Thin-layer chromatography combined with diode laser induced desorption/atmospheric pressure chemical ionization mass spectrometry

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Contents

Contents

Abstract

An analytical technique to combine TLC with MS was developed. The initial study was carried out on a graphite plate, which functions as a photon absorbing material. A continuous wave diode laser replaced traditional pulsed lasers as the desorption source, which was employed for this purpose for the first time. The thermally desorbed analyte molecules are ionized in the gas phase by a corona discharge device at atmospheric pressure and detected subsequently by a mass spectrometer, by which both essential processes — the desorption and the ionization of analyte molecules, which are often performed in one step - are separated. The technique was subsequently applied to thin-layer chromatography (TLC) to realize the combination of TLC and mass spectrometry. A graphite suspension was employed to couple the laser energy and improve the desorption efficiency. In this case, the necessary power density for desorption was decreased by two orders of magnitude. In addition, a TLC plate-scanning device was developed, by which the chromatography on a TLC plate can be recovered, and rapid screening for numerous analytes on a TLC plate can be achieved. The device can also be applied for the identification of unknown compounds or to recognize overlapping sample spots. Finally, a quantification method for this system was developed. An internal standard was added into the mobile phase to yield a ‘background’ signal, which was used as a reference signal for the quantification. In this way, a wide range of compounds can be chosen for this purpose.

1. Introduction

1.1 Projective intention

Thin-layer chromatography (TLC), as an economic and handy chromatographic separation technique, has been extensively used for many years. The attractive features of TLC include parallel sample processing for high sample throughput; accessibility of the sample for post-chromatographic evaluation free of time constraints; detection in the presence of the stationary phase independent of mobile phase properties; and the stationary phase is normally used only once. It is generally agreed that thin-layer chromatography is most effective for the low-cost analysis of samples requiring minimal sample clean-up, or where thin-layer chromatography allows a reduction in the number of sample preparation steps (e.g. the analysis of samples containing components that remain sorbed to the stationary phase). Thin-layer chromatography is also preferred for the analysis of substances with poor detection characteristics requiring post-chromatographic treatment for detection. Since all sample components are located in the chromatogram, thin-layer chromatography is the most suitable technique for surveying sample properties. The popularity of TLC owes not only to the modest demands on instrumentation, but also to its sensitivity, general applicability, and, last but not least, its flexibility.

TLC can be viewed as the complementary technique in contrary to column chromatography, such as high-performance liquid chromatography (HPLC) due to their different attributes, resulting in a preference for one approach over the other independent of the application. However, the separated analytes are mostly detected with visual methods such as staining techniques, ultraviolet (UV) absorption and fluorescence

extinction, which causes the inability to identify the analytes and a low specificity with partially overlapping substances in TLC. It does not obstruct a technique like HPLC because the combination of HPLC and mass spectrometry (MS) has been developed to a reliable and robust technique in the last years. Obviously, mass spectrometry is an excellent technique for identification. Therefore, the full complement of identification and quantification tools available to column liquid chromatography is the desirable goal for thin-layer chromatography. It somehow means an interface for scanning and recording in situ mass spectra.

The introduction of mass spectrometry is mostly realized by a 'scrape and elute' mode, i.e. scrape the stationary phase containing analytes; elute/extract analytes from the stationary phase to a solvent; finally introduce the solvent into the ion source of the mass spectrometer in the regular way. Analytical strategies of TLC-MS are still widely carried out in this mode at present. However, as described, the whole procedure is time consuming and destructive for chromatography. In fact, it is not a way to really 'couple' mass spectrometry to thin-layer chromatography, but just a sample preparation process for mass spectrometry. It is suitable for the analytical work that has strict time limitation in method development or have a requirement for post-chromatographic treatment. However, the advantage of high throughput analysis is lost. In contrast, a modern mass spectrometer coupled to a liquid chromatographic system can acquire data automatically and has large advantages in the analysis of complex samples. Moreover, several robust ionization methods have been developed for LC/MS coupling. However, its method development and separation process are normally time-consuming, and often not suitable for high throughput analysis. If a TLC/MS system can be realized 'instrumentally', it will have advantages of quick experimental procedure,

large capacity for sample handling, and powerful identification ability at the same time.

There are three major factors in an 'instrumental' TLC/MS system: sampling, transport and ionization. Sampling mode decides how to acquire analytes from the bulk of TLC material. Transport part delivers molecules/ions from the TLC plate, after they are somehow obtained, to the mass spectrometer. Finally, ionization has to be achieved for the mass spectrometric analysis. These three factors influence each other and determinate the structure and performance of an 'instrumental' TLC/MS system.

1.2 Conceptions

On an intact TLC plate during the scanning process, the acquirement of analyte molecules should be efficient (which means a high recovery rate of the analyte), fast, and possess high spatial resolution. One parameter effecting the performance is the scanning speed. Lower speed normally causes higher sample recovery but longer analysis time. There are two methods to acquire molecules from TLC: desorption with energy or extraction with a liquid. The latter is based on the same principle as the 'scrape-elute' mode, but with a probe to sample continuously and does not destroy the plate. The solvent containing extracted analytes is transferred to the ion source of the mass spectrometer and subsequently obtain the in situ mass spectra. The choice of the ionization method for such a device is normally electrospray ionization (ESI). However, such a sampling mode involves the choice of solvent, which is critical for the extraction efficiency, and an inevitable diffusion of the analyte molecules on the TLC plate due to the use of the liquid. A desorption with energy has inherent advantages in respect to spatial resolution. In fact, it is more widely used compared to the extraction mode. Several methods to provide the

desorption energy exist, such as thermal desorption, laser desorption or desorption with fast atoms or ions. Except for thermal desorption, which was only used in a few studies in the early stage of TLC-MS coupling, the other desorption methods can also be used to perform ionization of the desorbed molecules in situ. In the applications adopting these methods on a piece of TLC plate (i.e. a small area cut from an entire TLC plate), ionization can actually also be realized by energy. Then ionisation takes place nearly at the same time as the desorption. However, it is not suitable for a plate scanning system, in which the transfer of desorbed molecules or ions from the surface of the TLC plate to the ion source have to be carried out since the ion source of a mass spectrometer is not big enough to contain a whole TLC plate. Unfortunately, the transfer of ions is more complicated than the transfer of molecules, which require major modifications to the mass spectrometer and decrease the applicability of such a device. Accordingly, an ideal sampling method should only desorb molecules for more convenient transport, and the ionisation should happen in the ion source of mass spectrometry. Obviously, this involves the decoupling of the desorption and ionization step.

The transport part is necessary for scanning an intact TLC plate. In an extraction based system, this part is relatively simply because analytes are contained in liquid. Such a transport part normally means a tube connecting the sampling probe to the ion source of the mass spectrometer. In an energy desorption based system, if not to modify the mass spectrometer, analytes are transferred with a gas flow. Before the appearance of atmospheric pressure ionization (API) techniques, the desorbed materials were transferred under vacuum or reduced pressure. This results in the requirement of pumping time for the correct pressure and more manufactural work for a sealing system. The API techniques allow a simpler design and more flexible installation of transport part.

However, transport under atmospheric pressure also causes more sample losses. Partially, these losses can be decreased by heating the transport lines.

The ionization method critically decides the performance of a TLC/MS interface. As a matter of fact, all popular ionization methods for mass spectrometry have been employed in TLC/MS, which will be discussed in detail in the next chapter. An API method has the advantage to be a 'soft' ionization technique with fewer fragments, simpler sample manipulation and, in our case, easier instrumental design and experimental arrangement. The most popular API techniques include ESI, atmospheric pressure chemical ionization (APCI) and AP-matrix-assisted laser desorption /ionization. These techniques are chosen for a certain application in dependence on the analytes or the purpose of the investigation. The combination of laser desorption and APCI was recently developed, in which the desorption by laser and ionization by APCI were well decoupled. This combination can be easily incorporated into TLC/MS system. Such a system benefits from the high spatial resolution of the laser, simple transfer of analyte molecules, compatibility with modern mass spectrometric systems and less fragmentation under atmospheric pressure. One drawback of such a system is that the cost for a traditional pulsed laser system is relatively high, which somehow counteracts the advantage of TLC in the low costs. The size of the laser system is also not ideal for a miniaturization of the whole analytical system.

Accordingly, another choice of the laser system was done here: the application of diode lasers. Diode lasers are reliable, compact, costs effective, easy to use and have a high efficiency. It can be anticipated that they play a more important role in the future. For the purpose of desorption, a continuous wave (cw) diode laser can continuously desorb molecules with the correct power and therefore keep desorption process

uninterrupted. However, diode lasers have never been used for laser desorption in the past. To introduce diode lasers into this scope, a graphite substrate was applied here to improve the absorption efficiency of laser irradiance. In doing so, the necessary power density for laser desorption decreased by two orders of magnitude. As a consequence, graphite-assisted diode laser induced desorption/atmospheric pressure chemical ionization can successfully be used for a TLC/MS interface system with plate scanning.

The experimental work of this thesis starts with studies on graphite plate targets for the testing of the feasibility of the method and basic optimization. Then, the studies on TLC pieces cut from the entire plates with graphite suspension covered are carried out to investigate the real situation with the appearance of TLC material. Subsequently, a plate scanning system is built up based on the results of above studies. Finally, a quantification method is developed for this system.

2. Theoretical Background

2.1 Basic techniques involved in this work

This work is focused on the establishment of a combination between a chromatography technique and mass spectrometry, and cw diode lasers are employed for laser desorption for the first time. To give a general impression on the basic techniques involved, they are introduced as follows.

2.1.1 Diode laser

Since 1962, the first diode laser was reported by Nathan et al,[1] many types of diode lasers emitting in the near-infrared or far-red region have been developed. Recently, green and blue diode lasers become commercially available. Diode lasers are widely used in bar-code readers, compact disks and laser printers.

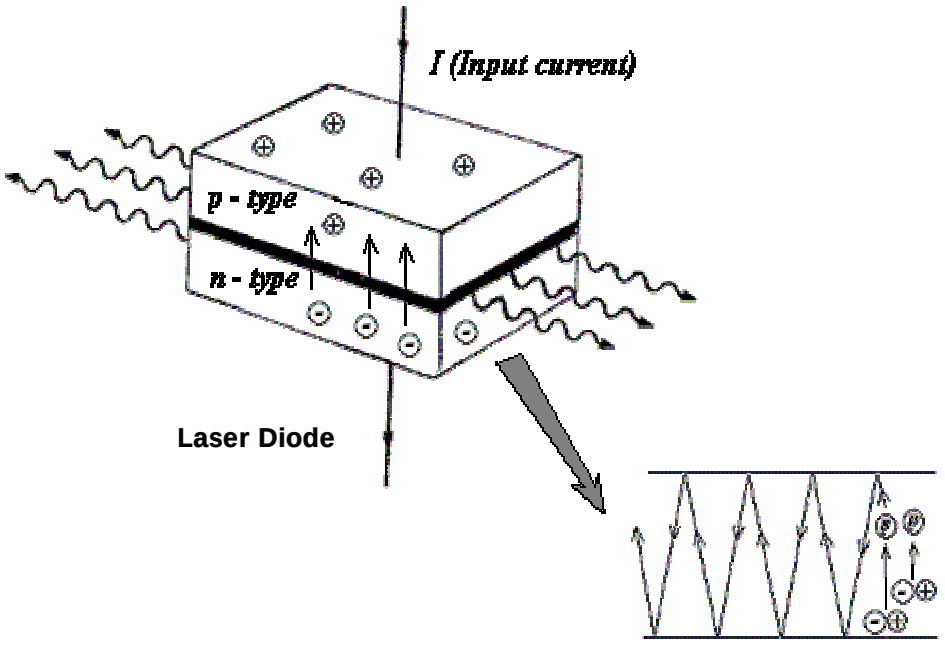

Figure 2-1. Mechanism of LED emitting

In a diode laser the lasing medium is a semiconductor p-n junction similar to that found in a light emitting diode (LED). As shown in Fig. 2-1, when a diode is forward biased, holes from the p-region are injected into the n-region, and electrons from the n-region are injected into the p-region. If electrons and holes are present in the same region, they may radiatively recombine—that is, the electron "falls into" the hole and emits a photon with the energy of the band gap. This is called spontaneous emission. Under suitable conditions, the electron and the hole may coexist in the same area for quite some time (on the order of microseconds) before they recombine. If a photon of exactly the right frequency appears within this time period, recombination may be stimulated by the photon. This causes another photon of the same frequency to be emitted, with exactly the same direction, polarization and phase as the first photon. In a laser diode, the semiconductor crystal is shaped somewhat like a sheet of paper—very thin in one direction and rectangular in the other two. The top of the crystal is n-doped, and the bottom is p-doped, resulting in a large, flat p-n junction. The two ends of the crystal are cleaved so as to form perfectly smooth, parallel edges; two reflective parallel edges are called a Fabry-Perot cavity. Photons emitted in precisely the right direction will be reflected several times from each end face before they are emitted. Each time they pass through the cavity, the light is amplified by stimulated emission. Hence, if there is more amplification than loss, the diode begins to "lase".[2,3]

Fig. 2-2 shows a picture of a diode laser. It was taken by a camera mounted onto a microscope. The laser diode (circled part) was mounted on a copper stage, which is helpful for heat elimination. The size of the laser diode is about 0.8 x 0.8 mm^2. As introduced above, the laser beams exit the diode in two dimensions, in this picture, upward and downward. The upward laser beam is the laser output, and the downward laser beam,

called ‘reference beam’ is monitored by a photo diode, so that the power output of a diode laser is accurately tuneable.

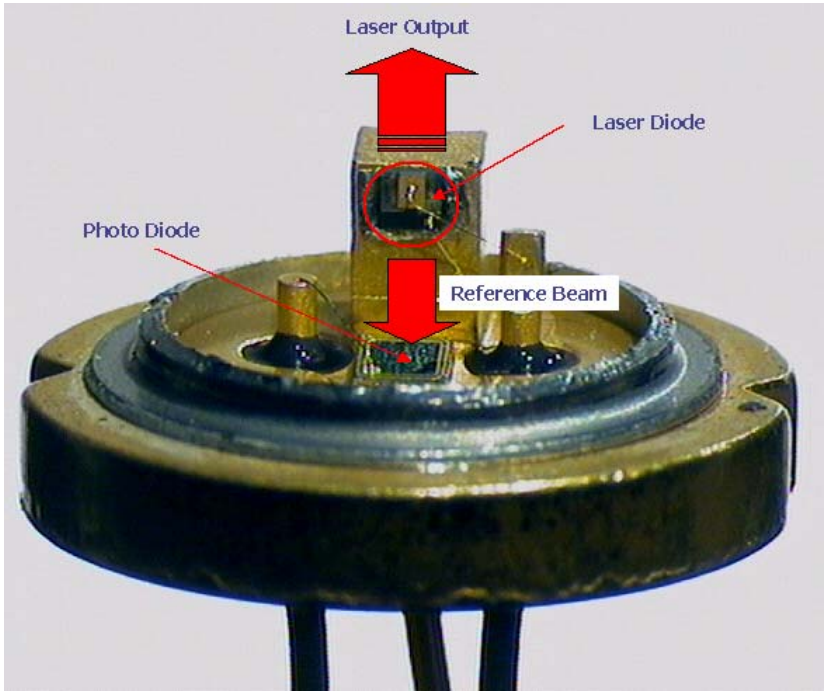

Figure 2-2. Pictures of a real diode laser

Due to reliability, compactness, tunability, long lifetime, and easy operation and maintenance, diode lasers have been used successfully in analytical spectroscopy, such as molecular absorption spectroscopy, fluorescence spectroscopy, atomic absorption spectroscopy and optogalvanic spectroscopy.[4-8] However, diode lasers have never been applied in the laser desorption technique until now. Generally, diode lasers can be distinguished into two categories: single-mode and multimode.[4] Single mode diode lasers are mostly employed in atomic absorption spectroscopy. Compared with hollow cathode lamps, which are the classic light sources in atomic absorption spectroscopy, single-mode diode lasers have the advantages of higher light intensity, narrower linewidth, more

stable output power, and smaller dimension. However, they only have output powers of up to 150 mW, which are not suitable for our application. Accordingly, all diode lasers used in this work are multimode diode lasers. Today, there are multimode diode lasers with powers up to 50 W commercially available. This power is sufficient for laser desorption.

2.1.2 Atmospheric pressure chemical ionization mass spectrometry (APCI/MS)

The first reported use of chemical ionization at atmospheric pressure coupled to mass spectrometry is the work by Shahin.[9,10] He used a discharge chamber with a platinum wire as anode to study ion-molecule reactions. The group of Horning developed an atmospheric pressure ionization source, in which a radioactive ^{63}Ni foil is used to initiate gas-phase ionization.[11] Later, when they coupled liquid chromatography (LC) to mass spectrometry, the use of a corona discharge needle was introduced.[12-15]

APCI is an ionization technique for mass spectrometry (MS) that uses gas-phase ion-molecule reactions at atmospheric pressure. The method is analogous to chemical ionization (CI), which is normally used in gas chromatography/mass spectrometry (GC/MS). The APCI technique is mainly applied to polar and ionic compounds with moderate molecular weight and generally gives monocharged ions. It is usually coupled with liquid chromatography, especially high-performance liquid chromatography (HPLC), and has become a popular ionization source for mass spectrometry in these years.[16,17]

The schematic diagram of an APCI source is shown in Fig. 2-3.[18,19] The analyte in solution from a direct inlet probe or a liquid chromatography eluate is introduced directly into a pneumatic nebulizer where it is converted into a thin fog by a high-speed nitrogen beam. The droplets are

then transported by the gas flow through a heated quartz tube called a desolvation/vaporization chamber. The heat transferred to the spray droplets allows vaporization of the mobile phase and of the sample in the gas flow. The temperature of this chamber is controlled, which makes the vaporization conditions independent of the flow and from the nature of the mobile phase. After desolvation, the solvent and analyte molecules are carried along a corona discharge electrode where ionization occurs.

