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Parallel Electrophoretic Depletion, Fractionation, Concentration, and Desalting of 96 Complex Biological Samples for Mass Spectrometry

James B. Harkins, IV, Benjamin B. Katz, Salvador J. Pastor, Peter Osucha, Dean G. Hafeman, Charles E. Witkowski, II, and Jeremy L. Norris*

Protein Discovery, Incorporated, 418 South Gay Street, Suite 203, Knoxville, Tennessee 37902

The preparation of complex biological samples for high-throughput mass spectrometric analyses remains a significant bottleneck, limiting advancement of the capabilities of mass spectrometry (MS) and ultimately limiting development of novel clinical assays. The removal of interfering species (e.g., salts, detergents, and buffers), concentration of dilute analytes, and the reduction of sample complexity are required in order to maximize the quality of resultant MS data. This study describes a novel sample preparation method that makes use of electrophoresis to prepare complex biological samples for high-throughput MS analysis. The method provides for integration of key sample preparation steps, including depletion, fractionation, desalting, and concentration. The prepared samples are captured onto a monolithic reversed-phase capture target that can be analyzed directly by a mass spectrometer. Up to 96 individual samples are simultaneously prepared for MS analysis in under 1 h. For standard proteins added to serum, this method provides femtomole level sensitivity and reproducible label-free detection (coefficient of variation <30%). This study demonstrates that this electrophoretic sample preparation system permits high-throughput sample preparation for mass spectrometric analysis of complex biological samples, such as serum, plasma, and tissue extracts.

Mass spectrometry (MS) is fundamental to the analysis of a wide range of molecules due to its superb sensitivity and selectivity.[1−3] Soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) now permit the accurate and precise measurement of the molecular mass of pharmaceuticals, metabolites, nucleic acids, and proteins, allowing for the identification, characterization, and quantitation of these biologically important analytes. Over the past decade, the capabilities of MS instrumentation dedicated to these applications has progressed significantly, now making it possible to analyze thousands of biological samples in a single day.[4] Despite the tremendous improvements in MS instrumentation, the presence of interfering chemical and biological materials compromise and can prevent MS analysis of specific analytes of interest. Taking advantage of the high-throughput capabilities of modern mass spectrometers is difficult without rapid and high-quality sample preparation techniques.[3,5] To fully exploit the analytical and economic advantages of modern mass spectrometry techniques in both biomedical research and clinical testing, high-throughput, high-fidelity, automated sample preparation methodologies are needed.[6]

Biological samples that contain chemical contamination require a number of routine, often manual, sample preparation tasks prior to mass spectrometric analysis. The concentrations of salts, lipids, and nucleic acids in physiological samples as well as common laboratory reagents such as buffers and detergents contribute significantly to the overall molecular content of a biological sample and can cause significant problems for mass spectrometric analysis.[7,8] Interfering chemical species must be removed, or their concentrations reduced significantly, to obtain the highest sensitivity and dynamic range for the analytes of interest. There are a number of devices and techniques that are helpful in the removal of salts and surfactants, the most common of which are based on some variation of solid-phase extraction (SPE), liquid−liquid extraction (LLE), or protein precipitation.[9−12] In SPE, the sample is permitted to interact with a surface that has affinity for the analyte of interest, with reversed-phase resin being the surface used most frequently. The unwanted chemical species are washed away, leaving the analyte bound to the surface. Application of an optimal volume of the proper eluent releases the analyte to form a concentrated solution, which leads to a lower limit of detection

* To whom correspondence should be addressed. Phone: 865-521-7400. Fax: 865-521-3548. E-mail: jeremy@proteindiscovery.com.

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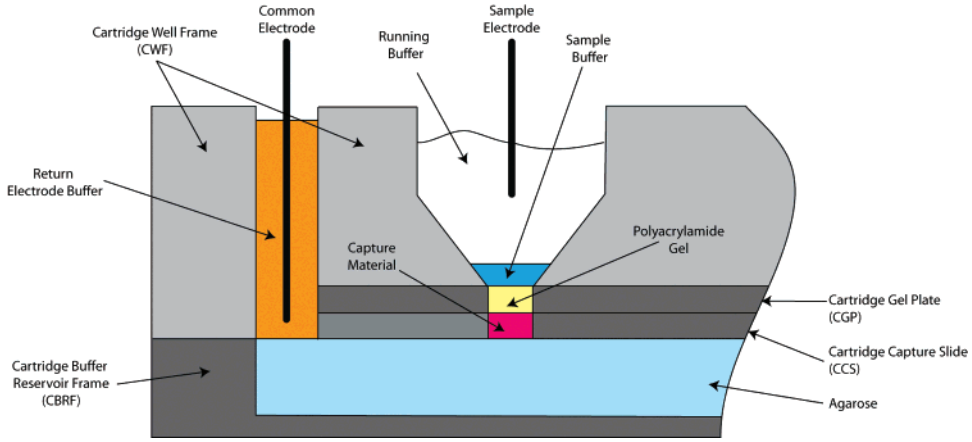

Figure 1. Schematic of the electrophoretic sample preparation device. Samples are introduced into the top of the well. A direct current is passed through the system via the electrode in the well. Analytes with a charge matching the polarity of the sample electrode migrate from the well, through a polyacrylamide gel, and are captured onto the reversed-phase capture material located directly below the gel. Following capture, the device is disassembled to allow direct analysis of the capture surface by mass spectrometry.

upon analysis by MS. Numerous SPE devices designed for small-molecule, metabolite, and protein and peptide sample preparation are commercially available. A significant disadvantage of these devices is that they are often not integrated with the other sample preparation steps and therefore introduce problems associated with sample transfer, such as sample loss and irreproducibility. Furthermore, SPE is often performed manually, adding costs of labor and limiting sample throughput. LLE has the advantage of being easy to develop and inexpensive to implement on a small scale. However, the precision and reproducibility is suboptimal, it generates a large amount of organic waste, and is also not easily adapted to automation, making high-throughput sample preparation less practical. Protein precipitation, though widely used because of simplicity, has limited utility due to poor selectivity. The enriched fractions of samples prepared by protein precipitation often retain significant amounts of endogenous compounds. Thorough method development for a particular analyte involves exploring each of these techniques, and then balancing speed, cost, and specificity to find an adequate solution.

