Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 803 (2004) 101–110
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Optimization of reversed-phase microcapillary liquid chromatography for quantitative proteomics

Hookeun Lee [a], Eugene C. Yi [a], Bo Wen [b], Timothy P. Reily [c], Lance Pohl [d], Sidney Nelson [b], Ruedi Aebersold [a], David R. Goodlett [a,*]

[a] *Institute for Systems Biology, Seattle, WA, USA*
[b] *Department of Medicinal Chemistry, University of Washington, Seattle, WA, USA*
[c] *Toxicology Department, Bristol-Myers Squibb, Syracuse, NY, USA*
[d] *Laboratory of Molecular Immunology, Molecular and Cellular Toxicology Section, NHLBI, NIH, DHHS, Bethesda, MD, USA*

Abstract

Currently, the field of shotgun proteomics relies primarily on the separation of peptides by reversed-phase microcapillary chromatography (RP-μLC) combined with either electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) and tandem mass spectrometry (MS/MS) for protein identification as well as quantification. For this purpose we herein describe construction of a RP-μLC–ESI column-emitter along with optimized μLC conditions for using the device to quantify pair-wise changes in protein expression via the isotope coded affinity tag (ICAT™) method that also maximize peak capacity. These optimized RP-μLC parameters required a balance be reached between the disparate needs of *quantification* which requires good peak shape and *identification* (i.e. proteome coverage) of proteins via peptide collision induced dissociation (CID) which requires peak capacity be maximized. A complex biological sample from a study of murine acetaminophen toxicity in hepatocyes was chosen for method development because of the high level complexity, but the biological results are not the focus of this manuscript.

Keywords: Optimization; Quantitative proteomics

1. Introduction

Quantitative proteome profiling, identifying the proteins having abundance changes resulting from external or internal perturbations of a cell or tissue, is an important strategy for interpretation of gene function in biological systems. Traditional quantitative proteome analysis has been carried out by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separation of proteins and the subsequent mass spectrometric identification of the isolated proteins [1]. In the 2D-PAGE methodology, protein quantification is achieved by comparing differential staining of proteins in the polyacrylamide gels. One problem with this popular approach is that a stained spot of a gel may contain multiple proteins making it difficult to track the protein responsible for differential staining.

Recently developed methods for pair-wise protein quantification employing stable isotope labeling [2–7] of proteins (or peptides) circumvent the problem of which protein is responsible for changes in expression by measuring changes in protein expression directly from peptides. These methods utilize liquid-phase fractionation of peptides by chromatography or electrophoresis prior to mass spectrometry based methods for identification and quantification. In these methods, the abundance changes of individual proteins between two different samples are measured by comparing the area under the curve for the single ion current trace for each of the two peptides having identical sequence but different mass due to the substitution of so-called heavy for normal or light isotopic elements such ^{2}H for ^{1}H, ^{15}N for ^{14}N or ^{13}C for ^{12}C.

While there are several published methods for use of stable isotope labeling to measure pair-wise changes in proteomes, our preferred method uses the isotope-coded affinity tag (ICAT) reagents [2]. The ICAT reagent consists of a thiol-reactive group capable of specifically binding

* Corresponding author. Tel.: +1-206-732-1277; fax: +1-206-732-1299.
E-mail address: goodlett@systemsbiology.org (D.R. Goodlett).

1570-0232/$ – see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2003.09.005

to cysteine, a polyether linker synthesized in an isotopically normal form (i.e. d(0)-ICAT reagent) or heavy form where eight deuteriums replace eight hydrogens (i.e. d(8)-ICAT reagent), and a biotin affinity tag allowing the tagged peptides to be purified. The current ICAT method using reversed-phase microcapillary chromatography (RP-μLC)–electrospray ionization (ESI) tandem mass spectrometry (MS/MS) is shown in Fig. 1. After separate labeling reactions the two protein solutions are combined and subjected to proteolysis with trypsin. At this point in the procedure, the ICAT reagent-labeled peptides of the same sequence from the two samples are chemically identical and behave similarly in the subsequent separation stages providing an internal standard throughout any further sample processing. In the RP-μLC–ESI–MS/MS analysis, the mass spectrometer initially acquires a mass spectral survey scan of all peptides eluting at a specific point in time from the RP-μLC column. The instrument control software then sequentially selects peptide ions of a specific mass-to-charge (*m*/*z*) ratio for collision-induced dissociation (CID) in descending order of signal intensity. The acquired CID spectra are interpreted using sequence database search tools into peptide sequences to identify proteins from which the sequenced peptides originated [8,9]. For each sequenced ICAT reagent-labeled peptide, a chromatographic elution profile is constructed by arranging the peptide peaks detected in the MS scans in the order of acquisition time. The abundance ratio of each protein is calculated comparing the areas of the elution profiles of a specific ICAT-labeled peptide pair. This quantification procedure

