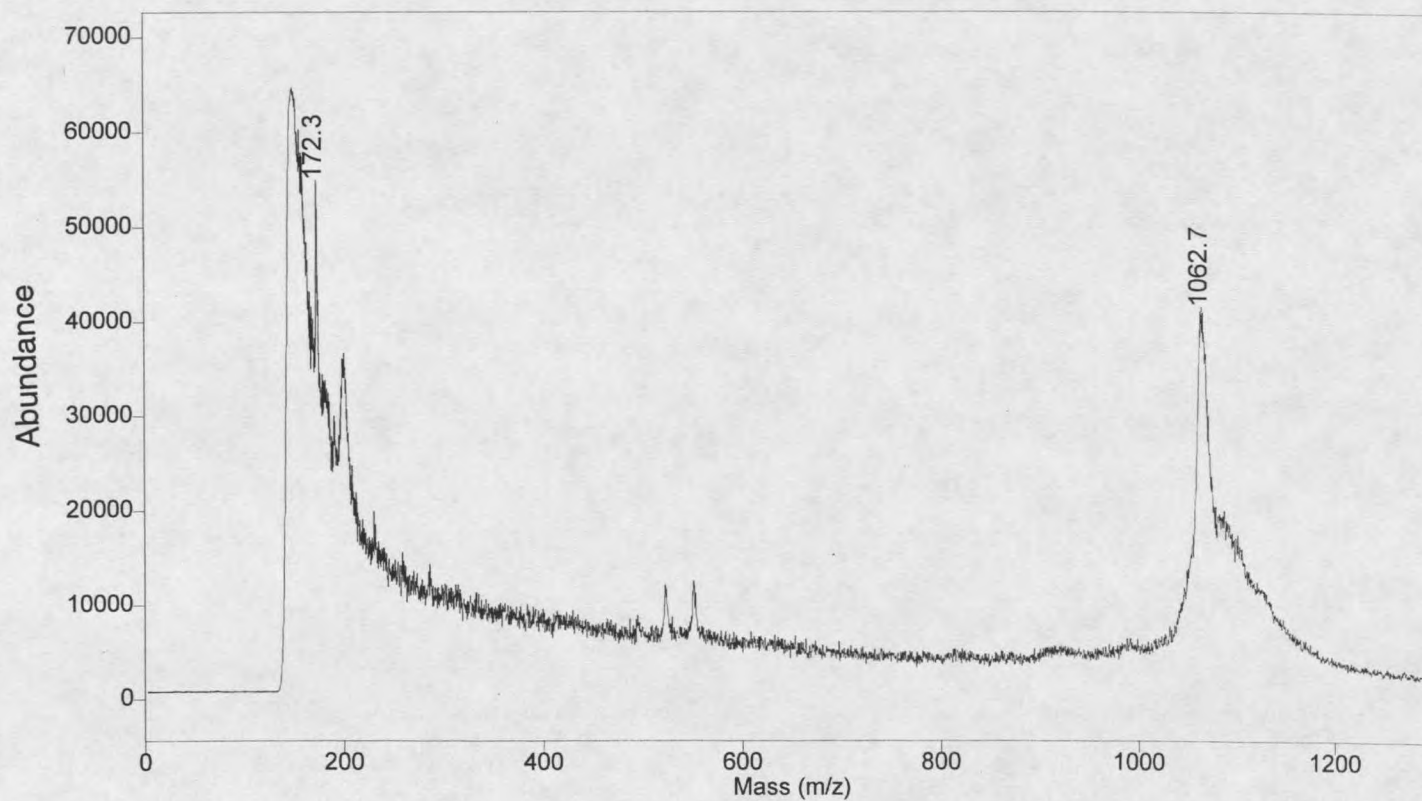


Figure 29. Mass spectrum of bradykinin (mw= 1,060.5) with saturated α -cyano-4-hydroxy cinnamic acid and with glycerol (1/1, v/v) as the matrix.

to immobilize the SALDI solid powder without substantially degrading the SALDI mass spectra. The ratios for these components in the SALDI suspension affects both mass resolution and sensitivity. In order to obtain the best SALDI mass spectra, varying ratios of the three materials were studied.

In a series of experiments, SALDI mass spectra were recorded using different amounts of glycerol in the SALDI suspension sample. The amount of activated carbon powder and the amount of sucrose were kept the same in these experiments. The sample preparation was as follows. An approximately equal volume of activated carbon powder was mixed with varying concentration of glycerol in methanol solution. The suspension was mixed using a vortexer. One microliter of the SALDI suspension was applied to a sample well. Then, 0.50 μL of a 5.0 % sucrose in water (w/w) solution was added. Finally, 1.0 μL of a bradykinin solution (1 nmole) in water was added to the well. Figure 30 shows the SALDI mass spectra of bradykinin by adding different concentrations of glycerol/methanol solution to the SALDI sample. The concentrations of glycerol in methanol were 15%, 30%, 60% by volume. The corresponding ratios of glycerol to activated carbon are 0.75, 1.50, and 3.0 by weight. It is seen that the width of the protonated bradykinin ion peak is decreased when the ratio of glycerol to activated carbon is increased. The mass spectrum with the 3.0 ratio of glycerol to activated carbon shows the best mass resolution. However, the intensity of protonated bradykinin ions in the mass spectrum with the 3.0 ratio is the lowest. It is tentatively concluded that the mass resolution is improved by adding more glycerol to the SALDI suspension. However, at the same time, the intensity of the analyte ions is decreased. In order to achieve the optimum result, both mass resolution and sensitivity must be considered.

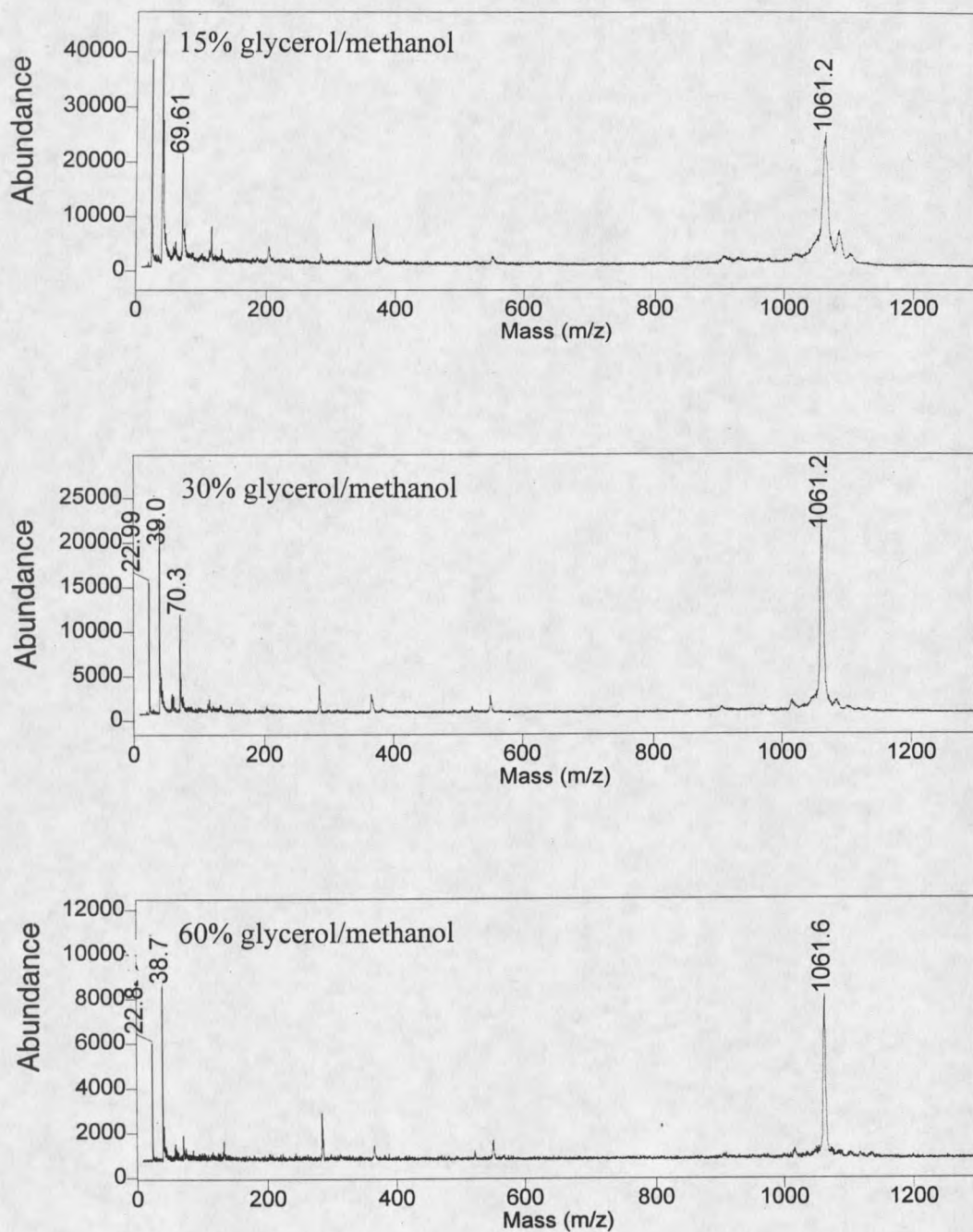


Figure 30. Activated carbon/SALDI mass spectra of bradykinin ($m_w = 1,060.5$) using different concentration glycerol to prepare the SALDI suspension: 15% glycerol/methanol (LP= 380, SA= 42), 30% glycerol/MeOH (LP= 380, SA= 55) , and 60% glycerol/MeOH (SA=380, SA= 46) from top to bottom. The ratios of glycerol to activated carbon (w/w) are 75%, 150%, 300%, respectively.

It is considered that the 1.5 ratio of glycerol to activated carbon in the SALDI sample gives the mass spectra with the best compromise between mass resolution and analyte ion intensity. The mass resolution with the 1.5 ratio is almost as good as that with the 3.0 ratio of glycerol to activated carbon. Furthermore, the intensity of the protonated bradykinin ions is very similar. In addition, by using the 1.5 ratio of glycerol to activated carbon, the alkali adduct ions of bradykinin are reduced relative to the 0.75 ratio. The 1.5 ratio was obtained by adding a 30% glycerol in methanol solution to the SALDI suspension. This concentration was used for most subsequent studies in this work.

Another factor that affects the SALDI mass spectra is the concentration of the sucrose "adhesive". One might ask at what sucrose concentration the SALDI mass spectra will start to degrade. Several series of experiments with varying concentrations of sucrose in the SALDI suspension were performed. The sample preparation (method D) was as follows. An approximately equal volume of activated carbon powder was mixed with 50% glycerol/methanol. This suspension was mixed using a vortexer. One microliter of the suspension was deposited into a sample well, and 1.0 μL of an aqueous sucrose solution was then added. Finally, 1.0 μL of a bradykinin (1.0 mM) solution in water was added. The procedure was repeated with sucrose concentrations from 5.0 to 60% (w/w). Figure 31 shows the SALDI mass spectra of bradykinin from one such series. The concentrations of sucrose in water were 5.0%, 25%, and 50%. After evaporation of the water, this corresponded to a sucrose to glycerol ratio of approximately 0.08, 0.16, and 0.32 (w/w), respectively. The width of the protonated bradykinin peaks are almost the same in the three mass spectra. However, the intensity of the protonated bradykinin molecular ion peak

decreases as the sucrose concentration increases. Similar results were obtained using sample preparation method C. It is concluded that the sucrose additive does not affect the SALDI mass spectra very much. The lowest concentration 5.0% (w/w), sucrose in water, is sufficient to largely immobilize the SALDI solid powder. For this reasons, 1.0 μ L of 5.0% (w/w) sucrose in water solution was added to the SALDI matrix system in most subsequent studies.

A significant advantage of adding sucrose to the SALDI suspension is due to the fact that the viscosity is increased and the volatility is lower. For this reason, the analyte sample remains in the ion source for a longer time without any degradation. This has otherwise been a problem (Dale et al., 1996). This is illustrated in Figure 32. The top spectrum in Figure 32 shows the SALDI mass spectrum of angiotensin II without added sucrose and after the sample has been in the ion source for 30 minutes. This sample was the same as that used for the top mass spectrum in Figure 24, but the Figure 24 spectrum was obtained within 5 minutes after the sample plate had been injected into the ion source. Abundant alkali adduct ions of angiotensin II are observed after 30 minutes under vacuum. Also, the mass resolution has greatly deteriorated. In contrast, the SALDI mass spectrum of angiotensin II at the bottom of Figure 32 was also obtained after 30 minutes in the ion source; however, in this case sucrose had been added to the SALDI sample. It is seen that the mass resolution is still nearly as good as after 5 minutes (compare the bottom mass spectrum in Figure 240. Also, much lower alkali adduct ions are observed. However, the total ion yields are lower after 30 minutes in vacuum. Presumably, this is due to an increase in the sucrose concentration in the sample. It was observed that the resolution of the SALDI spectrum tended to degrade

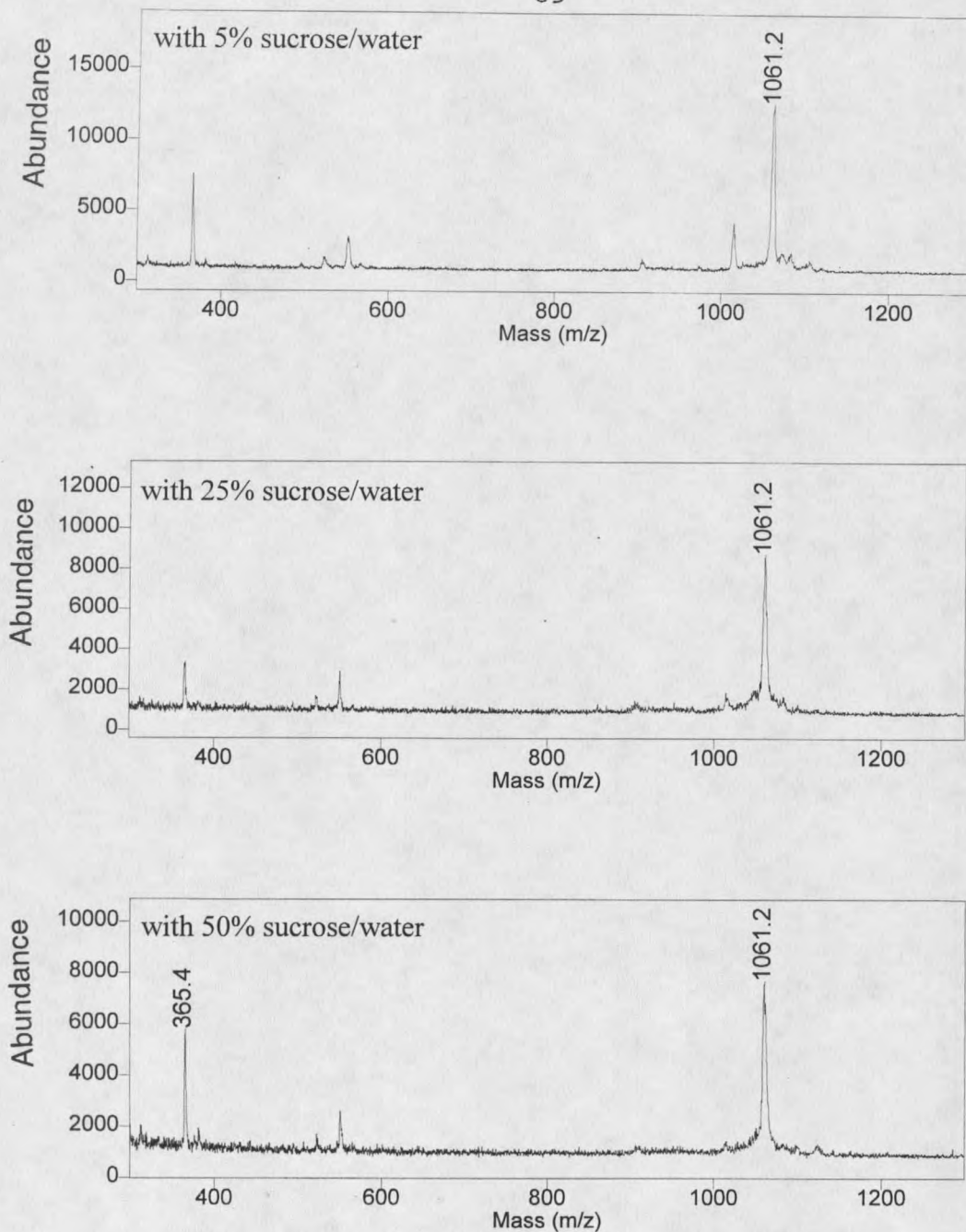


Figure 31. Activated carbon/SALDI mass spectra of bradykinin (mw= 1,060.5) using different concentrations of sucrose in water to immobilize activated carbon powder: 5% sucrose/water (LP= 400, SA= 58), 25% sucrose/water (LP= 400, SA=38), and 50% sucrose/water (LP= 400, SA= 44) from top to bottom.

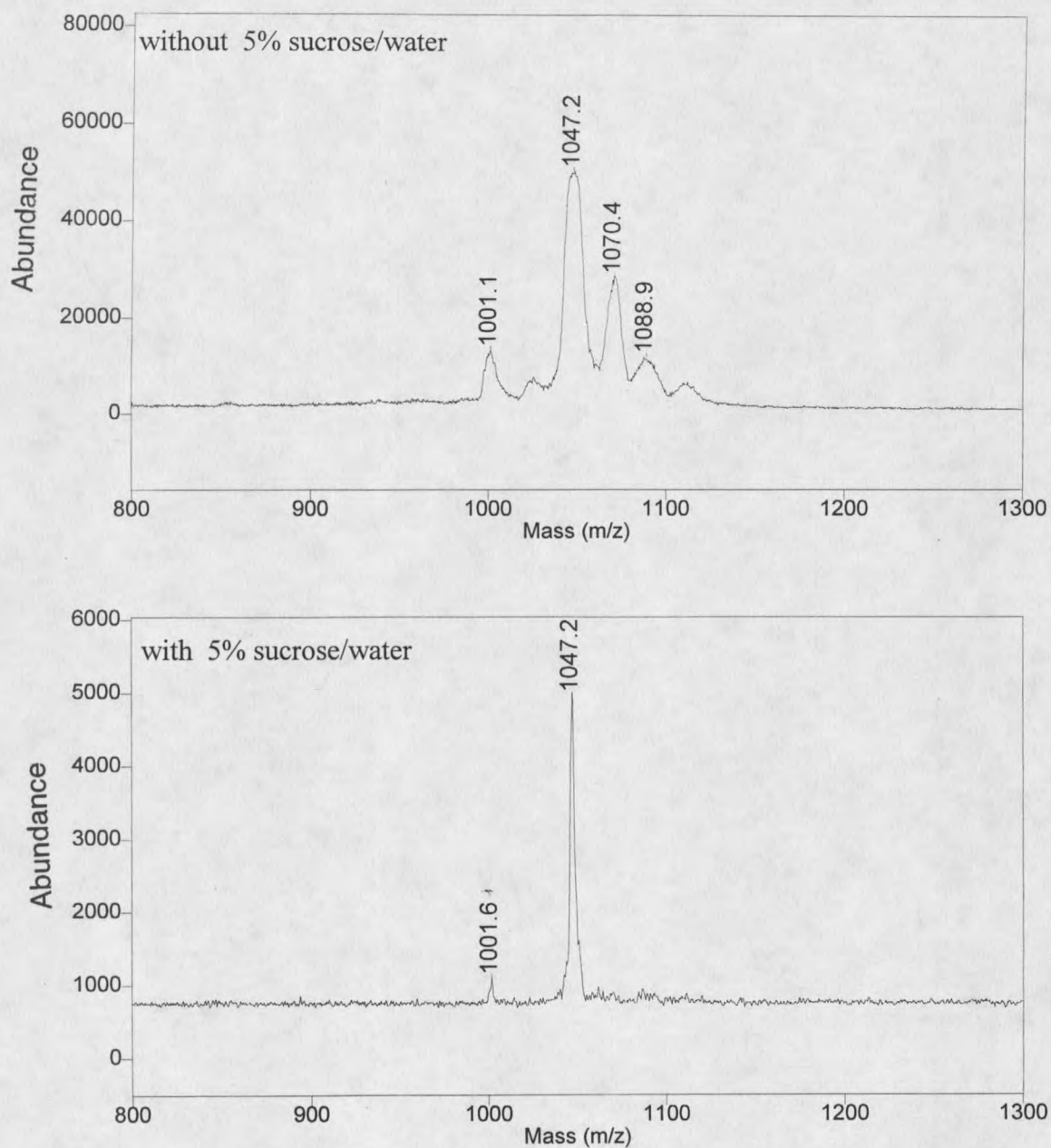


Figure 32. The same sample as for Figure 24. However, the mass spectra were recorded after the sample had been left in the ion source for 30 minutes. top (LP= 400, SA= 29). bottom (LP= 445, SA= 24)

as samples sat in the vacuum chamber of the mass spectrometer. This presented a real limitation since for the best reproducible data only a small number of samples could be introduced on the sample plate. However, we found that the addition of 5% (w) sucrose to immobilize the SALDI solid powder also stabilized the signals against degradation caused by exposure to the vacuum.

From the studies above, it was concluded that near the ideal proportion of SALDI solvent plus SALDI adhesive is sucrose/glycerol/MeOH= 5/28/67 (w/w/v). Then, roughly equal volume (0.10 mL of activated carbon powder has an approximate weight of 25 mg.) of activated carbon was mixed with the SALDI solvent (0.10 mL).

Detection Limit of SALDI

In the early SALDI work, it was found that the detection limit for typical analytes was about 1.0 pmole (Sunner et al., 1995). In these initial experiments, 1.0 μ L of an analyte solution was added to 0.50 μ L of glycerol with graphite powder sprinkled on the sample surface. It was found that the intensity of the analyte ions was roughly proportional to the concentration in the analyte solution. The more concentrated analyte in the sample, the more intense signals were obtained in the SALDI mass spectra. It was argued, therefore, that sensitivity can be increased by concentrating the analyte in a very small volume of SALDI suspension. The concentrating procedure used was as follows. The SALDI suspension was made by mixing roughly equal volumes of activated carbon powder and 50% glycerol/methanol. Two μ L of this suspension was then mixed with 1.0 μ L of 5.0% sucrose in water. About 0.10 μ L of the resulting mixture was applied to a sample well. Then, 0.10

μL of analyte solution was carefully added to this SALDI matrix. Bradykinin and angiotensin II were used as the analytes in these experiments. A series of analyte dilutions were made. The detection limits of bradykinin and angiotensin II were obtained when $0.10\ \mu\text{L}$ of a $10^{-6}\ \text{M}$ analyte solution was used. This corresponds to 100 fmole of peptides. However, much lower detection limits are possible because the volume of SALDI suspension needed to obtain spectra is much less than $0.10\ \mu\text{L}$. Using a needle tip, a fraction of the SALDI suspension was moved from the original sample well to another sample well. The amount transferred was estimated by weighing the original mixture and was found to be about one tenth of the original sample. The laser focus was not easily seen because the sample was so small. A label was therefore made on the TV monitor screen. It was then easy to move the laser focus to the small sample. The absolute amount of analyte in the small sample was $10^{-7}\ \text{L} \times 10^{-6}\ \text{M} \times 10^{-1} = 10^{-14}$ mole. The ratio of signal to noise was about three with this amount. It is concluded that the SALDI detection limit is lower than 10 fmole of peptide.

Mass Range of SALDI

The largest molecule that has been observed in this SALDI work is myoglobin (mw=16,951) as shown in Figure 33. Efforts were made to obtain SALDI spectra also of bovine albumin, trypsin, and urease, but all were unsuccessful. For proteins, the SALDI upper mass limit remains at less than 20 kDa. The reason for this is not understood. It is of interest that multiple charged ions of proteins are commonly observed, but the mechanism behind this is unknown.

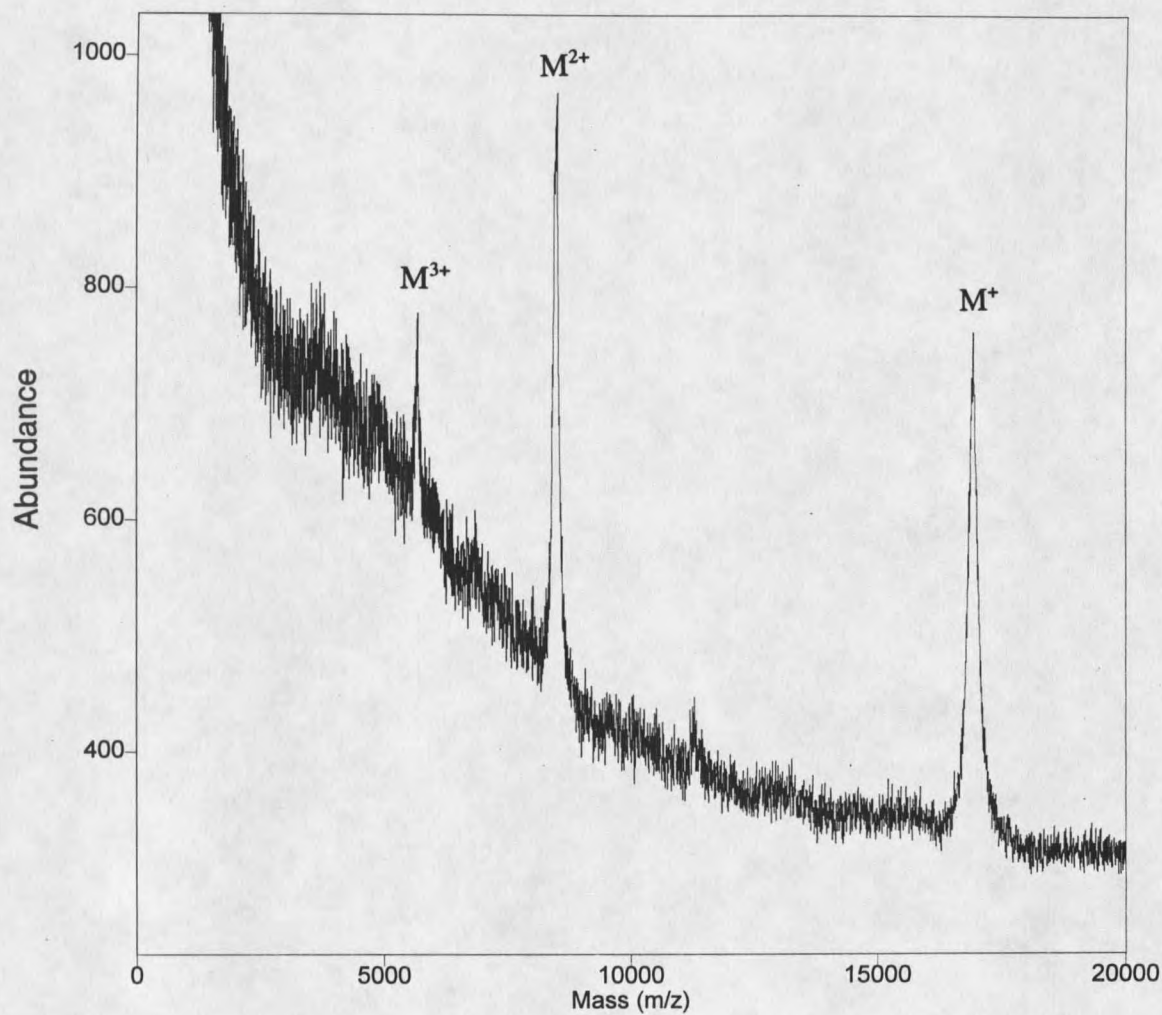


Figure 33. Activated carbon/SALDI mass spectrum of myoglobin (mw= 16951).

CHAPTER 4

SALDI SOLVENT EFFECTS

Gas-Phase Basicity Effects in SALDI

In FAB, gas-phase basicity is an important factor in determining mass spectra. Since all liquid matrices used in FAB can be used also as SALDI solvents, the gas-phase basicity of the SALDI solvent may be expected to have similar effects on the SALDI mass spectra as they have in FAB. In this section, studies of gas-phase basicity effects in SALDI will be described.

To investigate how important the gas-phase basicity of the SALDI solvent is for the SALDI spectra, asparagine was used as the analyte. If extensive proton transfer reactions occur in the SALDI ionization process, the intensity of protonated asparagine molecular ions should be affected by the gas-phase basicity of the SALDI liquid. Two SALDI solvents were used in this study, DEA and glycerol. The gas-phase basicity of DEA is 220 kcal/mol, and the gas-phase basicity of glycerol is 195 kcal/mol (Sunner et al., 1995). In the early SALDI results, it was shown that protonated DEA dominated the SALDI mass spectrum although only 0.25% DEA was added to glycerol (see Figure 6). It seems that most of the protons are transferred to DEA from glycerol because of the higher gas-phase basicity of DEA.

In this study, different amounts of DEA were added to glycerol to give mixed SALDI solvents. Figure 34 shows a semi-log plot of the intensity of protonated asparagine in the SALDI mass spectra versus the DEA concentration in glycerol. The intensity of protonated asparagine is seen to decrease with increasing DEA concentration. When the effective gas phase basicity of the SALDI solvent is increased, the intensity of protonated analyte decreased. This demonstrates that the gas-phase basicity of the SALDI solvent affects the mass spectra. When the gas phase basicity of the SALDI solvent is increased, the analyte molecules have less chance to compete for protons. Figure 35 shows a negative ion mode SALDI mass spectrum of asparagine using DEA as the SALDI solvent. The de-protonated asparagine molecules dominate the mass spectrum. Presumably, this is a result of transfers of protons from asparagine to the $(\text{DEA}+\text{H})^+$ ions.

A gas-phase basicity effect in SALDI has been observed also for large molecules like cytochrome C. In these experiments, TEA, DEA, and glycerol were used as the SALDI solvents. TEA has the highest gas-phase basicity among these three SALDI solvents. Figure 36 shows the SALDI mass spectra of cytochrome C using these three SALDI solvents. There is a dramatic decrease in the number of multiple charges when the gas-phase basicity of the SALDI solvent is increased from glycerol to TEA. In glycerol, the maximum number of multiple charges is as high as five. However, in TEA and DEA, the maximum number of multiple charges is two. It seems that when the gas phase basicity of the SALDI solvent is higher, fewer protons can be attached to the cytochrome C molecules. In other experiments, a 10⁻⁴% DEA in GI (v/v) solvent was used. Although the DEA concentration was very low, the maximum number of the cytochrome C multiple charges was still observed to be two

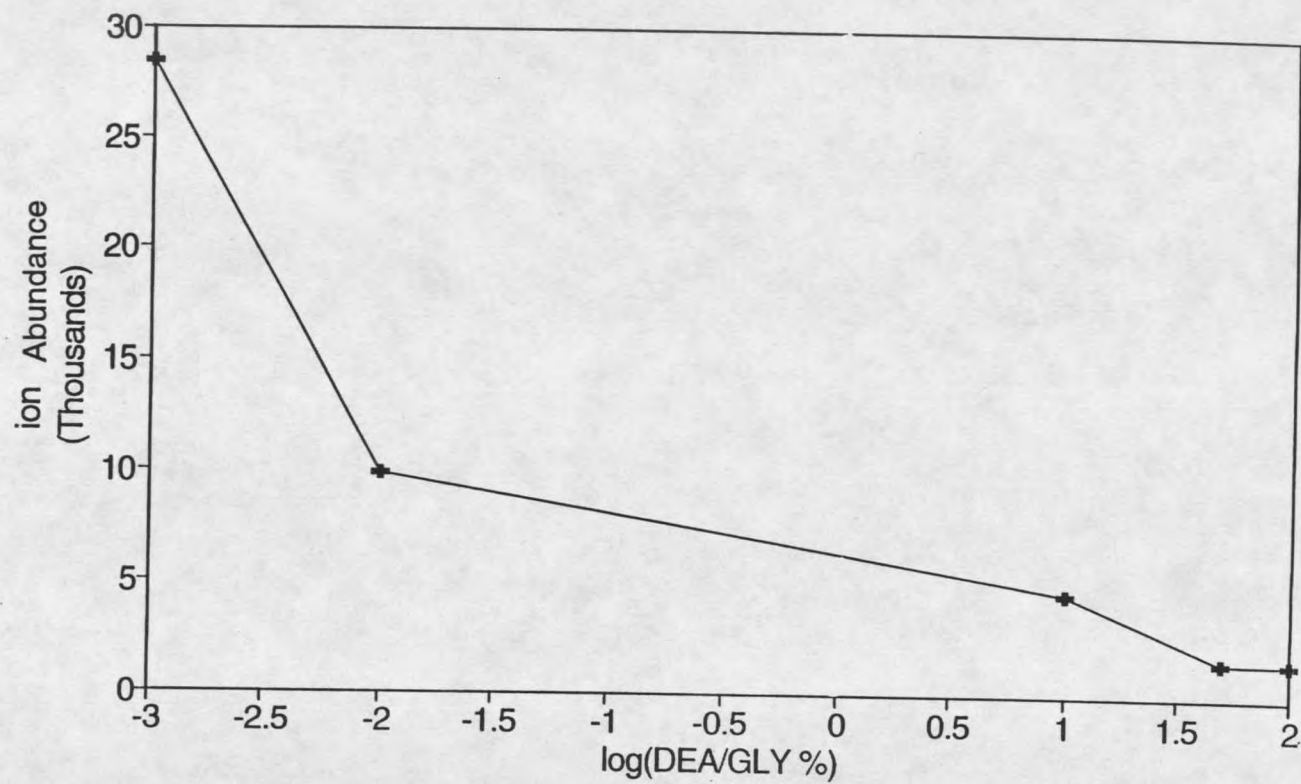


Figure 34. Semilog plot of the intensity of asparagine pseudo-molecular ions in the SALDI mass spectra versus DEA/GI concentration in the SALDI matrix.