The typical biological sample contains multiple molecular species that range in concentration over many orders of magnitude. The most extreme example, human blood plasma, contains clinically important protein components that range in concentration from more than 50 mg·mL^{-1} of serum albumin down to less than 1 pg·mL^{-1} of interleukin-6.[13] Accordingly, the most critical sample preparation task one must perform is to reduce the sample complexity through fractionation of the sample or depletion of specific molecular components. The method most commonly used to reduce sample complexity prior to MS analysis is chromatography. This technique typically employs nonspecific separation methods such as reversed-phase and ion-exchange chromatography but can also include affinity purification methods that allow for the removal or enrichment of selected analytes from the mixture.[14−16] Chromatographic methods have the advantages of being amenable to automation and providing high-resolution separation of complex samples. Chromatographic techniques can be applied in series to single samples, providing a multidimensional analysis of the sample of interest.[17,18] Recent examples demonstrate that with high-performance chromatographic methods, analytes can be detected over a dynamic range greater than 8 orders of magnitude.[19] A major disadvantage is that chromatography is time-consuming and requires highly trained laboratory staff to perform the experiments. The time required for the measurement of a single sample, the lack of reproducibility, and the cost of using liquid chromatography-based methods limits the utility of the technique for large-scale discovery projects requiring hundreds of samples.[20] Methods for the reduction of sample complexity that can be performed rapidly and reproducibly with many samples in parallel overcome these issues and will contribute significantly to the application of mass spectrometry in biomedical applications.

This study introduces an automated approach to the depletion, fractionation, concentration, and desalting of biological samples that increases the efficiency and reproducibility of sample preparation for mass spectrometry. The method utilizes parallel electrophoresis through a series of thin-layer supports, to simultaneously prepare 96 independent samples for MS analysis. Figure 1 shows a schematic of an electrophoretic sample preparation system that can be used to process a single sample. At a given buffer pH, the analyte of interest will migrate toward the anode or cathode, depending on the charge of the analyte under the conditions of the experiment, resulting in binary fractionation. Those analytes found in the target pH fraction first migrate through a thin separation layer, which further fractionates analytes based on their native electrophoretic mobilities. Those molecules that pass completely through the separation layer are captured on a three-dimensional, reversed-phase, porous capture site, which can then be analyzed by MALDI MS or extracted for analysis by ESI

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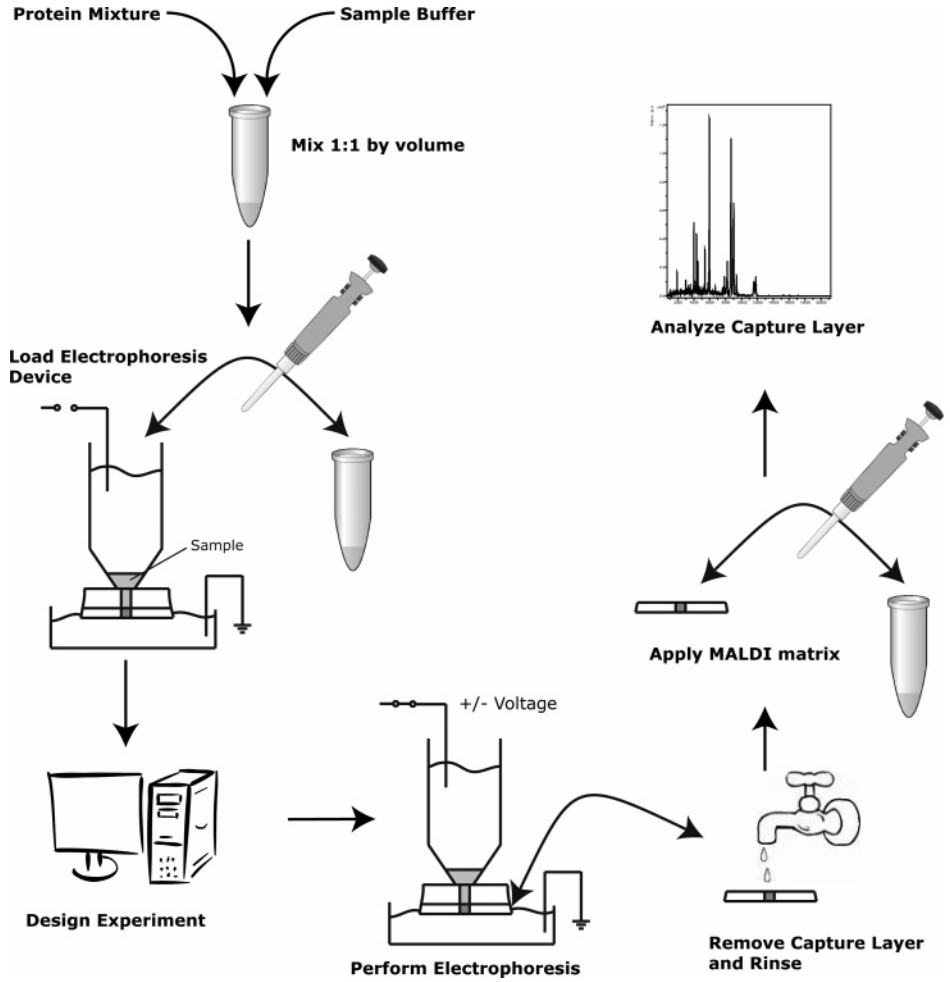

Figure 2. MALDI MS sample preparation using electrophoresis. The sample is mixed with sample buffer and loaded into the electrophoretic sample preparation cartridge. The experiment is designed and conducted using a software-controlled user interface. The device is disassembled, and the capture surface is removed. The capture surface is washed, MALDI matrix is applied, and the capture layer is analyzed directly using MALDI MS.

(Figure 2). The unique features of the method make it ideally suited for the analysis of low molecular weight proteins and peptides found in complex matrixes such as plasma. The sample preparation format lends itself to the preparation of many samples in parallel (e.g., 96, 384, etc.) without sacrificing performance. This paper will describe the novel electrophoretic sample preparation system, demonstrate its analytical performance parameters, and present the MS analysis of peptides and proteins from human serum, plasma, and tissue subsequent to sample preparation using the methodology.

EXPERIMENTAL SECTION

All sample preparation steps were performed using a system comprising an electrophoretic controller and single-use 96-well electrophoresis cartridges (Protein Discovery, Inc., Knoxville, TN). The components of this system are described below.

Electrophoretic Controller. A computer-driven controller provided independent, parallel, preprogrammed electrophoretic control for 96 samples. Utilizing potential control or current control, the analysis could be programmed for periods of time or total coulombs of electrophoretic charge per channel. The current and voltage were recorded for each channel in order to validate the actual electrophoretic conditions for each independent channel and to show any resistance changes occurring during electrophoresis. Parallel electrophoresis in 96 sample cartridges arranged in microplate format was accomplished utilizing 96 platinum electrodes powered independently by the controller.

