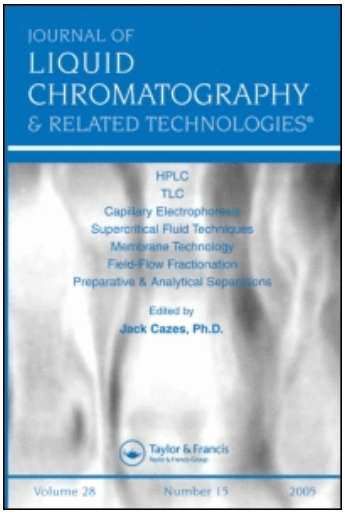

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PLANAR CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY

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ABSTRACT

Applications of thin-layer chromatography/mass spectrometry are expanding rapidly due to commercial availability of the devices, and improved understanding of the procedures required to measure good quality mass spectrometric data. Several of the common approaches to TLC/MS coupling are pursued in our laboratory; recent focus has been on techniques of sample preparation and plate treatment that allow direct TLC/MS analysis to be completed on almost any instrument. Specific examples to be covered are the direct derivatization of alkaloid samples on Empore TLC plates for volatilization into an electron/chemical ionization source, development and concentration of thin-film fluorescent dyes for analysis by liquid secondary ion mass spectrometry (LSIMS), and the use of a CCD-based imaging system to explore the integration of optical and mass spectrometric information for the characterization of samples separated by thin layer chromatography.

Introduction

A number of recent reviews chronicle the development of thin-layer chromatography coupled with mass spectrometry over the past twenty years [1-3]. Solutions to technical problems have long been possible, and a number of ingenious designs for interfaces have been described. In an era of modern instrumentation, integrated analytical systems dominate the market, and a commercial dedicated TLC/MS system is not yet available. With the production of one-dimensional thin-layer chromatogram scanning accessories (based on the direct insertion probe)

for at least two commercial mass spectrometers, the number of TLC/MS applications described in the literature has risen markedly over the past few years. Most of the "system integration" in these applications is derived from the capabilities already in place for gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry. The specificity and sensitivity associated with mass spectrometric detection is the driving force behind these applications. Recent work involves the separation of drug-related compounds, and the separation of mixtures derived from natural products, with analysis by fast atom bombardment mass spectrometry [4-6].

Several years ago, we described a grand-scale imaging mass spectrometer designed specifically for TLC/MS [7]. This instrument remains unique in its capabilities; although a similar instrument was constructed for specific purposes within an industrial laboratory. More recently, we have shown that an imaging SIMS instrument designed for more classical pursuits could indeed be used for high spatial resolution and high mass resolution imaging of samples on thin-layer chromatograms. However, most TLC/MS will be carried out with instruments that are not equipped with specialized accessories, or not originally configured as imaging analyzers. There is therefore considerable interest in the development of procedures through which samples separated by TLC can be analyzed by mass spectrometry using the standard inlet systems available on most mass spectrometers. This coupling involves relatively small portions of the chromatogram that can be attached to the end of a direct insertion probe, and with TLC media that can be easily manipulated. Empore medium for thin-layer chromatography is such an easily handled medium, and we have spent some effort over the past year or so investigating its applications in TLC/MS.

In another series of experiments, we have studied the use of optical spectroscopy in conjunction with TLC/MS. This work has involved the grand-scale instrument referenced above, as well as new designs for accommodation of an optical probe in standard sources for mass spectrometers. In addition to specific work in TLC/MS, optical spectroscopy provides analytical information of relevance to the fundamental mechanisms of sputtering by an energetic primary particle beam.

Experimental

Two sector-based mass spectrometers were used in these experiments. The VG-70SEQ mass spectrometer is a hybrid-configuration instrument consisting of an electric sector/magnetic sector with a collision cell and a quadrupole mass analyzer (EBqQ). The VG-70SEQ is equipped with a cesium ion gun for liquid secondary ion mass spectrometry (LSIMS). Cesium ions are accelerated into the source with 35 keV ion energy, and the current to the gun was held between 2 and 5 A. The mass range was scanned from 50 to 800 Daltons at a scan rate of 1.5 s/decade, and a mass resolution of 1500. The second sector instrument used was a VG-70SE, and is an EB geometry, equipped with a xenon atom gun for fast atom bombardment (FAB). The xenon atoms are formed with 8 kV energy and a source current of 1 mA. The mass range was scanned from 100 to 800 Daltons, the scan rate was 1.5 s/decade, and the mass resolution was set at 1000. For electron ionization (EI), the electron energy was 70 eV and the source temperature was 200°C. The direct insertion probe with sample cup was used as the inlet system. For experiments with the optical detector, the system was set up as previously described [8].