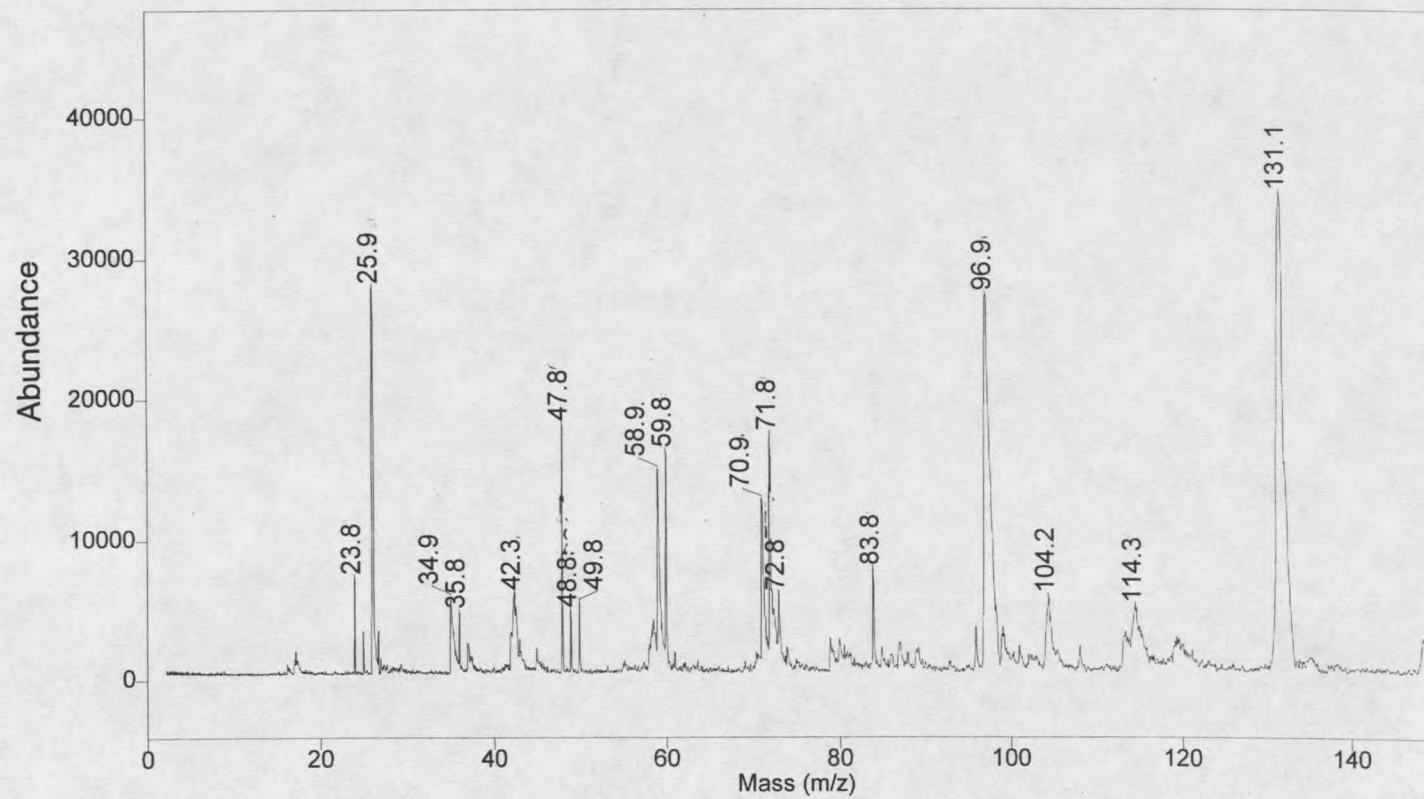


Figure 35. Negative ion activated carbon/SALDI mass spectrum of asparagine (mw= 132.1, 0.1 nmol). LP= 405. SA= 28.

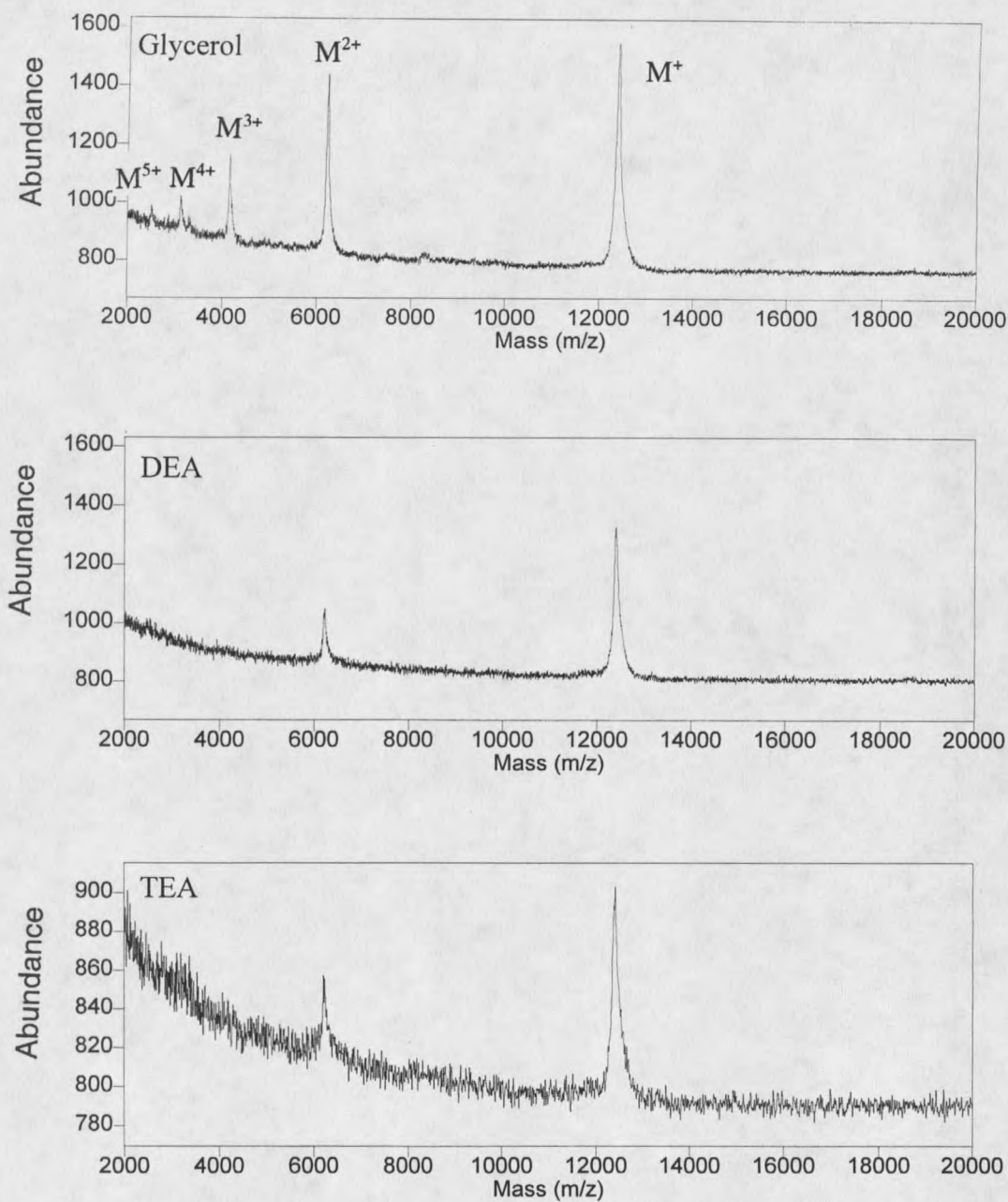


Figure 36. Activated carbon/SALDI mass spectra of cytochrome C (mw= 12,360). The SALDI solvents are Gl, DEA, and TEA.

(results not shown). It is surprising that multiple charging was suppressed by this small amount of DEA. However, the total intensity of the cytochrome C peak was not affected much by the gas phase basicity of the liquid matrix. Another observation is that the intensity of multiple charged cytochrome C changed with the time that the laser beam hit the same sample spot. Figure 37 shows the SALDI mass spectrum of cytochrome C obtained immediately after the sample was inserted in the ion source. The maximum charge number is three. The mass spectrum obtained after the laser pulses had been focused onto the sample spot for 40 minutes is shown at the bottom of Figure 37. The peaks of M^{4+} and M^{5+} are not quite intense. In addition, the intensity of M^{3+} had increased. The doubly charged ion peak, M^{2+} , dominates the mass spectrum. Presumably, impurities on the surface of the SALDI solid were cleaned off by the laser beam. The cytochrome C may then have a better chance to compete for protons. In contrast, when DEA or TEA was used as the SALDI solvent, the maximum charge number remained constant with irradiation time.

Figure 38 shows the SALDI mass spectrum of angiotensin II with DEA as the SALDI solvent. As with glycerol, only singly protonated angiotensin II is observed. Angiotensin II, is likely to be too small to obtain more than one proton in SALDI process. The intensity of protonated angiotensin II was not appreciably changed by the high gas-phase basicity of DEA. However, more abundant alkali adduct ions were observed with DEA than with glycerol.

It has been seen that the gas phase basicity of the SALDI solvent has a strong effect on the SALDI spectra. For small molecules, de-protonation of the analyte molecules by a high gas-phase basicity SALDI solvent is observed. For medium size molecules like peptides, the

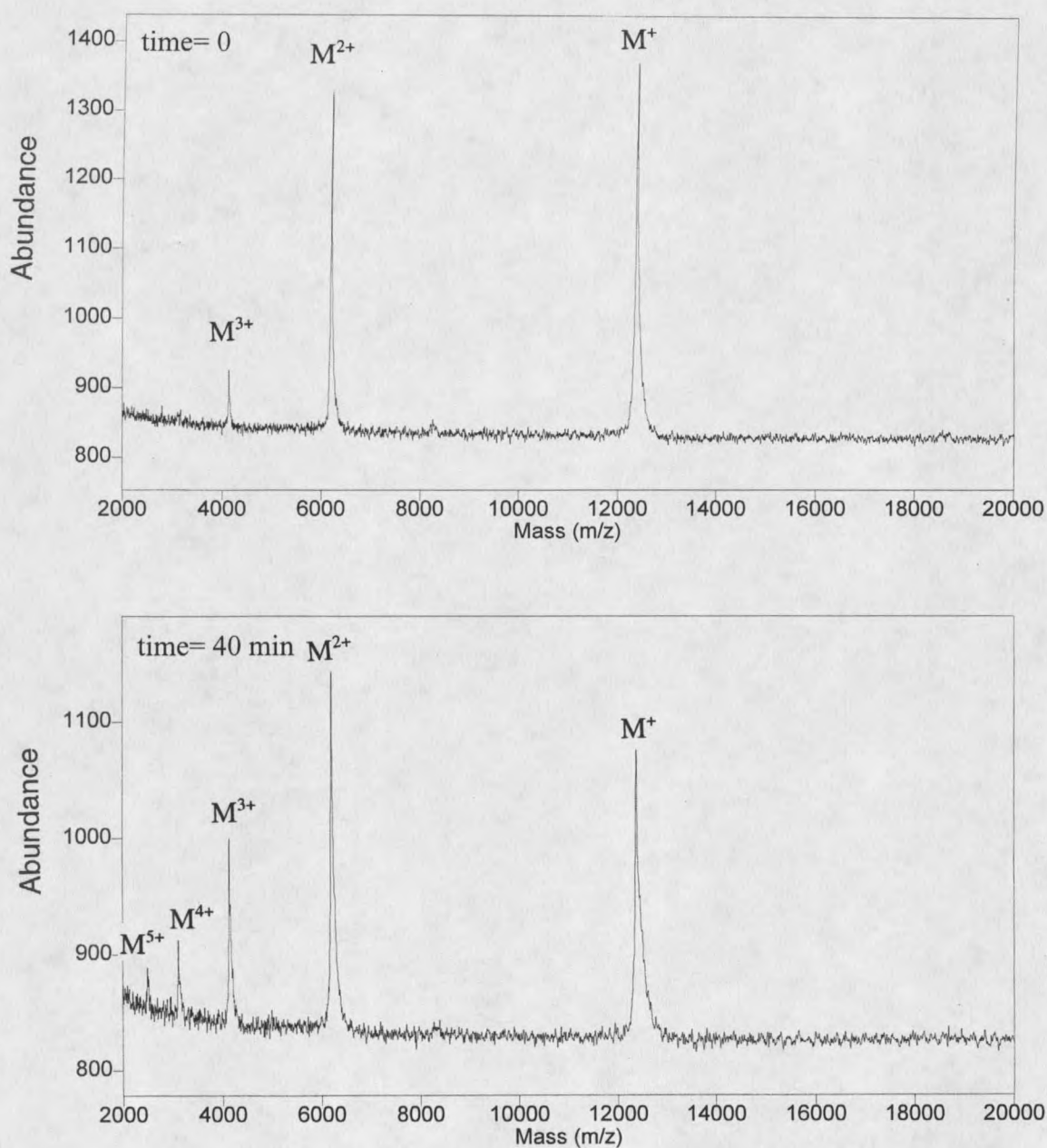


Figure 37. Activated carbon/SALDI mass spectra of cytochrome C (mw= 12,360). Spectra were obtained in the few minutes (top) and after 40 minutes (bottom) of irradiating the same spot with the laser.

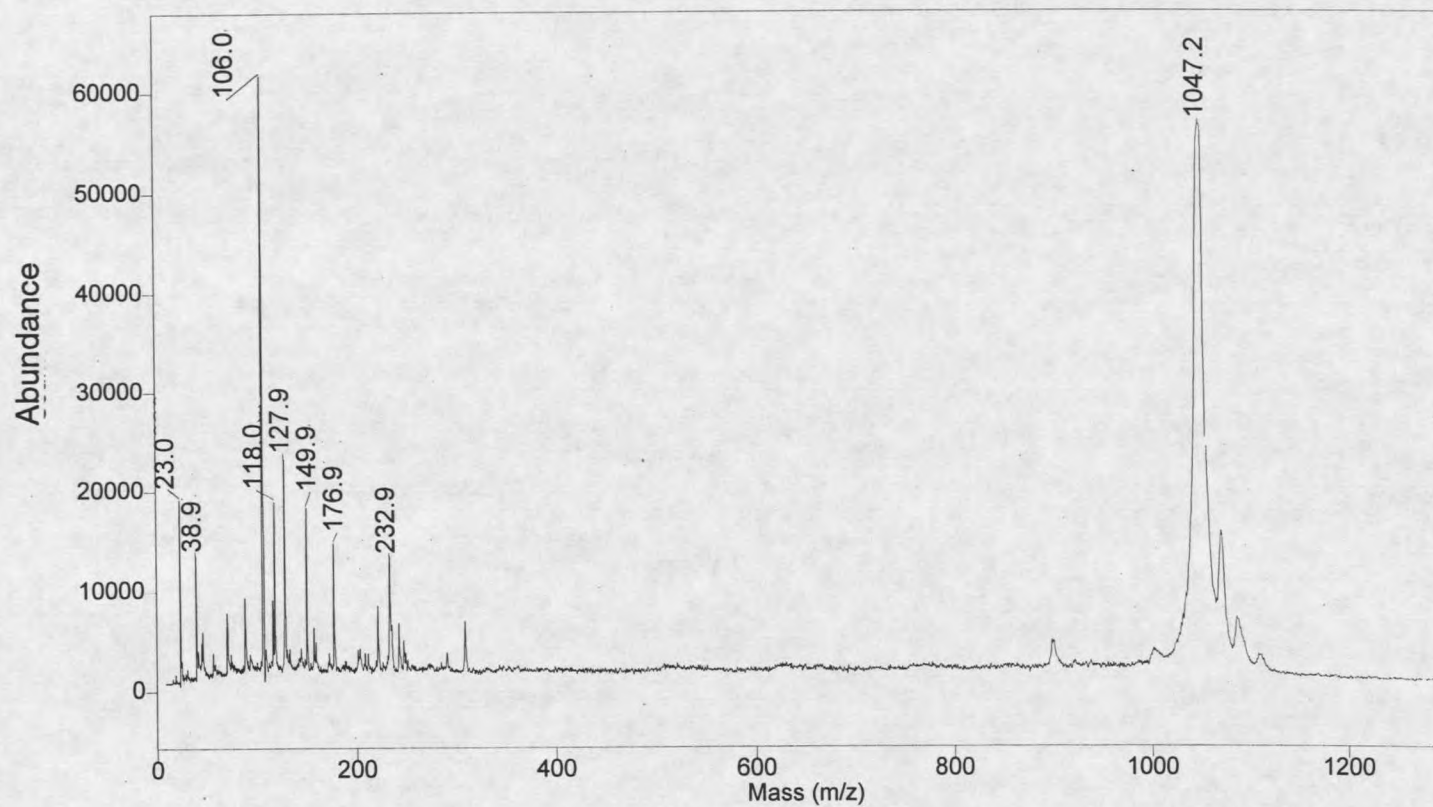


Figure 38. Activated carbon/SALDI mass spectrum of angiotensin II (mw= 1046.2) using DEA as the SALDI solvent.

sensitivities were not affected. However, more abundant alkali adduct ions were observed in the mass spectrum when DEA was used as the SALDI solvent than with glycerol. For large molecules like cytochrome C, the multiple charge distribution is shifted. Higher gas phase basicity of SALDI solvents reduces the possibility of cytochrome C molecules obtaining more than two protons during the SALDI process.

Salt Effects in SALDI

SDS (sodium dodecyl sulfate) is a common surfactant used to solubilize hydrophobic analytes in general and in particular in gel electrophoresis. At least trace amounts of SDS are commonly present in biochemical samples. In order to understand how this may affect SALDI spectra, different concentrations of SDS added to the SALDI solvents were used. SALDI sample preparation "method C" (see Chapter 2, Experimental) was used in the following experiment, unless otherwise noted. For comparison, the top spectrum in Figure 39 shows the SALDI mass spectrum of bradykinin without adding any SDS to the SALDI glycerol solvent. Alkali adduct ions of bradykinin are absent in the mass spectrum. The middle spectrum in Figure 39 shows the SALDI mass spectrum of bradykinin (1.0 nmole) with 2.5% SDS added to the glycerol. The sodiated bradykinin ion peak now has almost the same intensity as that of the protonated bradykinin ions. A peak corresponding to two sodium ions attached to bradykinin is also observed. The mass resolution is similar to that of the top spectrum. The bottom spectrum in Figure 39 is the SALDI mass spectrum of bradykinin with 5.0% SDS added to the SALDI solvent. Sodiated bradykinin ions dominate this mass spectrum. The peak with two sodium ions attached to bradykinin now has higher

intensity than in the 2.5% SDS spectrum. However, the mass resolution is still similar to that seen in the previous spectra. This shows that high SDS concentrations do not appreciably affect the mass resolution, but very abundant sodium adduct ions of the analyte molecules are observed at high SDS concentrations. It can also be seen that the total ion yield decreases with increasing SDS concentration. Furthermore, a higher laser power was required with 5.0% SDS in the SALDI solvent to obtain a spectrum.

With SDS added as shown above to the SALDI solvent, the mass resolution did not degrade. However, when sodium chloride was added to the SALDI solvent, the mass resolution was affected. This is illustrated in Figure 40 that shows the SALDI mass spectrum of bradykinin without (top) and with (bottom) 1.25% NaCl added to the SALDI solvent. With 1.25% NaCl added, protonated bradykinin ions are not observed in the mass spectrum, and sodiated bradykinin ions $((M)Na^+ (m/z = 1083), ((M-H)^- Na^+)Na^+ (m/z = 1105))$ dominate. Furthermore, the mass resolution is quite poor. The intensity of sodium adduct ions increases by the addition of sodium salt like SDS or NaCl. However, the mass resolution is not affected by SDS, but does deteriorate with NaCl addition. For the purpose of increasing the sodium adduct ions of analytes, SDS is therefore the best choice.

Acid Effects in SALDI

In MALDI, most of the matrices used are acids. In FAB, acids are often helpful to solubilize analytes. In this section, the effects of acids on SALDI spectra will be discussed. In most cases, glycerol is used as the SALDI solvent. Glycerol is a neutral compound. Whether acid is helpful for increasing the sensitivity in SALDI or not is of considerable

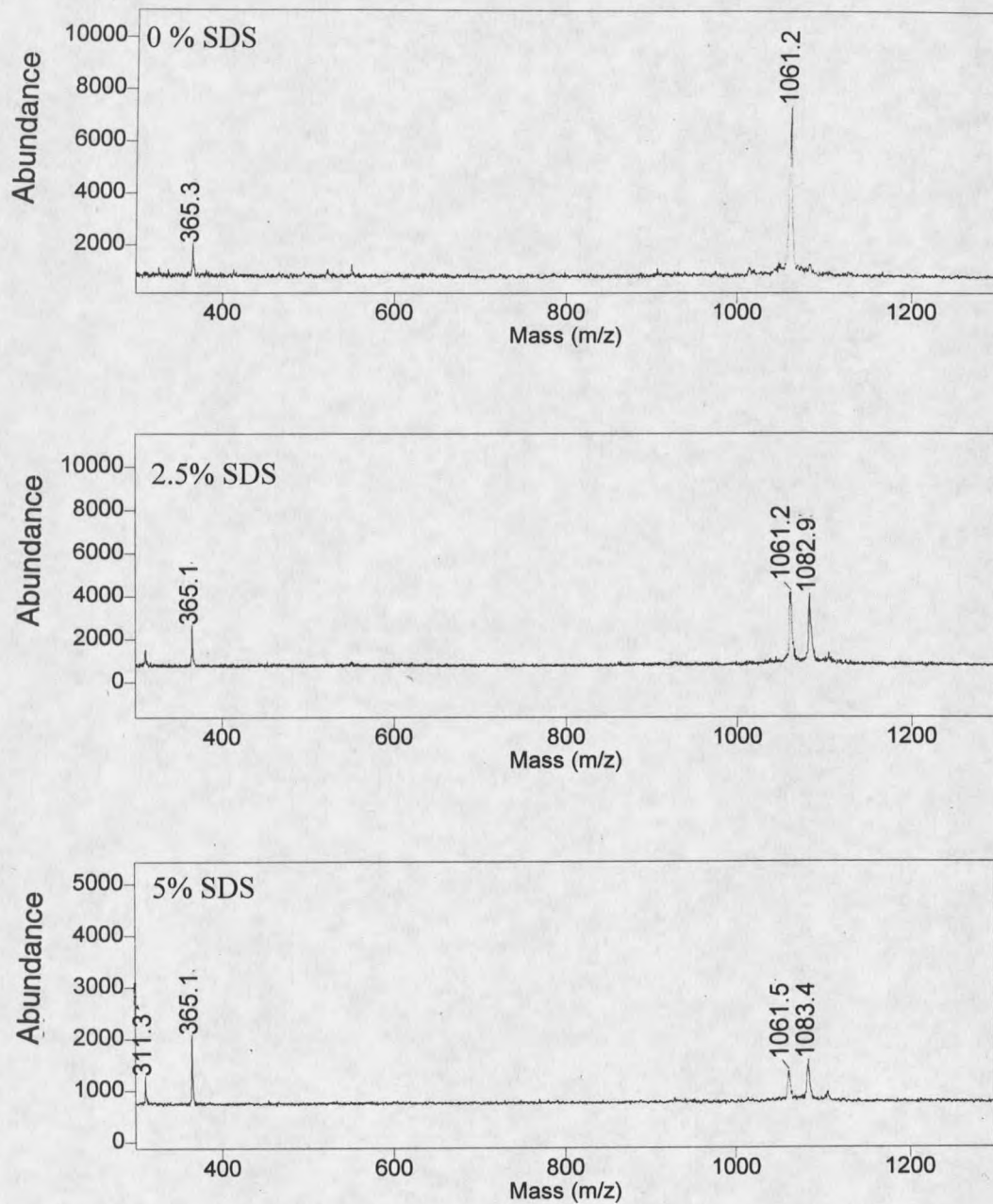


Figure 39. Activated carbon/SALDI mass spectra of bradykinin (mw= 1,060.5) with varying concentrations of SDS in the SALDI suspension: 0% SDS (LP= 415, SA= 22), 2.5% SDS (LP= 415, SA= 36), and 5% SDS (LA= 455, SA= 65) from top to bottom.

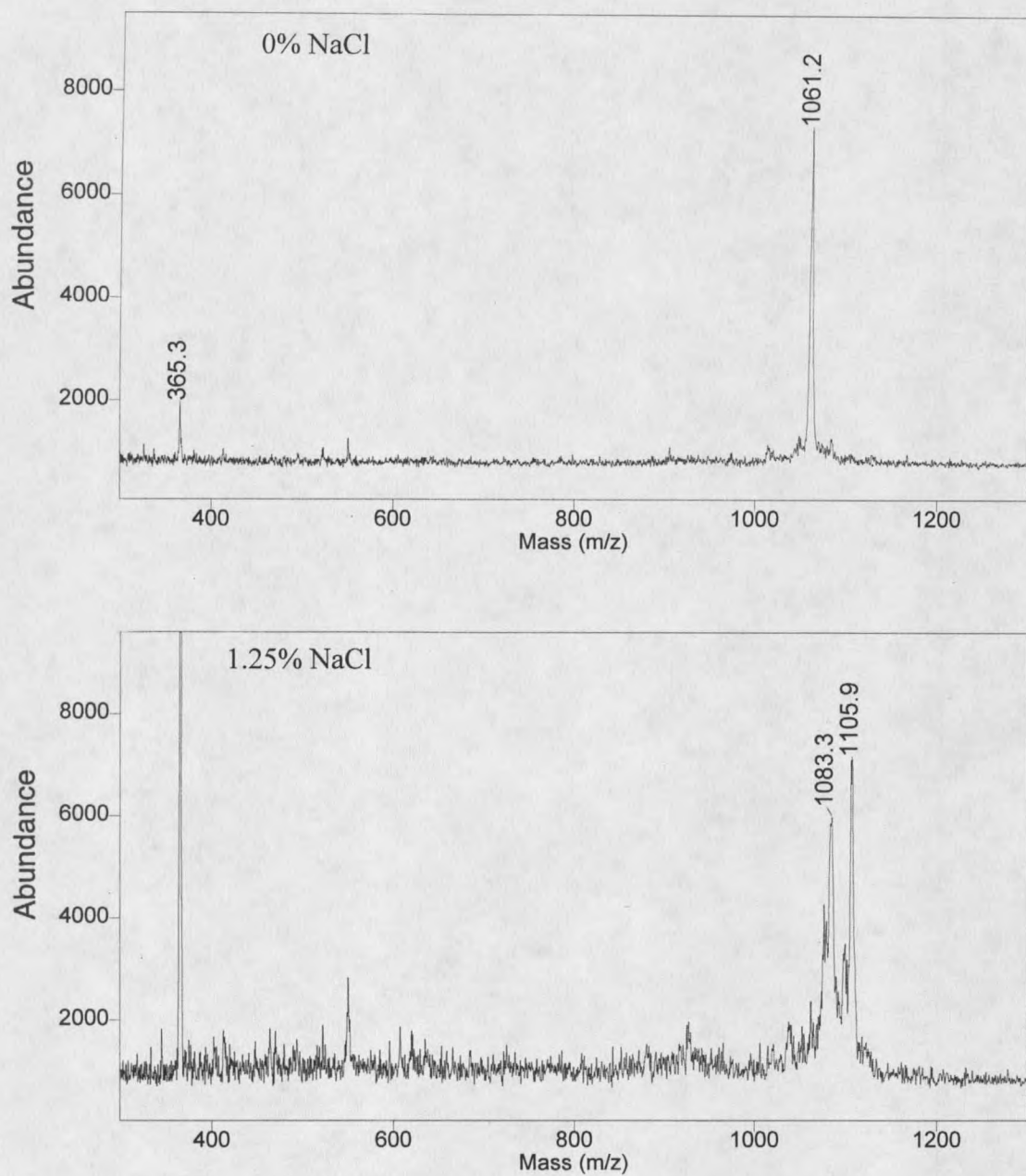


Figure 40. Activated carbon/SALDI mass spectra of bradykinin (mw= 1,060.5) with various concentrations of NaCl in the SALDI suspension: 0% NaCl (top, LP= 415, SA=22) and 1.25% NaCl (bottom, LP= 410, SA=8).

interest. In a series of experiments, varying concentration acetic acid was added to the SALDI solvent. Sample preparation "method D" (see Chapter 2, Experimental) was used as described earlier. For comparison, the top spectrum in Figure 41 shows the SALDI mass spectrum of bradykinin without added acid. The middle spectrum in Figure 41 shows the same mass spectrum but with 0.30% acetic acid added to the SALDI solvent. The mass spectrum did not change, compared with the top spectrum. The bottom spectrum in Figure 41 is the SALDI mass spectrum of bradykinin with 2.5% acetic acid in the SALDI solvent. It is clearly seen that the intensity of protonated bradykinin ions has drastically decreased. Furthermore, the mass resolution in the molecular ion region becomes worse. Figure 42 shows another example of the effect of adding acetic acid to the SALDI solvent. For comparison, the top spectrum in Figure 42 shows the SALDI mass spectrum of angiotensin II without added acid. Sodium adduct and potassium adduct ions of angiotensin II molecules are observed in this mass spectrum. The middle spectrum in Figure 42 is the SALDI mass spectrum of angiotensin II with 0.3% acetic acid in the SALDI solvent. The intensity of protonated angiotensin II ions is almost identical to that of top spectrum. However, the intensities of the alkali adduct ions have increased. The bottom spectrum in Figure 42 shows the SALDI mass spectrum of angiotensin II with 2.5% acetic acid in the SALDI solvent. The intensities of the alkali adduct ions have increased further. The mass resolution has become worse. Furthermore, the total intensity of analyte ion has decreased. To conclude, 0.3% acetic acid in the SALDI solvent did not significantly affect the SALDI mass spectra. However, when the acetic acid concentration was increased to 2.5%, there were serious effects on the SALDI mass spectra. The intensity of analyte ions and the mass resolution

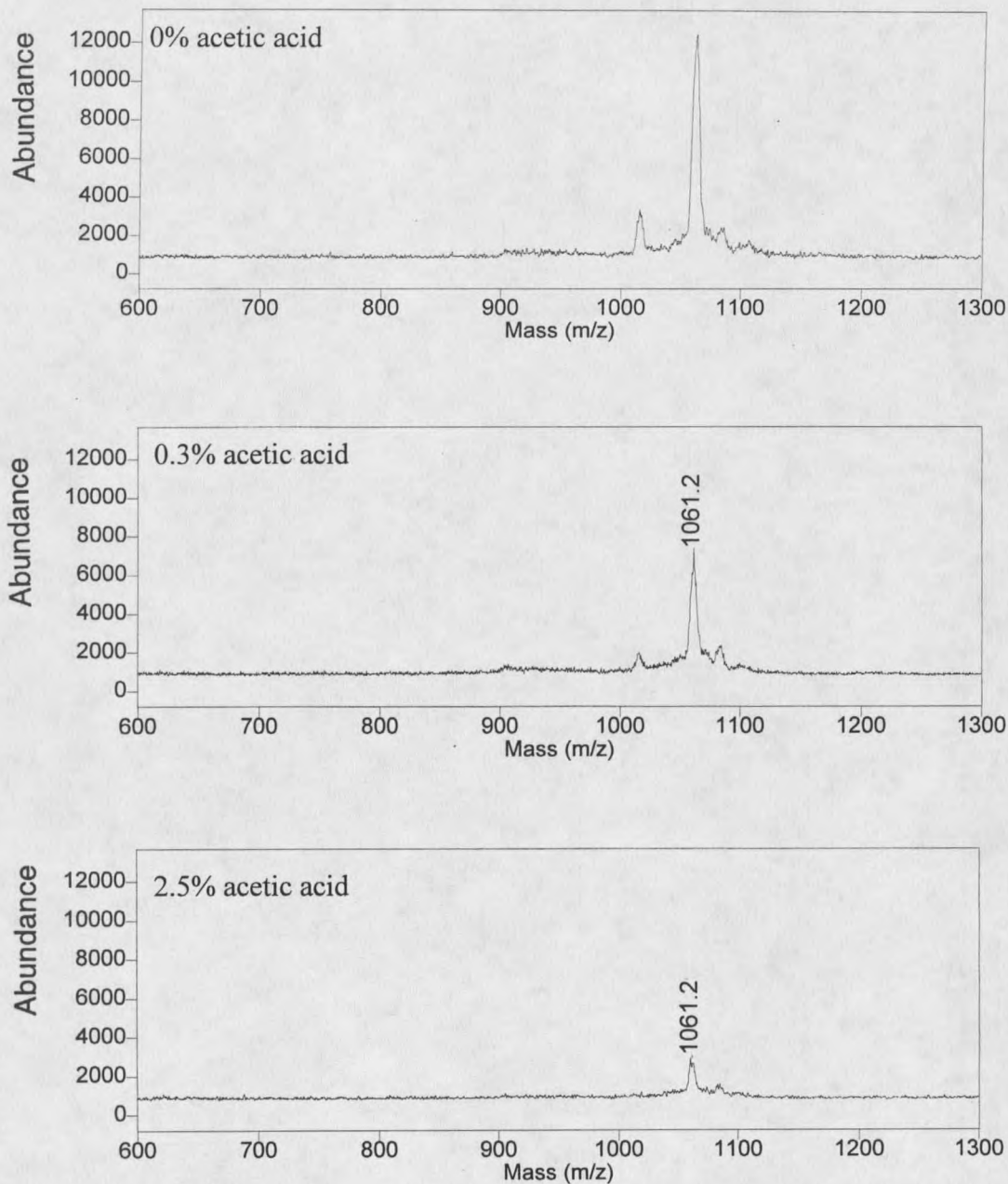


Figure 41. Activated carbon/SALDI mass spectra of bradykinin ($mw = 1,060.5$) with varying concentrations of acetic acid in the SALDI suspension: 0% acetic acid (LP= 400, SA= 32), 0.3% acetic acid (LP= 400, SA= 23), 2.5% acetic acid (LP= 400, SA=32) from top to bottom.

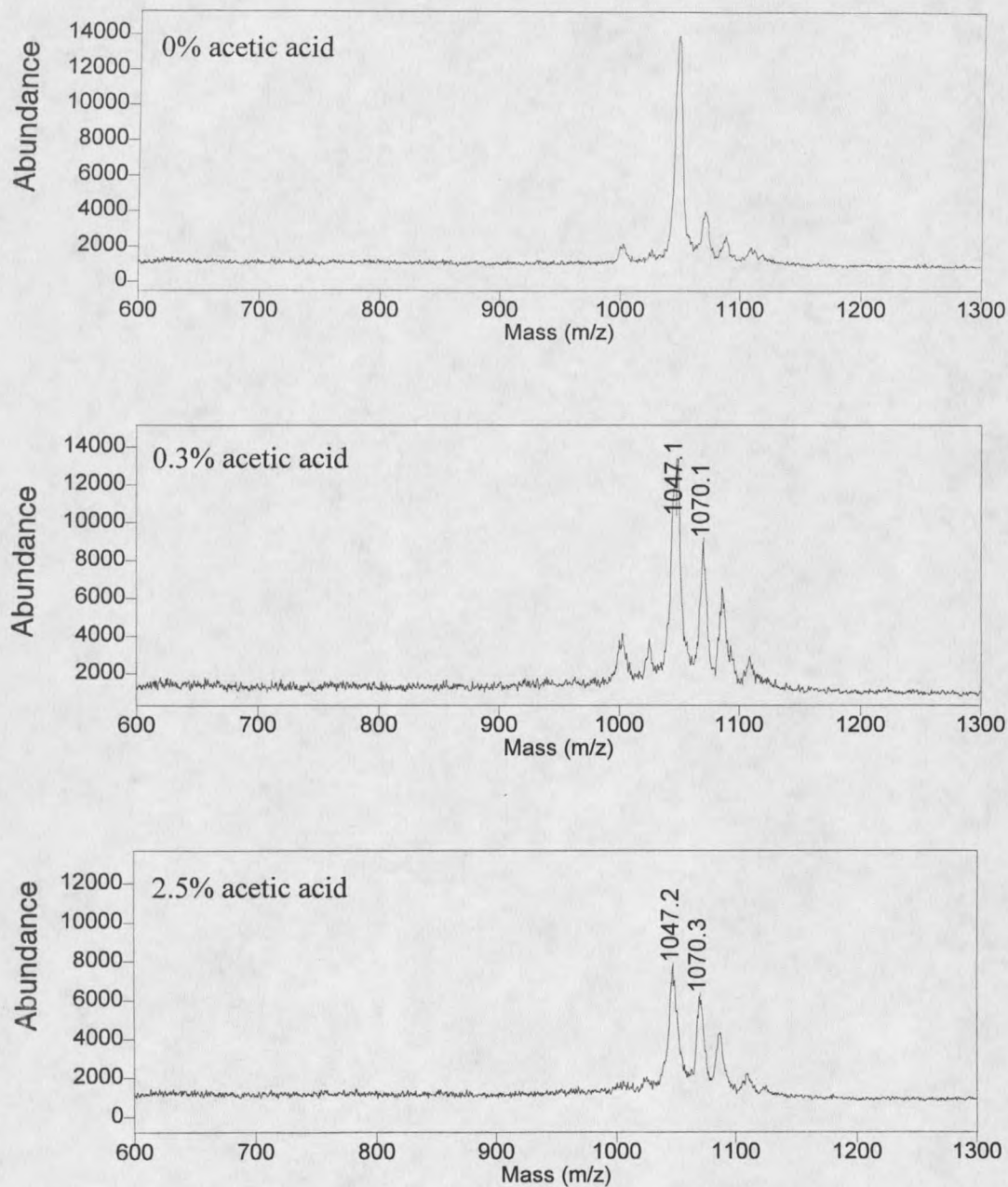


Figure 42. Activated carbon/SALDI mass spectra of angiotensin II ($mw=1,046.2$) with varying concentrations of acetic acid in the SALDI suspension: 0% acetic acid (LP= 400, SA= 34), 0.3% acetic acid (LP= 400, SA=18), and 2.5% acetic acid (LP= 400, SA= 28) from top to bottom.

were worse. Furthermore, very abundant alkali adduct ions were observed.

