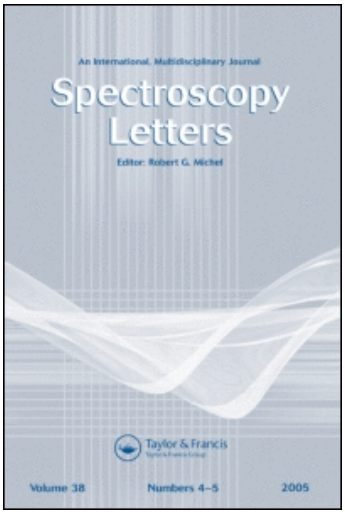

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Fast-Atom Bombardment Mass Spectrometry Coupled to Planar Gel Electrophoresis

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FAST-ATOM BOMBARDMENT MASS SPECTROMETRY COUPLED TO PLANAR GEL ELECTROPHORESIS

Keywords: FAB-MS, electrophoresis, interface, peptides

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ABSTRACT

An interface system between planar gel electrophoresis and mass spectrometry is described that includes a means of rapid electrophoretic gel disruption, concentration of extracted sample in a pre-column, and transfer of the sample molecules to the continuous flow FAB source of a mass spectrometer. Spatial resolution achieved on the surface of the gel is on the order of 2 mm diameter for a spot, and 4 mm^2 for a band. Sensitivities to 50 picomoles have been demonstrated for the analysis of small peptides with continuous-flow fast atom bombardment mass spectrometry.

INTRODUCTION

Electrophoretic techniques that use gel support media, such as polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE, polyacrylamide gel isoelectric focussing (PAGIF) and agarose gel isoelectric focussing (AGIF), have been developed that (when used alone or in combination) enable the researcher to characterize proteins based on physicochemical properties. There is a problem, however, facing the researcher using modern, high-resolution gel electrophoresis; that problem is detection and identification of the samples after the electrophoresis has taken place. Traditionally, stains have been used that bind to the sample,

imparting a color to the band. There has been a tremendous effort to find suitable protein stains with the necessary detection capabilities and sufficient dynamic range. Perhaps the most widely used stain in the last ten or fifteen years has been the Coomassie series, including Coomassie Blue R-250 and Coomassie Blue G-250.[1] These stains possess detection limits of about 50-60 ng/mm2, and a linear response over a 20 to 30 fold concentration range. Silver staining, which has gained widespread use since 1980, is approximately 20-200 times more sensitive (with a similar dynamic range) than the Coomassie stains for proteins. Detection limits of 30-100 pg/mm^2 are now attainable.[2] The advent of microcomputers has revolutionized the optical methods of detection in gel electrophoresis. Scanning densitometers, utilizing solid state photodiode arrays and operating in both reflectance and transmittance modes, are commercially available and widely used.

No matter how elaborate these optical detection methods are, however, the information obtainable using them is very limited. That is, the detector may measure where a protein band is located, but cannot directly yield any further information about that protein such as molecular weight or structural information. Although the electrophoresis itself may provide an estimate of the molecular weight of the protein, these determinations are limited by the resolution of the electrophoretic method and internal inconsistencies that may be present in the gels, thereby providing molecular weights with errors on the order of +/- 2-5%. This may seem like a small amount of error, but for a small protein of 50,000 amu, the error range is +/- 1000-2500 amu. When sequence information of the protein is desired, this translates to an error of about 8-20 amino acid residues, an error that is generally unacceptable.

It is clear that a more information-rich detection scheme is needed for gel electrophoresis. Mass spectrometry can provide that needed information. The analysis of large, non-volatile, biological compounds by mass spectrometry has seen great advances in the last ten years, with the introduction of fast atom bombardment mass spectrometry and, more recently, electrospray[3] and ionspray[4] ionization; these methods, especially electrospray and ionspray, are capable of obtaining the molecular weight of a protein to within 1 dalton to a mass limit of 200,000 amu. Also, tandem mass spectrometry (MS/MS) has been shown very effective in yielding sequence information of proteins[5,6], although not yet for these ions of very high masses.

