



Surface substrates for high performance laser desorption ionization mass spectrometry and their interfacing to chromatography
by Mei Han

A thesis submitted in partial fulfillment of the requirements of the degree of Master of Science in Chemistry
Montana State University
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Abstract:

Surface substrates for high sensitivity Surface-Assisted Laser Desorption Ionization (SALDI) mass spectrometry has been developed. This new technique uses an immobilized thin layer of activated carbon (AC) particles. The use of these substrates for the interfacing to chromatography was demonstrated.

A mass resolution of 1400 (m/A_m) for crystal violet in reflecting mode time-of-flight (TOF) mass spectrometry and more than 1000 for bradykinin in linear mode were obtained. The sensitivity was in pico- to femtomole range. The detection limit for crystal violet was less than 10 fmol and about 0.5 pmol for bradykinin, developed on a TLC plate. The ion source contamination problem for SALDI was solved. With the substrates developed here, sample preparation is extremely simple; analyte solutions are pipetted directly onto the substrate.

The application of AC substrates to the analysis of amino acids, peptides and organic compounds have been demonstrated. The combination of TLC with AC-SALDI substrates has been developed. The AC substrate can "store" chromatograms for more than six months. HPLC/SALDI using the AC substrates was also demonstrated.

SURFACE SUBSTRATES FOR HIGH PERFORMANCE LASER
DESORPTION IONIZATION MASS SPECTROMETRY AND THEIR
INTERFACING TO CHROMATOGRAPHY

By

Mei Han

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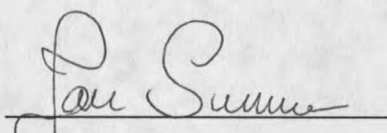
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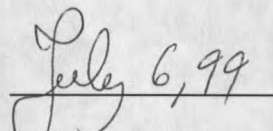
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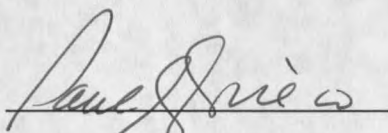
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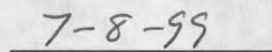

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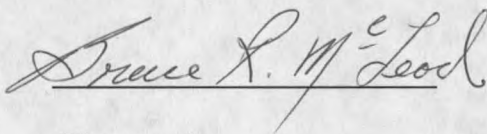
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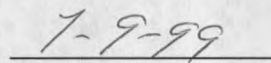

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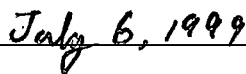
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ABSTRACT

Surface substrates for high sensitivity Surface-Assisted Laser Desorption Ionization (SALDI) mass spectrometry has been developed. This new technique uses an immobilized thin layer of activated carbon (AC) particles. The use of these substrates for the interfacing to chromatography was demonstrated.

A mass resolution of 1400 ($m/\Delta m$) for crystal violet in reflecting mode time-of-flight (TOF) mass spectrometry and more than 1000 for bradykinin in linear mode were obtained. The sensitivity was in pico- to femtomole range. The detection limit for crystal violet was less than 10 fmol and about 0.5 pmol for bradykinin, developed on a TLC plate. The ion source contamination problem in SALDI was solved. With the substrates developed here, sample preparation is extremely simple; analyte solutions are pipetted directly onto the substrate.

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CHAPTER 1

INTRODUCTION

General

Since 1907, when J.J. Thomson constructed the first mass spectrometer for determination of mass-to-charge ratios of ions (Watson et al., 1997), mass spectrometry has become an increasingly popular technique for providing information on the elemental composition of samples and on the structures of inorganic, organic and biological molecules. The first generation of mass spectrometers was dominantly single focusing magnetic sector instruments. With the development of high mass resolution double-focusing instruments, in which ions of the same mass are focused for both direction and velocity, the exact mass of compounds could be obtained, which was very useful for identification of organic compounds.

There are two main types of ion sources in mass spectrometry, gas phase ion sources and desorption ion sources (Busch et al., 1982). In a gas phase ion source, the analyte is first vaporized and then ionized, so it is necessary for compounds to be volatile and thermally stable. Both electron impact (EI) and chemical ionization (CI) are gas phase

ion sources (Busch et al., 1982). With the increasing requirement for analyzing large, involatile and thermal labile molecules from biochemistry, several desorption (also called desorption/ionization) methods were developed as a new branch of mass spectrometry. In these ionization methods, the molecules in a solid or liquid sample are directly converted to gaseous ions in the ion source. These methods greatly advanced mass spectrometry, making it capable of analyzing nonvolatile and thermally fragile molecules, such as polypeptides, proteins and other high-molecular-weight biomolecules.

Desorption ionization is a general term which includes field desorption (FD), plasma desorption (PD), secondary ion mass spectrometry (SIMS), laser desorption (LD), fast atom bombardment (FAB), electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) mass spectrometry. FD was the first desorption ionization method. It was developed in 1969 by Beckey et al. (Beckey et al., 1969). In this method, analytes are placed on a carbon or silicon-coated fine wire. After the wire is inserted into the sample compartment, ionization takes place by applying a high potential to the wire. In 1974, PD mass spectrometry was introduced by Macfarlane and Torgerson (Macfarlane et al., 1974). It uses very high energy fission fragments from ^{252}Cf to desorb and ionize molecules in solid-film samples. SIMS, which was developed during the middle 1970's, uses an energetic ion beam (usually several keV) to ionize molecules from the surface of a solid sample (McHugh et al., 1975). SIMS is widely used as a sensitive method of elemental analysis, capable of microscopic resolution and depth profiling (McHugh et al., 1975). LD mass spectrometry appeared in the late 1970's (Cotter et al., 1987). In this method, a high-energy laser beam is used to desorb

sample molecules from surfaces. The thermal degradation of analyte molecule is greater in this method than in other desorption methods. With the development of yet another kind of desorption method, FAB or "Liquid SIMS" in 1981 (Kerr et al., 1982), it become practical to obtain mass spectra of polar high-molecular-weight compounds. In FAB mass spectrometry, an energetic neutral atom beam is used to gently ionize compounds from the surface of a liquid matrix solution. The two "new comers" in the mass spectrometry family are ESI and MALDI. In ESI mass spectrometry, an analyte solution is pumped through a stainless steel hypodermic needle, which is held at a high electric potential. As the liquid flows out of the capillary end, it is dispersed by Coulomb forces into a very fine spray of charged droplets. With the evaporation of solvent, ions are desorbed and are then transported into the ion source. In MALDI mass spectrometry, a UV-absorbing material (referred to as a "matrix") is mixed with analyte to assist the desorption process and the mixture is irradiated with a pulsed laser. ESI and MALDI have made great contributions to the analysis of large involatile biomolecules.

Our research is based on a liquid/solid matrix assisted laser desorption ionization method. Therefore, a brief history of LD mass spectrometry and the development of liquid matrix desorption ionization methods will be reviewed first and then the instrumentation of mass spectrometry will be discussed.

History of Laser Desorption Mass Spectrometry (LDMS)

The first laser desorption mass spectrometry experiment was performed by Vastola et al. (Vastola et al. 1968) in late 1960's. In this method, a 100 mJ ruby laser with a pulse

width of 500 ns was employed to produce ions from organic salt samples. Ions in the range from 200 to 300 daltons were detected in a time-of-flight mass spectrometer. In the late 1970's and early 1980's, with the development of the "microprobe", small organic molecules were analyzed by this method (Hillenkamp et al., 1975; Hercules et al., 1982 & 1983). Posthumus et al. (Posthumus et al., 1978) demonstrated in 1978 that pulsed laser induced thermal desorption ionization mass spectrometry could analyze thermally labile compounds, such as oligosaccharides, glycosides, amino acids, peptides, steroids and antibiotics. In the next few years, a number of biomolecules were detected by LD/MS. However, the upper mass limit was about 2,000 Da (Lindner et al., 1985). In 1988, Tanaka in his noteworthy paper claimed a breakthrough for detecting large molecules, as the maximum molecular weight was extended to over 30,000 Da (Tanaka et al., 1988). At the same time, MALDI was developed by Karas and Hillenkamp (Karas et al., 1988), who extended the molecular weight range from a few thousand to several hundred thousand daltons. In MALDI, a solid matrix was used, while in Tanaka's work, a liquid matrix was used to facilitate the desorption ionization process. The function of the matrix in both methods is to transfer energy from the laser beam to the analyte. This results in the sublimation of the analyte as ions into a time-of-flight mass spectrometer (Noble, 1995). A more detailed discussion about solid and liquid matrices will be found in the following section.

From Solid to Liquid Matrix and SALDI

MALDI-MS is a rapidly growing technique because of its unique advantages for analyzing biomolecules. In MALDI, a large excess of a solid radiation-absorbing material, called a matrix, is mixed with the analyte solution. The molar ratio of matrix to analyte ranges from 100:1 to 50,000:1 (Hillenkamp et al. 1991). Once the solvent evaporates, the matrix and analyte form a coprecipitate, which is introduced into the ion source. The matrix is believed to serve two functions: Transferring energy from the laser beam to the analyte and isolating the analyte molecules from each other. In order to get good mass spectra, the matrix molecules must have a high laser absorptivity (Hillenkamp et al., 1991). It is also critical that the matrix forms a fine homogenous co-crystalline solid as a result of solvent evaporation. The buffers, salts and surfactants, most commonly used in biological sample preparations, will often affect the crystal formation process and may cause low sensitivity (Shaler et al., 1996). The analyte is often unevenly distributed in the sample, and this causes "hot spots", where good quality mass spectra can be obtained. A lot of time is often required to search for these particular spots. If the sample contains low-volatility solvents such as glycerol, it is difficult to dry and will often fail to form crystals. A liquid matrix, would have advantages, however. It can dissolve analytes and allow them to diffuse to the sample surface to help replenish analyte between laser shots. It would not have the inhomogeneous cocrystallization problem. For these reasons, many efforts have been

made to use liquid matrices. The first group to use a liquid matrix was Tanaka and co-workers, as already mentioned (Tanaka et al., 1988). In their experiment, they suspended an ultrafine cobalt powder ($\phi = 30$ nm) in glycerol. Proteins and polymers with molecular weight up to 30 kDa were observed. However, the mass resolution was poor with $m/\Delta m$ about 20. Subsequently, there were some different methods used for liquid matrices. Zhao et al. (Zhao et al., 1991) used nitrobenzyl alcohol matrix (NBA) mixed with methanol and water. This was applied to a fibrous material substrate. They obtained signals from proteins, with a mass up to 97,000 Da. Cornett et al. (Cornett et al., 1992 & 1993) used a strongly absorbing organic compound, rhodamine 6G, dissolved in glycerol, to get signals from peptides and proteins when irradiating with 532 nm and 337 nm laser beams. Mass spectra of compounds over 250 kDa were obtained, but the mass resolution was less than the mass resolution obtained from solid matrix (Cornett et al., 1993).

Liquid matrices have also been used in IR-MALDI. Hillenkamp and co-workers (Overberg et al., 1990) used glycerol as a liquid matrix to obtain a spectrum from lysozyme (14 kDa). More recently they reported mass spectra of a range of proteins using glycerol as the matrix and obtained very high mass resolution (Berkenkamp et al., 1997).

In 1995, Sunner and co-workers (Sunner et al., 1995) used glycerol and triethanolamine as liquid matrices as in a new desorption ionization method. Graphite particles were added to glycerol and methanol, and analyte solution was subsequently added. High quality mass spectra were obtained by using a UV laser at 337 nm. They called this method "Graphite-Surface Assisted Laser Desorption Ionization" (Graphite-

SALDI). Using Sunner's method, Dale et al. (Dale et al., 1996) demonstrated the potential of the method. They applied their method to low molecular weight proteins, carbohydrates and polymers. The wide range of applications, high sensitivity (5-50 fmol) and a relatively simple sample preparation procedure made them believe that graphite SALDI (they used a different term, "graphite with liquid matrix") has great potential and can be used as a general procedure for intermediate weight molecules. In their latest paper, they applied this method to the analysis of varnishes used for artwork (Zumbuhl et al., 1998).

More recently, Sunner's group developed a method to couple TLC with SALDI (Chen et al., 1998). These methods will be discussed in more detail on Chapter 3.

Another variation of SALDI, cryo-IR-SALDI, has also been presented by the Sunner group (Kraft et al., 1998). In this method, analytes such as proteins and peptides were dissolved in a liquid solvent consisting of water and glycerol (33:67, w/w) at room temperature. A few μL of the solution was pipetted onto the probe tip. A small amount of an organic solid powder ("SALDI" solid) was then placed onto the liquid surface with a spatula, and the excess dry powder was blown off the sample. A suspension was formed and was cooled by immersing the probe tip in liquid nitrogen. Mass spectra of peptides and proteins were reliably obtained. The mass range was extended beyond 10^5 Da.

Overall, because of the homogeneity of the liquid matrices, they eliminate the problems associated with inhomogeneous crystallization in MALDI. Liquid matrices also have an evenly distribution over the sample which improves shot-to-shot

reproducibility, and much time is saved from searching for "hot spots". For these reasons, liquid matrix is gaining more attention.

A liquid matrix was used in our research. First, the instrumentation was reviewed in the following section.

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

There are presently many different kinds of mass spectrometers being used. However, they basically all have the same main components: inlet system, ion source, mass analyzer, detector, and data system. The ion path should be in a vacuum system that maintains a low pressure ($\leq 10^{-5}$ torr) in order to minimize ion/molecule collisions. The function of the inlet system is to introduce the sample to the mass spectrometer ion source. The ion source is the place where the ionization occurs by applying a certain amount of energy. The mass analyzer separates ions according to their mass-to-charge ratio. There are several different types of mass analyzers: magnetic sectors (single and double focussing), quadrupole, time-of-flight, ion trap and FT-ICR. The detector is an electronic device that converts the impact of the ion beam into an electrical signal (current or voltage). The signal is sent to the data system that measures, records, and analyzes data.

Among the mass analyzers, the time-of-flight (TOF) is currently gaining much attention. The time of flight mass spectrometer (TOF-MS) for practical use was originally described by Wiley and McLaren (Wiley et al., 1955). This mass analyzer has many advantages compared to the quadrupole and magnetic sector instruments, such as high ion transmission, ability to obtain a complete mass spectrum in a few microseconds, and a high scan speed. With pulsed laser desorption ionization methods,

time-of-flight MS can be used to analyze nonvolatile, thermal unstable and very large molecules such as proteins.

The following section will discuss the instrumentation of TOF-MS.

The Laser

In a TOF mass spectrometer, a pulsed laser is usually used as an energy source to desorb and ionize analyte molecules into the gas phase for mass spectrometric analysis. The heating rate of the sample induced by laser irradiation is high enough, about 10^8 - 10^{12} K/s (Levis et al., 1994), that labile biomolecules in a condensed phase to be desorbed before they decompose. Generation of ions with molecular weights as high as hundreds of thousands of dalton is possible. By using a pulsed laser in TOF mass spectrometry, ions can be emitted during a short time interval, 3-20 ns. All ions are emitted during the pulse.

There are a variety of lasers available with varying pulse lengths (continuous wave to fs), irradiance (50 kWcm^{-2} to 100 MWcm^{-2}) and wavelengths (151 nm to $10.6 \mu\text{m}$). Among them, UV and IR lasers are most commonly used for laser desorption. A nitrogen UV laser with a pulse length of 3 ns and wavelength for 337 nm was used in our system.

Basic Principles

A time-of-flight mass spectrometer separates and measures the masses of ions by the differences in transit time through a field-free drift region. The pulsed laser source produces the ions in a pulse, and an electric field is applied that extracts the ions and

accelerates them into the flight tube, which is a field free region. All ions will have received the same kinetic energy, zeV , after they have been accelerated out of the source, where z is the number of charges, e is the charge of an electron and V is the accelerating voltage. Then:

$$\frac{1}{2} mv^2 = zeV \quad (1)$$

In equation (1), m is the mass of the ion, and v is the velocity of the ion. Rearrange the equation to solve for v :

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

From equation (2), we can see that v is inversely proportional to square root of m/z . Thus, lighter ions will take less time to arrive at the detector than heavier ones. The time-of-flight (t) will equal the length (L) of the flight tube divided by the velocity (v). Substituting into equation (2), we find:

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

Rearranging the above equation, we obtain,

$$m/z = 2zV (t/L)^2 \quad (4)$$

By measuring the flight time for known L , z and V , it is easy to calculate the mass of the ion. To obtain the flight time, the time of ion formation must be known. The time-of-flight mass spectrometer uses pulsed laser ionization so that the time between ion formation and arrival at the detector can be measured. Flight tube lengths ranging from 15 cm to 8 meters have been used. Typically, the time-of-flight range from 5 to 100 microsecond and the accelerating voltage (V) from 3 kV to 30 kV (Cotter, 1994).

Figure 1 shows the schematic diagram of TOF mass spectrometer.

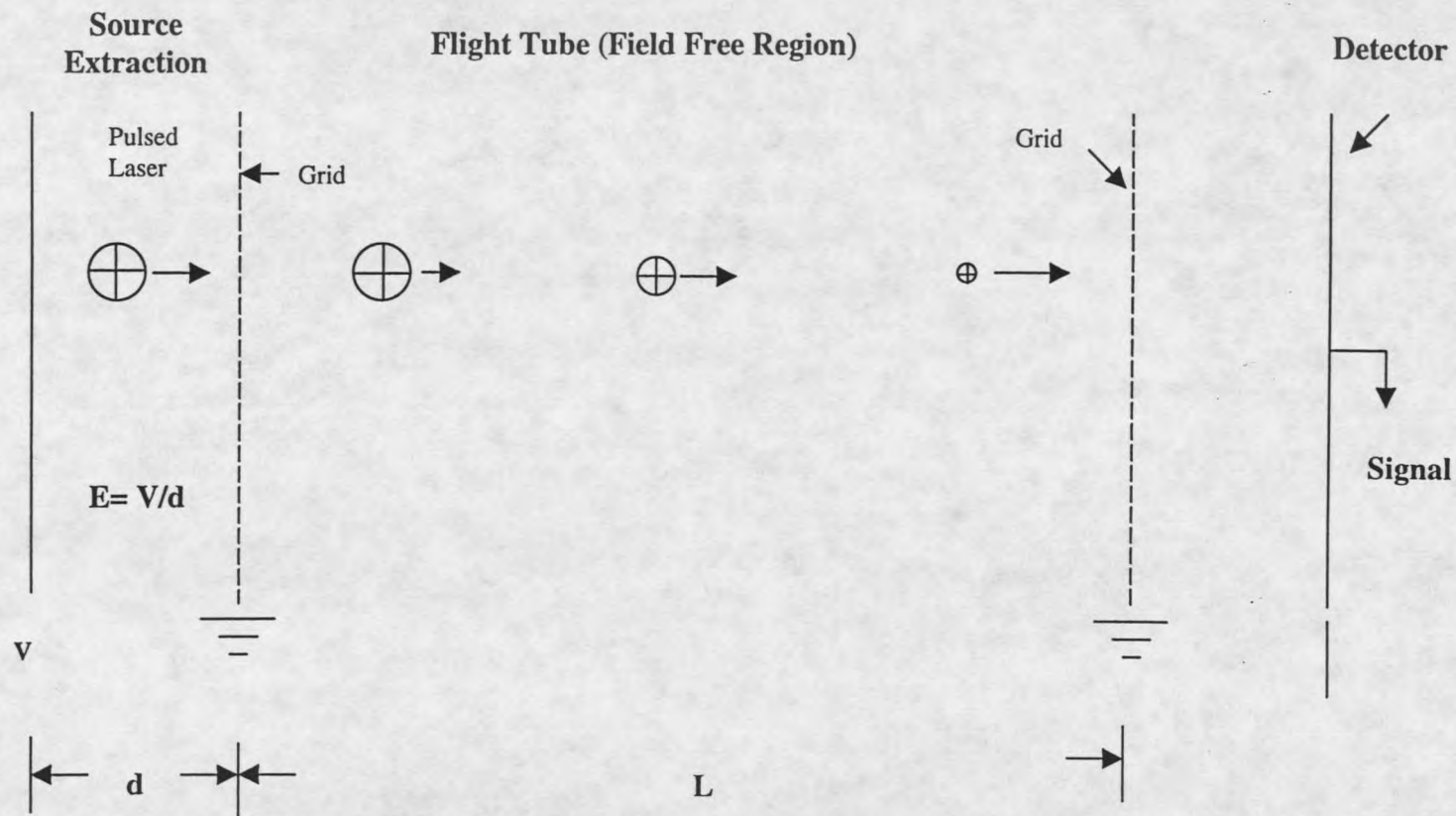


Figure 1. Schematic diagram of a linear time-of-flight mass spectrometer.

Ion Detectors for TOF Mass Spectrometer

Three kinds of detectors are regularly used in mass spectrometers: discrete dynode electron multipliers, continuous dynode electron multipliers (Channeltron) and microchannel plates (MCP). In a discrete electron multiplier, the ion beam from the mass analyzer strikes a conversion dynode. At this dynode, ions are converted to electrons as the momentum of an ion causes several electrons to be ejected from the surface. The ejected electrons are accelerated by the electrical potential difference toward the second dynode. As they hit the surface, they eject more electrons. By repeating this process, the numbers of electrons continue to increase. This multiplication effect causes a gain of 10^3 to 10^6 .

Another type of electron multiplier is the continuous dynode, also called Channeltron. The operation principle is the same as for the discrete electron multiplier. Unlike the discrete electron multiplier, the channeltron has a continuous electron emissive surface. An electrical resistive material is coated on the inside surface of a "horn" in order to let the large potential difference (about 2 keV) become evenly distributed from one end to the other.

A microchannel plate has a flat plate surface exposed to the incoming analyte ions. The plate consists of an array of about 10^6 hollow glass fibers (10-40 μm in diameter). The inside of each fiber is coated with a electron emissive resistive material. When a high voltage, E_b , is applied between the two surfaces of the microchannel plate, each fiber acts as an individual electron multiplier. Similar to the Channeltron, a potential gradient develops along the length of the microchannel. Ions strike the inner wall of a

channel on the input side and several secondary electrons are emitted. The output electrical signals are typically recorded on an oscilloscope.

MCP with two microchannel plates in a so-called Chevron arrangement is used in our TOF mass spectrometer.

Data System

The data system converts the electric signals from the electron multiplier into mass spectra graphs and tables.

Because pulsed ionization techniques usually produce a huge number of ions in each time-of-flight cycle, it is necessary to have a high-speed recording system to process data. A transient recorder or a high-speed Analog-to-Digital (A/D) converter can digitize and record the entire spectrum. In PerSeptive system, the output signals are transported to an oscilloscope, and the digitized signals are sent to a computer for additional processing. The Tektronix instrument can sample and digitize the detector signal as often as every 2 ns into a maximum 50,000 channels, giving a flight time observation window of 100 μ s. A Windows program, Graphical Relational Array Management System (GRAMS), is a general laboratory data management program and is used to convert the intensity versus time data recorded on the oscilloscope to intensity versus mass.

Advantages and Disadvantages of TOFMS

The TOF mass spectrometer is affordable and simple in design and construction. It does not have a theoretical upper mass limit. It also has a high sensitivity because it

transmits ion efficiently, and an entire mass spectrum can be recorded from every single laser shot.

Historically, the major drawback of a TOF mass spectrometer has been a relatively low mass resolution due to a distribution of flight times for ions with the same mass. The reasons for this broadening are the spreading of time, space, and kinetic energy (Cotter et al., 1992). The following sections will discuss these three effects and methods used to compensate for them in order to improve the resolution.

Temporal, Spatial, and Kinetic Energy Distribution in TOFMS

(1) Temporal Distribution

Ideally, the ions in TOF-MS should be formed simultaneously. However, sometimes two ions are formed at slightly different times. Even though they obtain the same velocity, they will arrive at the detector at different times. This is referred to as the temporal distribution of ion flight times.

(2) Spatial Distribution

The initial spatial distribution is the result of ions formed at different positions along the direction of the electric field. Ions formed toward the rear of the ion source, will start at a higher electric potential and will be accelerated to a higher kinetic energy. Therefore, they have a shorter flight time. This is referred to as spatial distribution.

(3) Kinetic Energy Distribution

If two ions with the same mass are formed at the same time and in the same position but with different initial kinetic energies, they will arrive at the detector at different times. If the two ions have the same initial kinetic energy but move in opposite directions, one ion will travel against the field, stop, turn around and accelerate to the same kinetic energy as the other ion. The two ions will have different flight times. This is called "turn-around time". A lower mass resolution results. In laser desorption, ions are produced at the surface of sample rather than in the gas phase, and "turn-around time" can be neglected.

Methods for Improving Mass Resolution in TOFMS

From the discussion above we can see that in order to improve mass resolution, one has to find a way to minimize or correct for the temporal, spatial and the kinetic energy distributions. To minimize the temporal distribution, an ultrashort pulsed laser can be used to shorten the time window of ion formation. By using low extraction fields, the spatial distribution can be minimized. In laser desorption, ions are produced at the surface rather than in the gas phase, and the spatial spread of ion formation is negligible. The kinetic energy distribution can be compensated for by using a reflectron (see below) (Mamyrin et al., 1966 & 1973). The effect of turn around time and kinetic energy variations can be compensated for by "time-lag focussing" or delayed ion extraction (DE). Next, the techniques of reflectrons and time-lag-focusing (or DE) will be reviewed.

Reflectron TOFMS

The “reflectron” was invented by Mamyrin in 1966 (Mamyrin et al., 1966) in order to compensate for the initial kinetic energy distribution. The reflectron, which is located at the end of the flight tube, contains a series of rings that have potentials that increase gradually along the rings. Ions travel through the flight tube and into the reflectron until they reach zero kinetic energy. They then turn around and accelerate back through the reflectron and into the flight tube. Because ions with higher energies will travel further into the reflectron than ions with lower energies, both of them can be made to arrive at the detector at approximately the same time. This design greatly improved the mass resolution from $m/\Delta m = 300$ to 3500 (Mamyrin et al., 1966) and made TOF mass spectrometers more widely used. However, it did not work well for large molecules. In general, as the molecular weight increases, the time frame for fragmentation increases (Chait et al., 1983; Demirev et al., 1987). In linear mode mass spectrometers, ions and neutrals produced by the dissociation of molecular ions have the same velocity and they arrive the detector at the same time. In the reflectron, however, the neutral fragment will not be reflected. Fragment ions are reflected, but their kinetic energy is less than that of the parent ion. Therefore, these ions will be poorly focused and have low mass resolution. Thus, when there is a substantial metastable fragmentation the reflectron is less sensitive than that linear mode mass spectrometer. Another problem with the reflectron is due to the “plume effect” (Ingendoh et al., 1994). With increasing acceleration fields, the width of initial kinetic energy distribution is greatly increased because of collision processes in the expanding plume above the sample surface. This

results in a lower mass resolution. This can not be corrected by the reflectron; however, delayed ion extraction can solve this problem.

Time-Lag-Focusing or Delayed Ion extraction (DE)

In continuous ion extraction mode, the accelerating voltage is applied all the time. The ions formed are immediately extracted through the high density plume produced by the ablated material from the sample. Ions will collide in the dense ion/neutral plume, therefore lowering the mass resolution. Many different techniques (Wiley et al., 1955; Browder et al., 1981; Moller et al., 1984; Muga et al., 1987; Kinsel et al., 1991; Whittall et al., 1995; Juhasz et al., 1996) have been used to correct for this problem. Among them, "Time-Lag-Focussing" or "Delayed Ion Extraction" has been the most successful. In this technique, the ion formation is decoupled from the ion extraction step and the ions are allowed to spread out in the field free ion source before the extraction field is switched on. In other words, it employs a time delay between the end of the ionization pulse and the start of the extraction pulse. At the end of the time delay, the plume has become less dense, and the energy loss of the ions from collision with neutrals is minimized. By using this technique, the mass resolution was improved to 7,500 for oligonucleotides in reflecting mode according to Juhasz et al. (Juhasz et al., 1996).

Objective of the thesis

Development And Application Of Activated Carbon (AC) Substrates For Assisted Laser Desorption Ionization (SALDI)

The major objective of this thesis was to improve the sensitivity and mass resolution in the existing "Surface-Assisted Laser Desorption Ionization (SALDI)" Mass Spectrometry methodology developed in our group and to develop the application of SALDI to chromatography. MALDI and other established laser desorption ionization methods, have a high background of matrix ions which affects the analysis of low molecular weight compounds. This is due to UV-absorbing properties of the matrix. To avoid this problem, a non-UV-absorbing substance such as graphite or activated carbon was used in SALDI and a low matrix ion interference was observed (Sunner et al., 1995; Chen et al., 1997; Chen et al., 1998). However, the solid used was a very fine powder, and as the solvent evaporated in the vacuum chamber of the mass spectrometer, the powder dried and spread in the ion source. Previous work on immobilizing the powder used a non-volatile component (a saccharide) with adhesive-like properties that was added to the analyte matrix suspension. However, some ion source contamination still occurred. It was thus necessary to find a better way to immobilize the powder.

The new desorption ionization method by immobilizing a very thin layer of AC, it was also considered that the sensitivity might be dramatically improved and sample handling simplified.

In the previously developed TLC-SALDI method, the analyte was extracted by a liquid matrix (glycerol/methanol) to the surface of the TLC plate where the analyte was adsorbed on activated carbon powder. The intact plate was brought to the mass spectrometer. However, the mass resolution suffered from surface-charging (Chen et al., 1998) and the sensitivity suffered from inefficient analyte extraction.

An interface method, therefore, had to be developed that offers an electrically conducting path from the AC substrate to the ion source sample plate and in which the extraction efficiency is maximized. The application of such an interface as a continuous fraction collector in chromatography was to be explored.

CHAPTER 2

EXPERIMENTAL SECTION

Chemicals and Materials

Aluminum backed TLC plates pre-coated with silica gel 60 (surface area 550 m²/g, without fluorescent indicator) were purchased from Merck Company (Darmstadt, Germany). The thickness of the silica gel layer was 0.20 mm. Aluminum backed TLC plates with 0.25 mm thickness precoated with Silica G and with fluorescent indicator F₂₅₄ were from Whatman (Cat no. 4420 222). Plastic backed TLC plates pre-coated with silica gel (with fluorescent indicator F₂₅₄) were graciously supplied by Dr. Dratz group. The thickness of the silica gel was 0.10 mm. Activated carbon powder (Darco G-60, 100 mesh) was purchased from Aldrich. Victoria blue was obtained from Matheson Coleman & Bell. Methyl green and red food color was purchased from Allied Chemical Corporation. Ninhydrin was purchased from Baker. The aluminum foil, spray adhesive Super 77 (3M Company, Industrial Tape and Specialties Division), and carbon conductive adhesive double sticky tape was purchased from Ted Pella, Inc. (Redding,

CA). Glass fiber filter paper (Grade: 111; Size: 5.5 cm) was purchased from Fisher. Glycerol (99.5%, ACS reagent grade) was obtained from Fisher and acetic acid (99.7%) was purchased from EM Science. All other organic solvents, such as methanol, ethanol and acetone were obtained from Fisher. Caffeine was obtained from Mallinckrodt Chemical Works, lauryldimethylamine oxide (LDAO) was from Hoechst, crystal violet (dye content: 90%), sodium dodecyl sulfate (SDS), all amino acids and peptides were obtained from Sigma.

TLC Separation

The aluminum-backed TLC plates pre-coated with silica gel 60 (without fluorescent indicator) were used for most experiments. Some of the experiments used plastic-backed TLC precoated with silica gel for method development. Aluminum-backed TLC plates with 0.25 mm precoated Silica G and F₂₅₄ fluorescent indicator were used for caffeine analysis. The dyes, amino acids and peptides were dissolved in methanol. Caffeine and saccharides were dissolved in deionized water. Food color mixture was diluted with deionized water. Unless otherwise stated, the developing process was performed in a vertical glass developing chamber with several slot inside. In experiments where the plates were developed in a horizontal position, a large glass developing chamber was used.

A suspension "C" was made by adding 25 mg of AC to 0.10 mL of a mixed glycerol-methanol at 1:1 volume ratio, then it was mixed with a solution of 5% (w/v) sucrose in water in a 1:8 (v/v) ratio.

The following solvent systems were used for TLC plate development. For dyes, methylethylketone-acetic acid-isopropanol (40:40:20 v/v/v) (Scheppe et al., 1977) was used. For peptides and amino acids, water-methanol-acetic acid (44:50:6, v/v/v) was used (Dunphy et al., 1988). All separations were carried out at room temperature. Ninhydrin (0.20%) in ethanol was used for visualization of peptides and amino acids. The ninhydrin solution was sprayed onto the TLC plate, the ethanol solvent was allowed to evaporate for several minutes, and the plate was heated using a hair dryer or a hot plate.

Mass Spectrometer

A Voyager RP time-of-flight (TOF) mass spectrometer (PerSeptive Biosystems, Framingham, MA) was used for all experiments. This instrument is equipped with a nitrogen laser emitting a 3 ns pulse at 337 nm (CVI Laser, Newton, MA). The maximum energy is 400 μ J per pulse. The mass spectrometer has a 1.4 m vertical flight tube with both linear and reflecting modes. It has a single stage reflector and dual stage ion source with continuous ion extraction. The $2\frac{1}{2} \times 2\frac{1}{2}$ in. (1 inch = 2.54 cm) stainless steel sample plate is attached to a computer-controlled XY stage that allows for precise manipulation of the sample relative to the fixed laser focal position inside the source.