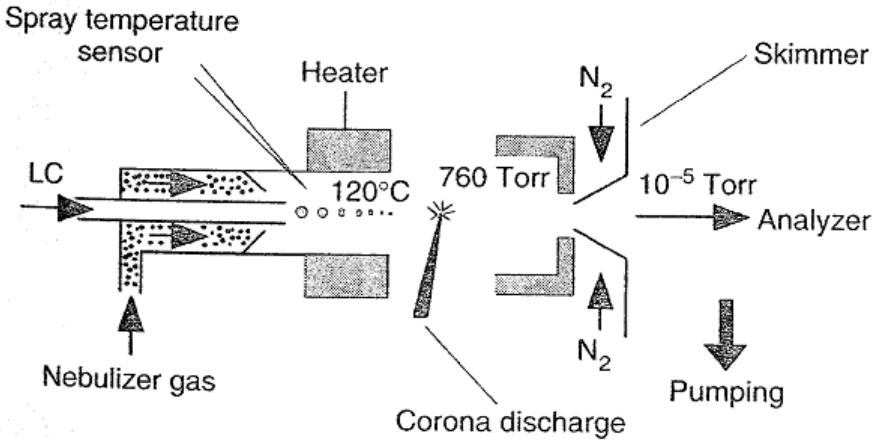

Figure 2-3. Schematic diagram of an APCI source[18]

The ionization processes in APCI are equivalent to the processes that take place in CI but all of these occur under atmospheric pressure. In the positive ion mode, either proton transfer or addition of reactant gas ion can occur to produce the ions of molecular species, depending on the relative proton affinities of the reactant ions and the gaseous analyte molecules. In the negative mode, the ions of the molecular species are produced either by proton abstraction or adduct formation. Typically, the corona discharge is formed by electron ionization primary ions such as $N^{\bullet+}$ or $O_2^{\bullet+}$. Then, these ions collide with vaporized solvent molecules to form secondary

reactant gas ions. As shown below, it is a typical proton transfer process. Here, M is the analyte, and H_3O^+ plays the role of the reactant gas ion.

$$N_2^{\bullet+} + 2N_2 \rightarrow N_4^{\bullet+} + N_2$$
$$N_4^{\bullet+} + H_2O \rightarrow H_2O^{\bullet+} + 2N_2$$
$$H_2O^{\bullet+} + H_2O \rightarrow H_3O^+ + OH^{\bullet}$$
$$H_3O^+ + M \rightarrow [M + H]^+ + H_2O$$

Because the ionization of the substrate occurs at atmospheric pressure and thus with a high collision frequency, it is very efficient. Furthermore, the moderate desolvation and vaporization of the droplets considerably reduce the thermal decomposition of the analyte. The result is that the ionization step yields predominantly ions of molecular species with few fragments. In comparison to other ionization techniques working under vacuum, APCI can be regarded as a 'soft' ionization technique.

2.1.3 Thin-layer chromatography (TLC)

Thin-layer chromatography is a type of chromatography in which the stationary phase is a thin layer of an adsorbent, e.g. silica gel, coated on a rectangular plate and the mobile phase is typically a solvent mixture. For analytical purposes, samples are applied in the form of spots at a point near one edge of a plate. In preparative work, the samples are streaked, often by means of a special device (applicator, streaker) on a line parallel to that edge. The chromatograms are developed in a closed vessel (chamber) by allowing the edge of the plate to dip into the mobile phase, which then advances past the sample to a paralleled line (solvent front) near the opposite edge. The detection is carried out after the TLC plate is dried in order to remove the residues of the solvents from the sorbent. Since most substances resolved by TLC are colorless, visualization step is used to find the position of the analyte, such as staining techniques,

ultraviolet (UV) absorption and fluorescence extinction. Automated and more convenient detection is based on UV/visible (VIS) absorption or fluorescence imaging allowing the quantification of the analytes. Normally, TLC detection methods are nondestructive and permit further analysis of the separated substances.[20-22]

The attractive features of TLC include parallel sample processing for high sample throughput; accessibility of the sample for post-chromatographic evaluation free of time constraints; detection in the presence of the stationary phase independent of mobile phase properties; and the stationary phase is normally used only once. As an economic and handy chromatographic separation technique, TLC has been extensively used for many years not only because of the modest demands on instrumentation, but also due to its sensitivity, general applicability, and its flexibility.[23-25] It is generally agreed that TLC is most effective for the low-cost analysis of samples requiring minimal sample clean-up, and it is also preferred for the analysis of substances with poor detection characteristics requiring post-chromatographic treatment for detection. Since all sample components are located in the chromatogram, TLC is the most suitable technique for surveying sample properties.

2.2 TLC-MS

The major inherent drawback to the usual detection techniques of TLC is the inability to identify the analytes and have a low specificity with partially overlapping substances. Recently, mass spectrometry has become the method of choice for identification of compounds in conjunction with TLC techniques, which was first realized by Kaiser.[26] In his work, a small H_2-O_2 flame was used to desorb samples directly from a silica plate, and then desorbed materials were simultaneously swept into a mass spectrometer by Helium.

In the last few decades, a series of attempts have been made to couple TLC and MS.[23,24,26,27] There are two approaches to remove the analyte molecules from the TLC plate. One possibility is to extract the analytes with a solvent either directly from the TLC plate or after removing a part of the stationary phase that contains the separated analyte. Typically, the extraction step is then followed by gas chromatography /mass spectrometry (GC/MS) analysis. Unfortunately, this extraction method can destroy the chromatographic integrity and it is also quite time consuming. The other basic technique is the desorption of analytes with energy, such as secondary ion mass spectrometry (SIMS), fast atom bombardment (FAB) or laser desorption/ionization and some related techniques. These techniques need fewer experimental steps, however, due to an intrinsic coupling of desorption and ionization of the analytes they do have high requirements for the instrumentation.

2.2.1 Electron impact (EI) and Chemical ionization (CI)

EI is the classical ionization technique in mass spectrometry, in which a gaseous sample is bombarded by electrons usually generated from a tungsten filament.[28,29] Because the pressure inside the ion source is kept low, the technique induces extensive fragmentation. Ion-molecule reactions do not occur, e.g. a $[M+H]^+$ signal due to proton transfer is not observed. The application of EI is restricted to the thermally stable samples with low molecular masses.[18] Because EI is a popular ionization method for GC/MS, the coupling is generally performed by using a GC mass spectrometer system. There are two methods to introduce analytes into the EI ion source after removing the stationary phase containing the analyte. In the first method, the analyte is extracted with a solvent, and then the solvent is injected as a liquid phase. This method is technically undemanding and efficient, but involves extraction and concentration

steps, which is usually time-consuming.[30,31] In another method, the TLC piece is put on a heating probe. The analyte absorbed in the stationary phase is thermally evaporated and injected in its gaseous phase. The method has less experimental steps but is restrictedly for polar or involatile compounds on silica gel plates because of the strong absorption of such analytes to silica.[32] One solution for the problem is the use of less absorbing stationary phase, such as polyamide TLC plates.[33-35]

The basic principle of CI is similar to that of APCI, but ion-molecule reactions happen in much lower pressure (about 10^{-3} Pa).[36] Compared with EI, it yields less fragmentation and often gives information about the molecular weight of the analyty.[18] CI is not widely used in combination with TLC, but very often used in GC/MS. A prominent work about TLC-CI/MS was performed by Ramaley et al.[37,38] A remarkable advance in this work was that they developed a device for plate scanning, in which a chamber containing a TLC plate was put on a stepping motor to provide motion. The analyte on the TLC plate was desorbed with a quartz-halogen projection lamp or a CO_2 laser. The desorbed molecules were swept by a CI regent gas to the ion source of the mass spectrometer after passing through a heated glass tube.

2.2.2 Secondary ion, fast atom bombardment and liquid secondary ion mass spectrometry (SIMS, FABMS and LSIMS)

SIMS analyzes the secondary ions emitted when a surface is irradiated with an energetic primary ion beam, such as $^{2}He^{+}$, $^{16}O^{+}$, or $^{40}Ar^{+}$.[39,40] FABMS[41] and LSIMS[42] are techniques that consist of focusing a high-energy primary current beam of neutral atom/molecules or ions on the sample, respectively. Essential features of these two techniques are the use of a non-volatile liquid matrix, e.g. glycerol, which is not used in SIMS. The purpose of the matrix is to induce ionization and refresh the surface.

Compared with EI, these ionization techniques are 'soft' and produce only few fragments. They are more appropriate for polar or thermally unstable substances than EI.[18]

The use of TLC in combination with these ionization techniques was realized for a whole range of analytes, including drugs and their metabolites, pesticides, natural products, synthetic chemicals and dyes.[26] Because these ionization techniques can be applied on solid surfaces directly, the approach of extraction with solvent is not necessary. The normal procedure of the combinations includes the removal of the stationary phase with substrate; condensation with a solvent, such as methanol, for analytes with small amount or in a diffuse spot, which is applied around the sample spot; application of matrix (for FABMS and LSIMS); and subsequent mass spectroscopic analysis.[43-45] Suzuki et al. developed an improved condensation technique, in which the area including the desired spot was cut, and a trapezoidal shape was scribed, out of which the stationary phase was scratched. A small amount of methanol was deposited on the lower base of the trapezoid, which causes the migration of the analyte to the upper base by penetration of methanol. Finally, the analyte was concentrated in a line of 0.5-1 mm.[46] Nakagawa et al. designed a simple device allowing a stripe of the TLC plate to be scanned by a LSIMS source.[47] It supplies the information of chromatography, but has just a few modifications of the ion source at the same time.

2.2.3 Electrospray ionization (ESI)

ESI is another popular ionization method for mass spectrometry, suitable for large bio-molecules or synthetic polymers. Like APCI, it also works under atmospheric pressure and enables LC/MS coupling. An ESI source consists of a very fine needle and a series of skimmers. A high potential is

applied to the needle. The sample solution is sprayed into the source chamber to form droplets. The droplets carry charges when they exit the capillary. After the solvent is vaporized, the droplets disappear and leave highly charged analyte molecules.[48,49] In contrast to APCI, ESI normally produces multiply charged ions, which allows the analysis of high molecular weight molecules using analysers with a lower nominal mass limit. However, the sample must be soluble in low boiling solvents and stable at very low concentrations, i.e. 10^{-2} mol/l. Very often HPLC methods have to be modified for ESI when they use high concentrations of buffers.[18]

The first work about TLC-ESI coupling was the development of a micro solvent extraction device.[50] The device composes of an inner capillary with a sheath of absorbent material that is contained by a metal tube. A suitable solvent is transferred through the capillary to the area containing analyte on the TLC plate, diffuses a short distance, and then drawn up by capillary action into the absorbent material. The analyte is subsequently eluted from the sorbent for ESI-MS analysis. Luftmann presented another device based on a similar principle, but without absorbent material. Furthermore, the solvent was injected continuously, which enable direct coupling with an ESI mass spectrometer to perform 'on-line' analysis.[51] Berkel, et al. built a computer-controlled movable probing system that allows 'on-line scanning' of the whole TLC plate and, as a result, the investigation of unknown mixtures.[52] Apart from the 'extraction /transmission' mode mentioned above, another approach that realizes electrospray ionization directly on the edge of the plate was developed.[53] Here, the electrospray process happens at one sharp end of a TLC strip, which is connected to a power supply.

2.2.4 Laser desorption/ionization (LD/I), matrix-assisted laser desorption/ ionization (MALDI) and surface-assisted laser desorption/ionization (SALDI)

In a laser desorption/ionization process, laser pulses desorb material from the surface and create a microplasma of ions and neutral molecules, which may react among themselves in the dense vapor phase near the sample surface. The laser pulse realizes both the vaporization and ionization of the sample.[18] Because of the small sampling area of the laser, the introduction of laser systems to TLC gives the advantage of a higher spatial resolution, and also the possibility of repeatable analysis due to the unchanged bulk material. However, laser desorption/ionization always produces fragmentation products, especially when a UV laser is applied.[54] A partial solution of the problem is the use of an infrared (IR) laser for desorption followed by multi-photon ionization (MPI).[55] The LD/I process depends critically on the specific physical proprieties of the surface. Therefore, more laser power is required to desorb analytes from a TLC plate compared with desorption from a metal plate (sample-substrate affinity).[56]

Recently, MALDI is widely used in the analysis of large biological molecules such as proteins or DNA fragments.[57] Differing from other LD/I techniques, MALDI involves the use of a matrix. The analyte molecules are dispersed in a solid matrix crystal so that they are isolated from each other. The rapid heating by the laser beam causes localized sublimation of the matrix crystals together with analyte molecules. It is the matrix that absorbs the most energy so that little internal energy is transferred to the analyte molecules.[58,59] However, the ionization process in MALDI is still not fully understood,[60] and the most widely accepted ion formation mechanism involves gas-phase proton transfer in the expanding plume with photoionized matrix molecules, as shown in Fig. 2-4. MALDI is a soft ionization technique and allows the determination of the molecular weight of molecules up to 500 kDa. Moreover, the MALDI process is

independent of the absorption properties and size of the analyte so that it is not necessary to adjust the laser wavelength to match the analyte.[18]

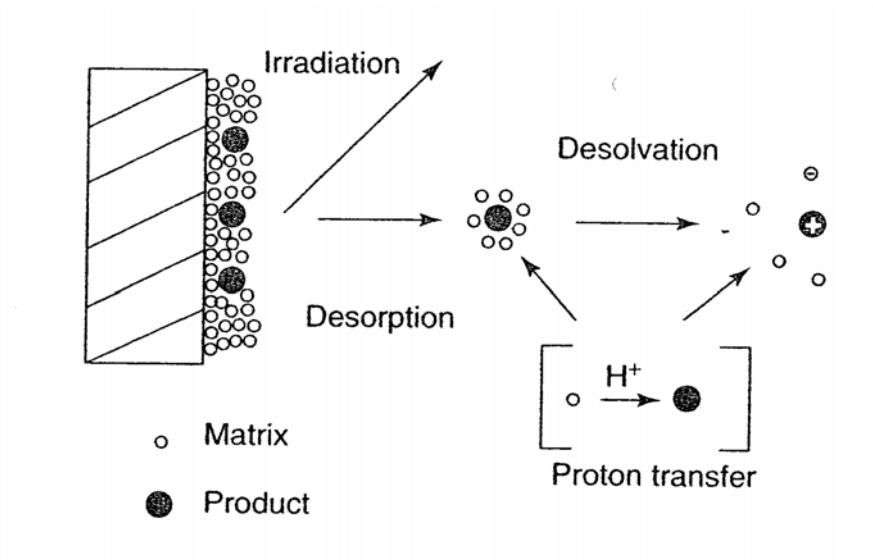

Figure 2-4. Diagram of the principle of MALDI[18]

This technique was also introduced to TLC recently.[61] However, one problem, compared with normal MALDI on a metal substrate, is that the analyte molecules must be 'extracted' to the surface of the plate from the bulk of the TLC material before they enter the matrix crystal. For this purpose, an 'extraction' solvent is applied on the sample spot after separation. Then, matrix is added to the extraction solvent gradually after the extraction step is finished, and at last forms a structure as normal MALDI.[62-64] However, the lateral diffusion of separated compounds is inevitable in this method due to the 'extraction' solvent. Therefore, a protocol was developed to solve the problem. A matrix layer is prepared on a stainless steel plate first, and then the layer is transferred onto the developed TLC plate prewetted with the 'extraction' solvent. With a gentle pressure, the analyte is incorporated into the matrix structure, and then the stainless steel is discarded, followed by laser

desorption/ionization.[65,66] More recently, a hybrid plate with a silica gel layer and an adjacent MALDI layer on a normal backing was developed to obtain nearly 100 % analyte recovery.[67] In this method, after analytes are developed in the silica gel layer, the plate is rotated 90°, and the separated compounds are eluted to the MALDI layer, followed by normal MALDI analysis.

Another similar method, named surface-assisted laser desorption /ionization (SALDI) was developed lately.[68,69] Instead of using a matrix, particle suspensions are employed on the plate surface to couple the laser energy resulting in a thermal induced desorption/ionization process. Activated carbon or graphite particles are normally used in the technique, and the latter with a diameter of 2 μm shows the best capability for signal yield.[70] SALDI can also be performed with a pencil drawing on a TLC plate.[69] The extracting step is not necessary in the method, and it opens the possibility to choose lasers with different wavelength because particle suspensions have an almost wavelength independent absorption ability.[71]

2.2.5 Plate scanning device

Most methods to couple TLC with MS are based on the 'scrape and elute' mode, but an interface for TLC plate scanning is attractive for TLC-MS. A plate-scanner coupled with a mass spectrometer gives the chance to reconstruct the chromatogram based on either single ion monitoring or the total ion current. If the plate is scanned along the lane in which the sample is developed, the detection can be carried out without the information of migrating distances for all the analytes. It is possible to detect component of unknown samples and overlapping spots. Moreover, the coloration step is not necessary in this mode. Furthermore, the scan can be also carried out orthogonally to the direction of development and on the level corresponding to a special substance. Since many samples are developed

parallelly on a TLC plate, the 'screening' of the substance in samples can be carried out with a high-throughput.

For any scanner, the TLC plate can be moved linearly past the source of desorption or extraction, or the source can be moved linearly along the plate. The former is a simpler method and has the fewest disadvantages. Furthermore, the ionization of desorbed or extracted sample molecules also can happen before or after transfer, i.e. transporting ions or molecules. The first solution is cumbersome, requiring major modifications to the spectrometer but provides better spectra. The other solution is simpler, and its setup can be coupled with a wide variety of mass spectrometers, but it includes the danger of samples loss or breakdown during transfer.

Because there were no AP ionization methods for mass spectrometry several years ago, the transfer of desorbed materials was complicated. It was necessary to maintain vacuum in the transferring tube and the chamber in which the plate was placed. Furthermore, there was a pumping time necessary for each analysis after the plate was placed inside the chamber and before the mass spectrometer was started. Ramaley et al. built up such a device that was briefly described in the chapter 2.2.1.[37,38] Another scanner was designed based on liquid secondary ionization,[47] by which a stripe of TLC plate can be scanned. Because desorption and ionization happen simultaneously in liquid secondary ionization, ions are transferred rather than molecules, which is different from the case in Ramaley' work. As a result, the ion source had to be modified.