The alkaloids morphine and codeine were purchased from Alltech in 1 mg/mL ampules. Noscapine and papaverine were purchased from Sigma. For comparative studies, Empore™ sheets and Merck HPTLC aluminum-backed plates were considered. The Empore™ sheets are composed of 90% fibrillated polytetrafluoroethylene (PTFE) and 10% silica gel or bonded stationary phase silica gel particles; Empore is a trademark term, but we will delete the designation in the following discussions. Three varieties of Empore sheets were purchased from Varian & Associates: silica gel, C_8-modified and C_{18}-modified. The Merck plates have a normal phase silica gel stationary phase, and were purchased from EM Science. Each TLC plate incorporated a fluorescent indicator, and a hand-held UV lamp was used to help indicate the location of sample bands. Iodoplatinate, a common indicator for alkaloids, was also used to develop a sample spot color. Each TLC sheet or plate was spotted with 10-13 micrograms of

the model compound with a micropipet. After adsorption, or after development, the spot was removed from the TLC plate using a single hole paper punch to produce a disc 5 mm in diameter. This disc is about the same area as the standard FAB probe tip on the VG instruments, and the disc was attached with double-sided tape to the FAB probe. Approximately 50-100 microliters of the FAB solvent was added to the surface; these relatively large volumes are necessary to thoroughly wet the surface and to establish an uniform potential gradient on the sample. All of the following (v/v) solvent matrices were examined in FAB: *meta*-nitrobenzylalcohol (mNBA), 20/80 hexane/thioglycerol, 50/50 methanol/thioglycerol, 67/33 mNBA/methanol, 50/50 methanol/glycerol, 5/95 pyridine/glycerol, 5/95 dimethylsulfoxide/glycerol, and finally 5/95 triethylamine/glycerol. Full results of these comparative evaluations are provided elsewhere [9]; a summary of the best results is provided here. Empore silica gel, C_8-modified and C_{18}-modified Empore sheets were examined using each of these solvent/matrices. Empore silica gel was compared directly to Merck HPTLC silica gel for extraction efficiency and limit of detection for the model compounds.

Separations for the alkaloid model compounds were carried out on a 6 x 2.5 cm strip of the Empore silica gel or a 6 x 4 cm strip of the Merck aluminum-backed silica gel HPTLC plate via normal ascending development. The mobile phase consisted of 1,4-dioxane, hexane, ethanol, and dimethylamine in a volume ratio of 44:48:4:4. The development time for the Empore silica gel was about 20-30 min, and about 15-20 min for the Merck silica gel HPTLC plate. The literature method for TLC separation of these alkaloids required modification to provide equivalent separations for both Empore and standard HPTLC plates. To introduce the chromatograms into a standard EI source, the Empore material was rolled up and inserted into the direct insertion probe cup. Alternatively, the excised sample was cut into a small strip, 4 mm x 1 mm, and then inserted lengthwise into the cup. Extraction solvents used for direct removal of the sample from the Empore included methanol and dimethyl sulfoxide. Approximately 10 microliters of the solvent was delivered into the sample cup, and after extraction, was evaporated into the source.

Data acquisition was begun immediately before the sample was introduced into the source to ensure that all of the compound signal was recorded. The direct insertion probe was not heated during these experiments. For derivatization reactions on the Empore, 10 g of morphine was applied to the silica gel and C_{18}-silica gel surfaces of the Empore. The spot was removed as described above, and the entire disk was placed into a 1 mL vial. Next, 200 microliters of BSTFA/1% TMCS (bis-trimethylsilytrifluoro-acetamide/trimethylchlorosilane) was added to the vial and the mixture heated for 20 min at 70°C. Later the spot was trimmed into a strip as above, inserted into the probe cup, and the probe heated to 400°C to evaporate the volatile derivative directly from the silica gel.

For concentration experiments with HPTLC separation of the fluorescent dyes, standard silica gel plates were used, and methanol was used as the developing solvent in an ascending mode. Development time for separation of two- or three- component mixtures was approximately ten minutes, depending on the degree of separation required. After the edge of the chromatogram was cut into the repetitive wedge pattern (see following discussion), the chromatogram was rotated ninety degrees, and solvent applied in a descending mode to concentrate the sample into the wedges. Measurement of the mass spectra was completed on the VG70SEQ instrument as described above for the alkaloid samples. The fluorescent dyes were obtained from Molecular Probes, Inc.