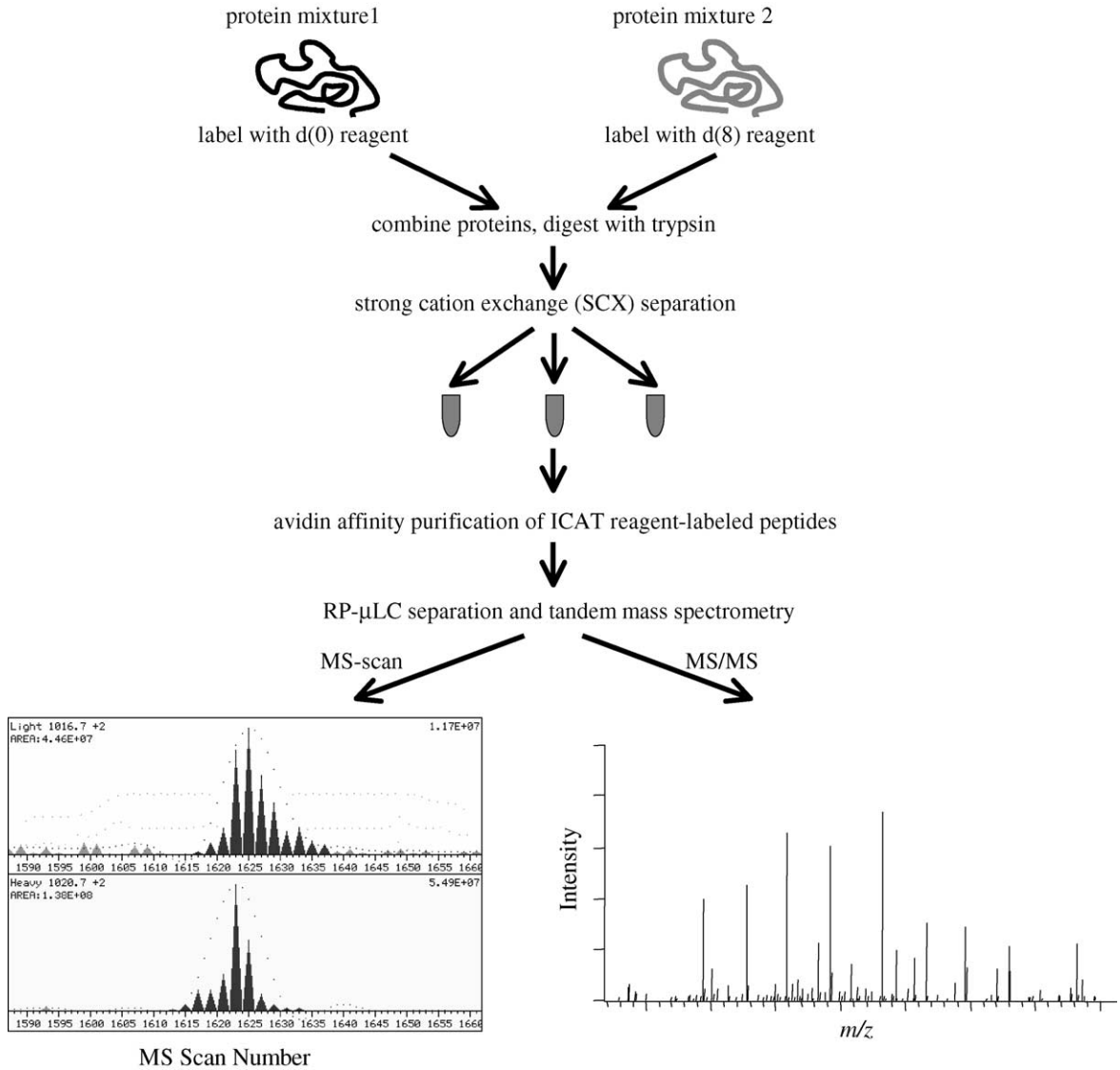


Fig. 1. Strategy for isotope-coded affinity tag (ICAT) reagent-based quantitative proteome analysis. Two protein samples to be compared are separately labeled with either d(0) or d(8) ICAT reagent. Following digestion with trypsin, ICAT-labeled peptides are fractionated by cation ion-exchange chromatography. Each fraction is subjected to avidin affinity chromatography to selectively isolate ICAT reagent-labeled peptides, which are then analyzed by RP-μLC–ESI–MS/MS.

based on chromatographic elution profiles provides better accuracy and precision than the quantification based on intensity alone at a single point in the chromatographic elution profile. Also it practically resolves the problem arising from different elution times between light and heavy ICAT reagent-labeled peptides due to deuterium isotopic effect [10].

Reliance on the chromatographic elution profile as a means of quantification from area under the curve of ICAT-labeled peptide pairs produced from single ion current traces for each peptide, means that it is essential to achieve high-resolution separations to obtain high accuracy and dynamic range for peptide quantification. To this end, the design and the operation of RP-μLC system should be optimized to minimize band broadening which can result in poorer analyte concentration worsening limit of detection and preventing quantification due to an inability to locate where a "peak" begins and ends. In this study, we present a unique RP-μLC setup and discuss the effects of solvent composition gradient on identification/quantification of complex mixtures of ICAT-reagent labeled peptides from a biological tissue, murine
liver.

2. Experimental

2.1. Materials and reagents

The ICAT reagent was purchased from Applied Biosystems (Foster City, CA, USA). Formic acid, HPLC-grade acetonitrile and water were from Fisher Scientific (Fair Lawn, NJ, USA). Polyimide-coated fused-silica capillaries (50 and 75 μm i.d.) were from Polymicro Technologies (Phoenix, AZ, USA). Capillary tubing (100 μm i.d.) with an integrated borosilicate frit (Integrafrit) were obtained from New Objective (Cambridge, MA, USA), Magic C18 resins (5 μm, 100 and 200 Å pore) were from Michrom BioResources (Auburn, CA, USA) and a microtee and a microcross from Upchurch Scientific (Oak Harbor, WA, USA).