Each individual sample was viewed with a video camera and monitor, and this allowed the desorption site to be selected by visual inspection.

For dyes, the mass spectrometer was run in reflecting mode, the accelerating voltage was 28 kV, the grid voltage was set at 70%, and the guide wire voltage was set at 0.1% of the accelerating voltage. All the other analytes, for both positive and negative ions, was run in linear mode in order to improve sensitivity. The accelerating voltage was set at 30 kV, the secondary grid voltage was set at 70%, and 0.1% guide wire voltage. Approximately 30 single shot mass spectra were averaged on a 500 MHz digitizing oscilloscope, TDS 520A (Tektronix, Wilsonville, OR, USA). Spectra were downloaded to a personal computer and mass calibrated using PerSeptive and GRAMS (Galactic Software, Salem, NH) software. Two mass calibration methods were used, internal and the default mass calibration. Internal mass calibration used two known peaks in each mass spectrum as calibration points. Default calibration was executed by the software and was based on the accelerating voltage, the length of the flight tube, etc. Internal mass calibration was used unless otherwise noted.

Excised pieces of TLC plates or AC substrates were attached to the MALDI stainless steel sample plate by using double-sided sticky tape. The samples were air-dried and the plate was introduced into the TOF-MS instrument and brought under vacuum by a pneumatic system.

CHAPTER 3

DEVELOPMENT OF SALDI METHOD

Introduction

As described in Chapter 1, the first group to use a liquid matrix for laser desorption was Tanaka et al. (Tanaka et al., 1988). This was a groundbreaking work in laser desorption mass spectrometry. Mass spectra from very high molecular weight molecules were obtained for the first time, however, the mass resolution was very low (about 20). Inspired by Tanaka's method, several groups have tried to improve the sensitivity and mass resolution of the liquid based matrix method. In 1991, Zhao et al. used nitrobenzyl alcohol (NBA) matrix to analyze proteins (Zhao et al., 1991). The detection limit was claimed to be 5 fmol for a single-shot mass spectrum of albumin. Cornett et al. obtained mass spectra of peptides and proteins (Cornett et al. 1992 & 1993) using a strongly absorbing organic compound. However, the mass resolution was less than the mass resolution obtained from solid matrix (Cornett et al., 1993).

In 1995, Sunner and co-workers (Sunner et al., 1995) developed a new laser desorption ionization method, "Surface Assisted Laser Desorption Ionization (SALDI)".

In this method, a 5% glycerol: methanol (v/v) solution was made and mixed with an equal volume of dry graphite powder using a vertexer. One μL of the slurry was pipetted onto the probe well. Graphite powder was used to help the laser light absorption and the ionization process. Good quality mass spectra were obtained. However, they reported an absolute sensitivity that was significantly lower than for conventional MALDI, and their mass resolution was only about 300 ($m/\Delta m$).

One year later, a group in Switzerland (Dale et al., 1996) explored the great potential of this method. Dale et al. used a somewhat different procedure for making the suspension. The composition of the liquid matrix was changed and the graphite/liquid matrix mixing time were extended in their procedure. They made a 30% glycerol/methanol (v/v) matrix solution, and gradually added an approximately equal volume of 2 μm graphite particles. The mixture was shaken for 1 hour to ensure a good mixing of graphite and liquid matrix. About 1 μL of this mixture was applied to the sample holder and the methanol evaporated. The analyte solution was deposited on top of the graphite/liquid matrix layer. They applied SALDI to many different kinds of analytes, such as oligosaccharides and organic polymers and claimed a detection sensitivity in the femtomole range for lower molecular weight peptides and oligosaccharides. They concluded that this method can be used as a widespread tool for the mass spectrometry of intermediate weight compounds. Dale et al. also improved the mass resolution to about 480 ($m/\Delta m$) for cationized polyethylene glycol (PEG).

More recently, Sunner and co-workers developed a method to couple TLC with SALDI (Chen et al., 1998). They made three different matrix preparation (suspensions)

using activated carbon. All suspensions contain AC, glycerol, sucrose, methanol and water, but the relative concentrations vary. Among the three suspensions, suspension "C" was the latest developed. It was made by mixing 25 mg of activated carbon powder with 0.10 mL of a glycerol methanol mixture (1:1, v/v). This suspension was then mixed with a 5% (w/v) sucrose in water solution in a 1:8 volume ratio. This suspension (0.50 μ L) was deposited analyte onto the TLC plate such that the carbon powder evenly covered the gel surface. In this way, the analyte was extracted from the TLC plate. The intact TLC plate was then carefully cut and attached to the ion source sample plate with double-sided tape. This method, using suspension "C", proved to give the best mass spectra. Good mass spectra, with few background ions, were obtained for a wide range of organic compounds and small peptides (Chen et al., 1998). A problem with the method was the lack of electrical contact between the plastic-backed TLC plate and the sample plate. This resulted in a relatively low resolution because of surface charging.

In this work, a new SALDI method will be presented that has a higher mass resolution and a better sensitivity. Instead of using graphite, we use activated carbon to help the desorption ionization process. The porous surface of activated carbon will help the extraction and desorption ionization processes (Chen et al., 1997). First the general properties of activated carbon will be introduced, and then the new method will be shown in the following chapters.

Activated Carbon

Activated carbon has been used as an adsorber of organic compounds in industry for a long time, especially for adsorptive removal of pollutants from gases or aqueous solutions. The term "activated carbon" is a trade name for a family of carbonaceous adsorbents with a highly crystalline form and extensively developed internal pore structure. It is defined as follows (Test methods for activated carbon, European Council of Chemical Manufacturers' Federations/ CEFIC, Brussels (1986)): "Activated carbons are non-hazardous, processed, carbonaceous products, having a porous structure and a large internal surface area." These materials can adsorb a wide range of substances, they are able to attract molecules to their internal surface, therefore they are also called adsorbents.

Activated carbons are classified by their pore size into three categories by the International Union of Pure and Applied Chemistry (IUPAC). If the average pore size is less than 1 nm, the activated carbon is referred to as "Microporous". If it is ranging from 1 to 25 nm, it is referred to as "Mesoporous" activated carbon, and if it larger than 25 nm, it is referred to as "Macroporous" activated carbon. The smaller the particle size of the activated carbon, the better the access to the surface area and the faster the rate of the adsorption process (<http://www.activated-carbon.com>). The adsorption of molecules from solution depends on the nature of the activated carbon. The kinetics of

diffusion and absorption processes have been reviewed (Snoeyink et al, 1968; Digiano et al., 1969) and is beyond the scope of this paper.

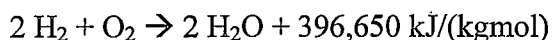
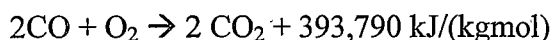
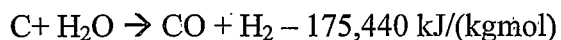
Activated carbon has been commercially produced from various starting materials, such as sawdust, wood, coconut shells and carbon black (Mattson et al., 1971) The properties of activated carbon vary depending on the manufacturing process and the starting material. Two main "activation" techniques are most principally used by the manufacturers: Chemical activation and steam activation (<http://www.activated-carbon.com>, Carbon Link Limited Company bulletin). The following section will discuss them individually.

Chemical Activation

This technique is generally used with wood-based raw materials. The raw material is treated with a strong dehydrating agent, phosphoric acid (H_3PO_4) or zinc chloride (ZnCl_2), then heated to 500-800 °C to activate the carbon. The product is then washed, dried and ground to the needed powder.

Steam Activation

This technique is generally used with coal and coconut shells as raw materials. Initially, the raw material is kept at a high temperature, 800-1100 °C. In the presence of steam, some reactions will occur:



A small amount of air is added to oxidize CO and H₂ without reacting directly with the carbon. The activated carbon product is then screened, de-dusted and sorted them according to the particle size. Activated carbon produced by this method has a fine pore structure, and is usually used for the adsorption of compounds from both liquids and gases.

In our experiment, a DACO-G 60 activated carbon was used. This material was activated by the steam technique. It was then washed with strong mineral acid and rinsed with water (Aldrich Technical bulletin). Its particle size is around 5-10 μm and its total surface area is 1 m^2/g (dry basis). It is a microporous activated carbon. This kind of activated carbon was used through this research.

CHAPTER 4

IMMOBILIZED ACTIVATED CARBON FOR SALDI

Introduction

Surface-Assisted Laser Desorption Ionization (SALDI) mass spectrometry is a new desorption ionization method developed by the Sunner group (Sunner et al., 1995). Desorption of macromolecules directly from solid surfaces may have great advantages. Because all the analyte molecules may be deposited on the surface and desorbed as ions, the sensitivity is potentially very high. By carefully extracting ions from a well-defined electrical potential, the mass resolution also could be high (Sunner et al., 1995).

In graphite-SALDI, graphite particles with a size distribution in the 10-150 μm range are used to make slurry with an analyte solution. Even though good-quality mass spectra were obtained by this method, the sensitivity and mass resolution, while good, were not as high as for MALDI. The sensitivity was in the pico- to nanomole range, and the mass resolution was about 300 (Sunner et al., 1995). With graphite, it was often difficult to find the ion signal; one had to move the laser focus over the sample in order to find a "sweetspot". In an effort to find new surface-assisting materials, that would

reliably give mass spectra with high sensitivity and good mass resolution, several different carbon materials were tried (Chen et al., 1997). Among them, activated carbon (AC) proved to be the best choice. There were several reasons. First, it was proven very easy to obtain mass spectra. Second, the sensitivity and mass resolution were about the same as with graphite (Chen et al., 1997). Third, AC-SALDI had less glycerol fragments in the mass spectra. AC is well known as an efficient absorber of organic compounds. It has a very large surface area due to its porous structure. This can be seen in the scanning electronic microscope (SEM) pictures in Figure 2. AC shows a very porous surface while graphite has relatively flat surfaces. It can be speculated that the advantages of the porous AC structure are as follows. First, AC can adsorb more liquid matrix, resulting in a "softer" desorption process than from graphite (Chen et al., 1997). Large molecules might then have a better chance to survive the desorption process. The second possible advantage of AC is that, because the surface of AC particles has been modified by oxidation (Mantell, 1968), functional groups such as -COOH and -OH are bound at the AC surface. With the irradiation by the laser, these functional groups might be decomposed to small gas-phase neutrals such as CO_2 or H_2O which may transfer momentum to the analyte molecules, therefore helping the desorption process. Because of its advantages, activated carbon was used almost exclusively in subsequent work. By comparing the mass spectra obtained using different size AC particles, it was found that smaller particles yielded a higher mass resolution than larger particles (Chen et al., 1997). Therefore, microporous particles with sizes from $5\text{ }\mu\text{m}$ to $10\text{ }\mu\text{m}$ (Darco 60) were used.

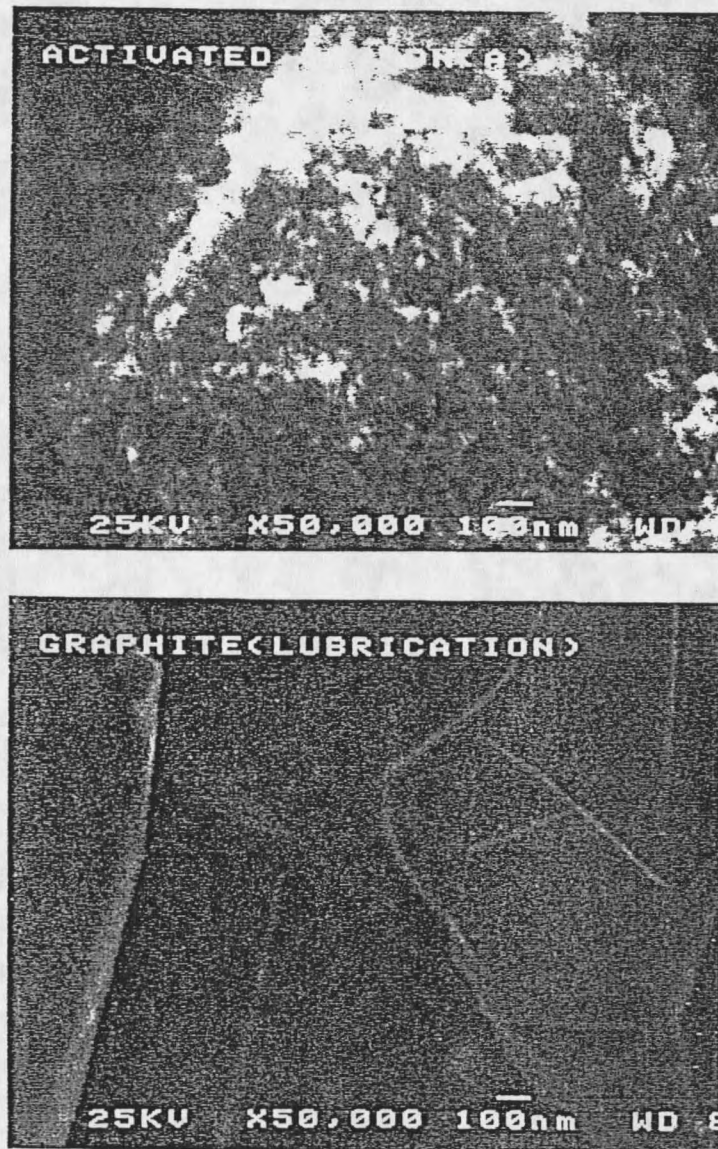


Figure 2. Scanning Electron Microscope (SEM) pictures of the surface of activated carbon (top) and graphite (bottom) (From Chen et al., 1997).

In their sample preparation, Chen et al. (Chen et al., 1997 & 1998) added 25 mg AC to 0.10 mL of a mixed glycerol-methanol solution (1:1, v/v) to get a slurry, then mixed this suspension with a solution of 5% (w/v) sucrose in water in a 1:8 volume ratio. A volume of 1 μ L of this slurry was pipetted into sample well and 1 μ L of analyte solution was added. The mass resolution and sensitivity were almost the same as for graphite-SALDI. A possible reason for the low sensitivity is that some of the analyte was adsorbed inside the porous structure of AC particles. Also, most of the analyte should be found inside the thick suspension sample. Only a small fraction of the analyte was accessible to the laser. A possible explanation for the low mass resolution is that because of the thickness of the AC suspension sample, the electrical potential in the sample surface may not be well defined. At the beginning of this project, it was considered that a way to solve these problems may be to use a monolayer of AC particles. A monolayer could be made by immobilizing the AC particles on a conducting surface. Most of the analyte would then be adsorbed closed to the surface of the sample. The electrical potential would also be well defined. There would be other advantages of immobilizing the AC particles. In suspension samples, AC particles tend to dislodge in the ion source, causing a serious ion source contamination problem. Because the particles are electrical conductors, they may cause electrical short-circuit problems. Of particular concern in the Perceptive instrument is the high voltage grid that is positioned only 2 mm above the sample plate. Therefore, immobilizing AC particles becomes very important both for improving the quality of the mass spectra and for protecting the instrument. Several methods were tried by Chen et al. (Chen et al., 1997), in order to avoid ion source contamination, such as sprinkling AC particles on a

double-sided sticky tape and mixing a dilute commercial adhesive with the SALDI suspensions. However, none of these methods yielded good mass spectra. In this work, a method for immobilizing AC particles in a thin layer (close to a monolayer) has been developed. The method yields good quality mass spectra with high mass resolution and excellent sensitivity.

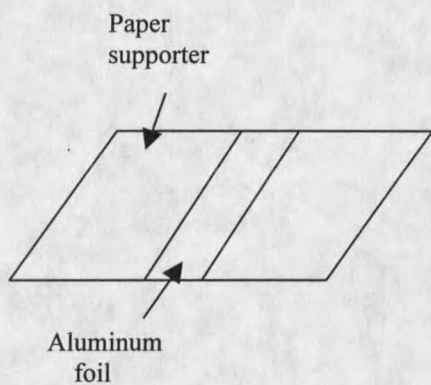
Procedures for Making Activated Carbon (AC) Substrates

There are two factors that should be considered when making activated carbon substrates (immobilized AC particles on a supporting surface). The first and most important is the technique used to immobilize the particles. In previous efforts to use an adhesive to bond the AC particles to a substrate essentially no analyte ions were obtained, presumably because most adhesives have a strong tendency to coat the μm -sized particles on all sides. The second consideration is that a monolayer of particles should be immobilized. Figure 3 (a-e) shows the process of making an AC substrate strip. A piece of aluminum foil, about 6×2 cm large and about $25 \mu\text{m}$ thick was cut from an aluminum sheet. This piece of aluminum foil was attached to a 8.5×11 inch paper with tape (Figure 3a). A commercial adhesive (Super 77) was sprayed to give an even coverage (Figure 3b). The white paper was folded such that only a 2-4 mm wide strip of the aluminum foil remained uncovered. AC particles were applied to the exposed and sticky area of the aluminum foil. The AC powder was pressed onto the sticky aluminum band for about 30 seconds using a spatula. Loose powder was then simply blown off (Figure 3d). A thin layer (close to monolayer) of AC particles was

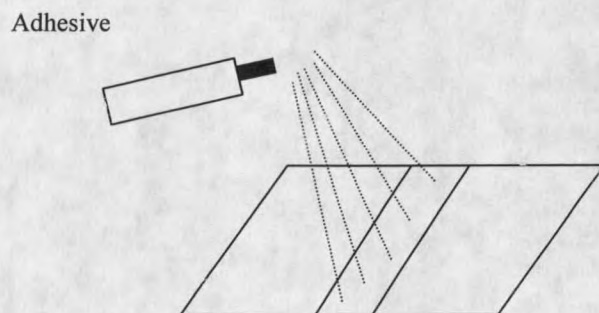
formed. An "AC strip" with desired width and length was cut from the paper with a pair of scissors. (Figure 9e). For some TLC applications (Chapter 5), it was required that the two ends (about 5 mm long) of the strip were free of AC particles.

Sample Preparation for SALDI with Immobilized Activated Carbon

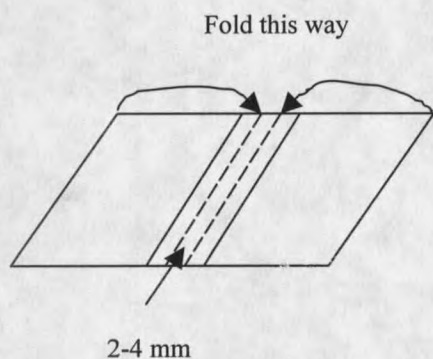
The sample preparation for this method is as follows: First, an aluminum backed AC substrate strip was made. It had a width 2-4 mm and a variable length, as discussed above (Figure 3). The strip was cut into smaller pieces, about 3 mm long each. A small piece of double-sided carbon tape, was first positioned inside the MALDI sample plate well, then an "AC patch" was attached to the tape and pressed down to ensure physical contact with the surface of the metal plate. The analyte solution, with a volume of 0.10 μL to 0.50 μL , was pipetted onto the small AC patch. Then 1 μL of a 20% glycerol in methanol (v/v) matrix solution was added. After 5-10 minutes at room temperature the methanol had evaporated, and sample plate was inserted into the time-of-flight mass spectrometer.



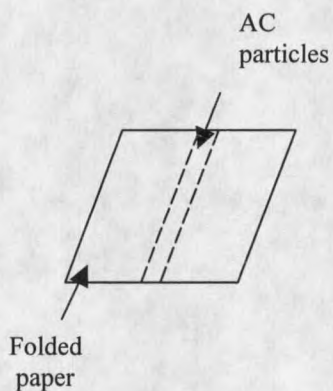
(a) A piece of aluminum foil was taped to paper



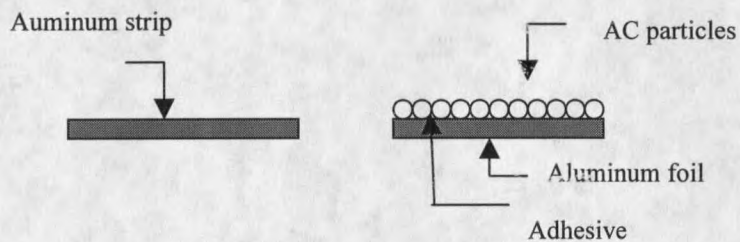
(b) Spray adhesive onto aluminum foil



(c) Fold the edges of the paper leaving a strip with a width of 2 to 4mm uncovered by the folded paper



(d) Add AC particles to strip, press down by spatula and shake off excess powder



(e) Cut strip out

Figure 3. Making AC substrate strips.

Results and Discussion

Several different types of compounds were tried with the immobilized AC substrates. Some representative mass spectra are shown in Figures 4 to 16. With the exception of Figures 12 and 13, default mass calibration was used. The time-of-flight mass spectrometer was operated in linear mode (unless otherwise noted). Figure 4 to 8 were obtained from amino acids and peptides, including L-arginine, DL-lysine, L-leucine, methionine enkephalin and bradykinin. Figures 9 to 10 were obtained from two saccharides, D(+)-glucose and D(+)-galactose. Figure 11 was obtained from caffeine. Figures 12 to 14 were obtained from the organic dyes, crystal violet, methyl green and a commercial red food color mixture. Figures 15 and 16 show the mass spectra of two surfactants, sodium dodecyl sulfate (SDS) and lauryldimethylamine oxide (LDAO). The mass spectra of these compounds will be discussed individually. Symbol " M^+ " represents the molecular ion, "G1" represents Glycerol, " MH^+ " represents the protonated analyte molecule, and cationized molecules also represented by " $Cat^+(M)$ ".

Immobilized AC-SALDI Mass Spectra of Amino Acids and Peptides

Mass spectra of amino acids and peptides have been obtained by many different ionization methods, such as EI (Heyns et al., 1963), CI (Milne et al., 1970), FD (Winkler et al., 1972), MALDI (Karas et al., 1988). Here, immobilized AC-SALDI was

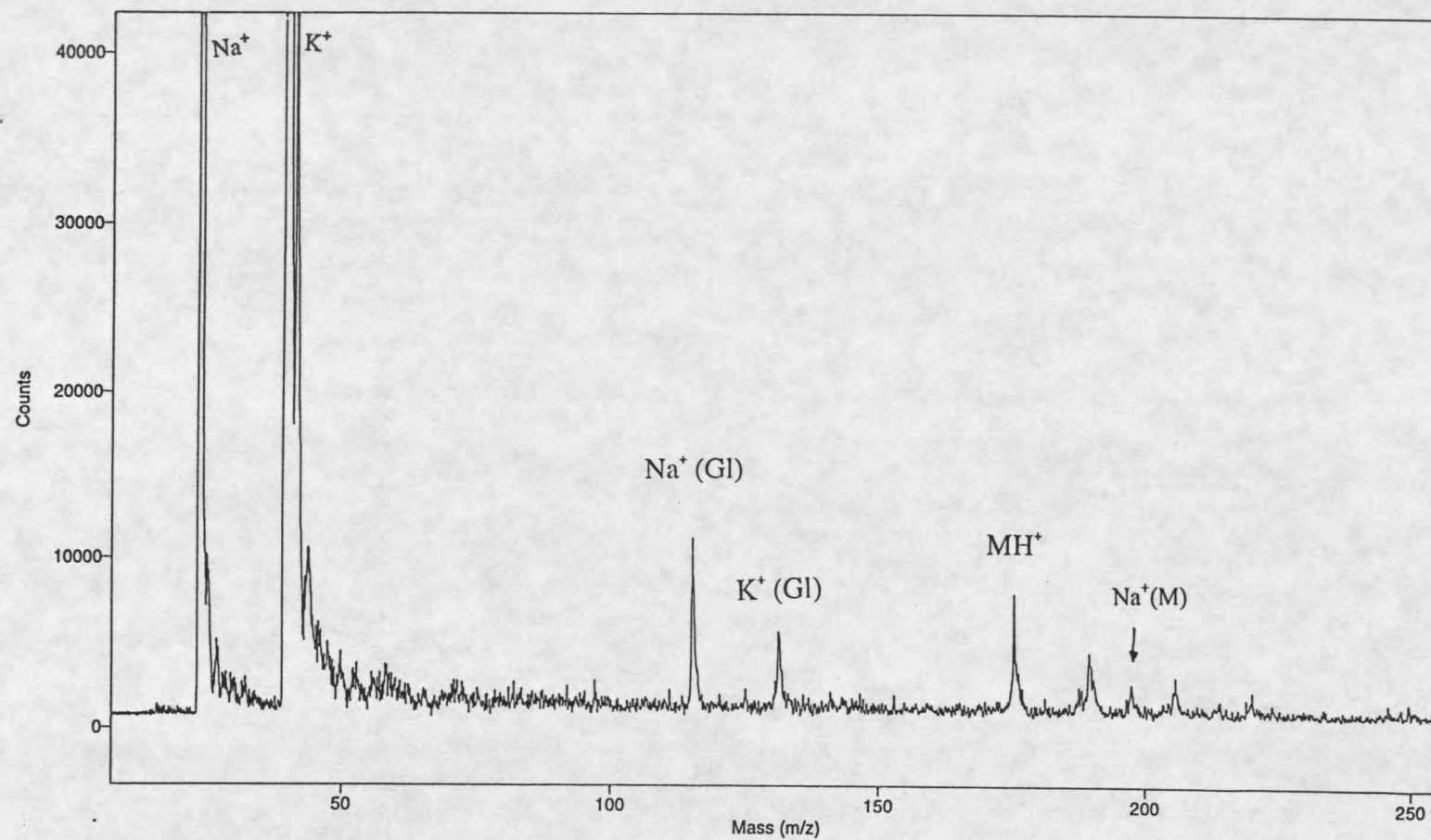


Figure 4. Immobilized AC-SALDI mass spectrum of L-arginine (MW = 174.2, 0.14 nmol).

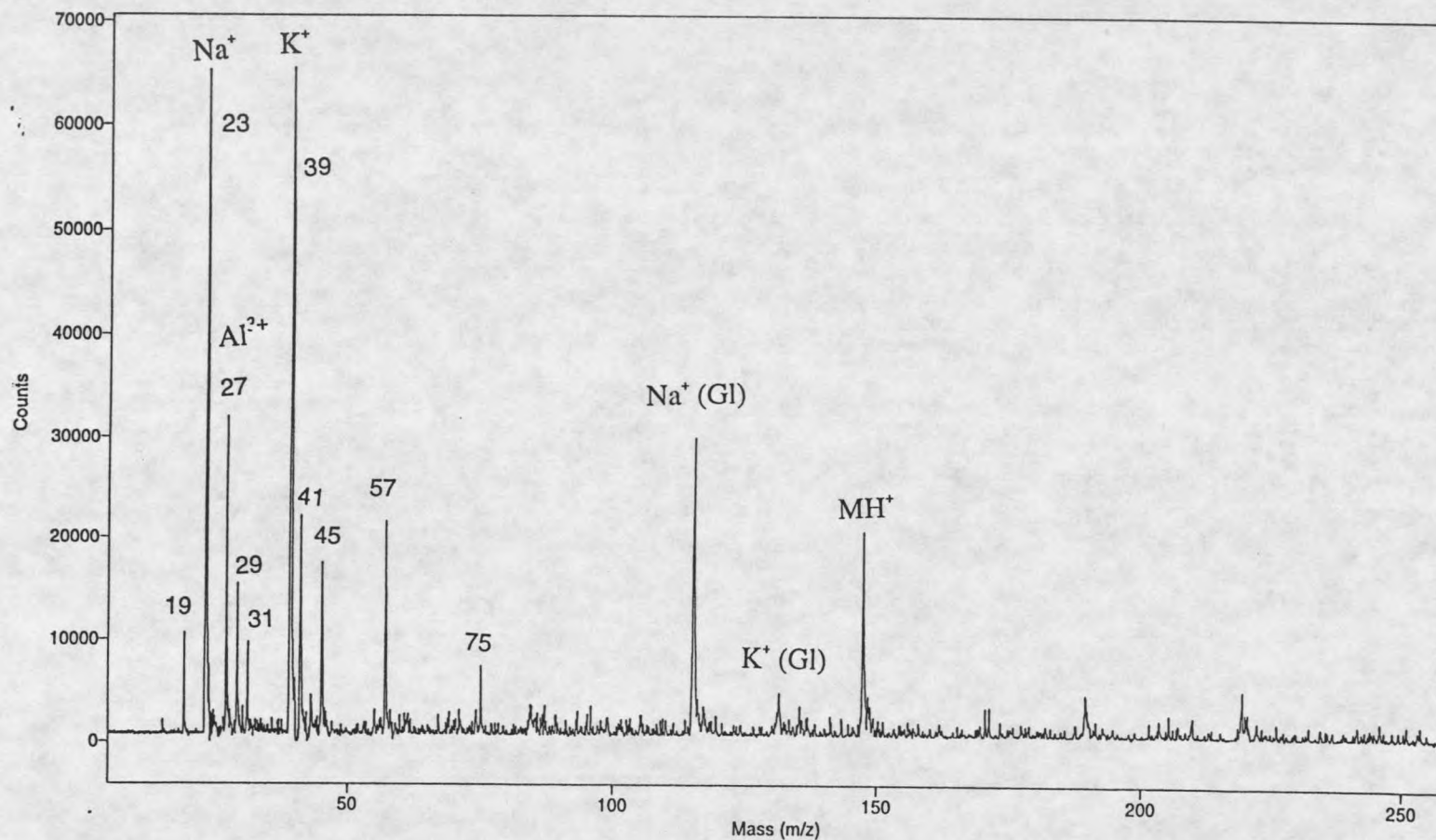


Figure 5. Immobilized AC-SALDI mass spectrum of DL-lysine (MW = 147.2, 1.8 nmol).