Results and Discussion

Direct Analysis of Compounds on Empore TLC Chromatograms

The original reference to the use of Empore chromatograms in TLC/MS is that of Minard and Long [10]. Their work involved a C_8-modified silica gel stationary phase and FAB-MS using matrices of thioglycerol/1% oxalic acid and glycerol/30% methanol. FAB mass spectra were measured for several dyes and two alkaloids, haemanthidine and haemanthamine. After some

years of experience with aluminum-backed silica gel HPTLC plates for TLC/MS, we were attracted to the ease of handling of the Empore plates. Empore plates were used in the imaging SIMS experiments described above, and we have also used Empore plates in an study of matrix effects in matrix-assisted laser desorption mass spectrometry [11]. Although there are distinct advantages with the use of Empore in terms of sample handling, wetting of the surface and extraction of the sample molecules can be difficult. The impetus behind the current investigation was a comparison of various extraction solvents and chromatography media (normal- or reverse-phase) for TLC/MS. We also report here the results of investigations that use electron ionization (EI) mass spectrometry in conjunction with rapid evaporation of sample molecules from Empore sheets. Finally, we show that a derivatizing agent, BSTFA/1% TMCS can be applied directly to a compound spot on the Empore chromatogram to form a volatile derivative suitable for subsequent EI analysis.

Each of the solvent mixtures described in the experimental section was examined for the ability to extract the sample molecules (the alkaloids noscapine, papaverine, morphine, and codeine) from Empore. The best overall results were obtained using the 50/50 (v/v) methanol/glycerol solvent mixture, with a mixture of 66/33 (v/v) mNBA/methanol also satisfactory in most cases. Most of the solvent mixtures examined (chosen because they have been previously used in other situations) did not work especially well. In general, the mixture must contain a substantial methanol component to wet the surface of the Empore.

The positive ion FAB spectra that follow reflect comparisons of the sputtering efficiencies from Empore silica gel and Merck HPTLC silica gel using the 50/50 (v/v) methanol/glycerol matrix and equivalent amounts of sample. Figure 1 compares the positive ion FAB mass spectra of 13 micrograms of papaverine on the Empore silica gel (top) with the mass spectrum obtained from the Merck silica gel (bottom). The $(M+H)^+$ ion of papaverine at m/z 340 is relatively intense, and is similar in intensity to the glycerol cluster ion at m/z 369 $(4G+H)^+$; no background subtraction is used to enhance the appearance of this mass spectrum. The presence of papaverine

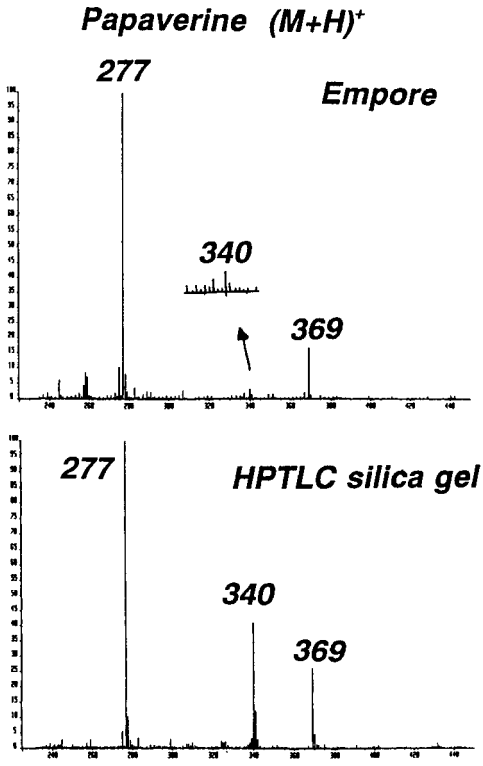

Figure 1. Comparison of the positive ion FAB mass spectra recorded from papaverine sputtered from Empore silica gel (top) and standard HPTLC silica gel plates (bottom).

can also be deduced from the mass spectrum obtained from the Empore silica gel (bottom), but the intensity of the protonated molecule is only slightly above the intensity of background ions. Figure 2 contains the mass spectra recorded from 12 micrograms of noscapine, with the $(M+H)^+$ ion expected at m/z 414. The protonated molecule of noscapine is clearly seen in the spectrum obtained from standard silica gel (top), but is barely visible above the background ions in the mass spectrum obtained from the Empore silica gel (bottom). The amounts of sample quoted are the total amounts of sample applied to the TLC plate before development. The surface density