Direct mass spectral analysis of hydrated gels is clearly problematic; electrophoretic gels cannot be inserted directly into the high vacuum. Stanley and Busch[7] transferred analytes from polyacrylamide gels to a nitrocellulose support in a blotting procedure prior to imaging mass spectral analysis. However, a difficulty with this approach is the great tenacity with which the sample molecules, especially large proteins, interact with the gel matrix. Numerous methods

have been employed over the years for the release and recovery of the sample from the gel. These include blotting techniques (such as the Southern or Western blot), electroelution into dialysis bags, electroelution into troughs, overnight sonication in an appropriate extraction solvent, and electrophoresis onto a dialysis membrane. The major obstacles preventing the interfacing of gel electrophoresis to mass spectrometry has been the strong retention of the analyte molecules within the gel matrix, and the time required to release the sample using the methods described above. We postulated that if the matrix could be physically disrupted in a rapid procedure, the analyte would be free to migrate out of the gel. Recently, Camilleri et al.[8,9] reported the extraction of samples from polyacrylamide gels, with subsequent analysis of the discrete samples by FAB mass spectrometry. However, the necessary sample preparation included an extended extraction of the gel with 9M acetic acid and crushing of the gels after 5 sample bands were excised out of the gel. The connection of the electrophoresis with the mass spectrometric analysis was no more direct than if the sample had been prepared by any other means. Similarly, Duffin et al.[10] have extracted biological compounds, such as angiotensin and certain subchains of bovine insulin, from within gels with sonication and a series of solvents, and have shown that FAB analysis of the discrete samples provided good quality mass spectra of these extracts. Even high power laser desorption can be insufficient for extraction, although recent results[11] suggest that conditions can be found that release intact molecular ions of nucleosides from dried agarose gels.

Therefore, an interface methodology was developed in this laboratory that utilizes localized mechanical disruption of the gel directly (with no transfer or blotting techniques), and rapidly releases the analyte molecules into appropriate solvents, while still retaining the spatial coordinates of the separation. The disruption is accomplished by microhomogenizers (either a piezoelectric sonicator or a rotor/stator type), commonly used for red blood cell disruption, for example. The time required for the complete disruption of a gel band was a significant figure of merit. In order for this methodology to be useful in the analysis of large 2-dimensional electropherograms, a target time of one minute for gel disruption, sample concentration, and mass spectrometric determination was set.

EXPERIMENTAL

The interface consists basically of a means of physically disrupting the gel (either by a rotor/stator homogenizer or a piezoelectric sonicator), a means of locally introducing solvent to the point of disruption, a concentration column, and transfer capillaries and valves that connect to the source of the mass spectrometer. In the current version of the interface, the

homogenization probe is surrounded by a sleeve so that solution cannot escape from the point of disruption, and so that chemical treatment of the gel to aid disruption is localized.

In order to determine whether the proposed experimental protocols involving the physical disruption would be feasible, initial probe experiments were conducted with minimal experimental variables. These experiments consisted of: standard solutions of the samples (Coenzyme B_{12} and angiotensin II, 1-2 mg/mL) were made in distilled water. A small piece of 2% agarose gel (made with Tris-acetate buffer, pH 7.8), approximately 0.5 cm square, was placed into each standard solution and allowed to stand at room temperature for 18 hours. This ensured uniform diffusion of the analyte into the gel matrix. The pieces of gel containing the analyte were then removed from solution, 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. The gel was then homogenized completely with the rotor/stator homogenizer, the Omni 1000, manufactured by Omni International, for a period of one minute. The resulting slurry was then passed through a syringe filter. To the filtered solution was added a few drops of glycerol to make the glycerol concentration approximately 10%. This solution was then analyzed by continuous-flow fast atom bombardment by immersing the flow-FAB capillary into the analyte solution. In this protocol, the mass spectra were obtained on a VG-70SE double-focussing mass spectrometer of EB geometry. In one determination (that of Coenzyme B_{12}), the multichannel acquisition mode the instrument was used to enhance the sensitivity of the analysis. No background subtraction was performed.

