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Journal of Chromatography A, 692 (1995) 275–290

JOURNAL OF
CHROMATOGRAPHY A

Review

Mass spectrometric detectors for samples separated by planar electrophoresis

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Abstract

Mass spectrometric detection for samples separated by planar chromatography (high-performance thin-layer chromatography and planar electrophoresis) has evolved over the past 10 years from concept to feasibility and to commercial availability. This review concentrates on the interface between planar electrophoresis and mass spectrometry. Although hardware aspects of the interface have been developed and refined over the past few years, and there are impressive demonstrations of feasibility, we are only beginning to exploit new methods in sample storage, preparation and reaction in conjunction with planar chromatographic separation, and new uses of complex multi-dimensional imaging data.

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1. Introduction

Complex mixture analysis is a proving ground for analytical methods that combine high-resolution separation techniques with equally powerful detection systems. Mass spectrometry (MS) has been used as the detection system for gas chromatography (GC) and liquid chromatog-

raphy (LC), and GC-MS and LC-MS are now optimized, ubiquitous tools for complex mixture analysis. Growing needs for separations of biological mixtures have catalyzed advances in high-performance liquid chromatography, capillary electrophoresis, affinity chromatography and planar forms of electrophoresis, including agarose and polyacrylamide gel electrophoresis (PAGE).

In devising the interface between the chromatograph and the mass spectrometer, the instrument designer is concerned with the sample flux available to the mass spectrometer. From this perspective, separation methods can be divided into “sequential-in-time” and “sequential-in-space” techniques.

Column methods (sequential-in-time) such as gas chromatography, liquid chromatography and capillary electrophoresis introduce fractions of the sample mixtures sequentially to the source of the mass spectrometer, and separation resolution is related to the difference in retention times that can be achieved. Coupling to the mass spectrometer can be on-line or off-line, although the on-line combination (despite the limitations placed on the operation of the mass spectrometric detector) is more popular. In planar chromatography (sequential-in-space), sample mixture components are distributed in x and y dimensions. The detector is focused randomly in time to any specified (x, y, z) coordinate for data measurement. Although the combination of planar chromatography with mass spectrometry can be on-line (and mass spectrometry can be used to monitor the progress of the spatial development), the most popular combination of mass spectrometry with planar chromatography has been in an off-line configuration.

Although the analytical attributes of planar chromatography have been ably reviewed [1–3], relatively few have advocated any expanded use beyond the applications areas in which these particular advantages were overwhelming. For thin-layer chromatography, early exposure of most scientists to a “low-tech” and “low-performance” version provided initial impressions at odds with current instrumentally based, higher performance versions of planar chromatography now available. However, planar forms of electrophoresis such as agarose gel electrophoresis and polyacrylamide gel electrophoresis were adopted readily by biochemists with the need to separate complex mixtures of biomolecules, and these methods have evolved into sophisticated separations systems with complex imaging detectors. Insulated within the biochemical community, the planar electrophoretic methods became isolated

from the mainstream of the analytical separations community, which focused instead on column-based methods of separations with great success. A fair proportion of that success was derived from the ease with which mass spectrometric detection could be enlisted to support the need to identify hundreds and sometimes thousands of separated components. Conversely, the lower respect afforded planar chromatography was also a function of the simple detector systems in common use. Although a number of sophisticated activation, derivatization and color development schemes have been developed, often with extraordinary sensitivity and excellent selectivity, the determination of the location of the spot on a planar chromatogram was usually based on visual observation of the spot on the uniform silica gel background. Although derivatization chemistry involved in detection methods evolved rapidly in sophistication, essential aspects of the instrumental spectrophotometric detection did not. Even with the development of multiple-wavelength measurements and instrumental UV-Vis scanners, the amount of analytical information was not substantially increased.

The predominant attraction of mass spectrometry with column chromatographic separations was that the detection was over-determined. This means that there is more information potentially available in the mass spectrum than is generally needed to identify the sample component at a particular retention time. The analyst could therefore be assured that almost any analysis could be successfully completed. If the chromatographic separation was insufficient, then the detector could provide needed independent information.

In planar chromatography, the need for an over-determined detection method is even more cogent. In planar chromatographic separations, the sample molecules are by definition present in a matrix of chromatographic support material, whether the matrix is silica gel in HPTLC or polyacrylamide or agarose gel in electrophoresis. Differential interfaces in GC-MS or LC-MS preferentially transport sample molecules to the source of the mass spectrometer, providing a

substantial sample enrichment. This is not possible in planar chromatography. The detection method must function *in situ* to provide a measurement related to the sample independently of the matrix. Spectrophotometric methods can accomplish this discrimination with relative ease, but do not provide enough information to identify the sample unambiguously. Mass spectrometry provides specific analytical information necessary to identify the compound, but must do so with the constraint that the sample and matrix contribute to the observed mass spectrum.

Some early work in planar electrophoresis (PE)–MS used procedures in which the individual sample bands were removed and the sample solution derived from those bands examined individually. In considering PE–MS, it is more appropriate to review the analytical aspects of planar chromatography. In planar chromatography, compounds are separated by their differential migration along the development axis or axes. The time domain in which the mass spectrometer must operate is independent of chromatographic development. Rather than have each component available in the mass spectrometer source only within the elution window, as in separation-in-time, a planar chromatographic detector can integrate the signal for any sample spot for as long as necessary. Analogously, shorter data acquisition intervals can be assigned to higher level components, or components of lesser interest. Samples cannot be “lost” in a planar chromatogram. Components of a mixture that will not separate under the chosen conditions are lost on the column in GC or LC, but can be easily retrieved from the point of initial sample deposition in planar chromatography, and may be eluted and detected using other analytical methods. Alternatively, the system can be viewed as a chromatographic clean-up of the non-partitioning material in which none of the matrix is discarded. As the chromatographic development time is not a factor in detector operation, any component in the chromatogram can be investigated in any order. This independence of access order is an advantage in complex mixture analysis when targeted components are sought, or when a priority ranking to

the compounds present can be assigned. A related advantage is the fact that the chromatogram can be rescanned many times in order to establish the required level of analytical accuracy in spatial profile and sample identification. Finally, the data measured in an off-line detection system for planar chromatography can not only be used to identify the components separated within the chromatogram, but can also be interpreted to provide on-line control for measurement of additional data, as in TLC–MS [4,5].

