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A Multidimensional Differential Proteomic Platform Using Dual-Phase Ion-Exchange Chromatography−Polyacrylamide Gel Electrophoresis/Reversed-Phase Liquid Chromatography Tandem Mass Spectrometry

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Differential proteomic analysis has arisen as a large-scale means to discern proteome-wide changes upon treatment, injury, or disease. Tandem protein separation methods are required for large-scale differential proteomic analysis. Here, a novel multidimensional platform for resolving and differentially analyzing complex biological samples is presented. The platform, collectively termed CAX-PAGE/RPLC-MSMS, combines biphasic ion-exchange chromatography with polyacrylamide gel electrophoresis for protein separation, quantification, and differential band targeting, followed by capillary reversed-phase liquid chromatography and data-dependent tandem mass spectrometry for quantitative and qualitative peptide analysis. CAX-PAGE provides high protein resolving power with a theoretical peak capacity of 3570, extendable to 7600, a wide protein mass range verified from 16 to 273 kDa, and reproducible differential sample comparison without the added expense of fluorescent dyes and imaging equipment. Demonstrated using a neuroproteomic model, CAX-PAGE revealed an increased number of differential proteins, 137, compared with 82 found by 2D difference gel electrophoresis. When combined with RPLC-MSMS for protein identification, an additional quantification step is performed for internal validation, confirming a 2-fold or greater change in 89% of identified differential targets.

Proteomic characterization reveals protein dynamics incomprehensible at the genetic level[1−4] and essential to understanding cellular function under normal or challenged conditions.[5,6] Application to the nervous system (neuroproteomics) in health and disease has recently begun;[7] however, general difficulties persist, limiting utility. Biological complexity is the principal concern.[8] The sheer number of proteins (50 000 or more)[9] and the wide dynamic concentration range overwhelm today's technology. Many rely on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for high-resolution protein separations.[10,11] Around since the mid 1970s, 2D-PAGE is the staple of proteomic analysis, owing to its high resolving power. However, gel-to-gel reproducibility is a problem when performing differential analysis by 2D-PAGE. 2D difference gel electrophoresis (2D-DIGE)[12−14] was introduced to alleviate this problem. Two samples are differentially labeled using matched cyanine fluorophores (Cy3 and Cy5) and combined for separation on the same gel. Differential quantification is determined by subtracting two images taken at distinct excitation and emission wavelengths for each fluorophore. Associated proteins are identified using 2D-PAGE databases or mass spectrometry, though sample mixing prevents independent mass spectrometry analysis. Another limitation is the expense of the cyanine dyes and fluorescence scanner employed. Further complications have led to the development of normalization protocols using a third cyanine dye (Cy2) for sample multiplexing,[15,16] and engineering

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of cysteine labeling saturation versions of Cy3 and Cy5 tags,[17,18] adding complexity and cost. Such limitations of 2D-PAGE technology have prompted some to explore alternative technologies.[19−23]

Of recent protein separation strategies, free-flow electrophoresis (FFE) is notable for effective sample fractionation by isoelectric point (pI) prior to either 1D-PAGE[24] or reversed-phase liquid chromatography (RPLC).[25] In the latter case, the large number of FFE fractions (80), combined with the high resolving power of RPLC, resulted in the large theoretical peak capacity of 6720, comparable to that of traditional 2D-PAGE (10^3−10^4). Other examples of mixed-mode liquid chromatography, as reviewed elsewhere,[19,26] include chromatofocusing,[27−31] liquid-phase isoelectric focusing,[30,32−34] size exclusion,[27,29] ion exchange,[35] and a combined size exclusion−strong cation-exchange medium,[36] all as first dimensions prior to RPLC. Still others have tried using capillary electrophoresis in a two-dimensional mode[37] or in combination with liquid chromatography.[38,39] All approaches provide reasonable resolving power; however, none as yet have met the needed capacity to resolve an entire proteome (>10^4).

It is apparent from these efforts that future proteomic studies will involve varying multidimensional separations.[9,10,23] Ion-exchange chromatography has often been employed in 2D separations, prior to PAGE[40,41] or orthogonal chromatographies.[11,42] One drawback to ion exchange is that a significant portion of protein from a biological sample will not bind to either a positively charged anion or a negatively charged cation exchanger due to incongruent protein surface charge. We hypothesized that more protein could be retained by combining cationic and anionic exchange media (CAX), either by placing columns in series or by mixing both media together. This principle had been previously demonstrated by El Rassi and Horvath with the separation of simple protein mixtures.[43] Remarkably, this idea received no further attention, likely because no pertinent application was then foreseen.

In translational proteomic applications, researchers are particularly interested in identifying protein expression differences or changes associated with a particular disease, injury, or treatment. This requires, in addition to proteome resolution, a means to detect and quantify differences between two or more samples (e.g., control and treated). The presented platform is designed specifically for this purpose. Combined cation−anionic exchange placed in series with polyacrylamide gel electrophoresis (CAX-PAGE) provides quantified selection of differential proteins, subsequently identified and further quantified by reversed-phase liquid chromatography tandem mass spectrometry (RPLC-MSMS). In this study, we evaluate CAX-PAGE/RPLC-MSMS by comparing the proteomes of cerebellum and cortex tissues, a model system for future application to biomarker discovery in brain injury paradigms. Retention, reproducibility, sample recovery, and resolving power are examined. Differential analysis is compared between CAX-PAGE and the current benchmark 2D-DIGE. Finally, the dual differential quantification strategy is verified using differential and nondifferential gel band pairs.

EXPERIMENTAL SECTION

Sample Preparation. Male Sprague−Dawley rats (five) purchased from Harlan (Indianapolis, IN) were acclimated for 7 days prior to sacrificing. The rats were then anesthetized with 4% isoflurane in a carrier gas of 1:1 O_2/N_2O (4 min) and were perfused with 0.9% saline transcardially prior to decapitation via guillotine. Cerebellum and cortex brain regions were dissected and transferred to microfuge tubes kept on dry ice. Sections were snap frozen in liquid nitrogen and then ground to a fine powder via mortar and pestle kept on dry ice. Powder was scraped into chilled microfuge tubes to which 0.1% SDS lysis buffer (300 μL) was added, containing 150 mM sodium chloride, 3 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethyleneglycol bis(aminoethyl ether) tetraacetic acid (EGTA), 1% ethoxylated octylphenol (all from Sigma-Aldrich, St. Louis, MO), one tablet of Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany), and 1 mM sodium vanadate (Fisher Scientific, Fair Lawn, NJ), with the sample solution brought to neutral pH using Tris-base (Sigma-Aldrich). Cell lysis was conducted over 3 h at 4 °C with hourly vortexing. Lysates were spun down at 14 000 rpm at 4 °C for 10 min to remove DNA, lipids, and particulates. Supernatants were then filtered through 0.1-μm Millipore Ultrafree-MC filters (Bedford, MA) for further clarification. Protein concentrations were determined via Bio-Rad DC Protein Assay

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(Hercules, CA), after which pooled ($n = 5$) 1-mg cortex and 1-mg cerebellum samples were prepared for differential comparison.

