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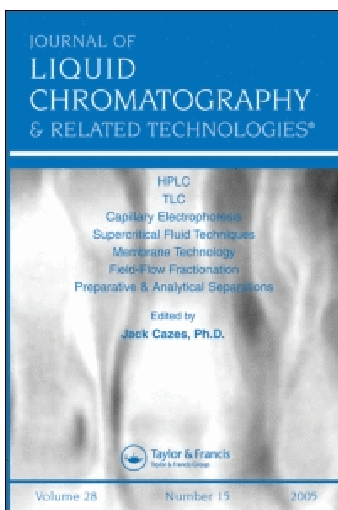

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Evaluation of Liquid Chromatography–Mass Spectrometry for Routine Proteome Analyses

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ABSTRACT

Many different separation strategies have been developed for conducting proteomic studies, but microcapillary reversed-phase liquid chromatography (μLC) coupled online to mass spectrometry (MS) has played an undeniably central role. We have conducted a study to evaluate two different common column configurations, along with two common stationary phase materials, for their ability to identify peptides from a complex proteome digest. A 10 cm long × 75 μm inner diameter (id)

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capillary column with an integrated electrospray tip, and a 30 cm long × 75 μm id capillary column in which the electrospray tip is separated from the separation capillary via a stainless steel union, both packed in-house with reversed phase C_{18}, 5 μm, 300 Å pore size particles, were evaluated for their ability to effectively resolve a complex mixture of tryptic peptides from a mouse cortical neuron proteome sample for online tandem MS (MS/MS) analysis. The results demonstrate that the continuous 10 cm long × 75 μm id capillary column with the integrated electrospray tip, enables the identification of a comparable number of peptides in a single μLC-MS/MS analysis of a proteome tryptic digestate. We further evaluated the relationship between the numbers of peptides identified vs. the amount of sample loaded onto a 10 cm long × 50 μm id column packed with 3 μm, 100 Å, C_{18} reversed phase particles. The results show that more than 100 peptides can be identified from as little as 5 ng of tryptic peptides loaded, and that the number of identified peptides did not significantly increase beyond the loading of 50 ng.

Key Words: Proteomites; Liquid chromatography; Mass spectrometry; Tandem MS; Peptide.

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INTRODUCTION

While representing a diverse field with a variety of objectives, one of the promises of proteomics research is the potential to identify a large number of proteins from complex protein samples such as cell, tissue, or serum.[1,2] The challenges to attain this goal range from the inherent complexity of the protein extracts that are being analyzed, to the tools available for performing the analyses. In addition to optimizing experimental chromatographic parameters, the most effective means to identify a large number of proteins in a complex mixture, is to fractionate the sample to simplify the complexity of the mixtures being analyzed, followed by an online separation procedure, coupled with tandem mass spectrometry (MS/MS) for peptide identification.[3] Most commonly, proteomic studies employ a strategy where proteins are digested, either chemically or enzymatically, to capitalize on the favorable solubility characteristics of peptides compared to proteins, with the concurrent benefit that peptides can be fragmented by MS/MS and identified by comparing the peptide fragmentation pattern to those hypothetically generated from a protein sequence database.[4]

Though the general scheme for conducting proteomic studies may appear straightforward, several factors need to be considered for conducting successful proteomic measurements. For example, since it is presently impossible to ascertain the number of different proteins expressed within a cell system at any

given time, it can never be known what percentage of the total proteome has been identified. In addition, the dynamic range of protein concentration in a cellular extract exceeds the capabilities of current mass spectrometry (MS) instrumentation.[5] To identify low abundance proteins, the complex peptide mixture must be fractionated (frequently into 10–200 fractions) prior to microcapillary reversed-phase liquid chromatography (μLC)-MS/MS analysis to simplify, at any given time, the sample being analyzed. The effectiveness of any fractionation scheme coupled with μLC-MS/MS analysis, is typically determined by a "diminishing returns" plateau in which the increase in the number of unique proteins being identified per fraction diminishes as the number of fractions being analyzed increases.