As PE–MS develops, its conceptual origin in TLC–MS becomes more clear. Many of the hardware approaches are similar. Yet there are some differences that are emphasized in the coupling. An agarose or a PAGE gel contains a great deal of water that must be (1) removed before the gel itself can be placed inside the vacuum system for sampling or (2) the sample molecules must be removed or transferred from the gel matrix. Both of these methods have been pursued in PE–MS, although the latter is preferred. The biomolecules typically separated in planar electrophoresis are high-mass molecules, and will require use of a mass spectrometer that can operate in the appropriate mass range. These biomolecules may also require special handling to preserve their integrity or higher order structures. Finally, the complexity of the mixtures separated by two-dimensional planar electrophoretic methods may be high, exceeding even the hundreds of components separated in a capillary GC–MS run. The sample preparation and handling and the data collection and handling systems must be adequate for this task.

2. Experimental

Experiments in PE–MS and TLC–MS have used several different instruments [6–10] described briefly here; this paper reviews concepts and does not repeat previously published results. In brief, gel disruption methods used mechanical or ultrasonic homogenizers, and continuous-flow fast atom bombardment (FAB) with a VG-70SEQ double-focusing hybrid mass spectrometer. For matrix-assisted laser desorption ioniza-

tion (MALDI) mass spectra recorded with a Fourier transform (FT) mass spectrometer, a Nicolet FTMS-2000 instrument interfaced with a Quanta-Ray DCR-11 Nd:YAG laser was used [11]. Using the 266-nm line (frequency-quadrupled), the laser can fire only in the *Q*-switched mode (10-ns pulse width), and energies range from 2 to 20 mJ per pulse. With the 355-nm line (frequency-tripled) in the *Q*-switched mode, laser energies range from 20 to 60 mJ per pulse. In all instances, the laser beam diameter at the substrate surface was approximately 0.2 mm. For MS–MS experiments with the FT-MS, collision-induced dissociation (CID) was accomplished by isolating an ion of interest and then accelerating that ion into argon collision gas, which was maintained at a static pressure of approximately 3×10^{-6} Torr. Translational collision energies were in the range 50–100 eV (laboratory). For MALDI experiments with a time-of-flight (TOF) mass spectrometer, mass spectra were acquired on a custom-built instrument that can be operated in both the linear and reflected ion modes [12]. The TOF mass spectrometer consists of a 1.4-m long drift tube that houses a custom-built ion source and a Chevron microchannel plate detector. A Laser Science VSL-337ND nitrogen laser provides 3-ns pulses of 337-nm light, which are steered and focused so that the 3×8 mm beam is reduced to approximately 0.126 mm^2 in area. Power densities of 10 MW/cm^2 are typically used for MALDI mass spectra. Typically, the ion output from 20–30 laser shots are summed using an oscilloscope, and then transferred to a computer for further data analysis. Instruments used in other researchers' work are usually commercial instruments described in the appropriate references.

3. Evaluation of results

The coupling of chromatography with mass spectrometry is driven by the ability of an integrated system to provide more information than is necessary to solve most analytical problems. Ideally, one measures only enough information (with the minimum necessary resolu-

tion, the maximum possible speed, the highest possible sensitivity, the lowest possible cost and with accuracy, precision and reliability) to solve the problem at hand. However, the ideal problem answered with the ideal solution process is an imaginary construct. We accept less than exemplary separation and less than exemplary detection only because we use the synergistic nature of the combined information to derive a solution. Specifically, in PE–MS, the information that we want is sample band location, sample band homogeneity and sample mass. Further, we often know a great deal about the sample to begin with. We use the mass information from the mass spectrum to support our initial suspicions, and we can use sample reactions and derivatizations to provide further circumstantial evidence for sample identity. We shall therefore concentrate on the various aspects of the PE–MS coupling, following the order (1) chromatographic and spectroscopic resolution, (2) sample preparation, (3) sample reactions and derivatizations and (4) data handling.

3.1. Chromatographic resolution

How can mass spectrometry be used to increase the "resolution" of the chromatography? Can we use the information present in the mass spectrum to discern the presence of unresolved spots in a chromatographic spot or band? As in TLC–MS, use of the mass-resolved information in PE–MS allows unresolved spots on a planar electropherogram to be identified. The practice of using mass spectral data to establish the homogeneity of a sample band in PE–MS is shown in Fig. 1, redrawn from the data of Fenselau and Vestling [13]. The right-hand side shows the more general expectation that ions from samples in spot A and spot B will trace different *xy* profiles. The MALDI data shows a simple measurement of the molecular ions of two different proteins in a single electrophoretic band, showing the presence of unresolved sample components. In general, the presence of *n* different components is shown by the presence of *n* separate molecular ion masses. Fragment ions of common masses or background ions