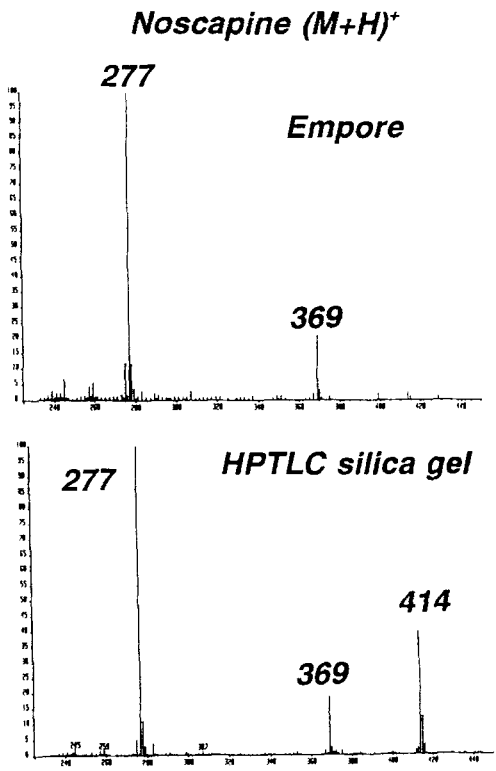

Figure 2. Comparison of the positive ion FAB mass spectra recorded from noscapine sputtered from Empore silica gel (bottom) and standard HPTLC silica gel plates (top).

after development, the amount of sample within the footprint of the primary particle beam, and the amount of sample remaining after analysis all factor into the actual amount of sample consumed to produce the mass spectrum. The estimated densities range from 1 microgram/mm^2 for morphine to 0.3 microgram/mm^2 for noscapine on Empore, and for Merck HPTLC the results ranged from 2 microgram/mm^2 for morphine to 1 microgram/mm^2 for noscapine. Estimates of the sample consumption rate and the remaining sample lead established from other experiments suggest that the actual amount of sample that produces the mass spectra shown is on the order of 50-100 ng.

An inability to use **all** of the sample molecules to generate signal in FAB led to our investigation of other methods that are more efficient in the production of measurable ion current from the sample molecules. Direct evaporation of relatively volatile samples from TLC plates, with subsequent analysis by electron ionization (EI), is an idea that originates in the very first couplings of thin-layer chromatography with mass spectrometry. This procedure was evaluated here for both the Empore sheets and the Merck HPTLC plates with codeine as a model compound. A blank control chromatogram strip was used in both instances to establish the spectral background. The silica gel HPTLC plate was spotted with 10 micrograms of codeine, dried, and the silica gel containing the sample was scraped from the plate and transferred to the sample cup of the direct insertion probe. Ten microliters of methanol was then added to the sample cup as the extraction solvent. The same procedure was completed for the Empore, except that the entire spot was removed as a disc, clipped into smaller pieces and the pieces transferred into the sample cup. Figure 3 contains the mass spectrum of codeine using the direct evaporation method with analysis by electron ionization mass spectrometry. The molecular ion for codeine at m/z 299 is present in the mass spectra from both the Empore sheets (top) and the Merck plates (bottom). The fragment ion at m/z 229 present in both mass spectra is the loss of C_4H_6O from the molecular ion. Ions at m/z 77, m/z 105, and m/z 163 were found in the mass spectra from the blank, and the ion at m/z 149 is probably due to the presence of a phthalate contaminant. The lower limit of detection for direct evaporation of samples from Empore is 2 micrograms for codeine. This limit of detection was derived from the fact that a strip approximately 1/5 the size of the sample spot was actually examined. Thus ratio means that 20% of the 10 micrograms, or 2 micrograms, of the original sample was examined. Establishing the limit of detection from the Merck silica gel is somewhat more difficult because scraping and transfer is not quantitative, but a similar limit of detection is established. Again, these are full-scan mass spectra and no background subtraction is used.