The second experimental protocol, developed after the above preliminary results were obtained, consisted of the following procedures. Approximately 10 micrograms of analyte were introduced into the 2% agarose gel matrix (made with Tris-phosphate buffer, pH 7.6) by the application of 50 volts across the gel for approximately 5 minutes. The appropriate analyte band was then excised out of the gel and placed into a vial, to which was added 5-10 mL of 0.1M KI. (In later experiments, a stainless steel isolation sleeve was used to select a particular band from within the gel, eliminating the excision step.) In either case, the gel was then homogenized completely in 30-60 seconds by the piezoelectric sonicator probe, Model Virsonic 50 manufactured by Virtis, Inc. The resulting slurry was passed through a 0.5 micron in-line screen filter, and then through a microvolume (16 uL) C18 guard column. This concentrated the analyte molecules onto the head of the column, while allowing the buffer salts and KI to pass through to waste. The analytes were then eluted from the concentration column by using a uLC500 syringe pump to backflush with the flow-FAB mobile phase (80:10:10, MeOH:H_2O:glycerol), and thereby transferring the analyte molecules into the source of the mass spectrometer. Continuous-

flow FAB analysis was performed on a VG-70SEQ hybrid mass spectrometer of EBQQ geometry. The primary beam consisted of 35 keV Cs^+ ions. The standard VG dynamic FAB probe was used, with a 50 micron ID capillary. The source block of the instrument was heated to 40 °C. In some instances, several scans were averaged to produce the given spectra.

RESULTS AND DISCUSSION

As mentioned previously, the major obstacle preventing the direct coupling of gel electrophoresis with mass spectrometry has been the difficulty encountered in extracting sample molecules from within the gel matrix once the electrophoresis has been performed. Other researchers have succeeded in removing analyte molecules from gel matrices and subsequently performing mass spectral analyses on the compounds; however, their experimental procedures were time-intensive and destroyed any spatial integrity of the electrophoresis. An interface device was envisioned in this laboratory that would utilize localized mechanical disruption of the gel directly (with no transfer or blotting techniques), thereby releasing the analytes into appropriate solvents, yet still retaining the spatial coordinates of the separation. The disruption would be accomplished by micro-homogenizers (either a piezoelectric sonicator or a rotor/stator type), commonly used for red blood cell disruption, for example. The location of the sample bands to be disrupted would be accomplished through the incorporation of computer-controlled optical detection. The time required for the complete disruption of a gel piece was hoped to be significantly less than other reported techniques.

Initial experiments were performed with a small rotor/stator homogenizer in order to determine the feasibility of the interface methodology. Later experiments used a miniature ultrasonic homogenizer originally developed for cell disruption. Use of this disrupter is preferred due to its smaller size (3 mm tip diameter as compared to 7 mm for the rotor/stator device) and easier cleaning between samples.

The first model compound analyzed using this interface method was Coenzyme B_{12}. Figure 1 shows the positive ion flow-FAB mass spectrum obtained for the compound extracted out of the agarose gel. This spectrum is very similar to that of the standard material. It should be noted that these two spectra were obtained using the MCA mode with magnet scanning to enhance the sensitivity of the analysis, and that the mass spectra are not background-subtracted. The presence of the protonated molecule at m/z 1580 is not noted; however, a signal from the significant fragment ion at m/z 1331 is seen in the spectrum. This fragment arises from the loss of the 5'-deoxyadenosyl group from the protonated molecule. An additional loss of the ribofuranyl group yields the ion at m/z 1070. Several sodium adduct ions are also seen.

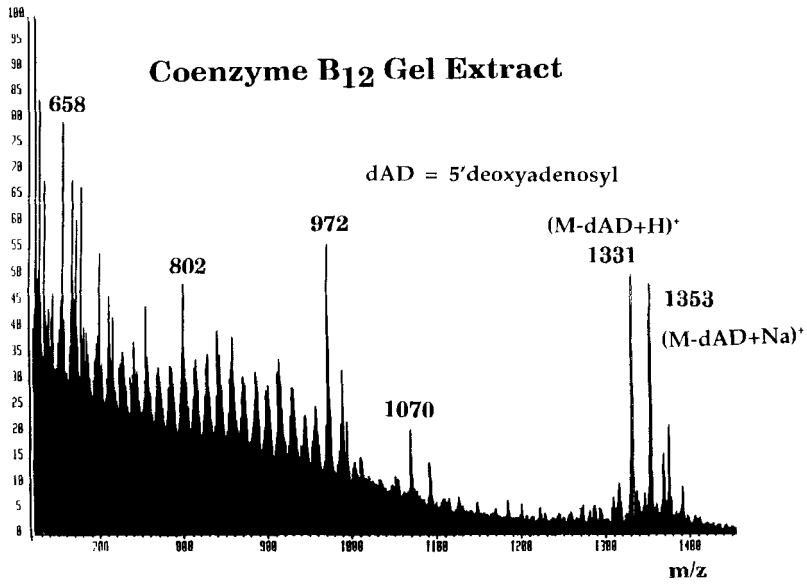

FIG 1 Positive ion fast atom bombardment mass spectrum of coenzyme B_{12} extracted from an agarose gel.