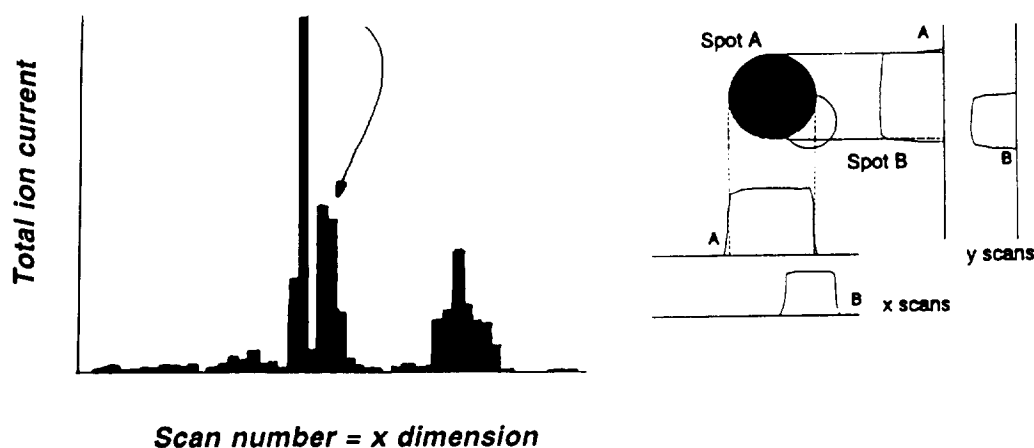


Fig. 1. Expected variations in mass-selected ions from samples A and B with x and y dimensions are shown on the right. The left portion shows PE-MS data with MALDI ionization (adapted from Ref. [13]); total ion current is plotted against scan number in the x -dimension. The presence of two concurrent masses (indicated by arrow; M_r 10 026 and 11 001), representing two different molecular species, at a given band signifies unresolved components.

cannot be used to deconvolute overlapped spots. The same conceptual experiment is carried out in GC-MS and LC-MS when time-resolved traces of mass-selected ions are reconstructed from the full data set measured. This deconvolution helps to identify all of the ions that belong together in the mass spectrum of a single component (they should exhibit identical time traces) and helps to emphasize unresolved peaks or background ions (distinctive and different time behaviors). In planar chromatography, the conceptual basis of the experiment is exactly the same, although the time base is replaced by a linear spatial dimension. Two such spatial dimensions (x and y) can be used independently to increase the effective resolution of the chromatographic separation and to establish sample-band homogeneity. The PE-MS data shown consist only of data measured along one linear dimension.

In PE-MS, the spatial dimensions of the area from which ions are generated should be much smaller than the size of the sample spot or the sample band. In particle-induced desorption ionization [FAB and liquid secondary ion mass spectrometry (LSIMS)], which has been used in TLC-MS, the diameter of the probe beam is limited to a diameter of 0.1–1 mm. The larger size is appropriately used for sputtering sample molecules from single excised spots, while the

smaller size is used for imaging across sample spots with xy dimensions of 1 mm or more. With microprobe mass spectrometers, image fields of 100–200 μm have been used to examine samples in TLC spots [14,15]. In PE-MS, sample bands are of approximately the same size, and similar FAB and LSIMS ion sources can be used. For MALDI (see below), the diameter of the laser beam should also be about 0.1–0.3 mm, and this spot size is readily achieved with commercial MALDI instruments.

3.2. Spectroscopic resolution

What about mass resolution in the mass spectrometer used for PE-MS? Mass resolution is closely related to the accessible mass range of the instrument, and we shall consider these issues together. The sieving properties of an electrophoretic gel provide separation (and some crude molecular mass information) for sample molecules with molecular masses from 5000 to over 200 000. The mass spectrometric detector must be able to deal with molecules within this mass range. For singly charged ions, the TOF mass spectrometer is the sole viable instrumental option. We therefore briefly review the capabilities of this spectrometer with respect to resolution and mass range. In a TOF mass

spectrometer, ions should be created at the same time, in the same place and with the same amount of kinetic energy. A pulsed ionization source such as a laser is conventionally used. The ions are extracted from the source and sent down the flight tube of the instrument. Ions of different mass but with the same kinetic energy will travel at different velocities, and the time of their arrival at the detector at the end of the flight tube will correlate with their masses. Although very high-mass ions will be traveling slowly, they will eventually reach the detector. Therefore, if a detector signal can be generated that heralds their arrival, even very high-mass ions can be analyzed in a TOF mass spectrometer. The mass resolution is limited by the ideality of the initial constraints of ion formation at the same time, the same place and with the same energy. In general, the mass resolution of the TOF mass spectrometer is relatively low (under 1000) and the mass peaks are very wide.

We consider if the relatively low mass resolution of a TOF instrument is an impediment in biomolecular analysis. For higher mass biomolecules, such as proteins, the ability to determine molecular mass within even 10–15 u is sufficient to establish the congruence between the expected mass and the observed mass, or to determine a mass difference from the expected mass that corresponds to any of the common biotransformations such as acetylation or phosphorylation. As the MALDI ionization process [16,17] produces molecular ions almost exclusively, an ion in the high-mass end of the mass spectrum is almost certainly a molecular ion. Further, if there are two signals present, there are most likely two compounds present in the interrogated band (and then we know that the spot is not homogeneous, as described above), as the lower mass ion is seldom a fragment of the higher mass ion. Proteins often analyzed by MALDI TOF dissociate primarily by loss of complete amino acid residues, and the mass shifts that this loss encumbers is easily discerned by the TOF mass analyzer. For most situations, the relatively low resolution of the TOF mass analysis is not debilitating.