Reagents. Running buffer was prepared using 2-(*N*-morpholino)ethanesulfonic acid (MES) purchased from Fisher Scientific (Pittsburgh, PA). The running buffer contained 689 mM MES adjusted to pH 5.35 with 50% NaOH solution in water from Sigma (St. Louis, MO). The sample buffer contained 45 mM octyl β-D-glucopyranoside and 30% (w/v) sucrose in running buffer. All of the reagents for the sample buffer were purchased from Sigma (St. Louis, MO). The return solution was prepared using 1.5 M sodium sulfate (Sigma, St. Louis, MO), 250 mM sodium bicarbonate (Sigma, St. Louis, MO), and 250 mM boric acid (Fisher Scientific, Pittsburgh, PA). ACTH fragment 18−39, trypsinogen, insulin, and cytochrome *c* were purchased from Sigma (St. Louis, MO). Insulin B-chain was purchased from Anaspec (San Jose, CA). Serum and plasma were purchased from Innovative Research, Inc. (Southfield, MI). Rat liver was obtained from Vanderbilt University; the liver was collected under IRB approved protocols.

Cartridge Design. A cartridge comprises 96 individual sample cartridge wells in a standard 96-well microplate format. Each well

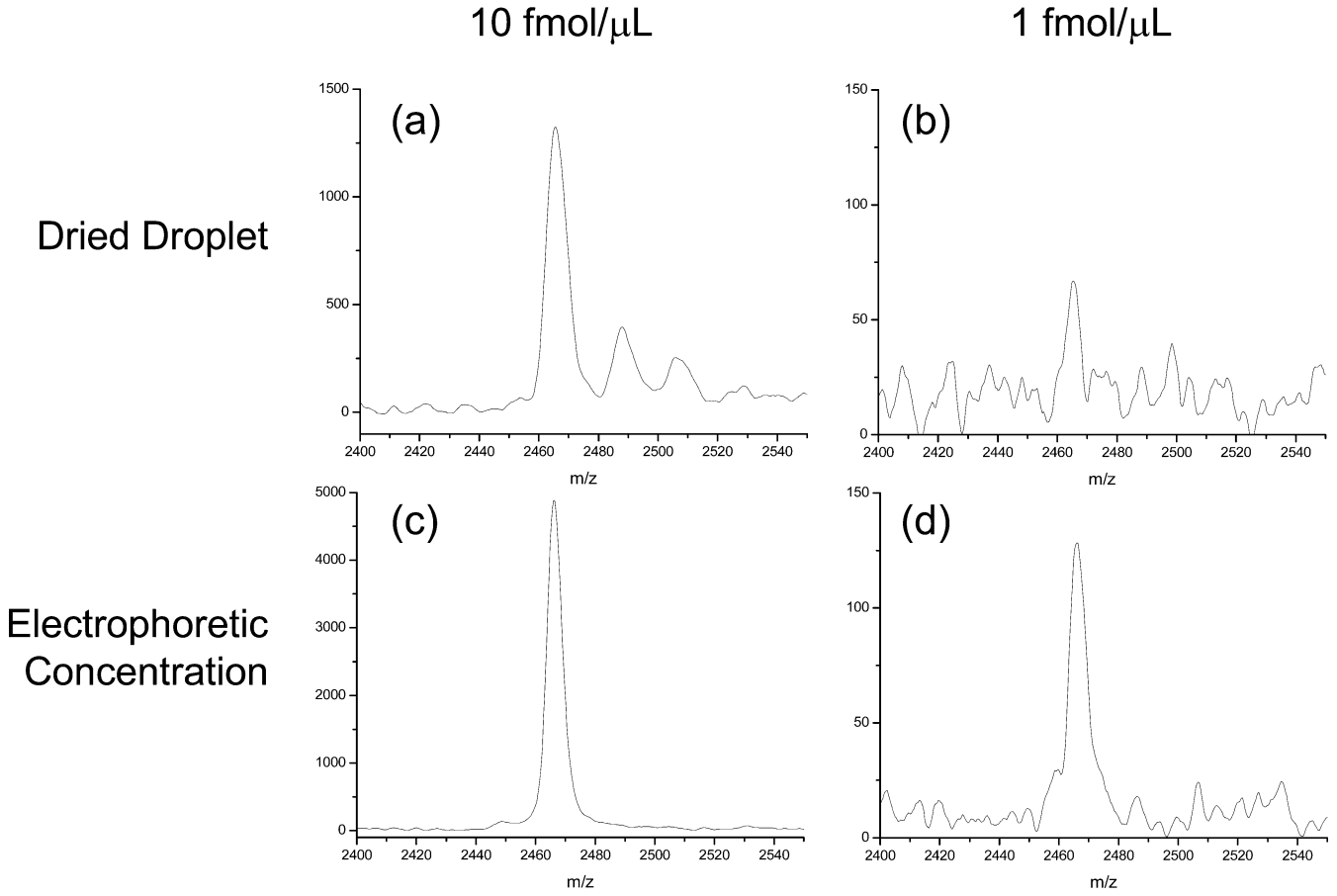

Figure 3. Concentration and desalting of dilute proteins. The protein, ACTH [18−39], was reconstituted in phosphate-buffered saline (PBS) and prepared for MALDI analysis using the dried droplet technique and using electrophoretic concentration. (a) A 10 fmol/μL solution of ACTH in PBS was prepared for MALDI analysis on a conventional stainless steel target using the dried droplet technique. (b) Similarly, a 1 fmol/μL solution of ACTH in PBS was prepared using the dried droplet technique. (c) Ten microliters of a 10 fmol/μL solution and (d) 10 μL of a 1 fmol/μL solution of ACTH in PBS were concentrated and desalted using electrophoresis. Electrophoretic concentration allowed for the desalting, recovery, and mass analysis of dilute protein samples.

of the cartridge is an independent electrophoretic separation chamber, including a polyacrylamide gel layer and a reversed-phase capture layer (Figure 1). The interior layers are pressed together to form a tight seal that isolates each well mechanically, chemically, and electrically. Independent operation of each of the 96 cartridge cells by current (current control mode) or potential (potential control mode) is accomplished by the microprocessor controller. By specifying the desired experiment in software, the experimental parameters of time, current/potential, and polarity of the experiment may be varied for each of the 96 wells. The sample is mixed with a high-density sample buffer solution so that the sample is in contact with the acrylamide gel layer when the current is applied. A volume of 10 μL of sample is loaded into each well of the cartridge. Under an appropriate set of experimental conditions, analytes of interest will migrate through the gel layer.

Inside the cartridge, a 12% acrylamide/bisacrylamide gel plug mediates electrophoretic migration of the analytes, providing separation. High-mobility species in the sample migrate at a faster rate through the gel layer than low-mobility species. In effect, the gel facilitates a low-resolution separation of proteins, depleting high molecular weight biomolecules such as those found in plasma, while maintaining fast separation and minimizing sample loss. A ratio of 19:1 acrylamide/bisacrylamide gel is used to allow efficient electrophoretic migration of low molecular weight analytes, while retarding high molecular weight, high-abundance proteins. In addition, by controlling the separation time (total accumulated charge), pH of the system, and the magnitude and polarity of the applied potential, the nature and number of captured analytes can be optimized.