Angiotensin II (human) was also chosen as a model compound for these initial experiments. The positive ion flow-FAB mass spectrum of this compound is shown in Figure 2. An abundant protonated molecular ion at m/z 1047 is observed, in addition to lower abundance sequence fragment ions. The spectrum obtained for angiotensin II extracted out of agarose gel is very similar to that of the standard.

These results of these initial experiments gave evidence that the extraction procedures in the envisioned interface methodology would, in fact, work. Therefore, improvements were made to the experimental procedures as described in the Experimental section. Perhaps the most important change in procedures was the utilization of the piezoelectric sonicator probe instead of the rotor/stator homogenizer, which yielded a gel slurry with large gel particles (on the order of 0.5 mm in size); these gel pieces quickly plugged the in-line screen filter, so that the filter had to be cleaned or replaced after each sample band was homogenized. The rotor/stator design is also much more difficult to clean due to its close-tolerance moving parts; cleaning had to be meticulous to ensure no memory effects. On the other hand, the piezoelectric sonication probe

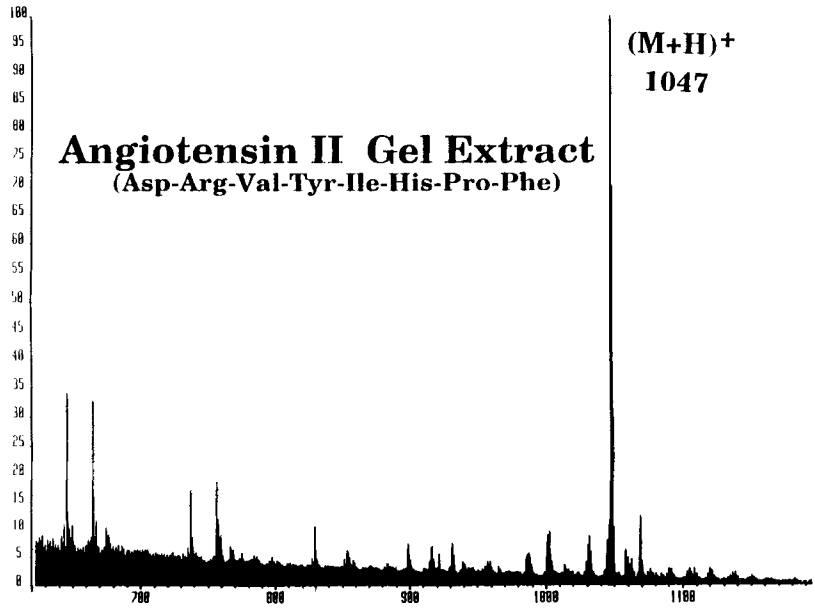

FIG 2 Positive ion fast atom bombardment mass spectrum of angiotensin II extracted from an agarose gel.

can be cleaned with a stream of distilled water, due to the probe's polished titanium surface and lack of moving parts. Another factor in the favor of the piezoelectric sonicator lies in the agarose gel itself. When an agarose gel is prepared with Tris-phosphate buffer, the gel matrix is slowly dissolved by potassium iodide.[12] The combination of the sonication and the use of the potassium iodide solution enabled complete dissolution of the agarose gel in approximately 30 seconds, with no visible gel pieces. Because of this, the in-line screen filter needed much less cleaning.