Higher mass resolution can be achieved if

another method of mass analysis is used. The FT ion cyclotron resonance mass spectrometer can measure the masses of stored ions with extraordinary accuracy. Dunphy [11] has described the coupling of MALDI with FT-MS in the analysis of samples in planar electropherograms. Moderate mass resolutions of 5000–10 000 were achieved in these analyses, but the masses that could be stored in the cell were restricted to below 2000 u because of the strength of the magnet in the instrument. Several workers have described the use of ion re-measurement techniques that make it possible to store mass-selected ions for extended periods of time, generating mass resolutions of several hundred thousand [18–20]. The ability to perform this experiment with ions generated from sample molecules separated in a planar chromatogram is particularly attractive as the separation is complete, and either a few or many of the ions can be so examined at the leisure of the investigator. The ability to store ions of very high mass is problematic at this point. For a singly charged ion, the limit on the magnetic field strength of even a 7 T superconducting magnet restricts the mass of the ion that can be stored in a cell of reasonable dimensions to perhaps 10 000 u (this will vary from instrument to instrument). The future contribution of FT-MS will be in its ability to provide accurate mass measurements rather than extremely high mass ranges, and in the ability to pursue multiple stages of collision-induced dissociation for mass-selected parent ions. Exact mass differences for ions of high masses, if they can be determined with sufficient accuracy, can unambiguously indicate the nature of the loss, and therefore provide specific structural information. The exact mass measurement is not used to derive the empirical formula of an ion. Rather, the exact mass difference between two high-mass ions is used to define the atomic composition of the group involved in the dissociation or fragmentation. As large-mass biomolecules such as proteins often dissociate in regular patterns involving sequential losses of single amino acid residues, the exact mass difference resolution required is actually fairly modest.

If electrospray ionization [21,22] is used, multiply charged ions (usually $[M + nH]^{n+}$) can be generated, and the effective mass range of the analyzer is increased proportionately by a factor of n . PE-MS using electrospray ionization has been reported [23], and with multiple reports of electrospray ionization coupled with FT-MS, PE-MS with electrospray will be a popular avenue for near future applications (see the next section).

3.3. Sample preparation

There are two aspects of the preparation of the sample considered. The first is the manipulation of the electropherogram itself into a form compatible with the mass spectrometer's requirements, and the second is the incorporation of the matrix that allows the ionization process to succeed. A distinction is drawn here between methods that attempt to analyze the gel in x or xy dimensions, and those that elute the sample components from the gel and then present a discrete sample solution to the mass spectrometer for analysis. There are ample examples of the latter sort [24–30], including the work of Camilleri et al. and other early workers. More recent work that also involves the preparation of discrete sample solutions includes the investigation [11] of the "freeze-squeeze" method [31,32], originally described for the rapid recovery of long DNA strands from agarose gels, gel disruption and homogenization methods [33,34] used in conjunction with flow-FAB ionization.

A water-rich gel can be placed under vacuum, but as the gel shrinks with dehydration, the spatial separation of sample components is adversely affected, and analysis of whatever is left is unlikely to provide interpretable mass spectra. The very first attempts at PE-MS [35] used plasma desorption ionization (very fast and very heavy ions derived from a tandem accelerator, or present as fission fragments of a radioactive nuclide, are used to sputter molecules and ions from a thin sample surface) to determine mass spectra for protein mixtures separated by gel electrophoresis and then transferred to a nitrocellulose membrane with an electroblotting pro-

cedure. The use of nitrocellulose membranes and electroblotting procedures is well known within the bioanalytical community. The next foray into PE-MS used secondary ion mass spectrometry (SIMS) to analyze samples separated in paper and cellulose acetate electropherograms, and in these instances no transfer or blotting procedure was necessary [36]. With agarose gel electrophoresis and PAGE, the standard blotting procedures using capillary blots, vacuum blots and electroblots were further developed [37,38]. Within the limits of the SIMS ionization and the mass range of the quadrupole mass filter (below 2000 u), the experiment was successful in first demonstrating the spatial fidelity of the transferred material, the two-dimensional imaging capabilities of the PE-MS combination and the use of mass spectral information to deconvolute overlapped compounds in a single electrophoretic band. Additional work studied various quantitative aspects of sample sputtering from paper and cellulose acetate electrophoretic membranes, and the use of digestion reactions of larger peptides on the transfer membrane [39].

Limitations of Southern and Western blots for PE-MS soon became apparent, and extensive experience suggested that electroblots were preferred for reasons of transfer efficiency and preservation of spatial fidelity. Reports of the electroblotting technique in PE-MS appeared almost simultaneously from a number of research groups [11,40–44]. The electroblot procedure is still being developed for PE-MS [45], now with plasma desorption ionization and samples in the mass range up to 14 000 u. Experience suggests that the transfer efficiency with electroblotting techniques is in the range 60–90%, and that the spatial fidelity can be preserved for scanning and imaging experiments. Heating of the gel during transfer must be carefully monitored, but standard electrotransfer cells control this potential problem. PE-MS routinely uses electrotransfer methods, in contrast to the dot blot and capillary blot methods developed earlier [39,46]. Fig. 2A shows the general method used in small gel electrotransfers, illustrating the spatial fidelity that is maintained in the xy plane.

The nature of the membrane to which the

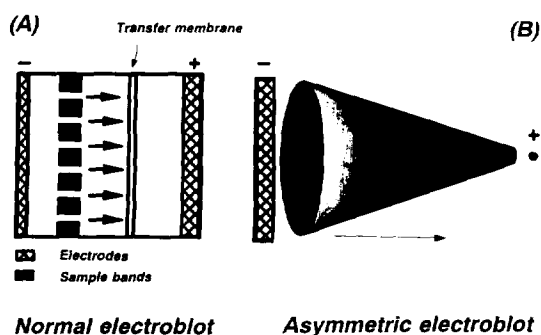


Fig. 2. (A) Normal electroblot procedure. (B) Asymmetric electroblot used to generate a variation in matrix-to-sample ratio along the dimension indicated by the arrow.

sample is transferred is important. Early work used nitrocellulose membranes, following standard biochemical protocols, and the early observation that ion signals for high-mass biomolecules from such surfaces were of greater intensity than other surfaces such as metals. Recently, a number of other membrane surfaces have been used in PE-MS, including nylon and poly(vinylidene difluoride) (PVDF) [47–50]. Although these materials are new to mass spectrometrists using MALDI, these and a host of other materials are well known in biological applications. The membrane or modified membrane material must be able to blot the sample compound, and it must also be compatible with the matrix molecules used in MALDI. The matrix-to-sample ratio may often be 1000:1 to 10 000:1. Methods for application of the MALDI matrix, an integral part of sample preparation, are considered next.

