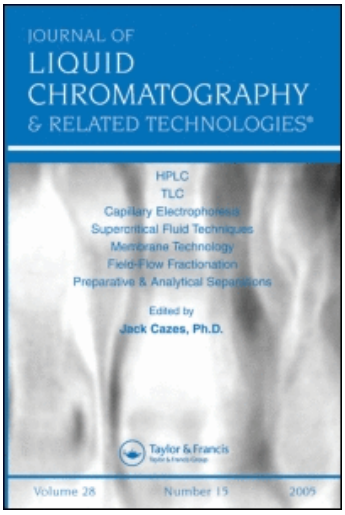

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ANALYSIS OF PLANAR CHROMATOGRAMS BY FAST ATOM BOMBARDMENT AND LASER DESORPTION MASS SPECTROMETRY

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Summary

The applications of fast atom bombardment mass spectrometry coupled with thin-layer chromatography in a study of phospholipids, alkaloids derived from bloodroot rhizomes, and diuretics are summarized. Use of the mass spectrometric data to trace compounds via characteristic fragment ions increases the effective resolution of the chromatographic resolution. Quantitative values derived in TLC/FAB experiments for the diuretic amiloride hydrochloride is linear over two orders of magnitude encompassing the clinically useful range. The use of a laser desorption ionization method and Fourier transform mass analysis for TLC plate detection is also described; matrix and concentration effects are significant in this case, but several successful applications are described. An interface device for the linkage

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of planar electrophoresis with mass spectrometry is also described. Molecules within an agarose gel are released within a few seconds into a transfer line that terminates in a continuous flow FAB source.

Introduction

Developments in the use of mass spectrometric detection for planar chromatography, including thin layer chromatography (TLC) and planar electrophoresis, have been recently reviewed.[1-2] A number of research groups now routinely use fast atom bombardment (FAB)[3,4], liquid secondary ion mass spectrometry (SIMS), and laser desorption (LD)[5] mass spectrometry in the analysis of sample mixtures separated by thin layer chromatography. The latter experiment is carried out with commercial laser microprobe instruments. With FAB and SIMS, custom-built systems have been used for full two-dimensional measurements.[6,7] Several commercial manufacturers offer interface devices between TLC and standard organic mass spectrometers. Operation of mass spectrometers in a variety of ionization modes, and in an array of mass measurement experiments, is a reliable and straightforward procedure. The opportunities for linking such a powerful detection system to planar chromatography are clear. The analytical advantages to doing so arise from the same balance of capabilities for separation and detection that has made gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry powerful, general-purpose analytical instrumental techniques. This paper provides an overview of the capabilities, both qualitative and quantitative, of thin layer chromatography/mass spectrometry (TLC/MS) and planar electrophoresis/mass spectrometry (PE/MS).

Experimental

TLC/FAB data described in this paper were recorded with the instrument that we had constructed specifically for the purpose of exploring the coupling of thin layer

chromatography with secondary ion or fast atom bombardment mass spectrometry. In this system, the point of focus of the primary ion beam at the xy surface of the chromatogram overlaps with the point of secondary ion extraction into the quadrupole mass analyzer. This point of instrument focus remains fixed. Sample spots are physically brought into or out of the point of focus with a manipulator that provides movement of the sample plate in the xy plane. Spatial profiles are generated by moving a manipulator in the x and y directions while monitoring the abundance of the selected ions. Three-dimensional images for organic ions on the surface of the chromatogram are produced by plotting the spatial dimensions x and y versus ion intensity. Mass spectra were measured using a modified Extrel C-50 quadrupole mass spectrometer and a saddle field discharge neutral particle source mounted on a vacuum flange at an angle of 45 degrees relative to the sample plane. Primary ion current equivalence was maintained at approximately 1-2 nA at the surface for argon atoms as the primary neutral particle. Mass spectra were acquired with modified ASYST software, which also generated the three-dimensional images using standard plotting routines. In some cases, spectra were recorded directly by an x-y recorder.

TLC-based separations followed, for the most part, procedures already described in the literature. Phosphatidylcholines were obtained from the Sigma Chemical Company, and prepared as standard solutions in chloroform at a concentration of one microgram per microliter. Thin-layer chromatography was performed on aluminum-backed silica gel 60 TLC plates containing a fluorescent indicator (EM Science). The plates were pre-eluted with a 70:30 v:v methanol:chloroform solvent mixture, and subsequently developed with the same solvent mixture following application of 5 microliters of a solution containing 1.6 micrograms of each of distearoyl, dipalmitoyl, and dioctanoyl fatty acids. In each case, the L-alpha compounds were of synthetic origin, and no impurities were noted in the TLC separation. The solvent front was allowed to migrate 5 cm from the origin. R_f values were measured as 0.80, 0.40, and

0.30, respectively, for the three compounds listed. For imaging experiments with the TLC/FAB instrument, the TLC plates were positioned on the sample platform such that an x-scan by one of the translation stages corresponded to the axis of chromatographic development. Sorbitol was used as the extraction and sputtering matrix. Two minutes were required to scan the entire 5 cm length of chromatogram development.

The bloodroot extract study began with 28 g of bloodroot rhizomes that were obtained locally. This material was dried in the oven at 100° C for 48 hours. The dried material (approximately 7 g) was pulverized with a mortar and pestle, extracted with chloroform (4 x 60 mL), and the combined solvents evaporated to yield 0.46 g of dried extract material. This extract was then dissolved in 5 mL of chloroform. For subsequent measurements, 5 microliter aliquots of this solution were used directly without further processing. Thin-layer chromatography was performed on aluminum-backed silica gel 60 plates containing a fluorescent indicator. The TLC plates were pre-eluted and then developed with a 2:2:7 (v:v:v) benzene: ethyl acetate: hexane solvent mixture. Both one- and two-dimensional TLC separations were completed to produce a number of TLC plates that were then analyzed by FAB mass spectrometry. In addition, a standard sample of sanguinarine chloride was obtained from Aldrich, prepared into a stock solution with a concentration of 1 microgram per microliter, and used for co-chromatography in adjacent lanes to the extract sample, and directly for exact mass and daughter ion MS/MS experiments. For TLC/FAB, sorbitol was used as the extraction matrix. Exact ion masses were measured on a ZAB-HF mass spectrometer with a resolution of 12,000 and an accelerating voltage of 8 kV. Daughter ion MS/MS spectra were recorded on a Finnigan MAT TSQ-70 triple quadrupole mass spectrometer, with argon at a gauge pressure of 1 millitorr as the collision gas for collision-induced dissociation, and a collision energy of 30 eV. For both the exact mass measurements, and the daughter ion MS/MS experiments, a

glycerol matrix containing a small amount of thioglycerol and dimethylsulfoxide was used as the liquid solvent from which the sample was sputtered.