Below the gel layer is a cartridge capture slide. The capture material consists of a C8 chromatographic media immobilized in a monolithic polymer matrix having hydrophobic properties similar to a C8 to C18 reversed-phase capture surface. When analytes are electrophoretically driven to the surface of the capture slide, they are bound to the capture material. The capture slide is removed from the cartridge and washed in an aqueous solution to remove water-soluble contaminants such as buffer salts. Analytes are liberated from the reversed-phase surface in a single step when an aqueous/organic solvent is applied to the surface. The elution solution may contain a MALDI matrix for direct analysis from the capture layer, or samples may be eluted for off-line analysis by ESI MS. For MALDI MS, the cartridge capture slide is introduced directly into a MALDI mass spectrometer for analysis of the captured analytes using a specially designed target adapter. The capture layer is molded from carbon-doped PEEK, a chemically inert, electrically conductive polymer to prevent charging inside the mass spectrometer as ions are desorbed from

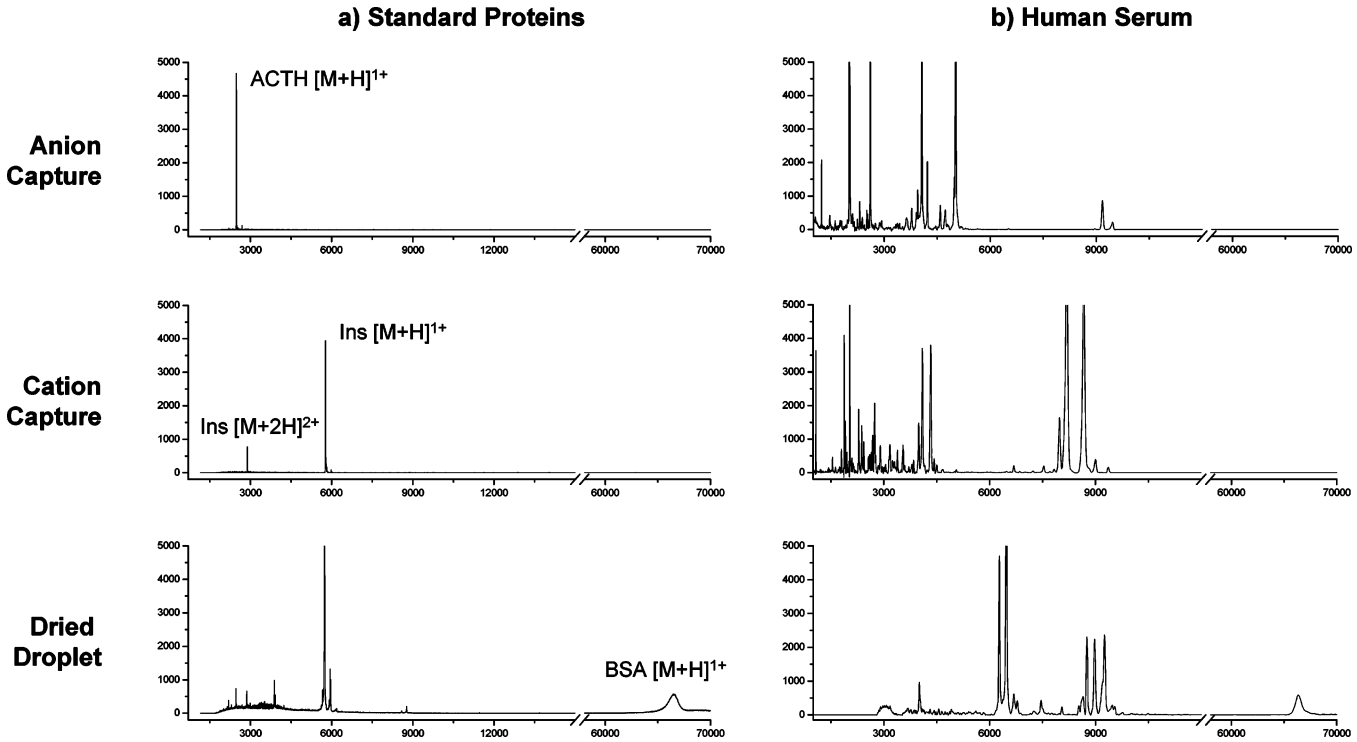

Figure 4. Simultaneous protein fractionation and depletion of high molecular weight proteins. Electrophoretic protein capture at a specific pH allows for the fractionation of low molecular weight proteins while simultaneously removing higher molecular weight species. (a) Shown is the selective separation of anions (ACTH, pI = 4, 1 pmol) and cations (insulin, pI = 7, 1 pmol) at pH 5.35 with simultaneous depletion of serum albumin (145 pmol) from a mixture of standard proteins. (b) This principle is further demonstrated using 5 μL of human serum.

the capture surface. The monolithic material is cast into 1 mm diameter holes in the conductive polymer capture slide that are arranged in 96-well format.

The bottom two layers of the cartridge consist of an internal support frame for increasing structural rigidity and a bottom cartridge well frame that provides the molding for the return wells. The lower portion of the cartridge uses a 1% agarose media in a conductive solution to provide the electrical return path for each well. Through the return paths, the controller is able to provide real-time feedback of current and voltage to closely monitor and control experimental parameters.

Sample Handling Protocol. The protocol used to prepare samples for MALDI analysis is shown in Figure 2 and described in the following paragraphs.

Cartridge Preparation. Prior to sample addition, the wells of the 96-well cartridge were filled with 300 μL of running buffer. The solution in the return reservoirs was replaced with high-conductivity return solution.

Sample Preparation. The samples were stored at −80 °C until use. The samples were thawed at room temperature, thoroughly mixed 1:1 with sample buffer, and sonicated for 5 min. Twenty microliters of the sample solution was pipetted into each well, and the cartridge was loaded into the electrophoresis device.

Instrument Parameters. Experimental design was accomplished using a graphical software interface that allows for independent parameters to be applied to each of the 96 cartridge channels. At a given pH, proteins having a lower pI will carry a net negative charge. To move those proteins though the gel layer and capture them onto the reversed-phase layer below, the polarity of the system must be such that the anode is the common electrode and cathode is in the well. Upon the application of electrical current, the anionic proteins will migrate through the gel toward the capture surface. This mode of operation is called anion capture mode (ACM). If the polarity is reversed, only the proteins having pIs above that of the operating pH will migrate through the gel layers and be captured. With the use of this convention, this mode is subsequently referred to as cation capture mode (CCM). The device was programmed to accumulate the protein samples in ACM or CCM via the user interface. The appropriate time was set to run the wells for 2 C of total charge for peptides and proteins.

Buffer Replacement. If the charge setting was greater than 0.5 C of accumulated charge, the run was paused at 0.5 C to change out 200 μL of running buffer from each well with fresh running buffer to maintain consistent pH levels throughout the run. Similarly, if more than 1 C of charge is accumulated, the run was paused again at 1 C to replace the entire contents of the wells with 300 μL of fresh running buffer.