Selection of the appropriate MALDI matrix is discussed in detail elsewhere [51–54], and only a brief summary is provided here. There is more than sufficient energy in the incident laser beam to degrade a biological molecule. In simple terms, the role of the matrix is to act as an energy buffer, absorbing most of the energy from the laser beam and converting it into an appropriate mix of thermal energy that leads to vaporization of the matrix and encapsulated sample molecules, and electronic or photo-excitation that leads to ionization of the sample molecules. Useful matrix materials therefore

exhibit a high molar absorptivity at the wavelength chosen, an intermediate volatility and photoexcitation or charge-transfer properties in a useful energy range. In addition, as discussed in detail in the original literature in this area, the matrix and the sample molecules should co-crystallize on the surface so that an efficient energy coupling can occur. Empirically, researchers find matrices that work and continue to use them while searching for others that may work better. Predictions based on a detailed understanding of the mechanisms in the laser-energized micro-volume are beyond our current understanding of the process, echoing a situation that developed in FAB-MS some 15 years ago.

Of relevance to PE-MS and the transfer of materials to a blotting membrane is the question of when and how the matrix is to be applied to the membrane. Strupat et al. [50] have recently completed a study of the mass spectra that result from different methods for application of the MALDI matrix, including whether the matrix solution is applied to a dried or a partially dry membrane. They also studied the use of membranes of different types, including five PVDF membranes differing in pore sizes and surface areas. Superior results in terms of widths of the signals measured are found for PVDF membranes with higher specific areas, and better signal intensity is found when the matrix solution is applied to a membrane that is only partially dried. In each case, the solution in which the matrix compound is dissolved must be able to wet the membrane and distribute the matrix so that enhancing crystallization can occur. Scanning electron micrographs of membranes so prepared emphasize the importance of homogeneous, ultra-fine crystal formation. Of course, it is not possible to examine each membrane individually before mass spectrometric analysis, and a standard protocol is established and then followed with expectations of success. As a number of other membrane materials are investigated for use in MALDI [17,55], it can be expected that they will be used for PE-MS applications also.

Some of the vagaries of this procedure can be removed if the transfer medium is designed

ahead of time to enhance the desorption. Hutchens and Yip [56] have described surfaces in which energy-absorber molecules are “tethered” to the surface, leading to an enhancement in the laser-induced desorption of subsequently adsorbed sample molecules. In addition, these surfaces can also be used as part of the sample clean-up itself, using well known methods of affinity chromatography. These developments are adaptations of the practice of using modified adsorbent materials in thin-layer chromatography [57,58]. In almost all instances, the high level of background signal at lower masses in MALDI mass spectra (derived primarily from the matrix itself and its degradation products) can be limiting when use is made of reactions that generate fragment or product ions that lie within the same mass region. Changes in the matrix-to-sample ratios may make this problem more tractable, and MS–MS can potentially isolate the collision-induced dissociations of a mass-selected parent ion from such background noise in the mass spectrum.

In practice, the mass spectrum is modified most simply by changing the matrix-to-sample ratio, and this has led to the proposal for transfer described here. In the usual procedures, the sample molecules are transferred to the membrane, and then a matrix solution is syringed on to the surface of the membrane. If the initial selection of matrix-to-sample molecule ratio is unsatisfactory, then the sample membrane can be withdrawn from the mass spectrometer and additional matrix can be applied prior to a second analysis. Matrix molecules can be placed ahead of time in the transfer membrane material at a known and homogeneous concentration. With the matrix already present, the problem of finding the appropriate matrix-to-sample ratio can be simplified by use of an asymmetric electroblot procedure, as illustrated in Fig. 2B. As before, the concept for this transfer has its origins in earlier work on transfer procedures developed for TLC–MS. The band shown on the left in Fig. 2B is induced to move under the influence of a potential gradient established between a planar or curved electrode and a point electrode, as shown. The potential gradient

causes a movement of the charged sample molecules towards the point electrode in an asymmetric fashion, and clearly there has to be a dimensional asymmetry in the physical set-up also. The transfer generates an analogous concentration gradient. The darker shading shown represents a higher sample concentration nearer the point electrode achieved at some point during the transfer. Note that transfer is not carried through to completion. If the matrix is homogeneously distributed in the transfer medium, then the matrix-to-sample ratio decreases along the linear path to the point electrode. As the matrix-to-sample ratio is critical in generating a high-quality MALDI mass spectrum, the ability to acquire rapidly a series of mass spectra (at different matrix-to-sample ratios) with focusing of the incident laser beam at different points along that gradation line can be advantageous. The range of matrix-to-sample ratios that can be accommodated depends both on the time allocated for the transfer and the dimensions and spacing of the electrodes, but a factor of 10–100 should be readily achieved.