While many different separation strategies have been employed in proteomic studies, μLC-MS/MS analysis is common to a vast majority of global proteomic analyses.[6] Two different, yet common column configurations, as well as two different common C_{18} stationary phases, were presently evaluated for their ability to identify peptides within a complex proteome digest. The evaluation relied not on theoretical calculations (i.e., theoretical plates/meter, etc.), but on the number of peptides identified in a single μLC-MS/MS analysis of the same sample with the various column configurations. Such an experimental approach is practical, because proteomic investigations are most concerned with the number of peptides that can be identified from complex mixtures. Importantly, we selected materials and instrumentation that are readily available from commercial vendors commonly used in proteomic labs. In addition, using a tryptic digest of a cortical neuron protein isolate, we sought to find a relation between the amount of sample injected on the column and the numbers of peptides that can be identified. The results show that more than 100 unique peptides can be identified from as little as 5 ng of the cortical neuron protein digest. As the amount of sample loaded on the column was increased, so did the number of peptides/proteins identified, however, the increase in identified number of peptides did not increase significantly beyond the loading of 50 ng. These results suggest that significant numbers of peptides/proteins can be identified from as little as 5 ng of sample, enabling even limited amounts of a cell digest (i.e., 1 μg total) to be fractionated prior to μLC-MS/MS analysis.

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EXPERIMENTAL

Materials

Ammonium bicarbonate (NH_4HCO_3), *tris*[hydroxymethyl]aminomethane, Triton X-100, NaF, and sodium orthovanadate were purchased from Sigma (St. Louis, MO). Formic acid was from Fluka (Milwaukee, WI).

HPLC grade acetonitrile (ACN) was obtained from EM Science (Darmstadt, Germany). The bicinchonic acid (BCA) protein assay reagent kit was from Pierce (Rockford, IL). PD-10 columns (Sephadex G-25 M) were purchased from Amersham Biosciences (Uppsala, Sweden). Waters Oasis MCX 1 mL extraction cartridges were from Waters Corporation (Milford, MA). Luna C_{18} (3 µm diameter, 100 Å pore size) and Jupiter C_{18} (5 µm diameter, 300 Å pore size) stationary phases were obtained from Phenomenex (Torrence, CA). Stainless steel internal unions and screens were purchased from VICI Valco Instruments (Houston, TX). PEEK tubing (380 µm id) was from Upchurch Scientific (Oak Harbor, WA). Ultrapure water was obtained from a Barnstead Nanopure water purification system (Dubuque, IA).

Proteome Sample Preparation and Digestion

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Primary cortical neuron cultures were established from newborn pups of a p53 wild-type mouse strain as described previously.[7] Briefly, cortical brain tissue was excised, trypsinized, and dissociated by trituration to obtain single cells. Cells were then plated onto poly-*D*-lysine-coated cultureware and maintained in Neurobasal medium with B27 supplements (Invitrogen, Carlsbad, CA). Under these conditions, cultures contain greater than 95% neurons as assessed by neurofilament immunostaining. Cultures were maintained for 4 days and lysed in the culture dishes with a lysis buffer containing 50 mM Tris–HCl (pH 8.5), 2% Triton X-100, 10 mM NaF, and 1 mM sodium orthovanadate. The lysed neurons were scraped into an Eppendorf tube and sonicated 5 times (10 sec each) (Branson digital sonifier 250, Danbury, CT) on ice. The lysate was centrifuged at 15,000*g* for 15 min at 4°C. The supernatant was collected and desalted into 50 mM NH_4HCO_3, pH 8.3, using a PD-10 column. Protein concentration was determined by the BCA assay. An aliquot of lysate containing 200 µg of solubilized protein, was digested overnight at 37°C with sequencing grade modified trypsin (Promega, Madison, WI), at a ratio of 50 : 1 (w/w, protein-to-trypsin). The digestion reaction was terminated by boiling the sample in a water bath for 10 min. The sample was acidified to 1% formic acid and desalted using a 1 mL Oasis MCX extraction cartridge. The desalted digestate was lyophilized and stored at −80°C.