CHAPTER 5

APPLICATIONS OF SALDI

Analysis of Biochemicals

Most biochemicals are sensitive to pH changes. Keeping conditions as close as possible to physiological conditions may sometimes be important for analysis. In MALDI, the most common matrices are acids. For compounds that are sensitive to acids, these matrices cannot be used. In MALDI, the biochemicals need to co-crystallize with the matrix in order to be analyzed. However, not every biochemical compound has the ability to form good co-crystals with a given matrix. When the co-crystallization process does not work, no MALDI spectra are obtained. In most unknown biochemical samples, there are many compounds present in the solution. Such mixture often has a problem with forming co-crystals because of interference from other analytes. The result is that some analytes are suppressed by other analytes, and will not be observed in the MALDI mass spectrum. For example, enzymatic digest products are difficult to separate into pure peptides, and several peptides are typically present in digests to be analyzed. Furthermore, no unique MALDI matrix is ideal for every peptide or protein. A trial and error search for a special matrix compound is often needed.

In SALDI, there is no co-crystallization process. The analyte solution is directly analyzed from the SALDI suspension. Thus, the suppression effect is not expected to be as serious as in MALDI. The most commonly used SALDI liquid matrix is glycerol. It is almost universally useful for biochemicals because glycerol is polar and neutral. Thus, for many biochemicals that are sensitive to high or low pH, SALDI is potentially the analysis method of choice. SALDI also appear to tolerate low levels of SDS which can be used to solubilize hydrophobic materials. SALDI is especially useful for the analysis of enzymatic digests of proteins because of its low suppression effects and clean background ion spectrum. The SALDI results from peptides and mixtures of peptides, from digests, will be discussed. The results from the SALDI analysis of polyamino acids will also be presented.

Experimental

For tryptic digestion, a 0.10 mL of cytochrome C (800 μ M) solution was made, and ammonium bicarbonate (1.0 M) was added in order to adjust the pH value to about 7.5 to 8.0. Two microliters of a trypsin (0.010 g/mL) aqueous solution was added to the cytochrome C solution. The final ratio of enzyme to substrate was 1:50 (w/w). The digestion proceeded at 37°C for 20 hours (Lin, 1992).

For carboxypeptidase Y digestion of bradykinin, a 2.34 mM bradykinin solution was made in 6 M urea in water. Two microliters of a CPY (1.0 mg/mL) aqueous solution was then added to 0.10 mL of the bradykinin solution. This digestion reaction proceeded at 37°C. After the first ten minutes, two amino acids had been cleaved from the C-terminal. The reaction was stopped by adding 0.010% TFA.

Results and Discussion

Figure 43 shows a SALDI mass spectrum of a mixture of melittin (0.12 nmole), bradykinin (0.12 nmole), and methionine enkephalin (0.12 nmole) in one microliter solution. The SALDI solid was graphite. Although equal concentrations of peptides were added to the analyte solution, quite different intensities of analyte signals are observed. The intensity of protonated mellitin molecular ions is higher than those of the sodium adduct ions of methionine enkephalin or protonated bradykinin ions. No protonated methionine enkephalin molecular ions are seen in the spectrum, but sodium and potassium adduct ions of methionine enkephalin are observed. For confirmation, pure methionine enkephalin was analyzed by SALDI. The mass spectrum is shown in Figure 44. Again, no protonated molecular ions are observed. Thus, interference by the other peptides was ruled out as a cause for the observation of the sodium adduct ions only, and not of the protonated molecule. Two factors may explain this observation. First, methionine enkephalin may have a higher affinity then bradykinin and angiotensin II for sodium ions. Because sodium ions are present on the surface of the SALDI solid, sodiated peptide ions are easily formed. Second, the proton affinity of methionine enkephalin may be lower than that of glycerol. The peptide can then not compete with the glycerol for protons. For a comparison, Figure 45 shows a MALDI mass spectrum of the same mixture. Both cationized and protonated methionine enkephalin are seen in the mass spectrum. Again, the three peptides ion peaks had different intensity even though equal concentrations of peptides were present. The sodiated methionine enkephalin ion shows the most intense peak among the three peptides. Different sensitivities are not surprising because the intensities of analytes in MALDI spectra depend on the

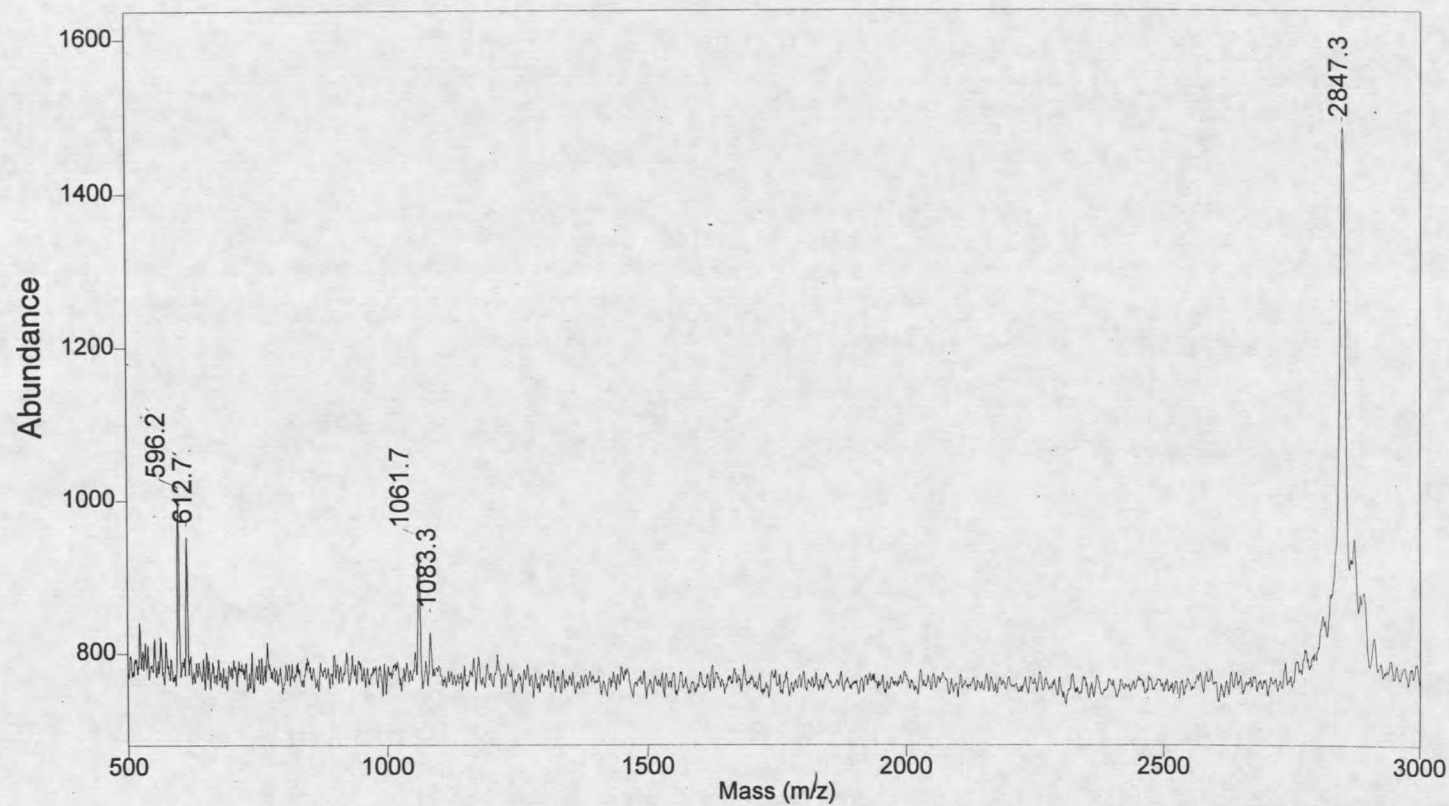


Figure 43. Graphite/SALDI mass spectrum of peptide mixture. The mixture includes methionine enkephalin (mw= 573.7, 0.1 nmole), bradykinin (mw= 1,060.5, 0.1 nmole), and mellitin (mw= 2,846.5, 0.1 nmole). LP= 386. SA= 24.

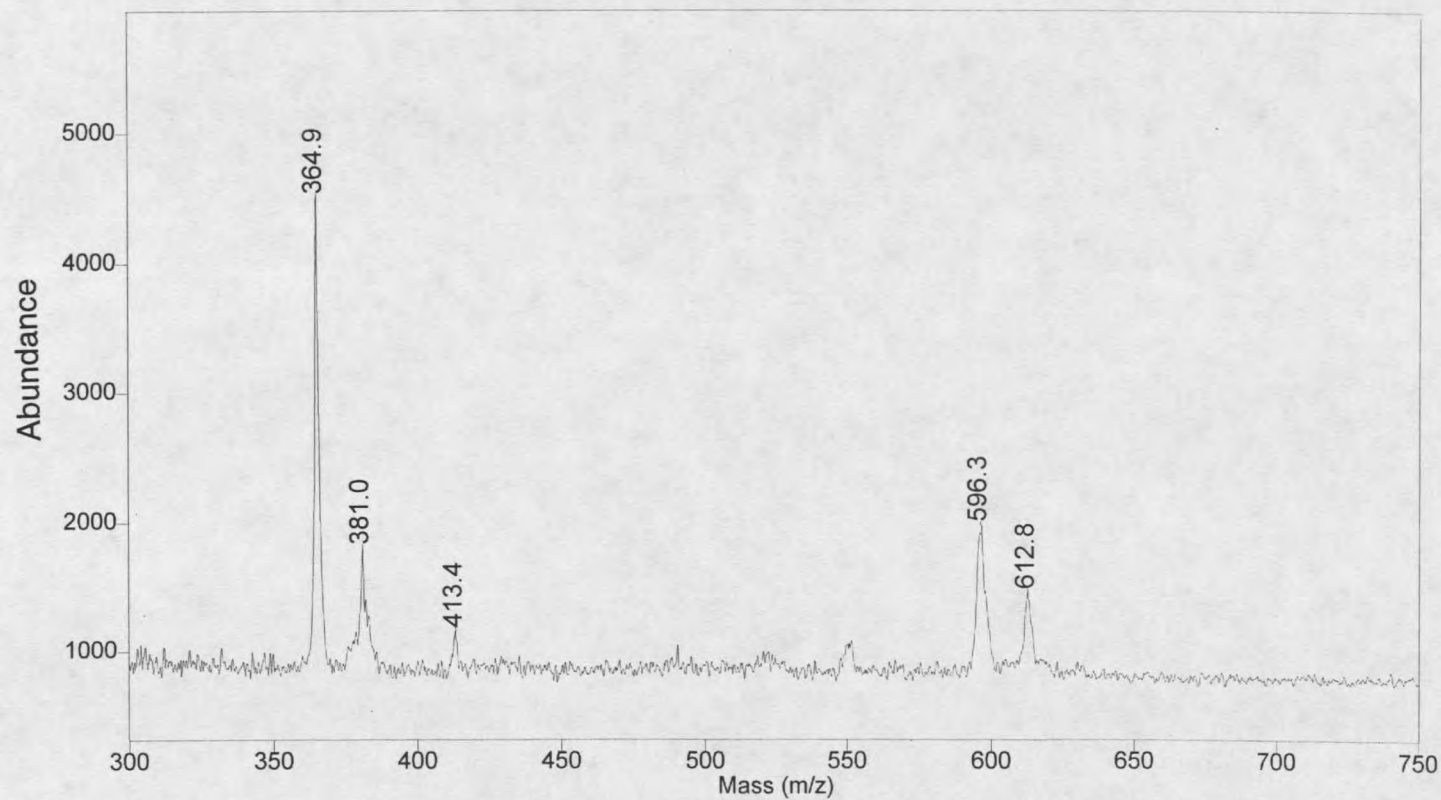


Figure 44. Graphite/ SALDI mass spectrum of methionine enkephalin (mw= 573.7, 0.1 nmole). LP= 344. SA= 85.

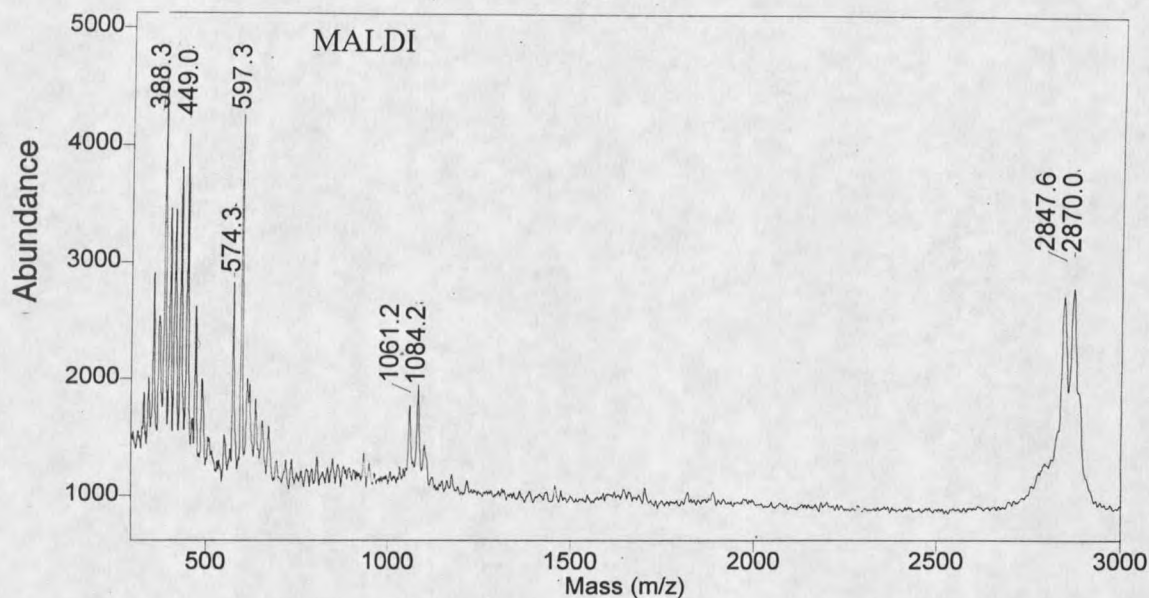


Figure 45. MALDI mass spectrum of a peptide mixture. The mixture includes methionine enkephalin (mw= 573.7, 0.1 nmole), bradykinin (mw= 1,060.5, 0.1 nmole), and mellitin (mw= 2,846.5, 0.1 nmole). LP= 292. SA= 45.

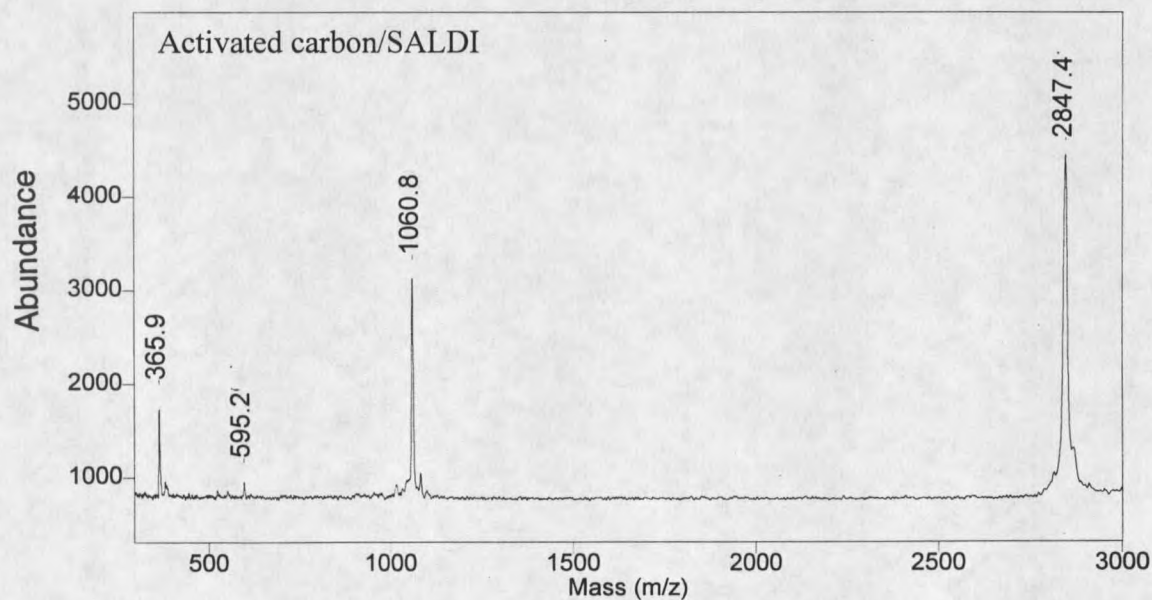


Figure 46. Activated carbon/SALDI mass spectrum of a peptide mixture. The mixture includes methionine enkephalin (mw= 574, 0.1 nmole), bradykinin (mw= 1,060.5, 0.1 nmole), and mellitin (mw= 2,846.5, 0.1 nmole). LP= 344. SA= 79.

on the crystallization process. The co-crystals may not have contained the same amounts of the three peptides. Thus, an inhomogeneous composition of the crystals may have resulted in the unequal intensities. Figure 46 shows the SALDI mass spectrum of the same sample used in Figure 43, but with activated carbon as the SALDI solid. The intensity of the methionine enkephalin peaks was very weak. A comparison of this spectrum with the graphite/SALDI spectrum (Figure 43) shows that the signal of sodiated methionine enkephalin was greatly suppressed in activated carbon SALDI. The reason is unknown. One may suspect that the porous surface composition of activated carbon was a factor, however.

Figure 47 shows a graphite SALDI mass spectrum of a tryptic digest of cytochrome C. Trypsin specifically cleaves peptide bonds at the C-terminal end of lysine and arginine. Sodiated peptides dominated the mass spectrum due to the addition of a sodium salt (0.010% SDS). The 0.010% SDS was added into the SALDI matrix to increase the intensity of sodium adduct ions. SDS is a better additive than other sodium salts such as sodium chloride. This has been discussed in the Chapter 4, "Salt Effects in SALDI". Of 16 tryptic peptides with two or more amino acids (Table 5), 14 were easily identified in this spectrum. Only the amino acid sequence #14-22 peptide was missing in this spectrum. Because of the low ion matrix background in SALDI, identifying digest products was comparatively easy.

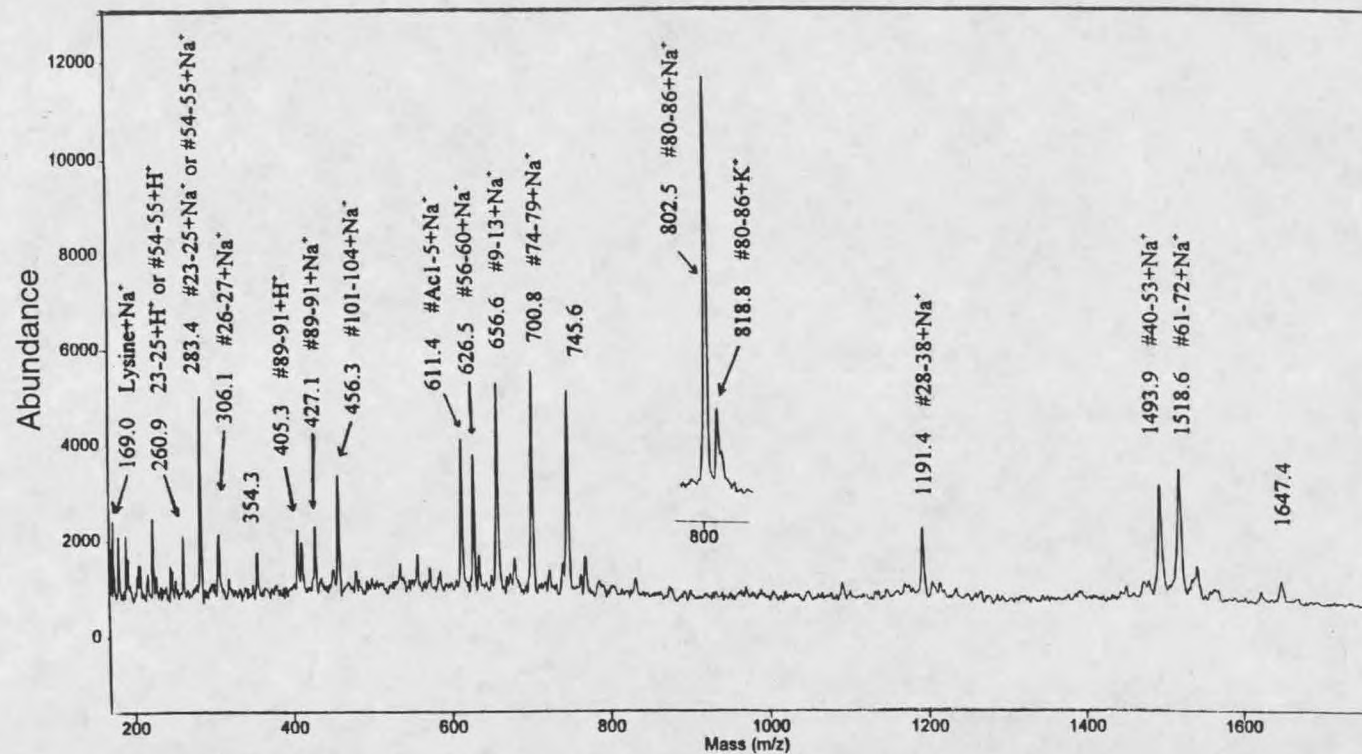


Figure 47. Graphite/SALDI mass spectrum of tryptic digest of cytochrome C. 0.01% (w/w) SDS/H₂O was added to the SALDI suspension. LP= 352. SA= 51.

Table 5. Tryptic peptides of cytochrome C.

From-	To	Mass	Sequence
8-	8	146.19	K
39-	39	146.19	K
73-	73	146.19	K
87-	87	146.19	K
88-	88	146.19	K
100-	100	146.19	K
6-	7	203.24	GK
23-	25	260.29	GGK
54-	55	260.29	NK
26-	27	283.33	HK
89-	91	404.42	TER
101-	104	433.42	ATNE
1-	5	546.58	GDVEK
56-	60	603.72	GITWK
9-	13	634.77	IFVEK
74-	79	677.80	YIPGTK
80-	86	779.01	MIFAGIK
92-	99	677.80	EDLIAYLK
14-	22	1016.15	CAQCHTVEK
28-	38	1168.32	TGPNLHGLFGR
40-	53	1470.55	TGQAPGFTYTDANK
61-	72	1495.66	EETLMEYLENPK

Carboxypeptidase Y (CPY) hydrolyzes the peptide bond adjacent to the free carboxyl terminal group in a protein or peptide. Figure 48 shows the CPY digestion reaction. Figure 49 shows the SALDI mass spectrum of bradykinin digested by CPY. The reaction was stopped after 10 minutes. The ion M_1^+ ($m/z=904$) is the peptide residue obtained by losing the C-terminal arginine. The cleavage of the first amino acid, arginine, is seen to be nearly complete. In the low mass range, the ion at $m/z=175$ is due to free arginine. Also, the second amino acid, phenylalanine, is seen to be removed from the peptide by the appearance

of the M_2^+ ion at $m/z = 757$. For the CPY digestion, bradykinin

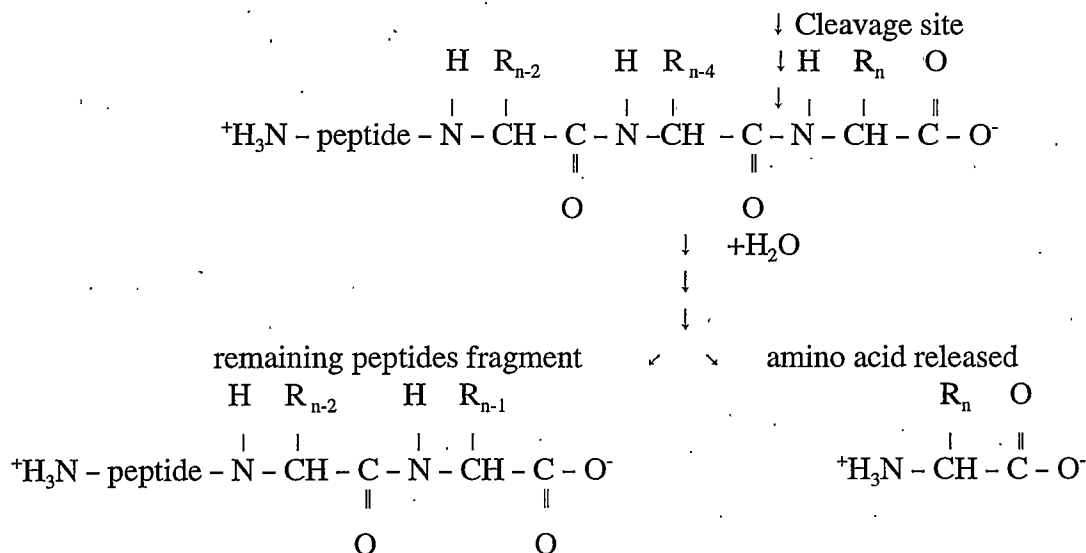
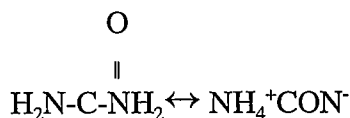


Figure 48. Peptide bond hydrolysis by carboxypeptidase.

was dissolved in a highly concentrated aqueous urea solution (6.0 M) to increase the solubility. In the low mass range in Figure 49, strong protonated urea ($m/z = 61$) and sodiated urea ions ($m/z = 83$) peaks are seen. The peak at $m/z = 948$ is due to M_1^+ plus a cyanate ion adduct. Presumably, carbamylation occurred and the cyanate ion attached to the amino group in the reaction.



Cyanate ion adducts were observed for almost every bradykinin fragment formed.

CPY digest products were collected every 10 minutes from time = 0 to time = 110 minutes. In each fraction, the reaction was stopped by adding 0.01% TFA. The twelve fractions were then mixed together. Figure 50 shows the SALDI mass spectrum of this

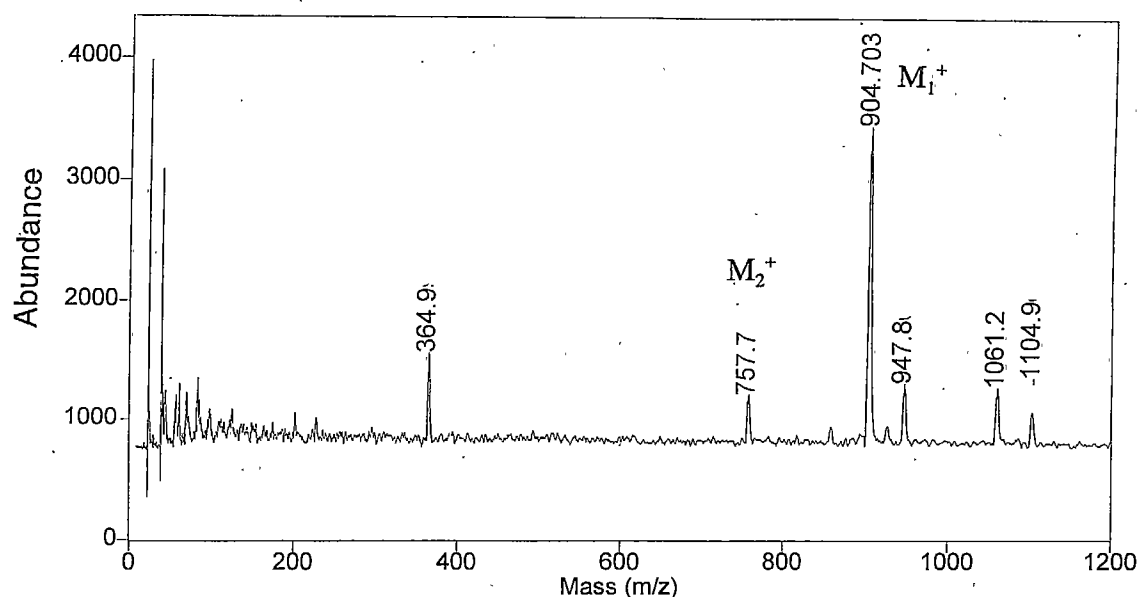


Figure 49. Activated carbon/SALDI mass spectrum of carboxypeptidase Y digest of bradykinin. The digest reacted for 10 minutes. The sequence of bradykinin is Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. LP= 402. SA= 40.

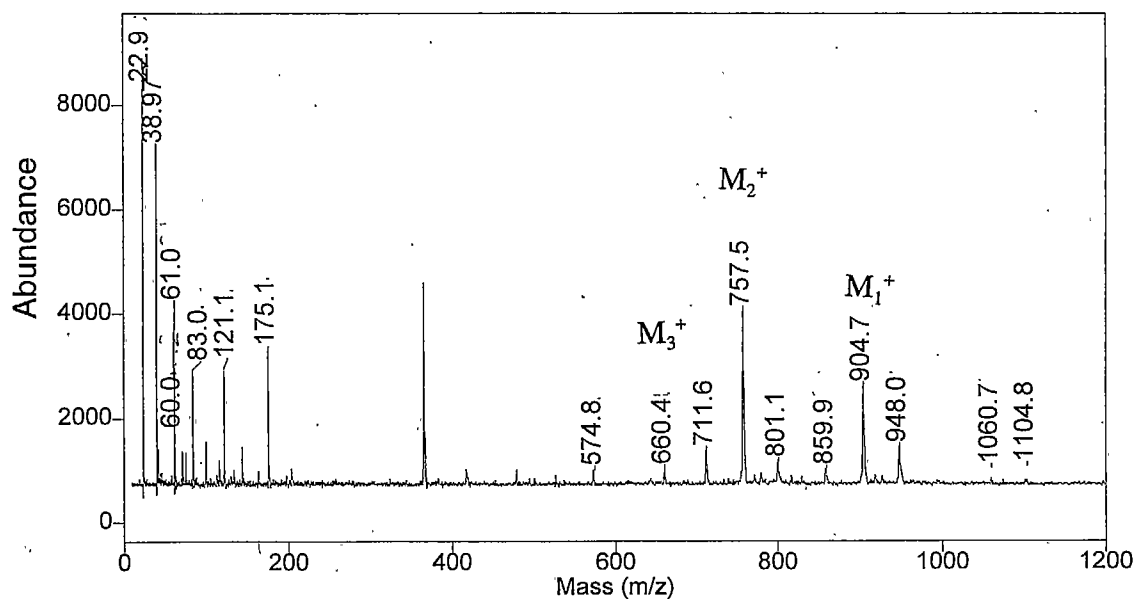


Figure 50. Activated carbon/SALDI mass spectrum of carboxypeptidase Y digest bradykinin. The digest reacted for 110 minutes. Fractions were collected every 10 minutes. The spectrum was obtained from the combination of the 12 product fractions. LP= 372. SA= 65.

peptide mixture. In this digestion, four amino acids were removed, arginine, phenylalanine, proline, and serine as seen by the sequence of ions at $m/z=904$ (M_1^+), $m/z=757$ (M_2^+), $m/z=660$ (M_3^+) and $m/z=573$ (M_4^+). The ions at $m/z=947$, $m/z=800$, and $m/z=711$ are the urea adduct ions of M_1^+ , M_2^+ , and M_3^+ (with a water molecule loss), respectively.

Figure 51 shows another example of peptides mixture analysis by SALDI. This mass spectrum is of polyalanine with a molecular weight distribution between 1000 and 5000 Da. The mass separation between each successive peak is 71 Da.

The successful application of SALDI to peptide analysis has been demonstrated. In particular, SALDI is a good analysis method for peptide mixtures such as obtained in enzymatic digest due to low matrix background effects. Finally, polyamino acids like polyalanine can be analyzed by SALDI.

Analysis of Polymers and Porphyrins

In addition to biochemical analysis, SALDI can be applied to the analysis of many types of compounds. This will be exemplified here with SALDI mass spectra of polymers first presented. Figure 52 shows SALDI mass spectra of polyethylene glycol (PEG) 200, 400, 1500, 4600, and poly(propylene glycol) (PPG) 3000. Characteristically, only sodium and potassium adduct ions are observed in these mass spectra. This is due to the low proton affinity of these polymers. One of spectra in Figure 52 is the SALDI mass spectrum of PEG 200. The spectrum consists of two series of peaks. In each series of PEGs, the mass differences between consecutive peaks is 44 Da. The two series are separated by 16 Da. The 16 mass unit difference is due to the difference between the sodiated PEG and potassiated

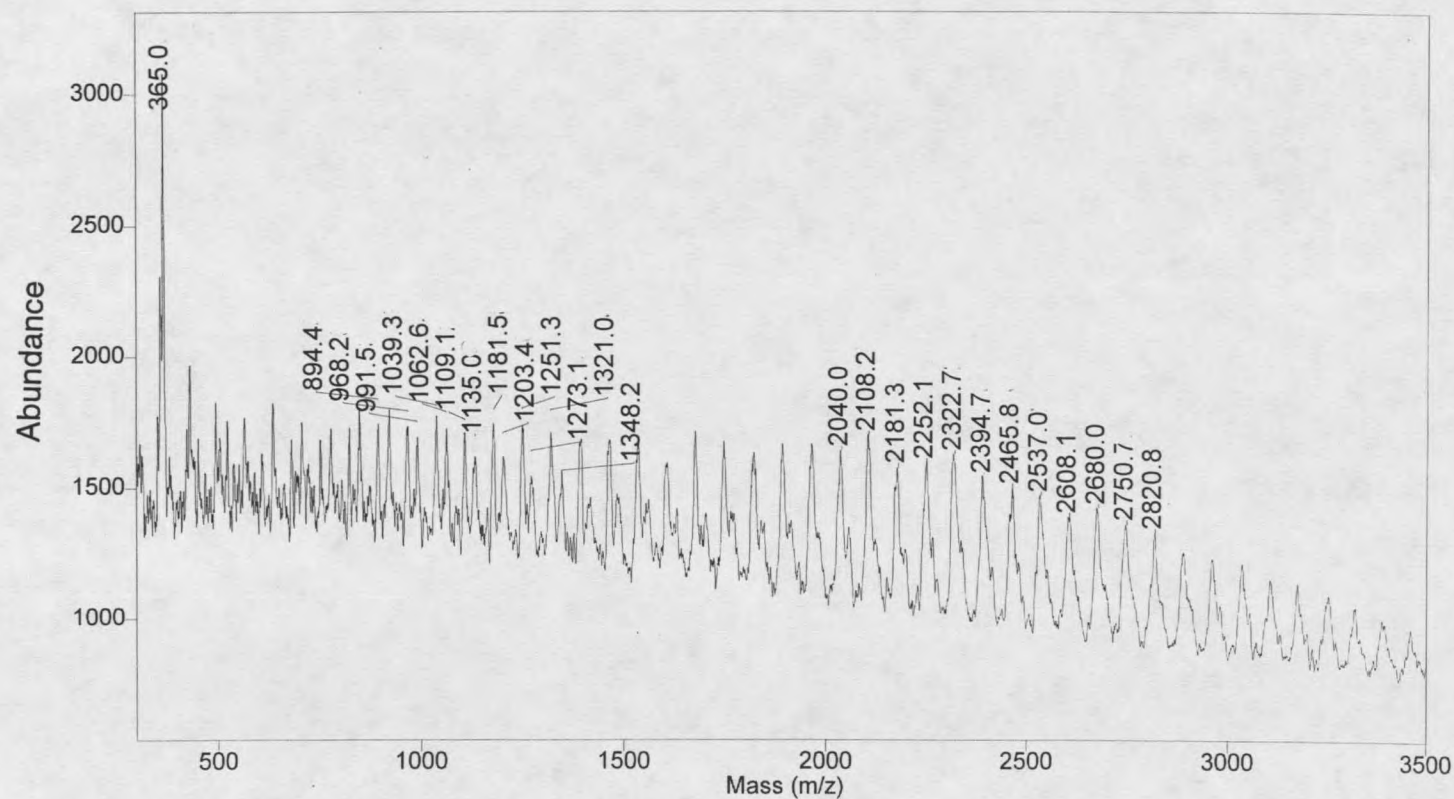


Figure 51. Activated carbon/SALDI mass spectrum of polyalanine (mw= 1,000-5,000, 0.01 mg). LP= 428. SA= 66.