Besides the fact that API techniques are softer than vacuum ionization techniques, API techniques give also the benefit of easier sample handling. With API techniques, TLC plates can be placed on a movable stage directly without a sealed chamber and additional waiting time for vacuum. Disadvantage is the possible loss of sample molecules during transfer. A scanner based on ESI was developed recently, in which analytes were

extracted and transported with a solvent.[52] In this case, the sample recovery can be improved by choosing the suitable solvent and decreasing scan speed.

In all the experimental setups described above, the sample molecules were removed from the TLC material by thermal or laser desorption, bombardment with ions or extraction with solvent. Among them, laser desorption have the advantage of a smaller sampling area, which results in a higher spatial resolution. If the laser beam is conducted by an optical fibre, the experimental arrangement is even more flexible. However, in the techniques adopting traditional pulsed lasers, desorption and ionization are not decoupled, and both happen 'in situ' on the TLC plate. In this case, ions rather than molecules have to be transported. Moreover, the output of a traditional pulsed laser is usually unstable from pulse to pulse. It possibly varies from a power density high enough for ionization or even fragmentation of sample molecules to a level not enough for desorption without changing any working parameters. The decoupling of desorption and ionization is therefore quite difficult for pulsed lasers. These disadvantages can be eliminated by using a cw diode laser. A cw diode laser has very stable and accurately tuneable power output. In combination with the adequate irradiation time, the energy density can be accurately set to desorb molecules without ionization and less fragmentation.

3. Diode laser induced desorption in combination with APCI/MS on graphite substrate

Diode lasers are reliable, compact, costs effective, easy to use and have high efficiency. According to inherent advantages of a diode laser, it can be anticipated that they will be extensively used in routine analytical tasks in the future.

Compared with pulsed lasers, cw diode lasers have very stable and accurately tuneable power outputs, which is an important preconditions for experimental studies of laser desorption/ionization. However, diode lasers have never played any role in this scope until now. Most diode lasers emit in the near-infrared range, and few materials resonantly absorb in this range. The power output of a cw diode laser is also not capable to induce ionization. Accordingly, if wanting a diode laser is going to be used for laser desorption/ionization, more power from the laser beam should be coupled to the surface and the desorption and ionization steps should be decoupled.

A graphite surface can work well as a photon absorbing material. Kim et al applied MALDI to a graphite target that works as an energy transfer mediator for visible light.[72] This group showed that the graphite surface also works well for a near-infrared laser beam. A desorption of biochemical molecules with moderate molecular weight deposited on a graphite plate can be realized with a power density in the order of 10^4 W/cm^2, which is 100 times lower than the power density which is needed to desorb from a white surface.[18]

The decoupling of desorption and ionization was realized by LD-APCI technique, which was recently presented by Coon et al.[73-76] As shown in Fig. 3-1, a corona needle was positioned above the target to ionize the desorbed molecules rather than normal AP-MALDI process. In this work,

the signal intensities were enhanced by 2 to 3 orders of magnitude for different analytes when compared with measurements without corona discharge, which means that the desorption and ionization steps were decoupled.

Based on above theories, a study to evaluate the applicability of desorption from a graphite surface by a continuous wave diode laser is presented here.

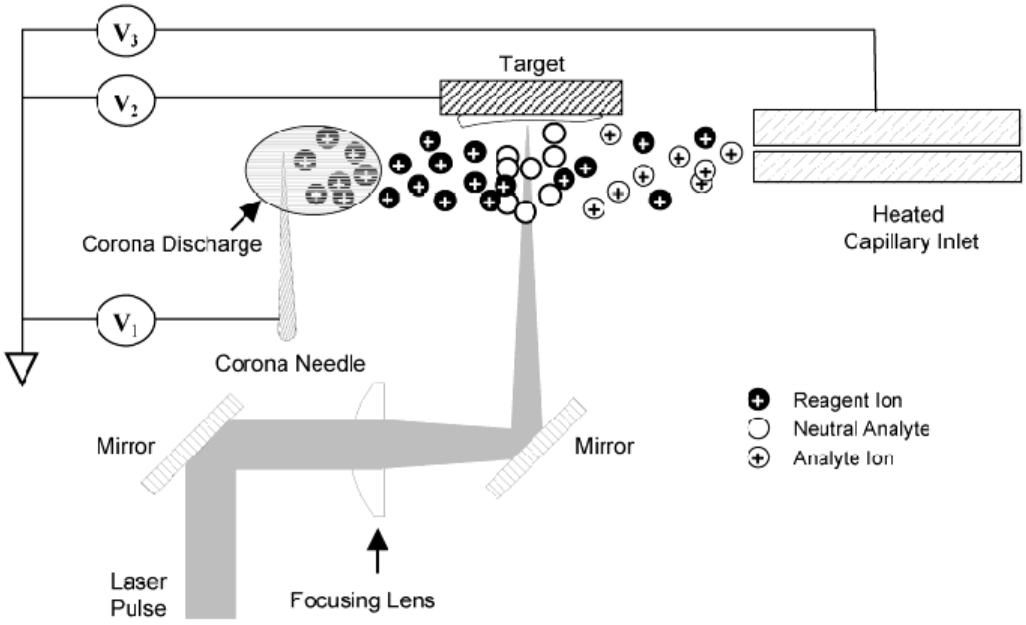

Figure 3-1. Schematic representation of LD-APCI source[73]

3.1 Chemicals and sample preparation

The first evaluation was accomplished with reserpine (from Sigma), a calmative and antihypertensive drug. It is often used for the calibration of the LC-MS system and is accordingly suitable to give a well-defined signal (for structure, see Fig. 3-2).

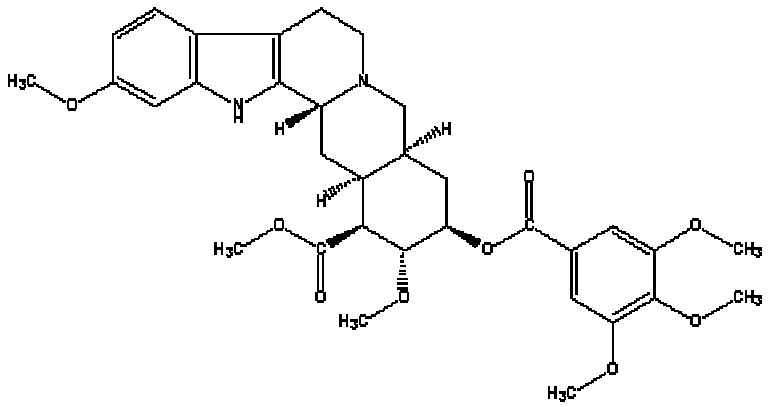

Figure 3-2. Structure of reserpine

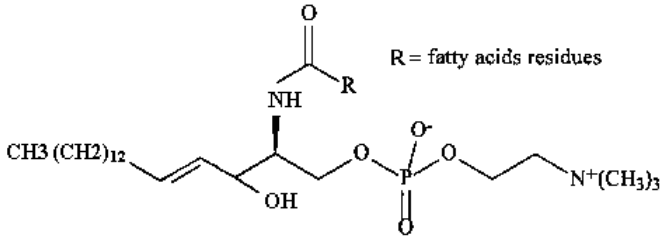

Figure 3-3a Structure of sphingomyelin

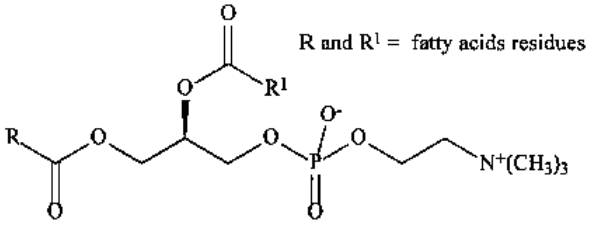

Figure 3-3b Structure of lecithin

Sphigomyelin (SPM, from bovine brain) and Lecithin (L-α-phosphatidylcholine, PC, from fresh egg yolk) were also employed as analytes (both from Sigma). They are the most abundant phospholipids inside living bodies. Sphingomyelin is a group of phosphosphingolipids made by transferring choline from lecithin to a ceramide, which make up a

significant portion of the membrane lipids of the myelin sheath of nerve tissue. Lecithin is a group of glycerophospholipids that are involved in the metabolism of several lipid compounds. Determination of the lecithin/sphingomyelin ratio in the amniotic fluid is a viable method of monitoring and assessing fetal lung maturity. This is particularly important in high-risk pregnancies, for which early confinement is to be expected. They both are a group of phospholipids that have similar structures but different masses based on the length of respective carbon chain and the amounts of unsaturated carbon bonds, as shown in Fig. 3-3a, b.

Other chemicals used in this study include methanol (from Merck, Germany), acetic acid (from Merck, Germany), chloroform (99.8 %, from Aldrich), n-Hexane (for GC, from Merck, Germany), isopropanol (for GC, from Merck, Germany) and ethanol (99.8 %, for chromatography, from Merck, Germany). No further treatment or purification was performed for the materials.

The reserpine solutions were prepared by dissolving reserpine in an aqueous solution of 50 % methanol with 0.1 % acetic acid. The sphingomyelin and the lecithin solutions were prepared by dissolving their powders in a mixture of chloroform/methanol/n-hexane (13:18:69) and ethanol/n-hexane (70:30), respectively.

3.2 Design of ion source and experimental setup

Fig. 3-4 shows a picture and its sketch of the ion source used for the following experiments. A stainless steel target with a diameter of 4 mm was mounted on a xy-stage (DS40-xy, Newport, USA). The distance of the target to the front of the heated capillary inlet (transfer capillary) of a LCQ Classic ion trap mass spectrometer (ThermoFinnigan, USA) and the position in reference to the axis could be changed by the xy-stage. A potential of 1.5 kV was applied to the target by an additional power supply

in order to improve the ion transmission efficiency. The corona needle was positioned on the axis of the transfer capillary and above the rear edge (in reference to the capillary inlet) of the target. The needle could also be moved along the axis, therefore allowing an adjustment of the needle-to-plate distance for optimal APCI conditions. The standard APCI power supply of the LCQ system was connected to the needle to generate the corona discharge. A diode laser (GBL981000G from Roithner Lasertechnik) with a wavelength of 985 nm and a maximum power output of 1 W was used to desorb the analyte molecules. For all experiments the laser beam was focused on the surface with a calculated spot diameter of approximately 0.1 mm, resulting in a maximum power density in the order of 10^4 W/cm^2.

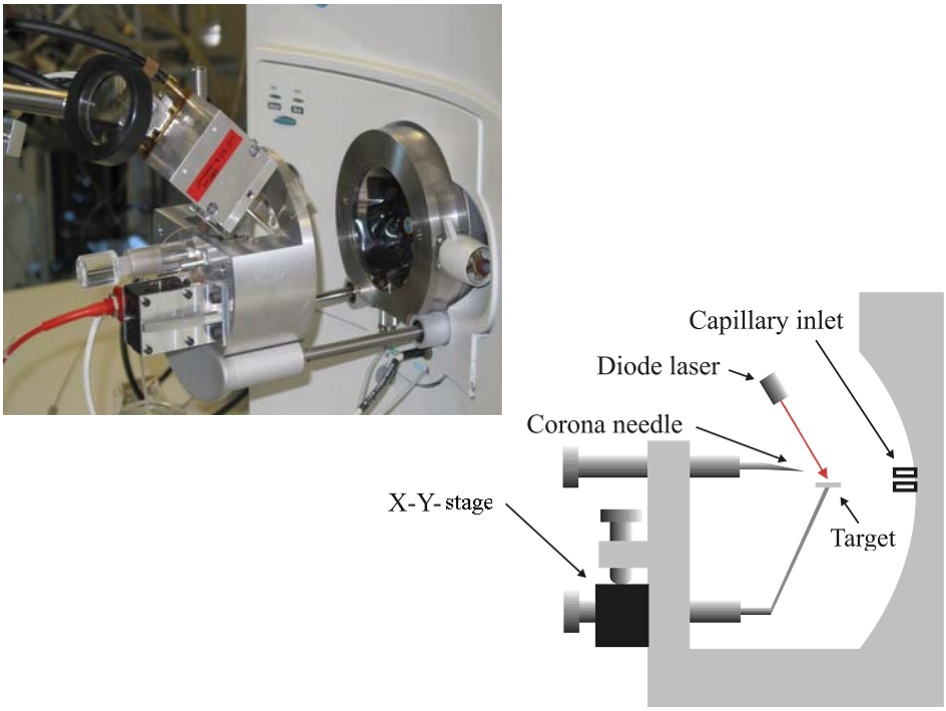

Figure 3-4. Ion source

The parameters of the mass spectrometer were optimized according to the analytes, individually. For reserpine, the capillary voltage was set to 6 V, the tube lens voltage offset was set to 57 V; for sphigomyelin and lecithin, they are 41.5 V and 43 V, respectively. The capillary temperature was maintained at 150 °C in all cases. The ion injection was set to AGC (Automatic Gain Control) mode, because the synchronization between laser and mass spectrometer is not necessary due to the use of a continuous wave diode laser system.

3.3 Optimization of experimental conditions

The first step of the following experiments was to find the best position of the corona discharge in reference to the entrance of the transfer capillary in order to realize ionization of desorbed molecules and an efficient transportation by the electric field. A short distance between the target and the inlet of the mass spectrometer is helpful to improve the transmission efficiency. However, if the target is too close to the capillary inlet, electric arc will appear in between. A distance of 3 mm from the front edge of the target to the inlet was evaluated as the optimized parameter at last whereas the target was 3 mm underneath the axis of the transfer capillary. This ensured the cloud from desorbed materials formed on the axis. It was found that the target can influence the corona discharge if it is too close to the axis of the electric field. The tip of the corona needle was positioned on axis and above the rear edge of the target, 1-2 mm offset from the spot of the laser beam, which ensured that the electric field contained the whole region of the desorbed molecules in gas phase. It has to be noted that when the corona needle is moved above the front part of the target, only a very weak sample signal can be measured, and when the position is beyond the front edge, no signal can be obtained. Such a geometry layout assures that a well-build electric field is set to form the steady ion current to carry

ionized sample molecules into the mass spectrometer. In other studies about LD-APCI,[73,74] long distance among each part of the source (1.5 or 3 cm from heated capillary inlet to the tip of corona needle) was applied and for efficient ionization in such a distance, a high discharge voltage (5.3 or 8.1 kV) was utilized. In our case, a compact source layout was employed to improve the transmission efficiency, and the discharge voltage was dropped to around 3 kV, which results in a current of 0.5 μA. Higher discharge currents were also used, but no obvious enhancement of the signal intensity could be observed, and the risk of a plasma breakthrough between corona needle and target is high. A potential of 1.5 kV on the target was found to enhance desorption and also improve ion transmission efficiency.

3.4 Analysis of a compound with moderate molecular weight

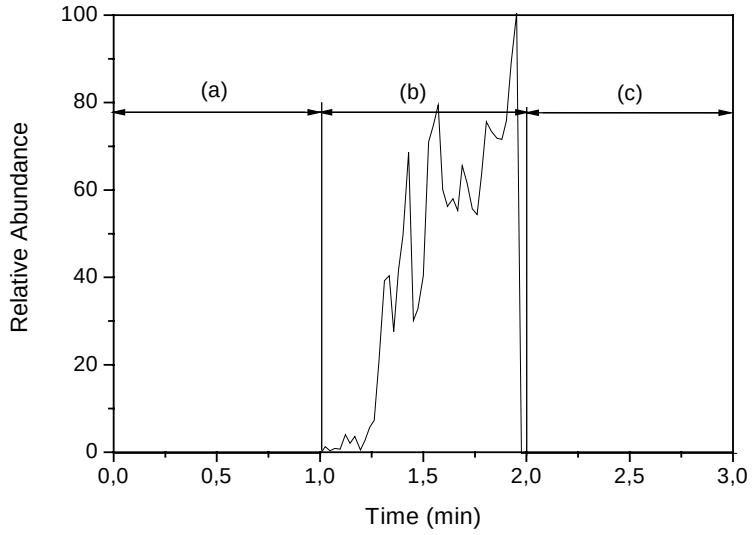

Figure 3-5. Total ion counts obtained with (a) only corona discharge on (b) both corona discharge and laser on (c) only laser on.

A reserpine solution of 2 µl (0.6 µg reserpine) was deposited on a piece of graphite plate that was placed on the metal target. The 1 W diode laser with maximal power output was used to desorb sample molecules after the sample solution was dried for 5 min at room temperature. The total ion counts in the m/z range from 150 to 700 were measured by the mass spectrometer.

As shown in Fig. 3-5, no signal of reserpine was measured when only the corona discharge was in operation (a), obviously due to the fact that reserpine was not evaporated from the surface of the graphite plate. After about 1 min, also the diode laser was switched on (b), and an intensive signal appeared. Finally, after about 2 minutes, the corona discharge was switched off (c) and the signal intensity decreased immediately to zero. Therefore, analyte ions can only be measured when both the laser and the corona discharge are in operation. It is obvious that the increase of the signal initiated by laser irradiation was less slow than the decrease caused by stopping the corona discharge. The slow increase of the signal might be explained by the time of the heat transfer. The instantaneous decrease is due to the fact that the ionization process is stopped and no more ions enter the mass spectrometer.

In the case when only the laser was on (c), it is obvious to assume that the power from the diode laser was not high enough for ionization. However, even if a few sample molecules are ionized, the absence of an electric field in the entrance region of the mass spectrometer resulting from the corona discharge reduces the transmission efficiency of ions. Therefore these ions cannot be detected. In order to clarify this, the voltage of the corona discharge was decreased to the extent that the corona discharge expired, but attendant ions could still be transported into the capillary inlet. Nevertheless, there was still no measurable signal. Therefore, desorption and ionization are well decoupled in this technique.