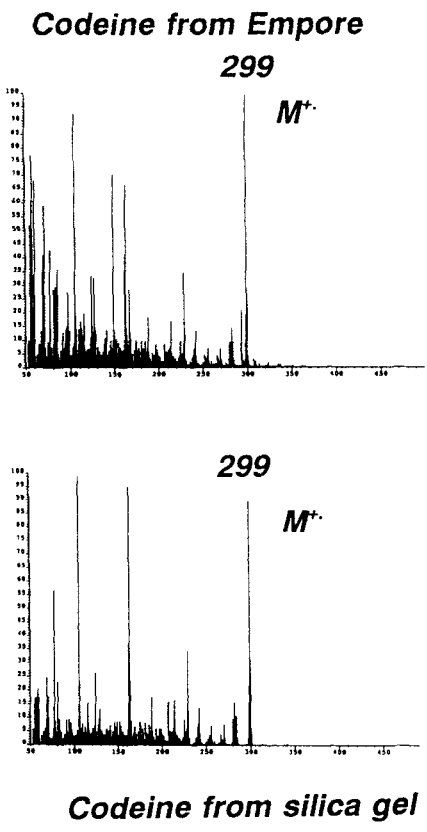

Figure 3. Electon ionization mass spectrum obtained for codeine evaporated directly from a portion of a standard HPTLC silica gel thin-layer chromatogram.

Morphine is difficult to analyze and to recover by gas chromatography, and is usually derivatized to obtain a more stable and a more volatile derivative that produces a higher quality electron ionization mass spectrum. Morphine contains two hydroxyl groups and will react with two trimethylsilyl (TMS) groups to produce an ion at m/z 429, while reaction with only one TMS group gives an ion at m/z 357. Each TMS group adds 72 daltons to the mass of the molecule, representing replacement of the active hydrogen on the hydroxyl group with 73 daltons

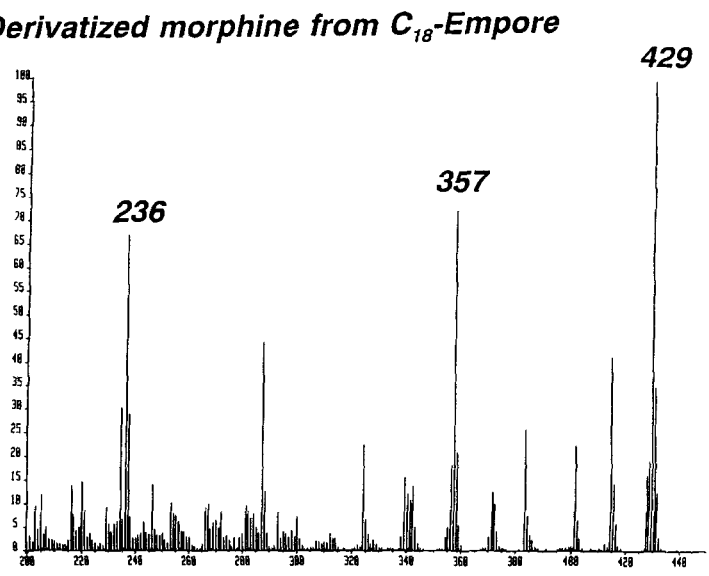

Figure 4. Electron ionization mass spectra measured after direct evaporation of *in situ* derivatized morphine on an Empore chromatogram.

representing the $(CH_3)_3Si$ unit. The mass spectrum of a derivatized standard contains a fragment ion at m/z 236 that is the result of loss of both OTMS groups, and cleavage of the N-C bond. We show here that derivatization with TMS can be carried out directly on Empore silica gel, C_8-modified and C_{18}-modified TLC sheets. After the derivatized sample was transferred to the mass spectrometer, the direct insertion probe was heated to 400°C. Figure 4 contains the mass spectrum for the TMS derivative of morphine derivatized directly on Empore C_{18} modified silica gel. The expected ions $(M+2TMS)^+$ and $(M+TMS)^+$ ions are present in the spectrum. A phthalate contaminant at m/z 149 is present, as is the characteristic fragment ion of TMS derivatives, $(C_3H_8Si)^+$ at m/z 73. Mass spectra of similar quality can be measured by direct evaporation of the derivatized sample molecules on the C_8-modified silica gel. Overlapped components in spots on the Empore C_8-modified silica gel sheets can be identified by the

summed mass spectra of the compounds evaporated into the EI source. Noscapine and papaverine were prepared as overlapping spots (10 micrograms of each) on a TLC plate. The hole punch was used to remove the sample, and the mixture was transferred to the sample cup. Ten microliters of methanol was used for the extraction of both of the compounds present in the spot. The mass spectrum of the mixture contains ions for both the papaverine, $M^{+\cdot}$ (m/z 339), and noscapine (m/z 220, representing [M-193]). Spatial resolution is only dependent on the device that is used to excise the sample (see following section), and determination of the number of overlapping spots is dependent only on the ability to identify the presence of ions from two or more sample components in the mass spectrum.