Microcolumn Packing and Column Connection

Capillary columns of various lengths and inner diameters were slurry packed with different alkyl-bonded stationary phases. To pack long microcolumns (i.e., 30 cm long), one end of the fused-silica microcapillary (75 µm id × 360 µm od, Polymicro Technologies, Phoenix, AZ) was connected

to a stainless steel union using PEEK tubing (380 μm id), wherein a stainless steel screen with 2 μm pore size was positioned to hold the packing particles. The other end of the capillary was connected to a stainless steel packing vessel. Jupiter C_{18}-bonded silica particles (5 μm diameter, 300 Å pores) were suspended in isopropanol and sonicated in a water bath for 1–2 min. After sonication, the slurry was immediately introduced into the packing vessel connected to a slurry packing pump (Model 1666, Alltech Associates, Deerfield, IL). Using isopropanol as the packing solvent, the particles were packed into the capillary at ~6000 psi. Bifunctional microcapillary electrospray columns, that act as both separation column and electrospray tip, were also slurry packed. To construct the continuous μLC-electrospray columns, one end of the microcapillary (75 μm id × 360 μm od) was flame-pulled to construct a fine id tip (i.e., 5–7 μm), against which stationary phase particles were slurry packed as described. After the column was packed, the inlet end of the column was connected to a stainless steel union into which a 2 μm pore size stainless steel screen was placed using PEEK tubing.

The μLC columns were connected through a 20 μm id fused-silica transfer line directly to the outlet of the sample injection valve of an Agilent 1100 capillary LC system (Agilent Technologies, Paolo Alto, CA). For the 30 cm microcolumns, an unpacked 5 cm electrospray tip was separately constructed from fused silica (50 μm id × 360 μm od) and connected to the outlet end of the packed column through a stainless steel union (Fig. 1). The columns were coupled on-line with an ion-trap (IT) mass spectrometer (LCQ Deca XP, ThermoFinnigan, San Jose, CA) to analyze tryptically digested proteome samples obtained from mouse cortical neurons.

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Reversed-Phase: μLC-MS/MS

Thirty centimeter and 10 cm × 75 μm id microcolumns packed in-house with different C_{18} stationary phases were used in this study. Before sample injection, the cortical neuron tryptic digest was dissolved in 5% ACN and 0.1% formic acid. Different quantities of the digestate, ranging from 2.5 μg to 5 ng, were loaded onto the columns and the peptides were eluted with a two-step linear gradient generated from solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in ACN) over 180 min: 0–35% B for 140 min and 35–85% B for 40 min, at a flow rate of 500 nL/min. To evaluate the online separation efficiency and protein identification capability for the different microcolumns, the IT mass spectrometer was operated in a data-dependent MS/MS mode, e.g., one MS scan followed by three MS/MS scans, and dynamic exclusion was applied to avoid repeated fragmentation of the same precursor ion whose CID spectrum had already been acquired. The MS