For TLC/FAB of the diuretic samples, the six diuretic drugs were used as received from Sigma. These compounds were dissolved together in methanol, spotted in quantities of 0.5 micrograms each, and eluted with ethyl acetate:water (100:1.5 v:v) on normal-phase, aluminum-backed silica gel HPTLC plates containing a fluorescent indicator. The R_f values determined for the diuretic drugs were: amiloride hydrochloride (AMI) 0.00, furosemide (FUR) 0.38, hydrochlorothiazide (HCT) 0.63, chlorthalidone (CTA) 0.71, hydroflumethiazide (HFM) 0.80, and trichoromethiazide (TCM) 0.91.

For experiments in electrophoresis/mass spectrometry, a standard solution of coenzyme B_{12} (2.0 mg/mL) was prepared. A potential was applied to migrate the sample molecules into an agarose gel. Uniform diffusion of the analyte into the gel matrix was ensured by letting the gel stand for about eighteen hours. The portion of the gel containing the analyte was washed with distilled water to remove any residual analyte from the surface of the gel, and placed in a sample vial. Approximately 1 mL of H_2O was added to each vial, and a recently constructed probe used to release the sample molecules from the gel into a transfer line. Samples required about one minute to prepare for transfer to the mass spectrometer. The gel with the compound present was disrupted using a small probe that produces a fine suspension of gel particles and sample molecules. A small particle filter was used in line to remove the relatively large particles from the slurry produced. A measured amount of glycerol was added to the filtrant to yield a 10% glycerol concentration. The sample was introduced into the mass spectrometer by immersing the flow-FAB probe capillary into these solutions. The entire sample preparation operation (location, preparation, filtration, matrix addition and transfer) took less than 5 minutes to complete. Continuous-flow FAB analysis was performed on a VG-70 SE mass

spectrometer. The standard VG dynamic FAB probe was used, with a 50 micron ID, 500 micron OD capillary. No background subtraction of the spectra was performed. Spectra were taken once stable ion signal was obtained, approximately one minute after the capillary was introduced into the sample solution.

For laser desorption experiments, a FT-2000 Fourier Transform mass spectrometer was used for the mass analysis. Standard instrument conditions were used for recording of mass spectra, exact mass measurements, and daughter ion MS/MS spectra. A Nd:YAG laser was used in a pulsed mode for desorption. Experimental parameters that were varied include the laser pulse width and the laser wavelength. The laser wavelength was either 266 nm or 1064 nm. The focus of the laser was varied slightly to empirically increase sensitivity, but in general the laser spot size on the surface of the direct insertion probe or the chromatogram was 0.2 mm^2. For MS/MS experiments, collision-induced dissociation was accomplished by isolating the ion of interest, and then accelerating the ion into argon collision gas maintained at a static pressure of approximately 3×10^{-6} torr. For all of the experiments outlined here, the collision energies were in the range of 50-100 eV. The use of the laser desorption ionization method and the FTMS instrument for the analysis of TLC plates and electropherograms is described more fully elsewhere.[8] Solutions of biological compounds tested were made in compatible solvents: peptides were dissolved in methanol/acetic acid 1:1 (v:v), bile acids and bile salts were dissolved in ethanol, PTH-amino acids were dissolved in methanol/acetonitrile 1:1 (v:v), and nucleosides and nucleotides were dissolved in water. Five microliters (corresponding to 5 micrograms of analyte) was deposited into the TLC matrix and steel surface with a microsyringe and the solvent was allowed to evaporate. For TLC matrices the sample spot size was 4-5 mm^2. No development was carried out in the preliminary detectability studies. Biological compounds were obtained from Sigma or Pharmacia and used without further purification. For best comparisons, TLC sheets

from the same manufacturer (Analytichem) were used. Empore TLC sheets are composed of 90% TLC adsorbent dispersed in a perfluorinated polymer matrix, and are approximately 1 mm in thickness. Sample material is estimated to penetrate approximately 50% of TLC sheet thickness. For mass spectral analysis, a 3/4" diameter circular piece of Empore TLC sheet was cut out and affixed to the direct solids autoprobe of the FTMS. Spatially-resolved mass spectral data could be obtained by rotating the probe through 360 degrees, as well as moving the probe in the z direction relative to the laser beam. For comparison purposes, mass spectra of all compounds tested were acquired from stainless steel probe surfaces at similar concentrations and laser conditions. For gel electrophoresis experiments, 1 x 1 x 0.2 cm pieces of intact agarose gel were soaked in a 1.0 mg/mL solution of analyte. The gels were washed, and then dried in the sample lock of the mass spectrometer before the mass spectra were measured.