However, in spite of all the advantages offered by the piezoelectric sonication homogenization probe, there was still some trepidation concerning its use. Our hesitation was due to the possible detrimental effects of the sonication on large macromolecules, such as proteins or DNA strands. An examination of the literature revealed that such degradations of large molecules are possible.[13] Upon ultrasonic radiation for 38 hours, a solution of polymethyl methacrylate (initial molecular weight: 547,000 amu) was degraded so that the molecular weight of the breakdown products approached a lower limit of 47,000 amu. Since the researchers

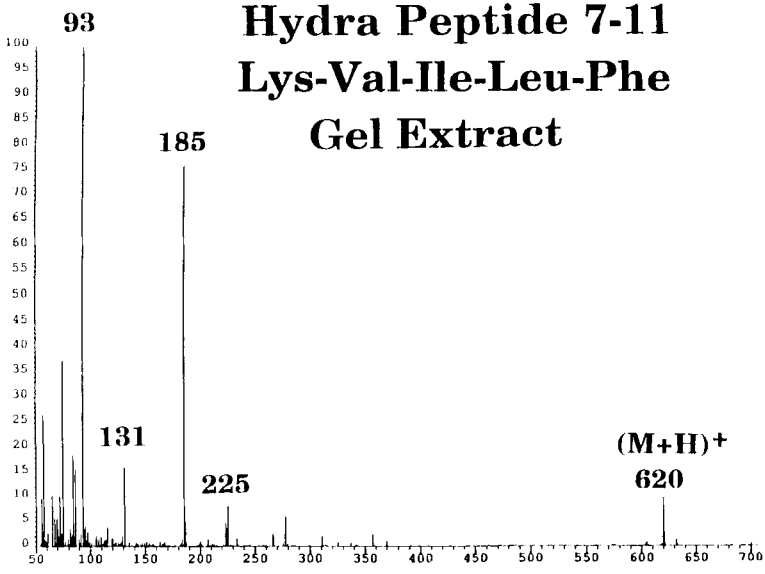

FIG 3 Positive ion fast atom bombardment mass spectrum of hydrapeptide 7-11 extracted from an agarose gel.

reported that the chemical structure of the molecule does not play a significant part in the liability to ultrasonic breakdown, we can extend these data to proteins or DNA strands, and expect similar tendencies for ultrasound-induced degradation. However, it was also reported that the degradation was caused by cavitation, the explosion of microscopic bubbles in solution, rather than the ultrasound itself. If the intensity of the ultrasonic radiation is kept low enough so as to minimize cavitation, one can expect the structures to be unperturbed. In fact, Melville and Murray[13] measured a constant molecular weight of over 200,000 amu for 38 hours of sonication under non-cavitating conditions. Since our expected time of ultrasonic radiation is about 1 minute, we can expect little degradation of the large molecules. Ironically, there recently appeared an article[14] reporting the use of ultrasound radiation to aid in the agarose gel electrophoresis of DNA fragments. The researchers observed no degradation of the DNA strands which were up to 23 kilobases (~ 500,000 amu) in length. They also reported that ultrasound radiation resulted in a decrease of DNA-agarose gel affinity, a conclusion which bodes well for our experiment.

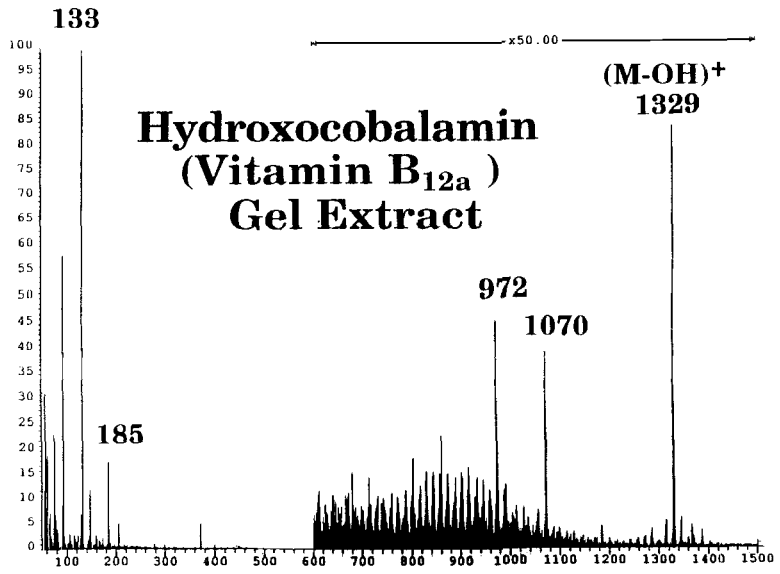

FIG 4 Positive ion fast atom bombardment mass spectrum of hydroxocobalamin extracted from an agarose gel.