Anion/Cation-Exchange Chromatography. A Bio-Rad Biologic DuoFlow system with QuadTec UV detector and BioFrac fraction collector was used with Uno series SAX (Q1) and SCX (S1) prepacked ion-exchange columns. For CAX chromatography, S1 and Q1 columns were placed in series. Buffers consisted of ice cold 20 mM Tris-HCl (pH 7.5 molecular biology grade, Fisher Scientific) in HPLC water (Burdick & Jackson, Muskegon, MI) (mobile phase A). A two-step elution gradient was performed with 1 M NaCl (Fisher Scientific, crystalline 99.8% certified) (mobile phase B) at a flow rate of 1 mL/min with a linear transition from 0 to 15% B in 12.5 mL, followed by 15 to 50% B in 7 mL. The composition was held at 50% B for 2 mL and then reequilibrated to 0% B in 1 mL. An optimized three-step gradient was used for differential analysis. At a flow rate of 1 mL/min, the first linear transition was from 0 to 5% B in 2.5 mL, from 5 to 15% in 9 mL, and followed by 15 to 50% in 10 mL. Again the composition was held at 50% B for 2 mL and reequilibrated to 0% B in 1 mL. UV chromatograms were collected at a wavelength of 280 nm. Twenty-five 1-mL fractions were autonomously collected via the BioFrac fraction collector into 1.5-mL screw-cap microfuge tubes (RPI, Mt. Prospect, IL) kept on ice.

1D-SDS−PAGE. Fractions collected during ion-exchange chromatography were concentrated via Millipore YM-30 centrifugal filters, which were demonstrated to retain proteins of >5 kDa. Each fraction was spun through filters prewashed with 500 μL of HPLC water as two 500-μL sequential portions at 13 500 rpm for 20 min. Laemmli sample buffer (Bio-Rad, with 5% BME) was added to the retentate and incubated for 10 min prior to collection by centrifugation at 3500 rpm for 3 min. The supernatant for each fraction was boiled at 85 °C for 2 min and then loaded onto an Invitrogen Novex 10−20% gradient, 1 mm wide, 10-well gels in a Tris-glycine buffer system (Carlsbad, CA) alongside a lane of Amersham Biosciences Rainbow Marker (Piscataway, NJ) for initial studies. Differential analysis between cerebellum and cortex tissues was performed by pairing fractions for loading side by side (i.e., cerebellum fraction 1 next to cortex fraction 1, etc.) on Bio-Rad Criterion 10−20% gradient, 1-mm 18-well gels in a Tris-glycine buffer system.

Protein Recovery and Retention of CAX. Protein recovery was evaluated with SAX, SCX, and combined-phase CAX. A constant protein amount (750 μg) in a 100-μL injection of the previously described cerebellum lysate was loaded. A 1 mL/min isocratic flow was maintained for 9 min to allow unretained proteins to flow through. The mobile-phase composition was then increased in 1 min and held for 9 min at 50% B to elute bound proteins collected in a normal gradient run. This was followed by an additional increase to 100% B in 1 min, which was held for another 9 min to check for additional protein. Throughout, UV absorbance was monitored at 280 nm, and 1-mL fractions were collected, each concentrated using Millipore YM-30 centrifugal filters and analyzed via Bio-Rad DC protein assay.

CAX-PAGE Coomassie Blue Imaging. Gels were visualized by regressive staining using concentrated Bio-Rad Coomassie Blue R250 for 20 min and destained in 40% HPLC grade ethanol (EM Science, Gibbstown, NJ)/10% acetic acid (ACS Plus grade, Fisher) for ~2 h. Images were captured with an Epson 1640 XL flatbed scanner (Long Beach, CA) and saved as eight-bit TIFF files. Differential analysis of Coomassie Blue-stained gels was performed using Phoretix 1D (Nonlinear Dynamics, Newcastle, U.K.) gel image analysis software. Band intensities were automatically calculated and manually verified for bands above a preset threshold. Intensities were output to Excel (Microsoft, Redmond, WA) for differential evaluation. Manual confirmation was aided by superimposing cerebellum lanes false colored red over adjacent cortex lanes false colored green, creating gradient color lanes for each fraction. Image contrast was improved by adjusting RGB color balance to emphasize mid-tones over shadows.

2D-DIGE. Cerebellum and cortex samples (1 mg each) were prepared from the same pooled material used for CAX-PAGE. Each was adjusted to 2% SDS, followed by TCA precipitation. The pellet was air-dried and resuspended in 150 μL of pH 8.8 urea lysis buffer. Benzonase Nuclease (Novagen, Madison, WI) and 5 mM magnesium chloride (Fisher) were added, incubating the mixture for 30 min on ice to degrade nucleic acids. The solution was clarified by centrifugation with a Beckman Coulter (Fullerton, CA) Airfuge at 100 000 g for 30 min. The supernatant was dialyzed against the urea lysis buffer overnight at room temperature. A 50-μg portion of cortex and cerebellum lysate was labeled with Cy3 and Cy5 minimal dyes (Amersham Biosciences), respectively, using the manufacturer's suggested protocol. Cyanine-labeled samples were combined with 275 μg each of unlabeled cortex and cerebellum lysates. The solution was adjusted to 0.2% IPG pH 3−10 buffer (Amersham Biosciences) and 100 mM DTT with a trace of Orange G stain (Fisher). An 18-cm nonlinear pH 3−10 IPG strip (Amersham Biosciences) was rehydrated in the mixed sample under oil overnight at room temperature. Proteins were focused on the strip at 8 kV until migration was complete (65 kV h). Proteins in the strip were reduced with 100 mM DTT in the reaction buffer, 50 mM pH 6.8 Tris-HCl, 6 M urea, 30% glycerol, and 2% SDS. Alkylation was performed with 2.5% iodoacetamide in the same reaction buffer. The strip was mounted atop a Bio-Rad precise 8−16% tris-glycine gel and run for 6 h at 25 mA and 24 °C. Separate Cy3 and Cy5 images were collected on an Amersham Typhoon 8600 fluorescence imager and processed with Phoretix 2D software (Nonlinear Dynamics).