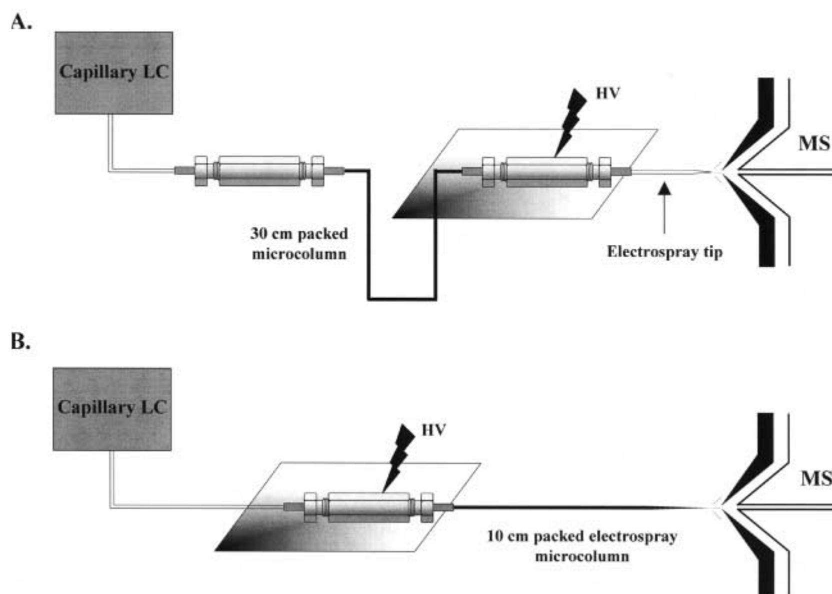

Figure 1. Schematic diagrams of the column connections for online microcapillary LC-MS/MS proteome analysis. (A) A 30 cm long column is connected to an LC system via a union and the other end of the column is connected to a short electrospray tip via a stainless steel union. (B) A 10 cm long column is connected to the LC system via a stainless steel union.

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spectrum for the molecular ions was acquired with 2 microscans at the mass range of m/z 300–2000, and the CID spectrum for the fragment ions was acquired with 3 microscans. The voltage and temperature of the capillary for the ion source were 10 V and 180°C, respectively.

Protein Identification

The raw MS/MS data acquired on the IT mass spectrometer were searched using SEQUEST (ThermoFinnigan, San Jose, CA) against a mouse protein database from the National Center for Biotechnology Information (http://www.ncbi.nih.gov). Only tryptic peptides with Xcorr ≥ 1.9 for singly charged ions, ≥ 2.0 for doubly charged ions, and ≥ 3.0 for triply charged ions, and DeltaCn ≥ 0.1, were considered for legitimate peptide identification.

RESULTS AND DISCUSSION

Two different column configurations were prepared and tested for their ability to separate and identify peptides in the analysis of a complex mixture using μLC-MS/MS, as shown in Fig. 1. The first configuration employed a 30 cm long by 75 μm id (360 μm od) capillary packed with 5 μm C_{18} stationary phase with 300 Å pore size. A steel screen with 2 μm pores was used to retain the packing material within the capillary. This capillary was connected to a stainless steel union using PEEK tubing (380 μm id) and a short electrospray tip that had been flame-pulled to form a fine id tip (5–7 μm) was connected to the outlet of this union. The second configuration employed a 10 cm long by 75 μm id capillary that was packed with the same C_{18} stationary phase. The end of this capillary, however, was flame-pulled to construct a fine id tip (i.e., 5–7 μm) against which the packing material is retained. Both capillary columns were connected to identical HPLC systems via a stainless steel union. An equal amount (i.e., 2.5 μg) of a cortical neuron tryptic digestate was loaded onto each column and analyzed by μLC-MS/MS. The base-peak chromatograms (BPCs) for each μLC-MS/MS analysis are shown in Fig. 2. There are obvious differences in the overall BPCs between the two analyses, particularly in the earlier stages of the μLC-MS/MS analysis. A comparison of the reconstructed ion chromatogram (RIC) for the peptide at mass-to-charge (m/z) ratio 1008.3, shows that the peak width for this particular peptide is essentially identical in both analyses. In addition, the overall peak capacity for the 10 cm continuous capillary column/spray tip configuration was comparable, within experimental error, to the 30 cm capillary column configuration in which a union was used to connect the column and the spray tip.