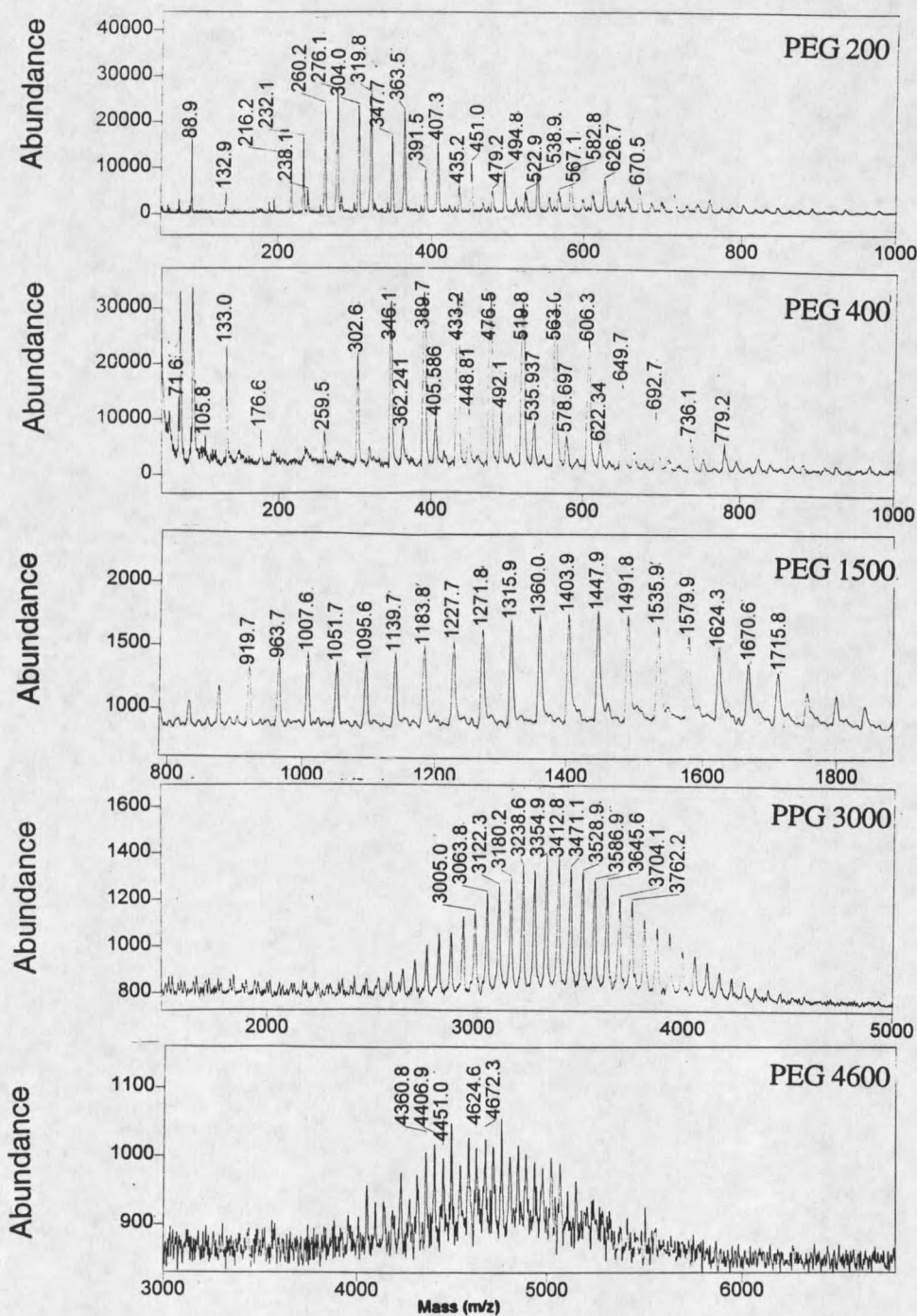


Figure 52. Activated carbon/SALDI mass spectra of PEGs: PEG 200, PEG 400, PEG 1500, PPG 3000, PEG 4600. LP= 390 and SA= 70 in PEG 200, LP= 386 and SA= 15 in PEG 400, LP= 598 and SA= 198 in PEG 1500, LP= 364 and SA= 141 in PPG 3000, and LP= 398 and SA= 31 in PEG 4600.

PEG of the same oligomer. In PPG 3000, the mass differences between consecutive peaks is 58 Da. However, this peak pattern was only found for PEG 200 and PEG 400. In the spectra of PEG 1500, PPG 3500, and PEG 4600, all major ions were due to sodiated polymers, and the mass difference between successive peak was 44 Da in PEGs. The potassiated PEG and PPG peaks were very weak or absent. This might be due to the higher sodium affinity of PEG 1500, 4600, and PPG 3500.

The non-polar polymer, polystyrene (PS), was also analyzed by SALDI. The PS was first dissolved in pure toluene. The SALDI solvent was composed of sucrose/glycerol/MeOH (5/28/67). This solution (0.10 mL) was mixed with an equal volume of dry activated carbon powder. One microliter of the suspension was applied to a sample well. Then, 1.0 μL of PS in toluene solution was added. Although the polarities of toluene and glycerol are very different, this did not seem to cause any problem in the analysis of PS. At first, no polystyrene ions were observed. However, ions were obtained by adding metal ions like Ag^+ and Cu^{2+} to the SALDI sample. The top spectrum in Figure 53 shows the SALDI mass spectrum of PS ($M_{\text{ave}}=2,727$) obtained when 0.50 M AgNO_3 had been added to the SALDI suspension. Each peak observed was a silver adduct ion of a PS oligomer. The middle spectrum in Figure 53 is the SALDI mass spectrum of PS obtained ($M_{\text{ave}} = 2,727$) by adding CuSO_4 (0.50 M) to the SALDI suspension. All the ions observed are copper adduct ions of PS oligomers. The bottom spectrum in Figure 53 shows the SALDI mass spectrum of PS ($M_{\text{ave}}=2,727$) obtained by adding a mixture of Ag^+ and Cu^{2+} to the SALDI matrix. Both silver and copper adduct ions of PS are seen. Copper adduct ions ($M_n+63.5$) are next to the corresponding silver adduct ions ($M_n+107.87$) separated by 44 Da. These two series metal

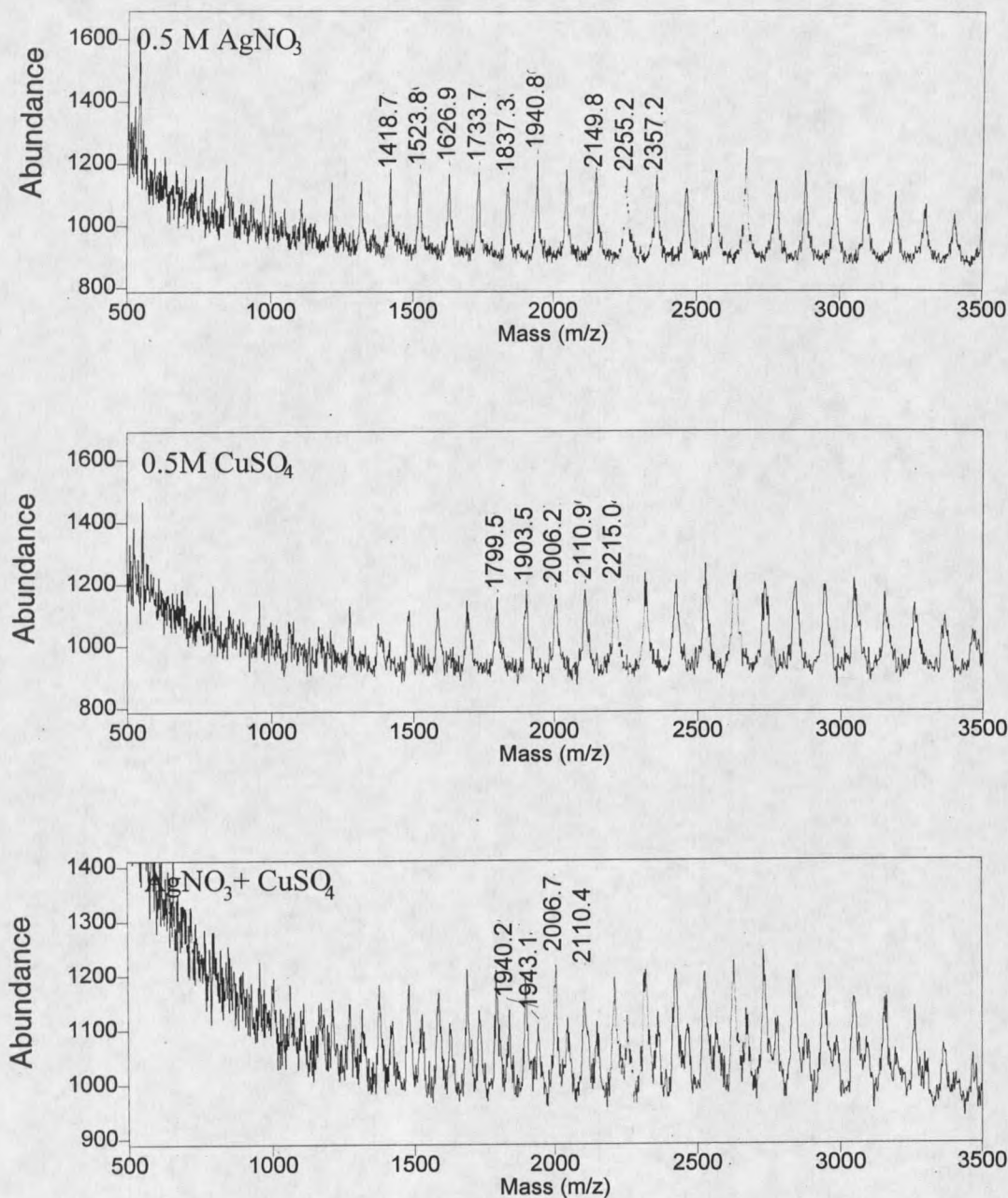


Figure 53. Activated carbon/SALDI mass spectra of polystyrene ($\text{mw}_{\text{ave}} = 2,727$). 0.5 M silver nitrate in the SALDI suspension (top). SA= 81. Activated carbon/SALDI mass spectrum of polystyrene ($\text{mw}_{\text{ave}} = 2,727$). 0.5 M copper sulfate in the SALDI suspension (middle). Activated carbon/SALDI mass spectrum of polystyrene ($\text{mw} = 2,727$). SA= 55. 0.5 M silver nitrate and copper sulfate in the SALDI suspension (bottom). SA= 96. LP setting was 384 for all spectra.

adduct ions in the mass spectrum can be identified by comparing with the top (with silver ion only) and middle spectra (with copper ion only) in Figure 53. In general, adding transitional metal ions to the SALDI suspension makes possible the analysis of polymers that will not give protonated ions.

SALDI was used also for the analysis of porphyrins. Figure 54 and Figure 55 show the SALDI mass spectra of 5, 10, 15, 15, 20-tetrakis (pentafluorophenyl)-21H, 23H-porphine iron (III) chloride (mw= 1063.85) and 5, 10, 15, 20-tetraphenyl-21H, 23H-porphine cobalt (II) (mw= 671.67), respectively. Only sodiated porphyrin ions are observed, but no protonated porphyrins. In addition, the intensity of the sodium adduct ions of iron-deficient porphyrin (m/z=1031) is quite high. However, in Figure 54, no sodiated porphyrin ions are observed. It is demonstrated that porphyrin can easily be analyzed by SALDI. Whether protonated or cationized molecular ions are observed depends on the affinities of protons and sodium ion of the respective porphyrins.

Analysis of Volatile Organic Compounds

A unique characteristic of activated carbon is its high absorption capacity due to the porosity of its surface. Based on this absorption capacity, activated carbon can be used as the adsorbent to trap volatile compounds. The potential to trap volatile organics on the porous surface of activated carbon and then analyze the organic compounds directly by SALDI was investigated.

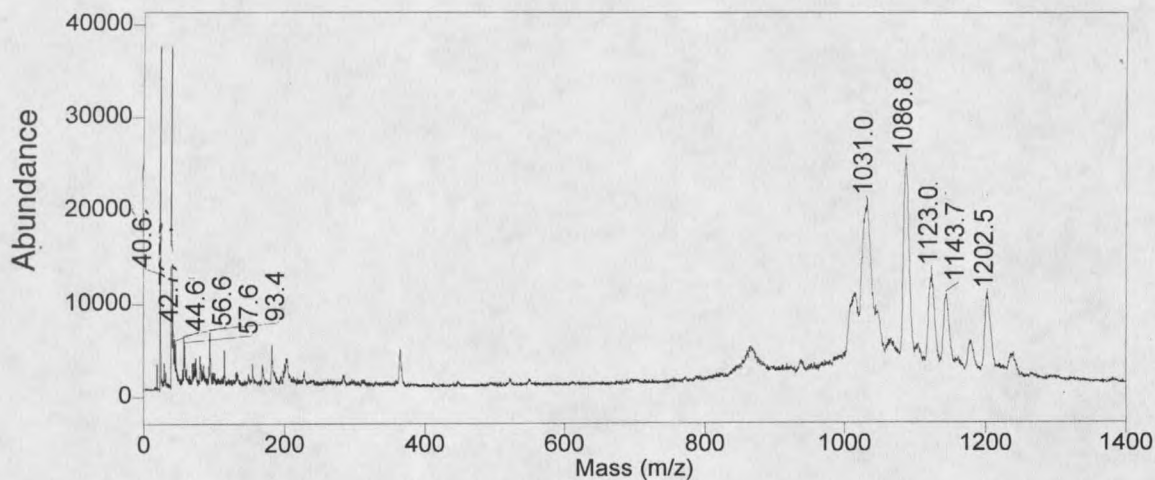


Figure 54. Activated carbon/SALDI mass spectrum of 5, 10, 15, 20-tetrakis(pentafluorophenyl)-23H-porphine iron (III) chloride (mw= 1,063.85). LP= 378. SA= 59.

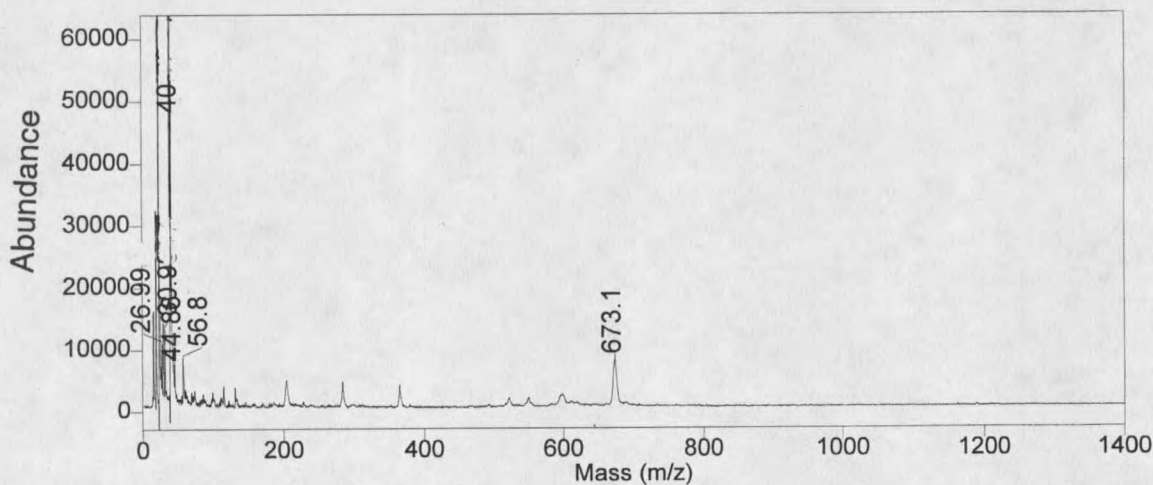


Figure 55. Activated carbon/SALDI mass spectrum of 5, 10, 15, 20-tetraphenyl-23H-porphine cobalt (II) (mw= 671.67). LP= 440. SA= 26.

The utilization of the absorption capacity of activated carbon for direct SALDI analysis was first demonstrated using azulene as the analyte. Figure 56 shows a SALDI spectrum of azulene (mw= 128). After the SALDI suspension was applied to the sample well, 1.0 μ L of a 0.10 M azulene in chloroform solution was added. A very strong azulene molecular ion signal was observed in the mass spectrum. Azulene dimers are also seen. Only two other peaks, sodium and potassium ions show up in the low mass range. Due to the stability of azulene molecular ions, these are more abundant than protonated azulene by a factor 5. Figure 57 shows the MALDI spectrum of azulene. In this spectrum, azulene molecular ions are again observed; however, the intensity is lower than in the SALDI spectrum. Some of the azulene molecules were likely trapped in the matrix crystals. However, in efforts to analyze azulene by direct laser desorption, i.e. without adding any matrix, no azulene signals were obtained (Figure 58). It was observed that the blue azulene crystals had completely disappeared when the sample plate was ejected from the ion source chamber after the analysis. Azulene would seem to have totally evaporated in the vacuum when no MALDI matrix or activated carbon was present to hold the molecules in the sample. Figure 59 shows the SALDI mass spectrum of naphthalene (mw= 128). A intense analyte signal is observed, and the naphthalene dimer ion peak is also seen. Sodium and potassium ions dominated the mass spectrum in the low mass range. Figure 60 shows the LD mass spectrum of naphthalene. Again, if no SALDI or MALDI matrix was added to the sample, no analyte ions were observed in laser desorption analysis.

Pyridine is considerably more volatile than azulene or naphthalene due to its low molecular weight. To analyze pyridine by LD (at room temperature) would be nearly hopeless. Also,

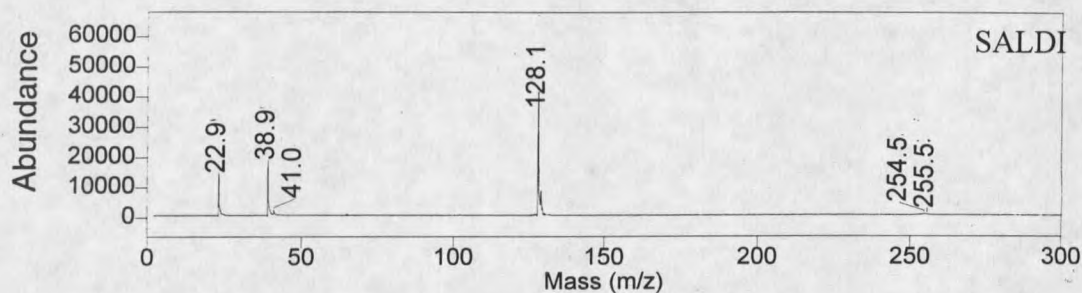


Figure 56. Activated carbon/SALDI mass spectrum of azulene (100 nmole, mw= 128.16). LP= 356. SA= 255.

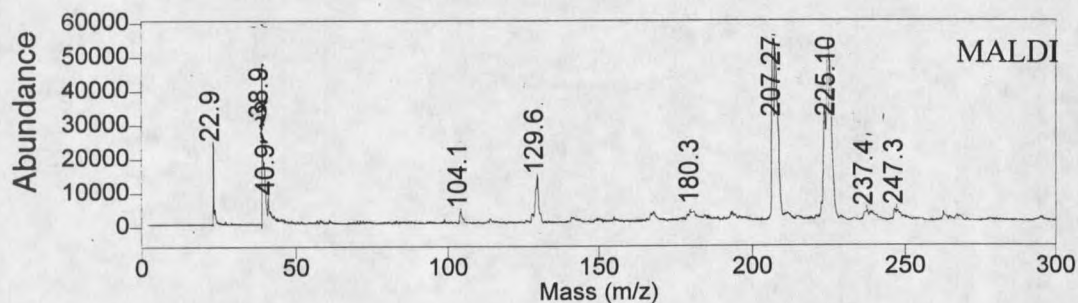


Figure 57. MALDI mass spectrum of azulene (100 nmole, mw= 128.16). LP= 262. SA= 33.

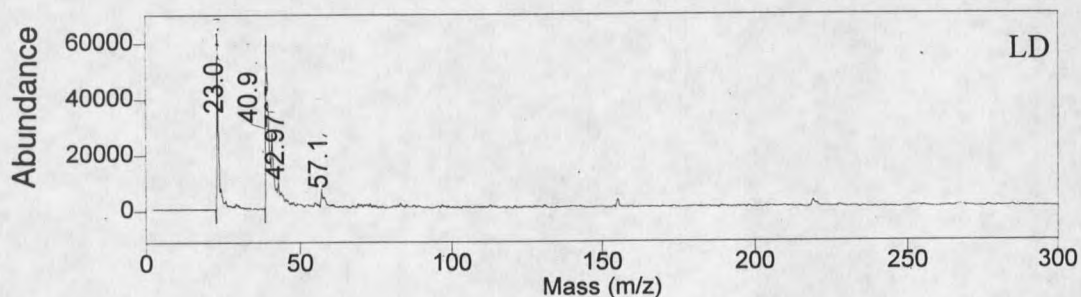


Figure 58. LD mass spectrum of azulene (100 nmole, mw= 128.16). LP= 348. SA= 7.

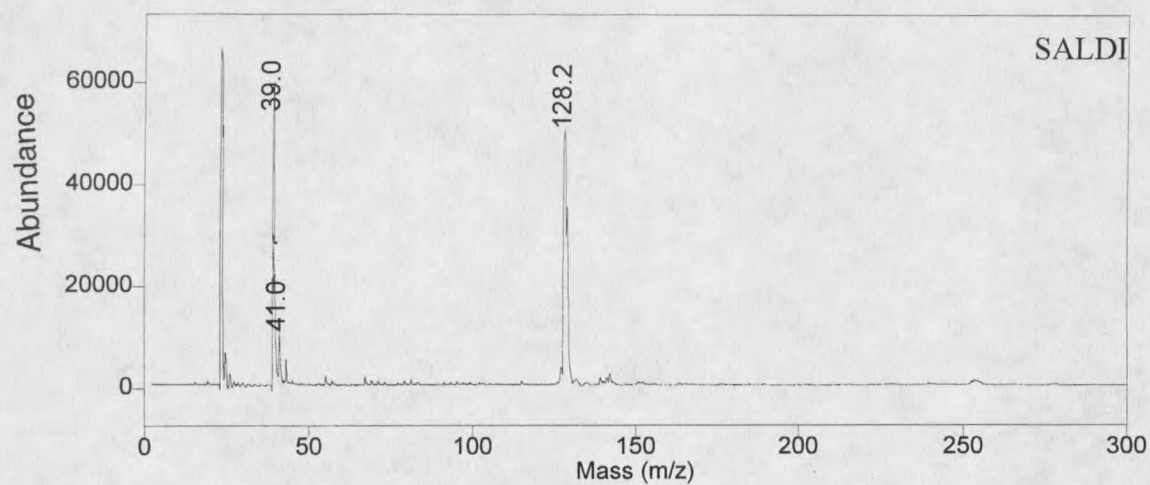


Figure 59. Activated carbon/SALDI mass spectrum of naphthalene/chloroform (mw= 128.16, 1000 nmole). LP= 354. SA= 113.

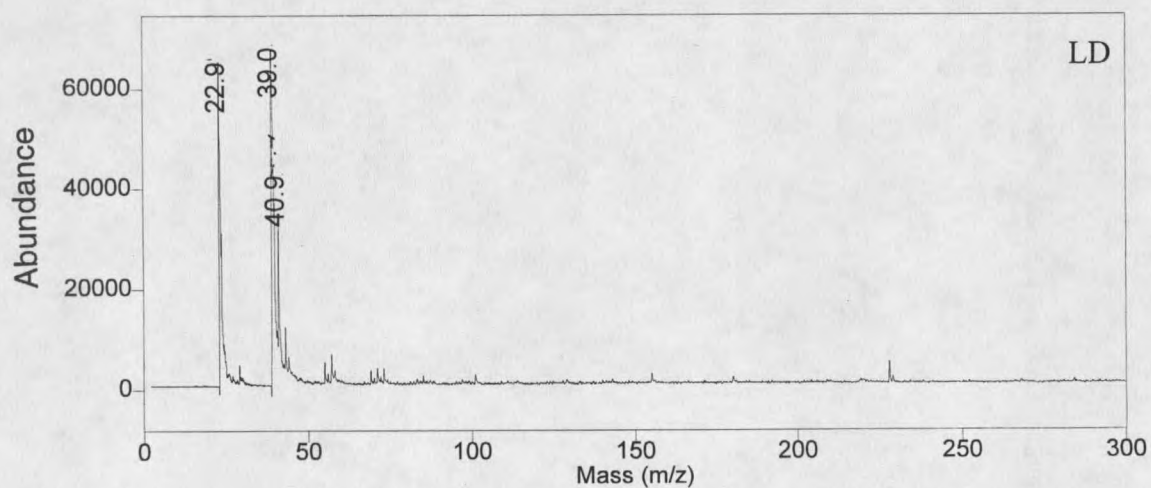


Figure 60. LD mass spectrum of naphthalene/chloroform (mw= 128.16, 1000 nmole). LP= 338. SA= 53.

in MALDI, no pyridine signal can be seen. However, pyridine can be adsorbed on the surface of activated carbon. Figure 61 shows the SALDI mass spectrum of pyridine ($m_w = 79$, m/z (MH^+) = 80). Pyridine is the smallest volatile compound for which SALDI spectra have been obtained. Efforts were made to analyze also ammonium hydroxide, acetonitrile, chloroform, and toluene by SALDI, but no analyte signals were observed in those SALDI mass spectra. There are at least two possible explanations for this: (1) high volatility (2) low gas phase basicity. Ion-molecule reaction occur during the SALDI process. If analyte molecules do not have a sufficiently high gas phase basicity, no protonated molecules will be observed in the SALDI mass spectra even though the analytes might have been adsorbed on the surface of the activated carbon. Similarly, compounds that have a high ionization energy will not be observed as M^+ ions in the SALDI mass spectra.

In order to further investigate the role of the absorption ability of activated carbon for the SALDI spectra, the intensity of the azulene mass peak was studied as a function of the concentration of azulene. The result is shown in Figure 62. The intensity of the analyte signal increased with the azulene concentration up to about 0.10 M. The intensity then stabilized above 0.10 M. A reasonable explanation is that once the surface area of activated carbon was saturated by azulene, the intensity of the azulene ion could not be further increased even though a more concentrated azulene solution was used. The surface area was not large enough to trap all the analyte molecules. An alternative explanation is that ion detector was saturated. Thus, the actual ion counts might have been higher than observed.

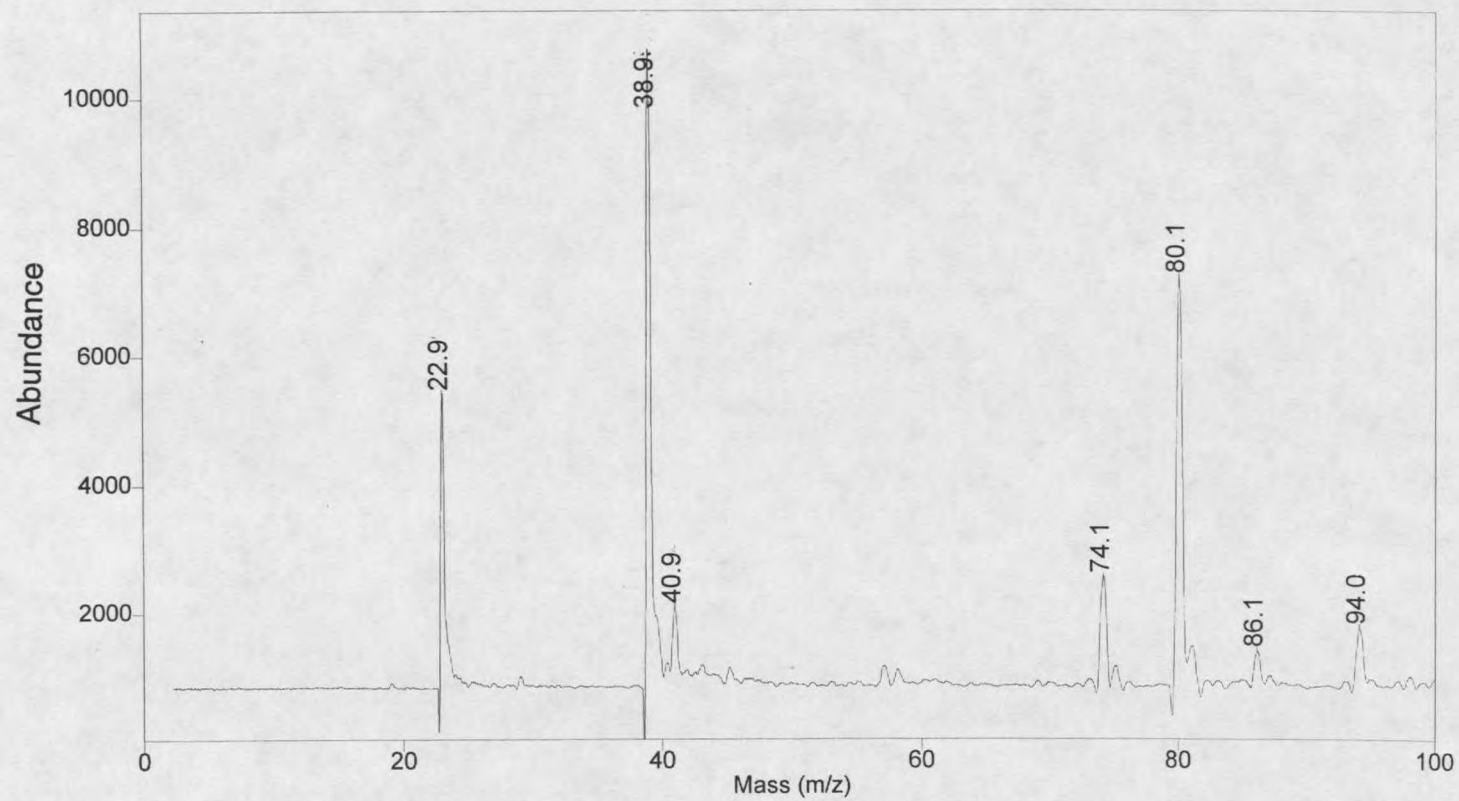


Figure 61. Activated carbon/SALDI mass spectrum of pyridine (mw= 79.09). LP= 468. SA= 53.

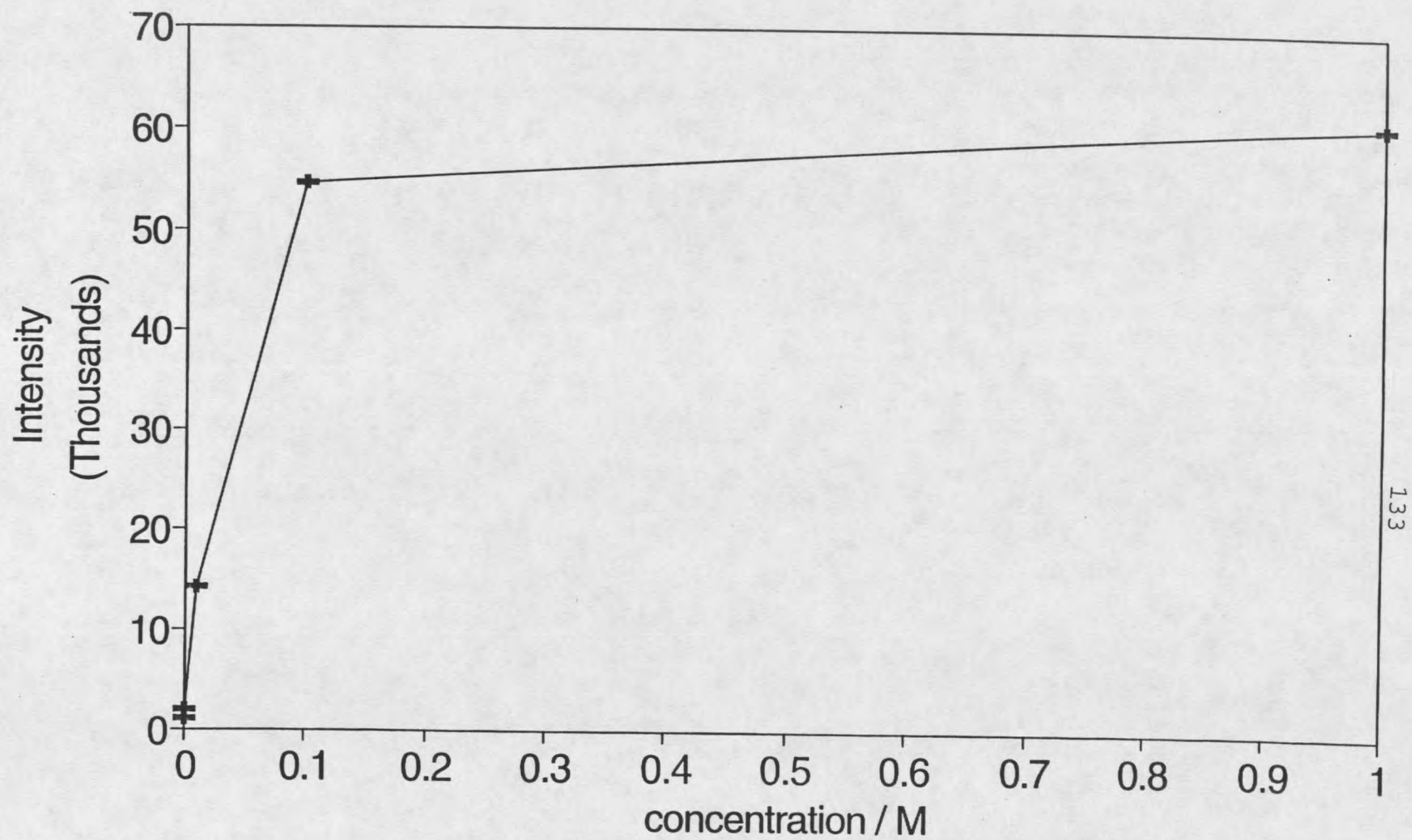


Figure 62. The intensity of azulene molecular ions versus the concentration of azulene based on the SALDI mass spectra.

CHAPTER 6

SPE (SOLID PHASE EXTRACTION)/SALDI

In addition to volatile organics, activated carbon also can be used for adsorbing non-volatile organics. In fact, activated carbon is one of the adsorbents commonly used in solid phase extraction (SPE). The combination of SPE with SALDI has been successfully developed, and will be presented in the following section.