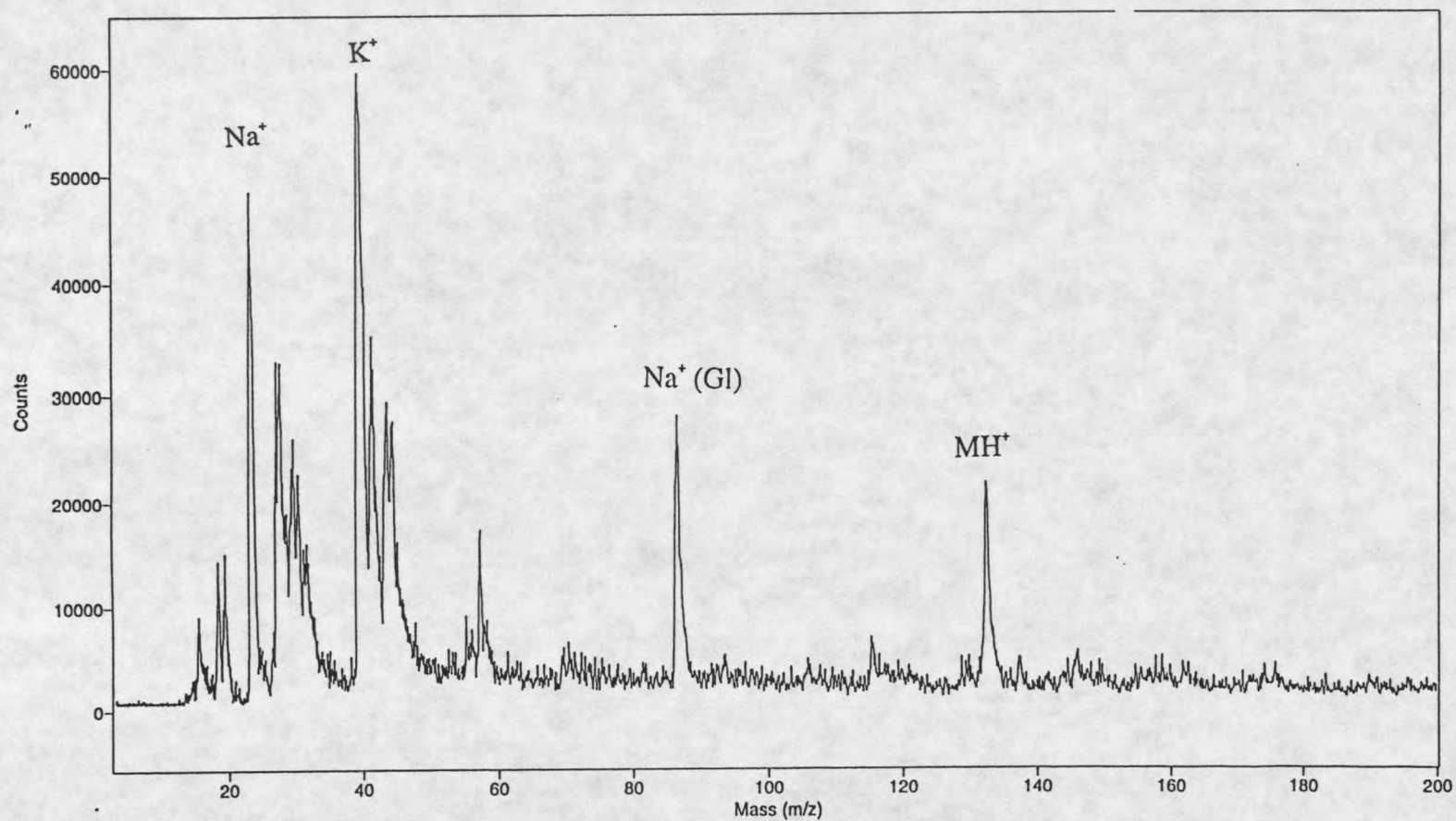


Figure 6. Immobilized AC-SALDI mass spectrum of L-leucine (MW = 131.2, 10 nmol).

used to analyze three different amino acids and two peptides. Figure 4 was obtained from 0.50 μL of a 0.28 mM (0.14 nmol) L-arginine (MW = 174.2) methanol solution. An MH^+ peak ($m/z = 175.2$) and a $\text{Na}^+(\text{M})$ peak ($m/z = 197.2$) were observed. Peaks at $m/z = 115$ and 131 are due to $\text{Na}^+(\text{Gl})$ and $\text{K}^+(\text{Gl})$. Peaks at $m/z = 189$ and 205 are due to impurities. For Figure 5, 0.50 μL of a 3.6 mM (1.8 nmol) solution of DL-lysine was applied to the AC patch, and a mass spectrum was obtained. The dominant analyte ion peak is MH^+ ($m/z = 148.2$). The spectrum has more background ion peaks below $m/z = 80$ than the spectrum of arginine in Figure 4. These peaks were due to glycerol fragments and appeared at $m/z = 75, 57, 45, 31, 29$ and 19. These glycerol peaks shown up also in FAB and graphite-SALDI (Sünner et al., 1995). In immobilized AC-SALDI, these peaks are only seen for the first few laser shots. Peaks at $m/z = 23, 27, 39, 41$ are due to $\text{Na}^+, \text{Al}^+, {}^{39}\text{K}^+$ and ${}^{41}\text{K}^+$. The relatively higher intensity observed at the spectrum is due to the saturation of detector at a strong signal of $m/z = 39$. The L-leucine mass spectrum in Figure 6 was obtained by applying 0.50 μL of a 0.0211 M (10 nmol) solution to the AC strip. A strong MH^+ ($m/z = 132.2$) peak was observed. The broad Na^+ ($m/z = 23$) and K^+ ($m/z = 39$) peaks are due to metastable ion decomposition. When a lower concentration L-leucine solution was applied to the patch, no MH^+ peak was observed, because of overlapping with $\text{K}^+(\text{Gl})$ which has a strong and wide peak at $m/z = 131$. Among the three mass spectra in Figures 4-6, L-arginine is the only one which has both MH^+ and $\text{Na}^+(\text{M})$ peaks. This can be explained by the relative high proton affinities of DL-lysine and L-leucine. Therefore, no sodiated ions were observed. Figure 7 was obtained from methionine enkephalin (0.50 μL , 1.0 mM; 0.50 nmol). A

$\text{Na}^+(\text{M})$ peak at ($m/z = 596.7$) was observed instead of a MH^+ peak. There are probably two reasons for this observation. First, the sodium ion affinity is higher than for the other amino acids and bradykinin. Second, the proton affinity of methionine enkephalin may be lower than that of glycerol. The peptide can not compete with glycerol for protons. Figure 8 shows a mass spectrum from 0.10 μL , 0.547 mM (0.0547 nmol) bradykinin applied to the AC strip. A strong MH^+ ($m/z = 1061.2$) was observed. The sensitivity of methionine enkephalin is relatively low compared to bradykinin. This was also observed in the former SALDI work by using AC suspension (Chen et al., 1997). It was suspected that the AC porous structure suppressed the sodiated methionine enkephalin. The reason is unknown.

Immobilized AC-SALDI Mass Spectra of Saccharides

Mass spectra were obtained of two saccharides. Figure 9 and Figure 10 were obtained from 3.19 nmol of D(+) glucose and 0.18 nmol D(+) galactose by applying 0.50 μL , 6.38 mM and 0.50 μL , 0.39 mM analyte solutions, respectively, to the AC substrates. Both saccharides have a molecular weight of 180.2, and both of them formed $\text{Na}^+(\text{M})$ ($m/z = 203.2$) and $\text{K}^+(\text{M})$ ($m/z = 219.2$). The reason is the same for methionine enkephalin. The sensitivity for D(+) galactose is much better than for D(+) glucose. This can be possibly explained by their structures. Both compounds have six-membered rings formed by hydroxy aldehydes. Glucose exists largely in the two possible structures, α -glucose and β -glucose. The β form, is more stable (Lowry et al., 1981). In this structure, the two hydroxyl groups are not in the same side, it is hard for

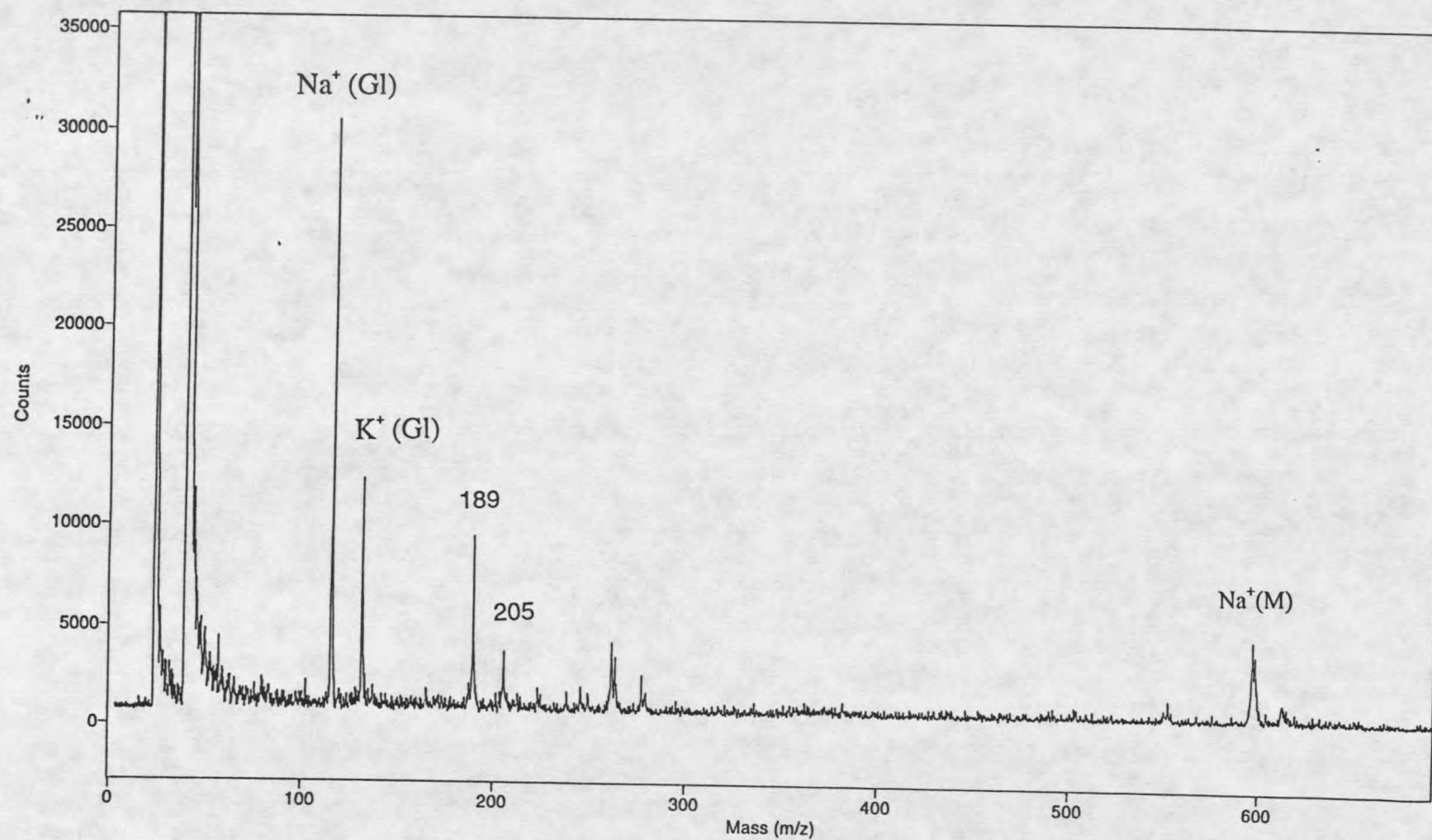


Figure 7. Immobilized AC-SALDI mass spectrum of methionine enkephalin (MW = 573.7, 0.5 nmol).

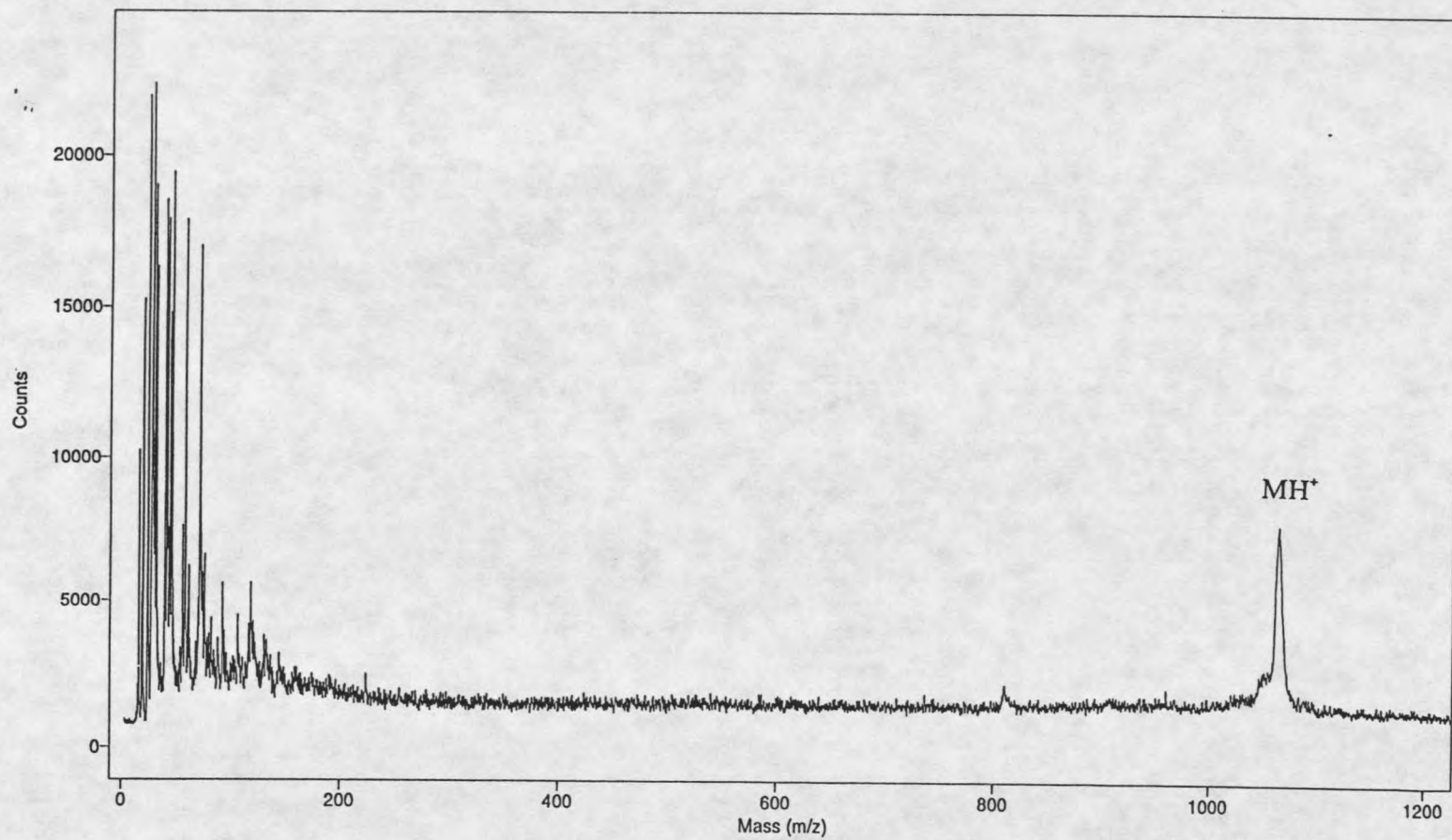


Figure 8. Immobilized AC-SALDI mass spectrum of bradykinin (MW =1060.2, 0.0547 nmol).

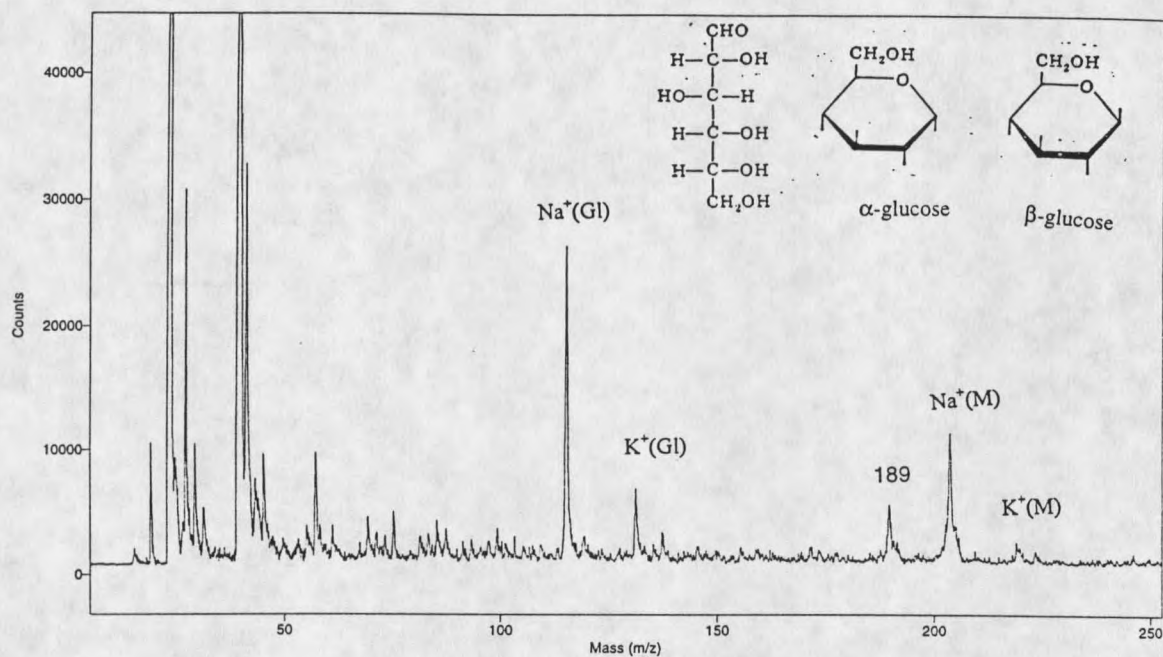


Figure 9. Immobilized AC-SALDI mass spectrum of D(+)-glucose (MW = 180.2, 3.19 nmol).

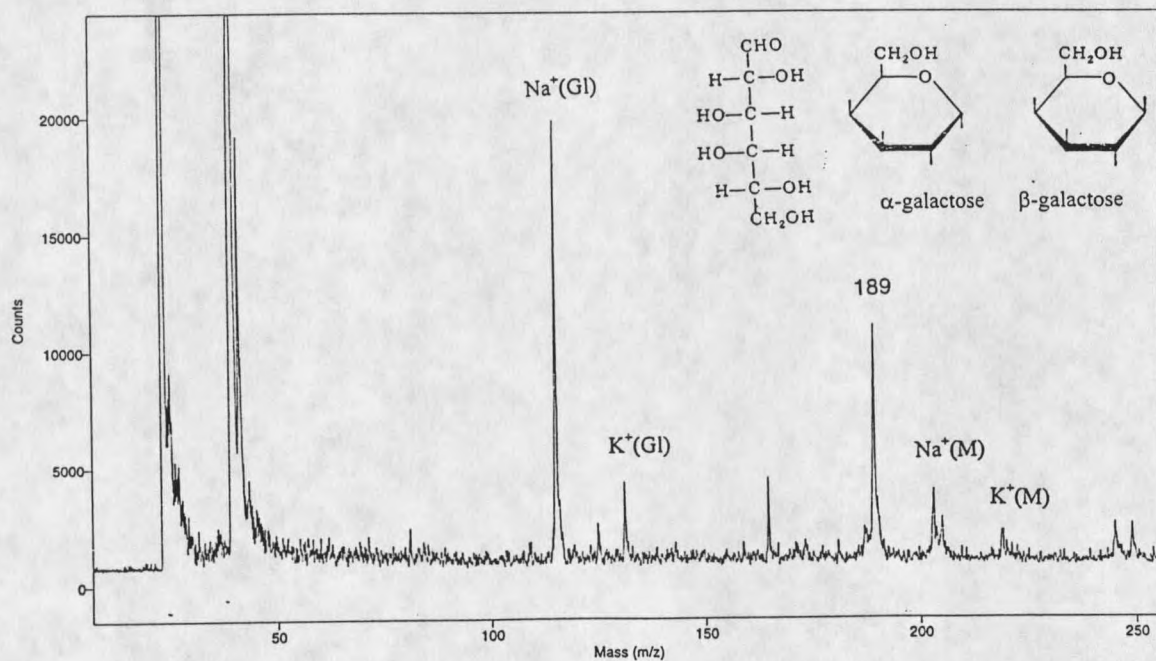


Figure 10. Immobilized AC-SALDI mass spectrum of D(+)-galactose (MW = 180.2, 0.18 nmol).

both of them to bind one Na^+ ion at the same time. In contrast, both α and β galactose have two hydroxyl groups in the same side, therefore, it may be easy for them to bind with Na^+ at the same time.

Immobilized AC-SALDI Mass Spectrum of Caffeine

Caffeine is a drug that affect the central nervous system. There are many developed mass spectrometry (MS) methods for detecting caffeine, such as liquid chromatography with MS (Midha et al., 1977) and EI-MS/MS (Ligroin et al., 1991). Here, A mass spectrum of 0.50 nmol (0.50 μL 1.0 mM) caffeine in water solution was detected by immobilized AC-SALDI and a strong MH^+ ($m/z = 195.4$) was observed (Figure 11).

Immobilized AC-SALDI Mass Spectra of Organic Dyes

Dyes are examples of thermally labile and fragile compounds of commercial value (Scheifers et al., 1983). A variety of mass spectrometry analysis techniques, such as FD (Mathias et al., 1976), FAB (Barofsky et al., 1983), SIMS (Scheifers et al., 1983) and LD (Novak et al., 1985) can be used. The immobilized AC-SALDI mass spectrum of crystal violet 0.50 pmol (204 pg) is shown in Figure 12. The S/N ratio is about 335. Thus, the detection limit, calculated for $S/N = 3$, is 1.5 fmol (about 0.6 pg). Because the SALDI signal is found to be proportional the square root of the amount of analyte (Chapter 5), a detection limit from the theory is 1 fmol. Thus, this result matched it very well. This compares very favorably with the detection limit by SIMS which was 10 ng of methylene blue supported on a silver foil (Scheifers et al., 1983). A strong M^+ peak ($m/z = 372$) was observed. The lower mass peak at $m/z = 356$ is due to loss of CH_4 . As

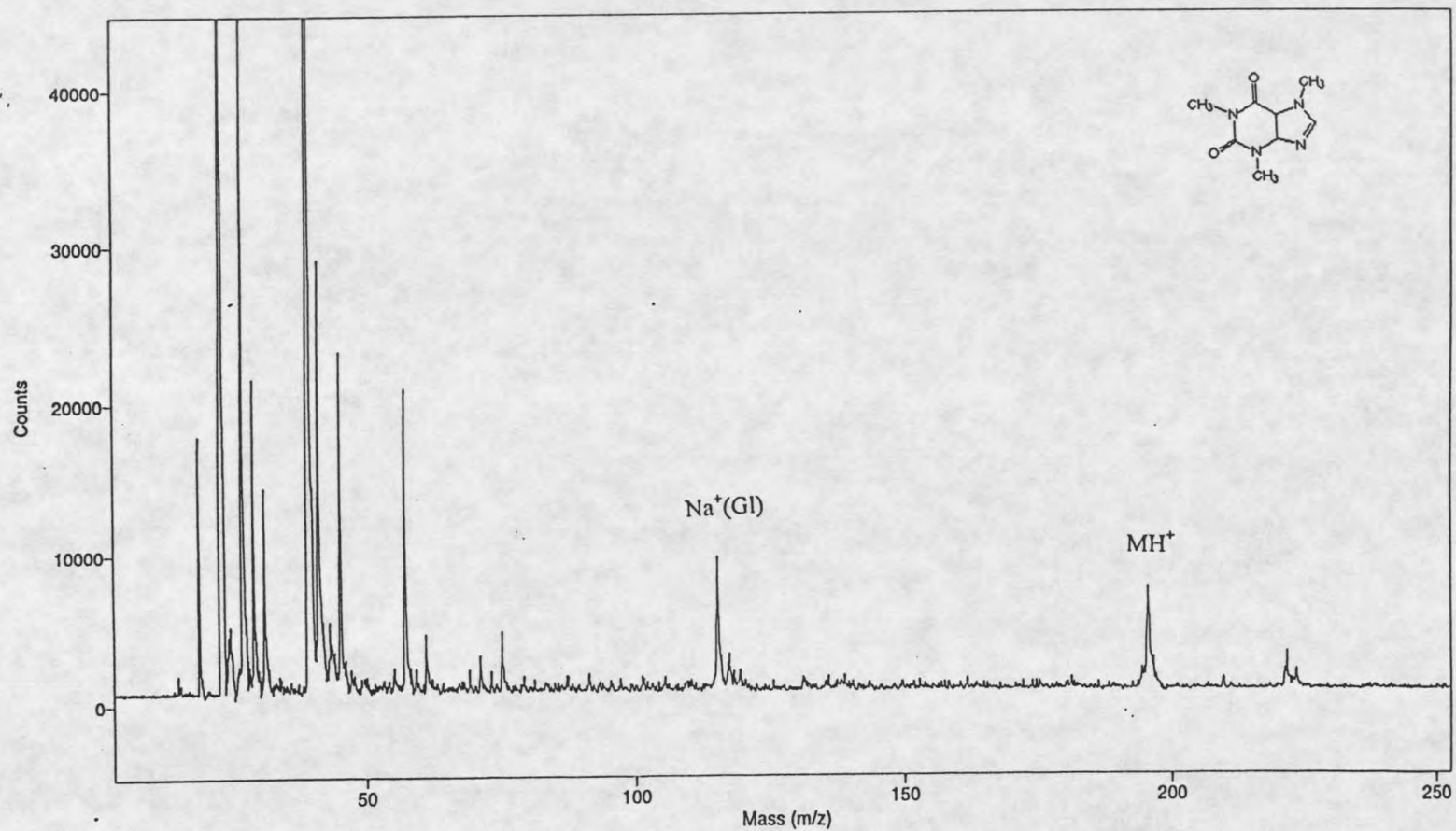


Figure 11. Immobilized AC-SALDI mass spectrum of caffeine (MW = 194.4, 0.5 nmol).

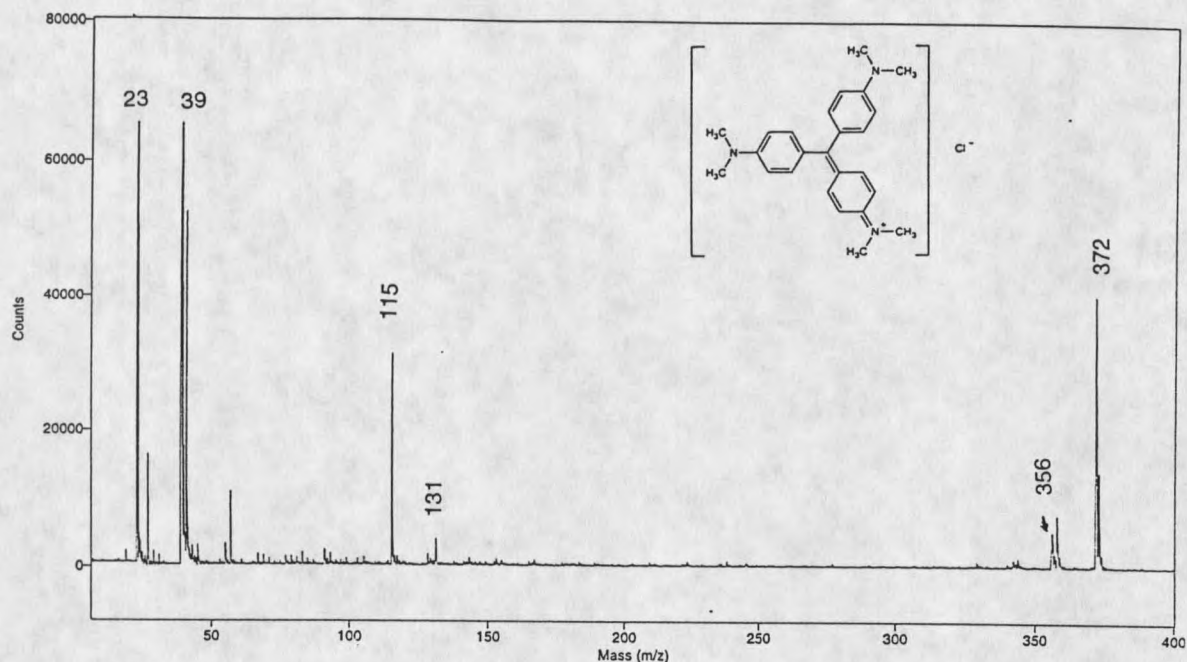


Figure 12. Immobilized AC-SALDI mass spectrum of crystal violet (0.5 pmol; 0.5 μ L, 10^{-6} M) (reflecting mode).

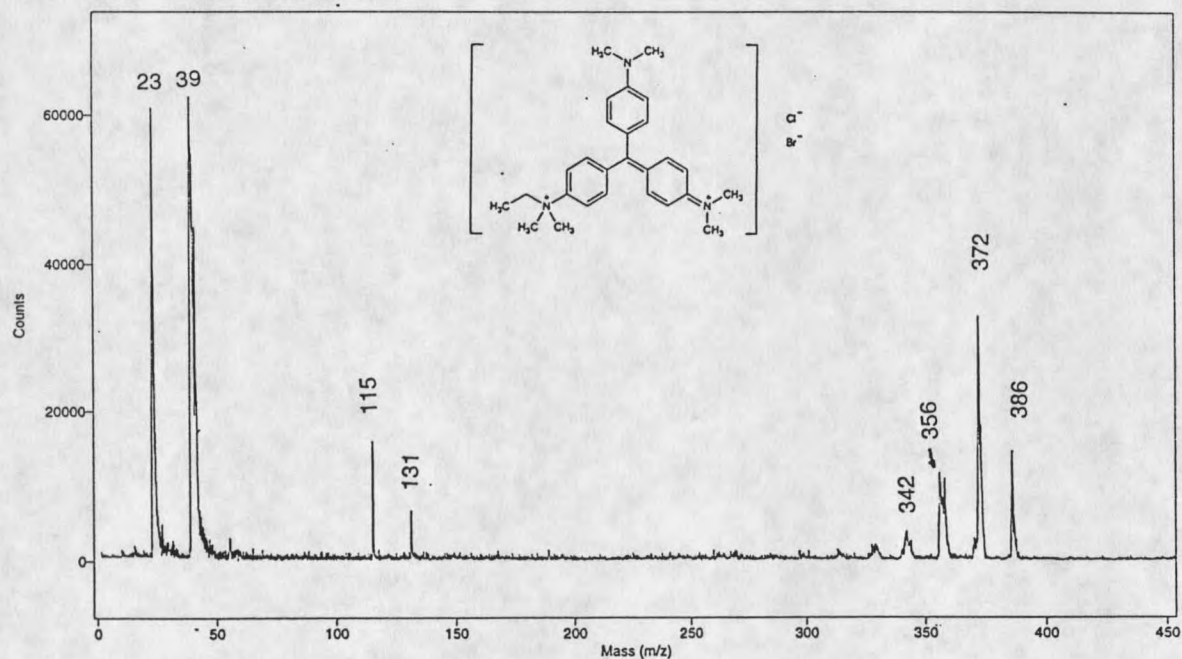


Figure 13. Immobilized AC-SALDI mass spectrum of methyl green (0.078 nmol; 0.5 μ L, 0.156 mM) (reflecting mode). Structure obtained from Merck Index.

for Figure 5, the relatively high intensity of the " K^+ " isotope peak ($m/z = 41$) is due to saturation of the detector at $m/z = 39$. The mass spectrum in Figure 13 was obtained from 0.078 nmol (0.50 μ L, 0.156 mM) methyl green. It is difficult to obtain M^+ ions ($m/z = 387$) from this compound due to a strong cationic dealkylation (Scheifer et al., 1983). Fragments ions at $m/z = 386, 372, 356$ and 342 were due to loss of H^+ , CH_3^+ , CH_4 followed by CH_3^+ and $CH_3CH_2NH_2^+$. Figure 14 shows a negative ion mass spectrum of a diluted (1:10) commercial red food color sample. This mixture include FD&C Red No. 40, FD&C Red No. 3 and some additives such as propyleneglycol and propylparaben. Both M^- for FD&C Red 40 ($m/z = 450.4$) for and FD&C Red 3 ($m/z = 833.9$) are seen in Figure 14.

Immobilized AC-SALDI Mass Spectra of Surfactants

SDS and LDAO surfactants are widely used in biochemistry to solubilize peptides and proteins (Rosinke et al., 1995). Because mass spectrometry is a good method for identifying those compounds, knowing the effects of these surfactants on the mass spectra is very important. Figure 15 shows a negative mode mass spectrum of SDS. An M^- ($m/z = 265.4$) was observed. Figure 16 shows a series of mass spectra of LDAO obtained at different concentrations. It is seen that, the lower the concentration, the fewer the peaks (Figure 16, a-d). The peaks of $m/z = 230.4, 253.4, 268.4, 460.3$ and 481.8 were due to MH^+ , $Na^+(M)$, $K^+(M)$, $[2M+H]^+$ and $[2M+Na]^+$. Other peaks at $m/z = 259, 489, 511, 517$ probably came from impurities.

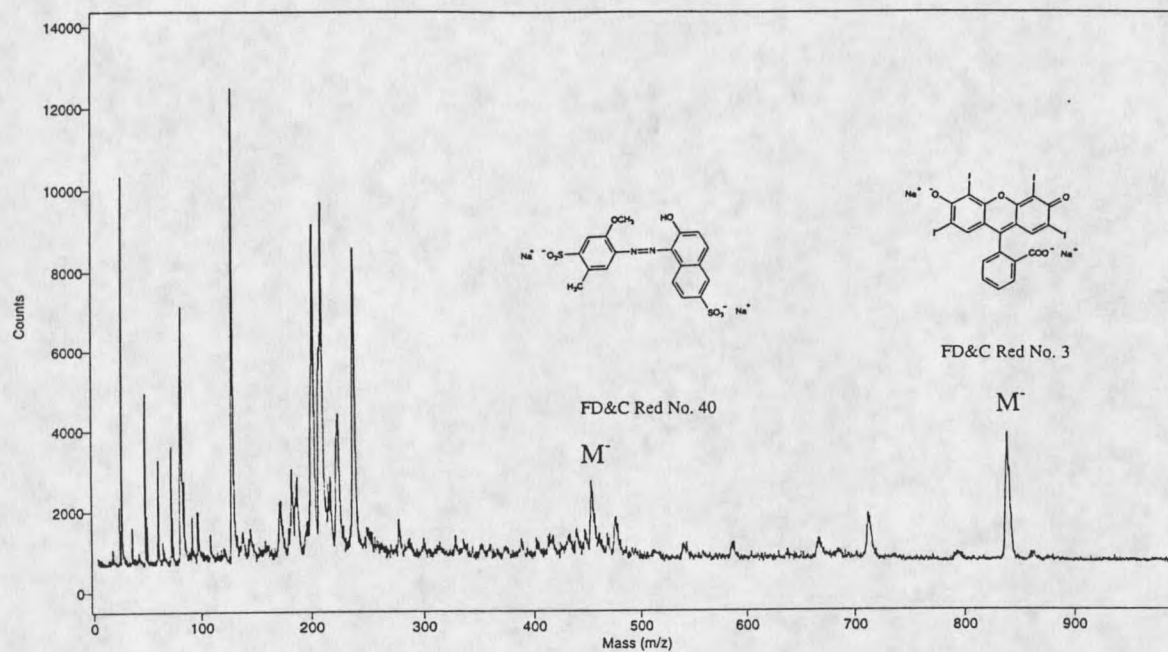


Figure 14. Immobilized AC-SALDI mass spectrum of FD&C Red No. 40 and FD&C Red No.3 mixture (1:10 dilution).