2.2. Preparation of ICAT reagent-labeled peptides

Two samples each containing 500 μg of liver protein from 8-week-old SJL mice treated with either acetaminophen (300 mg/kg) or saline were separately labeled using the original d(0) and d(8) ICAT reagents. The two protein mixtures were combined, digested, fractionated by strong cation exchange (SCX) HPLC, and purified by avidin affinity chromatography as described [11]. A single SCX fraction of ICAT-labeled peptides was selected for method optimization, dried by vacuum centrifugation and brought up in 12 μl of 0.1% formic acid ready for injection onto the μLC column.

2.3. Microcapillary liquid chromatography

A schematic overview of the RP-μLC system is shown in Fig. 2. The system consists of a binary HP1100 pump from Agilent Technologies (Wilmington, DE, USA), a Famos micro-autosampler from Dionex LC packings (San Francisco, CA, USA), a six-port switching valve integrated on LCQ DecaXP ion trap mass spectrometer (ITMS) from Thermo Finnigan (San Jose, CA, USA), a precolumn

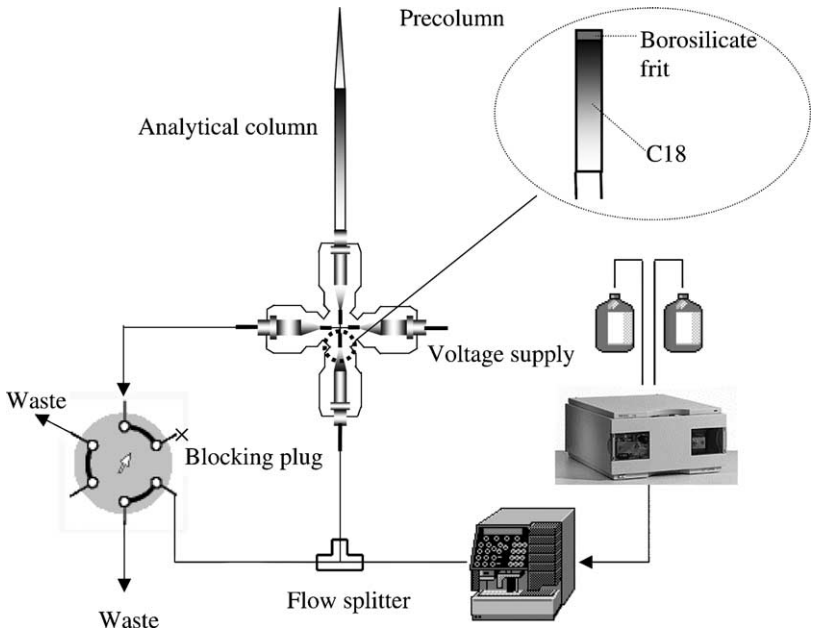


Fig. 2. Schematic representation of reversed-phase microcapillary liquid chromatography system.

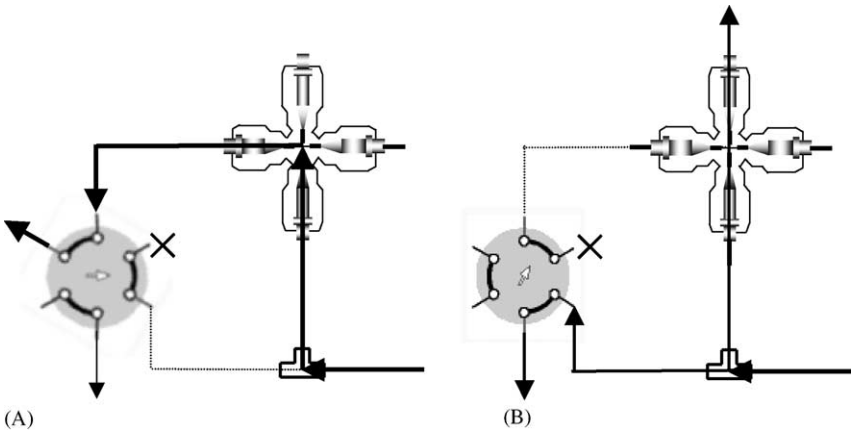
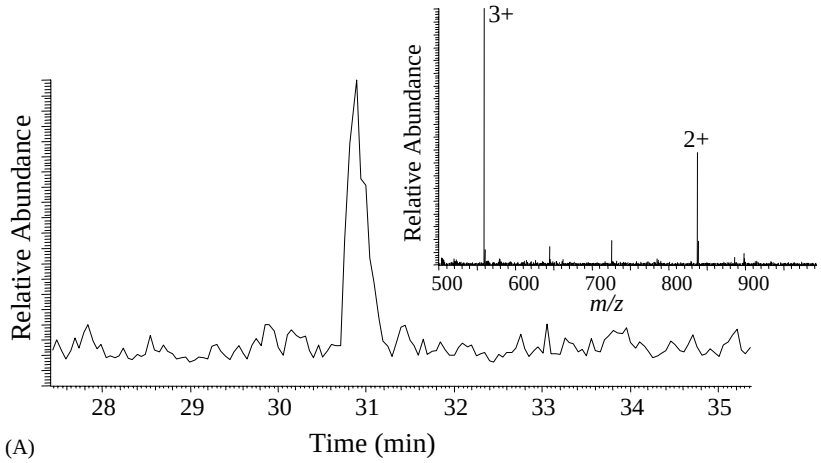


Fig. 3. Flow paths and valve configurations at (A) sample loading and (B) separation stages.

(100 μm i.d. × 1.2 cm length) and a microcapillary column (75 μm × 15 cm) that also serves as the ESI emitter.