Use of the matrix in MALDI allows the mass spectra of high mass biomolecules to be recorded, but the addition of the matrix is not without complicating consequences. In particular, the lower mass region of the mass spectrum is congested, filled with ions arising from the matrix molecules, and from degradation of the matrix molecules on interaction with the incident laser beam. Fig. 3 illustrates this characteristic for the positive-ion MALDI mass spectrum of cytochrome *c* recorded from a matrix of α -cyano-4-hydroxycinnamic acid. The high-mass region of the mass spectrum is unobscured, containing singly, double and triple charged forms of the molecular ion for this sample. The region below m/z 1000 is uninterpretable; the range of mass obscured varies with the matrix and with the conditions of laser ionization. The consequence is that spectral interpretation must occur exclusively in the high-mass range, and that derivatizations or sample reactions (see the next section) should preferably lead to higher mass product ions. An alternative approach is to isolate the ion of interest in an MS–MS experi-

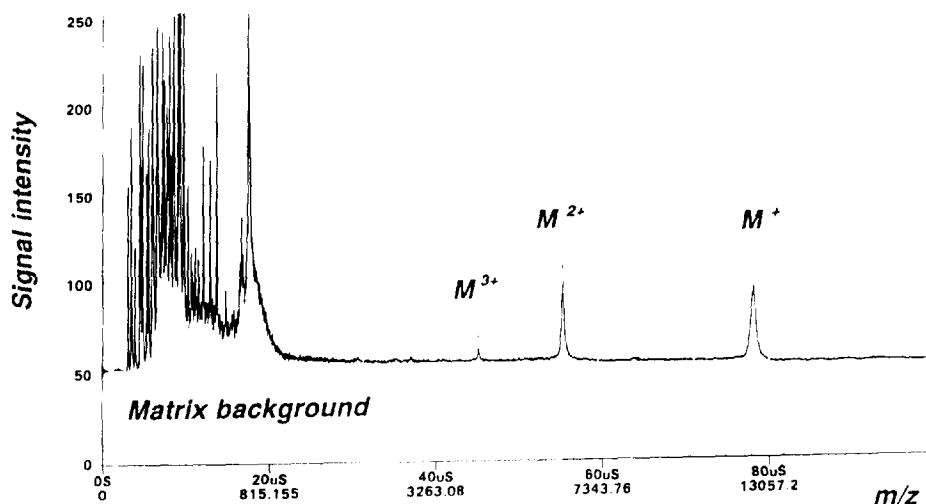


Fig. 3. Positive-ion MALDI mass spectrum of cytochrome *c* showing the spectral background generated from the use of the energy-absorbing matrix (cinnamic acid).

ment, and then to create lower mass ions in collision-induced dissociations or surface-induced dissociations. The FT-MS instrument described earlier is easily used for these MS–MS experiments.

The emphasis so far has been on MALDI. The earliest work on PE–MS used FAB and LSIMS, and the choice of solvent matrix was not as critical. The solvent did have to dissolve the sample from the transfer membrane, or from the electrophoretic gel itself, but most of the standard solvent matrices used in FAB and LSIMS were adequate. For the plasma desorption ionization work reported, the passage of the fast incident particle through the sample solution placed on a nitrocellulose support often provided an interpretable signal. As the direction of the incoming beam was not specified in these experiments, the scanning and imaging experiments could not be completed except through excision of the sample band and examination of discrete individual samples.

3.4. Sample reactions and derivatization

The heritage of TLC–MS in the development of PE–MS is seen again in the use of surface derivatization reactions [59] that can be used to

enhance ion signals, to highlight the presence of certain structural features within the target molecule or to degrade the sample molecule controllably, as in the digestion of larger proteins into smaller peptide fragments. A number of functional-group-specific reactions have been developed for use in mass spectrometry; many of the reactions used in FAB and electrospray also work [60] in MALDI. Derivatization labels used make known and predictable changes in the mass spectrum and, in fact, also within the MS–MS spectrum. The expected mass changes are generally below 100 u per reaction. If several sites react, then the expected mass change is multiplied accordingly. An exemplary reaction is that of the amino groups in peptides with 2-iminothiolane [61]. The mass shift is easily discernible with even lower resolution TOF mass analyzers. Cohen and Chait [62] have described a clever application of this derivatization reaction that documents the accessibility of lysine residues in a globular protein to derivatization. Reagents that introduce small, recognizable cleavages can also be useful in establishing the presence or absence of certain functional groups in sample molecules held at the surface of a planar chromatogram. A match between an expected and an observed mass shift is a necessary but not a sufficient condition for establishing structure. An exact

mass match between the expected and the observed change is, in contrast, a very specific indication for the proposed structural feature.

A number of reactions are already in place that can be used as probes of biomolecular structure, including enzymatic and non-enzymatic protein digestion reactions, sequencing chemistry such as the Edman reaction and various oxidation and reduction reactions, especially those that involve the sulfur-containing amino acids [63]. Of special interest to PE-MS is the fact that these reactions can be carried out sequentially, and under conditions independent of the chromatographic separation itself. The gel or the transfer membrane can be removed from the mass spectrometer and incubated with a digestion enzyme; the products can then be analyzed by mass spectrometry [13,47]. Cytochrome *c* supported on a PVDF membrane was incubated with trypsin [47] to yield a distribution of tryptic peptides that can be used to map the primary sequence. A much wider variety of such derivatization/digestion reactions can be expected to be exploited in the near future with growing research in PE-MS. Of particular interest will be the ability to analyze a sample held on

a membrane, then expose it to a specific substrate or co-factor and subsequently to identify differences in the mass spectrum that correspond to expected interactions.