Fig. 3-6a shows the spectrum from one single scan, which gives an intensive molecular peak with a low background and no fragments. After decreasing the concentration of the analyte by a factor of 10 (60 ng, i.e. 100 pmol), the molecular ion of reserpine was still observed as the base peak with intensity about 10 times lower. However, the background in the low mass range was higher, and peaks from oxidized products ($[M+H+16]^+$, $[M+H+32]^+$, etc.) became more obvious. Fig. 3-6b shows the spectrum, which resulted from the average of 10 individual mass spectra.

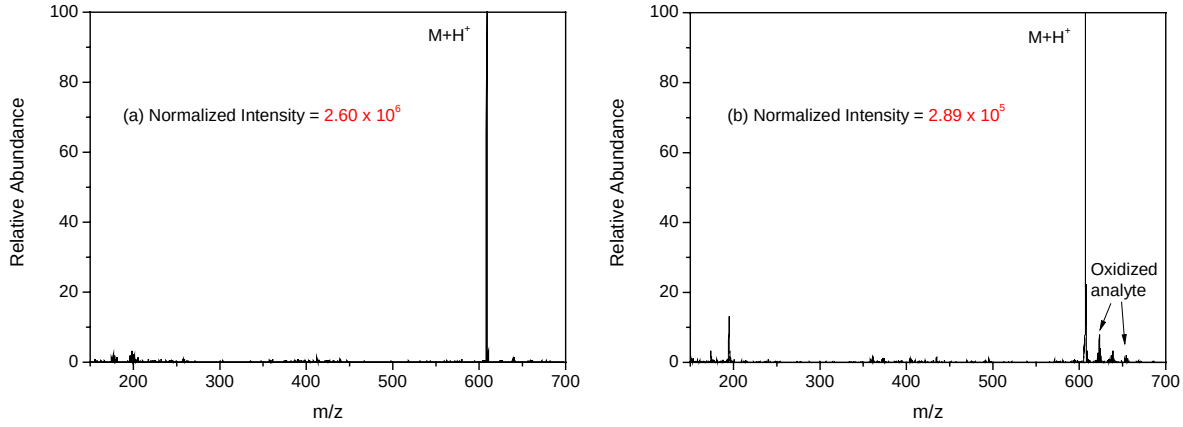

Figure 3-6. Mass spectra of reserpine with sample amount of (a) 600 ng and (b) 60 ng.

In the initial work, the maximum output, i.e. 1 W was used, which worked well to desorb molecules. Subsequently, the laser power was decreased step by step to investigate the influence on the signal. As shown in Fig. 3-7and Table 3-1, analyte signals can be obtained if laser power was higher than 0.40 W, which corresponds to a power density of 5 x 10^3 W/cm^2. Below that, no stable analyte signals were obtained. The peak intensity decreased with the decrease of laser power, and signal duration increased accordingly. The integral area of analyte signal during the measurements increased with power when it was lower than 0.73 W, and

decreased while the laser power was higher. Therefore, 0.73 W is an optimized power for some experiments in which the exhaustion of sample is necessary.

Table 3-1. Influence on sample signals by changing laser output

Power (W)	0.40	0.47	0.53	0.60	0.67	0.73	0.80	0.87	0.93	1.00
Peak Intensity	$7.47x10^3$	$1.66x10^4$	$4.78x10^4$	$1.27x10^5$	$5.28x10^5$	$9.27x10^5$	$1.90x10^6$	$2.10x10^6$	$2.56x10^6$	$2.60x10^6$
Integral Area	$2.44x10^4$	$1.01x10^6$	$2.00x10^6$	$9.21x10^6$	$3.91x10^7$	$7.54x10^7$	$5.45x10^7$	$4.87x10^7$	$3.79x10^7$	$3.56x10^7$
Signal Time (min)	9.41	14.82	7.06	5.26	4.76	4.46	3.09	2.38	1.93	1.41

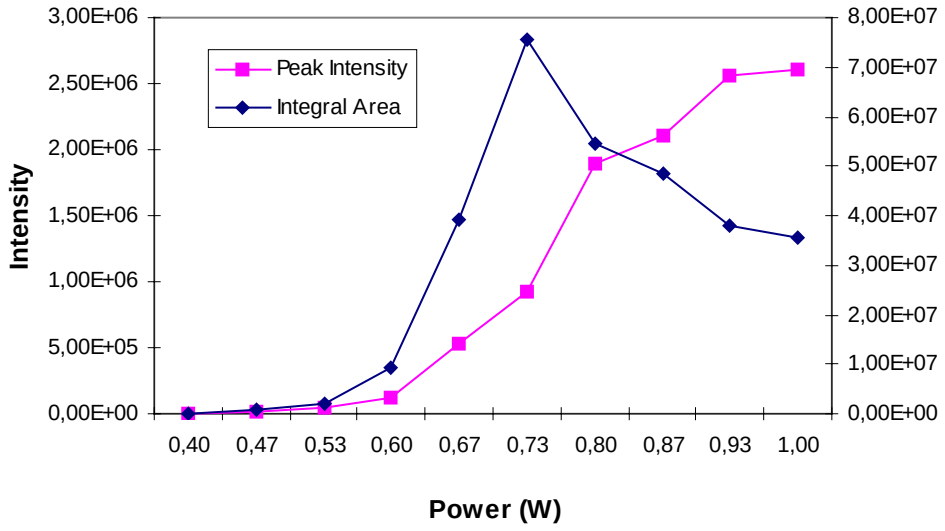

Figure 3-7. Peak intensity and integral peak area as a function of the laser output power

3.5 Analysis of complex samples

Further experiments on graphite plate were carried out with two phospholipids — sphingomyelin and lecithin. Both are mixtures of a group of compounds with similar structures, and their spectra are more complicated than that of reserpine. In this study, 1 µl sample solutions of sphingomyelin and lecithin (each containing 100 ng analyte) were used. The spectra are shown in Fig. 3-8, 3-9. In the spectra, the expression, such

as 'Frag (24:1 SPM)', means a fragmental peak in which the branched chain has 24 carbon atoms (another branched chain has fixed 15 carbon atoms, see Fig. 3-3a), and the amount of unsaturated carbon bonds is 1. 'RCOO+58' is a secondary fragmental peak resulting from the loss of the head group and an additional branched chain.

In the spectrum of sphigomyelin, no molecular but only fragmental signals were acquirable, which is regular in APCI-MS.[77] The fragments were produced after losing the phosphocholine group (head group, MW=183), which was observed in the low mass range. In some cases, they will go on to lose a H_2O. The whole procedure is deduced as shown in Fig. 3-10.

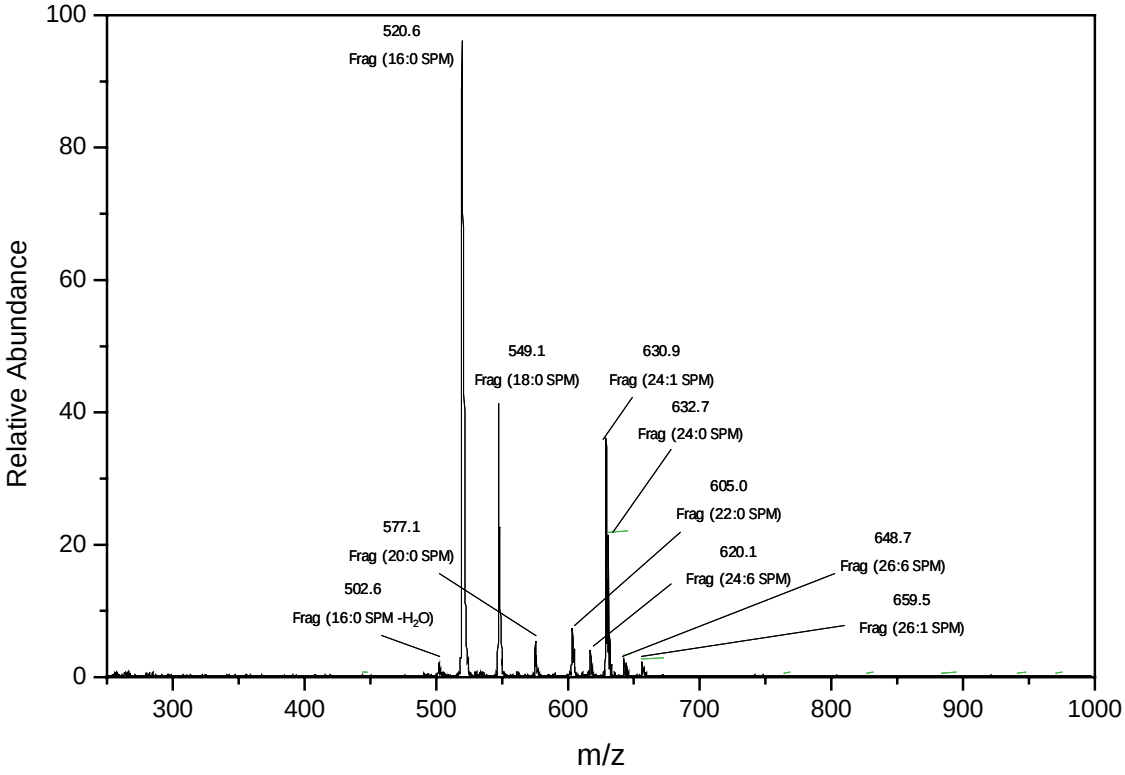

Figure 3-8. Mass spectrum of sphingomyelin

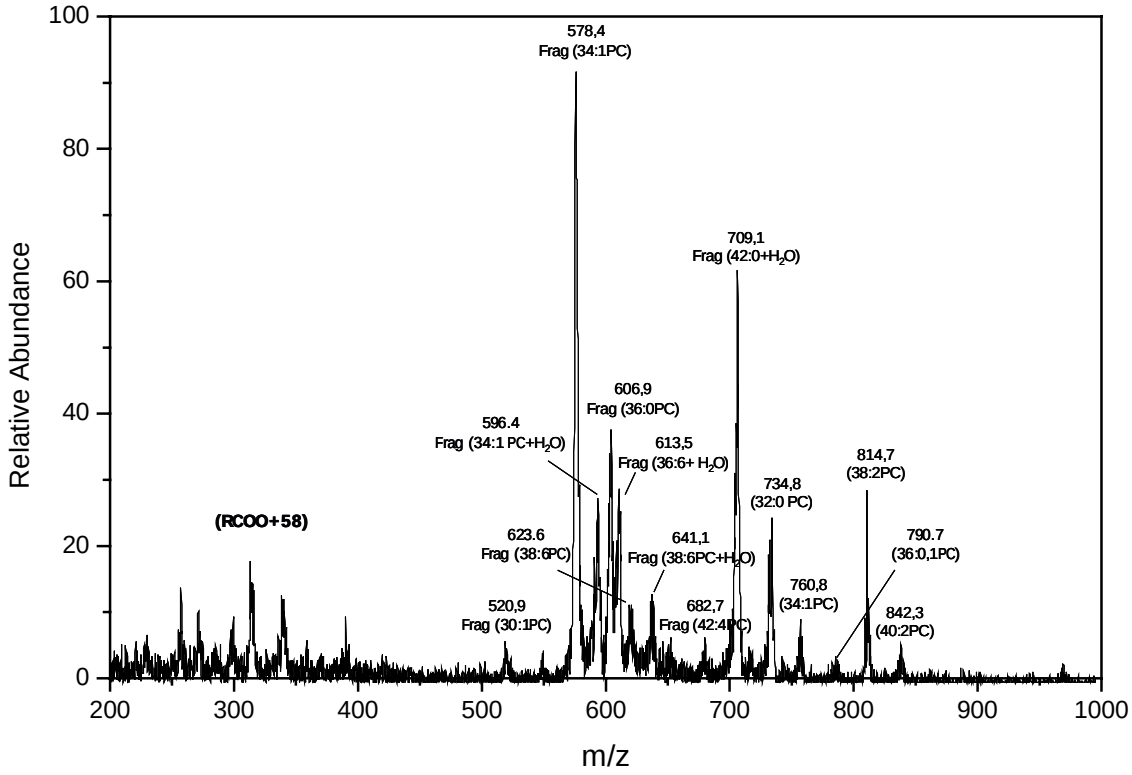

Figure 3-9. Mass spectrum of lecithin. Here, 'PC' is the abbreviation of phosphatidylcholine (lecithin).

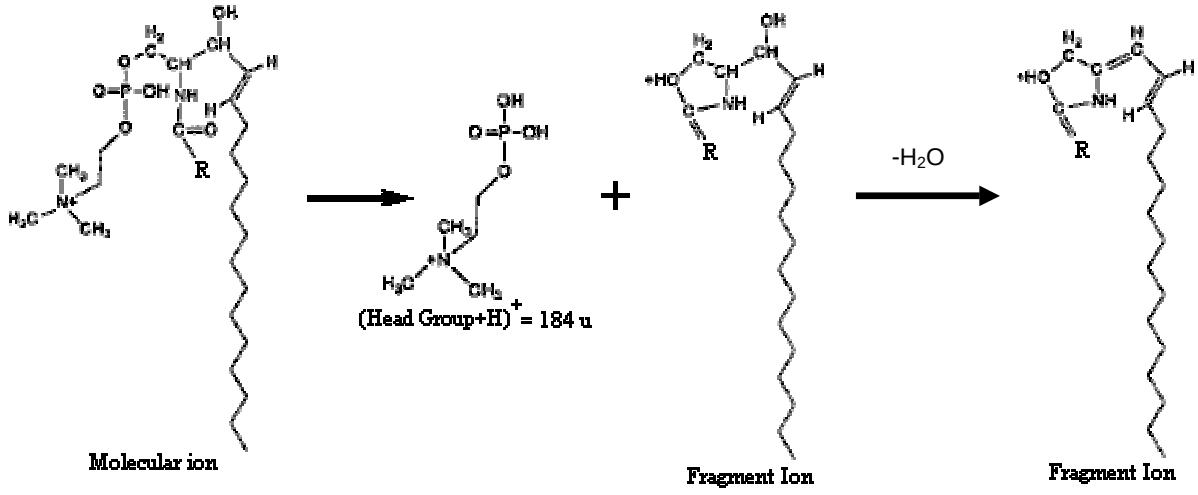

Figure 3-10. Fragmentation of sphingomyelin molecules

Compared with the spectrum of sphingomyelin, the background noise is higher in the spectrum of lecithin. Furthermore, molecular ions (ions without the label 'Frag') were observed in the higher mass range. Another difference is that the secondary fragments were also observed in the low mass range. These ions can be assumed to formed from the molecular ions by loss of the phosphocholine group and subsequently the loss of one side chain, i.e. RCOO+58. Here, RCOO is the fatty acid, and 58 is the mass of glycerol backbone. To prove that, a MS/MS experiment was carried out with m/z 596, which can be assumed to be the hydrated fragment of 34:1 lecithin after losing the head group. The fragmentation process is shown in Fig. 3-11.

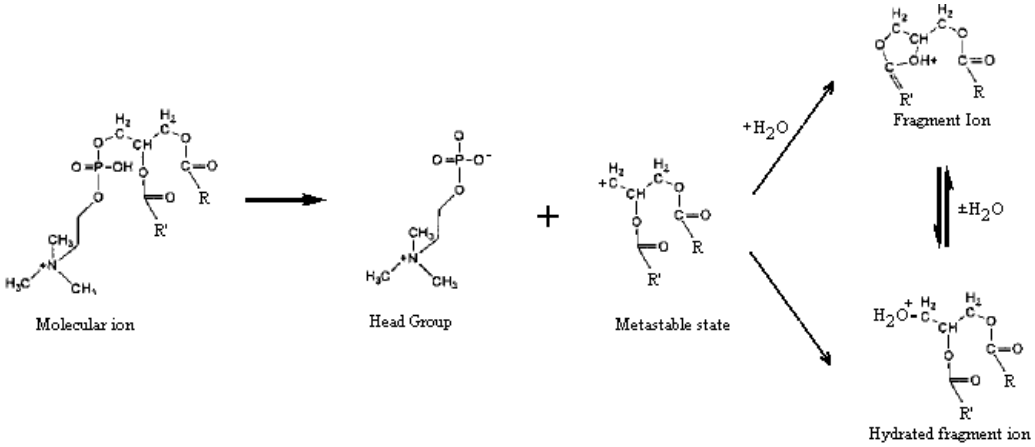

Figure 3-11. Fragmentation of lecithin molecules.

The MS/MS spectrum is shown in Fig. 3-12. The collision energy was set to a value that was not so high to destroy all the parent ions, so that the signal from the parent ions (m/z 596) could still be observed. As expected, a signal at m/z 578 was observed, which was the result of the parent ion losing a H_2O. This signal was also observed in the spectrum of lecithin (Fig. 3-9) as the base peak. The parent ion can also lose one of two fatty acids in the side chains to form two kinds of secondary fragment ions, i.e.

palmitic acid residue + 58 (resulting in m/z 313) and oleic acid residue + 58 (resulting in m/z 339), as shown in Fig. 3-13. These secondary fragment ions are responsible for the signals labelled RCOO + 58 in the low mass range of the lecithin spectrum. Further proofs for the statement is the fact that the fatty acid ions corresponding to the secondary fragment ions were also observed in the MS/MS spectrum, i.e. oleic acid ion (m/z 283) and palmitic acid ion (m/z 257). Two signals with m/z ratios of 283-18 and 257-18 were also observed. They belong to the fatty acid ions after losing H_2O groups. In this case, the signal at m/z 596 was proved to result from the hydrated fragment of 16:0-18:1 lecithin. The structures of other species can also be confirmed with such a MS/MS method.