Method for Sample Concentration in TLC/MS

Several previous workers in the field of TLC/MS have described concentration methods that counteract the natural band diffusion that occurs as TLC plates are developed. For instance, a sample spot can be encircled with a ring of solvent [12]. As the solvent diffuses through the silica, solvent at the perimeter of the spot carries sample towards the center. The more concentrated spot is then analyzed by mass spectrometry. In another form of sample concentration, an ammonium chloride prism is used to concentrate a sample that is later analyzed by chemical ionization mass spectrometry [13]. We have used with success a simple concentration method that also fulfills the need for generating a portion of the chromatogram with a physical scale consistent with the size of the FAB probe stage, and which allows a progressive change in the spatial resolution that can be attained in the detection of overlapped components on TLC plates. Figure 5 illustrates the general idea of this approach. Development of the chromatogram occurs in the x direction as usually practiced. Either one or two lanes of the sample mixture can be developed; if sample visualization is detrimental to the mass spectrometric analysis, two lanes (one treated with developer and one not) are used. After the development of the chromatogram in the x-dimension is complete, the edge of the plate is cut in a regular pattern

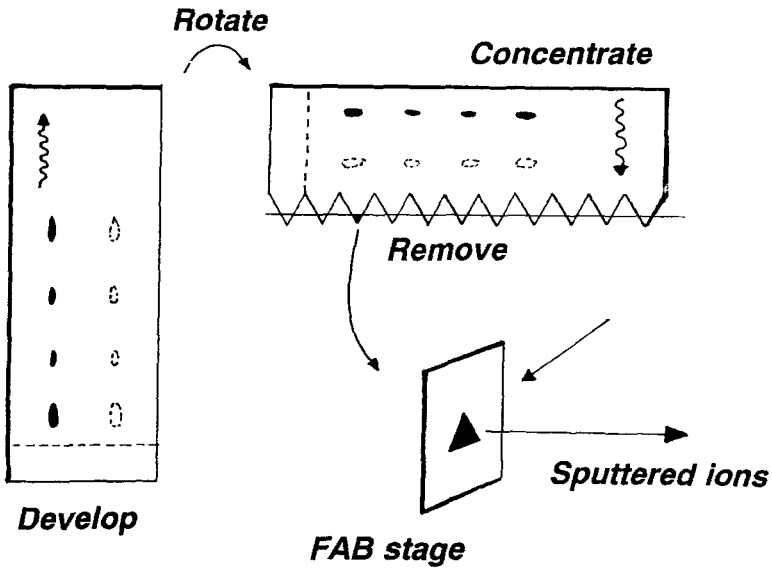

Figure 5. Concentration method for samples separated by TLC with subsequent analysis by direct insertion probe fast atom bombardment.

as shown. "Pinking" shears do an excellent job with aluminum-backed or Empore plates, and provide the proper dimensions for the FAB direct insertion probe. Rotation of the plate ninety degrees, and then the application of solvent in a descending mode concentrates the sample spot into the wedge formed by the shears, and could (in principle) be used to elute the sample from out of the chromatogram completely. However, in the situations described here, the sample spot is only concentrated within the sample wedge, which is an equilateral triangle with a side length of 2 mm. After the sample concentration is complete, a cut along the indicated line is made, resulting in a number of small triangles that are each of the proper size to fit on the probe tip. Without knowing the value of sample R_f, each of the TLC portions can be investigated. However, in the general case in which the sample R_f is known, the wedge containing the sample, and perhaps adjacent wedges are analyzed.