General

The solid phase extraction (SPE) method is used to concentrate trace amounts of organics from dilute solutions. The analytes are extracted by passing the dilute solution through a filtration tube that contains the adsorbent material. After the extraction step, the adsorbed organics are typically eluted by using a solvent with a proper polarity. The eluted solution is collected and can be further concentrated by repeating the procedure. The concentrated solution can be analyzed by gas chromatography (GC) or high performance liquid chromatography (HPLC) for qualitative or quantitative analysis. However, analytes with high polarity and low volatility need to be derivatized before analyzed by GC. This procedure of elution followed by derivatization and chromatography analysis is quite time-consuming (Cai et al., 1993; Bauw et al., 1991; Battista et al., 1989; Corcia et al.,

1989). In addition, much care has to be taken in order to avoid excessive sample loss. For qualitative analysis, the procedure seems unnecessarily complicated.

SPE/SALDI

Mass spectrometry can provide confident qualitative information on the molecular weights of analytes. Furthermore, analytes do not have to be volatile for ionization methods such as SALDI. Without requiring elution after concentrating trace organics on the adsorbents or derivatization reactions for increasing the volatility of analytes, SALDI would seem to have promise to directly detect trace organics from the adsorbents. Here, a fast screening technique developed by combining SALDI with SPE is presented.

The basic procedures used to combine SPE with SALDI is as follows. Activated carbon or carbopack was used both as the adsorbent material in SPE and as the SALDI solid. Thus, once trace organics were adsorbed on the activated carbon in SPE, the activated carbon were directly mixed with the SALDI solvent for mass spectrometry analysis. In this combination of SALDI with SPE, the elution procedure is not necessary. Qualitative information such as analyte molecular weight information can be provided in a shorter and faster procedures than in the traditional SPE method. Moreover, SPE/SALDI will be shown to be sensitive. Finally, it will demonstrated that SPE/SALDI also can be used for quantitative analysis.

Sample Preparation of SPE/SALDI

Method

Figure 63 shows the SPE extractor design for small volume extraction. The extractor is composed of three parts, a vis-1 single SPE tube processor, a filtration tube, and a tip. The vis-1 single SPE tube processor has a plunger that is mounted on a screw. This allows for fine control of the extraction flow rate by rotating the screw. The diluted solution was pulled into the filtration tube. Two sizes of filtration tubes were available: 3 mL and 6 mL. A piece of polyethylene frit was packed in the very front of the tip to prevent adsorbent material from leaking out of the tip. Approximately 2 mg of adsorbent was packed in the very front of a tip. Two milligrams of adsorbent was ideal for a volume of dilute solution between 3 mL and 30 mL. If more than 30 mL of dilute solution was analyzed in SPE, more than 2 mg of adsorbent was packed. To tightly connect the tip to the filtration tube, roughly 0.5 cm of the tip was cut out from the bottom big opening of the tip. The tip could then be tightly connected to the small opening of the filtration tubes. The dilute solution was added into the filtration tube. Next, the vis-1 single SPE tube processor was connected to the tube. The solid phase extraction proceeded by slowly rotating the knurled knob on the single tube processor for flow rate control. The flow rate was kept at about 0.2 mL per minute (for a 3 mL to 30 mL solution volume). Higher flow rates were used if the solution volume was larger than 30 mL. For example, when 300 mL of extraction solution was used, the flow rate was adjusted to about 2 mL/min. For large extraction volumes, instead of the single tube processor, a syringe pump was used to control the flow rate. Various sizes of plastic syringes

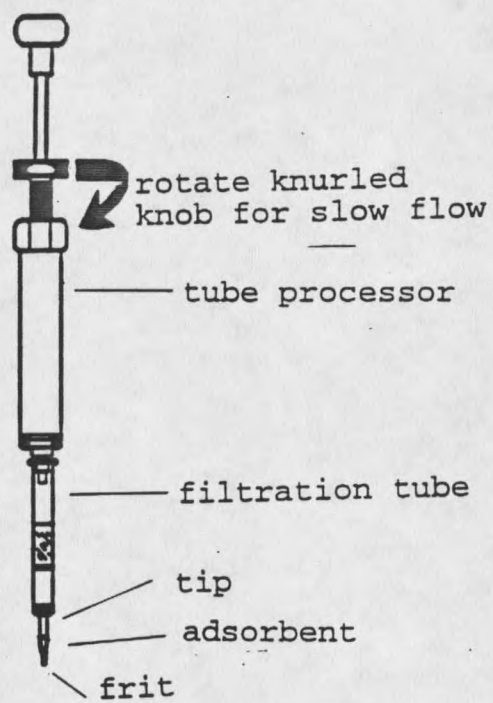


Figure 63. SPE extractor for low volume extraction solution.

were used to accommodate large volumes of solution. A tip with packed adsorbent material was connected to the syringe. The flow rate was increased to more than 2 mL/min if the sample volume was 300 mL or more. After the solid phase extraction was completed, the single tube processor, the filtration tube, and the tip were disconnected. The adsorbent material was dried for 10 minutes. After drying, the adsorbent material was easily removed. It was then mixed with roughly 8 μ L of SALDI solvent. The SALDI solvent was 15% sucrose/glycerol (w/w). The resulting solution was diluted by a factor of three using methanol. Finally, 1 μ L of the resulting mixture was pipetted onto one of wells on the sample plate for SALDI/MS analysis.

Summary of SPE/SALDI Procedures

The procedures for SPE/SALDI can be summarized as follows.

1. Pull sample solution into a filtration tube which is connected to a tip containing adsorbent material.
2. Connect the tube to a processor or a syringe pump for controlling the flow rate. Then, run the extraction experiment.
3. Disconnect the tip from the filtration tube, let the adsorbent dry for 10 minutes, and remove the adsorbent from the tip.
4. Mix the adsorbent with SALDI solvent, and apply this mixture to a well on a sample plate and analyze by SALDI.

Results and Discussion

The adsorbent material in SPE and the SALDI solid in SALDI was activated carbon or carbopack. Carbopack was reported by Supelco to have a high adsorbent capacity toward nitrogen-containing compounds. However, nearly identical results were obtained with either activated carbon or carbopack. Since activated carbon was very inexpensive compared to carbopack, activated carbon was used in this study for most of experiments. In the following, the SPE/SALDI results from nitrogen-containing organics such as diuretics and herbicides and biochemicals such as amino acids and peptides will be shown. Most of the experiments were focused on qualitative analysis. However, quantitative results were also obtained by SPE/SALDI.

Qualitative Analysis

Analysis of Diuretics. Diuretics are drugs that are abused in sports that involve weight categories. They are used to deliberately reduce weight prior to a competition, or to dilute urine specimens (Herraez-Hernandez et al., 1992). If athletes take diuretics before the competitions, the drugs can be found in their urine samples. Only the original molecules of diuretics, but no metabolic products can be found in the urine. Most diuretics are polar and with negligible volatility. Therefore, they need to be derivatized before analysis by traditional methods such as GC or GC/MS. However, polar and low volatility compounds can be directly analyzed by SPE/SALDI without derivatization. Figure 64 shows the SPE/SALDI spectra of several different diuretics: hydroflumethazide (mw= 331.29), dichlorphenamide (mw= 305.16), furosemide (mw= 330.7), bumetanide (mw= 364.42), and

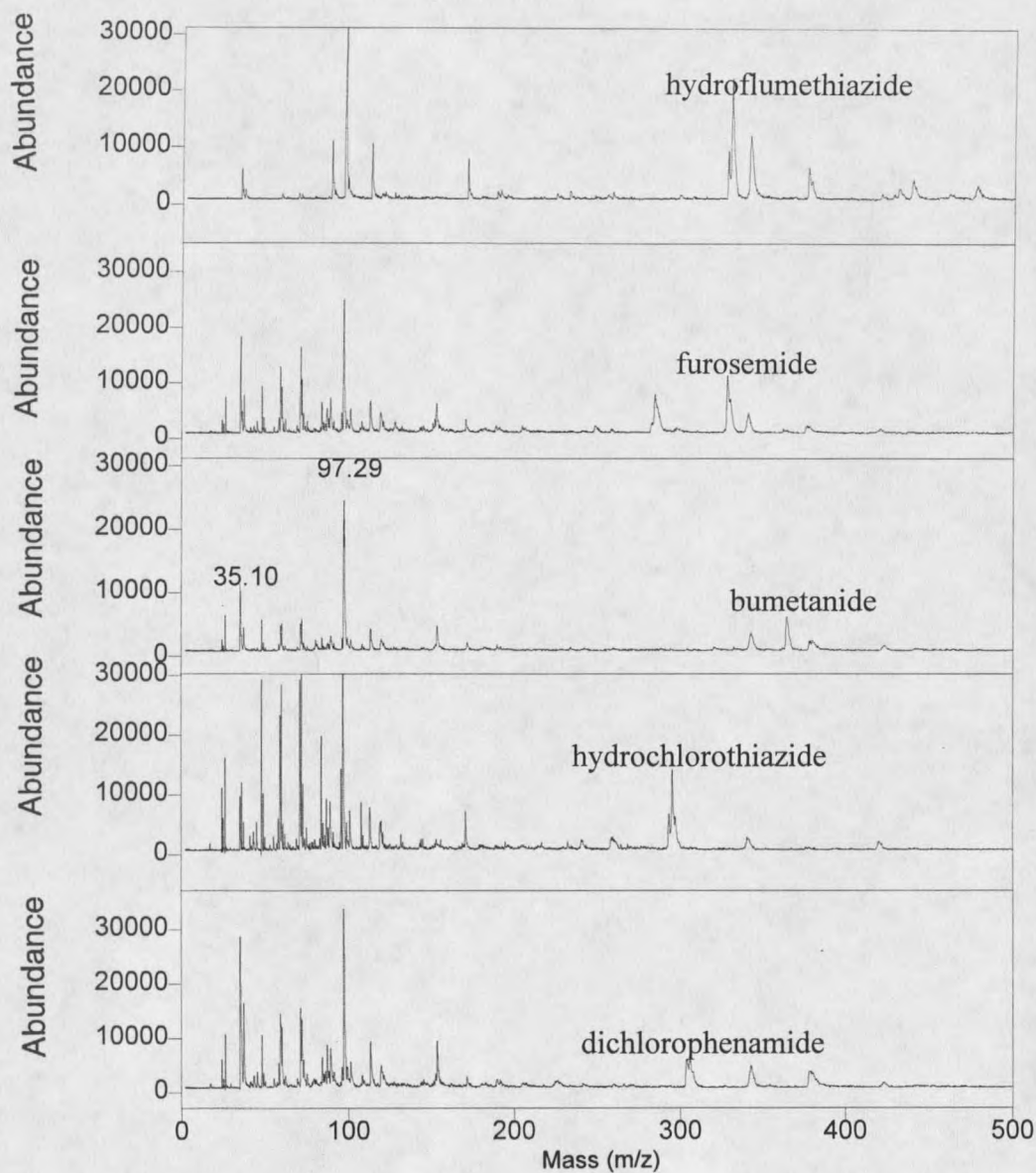


Figure 64. SPE/SALDI mass spectra (negative ion mode) of hydroflumethiazide (mw= 331.29, 3.0 μ g, LP= 432, SA= 56), dichlorophenamide (mw= 305.16, 30 μ g, LP= 366, SA= 39), furosemide (mw= 330.7, 30 μ g, LP= 424, SA= 51), bumetanide (mw= 364.42, 20 μ g, LP= 420, SA= 64), hydrochlorothiazide (mw= 297.72, 20 μ g, LP= 408, SA= 28). Analyte was added to 3.0 ml of pure water for each spectrum.

hydrochlorothiazide (mw= 297.72). These diuretics can easily lose a proton or capture an electron. Thus, they were observed in the negative ion mode. No diuretic molecular ions were observed in positive ion mode. In the low mass range of negative ion SALDI mass spectra, a series of carbon cluster peaks with 12 mass unit difference were always observed like ions at $m/z=48$ (C_4^-), 60 (C_5^-), 72 (C_6^-) etc.. These carbon clusters are in the mass range below $m/z=100$. Therefore, no serious interference was caused for identifying analytes. In addition, chloride ions were observed in all the negative ion mode mass spectra. These chloride ions may come from impurities in activated carbon or the sample solutions. The deprotonated molecule of sucrose, $(M-H)^-$ at $m/z=341$ and $(M+Cl)^-$ at $m/z=377$ were observed. After all the background ions in Figure 64 were identified, the analytes ions were easily recognized. Figure 65 shows the SPE/SALDI spectra obtained from a mixture solution of four diuretics. The diuretics are hydroflumethiazide, dichlorophenamide, bumetanide, and hydrochlorothiazide. Ten micro liters of a 10 mg/mL diuretic solution of each was spiked into 6 mL pure water for a final concentration of $17\text{ }\mu\text{g/mL}$. Different intensities for each diuretic were observed in the SALDI mass spectrum. Different diuretics show different sensitivities in SPE/SALDI. Hydroflumethiazide had the highest sensitivity. The absolute detection limits for these diuretics in solution (spiking analytes into 3 mL of water) are presented in Table 6.

The absolute detection limits were calculated from SALDI mass spectra by extrapolating to a signal to noise ratio of 3. Sample precleaning would, however, be required in a real urine sample to get rid of components such as proteins before this SPE/SALDI analysis can be continued.

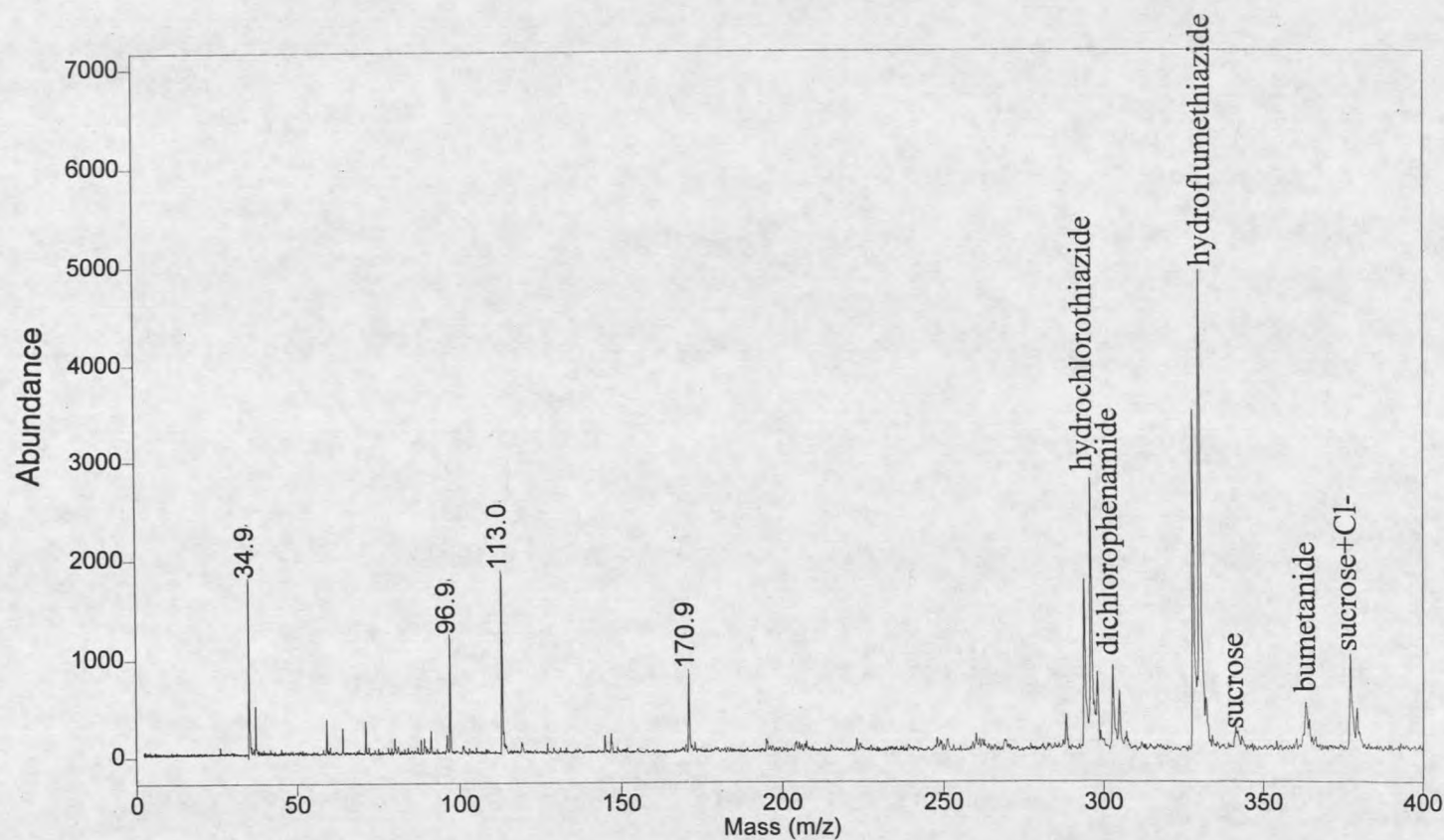


Figure 65. SPE/SALDI mass spectrum (negative ion mode) of diuretics spiked into pure water. The diuretics were hydroflumethiazide (mw= 331.29), hydrochlorothiazide (mw= 297.72), bumetanide (mw= 364.42), and dichlorophenamide (mw= 305.16). 100 μ g of each diuretic was added to 6.0 ml pure water. LP= 365. SA= 76.

Table 6. Absolute Detection limits of diuretics in a 3 mL volume of water in the analysis of SPE/SALDI.

Diuretics	Detection limit (ng)
Hydroflumethiazide	70
Dichlorophenamide	600
Furosemide	400
Bumetanide	700
Hydrochlorothiazide	500

Analysis of Herbicides. Triazines are the most common herbicides used on crops. Triazines are frequently found in environmental samples such as soil or water. The maximum allowable concentration for atrazine in potable water is 3 µg/mL. The first observed adverse health effects begin to appear in the milligram per liter water range (Cai et al., 1993). Triazines can be detected by liquid-liquid phase extraction or liquid-solid phase extraction, followed by HPLC or GC analysis. Figure 66 shows SPE/SALDI mass spectra of different triazines: prometryn (mw= 241.37), atrazine (mw= 215.68), ametryn (mw= 227.35), and prometon (mw= 225.29). The prometryn sample was extracted from 6 mL of a 16.7 ppm solution. The other triazines were extracted from 3 mL of 10 ppm. solutions. Table 7 shows the absolute SPE/SALDI detection limit for each herbicide.

Table 7. Absolute SPE/SALDI detection limits for triazine in pure water solution.

Triazine	Detection limit (ng)
prometryn	200
atrazine	2500
ametryn	5000
prometon	300

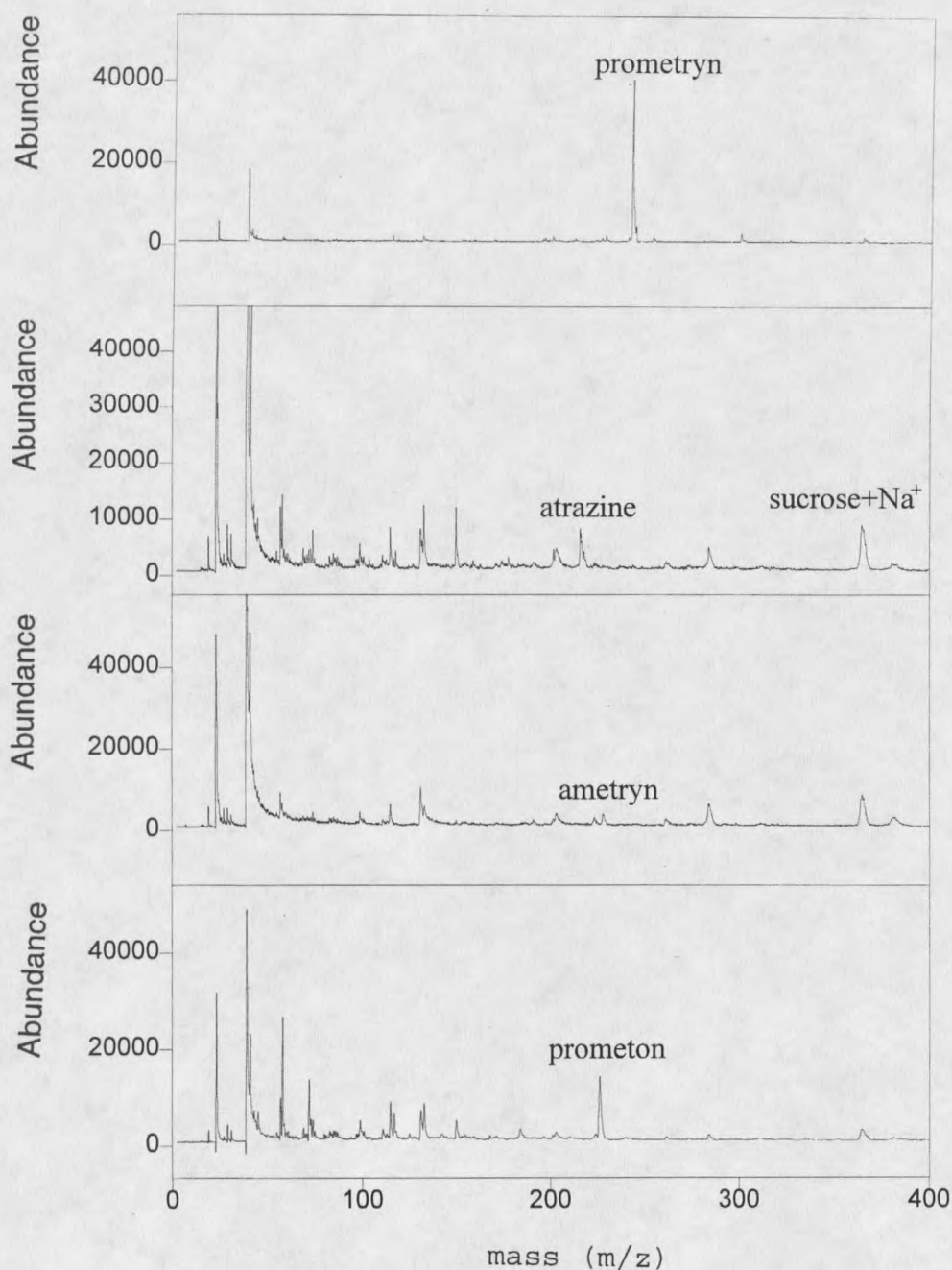


Figure 66. SPE/SALDI mass spectrum of prometryn (mw= 241.37, 50 μ g, LP= 410, SA= 50), atrazine (mw= 215.68, 30 μ g, LP= 420, SA= 57), ametryn (mw= 227.35, 3.0 μ g, LP= 432, SA= 50), prometon (mw= 225.29, 3.0 μ g, LP= 400, SA= 44). The analyte was added to 3 mL of pure water.

Prometryn has the highest sensitivity among the four herbicides. The spectrum in Figure 67 was obtained from 300 mL of a 3 ppm prometryn solution. The absolute detection limit is 200 ng. It was calculated by extrapolating to a signal-to-noise ratio of 3. To simulate more realistic conditions, triazines were spiked into a water sample obtained from river water (Hyalite stream, Bozeman, Montana). Ten microliters of a 10 mg/mL of each triazine was added to 3 mL river water. Figure 68 shows the SPE/SALDI mass spectrum of the river water sample before triazines were spiked. The spectrum looks clean aside from the matrix peaks. Figure 69 shows the SPE/SALDI spectrum after spiking the four triazines into the river water sample. Four triazines were observed in this spectrum, but the peak intensity for each triazine is different due to the different sensitivity. Also, the spectrum shows that when more than one triazine exist in a sample solution, all the triazines were still absorbed on the surface of activated carbon and detected by SALDI.

Analysis of Amino Acids and Peptides. SPE/SALDI can be applied also to biochemical analysis. That is, this technique can concentrate biochemicals like amino acids and peptides on the surface of activated carbon. In biological fluids, some amino acids and peptides play an important role for diagnosing diseases (Espat et al., 1995; Candito et al., 1994; Castillo et al., 1995; Tameda et al., 1993; Sperl et al., 1994). Whether a specific amino acid is present in biological fluids or not is therefore significant. A fast screening method such as SPE/SALDI could be very useful for obtaining a preliminary result in a short time. Figure 70 shows a negative ion SPE/SALDI spectrum of phenylalanine spiked into 3 mL of water at a concentration of 3 $\mu\text{g/mL}$. The absolute detection limit of 0.3 μg was calculated using

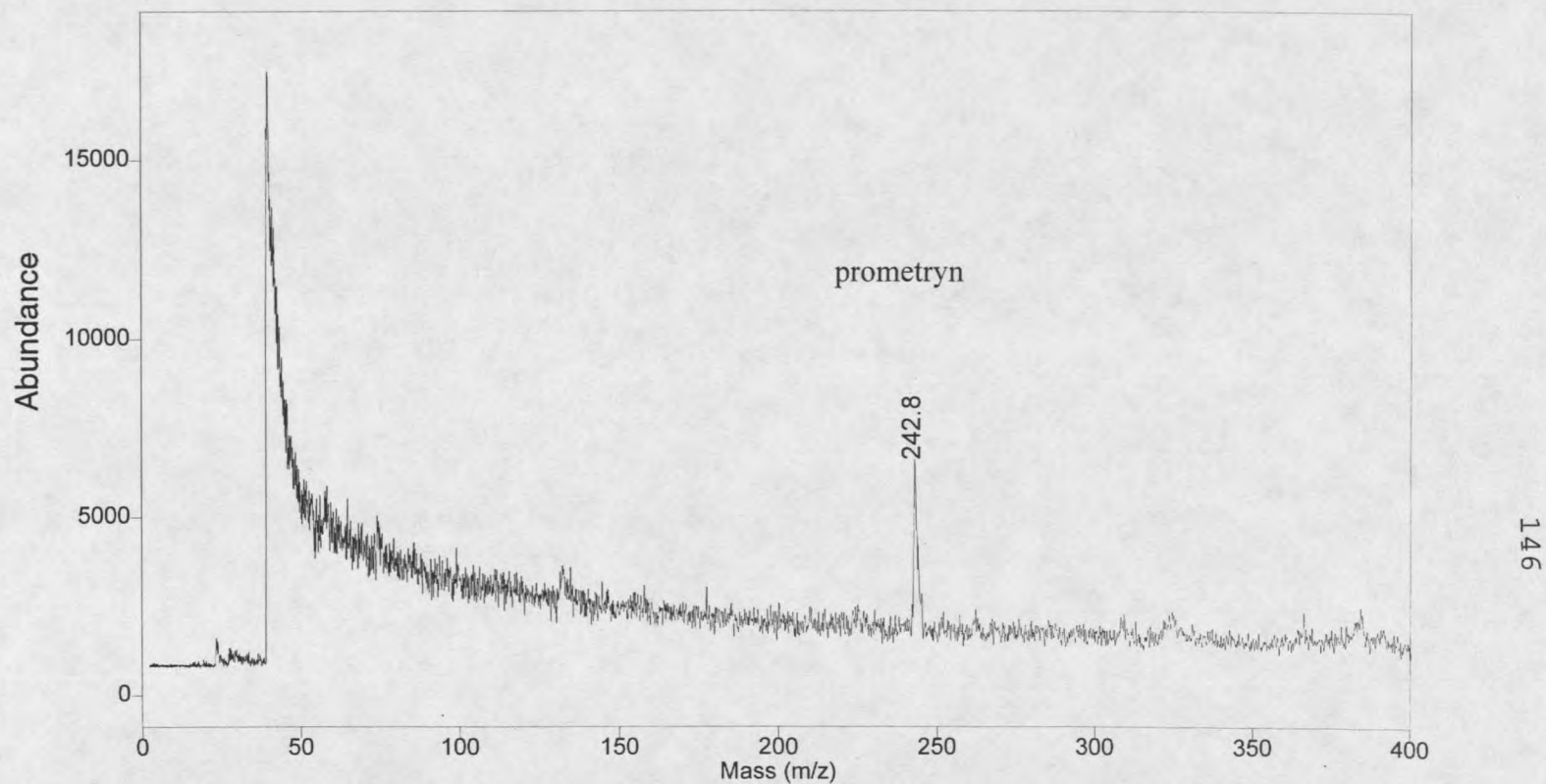


Figure 67. SPE/SALDI mass spectrum of prometryn (mw= 241.37). 1000 μ g of prometryn was spiked to 300 ml of pure water. LP= 465. SA= 33.

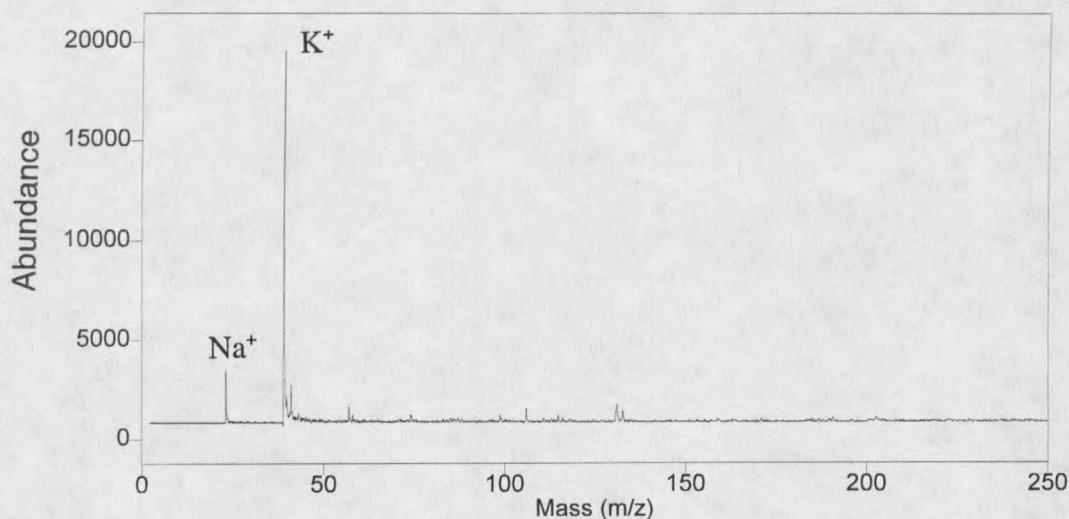


Figure 68. SPE/SALDI mass spectrum of the river water sample. LP= 400. SA= 51.

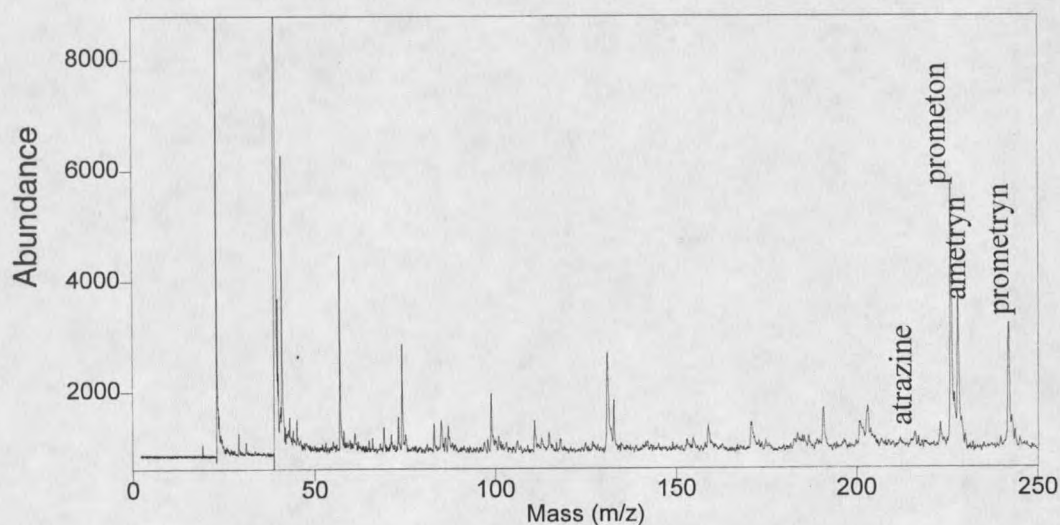


Figure 69. SPE/SALDI mass spectrum of triazines spiked into river water. The triazines include prometryn (mw= 241.37), atrazine (mw= 215.68), ametryn (mw= 227.35), prometon (mw= 225.29). One hundred micro grams of each triazine were added to 3 mL river water. LP= 400. SA= 139.

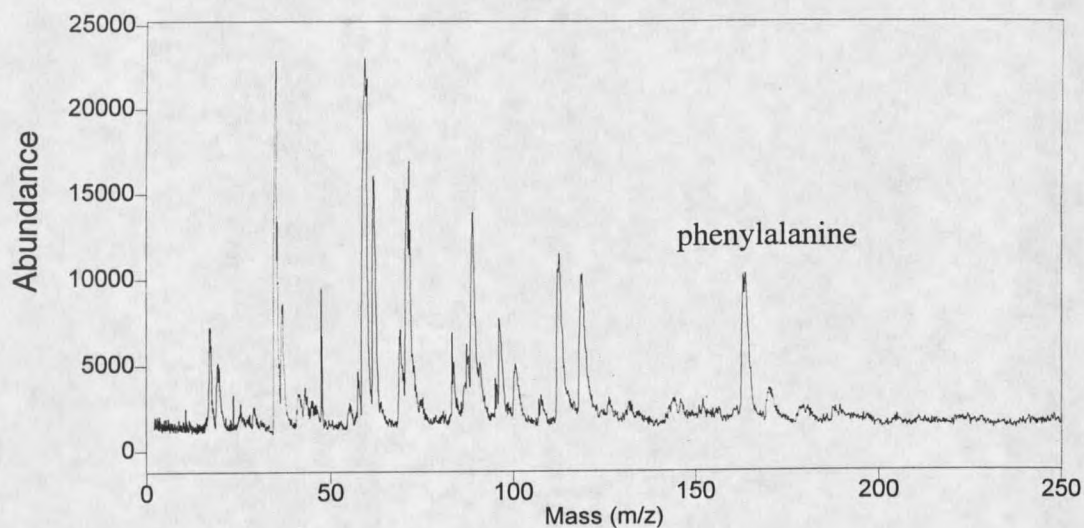


Figure 70. SPE/SALDI mass spectrum (negative ion mode) of phenylalanine. 100 μ g of phenylalanine was spiked into 3.0 ml of pure water. LP= 388. SA= 55.