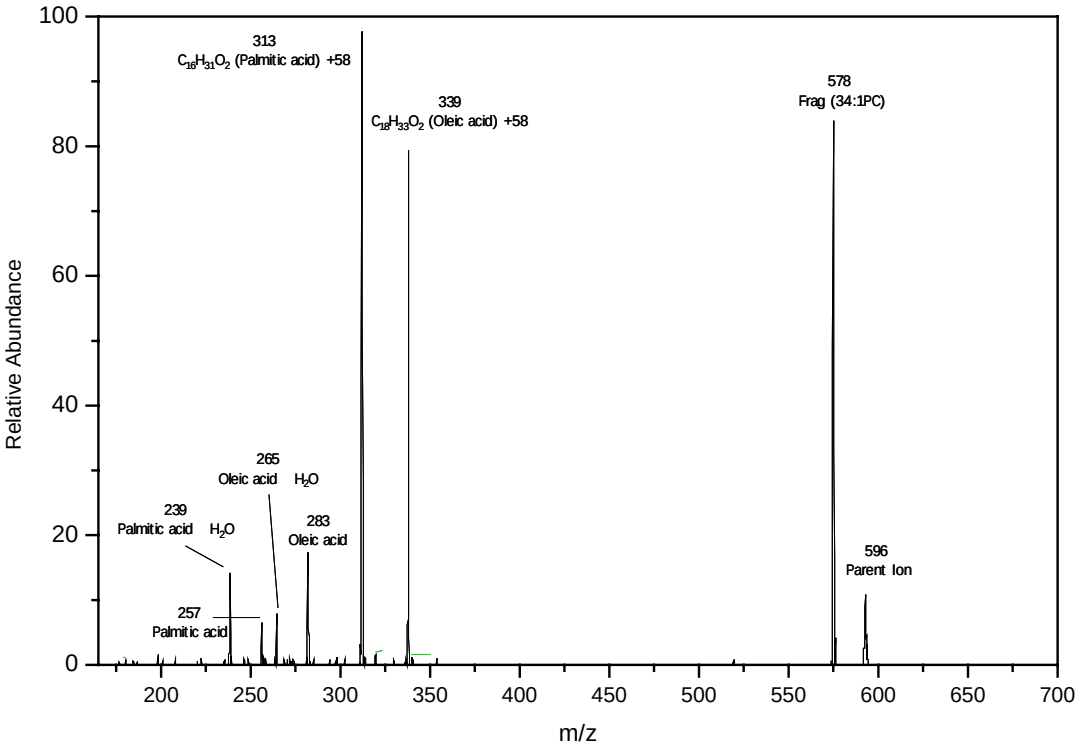

Figure 3-12. MS/MS spectrum at the peak of 596

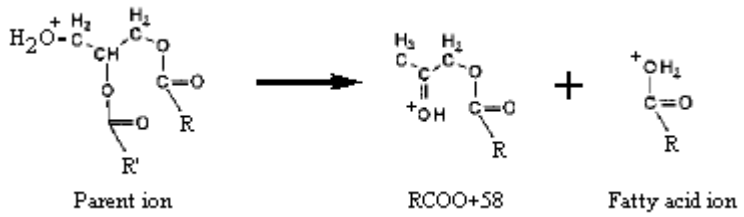

Figure 3-13. Fragmentation by MS/MS.

In this study, diode laser induced desorption were performed on a graphite target, which was applied as a photon absorber to couple the near-infrared laser power. In combination with APCI-MS, biochemicals with moderate molecular weight can be well desorbed /ionized. Compared with traditional laser desorption/ionization, a 100 times lower power density was applied. The decoupling of desorption /ionization gives the opportunity to apply continuous wave diode lasers in laser desorption techniques. The decoupling also allows an individual optimization of these two steps. The results and experiences from the work presented in this chapter gave important instructions for the following steps of the experimental work.

4. Thin-layer chromatography combined with diode laser desorption/APCI mass spectrometry

In the last few decades mass spectrometry coupled to separation techniques like liquid and gas chromatography has become the most versatile detection technique in many fields of applications. Coupling of mass spectrometry to thin-layer chromatography (TLC), which has been extensively used for many years as an economic and handy separation technique, is still a challenge. Thin layer chromatography in conjunction with mass spectrometric detection could be a useful tool for rapid screening in many fields of applications.

Laser desorption (LD) allows to sample in a small and defined area and therefore guarantees higher spatial resolution than the TLC separation itself. This property offers the possibility to characterize partially overlapping components after separation on TLC plates. In order to desorb analytes from a commercially available TLC plate, the power density of the radiation should be about 10^6 W/cm^2 and not exceed 10^7 W/cm^2.[78] This power density is valid for desorption of analytes from a white surface. According to the initial study demonstrated before, a graphite surface can function well to couple the laser power, resulting in a 100 times lower requirement for power density. This advantage also can be applied to a TLC plate, if covering it with a graphite suspension, which has an almost wavelength independent absorption of the laser radiation.[71] Such as in the initial experiment, graphite particles on the surface absorb the laser power and convert it to heat, causing a rapid evaporization under the laser spot.[79] Graphite has the advantage that not only the absorption coefficient is relatively high, but also the heat conductivity.

In this study, a graphite suspension was applied on a piece of TLC plate after depositing the analyte. The TLC piece was obtained by cutting it directly from a commercial TLC plate. LD-APCI/MS was carried out in the same way as on the graphite plate. However, in this case the sample molecules are underneath the graphite layer. Furthermore, the affinity between the analyte molecules and the TLC materials also hampers the desorption. In addition to the 1 W laser used before, a more powerful 4 W laser was also applied to probe the influence under higher laser power density.

4.1 Preparation of graphite-covered TLC plates

The graphite suspension was prepared by diluting glycerol (99.5 %, photometric grade) in methanol and afterwards adding graphite powder. For obtaining a well-mixed suspension, the mixture was then mechanically shaken for 30 min.

The purpose of the presented paper is to evaluate the applicability of desorption from a TLC-plate by continuous diode laser radiation. Therefore, the samples were deposited on small pieces of TLC plates without actual separation. The pieces were obtained by cutting a commercial TLC plate (glass, 20 cm x 20 cm, Merck Art. 5721, Germany) to a size of 3 mm x 3 mm. Afterwards, a TLC-piece was fixed onto the sample holder by using double-sided adhesive tape. The sample solution was deposited on the TLC piece, and subsequently dried for 5 min at room temperature. 2 µl graphite/methanol/glycerol mixture was topped on the TLC piece and left in room air for 10 min in order to obtain a dry and flat surface. Finally, the experiment was started and the protonated analyte was detected by the LCQ Classic ion trap mass spectrometer.

4.2 Analysis on graphite-covered TLC plates

Reserpine (2.5 nmol) was used as the model compound for all investigations in the positive ion mode. β-Alanine (Merck, Germany) was used in the negative ion mode. It is a crystalline amino acid, which is a constituent of many proteins (for structure, see Fig. 4-1).

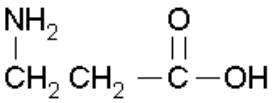

Figure 4-1. Structure of β-Alanine

Except for an additional 4 W diode laser (SLD326B-24 from SONY with a wavelength of 807 nm), the instruments used in this study were the same as those in the initial study. The parameters of the mass spectrometer and the experimental condition were also the same and were based on the optimized conditions found in the initial work.

4.2.1 Positive ion mode

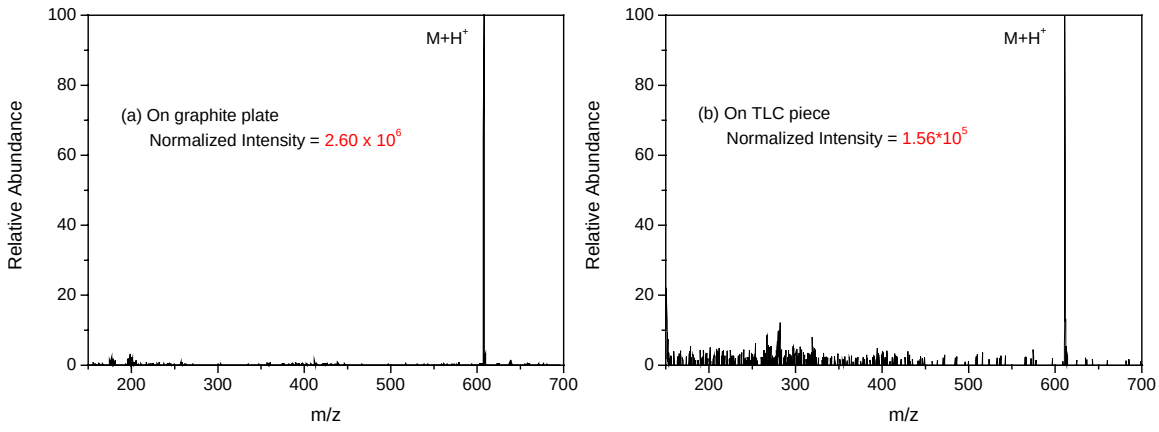

Figure 4-2. Mass spectrum of reserpine (a) on graphite plate; (b) on a piece of TLC plate

The experimental demands when working with TLC plates are more complicated and challenging than with graphite plates. It is inevitable to cause a higher background noise and a decreased spectral resolution. Furthermore, the signal intensity will also decrease because the desorption of the analyte molecules is more difficult. Fig. 4-2a, b shows the difference between the measurement on a graphite plate and on a piece of TLC plate with the same laser power. For the latter, the peak intensity is decreased about 16 times. The background noise is more pronounced, however, it is limited to the low mass range and consequently do not disturb the analyte signal. Furthermore, the graphite suspension also gives a background noise. Glycerol related ions, including glycerol ions, and glycerol/C_n^+ cluster were observed especially in the lower mass range (as shown in Fig. 4-3), which was also reported in other work.[80]

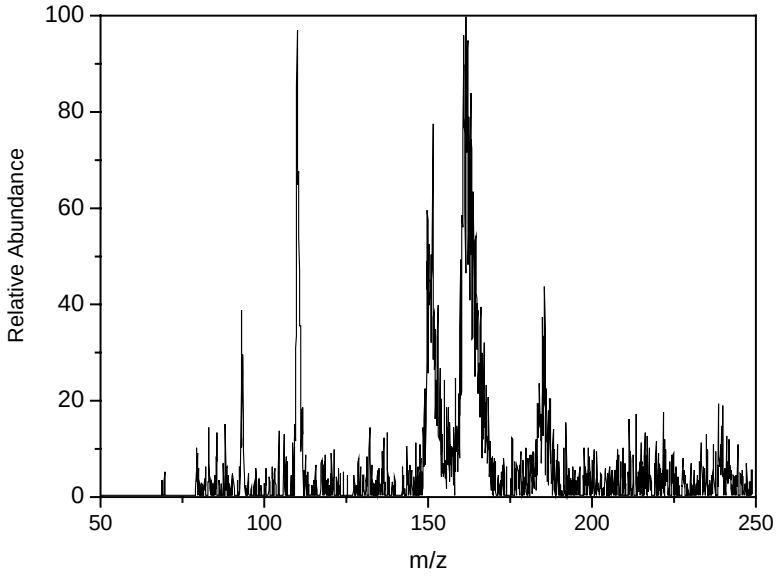

Figure 4-3. Background noises in the low mass range

4.2.2 Negative ion mode

Molecules with acidic groups or electronegative elements can easily produce negative ions. Benefits of the negative ion mode APCI are efficient ionization, higher sensitivity and less fragmentation. There is also a greater selectivity for certain environmentally or biologically important compounds. Here, β-Alanine (0.1 µg) was chosen as a test compound in the negative ion mode APCI.

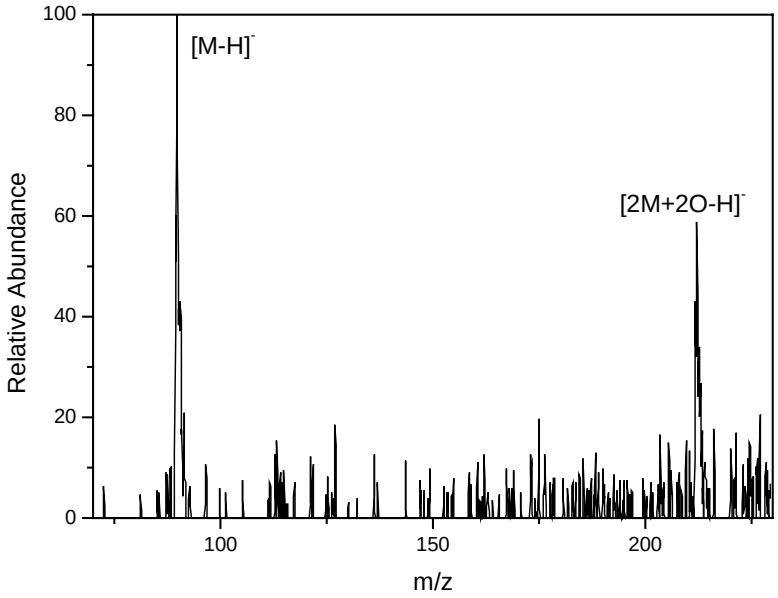

Figure 4-4. Spectrum of β-Alanine in negative APCI

The analysis was first carried out in the positive ion mode. However, no signals were observed in this case. Therefore, the mass spectrometer was switched to the negative ion mode, and an intensive signal from β-Alanine and a related ion signal were observed, as shown in the spectrum (see Fig. 4-4). Except for the quasi-molecular ion $[M-H]^-$, the dimer of the oxidized product of $[2M+2O-H]^-$ was also observed.

4.3 Influence of glycerol

The purpose of glycerol was described several times. One of the reasons is that the viscosity of glycerol is necessary to get a homogenous surface. The results of the investigation of the signal dependence on the glycerol concentration are presented below. Graphite suspensions were prepared by diluting glycerol in methanol, and thereafter adding 14 mg/ml graphite powder. Different proportions of glycerol and methanol (60/40, 50/50, 30/70 and 0/100) were used.

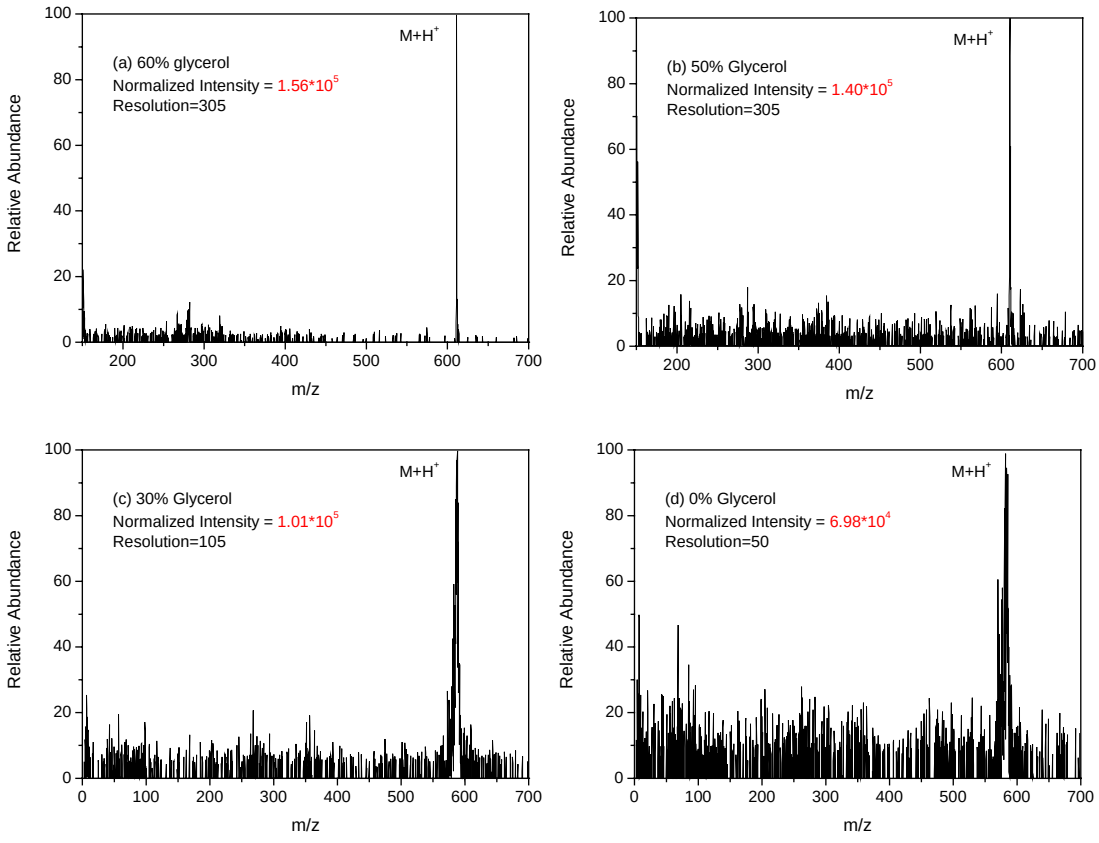

Figure 4-5. Influence of glycerol on the analyte signal.

Fig. 4-5 shows 4 mass spectra measured with different amounts of glycerol. With the decrease of the amount of glycerol also the signal intensity decreases. Although the protonated molecular ion of reserpine could still be obtained without glycerol (Fig. 4-5d), the intensity was about 2 times weaker. In addition, the background noise increases when compared to those with 60 % glycerol (Fig. 4-5a). Therefore, higher glycerol concentration is helpful to obtain a sharp signal peak, which corresponds to a high mass spectrometric resolution. When the amount of glycerol was less than 30 %, the resulting graphite layer had not a glossy and uniform surface. Obviously, this led to the reduced mass spectrometric resolution.

4.4 Influence of the amount of graphite

The purpose of graphite is to absorb the laser power and to convert it into heat, causing rapid thermal desorption of the analyte from the TLC plate. Differing from other techniques in which pulsed lasers are employed, here graphite is not only for the purpose of improving the experimental results, but is necessary to obtain a signal.