A slurry packing procedure was employed to prepare the columns. Two C18 slurries of 100 Å pore C18 and 200 Å pore C18 were prepared by placing ∼5 mg of C18 resin in a small vial, and adding 1 ml of methanol and a mini stirring magnet to each. For the microcapillary column, one end of 75 μm i.d. fused-silica capillary was manually pulled to a fine point ∼5 μm at the tip in flame. The resulting needle-like tip served as a frit holding C18 resin in place and as the



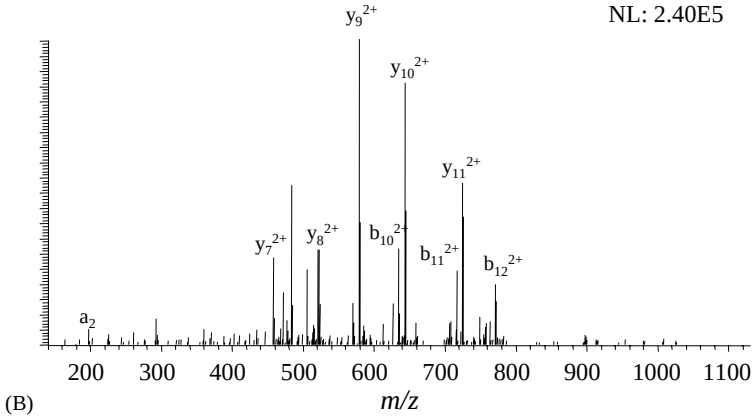


Fig. 4. Sequence analysis of 10 amol of neurotensin. (A) Base peak chromatogram showing the elution of the neurotensin peak and MS scan obtained at peak maximum. (B) MS/MS spectrum of the $[M + 3H]^{3+}$ ion for neurotensin, 559 *m/z*.

point from which ESI originated. For the precolumn, 100 μm i.d. fused-silica capillary with an integrated frit prepared by sintering bare silica resin at the tip with a mircotorch was used. Both columns were packed using a pneumatic pump from Brechbuehler, Inc. (Spring, TX, USA) at constant helium gas pressure of 1500 psi. The ESI device shown in Fig. 2 sat on a micromanipulation stage from Brechbuhler (Spring, TX USA).

Flow paths and valve configurations at sample loading and separation stages are shown in Fig. 3. A sample volume of 0.1 μl was loaded onto the precolumn with 5% solvent B at an unsplit flowrate of 5 μl/min in 5 min. After sample loading and cleanup, binary solvent composition gradients with solvent A (100% water/0.1% formic acid) and solvent B (100% acetonitrile) were started with a valve switch enabling a split flow (∼200 nl/min) into both columns. Linear binary gradients of 5, 10, 13, 15, 20–32% solvent B were generated over 150 min, followed by isocratic elution at 70% B for 4 min as shown in Fig. 4. The total analysis time for a single μLC–MS/MS run was 178 min from injection to end of isocratic wash. The above procedure including sample loading, valve-switching, and solvent composition gradient elution is fully automated and controlled from the ITMS computer.

2.4. Mass spectrometric analysis and data analysis

Eluting peptides were selected for collision induced dissociation (CID) during a procedure that alternated between a MS scan over the *m/z* range 400–1800 and a MS/MS scan in which the single most abundant peptide ion was subjected to CID. Each scan cycle lasted an average of ∼1.6 s. The specific *m/z* value of the peptide fragmented by CID was excluded from reanalysis for 3 min. For peptide identification, all tandem mass spectra were analyzed using SEQUEST, a computer program that performs the correlation of experimental data with theoretical spectra generated from known protein sequence [12]. Tandem mass spectra of peptides having a probability score of at least 0.99 were considered correctly identified [13]. Peptide quantification was performed using the XPRESS program as described [14].

3. Results and discussion

3.1. Design of RP-μLC system

The RP-μLC system as shown in Fig. 2 is equipped with a precolumn which enables sample cleanup and speedy sample loading by utilizing a high flowrate (5 μl/min). The primary role of the precolumn is to extend the lifetime of the microcapillary analytical column and secondarily to increase sample throughput in proteome-wide analysis by decreasing overall cycle time. In the first instance the precolumn functions as a last on-line sample cleanup prior to injection on to the analytical column and in the second instance to allow loading of the sample at a higher flow rate than would be otherwise possible (Fig. 3); e.g. μl/min rather than nl/min at which the separation takes place on the analytical column. The setup is similar to previously described systems [15,16] integrating a precolumn and a microcapillary analytical column as one piece. However, by separating the two columns with a small dead volume (∼50 nl), it is more practical to assemble, and replace components as necessary. This robust device is routinely used in our automated setup for 50 analyses prior to failure. In addition, the dimensions of the precolumn may be easily increased or decreased