In−Gel Digestion. Gels were thoroughly rinsed with HPLC water. Target differential bands were excised and dissected into four cubes and placed in 0.5-mL tubes. Each was washed with HPLC water and then 50% 100 mM ammonium bicarbonate (Fisher)/50% acetonitrile (Burdick-Jackson, HPLC grade). Pieces were dehydrated with 100% acetonitrile and dried by Speedvac (ISS110, Thermo Savant, Milford, MA). Cubes were rehydrated with 50 μL of 10 mM dithiothreitol (Calbiochem, San Diego, CA) in 50 mM ammonium bicarbonate and incubated for 30 min at 56 °C. Dithiothreitol was replaced by 50 μL of 55 mM iodoacetamide (Calbiochem) in 50 mM ammonium bicarbonate and reacted for 30 min in the dark at room temperature. Gel pieces were washed with 50 mM ammonium bicarbonate and dehydrated with 100% acetonitrile followed by Speedvac. Rehydration was performed with 15 μL of a 12.5 ng/μL trypsin solution (Promega Gold, Madison, WI) for 30 min at 4 °C, and then 20 μL of 50 mM ammonium bicarbonate was added and left at 37 °C overnight for digestion. The supernatant and two 50% acetonitrile/5% acetic acid extractions were placed into a new tube. The peptide extract was

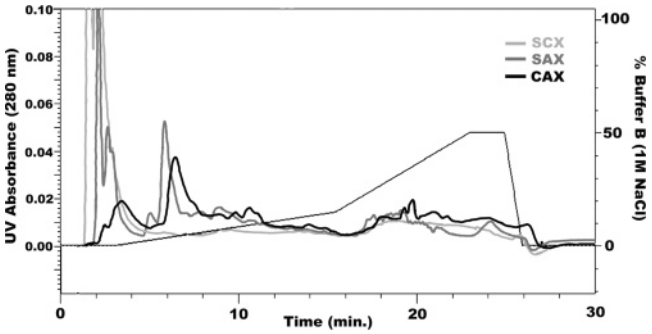

Figure 1. Tandem CAX chromatography with superior protein retention. SCX, SAX, and tandem CAX chromatograms of 1 mg of rat cerebellum lysate are shown overlaid with the same 280-nm absorbance scale. A 5-fold absorbance reduction is observed during flow-through (1−5 min) for CAX, as compared to SCX or SAX.

dried by Speedvac and resuspended in 20 μL of 4% acetonitrile/0.4% acetic acid.

Capillary RPLC-MSMS. Capillary RPLC tandem ion trap mass spectrometry[44] was employed for protein identification as described previously with some modifications.[45] Nanoflow reversed-phase chromatography was performed with a 100 μm i.d. × 5 cm capillary column packed in-house with Agilent (Palo Alto, CA) 3-μm C-18 particles behind an Upchurch 0.5-μm PEEK microfilter assembly. The integrated polymerized frit was replaced with a pulled emitter made from 25-μm-i.d. capillary affixed to the other end of the microfilter assembly. Thirty-minute gradients, 4% HPLC acetonitrile/0.4% acetic acid (Fisher, Optima grade) to 60% acetonitrile/0.4% acetic acid, were used to elute tryptic peptides. Tandem mass spectra were collected on a ThermoElectron (San Jose, CA) LCQ Deca XP-Plus using data-dependent analysis.[19] Collected data were searched against the trypsin indexed complete NCBI RefSeq mammalian database filtered for rat taxonomy using ThermoElectron Bioworks Browser (version 3.1). We report protein identifications made with two or more peptides matched with strict cross-correlation values of Xc ≥ 1.8, 2.5, and 3.5 for +1, +2, and +3 charge states, respectively.[46] Data filtering was performed with DTASelect, and cerebellum versus cortex MSMS data were compared using Contrast software.[46]

RESULTS AND DISCUSSION

CAX Chromatography. The majority of proteins in biological samples, such as tissue lysates or body fluids, retain regions of significant charge on their external surfaces when at physiological pH. Though regions of external charge act independent of net charge,[47] the improvement associated with CAX chromatography can generally be explained through retention of both positively and negatively charged proteins rather than predominantly those of one net polarity. Figure 1 illustrates the difference between SCX, SAX, and CAX chromatography of a complex rat brain cortex tissue lysate. With single ion exchangers, a significant portion of the proteome is unretained, evidenced by a large peak (flow-through) at the beginning of the chromatograms and verified by SDS−PAGE. CAX binds most proteins, leaving the few with low charge density in the flow-through, which partially resolve through hydrophobic interactions[48] in the first four fractions (4-mL window). A two-stage gradient (0−15% B in 12 min, 15−50% B in 7 min) was optimized based on uniform UV absorption throughout the chromatogram, which was presumed to distribute protein for maximal resolution evenly across 25 1-mL fractions, a volume selected for compatibility with column flow rate, the fraction collector, and CAX half-height peak width (∼0.25 mL).

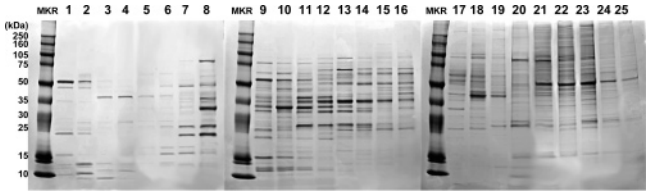

Figure 2. Rat cerebellum proteome visualized on 1D-PAGE following CAX fractionation. One milligram of rat cerebellum brain lysate was divided into 25 CAX fractions, each resolved further by 1D-polyacrylamide gel electrophoresis (10−20% acrylamide). Protein bands were then visualized by Coomassie Blue staining. MKR indicates molecular weight marker lanes.