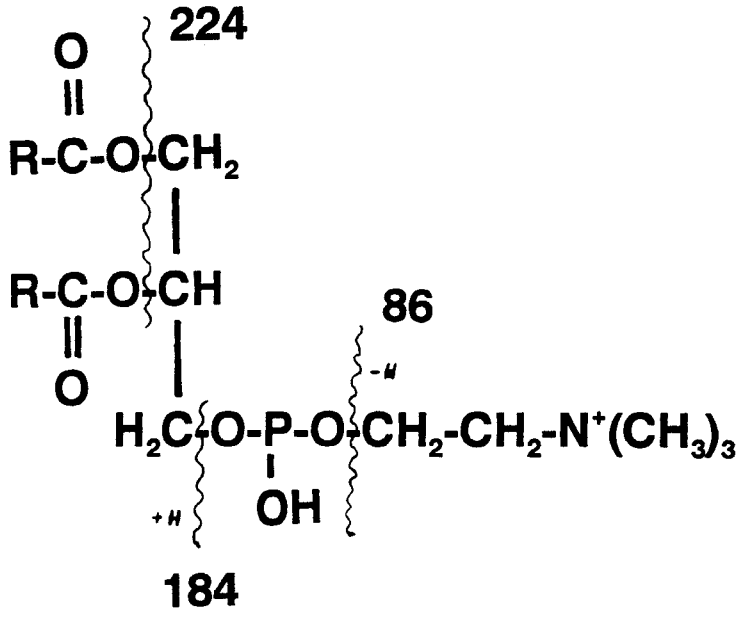
Results and Discussion

This section is divided into three categories corresponding to the three main areas in which mass spectrometry has been used as a detection system for planar chromatography in our laboratory. The first part concentrates on some results obtained with TLC/FAB, including a short study of phospholipids, an exploration of the alkaloid compounds extracted from bloodroot rhizomes, and finally, a qualitative and quantitative study of diuretic drugs separated by thin-layer chromatography and detected by negative ion fast atom bombardment mass spectrometry. The potential of laser desorption ionization in conjunction with Fourier transform mass spectrometry for TLC and planar electropherogram analysis is outlined. Finally, extension of these ideas to the interface between planar electrophoresis and mass spectrometry is then presented. An interface device that allows the direct sampling of biological compounds from within aqueous agarose or polyacrylamide gels is briefly described.

Thin-layer chromatography/fast atom bombardment mass spectrometry

Phospholipids: Shortly after the introduction of fast atom bombardment mass spectrometry as an ionization technique for the determination of the mass spectra of non-volatile and thermally fragile molecules, the first report of the FAB mass spectra of phospholipids appeared, underscoring the importance of reliable and sensitive analytical methods for these compounds. Jensen and Gross[9] have produced a review article with extensive references on the use of mass spectrometry for the structural determinations of phospholipid molecules. Many of the methods highlighted in that review involve the use of FAB as the ionization method of choice. The potential of TLC/FAB and TLC coupled with secondary ion mass spectrometry was also recognized at an early stage. In the work of Kushi[10], Pahlsson[11], and Tamura[12], the TLC/FAB analysis was not a direct combination. Rather, the analyte bands or spots on the TLC plate were identified by a separate and independent method. Then, the sample within the silica was physically removed from the aluminum backing plate, and transferred to a separate direct insertion probe. The normal FAB solvent glycerol was added to the tip of the probe to extract the material from the silica and to support the persistence of the sputtering.

The positive ion FAB mass spectra of the three phosphatidylcholines that form the basis of this study contain, as expected, signals for the protonated molecules of each compound. In addition, there are a series of ions at lower masses that are characteristic of the phosphatidylcholine structural backbone. The origins of these ions, also observed by other workers who have recorded the FAB mass spectra of these compounds, are given in Scheme 1. The ions of particular interest here are those at m/z 86, m/z 184, and m/z 224. In addition to measurement of a complete mass spectrum at each indicated spot on the TLC plate, the plate can be moved while monitoring the abundances of these class-specific ions. By way of example, Figure 1a illustrates the result for an x-scan along the axis of development for the



Scheme 1

Characteristic fragment ions from phospatidylcholines

characteristic ions at m/z 86 and m/z 184, and for the protonated molecule of distearoyl-phosphatidylcholine at m/z 790. The translation stage was moved continuously at a rate of 200 microns per second. Each scan of the mass spectrometer covered a ten dalton mass range window around the mass of the ion of interest, and was complete in 0.5 s. The traces of intensity are those recorded directly by an xy recorder. The location of the distearoyl-phosphatidylcholine is established, as is the presence of a second related compound at another spot on the TLC plate. In this case, the other compound was in fact two overlapping spots from the dipalmitoyl and the dioctanoyl phosphatidylcholines. That there are indeed two overlapping spots is shown in Figure 1b, which traces the intensity of the common ion at m/z 184 along

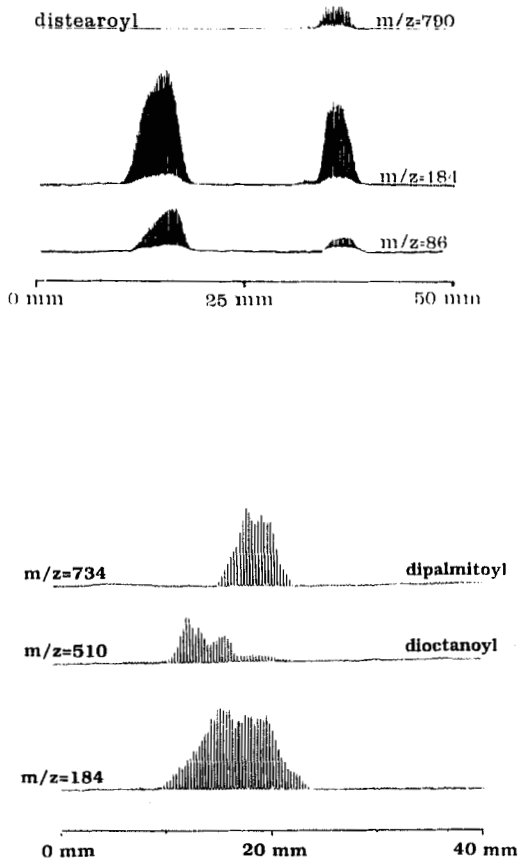


Figure 1. a) Ion intensity traces for a one-dimensional x-scan while monitoring characteristic fragment ions at m/z 86, m/z 184, and the protonated molecule of distearoyl-phosphatidylcholine at m/z 790. b) Expanded scale for the trace of the ion intensity at m/z 184 and the protonated molecules of dipalmitoyl- (m/z 734) and dioctanoyl- (m/z 510) phosphatidylcholine, illustrating the ability of mass spectrometry to identify discrete components in overlapping spots.

with the protonated molecules for each of these two compounds across an expanded x scale. Again, a ten dalton mass range around the mass of the ion of interest was scanned by the mass spectrometer, while moving the translation stages at a continuous rate of 200 microns per minute. The spatially discrete maxima for the traces of the intensity of the protonated molecules are clearly observed, as is the dual maximum in the intensity trace for the ion at m/z 184. When fluorescence or iodine staining was used to visually evaluate this combined spot, the only tenable conclusion was the presence of a single spot with as slight degree of tailing. The specific information available from the evaluation of the spot by mass spectrometry provides the separation of the chromatographically unresolved compounds. The diameters of the spots for these compounds as derived from the scale in Figure 1b are enlarged due to the fact that the diameter of the primary particle footprint on the surface of the chromatogram is a large fraction of the spot diameter itself, leading to an apparent broadening of the signal. No diffusion of the sample components within the sorbitol matrix is occurring, as has been shown explicitly in independent experiments.[13]

The spatially resolved experiment described above is no different in principle from the reconstructed ion profile generated from a set of data recorded in gas chromatography coupled with mass spectrometry. Advantages accrue from the fact that the experiment can be repeated in both the x and the y axes to provide complete spot profiling, and that the mass spectra can be measured after separation has been completed. Extended signal integration times provide higher sensitivity for characterization of low-level samples on the TLC plate.