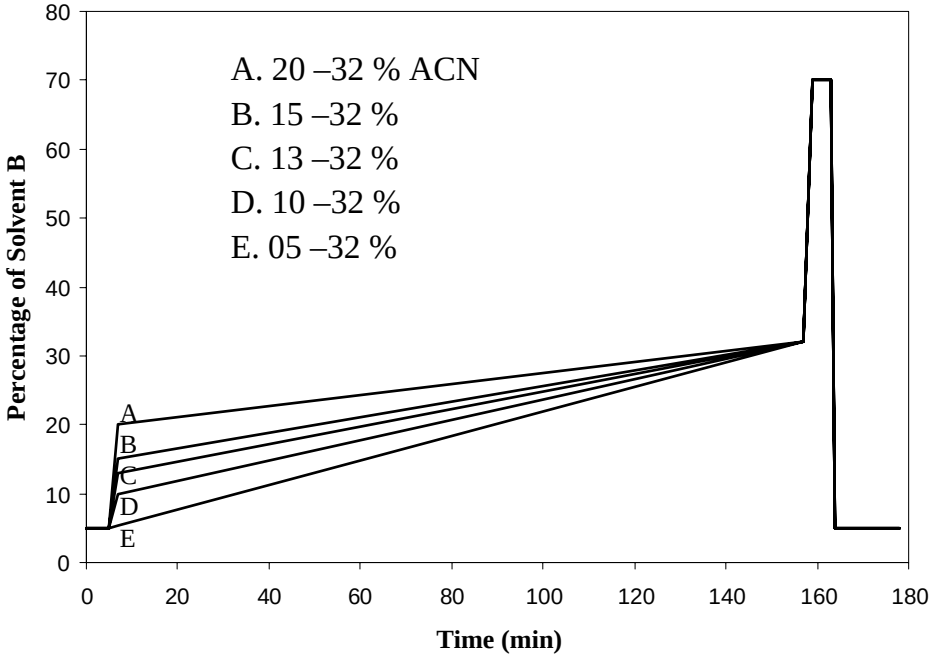


Fig. 5. Solvent composition gradient curves.

without any influence on separation efficiency of the micro-capillary column. As shown in Fig. 4, the sensitivity of the RP-μLC system was demonstrated by on-column injection of 10 amol of a standard peptide producing a S/N ≅ 40 for $[M + 3H]^{3+}$ ion (Fig. 4A) that produced a tandem mass spectrum (Fig. 4B) of sufficient quality to allow the peptide to be identified in a database search using SEQUEST [17].

3.2. Effects of gradient slope and organic modifier composition

Binary solvent gradient elution has been widely used in reversed-phase chromatography for the separation of complex mixtures of peptides that vary greatly in overall chemical composition; e.g. from very hydrophobic to very hydrophilic and from high to low isoelectric point. As mentioned in the introduction optimization of the gradient elution to obtain highly resolved peptide elution profiles is essential in our quantitative proteome analysis scheme (Fig. 1). Poorly optimized conditions will lead to excessive band broadening the result of which will be poor quantification via area under the curve measurements of single ion current traces of ICAT-labeled peptides.

To optimize RP-μLC conditions we applied five different gradient elution profiles (Fig. 5) each with a slightly different slope for the μLC-MS/MS analysis of a single

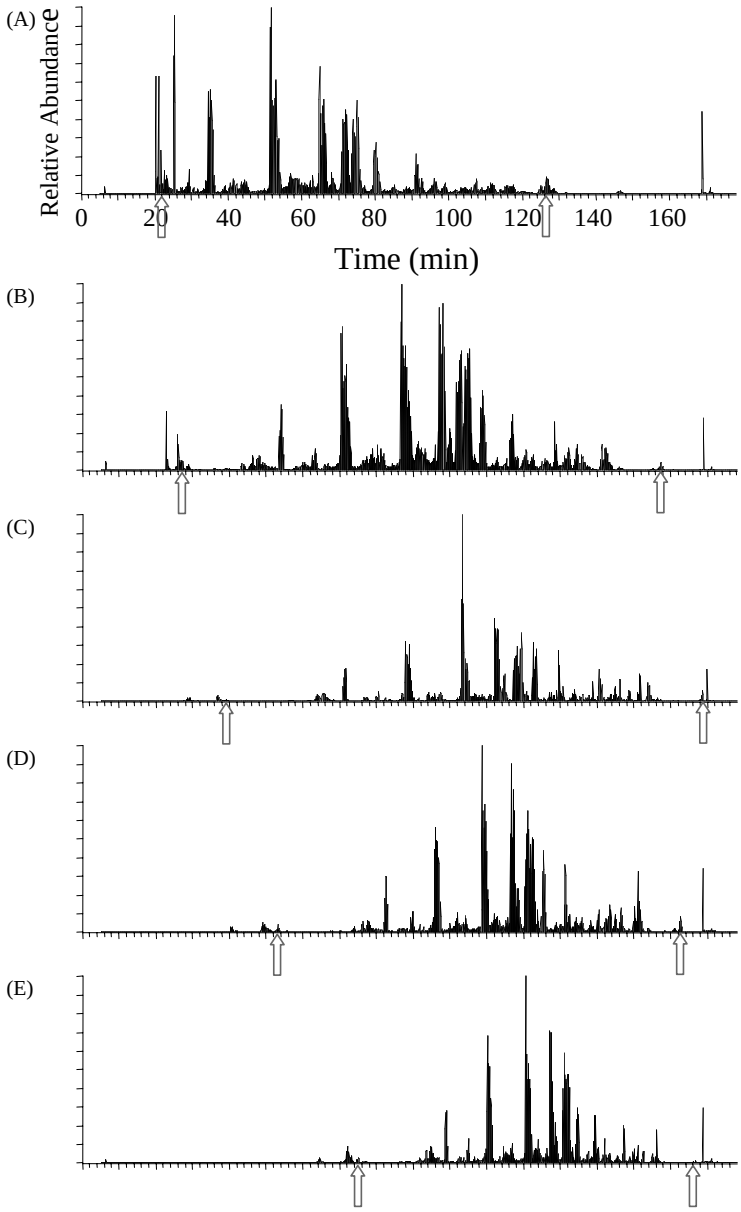


Fig. 6. Base peak chromatograms of ICAT reagent labeled peptides obtained at the various gradient elution. Linear binary gradients from (A) 20%, (B) 15%, (C) 13%, (D) 10%, (E) 5%, acetonitrile to 32% were generated over 150 min. The block arrows indicate the retention times of two peptides, CYAAYR and PFTLDDVQYMIFHTPFCK, eluting at the beginning and ending of the gradient elution, respectively.