The repetitive withdrawal and reinsertion of sample membranes into the source of the mass spectrometer is not an elegant way of completing these surface reaction experiments. A surface tracking probe has been devised for use in TLC-MS [64,65]. Derivatization reagents are delivered to the surface of the planar chromatogram in a flow-injection mode, and real-time continuous analysis of the surface by mass spectrometry provides direct spectral evidence for the new products expected of the derivatization. Also demonstrated, and of particular importance in PE-MS of complex biomolecules, is the ability to follow reactions that are reversible either with time or with the addition of a second reagent. The conceptual design for this surface capillary is shown in Fig. 4.

Although preliminary results show that normal staining procedures are not compatible with mass spectrometric detection, staining with colloidal gold particles can be used, although the quality of the measured mass spectrum suffers, and the

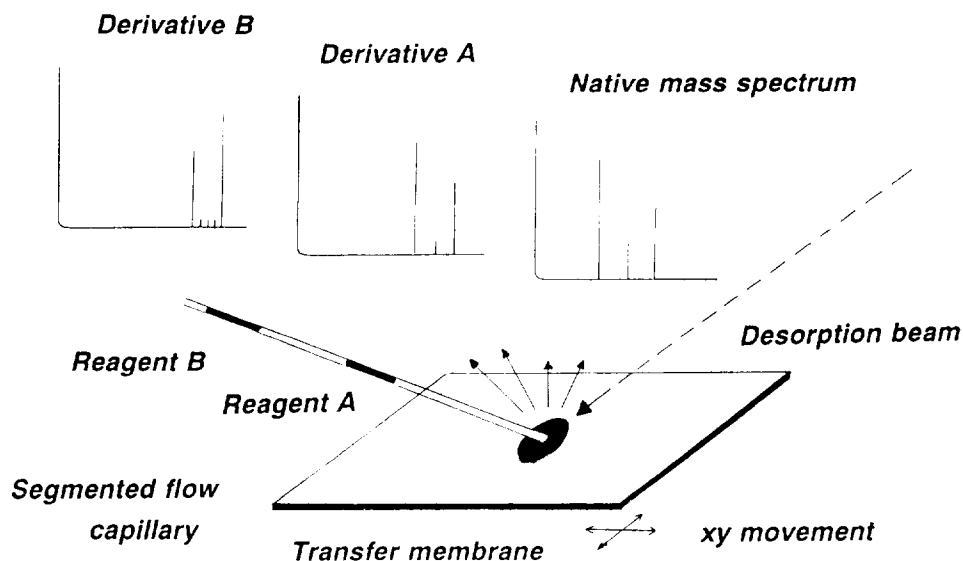


Fig. 4. A segmented flow capillary can bring reagents to the surface to a transfer membrane for in situ derivatization/digestion reactions monitored in real time as the sample is analyzed. The native mass spectrum should change to the spectrum of derivative A, and then to the mass spectrum of derivative B, with the mass differences reflecting the expected structural changes.

potential exists for the formation of adduct ions between the dye and the sample molecules [50]. The use of mass spectrometry may help to provide insight into the interaction between such dyes and biological sample molecules on the membrane.

3.5. Data handling

Most modern data systems deal easily with data sets modelled in three dimensions. GC–MS data sets contain m/z information and relative intensity information as a function of time or scan number, and clever graphical displays make it straightforward to discern information. One-dimensional mass spectral scanning in planar chromatography similarly leads to three-dimensional data sets in which the time dimension is replaced by a linear dimension of sample movement. Data manipulation and display are accomplished with the same processing routines already in widespread use. True imaging requires treatment of data in four dimensions, viz., m/z , relative intensity and x and y dimensions. Ion microprobes and ion microscopes are integrated with data handling systems that deal with this higher level of data. Concerns of additional storage capability within the data system and multi-dimensional data displays have already been satisfactorily addressed. To catalyze the development of commercial PE–MS systems, it only remains for the manufacturers to import this already developed technology base into their present data systems.

There is a larger problem at hand than the higher order or sheer bulk of the data generated in PE–MS. In off-line PE–MS, the separation is completed before detection of the bands is pursued. Mass spectrometry is only one of a number of methods that can be used for spot location and sample band detection. The list of analytical methods that can be used includes visual analysis, UV–Vis spectrophotometry, spectrofluorimetry, optical microscopy techniques, electron microscopy techniques, electron spectroscopy for chemical analysis (ESCA), Auger spectroscopy, reflectance infrared spectroscopy, radioimaging methods, near-infrared analyses and mass spectrometry in several forms,

including secondary ion mass spectrometry, fast atom bombardment, and laser desorption ionization. Coordinated sample positioning and manipulation are central to each of these methods. Detection of sample bands in planar electrophoresis has traditionally been accomplished with optical detectors. Detection schemes range in complexity from simple visible staining procedures and hand-held ultraviolet lamps to sophisticated scanning densitometers and image analysis systems. These optical methods have proved themselves reliable and indispensable, with typical detection limits in the low nanogram range. New optical technologies, including solid-state multi-channel optical devices and charge-coupled detectors, provide even higher performance. In PE–MS, the goal is to afford priority to the sample, and to bring a number of independent analytical methods to the problem. As optical detection methods are among the most sensitive, and are non-destructive, it is logical to begin with such methods, and then to use the mass spectrometry when sample identity must be confirmed with more specific information. There is a substantial increase in the certainty of detection as more independent analytical methods are used. However, the optical image can also be used to guide the mass spectrometric analysis. In discrete analyses, this is already the case; the sample spots are independently located by color development or quenching, and that particular sample area is examined with mass spectrometry. More sophisticated control and analysis are possible when the optical detector and the mass spectrometric detector operate in unison. This combination has been demonstrated in TLC–MS [15] and application to PE–MS will certainly occur in the near future.