Coupling to 1D-PAGE. Following CAX chromatography with 1D-PAGE further resolved the brain lysate by protein mass. Microtube centrifugal filters were used to reduce the 1-mL fractions to 15 ± 5 μL to which 20 μL of 2× sample buffer was added for gel loading. Random fractions would on occasion run slowly through the filter, potentially due to membrane pore size variability, though no effect on protein retention was observed. Figure 2, visualized by Coomassie Blue stain, revealed that optimizing the CAX gradient based on uniform UV absorption resulted in high protein density toward the end of the CAX separation. The significant band overlap necessitated gradient reoptimization for more effective separation and differential analysis.

CAX-PAGE Reproducibility. The reproducibility of separations is often a limiting factor for differential analysis, particularly with 2D-PAGE.[49,50] The reproducibility of CAX-PAGE was evaluated with triplicate runs of the same rat cerebellum sample. Sequential chromatograms shown in Figure 3a overlap without significant deviation. Next, three groups of fractions spaced evenly at the beginning (1, 4, and 7), middle (10, 13, and 16), and end of the separation (18, 20, and 24) were loaded in triplicate onto 1D-PAGE (Figure 3b) and showed identical protein complements and an average intensity correlation of 94% (Phoretix 1D software). Run-to-run separation remained consistent when the experiment was repeated at a later date (data not shown); however, a nonuniform shift in retention time relative to the data in Figure 3 was observed. Peak shifting is typical of column chromatography and occurs from environmental, buffer, and column aging factors. Thus, runs must be performed sequentially when comparing samples.

CAX-PAGE Protein Recovery and Retention. Though ion exchange is known to provide high protein recovery, the pos-

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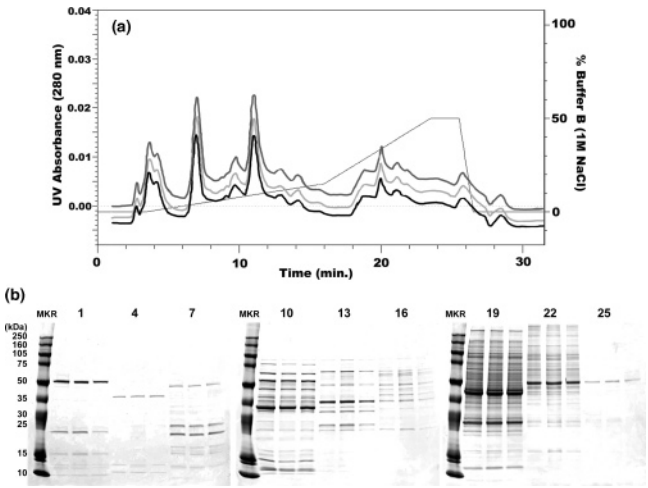

Figure 3. Reproducibility of CAX-PAGE protein separations. (a) Chromatogram of rat cerebellum brain tissue lysate (1 mg of protein) run sequentially in triplicate by CAX. (b) Selected fractions (paired as indicated) from the three replicate CAX runs resolved and visualized side by side on 1D-PAGE. Protein complement remained constant while band intensity varied on average by only 6%.

sibility of exacerbated protein loss was of concern when SAX and SCX phases were combined. A protein recovery of 88 ± 6% after CAX was determined by protein assay while accounting for the buffer change from initial lysate to CAX fractions. Unexpectedly, the sample recovery observed for SAX and SCX was lower than that of CAX, suggesting some variability between protein assay measurements. Using densitometric analysis after 1D-PAGE, microfiltration alone showed an 11 ± 5% sample reduction, the major source of protein loss for this method. In general, reported 2D separations do not discuss protein recovery, with the exception of FFE-IEF shown to have 87.6% protein recovered.[22] With 2D-PAGE, significant protein loss is known to occur, particularly for proteins above 100 kDa, with pI values outside of the 3−10 range and those too hydrophobic for solubalizition in the urea/chaps IPG buffer.[10,11,61]

CAX chromatography showed increased protein retention over SAX and SCX. In practice, 88% of recovered protein was retained by CAX for gradient elution in comparison with 66% for SAX and 47% for SCX as determined by protein assay. Peak area calculations proved similar, with CAX having had the largest retained peak area of 10 (84% of total area) compared with 5.2 (55%) for SAX

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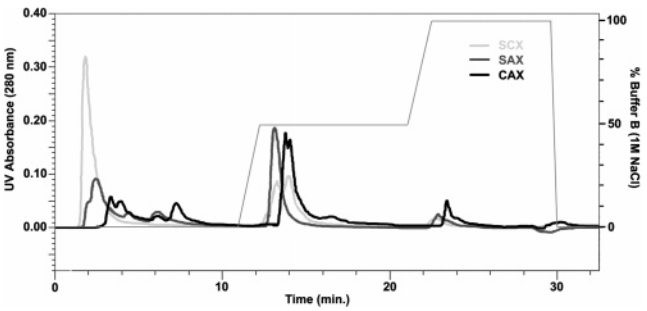

Figure 4. Recovery and retention of CAX-PAGE separation. Chromatogram of rat cerebellum tissue lysate (750 μg) performed with SCX, SAX, and CAX with two-step elution processes.

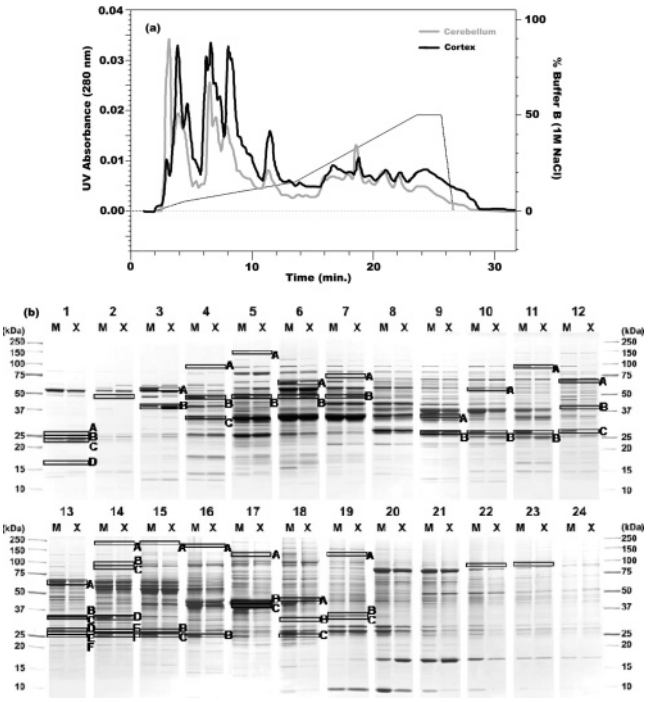

Figure 5. Comparison of rat cerebellum and cortex proteomes via sequential CAX and side-by-side 1D-PAGE. (a) Overlay of cerebellum and cortex CAX chromatograms at 280 nm. (b) Side-by-side (M, cerebellum on left; X, cortex on right) pairing of 25 fractions run on 1D-PAGE. Boxed bands were excised for protein identification; note letter labeling for correlation with Tables 1 and 2.

and 5.7 (37%) for SCX. Increased retention, the motivation for CAX, affords the ability to evenly distribute complex protein mixtures across an expandable number of fractions using gradient optimization (see Figure 4).