Slide Preparation and MALDI MS. At the conclusion of the protein accumulation step, the capture slide was removed from the cartridge, rinsed with deionized water, and soaked in 0.1% trifluoroacetic acid (Sigma, St. Louis, MO) for 45 min. The slide was then dried for 10 min, and matrix was spotted onto each of the 96 reversed-phase capture sites. Two 500 nL aliquots of saturated α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) in 50/50 acetonitrile/0.1% TFA were pipetted onto each reversed-phase capture site. The matrix was allowed to dry between additions. Following the second application of matrix, the capture slide was vacuum-desiccated for 10 min before MALDI MS analysis on an Autoflex III (Bruker Daltonics, Inc., Billerica, MA).

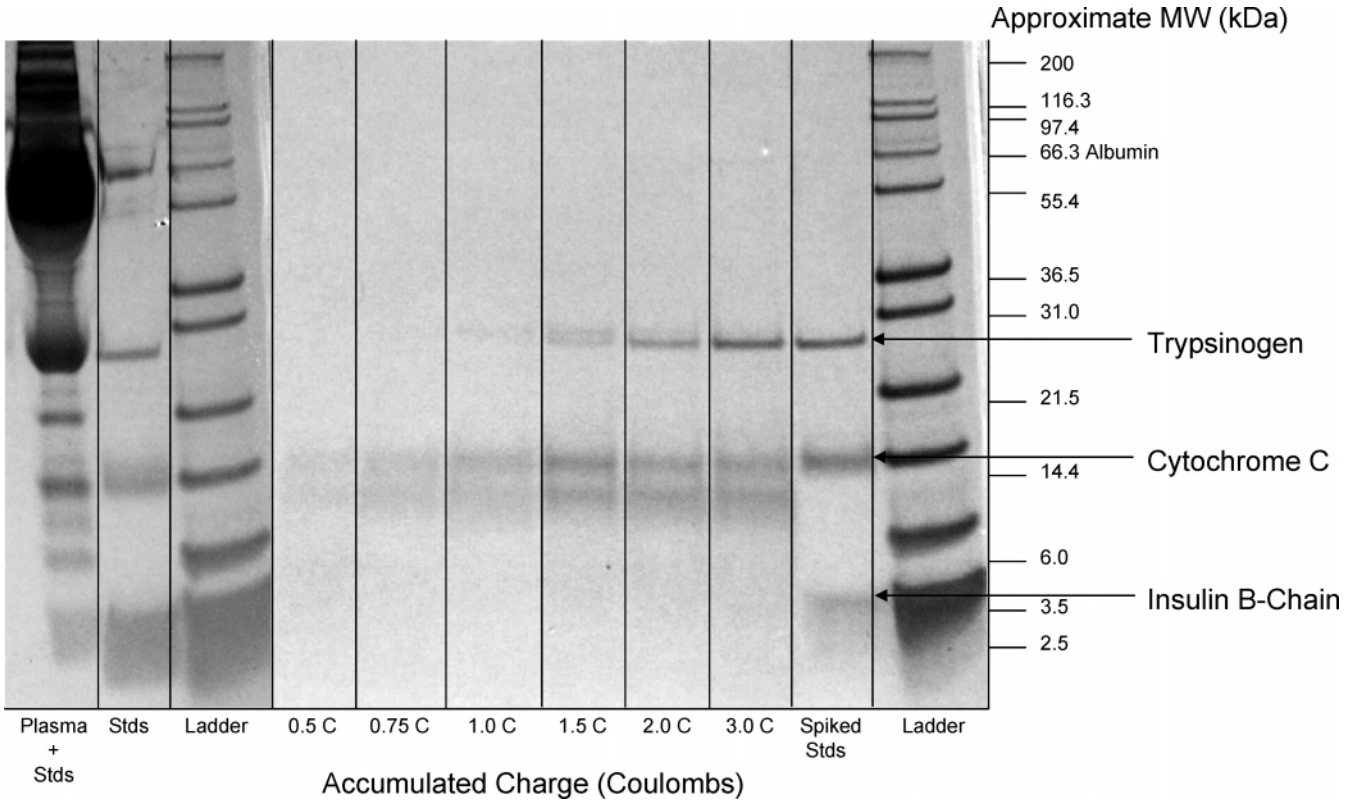

Figure 5. Depletion of high molecular weight proteins. The depletion of high molecular weight proteins from raw serum samples was confirmed using 1D gel electrophoresis.

Data Processing and Analysis. All mass spectra were baseline-subtracted and internally calibrated using the msbackadj and msalign functions provided by Matlab 7.1 and the corresponding Bioinformatics Toolbox (Mathworks, Natick, MA). The data in Figure 6 are reported as a percentage of the total ion current (TIC) between m/z 2000 and 20 000. Peak areas were computed using ProTS Data (Biodesix, Steamboat Springs, CO). Data are plotted using OriginPro 7.0 (OriginLab Corp., Northhampton, MA). Coefficients of variation were calculated using Microsoft Excel (Microsoft Corp., Redmond, WA).

Gel Electrophoresis. Depleted protein fractions were prepared using electrophoretic protocols detailed above. Six different protein fractions were prepared by accumulating 0.5, 0.75, 1.0, 1.5, 2.0, or 3.0 C of charge for the sample loaded into each well; each fractionation step was preformed in six replicate wells. The sample consisted of 5 μL of undiluted serum mixed with 1200 ng of insulin B-chain, 600 ng of insulin, 300 ng of cytochrome *c*, and 300 ng of trypsinogen. The sample was then mixed 1:1 with sample buffer and loaded into the well. Following electrophoretic preparation, the monolithic capture sites were extracted twice using 20 μL of 75/25 acetonitrile/0.1% TFA; the two extracts were combined. The extracted protein from six replicates were pooled into a single microtube and evaporated to dryness using a SpeedVac. The fractions were analyzed by one-dimensional (1D) SDS polyacrylamide gel electrophoresis using Novex Tris−glycine minigels (Invitrogen, Carlsbad, CA) as directed by the manufacturer. For comparison, the mixture containing serum and protein standards (diluted 1:10 to avoid overloading gel), the standard mix, and a commercial standard protein ladder (Mark 12, Invitrogen) were run directly by gel electrophoresis without electrophoretic sample preparation. The gels were stained using a colloidal blue staining kit (Invitrogen, Carlsbad, CA) as directed by the manufacturer.

RESULTS AND DISCUSSION

Concentration and Desalting of Dilute Protein Solutions. Sample concentration strictly determines whether physical measurements of a particular analyte are possible. If the sample is found to be too dilute for the measurement to proceed, steps are taken to increase the concentration. The physiological level of salt found in samples derived from biological sources imposes further limits on the analysis, and concentrating the samples often results in concentration of the associated salts, counteracting the benefit of increased analyte concentration. A strategy for removing interfering salts and concentrating dilute proteins using electrophoresis is described below.