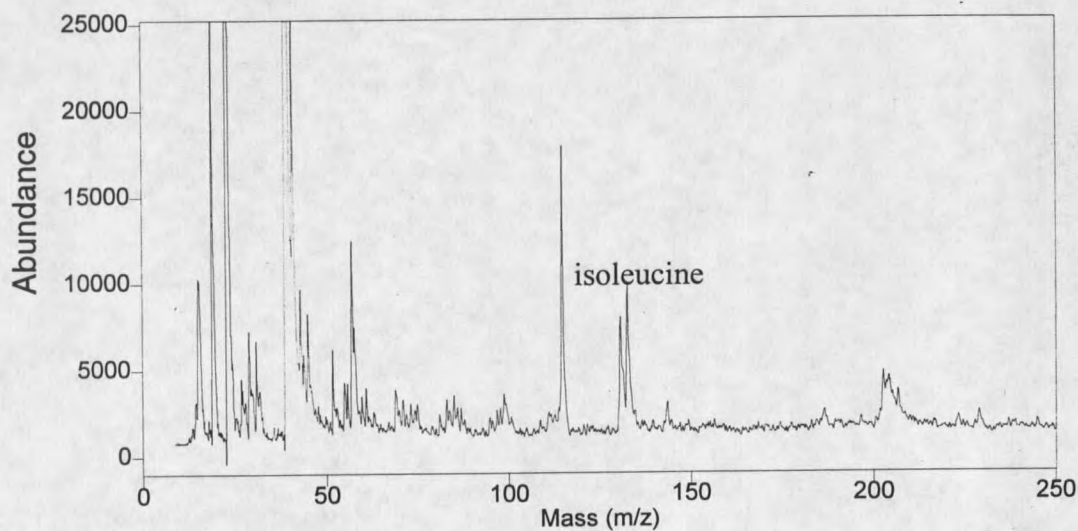


Figure 71. SPE/SALDI mass spectrum of isoleucine (mw= 132.08). One ml of isoleucine solution (0.052 mg/ml) was extracted by SPE. LP= 474. SA= 46.

a signal-to-noise ratio equal to three. Without concentrating the sample solution by SPE, no analyte signal was observed in the SALDI mass spectrum. Figure 71 shows the SALDI mass spectrum of isoleucine ($m_w = 132.08$) obtained from extracting 1 mL of a 0.052 mg/mL isoleucine solution. In addition to amino acids, peptides can be analyzed by this method. Angiotensin II is an octapeptide that is produced by the kidneys and increases blood pressure. Figure 72 shows the negative ion SPE/SALDI spectrum of angiotensin II. The concentration of angiotensin II in the original solution was 3.3 $\mu\text{g/mL}$, and the volume was 3 mL. The absolute detection limit was estimated to be 70 pg. The negative ion SPE/SALDI mass spectra of phenylalanine and angiotensin II showed more intense analyte peaks than those in positive ion mode.

Quantitative Analysis

In addition to a fast screening method for qualitative purpose, SPE/SALDI has potential to be a routine quantitative analysis method. Figure 73 shows a calibration curve for the intensity of hydroflumethiazide molecular ions in SPE/SALDI mass spectra versus the concentration in the original solution. It is seen that the intensity of molecular ions has a linear dependence on concentration. The correlation coefficient is 0.991.

The sensitivity of SPE/SALDI obtained were found to depend on three factors: (1) liquid flow rates through the absorbent (2) amount of the absorbent material (3) method of packing. It was important to control the flow rate. If the flow rate was too fast, the sensitivity decreased due to analyte not having enough contact with the adsorbent material. If the flow rate was too slow, the analyte achieved an equilibrium condition with extraction solution and

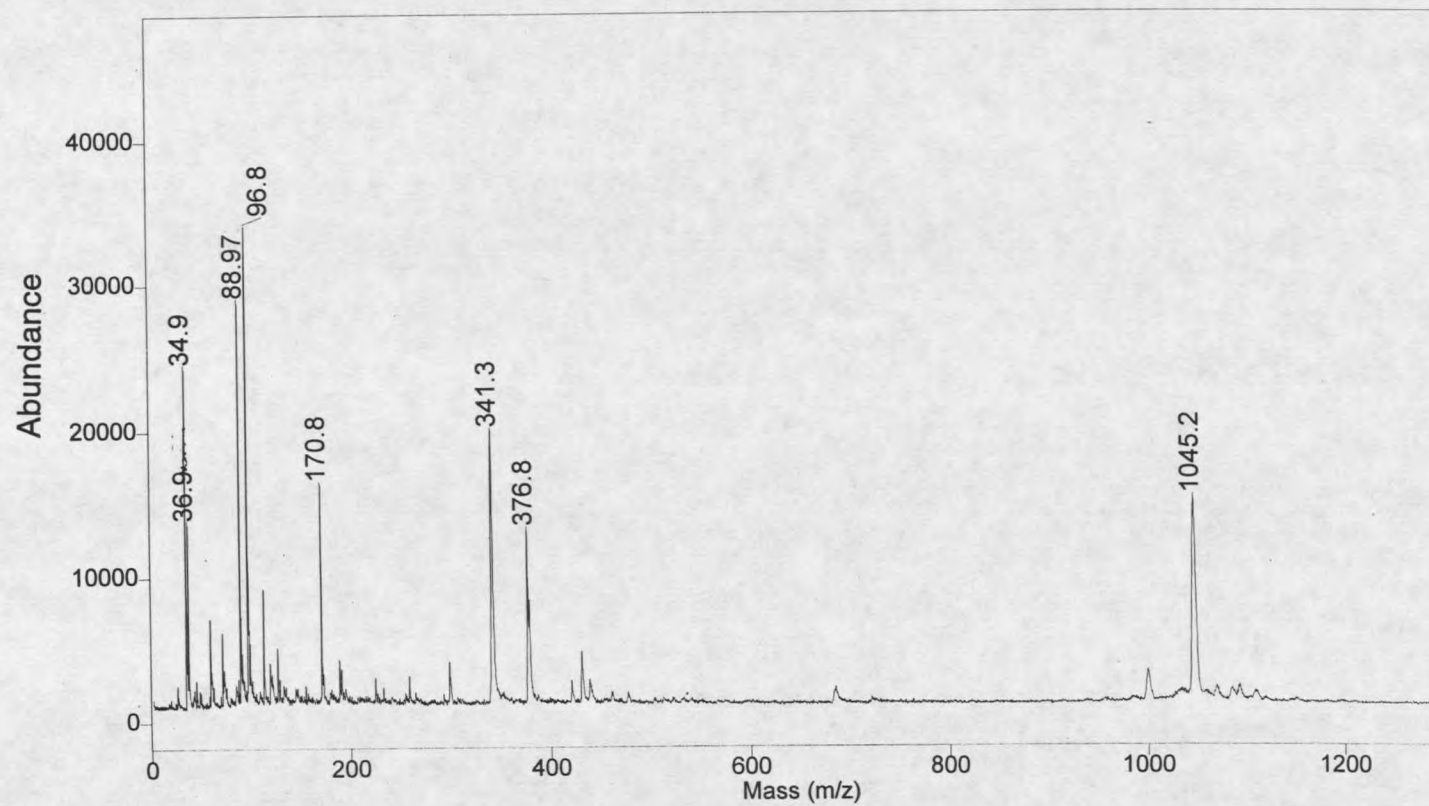


Figure 72. SPE/SALDI mass spectrum (negative ion mode) of angiotensin II (mw= 1,046.2). Angiotensin II (10 nmole) aqueous solution was extracted by SPE. LP= 460. SA= 93.

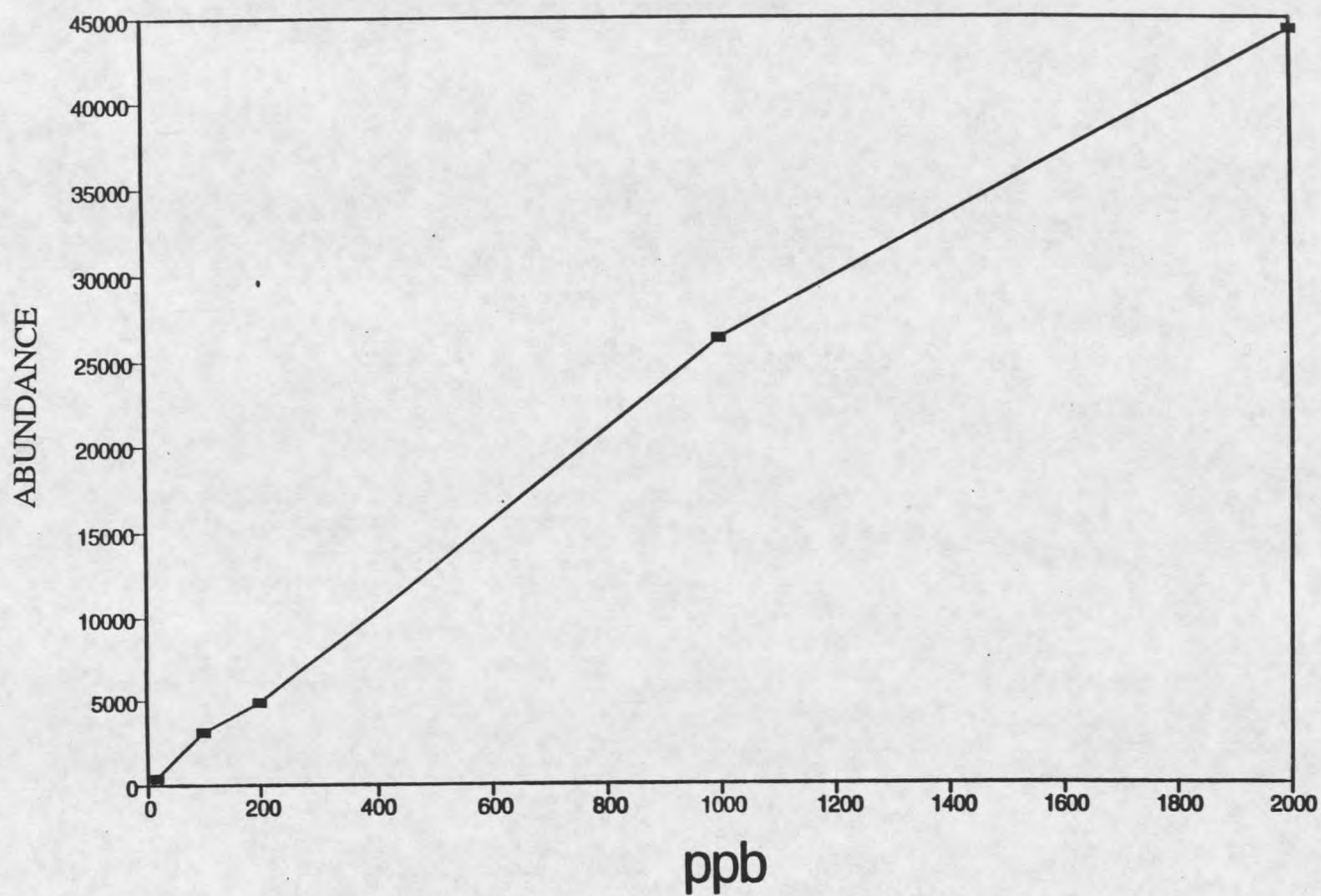


Figure 73. The intensity of hydroflumethiazide molecular ions in SPE/SALDI versus the concentration of hydroflumethiazide in SPE/SALDI results.

adsorbent materials below the saturation limit. In this case, trace organics eluted with the aqueous solution. When the volume of the extraction solution was increased, the flow rate had to be increased. The second important factor was using the correct amount of adsorbent material. For 30 mL or less solution, 2 mg of activated carbon was used. For large volumes of sample solution, it was necessary to use more adsorbent material. The limiting factor was the available surface area of activated carbon for analyte. Too large a volume of solution resulted in sample loss. This was due to the surface area of activated carbon was insufficient to adsorb all the trace organics from the extraction solution. The third important factor was the method of packing of adsorbent material. The material should be tightly packed. Otherwise, activated carbon was lost with the sample solution when the flow rate was high.

It is concluded that SPE/SALDI is a fast, convenient, and sensitive technique for application in either environmental chemistry or biochemistry. Moreover, it is not only a qualitative method, but also a quantitative one.

SPME (Solid Phase MicroExtraction)/SALDI

General

Solid phase microextraction (SPME) was first developed by Pawliszyn and Arthur (Arthur et al., 1990 & 1992). SPME is version of solid phase extraction. The technique used both for trace non-volatile and volatile organics. The procedures for SPME are as follows (Eisert et al., 1995; Berg et al., 1993; Chai et al., 1993). First, a fused silica fiber coated with a stationary phase for extraction is exposed to a sample solution for a given time. Trace organics diffuse to the stationary phase from the sample solution during the time of fiber

exposure. The fiber is then withdrawn from the sample and introduced into a GC-injector, where thermal desorption occurs, and analytes are released. The technique is rapid, convenient, and inexpensive (Arthur 1990 & 1992).

In this section, a new SPME method will be presented. Instead of using a fused silica fiber coated with stationary phase for the microextraction, a single activated carbon pellet was used as the stationary phase. SALDI was then used for desorbing analytes from the stationary phase instead of using thermal desorption. This new technique is called SPME/SALDI.

Sample Preparation of SPME/SALDI

An activated carbon pellet was used as the adsorbent in SPME. The pellets were designed for use in liquid-phase adsorption systems. The procedures for SPME/SALDI are as follows. One activated carbon pellet was added into a sample solution, and agitated by a vortexer for 8 minutes. Next, the activated carbon pellet was removed from the sample solution. The pellet then was ground into a fine powder. The powder was then mixed with 4 μL of SALDI solvent (sucrose/glycerol/methanol = 5/28/67, w/w/v). One microliter of this mixture was applied to the sample plate of SALDI.

Results and Discussion

Activated carbon is a good absorbent for organics in SPE and should be a suitable stationary phase also in SPME. Activated carbon pellets were chosen as stationary phase adsorbents because the pellets were designed for liquid phase extraction. The activated carbon pellet were about 0.40 cm long and the diameter is about 0.08 cm. Figure 74 shows

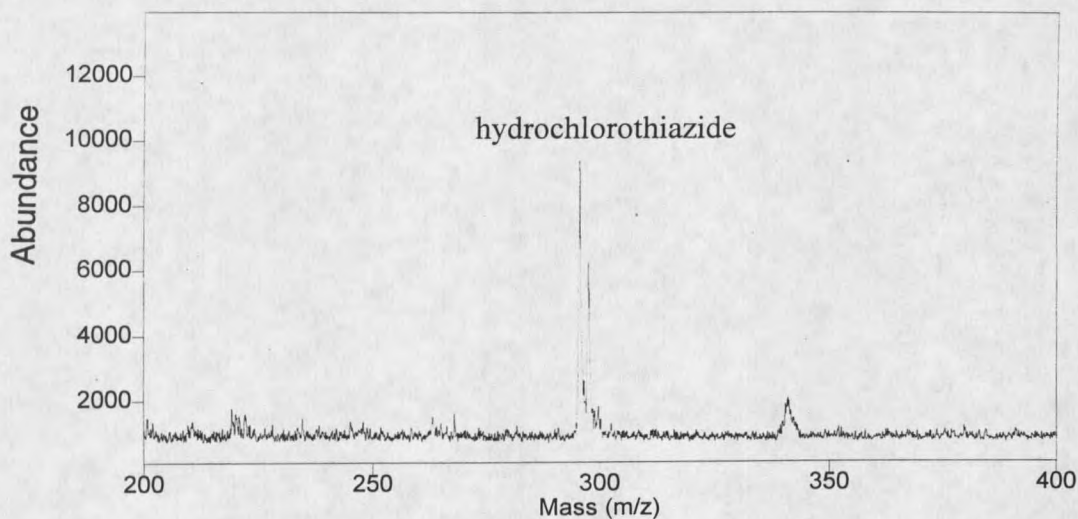


Figure 74. SPME/SALDI mass spectrum (negative ion mode) of hydrochlorothiazide (mw= 297.72, 50 μ g analyte in 0.5 ml solution). LP= 500. SA= 28.

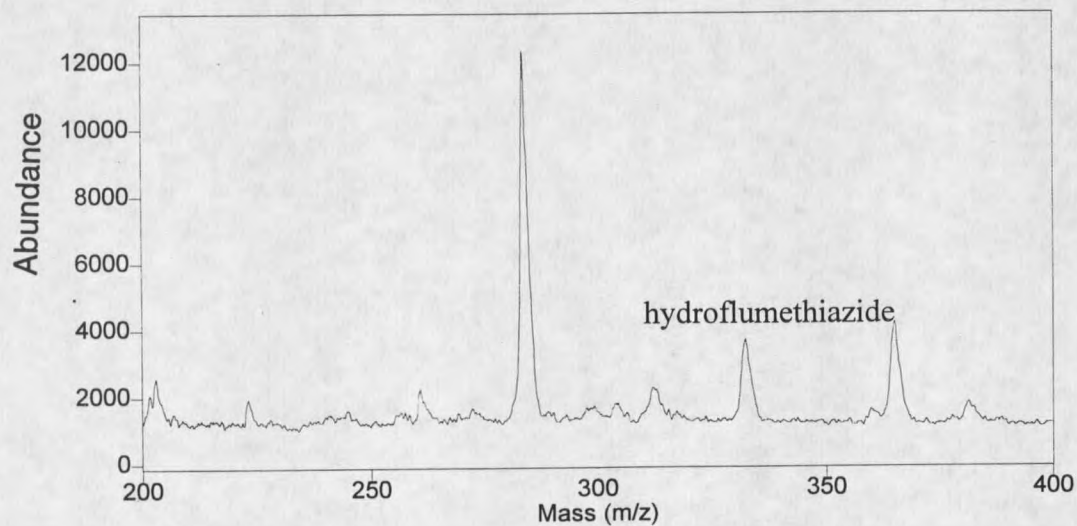


Figure 75. SPME/SALDI mass spectrum of hydroflumethiazide (mw= 331.29, 1 μ g analyte in 0.6 ml solution). LP= 400. SA= 155.

the SPME/SALDI mass spectrum obtained of hydrochlorothiazide from 0.50 mL of a 100 $\mu\text{g/mL}$ solution. The molecular ions of hydrochlorothiazide are observed in the negative ion mode mass spectrum. The absolute detection limit is estimated to be 3 μg . Figure 75 shows the spectrum obtained from 0.60 mL of hydroflumethiazide aqueous solution (1.0 μg) by SPME/SALDI. The absolute detection limit is estimated to be 0.10 μg .

The adsorption-time profile was obtained by monitoring the intensity of analyte molecules as a function of exposure time. In Figure 76, an activated carbon pellet was exposed and stirred in a 0.50 mL of dichlorothiazide solution (0.50 mg/mL) with different times. In the first 8 minutes, the rapid adsorption of dichlorothiazide on the activated carbon pellet occurred. After this time, the rate of adsorption slowed. Thus, the adsorbent needed to be exposed to the sample solution for eight minutes more to give analytes enough time to be adsorbed onto the adsorbent.

The quality of the SALDI mass spectra depended on the powder size of activated carbon after the activated carbon pellet was ground. The best SPME/SALDI results were achieved with a small powder size. It is required in SALDI that the diameter of the solid powder must be small enough to allow the SALDI to occur. Thus, when the activated carbon pellet could not be ground into a very fine powder, it was difficult to obtain good SPME/SALDI results, although sufficient trace organics were adsorbed on the activated carbon pellet.

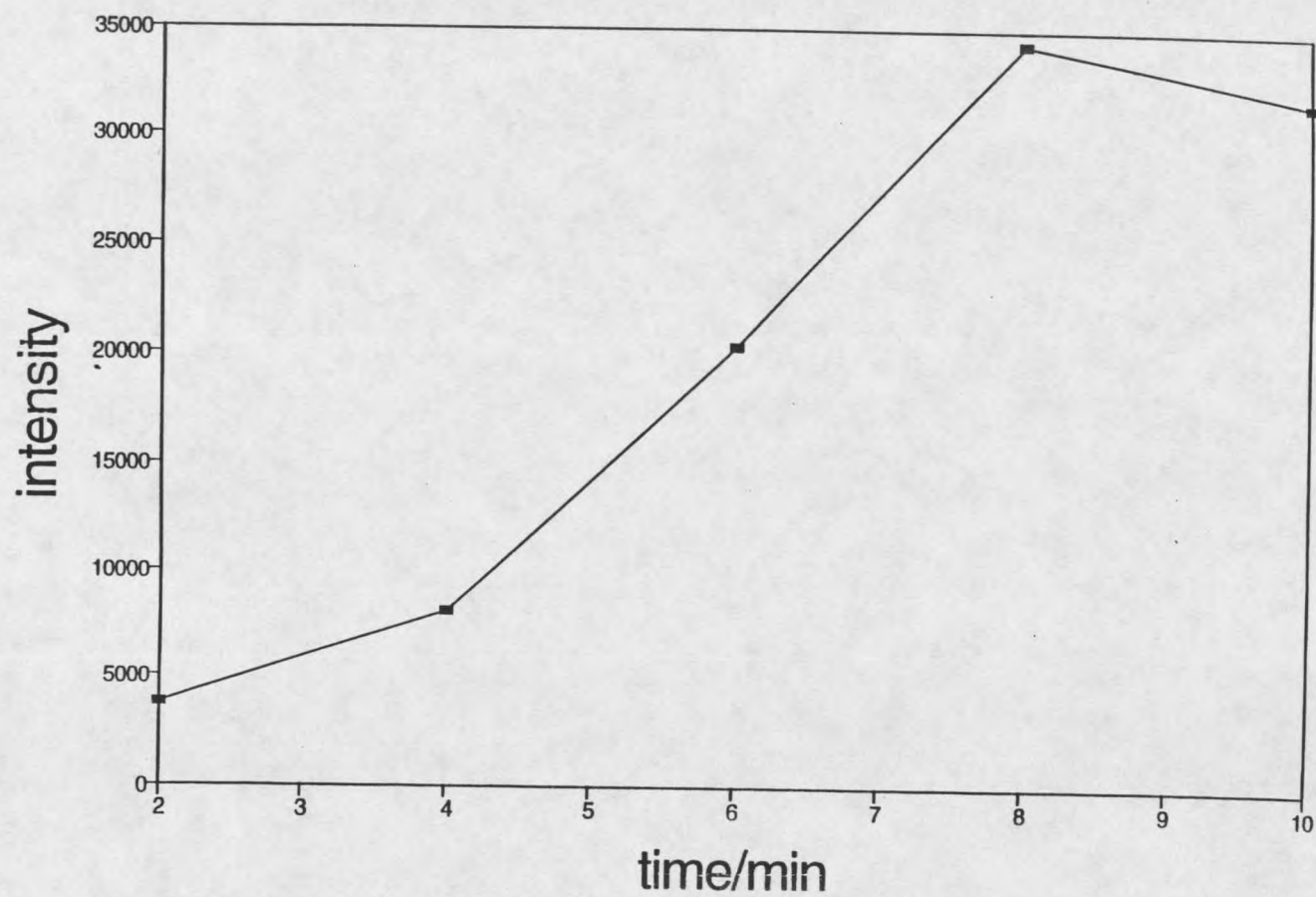


Figure 76. The intensity of dichlorothiazide molecular ions in SALDI versus the extraction time in SPME.

CHAPTER 7

THIN LAYER CHROMATOGRAPHY (TLC)/MS

Introduction

Thin Layer Chromatography (TLC) is a common separation and purification method that is extensively used for organics, amino acids, peptides, and lipids (Skoog, 1992). A TLC plate consists of a separation medium coated on a solid support. The mobile phase moves through the stationary phase by capillary action when the plate is "developed". After completed separation, the analytes are "visualized" or otherwise located on the plate. Common visualization methods are to expose the plate to iodine vapor or to spray the plate with a ninhydrin solution. Thus, analytes are identified primarily on the basis of their location on the plate, or the so-called R_f (Retardation Factor) value. The retardation factor in TLC is defined by

$$R_f = d_R/d_M$$

where d_R is the linear distance measured from the location of the sample spot to the original line, and d_M is the linear distance measured from the solvent to the original line (Skoog, 1992). The measurement of d_R is based on the position of maximum intensity. In order to positively identify unknown analytes on a TLC plate, another analytical method must be used. Mass spectrometry would be an ideal detector for TLC because mass spectrometers

can provide information about structures and molecular weights for analyte molecules. Many types of ionization methods have been tried in combination with TLC, such as EI, CI, PD, FAB, LD, and MALDI (Busch, 1991).

When TLC is directly coupled to mass spectrometry, certain problems can be predicted to occur. First, not only analytes are present on the TLC plate, but also residual solvents, reagents used for locating sample spots, etc. Second, the method used to extract analytes, adsorbed on the silica gel, is expected to be critical to obtaining successful TLC/MS results. Two methods have been used to release analytes from TLC plates: evaporation at elevated temperatures and solvent extraction (Busch, 1991). Using the first method, a TLC plate can be directly positioned in the ion source of a mass spectrometer. The plate is heated in the source, and high volatility compounds are released and detected by EI or CI. However, for nonvolatile and thermally labile compounds, this method does not work. Instead, solvent extraction must be used to release analytes from the TLC plate. Successful detection of TLC separated analytes by FAB, PD, and MALDI after solvent extraction has been reported (Busch, 1991).

Here, previous efforts to interface TLC with mass spectrometry will first be reviewed. Second, a new mass spectrometric interface technique, TLC/SALDI, will be presented. It will be seen that the sample preparation in TLC/SALDI is easy, and that the results are very promising.

TLC/EI (or CI) MS

In the early 1980's, many groups combined TLC directly with gas-phase ionization methods, such as electron ionization (EI) and chemical ionization (CI). These experiments were recently reviewed (Busch, 91). The most common method was to remove the solid phase containing the analyte from the TLC plate. In the form of a powder, the solid phase was then introduced into the ion source and heated in-situ (Durden et al., 1980; Kraft et al., 1981). In a more elaborate scheme, plate scanners and CO₂-laser-induced neutral desorption were also used (Ramaley et al., 1983 & 1985). In either case, high volatility compounds were released from the stationary phase by thermal desorption and subsequently ionized by EI or CI. The only compounds that can be analyzed by these methods are those which can be thermally desorbed from the TLC plate without decomposition. For example, it was reported that amino acids could not be detected (Ramaley et al., 1985). Desorption temperatures up to 300°C may be necessary. For some compounds that bind very strongly to silica, even a temperature of 600°C may not be sufficient (Busch, 1991). Despite its problems, the thermal desorption method has been successfully used for a wide variety of organic compounds, such as phenols, steroids, nucleoside, biogenic amines, and amino acids (Busch, 91). Sensitivity is typically reported to be in the µg level (Ramaley et al., 1985).

TLC/PDMS

Plasma desorption mass spectrometry is based on the passage of very high energy (MeV) particles through a thin layer of sample material. The events generate sputtered neutral

molecules, electrons, photons, positive and negative ions. An off-plate TLC/MS method was based on eluting analytes from the TLC stationary phase, and electrospraying the eluate onto a thin target support (Krueger et al., 1977; Danigel et al., 1985). However, this indirect method was time-consuming and inconvenient (Krueger et al., 1977; Danigel et al., 1985).

TLC/LSIMS or FAB

In LSIMS or FAB, analytes are dissolved in a low vapor pressure liquid solvent such as glycerol, and the solution is directly introduced into the ion source. A high energy particle beam is used to desorb intact analyte molecules from the condensed phase to the vacuum. Thus, analytes are not required to be in the gas phase before ionization. FAB is a much softer ionization method than EI or CI. For TLC interfacing, the problem is how to effectively extract the analytes from the TLC solid phase. Many groups have reported results on TLC/FAB (Dunphy et al., 1988; Oka et al., 1992; Busch et al., 1993). Most groups used FAB matrices such as glycerol, thioglycerol, and triethanolamine as the extraction solvent. The viscous extraction solvent was deposited directly on the sample spot on the TLC plate. The whole plate or section was then inserted into the FAB ion source of a mass spectrometer. The extraction procedure always caused problems with spatial diffusion of analytes and sample spot spreading. Because all FAB matrices have a high viscosity, analyte diffusion is not as serious a problem as when organic solvents are used for extraction. Furthermore, because of the low volatility of the FAB matrices, there is enough time for analyte molecules to diffuse into the liquid solvent before it evaporates. The best sensitivities reported for TLC/FAB are with range of 100 pg-10 ng (Duffin et al., 1988; Busch et al., 1992). Also, the

method makes direct imaging TLC possible. The whole TLC plate can be positioned into the FAB ion source, if the pressure problem inside the source chamber is solved by using a high capacity pump. A special, large ion source chamber must be built for this purpose.

TLC/LD

An ionization method that does not require an extraction step is laser desorption. Laser beams can penetrate into the silica gel and provide enough energy to desorb small molecules from the condensed phase. Thus, the laser beam can be focused directly onto the intact TLC plate. Because no liquid matrix is added, the whole TLC plate can be introduced into the ion source without the need for a high capacity vacuum pump. In this way, a direct MS analysis of compounds on the TLC can be performed without the need for extraction solvents. Hercules et al. described such a TLC/LD system (Hercules et al., 1983; Novak et al., 1985; Rogers et al., 1993; Fanibanada et al., 1994). However, TLC/LD is only suitable for low molecular weight compounds. Intact large molecules do not survive the direct laser irradiation. Furthermore, LD induces significant fragmentation and suffers from poor reproducibility. Sensitivity is about 1 μ g (Rogers et al., 1993).

TLC/MALDI

MALDI is a much softer ionization method than LD and can also be used for large, thermally labile molecules. However, sample preparation for TLC/MALDI is complicated because not only must the analytes be extracted from the silica gel, but they must also co-crystallize with the MALDI matrix. However, forming the analyte/matrix co-crystals on

the TLC plate has proven to be difficult. For example, the correct ratio of matrix to analyte is critical for obtaining high sensitivity, high quality mass spectra, and controlling this ratio is difficult. It is difficult to know how much analyte is present on the TLC plate when unknown samples are analyzed. Thus, MALDI matrix addition tends to be a trial and error exercise. Hercules and coworkers tried two different approaches (Gusev et al., 1995a & 1995b). In the first procedure, they added an extraction solvent on the TLC plate to extract and concentrate the analytes close to the surface. A MALDI matrix solution was then deposited onto the same plate. As a result of evaporation, analyte/matrix co-crystals were formed on the surface. A piece of the TLC plate was cut out and subjected to MALDI analysis. However, the two solvents deposited on the TLC plate always caused a serious analyte spreading problem and lowered the sensitivity. Hercules et al. developed an alternative sample preparation procedure in an effort to reduce the spreading of analyte (Gusev et al., 1995b). A matrix crystal layer was first generated on a different substrate from which it was transferred to the TLC plate. The transfer was accomplished by pressing the matrix onto a previously wetted TLC plate using very high pressure. With this method, the absolute detection limit, calculated at a signal-to-noise ratio equal to three, was reported to be in the picogram range. Mass spectra of peptides, such as bradykinin and angiotensin II, were obtained. Direct imaging of the TLC plate was demonstrated. However, the sample preparation is complicated and we were not able to reproduce the results in our laboratory. Furthermore, the high MALDI matrix background in the low mass range often makes it impossible to identify low molecular weight analytes. This is unfortunate because TLC is usually used to separate small molecules.

TLC/SALDI

Each ionization technique used in the past has had serious limitations for TLC/MS interfacing. The ideal technique should have advantages such as easy sample preparation, clean matrix background, low detection limits, an extended mass range, and the capacity for direct imaging. Here, the interfacing of SALDI with TLC will be described. It will be seen that this approach has all the necessary advantages for a practical TLC interface method.

SALDI has real advantages that makes it promising for TLC analysis. First, SALDI is a relatively soft ionization method, so there is little or no molecular fragmentation. Second, there is no requirement to form co-crystals of matrix and analyte because the analytes should be solvated in the liquid SALDI solution. Third, there is not a problem of attaining the correct ratio of matrix to analyte. In SALDI, the glycerol solvent appears to be nearly universally applicable to all classes of compounds, and there is no need to search for a suitable matrix. For these reasons, the SALDI sample preparation for TLC is expected to be easier than for MALDI. Since TLC is usually used to separate low molecular weight compounds, it is important to have a "clean" mass spectrum in the low mass range with little or no matrix background. As described in previous sections, the matrix background in SALDI can be very low. Also, larger molecules like peptides can be analyzed by SALDI. The mass range is wider than for TLC/FAB and about the same as for TLC/MALDI.

Experimental

Silica gel 60 (with or without a fluorescent indicator) pre-coated TLC plates with a plastic backing were purchased from EM reagents (Germany). The silica layer thickness was 0.20

mm. The developing solvent used for peptide and amino acid separations was water/methanol/acetic acid (99.7%) = 44:50:6 (v/v/v) (Dunphy et al., 1988). The reagent used for visualization of peptides and amino acids was 0.20% ninhydrin in ethanol. This reagent was applied to the whole TLC plate after it had been developed and dried. The ninhydrin-treated plate was then heated by a hot air gun for visualization. The center of the bradykinin sample spot was observed at $d_R = 0.40$ cm from the application spot when the developing distance was $d_M = 2.60$ cm. This corresponds to $R_f = 0.19$. Angiotensin II had an R_f value of 0.17 and arginine has an R_f value of 0.58 under identical conditions. The developing solvent used for diuretics was acetone/methanol = 1:1. Iodine vapor was used for visualization. To avoid interference from the visualization reagents, a pair of TLC plates with the same samples were developed in parallel. One was used for locating the analytes and the other one for the TLC/MS experiments. The SALDI suspension was composed of glycerol, sucrose, and 2 μ m activated carbon powder. The composition of the SALDI matrix system varied between the different approaches tried. The composition of the SALDI suspension for the first approach was as follows: 25 mg of activated carbon was added to 0.10 mL of sucrose/glycerol/methanol (5:28:67, w/w/v), and this solution was mixed with 0.10 mL 5.0% sucrose in water. This mixture was diluted by adding 0.20 mL methanol. This SALDI suspension solution will be called SALDI suspension "A" in the following to simplify the description. SALDI suspension "B" was prepared as follows: 25 mg of activated carbon was mixed with 0.40 mL of solvent made by mixing 0.10 mL of 50% glycerol/methanol (v/v) with 0.30 mL of 5% sucrose/water (w/w). Finally, SALDI suspension "C" was made by adding 25 mg of activated carbon powder to 0.90 mL of a

solvent made by mixing 0.10 mL of 50% glycerol in methanol (v/v) with 0.80 mL of 5% sucrose in water (w/w).

When the sample was located by a visualization reagent, the spot was circled by a pencil. The specific spot was then cut off from the TLC plate keeping the silica gel and backing intact. Then, the extraction solution and the SALDI suspension were added. After the small section of the TLC plate had been dried in air for 10 minutes, it was attached to the sample tray using a double-side tape. The tray was then inserted into the ion source for SALDI analysis. If not more than only 1 to 3 sample spots were extracted, the whole TLC plate could be attached to the sample tray without causing too high a pressure in the ion source.

Results and Discussion

The matrix background in TLC/SALDI mass spectra is shown in Figure 77. Only SALDI suspension "A" was applied to the TLC plate, but no analytes. A very clean spectrum is observed. The only intense peaks are due to Na^+ ($m/z=23$), K^+ ($m/z=39$), the sodium ion adduct with glycerol ($m/z=23+92$), and the sodium ion adduct with sucrose ($m/z=23+342$). The peak at $m/z=203$ is due to the sodium ion adduct with glucose, a fragment of sucrose. It is noteworthy that essentially no protonated glycerol or fragments of protonated glycerol are observed. A series of low-intensity peaks can be seen between mass 200 and 300 (inset spectrum in Figure 77). These ions probably originate from the silica gel binder. The intensities of these ions are low compared with the major ions. However, it was found that the intensity increased with increasing laser power.

Several approaches were explored to extract analytes from the silica gel on the TLC plate.