Five different graphite concentrations 84 mg/ml, 42 mg/ml, 14 mg/ml, 5.6 mg/ml and 2 mg/ml were investigated to obtain an optimized result. A high concentration of graphite leads to a high absorption of the laser power, but on the other hand, a thick graphite layer keeps part of the analyte inaccessible to laser desorption. As indicated in Table 4-1, the signal intensities were smaller and the resolution was lower measured with 84 and 42 mg/ml graphite concentration than that with 14 mg/ml. Furthermore, at higher graphite concentrations the danger of igniting the graphite layer increases. Strong analyte signals and ‘clear’ spectra were obtained with a concentration of about 14 mg/ml. Analysis at graphite

concentrations lower than 14 mg/ml were performed as well, however, the signal intensity started to decrease rapidly due to reduced absorption of laser power. In addition, the intensity of background signals stayed almost constant. When the graphite concentration was decreased to 2 mg/ml, only a faint gray shadow was visible on the surface of the TLC plate. Only a weak signal could be obtained in this case because the surface was not dark enough to couple necessary laser power for desorption. The data measured with a graphite concentration of 2 mg/ml shown in Table 4-1 are obtained by averaging 100 spectra. The average intensity is about 100 times weaker than that at 14 mg/ml graphite concentration, but the peak resolution is higher.

Table 4-1. Influence of graphite on analyte signals

Graphite Concentration (mg/ml)	Intensity	Resolution	Ignited
84	1.27×10^5	60	Yes
42	1.17×10^5	100	Yes
14	1.56×10^5	305	No
5.6	8.54×10^4	305	No
2*	1.22×10^3	610	No

* Average of 100 spectra

The choice of different graphite and glycerol proportion is a critical parameter for the desorption process. If only methanol and graphite are used, methanol penetrates quickly into the porous surface of silica gel, and graphite powder has not enough time to diffuse and form a smooth layer. Glycerol keeps the graphite powder in the liquid phase and results in a uniform distribution. In general, a more homogeneous surface was

obtained when more glycerol is used. If the glycerol amount is small, the surface was not suitable for desorption. If the graphite concentration was less than 2 mg/ml, the resulting surface is not dark enough to absorb adequate power for desorption, and no stable sample signal could be obtained.

4.5 Influence of laser power

In the beginning, the measurements were carried out by using the maximum output of the 1 W diode laser. Interestingly, the analyte signal disappeared even if the laser power was just reduced by 10%. Therefore, 1 W is presumably near to the 'threshold' for desorption for this method. A stronger 4 W diode laser was utilized to reach higher signal intensity in the following experiments. As shown in Table 4-2, experiments were performed with the laser power of 4 W and 2 W. Spectra with better resolution and similar intensity were obtained at 2 W. The result at 4 W shows a marginal improvement in signal intensity, which was often cancelled by poor sample-to-sample reproducibility, but resulted in a notable increase of background noise and a decrease in mass spectrometric resolution. Furthermore, a very strong laser power causes much more material desorbed simultaneously including impurities from silica gel. Too many ions introduced into the ion trap can result in 'space charge effects', which broadens analyte peak and causes mass errors. Laser power also has an influence on the duration of the signal. It is reasonable that lower power causes longer signal duration. The duration of the signal measured at 4 W is much shorter than that at 2 W, which means more sample molecules are vaporized in the same time range at 4 W than at 2 W, but the signal intensity was nearly the same. One explanation for this observation is that many ions might be lost during transmission.

Table 4-2. Influence of laser power on analyte signals

Laser Power (W)	Intensity	Duration of Signal (min)	Resolution
4W	2.20×10^5	1.5	150
2W	2.17×10^5	4.5	205
1W	1.56×10^5	6	305

A novel technique, thin-layer chromatography combined with diode laser induced desorption/atmospheric pressure chemical ionization, is demonstrated in this study. The use of graphite suspension and the decoupling of desorption and ionization allows diode lasers to be used in TLC-MS for the first time. It supplies a possible fast and easy separation and detection method in the future. Graphite suspension plays a role of energy absorber and transfer medium, and it is proved to be a suitable material to couple the power of the continuous diode laser. Glycerol is used to improve signal intensity and decrease background noise, and it is important for obtaining a satisfying graphite layer. A corona discharge is employed to ionize the molecules desorbed by diode laser that is not strong enough to achieve the ionization step. Matrix related peaks are limited at low mass range, and no fragments are observed.

5. A new interface to couple mass spectrometry with thin-layer chromatography for full-plate scanning

A TLC plate-scanner coupled with the mass spectrometer gives benefits for rapid screening and high-throughput analysis. It is also useful for the detection of unknown substances and overlapping spots.

In the preceding study,[81] a method of TLC-MS by using a diode laser to desorb molecules and a corona needle to ionize the desorbed molecules with the assistance of graphite was demonstrated. The decoupling of desorption and ionization enable the transfer of the desorbed molecules for a certain distance, followed by the ionized in the ion source of the mass spectrometer without any modification of the spectrometer. Because the ionization is realized by an AP technique, the transporting system can work without a requirement for vacuum environment. All these advantages can be applied to a plate scanning system, which is uncomplicated but highly efficient.

Accordingly, a plate scanning system based on the same principle as described before was built by using a gas jet pump for the transport of the analyte molecules and a motorized movable stage for scanning. An optical fibre was used to guide the laser beam, which makes the experimental arrangement more flexible. The loss of molecules during transportation can be controlled by heating up the transport part. As described before, graphite can effectively reduce the demand for laser power and improve desorption efficiency. In this case, the graphite layer was produced by spraying the graphite suspension with a sprayer on the TLC plates after development. However, for some measurements, if a faster analysis is required, the pretreating procedure is not appropriate. Therefore, a stronger diode laser was employed, which could desorb molecules without the

assistance of graphite. Two desorption modes: direct LD and LD with graphite-assistance are tested in this study.

5.1 Instrumental setup of the plate scanning system

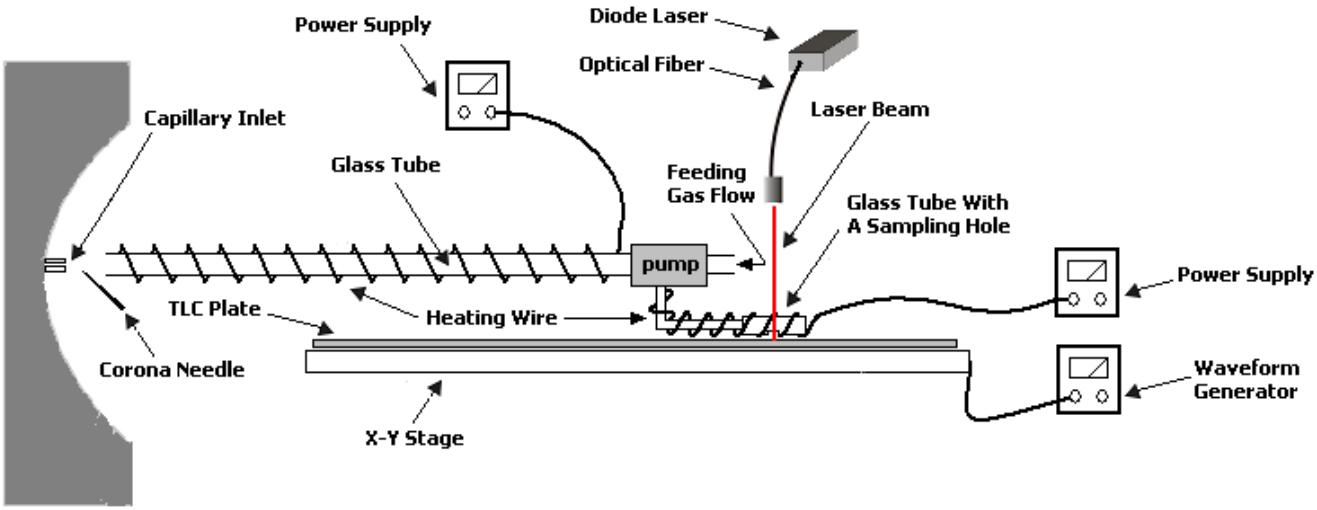

Figure 5-1. Schematic diagram of the experimental arrangement

A sketch of the experimental arrangement used for the following experiments is shown in Fig. 5-1. The TLC plate was placed on a motor operated xy-stage that was controlled by a waveform generator (FG-5000, WAVETEK). A short glass tube (2.5 cm in length, 5.5 mm i. d., 7.5 mm o. d.) was installed 2 mm above the plate. An inlet hole with a diameter of 2 mm was located at the bottom side of the short glass tube for sampling the desorbed molecules. The tube was heated up to 200 °C with a coiled heating wire. One end of the glass tube was closed, and the other was connected to a gas jet pump. A gas flow of 1.5 l/min N_2 was introduced to the pump to supply a suction flow of 1.1 l/min. The gas was then

transported to a transfer glass tube of 10 cm length and 7.5 mm inner diameter, which was positioned several millimeters in front of the capillary inlet of a LCQ Classic ion trap mass spectrometer (ThermoFinnigan, USA). A coiled heating wire maintained the temperature of the transfer tube at 350 °C to reduce the sample loss during transfer.

A laser beam from a diode laser (OTF 30P-40 from OPTOTOOLS) with a wavelength of 808.8 nm guided by an optical fiber, was aligned through the sampling inlet and desorbs analytes from the surface of the TLC plate. The laser beam with a maximum power of 16.8 W was focused on the surface with a calculated spot diameter of 0.05 mm resulting in a maximum power density in the order of magnitude 10^6 W/cm^2. The desorbed molecules were transferred to the area in front of the heated capillary inlet of the mass spectrometer and then ionized by a corona needle with a discharge current of 1 μA and a potential of 4 kV supplied by the LCQ system. The parameters of the mass spectrometer were optimized for each analyte, individually, as in the initial work.

5.2 Chromatography

The analytes were separated on commercial silica gel TLC plates (aluminum-backed, 5 cm x 10 cm or 10 cm x 10 cm, Merck, Germany). The separation was carried out with a chloroform/methanol/water (20:7:1) solvent system for 15 min at room temperature. If multi-samples were developed on the same plate simultaneously, the track distance in between was 1 cm. In the experiments with graphite-assistance, the graphite layer was produced by spraying the graphite suspension with a sprayer on the TLC plates after development, which can be monitored with a microscope. The graphite suspension was prepared by adding graphite powder in

isopropanol (from Merck, Germany), followed by mixing for 5 min with an electromagnetic stirrer.

5.3 Scanning on untreated TLC plates

5.3.1 Scanning of a single compound after development

2 µl reserpine solution (0.6 µg) was used in the first experiments to check the feasibility of the interface. After the developed TLC plate was dry, the plate was mounted on the x-y stage. The scan was carried out with a velocity of 0.6 mm/s. In the previous work, a 1 W diode laser was employed as the desorption source. Using this experimental setup desorption from a white surface was not possible. However, the diode laser used in the present experiments had a maximum power of 16.8 W, which resulted in a power density of 10^6 W/cm^2. This power density also enables laser desorption of analytes from a white TLC plate. As shown in Fig. 5-2a, b, besides the chromatographic information of reserpine ion counts, also more specific information on the sample spot was obtained from the spectrum.

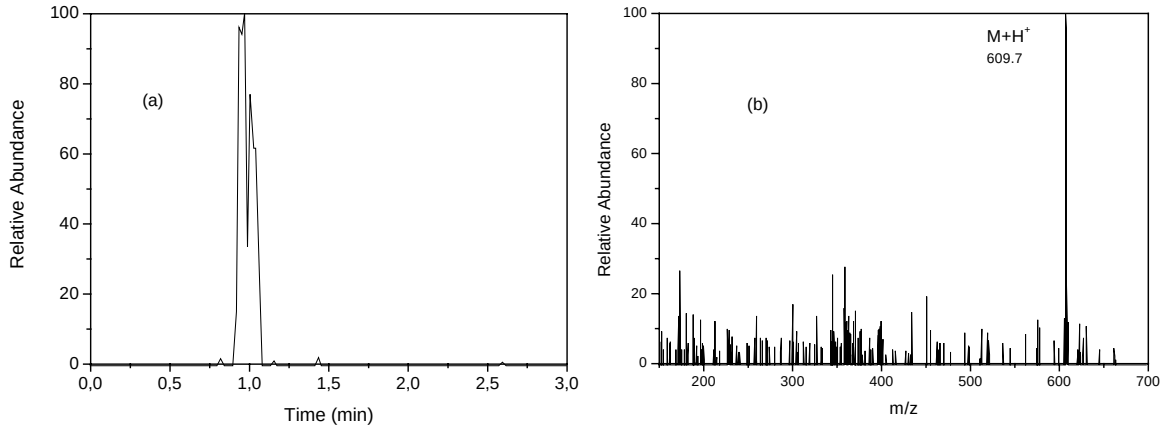

Figure 5-2. (a) Chromatogram of reserpine ion counts during a scan; (b) Mass spectrum of reserpine on the sample spot after development.

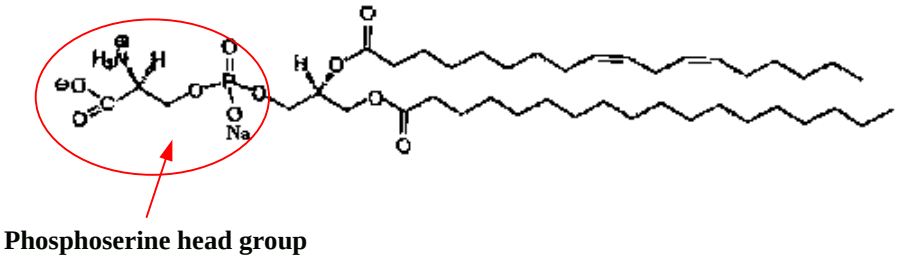

Figure 5-3. Structure of 18:0-18:2 phosphoserine

Besides reserpine, a phosphoserine (in sodium salt, $C_{42}H_{77}NO_{10}PNa$, full name 1-Stearoyl-2-Linoleoyl-*sn*-Glycero-3-[Phospho-L-Serine], 18:0-18:2 PS) was also employed. It is also a phospholipid and has a similar structure as lecithin except that it contains a serine group rather than choline group. 18:0-18:2 represents two fatty acid residues both with 18 carbon atoms and one with two double bonds in the carbon chain as shown in Fig. 5-3. In APCI mode the molecule easily loses the head group (phosphoserine group). The sample solution of 18:0-18:2 phosphoserine was prepared by dissolving its powder in an aqueous solution of 50 % methanol with 0.1 % acetic acid.

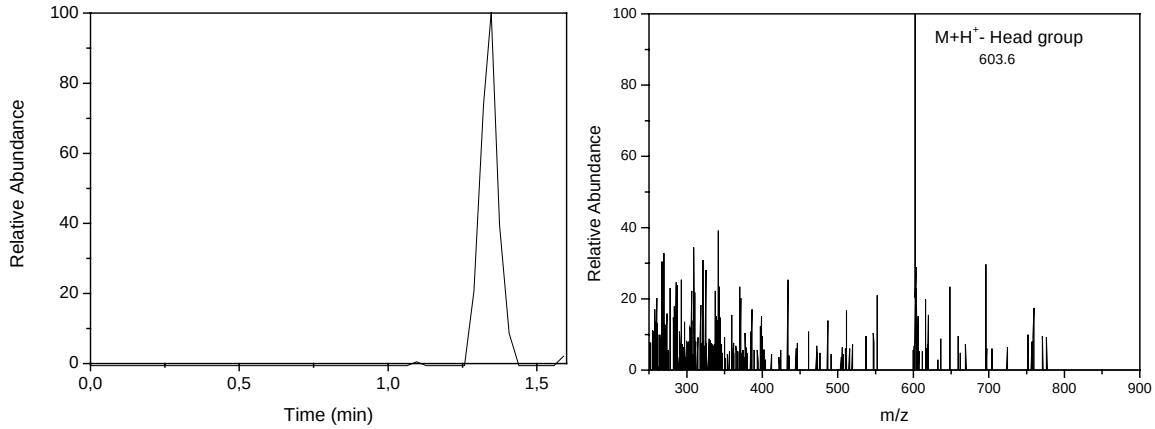

Figure 5-4. (a) Chromatogram of 18:0-18:2 PS ion counts; (b) Spectrum of 18:0-18:2 PS on the sample spot after development.

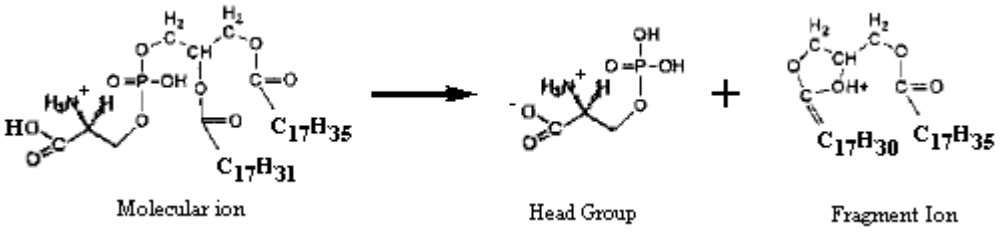

Figure 5-5. Fragmentation of 18:0-18:2 phosphoserine.

5.3.2 Scanning of a mixture after separation

The measurements discussed before by using a single compound after separation demonstrated that the system can well desorb, transport and ionize analyte molecules during a scanning process. The subsequent experiment was carried out by using a complex mixture of sphingomyelin and lecithin. For this purpose, 1µl SPM solution (100 ng), and 1 µl PC solution (500 ng) and a mixture of 1 µl SPM/PC (100 ng sphingomyelin and 500 ng lecithin) was developed on a TLC plate.

The first scan was carried out along the direction of development on the lane of SPM/PC mixture, as shown in Fig. 5-6a. A TIC-signal with two peaks from sphingomyelin (SPM) and lecithin (PC) measured during the scan as well as the related mass spectra are shown in Fig. 5-6b-d. As mentioned before, sphingomyelin and lecithin are mixtures of a group of glycerophospholipids. The different species of sphingomyelin as well as lecithin cannot be separated by conventional TLC techniques because all species in the group have nearly the same migrating distance so that they are located in the same sample spot. The mass spectra depicted in Fig. 5-6c, d show that sphingomyelin and lecithin can easily be identified. As shown in the spectra, signals of different molecular species of sphingomyelin and lecithin were obtained at the same time although they

were overlapping in chromatography, which in this case enhances the ability of analysing complex samples. Scans on the other two lanes (for SPM and PC, individually) were also performed. The signals from SPM and PC were also observed on the same level of the plate such as they were observed in the mixture.