Bloodroot alkaloids: The benzophenanthridine alkaloids are a diverse collection of natural product alkaloids with significant pharmacological activity. The extract of the plant Sanguinaria canadensis, or bloodroot, contains several benzophenanthridine alkaloids, principally sanguinarine itself. This plant extract has been used in various

medications for over 100 years, and sanguinarine has most recently been used as an ingredient in oral rinses and toothpastes due to its effectiveness in decreasing the extent of plaque formation. Conversely, elevated levels of sanguinarine have been linked to heart disease, glaucoma, and cancer. The chemical separation of the components from an extract of the bloodroot has continually been refined, but is made difficult by the presence of the related alkaloids chelerythrine and protopine. Initial work using paper chromatography and electrophoresis gave inconsistent results.[14,15] Several liquid-chromatographic-based methods have been described, with both normal and reverse phase column operation, and with ultraviolet absorbance as the detection system.[16-18] A pulsed-polarographic analytical method has also been suggested.[19] Apparently, the most consistently useful method is that based on silica gel thin-layer chromatography. Reliable quantitation was achieved only with greater than 10 micrograms of the sanguinarine, and extensive sample processing was required to provide a relatively clean sample for the TLC separation. In beginning this work, we undertook a survey of the literature on bloodroot analysis, and uncovered the discrepancy that a crystal structure study[20] found that the primary component in the bloodroot extract to be oxysanguinarine (molecular weight 348) while a liquid chromatographic separation and analysis[16] determined sanguinarine itself (molecular weight 332) to be the primary component. The purpose of the project described briefly here was to employ a variety of mass spectrometric techniques, including TLC/FAB, high-resolution mass spectrometry, and daughter ion MS/MS experiments, to identify and differentiate some of the components in the bloodroot extract, and to attempt to resolve the uncertainties evident in the literature.

Figure 2 is the expanded mass range portion of the positive ion FAB mass spectrum of an aliquot of the total extract solution in two common FAB solvent matrices, namely 3-nitrobenzyl alcohol (Figure 2a) and thioglycerol (Figure 2b). No other ions other than those attributable to background could be observed in the mass

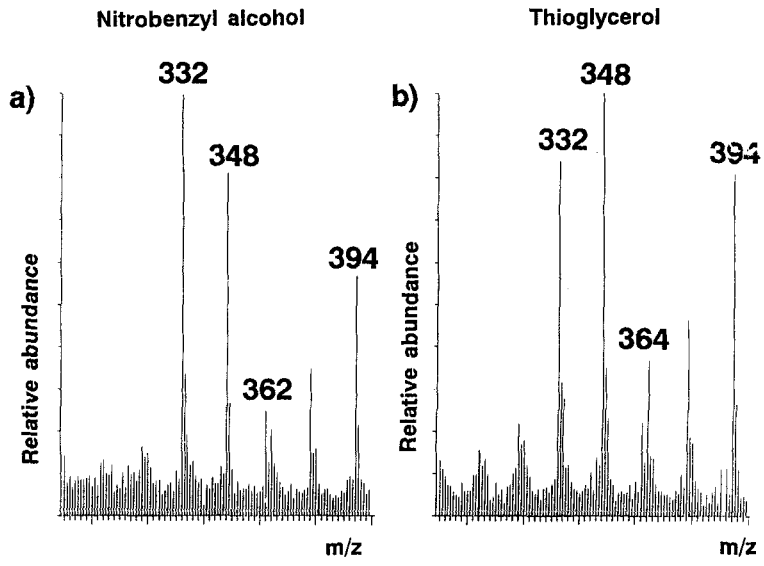


Figure 2. Positive ion FAB mass spectrum illustrating ,olecular ions of the predominant alkaloids extracted from bloodroot. Matrices are a) nitrobenzylalcohol and b) thioglycerol.

spectrum. Other than the correlation between the ions observed, which are all attributed to the molecular ions of the compounds in the sample, an interesting difference is the shift in the relative abundances of the ions at m/z 362 and m/z 364. The ability of various FAB solvents to reduce organic molecules has been documented, and reductions typically involve addition of hydrogens to the sample molecule to form species such as $(M+2H)^+$ or $(M+3H)^+$. Thioglycerol, like the more widely used glycerol solvent, is a reducing matrix whereas nitrobenzylalcohol is much less so. That the benzophenanthridine alkaloids are reducible is shown by the use of the electrochemical reduction for a detection method, as noted previously. The increase in the abundance of the ion at m/z 364 in thioglycerol suggests that the indigenous

species has a molecular weight of 362. Liquid chromatographic analysis of the extract had indeed identified chelirubine as present, and this compound does exhibit a molecular weight of 362 atomic mass units.

The nominal masses of the prominent ions observed in the positive ion FAB mass spectrum of the crude extract indicate the number of alkaloids present in the abstract, since each ion is assumed to correspond to a protonated molecule. For each nominal mass, several alkaloid compounds of isomeric structures may be present, and different isomers have indeed been idenitified in previous work on the alkaloids extracted from the family *Papaveracae* and genus *Sanguinaria* to which the bloodroot plant belongs. To ascertain the identity of the material producing a molecular ion at m/z 332, a thin-layer chromatographic separation of the extract was compared to a concurrent chromatographic separation of a known solution of sanguinarine chloride. The sanguinarine chloride standard produces two distinct spots in its lane of development that exhibited the characteristic yellow-orange coloring characteristic of sanguinarine. The origin of two spots is yet unclear, especially as both of these spots provided a FAB mass spectrum with a base peak of m/z 332, as expected from sanguinarine. Changes in the counter ion may be reflected in the different chromatographic behavior. In any case, both spots are also observed in the TLC separation of the crude extract. FAB mass spectra were recorded for the uppermost spot that had an R_f value corresponding to that of the sanguinarine standard, and this spectrum is compared to that recorded from the spot of the standard itself in Figure 3. The dominance of the ion at m/z 332 is consistent with the suggestion that sanguinarine is a major component of the spot for the extract but the presence of higher mass ions not observed in the mass spectrum of the standard suggests that the separation is still not complete. The daughter ion MS/MS spectra of the parent ion at m/z 332 was measured directly from the extract, and from the standard of sanguinarine. The two daughter ion MS/MS spectra are compared in Figure 3.