Differential Expression Analysis. The potential of CAX-PAGE is realized during differential expression profiling. Chromatographic differences are observed in Figure 5a between cerebellum and cortex lysates sequentially separated by CAX. For differential analysis, fractions from each run are paired and run side by side on 1D-PAGE (Figure 5b), whereby problems of gel-to-gel reproducibility are avoided through comparing matching fractions on the same gel. Side-by-side fraction pairing also allows for direct visualization of differential expression using simple, cost-efficient, visible stains (e.g., Coomassie Blue or silver).[40] Fluorescent stains such as Sypro Ruby and Deep Purple also work well, though they require a more expensive fluorescence scanner (three times the cost of the liquid chromatography system). The

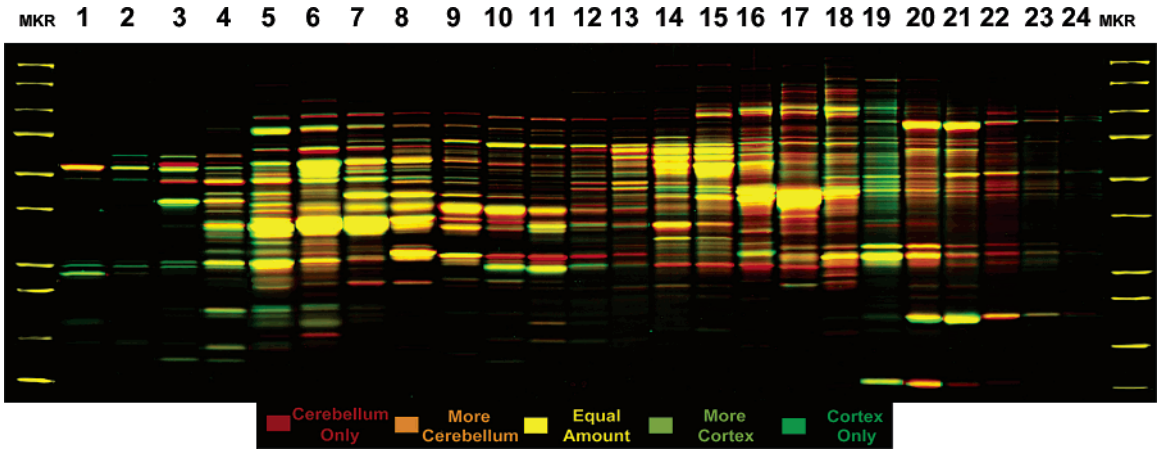

Figure 6. Colorized rat cerebellum−cortex differential proteome display after CAX-PAGE. The colorized display was performed by overlaying adjacent lanes from Figure 5b false-colored red for cerebellum and green for cortex. Color aids in manual inspection of the differential separation.

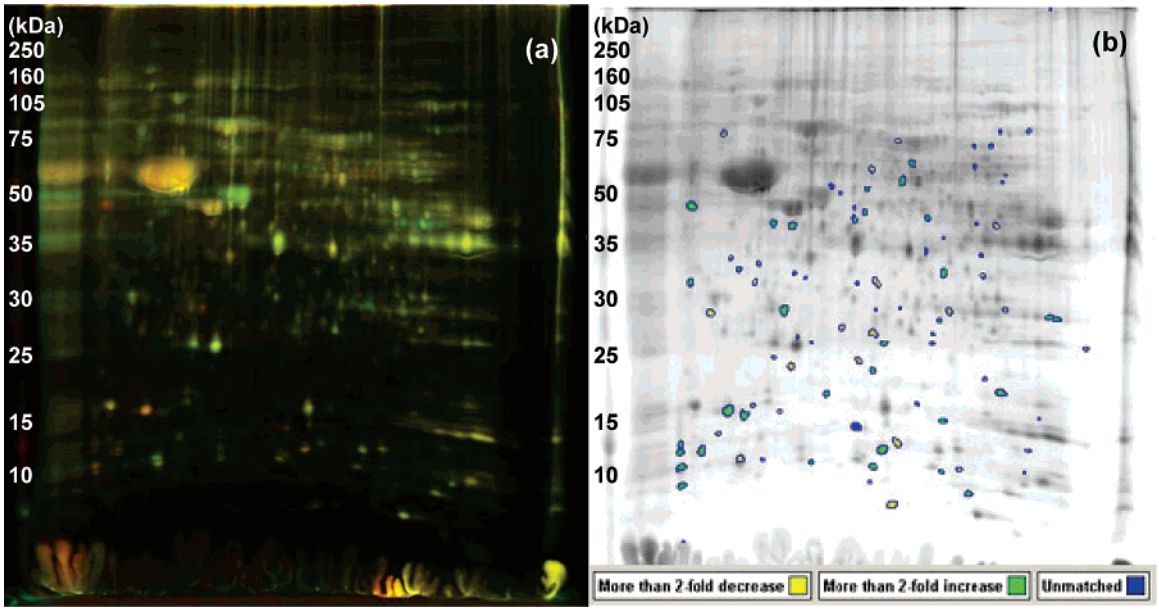

Figure 7. Rat cerebellum−cortex differential proteome display using 2D-DIGE. 2D-DIGE display of the pooled cerebellum and cortex lysates used with CAX-PAGE. (a) False-color overlay of cortex Cy3 (green) and cerebellum Cy5 (red)-labeled DIGE images. (b) Results of 2D differential software analysis comparing cortex and cerebellum tissue. Spots with a 2-fold difference between samples are indicated by yellow for greater in cortex and green for greater in cerebellum, while blue indicates spots found only in one sample.