SCX fraction of ICAT reagent labeled peptides estimated to contain several hundred peptides. As shown in Fig. 5, starting acetonitrile composition was varied over a defined time. Thus, the rate of change of acetonitrile composition (i.e. slope of gradient) for each gradient tested was slightly different. The resulting base peak chromatograms (BPCs) of the μLC–MS/MS analyses obtained from the gradient elution by injecting 0.1 μl of the sample are shown in Fig. 6. In Fig. 6A–E, shifts in peak elution times as a result of the different gradient elution profiles indicate the expected dependence of peptide elution on the acetonitrile composition. Also similarities in relative intensities, shapes, and elution orders of the peaks show that the effect of the selected gradient conditions on separation selectivity was negligible.

To assess the resolution achieved from the different conditions tested, we used the change in elution time between of two peptides, CYAAYR and PFTLDDVQYMIFHTPFCK, one of which eluted early and the other late from the RP-μLC column. The plot of change in elution time between these two peptides shown in Fig. 7 demonstrates how the separation in time between these two peptides increases as beginning acetonitrile composition increases from 5 to 15%, i.e. separation increases as slope of acetonitrile gradient decreases. For the gradient run of 20–32% solvent B (Fig. 6A), the peptide elution range was drastically decreased because the gradient started from an excessively high acetonitrile composition that forced concurrent elution of hydrophilic peptides and resulted in unresolved elution profiles in the range of 20–25 min.

In these studies using μLC–ESI–MS/MS to analyze complex mixtures of peptides, the shallower gradient elution profiles increased the overall peptide elution range enabling more CID spectra to be acquired; i.e. higher potential proteome coverage could in theory be obtained. However, it was accompanied by broadening of peptide elution profiles. The band broadening effect induced by changes of gradient slope is shown in Fig. 8. At the shallower gradients (Fig. 8A and B), the peak widths are broader and the intensities (peak heights) are decreased. These changes in peak shape resulted in variations in the measured ICAT abundance ratio from the average values. Therefore, excessive band broadening arising from the shallow gradient needed to be avoided to maintain accurate peptide quantification. As expected this trend was observed during analysis of two other complex SCX fractions from the sample of murine liver purified ICAT-labeled peptides.

3.3. Gradient elution developed at high acetonitrile composition

For the gradient of 20–32% solvent B (Fig. 6A), peptides eluted in the range of 20–25 min were not retained well and were poorly resolved because of the abrupt gradient change from 5 to 20% B in 2 min. Fig. 8 shows ion chromatograms of an ICAT-reagent labeled peptide pair eluted at 21 min for the various gradient conditions. Comparing the extremes of gradients used one can note in Fig. 9 that the ion chromatogram obtained at a gradient of 5–32% solvent B (Fig. 9B) shows more symmetry than those obtained at 20–32% solvent B (Fig. 9A). Because peptide quantification is based on single ion current chromatograms of individual peptides reconstituted from multiple MS scans with tandem MS scans of different peptides interjected between them, quantification from a smaller number of MS scans as shown in Fig. 9A will be influenced more by fluctuations of peptide ion intensities in each MS survey scan and produce less accurate quantification. Thus, it is desirable to have a trade off between excessive band broadening and too narrow a chromatographic peak width.

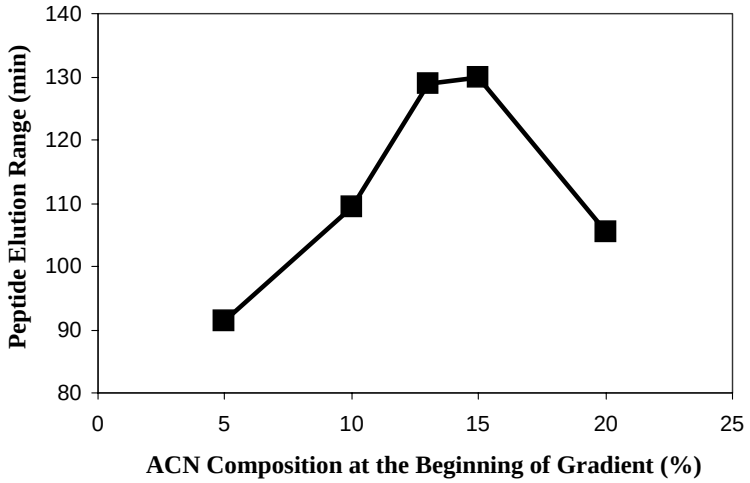


Fig. 7. Plot of peptide elution ranges of the gradient elution.