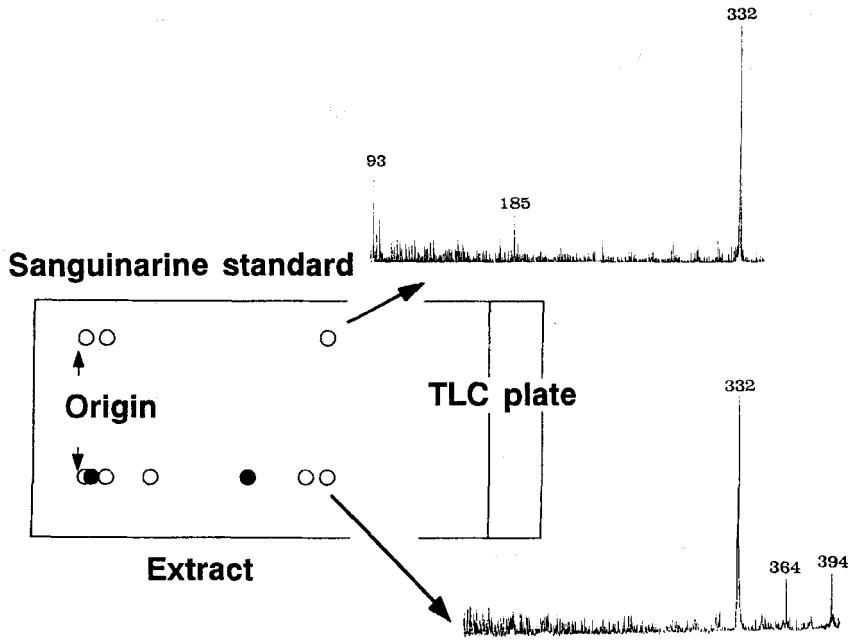


Figure 3. Comparison of the positive ion FAB mass spectra of the spots coresponding to the standard of sanguinarine, and that recorded from the indicated spot on the TLC plate (inset).

These two spectra are virtually identical. The predominant daughter ions in the MS/MS spectrum are m/z 317 (loss of a methyl group), m/z 304 (loss of 28 atomic mass units, presumably carbon monoxide), m/z 274 (loss of 58, best thought of as the elements C_3H_6O, although in what form is unknown), and an ion at m/z 246 (resulting from further loss of 28 from the ion at m/z 274). Significantly, no daughter ion representing loss of sixteen daltons is observed, lending support to the assumption that the ions observed in the mass spectrum of the extract are the protonated molecules of discrete compounds. Finally, an exact mass measurement of the ion at

m/z 332 provided an empirical formula consistent with sanguinarine (calculated 332.0923, measured 332.0918). Based on the R_f value in TLC, the FAB mass spectrum recorded directly from the TLC plate, the identity of the daughter ion MS/MS spectrum, and the consistency of the exact mass measurement, we conclude that the ion at m/z 332 does indeed arise from sanguinarine.

Attention then turned to the identity of the ion at m/z 348. Assuming equal sampling probabilities, the compound at m/z 348 is present in the extract at about the same level as the sanguinarine itself. There are five benzophenanthradine alkaloids with a nominal mass of 348 daltons, and two of these (oxysanguinarine and chelerythrine) have been variously identified in the extract of the bloodroot plant. The TLC separation was not definitive. Two spots provided FAB mass spectra with a base peak of m/z 348. However, even the supposedly pure sanguinarine standard provided a two-spot TLC separation. With no commercial standards of these compounds possible, direct comparisons of the individual R_f values was not possible. Exact mass measurement of the ion at m/z 348 sputtered directly from an aliquot of the unseparated mixture is helpful, however. The calculated exact mass of oxysanguinarine is 348.0867 daltons; the exact mass of the ion from chelerythrine is 348.1231. The exact masses measured for the two ions at a nominal mass of 348 are 348.0884 and 348.1232, corresponding to the calculated masses of oxysanguinarine and chelerythrine respectively. These ion masses were measured directly from the extract, and assuming no sampling discrimination in the FAB analysis, the relative proportion of these two compounds is about 10:1.

Taken as a whole, these data show that the predominant alkaloids present in the bloodroot extract are sanguinarine and oxysanguinarine, in approximately equal amounts, and that chelerythrine is present at about 1/10 the level of these first two components. Further investigations into the composition of this extract is clearly justified to ascertain the origin of the two spots on the TLC plate for the supposedly

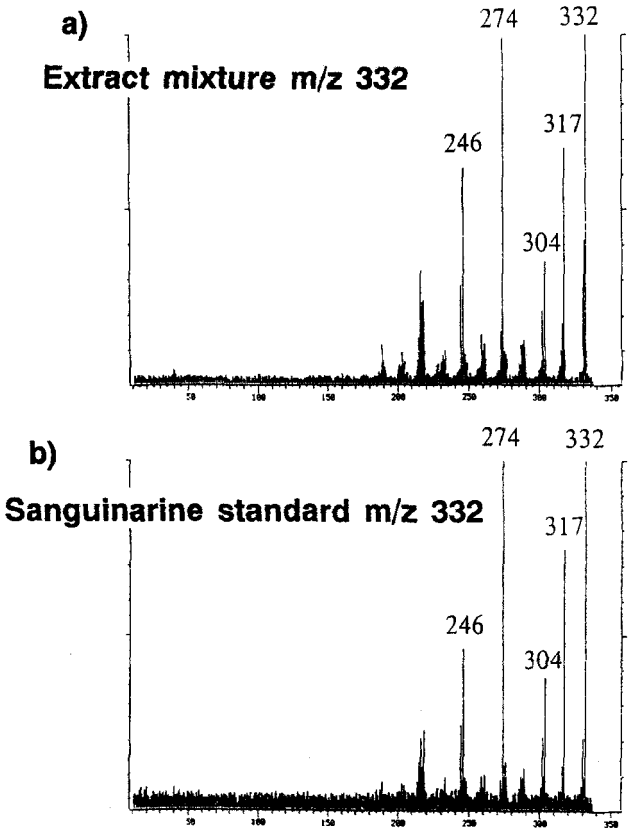


Figure 4. Daughter ion MS/MS spectra of a) the ion at m/z 332 derived directly from the extract of bloodroot, and b) the standard of sanguinarine (parent ion at m/z 332) .

pure standard, and the identity of the higher mass components apparent in the TLC/FAB mass spectrum of the extract sanguinarine spot.