Phoretix software was able to automatically identify gel lanes having a clear boundary along the *x*-axis; however, band height was sometimes more difficult to distinguish, requiring manual verification. A threshold of a 2-fold difference in band density between cerebellum and cortex data was used to generate a list of target bands for further analysis, whereby the mass spectrometry workload was minimized.

CAX-PAGE Differential Colorization. A false-colorization scheme can also be used to aide manual inspection of differential expression, creating images (Figure 6) similar to those produced with 2D-DIGE (Figure 7a). The colorized image was generated by converting adjacent cortex and cerebellum lanes into green and red, respectively, and superimposing the two. The human eye is adept at recognizing slight color shifts (away from yellow at equal expression) more so than recognizing slight changes in gray band intensity. The colorization map was useful for confirming Phoretix 1D output, particularly in cases of band overlap.

Comparing Differential Analysis by CAX-PAGE and 2D-DIGE. Analysis of the same cortex and cerebellum tissue lysates was performed by 2D-DIGE as a benchmark for evaluating CAX-PAGE. The Cy3 and Cy5 images shown overlaid in Figure 7a were compared using Phoretix 2D image analysis software with the result illustrated in Figure 7b. Using 2D-DIGE, 45 spots were discerned as more than twice as prominent in cerebellum and 37 spots were more than twice as prominent in cortex (Figure 7b) for a total of 82 differential protein targets. In comparison, CAX-PAGE revealed 105 band intensities more than twice as prominent in cerebellum and 41 bands more than twice as prominent in cortex for a total of 146 targets.

Proteins of high concentration pose a problem by masking less abundant proteins in both techniques. Those that show up as large spots with 2D-DIGE, separate across multiple fractions with CAX-PAGE (confirmed by RPLC-MSMS analysis), presenting an additional problem by increasing the number of apparent targets.

Nine of the 146 band pairs were redundant, reducing the number of targets to 137, still 67% more than observed by 2D-DIGE.

CAX-PAGE provided an improved mass range for differential analysis when compared with 2D-DIGE. Of the 137 differential targets, 13 were at a mass of 100 kDa or greater. In comparison, none of the differential targets uncovered by 2D-DIGE were above 100 kDa. The ability to discern differences at high mass is particularly relevant in brain injury paradigms where cytoskeletal proteins of great mass (e.g., MAPs and spectrins) are particularly prone to proteolysis associated with neuronal death after brain injury.[51−53]

In practice, we found numerous difficulties using 2D-DIGE that were absent when using CAX-PAGE. Signal intensity differed somewhat between the two cyanine dyes as noted by others,[17] giving a bias toward green or red from one gel to the next. Known problems with the stoichiometric ratio of protein to dye have been cited,[7] possibly explaining the observation. As well, more background was detected at the emission wavelength for Cy3 over Cy5, making fainter spots more difficult to discern. Rapid photobleaching made the technique difficult to use,[41] as sensitivity was quickly effected. This potentially is improved with the new saturation 2D-DIGE dyes.[54] Overall, upon comparing the two techniques, CAX-PAGE showed improved differential determination in this initial study, illustrating that CAX-PAGE can identify differential targets in a robust and cost-effective manner, and outperforms 2D-DIGE for analysis of high-mass proteins.

Resolving Power of CAX-PAGE. The most common means for comparing multidimensional separations is the use of theoretical peak capacity (n_c).[55] For 2D-PAGE, total n_c can be determined from the final spot dimensions (*x*- and *y*-axis width values) divided into the length of separation for each axis. From Figure 7, the *x*-axis n_c was 73.5 and *y*-axis n_c 74.0. This generates a theoretical total n_c of 5440 for 2D-DIGE, about the average for 2D-PAGE as cited in the literature ($10^3 - 10^4$).[22,56]

CAX-PAGE has an *x*-axis n_c equal to the fraction number, in this case 25, about one-third that of IEF, but CAX-PAGE has twice the peak capacity of 2D-PAGE along the *y*-axis at 143 due to the narrower band height achieved as a result of the larger *x*-axis width and the 1D-PAGE stacking gel region. Despite a shorter gel length, greater *y*-axis n_c of CAX-PAGE partially compensates for the small fraction number, producing a total n_c of 3570, which is 34.4% shy of that calculated for 2D-DIGE. However, calculating peak capacity based on working area, a rectangular separation space beyond which no proteins migrate, brings the values for CAX-PAGE and 2D-DIGE closer at 3120 and 4030, respectively. A recent improvement to CAX-PAGE has been shown using larger format commercial gels with an increased *y*-axis n_c of 211, and if combined with an expansion of CAX separations to 36 fractions (two gels per sample), a theoretical peak capacity of 7600 can be achieved.

CAX-PAGE resolution is comparable with other published 2D protein separation techniques,[21,22,28,30,31,57] that have first-dimension n_c values between 15 and 80 and second-dimension n_c values around 100 for combined values on the order of 10^3. In practice, all of these techniques can be viewed as complementary rather than exclusive, as they use different physical properties for separation, and none fully resolve an entire proteome. However, in the specific application of biomarker discovery, for which CAX-PAGE was developed, separation of an entire proteome is not necessary as only the most prominent proteins that demonstrate a clear expression change are of interest. CAX-PAGE in this case is advantageous as a protein separation and selection technique placed prior to RPLC-MSMS protein identification.

Differential Quantification and Protein Identification by Capillary RPLC-MSMS. A notable advantage of CAX-PAGE over 2D-DIGE[7,58] is the maintenance of spatial separation between samples, such that a second means of protein quantification can be performed. This is advantageous since 2D protein separations, having theoretical peak capacities of 10^3, are unlikely to fully resolve each protein into a single gel spot, band, or chromatographic fraction. This leaves doubt as to which identified protein is actually differentially expressed,[10,11] a common problem not generally addressed. In our platform, secondary quantification by MSMS verifies the identity of the differentially expressed protein.

To proceed, three logical assumptions were made with regard to the MSMS data. The first, (i), was that a protein producing a visible band would be identified by two or more peptides using strict Sequest cross-correlation values,[46] since the detection limit of Coomassie stain and dynamic exclusion MSMS are similar. The second assumption, (ii), was that proteins producing a 2-fold difference in band density would have similar or greater expression relative to background proteins. The last, (iii), was that only a differentially expressed protein (2-fold or greater difference) would exhibit a discernible change in peptide coverage[59,60] between the two samples.