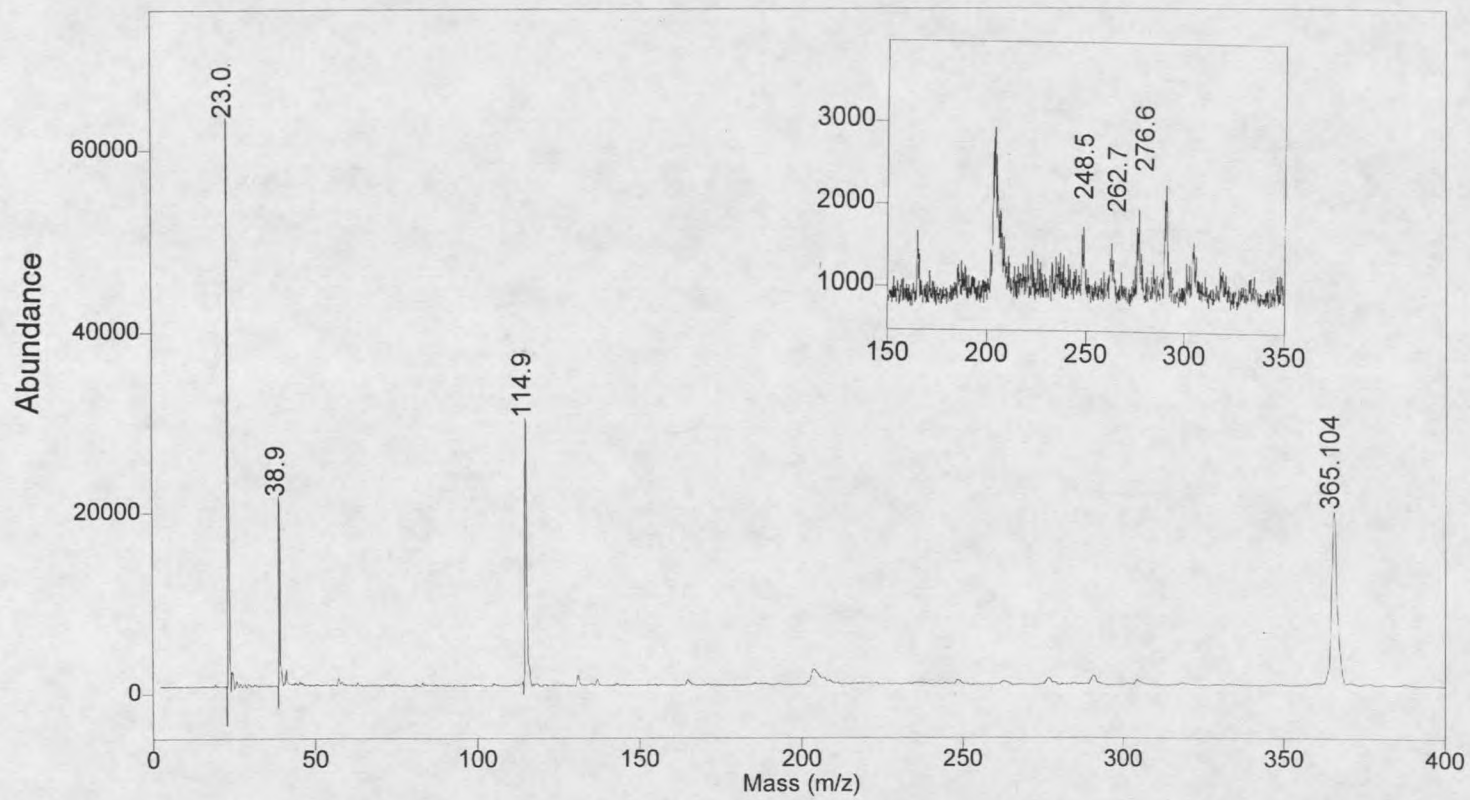


Figure 77. Mass spectrum of SALDI suspension on TLC plate. LP=470. SA=32

The first approach used volatile organic solvents for extraction. Five μL of an organic solvent, such as ethanol or methanol, were added to the TLC sample spot in an effort to extract analytes from the interior of the silica gel to the surface. However, once the solvent was applied, analytes started to diffuse away from the sample spot. To reduce diffusion, the extraction solvent therefore had to be applied gradually. It was necessary to continue to add solvent because it was found that if the sample spot dried before 1.0 μL of the SALDI suspension "A" was added, most the analytes were reabsorbed on the silica gel and the extraction process was unsuccessful.

Figure 78 shows a TLC/SALDI spectrum of bradykinin obtained by using organic solvent extraction. The extraction solvent was 70% ethanol in water. The bradykinin peak is seen to be quite broad, and strong sodium adduct peaks are also observed. The fast diffusion rate of the peptide in the extraction solvent diluted the analyte on the TLC plate. Partly for this reason, the sensitivity was low. Moreover, if the extraction process was not performed correctly, the analyte signal was often absent. In an effort to increase the extraction efficiency, a few holes were punctured on the back of the TLC plate, opposite to the analyte spot. The eluting solvent was then added through the back side of the plate. However, back-side elution was discovered to have the same problems as the previous procedure. Lateral diffusion was still fast. If the elution procedure was not performed correctly, intense sodium adducts were observed, and resolution was low. Figure 79 shows the best TLC/SALDI spectrum of bradykinin obtained by this approach. However, such spectra were not obtained consistently. Furthermore, by puncturing holes the TLC plate was destroyed.

There are important similarities between SALDI and FAB. SALDI solvents and FAB

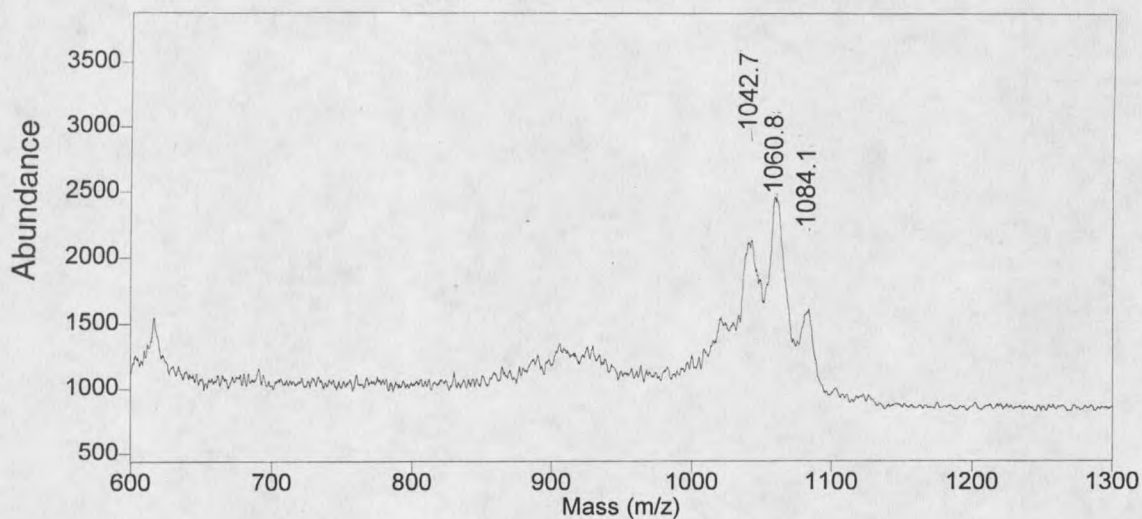


Figure 78. SALDI mass spectrum of bradykinin (1 nmole, mw= 1,060.5) on TLC plate. The analyte was extracted from silica gel, and SALDI suspension "A" was added. LP=415. SA=129.

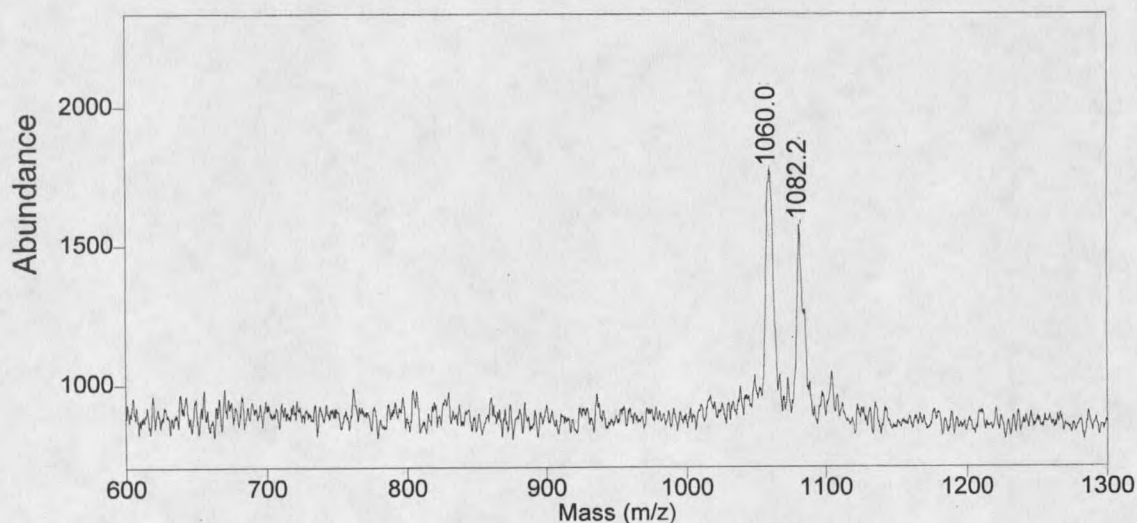


Figure 79. TLC/SALDI mass spectrum of bradykinin (1 nmole, mw= 1,060.5). The analyte was eluted onto the TLC surface by 70% EtOH/H₂O. SALDI suspension "A" was added. LP= 425. SA= 29.

matrices are typically the same compounds. Indeed, the sample preparation in SALDI can be quite similar to that used in FAB. As in the TLC/FAB experiments, the SALDI solvent such as glycerol can be used as extraction solvent instead of a high volatile organic solvent. The matrices used in FAB and SALDI have a high viscosity, so that the diffusion rate is expected to be much slower than in organic solvents. The extraction liquid is also used as the SALDI solvent, so its pressure will not adversely affect the SALDI results. The sample preparation consisted of adding 0.50 μL of a SALDI solvent like glycerol onto the sample spot immediately followed by 0.50 μL of SALDI suspension "A". Analytes are free to diffuse into the SALDI solvent and re-adsorb on the activated carbon powder. In a series of experiments, the analyte solution was spotted onto the TLC plate, but the plate was not developed. The sample spot was then dried using a hot air gun, and the SALDI solvent and suspension solution applied as described above. Figure 80 shows the spectrum of angiotensin II obtained by this procedure. It is seen that the mass resolution is improved and the intensity is much higher than with volatile organic solvent extraction. Moreover, the spectra were easy to obtain and reproduce.

It is seen in the top spectrum in Figure 80 that the angiotensin II sodium adduct ions dominate the mass spectrum (SALDI suspension A was used, see experimental section). The abundance of sodium adduct ions depends on the amount of glycerol. With a low glycerol to activated carbon ratio, the sodium adduct ions likely will dominate the mass spectrum. This phenomenon was previously observed in the development of SALDI and is described in Chapter 3. In order to decrease the intensity of sodium adducts, a higher ratio of glycerol to activated carbon should be used. The bottom of Figure 80 shows an improved mass spectrum

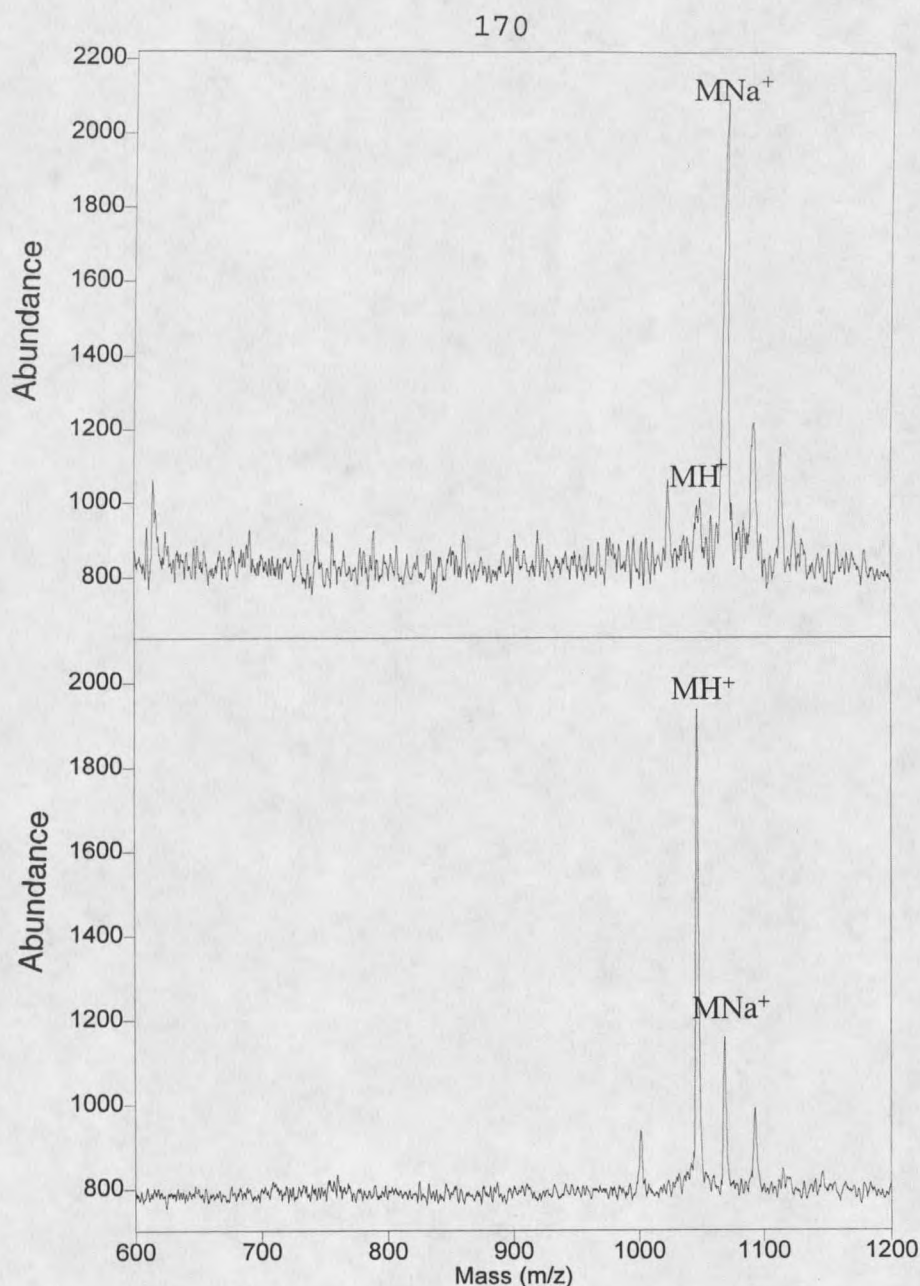


Figure 80. TLC/SALDI mass spectra of angiotensin II (0.5 nmole). Angiotensin II was extracted from the silica gel by adding 0.50 μ L of glycerol and subsequently adding 0.50 μ L of SALDI suspension A (Top spectrum, LP= 445, SA= 12). One microliter of glycerol and 0.5 μ L of SALDI suspension B were added to the sample spot (Bottom spectrum, LP= 445, SA= 49).

of angiotensin II. In this case, the TLC plate was developed. Half a microliter of glycerol was used for extraction. The more dilute SALDI suspension "B" (see Experimental) was used. This spectrum shows that the intensity of sodium adduct ions is now much lower.

The glycerol extraction method used above still suffered from some problems. When the SALDI solution was added onto the glycerol on the sample spot, most of the activated carbon was rapidly spread out to the edge of the original glycerol droplet. Activated carbon is necessary for the SALDI process. Therefore, the analyte signal could only be obtained along the edge of the activated carbon powder circle. Also, if the layer of activated carbon powder was too thick, no analyte signal was observed. Too much activated carbon material might have inhibited analyte diffusion from the silica gel to the surface. The uneven distribution of carbon powder made it difficult to find a spot to obtain a good mass spectrum. A modified approach was developed to solve the problem of uneven carbon powder distribution. A more diluted SALDI solution, 0.1 μ L of SALDI suspension "C" (see Experimental), was added to the sample spot followed by glycerol liquid matrix. In this way, a more even distribution of activated carbon powder was obtained, and it was easier to obtain analyte spectra. Mass spectra of bradykinin (top) and angiotensin II (bottom) are shown in Figure 81.

Experiments were performed to find the optimum activated carbon concentration in the SALDI matrix system. Angiotensin II mass spectra obtained with different ratio of solutions "1" and "2" as the SALDI suspension are shown in Figure 82. Solution "1" is composed of equal volumes of activated carbon powder and 50% glycerol in methanol solution. Solution "2" is 5.0% (w/w) sucrose in water. The mass spectrum obtained with a ratio 1/8 of "1" to

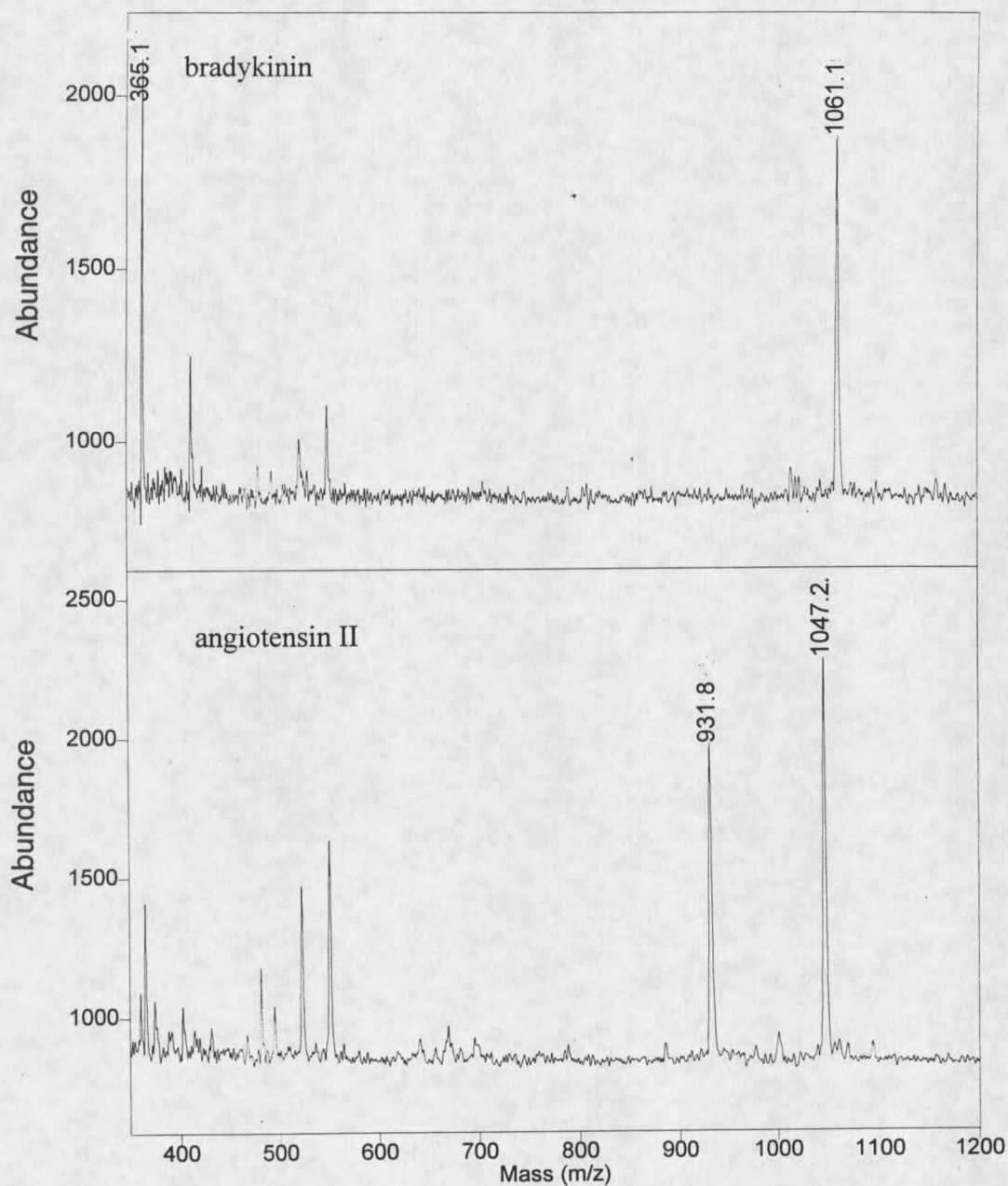


Figure 81. SALDI mass spectra of bradykinin (2.5 nmole, mw= 1,060.5) (top, LP= 425, SA= 37) and SALDI mass spectrum of angiotensin II (0.5 nmole, mw= 1,046.2) (bottom, LP= 450, SA= 43). SALDI suspension C (0.5 μ L) was first applied to the sample spot, and then 0.5 μ L of glycerol was added.

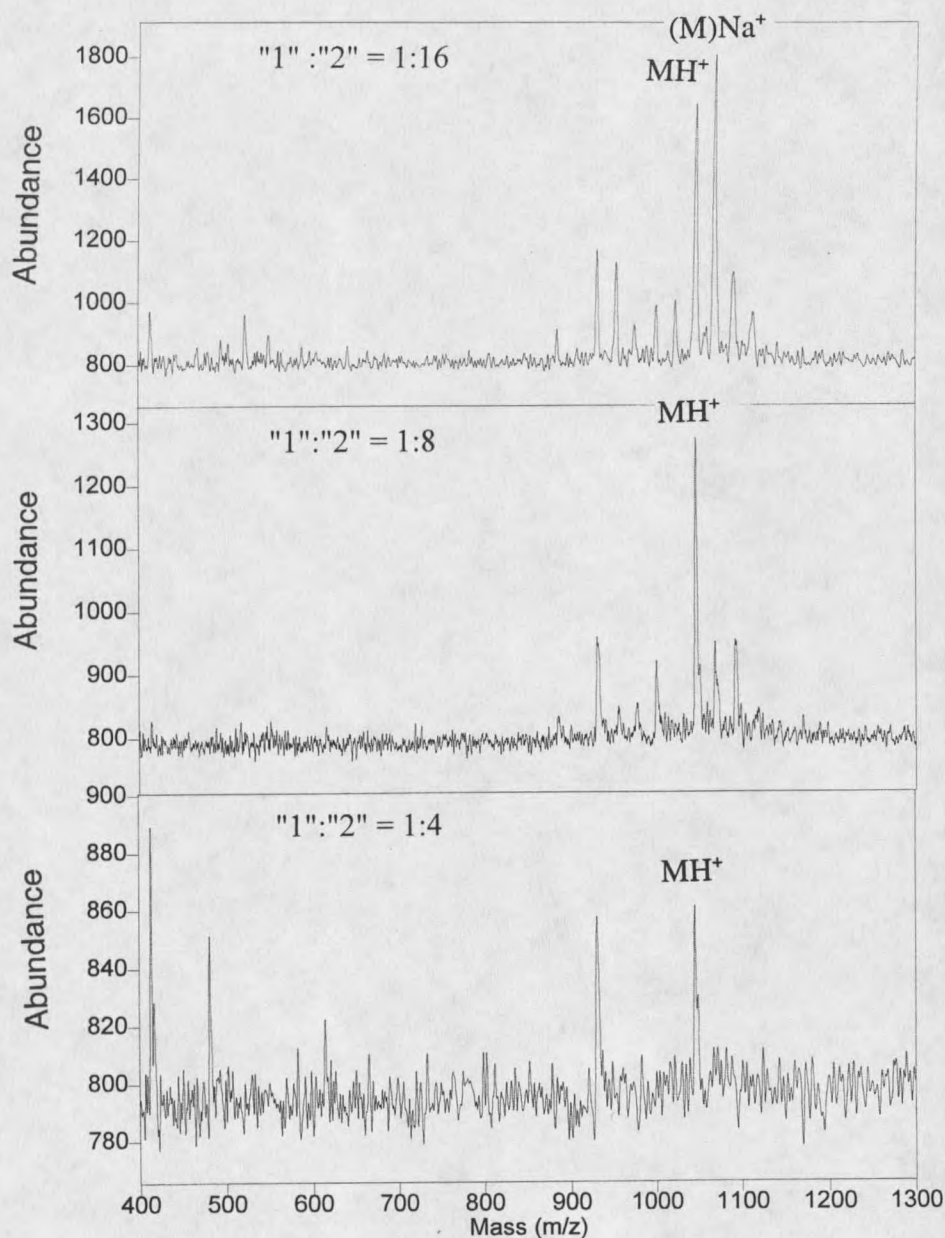


Figure 82. SALDI mass spectra of angiotensin II (0.5 nmole, $mw = 1,046.2$) on TLC plate by adding different ratios of "1" and "2" solution. "1" solution was made by mixing 25 mg of activated carbon powder with 50% glycerol/methanol. Solution "2" was made by 5% sucrose/water. (top, $LP = 450$, $SA = 43$; middle, $LP = 570$, $SA = 19$; $LP = 530$, $SA = 69$).

"2" shows the best result among the three spectra in Figure 82. The intensity of angiotensin II sodium ion adduct is decreased when the ratio of solution "1" to "2" is increased. Thus, the 1/8 ratio of solution "1" to "2" shows a fair mass resolution with a minimum of sodium adduct ion of analyte.

Figure 83 shows the mass spectra of angiotensin II without (top) and with (bottom) spraying ninhydrin on the TLC plate. The peptide was developed on the TLC plate and analyzed using the same approach as used for Figure 81. The protonated angiotensin II is intense, and few sodium adduct ions show up in the top mass spectrum. However, when the angiotensin II TLC spot was sprayed with ninhydrin for visualization and then analyzed by SALDI, the protonated angiotensin II ions were practically absent, and several new ion peaks appeared (bottom spectrum in Figure 83).

Liquid matrices other than glycerol were used to extract the peptides, and mass spectra were similar to those obtained using glycerol. For example, Figure 84 shows an angiotensin II mass spectrum for which Dithiothreitol was used as the extraction solvent. Dithiothreitol also has high viscosity and low vapor pressure. More abundant sodium adduct ions of the peptide were obtained than when using glycerol. With the same sample preparation, it seems that glycerol has a higher tolerance for sodium ions than other liquid matrices. Busch and DiDonato reported that the matrix threitol can be used to reduce the lateral spreading of analytes in TLC/FAB (DiDonato & Busch, 1986). The authors also reported that threitol had a higher extraction efficiency for analytes from the silica gel. In this study, this matrix was used as the extraction solvent in TLC and as the liquid matrix in SALDI. Threitol is solid at

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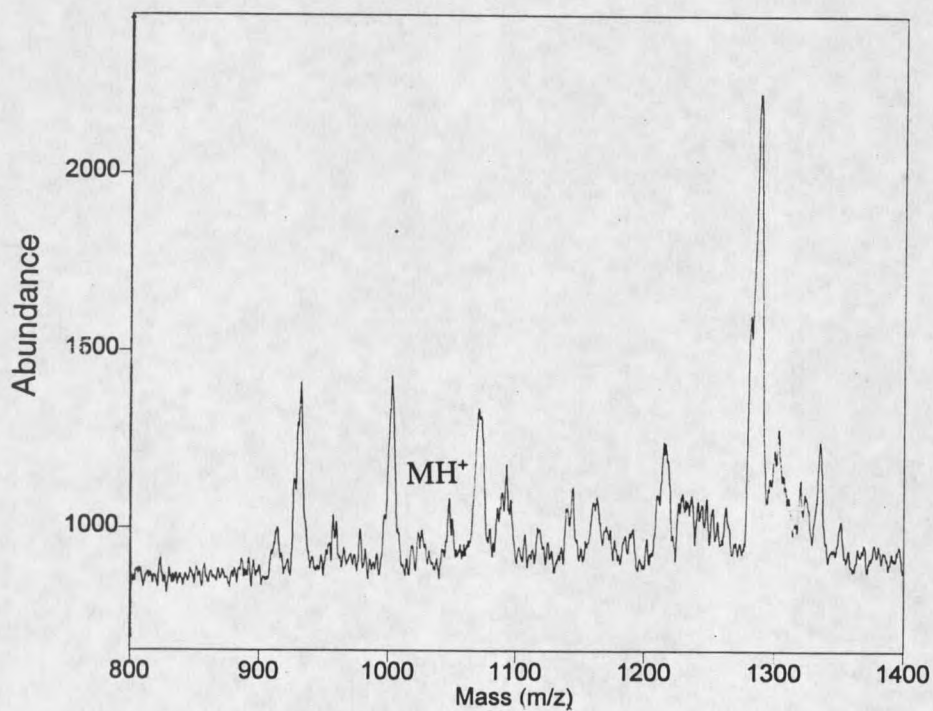
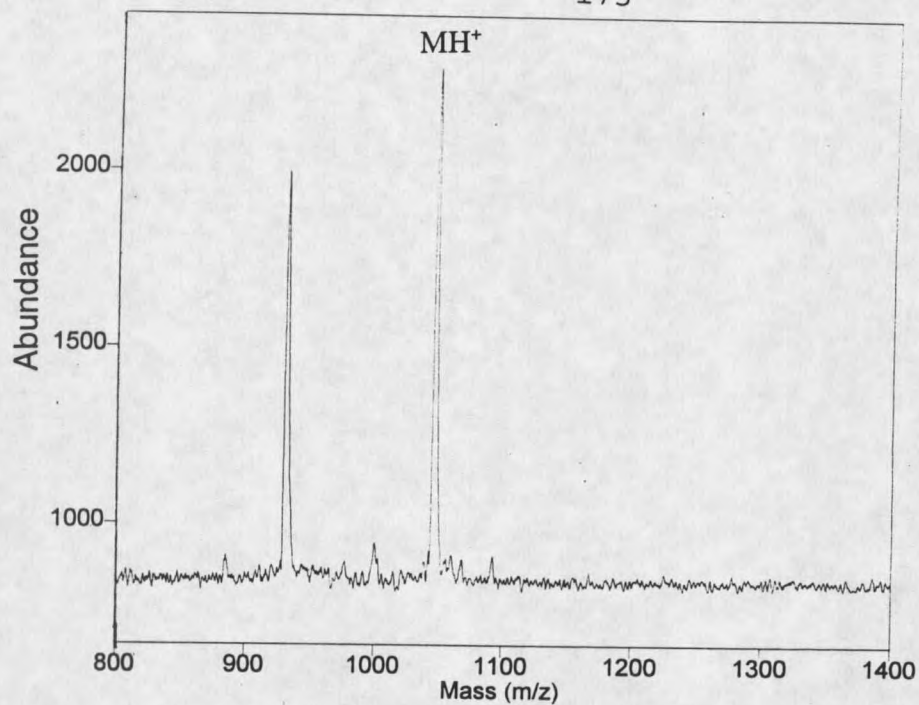


Figure 83. SALDI mass spectra of angiotensin II (0.5 nmole, mw= 1,046.2) on TLC plate without ninhydrin (top) and with ninhydrin (bottom).

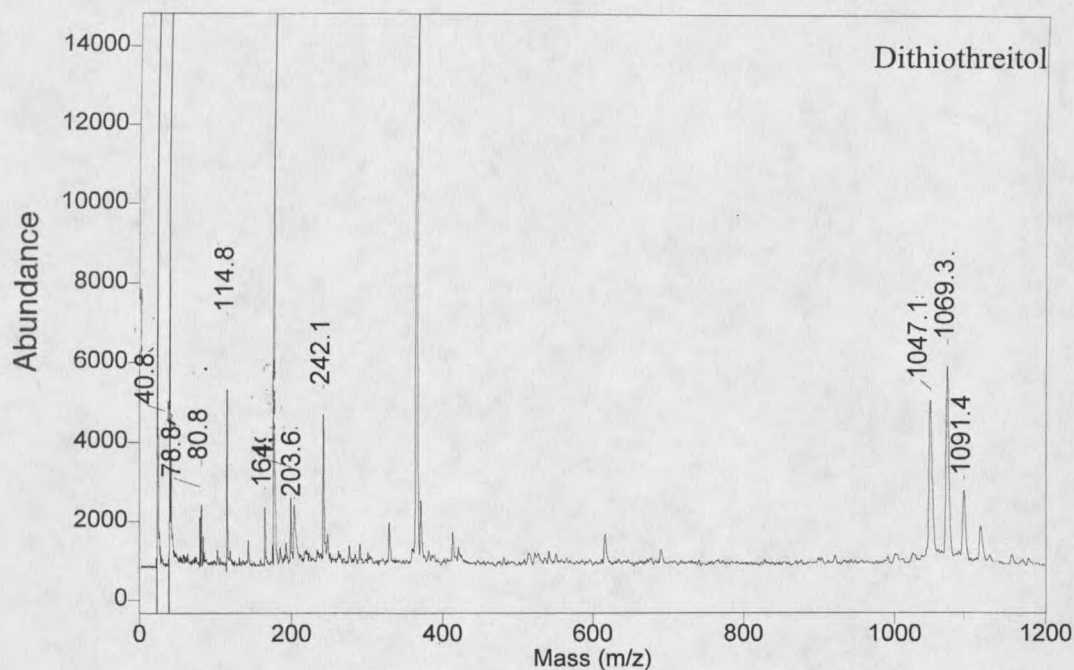


Figure 84. SALDI mass spectrum of angiotensin II (0.5 nmole, mw= 1,046.2) on TLC plate. Dithiothreitol was the extraction solvent. LP= 405. SA= 25.

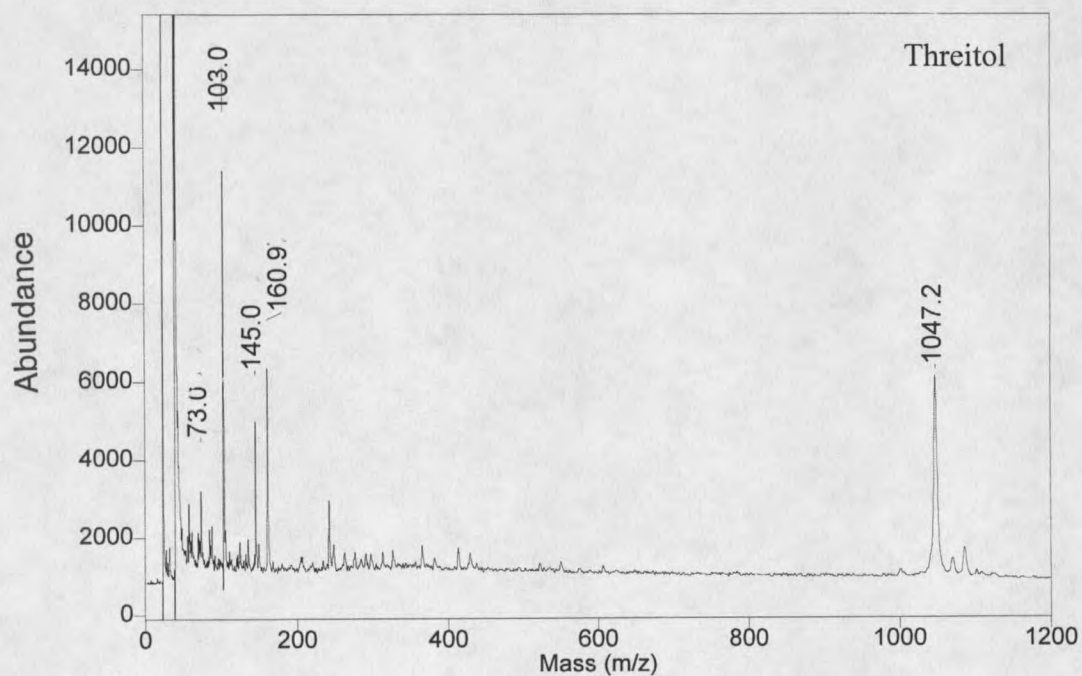


Figure 85. SALDI mass spectrum of angiotensin II (0.5 nmole, mw= 1,046.2) on silica gel plastic-backed TLC plate. Threitol was the extraction solution. LP= 405. SA= 73.

room temperature, and it was heated to a liquid before being used as the extraction solvent. The sample preparation was the same as that used for the spectra in Figure 81 except that threitol was used instead of glycerol. The angiotensin II TLC/SALDI mass spectrum is shown in Figure 85. The mass spectrum is seen to be similar to that obtained when using glycerol extraction solvent. The intensity of the sodium adduct ion of angiotensin II is low, and the intensity of protonated angiotensin II ions is similar to that observed in the glycerol spectrum. There is no evidence that threitol has a higher extraction efficiency than glycerol for this analyte. Since both matrices give similar intensity peaks, this may be due to the fact that they have similar elution strengths toward peptides (Harris, 1991). In order to further understand what determines the extraction efficiency in TLC/SALDI, the matrix viscosity was varied by adding different amounts of sucrose to glycerol. Figure 86 shows a plot of the intensity of the protonated angiotensin II ion peak versus the concentration of sucrose in glycerol. It is seen that when the concentration of sucrose was increased from 0% to 12.5%, the intensity of the analyte signal was increased. However, when the concentration of sucrose in glycerol was increased further to 50%, the analyte signal decreased to a very low level. This demonstrates that using a matrix with a correct viscosity can help to improve the extraction efficiency because the analyte diffusion rate along the TLC plate is lower. However, too high a viscosity apparently also limited the extraction efficiency. This may be due to a slow diffusion rate of analyte from the silica gel to the TLC surface.