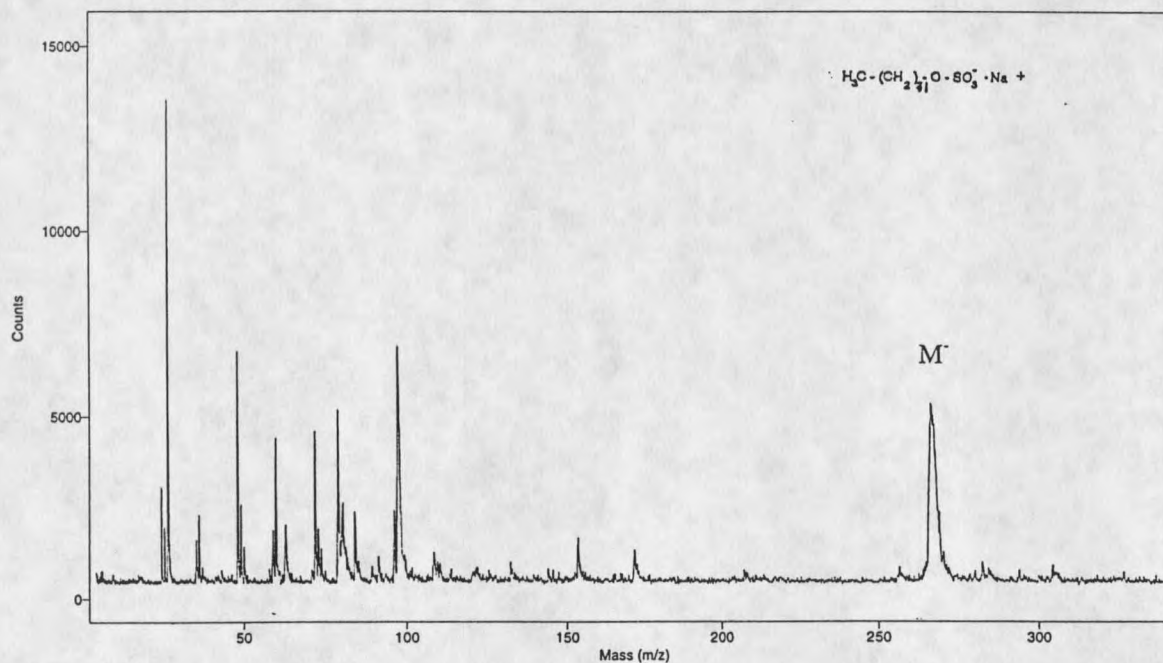


Figure 15. Immobilized AC-SALDI mass spectrum of SDS (1 µg).

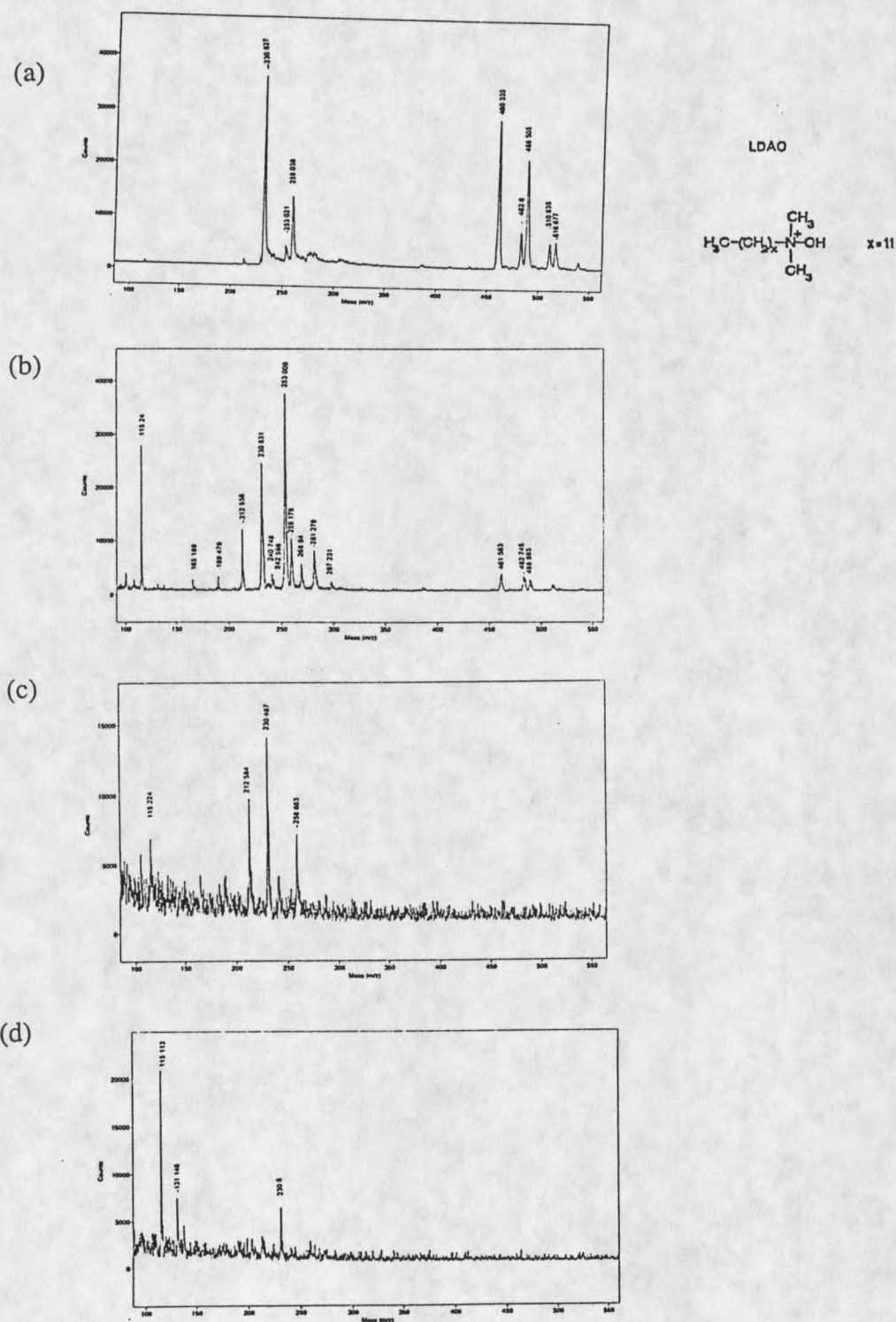


Figure 16. Immobilized AC-SALDI mass spectra obtained from different concentrations (w/v) of LDAO (MW = 229.4) aqueous solution (a) 30% LDAO (b) 3% LDAO (c) 0.3% LDAO (d) 0.003% LDAO.

Effects of Adding Metal Salts

Alkali salts are common contaminants and may be present in solvents, adhesives, and activated carbon particles. In order to study how the salts affect the SALDI mass spectra, some metal salts were added in a series of experiments. Three different methods of adding the salts (a-c) and different concentrations of the salts solutions were used. Caffeine, a basic drug, was used as a model analyte for these experiments. All experiments were performed with a 10 mg/ml (0.047 M) caffeine in water solution. External mass calibration was used.

First, different methods of adding salts to the AC substrates were tried. In method a, a caffeine solution was mixed with the same volume of 0.01 M NaCl; 1 μ L of this mixture was pipetted onto the AC patch, and 1 μ L of 20% glycerol in methanol was added. No good quality mass spectrum was obtained. In a second experiment, the same procedure was used except that the caffeine solution was mixed with the NaCl solution at a 2:1 (v/v) ratio. This time, an intense sodium caffeine adduct ($m/z = 217$) peak was observed (Figure 17). In method b, 0.50 μ L of the caffeine solution was first pipetted onto the AC patch, then 0.50 μ L 0.01 M NaCl was added. Finally, the glycerol matrix solution was added as before. By this method, good quality mass spectra were obtained (Figure 18). The intensity of the sodium caffeine adduct peak ($m/z = 217$) is much higher than that of the protonated peak ($m/z = 195$). In method c, the same procedure

was used as in method b except that the NaCl solution was applied first, before the caffeine solution. A very noisy mass spectrum was obtained (not shown) by this method. These results were reproducibly obtained samples.

The above experiments were repeated, but using another salt, 0.10 M AgNO₃. Unlike NaCl, as seen in Figures 19 and 20, the protonated peaks were more intense than the cationized peaks. With method a, in which the caffeine solution was mixed with 0.10 M AgNO₃ in a 1:1 ratio (v/v), a noisy mass spectrum was obtained (Figure 19). As can be seen in Figure 19, the intensity of MH⁺ (m/z = 195) is slightly higher than of the Ag⁺(M) isotope peaks (m/z = 301, 303). The same observation was made with method b, Figure 20. It is seen in Figure 20 that the intensity of MH⁺ (m/z = 195) is about 3 times higher than that of Ag⁺(M) (m/z = 301, 303). A sodium caffeine adduct peak is also observed (m/z = 217). Peaks at m/z = 221, 248 250 and 253 were probably due to impurities. With method c, the mass spectrum was very similar, but of inferior quality.

The concentration dependence of the salt effects on the mass spectra was also studied. Three different concentrations of NaCl (1 M, 0.10 M, 0.01 M) and different amounts of AgNO₃ (5 nmol, 50 nmol, 100 nmol) were used. It was found above that, method b was the best choice for getting good quality mass spectra for both NaCl and AgNO₃ so this method was used. The highest concentrations of NaCl, 1 M and 0.10 M, did not yield good mass spectra. The lower concentration, 0.01 M NaCl, did yield a good sodium adduct (m/z = 217) ion peak, as shown in Figure 18. With AgNO₃, mass spectra were obtained from all three different amounts (Figure 21, a-c). By comparing the spectra, one can see that the intensity of the cationized caffeine peaks (m/z = 301, 303) increased with increasing amount of AgNO₃. However, the cationized peak still

had a lower intensity than that the protonated peak ($m/z = 195$). In these experiments, it was not found that the salt addition improved the sensitivity dramatically, as Dale et al. reported (Dale et al., 1996).

From the sodium salt addition experiments, one can see that, if the concentration of NaCl is higher than of the analyte and if the salts were applied prior to the analyte, good quality mass spectra were not obtained. Possibly the AC particles had already been "saturated" by the salts, and were not able to efficiently adsorb the analyte. Therefore, the order of adding salt and analyte to the AC substrate is very important for the quality of the mass spectra.

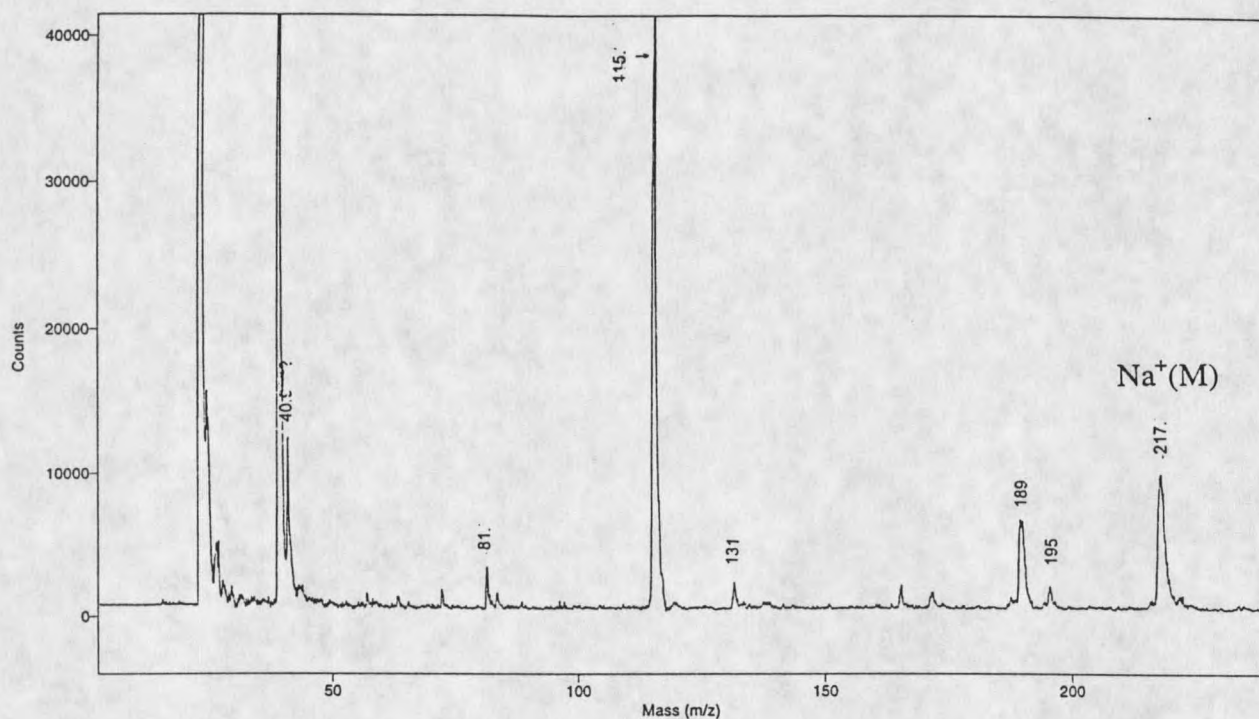


Figure 17. Method a. Caffeine (10 mg/ml, MW = 194.2) was mixed with 0.01 M NaCl (2:1), 1 μL mixture was applied to AC patch.

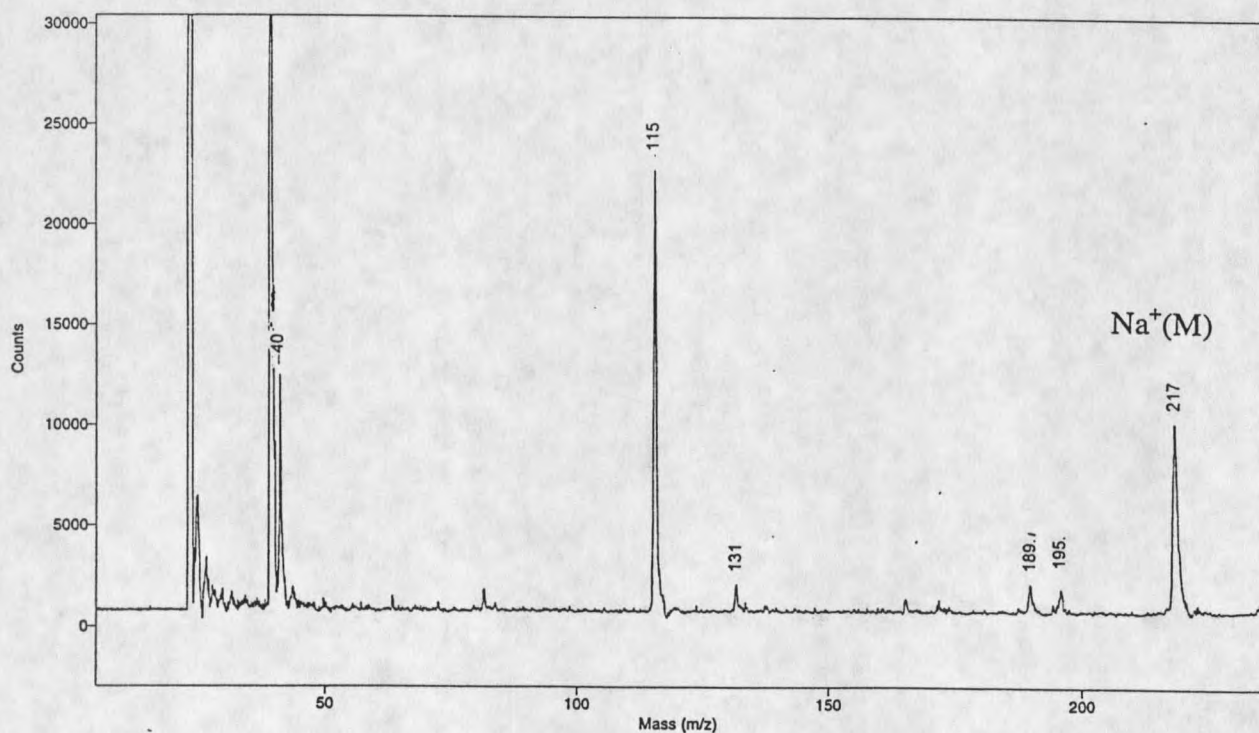


Figure 18. Method b. A volume of 0.5 μL Caffeine (10 mg/ml, MW = 194.2) was pipetted onto AC patch, then 0.5 μL 0.01 M NaCl was added.

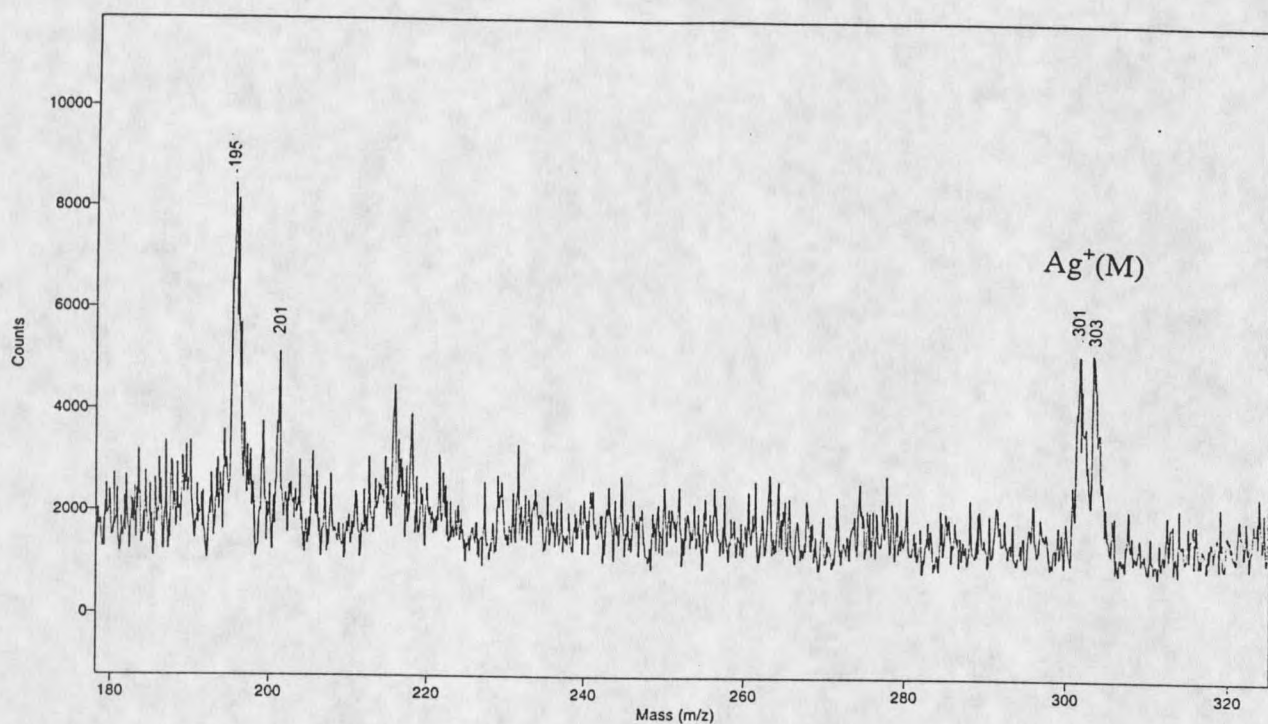


Figure 19. Method a. Caffeine (10 mg/ml, MW = 194.2) was mixed with 0.1 M AgNO₃ (1:1), 0.5 μ L mixture was applied to AC patch.

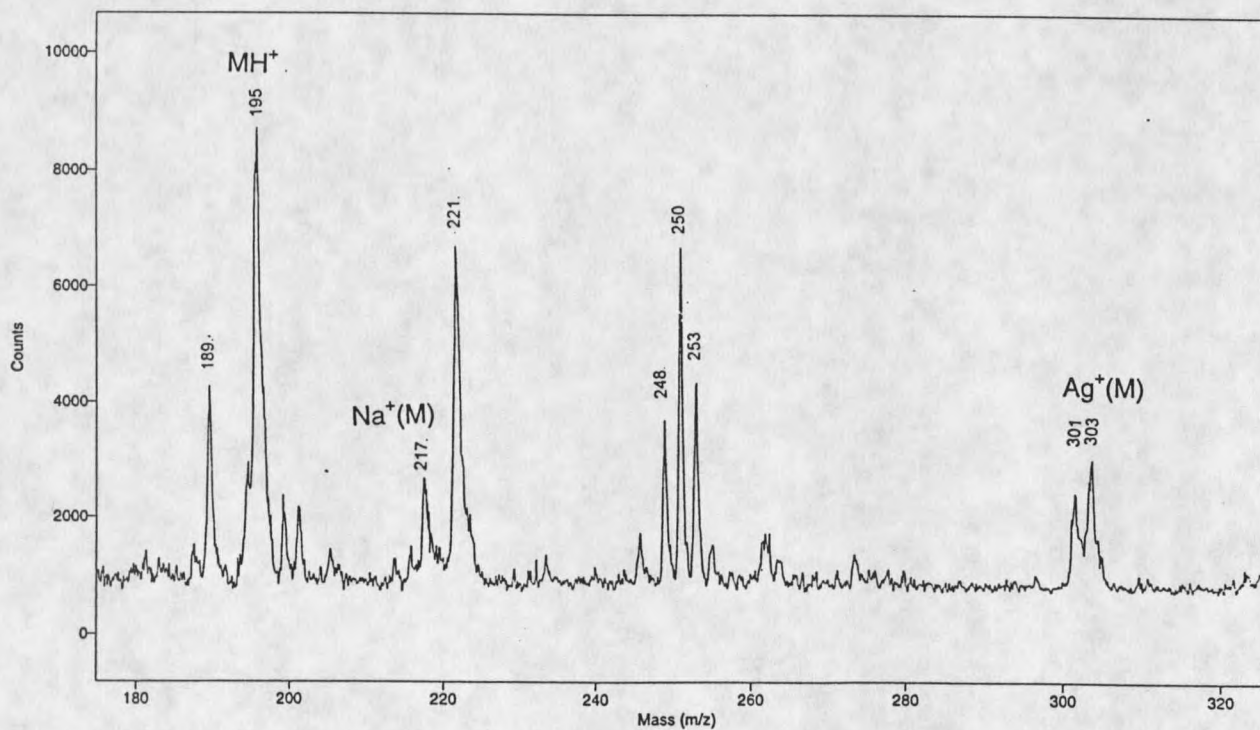


Figure 20. Method b. A volume of 0.5 μ L Caffeine (10 mg/ml, MW = 194.2) was pipetted onto AC patch, then 0.5 μ L 0.1 M AgNO₃ was added.

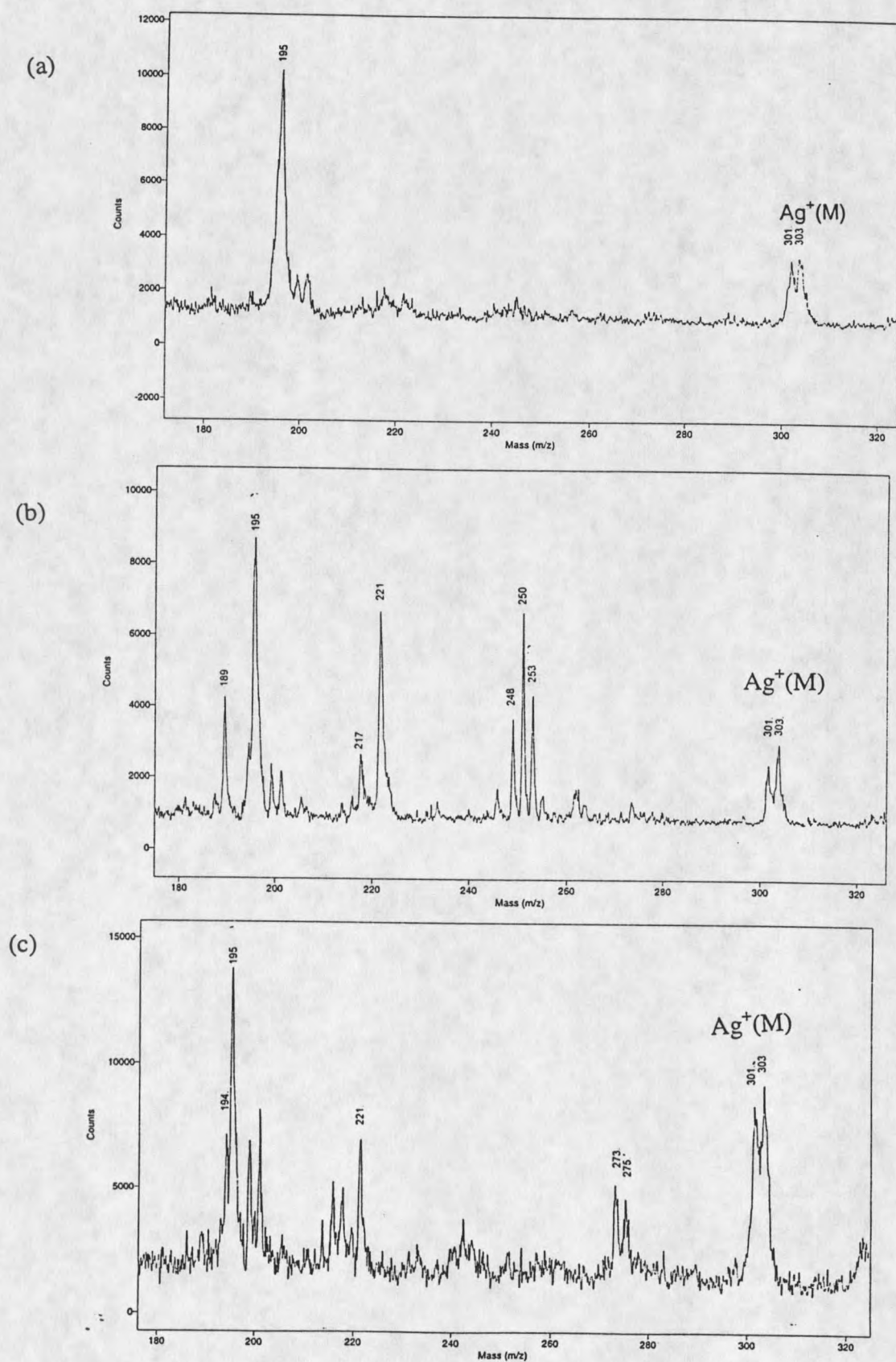


Figure 21. Effect of different amounts of AgNO_3 . (a) 0.5 μL 0.01 M (b) 0.5 μL 0.1 M (c) 1 μL 0.1 M.

Effect of Varying the Amount of Glycerol

The functions of the liquid matrix in LD was discussed in Chapter 1. In previous SALDI work (Sunner et al., 1995; Chen et al., 1997), it was found that the ratio of graphite to glycerol influenced not only the quality of the mass spectra, but also the relative importance of analyte protonation to cationization (Chen et al., 1997). When the carbon to glycerol ratio was high, cationization was preferentially observed, and when the carbon to glycerol ratio was low, protonation was preferentially observed. Dale et al. Reported that an excess of liquid matrix minimized thermal decomposition of analyte during the laser-induced desorption process, and that a low concentration of liquid matrix can cause more fragmentation.

The effect on the SALDI mass spectra of different amount of glycerol deposited on the AC strip was studied in a series of experiments. The AC strip was cut into small patches, about 3 mm×3 mm, and attached to the TOF sample plate. A 0.50 μL volume of a 10^{-4} M crystal violet solution was pipetted onto each patch. The patches were covered with 1 μL of 10%, 20%, 50% glycerol in methanol solutions. After 5-10 minutes, to let the methanol evaporate, the patches were brought to the mass spectrometer. The more glycerol used, the longer it takes to reach the required vacuum. For example, it takes about 10 minutes to reach 10^{-6} Torr when using the 20% glycerol matrix solution but it takes about 20 minutes when using 50% glycerol matrix solution.

The mass spectra obtained are shown in Figure 22, a-c. The M^+ ion ($m/z = 372$) are observed in all of them. The peaks at $m/z = 356, 342, 329$ and 313 were due to

fragmentations, the peaks at m/z 100, 102, 189 and 260 were due to impurities. Peaks at $m/z = 59$ and 75 were due to glycerol decomposition as discussed in Figure 5. From these spectra one can see that less glycerol leads to more fragmentation (Figure 22a). This is in agreement with the observations of Dale (Dale et al., 1996) and of Chen (Chen et al., 1997). Unfortunately, excess glycerol did not guarantee that a high quality mass spectrum was obtained (Figure 22c). In our experience, it is very hard to obtain a good resolution mass spectrum with either too little or too much glycerol. Only intermediate amount of glycerol (20% glycerol in methanol matrix solution) will give a good quality mass spectrum with respect to both intensity and resolution (Figure 22b). The reason is unknown.

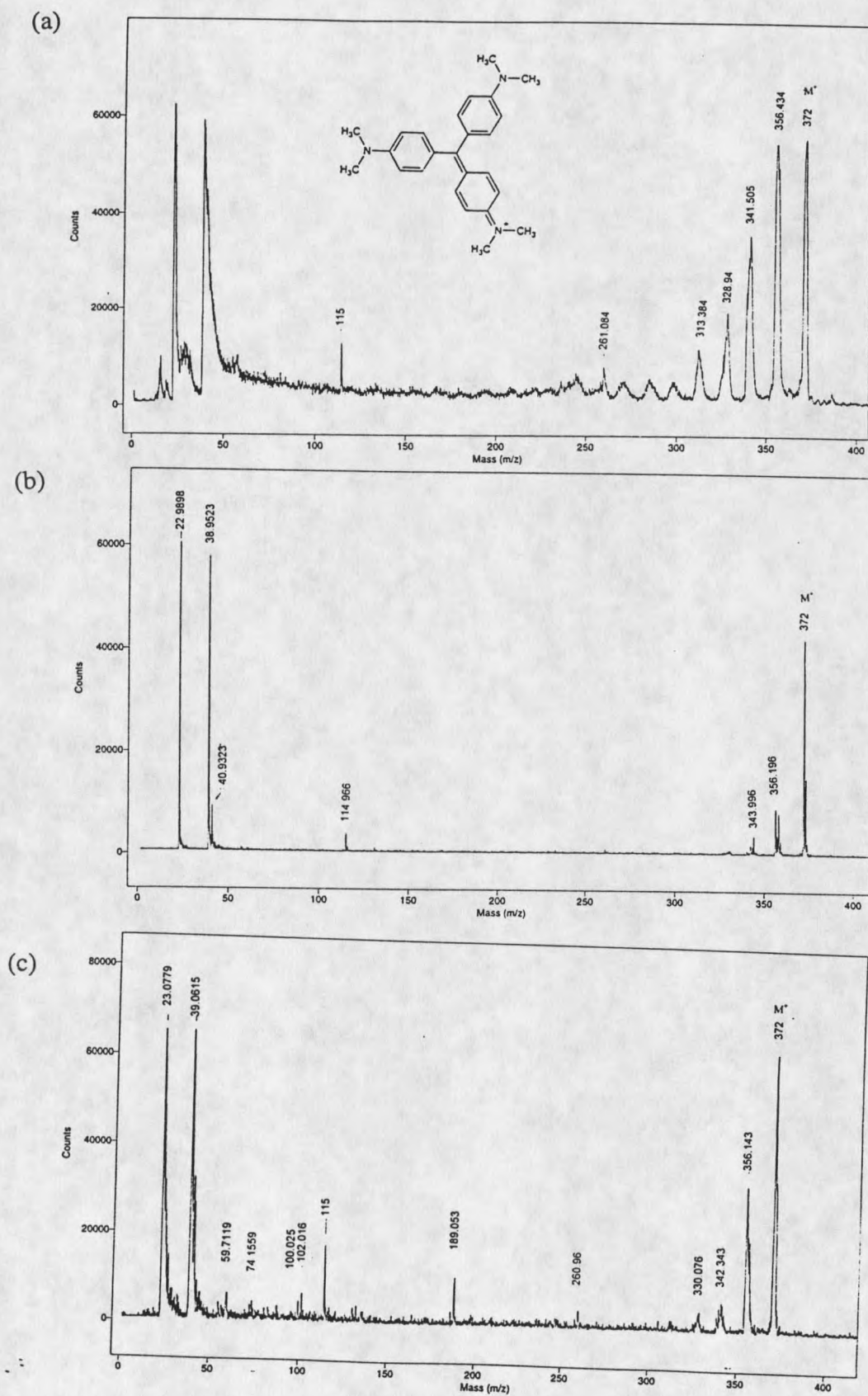


Figure 22. Different amounts of glycerol were applied to 0.5 μ L 10^{-4} M crystal violet sample patches (a) 10% glycerol (b) 20% glycerol (c) 50% glycerol.

Conclusions

From the results above, it is clear that immobilized AC-SALDI can be used to detect different kinds of small molecules, such as amino acids, peptides and organic compounds. The sensitivity of the method is in the picomole and femtomole range (Figure 12). For some compounds, such as crystal violet, the detection limit can be as low as 1.5 fmol at $S/N = 3$. It is better than the sensitivity of graphite-SALDI (Sunner et al., 1995), which is in pico- to nanomole range. The mass resolution can be improved to $m/\Delta m \approx 900$, observed for crystal violet. For other compounds the mass resolution varied from 400-800. By incorporating modern techniques, such as delayed ion extraction (Jushasz et al., 1996), the mass resolution is likely to reach several thousand, at least. The sample preparation process is simple and fast. AC substrate could be premade in bulk quantities and the analyte solution is simply added. There is no critical sample preparation step as in MALDI. It can be used as a routine analytical technique.