Utilizing the second experimental protocol, two more model compounds were studied. Figure 3 contains the positive ion flow-FAB mass spectrum of the sample pentapeptide, hydrapeptide 7-11. A very abundant signal corresponding to the protonated molecular ion at m/z 620 is observed, as well as smaller characteristic fragment ions. The ion at m/z 133 is due to the Cs^+ from the primary ion beam. Approximately 10 micrograms of peptide was analyzed in this instance.

The last model compound used to demonstrate the performance of this interface was hydroxocobalamin (Vitamin B_{12a}). Figure 4 shows the flow-FAB mass spectrum of the compound extracted out of agarose gel. Typical for the mass spectra of such cobalamin analogs, a signal for the protonated molecular ion is not seen. However, an abundant fragment ion at m/z 1329 is observed, corresponding to the loss of a hydroxyl group. More accurately, this corresponds to the loss of neutral water from aquocobalamin (the form in which hydroxocobalamin exists in aqueous solutions). Characteristic fragment ions are also seen at m/z 1070 and m/z 972.

In order for this interface technique to gain widespread acceptance, significant sensitivity must be obtainable so that a useful mass spectrum can be generated for the small amounts of

sample present in actual electrophoretic runs, usually in the low- to mid-nanogram range (picomoles of material). Full-range mass spectra of the compound, Vitamin B_{12a}, have been obtained at a signal-to-noise of 3:1 (m/z 1329) for 10-20 ng of sample, corresponding to about 10 pmole sensitivity. This level of sensitivity is sufficient for analysis of one-dimensional gel electropherograms, which typically contain 50 ng of sample. However, another two orders of magnitude or so are needed for analysis of 2-dimensional gels, which typically contain much less sample and more complex mixtures. We hope to achieve that sensitivity with continued refinement of the interface and experience with the flow-FAB techniques, and future adaptation of this interface to electrospray ionization mass spectrometry. Extensions of the method to polyacrylamide gel electrophoresis is underway.

CONCLUSIONS

An interface device for the direct coupling of gel electrophoresis with mass spectrometry has been described. The design has many advantages over previous reports of the mass spectral analysis of compounds contained in gel electropherograms. First, transfer operations, which are not always quantitative, are not necessary; the compounds entrapped in the gel matrix are accessed directly. Second, the time required for disruption of the gel, release of the sample material into the flow stream, concentration of the sample, and transfer of the sample in a concentrated bolus to the source of the mass spectrometer is about 3 minutes. Also, the sensitivity obtained with this methodology is greater than previously reported; full-range fast atom bombardment mass spectra are obtained on 15 pmole of analyte. In addition, since the extraction of the sample takes place at atmospheric pressure outside of the vacuum of the mass spectrometer, many different solvents can be locally introduced onto the gel, obviating the need for non-volatile extraction solvents. Also, the extraction procedure is compound-independent, since the release of the analyte molecules is accomplished by a mechanical disruption of the gel matrix in which the molecules are entrained. In other words, larger analyte molecules can be released as easily, and in the same amount of time, as the model compounds described here. The ultrasonic homogenizer has been successfully utilized to release several variants of hemoglobin from agarose gels. Finally, the versatility of the transfer process will enable the utilization of any ionization technique that is adaptable for analysis of a liquid flow stream, including, but not limited to, flow-FAB, electrospray, ionspray, thermospray, and laser desorption. Substantial work remains to be done, including studies of extractions and transfer efficiency for a wide range of molecular size, and the incorporation of other concentration and separation variations within the interface. However, the present design has already met reasonable goals of speed and sensitivity.

Simplicity in construction and ease of use should aid in the rapid development and proliferation of similar devices. While capillary zone electrophoresis has met with success in bioanalytical separations, planar gel electrophoresis will continue to dominate the field, and a need for improved detection methods will continue. Further, while mass spectrometry suffers in terms of absolute sensitivity when compared to other detection methods used in capillary zone electrophoresis, mass spectrometry has already demonstrated sensitivities equal to the detection methods currently used for planar electrophoresis, with the significant added advantage of specific mass spectral information.

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