The results presented in Fig. 2 demonstrate that a 10 cm long continuous capillary electrospray (ESI) tip column packed with 5 μm C_{18} stationary phase can separate a complex mixture and identify a large number of peptides. The question that needs to be answered is, what is the optimum sample size that has to be injected to give the largest number of peptide/protein identifications, and what is the relationship between the amount of sample injected and the number of species identified by μLC-ESI-MS/MS? Injections of 500, 100, 25, and 5 ng of the cortical neuron digest were made onto a 10 cm × 50 μm id column packed with C_{18}, 3 μm, 100 Å reversed phase particles, and the number of peptides identified was determined. As expected, the number of peptides identified and the overall signal intensity decreased as the amount of sample loaded onto the column decreased (Fig. 3). What is intriguing, however, is that a substantial number of peptides and proteins can be identified when as little as 25 ng (374 peptides/195 proteins) and even 5 ng (139 peptides/77 proteins) of sample were analyzed using the μLC-MS/MS configuration. Typically, a minimum of 1 μg of complex proteomic sample

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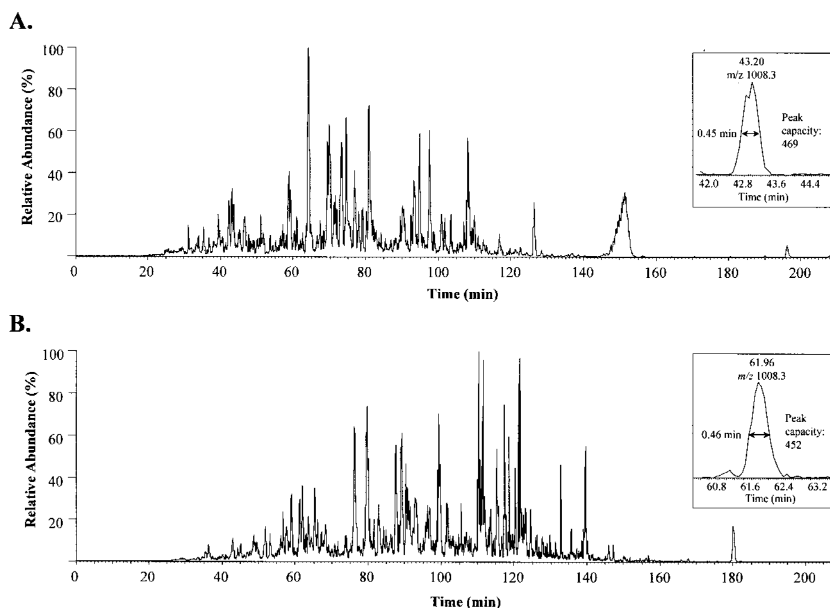

Figure 2. Base-peak chromatograms of the separation of cortical neuron protein digest using a 10 cm column (A) and 30 cm column (B), both packed with C_{18}-bonded 5 μm particles having 300 Å pore size. About 2.5 μg of cortical neuron protein digest were injected onto each column. Peptides were eluted at a flow rate of 0.5 μL/min with a two-step gradient of 0–45% B for 160 min and 45–85% B for 50 min, and detected by data-dependent MS/MS. Other experimental conditions are given in text.

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is used for protein digestion and analysis by μLC-MS/MS analyses; however, the configuration described above is sensitive enough to analyze several hundred nanograms of proteome samples as demonstrated by the signal intensity, peak complexity, and the number of peptides identified in Fig. 3(A) and (B). The quality of the MS/MS data that can be acquired from as little as 5 ng of a complex mixture analyzed using the described μLC-MS/MS configuration is shown in Fig. 4. The entire BPC is shown (bottom), along with a single MS scan (middle), and the MS/MS spectrum of the selected peptide (top). The CID spectrum possesses an excellent signal to noise ratio and is found to unambiguously correspond to a tryptic peptide from tubulin β3.