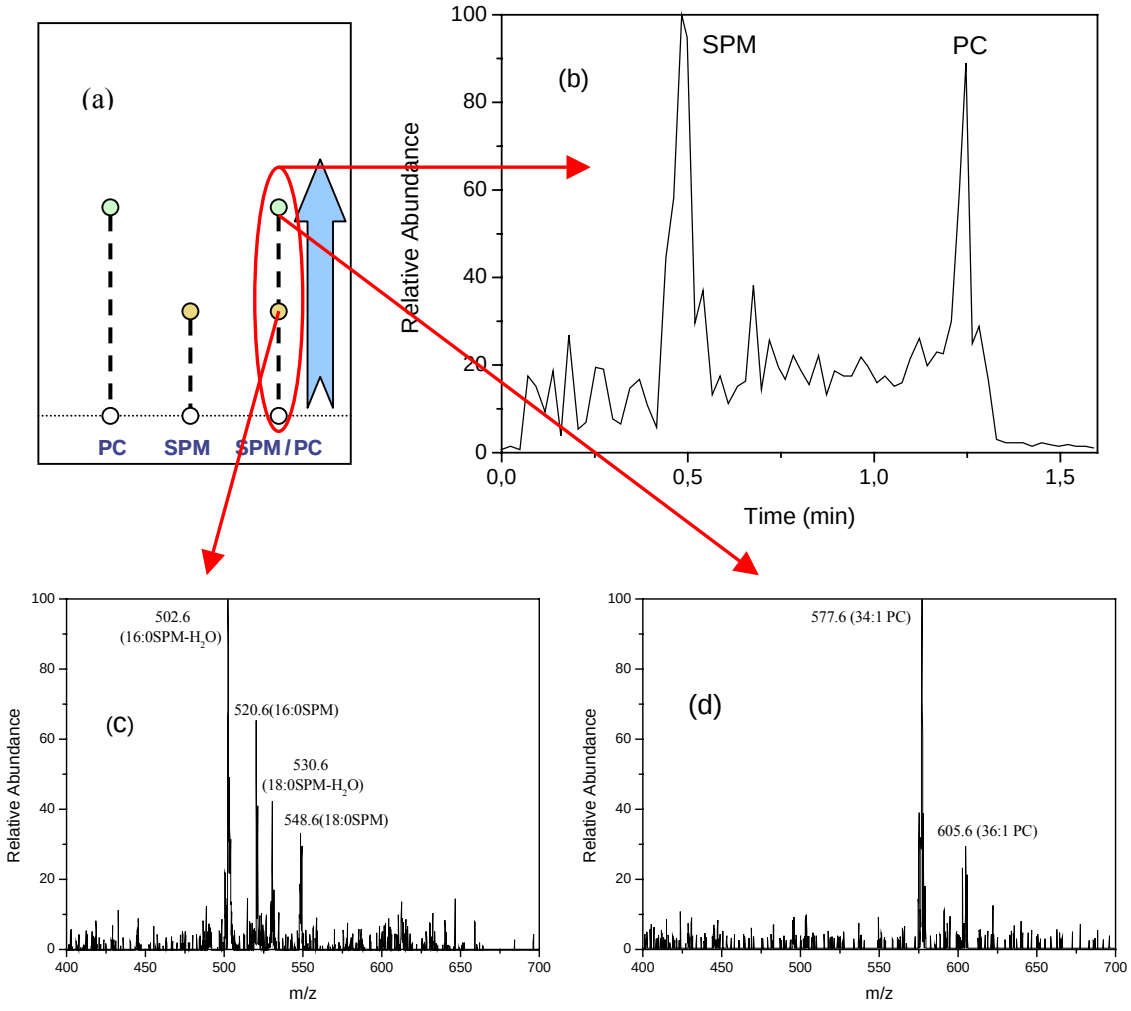

Figure 5-6. Results from a scan on an untreated TLC plate. (a) Schematic diagram of the experiments; (b) Total ion chromatogram (TIC); (c) Mass spectrum of sphingomyelin; (d) Mass spectrum of lecithin.

5.4 Scanning on the TLC plate with graphite assistance

5.4.1 Graphite covered plates

A high power density of laser beam is not always desired because it may cause fragmentation and destroy the TLC layer. Therefore, similar experiments were carried out on surfaces covered with five different graphite particle densities. The graphite suspension was sprayed on the TLC plates and the graphite densities were determined by measuring the gray scale of photos taken with a camera mounted on a microscope, as shown in Fig. 5-7.

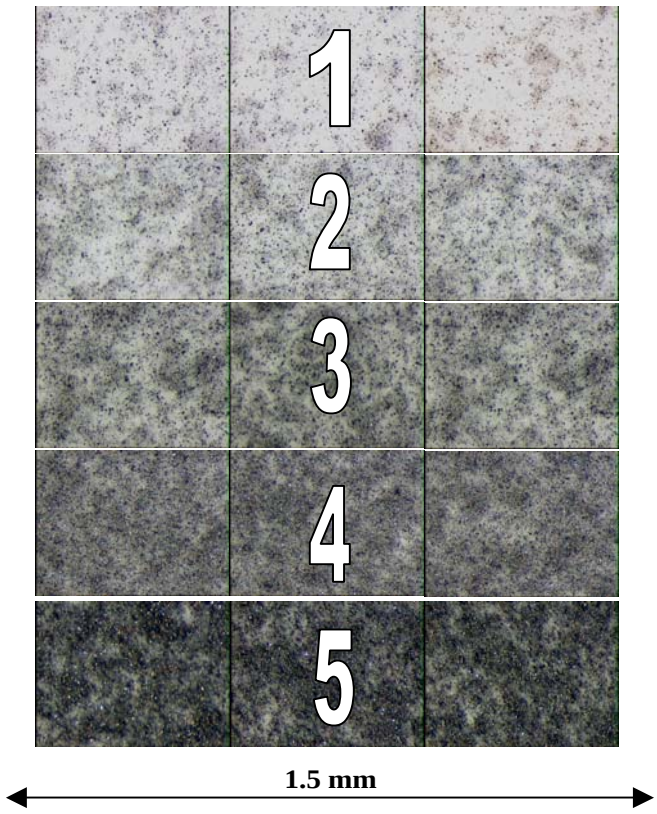

Figure 5-7. Pictures take from the surfaces of the TLC plates with a camera mounted on a microscope.

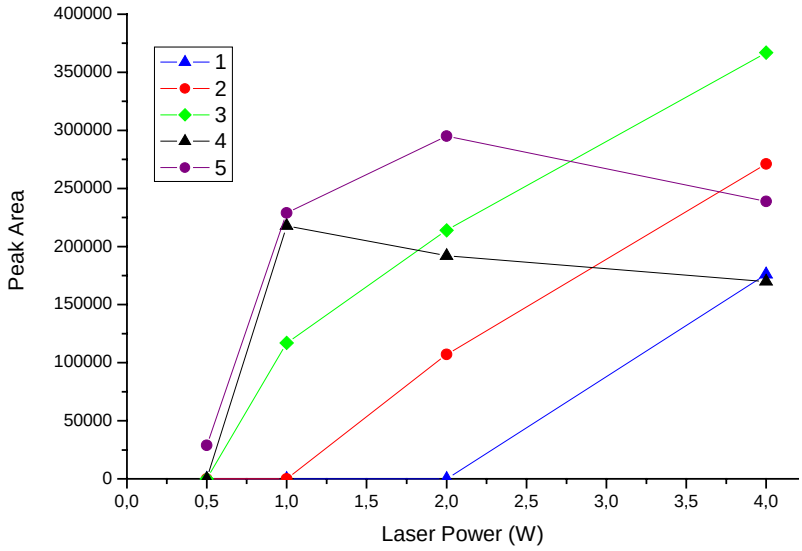

Figure 5-8. Peak area of the SPM signal on the TLC plates covered with different graphite particles densities and scanned with different laser powers

TLC plates with 5 different grey scales were scanned with different laser power densities and a scan velocity of 0.6 mm/s. The signal area measured during these experiments is shown in Fig. 5-8 as a function of the laser power density. The SPM signal was first obtained when the laser power was higher than 500 mW focused on a spot with a diameter of 50 µm (power density: 2.5×10^4 W/cm^2) and when the TLC plate with the highest graphite density was used. Therefore, the necessary power density is about 30 times lower than that measured when a TLC plate was used without graphite coating. A power of 4 W (power density: 2×10^5 W/cm^2) was enough to measure an intensive signal when the plate with the grey scale value 1 was used. Increasing graphite particle density results in a reduced demand on laser power density for desorption. For the relatively 'light-coloured' plates with the grey scale values 1, 2 and 3 the peak area

increased with higher laser power. However, for the darker TLC plates 4 and 5, the peak area decreased with higher laser powers. Ignited tracks were observed on these two plates at the sites where the laser beam was scanned. Obviously, these conditions caused fragmentation of the analytes and reduced signal intensity.

5.4.2 Graphite embedded plates

The requirement for desorption energy can be well reduced on a graphite covered TLC plate, and the method can be applied to all kinds of commercial TLC plates, but it results in more laboratorial work for the preparation of graphite layer at the same time. Because visualization methods, such as staining techniques, are mostly used to locate sample spots on TLC plate, commercially available TLC plates are white, and graphite particles have to be applied on the surface of a plate. However, a 'grey' plate with graphite particles embedded in TLC materials probably supplies the similar function as a graphite covered plate.

A quick protocol was adopted to investigate the feasibility of the detection on such a plate with simple tools. Graphite particles were added into silica gel 60 particles with a quantitative ratio of 2:100. Afterwards, 4 ml water was added into 1 g of such a particle mixture, and then it was mixed by a glass rod to form slurry. The slurry was deposited on a piece of glass (5 cm x 10 cm) and then pressed over with a glass rod horizontally. Two ends of the glass rod were covered with teflon tubes to form a space between the glass tube and the table surface. The space ensured the slurry distributed homogeneously on the plate after the glass rod was pushed over from one edge to another of the plate, as shown in Fig. 5-9. The plate was subsequently dried in an oven for 15 min under 150 °C. Finally, the plate had a grey and a relatively smooth surface.

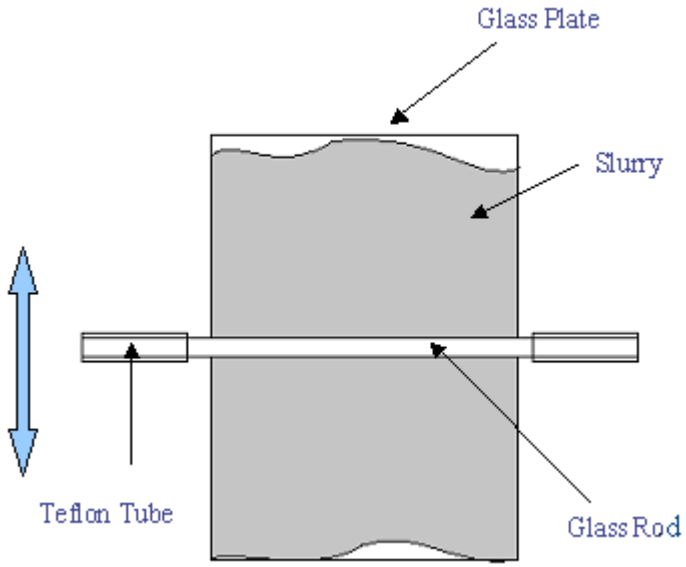

Figure 5-9. Schematic diagram about the fabrication of home-made TLC plate

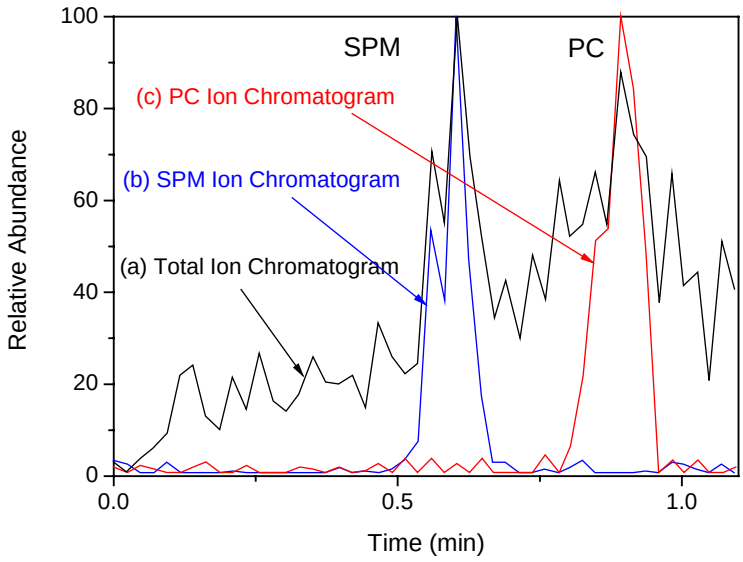

Figure 5-10. Chromatograms measured by scanning of graphite embedded TLC plate

A sample of SPM/PC mixture was deposited and developed on such a plate. The chromatograms of the individual analytes and the total ion chromatogram are shown in Fig. 5-10. Compared with the chromatogram obtained from a commercial TLC plate, the background noise was higher in the total ion chromatogram, but the chromatograms based on the individual analyte were still clear and showed no interference from background. The spectra are shown in Fig. 5-11 a, b, and there are no obvious differences with those obtained from a commercially available TLC plate covered with graphite particles.

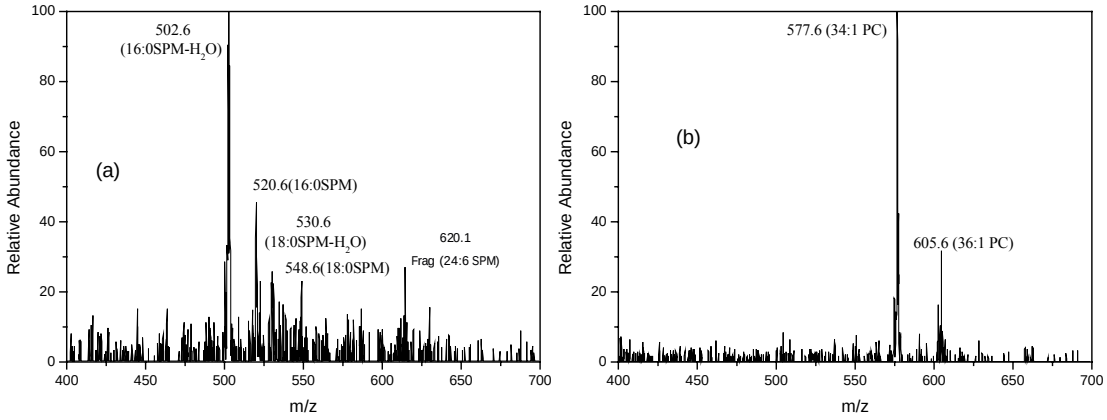

Figure 5-11. Spectra measured by scanning a graphite embedded TLC plate. (a) sphingomyelin; (b) lecithin.

In this work, the plate was built up without further technical tool for TLC plate making and not based on an optimized protocol, but the experimental results had no unacceptable drawback and proved that the approach was feasible. It is reasonable to anticipate that it works better with a more sophisticated instrumentation for plate preparation. However, only after commercial graphite embedded TLC plates had become available, laser

desorption on such TLC plates can benefit from both reduced requirement for laser power and a quicker experimental procedure.

5.5 Influence of scanning speed

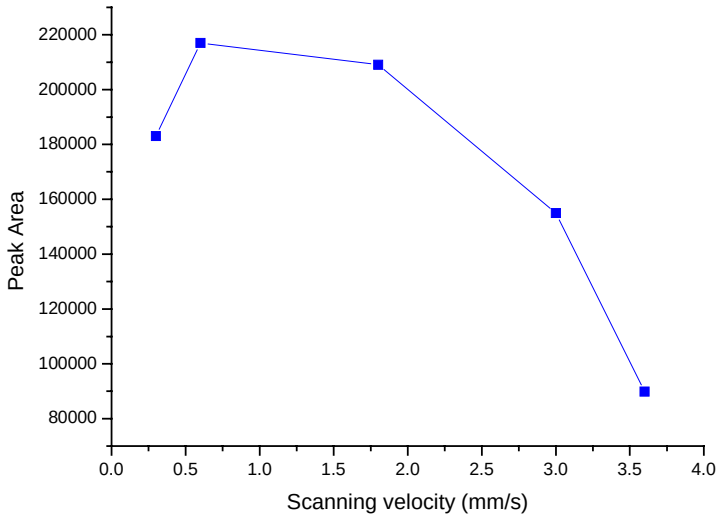

Figure 5-12. Peak area of the SPM signal at different scanning speeds

The scanning speed is an important factor for the system. Higher speed causes faster analysis, but possibly results in an incomplete desorption of analyte molecules. A low scanning speed can theoretically give benefits on sensitivity and spatial resolution, but needs more time for a scan. A compromise is necessary between speed and sensitivity. To probe the influence of scanning speed on the signal, 5 samples with identical amount of sphingomyelin (100 ng) on a TLC plate were developed and then scanned with different velocity: 0.3 mm/s, 0.6 mm/s, 1.8 mm/s, 3.0 mm/s, 3.6 mm/s. As shown in Fig. 5-12, except for the data spot at 0.3 mm/s, the peak area decreases with higher scanning speed. This result is interpretable to be a result of an insufficient evaporation of analyte molecules at higher

scanning speed. In this case, the sample spot was irradiated by the laser beam for a shorter period of time, and not enough energy was conducted to the analyte molecules. However, the wall of the transport system keeps capturing analyte molecules for a longer time at lower scanning speeds. This can cause more sample loss during transfer. If a more sufficient desorption cannot compensate the larger sample losses at slower scan rates, the signal peak area will decrease, such as in the case of 0.3 mm/s. Therefore, scanning speed of 0.6 mm/s was chosen for the following studies. This speed is much higher than the speed selected for the plate scanner based on ESI, which has to be operated at very low scanning speeds (several tens μm/s),[52] since the extraction of the analyte from TLC material is a much more time-consuming step than desorption with energy.