Diuretics: High-performance thin-layer chromatography (HPTLC) is a viable alternative technique to liquid chromatography in the separation of diuretic drugs, and offers several advantages including, in general, a time savings and the ability to perform simultaneous multiple sample analysis.[20]

A common shortcoming with TLC separations, however, lies in the low specificity of the detection methods generally available. Mass spectrometry offers a high degree of specificity, as shown in the above examples, and reiterated here. This section of the paper discusses the use of high performance thin-layer chromatography with fast atom bombardment mass spectrometry, in the separation of six diuretics: hydrochlorothiazide (HCT), hydroflumethiazide (HFM), trichloromethiazide (TCM), chlorthalidone (CTA), amiloride hydrochloride (AMI), and furosemide (FUR).

With the mass spectra of the diuretics measured, and the characteristic ions identified, one- or two-dimensional imaging analysis of the developed thin-layer chromatogram can be easily accomplished by monitoring the protonated (positive ion mode) or deprotonated (negative ion mode) molecular ion of each particular diuretic as a function of x and y directions (or x direction for 1-D imaging). Such an experiment in two dimensions leads to the spectral image given in Figure 5. This image is recorded in the negative ion mode, and, since it had been established that amiloride hydrochloride does not give an intense negative ion signal, its absence is not unexpected. The complementary image in the positive ion mode of analysis provides the appropriate signal for the amiloride hydrochloride at the appropriate R_f coordinates. Of particular interest in this study was the ability of the TLC/FAB experiment to provide quantitative information about the amount of diuretic sample in the spot. Accordingly, we measured the signal response curve for the protonated molecule of amiloride hydrochloride as sputtered directly from a TLC plate. The calibration curve so obtained has a linear (correlation coefficient of 0.997) dynamic range of approximately two orders of magnitude encompassing the range of expected urinary AMI concentrations from about 10 ng to 1000 ng. At the lower limit of detection, the signal-to-noise ratio for the intensity of the protonated molecule versus the level of background signal from the matrix determines the limit. We rely on a signal-to-noise ratio of at least 5. The level of background signal from the matrix is

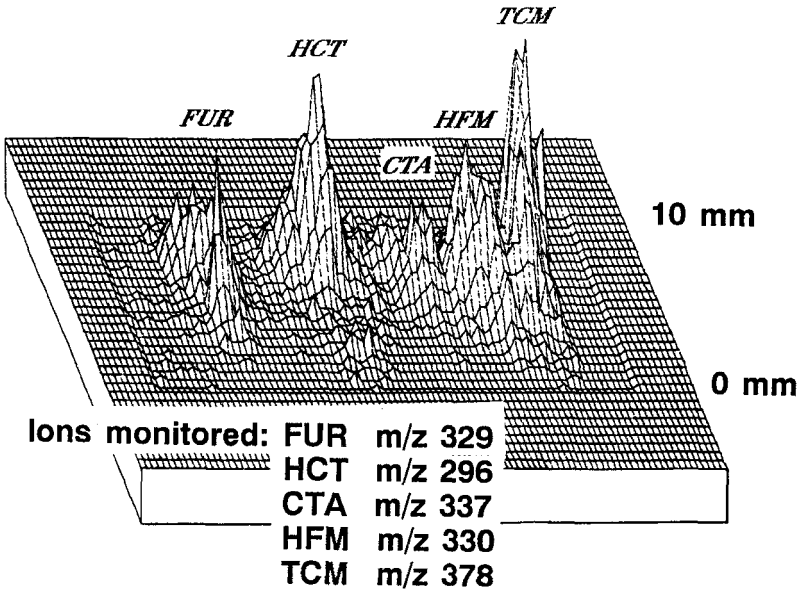


Figure 5. Two-dimensional image of the negative ions generated from a mixture of diuretic drugs separated by thin-layer chromatography. The intensities of the indicated masses were monitored in the appropriate xy regions, and the identity of each spot is given.

determined by the uniformity of the extraction solvent deposition on the surface of the plate (an electrospray method is preferred) and the chemical nature of the solvent (threitol was used in this case). Changes in solvent, and changes in deposition and instrument operating parameters can yield a limit of detection about ten times lower, but with a concomitant decrease in the length of time that the signal can be observed for imaging purposes. At the upper limit of the calibration curve, the signal intensity is observed to bend over as in a Beer's law plot. We attribute this plateau in response versus sample amount to a limit on the efficiency of extraction of the sample

molecule from the chromatographic matrix and efficient transport of the sample molecules to the surface from which sputtering occurs. This upper limit will again vary with sample identity, solvent choice, and instrumental conditions, but several years of experience have shown that the plateau range is generally to be encountered in the 1-50 microgram range for the time scales of our measurement scheme. The calibration curve for amiloride hydrochloride was recorded with data that represent the average of three replicate experiments with a standard error of 3-5% in each data point.