The number of peptides that can be identified vs. the amount of sample injected was further evaluated. Various amounts (5–1000 ng) of the same cortical neuron digestate was loaded onto the column and the number of unique peptides and proteins identified in the μLC-MS/MS analysis are shown

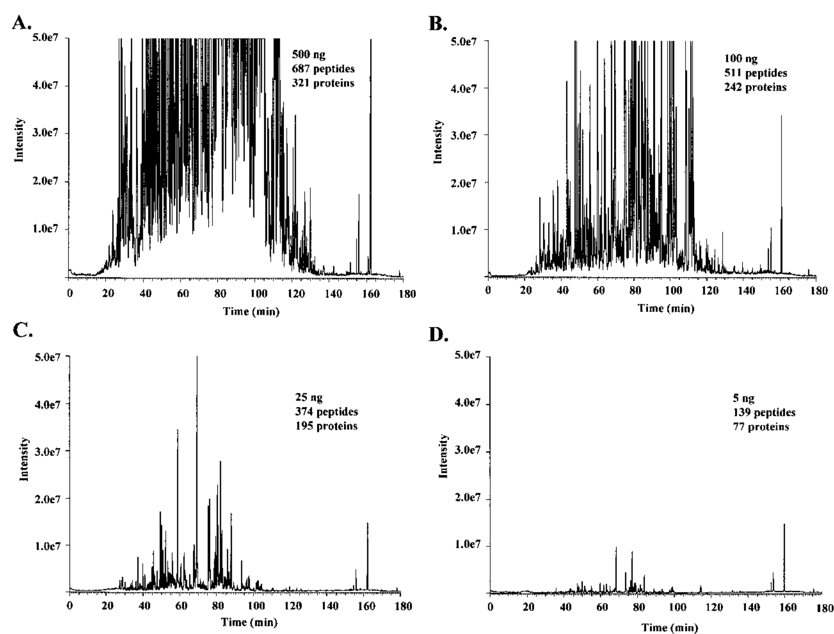

Figure 3. Base-peak chromatograms of different amounts of cortical neuron protein digest, 500 ng (A), 100 ng (B), 25 ng (C) and 5 ng (D) injected onto a 50 μm id × 100 mm μLC-electrospray column packed with 3 μm C_{18}-bonded particles. Other experimental conditions are given in text.

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in Fig. 5. Approximately 4-fold as many peptides, and 3-fold as many proteins, were identified as the amount of sample loaded increased from 5 to 50 ng; increasing the amount of sample loaded by another 10-fold (from 50 to 500 ng), however, resulted in only a ~1.4 and ~1.37 increase in the number of peptides and proteins identified, respectively. Quite remarkably, increasing the amount of sample loaded to 1000 ng resulted in only a 1.07- and 1.03-fold increase in the number of peptides and proteins identified, respectively.

CONCLUDING REMARKS

One of the many critical needs for the proteomic analysis of complex mixtures using MS-based tools is sensitivity.[8] While the development of more sensitive MS instruments is an obvious way to increase sensitivity, the chromatography associated on-line with the MS analysis plays a critical role in this area.[9]