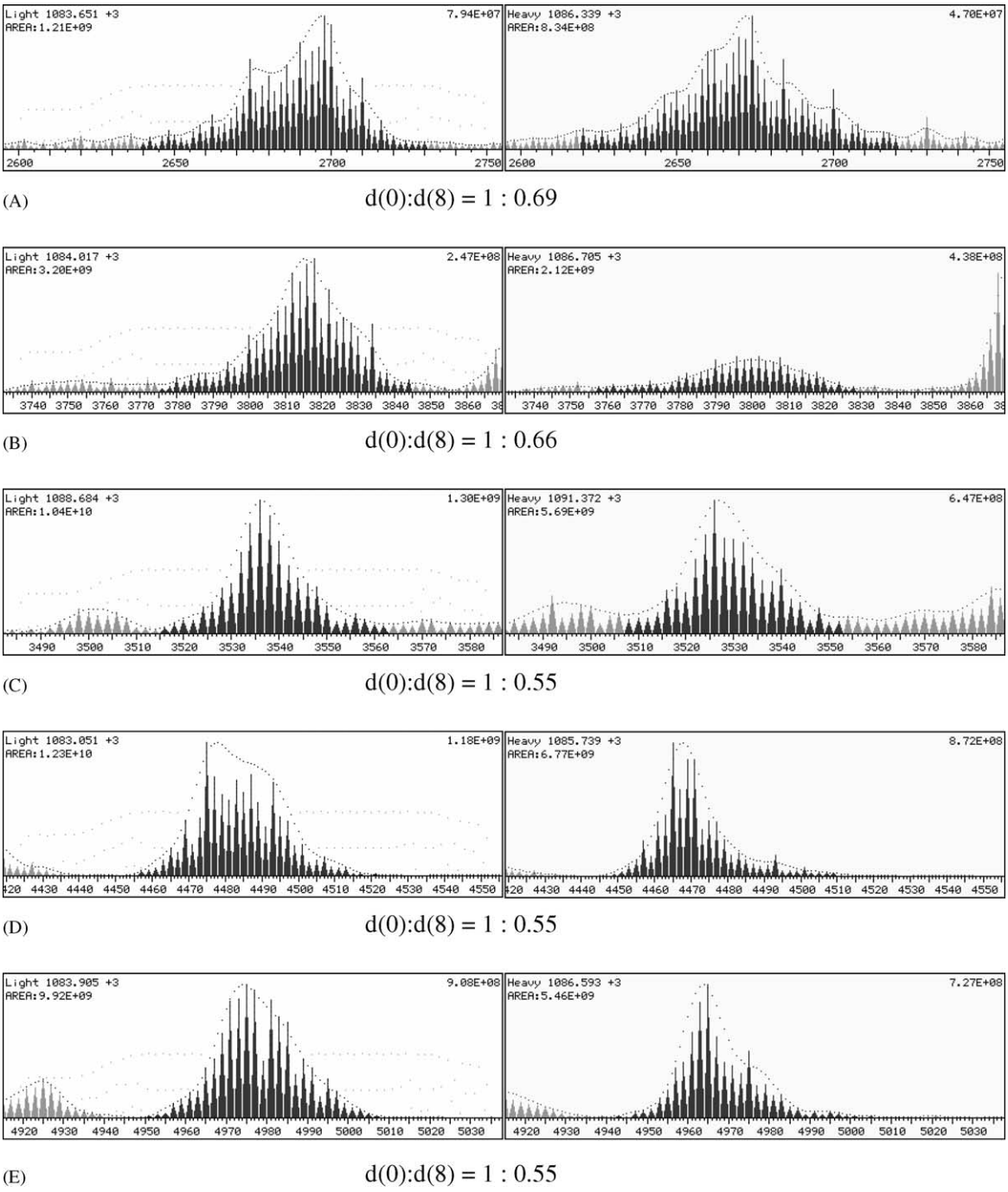


Fig. 8. Quantification analysis of the ICAT reagent labeled peptide, the sequence of VVGYFVSGCDPTIMGIGPVPAINGALKK obtained at (A) 20–32% ACN gradient, (B) 15–32%, (C) 13–32%, (D) 10–32%, and (E) 5–32%.

3.4. Effect of organic modifier composition range on total μLC–ESI–MS/MS analysis time

In gradient elution of peptides from RP-μLC columns, the range of organic modifier composition change should be selected to separate as many different peptides as possible while maintaining high chromatographic resolution and to achieve as low an overall HPLC cycle time as possible. Because elution of peptides depends on the acetonitrile composition as shown in Fig. 7, the elution times of peptides could be adjusted without any changes in resolution by maintaining a constant gradient slope. In Fig. 10, two chromatograms obtained at 5–32% solvent B over 150 min and 13–32% B over 106 min, respectively, are presented. In both runs, comparable resolution and elution time range were obtained by applying the identical gradient slope (0.18% acetonitrile/min). However, it is noted that the shifts in elution time in Fig. 10B reduced the total analysis time by 40 min.

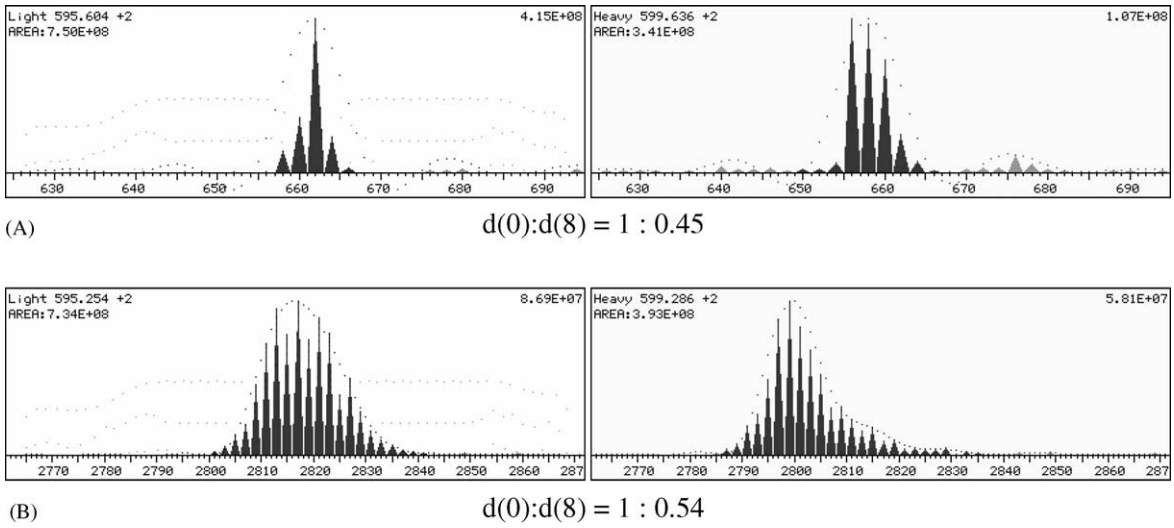


Fig. 9. Ion chromatograms of an ICAT reagent labeled peptide pair having identified sequence of CYAAYR obtained at (A) 20–32% ACN gradient and (B) 5–32% ACN gradient elution.