Thin-layer chromatography/laser desorption mass spectrometry

Laser desorption/ionization has been reported as a way of obtaining mass spectra directly from thin-layer chromatography (TLC) plates with no special sample preparation. Hercules and co-workers have used direct laser desorption/ionization with a Nd:YAG laser at 266 nm to detect organic compounds from polyamide TLC plates.[5] Differences in detectabilities for the same compound were noted between sputtering from polyamide TLC plates and zinc foils. Previous work in the coupling of laser desorption mass spectrometry with TLC has used a commercial laser microprobe instrument based on a time-of-flight mass analysis. Our particular interest in this study was to explore the capabilities of a Fourier transform mass spectrometer for TLC/LD. Two attributes of the Fourier transform mass spectrometer catalyze this study. The first is its combination of all of the capabilities that one would expect in a general purpose analytical and organic mass spectrometer, including the ability to record positive and negative ion mass spectra, capabilities for exact mass measurement, and the ability to carry out daughter ion MS/MS experiments. Countering the broad instrumental capabilities are requirements on sample size and pressures that must be obtained within the instrument, although these latter concerns are lessened in the dual cell design of the FT-2000 instrument. The second attribute of Fourier transform mass spectrometry was the recent commercial introduction of the

sample translation stage that has the potential to make the FT mass spectrometer into a microprobe based instrument, with obvious extensions to the direct analysis of planar chromatograms by laser desorption mass spectrometry. We were therefore especially interested in the instrumental and chromatographic factors affecting laser desorption/ionization of biological compounds directly from TLC plates, using the same instrumental configuration in all experiments, but varying TLC substrates, sample preparation, and the laser power and wavelength used. From this data, it is possible to carefully design experiments to increase the detectability of problematic compounds as well as to optimize the analysis of selected analytes. The full analysis of the study is presented elsewhere[8], but a brief summary of the results now in hand is appropriate in this overview.

A general survey of compound detectability was completed using small peptides, bile acids and bile salts, nucleosides and nucleotides, and phethylthiohydantoin-derivatives of amino acids asmodel compounds. Desorption thresholds (laser power required to produce a detectable ion signal in the mass spectrometer) were not explicitly determined from this study. However, it was found that with the same laser wavelength, greater powers were generally required to obtain comparable signal-to-noise ratios from TLC media relative to stainless steel substrates. For instance, bile acids and bile salts were not detected easily from stainless steel using the lower power long-pulse mode of the 1064 nm line of the laser. A mass spectrum for these compounds could only be detected at the highest available powers of the Q-switched mode when contained in TLC substrates. One factor that must be considered in comparing desorption thresholds is the dilution factor of the analyte in the relatively thick TLC matrix as opposed to stainless steel substrates. To obtain equivalent sample fluxes in TLC, a degree of depth profiling is required with the higher laser powers. Further, since surface reflectivity is a determinant of local surface heating, and TLC plates are not highly reflective surfaces, higher power

densities for desorption may compensate for a less efficient energy transfer into the molecule on the surface of the TLC. The factors of laser power density and compound detectability are fully discussed elsewhere.[8]

The influence of laser wavelength is also significant. For instance, bile acids, bile salts, and peptides are not detected at the 5 microgram level under any conditions with the 266 nm Q-switched laser pulses. PTH-amino acids, nucleosides and nucleotides, however, are very easily detected, even from silica gel surfaces. Nucleosides and nucleotides gave detectable signal with the 1064 nm line of the laser from C18-modified TLC surfaces, but use of the 266 nm line gave better results even from the unmodified silica gel surface, otherwise the most difficult of the surfaces from which to desorb compounds. Success with the 266 nm line of the laser correlates with compounds that absorb radiation strongly around 266 nm. Such a correlation is the basis of matrix-assisted laser desorption mass spectrometry.[21,22] More model compound evaluations must be completed to test the breadth of the correlation. In addition, the effect of the fluorescent binders in the TLC plate, if any, must be determined. It may ultimately be possible to modify the TLC surface with a chromatographically inert substance, such as the fluorescent material in the binder, that will facilitate desorption at the wavelengths and power densities accessible in the present instrument.

The nature of the substrate also exerts a significant effect on the ability to measure an LD mass spectrum. One relevant example of such an effect is given here. A careful study was performed to test this effect by soaking to saturation circular pieces of several different types of TLC media. In this case, C8-, C18, and unmodified silica gels were used. By soaking to saturation, concentration effects are removed, and a direct comparison can be made. The model compound chosen was 1-methyl guanosine (1.8 microgram per microliter standard solution); the relative abundances of the ions at m/z 164 (deprotonated base) and m/z 133 (fragment ion

from the deprotonated base) in the negative ion mode for 10 laser shots under identical laser conditions were measured. The probe was rotated to a new position for each laser shot. Shot-to-shot reproducibility is poor (ion abundances vary by up to 25%), but the ability to record the complete mass spectrum for each shot is a tremendous advantage. There is a statistically significant difference between the alkyl-derivatized and silica gel TLC plates. We believe that differences in the binding characteristics of the compound on the TLC media are responsible for the differences observed.

Electrophoresis/mass spectrometry

We have developed in interface device for the combination of planar electrophoresis with mass spectrometry, and demonstrate here the use of this device in the release of biological samples directly from electrophoretic gels. This interface device allows direct coupling of gel electrophoresis in its various forms to mass spectrometry. Here we describe the use of FAB as the ionization method. Electrospray could be as easily used as the ionization method; in fact, this ionization method would be more suited to the molecular weights of compounds normally separated by electrophoresis. The first report of an interface between gel electrophoresis and mass spectrometry was that of Stanley and Busch[23], who reported spatially resolved secondary ion mass spectral data from gels, but with intermediate blotting procedures to transfer sample material from the water-rich gel to a nitrocellulose backing compatible with the vacuum of the mass spectrometer.

Various forms of gel (agarose or polyacrylamide) electrophoresis are utilized in virtually every biochemical laboratory for samples of molecular weights of several thousand to several hundred thousand daltons. The coupling of mass spectrometry with these gel electrophoretic techniques would benefit a great number of researchers searching for a more selective and information-rich detection method. Recently Camilleri et al.[24,25] reported the extraction of samples from polyacrylamide gels, with

subsequent analysis of the discrete samples by FABMS. Sample preparation included an extended extraction of the gel with strongly acidic solvents and crushing of the gels after sample bands were excised out of the gel. It is generally agreed that relatively drastic means are required to release large biological samples from gel matrices in which they are encapsulated. The interface device, described in detail elsewhere,[26] provides for the quick release of samples from within the electrophoretic gel. Transfer solvent is brought to the surface with a supply capillary at a rate of about 5 microliters per minute, and then passed through an online filter into the transfer line to the source of the mass spectrometer. The supply flow is regulated by a syringe pump.. Transfer line flow is regulated by the pressure difference into the vacuum of the mass spectrometer. The interface device is mounted on a vertical travel stand. In this way, individual bands of material separated within the gel can be selected for mass spectrometric analysis. Independent means of sample detection can be correlated into the system so that detection of a sample band triggers the operation of the mass spectral sampling device.