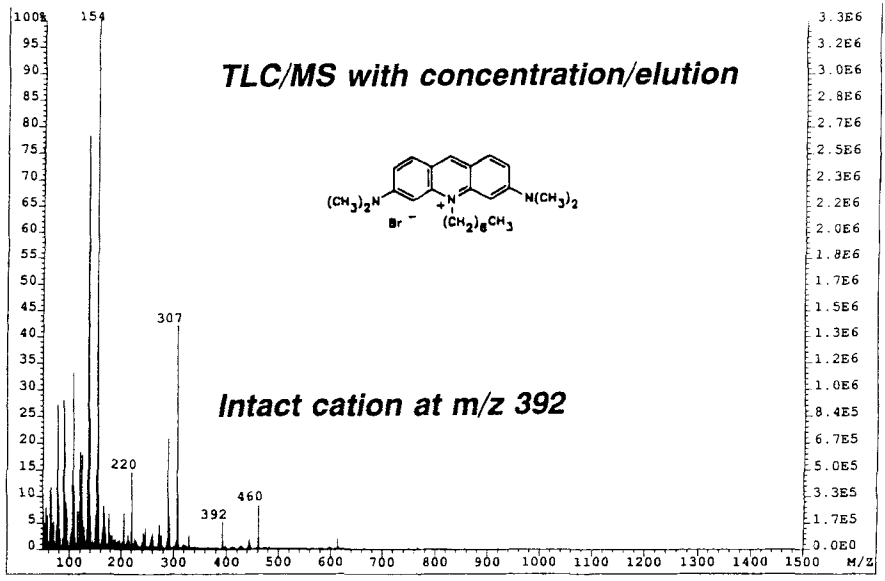

Figure 6. Positive ion FAB mass spectrum for a lower molecular weight laser dye concentrated into a wedge placed on the direct insertion probe stage.

The wedge is placed on the probe tip and held there by a thin film of FAB solvent, or by a dab of silver paint or other conducting adhesive. Solvent is added to the top of the sample, and direct sputtering provides a FAB mass spectrum of the sample molecules concentrated within that wedge. We are investigating a number of fluorescent dyes by capillary zone electrophoresis and mass spectrometry to determine purity prior to other uses in thin film sensor preparation. Thin-layer chromatography provides a rapid check for sample purity. The positive ion FAB (LSIMS) mass spectrum of a laser dye from the HPTLC (aluminum backing) is shown in Figure 6. The migrated sample band was concentrated using the technique described above. Figure 7 is the positive ion FAB mass spectrum of a laser dye at a different R_f on the same TLC plate.

This relatively simple procedure provides estimated concentration factors of 10-20 fold, depending on the original size of the sample spot, and the degree to which the sample is collected

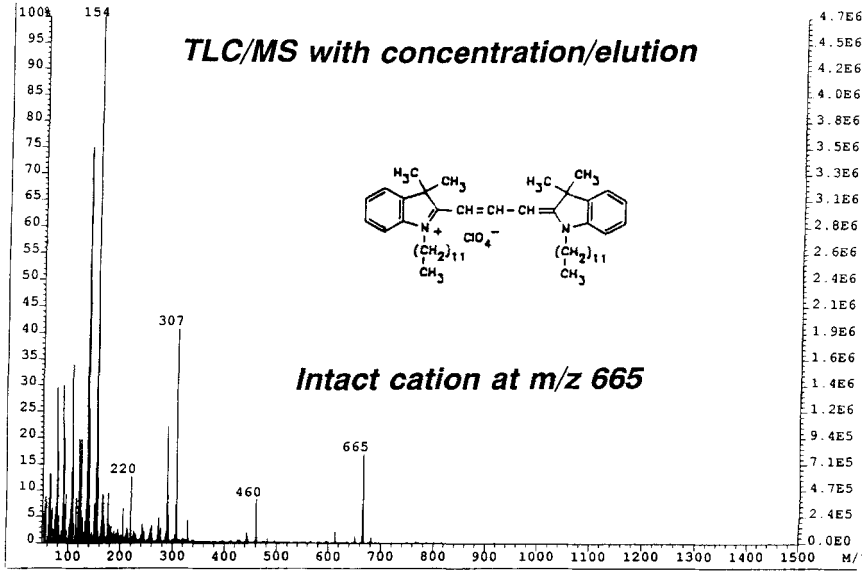

Figure 7. Positive ion FAB mass spectrum for a higher molecular weight laser dye concentrated into a wedge placed on the direct insertion probe stage.

in the tip of the wedge. In contrast to the solvent encirclement procedure described above, it is not necessary to know the location of the sample spot beforehand. Additionally, in contrast to scraping methods that can compromise analytical measurements because the degree to which the sample has been removed from the plate is unknown, this simple physical procedure provides a uniform set of concentration wedges of equivalent size, and the quantitative distribution of the sample molecules can be monitored with greater ease. The primary advantage is that we can easily adapt TLC/MS to a higher performance mass spectrometer that provides capabilities beyond that of the imaging TLC/MS system. For example, two dimensional imaging and direct analysis can be performed on the quadrupole-based single stage mass spectrometer. Once compounds deserving of further study are identified, then the chromatogram (preserved intact in the first analysis) can be manipulated and samples concentrated into wedges subjected to mass