Solutions of the standard protein, ACTH [18−39], in phosphate-buffered saline (PBS), were prepared in concentrations of 10 and 1 fmol/μL. Figure 3a shows the MALDI analysis of 1 μL of a 10 fmol/μL solution prepared using the dried droplet technique.[21] In this example, the presence of PBS negatively affected the sample preparation causing a decrease in overall sensitivity and an increase in the abundance of sodium and potassium adducts. Figure 3b shows the MALDI analysis of a 1 fmol/μL solution of ACTH using the dried droplet technique. Due to the interference of the buffer, it was not possible with the dried droplet technique at this concentration to measure ACTH with a signal-to-noise

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greater than 3. Parts c and d of Figure 3 show the MALDI analysis of 10 and 1 fmol/μL solutions of ACTH in PBS prepared using electrophoretic concentration and desalting. Ten microliters of ACTH, 100 and 10 fmol of total protein in Figure 3, parts c and d, respectively, were loaded into the electrophoretic cartridge and concentrated onto the capture surface. The conical shape of the well concentrated ACTH onto the 1 mm diameter reversed-phase capture surface. Since the capture surface had negligible affinity for the ionic compounds contained in PBS, these ions did not bind to the porous surface. After ACTH was accumulated, the capture slide was washed by immersion in 0.1% TFA to further remove salts. The MALDI matrix was then applied to the capture surface to liberate the captured protein and prepare the sample for MALDI analysis. The advantage of electrophoretic sample preparation is that the dilute protein solution was concentrated to a small area and PBS was removed. In this example, those benefits combined to yield a 5−10× increase in signal-to-noise and the ability to extend the limit of detection to lower concentrations.

Removal of High Molecular Weight Proteins and pI-Based Fractionation. Reduction of sample complexity is critical to the analysis of components of mixtures present in low abundance. The electrophoretic sample preparation method described herein allows for the fractionation of low molecular weight proteins based on isoelectric point (pI) while also depleting high molecular weight proteins.

As an example of this principle, a mixture of standard proteins was analyzed both before and after electrophoretic sample preparation. Proteins having distinct physical properties were selected for this example: bovine insulin (MW 5734, pI 7.6), human ACTH [18−39] (MW 2466, pI 4.1), bovine serum albumin (BSA) (MW 66 472, pI 5.7).[22] The mixture, comprised of a total amount of 145 pmol of BSA and 5 pmol each of insulin and ACTH, was loaded into two different electrophoretic wells. The two wells were run at opposite polarity maintaining conditions of constant current at 0.5 mA for 1.1 h (2 C of total charge). At this operating pH, insulin was positively charged, whereas ACTH was negatively charged; therefore, these two proteins were captured in CCM and ACM, respectively. Although albumin had a net positive charge, it was retained by the polyacrylamide gel layer due to its low electrophoretic mobility. This protein did not reach the capture slide during the same time required to capture the smaller, more mobile peptides. Therefore, by controlling the time of the experiment, albumin was depleted from the sample. After disassembly of the cartridge and addition of MALDI matrix, the captured proteins were measured by MALDI MS.

Figure 4a shows the results from this experiment. Insulin was captured, and both the singly and doubly charged states of insulin were observed when the mixture was subjected to CCM prior to MALDI analysis. Whereas insulin was clearly observed, the other cationic protein, BSA, was excluded because of its low electrophoretic mobility. Although ACTH has high electrophoretic mobility due to its low molecular weight, at the operating pH ACTH was anionic; therefore, this peptide did not migrate toward the capture surface and was excluded from protein capture. ACTH was only observed when the sample was subjected to ACM, in which case insulin was excluded. For comparison, the standard

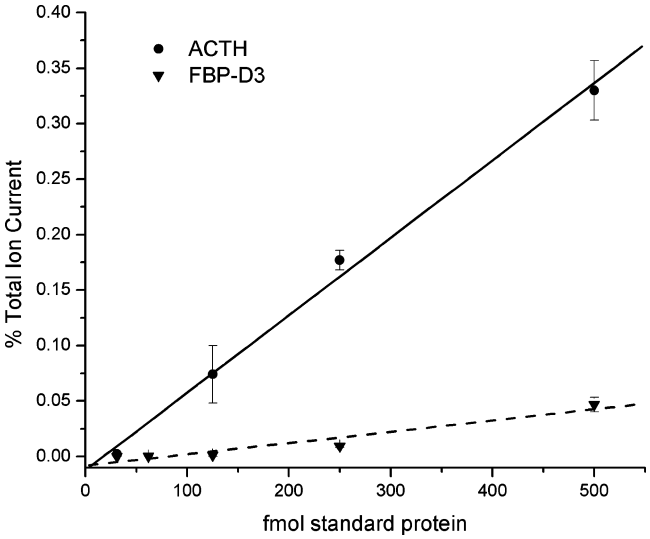

Figure 6. Instrument response is linear with concentration. Dilution series of standard proteins spiked into serum were analyzed using electrophoretic concentration. The MALDI response as a function of total standard protein loaded into the electrophoresis well was linear across 2 orders of magnitude. The intensities of the protein standards were normalized to the total ion current (TIC) for each sample.

mixture was analyzed directly using the dried droplet technique. This example demonstrated the use of this electrophoretic sample preparation technique to affect protein separation based on the pI of the analyte in a way that was directly compatible with MALDI MS.

To further demonstrate the principles of fractionation of the low molecular weight proteome and depletion of high molecular weight proteins, human serum was prepared for MALDI analysis using the same strategy applied to the standard mixture. The direct analysis of unprocessed human serum by MALDI MS allowed for only a limited number of proteins to be analyzed. The presence of highly abundant proteins in unprocessed human serum prevented the measurement of the lower abundance species due to ion suppression effects and limited the dynamic range of MALDI MS.[23] Since many of the high-abundance proteins in human serum are high molecular weight species, these species were separated from the low molecular weight proteins according to their electrophoretic mobility. Figure 4b shows the fractionation of human serum with the corresponding depletion of human serum albumin.

Comparison of ACM and CCM sample preparation showed that these two fractions are comprised of a distinct subset of the low molecular weight proteome. Since these fractions were distinguished by the pI of the proteins, these fractions were unique. Low molecular weight peaks observed in the dried droplet preparation that were not observed in the ACM of CCM fractions can be assigned to multiple charge states of depleted high molecular weight proteins or have electrophoretic mobility too low to be efficiently transported under the conditions of this experiment. Additionally, as in the example provided by the standard proteins, the high molecular weight proteins (e.g., albumin) that account for the majority of ion current in the direct analysis were absent from the spectrum.

(22) Protein isoelectric points (pI) were calculated using Compute pI/Mw tool (ExPASy, S.I.o.B., http://www.expasy.org/tools/pi_tool.html).