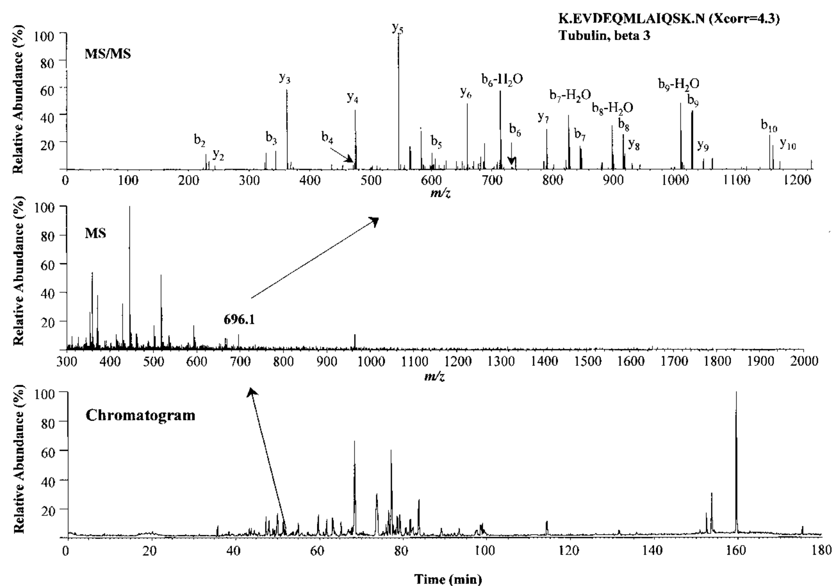

Figure 4. Peptide identification from µLC-MS/MS analysis of as low as 5 ng of cortical neuron protein digest loaded onto a 50 µm id × 100 mm µLC-electrospray column packed with 3 µm C_{18} stationary phase.

Other studies have evaluated the use of long (i.e., >80 cm) microcapillary columns and the effect of various parameters, such as id and solvent flow rate, on the sensitivity of the measurement of complex proteome samples.[10] The use of long capillaries, however, requires high pressure (i.e., ~10,000 psi) for both packing the microcolumn and for gradient development through the packed microcolumn. The present study was designed to evaluate capillary columns that possess dimensions, and stationary phase material that are amenable to common commercially available LC equipment that are within the operation capability of a majority of MS-based proteomic laboratories. In addition, the focus of this study was not to evaluate the characteristics of the separation per se, but to evaluate the results based on the endpoint of the entire process; that being the number of identified peptides via database searching of the resulting data from a routine µLC-MS/MS analysis.

The identification of as many proteins as possible within a complex mixture is one of the goals of proteomics.[11] Such global proteomic analyses require sample fractionation by at least two orthogonal separation methods prior to MS analysis to achieve the optimum results.[12] The results provided in Figs. 3–5, demonstrate that a significant number of peptides and proteins can

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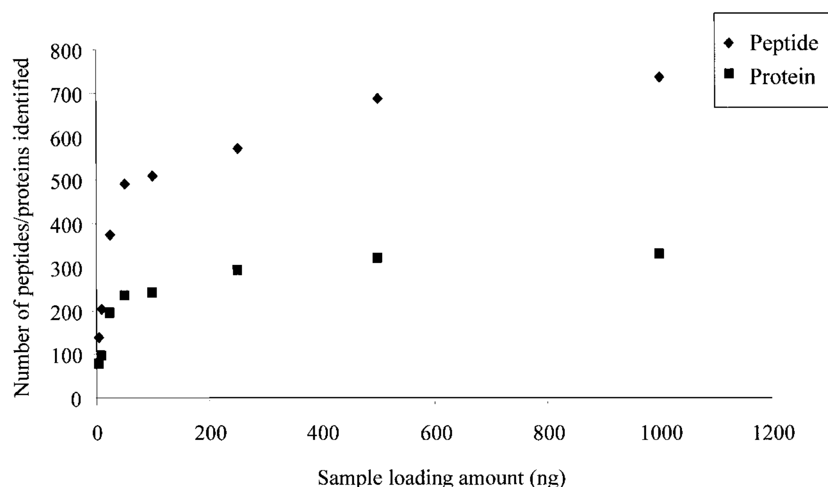

Figure 5. A plot of sample loading vs. number of unique peptides and proteins identified from various loading amounts of cortical neuron protein digest. Experimental conditions are as in Fig. 3.

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be identified from as little as 5 ng of sample. While increasing the amount of sample to approximately 50 ng had a significant positive effect on the number of peptides identified, increasing the amount of sample loaded resulted in a diminishing return on the number of identified peptides/proteins. This trend suggests that two-dimensional fractionation is practical to limited amounts of a cell digest (i.e., 1 μg total), and can still be accomplished when dealing with very small amounts of material (i.e., <100 ng), as long as the transfer between the two separations is efficient. In cases where the amount of sample is not limiting, a larger number of samples can be collected from the first fractionation dimension and, subsequently, analyzed by μLC-MS/MS.[13]

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