Another factor that affects the TLC/SALDI results is the vapor pressure. Thioglycerol is a known FAB solvent. Figure 87 shows the angiotensin II TLC/SALDI mass spectrum

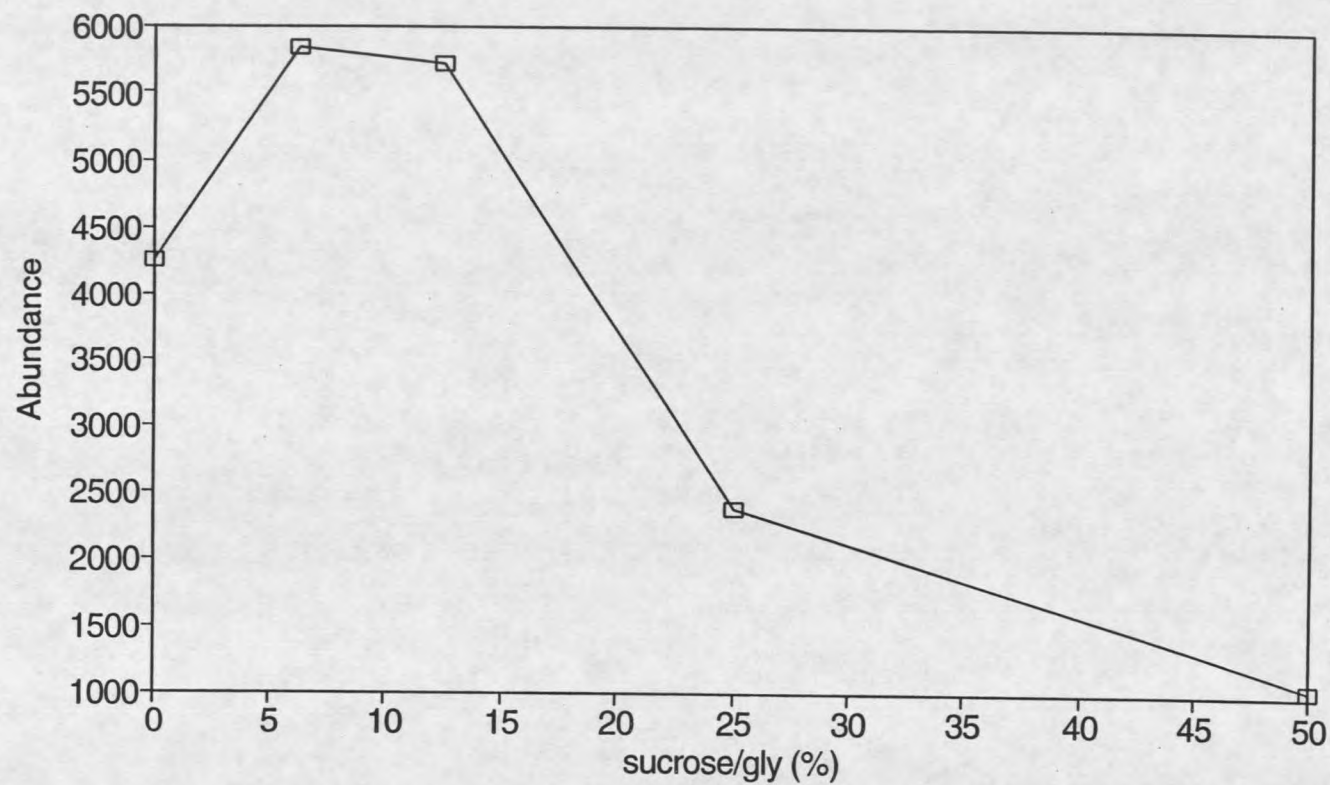


Figure 86. The intensity changes of bradykinin pseudo-molecular ions in TLC/SALDI versus the different concentrations of sucrose as the extraction solvent.

obtained with thioglycerol as the combined extraction and SALDI solvent. The intensity of the protonated angiotensin II ion peak is low, but abundant sodium adduct are observed. Thioglycerol has a significantly higher vapor pressure than either glycerol or threitol. This might be the reason for the weak protonated angiotensin II signal. When the TLC plate was put into the ion source chamber, most of the thioglycerol evaporated, and little liquid solution was left on the TLC plate. Thus, most of the analytes were possibly unable to diffuse to the surface. This is supported by the previous study which showed that if there was not enough SALDI solvent present on the sample, the intensity of alkali ion adducts increased in the mass spectra. It is clear that choosing a liquid matrix in TLC/SALDI with the proper viscosity and vapor pressure is critical to obtain the best possible mass spectra. Glycerol and threitol seem near-ideal in TLC/SALDI analysis.

In the TLC experiments described so far, analyte solutions were spotted onto the TLC plate. Next, SALDI spectra from actual TLC separation experiments will be shown. A solution of bradykinin and arginine was separated on the TLC plate, see Experimental. Ninhydrin was used for visualization. The TLC plate was cut and squares approximately 3 x 3 mm large containing the sample spots were used for subsequent procedures. SALDI suspension "C" was applied to each square followed by pure glycerol, Figure 88 shows the TLC/SALDI mass spectrum of TLC separated arginine. The bradykinin mass spectrum is similar to the mass spectrum in Figure 81.

The TLC/SALDI method is useful not only for biochemicals, such as peptides or amino acids, but also for organic analytes. To illustrate the application of TLC/SALDI to organics, a hydrochlorothiazide (mw= 297.72) solution was separated on the TLC plate by developing

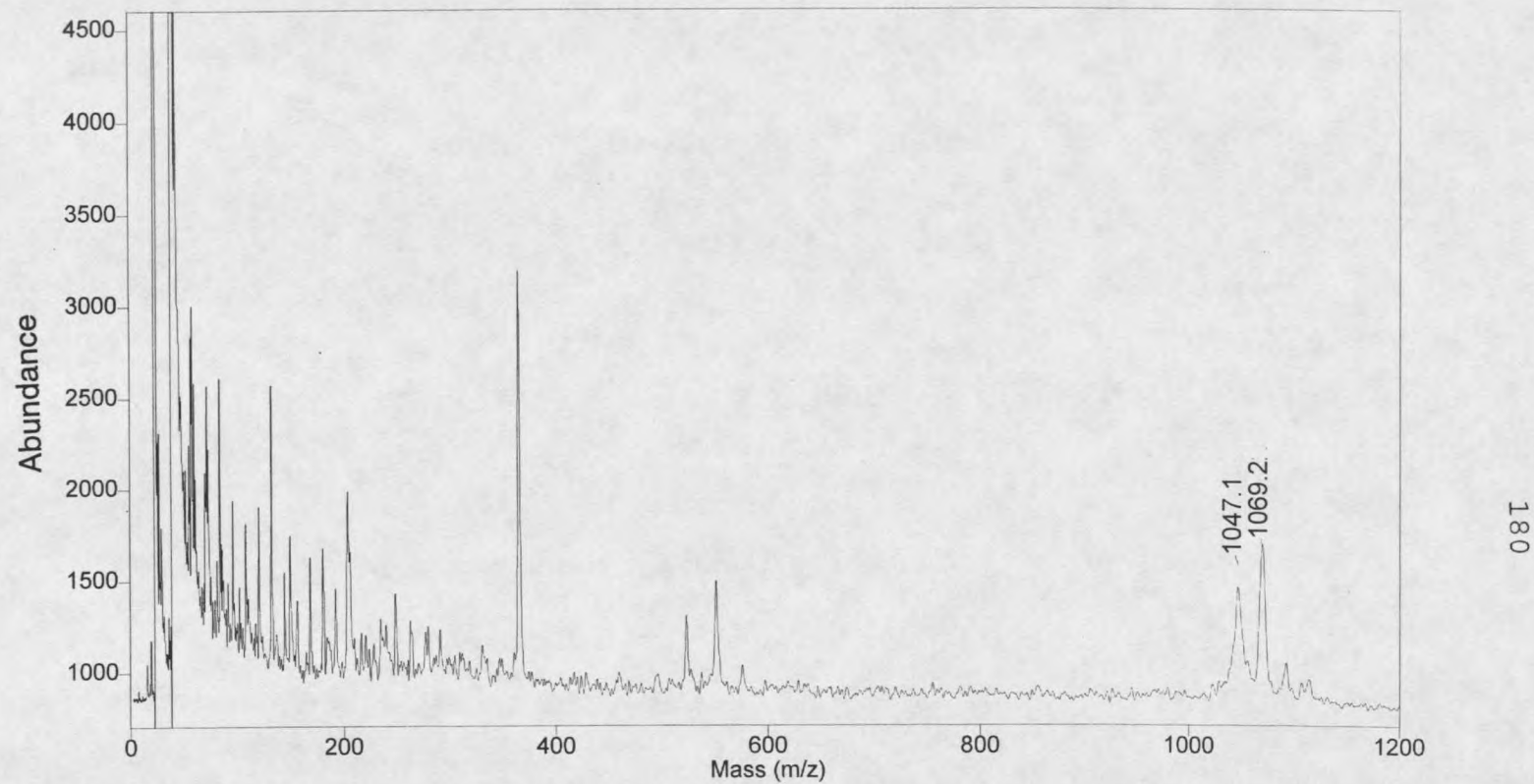


Figure 87. SALDI mass spectrum of angiotensin II (0.5 nmole, mw= 1,046.2) on silica gel plastic-backed TLC plate. Thioglycerol was the extraction solution. LP= 370. SA= 43.

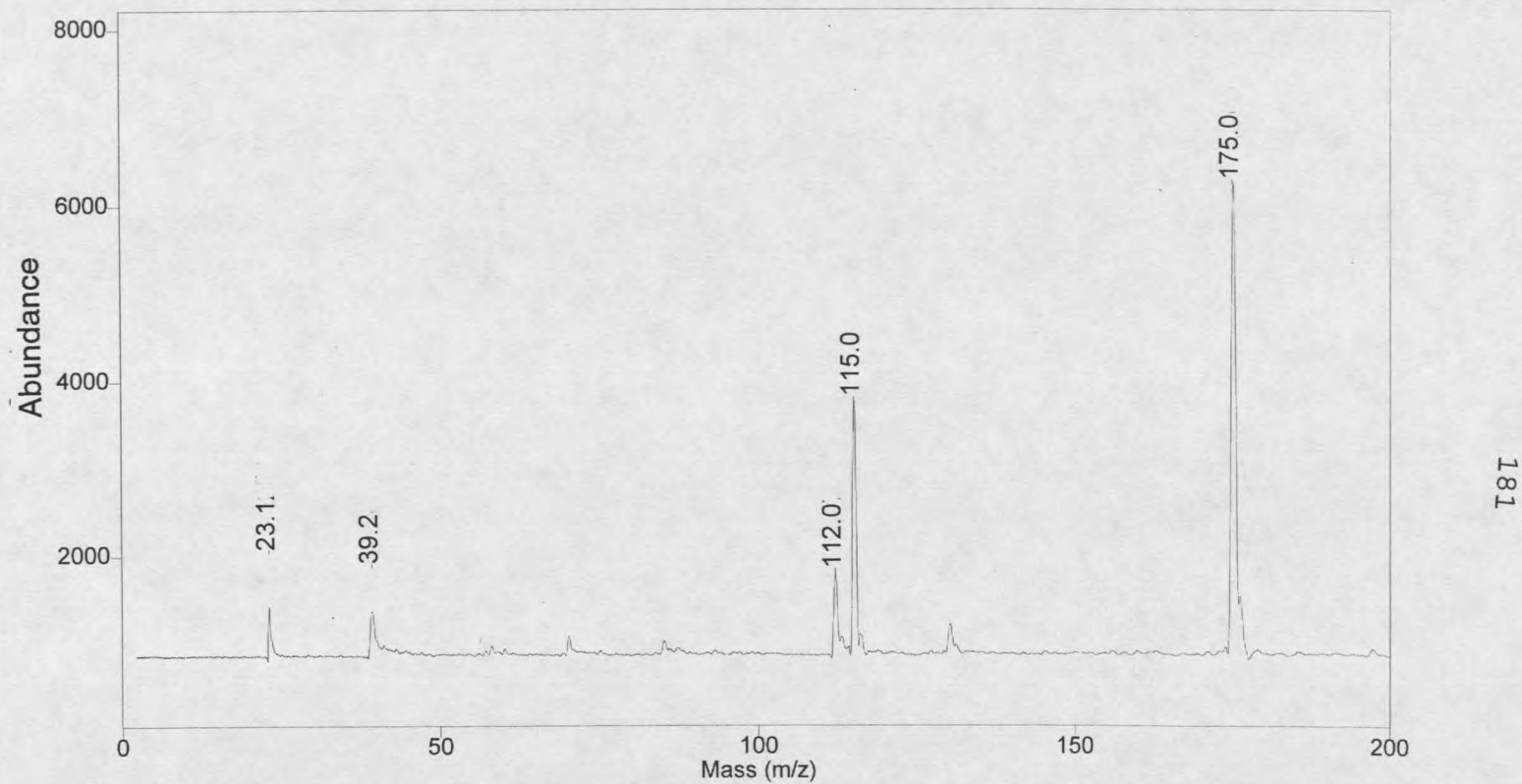


Figure 88. SALDI mass spectrum of arginine (100 nmole, mw= 174.1) on silica gel plastic-backed TLC plate. Glycerol was the extraction solution. LP= 425. SA= 197.

with a methanol/acetone (1/1, v/v). The R_f value for hydrochlorothiazide was 0.82. Figure 89 shows the resulting mass spectrum. Because of the low ion background in SALDI, this low molecular weight compound peak is easily observed. This spectrum was obtained in negative ion mode because hydrochlorothiazide is easily ionized by proton abstraction. The detection limit is in μg range.

In the experiments above, the silica gel remained intact on the TLC plate. This is not required. Instead, the silica powder may be scratched from the sample spot, mixed directly with a SALDI suspension, and analyzed by mass spectrometry. Results are shown for bradykinin and hydrochlorothiazide in Figure 90. Surprisingly, these spectra are not as good a quality as those obtained in direct analysis of intact TLC plates. Also, abundant sodium ion adducts are observed in the mass spectra.

An interesting observation was made when a pencil line happened to cross on the sample spot area and glycerol was added on the top of the line. SALDI mass spectra were obtained even though the amount of carbon material from the pencil line was very small and estimated to be about 1 μg . TLC/SALDI spectra of bradykinin and hydroflumethiazide obtained in this way are shown in Figure 91. Abundant sodium adducts of bradykinin are observed in the mass spectrum. The mass resolution is worse than with activated carbon. These results prove that already extremely small amounts of carbon powder are sufficient to obtain SALDI mass spectra.

In conclusion, sample preparation for TLC/SALDI is very easy compared to that for TLC/MALDI. TLC/SALDI seems near-ideal for peptide analysis. TLC/SALDI has a similar mass range to that of TLC/MALDI. However, in contrast to TLC/MALDI, the matrix

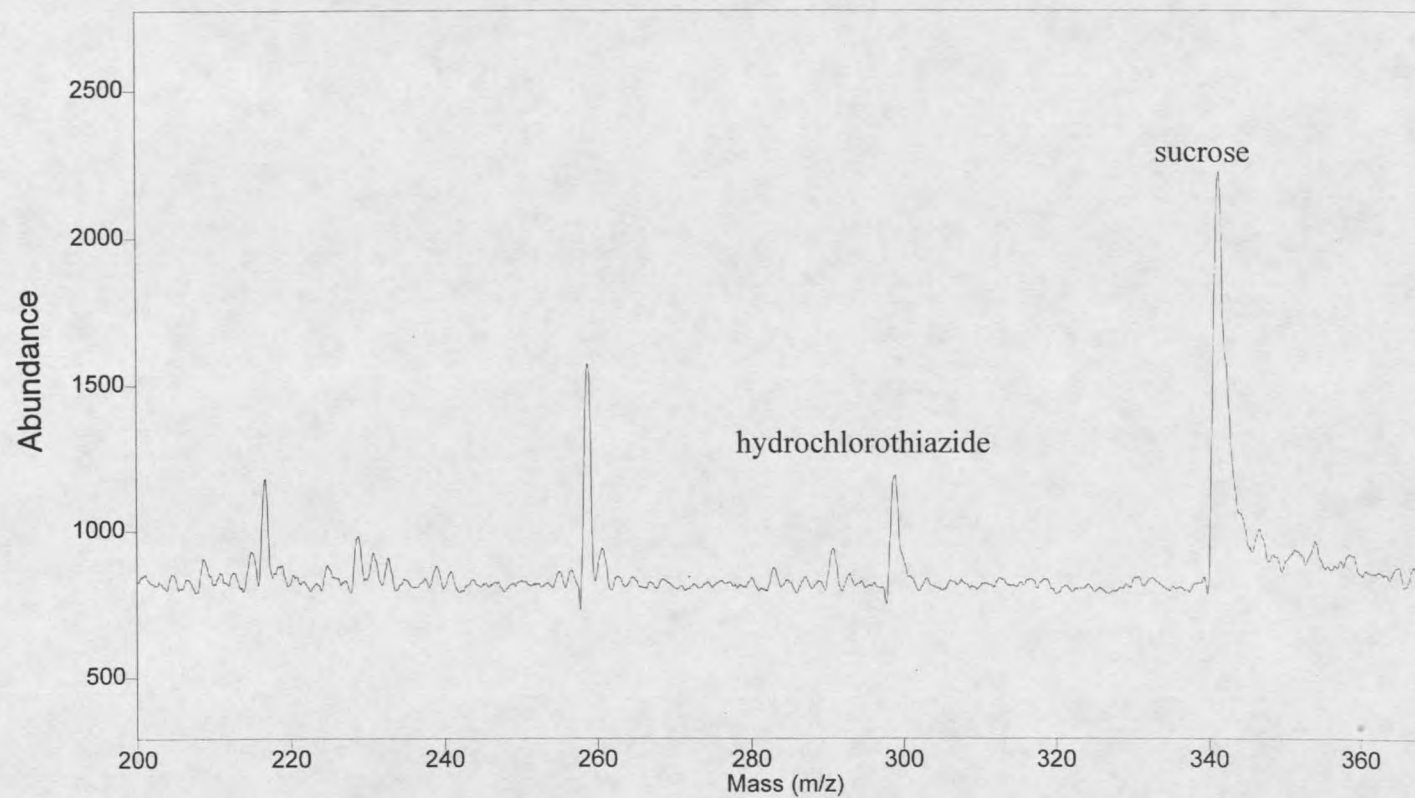


Figure 89. SALDI mass spectrum of hydrochlorothiazide (1 μ g, mw= 297.72) on TLC plate (negative ion mode). LP= 490. SA= 91.

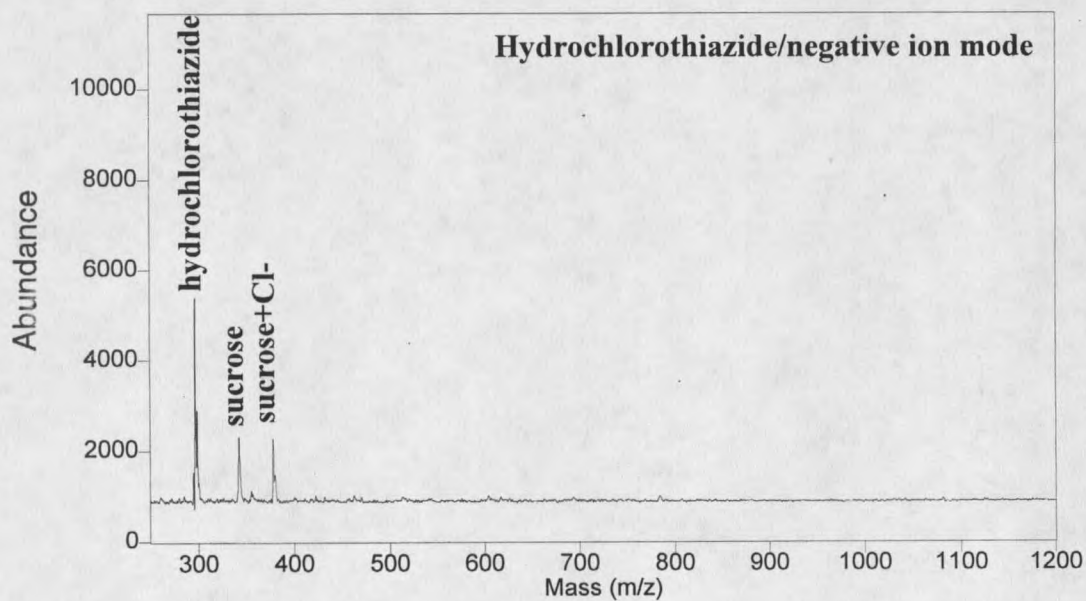
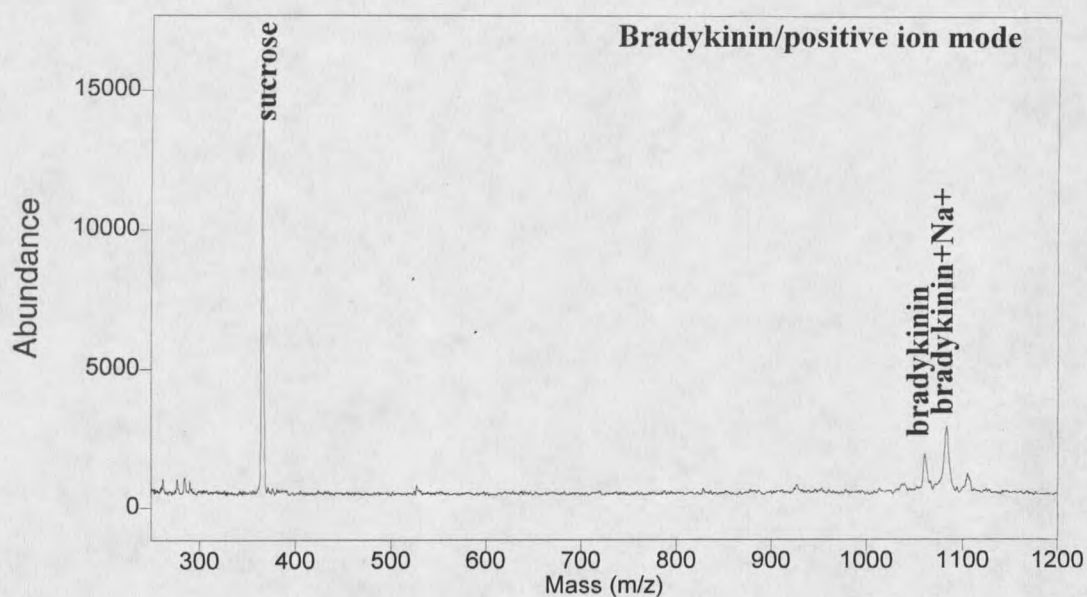


Figure 90. SALDI mass spectra of bradykinin (1 nmole, mw= 1,060.5) and hydrochlorothiazide (10 μ g, mw= 297.72) on TLC plate scratched from the TLC plate and mixed with SALDI suspension. LP= 515. SA= 51.

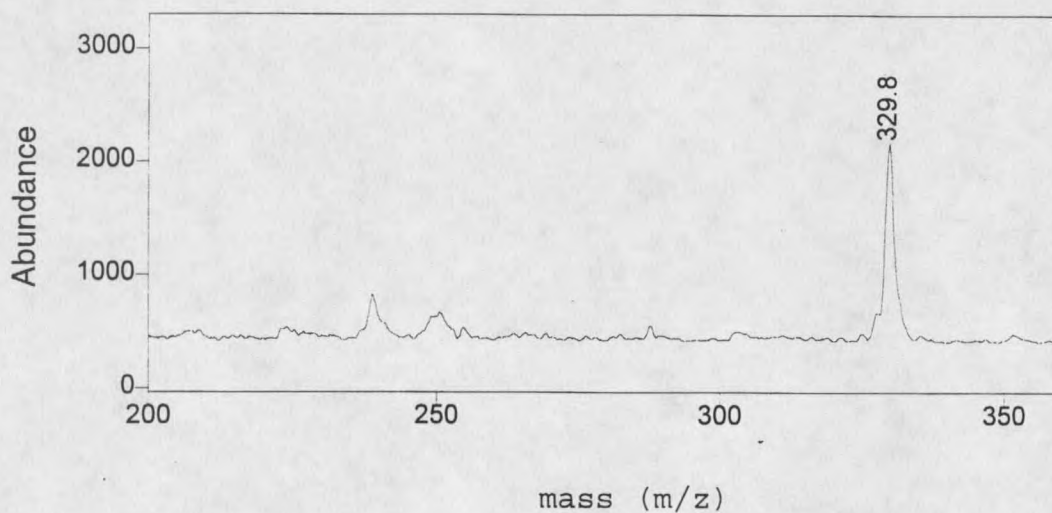
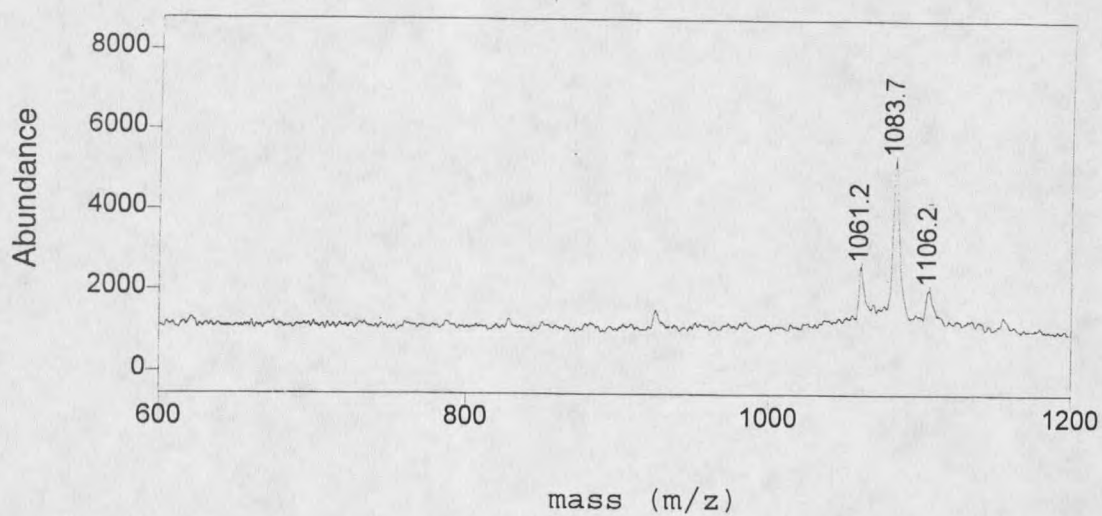


Figure 91 SALDI mass spectra of bradykinin (0.5 ng, mw= 1,060.5, LP= 455. SA= 18) and hydroflumethiazide (LP= 515, SA= 51) on silica gel plastic-backed plate. Activated carbon was added by drawing a line with a pencil over the sample spot.

background is very low. Therefore, it is very easy to identify low molecular weight compounds. Imaging of TLC by SALDI should be possible if a high capacity vacuum pump were connected to the ion source. The best sensitivity for bradykinin and angiotensin II was 5 ng of peptide separated on the TLC plate. The amount of material actually used was much lower than this value since the diameter of a sample spot is about 2 mm and only a laser focus spot (10 μ m) diameter is needed to obtain spectra.

CHAPTER 8

CONCLUSIONS

In this chapter, the major results and conclusions of this thesis are discussed. In addition, future prospects of SALDI are described.

Summaries of Results and Conclusions

Development of SALDI

The major part of this thesis is the development of a new laser desorption ionization method, SALDI. The unique characteristics of SALDI are: (1) low matrix background ions are observed in the low mass range (2) easy sample preparation. (3) excellent spectra from liquid solution. Instead of using MALDI matrices in laser desorption, a SALDI suspension is used. The SALDI suspension is composed of three major parts: (1) SALDI solid (2) SALDI solvent (3) SALDI "adhesive". The SALDI solid plays the role of energy transfer medium that provides enough energy for the desorption ionization of analytes. Graphite and activated carbon powder are the most common SALDI solids. Analytes are dissolved in the SALDI solvent for SALDI analysis. Presumably, the SALDI solvent also acts as a cooling medium that prevents too much energy input into analyte molecules. Without adding SALDI solvent to SALDI suspension, poor intensity and low mass resolution spectra of analytes were obtained. Furthermore, it seems that the SALDI solvent is the major proton donor according

to the study of the importance of the gas phase basicity of SALDI solvent. Glycerol can apparently be used as a universal SALDI solvent. It works well for both polar and nonpolar analytes. SALDI "adhesive" is added to make particles adhere to each other. To reduce contamination problems caused by the SALDI solid spreading in the ion source of the mass spectrometry, sucrose was used in this work as the SALDI "adhesive". A characteristic of SALDI mass spectra is that alkali cation adduct ions of analytes can be easily observed. However, the alkali adduct ions can be reduced by controlling the amount of SALDI solvent in the SALDI suspension. SALDI can also improve the drawback of a high matrix background in MALDI. This is due to low extent of fragmentation of SALDI solids and to the fact that non-UV absorbing SALDI solvents were used. Furthermore, analytes are directly mixed with the SALDI suspension without crystallization requirements. This eliminates the problems caused in MALDI by the requirement of co-crystallization such as suppression effects in the crystals. The mass resolution of SALDI is about 300. It is almost identical with that of MALDI. The sensitivity of peptides such as bradykinin and angiotensin II in SALDI can be as low as 10 fmole. This is also competitive with MALDI. The SALDI mass range is less than 20,000 Da which is considerably lower than MALDI. The desorption ionization process in SALDI is harder than that in MALDI according to the mass spectra of adenosine in PSD mode.

SALDI Solvent Effects

The gas phase basicity of the SALDI solvent is a significant factor in determining the intensities of protonated analyte ions for small molecular weight molecules like amino acids. Asparagine in different gas phase basicities SALDI solvents was studied. The intensity of

protonated asparagine ion peak was decreased with increasing gas phase basicities of the SALDI solvents. This is due to proton transfer from the lower gas phase basicity analyte molecules to higher gas phase basicity solvent molecules. However, no gas phase basicity effect was observed for peptides. For larger size molecular weight analyte like cytochrome C, increasing gas phase basicities of the SALDI solvents had an effect on the multiple charged distribution.

The effects of adding salts in SALDI were studied. Adding a high concentration SDS did not change the mass resolution of analyte molecules. However, more abundant sodium adduct ions of analytes were observed. However, the mass resolution was poor with extra NaCl added to the analyte solution. This shows that SALDI can tolerate substantial SDS, but little NaCl.

Acids were added to the SALDI solvent, acetic acid 0.3% in SALDI solvent did not change the mass spectra much, except that more abundant alkali adduct ions of analyte molecule ions might be observed. However, when the concentration of acetic acid in SALDI solvent was increased to 2.5%, the intensity of protonated analyte ions was decreased. For an analyte like angiotensin II, more abundant alkali adduct ions of analyte molecules were observed with increasing acid concentration.

Applications of SALDI

Because of the low ion background interference and negligible SALDI suspension effects, SALDI is especially useful for the analysis of enzymatic digest products. Almost every peptide in a mixture sample could be observed in a same SALDI mass spectrum. Tryptic digest products and CPY digest products have been used to demonstrate these advantages.

SALDI is very good analysis method for analytes with the size of peptides.

SALDI also can be used to analyze polymers. Because of the low proton affinity and the high alkali ion affinity of many polymers, the alkali adduct ions of polymers may be the dominant ions observed in the mass spectra. SALDI solids, such as graphite and activated carbon, have an abundant source of alkali ions on the surface. Thus, no extra alkali salts were needed to add to the SALDI suspension. For nonpolar polymers like polystyrene, it is necessary to add transition metal ions like Ag^+ and Cu^{2+} to the SALDI suspension. These non-polar polymers have higher affinities toward transitional metal ions. Otherwise, no ions of these polymers were observed in the mass spectra.

Activated carbon powder is one of the best SALDI solids. It has the characteristic of adsorbing trace organics on the surface. Thus, volatile organics can be trapped on the surface of activated carbon directly followed by SALDI analysis. Pyridine is the smallest volatile compound that has been observed in SALDI.

SPE/SALDI

SPE is a method for concentrating trace amounts of organics on an SPE adsorbent by eluting a large volume of dilute solution through the SPE adsorbent. Activated carbon can be used one of the SPE adsorbents. Activated carbon is also a SALDI solid. Thus, these two methods were combined without much compromise. SPE/SALDI has the advantages of being fast, simple, and convenient. To obtain the optimum result, elution flow rate, amount of adsorbent material, and method of packing needed to be optimized. SPE/SALDI is not only a qualitative method, but also a quantitative method. Trace organics like diuretics and herbicides in dilute solution have been analyzed by SPE/SALDI. The results were

promising. In addition, SPE/SALDI could be used in the analysis of biochemicals such as amino acid and peptides.

SPME has also been combined with SALDI. A small activated carbon pellet was used as the adsorbent. After the analyte had been adsorbed on the pellet, the pellet was ground to a fine powder and mixed with SALDI solvent and "adhesive" for SALDI analysis.

TLC/SALDI

Previous efforts on the development of TLC/MS were reviewed in Chapter 7. The development of new technique of TLC/SALDI was described. TLC/SALDI has the advantages: (1) easy sample preparation, (2) high reproducibility, (3) ideal for low molecular weight analysis due to low matrix background and soft ionization. TLC/SALDI has the potential to become a routine analysis technique. Peptides, amino acids, and organics have all been successfully used as the analytes in this technique.

Future Prospects of SALDI

One of the important future goals in SALDI is the development of a liquid chromatography (LC) system combined with SALDI. Since a liquid phase suspension is used in SALDI, combining SALDI with LC does not have the problem that occurs with the solid MALDI matrices. If LC-SALDI can be successfully developed, it may become useful for biological and biochemical analytes such as in biological fluid system.

Although many models for laser desorption have been proposed, the desorption ionization process is still not well understood. Because liquid suspension is used in SALDI, there is an opportunity to understand the process. For example, mass spectra obtained with different

phases of SALDI suspension, liquid phase and solid phase (by freezing the suspension) can be compared. Such studies would be helpful for further understanding the laser desorption process.

The weakness of SALDI compared with MALDI is the mass range. This is probably due to the inefficient desorption process so that intact large analyte molecules cannot be desorbed before the occurrence of fragmentation. Thus, if the desorption process can be improved, the mass range in SALDI can be extended. A more efficient SALDI solid should be searched for solving the problem.

It has been proven that SALDI "adhesive" can reduce the contamination caused by that SALDI suspension. However, the ideal would be to completely get rid of the problem. Thus, a new method for depositing the SALDI solid into the SALDI suspension should be developed. A new SALDI solid that has the function of activated carbon, but no contamination problem is another solution for this problem.

To conclude, SALDI is a newly developed method. However, from the discussion above, it has a potential to be near-ideal LC /MS system. Furthermore, the mechanistic studies in SALDI will be helpful to understand the laser desorption ionization process. However, additional studies are needed before SALDI can become an accepted and established technique.

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