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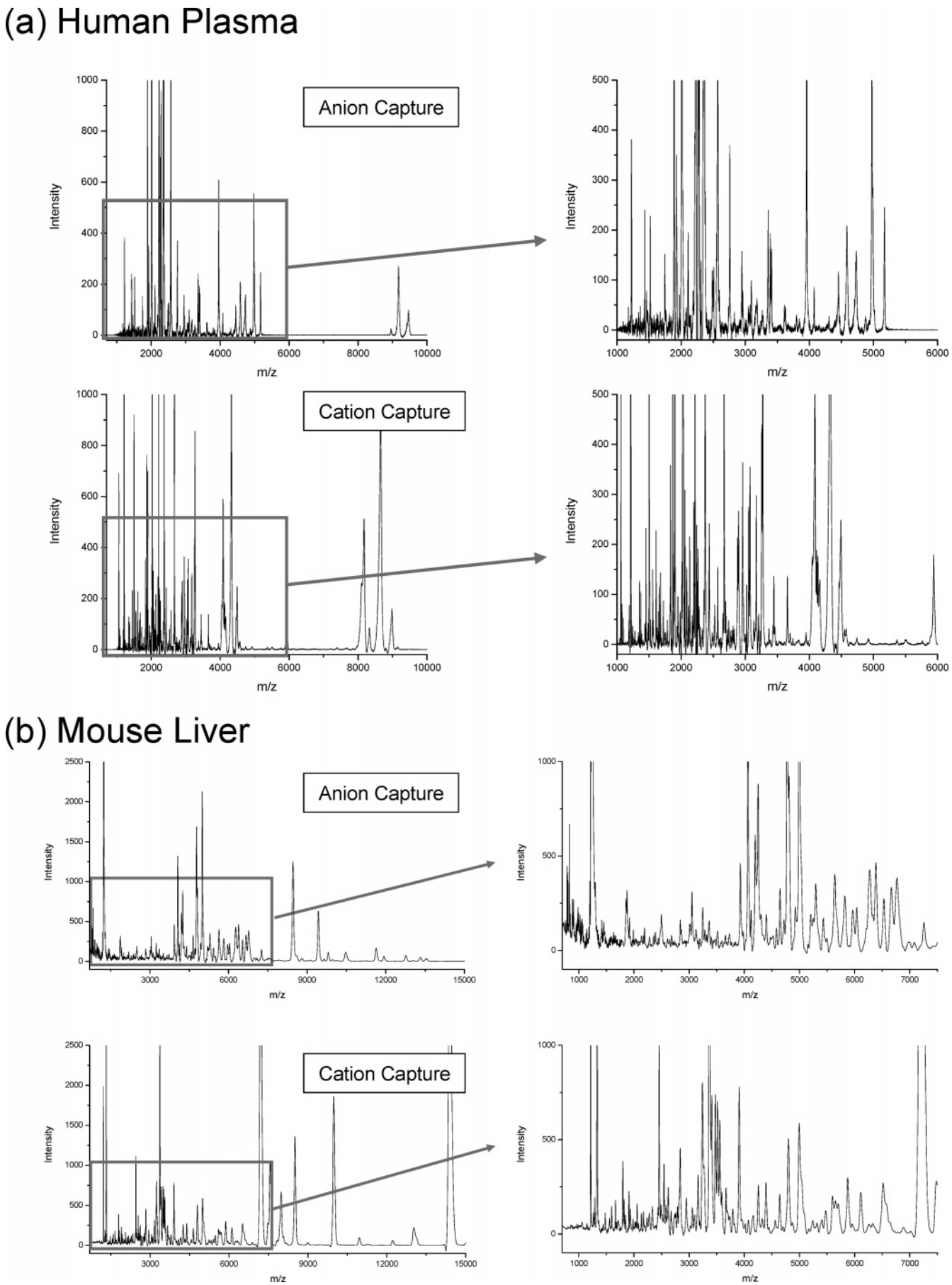

Figure 7. Fractionation, desalting, and depletion of clinical samples for MALDI MS. Two different biological samples, human plasma and mouse liver extract, were subjected to electrophoretic sample preparation and analyzed by MALDI MS.