A global coordinate system describes the distribution of sample molecules in the xy plane of chromatographic separation, and may also expand to include the z -dimension of depth as well. As detection methods for planar electrophoresis evolve into integrated systems that include optical spectroscopy in addition to mass spectrometry, several aspects of the global coordinate system will increase in importance. The first is the ability to calibrate in space across multiple separations as well as we can presently calibrate

in time. With automated injection systems and very reproducible flow and temperature profiles, a retention time reproducibility of less than 1% can be readily achieved. It is fair to say that the same level of reproducibility has not yet been achieved in planar separations. However, automated methods for sample application and chromatogram development will ultimately have the same effect in planar chromatography as in column chromatography, and incorporation of cross-axis bar codes into the electropherogram itself will allow for both sample identification and *xy* axis calibration. The bar code calibrant/identification is also important in the development of parallel detection systems that operate simultaneously (as in optical detection concurrent with mass spectrometric analysis), and repetitive analyses in which the sample chromatogram surface is sequentially treated with reagents, and re-analyzed, or when the time evolution of the sample signal is followed in kinetic or degradation studies.

The use of chemometrics in the processing of imaging data has grown in direct proportion to increases in computing power, and we can also reasonably expect that these methods will be used to extract information from electrophoretic images. Graphical overlays of optical with mass spectroscopic data may provide new insights into electrophoretic separations, even as the physical scale with which mass spectra data can be measured are reduced; this has been the case with high spatial resolution TLC-MS. It is probable that the usual display of mass spectral information on an *xy* plot of *m/z* value and relative intensity will be displaced by more useful graphical constructs (consider the many means with which multi-dimensional NMR data are displayed). The need for enlightenment in explaining mass spectrometry to biochemists and vice versa will proliferate accordingly.

4. Conclusions

The development of mass spectrometric detectors for planar electrophoresis has followed a course charted previously for the development of the interface between thin-layer chromatography

and mass spectrometry. Despite false starts and more than a fair share of misleading claims in the early literature of PE-MS, one can reliably foresee the specific developments in PE-MS in parallel analogy to TLC-MS. A list of significant developments is given in Table 1. The scanning capabilities just now becoming evident in PE-MS will be supplanted by imaging capabilities that allow a full two-dimensional characterization of the separated bands. Experimental methods for sample preparation, reaction and manipulation will become noticeably more sophisticated as users realize that the separation matrix itself can be used innovatively as a support and as a tool. The widespread consensus that small amounts of sample are difficult to manage is an outdated holdover from our insistence on trying to handle those samples in liquid form and in liquid streams. We shall most certainly develop methods that fully use the chemistry that has been developed for sample digestions and derivatizations, and in so doing we shall take advantage of the ability inherent in planar chromatography to carry out these reactions repetitively, simultaneously and sequentially. In so doing, we shall uncover experimental protocols that can lead to extraordinary specificity at very high sensitivity. Operations at the picomole to femtomole level will become the normal and expected level of analysis for targeted analyses. In proving the validity of these measurements, we shall be forced on the other hand to develop data systems, imaging systems and analysis systems that can manage 1000 times as much information as we confront today. As is the case of TLC-MS today, we shall seek to integrate many different types of spectroscopic data and analytical measurement in one global coordinate system, and then to search for correlations and patterns in those data. Time, removed from consideration in the "off-line" nature of the planar chromatographic separation itself, will reassert an impact as multi-dimensional data are also searched repetitively for relevant changes. Distinctions between TLC-MS and PE-MS will eventually disappear as we construct a seamless, automated analytical approach that takes full advantage of the particular advantages of planar chromatography for analytical measurements.

Table 1
Relevant developments in planar electrophoresis–mass spectrometry

| Year | Transfer method ^a | Ionization ^a | Ref. |
|------|------------------------------|-------------------------|-------|
| 1986 | Capillary, electroblot | PD | 35 |
| 1987 | Capillary blot | Imaging SIMS | 37 |
| 1988 | Southern, Northern | Imaging SIMS | 38 |
| 1989 | (Diffusion) | (FAB) | 24,25 |
| 1990 | Disruption | Flow FAB | 26 |
| | Disruption | FAB | 27 |
| | Electroelution | MALDI (FT-MS) | 11 |
| | Capillary blot | MALDI | 46 |
| 1991 | Electroelution | MALDI (FT-MS) | 41 |
| | Freeze–squeeze | FAB | 40 |
| | (Electroelution) | (HPLC–LSIMS) | 28 |
| 1992 | Electroblot (PVDF) | MALDI (IR, TOF) | 43 |
| | – | MALDI (UV) | 53 |
| | – | (MALDI) | 30 |
| 1993 | Electroblot (PVDF) | Scanning MALDI | 13 |
| | Nitrocellulose | PD | 45 |
| | Electroelution | Electrospray | 23 |
| 1994 | Electroblot (PVDF) | Scanning MALDI | 47 |

^a Terms in parentheses indicate that the samples were analyzed individually after removal from the electropherogram.

Most important, we shall finally take advantage of our expertise in materials science and nanofabrication to reduce the scale of our separation technology to the scale at which the analytical methodology already operates. Planar chip electrophoresis is seductively feasible. The growth curve for PE–MS is in the exponential stage; the pathway is marked, and the games are open.

Acknowledgements

The National Science Foundation and the National Institutes of Health provided early support for our work in TLC–MS and PE–MS. I would especially like to thank Unilever Research and Monsanto for their support in this work, and the Georgia Institute of Technology for supporting patent applications. Several graduate students (past and present) have worked on these projects over the years, including Michele Stan-

ley-Schilling, Steve Brown, Jocelyn Dunphy (working through Oak Ridge Associated Universities) and Michael Bartlett. Kevin Schey and Dan Knapp at the Medical University of South Carolina have graciously provided access to MALDI–TOF instrumentation.

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