CHAPTER 5

THIN LAYER CHROMATOGRAPHY – SALDI/MASS SPECTROMETRY

Introduction

Chromatography is known as a powerful separation method that is widely used in many branches of science (Fried et al., 1996). The word chromatography was derived from the Greek word “chroma” meaning “color ” and “graphein” meaning “to write ”. Chromatography was first developed by the Russian botanist, Michael Tswett (Tswett et al., 1903) who found that various plant pigments such as chlorophylls and xanthophylls can be separated by passing a solution of the pigments in petroleum ether through a finely packed calcium carbonate glass column. Subsequently, several different kinds of chromatography have gradually been developed.

Thin layer chromatography (TLC), a method that separates analytes according to their polarities, is a simple and fast separation method used in many laboratories. It was introduced by Stahl in the 1950's (Stahl et al. 1956 & 1958 & 1959). Stahl made great contributions to procedures, standardization of materials, nomenclature, documentation,

applications, and solvent system selection. His book "Thin Layer Chromatography" (Stahl et al., 1969) is still being used as a classical TLC laboratory handbook.

A TLC plate makes use of a thin (0.1-2.0 mm) and flat layer of a sorbent material, such as silica gel, alumina, cellulose or polyamides, that is either self-supporting or is coated on a glass, plastic, or metal surface. The sorbent material is called the "stationary phase". To accomplish a TLC chromatographic separation, a liquid transporter, which is called the "mobile phase", is also needed. An analyte solution is applied to the plate "origin" either as a small spot or a narrow zone (Figure 23). After the sample solvent has evaporated from the plate into the air, the plate is put into a sealed developing chamber with one end of the plate immersed in the mobile phase. This so-called "developing solvent" move through the stationary phase by capillary force. Before the solvent front reaches the opposite end the TLC plate, the plate is removed and dried. The separation of the analytes takes place as every component in the sample has the same developing time but has different migration distances (Figure 23). There are several methods to visualize the components. Colored compounds can, of course, be seen directly in ambient light. For colorless compounds, there are basically two categories: Compounds, that can show self-absorption at 254 nm, can be visualized by using a TLC plate with a "fluorescence indicator" in the sorbent. A quenched fluorescence at the sample spot will be found under a UV lamp. If the compounds can be excited by 365 nm light, to produce fluorescence, they can be detected directly under the UV lamp. Other methods of visualization are based on chemical reactions. For example, amino acids or peptides can be visualized by ninhydrin. When amino acids or peptides have been separated on the TLC plate, the plate is first dried, and a 0.2%

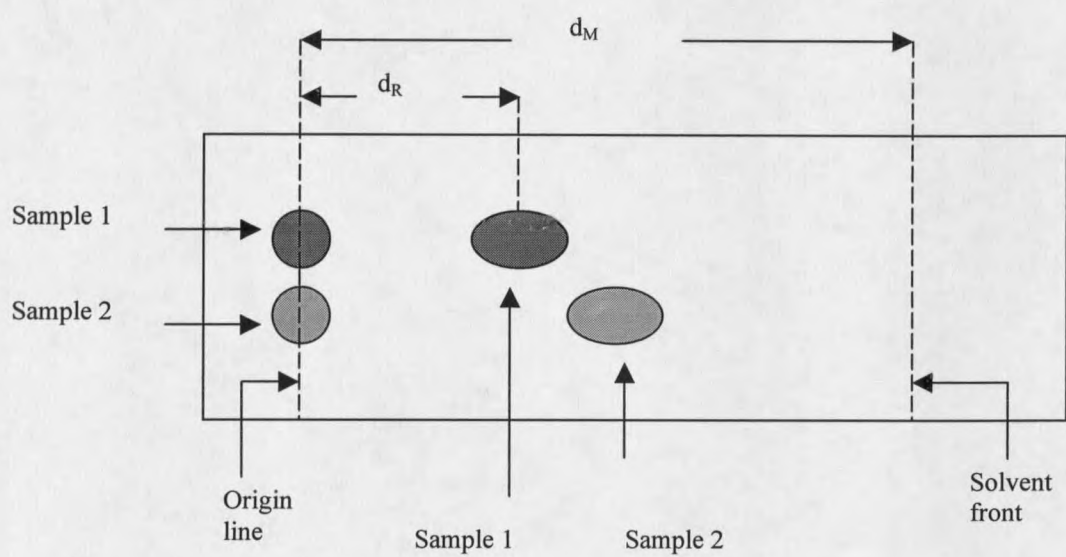


Figure 23. The thin-layer chromatographic parameters used in the calculation of the retardation factor, R_f .

ninhydrin in ethanol solution is sprayed onto the plate. After the ethanol has evaporated, the plate is heated. The analyte spots will show a purple color. This is a characteristic reaction for visualizing amino acids or peptides. There are other reagents, such as iodine vapor, 50% aqueous sulfuric acid, rhodamine B, etc. that are used to visualize a range of colorless organic compounds (Fried et al., 1986).

With the rising popularity of High Performance Liquid Chromatography (HPLC) in the 1970's, the rapid growth of TLC slowed down. HPLC is widely used because of its high sensitivity, ready adaptability to accurate quantitative determinations, and widespread applicability to different substances. Although both HPLC and TLC are capable of separating the same type of compounds, HPLC is considerably more efficient. The number of theoretical plates in conventional TLC is around 600 (Fried et al., 1986). Typical HPLC columns (25 cm in length, 4.6 mm in inside diameter and packed with 5 μ m particles), however, contain 40,000 to 60,000 theoretical plates per meter (Skoog et al., 1998). There is a misconception that TLC will be replaced by HPLC, but this is actually not correct. TLC still has its unique advantages compared to HPLC. The most important fact is that in TLC, the mobile phase totally evaporates before detection, and this increases the choices of mobile phases. It can be a single solvent, or it can be a mixture of several solvents with certain ratios. When choosing a solvent for HPLC, the UV absorbing properties, purity, and acid/base properties of the mobile phase must be considered. Therefore, the development and detection steps in TLC are much more flexible and versatile than in HPLC. Also, separation of samples in parallel leads to a much higher sample throughput (short analysis time) and less cost per analysis in TLC. Because the samples are run simultaneously under identical

conditions, it is easy to handle data statistically. Because the stationary phase is used only once, the TLC system provides simultaneous sample separation and cleanup. Thus, the sample to be analyzed by TLC requires a smaller number of sample preparation steps than for HPLC. In addition, all the components of the sample will show up on the TLC plate while in HPLC only those portions of the sample that manage to pass through the column can be detected. Thus, there is not a problem of "missing" a substance during the analysis process as in HPLC. Finally, it is easy to perform 2-dimensional, or even multidimensional separations in TLC (Poole et al., 1995). This is impossible in HPLC when using a single column.

With the appearance of High Performance TLC (HPTLC) and recent improvements in modern instrumentation for TLC and HPLC (Poole et al. 1994), the number of theoretical plates was increased to 5000 or more (Fried et al., 1986). HPTLC is defined as TLC that incorporates a combination of improvements in several aspects of TLC (Castanzo, 1984). These improvements include optimized plate coating materials, the method of applying sample to the plate, and novel procedures for conditioning the TLC plate. In some respects, TLC gives better selectivity and efficiency than HPLC (Costanzo et al., 1984). However, this does not mean that TLC can replace HPLC. In reality, TLC and HPLC are complementary separation techniques. TLC has difficulty with the analysis of volatile compounds and compounds sensitive to air or light. TLC sometimes can serve as a guide to the development of optimal conditions for column liquid chromatography.

Overall, TLC is a very useful separation technique due to its advantages, such as simplicity, fast separations, good separation of mixtures, and cost effectiveness. About

1000 scientific papers on TLC (Fried et al., 1986) appear each year ranging over a variety of sciences. Numerous chemical and biological journals, such as *Journal of Chromatography*, (Parts A and Part B), *Journal of Chromatographic Science*, *Chromatographia*, *Analytical Chemistry*, and *Analytical Biochemistry*, *Planar Chromatography*, regularly publish articles on TLC. *Analytical Chemistry* reviews new advances in the theory and practical applications of TLC methods every two years (Sherma et al., 1998).

TLC methods have been successfully used in many research and development areas, for example in the drug industry for the determination of product quality. It has also been widely used in clinical laboratories, environmental analysis, food analysis and forensic toxicology. Fried and Sherma give a detailed description of the applications in their book (Fried et al., 1996). TLC is used in bacteriology to study microbial lipid compositions and microbial lipases. In plant science, numerous compounds in plants are detected by TLC. In parasitology, TLC has been used as analytical and preparative techniques (Fried et al., 1996). Due to these many application areas, it has been estimated that at least as many analyses are performed by TLC as by HPLC (Touchstone et al., 1993). In addition, since the coupling of TLC with spectroscopic methods (such as TLC-UV-VIS, TLC-MS, and TLC-FTIR) has been developed, the applications have dramatically improved.

In our work, we are interested in the coupling of TLC to Mass Spectrometry. First, the basic principles of TLC will be introduced in the following section.

Theory of TLC

The TLC separation needs an interaction of analytes in both the stationary phase and the mobile phase. The stationary phase is usually inorganic (sometimes organic material with porous structure and relatively high specific surface areas are used). The mobile phase in TLC can be any kind of solvent or solvent mixture system.

The term "retardation factor" (R_f) is used to characterize the migration of an analyte in TLC. It is defined as $R_f = d_R / d_M$ (Skoog, 1998), where d_R refers to the linear distance that a substance has traveled from the original sample spot ("origin line") and d_M refers to the linear distance that the solvent has traveled from the analyte deposition position (Figure 23). Values of R_f can vary from 1 to 0. When $R_f = 1$, the solute moves the same distance as the solvent front and when $R_f = 0$, the solute does not move at all.

TLC Coupled to Mass Spectrometry (MS)

As discussed above, TLC is popular as a separation tool because of its simplicity and effectiveness. However, it is difficult to obtain structural information from an unknown in TLC. If TLC is coupled with an appropriate spectrometric method, such as mass spectrometry (MS), the combination will provide a powerful system for both separation and identification. Several different mass spectrometry ionization methods have been used, such as TLC coupled to laser desorption (LD) (Novak et al., 1985), plasma desorption (PD) (Macfarlane et al., 1983), electron impact (EI) (Down et al., 1975; Henion et al., 1983), chemical ionization (CI) (Ramaley et al 1983 & 1985), fast atom

bombardment (FAB) or liquid secondary ion mass spectrometry (LSIMS) (Chang et al., 1984; Oka et al., 1992), matrix assisted laser desorption ionization (MALDI) (Gusev et al., 1995) and surface assisted laser desorption ionization (SALDI) mass spectrometry (Chen et al., 1998). These methods are based on two different approaches: direct coupling or indirect coupling. The direct coupling methods use intact TLC plates in the mass spectrometer ion source. This approach was used, for example, by Hercules et al. (Hercules et al., 1985) in their laser desorption method and by Chen et al. (Chen et al., 1998) in their SALDI method. The indirect coupling involves removing the solid phase from the TLC plate. Either the analytes are extracted from the solid phase, or the solid phase is transferred directly to the ion source. These methods will be reviewed in more detail in the following section. The detection limits reported below for these different methods should be compared to the 3 pg detection limit for crystal violet obtained in the present work.

TLC/EIMS

Using electron impact mass spectrometry to detect analytes from TLC separations was the first way of combining TLC and MS. Direct thermal evaporation is used to transport sample molecules into the mass spectrometer ion source for ionization and analysis (Down et al., 1975, Henion et al., 1983). The silica gel can be scraped off the TLC plate and the analyte extracted by a solvent (Down et al., 1975). Alternatively, a portion of the solid phase from the TLC plates can be placed into a insertion probe. Kraft et al. (Kraft et al., 1981) used polyamide TLC plates, instead of silica gel, and found that different kinds of small molecules could be detected, such as phenols,

steroids, and nucleosides. The major limitation of EIMS is that the analyte must be volatile and thermal stable. The detection limit for organic compounds is in the nanogram range (Kraft et al., 1981).

TLC/CIMS

CI and EI can use the same interface methods with TLC. The difference is that CI uses a reagent gas to accomplish the ionization. In the work of Ramaley, a TLC plate scanner was used and a pulsed CO₂ laser was employed to desorb analytes in selected spots. Chemical ionization reagent gas at 1 atm swept desorbed materials through a heated inlet system into the ion source of a quadrupole mass spectrometer (Ramaley et al 1983 & 1985). By using this system, good peak shapes and high mass resolution were obtained. The detection limit for phenanthrene was reported to be 10 ng. However, more polar compound are hard to detect with this method, and compounds with molecular weights over 300 were not observed consistently (Ramaley et al. 1985).

TLC/PDMS

TLC/PDMS use ²⁵²Cf plasma desorption mass spectrometry as a detector for non-volatile and thermally unstable biomolecules separated by TLC (Krueger et al., 1977; Macfarlane et al., 1983; Danigel et al., 1985). This ionization method is based on the passage of a very-high-energy (MeV) particle beam through a thin layer of sample material. This generates sputtered neutral molecules, as well as positive and negative ions. Krueger (Krueger et al., 1977) and Danigel (Danigel et al., 1985) indirectly coupled TLC to PDMS. A secondary extraction solvent was used to remove the analyte

from TLC plate. The extracting solution was then electrosprayed onto a thin aluminum target support foil. The detection limit for chloranphenicol and reserpine was 100 ng (Krueger et al., 1977). Very few labs use PD/MS anymore.

TLC/LDMS

Laser desorption ionization mass spectrometry has been widely used because of its convenient sample preparation and ability to directly analyze TLC samples. Thermal energy is transmitted to the surface of an intact TLC plate by a laser beam. Analytes are desorbed and ionized by this energy. No extraction solvents are used. Novak and Hercules (Novak et al., 1985) used a microprobe system, LAMMA-1000 (Nd:YAG laser, 265 nm), to sputter triphenylmethane dyes from a HPTLC plate. However, the mass spectra obtained directly from TLC plates required a high laser power that resulted in extensive fragmentation of ions. Also, the analyte ions at $m/z < 100$ were obscured by background peaks. Hercules and co-workers (Kubis et al., 1989) then used polyamide TLC plates instead of silica gel plates in order to observe low mass fragment ions ($m/z < 150$). Rogers et al. (Rogers et al., 1993) used LD to detect caffeine and theophylline, by cutting out the analyte spot from the TLC plate and attaching it to an insertion probe. In summary, LD combines with TLC without disturbing the TLC sorbent surface. No liquid matrix or extraction solvent is required. However, LD/TLC method only worked well for thermally stable small molecules (Novak et al., 1985, Rogers et al., 1993) and many molecular fragments are produced. The reproducibility is not very good (Novak et al., 1985). The detection limit is in the nanogram range (Rogers et al., 1989).

TLC/FABMS (TLC/LSIMS)

Fast atom bombardment ionization (FAB) mass spectrometry, also called liquid secondary ion mass spectrometry (LSIMS), is a widely used method for nonvolatile or thermal labile compounds. FAB is based on sputtering sample molecules from a liquid surface by the impact of a high kinetic energy (several keV) xenon or argon atom beam. The analyte is ionized, while in the condensed state or during the desorption process. A liquid matrix, such as glycerol, thioglycerol or triethanolamine, is used to continuously provide sample molecules to the surface. When coupled to TLC, the liquid matrix also extracts the analytes from the TLC gel. FAB is a relatively "soft" ionization method that induces little fragmentation. A wide range of analytes has been detected by TLC/FAB, such as drugs and drug metabolites (Chang et al., 1984; Oka et al., 1992), lipids (Kushi et al., 1985; Dunphy et al., 1988), peptides (Brown et al., 1988), and dyes (Oka et al., 1992). Pieces of intact TLC plates can be placed directly in the ion source by using a double-sided sticky tape (Chang et al., 1984) or by using a dedicated TLC plate holder (Oka et al., 1994). A problem with TLC/FAB is that when a liquid matrix is used to extract an analyte from a spot on the TLC plate, the spot spreads out. This results in lower sample concentration and loss of chromatographic resolution. Oka et al. (Oka et al., 1992) developed a method to concentrate the analyte by selectively removing the silica around the spot of interest, in a trapezoidal shape. They then used methanol, which is a high-eluting-strength solvent, to "force" the analyte to migrate to the narrow end of the remaining silica gel. The sensitivity was improved by this

method. However, its success depended on the solvent extraction efficiency. The sensitivity of TLC/FAB is in nanogram range (Busch et al., 1992).

TLC/MALDI/MS

Matrix-assisted laser desorption ionization (MALDI) is another "soft" ionization method. It is able to ionize fragile low and high molecular weight compounds without significant fragmentation. The coupling of TLC to MALDI was demonstrated by Hercules group in 1995 (Gusev et al., 1995). The method development occurred in two stages. The first stage used "direct matrix deposition" (Gusev et al., 1995). In this method, a sample was first separated on aluminum backed silica gel (or cellulose) TLC plate. After drying, the extraction solvent was sprayed on the surface of the silica gel. This served to extract the analyte, which was located throughout the depth of the gel. Finally the matrix solution was applied on top. After 10-15 minutes of solvent evaporation and matrix crystallization, the TLC plate was placed in the mass spectrometer for analysis. The detection limit at $S/N = 3$ was 2-4 ng for bradykinin, angiotensin and enkephalin derivatives. However, this method of sample preparation suffered from analyte spreading in the TLC plate during solvent extraction and matrix crystallization. Therefore, "indirect matrix deposition" (Mehl et al., 1997 & 1998), was developed in the second stage. The matrix layer was first formed on a separate smooth inert planar substrate. The extraction solvent was again sprayed onto the TLC plate in order to transfer analytes to the top of the TLC gel. After 5 minutes, the MALDI matrix substrate layer and the TLC plate were positioned face to face and a high pressure (2.0 kgmm^{-2} to 7.5 kgmm^{-2}) was applied. This pressure facilitated the transfer of matrix to

the top of the TLC plate. The TLC plate was then brought to the mass spectrometer. This method provided a good sensitivity and minor fragmentation. A low detection limit in the femtomole range for oligonucleotides separated on a cellulose TLC was achieved (Mehl et al, 1998). However, the sample preparation procedure is complicated, and the TLC plate needs prewashing prior to use. Several experimental parameters, such as the selection of extraction solvent, the extraction time and the pressure applied affect the results. A very even matrix crystal distribution is also needed. To obtain good mass spectra by this method can be very challenging (Chen et al., 1997). Also because of an intense matrix background, it is difficult to study analytes with a molecular weight lower than 400 Da.

Early TLC/SALDI

Sunner's group recently demonstrated yet another method for the direct interfacing of TLC with mass spectrometry (Chen et al., 1998). Activated carbon particles were used to extract the compounds. A liquid matrix, glycerol, was added to help the extraction. Cut-out pieces of the TLC plate were introduced into the mass spectrometer, and mass spectra were obtained from the intact plate surface. A "clean" background was obtained in the low mass range. This is a very important advantage because most TLC separations are for small molecules. A wide range of organic compounds and some peptides were detected. The detection limit for bradykinin from a developed plate was

approximately 25 ng. The mass resolution varied from 500 to a low 100 due to surface charging (Chen et al., 1998).

TLC-SALDI

There were obvious limitations to the early TLC/SALDI work. The mass resolution was relatively low and variable because of surface charging. The sensitivity was lower than for TLC/MALDI (Mehl et al., 1998). In order to overcome these problems, the use of the immobilized activated carbon substrates, described in Chapter 4, for TLC interfacing, was studied in the present work. The idea was that analytes separated on a TLC plate, should be transferred to the activated carbon storage strip. Because aluminum foil is used as a substrate, surface charging might not be problem. A very high sensitivity and good mass resolution have been obtained for the AC strip, as discussed in Chapter 4.

During the method development process, several different geometric arrangements of the AC strip and the TLC plate were tried, in order to achieve the best possible results. Both plastic-backed TLC plates, precoated with 0.10 mm silica gel with fluorescent indicator, and aluminum-backed TLC plates pre-coated with 0.20 mm silica gel 60 without indicator, were used. Aluminum and glass fiber filter paper, as substrates for the AC strips, were compared. Triphenylmethane dyes such as victoria blue and crystal violet with their strong colors were found to be convenient choices as model analytes in the initial studies of coupling TLC with SALDI mass spectrometry.

Method Development

As described above, Chen et al. (Chen et al., 1998) in their early SALDI work used AC to obtain mass spectra from intact TLC surfaces. However, the sensitivity was relatively low. Thus, the detection limit was 25 pmol of bradykinin from a developed TLC plate. One possible reason is that the extraction process might be inefficient. To examine the diffusion process, a light microscopy study was done using victoria blue. A solution of victoria blue was first deposited onto the plastic-backed TLC plate and the plate dried. A thin layer of AC suspension was applied to the gel surface above the colored spots. In the microscope, the plate was illuminated from below. A white ring, showing the removal of the dye, was seen around each particle of activated carbon while further away from the AC particles the blue dye was seen to still remain in the silica gel. The pattern clearly reflected an inefficient diffusion process. It was estimated that $< 10\%$ of the dye had been adsorbed. Clearly this caused a lowered sensitivity. A second reason for the low sensitivity was probably that the analyte was adsorbed mostly on the undersides of the AC particles which were in contact with the TLC gel. When the laser irradiated the intact TLC plate, only the analyte on the upper side of the AC particles may have been desorbed.

TLC/SALDI Using Passive Transport of Analytes to the AC Particles

In order to find a method with a more efficient passive diffusion process, several AC/TLC geometries were studied. The plastic-backed TLC plates, precoated with 0.10 mm silica gel, were used for this work. Experiment was performed to observe dye

adsorption directly from the liquid flow during TLC development. As illustrated in Figure 24, a narrow band of victoria blue was deposited on the TLC plate, and two spots of activated carbon suspension "C" (see Experimental) were applied to the gel surface about 1 cm above the dye band. The plate was then developed. The victoria blue band was seen to pass between the suspension spots only, leaving two white "tracks" after passing the activated carbon spots (Figure 24). The dye was either adsorbed by the activated carbon or it bypassed the suspension spots due to avoiding the glycerol or sucrose present in suspension "C". Therefore, the experiment was repeated, except that an AC suspension without glycerol or sucrose was used. The same amount of AC particles was added to ethanol suspension (25 mg AC in 0.10 mL ethanol). The identical observation was made as in Figure 24. This showed that the dye was indeed adsorbed by the AC.

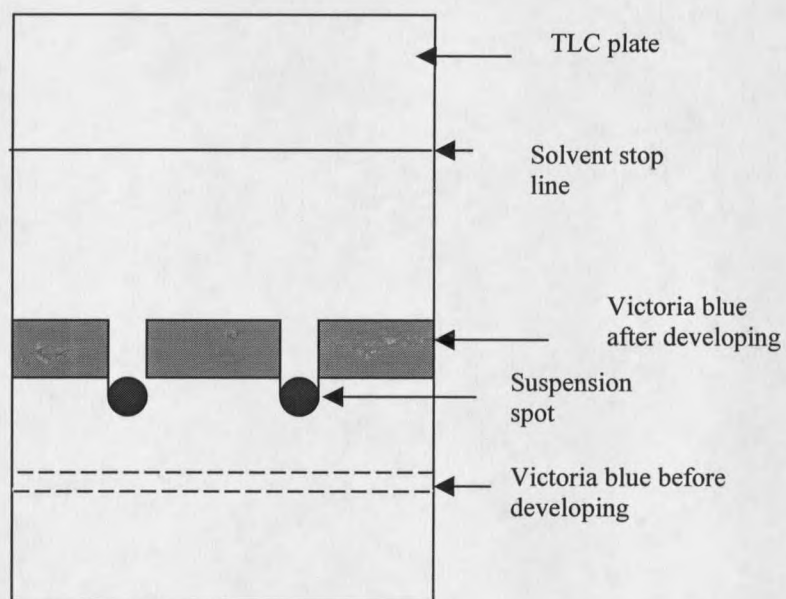


Figure 24. Victoria blue passing through the suspension diagram.

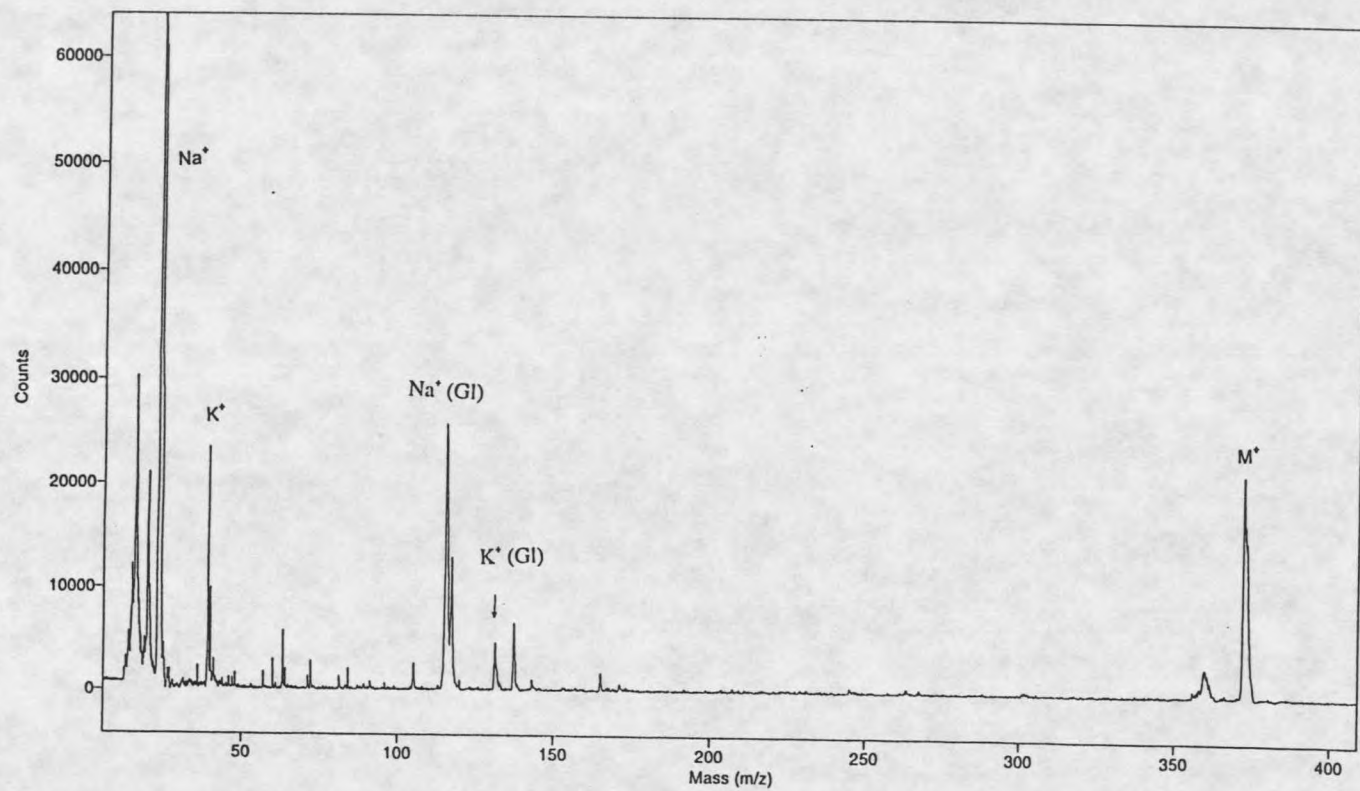


Figure 25. Crystal violet mass spectrum obtained from a TLC plate using method "A" (bottom layers of AC particles).

It was shown in the above experiments that the analytes can be adsorbed onto activated carbon directly from the liquid flow during TLC plate development. However, in order to obtain mass spectra from the AC-TLC system, the method had to be further developed. Several different approaches were studied as follows. The suspension used above was barely fluid and it was difficult to pipette to the TLC plate. Therefore, a more dilute suspension was made by adding 7.5 mg AC to 0.10 mL ethanol. In this method, referred to here as method "A", 0.50 μL of a 1.0 mM crystal violet solution was first pipetted onto the TLC plate, then about 2.0 μL of the suspension was deposited on top of the TLC gel about 1 cm above the crystal violet spot. A thick layer of AC particles was formed. The TLC plate was developed. Again the dye was seen to be adsorbed as it passed below the AC suspension spot. Next, an effort was made to obtain mass spectra. After solvent evaporation, 0.50 μL glycerol was added to the AC spot and left to seep into the AC for about 30 seconds. The area of the TLC plate was carefully cut out by sharp scissors and attached to the MALDI stainless steel sample plate using double-sided sticky tape. However, no analyte ion signal was obtained. It was considered that the dye was probably adsorbed mainly in the bottom layers of the activated carbon particles. To see whether this was the case, the top layers of AC particles were scraped off, leaving a thin bottom layer closest to the gel surface. Glycerol was added, and a very strong signal of the crystal violet molecular ion peak (Figure 25) at $m/z = 372$ was observed. (Figure 25). A fragment peak at $m/z = 357$ was due to loss of $\text{CH}_3^\bullet$. Peaks at $m/z = 115$ and $m/z = 131$ are due to sodium and potassium adducts with glycerol. Intense Na^+ ($m/z = 23$) and K^+ ($m/z = 39$) ion peaks were also observed. The suspension

was then further diluted by a factor of 2.5, and 1.0 μL of this new suspension was deposited onto the TLC plate. A thin layer of AC was formed. The plate was developed, and as before the dye was adsorbed. This time, however, mass spectra were obtained without having to scrape off the top AC layer. From these experiments, one can see that the amount of activated carbon powder deposited is an important factor in order to obtain mass spectra.

Unfortunately, the mass peaks obtained in the experiments above were broad and the mass resolution was only about 300-400. This was probably due to bad electrical contact between the AC particles, the plastic substrate of TLC plate, and the ion source sample plate, causing surface charging. Therefore, aluminum-backed TLC plates, pre-coated with 0.20 mm silica gel, were used in subsequent experiments. In method "B", the same procedures were used but to keep the activated carbon powder from dislodging from the gel surface, the plate was developed horizontally in a large developing chamber (Figure 26). One end of the TLC plate was bent down to make contact with the developing solvent. Unfortunately, and in contrast to the previous experiments, most of the dye was not adsorbed but passed through, below the applied AC suspension. It became clear that this result came from changing the TLC plates. The difference was probably due to the two plates having different gel particle sizes and thickness of the silica gel layer. Visual inspection under a microscope showed that the plastic-backed TLC plate had a granulated gel structure while the silica gel 60 on the aluminum-backed TLC plates appeared to be homogenous. Also, the silica gel layer of the aluminum-backed TLC plates was 0.10 mm thicker than that of the plastic-backed TLC plates.

These factor apparently changed the adsorption properties. The experiment was repeated using other silica 60 aluminum backed TLC plates, with a gel thickness of both 0.20 mm and 0.25 mm, and, the same result was obtained. In order to determine if the adsorption efficiency was affected by the thickness of the gel layer, another experiment was performed. On a 0.20 mm aluminum-backed TLC plate, some of the silica gel was scraped off in a small area, leaving a thin layer (about 0.10 mm). The resulting depression was filled with loose activated carbon powder (Figure 27a). Performing the same developing procedure as above, it was found that some of the dye still passed through underneath the AC spot. A control experiment was performed without AC particles. The dye still moved from the thick layer of silica gel through the thin layer and to the thick layer on the opposite side (Figure 27b). Thus, changing the thickness of the silica gel did not significantly affect the efficiency of adsorption onto AC. Clearly, it was the different silica gel structure that changed the diffusion process. The solvent flow through the silica gel is much faster than the analyte diffusion toward the AC particles. Therefore, the analyte tend to pass through underneath the AC particles.

The extraction process used above relies on a passive transport process. In addition to the problems encountered with silica gel 60, there is another problem in that the analytes are still adsorbed on the undersides of the AC particles. This was a critical point in the developing work. The higher-quality, more homogeneous gel in these plates made it less likely that analytes that passed through the gel underneath a layer of AC would actually reach the AC particles and become adsorbed. It was, however, decided that the higher quality aluminum-backed TLC plate should be used during the following

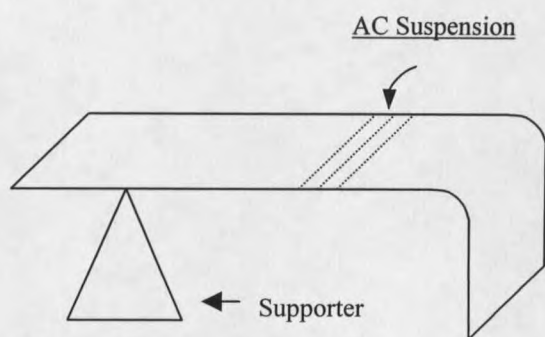


Figure 26. Horizontal development using method "B".