To evaluate the protocol, two protein groups were selected for RPLC-MSMS analysis: (1) a random selection of differential band pairs, as listed in Table 1, the CAX-PAGE differential target list; and (2) a random selection of nondifferential band pairs of similar intensity as listed in Table 2. In total, 85% of MSMS runs fulfilled the first assumption i, irrespective of whether the band was differential or not. The 15% not conclusively identified were generally low-intensity bands. Enhanced mass spectrometers, such as the new linear ion traps, should improve protein identification in these cases.

Assessing assumptions ii and iii, we compared how often MSMS quantification matched, did not match, or was inconclusive (<2 peptide difference) when compared with band density data. With the differential target group (Table 1), both peptide number and band density reflected higher expression in the same tissue 89% of the time. Inconclusive MSMS quantification occurred 7% of the time, and only one case (4%) showed quantification that did not match. The matching rate of 89% demonstrated the effectiveness of dual quantification for internal verification of a differential protein's identity. In contrast, band density and MSMS quantification in the nondifferential target group (Table 2) were just as likely to match (28%) as mismatch (28%) with most showing an inconclusive determination (44%), demonstrating that the quantification correlation observed for the differential target group was not a random occurrence.

Summary of Differential Findings. The differentially identified proteins shown in Table 1 fit into three distinct protein classes known to be prominent in the brain,[58] listed here in order of prevalence: (1) metabolic enzymes such as α-enolase, pyruvate kinase 3, transketolase, GMP synthase, fatty acid synthase, etc.; (2) neuronal function proteins such as calbindins 1 and 2, translin,

Table 1. Quantification and Identification Results of Gel Band Pairs Showing Greater Than a Twofold Difference in Intensity between Cerebellum and Cortex—Differential Target List

gel data			MSMS data					data base search results		
excised band	gel band MW	% M to X diff[a]	expressed greater by MSMS[b]	no. peptides in M	M % protein covered	no. peptides in X	X % protein covered	ID'd protein MW	rat protein identified	accession no.
2	46.3	2094	X	0	0	2	5.3	47.2	α-enolase (enolase 1)	NP_036686.1
3A	53.0	−8256	M	12	22.2	0	0	57.8	pyruvate kinase 3	NP_445749.1
4A	72.5	−391	X	4	5	0	0	76.7	transferrin	NP_058751.1
5A	148.3	−365	M	6	3.5	3	2	180.1	amylo-1,6-glucosidease	XP_342332.1
6A	61.3	−101	M	10	14.5	5	8.3	71.2	transketolase	NP_072114.1
6C	15.1	−288	M	3	16.2	0	0	15.9	coactosin-like 1	XP_341701.1
7A	71.7	−178	M	3	4.4	0	0	76.7	transferrin	NP_058751.1
			M	2	3.6	0	0	70.8	GMP synthase	XP_215574.2
10A	53.8	−143	M	11	17.7	7	13.1	57.8	pyruvate kinase 3	NP_445749.1
			M	3	5.9	0	0	58	WD repeat containing protein 1	XP_341229.1
10B	27.5	−359	M	6	23.3	3	14.4	31.4	calbindin 2	NP_446440.1
11A	88.7	−168	M	3	3.5	0	0	95.3	trans elongation factor 2	NP_058914.1
11B	27.4	−443	M	9	22.5	7	29.9	31.4	calbindin 2	NP_446440.1
12A	61.5	−634	M	6	9.1	0	0	68.7	albumin	NP_599153.1
12C	27.5	−308	M	2	10.3	0	0	28.8	chloride intracellular channel 4	NP_446055.1
			M	2	8.3	0	0	25.6	platelet-activating factor acetylhydrolase	NP_446106.1
			M/X	5	16.2	5	21.4	31.4	calbindin 2	NP_446440.1
13D	28.1	−740	X	3	7.7	5	19.2	31.4	calbindin 2	NP_446440.1
13E	26.1	−1135	M	5	17.2	0	0	30	calbindin 1	NP_114190.1
13F	24.9	768	M/X	1	7.8	2	13.2	23.2	ρ-GDP dissociation inhibitor α	XP_340776.1
14A	222.5	−2170	M	6	3	0	0	273	fatty acid synthase	NP_059028.1
14B	100.0	−186	M	3	3.7	0	0	105.6	hexokinase 1	NP_036866.1
			M	2	1.8	0	0	118	insulinase (insulusin)	NP_037291.1
14C	89.2	−631	M	8	10.5	1	1.4	96.7	brain glycoprotein phosphorylase	XP_342543.1
14E	27.5	−910	M	3	12.3	0	0	30	carbonyl reductase	NP_062043.1
14F	25.8	−491	M	7	17.2	3	11.1	30	calbindin 1	NP_114190.1
15A	235.9	−208	M	5	2.1	0	0	273	fatty acid synthase	NP_059028.1
15C	26.1	−172	M	6	27.6	3	13.4	30	calbindin 1	NP_114190.1
16A	236.5	−215								
16B	25.9	−240	M	9	21.5	3	13.4	30	calbindin 1	NP_114190.1
			M	2	5.4	0	0	30	cerebellar Ca-binding protein	NP_114190.1
			M	3	16.2	0	0	26.2	translin	NP_068530.1
17A	119.3	−172	M	2	1.8	0	0	145.9	Ca-dependent activator secretion protein	NP_037351.1
18B	21.7	−586								
18C	20.1	−171	M	7		4		27	TYR 2-monooxygenase (14−3−3) ζ,η,θ	NP_062249.1
			M	2	7.3	0	0	30	calbindin 1	NP_114190.1
19A	116.4	−220	M/X	0	0	3	2.2	198.8	microtubule-associated protein 2	NP_037198.1
- -	150.0	-	M/X	3	12.6	0	0	42	brain creatin kinase	NP_036661.2
19B	22.7	227								
19C	21.6	−581	M	3	12.1	0	0	33	carbonic anhydrates 8	XP_226204.2
22	93.4	−199								
23	96.2	−297								

[a] Greater band intensity is indicated as a positive value in cortex and a negative value in cerebellum. [b] M indicates two or more peptides found for cerebellum over cortex; X indicates the opposite; M/X indicates a one- or no-peptide difference between tissues for that protein.

transferrin, etc.; and (3) cytoskeletal proteins such as chloride intracellular channel 4 and MAP2. Proteins were identified over a wide molecular mass distribution from 16 to 273 kDa. This is notably better than 2D-PAGE, which underrepresents proteins above 120 kDa due to poor diffusion into the IPG strip[7,61] and far exceeds the current mass range of top-down mass spectrometry approaches.[62] Another potential CAX-PAGE advantage is that hydrophobic membrane proteins are readily soluble in the loading buffer used, a known problem with 2D-PAGE.[61] However, this was not confirmed by this study, likely because membrane proteins are generally of low abundance and only 53 bands were analyzed by RPLC-MSMS. Future studies employing a membrane protein subproteome would be better able to address this point.