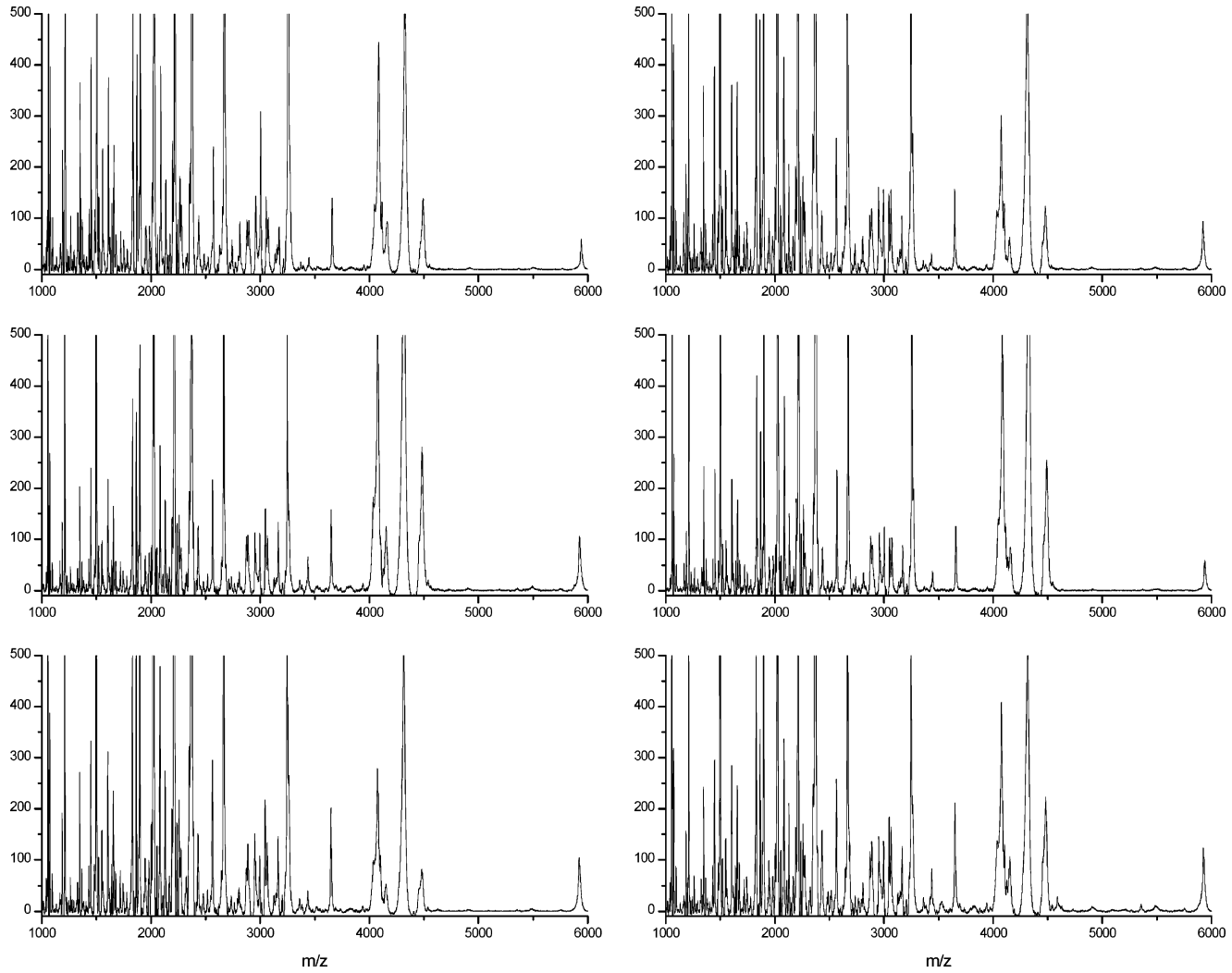

Figure 8. Reproducibility of electrophoretic sample preparation/MALDI MS. The MALDI profile of six replicate electrophoretic sample preparation fractions of human plasma. Samples were prepared using CCM, passing 1.0 C of electrophoretic charge.

The depletion of serum albumin from the sample was confirmed by off-line extraction of the capture surface and 1D gel electrophoresis (Figure 5). The depletion of high molecular weight proteins from raw serum samples was confirmed for up to 3.0 C of accumulated charge in CCM fractions using 1D gel electrophoresis. Human serum samples were spiked with known amounts of protein standards and subjected to electrophoretic sample preparation for 0.5−3.0 C of accumulated charge. Additionally, unprocessed human serum (diluted 1:10) containing spiked standards, protein standards, and a commercial molecular weight ladder were analyzed for reference. Under these conditions, electrophoretic sample preparation effectively removes proteins larger than trypsinogen (23.9 kDa). The high molecular weight proteins that comprise human serum are observed in the undepleted sample. However even at extended run times (>2.0 C), these proteins, including serum albumin, are not observed in the extracts of proteins from the capture slide, though the results in Figure 4 demonstrate a significant amount of low molecular protein was captured.

Sensitivity, Reproducibility, and Linear Dynamic Range in Complex Samples. Standard proteins were used to assess the sample preparation for proteins measured from a complex mixture. The standard proteins ACTH and FBP were spiked to known concentrations into separate vials of human serum. These spiked samples were prepared for MALDI analysis as described using electrophoretic sample preparation. The standard peak areas as a percentage of the TIC (m/z 2−20 000) were used to construct a response curve as a function of protein concentration. It was found that the response of the instrument with respect to protein concentration for ACTH and FBP spiked into human serum was linear over approximately 2 orders of magnitude with R^2 equal to 0.99 and 0.96, respectively (Figure 6). The limit of detection for these proteins was found to be 1 fmol of total ACTH and 5 fmol of total FBP in human serum using this protocol. The reproducibility of the assay was determined by spiking ACTH and FBP into human serum to a final concentration of 333 fmol/μL of each component. Five microliters of the spiked serum solution was electrophoretically concentrated onto the capture surface for 2.0 C of total charge. Twelve independent sample preparations were measured. The coefficients of variation of ACTH and FBP measured from human serum are 30% and 19%, respectively.

Integrated Electrophoretic Sample Preparation Enhances Profiling by MALDI MS. Molecular profiling by mass spectrometry has helped discover potential biomarkers[24,25] and stratify patients into clinically useful groups[26−28] and has provided new information about the biological changes that occur as a result of

disease progression.[29−31] The technique is fast, requires minimal amount of sample, and provides rich data to the researcher or clinician. A disadvantage of the technique is that the protocols for sample collection, and methodologies for sample preparation, are still being developed. To date, there is still much to learn about how to improve on these techniques. Sample preparation steps performed using conventional technologies are time-consuming, difficult to automate, and require large volumes of sample (>100−1000 μL). The technique described here for electrophoretic sample preparation integrates the steps of depletion, fractionation, desalting, and concentration and, therefore, has much to offer this field by providing a workflow that combines all of these necessary steps while requiring minimal amounts of (<10 μL) of sample. These key steps are integrated into an automated platform that enables both high sample throughput and high data reproducibility.

Application of this electrophoretic sample preparation technique for MALDI profiling of complex biological samples was demonstrated for both human plasma and mouse liver extract. Two different fractions from human plasma and mouse liver extract were prepared for MALDI MS analysis by electrophoretic sample processing. Each sample was subjected to both ACM and CCM for 2.0 C of total accumulated charge, followed by MALDI MS analysis. Figure 7 shows the resulting MALDI spectra. As in the example shown in Figure 4, these complex mixtures are known to contain proteins spanning a wide variety of molecular weights, some of which severely suppress ionization of the low molecular weight analytes in the sample. The electrophoretic sample preparation technique described herein produced the effective depletion of proteins having molecular weights greater than ca. 25 kDa while providing a means to remove salts and concentrate the fraction of interest prior to MS analysis. This allowed for the detection of hundreds of ions in each fraction. Figure 8 shows six independent preparations of human plasma using ACM that were profiled using MALDI MS. The resulting spectra exhibited similarity in both the number of peaks in the MALDI profile and the absolute and relative intensities of these peaks.

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CONCLUSION

Experiments were performed to demonstrate the principles and parameters of operation and to highlight the performance characteristics of a novel electrophoretic sample preparation system. Electrophoretic depletion, concentration, fractionation, and desalting of samples were performed in a 96-well cartridge, which is activated within a 96-channel, independently controllable, electrophoresis instrument. We demonstrate that the electrophoretic system described herein may be used to reproducibly remove high-abundance, high molecular weight proteins that have limited electrophoretic mobilities. The system was also shown to fractionate proteins according to pI and concentrate analytes quantitatively onto a slide designed to fit directly into a commercially available MALDI MS. Integrated concentration and desalting of proteins by this methodology was shown to enhance sensitivity. The measurement of unlabeled standard proteins in human serum was demonstrated to be reproducible with coefficients of variation less than 30%. Furthermore, the system was shown to be useful for the reproducible preparation of human plasma and mouse liver extracts for MALDI profiling. In all cases, the total time for the preparation of a set of 96 samples using this method was approximately 1 h. Future work will focus on both top-down and bottom-up identification of proteins captured using this electrophoretic sample preparation method to enable faster discovery, identification, and validation of biomarkers by MS. Additional work will also focus on the development of quantitative small-molecule assays from tissue, plasma, and other relevant biological fluids, further taking advantage of the ability to deplete and concentrate dilute samples in high throughput.

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This article was released ASAP on March 11, 2008. Figure 4 was replaced, and the correct version was posted March 12, 2008.

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