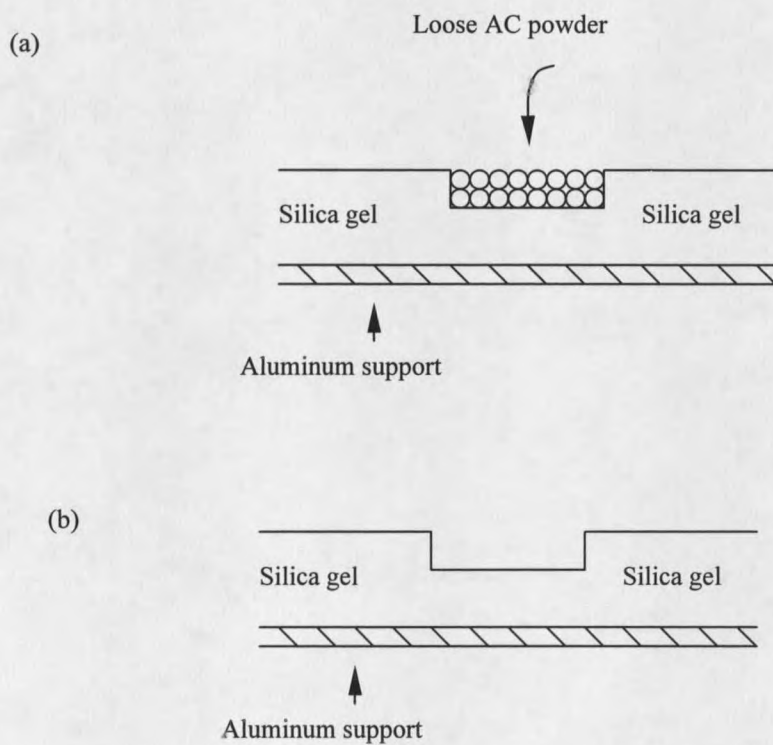


Figure 27. Effect of silica gel thickness experiment.

method developments. Because of its homogenous silica gel distribution, a better separation can be achieved. It was also decided to abandon the efforts to use AC deposited onto the gel surface. It became clear that it would be necessary to design a TLC/SALDI interface that relied on active transport of analytes to the activated carbon. These efforts are described in the next section.

TLC/SALDI Using Active Transport of Analytes to the AC Particles

In order to force the TLC analytes to flow toward the AC particle bed, a new experiment, method "C", was performed. Crystal violet was pipetted onto the TLC sample deposition line. Two lines were drawn perpendicular to the direction of TLC development and 1 cm apart. The silica gel between these two lines was removed, leaving a bare aluminum surface. Thus, a gap in the gel, with a width of 1 cm, was formed. The gap was filled with loose activated carbon particles. The TLC was developed horizontally as above. After all the dye had disappeared into the band of activated carbon particles, the TLC plate was carefully removed from the chamber and the solvent evaporated at room temperature. A 0.50 μL volume of glycerol was added to the AC bed. The analyte spot was carefully cut out and attached to the MS sample plate. A mass spectrum with good mass resolution was now obtained (Figure 28). The isotope peaks of M^+ at $m/z = 372$ and 373 were clearly resolved ($m/\Delta m \approx 800$). The improved mass resolution was likely due to the electrical contact between AC particles, the aluminum support of the TLC plate, and the ion source sample plate. These results also showed that the ability of the AC to adsorb analytes had not been compromised by some constituents in the gel in the aluminum-backed TLC plates. In additional

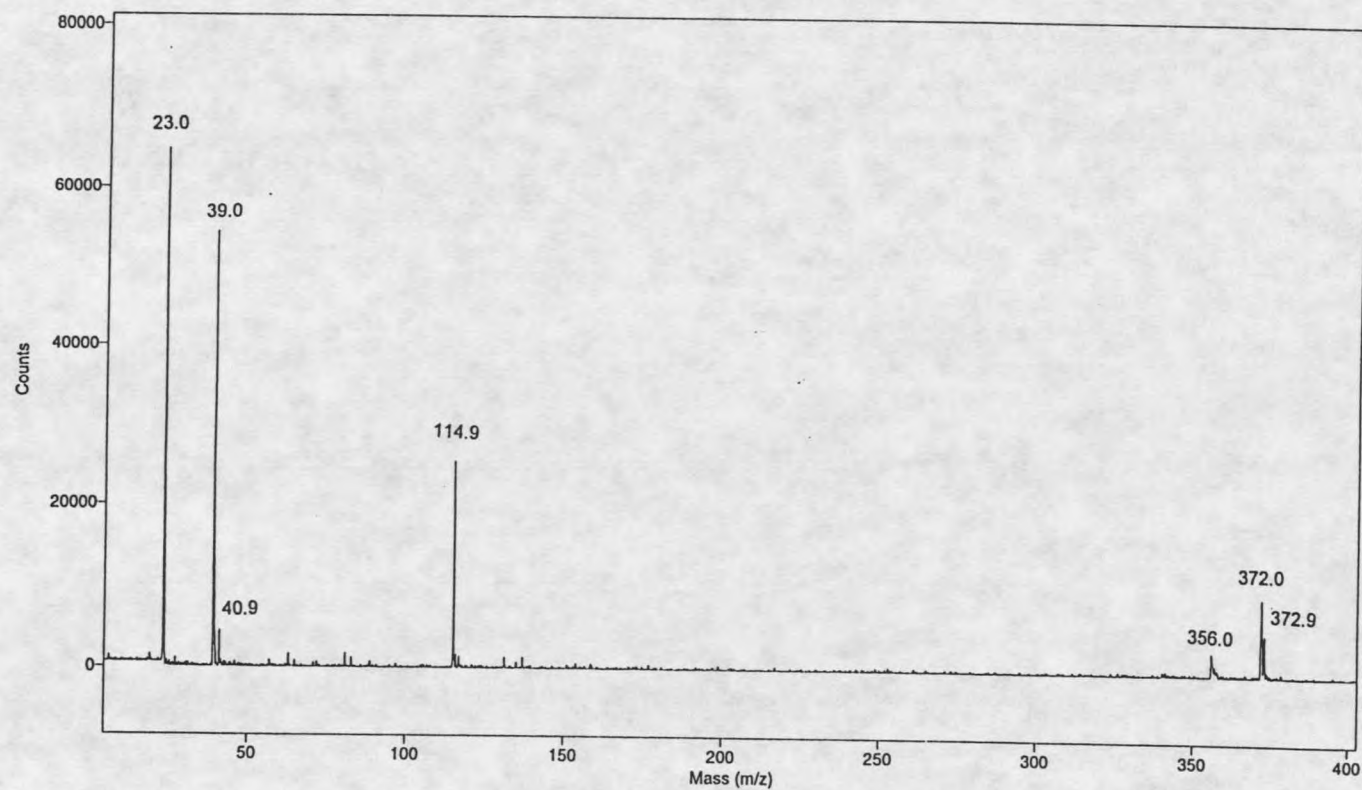


Figure 28. Crystal violet mass spectrum using method "C".

experiments, the same procedure was repeated except that the AC particles filled the gap only half way through the gap instead of the whole gap. Essentially the same mass spectra was obtained.

There were two major problems with the active transport method, "C". First, the loose AC powder causes serious ion source contamination (Chapter 4). Second, the horizontal developing process was inconvenient. The powder was easy to dislodge from the gap when the TLC plate was removed from the developing chamber. Therefore, an arrangement for immobilizing the AC powder to the TLC support was considered next.

In method "D" (Figure 29), a gap in the gel was first made, as in method "C". Then a "mask", which was made by cutting out a 1 cm wide, variable length, rectangular area from a paper board, was used to cover the TLC plate. The open rectangular area was positioned above the gap. Contact adhesive was then sprayed onto the assembly. After removing the mask, AC particles were added to the adhesive, and pressed into the adhesive with a spatula for about thirty seconds. The loose powder was blown off. The same vertical developing procedure was performed as in method "A". It was found that the crystal violet was adsorbed. However, the adhesive was very sticky at the edges of the gap in the TLC plate, and when applying the AC powder to the sticky band, it was difficult to keep the plate surface clean from AC.

Indirect coupling (i.e. the removal of analytes from the TLC plate prior to MS sample plate) of TLC to SALDI/MS was considered next. In method "E" (Figure 30), an AC strip as described in Chapter 4 (Figure 3) with a width of 2-4 mm and the same length as the width of the TLC plate was made. A gap in the gel was made as described in method "C", except the width of the gap was 2-4 mm. The adhesive was sprayed over

the gap, using a same-sized "mask". The aluminum side of AC strip was then stuck to the adhesive by pressing down the strip. The AC particles faced up. The assembly was vertically developed. However, most of the crystal violet was seen to "stop" at the edge of the gel facing the AC strip. This was probably due to the capillary forces of the AC bed being too weak to "pull" the solvent through.

Finally, method "F", was developed. The method was identical to "E" except that the AC strip was turned up-side-down. The strip was made with a width of 2-4 mm, and the length was 10 mm longer than the width of the TLC plate. The two ends (about 5 mm on each side) of the strip were free of AC particles. The same gap in the gel was made as in method "E". The strip was pushed up against the edge of the gel, with the AC side facing down towards the aluminum support. The two ends of the AC strip were folded over the edge towards the back of the TLC plate, as shown in Figure 31(a-d). The plate was developed. In contrast to method "E", the solvent was found to pass through the AC bed. The dye analyte migrated into the strip and was adsorbed by the AC. Like the water that is "pulled in" between two parallel glass plates by the affinity between water and glass, the two parallel "plates" of aluminum foil and adhesive probably served to increase the strength of the capillary forces. The assembly was dried and the AC strip removed. Glycerol liquid matrix was added. However, it was found that it was difficult to wet the strip with pure glycerol after development. In contrast, freshly prepared strips wet easily. The reason is unknown. A 20% glycerol in methanol worked much better. The methanol was allowed to evaporate for 10-15 minutes in ambient air, and the plate inserted into the ion source. Good quality mass spectra with a strong analyte ion signal, "clean" background, and good mass resolution, were obtained.

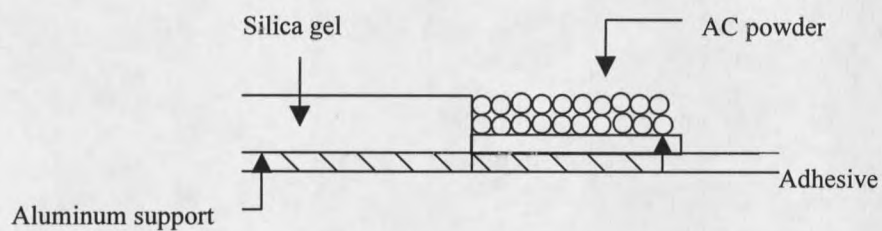


Figure 29. Method "D".

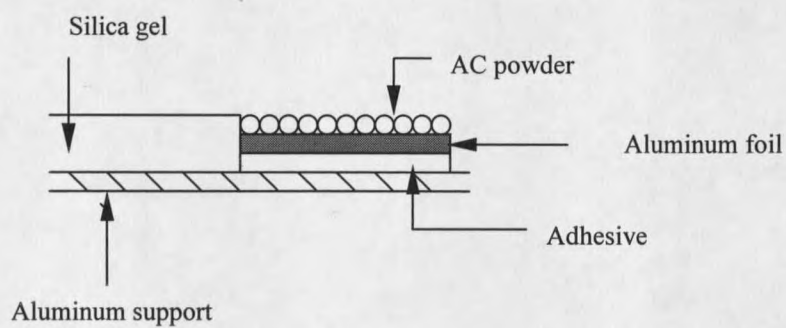


Figure 30. Method "E".

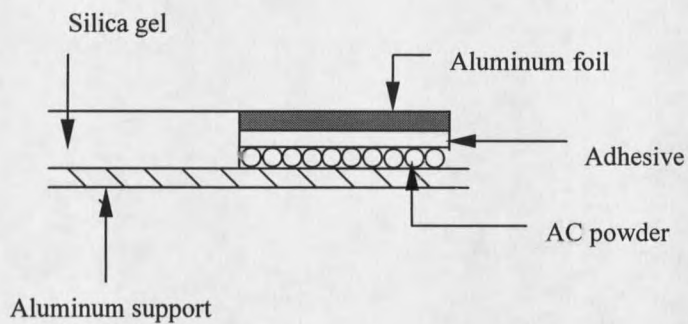
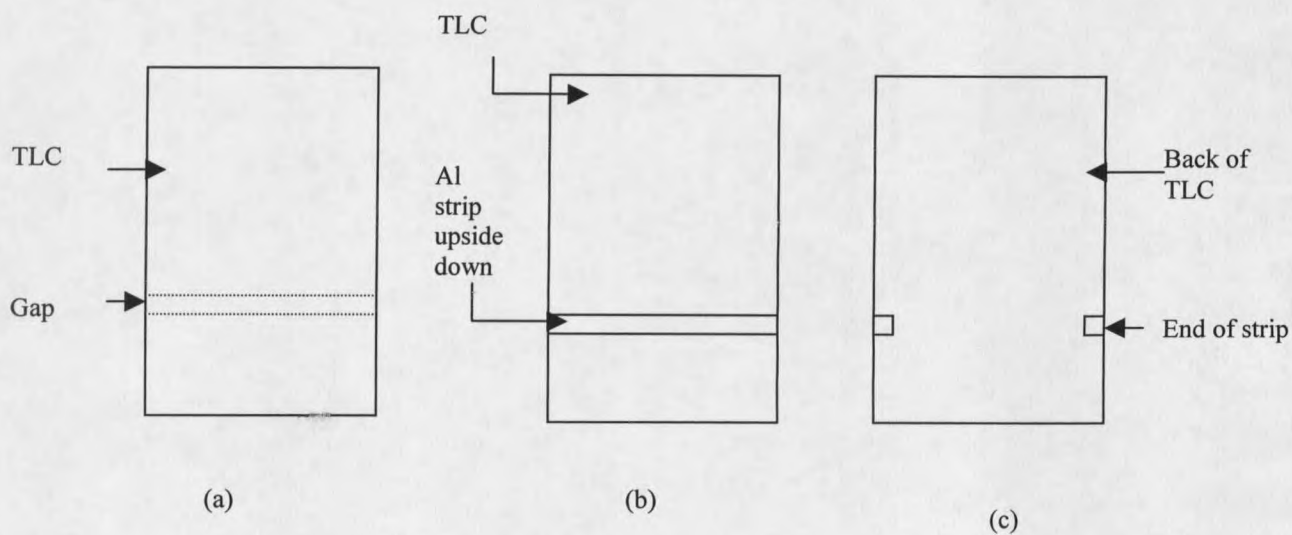


Figure 31. Method "F" (standard method).

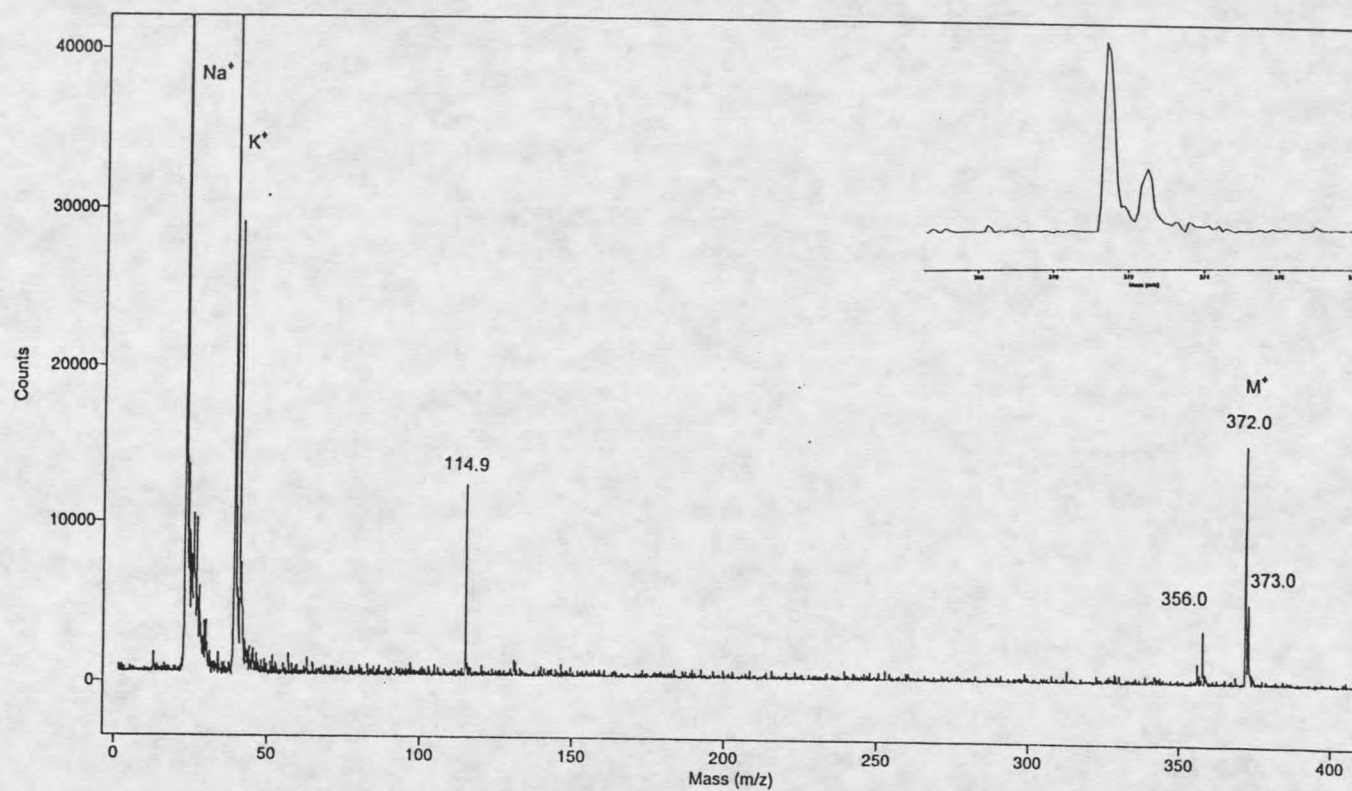


Figure 32. Crystal violet mass spectrum using method "F".

The mass spectrum of crystal violet is shown in Figure 32. The isotope peaks at $m/z = 372.0$ and 373.0 are well resolved. This method ("F") will be referred to as the "standard method" in the following.

The "standard" method (Figure 31) has three clear advantages over the old TLC/SALDI method (Chen et al., 1998). First, it solved the surface charging problem, and this improved mass resolution and mass accuracy. Second, the analyte was actively transported by liquid flow to the AC particle bed, improving the sensitivity. The third and possibly most important advantage is that the desorption efficiency was improved. In the old SALDI method, the analytes were adsorbed on the underside of the AC particles (Figure 33a) where they are not accessible to the laser light (Figure 33b). In the new SALDI method, the AC strip was positioned faced down and the analytes, diffused directly towards the surface of the AC strips (Figure 33c). After developing, the AC strip was turned over and the top surface of the AC particles was positioned towards the laser light (Figure 33d).

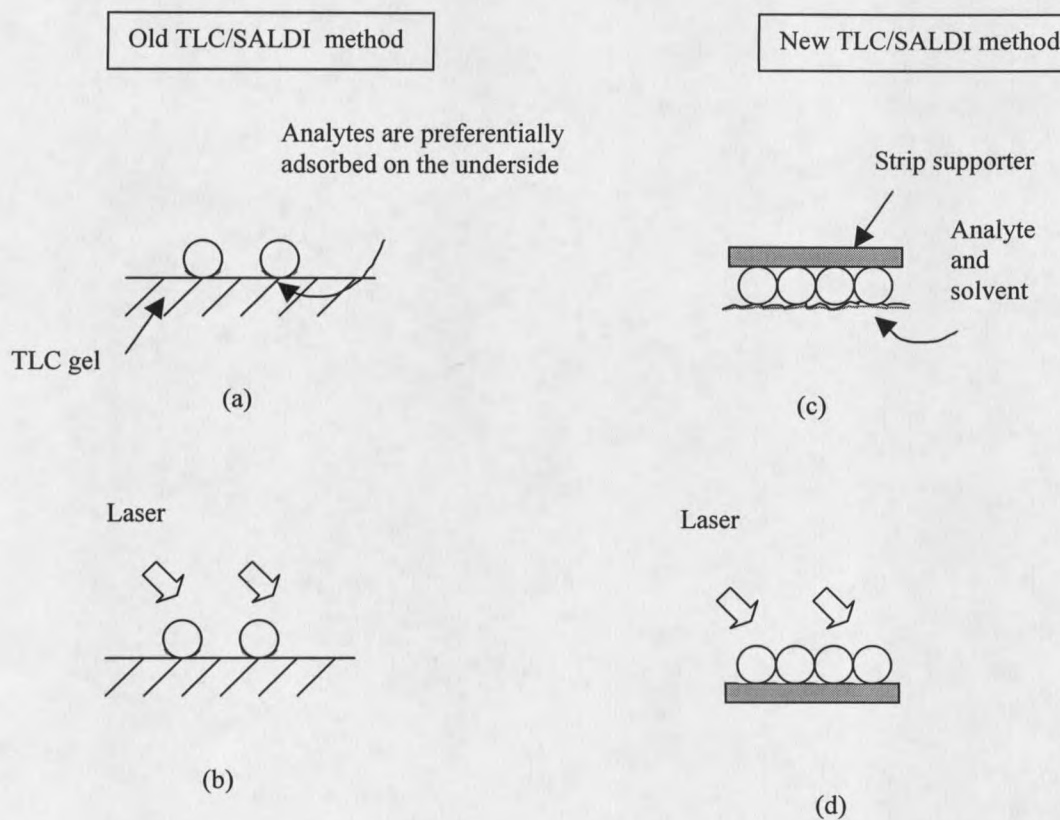


Figure 33. Comparison of the old and new TLC/SALDI methods.

Effect of Changing the AC Support Material

In the experiments described above, the AC particles were immobilized on aluminum foil. Then aluminum foil was considered because it is electrical conductive, flexible, easy to obtain, and cost-effective. A few experiments were performed using a glass fiber filter paper support material, and they will be discussed here.

A glass fiber filter paper support was considered because this material has good permeability compared to aluminum foil, which is not permeable at all. It was expected that the glass fiber might achieve a better extraction efficiency because strong capillary forces should promote the motion of the solvents into the AC strip assembly. In a comparative study, a pair of AC strips were made; one using the aluminum support and the other using the glass fiber paper support. They were attached to a pair of TLC plates. The same amount of crystal violet (0.10 μ L, 0.10 mM) was applied to each plate, and the plates were developed using identical conditions. The strips were removed and dried and 20% glycerol in methanol was added. The same laser intensity was used for both. Two SALDI spectra were obtained in reflecting mode (Figure 34a and Figure 34b). First of all, it is seen that both strips gave good mass spectra. Both had a "clean" background, strong M^+ ($m/z = 372$) ion signal, and a good mass resolution. From the expanded-scale M^+ peak, shown in the inset in Figure 34a one can see, that the mass resolution for the glass fiber filter paper support is actually better. However, often it was much worse. This variability was probably due to poor electrical conductivity and surface charging. The analyte ion signal from the glass fiber support strip is less intense

than from the aluminum support strip. This can be explained by the fact that the glass fiber filter paper is very absorbent. Not only will the dye be adsorbed on the AC but it will also be absorbed by the glass fiber filter paper. The violet color could indeed be seen at the back of the strip, on the paper side. The mass spectrum in Figure 34b shows many small "spikes". This is due to averaging of just a few laser shots (5). The reason for selecting this particular mass spectrum was its high mass resolution.

Aluminum foil was chosen as the support material for AC substrate strips for the remainder of this work because aluminum has less of a problem with surface charging.

Effect of Laser Pulse Energy

Several series of experiments were performed in which the laser pulse energy was varied. Representative mass spectra are shown in Figure 35, a and b. Both spectra were obtained from the same sample but at different laser pulse energies. As shown in Figure 35, the relative abundance of the ions varied with the laser pulse energy. High laser pulse energy caused more glycerol fragmentation and more carbon background than a lower laser pulse energy. The best mass resolutions was obtained just above threshold. After the sample had been irradiated with high energy laser pulses, it was very difficult to again obtain an analyte ion signal. This was probably due to a high energy "burn out" of the analyte in the irradiated spot.

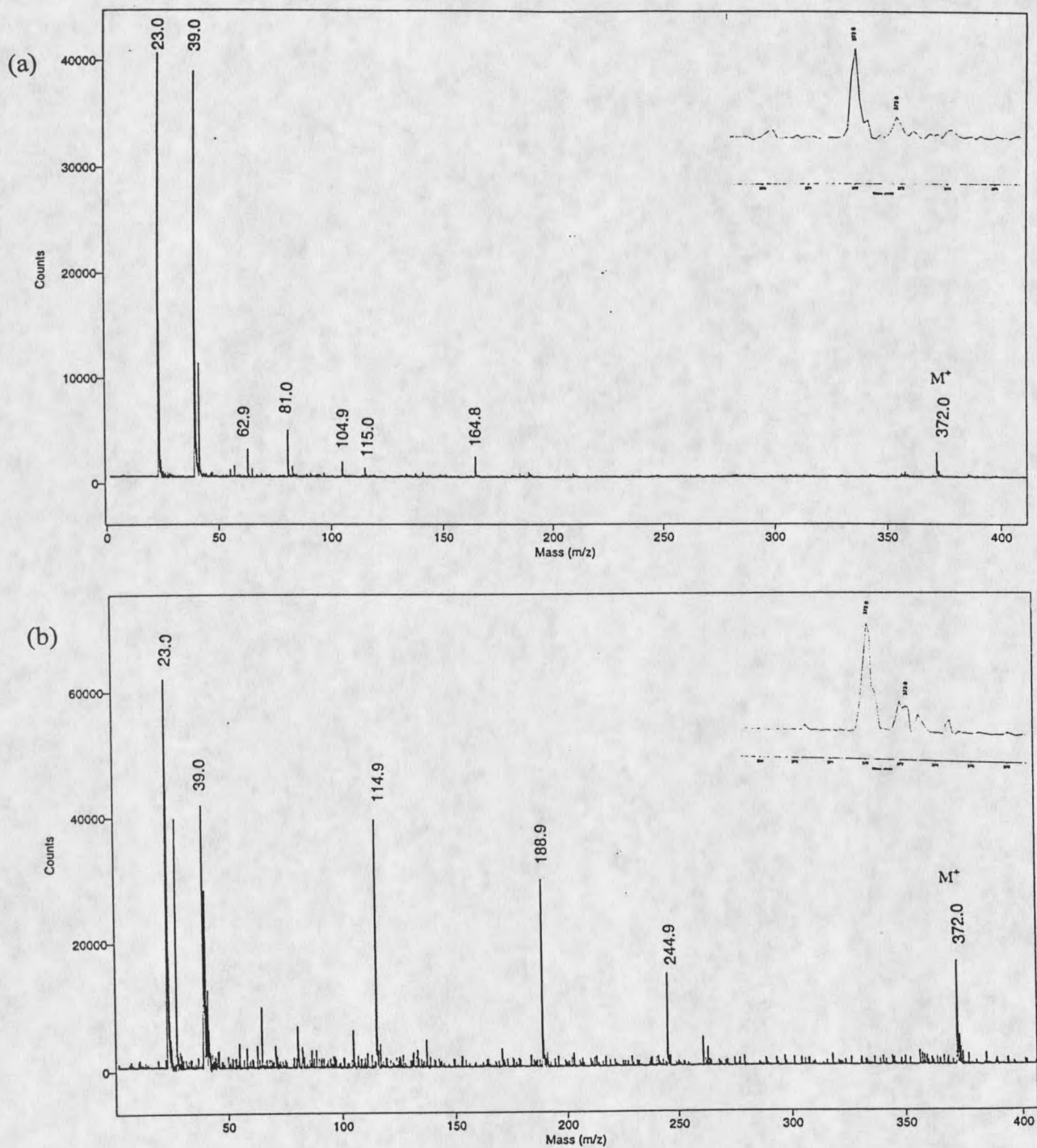


Figure 34. (a) Crystal violet (0.10 μ L, 0.10 mM) mass spectrum from an AC strip on glass fiber filter paper support (b) Crystal violet (0.10 μ L, 0.10 mM) mass spectrum from an AC strip on aluminum support.

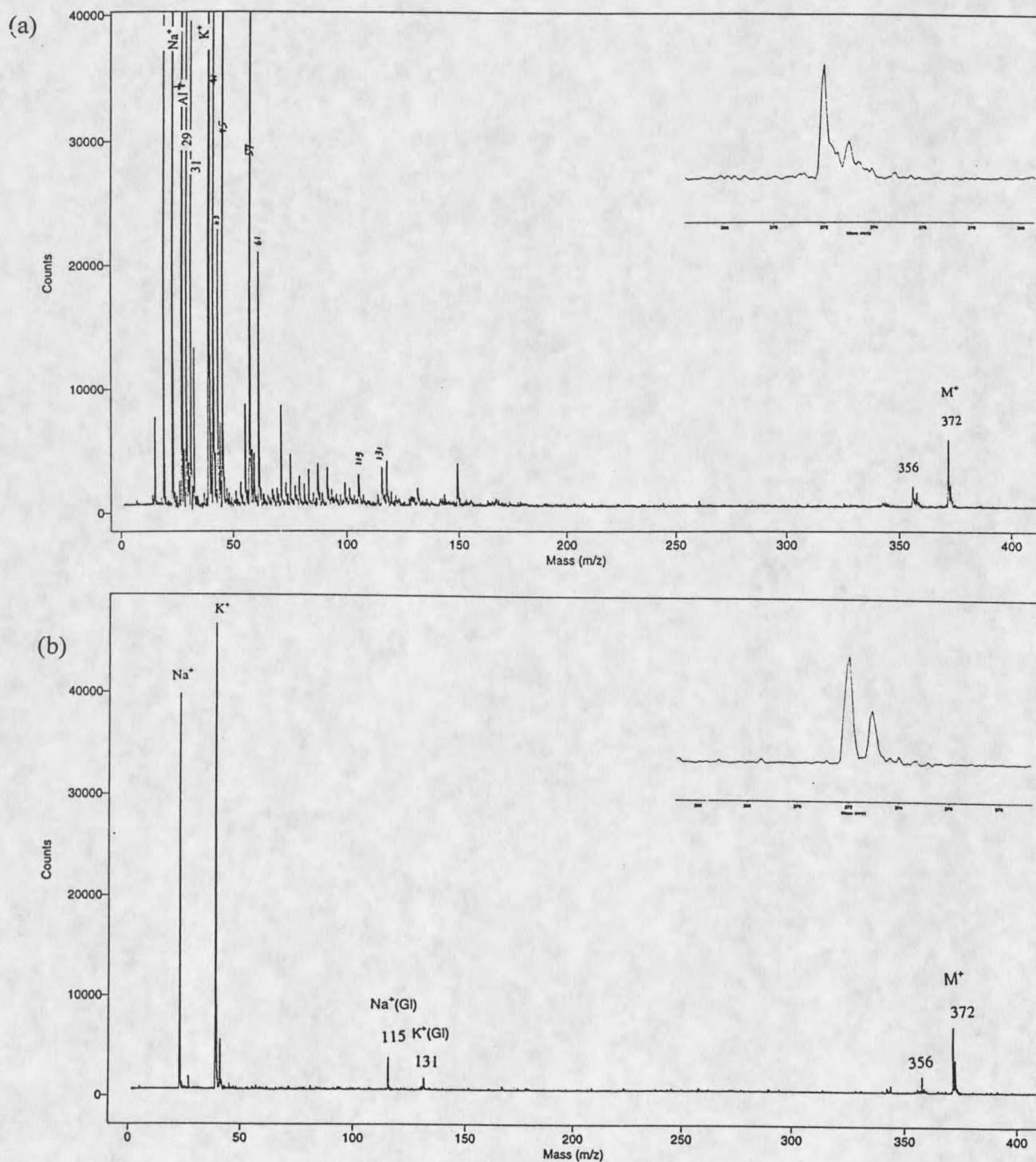


Figure 35. (a) Crystal violet (0.50 μ L, 0.0010 mM) mass spectrum from aluminum foil support AC strip with high laser pulse energy (b) Crystal violet (0.50 μ L, 0.0010 mM) mass spectrum from aluminum foil support AC strip with low laser pulse energy.

Sensitivity

Experiments were performed to determine the sensitivity of the TLC/SALDI method developed in this work. A crystal violet stock solution was diluted to 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M. A 0.10 μ L volume of these solutions was applied to each of a series of TLC plates. When the concentration was too low to see the dye on the plate, a higher concentration crystal violet solution was developed in a parallel lane to in order to visualize the analyte transport. Figure 36 shows the analyte ion region for amounts of crystal violet from 100 pmol to 10 fmol. A good analyte ion signal was obtained from 0.10 pmol and the detection limit ($S/N = 3$) is below 10 fmol. The M^+ peak intensity is plotted versus the amount applied to the TLC plate in a double-logarithmic diagram in Figure 37. A straight line was obtained by with the following equation:

$$y = 0.5 x + 3.5$$

where $y = \log(\text{intensity})$ and $x = \log(\text{amount crystal violet/pmol})$. This suggests that the ion abundance has a square root dependence on concentration. For another compound, bradykinin, the detection limit was calculated to be 0.50 pmol (about 0.50 ng) with an $S/N = 3$. This is better than the 2-4 ng detection limit reported for bradykinin in TLC/MALDI (Gusev et al., 1995).