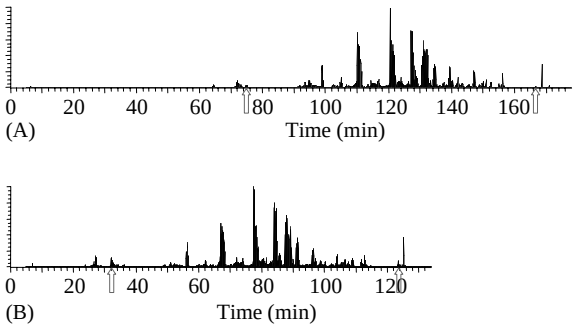


Fig. 10. Base peak chromatograms obtained at (A) 5–32% solvent B over 150 min and (B) 13–32% B over 106 min. The block arrows indicate the retention times of two peptides, CYAAYR and PFTLDDVQYMIFHTPFCK.

4. Conclusions

In quantitative proteome analysis based on the ICAT-labeled peptide method or other methods incorporating stable (heavy and light) isotopes into peptides for quantification, band broadening occurring during μLC separations is an important phenomenon to minimize if one wants to achieve accurate relative abundance ratios. In this study, we confirmed that peptide elution from very complex samples mainly depends on acetonitrile composition in RP-μLC separation, and demonstrated the need for controlling the parameters of the gradient elution including the slope of gradient and solvent composition range. With our RP μLC setup, it is observed that the gradient slope should be at least 0.13 (percentage of acetonitrile/min) to prevent excessive peak broadening which will introduce errors in quantification. Thus, the conditions shown in Fig. 10B provided the optimum compromise between maintaining peak shape for quantification and analyte separation for proteome coverage. Ideally, in the future we will tailor the linear gradient elution of ICAT-labeled peptides for each of the many SCX fractions (∼50 are collected in the ICAT method) each of which contains a complex mixture of hundreds of peptides that vary greatly in chemical composition between SCX fractions.

Acknowledgements

The authors acknowledge support from NIEHS P30ES-07033 to SDN and NIDA P30 DA015625 P51 to RA.

References

[1] R. Aebersold, D.R. Goodlett, Chem. Rev. 101 (2001) 269.
[2] S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, R. Aebersold, Nat. Biotechnol. 17 (1999) 994.
[3] Y. Oda, K. Huang, F.R. Cross, D. Cowburn, B.T. Chait, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 6591.
[4] O.A. Mirgorodskaya, Y.P. Kozmin, M.I. Titov, R. Korner, C.P. Sonksen, P. Roepstorff, Rapid Commun. Mass Spectrom. 14 (2000) 1226.
[5] D.R. Goodlett, A. Keller, J.D. Watts, R. Newitt, E.C. Yi, S. Purvine, J.K. Eng, P. von Haller, R. Aebersold, E. Kolker, Rapid Commun. Mass Spectrom. 15 (2001) 1214.
[6] J. Ji, A. Chakraborty, M. Geng, X. Zhang, A. Amini, M. Bina, F. Regnier, J. Chromatogr. B: Biomed. Sci. Appl. 745 (2000) 197.
[7] H. Zhou, J.A. Ranish, J.D. Watts, R. Aebersold, Nat. Biotechnol. 20 (2002) 512.
[8] D.R. Goodlett, E.C. Yi, Funct. Integr. Genom. 2 (2002) 138.
[9] T.J. Griffin, D.K.M. Han, S.P. Gygi, B. Rist, H. Lee, R. Aebersold, J. Am. Soc. Mass Spectrom. 12 (2001) 1238.
[10] R. Zhang, C.S. Sioma, R.A. Thompson, L. Xiong, F.E. Regnier, Anal. Chem. 74 (2002) 3662.
[11] N.S. Baliga, M. Pan, Y.A. Goo, E.C. Yi, D.R. Goodlett, K. Dimitrov, P. Shannon, R. Aebersold, W.V. Ng, L. Hood, Proc. Natl. Acad Sci. U.S.A. 99 (23) (2002) 14913.
[12] J.R. Yates III, A.L. McCormack, J. Eng, Anal. Chem. 68 (1996) 534A.

[13] A. Keller, A.I. Nesvizhskii, E. Kolker, R. Aebersold, Anal. Chem. 74 (2002) 5383.
[14] D.K. Han, J. Eng, H. Zhou, R. Aebersold, Nat. Biotechnol. 19 (2001) 946.
[15] H.D. Meiring, E. van der Heeft, G.J. ten Hove, A.P.J.M. de Jong, J. Sep. Sci. 25 (2002) 557.
[16] L.J. Licklider, C.C. Thoreen, J. Peng, S.P. Gygi, Anal. Chem. 74 (2002) 3076.
[17] H. Lee, E.C. Yi, R. Aebersold, D.R. Goodlett, Proceedings of the 50th ASMS Conference on Mass Spectrometry and Allied Topics, TPA036, Orlando, FL, USA, June 2002.