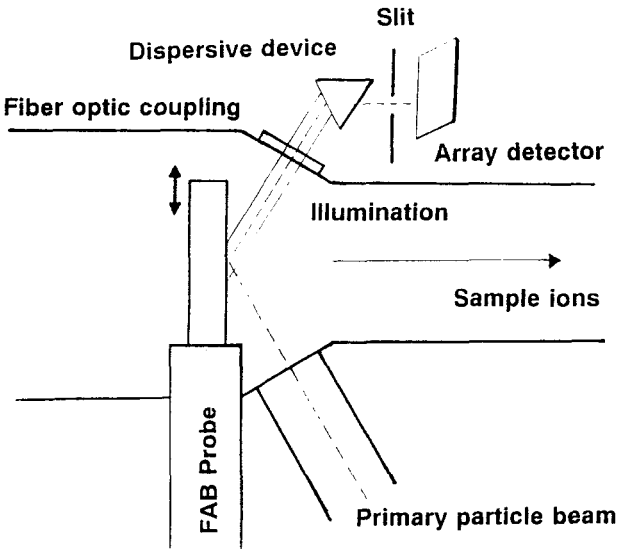

Figure 8. General design for an optical spectroscopic detection system integrated with a fast atom bombardment source.

spectrometric analysis on the second instrument. Examples of more sophisticated experiments that can be carried out in this second level of analysis include positive and negative ion analysis, high resolution mass measurements, and MS/MS experiments. For instance, the positive ion FAB mass spectra of the fluorescent dyes illustrated in the previous two figures are recorded at a mass resolution of about 1500, which allows the empirical formula of some of the intriguing lower mass ions in the mass spectrum to be established.

Optical Spectroscopy in Conjunction with Mass Spectrometry

As mentioned, we have fitted a charge-coupled device camera to the imaging SIMS instrument, replacing the mass spectrometer detector [8]. While the chromatogram remained in place on the manipulators, and within the same coordinate system, both optical and mass

spectrometric analytical data could be recorded. A more recent paper provides perspective for the limits of detection that can be achieved with these two detection modes [14]. Ultimately, the goal is to develop and analytical system with enough power to control and integrate the disparate data; realistically, we are several years from achieving that goal. For the CCD-camera attached to the quadrupole-based SIMS system, detection limits into the low picogram range could be established in favorable situations, based on simple contrast difference measurements made at the surface of the thin-layer chromatogram. CCD technology has evolved significantly over the past few years. CCD-detectors are now much less expensive, more powerful, smaller, and now fully integrated into imaging spectrophotometers. Our TLC/MS work with the fluorescent laser dyes (Figures 6 and 7) emphasize the need to integrate optical spectroscopy with mass spectrometric detection. Figure 8 illustrates the general design for optical spectroscopy integrated into a standard FAB source. Fiber optic coupling for light leaving the sample is used, and is consistent with the space available within the source. The two-dimensional CCD array detector allows spatial resolution in one axis (up to about 6 mm), and wavelength or intensity information is dispersed on the other axis of the array. Simple contrast measurements in real time might be used to control movement along the axis of the direct insertion probe movement. Such control, for instance, would be used to record a mass spectrum when the optical detector indicates that a spot is present. With a more sophisticated design that provides wavelength selectivity, an integrated analytical system would record optical spectra and mass spectra as a function of R_f.

Conclusions

Direct sputtering of most organic samples from Empore chromatographic media is not as efficient as is sputtering from aluminum-backed TLC plates. This lowered efficiency is due in some part to lower sample densities, but is also due to the fact that the surface of Empore is extremely difficult to wet, and an extraction process can only occur if the solvent is allowed to penetrate thoroughly within the thickness of the chromatographic medium. However, we show

that morphine, for example, can be directly derivatized on Empore C_{18}-modified silica gel with the TMS-reagent BSTFA/1%TMCS. The more volatile sample derivative is more easily evaporated into the ionization source from the direct insertion probe. Concentration of samples separated in TLC into a wedge that fits directly onto the direct insertion FAB probe of commercial mass spectrometers is described. Addition of a small amount of extraction solvent to the portion of the TLC plate held on the probe provides a high quality FAB mass spectrum. Most importantly, the physical dimensions of the wedge can be varied to meet the required concentration factors or spatial resolution. Finally, a design for the conjunction of optical spectroscopy with TLC/MS is developed with a focus on integration of the measured spectroscopic data and on-line control of chromatogram movement and mass spectral measurement.

Acknowledgements

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