Figure 6 shows the positive ion flow-FAB mass spectrum of coenzyme B_{12}. There was no $(M+H)^+$ ion at m/z 1580 present; however, a significant fragment ion at m/z 1330 is seen. This fragment arises from the loss of the 5'-deoxyadenosyl group from the pseudomolecular ion. An additional loss of the ribofuranyl group yields the ion at m/z 1069. The mass spectrum of the sample from the electrophoresis gel is identical to that of the standard, also shown in Figure 6. The attractiveness of this interface approach is its versatility. The transfer capillary can be interfaced to a variety of ionization techniques, including a flow-FAB probe, an electrospray source, or possibly even a quadrupole ion trap. Also, because release of the sample molecules takes place at atmospheric pressure, many different solvents can be locally introduced onto the gel through the solvent inlet capillary, as needed to release the sample. The interface device described here, and other details of the

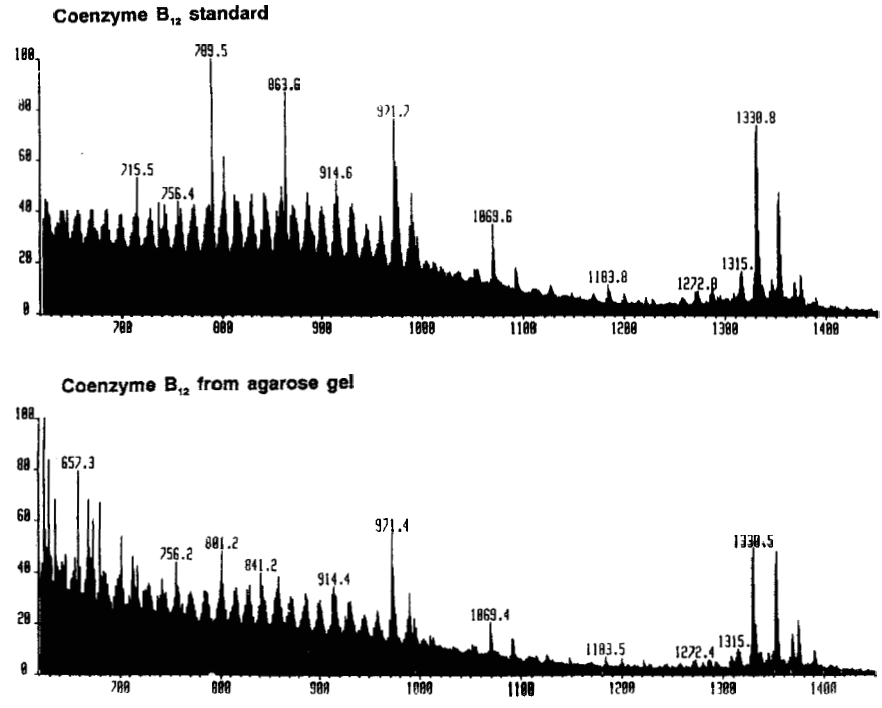


Figure 6. Positive ion continuous flow FAB mass spectrum of coenzyme B_{12} released directly from a planar electrophoretic gel (agarose).

successful coupling of planar electrophoresis with mass spectrometry, are the subject of patent disclosure.

An alternative to the use of an interface probe and transfer of the sample in a short capillary to the source of a mass spectrometer is the direct analysis of the gel material itself. Within the present limits of the instrumentation that we are using, this requires that the majority of the water be removed from the gel. We have completed preliminary experiments with the laser desorption Fourier transform mass spectrometer described above for direct LD of samples in dried agarose gels. A small

portion of the gel containing the sample of interest was affixed to the end of the direct insertion probe and the water removed in the pump out line. A wire mesh over the surface of the gel kept the gel in place, and minimized contamination of the inlet line. The compounds 1-methylguanosine and deoxyadenosine were again used as model compounds since their desorption behaviors under a variety of laser conditions (as outlined above) were known. Laser conditions were ultimately found that allowed the direct desorption of these compounds from the dried gel. Negative ion mass spectra were obtained with the Q-switched laser mode and a 266 nm wavelength. Detection limits were high (on the order of 10 micrograms); however, sporadic results could be obtained at sample levels 10-100 times lower. Our experience has shown that much lower detection limits are attainable if desorption occurs directly from metal surfaces, such as the stainless steel plates usually supplied with the direct insertion probe of the mass spectrometer. Consequently, we are developing transfer (blotting) methods to place components from the dried gel on metal surfaces that can subsequently be studied by laser desorption mass spectrometry.

Conclusions

The difficulties in coupling of planar chromatography to mass spectrometry are no more insurmountable than those encountered in coupling gas chromatography or liquid chromatography to mass spectrometry. In the development of GC/MS and LC/MS, the expanding applications of the separation methods demanded detection systems of higher performance, and the success of those integrated systems is attributed to a balance between separation power and detector specificity. Systems of similar overall capabilities can be expected as capillary zone electrophoresis and supercritical fluid chromatography, and the planar forms of chromatography such as high-performance thin-layer chromatography and planar electrophoresis, are similarly coupled to mass spectrometry. The rate-determining step in these developments is

our inventiveness in devising interfaces, and not in the potential demand for the results that these systems may provide. This overview describes our forays into TLC/FAB mass spectrometry and planar electrophoresis/mass spectrometry. The breadth of interface devices for TLC in general has been recently reviewed[27]; after an initial few years of feasibility studies, and the initial reluctance to adopt new practices, TLC/MS is now in place in dozens of research laboratories, and with commercial availability over the past year, in increasing numbers of applications laboratories as well. We expect the same growth for planar electrophoresis/mass spectrometry, and further expect that this will occur in parallel, rather than in competition, with continued developments in mass spectrometric detection of samples separated by capillary zone electrophoresis and its variants. New initiatives such as the human genome project, and continued pressure on analytical techniques relevant to biotechnology will create a broad base of users that migrate from their extensive experience in planar electrophoresis to adopt the mass spectrometer as a new detector of vastly increased selectivity. One distinct challenge that remains in the coupling of electrophoresis and mass spectrometry is whether mass spectrometry can be made to routinely exhibit the sensitivity needed to identify unknown compounds at the low levels relevant to the problems of this community of users.

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