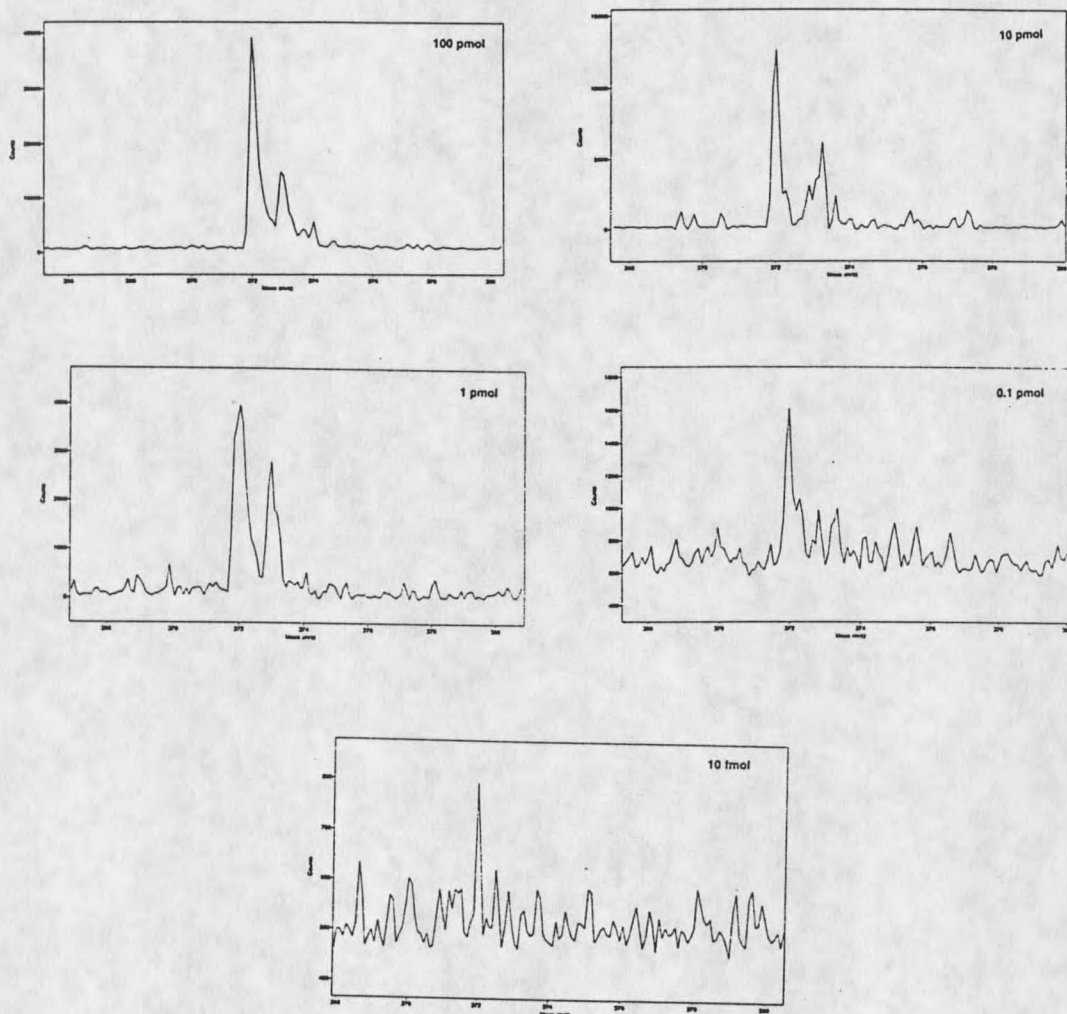


Figure 36. Crystal violet mass spectra from different amounts of the compound.

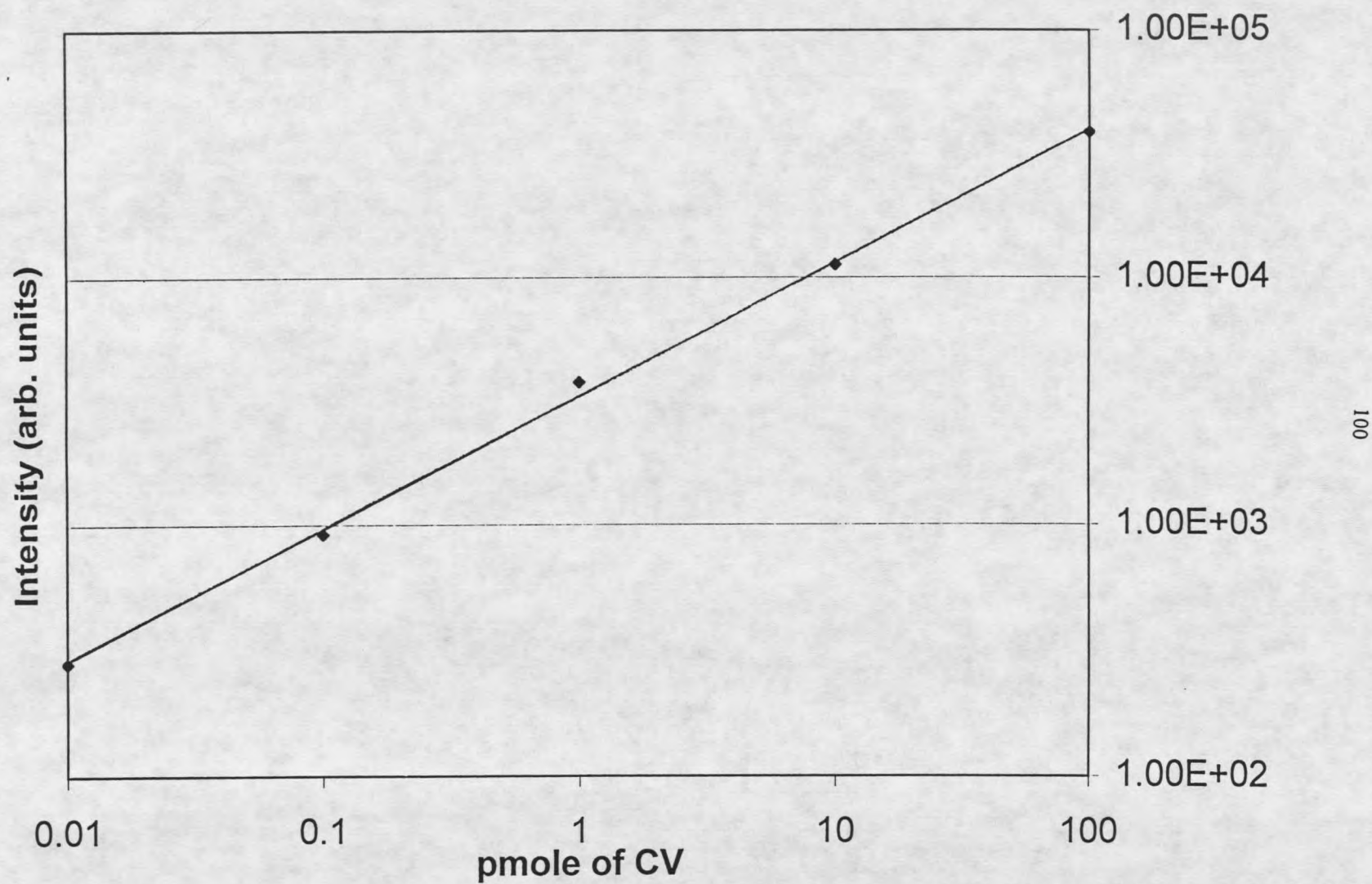


Figure 37. M^+ peak intensity for crystal violet as a function of amount of crystal violet developed on TLC plate.

Storage

The most characteristic property of this method is the storage. As we know, a chromatogram can not stay very long in the TLC plate because of the interaction between the absorbent and analyte. Some studies were performed on this indirect coupling TLC to MS method for "storing" the chromatogram. A tracking experiment for a half year was done in our research. Six parallel samples were made by developing 0.50 μL of 10^{-3} M crystal violet simultaneously. The strip was dried and 20% glycerol matrix solution was added. The samples were monitored for six months. The mass spectra were taken once a week at the beginning, after one month later, they were taken once a month for five months. Figure 38a and 38b show one of the samples. By comparing mass spectra from experiment starting (Figure 38a) and the spectra from six months later, no significant intensity decrease in the later case have been found. And the glycerol matrix solution was only added once more during this period.

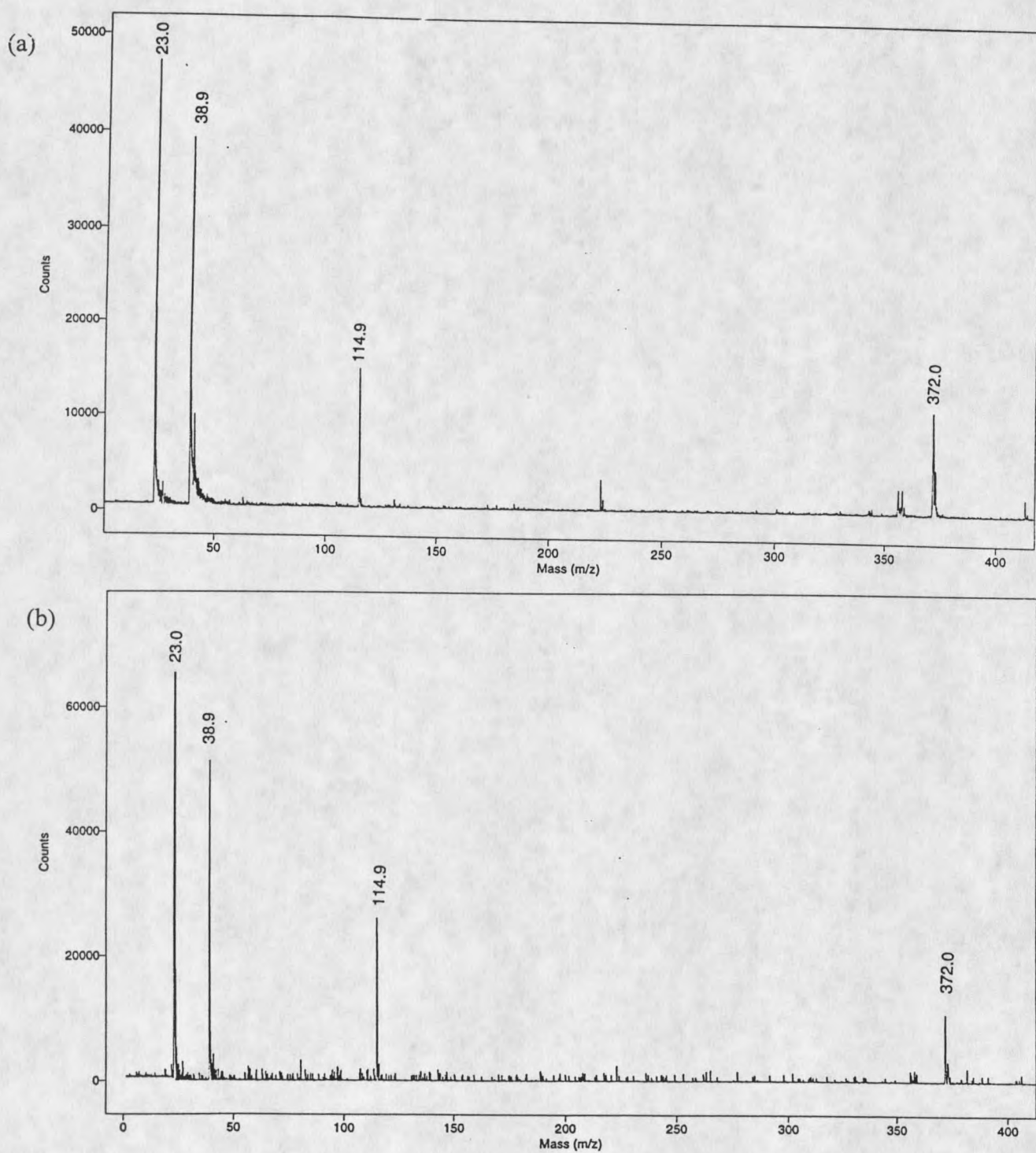


Figure 38: (a) Crystal violet mass spectrum from "fresh" sample (b) Crystal violet mass spectrum from "old" (half year later) sample.

Applications

Separation of Two Dyes

One of the advantages of TLC is that it is a simple method. The reconstruction of TLC chromatograms from the AC strips was studied in a separation experiment. A two-dye mixture of crystal violet and victoria blue was prepared for TLC separation. A 0.50 μL of 1:1 volume ratio of 5.3 mM victoria blue and 1.0 mM crystal violet dye mixture was applied to the TLC plate for a first developing process. The plate was brought out of the developing chamber, and the solvent was evaporated at the room temperature. The plate was then rotated 90 degrees, the AC strip was attached to the plate using the standard method described above (Figure 39). The plate was developed a second time, using a high eluting strength solvent (100% ethanol) to transfer the analyte to AC bed. The strip was detached, dried, and enough of a 20% glycerol in methanol matrix solution was added to cover the strip. The sample plate was brought to the mass spectrometer and mass spectra were obtained at different positions along the strip. Figure 40a shows a mass spectrum obtained at the crystal violet end, and Figure 40b shows a mass spectrum obtained at the victoria blue end. The mass spectrum in Figure 40c was obtained from an intermediate region. The peak at $m/z = 372$ is M^+ for crystal violet and $m/z = 470$ is M^+ for victoria blue. In Figure 40b, a small crystal violet peak ($m/z = 372$) is observed and in Figure 40c, both of them can be seen. The reason for both peaks being present the TLC were not able to totally separate the two dyes. In

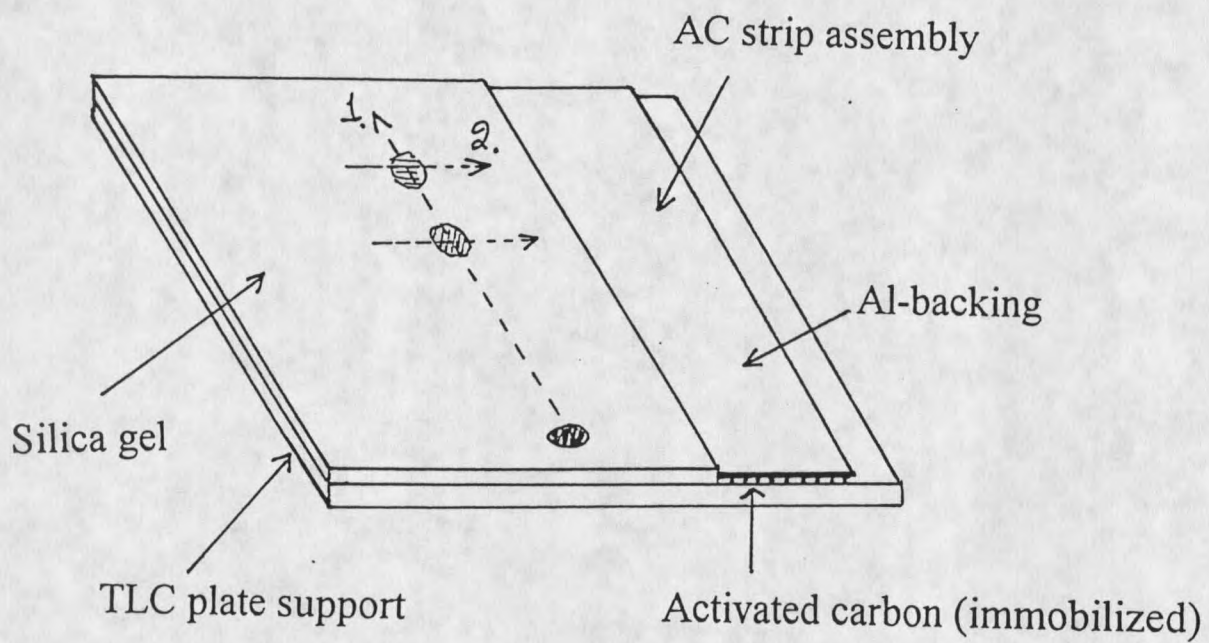


Figure 39. Two dimensional separation assembly diagram of two dyes mixture.

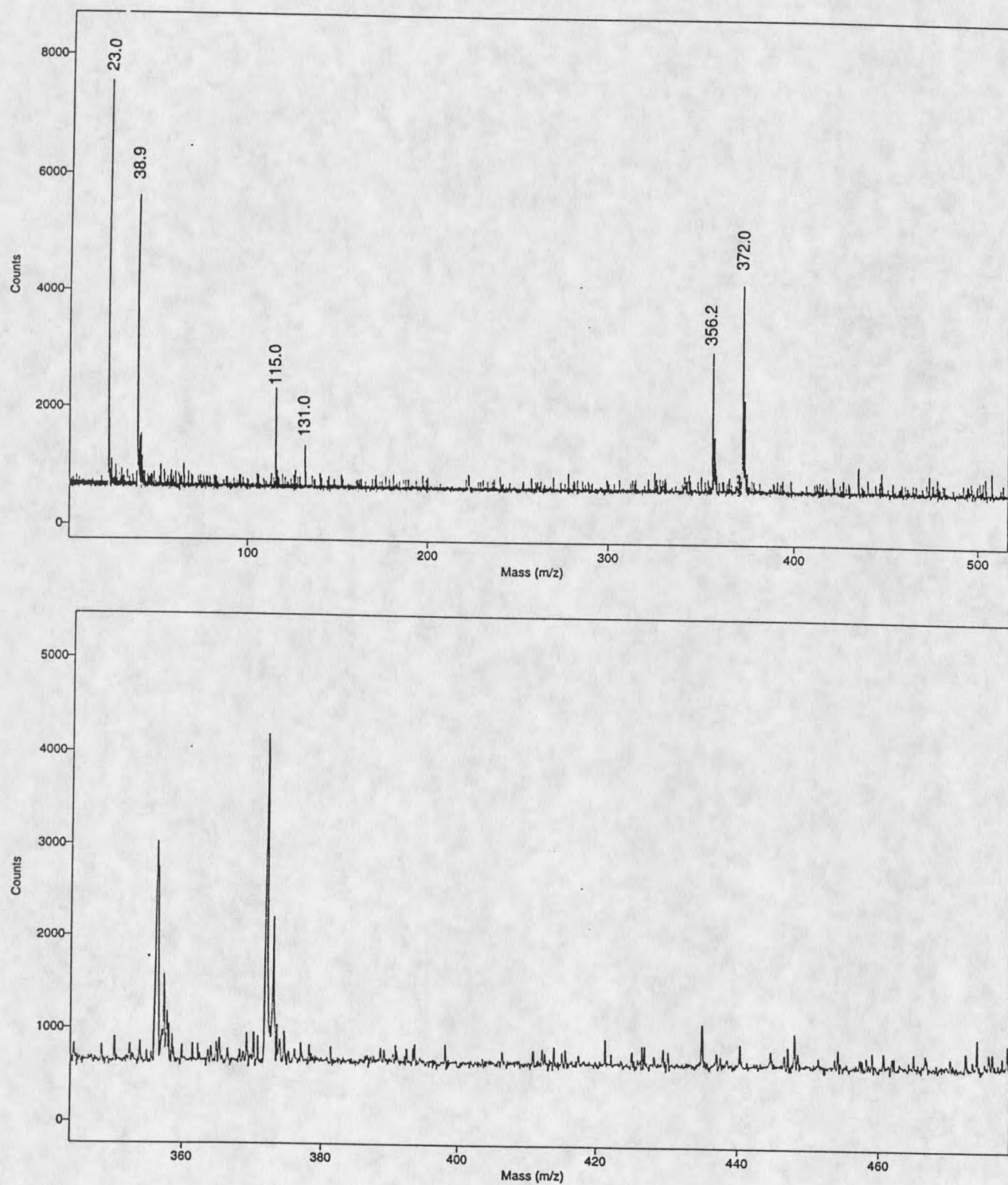


Figure 40: (a) Crystal violet mass spectrum from dye mixture separation experiment (crystal violet side). Lower frame shows the expanded mass range from $m/z = 350$ to 470.

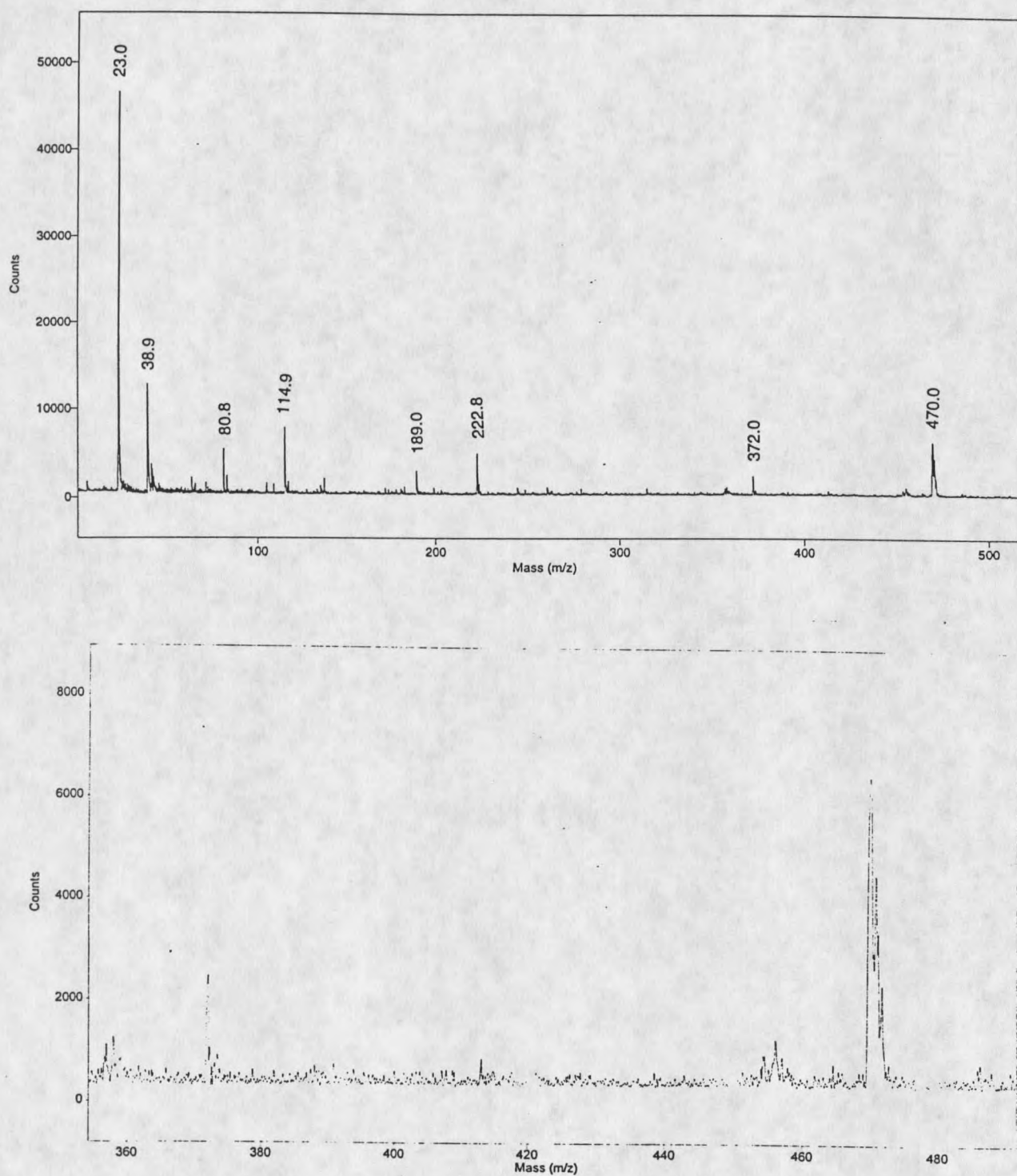


Figure 40: (b) Victoria blue mass spectrum from dye mixture separation experiment (victoria blue side). Lower frame shows the expanded mass range from $m/z = 360$ to 480 .

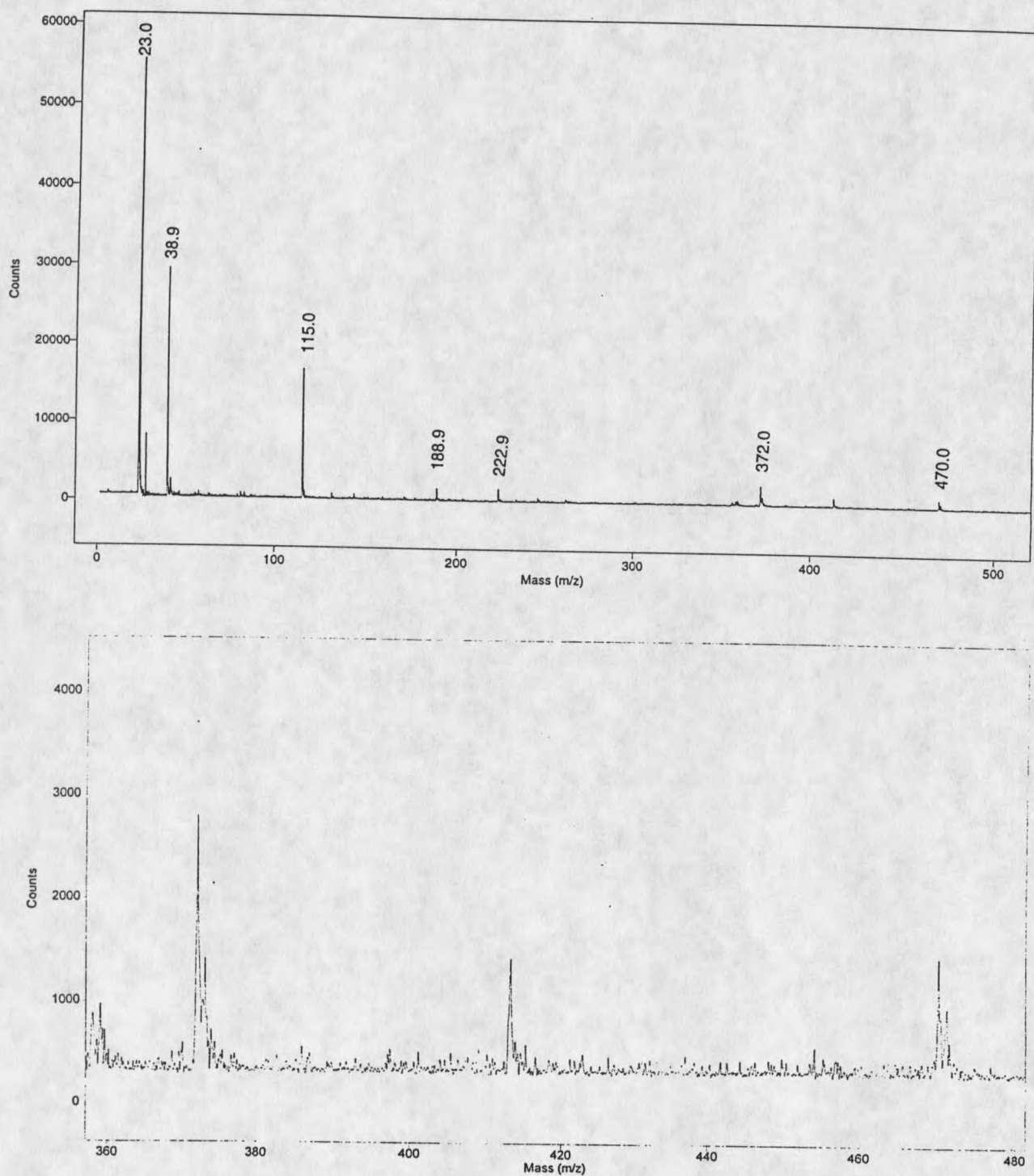


Figure 40: (c) Crystal violet and victoria blue mass spectra from dye mixture separation experiment (crystal violet and victoria blue edge). Lower frame shows the expanded mass range from $m/z = 360$ to 480.

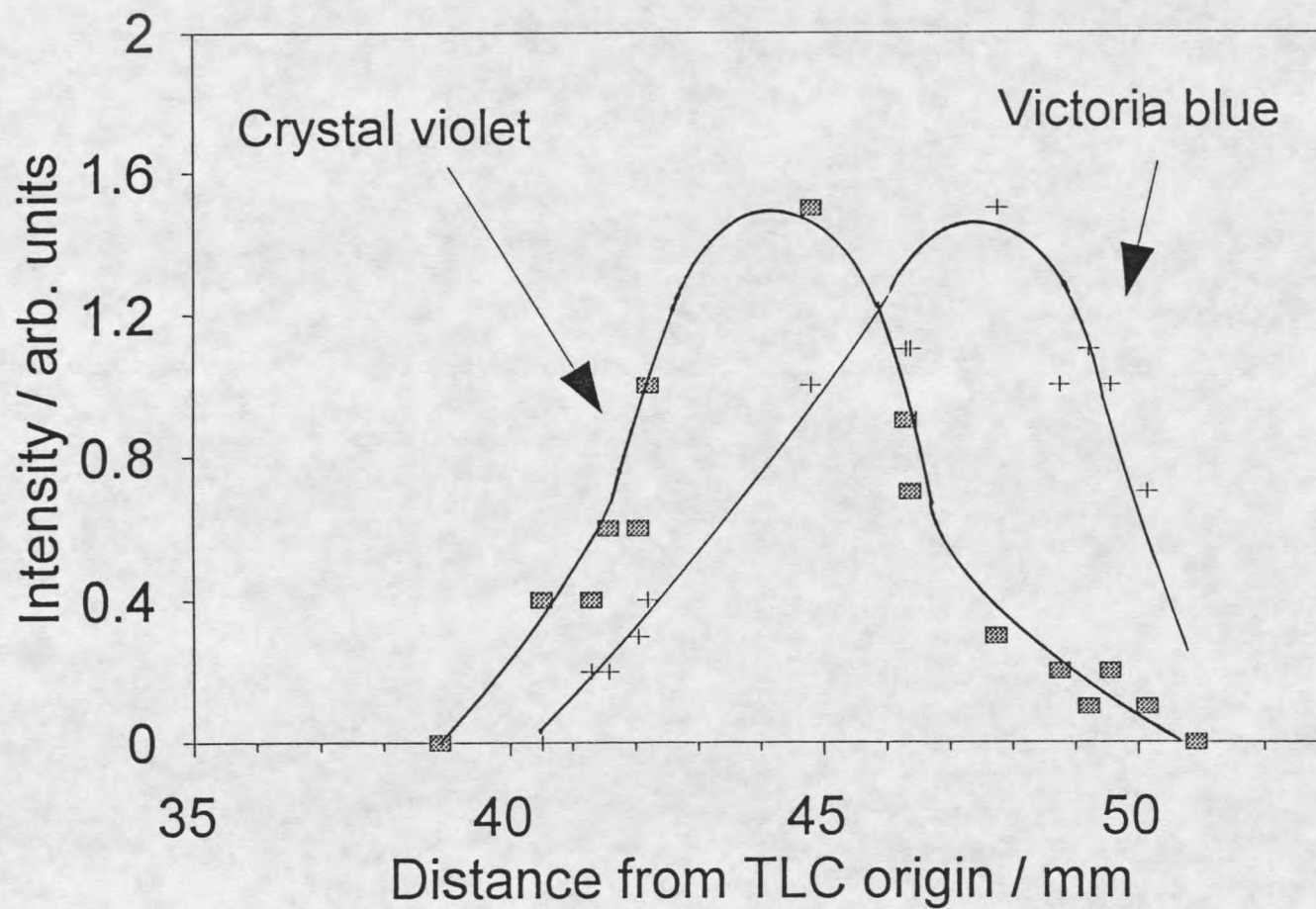


Figure 41: TLC separation profile of crystal violet and victoria blue dye mixture separation experiment.

particular significant tailing was seen for victoria blue. This is verified by the chromatogram profile (Figure 41). By using this AC strip, even the TLC separation is not very complete, a mass spectrum still can be obtained.

Different Analytes

Several different kinds of analytes were analyzed with TLC/SALDI, such as amino acids, peptides and organic compounds. Some representative mass spectra were shown on Figures 42 to 45. Internal mass calibration was used. For caffeine, a TLC plate with fluorescent indicator was used, and for amino acids and peptides, a 0.2% ninhydrin in ethanol solution was used for visualization.

Figures 42-45 shows mass spectra obtained from bradykinin, victoria blue, methionine enkephalin and caffeine. Among those peaks, $m/z = 23, 39, 115, 131$ were due to Na^+ , K^+ , $\text{Na}^+(\text{Gl})$, $\text{K}^+(\text{Gl})$; $m/z = 19$ was come from Gl fragmentation; $m/z = 65, 83, 99, 105$ probably due to carbon cluster in high laser power and $m/z = 137, 189$ were come from impurities. Figure 42 was obtained from bradykinin. An isotope peak obtained from protonated bradykinin ($m/z = 1060.57$, $m/z = 1061.71$). More background peaks were seen due to a higher laser power was applied. Figure 43 was obtained from victoria blue. A molecular ion peak ($m/z = 470$) was observed. Figure 44 was obtained from methionine enkephalin, a good sodinated isotope peak was observed ($m/z = 596.7$ and $m/z = 597.9$). A peak at $m/z = 619.3$ probably due to $\text{Ca}^{2+}(\text{M})$. Figure 45 was for caffeine development, for this experiment, 95% ethanol was used to "transfer" caffeine to strip, a protonated peak ($m/z = 195.2$) and a sodinated peak ($m/z = 217$) were observed. Some proteins such as myoglobin, bovine serum albumin, Cyc C

have been tried, but no good signals were obtained. This probably because the proteins bound to AC powder too tight, it is hard for them to be desorbed from the activated carbon surface. However, TLC is not a good separation tool for analyzing protein.