Remaining Challenges. Relative to other 2D separations, CAX-PAGE can be difficult to automate, mainly due to the sample concentration between CAX and 1D-PAGE. An envisioned solution is to use smaller i.d. ion-exchange columns to provide increased column efficiency and reduced fraction size for direct gel loading.

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Table 2. Quantification and Identification Results of Gel Band Pairs Showing Less Than a Twofold Difference in Intensity between Cerebellum and Cortex—Nondifferential Target List

gel data			MSMS data					data base search results		
excised band	gel band MW	% M to X diff[a]	expressed greater by MSMS[b]	no. peptides in M	M % protein covered	no. peptides in X	X % protein covered	ID'd protein MW	rat protein identified	accession no.
1A	25.9	64	M	5	20.7	3	10.6	25.9	glutathione *S*-transferase	NP_058710.1
1B	24.7	81	M/X	4	15.3	5	20.7	25.9	glutathione *S*-transferase	NP_058710.1
1C	23.2	36	M/X	2	5.6	3	10.6	25.9	glutathione *S*-transferase	NP_058710.1
1D	16.4	77	X	2	10.5	6	25.6	22.1	peroxiredoxin 5 precursor	NP_446062.1
3B	39.3	90	M/X	7	18.2	6	12.8	46.3	glutamate oxaloacetate transaminase 1	NP_036703.1
4B	45.6	−53	M	7	11.5	3	7.6	47.2	α-enolase (enolase 1)	NP_036686.1
4C	32.3	−34	M	5	21.5	3	9	34.7	pyridoxal kinase	XP_342113.1
5B	47.1	−17	M/X	5	9.7	4	7.6	47.2	α-enolase (enolase 1)	NP_036686.1
6B	46.3	28	X	3	7.6	5	7.6	47.2	α-enolase (enolase 1)	NP_036686.1
7B	47.8	70	X	3	5.3	10	14.5	47.2	α-enolase (enolase 1)	NP_036686.1
9A	34.5	−85	X	3	11	5	15.2	39.5	aldolase	NP_036629.1
9B	27.4	−32	X	2	8.3	5	24.4	28.9	phosphoglycerate mutase 1	NP_445742.1
12B	33.4	−5								
13A	60.9	−85	M/X	9	17.9	8	16.3	68.7	albumin	NP_599153.1
13B	33.6	−16	M/X	5	15.6	6	18.3	36.6	lactate dehydrogenase B	NP_036727.1
13C	32.2	51	M	5	16.9	3	9.5	35.6	malate dehydrogenase B	NP_112413.2
14D	33.6	−73	M/X	8	20.1	7	21	36.6	lactate dehydrogenase B	NP_036727.1
15B	27.8	0	M	4	14.4	1	5.9	31.4	calbindin 2	NP_446440.1
17B	35.5	−12	M/X	7	19.1	8	15.7	42	brain creatine kinase	NP_036661.2
17C	29.5	10								
18A	34.3	−67								

[a] Greater band intensity is indicated as a positive value in cortex and a negative value in cerebellum. [b] M indicates two or more peptides found for cerebellum over cortex; X indicates the opposite; M/X indicates a one- or no-peptide difference between tissues for that protein.

CAX-PAGE automation would then be similar to other 2D techniques that use fraction collection between dimensions. Protein immobilization within a gel matrix may also be viewed as a difficulty for automation; however, high-throughput staining, robotic band excision, and robotic digestion have already been developed for 2D-PAGE and would work equally well for CAX-PAGE. After robotic digestion, samples are autonimously placed into 96-well plates that interface with a capillary RPLC-MSMS autosampler.

Multiplexing large numbers of samples may prove difficult by CAX-PAGE/RPLC-MSMS. The platform works as long as the same fraction from each sample is grouped on a single gel (i.e., fraction 1 from each sample on gel 1, etc.). The maximum number of multiplexed samples is determined by the number of lanes within a single gel (up to 19 samples with large-format 20-well gels—1 lane for protein mass makers). In preliminary experiments, we have successfully multiplexed three samples.

CAX-PAGE/RPLC-MSMS in comparison with other separations strategies does not provide a direct measure of pI, which would be useful when 2D maps are employed for protein identification, as often done with 2D-PAGE. Preliminary investigation shows an apparent correlation between CAX elution and pI, though the precision was low. Foreseeably, a CAX fraction could be assigned a pI range as a means to confirm protein identity. On the other hand, CAX-PAGE provides protein mass, another good parameter to confirm protein identity that is not determined using 2D proteins separations with RPLC as the second dimension.

Dynamic range is a major complication for differential analysis, irrespective of the platform.[63] RPLC-MSMS performed in data-dependent mode has a low dynamic range due to possible signal saturation and poor ionization that can prevent triggering of MSMS scans. This negates possible CAX-PAGE improvements in protein detection using more sensitive stains. If differential analysis was performed exclusively by CAX-PAGE, then single peptide information could be used to identify proteins; however, the false positive rate would increase as the differential protein cannot clearly be distinguished from background proteins. Use of more sample, possible with the high capacity of CAX, could also help in detecting less abundant proteins up to a point. More sensitive MSMS analysis methods and instrumentation will ultimately aide identification of lower abundant proteins.

CONCLUSIONS

A novel approach was presented based on combining bipolarity ion-exchange chromatography in tandem with gel electrophoresis for protein separations, followed by capillary reversed-phase liquid chromatography online with tandem mass spectrometry for targeted peptide analysis. The platform is straightforward to perform, utilizing cost-effective traditional visualization stains and two quantification steps for internal verification of differential protein determinations. The platform was demonstrated for differential analysis comparing between cerebellum and cortex tissues, a test model for biomarker discovery in brain. Future

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efforts are focused on improving chromatographic efficiency for direct coupling with larger format 1D-PAGE and applying the platform to biomarker discovery for clinical diagnostics of traumatic brain injury, stroke, and substance abuse.[7,36,64−69]

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