5.6 Rapid screening

One of the important applications of the TLC technique is rapid screening for specific substances in complex samples. This can be conveniently realized with the device described here. To demonstrate the feasibility of the technique, the screening of SPM in a SPM/PC mixture was performed. One reference sample containing only SPM and four synthetic samples containing SPM/PC mixtures were developed simultaneously on a TLC plate. The SPM concentration was identical in all 5 samples. First, the migrating distance of SPM can be identified by scanning the reference sample along the migrating direction. Then, the scan was continued from the center of the SPM spot in the reference sample in orthogonal direction of the development, as shown in Fig. 5-13a. SPM signals were obtained from all synthetic samples at the same migrating distance of the plate. The signal areas (as shown in Fig. 5-13b) of the 4 mixed samples were observed to be close to that of the reference sample. The average relative

error is 7.0 %. If the migrating distance of the analyte is known, as it is usually the case for screening tasks, the overall measurement time can be quite short. For the conditions chosen here it was about 1 min. However, when the scanning velocity would be increased or sample displacement decreased the time necessary for such a screening run could be even shorter.

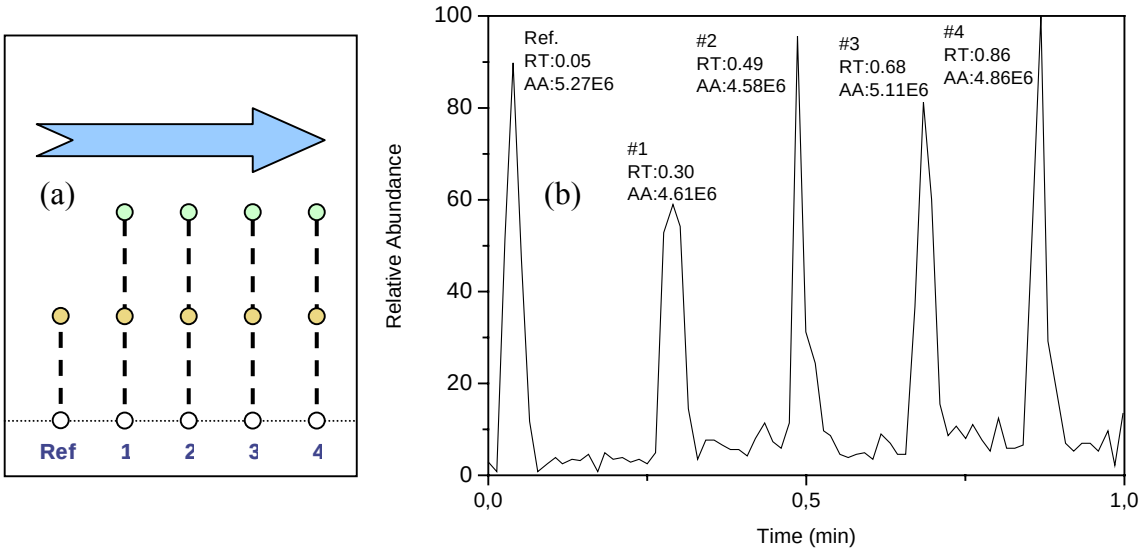

Figure 5-13. (a) Schematic diagram of rapid screening; (b) Total ion chromatogram of a SPM screening experiment. Ref. and #1, #2, #3, #4 means reference sample and four synthetic samples; RT is the retention time; AA means integral peak area.

In this chapter, a novel interface to realize the coupling of TLC and MS was demonstrated. A diode laser, which is compact, easy to use and cost effective, was employed as the laser desorption source. The TLC plate was placed on a moving x-y stage to realize full-plate scanning. Two scan modes were used: a scan along the direction of the analyte development, which is appropriate for detecting all the

components in one sample and a scan across several sample spots orthogonally to the direction of development, which is suitable for high throughput analysis of the same compound or compounds with the same R_f from several samples. The mass spectrometer was coupled to provide an identifying ability to TLC technique, which is important for the analysis of unknown substances or overlapping spots as well as to improve the reliability of the analysis. A gas jet pump connected with heated glass tubes enabled transportation of the desorbed analyte molecules to the ionization region.

The measurement was carried out on both non-pretreated and graphite-coated TLC plates. Using the graphite-coated surface, the necessary laser power for desorption decreased down to 10^4 W/cm^2, about 30 times lower than that needed for the desorption from white surfaces.

6. Quantification with the plate scanning device

6.1 Quantification in mass spectrometry

The goal of quantitative analysis in mass spectrometry is the correlation the intensity of the signals with the quantity of the compound present in the sample. Generally, an external or an internal standard is applied for this purpose.

In an external standard method, a synthetic sample containing a known quantity of the molecule to be measured (M_{ste}) is introduced into mass spectrometer, and then the signal intensity (I_{ste}) is recorded. Under the same analytical conditions, an equal volume of the solution containing the analyte molecules to be quantified (M_x) is introduced into the mass spectrometer and its response signal intensity (I_x) is measured. Because the volumes that are introduced are equal, there is a proportionality between the response intensities and the quantities as long as the response signal intensity remains linear with respect to the concentration and as long as the signal intensity is zero at zero concentration:

$$M_x = I_x \bullet M_{ste} / I_{ste}$$

Because this linearity is not always good over a wide concentration range and for all ionization methods, verification using a calibration curve is necessary. In order to do this, equal volumes of a series of synthetic samples containing an increasing quantity of the molecules to be measured are introduced into the mass spectrometer and the intensities of their response signals are recorded. In fact, the visual quantification method in TLC is also an external method.

An internal standard method allows the elimination of various error sources compared with an external standard method. In this method, an internal standard with chemical and physical properties as close as possible to the properties of the molecules to be measured is added into the sample. A calibration curve is constructed by measuring synthetic samples containing the same known quantity of the internal standard and increasing quantities of the compound to be measured. This allows a mathematical relationship to be obtained between the intensities of the signals corresponding to the compound to be analyzed and the internal standard (I_x/I_{sti}) and the quantity of compound present in sample (M_x). The measurements are then carried out with the unknown samples after a constant quantity of internal standard had been added to them. Internal standards can be classified into three categories: structural analogs that are labeled with stable isotopes, structural homologs and compounds from the same chemical family.

6.2 Quantification in TLC-MS

In TLC-MS, often the quantification is done off-line and represents a complicated and time-consuming procedure. The sample spot has to be located by a visualization method first. Then, the stationary phase containing the analyte has to be removed from the plate followed by extracting the analyte with a solvent. Finally, the usual quantification procedure has to be carried out for the analyte solution.[30] Another approach is the extraction of the analyte directly from the plate with a liquid[51] and the direct transport of the extraction liquid to a mass spectrometer. However, the measurement is still performed intermittently from one sample spot to another, which is not really suitable for high throughput analysis. In contrast, the experimental setup presented here is

able to provide a quick and continuous on-line quantitative measurement technique. Nevertheless, reliable quantification is a critical aspect using desorption techniques. Obviously, the surface condition is an important factor for laser desorption. Especially on TLC plates, the inhomogeneity of the microscopic surface condition at different locations results in differences in the desorption efficiency of analyte molecules. Consequently, the use of an internal standard is highly beneficial. The ideal internal standard would be the application of isotopically labelled analyte species (e.g. ^{13}C-labelled) because they are located in the same position as the analyte after separation. However, just very few isotopically labelled biomolecules are commercially available. Another possibility is the use of structural homologs, e.g. other phospholipids, as internal standards. However, even slightly different structures can lead to a separation from the analytes during the TLC run. To overcome the problem, a suitable reference compound was added into the mobile phase. After the development of the sample this compound was distributed homogeneously over the whole area below the solvent front on the plate. The 'background' signal of this compound was then used as an internal standard. In this way, the chromatographic property of the internal standard is no longer important and a wide range of compounds can be chosen for this purpose. Here, reserpine (RSP) was used as the internal standard (0.6 g/l in the mobile phase), a compound that is often used for calibration of LC-MS systems.

6.3 Quantification for the TLC plate scanning system

In a normal quantification procedure, the ratio between the signal area of analyte (A_x) and the signal area of the internal standard is used to build a calibration curve. In this work, the internal standard yields a steady signal.

As shown in Fig. 6-1, one possibility is to use the integral area of the internal standard (A_{st}) during the appearance of the analyte signal. However, this area is subject of change due to the chromatographic reproducibility, i.e. it will change with the width of the analyte signal although its signal area might be the same. To reduce this kind of deviations, the average intensity of the internal standard (I_{st}) was used, which is defined here as the integral area during the appearance of the analyte signal divided by the number of scans (n) that is recorded by mass spectrometer:

$$I_{st} = A_{st} / n$$

A calibration curve is constructed based on the ratio between the signal area of analyte and the average intensity of the internal standard (A_x/I_{st}).

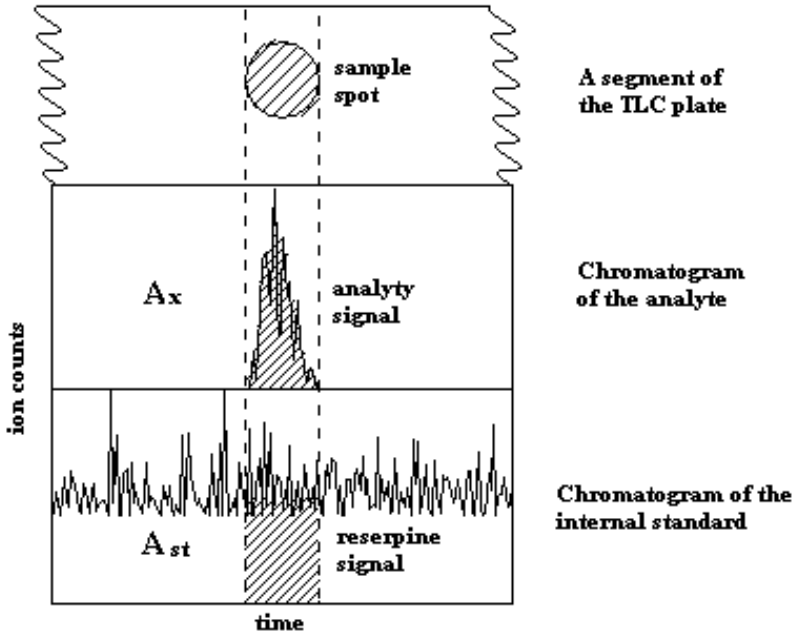

Figure 6-1. Schematic diagram about the quantification strategy

For this study, sphingomyelin was chosen as the analyte. Its strongest signal (*m/z* 502.6) was employed as the basis to calculate the signal area. For each point in the calibration curve five synthetic samples with the same SPM concentration were measured. The results are listed in Table 6-1.

Table 6-1. Results in quantification

Concentration (µg/µl)	0.2	0.4	0.6	0.8	1.0
A_x	$4.82x10^5$±6.9%	$1.54x10^6$±8.0%	$1.98x10^6$±8.7%	$2.27x10^6$±9.1%	$2.94x10^6$±10.0%
A_x / I_{st}	19.03±6.7%	64.13±6.2%	102.26±5.8%	143.52±4.7%	189.61±3.9%

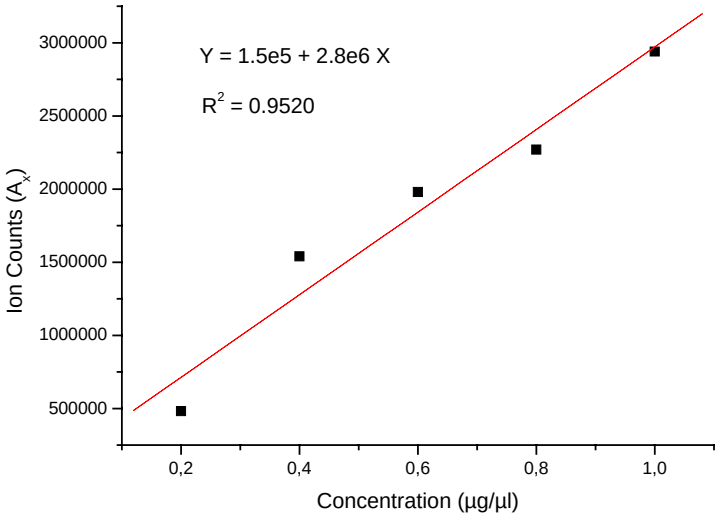

Figure 6-2. Calibration curve for the peak area of SPM

At first, the calibration curve was constructed only based on the signal area of SPM (A_x) without involving the signal of reserpine. It is actually an external method. As shown in Fig. 6-2, it shows a poor linearity (R^2 = 0.9520). However, if the calibration curve was constructed based on the ratio of the signal area and the average intensity of RSP (A_x/I_{st}), a good

linear correlation (R^2 = 0.9991) could be obtained (see Fig. 6-3). Besides the benefit of a better linear correlation, the use of A_x/I_{st} also improves the reproducibility of the method compared with the use of only A_x, which is especially important for a laser desorption technique.

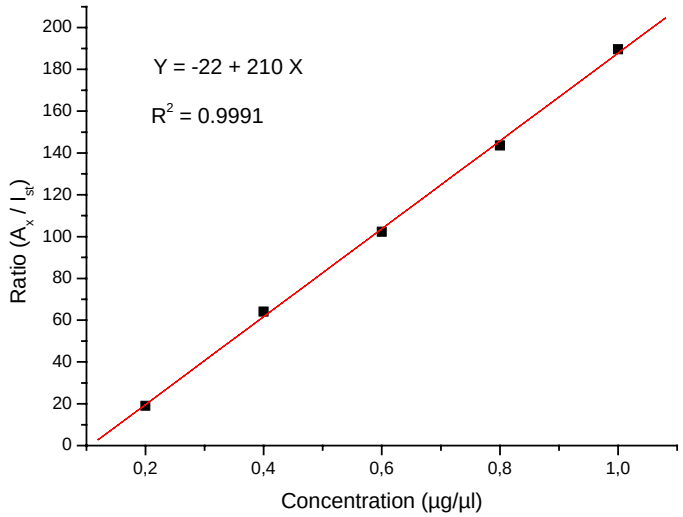

Figure 6-3. Calibration curve for the ratio of peak area of SPM and average intensity of reserpine.

The internal standard used here shows no structural conformity with the analyte. In principle, also isotopically labelled compounds or structural homologs could be used as internal standards in the same way as described here, which possibly would even result in a better accuracy. However, since a relatively large amount of internal standard is necessary for providing enough signal intensity, this kind of internal standard application would not be suited for high-cost internal standard materials.

Although the device is mainly designed for qualitative work, also analyte quantification was carried out. The internal standard was

added into the mobile phase to yield a 'background' signal, which was used as a reference signal for the quantification. This method has advantages in linear correlation and reproducibility compared with an external method.

7. Conclusions

A novel interface for TLC/MS, in which a full-plate scanning can be carried out on a TLC plate to recover the chromatographic information and real-time acquire mass spectra for separated analytes, was developed. In this system, a compact, easy to use and cost effective diode laser was employed to desorb molecules into the gas phase. The desorbed molecules were transferred by gas flow to the area in front of the heated capillary of the mass spectrometer and subsequently ionized by a corona discharge at atmospheric pressure. This system enables fast detection, simple sample preparation, high throughput analysis, the capacity for identifying unknown compounds and compatibility for most modern LC/MS systems. An optional plate pretreating method with graphite assistance was employed to decrease the requirement for desorption power density and consequently increased the possible choices for the laser source.

The first part of the work was carried out on a graphite target to investigate the feasibility of the application of a diode laser induced desorption. At the same time, basic optimizations of the mass spectrometer and the experimental arrangement were performed. A simple ion source with tunable geometric parameters was development for this purpose. The results showed a diode laser can be well applied for laser desorption with the assistance of graphite, and a power density in the order of 10^4 W/cm^2 was sufficient for desorbing biochemical analytes with moderate molecular weight. This power density is two orders of magnitude lower than the necessary power density for a white surface.

The experimental work was then continued using a TLC piece to evaluate the desorption behavior of the analytes with the presence of TLC material. A graphite suspension containing graphite /glycerol /methanol was

adopted as matrix to function as an energy absorber and transfer medium. The work showed that the technique was able to directly acquire molecules from the bulk of TLC material and an additional extraction step was not necessary.

A plate scanning system was subsequently developed based on the same principle to realize the projective intention. Different samples containing only a single compound or a complex mixture were scanned after chromatographic separation and promising results were obtained. Both vertical and horizontal scan modes were carried out for different applications: lane after lane scanning for the analysis of complex or unknown samples; or scanning across several lanes orthogonally for high throughput analysis. The graphite assisted desorption was still advantageous in this case, which resulted in a 30 times lower requirement for power density. Furthermore, homemade TLC plates with embedded graphite particles were prepared based on a simple protocol. Despite the simple preparation procedure, the TLC plates showed good separation and desorption properties. If TLC plates with embedded graphite particles would become commercially available in the future, laser diode desorption-APCI-MS analysis would be facilitated and the pretreating time shortened.

At last, a suitable quantification method was developed. The internal standard was added into the mobile phase and distributed homogenously on the plate after development. This procedure reduces the error resulting from the different surface conditions on the plate, and more important, abandoned the requirement of the internal standard to have identical chromatographic property as the analyte molecules.

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1. Thin-layer chromatography combined with diode laser desorption/atmospheric pressure chemical ionization mass spectrometry (Peng S, Ahlmann N, Kunze K, Nigge W, Edler M, Hoffmann T, Franzke J. Rapid Commun. Mass Spectrom. 2004, 18: 1803)
2. A new interface to couple thin-layer chromatography with laser desorption/atmospheric pressure chemical ionization mass spectrometry for plate scanning. (Accepted by Rapid Commun. Mass Spectrom.)

Poster

Thin-layer Chromatography Combined with Diode Laser Induced Desorption /Atmospheric Pressure Chemical Ionization Mass Spectrometry
Deutsche Gesellschaft für Massenspektrometrie (DGMS), 2005