Conclusions

From above method development and applications can see, TLC-SALDI is a new good analytical tool for small molecules. Mass spectra with good mass resolution and sensitivity were obtained. The two dimensional assembly is easy for transferring separation chromatograms to the strip. The strip can "store" chromatograms. This makes more convenience for remote analysis.

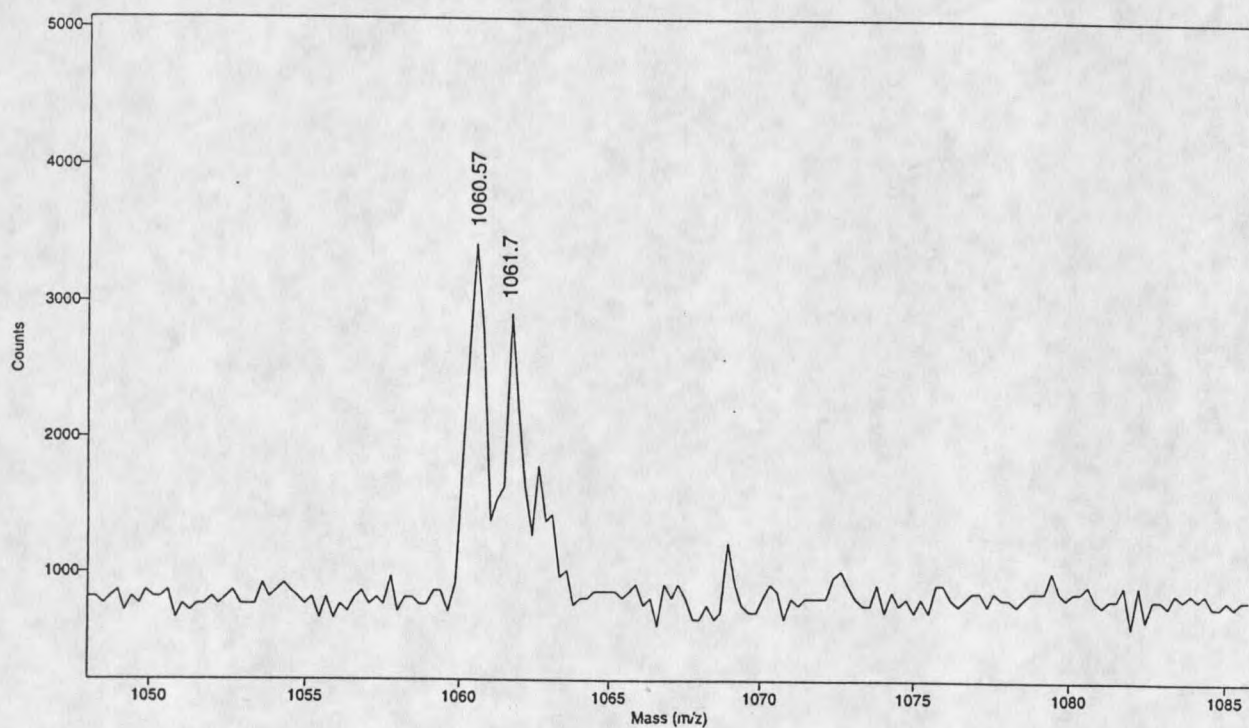
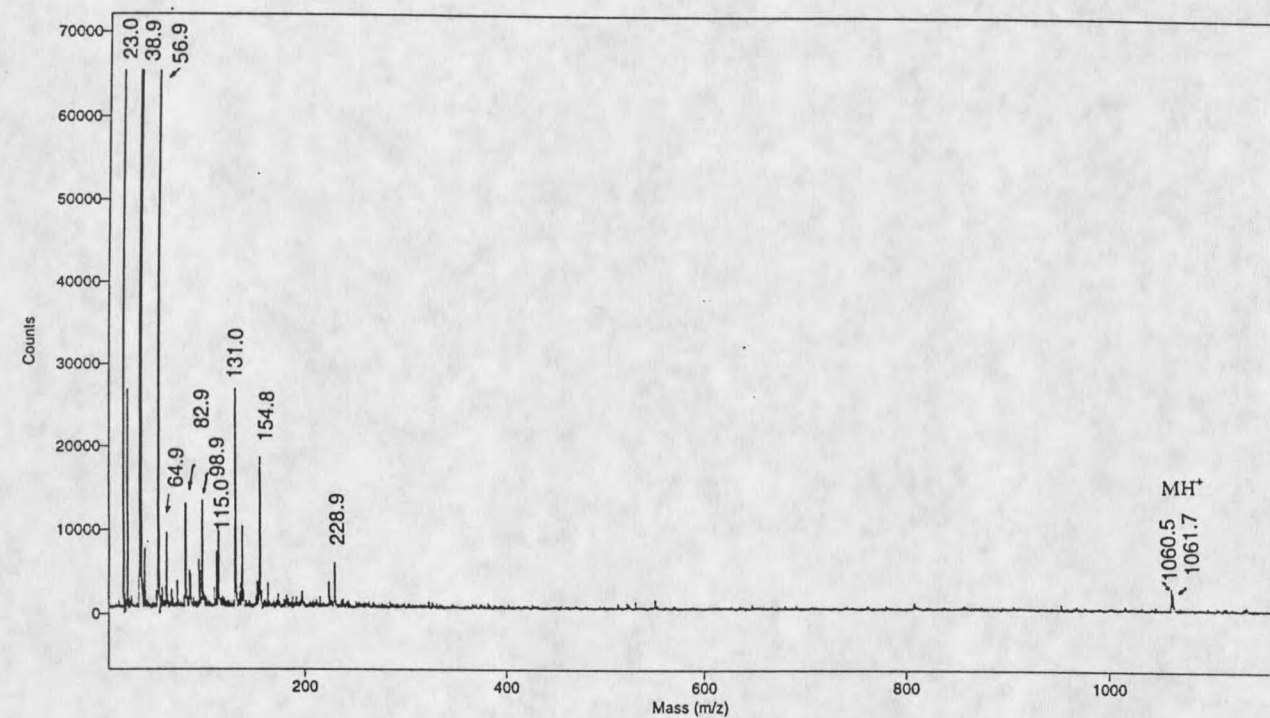


Figure 42: TLC/SALDI mass spectrum of bradykinin (MW = 1059.5, monomer, 0.27 nmol).

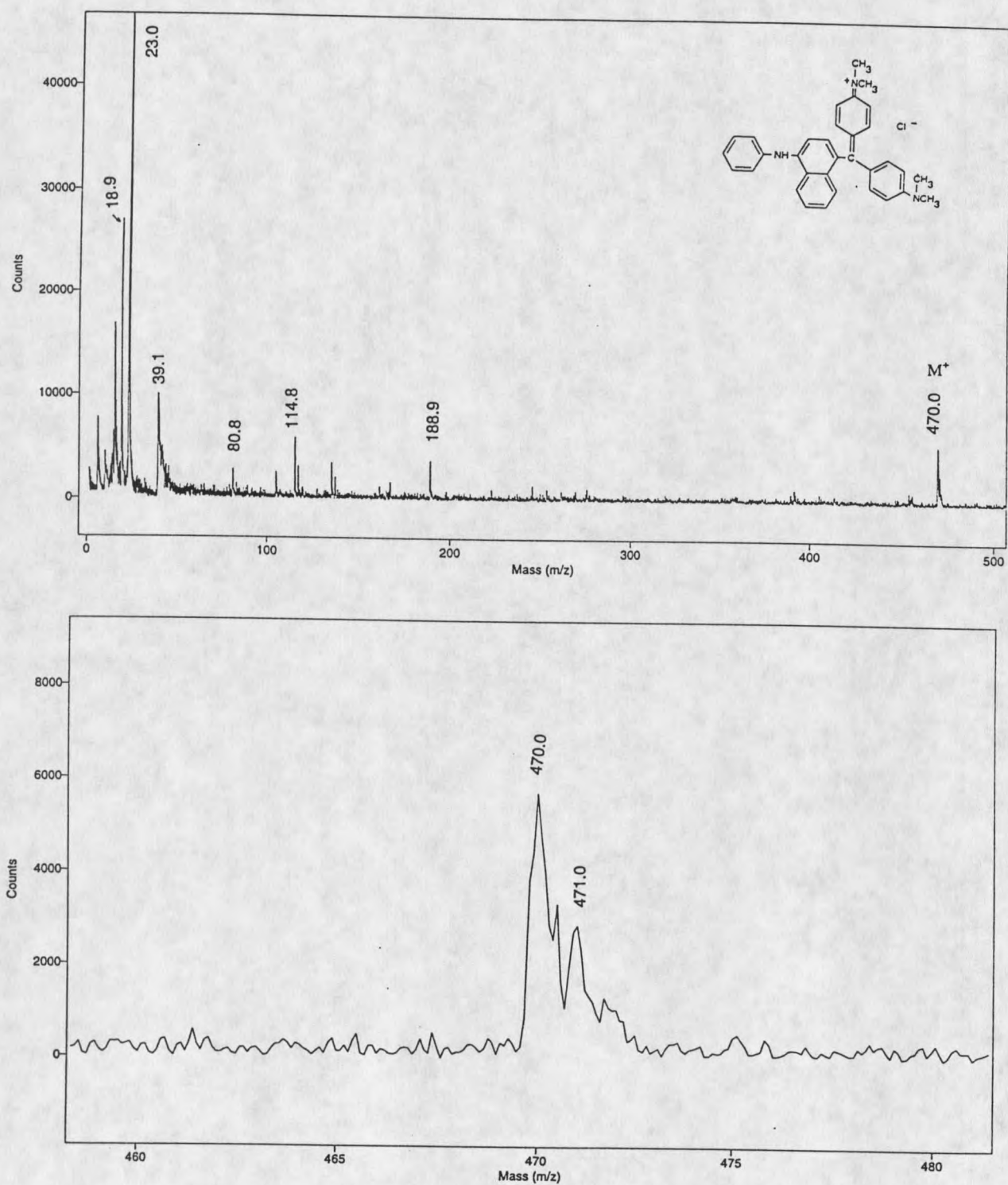


Figure 43: TLC/SALDI mass spectrum of victoria blue (0.5 nmol).

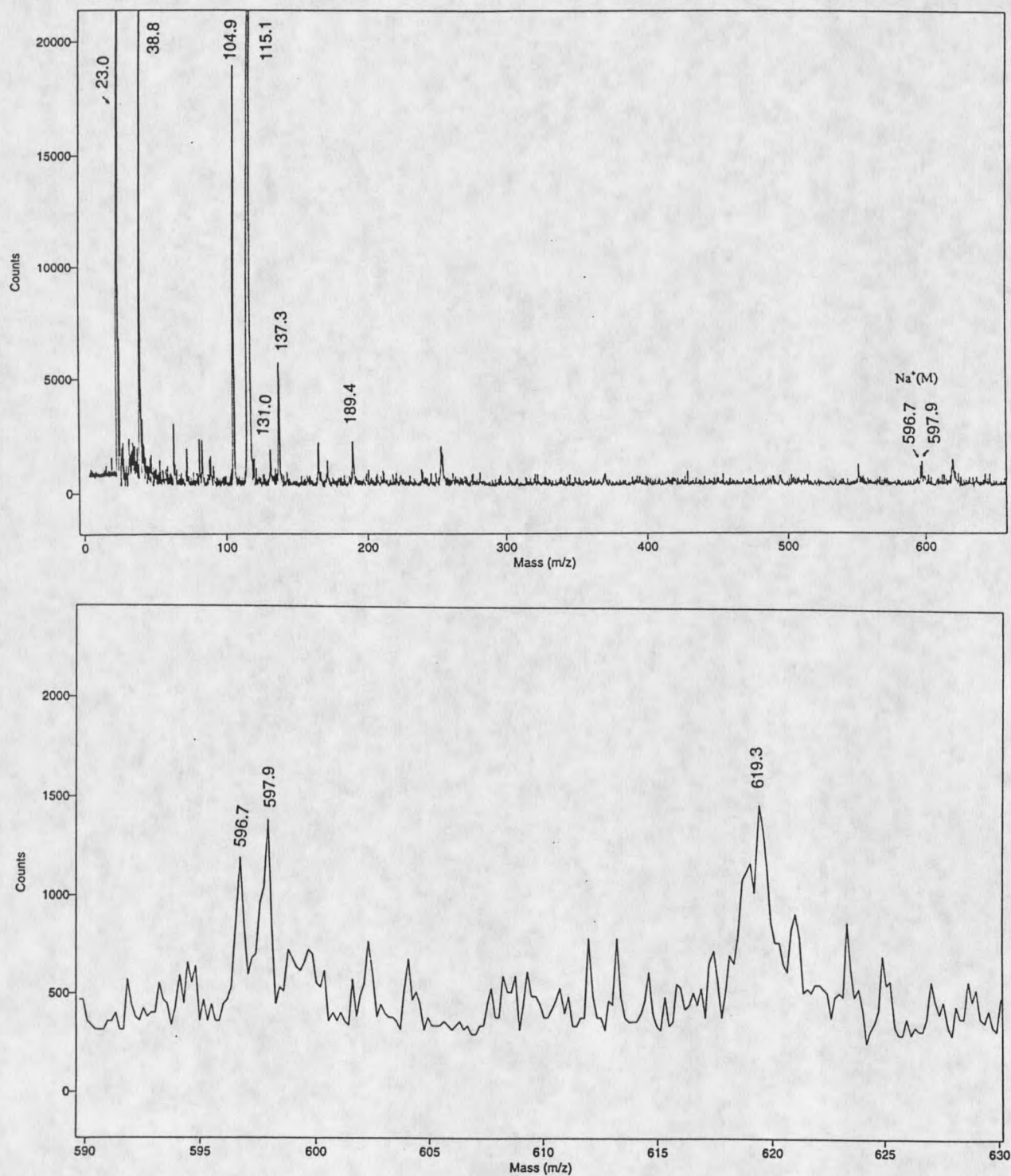


Figure 44: TLC/SALDI mass spectrum of methionine enkephalin (MW = 573.6, 2 nmol).

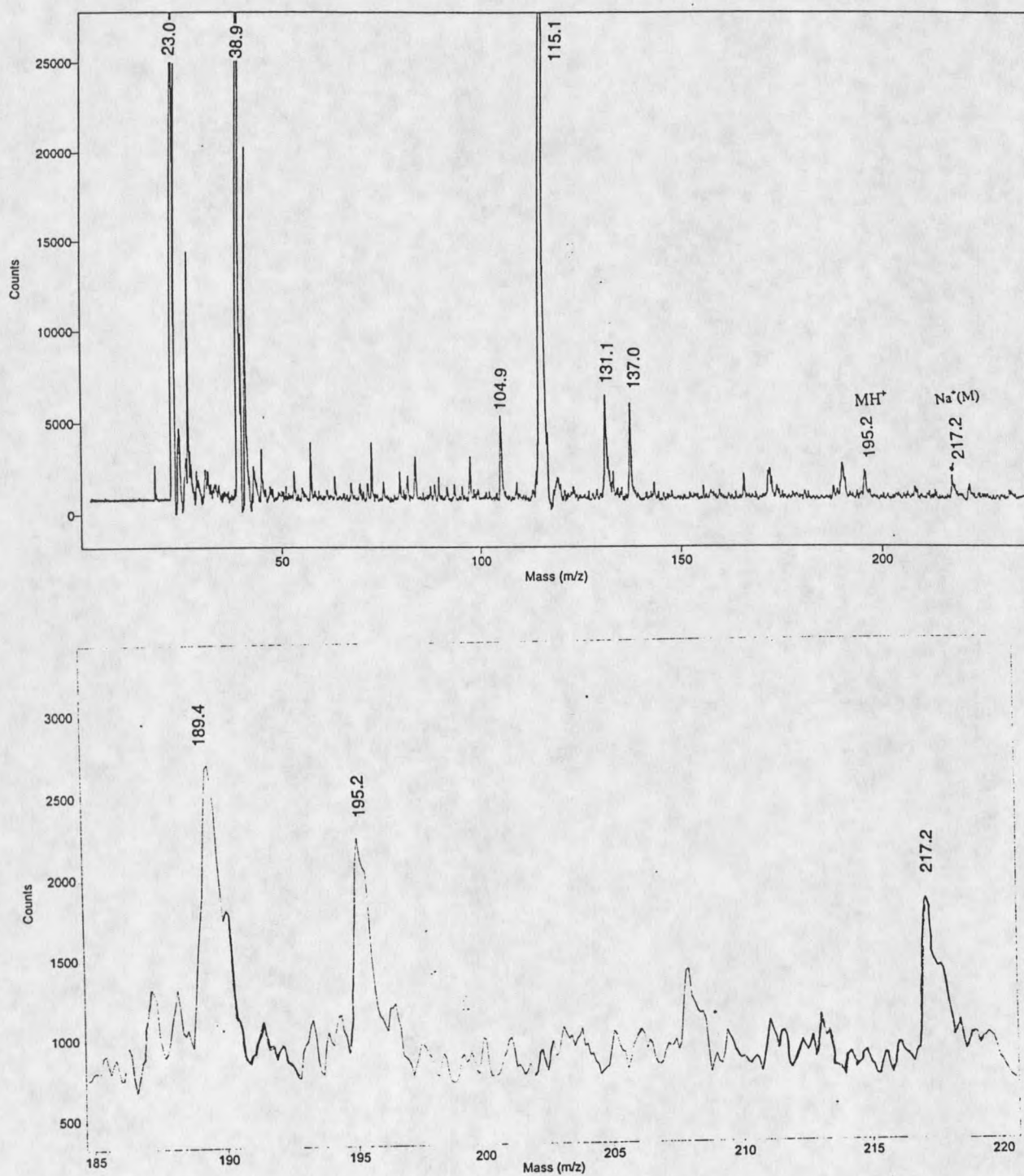


Figure 45: TLC/SALDI mass spectrum of caffeine (MW = 194.2, about 1 μg).

CHAPTER 6
DEMONSTRATION OF
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/SALDI MASS
SPECTROMETRY

Introduction

High performance liquid chromatography (HPLC) is a widely used analytical separation tool because of its sensitivity, ready adaptability to accurate quantitative determinations and its ability to separate nonvolatile or thermally unstable species. It is used to analyze many kinds of compounds, such as amino acids, proteins, nucleic acids, hydrocarbons, drugs, steroids, etc. (Skoog et al., 1998). HPLC coupled to MS is a very powerful analytical tool for both separation and identification of the components in a complex sample. The interfacing of HPLC to immobilized activated carbon-SALDI is potentially both interesting and useful. Two demonstration experiments were performed in our lab in order to show the potential of this method.

Experimental and Instrumentation

For the HPLC work, a Shimadzu LC-6A liquid chromatograph was used. It has two pumps, a sampling loop injector and an ABI Analytical Kratos, Spectroflow 757 UV-VIS absorbency detector. An Adsorbosphere HS C18 column was purchased from Alltech. The mobile phase for crystal violet was $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (60:40) and the mobile phase for caffeine was 10 mM KH_2PO_4 , pH 4.8: $\text{CH}_3\text{CN}:\text{THF}$ (94:4:2). The flow rate was 1.0 ml/min.

Procedure

A volume of 20 μL of analyte was injected into the injector. After separated in the column, The fractions were collected on the AC substrate (Chapter 4), by continuously moving the elute capillary along the AC strip. The solvents were evaporated in ambient air, 20% glycerol in methanol was added to the strip, and the sample was brought to the TOF-MS.

Results and Discussion

Two mass spectra were obtained from crystal violet and caffeine (Figure 46 and Figure 47). The M^+ ($m/z = 372$) was obtained from crystal violet and $[\text{M} + \text{H}]^+$ ($m/z = 195$) was obtained from caffeine. The spectra signal did not get a very strong signal as it did in TLC/SALDI, that probably because of the huge amount of mobile phase dilute

the sample. Additional research is needed to further develop this method, such as making a spiral track on a small disc (Figure 49). The HPLC fraction will be collected on to the track continuously by slowing rotating the disc. Thus, the chromatogram was continuously record on to the track.

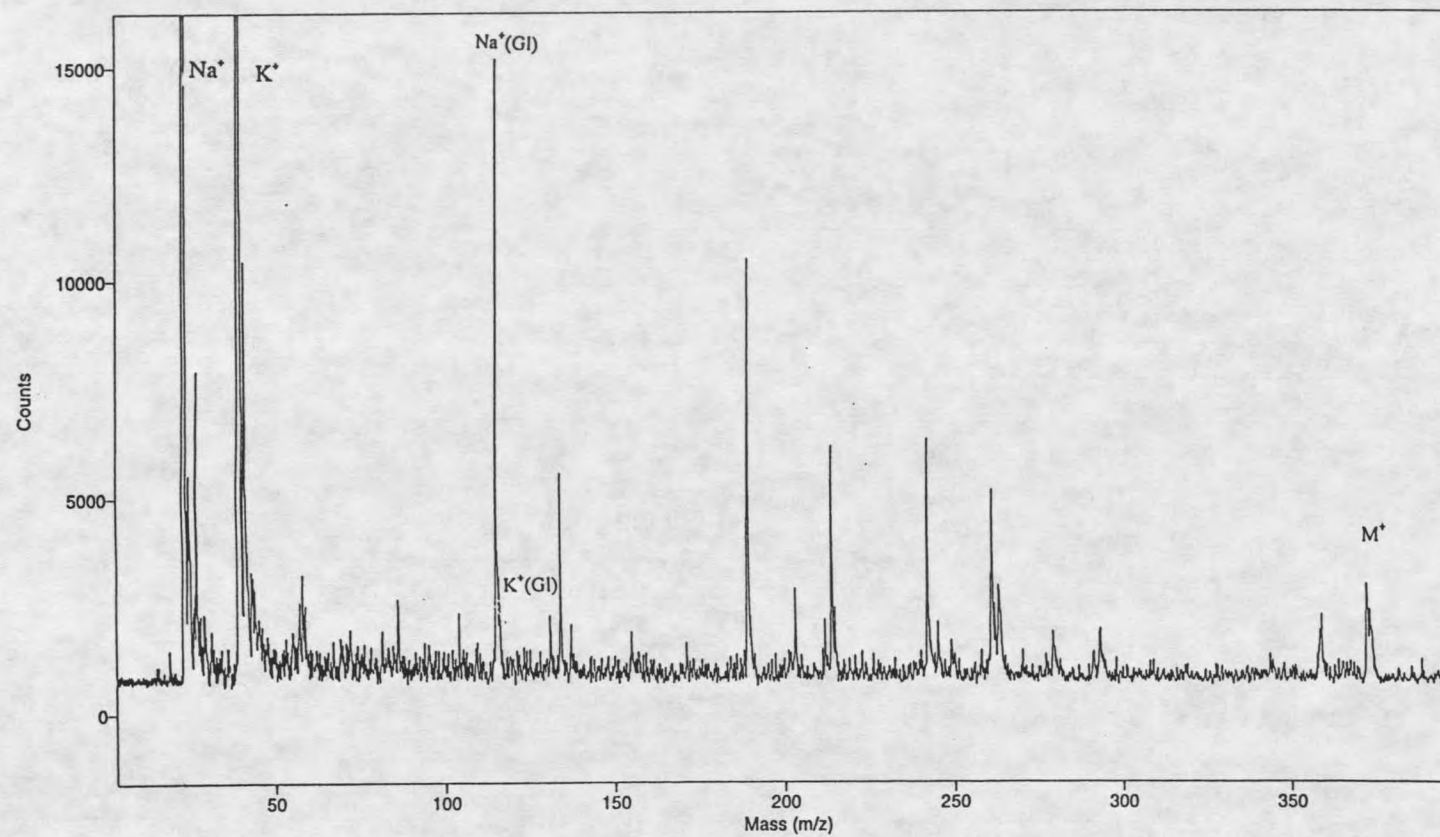


Figure 46. Crystal violet from HPLC fraction.

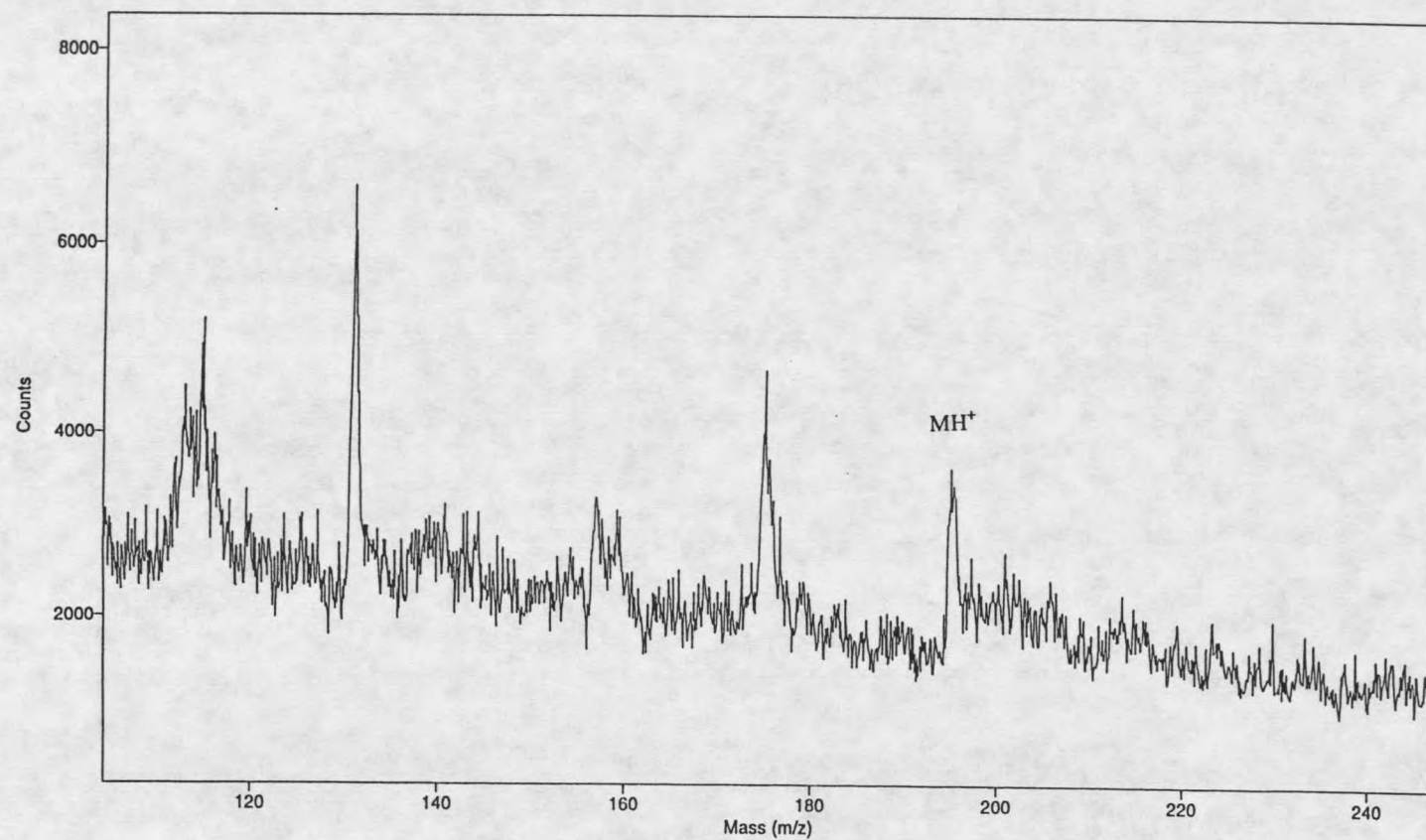


Figure 47. Caffeine from HPLC fraction.

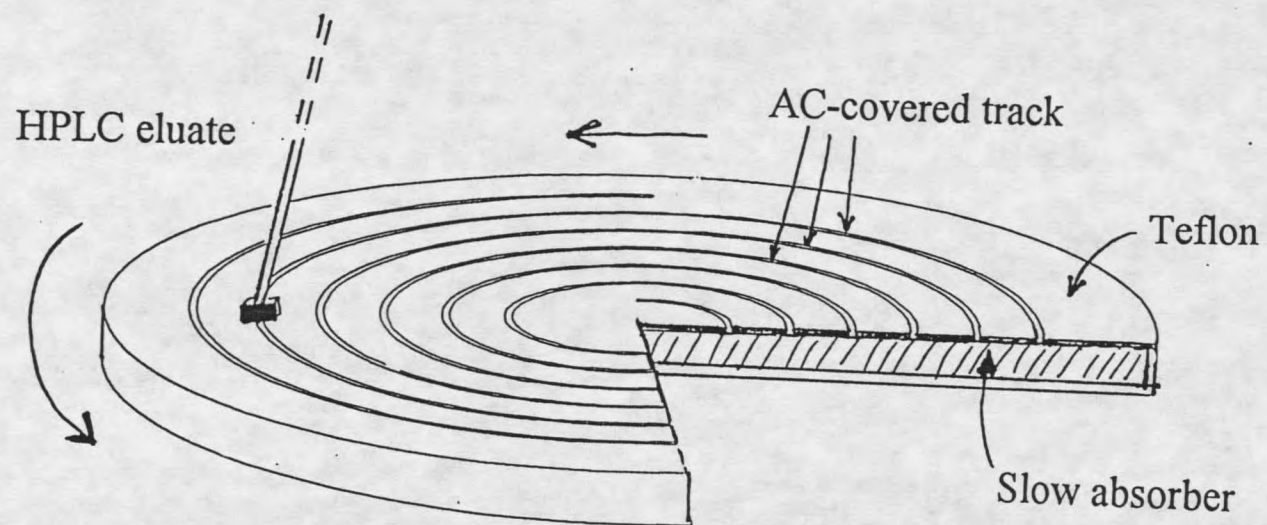


Figure 48. Continuous fraction collector of HPLC.

CHAPTER 7

CONCLUSIONS

Summaries of Results and Conclusions

A major result of this thesis is the improvement of sensitivity and resolution of "Surface-Assisted Laser Desorption/Ionization (SALDI)" mass spectrometry. The unique advantages of SALDI are as follows. First, it has a relatively "clean" background at the low mass range comparing to MALDI. Second, the sample preparation process is easy and the same sample preparation can be used for essentially all analytes. Third, good quality mass spectra are obtained directly from liquid solutions. The new improved SALDI method using immobilized AC substrates have additional advantages. First, since the AC particles are immobilized by adhesive on the aluminum support, ion source contamination is virtually eliminated. Second, by using a very thin layer of AC, the sensitivity was improved. Third, by using AC strip, good electrical contact from aluminum solved the surface charging problem. This resulted in improved mass resolution. Fourth, the elimination of sucrose binder further simplified the mass spectrum at low mass.

Immobilized AC-SALDI

Several different types of analytes have been studied by the immobilized AC-SALDI method. It is especially useful for analyzing small molecules. The sensitivity of the method is in the picomole and femtomole range. For some compounds, such as crystal violet, the detection limit can be as low as 1.5 fmol at $S/N = 3$. This is better than the sensitivity of graphite-SALDI (Sunner et al., 1995), which is in the pico- to nanomole range, and this is as good as conventional MALDI. The mass resolution has been improved to $m/\Delta m \approx 900$, observed for crystal violet. For other compounds, the mass resolution varied from 400-800, about a factor of 2-3 better than graphite-SALDI. By incorporating modern techniques, such as delayed ion extraction (Jushasz et al., 1996), the mass resolution is likely to reach several thousand. The sample preparation process is simple and fast. AC substrates could be manufactured in bulk quantities and the analyte solution is simply added. There is no critical sample preparation step, as in MALDI. AC substrate SALDI could be used as a routine analytical MS technique.

TLC/SALDI

In TLC-SALDI, the major improvement is the change from passive diffusion to active transport. The sensitivity and desorption efficiency have been dramatically improved. A mass resolution over 1400 for crystal violet in reflecting mode and more than 1000 for bradykinin in linear mode was obtained. A detection limit of less than 10 fmol for crystal violet and 0.50 pmol for bradykinin developed on the TLC plate was demonstrated. This is better than TLC/MALDI (Mehl et al., 1998) for which a

detection limit for bradykinin of 2-4 pmol and in femtomole range for carcinogen-adducted oligonucleotides. On the AC substrate strip, a chromatogram can be stored for more than six months without significant loss of sensitivity. This ability has many potential application, for example, to remote analysis.

HPLC/SALDI

Two short demonstrations of HPLC/SALDI showed the possibility of interfacing HPLC with SALDI. Further work is needed develop this application.

Future Prospects of SALDI

One of the important future goal in SALDI is the development of methods to combine other analytical techniques with SALDI, such as SDS-PAGE, CE and HPLC. If these methods can be successfully developed, they may become useful analytical techniques for analyzing biological and biochemical samples.

The major weakness of immobilized AC-SALDI is its relatively low mass range compared to MALDI. This is probably due to the strong bond between the large molecules and AC particles. A more efficient SALDI solid should be considered for solving this problem.

In conclusion, we believe that SALDI has a bright future. The attractive features such as clean background, good sensitivity and mass resolution and simple sample

preparation process make it complementary to other desorption ionization methods and unique in its applications.

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